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Cancer Therapeutic Targets

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John L. Marshall
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With 51 Figures and 18 Tables

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Division of Hematology & Oncology
Georgetown Lombardi Comprehensive Cancer Center
Georgetown University
Washington, District of Columbia, USA

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Preface

Our understanding of cancer is undergoing a dramatic revolution. As we explore deeper into the biology of each cancer, we enter even more complex worlds. While each new open door provides critical novel insights and exciting new targets, we are exposed to even more questions and deeper levels of complexity. As a practicing physician, I have found it very difficult to keep up with the new science. Each month too many new genes, new proteins, new levels of defining the different types of cancers, and new treatments to keep up with. I also try to stay up to date with the preclinical literature, but here too, I could not judge which new targets were actually important and which were simply a discovery with little clinical impact. I found myself constantly asking my colleagues to "interpret" the literature for me as I did not have an adequate context to judge the science on my own. It was my personal, muddled understanding of the very science I was meant to be an "expert" in that inspired me to create this book.

The goal was to create a "go to" reference with the latest science on cancer targets, their relevance to clinical medicine, and their connections to other cancer targets, written by the true experts in the field. Our target readers are clinicians, clinician scientists, and all allied scientists in the drug discovery and drug development fields. To be most effective and valuable, this text should be revised and updated on a near annual basis, given the pace of discovery in our field. We hope that you will find this a useful resource and that we can continue to maintain the information in the most up-to-date state for years to come.

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John L. Marshall M.D.

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Dr. Marshall received his training at Duke University, the University of Louisville, and Georgetown University. Dr. Marshall is an internationally recognized expert in new drug development for GI cancer, with expertise in phase I, II, and III trial design, and has served as Principal Investigator for more than one hundred clinical trials. While he has an interest in many areas of cancer research, his primary focus has been on the development of vaccines to treat cancer. Dr. Marshall has become an outspoken advocate for GI cancer patients and the importance of clinical research participation. Most recently, he has established the Otto J. Ruesch Center for the Cure of GI Cancers, an organization solely focused on improving the lives of GI cancer patients through innovative research, personalized medicine, and focused advocacy.

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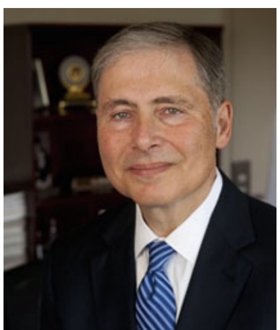

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Part I

Immunology

AKT

1

Timothy A. Yap and Johann S. de Bono

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Abstract

AKT, also known as protein kinase B and RAC-PK, was first discovered as an oncogene transduced by the acute transforming retrovirus (AKT-8), which is known to cause leukemia in mice. AKT is the major downstream target of phosphatidylinositol 3-kinase (PI3K), which can be activated by receptor tyrosine kinases in response to various growth factors. AKT is a serine/threonine kinase located at the apex of a cascade of signaling pathways. Deregulated AKT signaling is implicated in cancer cell growth, proliferation, and survival. Novel antitumor strategies have now been developed to target AKT and key downstream targets in the clinic.

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AKT • A-443654 • Assessment • AT13148 • AT7867 • CCT128930 • GSK690693 • Predictive biomarkers • Therapeutics • Fluorescence in situ hybridization (FISH) • Phosphatidylinositol 3-kinase (PI3K) • Protein kinase B. *See* AKT

AKT, also known as protein kinase B and RAC-PK, was first discovered as an oncogene transduced by the acute transforming retrovirus (AKT-8), which is known to cause leukemia in mice (Hennessy et al. 2005; Yap et al. 2008). AKT is the major downstream target of phosphatidylinositol 3-kinase (PI3K), which can be activated by receptor tyrosine kinases (RTKs) in response to various growth factors (Hennessy et al. 2005; Yap et al. 2008). AKT is a serine/threonine kinase located at the apex of a cascade of signaling pathways. Deregulated AKT signaling is implicated in cancer cell growth, proliferation, and survival. Three members of the AKT family have been isolated, which have been termed AKT1 (PKBα), AKT2 (PKBβ), and AKT3 (PKBγ) (Murthy et al. 2000). Despite being the products of different genes, these proteins are closely related, with up to 80% amino acid homology (Yang et al. 2004). Each isoform bears a pleckstrin homology (PH) domain of approximately 100 amino acids in the N-terminal region, a central kinase catalytic domain containing a threonine residue (Thr308 in AKT1), and a hydrophobic C-terminal tail containing a second regulatory phosphorylation site (Ser473 in AKT1) (Hennessy et al. 2005).

Biology of the Target

The activated PI3K generates the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP_3) from the substrate phosphatidylinositol 3,4-bisphosphate (PIP_2). PIP_3 functions as a high-affinity binding ligand to recruit PH domain-containing proteins to the inner surface of the cell membrane, including AKT (Andjelkovic et al. 1997; Frech et al. 1997). AKT is negatively regulated by the tumor-suppressor protein/lipid PTEN (phosphatase and tensin homologue deleted on chromosome 10) through the dephosphorylation of PIP_3 (Maehama and Dixon 1998; Myers et al. 1998). Importantly, the binding of the PH domain of AKT to PIP_3 is the rate-limiting step in the activation of AKT. This recruitment of AKT to the plasma membrane drives a conformational change in the protein, enabling the activation loop of AKT to undergo phosphorylation by the constitutively active phosphoinositide-dependent kinase 1 (PDK1) at threonine 308 (in AKT1) (Engelman et al. 2006). Further phosphorylation of AKT at the hydrophobic C-terminal domain (serine 473) in AKT1 is required for full activation (Hennessy et al. 2005). While the Thr308 kinase has been confirmed as PDK1, the identity of the serine 473 (Ser473) kinase (termed PDK2) is less clear. Various kinases have been implicated, including the mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov et al. 2005). Following activation, AKT translocates to

the cytoplasm and nucleus, phosphorylating various downstream substrates involved in the regulation of a range of cellular functions, including cell growth, proliferation, and survival.

Target Assessment and Role of the Target in Cancer

AKT is a central node in a complex cascade of signaling pathways, with cross talk and feedback loops, which influence the regulation of this kinase. Aberrant AKT hyperactivation frequently occurs in cancer due to a number of mechanisms affecting upstream regulators, e.g., the overexpression of HER2 in breast carcinoma (Bellacosa et al. 2005; Tokunaga et al. 2006). Genetic amplification or mutation of *PIK3CA*, which encodes the p110α catalytic subunit of PI3K, is also a frequent event in human cancers, including colorectal, breast, gastric, brain, ovarian, and cervical tumors (Broderick et al. 2004; Campbell et al. 2004; Samuels et al. 2004; Shayesteh et al. 1999). These genetic aberrations result in PI3K upregulation, deregulation of AKT activation, and oncogenic transformation (Sarker and Workman 2007). *PTEN* is the second most commonly mutated tumor-suppressor gene and has been implicated in glioblastoma and endometrial and prostate cancers (Vivanco and Sawyers 2002). Recent studies suggest that *AKT* amplification may also be a frequent event in human cancers, although *AKT* mutations are rarely found (Fresno Vara et al. 2004; Soung et al. 2006). Crucially, there is currently no clinically available FDA-approved test for genetic aberrations of AKT.

Predictive Biomarkers

An important factor that may determine the success of PI3K/AKT pathway inhibitors is the incorporation of predictive biomarkers for the selection of appropriate patients to enrich for antitumor responses. Solid tumors possess distinct underlying molecular aberrations, which may be matched with selective anticancer agents, for example, *AKT2* gene amplification or mutations (Fresno Vara et al. 2004; Soung et al. 2006). PTEN loss, detected with techniques such as immunohistochemistry and fluorescence in situ hybridization (FISH) for genomic loss, may also potentially be predictive of antitumor efficacy to inhibitors of the PI3K/AKT signaling pathway or other key targets (Yap et al. 2010a). A phase II study of the mTOR inhibitor everolimus (Novartis) in patients with endometrioid endometrial cancer provided early evidence that PTEN loss may potentially enrich for antitumor responses to PI3K/AKT pathway inhibitors. Other tumor types to consider targeting with this approach are prostate cancer and glioblastoma. Recently, a gene expression signature for PTEN loss, which correlated with adverse outcomes in prostate, breast, and bladder cancers, was developed. It is hypothesized that such molecular profiles may be monitored following treatment and even repeated upon disease progression, in order to dissect mechanisms of drug resistance.

It is crucial for such biomarkers to be analytically validated and clinically qualified in parallel with the development of such novel therapeutics. Currently, there are still no approved predictive biomarkers to select patients for inhibitors of the PI3K/AKT pathway.

In summary, AKT inhibitors may thus potentially be effective as single agents in certain genetically defined cancers, but these tumor types and molecular aberrations are yet to be defined in large randomized clinical trials. Other potential groups of molecularly defined tumors may include HER2-amplified breast cancers, malignancies with *PIK3CA* mutations, and PTEN-deficient cancers (Courtney et al. 2010). Conversely, there are also now data which suggest that cancers with KRAS mutations may be potentially resistant to AKT inhibitors (Courtney et al. 2010). In such scenarios, it is likely that combination strategies involving horizontal and vertical pathway inhibition may be appropriate, such as the pairing of an AKT inhibitor with a MEK inhibitor.

High-Level Overview

Therapeutics

The rapamycin analogues (CCI-779, RAD-001) that inhibit the mTOR kinase have provided proof of principle that the PI3K/AKT pathway may be successfully targeted for clinical use in cancer (Hennessy et al. 2005). These compounds have shown promising evidence of clinical efficacy in a range of tumor types.

AKT is an attractive target for cancer therapy because it sits at the apex of the PI3K/AKT pathway where the latter diverges and integrates with signals from other important pathways. Targeting AKT may thus potentially inhibit this pathway more globally and be less susceptible to feedback loops, when compared with single branch pathways downstream (Hennessy et al. 2005).

Different classes of small molecule AKT inhibitors with varying potencies and specificities for the different AKT isoforms have now been developed. These include ATP-competitive, phosphatidylinositol analog, pseudosubstrate, and allosteric inhibitors, as well as ones with unknown mechanisms of action (Yap et al. 2008). Clinical candidates targeting AKT have only recently entered phase I studies, and clinical trial results have not been formally published.

Preclinical Summary

The preclinical profiles of several AKT inhibitors have recently been disclosed, including GSK690693, A-443654, AT7867, AT13148, and CCT128930. GSK690693 is a novel ATP-competitive pan-AKT kinase inhibitor (Rhodes et al. 2008). It is selective for all three AKT isoforms versus the majority of kinases assessed in a broad panel, though 13 other kinases demonstrated IC_{50} values $\leq$100 nmol/L, including those from the AGC kinase family. GSK690693

demonstrated antiproliferative activity in vitro and in vivo and induced apoptosis in vitro. It also demonstrated antitumor activity in mice bearing established human SKOV-3 ovarian, LNCaP prostate, and BT474 and HCC-1954 breast carcinoma xenografts.

A-443654 is a pan-AKT inhibitor, with equal potency against all three AKT isoforms with activity observed both in vitro and in vivo. This compound has been co-crystallized with PKA and was shown to have 40-fold selectivity for AKT1 versus PKA (Luo et al. 2005). This raises the potential issue of selectivity for AKT versus other AGC kinases. ATP-competitive inhibitors of AKT often inhibit other AGC kinases such as p70S6 kinase, PKA, and Rho kinase. The potential for antitumor effects versus the undesired toxicity of these selectivity profiles remains to be determined in clinical trials. Until recently, it was not possible to co-crystallize compounds with native AKT, and instead, surrogates, such as wild-type PKA or a mutant form in which ATP-site residues are mutated to generate a PKA-AKT chimera, have provided a useful strategy. An alternative technique has recently been developed for the generation of phosphorylated, AKT-inhibitor crystal structures, which represents an important step forward in the development of ATP-competitive AKT inhibitors and also provides a potential explanation for the selectivity of A-443654 for AKT versus PKA (Yap et al. 2008).

AT7867 is a novel and potent inhibitor of both AKT and the downstream kinase p70S6 kinase and protein kinase A (Grimshaw et al. 2010). This compound was shown to inhibit AKT and p70S6 kinase activity, with growth inhibition and cellular apoptosis observed in multiple cell lines. Importantly, robust pharmacodynamic effects and induction of apoptosis were shown in the PTEN-deficient U87MG human glioblastoma xenograft, with tumor growth inhibition observed at the same doses. These results have supported the novel approach of targeting both AKT and p70S6K for the development of a potent anticancer monotherapy.

AT13148 is a novel small molecule inhibitor of AKT developed from a series identified through fragment-based screening linked to high-throughput x-ray crystallography (Lyons et al. 2007). This oral agent inhibits downstream biomarkers of the AKT pathway both in vitro and in vivo and inhibits tumor growth in vivo. Interestingly, AT13148 also inhibits the related AGC kinases: p70S6 kinase, ROCKII, and PKA. In vivo studies with mouse xenograft models demonstrated that AT13148 has antitumor effects at 40–50 mg/kg. AT13148 was found to be especially effective in the *PTEN*-deficient MES-SA uterine sarcoma tumor xenograft.

CCT128930 was recently disclosed as a novel ATP-competitive AKT inhibitor discovered using fragment and structural-based approaches (Yap et al. 2011). It is a potent advanced lead pyrrolopyrimidine compound exhibiting selectivity for AKT over PKA, achieved through the targeting of a single amino acid difference. CCT128930 was shown to inhibit the phosphorylation of AKT downstream targets in the *PTEN*-deficient U87MG human glioblastoma xenografts, confirming pharmacodynamic target inhibition. In addition, significant suppression of pThr246 PRAS40 fluorescence in CCT128930-treated mouse whiskers in vivo and ex vivo-treated human hair follicles was observed, with minimal changes in total PRAS40

levels. Antitumor growth inhibition was demonstrated in U87MG and HER2-positive, *PIK3CA*-mutant BT474 human breast cancer xenografts, consistent with the pharmacodynamic biomarker changes observed.

An alternative strategy for AKT drug discovery has been through the development of allosteric inhibitors of AKT. A series of inhibitors were discovered following the screening of a 270,000 compound library for inhibitors of full-length AKT1, 2, and 3. Two were shown to inhibit the phosphorylation and activation of the corresponding AKT isoforms by PDK1, which phosphorylates Thr308 of AKT. Additional analyses led to the hypothesis that these inhibitors bind to a site on AKT, which is formed only in the presence of the PH domain, and that binding to AKT induces the formation of an inactive conformation. These allosteric inhibitors showed high selectivity for AKT isoforms versus other kinases and demonstrated that inhibition of AKT1 and 2 selectively sensitized tumor cells but not normal cells to apoptotic stimuli, indicating a potential therapeutic strategy for cancer treatment. Furthermore, in vivo activity has been demonstrated with this class of AKT inhibitors. The allosteric inhibitor MK-2206 (Merck & Co., Inc) is currently in phase I clinical trials and is described in the next section.

Clinical Summary

There are currently different chemical classes of AKT inhibitors with a range of potencies and selectivities for the different AKT isoforms in development. These include phosphatidylinositol analogs, pseudosubstrate compounds, ATP-competitive small molecules, and allosteric inhibitors (Yap et al. 2008).

The first class of inhibitors to be developed are lipid-based AKT inhibitors which interact with the PIP_3-binding PH domain of AKT (Yap et al. 2008). An often cited example is perifosine, an oral alkyl-phosphocholine compound which inhibits the translocation of AKT to the cell membrane, preventing the activation of AKT. It is actually believed that perifosine targets cellular membranes, modulating membrane permeability, membrane lipid composition, phospholipid metabolism, and mitogenic signal transduction, resulting in cell differentiation and inhibition of cell growth. This approach has demonstrated antitumor activity in vitro and in vivo. Additive and synergistic effects of perifosine in combinatorial regimens with conventional chemotherapies have been observed. Despite these promising preclinical studies, data from early phase I and II clinical trials of single-agent perifosine in a wide range of advanced cancer types have been disappointing, with only modest responses seen. Gastrointestinal and constitutional toxicities have also limited its development, resulting in the discontinuation of a phase II study of perifosine in patients with advanced pancreatic cancer.

Perifosine was shown to have some activity in patients with advanced sarcoma. A retrospective study of 60 evaluable patients from three phase I and four phase II studies demonstrated an overall clinical benefit rate of 50%, which compares favorably to that observed with the mTOR inhibitors in sarcoma (Yap et al. 2008).

Single-agent perifosine also demonstrated antitumor activity in patients with advanced multiple myeloma and Waldenstrom's macroglobulinemia.

Multiple combination strategies have also now been pursued, including chemotherapies and other novel targeted therapeutics, in order to enhance cytotoxicity and overcome drug resistance (Yap et al. 2008). Phase I studies have now confirmed that these combinations are well tolerated with different agents, including radiation, taxanes, gemcitabine, sunitinib, and sorafenib, with further studies underway.

The D-3-deoxy-phosphatidyl-myo-inositol PX-316 is an AKT PH domain inhibitor (Yap et al. 2008). PX-316 was well tolerated in vivo when administered intravenously with no hemolysis or hematological toxicities. It was shown to have antitumor activity against human MCF-7 breast cancer and HT-29 colon cancer xenografts in vivo.

API-2/TCN (triciribine phosphate monohydrate, VD-002) is a synthetic small molecule that was previously assessed as a cytotoxic agent in phase I and II clinical studies in the 1980s and 1990s (Yap et al. 2008). However, a poor toxicity profile and minimal efficacy, such as hyperglycemia and hypertriglyceridemia, precluded the further development of this agent. A recent screening of the National Cancer Institute Diversity Set for compounds that inhibited the growth of an AKT2-transformed cell line has renewed interest in this agent with the detection of API-2/TCN in this screening set. API-2/TCN suppresses the phosphorylation of AKT at both Ser473 and Thr308, indicating that it is an inhibitor of the AKT signaling pathway, but not directly of AKT. API-2/TCN demonstrated suppression of AKT signaling in vitro and exhibited antitumor activity in cancer cell lines with elevated AKT in mouse xenografts, including ovarian and pancreatic tumors. It will be important to investigate if the previously observed toxicities of hyperglycemia and hypertriglyceridemia were due to AKT2 inhibition. Phase I trials are currently underway assessing lower doses of API-2/TCN by weekly IV infusions in patients with advanced tumors with high phosphorylated AKT expression.

ATP-competitive inhibitors that have been recently disclosed are GSK690693, A-443654, AT7867, AT13148, and CCT128930 (www.clinicaltrials.gov). An intravenous formulation of GSK690693 was assessed in a phase I study in patients with solid tumors or lymphoma. The 14 patients enrolled stayed on study between 4 to at least 30 weeks (Yap et al. 2008). GSK690693 was well tolerated at weekly doses of 25, 50, 75, and 115 mg, while PK parameters (C_{max} and AUC) increased with dose. Surrogate pharmacodynamic analyses included blood glucose levels and AKT pathway biomarkers in peripheral blood mononuclear cells using an ELISA-based assay. Transient drug-related increases in blood glucose levels above 250 mg/dL were observed in two patients in the 50 mg and 75 mg weekly cohorts. An oral formulation of this compound, GSK2141795, is currently being assessed in a phase I clinical trial in patients with solid tumors and lymphoma (www.clinicaltrials.gov).

As discussed, an alternative strategy for AKT drug discovery, which avoids the issue of kinase selectivity, is the development of allosteric inhibitors of AKT (Yap et al. 2008). These inhibitors bind to a site on AKT, which is formed only in the presence of the PH domain, and that binding to AKT promotes the formation of an inactive conformation. As allosteric inhibitors, these compounds demonstrate

excellent selectivity for AKT isoforms versus other kinases, and in vivo activity has recently been reported with this class of AKT inhibitor (Bilodeau et al. 2008).

MK-2206 is a potent, allosteric Akt1/2/3 inhibitor, with wide preclinical antitumor activity and a long terminal elimination half-life ($t_{1/2}$) (Yap et al. 2010b). In view of the long $t_{1/2}$, an alternate-day dosing schedule was pursued initially in the first-in-man study. Overall, MK-2206 was well tolerated in this schedule, with the main drug-related toxicities including rash nausea, fatigue, and transient hyperglycemia. Doses of MK-2206 tested included 30 mg, 60 mg, 75 mg, and 90 mg QOD, with dose-limiting rash and mucositis observed at 75 mg and 90 mg QOD. This established the maximum tolerated dose at 60 mg QOD. The pharmacokinetic profile of MK-2206 was dose proportional, with MK-2206 concentrations exceeding a statistically determined pharmacokinetic target for significant inhibition of pSer473 AKT in blood that was maintained over the entire dosing interval in patients in the 60 mg cohort. Importantly, the pharmacodynamic analyses of pSer473 AKT and downstream substrate phosphorylation confirmed target modulation in both tumor and surrogate tissue. Minor tumor regressions were also observed following single-agent MK-2206 administration. MK-2206 is currently being evaluated in combination with a range of chemotherapies and other targeted agents, as well as in selected populations of patients with distinct molecular aberrations. An alternative single-agent weekly schedule is also being assessed in view of the long half-life of MK-2206, in order to evaluate pulsatile drug dosing. This weekly dosing schedule may potentially minimize drug toxicity and maximize tumor blockade and antitumor activity.

Anticipated High-Impact Result

AKT inhibitors are currently being assessed in early-phase clinical trials in patients with selected molecular aberrations and also in multiple combination studies. Combinatorial strategies involving targeted agents may be categorized into horizontal and vertical blockade of their respective signaling pathways. Examples of potential targets for horizontal combination with AKT blockade include Ras, Raf, and Mek inhibitors. Both PI3K/AKT and Ras/Raf pathways play pivotal roles in signal transduction and malignant progression. It therefore makes rational sense to block both signaling cascades simultaneously. There is currently a phase Ib combination trial involving the AKT inhibitor MK-2206 and the MEK inhibitor AZ6244 (AstraZeneca) and results are expected soon.

The vertical blockade of the PI3K pathway with multiple agents also holds great promise in view of the feedback signaling loops that may develop following the inhibition of components of the pathway. Potential combination partners for AKT along the PI3K pathway include the insulin-like growth factor-1 receptor (IGF-1R), epidermal growth factor receptor, HER2, and mTOR. A recent phase I study combined the oral mTOR inhibitor ridaforolimus (Merck & Co., Inc) and the IGF-1R antibody dalotuzumab (Merck & Co., Inc) in patients with advanced solid tumors. Encouragingly, this combination was well tolerated, with promising

antitumor activity, including responses in patients with ER-positive breast cancer. Various AKT studies are already underway, including MK-2206 and trastuzumab (Roche) and lapatinib (GlaxoSmithKline) in advanced solid tumors, including HER2-positive tumors (Di Cosimo et al. 2010).

In the future, with a better understanding of the underlying biology of the individual isoforms of AKT, it may be essential that the development of selective inhibitors against each isoform may be necessary for maximal antitumor benefits and minimal toxicities.

Conclusion

AKT sits at the apex of key signaling cascades and is implicated in oncogenesis and malignant progression in a range of tumor types. The development of potent inhibitors against this key serine/threonine kinase is thus likely to represent an important antitumor therapeutic strategy. It remains to be determined if this will be in combination with other agents or as monotherapies in molecularly selected populations of patients. Multiple clinical trials of AKT inhibitors are now well underway and results are anticipated imminently.

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2 Anti-4-1BB/4-1BBL

Ezra A. Bernstein and Yvonne Saenger

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Abstract

H4-1BB, the human homologue of 4-1BB, is a 256 amino acid, 27 kDa transmembrane receptor glycoprotein first identified in screens for receptors on mouse concanavalin A-activated helper and cytotoxic T-cell lines (Vinay and Kwon 2006). It was then isolated from PMA plus ionomycin-treated human peripheral T-cell cDNA libraries (Zhou et al. 1995). In humans, 4-1BB maps to chromosome 19p13.3 (Alderson et al. 1994). It is also commonly referred to as CD137, induced by lymphocyte activation (ILA), and tumor necrosis factor receptor superfamily member 9 (TNFRSF9). The receptor, 4-1BB, and its ligand,

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4-1BBL, are members of the tumor necrosis factor and tumor necrosis factor receptor superfamilies, respectively (Vinay and Kwon 2012). However, unlike other members of the tumor necrosis factor superfamily, the ecto-domain of 4-1BB forms a homotrimer with an extended, three-bladed propeller structure (Won et al. 2010). 4-1BB also has a cysteine-rich extracellular domain, a transmembrane region, and a cytoplasmic domain that contains a tyrosine kinase p56^{lck} binding site (Vinay and Kwon 2006).

Keywords
Anti-4-1BB monoclonal antibody (mAB) • H4-1BB

Target: 4-1BB

H4-1BB, the human homologue of 4-1BB, is a 256 amino acid, 27 kDa transmembrane receptor glycoprotein first identified in screens for receptors on mouse concanavalin A-activated helper and cytotoxic T-cell lines (Vinay and Kwon 2006). It was then isolated from PMA plus ionomycin-treated human peripheral T-cell cDNA libraries (Zhou et al. 1995). In humans, 4-1BB maps to chromosome 19p13.3 (Alderson et al. 1994). It is also commonly referred to as CD137, induced by lymphocyte activation (ILA), and tumor necrosis factor receptor superfamily member 9 (TNFRSF9). The receptor, 4-1BB, and its ligand, 4-1BBL, are members of the tumor necrosis factor and tumor necrosis factor receptor superfamilies, respectively (Vinay and Kwon 2012). However, unlike other members of the tumor necrosis factor superfamily, the ecto-domain of 4-1BB forms a homotrimer with an extended, three-bladed propeller structure (Won et al. 2010). 4-1BB also has a cysteine-rich extracellular domain, a transmembrane region, and a cytoplasmic domain that contains a tyrosine kinase p56^{lck} binding site (Vinay and Kwon 2006).

4-1BB, primarily an inducible co-stimulatory molecule, is expressed on activated CD8^{+}and CD4^{+} T cells, but it has also been shown to be expressed on activated dendritic cells (DCs), monocytes, neutrophils, B cells, and natural killer (NK) cells (Vinay and Kwon 2011). It is also constitutively expressed on primary human monocytes, blood vessel endothelial cells, human follicular dendritic cells, and CD4^{+}CD25^{+} regulatory T cells. Its ligand, 4-1BBL, is primarily expressed on antigen-presenting cells (APCs) including activated B cells, macrophages, and dendritic cells (DCs), but it is also expressed on some T cells (Lee and Croft 2009). 4-1BBL is a 34 kD type II membrane glycoprotein. Given its MW of ~97 kD in reducing conditions, it is believed to be a disulfide-linked homodimer (Vinay and Kwon 2006). Ligation of 4-1BB by 4-1BBL or an agonistic anti 4-1BB antibody can activate anti-apoptotic pathways and lead to cell survival (Lee and Croft 2009).

Intriguingly, 4-1BB has also been shown to be expressed within tumor vessel walls either on the endothelial cells or vascular smooth muscle cells or both (Broll

and Richter 2001). 4-1BBL has been shown to be expressed on the tumor cells themselves (Wang et al. 2008). The exact biological effect of this localization is unknown, but it is thought that constitutive expression of 4-1BB within tumor vessel walls could be implicated in angiogenic activity.

Biology of the Target

Ligation of 4-1BB by 4-1BBL or an agonistic anti 4-1BB antibody can activate the PI3K, PKB (Akt), and NF-KB pathways and upregulate expression of anti-apoptotic Bcl-2 family members (Lee and Croft 2009). This can lead to increased numbers of CD8+ T cells, NK cells, and other immune cells – all of which are important for an effective antitumor immune response. In addition to these immune-stimulatory effects, ligation can also have an immune-regulatory role. The regulatory role is not completely understood but may occur through a few different mechanisms (Vinay and Kwon 2006). Ligation of CD4+CD25+ Tregs could cause their expansion and result in an increase in their suppressive role. Ligation of dendritic cells could simulate them to release suppressive cytokines. Ligation of regulatory CD8+ populations could cause their expansion and result in an increase in their suppressive role via TGF-B and IFN-y (Vinay and Kwon 2006). 4-1BB has also been shown to suppress humoral immunity (Vinay and Kwon 2012).

The regulatory effects of 4-1BB ligation are important and have been targeted to treat some autoimmune diseases. However, the immune-stimulatory effects of 4-1BB are better understood. The activation of cytotoxic T cells and NK cells are important for an antitumor immune response; thus, agonistic 4-1BBL mAb offers a rational therapeutic approach.

Target Assessment

Assessing expression levels of 4-1BB/4-1BBL is not commonly done, but it has been successfully done. 4-1BBL levels in the serum of patients can be measured using a standard ELISA protocol. Hentschel et al. (2006) successfully used a polyclonal mouse anti-4-1BBL antibody. 4-1BB levels expressed on the surface of cells can be evaluated using immunohistochemistry or flow cytometry. Anderson et al. (2012) successfully used clone BBK-2, a commercially available mouse monoclonal anti-CD137 antibody. While 4-1BB/4-1BBL levels can be assessed, there is still a great deal of research needed to further understand and develop this target for clinical practice and define the role of 4-1BB/4-1BBL as a diagnostic, prognostic, and/or predictive marker.

Role of the Target in Cancer

Rank: 5

High-Level Overview

Diagnostic, Prognostic, and Predictive

Currently, detection and measurement of 4-1BB or 4-1BBL levels is not used as a diagnostic, prognostic, or predictive marker for any cancers. Current studies are investigating correlations between expression/expression levels and tumors to better establish diagnostic, prognostic, and predictive value. 4-1BBL is beginning to be seen as an applicable prognostic or predictive marker in certain malignancies such as acute myeloid leukemia (AML). In AML, for reasons that are not fully understood, 4-1BBL in the serum correlated with unfavorable subtypes and high BM-blast counts. Serum 4-1BBL levels also correlated significantly with the probability of obtaining complete remission and the probability of remaining in complete remission (Hentschel et al. 2006). Additionally, 4-1BB **is** expressed by a select group of hematolymphoid tumors, including classical Hodgkin lymphoma, T-cell and NK/T-cell lymphomas, and follicular dendritic cell neoplasms (Anderson et al. 2012). Thus, it could be a possible diagnostic marker for these diseases. It is also **possible** that 4-1BB/4-1BBL levels within the immune microenvironment can be used as a prognostic and predictive tool for other cancers, such as diffuse large B-cell lymphoma (Alizadeh et al. 2011). However, the clinical application of 4-1BB or 4-1BBL as a diagnostic, prognostic, or predictive value is not in the immediate future.

Therapeutics

Given the ability of 4-1BB/4-1BBL to modulate immune activity by upregulating survival genes, enhancing cell division, inducing cytokine production, and preventing activation-induced cell death in T cells, much effort has gone into targeting and stimulating 4-1BB therapeutically (Vinay and Kwon 2012). Therapeutic efforts have largely been focused on murine models using an agonistic anti-4-1BB monoclonal antibody (mAb), but recently the use of a recombinant adenovirus as a means of delivering 4-1BBL has also been explored (Lee and Croft 2009). The efficacy of using anti-4-1BB mAb as a therapeutic was first established by eradicating large tumors in mice, including the poorly immunogenic Ag104A sarcoma and the highly tumorigenic P815 mastocytoma line (Melero et al. 1997). In another study of a murine myeloma model, anti-4-1BB mAbs induced complete eradication of established s.c. NS0-derived tumors (Murillo et al. 2008). However, mixed findings have been obtained. Some tumors do not respond as well to anti-4-1BB mAbs. Much of the efficacy in 4-1BB mAb therapy is through a $CD8^+$ T-cell-led immune response. In poorly immunogenic tumors, including C3 tumor, TC-1 lung

carcinoma, and B16-F10 melanoma, established solid tumors or metastases were refractory to treatment by anti-4-1BB mAb. This was found to be due to immunological ignorance, rather than anergy or deletion (Melero et al. 1997). This was confirmed in another study where mice with a P1A-expressing plasmacytoma were treated with P1A-specific CD8+ CTL (P1CTL) in conjunction with either anti-4-1BB mAb or control IgG. When compared to the control mice treated with IgG, mice that were treated with anti-4-1BB mAb exhibited markedly enhanced tumor rejection, delayed tumor progression, and prolonged survival (May et al. 2002).

Anti-4-1BB mAb as a single agent has certainly proven effective in some preclinical models enhancing an immune-mediated tumor attack, but combination therapy with other immune therapy, chemotherapy, or radiation therapy has shown synergy and enhanced efficacy. When 4-1BB mAb was administered in combination with trastuzumab, a monoclonal antibody targeting human epidermal growth factor receptor 2 (Her2), breast cancer cells (including an intrinsically trastuzumab-resistant cell line) were killed more efficiently both in vitro and in vivo in xenotransplant models of human breast cancer (John et al. 2012). In another study, 4-1BB mAb was administered in combination with an oncolytic adenovirus. Again, combination therapy significantly reduced the growth of established subcutaneous tumors relative to either treatment alone. Furthermore, when the oncolytic adenovirus and 4-1BB mAb were administered in conjunction with T-cell and NK-cell depletion, efficacy was reduced. This confirmed the importance of these cells in an effective therapeutic response (John et al. 2012).

Radiotherapy has been used in combination with immune therapy to enhance the effect by lysing tumor cells and exposing tumor-associated antigens. In a study testing this, anti-4-1BB mAb was combined with radiotherapy in a murine model with high-grade glioma. The combination of radiation and anti-4-1BB therapy resulted in complete tumor eradication and prolonged survival in six of nine (67%) mice with established brain tumors ($P = 0.0009$). Five of six (83%) long-term survivors in the combination group demonstrated antitumor immunity by rejecting challenge tumors (Newcomb et al. 2010).

Chemoimmunotherapy, the combination of chemotherapy and immunotherapy, may seem counterintuitive since the chemotherapy depletes immune cells that immune therapy promotes, but studies have shown synergy and enhanced efficacy with chemoimmunotherapy. If the chemotherapy is administered prior to the immunotherapy, the chemotherapy can create an environment for homeostatic lymphoproliferation and eliminate some of the suppressive immune networks (Kim et al. 2009). Anti-4-1BB and cisplatin showed synergistic anticancer effects in a CT-26 colon carcinoma model. It produced complete regression in >60% of mice with either preventive or therapeutic treatment. The tumor-free mice formed long-lasting $CD8^+$ T-cell-dependent tumor-specific memory. Anti-4-1BB was able to induce rapid repopulation of T and B cells from cisplatin-mediated lymphopenia and differentiation and expansion of IFN-gamma$^+$ CD11c$^+$CD8$^+$ T cells. Combination therapy produced almost twice as many lymphoid cells as anti-4-1BB alone (Kim et al. 2008). In another study, anti-4-1BB mAb in combination with 5-fluorouracil was evaluated in treating renal cell carcinoma, a very difficult malignancy to treat.

Either treatment alone had little effect, but combination therapy with anti-4-1BB mAb and 5-FU eradicated the tumors in more than 70% of mice (Ju et al. 2008).

There are many other ways that anti-4-1BB mAb is being used as a therapeutic. It has frequently been evaluated in combination with a dendritic cell vaccine in poorly immunogenic tumors. It has also been used as an in vitro co-stimulatory agent for adoptive T-cell transfer where it was shown to significantly increases T-cell yield and amplify antitumor responses (Kroon et al. 2007).

Many studies have confirmed the therapeutic benefit of anti-4-1BB mAb and the enhanced efficacy and synergistic effects of combination therapy. However, as mentioned previously, 4-1BB has dual stimulatory and regulatory pathways that are still not completely understood, so undesirable side effects could occur. In one study using naive mice, anti 4-1BB mAb led to the development of a series of immunological anomalies including splenomegaly, lymphadenopathy, hepatomegaly, multifocal hepatitis, anemia, altered trafficking of B cells and CD8 T cells, loss of NK cells, and a tenfold increase in bone marrow cells bearing the phenotype of hemopoietic stem cells. These events were dependent on CD8 T cells, TNF-alpha, IFN-gamma, and type I IFNs (Niu et al. 2007). Research is now also focusing on optimizing 4-1BBL and using different methods of delivery to avoid some of the toxicity associated with 4-1BB mAb (Schabowsky et al. 2009).

Preclinical Summary

There has been a large amount of preclinical work investigating the efficacy of the agonistic anti-4-1BB mAb. The great majority of this work has been in murine models. This work has yielded a great deal of knowledge regarding the immune-modulating abilities and immune-modulating mechanisms of 4-1BB ligation as well as insights into the specific cells involved in the antitumor immune response. 4-1BB mAb has been shown to work alone as a single agent but to be even more efficacious when used in combination with other immunotherapies, radiotherapies, or chemotherapies. In vivo murine models have shown that the immune response from anti-4-1BB mAb is primarily mediated through $CD8^+$ T cells and NK cells. More recent preclinical studies are focusing on optimizing combination therapy, developing more potent less toxic variants of anti-4-1BB mAb, and exploring new methods of delivery.

Clinical Summary

At this point, there has not been a large amount of published clinical work investigating the use of anti-4-1BB. Far more preclinical work has been done. Anti-4-1BB mAb marketed as Urelumab, or BMS-663513 by Bristol-Myers Squibb, has been investigated in a few phase 1 trials. In a phase 1 trial completed in 2007, toxicity was not an issue, but partial responses were only seen in 6% of the melanoma patients. Although, 17% of melanoma patients and 14% of renal cell patients had stable

disease at 6 months or longer (Sznol et al. 2008). There is one more phase 1 study currently ongoing to test the safety, tolerability, pharmacokinetics, and immune regulation in patients with advanced and/or metastatic solid tumors. This study is estimated to finish on September of 2014 (Clinical trial database 2012).

Anticipated High-Impact Results

- Development and use of 4-1BB as a diagnostic, prognostic, and predictive marker
- Clinical study establishing efficacy in human subjects
- Evaluating combination therapy of Anti-4-1BB mAb with other immunotherapies, radiotherapies, or chemotherapies within a clinical setting

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Anti-B7-H4

3

Jun Wang and Lieping Chen

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Abstract

B7-H4, also known as B7x or B7S1, is a new member of the B7 family of immune co-signaling molecules. Despite the wide distribution in mRNA levels, B7-H4 protein expression is generally absent in peripheral tissues but is increased on many cancer cells or tumor-associated immune cells. In addition, B7-H4 up-regulation has shown to be associated with diseases progression and/or outcome

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in some cancers. The unique expression of B7-H4 in the tumor microenvironment and its potential immune inhibitory functions on both innate and adaptive immune responses represents a novel target for the next-generation cancer diagnosis and immunotherapy.

Keywords

B7 homolog 4 (B7-H4) • Antigen-dependent induction • Downstream gene target of p70S6K • ELISA • Immunohistochemistry • Cancer • Marker for pancreatic ductal adenocarcinoma (PDA) • Neutrophil progenitor cell proliferation • Preclinical studies • Shield for immunosurveillance evasion • Soluble form • Staining in tumor endothelial vasculature • T-cell inhibition • T regulatory cell trafficking • Therapeutics for autoimmune diseases and islet transplantation • B7S1[1–3]. *See* B7 homolog 4 (B7-H4) • B7x. *See* B7 homolog 4 (B7-H4)

Target: B7 Homolog 4 (B7-H4)

B7-H4, found on 2003 and also known as B7x or B7S1[1–3], is a new member of the B7 family of immune co-signaling molecules identified by protein sequence analysis and comparative molecular modeling (Sica et al. 2003; Zang et al. 2003; Prasad et al. 2003). Similar to the other B7 members, the B7-H4 molecule is a type I transmembrane protein with one Ig-like V-set domain and one Ig-like C2-set domain. Its transmembrane domain is followed by a very short intracellular tail of two amino acids. B7-H4 shares approximately 25% amino acid homology in the extracellular domain with other B7 family members. The mouse and human B7-H4 shares about 87% identity in their amino acid sequences, suggesting this gene is highly conserved evolutionarily (Sica et al. 2003; Yi and Chen 2009). Although initially proposed to be a GPI-anchored protein, further studies show that it might not be the case (Choi et al. 2003).

Despite the wide distribution in mRNA levels, B7-H4 protein expression is generally absent in peripheral tissues except various normal epithelia (Sica et al. 2003). However, aberrant B7-H4 protein expression was observed on several adenocarcinomas, including ovarian (Choi et al. 2003; Salceda et al. 2005; Kryczek et al. 2006a, 2007; Simon et al. 2006), renal (Krambeck et al. 2006; Thompson et al. 2008), prostate (Zang et al. 2007), breast (Salceda et al. 2005; Simon et al. 2006; Tringler et al. 2005), pancreatic (Awadallah et al. 2008), and lung cancers (Choi et al. 2003; Sun et al. 2006). Interestingly, B7-H4 is upregulated on the surface of tumor macrophages in patients with ovarian cancer, upon stimulation with IL-6 and IL-10 and might contribute to the suppression function of this novel cell population (Kryczek et al. 2006a, b, 2007). A soluble form of B7-H4, possibly acting as a decoy factor for B7-H4 signaling, is also found in the serum and ascites fluid of patients with autoimmune diseases (Azuma et al. 2009) and cancers (Simon et al. 2006; Thompson et al. 2008). These clinical studies indicate its importance in association with disease severity and outcome. In addition, B7-H4 binds a putative receptor on activated T cells, which is distinct from other receptors in B7 family

(Sica et al. 2003; Prasad et al. 2003) and also BTLA. Ligation of B7-H4 inhibits antigen-dependent induction of T-cell proliferation, activation, and cytokine production by cell cycle arrest (Sica et al. 2003). Besides its role in adaptive responses (mildly augmented Th1 responses) (Suh et al. 2006), B7-H4-deficient mice also show augmented neutrophil-mediated innate immunity (Zhu et al. 2009). Moreover, B7-H4 also promotes the malignant transformation of epithelial cells by protecting them from apoptosis (Salceda et al. 2005). Taken together, the unique expression of B7-H4 in the tumor microenvironment and its potential immune inhibitory effects that foster tumor growth represent a new target for cancer diagnosis and therapy.

Biology of the Target

The role of B7-H4 in tumor immune evasion is yet to be elucidated. It has been shown that immobilized B7-H4Ig or cell-associated B7-H4 has a profound inhibitory effect on the proliferation of T cells activated by TCR signaling and significantly reduces CTL cytotoxicity as well as IFN-γ and IL-2 secretion (Sica et al. 2003; Zang et al. 2003; Prasad et al. 2003). T-cell inhibition is due to cell cycle arrest at the G0/G1 phase (Sica et al. 2003). Administration of B7-H4Ig into mice impairs antigen-specific T-cell responses, whereas blockade of endogenous B7-H4 by specific mAb promotes T-cell responses (Sica et al. 2003; Prasad et al. 2003). A recent study identified that B7-H4 is also constitutively expressed on human bone marrow-derived mesenchymal stem cells (hBMSCs) and is responsible for the suppressive effect of hBMSCs on T-cell activation (Xue et al. 2010). In addition, Zhu et al. characterized a new role of B7-H4 in inhibiting neutrophil progenitor cell proliferation (Zhu et al. 2009). However, whether and how B7-H4 exerts its function on the innate immune responses during tumor pathogenesis or progression still needs further investigation.

B7-H4 shows aberrant expression in many human cancers, and this expression is associated with disease progression and patient outcome. Because B7-H4 is not detected in the majority of normal tissues and cells (Choi et al. 2003), constitutive expression of B7-H4 on human cancers may play a role in tumor immune escape. Other studies also show that increased B7-H4 expression on tumor cells correlated with both decreased apoptosis and enhanced tumor growth (Salceda et al. 2005). This was confirmed in mice and even in mice without adaptive immunity (Salceda et al. 2005), suggesting that, besides the inhibitory roles to immune responses, B7-H4 on human tumors might act as a molecular shield to evade immunosurveillance. However, engagement of B7-H4 on EBV-transformed B cells or B-cell lymphoma cells induced apoptosis or cell cycle arrest, respectively (Park et al. 2009). Furthermore, B7-H4 expression is not limited to tumor cells. It was highly expressed on tumor-associated macrophages in the ascites of ovarian cancer patients, and those B7-H4+ macrophages suppressed tumor-associated antigen-specific T-cell immunity (Kryczek et al. 2006a). Silencing of B7-H4 by siRNA restored the function of macrophages to stimulate antigen-specific T cells and led to tumor regression in vivo (Kryczek et al. 2006a). In addition, B7-H4+ cells spontaneously

produce CCL22 chemokine that facilitate T regulatory cells (Treg) trafficking into tumor (Kryczek et al. 2007). Further studies showed that tumor-associated Treg can trigger APC, including macrophages, to produce IL-6 and IL-10, which are responsible for stimulating B7-H4 expression on macrophages in an autocrine manner (Kryczek et al. 2006b). In a recent study, B7-H4 was found to be upregulated on murine splenic macrophages stimulated by IL-10/TGF-beta and could induce Treg differentiation from CD4+ CD25- T cells in vitro (Cao et al. 2010). Besides, B7-H4Ig significantly promotes IL-10 production in mouse splenic macrophages in vitro (Shvets et al. 2009) and in the serum of ConA-treated mice (Xu et al. 2010). Therefore, a mechanistic link among IL-10, B7-H4, Treg, and macrophages with inhibitory effects might form a complicated suppressive network in the tumor microenvironment that can be targeted therapeutically.

Target Assessment

B7-H4 can be measured in the serum or other body fluids by ELISA (Simon et al. 2006) and on tissues using immunohistochemistry or FACS (Choi et al. 2003; Kryczek et al. 2006a, 2007; Krambeck et al. 2006; Zang et al. 2007). Such antibodies for human B7-H4 are commercially available for basic or clinical research. It is important to emphasize that the anti-B7-H4 antibodies binding to formalin/paraffin-fixed tissues for immunohistochemistry are also available (Tringler et al. 2005), which is helpful for large-scale retrospective study.

Role of the Target in Cancer

Rank: “unknown” to 10

Unknown to-1-2-3-4-5-6-7-8-9-10: 4

To date, studies regarding B7-H4 potentiate its inhibitory effects on the immune system and its significance in cancer diagnostics according to the clinical association and the aberrant expression pattern in human tumors. However, the real function of B7-H4 on cancer pathogenesis or development is pretty unclear. Further studies on animal tumor models and human patients, both mechanistically and therapeutically, could better elucidate the role of B7-H4 as a target in cancer.

High-Level Overview

Diagnostic, Prognostic, and Predictive

B7-H4 is overexpressed in several human tumors including ovarian, renal, prostate, breast, pancreatic, and lung cancers. In addition, several data suggest the pathogenic relevance of B7-H4 in tumors and its importance in diagnosis and/or prognosis.

In an initial study, 22 out of 26 ovarian carcinomas were found to express high levels of B7-H4, either in the cytoplasm or in the plasma membrane (Choi et al. 2003). Further studies with more samples also demonstrated the overexpression of B7-H4 protein in serous, endometrioid, clear cell ovarian carcinomas, and the relative absence in normal tissues as well as in most mucinous ovarian cancers or other benign gynecologic lesions (Simon et al. 2006). In addition, ovarian tumor-associated macrophages also express B7-H4, induced by tumor environmental IL-6 and IL-10 stimulation, and are responsible for its suppressive function (Kryczek et al. 2006a). In a follow-up study, Ilona et al. characterized that both Treg and macrophage B7-H4, but not tumor B7-H4, were negatively associated with patient outcome (Kryczek et al. 2007). Interestingly, sera B7-H4 is much higher in serous or endometrioid ovarian cancers than those in mucinous histotypes (Simon et al. 2006). However, whether and how serum B7-H4 is derived from tumor and/or other cells is still unknown. Several studies also suggest that B7-H4 could be a promising new biomarker for ovarian carcinoma and might improve the diagnosis, the prognosis, and the prediction of response to chemotherapy when used in combination with traditional tests (Simon et al. 2006).

B7-H4 protein is also highly expressed in breast cancer (Salceda et al. 2005; Tringler et al. 2005). In a large cohort study, B7-H4 was detected in 165 of 173 (95.4%) primary breast cancers and in 240 of 246 (97.6%) metastatic breast cancers, while there was minimal expression on normal breast epithelium (Tringler et al. 2005). In addition, B7-H4 staining intensity was greater in invasive ductal and lobular carcinomas than normal tissue (Tringler et al. 2005). However, similar to that found in high-risk uterine endometrioid adenocarcinomas, there was only a statistically significant adverse association between B7-H4 expression in invasive ductal carcinomas and tumor T-cell infiltration, while not in regard to tumor grade or stage (Tringler et al. 2005). A recent study indicates that B7-H4 might act as the downstream gene target of p70S6K, a serine/threonine kinase regulated by the PI3K/mTOR pathway, to control cell cycle, growth, and survival of breast cancer cells (Heinonen et al. 2008). In a haplotype analysis, B7-H4 polymorphisms are found to be associated with sporadic breast cancer risk and prognosis in Chinese Han women (Zhang et al. 2009).

In renal cell carcinoma (RCC), 59.1% (153 of 259) RCC tumor specimens exhibited strong high B7-H4 staining, and tumor cell B7-H4 expression was associated with adverse clinical and pathologic features, including constitutional symptoms; tumor necrosis; advanced tumor size, stage, and grade; and, most importantly, the poor survival of patients (Krambeck et al. 2006). In addition, 81.5% specimens showed strong B7-H4 staining in the tumor endothelial vasculature (Krambeck et al. 2006). Along with other markers like B7-H1, IMP-3, CXCR3, p53, and also B7-H3, B7-H4 has been associated with disease progression of RCC after nephrectomy (Crispen et al. 2008). Furthermore, RCC patients are also more likely to have detectable soluble B7-H4 compared with healthy controls, and the levels positively associate with tumor stage (Thompson et al. 2008). Thus, B7-H4 might be a potential prognostic marker and a target for immunotherapy of RCC.

B7-H4 expression was also observed in lung cancers (5 of 16) (Choi et al. 2003). Compared to B7-H3, B7-H4 showed little higher expression rate in non-small cell lung cancers (43% vs 37%) (Sun et al. 2006). However, there was significant positive correlation between B7-H4 and B7-H3 expression, and patients with high expression of B7-H3 or B7-H4 were more likely to have lymph node metastasis (Sun et al. 2006). In addition, B7-H4 and B7-H3 were found to be highly expressed (>90%) in human prostate cancer and associated with disease spread at the time of surgery and poor outcome (Zang et al. 2007). In pancreatic ductal adenocarcinoma (PDA), 92% of tumor sections were found to be B7-H4 positive, even greater than p53 (Awadallah et al. 2008). B7-H4 is a potential marker for diagnostic use for PDA in resected and endoscopic ultrasound-guided fine-needle-aspirated specimens despite the problematic expression of B7-H4 in benign/normal cells (Awadallah et al. 2008). In addition, nondividing brain tumor cells, a subset of brain tumor stemlike cells (Yao et al. 2008), and Brenner tumors (Yee et al. 2010) also have positive B7-H4 staining.

B7-H4 showed similar expression rate as B7-H1 in several cancers including ovarian (85% vs 87%) cancers, breast cancers (95% vs 75%), and lung cancers (31% vs 97%). However, B7-H4 is not detectable at all in melanoma (0 of 17), which is unlike the broader expression of B7-H1 (22 of 22) (Choi et al. 2003), suggesting a nonredundant potential of these B7 members in cancer diagnosis or therapy.

Therapeutics

Due to its potent inhibitory effects on both adaptive and innate responses, enhancement of B7-H4 effects has been the therapeutic target for autoimmune diseases (Azuma et al. 2009) and islet transplantation (Yuan et al. 2009), while the inhibition of B7-H4 effects is considered for bacterial infection (Zhu et al. 2009) and, potentially, for cancer immunotherapy. Although its expression is relatively absent in most normal tissues, B7-H4 is upregulated in several tumor cell types and is shown to be associated with disease progression and/or outcome. However, the detailed mechanisms of B7-H4 in tumor pathogenesis and development still remain unclear. In addition, further studies to identify the receptor and the key factor upstream or downstream of B7-H4 signaling will aid in understanding B7-H4 function. As such, developing blocking reagents like tagged B7-H4 proteins or anti-B7-H4 blocking antibodies as well as carefully evaluating their potential therapeutic effects will provide additional reagents for treating human cancer.

Preclinical Summary

Extensive preclinical studies support a role of B7-H4 molecule as a target for immunotherapy. These works have shown consistent results suggesting an inhibitory role of B7-H4 in immune responses, which is not restricted to T cells. Strategies

include injection of DNA, viral delivery, application of fusion protein or decoy protein, knockout animals and siRNA gene silence, etc. Moreover, several reports also suggest an additional role of B7-H4 signaling on tumor cells that might be important for tumor growth, either in vitro or in vivo. Further studies regarding the potential role of B7-H4 in tumor pathogenesis and development, especially the role in mice spontaneous tumor models and human cancer models, are of great value and increasing importance.

Clinical Summary

No clinical data is available so far for targeting B7-H4 in cancer immunotherapy.

Anticipated High-Impact Results

Identification of the Natural Receptor/Ligand for B7-H4

Identifying the counter-receptor for B7-H4 will aid in understanding B7-H4 function and also is important for targeting of B7-H4 pathway. Since B7-H4 shows inhibitory roles on both adaptive and innate immune responses, blockade the interaction of B7-H4 with its counter-receptor might represent an attractive approach in cancer immunotherapy.

Upstream or Downstream Pathways for B7-H4 Signaling

Characterization of B7-H4 signaling will definitely provide important information for B7-H4 function and help in rational design of immune modulation for cancer therapy.

The Potential Role and Mechanisms of B7-H4 in Tumor Pathogenesis

It is very unclear whether and how B7-H4 play a role in cancer development. Investigating the function and mechanisms of B7-H4 in tumorigenesis, and the potential therapeutic effects of B7-H4 pathway in tumor models, could better evaluate the significance of B7-H4 as cancer targets and make B7-H4 more likely to be important in cancer diagnosis and/or therapy.

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Anti-CD40/Anti-CD40L

4

David Kotlyar and Anthony Leonardi

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Abstract

CD40 receptor and its ligand CD40L have a multifactorial role in inflammation, angiogenesis, and tumor modulation. High levels of soluble CD40L alone are associated with increased angiogenesis and systemic inflammation. High levels of the ligand alone are also associated with poorer tumor outcomes. However, increased levels of both the ligand and receptor are associated with increased tumor antigen presentation by antigen presenting cells and have an antitumor effect. The combination of receptor stimulation with agonist antibody and IL-2

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has been shown to potentiate antitumor effect, increase NK cell activity against tumors, and delete regulatory T cells. In clinical trials with agonist antibody alone, there was a modest effect with patients with melanoma having the best outcomes, with a minority of patients having some responses. Cytokine release syndrome was the most common adverse event. Rational combinations of cytokines with the use of novel immunotherapies targeting CD40 may show promise in the treatment of neoplastic disease.

Keywords
CD40 ligand (CD40L) • CD40 receptor • CD40L • Chronic lymphocytic leukemia (CLL) • Dacetuzumab • Lucatumumab

Target

CD40L is a costimulatory molecule within the TNF superfamily and is also known as CD154. The molecule is canonically expressed by T Follicular Helper Cells in order to stimulate B cells, but has also been found on a variety of hematopoietic cells including basophils, macrophages, mast cells, NK cells, platelets, and nonhematopoietic cells including endothelial cells, epithelial cells, and smooth muscle (Hassan et al. 2015). However, it is mainly presumed to be a functional marker in CD4+ T cells and platelets despite having expression on these other tissues (Aloui et al. 2014).

When activated, both T cells and platelets will proteolytically cleave membrane-bound CD40L to make a soluble form called sCD40L (El Fakhry et al. 2012). The soluble form of the protein behaves like a proinflammatory cytokine, where it can agonize distant targets systemically (Aloui et al. 2014). As a testament to this behavior, high levels of sCD40L have been found in auto-immune conditions such as Crohn's disease, systemic lupus erythematosus, and inflammatory bowel disease (Matthies et al. 2006).

CD40L is able to bind additional receptors including αIIbβ3, α5β1, and αMβ2 integrins, which are expressed on platelets (El Fakhry et al. 2012). The ligation of these integrins precipitates further activation, and they are typically agonized by fibrinogen and Von Willebrand factor (El Fakhry et al. 2012). CD40L has been implicated in the diseases of atherosclerosis, hypercholesterolemia, and unstable angina as high serum levels have been observed in individuals with the conditions (Matthies et al. 2006).

Biology of Target

Functionally, CD40L promotes inflammation by agonizing its target CD40 on a multitude of cell types. It is upregulated in CD4+ cells following TCR stimulation and promotes both an innate (Macrophages) and adaptive (B Cell) response. The

soluble form is released by platelets and T Follicular Helper cells after their activation and contributes to thrombosis and inflammation (Aloui et al. 2014). Soluble CD40L is implicated in transfusion-related acute lung injury (TRALI) after its release by transfused platelets, thereby causing a systemic proinflammatory and prothrombotic effect (Aloui et al. 2014). The promiscuous binding the ligand has between different receptors expressed on platelets (integrins) and B cells (CD40) exemplifies the ligand's dual role (El Fakhry et al. 2012). Outside of the physiological role, it contributes to vascular disease through several mechanisms, including decreased NO production and the induction of tissue factor (TF), which promotes thrombus formation (Aloui et al. 2014). CD40L in the membrane bound form causes NFk-B activation in endothelial cells, the production of proinflammatory cytokines, and the production of matrix metalloproteinases (Aloui et al. 2014).

It is important to consider the mechanisms contributing to proliferation for each histology given the diverse role of the ligand and its receptor not only between cell types, but also potentially between patient's tumors. TNF family members are known not only to trigger apoptosis and differentiation but also to promote tumor survival and proliferation on the very same cell types before the cell's transformation into cancer (Chen et al. 2010). Likewise, a single TNF molecule may promote tumor death, but also promote a change in the immunological compartment in the microenvironment that dampens T cell response (Kerkar et al. 2013; Klebanoff et al. 2016).

Once introduced within the tumor microenvironment, CD40L may have different effects on different cell types. APCs may become primed to secrete chemokines and cytokines and upregulate MHC molecules (Hassan et al. 2015). Platelets and tumor stroma may become activated to increase clotting and inflammation (Hassan et al. 2015).

Target Assessment

As an example, for protocols with transfection of CD40L to CLL cells, patients received a single dose of autologous CLL cells that had been modified to express the ligand and IL-2 through electroporation or adenovirus (Okur et al. 2011). Each patient received a fixed dose of autologous cells expressing IL-2, then subcutaneous injections of cells with increasing levels of expression of CD40L (Okur et al. 2011). To evaluate the effect of the CLL vaccination, flow cytometry of the WBC populations by blood was performed before and following vaccination and compared (Okur et al. 2011).

Role of the Target in Cancer

CD40 Ligand/Antibody

All cancers: **7/10**

Diagnostic, Prognostic, and Therapeutic Potential of CD40L

Prognostic Aspects

The expression of CD40 ligand in some tumors has been correlated with a lower survival, including that seen in melanoma and renal cell carcinoma (Hassan et al. 2015). In melanoma, there were no differences in prognostic criteria but did have a significantly shorter progression free survival (van den Oord et al. 1996) (see Fig. 1). Overall 41 of 71 cases showed CD40 positivity, and positivity was generally observed in the cytoplasm with less staining seen in cellular membranes (van den Oord et al. 1996).

For renal cell carcinoma, out of 24 tumor specimens, CD40 ligand expression was directly correlated with disease stage in patients (Bussolati et al. 2002). Tumor samples were also shown to release soluble CD40 ligand, which promoted the activity of the pro-angiogenic protein platelet-activating factor (PAF) (Bussolati et al. 2002). Lung, B cell lymphomas, and ovarian tumors have also been shown to have increased soluble CD40L.

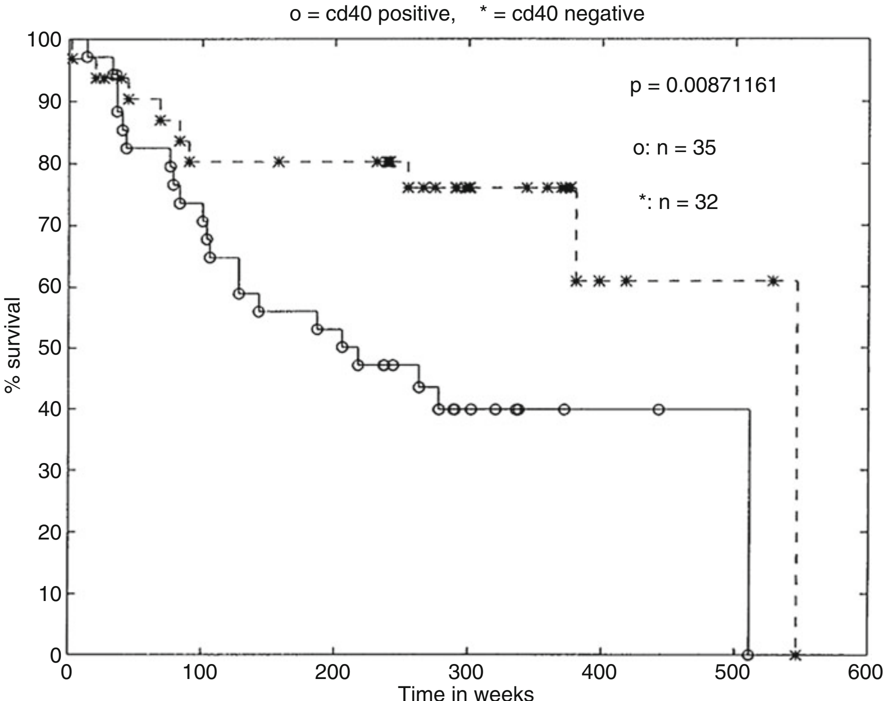

Fig. 1 Tumor free survival in 35 metastatic melanoma patients expressing CD40 in the vertical growth phase (O) compared with 32 patients lacking CD40 (*) shows a significantly shorter tumor-free survival for the first group of patients ($P < 0.01$) (van den Oord et al. 1996. Reproduced with permission)

However, the CD40 receptor and CD40 ligand (also known as CD154) has been shown to have both pro- and antitumor activity. The increased expression of soluble CD40L may in fact be due to downregulated expression of membrane-bound CD40, which may induce tumor resistance to CD40L-mediated killing (Hassan et al. 2015). In gliomas, expression of CD40/CD40L was inversely correlated with disease stage with stage III gliomas showing higher amounts of mRNA for CD40/CD40L (Chonan et al. 2015). High expression of CD40 and CD40L was also associated with longer overall survival (Chonan et al. 2015). Benefit was also seen in high grade serous ovarian cancer as well as low grade serous, but not serous borderline ovarian tumor cells.

Increased expression of CD40L also was correlated with increased programmed death receptor ligand-1 expression (Zippelius et al. 2015). In particular, monocytes and macrophages showed upregulation of PD-L1 with exogenous CD40L (Zippelius et al. 2015). Inhibition of PD-L1 along with CD40L resulted in synergistic killing of colon and breast tumor xenografts (Zippelius et al. 2015).

CD40L has been hypothesized to have similar function to the pro-death domains of Fas and TNF superfamily ligands such as TNF-related apoptosis-inducing ligand (TRAIL) (Eliopoulos et al. 2000). Interestingly, when carcinoma cells were exposed to a monomer ligand to CD40, cells were unaffected but when exposed to a trimer, there was CD40L-mediated killing (Eliopoulos et al. 2000). The critical step may involve CD40L (CD154) crosslinking, and the relative amounts of CD40L may be important for the initiation of cell killing (Eliopoulos et al. 2000). In addition, the supplementation of an antibody to CD40 with IL-2 or IL-15 has been shown to mediate increased regulatory T cell deletion, enhancing the tumoricidal effect of an antibody to CD40L (Weiss and Wiltout 2014).

The interaction between CD40L and CD40 is also crucial for immune system function and may also thus play a role in prognosis. In particular, this interaction is important for B cell class switching B and T cell proliferation as well as activation of antigen presenting cells (Hassan et al. 2015). There is also NK cell activation through indirect activity on APCs (Hassan et al. 2015).

Therapeutic Aspects

Preclinical

Ligands against CD40, including antibodies against CD40, have been shown to have indirect tumoricidal activity in mouse models, including mechanisms such as the activation of antigen presenting cells causing upregulation of tumor killing macrophages (Vonderheide and Glennie 2013). There have been a multitude of murine studies showing efficacy of both CD40 ligand and antibodies against CD40 having antitumor effect.

In a mouse model of lymphoma of BCL1, A31, A20, and EL4 tumors, an agonistic antibody against CD40 was shown to have dramatic antitumor activity, eliminating IgM production from the tumor cells and curing mice with higher levels of tumor inoculum (Tutt et al. 2002). Another study of lymphoma revealed enhancement of cancer vaccine efficacy with the use of anti-CD40 in combination with a

cancer idiotype antigen, also dependent on CD8+ effector T cell function (Carlring et al. 2012). In a study of a murine model of CLL, the use of a CD40 agonist significantly enhanced the efficacy of an anti-CD20 antibody, which normally elicited weak responses alone (Jak et al. 2011).

In a model of melanoma, the use of anti-CD40L and CpG nucleotides conjugated to pegylated liposomes showed excellent efficacy with a good safety profile, when directly injected into tumor (Kwong et al. 2011). For a mesothelioma model, injection of Il-2 was found to have good antitumor efficacy of local tumors, but the addition of anti-CD40 was required for responses that eradicated distal tumors (Jackaman and Nelson 2012). The combination of IL-2 and anti-CD40 was also shown to be vital, as IL-2 also enhanced NK cell infiltration of tumor, and when animals were depleted of NK cells, efficacy was reduced, and tumors frequently recurred, indicating the importance of this cell subset in maintaining cure (Jackaman et al. 2012). The interplay between CD4+ T cells and tumor-specific CD8+ T cells, as well as maturation of tumor-specific CD8+ T cells (with upregulation of CD44), was crucial for eradication of distal tumor growth (Jackaman and Nelson 2012). A separate study examining rational combinations of 14 different immune interventions found the specific combination of anti-CD40, IL-2, and IL-12Fc was most efficacious in causing regression of adenocarcinoma caused by SV40 in TRAMP mice (Bransi et al. 2015).

Inhibitory cells within tumors may dampen the effect of anti-CD40, and so complementary techniques have shown promise. In one model of fibrosarcoma, the combination of an anti-VEGF agent, sunitinib, and anti-CD40 was shown to enhance effector CD8+ T cell infiltration of tumors (Hooren et al. 2016). In addition, the combination of checkpoint inhibitors such as anti-PD1 in combination with CD40 ligands has shown promise. In a pancreatic cancer murine model, the combination of CD40 agonist antibodies with anti-PD1 resulted in improved T cell infiltration and separation of tumor from normal pancreatic tissue (Luheshi et al. 2016). In addition, a recent report has demonstrated that the combination of chemotherapy with gemcitabine and nab-paclitaxel and agonistic antibody against CD40 was shown to cause durable regression of pancreatic tumors in mice (Byrne and Vonderheide 2016). Neither agent alone was able to achieve such regression (Byrne and Vonderheide 2016).

Clinical

There have been over 10 clinical trials using CD40L as an antitumor agent (see Table 1) (Hassan et al. 2015). The first treatment, a phase I dose-escalation study with recombinant CD40L in 32 patients, showed excellent tolerability, with the most significant adverse event being elevated transaminases (Vonderheide et al. 2001). Out of 32 patients with a variety of solid tumors and non-Hodgkin lymphoma (NHL), 12 had stable disease, one had a partial response, and one had a durable remission (Vonderheide et al. 2001).

Another phase I study transfused genetically modified autologous T cells to patients with chronic lymphocytic lymphoma (CLL)(Wierda et al. 2010). In their first study, nine patients had their own cancerous B cells transduced with a

Table 1 Clinical trials utilizing agents influencing the CD154/CD40 system

Treatment	Cancer type	No. of patients[a]	Clinical response
CD 154			
Recombinant CD 154	Advanced solid tumors, inter. or high grade NHLs	32	CR (1 patient) PR (1 patient) SD (12 patients)
Ex vivo transduction of chimeric CD 154 (human/ murine)	CLL	9	Decreased ALC and lymphadenopathy (most patients)
AdV[b] -chimeric CD154 (human/murine)	CLL	15	PR (3 patients) SD (7 patients) Durable response (6 patients[c])
Oncolytic virus-CD154	Adv. solid tumors	9	SD (3 patients) PD (3 patients) Anti-tumor effect (5 of 6 evaluable patients)
Anti-CD40 mAbs			
CP-870,893	Adv. solid tumors	29	PR (14% of patients) SD (24% of patients)
	Adv. solid tumors	27	SD (26% of patients)
Dacetuzumab	MM	44	SD (9 patients)
	NHL	50	CR (1 patient) PR (5 patients) SD (13 patients)
	CLL	12	SD (5 patients)
Lucatumumab	MM	28	PR (4% of patients) SD (43% of patients)
	CLL	26	PR (1 patient) SD (17 patients)

Reproduced with permission Hassan et al. (2015)
Abbreviations: *ALC* absolute lymphocyte count, *CR* complete response, *PD* progressive disease, *PR* partial response, *SD* stable disease
[a]No. of patients enrolled
[b]Replication-deficient adenovirus
[c]These patients did not require additional treatment for over 6 months (two patients even for more than 12 months)

replication defective adenovirus to express a CD40L-like membrane protein (ISF35, a humanized membrane stable modified version) (Wierda et al. 2010). Two patients had a sustained reduction in lymph node size which lasted several months after multiple infusions of the ISF35 augmented cells (Wierda et al. 2010).

In the second phase I trial for the treatment of chronic lymphocytic leukemia (CLL), a disease replication-deficient adenovirus coding for a ISF-35 (humanized CD40L-like particle) was directly injected into tumor-containing lymph nodes (Castro et al. 2012). The phase I study was a typical 3+3 dose-escalation study, and lymph nodes in the axilla being more than 2×2 cm were injected (Castro

et al. 2012). The majority of patients showed an increase in the number of T cells, and of the fifteen patients in the study, six did not need further treatment for 6 months and three had a partial response (Castro et al. 2012).

Another phase I study explored the use of a CD40L expressing adenovirus in multiple advanced solid tumors (Pesonen et al. 2012). In the trial, the researchers constructed a "double-targeted" adenovirus with the hTERT promoter which expressed CD40 ligand (Pesonen et al. 2012). Nine patients had the viral payload injected directly into the tumor, and six of the nine were treated with concomitant cyclophosphamide (Pesonen et al. 2012). Of these nine patients, six were evaluable by CT (evaluated by RECIST criteria) prior and after treatment, and three responded with stable disease while three had progressive disease (Pesonen et al. 2012). The three other patients had their disease assessed with PET/CT, one showed mixed response (MMR by PERCIST criteria), and two had stable disease (SMD) (Pesonen et al. 2012).

Another phase I trial examined the use of the CD40 antibody CP-870,893 on a variety of advanced solid tumors including melanoma (15 patients), non-small cell lung cancer (NSCLC – five patients), sarcoma (three patients), and six other patients with a variety of other advanced solid malignancies (Vonderheide et al. 2007). Limiting toxicities included cytokine release syndrome and grade three headache (Vonderheide et al. 2007). While a previous phase 1 trial from the same group did not show efficacy (Ruter et al. 2010), this trial showed that, of the patients with melanoma, 4 of 15 (27%) had a partial response, and stable disease was observed in 7 of 29 patients (24%) (Vonderheide et al. 2007). A third trial with CP-870,893 combined with carboplatin and paclitaxel showed some activity in multiple advanced solid tumors (Vonderheide et al. 2013). Thirty-two patients were treated, 25 of which had advanced melanoma (Vonderheide et al. 2013). The most common adverse effects included cytokine release syndrome, fatigue, cytopenias, and nausea (Vonderheide et al. 2013). There was one case of fatal intracranial hemorrhage (Vonderheide et al. 2013). In terms of efficacy, six of 30 evaluable patients (20%) had a partial response (Vonderheide et al. 2013). A fourth trial with the compound and gemcitabine was a phase I trial for pancreatic ductal adenocarcinoma (Beatty et al. 2013). Of 22 chemotherapy-naïve patients with PDA, 85% had cytokine release syndrome, with other side effects including cytopenias, nausea, vomiting, and liver test abnormalities (Beatty et al. 2013). Four patients had partial response as per RECIST criteria and 11 had stable disease (Beatty et al. 2013).

A separate trial assessing the safety and efficacy of Dacetuzumab, another monoclonal antibody raised against the CD40 receptor, showed good safety in patients with multiple myeloma (Hussein et al. 2010). Toxicities related to treatment included cytokine release syndrome, elevated liver enzymes, ocular inflammation, and headache (Hussein et al. 2010). Use of premedications including steroids reduced cytokine release syndrome frequency from 56 to 17% (Hussein et al. 2010). Nine of 44 patients (~20%) had stable disease, while the remainder had progressive disease (Hussein et al. 2010). The same antibody was also tested in patients with non-Hodgkin's lymphoma (NHL) and showed a good safety profile with the most significant adverse effects being cytokine release syndrome,

cytopenias, ocular inflammation, and pleural effusion in several (Advani et al. 2009). Overall, there was a response rate of six patients out of 50 (12%) (Advani et al. 2009). Stable disease was observed in 13 of 50 patients (26%) (Advani et al. 2009). Activity was also demonstrated in chronic lymphocytic leukemia (Furman et al. 2010).

Lucatumumab, a monoclonal, fully human antibody against CD40, has also been tested in several phase 1 trials (Bensinger et al. 2012). In the first trial of multiple myeloma patients, 28 patients received 1 to 2 cycles of treatment with the antibody (Bensinger et al. 2012). In terms of adverse events, most consisted of infusion reactions, with the most serious adverse consisting of cytopenias, elevated liver enzymes, electrolyte abnormalities, and elevated lipase (Bensinger et al. 2012). Of 23 evaluable patients, one had a partial response, while 12 had stable disease (Bensinger et al. 2012). The median time of disease stabilization was 61 days, while five patients had disease stabilization of greater than 5 weeks (Bensinger et al. 2012). In a separate trial for patients with CLL, 26 patients with relapsed disease received weekly lucatumumab for four weeks (Byrd et al. 2012). Of these patients, four developed grade 3 and 4 elevated amylase and lipase levels (Byrd et al. 2012). In terms of efficacy, 17 patients (65%) had stable disease for a mean time of 76 days, while one patient had partial response with duration of 230 days (Byrd et al. 2012).

Conclusion

Rational combinations of cytokines with novel immunotherapies hold great promise in not only slowing tumor growth and promoting increased disease-free survival, but also potentially causing durable immunological responses, which may dramatically improve and lengthen both quality of life and overall survival in metastatic cancer with historically dismal prognoses. Translating advances such as the use of CD40 ligand will be critical in the continuation of steady improvements in meaningful advancements in clinical oncology.

High Impact Points

- CD40 ligand and CD40 have a multifactorial role in inflammation, angiogenesis, and tumor modulation.
- High levels of CD40 ligand together with high levels of nonmutated CD40 receptor were associated with longer survival in patients with gliomas and ovarian cancer.
- The concomitant administration of CD40 agonist antibody with cytokines, specifically IL-2 and IL12-Fc, has been shown to strongly potentiate antitumor effect in animal models.

- The combination of CD40 agonists with antiangiogenic agents, as well as with immunotherapy with checkpoint inhibitors, has also shown promise in tumor models.
- In clinical trials with CD40 agonists alone, limited clinical benefit has been observed in patients with the exception of melanoma.
- Rational combinations of cytokines with agonists targeting CD40 may hold significant promise for the future treatment of tumors.

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5 Anti-Idiotype Antibodies

John M. Timmerman

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Abstract

The tumor-specific immunoglobulin (Ig) expressed by B-cell malignancies can serve as a target for passive or active immunotherapy. This target, referred to as the "idiotype" (Id), is composed of the unique antigenic determinants in the variable regions of the clonal Ig heavy and light chains expressed by the tumor cells (Timmerman and Levy, Clin Lymphoma 1:129–139, 2000). This protein target has the unusual nature of being unique in every B-cell cancer (Fig. 1). The human immune system contains billions of different B cells, each with a unique Ig polypeptide sequence resulting from the physiologic rearrangement and somatic mutation of gene segments during B-cell differentiation. The Ig is expressed on the B-cell surface to serve as an antigen receptor (B-cell receptor, BCR).

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Introduction to B-Cell Idiotype as a Cancer Antigen Target

The tumor-specific immunoglobulin (Ig) expressed by B-cell malignancies can serve as a target for passive or active immunotherapy. This target, referred to as the "idiotype" (Id), is composed of the unique antigenic determinants in the variable regions of the clonal Ig heavy and light chains expressed by the tumor cells (Timmerman and Levy 2000). This protein target has the unusual nature of being unique in every B-cell cancer (Fig. 1). The human immune system contains billions of different B cells, each with a unique Ig polypeptide sequence resulting from the physiologic rearrangement and somatic mutation of gene segments during B-cell differentiation. The Ig is expressed on the B-cell surface to serve as an antigen receptor (B-cell receptor, BCR). When a normal B cell undergoes malignant transformation, these sequences are maintained by the malignant clone and can thus serve as a tumor-specific antigen (Fig. 1). Mature B-cell malignancies such

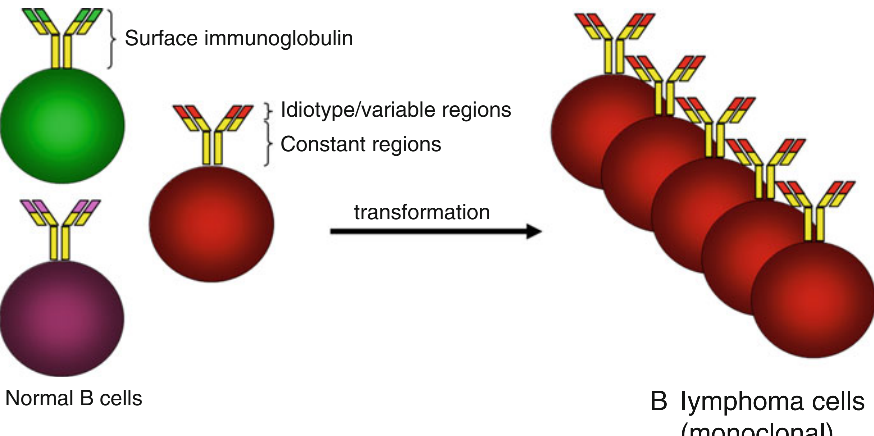

Fig. 1 Idiotype as a tumor antigen specific for B-cell lymphoma. "Idiotype" (Id) is the collection of unique antigenic determinants in the variable regions of a B-cell clonal immunoglobulin. In B-cell malignancies, the Id can serve as a tumor-specific antigen target

as non-Hodgkin lymphomas (NHL), chronic lymphocytic leukemia (CLL), and multiple myeloma are the most common B-cell cancers bearing this target. Despite the potential for exquisite tumor specificity and a few spectacular successes in cases of NHL treated with monoclonal anti-Id antibodies or Id protein vaccines, tumor Id represents a technically challenging target given the need to develop individualized therapies for each patient's tumor.

Biology of B-Cell Receptor Idiotypes

Mature normal B cells and their malignant counterparts NHL and CLL express idiotypic Ig on their cell surfaces, while antibody-secreting plasma cells and their malignant equivalent multiple myeloma express the protein only in the cytoplasm. Id proteins expressed by B-cell malignancies contain structures that can be recognized by antibodies as well as by $CD4^+$ and $CD8^+$ T cells, the latter in the form of peptides bound to class II and class I MHC molecules, respectively (Fig. 2) (Timmerman and Levy 2000; Timmerman 2004; Weng et al. 2011). Cross-linking of the surface Ig on a normal B cell by antigen initiates a complex signal transduction cascade that results in either B-cell activation or apoptosis, depending on the activation state of the cell and the timely delivery of appropriate "co-stimulatory"

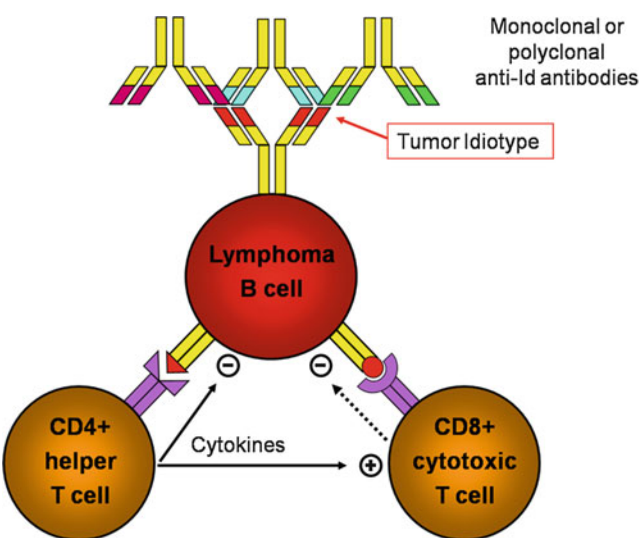

Fig. 2 Potential for multiple levels of tumor attack by Id-directed immunotherapies. Passive anti-Id monoclonal antibodies require tumor Id expression at the cell surface. Active Id vaccination can induce polyclonal anti-Id antibodies as well as CD4+ and CD8+ T cells recognizing Id peptides in association with MHC molecules on the cell surface. *MHC* major histocompatibility complex

signals (Carey et al. 2000; Niiro et al. 2002). Anti-Id antibodies can similarly cross-link Ig/Id on the surface of B-cell NHLs and result in growth inhibition and/or apoptosis (Eeva and Pelkonen 2004; Vuist et al. 1994; Varghese et al. 2009). BCR signal transduction pathways that appear to underlie this response are outlined in Fig. 3 and begin with the association of surface Ig with several tyrosine kinases, including syk, lyn, and btk, and activation of the phospholipase C, RAS, and PI3 kinase pathways. In certain NHL cells, this can also result in G1 cell cycle arrest of cell death via apoptosis, but the precise mechanisms for most tumor types are not fully understood. Anti-Id antibodies may also recruit immunologic effectors to destroy tumor cells indirectly via antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity.

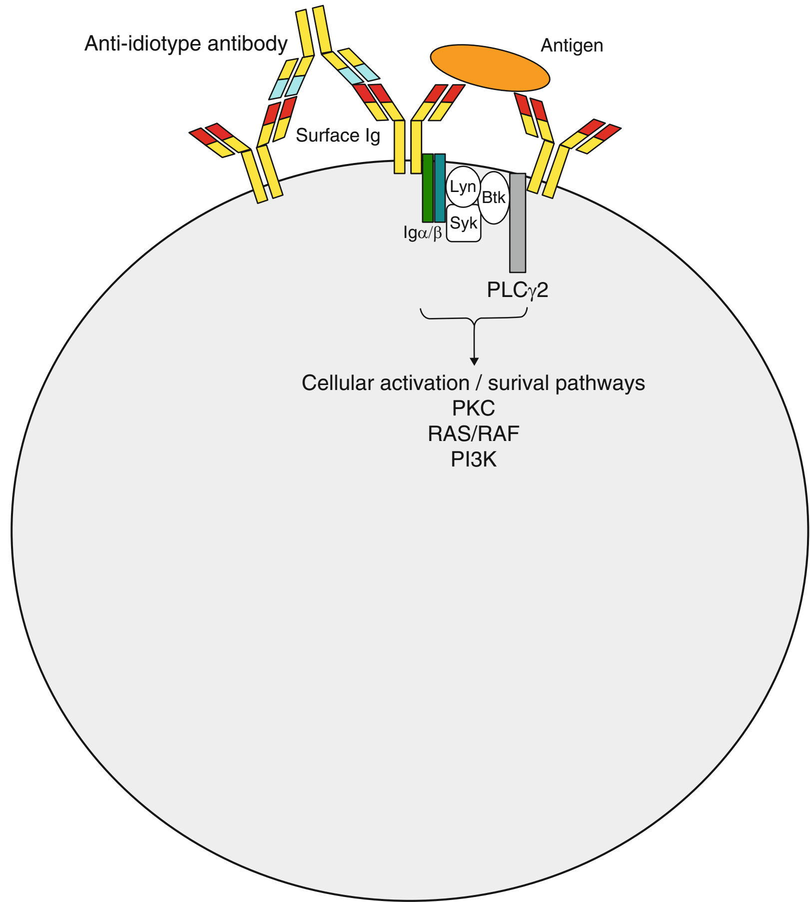

Fig. 3 Signal transduction through the B-cell receptor. Like antigen, anti-Id antibodies engage surface Ig, leading to signal transduction events that can promote growth arrest or apoptosis

Target Assessment

Tumor Id is readily measured on the tumor cell surface as Ig by flow cytometry or immunohistochemistry, but these tests measure the conserved framework regions of the Ig, not the truly tumor-specific idiotypic regions. These are best characterized by complete sequencing of the tumor's Ig variable region genes. While this yields a tumor-specific marker that can be used to monitor lymphoma therapy via PCR (Davis et al. 1998), it is not yet possible to predict the biologic behavior of individual tumor Id proteins in response to anti-Id therapy.

Role of the Target Antigen in Cancer

There are currently no approved Id-directed therapies for B-cell cancers, and thus the role of this target in modern cancer therapy remains unknown. As discussed below, several recent phase III trials of Id vaccination in follicular lymphoma yielded disappointing results. However, given the combined prevalence of NHL, CLL, and myeloma, Id-directed therapy could potentially gain a role as a tumor-specific therapy with few off-target side effects.

High-Level Overview

Therapeutics

There have been four different Id vaccines brought forward by commercial entities for phase II and phase III clinical testing, though none have achieved marketing approval: MyVax® (Genitope, Inc.), FavId/Mitumprotimut-T®(Favrille, Inc.), BiovaxID® (Accentia/Biovest, Inc.), and IdioVax® (CellGenix, Inc). Only the latter two remain under active development.

Preclinical Summary

Id-directed therapies have been tested in patients since 1981, so clinical results (described below) have largely trumped preclinical findings in terms of therapeutic relevance (Timmerman and Levy 2000). Nonetheless, several recent preclinical results bear mentioning. First, monoclonal anti-Id antibodies continue to display the potential for strong direct antiproliferative effects against B-cell lymphomas, in contrast to most other anti-lymphoma antibodies, which function largely through ADCC (Varghese et al. 2009). Second, studies in syngeneic mouse lymphoma models have shown that alternative chemical conjugation techniques for linking Id to carrier proteins can markedly improve the ability of Id-KLH vaccines to elicit anti-idiotypic antibody and T-cell responses (Betting et al. 2008; Kafi et al. 2009). Thus,

both passive and active anti-Id immunotherapies appear to still hold untapped value for therapy against B-cell malignancies.

Clinical Summary

Passive Anti-Id Monoclonal Antibody Therapy

The first successful therapeutic use of monoclonal antibodies against human cancer was carried out by Ronald Levy and colleagues at Stanford University, who treated B-cell lymphoma with custom-made murine antibodies recognizing tumor Id (Miller et al. 1982). Stevenson and colleagues had laid the groundwork for this innovation through demonstration that Id could serve as a vulnerable antibody target by using polyclonal anti-Id antiserum to treat a guinea pig B-cell leukemia (Stevenson et al. 1977). Levy moved this principle forward into humans (Hall 1997) by developing the technique of fusing patient lymphoma cells with myeloma cells (rescue hybridization), yielding stable cell lines secreting large amounts of tumor-specific Id protein (Levy and Dilley 1978), which was then used to immunize mice and generate mouse antihuman Id monoclonal antibodies. This tailor-maid therapy worked spectacularly well in the first patient treated, a man with advanced, refractory follicular lymphoma, resulting in a rapid complete remission (Miller et al. 1982). Over a period of 12 years, 34 patients were treated with 45 of these custom-made mouse or rat antibodies, and objective tumor regressions were seen in 68% of cases (Timmerman and Levy 2000; Davis et al. 1998; Maloney et al. 1992). In eight cases, these regressions were complete, and some patients have been apparently "cured," as they remain tumor-free without additional treatments many years later. The mechanism of action for these murine anti-Id antibodies is believed to have been growth-inhibitory signal transduction resulting from BCR cross-linking, based on efficacy correlating with the ability of the antibodies to elicit tyrosine phosphorylation in target tumor cells (Vuist et al. 1994). IDEC Pharmaceuticals (San Diego, CA, USA) was founded with the intent of commercializing this procedure. A panel of 199 anti-Id mAbs recognizing the Id of 67 follicular lymphomas was generated, and 20 of these recognized more than one lymphoma (Miller et al. 1989). However, these 20 "shared anti-Ids" recognized only one-third of B-cell lymphomas and thus did not avoid the overall need for customized therapy. Moreover, several patients recurred with tumor cell populations that no longer expressed the target of the therapeutic antibody because of downregulation or mutation of their surface Id (Maloney et al. 1992; Meeker et al. 1985). Eventually, the patient-specific anti-Id approach proved to be prohibitively complex and expensive and was abandoned in favor of developing the anti-CD20 antibody rituximab (Reff et al. 1994). Despite the revolutionary efficacy achieved by rituximab in the treatment of B-cell lymphoma, it is intriguing to consider that customized anti-Id antibodies appear to have been even *more* effective against follicular lymphoma. Thus, many believe that this approach should be revisited if and when it becomes more technologically feasible.

Active Vaccination Against Id in Lymphoma

An alternative to passive administration of anti-Id sera is active vaccination with tumor-derived Id proteins, first pioneered in murine plasmacytomas by Lynch et al. (1972). Id protein can be isolated from autologous tumor cells (or the serum of myeloma patients) and formulated into a custom-made therapeutic tumor vaccine. The Id is usually chemically conjugated to the highly immunogenic carrier protein keyhole limpet hemocyanin (KLH) rendering it more immunogenic and injected subcutaneously along with an immunologic adjuvant to evoke tumor-specific antibody and T-cell responses. Active Id vaccines offer the potential to elicit multiple levels of attack against lymphoma cells, including a polyclonal antitumor antibody response and $CD4^+$ and $CD8^+$ T cells (Fig. 2). This broad antitumor response elicited by active vaccination might have advantage over passive antibody therapies, overcoming Id-escape variants and also encompassing immunologic memory, serving to carry out ongoing surveillance against tumor cells.

Results from phase I/II clinical trials of Id immunization for follicular lymphoma using hybridoma-derived Id have included the induction of tumor-specific anti-Id immune responses that correlate with improved disease-free and overall survival (Hsu et al. 1997; Kwak et al. 1992), achievement of molecular complete remissions (bcl-2 negative PCR status) and favorable progression-free survival (PFS) using Id-KLH plus granulocyte-macrophage colony-stimulating factor (GM-CSF) (Bendandi et al. 1999; Inoges et al. 2006; Barrios et al. 2002; Yanez et al. 2008), and durable tumor regressions following immunization with Id protein-loaded autologous dendritic cells (Hsu et al. 1996; Timmerman et al. 2002).

However, limitations of the rescue hybridoma method include a production failure rate as high as 15%, the need for viable tumor cells for cell fusion, nonuniformity of the Id product (IgG, IgM, or other isotypes expressed by the tumor), and the instability of Id secretion by tumor hybridomas over time. An alternative technique, “molecular rescue,” employs PCR amplification of the tumor-specific variable region Ig sequences from small numbers of tumor cells for cloning into expression vectors to produce recombinant Id proteins. Such recombinant Id proteins have been produced in a colorful collection of expression systems including mammalian cells (Timmerman et al. 2009), insect cells (Redfern et al. 2006), bacteria (Bertinetti et al. 2006a, b), tobacco plants (McCormick et al. 1999), and cell-free in vitro translation systems (Kanter et al. 2007), greatly decreasing the time required to produce tumor Id proteins.

Despite promising results in the above early phase clinical trials, the performance of first-generation Id vaccines in controlled clinical trials has been disappointing. Table 1 summarizes the three large randomized phase III clinical trials of Id-KLH plus GM-CSF vaccination performed in patients with advanced stage, indolent follicular NHL, each with the aim at prolonging the time to progression (TTP) after initial systemic cytoreductive therapy. All three enrolled previously untreated patients (prior local radiation therapy to a single site was permitted), but there were important differences between the trials, including the method of Id production, the pre-vaccine cytoreduction therapy employed, and the clinical response requirement to be eligible for vaccination.

Table 1 Phase III trials of idiotype vaccination for follicular lymphoma

	Genitope	Favrille	NCI/Biovest
Year opened/ reported	2000/2008	2004/2009	1999/2009
Randomized	$n = 287$	$n = 349$	$n = 117$
Method of Id production	Mammalian cells (recombinant IgG3)	Insect cells (recombinant IgG1)	Rescue hybridoma (IgG or IgM, according to tumor)
Cytoreductive therapy	CVP × 8	Rituximab	PACE to best response
Response requirement	CR, CRu, or PR	CR, PR, or stable	CR or CRu
Randomization: vaccine vs. control	2:1	1:1	2:1
Vaccine	Id-KLH + GM-CSF × 7	Id-KLH + GM-CSF × 6, then every 3 months	Id-KLH + GM-CSF × 5
Control therapy	KLH + GM-CSF	Placebo + GM-CSF	KLH + GM-CSF
Results	No advantage to vaccine	No advantage to vaccine	Advantage to vaccine
	Median TTP 19.1 vs. 23.3 months	Median TTP 9.0 vs. 12.6 months (no difference when adjusted for prognostic factors)	Median TTP 44.2 vs. 30.6 months
	Longer TTP in those with anti-Id antibody responses		Improvement limited to IgM+ tumors
References	Hsu et al. (1997)	Bendandi et al. (1999)	Barrios et al. (2002)

NCI US National Cancer Institute; *XRT* radiotherapy; *LN* lymph node; *FNA* fine needle aspiration; *BM* bone marrow; *CVP* cyclophosphamide, vincristine, and prednisone; *PACE* prednisone, doxorubicin, cyclophosphamide, and etoposide; *CR* complete response; *CRu* complete response unconfirmed; *PR* partial response; *Id-KLH* idiotype coupled to keyhole limpet hemocyanin; *GM-CSF* granulocyte-macrophage colony-stimulating factor; *TTP* time to progression

The Genitope trial utilized recombinant Id protein produced in mammalian cells (MyVax®), traditional CVP chemotherapy, and vaccinated patients who had achieved at least a partial response to therapy (Levy et al. 2008). However, there was no TTP advantage found to Id-KLH vaccine vs. control. As observed in previous phase II trials (Hsu et al. 1997; Weng et al. 2004), subjects mounting anti-Id antibody responses had significantly improved progression-free survival, but whether this reflects a therapeutic effect or underlying intrinsic host or tumor differences remains unknown.

The Favrille trial employed recombinant Id protein produced in insect cells (FavId/Mitumprotimut-T®), rituximab anti-CD20 mAb cytoreduction, and required only stable disease or better prior to vaccine therapy (Freedman et al. 2009). Again, there was no TTP advantage among Id-KLH vaccine vs. control patients when adjusted for clinical prognostic factors. It has been theorized that rituximab might have severely impaired the ability to mount anti-Id antibody responses, thus eliminating an important component of anti-Id immunity.

The most recently reported trial, begun initially at the US National Cancer Institute (NCI) and then sponsored by Biovest, Inc., was the only trial to use Id produced by the traditional rescue hybridoma method (BiovaxID®). This vaccine had been piloted by Kwak and colleagues at the NCI, who found that inclusion of locally administered GM-CSF with Id-KLH vaccines elicited T-cell anti-Id immunity (Bendandi et al. 1999; Kwak et al. 1996). This phase III trial also differed from the other two in requiring patients to have achieved complete remission after aggressive chemotherapy in order to be eligible for vaccination. These requirements contributed to delayed accrual to the trial, which was terminated well before meeting its pre-stated accrual goal of 250 randomized subjects; only 117 subjects were randomized among 234 enrolled. Among those randomized to Id-KLH vaccine, median TTP was prolonged by 13.6 months (44.2 vs. 30.6 months) (Schuster et al. 2009). However, in subsequent analyses, it was found that only those vaccinated patients whose tumor Id was an IgM had improved TTP; those whose tumor Ids were IgG did not have prolonged tumor-free survival (Schuster et al. 2010). Earlier studies in mice had suggested that IgM Id proteins were immunogenic, while IgG Id proteins could be tolerogenic (under adjuvant-free, unconjugated conditions) (Reitan and Hannestad 2002), raising the possibility that the isotype of the Id protein immunogen might influence the outcome of therapeutic Id vaccination in humans. However, this association, the result of an apparently unplanned retrospective analysis, derives from a small sample size and requires confirmation in an independent data set (Inoges et al. 2006; Weng et al. 2004). KLH conjugation and coadministration of the GM-CSF adjuvant should have negated the influence of the Id isotype on anti-Id immune responses. Furthermore, the lack of significant benefits in IgG Id-KLH-vaccinated subjects in this study is incongruous with the impressive previous phase II results with this vaccine at the NCI (Bendandi et al. 1999), which included IgG+ subjects.

The overall conclusion that can be drawn from these three randomized phase III trials is that none of these “first-generation” Id vaccines was as clinically potent as expected. Several variables, including differences in the source and isotype of Id protein, pre-vaccine cytoreductive therapies, and the level of residual tumor, make it difficult to attribute the failure of the Genitope and Favrille trials to a single factor. For now, the Biovest rescue hybridoma vaccine, especially of IgM isotype, certainly deserves further study. One could speculate whether recombinant Id vaccines might have performed better clinically had they utilized an IgM backbone. Another factor contributing to the disappointing clinical results could relate to the glutaraldehyde Id-KLH cross-linking step’s capacity to damage important immunogenic epitopes in Id proteins (Betting et al. 2008; Kafi et al. 2009).

Active Vaccination Against Id in Myeloma

Since myelomas express high levels of tumor-specific Ig, they too are potential targets for active Id vaccine therapy (Reichardt et al. 1997; Bogen et al. 2006). As

they lack surface Id expression, the goal is induction of an Id-specific $CD8^+$ cytotoxic T-cell response (Weng et al. 2011). Numerous preclinical investigations have demonstrated the capacity to generate class I or class II MHC-restricted T-cell immunity to myeloma Id-derived peptides (Li et al. 2000; Hansson et al. 2003; Kim et al. 2003; Abdalla et al. 2007a, b). Phase I/II clinical trials of Id vaccination in myeloma have yielded some hints to efficacy (Reichardt et al. 1999; Rasmussen et al. 2003; Hansson et al. 2007; Abdalla et al. 2008; Yi et al. 2010). To date however, no randomized, controlled trials have been performed to demonstrate clinical benefits. Refining strategies to overcome myeloma-induced host immune suppression barriers is an ongoing goal of current research.

Anticipated High-Impact Results

The largely disappointing performance of Id vaccines in recent phase III trials has highlighted several challenges facing the development Id-directed therapy:

1. It will be important to understand the reasons for the failures of first-generation Id vaccines; was this due to inadequate tumor cytoreduction, rituximab-mediated B-cell depletion, method of Id production, suboptimal KLH conjugation, or Id protein isotype?
2. The weak immunogenicity of Id-KLH vaccines should be improved. This might be overcome by using alternative carrier protein conjugation techniques (i.e., maleimide chemistry). Host anti-Id immune responses to Id vaccines might be further boosted by inclusion potent adjuvants (such as toll-like receptor agonists) and reversal of tumor-induced immune suppression through targeting immune checkpoints such as CTLA-4, PD-L1/L2, and 4-1BB or via neutralization of regulatory T cells and their cytokine products (IL-10, TGF-b).
3. Given the historically remarkable efficacy of murine anti-Id passive antibody therapy, and the availability of new antibody engineering technologies, one can ask whether it is now time to revisit this approach and produce patient-specific human anti-Id antibodies.

Continuing advances in molecular immunology should allow these challenges to be met, offering new generations of anti-Id therapies targeting B-cell cancers.

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Anti-Programmed Death 1 (PD1)

Gordon J. Freeman and Arlene Sharpe

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Abstract

Programmed death-1 (PD-1) is a cell-surface receptor expressed on activated T and B cells, NK, NKT cells, and some myeloid cells (Okazaki and Honjo, Int Immunol 19:813–824, 2007; Keir et al., Annu Rev Immunol 26:677–704, 2008). Upon ligand binding, PD-1 delivers an inhibitory signal that attenuates T-cell receptor (TCR) signaling. PD-1 signaling results in reduced T-cell activation and effector function (Okazaki and Honjo, Int Immunol 19:813–824, 2007; Keir et al., Annu Rev Immunol 26:677–704, 2008). The PD-1 ligand PD-L1 is expressed on many tumors and is an important component of the immunosuppressive tumor

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microenvironment (Brown et al., J Immunol 170:1257–1266, 2003; Dong et al., Nat Med 8:793–800, 2002; Driessens et al., Immunol Rev 229:126–144, 2009; Wang and Chen, Curr Top Microbiol Immunol, n.d.).

Keywords
Antigen-presenting cells (APCs) • Phosphatase and tensin homolog (PTEN) • Phosphatidylinositol-3-kinase (PI3K) activity • Programmed death-1 (PD-1) • Antigen receptor signaling • Assessment • Biology of • Clinical trials • In cancer • In hematologic malignancies • Prognosis • PTEN, loss of • T-cell death • Therapeutics • T-cell receptor (TCR) signaling

Target: Programmed Death-1 (PD-1)

Programmed death-1 (PD-1) is a cell-surface receptor expressed on activated T and B cells, NK, NKT cells, and some myeloid cells (Okazaki and Honjo 2007; Keir et al. 2008). Upon ligand binding, PD-1 delivers an inhibitory signal that attenuates T-cell receptor (TCR) signaling. PD-1 signaling results in reduced T-cell activation and effector function (Okazaki and Honjo 2007; Keir et al. 2008). The PD-1 ligand PD-L1 is expressed on many tumors and is an important component of the immunosuppressive tumor microenvironment (Brown et al. 2003; Dong et al. 2002; Driessens et al. 2009; Wang and Chen n.d.). PD-1 expression is upregulated on tumor-infiltrating lymphocytes and makes them receptive to PD-1 ligands expressed in the tumor microenvironment (Ahmadzadeh et al. 2009). Blockade of the PD-1 pathway results in enhanced T-cell proliferation and effector function and shows promising results in early clinical trials of cancer immunotherapy.

Biology of the Target

PD-1 (CD279) is a monomeric immunoglobulin (Ig) superfamily member with an N-terminal IgV-like domain, an approximately 20-amino-acid stalk separating the IgV-like domain from the plasma membrane, a transmembrane domain, and a cytoplasmic domain with an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). PD-1 is inducibly expressed on peripheral CD4 and CD8 T cells, NKT cells, B cells, monocytes, and some dendritic cell (DC) subsets upon their activation (Okazaki and Honjo 2007; Keir et al. 2008).

PD-1 has two ligands: PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273) (Okazaki and Honjo 2007; Keir et al. 2008; Wang and Chen n.d.). The two PD-1 ligands have IgV-like and IgC-like extracellular domains similar to other B7 family members. PD-L1 and PD-L2 differ in their affinities for PD-1, with PD-L2 having a threefold higher affinity for PD-1. B7-1 is an additional binding partner for PD-L1 but does not bind to PD-L2. PD-L1 is broadly expressed on hematopoietic and

non-hematopoietic cells. PD-L1 is constitutively expressed on B cells, DCs, macrophages, BM-derived mast cells, and T cells and further upregulated upon their activation. Constitutive expression of PD-L1 is higher in mice than in humans. PD-L1 can also be expressed on a wide variety of non-hematopoietic cell types, including vascular endothelial cells, fibroblastic reticular cells, epithelia, pancreatic islet cells, astrocytes, neurons, and in cells at sites of immune privilege including trophoblasts in the placenta and retinal pigment epithelial cells and neurons in the eye. PD-L2 is inducibly expressed on DCs, macrophages, peritoneal B1 B cells, memory B cells, and cultured bone marrow (BM)-derived mast cells.

The expression of PD-L1 and PD-L2 is regulated by the inflammatory milieu. PD-L1 expression is strongly upregulated by interferon-a, interferon-b, and interferon-g on a wide variety of cell types including tumor cells. PD-L2 expression is strongly upregulated by IL-4 and less strongly by interferons (IFNs). The JAK/STAT, MAP kinase, and PI3K/AKT pathways mediate IFN-signaling, and recent studies indicate that the JAK/STAT and MAP kinase signaling pathways are involved in IFN-induced PD-L1 expression. Studies using pharmacological inhibitors show that PD-L1 expression in cell lines is decreased when MyD88, TRAF6, and MEK are inhibited. JAK2 has also been implicated in PD-L1 induction.

Constitutive and immune-induced PD-L1 expression may be relevant in the tumor microenvironment. Loss of phosphatase and tensin homolog (PTEN) increases PD-L1 expression in tumors by a posttranscriptional mechanism (Parsa et al. 2007). Loss or inhibition of PTEN, a cellular phosphatase that modifies phosphatidylinositol 3-kinase (PI3K) and Akt signaling, is one of the most frequent alterations in cancer and may contribute to constitutive tumor expression of PD-L1. Inhibition of PI3K or Akt decreases PD-L1 expression in tumor cells. Immune-mediated increases of PD-L1 expression may be seen in metastatic melanoma where a feedback loop may exist in which immunogenic tumors stimulate an antitumor immune response and T-cell production of IFN-g, leading to upregulated PD-L1 expression on the tumor (Taube et al. 2012).

PD-1 is induced by TCR or BCR signaling and remains high in the setting of persistent antigen stimulation, either self or foreign. PD-1 is upregulated after T-cell activation, but declines in the later stages of a successful immune response as antigen is cleared and the TCR is no longer engaged. If antigen is not cleared and T cells are repetitively stimulated, such as in a chronic infection or cancer, PD-1 expression remains high and T cells may enter a state of reduced effector function and proliferative capacity, termed “T-cell exhaustion” (Barber et al. 2006). Thus, expression of PD-1 indicates receptivity for inhibition by engagement of PD-1 with PD-L1 or PD-L2. PD-1 is a marker for exhausted T cells, but does not exclusively indicate that a T cell is exhausted. The common gamma chain cytokines IL-2, IL-7, IL-15, and IL-21 also can induce PD-1 expression on T cells, suggesting potential synergies in combination therapies using these cytokines and PD-1 blockade. NFATc1 is a critical factor for induction of PD-1 expression in T cells, as evidenced by the marked reduction in PD-1 expression by the calcineurin inhibitor cyclosporine A and the NFAT-specific inhibitor VIVIT. IFNs can induce PD-1 upregulation in macrophages, and ligation of TLR2, TLR3, or TLR5 can induce PD-1 in some myeloid DCs.

In association with antigen receptor signaling, engagement of the PD-1 receptor by its ligands delivers inhibitory signals that regulate the balance among T-cell activation, tolerance, and immune-mediated tissue damage. T-cell activation is a summation of positive and negative signals, and strong costimulatory signals generally dominate during the early stages of an immune response. The PD-1 pathway serves to limit the extent of an immune reaction and exerts important inhibitory functions in the setting of persistent antigenic stimulation such as during encounter with self-antigens, chronic viral infections, and tumors. The PD-1 pathway controls multiple tolerance checkpoints that prevent autoimmunity: inhibiting initial activation of self-reactive T cells, restraining effector T cells, and promoting induced regulatory T-cell development and function (Keir et al. 2008). Both tumors and pathogens have evolved strategies to evade immune responses via this pathway. The PD-1:PD-L pathway contributes directly to T-cell exhaustion and the suppressive tumor microenvironment, as well as lack of viral control during chronic infections.

PD-1 engagement can inhibit T-cell proliferation, cytokine production, cytolytic function, and survival. Engagement of PD-1 by ligand alters membrane-proximal signaling events in T cells. PD-1 engagement recruits phosphatases, particularly SHP2, to the phosphorylated ITSM motif of the PD-1 cytoplasmic domain, resulting in dephosphorylation of proximal signaling molecules including ZAP70, PKCq, and CD3z and attenuation of the TCR/CD28 signal (Riley 2009). This reduced TCR signal also results in shorter interaction times with antigen-presenting cells (APCs) or target cells expressing PD-1 ligands, thereby reducing T-cell responses. In addition, PD-1 engagement inhibits the induction of phosphatidylinositol-3-kinase (PI3K) activity and downstream activation of Akt. The effects of PD-1 on Akt activation may explain why IL-2 can rescue T cells from PD-1 inhibition. IL-2 can trigger Akt activation through STAT5 and thereby circumvent PD-1-mediated inhibition of Akt activation. PD-1 ligation also inhibits Erk activation, but this effect can be overcome through cytokine receptor signaling, particularly cytokines that activate STAT5, such as IL-2, IL-7, and IL-15. PD-1 engagement also prevents expression of transcription factors associated with effector cell function, including GATA-3, T-bet, and Eomes, and inhibits induction of the T-cell survival factor bcl-xl. In addition, PD-1 engagement upregulates expression of the basic leucine zipper transcription factor, ATF-like (BATF), a transcription factor in the AP-1 family, which can impair T-cell proliferation and cytokine secretion.

While far less is known about the function of PD-1 on B cells, macrophages, and DC, emerging data indicate that PD-1 also inhibits function of these cell types. Thus, the PD-1 pathway may inhibit tumor immunity by multiple mechanisms.

Target Assessment

The expression of PD-1 and its ligands can be assessed on cells and in tissues on the mRNA and protein levels. Antibodies to PD-1 and its ligands can be used to evaluate the expression of these molecules by flow cytometry or immunohistochemistry. PD-1 monoclonal antibodies (mAbs) that work well in immunohistochemistry of

paraffin-embedded sections have been reported (Taube et al. 2012; Dorfman et al. 2006; Xerri et al. 2008). Quantitative PCR also can be used to evaluate expression of PD-1 and its ligands.

Role of the Target in Cancer

Rank: 10

The role of PD-1 as a therapeutic target in cancer has been validated in several phase I clinical trials in patients with solid organ tumors and hematologic malignancies. Approximately 35% of patients had an objective response with a low rate of severe adverse events. These data support the important role for anti-PD-1-blocking antibodies in cancer immunotherapy. Phase II trials are being aggressively pursued with anti-PD-1 alone and in combination with other therapies such as anti-CTLA-4. Other agents that target PD-1 and its ligands are in phase I clinical trials, including anti-PD-L1 mAb and PD-L2-Ig.

High-Level Overview

Diagnostic, Prognostic, and Predictive

Studies relating PD-L1 expression on tumors to disease outcome show that high PD-L1 expression strongly correlates with unfavorable prognosis in kidney, ovarian, bladder, breast, gastric, and pancreatic cancer but not small cell lung cancer (Thompson et al. 2007). In contrast, in patients with metastatic melanoma, PD-L1 expression on metastatic tumors correlated with improved survival, which is thought to reflect an inflammatory tumor microenvironment (Taube et al. 2012). In melanomas, most tumor-infiltrating T lymphocytes (TIL) specific for tumor antigen express PD-1 as well as CTLA-4 and are functionally impaired (Ahmadzadeh et al. 2009). In a small sample set, PD-L1 expression on tumors was correlated with therapeutic response to PD-1 blockade (Brahmer et al. 2010).

Therapeutics

Both blocking antibodies and gene knockdown approaches are being developed as strategies to reduce function of the PD-1 pathway and thereby enhance tumor immunity. A monoclonal antibody (originally termed BAT or CT-AcTibody) developed against the Daudi B-cell tumor was found to have immune stimulatory activity and therapeutic efficacy against transplanted human neoplasias in immunodeficient mice (Hardy et al. 1997). This mAb was found to recognize PD-1 and has been developed as CT-011, a humanized IgG1 monoclonal antibody (Berger et al. 2008). Fully human PD-1 mAb (MDX-1106 = BMS-936558 = ONO-4538) with a human IgG4 Fc has also been developed (Brahmer et al. 2010). Phase I trials have been

reported with MDX-1106 focused on solid tumors, while CT-011 trials are focused on hematologic malignancies. The mechanisms of action of CT-011 and MDX-1106 may differ somewhat based on the effector activity of their Fc regions. Additional PD-1 pathway antagonists have entered phase I clinical trials. These include a third anti-PD-1 mAb (MK-3475), two anti-PD-L1 mAbs (MDX-1105, MPDL3280A), and PD-L2-Ig (AMP-224). Gene knockdown approaches using siRNA to reduce expression of PD-1, PD-L1, or PD-L2 also result in enhanced T-cell proliferation and antitumor activity and represent an alternative therapeutic strategy (Borkner n.d.).

Preclinical Summary

In humans, PD-L1 expression has been shown in a wide variety of solid tumors, including breast, lung, colon, ovarian, melanoma, bladder, liver, salivary, stomach, gliomas, thyroid, thymic epithelial, head, and neck (Brown et al. 2003; Dong et al. 2002). The expression of PD-L1 on many tumor types provided impetus for investigation of the role of PD-1 and its ligands in the regulation of tumor immunity. Studies in animal models demonstrate that PD-L1 on tumors inhibits T-cell activation and lysis of tumor cells and in some cases leads to increased tumor antigen-specific T-cell death (Driessens et al. 2009; Wang and Chen n.d.). Increased T-cell death in the presence of PD-1 signaling is likely a secondary effect accounted for by reduced growth factor and pro-survival gene expression. Thus, despite its name, PD-1 does not directly transduce a death signal in the fashion that the Fas pathway does. Further studies in mice indicate that tumor-associated APCs (such as myeloid and plasmacytoid DC) also use the PD-1:PD-L pathway to impair tumor-specific T-cell responses. In melanomas, most TILs specific for tumor antigen express PD-1 as well as CTLA-4 and are functionally impaired (Ahmadzadeh et al. 2009). PD-1 has been used as a biomarker to sort TILs from melanomas and found to enrich for melanoma antigen-specific T cells.

The PD-1 pathway also plays a role in hematologic malignancies. PD-1 is highly expressed on germinal center-associated T cells in normal reactive lymphoid tissue, consistent with reported expression on T follicular helper cells (Dorfman et al. 2006). PD-1 is highly expressed on neoplastic T cells of angioimmunoblastic lymphoma and other T-cell tumors of T follicular helper cell origin (Dorfman et al. 2006). PD-L1 is expressed on the associated follicular dendritic cell network (Dorfman et al. 2006; Xerri et al. 2008). PD-1 is weakly expressed on neoplastic B cells of small lymphocytic lymphoma and chronic lymphocytic leukemia and further upregulated by CD40 engagement (Xerri et al. 2008). PD-1 expression is rare on other types of lymphoid malignancies (Dorfman et al. 2006; Xerri et al. 2008). In nodular lymphocyte predominant Hodgkin's lymphoma, the neoplastic lymphocytic and/or histiocytic cells express PD-L1, and the reactive T cells surrounding the neoplastic cells express PD-1. Nodular sclerosing Hodgkin's lymphoma and mediastinal large B-cell lymphoma often have chromosomal amplifications in the 9p24.1 chromosomal region encoding PD-L1 and PD-L2 (Green n.d.). This increased copy number results in increased PD-L2 and PD-L1 expression in these tumors. In addition, some B-cell lymphomas have chromosomal translocations of the MHC

class II transactivator (CIITA) promoter with PD-L1 or PD-L2, resulting in constitutive expression of PD-L1 or PD-L2 (Steidl n.d.). Blockade of the PD-1 pathway restored the IFN-g-producing function of infiltrating T cells in Hodgkin's lymphoma. PD-L1 is expressed on multiple myeloma cells, but not on normal plasma cells. T-cell expansion in response to myeloma cells is enhanced in vitro by PD-L1 blockade. Blockade of PD-1 on NK cells with CT-011 resulted in increased NK cell cytotoxicity against multiple myeloma target cells. PD-1 and PD-L1 also are expressed on CD4 T cells in HTLV-1-mediated adult T-cell leukemia and lymphoma. These tumor cells are hyporesponsive to TCR signals, and PD-1 blockade increased their expression of TNF-a, but not IFN-g.

PD-1 blockade may be beneficial in combination therapeutic strategies, when given in conjunction with other immunomodulatory therapies, tumor vaccines, or targeted cancer therapies. Coadministration of PD-1 and CTLA-4 mAbs resulted in enhanced tumor eradication and tumor immunity in animal models (Curran n.d.). PD-1 and CTLA-4 also have been given together with immunostimulatory therapies (e.g., CpGs, GM-CSF, IL-15, anti-41BB), and these combinations increase antitumor activity and survival (Yu n.d.). In addition, PD-1 blockade can enhance responses to therapeutic vaccination. PD-1 is expressed on tumor antigen-specific human T cells following vaccination with tumor peptide antigen. Blockade of PD-1 with mAb during in vitro stimulation with melanoma peptide increased the numbers and effector activity of tumor-specific human T cells (Wong et al. 2007). PD-1 blockade did not change the percentage of apoptotic antigen-specific human T cells, indicating that the increase in number was due to increased proliferation, not decreased death. PD-1 blockade also limited the inhibitory capacity of $CD4^{+}CD25^{hi}$ T reg. Cyclophosphamide depletes Treg, and combination therapy of cyclophosphamide, PD-1, and a tumor vaccine was effective for eradication of TC-1 tumor expressing the HPV E7 oncogene (Mkrtichyan n.d.). The finding that certain cancer chemotherapeutic agents (e.g., cisplatin) can upregulate PD-L1 provides a rationale for exploration of additional combinatorial therapies.

Clinical Summary

A phase I trial of CT-011 in 17 patients with advanced-stage hematologic malignancies found that a single dose of drug was safe and well tolerated up to the maximum tested dose, 6 mg/kg (Berger et al. 2008). One-third of the patients showed clinical benefit with one complete remission in a patient with follicular lymphoma. A phase I trial of MDX-1106 in 39 patients with treatment-refractory solid tumors (melanoma, colon, lung, kidney) found that a single dose of drug was safe and well tolerated with no dose-limiting toxicities after a single dose of up to 10 mg/kg (Brahmer et al. 2010). Patients with clinical benefit were eligible for repeated therapy and 12 received additional doses. One durable complete response in a metastatic colorectal cancer and two partial responses in melanoma and renal cell cancer were seen. Responders have remained in remission for over 1 year after therapy ended (Pardoll 2012). In additional clinical trials where treatment was extended, 31–41% of patients

had objective responses (Pardoll 2012; McDermott et al. 2011; Sznol et al. 2010). Among nine patients studied for PD-L1 expression on their tumors, three of four patients with cell-surface PD-L1 had tumor regressions, but zero of five patients without surface PD-L1 expression on tumor had clinical responses (Brahmer et al. 2010). This suggests that PD-1 blockade may work best with PD-L1-expressing tumors and that PD-L1 expression on the tumor may be a predictive biomarker for responsiveness to PD-1 blockade. One serious adverse event, inflammatory colitis, was seen in a patient who received five doses (Brahmer et al. 2010). Two patients developed grade 2 polyarticular arthropathies requiring steroids. One had a history of Lyme arthritis and the other a preexisting antinuclear antibody titer >1:1,000. Given the immunostimulatory mechanism of action of the drug, this may suggest increased caution and monitoring in patients with preexisting autoimmunity. Twenty-four hours after drug dosing, T-cell numbers in peripheral blood declined moderately. B- and NK cell numbers were unaffected. T-cell numbers rebounded from days 2 through 29 and declined from days 29 through 85. This might reflect movement of T cells from blood into tumor and tissue sites.

Anticipated High-Impact Results

Therapeutic benefit with limited adverse events in a significant proportion of solid and hematologic malignancies, particularly PD-L1-expressing tumors.

- Phase II/III clinical trials in melanoma, renal, lung, prostate, colon, and hematologic malignancies

Additional therapeutic efficacy and increased response rates in combination with other immunomodulatory therapies, cancer-targeting drugs, and tumor vaccines.

- PD-1 pathway blockade plus anti-CTLA-4 (ipilimumab)
- PD-1 pathway blockade plus blockade of other immunoinhibitory pathways (e.g., LAG-3, TIM-3, BTLA)
- PD-1 pathway blockade plus immunostimulatory pathway activation (e.g., OX40, 4-1BB, TLR, IL-2)
- PD-1 pathway blockade plus targeted kinase inhibitors (e.g., B-Raf)
- PD-1 pathway blockade plus radiation therapy
- PD-1 pathway blockade plus cancer chemotherapy (e.g., cisplatin)
- PD-1 pathway blockade plus tumor vaccines and other approaches to initiate an antitumor immune response that can be enhanced by PD-1 pathway blockade

Development of biomarkers to identify patients who will respond to PD-1 pathway blockade.

- Further work is needed in large cohorts to determine how well PD-L1 expression on tumors correlates with responsiveness to PD-1 pathway blockade.

- Additional biomarkers need to be determined.

Anti-PD-1, anti-PD-L1, and PD-L2Ig block different receptor/ligands interactions and may differ in their efficacy and safety profiles.

- Studies needed to compare efficacy and safety in different types of tumors.

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B7.1

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Abstract

Compared to oncogenes and tumor-associated antigens (TAAs), B7-1 is not a traditional cancer target as it is not expressed on the vast majority of solid tumors (Lenschow et al., Annu Rev Immunol 14:233–258, 1996). Nevertheless, B7-1 has been shown to be essential for the induction of T-cell responses, which play a central role in mediating tumor immunosurveillance and immune-mediated tumor regression (Ward and Kaufman, Int Rev Immunol 26:161–196, 2007). B7-1 consequently plays an important role in cancer vaccines that focus on harnessing the potential of the host immune system to recognize and eradicate malignant tumors.

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Target: B7-1

Compared to oncogenes and tumor-associated antigens (TAAs), B7-1 is not a traditional cancer target as it is not expressed on the vast majority of solid tumors (Lenschow et al. 1996). Nevertheless, B7-1 has been shown to be essential for the induction of T-cell responses, which play a central role in mediating tumor immunosurveillance and immune-mediated tumor regression (Ward and Kaufman 2007). B7-1 consequently plays an important role in cancer vaccines that focus on harnessing the potential of the host immune system to recognize and eradicate malignant tumors.

The generation of an efficient T-cell immune response requires two signals (Driessens et al. 2009). The process is initiated when a T-cell receptor engages its cognate antigen through interaction with the peptide–major histocompatibility complex (MHC) on antigen presenting cells (APCs). The second signal – or costimulatory signal – is an antigen independent signal that is delivered by the binding of a cell surface molecule on the APC to its ligand(s) on the T-cell. B7-1 is one of several distinct molecules capable of providing the second signal critical for T-cell activation and proliferation through its interaction with CD28 on the surface of the T-cell (Ward and Kaufman 2007). The combination of signal 1 and 2 initiates a cascade of events resulting in cytokine production and release, entry into the cell cycle, and blockade of apoptosis (Lenschow et al. 1996). T-cells that encounter antigen in the absence of sufficient costimulation typically become anergic, rendering them nonresponsive (Ward and Kaufman 2007).

Biology of the Target

B7-1 is a member of the B7/CD28 family and the immunoglobulin superfamily that is encoded by chromosome 3 in humans (Greenwald et al. 2005). Expression of B7-1 is restricted to APCs such as dendritic cells (DCs) and B-cells and functions as both a costimulatory and coinhibitory factor through its interaction with CD28 and CTLA-4, respectively (Greenwald et al. 2005). The binding of B7-1 to CD28, in conjunction with antigen-receptor signaling, promotes T-cell activation, growth factor production, and cell proliferation and survival. To downregulate the T-cell response and maintain a threshold of activation, CTLA-4 expression on the T-cell is induced 24–48 h after initial T-cell activation (Lenschow et al. 1996). B7-1's affinity for CTLA-4 is significantly higher than for CD28; the subsequent binding of B7-1 to

CTLA-4 results in attenuation of antigen-receptor signals, decreased cell activation, inhibition of cell proliferation, and possible cell death (Driessens et al. 2009). Studies in mice have shown that costimulation mediated by B7-1/CD28 is critical for generation of an effective immune response and that the absence of this costimulation can result in antigen-specific immune tolerance (Lenschow et al. 1996). As a result, the costimulatory activity of B7-1/CD28 binding has been targeted as an immunotherapeutic strategy for the treatment of cancer and has been evaluated both in the preclinical and clinical settings.

Target Assessment

Due to tolerance for self-antigens that is acquired by the immune system in its developmental stages, TAAs are typically either weakly immunogenic or functionally non-immunogenic. Moreover, efficient T-cell activation requires that both the antigen-specific signal and costimulatory signal be present on the same cell; however, costimulatory molecules, including B7-1, are not expressed on the vast majority of solid tumors (Driessens et al. 2009). Therefore, although tumors expressing MHC are capable of delivering antigen-specific signals to T-cells, presentation by tumors of TAAs to the host immune system in the absence of appropriate costimulation often results in T-cell anergy. Efforts have consequently been focused to develop immunotherapeutic strategies that present TAAs to the host immune system to achieve far greater activation of T-cells than that which normally occurs. These strategies have included increasing the expression of costimulatory molecules, including B7-1, on the tumor as well as on professional APCs.

Role of the Target in Cancer

Rank: 4 (medium)

Nonviral gene therapy using DNA plasmids encoding B7-1 fusion proteins has demonstrated the proof of concept of inducing B7-1 expression on tumor cells as an immunotherapy for cancer. Using transplantable murine melanoma and sarcoma cells, several groups showed that induction of B7-1 on some tumor cell types by transfection was sufficient to induce CD8 T-cell-mediated rejection of these tumors (Zang and Allison 2007). In some cases, the introduction of B7-1 into tumor cells protected animals from further challenge with parental tumors that had not been engineered to express B7-1 (Zang and Allison 2007). Although these studies demonstrated that some tumors modified to express B7-1 can provide both signals 1 and 2 to activate naïve T-cells, this activation was shown to occur primarily in an indirect manner, in a process that included shedding of tumor antigens for uptake, processing, and presentation by professional APCs (Huang et al. 1996). This study concluded that even with the addition of B7-1, tumors were significantly less efficient at priming naïve cytotoxic T lymphocytes (CTLs) than host APCs (Huang et al. 1996). Efforts have since focused on optimally integrating B7

costimulation, either alone or in combination with other costimulatory molecules and/or tumor antigens to enhance the ability of host APCs to activate T-cells to generate immune responses against TAAs. These studies have been performed in both the preclinical and clinical settings utilizing a number of vaccine platforms.

High-Level Overview

Little attention has been given to the host immune system in terms of prognosis and/or potential response of cancer patients to therapy; however, it has long been known that the immune system plays a role in cancer. For example, patients with immunosuppressive disorders or individuals undergoing immunosuppressive treatments to reduce transplantation rejection have a higher incidence of cancer (Jochems and Schlom 2011). Moreover, a number of studies on various carcinomas, including colorectal, hepatocellular, gallbladder, pancreatic, esophageal, ovarian, endometrial, cervical, bladder, and urothelial, have shown a strong association between the presence and level of tumor-infiltrating immune cells and prognosis (Jochems and Schlom 2011). Cancer cells have also been shown to evade recognition by the immune system by downregulating antigen expression, components of the antigen processing and presentation machinery, MHC molecules, and costimulatory molecules (Dunn et al. 2002). Additionally, tumors have been reported to produce immunosuppressive factors such as TGF-beta and IL-10, which ultimately inhibit the activation of the various components of the immune response (Dunn et al. 2002). Taken together, these observations support a role for the immune system in controlling tumor growth and form the rationale for developing immunotherapeutic interventions against cancer.

Advances in the understanding of the immune system and its interaction with both host and tumor cells have provided a number of new strategies for improving antitumor immunity. Cancer vaccines constitute a unique therapy in that they initiate a dynamic process, activating the host's immune response to result in significantly greater activation of T-cells than that which normally occurs. In order for the immune system to mount an effective antitumor T-cell response, an adequate number of high-avidity T-cells specific for the antigens expressed in the malignancy must be activated (Hodge et al. 2005). Enhanced levels of T-cell costimulatory molecules have been shown to produce high-avidity CTL, which are able to recognize low levels of peptide presented in the context of the MHC molecules on the surface of the tumor and thereby kill tumor targets (Hodge et al. 2005). Inclusion of costimulatory molecules, including B7-1, has been important in the development of a variety of immunotherapeutic strategies.

Therapeutics

One of the primary goals of cancer immunotherapy is to generate a robust T-cell-mediated response capable of recognizing and destroying cancer cells. As

costimulatory molecules, including B7-1, are essential in mounting an efficient T-cell response, it was hypothesized that inclusion of B7-1 in the development of cancer immunotherapies would be beneficial. A number of preclinical and clinical studies have demonstrated the benefits of including the costimulatory molecule B7-1, alone and in combination with other costimulatory molecules and TAAs, in the development of therapeutic cancer vaccines.

Preclinical Summary

Preclinical investigation of recombinant viral vectors carrying the B7-1 transgene has been extensive. Recombinant vector-based vaccines are highly immunogenic, inducing an inflammatory response directed mainly toward vector proteins. This inflammatory response, in turn, leads to an increased immune response against the genes of interest that have been inserted into the vector. Viral vectors have large genomes that can accommodate the insertion of multiple transgenes. The use of adenoviral vectors carrying B7-1 alone or B7-1 in combination with TAAs, other costimulatory molecules, and/or cytokines has been described in detail, and antitumor effects have been reported in a variety of models (Sharma et al. 2009). Similar studies using lentivirus vectors, herpes simplex virus-1 vectors, and recombinant retroviruses carrying the B7-1 transgene, in combination with other cytokines such as GM-CSF, IL-2, IL-12, and IL-18, have demonstrated significant T-cell-mediated immune responses against tumor cells in vitro and in vivo (Chan et al. 2005; Fukuhara et al. 2005; Tseng et al. 2005).

Using recombinant poxvirus vectors to deliver an array of costimulatory molecules either by direct vaccination or intratumoral administration has been shown to be safe and effective in preclinical and clinical studies. Because most tumors do not express B7-1, investigation has focused on the efficacy of delivering a TAA (signal 1) combined with B7-1 (signal 2) to optimally activate antigen-specific T-cells. Poxviral vectors such as recombinant vaccinia (rV) (Hodge et al. 1994), modified vaccinia Ankara (MVA) (Hodge et al. 2009), and the avian-derived recombinant fowlpox (rF) (Hodge et al. 1999) and canarypox (ALVAC) (Hodge et al. 1997) have been used extensively in B7-1 immunotherapy. These poxviral vectors are large enough to allow insertion of multiple transgenes and have been shown to efficiently infect APCs, including murine and human B-cells and DCs (Hodge et al. 2001). Also, because poxviral vectors replicate in the cytoplasm and do not integrate into host DNA, their safety profile has been well documented.

The availability of multiple costimulatory molecules led to the generation of viral constructs expressing multiple costimulatory molecules. Vaccines that deliver multiple costimulatory molecules in the same vector have been shown to induce an even stronger signal 2. Triad of costimulatory molecules (designated TRICOM) is a recombinant vaccine consisting of the costimulatory molecules B7-1, intercellular adhesion molecule 1 (ICAM-1), and lymphocyte function-associated antigen 3 (LFA-3). ALVAC, rV, rF, and rMVA vectors containing this combination of costimulatory molecules have been described. The combination of B7-1, ICAM-1,

and LFA-3 raises T-cell activation to far greater levels than can be achieved with any 1 or 2 of these molecules (Hodge et al. 1999, 2005). Preclinical studies have demonstrated that TRICOM vaccines increase T-cell responses to TAAs and induce antitumor responses through the generation of high-avidity CTLs and memory T-cells (Hodge et al. 2005).

The use of whole tumor cells manipulated to express B7-1 has also been explored preclinically as an immunotherapeutic strategy for the treatment of cancer (Mitchell 2002). Whole tumor cell vaccines (WTCVs) utilize the entire tumor-antigen repertoire, present an alternative to antigen-defined vaccination strategies, and bypass the necessity of tumor-antigen identification. Tumor cells are engineered to express B7-1 using viral or nonviral gene therapy, followed by high-dose gamma radiation to prevent tumor cells from proliferating and metastasizing within the host. A study by Briones et al. observed that lymphoma cells infected with a B7-1-expressing rMVA vector used as a WTCV in mice significantly increased survival through a $CD4^+$ and $CD8^+$ T-cell-mediated mechanism (Briones et al. 2003). Similar studies in preclinical models of AML, colon, lung, and renal carcinoma and melanoma have further demonstrated the antitumor efficacy of using the B7-1 costimulatory molecule as part of a WTCV (Mitchell 2002).

Another vaccine strategy that has been explored preclinically employs B7-1 costimulation along with modified APCs. DCs are able to mediate T-cell recognition and responsiveness through the processing and presentation of antigens, the abundance of costimulatory molecules present on their cell surface, and the ability to differentiate or mature in response to a variety of stimuli. These qualities make DCs an attractive modality for cancer immunotherapy. Many studies have focused on exploiting the natural expression of B7-1 on the cell surface of DCs in combination with the forced expression of TAAs. For example, Tsang et al. reported that human DCs infected with a recombinant avipox vector containing CEA and B7-1 activated a higher level of antigen-specific immune response than a vector containing CEA alone (Tsang et al. 2001). Also, a study investigating TRICOM-infected murine DCs showed that T-cell activation was significantly enhanced in vitro and in vivo (Hodge et al. 2000). Another study found that TRICOM infection of human DCs resulted in enhanced peptide-specific T-cell activation in vitro (Zhu et al. 2001).

Clinical Summary

More than 20 clinical trials have investigated B7-1 as an immunostimulant, delivered alone or in combination with other costimulatory molecules and/or tumor antigens. Autologous or allogeneic whole tumor cell vaccines, DC vaccines, and local and systemic administration of recombinant poxviral vectors that exploit B7-1 costimulation have been investigated in the clinical setting.

Fishman et al. have reported a single-arm, single-center phase II trial using a whole tumor cell vaccine modified to express B7-1 as a treatment for renal cell carcinoma (Fishman et al. 2008). Autologous tumor was transduced ex vivo with a recombinant ALVAC vector containing B7-1 (ALVAC-hB7-1), irradiated and

administered subcutaneously, followed by a schedule of subcutaneous IL-2. Of the 60 patients enrolled, 3% had a complete pathologic response, 5% had a partial response, 64% had stable disease, and 28% showed disease progression. In another study, a whole tumor cell vaccine employing a B7-1-modified, HLA-A2-matched, allogeneic breast cancer cell line was administered to 30 women with previously treated stage IV metastatic breast cancer (Dols et al. 2003). This immunization strategy proved to be safe and feasible and induced tumor-specific immune responses in a minority of patients; however, no objective tumor regressions were observed. Finally, in a study by Raez et al., patients with stage IIIB/IV non-small cell lung carcinoma (NSCLC) were immunized with a B7-1, HLA-A1, or HLA-A2 plasmid-transfected allogeneic NSCLC whole tumor cell vaccine (Raez et al. 2003). IFN-γ-secreting CD8 T-cells increased in 14 of 15 patients, and a clinical response was observed in 5 patients.

Three studies investigating the use of autologous DCs infected with TRICOM vectors have been completed in patients with advanced or metastatic CEA-expressing carcinomas (Morse et al. 2013). Four other phase I/II studies are actively recruiting patients with prostate, bladder, and CEA-expressing solid tumors. An additional phase II randomized study examining docetaxel and prednisone with or without vaccine in metastatic castration-resistant prostate cancer (mCRPC) was recently completed and evaluated overall survival as the primary endpoint, with secondary endpoints including time to progression, prostate-specific antigen (PSA) response, and immune response (Heery et al. 2012).

Intratumoral delivery of vectors encoding B7-1 has also been translated to the clinical setting, including a completed phase I trial of intralesional rV-B7-1 vaccine in the treatment of malignant melanoma (Kaufman et al. 2005). In this study of 12 patients, an increase in the frequency of gp100 and T-cells specific to a known melanoma TAA was observed, as well as increased CD8 and IFN-γ expression in the tumor microenvironment. A phase I trial of intralesional rV-TRICOM vaccine in the treatment of malignant melanoma (Kaufman et al. 2001) and a phase II study using an rF vaccine containing TRICOM have been completed. Moreover, a phase Ib study to determine the efficacy of intratumoral administration of ALVAC-hB7-1 in patients with surgically incurable melanoma is ongoing.

Systemic delivery of B7-1-containing vaccines for treatment of a variety of carcinomas has been investigated clinically. Zajac et al. conducted a phase I/II clinical trial of a vaccinia virus expressing multiple HLA-A0201-restricted tumor-associated epitopes (MART-1, gp100, and tyrosinase) and costimulatory molecules (B7-1 and B7-2) in patients with metastatic melanoma (Zajac et al. 2003). Of 17 patients who completed the study, 3 displayed regression of individual metastases, 7 had stable disease, and 7 had progressive disease. In a recently reported phase II, randomized, multicenter trial combining an ALVAC vector expressing B7-1 and CEA administered with a standard chemotherapy regimen, 40.4% of 118 patients with metastatic colorectal cancer showed an objective clinical response (Kaufman et al. 2008). Currently, an ALVAC vector containing the NY-ESO-1 TAA combined with TRICOM is being investigated in an open phase I study for women with stage II, III, or IV ovarian epithelial, fallopian tube, or peritoneal carcinoma.

Eleven phase I and II trials investigating the efficacy of rV/F-TRICOM vaccines have been completed since 2001, and results from additional trials are pending. These studies in lung, prostate, breast, colorectal, pancreatic, liver, gastric, ovarian, and head and neck cancer have demonstrated the safety of systemic and local administration of pox virus vaccines containing a triad of costimulatory molecules (B7-1, ICAM-1, LFA-3) in advanced-stage disease. Data from these studies, which combined rV/F-TRICOM vaccines with chemotherapy, radiation, hormonal therapy, and adjuvants such as IL-2 and GM-CSF, have demonstrated the safety and efficacy of these combinations in inducing an immune response. Second-generation vaccines combining TRICOM with TAAs to induce a more specific T-cell population are also being tested clinically. In a series of phase I and II clinical trials, PANVAC (CEA/mucin 1-TRICOM) (Madan et al. 2007) and PROSTVAC (PSA-TRICOM) (Madan et al. 2009) demonstrated significant safety, increased antigen-specific immune response, and preliminary evidence of increased survival. For example, PROSTVAC combined with docetaxel has been reported to increase time to progression in mCRPC by 2.4 months compared to docetaxel alone (6.1 months vs. 3.7 months, respectively) (Arlen et al. 2006). In another study combining PROSTVAC with radiotherapy to treat localized prostate cancer, 76.5% of patients showed a ≥3-fold increase in PSA-specific T-cells versus 0% in the radiotherapy-only arm ($P < 0.0005$) (Gulley et al. 2005). Moreover, in a different study of PROSTVAC in mCRPC, Madan et al. reported that patients given nilutamide after disease progression on PROSTVAC had a median overall survival of 6.2 years compared to 3.7 years for patients given vaccine following progression on nilutamide ($P = 0.045$) (Madan et al. 2008). Most recently, in a randomized, placebo-controlled, multicenter trial in patients with progressive mCRPC, patients who received PROSTVAC had a greater 3-year overall survival rate than control patients (30% vs. 17%) and a significantly longer median overall survival (25.1 vs. 16.6 months, respectively; $P = 0.0061$) (Kantoff et al. 2010). Another multicenter trial of PROSTVAC in mCRPC also demonstrated enhanced median overall survival in this patient population (Gulley et al. 2010). Based on the results of these studies, a randomized, double-blind, placebo-controlled, phase III study is ongoing. Studies of TRICOM vaccines that utilize the costimulatory activity of the B7-1 molecule are building a strong rationale for the clinical use of B7-1 as part of immunotherapy to treat a variety of malignancies.

Anticipated High-Impact Results

PROSTVAC, a vector-based platform utilizing the triad of costimulatory molecules (B7-1, ICAM-1, and LFA-3) in combination with the TAA PSA, has already demonstrated an increased median overall survival of 8.5 months in patients with mCRPC in a phase II clinical trial (Kantoff et al. 2010; Gulley et al. 2010). If those results are replicated in the randomized, double-blind, placebo-controlled phase III trial, PROSTVAC (Bavarian Nordic, Kvistgaard, Denmark) will be a promising candidate for approval by the US Food and Drug Administration (FDA) as a

therapeutic cancer vaccine. The rationale for approving PROSTVAC is supported by the recent FDA approval of the first therapeutic cancer vaccine, sipuleucel-T (Dendreon Corp., Seattle, WA, USA), which is an autologous DC vaccine that demonstrated an increase in median overall survival of 4.3 months compared to placebo in an integrated assessment of two identical phase III trials in mCRPC (Higano et al. 2009).

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8 Bacterial Vaccines

Paulina Chorobik and Joanna Bereta

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Abstract

A contemporary approach to bacterial cancer immunotherapy takes advantage of substantial progress in the understanding of the tumor-immune system interplay, as well as the recent advances in genetic engineering. Safe, targeted therapies are being developed, in which genetically-modified pathogens are designed to trigger effective anti-tumor immune response. Here we describe the bacterial strains intended for cancer immunotherapies, the genetic modifications that attenuate their pathogenicity but strengthen anti-tumor potential and the desirable mechanisms of actions. The only FDA approved *Bacillus Calmette-Guerin*-based cancer therapy as well as numerous examples of ongoing clinical trials involving different bacterial strains are presented.

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Target

Over a century ago, Dr. William Coley for the first time intentionally administered bacteria to patients to treat inoperable tumors. Thanks to the dedication of a lifetime to perfect this therapy, which in many cases led to complete tumor eradication, Dr. Coley was acknowledged as the Father of Cancer Immunotherapy (Richardson et al. 1999). Since that time, the major principle of bacterial anticancer therapy has not changed much and is based on systemic application of pathogenic bacteria to alert the immune system and induce a potent, specific, antitumor immune response, resulting in the inhibition of tumor growth.

Despite its initial success, bacterial antitumor therapy became underexploited and neglected throughout the years of the domination of chemo- and radiotherapy, which were easily manageable due to the simplicity of the effector nature (radiation or chemotherapeutics) and relatively rapid assessment of the effectiveness of treatment. However, acute toxicity and other adverse effects of conventional tumor therapies call for a continuous search for less devastating alternatives.

Among biological therapies, the application of live bacteria may meet these expectations, especially as some bacterial species spontaneously colonize solid tumors. In consequence, this natural tumor targeting may reduce systemic adverse effects compared to other therapies.

Bacteria are applied in cancer treatment to address at least one of the following: (*i*) specifically target tumor tissue, (*ii*) preferentially deliver therapeutic agents to tumor tissue to limit systemic toxicity, and (*iii*) break the immune suppression, disarm mediators which promote tumor progression, and boost antitumor immunity.

Since the first attempts of bacterial cancer therapy, the advances in genetic engineering have enabled the attenuation of pathogen virulence and thus the application of live instead of killed microorganisms. The viability of therapeutic bacteria extends the potential benefits of treatment as it guarantees the features that are unique for bacteria-based therapy, described in the next section. Weakening of the virulence allows for a repeated dose scheme as long as the immunogenicity of the attenuated strain is balanced to guarantee its proliferative and stimulatory capacities, along with the safety of treatment.

With the aid of genetic engineering, bacteria may also acquire novel therapeutic features. The term “bacterial vaccine vector” was coined to underline the use of bacteria as a vehicle for the delivery of therapeutic molecules.

Biology of the Target

The live-attenuated bacteria-based approach represents a versatile therapeutic option for cancer therapy, as it provides the complex and complete array of immunostimulatory effects that address the drawbacks of many single-agent therapies. Its additional advantage over small molecule therapeutics, as well as over antibody- or cell-based immunotherapies, is that most of the bacteria under consideration are self-propulsive and actively penetrate tumor tissue, including necrotic and hypoxic regions. Live bacteria represent a therapeutic unit that can be robustly multiplied in vitro in relatively simple growth media. Moreover, they retain the ability to replicate and efficiently produce proteins after being applied to patients. Intracellular pathogens trigger a cellular type of immune response with a Th1 cytokine profile, essential for effective antitumor effects. Therefore, predominantly intracellular microorganisms, examples presented in Table 1, are applied in preclinical studies or clinical trials.

The natural adjuvant-like properties of bacteria may further be augmented by genetic modifications that arm bacteria with heterologous molecules. Virtually any protein of bacterial, viral, or eukaryotic origin whose activity does not depend on posttranslational modifications can be produced by a bacterial vector. Depending on the adopted strategy, this additional effector molecule is synthesized outside the host cells or is delivered into host cells during the intracellular phase of infection. For example, when bacteria capable of expressing a tumor antigen invade and survive inside the antigen presenting cells, such as dendritic cells (DCs) and macrophages, they can produce and deliver a tumor protein to the MHC-I antigen presentation

Table 1 Examples of bacteria candidates for cancer therapy

Species	Gram staining	Motile	Oxygen tolerance	Intracellular phase of infection
Clostridium sp.	Positive	Vegetative forms are motile	Anaerobe	Not applicable
Listeria monocytogenes	Positive	Yes	Facultative anaerobe	Facultatively intracellular, actively escape from phagosome to the cytosol of the infected cell
Mycobacterium bovis BCG	Positive	No	Obligate aerobe	Facultatively intracellular; survive and replicate in vacuoles of phagocytes
Salmonella enterica, sv. *typhimurium* and *typhi*	Negative	Yes	Facultative anaerobe	Facultatively intracellular, survive and replicate in vacuoles of phagocytes and also in the cytoplasm of epithelial cells
Shigella flexneri	Negative	No	Facultative anaerobe	Facultatively intracellular

pathway. This approach resulted in enhanced CTL responses to tumor antigens after the application of attenuated bacterial vaccine vectors to tumor-bearing animals (in the case of *S. typhimurium*) or to either animals or humans (in the case of *L. monocytogenes*).

The suppressed pathogenicity is a prerequisite for a modern bacterial cancer therapy. It can be achieved through: (*i*) direct modification of virulence factors or (*ii*) indirectly by modifying bacterial metabolic pathways.

The first approach involves mitigating virulence factors that directly damage host cells or activate robust and devastating immune responses during the natural course of infection. Virulence factors that are often targets for attenuation belong to ubiquitous pathogen-associated molecular pattern (PAMP) molecules, e.g., LPS, or are genus specific, e.g., *Listeria monocytogenes* ActA, which mediates actin polymerization and intracellular motility or *Clostridium novyi* α-toxin, which destroys host cell cytoskeleton through the modification of small GTP-binding proteins. Another approach of attenuation involves modifications of metabolic capabilities to impair bacterial growth and limit or redirect bacterial spread in the infected organism through the addiction of bacteria to external sources of missing metabolites. The concomitant effect of the disruption of a bacterial metabolic pathway is an accumulation of bacteria in tumor tissue which is a rich source of metabolites. Indeed, the metabolic attenuation of bacteria is indicated as a possible cause of their preferential tumor colonization.

Historically, attenuation was achieved by a nondirected method based on the exposure of a pathogen to mutagenic factors and/or selection of less virulent strains in vitro under special conditions or in vivo in nonhost species. For example, Bacillus Calmette-Guérin (BCG), the vaccine strain of *Mycobacterium bovis*, was developed after in vitro selection in a special culture medium. At present, most often, although not exclusively, a site-directed mutagenesis that disrupts a chosen vital gene is used for the development of therapeutic bacterial strains.

Attenuated *S. typhimurium* strains were obtained by the disruption of genes coding for: (*i*) enzymes involved in amino acid synthesis (leucine and arginine auxotrophic strain, aromatic amino acid synthesis defective *aroA* and *aroD* mutant strains), (*ii*) enzymes involved in purine synthesis (*purI* or *purD* mutant strains), and (*iii*) global transcriptional regulatory factors essential for the synthesis of numerous enzymes of catabolic pathways (*cya* and *crp* mutant strains). Genetically modified therapeutic strain *Clostridium novyi*-NT (NT stands for nontoxin) was obtained by the disruption of the genes coding for the lethal α-toxin. Moreover, *Clostridium* sp. is strictly anaerobic; therefore, the spores which are administered to the animal or human organism do not multiply effectively unless they reach hypoxic areas of the tumor. The attenuation of a modified *Listeria monocytogenes* strain, which is currently in Phase 2 clinical trial, limits bacterial survival in vivo due to the inactivation of *actA* gene encoding a major virulence factor (Wallecha et al. 2009). *Shigella flexneri aroA* mutant has also been used in preclinical studies (Galmbacher et al. 2010).

Target Assessment

A couple of dozen treatment regimens are currently under evaluation in Phase 1 safety studies. The bacterial inocula are administered based on the number of colonies formed on solid media (CFU, colony forming units). Maximum tolerated doses (MTD) are determined for each therapeutic strain or its novel derivative. The complete blood cell count (CBC) and blood biochemical assays are performed to monitor the patient status after bacteria administration. To evaluate the level of tissue colonization and biodistribution as well as to monitor bacteremia, the level of bacteria in the tissues, blood, urine, and stool is measured by cultivating bacteria from specimens on proper selective media. Serum levels of cytokines and chemokines such as IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, TNF, IFNγ, MCP-1, MIP-1α, and GM-CSF can be measured to assess the biological activity of the treatment or indicate the risk of cytokine-release syndrome (CRS). If relevant, the presence of antigen-specific T cells in peripheral blood is examined by ELISpot assays to assess the efficacy of bacterial vectors that express tumor antigen. Due to the differences in immune responses and severity of toxic effects, which are specific to different bacterial species tested, the dose-limiting adverse events are defined for each study, according to common toxicity criteria.

Role of the Target in Cancer

Rank: 10

Apart from a single example of *Bacillus Calmette-Guérin* (BCG) already used for over three decades for bladder cancer treatment (described below), applications where bacteria are used as antitumor agents are in most cases still in the preclinical phase of development and are thus categorized as experimental immunotherapies.

The primary concept of immunotherapy is to overcome the tumor immunosuppressive microenvironment and induce efficient tumor-specific immunity, including memory responses that would protect patients from tumor recurrence and metastatic disease. Bacteria are well suited for this purpose as they are the most potent activators of the immune system due to the engagement of numerous alarm-sensing pattern recognition receptors (PRR) including the majority of Toll-like receptors (TLRs) by bacterial pathogen-associated molecular patterns (PAMPs) such as cell wall components (LPS, lipopeptides), unmethylated CpG, flagellin, outer membrane proteins, and others. Additionally, an administration of intracellular bacteria is supposed to skew the immune response toward the desirable Th1 type. Therapeutic bacteria are often genetically modified and serve as vectors delivering gene coding for proteins of choice.

There are two major approaches to bacteria-based therapy. In the first one, bacteria engineered to express a given tumor antigen (TA) serve as its additional rich source delivered simultaneously with the strong bacterial alarm signals. In this

approach, bacterial invasion of the tumor is not necessary. The second approach intends the accumulation of bacteria in the tumor. By infecting and disrupting cancer cells, bacteria may increase the cross-presentation of TAs by dendritic cells via increasing the availability of TAs as well as providing or inducing multiple signals for the maturation and activation of DCs. These comprise: (*i*) ligands for all TLRs as well as other danger signal receptors, including tumor cell-derived damage-associated molecular patterns (DAMPs) such as heat shock proteins, nucleotides, nuclear proteins, and bacterial PAMPs, and (*ii*) proinflammatory cytokines and, in case of using intracellular microorganisms, also type I interferon. By infecting tumor-associated immune cells which are partially responsible for immunosuppressive microenvironment, bacteria may help in breaking anergy or tolerance toward tumor antigens. The natural effects of bacterial infection are often strengthened by their genetic modifications, leading to the expression of: (*i*) proapoptotic proteins that increase death rate (e.g., apoptin, TRAIL), (*ii*) proteins that modulate immune response (e.g., IL-2, IL-18, TNF, LIGHT), (*iii*) molecules that modulate the tumor microenvironment (e.g., ▶ IDO shRNA), (*iv*) molecules that modulate intracellular signaling (e.g., STAT3 shRNA), or (*v*) a combination of the above.

In contrast to mice, where preferential tumor localization of bacteria has been proven, insufficient tumor colonization in humans has been identified as the major limitation in developing effective therapies. Antibody-fragment-based targeting of bacteria toward cancer cells is proposed to overcome this drawback (Bereta et al. 2007; Massa et al. 2013).

High-Level Overview

Diagnostic, Prognostic, and Predictive

An increased number of suppressive immune cells in peripheral blood and in the tumor are negative prognostic factors for numerous malignancies. High frequency of immature MDSCs is correlated with poor prognosis in melanoma and gastrointestinal, lung, and breast cancers (Gabrilovich et al. 2012; Solito et al. 2014; Weide et al. 2014), and increased tumor infiltration with $FOXP3^+$ regulatory T cells predicts reduced survival of breast, cervix, gastric, kidney, ovary, and pancreatic cancer patients (Martin et al. 2010).

Two main therapeutic objectives of bacterial anticancer therapies are (*i*) the delivery of multiple immunostimulatory signals to resolve chronic inflammation and (*ii*) the augmentation of effective tumor-specific cellular immune responses. Effective intervention should inhibit the accumulation of suppressive cells in the tumor and overcome immunosuppressive tumor microenvironment consisting of soluble factors promoting tumor progression, released by both tumor cells and immunosuppressive cells. The activation of the innate immune Toll-like receptors with bacteria-derived ligands stimulates the proliferation of hematopoietic stem cells as well as the synthesis of proinflammatory cytokines. Upon acute exposure to the cytokines, cells of monocytic lineage undergo differentiation and activation

(Goldszmid et al. 2014) and may result in repopulation of tumors with myeloid cells without a suppressive phenotype.

The specific predictive biomarkers for bacterial anticancer therapies are not yet defined as most of them are only in Phase 1 safety studies. The main candidates for predictive factors are the serum levels of proinflammatory Th1 cytokines and additionally, if the therapeutic bacteria express a tumor antigen, the antigen-specific T-cell responses. The only approved bacterial anticancer agent is Bacillus Calmette-Guérin vaccine for the treatment of superficial bladder cancer. Recently, candidate predictive factors of intravesical BCG therapy for non-muscle-invasive bladder cancer have been evaluated (Lima et al. 2012). The results indicate that a higher number of tumor-associated macrophages (TAMs) in the tumor and surrounding tissue are associated with shorter relapse-free survival (RFS), whereas higher IL-2, IL-6, and TNF levels in urine are associated with longer RFS. The predictive value of urine levels of other cytokines, such as IFNγ or GM-CSF, is inconsistent and requires further research (Lima et al. 2012).

Therapeutics

Over three decades ago, intravesical BCG therapy for the management of bladder cancer superseded cystectomy. It is currently the standard therapy applied after transurethral lesion resection of intermediate- and high-risk non-muscle-invasive bladder tumors of Ta and T1 stages and for the management of carcinoma in situ (Kawai et al. 2013). The exact immunological mechanism is not clear; however, the therapeutic effect was ascribed to the infection of urothelial and bladder cancer cells which trigger an immune response, evidenced by the presence of cytokines in the urine. Moreover, the antitumor effect of BCG was shown to be dependent on T cells, NK cells, and neutrophils (Kawai et al. 2013).

BCG is administered as an induction and maintenance therapy and effectively reduces cancer recurrence and progression. During the induction therapy, one dose is given weekly for 6 weeks, but evidence shows that for an improved response, the therapy should be prolonged (Kamat and Porten 2014). The optimal frequency and duration of the maintenance therapy are still under investigation, although the protocol described by Lamm and coworkers has been suggested to be optimal (Kamat and Porten 2014; Lamm et al. 2000). The regimen consists of a 6-week induction therapy with intravesical and percutaneous administrations, followed by three weekly intravesical and percutaneous treatments at 3 and 6 month and then every 6 months, for a total of 3 years from the start of induction therapy (Lamm et al. 2000). The clinical efficacy is high and reaches 83% overall 5-year survival, with median 76.8 months of recurrence-free survival (Lamm et al. 2000). Despite the high efficacy, some patients do not respond to BCG. Moreover, local and systemic adverse events tend to escalate during the maintenance therapy, in severe cases forcing therapy cessation. These drawbacks prompted further development of BCG anticancer therapy toward genetically modified strains which produce

recombinant cytokines or application of BCG-derived particles instead of whole live organisms.

Preclinical Summary

The idea of bacteria-based immunotherapy is gaining more and more interest. In addition to bacteria strains, listed in Table 1, especially well suited for tumor therapy due to their intracellular lifestyle, the usefulness of extracellular bacteria such as *Escherichia coli* (St Jean et al. 2008) and certain lactic acid bacteria from the *Lactococcus*, *Lactobacillus,* and *Bifidobacterium* genera (Tangney 2010) has also been tested in preclinical studies, although to a lesser extent.

The preclinical research addresses two major issues: (*i*) Does the expression of a given tumor antigen (TA) in the context of bacteria induce a more effective antitumor immune response than the antigen administered solely? (*ii*) How does the expression of additional modifiers at the tumor site affect antitumor immune response? In both cases a gene of interest may be placed under a bacterial promoter, and the bacterial transcriptional, translational, and secretory machineries are used to express and deliver a heterogeneous protein. Alternatively, a gene coding for a protein or regulatory RNA is placed under a strong eukaryotic promoter such as CMV, and bacteria are used simply as vehicles that deliver the gene to the mammalian cell expression machinery.

The response to the following bacteria-coding TAs has been studied in mouse models: protein E7 of human papillomavirus type 16 (HPV-16 E7), tyrosinase-related protein 2 (TRP2), prostate-specific antigen (PSA), cancer/testis antigen 1 (NY-ESO-1), mesothelin, and survivin.

The applicability of the following modifiers has been studied in mouse models: (*i*) effector proteins enhancing presentation of TAs through induction of tumor cell death and TAs processing (TRAIL, apoptin, FasL, Noxa), *(ii)* effector molecules modifying immune response (TNF, IL-2, IL-18, CCL21, LIGHT, ► IDO shRNA, STAT3 shRNA), and *(iii)* molecules triggering antiangiogenic response (VEGFR-2) (Chorobik et al. 2013; Wood and Paterson 2014).

Salmonella and *Listeria* are the genera most commonly used in the preclinical studies. Bacteria of genus *Listeria* are predominantly exploited as vectors producing TAs inside antigen presenting cells. Hence, TAs are delivered in the context of bacterial immunostimulatory molecules to boost the immune response. As selected attenuated *Salmonella* strains accumulate preferentially in solid tumors over internal organs at a ratio of 1000:1, they are well suited for the delivery of therapeutic molecules locally to tumor tissue. This approach offers the advantage of the delivery of the molecules that are too toxic for systemic administration or the factors that for any reason would bring benefit when accumulated mostly in the tumor. Both *Listeria* and *Salmonella* in various experimental settings have been shown to induce strong tumor-specific immune responses leading to delay in tumor growth or even tumor eradication (Brockstedt et al. 2004; Chorobik et al. 2013; Wood and Paterson 2014).

Tumor growth inhibition after systemic or intratumoral administration of attenuated *S. typhimurium* to tumor-bearing mice has been associated with the shift in the phenotype and activity of intratumoral MDSCs indicated by the upregulation of maturation and activation markers (MHC class II and co-stimulatory molecules), their reduced suppressive capacity, and increased TNF secretion (Hong et al. 2013; Kaimala et al. 2014). Moreover, the frequency of regulatory T cells in tumors has also been reduced (Hong et al. 2013). Unfortunately, the very promising preclinical studies do not always translate into encouraging clinical trials. For example, the colonization of tumors in humans by attenuated *S. typhimurium* proved to be infrequent and insufficient for the therapeutic effect, which is in contrast to a high level of tumor colonization in mice susceptible to systemic *S. typhimurium* infection. This may result from significant differences in immune systems between humans and model mouse strains. NRAMP1 (natural resistance-associated macrophage protein-1) is important for the fate of intracellular bacteria such as *Mycobacterium* and *Salmonella*, which may survive and replicate inside phagocytes. NRAMP1 is absent in two major model mouse strains used for tumor therapy studies, namely, Balb/c and C57Bl/6, and this may explain the differences in the extent of bacterial tumor colonization between mice and humans. Hopefully, research on the mechanisms of bacterial infections, as well as on differences in the immune response between different species and strains, will help to bring the promising therapies to the clinic.

Clinical Summary

Since its approval in 1990, the BCG repeated intravesical instillations for treatment of non-muscle-invasive bladder cancer and inoperable bladder carcinoma in situ appear to have been one of the most effective cancer immunotherapies. The administration of BCG as a single agent against melanoma or colorectal or lung cancer has not been proven to be more effective than conventional therapies, but its use as a potential adjuvant for various cancer vaccines has been proposed.

The safety and therapeutic applicability of novel genetically modified bacteria species are being evaluated in Phase 1 or 2 clinical trials. *L. monocytogenes* therapeutic strains of series CRS are attenuated derivatives of wild-type *L. monocytogenes* 10403S obtained by the deletion of two genes: *actA* coding for a major virulence protein, actin assembly inducing protein, which mediates intracellular *Listeria* motility, and internalin B, which mediates the invasion of nonphagocytic cells. Clinical trials of the CRS-100 strain (ANZ-100, ClinicalTrial.gov identifier NCT00327652) have proven its safety. Further genetic modifications leading to the production of TAs by *Listeria* have been introduced, and the potentially improved efficacy of new strains is under clinical evaluation. The examples of ongoing clinical trials involving different bacterial species and strains are listed in Table 2.

Table 2 Examples of ongoing clinical trials on anticancer immunotherapy with live-attenuated bacteria

Therapeutic strain	Title/status[a]/identifier[b]	Tumor type/study objectives and outline/result
Clostridium novyi-NT	Safety study of intratumoral injection of *Clostridium novyi*-NT spores to treat patients with solid tumors that have not responded to standard therapies **O** NCT01924689	Advanced solid tumor malignancies Safety and tolerability of intratumoral administration of *C. novyi*-NT spore; additional outcome measures: antitumor activity, bacterial load in the blood, and host immune response
Listeria monocytogenes ADXS11-001	A phase II evaluation of ADXS11-001 in the treatment of persistent or recurrent squamous or non-squamous cell carcinoma of the cervix **O** NCT01266460	Cervix carcinoma Three intravenous administrations (every 28 days) of bacteria expressing HPV-16-E7 tumor-associated antigen fused to fragment of listeriolysin O (LLO); adverse effects, progression-free and overall survival, objective tumor response, and serum cytokine assessment
Listeria monocytogenes ADXS11-001	BrUOG 276: a phase I/II evaluation of ADXS11-001, mitomycin, 5-fluorouracil (5-FU), and IMRT for anal cancer **O** NCT01671488	Invasive primary squamous, basaloid, or cloacogenic carcinoma of the anal canal Safety study of four intravenous doses given once every 28 days in combination with standard chemoradiation (mitomycin, 5-FU, and IMRT); evaluation of 6-month clinical complete response rate, progression-free and overall survival, peripheral, and histologic markers of immune response (T-cell infiltration)
Listeria monocytogenes ADXS11-001	A phase I, dose-escalation trial of recombinant *Listeria monocytogenes* (Lm)-based vaccine encoding human papillomavirus genotype 16 target antigens (ADXS11-001) in patients with HPV-16 +ve oropharyngeal carcinoma **O** NCT01598792	Oropharyngeal carcinoma (HPV-16 positive) Safety study combined with the assessment of vaccine-induced T-cell responses
Listeria monocytogenes ADXS11-001	Window of opportunity trial of neoadjuvant ADXS 11-001	Newly diagnosed squamous cell carcinoma of

(*continued*)

Table 2 (continued)

Therapeutic strain	Title/status[a]/identifier[b]	Tumor type/study objectives and outline/result
	vaccination prior to robot-assisted resection of HPV-positive oropharyngeal squamous cell carcinoma **O** NCT02002182	stage I–IV (T1-3, N0-2b) of the oropharynx Safety and efficacy study of two intravenous ADXS11-001 infusions prior to tumor resection; induction of HPV E6/E7 antigen-specific CTLs in peripheral blood will be assessed
Listeria monocytogenes CRS-100 (currently ANZ-100)	Phase 1 dose-escalation study of safety and tolerability of intravenous CRS-100 in adults with carcinoma and liver metastases **O** NCT00327652	Carcinoma and hepatic metastases Safety study to assess maximum tolerated dose of single intravenous administration of *L. monocytogenes* CRS-100 strain, a derivative of wild-type 10403S strain, attenuated due to deletion of two virulence factors: ActA and internalin B (Δ*actA*/Δ*inlB*) Six patients with colorectal cancer (CRC), two with pancreatic ductal adenocarcinoma (PDA), and one melanoma patient, all with liver metastases, received a single intravenous dose (one of three escalating doses) to determine maximum tolerated dose; all doses were well tolerated; reported adverse events associated with cytokine release were transient Decrease of lymphocytes and NK cells numbers in peripheral blood was observed (Le et al. 2012)
Listeria monocytogenes CRS-207	A phase 1B study to evaluate the safety and induction of immune response of CRS-207 in combination with pemetrexed and cisplatin as front-line therapy in adults with malignant pleural mesothelioma **O** NCT01675765	Malignant pleural mesothelioma CRS-207 is derived of CRS-100 modified to produce tumor-associated antigen – human mesothelin under the control of bacterial *actA* promoter; bacteria will be administered twice in two cycles, in combination with

(*continued*)

Table 2 (continued)

Therapeutic strain	Title/status[a]/identifier[b]	Tumor type/study objectives and outline/result
		six cycles of chemotherapy; adverse events, induction of antigen-specific immune response, objective tumor response, time to progression, and serum mesothelin will be assessed
Listeria monocytogenes CRS-207	A phase 2, randomized, multicenter, open-label study of the efficacy and immune response of the sequential administration of GVAX pancreas vaccine alone or followed by CRS-207 in adults with metastatic pancreatic adenocarcinoma **O** NCT01417000	Pancreatic cancer (malignant adenocarcinoma of the pancreas) Assessment of safety and immune response to combined treatment with cyclophosphamide, GVAX Pancreas vaccine, and CRS-207, *L. monocytogenes* bacteria expressing tumor-associated antigen, mesothelin
Listeria monocytogenes CRS-207	A phase 2B, randomized, controlled, multicenter, open-label study of the efficacy and immune response of GVAX pancreas vaccine (with cyclophosphamide) and CRS 207 compared to chemotherapy or to CRS-207 alone in adults with previously treated metastatic pancreatic adenocarcinoma **O** NCT02004262	Metastatic pancreatic cancer Overall survival and adverse events of CRS-207 therapy alone or combined therapy: cyclophosphamide + GVAX Pancreas vaccine + CRS-207 will be assessed
Listeria monocytogenes CRS-207	A phase 1, open-label, dose-escalation, multiple-dose study of the safety, tolerability, and immune response of CRS-207 in adult subjects with selected advanced solid tumors who have failed or who are not candidates for standard treatment **C** NCT00585845	Treatment-refractory mesothelioma, pancreatic ductal adenocarcinoma (PDA), non-small cell lung cancer (NSCLC), or ovarian cancer Safety study to determine dose-limiting toxicities; 17 patients (five mesothelioma, seven PDA, three NSCLC, and two ovarian cancer patients) received up to four doses; all applied doses were well tolerated; mesothelin-specific $CD8^+$ T-cell responses were induced in six out of ten evaluated patients, but the

(*continued*)

Table 2 (continued)

Therapeutic strain	Title/status[a]/identifier[b]	Tumor type/study objectives and outline/result
		response did not correlate with survival; 37% of patients lived for at least 15 months (Le et al. 2012)
Listeria monocytogenes ADU-623	Phase I study of safety and immunogenicity of ADU-623, a live-attenuated *Listeria monocytogenes* strain (ΔactA/ΔinlB) expressing the EGFRvIII-NY-ESO-1 vaccine, in patients with treated and recurrent WHO grade III/IV astrocytomas **O** NCT01967758	Grade III or grade IV astrocytic tumors Safety, tolerability, and immunogenicity of four intravenous doses (every 21 days) of *L. monocytogenes* strain ADU-623 producing EGFRvIII and NY-ESO-1 tumor antigens; maximum tolerated dose, tumor burden, and humoral and cellular immune responses will be determined
Salmonella typhimurium VNP20009	A phase I trial of a live, genetically modified *Salmonella typhimurium* (VNP20009) for the treatment of cancer by intravenous administration **C** NCT00004988	Advanced and/or metastatic solid tumors Safety study of a single intravenous injection of attenuated *S. typhimurium* VNP20009 strain, modified to preferentially colonize tumors; evaluation of dose-related toxicities, selective replication in tumors, and antitumor effects Twenty-four patients with metastatic melanoma and one patient with metastatic renal cell carcinoma received a single intravenous dose, in either of three escalating doses to determine the maximum tolerated dose. Dose-limiting toxicities were observed for the highest dose. Tumor colonization was observed only in three patients; none of the patients experienced objective tumor regression
Salmonella typhimurium VNP20009	A phase I trial of a live, genetically modified *Salmonella typhimurium* (VNP20009) for the treatment of cancer by intratumoral	Advanced or metastatic solid tumors Determination of safety, efficacy, and maximum tolerated dose of intratumoral VNP20009 injection

(*continued*)

Table 2 (continued)

Therapeutic strain	Title/status[a]/identifier[b]	Tumor type/study objectives and outline/result
	injection **C** NCT00004216	Maximum tolerated dose has not been reached
Salmonella typhi Ty21a	VXM01 phase I dose-escalation study in patients with locally advanced, inoperable, and stage IV pancreatic cancer to examine safety, tolerability, and immune response to the investigational VEGFR-2 DNA vaccine VXM01 **O** NCT01486329	Stage IV pancreatic cancer Determination of dose-limiting toxicities and the maximum tolerated dose, immune response, tumor staging according to RECIST criteria, after oral administration of VXM01 – *S.* Typhi Ty21a strain carrying VEGFR2 coding sequence under the control of eukaryotic promoter in order to deliver cDNA to monocytes and dendritic cells for the antigen presentation
Mycobacterium bovis – Bacillus Calmette-Guérin (Tice strain, Chicago Research Laboratories)	The study conducted in the Surgery Branch of the National Cancer Institute (Bethesda, Maryland) from 1967 to 1970 or in the Division of Oncology, Department of Surgery, UCLA School of Medicine (Los Angeles, California) from 1971 to 1974 (Morton et al. 1974) **C**	Patients with recurrent melanoma, known residual disease, or a high risk of developing recurrence 36 patients with intracutaneous lesions, treated with intratumoral injections, were the most likely responders to BCG when compared to the patients treated by any other investigated route or to the patients with subcutaneous or visceral metastatic lesions. In the group of 36 patients, 91% of injected lesions underwent complete regression, 17% of patients had regression of not injected melanoma nodules, and 31% of patients remained free of disease from 6 to 74 months following the therapy (Morton et al. 1974)
Mycobacterium bovis – Bacillus Calmette-Guérin in combination with ipilimumab	A phase I study of intralesional Bacillus Calmette-Guérin (BCG) and followed by ipilimumab therapy in patients with advanced metastatic melanoma **O** NCT01838200	Metastatic melanoma Safety study of one intralesional BCG injection followed by four intravenous ipilimumab injections every 3 weeks, starting on day 36 after BCG; clinical efficacy and tumor-specific immune responses will be

(*continued*)

Table 2 (continued)

Therapeutic strain	Title/status[a]/identifier[b]	Tumor type/study objectives and outline/result
		assessed; ipilimumab is an anti-CTLA-4 monoclonal antibody approved for melanoma treatment
Mycobacterium bovis – Bacillus Calmette-Guérin	The SILVA study: Survival in an International Phase III Prospective Randomized LD Small Cell Lung Cancer Vaccination Study with Adjuvant BEC2 and BCG **C** NCT00006352	Limited stage small-cell lung cancer (SCLC); patients must have achieved clinical response to first-line chemotherapy with no evidence of progression or relapse Determination of safety, progression-free survival and the patient's quality of life; patients received five intradermal doses of BCG and monoclonal antibody BEC2 every 2 weeks; BEC2 is an anti-idiotypic antibody that mimics ▶ GD3 (ganglioside expressed on tumor cells); vaccination had no impact on outcome of patients nor quality of life (Giaccone et al. 2005)
Mycobacterium bovis – Bacillus Calmette-Guérin in combination with Melaxin (autologous dendritoma vaccine)	Phase II, open-label trial in patients with stage IV malignant melanoma using Melaxin as a cancer vaccine in conjunction with BCG **C** NCT00671554	Melanoma Safety and response to treatment were assessed; all three enrolled patients had progression of the disease within 18 months post study completion

[a]Status: **O**, ongoing; **C**, closed (completed or terminated)
[b]Identifier according to www.clinicaltrials.gov database (2014-07-31)

Anticipated High-Impact Results

The perspectives for the development of effective bacterial cancer therapies are within the scope of four vital areas.

1. **Modification of bacterial metabolism or application of targeting molecules in order to promote preferential tumor localization and colonization**
   The evidence from preclinical studies on *Salmonella* shows that co-localization of bacteria and tumor antigen is important for therapeutic efficacy, and the intratumoral injection of bacteria more efficiently inhibits tumor growth than

systemic administration. The increased bacterial ability to selectively target tumors and infect predominantly cancer cells rather than normal cells was demonstrated to improve therapeutic efficacy (Bereta et al. 2007; Massa et al. 2013). Hence, a lack of tumor regression after *S. typhimurium* VNP20009 intravenous infusion to patients was initially thought to result from inefficient tumor colonization. However, it was only slightly improved when the bacteria were injected directly into the tumor (Nemunaitis et al. 2003), indicating that bacterial tumor targeting in humans is an issue more complex than expected. It is possible that the increased survival of bacteria in tumor or the increased retaining of bacteria at the tumor site or higher rate of infection of tumor cells or enhanced damage in the tumor tissue due to bacterial invasion of cancer cells, achieved by genetic modifications of bacteria, could improve the clinical outcome.

2. **Delivery of new tumor antigens or novel molecules with immunostimulatory potential or the combinations of the above**
3. **Personalization of treatment according to clinical status and genetic profile including the genes with predictive value**
   Genetic polymorphism may have the prognostic value for bacterial anticancer therapies, as it was already proposed for BCG treatment of bladder cancer. The allele variabilities of inflammation-associated genes (IL-8, TNF, IL-6, TGFβ, COX-2, NF-κB) and factors that influence innate defense to intracellular parasites, such as NRAMP1 (SLC11A1), were associated with differences in the risk of recurrence (Lima et al. 2012).
4. **Standardization of novel treatment regimens favoring the effectiveness of immunotherapy**
   The superior preclinical efficacy of early intervention, i.e., the administration of bacteria soon after tumor implantation, rather than to animals with large tumors, as well as the efficacy of BCG in recurrence of superficial bladder cancers, supports the notion of bacterial immunotherapy as the therapeutic option for patients with minimal tumor burden.

   Repeated administrations of bacterial therapeutics may help to overcome tumor tolerance, as they would constantly deliver a danger signal consisting of PAMPs and DAMPs that stimulate the maturation of antigen presenting cells and effective T-cell responses. The maintenance therapy could bypass the drawback of single tumor antigen-specific approaches, as they inevitably drive the selection of a novel antigen repertoire. Sustained treatment with nonspecific agents should not accelerate the selection of novel tumor cell variants but rather promote effective tumor recognition and killing. The concept of repeated vaccination aimed at the delivery of a danger signal co-localized with antigen has been proposed by Polly Matzinger as the remedy for low effectiveness of tumor vaccines (Matzinger 2012). The clinical experience with BCG treatment for bladder cancer proved the efficacy of repeated vaccination localized to the tumor lesion.

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Brachyury

9

Claudia Palena

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Abstract

The switch of human carcinoma cells from an epithelial to a mesenchymal-like phenotype is now being recognized as a potentially important process along the metastasis of solid tumors. The phenotypic switch, termed epithelial-to-mesenchymal transition (EMT), has also been associated with resistance of carcinoma cells to chemotherapy, radiation, and some small-molecule-targeted therapies. Brachyury is a T-box transcription factor that has recently been identified as a driver of the EMT process in human carcinoma cells. Overexpression of brachyury in epithelial cancer cells has been shown to drive the acquisition of a mesenchymal-like tumor phenotype, a more migratory and invasive phenotype, and a greater resistance to chemotherapy and radiation. Preferentially expressed in human tumor tissues while being absent in the majority of adult normal tissues,

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brachyury exhibits a highly tumor-associated pattern of expression and has now been correlated with poor prognosis in multiple tumor types. Preclinical data as well as evaluation of antigen-specific responses in the blood of cancer patients have demonstrated that brachyury is an immunogenic molecule. Based on those results, recombinant vaccine vectors targeting the transcription factor brachyury have been developed and are undergoing clinical evaluation in patients with advanced carcinomas. We anticipate that these vaccines could help eliminate tumor cells highly metastatic and prone to survive most of the currently available therapeutic agents.

Keywords

Brachyury • Chemotherapy/radiation resistance • Epithelial-to-mesenchymal transition (EMT) • Expressed sequence tag (EST) clusters • Identification of cancer cells • In cancer • Recombinant *Saccharomyces cerevisiae* • RT-PCR • Tumor dissemination and metastasis • T-box proteins • Transcription factor T (TFT) (*see* brachyury)

Introduction

The gene encoding for brachyury, also known as “T” or “Transcription Factor T (TFT)”, was initially identified in mice heterozygous for a mutation in this gene, which are characterized by an arrest in mesoderm formation (Herrmann et al. 1990). Homologues of brachyury have since been identified in a vast variety of multicellular organisms, including humans (Edwards et al. 1996). Brachyury is a member of a large family of transcription factors, the T-box family, characterized by a conserved DNA-binding domain of approximately 200 amino acids, designated as the T-box (Kispert and Herrmann 1993). In general, T-box proteins play essential roles during early development (Showell et al. 2004); the expression of brachyury, in particular, is known to be required for the formation and organization of mesoderm during the development of vertebrates (Kispert et al. 1994). In the mouse, for example, brachyury is only transiently expressed in a highly tissue-specific manner, restricted to the notochord and early mesoderm cells in the embryo (Wilkinson et al. 1990).

A computer-based differential display of expressed sequence tag (EST) clusters in the Unigene human database recently identified brachyury as a gene highly represented in tumor-derived libraries and rarely observed in normal tissue-derived libraries (Palena et al. 2007). Brachyury-encoding ESTs were enriched in libraries derived from lung carcinoma cell lines, germ cell tumors, chronic lymphocytic leukemia B cells, and breast cancer (Palena et al. 2007). Subsequent studies demonstrated high expression of brachyury mRNA in a variety of human carcinomas, including tumors of the lung, breast, colon, small intestine, stomach, kidney, bladder, uterus, ovary, testis, and prostate. Additionally, overexpression of brachyury mRNA was reported in cell lines of the lung, colon, and prostate cancers, but not in the majority of normal tissues evaluated, with the exception of the testis (Palena

et al. 2007). The expression of brachyury has also been reported in human teratocarcinoma lines (Gokhale et al. 2000), and brachyury gene duplication has been associated with susceptibility to familial chordoma (Yang et al. 2009). Immunohistochemical studies of multiple human normal and cancer tissues using a brachyury-specific monoclonal antibody have validated the RT-PCR results. Brachyury protein is detectable in human tumor tissues but not in the majority of human normal tissues, thus reinforcing the concept that brachyury is a tumor-associated molecule (Roselli et al. 2012).

Biology of the Target

A work by Fernando and colleagues (Fernando et al. 2010) recently demonstrated that brachyury functions as a master regulator of the epithelial-to-mesenchymal transition (EMT) in human carcinoma cells. EMT is a reversible process during which cells switch from a polarized, epithelial phenotype into a highly motile, mesenchymal phenotype (Thiery and Sleeman 2006; Kalluri and Weinberg 2009). While EMT is a normal process during embryogenesis and organogenesis, numerous observations now support the concept that EMT also plays an essential role in the progression of carcinomas (Thiery 2002). During the metastasis of carcinomas, tumor cells must undertake a series of sequential steps that will allow them to detach from the primary tumor mass and to finally reach the distant sites of metastasis. By undergoing EMT, tumor cells can acquire the ability to move and to invade the surrounding tissues, two fundamental properties for tumor dissemination. In addition, several reports are now indicating that tumor cells undergoing EMT also acquire stem cell-like features and mechanisms of resistance to cell death (Arumugam et al. 2009; Vega et al. 2004). The induction of EMT in various cancer cell lines, for example, has been shown to positively correlate with resistance to radiation (Kurrey et al. 2009), chemotherapy (Yang et al. 2006), and epidermal growth factor receptor (EGFR) kinase inhibitors (Thomson et al. 2005). Moreover, mesenchymal-like tumor cells with markers of tumor stemness (CD44+/CD24low) have been observed among residual breast cancer cell populations that survived conventional therapies (Creighton et al. 2009), an observation that strengthens the link between EMT, cancer stem cells, and therapeutic resistance.

A work conducted by Fernando and colleagues (Fernando et al. 2010) demonstrated that the upregulation of brachyury in human epithelial cancer cells results in morphological changes representative of EMT, including the acquisition of a fibroblast-like morphology, the loss of the epithelial markers E-cadherin and plakoglobin, and enhanced levels of the mesenchymal proteins fibronectin, N-cadherin, and vimentin (Fig. 1a). As a consequence of this phenotypic switch, human carcinoma cells undergoing brachyury-mediated EMT acquire enhanced motility and the ability to invade the basal membrane and extracellular matrix (ECM) (Fig. 1b). In additional experiments, the stable silencing of brachyury expression in brachyury-positive human carcinoma cells (lung cancer H460 cells) resulted in downregulation of mesenchymal markers, upregulation of epithelial

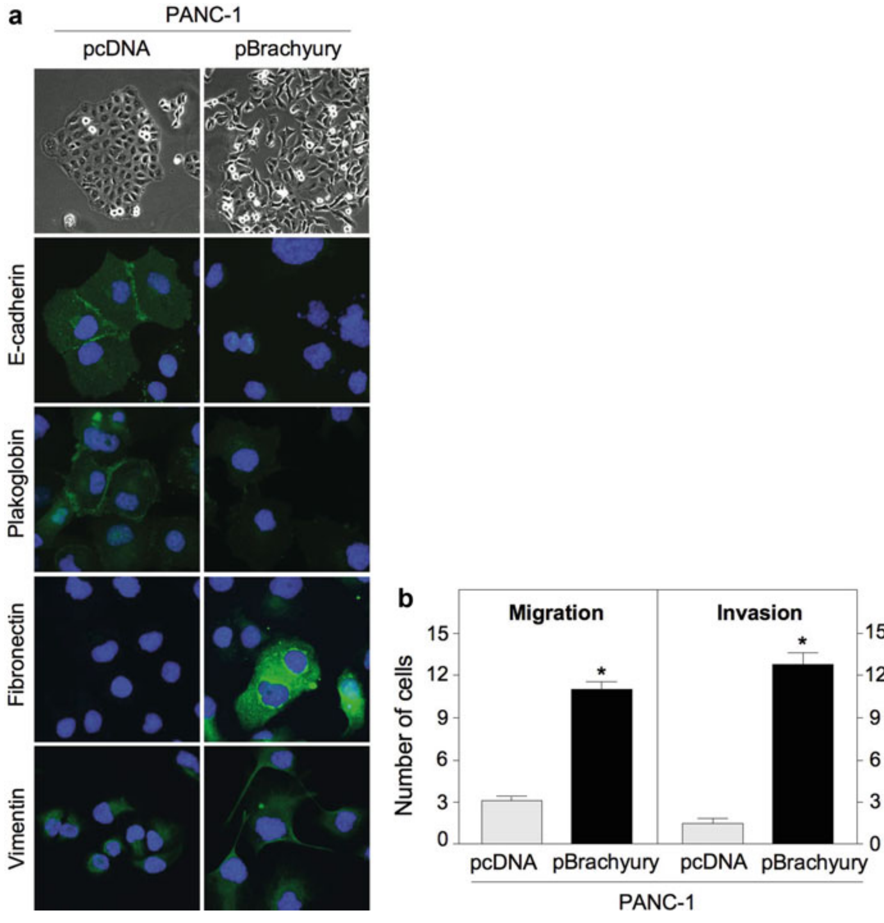

Fig. 1 Brachyury induces epithelial-to-mesenchymal transition (EMT) in human carcinoma cells. (**a**) Pancreatic carcinoma PANC-1 cells were stably transfected with a control pcDNA or a vector encoding for full-length Brachyury (pBrachyury). *Top panels*: bright field images of cells grown on plastic surface. *Bottom panels*: immunofluorescence analysis of EMT markers in cells grown on cover glasses. The *green signal* represents the staining of the corresponding protein, and the *blue signal* represents the 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei. (**b**) In vitro cell migration and extracellular matrix (ECM) invasion assays (Reproduced from Fernando et al. (2010))

markers, and significant loss of cell migration and invasion (Fernando et al. 2010). Inability to invade the ECM after brachyury inhibition was concomitant to a significant reduction in the expression of genes encoding for matrix metalloproteinase-2 and -24 (MMP-2, MMP-24), proteins previously shown to be overexpressed in tumors and to play a role in cancer progression (Ji et al. 2005; Guo et al. 2007). Therefore, reduction of brachyury expression in H460 tumor cells induced changes reminiscent of a mesenchymal-epithelial transition (MET). Xenograft experiments conducted in immunocompromised mice demonstrated that

brachyury expression does not affect the growth of the primary tumor, as brachyury-inhibited H460 cells grew as subcutaneous tumors at rates similar to the control, brachyury-high, H460 cells (Fig. 2a). Inhibition of brachyury, however, resulted in a significant impairment on the ability of H460 cells to disseminate from the subcutaneous tumor to the site of metastasis (Fig. 2b). Moreover, brachyury-inhibited tumor cells had a reduced ability to establish experimental lung metastasis after intravenous injection (Fig. 2c).

Overexpression of brachyury in human carcinoma cells has also been shown to attenuate cell cycle progression in vitro and to correlate with lower levels of cyclin D1 expression (Fernando et al. 2010). A lower proliferation rate of mesenchymal-like tumor cells may be protective against radiation-induced or chemotherapy-

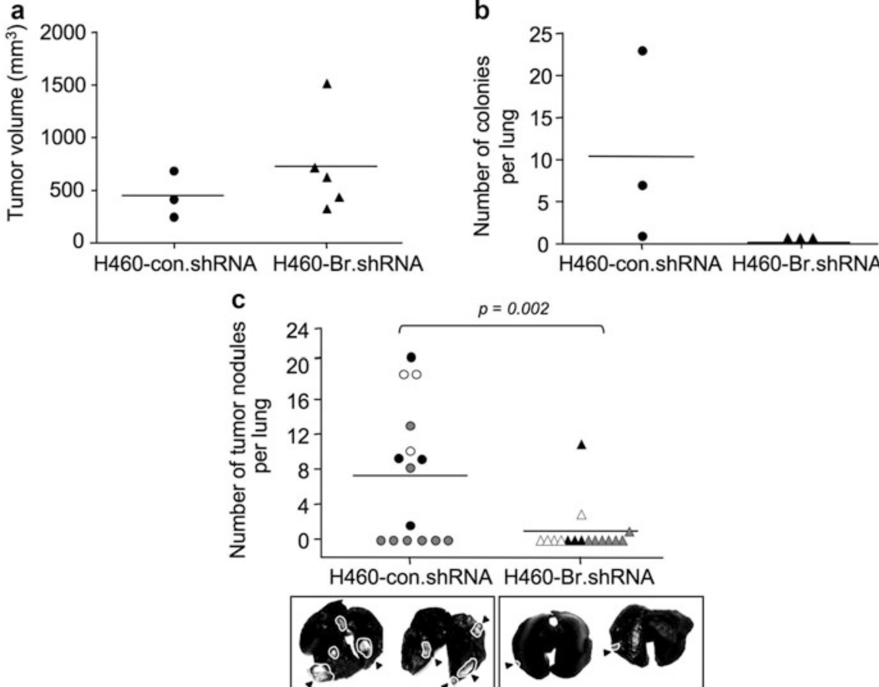

Fig. 2 Brachyury controls tumor dissemination and metastasis. (**a**) H460 cells stably transfected with control shRNA (*circle*) or Brachyury-specific shRNA (Br.shRNA, *triangle*) vectors were injected subcutaneously in nude mice. Graph shows tumor volumes at day 15 post-tumor implantation. (**b**) Lungs from animals bearing subcutaneous tumors were collected, homogenized, and cultured in puromycin-containing medium. Graph shows visible colony counts. (**c**) Mice were inoculated with H460 cells transfected as indicated via the tail vein. Forty-five days after tumor implantation, animals were euthanized and lungs were evaluated for tumor nodules. Graph shows results from three independent experiments. Experiments 1–3 are denoted by *black*, *gray*, and *white circles* (con.shRNA) and *triangles* (Br.shRNA), respectively. Two representative lungs from each group are shown for comparison. *White outlines* and *black arrowheads* point to tumor masses (Reproduced from Fernando et al. (2010))

induced genotoxic stress. Overexpression of brachyury in human carcinoma cells has been shown to enhance resistance of tumor cells to chemotherapy and radiation, while silencing of brachyury in tumor cells that naturally express high levels of this transcription factor has resulted in enhanced susceptibility to both types of therapeutics, in vitro (Huang et al. 2013). These results are in agreement with previous reports demonstrating a direct correlation between the acquisition of a mesenchymal phenotype by carcinoma cells and enhanced resistance to a variety of cell death-inducing signals. For example, induction of oxaliplatin resistance in colorectal cancer cell lines by chronic exposure to oxaliplatin has been reported to induce a phenotypic change indicative of EMT (Yang et al. 2006). Similarly, paclitaxel resistance and radioresistance in ovarian cancer cells have also been associated with a switch from an epithelial to a mesenchymal phenotype (Kurrey et al. 2009; Kajiyama et al. 2007). Collectively, these results demonstrate that the transcription factor brachyury confers on epithelial tumor cells a mesenchymal-like phenotype, migratory and invasive abilities, resistance to cell death mechanisms, and enhanced metastatic propensity. In additional studies, we have demonstrated that tumor cells undergoing EMT via brachyury overexpression secrete IL-8, which, in turn, can induce brachyury expression in carcinoma cells (Fernando et al. 2011; Palena et al. 2012).

Target Assessment

Reverse transcription-polymerase chain reaction (RT-PCR) studies have demonstrated enhanced expression of brachyury mRNA in the following human carcinomas: lung, breast, colon, small intestine, stomach, kidney, bladder, uterus, ovary, testis, and prostate, as well as in chronic lymphocytic leukemia cells, while its expression was undetectable or extremely low across adult normal tissues examined, with the major exception of the testis (Palena et al. 2007; Hamilton et al. 2012). As an immunologically privileged site, it is anticipated that the testis would not be a target of brachyury-specific T cells generated postvaccination against brachyury (see below).

Role of the Target in Cancer

Rank: 9 (high).

Multiple reports support the involvement of EMT in the progression of human carcinomas. The downregulation of epithelial E-cadherin, for example, has been previously associated with tumor progression and metastasis (Onder et al. 2008). Similarly, the switch from epithelial E-cadherin to mesenchymal N-cadherin (low expression of E-cadherin and high expression of N-cadherin) in carcinomas has been associated with poor prognosis (Gravdal et al. 2007). Other transcription factors that regulate EMT have been identified, including twist, snail, and slug, among others, which have been shown to drive the switch of carcinoma cells from an epithelial to a mesenchymal phenotype in various types of human carcinomas. The expression of

some of these EMT transcription factors has also been associated with tumor progression in several types of cancers. For example, snail overexpression correlates with breast or cervical cancer progression (Moody et al. 2005; Elloul et al. 2005), and overexpression of twist was reported in prostate cancers of high Gleason score or in cervical carcinomas from patients with a poor disease outcome (Shibata et al. 2008; Kwok et al. 2005). However, unlike brachyury, mRNA encoding for the EMT transcription factors twist, snail, and slug can be detected at high levels in multiple human normal tissues, setting apart brachyury as a tumor-associated target for therapeutic interventions against metastasis. Real time PCR analyses in multiple human lung tumor tissues have also demonstrated brachyury mRNA expression in approximately 35% of Stage I lung cancers and approximately 60% of Stages II–IV lung cancers, thus indicating an association of brachyury with lung tumor stage and a potential association of brachyury with lung tumor progression (Fernando et al. 2010). Brachyury expression was also evaluated by immunohistochemical analysis in tumor tissues obtained from 748 cases of colorectal cancer (Kilic et al. 2011). The authors demonstrated expression of brachyury in $>80\%$ of colon cancer samples and a statistically significant association ($p = 0.034$) of brachyury staining with a poor 25-year survival outcome in early stage colorectal cancer patients (T1-2N0M0, Dukes A, $n = 191$), reinforcing the idea that brachyury expression in epithelial tumors is associated with more aggressive disease.

High-Level Overview

Several strategies can be envisioned to specifically target tumor cells undergoing EMT. As this process may be initiated and or maintained via signaling events mediated by cytokines, growth factors, and/or other components of the tumor microenvironment, specific inhibitors of those signaling pathways could be used to revert the phenotype of mesenchymal tumor cells into a more epithelial one (Palena et al. 2011). For example, fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin-like growth factors (IGFs), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-ß), tumor necrosis factor-alpha (TNF-α), Wnt ligands, and components of the ECM signaling through surface integrins have all been implicated in the induction of EMT (Palena et al. 2011). One limitation of this strategy, however, is that signaling events that initiate and/or maintain EMT in tumor cells may be multiple and redundant, thus reducing the effectiveness of the approach. Because the multiple signaling events may ultimately converge to upregulate the expression of EMT transcription factors, these molecules appear as attractive targets. Targeting of transcription factors is however difficult, and these molecules are currently regarded as "undruggable" with canonical small molecule-targeted therapeutics. Moreover, transcription factors cannot be targeted by modalities that depend on the cell-surface expression of the target, such as monoclonal antibodies. Alternatively, T cells are able to recognize short peptide fragments derived from the antigen, which are presented on the cell surface as complexes with the major histocompatibility (MHC) class I and II

molecules. Thus, T cells induced by cancer vaccines may be a suitable means to target tumor cells that overexpress the transcriptional regulator of EMT, brachyury.

Evidence on the role of the immune system in limiting tumor growth and progression in humans is linked to observations indicating a positive correlation between the presence of tumor-infiltrating CD8+ T cells and good prognosis in various types of cancer. In colorectal cancer, for example, significantly higher levels of early memory and effector memory CD8+ T-cell infiltrates positively correlate with good clinical outcome, defined as the absence of metastatic invasion, less advanced pathological stage, and increased survival (Pages et al. 2005; Galon et al. 2006; Jochems and Schlom 2011). Altogether, these observations support a role for the immune system in controlling tumor burden and form the rationale for the development of vaccine-based interventions against cancer that rely on the stimulation of an effective antitumor immune response in the host (Palena and Schlom 2010; Palena et al. 2011). It is expected that a long-lasting immune response directed against brachyury will eradicate a subpopulation of tumor cells undergoing EMT, therefore reducing the dissemination of the tumor and/or the establishment of chemotherapy-resistant tumors. It is important to point out that the phenomenon of EMT might only involve a few cells at the invasive front of a tumor mass. Therefore, unlike other antitumor therapies aimed at targeting the bulk of the tumor mass, targeting of tumor cells undergoing EMT could constitute a conceptually novel antitumor strategy aimed at reducing metastasis by eliminating tumor cells that exhibit a more aggressive and drug-resistant phenotype.

Diagnostic and Prognostic

The identification of cancer cells with high metastatic potential or resistance to cell death within a preneoplastic lesion or a primary tumor mass would be extremely important in identifying patients with more aggressive tumors. The use of immunohistochemistry with a monoclonal antibody directed against brachyury may be thus useful in identifying such tumor cell populations and, consequently, in selecting patients at high risk for metastasis that may require more aggressive treatment regimens. Recent advances in the detection of circulating tumor cells (CTCs) in the blood of cancer patients have demonstrated an association between the presence of CTCs in the blood and poor outcome in certain types of cancer (Miller et al. 2010). Further evaluating the expression of brachyury in CTCs by either flow cytometry or polymerase chain reaction (PCR) methodology would help in understanding whether the expression of this transcription factor and, in consequence, a more mesenchymal phenotype in CTCs associates with a poorer disease outcome.

Therapeutics

As a regulator of EMT in human carcinoma cells, the transcription factor brachyury is expected to participate in the control of two critical aspects of tumor progression:

metastatic dissemination and the acquisition of chemotherapy/radiation resistance. The highly selective expression of brachyury in human carcinomas and its potential relevant role in tumor progression make this molecule an excellent target for cancer therapy. It can be hypothesized that, if employed at early stages of disease, strategies targeting brachyury-positive tumor cells might be effective at reducing or eliminating tumor cells with a more metastatic phenotype within a tumor mass, thus preventing the dissemination of tumor cells from the primary site. Moreover, once tumor cells have disseminated, the targeting of brachyury-positive tumor cells could also result in the alleviation of tumor resistance, thus improving the efficacy of conventional antitumor therapies such as radiation and chemotherapy.

Preclinical Summary

By using a MHC-peptide-binding prediction algorithm, a human HLA-A2 binding brachyury 9-mer peptide has been identified, WLLPGTSTL, and has been successfully employed to expand, in vitro, human brachyury-specific CD8+ T cells from the blood of both normal donors and cancer patients (Palena et al. 2007). T cells generated with this peptide were able to efficiently lyse peptide-pulsed target cells, even at low peptide concentrations, as well as tumor targets endogenously expressing brachyury. Tumor cell lines used as targets included the lung carcinoma cells H441 (HLA-A02+/brachyury+) and NCI-H460 (HLA-A2-/brachyury+), the colorectal carcinoma line SW1463 (HLA-A02+/brachyury-), and the pancreatic carcinoma cells AsPC-1 (HLA-A02-). T cells expanded with the brachyury peptide were highly efficient at killing lung H441 tumor cells, while no lysis was observed against the other cell lines, thus indicating that the lysis was MHC-restricted and antigen-specific (Fig. 3). These results have demonstrated that a T-box transcription

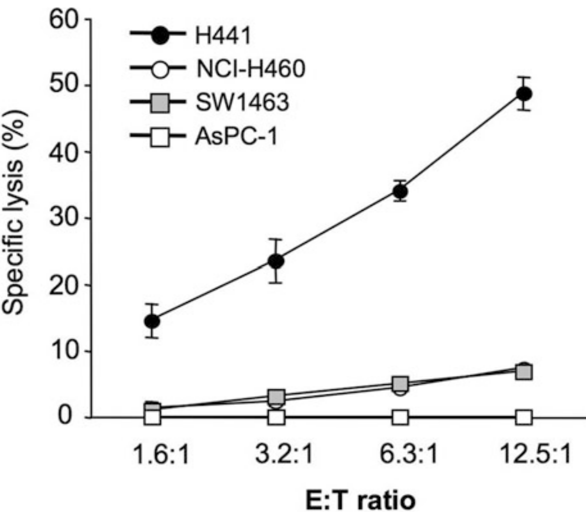

Fig. 3 Brachyury-specific T cells lyse brachyury-positive tumor cells. Brachyury-specific, cytotoxic T lymphocytes were used as effectors against various tumor targets in an ^{111}In 16-h release assay, as indicated. The following tumor cell lines were used: H441 (HLA-A2+/brachyury+), NCI-H460 (HLA-A2-/brachyury+), SW1463 (HLA-A2+/brachyury-), and ASPC-1 (HLA-A2-) (Reproduced from Palena et al. (2007))

factor and a molecule implicated in EMT can be a potential target for human T-cell-mediated cancer immunotherapy. The findings have thus provided the rationale for the development of a therapeutic vaccine against brachyury-positive tumor cells, which are expected to exhibit high metastatic propensity.

Clinical Summary

A phase I clinical trial employing a recombinant *Saccharomyces cerevisiae* (yeast) – brachyury vaccine has been completed in patients with advanced carcinoma (Open Label Study to Evaluate the Safety and Tolerability of GI-6301 n.d.).

Anticipated High-Impact Results

If it is demonstrated that brachyury-based vaccines can generate human T-cell responses in cancer patients, such vaccines may have a major impact on the prevention and/or control of metastasis and drug resistant populations for a range of human tumor types. The US Food and Drug Administration (FDA) approval of a therapeutic vaccine for prostate cancer (Provenge, Sipuleucel-T, Dendreon Corporation) and the promising results with the use of other vaccines such as PSA-TRICOM encoding for prostate-specific antigen and the triad of costimulatory molecules B7-1, ICAM-1 and LFA-3 (TRICOM) (Prostvac, Bavarian Nordic) (Kantoff et al. 2010) for the therapy of solid tumors pave the way for the development of a brachyury-based vaccine. Vector-based vaccine platforms expressing brachyury are currently in development. Perhaps the most appropriate therapeutic application for a brachyury-based vaccine will be in the neoadjuvant or adjuvant settings. If and when a long-term safety profile for such vaccines is established, along with evidence of patient benefit in clinical trials, it is possible that brachyury-based vaccines could also be employed, in the long term, in the preneoplastic and/or the preventive setting.

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CCL21

10

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Abstract

Chemokines that attract both dendritic cells (DC) and lymphocyte effectors can aid in cancer immunotherapy by enlisting host immune cells to recognize tumors of low immunogenicity. CCL21 mediates the recruitment and co-localization of naive lymphocytes and antigen-stimulated DC into T-cell zones of secondary lymphoid organs, facilitating T-cell activation. In this chapter, we discuss CCL21 as a novel agent to boost immune responses against cancer. Based on the findings on CCL21, it is anticipated that rational combinations with other treatment

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modalities will improve the therapeutic efficacy of this chemokine and antitumor benefit in a broad range of solid tumors.

Keywords
Chemokine • CCL21 • T lymphocytes • Dendritic cells • Immune activation • Anti-tumor immune activity • Lung cancer

Target

CCL21

Biology of the Target

Chemokines, a group of homologous, yet functionally divergent proteins, directly mediate leukocyte migration and activation and play a role in regulating angiogenesis. They also function in maintaining immune homeostasis and secondary lymphoid organ architecture. Secondary lymphoid chemokine (CCL21) (also known as thymus-derived chemokine 4, 6Ckine, or Exodus 2) is expressed by high endothelial venules, lymphoid endothelial cells, stromal cells within T-cell areas of the lymph nodes, spleen, and Peyer's patches. Acting through the G-protein coupled CCR7 transmembrane receptor, CCL21 mediates the recruitment and colocalization of naive lymphocytes and antigen-stimulated dendritic cells (DC) into T-cell zones of secondary lymphoid organs, facilitating T-cell activation (Cyster 1999; Gollmer et al. 2009). T-cell activation in vivo occurs in a lymphoid milieu that presents chemotactic and T-cell receptor signals concurrently. The T-cell zone chemokines such as CCL21 are bound to the surface of lymph node DC. Contact with antigen-presenting cells bearing CCL21 chemokine costimulates T cells by a two-step contact mechanism. T cells initially form an antigen-independent "tethered" adhesion on CCL21-bearing antigen-presenting cells. The formation of these tethers supersedes T-cell receptor signaling and immunological synapse formation. However, chemokine-tethered T cells are hyperresponsive to subsequent contacts with antigen-presenting cells. Thus, T cells are costimulated "in trans" and sequentially after initial engagement with their CCL21-rich environment (Friedman et al. 2006). In addition to inducing chemotactic migration, CCL21 costimulates expansion of $CD4^+$ and $CD8^+$ T cells and induces Th1 polarization. The immune suppressor cell population, $CD4^+CD25^+$ regulatory T cells are hyporesponsive to CCL21-induced migration and unresponsive to CCL21 costimulation. These functions of CCL21 to both attract naive T cells as well as costimulate their proliferation, differentiation, and activation suggest that CCL21 is a pivotal molecule for priming T-cell responses and has therapeutic implications for local delivery of CCL21 (Flanagan et al. 2004). The antitumor effectors NK and NKT cell subsets also express the CCR7 receptor and are chemoattracted by CCL21.

Target Assessment

CCL21 can be quantified in samples by high sensitivity RT-PCR and by ELISA using commercially available antibodies from R&D Systems (Minneapolis, MN). For the phase I clinical trials, following CCL21 gene modification of DC, CCL21 production is quantified by ELISA. Tissue expression of CCL21 can be evaluated by immunocytochemistry. CCL21 determinations have not been standardized or validated for clinical practice yet.

Role of CCL21 in Cancer

CCL21 priority ranking is 13 among the list of 20 National Cancer Institute ranked biological agents with high potential for use in cancer therapy. Generation of an antitumor immune response requires the coordinate interaction of NK, T, and DC effectors. There is a paucity of these effectors in the tumor. One regimen to initiate antitumor responses is through the use of chemokines that induce both efficient recruitment and strong activation of effector cells in the tumor mass. The rationale for the use of CCL21 for immune therapy against solid tumors is that CCL21 modulates host immune responses by recruiting and colocalizing NK, DC, and T-cell effectors to mediate antitumor activity.

High Level Overview

One of the challenges in developing immunotherapy for cancer is enlisting the host response to recognize tumors of poor immunogenicity. Effective antitumor responses require antigen-presenting cells (APC), lymphocyte, and NK effectors. Although cancer cells express tumor antigens, the limited expression of major histocompatibility complex (MHC) antigens, defective transporter associated with antigen processing, and lack of costimulatory molecules make them ineffective APC. Effective anticancer immunity can be achieved by recruiting professional host APC for tumor antigen presentation to promote specific T-cell activation. DCs are uniquely potent APCs involved in the initiation of immune responses. Serving as immune system sentinels, DCs are responsible for Ag acquisition in the periphery and subsequent transport to T-cell areas in lymphoid organs where they prime specific immune responses. Thus, chemokines that attract both DC and lymphocyte effectors could serve as potent agents in immunotherapy. The rationale for utilizing CCL21 in cancer therapy is to facilitate the colocalization of DC, T, NK, and NKT cells to orchestrate effective cell-mediated immune responses in the tumor microenvironment. CCL21 may be distinctly advantageous because of its capacity to elicit a type 1 cytokine response in vivo that promotes antitumor activity. Intratumoral infiltration of T lymphocytes and DC in lung cancer has been shown to be associated with a better patient outcome. NK cells and NKT cells induce antitumor responses, and the recruitment of NK and NKT cells by CCL21 augments antitumor activity

because these effectors can recognize tumor targets in the absence of MHC expression. In addition to expressing the CCR7 receptor, NK, NKT cells, and Th1 cells express the CXCR3 receptor and migrate in response to the CXCR3 ligands CXCL9, CXCL10, and CXCL12. CXCL9 and CXCL10 are potent inhibitors of angiogenesis. CCL21 induces CXCL9, CXCL10, and IL-12 from monocytes, DC, and stromal cells. The induction of IL-12, CXCL9, and CXCL10 further amplifies the antitumor responses of CCL21 in the recruitment of CXCR3 expressing effectors and inhibition of angiogenesis. The ability of CCL21 to inhibit angiogenesis has added further support for its use in cancer therapy.

Diagnostic, Prognostic, and Predictive

Diagnostic tests for CCL21 expression and protein concentrations in samples can be performed by RT-PCR and ELISA. Tissue expression of CCL21 can be assessed by immunocytochemistry. Based on the preclinical data, high levels of CCL21 expression in tumors may be indicative of immune reactivity and serve as a prognostic marker for patient survival. Immune effects of CCL21 can be monitored by antigen-specific IFN-γ T lymphocyte ELISPOTS, ELISA, or RT-PCR for Th1 cytokines and immunocytochemistry for T lymphocytes and DC effector cell infiltrates.

Therapeutics

CCL21 is being developed as an anticancer therapeutic agent. Current phase I clinical trials are in lung cancer and melanoma, but as the preclinical data warrants in other tumor models, this form of therapy may be extended to include other solid cancers.

Preclinical Summary

The development of intratumoral therapies to effectively augment local and *systemic* antitumor immunity in lung cancer can lead to a paradigm shift in the current forms of therapy. In preclinical model systems, intratumoral administration of DC led to both local and systemic antitumor responses (Sharma et al. 1997). This form of therapy can be augmented by utilizing intratumoral administration of genetically modified DC overexpressing certain cytokine genes (Miller et al. 2000). Congruent with this overall concept, the intratumoral administration of recombinant CCL21 mediated T-cell-dependent antitumor responses (Sharma et al. 2000). In immune competent mice, intratumoral CCL21 injection led to a significant increase in CD4 and CD8 T lymphocytes and DC infiltrating both the tumor and draining lymph nodes. Studies performed in CD4 and CD8 T-cell knockout mice revealed a direct therapeutic requirement for both CD4 and CD8 T-cell subsets for CCL21-mediated tumor regression. Based on these results, experiments were performed to evaluate

the tumorigenicity of CCL21 gene-modified murine lung cancer cells. In all three tumor models, subcutaneous implantation of retroviral-mediated CCL21 gene-modified lung cancer cells led to T-cell-mediated tumor eradication. Because the levels of CCL21 required for tumor rejection can be achieved by using CCL21-transduced DC as the transfer vehicle, the intratumoral injection of DC overexpressing CCL21 in the transplantable and spontaneous bronchoalveolar cell carcinoma models of lung cancer was evaluated (Yang et al. 2004, 2006). These studies demonstrated that DCs expressing CCL21 are highly effective means to achieve intratumoral delivery of CCL21 in murine models. There was anticipation that this therapy would be most effective in creating a "lymph node-like" environment at the tumor site. In fact James Mule reported that DC overexpressing CCL21 demonstrated effective antitumor immunity in lymphotoxin knockout mice that lacked lymph nodes (Kirk et al. 2001). Thus, based on the initial studies documenting the antitumor properties of CCL21 and gene-modified DC, this approach was adopted by other investigators who have reported immune-dependent, antitumor properties of CCL21 in a variety of tumor models, including lung (Sharma et al. 2000), colon (Vicari et al. 2000), melanoma (Kirk et al. 2001a, b; Novak et al. 2007), prostate (Yousefieh et al. 2009) {Turnquist 2007, #19383}, breast {Ashour 2007, #19378}, and liver (Liang et al. 2007). CCL21 acted as an adjuvant for TERT-DNA vaccine in a breast cancer model and showed immunologically mediated regression of pancreatic tumors in mice upon intratumoral delivery. CCL21 also enhanced the therapeutic efficacy of adoptive T-cell transfer in a murine model of melanoma. In all models, CCL21 demonstrated potent regression of tumors, which was shown to be dependent on host T-cell immunity. All these studies reaffirmed the antitumor efficacy of CCL21 further supporting the rationale to proceed with clinical investigations of this chemokine.

Clinical Summary

Based on the preclinical model systems, a clinical trial was initiated using intratumoral injection of CCL21 gene-modified autologous DC in lung cancer. The intratumoral route of DC administration is used to activate specific immune responses within the tumor microenvironment and, in addition, to generate systemic immunity. Several studies suggest (Sharma et al. 1997; Tatsumi et al. 2003) that intratumoral DC administration may be particularly effective as an antitumor strategy. Lung cancer patients have decreased numbers of circulating competent DC; thus, injecting DC within the lung tumor site may be a particularly effective approach. A correlation exists between the number of tumor-infiltrating DC and survival in cancer patients. In fact, there is a relationship between tumor-infiltrating DC aggregation and apoptosis in situ in human non-small cell lung cancer (NSCLC). This is consistent with recent studies indicating that attraction and activation of DC at the site of tumor elicits potent antitumor immunity (Lapteva et al. 2009). Dieu-Nosjean et al. (2008) have identified ectopic lymph node or tertiary lymphoid structures within human NSCLC specimens and demonstrated a correlation of

their cellular content with clinical outcome. These structures have been referred to as tumor-induced bronchus-associated lymphoid tissue, which are follicle-like and contain germinal centers, similar to those in secondary lymphoid follicles of lymph nodes. The density of DC-Lamp, mature DC within these structures, is a predictor of long-term survival in lung cancer patients (Dieu-Nosjean et al. 2008). These findings suggest that tumor-induced bronchus-associated lymphoid tissue has clinical relevance and participates in the host's antitumor immune response, and they are consistent with previously reported preclinical and clinical data (Zeid and Muller 1993; Kirk et al. 2001; Coppola and Mule 2008). For example, in murine tumor models, Mule (Kirk et al. 2001) reported that DC genetically modified to secrete CCL21 can produce lymphoid cell aggregates and, importantly, prime naive T cells extranodally within a tumor mass, resulting in the generation of tumor-specific T cells and subsequent tumor regression (Kirk et al. 2001a, b). Thus, the intratumoral approach may achieve tumor antigen presentation by using the tumor as an in vivo source of antigens for DC. In contrast to immunization with purified peptide antigen(s), autologous tumor has the capacity to provide the activated DC administered at the tumor site access to the entire repertoire of available antigens in situ. This may increase the likelihood of a response and reduce the potential for tumor resistance because of phenotypic modulation. On the basis of preclinical results, a phase I clinical evaluation has been initiated at the University of California Los Angeles (in collaboration with the National Cancer Institute – Rapid Access to Intervention Development program) in patients with advanced-stage NSCLC. The safety and clinical activities of the intratumoral administration of autologous DC transduced with a replication-deficient adenoviral vector to express CCL21. A GMP grade AdCCL21 replication-deficient virus (Baratelli et al. 2008) has been made available through the RAID program to conduct the phase I clinical trial. Human DCs transduced with advenovirus-CCL21 produce CCL21 to attract T cells and DCs. Preliminary findings demonstrate tumor-specific systemic immune responses as assessed by the IFN-γ T-cell ELISPOT. Multiplex assessment of plasma cytokines before and after therapy in these patients revealed induction of IL-2, IFN-γ, IL-12, and CXCL10. Immunohistochemistry of posttumor biopsies revealed an influx of CD4-expressing tumor-infiltrating lymphocytes.

In melanoma, Mule's group have genetically modified human tumor lysate pulsed (TL) DC to secrete human CCL21 that, similar to preclinical studies, could potently recruit naive human CD4 and CD8 T cells. They showed for the first time that TL-DC secreting the CCL21 could significantly enhance the level or number of tumor antigen-specific T cells to at least 2 specific melanoma peptides (i.e., melanoma-associated antigen recognized by T cells [MART-1] and gp100). Thus, TL-DC-producing CCL21 served as a vehicle for both recruiting naive T cells and enhancing the production of tumor-specific T cells. These data in human have provided the feasibility for an ongoing clinical trial (in collaboration with the National Cancer Institute – Rapid Access to Intervention Development program) in melanoma patients at the Moffitt Cancer Center. A phase I clinical trial has been initiated to assess the toxicity, immune responses, and antitumor clinical responses in human leukocyte antigen-A*0201-positive patients with chemotherapy-naive metastatic

melanoma receiving escalating doses of adenoviral CCL21-transduced DC matured ex vivo with a cytokine cocktail and pulsed with MART-1/gp100/NY-ESO-1 class I peptides and keyhole limpet hemocyanin. In this study, patients are receiving the vaccine intradermally at multiple sites. To date, 12 patients (the first two of three dose cohorts) have been treated, and early results show indications of the known chemotactic activity of CCL21 through the accumulation of CD3 expressing T cells in biopsies of one of the several injection sites.

Anticipated High Impact Results

The results of the ongoing phase I studies in lung cancer and melanoma are promising, and future trials could assess the combined efficacy of DC-AdCCL21 with radiation, chemotherapy, or targeted therapy regimens. Based on the findings on CCL21 thus far, it is anticipated that the rational combinations with other treatment modalities will improve the therapeutic efficacy of this chemokine and antitumor benefit in a broad range of solid tumors.

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CD4+ T Cells

11

Frederick J. Kohlhapp and Andrew Zloza

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Abstract

CD4+ T cells are components of the adaptive immune system that have a diverse repertoire of functions, which are defined by the production of specific cytokines and expression of distinct intracellular transcription factors and surface chemokine receptors. The functional diversity of T cells is demonstrated by the association of certain CD4+ T cell types (including Th1 CD4+ T cells) with positive cancer prognosis and other CD4+ T cell types (including T regulatory and Th2 CD4+ T cells) with a negative cancer prognosis. While the presence of CD4+ T cell subtypes correlates with tumor progression, the precise role of CD4+ T cells in such progression remains uncertain based on the indirect role that CD4+ T cells often play in helping or suppressing other immune cell types (including CD8+ T

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cells, dendritic cells, NK cells, and myeloid-derived suppressor cells). Clinical therapies focusing on generating anti-tumor CD4+ T cell responses have been met with limited success. However, new approaches including Chimeric Antigen Receptors (CARs) may be increase the viability of CD4+ T cells as a potential therapeutic modality in the treatment of cancer.

Keywords
CD4+ T cells • Adoptive transfer • Clinical monitoring • Evaluation • Prognosis • Subsets • Therapy • Tumor progression • Types • Unique aspect of • Major histocompatibility complex II (MHC-II) • Regulatory T cells • T cell receptor (TCR) • T follicular helper (Tfh) • T helper 1 (Th1) cells • T helper 17 (Th17) cells • T helper 2 (Th2) cells • T helper 22 (Th22) cells • T helper 9 (Th9) cells

Target

CD4+ T cells originate from precursors in the bone marrow, which migrate to the thymus for maturation. They are a major component of the adaptive immune system responsible for a diverse set of immunostimulatory and immunosuppressive functions. CD4+ T cells are mainly categorized by the cytokines they produce and a combination of transcription factors, transduction activators, and chemokine receptors they express. Exposure of CD4+ T cells to specific cytokine milieus results in their conversion to a specific subtype and thus their acquisition of specific functional capabilities.

Th1 (T helper 1) CD4+ T cells are characterized by production of IFN-γ and expression of transcription factor T-bet (Zhu et al. 2010). Their main function is to “help” CD8+ T cells by providing stimulatory signals which lead to CD8+ T cell activation, differentiation, response, and memory formation. CD4+ T cells differentiate into Th1 CD4+ T cells when exposed to IL-12 and IFN-γ, which results in their expression of transcription factor T-bet, signal transduction activators STAT1 and STAT4, and chemokine receptors CCR5 and CXCR3. In the context of cancer, Th1 cells generally indicate a positive prognosis and promote antitumor responses, especially through CD8+ T cells, which are critical immune mediators of the antitumor response (see Chap. 12, “► CD8 T Cells”).

Th2 (T helper 2) CD4+ T cells are characterized by production of IL-4 (as well as IL-3, IL-5, and IL-13) and expression of transcription factor GATA-3 (Zhu et al. 2010). Their main function is to “help” the humoral response by promoting B cell differentiation and antibody production. CD4+ T cells differentiate into Th2 CD4+ T cells when exposed to IL-4, which results in their upregulation of transcription factor GATA-3, signal transduction activator STAT6, and chemokine receptors CCR4 and CRTh2. In the context of cancer, a Th2 cell infiltrate or gene signature within a tumor correlates with a worse prognosis.

Th17 (T helper 17) CD4+ T cells are characterized by production of IL-17 and IL-22 and expression of transcription factor RORγt (Zhu et al. 2010; Muranski and Restifo 2013). Their main function is in mucosal host defense against extracellular

pathogens. CD4+ T cells differentiate into Th17 CD4+ T cells when exposed to TGF-β along with some permutation of IL-6/IL-21/IL-1β/IL-23, which results in their upregulation of transcription factors RORγt and RORα, signal transduction activator STAT3, and chemokine receptors CCR4 and CCR6. In the context of cancer, Th17 cells infiltrate the tumor microenvironment; however, whether these cells promote pro-tumor or antitumor immune responses is the subject of continued debate.

Treg (T regulatory) CD4+ T cells are characterized by production of TGF-β and IL-10 and expression of transcription factor Foxp3 (Zhu et al. 2010; Josefowicz et al. 2012). Their main function is to suppress inflammatory immune responses. CD4+ T cells differentiate into Treg CD4+ T cells when exposed to TGF-β, which results in their upregulation of transcription factor Foxp3. Tregs play a significant role in suppressing antitumor responses.

Tfh (T follicular helper) CD4+ T cells are characterized by their location within the lymph node and secretion of IL-21 (Tangye et al. 2013). Their main function is to help B cells. When CD4+ T cells differentiate into Tfh CD4+ T cells, they upregulate transcription factors Bcl-6 and IRF-4; signal transduction activators STAT1, STAT3, and STAT4; and chemokine receptor CXCR5. In the context of cancer, a Tfh cell infiltration in the tumor microenvironment correlates with a better prognosis suggesting an antitumor function for this CD4+ T cell subset.

Th22 (T helper 22) CD4+ T cells are characterized by production of IL-22 (and IL-13 and TNF-α, but not IL-17) and expression of transcription factor AHR (Trifari et al. 2009; Protti et al. 2015). Their main known function is in instructing mesenchymal and epithelial cells primarily within the skin. CD4+ T cells differentiate into Th22 CD4+ T cells when exposed to IL-6 and TNF-α, which results in the upregulation of transcription factors AHR and RORγt; signal transduction activators STAT1, STAT3, and STAT5; and the chemokine receptors CCR4, CCR6, and CCR10. While recently recognized as a subset of CD4+ T cells, Th22 cells correlate with advanced tumor staging and a worse prognosis.

Th9 (T helper 9) CD4+ T cells are characterized by production of IL-9 (and IL-10) and expression of transcription factors PU.1 and IRF4 (Muranski and Restifo 2013; Protti et al. 2015). They are increased in the peripheral blood of patients with allergic responses and their function and regulation is under active study. CD4+ T cells are thought to differentiate in Th9 CD4+ T cells when exposed to IL-4 and TGF-β, which results in the upregulation of transcription factors PU.1 and IRF4 and transduction activator STAT6. The role of Th9 CD4+ T cells in cancer is currently under investigation; however, several mouse studies have demonstrated that Th9 CD4+ T cells produce antitumor responses.

Biology of the Target

CD4+ T cells recognize peptide antigens presented within the context of major histocompatibility complex II (MHC-II) molecules by antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages. Upon recognition of an

antigen, CD4+ T cells become activated and undergo effector differentiation (often to distinct subsets described here in the Target section), which confers varying functions to them according to the priming conditions (mainly, cytokines) within which they are present.

A unique aspect of CD4+ T cells lies in the vast diversity of their T cell receptor (TCR). The TCR is composed of a β and α chain, and studies have reported the existence of over 10^6 β chains which pair with over 25 distinct α chains (Arstila et al. 1999). This diversity in individual TCR chains is achieved through the structure and composition of the TCR. The TCR is composed of a constant and variable region. Within the variable region, there are hypervariable sites referred to as complementarity determining regions (CDRs) (Nikolich-Zugich et al. 2004). These regions are the result of somatic recombination and are responsible for TCR diversity. The vast diversity of TCRs ensures the ability of the T cell population as a whole to respond upon recognition of a nearly infinite number of antigens. However, in the generation of such diversity by somatic recombination, some TCRs produced are not functional and some TCRs recognize self-epitopes (recognition through which could result in autoimmunity). To ensure that CD4+ T cells are functional but do not lead to autoimmunity, CD4+ T cells (like CD8+ T cells) undergo "thymic education," a maturation process broken down into distinct phases in which CD4+ T cells undergo positive and negative selection. Positive selection ensures that the CD4+ T cells that enter the periphery have functional T cell receptors capable of recognizing antigens presented on MHC-II, while negative selection ensures that strongly autoreactive T cells are deleted to limit autoimmunity (Takahama 2006).

However, given the potentially detrimental ramifications of autoimmunity, a series of several additional checkpoints exist to prevent inappropriate CD4+ T cell activation. Specifically, optimal CD4+ T cell activation requires three distinct signals. Signal one is TCR stimulation provided to the CD4+ T cell upon its recognition of a cognate antigen (an antigen for which its TCR is specific) presented in the context of MHC-II, usually by a professional APC. Signal two is delivered by surface "costimulatory" molecules upregulated on the APC (such as B7-1 and B7-2) that provides additional binding and signaling to receptors (such as CD28) on the CD4+ T cell, leading to further CD4+ T cell activation (Lenschow et al. 1996). Signal three, provided by the cytokine milieu and soluble factors directs the subtype of CD4+ T cell response (Curtsinger et al. 2010). While signals one and two are critical for CD4+ T cell activation through initiating proliferation, IL-2 production, and acquisition of effector function, signal three regulates the specific transcription factors, signal transduction activators, and chemokine receptors expressed by a CD4 + T cell and ultimately is responsible for the distinct subtype, and thus, function of that CD4+ T cell. It is important to note that inflammatory stimuli lead to signals two and three, and therefore, cognate antigen presentation alone is not sufficient for a CD4+ T cell response but rather the inflammatory context in which an antigen is presented provides signals critical to CD4+ T cell functionality (Zhu et al. 2010).

Role of the Target in Cancer

Rank: "unknown" to 10.

Unknown to 1-2-3-4-5-6-7-8-9-10: 7.

CD4+ T cell function within the tumor microenvironment is determined in large part by its specific subset (Th1, Th2, Treg, etc.) and its effector differentiation status. While some general immune roles have been attributed to certain subsets (see the "Target" section in this chapter), the specific role that each of these CD4+ T cell subsets plays in the context of cancer remains understudied, especially in regards to the non-Treg subsets. One reason for this is the fact that CD4+ T cells are generally not direct effectors but rather indirect mediators of anti- or pro-tumor responses, and thus their specific role is often not clear. Further, CD4+ T cell plasticity (ability to convert from one subset to another) makes understanding these cells a complex undertaking.

Further complexity in assessment of the role of CD4+ T cell subsets in cancer is provided by the fact that CD4+ T cells (like CD8+ T cells) undergo a maturation process that eliminates those cells that possess the greatest ability to bind and respond to tumor antigens. This is the case because tumor antigens are mainly a subset of self-antigens (often harboring minor mutations or simply being overexpressed) and negative selection in the thymus results in the deletion of CD4+ T cells with TCRs that demonstrate high affinity for self-antigens (and thus, tumor antigens). This means that the CD4+ T cells that are capable of mounting an antitumor response have TCRs that have a reduced affinity for these tumor antigens, thus limiting CD4+ T cell activation, or requiring augmented costimulation (Stone et al. 2009). Further, studies in which CD4+ T cell TCRs exhibit high affinity for antigens (as is in the context of studies of the field of infection, or studies in the cancer field where transplantable tumors are forced to express non-self-antigens) may not progress the understanding of how these same CD4+ T cell subsets respond in the context of a tumor (i.e., self-) antigen.

High-Level Overview

Diagnostic, Prognostic, Predictive

Initially the finding of an immune infiltrate within the tumor was perceived to be an indication of an active immune response that would lead to a better prognosis. There is indeed evidence that tumors, which present with immune infiltrates are associated with a better prognosis than tumors which present without an immune infiltrate (Fridman et al. 2012). This may be in large part associated with the idea that antitumor therapies (and in particular immunotherapies) boost the response that is present in the tumor but do not create a *de novo* response in tumors where one does not exist. However, given the duality of the immune system in regards to having both

stimulatory and suppressive effects on immune responses and combined with differences in the immune response based on tissue type, it is not surprising that prognosis based on the immune infiltrate requires further dissection in order to be clinically relevant.

In studies where the subsets constituting the CD4+ T cell repertoire were taken into account, rather than total CD4+ T cell presence, gene signatures of the CD4+ T cell differentiation have served as a barometer that correlates with tumor progression. Generally, a Th1 CD4+ T cell infiltrate, determined through prognostic gene signatures, has been associated with prediction of a good prognosis, while both Th2 CD4+ T cell and Treg CD4+ T cell gene signatures have been associated with a worse prognosis (Fridman et al. 2012). However, in some cancers (including breast cancer (Yoon et al. 2010) and Hodgkin's lymphoma (Schreck et al. 2009)), increased Th2 CD4+ T cell responses have been shown to indicate a positive prognosis. Additionally, the impact of Tregs on prognosis has been complicated by studies within the same cancer type showing opposing results. Findings from studies of Th17 CD4+ T cells have likewise reported infiltration of tumors with Th17 cells to have both good and poor prognoses, based on different tumor types (Fridman et al. 2012). Interestingly, less well-studied CD4+ T cells subsets may prove to have prognostic potential. Specifically, Tfh CD4+ T cell infiltration has been correlated with a positive prognosis in both breast and colon cancer patients (Gu-Trantien et al. 2013; Bindea et al. 2013). Further, elevated levels of Th22 CD4+ T cells have been shown to correlate with negative prognoses and progressed tumor staging in gastric cancer (Liu et al. 2012), pancreatic cancer (Xu et al. 2014), and hepatocellular carcinoma (Qin et al. 2014). Whether these CD4+ T cells subsets will turn out to be prognostic indicators remains to be seen. Recent studies using mouse tumor models have also shown roles for Th9 CD4+ T cells; however, their clinical importance for prognosis in patients remains an open question (Muranski and Restifo 2013). An area for upcoming research may be investigating such subsets as clinical predictors of tumor therapy response, particularly for treatments utilizing immunotherapies, where typical clinical outcomes are not recognized for extended periods.

Target Assessment

Clinical monitoring of CD4+ T cells is based on (1) determining effector responses and persistence of CD4+ T cells, (2) analyzing the TCR repertoire of CD4+ T cells, and (3) determining CD4+ T cell gene signatures (of transcription factors, chemokine receptors, and cytokines indicative of CD4+ T cell function) (Macchia et al. 2013). This monitoring is employed for treatment follow-up especially with immunotherapies in order to gauge effectiveness.

CD4+ T cell effector response monitoring is assessed using ELISPOT and flow cytometry to determine functional cytokine production. Cytokine production usually focuses on cytokines associated with specific CD4+ T cell subsets, mainly IFNγ (expected from Th1 cells), IL-17 (from Th17 cells), IL-4 (from Th2 cells), and

TGF-β (from Treg cells) (Clay et al. 2001). For such studies, isolated peripheral blood mononuclear cells (PBMCs) from patient blood samples are stimulated with autologous (same patient) tumor lysate or HLA-matched tumor cell lines (Wen et al. 2002). While the types of cytokines produced are major indicators for the overall effectiveness of CD4+ T cell responses, surface receptors signifying differentiation and exhaustion are critical in determining CD4+ T cell persistence. CD4+ T cell differentiation from naïve to effector to memory confers added longevity and functionality to CD4+ T cells. Therefore, it is critical for therapy responses to determine differentiation not only to ensure that naive CD4+ T cells are activated into effector cells but that these effector CD4+ T cells become memory CD4+ T cells to afford long-term protection. Further, CD4+ T cell surface receptors indicating an antigen experienced/memory phenotype indicate better antitumor responses (Galon et al. 2006). Adoptive transfer immunotherapies require T cell activation regimens that promote proliferation and effector function. However, as a part of a negative feedback loop in response to the activation, T cells upregulate surface receptors that limit T cell responses associated with exhaustion, such as PD-1, Tim-3, Lag-3, and CTLA-4. Therefore, surface receptors for CD4+ T cell exhaustion may be used as a surrogate for therapeutic efficacy of these therapies (Kalos and June 2013).

Additionally, CD4+ T cell responses are monitored through the persistence and proportion of specific TCRs or clonotypes of the polyclonal CD4+ T cell population. Peptide-MHC complexes allow for the identification of tumor-specific CD4+ T cells, which can be used for longitudinal monitoring of either adoptive transfer immunotherapies or endogenous CD4+ T cell responses to antitumor therapies (Macchia et al. 2013; Kalos 2011). However, the disadvantage with peptide-MHC complex monitoring is the relatively low sensitivity of detection limited by flow cytometry as well as the requirement for *a priori* knowledge of the CD4+ T cell antigen. Additionally, CD4+ T cell responses can be monitored using TCR tracking which identifies specific TCRs based on genomic rearrangement. This method is effective for clonal tracking in adoptive therapy regimens; however, it is limited based on its inability to monitor polyclonal responses. To monitor polyclonal responses through TCR expression, spectratyping is used. This method uses high-throughput analysis to determine usage of specific TCRs to determine the skewing of the TCR repertoire based on the expansion of responding T cell populations (Kalos 2011). This works well with adoptive transfer and immune reconstitution therapies; however, spectratyping is semiquantitative and does not identify specific TCRs (Kalos 2011). High-throughput DNA sequencing is effective for monitoring polyclonal CD4+ T cell responses by determining the precise TCRs utilized in a time-effective manner. However, this analysis is currently expensive based on the amount of data generated and the bioinformatics that is required to analyze the data for meaningful conclusions (Robins 2013).

CD4+ T cell gene signatures within tumors are determined through qPCR arrays. The targets for the qPCR arrays were generally identified based on mRNA microarray analysis. In which the levels of CD4+ T cell-associated mRNAs (such as transcription factors, chemokine receptors, and cytokines) were assessed against overall patient prognosis to identify a CD4+ T cell gene signature that predicts the

disease course. Experimentally the use of CD4+ T cell gene signatures has been shown to correlate with prognosis; however, its clinical effectiveness needs to be assessed especially against proven standards of histopathology and tumor staging (Fridman et al. 2012).

For the evaluation of CD4+ T cells in the context of immunotherapy, the use of standard Response Evaluation Criteria in Solid Tumors (RECIST) has been questioned given the differences in action and mechanism when comparing immunotherapies with chemotherapies. Effective chemotherapies have generally shown either partial or complete responses quickly, which provided the framework for the design of RECIST (Weber et al. 2011). However, effective immunotherapies have shown significantly delayed partial or complete responses with continued tumor growth for months prior to regression. Therefore, new evaluation techniques must be explored for assessment of targets related to immunotherapies (Weber et al. 2011). Further, the tumor microenvironment specifically needs to be probed as responses from draining lymph nodes and peripheral blood do not always correlate with the events occurring within the tumor microenvironment itself.

Therapy

CD4+ T cells function in large part to support the immune functionality of other cells (including CD8+ T cells and B cells). Therefore, attributing responses to CD4+ T cells in the context of therapies is complex. Some CD4+ T cells, especially Th1 cells, have been shown to promote the efficacy of other cancer therapies that utilize the immune system (Protti et al. 2015). Both DC vaccines and peptide-based vaccines designed to elicit an inherent CD4+ T cell response have shown clinical responses in melanoma. Further, a DC vaccine designed to present a combination of MHC-I and MHC-II epitopes to elicit a coordinated CD8+ T cell and CD4+ T cell response, respectively, has demonstrated better clinical efficacy then the control DC vaccine loaded with MHC-I epitopes alone (Aarntzen et al. 2013). However, there are conflicting reports about the benefits of vaccinating with peptides designed to stimulate CD4+ T cell responses (Protti et al. 2015). Whether this is a difference based on the vaccine design or the variability of the protocols used in the clinical studies remains to be explored.

Another manner in which CD4+ T cell-based therapies can be employed beyond stimulating inherent CD4+ T cell responses is through the adoptive transfer of CD4+ T cells specific for tumor antigens. In one such study targeting the CD4+ T cell NY-ESO-1 epitope, transferred CD4+ T cells were shown to be clinically effective against melanoma in one out of nine patients (Hunder et al. 2008). A similar case was observed in a patient with metastatic cholangiosarcoma, who was found to have endogenous antitumor CD4+ T cells in her blood specific for the antigen ERBB2IP (Tran et al. 2014). These antitumor-specific CD4+ T cells were isolated and expanded *ex vivo* for adoptive transfer, which led to a significant reduction in tumor burden. In other studies, CD4+ T cells have been shown to improve adoptive transfer therapy regimens of tumor infiltrating lymphocytes isolated from melanoma patients. This is

thought to be through a costimulatory mechanism during TIL cocultivation (Antony et al. 2005). Surprisingly, traditional therapies, such as chemotherapy and radiation that were thought to be immunosuppressive are being reevaluated to promote immunogenicity through the pronounced ability of these therapies to induce cell death. Preliminary evidence indicates that the Th1 CD4+ T cell response can be promoted by rationally designed chemotherapy and radiation therapies to promote antitumor immune responses (Zeng et al. 2013; Andre et al. 2014; Ramakrishnan and Gabrilovich 2013).

Successes in studies utilizing CD4+ T cells targeting as a therapy indicate that CD4+ T cells may be used as an effective therapy; however, there are several limitations that need to be considered, including: (1) CD4+ T cell activation and stimulation requires fine tuning since under known and unknown conditions (e.g., specific cytokine milieu), CD4+ T cells differentiate into a variety of subtypes with varying antitumor potential (from antitumor Th1 cells to pro-tumor Tregs) and (2) CD4+ T cells harnessed for adoptive cell therapy do not expand as rapidly as CD8+ T cells making it difficult to generate the number of CD4+ T cells required under current adoptive transfer immunotherapy protocols (Muranski and Restifo 2009). Such technical difficulties combined with the limited evidence of direct antitumor responses must be overcome prior to the adoption of CD4+ T cell targeting as a viable option therapy against cancer (Protti et al. 2015).

Preclinical Summary

CD4+ T cells have been demonstrated to be key indicators of tumor progression. The effector differentiation type of CD4+ T cells that infiltrates the tumor has been shown to have prognostic value in several tumor types (Fridman et al. 2012). CD4+ T cell help is thought to be instrumental in promoting antitumor immune responses, including in enhancing tumor protection by promoting antitumor CD8+ T cell responses in the context of both peptide and DC vaccination (Protti et al. 2015). Further, CD4+ T cells may improve CD8+ T cell adoptive immunotherapy through increasing CD8+ T cell function, memory differentiation, and persistence (Antony et al. 2005). Finally, the abscopal effect of radiation and the immunogenicity of chemotherapy may improve antitumor responses through effects on CD4+ T cells that go beyond Treg depletion (Zeng et al. 2013; Ramakrishnan and Gabrilovich 2013). However, it remains to be seen whether CD4+ T cell therapies can be clinically translated towards improved tumor responses in therapeutic settings.

Clinical Summary

To elicit better antitumor responses, emerging DC vaccines and peptide vaccines have been designed to likewise target CD4+ T cells in addition to their traditional efforts at targeting CD8+ T cells. Initial clinical results have been mixed in which addition of CD4+ T cell epitopes has resulted in both improvement of and minimal effect on clinical responses (Protti et al. 2015). Rational vaccine design has attempted to

generate cooperative CD4+ T cell and CD8+ T cell responses using long peptides that encode for both CD4+ T cell and CD8+ T cell epitopes within the same protein (Melief and van der Burg 2008). This design has demonstrated complete clinical responses to vulvular intraepithelial neoplasia establishing a clinical precedence for this approach to be further examined (Kenter et al. 2009).

Adoptive transfer of CD4+ T cells specific for tumor antigens has shown clinical responses in a minority of patients. CD4+ T cells specific for NY-ESO-1 were shown to cause complete remission in one out of nine patients for a period of over 2 years (Hunder et al. 2008). Further, the identification of endogenous CD4+ T cells specific for a tumor antigen expressed by choloangiosarcoma, ERBIPP2, led to the isolation and expansion of these cells for adoptive transfer. The one patient included in this study showed a partial clinical response (Tran et al. 2014). Therefore, in contrast to other studies which have not demonstrated improved clinical outcomes, these studies serve as a proof-of-principle suggesting the need for further understanding of CD4+ T cell subtypes, differentiation, and functionality. Further, therapies targeting changes in CD4+ T cells or employing certain CD4+ T cell subsets may lead to future clinical success.

Anticipated High-Impact Results

- Vaccinations and therapies used to promote immune responses against the tumor should incorporate mechanisms in which to skew CD4+ T cells differentiation towards antitumor response-promoting subsets (e.g., Th1 and possibly Th17 effector types). Based on the significance of these subsets in predicting good prognoses, shifts towards such subsets could indicate better overall antitumor immune responses.
- Further understanding of the indirect effects of CD4+ T cells on antitumor responses (through helping CD8+ T cells and through interacting with APCs) will allow for rational design of immunotherapies that target CD4+ T cells. CD4+ T cells are known to be important for the immunogenic properties of chemotherapy, radiation, adoptive transfer, and vaccination; however, the manner in which CD4+ T cells contribute is not fully understood. Identification of these CD4+ T cell-driven antitumor immunogenic responses may allow for therapeutic manipulations that rescue antitumor CD4+ T cell responses lost due to immunosuppression.
- The use of CD4+ T cells has been difficult (more than CD8+ T cells) for adoptive transfer immunotherapy given the HLA diversity, reduced proliferation, and varied effector responses of CD4+ T cells. However, understanding and overcoming these limitations may provide for better antitumor responses as evidenced by several studies in which adoptive transfer of tumor-specific CD4+ T cells resulted in positive clinical responses (Hunder et al. 2008; Tran et al. 2014). Future successes related to adoptive transfers of CD4+ T cells may employ combination techniques which engage specific differentiation states (e.g., tissue resident

memory cells) into environments skewed for development of desired CD4+ T cell subsets.

- A potential breakthrough in adoptive transfer of CD4+ T cells could come from manipulation of these cells to express chimeric antigen receptors (CARs). CARs are rationally designed receptors in which the extracellular domains of proteins known to bind extracellular proteins solely or known to be overexpressed by cancer cells are fused to the intracellular domain of the TCR (Barrett et al. 2014). This results in CD4+ T cell activation through the TCR signaling pathway upon recognition of the extracellular domain of the tumor-specific protein, rather than relying on endogenous TCR recognition of cognate antigens presented on MHC-II (Barrett et al. 2014). This technology may overcome the limitation of HLA diversity since recognition of MHC-II is no longer required. Here, the number of CD4+ T cells available for adoptive transfer may not be an issue because CARs could be added to any CD4+ T cells (or subset of cells), thus avoiding the limitations of using only tumor antigen-specific CD4+ T cells.
- Future efforts in utilizing CD4+ T cells for direct tumor regression may focus on the development of cytotoxic CD4+ T cells. Such direct antitumor effects from CD4+ T cells have been reported in mouse models (Quezada et al. 2010), and importantly a recent study has likewise shown cytotoxic CD4+ T cells are induced during ipilimumab treatment in advanced melanoma patients (Kitano et al. 2013). This demonstrates the potential and importance for cytotoxic CD4+ T cells in future tumor immunotherapies.

Cross-References

► CD8 T Cells
► Interleukin-12
► Tregs
► TGF Beta Receptors

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CD8 T Cells

12

David Kotlyar

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Abstract
The harnessing and purification of CD8+ T cells as a novel therapy for neoplastic diseases is a recent innovation. With improvements in the understanding of T cell biology, the identification of specific subsets of CD8+ T cells has been shown to be superior at suppressing tumor activity both *in vitro* and *in vivo*. Recent clinical trials using both purified tumor infiltrating lymphocytes (TILs) as well as the novel chimeric antigen receptor T cells (CAR T cells) have shown great promise in the treatment of a variety of neoplasias including melanoma, and leukemias. Many clinical trials are now ongoing to elucidate the potential of these new therapies.

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Target

T lymphocytes are white blood cells, specializing in adaptive and cell-mediated immunity, which are educated and released from the thymus (Abbas et al. 2012). Initial progenitor cells from the bone marrow migrate to the thymus, where they undergo thymic education where T cells develop high specificity for a specific antigen and self-reactive T cells are deleted (Restifo et al. 2008). After thymic education, CD8+ T cells consist of a restricted class of thymocyte-educated lymphocytes which continuously sample antigen throughout the body, are activated by interactions with antigen presenting cells (APCs), and then have a specific function (Restifo et al. 2008).

Initially these cells are *naïve* T cells prior to development in specific secondary lymphoid tissues such as lymph nodes, the spleen, and mucosal associated tissues (such as the gut and tonsils) (Murphy et al. 2012). These cells later act as *effector T cells* or *memory T cells*. Effector T cells kill other cells which are infected or are aberrant (including tumor cells) with anomalous antigen (Abbas et al. 2012). Memory T cells retain the ability to divide later in the presence of a pathogenic antigen (Abbas et al. 2012).

It is common for activated host T cells to be present and function against host malignancies (Gajewski 2012). In the tumor microenvironment, tumor infiltrative lymphocytes (TILs) oppose the stromal cells which act as support cells for metastatic disease (Gajewski 2012; Feig et al. 2012). In addition, the interaction of programmed death receptor ligand-1 (PDL-1) on tumor cells may directly interact with the receptor on activated T cells, programmed death-1 (PD-1) (Gajewski et al. 2006). The effect of this interaction is likely to degrade T-cell activity (Gajewski et al. 2006).

The presence of CD8+ effector and memory T cells in the tumor environment predicts better prognoses, and these cells have been shown to be a prognostic factor in multiple tumors, including tumors of colorectal, ovarian, breast, and melanoma origin (Gajewski 2012). A robust TIL milieu also predicts an improved response to nascent immunotherapies such as ipilimumab for melanoma (Gajewski 2012).

The presence of these lymphocytes however, leads to another question, namely, why are TILs not capable of eradication of tumor? One important concept is that of “immune paralysis” by both direct tumor effects and indirect effects such as cytokine modifications and altered cell-cell signaling by tumor-associated “support” cells (Wallner et al. 2012). Direct effects include downregulation of tumor MHC-class I expression, as well as inhibitory cell cross talk such as CTLA-4 activation, and PDL-1 and PD-1 interaction (Gajewski 2012; Wallner et al. 2012). Indirect

inhibitory effects include aberrations in intracellular processing of TILs such as poor expression of perforin and interferon gamma that may result from interactions with increased numbers of T-regulatory cells (Harlin et al. 2006). However, stimulation of these T cells in vitro can reenergize the immune system and lead to regression and durable responses in selected patients (Wallner et al. 2012).

Biology of Target

The use of adoptive cell therapy (ACT) has shown promise in melanoma and has been studied in various tumors in humans, including melanoma, renal cell carcinoma, testicular cancer, and non-small cell lung cancer (Restifo et al. 2008). ACT begins with the isolation of autologous T cells, then proceeds with in vitro identification of these cells as those with antitumor activity, and then in vitro activation of these cells followed by reinfusion into the patient (Rosenberg 2011). These cells can be grown outside of the body to up to 31.5 X 10^9 cells (Dudley et al. 2013) cells and then reintroduced. In addition these infusions are paired with therapies for the patient, including lymphodepletion which acts to alter autoregulation, reduce numbers of regulatory T cells, and also reduce the "sink" for vital cytokines such as IL-15 and IL-17 which enhance lymphocyte survival (Rosenberg 2011; June et al. 2012). While both CD4+ and CD8+ cells from TILs may have some antitumor effect, CD4+ cell subsets may have immunosuppressive activity, while CD8+ cells are consistently of the effector subtype (Restifo et al. 2008).

Another potentially groundbreaking type of CD8+ T cell is the chimeric antigen receptor (CAR) T cells. For these cells, lymphocytes are taken from the peripheral blood and then modified using retroviral or lentiviral vectors (June et al. 2012). The modifications cause an expression of a T-cell receptor with affinity to a defined tumor antigen. Heavy and light chain variable regions of antibodies were found to be linkable to the constant regions of the T-cell receptor (Restifo et al. 2008). In addition these cells can be dramatically augmented through the linkage of intracellular signaling domains from co-stimulatory molecules (Restifo et al. 2008; June et al. 2012).

Target Assessment

For TIL protocols, in general, patients were required to have at least a 2-cm metastatic lesion which could be effectively removed (Rosenberg 2011). The purpose of this was to harvest the TILs from the specimen (Rosenberg 2011).

For TIL extraction, portions of removed tumor 1–3 μl were cultured, and cultures were expanded through the use of IL-2 (Dudley et al. 2013). These cells were expanded using peripheral blood mononuclear lymphocytes, anti CD-3, and IL-2 (Dudley et al. 2013). In the study that used irradiation first to cause lymphodepletion, patients received cyclophosphamide and fludarabine prior to infusion, and those irradiated had total body irradiation (Rosenberg et al. 2011). TILs were grown in

high-dose IL-2, separated into multiple wells, and once shown to have antigen specificity against tumor (by means of checking IFN-gamma levels), were selected for, and rapidly expanded (Rosenberg et al. 2011). Patients then received IL2 and infusions of TILs as well as infusions of CD34+ stem cells (Rosenberg et al. 2011).

CAR T cells can be assessed for persistence using flow cytometry and PCR. PCR for DNA for the chimeric T-cell receptor showed an initial doubling time in a chronic lymphocytic leukemia (CLL) patient of approximately 1.2 days with later half-life of elimination of 31 days (Porter et al. 2011). Cells were found to persist almost 6 months (Porter et al. 2011). In cases of acute lymphocytic leukemia (ALL), similar numbers were seen with greater than 1,000-fold increase of CAR T cells along with persistence peripherally and in CSF of over 6 months (Grupp et al. 2013).

Role of the Target in Cancer

Rank: TIL
All cancers 3/10
Use in melanoma: 8/10
Rank: CAR T cells
All cancers 10/10

Diagnostic, Prognostic, and Therapeutic Potential of CD8+ T Cells

CD8+ T cells are not usually used as a diagnostic tool for tumors with exception of some rare hematological malignancies. In terms of prognostic features of CD8+ T cells, however, the type of T cell used is highly important.

A crucial factor in TIL therapy is the differentiation state of the individual T cell. CD8+ T cells can be thought of as a spectrum of differently differentiated effector cells which express different cytokines, surface markers, and behaviors (Restifo et al. 2008). Naive CD8+ T cells will differentiate into early, middle, and late effector cells (Gattinoni et al. 2012). While there is still considerable uncertainty into how T cells compartmentalize to each state, what is known is that CD8+ T cells that are terminally differentiated showed the worst performance in vivo (Gattinoni et al. 2005).

It is thought that “younger” CD8+ T cells (which produce CD62L, CCR7, CD28, and CD27) are more responsive to cytokine signaling than more differentiated cells and also may be less prone to senescence with longer telomere length (Gattinoni et al. 2012). Higher levels of CD27+ CD8+ T cells in particular were associated with better response in recipients of TILs, and in terms of measurement, lower levels of proapoptotic molecules such as BID, BAD, and FAS ligand are associated with “younger” CD8+ T cells and better responses (Restifo et al. 2008).

Selectively enhancing this “younger” subset prior to infusion of TILs may be of great benefit. First, cytokines other than IL-2 are being explored in the expansion of TILs to avoid excessive differentiation of the TIL pool (Gattinoni et al. 2012). The

use, in particular of either IL-15 or IL-21, may cause expansion of the TIL pool while maintaining more of a central memory phenotype, and cells grown in vitro in IL-15 instead of IL-2 have shown better antitumor responses (Gattinoni et al. 2012; Hinrichs et al. 2008; Klebanoff et al. 2005). In addition, pharmacotherapeutics for other purposes such as diabetes and antirejection have been found to potentiate the memory T-cell phenotype. In particular, it has been found that the use of rapamycin specifically increases the number of memory T cells and improves their antitumor properties, although this should be tempered in that mTOR inhibition also seems to upregulate regulatory T cells and T-cell anergy (Araki et al. 2010; Chi 2012). Metformin may also represent a promising new drug which increases the number of CD8+ memory T cells in mice (Pearce et al. 2009). The use of these compounds offers great promise.

In terms of therapeutic potential, as mentioned above, TILs have been used most effectively in melanoma, where responses have ranged from 40% to 70% in pretreated patients, with durable, complete responses in up to 10–40% (Rosenberg et al. 2011; Besser et al. 2013; Vacchelli et al. 2013). TILs have been surgically extracted in both melanoma and in renal cell carcinoma (Vacchelli et al. 2013). In tumors other than melanoma, there has yet not been a significant clinical benefit observed. However, trials are ongoing for breast cancer with TILs targeting CD3 and ERBB2, HPV+ carcinoma, EBV positive cytotoxic T lymphocytes, and Merkel cell carcinoma, in addition to further trials with melanoma, including trials using ipilimumab (Vacchelli et al. 2013).

For CAR T cells, potentially any tumor antigen can be targeted, which offers huge potential for their future use, assuming delivery and safety issues are managed appropriately (June et al. 2012; Vacchelli et al. 2013). Trials are ongoing for AML, CML, MDS, multiple myeloma, ALL, CLL, other lymphomas, mesothelioma, pancreatic adenocarcinoma, melanoma, and ovarian carcinoma (Vacchelli et al. 2013). Monotherapy or combination use with agents such as ipilimumab (anti-CTLA4), nivolumab, and lambrolizumab (anti-PDL1) offers great promise (Hamid et al. 2013; Hodi et al. 2010; Wolchok et al. 2013).

Preclinical Data

Optimization of TILs is ongoing. The effectiveness of the TIL therapy is dependent on both the degree of engraftment and the persistence of its antitumor effect (Gattinoni et al. 2012). The degree of persistence of a T cell is directly related to the state of its differentiation (Gattinoni et al. 2012). Lymphocytes which are "young," grown in culture for an abbreviated amount of time, and which have longer telomeres have been shown to be more effective in their antitumor responses when infused (Rosenberg et al. 2011; Schwartzentruber et al. 1994). Murine experiments have shown that T cells which are more naive show better engraftment, persistence, and tumor destruction (Gattinoni et al. 2012, 2005; Klebanoff et al. 2005). In addition, the medium used to expand T cells is critical with concomitant IL-15 and IL-2, causing less differentiation of T cells than the use of IL-2 alone (Gattinoni et al. 2012).

Preclinical work on CARs is also rapidly developing. Over the past 10 years, the field has rapidly advanced, with incremental improvements in the design of the CAR T cell (Brentjens and Curran 2012). One major issue is optimization of the intracellular domains often associated with co-stimulatory receptors such as CD28. For B-cell malignancies, the use of anti-CD19 directed CARs is useful as it is usually found on most B cells and B-cell malignancies (Kochenderfer et al. 2012). Anti-CD19 CAR cells eradicated disseminated lymphoma in mice after total body irradiation and also eradicated most B cells, after the CARs rapidly expanded in mice, but normal B cells returned after a later time (Cheadle et al. 2010). A second study with anti-CD19 CAR cells showed similarly impressive results with eradication of both intraperitoneal and large subcutaneous lymphomas in mice after initial total body irradiation (Kochenderfer et al. 2010a). Another murine experiment showed good results with CARs specifically targeted to both the tumor target (gp100) and to the tumor stroma and vasculature target (VEGFR2) (Chinnasamy et al. 2013). Further animal models on other tumor types are ongoing.

Clinical Data

Clinical trials with the use of TILs have shown impressive results in melanoma. In melanoma specifically, the use of cytoreductive therapy with lymphodepleting (but not myelodepleting) agents followed by infusion of expanded TILs resulted in objective response of 50% of patients with metastatic melanoma (Dudley et al. 2005). Of these, nearly 10% had complete response, and mean responses were 11.5 months, with some having more durable remissions (Dudley et al. 2005). In another study, even when other immunotherapies, such as ipilimumab, were tried and failed, about 40% of patients had some objective response, and about 10% had complete response with a mean survival time of 15.2 vs 9.8 months (Besser et al. 2013). Another trial at the NCI showed an objective response rate as high as 72% when TILs were combined with 12 Gy of radiation and complete response rates of 22% with most of these being durable responses of over 3 years (Rosenberg et al. 2011). In another study, the use of “young” TILs, with a short culture time and without CD8+ T cells being specifically extracted, showed improved survival in a subset of patients with TILs extracted from subcutaneous melanomas (Dudley et al. 2013). Overall 35% of patients responded to the “young” TILs as compared to 20% of the patients with TILs cultured for a longer time and enriched in CD8+ T cells (Dudley et al. 2013). However these results were not significant due to low numbers of patients in the trial (69 pts.) (Dudley et al. 2013).

Groundbreaking results in the treatment of acute lymphoid leukemia were reported recently: two patients with refractory ALL had responses after receiving CAR T cells which express anti-CD19 (Grupp et al. 2013). The cells expanded more than 1,000-fold after transfusion (Grupp et al. 2013). In one patient, she emerged with a durable response, while the other relapsed with a leukemia that was CD19 negative (Grupp et al. 2013). In another patient with refractory CLL, the patient was put into durable remission after receiving CAR T cells (Porter et al. 2011). At the National Cancer

Table 1 Active anti-CD19 clinical trials

Center[a]	Clinical setting	Malignancies treated	Conditioning regimen	Method of genetic modification	Co-stimulatory domain or viral specificity	ClinicalTrials. gov reference
Baylor College of Medicine	Autologous transplant or no transplant	Lymphoma and CLL	Cyclophosphamide or none	Gammaretrovirus	Patients receive a mix of CAR T cells with or without a CD28 domain	NCT00586391
Baylor College of Medicine	Allogeneic transplant	Persistent B- cell malignancies or prophylaxis for high risk of relapse	None	Gammaretrovirus	CD28 co-stimulatory domain, multi-virus-specific T cells	NCT00840853
Baylor College of Medicine	Autologous transplant or no transplant	Lymphoma and CLL	Cyclophosphamide or none	Gammaretrovirus	Patients receive a mix of EBV-specific CAR T cells and CAR T cells with a CD28 domain	NCT00709033
Children's Hospital of Philadelphia	No transplant or allogeneic transplant	Pediatric ALL or lymphoma	Variable chemotherapy	Lentivirus	4-1BB co-stimulatory domain	NCT01626495
City of Hope	APBCST	Intermediate-grade B-cell lymphoma	T cells infused 2 days after APBSCT	Lentivirus	No co-stimulatory domain	NCT01318317
Fred Hutchinson Cancer Center	After allogeneic transplant	Persistent B cell malignancies or prophylaxis for high risk of relapse	None	Lentivirus	CMV or EBV-specific central memory-derived T cells. CAR with CD28 domain	NCT01475058
MD Anderson	Autologous transplant	B-cell lymphoma and CLL	T cells infused day 2–7 after APBSCT	Transposon	CD28 co-stimulatory domain	NCT00968760
MO Anderson	Allogeneic transplant	Prophylaxis or active meligancies	None	Transposon	CD28 co-stimulatory domain	NCT01497184
MD Anderson	Cord blood transplant	Prophylaxis or active malignancies	None	Transposon	CD28 co-stimulatory domain	NCT01362452

(*continued*)

Table 1 (continued)

Center[a]	Clinical setting	Malignancies treated	Conditioning regimen	Method of genetic modification	Co-stimulatory domain or viral specificity	ClinicalTrials.gov reference
Memorial Sloan Kettering Cancer Center	Allogeneic transplant	Pediatric ALL	Variable chemotherapy	Gammaretrovirus	EBV-specific, ant-CD19 CAR T cells with a CD28 domain	NCT01430390
Memorial Sloan Kettering Cancer Center	No transplant	Relapsed/refractory CLL and indolent lymphomas	Cyclophosphamide	Gammaretrovirus and lentivirus	Mix of CAR T cells with CD28 or 4-1BB domains	NCT00466531
Memorial Sloan Kettering Cancer Center	No transplant	CLL consolidation after prior chemotherapy	Cyclophosphamide	Gammaretrovirus	CD28 co-stimulatory domain	NCT01416974
Memorial Sloan Kettering Cancer Center	No transplant	Adult ALL	Either ALL re-induction or cyclophosphamide	Gammaretrovirus	CD28 co-stimulatory domain	NCT01044069

Notional Cancer Institute	No transplant	Any adult B-cell malignancy	Fludarabine + cyclophosphamide	Gammaretrovirus	CD28 co-stimulatory domain	NCT00924326
National Cancer Institute	Allogeneic transplant	Any adult B-cell malignancy after transplant	None	Gammaretrovirus	CD28 co-stimulatory domain	NCT01087294
National Cancer Institute	No transplant or allogeneic transplant	Pediatric ALL or lymphoma	Fludarabine + cyclophosphamide	Gammaretrovirus	CD28 co-stimulatory domain	NCT01593696
University College London	Allogeneic transplant, multiple European sites	Pediatric ALL, prophylactic or MRD	Fludarabine for all: vincristine and dexamethasone added for MRD	Gammaretrovirus	EBV-specific, no co-stimulatory domain. EBV cell vaccination	NCT01195480
University of Pennsylvania	No transplant	Adult B-cell leukemia or lymphoma	Variable chemotherapy	Lentivirus	4-1BB co-stimulatory domain	NCT01029366

ALL acute lymphoblastic leukemia, *APBSCT* autologous peripheral blood stem-cell transplant, *CAR* chimeric antigen receptor, *CLL* chronic lymphocytic leukemia, *CMV* cytomegatovirus, *EBV* Epstein-Barr virus, *MRD* minimal residual disease

[a]Sources from the ClinicalTrials.gov website and communication with the principle investigators of the studies

Institute, a patient with advanced follicular lymphoma, having failed other therapies, with the exception of partial remission with the EPOCH-R chemotherapy regimen, had a complete response after receiving anti-CD19 CAR cells that had duration of response of at least 36 weeks (Kochenderfer et al. 2010b). These CAR cells contained both anti-CD19 that was fused to a portion of the extracellular domain of CD28 and the transmembrane portion of CD28 as well as the cytoplasmic portion of CD28 (Kochenderfer et al. 2010b). A second trial of anti-CD19 CAR cells given to eight patients with refractory B-cell malignancies resulted in six of eight having remissions (Kochenderfer et al. 2012). See Table 1 for a list of ongoing trials with anti-CD19 CAR T cells (Kochenderfer and Rosenberg 2013).

High-Impact Results

- CD8+ T cells can be harnessed as a cellular therapy to reduce tumor burden and provide durable responses in selected patient populations with cancer.
- Two major types of T cell therapies include the use of tumor infiltrating lymphocytes (TILs) and chimeric antigen receptor (CAR) T cells.
- More naïve, “younger” T cells may provide more potent responses.
- Durable complete responses have been observed with the use of TILs in patients with melanoma, with responses from 10–40%.
- Durable complete responses have been observed in pediatric leukemia and in adults with lymphoma with the use of CAR T cells.
- The field is rapidly expanding, and many trials are ongoing to elucidate the full potential of CD8+ T cell therapy.

Conclusion

CD8+ T cells have been used for protocols involving both tumor-infiltrating lymphocytes (TILs) and newly applied and modified with gene therapy approaches as chimeric antigen receptor (CAR) cells. Tumor infiltrating lymphocyte protocols have greatly benefited patients with melanoma, with up to 70% of patients in some trials showing benefit and showing complete responses in 20–30%. CAR cells represent a possible revolution in cell therapeutic technology and may represent the next evolution of bone marrow transplant. In addition, the possible targeting of any cancer antigen with CAR cells belies the tremendous potential of this nascent technology.

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CEA

13

Marion Hartley and John L. Marshall

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Abstract

Carcinoembryonic antigen (CEA) was first described in 1965 by Gold and Freedman (Gold and Freedman, J Exp Med 122:467–481, 1965a; J Exp Med 121:439–462, 1965b). This 180,000 Da glycoprotein earned its name due to its presence in neoplastic and embryonic gastrointestinal tissues (Martin et al., Cancer 37:62–81, 1976). CEA is overexpressed on most adenocarcinomas of the colon, rectum, stomach, and pancreas, as well as breast cancers and non-small cell lung cancers, and as such is a "tumor-associated antigen" (TAA) (Chevinsky, Semin Surg Oncol 7:162–166, 1991). It has also been identified in small amounts on normal adult colonic mucosa (Fritsche and Mach, Immunochemistry 14:119–127, 1977). The family belongs to the immunoglobulin superfamily and resides on the long arm of chromosome 19. CEA protein shares significant amino acid homology with a nonspecific cross-reacting antigen that is found on normal granulocytes (Engvall et al., Proc Natl Acad Sci U S A 75:1670–1674, 1978). As an intercellular adhesion molecule, CEA may contribute to the formation of metastasis; there is a correlation between serum CEA in patients with cancer and the incidence of hepatic metastases, but this could also simply reflect tumor burden (Steele et al., Ann Surg 196:162–169, 1982; Yeatman et al., Ann Surg 210:505–512, 1989). In normal colonic epithelium, CEA is localized to the luminal surface, an arrangement that suggests that it contributes to spatial orientation of colonocytes and that it may also function to preserve the adult gut mucosal barrier (Marshall, Oncology (Williston Park) 19:1557–1565, 2005). In tumor cells, however, CEA is irregularly distributed throughout the cell membrane.

Keywords

Carcinoembryonic antigen (CEA) • Tumor-associated antigen (TAA) • Adenocarcinoma • Vaccine • Poxvirus • ALVAC • TRICOM • PANVAC • Vaccinia • Dendritic cell • Costimulation

Carcinoembryonic antigen (CEA) was first described in 1965 by Gold and Freedman (Gold and Freedman 1965a, b). This 180,000 Da glycoprotein earned its name due to its presence in neoplastic and embryonic gastrointestinal tissues (Martin et al. 1976). CEA is overexpressed on most adenocarcinomas of the colon, rectum, stomach, and pancreas, as well as breast cancers and non-small cell lung cancers, and as such is a "tumor-associated antigen" (TAA) (Chevinsky 1991). It has also been identified in small amounts on normal adult colonic mucosa (Fritsche and Mach 1977). The family belongs to the immunoglobulin superfamily and resides on the long arm of chromosome 19. CEA protein shares significant amino acid homology with a nonspecific cross-reacting antigen that is found on normal granulocytes (Engvall et al. 1978). As an intercellular adhesion molecule, CEA may contribute to the formation of metastasis; there is a correlation between serum CEA in patients with cancer and the incidence of hepatic metastases, but this could also simply reflect

tumor burden (Steele et al. 1982; Yeatman et al. 1989). In normal colonic epithelium, CEA is localized to the luminal surface, an arrangement that suggests that it contributes to spatial orientation of colonocytes and that it may also function to preserve the adult gut mucosal barrier (Marshall 2005). In tumor cells, however, CEA is irregularly distributed throughout the cell membrane.

A role for CEA in clinical medicine first became a consideration in 1969 when Thomson and colleagues developed a radioimmunoassay for measurement of CEA in the circulating blood system (Thomson et al. 1969). The development of a number of sensitive and reproducible radioimmunoassays for CEA ensued; these assays were capable of detecting nanogram quantities of CEA (Gold and Freedman 1975). By 1986, three commercialized kits for the analysis of CEA in serum were readily available: a Hoffman-LaRoche radioimmunoassay (direct and indirect) and two Abbott Laboratories' assays (a solid-phase radioimmunoassay and an enzyme immunoassay) (Fletcher 1986; Dierksheide 1981). However, a problem soon became clear: CEA measurements varied between laboratories and between assays. Thus, to minimize these discrepancies, guidelines were recommended for the clinical analysis of CEA (Dierksheide 1981; Taylor et al. 1977): the use of different assay methods interchangeably was to be avoided, and any clinical laboratory running an assay was to ensure strict validation of the assay and establish its individual "range of normal" (Fletcher 1986).

After the Clinical Laboratory Improvement Amendments (CLIA) were passed by the US Congress in 1988 (http://www.cms.gov/clia), only accurate, reliable, and reasonably straightforward assays were acceptable for clinical use, and CEA levels in clinical samples could be run only in CLIA-accredited labs. Although not identical, the majority of today's CLIA-certified CEA assays do share commonalities, and any differences between laboratory assays should be canceled out by calculating the range of normal using each individual assay. CLIA-accredited assay types now include two-site immunoenzymatic sandwich assays and a range of chemiluminescence immunoassays; numerous test-kits are commercially available (Locker et al. 2006; Moertel et al. 1993).

The Use of CEA as a Therapeutic Target in Oncology Practice

CEA is a circulating marker of tumor burden and response to treatment, and as such, assays that measure CEA levels in serum are widely used in oncology practice. Circulating CEA levels are used (1) to set a baseline at the beginning of patient therapy, (2) to detect cancer recurrence, (3) to get an idea of response to therapy, and (4) as follow-up. CEA has been linked to cancer metastasis, apoptosis, and chemotherapy resistance.

CEA has also been the target for therapeutic development for more than 10 years, with the primary emphasis being immunotherapy. This is discussed in the "Therapeutics" section below.

Target Assessment

Measurement of CEA in serum and on tissues, using immunohistochemistry, is FDA approved and commonly employed in the diagnosis and treatment of colon cancer and other adenocarcinomas. It is important to emphasize that measured levels of CEA may differ between laboratories and countries, hence the essential use of "range of normal" calculations for each individual assay.

Role of the Target in Cancer

Rank: "unknown" to 10
Unknown-1-2-3-4-5-6-7-8-9-10: 7

- A large number of adenocarcinomas overexpress CEA, and as such, CEA levels in the circulation are widely used as a serological marker for the assessment of tumor treatment response. However, this approach is not successful for *all* cancers and is not quantitative (7).
- Although an elevated preoperative CEA level is a poor prognostic sign (Locker et al. 2006), CEA has not proven itself useful as a screening test (6).
- Due to CEA's association with, and wide distribution in, human tumors, the protein has the characteristics of an ideal target for vaccine therapy. However, further study is still required before the best strategy for the clinical application of CEA-directed vaccines can be defined (Marshall 2003) (8).

High-Level Overview

Diagnostic, Prognostic, and Predictive

CEA measurements are used routinely and are recommended for three different colon cancer indications: preoperative assessment, follow-up monitoring of potentially cured patients, and an assessment of response to therapy. CEA is not routinely used for other cancers but may hold similar benefits in other GI cancers, as well as breast and lung cancers. A rising CEA value suggests cancer recurrence or progression, whereas a falling value suggests benefit.

For colorectal cancer, it is recommended that a CEA test be ordered preoperatively if it can assist in staging and surgical planning. Frequent monitoring of CEA postoperatively may allow for identification of patients with metastatic disease for whom surgical resection or other localized therapy might be potentially beneficial. The 2006 American Society of Clinical Oncology (ASCO) recommendations are that "Postoperative CEA levels should be performed every 3 months for stage II and III disease for at least 3 years if the patient is a potential candidate for surgery or

chemotherapy of metastatic disease" (Locker et al. 2006). CEA is the marker of choice for monitoring the response of metastatic disease to systemic therapy. Failure of CEA to return to normal levels after surgical resection is indicative of inadequate resection of occult systemic disease. Due to a lack of sensitivity in the early stages of colorectal cancer, analysis of CEA is not recommended for population screening.

The European Group on Tumor Markers (EGTM) stated that CEA testing should be done every 2–3 months in patients with stage II and III colon cancer who may be candidates for liver resection in the case of metastatic disease. CEA is thus considered to be (1) a valuable component of postoperative follow-up, (2) the most frequent indicator of recurrence in asymptomatic patients, (3) more cost-effective than radiology for the detection of potential curable recurrence, and (4) the most sensitive way of detecting early liver metastases.

Three meta-analyses confirm that testing for CEA levels as part of a follow-up program results in a reduction in mortality (Locker et al. 2006). Figueredo et al. (2003) carried out one of these meta-analyses and concluded that the incidence of asymptomatic recurrence is significantly less common in patients who are more comprehensively followed up. It was suggested that follow-up programs that include CEA measurements and liver imaging demonstrate significant impact on overall survival (relative risk [RR] = 0.71; 95% CI = 0.60–0.85; $P = 0.0002$).

In a separate meta-analysis, Rosen et al. (1998) compared patient outcomes reported in studies published between 1972 and 1996, comprising a total of 2300 patients. In this analysis, the cumulative 5-year survival rate was 72.1% for patients who were comprehensively followed up compared to 63.7% for less intensively followed up control groups ($P \leq 0.0001$). Economic analyses suggest that intensive follow-up incorporating CEA testing is cost-effective compared with conventional follow-up.

In 2006, ASCO guidelines recommended that, in addition to CEA testing every 3 months, annual CT of the chest and abdomen should be performed for 3 years after primary therapy for patients who are at high risk of recurrence and who could be candidates for curative-intent surgery. ASCO recommended that CEA is currently the only marker of choice for monitoring the response of metastatic disease to systemic therapy. An elevated preoperative CEA is a poor prognostic sign and correlates with reduced overall survival after surgical resection of colorectal carcinoma (Locker et al. 2006).

A study reported in a 2013 ASCO Annual Meeting abstract compared the proportion of patients originally diagnosed from stages I through III colorectal cancer that experienced disease recurrence following four different approaches to follow up (ref). The authors demonstrated that doing a CT scan and CEA analysis within the first 1–2 years after surgery was equally as useful as more intensive intervention. The study findings suggest that a single evaluation at 12–18 months after surgical intervention allows for detection of operable, potentially curable, recurrent colorectal cancer in the majority of patients. Due to the fact that our current standard, including that prescribed in clinical trials, entails a much longer (in some cases, up to 5 years) and more intensive follow-up, this is a potentially practice-changing and cost-effective finding.

Therapeutics

Although CEA is an oncofetal tumor antigen, normally expressed in fetal colon, it is present in saliva, feces, serum, colonic mucosa, and fluid from colonic lavages in adults. CEA is overexpressed in a high percentage of adenocarcinomas, particularly those of endodermal origin (e.g., the non-small cell lung, stomach, colon, rectum, and pancreas). As such, it is considered a shared (public), non-mutated oncofetal self-antigen. Due to its association with malignancy and wide distribution in human tumors, CEA has the characteristics of an ideal target for vaccine therapy (Marshall 2003; Wang et al. 2008) and has been the target for therapeutic development for more than 10 years (Marshall 2003; Wang et al. 2008). However, a major barrier to the use of CEA as a vaccine is that the antigen is not just present on tumor tissues but is also normally expressed in adult life, causing the immune system to have natural tolerance to CEA (Marshall 2003, 2005). To be successful, CEA vaccines need to overcome this immune tolerance and trigger a significant immune response against CEA-expressing cancer cells. Thus, CEA needs to be shifted from a non-immunostimulatory to an immunostimulatory state.

Garnett et al. (2006) discuss that stimulation and activation of T cells are essential for a successful adaptive immune response to an antigen; a sufficient immune response to a TAA, such as CEA, may result in direct attack of the tumor. Thus, the goal of CEA-related cancer immunotherapy is to generate T lymphocytes specific for CEA and elicit an immune response capable of tumor destruction. Cancer vaccines have been developed that alter the context in which CEA is presented to the immune system, resulting in activation of CEA-specific T cells that do not occur naturally in the host.

Preclinical Summary

Extensive preclinical work aiming to define and optimize the role of CEA as a target for immune therapy has been carried out, and positive results have been consistently published. Strategies include injection of naked DNA, incorporation of viral vectors and costimulatory molecules, addition of the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), prime and boost strategies, and dendritic cell loading. A major limitation in immunotherapy studies of human cancer is the general lack of appropriate preclinical models, increasing the importance of human trials and immune monitoring (Marshall 2003).

Some noteworthy preclinical studies.

DNA Vaccines

► DNA vaccine comprise a bacterial plasmid containing genes that are under the control of a strong eukaryotic promoter (this includes pathogens, allergens, or tumor antigens). Naked DNA vaccination (i.e., plasmid DNA in saline) has been preclinically tested. In one study, a plasmid encoding the full-length human CEA

gene was injected intramuscularly into mice; this induced CEA-specific humoral and cell-mediated immune responses. The ► DNA Vaccine was also shown to immunoprotect mice against CEA-expressing colon tumor progression (Conry et al. 1994). The safety of DNA vaccine administration to humans has been questioned: one concern is the possible induction of anti-DNA antibodies, as observed in patients who have systemic lupus erythematosus (SLE). However, animal studies have shown that vaccination with purified DNA does not induce anti-DNA antibodies (Katsumi et al. 1994).

Viral Vectors for Antigen Delivery

Poxviruses such as the vaccinia virus are particularly well suited as vectors for therapeutic cancer vaccines. They are easily engineered, accommodate large inserts of foreign DNA, allow posttranslational modification of expressed proteins, replicate accurately without helper viruses, stimulate potent immune responses, and have been extensively tested in humans as smallpox prophylaxis (Marshall 2003; Horig et al. 2000). Vaccinia virus expressing CEA was found to be effective in the treatment of established CEA-expressing tumors in a colon carcinoma mouse model and was associated with the development of anti-CEA antibody titers and T-cell responses (Kantor et al. 1992a); the vaccine was most effective in the prevention of CEA-expressing tumor growth when mice had undergone preimmunization. The same vaccine was also tested in a nonhuman primate model; toxicity was found to be minimal and the monkeys produced CEA-specific T-cell responses after vaccination (Horig et al. 2000; Kantor et al. 1992b).

In preclinical study, ALVAC-CEA vaccine (a vaccine containing a canary pox virus (ALVAC) combined with the CEA human gene) demonstrated protective and antitumor activity in mice (Hodge et al. 1997).

Co-stimulation

TRICOM® (triad of costimulatory molecules) vaccine contains three costimulatory molecules: B7.1, ICAM-1, and LFA-3. Preclinical studies showed that TRICOM recombinant vaccinia virus induced much greater antigen-specific T-cell proliferation and antitumor immunity than cells injected with any one or two of the listed costimulatory molecules alone (Hodge et al. 1999). Aarts et al. (2002) demonstrated in mice that continued boosting with recombinant fowl pox (rF)-CEA vaccine was important for the maintenance of CEA-specific T-cell response and boosting with the rF-CEA plus TRICOM was superior to boosting with rF-CEA alone. Initial vaccination with recombinant vaccinia (rV)-CEA/TRICOM followed by boosting with rF-CEA/TRICOM was more effective at inducing CEA-specific T-cell responses than homogeneous vaccination with rF-CEA/TRICOM. The combination of vaccination with cytokines, GM-CSF and IL-2, further enhanced the antitumor activity of CEA/TRICOM vaccines.

Dendritic Cells and CEA

Dendritic cells are the most potent type of antigen-presenting cells and are essential to prime the adaptive immune response (Wang et al. 2008; Song and Kim 2004). The identification of several dendritic cell growth factors, such as GM-CSF and interleukin 4 (IL-4), has permitted dendritic cell expansion and activation in vitro (Kea 1992; Romani et al. 1994; Sallusto FaL 1994). For example, large numbers of dendritic cells were generated in mouse bone marrow cultures that were supplemented with granulocyte-macrophage colony-stimulating factor (Kea 1992).

Clinical Summary

A large body of clinical data testing the various CEA-based vaccines in patients with cancer now exists (Marshall 2003; Wang et al. 2008; Horig et al. 2000). Several early Phase I and II studies have been completed that demonstrate safety and consistent immune stimulation. Larger Phase I/II trials have been completed combining vaccines with chemotherapy (Arlen et al. 2006; Kaufman et al. 2008; Quoix et al. 2011). A recent Phase II trial of vaccines with dendritic cell loading has completed accrual and results are pending (https://clinicaltrials.gov, identifier: NCT00103142). A larger Phase III second-line treatment study randomized patients with pancreatic cancer to receive either CEA-based vaccines or standard treatment. The trial was completed with negative results (https://clinicaltrials.gov, identifier: NCT00088660). A data summary indicated that the vaccines used were highly safe, no autoimmunity was seen, and immune responses were regularly observed, specific to CEA peptides. For example, Foon and co-workers reported the development of humoral and T-cell immunity to CEA as a result of immunization with a CEA anti-idiotype vaccine (Baral et al. 2003; Foon et al. 1999). Data from multiple trials suggest that patients with lower tumor burden, less chemotherapy exposure, and better performance status reap greater benefit than patients who do not fit those criteria. CEA-based vaccines that have been tested in human trials include ALVAC-CEA, Vaccinia-CEA, TRICOM-CEA, PANVAC (vaccinia-CEA-MUC-1-TRICOM), and PANVAC with dendritic cell loading (Marshall 2003; Wang et al. 2008; Horig et al. 2000). Isolated monocytes from individual patient peripheral blood can generate dendritic cells, which can then be expanded ex vivo, pulsed with an antigen, and then readministered to the same patient as a dendritic cell vaccine (Romani et al. 1994).

ALVAC-Based CEA Vaccines (Marshall 2005)

Fowl pox and canary pox viruses are members of the poxvirus family, in the genus *Avipoxvirus*; they are pathogenic in birds but are unable to replicate in mammalian cells. Avipox viruses are able to bring about strong T-cell immune responses in humans without concurrent production of strong neutralizing

antibodies. Thus, repeated immunizations are possible (Paoletti 1996). We conducted the first Phase I trial with a canary pox-CEA (ALVAC-CEA) vaccine, in 15 patients with advanced cancer (Marshall et al. 2000). Patients were treated with three injections of ALVAC-CEA 4 weeks apart. This study showed that ALVAC-CEA vaccine was safe to use in patients with advanced cancer; the main toxicities were mild skin reaction and mild injection site soreness. Regarding tumor response, a statistically significant increase in cytotoxic T-lymphocyte (CTL) response precursors was observed in approximately two out of three patients. This study demonstrated that ALVAC-CEA could induce CEA-specific CTL responses, but no objective antitumor effect in patients with advanced CEA-expressing cancer was observed.

Postvaccination CEA-specific T-cell counts were significantly higher when the cytokine, GM-CSF, was added to the ALVAC-CEA vaccine regimen. However, the cytokine, IL-2, did not show any enhancement effect on T-cell counts (Aarts et al. 2002).

Von Mehren et al. (2000) treated 39 cancer patients with a dual gene recombinant avipox vaccine, which contained both CEA and the costimulatory molecule B7.1. Most of the patients had advanced colorectal cancer, although some had breast, lung, thyroid, gall bladder, esophagus, pancreas, or appendix cancer. Patients received intradermally injected recombinant vaccine based on a dose escalation design (1.0×10^8 [$n = 6$], 2.5×10^7 [$n = 3$], and 4.5×10^8 [$n = 30$] pfu) every other week for 8 weeks. Patients without disease progression at 8 weeks continued to receive monthly boost injections until disease progression. Most patients tolerated the treatment well. Injection site erythema, swelling, myalgia, and flu-like symptoms were the most common toxicities. Thirty patients received at least four injections of vaccine (8 weeks), which made them evaluable for response. There were no partial or complete responses, although eight patients (seven with colorectal and one with pancreatic cancer) had stable disease at 8 weeks, which continued from one to seven boost vaccinations. Six patients with elevated serum CEA values at baseline exhibited a decline in their levels lasting 4–12 weeks. All six patients were in the stable disease group. It was concluded that recombinant avipox vaccine containing CEA and costimulatory molecule B7.1 gene was safe to use in patients with advanced CEA-expressing adenocarcinomas and was associated with the induction of a CEA-specific T-cell response. Disease stabilization was seen in 27% of all evaluated patients.

DNA Vaccines

In one of the first ► DNA Vaccines clinical trials, Conry et al. used CEA DNA along with hepatitis B surface antigen (HBsAg) DNA in a plasmid vector to vaccinate 17 patients with metastatic colorectal cancer (Conry et al. 2002). No patients had serological evidence of hepatitis B, and all were at least 4 weeks from their last chemotherapy or radiotherapy.

Patients received intramuscular (IM) escalating doses of 0.1, 0.3, and 1.0 mg of vaccine. Groups further received 0.3, 1.0, or 2.0 mg doses every 3 weeks after the

dose escalation. Toxicity was isolated to local tenderness at the injection site, fatigue, and elevated creatinine kinase. Elevated C-reactive protein (CRP) was found in seven patients, but this was more consistent with progression of disease than vaccination. Despite immunization, only four patients developed a lymphoproliferative response to CEA. It was noted that 12 patients experienced disease-free survival, which switched to progressive disease at 9 weeks of follow-up (Conry et al. 2002).

In another more recent clinical trial, CEA66 DNA (a plasmid DNA vaccine encoding a truncated form of human CEA fused to a T-helper epitope) was delivered three times intradermally at 2 mg or intramuscularly at 8 mg by Biojector® to patients with colorectal cancer. Prior to the first vaccination, all patients received intravenous cyclophosphamide (300 mg/m^2). Subcutaneous administration of GM-CSF accompanied each vaccination. All patients completed the vaccine regimen and were evaluable. No grade 3 or 4 adverse events were observed, and grade 1 and 2 toxicities included injection site reactions, headache, joint pain, muscle pain, chest tightness, and fatigue. Administration of CEA66 DNA vaccine to patients in combination with GM-CSF was well tolerated, and no autoimmunity was detected (Staff et al. 2011). The lack of autoimmunity is in agreement with DNA vaccine findings from animal studies (Katsumi et al. 1994).

Co-stimulation

We conducted a Phase I trial using recombinant CEA-TRICOM vaccines in 56 patients with metastatic CEA-expressing adenocarcinomas. Primary tumor sites included the GI (chiefly colorectal – more than half the patient population), lung, breast, and ovary. Patients were divided into cohorts and received variable vaccination regimens based on a backbone of recombinant fowl pox vector (rF)-CEA(6D)-TRICOM with or without recombinant vaccinia vector (rV) CEA(6D)-TRICOM, (rF)-CEA(6D)-TRICOM boosting, or addition of GM-CSF (Marshall et al. 2005). We demonstrated that TRICOM-CEA pox vaccines are safe to use alone or in combination with other biologics in patients with advanced cancer. TRICOM-CEA pox vaccines are able to generate significant CEA-specific immune responses, and they seem to have clinical benefit in some patients with advanced cancer. The impact of adding GM-CSF to the vaccine regimens was not clear in this study. A vaccine booster in the same scenario had no effect.

PANVAC (Wang et al. 2008)

Kaufman et al. (2007) reported a Phase I study using PANVAC in patients with advanced pancreatic cancer. Vaccinia vector expressing CEA, ▶ MUC-1, and TRICOM was named PANVAC-V, and fowl pox vector expressing CEA, MUC-1, and TRICOM was named PANVAC-F. Ten patients received PANVAC-V priming followed by three booster vaccinations with PANVAC-F. GM-CSF was also used as

a local adjuvant after each vaccination and for three consecutive days thereafter. Monthly booster was given if a patient had no disease progression. There was no significant toxicity; the majority of side effects were low-grade injection site reactions or constitutional symptoms. Anti-CEA-specific antibody was detected in 50% of patients, and a heightened antigen-specific T-cell response was observed in 63% of patients. The PANVAC recombinant vaccinia expresses two TAAs: CEA and MUC-1. For those patients who had an anti-TAA-specific response, a significant increase in overall survival was noted compared with those who did not have such a response. However, a company-sponsored Phase III trial using PANVAC as second-line treatment in metastatic pancreatic cancer failed to show benefit (https://clinicaltrials.gov, identifier: NCT00088660).

Anticipated High-Impact Results

Currently anticipated clinical trial results:

- In one randomized Phase II trial, 72 patients with completely resected hepatic or pulmonary metastases, secondary to colorectal cancer, are undergoing treatment with adjuvant vaccine therapy comprising PANVAC-V and PANVAC-F, administered with autologous dendritic cells (Arm 1) or with sargramostim (GM-CSF) (Arm 2). Two-year disease-free survival is being assessed, as well as the rate and magnitude of immune response, as determined by ELISpot. Comparisons between the two arms will be made (Michael A Morse, MD; Duke University, North Carolina) (https://clinicaltrials.gov, identifier: NCT00103142).
- In another trial being carried out at Ohio State University, a vaccine therapy, GM-CSF, and interferon alfa-2b combination is being administered to patients with locally advanced or metastatic cancer that expresses CEA. The purpose of this Phase I study is to observe the side effects and best dose of interferon alfa-2b when given together with vaccine therapy and GM-CSF in the treatment of patients with CEA-expressing locally advanced or metastatic cancer (Arthur G. James, MD) (https://clinicaltrials.gov, identifier: NCT00217373).
- The objective of an open-label pilot study being carried out at the NCI is to evaluate the safety and tolerability of PANVAC-V and PANVAC-F in combination with Sargramostim in adults with metastatic carcinoma. The intention is also to document the immune response to the vaccines and any antitumor responses that may occur (James L Gulley, MD, NCI) (https://clinicaltrials.gov, identifier: NCT00088413).

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CTLA-4

14

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Abstract

This is a brief summary of the biologic basis for use of CTLA-4 blockade in the treatment of cancer, specifically melanoma and renal cell carcinoma. Included is the biology of CTLA-4, the preclinical and clinical studies to date. Lastly, the future goals and directions of CTLA-4 are discussed including combination with newer immunotherapies.

Keywords

CTLA-4 • Immunotherapy • Ipilimumab • T cell

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Target: Cytotoxic T Lymphocyte Antigen-4 (CTLA-4)

Cytotoxic T lymphocyte antigen-4(CTLA-4) is a transiently expressed protein on the surface of activated T cells that plays a significant role in regulating T-cell responses. CTLA-4 signaling inhibits T-cell activation by decreasing cytokine production and blocking cell cycle progression. CTLA-4 is commonly referred to as a T-cell co-inhibitory molecule or a T-cell checkpoint since the net effect of CTLA-4 activity is to suppress T-cell responses. In 2011, a humanized monoclonal antibody to CTLA-4, ipilimumab, was approved by the FDA for the treatment of advanced melanoma (Hodi et al. 2010). Clinical trials demonstrated that CTLA-4 blockade improved overall survival in patients with advanced melanoma compared to peptide vaccine or dacarbazine chemotherapy (Hodi et al. 2010; Robert et al. 2011). Recent reports with longer periods of follow-up have demonstrated a durable response associated with administration of anti-CTLA-4 therapy (Prieto et al. 2012). Combined, these findings have firmly established CTLA-4 as an important target for immunotherapy.

This chapter will focus on the role of CTLA-4 as a therapeutic target, specifically through the use of anti-CTLA-4 monoclonal antibodies, in clinical oncology. There will be a brief discussion of the molecular structure and biologic function of the target, the proposed mechanism of action, and unique pharmacokinetics of ipilimumab. The current role of ipilimumab in cancer with attention to pertinent preclinical and clinical data, including the potential of ipilimumab as an immunologic adjuvant for cancer vaccines, will be covered. Potential predictive biomarkers of therapeutic response to ipilimumab, the type and management of adverse effects observed in patients treated with ipilimumab, and new approaches to assess clinical responses in patients treated with ipilimumab will also be discussed. Finally, the potential impact and future directions of research into the optimal use of CTLA-4 as a therapeutic target will be presented.

Biology of the Target

CTLA-4 plays a central role in T-cell homeostasis. T-cell activation requires that the T-cell receptor recognize its cognate antigen as processed peptide bound to major histocompatibility complex (MHC) classes I and II molecules for $CD8^+$ and $CD4^+$ T cells, respectively. In order to fully activate a T cell that has recognized its antigen, a second, or co-stimulatory, signal is also needed. This is delivered by CD28, which is expressed on the T-cell surface and binds to its ligands, CD80 and CD86 (also known as B7.1 and B7.2). CD28 cooperates with the T-cell receptor to induce cytokine production and cell cycle progression, promoting activation and differentiation of effector T cells. CTLA-4 is mobilized to the cell surface after T-cell activation and competitively binds to CD80 and CD86 effectively reducing T-cell co-stimulation and reducing cytokine production and cell cycle progression. CTLA-4 and CD28 share significant homology that permits cross-reactivity with CD80 and CD86. Thus, CD28 and CTLA-4 serve as an internal rheostat to modulate T-cell

activation and suppression allowing a rapid burst of effector T-cell activity while preventing excessive immune system activation or autoimmunity. The pivotal role of CTLA-4 in regulating T-cell responses has been observed in CTLA-4 knockout mice. In these models animals consistently develop overwhelming lymphoproliferation and succumb to multi-organ system failure by the fourth week of life (Tivol et al. 1995), supporting the essential role of CTLA-4 in suppressing immune reactivity.

CTLA-4 is a 41–43 kDa member of the immunoglobulin superfamily and the gene is located on chromosome 2q33. CTLA-4 is expressed on activated T cells, including both effector and regulatory populations. The CTLA-4 protein is comprised of an extracellular domain, a transmembrane component and an intracellular tail. The extracellular domain has a great deal of homology with CD28, which allows competitive binding with CD80 and CD86. There are variants of the intracellular domain that influence normal physiologic function, and these variants have been associated with a variety of clinical autoimmune syndromes. The intracellular domain is known to interact with multiple different proteins, and signaling exhibits an overall inhibitory effect on T-cell activation by blocking cytokine production (e.g., IL-2) and cell cycle progression. While CD28 is expressed on naive T cells, CTLA-4 expression is restricted to activated T cells, and together, they regulate T-cell activation and tolerance. Studies have suggested that CTLA-4 has a higher affinity for B7.1 compared to CD28, and this may be a mechanism through which CTLA-4 blocks T-cell co-stimulation, although other mechanisms may also be responsible (Sharma et al. 2011). CTLA-4 is also expressed in regulatory T cells although the functional significance of this is uncertain.

Since cancer cells can be recognized by activated T cells, it is possible that CTLA-4 signaling might be, at least in part, responsible for inducing tolerance in tumor-reactive T cells in a host with an established cancer. Blockade of CTLA-4 has been shown to prevent tolerance and promote tumor regression in murine tumor models (van Elsas et al. 1999). These animal models, as well as other preclinical data presented below, resulted in the clinical trials of humanized anti-CTLA-4 monoclonal antibody in patients with advanced melanoma. The clinical trials demonstrated a therapeutic benefit but also defined a distinct pharmacokinetic profile that was associated with unique immune-related toxicities and delayed kinetics of clinical response that poses novel challenges for managing patients treated with anti-CTLA-4 therapy.

Target Assessment

CTLA-4 expression can be measured by immunohistochemical staining as well as reverse transcription polymerase chain reaction (RT-PCR) in human tissue samples. Levels of CTLA-4 can also be measured by flow cytometry on T cells acquired from patients through peripheral blood draw. While these methods have been used in the research setting to evaluate levels of CTLA-4, they are not routinely used in the

clinical setting prior to or during anti-CTLA-4 therapy. At present there are no clinical practice guidelines for its measurement outside of the research setting.

Role of Target in the Cancer

Rank: 10

Ipilimumab has been shown in multiple phase III trials to prolong survival in stage IV melanoma, and as such it is currently FDA approved for therapeutic use in these patients. Ongoing research includes its combined use with other biologic and immunologic therapies. Additionally, work is ongoing to evaluate the potential therapeutic benefit in renal cell carcinoma and other solid organ malignancies.

High-Level Overview

Diagnostic, Prognostic, and Predictive

Anti-CTLA-4 treatment, like other forms of immunotherapy, has the potential to induce durable therapeutic responses but is also associated with significant toxicity. Thus, identifying predictive biomarkers of response and/or toxicity is a high priority. At present, there are no validated biomarkers of response to ipilimumab therapy, although investigation into putative predictive and prognostic biomarkers is ongoing.

Multiple retrospective analyses have been conducted looking at laboratory and immunologic markers before and during anti-CTLA-4 therapy. One promising biomarker is the absolute lymphocyte count (ALC). In one large pooled analysis ($n = 444$), clinical activity (CR, PR, or SD) after treatment with ipilimumab was associated with a greater mean rate of change in the ALC over the course of treatment (Berman et al. 2009). In a single institution, retrospective review of 51 patients, ALC was recorded after two doses of ipilimumab. Patients with an increase in ALC (ALC>1,000/μL) were found to have improved clinical response and prolonged overall survival (Ku et al. 2010). These findings prompted closer evaluation of these lymphocytes to attempt to identify subsets of cells that were prominent in patients responsive to treatment with anti-CTLA-4 therapy. In a study of 35 patients, clinical benefit was found to correlate with increased numbers of $CD8^{+}$ lymphocytes (Yang et al. 2010).

Additional research into a number of immune markers is ongoing. Inducible costimulator (ICOS) is a member of the immunoglobulin gene superfamily and has been found to be expressed with increased frequency on T cells in patients after treatment with ipilimumab and was also correlated with improved overall survival (OS) in one study (Chen et al. 2009). Human leukocyte antigen-D related (HLA-DR) (a class II MHC molecule) and CD45RO (a marker of memory T cells) have both been evaluated and found to be increased following treatment with CTLA-4 blockade. To date, however, only one small study ($n = 12$) has correlated

expression of these molecules with improved clinical response (Comin-Anduix et al. 2008). Each of these molecules has in common that they are markers of increased T-cell activation.

Another area of investigation has focused on differences in the host immune system that may account for response to anti-CTLA-4 therapy. One particular marker of interest has been polymorphisms in the CTLA-4 gene. There is evidence for an association between specific CTLA-4 genotypes and susceptibility to autoimmune diseases (Gough et al. 2005). Given our growing knowledge of the relationship between autoimmune-like toxicities and clinical response to anti-CTLA-4 therapy, there is an obvious interest in determining which polymorphisms might be associated with clinical response. One study of 152 patients with stage IV melanoma receiving therapy with ipilimumab evaluated this question. Genotyping of seven common single nucleotide polymorphisms (SNPs) of the CTLA-4 gene was conducted and clinical response data were collected and evaluated. Results showed that 15.1% of patients had complete or partial response to therapy. Clinical response was associated with three specific polymorphisms, while the other SNPs identified did not show a statistically significant difference between responders and nonresponders (Bruneis et al. 2008). In another small study of 19 patients, genetic polymorphisms associated with low versus high CTLA-4 expression were evaluated. In this study, 4 out of 19 patients had the allele with lower expression of CTLA-4 and 3 out of 4 of these patients developed autoimmune side effects after therapy and 2 out of 4 (50%) had relapse of their disease. On the other hand, 10 out of 15 (67%) patients with alleles correlating to higher levels of CTLA-4 expression developed disease relapse and only 33% developed autoimmune-like symptoms (Sanderson et al. 2005). These data require further prospective validation in a larger population before being applied in the clinical setting.

Preclinical Summary

Tumor cells have the capacity to evade normal immune regulation. Cancer immunotherapy has focused on modulating this response (e.g., IL-2 therapy), inducing an active tumor-specific response (e.g., vaccine therapy) or providing the patient with activated immune effector cells (e.g., adoptive T-cell therapy). Investigations of T-cell checkpoints, including CTLA-4, provide additional evidence for the potent antitumor activity possible by modulating T-cell responses through CTLA-4 blockade. Early murine models demonstrated that monotherapy with anti-CTLA-4 antibodies induced tumor regression in thymoma, prostate carcinoma, fibrosarcoma, and lymphoma murine models (Kwon et al. 1997). In less immunogenic tumors, monotherapy with CTLA-4 blockade did not induce tumor regression. However, coupling of anti-CTLA-4 antibodies with GM-CSF was able to induce tumor regression in non-immunogenic tumors, such as B16 melanoma (van Elsas et al. 1999; Hurwitz et al. 1998). The combination of anti-CTLA-4 therapy and GM-CSF resulted in a significant increase in the ratio of effector (T_{eff}) to regulatory

(T_{regs}) T cells, affecting the suppressive function of T_{regs} in murine models (Quezada et al. 2006).

There has been considerable preclinical investigation into the mechanisms of the antitumor activity induced by CTLA-4 blockade. To date, there is convincing data that CTLA-4 blockade acts on effector T cells as demonstrated by cotransfer experiments in which CTLA-4 expression by normal effector $CD4^+$ T cells completely abrogates expansion of CTLA-4-deficient, antigen-specific $CD4^+$ T cells (Corse and Allison 2012). CTLA-4 blockade has also been shown to enhance effector memory $CD8^+$ T-cell responses (Pedicord et al. 2011). CTLA-4 blockade may, however, also expand the FoxP3+ regulatory T-cell population, which is known to inhibit host antitumor immunity (Kavanagh et al. 2008). The impact of CTLA-4 blockade on T_{regs} is controversial with some studies showing a decrease in T_{regs} and others suggesting an increase in the different regulatory T-cell populations (Reuben et al. 2006; Maker et al. 2005a). A potential explanation may be that the impact of CTLA-4 blockade may differentially inhibit both T-cell populations and ultimately influence the ratio of effector to regulatory T cells. Further investigation is needed to better understand how CTLA-4 inhibition mediates tumor rejection.

Anti-CTLA-4 treatment has shown significant synergistic therapeutic effects when used in combination with other immunotherapy agents or conventional cancer therapies. For example, in a transgenic adenocarcinoma of the mouse prostate (TRAMP)cancer model, anti-CTLA-4 antibody was combined with IL-15 and an additive therapeutic effect was seen (Yu et al. 2012). In this model, the combination of IL-15 with anti-CTLA-4 and anti-programmed death-1 (PD-1) treatment resulted in significant survival benefit, which was also associated with the induction of prostate-specific $CD8^+$ T-cell responses. The investigators also noted a significant decrease in T_{regs} and $CD8^+$ suppressor T cells when all three agents were used. Cryoablation is a form of thermal ablation used to destroy tumor tissue and activate tumor-specific T cells through the release of tumor-associated antigens. In the TRAMP model, CTLA-4 blockade and cryoablation prevented the outgrowth of secondary tumors and was associated with an increase in the ratio of antigen-specific $CD8^+$ T cells to T_{regs}, which was not seen in cryoablated animals treated without anti-CTLA-4 treatment (Waitz et al. 2012). CTLA-4 blockade has also shown promise in combination with increased T cell co-stimulation. 4-1BB is transiently expressed by $CD8^+$ T cells after activation and promotes T-cell survival and has been shown to have some activity against the poorly immunogenic murine B16 melanoma. When combining an activating antibody to 4-1BB with CTLA-4 blockade and Flt3 ligand, a synergistic effect against B16 was observed. In this study 4-1BB treatment was associated with an increase in tumor-infiltrating $CD8^+$ T cells and a decrease in T_{regs}, while anti-CTLA-4 treatment was associated with an increase in tumor-infiltrating $CD4^+$ effector T cells (Curran et al. 2011). Anti-CTLA-4 antibody has also shown augmented tumor rejection when combined with an antibody against the glucocorticoid-induced tumor necrosis factor receptor (GITR) in an animal model (Mitsui et al. 2010). Importantly, the combination of CTLA-4 and PD-1 blockade was twice as effective in rejecting B16 melanoma tumors and was associated with a significant expansion of tumor-infiltrating effector T cells and a reduction

in T_{regs} and myeloid-derived suppressor cells (Curran et al. 2010). CTLA-4 blockade may also promote the therapeutic activity of adoptively transferred $CD4^+$ and $CD8^+$ T cells (Quezada et al. 2010). These preclinical findings are now being explored in the clinical setting with several early phase clinical trials in development.

Clinical Summary

Ipilimumab is the first agent designed to block CTLA-4 and has demonstrated an improvement in overall survival in patients with advanced melanoma in two prospective, randomized clinical trials (Hodi et al. 2010; Robert et al. 2011). The use of anti-CTLA-4 treatment represents not only a novel immunotherapy but a paradigm shift in the approach to immunotherapy based on its unique pharmacokinetic profile and types of adverse events observed with treatment. Anti-CTLA-4 therapy targets the host immune response to tumors rather than directly targeting tumor cells. The exact mechanism through which anti-CTLA-4 mediates tumor regression is unknown but is presumably mediated by a shift toward more activated, tumor-reactive T cells being available to recognize and kill tumor cells. Ipilimumab is the first of its class to receive FDA approval due to compelling phases II and III data supporting a clinical benefit in patients with metastatic melanoma. In addition to CTLA-4, there are several other T-cell checkpoint molecules, and these are being actively investigated as targets for immunotherapy. Notably, the PD-1 receptor on T cells also functions as a negative regulator of T-cell function, and monoclonal antibodies that block PD-1and its ligand, PD-1 ligand (PD-L1), are in clinical development with early phase clinical studies demonstrating clinical activity in melanoma, renal cell carcinoma, non-small cell lung cancer, and ovarian cancer.

Therapeutics

Ipilimumab is a humanized anti-CTLA-4 monoclonal antibody and has demonstrated significant benefit in clinical trials for the treatment of advanced melanoma. In a phase II dose-escalation trial, 0.3 milligrams (mg)/kilogram (kg), 3 mg/kg, and 10 mg/kg doses of ipilimumab were evaluated in 217 patients with unresectable melanoma. The best overall response rates (BORRs) were 0%, 4.2%, and 11.1%, respectively, at the three doses tested (Wolchok et al. 2010). In another phase II clinical trial of 115 metastatic melanoma patients combining ipilimumab with budenoside (used to modulate immune-related adverse events, or irAEs) or placebo, BORR was estimated between 12.1% and 15.8%, findings consistent with the previously mentioned phase II data. In this trial disease control rate (DCR), defined as complete response (CR), partial response (PR), and stable disease (SD), was estimated between 31% and 35.1%. The use of this endpoint is clinically significant because anti-CTLA-4 therapy may have a delayed benefit in patients and previously accepted early response assessments may not entirely reflect the benefit of this therapy in the clinical setting. In a third phase II single-arm trial, the 10 mg/kg

dose was again used and BORR was only 5.8% (O'Day et al. 2010). In this trial, however, the DCR was 27%, suggesting that longer periods for evaluation of clinical response are needed to assess the effect of ipilimumab. A long-term follow-up analysis of 177 patients enrolled on three different phase II protocols was conducted and estimated overall 5-year survival rates of 13–25% were reported, confirming a probable survival benefit with long-term follow-up (Prieto et al. 2012).

A prospective, placebo-controlled phase III trial of 676 previously treated patients with unresectable stage III or IV melanoma compared the combination of ipilimumab (3 mg/kg) with an HLA-restricted gp100 peptide vaccine compared to monotherapy with either agent alone. Overall survival (OS) in both the ipilimumab monotherapy (median survival 10.1 months) and the ipilimumab plus gp100 vaccine (10.0 months) groups exceeded that of patients who received the gp100 peptide vaccine alone (6.4 months) (Hodi et al. 2010). The major side effects consisted of immune-related adverse events (irAEs), such as dermatitis, colitis, hepatitis, and endocrinopathies with 10–15% of patients experiencing grade III or IV irAEs. The results of this trial led to FDA approval of the drug at the 3 mg/kg dose with dosing every 3 weeks for four total doses.

A subsequent phase III randomized clinical trial compared ipilimumab (10 mg/kg) and dacarbazine (DTIC) (850 mg/m^2 of body surface area) to DTIC and placebo. In this trial of 502 previously untreated melanoma patients, the group receiving ipilimumab + DTIC had longer OS (11.2 months v. 9.1 months) and higher survival rates at 1 year, 2 years, and 3 years of follow-up (Robert et al. 2011). The hazard ratio for patients receiving ipilimumab in combination with DTIC was 0.72 ($p < 0.001$). In this study 41.7% of patients receiving ipilimumab experienced grade III/IV irAEs, compared to 6% in the group treated with placebo. These side effects were managed with medical therapy, and no drug-related deaths were reported.

Ipilimumab is associated with a unique toxicity profile characterized by irAEs in which activated T cells mediate autoimmune-like pathogenicity in nontarget tissues. The most common organ toxicity observed has been in the skin, with symptoms ranging from mild rash to potentially fatal toxic epidermal necrolysis, and the colon, with symptoms ranging from mild diarrhea to autoimmune enterocolitis with bowel perforation. Other side effects include autoimmune hepatitis, hypophysitis and other endocrinopathies, uveitis, episcleritis, and peripheral and optic neuritis. In a retrospective evaluation of 56 patients, clinical response rates were evaluated and compared between groups based on specific irAEs. In patients with grade III or IV toxicity, 36% experienced a clinical response, compared to 5% in patients without autoimmune toxicity (Attia et al. 2005). In a study of 25 patients receiving ipilimumab at 10 mg/kg dosing, 48% of patients experienced grade II or greater irAEs. Overall, 16% of patients relapsed; however none of the patients with relapse had experienced an irAE (Weber et al. 2009). Another retrospective review of 198 patients with melanoma and renal cell carcinoma found that in patients with ipilimumab-induced enterocolitis, the objective tumor response rate was 36% in the melanoma group and 35% in the renal cell carcinoma (RCC) group, compared to 11% and 2%, respectively, among patients without enterocolitis (Beck et al. 2006). Despite these small clinical reports, the relationship between irAEs and clinical

response with ipilimumab is not firmly established and is an area of ongoing investigation.

While the development of irAEs was unexpected when CTLA-4 blockade was developed, there are emerging methods for managing these effects and the majority of side effects are not dose-limiting. The use of low-dose corticosteroids has been the mainstay of clinical management. Rarely patients require high-dose corticosteroids or other immunosuppresive agents to control autoimmune events. Occasionally, toxicity becomes severe and additional diagnostic or therapeutic intervention may be indicated. Adverse events, much like therapeutic response, may manifest late, up to several weeks or even months after exposure to ipilimumab and often wax and wane over several weeks before resolving. Clinical management algorithms for ipilimumab-related toxicity have been published (Ott et al. 2012).

Mild cases of ipilimumab-related enterocolitis can be easily managed with antidiarrheal agents, such as loperamide. In a large review of 700 ipilimumab-related cases of enterocolitis, only 4 cases of perforation were reported (Freeman 2012). In cases of severe colitis, other causes should be ruled out (e.g., *C. difficile* colitis) and careful evaluation for perforation needs to be considered (e.g., computed tomography scanning or endoscopy). If perforation has not occurred, treatment with high-dose corticosteroids or infliximab in resistant cases has been useful. The management of hepatic toxicity requires careful attention to liver function enzymes, early institution of corticosteroids, and, in severe cases, hospitalization and high-dose corticosteroids with daily monitoring of liver enzymes. In refractory cases, mycophenolate mofetil, tacrolimus, and infliximab have shown benefit. Hypophysitis is an autoimmune reaction of the pituitary gland and can manifest with fatigue, myalgia, headaches, visual disturbances, decreased libido, weakness, asthenia, anorexia, and constipation. This is much less common than other ipilimumab-related side effects. Patients with suspected hypophysitis should have an MRI of the brain as an enlarged pituitary gland is a typical finding and reimaging can document resolution of this rare event. In described cases of hypophysitis after treatment with ipilimumab, appropriate hormone replacement is required and may be permanent, and early referral to an endocrinologist is recommended (Ott et al. 2012).

An important observation in patients treated with ipilimumab has been the delayed onset of autoimmune side effects and therapeutic responses. This likely reflects the pharmacokinetics of ipilimumab activity in which T cells must be activated, travel to sites of tumor growth or end-organ toxicity, and mediate a local immune response and inflammation. This process takes time and differs from standard cytotoxic chemotherapy, which typically induces immediate tumor cell death. The long-term analysis of 177 patients enrolled in various combined immunotherapy protocols confirmed the delayed response in many patients treated with ipilimumab. In fact, this analysis demonstrated higher CR rates than had previously been reported by the original trial authors due to patients that became complete responders over time despite tumor persistence or even growth in the initial posttreatment period. This finding was noted across all study protocols included and follow-up extended up to 99 months (Prieto et al. 2012).

The delay in clinical response noted by treating physicians has prompted many involved in clinical immunotherapy research to suggest alternative metrics for evaluating response to therapy than the widely used WHO and response evaluation criteria in solid tumor (RECIST) criteria. In one study evaluating phase II clinical trial data available to date, the clinical response to ipilimumab monotherapy was described in four distinct response patterns: (1) regression of baseline lesions without new lesions, (2) durable stable disease (sometimes followed by very slow, gradual decline in total tumor burden), (3) response after an initial total increase in tumor burden, and (4) response in the presence of new lesions (Wolchok et al. 2009). The latter two categories represent a deviation from previously accepted standard response criteria. In the previously mentioned analysis of 177 patients enrolled in various phase II trials, the average time to CR among patients treated with ipilimumab was 30 months, and many of these patients were initially designated as SD or PR by WHO criteria following treatment (Prieto et al. 2012). A new system of clinical monitoring has been proposed called “immune-related response criteria” (ir-RC) (Wolchok et al. 2009). The ir-RC assess tumor burden by measurements that include both existing and new lesions as well as additional time points in the evaluation of the change in tumor burden. At this time these response criteria require formal validation before being utilized in a clinical setting. These observations, however, mandate caution in the evaluation of patients following ipilimumab treatment using the established response metrics.

Anticipated High-Impact Results and Future Directions

Ipilimumab is the first T-cell checkpoint inhibitor approved for the treatment of cancer, and randomized clinical trials have demonstrated a survival benefit in patients with advanced melanoma. Future directions will almost certainly include studies of ipilimumab in other types of cancer and in combination with conventional cancer therapeutics and other immunotherapy agents. Ipilimumab is currently being evaluated in the adjuvant setting for patients with stage III melanoma in a multi-institutional cooperative group’s clinical trial comparing two doses of ipilimumab (3 mg/kg and 10 mg/kg) to standard interferon-α treatment. Patients may have inherently stronger immune responses in the adjuvant setting when complete absence of measurable tumor has been achieved since established tumor suppression mechanisms may be weak or absent.

Several combination studies have been or will be conducted with regimens that include ipilimumab. A combination therapy that has generated a great deal of interest is ipilimumab with IL-2. In a phase I/II study of 36 metastatic melanoma patients, IL-2 was administered with a dose escalation of ipilimumab (from 0.1 to 3 mg/kg) (Maker et al. 2005b). In this study the objective response rate was 22%. However, at a median follow-up of 84 months, the median survival was 16 months and 5-year survival rate was 25%. Additionally, the CR rate associated with this particular combination was 17% in the previously cited long-term follow-up analysis (Prieto et al. 2012). This data is emerging and warrants further study.

The combination of anti-CTLA-4 and anti-PD-1 is another new area of research that has generated a significant amount of interest. Similar to CTLA-4, PD-1 has an inhibitory effect on T-cell activation, and PD-1 knockout mice demonstrate similar autoimmunity and splenomegaly to CTLA-4 knockout mice, suggesting loss of T-cell tolerance and highlighting the role of PD-1 in T-cell homeostasis. While both CTLA-4 and PD-1 activation block CD3/CD28-mediated glucose metabolism and Akt phosphorylation, recent work has demonstrated that they do so through different signaling pathways. CTLA-4 signals through tyrosine phosphatase (SHP-2) and serine/threonine phosphatase (PP2A), the latter of which plays an essential role in phosphorylation of Akt. In contrast to CTLA-4, PD-1 was found to directly suppress PI3K, which subsequently inhibits Akt (Parry et al. 2005). While the details of these two immune regulators remain under review, their differing mechanisms suggest a potential for therapeutic synergism. In murine models, the combination of CTLA-4 and PD-1 blockades has induced greater tumor rejection than that observed with blockade of either CTLA-4 or PD-1 alone (Curran et al. 2010). Clinical trials of anti-CTLA-4 and anti-PD-1 therapy are anticipated in the near future.

BRAF is a gene mutated in approximately 50% of all cutaneous melanomas. In BRAF mutant tumors, the mitogen-activated protein kinase (MAPK) pathway is constitutively activated, resulting in increased cellular proliferation. Vemurafanib is a selective BRAF inhibitor used in the treatment of melanoma with a confirmed BRAF mutation. Studies evaluating melanoma tissue before and after exposure to BRAF have shown an increase in both $CD8^{+}$ and $CD4^{+}$ T cells, with a greater increase noted among $CD8^{+}$ lymphocytes (Wilmott et al. 2012). One proposed mechanism of BRAF inhibitors is that they suppress the immunosuppressive effects of tumor cells and increase T-cell recognition. Thus, clinical trials are underway to combine BRAF inhibitors and ipilimumab in patients with metastatic melanoma.

The abscopal effect occurs when radiation to a tumor site results in regression of tumor at distant sites, a process that may occur through induction of systemic antitumor immunity at the site of radiation. A recent clinical report suggested an abscopal, synergistic effect between ipilimumab and radiation therapy in a patient with melanoma (Postow et al. 2012). The patient in this report received ipilimumab on clinical trial and was found on follow-up CT scan initially to have stable disease but then showed evidence of progression in the year following treatment. Ultimately the patient became symptomatic from her metastatic disease and palliative radiation was given to a paraspinal metastatic focus that was causing her pain. One month after radiation, her follow-up CT scan showed no change in disease burden and additional ipilimumab was given. In CT scans 3 and 8 months following this additional dose of ipilimumab, the patient was noted to have regression of disease both at the area of radiation and in other distant sites of metastases. In this patient the effect of ipilimumab and radiation was determined by measuring NY-ESO-1, an antigen expressed in many melanomas that was confirmed by tissue analysis of a metastatic focus in this patient. This patient had increased titers of antibodies to NY-ESO-1after ipilimumab as well as after radiation therapy. Additionally, investigators noted an increase in $CD4^{+}$ICOS cells and an increase in HLA-DR expression following radiotherapy (Postow et al. 2012). This clinical report and associated molecular

changes suggest that local radiation releases tumor antigens and induces a local immune response which is compounded by exposure to ipilimumab. This effect warrants further study.

In summary, anti-CTLA-4 therapy represents the newest addition to immunotherapy for the treatment of advanced melanoma. While the mechanism of antitumor activity is not completely understood, further investigation into how CTLA-4 blockade induces tumor regression is a high priority. The future will likely include clinical evaluation of CTLA-4 blockade in other cancers, in combination with other immunotherapy agents, such as IL-2 and anti-PD-1, cytotoxic chemotherapy, targeted therapy, such as BRAF inhibitors and c-kit inhibitors, and radiation therapy. Evaluation of CTLA-4 blockade in the adjuvant setting is also in clinical development. Additional studies of host and tumor biomarkers will also be an important goal of clinical research with ipilimumab, and these studies should help delineate the mechanism of action for this agent and might identify predictive biomarkers of response to help select the patients most likely to respond to this treatment. CTLA-4 is an important target for the immunotherapy of cancer.

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Dendritic Cells

15

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Abstract

In 1868, Paul Langerhans discovered the first immune cell during a study of the skin. The cells he discovered, dendritic cells (DCs), were named for their long projections that made them resemble the dendrites of nerve cells, which was the function Langerhans assigned to them at that time. The true role of DCs as powerful antigen-presenting cells (APC) was elucidated by Ralph Steinman in 1973, in studies that ultimately led to the award of a Nobel Prize in 2011. These cells play a vital role in our immune system, covering both innate and adaptive immune responses, and come from a variety of lineages and have various locations (Kuby et al., Immunology, 6th edn. W.H. Freeman, New York, 2007).

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Target

In 1868, Paul Langerhans discovered the first immune cell during a study of the skin. The cells he discovered, dendritic cells (DCs), were named for their long projections that made them resemble the dendrites of nerve cells, which was the function Langerhans assigned to them at that time. The true role of DCs as powerful antigen-presenting cells (APC) was elucidated by Ralph Steinman in 1973, in studies that ultimately led to the award of a Nobel Prize in 2011. These cells play a vital role in our immune system, covering both innate and adaptive immune responses, and come from a variety of lineages and have various locations (Kuby et al. 2007).

In humans, DCs have two major subsets: myeloid DCs (mDCs, also known as conventional DCs) and plasmacytoid DCs (pDCs) (Palucka and Banchereau 2012). Further, there are several major categories of myeloid DCs recognized, including Langerhans cells, interstitial DCs, and monocyte-derived DCs. Langerhans cells are located in the epidermis of the skin, and interstitial DCs are found in the interstitial spaces of nearly every organ except the brain. Monocyte-derived DCs migrate from the blood into the tissues and from there travel through the lymphatic system or reenter the bloodstream. They typically express CD11c but lack expression of the monocyte marker CD14. Plasmacytoid DCs have a plasma cell morphology, express CD123 (IL-3 receptor), lack expression of myeloid markers, and play a central role in activation of innate immune responses, whereas myeloid or monocyte-derived DCs efficiently activate T- and B-cell responses and drive adaptive immunity and the establishment of immunological memory. All categories of DCs bear major histocompatibility complex (MHC) class I and II molecules, the B7 family of costimulatory molecules, including CD80 and CD86, and CD40 (Kuby et al. 2007).

Biology of the Target

The versatility of DCs is showcased by their ability to direct both innate and adaptive immune responses. DCs link immune responses by transmitting information gathered from invading pathogens to lymphocytes, mounting a very specific immune response to eliminate the infection. Further, DCs have the ability to initiate both arms of the adaptive immune response, cellular and humoral, making them central figures in the immune system (Palucka and Banchereau 2012).

As part of the innate immune response, DCs can act directly on pathogens by generating reactive oxygen species and nitric oxide. They have also been reported to produce antimicrobial peptides. In addition, plasmacytoid DCs are potent producers of type I interferons, which inhibit viral replication in virally infected cells nearby

(Kuby et al. 2007). Interferons expressed by plasmacytoid DCs also activate myeloid DC, thus providing a bridge between innate and adaptive immunity.

One of the major functions of DCs is to trigger a highly specialized T-cell response, and they prove to be very effective (Parham 2009). Adaptive immune responses begin when immature dendritic cells survey the body for foreign particles or abnormal cells. DCs have many receptors that recognize pathogen cell surface components, including phagocytic receptors (e.g., mannose receptor) and pattern recognition receptors (e.g., Toll-like receptors) (Parham 2009). Upon activation, DCs are very efficient at taking up antigen whereupon they migrate to a draining lymph node for antigen presentation to T cells (Parham 2009). DCs may use three methods to engulf antigens or abnormal cells. They may use phagocytosis (engulfing whole particles or cells), internalization by endocytosis (receptor mediated), or pinocytosis (engulfing liquid). After ingestion, these APCs are activated and digest the antigen in specialized cytoplasmic vesicles making smaller fragments to load on MHC class I and MHC class II molecules (Kuby et al. 2007; Weinberg 2007). Lipid antigens are processed differently and are presented on a nonclassical MHC molecule of the CD1 family (Palucka and Banchereau 2012). DC activation also causes an increase in expression of CCR7, a receptor for CCL19 and CCL21, chemokines that are secreted in secondary lymphoid tissue. CCL21 is also thought to promote DC maturation, leading to a functional shift from antigen uptake to antigen processing and presentation, which is accompanied by an increase in expression of MHC class I and II molecules and increased expression of costimulatory molecules (Parham 2009). By presenting antigens on MHC class II molecules, DCs activate naïve $CD4^+$ T-helper (Th) cells, which undergo clonal expansion and differentiation into effector T cells (Parham 2009). DC presentation of processed antigen in association with MHC class I molecules similarly activates and expands $CD8^+$ antigen-specific cytotoxic T lymphocytes (CTL) with the capacity to kill tumor cells directly. The activation of tumor antigen-specific CTL is regarded as central to the stimulation of effective antitumor immunity following DC vaccination.

In addition to driving primary T-cell responses, DCs play an important role in humoral immunity. They can either directly interact with B cells, or indirectly, by activating T-helper cells that in turn activate B cells. They are also capable of interacting with NK cells, phagocytes, and mast cells (Palucka and Banchereau 2012).

Immature or nonactivated DCs also present self-antigens to T cells, promoting peripheral immune tolerance of the normal tissues in the body. In the thymus, presentation of normal peptides by DCs will induce T cells to either go through T-cell deletion or become regulatory T cells (Palucka and Banchereau 2012).

Target Assessment

For clinical purposes, target assessment is based on validation of DC manufacture and release criteria for DC vaccines in clinical trials, followed by assessment of immune responses and correlation with clinical responses.

The release of DC vaccine for clinical use requires assessment of sterility, integrity (i.e., viability), and potency. As it is impractical to test directly the potency of a DC vaccine, surrogate assays are usually performed. These assays may embody analysis of DC phenotype, on the assumption that phenotype will reflect function, or may embody a true functional surrogate for biological activity and immunogenicity. The simplest functional surrogate is a test for the ability of DCs to stimulate a mixed lymphocyte reaction, usually measured by allogeneic T-cell proliferation.

The current standards for assessment of immune responses in patients receiving DC vaccines include ELISPOT assays for enumeration of tumor antigen-responsive T cells that express the cytokine of choice – IFNγ expression by $CD8^+$ T cells is widely regarded as a valid surrogate for CTL activity (which can also be measured with ELISPOT assays for granzyme B release). ELISPOT assays may also test for other T-cell cytokines, including IL-4 (Th2 response), IL-10 (Treg response), IL-17 (Th17 response), and TNFα (Th1/Th17 response). T-cell responses can also be directly measured in vivo, by conducting delayed-type hypersensitivity skin tests with tumor antigen, either as free antigen or as antigen-loaded DC vaccine. If available, autologous, irradiated tumor cells or tumor lysate from the patient may also be used for skin tests, thus affording a direct test of vaccine-induced immune reactivity against the tumor. However, there may be safety concerns with the administration of tumor cell preparations directly to the patient, as it is difficult to assure non-viability of the test material.

Measurement of clinical end points is more problematic, as it is far from clear which end points are appropriate for DC vaccine trials (see section "Clinical Summary" for further discussion). The current standard follows the published Response Evaluation Criteria in Solid Tumors (RECIST), but there is some concern that RECIST may not always provide an accurate indication of long-term outcomes to active immunotherapy, where immune responses may take time to impose control on tumor progression. Progression-free survival, time to recurrence, and overall survival are probably the most objective clinical end points. Biomarker end points can serve as surrogates, when available (e.g., PSA in prostate cancer, CA125 in ovarian cancer).

Role of the Target in Cancer

Rank: "unknown" to 10

Unknown to 1-2-3-4-5-6-7-8-9-10: 7

Immunosuppressive or dysfunctional DCs have been associated with a number of malignancies (Kalinski et al. 2009), but their contribution to tumor progression is not clear. While it is probable that the multiple mechanisms contributing to immune suppression in the tumor microenvironment are likely to have a significant impact on DC function, it is less obvious that DCs in turn contribute to disease pathogenesis. Tumor-associated DCs may have a significant impact on Treg differentiation and expansion, through well-documented mechanisms such as expression of IDO or inhibitory members of the B7 family of accessory molecules, notably B7-H1. The

question of whether DC function can be modified in vivo, to promote stimulation of antitumor immunity, has not received the attention that the potential therapeutic impact merits. For example, inhibition of IDO, which is increasingly recognized as a fulcrum of immune regulation, may have a substantial impact on DC function. In one interesting study, treatment ex vivo of dysfunctional DCs from myeloma patients with an inhibitor of p38 MAPK signaling was sufficient to restore their ability to activate all reactive and tumor antigen-specific T cells (Wang et al. 2006).

The potential role of DCs in therapeutic applications has been studied extensively over the past two decades and is given further attention in the following section.

Therapeutics

DCs are sometimes called "nature's adjuvant" due to their ability to enhance antitumor immunity on their own (Kirkwood et al. 2012). Using dendritic cells as the weapon of choice from the immunologic arsenal, the goal of DC vaccination is to induce tumor-specific effector cells that decrease tumor burden and induce immunological memory to prevent tumor recurrence (Palucka and Banchereau 2012).

Manufacturing a DC vaccine is a multistage process and involves key decisions regarding the choice of DC subtype, the DC culture techniques, and the methods used for loading or expression of tumor-associated antigens. Differences in any stage produce differences in each DC vaccine and may account for the variability seen in clinical responses between clinical trials (Copier et al. 2011). DC vaccines can be prepared from pDCs, mDCs, $CD34^+$ hematopoietic stem cell-derived DCs, or $CD14^+$ monocyte-derived DCs. Although vaccination with both pDCs and mDCs may generate stronger antitumoral immunity than vaccination with monocyte-derived DCs, their numbers in circulation are very low, and therefore monocyte-derived DCs have been most widely used for clinical trials of DC vaccination (Copier et al. 2011; Schreibelt et al. 2010). The formulation used to treat the DCs in vitro is crucial in producing their phenotype and function using various cytokines or Toll-like receptor (TLR) ligands (Palucka and Banchereau 2012), as discussed below.

$CD14^+$ monocyte-derived DCs are typically generated following leukapheresis of the patient, in some cases succeeded by monocyte purification (e.g., using the clinically approved Miltenyi CliniMACS system). The standard practice for differentiation of immature DC involves culture of monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, but techniques for DC maturation vary considerably. As a general rule of thumb, inflammatory cytokines and/or ligands for TLR efficiently promote DC maturation, but the phenotype and function of the DC will vary according to the method of choice, and the type of T-cell response that is induced will also vary in phenotype and function. The current consensus in the field is that DC should be robust inducers of $CD4^+$ Th1 T-helper cells and $CD8^+$ CTL responses, and most approaches to the preparation of DC vaccines are adopted with that principle in mind. Monocyte-derived DCs express TLR 1–6 and TLR8, and maturation can be induced by TLR

ligands such as double-stranded RNA species (e.g., poly I:C, that binds TLR3) or bacterial lipopolysaccharide (that binds TLR4). Other TLR ligands that have been widely used experimentally include unmethylated bacterial CpG DNA, which binds TLR9, but TLR9 expression is confined to the plasmacytoid DC subset in humans. TLR signaling through the adapter molecule MyD88 upregulates costimulatory molecules, CCR7 (which is important for DC migration to draining lymph nodes) and CD83 (a marker for DC maturation). Inflammatory cytokines have also been widely used to induce maturation, typically involving a cocktail of TNFα and IL-1β, often combined with PGE_2 and IL-6. PGE_2 has both positive and negative effects on DC function, and its use is a subject of some debate. Various DC maturation stimuli can upregulate CCR7 expression, but these DC are not responsive to CCL19 and CCL21 unless the maturation conditions include PGE_2. These studies clearly show that PGE_2 is important for licensing responsiveness to CCR7 ligands and migration to draining lymph nodes. PGE_2 can also directly induce CCR7 expression, indicating that PGE_2 has a dual role in facilitating DC migration, regulating both the expression and activity of CCR7. On the other hand, PGE_2 (in combination with TNFα) induces expression of indoleamine 2,3-dioxygenase (IDO), which is an important mechanism of immune suppression and expansion of regulatory T cells.

Type I interferons (IFNα/β) and IFNγ have also been favored (sometimes in combination with anti-CD40 or soluble CD40L), by virtue of their ability to induce high levels of IL-12p70 expression and generate DC that efficiently stimulate Th1 and CTL responses (Kalinski et al. 2009).

As a rule of thumb, tumor antigen loading of DC is more efficient when the DC are immature, and antigen presentation is most efficient when loading is followed by DC maturation. However, there are numerous variations on this theme, starting with the choice of antigen formulation, which may take the form of autologous or allogeneic tumor lysates, recombinant tumor antigen, synthetic peptide fragments, DNA or RNA vectors, or recombinant viral vectors (e.g., adenovirus, adeno-associated virus, lentivirus). Tumor antigen may be introduced to DC by one of these means at any point during the preparation of DC vaccines.

The route chosen for administration of the DC vaccination can vary as well, including intratumoral, subcutaneous, intravenous, intranodal, or intradermal (Copier et al. 2011). Conflicting results from experimental models and clinical trials have not permitted the field to arrive at a consensus regarding the optimal route of DC vaccination, which in any event may be variable according to the clinical setting.

Mixed clinical results from DC vaccination trials suggest that “nature’s adjuvant” may need an adjuvant of its own. DC-driven immune responses are subject to immune regulation imposed by the tumor microenvironment (Copier et al. 2011). Tumors can inhibit antigen presentation through various mechanisms, and can interfere with DC maturation through secretion of IL-10, resulting in antigen-specific anergy. DC vaccine-stimulated T-cell responses can be inhibited by tumor-infiltrating Treg and a spectrum of myeloid suppressor cells, including macrophages, DCs, and myeloid-derived suppressor cells. A burgeoning appreciation of the crucial role of tumor-associated immune suppression in determination of responses to DC

vaccination and other tumor vaccines has fueled the search for adjuvant treatments that allow greater penetration of antitumor immunity. Tumor-infiltrating Treg is widely recognized as a major barrier to effective antitumor immunity, and adjuvants for DC vaccination that are currently under investigation include Ontak (denileukin diftitox, a recombinant fusion protein made of IL-2 and diphtheria toxin which has been used to deplete CD25-expressing Treg), low-dose cyclophosphamide, which has a long history for its ability to potentiate cell-mediated immune responses through depletion of Treg, and daclizumab, an anti-CD25 monoclonal antibody which can also target Treg. Myeloid-derived suppressor cells are also recognized as important mediators of immune suppression in the tumor microenvironment, and gemcitabine reportedly has activity against this cell subset (Copier et al. 2011).

Ipilimumab (anti-CTLA-4 monoclonal antibody) has recently been FDA approved for treatment of melanoma, and may have value as an adjuvant for DC vaccination, by virtue of its ability to block negative signaling to T cells mediated by CTLA-4 engagement of B7.1 and B7.2 on antigen-presenting cells (including DCs). Other drugs that have drawn attention as DC vaccine adjuvants include the thalidomide analogs lenalidomide and pomalidomide, both of which have shown the ability to enhance vaccine responses in experimental models (Liu and Dalgleish 2012). Sunitinib, a novel receptor tyrosine kinase inhibitor approved for treatment of metastatic renal cell carcinoma, has potential to alleviate tumor-associated immune suppression and boost responses to DC vaccination (Liu and Dalgleish 2012). Imatinib mesylate, which binds BCR-ABL and c-KIT, and is an effective treatment for BCR-ABL^{+} chronic myeloid leukemia, may also have activity in overcoming tumor-associated T-cell tolerance and enhancing vaccine efficacy. Studies in animal models showed that imatinib decreased Treg frequencies and enhanced antitumor immune responses to DC vaccination against imatinib-resistant BCR-ABL-negative lymphoma (Larmonier et al. 2008) and that imatinib activated CD8^{+} T cells and induced Treg apoptosis in a gastrointestinal tumor model through c-KIT inhibition and diminished IDO expression (Balachandran et al. 2011). As IDO is increasingly perceived to be central to immune suppression in the tumor microenvironment, agents that inhibit its activity may have a crucial role to play as adjuvants to DC vaccination – none are currently available for clinical use, although competitive inhibitors such as 1-methyl tryptophan and other small molecule inhibitors such as INCB024360 (Incyte Corp.) are under clinical investigation.

DC vaccination has also been tested in combination with IL-2, but there is no evidence that IL-2 has improved the efficacy of DC vaccines – on the contrary, several clinical trials have reported elevations in the frequency of peripheral blood Treg following IL-2 treatment, which may limit antitumor immunity. Based on animal studies, other cytokines, notably IL-7 and IL-15, may be more effective adjuvants for DC vaccination, but these await clinical testing in cancer patients. Although there is a limited range of drugs that are approved for clinical use, this is an active area of research that may be critical for the clinical success of DC vaccination. The history of cancer therapy has shown that monotherapy has rarely been successful, and DC vaccination is unlikely to be an exception.

Preclinical Summary

Studies in animal models have clearly shown the therapeutic potential of DC vaccination. However, the translational step to clinical efficacy has foundered on the inconsistent and usually disappointing results from clinical trials. Part of the problem rests with the question of whether mouse models of cancer provide an accurate representation of human disease – there are several excellent models, but any given model, be it a transgenic tumor-developing animal or a transplantable tumor model, suffers from uniformity. That consistency is an asset for experimental purposes in inbred mouse strains, but it is not representative of the heterogeneity of human disease. That heterogeneity represents a major barrier for translational and therapeutic success. Notwithstanding this obvious limitation, animal models retain utility for investigation of basic principles of DC biology and DC vaccine design. Areas of emphasis for preclinical research include optimization of antigen loading and expression, particularly with respect to in vivo targeting of DC, elucidation of mechanisms of DC polarization through costimulation and modulation of signaling pathways, studies on DC migration and trafficking, and optimal direction of antitumor effector T-cell responses.

Clinical Summary

DC vaccination trials started in the 1990s for melanoma and follicular lymphoma and showed initial signs of promise (Kirkwood et al. 2012). In one clinical trial for metastatic melanoma, patients were given an intranodal injection of semimature monocyte-derived DCs. All patients had a Th1 T-cell response to neoantigen keyhole limpet hemocyanin (KLH) and developed a tumor-specific CD8 T-cell response (Gilliet et al. 2003). While DC vaccination consistently shows evidence of T-cell responses to tumor antigen, including generation of CTL responses to autologous tumor cells, demonstration of clinical responses has been more elusive. The majority of clinical responses encompass stabilization of disease, rather than partial or complete responses (i.e., reduction in tumor burden). A review of DC vaccine trials for treatment of melanoma reported an overall clinical response rate of 30%, from a total of over 600 patients (Engell-Noerregaard et al. 2009), but partial or complete responses were recorded for only 9% of patients. Although inclusion of stable disease as a clinical response is open to question, stable disease was associated with the use of peptide antigens, use of any adjuvant or helper antigen, and induction of antigen-specific T-cell responses. In contrast, there was no significant correlation between partial or complete responses and any of these parameters (Engell-Noerregaard et al. 2009). There is a strong case for arguing that overall survival is the most reliable parameter of clinical efficacy. Early reduction in tumor burden may not be observed, and it is even possible that tumor size may increase by virtue of inflammatory processes associated with lymphocyte infiltration and the buildup of antitumor immunity, thus confounding determination of progression-free survival time (Palucka and Banchereau 2012).

The case for employing overall survival as an end point was reinforced by the experience with sipuleucel-T (Provenge®; Dendreon Corporation) which was the first (and so far the only) active cellular therapy for cancer to receive FDA approval. Sipuleucel-T consists of autologous peripheral blood mononuclear cells cultured with a recombinant fusion protein containing GM-CSF (which can induce DC differentiation from monocytes) and prostate-specific membrane antigen. Three infusions are delivered back to patients, 2 weeks apart. A pivotal placebo-controlled Phase III trial in prostate cancer patients with progressive disease after androgen ablation revealed that those in the sipuleucel-T group had a median overall survival of 25.8 months, versus 21.7 months in the placebo group (Kantoff et al. 2010). However, treatment assignment had no effect on the time to tumor progression, and only 1 of 341 patients in the sipuleucel-T group had a partial tumor response (3% showed a 50% reduction in PSA on readings taken at least 4 weeks apart). These observations indicate that an improvement in overall survival can be achieved without a measurable antitumor effect, underlining the point that traditional short-term parameters may not be appropriate for active cellular immunotherapies, including DC vaccination. However, the finding that a benefit in overall survival could be achieved without discernable antitumor activity remains a surprising observation and has raised some questions regarding clinical trial design, particularly with respect to the placebo control group (Longo 2010; Huber et al. 2012).

One of the major reasons that greater consistency of clinical responses has not been achieved is the lack of consensus over the optimal approach to DC vaccination, which has embodied a plethora of different formulations, targeting many different antigens. A second reason that may account for the relatively disappointing results probably rests with the limitations of clinical trial design. The majority of Phase I and Phase II clinical trials have targeted advanced, metastatic disease in patients with poor performance status and often with compromised immunity, either through high disease burden or extensive prior treatment with chemotherapy or radiotherapy. Notwithstanding the occasional case reports of remarkable clinical responses following DC vaccination as monotherapy in patients with stage IV disease, it is unlikely that DC vaccines have a future as salvage therapy. A more optimal approach would be to vaccinate patients with minimal residual disease following first-line treatment of surgical debulking plus chemotherapy/radiotherapy, with the goal of preventing disease recurrence or progression. In most cases, it may also be more practical to view cancer as a chronic disease that can be managed through continuing therapy, rather than pursuing the utopian but probably unrealistic goal of an outright cure.

Anticipated High-Impact Results

- The current consensus is that an effective DC vaccine should stimulate a tumor antigen-specific high avidity $CD8^+$ CTL response and that the CTL response should be supported by a Th1 polarized $CD4^+$ T-cell response. However, this model has come into question – some experimental studies in animal models have

suggested that a $CD8^+$ CTL response is not an absolute requirement for DC vaccine efficacy. Furthermore, recent investigations have suggested that a Th17 response, rather than a Th1 response, may have more potent antitumor activity in some settings (Zou and Restifo 2010), thus providing a rationale for formulation of DC vaccines that induce Th17-polarized $CD4^+$ T-cell responses.
- Vaccine DC should be endowed with the capacity to migrate to locoregional draining lymph nodes, through expression of CC7 and licensing of migratory capacity. While this may not be an absolute requirement, lymph node migration is likely to prove highly desirable.
- It is increasingly apparent that, no matter how immunogenic the DC formulation may be, DC vaccination is likely be clinically ineffective in the face of profound immune suppression in the tumor microenvironment. The design of combinatorial treatment regimens, encompassing adjuvants that can at least in part alleviate tumor-associated immune suppression, is a crucial field of investigation. Clinical testing of drugs that can inhibit or deplete tumor-infiltrating Treg and/or myeloid suppressor cell populations has current priority.
- Rational clinical trial design for DC vaccination will also be important. Phase I and II clinical trials in patients with minimal tumor burden are more likely to yield meaningful clinical responses than the use of DC vaccination as salvage therapy for patients with metastatic and bulky disease.
- Overall survival is probably a better end point for efficacy of DC vaccines and active cellular therapies than short-term parameters such as recording of stable disease (progression-free survival) or tumor regression. This raises the practical problem that overall survival as an end point may take years to achieve, and thus the need for appropriate surrogate end points is a pressing issue for clinical trial design.

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DNA Vaccines

16

Eric S. Bradley and Douglas G. McNeel

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Abstract

Immune based approaches to the treatment of cancer have demonstrated remarkable success in recent years. Many of these approaches, including T cell checkpoint inhibitors and adoptive T cell therapies, aim to amplify or modify the function of lymphocytes specific for tumor cells. Given this success, there has been renewed interest in the use of active immunotherapies, or vaccines, to generate and/or amplify tumor target-specific immunity, as these types of treatments are well poised to combine with other immune modulating therapies. While many anti-tumor vaccine approaches are being investigated, DNA vaccines

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offer particular advantages in terms of ease and cost of manufacturing, stability, and absence of infectious components. DNA vaccines have been approved as therapies for non-human diseases, including canine melanoma. However, early clinical trials in human patients were disappointing, with limited clinical responses. Since those initial studies many advances have been made in regards to the delivery and efficacy of DNA vaccines, seeking to enhance their therapeutic efficacy. Second generation vaccines are now undergoing clinical evaluation targeting a variety of antigens and in a variety of different approaches. We review here this approach specifically as an anti-cancer therapy.

Keywords
DNA vaccines • Cancer • Active immunotherapy

Target

At its simplest level, a DNA vaccine is an engineered bacterial plasmid containing a sequence that encodes a specific protein of interest downstream of either a viral or eukaryotic promoter. The use of plasmid DNA as a way to induce gene expression in vivo was first discovered by Jon Wolff and associates in 1990. They found that when a "naked" plasmid containing cDNA encoding a protein was injected into the muscle of rats, that DNA could be taken up by cells and expressed (Wolff et al. 1990). Soon after this discovery, studies were conducted identifying that plasmid DNA-encoded proteins could elicit immune responses, providing rationale for their use as vaccine vectors (Liu 2011). Initial studies evaluated DNA vaccines for their use against viral diseases, including HIV-1 and influenza, and in antitumor models, with some success in animal studies (Berglund et al. 1998; Epstein et al. 2002). However, early clinical trials were disappointing, with limited clinical responses (MacGregor and Boyer 1998). Since those initial studies, many advances have been made in regard to the delivery and efficacy of DNA vaccines, seeking to enhance their therapeutic efficacy. Second-generation vaccines are now undergoing clinical evaluation targeting a variety of antigens and in a variety of different approaches. We review here this approach specifically as an anticancer therapy.

As a means of targeting specific tumor antigens, DNA vaccines offer many advantages over other methods of antigen delivery. One advantage is that DNA vaccines do not encode foreign antigens, unavoidable for viral or bacterial methods of delivery, and therefore do not carry risk of eliciting neutralizing immune responses to the vector itself; this is unavoidable with most viral and bacterial vectors. The absence of an infectious agent also eliminates safety concerns associated with attenuated viruses or bacterial vectors (Berkhout et al. 1999). A specific advantage that DNA, viral, and bacterial vaccines have over peptide/protein vaccines is that they are not major histocompatibility complex (MHC) restricted. They can encode the entire target antigen which is then presented through the endogenous pathway. This then allows for the presentation of peptides on the major

histocompatibility complex I (MHC I) and subsequent CD8 T-cell recognition. In regard to cancer therapeutics, the direct CD8 T-cell recognition is ideal in that they are the primary cells capable of antigen-specific tumor cell lysis. Comparatively, protein vaccines are processed through the exogenous pathway, presented on the major histocompatibility complex II (MHC II), and do not necessarily lead to a CD8-biased T-cell response. While not specific to DNA vaccines, the inclusion of a defined antigenic target provides a means of in vivo biological assessment for immune response. This is unlike whole cell vaccines in which one does not know a priori the antigenic target. Another major upside to DNA vaccines is that they have a relatively low cost of production. This financial advantage can be attributed to the ease with which a DNA vaccine can be produced, its chemical stability in solution, and its capability to be easily modified. The aforementioned characteristics allow for DNA vaccines to be “off the shelf” as compared with autologous cellular vaccines like dendritic cell vaccines and hence do not require the culture and reinfusion of specific cells, greatly reducing cost and potential safety/infection concern. While a potential disadvantage to DNA vaccines is that they are less effective at transducing cells, recent research efforts have focused on elucidating techniques to enhance transfection efficiency. This has prompted efforts to deliver DNA vaccines using techniques to increase the transfection of host cells, use of multiple repetitive immunizations, combine DNA vaccines with adjuvants, and couple DNA vaccines with other immune-modulating therapies or other methods of immunization (Khan 2013).

Biology of the Target

The mechanism by which DNA vaccines elicit an immune response involves either direct transfection of professional or nonprofessional antigen-presenting cells (APCs) and/or cross presentation of encoded antigens produced by bystander cells to professional APC (Shedlock and Weiner 2000). Specifically, the gene sequence of the antigen of interest is inserted into a plasmid DNA backbone downstream of the promoter. It is then purified and injected into the recipient, typically either intradermally or intramuscularly. While the precise mechanism of uptake has not been determined, plasmid DNA is able to enter myocytes, intradermal cells, or APCs and migrate to the nucleus of these cells (Salman et al. 2001). After transfection, the gene of interest is transcribed and translated by the host cell machinery, and the resultant protein is processed and presented to the immune system via the endogenous presentation machinery, primarily via MHC I. Professional APCs are also required for presentation of peptides on MHC II following uptake of antigens secreted from transfected cells via cross presentation (Shedlock and Weiner 2000). APCs travel through the lymphatic system and present the antigens to naïve T cells at regional/draining lymph nodes. While the major interest in DNA vaccines has been their ability to activate CD8+ T cells via presentation through the MHC class I pathway, CD4 T-helper cells can also be activated via presentation through MHC class II, and this can lead to secretion of cytokines favoring further T-cell and/or

B-cell activation. Secreted antigens can also be captured by high-affinity immunoglobulins on the surface of B cells. DNA vaccines can thus effectively activate both T- and B-cell adaptive responses.

In addition to encoding an antigen, the presence of cytoplasmic DNA, and bacterial DNA in particular with unmethylated CpG motifs, likely serves as an immune-stimulating event via intracellular DNA sensors and toll-like receptor 9 (TLR9) activation. This provides the “adjuvant” effect or second signal, leading to the establishment of a functional adaptive immune response rather than a sterile tissue damage response (Hemmi et al. 2000). Several recent studies have demonstrated that TLR-9-mediated signaling, while important for an adjuvant effect through extracellular sensing of DNA, is not exclusively involved in the recognition and primary adjuvant effects of DNA vaccines (Sasaki et al. 2002; Ishii et al. 2008; Castaldello et al. 2010). Those key adjuvant effects can be attributed to various cytosolic DNA recognition molecules such as DNA-dependent activator of IFN-regulatory factors (DAI), RNA helicase retinoic acid-inducible gene-I (RIG-I), and LRRFIP1 acting through TANK-binding kinase-1 (TBK1) and stimulator of interferon genes (STING) (Tojima et al. 2000; Ishikawa and Barber 2008). More specifically, TBK1 and STING are crucial for an innate immune response by inducing type 1 interferon production within the TLR-dependent and TLR-independent pathways (Coban et al. 2011).

Target Assessment

Obviously, the choice of antigenic target, vector construct, adjuvant, route of administration, schedule, and use with other therapies are important scientific decisions in the translation of DNA vaccines into human trials. For purposes of clinical trial use, however, the assessment of DNA vaccines as related to specific release criteria for manufactured lots includes evaluations of identity, purity, integrity, quantity, sterility, safety, potency, and some assessment of immunogenicity. For clinical trials in the USA, the US Food and Drug Administration (FDA) has published guidance with respect to these specific categories, as detailed below.

The identity of a DNA vaccine can be deduced using several different techniques. Restriction digest analysis can provide the most rapid “fingerprint,” while direct sequencing provides a direct identification of the specific DNA coding sequence. Concentration can be measured by UV absorbance, and the integrity of DNA (in terms of supercoiled, open circle, or linear form) can be determined by gas chromatography and gel electrophoresis. Because plasmid DNA is typically manufactured by *E. coli*, other assessments of purity in terms of contaminating RNA, bacterial proteins, and especially contaminating endotoxins are typically made. Sterility is assessed by culture of the final plasmid lot for contaminating bacterial or fungal species using standard methods.

As with all therapeutics, assessments of safety are required prior to administration of any DNA vaccine to humans. Potential safety issues can result from the plasmid

DNA itself, its means of administration, or the immune response elicited by the DNA vaccine. An initial concern for DNA vaccines was the possibility of genomic integration. Tissue distribution studies have demonstrated that plasmid DNA can be transported through the blood and lymph systems and can remain present at the site of immunization (La Cava et al. 2000). However, studies have shown that genomic integration occurs at such a low level that spontaneous mutations are a more likely occurrence; hence, this specific concern is negligible (Nichols et al. 1995; Martin et al. 1999; Ledwith et al. 2000). Given these findings, tissue distribution studies are no longer required by FDA unless methods that can affect uptake or DNA persistence are employed. The main potential toxicities from DNA immunization are from immune responses elicited to off-target tissues that express the targeted antigen. Given this, standard assessments of toxicity to tissues expressing the targeted antigen are required.

Assessments of potency and immunogenicity typically include measures of immune response to the intended target. These assessments include measures of CD8+ and CD4+ T-cell or antibody responses to the antigenic target. T-cell responses can be measured in terms of antigen-stimulated cellular proliferation, cytokine expression, cytolytic activity, or expression of other activation markers. The preferred measure of immune response may differ with respect to the intended application. For example, an antibody response to an intracellular antigen may be of less relevance than a measure of cytolytic activity to cells expressing the targeted antigen.

Role of the Target in Cancer

Rank: “unknown” to 10

Unknown to 1-2-3-4-5-6-7-8-9-10: 7

In recent years, there has been a marked increase in pharmaceutical vaccine research and evaluation of plasmid-based genetic vaccines. Their low production cost, chemical stability, east of production, and promising results in animal models have led to this interest in the clinical translation of these vaccine constructs for the treatment of infectious diseases as well as cancer. Compared with other vaccine delivery approaches, the ability to easily adapt genetic vaccines to urgent global health threats suggests that this immunization approach is ideally suited to confront international health challenges.

In the case of antitumor vaccines, while there have been many successes in the treatment of animal tumor models with DNA vaccines, the clearest example of its veterinary success was the development of ONCEPT for treatment of canine melanoma. This USDA-approved vaccine, which is a DNA vaccine encoding human tyrosinase, was shown to be efficient at eliciting an immune response against the tyrosinase protein in dogs with melanoma and was associated with a significant long-term survival compared with historical controls (Bergman et al. 2003).

Although there have been no FDA-approved DNA vaccines for use in humans, several companies (including Inovio, Novartis, and Profectus) have made significant

strides in the development of DNA vaccines and evaluation of methods to improve their delivery and are committed to the clinical development of these therapies. From these advances, it is clear that DNA vaccines will play a key role in future cancer therapeutics.

High-Level Overview

Diagnostic, Prognostic, Predictive Overview

To date, there are no known uses for DNA vaccines themselves as a means of diagnosis or as prognostic or predictive biomarkers for other treatment approaches. Furthermore, while several groups are evaluating whether specific laboratory parameters might determine whether a particular individual might respond to a specific DNA vaccine, no standard approach or method has yet been identified.

Therapeutics

The primary purpose of DNA vaccines are as therapeutic agents. However, the decision for determining whether to pursue a particular DNA vaccine as a therapeutic vaccine involves many choices, most of which center around preclinical measures of potential efficacy. In regard to the DNA plasmid itself, identification of the target antigen and its biological relevance is of principal importance. The ideal antigen is expressed only by the target cell or pathogen with no expression in normal tissues. These antigens may represent normal or mutant proteins. A recent consensus panel identified several key considerations in the choice of tumor antigens for inclusion in vaccines: (a) therapeutic function, (b) evidence of immunogenicity, (c) role of the antigen in oncogenicity, (d) tumor/tissue specificity, (e) expression level and percent of antigen-positive cells, (f) stem cell expression, (g) number of patients with antigen-positive cancers, (h) number of antigenic epitopes, and (i) cellular location (Cheever et al. 2009).

The molecular structure of the delivery vector and included transcription/translation elements are other factors that can affect the efficacy of a DNA vaccine. Several studies have shown that viral-derived promoters like CMV and SV40 drive higher levels of antigen expression. However, evidence also suggests that viral promoters can be silenced a few days after delivery, partly due to anti-interferons released as part of the innate immune response. This has led to a focus on strong eukaryotic promoters, such as desmin (Kwissa et al. 2000). It has also been found that inclusion of polyadenylation signals, nuclear transport signals, and intronic sequences requiring RNA splicing can favor increased expression (Huang and Gorman 1990).

Carrier, adjuvant, route, and schedule of immunization are other variables that need to be considered. The commonly used route of administration early in DNA vaccine research was through intramuscular injection. Since that time, several studies have suggested this method is inefficient in comparison to other delivery methods

(Graham et al. 2013). Efforts to increase transfection efficiency, in particular through nanoparticle coating and electroporation, have been particularly successful in the past decade and are continuously being optimized (Sardesai and Weiner 2011). In more recent years, many particularly successful adjuvants for DNA vaccines have been identified (Kojima et al. 2002; Spies et al. 2003; Petrovsky and Aguilar 2004; Coban et al. 2005). Vaccine adjuvants function through a variety of different mechanisms such as innate immune activation, chemotaxis, or by increasing antigen uptake and presentation by APCs. Preclinical studies frequently consider many of these variables (carrier, route, schedule, adjuvant, as well as antigen choice and plasmid structure) together in designing and evaluating optimal antitumor vaccines.

Preclinical Summary

As described above, studies performed in the 1980s demonstrated that plasmid DNA, administered directly to rodents, could transfect cells in vivo leading to protein expression of an encoded gene product and ultimately lead to immune responses to that target (Wolff et al. 1990). Early clinical trials, primarily in infectious disease models, with this same general approach demonstrated less robust immune responses than what had been observed in preclinical studies. Hence, much of the focus of preclinical research over the last two decades has been on means to increase the immune response following DNA immunization. Most of these efforts, summarized below, have concentrated on:

1. DNA construct alteration to optimize expression of the gene product or target its expression to defined cell types
2. Routes of DNA delivery to target dendritic cells
3. Means to increase cell transfection at the site of delivery
4. Use of different adjuvants to shape the resulting immune response

Beyond these methods, many groups have studied DNA vaccines in combination with other traditional and immune-based therapies to determine if the magnitude of effect can be increased using these therapies in combination.

In order to obtain high-level expression of the encoded antigen, design of the DNA plasmid plays a major role in the subsequent immune response. Key among these considerations are the use of promoters and enhancers to enable efficient transcription, polyadenylation sequences to stabilize the transcripts, inclusion of optimized coding sequences, and utilization of Kozak sequences to enhance ribosomal translation. The cytomegalovirus (CMV) immediate early enhancer-promoter has been found to be particularly useful relative to other promoters. For example, in one study a plasmid expressing HIV-1 Gag/Env and including a CMV promoter/enhancer was compared to a similar plasmid containing the endogenous AKV murine leukemia long terminal repeat. The plasmids were injected into a nonhuman primate model, and analysis revealed a higher immune response for the CMV promoter in regard to expression of Gag- and Env-specific humoral and T-cell

proliferation. In another study, the CMV promoter demonstrated increased immune responses relative to other tissue-specific promoters, including dendritic cell (DC) or keratinocyte-specific promoters (Galvin et al. 2000; Huang and Gorman 1990). In regard to the use of strong eukaryotic promoters, a study done by Kwissa et al. directly analyzed and compared Hep B surface antigen (HBsAG) expression and found that both viral (CMV) and eukaryotic (desmin) promoters expressed HBsAG equally well and could prime immune responses equally well (Kwissa et al. 2000). However, others have demonstrated that the CMV promoter can be silenced in vivo, likely due to DNA methylation changes or histone modifications (Reyes-Sandoval and Ertl 2004). Hence, alternatives to CMV-derived control elements continue to be explored.

Several studies have shown that inclusion of an intron in the plasmid downstream of the promoter can increase the stability of the mRNA transcription product and subsequently increase expression (Chapman et al. 1991). Typically a strong transcriptional termination sequence is also included, such as the rabbit beta-globulin polyadenylation sequence or the SV40 termination element, to further increase mRNA stability (Lanoix and Acheson 1988). Furthermore, alterations to the DNA plasmid can be performed to increase protein translation, including the use of a Kozak sequence downstream of the promoter (Kozak 1987, 1990, 1991). A Kozak sequence is one located upstream of an AUG initiator codon ((gcc)gccRccAUGG) that influences its recognition by eukaryotic ribosomes to increase translation initiation. Some eukaryotic genes do not contain a Kozak sequence, and by insertion of the sequence it has been shown that the expression levels of the genes can increase (Gill et al. 2010). Finally, tRNA specific for individual codons are present in differing amounts and vary per species. Consequently, altering the plasmid to include only preferred codons has been shown to increase protein expression (Kozak 1997).

As was stated in the Therapeutics section above, intramuscular injection was the dominant means of DNA delivery given experience with intramuscular vaccines. Most importantly were the findings that DNA could directly transfect muscle cells and lead to protein expression in muscle tissues in early rodent studies (Wolff et al. 1991). A study by McCluskie et al. identified that the type of immune response could vary depending on the route of immunization in both a mouse model and nonhuman primate model (McCluskie et al. 1999). Specifically, they examined immune responses following immunization with a hepatitis B surface antigen (HBsAg)-encoding plasmid using eight different injection routes (intravascular (IV), intramuscular (IM), intradermal (ID), subcutaneous (SC), etc.), six non-injection routes (intranasal inhalation (INH), intranasal instillation (INS), ocular (Oc), etc.), and a gene gun (GG) approach. In mice they detected significant antibody responses to HBsAg following IM, IV, SC, and ID routes of delivery. All four of those routes also induced cytolytic T cells (CTL). In comparison to the GG delivery, direct IM delivery resulted in the highest antibody titers. The responses by IM were predominantly IgG2a (T_H1-like), and high titers were retained after a single immunization. They then used the ID, IM, and GG methods delivered at 0, 12, and 24 weeks to discern which resulted in the most robust immune response in a

nonhuman primate. In this model, antibody titers were found to be highest following GG delivery, followed by intramuscular delivery, and both greater than intradermal delivery. However, titers declined over time, even after a boost. They concluded that the route of administration of plasmid DNA differently influences the quantum and nature of immune responses elicited in different species. Consequently, results obtained in mice may not be predictive of responses in primates or future human clinical trials. These findings also suggested that efforts to increase the efficacy of vaccine delivery are paramount.

Alternative physical delivery methods have been another area of study aimed at enhancing transfection of host antigen-presenting cells by DNA vaccines. Recent innovations have included needle-free approaches, such as particle bombardment and high-pressure-mediated delivery, as well as dermal patches and electroporation (EP) (Jiao et al. 1993). Particle bombardment approaches use a high-pressure stream to deliver the DNA plasmids on microscopic heavy metal beads, while high-pressure delivery administers vaccines by forcing liquid through a tiny hole to create a small stream that is able to penetrate the skin. The dermal patch delivery method utilizes a patch with microneedles or a needle-free approach utilizing patches coated with nanoparticles composed of plasmids and a synthetic polymer (Ferraro 2011). A very promising and well-studied method that has demonstrated increased plasmid uptake at the site of administration is delivery followed by EP. Electroporation applies brief electric pulses to induce transient and reversible permeabilization of the cell membrane. The technique is now being used in several clinical studies, not only with DNA vaccines but also various drugs for a variety of targets (Gehl 2003). Mechanistic studies have shown that electroporation induces the destabilization of the cellular membrane by transmembrane voltage that then creates transient pores allowing for passage of macromolecules present at the time of administration (Cukjati et al. 2007). The end result is a 100–1000-fold increase in plasmid delivery. A preclinical study done in C57 BL/6 mice found that administration by intramuscular electroporation of a synthetic CpG with the encoded antigen hPSA increased antitumor responses and prevented tumor occurrence in 54% of treated animals (Ahmad 2012). Vaccination resulted in anti-hPSA antibody production and significant production of IFN-y.

The use of adjuvants, or other agents that might affect the resulting immune response, either encoded by the DNA vaccine or co-delivered with the DNA vaccine, have also been explored. One of those methods is the inclusion of plasmids that encode immunomodulatory proteins. These genes can encode an adjuvant (or immune-modulating protein) that is secreted into the surrounding tissue to recruit or affect local APCs. One adjuvant commonly used with DNA vaccines, either encoded by a plasmid or delivered as a protein, is granulocyte-macrophage colony-stimulating factor (GM-CSF) (Disis et al. 2003). GM-CSF is a white blood cell growth factor, and one study has shown that when used in combination with a DNA vaccine for the rabies virus, it increased CD4+ T-cell responses and antibody production relative to the target antigen DNA vaccine alone (Xiang and Ertl 1995).

Finally, the use of DNA vaccines in combination with other therapies, including other vaccine approaches, has also been investigated. Prime-boost strategies have

been shown to be useful in inducing a more robust immune response by priming the immune system to a target antigen delivered by one vector and then boosting in a selective manner by re-administering the antigen with a second distinct vector. The heterologous prime-boost strategy can result in higher numbers of antigen-specific CD4+ and CD8+ T cells (McShane et al. 2001; Fiorino et al. 2013). Most studies done evaluating prime-boost strategies have targeted infectious antigens such as tuberculosis, HIV, and malaria (McShane et al. 2001; Amara et al. 2002; Gilbert et al. 2002). A study conducted targeting malaria using DNA- and vaccinia-based vaccines for the pre-erythrocytic malarial antigen showed a five- to tenfold higher response relative to just the DNA or vaccinia vaccines alone (McConkey et al. 2003). In addition, DNA vaccines have been explored in combination with other immune-modulating therapies and other conventional antitumor therapies. For example, studies by Kast et al. used DNA vaccines specific for the prostate stem cell antigen (PSCA) and the prostate-specific membrane antigen (PSMA) in prime-boost strategies with other vectors encoding these antigens (Garcia-Hernandez et al. 2008; Yang et al. 2001). In a study utilizing PSCA as the target antigen, mice were immunized with a plasmid encoding mPSCA followed by a boost with a Venezuelan equine encephalitis replicon particle (VRP) encoding mPSCA. The investigators observed the generation of PSCA-specific T cells, infiltration of prostate tumors by T cells, the absence of obvious autoimmunity, and prolonged survival of these tumor-bearing mice compared with controls (Gilbert et al. 2002). Similarly, in a study by Goldberg et al., the investigators found that priming first with DNA encoding tyrosinase, and then boosting with alphavirus replicon particles encoding tyrosinase, resulted in better immune response and tumor protection against melanoma than vaccination with DNA alone (Goldberg et al. 2005).

Clinical Summary

The first clinical trials using direct transfer of DNA as an immunotherapy for cancer took place in the 1990s. One of the first trials investigated the transfer of DNA, complexed in a liposome formulation, and encoding a foreign major histocompatibility antigen (HLA-B7) directly intratumorally in patients with stage IV melanoma (Nabel et al. 1996). The investigators reported that the DNA plasmid, and mRNA encoding HLA-B7, could be detected in treated nodules and that treatment led to increases in tumor-infiltrating lymphocytes. This study effectively demonstrated that plasmid DNA could be taken up by human tissues at the site of delivery and could be used to express a specific target molecule. In 2002 some of the first trials using delivery of plasmid DNA encoding tumor-associated antigens (e.g., idiotype antigen for B-cell lymphoma or carcinoembryonic antigen (CEA) for colorectal carcinoma) were reported (Conry et al. 2002; Timmerman et al. 2002). In one of these first pure immunization trials, reported by Conry and colleagues, the investigators immunized patients with colorectal cancer three times at 3-week intervals intramuscularly in a dose-escalation fashion (0.1, 0.3, and 1.0 mg per dose) with plasmid DNA encoding CEA (as a tolerant self-antigen) and hepatitis B surface antigen (HBsAg, as a

non-tolerant foreign antigen). Antibody responses were detected to HBsAg but not CEA, although lymphoproliferative responses specific for CEA were elicited in 4 of 17 patients. While no clinical responses were observed in these patients with widespread metastatic disease, this trial effectively demonstrated that plasmid DNA could elicit immune responses to encoded antigens, including “self” antigens. Since these early trials, over 40 clinical trials using plasmid DNA vaccines have been conducted over the last decade for specific malignancies using tissue-specific antigen targets, or for multiple malignancies using DNA plasmids encoding commonly shared antigenic targets, as we have recently reviewed (Colluru et al. 2013).

The majority of DNA vaccine trials conducted to date have been phase I trials, designed to answer questions about safety, the target encoded (whether immune response can be elicited to different specific targets), dose, route (typically intramuscular or intradermal routes of delivery), and the schedule of immunization. The primary endpoints of these trials have generally been safety and evidence of immune response to the encoded target antigen. Uniformly, these trials have not demonstrated significant toxicity. Most of the trials that have been reported have demonstrated variable results in terms of immune responses elicited and occasional objective disease responses in patients with metastatic disease (Tagawa et al. 2003; Gnjatic et al. 2009; Norell et al. 2010; Weber et al. 2011). The observation by one group that patients with metastatic melanoma did not develop immune responses to DNA immunization targeting gp100 that had been observed in other trials (Rosenberg et al. 2003; Cassaday et al. 2007) has led many investigators to explore second-generation vaccines in patients with earlier stages of disease (Triozzi et al. 2005; Nemunaitis et al. 2006; McNeel et al. 2009). Others have explored the use of different cytokines or other molecules as vaccine adjuvants, as described above. Still others have explored methods to improve DNA uptake at the time of immunization using particle-mediated delivery devices or electroporation (Low et al. 2009; Ginsberg et al. 2010). Finally, others have investigated DNA vaccines in heterologous prime-boost approaches with other DNA vaccines or with other means of antigen delivery (Mincheff et al. 2000).

To date, it remains unknown whether there are optimal antigens and adjuvants for DNA vaccines in humans and whether the route and schedule of vaccination might differ with respect to the antigen targeted. Preclinical studies as described above suggest there may be an advantage to improved methods of DNA delivery such as by electroporation, at least in terms of the frequency and magnitude of immune responses elicited. There is growing consensus that using vaccines, whether DNA vaccine or by other means of antigen delivery, is preferred in earlier stages of cancer progression rather than in patients with bulky metastatic disease (Bilusic and Gulley 2012). As highlighted above, results from multiple phase I trials have demonstrated that DNA vaccines appear to be safe, can transfect human cells and lead to antigen expression, and can elicit humoral and cellular immune responses to target antigens, including tumor-associated “self” proteins. While there have been occasional and anecdotal clinical responses observed, to our knowledge there is only one randomized phase II trial underway using a DNA vaccine for the treatment of cancer, and this is in patients with early recurrent prostate cancer (NCT01341652). Consequently, it is

premature to conclude whether these vaccines have clinical efficacy for multiple malignancies. Ongoing and future studies will continue to explore DNA vaccines, and we anticipate multiple phase II clinical trials over the next decade evaluating multiple antigens, routes, and schedules and with different adjuvants and delivery methods. These will be critical studies to ask the important question of whether these vaccines elicit antitumor responses as have been observed in preclinical studies. Moreover, given the safety observed, evidence of immunogenicity in early clinical trials, and given the manufacturing and storage cost advantages described earlier, we anticipate that DNA vaccines will be easily combined with other immune-modulating therapies, and this will be a further direction for future clinical trials.

Anticipated High-Impact Results

- DNA vaccines offer multiple advantages over other types of antigen delivery approaches in terms of safety, sterility, stability, and cost and are not restricted to particular MHC types.
- The USDA approval of DNA vaccines as treatments for infectious disease and cancer in larger animals serves as evidence of their potential efficacy and commercial viability for the treatment of human diseases.
- The features above suggest that DNA vaccines could be easily incorporated into the standard armamentarium of treatments for human cancer and/or combined with other therapies.
- Current efforts in preclinical models seek to improve DNA vaccines themselves by using methods to increase delivery to host antigen-presenting cells, increase expression of target genes, and deliver appropriate adjuvant signals and by combining them with other immune therapies and conventional antitumor therapies.
- Preclinical studies to investigate the mechanism of action of DNA vaccines, and increase their therapeutic efficacy, will translate to improved therapies entering clinical trials over the next decade.

Cross-References

Other Vaccine Approaches**:**
▶ Bacterial Vaccines
▶ Dendritic Cells
▶ Peptide Vaccine: Overview
▶ Proteins (Mesothelin)
▶ Whole-Cell Vaccines

Targets for DNA Vaccines:
▶ AR, Overview
▶ gp100

▶ HER2/neu
▶ PAP
▶ PSA
▶ Survivin

Selected Combination Approaches Used with DNA Vaccines:
▶ Anti-Programmed Death 1 (PD1)
▶ GM-CSF and Whole Cells

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EGFR, Immunology

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Louis M. Weiner and Christina Wu

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Abstract

Epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane protein and a receptor tyrosine kinase. It is a member of the human epidermal receptor (HER) family, which includes EGFR (or HER1), HER2, HER3, and HER4.

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EGFR is expressed in a variety of cell types, but primarily cells of epithelial, mesenchymal, and neuronal origin. It has been shown to be critical in the development of epithelial cancers, and EGFR overexpression or constitutive action can promote angiogenesis and metastasis.

Keywords
Colorectal cancer (CRC) • Epidermal growth factor receptor (EGFR) • Assessment • Biology • Breast cancer • Head and neck cancer • In vitro studies • Ligand binding • NSCLC • Pancreatic cancer • Preclinical research • Prognostic role • Therapeutics • Immunohistochemistry (IHC) • Non-small cell lung cancer (NSCLC) • Pancreatic cancer

Target: Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane protein and a receptor tyrosine kinase. It is a member of the human epidermal receptor (HER) family, which includes EGFR (or HER1), HER2, HER3, and HER4. EGFR is expressed in a variety of cell types, but primarily cells of epithelial, mesenchymal, and neuronal origin. It has been shown to be critical in the development of epithelial cancers, and EGFR overexpression or constitutive action can promote angiogenesis and metastasis.

Biology of the Target

EGFR is a classic membrane-bound tyrosine kinase receptor that is composed of an extracellular ligand-binding domain and an intracellular tyrosine kinase domain. It has ligands that include: epidermal growth factor (EGF), transforming growth factor-a (TGF-a), amphiregulin, heparin-binding-EGF (HB-EGF), b-cellulin, and epiregulin (Yarden and Sliwkowski 2001). On ligand binding, EGFR forms homo or heterodimers with other HER family receptors, leading to autophosphorylation of tyrosine residues in the intracellular domain and affecting signaling pathways involved in survival signaling, proliferation, angiogenesis, and metastasis. EGFR can affect various signaling cascades important in carcinogenesis, most importantly the RAS-RAF mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C pathways. Of significance, downstream proteins/kinases of the EGFR signaling pathway e.g., KRAS, BRAF, and PI3K appear to play an integral role in tumor carcinogenesis and are studied as potential biomarkers and targets for drug therapy.

Target Assessment

EGFR can be detected on tumor tissue by a standardized FDA-approved immunohistochemistry (IHC) assay. Tumors are considered to be positive for EGFR on IHC when 1% or more cells are positive for EGFR membrane staining. Interestingly, the

intensity of EGFR expression by IHC is not predictive of response to anti-EGFR therapy. Thus, the IHC assay is not used to guide diagnosis and treatment of cancers. There is ongoing research assessing EGFR gene copy number with chromogenic in situ hybridization and fluorescent in situ hybridization (FISH) (Laurent-Puig et al. 2009).

In NSCLC, adenocarcinomas with somatic EGFR mutations at exons encoding the kinase domain (exons 18–21) are responsive to EGFR tyrosine kinase inhibitors (TKI). EGFR mutation is tested by either polymerase chain reaction (PCR) or amplification refractory mutation system (ARMS).

Role of the Target in Cancer

Rank: 10

High Level Overview

EGFR is crucial for epithelial cancer development and has been utilized as a target for cancer therapy in advanced pancreas cancer, NSCLC, breast cancer, colorectal cancer (CRC), and head and neck cancer. The most intriguing developments have occurred both in metastatic CRC (mCRC) and advanced NSCLC, as predictive markers for treatment effectiveness have been discovered. The wild-type or mutant status of KRAS, a small GTP protein downstream of EGFR, has been found to be predictive of anti-EGFR monoclonal (mAb) efficacy. ASCO published a provisional clinical opinion recommending that all metastatic CRC be tested for KRAS status in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory and that if KRAS mutation on codon 12 or 13 is detected, the patient should not receive anti-EGFR therapy (Allegra et al. 2009). Much research is in progress to better characterize the individual KRAS mutations with response to anti-EGFR therapy and to discover other predictive biomarkers. In the treatment of advanced NSCLC, the phase III BR.21 trial compared erlotinib to placebo and demonstrated survival benefit (Shepherd et al. 2005). However, more importantly, the exploratory multivariate analysis identified features of female sex, Asian ethnicity, nonsmoker status, and adenocarcinoma histology as favorable predictive factors. Subsequent studies were able to associate these patient features with EGFR mutations on exons 18–21. A Japanese phase III trial enrolled patients with the drug-sensitizing EGFR mutations and randomized them to chemotherapy or gefitinib and was able to demonstrate improved survival and less toxicity with the gefitinib treatment arm (Mitsudomi et al. 2010). In metastatic breast cancer, lapatinib, a dual EGFR and HER2 TKI, in combination with capecitabine or letrozole provides survival benefit to patients with HER2-positive breast cancers (Johnston et al. 2009; Geyer et al. 2006). Improved understanding of the EGFR-signaling pathway should lead to personalized, targeted therapy for patients.

Diagnostic, Prognostic, and Predictive

EGFR detection by IHC on tumor tissue has not been found to be diagnostic and thus is not utilized in establishing the diagnosis of cancer or differentiating the type of cancer. Some studies support the prognostic role of EGFR by IHC, but it is not regularly used in clinical practice. There is evidence to suggest that the mutation status of KRAS and BRAF, both proteins downstream of the EGFR signaling pathway, may be prognostic in colorectal cancer (CRC) (Rizzo et al. 2010). The intensity of EGFR expression by IHC is not predictive of treatment response to anti-EGFR therapy. However, KRAS mutation is a well-established negative predictive biomarker of anti-EGFR mAb in metastatic CRC. Currently, the EGFR gene copy number and EGFR ligands, e.g., amphiregulin and epiregulin, are being studied as possible predictive biomarkers in metastatic CRC (Laurent-Puig et al. 2009; Jacobs et al. 2009). In NSCLC, an EGFR mutation at exon 18–21 is predictive of response to EGFR TKIs (Shepherd et al. 2005; Mitsudomi et al. 2010).

Therapeutics

Two classes of drugs have been developed to inhibit EGFR signaling. Both mAb and TKI of EGFR have clinical utility in the treatment of some malignancies. The EGFR-directed mAb targets the extracellular EGFR to prevent ligand binding and downregulate EGFR signaling. The two clinically approved mAbs are cetuximab, a human-mouse chimeric IgG1 antibody, and panitumumab, a human IgG2 antibody. Cetuximab is FDA-approved for use in head and neck tumors and metastatic CRC. Panitumumab is approved for treatment in metastatic CRC. TKIs inhibit EGFR autophosphorylation and thus inhibit downstream signaling. The three TKIs that are used clinically are erlotinib, gefitinib, and lapatinib. Erlotinib is FDA-approved for the treatment of advanced NSCLC and in patients with advanced pancreatic cancer. Gefitinib is no longer prescribed in the USA, unless the patient with NSCLC has previously responded to it. Lapatinib, a dual tyrosine kinase inhibitor of EGFR and HER2, is FDA-approved for use in combination with capecitabine in the treatment of metastatic HER2-positive breast cancer and in combination with letrozole in metastatic breast cancer that is HER-2 positive and hormone receptor (HR)-positive.

Preclinical Summary

The activation of EGFR contributes to the transformation of tumor cells and alters survival signaling, proliferation, angiogenesis, and metastasis. The EGFR signaling pathway has been found to be activated in various cancers and this may be due to either overproduction of ligands or increased activation/expression of the receptors due to mutation or amplification. For example, ligand TGF-ais expressed in lung, ovary, and colon tumors and correlates with poor prognosis (Salmon et al. 1995).

Overexpression of EGFR is noted in head and neck, breast, bladder, prostate, kidney, and NSLC (Irish and Bernstein 1993; Gorgoulis et al. 1992). In an overview analysis of studies that examined EGFR IHC expression with disease prognosis, EGFR was found to be a strong poor prognostic indicator for head and neck, ovarian, cervical, bladder and esophageal cancer (Nicholson et al. 2001). Thus, two classes of drugs, mAb and TKI, were designed to target the EGFR pathway. In vitro studies with cetuximab demonstrate dimerization and internalization of EGFR and also perturbation of the cell cycle progression, inducing a G1 arrest [reviewed in (Ciardiello and Tortora 2001)]. Erlotinib reversibly inhibits the kinase activity of EGFR and autophosphorylation in vitro. It inhibits colon cancer cell line proliferation and blocks cell cycle at G1.

Currently the preclinical research of EGFR signaling is focused on identifying the mechanisms of resistance to anti-EGFR therapy. Astsaturov et al. employed a siRNA library-based synthetic lethal screen to identify 61 genes that contribute to EGFR-targeted therapy resistance (Astsaturov et al. 2010). Hence, the EGFR resistance phenotype is complex and not likely to be addressed by simply targeting one or two nodes in a pathway. Multiple pathways have been found to be activated in cancer cells that are resistant to anti-EGFR therapy. In cancer cell lines that have developed resistance to cetuximab, increased activation of HER2, HER3, and vascular endothelial growth factor (VEGF) has been demonstrated [reviewed in (Wheeler et al. 2010)]. Tumors may also develop an oncogenic shift to other receptors or kinases, such as insulin-growth factor receptor, Src family kinases, and MET. PTEN, BRAF, and PI3K mutations have been identified as possible determinants for poor response to therapy. Also, increased EGFR ubiquitination and decreased cell surface expression is postulated as a method for EGFR to evade mAbs. In NSCLC, an acquired T790M mutation on EGFR alters drug binding, and thus drugs designed to target this mutation have been developed (Zhou et al. 2009).

Clinical Summary

Anti-EGFR therapy is used in the treatment of a wide variety of cancers: CRC, pancreatic cancer, NSCLC, breast cancer, and head and neck cancer.

Colorectal Cancer

In metastatic CRC, cetuximab and panitumumab are utilized either as monotherapy or in combination with chemotherapy to treat tumors that were KRAS wild-type. Three phase III trials have examined KRAS status and the efficacy of anti-EGFR mAb therapy in the first-line setting. The CRYSTAL trial compared 5-fluorouracil and irinotecan (FOLFIRI) to FOLFIRI and cetuximab. There was an improvement in overall survival (OS) from 20 to 23.5 months ($p = 0.009$), as well progression free survival (PFS) from 8.4 to 9.9 months ($p < 0.001$) with the addition of cetuximab to FOLFIRI in the KRAS wild-type population (Van Cutsem et al. 2009). The COIN

trial randomized patients to three arms: continuous 5-fluorouracil (5FU) and oxaliplatin therapy; continuous 5FU, oxaliplatin, and cetuximab therapy; and intermittent 5FU and oxaliplatin therapy. Results presented at the 2010 ASCO meeting revealed no survival benefit with the addition of cetuximab to chemotherapy (Maughan et al. 2010). The PRIME study, compared 5FU and oxaliplatin (FOLFOX) to FOLFOX and cetuximab and showed an improvement in PFS from 8 to 9.6 months ($p = 0.02$) with the addition of cetuximab, but no OS improvement (Douillard et al. 2010). In the second-line setting for metastatic CRC, Peeters et al. compared FOLFIRI to FOLFIRI and cetuximab in patients with KRAS wild-type tumors (Peeters et al. 2010). There was an improvement in PFS from 3.9 to 5.9 months ($p = 0.004$) and in response rate (RR) from 10% to 35% ($p < 0.001$) but no OS benefit. Analyzed altogether, these trials show that anti-EGFR therapy does improve PFS but not OS, and the modest benefit is at great expense and moderate toxicity.

Pancreatic Cancer

In the setting of metastatic and locally advanced pancreatic cancer, the addition of erlotinib to gemcitabine was shown to lead to minimally improved survival in a phase III trial reported by the National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG) (Moore et al. 2007). Erlotinib plus gemcitabine therapy, when compared to gemcitabine alone, improved median survival from 5.91 to 6.24 months ($p = 0.038$), and PFS was increased from 3.55 to 3.75 months ($p = 0.03$). EGFR status by IHC was not associated with response or disease progression.

Non-small Cell Lung Cancer

In NSCLC, erlotinib improves survival in the second- and third-line setting, with superior efficacy in adenocarcinomas that contain an EGFR mutation at exons 18–21. The NCIC-CTG conducted the phase III BR.21 trial for patients with advanced NSCLC who had received prior chemotherapy, and patients were randomized to either erlotinib or placebo[iv]. The erlotinib treatment arm had an improved RR of 8.9% versus <1% ($p < 0.01$) and improved median overall survival of 6.7 versus 4.7 months ($p < 0.01$). Exploratory multivariate analysis identified features such as Asian ethnicity, female sex, adenocarcinoma histology, and never-smoker status as predictive factors for survival. A prospective trial by the Spanish Lung Group assessed EGFR mutations in 2,105 patients and reported that EGFR mutations at exons 19 and 21 were most common in tumors with adenocarcinoma histology, women, and nonsmokers (Rosell et al. 2009). Only patients with EGFR mutations were then treated with erlotinib, and their PFS and OS were 13 months and 27 months, respectively. The Japanese phase III WJTOG3405 study, wherein only patients with drug-sensitizing EGFR mutations were randomized to gefitinib or cisplatin-docetaxel, demonstrated improved PFS with less toxicity in the gefitinib

arm, as compared to the chemotherapy arm (Burtness et al. 2005). Likewise, the phase III Iressa Pan-Asia Study (IPASS) randomized nonsmoker patients with advanced NSCLC that were adenocarcinoma, to gefitinib or carboplatin-paclitaxel and there was improved 12-month rates of PFS of 24.9% versus 6.7% ($p < 0.001$) for the gefitinib arm (Mok et al. 2009). The EGFR gene mutation was a predictor for better outcome with gefitinib treatment. Gefitinib is not approved for use in the USA, but the data from the phase III trials have been extrapolated to clinical practices with erlotinib.

The addition of erlotinib to chemotherapy in the phase III TALENT trial (gemcitabine-cisplatin) and phase III TRIBUTE trial (carboplatin-gemcitabine) did not show improvement in overall survival in unselected patient populations (Gatzemeier et al. 2007; Herbst et al. 2005). Erlotinib versus placebo as maintenance therapy for the treatment of NSCLC in unselected patients was studied in the phase III SATURN trial. There was modest improvement in overall survival from 11.1 to 12.3 weeks ($p < 0.0001$) in the erlotinib arm (Capuzzo et al. 2010). Again, notably improved efficacy was seen in patients with EGFR mutations.

The FLEX phase III trial assessed the efficacy of adding an anti-EGFR monoclonal antibody to chemotherapy in patients with advanced NSCLC in the first-line setting. Cetuximab was given in addition to cisplatin-vinorelbine, and the combination therapy improved overall RR from 29% to 36% ($p = 0.01$) and the OS rate from 10.1 to 11.3 months ($p = 0.044$) across all histological subtypes (Pirker et al. 2009). However, the BMS-099 phase III trial comparing taxane-carboplatin (TC) to TC and cetuximab did not show any improvement in PFS or OS with cetuximab, only an increase in RR from 17.2% to 25.7% ($p = 0.07$) (Lynch et al. 2010). Thus, the benefit of adding cetuximab to chemotherapy in the first-line treatment of advanced NSCLC is still unclear.

Breast Cancer

Lapatinib is FDA-approved for the treatment of metastatic HER2-positive breast cancer, either in combination with capecitabine for tumors refractory to prior chemotherapy and trastuzumab treatment or as first-line treatment with letrozole in postmenopausal women. Johnston et al. performed a phase III trial of letrozole alone vs. letrozole combined with lapatinib as first-line therapy in postmenopausal women with hormone receptor (HR)-positive cancer. The addition of lapatinib improved median PFS from 3 to 8.2 months ($p = 0.019$) for HR- and HER2-positive patients, and the improvement was notably not seen in HER2-negative patients (Capuzzo et al. 2010). Geyer et al. reported a phase III trial of capecitabine vs. capecitabine and lapatinib in the treatment of metastatic breast cancer that was HER2-positive and had progressed on prior treatment of anthracycline, taxane, and trastuzumab. The addition of lapatinib improved median PFS from 4.4 months to 8.5 months ($p < 0.001$) (Ciardiello and Tortora 2001). Preclinical studies have shown that dual blockade of HER2 may be more effective than single agent alone, thus Blackwell et al. conducted a phase III trial comparing lapatinib alone to lapatinib and trastuzumab in patients

with HER2-positive, trastuzumab-refractory advanced breast cancer. The PFS was improved from 8.1 to 12 weeks ($p = 0.008$) with the combination regimen (Blackwell et al. 2010). It remains untested as to whether patients may have benefited from trastuzumab alone without the addition of lapatinib. However, this study does offer a chemotherapy-free option for patients whose cancers have worsened on trastuzumab.

Head and Neck Cancer

In the treatment of head and neck cancers, cetuximab is FDA-approved as a radiation-sensitizing agent in locally-advanced disease, and for patients with recurrent or metastatic disease. Bonner et al. reported a phase III trial of high-dose radiation with and without cetuximab. The median duration of locoregional control was improved from 14.9 to 24.4 months ($p = 0.005$) with the addition of cetuximab, and median survival improved from 29.3 to 49 months ($p = 0.03$) (Bonner et al. 2006). However, there are no trials comparing chemoradiation with platinum-based therapy to cetuximab and radiation therapy. Cetuximab has also been studied in combination with platinum-based chemotherapy for the treatment of recurrent or metastatic disease. An ECOG phase III study assigned patients to receive cisplatin and placebo or cisplatin and cetuximab. Response rates were improved from 10% to 26% ($p = 0.03$) with the addition of cetuximab but there was no survival benefit (Burtness et al. 2005). The phase III EXTREME trial studied first-line treatment of recurrent or metastatic head and neck cancer. Patients were randomized to 5FU and platinum-based chemotherapy or 5FU, platinum-based chemotherapy, and cetuximab. There was an increase in the median OS from 7.4 to 10.1 months ($p = 0.004$) with the addition of cetuximab to chemotherapy and a longer PFS from 3.3 to 4.5 months ($p < 0.001$) (Vermorken et al. 2008).

Anticipated High Impact Results

Although clinical trials have shown that anti-EGFR therapy may benefit patients, clinical responses are often modest at best and can be associated with increased toxicity and high cost. Further efforts in improving treatment efficacy need to be made in identifying the patients who are most likely to benefit from therapy. For instance, the presence of KRAS mutations in metastatic CRC is a negative predictive biomarker for response to anti-EGFR therapy. However studies have shown that some KRAS mutations may still respond to anti-EGFR therapy (De Roock et al. 2010). Conversely, not all KRAS wild-type CRC respond to anti-EGFR therapy. Thus, other predictive biomarkers are being explored: EGFR gene copy number, other EGFR mutations, EGFR ligands, PTEN loss, BRAF status, and PI3K status.

Better understanding of the EGFR signaling network offers the prospect of overcoming resistance to anti-EGFR therapy through the use of anti-EGFR-based

combinations that address resistance mechanisms. For example, HER2, HER3, and VEGF may be upregulated or activated in cell lines resistant to cetuximab, and thus mAb to these targets such as trastuzumab, UE-1287, and bevacizumab, respectively, would be good drug candidates. Also, BRAF and PI3K inhibitors are being explored in mCRC. The Src pathway is activated in cetuximab-resistant cell lines, and thus dasatinib, a Src inhibitor, is being studied in clinical trials with mCRC and NSCLC. The MET-signaling pathway is upregulated with anti-EGFR TKI therapy, and so ARQ 197, a cMET inhibitor, is being combined with erlotinib in NSCLC in a phase III trial. Thus, a number of interesting and potentially useful combinations remain to be studied to improve treatment outcomes.

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Fc Gamma R

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Tyler R. Simpson and James P. Allison

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Abstract

Monoclonal IgG antibodies targeting tumor antigens and receptors, such as CD20 (rituximab), erbB-2 (trastuzumab), and epidermal growth factor receptor (cetuximab), have shown remarkable activity in the clinic against a wide range of malignancies and are now part of standard treatment protocols. The activity of IgG antibodies is regulated in part by a diverse family of Fc gamma receptors (FcγR), which bind to the constant Fc region of IgG antibodies. FcγR are expressed on various cell types of hematopoietic origin, thus linking the diversity and specificity of antibodies to various immunological activities. Many of the

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antibodies used in the clinic activate FcγR on macrophages and NK cells, leading to tumor cell phagocytosis and lysis. More recent data have also highlighted the involvement of the FcγR system in potentiating chronic inflammation and de novo carcinogenesis and have also suggested that polymorphisms in FCGR genes can influence the outcome of antibody therapy. FcγR biology is complex, owing to the expression of multiple FcγR classes in overlapping expression patterns, all of which differ in their capacity to bind to various antibody isotypes. Nonetheless, a more complete understanding of FcγR biology is beginning to emerge, which will help guide the development of the next generation of antibody therapies.

Keywords
Immunology • Immune system • Immunotherapy • Oncology • Autoimmunity • Cancer • Fc gamma receptor • B cell • T cell • NK cell

Target

Fc gamma receptors (FcγR) are a network of receptors, which recognize the Fc region of antibodies, thereby linking the diversity and specificity of the antibody variable region to a plethora of downstream effector functions. Six FcγR have been identified in humans: FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA, and FcγRIIIB (Nimmerjahn and Ravetch 2008). All FcγR are members of the immunoglobulin superfamily. With the exception of FcγRIIIB, which is linked to the membrane through glycosylphosphatidylinositol, all FcγR are type I transmembrane proteins (Ravetch and Perussia 1989; Salmon et al. 1995). FcγRI binds to antibodies with high affinity and can therefore associate with monomeric antibodies. In contrast, the interactions between FcγRIIA/B/C and FcγRIIIA/B with IgG are considerably weaker (Bruhns et al. 2009). These low affinity FcγR can only engage antibody when it is complexed with antigen. With the exception of FcγRIIB, which transduces inhibitory signals, all FcγR transduce activating signals.

FcγR are widely expressed on cells of hematopoietic origin in overlapping expression patterns (Fig. 1). Macrophages, neutrophils, and dendritic cells (DC) express FcγRI, FcγRIIA, and FcγRIIB (Repp et al. 1991; van de Winkel and Anderson 1991; Fanger et al. 1996; Nimmerjahn et al. 2005; Veri et al. 2007; Guilliams et al. 2014; ▶ Chap. 15, “Dendritic Cells”). Neutrophils also express FcγRIIC and FcγRIIIB, whereas macrophages also express FcγRIIC and FcγRIIIA (Fleit et al. 1982; Ravetch and Perussia 1989; van de Winkel and Anderson 1991; Salmon et al. 1995; Nimmerjahn and Ravetch 2005; van der Heijden et al. 2012; Guilliams et al. 2014). Thus, macrophages and neutrophils express a gamut of FcγR. In contrast, FcγR expression is more restricted on NK cells, which only express FcγRIIC and FcγRIIIA, and on B cells, which only express FcγRIIB (Ravetch and Perussia 1989; Metes et al. 1998; Nimmerjahn and Ravetch 2005; Veri et al. 2007; van der Heijden et al. 2012; ▶ Chap. 35, “NK Cells”). Lastly, eosinophils, basophils, and mast cells express FcγRIIA in addition to other FcγR molecules (Zhao et al. 2006; Richards et al. 2008; Cassard et al. 2012).

Name	FcγRI	FcγRIIA	FcγRIIB	FcγRIIC	FcγRIIIA	FcγRIIIB
Structure						
Type	Activating	Activating	Inhibitory	Activating	Activating	Activating
Gene	*FCGR1A*	*FCGR2A*	*FCGR2B*	*FCGR2C*	*FCGR3A*	*FCGR3B*
CD	CD64	CD32A	CD32B	CD32C	CD16A	CD16B
Affinity	High	Low/Medium	Low/Medium	Low/Medium	Low/Medium	Low/Medium
Binds to	IgG1, IgG3, IgG4	All isotypes	All isotypes	All isotypes	All isotypes	IgG1, IgG3
Isotype preference	IgG1, IgG3 > IgG4	IgG1 > IgG3 > IgG2 > IgG4	IgG3, IgG4 > IgG1 > IgG2	IgG3, IgG4 > IgG1 > IgG2	IgG3 > IgG1, IgG4 > IgG2	IgG3 > IgG1
Expression on common immune subsets	Mac, DC, NK, Neut	Mac, DC, NK, Neut	B cell, DC, Mac, Neut	NK, Mac, Neut	NK, Mac	Neut
Ortholog in mouse	FcγRI	FcγRIII	FcγRIIB	-	FcγrIV	-

α chain
γ chain
ITAM
ITIM
GPI
Cell membrane
Mac: Macrophage
Neut: Neutrophil
DC: Dendritic cell

Fig. 1 Distribution and antibody-binding characteristics of FcγR in human

Murine cells express four FcγR: FcγRI, FcγRIIB, FcγRIII, and FcγRIV (Uchida et al. 2004; Nimmerjahn and Ravetch 2007; Nimmerjahn et al. 2010). Murine FcγRI and FcγRIIB are orthologous to their human counterparts, whereas FcγRIII and FcγRIV are most similar to human FcγRIIA and FcγRIIIA, respectively. There is no mouse ortholog of FcγRIIIB. In general, mouse FcγR follow a similar distribution to their human counterparts (Fig. 2). Murine macrophages, neutrophils, and DCs express FcγRIIB and FcγRIII (Nimmerjahn et al. 2005; Williams et al. 2012; Guilliams et al. 2014). Macrophages and DCs also express FcγRI, whereas macrophages and neutrophils also express FcγRIV (Nimmerjahn et al. 2005, 2010; Giorgini et al. 2008). Similar to their human counterparts, FcγRIII and FcγRIIB are the sole FcγR expressed by murine NK cells and B cells, respectively (van de Winkel and Anderson 1991; Schiller et al. 2000). FcγR expression on these cells can also change in response to the local cytokine environment. For instance, GM-CSF and IFN-γ upregulate FcγRI expression on neutrophils and DCs, respectively (Repp et al. 1991; Reff et al. 1994; Fanger et al. 1996; Carson et al. 2001; Manches

Name	FcγRI	FcγRIIB	FcγRIII	FcγRIV
Structure				
Type	Activating	Inhibitory	Activating	Inhibitory
Gene	*Fcgr1*	*Fcgr2b*	*Fcgr3*	*Fcgr4*
CD	CD64	CD32	CD16	CD16-2
Affinity	High	Low/Medium	Low/Medium	Medium/High
Binds to	IgG2a, IgG2b	All isotypes	All isotypes	IgG2a, IgG2b
Isotype preference	IgG2a > IgG2b	IgG1 > IgG2b > IgG2a	IgG2a > IgG2b > IgG1	IgG2a > IgG2b > IgG1
Expression on common immune subsets	Mac, Neut, DC	B cell, Mac, Neut, DC	NK, Mac, Neut	Mac, Neut, DC
Ortholog in human	FcγRI	FcγRIIB	FcγRIIA	FcγRIIIA

Fig. 2 Distribution and antibody-binding characteristics of FcγR in mouse

et al. 2003; Dall'Ozzo et al. 2004; Kimura et al. 2007). In addition, FcγR expression patterns also differ subtly across macrophage and DC subsets (Repp et al. 1991; Fanger et al. 1996; Veri et al. 2007; Guilliams et al. 2014). Thus, FcγR expression can potentially vary across different tissues and tumors depending on the cellular composition and local cytokine milieu.

With the exception of FcγRIIA and FcγRIIC, which transduce signals through an immunotyrosine activation motif (ITAM) contained within the intracellular tail of the FcγR molecule, all other activating FcγR signal through an ITAM contained on an accessory signaling chain (Ra et al. 1989; Anderson et al. 1990; Kurosaki et al. 1992; Takai et al. 1994; Nimmerjahn et al. 2005; Williams et al. 2012). Depending on the cell type, the Fc binding alpha chain associates with and signals through a zeta chain (NK cells), beta chain (mast cells), or gamma chain dimer (macrophage, neutrophils). FcγR clustering by antibody-antigen complexes results in phosphorylation of the tyrosine residue in the ITAM by Src family kinases, leading to the recruitment and activation of the tyrosine kinase Syk (Kiener et al. 1993; Indik et al. 1995; Veri et al. 2007). Syk kinase in turn activates Ras, Rho, protein kinase C, and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), leading to activation of the MAPK pathway, as well as Phospholipase C γ (PLCγ) activation and mobilization of Ca^{2+} into the cytoplasm (Odin et al. 1991; Rankin et al. 1993; Ninomiya et al. 1994; Karimi and Lennartz 1998; Williams et al. 2012).

Activation of these pathways can be opposed by co-ligation of the inhibitory FcγR, FcγRIIB. FcγRIIB clustering results in phosphorylation of the immunotyrosine inhibitory motif (ITIM) in the intracellular tail of FcγRIIB, resulting in the recruitment of SHIP phosphatase and subsequent inhibition of PI3K, PLCγ, and Ca^{2+} signaling (Daëron et al. 1995; Ono et al. 1997; Zhang et al. 2007; Bibeau et al. 2009; Williams et al. 2012; Srivastava et al. 2013). Antibodies generate significantly greater inflammatory responses in FcγRIIB knockout mice deficient, demonstrating the importance of this receptor in regulating FcγR activation (Koene et al. 1997; Clynes et al. 1999).

Activation of FcγR by antibody-antigen complexes triggers diverse immunological processes, depending on the cell type and FcγR triggered (Shah et al. 2005; Tadmor et al. 2011; Scott et al. 2012; Weiner et al. 2012). Macrophages, DCs, and neutrophils phagocytose antibody-antigen immune complexes through multiple activating FcγR classes (Salmon et al. 1991; Fanger et al. 1996; Manches et al. 2003; de Visser et al. 2005). Activation of FcγRIIIB on neutrophils by immune complexes triggers the release of cytotoxic reactive oxygen species (Fossati et al. 2002; Andreu et al. 2010). Similarly, FcγRIII clustering on NK cells induces antibody-dependent cellular cytotoxicity (ADCC), resulting in the secretion of cytotoxic granules and target cell lysis (Barbera-Guillem et al. 1999; Coussens et al. 1999; Schiller et al. 2000; Manches et al. 2003; Dall'Ozzo et al. 2004; Andreu et al. 2010). Lastly, ligation of FcγRIIB on germinal center B cells and plasma cells results in apoptosis, which is thought to select for high affinity antibodies and regulate the size of the plasma cell compartment, respectively (Nimmerjahn and Ravetch 2008; Andreu et al. 2010).

Macrophages, neutrophils, and DCs express multiple activating FcγR classes as well as inhibitory FcγRIIB, suggesting that the overall balance of activating and inhibitory FcγR engaged dictates the final outcome of antibody engagement (Clynes et al. 1999; Boruchov et al. 2005; Nimmerjahn and Ravetch 2005; Bernatsky et al. 2006). In agreement, co-ligation of FcγRIIB on DCs inhibits upregulation of the antigen processing pathways triggered by FcγRIIA clustering (Gourevitch et al. 1995; Boruchov et al. 2005; Desai et al. 2007; Lu et al. 2008). Similarly, FcγRIIB clustering on macrophages inhibits phagocytosis of immune complexes by activating FcγR (Clynes et al. 1999; Nimmerjahn and Ravetch 2008). In contrast, co-ligation of FcγRIIIB on neutrophils enhances phagocytosis through FcγRIIA (Ravetch and Perussia 1989; Salmon et al. 1995). Thus, multiple activating FcγR can be inhibited by co-ligation of FcγRIIB, while FcγRIIIB can potentially enhance the activity of FcγRIIA and other activating receptors.

Adding further complexity to the FcγR network, antibody isotypes differ in their capacity to bind to and activate FcγR. Human IgG1 and IgG3 antibodies bind to activating FcγR with higher affinity than IgG2 and IgG4 isotypes (Bruhns et al. 2009). Similarly, mouse IgG2a binds strongest to activating FcγR, followed by IgG2b and IgG1 (Repp et al. 1991; van de Winkel and Anderson 1991; Fanger et al. 1996; Nimmerjahn et al. 2005; Veri et al. 2007; Guilliams et al. 2014). In contrast, mouse IgG1 antibodies have greater affinity for FcγRIIB than IgG2a and IgG2b antibodies. The overall capacity of antibody to trigger inflammation is

regulated by the ratio of activating to inhibitory FcγR (A/I ratio) engaged by the antibody (Fleit et al. 1982; Ravetch and Perussia 1989; van de Winkel and Anderson 1991; Salmon et al. 1995; Nimmerjahn and Ravetch 2005; van der Heijden et al. 2012; Guilliams et al. 2014). This has been experimentally demonstrated in a mouse model of melanoma metastasis. IgG2a antibodies have the highest A/I ratio followed by IgG2b and IgG1 antibodies, which is mirrored by their capacity to clear melanoma metastasis from the lung. Notably, mouse IgG1 antibodies have the lowest A/I and are the most sensitive to regulation by FcγRIIB, as evidenced by significantly greater antitumor activity in the absence of FcγRIIB (Ravetch and Perussia 1989; Metes et al. 1998; Nimmerjahn and Ravetch 2005; Veri et al. 2007; van der Heijden et al. 2012). Therapeutically, the A/I ratio can be enhanced by amino acid substitutions in the Fc region, which selectively enhance binding to activating FcγR and improve phagocytosis of tumor cells (Zhao et al. 2006; Richards et al. 2008; Cassard et al. 2012).

Interestingly, IgG2a and IgG2b antibodies appear to mediate tumor clearance in mice largely through interactions with FcγR expressed by macrophages and not NK cells (Uchida et al. 2004; Nimmerjahn and Ravetch 2007; Nimmerjahn et al. 2010). In agreement, IgG2a and IgG2b antibodies bind with the highest affinity to FcγRIV, which is expressed by macrophages and not by NK cells (Nimmerjahn et al. 2005; Williams et al. 2012; Guilliams et al. 2014). Similarly, a number of studies in autoimmune models have shown that IgG2a and IgG2b antibodies mediate inflammation through FcγRIV (Nimmerjahn et al. 2005, 2010; Giorgini et al. 2008). However, the human ortholog of FcγRIV, FcγRIIIA, is expressed on NK cells, suggesting that human NK cells have a greater capacity for ADCC compared to their mouse counterparts (van de Winkel and Anderson 1991; Schiller et al. 2000). This is in agreement with a number of studies demonstrating ADCC by human NK cells (Repp et al. 1991; Reff et al. 1994; Fanger et al. 1996; Carson et al. 2001; Manches et al. 2003; Dall'Ozzo et al. 2004; Kimura et al. 2007). Thus antibody therapy can potentially function through different mechanisms of action in mice and human, depending on the FcγR engaged.

Target Assessment

FcγR expression can reliably be measured by flow cytometry using fluorescently conjugated monoclonal antibodies against specific FcγR molecules. Monoclonal antibodies are available for many of the FcγR classes, including FcγRI, FcγRII, and FcγRIII in humans (Repp et al. 1991; Fanger et al. 1996; Veri et al. 2007; Guilliams et al. 2014) and FcγRI, FcγRIIB, FcγRIII, and FcγRIV in mice (Ra et al. 1989; Anderson et al. 1990; Kurosaki et al. 1992; Takai et al. 1994; Nimmerjahn et al. 2005; Williams et al. 2012). More recently, antibodies capable of distinguishing between structurally similar FcγR molecules, such as FcγRIIA and FcγRIIB in humans (Kiener et al. 1993; Indik et al. 1995; Veri et al. 2007) and FcγRIIB and FcγRIII in mice have been developed (Odin et al. 1991; Rankin et al. 1993; Ninomiya et al. 1994; Karimi and Lennartz 1998; Williams

et al. 2012), further increasing the specificity of available assays. FcγR expression can also be measured using PCR-based approaches relying on primer sets and probes specific to each FCGR gene (Daëron et al. 1995; Ono et al. 1997; Zhang et al. 2007; Bibeau et al. 2009; Williams et al. 2012; Srivastava et al. 2013). These approaches can also be used to distinguish between FCGR alleles, which have different antibody-binding characteristics and differ by a single nucleotide substitution/ amino acid (Koene et al. 1997; Clynes et al. 1999).

Role of the Target in Cancer

Rank: 5

In addition to their well-established role in regulating the activity of monoclonal antibody therapies, a growing body of evidence supports the notion that interactions between antibodies and FcγR can promote carcinogenesis. Work in preclinical syngeneic tumor models first demonstrated that growth of EL4, B16, and MC38 tumors was completely inhibited in μMT mice, which lack endogenous antibodies due to the absence of a B cell compartment (Shah et al. 2005; Tadmor et al. 2011; Scott et al. 2012; Weiner et al. 2012). Studies in a mouse model of premalignant skin lesions have provided further mechanistic insight. In this model, tumor development was significantly impaired in mice lacking T and B cells but could be restored by transferring serum from tumor-bearing animals into mice with premalignant lesions (Salmon et al. 1991; Fanger et al. 1996; Manches et al. 2003; de Visser et al. 2005). Later studies showed that naturally occurring antibodies in the serum, which recognized tumor antigens, deposited into malignant tissue, where they activated FcγR expressed on tumor-infiltrating macrophages, neutrophils, and mast cells (Fossati et al. 2002; Andreu et al. 2010). Lastly, tumor progression was significantly impaired in FcγR knockout mice, demonstrating the involvement of antibody-FcγR interactions in driving tumor growth.

FcγR activation can foster tumor growth through a number of potential mechanisms. This include triggering the release of tissue-remodeling enzymes, chymase, tryptase, and elastase from mast cells and neutrophils, which can stimulate fibroblast proliferation, angiogenesis, and tissue remodeling, leading to tumor growth and invasion into surrounding tissue (Barbera-Guillem et al. 1999; Coussens et al. 1999; Schiller et al. 2000; Manches et al. 2003; Dall'Ozzo et al. 2004; Andreu et al. 2010). Activation of FcγR on macrophages can also drive the development of a proinflammatory phenotypes characterized by expression of IL-1, which promotes cell proliferation and secretion of chemokines that attract immune cells into the tumor microenvironment, further potentiating inflammation (Nimmerjahn and Ravetch 2008; Andreu et al. 2010).

There is no direct evidence linking FcγR activation to the development of neoplasia in humans, although there are data supporting the involvement of naturally occurring antibodies in promoting tumor development. For instance, patients who have B cell mediated autoimmune disorders, such as rheumatoid arthritis and

systemic lupus erythematosus, are at an increased risk for developing lung and other types of tumors (Clynes et al. 1999; Boruchov et al. 2005; Nimmerjahn and Ravetch 2005; Bernatsky et al. 2006). Endogenous antibodies recognizing tumor antigens can also be detected in the serum of cancer patients (Gourevitch et al. 1995; Boruchov et al. 2005; Desai et al. 2007; Lu et al. 2008). Some studies have also shown that the concentration of antibodies in the serum recognizing tumor antigens correlates with disease status, suggesting an interplay between tumor growth and the humoral immune system (Dass et al. 1991; Das et al. 1995; Aziz et al. 1997). However, other studies have failed to detect a positive association and have actually observed an inverse correlation between serum autoantibody concentrations and tumor growth, suggesting that endogenous antibodies protect against tumor growth (Gourevitch et al. 1995; Mensdorff-Pouilly et al. 1996). In support of the notion that endogenous antibodies promote tumor growth, depletion of B cells by rituximab, a chimeric IgG1 antibody targeting CD20, reduced tumor growth and stabilized disease in a proportion of colon cancer patients (Barbera-Guillem et al. 2000; CD20). Further studies exploring FcγR expression and activation in tumors by naturally occurring antibodies could help elucidate the involvement of these molecules in tumor progression and inform the development of novel treatment strategies for cancer. Nonetheless, circumstantial evidence suggests a role for FcγR in cancer, warranting a ranking of 5/10.

High Level Overview

Diagnostic, Prognostic, Predictive

Polymorphisms in *FCGR* genes, which influence antibody binding and effector activity, can potentially be used as a predictive biomarker for antibody therapy. For instance, the *158 V* allele of *FCGR3A*, which codes for FcγRIIIA, binds to IgG1 antibodies with higher affinity than the *158 F* allele (Mensdorff-Pouilly et al. 1996; Koene et al. 1997; Bruhns et al. 2009; Schneider-Merck et al. 2010). Functionally, NK cells isolated from patients with the *FCGR3A V/V* genotype were more effective at lysing HNC cell lines in response to treatment with cetuximab, a chimeric IgG1 antibody targeting the epidermal growth factor receptor (EGFR)(López-Albaitero et al. 2009; ▶ Chap. 62, “EGFR, Growth Factors”). Similarly, NK cells purified from individuals with the *FCGR3A V/V* genotype lyse B cell targets at lower concentration of rituximab (Dall’Ozzo et al. 2004).

Polymorphisms in *FCGR2A* do not significantly impact binding to IgG1 antibodies, but they can influence binding to IgG2 antibodies (Parren et al. 1992; Richards et al. 2008; Bruhns et al. 2009; Schneider-Merck et al. 2010). The *FCGR2A 131H* allele interacts more strongly with IgG2 antibodies than the *131R* allele, suggesting that it mediates greater activity in response to treatment with IgG2 antibodies, such as panitumumab, a fully human IgG2 monoclonal antibody targeting EGFR (Bruhns et al. 2009; Shashidharamurthy et al. 2009). In support of

this assertion, neutrophils isolated from individuals with the *FCGR2A H/H* genotype demonstrated greater toxicity towards EGFR positive tumor cells in response to panitumumab treatment (Schneider-Merck et al. 2010). However, IgG2 antibodies bind weakly to FcγRIIIA and do not efficiently induce ADCC, suggesting that these antibodies primarily function by inhibiting tumor growth pathways (Bruhns et al. 2009; Schneider-Merck et al. 2010).

Given that the *FCGR3A V* allele is more active in vitro, patients who have the *FCGR3A V/V* genotype could potentially respond better to IgG1 antibody therapy than patients with the *FCGR3A F/F* genotype. In agreement, early retrospective studies of colon, head and neck (HNC), and lung cancer patients treated with cetuximab demonstrated an association between the high affinity *FCGR3A F/F* genotype and reduced disease progression (Zhang et al. 2007; Bibeau et al. 2009; López-Albaitero et al. 2009). Interestingly, the *FCGR2A H/H* genotype was also associated with improved progression free survival (Bibeau et al. 2009), even though these alleles do not significantly affect binding to IgG1 antibodies and ADCC activity (Parren et al. 1992; Richards et al. 2008; Bruhns et al. 2009; Schneider-Merck et al. 2010). Trastuzumab is a humanized IgG1 antibody targeting the receptor tyrosine kinase erbB-2 (▶ Chap. 23, “HER2/neu”). In early stage and metastatic breast cancer patients treated with trastuzumab, the *FCGR2A H/H* genotype was found to be associated with a greater pathological and overall response, respectively (Tamura et al. 2011). Positive associations between the *FCGR3A V/V* genotype and clinical responses were also observed in follicular (Cartron et al. 2002; Weng and Levy 2003) and diffuse large B cell lymphoma (DLBCL) patients treated with rituximab (Kim et al. 2006). Interestingly, the *FCGR2A H/H* genotype also responded favorably to rituximab treatment in DLBCL and other non-Hodgkin’s lymphomas (NHL)(Weng and Levy 2003; Paiva et al. 2008). Taken together, these studies suggested that *FCGR2A* and *FCGR3A* genotyping could be used as a predictive biomarker for identifying patients who would respond favorably to antibody therapy.

In contrast, subsequent studies have been unable to detect an association between *FCGR2A* and *FCGR3A* genotype status and clinical response to monoclonal antibody therapies. Three large studies in colorectal carcinoma failed to detect an association between *FCGR2A* and *FCGR3A* genotype status and progression free survival or overall response rate in patients treated with cetuximab (Zhang et al. 2007; Park et al. 2012b; Geva et al. 2014; Kjersem et al. 2014). A smaller study also failed to detect an association between *FCGR2A* and *FCGR3A* polymorphisms and response rate in HNC (Srivastava et al. 2013). Similarly, *FCGR3A* genotype status did not correlate with disease control in DLBCL (Váróczy et al. 2012; Liu et al. 2014) and follicular lymphoma patients treated with rituximab (Prochazka et al. 2011). In another study, *FCGR3A* polymorphisms did not associate with overall or progression free survival in a large cohort of NHL patients treated with rituximab (Pennell et al. 2008). Lastly, no association between clinical response and the *FCGR2A H/H* and *FCGR3A V/V* genotype could be detected in breast cancer patients treated with trastuzumab (Levy et al. 2011; Hurvitz et al. 2012).

These conflicting data could possibly arise from a number of different factors, including the patient populations being studied. For instance, *FCGR* polymorphisms may not influence outcome in patients who have reduced NK and monocyte/macrophage abundance, potentially due to increased disease burden or treatment with previous cytotoxic chemotherapy, which can deplete various immune populations expressing FcγR (Rafique et al. 1997; Petricevic et al. 2013). It is also important to note that *FCGR2A*, *FCGR2B*, *FCGR2C*, *FCGR3A*, and *FCGR3B* are encoded in a gene cluster on chromosome one (Lejeune et al. 2012). In a Caucasian population, *FCGR2A 131R* and *FCGR3A 158 F* alleles were shown to be linked to a unique *FCGR2C* polymorphism that drives expression of the inhibitory receptor FcγRIIB on NK cells, resulting in reduced cytotoxic activity (Lejeune et al. 2012; van der Heijden et al. 2012). Thus, patients who have the *FCGR2A 131R/R* and *FCGR3A 158 F/F* genotypes could potentially have a lower response to antibody therapy due to reduced NK cell activity and not diminished antibody binding to FcγRIIIA. Lastly, in Caucasian populations, the *FCGR2A* and *FCGR3A* genes exist in linkage disequilibrium, resulting in linkage of the *FCGR2A 131H* allele to the *FCGR3A 158 V* allele (Lejeune et al. 2008). This could account for the improved survival noted in some patients who were treated with IgG1 antibody therapy and who also expressed the *FCGR2A 131H/H* genotype, which does not significantly affect binding of IgG1 antibodies (Bruhns et al. 2009).

Currently, *FCGRA* polymorphisms do not appear to be a reliable predictive biomarker. Going forward, studies in larger patient cohorts combined with assessment of immune status, FcγR expression, and sequencing of the entire FCGR coding region could potentially reveal associations between FCGR genotype status and responses to antibody therapy. It will also be interesting to study the impact of *FCGR* polymorphisms on the activity of antibodies targeting immune checkpoints, a relatively new approach in oncology discussed in the next section.

Therapeutic

Antibodies targeting tumor-associated antigens and receptors can function through multiple mechanisms of action, including blockade of growth factor signals, complement-dependent cytotoxicity, and direct induction of apoptosis in tumor cells (Scott et al. 2012; Weiner et al. 2012). The majority of antibodies being used in the clinic contain an IgG1 backbone, suggesting that they also mediate tumor clearance through the induction of ADCC and phagocytosis. In agreement, in vitro studies have conclusively demonstrated that antibodies targeting tumor antigens and receptors, such as EGFR, erbB-2, and CD20, engage FcγR on NK cells (Reff et al. 1994; Carson et al. 2001; Manches et al. 2003; Dall'Ozzo et al. 2004; Kimura et al. 2007), macrophages (Manches et al. 2003; Pahl et al. 2014), and neutrophils (Hernandez-Ilizaliturri et al. 2003), resulting in tumor cell lysis and phagocytosis, respectively. These results have been confirmed by studies in murine tumor models, demonstrating that antibody therapy cannot effectively control tumor growth in FcγR knockout mice (Clynes et al. 2000; Nimmerjahn and Ravetch 2005; Minard-

Colin et al. 2008). Lastly, many solid tumors are robustly infiltrated by macrophage, neutrophil, and NK cell populations expressing FcγR, suggesting that the microenvironment is particularly efficient at depleting antibody-associated cells (Andreu et al. 2010; Simpson et al. 2013; Sandin et al. 2014) .

Until recently, antibody-FcγR interactions were mainly thought to mediate tumor clearance by inducing ADCC and phagocytosis. However, a more complex model, where the FcγR network orchestrates an integrated immune response against cancer, is beginning to emerge. For instance, activation of FcγR on NK cells significantly increases IFN-γ secretion, resulting in the upregulation of multiple pathways central to antigen processing and presentation on DCs, which can potentially enhance T cell priming towards tumor antigens (Srivastava et al. 2013). Activation of FcγR on DCs can also directly enhance antigen presentation (Regnault et al. 1999; Boruchov et al. 2005). A number of studies have also shown that antibody treatment elicits antitumor $CD8^+$ T cell responses in vivo, supporting the assertion that the FcγR network regulates the activity of the T cell compartment. For instance, in a humanized mouse model of cancer, cetuximab treatment enhances intratumoral $CD8^+$ T cell infiltration (Kubach et al. 2014). In HNC patients, cetuximab treatment increases the frequency of $CD8^+$ T cells recognizing EGFR (Kohrt et al. 2014). However, other mechanisms of action, such as enhanced antigen presentation due to internalization of antibody-receptor complexes, could also account for the enhanced $CD8^+$ T cell responses observed (Büschenfelde et al. 2002). Nonetheless, further studies exploring the downstream innate and adaptive immune responses triggered by FcγR-antibody interactions are warranted and could lead to the development of combinatorial therapies with improved efficacy.

In contrast to conventional therapies, which directly target cancer cells or certain aspects related to tumor cell growth, immunotherapy seeks to control cancer by modulating the activity of the immune system (Quezada et al. 2011). Antibodies targeting immune checkpoints have shown remarkable success in preclinical animal models and in the clinic (Hodi et al. 2010). Ipilimumab, a fully human monoclonal IgG1 antibody targeting the T cell proliferation checkpoint CTLA-4, recently received Food and Drug Administration approval for the treatment of metastatic melanoma (▶ Chap. 14, "CTLA-4"). Data from animal tumor models have highlighted a surprising and previously unappreciated link between FcγR and antibodies targeting CTLA-4 and other immune checkpoints.

Antibodies targeting murine CTLA-4 inhibit tumor growth in many syngeneic mouse cancer models (Leach et al. 1996; Kwon et al. 1999; van Elsas et al. 1999; Pedersen et al. 2006). As CTLA-4 negatively regulates T cell proliferation, initial hypotheses regarding its mechanism of action suggested that anti-CTLA-4 treatment expands tumor reactive effector T cells (van Elsas et al. 1999, 2001). However, further work demonstrated that anti-CTLA-4 treatment significantly increases the effector T cell (Teff) to regulatory T cell (Treg) ratio in the tumor microenvironment but not in secondary lymphoid organs (Quezada et al. 2006; ▶ Chaps. 11, "CD4+ T Cells," and ▶ "12, CD8 T Cells"). Given that Teff engage and lyse tumor cells whereas Treg suppress antitumor immunity, this elevated intratumoral Teff/Treg ratio is thought to be a major driver of tumor clearance (Quezada et al. 2011).

Recent experiments have provided further insight into the mechanism of action of anti-CTLA-4 treatment. In a series of experiments, anti-CTLA-4 treatment was found to elevate the intratumoral Teff/Treg ratio by depleting Treg from the tumor microenvironment (Simpson et al. 2013). This depletion was completely absent in gamma chain deficient mice lacking activating FcγR, demonstrating the involvement of the FcγR network in regulating the intratumoral Teff/Treg balance (Takai et al. 1994). Further experiments determined that Treg depletion was largely mediated by FcγRIV, which was specifically expressed by tumor-infiltrating macrophages, thus accounting for the tumor-specific depletion observed. Treg were depleted by virtue of their significantly higher densities of CTLA-4 on the cell surface, resulting in greater antibody binding and FcγRIV engagement. In another set of experiments, antibody isotypes were also shown to influence the activity of anti-CTLA-4 (Selby et al. 2013). IgG2a isotypes, which engage FcγRIV with high affinity, efficiently depleted Treg compared to IgG2b and IgG1 isotypes, which bind to FcγRIV with lower affinity (Nimmerjahn et al. 2005). Efficacy was absent in FcγRIV knockout mice (Simpson et al. 2013) and in mice treated with IgG2b and IgG1 versions of anti-CTLA-4 (Selby et al. 2013), confirming the involvement of Treg depletion and heightened Teff/Treg ratios in mediating antitumor immunity.

Ipilimumab is a fully human IgG1 antibody, suggesting depletion as a possible mechanism of action in the clinic (Hodi et al. 2010). In agreement, Treg were significantly reduced in tumor tissues recovered from bladder cancer patients treated with ipilimumab prior to surgery (Liakou et al. 2008). Nonetheless, these observations were confined to a single study. Further studies measuring Treg abundance in tissues recovered after therapy and their association with intratumoral FcγR expression could provide further evidence of involvement of the FcγR system in regulating the activity of ipilimumab and other antibody-based immunotherapies.

CTLA-4 is one of many immune checkpoints being studied in preclinical animal models and in the clinic. Antibodies targeting GITR and OX40, two checkpoints preferentially expressed on Treg, have also been shown to significantly reduce intratumoral Treg abundance (Hirschhorn-Cymerman et al. 2009; Cohen et al. 2010; Schaer et al. 2013; Anti-OX40). Recent experiments have confirmed depletion through an FcγR-dependent mechanism and have highlighted the involvement of Treg depletion in driving tumor regression (Bulliard et al. 2013, 2014). Other T cell checkpoints, such as PD-1, Tim-3, and LAG-3, are also preferentially expressed by tumor-infiltrating Treg (Park et al. 2012a; ▶ Chap. 6, "Anti-Programmed Death 1 (PD1)"). Thus, antibodies targeting these checkpoints could potentially induce tumor regression through FcγR-dependent Treg depletion. CD40, which is expressed on antigen-presenting cells (APC), regulates various aspects of APC differentiation and antigen presentation. Interestingly, tumor clearance by anti-CD40 antibody treatment is dependent on interactions with FcγRIIB, although it is unclear as to why interactions with FcγRIIB are required for activity (Li and Ravetch 2011). FcγRIIB could potentially display anti-CD40 antibodies in a conformation that maximizes target receptor cross-linking. Overall, it appears that the FcγR system can influence tumor clearance through multiple mechanisms of action, depending on the immune checkpoint being targeted.

The involvement of the FcγR system in regulating the activity of antibodies targeting immune checkpoints is a relatively new concept. It will be interesting to see whether the function of other antibodies targeting immune checkpoints require interactions with FcγRs. Nonetheless, FcγR interactions should be considered when devising antibody-based immunotherapies. Understanding FcγR and target molecule expression patterns in human malignancies and the activity of different antibody isotypes in preclinical animal models could help with selecting the most active immunotherapy in the correct patient population.

Preclinical Summary

Studies in mice deficient for specific FcγR genes have conclusively demonstrated that FcγR regulate the activity of monoclonal antibodies targeting various tumor antigens and receptors. These studies have also provided a theoretical framework for understanding antibody activity, based on the proportion of activating and inhibitory FcγR triggered by different antibody isotypes. Recent data have demonstrated an unexpected involvement of FcγR and the antitumor activity of the monoclonal antibodies targeting immune checkpoints such as CTLA-4, GITR, and OX40, which function by depleting immunosuppressive Treg. Studies in a murine model of squamous cell carcinoma have also suggested the involvement of the FcγR system in promoting de novo carcinogenesis in premalignant skin lesions through the release of proinflammatory cytokines and tissue-remodeling enzymes.

Clinical Summary

Many of the monoclonal antibodies used in the clinic are IgG1, suggesting that their activity is mediated in part through interactions with activating FcγR. In agreement, in vitro studies have conclusively shown that engagement of antibody by FcγR on NK cells and macrophages results in ADCC and phagocytosis of tumor cell targets, respectively. Polymorphisms in *FCGRA* genes have also been suggested to influence the outcome of antibody-based therapies targeting tumor antigens, although there is abundance of conflicting data, which could stem from the patient populations being studied and the genetics of the *FCGR* locus. Compared to murine tumor models, much less is known about the involvement of FcγR in driving inflammation and de novo carcinogenesis and whether antibodies targeting immune checkpoints deplete Treg through FcγR interactions.

Anticipated High Impact Results

- Studies in large patient populations, taking into account pretreatment status and sequencing the entire *FCGR* locus, revealing associations between *FCGR* polymorphisms and responses to antibody therapies.

- Clinical studies showing that antibody-FcγR interactions drive tumor progression by upregulating the expression of tissue-remodeling enzymes and inflammatory cytokines.
- Studies showing that ipilimumab and other antibody therapies targeting immune checkpoints deplete Treg through FcγR interactions and that this activity drives tumor regression.

Cross-References

► Anti-Programmed Death 1 (PD1)
► CD4+ T Cells
► CD8 T Cells
► CTLA-4
► Dendritic Cells
► EGFR, Growth Factors
► HER2/neu
► NK Cells

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Gangliosides

19

Phil Livingston and Govind Ragupathi

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Abstract

Gangliosides are acidic glycosphingolipids that are expressed on the cell surface where they may play a role in the metastatic process and have proven to be uniquely potent targets for antibody-mediated immune attack using cancer vaccines or monoclonal antibodies. Five gangliosides are important targets for cancer therapy: GM2, GD2, and GD3 expressed primarily on tumors of neuroectodermal origin such as neuroblastomas, sarcomas, and melanomas; fucosyl GM1 expressed on small-cell lung cancers; and sialyl Lewis^{a} (Le^{a}), also known as CA19.9, expressed on cancers of the colon, pancreas, and breast. Vaccines and especially monoclonal antibodies targeting GD2, GD3, and sLe^{a} are able to prevent tumor establishment or slow tumor growth in preclinical models, but regression of visible or palpable tumors has proven more difficult. Recently, this has proven possible with radioimmune or antibody drug conjugates targeting sLe^{a}. These types of monoclonal antibody conjugates may represent the future of ganglioside-targeted therapy. Randomized clinical trials with vaccines targeting GM2, GD2, and GD3 gangliosides have been negative to date. However, randomized clinical trials with monoclonal antibodies targeting GD2 (Unituxin^{TM}) in neuroblastoma patients have been positive, and Unituxin^{TM} is now FDA approved for treatment of high-risk neuroblastoma patients. Radioimmune or antibody drug conjugates targeting gangliosides have not yet been tested in the clinic.

Keywords

Gangliosides • Glycosphingolipids • Cancer Vaccines • Monoclonal Antibodies (mAbs) • GM2 • GD2 • GD3 • Fucosyl GM1 • sLe^{a} • CA19.9 • Antibody-drug Conjugates

The Target

Gangliosides are acidic glycosphingolipids that are expressed on the cell surface, each containing a lipid component and a carbohydrate chain. The lipid component is ceramide which is intercalated into the cell surface lipid bilayer, and the carbohydrate chain consists of glucose, galactose, N-acetylneuraminic (sialic) acid, and other sugar moieties. Five gangliosides are particularly important targets for cancer therapy: GM2, GD2, and GD3 expressed primarily on tumors of neuroectodermal origin such as neuroblastomas, sarcomas, and melanomas; fucosyl GM1 expressed on small-cell lung cancers (SCLCs); and sialyl Lewis^{a} (sLe^{a}), also known as CA19.9, expressed on cancers of the colon, pancreas, and breast. The structures of these five gangliosides on the cell surface are demonstrated in Fig. 1. Each is recognized quite specifically by monoclonal antibodies (mAbs) or antibodies induced by vaccines.

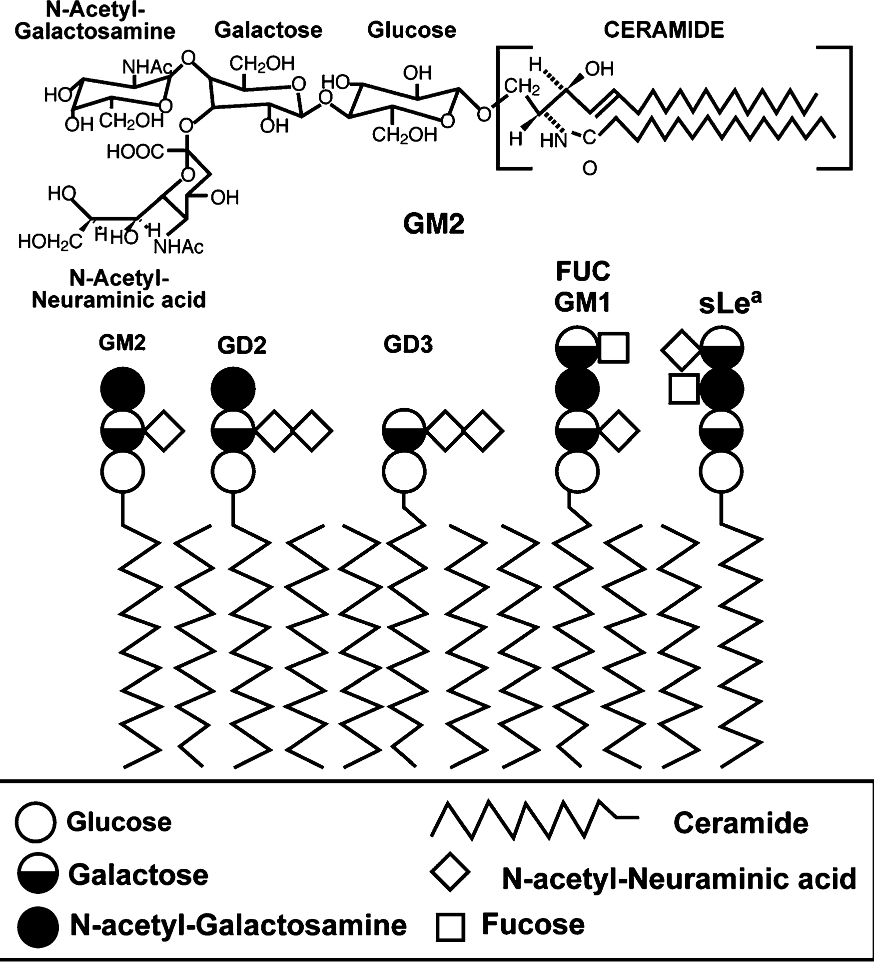

Fig. 1 Carbohydrate epitopes as they appear in five gangliosides that are overexpressed in the cancer cell surface lipid bilayer. The structure of GM2 is presented for comparison

Ganglioside Biology

Gangliosides are glycolipids and so not coded directly by the human genome. Instead, they are coded indirectly through a series of generally highly specific enzymes which build these structures sequentially. Gangliosides are believed to play a role in cell attachment and cell–cell interactions. The gangliosides GD2 and GD3 have been identified in adhesion plaques on the surface of human melanoma

and neuroblastoma cells and are postulated to be involved in attachment of human melanoma and neuroblastoma cells by interacting with and modulating receptors for extracellular matrix (Cheresh et al. 1986). Sialyl Lewis[a] (sLe[a]) antigen is a ligand for endothelial adhesion molecule E-selectin and therefore also thought to have an impact on metastatic potential. A recent study demonstrated that both GM2 and GD3 gangliosides purified from human melanoma cells impaired spontaneous maturation and induced apoptosis in human epidermal Langerhans cells, suggesting that these gangliosides may impede the host immune response by inducing Langerhans cell dysfunction in the tumor microenvironment (Bennaceur et al. 2006).

Target Assessment and Distribution

Gangliosides can be detected by immunohistochemistry on frozen sections (they are leached out by solvents in standard paraffin fixation) (Zhang et al. 1997) and by extraction in chloroform/methanol/water followed by thin-layer chromatography (Hamilton et al. 1993). sLe[a] is also commonly detected in serum where it is known as CA19.9 and used as a marker for disease progression, especially in malignancies of the gastrointestinal tract. Frozen sections from a variety of cancers and normal tissues have been screened by immunohistology using 40 monoclonal antibodies against 25 carbohydrate and protein antigens that were potential targets for immunotherapy. Antigens expressed on at least 50% of cancer cells in at least 60% of biopsy specimens were considered relevant targets. These five gangliosides were extensively expressed, generally in a lineage-specific pattern. While GM2 was also expressed on a variety of malignancies, GD2 and GD3 expressions were restricted to sarcomas, neuroblastomas, and melanomas (Zhang et al. 1997, 1998). Fucosyl GM1 expression was restricted to SCLC, and sLe[a] was expressed on SCLC and cancers of the breast, pancreas, and especially colon. Expression on normal tissues was largely restricted to the central nervous system for GM2, GD2, and GD3 (protected by the blood–brain barrier), peripheral neurons of the autonomic nervous system for GD2, and the lumens of various secretory organs for GM2 and sLe[a] (where immune effector mechanisms such as CDC and ADCC are not evident). Consequently, while gangliosides are clearly overexpressed in these selected malignancies, their cancer specificity and proven safety as targets for antibodies lie in their expression on normal tissues at sites largely inaccessible to antibodies and antibody-mediated effector mechanisms.

Role of the Target in Cancer

Rank: 5
While gangliosides may play a role in the metastatic process and in immune evasion, as described above, their presence in cancer cells largely reflects their cell of origin. The theoretical possibility that cancers could stop expressing a given ganglioside in response to immune attack (since it is probably not indispensable for malignancy) is one of the several reasons that polyvalent vaccines and mAb mixtures may be required.

High-Level Overview

Gangliosides such as GM2, GD2, GD3, and sLe[a] are acidic glycolipids that are overexpressed at the cell surface of several types of cancer where they may play a role in the metastatic process and in an immune invasion. In preclinical studies, they have proven to be uniquely potent targets for immune attack with monoclonal antibodies and vaccine-induced antibodies, also proven to be potent immunogens when conjugated to potent immunological carriers such as KLH and mixed with a potent immunological adjuvant such as QS-21. Monoclonal antibodies against GD3 and GD2 have induced major clinical responses or prolongation of disease-free interval in patients with melanoma or neuroblastoma. The extent of expression of GM2, GD2, and GD3 in the great majority of sarcomas and neuroblastomas makes these malignancies the focus for phase II and phase III trials targeting these gangliosides.

Diagnostic, Prognostic, and Predictive

Sialyl Lewis[a] (CA19.9 antigen) expression is high in the great majority of pancreatic, hepatocellular, and cholangiocellular cancers and also in gastric, colorectal, and occasional other cancers. In these cases, the CA19.9 antigen can frequently be detected in the blood. Since CA19.9 antigen is also expressed by the normal cellular antecedents of these malignancies, it can also be found in elevated levels in the blood in benign diseases such as obstructive jaundice, pancreatitis, hepatitis, and acute liver failure and some more chronic liver diseases. Consequently, measurement of serum CA19.9 which is now a standard blood test is not sensitive or specific enough to be used as a screening test for these cancers; it is used primarily as a tumor marker. It is used when symptoms such as abdominal pain, nausea, jaundice, and weight loss raise a possibility of these malignancies. Also, once a diagnosis of malignancy has been made if CA 19.9 is elevated, it can be used to monitor the patients' response to treatment and to watch for cancer recurrence.

Therapeutics

The carbohydrate components of gangliosides are the primary targets for the immune system, resulting primarily in antibody responses. As glycolipids, gangliosides may also be detected by a subpopulation of natural killer (NK) T cells in the context of CD1 as described for GD3 (Wu et al. 2003), but this recognition, its frequency, and its consequences remain mysterious at this time. Antibodies are ideally suited for eradication of circulating tumor cells and systemic micrometastases as demonstrated in a variety of preclinical mouse experiments (Nasi et al. 1997; Zhang et al. 1998; Sawada et al. 2011). Of the various types of antigens, gangliosides have been shown to be uniquely effective targets for antibody-mediated therapy in preclinical models and in the clinic, probably at least in part due to their intimate association with the cell surface lipid bilayer as shown in Fig. 1 (Ragupathi et al. 2005). Ganglioside antibodies, either passively administered or actively

induced by vaccines, mediate complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), inflammation, and phagocytosis by the reticuloendothelial system (opsonization). If antibodies of sufficient titer can be induced against cell surface antigens such as gangliosides, free tumor cells may be eliminated from the blood and lymphatic systems, and micrometastasis may be eradicated, thus making the establishment of new metastases no longer possible. Should this be achieved, aggressive local therapies such as surgery, radiation therapy, and intralesional treatments may result in long-term control of even metastatic cancers. The success of antibodies against gangliosides is demonstrated in preclinical studies and clinical trials, and FDA approval and wide use of mAbs such as Herceptin, Rituxan, and Erbitux, especially in the minimal disease (adjuvant) setting, support this approach.

Preclinical Studies with Vaccines and mAbs Targeting Gangliosides

The only syngeneic cancer models where these gangliosides are expressed are EL4 thymoma (expressing GD2) in C57BL/6 mice and Ab melanoma (expressing GD3) in Syrian golden hamsters. The ability of adoptively administered mAb 3F8 (targeting GD2) and of GD2–KLH plus QS-21 vaccine-induced antibodies to prevent EL4 recurrence and of R24 mAb administration to prevent Ab melanoma outgrowth after intravenous challenge has been described previously (Nasi et al. 1997; Zhang et al. 1998). Comparable efficacy in SCID mice challenged with xenogeneic neuroblastomas can be achieved by administration of mAbs against GM2, GD2, and GD3 (Wu et al. 2013) or with xenogeneic SCLC and colon cancers by administration of mAbs such as 5B1 against sLe^a (Sawada et al. 2011). In murine models, micrometastases and circulating tumor cells are readily eliminated with mAbs or vaccines targeting gangliosides. Neither approach is able to make visible or palpable experimental tumors consistently regress, a feat recently accomplished with HuMab-5B1 (targeting sLe^a) 1) as a radioimmunotherapy by attaching the antibody to either yttrium-90 (^{90}Y) or lutetium-177 (^{177}Lu) (Viola-Villegas, 2013, Houghton, 2015) or 2) as an antibody drug conjugate (unpublished observations). It is quite likely that approaches such as these will shortly prove more potent than either vaccines or unconjugated mAbs in the clinic as well.

Clinical Summary

Phase I and II Clinical Trials Targeting Gangliosides with Monoclonal Antibodies

Monoclonal antibodies have been widely utilized against gangliosides, especially GD3 mAbs against melanomas and GD2 mAbs against neuroblastomas. Seven studies with murine mAb R24 against GD3 from three different medical centers

have reported eight partial responses and two complete responses in the 103 patients treated (Nasi et al. 1997) for a 10% major response rate. Six studies with murine monoclonal antibodies 3F8 and 14G2a targeting GD2 have been described in patients with neuroblastoma or melanoma (Cheung et al. 1987, 1998; Handgretinger et al. 1992; Saleh et al. 1992; Murray et al. 1994, 1996; Yu et al. 1998). Five complete clinical responses and five partial responses were detected in 48 children with neuroblastoma and three partial responses in 42 melanoma patients. As a consequence of GD2 expression on nerves of the autonomic nervous system, the main side effect has been severe pain in most patients lasting for several hours after mAb infusion and requiring treatment with narcotics. To diminish the human anti-mouse antibody response, the Fab portion of ch14.18 was fused with a human Fc region and used in additional trials. Two complete responses and three partial responses were reported in 19 neuroblastoma patients, but no major responses were detected in 11 melanoma patients treated with ch14.18. The increased response rates in neuroblastoma patients compared to melanoma patients are not unexpected given the very high level of expression of GD2 in essentially all neuroblastoma patients but much lower level of GD2 in melanomas where (like GM2) GD2 is expressed on most melanomas but at only minimal levels. Nevertheless, the response rates are remarkable given the wide range of monoclonal antibody doses utilized in these phase I trials, the advanced stage of disease in most of the treated patients, and the short half-life of these antibodies after the initial several treatments as a consequence of human anti-mouse antibodies (HAMA).

These initial encouraging results and the extensive GD2 expression on neuroblastomas also led to studies targeting these antigens by human T lymphocytes through the use of humanized ch14.18–IL2 fusion antibodies or human T lymphocytes engineered to express chimeric 14.18 mAb variable region receptors (Murray et al. 1996). Evidence of clinical efficacy has been described in several neuroblastoma patients treated in phase II trials (Albertini et al. 2008; Pule et al. 2008; Shusterman et al. 2008). The third approach to targeting gangliosides has been with cancer vaccines.

Optimization of Cancer Vaccines Against Gangliosides

Gangliosides are poorly immunogenic when injected alone or mixed with immunological adjuvants. Immunization with anti-idiotype mAbs or DNA coding for mimotopes mirroring the form of gangliosides GD2 or GD3 has been described to induce immune responses against GD2 or GD3 and protection from tumor challenge in mice. A variety of approaches for increasing the antibody response against gangliosides have been explored, including the use of different immunological adjuvants, chemical modification of gangliosides to make them more immunogenic (Ritter et al. 1991), and conjugation to various immunogenic carrier proteins (Helling et al. 1994). The conclusion from these studies is that the use of a carrier protein plus a potent immunological adjuvant is the optimal approach. The optimal immunological adjuvant in each case has been the purified saponin fraction QS-21 obtained from the bark of *Quillaja saponaria* (Livingston and Ragupathi 2007). The optimal carrier protein was in each case keyhole

limpet hemocyanin (KLH). In the case of GM2–KLH, fucosyl GM1–KLH, and sLe^{a}–KLH, this was sufficient to induce a potent IgM and IgG response in >90% of vaccinated patients. In the case of GD2 and GD3, however, it was also necessary to stabilize the structures by forming lactones which contain internal rings between the two sialic acids and sometimes between sialic acid and the lactose core before consistent antibodies were induced. These vaccine conjugates are referred to as GD2L–KLH and GD3L–KLH. The pattern of IgM and IgG antibody induction following immunization with monovalent KLH-conjugate vaccines against GM2, GD2, and GD3 is demonstrated in Fig. 2 (Livingston and Ragupathi 2007). The antibodies induced by these vaccines also react well in flow cytometry assays with these antigens as they are naturally expressed on the cell surface, mediate potent complement-dependent cytotoxicity (CDC) in the presence of human complement, and mediate antibody-dependent cell-mediated cytotoxicity (ADCC).

Results of Randomized Clinical Trials Targeting Gangliosides

There has been only one randomized trial with a monoclonal antibody against a ganglioside target, chimeric monoclonal antibody ch14.18 against GD2. As reported by Yu et al. (2010), 226 neuroblastoma patients were randomized to receive standard therapy (13-cis-retinoic acid) or standard therapy plus ch14.18 (plus GM–CSF and IL2) after intensive induction and consolidation therapy. With median follow-up of 2.1 years after randomization, progression-free survival was significantly higher for patients randomized to the ch14.18 regimen ($p = 0.01$), with 2-year estimates of $66 \pm 5\%$ vs $46 \pm 5\%$. Preliminary OS was also significantly higher for the ch14.18 arm ($p = 0.02$; $86 \pm 4\%$ vs. $75 \pm 5\%$ at 2 years). FDA approval of ch14.18 (Unituxin^{TM}, dinutuximab) in the USA and Europe for use in high risk neuroblastoma patients took place in 2015.

Despite the high level of GD3 ganglioside expression in melanomas and of GM2, GD2, and GD3 in both neuroblastomas and various types of sarcomas, randomized trials with vaccines targeting these gangliosides or ganglioside mixtures have not been conducted in these disease settings. There have, however, been trials targeting GM2 (which is minimally expressed in most melanomas) in melanoma patients. After an encouraging, though statistically negative, single institution trial with a GM2/BCG vaccine in 122 AJCC stage III melanoma patients conducted in the adjuvant setting (Livingston et al. 1994), two larger multicenter randomized trials have been conducted. In the initial trial, 880 patients were randomized to receive either high-dose interferon for 1 year or vaccination with GM2–KLH plus QS-21 for 2 years. The trial was stopped after median follow-up of 18 months when interim analysis indicated the inferiority of the GM2 vaccine compared to high-dose interferon. The survival, difference between the two groups, had a P value of 0.01 at 1.5 years, 0.04 at 2.1 years, and 0.312 at 7.2 years (Kirkwood et al. 2004). The second trial was conducted in 1314 AJCC stage II melanoma patients, again in the adjuvant setting, and was again stopped early when the interim analysis revealed a decrease in overall survival, though not recurrence-free survival, with a P value of 0.03 at a median follow-up interval of 1.5 years but not at

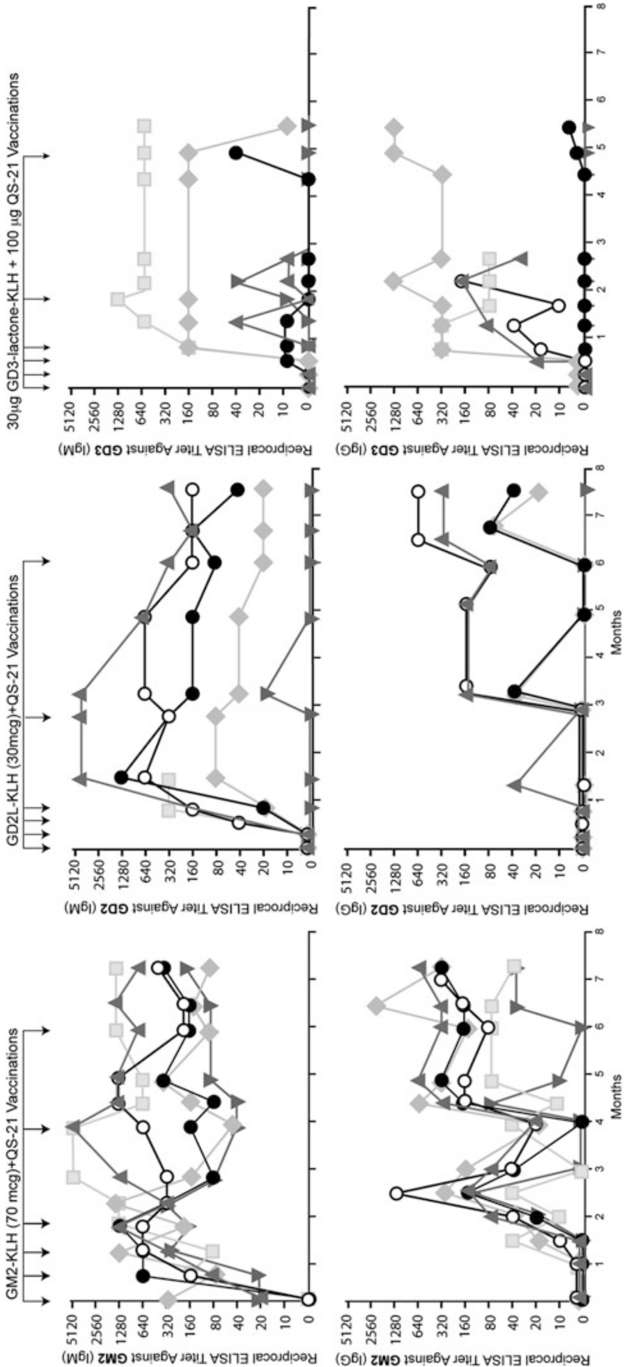

Fig. 2 IgM and IgG antibody responses after vaccination of three groups of melanoma patients with a monovalent ganglioside–KLH-conjugate vaccine + QS-21. Vaccinations are indicated by *arrows*. Each *line* indicates the ELISA antibody titers of a single patient

4 years (P value 0.25) (Eggermont et al. 2010). The low level of GM2 expression in most melanomas (less than 20% of melanoma cell lines can be lysed with human complement and monoclonal antibodies or immune sera against GM2) would explain lack of efficacy of the GM2 vaccine despite induction of high titer GM2 antibodies, but the apparent initial acceleration of recurrence was unexpected. It is however consistent with a significant body of studies demonstrating that inflammation caused by sublytic levels of cell surface complement activation may increase protein synthesis and alter growth patterns including stimulation of growth (Reiter et al. 1992). This same phenomenon has been described in preclinical studies with low-dose administration of monoclonal antibodies against GM2 and more recently against GD2, GD3, and even CD20 with Rituxan (Wu et al. 2013). Both the positive single randomized multicenter trial targeting GD2 in neuroblastoma with mAb ch14.18 and the two negative trials with the GM2–KLH vaccine in melanoma patients therefore mirror the results of preclinical studies. High levels of cell surface antibodies resulting in high levels of complement activation result in protection from tumor recurrence and prolonged disease-free survival, while very low levels are of no value and may be detrimental. This is the basis for restriction of antibody-mediated therapies to tumors expressing high levels of the target antigen and the use of polyvalent vaccines.

Anticipated High-Impact Trials

Treatment with polyvalent vaccines or highly reactive monoclonal antibodies and selection of tumors with uniquely high antigen expression may be required to maximize antibody efficacy and overcome concerns about sublytic levels of complement activation.

Monoclonal Antibodies

Anti-GD2

The trial with ch14.18 in neuroblastoma patients described above halted in 2009 when the benefit became clear. FDA approval of ch14.18 (UnituxinTM, dinutuximab) in the USA and Europe for use in high risk neuroblastoma patients took place in 2015. In Europe, the International Society of Paediatric Oncology (SIOP) is testing the ch14.18 regimen with IL2 administered subcutaneously instead of intravenously in an attempt to decrease toxicity. Also a humanized version of ch14.18 (Hu14.18K322A) has been tested in a phase I trial. It was humanized to induce less human anti-mouse antibodies (HAMA) and modified to be less toxic (less pain during infusion) with greater ADCC activity (Maris 2010; Navid et al. 2010). The phase I trial of Hu14.18K322A demonstrated that it was less immunogenic and induced less pain, permitting administration of higher doses (Navid, 2014).

Anti-GD3

Chimeric mAb KW 2871 against GD3 is currently in a phase II clinical trial in combination with interferon alpha in melanoma patients at the University of Pittsburgh Cancer Institute and the University of Chicago. Like humanized ch14.18, KW 2871 is prepared with diminished fucose content to increase ADCC.

Anti-sLe^a

Human mAb 5B1 (Sawada, 2011) derived from a patient immunized with our sLe^a-KLH vaccine is currently in Phase 1 clinical trials in patients with pancreatic cancer as ^{89}Zr-HuMab-5B1, a next generation PET imaging agent, and as unconjugated 5B1 as immunotherapeutic (O'Reilly, 2016). A therapeutic trial with ^{90}Y-5B1 is planned to start in early 2017.

Vaccines

Sarcomas and neuroblastomas express as much GD3 and far more GM2 and GD2 than melanomas. A randomized, multicenter phase II trial has been initiated at MSKCC and 12 other major medical centers by MabVax Therapeutics Inc. with a trivalent ganglioside vaccine in patients with resected stage IV sarcoma. The primary end points were impact on disease free and overall survival with a secondary endpoint of impact on circulating tumor cells measured with a PCR assay targeting GD2 synthase (Cheung et al. 2004). The study did not reach statistical significance for its first primary efficacy endpoint of a 50% improvement in progression free survival but has not yet accumulated a sufficient number of events to evaluate overall survival. Survival results from this study will be reported in late 2016 or early 2017. Also, a phase I/II trial in neuroblastoma patients with a bivalent vaccine containing GD2L–KLH + GD3L–KLH has been associated with a strikingly long disease free interval in very high-risk neuroblastoma patients (Kushner 2014). The primary end points for this study are toxicity and impact on neuroblastoma cells in the blood and bone marrow using the PCR assay.

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Glucocorticoid-Induced TNF Receptor (GITR)

20

Jedd Wolchok, Adam Cohen, and David Schaer

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Abstract

GITR is a type I transmembrane protein with significant homology, particularly within the cytoplasmic domain, to other tumor necrosis factor (TNF) receptor family members such as 4-1BB, OX40, and CD27. Initially identified following dexamethasone treatment of a murine T cell hybridoma line (Nocentini et al. 1997), GITR was subsequently characterized in human lymphocytes as a 241 amino acid, 25 kDa protein encoded by the *TNFSFR18* gene on chromosome 1p36. Since its identification, preclincal studies have demonstrated that ligation of GITR with agonist antibodies could results in clearance of established tumors.

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This has lead to interest in GITR as a target for immunotherapy with multiple agents currently in phase 1 development.

Keywords
GITR • Treg • Immunotherapy • Co-stimulation • TNFSFR18

Target: Glucocorticoid-Induced Tumor Necrosis Factor Receptor (GITR)-Related Gene

GITR is a type I transmembrane protein with significant homology, particularly within the cytoplasmic domain, to other tumor necrosis factor (TNF) receptor family members such as 4-1BB, OX40, and CD27. Initially identified following dexamethasone treatment of a murine T cell hybridoma line (Nocentini et al. 1997), GITR was subsequently characterized in human lymphocytes as a 241 amino acid, 25 kDa protein encoded by the *TNFSFR18* gene on chromosome 1p36. Mouse and human 24 GITR share 55% identity, and its name may be a misnomer, as dexamethasone had 25 no impact on GITR expression in human cells (Gurney et al. 1999).

Biology of the Target

GITR is expressed predominantly on T cells, natural killer (NK) cells, and NK-T cells, while its ligand (GITR-L) is expressed on antigen-presenting cells such as macrophages, dendritic cells, and B cells. GITR has low basal expression on resting CD4+ and CD8+ T cells but is significantly upregulated 24–72 h after T cell activation (Tone et al. 2003). The delayed expression pattern of GITR suggests that it does not play a predominant role in initial T cell priming, but instead exerts its effects at later time points. In fact, GITR knockout mice have intact T cell development and display normal priming (Ronchetti et al. 2002). Ligation of GITR provides a co-stimulatory signal to recently activated T cells, resulting in enhanced CD4+ and CD8+ T cell proliferation and effector function, particularly in the setting of suboptimal TCR stimulation (Tone et al. 2003; Ronchetti et al. 2004; Kanamaru et al. 2004). Additionally, under most circumstances, GITR stimulus promotes T cell survival, protecting T cells from activation-induced cell death (AICD) (Nocentini et al. 1997; Ronchetti et al. 2002), except in the setting of very strong TCR stimulus, such as with high doses of anti-CD3 antibody or certain alloreactive responses (Tone et al. 2003; Kanamaru et al. 2004; Muriglan et al. 2004). The effects of GITR signaling on NK or NK-T cells are less clear, with conflicting reports showing both activating and inhibitory effects on these cell subsets (Hanabuchi et al. 2006; Liu et al. 2008).

Like other members of the TNF receptor superfamily, GITR contains TNF receptor-associated factor (TRAF)-binding domains in its cytoplasmic tail, and its interactions with several TRAF molecules mediate downstream signaling from the

receptor. Similar to CD28, stimulation of GITR can cause activation of NF-kB and in addition has been shown to activate members of the MAPK pathway, including p38, JNK, and ERK. It is through these pathways, and their downstream events, that GITR ligation is in turn believed to enhance T cell survival by upregulating IL-2Rα, IL-2, and IFNg (Nocentini et al. 1997; Ronchetti et al. 2004; Esparza and Arch 2005). While GITR lacks canonical death domains in its cytoplasmic tail, it has been shown to bind to the death domain-containing protein Siva in Cos7 cells (Spinicelli et al. 2002). However, whether this interaction has a role in GITR's function in primary T cells has yet to be established.

In contrast to naïve CD4+ and CD8+ T cells, CD4+Foxp3+ regulatory T cells (Tregs) constitutively express GITR at high levels. This suppressive T cell subset, comprising 5–10% of circulating CD4+ T cells, serves to maintain peripheral tolerance by inhibiting "self"-specific adaptive immune responses, including antitumor responses. Initial in vitro studies suggested that GITR ligation with agonist anti-GITR monoclonal antibodies (mAbs) could render Tregs unable to suppress, leading to greater effector T cell (Teff) proliferation and function (Shimizu et al. 2002; McHugh et al. 2002). Subsequent in vitro studies using GITR$^{-/-}$ Tregs, however, suggested that this augmented Teff activation was a result of GITR ligation on the Teffs themselves, making them "Treg resistant," rather than an effect on the Tregs (Stephens et al. 2004). This was confirmed in murine tumor models, where in vivo GITR ligation using an agonist anti-GITR mAb or GITR ligand augmented Teff activity without global abrogation of Treg suppressive capacity (Nishikawa et al. 2008; Mitsui et al. 2010; Cohen et al. 2010). However, while peripheral Tregs do not appear significantly affected by GITR ligation, within the tumor, where GITR expression on Tregs is highest, Treg accumulation is markedly impaired. Several potential mechanisms for this have been identified, including impaired migration, intra-tumoral depletion, and lineage instability with loss of nuclear foxp3 expression (Cohen et al. 2010; Coe et al. 2010). The net effect is an augmented intra-tumor Teff/Treg ratio and improved Teff function, leading to tumor rejection, as discussed in more detail below.

Target Assessment

Fluorochrome-labeled monoclonal antibodies against both mouse and human GITR are widely available and allow for accurate assessment of GITR expression on immune cells by flow cytometry. While circulating soluble GITR-L can be measured by ELISA in serum or plasma of cancer patients, circulating soluble GITR has not been described.

Role of the Target in Cancer

Rank: 7

GITR expression has not been reported on cancer cells nor has it been shown to play a direct role in their underlying growth or survival. However, the ability of agonist

anti-GITR antibodies to potentially modulate both effector and regulatory T cells, particularly within the tumor microenvironment, suggests this may provide an important and novel approach to cancer immunotherapy, as described below.

High-Level Overview

The immune system contains checks and balances designed to maximize reactivity against foreign pathogens and minimize reactivity against self. In the setting of cancer, however, this system can be counterproductive, as tumors arise from normal host cells and most tumor antigens are in fact non-mutated, self-antigens. Thus, while there is now ample evidence that cancer patients have B and T cells capable of recognizing tumor antigens, the function of these cells is constrained by the barrier of immunologic tolerance against self.

One novel approach to overcoming this barrier is the use of immune-modulating antibodies. These antibodies do not target the tumor directly but rather alter the balance of immunity to more favor tumor killing. The most successful examples to date are antibodies blocking CTLA-4, a molecule expressed on T cells and involved in downregulating T cell responses following activation. Anti-CTLA-4 antibody treatment can induce durable responses in patients with advanced cancer and was recently shown to extend overall survival in melanoma patients in a randomized, phase III trial (Hodi et al. 2010). Other immune-modulating antibodies in clinical development include antagonists against PD-1 or PD-L1, two other inhibitory molecules which downregulate T cell activity, and agonists to CD40 or 4-1BB (CD137), two other TNF receptor family members involved in immune cell co-activation. Agonist antibodies targeting GITR have demonstrated preclinical activity in a number of tumor models. Targeting GITR holds particular promise because of its potential for dual immunomodulatory effects on both effector and regulatory T cells.

Diagnostic, Prognostic, and Predictive

GITR expression per se on immune cells has not been investigated as a potential prognostic factor in cancer patients. However, the presence of large numbers of intratumor Tregs, which constitutively express GITR and are a major target of anti-GITR antibodies, has been associated with a poorer prognosis in tumors of diverse histologies (Curiel 2007). Perhaps more important for prognostic purposes is the intra-tumor ratio of Teffs to Tregs. For example, higher Teff/Treg ratios within the tumor have been associated with more favorable disease-free and overall survival in patients with ovarian or hepatocellular carcinoma (Gao et al. 2007; Sato et al. 2005). Augmentation of this ratio is a consistent finding following treatment with anti-GITR or other immune-modulating antibodies in preclinical models (Cohen et al. 2010; Quezada et al. 2006) and could be a potential predictive biomarker of antitumor effects in patients, though this remains to be tested clinically.

Therapeutics

A number of approaches to target GITR have been investigated in preclinical models, including monoclonal or polyclonal agonist antibodies, recombinant GITR-ligand-Fc fusion proteins, plasmids encoding GITR-L, and dendritic cells engineered to secrete GITR-L or anti-GITR mAb. The most-studied and best-validated approach in murine models has been the DTA-1 agonist anti-mouse GITR mAb. This rat IgG2b antibody was initially developed by Sakaguchi and colleagues by immunizing rats with putative CD4+CD25+ Tregs. They then screened for Treg-specific antibodies and found one which could block Treg-mediated suppression in vitro, termed DTA-1 (Shimizu et al. 2002). DTA-1 was shown to bind to GITR on both Tregs and recently activated CD8+ and CD4+CD25- T cells (Shimizu et al. 2002). Subsequent studies confirmed DTA-1's ability to provide a co-stimulatory signal through GITR, as well as its ability to modulate both Teffs and Tregs in vivo and induce tumor rejection (described in more detail in the next section). The first antihuman GITR mAb (6C8) to be developed and shows similar agonist activity in vitro, including the ability to co-stimulate suboptimally activated human T cells and attenuate suppression by CD4+CD25+ Tregs in a coculture assay (GITR INC. 2007). A humanized version of this antibody (TRX518, GITR INC, Inc.) has been developed for clinical studies. Additional agents from Medimmune (MEDI1873) and Merck (MK-4166 in combination with Pembrolizumab).

Preclinical Summary

The first demonstration that in vivo GITR ligation could augment antitumor immunity was reported by Turk et al., using the poorly immunogenic B16 melanoma model. Mice bearing a B16 tumor on one flank had a second tumor inoculated on the opposite flank 6 days later. With control antibody treatment, both tumors grew unabated, but after administration of DTA-1, over 80% of mice rejected the second tumor (Turk et al. 2004). Subsequent work showed that DTA-1 could potently augment both vaccine-induced and endogenous immunity against melanoma tumor antigens, leading to tumor rejection in both the prophylactic and therapeutic settings (Cohen et al. 2006; Ramirez-Montagut et al. 2006). These antitumor effects have since been demonstrated in solid and liquid tumor models of diverse histologies, including sarcoma, renal cell, colon, breast, bladder, and lymphoma (Nishikawa et al. 2008; Coe et al. 2010; Ramirez-Montagut et al. 2006; Ko et al. 2005; Zhou et al. 2007; Houot and Levy 2009). Autoimmune toxicities have not been observed, other than mild-moderate hypopigmentation in melanoma models, due to cross-reactive immune responses against antigens shared by melanoma and normal melanocytes (Cohen et al. 2006).

Tumor rejection is lost in RAG$^{-/-}$ mice or if NK or T cells (especially CD8+ cells) are depleted, demonstrating that this is an immune-mediated effect (Ramirez-Montagut et al. 2006). In fact, GITR ligation therapy is more effective when initiated after a few days of tumor growth than when administered at the same time as tumor injection (Cohen et al. 2010; Ko et al. 2005). This implies a need for initial priming

by the tumor and is consistent with the timing of GITR upregulation after initial T cell activation. As described above, the antitumor effects are due to both enhanced co-stimulation of Teffs and impaired tumor accumulation of Tregs, Recent papers have investigated the shown that GITR ligation on Tregs appears alter their stability and programing, preventing their suppressive activity (Schaer et al. 2013). Likewise, the effects of DTA-1 has also been shown to skew Teff cells towards a Th9 phenotype as another mechanisms of GITR therapeutic activity (Kim 2015).

Despite this activity, in most murine models, treatment with anti-GITR mAb alone is unable to eradicate large, well-established tumors, with most studies showing loss of efficacy when therapy begins more than 7–14 days after tumor injection. Synergistic antitumor immunity, however, can be induced through a number of combination strategies, including tumor vaccines, chemotherapy, other immune-modulating antibodies (e.g., anti-CTLA-4 mAb), or adoptive cellular therapy, leading to rejection of even established, vascularized tumors (Mitsui et al. 2010; Ko et al. 2005, 2007; Liu et al. 2009; Cohen, Wolchok, unpublished). These studies provide both preclinical rationale and a framework for exploring these strategies in the clinical setting and suggest that the greatest efficacy with anti-GITR or other immune-modulating antibodies may be obtained when used as part of a rationally designed, integrated combination approach.

Clinical Summary

The first clinical trial of a GITR-targeting antibody (TRX518) opened in December 2010 (NCT01239134) and is ongoing. This is a phase I, dose-escalation study in patients with unresectable stage III or stage IV melanoma. The primary objectives are safety, tolerability, pharmacokinetics, and pharmacodynamics, including impact on immune parameters. A second follow-up multi does trial of TRX518 has recently opened as well (NCT02628574). A phase I trial of autologous dendritic cells transfected with mRNA encoding melanoma tumor antigens along with mRNA for GITR ligand (NCT01216436) is also recruiting. Patients with metastatic melanoma are eligible, and the primary and secondary objectives are safety and anti-melanoma immune responses, respectively. Recently, two additional phase 1 trials have initiated sponsored by Medimmune (MEDI1873, NCT02583165) and Merck (MK-416, NCT02132754) which are open to patients with advanced solid tumors. As of Jan 2015, no results had yet been reported from either study.

Anticipated High-Impact Results

- Additional mechanistic studies exploring how DTA-1 modulates tumor-infiltrating Tregs and induces loss of Foxp3 expression
- Further preclinical studies of GITR-targeted therapies combined with chemotherapy or other immune-modulating antibodies
- Safety and tolerability of antihuman GITR mAbs in phase I clinical trials

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GM-CSF and Whole Cells

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Glenn Dranoff and Kenneth F. May, Jr.

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Abstract

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a pleiotropic cytokine critical to immune homeostasis, capable of both pro-inflammatory and tolerance-inducing properties. GM-CSF has a variety of current and potential clinical applications, including use as a growth factor for myeloid cell recovery after chemotherapy, as a cancer vaccine adjuvant, and in combinatorial immunotherapy with monoclonal antibodies.

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 23 kDa secreted glycoprotein that is produced by a number of cell types, including T lymphocytes, macrophages, mast cells, pulmonary epithelial cells, fibroblasts, and endothelial cells. The GM-CSF receptor (CD116) is expressed primarily on precursor and mature myeloid cells and is composed of a unique α-subunit that determines cytokine specificity and a common signaling β-subunit that is shared with the interleukin-3 and interleukin-5 receptors. When engaged with the cognate cytokine, the GM-CSF receptor leads to activation of the JAK/STAT, PI-3 kinase, and MAP kinase pathways.

Biology of the Target

Originally discovered as a hematopoietic growth factor for myeloid cells, GM-CSF is now known to be required for pulmonary homeostasis through the modulation of surfactant uptake and catabolism by alveolar macrophages. GM-CSF also has pleiotropic effects in the generation and coordination of immune responses, as well as a role in immune pathology. The many functions of the cytokine include promoting the differentiation/maturation, proliferation, survival, and activation of macrophages and dendritic cells; recruitment and activation of granulocytes; upregulation of major histocompatibility complex and costimulatory molecules on antigen-presenting cells; and boosting of immune effector function. Interestingly, GM-CSF knockout mice show no defect in steady-state myeloid hematopoiesis, but manifest a pulmonary alveolar proteinosis syndrome and autoimmune pathology including a systemic lupus erythematosus-like disorder and insulitis. The combined deficiency of GM-CSF and interferon-gamma results in systemic chronic inflammation, opportunistic infections, and a high incidence of hematologic and solid tumors. These findings support a key role for GM-CSF in immune homeostasis, which reflects in part the ability of the cytokine to promote the efficient phagocytosis of apoptotic cells and the induction of $FoxP3^{+}$ regulatory T cells. GM-CSF may also contribute to hematopoiesis during times of organismal stress and may be critical to inflammatory pathology through the activation of myeloid cells (Hamilton 2008).

Target Assessment

GM-CSF can be detected in bronchoalveolar lavage and at low levels in the sera. However, antibodies to GM-CSF have been found in healthy donors, suggesting that most of the cytokine circulates as an immune complex. High titers of neutralizing anti-GM-CSF antibodies are present in most patients with pulmonary alveolar proteinosis and some individuals with inflammatory bowel disease. The biologic effects of the cytokine may thus be regulated in part through the levels of endogenous antibodies.

Role of the Target in Cancer

Rank of the Target: 8

The discovery of GM-CSF as a hematopoietic growth factor for the myeloid lineage has led to the application of the cytokine for the acceleration of myeloid cell recovery following cytotoxic chemotherapy. Because of its role in the coordination of immune responses, GM-CSF has also emerged as a prime target for evaluation in the manipulation of antitumor immunity. The ability of GM-CSF to improve tumor antigen presentation, resulting in enhanced cellular and humoral responses, has stimulated investigation of the cytokine as a cancer vaccine adjuvant (Metcalf 2010). Moreover, the capacity of GM-CSF to increase antibody-dependent cellular cytotoxicity has motivated testing of the cytokine in combination with antitumor monoclonal antibodies. GM-CSF is an established agent with ability to enhance hematopoietic recovery after cytotoxic therapy. It is a component of Provenge, the first FDA-approved cancer vaccine. A more general use in cancer immunotherapy will depend on optimizing the balance of pro-inflammatory and tolerance-inducing properties of the cytokine.

High-Level Overview

Therapeutics

As a growth factor, GM-CSF has been used to support myelopoiesis and decrease morbidity from infections following chemotherapy or bone marrow transplantation, although G-CSF is more widely used because of a more favorable safety profile. However, the potent immunomodulatory activities of GM-CSF have stimulated investigation into the potential use of the cytokine in cancer immunotherapy.

Preclinical Summary

Early preclinical studies demonstrated that vaccination with irradiated tumor cells engineered to secrete GM-CSF stimulated potent, specific, and long-lasting antitumor immunity. In this scheme, GM-CSF functions to enhance tumor antigen presentation by recruiting dendritic cells that phagocytose dying tumor cells, whereas protection against live tumor challenge is mediated through the coordinated activities of CD8 and CD4 T lymphocytes, NKT cells, and antibodies. The combination of irradiated, GM-CSF-secreting tumor cell vaccines and blocking antibodies to negative immune checkpoints, including CTLA-4 and PD-1, results in synergistic antitumor effects in murine models of melanoma and breast and prostate cancer. Other delivery strategies have also revealed the adjuvant activities of GM-CSF, including DNA vaccination, recombinant viral vectors, recombinant protein, and fusion proteins with tumor antigens, such as idiotype/GM-CSF for murine lymphoma. However, GM-CSF also promotes immunosuppressive myeloid cells and $FoxP3^+$ Tregs, which inhibit tumor immunity, suggesting that the optimal therapeutic use of the cytokine may require complementary strategies that antagonize the regulatory pathways, such as costimulatory blockade.

Clinical Summary

The administration of recombinant human GM-CSF protein following treatment of various hematologic malignancies has been assessed in a number of phase III clinical trials. A reduction in morbidity was observed with GM-CSF administration in the post-autologous or post-allogeneic bone marrow transplant setting (Nemunaitis et al. 1991, 1995). In a CALGB study, GM-CSF did not improve the response rate nor decrease myelosuppression-related side effects (Stone et al. 1995), while an ECOG study demonstrated reduced infectious toxicities and increased survival in acute myeloid leukemia (AML) patients receiving GM-CSF compared with placebo following chemotherapy (Rowe et al. 1995). Sargramostim (Leukine), a recombinant yeast-expressed human GM-CSF, is currently approved for use in boosting myeloid reconstitution to reduce infections (including fungal infections) following induction chemotherapy in AML, autologous and allogeneic bone marrow transplants, bone marrow transplant failure or engraftment delay, as well as for mobilization of hematopoietic progenitor cells for harvest.

The preclinical data for the therapeutic value of GM-CSF as an immune adjuvant has led to the clinical testing of a number of vaccination strategies employing GM-CSF. Of particular interest in this context was the recent approval by the FDA of sipuleucel-T (Provenge) for treatment of advanced prostate cancer patients. This represents the first therapeutic cancer vaccine approved for any cancer. Sipuleucel-T, an autologous cancer vaccine, is generated by first harvesting peripheral blood mononuclear cells (PBMC) from an individual patient. The PBMC (including antigen-presenting cells) are cultured for 36–44 h with PA2024, a protein consisting of GM-CSF fused with the tumor-associated antigen prostatic acid phosphatase. The

vaccine is then infused back into the patient for a series of three treatments administered every 2 weeks. Sipuleucel-T was approved by the FDA on the basis of the phase III IMPACT trial (Kantoff et al. 2010a), which demonstrated a survival advantage of 4 months and a 22% relative reduction in risk of death in asymptomatic or minimally symptomatic castration-resistant prostate cancer (CRPC) patients treated with sipuleucel-T compared with placebo. Interestingly, the vaccine did not affect progression of disease, which was similar in both vaccine-treated and placebo-treated patients.

A second promising immune therapy for advanced prostate cancer patients that incorporates GM-CSF is PROSTVAC-VF. This is a noncellular "off-the-shelf" vaccine that uses poxvirus vector engineered to contain prostate-specific antigen (PSA) plus three immune costimulatory molecules (B7.1, ICAM-1, and LFA-3), together designated TRICOM. Vaccinia-PSA-TRICOM is used for a priming immunization, followed by a series of boosting immunizations with fowlpox-PSA-TRICOM. Each immunization is administered with a subcutaneous injection of recombinant human GM-CSF. PROSTVAC-VF has been tested in several phase II trials, the most recent of which randomized 128 patients with minimally symptomatic metastatic CRPC to vaccine versus empty vector (Kantoff et al. 2010b). Vaccine-treated patients had longer median survival (25.1 vs. 16.6 months) and improved overall survival (30% vs. 17%) compared with control patients, without any difference in progression-free survival.

Idiotype vaccines in combination with GM-CSF have been tested in three randomized placebo-controlled phase III trials of patients with follicular lymphoma. These vaccine strategies consist of a tumor-specific idiotype protein conjugated with the immunogen keyhole limpet hemocyanin. One of these trials, comparing a hybridoma-derived vaccine BiovaxID plus GM-CSF with placebo plus GM-CSF, showed prolonged time-to-relapse in patients vaccinated following a 6-month chemotherapy-induced remission (44.2 months for vaccine versus 30.6 months for control arm; $p = 0.045$; HR = 1.6) (Schuster et al. 2011). Two other trials of recombinantly produced idiotype vaccine plus GM-CSF demonstrated no significant difference in progression-free survival between vaccine and control groups (Freedman et al. 2009; Levy et al. 2014). Analysis of humoral immune responses in patients from one of these trials suggested that patients developing anti-idiotype antibody responses had prolonged progression-free survival compared with patients not developing anti-idiotype antibodies (Ai et al. 2009).

The use of GM-CSF as a surgical adjuvant for resected stage IIIB, IIIC, IV, or mucosal melanoma has been evaluated in a large multi-cooperative group phase III trial (Lawson et al. 2015). Patients receiving 1 year of subcutaneous GM-CSF compared with placebo injections following surgical resection had a non-significant relapse-free survival (11.4 months versus 8.8 months; p = 0.131, hazard ratio 0.88) and overall survival (69.6 months versus 59.3 months; p = 0.528, hazard ratio 0.94).

GM-CSF has also been examined in the pediatric population in the treatment of high-risk neuroblastoma following high-dose chemotherapy with stem cell rescue. A phase III COG trial compared standard therapy of 13-cis-retinoic acid in this setting with and without the addition of GM-CSF, IL-2, and anti-GD2 monoclonal antibody.

Addition of immune therapy significantly prolonged event-free and overall survival such that randomization was stopped early for evidence of large early benefit (Yu et al. 2010).

Combinations of tumor peptide or protein vaccination with GM-CSF have yielded more variable results. A mixture of GM-CSF and HER2/neu protein or derived peptides administered to breast cancer patients with HER2/neu-expressing tumors was able to generate HER2/neu-specific T-cell and antibody responses (Disis et al. 2004). When given in conjunction with trastuzumab to metastatic breast cancer patients in a small phase I/II study, this vaccine gave a suggestion of clinical benefit as patients mounting T-cell responses greater than the median had prolonged survival compared with patients mounting T-cell responses less than the median (Disis et al. 2009). Recent work with melanoma peptide vaccination has demonstrated a potentially detrimental effect of GM-CSF. Earlier phase II studies had suggested a possible clinical benefit to advanced melanoma patients treated with MHC class I melanoma multipeptide vaccine, GM-CSF, and Montanide ISA-51 adjuvant (Slingluff et al. 2003, 2008). However, a subsequent multicenter randomized phase II trial revealed that the patients receiving GM-CSF in conjunction with multipeptide vaccine had lower CD8 and CD4 T-cell responses, compared with patients receiving no GM-CSF (Slingluff et al. 2009). Patient numbers were too small to adequately assess clinical outcomes. These findings question the role of GM-CSF as an adjuvant in peptide vaccination strategies.

Another immunotherapy scheme that has undergone intensive investigation involves GM-CSF-secreting tumor cell vaccines. These types of vaccines are variously prepared by (1) retroviral or adenoviral transfer of GM-CSF genes into irradiated autologous tumor cells, (2) stable transfection of plasmids containing GM-CSF genes into preestablished allogeneic tumor cell lines, or (3) utilizing a mixture of autologous tumor cells and a bystander cell line that secretes GM-CSF (Jinushi et al. 2008). Tested in a variety of tumor types, including melanoma, prostate cancer, renal cell cancer, non-small cell lung cancer, pancreatic cancer, breast cancer, ovarian cancer, and acute myeloid leukemia, these vaccines have shown immunogenicity but less pronounced clinical benefit. More significant clinical impact might be anticipated with combinatorial immunotherapy, based on data from preclinical models. One example of this was a small study of metastatic melanoma and ovarian cancer patients treated with irradiated autologous GM-CSF-secreting tumor cell vaccination, followed by the CTLA-4-blocking monoclonal antibody ipilimumab (Hodi et al. 2008). Significant clinical responses (partial responses and prolonged stable disease) and symptomatic improvement were observed in a majority of melanoma patients and several ovarian cancer patients. Interestingly, vaccinated melanoma patients in this study sustained none of the grade 3 and 4 autoimmune toxicities typically seen with CTLA-4 blockade, suggesting that GM-CSF may focus the immune response preferentially toward tumor antigens rather than autoantigens. Another example of combinatorial immunotherapy was reported in patients with acute myeloid leukemia or advanced myelodysplasia, who received autologous GM-CSF-secreting tumor cell vaccination following allogeneic myeloablative hematopoietic stem cell transplantation (Ho et al. 2009). In this study,

nine of ten patients that completed the vaccination schema had durable complete remissions without an increase in graft-versus-host disease, again suggesting that GM-CSF-based vaccination can augment antitumor immunity without exacerbating autoimmunity.

The complexity of manufacturing GM-CSF-secreting autologous tumor cell vaccines for individual patients motivated the development of allogeneic tumor cell vaccines derived from stable tumor cell lines that could be used to treat multiple patients. Allogeneic vaccines have also shown immunogenicity in several tumor types (prostate, breast, pancreas), although two phase III trials in advanced prostate cancer have failed to meet clinical end points for efficacy. This discordance between immunogenicity and clinical benefit underscores the need to address potential GM-CSF-stimulated regulatory pathways that may be dampening the effect of antitumor immune responses generated by vaccination.

Several recent clinical trials combining GM-CSF with CTLA-4 blockade have demonstrated the safety and efficacy of combinatorial immunotherapies. A randomized phase II clinical trial comparing the use of ipilimumab plus GM-CSF (sargramostim) versus ipilimumab alone in patients with unresectable stage III or stage IV melanoma demonstrated improved overall survival and less toxicity with ipilimumab plus GM-CSF compared with ipilimumab alone (Hodi et al. 2014).

Two phase I clinical trials testing GM-CSF-based vaccination strategies with CTLA-4 blockade have demonstrated the safety and feasibility of combinatorial immunotherapies. Both PROSTVAC-VF with coadministered GM-CSF plus ipilimumab (Madan et al. 2012) and GM-CSF-secreting allogeneic prostate cancer vaccine (GVAX) plus ipilimumab (Van den Eertwegh et al. 2012) showed evidence of immunologic and clinical activity in metastatic prostate cancer patients. Though these small studies require further validation, they provide enthusiasm for the partnering of therapies to target different facets of the immune response.

Lastly, another promising strategy uses GM-CSF secreting oncolytic herpesvirus vaccine injected directly into tumors. A phase III clinical trial testing the efficacy of the GM-CSF secreting oncolytic virus talimogene laherparepvec (T-VEC) versus subcutaneous GM-CSF in advanced melanoma patients demonstrated an improved overall response rate (26.4% versus 5.7%) and overall survival (23.3 months versus 18.9 months) in patient treated with T-VEC (Andtbacka et al. 2015).

Anticipated High Impact Results

There are a number of clinical settings in which randomized clinical trials have revealed the potential clinical benefits of incorporating GM-CSF as an immunomodulator for cancer therapy. However, understanding the mechanisms underlying GM-CSF-induced tolerance and immune suppression is a critical step to improving clinical outcomes. Several ongoing clinical trials are examining the contribution of GM-CSF to various cancer immunotherapeutic strategies. A randomized placebo-controlled phase III trial of PROSTVAC in metastatic prostate cancer patients (PROSPECT trial) contains study arms comparing PROSTVAC with or without

concurrent GM-CSF administration (ClinicalTrials.gov #NCT01322490). The results from these studies will help better define the biological and clinical role of this cytokine, particularly in determining whether GM-CSF can enhance the efficacy of other immunotherapies.

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22 gp100

Doug Schwartzentruber

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Abstract

Expression of Gp100 is most useful clinically as a diagnostic marker of melanoma. Its role as a therapeutic target is currently under investigation. The peptide gp100:209–217 (210 M) in Montanide ISA-51 (incomplete Freund's adjuvant) in combination with interleukin-2 (IL-2) demonstrated clinical activity which was significantly higher than IL-2 alone but most importantly established proof of biologic activity and relevance for future study (Schwartzentruber et al. 2011).

Keywords

Gp100 • Assessment • In cancer • In vitro and in vivo studies • Randomized multi-institutional study • Therapeutics • TIL • Interleukin-2 (IL-2) • Pmel17 • Tumor-infiltrating lymphocytes (TIL)

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Target: gp100

Gp100 is a nonmutated melanocyte differentiation protein located in melanosomes. It is expressed in melanoma, normal melanocytes (skin), and pigmented retinal cells. It is generally not expressed in other tumors or normal tissues (except in gliomas and low levels of normal brain tissue (Chi et al. 1997)).

Biology of the Target

Gp100 (also known as Pmel17) is a transmembrane protein of melanosomes (Berson et al. 2001). Melanosomes are subcellular organelles of melanocytes in which melanin pigment is synthesized and stored. Gp100 is expressed in the majority of melanoma tumors. Gp100 is recognized by antibodies (HMB45) and by T lymphocytes. Tumor-infiltrating lymphocytes (TIL) isolated from tumor metastases in patients with melanoma have recognized gp100-expressing tumor targets in vitro (as measured by various in vitro assays).

Target Assessment

Gp100 is measured by immunohistochemistry with the murine monoclonal antibody HMB45. This antibody is commercially available and is widely used in the diagnosis of melanoma. It is particularly useful in determining if there is metastatic spread to lymph nodes in conjunction with routine diagnostic stains. Frequently Melan-A/ MART-1 and S100 antibodies are utilized in conjunction with HMB45 for the diagnosis of melanoma.

Role of the Target in Cancer

Rank:

Diagnostic: 9 (HMB45 is diagnostic of melanoma if expressed on cancer cells)

Prognostic: Unknown (role in prognosis is not defined)
Therapeutic: 5 means further validation before ready for clinical use

Diagnostic, Prognostic, and Predictive

Gp100 expression as measured by the HMB45 antibody has been used as a diagnostic tool but has no reported prognostic value. Immunotherapies utilizing gp100-reactive TIL and IL-2 have resulted in clinical regression of tumor. However, gp100 is not a known predictive marker of clinical response.

Therapeutics

Gp100 is not currently an FDA-approved target for therapy.

Preclinical Summary

Studies of TIL and IL-2 have demonstrated clinical responses in patients with metastatic melanoma. In addition to enhancing our understanding of immunotherapies, TIL have served as biologic reagents in the laboratory to identify a number of melanoma antigens, such as gp100, MART-1/Melan-A, and tyrosinase. Various epitopes of the gp100 protein were identified using TIL from patients that responded to treatment (Kawakami et al. 1995, 1998). Peptides were then synthesized and clinical trials began in the mid-1990s. Many in vitro and in vivo studies identified gp100:209–217 (amino acid sequence ITDQVPFSV) as a relevant epitope for development in humans. It is recognized by T cells in the context of the MHC class I restriction element HLA-A2*0201, which is expressed in approximately 50% of the Caucasian population.

In order to improve the immunogenicity of gp100:209–217, an amino acid substitution was made that increased binding of the peptide to HLA-A2*0201 (Parkhurst et al. 1996). The modified peptide, gp100:209–217 (210 M), resulted in more circulating precursors reactive with gp100 compared to the native peptide (Rosenberg et al. 1999). It resulted in immunization of the great majority of patients when administered with incomplete Freund's adjuvant (IFA), as measured by a variety of in vitro assays (IFN-gamma secretion by in vitro sensitized PBMC, tetramer, and ELISPOT) (Rosenberg et al. 2005; Walker et al. 2004).

Other strategies to vaccinate with gp100 have been tried. Immunization utilizing peptides loaded on dendritic cells, peptide or protein delivered by viral vectors, or plasmid DNA injections have generally been less successful in generating circulating precursors to gp100 (Panelli et al. 2000; Rosenberg et al. 1998a, 2003a, b).

Clinical Summary

As mentioned above, a variety of approaches to vaccinate patients with metastatic melanoma have been utilized. Defined antigen vaccines (as opposed to whole tumor vaccines) have included peptide injections with immunoadjuvants (such as IFA), peptide pulsed on dendritic cells, peptide or protein delivered by viral vectors (vaccinia, adenovirus, and fowlpox), or DNA injection. Overall, they have resulted in infrequent clinical responses (3% of 422 patients), and responses have occurred at sites of disease that are primarily cutaneous and lymphatic (Rosenberg et al. 2004).

A recent study comparing gp100:209–217 (210 M) to another biologic agent ipilimumab noted a clinical response rate of 1.5% in 136 patients receiving the peptide vaccine alone (Hodi et al. 2010). The finding of this study supports the conclusion that vaccines by themselves are not sufficient to elicit meaningful clinical regression of metastatic melanoma, despite producing high levels of circulating immune cells.

In order to improve clinical efficacy, peptide vaccines have been combined with cytokines. The initial report of gp100:209–217 (210 M) in Montanide ISA-51 (incomplete Freund's adjuvant, IFA) plus IL-2 noted responses in 13 of 31 patients (42%) which was higher than had been seen historically with IL-2 alone (16%) (Rosenberg et al. 1998b). An expanded series from the same institution noted response rates of 22.3% vs. 12.8%, respectively (Smith et al. 2008).

To validate the above experience, a number of studies were begun. A series of three phase II studies with the same vaccine but varying schedules of IL-2 and a slightly lower dose of high-dose IL-2 were conducted (Sosman et al. 2008). Response rates varied between 12.5% and 23.8% in each cohort of 39–42 patients. The authors concluded that the vaccine plus IL-2 did not have higher clinical activity than IL-2 alone, but this observation was based on historical controls, as no IL-2 alone control was included (Sosman et al. 2008). A separate study intended to lower the side effects of IL-2, while preserving the immune augmentation of the vaccine, by giving low-dose IL-2 in combination with the vaccine (Roberts et al. 2006). The results of this study were disappointing, as there were no responders among 26 patients treated. However, they are in agreement with the observation that low-dose IL-2 is generally not effective in treating patients with metastatic melanoma.

One prospective randomized multi-institutional study comparing gp100:209–217 (210 M) in IFA plus IL-2 to IL-2 alone in 185 patients was recently completed (Schwartzentruber et al. 2011). It noted a greater than doubling of clinical response rates (16% vs. 6%, respectively, $p = 0.03$), increased progression-free survival (2.2 vs. 1.6 months, $p = 0.008$), and a trend for improved overall survival (17.8 vs. 11.1 months, $p = 0.06$) for the combination of vaccine and IL-2 (Fig. 1a, b). This study serves as proof of principle that gp100:209–217 (210 M) in IFA can augment the clinical response of IL-2 and suggests that further investigation with this peptide is warranted.

No randomized studies have been done in an adjuvant setting to assess the clinical efficacy of peptide vaccines in patients at high risk of recurrence. Gp100:209–217 (210 M) in IFA administered to patients with a history of melanoma who were clinically free of disease resulted in high levels of immunization but was not sufficient to prevent tumor recurrence (Rosenberg et al. 2005).

Anticipated High-Impact Results

- Studies of peptide vaccines with newer adjuvants, such as Toll-like receptor (TLR) agonists
- Combinations of vaccines with other immune stimulators, such as cytokines (IL-2, IL-15) or agonistic antibodies
- Combinations of vaccines with blockade of immunoregulatory pathways such as TGF-beta, IL-10, or CD4+/CD25+ regulatory cells

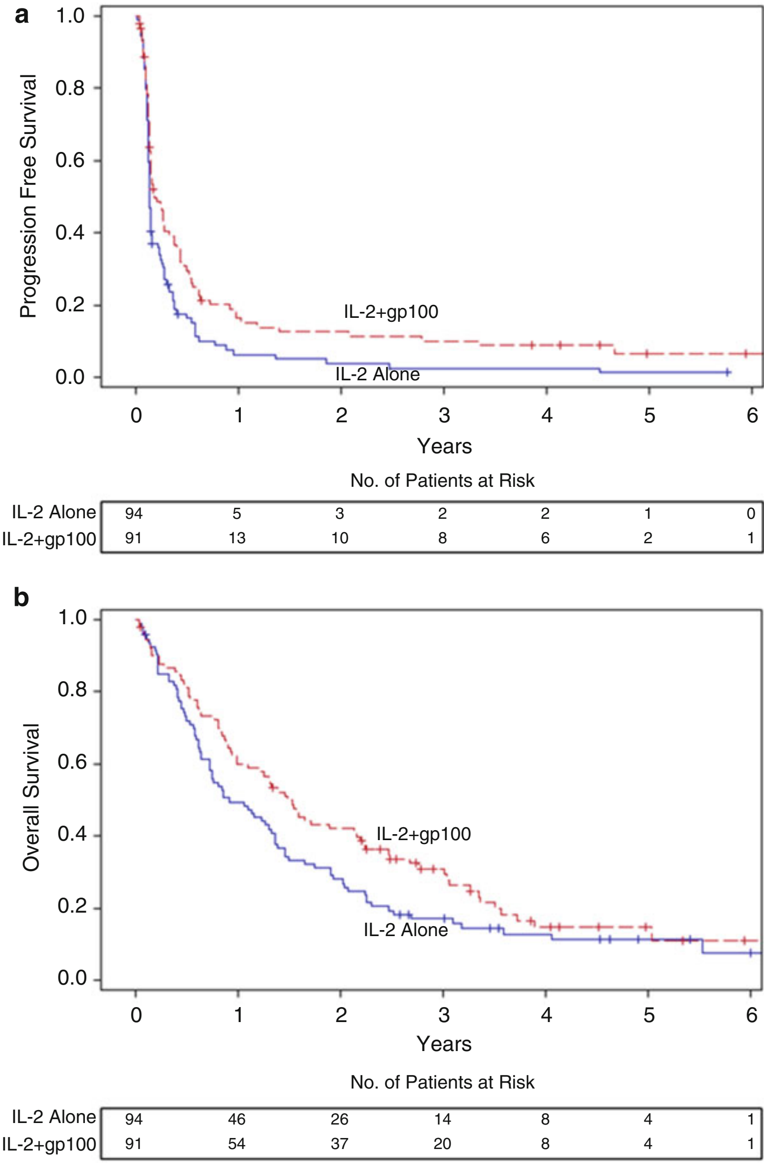

Fig. 1 (**a**) Patients receiving vaccine and IL-2 demonstrated improved progression-free survival. Median progression-free survival with vaccine was 2.2 months (95% CI, 1.7–3.9) compared to 1.6 months (95% CI, 1.5–1.8, $p = 0.008$) without vaccine. (**b**) Patients receiving vaccine and IL-2 demonstrated a trend for improved overall survival. Median survival with vaccine was 17.8 months (95% CI, 11.9–25.8) compared to 11.1 months (95% CI, 8.7–16.3, $p = 0.06$) without vaccine

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HER2/neu

23

Mary L. Disis and Megan M. O'Meara

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Abstract

HER2 is a 185 kDa transmembrane glycoprotein in the receptor tyrosine kinase family that also includes EGFR or HER1 (erbB1), HER3 (erbB3), and HER4 19 (erbB4). HER2 gene amplification and protein overexpression is seen in 20–25% of breast cancers. HER2 protein overexpression is an independent predictor of poor prognosis, thus, the receptor has become a focus for several novel types of targeted therapies. Some patients whose tumors overexpress HER2 develop immunity directed against the protein. Immunotherapeutic approaches aimed at generating or augmenting immunity to HER2 have been also been developed.

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Target: Human Epidermal Growth Factor Receptor 2 (HER2; *erb* B2/*neu*)

HER2 is a 185 kDa transmembrane glycoprotein in the receptor tyrosine kinase family that also includes EGFR or HER1 (erbB1), HER3 (erbB3), and HER4 (erbB4). HER2 was first identified in a rat neuro/glioblastoma models as an oncogene called neu with similarity to the epidermal growth factor receptor (Bargmann et al. 1986). The *erb* B2 (or *erb* B2/*neu*) proto-oncogene located on 17q21 encodes the HER2 protein. HER2 (*erb* B2) was named based on the similarity to the previously identified HER1 (protein encoded from *erb* B1 gene), and the *neu* refers to the human similarity to the originally discovered rat *neu* gene. HER2 consists of an N-terminal extracellular domain (ECD), a single transmembrane domain, and an intracellular domain (ICD). The ECD consists of four domains and is a site of ligand binding. The ECD can undergo proteolytic cleavage to release soluble HER2 ECD and can also be expressed as a truncated protein product via alternative splicing. The ICD consists of a tyrosine kinase (TK) domain, an intracellular regulatory domain, as well as multiple tyrosine residues that can be phosphorylated by another receptor tyrosine kinase through receptor dimerization. HER2 does not bind dedicated ligand directly, but rather favors heterodimerization with the other ligand-bound EGF/HER2-family receptors including most significantly HER3, leading to subsequent transduction of downstream signals; alternatively, ligand-independent homo- or heterodimerization can occur in the setting of HER2 overexpression (Hynes and MacDonald 2009). The ligand-induced or ligand-independent receptor dimerization triggers TK activity, tyrosine phosphorylation, recruitment of adaptor proteins, and finally activation of multiple downstream signaling pathways. Formation of different homodimers and heterodimers can activate a variety of signaling transduction pathways associated with cell growth, migration, and differentiation.

Biology of the Target

HER2 gene amplification is seen in 20–25% of breast cancers. In addition to breast cancer, elevated HER2 tissue levels and serum HER2 ECD levels have now been identified in other tumor types, including colorectal, esophageal, gastric, hepatic, ovarian, pancreatic, and prostatic cancers (Wu et al. 1993). Overexpression of the

protein can lead to activated signaling via PI3K/AKT pathway and RAS-MAPK pathway, as well as increased VEGF, leading to proliferation, invasiveness/motility, anti-apoptosis, and angiogenesis (Klos et al. 2006). Studies of the original rat *neu* oncogene reveal that it represented a mutant allele with a point mutation (V664E) in the transmembrane domain associated with constitutive TK activity and that this missense mutation was thought to be responsible for tumorigenicity of the protein (Segatto et al. 1988). In human tissues, however, it is believed the tumorigenic potential attributed to HER2 is due to overexpression of the protein rather than point mutation (Lemoine et al. 1990).

Overexpressed proteins are more likely to be immunogenic in cancer, and T cell and antibody immunity to HER2 has been demonstrated, such as in patients with HER2-overexpressing breast cancer (Disis et al. 1994). As described below, the immunogenicity of HER2 provides the rationale for therapeutic HER2 vaccination.

Target Assessment

HER2 assessment is part of the standard evaluation of new invasive breast cancers and biopsied recurrences for both prognostic and therapeutic objectives. Gene amplification has been correlated with protein overexpression, in one study the least amplification 4.1% seen in IHC-negative cases, 7.4% amplification seen in IHC 1+ cases, 23.3% amplification seen in IHC 2+ cases, and 91.7% amplification seen in IHC 3+ cases (Owens et al. 2004). However, protein overexpression has been seen in the absence of gene expression and vice versa, likely impacted by IHC tissue fixation and edge artifact issues versus FISH gene amplification heterogeneity issues (Ross et al. 2009). Given concerns for testing inaccuracy, all studies should be performed in an accredited lab, with two algorithms proposed by the American Society of Clinical Oncology/College of American Pathologists (ASCO-CAP): (1) HER2 expression testing by validated immunohistochemistry (IHC) followed by reflexive fluorescence in situ hybridization (FISH) gene amplification analysis if the IHC is equivocal or (2) HER2 gene amplification testing by validated FISH assay followed by repeat FISH testing or IHC if equivocal (Wolff et al. 2007). More recently, some groups suggest amending the guidelines to exclusively use FISH analysis, since IHC false negatives are seen in some paraffin tissues and FISH-negative status in metastatic breast cancer is associated with lack of responsiveness to trastuzumab or lapatinib (Sauter et al. 2009). Additionally, HER2 is one of the 21 genes measured by RT-PCR in the Oncotype DX™ microarray test as well as in other various multigene predictor tests for breast cancer that can help prognosticate and guide therapy. Other modalities for HER2 assessment have been studied including assessment of serum HER2 antibodies, shed HER2 ECD, and circulating tumor cell (CTC) HER2 by immunobead or FISH; nevertheless, these are not currently recommended outside of clinical trial settings. Also at this time, formal recommendations for assessment of HER2 gene amplification or protein overexpression in other HER2-positive tumor types have yet to be defined.

High-Level Overview

Diagnostic, Prognostic, Predictive

Knowledge of HER2 status is most prognostic and predictive in breast cancer and not as well described in other tumor types. HER2 status has been proposed to (1) provide prognostic information in newly diagnosed patients, (2) predict response to HER2-targeted therapies as well as possibly to chemotherapy and endocrine therapy, and (3) provide diagnostic or prognostic information when serum HER2 antibodies or shed HER2 ECD are identified.

HER2 overexpression is an independent predictor of shorter overall survival and time to relapse in patients with breast cancer and was demonstrated as an independent predictor of poor prognosis in 68 of 93 outcomes studies (Ross et al. 2009). HER2 overexpression may also identify those women who benefit from treatment with HER2-targeting therapies, such as trastuzumab and lapatinib. Several studies suggest increased benefit from anthracyclines in HER2-amplified/HER2-overexpressed tumors. It is not clear whether HER2 is a target of anthracyclines or more likely a surrogate for co-amplification of nearby genes; for example, in the prospective NEAT/BR9601 trial, it was shown that chromosome 17 centromere (Ch17CEP) duplication was a more powerful independent predictor of improved outcomes with anthracycline treatment than HER2 or TOP2A; (Bartlett et al. 2010) nevertheless, level II evidence suggests benefit of anthracyclines in HER2-positive patients. While controversial, at this time, ASCO guidelines do not support use of HER2 status in guiding use of taxane chemotherapy in the adjuvant setting, given mixed evidence of specific benefit in this population. Finally, HER2 overexpression is associated with resistance to endocrine therapies, namely, tamoxifen, although there are no official guidelines suggesting that HER2 status should change endocrine therapy management (Massarweh et al. 2008).

Circulating HER2 p185 ECD quantification may be associated with a more aggressive disease course, with 15% of unselected preoperative breast cancer patients and nearly 50% of metastatic breast cancer patients found to have markedly elevated levels of serum ECD (Krainer et al. 1997). Circulating HER2 ECD levels have been proposed but have yet to be validated as a surrogate for tissue measurement in attempts to monitor patients for early relapse or to monitor response to therapy. Given the controversy, serum ECD levels are not currently recommended for standard use in the clinical setting.

HER2-specific immunity may emerge in the future as an additional diagnostic, prognostic, or predictive factor. It has been hypothesized that serum autoantibodies to tumor antigens such as HER2 can be used as a cancer biomarker, especially since they can be detected even with minimal antigen exposure. Preliminary data suggests potential for development of a serum assay to evaluate the antibody response to a panel of tumor antigens including HER2 for breast cancer diagnosis (Lu et al. 2008). From a predictive standpoint, one study demonstrated the ability to detect human and T cell responses to HER2 in patients treated with trastuzumab, with a statistically significant association between anti-HER2 humoral response and clinical response

(Taylor et al. 2007). However, relationships between HER2 serum antibodies and cancer diagnosis or treatment response have yet to be demonstrated in larger populations.

Therapeutics

Many efforts have been made to develop effective therapeutic approaches to control epidermal growth factor pathway-associated tumorigenesis. Trastuzumab is a monoclonal IgG1 class humanized murine antibody that binds the extracellular portion of the HER2 transmembrane receptor, inducing antibody-dependent cellular cytotoxicity (ADCC). It was approved by the FDA for treatment of HER2-overexpressing metastatic breast cancer after a Phase III trial of trastuzumab plus standard chemotherapy demonstrated longer time to disease progression, higher response rate, longer response duration, and improved overall survival (median survival, 25.1 vs. 20.3 months) (Slamon et al. 2001).Notably, 2–16% of the patients receiving trastuzumab and chemotherapy had class III or IV cardiac dysfunction, highest in patients receiving anthracycline and trastuzumab concurrently, leading to recommendations for close cardiac monitoring in patients receiving HER2-targeted therapies. By 2006, four large multicenter trials studied the use of trastuzumab in the adjuvant setting (NSABP B-31, North Central Cancer Treatment Group N9831, Herceptin Adjuvant [HERA], and Breast Cancer International Research Group [BCIRG] 006 trials), revealing that disease-free survival time was 33–52% greater and the overall survival time was 34–41% greater when a 12-month course of trastuzumab was added to adjuvant chemotherapy (Ross et al. 2009). Furthermore, trastuzumab has been added neoadjuvant chemotherapy regimens with improved pathologic complete response rates (Buzdar et al. 2005).

Other HER2-targeting therapies are also under investigation. Trastuzumab-DM1 (T-DM1) is a HER2-targeting antibody-drug conjugate of trastuzumab with a potent antimitotic derivative of fungal toxin maytansine, shown to be safe in Phase I trials in advanced HER2-positive breast cancer, with Phase II and Phase III trials underway including used combination with other targeted therapies (Krop et al. 2010). Pertuzumab is a recombinant humanized monoclonal antibody which binds to the HER2 dimerization domain, preventing heterodimerization of HER2 with other HER receptors. It has shown clinical activity in breast and ovarian cancer and also appears safe with preliminary efficacy when used in combination with trastuzumab in trastuzumab-resistant breast cancer patients (Baselga et al. 2010). Lapatinib is an orally available small-molecule dual inhibitor of the EGFR and HER2 tyrosine kinases. In a Phase III trial comparing lapatinib plus capecitabine versus capecitabine alone in metastatic breast cancer patients who progressed on trastuzumab, an improvement was seen in median time to progression of 8.4 versus 4.4 months (Geyer et al. 2006). Moreover, there is evidence of beneficial synergy when lapatinib is given in combination with trastuzumab (Blackwell et al. 2010). Phase III trials testing lapatinib with or without trastuzumab in the adjuvant and neoadjuvant setting are still ongoing. Neratinib is an irreversible pan-erbB tyrosine

kinase inhibitor targeting HER1, HER2, and HER4, which has also shown clinical activity in advanced HER2-positive breast cancer although toxicity includes dose-limiting diarrhea (Burstein et al. 2010).

HER2 overexpression, while not as prognostically well defined in other tumor types, may be predictive of benefit from HER2-targeted therapies. For example, when trastuzumab was added to chemotherapy in advanced gastric cancer patients in the ToGA trial, the median OS increased from 11.1 to 13.5 months; of note, an interesting explorative analysis revealed that the median OS of IHC3 or IHC2+ and FISH-positive patients (versus IHC3+ or FISH positive) who had received chemotherapy plus trastuzumab increased to 16.0 months compared to 11.8 months for the patients on chemotherapy alone (Jorgensen 2010). Further studies using lapatinib in gastric cancer are in progress.

Since HER2 overexpression is associated with development of preexistent T and B cell immunity to HER2 in cancer patients, several groups have formulated vaccines targeting the HER2 tumor antigen as a novel HER2-targeted therapeutic modality. Vaccines can be composed of antigen-pulsed dendritic cells, tumor lysates/transfected cancer cells, viral/bacterial vector, peptides, or DNA formulations. In a Phase I clinical trial by Disis et al., 64 patients with stage III or IV breast, ovarian, or non-small cell lung cancer were vaccinated with CD4 T helper peptides from the HER2 ICD or ECD, using GM-CSF as an adjuvant. Ninety-two percent of the patients developed T cell immunity, and 89% developed epitope spreading which correlated with immunity to the protein itself, with 38% maintaining persistent immunity at 1 year (Disis et al. 2002). The same group more recently completed a Phase I/II study of combination of trastuzumab and a T helper peptide-based HER2 vaccine in 22 stage IV breast cancer patients, with preexisting immunity significantly boosted and maintained when adding vaccination to the priming of trastuzumab therapy (Disis et al. 2009a). While initial clinical trials have focused on heavily pretreated metastatic breast and ovarian cancer patients for safety purposes, HER2 tumor vaccines have ideally been designed for eradication of minimal residual disease or even chemoprevention. In Phase II clinical trials by Peoples et al., the E75 vaccine was given to disease-free node-positive and node-negative breast cancer patients with a statistically significant improvement in time to recurrence compared to the control arm (Peoples et al. 2008). The E75 vaccine was also given to men with high-risk prostate cancer, showing a potential increase in time to recurrence if vaccination was completed prior to PSA recurrence (Gates et al. 2009). Plasmid DNA vaccines plus adjuvants have some advantages over peptide vaccines, including adaptability to multi-antigen formulations and long-term stability. Trials are ongoing with HLA-unrestricted HER2 plasmid DNA vaccines as well. Lapuleucel-T is a cell-based immunotherapy containing autologous APCs loaded with a recombinant antigen including HER2/neu sequences linked to a GM-CSF domain, which was tolerated well and stimulates an immune response in a Phase I trial in patients with metastatic breast, colorectal, and ovarian cancer (Peethambaram et al. 2009). Despite concerns for autoimmunity, so far minimal toxicity has been seen with HER2 vaccines. Clearly, larger vaccine trials needed to better identify survival outcomes.

Preclinical Summary

Preclinical work on HER2 has led to the identification of new therapeutic targets being tested in the preclinical and clinical setting. For example, further understanding of HER2 signal transduction pathways has identified many potential trastuzumab resistance biomarkers, including increased PI3K activity, truncated p95 HER2, IGF1-R, and loss of PTEN, leading to evaluation of therapeutic interventions based on respective molecular mechanisms. A number of additional HER2-targeting kinase inhibitors are also in the preclinical stage and early clinical development, including dual and pan-HER TKIs. Another active area of translational research is in cancer immunotherapy, where a major current challenge is overcoming HER2 immunological tolerance by augmenting and extending tumor-specific cellular immunity in an immunosuppressive tumor microenvironment. Ongoing research with HER2-targeting cancer vaccines includes optimization for high-affinity epitope binding across HLA alleles, use of peptides that stimulate tumor antigen-specific Th1 immunity rather than an immunoregulatory response, and immunostimulatory modulation of the tumor microenvironment to enhance vaccine efficacy. Preclinical studies and early in-progress clinical trials include newer vaccine modalities with multivalent vaccines, adenoviral vector vaccines, and anti-idiotypic antibodies.

Clinical Summary

Since the discovery of HER2 as an oncogene, thousands of clinical studies have assessed the most appropriate diagnostics, its role as a prognostic/predictive marker, and clinical efficacy of HER2-targeted drugs. Debates are ongoing regarding the most accurate and feasible algorithm for determining HER2 status in breast cancer patients, with the ASCO-CAP guidelines as the standard of care at this time (Wolff et al. 2007). Extensive work has been performed investigating HER2 in attempts to improve prognostics and therapeutics, including correlation of gene expression analyses with clinical outcome to further define different HER2-positive subtypes in guiding treatment. The state of HER2-directed therapy is constantly changing. Evaluation is underway of HER2-targeted therapies given in combination with other new compounds including mTOR inhibitors, heat shock protein inhibitors, sheddase inhibitors, IGF-1R inhibitors, and anti-VEGF agents. Determination of prognostic and predictive value of HER2 and efficacy of HER2-targeted therapy in other non-breast tumor types remains an active area of clinical research, most recently in esophagus, gastric, uterine, bladder, and prostate cancer. There are many clinical trials utilizing anti-HER2 vaccine strategies as described above. Adoptive T cell therapy strategies against HER2 have been employed in patients with metastatic breast cancer, with HER2 antigen-specific T cells more easily expanded from patients previously primed with vaccination rather than from new donors (Disis et al. 2009b). Finally, the question remains whether HER2-targeted therapies may also benefit patients without HER2-overexpressing tumors. The NSABP B-31 trial using adjuvant trastuzumab plus chemotherapy revealed that a certain subpopulation

of patients, initially considered HER2 positive by local IHC and eventually considered HER2 negative by FISH, showed benefit to the addition of trastuzumab (Ross et al. 2009). Furthermore, the E75 peptide vaccine was shown to elicit immune responses even in patients with HER2-negative tumors (Peoples et al. 2008). Further studies may elucidate whether this is related to diagnostic difficulties or to other less-understood biologic pathways. As increased knowledge of the molecular mechanisms of HER2 is identified, more novel diagnostics and therapeutics will continue to emerge.

Anticipated High-Impact Results

- Determination of whether serum HER2 ECD levels will be useful as prognostic and predictive marker
- Clinical relevance of HER2 on CTCs
- HER2 as prognostic or predictive biomarker in other types of cancers
- Use of anti-HER2 therapies in other non-breast HER2-overexpressing tumors
- Studying combination of anti-HER2 therapies with other targeted therapies in breast cancer
- HER2 vaccines in the adjuvant setting, as part of a multivalent vaccine, as part of combination therapy, or as part of a chemoprevention vaccine

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Indoleamine 2,3-dioxygenase

24

Hatem H. Soliman

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Abstract

Indoleamine 2,3-dioxygenase (IDO1, EC 1.13.11.52, Gene ID NM_002164.4) is coded by the INDO (or IDO-1) gene situated on chromosome 8p12 in humans. The enzyme is a 407-amino acid heme-containing cytoplasmic protein that catabolizes tryptophan into N-formylkynurenine via cleavage and oxidation of tryptophan's pyrrole ring. The current form of IDO predominates in placental and marsupial mammals, whereas only less active prototypical IDO variants have been identified in chicken and fish genomes (Yuasa et al., Mol Evol 65:705–714, 2007). This adds credence to the important role IDO plays in the maintenance of

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placental pregnancy in mammals. In its normal physiologic role, IDO is important in modulating immune activation to antigenic challenges at mucosal surfaces in the digestive tract and lungs (Ciorba et al., J Immunol 184:3907–3916, 2010; Xu et al., Proc Natl Acad Sci U S A 105:6690–6695, 2008). The depletion of tryptophan in the tissue microenvironment by IDO exerts an antiproliferative effect on cancer cells and pathogens such as toxoplasmosis, trypanosomes, and chlamydia (Daubener and MacKenzie, Adv Exp Med Biol 467:517–524, 1999; Knubel et al., Faseb J 24:2689–2701, 2010). The gene is regulated by IFN-γ-responsive elements in its promoter region that bind activated STAT1, interferon regulatory factor-1 (IRF-1), and NF-kβ (Chon et al., J Biol Chem 271:17247–1752, 1996). IDO expression is detected in the brain, lungs, gut, kidneys, multiple tumor cell types, plasmacytoid dendritic cells (pDCs) within draining lymph nodes and the spleen, and human mesenchymal stem cells (Batista et al., Mol Imaging Biol 11:460–466, 2009; Gao et al., J Transl Med 7:71, 2009; Brandacher et al., Kidney Int 71:60–67, 2007; Munn et al., J Clin Invest 114:280–290, 2004; Uyttenhove et al., Nat Med 9:1269–1274, 2003; Ling et al., Cancer Res 74:1576–1587, 2014).

Keywords
Cancer immunotherapy • indoleamine 2 • 3-dioxygenase • amino acid metabolism • dendritic cells • tryptophan • kynurenine

Target

Indoleamine 2,3-dioxygenase (IDO1, EC 1.13.11.52, Gene ID NM_002164.4) is coded by the INDO (or IDO-1) gene situated on chromosome 8p12 in humans. The enzyme is a 407-amino acid heme-containing cytoplasmic protein that catabolizes tryptophan into *N*-formylkynurenine via cleavage and oxidation of tryptophan's pyrrole ring. The current form of IDO predominates in placental and marsupial mammals, whereas only less active prototypical IDO variants have been identified in chicken and fish genomes (Yuasa et al. 2007). This adds credence to the important role IDO plays in the maintenance of placental pregnancy in mammals. In its normal physiologic role, IDO is important in modulating immune activation to antigenic challenges at mucosal surfaces in the digestive tract and lungs (Ciorba et al. 2010; Xu et al. 2008). The depletion of tryptophan in the tissue microenvironment by IDO exerts an antiproliferative effect on cancer cells and pathogens such as toxoplasmosis, trypanosomes, and chlamydia (Daubener and MacKenzie 1999; Knubel et al.). The gene is regulated by IFN-γ-responsive elements in its promoter region that bind activated STAT1, interferon regulatory factor-1 (IRF-1), and NF-kβ (Chon et al. 1996). IDO expression is detected in the brain, lungs, gut, kidneys, multiple tumor cell types, plasmacytoid dendritic cells (pDCs) within draining lymph nodes and the spleen, and human mesenchymal stem cells (Batista et al. 2009; Gao et al. 2009; Brandacher et al. 2007; Munn et al. 2004; Uyttenhove et al. 2003; Ling et al. 2014).

Biology of the Target

The initial discovery of a tryptophan-catabolizing enzyme in the liver of mammals called tryptophan oxygenase (later renamed tryptophan 2,3 dioxygenase (TDO)) was made by Kotake and Masayama in 1936. Initially, it was thought that this enzyme was the sole enzyme responsible for the breakdown of L-tryptophan to the catabolite L-kynurenine. The fact that TDO is specific for the L-tryptophan stereoisomer led Yamamoto and Hayashi to search for another enzyme when they observed that mice could break down D-tryptophan as well. They discovered the enzyme responsible in 1967 from rabbit intestine homogenates and named it indoleamine 2,3 dioxygenase (IDO) (Yamamoto and Hayaishi 1967). Multiple investigators noted tryptophan metabolism was altered in various pathologic states, and subsequent studies demonstrated IDO could be induced by infectious agents, lipopolysaccharides, and interferon gamma (Spacek 1955; Yoshida and Hayaishi 1978; Yoshida et al. 1981; Yoshida et al. 1979). The immune regulating role of IDO was revealed in a groundbreaking paper by Munn et al. Immunocompetent mice pregnant with allogeneic or syngeneic concepti were fed an inhibitor of IDO known as 1-methyl-tryptophan. Mice with allogeneic fetuses experienced spontaneous abortions, while the syngeneic fetuses were unaffected (Munn et al. 1998). This demonstrated that IDO was important in preventing the maternal immune system from attacking paternal antigens expressed in the fetuses during pregnancy. The work also spurred interest in the role of IDO in tumor-mediated immune suppression.

In the tumor, high levels of IDO expression cause tumor-infiltrating lymphocytes to arrest in G1, become anergic, and die by apoptosis (Mellor et al. 2002). Low levels of tryptophan within the tumor causes an increase in uncharged tRNAs, and this activates the GCN2 kinase-mediated integrated stress response in T cells (Munn et al. 2005). Also, metabolites of tryptophan including kynurenine, 3-OH-anthranilic acid, and picolinic acid directly suppress tumor-infiltrating T cells (Frumento et al. 2002). Also IDO-expressing plasmacytoid dendritic cells in tumor-draining lymph nodes act to propagate systemic anergy toward tumor antigens by stimulating the proliferation of T regulatory cells via binding of their CTLA4 receptors (Baban et al. 2005).

Target Assessment

The level of IDO expression in tissues can be measured at the transcript level using various RT-PCR primers or microarray probes for IDO1 (probes ID JUC08002222-4 on Affymetrix Transcriptome v2.0). There are multiple commercially available antibodies which can detect IDO1 protein on Western blots or immunohistochemistry (clones 10.1, 1 F8.2, EPR1230Y). These assays do not indicate the enzyme's activity though. It is thought that measuring kynurenine, the downstream product of tryptophan catabolism, in tissue specimens may be a better marker of enzymatic activity. There are mass spectrophotometer/high-performance liquid chromatography assays in use at some research labs to measure this analyte in plasma. There are

also some antibodies that can detect kynurenine-modified proteins (clone 11 F9) in tissue specimens. No clinically validated assay methods are Western blots widely available for assessing IDO activity in cancer patients at this time.

Role of the Target in Cancer

Rank: 7

High-Level Overview

Diagnostic, Prognostic, and Predictive

The available data on IDO1 expression in tissues is primarily prognostic in many different cancers. There is data to indicate elevated IDO expression is an adverse prognostic factor in ovarian, endometrial, and colorectal cancers, melanoma, hepatocellular carcinoma, acute myelogenous leukemia, and lymphoma. In another study, elevated IDO expression in resected colon cancer tissue correlated with lower numbers of tumor-infiltrating CD3+ T lymphocytes and significantly higher risk of subsequent liver metastasis (Brandacher et al. 2006). Similarly, Okamoto et al. demonstrated IDO overexpression in serous ovarian cancer cells conferred a poorer prognosis (Okamoto et al. 2005). This association is less well established in breast and lung cancers (Creelan et al. 2013). It is unknown at this time if IDO expression is predictive for benefit from IDO inhibitors in development currently.

Preclinical Summary

Studies in Lewis lung cancer (LLC) tumor-bearing mice suggested IDO-expressing mononuclear cells in tumor-draining lymph nodes play a role in tumor-mediated immunosuppression (Munn et al. 2004). Uyettenhove et al. also demonstrated that the ability of DBA/2 syngeneic-immunized mice to reject P815B mastocytoma tumor grafts was nullified when those same grafts were transfected with IDO-expressing vectors (Uyttenhove et al. 2003). IDO expression is downregulated by the tumor suppressor gene Bin1, and its inactivation is frequently seen in melanoma, breast, prostate, and colon cancers (Muller et al. 2005). Loss of Bin1 activity allows overexpression of IDO when tumor cells are exposed to interferon γ, providing a mechanism for IDO overexpression in malignant tissues. Recently an autocrine-signaling loop has been described that also drives constitutive IDO expression in malignant tissues through activated STAT3, interleukin 6, and the aryl hydrocarbon receptor (AHR) which is activated by kynurenine (Litzenburger et al. 2014).

There are currently multiple drugs in clinical development which are known inhibitors of the IDO pathway. The most studied of these compounds in

development is indoximod (1-methyl-D-tryptophan). The preclinical data demonstrates the activity of indoximod in preventing T cell anergy in tumor-draining lymph nodes, delaying growth of transplanted Lewis lung cancer mouse xenografts, and working synergistically with various chemotherapeutic agents (doxorubicin, cyclophosphamide, paclitaxel) in regression of autochthonous breast tumors in MMTV-Neu mice (Muller et al. 2005). However, pharmacodynamics data with kynurenine/tryptophan plasma levels suggests that indoximod does not act directly on the enzyme. Rather, it appears to relieve the downstream effects of IDO-mediated tryptophan deprivation on lymphocytes by acting as a tryptophan mimetic in amino acid-sensing pathways that feed into the signaling of GCN2, WARS, and mTOR (Metz et al. 2012). Another important IDO inhibitor in clinical development is INCB024360. In this hydroxyamidine class, small molecule is an orally bioavailable direct inhibitor of the IDO1 enzyme. Preclinical data suggests the drug has nanomolar potency in biochemical assays of IDO activity. The drug is able to enhance T cell, NK cell, and dendritic cell activation in vitro. There is also in vivo activity in murine models showing similar tumor inhibition as a monotherapy and synergy with chemotherapeutic agents. This compound does show a significant impact on kynurenine/tryptophan levels in multiple preclinical models indicating it is a specific IDO1 inhibitor, with little activity on related enzymes such as IDO2 or TDO (Liu et al. 2010). Another compound which was incidentally found to have IDO inhibitor activity is the Bcr-Abl tyrosine kinase inhibitor imatinib which is used in the treatment of chronic myeloid leukemia (CML) and gastrointestinal stromal tumor (GIST) (Balachandran et al. 2011). Finally, a large number of other compounds such as ebselen, NLG919, and epigallocatechin gallate (EGCG, green tea extract) have exhibited inhibitory activity against IDO1 in various preclinical models (Terentis et al. 2010; Ogawa et al. 2012; Mautino et al. 2013).

Clinical Summary

Available phase 1 data on indoximod monotherapy demonstrated good oral bioavailability, a Tmax of ~2 h, t1/2 ~ 10.5 h, and Cmax levels of around 20–30 nM. The absorption of the drug plateaued at doses greater than 1600 mg. The maximum tolerated dose was not identified with dosing up to 2000 mg PO twice daily. The toxicity profile was tolerable with grade 1–2 fatigue, nausea, diarrhea, and anemia being the most common adverse events. A notable adverse event was de novo autoimmune hypophysitis, which was noted in three patients who had received prior checkpoint inhibitor therapy. The best response was a stable disease greater than six months in five patients. The drug did show activity in raising C-reactive protein levels and lowering circulating T regulatory cells but did not demonstrate a significant impact on circulating kynurenine levels (Soliman et al. 2012). Another phase 1 trial combining indoximod with docetaxel was presented which demonstrated no significant increase in the toxicity of docetaxel at 75 mg/m^2 when combined with indoximod dosed at 1200 mg PO twice daily. The objective response

rate was 18% with responses seen in two breast cancer patients, one non-small cell lung cancer patient and one patient with thymic cancer (Jackson et al. 2012). A randomized, placebo-controlled phase 2 of docetaxel +/− indoximod is underway in metastatic breast cancer.

Phase 1 data on INCB024360 also demonstrated good oral bioavailability, good tolerability at doses up to 700 mg PO twice daily, mild toxicities such as grade 1–2 fatigue, and nausea, and the best response was eight patients showing stable disease for > 16 weeks. The pharmacodynamic data did show 90% inhibition of plasma kynurenine levels at doses of 300 mg PO twice daily (Newton et al. 2012). Ongoing trials using INCB024360 include a combination phase 1/2 with ipilimumab in melanoma and monotherapy phase 2 trial in ovarian cancer. Plans are underway to combine INCB024360 with a PD-1 inhibitor and MK-3475 in solid tumors.

Anticipated High-Impact Results

- Results of ongoing phase 1 and 2 trials for indoximod, NLG919, and INCB024360 in combination with other agents (chemotherapy, vaccines, immunomodulators) in various indications (melanoma, ovarian, breast, pancreatic)
- Evaluating the ability of IDO inhibitors to improve response to checkpoint inhibitors such as MK-3475 and ipilimumab

Cross-References

▶ Dendritic Cells
▶ Tregs

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Integrins, Immunology

25

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Abstract

The role of integrins is important for tumor proliferation, angiogenesis, invasion and metastasis. Integrins are the principal receptors for binding most extracellular matrix (ECM) proteins. Integrins serve two major roles in cell function: cell adhesion and cell signaling. There are multiple mechanisms by which integrins can be assessed in the laboratory, and clinical trials with anti-integrin antibodies are ongoing. Tumor cells can use variable integrin expression to increase proliferation and promote metastasis and angiogenesis. There may be a role for

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integrin blockade in the treatment of tumor growth and metastasis, though successful human trials have not yet been achieved.

Keywords
Integrin • Therapeutic targets • Angiogenesis • Extra cellular matrix

Target

Integrins are the key principal receptors for binding most extracellular matrix (ECM) proteins, and virtually all animal cells express integrins. In the blood, integrins can also serve as a cell-cell adhesion molecule. There is a growing appreciation for the role that integrins play in cancer cell migration, invasion, and recruitment of blood supply in tumor progression, invasion, and metastasis.

Integrins are composed of two noncovalently associated transmembrane glycoproteins: α and β. Twenty-four αβ heterodimers have been identified, and they are categorized into three subfamilies based on the β unit. β1 integrins bind mostly to ECM proteins. β2 integrins are expressed on leukocytes. β3 integrins are expressed on platelets and megakaryocytes, endothelial cells, fibroblasts, and some tumor cells. Each α subunit contains β-propeller on the extracellular portion of the subunits. It is composed of seven 60-amino acid repeats that form the blades of the propeller plus three or four "EF hand" motifs, which bind divalent cations, such as calcium. About half of integrins also have a domain on the α subunit, termed the I domain, which interacts with either Mg2+ or Mn2+. For integrins containing an I domain, the I domain determines ligand specificity. For all other integrins, ligand specificity is determined by the combination of the extracellular domains of both the α and β subunits (Alberts et al. 2002; Plopper 2011).

Biology of the Target

Cell Adhesion

Integrins function to attach cells to extracellular matrix proteins and to other cells. Most cells express more than one type of integrin receptor, and each integrin can often bind multiple ECM proteins. Unlike signaling receptors, integrins bind their ligand with lower affinity and are significantly more densely located on the cell surface.

In order to function as a transmembrane connection to extracellular matrix proteins, integrins must interact with the cytoskeleton of the cell. After binding its extracellular ligand, the cytoplasmic tail of the β subunit will bind several anchor proteins. Anchor proteins include talin, α-actinin, and filamin, and these proteins connect the integrins to actin in the cell cytoskeleton. Most integrins

connect to actin filaments, though others, such as α6β4, bind intermediate filaments. This cytoplasmic anchoring is crucial to the functionality of integrins, as demonstrated by β subunits that have been manipulated with recombinant DNA to delete the cytoplasmic tails, which do not mediate strong adhesion (Plopper 2011).

There are two ways in which cells control the strength of integrin-ECM adhesion. Firstly, the integrin heterodimer can exist in an inactive state. “Affinity modulation” is a change in the conformation of the receptor that increases its affinity for its ligands. Secondly, cells can increase the number of integrins at a point of focal adhesion, known as “avidity modulation.” Signals that originate in the cytoplasm and modulate affinity and avidity are known as “inside-out” signaling (Alberts et al. 2002).

When integrin receptors cluster on the cell surface, their collective adhesion capacity increases the strength of the integrin-ECM bond. Focal contacts are the first integrin clusters at the leading edge of a migrating cell. Focal adhesions are mature focal contacts capable of resisting mechanical force.

Cell Signaling

1. *Outside-in Signaling*
   Integrins are not only structural proteins. Activation of integrins can induce global cell response as well as localized cytoplasmic responses. Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase involved in the localized cytoplasmic response of integrins. FAK is recruited by anchor proteins, and the clustered FAKs cross-phosphorylate one another, creating a phosphotyrosine docking site for Src cytoplasmic tyrosine kinases. Src proteins further phosphorylate the FAK proteins, which creates docking sites for many more intracellular signaling proteins (Plopper 2011).

   In addition, integrins can work together with conventional signaling receptors. Signaling receptors can increase the expression of integrins, while integrin signaling can increase the expression of conventional signaling receptors. Integrins and signaling receptors can work together to sustain activation of intracellular pathways, including the Ras/MAP kinase pathway. The importance of this interaction is highlighted by the fact that many cells will not proliferate and will, in fact, apoptose, if not attached to ECM proteins via integrins. This attachment-dependent growth is often lost in more invasive stages of cancer.
2. *Inside-out Signaling*
   It is less well understood how the cell regulates the activity of integrins via “inside-out signaling.” In some cells, the integrins remain in the adhesion-competent state. However, in other cell types, including leukocytes and platelets, integrins must be activated to the adhesive state. For examples, platelets induce the activation of their β3 integrin when exposed to damaged blood vessels or soluble signal molecules.

Target Assessment

There are several mechanisms by which integrins can be assessed in the laboratory. Integrins can be stained by immunohistochemistry with antibodies targeting specific integrin heterodimers, and labeled antibodies have been used in clinical studies to assess integrin distribution patterns in different disease and cancer states (Dearling et al. 2010; Gu et al. 2014; Trajkovic-Arsic et al. 2014). Integrins can also be immune precipitated. Cell attachment assays can be used to assess the function of integrins (Masur et al. 1993). Cell binding assays have been used to identify many of the known integrin ligands, including ECM proteins and members of the immunoglobulin superfamily, such as vascular cell adhesion molecule (VCAM-1) (Marciano et al. 2007).

Clinical trials are ongoing to treat tumors using antibodies that target integrins in vivo. So far, there have been trials for a humanized anti-αvβ3 monoclonal antibody, a pan anti-αv antibody, an anti- α5β1, and a cyclic RGD (arginine-glycine-aspartic acid) peptide inhibitor of αvβ3 and αvβ5. The RGD sequence is a common sequence at the site for interaction with ECM proteins on integrins. One mechanism for targeting integrins in clinic has been identifying unique sequences of amino acids that bind to a particular integrin, and using labeled probes that target this sequence. The RGD has been successfully targeted in several studies. Several groups have linked RGD amino acid sequence to a light-emitting solid lipid nanoparticle (SLN) and used it to assess biodistribution (Morales-Avila et al. 2012; Shuhendler et al. 2012; Wu et al. 2013).

Role of Target in Cancer

Rank: 4

Tumor cells' expression of integrins depends on the integrin's ability to augment essential tumorigenic properties, such as tumor proliferation, survival, and migratory capacity. Integrins such as αvβ3, αvβ6, and α6β4 that enhance tumor proliferation may be upregulated in cancer cells. Integrins can also promote the survival of cancer cells by increasing the expression of antiapoptotic genes, like BCL2, and downregulating the expression of proapoptotic genes, like BIM (Guo and Giancotti 2004).

Aberrantly expressed integrins enhance tumor proliferation by disrupting tumor cell adhesion. Whereas normal cells undergo apoptosis when they lose cell-cell contact, mutations in tumor cells allow them to circumvent these pathways and later replicate and metastasize to other sites. β1 integrin interferes with cell-to-cell adhesion by disrupting adherens junctions. This disruption can occur when β1 interacts with SNAIL/SLUG to downregulate E-cadherin expression. β1 can also disrupt cell adhesion by interacting with E-cadherin to attract ubiquitin and proteases to decrease E-cadherin levels via endocytosis (Guo and Giancotti 2004). Because β1 integrin disrupts cell adhesion, its presence has been shown to lead to tumor

recurrence and resistance to radiation therapy. Low levels of β1 integrin may be found in tumors that do not recur after surgery. Experiments have shown that inhibitory antibodies or short hairpin RNA can block the effects of β1 integrins, including the transition of tumor cells to a metastatic state. Flavopiridol, an integrin inhibitor, has also been shown to downregulate β1 integrins and prevent tumor survival (Barkan and Chambers 2011).

Integrins increase metastasis by promoting invasion. Integrins augment a tumor's ability to invade normal tissue by promoting epithelial to mesenchymal transformation (EMT). The integrins αvβ6 and αvβ8 increase TGF-β signaling, and TGF-β leads to EMT and interacts with Ras to enhance tumor cell invasion. Integrins also promote tumor cell migration by activating the ERK/MAPK and Jun amino-terminal kinase (JNK) pathways; these pathways promote metastasis by phosphorylating the cytoskeleton and modifying genes. The integrin αvβ6 promotes metastasis by disrupting hemidesmosomes in the cell membrane (Guo and Giancotti 2004). Integrins and integrin-linked kinase 1 (ILK1) can promote tumor cell invasiveness by activating proteases that degrade the basement membrane. The integrin αvβ6 allows tumor cells to invade tissues, specifically breast tissue, by activating phosphatidylinostitol 3-kinase (PI3K). The integrin αvβ3 enhances tumor cell invasion by promoting tumor cell adherence to platelets and leukocytes. Once bound to platelets or leukocytes, tumors gain access to new sites in the body and can metastasize (Guo and Giancotti 2004). Overexpression of ILK1 also increases the ability of tumor cells to survive and metastasize in breast, prostate, and ovarian cancers. When ILK1 is overexpressed, tumor cells begin anchorage-independent growth (Cortez et al. 2011).

Integrins also promote metastasis by upregulating angiogenesis. The αvβ3 integrin promotes tumor cell invasion by activating MMP2 and plasmin to break down the basement membrane and increase blood vessel formation. The αvβ3 integrin works with EGF, PDGF, and VEGF to allow tumor cell migration (Guo and Giancotti 2004). The integrin αvβ3 can also be upregulated by growth factors that promote angiogenesis. Importantly, this integrin is expressed on the blood vessels in the tumor tissue but not on the blood vessels in the healthy tissue (Danhier et al. 2012). ILK1 has been shown to increase the levels of vascular endothelial growth factor (VEGF), which increases blood vessel formation and the ability of cells to be invasive. ILK1 inhibits anoikis, which causes cells to die when they lose connections with the matrix; this further allows tumor cells to proliferate (Cortez et al. 2011).

RGD antagonists have also been shown to inhibit angiogenesis and tumor cell proliferation. RGD antagonists bind to an RGD sequence that is found in proteins that bind to the integrin receptor. Because these antagonists inhibit angiogenesis, the tumor cells are not able to receive the necessary oxygen or nutrients and die. However, it has not been proven that integrin blockade is sufficient to completely block angiogenesis in tumors. RGD antagonists have also been shown to upregulate integrin expression and proportionally improve a patient's response to radiotherapy (Danhier et al. 2012).

Therapeutics

Clinical trials for integrin antagonists began in the late 1990s and early 2000s, the earliest of which include the humanized anti-αvβ3 monoclonal antibody, vitaxin (MEDI-522), and later its successor, etaracizumab. While Phase I trials uniformly indicated low toxicity and showed slight efficacy in halting disease progression in a minority of patients with late-stage solid tumors, Phase II trial of etaracizumab showed no end-point survival impact, and further development ceased (Desgrosellier and Cheresh 2010).

Intetumumab (CNTO 95), a pan anti-αv antibody, showed low toxicity and slight antitumor activity in pilot and Phase I trials. Limited Phase II trials have been completed in metastatic melanoma and metastatic prostate cancer cohorts and yielded worse or nonsignificant end-point results in comparison to standard chemotherapeutic treatments and, in one study, worse progression-free survival and overall survival rates than placebo (O'Day et al. 2011; Heidenreich et al. 2013).

The first anti-α5β1 antibody to progress to clinical testing, volociximab (M200), has undergone multiple nonrandomized Phase II trials for metastatic melanoma, renal cell carcinoma, lung, ovarian, and pancreatic cancer with high rates of disease stabilization in small groups of patients (Almokadem and Belani 2012). Only one anti-integrin treatment, cilengitide, a cyclic RGD peptide inhibitor of αvβ3 and αvβ5, has passed into Phase III trials in treatment of brain tumors. After demonstrating tolerance across a range of dosages and mostly mild toxicity in Phase I, cilengitide induced little objective clinical response in Phase II studies of metastatic melanoma, lung, prostate, and pancreatic cancers. Overall, progression-free survival rates across all phase II studies were similar to that of standard chemotherapeutic treatments when tested as a single agent and in combination. However, all trials showed higher rates of disease stabilization in high-dose cohorts compared to low-dose cohorts (Millard et al. 2011).

Phase II success of cilengitide in glioblastoma multiforme (GBM) and glioma patients became the most exciting prospect for anti-integrin cancer therapy, after multiple Phase II studies showed modest clinical efficacy, either as single treatment or in combination with standard radiotherapy (RT)/temozolomide (Millard et al. 2011). Current treatment modalities of GBM and gliomas offer little benefit, with likelihood of local recurrence and fatality within 2 years. Under these clinical conditions, even modest efficacy of novel treatments is thoroughly explored. In one single-treatment study of 81 patients with recurrent GBM, 15% receiving high-dose cilengitide (2000 mg) had 6-month progression-free survival, on par with the efficacy of temozolomide in prior studies, and 9% had partial response; many of these patients were temozolomide refractory (Reardon et al. 2008). Studies testing cilengitide in combination with RT and concomitant temozolomide showed higher rates of progress-free survival over RT alone or RT + temozolomide (Stupp et al. 2010).

Preclinical research revealed that cilengitide sensitized tumors to radiotherapy and given the highly vascularized nature of brain neoplasms, these findings supported the use of cilengitide as a single or combination anti-angiogenic treatment.

The latter study also provided the first evidence that patients with methylated methylguanine methyltransferase (*MGMT*) gene promoter had significantly longer progression-free survival compared to patients with unmethylated MGMT, a result that had been anecdotally noted in other trials. For this reason, the largest anti-integrin testing to date, a multicenter, randomized, open-label Phase III trial, began in 2008 to test efficacy of cilengitide + RT + temozolomide versus RT + temozolomide alone in a population of patients newly diagnosed for GBM with methylated MGMT gene promoter. After failing to show improved overall survival or progression-free survival over control, the study was discontinued in 2013 (Stupp et al. 2014). These results may mark the end of the cilengitide era in GBM and glioma research, but various Phase II trials are still underway in the use of cilengitide in other types of cancer.

Preclinical trials have shown that the tumorigenic enhancing properties of ILK1 can be blocked. ILK1 activates proteases, which facilitate tumor invasion, and ILK1 correlates with the progression of some cancers, including breast, prostate, and ovarian cancer. Inhibition of ILK1 has been shown to halt tumor cell proliferation. Studies have demonstrated that small molecular inhibitors of ILK1 are especially useful in treating breast cancer because they specifically target and kill breast cancer cells and do not harm normal breast tissue cells (Cortez et al. 2011).

There are important caveats to note in the trials that have thus far been completed. Many Phase II trials showed trends toward clinical benefit but could not analyze for statistical significance due to low sample size. Furthermore, all studies used disease stabilization as end-point markers of clinical success, the validity of which is widely debated but nonetheless, commonly used. In the case of integrin blockers, which have been widely tested in a variety of cancers, comparing efficacy between these studies is difficult given that disease stabilization in one cancer type may have different prognostic value than in another. Many studies, however, successfully corroborated end-point results with decreased αv, α5, or β1 protein expression and increased levels of the given treatment in the tumor area. The variability in treatment success may be due, in part, to the target specificity and pharmacokinetics of the administered blocker.

Preclinical Summary

Given the diverse utility of integrins in cell survival, migration, invasion, proliferation, and cell-cell and cell-ECM adhesion, preclinical studies have aimed to elucidate their function within the tumor microenvironment. Research has focused on their role in activating tumorigenic signal transducers like focal adhesion kinase (FAK) and integrin-linked kinase (ILK), altering gene expression to promote metastatic success, tumor angiogenesis and lymphangiogenesis, and tumor cell motility and survival (Garmy-Susini 2010; Hudson and Stack 2010). Murine models, when treated with integrin antagonists or inhibitors of FAK or ILK, showed success in reducing tumor growth, invasion, angiogenesis, and metastasis. Transgenic mice with deleted integrin genes have demonstrated similar results.

Overall, in vivo and in vitro experiments indicate therapeutic benefit of integrin antagonists, with low apparent toxicity and mild efficacy for a wide range of cancers, including melanoma, glioblastoma, breast, prostate, pancreatic, ovarian, and colorectal (Desgrosellier and Cheresh 2010). Currently, integrin blockade is being further studied for anti-angiogenic and antimetastatic properties in cancer cell lines and in animal models.

Clinical Summary

The integrin-ligand interaction has been inhibited in clinical trials in two primary ways: targeted antibodies and RGD peptide mimetics. Based on preclinical demonstration of significant anti-angiogenic and antitumor activity, blockers of β1 and αv integrin, which regulate critical tumor cell behaviors in proliferation and metastasis and are upregulated in malignant tumors, have been the primary focuses of clinical trials. Other classes of inhibitors, like non-peptide RGD mimetics and disintegrins, are still in preclinical development or Phase I testing (Danen 2013).

Overall, results in clinical testing of anti-integrins have yielded modest efficacy in a variety of cancers and potential with further testing. Use in cancer-specific therapeutics showed particular promise, especially in GBM and gliomas, but momentum has stalled since the latest trials yielded disappointing results. Integrin antagonists may serve a role as a last line of treatment in late-stage and metastasized patients for whom standard treatments had no effect. So far, clinical trials have not proven their reliability as an alternative cancer therapy.

Anticipated High-Impact Results

- The current consensus is that integrins play an important role in tumor proliferation, angiogenesis, invasion, and metastasis.
- While preclinical trials have demonstrated a role for integrin blockade in the inhibition of angiogenesis and metastasis of tumors, phases II and III trials, integrin blockade has met with little success.
- Success in limiting tumor growth and metastasis in vitro and in murine models provides hope that integrin inhibition may be a useful therapeutic target in cancer.

Cross-References

► VEGF

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Interferon Alpha

26

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Abstract

Interferons comprise a family of cytokines subclassified as types I-III based on their structural and functional properties. Named for their ability to "interfere" with viral replication, interferons perform a host of other functions including immune cell activation, augmentation of antigen presentation and upregulation of major histocompatibility complex molecules. Signaling through IFN-α/β receptor (IFNAR) and signal transducer and activator of transcription (STAT) complexes,

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type I interferons result in the transcription of a variety of proteins with broad anti-viral and anti-tumor effects. In this chapter we briefly delineate the interferon biology before reviewing the clinical application of interferons in cancer particularly in melanoma and renal cell carcinoma.

Keywords
IFN • JAK • STAT • Melanoma • RCC • Kaposi's sarcoma

Target

Nagano and Kojima suggested that the inhibition of viral growth in tissue previously inoculated with inactivated virus was secondary to a "viral inhibitory factor" in 1954 (Nagano and Kojima 1954). However, credit for the discovery of interferon largely goes to Isaacs and Lindenmann who observed that heat-inactivated influenza virus inhibited the growth of live virus and coined the term "interferon" in their seminal 1957 paper (Isaacs and Lindenmann 1957). In the decades that followed, interferons were shown to have broad effects upon cellular protein synthesis, antiviral state, and proliferation, and host immunomodulatory effects. However, the potential for interferon in cancer therapy was only realized as interferon became available from large-scale in vitro production, ultimately through recombinant DNA; initially Tan and colleagues superinduced the interferon gene in fibroblasts (1977) from which purified, biologically active interferon was produced (Berthold et al. 1978). Subsequently, the isolation and cloning of interferon genes coding for IFN-α, IFN-β, and IFN-γ in the 1980s, together with recombinant DNA technology, enabled production of recombinant interferon in larger quantities required for clinical research purposes (Nagata et al. 1980). In this chapter, we will delineate the biology of IFN-α and its development as an active agent in multiple malignancies in both the adjuvant and advanced disease settings.

Biology of the Target

Interferons comprise a large family of structurally related molecules with diverse effects. Interferons are subclassified as types I and II according to their structural and functional properties. Type II IFNs (IFN-γ in humans) are released by Th1 cells. Signaling via the IFN-γ receptor (IFN-γR), IFN-γ recruits leucocytes to infected areas resulting in inflammation, stimulates macrophages to phagocytose engulfed bacteria, and upregulates the Th2 response.

Type I IFNs are structurally similar molecules including IFN-α, IFN-β, IFN-ω, IFN-ε, and IFN-k of which IFN-α, IFN-β, and IFN-ω are the most important ones in humans. Type I IFNs all signal via the IFN-α receptor (IFN-αR). Downstream signaling

effects are mediated through the JAK/STAT pathway and IFN regulatory factor (IRF)-9 which on binding with STAT1-STAT2 complex migrates to the nucleus and regulates expression of RNA-dependent protein kinases that regulate cell growth and differentiation and mediate the antiviral state (Kawamoto et al. 2004). Although related molecules like limitin signal through IFN-αR, they utilize alternative signal transduction pathways which account for their differential biological profile compared to IFN-α. Type I IFNs are produced in large quantities chiefly by the plasmacytoid dendritic cell in response to infectious and other noxious stimuli and serve to link the innate and adaptive immune response to infection (Tough 2004).

Of the various type I IFNs, IFN-α2 is the best described with immunoregulatory, antiproliferative, differentiation-inducing, proapoptotic, and antiangiogenic properties that have been documented in the setting of a variety of malignancies (Kirkwood et al. 2002). Three sub-species including IFN-α2α (Roferon-A; Roche Pharmaceuticals), IFN-α2b (Intron A; Merck), and IFN-α2c (Boehringer Ingelheim) are available commercially.

The antitumor activity of IFN-α was first appreciated in several hematological malignancies (hairy cell leukemia, multiple myeloma, and non-Hodgkin's lymphoma) and an expanding array of solid tumors (including melanoma, renal cell carcinoma, and Kaposi's sarcoma). Below, we review the development of IFN-α in these diseases Atzpodien et al. (2001); Groopman et al. (1984); Real et al. (1986).

Target Assessment

IFN-α's mechanism of action is thought to be immunomodulatory rather than directly cytotoxic or antiangiogenic. Tumors suppress native antitumor immunity though multiple mechanisms including constitutive activation of STAT3; elaboration of VEGF, IL-10, and TGFβ; recruitment of regulatory T (Treg) cells within the tumor microenvironment (TME); and expression of negative regulatory T-cell markers such as PD-L1 (Davar et al. 2012a). IFN-α also plays a critical role in T-cell recruitment and dendritic cell-mediated T-cell priming – an effect inhibited by tumor growth which may be reversed by IFN administration (Davar et al. 2012a).

The effects of IFN upon the tumor and TME can be quantified by looking for evidence of immune upregulation and/or increased effector T-cell function:

- Assessment of CD8+ T cell-infiltrate using quantitative immunohistochemistry – increased levels suggestive of immune upregulation
- HLA class I and II expression using quantitative immunohistochemistry – increased levels suggestive of immune upregulation
- CD8+ and CD4+ TIL function using CD107a degranulation and flow-based killing assays – increased levels suggestive of enhanced cytolytic function and tumor reactivity

Role of IFN-α in Cancer

IFN-α2b has well-established indications both in the adjuvant (melanoma) and advanced (RCC) disease settings. Adjuvant immunotherapy with high-dose IFN-α2B (HDI) improves relapse-free survival (RFS) and overall survival (OS) in 30% of patients with high-risk melanoma. European trials utilizing pegylated IFN-α in node-positive melanoma have demonstrated improved RFS with a survival benefit apparently restricted to a subgroup of patients with ulcerated disease.

In advanced renal cell carcinoma (RCC), the use of IFN-α is associated with a 15% objective response rate in select individuals – typically patients with low-volume pulmonary and/or soft tissue metastases with excellent performance status. These data support the ongoing use of IFN-α in these settings and are elaborated below.

High Level Overview

Prognostic and Predictive Factors

The identification of prognostic and predictive biomarkers in melanoma and RCC would enable us to individualize therapy and thereby improve the cost-benefit ratio, while minimizing toxicity. This is an area of active investigation in multiple disease settings (adjuvant, neo-adjuvant, and metastatic) in both tumor types.

Motzer and colleagues at Memorial Sloan-Kettering Cancer Center retrospectively analyzed 463 patients with advanced RCC who received IFN-α as first-line systemic therapy in six prospective clinical trials and identified five pretreatment clinical variables (time from initial RCC diagnosis to start of IFN-α therapy of less than 1 year, low Karnofsky performance status, serum lactate dehydrogenase greater than 1.5 times the upper limit of normal (ULN), low serum hemoglobin, and high corrected serum calcium), and used these to subclassify patients into three risk substrata: favorable (0 risk factors); intermediate (1–2 risk factors); and poor (≥3 risk factors) (Motzer et al. 2002). Patients in the favorable risk category had improved median survival (30 vs. 14 and 5 months for intermediate and poor risk patients), 1 year survival (83% vs. 58% and 20%), 2 year survival (55% vs. 31% and 6%), and 3 year survival (45% vs. 17% and 2%). The prognostic value of these factors was validated in 353 patients with metastatic RCC enrolled on immunotherapeutic trials at the Cleveland Clinic, where two additional factors that had independent prognostic value were identified: prior radiotherapy and the presence of hepatic, lung, and retroperitoneal nodal metastases (Mekhail et al. 2005).

In patients with melanoma, analysis of data obtained from patients compiled in the American Joint Committee on Cancer (AJCC) Melanoma Staging Database revealed six independent prognostic factors that were included in the 7th edition of the AJCC staging manual for melanoma revised in 2009 (Balch et al. 2009). These factors – increasing tumor thickness, presence of ulceration, high mitotic rate, greater lymph node burden, systemic disease (**number** of metastatic sites and **sites** of distant metastases), and LDH – independently predict outcomes. Several

prediction tools have been developed that use these factors to predict the risk of nodal metastases (Memorial Sloan Kettering Sentinel Node Metastasis prediction tool) and the 5- or 10-year survival (AJCC Individualized Melanoma Patient Outcome Prediction Tool) with a high degree of accuracy.

The role of ulceration as a factor that may predict response to adjuvant therapy with IFN is less clear. Aggregate data from European studies (EORTC 18952 and 18991) and the Sunbelt melanoma trial suggest that ulceration predicts response (McMasters et al. 2008; Atzpodien et al. 2004). US intergroup trials have required rigorous central pathology review beyond institutional pathology assessment for enrolled subjects do not support this conclusion. Hopefully, the new planned EORTC 18081 will rigorously evaluate this hypothesis and illuminate the role of ulceration as a predictor of therapeutic outcomes in intermediate/high risk melanoma patients treated with adjuvant pegylated IFN-α.

Autoimmune leukoderma (vitiligo like depigmentation) and thyroid dysfunction have been observed among patients treated with immunotherapy ranging from IL-2 and IFN-α to the more recent immunotherapies with immune checkpoint inhibitors like anti-CTLA4 and anti-PD1. Observations of increased frequency of antitumor response among patients who develop autoimmune phenomena following IL-2 therapy prompted the evaluation of autoimmunity as a potential surrogate marker of IFN-α response in adjuvant trials in the 1990s. Analyses of US Intergroup trials E2696 and E1694 suggested that HDI-induced autoimmunity correlated with improved survival (Tarhini et al. 2009). The strongest data in support of this linkage has come from the Hellenic Oncology Group trial 13A/98, where prospective clinical and serological data were gathered that support the conclusion that the development of autoimmunity is associated with improvements in RFS/OS (Gogas et al. 2006). Other candidate biomarkers including methylthioadenosine phosphorylase (MTAP) protein expression, YKL-40 (a mammalian chitinase-like protein) levels, S100B, melanoma-inhibiting activity (MIA), and tumor-associated antigen 90 immune complex (TA90IC) have also been retrospectively associated with outcomes and are reviewed in detail elsewhere (Davar et al. 2012a). However, prospective validation of these markers as predictors of response to adjuvant IFN-α is lacking.

Therapeutics

Role of IFN-α in Melanoma

Early trials of IFN-α in melanoma were conducted in patients with advanced disease and demonstrated modest clinical benefit. A series of phase I/II studies then tested a variety of doses and schedules and generally observed RR of 13–24%. Responses seen in advanced disease were accompanied by the observation of prolonged remissions in patients who had failed prior therapy and occasional late responses that have greater meaning in the context of similar delayed patterns of response with more recently approved immunotherapies such as anti-CTLA4 blocking antibodies – and the overall response rates of 16% and median durations of response that were

4–6 months did not lead to submission for regulatory approval in advanced inoperable melanoma (Creagan et al. 1987). Retrospective analyses suggest that patients with a lower tumor burden had a greater likelihood of response and spurred the evaluation of this agent in patients with micrometastatic disease at high risk of recurrence following surgical resection – “adjuvant therapy.”

The initial adjuvant trials used a high dose (~20 MU/m^2 thrice weekly) similar to that used in the early trials of advanced disease. ECOG’s high-dose regimen (HDI) (I.V. 20 MU/m^2 5 days a week for 4 weeks followed by S.C. 10 MU/m^2 3 days a week for 48 weeks) was tested against placebo in a randomized phase III trial (E1684) utilizing patients with deep primary tumors (T4N0M0) and/or regional lymph node metastases (TxN1-3 M0). In the original 1996 publication, the authors reported statistically significant improvements in both RFS and OS at a median follow-up of 6.9 years (Kirkwood et al. 1996). However, updated survival statistics published in 2004 at a median follow-up of 12.6 years reported a diminution of benefit – particularly on OS likely secondary to alternative causes of death in an increasingly older patient population (Kirkwood et al. 2004).

The significant attendant toxicity associated with the year-long HDI regimen (67% Grade III and 9% Grade IV toxicity in E1684) prompted consideration of lower dose regimens of IFN-α for this same indication. These regimens considered alternative end-points such as RFS and distant metastasis-free survival (DMFS) beyond the OS endpoint addressed in ECOG HDI trials. Regimens tested include the very-low-dose (1 MU S.C. every other day) tested in EORTC 18871 (stage IIB/III); low-dose (3 MU S.C. thrice weekly) evaluated in both stage II (T2-4N0M0) patients in Austrian and French Melanoma Cooperative Group trials and stage III patients in the WHO Melanoma Program Trial 16, UKCCCR AIM-High, Scottish and the 2010 German Dermatologic Cooperative Oncology Group (DeCOG) studies; and the intermediate-dose regimen tested with IFNα-2b in EORTC 18952 and Nordic IFN and pegylated IFN-α in EORTC 18991 (Davar et al. 2012a).

Although most of these trials have suggested improvement of RFS, none of the individual trials testing lower-dose regimens demonstrated any OS benefit. The 2008 DeCOG study demonstrated a survival benefit for the low-dose IFN-α arm but as the comparators included an IFN/dacarbazine arm in addition to placebo, the study was ipso facto, not capable of evaluating the survival benefit of low-dose IFN. These results are summarized in Table 1 (Davar et al. 2012a; Kirkwood et al. 1996).

The question whether the RFS benefit observed in E1684 was derived from the month-long intravenous induction phase of therapy or the full year of treatment has been raised from the pattern of benefit observed over time and the early separation of the relapse-free survival curves following therapy. This question has been evaluated in two phase III studies (HeCOG 13A/98 and ECOG E1697) and a phase II study. A trial of the Hellenic Oncology Group (HeCOG 13A/98) tested a modified dosage of IFN-α given as induction for 1 month or for 1 year, with the maintenance phase for 11 months, and showed no difference in either RFS or OS at a median of 61 months follow-up between the 1 month and 1-year treatment arms (Pectasides et al. 2009). ECOG and US Intergroup trial E1697 prospectively assessed whether intermediate-risk and high-risk melanoma patients (≥T3N1a-2aM0) benefit from just the

Table 1 Phase III studies of adjuvant IFN-α in high-risk resected melanoma

Study reference	Number of patients eligible for analysis	TNM stage	Therapy and IFN subspecies	Dose and schedule – treatment arm	Median follow-up at time of reporting (years)	DFS/RFS	OS	% node positive
High dose								
NCCTG 83–7052 (Davar et al. 2012a)	262	II–III (T2-4N0M0/ TanyN + M0)	IFNα-2a versus observation	I.M. 20 MU/m^2 thrice weekly for 4 months	6.1	NS	NS	61
ECOG E1684 (Kirkwood et al. 1996)	287	II–III (T4N0M0/ TanyN + M0)	IFNα-2b versus observation	I.V. 20 MU/m^2 5 days a week for 4 weeks → then → SC 10 MU/m^2 3 days a week for 48 weeks	12.6	HR: 1.38 (HDI vs. obs) (S)	HR: 1.22 (HDI vs. obs) (S at 6.9 years but NS at 12.6 years)	89
ECOG E1690 (Davar et al. 2012a)	642	II–III (T4N0M0/ TanyN + M0)	IFNα-2b – high dose versus low dose versus observation	High Dose: *Induction* – I.V. 20 MU/m^2 5 days a week for 4 weeks *Maintenance* – S.C. 10 MU/m^2 3 days a week for 48 weeks Low Dose: S.C. 3 MU/m^2 2 days a week for 2 years	4.3	HR: 1.28 (HDI vs. obs) (S) 1.19 (LDI vs. obs) (NS) RFS: 44% (HDI) versus 40% (LDI) versus 35% (obs)	HR: 1.0 (HDI vs. obs) (NS) 1.04 (LDI vs. obs) (NS) OS: 52% (HDI) versus 53% (LDI) versus 55% (obs)	74
ECOG E1694 (Davar et al. 2012a)	774	II–III (T4N0M0/ TanyN + M0)	IFNα-2b versus GMK vaccine	*Induction* – I.V. 20 MU/m^2 5 days a week for 4 weeks *Maintenance* – S.C. 10	2.1	HR: 1.49 (HDI vs. GMK) (S) RFS:	HR: 1.38 (HDI vs. GMK) (S) OS:	77

(*continued*)

Table 1 (continued)

Study reference	Number of patients eligible for analysis	TNM stage	Therapy and IFN subspecies	Dose and schedule – treatment arm	Median follow-up at time of reporting (years)	DFS/RFS	OS	% node positive
				MU/m^2 3 days a week for 48 weeks		25% (HDI) versus 39% (GMK)	78% (HDI) versus 73% (GMK)	
ECOG E2696 (Davar et al. 2012a)	107	II–IV	GMK vaccination with concurrent HDI (Arm A) versus GMK vaccination with HDI beginning D28 (Arm B) versus GMK vaccination alone (Arm C)	HDI: *Induction* – I.V. 20 MU/m^2 5 days a week for 4 weeks *Maintenance* – S.C. 10 MU/m^2 3 days a week for 48 weeks GMK vaccination: GM2-KLH/QS-21 on D1, 8, 15, 22 then weeks 12, 24, 36	2.4	HR: 1.75 (C vs. A) (S) 1.96 (C vs. B) (S) RFS: Not reached (A) versus 30.72 months (B) versus 14.85 months (C)	HR: not reported OS: Not reached (A, B or C)	Not Available
Italian Melanoma Intergroup (Davar et al. 2012a)	330	III (TanyN1-3 M0)	Intensified IFNα-2b (IHDI) every other month versus IFNα-2b for 1 year	IHDI: I.V. 20 MU/m^2 5 days a week for 4 weeks every other month for 4 cycles Standard HDI: *Induction* – I.V. 20 MU/m^2 5 days a week for 4 weeks	5.0	Median RFS: 47.9 months (IHDI) versus 35.6 months (HDI) (NS) 5 year RFS: 45.8% (IHDI) versus	Median OS: 88.7 months (IHDI) versus 82.6 months (HDI) (NS) 5 year OS: 60.1% (IHDI) versus	100

				Maintenance – S.C. 10 MU/m^2 3 days a week for 48 weeks		44.3% (HDI) (NS)	52.7% (HDI) (NS)	
Intermediate dose								
EORTC 18952 (Davar et al. 2012a)	1388	II–III (T4N0M0 or TanyN + M0)	IFNα-2b for 1 year versus 2 years versus observation	*Induction* – I.V. 10MU 5 days a week for 4 weeks *Maintenance* – S.C. 10MU 3 days a week for 1 year *OR* S.C. 5MU 3 days a week for 2 years	4.7	DMFI: HR: 0.93 (13 month vs. obs) (NS) 0.83 (25 month versus obs) (S)	DMFS: HR: 0.95 (13 month vs. obs) (NS) 0.85 (25 month vs. obs) (NS)	74
EORTC 18991 (Davar et al. 2012a)	1256	III (TanyN + M0)	PEG IFNα-2b for 5 years versus observation	*Induction* – S.C. 6 μg/kg/week for 8 weeks *Maintenance* – S.C. 3 μg/kg/week for 5 years	7.6	34.8 months (IFN) versus 25.6 months (obs); S	47.8% (IFN) versus 46.4% (obs); NS	100
Nordic IFN (Davar et al. 2012a)	855	IIB-IIIB (T4N0M0 or TanyN1-2 M0)	IFNα-2b for 1 year versus 2 years versus observation	Observation (A) versus S.C. 10MU 5 days a week for 4 weeks then S.C. 10MU 3 days a week for 1 year (B) versus S.C. 10MU 5 days a week for 4 weeks then S.C. 10MU 3 days a week for 2 years (C)	6.0	23.2 months (A) versus 37.8 months (B) versus 28.6 months (C) IFN versus obs & IFN 1 year versus obs (S); IFN 2 years versus obs (NS)	56.1 months (A) versus 72.1 months (B) versus 64.3 months (C) (NS)	81

(*continued*)

Table 1 (continued)

Study reference	Number of patients eligible for analysis	TNM stage	Therapy and IFN subspecies	Dose and schedule – treatment arm	Median follow-up at time of reporting (years)	DFS/RFS	OS	% node positive
Low dose								
Austrian Melanoma Cooperative Group (AMCG) (Davar et al. 2012a)	311	II (T2-4N0M0)	IFNα-2a versus observation	S.C. 3MU 7 days a week for 3 weeks → then → S.C. 3MU 3 days a week for 1 year	3.4	RFS/DMFS not reported Rate of relapse: (24.0% LDI vs. 36.3% obs)	Not available	0
French Melanoma Cooperative Group (FCGM) (Davar et al. 2012a)	499	II (T2-4N0M0)	IFNα-2a versus observation	S.C. 3MU 3 days a week for 18 months	>3	HR: 0.74 (LDI vs. obs) (S)	HR: 0.70 (LDI vs. obs) (S)	0
WHO Melanoma Program Trial 16 (Davar et al. 2012a)	444	III (TanyN + M0)	IFNα-2a versus observation	S.C. 3MU 3 days a week for 36 months	7.3	NS	NS	100

Scottish Melanoma Cooperative Group (Davar et al. 2012a)	96	II–III (T3-4N0M0/ TanyN + M0)	IFNα-2a versus observation	S.C. 3MU 3 days a week for 6 months	>6	NS	NS	Not Available
UKCCCR/ AIM HIGH (Davar et al. 2012a)	674	II–III (T3-4N0M0/ TanyN + M0)	IFNα-2a versus observation	S.C. 3MU 3 days a week for 24 months	3.1	NS	NS	Not available
DeCOG (Davar et al. 2012a)	840	III (T3anyN + M0)	IFNα-2a	S.C. 3MU 3 days a week for 18 months (A) versus 5 years (B)	4.3	5 years DMFS 81.9% (A) versus 79.7% (B) (NS)	5 years OS 85.9%(A) versus 84.9% (B) (NS)	Not available
DeCOG (Davar et al. 2012a)	444	III (TanyN + M0)	IFNα-2a	S.C. 3MU 3 days a week for 24 months (A) versus S.C. 3MU 3 days a week for 24 months + DTIC 850 mg/m^2 every 4–8 weeks for 24 months (B) versus observation (C)	3.9	HR: 0.69 (A) versus 1.01 (B) versus 1.0 (C)	HR: 0.62 (A) versus 0.96 (B) versus 1.0 (C)	100%
Very low dose								
EORTC 18871/DKG 80–1 (Davar et al. 2012a)	728	II–III (T3-4N0M0/ TanyN + M0)	IFNα-2b versus IFN-γ versus ISCADOR M® versus observation	IFN-α2b: S.C. 1MU every other day for 12 months IFN-γ: S.C. 0.2 mg every other day for 12 months	8.2	NS	NS	58

Key : *NS* not significant, *S* significant, *HR* hazard ratio, *DFS* disease-free survival, *OS* overall survival

induction phase of the HDI regimen, compared to observation. Patients were enrolled to receive standard HDI induction alone versus observation. After enrollment of 1150 of a planned 1420 patients, the study was closed for futility: neither RFS nor OS were likely to be affected (5-year survival rate 0.82 for IFN and 0.85 for observation) (Agarwala et al. 1697). A phase II study conducted by the Oxford University Hospitals under the aegis of the National Health Service Trust posed the same question regarding the role of the induction phase of the HDI regimen (Payne et al. 2014). Unlike in E1697, patients were randomized to either induction HDI alone or standard HDI for 1 year. Unlike HeCOG 13A/98, Oxford study investigators omitted patients with stage IIIA disease. In studying 194 patients with stage IIB/C and IIIB/C disease treated between 2003 and 2009, at a median of 39.5 months follow-up, investigators observed that both RFS and OS were improved in the standard HDI group compared to the induction alone group. The results of the Oxford and ECOG E1697 are concordant – underscoring the importance of 12 months of therapy. In considering the discordance between former two studies and HeCOG 13A/98, it is important to note that HeCOG 13A/98 was underpowered to detect RFS/OS differences of less than 15% that would have been relevant. Although the Oxford phase II study excluded stage IIIA patients while these were included in HeCOG 13A/98, it is unlikely that these patients accounted for this difference given that: these patients only accounted for 11% of HeCOG 13A/98 enrollment and substantial improvements in RFS/OS observed with pegylated IFN-α2b in stage IIIA disease in EORTC 18991.

The Italian Melanoma Intergroup tested a derivative hypothesis that IV induction therapy given in repetitive monthly cycles may be superior to conventional HDI given for 1 year. This randomized phase III study utilized a regimen of induction dose HDI given every other month for four cycles (intensified HDI, IHDI) compared against the year-long HDI regimen (Davar et al. 2012a). DeCOG evaluated the same hypothesis with a slightly less intensive regimen that utilized three cycles interrupted by 12-week breaks vis a vis the Italian Melanoma Intergroup's four cycles (Mohr et al. 2015). The Italian Melanoma Intergroup reported that RFS/OS were nonsignificantly improved compared to HDI at a median follow-up of 60 months. Conversely, DeCOG authors reported no significant differences in RFS/OS between IHDI and HDI arms – with RFS/OS rates actually being numerically greater in HDI arm. Both shorter schedules merit attention for the improved cost/benefit profile and quality of life benefits especially considering the similar toxicity profiles between HDI and IHDI arms. However, there are differences in maximum cumulative IFN-α2b dose between these two regimens and standard HDI: 1840 MIU/m^2 (HDI) vs. 1600 MIU/m^2 (Italian Melanoma Intergroup IHDI) vs. 1200 MIU/m^2 (DeCOG IHDI). Factoring in discontinuation rates (greater in IHDI arms), it is apparent that overall drug exposure is lower in IHDI regimens. Thus, any enthusiasm for truncated HDI regimens must be tempered by the limited survival data and inconsistent results across both Italian Melanoma Intergroup and DeCOG studies.

Prior adjuvant studies have almost exclusively focused on cutaneous melanoma given the relatively small numbers of noncutaneous melanoma seen at most US, European, and Australian centers. A Chinese group studied adjuvant HDI (compared

to observation or cisplatin/temozolomide) in patients with stage II/III resected mucosal melanoma (Lian et al. 2013). This phase II study was primarily powered to evaluate RFS in patients treated with cisplatin/temozolomide or HDI against observation and it was not powered for three-way comparisons. Although both cisplatin/temozolomide and HDI improved RFS and OS compared to observation, the RFS and OS benefits for cisplatin/temozolomide were greater than that for HDI. Pegylated IFNα-2b (Peg IFN) was developed with the hope that this formulation would allow therapeutic dose levels to be reached with once-weekly administration. Initial approval for PegIFN as treatment of hepatitis C has demonstrated that this formulation has antiviral efficacy. Given the compliance issues with HDI in induction therapy, there was interest in evaluating pegylated IFN-α as an alternative that might be therapeutically superior to HDI. The European Organization for Research and Treatment of Cancer (EORTC) trial 18991 examined peg IFN given subcutaneously at initial doses of 6 μg/kg/week for 8 weeks (induction) followed by 3 μg/kg/week for 5 years (maintenance) in 1256 stage III (TanyN + M0) patients. A small RFS increment with this treatment (4.5% absolute difference in the estimated 7-year RFS rate) and no significant differences in either OS or DMFS were observed at 7.6 years median follow-up (Davar et al. 2012a). Toxicity profiles with peg IFN were similar to HDI with an incidence of grade 3/4 fatigue (24%) and depression (10%) in EORTC 18991, compared to HDI. Subgroup analyses suggested that patients with ulcerated primaries and microscopic nodal metastases benefited disproportionately with improvements in OS and DMFS in addition to RFS. Although ulceration has long been recognized as an adverse prognostic factor in melanoma, its impact on the success of adjuvant IFN treatment is unclear, with some data suggesting that this is a predictor of treatment benefit with low- and intermediate-dose regimens including the EORTC 18991 Peg IFN regimen, while data from ECOG and US Intergroup trials of HDI do not reveal an effect (Eggermont et al. 2012). EORTC 18081 is a current trial that is prospectively testing this hypothesis in a randomized phase III trial comparing adjuvant peg IFN for 2 (rather than 5) years in patients with ulcerated primary melanoma (T2b-4bN0M0). At this time, pegylated IFN-α (Sylatron™, Merck Corporation) has received US Food and Drug Administration (US FDA) approval for adjuvant therapy of stage III melanoma with either microscopic or macroscopic nodal involvement following definitive surgical resection including complete lymphadenectomy.

Multiple retrospective analyses have confirmed the benefit of adjuvant IFN-α including two meta-analyses (Wheatley et al. 2003, 2007), a systematic review (Mocellin et al. 2010), and a pooled data analysis (Lens and Dawes 2002). Taken in aggregate, the evidence suggests that adjuvant HDI reduces relapse risk and improves RFS reliably by 18–30 %, with a smaller impact on OS. The pivotal trial E1684 and the subsequent largest trial of HDI compared against vaccine GMK showed improved mortality out to 10 years. Based on E1697 and Oxford phase II study, it is clear that the RFS/OS benefits of HDI are duration dependent with no benefit to shorter courses of therapy. These studies were done in an era prior to mutation testing being the norm and hence the differential effect of either HDI or peg IFN in mutationally defined subsets of melanoma has not been assessed in any

prospective study – although data from a Chinese phase II study of HDI in *BRAF* and *NRAS* mutant melanoma suggested that HDI provided substantial benefit in *BRAF* (but not *NRAS*) mutated patients (Wang et al. 2015). The previously referenced Chinese phase II study in mucosal melanoma supports the use of HDI in this high-risk cohort as well (Lian et al. 2013).

The impact of peg IFN appears in relation to RFS only, however, without any suggestion of an impact upon OS. Improvements have been noted in meta-analysis of individual patient data that suggested a differential impact that is greater in patients with ulcerated primary melanoma; the recent EORTC trial 18991 of peg IFN shows a differential impact in node-positive patients with microscopic disease and ulcerated primary melanoma. Unlike HDI, peg IFN has not demonstrated benefit in patients with larger burden palpable or gross nodal N2/3 disease whose risk of recurrence/death is substantively greater than for those with microscopic N1 disease. Peg IFN has therefore been suggested for patients with N1 disease who are either unwilling or unable to pursue the HDI regimen.

Role of IFN-α in RCC: Monotherapy in Advanced Disease

Similar to melanoma, biological therapy was pursued in renal cell carcinoma (RCC) given the observation of occasional spontaneous remissions, the documentation of tumor-associated antigens, lack of active chemotherapeutic options, and the empiric therapeutic successes with IL-2. Early studies focused on single-agent IFN-α at dosages between 8 and 18 MU/m^2 dose thrice weekly given by subcutaneous injection for advanced disease (Minasian et al. 1993). Although response rates were low (~10%), a small proportion of patients experienced durable remissions with long-term survival that prompted further trials. Below, we detail the development of IFN-α given alone or as part of a combination in metastatic RCC. The studies alluded to below are summarized in Table 2 (Davar et al. 2012b).

A 2005 Cochrane Database systemic review analyzed 644 patients in four pooled trials of IFN-α compared to a variety of nonimmunotherapy controls. Authors reported that IFN-α significantly reduced 1-year mortality (pooled overall HR of death of 0.74) and improved median OS by 3.8 months (Coppin et al. 2005). Although the controls used were dissimilar – medroxyprogesterone in three studies and vinblastine in 1 – the pooled outcome results presented compelling evidence for the superiority of IFN-α as a single-agent in advanced disease and led to the adoption of IFN-α as the reference control arm of subsequent trials.

Role of IFN-α in RCC: Combination Therapy in Advanced Disease

Subsequent efforts centered on combining IFN-α with other agents including immunomodulators (IL-2), chemotherapy (5-fluorouracil, vinblastine, cis-retinoic acid), targeted agents (sunitinib and sorafenib), and monoclonal antibodies (bevacizumab) to improve the therapeutic index with minimal additional toxicity.

Table 2 Phase III studies of IFN-α singly or in combination in advanced renal cell cancer

Study reference	Number of patients eligible for analysis	Design	Interventions Dose and schedule	Response rate	DFS/PFS/OS	MSKCC risk; histology; % postnephrectomy
Negrier et al. (Davar et al. 2012b)	425	Multicenter, phase III RCT	(1) HD IL-2 per protocol (2) SC IFN-α 18×10^6 IU/m^2 3 days/week × 10 weeks (induction) followed by 13 weeks (maintenance) (3) HD IL-2 per protocol with SC IFN-α 6×10^6 IU/m^2 during each IL-2 cycle	DCR: 10 weeks: 6.5% (group 1) versus 7.5% (group 2) versus 18.6% (group 3) 25 weeks: 2.9% (group 1) versus 6.1% (group 2) versus 13.6% (group 3)	Event-free Survival rates at 1 year: 15% (group 1) versus 12% (group 2) versus 20% (group 3)	Postnephrectomy 92–94%
McDermott et al. (Davar et al. 2012b)	193	Multicenter phase III RCT; stratified for site (liver/bone Y/N), PS 0 vs 1, nephrectomy status	(1) IL-2 5–15 MU/m^2/day s.c. + IFN-α2b 5 MU/m^2 thrice weekly (2) IL-2 1.8 MU/kg/day i.v.	HD IL-2 (22/95 = 23%) versus LD IL-2 + IFN (9/91 = 10%)	Not reported	Not reported
Negrier et al. (Davar et al. 2012b)	492	Multicenter, phase III RCT	(1) MPA# 200 mg daily (2) SC IFN-α 9×10^6 IU/m^2 thrice weekly for 10 weeks (3) SC intermediate dose IL-2 (5-day continuous infusion at 9×10^6 IU/m^2	RR (CR + PR): 12 weeks: 2% (group 1) versus 4% (group 2) versus 4% (group 3) versus 10% (group 4)	OS (months): 14.9 (group 1) versus 15.2 (group 2) versus 15.3 (group 3) versus 16.8 (group 4)	Clear cell 79%; postnephrectomy 96%

(*continued*)

Table 2 (continued)

Study reference	Number of patients eligible for analysis	Design	Interventions Dose and schedule	Response rate	DFS/PFS/OS	MSKCC risk; histology; % postnephrectomy
			daily) (4) –(2) + (3)	6 months: 2% (group 1) versus 8% (group 2) versus 4% (group 3) versus 7% (group 4)	PFS (months): 3.0 (group 1) versus 3.4 (group 2) versus 3.4 (group 3) versus 3.8 (group 4)	
AVOREN (Davar et al. 2012b)	649	Multicenter, phase III, placebo-controlled RCT	(1) SC IFN-α2b 9×106 IU/m^2 thrice weekly + Bevacizumab 10 mg/kg every 2 weeks (2) SC IFN-α2b 9×106 IU/m^2 thrice weekly + placebo	OR (CR + PR): Bev + IFN-α 31% versus IFN-α alone 13%	Median OS (months) in ITT (updated 2010): Bev + IFN-α 23.3 versus IFN-α alone 21.3 By MSKCC risk category: *favorable:* Bev + IFN-α 35.1 versus IFN-α alone 37.2 *intermediate:* Bev + IFN-α 22.6 versus IFN-α alone	MSKCC risk – intermediate (56%) and poor (9%); predominantly clear cell; postnephrectomy 99%

					19.3 *poor:* Bev + IFN-α 6.0 versus IFN-α alone 5.1	
CALGB 90–206 (Davar et al. 2012b)	732	Multicenter, phase III, open label RCT	(1) SC IFN-α2b 9×10^6 IU/m^2 thrice weekly + Bevacizumab 10 mg/kg every 2 weeks (2) SC IFN-α2b 9×10^6 IU/m^2 thrice weekly alone	OR (CR + PR) (2008): Bev + IFN-α 25.5% versus IFN-α alone 13.1%	OS (months) (updated 2010): Median Bev + IFN-α 18.3 versus IFN-α alone 17.4 By MSKCC risk category: *favorable:* Bev + IFN-α 32.5 versus IFN-α alone 33.5 *intermediate:* Bev + IFN-α 17.7 versus IFN-α alone 16.1 *poor:* Bev + IFN-α 6.6 versus IFN-α alone 5.7	MSKCC risk – intermediate (64%) and poor (10%); predominantly clear cell; prior nephrectomy 85%
MRC RE04/ EORTC GU 30012 (Davar et al. 2012b)	1006	Multicenter, randomized, phase III	(1) SC IFN-α 10×10^6 IU/m^2 3 days/ week	BORR at 3 years: 16% (1) versus 23% (2)	At 1 year: 67% (IFN-α) versus 67% (combination)	MSKCC risk group (58–60% medium, 17–19% high); prior nephrectomy 89–90%

(*continued*)

Table 2 (continued)

Study reference	Number of patients eligible for analysis	Design	Interventions Dose and schedule	Response rate	DFS/PFS/OS	MSKCC risk; histology; % postnephrectomy
			(2) combination therapy with IFN-α, IL-2, and 5-FU		At 3 years: PFS – 5.5 months (IFN-α) versus 5.3 months (combination) OS – 30% (IFN-α) versus 26% (combination)	
INTORACT (Davar et al. 2012b)	791	Multicenter, phase IIIb, open label trial	(1) Bevacizumab 10 mg/kg every 2 weeks + Temsirolimus 25 mg weekly (2) Bevacizumab 10 mg/kg every 2 weeks + SC IFN-α2b 9×10^6 IU/m^2 thrice weekly	ORR: 27% (1) versus 28% (2)	Median OS (months): Bev + Tem 25.8 versus Bev + IFN-α 25.5 HR Median PFS (months): Bev + Tem versus 9.1 Bev + IFN-α 9.3	MSKCC risk – intermediate (66%); predominantly clear cell; prior nephrectomy 85%

Given IL-2's ability to augment the cytotoxic effects of effector T-cells and IFN-α's ability to increase tumor recognition through upregulation of HLA class I and tumor associated antigen expression, the hope was advanced that the combination may be synergistic. IFN-α was combined with IL-2 in a variety of schedules including low-dose SC injections (Davar et al. 2012b; Atzpodien et al. 1995, 2004; Dutcher et al. 1997; Vogelzang et al. 1993; van Herpen et al. 2000; Atkins et al. 2001; Rathmell et al. 2004), intermediate-dose continuous IV infusions (Neidhart et al. 1991), and high-dose intravenous bolus administration (Rosenberg et al. 1989; Atkins et al. 1993; Sznol et al. 1990; Bergmann et al. 1993; Spencer et al. 1992; Budd et al. 1992). Although phase II data suggested that the IL-2/IFN-α combination doubled response rates, phase III data did not show any increase in either progression-free survival (PFS) or OS over the single agents (Davar et al. 2012b). Rates of grade 3–4 toxicity were greater than for either agent alone.

Combinations of IFN-α and conventional chemotherapy have been evaluated including IFN-α/5-fluorouracil (Falcone et al. 1993; Igarashi et al. 1999; Elias et al. 1996) and IFN/vinblastine (Neidhart et al. 1991; Fossa et al. 1992). While initial reports from phase II trials suggested an increase in activity with the combinations, this benefit has not been confirmed in phase III trials. The IFN-α/5-fluorouracil combination appears to be the most active with response rates ranging from 12% to 39% in two studies (van Herpen et al. 2000; Neidhart et al. 1991). However, when evaluated in the phase III setting against IFN-α alone, although combination therapy had greater response rates (23% vs. 16%), no overall survival advantage (3 year survival: 30% with IFN-α and 26% with combination) was noted with significantly greater Grade 3/4 toxicity in the combination therapy arm (53% vs. 36%) (Davar et al. 2012b).

Adding combination chemotherapy to the IL-2/IFN-α combination appears to be active (Spencer et al. 1992). An Italian group reported 29% partial response (PR) and 37% stable disease (SD) out of 51 patients who received IL-2/IFN-α in combination with bevacizumab and 5-fluorouracil/gemcitabine (BIC combination) at ASCO 2010 (Passalacqua et al. 2010). Notably, the 11% PR rate and 55% DCR in MSKCC high-risk group is encouraging and merits further evaluation in the phase III setting.

The success of the tyrosine kinase inhibitors sorafenib and sunitinib in treating metastatic RCC generated interest in IFN-α/TKI combinations. Early phase trials involved sunitinib and sorafenib with IFN-α administered at doses between 3 and 9 MU subcutaneously three times a week. The IFN-α/sunitinib combination had a response rate of 12%, similar to single-agent IFN-α but significantly less than the ~30% response rate observed with sunitinib alone (Motzer et al. 2009). Moreover, the combination had significant toxicity such that further evaluation has not been pursued.

The combination of IFN-α and sorafenib has been studied more extensively: 2 phase II studies have reported response rates of 19–33% with sorafenib (400 mg twice daily) and IFN-α2b (10 MU subcutaneously three times a week) (Gollob et al. 2007; Ryan et al. 2007). RAPSODY was a randomized, open-label phase II study of sorafenib plus low-dose IFN-α administered in one of two dose schedules (9 and 3 MU subcutaneously three times a week) for patients with advanced renal

cell carcinoma (Bracarda et al. 2013). Overall response rate was 26% with a complete response rate of 6%. While the lower-dose arm had a greater number of responses (3 complete and 14 partial vs. 9 partial) and better median PFS (8.6 vs. 7.9 months) the high-dose arm was associated with stable disease in a larger fraction of patients. In the absence of evidence for an impact upon survival or unequivocal phase III trial data, it is unclear what benefit the combination confers over sorafenib alone given the toxicity and required dose reductions in a majority of patients.

The anti-VEGF-A antibody bevacizumab given in combination with cytotoxic chemotherapy has improved PFS/OS in several solid tumors (colorectal cancer, non-small cell lung cancer, glioblastoma multiforme). Following reports of single-agent activity against RCC, this agent was combined with IFN-α for treatment of metastatic RCC. Two large randomized phase III studies of IFN-α/bevacizumab combination versus IFN-α alone were performed – Avastin and Interferon in renal cancer (AVOREN) and the Cancer and Leukemia Group B trial (CALGB 90206) (Davar et al. 2012b). Dosages of IFN-α and bevacizumab were 9 MU thrice weekly and 10 mg/kg, respectively. Both trials demonstrated that the combination improved ORR (AVOREN, 31.4% vs. 12.8%; CALGB 90206, 25.5% vs. 13.1%) and PFS (AVOREN, 10.4 vs. 5.5 months; CALGB 90206, 8.4 vs. 4.9 months) vis a vis IFN-α alone. These results prompted regulatory approval of the combination of IFN-α/bevacizumab for first-line therapy of advanced RCC (predominant clear cell histology).

The mammalian target of rapamycin (mTOR) pathway is downstream of the phosphoinositide 3-kinase (PI3K) and Akt pathways that are regulated by the PTEN tumor suppressor gene. mTOR pathway inhibition may inhibit tumor progression at multiple levels. The IFN-α/bevacizumab combination was a control arm in three major clinical trials designed to evaluate the mTOR inhibitor temsirolimus in patients with predominantly clear cell tumors: INTORACT (investigation of Torisel and Avastin combination therapy), TORAVA (Torisel and Avastin), and RECORD-2 (renal cell cancer treatment with oral RAD001 given daily). Preliminary data from the INTORACT trial revealed similar median PFS and OS in both arms and the authors concluded that the temsirolimus/bevacizumab combination was not superior to IFN-α/bevacizumab (Négrier et al. 2011). TORAVA enrolled patients in a 2:1:1 ratio to temsirolimus/bevacizumab, sunitinib or IFN-α/bevacizumab. Both PFS and response rate were greater in the IFN-α/bevacizumab treated group than in either the sunitinib or temsirolimus/bevacizumab treatment groups, with the latter associated with significant toxicity (36% incidence of Grade III/IV leading to 43% dropout) (Négrier et al. 2011). Survival data is not yet available.

In conclusion, combinations of IFN-α with IL-2 or conventional chemotherapy have not provided significant improvements in response rate or OS. On the basis of robust phase III data from two trials, the combination of IFN-α/bevacizumab can be recommended for previously untreated patients with advanced RCC and predominantly clear cell histology. However, no data presently allows us to decide between mTOR inhibitors (temsirolimus), TKIs (sunitinib, pazopanib), IFN-α/bevacizumab, and high-dose IL-2 in the first-line setting for patients with intermediate to favorable

categories of RCC (MSKCC risk status). However, in the challenging setting of poor-prognosis RCC, temsirolimus has demonstrated a survival advantage in a 3-arm phase III trial against IFN-α/temsirolimus and single-agent IFN-α leading to FDA approval for the specific indication of first-line treatment of poor-risk RCC (Hudes et al. 2007).

Role of IFN-α in RCC: Adjuvant Therapy in Resected High-Risk Patients

Similar to the experience in melanoma, trials of adjuvant IFN-α following nephrectomy were conducted to determine whether this agent might improve the RFS and OS. In the adjuvant setting after nephrectomy for localized RCC, seven randomized trials have been conducted demonstrating neither RFS nor OS benefit with therapy including IFN-α alone or as part of a combination (Messing et al. 2003; Porzsolt 1992; Passalacqua et al. 2007; Pizzocaro et al. 2001; Atzpodien et al. 2005; Clark et al. 2003; Aitchison et al. 2011). At this time, there is no evidence to support the use of adjuvant IFN-α alone or in combination for adjuvant therapy of high-risk patients following potentially curative surgery.

Two studies have demonstrated improved survival for patients with metastatic disease who received adjuvant IFN-α at 5 MU/dose thrice weekly given by subcutaneous injection following cytoreductive nephrectomy compared to IFN-α monotherapy without surgery (Mickisch et al. 2001; Flanigan et al. 2001). The contribution of interferon to the outcomes in these subjects has not been established, and it is not known whether the benefit of surgery would be seen if alternative approaches with TKI or mTOR inhibitor therapy were substituted for the interferon.

Role of IFN-α in Kaposi's Sarcoma

Prior to the 1980s, Kaposi's sarcoma (KS) was a rare malignancy of lymphatic endothelium that has more recently been shown to be caused by human herpes virus-8 (HHV-8) that generally affects middle-aged men of Mediterranean and Jewish descent and generally follows an indolent clinical course (classic KS). In the early 1980s, the incidence of KS skyrocketed, reaching epidemic proportions in a new demographic – previously healthy, homosexual men with the acquired immunodeficiency syndrome (AIDS), so-called AIDS-associated KS or epidemic KS. KS in these patients was more aggressive and widespread – affecting visceral organs in addition to the skin.

Following reports of remission in AIDS-associated KS patients treated with IFN-α, investigators sought to formally evaluate the recombinant formulation of IFN in this population. Initial reports established efficacy at both high ($20–50 \times 10^6$ U/m^2 I.V. or I.M. 5 days a week for 4 weeks) and low ($0.4–1 \times 10^6$ U/m^2 S.C. or I.M. 5 days a week for 4 weeks) dose levels in phase I and II clinical trials (Aitchison et al. 2011; Mickisch et al. 2001). Response rates averaged 30–40% and were

associated with higher dosages, higher pretreatment CD4 T-cell counts ($>200 \times 10^9$/L), and a lack of prior opportunistic infections (Krown 1991).

Although the work of Montagnier and Gallo led to the recognition of the human immunodeficiency virus (HIV) as the etiologic agent of AIDS by 1985, antiviral therapy was then in its infancy and the realization that antiretroviral therapy could inhibit HIV replication and thereby delay the progression to AIDS was not made until later. The rationale for combining IFN-α with antiviral agents was therefore based on the premise of synergistic suppression of HIV-1 replication. However, studies combining IFN-a with zidovudine reported a high rate of KS regression, even in patients with low CD4 counts whose tumors had not previously responded to single-agent IFN-α – especially interesting given that zidovudine lacked single-agent activity in treating KS (Krown et al. 1990; Fischl et al. 1991, 1996; Kovacs et al. 1989).

The realization that highly active antiretroviral therapy (HAART) prevents progression to AIDS and the development of AIDS-defining illnesses has radically altered the management of KS. Currently, HAART is recommended for patients with AIDS-related KS. In fact, the role of systemic treatment beyond HAART is minimal and confined to advanced KS. Indications for the addition of systemic chemotherapy to HAART include: widespread skin involvement (>25 lesions), widespread cutaneous KS unresponsive to local treatment, extensive lymphatic disease with significant edema, symptomatic visceral involvement, and immune reconstitution inflammatory syndrome (IRIS). The combination of HAART and IFN-α has not been assessed as trials of IFN-α/antiretroviral combinations generally predated the HAART era. A phase I trial of low-dose IFN-α (at two dose levels) in combination with the reverse transcriptase inhibitor didanosine reported responses in 40–55% of patients with no significant differences in survival between the treatment groups (Krown et al. 2002). The dose of IFN-α used was much lower than in prior studies. Moreover, antiretroviral monotherapy is not accepted practice (a standard that changed during the conduct of this trial). However, the results suggest that a single antiretroviral drug alone in combination with low-dose IFN-α is sufficient to induce remission in AIDS-related KS.

Currently, IFN-α is approved for the treatment of AIDS-related KS. However, when systemic chemotherapy is indicated, liposomal anthracyclines (doxorubicin or daunorubicin) and taxanes (paclitaxel) are generally preferred as first-line options with RR of 30–60%.

Anticipated High-Impact Results

Initial forays into cancer immunotherapy tested crude immunostimulants and nonspecific cytokines (IL-2 and IFN-α) that have yielded modest benefits in unselected patients. The immunogenicity of RCC and melanoma have provided the impetus to test immunological approaches both for metastatic disease and the earlier and perhaps more immunologically assailable adjuvant postoperative settings of

disease. The outcomes of immunotherapy in these two kindred tumors have shown diverse results.

In RCC, IFN-α has not demonstrated efficacy either alone or in combination with other active agents in the adjuvant setting. AVOREN and CALGB 90206 demonstrated improved ORR and PFS for the IFN-α/bevacizumab combination compared to IFN-α alone in metastatic RCC and established this combination as a reference standard for this setting of disease. Agents targeting PDGF-R/VEGF-R [sunitinib (Sutent®), sorafenib (Nexavar®), axitinib (Inlyta®), pazopanib (Votrient®), tivozanib, cediranib] and mTOR pathway [temsirolimus (Torisel®) and everolimus (Afinitor®)] have demonstrated benefits in relation to PFS in large phase III clinical trials and have largely supplanted the combination of IFN-α/bevacizumab. However, the increased survival (although not necessarily cure) in this patient population – with "survival migration" – underscores the value of rigorous clinical trials in this refractory setting of disease to determine the optimum sequence of therapies and evaluate the role of combinations. Other than IFN-α/bevacizumab, doublets have been associated with excessive toxicity and marginal or inferior benefit compared to sequential use of agents.

Aggregate data from multiple prior US and European intergroup studies (E1684, E1690, E1694, EORTC 18952, and EORTC 18991) demonstrates that adjuvant therapy with IFN-α in two separate schedules – HDI and intermediate-dose PegIFN – reliably improve RFS in patients with deep primary melanomas and/or nodal disease who are at intermediate to high risk of recurrence. Only HDI has demonstrated a significant impact on OS, although long-term follow-up past 10 years has showed that this benefit upon survival is not as durable as the effect upon relapse-free interval. Conversely, the benefits of PegIFN have been observed in relation to RFS, where the magnitude of this benefit upon RFS is less than that of HDI and no impact has been observed in relation to OS. The analysis of therapeutic benefit in relation to disease burden has shown that where the impact of HDI does not differ for microscopic and macroscopic bulky nodal disease, the effect of pegylated IFN-α appears restricted to patients with microscopic nodal disease and may be greater in patients whose primary melanoma demonstrates ulceration. Both HDI and pegylated IFN-α have received FDA approval for adjuvant therapy of node-positive disease.

Although IFN-α is approved for the treatment of AIDS-related KS, practitioners more recently have generally favored alternative chemotherapeutic regimens with greater response rates, such as liposomal anthracyclines and taxanes, when systemic chemotherapy is indicated.

In conclusion, the past decade has witnessed an explosion in our understanding of the molecular biology of RCC and melanoma, specifically in relation to the pathways relevant to their transformation and progression, and the role of the immune system in this cascade and the tumor microenvironment. Therapeutic agents that target pathways relevant to progression in melanoma (BRAF, MEK, and for some forms, C-Kit) and RCC (PDGF-R/VEGF-R/c-kit/mTOR) have been developed, and this understanding has resulted in a rapid increase in the number of phase III trials that have led to the registration of multiple new therapeutic agents and combinations. The next wave of clinical trials will attempt to harness the effects of interferon and

other immunotherapies together with molecularly targeted therapies that may have their influence upon the tumor microenvironment, and enable rational development of more effective and synergistic combinations with targeted agents. Current trials are exploring these questions.

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Interleukin-2

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Abstract

Interleukin-2 is an autocrine cytokine that activates T cell, B cell, NK cells, monocytes and oligodendrocytes. IL-2 binds to a heterotrimeric receptor and can mediate anti-tumor activity through expansion of cytotoxic effector T and NK cells and can mediate immune suppression through expansion of regulatory suppressor T cells. The factors that mediate clinical outcomes of IL-2 treatment are incompletely understood. Nonetheless, IL-2 has shown therapeutic benefit

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with FDA approvals for the treatment of patients with metastatic renal cell carcinoma and melanoma. An improved understanding of the biology of IL-2 and the ability to combine IL-2 with other immunotherapy agents suggest that IL-2 will continue to be a therapeutically useful cytokine. The identification of predictive biomarkers for IL-2 response is another high priority for the field.

Keywords
Interleukin-2 • Melanoma • Renal cell carcinoma • Treatment

Interleukin-2 (IL-2) is a cytokine that plays a major role in the modulation of the immune system. IL-2 was one of the first cytokines to be identified and evaluated in the clinical setting. Previous clinical studies established that IL-2 induces durable therapeutic responses in a subset of patients with metastatic melanoma and renal cell carcinoma. More recent preclinical studies have also suggested that IL-2 may play a role in the control of autoimmune diseases. The basis for this dual role in promoting and suppressing immune responses highlights the complex physiologic role of IL-2 as a regulatory cytokine. The therapeutic mechanisms of action for IL-2 as a form of tumor immunotherapy, however, are still incompletely understood.

In the late 1970s, IL-2 was identified as a T-cell growth factor that was critical for maintenance of viable T cells in vitro. Early clinical trials with adoptively transferred lymphocytes in patients with cancer were supported by administration of recombinant IL-2 to maintain T-cell viability in vivo. Subsequent preclinical and clinical trials demonstrated a direct antitumor effect of IL-2 in metastatic renal cell carcinoma and melanoma. The durability of clinical responses led to rapid FDA approval of IL-2 although the mechanism by which IL-2 mediates the antitumor activity was not known. The development of IL-2 and IL-2 receptor knockout mice suggested that IL-2 also played a role in maintaining peripheral tolerance as absence of IL-2 resulted in progressive autoimmunity and T-cell activation. Preclinical studies have now demonstrated that IL-2 may have potential as a therapeutic strategy in a variety of inflammatory and autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, diabetes mellitus, and graft-versus-host disease, among others. The identification of a regulatory CD4+ T-cell subset characterized by high levels of CD25 and FoxP3 expression and a functional role in T-cell suppression has provided new insight into the complex regulatory role of IL-2. New research is focusing on understanding how IL-2 regulates T-cell homeostasis, and clinical research highlighting predictive biomarkers of IL-2 response is providing provocative insights into how to optimize the therapeutic potential of this critical cytokine.

This chapter will focus on the role of IL-2 on the immune system and its application in clinical oncology. There will be a brief discussion of the molecular structure, targets, and proposed mechanisms of action. This will be followed by an outline of its current role as a cancer treatment and as an immunologic adjuvant for cancer vaccines and adoptive T-cell therapy.

Biology of the Target

IL-2 is a 15.5 kDa variably glycosylated protein comprised of four-bundled antiparallel alpha-helices (Malek and Castro 2010). IL-2 acts in an autocrine/paracrine manner by binding to an IL-2 receptor expressed on T cells, B cells, NK cells, monocytes, and oligodendrocytes resulting in cell proliferation and differentiation of effector functions, including cytokine secretion and enhanced cytotoxicity functions in T and NK cells. The pleiotropic effects of IL-2 are mediated by variable expression patterns of the IL-2 receptor complex on different target cells. Although IL-2 was originally thought to stimulate activation of the immune system through clonal expansion of effector lymphocyte populations, it is now clear that IL-2 also regulates the expansion of regulatory and suppressor lymphocyte populations as well. Thus, IL-2 is better regarded as a regulatory cytokine that functions to maintain immunologic homeostasis by influencing both effector and regulatory lymphocyte populations. Further, homeostasis is influenced by the local levels of IL-2 produced and the type, number, and cellular patterns of expression of the IL-2 receptor (IL-2R).

The IL-2 receptor (IL-2R) is a heterotrimeric complex composed of an alpha (CD25), beta (CD122), and common gamma (CD132) chain. The gamma chain is shared by the receptors for IL-4, IL-7, IL-9, IL-15, and IL-21, which make up the Type I cytokine-receptor family (Kim-Schulze et al. 2007). The alpha chain is primarily involved in cytokine binding and does not appear to have a role in signaling. The beta and gamma chains signal through the intracellular activation of the JAK/STAT pathway (Malek and Castro 2010). The heterotrimeric complex is considered the high-affinity IL-2R, and the CD25 component is rapidly internalized within 15 min of IL-2 binding to the receptor complex. This is in contrast to expression of C25 alone, in which binding of IL-2 results in negligible receptor internalization. The expression of the low-affinity IL-2R consists of the beta and gamma chains as a dimeric receptor complex and results in less rapid and more dampened signaling. While the beta and gamma chains are expressed on T, B, and NK cells, the alpha chain is restricted to T cells. Thus, the subunits can form low (α), intermediate (βγ), or high-affinity receptor (αβγ) complexes. The number of cells and IL-2R expression patterns can determine the functional outcome of IL-2 on the immune system.

The predominant source of IL-2 is activated Th1 CD4+ T cells (Kim-Schulze et al. 2007). Among the most important targets of IL-2 are T cells, B cells, and NK cells, although monocytes and oligodendrocytes are also activated by IL-2. The opposing effects of IL-2 are due, in part, to the differential expression patterns of the receptors on lymphocyte subsets. As an immune enhancer, IL-2 acts on high-affinity (αβγ) receptors that support the expansion, survival, and differentiation of helper CD4+ T cells, effector CD8+ T cells, and memory T cells. IL-2 also enhances NK cell proliferation, cytolytic activity, and secretion of other cytokines, such as INF-γ via an intermediate-affinity receptor (βγ). NK cells also acquire lymphokine-

activated killer (LAK) cell activity upon exposure to IL-2, giving these cells potent tumor lytic capabilities. IL-2 stimulates NKT cells to produce large amounts of INF-γ, IL-4, and mixed pro- and anti-inflammatory cytokines (Bessoles et al. 2008). Direct action on an intermediate-affinity receptor (βγ) also assists in B-cell proliferation and differentiation (Malek and Castro 2010).

As an immune suppressor, IL-2 promotes activation-induced cell death of T cells and suppression of self-reactive T cells. These responses are accomplished via induction of CD4+CD25+ T-regulatory cells (Tregs) and CD8+CD28-T-suppressor cells (Ts) (Shevach 2009; Kim and Cantor 2011). Tregs have high levels of the alpha-receptor chain (CD25) and the forkhead/winged helix transcription factor FoxP3. Although the exact mechanism of immune suppression is not defined, Tregs are thought to act by local release of suppressive cytokines, depletion of extracellular IL-2, and cytolysis of effector cells. Their role in the maintenance of tolerance and prevention of autoimmunity has been characterized through IL-2 and IL-2R knockout mice. In these models, depletion of Tregs results in increased autoimmunity from the unopposed action of CD8+ cytotoxic T cells (Contractor et al. 1998). These mice, as well as subsequent studies of Treg depletion, have demonstrated enhanced tumor rejection and improved responses to cancer vaccines in the absence of IL-2-regulated Tregs (Comes et al. 2006).

Although less is known about CD8+ T-suppressor cells, reports have demonstrated that these cells lack CD28 expression, have no cytotoxic activity, and have been found to suppress in an antigen-specific and MHC class-I-restricted manner. These cells act directly on antigen-presenting cells (APC) to decrease co-stimulatory molecule expression and increase inhibitory mechanisms (Kim and Cantor 2011).

Target Assessment

IL-2 can be measured by standard ELISA in serum, and the IL-2 receptor can be easily identified by multiparametric flow cytometry. Monoclonal antibodies targeting IL-2 and each individual component of the IL-2R complex are available for such analyses. In addition, cytokine and receptor levels can be quantitatively measured by PCR analysis of purified cell populations, Western blotting, and customized proteomics analysis. In current clinical practice, serum IL-2 and IL-2R complex levels are usually not measured prior to or during IL-2 treatment.

Role of Target in the Cancer

Rank: 10

The role of IL-2 as a cancer therapeutic has been validated in several clinical trials demonstrating a 15–20% objective response rate in patients with metastatic melanoma and renal cell carcinoma (Atkins et al. 1999). Further, long-term follow-up of patients achieving a complete response to IL-2 treatment suggests that patients are cured of disease as the majority remain alive without disease recurrence 15 years

later (Atkins et al. 2000; Fisher et al. 2000). These data support the important role for IL-2 in cancer immunotherapy and support the identification of predictive biomarkers to better select those patients who might benefit from IL-2 immunotherapy.

High-Level Overview

Diagnostic, Prognostic, and Predictive

There has been intense interest in the discovery of predictive biomarkers for better selection of patients likely to respond to IL-2 therapy. To date, several putative markers have been suggested, but none has been validated in prospective trials. Nonetheless, as our understanding of IL-2 has improved, the potential to identify predictive biomarkers has been slowly developing.

In a retrospective analysis, the clinical response to IL-2 in patients with renal cell carcinoma was associated with the presence of alveolar histological features in more than 50% of the specimen and an absence of papillary characteristics (Upton et al. 2005). Additionally, high levels of carbonic anhydrase IX (CAIX) expression were shown, in a retrospective analysis, to predict for response in patient with RCC treated with high-dose IL-2 (Atkins et al. 2005). This finding, however, has not been replicated in prospective series (Phan et al. 2001).

In melanoma, response to IL-2-containing regimens has been associated with HLA-CW7 phenotype; development of autoimmunity, such as vitiligo and autoimmune thyroiditis; as well as low pretreatment serum levels of IL-6 and C-reactive protein (CRP) and the height of rebound lymphocytosis following IL-2 treatment (Clement and McDermott 2009). Novel methods such as single nucleotide polymorphisms and gene expression profile may be important tools to help with response prediction. A defined polymorphism in the CCR5 gene (CCR5Δ32) was associated with decreased survival following IL-2 administration in patients with Stage IV melanoma compared to patients not carrying the deletion (Ugurel et al. 2008). This is a well described polymorphism in the general population and results in deletion of the final 32 C-terminus amino acids. The lack of clinical response in patients with the CCR5Δ32 polymorphism suggests that therapeutic responses to IL-2 depend on intact CCR5 signaling since this is impaired in T cells from patients harboring this genotype. Further prospective analysis of this marker is needed.

Other studies have focused on assessing the number, phenotypic characteristics, and functional status of $CD4^{+}FoxP3^{+}$ Tregs in melanoma and renal cell carcinoma patients undergoing standard high-dose IL-2 administration (Cesana et al. 2006). While the number of Tregs increased after exposure to IL-2 and remained elevated in patients with disease progression, patients who responded to IL-2 demonstrated a decrease in Tregs to normal levels within 4 weeks of completing IL-2 treatment. While these data were intriguing, it is not clear if this pattern can be used to develop a predictive algorithm for clinical monitoring. Using the same patient cohort, serum was evaluated by customized proteomic analysis to determine if specific proteins could identify therapeutic responders. In this study, elevated levels of pretreatment

serum vascular endothelial growth factor (VEGF) and fibronectin were associated with a lack of clinical response to IL-2 and decreased overall survival (Sabatino et al. 2009). The potential utility of VEGF and fibronectin as predictive biomarkers are being prospectively evaluated in the melanoma SELECT trial.

Therapeutics

Interleukin 2 (IL-2) plays a central role in the regulation of cell-mediated immunity through stimulation and proliferation of T cells, NK cells, and activated B cells. There is considerable preclinical data supporting the therapeutic role of IL-2 as a single agent and as an adjuvant to a wide range of immunotherapeutic strategies in the treatment of cancer. Human and murine lymphocytes can be activated by IL-2 to become cytotoxic, with a wide-ranging lymphokine-activated killer (LAK) cell activity, able to lyse both freshly NK cell-resistant isolated autologous and cultured allogeneic tumor cells (Belldegrun et al. 1988). More recently, it has been shown that most LAK cell activity mediated by blood lymphocytes is attributable to stimulation of NK cells by IL-2 (Burke et al. 2010). Both NK and LAK cell activity appear to have an active role in resistance to cancer development and progression. The systemic administration of LAK or NK cells followed by systemic IL-2 treatment is strongly supported by murine models, but further studies are needed in the clinic to determine the most susceptible tumors, optimal doses and combinations of agents, and schedule of drug and cell administration. While the role of LAK and NK cells is still in development, there has been more preclinical support for the effects of IL-2 alone or in combination with vaccines or adoptive T-cell administration as a logical strategy for maximizing therapeutic responses.

High-dose bolus IL-2 is approved for the treatment of patients with metastatic renal cell carcinoma and malignant melanoma. There is a highly consistent clinical response rate that includes a small cohort of patients with durable long-term responses. IL-2 is typically administered in selected centers with available physician and nursing expertise with an excellent safety record. The identification of predictive biomarkers to better select patients who might benefit from IL-2 has been a high priority, as previously mentioned.

Preclinical Summary

Early ex vivo studies in murine models demonstrated that using IL-2 alone could reduce or eliminate pulmonary metastases from methacholine-induced sarcoma and melanoma cell lines and that the antitumor effect was strictly related to the dose of administered IL-2 (Mazumder and Rosenberg 1984). In some animal models tumor eradication with IL-2 resulted in immunization against the tumor, while in other studies IL-2 was used as an adjuvant to enhance the efficacy of a vaccine (Shimizu et al. 1999). There have been innumerable reports further suggesting that IL-2 had adjuvant properties with a wide range of vaccine approaches, including peptide

vaccines, dendritic cell vaccines, recombinant viral vaccines, and autologous or allogeneic whole cell vaccines. The potential benefits of adoptively transferred tumor-specific T cells have been explored as a promising approach for the treatment of established cancers. Adoptive transfer into lymph-depleted hosts appears to have a major impact on tumor regression in both murine models and in early clinical trials (Gattinoni et al. 2006). The preclinical studies have provided several potential mechanisms for the improved therapeutic responses, including elimination of suppressive cells (e.g., Tregs), eliminating cellular cytokine sinks, improved availability and function of antigen-presenting cells, and increased effector T-cell differentiation, trafficking, and persistence. In these studies the adoptively transferred T cells are typically supported by adjuvant IL-2 administration. Although further research is needed to better define the clinical effects of adoptive T-cell therapy in cancer patients, IL-2 will likely play a role in augmenting the clinical response. In addition to mice, complete tumor regression of canine melanoma was observed in dogs after treatment with sequential tumor necrosis factor (TNF)-α and IL-2 (Moore et al. 1991). Thus in preclinical models, IL-2 has been shown to be an important antitumor agent alone and can serve as a critical immune adjuvant in a variety of immunotherapy platforms.

Clinical Summary

IL-2 received regulatory approval by the FDA for the treatment of metastatic melanoma in 1998. Following initial reports from the National Cancer Institute demonstrating objective responses in patients with advanced melanoma, a series of single-arm phase II clinical trials were conducted evaluating 270 patients with metastatic melanoma from 1985 to 1993 (Atkins et al. 1999). Patients were treated with 600,000 or 720,000 international units per kilogram administered intravenously every 8 h for up to 14 consecutives doses as clinically tolerated. A second treatment cycle was administered after 6–9 days of rest. These courses could be repeated every 6–12 weeks for patients demonstrating a therapeutic response. The mean number of courses administered was 1.4 with each course representing two cycles. Eighty-one patients received more than one treatment course. The overall response rate was 16% with 6% of patients achieving a complete response (Atkins et al. 1999). The median progression-free survival (PFS) for all responders was longer than 1 year, and 59% of complete responders remained progression-free at 7 years. No patients achieving a response lasting in excess of 30 months relapsed, suggesting that some patients may actually be cured of disease. In this series, more than 50% of the study population had been previously treated for metastatic disease, and prior therapy was associated with diminished response rates.

IL-2 was also granted FDA approval for the treatment of metastatic renal cell carcinoma in 1992 based on its ability to produce durable responses in a small number of patients (Fyfe et al. 1995). An update of the initial analysis of 255 patients treated in seven phase II clinical trials revealed an overall objective response rate of 15% with complete responses seen in 7% of patients (Fisher et al. 2000). Those

figures remained stable since initial reports. The median response duration for all objective responders was 54 months, and the median duration for all complete responses had not been reached at the time of the last analysis but was at least 80 months. This data together with other reported studies suggests that a small subset of patients could be eventually cured from disease. To date, the criteria used to select eligible patients with metastatic renal cell carcinoma for IL-2 therapy continues to be patient performance status, comorbid conditions, and predominantly clear cell histology of the primary tumor.

The combined use of cytotoxic chemotherapy and IL-2 for melanoma has been studied in an attempt to increase response rates. Although several phase II trials suggested a clinical benefit for biochemotherapy in the treatment of metastatic melanoma, a large prospective phase III clinical trials showed no survival advantage (Atkins et al. 2008). The phase III trial was a multi-institutional, randomized study that compared biochemotherapy, consisting of cisplatin, vinblastine, dacarbazine, low-dose IL-2, and interferon alpha-2b to chemotherapy alone (cisplatin, vinblastine, and dacarbazine). The biochemotherapy cohort had slightly higher response rates and progression-free survival compared to chemotherapy alone, but there was no difference in overall survival or in quality of response between the two arms (Ives et al. 2007). Furthermore, a meta-analysis of biochemotherapy clinical trials corroborated that despite improvement in response rates, there was no overall survival advantage for biochemotherapy regimens. Of note, the incidence of toxicity was higher in the biochemotherapy groups in most studies (Atkins et al. 2008; Ives et al. 2007).

There have been conflicting reports about the potential of IL-2 to act as an adjuvant in patients receiving cancer vaccines. In one of the largest, prospective, randomized clinical trials conducted to date, 185 patients with metastatic melanoma across 21 centers were randomized to treatment with an HLA-A2-restricted, modified gp100 peptide vaccine administered by subcutaneous injection followed by standard high-dose bolus IL-2 or IL-2 alone (Schwartzentruber et al. 2011). In this trial, there was a statistically significant increase in response rate from 9.7% in the IL-2 arm to 22.1% in the combination arm. In this study, a significant increase in progression-free survival was also reported for the combination group (Schwartzentruber et al. 2011). The study was not, however, powered for overall survival, and further studies are needed to confirm these initial findings.

The major toxicity related to IL-2 is due to capillary leak syndrome that occurs to varying degrees in most patients treated with IL-2. The manifestations include fever, chills, hypotension, peripheral edema, tachycardia, oliguria, and reversible organ-specific dysfunction, such as elevated liver enzymes, renal dysfunction, nausea, vomiting, diarrhea, thrombocytopenia, neutropenia, electrolyte depletion, and in some cases confusion or disorientation. The constitutional symptoms can usually be managed by pretreatment with acetaminophen and nonsteroidal analgesics, while the cardiovascular effects are usually managed by close observation, including

continuous telemetry and careful fluid management during and after treatment. Most patients recover quickly following cessation of treatment. Early reports of mortality were largely related to sepsis, and it is generally recommended that patients receive prophylactic gram-positive antibiotic coverage during active treatment. The underlying cardiac, pulmonary, and neurologic risks also mandate that patients be carefully screened prior to treatment with an MRI of the brain, cardiac stress test, and pulmonary function studies, whenever appropriate. IL-2 can be safely administered at experienced centers with trained staff.

Anticipated High-Impact Results

IL-2 has been validated as a therapeutic target by demonstrating durable long-term clinical responses in patients with metastatic melanoma and renal cell carcinoma. Although IL-2 has been approved since 1992, the exact mechanisms through which IL-2 mediates antitumor activity are not completely defined, and the role of IL-2 in maintaining T-cell homeostasis is likely more complex than previously realized. A new understanding of the regulatory nature of IL-2 biology under normal physiologic conditions and expanding translational research in tumor immunotherapy are suggesting promising directions for future investigation that could increase the impact of IL-2 in the treatment of cancer.

An important area of future study will undoubtedly be combination therapy in which IL-2 will be used concurrently or sequentially to promote more potent antitumor activity or as an adjuvant to enhance immunologic responses. The anti-CTLA4 monoclonal antibody, ipilimumab, was approved for the treatment of patients with advanced melanoma in 2011. This agent demonstrated a significant improvement in overall survival for patients with metastatic melanoma, yet the objective response rate was only 10.9% (Hodi et al. 2010). Thus, one logical combination to test would be the combination of high-dose IL-2 and ipilimumab. The BRAF inhibitor, vemurafenib, was also approved in 2011 for the treatment of advanced melanoma in patients harboring a mutation in BRAF (Chapman et al. 2011). This agent demonstrated significant improvement in both progression-free and overall survival with an objective response rate of 48%. However, of the 106 patients with an objective response in the phase III trial, only 2 were complete and drug resistance has been seen. Thus, another logical combination would be vemurafenib and high-dose IL-2. These combination clinical trials are anticipated in the near future. The remarkable clinical responses observed following adoptive T-cell therapy in patients with melanoma also warrant further evaluation (Rosenberg et al. 2011). To date, the majority of these studies have incorporated high-dose IL-2 as an adjuvant to maintain T-cell differentiation and persistence. The full impact of this therapy requires prospective, randomized clinical trials with survival endpoints.

The ability of IL-2 to expand regulatory T-cell populations has suggested that treatment may promote immune suppression, at least in some patients. This observation may allow IL-2 to be used for promoting immune suppression in cancer patients where immune activation is pathologic. For example, in a recently published observational cohort study, patients with chronic graft-versus-host disease (GVHD) that developed as a result of allogeneic hematopoietic stem cell transplantation performed for the treatment of hematologic malignancies were treated with low-dose subcutaneous IL-2 (Koreth et al. 2011). In this trial, 29 patients were treated, and objective partial responses were seen in 50% with responding patients also demonstrating an increase in Treg cell count. Glucocorticoid doses could be tapered by a mean of 60% in patients who received IL-2 for an extended period of time. None of the patients had progression of chronic GVHD or relapse of their baseline hematologic disorder. Two patients included in the trial developed thrombotic microangiopathy (TMA)-associated renal failure. Those patients were also being treated with sirolimus and tacrolimus, agents that are known to predispose to TMA. It was postulated that IL-2 could potentiate this side effect, and therefore patients receiving sirolimus and tacrolimus were subsequently excluded from the trial. TMA did not recur. There was no obvious associated immune impairment with low-dose subcutaneous IL-2.

An improved understanding of the basic biology of IL-2 and identification of predictive biomarkers are also important areas of current focus. As discussed, there are several putative biomarkers that have been identified, and these are in the process of prospective validation through the melanoma SELECT trial. The identification of several important mutational targets in melanoma cells, such as BRAF, c-KIT, and NRAS, has paved the way for new therapeutic options with targeted therapy. The role of IL-2 in patients whose tumors harbor these mutations has not been established yet and may be another important avenue of investigation in the future. Another approach to advancing our understanding of the basic biology is the use of computational modeling to provide testable predictions about biological or clinical outcomes. The use of such a model that incorporates the dynamics of IL-2 and IL-2R binding interactions and the endocytic trafficking of the cytokine-receptor complexes has resulted in establishing kinetic equations that have accurately described the binding, internalization, and post-endocytic sorting of IL-2 and its receptor in lymphocyte subsets (Fallon and Lauffenburger 2000). This model has made predictions on the activation status of T lymphocytes in vitro and may be a useful tool for identifying new and unexpected interactions between lymphocyte populations upon exposure to IL-2 in vivo.

In summary, IL-2 is one of the most clinically useful cytokines to date and has been approved for the treatment of patients with metastatic melanoma and renal cell carcinoma. Although the exact mechanism of IL-2-mediated antitumor activity is not fully defined, additional research has highlighted the complex role of this cytokine in maintaining T-cell homeostasis. Further investigation into combination regimens, identification of predictive biomarkers of response, and computational modeling of IL-2 effect will likely identify novel clinical indications for IL-2. Additional

investigation of the IL-2-related cytokines, such as IL-7, IL-12, and IL-15, will also be promising areas for future research.

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Interleukin-7

28

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Abstract

IL-7 and its cognate receptor are critical for normal development, function and maintenance of the immune system. Aberrant and excessive activation of the IL-7 signalling pathway can promote certain hematological malignancies. It is not clear if a potential therapeutic that targets and antagonizes IL-7 signalling would have efficacy as an anti-cancer agent for maligancies where this signalling pathway is abnormally activated. Conversely enhancing IL-7 signalling, through administration of exogenous IL-7 cytokine, may have an adjuvant therapeutic benefit in promoting the efficacy of other immune based therapies used to treat

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malignancies such as melanoma. It is unclear if prolonged use of exogenously administered IL-7 results in any adverse outcomes.

Keywords

Cytokine receptor-like factor 2 (CRFL2) • Interleukin-7 (IL-7) • Antagonizing signaling • CRFL2 • Diagnosis • Graft versus leukemic effect • Immunotherapies • JAK1 • Levels in serum and tissues • Maintenance and homeostasis of cells • Naïve and memory T cells • Phase I/II clinical trials • Receptor • Role in immune homeostasis • Systemic IL-7 therapy • TGFb1 • TGF-band T regulatory (Treg) cells • Therapeutics • Vaccination • Systemic IL-7 therapy

Target: Interleukin-7/Interleukin-7 Receptor

IL-7 is a homeostatic cytokine, and, along with its receptor, it is required for the development and maintenance of the immune system. Diverse cell types including stromal, epithelial, and fibroblast cells in the bone marrow, lymphoid organs, thymus, liver, gut, and skin produce IL-7. Additionally dendritic cells (DCs) and myeloid cells are capable of producing IL-7. Given the diversity of IL-7 producing cells, this cytokine's tissue distribution is widespread. Its cognate receptor (IL-7R), a heterodimer composed of an IL-7-specific alpha subunit (IL-7Ra) and the common gamma chain (g_c), has a much more restricted distribution. IL-7R is expressed on T cells, DCs, and pre-B cells. IL-7 signal transduction is achieved predominately through the activation of Janus kinase (JAK) and signal transducers and activators of transcription pathways, phosphatidylinositol 3-kinase, and protein kinase B pathways and also through the activation of src family kinases. The expression of both cytokine and receptor are tightly regulated. IL-7 levels rise during lymphopenia coincident with its diminished utilization. Receptor expression is downregulated by IL-7 binding, and in the case of T cells, it is downregulated following activation. The downstream effects of IL-7 signaling include promotion of cell cycling through repression of the cell cycle inhibitor $p27^{Kip1}$ and increased cell survival mediated by the induction of pro-survival molecules like Bcl-2. The physiology of IL-7 has been thoroughly reviewed (Palmer et al. 2008; Capitini et al. 2009).

Biology of the Target

IL-7 and IL-7R are essential for the development of T cells including gdT cells (Capitini et al. 2009). IL-7 signaling is critically required at the very early stages of thymocyte development and for the induction of T cell receptor (TCR) recombination mediated by Rag-1. In the absence of IL-7 or its receptor, humans develop a severe combined immunodeficiency characterized by a lack of T cells. IL-7 has also been shown to be important for the maintenance and homeostasis of naïve and memory T cells, NK cells, and DCs (Capitini et al. 2009; Vogt et al. 2009;

Vosshenrich et al. 2006). Signaling through the IL-7 receptor is required for the development and homeostasis of lymphoid tissue inducer (LTi) cells and LTi-like cells that are critical for the development of lymphoid organs (Schmutz et al. 2009). In mice IL-7 is also required for B cell development, but the role of IL-7 in human B cell development is not clear. IL-7R is expressed from the early pro-B cell stage in humans but is no longer expressed in mature B cells. Humans deficient in IL-7 or IL-7R have normal B cell numbers, but B cell function may be defective (Puel and Leonard 2000). This B cell defect may be secondary to more profound T cell defects.

Among the T cell population, IL-7R is most highly expressed by naïve T cells and memory T cells. In naïve T cells, IL-7 promotes cell survival and homeostatic proliferation through its modulation of cell cycle inhibitors and upregulation of pro-survival molecules. The elevated levels of IL-7 associated with chemotherapy, bone marrow conditioning, HIV-associated lymphopenia, and other acquired states of lymphopenia drives immune reconstitution.

Recent studies have clearly elucidated that mutations causing constitutive IL-7R activation are oncogenic, promoting transformation and tumor formation in T-cell acute lymphoblastic leukemia (T-ALL) (Zenatti et al. 2011). IL-7R mutations are found in about 10% of patients with T-ALL (Zenatti et al. 2011; Shochat et al. 2011). Approximately 18% of adult and 2% of pediatric T-ALL cases have activating mutations in JAK1 (Flex et al. 2008). About 10% of B cell ALL have mutations in JAK1/2 on genome-wide analysis. Although these JAK mutations in isolation do not cause transformation, they are often associated with aberrant expression of the heterodimeric receptor complex consisting of cytokine receptor-like factor 2 (CRFL2) and IL-7R (Malin et al. 2010; Harvey et al. 2010a). Approximately 5–10% of childhood ALL and about 60% of Down syndrome-associated B cell precursor ALL (BCP-ALL) have anomalies in CRFL2 (Shochat et al. 2011). A gain of function mutation in IL-7R is associated with the aberrant expression of CRFL2 in cases of BCP-ALL (Shochat et al. 2011). IL-7R is expressed in some cases of non-Hodgkin lymphoma, and elevated levels of circulating IL-7 have been found in patients with pre-B-ALL and B-cell chronic lymphocytic leukemia and HIV-associated lymphomas (Sasson et al. 2010). In vitro studies and xenotransplantation experiments have indicated that exogenous IL-7 can promote the growth of Sezary cells, pre-B, and T-ALL (Sasson et al. 2010; Silva et al. 2011). Indeed, some studies have suggested that the effectiveness of rapamycin in the treatment of mouse models of B-precursor leukemia is through the modulation of IL-7 downstream pro-survival signals (Brown et al. 2007). IL-7 signaling within the microenvironment of Hodgkin lymphoma has been implicated in the homeostasis of this tumor (Cattaruzza et al. 2009).

It is clear that mutant constitutively active IL-7R in some hematological malignancies is oncogenic; however whether overexpression of IL-7 cytokine or its exogenous administration can promote transformation or cancer progression is less clear. Although IL-7R is expressed on some adult solid tumors, its significance is not clear (Cosenza et al. 2002). IL-7 transcriptional upregulation in tissue and elevated serum levels of IL-7 have been described in cases of early-stage prostate cancer (Mengus et al. 2011). In breast cancer studies, IL-7 has been implicated to play a role

in endothelial cell proliferation by promoting tumor production of angiogenic factors (Al-Rawi et al. 2005). There is also some in vitro data associating IL-7 with lung cancer cell proliferation (Ming et al. 2012).

IL-7 Assessment

IL-7 expression levels in serum and in tissues are, as yet, not a part of any standard evaluation for malignancy. Analysis of IL-7R coding sequence has been used to link mutations in the receptor to T and B cell ALL, but until the possibility and efficacy of therapeutic targeting are evaluated, this remains a research and investigative tool.

Role of Target in Cancer

Rank for antagonizing IL-7 signaling in hematological malignancies: unknown. Although excessive signaling downstream of IL-7R may participate in the promotion and maintenance of some hematological malignancies, it is unknown if antagonizing this signaling will cause regression of the malignancies. The use of serum IL-7 level as a prognostic indicator needs to be further investigated.

Rank for the use of IL-7 in immunotherapies: 5.

Preclinical data supports a therapeutic role for IL-7 in promoting antitumor immunity, but these results still need to be validated in clinical trials.

High-Level Overview

Diagnostic, Prognostic, Predictive

Mutated IL-7R is found in some hematological malignancies and is oncogenic, but it is not diagnostic of any single malignancy. Aberrant CRLF2 (and by association IL-7R) expression is associated with a worse prognosis in childhood ALL (Harvey et al. 2010a, b; Cario et al. 2010; Yoda et al. 2010; Ensor et al. 2011). Elevated plasma IL-7 levels have been linked to a poor prognosis in myelodysplastic syndrome (Pardanani et al. 2011). In the case of solid tumors, some data indicate that higher tissue levels of IL-7 plus TGFb1 are prognostic indicators for prostate tumor progression and aggressiveness (Schroten et al. 2011). The potential for serum IL-7 levels to be predictive of early prostatic cancer compared to benign prostatic hypertrophy was highlighted by a recent study (Mengus et al. 2011).

Therapeutics

The therapeutic potential of targeting the IL-7 signaling pathway is dependent on which biological consequence is most relevant. *Promotion of IL-7 signaling* would

be extremely beneficial in reconstituting immunity after chemotherapy or bone marrow conditioning. Major complications of these treatments are infections that directly contribute to high rates of morbidity and mortality. Exogenous IL-7 could promote immune recovery and circumvent infectious complications. The biology exploited in this example is IL-7's capacity to promote expansion of naïve T cells and recent thymic emigrants.

IL-7's fundamental role in immune homeostasis can be further exploited in promoting antitumor immunity. As a sole agent, IL-7 is unlikely to be efficacious, but in combination with conventional therapies, which promote tumor killing and thereby potentially facilitate immune activation, it may have beneficial effects. Its most obvious potential is in promoting the efficacy of other forms of antitumor immune therapy. Vaccination has been used to promote antitumor immunity, but despite many studies demonstrating mixed preclinical success, clinical trials have only shown limited applicability as a modality of antitumor immune therapy. Vaccination can take the form of recombinant tumor antigen vaccines, modified tumor antigen vaccines that are linked to proinflammatory cytokines, whole tumor-cell vaccines that have been engineered to express proinflammatory cytokines, and adoptive transfer of autologous dendritic cells that have been loaded with tumor antigens or loaded with modified tumor antigens linked to cytokines (Melief 2008; Harzstark and Small 2009). The role of IL-7 in promoting the efficacy of these forms of immune therapy must still be explored.

Another form of immunotherapy relies on the adoptive transfer of antitumor T cells recovered from patients or the use of engineered T cells that are modified to recognize tumors (Rosenberg et al. 2008; Morgan et al. 2006). These cells are expanded ex vivo and then reinfused back into the patients. Systemic IL-7 therapy may be useful in promoting the expansion, survival, and antitumor activity of adoptively transferred T cells. Again these possibilities need to be explored.

An alternative adoptive cell therapy relies on a graft versus leukemic effect that occurs in some cases where allogeneic hematopoietic stem cells are transplanted for the treatment of certain leukemias. In these cases donor T cells and NK cells that have minor mismatches and recognize leukemic cells as foreign actively participate in removing residual tumor cells (Ringden et al. 2009). This graft versus leukemic effect confers a significant survival advantage. IL-7 has been implicated in graft versus host disease (GVHD) so it may have utility in promoting a graft versus leukemic effect . The efficacy in promoting this effect however may be overshadowed by possible severe side effects related to potentiating overt GVHD (Sinha et al. 2002). Infusions of donor lymphocytes are efficacious in promoting remissions in chronic myelogenous leukemia (Ringden et al. 2009). Systemic IL-7 therapy may be useful in this setting and also in potential therapies that utilize haploidentical NK cell transfusions.

Although systemic IL-7 therapy appears to be safe in most respects, its use needs to be approached with great care. It is possible that indiscriminate use of IL-7 might worsen some human hematological malignancies that express functional IL-7R. Indeed in these instances endogenous IL-7 might be promoting tumor growth, and IL-7R antagonists would be the relevant therapeutic rather than IL-7R agonists.

Therapeutically targeting/antagonizing IL-7R signaling is particularly relevant in cases of T cell and B cell ALL associated with a mutated and constitutively active IL-7R. In these cases, simply neutralizing IL-7 and or blocking IL-7/IL-7R binding would not be sufficient as the mutant receptor constitutively binds and activates JAK1 independent of IL-7. Another caveat to the use of IL-7 is its potential to promote overt autoimmunity (Calzascia et al. 2008). However, phase 1 clinical trials of IL-7 have not provided any evidence to support this possibility.

Therapeutics

Although it was cloned over 20 years ago, IL-7 has only recently entered the therapeutic arena. This likely reflects the time it has taken to fully appreciate its biology. The efficacy of recombinant *E. coli*-derived IL-7 is limited by the development of neutralizing anti-IL-7 antibodies (Rosenberg et al. 2006). This prompted the manufacture of a recombinant mammalian cell-derived fully glycosylated IL-7 (Beq et al. 2009). Trials examining the efficacy of this preparation are very limited.

The theoretical potential benefit of antagonizing IL-7 receptor signaling, for example, in the case of hematological malignancies that express functional receptor, has not been thoroughly examined. Consequently therapeutics antagonizing the IL-7 receptor signaling pathway have not been clinically developed. Only recently has a mutation driving IL-7R activation been linked to T and B cell ALL, and this may prompt the development of therapeutics that target the IL-7 signaling pathway.

Preclinical Summary

Animal studies have elucidated many interesting attributes of systemic IL-7 therapy. The ability of IL-7 to promote T cell survival and cycling has been exploited to reverse an unresponsive or anergic state in antitumor T cells and improve their function (Bendle et al. 2007; Li et al. 2006). In tumor models, IL-7 enhances the capacity of T cells to kill tumors by upregulating the expression of killer effectors like granzymes, and it promotes their ability to deregulate and liberate proinflammatory molecules and cytotoxic mediators (Pellegrini et al. 2009; Lynch and Miller 1994; Gattinoni et al. 2005). IL-7 was also shown to favor the differentiation of CD4 T cells toward a more aggressive phenotype called Th17 cells that secrete IL-17 (Pellegrini et al. 2009; Muranski et al. 2008; Martin-Orozco et al. 2009). In addition to expanding the T cell population, IL-7 can also promote T cell receptor diversity, thus broadening a T cell immune response (Sportes et al. 2008; Melchionda et al. 2005). This may be advantageous in circumventing tumor immune escape that may result as a consequence of mutations in tumor antigens. The ability of IL-7 to promote antitumor immunity has also been linked to its ability to render T cells refractory to several inhibitory factors. For example, T cells from animals systemically treated with IL-7 are refractory to the inhibitory effects of TGF-band T regulatory (Treg) cells (Pellegrini et al. 2009; Ruprecht

et al. 2005). Furthermore, IL-7 represses several T cell intrinsic molecules that normally function to keep the cell in a quiescent state including the E3 ligase Cbl-b and the transcription factor FoxO (Pellegrini et al. 2009). Studies have also shown that the efficacy of IL-7 can be enhanced with the concomitant use of synergistic cytokines like IL-15 and IL-21 (Melchionda et al. 2005; Liu et al. 2007).

Regarding the use of IL-7/IL-7R antagonist for the treatment of hematological malignancies, one group has shown that in a mouse model of pre-B ALL, the use of rapamycin caused tumor regression, and this was associated with a coincident decrease in IL-7-mediated survival signaling (Brown et al. 2007).

Clinical Summary

Three phase I/II clinical trials have addressed the safety of IL-7 as a cancer therapeutic (Rosenberg et al. 2006; Sportes et al. 2008; Moller et al. 1998). None of the trials reached dose-limiting toxicities, and in two studies examining the administration of recombinant IL-7, only short courses of cytokines were given in view of the development of neutralizing antibodies. None of the trials combined IL-7 with another clinically efficacious form of immunotherapy or conventional chemotherapy. With all these caveats and the fact that the studies were primarily designed to evaluate toxicity, minor antitumor efficacy was only observed in one study (Moller et al. 1998).

Two of the studies involved administration of recombinant IL-7 to patients with metastatic melanoma or other refractory malignancies. Both studies showed significant increases in CD4 and CD8 T cell numbers without a concomitant increase in the number of Treg cells (Rosenberg et al. 2006; Sportes et al. 2008). The third study used IL-7-secreting melanoma cells as a vaccine and demonstrated a marginal antitumor effect (Moller et al. 1998). It is clear that further trials are required to optimize the dose, combine treatment modalities, and extend the duration of therapies to maximize the therapeutic efficacy of IL-7.

Anticipated High-Impact Results

- The development of IL-7 signaling antagonists and evaluation of their efficacy in T and B cell ALL associated with mutant constitutively active IL-7R.
- Determine if IL-7 neutralization or receptor blockade is efficacious in hematological malignancies associated with high circulating levels of IL-7.
- Determine the relevance of IL-7 signaling in solid tumors expressing IL-7R.
- Explore adjuvant IL-7 immunotherapy with other antitumor modalities.
- Trials examining utility of IL-7 therapy administered at the time of targeted tumor killing to promote antitumor immunity and prevent recurrence.
- Awaiting results of pilot studies addressing utility of IL-7 combined with dendritic vaccinations in high-risk solid tumors.
- Trials of combination cytokine therapies to promote antitumor immunity.

- Awaiting results of several studies addressing the utility of IL-7 therapy in immune recovery post-tumor chemotherapy.
- Further studies addressing the utility of IL-7 tumor/serum levels in determining prognosis.

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Interleukin-12

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Abstract

Interleukin-12 (IL-12) is a heterodimeric cytokine and is a member of the larger family of IL-12-related cytokines. The other members of the IL-12 family include IL-23, IL-27, and IL-35. While IL-12 shares numerous structural features and molecular receptors with other members of its family, IL-12 is functionally unique (Vignali and Kuchroo, Nat Immunol 13:722–728, 2012). IL-12 was first recovered from an Epstein-Barr virus-transformed B lymphoblastoid cell line in 1989 as "natural killer-stimulating factor" (Kobayashi et al., J Exp Med 170:827–845, 1989). The following year, a separate group independently discovered IL-12 as "cytotoxic

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lymphocyte maturation factor" (Stern et al., Proc Natl Acad Sci U S A 87:6808, 1990). Since its discovery, a tremendous amount of research has been conducted to characterize the physiologic function of IL-12 and assess therapeutic potential of agonist and antagonistic targeting of IL-12 for the treatment of a variety of diseases.

Keywords
Interleukin-12 • Biology • Clinical summary • Diagnostics • Preclinical summary • Target assessment

Target: Interleukin-12

Interleukin-12 (IL-12) is a heterodimeric cytokine and is a member of the larger family of IL-12-related cytokines. The other members of the IL-12 family include IL-23, IL-27, and IL-35. While IL-12 shares numerous structural features and molecular receptors with other members of its family, IL-12 is functionally unique (Vignali and Kuchroo 2012). IL-12 was first recovered from an Epstein-Barr virus-transformed B lymphoblastoid cell line in 1989 as "natural killer-stimulating factor" (Kobayashi et al. 1989). The following year, a separate group independently discovered IL-12 as "cytotoxic lymphocyte maturation factor" (Stern et al. 1990). Since its discovery, a tremendous amount of research has been conducted to characterize the physiologic function of IL-12 and assess therapeutic potential of agonist and antagonistic targeting of IL-12 for the treatment of a variety of diseases.

Similar to other cytokines, IL-12 plays a role in regulating aspects of the immune response, including the development, expansion, homeostasis, and differentiation of lymphocytes. IL-12 is produced by natural killer (NK) cells, T cells, dendritic cells (DCs), and macrophages and is a critical cytokine for mediating innate immunity. IL-12 production can be triggered by microbial factors, which may explain the impact on innate immune responses. In addition, IL-12 helps determine the type and duration of adaptive immune responses, most notably by inducing the release of interferon gamma and maintaining Th1-type immune responses. IL-12 is also thought to contribute to the development of memory T cell responses, which are important for clearance of certain infectious diseases, and likely cancer cells as well.

Although the role of IL-12 in cancer is controversial, the cytokine appears to have a profound effect on the tumor microenvironment. IL-12 may act directly on tumor cells and can also influence infiltrating immune cells and tumor stromal cells. The effects of IL-12 usually result in lymphocyte recruitment into the tumor microenvironment and activation of tumor-infiltrating lymphocytes. The ability to enhance local interferon production likely also contributes to host antitumor immunity since interferon can upregulate tumor-associated antigen and major histocompatibility complex expression on tumor cells.

Preclinical studies have demonstrated antitumor activity with IL-12 treatment in a variety of different tumor models. Based on these reports, clinical trials evolved but were halted when unexpected serious adverse events were documented. While

further clinical investigation is warranted, IL-12 continues to be an attractive target for immuno-oncology drug development, and a variety of approaches, such as intratumoral injection, expression by recombinant viral vectors, and lower dosing in combination regimens, have been proposed. This chapter will briefly review the biology of IL-12, discuss the current status of clinical development, and describe the therapeutic potential of this target in oncology.

Biology of the Target

Biology of Interleukin-12

IL-12 is a 74-kDa heterodimeric cytokine composed of two disulfide subunits, a light α-chain (known as p35 or IL-12α) encoded on human chromosome 3 and a heavier β-chain (known as p40 or IL-12β) found on human chromosome 5. Biologically active IL-12 is called IL-12p70 and is composed of both subunits (Kobayashi et al. 1989; Stern et al. 1990; Carra et al. 2000).

The IL-12 p35 (α-chain) is significantly modified posttranslationally during biosynthesis of IL-12. These modifications are found on secreted IL-12 p35 but are not present on intracellular, immature IL-12. Free soluble α-chains have never been isolated. In contrast, a free soluble β-chain exists and is usually secreted in association with the active IL-12p70 complex. The IL-12 β-chain (p40) does not undergo significant modification in terms of molecular weight, isoelectric point, and posttranslational modifications, but there are minor differences that distinguish free from complexed p40. The p40 subunit is also used in the construction of mature IL-23, although the alpha chain is different between the two cytokines. Cells that secrete IL-12 express both the α-chain and β-chain genes, while most other cells only express the α-chain gene (Carra et al. 2000). Antigen-presenting cells (APCs), such as DCs, monocytes, macrophages, and B cells, secrete IL-12 when activated by pathogen-associated molecular patterns (PAMPs) via a Toll-like receptor (TLR). The production of IL-12 increases as more TLRs become activated (Gautier et al. 2005).

Biology of the Interleukin-12 Receptor

After secretion, IL-12 binds to an IL-12 receptor (IL-12R) in an autocrine or paracrine manner. Originally, the IL-12R was discovered as a single protein, but there was evidence that IL-12 bound to an isolated unit with a low affinity and eventually a second subunit was discovered. Currently, the evidence suggests that IL-12R is composed of two subunits – IL-12Rβ1 and IL-12Rβ2 – and both are required for IL-12 binding and signal transduction (Desai et al. 1992; Chua et al. 1994; Presky et al. 1996). The IL-12R activates the Janus kinase (JAK)-STAT pathway within cells expressing the receptor. The IL-12Rβ1 tightly binds to the IL-12 β-chain and associates with Tyk-1 (JAK family member) within the cell. The IL-12Rβ2 subunit is primarily responsible for transducing the signal via STAT4

and Jak-2. IL-12-mediated STAT4 signaling is also thought to promote t-bet expression, which helps to further drive Th1 polarization. The IL-12Rβ2 subunit also binds to the IL-12 heterodimer or IL-12 α-chain (Sinigaglia et al. 1999; Thierfelder et al. 1996). The IL-12R is mainly expressed on activated T cells, NK cells, and NK T cells (Desai et al. 1992; Kawamura et al. 1998). IL-12R has also been discovered at lower levels on DCs (Grohmann et al. 1998), macrophages (Grohmann et al. 2001), and B cells (Vogel et al. 1996).

Biology of the Interleukin-12 Family

IL-12 is the prototype cytokine of the IL-12-related cytokine family, which consists of both pro-inflammatory cytokines (e.g., IL-12 and IL-23) and inhibitory cytokines (e.g., IL-27 and IL-35). These cytokines share structural homology among heterodimer conformations and their respective receptors. All of the heterodimers of the IL-12 family consist of both α- and β-chains. The α-chains used are designated p19, p28, and p35. The β-chains utilized are p40 or Ebi3. Both IL-12 and IL-23 share the p40 β-chain, but p40 pairs with p35 to form IL-12 and pairs with p19 to form IL-23. On the other hand, Ebi3 can pair with p28 to form IL-27 or p35 to form IL-35. Similarly, the IL-12 family receptors also share common subunits. As mentioned previously, IL-12R is made up of IL-12Rβ1 and IL-12Rβ2 subunits. IL-23 also signals using IL-12Rβ1, but it is paired with the IL-23R subunit. On the other hand, IL-35 uses IL-12Rβ2 paired with gp130. In contrast, IL-27 is the furthest removed from the IL-12R and signals via gp130 and IL-27R subunits. All of these receptors mediate their function via the Jak-STAT signaling pathway. Surprisingly, despite the homology in structure, the IL-12 family of cytokines mediates unique physiologic functions, which ultimately help coordinately regulate lymphocyte activation and suppression (Vignali and Kuchroo 2012).

Biologic Function of Interleukin-12

The function of IL-12 depends on the concentration of IL-12, the expression levels of IL-12R, and the location of IL-12R expression. The IL-12/IL-12R axis plays a critical role in bridging innate immunity and adaptive cell-mediated immunity via T cells and IFN-γ, and it may also play a role in antiangiogenesis. DCs and phagocytic cells present antigens when priming T cell responses, and IL-12 released by conditioned APCs interacts with activated $CD4^+$ T cells, resulting in Th1 differentiation (Manetti et al. 1993; Hsieh et al. 1993; Macatonia et al. 1995). IL-12-mediated interferon production further potentiates the Th1 skewing and helps to promote activation of $CD8^+$ T cells. IL-12 also enhances the cytotoxic activity of T cells, NK cells, and NK T cells by stimulating the gene expression of cytotoxic granule-associated molecules (e.g., perforin and granzymes) and expression of adhesion molecules that promote chemotaxis of T cells into peripheral sites (Kawamura et al. 1998; Cesano et al. 1993; Bonnema et al. 1994; Ebert 2004).

Shortly after the discovery of IL-12, it was found that it plays another critical role by inducing IFN-γ production in NK cells and T cells after binding to their IL-12Rs (Chan et al. 1991; Micallef et al. 1996). Synergy between IL-12 and other lymphocyte-activating stimuli is an important factor in upregulating IFN-γ production in numerous cell types, often through independent and nonoverlapping mechanisms, allowing amplification of adaptive immunity (Walker et al. 1999). IL-12 cooperates with IL-2, activating the CD28 receptor to induce production of IFN-γ in T cells. Together, these cytokines have an additive effect on cytolytic activity and synergistic effect on IFN-γ production in T cells (Kubin et al. 1994; Gollob et al. 1999). IL-12 and IL-2 also work together on NK cells and similarly activate the CD28 receptor to increase IFN-γ expression (Chan et al. 1992; Ye et al. 1995).

IL-12 also collaborates with IL-18 (formerly IFN-γ-inducing factor, IGIF). IL-18 is similar in structure to IL-12 and was discovered to stimulate IFN-γ production with IL-12 in NK cells, activated T cells, Th1 cells, B cells, DCs, and macrophages. IL-12 increases the expression of IL-18Rs on T cells, NK cells, and B cells. Together, IL-12 and IL-18 synergistically induce IFN-γ production by these cell subsets (Micallef et al. 1996; Yoshimoto et al. 1997; Taoufik et al. 2003). IL-18 responsiveness on helper T cells appears to be dependent on IL-12. Further, it has been demonstrated that IL-18 binds to Th1 cells, as opposed to Th2 cells, and this is thought to help promote differentiation of the Th1 cells (Yoshimoto et al. 1998). Antigen-presenting cells, such as DCs and macrophages, also produce IFN-γ in response to IL-12 via STAT 4 (Frucht et al. 2001). Even though IL-12 alone induces low levels of IFN-γ production, both DCs (Fukao et al. 2000; Stober et al. 2001) and macrophages (Munder et al. 1998; Schindler et al. 2001) respond to the synergistic effects of IL-12 and IL-18 with greater cytokine production.

The action of IL-12 on DCs and macrophages is not limited to increasing IFN-γ production. IL-12 primes both DC and macrophage activation and acts in a positive feedback loop, to induce greater IL-12 production. The greater concentration of IL-12 intensifies its effects through an autocrine loop, similar to IL-2 activation of lymphocytes (Grohmann et al. 1998; Grohmann et al. 2001). IL-12 also plays a role in increasing macrophage antimicrobial activity, an effect that likely depends on IFN-γ production. A study found that IL-12 and mycobacterial infection work in synergy to increase tumor necrosis factor alpha (TNF-α) release from macrophages when IFN-γ is present (Xing et al. 2000). Furthermore, IL-12 and mycobacteria synergize to increase nitric oxide (NO) production by macrophages, but only when IFN-γ is available. IL-12-stimulated IFN-γ release was found to be required for increasing TNF-α and NO, both mediators of antimicrobial activity in macrophages, thus providing a correlation between IL-12, IFN-γ, and antimicrobial activity (Xing et al. 2000).

In summary, the main purpose of IL-12 is to drive the transition from innate immunity to cell-mediated adaptive immunity. IL-12 plays a major role in the directing the differentiation of T cells into a Th1 phenotype, which further potentiates cytotoxic $CD8^+$ T cell responses. While these functions have been well linked to eradication of infectious pathogens that require cell-mediated immunity, the role of IL-12 in cancer immune surveillance and eradication has been more controversial.

Nonetheless, the control of T cell-mediated immune responses by IL-12 suggests that the IL-12/IL-12R pathway may have major therapeutic potential and has been a high priority in the field of tumor immunotherapy.

Target Assessment

IL-12 is typically measured by a standard ELISA assay in serum (Gately et al. 2001), while IL-12R can be detected via immunofluorescence and flow cytometry of immune cells from peripheral blood mononuclear cells or tumor-infiltrating lymphocytes (Zola 2004). There are many diagnostic antibodies that can detect IL-12 and both subunits of the IL-12R that can be used to identify the molecules. In addition, numerous other molecular biology techniques to quantify IL-12 and IL-12R are available, including PCR analysis, Western blotting, proteomics, and more recently Nanostring technology. IL-12 and IL-12R levels are not normally measured prior to, during, or after IL-12 treatment, but some clinical trials have measured serum IL-12 and anti-IL-12 antibody levels, as well as serum IFN-γ levels during and posttreatment to confirm biologic activity.

Role of Target in the Cancer

Rank: 7

The potential for IL-12/IL-12R to serve as a target for tumor immunotherapy is very high and has been well demonstrated in numerous strategies in the preclinical setting. Further, early-phase clinical trials of IL-12 treatment in patients with advanced melanoma and renal cell carcinoma demonstrated some hints of therapeutic response, but toxicity was dose limiting. Given the recent advances in the field of tumor immunotherapy and improved understanding of the basic biology of IL-12 would favor further clinical development of this approach in oncology. Clinical trials with alternate dosing schedules and delivery methods may be especially promising, as well as combination studies with T cell checkpoint inhibitors and other evolving immunotherapy agents.

High-Level Overview

Diagnostic, Prognostic, and Predictive

At present, IL-12 is generally not considered in the diagnostic or prognostic work-up of patients with cancer. There are, however, several interesting potential ways in which IL-12 could be envisioned as a biomarker when considering which patients might benefit most from immunotherapy. In general, interferon-γ and related genes have been highly evaluated as predictors of immune response and therapeutic benefit from immunotherapy. Since IL-12 is involved in the regulation of interferon expression, it would not be surprising if IL-12 were eventually found to be an important

factor in prognostication or in predicting clinical responses. A study in women with cervical cancer, which is caused by the human papillomavirus (HPV), used IL-12 as an immunopotentiator of T cell function. Following IL-12 administration, there was a significant increased cell-mediated immune response to specific HPV antigens, specifically 16, E4, E6, and E7. The authors suggested that such antigen-specific responses might be useful as predictive biomarkers when treating patients with cervical cancer (Wadler et al. 2004).

The potential for IL-12 as a therapeutic approach to treating cancer has been supported by preclinical studies and early-phase clinical trials. Further studies will be needed before IL-12 is fully evaluated as a therapeutic candidate in oncology.

Therapeutics

Preclinical Summary

Early studies in mice suggested that administration of IL-12 could augment cytotoxic NK and T cell responses, induce the production of interferon-γ, and demonstrated therapeutic activity against several transplantable and metastatic murine tumors, including B16 melanoma, Renca renal cell adenocarcinoma, and M5076 reticulum cell sarcoma (Brunda et al. 1993). In these studies, the therapeutic effect was dose dependent and was lost when $CD8^{+}$ T cells were depleted. Comparable therapeutic responses were observed when IL-12 was engineered into recombinant vaccinia virus, but viral delivery was associated with no toxicity in the mice (Kaufman et al. 2002). IL-12 was also expressed in a Newcastle disease virus and demonstrated therapeutic activity against a murine hepatocellular cancer model (Ren et al. 2015). An adenovirus construct encoding murine IL-12 was evaluated in combination with an adenovirus encoding cytosine deaminase, in a murine Renca model of renal cell carcinoma (Hwang et al. 2005). In this study, mice that were given a single dose of both adenovirus vectors followed by intraperitoneal 5-fluorocytosine (which is converted to 5-fluorouracil by the virally expressed cytosine deaminase) demonstrated significant therapeutic responses and an increase in splenic NK cells and interferon-γ production. Collectively, these preclinical studies suggested that IL-12 treatment was associated with therapeutic responses against murine cancers and could be delivered as soluble cytokine or encoded in recombinant viral vectors alone or in combination strategies. These studies helped support the clinical development of IL-12 therapy for patients with cancer. In addition, a number of other novel approaches for IL-12 treatment and delivery have been evaluated in mouse models.

In an attempt to improve the induction of tumor-specific T cell responses, DCs encoding IL-12 were injected directly into the tumor microenvironment of murine Renca or MethA sarcoma tumors (Huang et al. 2012). In this model, therapeutic activity was seen and was associated with both the induction of tumor-specific $CD8^{+}$ T cell responses and infiltration of $CD4^{+}$ and $CD8^{+}$ T cells into the tumor microenvironment. An interesting point in this study was that combinations of different

cytokines, including IL-12, interferon α, and TNF-α, were not better than IL-12-transduced DCs. In an effort to avoid systemic toxicity of IL-12, another strategy has been the local delivery of IL-12 co-formulated with chitosan, a biocompatible polysaccharide derived from crustacean exoskeletons (Vo et al. 2015). Using mice with four spontaneously metastatic breast cancers, mice that received neoadjuvant intra-tumoral injections of chitosan/IL-12 experienced a significant improvement in long-term tumor-free survival compared to control animals. The therapeutic effect was also associated with DTH and tumor-specific T cell responses. The only adverse event reported was a transient and reversible leukopenia. Another approach to alternative delivery of IL-12 utilized adipose-derived mesenchymal stem cells transduced with a lentivirus expressing IL-12 (Li et al. 2015). In this study, A549 lung adenocarcinoma cells were cocultured with IL-12-expressing mesenchymal stem cells and found to decrease tumor cell motility, invasive ability, and Ki-67 expression. This approach has not yet been tested in a therapeutic setting.

An interesting approach would be to combine IL-12 with T cell checkpoint inhibitors that block the PD-1/PD-L1 pathway. Using a HER-2 murine model of breast cancer, tumor growth was completely inhibited by treating the mice with a whole-cell tumor vaccine with cell surface B7.1 molecules and GPI-anchored IL-12 expression and PD-L1 blockade (Bozeman et al. 2015). The therapeutic activity appeared to depend on both $CD4^+$ and $CD8^+$ T cells. Another interesting combination has been evaluated in mice using neoadjuvant treatment of 4T1 breast cancer with a Semliki Forest virus encoding IL-12 and attenuated salmonella therapy (Kramer et al. 2015). In this model, 90% tumor control was seen only when both treatments were used in combination. The use of adoptively transferred T cells, with or without genetic engineering, has been another promising approach in clinical development. Since IL-12 can regulate T cell responses, there has been interest in using IL-12 to enhance the therapeutic effectiveness of adoptively transferred T cell therapy. In one study, murine $CD8^+$ T cells were conditioned with IL-12 ex vivo and were found to be associated with a 10–100-fold increased persistence and improved antitumor activity against an established melanoma when delivered into lymphodepleted mice (Rubinstein et al. 2015). In another approach using a nonviral transposon system to genetically modify T cells, IL-12 was encoded in murine splenocytes isolated from C57BL/6 OT-1 mice (Galvan et al. 2015). The adoptive transfer of these IL-12-expressing splenocytes was able to home to B16 melanoma expressing ovalbumin and could mediate tumor regression.

Clinical Summary

IL-12 has been evaluated in nearly 60 clinical trials that began around 1995 and included studies of recombinant IL-12 and IL-12-expressing autologous fibroblasts targeting several types of cancer, including melanoma, renal cell carcinoma, and breast cancer (Lamont and Adorini 1996; Cohen 1995; Atkins et al. 1997; Leonard et al. 1997). These trials used several different doses, routes of administration, and schedules. In an initial phase I clinical trial, a single priming dose of IL-12 was used,

but this was omitted in a phase II trial and resulted in serious adverse events in 12 of 17 patients, and two treatment-related deaths occurred, resulting in the cessation of IL-12 trials (Cohen 1995). Over a range of studies, the maximum tolerated dose was generally defined between 250 and 500 ng/kg, and the use of an initial priming dose was associated with reduced toxicity (Portielje et al. 1999). The adverse events associated with IL-12 treatment included systemic flu-like symptoms, such as fever, chills, fatigue, arthromyalgias and headaches, bone marrow, and liver dysfunction. In addition, some patients also experienced oral mucositis, colitis, and stomatitis, suggesting a more profound inflammation of mucosal surfaces (Cohen 1995; Atkins et al. 1997; Leonard et al. 1997). The side effects were thought to be related to induction of secondary cytokines, such as IFN-γ and TNF-α, and chemokines, such as IP-10 and MIG (Haicheur et al. 2000). There were a few clinical responses seen in these early studies, which were generally associated with sustained levels of IFN-γ (Wadler et al. 2004). The overall response/adverse event ratio, however, was not particularly strong, and so IL-12 monotherapy studies quickly fell out of favor. There was some attempt to combine IL-12 with other agents, most notably IL-2 for patients with melanoma and renal cell carcinoma, but these studies did not demonstrate durable responses (Weiss et al. 2007).

Despite the lack of success of these early clinical studies, IL-12 has shown more promise in several clinical trials in patients with cutaneous T cell lymphoma (CTCL), HIV-associated Kaposi's sarcoma, and non-Hodgkin's B cell lymphoma (NHL). CTCL is an interesting disease characterized by low levels of Th1 cells and decreased secretion of IFN-γ, suggesting that IL-12 could be especially useful. In a study of ten CTCL patients, eight responses were observed, including two complete responses, when IL-12 was given at doses of 50–300 ng/kg by subcutaneous or intralesional injection twice weekly (Rook et al. 1999). Patients with recurrent or refractory NHL were treated with IL-12 with 11 subjects receiving 250 ng/kg by intravenous delivery daily for 5 days every 3 weeks after a priming dose and 21 subjects receiving 500 ng/kg by subcutaneous injection twice weekly (Younes et al. 2004). Six of 29 evaluable patients (21%) had an objective clinical response. IL-12 was also tested at increasing doses in patients with recurrent NHL in combination with rituximab given at a dose of 375 mg/m^2 (Ansell et al. 2002). In this trial, 69% of subjects exhibited an objective response with a trend toward higher response rates in patients who had higher doses of IL-12. This, however, was difficult to distinguish from responses with rituximab alone. IL-12 has also been tested alone and in combination with pegylated liposomal doxorubicin in patients with HIV-related Kaposi's sarcoma with promising early results (Little et al. 2007). Randomized clinical trials, however, are needed to confirm the potential benefit of IL-12 in these patients.

In order to improve the therapeutic index with IL-12, a number of alternative approaches have entered clinical trials. An IL-12 plasmid incorporated into a nanoparticle composed of synthetic polyethyleneglycol-polyethyleneimine-cholesterol lipopolymer, designated eGeN-001, has been tested by intraperitoneal delivery in patients with ovarian or peritoneal carcinomatosis although no responses were reported (Lenzi et al. 2007). A phase I dose-escalation trial of eGen-001 in women

with recurrent ovarian cancer enrolled 13 patients for four weekly intraperitoneal infusions (Anwer et al. 2010). The treatment was well tolerated with low-grade abdominal pain and discomfort, the most common side effect cited. Six patients demonstrated a drop or stable CA-125 levels with 31% response rate. In a similar trial, patients with recurrent platinum-sensitive ovarian cancer received eGen-001 with carboplatin and docetaxel (Anwer et al. 2013). An objective response rate of 50% was reported with 17% complete responses and an additional 42% of patients with stable disease. While these initial studies appear promising, they are complicated by small sample sizes and lack of randomized designs, thus limiting the impact of the results.

Another novel strategy being developed for IL-12 therapy is the intra-tumoral delivery of an IL-12 plasmid, which delivers IL-12 to the local tumor microenvironment while preventing systemic toxicity. The approach was initially tested with promising results in preclinical models, but early-phase clinical trials in melanoma were complicated by low response rates thought to be due to low gene transfer efficiencies (Heinzerling et al. 2005; Mahvi et al. 2007). To improve the efficiency of gene transfer, the addition of electroporation to the plasmid delivery is thought to enhance gene delivery and increase therapeutic responses. A phase I dose-escalation clinical trial of electroporated IL-12 plasmids was completed in 24 patients with metastatic melanoma (Daud et al. 2008). Treatment was associated with minimal injection site pain, local IL-12 production, tumor necrosis, and 53% clinical response rates. Based on these promising results, additional trials in other cancers, including Merkel cell carcinoma, are underway.

As mentioned in preclinical studies, IL-12 may be useful in enhancing the activity of adoptively transferred T cells. There are already two melanoma studies in which genetically modified lymphocytes designed to secrete IL-12 are being tested. In one trial, 33 patients with metastatic melanoma were treated with IL-12-transduced TILs in which the IL-12 gene was driven by the nuclear factor of activated T cell (NFAT) promoter (Zhang et al. 2015). Sixty-three percent of patients (10/16) demonstrated an objective response, but therapeutic effects were generally short with few cells persisting beyond 4 weeks, and higher cell doses were associated with significant adverse events, including high fevers, hemodynamic instability, and hepatic dysfunction. The authors concluded that further refinements were needed before pursuing this approach further.

Other strategies for local IL-12 delivery include innovative approaches for recombinant viruses encoding the IL-12 gene. Using a novel RheoSwitch Therapeutic System® (RTS®), an adenovirus was engineered with two unstable heterodimeric receptor proteins that bind to an inducible promoter that regulates IL-12 expression (Linette et al. 2013). This system utilizes an oral small molecule activator ligand (INXN-1001), which enhances stable conformation of the heterodimeric proteins enabling induction of IL-12 transcription. This system was being evaluated in several clinical trials for patients with advanced melanoma and breast cancer. Dendritic cells can be fused to tumor cells as a vaccine strategy, and this allows presentation of an array of potential tumor-associated antigens from individual tumors by professional antigen-presenting cells. This approach is being

used for a variety of cancers in combination with IL-12 cytokine treatment, although clinical data awaits maturation (Avigan et al. 2012). Finally, IL-12 can be included in the development of immunocytokine therapy, in which IL-12 is fused to a monoclonal antibody with tumor antigen specificity. This approach theoretically allows better tumor targeting to the tumor microenvironment while reducing the systemic toxicity of IL-12. In a phase I trial, the immunocytokine termed AS1409, which targets the extra-domain B of fibronectin and is linked to IL-12, was tested in 13 patients with melanoma and renal cell carcinoma (Rudman et al. 2011). The treatment was well tolerated with one objective response and five patients with stable disease. NHS-IL12 is a fusion protein consisting of two IL-12 molecules conjugated to a humanized monoclonal antibody targeting necrotic portions of tumor due to its high affinity to single- and double-stranded DNA. This agent is entering into phase I clinical testing.

Anticipated High-Impact Results

Although IL-12 has clearly demonstrated the therapeutic activity against cancer in a variety of preclinical models, the clinical development of IL-12 has been slow and hampered by initial reports of excessive toxicity. Thus, it is highly anticipated that IL-12 will require a novel development pathway in oncology, and a variety of possible suggestions have been confirmed in preclinical models but await clinical investigation. The following represent important high-impact results that might be anticipated in the next few years:

- Better understanding of the differences between IL-12 and other IL-12-related cytokine family members, which may help guide more rationale clinical drug development.
- Additional preclinical development of novel combinations that include IL-12 as part of the treatment strategy.
- IL-12 will likely be included as a putative predictive biomarker of immunotherapy response with other agents in clinical development, and attention to the impact of IL-12 in large biomarker trials will be of high priority.
- The use of IL-12 in an ex vivo or local delivery manner to promote other forms of tumor vaccines and adoptive T cell transfer will likely be in clinical trials.

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Interleukin-15

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Abstract

Interleukin-15 (IL-15) is a gamma-C (γC) cytokine that stimulates the differentiation and proliferation of T, B, and natural killer (NK) cells. Many clinical and preclinical studies have focused on exploiting memory CD8 T cells and NK cells to treat cancer due to their ability to recognize tumor cells, be rapidly activated, and produce many antitumor cytokines, cytotoxic granules, and surface ligands that promote cell death. Memory CD8 T and NK cells respond robustly to IL-15 compared to naive and effector T cells and are thought to be the primary target of IL-15's function in vivo. In response to IL-15 or therapeutic soluble IL-15/IL15 receptor complexes, CD8 T cells and NK cells increase production of cytotoxic granules and migratory capacity which promotes antitumor immunity. Thus,

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IL-15 plays a critical role in the immune system's ability to eliminate tumor cells and has proved to be a promising target for cancer immunotherapy.

Keywords
Interleukin-15 • Metastatic renal cell carcinoma • Malignant melanoma • Cytokine therapy • Adoptive cell therapy • NK cells • T cells

Despite being discovered in 1994, clinical evaluation of IL-15 as a vaccine adjuvant and interventional therapy has only recently gained momentum. In 2007 the United States National Cancer Institute (NCI) Immunotherapy Agent Workshop ranked IL-15 as the top priority of potential immunotherapy agents for clinical investigation.

This chapter will discuss the potential for IL-15's use in tumor therapy by reviewing preclinical findings and highlighting recently initiated clinical studies. IL-15's effect on the tumor-specific immune response will be compared to that of the widely used cytokine therapy IL-2.

Biology of the Target

IL-15 is a member of the gamma-C (γC) cytokine family, a group of cytokines that signal through a shared common gamma receptor subunit. Members of the γC cytokine family include IL-2, IL-4, IL-7, IL-9, and IL-21. Despite similar biological properties to IL-2, IL-15 and IL-2 share no structural homology. IL-15 and IL-2 signal through a heterotrimeric receptor complex comprised of a shared beta (IL-2/15Rβ) and gamma (γC or CD132) subunit along with their unique alpha subunits, IL-15Rα (CD215) and IL-2Rα (CD25), respectively. IL-15Rα and IL-2Rα share structural homology; however, IL-15 can bind to IL-15Rα (1,000-fold higher than IL-2 to IL-2Rα) with very high affinity even in the absence of IL-2/15Rβ and γC subunit (Wang et al. 2009). Due to this high-affinity interaction, IL-15 possesses a unique functional characteristic in that it can act on cells through *cis* and *trans* signaling mechanisms. IL-15 is expressed on the surface bound to IL-15Rα predominantly by monocytes, macrophages, dendritic cells, and stromal cells and can be *trans*-presented to IL-15Rβ and γC chains on neighboring T cells and NK cells (Dubois et al. 2002). IL-15/IL-15Rα expression can be upregulated by type I interferons (e.g., IFN-α) and toll-like receptor agonists (Mattei et al. 2001). In addition to being expressed together on the cell surface, IL-15/IL-15Rα complexes can be detected in the serum (Bergamaschi et al. 2012). Soluble IL-15/IL-15Rα complexes or free IL-15 can bind to and signal through IL-15Rβ/γC. The biological effects of IL-15 on CD8 T cells and NK cells are mediated through signaling of the Jak1 and Jak3 pathways associated with the IL-15Rβ and γC chains, respectively, which subsequently induce activation of STAT3 and STAT5. Downstream targets of STAT3/STAT5 Bcl-2, MAP kinase pathways, and the PI3K-Akt pathways can prevent apoptosis and induce proliferation and differentiation of CD8 T cells and NK cells. Preclinical studies demonstrate that in vivo administration of IL-15 or

IL-15/IL-15Rα complexes selectively induces robust expansion and enhances cytotoxicity of memory CD8 T cells, NK cells, and NKT cells (Stoklasek et al. 2006; Rubinstein et al. 2006). IL-15 complexed with IL-15Rα enhances the potency of IL-15 in vivo by increasing its affinity for IL-15Rβ/γ subunits, its half-life, and its bioavailability (Dubois et al. 2002; Stoklasek et al. 2006; Bergamaschi et al. 2008).

Target Assessment

IL-15 mRNA transcripts are readily detectable by PCR in many tissues but mRNA levels do not correspond to level of detectable IL-15 protein. With the current available methods of detection, free IL-15 is relatively undetectable in human serum, while only IL-15/IL-15Rα complexes are present at detectable levels in the sera. IL-15/IL-15Rα complexes can be detected by enzyme-linked immunosorbent assay (ELISA) in metastatic melanoma patients, and soluble IL-15/IL-15Rα levels are increased transiently after non-myeloablative chemotherapy (cyclophosphamide/ fludarabine) or ablative total body irradiation (2–12Gy) (Bergamaschi et al. 2012; Dudley et al. 2008). However, expression of surface IL-15Rα can be detected independently by flow cytometry using commercially available monoclonal antibodies.

Role of Target in Cancer

Rank: “unknown” to 10
Unknown to 1-2-3-4-5-6-7-8-9-10: 8

High-Level Overview

Diagnostic, Prognostic, and Predictive

The value of IL-15 as a prognostic indicator is unclear and likely dependent on the type of cancer and disease stage. In the case of some leukemias, e.g., T-cell large granular lymphocyte leukemia (T-LGL), adult acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and multiple myeloma (MM), detection of high serum concentrations of IL-15 may correlate with disease progression as IL-15 has the ability to induce proliferation and enhance survival of malignant lymphocytes (Pappa et al. 2007; Chen et al. 2012). Other reports suggest levels of IL-15 in the serum after hematopoietic stem cell transplants are predictive of GVHD and relapse (Thiant et al. 2010). However, the presence of IL-15 might also be predicative of engraftment of stem cell transplants and expansion of adoptive cell therapies. For example, the presence of IL-15 in serum 15 days after autologous hematopoietic stem cell transplant correlates with overall survival and progression-free survival in

non-Hodgkin's lymphoma by supporting the expansion and cytotoxic function of NK cells (Porrata et al. 2010).

Therapeutics

Unlike IL-2, IL-15 is not currently approved for the treatment of malignancies. Its efficacy as a cancer therapy has not been thoroughly validated through clinical trials. However, results from preclinical studies demonstrate the efficacy of IL-15 as a vaccine adjuvant and immunotherapeutic to enhance cytotoxicity of T cells and NK cells and induce tumor regression (Waldmann 2006). These results along with its functional similarities to IL-2, ability to inhibit IL-2-mediated AICD, and selectivity for cytotoxic cell populations provide strong evidence that IL-15 immunotherapy could prove to have greater clinical efficacy than IL-2 in the treatment of metastatic melanoma or metastatic renal cell carcinoma (mRCC). In clinical settings, only 10–15% of patients exhibit complete responses to IL-2 therapy. In addition, high-dose IL-2 therapy has significant toxicities associated with it and safe administration requires a certain level of expertise. IL-2 therapy also commonly causes capillary leak syndrome, which results in accumulation of fluid in the extravascular space (Schwartz et al. 2002). Conversely, IL-15 is not associated with capillary leak syndrome and may prove to have less toxicity than IL-2.

Several considerations should be made when designing studies to evaluate the clinical efficacy of IL-15 therapy in cancer. Increased IL-15Rα is detected in patients with T-LGL (Chen et al. 2012). Peripheral blood mononuclear cells (PBMC) from some T-LGL patients exhibit enhanced proliferation in the presence of IL-15 compared to healthy donor PBMCs. Since IL-15 exerts potent selective effects on cells of hematopoietic origin, it should be considered that treatment of hematological malignancies with IL-15 could potentially contribute to their pathogenesis. To this end, the efficacy of blocking IL-15 signaling through anti-CD122 (anti-IL-2/15Rβ) therapy is being assessed in T-LGL (NCT00076180).

IL-15 can signal through PI3K-Akt and MAP kinase pathways, and combining IL-15 with current therapeutic strategies for mRCC like mTOR (mammalian target of rapamycin) inhibitors (used to treat metastatic renal cell carcinoma) could reduce the effectiveness of IL-15 by inhibiting the activation of some of IL-15's downstream targets.

Preclinical Summary

The effectiveness of IL-15 as both a vaccine adjuvant and means to enhance tumor-specific immune responses has been demonstrated in numerous preclinical models. Using murine models of colorectal cancer and metastatic melanoma, IL-15 has been shown to directly act on NK cells and CD8 T cells to limit tumor growth (Klebanoff et al. 2004; Kobayashi et al. 2005). The antitumor effects of IL-15 are largely dependent on NK cells. *Trans*-presentation of IL-15 to NK cells within the tumor

microenvironment is essential and impacts their ability to lyse tumor cells and can protect them against tumor-mediated immunosuppression (Muller 2012; Liu et al. 2012). In a number of tumor models, tumor-specific CD8 T cells respond poorly to tumor antigens and are either exhausted or anergic. In spite of this, IL-15 has been shown to reverse CD8 T-cell anergy to self-antigens and restore their ability to respond to established tumors (Teague et al. 2006). In addition, IL-15 plays a critical role in the generation of CD8 T-cell responses, and its potential use in conjunction with vaccines has been explored in animal models (Steel et al. 2012; Waldmann 2006). These studies ascertain that IL-15 expressed on DCs during priming mediates the generation of long-lived high avidity, cytotoxic CD8 T cells and can overcome the need for CD4 help. Furthermore, in nonhuman primate models, administration of IL-15 along with peptide vaccines induces long-lived memory CD8 T cells that confer protection against simian immunodeficiency virus (SIV) and was shown to be superior to IL-2 as an adjuvant (Berzofsky 2012; Oh et al. 2003). Collectively, these studies provide promise for the potential use of IL-15 in combination with DC-peptide vaccines in cancers.

In preparation for the use of IL-15 as a tumor immunotherapeutic in clinical trials, the safety, pharmacokinetics, effective dosage regimens, and immunologic effects of soluble IL-15 were assessed in nonhuman primates. Human and macaque IL-15 share a 97% sequence homology suggesting a suitable preclinical model for the assessment IL-15 safety and toxicity. Doses up to 200 mg/Kg of recombinant human IL-15 have been evaluated for toxicity. Administration of 10 mg/Kg of soluble recombinant human (rh)IL-15 by either subcutaneous injection or intravenous infusion has a half-life ($t_{1/2}$) of 0.92–1.31 h and is undetectable after 24 h, thus increasing the probability for a daily dosage regimen (Berger et al. 2009; Waldmann et al. 2011). Alternatively, the short half-life of IL-15 can be potentially augmented through the use of IL-15/IL-15Rα complexes, and its clinical investigation is warranted. The activity of IL-15 in vivo is enhanced when administered as a complex of IL-15 and IL-15Rα. In murine models of metastatic melanoma and colorectal cancer, IL-15 treatment prolonged survival, decreased tumor burdens, and enhanced CD8 T cell and NK cell function. What's more, these effects were further enhanced by treatment with IL-15/IL-15Rα complexes (Stoklasek et al. 2006; Bessard et al. 2009). Thus the use of soluble IL-15 in a clinical setting may not prove to be as effective as preclinical studies suggest. However, IL-15/IL-15Rα complexes could yield more success in achieving effective antitumor responses.

Clinical side effects of daily IL-15 administration include weight loss, diarrhea, emesis, and loss of appetite. IL-15 induced severe (Grade 3/4) neutropenia in the blood likely due to redistribution of neutrophils from circulation to peripheral tissues (Waldmann et al. 2011). IL-15 administered at higher doses results in enlargement of lymph nodes, spleen, and liver that is associated with an increased numbers of leukocytes. Furthermore, administration of IL-15 induced robust proliferation and accumulation of NK cells and memory CD8 and CD4 T cells (Berger et al. 2009; Waldmann et al. 2011; Sneller et al. 2011). More importantly these studies show that the overall toxicity of IL-15 is less than that of IL-2 and thus a potentially safer therapy.

Clinical Summary

Until recently, the availability of clinical grade IL-15 has constrained its use in immunotherapy clinical trials. However, the US National Cancer Institute (NCI) began manufacturing GMP recombinant human IL-15, and clinical evaluation of IL-15 therapy has recently begun. At this time, evaluation of the IL-15 therapy in metastatic malignant melanoma and mRCC is limited to phase I and II trials that will assess safety and optimal dosing regimens of intravenous recombinant human IL-15 (NCT01021059, NCT01572493). Additionally, these trials will determine if intravenous administration of IL-15 can boost reconstitution of transferred tumor-reactive cells following adoptive cell therapy in several cancers. For instance, in metastatic melanoma patients, reconstitution will be measured after daily intravenous infusions of IL-15 for 10–12 days following lymphodepleting chemotherapy (cyclophosphamide/fludarabine) and adoptive cell therapy (ACT) of tumor-infiltrating lymphocytes (TIL) (NCT01369888). The ability of IL-15 to limit regulatory $CD4^+$ T-cell reconstitution will also be assessed. An additional study will establish the minimum efficacious (MED) and maximum tolerated dose (MTD) of IL-15 to enhance the acute expansion of haploidentical donor NK cells to target refractory myelogenous leukemia (AML) following lymphodepleting chemotherapy (NCT01385423). The secondary objectives in both of these studies include evaluation of tumor responses and IL-15 pharmacokinetics. Currently, IL-2 is administered to refractory AML patients following NK cell adoptive transfer but unfortunately, NK cell expansion remains poor in a number of patients. However, preparative regimens of high-dose cyclophosphamide/fludarabine prior to ACT induced increased serum concentrations of IL-15 and enhanced NK cell expansion (Miller et al. 2005). IL-15 may also enhance the expression of NK-activating receptors NKp30, NKp46, NKG2C, and NKG2D and thus increase NK cell cytotoxicity in AML patients (Szczepanski et al. 2010).

IL-15 has been previously investigated as a potential adjuvant in HIV-1 DNA vaccines (NCT00775424, NCT00115960, NCT00528489, and NCT00195312), and a study of its efficacy in boosting dendritic cell vaccines to melanoma was recently initiated (NCT01189383). Patients with resected stage IIIc and stage IV melanoma will receive autologous dendritic cells (DC) manufactured with GM-CSF, IL-15, KLH, and melanoma antigens and then activated with CD40 and LPS. The quality of the melanoma-specific immune response induced by four doses of the IL-15 DC vaccine will be evaluated by assessing the generation of long-lived, protective, melanoma-specific memory CD8 T cells as well as their ability to contribute to tumor regression and disease-free survival rates.

A more recent study aims to expand the evaluation of IL-15 immunotherapy to a broader range of advanced solid tumors (NCT01727076). Results from these studies are expected to yield valuable information regarding IL-15's immunotherapeutic potential, and if clinical data supports earlier preclinical findings, it can potentially replace IL-2 as the standard cytokine therapy for metastatic malignant melanoma and mRCC.

Anticipated High-Impact Results

- Establish pharmacokinetic parameters to determine safe and low-toxicity IL-15 dosing regimens.
- Determine if IL-15 monotherapy can induce effective antitumor responses in metastatic melanoma and mRCC.
- Enhance ACT engraftment and antitumor responses using IL-15 immunotherapy post-transfer.
- The addition of IL-15 as a therapeutic vaccine adjuvant to induce effective persistent antitumor responses to melanoma and mRCC.
- Investigate the use of IL-15/IL-15Rα complexes to improve efficacy of IL-15 therapy.

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Interleukin-21

31

Rosanne Spolski and Warren J. Leonard

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Abstract
Interleukin-21 is a type I cytokine with actions on both lymphoid and myeloid cells. Soon after its discovery, IL-21 was shown to induce cytotoxic activity in both $CD8^+$ T cells and NK cells, leading to the elucidation of its antitumor activity in animal models. This preclinical work led to the inclusion of IL-21 in human clinical trials in patients with advanced solid tumors, including melanoma and renal cell carcinoma. Encouraging results were seen with the use of IL-21 as a single agent, and it is possible that IL-21 might yield enhanced results as a component of combination therapy or in adoptive immunotherapy approaches for the generation of $CD8^+$ T cells with potent antitumor activity.

Keywords
IL-21 • Anti-tumor • $CD8^+$ T cells • NK cells

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Target: Interleukin-21 (IL-21)

Interleukin-21 (IL-21) is a four-α-helical bundle type I cytokine with a molecular weight of approximately 15.6 kDa that signals through a heterodimeric receptor composed of a unique IL-21R chain and the common cytokine receptor gamma chain, γ_c (Spolski and Leonard 2008; Parrish-Novak et al. 2000; Ozaki et al. 2000). γ_c is encoded by the gene that is mutated in humans with X-linked severe combined immunodeficiency (XSCID) (Noguchi et al. 1993) and is also shared by the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (Spolski and Leonard 2008). IL-21 induces the activation of the JAK1 and JAK3 (Janus associated tyrosine kinases 1 and 3), to mediate the activation of the STAT (signal transducer and activator of transcription) protein STAT3 and to a lesser extent STAT1 and STAT5 (Zeng et al. 2007; Konforte and Paige 2006). IL-21 has structural homology with IL-15, IL-2, and IL-4 (Parrish-Novak et al. 2000). IL-21 is expressed by $CD4^+$ populations of T cells and NKT cells (natural killer T cells) and has pleiotropic effects within the immune function, with broad expression of the IL-21R on a wide array of target populations including lymphoid cells, myeloid cells, epithelial cells, and keratinocytes (Spolski and Leonard 2008).

Biology of the Target

IL-21 has profound effects on both the innate and adaptive immune system (Spolski and Leonard 2008). It plays a critical role as an inducer of terminal B-cell differentiation and antibody production through its induction of the master transcription factor BLIMP1 (B-lymphocyte maturation protein 1), although BLIMP1 is now known to exert broader actions than just in B cells (Ozaki et al. 2004). Moreover, defective signaling by the combination of IL-21 and IL-4 appears to explain the basis for defective B-cell function in humans with XSCID (Ozaki et al. 2002). IL-21 also induces the proliferation and activation of both $CD8^+$ T cells and NK cells, either alone or in combination with other cytokines (Zeng et al. 2005; Brady et al. 2004). Moreover, IL-21 plays a major role in the differentiation of IL-17-producing $CD4^+$ T cells, a population involved in mediating inflammatory responses (Korn et al. 2007; Zhou et al. 2007). Although IL-21 has these immunostimulatory functions, it also has direct immunosuppressive actions on B cells by inducing apoptosis (Ozaki et al. 2004; Jin et al. 2004; Kasaian et al. 2002) as well as on dendritic cells (Brandt et al. 2003), and IL-21 is also known to be an inducer of IL-10 (Spolski and Leonard 2010; Pot et al. 2009), a potent suppressive cytokine. Although endogenous IL-21 plays no apparent role in antitumor activity (Sondergaard et al. 2009), IL-21 has potent antitumor activity mediated by its effects on $CD8^+$ T cells and NK cells (Spolski and Leonard 2008).

Target Assessment

IL-21 levels can be measured in the serum by commercially available ELISAs and have also been qualitatively assessed in tissue samples by immunohistochemistry.

Role of the Target in Cancer

Rank: 5

Editors' note: The editors have assigned IL-21 a ranking of 5. While there is considerable preclinical data supporting a therapeutic effect of IL-21 in cancer treatment and early phase clinical trials have demonstrated an acceptable safety profile, there is so far little evidence of clinical activity with IL-21 as a single agent. The pleiotropic effects of IL-21 might allow for use in specific contexts, such as part of combination regimens. This potential for a therapeutic role, however, must await a better understanding of IL-21 biology and further clinical development. The authors neither agree nor disagree with the assigned Rank, as we do not know the criteria for making such an assessment.

High-Level Overview

IL-21 has possible therapeutic potential in cancer based on a number of studies. First, IL-21 is proapoptotic for a number of cells, including B cells, and thus has potential to eliminate certain tumor cells based on this effect. Second, IL-21 exerts a range of actions on T cells and NK cells, through which it can modulate antitumor activity. This combination of actions of IL-21 is the basis for anticancer activity for this cytokine.

Preclinical Summary

IL-21 has been studied in a number of animal tumor models, and these studies have confirmed the potent antitumor activity of this cytokine (Skak et al. 2008). Although endogenous IL-21 seems to play no role in antitumor activity, exogenously administered IL-21 could lead to the inhibition of growth of large pre-established melanomas (Wang et al. 2003). This effect was mediated primarily by NK cells and only minimally by $CD8^+$ T cells. Intraperitoneal administration of IL-21 was more effective than either IL-2 or IL-15 in prolonging the survival of mice bearing thymomas, and this survival was associated with the persistent presence of a memory $CD8^+$ T-cell population (Moroz et al. 2004). Intra-tumor injection of IL-21 was more effective than subcutaneous injection at inhibiting melanoma growth and increasing survival, and this delivery method augmented local activation of tumor infiltrating

CD8$^+$ T cells (Sondergaard et al. 2010). IL-21 was also highly effective at eradicating large melanomas when administered in combination with a tumor-antigen-specific vaccine, and this effect could be amplified by combination treatment with both IL-21 and IL-15, consistent with synergistic effects of these cytokines on CD8$^+$ T-cell proliferation (Zeng et al. 2005). Combination therapy of IL-21 in conjunction with chemotherapeutic drugs showed additive effects on tumor inhibition but only when IL-21 treatment was delayed relative to drug treatment (Skak et al. 2009).

In addition to studies demonstrating the powerful antitumor effects of exogenously administered IL-21, attention has also been focused on using IL-21 to engineer more effective tumor-specific CD8$^+$ T cells for adoptive immunotherapy.

Human CD8$^+$ T cells engineered to constitutively overexpress IL-21 had enhanced ability to eradicate lymphomas in immunodeficient mice (Markley and Sadelain 2010). Tumor-specific CD8$^+$ T cells that were primed in vitro in the presence of IL-21 and then adoptively transferred into mice displayed a superior ability to eradicate large melanomas, as compared to cells primed with either IL-2 or IL-7 or IL-15, and these IL-21-primed cells displayed a less differentiated phenotype and exhibited enhanced persistence in vivo (Hinrichs et al. 2008).

Clinical Summary

Since its approval for clinical trials in 2005, IL-21 has been evaluated in a number of Phase I and Phase II trials for malignant melanoma, renal cell carcinoma, and non-Hodgkin's lymphoma (Hashmi and Van Veldhuizen 2010). In the initial dose-escalation studies, IL-21 was found to have significant biological activity on both CD8$^+$ T-cell and NK-cell populations, even at the lowest dose tested (Frederiksen et al. 2008). In keeping with the results from animal models, IL-21 was well tolerated with only minimal adverse effects that include fatigue, fever, and electrolyte abnormalities. In contrast to IL-2, IL-21 has not been observed to induce capillary leak syndrome. In one completed Phase II trial for metastatic melanoma in Australia, there were 4% complete and 4% partial responses as well as 37% in whom disease was stable (Davis et al. 2009). An ongoing Phase II trial in Canada for metastatic melanoma has shown 29% partial responses and 33% in stable disease (Hashmi and Van Veldhuizen 2010). An ongoing Phase II study for renal cell carcinoma in which IL-21 was used in conjunction with sorafenib showed 21% partial responses and 61% in stable disease (Hashmi and Van Veldhuizen 2010). In summary, although the complete response rates for melanoma are lower for IL-21 than those achieved with IL-2 treatment (14%), there is hope and promise for the use of IL-21 in the combination therapies or in the adoptive immunotherapy strategies that have been highly successful in the preclinical animal studies. A Phase I clinical trial combining IL-21 and cetuximab in metastatic colorectal cancer has now also been completed, with a total of 15 patients (Steele et al. 2012). A maximum dose of 100 ug/kg was used and was well tolerated with adverse events $\leq$ grade 2, and a total of 60% of the patients had stable disease. A number of T-cell and NK-cell activation markers were

evaluated to confirm immune activation, including dose-dependent induction of soluble IL-2Rα expression.

Anticipated High-Impact Result

More information will be forthcoming from ongoing clinical trials, but it is difficult to predict with certainty how Phase II trials will extend to Phase III clinical trials and beyond.

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Conflict of interest statement: R.S. and W.J.L. are inventors on patents and patent applications related to IL-21.

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Lymphocyte Activation Gene 3 (LAG-3) 32

Joel C. Sunshine and Evan J. Lipson

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Abstract

Lymphocyte activation gene 3 (LAG-3, CD223) is a protein expressed on the surface of activated T cells, regulatory T cells (Treg), natural killer (NK) cells, B cells, and plasmacytoid dendritic cells. LAG-3 signaling inhibits T cell activation and enhances regulatory T cell function (Camisaschi et al. 2010; Grosso et al. 2007; Joosten et al. 2007; Park et al. 2012). Like other molecules such as cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), and T cell immunoglobulin mucin-3 (TIM-3), LAG-3 has attracted interest in oncology for its role as a negative regulator of T cell activation – an immunological

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"checkpoint" – that may play a role in helping tumors evade effective immune surveillance.

This chapter will present a brief discussion of the molecular structure and biologic function of LAG-3 as a therapeutic target. The current role of LAG-3 in cancer with attention to pertinent preclinical and clinical data will be described. Finally, the potential impact and future directions of research into the optimal use of LAG-3 as a therapeutic target will be presented.

Keywords
LAG-3 • Immunotherapy • T cell • Cancer

Biology of the Target

LAG-3, a 51 kDa member of the immunoglobulin superfamily, plays a critical role in the regulation of T cell function. T cell activation requires two main signals: Signal 1 is transmitted through an antigen-specific interaction between the T cell receptor (TCR) and an antigenic peptide presented in the context of major histocompatibility complex (pMHC). The second signal is transmitted through costimulatory molecules that modify the T cell response. When the T cell receives signal 1 in the context of costimulation via signal 2, the T cell responds by proliferating, acquiring effector function, and migrating to sites where the antigen is expressed. However, the stimulatory effect of Signal 2 can be thwarted, as a number of immunological checkpoint molecules are also present which inhibit T cell activation. Physiologically, these regulatory molecules prevent an over-exuberant response which would otherwise lead to autoimmunity. Among these checkpoints is CTLA-4 (Walunas et al. 1994), which serves as a major modulator of central inhibition, as well as PD-1 (Keir et al. 2008), TIM-3 (Sabatos et al. 2003), and LAG-3 (Grosso et al. 2007), which may have stronger roles in promoting peripheral tolerance.

The gene-encoding LAG-3 is located on the distal part of the short arm of chromosome 12. LAG-3 is a type I transmembrane protein with 4 extracellular immunoglobulin-superfamily domains, with conserved structural motifs found throughout (Triebel et al. 1990). Although it is a structural homologue of CD4, it shares less than 20% sequence identity. Importantly, the IgG domain farthest from the membrane (D1) contains a unique extra loop not present on any CD4 molecule identified at this point. On the cytoplasmic side, LAG-3 has a short intracellular portion with a unique conserved "KIEELE" motif which is required for LAG-3 modulation of T cell function and which plays a key role in regulating the expansion of activated T cells (Workman and Vignali 2003). The extracellular component of LAG-3 can be cleaved by TNF alpha converting enzymes (TACE). Cleavage appears to be important for termination of LAG-3 signaling and abrogation of the negative regulatory effect of LAG-3, as expression of a non-cleavable form of LAG-3 results in an irreversible defect in T cell function (Li et al. 2007).

Like CD4, LAG-3 binds to MHC class II molecules, but does so with several orders of magnitude higher affinity (60 nM for LAG-3 vs. 10^{-4} M for CD4) and on a different binding site (Huard et al. 1995, 1997). In addition, LAG-3 is expressed only on activated T cells and colocalizes with CD8 and CD3/TCR complexes. It is five to eightfold overexpressed on CD8 vs CD4 cells (Hannier and Triebel 1999; Woo et al. 2010), suggesting that it may have a primary role in the regulation of CD8+ effector T cells. The majority of LAG-3 is retained intracellularly and rapidly translocated to the cell surface on stimulation via protein kinase C-dependent signaling (Bae et al. 2014).

LAG-3 appears to play a critical role in mediating peripheral tolerance to both self and tumor antigens primarily via its effects on CD4+ T cells, CD8+ T cells, and Tregs. CD4+ effector T helper cells that express LAG-3 have reduced proliferative capacity (Huang et al. 2004). High LAG-3 expression on "exhausted" CD8+ T cells contributes to their unresponsive state, limits CD8+ T cell antitumor responses, and maintains tolerance to self and tumor antigens (Grosso et al. 2007). LAG-3 expression on Tregs results in increased suppressive capacity in FoxP3+ CD8+ (Joosten et al. 2007), CD4+ CD25+ (Camisaschi et al. 2010), and FOXP3+ CD4+ cells in the tumor microenvironment, in the context of a broad increase in the expression of multiple other co-inhibitory receptors (Park et al. 2012). These data, when taken together, suggest that blocking LAG-3 might enhance antitumor responses by promoting T cell activity and reducing the suppressive effects of Tregs in the tumor microenvironment.

Given the critical role LAG-3 appears to play in peripheral tolerance, it is perhaps surprising that LAG-3 knockout mice exhibit minimal immunological sequelae, with no evidence for autoimmunity in non-autoimmune-prone strains (Okazaki et al. 2011). However, in non-obese diabetic (NOD) mice that develop autoimmune diabetes, LAG-3 deficiency exaggerated the onset and penetrance of type I diabetes mellitus (Okazaki et al. 2011). This may be due to the presence of other immunological checkpoints involved in peripheral tolerance such as PD-1. Indeed, dual knockout mice (LAG3-/-, PD1-/-) experience lethal autoimmune myocarditis and autoimmune infiltrates in multiple organs (Okazaki et al. 2011).

While the interaction between T cell surface-expressed LAG-3 and MHC class II downregulates T cell activity, and cleavage of LAG-3 shuts off LAG-3 signaling at the T cell level, the soluble form of LAG-3 appears to be able to interact with class II MHC and stimulate dendritic cells, thereby enhancing immune responses (Subramanyam et al. 1998). These data lead to preclinical testing of recombinant soluble LAG-3-Ig as an adjuvant for viral or cancer vaccines as well as a therapeutic in cancer. More recently, however, studies have suggested that signaling through MHC II via the LAG-3 interaction likely contributes to the ability of melanoma tumors to evade destruction by the immune system. Plasmacytoid dendritic cells (pDCs) are often tolerogenic, and LAG-3+ pDCs are enriched in the tumor microenvironment. The MHC II/LAG-3 interaction results in activation of these tolerogenic DCs, enriched secretion of IL-6, and leads to a more suppressive tumor microenvironment (Camisaschi et al. 2014). Additionally, MCH II expression on melanoma cells and activation of MHC II signaling by either soluble LAG-3 or cells expressing LAG-3 results in protection of the MHC II+

melanoma cells from FAS-mediated and drug-induced apoptosis. This suggests that the LAG-3/MHC II interaction results in a bidirectional immune escape pathway in melanoma, reducing the activity of tumor infiltrating lymphocytes and directly protecting the tumor from apoptosis. This hypothesis supports the idea that one approach to treating melanoma might be blockade of the LAG-3 MHC II interaction (Hemon et al. 2011).

Target Assessment

LAG-3 expression can be measured on the surface of T cells in the peripheral blood by flow cytometry (Camisaschi et al. 2010, 2014; Grosso et al. 2007). In tissue samples, LAG-3 expression can be assessed by immunohistochemistry (Camisaschi et al. 2014). At present, there are no guidelines for measuring LAG-3 expression in the clinic, outside of a research setting.

Role of the Target in Cancer

Rank: 7

High-Level Overview

Diagnostic, Prognostic, and Predictive

At present, there are no clinically validated biomarkers of response to LAG-3 therapy. One active area of investigation involves the evaluation of LAG-3 expression on tumor infiltrating lymphocytes.

Preclinical Summary

Blocking antibodies against LAG-3 have been developed and tested as a way of generating a productive immunological response against tumors. LAG-3 blockade in vitro on human T cells results in increased CD4+ and CD8+ T cell proliferation, increased expression of activation antigens such as CD25 and CD69, and secretion of T_H1 type pro-inflammatory cytokine production when challenged with antigen (Huard et al. 1994; Macon-Lemaitre and Triebel 2005). LAG-3 blockade in mice results in enhanced T cell proliferation and effector function in a self-tolerance model where mice express a protein from influenza as a self-antigen (Grosso et al. 2007). In addition, LAG-3 blockade in mice in combination with antitumor vaccination results in increased accumulation of prostate tumor-specific CD8+ T cells, increased cytolytic activity of those tumor-targeted endogenous CD8+ T cells, and delayed tumor outgrowth and tumor disruption (Grosso et al. 2007).

Preclinical data suggest that combinatorial therapy involving blockade of LAG-3 and other co-expressed immunoregulatory molecules may be synergistic. T cells become progressively inactivated ("exhausted") after continual exposure to antigen, and these exhausted T cells are characterized not only by the expression of LAG-3, but also of other

immune checkpoints such as CTLA-4 and PD-1. Extensive co-expression of PD-1 and LAG-3 on tumor-infiltrating CD4+ and CD8+ T cells has been shown in transplantable melanoma, colorectal adenocarcinoma, and fibrosarcoma tumors, and in tumor samples from patients with melanoma and ovarian cancer (Woo et al. 2012; Goding et al. 2013; Grosso et al. 2009; Gros et al. 2014). In mice, dual anti-LAG-3/anti-PD-1 antibody therapy is able to cure most mice of colorectal adenocarcinoma and fibrosarcoma tumors that are largely resistant to single antibody treatment (Woo et al. 2012). In addition, dual anti-LAG-3/anti-PD-1 therapy has been shown to induce tumor regression in recurrent melanoma tumors in mice without the need for depletion of tumor-specific Tregs (Goding et al. 2013). These data indicate that dual therapy might be of great utility in the clinic.

Because LAG-3 binds MHC class II with high affinity, and early data suggested that soluble LAG-3 could stimulate DCs, recombinant soluble LAG-3-immunoglobulin (LAG-3-Ig) has also been developed and tested in preclinical models. LAG-3-Ig was shown to function in vitro and in vivo as an activator of DCs. It can enhance the ability of DCs to mediate a T_H1 response (Andreae et al. 2002) and can control tumor growth and mediate tumor regression in mice (Prigent et al. 1999). Because LAG-3-Ig activates DCs, it has been tested as a vaccine adjuvant. LAG-3-Ig enhances a humoral response to hepatitis B surface antigen (HBsAg), potentiates a CD8+ T cell response to ovalbumin (OVA), and helps prevent mammary cancer when combined with a DNA vaccine in mice (Cappello et al. 2003; El Mir and Triebel 2000). Co-administration of LAG-3-Ig with HBsAg or OVA results in induction of peptide-specific cytotoxic T lymphocytes, activation of the proliferative response and Th1-type cytokine production of splenic T cells, and higher titer antibody production than with antigen alone (El Mir and Triebel 2000). DNA vaccination against Her2/*neu* by itself slows but does not provide durable protection against mammary tumor progression in mice, but addition of soluble LAG-3 results in durable tumor protection (Cappello et al. 2003). These encouraging preclinical results spurred clinical studies using LAG-3-Ig.

Clinical Summary

LAG-3 antibodies have been developed for clinical testing (BMS-986016, Bristol-Myers Squibb, Princeton, NJ, USA) (Table 1). No study results have been published to date, but blockade of LAG-3 is now being studied in two phase I clinical trials in oncology. It is being used as a single agent for leukemia/lymphoma in a dose-escalation study for patients with treatment-refractory chronic lymphocytic leukemia, lymphoma, and multiple myeloma (NCT02061761). A separate phase I trial is testing anti-LAG-3 alone and in combination with anti-PD-1 in advanced solid tumors (NCT01968109).

In addition to its use in combination with immune checkpoint blockade agents, future trials involving anti-LAG-3 or LAG-3-Ig may include cytotoxic chemotherapy, radiation, antitumor vaccines, or small molecule inhibitors of signaling pathways such as BRAF or MEK.

Table 1 Summary of clinical trials testing anti-LAG-3 or LAG-3-Ig in patients with cancer

Agent	Study	Phase	Comments
Anti-LAG-3 (BMS-986016)	With and without anti-PD-1 (nivolumab) in the treatment of solid tumors (NCT01968109)	I	Recruiting
	In chronic lymphocytic leukemia (CLL), hairy cell leukemia (HL), and non-Hodgkin's lymphoma (NHL) (NCT02061761)	I	Recruiting
LAG-3-Ig (IMP321)	In HLA-A2-positive stages II–IV melanoma patients (NCT01308294)	I	Recruiting
	With gemcitabine for treatment of advanced pancreatic cancer (NCT00732082)	I	Terminated
	Plus first-line paclitaxel in metastatic breast carcinoma (NCT00349934)	I	Completed (Brignone et al. 2010)
	In metastatic renal cell carcinoma (NCT00351949)	I	Completed (Brignone et al. 2009)

Soluble LAG-3 (LAG-3-Ig, IMP321, Immutep, Paris, France) is also being tested in clinically. IMP321 has been studied in multiple phase I studies as an adjuvant for influenza (Brignone et al. 2007a) and hepatitis B vaccines (Brignone et al. 2007b). In addition, IMP321 was studied in a phase I dose escalation study in patients with advanced renal cell carcinoma (Brignone et al. 2009). No objective responses were seen, but IMP321 was well tolerated and induced effector memory CD8+ T cell activation, and a significant dose-response relationship was observed: 7 of 8 (88%) patients who received higher doses of IMP321 demonstrated stable disease at 3 months versus only 3 of 11 (27%) patients treated in the lower-dose cohort ($P = 0.015$). This study was then followed by another phase I trial testing IMP321 in combination with taxane-based chemotherapy for the treatment of metastatic breast cancer (Brignone et al. 2010). The study demonstrated an objective response rate of 50% vs. a historical response rate of only 25%.

IMP321 is also being investigated as an adjuvant in antitumor vaccines. For example, one trial is evaluating a combination of a peptide vaccines and IMP321 and Montanide adjuvants for patients with HLA-A2+ stage II–IV melanoma (NCT01308294). Results from this trial have not yet been reported.

Anticipated High-Impact Results

- A Phase 1 Dose Escalation and Cohort Expansion Study of the Safety, Tolerability, and Efficacy of Anti-LAG-3 Monoclonal Antibody (BMS-986016) Administered Alone and in Combination With Anti-PD-1 Monoclonal Antibody (Nivolumab, BMS-936558) in Advanced Solid Tumors (NCT01968109)

- A Phase 1 Dose Escalation and Cohort Expansion Study of the Safety, Tolerability, and Efficacy of Anti-LAG-3 (BMS-986016) in Relapsed or Refractory Chronic Lymphocytic Leukemia and Lymphomas and Multiple Myeloma (NCT02061761)

Cross-References

▶ Anti-4-1BB/4-1BBL
▶ Anti-CD40/Anti-CD40L
▶ Anti-Programmed Death 1 (PD1)
▶ B7.1
▶ CTLA-4
▶ Indoleamine 2,3-dioxygenase

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MART-1

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Brendan D. Curti

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Abstract

MART-1 (melanoma antigen recognized by T cells) is also known as Melan-A. It has gained wide acceptance as an immunohistochemical marker to diagnose melanoma in biopsy material. MART-1 is not expressed in tissues lacking melanin pigment. Cellular and humoral immune responses against MART-1 have been detected in patients with melanoma and substantial efforts are ongoing to develop MART-1 as a therapeutic target in conjunction with T-cell checkpoint antibodies, cellular immunotherapy and peptide vaccines using a variety of adjuvants.

Keywords

Ipilimumab • Melan-A. *See* Melanoma antigen recognized by T cells (MART-1) • Melanoma antigen recognized by T cells (MART-1) • Animal models • Antigens • Gene • Human immune responses • Immunohistochemical staining panels •

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MART-1 (melanoma antigen recognized by T cells) is also known as Melan-A. It is a lineage-specific protein present on melanocytes. The protein consists of 118 amino acids (13 kDa) and has a strongly hydrophobic domain. It is a transmembrane protein and present in the endoplasmic reticulum and *trans*-Golgi network of melanocytes in the skin and retina. It is a useful immunohistochemical marker in the diagnosis of melanoma on biopsy specimens but is also present in benign melanocytes. Due to the absence of MART-1 expression on nonpigmented tissues, there has been significant effort to use it as a target for vaccine and cellular immunotherapy in patients with melanoma.

Biology of the Target

Although the gene for MART-1 was first cloned in (Kawakami et al. 1994a), its function is still unknown. MART-1 protein associates with other melanosomal proteins such as OA1, which are involved with melanosome transport and biogenesis. When MART-1 is inactivated, the OA1-MART-1 complex is destabilized and results in ocular albinism. Based on these findings, MART-1 may serve as an escort protein in the early stages of melanosome formation.

Despite a lack of understanding of its function, the importance of MART-1 in the immune response to melanoma has been recognized since its discovery. The gene for MART-1 was originally determined by examining the cDNA from melanoma cell lines and studying the reactivity of tumor-infiltrating lymphocytes (TIL) obtained from surgical specimens in HLA-A2$^+$ patients with metastatic melanoma. When non-MART-1-expressing HLA-A2+ cell lines were transfected with MART-1 cDNA, TIL clones were identified that produced interferon-γ when exposed to the MART-1-expressing target cells. MART-1- and HLA-A2-expressing melanoma cells were lysed by the same TIL clones. The genes for the T-cell receptor (TCR) of the TIL were examined and found to have restricted Vα and Vβ gene sequences, confirming the specificity of the TIL for MART-1. The nonamer and decamer peptides of MART-1 recognized by the TCR map to amino acid residues 27–35 and 26–35, respectively. These peptides are part of the transmembrane portion of the protein, yet they have distinctly different conformations. Greater than 90% of TIL derived from melanoma deposits in HLA-A2$^+$ patients recognize one or both of these peptides Reviewed in (Romero et al. 2002). The MART-1 27–35 epitope is believed to be the immunodominant peptide for immunological response against melanoma (Kawakami et al. 1994b). An alternative hypothesis has been proposed to account for the observation that a high proportion of melanoma patients (and normal individuals) have T cells that recognize MART-1, yet the melanoma is not eliminated by the T-cell response. The immunogenic peptides of MART-1 in conjunction HLA-A2 bind weakly to the TCR, thus it is likely that tolerance to MART-1 is

induced during thymic processing and selection of T cells. These MART-1-responsive T cells persist after thymic selection because of the weak binding of MART-1 peptide to the TCR. When melanomas express MART-1, the T-cell response may be weak, because MART-1 immunogenic peptides presented to the immune system by the melanoma cells are interpreted as self. As discussed below, there are vaccine and cellular immunotherapies with the potential to break tolerance to MART-1 and mount a more effective immune response to melanoma. Alternatively, malignant melanocytes may develop resistance to attack by cytotoxic T lymphocytes by overexpressing proteins associated with survival and resistance to apoptosis such as NF-kB and Bcl-2 family members.

T cells that recognize these peptides can also be isolated and expanded from the peripheral blood of patients with melanoma. Although most of the immunobiology of MART-1 has been studied in patients with the HLA-A2 haplotype, the same MART-1 epitopes are recognized by T cells in patients expressing HLA-B44 and HLA-B45. Surprisingly, approximately 70% of healthy HLA-A2^{+} individuals with no history of melanoma have CD8^{+} T cells that recognize a MART-1 peptide.

Knowledge of the amino acid sequence and protein chemistry of the dominant antigenic epitopes of MART-1 has been useful in developing synthetic peptides for therapeutic vaccine trials (reviewed below) and synthetic tetramers that can be used as reagents for immunological monitoring.

Target Assessment

MART-1 is not a prognostic factor and is not measurable in the peripheral blood. MART-1 is commonly used in immunohistochemical staining panels to diagnose melanoma in conjunction with S-100 and HMB-45. MART-1 is an excellent target for therapeutic development since it is present in a high percentage of melanomas and immune responses (albeit ineffective in controlling the tumor) are already present in many patients at baseline and because it is expressed only in pigment-making cells. The T-cell immune response to MART-1 can be measured with tetramer assays. Although assessment of immune response is important in developing any immunotherapy, the correlation of immune response to MART-1 with regression of melanoma is inconsistent in most human clinical trials, a finding similar to many other assays for melanoma antigens.

Role of the Target in Cancer

Rank: 8

MART-1 is already established as widely used immunohistochemical diagnostic test in surgical pathology to analyze specimens suspected to be melanoma. There is no direct correspondence between MART-1 expression and prognosis in melanoma; however, it can be used in conjunction to other markers to detect circulating tumor

cells and melanoma micrometastases, which have prognostic significance. MART-1 has significant potential as a therapeutic target for vaccines and cellular therapy.

High-Level Overview

Diagnostic, Prognostic, and Predictive Uses

MART-1 is not a useful predictive marker in melanoma. As described above, it is useful in the immunohistochemical analysis of biopsy specimens suspected of being melanoma in conjunction with other markers such as gp100 and HMB-45. T-cell responses to MART-1 can be assessed with tetramer and are commonly used in analyzing immune responses in clinical trials that have been performed using MART-1-targeted therapy.

Therapeutics

MART-1 has been used as a target for inducing antitumor immune responses in patients with melanoma since 1994, when the specificity of MART-1-specific TIL clones was recognized in HLA-A2^{+} patients (Cole et al. 1994). There have been numerous MART-1 clinical trials involving peptide vaccines, irradiated MART-1-expressing melanoma cell line vaccines, T cells transduced with a TCR for MART-1, TIL, peptide-loaded dendritic cells (DC), tumor-cell-loaded DC (Palucka et al. 2006) and combinations including peptide vaccines with a variety of adjuvants, and DC vaccines plus anti-CTLA-4 therapy (Ribas et al. 2009). Tumor regressions, some of which are durable, have been observed with each of these immunotherapy platforms. Objective response rates for MART-1-based immunotherapy range from 3% for peptide vaccines to over 50% for TIL-based approaches. This broad range of response is comparable to immunotherapy directed at other known melanoma antigens (e.g., gp-100, MAGE-A3, NY-ESO-1, and others), but MART-1-directed therapy does not appear more effective than other targeted approaches in melanoma. The more central issue with antigen-specific immunotherapy is that even though good targets can be defined, other aspects of immune response include breaking tolerance, sustaining cytolytic responses, developing effective immunological memory, and overcoming the inhibition of immune response mediated through regulatory T cells.

Preclinical Summary

There is an extensive preclinical literature of using and assessing MART-1-targeted therapy in animal models and ex vivo analysis of human immune responses to this antigen. Recent work has focused on understanding why immune response to this commonly expressed target is suboptimal in controlling melanoma. For instance, Li et al. showed that TIL after undergoing rapid expansion protocols had markedly

reduced CD28 expression, decreased responsiveness to restimulation with MART-1 peptide and increased apoptosis (Li et al. 2009). These problems could be overcome by growing the TIL in IL-15 and IL-21. This work has potential for improving and maintaining T-cell responses to other antigens and other tumor types.

Clinical Summary

MART-1 is a useful and commonly used component of the immunohistochemical staining panels to confirm the diagnosis of melanoma in routine pathology assessment. The clinical targeting of MART-1 to treat established melanoma remains experimental, although it has been studied for over 15 years. The best strategy for inducing consistent and durable immune responses against MART-1 is unknown, but this criticism can be applied to all other tumor antigens that have been tested in clinical trials thus far.

Although vaccination to achieve clinically significant antitumor effects in humans remains a work in progress, a recent observation in patients with melanoma treated with ipilimumab described by Klein et al. helps to affirm the importance of immune responses to MART-1. Patients who achieved regression of melanoma after ipilimumab immunotherapy had infiltration of regressing tumor nodules with MART-1-specific CD8+ T cells (Klein et al. 2009). Some individuals displayed a 30-fold increase of MART-1-specific T cells in the peripheral blood and in skin biopsies taken from areas of erythematous rash induced by ipilimumab. This finding suggests that MART-1 is central to an effective immune response in melanoma and may also be linked to some of the autoimmune toxicities induced by ipilimumab. There is another important aspect to the ipilimumab work that is applicable to MART-1 and other tumor antigens, namely, that effective immune responses to cancer require T cells with enhanced effector and memory function. The main pathways that influence T-cell survival, effector function, and memory after exposure to antigen are CTLA-4, PD-1, OX40, and 4-1BB. Antagonists to CTLA-4 and PD-1 and agonists to OX40 and 4-1BB used in conjunction with vaccines to MART-1 and other tumor-specific antigens have great potential for therapeutic development in melanoma.

Immune responses to other melanoma antigens such as gp-100, NY-ESO-1, and MAGE-A3 in conjunction with MART-1 may result in more robust clinical responses. There are many clinical trials studying antigen combinations using multivalent peptide vaccines, ex vivo antigen presentation with adoptive transfer of “educated” cytotoxic T cells, and adoptive transfer of T cells with engineered TCRs to recognize MART-1 and other relevant melanoma antigens. These more advanced antigen presentation platforms could be used in conjunction with cytokines like IL-15 or IL-21 to increase central memory T cells without increasing the number or activity of regulatory T cells, which can dampen immune response as is known to occur after IL-2 administration.

A deeper understanding of the signals that promote T-cell survival and activity after antigen exposure and reagents to modify those signals are likely to result in

more consistently effective immunotherapy for melanoma and other malignancies. Targeting MART-1 and other tumor antigens administered with biological agents to influence T-cell behavior shows great promise in unlocking the potential of vaccines for melanoma and other solid tumors.

Anticipated High-Impact Results

- DC pulsed with peptides for MART-1/gp100/Tyrosinase/NY-ESO-1/MAGE-3 in conjunction with lymphodepletion and autologous lymphocyte infusion, Weber et al.
- Phase II trial of extended dose anti-CTLA-4 antibody ipilimumab (formerly MDX-010) with a multi-peptide vaccine for resected stages IIIC and IV melanoma, Weber et al.
- Phase II Randomized Study of a Lymphodepleting Conditioning Regimen Comprising Cyclophosphamide, Fludarabine Phosphate, and Total-Body Irradiation Followed by Anti-MART-1 and Anti-gp100 T-Cell Receptor Gene-Engineered Autologous Peripheral Blood Lymphocytes, High-Dose Aldesleukin, and gp100:154–162 or MART-1:26–35 (27L) Peptide Vaccination in Patients With Metastatic Melanoma, Rosenberg, et al.

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MUC1

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Abstract

MUC1 mucin was the first molecule expressed by human tumor cells to be shown as a target for human antibodies and T cells. It belongs to a large family of shared, non-mutated tumor associated antigens differentially expressed on tumors versus normal cells. MUC1 is overexpressed and abnormally glycosylated on over 80% of human tumors including all epithelial adenocarcinomas, multiple myelomas and some B and T cell lymphomas. As such, it is an almost universal tumor antigen and an attractive target for immunotherapy. MUC1 has been tested as a vaccine antigen in many preclinical cancer models and in clinical trials. It has also

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been targeted with antibodies specific for its tumor form. In addition to being a specific tumor target for the immune system, MUC1 has important tumor promoting functions that could be targeted with other forms of therapy.

Keywords
Antibody-dependent cell-mediated cytotoxicity (ADCC) • Europe Group on Tumor Markers (EGTM) • MUC1 • Abnormal expression • Assessment • Clinical trials of • Diagnosis • Hypoglycosylation of • Immunobiology of • Monoclonal antibodies • mRNA isoforms • TCR • Therapeutics • VNTR • Variable number of tandem repeats (VNTRs)

Target

MUC1 (other designations: CD227; polymorphic epithelial mucin, PEM; peanut-reactive urinary mucin, PUM; CA 15-3; CA 549; DF3, episialin; H23) is a high molecular weight (>200,000 Da) type I transmembrane glycoprotein. MUC1 is expressed primarily by epithelial cells in low levels, on apical surfaces only, and extensively glycosylated with O-linked and N-linked glycans. Activated and memory T cells can also be $MUC1^{+}$. MUC1 overexpression, loss of polarization, and hypoglycosylation characterize its expression on human epithelial cancers and cancer stem cells (Engelmann et al. 2008; Fatrai et al. 2008) where it serves as a tumor antigen. Abnormal MUC1 expression has been used as a target for cancer immunotherapy in many preclinical and clinical studies and as a serum marker for monitoring cancer recurrence (Vlad et al. 2004; Hattrup and Gendler 2008). MUC1 has been ranked as the No. 2 cancer vaccine target antigen among 75 other cancer antigens in a pilot project initiated by the National Cancer Institute for the purpose of accelerating cancer translational research (Cheever et al. 2009).

Biology of the Target

MUC1 is encoded by the *MUC1* gene located on human chromosome 1q21. Multiple MUC1 mRNA isoforms can be produced through alternative splicing resulting in full-length isoforms that have all seven exons and short isoforms that are devoid of certain (partial or full) exon(s). Most published studies on MUC1 concern the full-length isoforms. Full-length MUC1 is a heterodimer composed of the extracellular α-subunit and the transmembrane β-subunit assembled through non-covalent binding. The a-subunit contains a region of a variable number of tandem repeat (VNTR) characteristic of all members of the mucin family. MUC1 repeats are 20-amino acid long and have the sequence PDTRPAPGSTAPPAHGVTSA. The number of repeats is allelically determined and varies from 25 to over 125 per molecule (Vlad et al. 2004). Most MUC1-specific monoclonal antibodies or T cells recognize or target the VNTR region of the α-subunit because of

the unique structure and high immunogenicity of this region and differences between normal and tumor cells. This subunit is highly O-glycosylated (especially in the VNTR) when expressed on normal cells (though N-glycosylation can also occur) and severely underglycosylated on cancer cells. Because this subunit can interact with adhesion and other molecules on neighboring cells, it plays an important role in cancer cell growth and metastasis. The β-subunit contains a short extracellular domain, a transmembrane domain, and a cytoplasmic domain and is involved in several signal transduction pathways such as ERK, SRC, Ras/MAPK, and NF-kB pathways, through interacting with intracellular molecules such as b-catenin, GRB2, ERa, p53, IKKb, and IKKg (Theodoropoulos and Carraway 2007; Kufe 2009). The b-subunit may also inhibit apoptosis of cancer cells through several different mechanisms and contribute to the tumor microenvironment (Bafna et al. 2010; Behrens et al. 2010). As for short MUC1 isoforms, more than 100 cDNA isoforms have been reported, but only few of them have been confirmed to have protein products whose biological functions have not yet been elucidated (Zhang et al. 2013).

Besides the polymorphism in the numbers of tandem repeats, multiple single-nucleotide polymorphisms (SNPs) have been observed in the *MUC1* gene. One of them is the A/G SNP in exon 2 (rs4072037). The frequency of A SNP or G SNP varies in different ethnic groups. The A SNP is associated with shorter VNTR region, while the G SNP is correlated with longer VNTR region, which may contribute to the higher MUC1 serum level in healthy people and cancer patients with G/G SNPs than those with A/A SNPs. The A/G SNP can affect the site selection during MUC1 mRNA splicing and may produce protein products with different N-terminals. These variations may affect MUC1 function for which there is currently only limited evidence. For example, A/A allele has been reported to be associated with higher risk of gastric cancer (Xu et al. 2009; Jia et al. 2010).

Target Assessment

MUC1 can be measured in serum by ELISA or RIA and on tissues using immunohistochemistry. In 1997, FDA approved using Truquant BR radioimmunoassay for measuring serum MUC1 to monitor the recurrence of stage II and stage III breast cancer. Importantly, numerous anti-MUC1 antibodies can distinguish between the normal MUC1 and the tumor MUC1, which could be used to improve tissue immunohistology and provide more informative pathology reports (1998). These antibodies have not yet been incorporated into standard diagnostic tests.

Role of the Target in Cancer

Rank: 10

It is an oncogene through its cytoplasmic domain; it promotes inflammatory pro-tumor microenvironment; it promotes cell adhesion and deadhesion driving metastatic potential; it is a tumor antigen; and all its functions could be targeted with pharmacological or biological therapy.

High-Level Overview

Diagnostic, Prognostic, and Predictive

MUC1 has been used as a marker for diagnosis of the breast, ovarian, pancreas, and prostate cancers. MUC1 is one of the most frequently measured tumor antigens and the most widely used tumor marker in breast cancer. MUC1 level is associated with tumor burden and metastatic status. Serum MUC1 concentrations are not elevated in patients with primary cancers, but over 70% of patients with metastasis show increased MUC1 level. Though there are MUC1 antibodies that recognize different epitopes, combined usage of different antibodies does not show any improvement of sensitivity. Combination of MUC1 and CEA is recommended to provide more diagnostic information, but due to low sensitivity, they are not recommended for early diagnosis or screening. Increased MUC1 concentration in serum of patients after treatment as an index has been reported to be superior to clinical or radiological indication in 40–55% patients. Europe Group on Tumor Markers (EGTM) recommends monitoring of circulating MUC1 levels for early diagnosis of recurrence and distant metastasis in breast cancer patients (Molina et al. 2005). Circulating MUC1 levels are higher in patients with progressive breast cancer than those in remission. EGTM recommends measuring MUC1 concentration prior to every chemotherapy course and at least every 3 months for patients who are receiving hormone therapy. At least 25% increase of the previous value is considered a significant increase. There are also reports that preoperatively elevated MUC1 level is associated with adverse outcome though conflicting reports also exist. To improve the test, combination of MUC1 levels with CEA levels and with other patient-specific factors is recommended (Molina et al. 2005). The American Society of Clinical Oncology (ASCO), however, does not recommend the FDA-approved MUC1 serum assay for routine clinical use due to the lack of evidence of clinical benefit.

Besides circulating MUC1, anti-MUC1 antibodies are also reported to be present in cancer patients. The presence of MUC1-specific IgG at diagnosis is correlated with better survival and with good prognosis (Reuschenbach et al. 2009).

Therapeutics

In the past more than 20 years, MUC1 has been used as a target for cancer immunotherapy. Passive (antibodies) as well as active (vaccines) immunotherapies have been deployed to treat solid tumors such as the breast, ovarian, pancreatic, colon, lung, skin cancers, and blood malignancies such as multiple myeloma (Vlad et al. 2004; Tang and Apostolopoulos 2008; Tang et al. 2008).

Multiple MUC1-specific monoclonal antibodies have been generated, naked as well as isotope labeled, and used in treatment of cancers. Several phase I/II and III clinical studies using ^{90}Y-HMFG1 antibody have been done in ovarian cancer patients (Oei et al. 2008). Two phase II studies showed increased survival rate (70% 5 years and 78% >10 years) leading to two phase III studies. Neither of the

phase III trials showed improved overall survival though there was a difference in time to disease recurrence. Recently, a humanized anti-MUC1 HMFG1 antibody, AS1402 (formerly Therex, R1550), was generated that recognizes PDTR epitope in the MUC1 VNTR region. It can induce antibody-dependent cellular cytotoxicity (ADCC) against cancer cells that express abnormal MUC1. A phase I study using naked AS1402 has been done in advanced on metastatic breast cancer patients (Pegram et al. 2009). The results showed that the antibody was well tolerated and safe at a dose as high as 16 mg/kg administered intravenously. Five patients (22.7%) showed prolonged stable disease. A phase II study with AS1402 plus letrozole in postmenopausal women with locally advanced or metastatic breast cancer was started but soon discontinued in 2009 because early evaluation suggested that the trial was unlikely to give sufficiently positive efficacy findings.

In view of discouraging results with the antibodies, it is important that MUC1 can activate T cells. As a result of its tandem repeat structure, T cells can recognize MUC1 in an MHC-unrestricted manner and MHC-unrestricted killing of MUC1-expressing tumors has been observed (Finn 1992; Vlad et al. 2004). Most carcinoma cells express hypoglycosylated MUC1, and the MHC-unrestricted recognition and killing of MUC1-expressing carcinoma cells by T cells with MUC1-specific TCR suggests a potentially broadly applicable adoptive T cell therapy. In vitro and in vivo studies have shown the potential of MUC1-specific TCRs in cancer immunotherapy (Alajez et al. 2005; Chen et al. 2009).

As one of the top cancer vaccine antigen candidates (Cheever et al. 2009), MUC1 proteins, peptides, glycopeptides, or DNA have been delivered as vaccines with adjuvants such as DCs, cytokines, and toll-like receptor (TLR) ligands. MUC1-specific humoral and cellular responses have been elicited in various preclinical and clinical studies. Results of two phase I/II clinical trials in pancreatic cancer patients have been reported. One used MUC1 100mer peptide (five tandem repeats from the VNTR) plus SB-A2 adjuvant. The results showed that the vaccine was safe and capable of inducing IgG and T cell responses in some patients. Two of 15 patients were disease-free at 32 and 61 months of follow-up. The other trial used MUC1 100mer peptide-loaded DC as vaccine (Lepisto et al. 2008). The vaccine was well tolerated and nontoxic in 12 pancreatic and biliary cancer patients enrolled. The patients were followed for over 4 years and four of the 12 patients were alive and disease-free at 5 years. In another study with 20 pancreatic cancer patients, MUC1 100mer loaded DCs and MUC1+ tumor cell line activated lymphocytes were injected into patients intradermally and intravenously, respectively, between two and 15 times. One patient with multiple lung metastasis showed complete response and another five patients had stable disease (Kondo et al. 2008). A mannan-MUC1 (100mer) vaccine was used for a pilot phase III trial in 31 stage II postmenopausal breast cancer patients. There were no relapses in the vaccinated group after 8 years, while four of 15 patients treated with placebo had relapsed. A phase IIB trial of Stimuvax MUC1 cancer vaccine, which consists of MUC1(25aa) lipopeptide BLP25 and immunoadjuvant (monophosphoryl lipid A and three other lipids), showed some efficacy though nonsignificant (a median OS of 30.6 months versus 13.3 months) in patients with stages IIIB and IV non-small cell lung cancer (NSCLC). Two large

phase III trials (START and INSPIRE) of the same vaccine are ongoing in NSCLC patients. In a pilot study using a heptavalent vaccine containing GM2, Globo-H, Lewis Y, Tn(c), STn(c), TF(c), and Tn-MUC1 conjugated to KLH with QS21 as adjuvant, Tn-MUC1 was one of the two most potent immunogens with eight of nine ovarian carcinoma patients showing serologic responses (Sabbatini et al. 2007). A phase II study using MVA-MUC1-IL-2(TG4010) with or without cytokines in renal cell cancer (RCC) patients (Oudard et al. 2011) showed that the vaccine is well tolerated with no serious adverse events. Although no partial or complete clinical responses occurred, MUC1-specific CD4 and CD8 T cells were elicited in six and four patients, respectively.

Preclinical Summary

Several tumor models have been established based on human MUC1 transgenic mice for testing MUC1-specific immunotherapy. Different forms of the MUC1 antigen (peptides, glycopeptides, DNA) have been used with or without certain adjuvants. Most vaccines could elicit humoral, cellular, or both immune responses effective in eliminating tumor cells. With an ever-increasing understanding of the immunobiology of MUC1, optimal epitope selection or modifications can be made to increase tumor specificity and immunogenicity of new MUC1 vaccines (Ryan et al. 2010, 2009). In addition to cancer, hypoglycosylation of MUC1 is an early change in tissues with chronic inflammation, such as inflammatory bowel disease (IBD). MUC1 glycopeptide vaccine was reported to induce MUC1-specific IgG and CTL responses in a mouse model of spontaneous IBD models, changing the microenvironment and delaying IBD as well as preventing progression to colitis-associated colon cancer (CACC) (Beatty et al. 2010; Weiner et al. 2010).

Clinical Summary

Since the first clinical trial of a MUC1 vaccine in advanced pancreatic cancer patients that opened in 1993 (Goydos et al. 1996), ten of clinical trials of MUC1-based vaccines or MUC1-targeted immunotherapy have been done and many more are currently in progress. Very few have advanced to the phase III stage and thus no MUC1-based therapy has yet been approved for routine clinical use. Adoptive therapy has employed naked or labeled MUC1-specific antibodies, and the vaccines have used MUC1 peptides, glycopeptides, lipopeptides combined with different adjuvants (poly ICLC, IL-2, KLH, QS21, dendritic cells), or MUC1 DNA delivered in plasmid or viral vectors.

Anticipated High-Impact Results

Antitumor efficacy seen in animal models has not yet been recapitulated in clinical trials. Increased understanding of the immunosuppressive nature of the tumor microenvironment provides multiple mechanistic explanations for this discrepancy as well as additional targets for combined therapy that would improve the outcome of immunotherapy. Antitumor efficacy of adoptive immune therapy with anti-MUC1 antibodies and MUC1-specific TCR-transduced immune cells could also be enhanced by targeting epitopes that are more tumor specific. Lastly, the excellent safety profile of MUC1-based immunotherapeutic reagents and vaccines allows consideration for their use in the future for prevention of cancer in high-risk individuals before the tumor-driven immunosuppressive microenvironment gets established.

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NK Cells

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William Richard Childs and Jeremy M. Pantin

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Abstract

Natural killer (NK) cells are innate immune lymphocytes that express CD56 and lack CD3 surface antigens. NK cells do not require the presence of specific tumor antigen for the recognition and killing of cancer cells. NK cell recognition of tumor targets is regulated through a balance of activating and inhibitory signals. NK cells also have the ability to directly kill target cells through antibody-dependent cell cytotoxicity (ADCC) via the membrane Fc-γ receptor III (CD16) which binds to IgG antibodies and can also indirectly induce tumor apoptosis through cytokine secretion, directly through the perforin-granzyme

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pathway, or through death-receptor ligands such as TRAIL or Fas ligand expressed on their cell surfaces.

Tumors with low HLA class I expression are more susceptible to NK cell cytotoxicity. Adoptive infusion of allogeneic NK cells in patients who lack MHC class I molecules for one or more KIRs present in the donor may overcome NK cell-mediated KIR inactivation. Agents that increase surface expression of cellular death-receptors which render tumors more susceptible to NK cell cytotoxicity include bortezomib and depsipeptide. In vitro expanded autologous NK cells isolated from patients with cancers have been shown to exhibit significantly more cytotoxicity when tumors were pretreated with bortezomib compared with untreated tumor controls. Combining adoptive NK cell transfer with monoclonal antibody therapy could augment NK cell-mediated ADCC.

Keywords
Antibody-dependent cell cytotoxicity (ADCC) • Chimeric antigen receptors (CAR) • Interleukin-2 (IL-2) • Killer immunoglobulin-like receptors (KIRs) • Natural killer (NK) cells • ADCC • CAR • Clinical trials • Effector assessment • IL-2 activation • In cancer • In vitro and in vivo susceptibility • KIR-mediated inactivation of • Tumor susceptibility

NK Cell Targets: Effector

Natural killer (NK) cells are innate immune lymphocytes that express CD56 and lack CD3 surface antigens(Caligiuri 2008). Unlike antigen-specific T cells, NK cells do not require the presence of a specific tumor antigen for the recognition and killing of cancer cells. NK cell recognition of tumor targets is regulated through a balance of activating and inhibitory signals. NK cells also have the ability to directly kill target cells through antibody-dependent cell cytotoxicity (ADCC) via the membrane receptor, Fc-γ receptor III (CD16), which binds to the Fc portion of IgG antibodies. In addition, NK cells can indirectly induce tumor apoptosis through cytokine secretion or directly through the perforin-granzyme pathway or through death receptor ligands, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or Fas ligand expressed on their cell surfaces, which directly trigger tumor death via their respective receptors(Srivastava et al. 2008).

Biology of the Effector

The mechanism by which an NK cell recognizes a target cell, with subsequent activation or inhibition of killing, is complex. Under physiological circumstances, NK cell recognition of target cells is predominantly mediated by paired inhibitory

and activating signals through NK receptors (NKR) (Lanier 1998), as well as various adhesion and co-stimulatory molecules. The best characterized activating and inhibitory receptors belong to the killer cell Ig-like receptor (KIR) superfamily, which primarily recognize class I human leukocyte antigens (HLA) A, B, and C and the C-type lectin CD94/NKG2 heterodimers which are ligated by HLA-E (Lopez-Botet et al. 2000).

Even in the presence of an activating ligand, inhibitory ligands can initiate overriding signals that culminate in a net suppression of NK cell function. The inactivation of NK cells by self-HLA molecules is thought to be a mechanism by which malignant cells evade host NK cell-mediated immunity. This may limit the ability of both endogenous and adoptively infused autologous NK cells to induce antitumor effects against tumors.

The killer cell immunoglobulin-like receptors (KIRs) with two Ig domains (KIR2D) bind HLA-C molecules: KIR2DL2 and KIR2DL3 recognize HLA-C “group 1” allotypes that possess the amino acid residues Ser and Asn at positions 77 and 80 in the alpha-1 helix of the HLA-C molecule. KIR2DL1 recognizes HLA-C “group 2” molecules that possess Asn77 and Lys80 in the alpha-1 helix. KIR3DL1 has three Ig domains that recognize an epitope shared by HLA-Bw4 alleles and HLA A24. Finally, KIR3DL2, a homodimer of molecules with three Ig domains, recognizes HLA-A3 and HLA-A11 (Borrego et al. 2002; Sun 2003; Natarajan et al. 2002).

Human NK cells can also lyse Ab-coated target cells through the process of Ab-dependent cell cytotoxicity (ADCC). A large proportion of human NK cells express CD16, the low-affinity Fc-γ receptor IIIa (FCGR3A) which binds to the constant (Fc) region of immunoglobulin. When CD16 engages Fc on antibody-coated tumor cells, NK cell degranulation and perforin-dependent killing occur (Sulica et al. 2001).

Spontaneous NK cytotoxicity can be triggered by the activating receptors NKG2D and leukocyte adhesion molecule DNAX accessory molecule 1 (DNAM-1). Ligands for NKG2D belong to two distinct families, the MHC class I chain-related (MIC) antigens (MICA and MICB) (Steinle et al. 2001) and the UL16-binding proteins (ULBPs) (Sutherland et al. 2002). These ligands may be present at low surface density on normal tissues, but may be upregulated during malignant transformation, leading to NK cell activation and cytotoxicity (Bauer et al. 1999; Elishmereni et al. 2008).

NK cells lyse tumor targets indirectly through cytokines or directly through perforin/granzyme or surface-expressed Fas ligand and/or TRAIL which directly trigger death receptor pathways leading to tumor apoptosis (Arase et al. 1995; Smyth et al. 1999; Kayagaki et al. 1999). Death receptors expressed on tumor cells which may be activated by NK cells leading to cytotoxicity include Fas, TNFR1, TRAIL-R1/DR4, TRAIL-R2/DR5, DR3, and DR6, all of which share a conserved death domain that is triggered by adaptor molecules that activate executioner caspases and initiate apoptosis.

Effector Assessment

The receptors and ligands which regulate NK cell activity are diverse. This makes multicolor flow cytometry-based analysis a highly attractive technique for detailed phenotypical and functional evaluation of NK cells (Bjorkstrom et al. 2010). NK cell cytotoxicity of tumor targets in vitro is usually assessed through standard [51] Cr-based cytotoxicity assays or more recently flow-based assays in which tumor targets are assessed for expression of annexin V and/or 7AAD as markers for apoptosis and cell death, respectively. More recently, in vivo assays of human NK cell ADCC in immunodeficient mice have been described (Shiokawa et al. 2010).

Role of the Effector in Cancer

Although innate immunity has been hypothesized to be important in tumor immune-surveillance, the exact role NK cells play in protecting the host from malignancy is unknown. However, both indirect and direct evidences support a role for NK cells against cancer. Indirect support for tumor immune-surveillance by NK cells includes the observation that survival in cancer patients is positively correlated with infiltration of NK cells in the tumor (Albertsson et al. 2003) and the observation that the risk for cancer may be increased in individuals with low levels of NK cell cytotoxicity (Imai et al. 2000). Direct support for tumor immune-surveillance by NK cells includes the observation that tumor formation is increased in NKG2D and DNAM-1 knockout mice that have defective NK cell immunity (Iguchi-Manaka et al. 2008; Guerra et al. 2008).

High-Level Overview

Diagnostic, Prognostic, and Predictive

The in vitro and in vivo susceptibility of tumors to NK cell killing is highly variable and is not necessarily related to the specific tumor histology. Tumors with low to absent HLA class I expression (e.g., neuroblastoma) tend to be more susceptible to NK cell cytotoxicity, likely as a consequence of the tumor lacking the ligands which inhibit NK cell function through their inhibitory KIR. In contrast, tumors with high surface expression of molecules that serve as ligands for NK cell-activating receptors, such as ULBPs, MICA/B, CD155, etc., may be more susceptible to lysis by NK cells, especially when they lack surface expression of molecules which serve as ligands for NK cell inhibitory receptors (i.e., MHC class I, HLA-E, etc.).

Therapeutics

Overcoming KIR-Mediated Inactivation of NK Cells

The adoptive infusion of allogeneic NK cells in cancer patients who lack MHC class I molecules for one or more KIRs present in the donor (so-called KIR-mismatched NK cells) has recently been explored as a method to overcome NK cell-mediated inactivation as a consequence of KIR (Miller et al. 2005). A pilot study of a haploidentical NK cell infusion following cyclophosphamide and fludarabine in children with AML in remission documented transient NK cell engraftment in all ten treated patients. Remarkably, with a median follow-up of 964 days, the 2-year event-free survival estimate was 100% (Rubnitz et al. 2010). Based on these favorable data, these investigators are exploring the efficacy of KIR-mismatched allogeneic NK cells in a phase II trial as consolidation therapy to decrease relapse in children with AML. In adult patients, a phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer demonstrated only transient donor chimerism and prolonged neutropenia. Although the infusions were tolerated, toxicities from the conditioning regimen and a lack of tumor responses limit this approach (Geller et al. 2011). The use of allogeneic NK-92 cell line, which lacks KIR, is being explored as cellular therapy for metastatic melanoma and renal cell carcinoma. The feasibility and safety of this approach was investigated in a phase I clinical trial (Arai et al. 2008).

Although these results are promising, outside the setting of an allogeneic transplant, KIR ligand-mismatched NK cells might be of limited therapeutic use as differences in MHC molecules would eventually lead to their rejection by the patient's immune system. Alternative methods to potentiate autologous NK cell tumor cytotoxicity include the use of KIR antibodies to disrupt the function of specific inhibitory KIRs (Romagne et al. 2009) or genetic manipulation of NK cells to knock down expression of NK cell inhibitory receptors. Preclinical studies have shown that siRNA knockdown of the NKG2A inhibitory receptor enhances NK cell tumor cytotoxicity in vitro and augments the antitumor effects of adoptively transferred NK cells in tumor-bearing hosts (Furutani n.d.).

The blocking of NK cell MHC class I-specific KIR2DL1/2/3 inhibitory receptors with the monoclonal Ab IPH-2101 increases NK cell cytotoxicity in vitro and is currently being evaluated in a number of human clinical cancer trials (see below).

Enhancing Tumor Susceptibility to NK Cell Killing

An alternative approach to offset KIR ligand inhibition that augments NK cell tumor killing would be to render tumor cells more susceptible to NK cell tumor attack. Agents that increase the surface expression of cellular death receptors which render tumors more susceptible to NK cell cytotoxicity include the proteasome inhibitor bortezomib and the histone deacetylase inhibitor depsipeptide (Sayers et al. 2003). Pretreatment of malignant cells in vitro with depsipeptide or bortezomib enhances TRAIL-mediated NK-tumor cytotoxicity by upregulating tumor surface expression of the TRAIL death receptor DR-5. Furthermore, bortezomib also enhances

perforin-/granzyme-mediated NK-tumor cytotoxicity through drug-induced augmentation of tumor caspase 8 activity (Lundqvist et al. 2006).

Directing Chimeric Antigen Receptor-Transduced NK Cells Against Tumor-Specific Antigens

In contrast to T cells, NK cells have historically been difficult to transduce with viral vectors. By modifying experimental conditions, investigators have recently improved transduction efficiency of NK cells to the range of 30–50%. The development of an efficient method to genetically modify NK cells has led to studies exploring whether transduction of NK cells with chimeric antigen receptors (CAR) specific for tumor antigens could be used to augment NK cell tumor cytotoxicity. It is shown that successful transduction of ex vivo expanded NK cells with a CD19 CAR can be achieved using a lentiviral vector, with CD19 CAR-transduced NK cells exhibiting enhanced antigen-specific cytotoxicity against CD19-expressing B-cell malignancies compared to nontransduced NK cells. These findings provide both a method and rationale for clinical trials exploring the antitumor effects of adoptively infused CD19 CAR lentiviral vector-transduced NK cells in patients with refractory B-cell malignancies (Boissel et al. 2009; Ramanathan et al. 2008).

Transduction of NK cells to express a CAR for HER-2, which is frequently overexpressed on carcinomas, leads to NK cell activation against HER-2-positive tumor cells, including autologous HER-2-expressing tumors. Transduction of NK cells with a HER-2 CAR, but not a mock-transduced counterpart, efficiently eradicates tumor cells in RAG2 knockout mice bearing HER-2-expressing tumors (Kruschinski et al. 2008). Taken together, these data indicate that expression of tumor-specific CAR in NK cells can be used as a strategy to override NK cell inhibitory receptors and direct NK cell cytotoxicity specifically against tumor cells.

Enhancing NK Cell-Mediated ADCC

Antibody-dependent cell cytotoxicity (ADCC) is proposed as a mechanism that contributes at least in part to the efficacy of tumor-directed monoclonal antibody (mAb) therapies, such as rituximab and ofatumumab (both anti-CD20 mAbs) and trastuzumab (anti-Her2/Neu mAb). In vitro, rituximab-mediated ADCC is mediated in a large part by activated NK cells (Golay et al. 2003). Improving ADCC responses is desirable because it is thought to be an important antitumor mechanism for some antibody-based therapies. Combining adoptive NK cell transfer with mAb therapy could be used as a method to augment NK cell-mediated ADCC. Recent data also suggest blocking NK cell KIR with mAb can be used as a method to augment NK cell-mediated ADCC (Binyamin et al. 2008).

Preclinical Summary

Methods to enhance the antitumor effects of adoptively transferred NK cells by increasing target susceptibility to NK cell cytotoxicity or by interfering with NK cell inhibitory signaling are currently being explored.

In vitro studies demonstrate that pretreatment of malignant cells with bortezomib significantly enhances NK cell-mediated tumor cytotoxicity by sensitizing cancer cells to TRAIL. Murine studies have also established that bortezomib treatment sensitizes tumors in vivo to killing by adoptively infused syngeneic NK cells; murine renal cell carcinoma line (RENCA) tumors in BALB/c mice grow significantly slower, and survival is prolonged when syngeneic NK cell infusions are given following bortezomib treatment compared to mice receiving NK cell infusions alone or bortezomib alone. This antitumor effect is further potentiated by eradicating T-regulatory cells prior to adoptive NK cell infusion and by administering interleukin-2 after adoptive NK cell infusion (Lundqvist et al. 2009). These findings have been translated into a clinical trial exploring whether the antitumor effects of adoptively transferred autologous NK cells can be augmented against a variety of malignancies which have been sensitized to NK-TRAIL cytotoxicity with bortezomib (ClinicalTrials.gov Identifier: NCT00720785).

IPH-2101 (formerly 1-7 F9), which is being developed by Innate Pharma, is a fully humanized IgG4 anti-KIR monoclonal antibody. Preclinical studies demonstrate that IPH-2101 selectively binds to KIR2DL1, 2, and 3 and KIR2DS1 and 2, which augments NK cell-mediated lysis against tumor targets expressing the ligands for one or more of these inhibitory KIR (Romagne et al. 2009).

Clinical Summary

NK cells activated with cytokines, such as interleukin-2, have been used since the 1980s as adoptive immunotherapy against cancer. IL-2 activates lymphocytes including NK cells enhancing their capacity to lyse tumor cells. To date, most clinical studies of adoptive NK cell transfer have utilized short-term (12–16 h) IL-2-activated NK cells. Because IL-2 alone is ineffective in expanding NK cells in vitro, the relatively low numbers of NK cells obtained for infusion following short-term IL-2 activation may limit the full therapeutic impact of this approach. Recently, investigators have developed a number of different novel ex vivo expansion protocols that utilize irradiated EBV-LCL or K562 feeder cells to expand NK cells in vitro (Voskens et al. 2010; Berg et al. 2009). Preclinical studies show that ex vivo expanded NK cells are phenotypically and functionally different than short-term IL-2-activated NK cells, having increased expression of natural cytotoxicity receptors, NKG2D and TRAIL, and greater TRAIL-mediated tumor cytotoxicity compared to IL-2-activated NK cells. Importantly, despite extensive ex vivo proliferation, expanded NK cells appear to maintain similar longevity in vivo as non-expanded short-term IL-2-activated NK cells. The recent ability to expand NK cells in vitro now provides the opportunity to explore the antitumor efficacy of infusing large numbers of activated NK cells in patients with advanced malignancies.

Alloreactive or KIR-incompatible NK cells also demonstrate enhanced graft versus tumor effects against AML in HLA haplotype-mismatched hematopoietic transplantation (Symons et al. 2010) and are currently being explored as a tool for

adoptive immunotherapy for cancer patients, both within and outside the context of an allogeneic hematopoietic stem cell transplant.

Anticipated High-Impact Results

In phase I clinical trials in patients with acute myeloid leukemia and multiple myeloma treated with IPH-2101, activation of NK cells was observed and IPH-2101 exhibited a good safety profile. This agent is currently being evaluated in phase II clinical trials to assess single-agent IPH-2101 in patients with smoldering myeloma or IPH-2101 given either alone or in combination with lenalidomide in patients with multiple myeloma (ClinicalTrials.gov Identifiers: NCT00999830, NCT01217203). Additional studies will be needed to define whether clinically meaningful antitumor effects can be induced by IPH-2101 against solid tumors and/or hematological malignancies.

In vitro expanded NK cells isolated from patients with metastatic cancers or hematological malignancies have been shown to exhibit significantly more cytotoxicity when tumors were pretreated with bortezomib compared with untreated tumor controls. These findings suggest that drug-induced sensitization to TRAIL could be used as a novel strategy to potentiate anticancer effects of autologous adoptively infused NK cells in patients with cancer. This approach is being studied in a nonrandomized, phase I, dose-escalating study designed to evaluate the safety and the antitumor effects of escalating doses of adoptively infused ex vivo expanded autologous natural killer (NK) cells against metastatic cancers or hematological malignancies sensitized to NK-TRAIL cytotoxicity with bortezomib (ClinicalTrials.gov Identifier: NCT00720785).

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P53, Immunology

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Abstract

P53 is one of the most frequently mutated suppressor genes in human cancers. P53 normally functions as a transcription factor that is stabilized and activated by various genotoxic and cellular stress signals leading to the cell cycle arrest and apoptosis of damaged cells. P53 is often referred to as "the guardian of the genome." In most cancers, p53 becomes functionally deficient. In addition, mutant p53 may acquire dominant negative activity and oncogenic properties. P53 remains an attractive target for cancer therapy, and strategies for targeting p53 include gene therapy to restore its functions, inhibition of p53-MDM2 interaction, restoration of wild-type p53, p53 based vaccines and targeting of the p53 family of proteins. Some of these therapies are in clinical trials. Novel strategies for p53-targeted therapy are under development.

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Target: Tumor Protein 53 (TP53/p53)

Tumor protein 53 (TP53), more commonly referred to as p53, functions as a tumor suppressor. It is expressed by all nucleated mammalian cells and encoded by the *TP53* gene located in humans in the short arm of chromosome 17 (17p13.1). Although p53 is a 392 amino acid residue protein with a mass of 43.7 kDa, its apparent molecular weight (M_r) in gel electrophoresis under denaturing conditions is 53 kDa. *TP53* consists of 11 exons with exons 2–9 encoding p53. Extensive information about the molecular biology of p53 in human cancer is found on several web sites, two of which are www.iarc.fr/p53 and www.p53.free.fr.

Biology of the Target

p53 is a transcription factor with tumor suppressor activity (Levine and Oren 2009; Soussi 2003a; Olivier et al. 2009). It regulates critical genes controlling the cell cycle, angiogenesis, autophagy, and apoptosis. Loss of p53 function is the most common event associated with oncogenesis in humans. p53 is comprised of seven domains, each responsible for a distinct cellular function, such as transcription factor activation, apoptosis, nuclear signaling, oligomerization, or DNA binding. Several posttranslational modifications involving phosphorylation, acetylation, and sumoylation are key events in defining the functional activities of p53. P53 belongs to a family of proteins with a high degree of structural similarity which also includes p73 and p63. All three members have a central DNA binding domain (DBD) that binds to response elements of target genes. The N-terminal transcription activation domain for positive (e.g., p300/CBP and TAFII40/60) or negative regulators (e.g., MDM2 and MDMX) of gene transcription. MDM2 inhibits the activation domain by initiating proteasomal degradation via the E3 ubiquitin ligase. Thus, wild type (WT) p53 has a short half-life in normal cells. MDMX/MDM4, is a member of the MDM2 family but it lacks ligase activity. It regulates p53 by forming heterodimers with MDM2 family members, which have enhanced ligase activity. Stress signals resulting from chemical or physical DNA damage lead to p53 activation. Activated p53 downregulates expression of MDM2, thereby reducing its own degradation rate, blocking replication and the cell cycle, which allows DNA repair to proceed. If repair is not successful, p53 initiates cell death or apoptosis. Such elimination of damaged cells ensures genetic stability.

Four distinct mechanisms can lead to a loss of p53 function. The most common is a missense mutation or deletion in *TP53*, particularly in exons 5–8, which encode its central DNA-binding domain (DBD). Missense or frameshift mutations or deletions in *TP53* exons and introns are found in upwards of 90% of most types of human cancer. The literature on genetic alterations in p53 in human cancer is voluminous and the subject of many web sites with useful further links, such as www.iarc.fr/p53 and www.p53.free.fr. Other mechanisms independent of a genetic alteration in *TP53* which lead to loss of p53 function include the deregulation of p14ARF, an alternative reading frame variant of the cell cycle kinase inhibitor CDKN2A gene, overexpression of MDM2, and expression of human papillomavirus (HPV) E6, which is an E3 ubiquitin ligase and promotes p53 degradation.

Target Assessment

Due to its short half-life, WTp53 is essentially non-detectable in normal tissues and cells, with the exception of the thymus and mitogen-activated T lymphocytes. In most tumors, p53 loss of function is associated with an increase in its half-life and results in its accumulation (erroneously referred to as overexpression). Intact p53 molecules accumulating in tumors are detectable by immunohistochemistry (IHC) and immunoblot assays using p53-specific antibodies (Abs). Comprehensive analyses aimed at identifying genetic alterations in p53 require genomic DNA sequence analysis of all 11 exons and the intron/exon junctions, as shown for head and neck cancer (Balz et al. 2003). Loss of p53 function of tumors due to *TP53* frameshift mutations and deletions, which result in truncated p53 molecules or HPV E6- or enhanced MDM2-mediated degradation, is not readily identified based on IHC or immunoblot assays. Some of the p53-mutated ptoteins acquire new oncogenic functions [“gain of function”: GOF] that contribute to increasing cell proliferation, invasion, angiogenesis, genomic instability and chemoresistance in human cancers (Masciarelli et al. 2014). Further, mutations in p53 protein determine distinct interactions of the mutated p53 with p73/p63, resulting in different functional cell responses (Ferraiuolo et al. 2016).

Role of the Target in Cancer

Rank: 9.

High-Level Overview

p53 is an excellent target for use in cancer diagnosis, prognosis, and therapy.

Diagnostic, Prognostic, and Predictive

The majority of human tumors that are positive for p53 expression by IHC are associated with a high tumor grade/stage, reduced sensitivity to conventional chemo-/radiotherapies, and poor prognosis (Hong et al. 2010; Soussi 2003b; Hoffmann et al. 2008). Genetic analyses of *TP53* in tumors, which only sequence exons 5–8 (the p53 DBD), generally identify alterations in about half of the tumors tested. However, analyses of *TP53* coding exons 2–9, as well as intron/exon junctions, tend to identify alterations in upward of 80–90% tumors tested. Since tumors containing mutations of *TP53* tend to be more invasive and metastatic, these results can influence patient selection for therapies, as noted in head and neck cancer (Balz et al. 2003; Petitjean et al. 2007). Based on their p53 status, cancer patients may be stratified to different drug or radiation treatments. Tumor recurrence may be predicted by the presence of genetic alterations in p53 detectable in tumor margins (Poeta et al. 2009). In addition, the presence of Abs to p53 in the peripheral circulation of up to 20% of subjects with solid cancers is nearly always associated with the subjects having p53$^+$ tumors (Sangrajrang et al. 2003a). High titers of p53 Abs in the sera of patients may also serve as a prognostic marker. For example, in subjects at high risk for developing lung cancer based on their tobacco use, p53 seropositivity predicts poor prognosis (Sangrajrang et al. 2003b).

Therapeutics

Therapeutic targeting of p53 includes vaccinations against p53, Ab-based treatments, restoration of p53 functions, and activation of WTp53 in tumors (reviewed in Hong et al. 2014). Much effort has been invested in the development of p53-based vaccines aimed at inducing p53-specific T-cell-mediated antitumor responses (DeLeo and Whiteside 2008). The constraints of HLA allele restriction of antigen-specific CD8$^+$ cytotoxic T lymphocytes (CTL) and CD4$^+$ T helper (T_h) cells make it difficult to identify whether specific p53 codon mutations are immunogenic and can induce p53-specific T cells in patients with cancer (Ito et al. 2007). Consequently, the majority of studies aimed at either identifying p53-specific T-cell-mediated immune responses or developing p53-based vaccines focus on non-mutated epitopes that can be derived from the non-mutated regions of genetically altered p53 molecules or WTp53 expressed in tumors. The list of p53-based vaccines includes (i) single epitope- or multiple epitope-based peptide or polypeptide vaccines using dendritic cells (DC) and/or various adjuvants as delivery systems (DeLeo and Whiteside 2008; Ito et al. 2007; Hoffmann et al. 2002; Leffers et al. 2009; Speetjens et al. 2009; Antonia et al. 2006) and (ii) replication-defective viral constructs encoding WTp53 delivered directly in dendritic cells (Antonia et al. 2006). These vaccines are expected to increase the frequency and antitumor reactivity of these cells. The clinical trials testing these therapeutic vaccines in cancer patients are in progress (Cheok et al. 2011; DeLeo and Whiteside 2008).

Although Abs recognizing non-cell surface antigens, such as p53, primarily have a diagnostic/prognostic value, recently employed cationic lipid vehicles or recombinant single-chain Ab fragments have confirmed that intracellular delivery of p53-specific Abs or intact WTp53 molecules to tumor cells has antitumor effects (Weill et al. 2008; Hansen et al. 2007).

p53-based gene therapies aim at the restoration of p53 functions in the hope of reversing the progression of preneoplastic lesions or arresting tumor growth. Gene replacement therapy was initiated in the 1990s by intratumoral injection of replication-defective adenoviral constructs (Cheok et al. 2011). Therapies with recombinant adenoviral vectors encoding WTp53 or with modified vaccinia Ankara constructs are in clinical trials (Stegh 2012). Another variant gene therapy approach utilizes lytic viruses that can only replicate in cells expressing mutated p53. TP53 delivered as gene therapy is the only currently approved p53-based treatment (Cheok et al. 2011).

A promising approach makes use of small molecular weight compounds (SMWC) to restore p53 function or activate WTp53 (Chen et al. 2010; Mandinova and Lee 2011; Cheok et al. 2011). For example, screening for agents that selectively induce apoptosis of cells expressing mutant p53 can be accomplished by comparing the LD50 of an agent against two tumor cell lines, one a parental p53 null and the other line transfected with a construct encoding a mutant p53 molecule, usually one with a mutation in the DBD. Using this basic approach, agents that belong to one of three classes of p53 modulators are known. The first includes agents that activate WTp53. Most of these agents (e.g., MI-219, MI-319, nutlins, or SAH-p53-8) inhibit the p53/MDM2 and p53/MDM2-HDMX interactions that promote p53 degradation. The second class, conceptually the most challenging, consists of compounds that presumably can bind directly to mutant p53 molecules, alter their conformation, and restore their normal functions. This class includes CP-31398, PRIMA-1, MIRA-1, and the peptide RI-TAT-p53C' (Ferraiuolo et al. 2016). The exact mechanisms of these compounds are presently unknown and could vary with each agent. The third class includes compounds that activate members of the p53 family of transcription factors, TP73 and TP63, which can substitute for p53 in controlling the cell cycle. Presently, only one compound in this class has been identified, RETRA, which enhances expression of TP73.

Preclinical Summary

Extensive in vitro and in vivo preclinical studies have established the efficacy of various cellular, viral, and SMWC p53-based therapies for cancer. They employ murine and human tumor cells and primary or transplantable syngeneic murine tumor models in immunocompetent mice and human tumor-derived xenografts in immunodeficient mice. In addition to murine and human tumor cell lines or xenografts expressing WTp53, mutant p53, or p53−/−, the p53 isogenic HCT116 colon carcinoma cell panel developed by Bunz et al. (1998) is often used in the discovery phase and in subsequent in vivo evaluations of the therapeutic efficacy of SMWC

p53 modulators. This panel consists of the parental HCT116 cell line which is homozygous for WTp53 (+/+) and two p53 knockout variants, p53+/− and p53−/−. Their use permits a direct assessment of *TP53* gene dosage on the efficacy of a therapy.

Immune responses to p53 in tumor-bearing mice and humans with cancer have made p53 an attractive candidate for development of cancer immunotherapy. The development of p53-based vaccines is slow, in part, because the immune system has evolved numerous mechanisms that enable it to distinguish "self" from "nonself" and avoid autoimmune side effects. Since tolerance to "self" p53 epitopes exists in normal hosts, only low to intermediate affinity p53-specific T cells are detected (Theobald et al. 1997; Lauwen et al. 2008). The high-affinity "self" p53-specific T cells, presumed to be the most effective in antitumor responses, are deleted to avoid autoimmunity and can only be induced in p53 null mice and transgenic mice. In addition to tolerance, multiple mechanisms leading to p53 loss of function in tumors confound direct evaluations of whether a tumor cell processes and presents a sufficient level of HLA/p53-derived peptide complexes for recognition by T cells (Theoret et al. 2008; Andrade Filho et al. 2010). Despite these issues, p53-based vaccines are effective in controlling tumor growth in syngeneic murine tumor model systems in the therapeutic and prevention settings (Mayordomo et al. 1996; Vierboom et al. 1997). A variety of vaccines comprised of p53 peptides pulsed onto DC or admixed with chemical adjuvants, as well as nonviral plasmids and viral constructs encoding p53 epitopes or the intact protein, have been evaluated in numerous transplantable mouse tumor model systems and shown to have efficacy in limiting tumor growth. More recently, combinations of p53SMWC and p53-based vaccines were used for treatment of methyl cholantrene (MCA)-treated mice albeit with disappointing results, as the mice survival was not better relative to vaccines alone or SMWC alone (Zhang et al. 2016).

The availability of p53 knockout and p53 transgenic HLA-A2 mice models not only permitted researchers to establish the evidence for p53 tolerance but also to obtain high-affinity T-cell effectors against HLA-A2-restricted, human p53-derived epitopes. Based on evidence that these effectors did not recognize normal tissue cells, the potential of using genetically modified T cells expressing high-affinity p53 T-cell receptors (TCR) was developed for adoptive therapy of patients with cancer (Theobald et al. 1997; Theoret et al. 2008). Their clinical use is delayed, however, due to concerns for selection of tumors suitable for targeting based solely on IHC evidence for p53 positivity.

In preclinical in vitro and in vivo assays, the latter involving transplantable as well as primary mouse tumor systems and human tumor xenografts in immunodeficient mice, p53 SMWC have been shown to be effective in controlling the growth of murine and/or human tumors expressing either WTp53 or mutant p53 and primary murine tumors (Cheok et al. 2011; Tang et al. 2007; Rao et al. 2008). However, the effect of these p53 SMWC on the host immune system needs evaluation. SMWC effects on T cells could be critical, since these cells drive antitumor immune responses and activated T cells express elevated levels of WTp53. In this respect, highly proliferating T cells, including p53-specific T cells, can become targets of the

SMWC and be eliminated. Consequently, p53 SMWC modulators selected for their ability to target directly or indirectly p53 molecules in tumors and induce apoptosis also have the potential to interfere with induction and maintenance of antitumor immune responses.

Clinical Summary

The National institutes of Health database of clinical trials targeting p53 lists 267 trials, 99 of which are currently open for accrual. The majority are testing the p53 usefulness for patient stratification to different oncological therapies. The other trials for treatment of hematologic and solid malignancies include gene therapy, p53-based vaccines, inhibition of the p53-MDM2 interactions, and/or activation of WTp53 in tumors by SMWC. Of the current trails, those involving anti-p53 vaccines and blocking of p53-MDM2 interactions are the most numerous. The therapeutic vaccines currently in clinical trials are of an advanced design, utilizing mixtures of overlapping, long synthetic peptides representing the p53 region harboring most of the known HLA class I and II epitopes recognized by T cells of cancer patients and delivered with an adjuvant (Leffers et al. 2009; Speetjens et al. 2009). While this vaccine induces T helper type I responses in the majority of patients, its effects on survival have yet to be determined. Another type of promising vaccines now in phase I and II clinical trials utilizes adenovirus WTp53 construct-transduced DC with a goal of generating strong and polarized p53-specific immune responses as well as increasing tumor sensitivity to chemotherapy (Antonia et al. 2006).

Several phase I trials are testing various pharmacologic agents modulating the p53-MDM2 pathway that have shown efficacy with no toxicity in preclinical studies (Cheok et al. 2011). Both inhibitory agents (e.g., nutlin-3, which displaces p53 from the complex with MDM2) and p53-activating molecules (e.g., MDMX, which stabilizes p53) are promising because of their potential to restore WTp53 activity. Overall, one expects that the initial clinical trials of pharmacological and immunological strategies to restore functions of mutant p53 validate the preclinical results demonstrating that they can control cancer progression.

Anticipated High-Impact Results

As p53 remains a high-impact target for oncologic diseases, an accelerated translation of promising preclinical results to the clinic will continue in the near future. The expectations for the success of clinical trials targeting p53 and opportunities they create might provide a paradigm shift in therapy of cancer in the following ways:

- p53 status of patients with cancer will become the major factor in selecting oncological therapies and determining prognosis.

- Efforts to restore normal p53 functions in patients with cancer using one or several pharmacologic agents will accelerate, and this strategy will become approved by regulatory agencies.
- Therapeutic p53-based vaccines optimized for efficacy in eliciting robust and long-lasting antitumor responses will prolong patient survival.
- Prophylactic p53-based vaccines will be developed and translated to clinical trials.

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PAP

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Abstract

Prostatic acid phosphatase (PAP) is a 100 kDa glycoprotein synthesized by well-differentiated prostatic gland columnar epithelia and secreted in large quantity in seminal fluid (Hassan et al., Expert Rev Anticancer Ther 10:1055–1068, 2010). PAP is present in elevated concentrations in the serum of men who have prostate cancer or other prostatic diseases (Hassan et al., Expert Rev Anticancer Ther 10:1055–1068, 2010). The PAP gene is located along the long arm of chromosome 3 at locus 21 (3q21), and the protein product exists as intracellular, transmembrane, and secreted forms, with slightly different biochemical properties differentiating each of these (Hassan et al., Expert Rev Anticancer Ther 10:1055–1068, 2010; Solin et al., Biochem Biophys Acta 1048:72–77, 1990).

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Target: Prostatic Acid Phosphatase (PAP)

Prostatic acid phosphatase (PAP) is a 100 kDa glycoprotein synthesized by well-differentiated prostatic gland columnar epithelia and secreted in large quantity in seminal fluid (Hassan et al. 2010). PAP is present in elevated concentrations in the serum of men who have prostate cancer or other prostatic diseases (Hassan et al. 2010). The PAP gene is located along the long arm of chromosome 3 at locus 21 (3q21), and the protein product exists as intracellular, transmembrane, and secreted forms, with slightly different biochemical properties differentiating each of these (Hassan et al. 2010; Solin et al. 1990). Expression of the secretory form of human PAP has been evaluated using Northern blot analysis which confirmed high levels of expression in benign prostatic hyperplasia (BPH), prostatic carcinoma, and the androgen-dependent human prostate cancer cell line LNCaP (but not in the androgen-insensitive human prostate cancer cell line PC-3 or in non-prostatic malignancy) (Solin et al. 1990). The transmembrane form of PAP is expressed in non-prostatic tissues such as the brain, kidney, liver, lung, muscle, placenta, salivary gland, spleen, thyroid, and thymus (Quintero et al. 2007).

Biology of the Target

Prostatic acid phosphatase is one member of the acid phosphatases, a group of tissue isoenzymes widely distributed throughout the body, functioning to hydrolyze organic monophosphate esters (Taira et al. 2007). At the molecular level, PAP acts as a differentiation-associated protein tyrosine phosphatase, dephosphorylating the human epidermal growth factor receptor-2 (c-ErbB-2/HER-2/neu), thus decreasing the growth and tumorigenicity of prostate cancer cells (Hassan et al. 2010; Lin et al. 2001). Downregulation of cellular PAP results in hyperphosphorylation of the tyrosine residues of ErbB-2, activation of the mitogen-activated protein (MAP) kinase signaling cascade, and resultant androgen-independent stimulation of prostate cancer cell growth (Hassan et al. 2010; Meng et al. 2000). The secretory form of PAP may be elevated in the serum of prostate cancer patients despite the low expression of intracellular PAP in advanced prostate cancer tissue (Hassan et al. 2010).

Target Assessment

PAP can be quantified in the serum using either enzymatic reactions or radioimmunoassay (RAI). Most enzymatic reactions detect multiple acid phosphatase isoenzymes, have low sensitivity, are technically complicated by being unstable at room temperature, and are subject to diurnal variability (Taira et al. 2007). Of all of the acid phosphatase enzymatic tests, the most specific for PAP was developed by Roy et al. and utilizes thymolphthalein (Taira et al. 2007). Radioimmunoassay has improved sensitivity for prostatic acid phosphatase compared to the enzymatic tests; however an elevated serum PAP level by RAI is not specific for prostate cancer. Such elevations have been noted in both benign prostatic conditions (benign prostatic hypertrophy and prostatitis) and non-prostatic disease states (e.g., Paget's disease, hyperparathyroidism, Gaucher's disease, multiple myeloma, and non-prostatic malignancies with hepatic or osseous metastases) (Taira et al. 2007; Romas 1983).

Role of the Target in Cancer

Rank: Unknown-1-2-3-4-5-6-7-8-9-10: 8

Ranking is based on the clinical utility of PAP as a target for prostate cancer-specific immunotherapies and the evolving role for PAP as a prognostic marker in prostate cancer [see "High-Level Overview" section below].

High-Level Overview

Diagnostic, Prognostic, and Predictive

From the 1940s through the 1980s, PAP was the only available biomarker for prostate cancer, serving both as a screening tool and as a marker for response to treatment (Hassan et al. 2010; Taira et al. 2007). Due to its low sensitivity compared with the prostate-specific antigen (PSA) test, PAP has, since the 1980s, been replaced by PSA for the purposes of prostate cancer screening, staging, and monitoring of disease recurrence (Stamey et al. 1987; Johnson et al. 1976; Reif et al. 1973). Recent studies suggest that PAP may have prognostic value for patients with intermediate- and high-risk prostate cancer in terms of predicting biochemical recurrence and disease-free survival following definitive local control (Moul et al. 1998; Han et al. 2001; Dattoli et al. 2007). Moul et al. evaluated preoperative PAP levels in 295 patients who underwent radical prostatectomy between 1990 and 1996. While serum PAP was not able to predict pathologic stage (organ confinement or margin status), it was found to be an independent predictor of biochemical recurrence (defined as two successive PSA measurements >0.2 ng/mL). Biochemical recurrence-free survival rate at 4 years was 78.8% for men with PAP <3 ng/mL compared with 38.8% for those with PAP of ^{3}3 ng/mL; this difference remained

statistically significant regardless of preoperative PSA level (Moul et al. 1998). In a study by Han et al., serum PAP measured in 1,681 men before radical prostatectomy was again found to be a statistically significant, independent predictor of biochemical recurrence, defined as a PSA value of >0.2 ng/mL. Five- and 10-year biochemical recurrence-free survival rates for men with preoperative PAP <0.4 U/L were 87% and 77%, respectively, compared with 63% and 44% for men with preoperative PAP values of >0.5 U/L (Han et al. 2001). Dattoli et al. evaluated the prognostic significance of pretreatment serum PAP levels in 161 prostate cancer patients who received external beam radiation (41 Gy) followed by palladium 103 (Pd-103) brachytherapy; pretreatment serum PAP was found to be a stronger predictor of biochemical failure (defined as PSA>0.2 ng/mL) compared to Gleason score and pretreatment PSA (Dattoli et al. 2007). In a review by Roach et al. of the long-term survival of 1,557 patients enrolled onto four different randomized phase III Radiation Therapy Oncology Group (RTOG) trials, multivariate analysis revealed that elevated pretreatment serum PAP was associated with a reduction in disease-specific survival.

Therapeutics

Prostate cancer is the most common non-cutaneous malignancy in American men and remains the second leading cause of cancer-related deaths in this population (McNeel et al. 2009; Fong et al. 1997). Following definitive local surgery or radiotherapy, the disease recurs in approximately 20–30% of patients (McNeel et al. 2009). Prostate cancer has long been the focus of several efforts aimed at developing active immunotherapies, or antitumor vaccines, that might alter the natural progression of the disease once it recurs following initial local therapy. Prostatic acid phosphatase is considered a prostate tumor antigen and has been utilized as the target for many of these immunotherapies. The usefulness of PAP as a target for prostate cancer immunotherapy derives in part from the relative prostate specificity of the secretory form of this protein and the existence of a rodent homologue, thus providing an appropriate preclinical animal model (McNeel et al. 2009). Immunological strategies utilizing PAP as a target have included vaccination with either recombinant vaccinia virus expressing human PAP (Fong et al. 1997), plasmid DNA encoding full-length human PAP cDNA (co-administered with granulocyte-macrophage colony-stimulating factor, GM-CSF) (McNeel et al. 2009; Johnson et al. 2006, 2007), or autologous antigen-presenting cells (APCs) primed ex vivo with the recombinant fusion protein PA2024, consisting of PAP fused to GM-CSF (Sipuleucel-T, Provenge[â], Dendreon Corporation, Seattle, WA) (Burch et al. 2000; Small et al. 2000).

Preclinical Summary

The immunogenicity of recombinant vaccinia virus vectors expressing PAP has been evaluated by Fong et al. (1997). Copenhagen male rats were immunized with recombinant vaccinia virus vectors expressing either rat PAP(vv-rPAP) or human PAP (vv-hPAP).

The investigators demonstrated T-cell proliferative responses when splenocytes from either of these two rat groups were challenged with human PAP, suggesting an immunologic response had been generated to cross-species reactive epitopes. Following immunization with recombinant vaccinia viral constructs, cellular cytotoxicity was assessed using a standard 4-h ^{51}Cr release assay. Cytotoxic T-lymphocyte (CTL) activity against the rat PAP expressing tumor cell line AT-3 was seen only in those animals immunized with vv-hPAP and not in those immunized with vv-rPAP or control vaccinia vectors. This activity was able to be blocked using anti-MHC class I antibody, verifying the generation of an antigen-specific, MHC class I restricted, T-cell response (Fong et al. 1997). While these preclinical evaluations have been encouraging, concerns over preexisting immunity to the vaccinia vector, as well as the generation of vaccinia-specific immune responses which may preclude repeated immunizations (Johnson et al. 2007), have caused this strategy to be largely superseded by immunization with either plasmid DNA encoding human PAP or autologous APCs primed ex vivo with the fusion protein PA2024.

Preclinical work utilizing PAP-specific plasmid DNA vaccines has demonstrated immunological efficacy with minimal toxicity. In a report from 2006, Johnson et al. demonstrated that repeated immunizations of male Lewis rats with a GMP-grade, plasmid DNA vaccine encoding human PAP was safe and able to elicit both cell-mediated and humoral immune responses (Johnson et al. 2006). None of the animals displayed toxicity as assessed by examination of tissue histology, laboratory testing of blood counts or chemistry panel, or serial measurement of animal weight. Immunization with the vaccine was shown to generate antigen-specific, dose-dependent IgG1 and IgG2 antibody responses as well as PAP-specific T cells (Johnson et al. 2006). In a subsequent study by the same group, it was shown that Lewis rats immunized with a plasmid DNA vaccine encoding human PAP developed PAP-specific, interferon-gamma secreting CD4+ and CD8+ T cells as measured by in vitro T-cell proliferation assays and cytokine release enzyme-linked immunosorbent assays. Most importantly, repeated immunization with plasmid DNA vaccine encoding the rat PAP homologue resulted in an autologous, Th1-predominant immune response with antigen-specific CD4+ and CD8+ T cells, demonstrating that this immunization strategy was capable of breaking self-tolerance and inducing an autoimmune T-cell response (Johnson et al. 2007).

Immunization with autologous antigen-presenting cells (APCs) primed ex vivo with various tumor-associated antigens has been a strategy employed to induce therapeutic immunity to different malignancies (Burch et al. 2000; Small et al. 2000). Dendritic cells are APCs capable of inducing an antigen-specific immune response from an otherwise naive T cell (Burch et al. 2000). Preclinical studies in rats demonstrated that immunization with autologous dendritic cells loaded ex vivo with the fusion protein PA2024 is able to generate PAP-specific T-cell immune responses in vivo (Small et al. 2000).

Clinical Summary

McNeel et al. reported in 2009 results of a combined phase I/II trial evaluating the safety and efficacy of a PAP plasmid DNA vaccine in stage D0 prostate cancer patients (McNeel et al. 2009). The vaccine was found to be safe without any grade

three or four toxicity related to the investigational agent. Immunological activity was noted in the generation of antigen-specific T-cell responses in ten out of 22 patients (41%) as evidenced by either antigen-specific T-cell proliferation or CD8+ interferon-gamma ELISPOT assays (McNeel et al. 2009; Becker et al. 2010). Clinically, there was a statistically significant increase in the median PSA doubling time from a pretreatment baseline of 6.5–8.5 months on treatment and then 9.3 months at 1 year posttreatment (McNeel et al. 2009). Subsequent analysis revealed that of the eight patients who had experienced at least a 200% increase in PSA doubling time, six patients had evidence of durable PAP-specific interferon-gamma secreting T cells (Becker et al. 2010).

In 2000, Small et al. reported results of a combined phase I/II trial of Sipuleucel-T (Provenge[â], Dendreon Corporation, Seattle, WA) in men with castration-resistant prostate cancer (CRPC) (Small et al. 2000). Twelve patients with CRPC were enrolled in the phase I study, all of whom had metastatic disease; median PSA of patients in the phase I was 209 ng/mL. Nineteen patients with stage D0 prostate cancer were enrolled in the phase II portion, with a median on-study PSA of 14.5 ng/mL. Sipuleucel-T was well tolerated with fever being the most common adverse event, occurring in 15 of the 102 total infusions (14.7%) (Small et al. 2000). Two of these febrile reactions were scored as grade three according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE). Evaluation of immunological efficacy showed PA2024-specific T-cell proliferative responses to be present in all patients following infusion of Sipuleucel-T, with maximal responses occurring after two or three infusions; no patient had baseline pretreatment PA2024-specific T-cell proliferative responses. 38% of patients examined developed Th1-type immune responses to PAP, as assessed by T-cell interferon-gamma production (Small et al. 2000). Three patients had at least a 50% decline in PSA, with an additional three patients experiencing a decline in PSA of between 25% and 49%. No objective radiographic responses were seen. Median time to disease progression was 12 weeks for the phase I patients and 29 weeks for the phase II patients (Small et al. 2000).

In 2010, Kantoff et al. published results of a randomized, double-blinded, placebo-controlled multicenter phase III trial of Sipuleucel-T in patients with metastatic castration-resistant prostate cancer (CRPC) (Kantoff et al. 2010). Compared to the placebo arm, patients in the Sipuleucel-T arm had a statistically significant 22% relative reduction in the risk of death, corresponding to a 4.1-month improvement in median overall survival (25.8 months in the Sipuleucel-T arm vs. 21.7 months in the placebo group). Interestingly, despite the improvement in the primary end point of overall survival, there was no difference between the two arms in time to objective disease progression (Kantoff et al. 2010).

In summary, the clinical data with prostatic acid phosphatase which targeted immunotherapies has shown that they are well tolerated, are able to induce antigen-specific immune responses, and, in the case of Sipuleucel-T, have a demonstrated ability to alter the kinetics of prostate cancer progression, resulting in improvements in overall survival.

Anticipated High-Impact Results

Long-term analysis of survival and immunological efficacy following immunization with PAP-specific plasmid DNA and/or dendritic cell vaccines.

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Peptide Vaccine: Overview

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Patrick M. Dillon and Craig L. Slingluff

Conflicts
Dr. Slingluff is a member of the Scientific Advisory Board for Immatics and is an inventor on patents for peptides used in cancer vaccines, held by the University of Virginia Licensing and Ventures Group. Dr. Dillon has no conflicts.

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Abstract

"Peptide antigens are presented by class I HLA molecules and have been studied for use in cancer vaccines. Short peptides from 8 to 11 amino acids have been able to elicit immune responses in both animal and human studies historically, although none have been approved in the clinic. More recently, long peptides, phosphopeptides, helper peptides, hybrid peptides and peptide cocktails are being explored for use in vaccine therapies of cancer. Antigenic peptides have been found for every cancer type and the peptide vaccines have been tested in nearly all major cancer types. A few of the most important peptide vaccine studies are highlighted in this overview."

Keywords

Helper T cells • Human leukocyte antigen • Peptide vaccine • Synthetic peptides

Target

Cancer-associated proteins can serve as targets for cancer identification, biomarker development, and immune therapies. Those proteins in whole or as shorter peptide fragments may be used for cancer vaccines. Vaccines against cancer-causing viruses have been highly successful, such as the human papillomavirus and hepatitis B vaccines which have dramatically decreased the burdens of cervical cancer and hepatocellular cancer, respectively. Since the identification of cancer antigens nearly four decades ago, a wide array of cancer-associated protein antigens have been discovered. Many of the relevant proteins have been defined and evaluated. Furthermore, short peptide epitopes (8–11 amino acids) representing the minimal immunogenic components of a protein have been characterized for many of the cancer proteins known to date. The short peptides offer a specific target for antitumor immunity and are generally easy to synthesize. They have predictable structural and stability characteristics (Slingluff et al. 2006). Longer peptides (up to 50 amino acids), phosphopeptides, helper peptides, hybrid peptides, and peptide cocktails are some of the advances being applied to vaccine therapies for cancer.

Biology of the Target

A cloning technique to identify genes and peptides of tumor-associated antigens from melanoma was first reported in 1991 (van der Bruggen et al. 2007). Since then, mass spectrometric techniques, coupled to functional assays, have identified antigenic MHC-associated peptides directly from the cell surface (Cox et al. 1994). All these advancements led to identification of numerous immunologically relevant antigens including several which are posttranslationally modified (Skipper et al. 1996; Cobbold et al. 2013; Depontieu et al. 2009; Mohammed et al. 2008). The immunogenic peptides are characterized by MHC class I or II recognition and in

some cases can be characterized by their activity in clinical trials. Only a fraction of the known cancer-associated peptides have been tested clinically, to date.

The peptide antigens recognized by $CD8^+$ T cells are presented (restricted) by class I human leukocyte antigen (HLA) molecules, so most peptide sequences can be recognized only in patients with a matching HLA type. This constraint limits single peptide vaccines to a subset of the population with matching HLA type. Fortunately, multi-peptide vaccines and individualized vaccines may circumvent the HLA restriction. Additionally, the wide breadth of cancer-specific peptides increases the applicability. Most full length proteins contain more than one epitope, so there is potential to generate multiple epitopes from a single protein. Furthermore, a majority of proteins in mammals contain epitopes recognizable by both cytotoxic T lymphocytes ($CD8^+$) and also by helper T cells (typically $CD4^+$). Thus, there is potential to manipulate both components of the T cell immune response.

Target Assessment

Patients' tumors can be assessed for expression of the target peptide antigen. For nonmutated antigens, immunohistochemistry of the tumor can test whether the source protein is expressed or flow cytometry of hematologic malignancies. For mutated antigens, genetic sequencing is typically required. To determine whether the peptide target is expressed, HLA typing can be performed on the patient's peripheral blood lymphocytes. However, some tumors can escape immune recognition by downregulating HLA molecule expression or by acquired defects in antigen processing machinery in the tumor cells. Definitive assessment of peptide antigen expression can be determined either by mass spectrometry or by testing whether T cells reactive to the test antigen recognize the patient's tumor cells; however, these definitive approaches are challenging to apply on a routine basis. Assessment of phosphoproteins, nuclear proteins, and secreted and extracellular matrix proteins is also possible by chromatography and mass spectrometry based techniques. Mutated proteins for targeting can be detected by routine PCR and next generation sequencing.

Role of the Target in Cancer

The role of peptides as cancer therapeutics is mostly limited to research applications. As mentioned above, the preventative vaccines for HPV and hepatitis B both employ whole proteins rather than peptides for vaccination, although a peptide-based HPV vaccines using the L2 capsid protein is reported. Also, clinical trials of peptide vaccines have induced promising clinical benefits: vaccination with a gp100 peptide has improved clinical outcome when added to high-dose IL-2 for melanoma, and vaccination with HPV peptides has induced high rates of clinical regression of vulvar neoplasia (Kenter et al. 2009; Schwartzentruber et al. 2011). A related immunotherapy, Sipuelucil-T, utilizes a fusion prostatic acid phosphatase-GM-CSF protein for the manufacture of educated antigen-presenting cells and was FDA approved in 2010.

A few peptide-based vaccines are in late stage clinical trials and may achieve approvals from regulatory authorities. Specifically vaccines for melanoma, breast cancer, and renal cell carcinoma are in late stage development. It is not yet clear whether peptide vaccines will ultimately gain approvals in a preventative setting, an adjuvant setting, or an advanced disease setting. Studies are ongoing to evaluate efficacy in all three settings and in various combinations with other cancer therapeutics.

High Level Overview

Diagnostic, Prognostic, Predictive

In patients with advanced disease who achieve measurable clinical response to peptide vaccines, there tends to be a degree of durability. Intriguingly, some patients without a RECIST measurable response enjoy long duration stability and some patients with so-called pseudoprogression go on to derive late benefits. No predictive biomarkers exist beyond the HLA typing which is required to select patients for specific peptides. There are limited data on IL-6 as a prevaccine biomarker of response to peptide vaccines, but much more work needs to be done to validate this (Hazama et al. 2014). In some cases, the expression of the parent protein of a vaccinating peptide may be useful, but expression is often poorly predictive of response and may even associate with a tolerogenic response.

Therapeutics

Peptides are easily synthesized and purified. They have good stability when stored at $-70°$. Their safety is well established in over 100 human trials. They have proven to be effective at inducing both CD8 and CD4 T cell responses in vivo in humans, and it is feasible to perform direct monitoring of the T and B cell responses to peptide vaccination (Slingluff et al. 2006). Additionally, repeated boosting is feasible and may enhance the magnitude and duration of T cell and B cell responses. In fact, by careful monitoring it has been observed that in some cases the proportion of vaccine-specific $CD8^{+}$ cells has exceeded 1% of the circulating $CD8^{+}$ T cells (Speiser et al. 2005). Types of peptides for vaccination are discussed below.

Short Peptides

Immune response rates for peptide vaccines have been reported as high as 100%, although definitions of response vary. Similarly, some MHC-presented peptides have not proven to be immunogenic, and several strongly immunogenic peptides have failed to induce clinically meaningful responses. One reason for limited response may be that short peptides can bind to numerous types of cells but only a minority of cells with binding potential are professional antigen-presenting cells. Nonspecific

binding to cells such as fibroblasts can result in tolerance, especially when costimulation is absent (Loschko and Krug 2012). Also if a tumor antigen is expressed on any normal cells at low levels, then preexisting tolerance may exist and hinder vaccine response.

Tumor antigens include the differentiation antigens, cancer testis antigens, overexpressed antigens, and some unique antigens which arise from somatic mutations in tumor. A listing of 403 well-characterized peptides and potential peptides for vaccine therapy is available (Vigneron et al. 2013) as well as a NCI review of characterized antigens (Cheever et al. 2009).

Well-known differentiation antigens include Melan-A/MART-1, gp100, and tyrosinase for melanoma, and PSA and PSMA for prostate cancer. Despite being self-antigens, these can all elicit strong T cell responses under special circumstances. The cancer testis antigens include MAGE-A1, MAGE-A3, NY-ESO-1, and others. These antigens are expressed in many tumors and in both male and female germ line cells but not in other normal tissues. Their aberrant reexpression in cancer is likely due to promoter hypomethylation by well-described mechanisms (Vatolin et al. 2005). Since most germ line cells do not express MHC molecules on their surface, there is little risk of germ cell targeting. Overexpressed antigens include HER2/Neu, MUC-1, CEA, p53, survivin, or telomerase. These may present in many normal cells but typically at low levels. Their overexpression on cancer cells can make them reasonable immunologic targets.

Human T cells typically can recognize unique antigens from mutated proteins as well as self-antigens. Antigens from mutated proteins may be more effective immunogens because of their unique restriction to tumor cells and low preexisting host tolerance. Shared or self-antigens have been the principal antigens used in cancer vaccines, but as technologies for genome sequencing have become more accessible, unique antigen discovery and delivery on an individual basis is now feasible.

Long Peptides

While vaccination with short peptides (8–10 amino acids) often shows early immunogenicity, there is concern that short peptides may ultimately induce tolerance due to binding to nonprofessional antigen-presenting cells which cannot produce costimulatory signals. Full length proteins frequently harbor multiple HLA class I and II epitopes. Thus, vaccines with whole proteins or peptides >25 amino acids in length may be expected to have immunogenicity across HLA types. An illustrative example of long peptides was a study in end-stage cervical cancer (Kenter et al. 2008) using nine peptides up to 35 amino acids in length. T cell responses to most of the peptides were observed, and clinical responses were seen in 15 of 19 patients, with nine complete regressions.

Several other vaccines have been tested using long peptides. For example, a p53 synthetic long peptide vaccine resulted in 9 of 10 patients showing immune response. Surprisingly, most of the responding cells were $CD4^{+}$ rather than $CD8^{+}$. Another recent example (Kakimi et al. 2011) tested a 20-mer NY-ESO-1 peptide along with OK-432 and Montanide ISA-51. The vaccine resulted in specific CD4

and CD8 T cell responses, as well as NY-ESO-1 antibody induction in 9 of 10 patients with advanced solid tumors. Three of 10 patients had stable disease as their best clinical response.

Helper Epitopes

Helper T cells likely play roles in the induction of cytotoxic lymphocyte responses, both in augmenting activation of dendritic cells and in regulating responses. Helper T cells also enhance cytokine production, which supports antigen presentation and $CD8^+$ T cell stimulation. Early studies demonstrated that adoptive transfer of $CD4^+$ helper T cells may be clinically beneficial. Likewise, $CD4^+$ T cell depletion blocked immune response to vaccines. Numerous helper epitopes are now characterized and many of them exist within known cancer-associated proteins and often adjacent to epitopes recognized by $CD8^+$ T cells.

One of the earlier helper epitopes tested was PADRE which is a universal helper T cell epitope which has been combined with dozens of other epitopes in several cancer vaccines and vaccines for infectious diseases (Alexander et al. 1994). Several groups have shown increases in PADRE-specific CD4 T cells after vaccinations. Similar studies with keyhole limpet hemocyanin (KLH) and tetanus toxoid helper peptide have been reported. Both have produced immune responses in over 90% of patients, and the responses are predominantly Th1 dominant.

The largest helper peptide trials to date were performed in melanoma patients using a combination of six helper peptides from differentiation antigens and cancer testis antigens. In phase II trials, there were several clinical responses and fair rates of immunogenicity (>80%) (Slingluff et al. 2008). Epitope spreading was observed as well as antibody production. Responses were sustained more than 6 months after completion of a vaccination series, which is suggestive of immune memory. Vitiligo was induced in 10% of the patients, and delayed type hypersensitivity responses were seen in 29%. The helper peptides are longer than the minimal epitopes for $CD8^+$ T cells (14–23 residues) and thus may require uptake by dendritic cells and presentation in association with Class II MHC molecules. Thus, they may be selectively presented by professional APC's.

Peptide Cocktail Vaccines

Multiple peptides can be copresented without significant diminution of the response to any of the individual peptides. Several clinical trials have confirmed that finding and even suggested that cocktails of multiple peptides induce significantly greater proportions of T cells reactive to vaccine antigens than single peptides alone.

Several studies have evaluated immunologic and clinical outcomes after vaccination with mixtures of helper peptides with MHC class I-restricted peptides (type I peptides). In a metastatic non–small cell lung cancer, the combination of nine type I peptides and the PADRE helper peptide resulted in an encouraging median survival of 17.3 months and one complete response (Barve et al. 2008). In hormone sensitive prostate cancer, a mixture of 11 type I peptides and two helper peptides was performed and showed a slowing of PSA rise in 21% of patients (Feyerabend

et al. 2009). Likewise a mixed vaccine in renal cell carcinoma suggested clinical responses (Walter et al. 2012).

However, the only prospective randomized trials have found paradoxically that combination of helper peptides and class I associated peptides failed to improve the CD8 T cell response. These were both large multicenter prospective randomized trials; in one of those trials, the combination significantly diminished the CD8 T cell response, but the CD4 T cell response was maintained (Slingluff et al. 2011). Those findings are not yet explained and may be related to the nature of the adjuvants used or other factors. Additional studies are warranted to develop optimal ways to combine peptides for both helper and cytotoxic T cells.

Hybrid Peptides

Hybrid-type vaccines are peptides comprised of two epitopes fused together. Examples include the Ii-Key/HER-2/neu hybrid peptide and a MAGE-A4 helper/type I fused peptide. The Ii-Key/HER2/neu fuses the li-key 4-mer peptide and HER-2/neu (776–790) helper epitope (Perez et al. 2010). It is reported that the Ii protein catalyzes direct charging of MHC class II epitopes to the peptide-binding groove, bypassing intracellular epitope processing. Lower frequencies of regulatory T cells are reported as well as lower TGF-β compared with the nonfused HER-2/neu peptide. For the MAGE-A4/helper hybrid, a phase II study is ongoing.

Phosphopeptides

Since signal transduction is an effective target for small molecule drugs in oncology, it is logical to pursue methods to block the activity of bioactive phosphoproteins. Thus, peptides that contain phosphoserine or phosphotyrosine residues have been developed for vaccine therapy. A study with two of these agents is ongoing (NCT01846143).

Preclinical Summary

The first peptide vaccination experiments in mice were performed in the late 1980s and showed that short peptides confer T cell recognition. Synthetic peptides were able to prevent tumor outgrowth in several different mouse models. The early studies were limited to viral epitopes and mutated self-epitopes with strong binding affinity for MHC class I and were delivered in mineral oil preparations. Disappointingly, the human follow-up studies with short peptides failed to achieve the same degree of success observed in the mice. There was even evidence that vaccination led to functional deletion of antigen-specific T cells in humans. Improved vaccine designs and adjuvants have mostly addressed this concern, but it remains a risk to any new vaccine formulation.

Despite their differences, mouse studies still inform and generally precede human vaccine trials. Key advances in preclinical vaccine research include use of longer peptides requiring dendritic cell processing, new adjuvants, and the use of

immunocompetent mouse models. Preclinical studies have informed the development of whole classes of adjuvants from pathogen-associated molecular patterns (PAMPs). Use of PAMP adjuvants allows for recognition by pattern recognition receptors such as the Toll-like receptors which results in the activation and maturation of antigen-presenting cells and low likelihood of tolerogenic responses (van Hall and van der Burg 2012). Animal models have also advanced the understanding of the tumor microenvironment response to peptide vaccines, and the models have also provided platforms for measuring peptide recognition, stability, distribution, and immunogenicity. Several polymer and nanotechnology-based delivery systems for vaccines have also recently been validated in animal models.

Clinical Summary

Renal Cell Carcinoma

An exciting advance in peptide vaccines is the IMA901 renal cell vaccine which consists of nine tumor-associated peptides. The peptides were identified from comparison of RNA and HLA ligands in normal and tumor tissue. Mass spectrometry techniques were used to identify peptides found naturally presented by renal cell carcinomas. In the phase I study of 28 patients with RCC, the vaccine was given with GM-CSF and the hepatitis B core antigen as a helper. There was one partial response and 11 patients with stable disease. In the phase II study of 68 patients with or without cyclophosphamide (Walter et al. 2012), there was one complete response and two partial responses. Among immune responders in that trial, cyclophosphamide was associated with prolonged survival. The study showed a 20% decrease in T-reg numbers with cyclophosphamide which suggests a benefit to CTL generation. Their studies also identified potential biomarkers of immune response, namely APOA1 and CCL17. Since reduced APOA1 levels are found in many types of cancer and are associated with chronic inflammation, it is suspected that this may be a marker of suppressed adaptive immune response.

Breast Cancer

Her-2/neu, a 185 kD gene product of erbB2/neu proto-oncogene, is overexpressed in 20% of breast cancer patients. Her-2/neu is weakly detectable on epithelial cells of normal tissues. Her-2/neu has no direct ligand, instead it has mitogenic activity when heterodimerized with HER3 (ErbB3). Her-2/neu has become an attractive target for immunotherapy due to those properties. Indeed, the HER2/neu targeting antibodies, trastuzumab and pertuzumab, have been dramatically successful in treating both primary and metastatic HER2^{+} breast cancer.

To date, several type I and type II epitopes to HER2 have been identified and tested (Disis et al. 2009).

Disis et al. reported 92% immune response rate to a mixture of three helper peptides derived from HER2/neu in a phase II study given with GM-CSF. They also observed epitope spreading. They were later able to show that the combination of that vaccine with trastuzumab was safe and immunogenic (Disis et al. 2009). Additionally, a phase II study with a HLA-A2 restricted peptide from the extracellular domain of HER2/neu (E75) was immunogenic and was associated with lower risk of recurrence in high risk patients (Holmes et al. 2011). Over a dozen trials are currently ongoing using various HER2/neu derived peptides with various adjuvants in various patient subsets.

Melanoma

Peptide vaccines for melanoma may be the most extensively studied area in cancer vaccine research with over 100 melanoma antigens recognized by T cells having been described to date. The melanoma vaccines have induced objective clinical tumor regressions in only 3–5% of patients as single agent therapies for advanced melanoma and some large vaccine trials have yielded negative results. To highlight a few accomplishments, a vaccine containing a gp100 peptide in Montanide ISA-51 improved progression free survival in a phase III randomized prospective trial when combined with IL-2, compared to IL-2 alone. However, a combination of the same gp100 peptide vaccine with CTLA4 blockade was associated with a slightly worse clinical outcome than use of CTLA4 blockade alone (Hodi et al. 2010). Likewise, a trial of a melanoma ganglioside-KLH vaccine compared with observation was stopped early as the vaccine arm was performing worse than the observation arm. Arguably, many of the melanoma vaccine trials over the last two decades have included GM-CSF as a stimulator of APCs, and since GM-CSF is now shown to have limited adjuvant value, this may cloud some of the existing efficacy data.

On a positive note, the small subset of patients who do achieve clinical responses to melanoma vaccines usually get prolonged durability similar to the durability seen with IL-2 or ipilimumab therapy. Additionally, humoral responses are frequently seen in melanoma vaccine studies, and these may augment the strength and breadth of response. It is possible that features of the vaccine adjuvant may be critical determinants of the ability of peptide vaccines to induce and to maintain T cell responses. As new checkpoint inhibiting agents enter the therapeutic arena in patients with advanced melanoma, there may be value in combining them with vaccines. There are already studies underway to test such combinations in the adjuvant setting. Currently, the only FDA approved adjuvant therapy for melanoma is high-dose interferon, which has toxicity limitations. Since peptide vaccines are a low toxicity alternative, they may have value in the adjuvant setting, especially in the setting of low tumor burden.

Lymphoma

Two studies of idiotype peptides for vaccination in follicular lymphoma resulted in statistically significant improvements in disease free survival (Schuster et al. 2011). While there had been prior negative studies, the recent successes observed do support the potential use of mutated antigens in hematologic malignancies. The idiotype is the antigenic determinant of the hypervariable region of immunoglobulin, and it is a clone-specific antigen. Thus, the idiotype is an excellent target for which there is no preexisting self-tolerance. The finding of a 13-month extension in DFS ($p < 0.05$) in the follicular lymphoma trials is encouraging (Schuster et al. 2011).

Anticipated High Impact Results

Selected peptide vaccines have demonstrated inducible tumor recognition and eradication in subsets of patients. No peptide vaccine has yet achieved tumor regression across a large cohort of human cancer patients. Nevertheless, tumor control and prevention in the correct context is possible and potentially durable. The peptide vaccines do continue to be developed as both stand-alone therapies and in combination with other cancer therapies.

Anticipated future directions include the expected outcomes of several long-peptide vaccine studies, the new phosphopeptide studies, and several idiotype-based peptide antigen studies. Peptide antigens have been incorporated into adoptive cell strategies. Nanoscale platforms to bring peptides in close approximation to the adjuvants and cytokines necessary for robust immune response have been tested and may aid in overcoming tolerogenic responses.

It is also expected that with tumor sequencing becoming widespread, we may see the identification of additional potential antigens. Sequencing may also open the door to individualized peptide vaccines to each patient's uniquely mutated proteome. Indeed, studies are already underway to exploit the mutant proteome for both vaccine and antibody targeting.

Advances are expected in vaccine adjuvants. Adjuvants for peptide vaccines have not been optimized. Incomplete Freund's adjuvant (IFA), for example, aids the induction of antibody responses to protein vaccines, but it appears to be suboptimal for induction of T cell responses to short peptides. Toll-like receptor agonists and other activators of innate immunity have proven benefit as adjuvants, either alone or in combination with IFA, and studies are needed to assess whether optimized adjuvants may improve T cell responses and clinical benefit of peptide vaccines.

A challenge for both peptide vaccines and other immunotherapies remains the lack of T, B, or NK cell infiltration at the sites of many tumors. It is known that several mechanisms contribute to tumor microenvironment hostility toward effective immune cell infiltration. Thus, while immune responses can be generated in up to 100% of vaccinated patients, it remains unclear how to best steer responding lymphocytes to sites of disease. Some local therapies such as radiation and certain ablative therapies can alter the tumor microenvironment to support T cell infiltration;

thus, there is a rationale for combination of cancer vaccines with local therapies. Potential combinations in development include PD-1 blockade, CTLA-4 blockade, tyrosine kinase inhibitors, radiation therapy, and other novel therapies. With these ongoing efforts, it is likely that peptide-based vaccine approaches will find places alongside other immunotherapies in the future.

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Cross-References

- ▶ Anti-Idiotype Antibodies
- ▶ Anti-Programmed Death 1 (PD1)
- ▶ APC
- ▶ CD4+ T Cells
- ▶ CD8 T Cells
- ▶ CEA
- ▶ CTLA-4
- ▶ Dendritic Cells
- ▶ GM-CSF and Whole Cells
- ▶ gp100
- ▶ HER2/neu
- ▶ Interleukin-2
- ▶ MART-1
- ▶ MUC1
- ▶ NK Cells
- ▶ P53, Immunology
- ▶ PAP
- ▶ PSA
- ▶ TGF Beta Receptors
- ▶ Tregs
- ▶ TLR7 and TLR8, Resiquimod, and 852A
- ▶ TLR9
- ▶ Tyrosinase: Overview

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Proteins (Mesothelin) 39

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Abstract

Mesothelin is a glycosyl-phosphatidylinositol (GPI)-anchored glycoprotein present on the surface of many malignant cells originally identified as the target of a monoclonal antibody raised against a human ovarian cancer cell line (Chang and Pastan 1996). Normal expression of mesothelin is primarily restricted to mesothelial cells lining the pericardium, peritoneum, and pleura. However, mesothelin is

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expressed on nearly all mesotheliomas and pancreatic adenocarcinomas; commonly on ovarian cancers, non-small-cell lung cancers, squamous cell carcinomas, and acute myeloid leukemias; and less frequently on other cancer cells including colorectal, esophageal, and gastric cancers (Chang and Pastan 1996; Hassan et al. 2004; Hassan and Ho 2008; Steinbach et al. 2007). In total, mesothelin is expressed on nearly a third of human malignancies. The mesothelin gene consists of 17 exons located on the human chromosome 16p13.3 (Chang and Pastan 1996; Hassan et al. 2004). It encodes a 71-kDa precursor protein that is proteolytically processed into a shed 31-kDa N-terminal fragment – megakaryocyte-potentiating factor (MPF), a protein shown to stimulate megakaryocyte colony-forming activity of IL-3 in mouse bone marrow cell culture (Kojima et al. 1995), and the surface-bound 40-kDa C-terminal fragment – mesothelin. Elevated levels of soluble mesothelin have been detected in the sera of patients with mesotheliomas and ovarian and pancreatic cancers (Scholler et al. 1999; Hassan et al. 2006; Johnston et al. 2009). Studies are underway to determine if serum levels of mesothelin can serve as a marker of tumor burden or as a marker for diagnosis and follow-up of patients with mesothelin-expressing tumors. Spontaneous mesothelin-specific antibody responses have been detected in patients with mesotheliomas and ovarian cancers and less commonly in patients with pancreatic cancers (Johnston et al. 2009; Hellstrom et al. 2008; Ho et al. 2005). Although antibody responses are less common in pancreatic cancer patients, spontaneous mesothelin-specific T cell responses have been measured in approximately 50% of patients with pancreatic cancers (Johnston et al. 2009). Furthermore, early studies suggest that enhanced post-immunotherapy mesothelin-specific T cell responses may be associated with improved survival in pancreatic cancer patients receiving a whole tumor cell vaccine (Thomas et al. 2004; Laheru et al. 2008; Lutz et al. 2011). Due to the immunogenicity of mesothelin, its restricted expression in normal tissues and high expression on the surface of multiple cancers, mesothelin is considered an attractive target for immunotherapy (Hassan et al. 2004; Hassan and Ho 2008). For this reason, both T cell-mediated and antibody-mediated treatment strategies specifically targeting mesothelin are under clinical development.

Keywords

CanScript • Mesothelin • CRS-207 vaccine • Gene • Granulocyte-macrophage colony-stimulating factor • Immunostaining • Immunotherapy • In tumors • MESOMARK assay • MORAb-009 • Phase I clinical studies • Preclinical studies • Soluble • Variant • Soluble mesothelin • Soluble mesothelin-related protein (SMRP) • SS1P

Target: Mesothelin

Mesothelin is a glycosyl-phosphatidylinositol (GPI)-anchored glycoprotein present on the surface of many malignant cells originally identified as the target of a monoclonal antibody raised against a human ovarian cancer cell line (Chang and

Pastan 1996). Normal expression of mesothelin is primarily restricted to mesothelial cells lining the pericardium, peritoneum, and pleura. However, mesothelin is expressed on nearly all mesotheliomas and pancreatic adenocarcinomas; commonly on ovarian cancers, non-small-cell lung cancers, squamous cell carcinomas, and acute myeloid leukemias; and less frequently on other cancer cells including colorectal, esophageal, and gastric cancers (Chang and Pastan 1996; Hassan et al. 2004; Hassan and Ho 2008; Steinbach et al. 2007). In total, mesothelin is expressed on nearly a third of human malignancies. The mesothelin gene consists of 17 exons located on the human chromosome 16p13.3 (Chang and Pastan 1996; Hassan et al. 2004). It encodes a 71-kDa precursor protein that is proteolytically processed into a shed 31-kDa N-terminal fragment – megakaryocyte-potentiating factor (MPF), a protein shown to stimulate megakaryocyte colony-forming activity of IL-3 in mouse bone marrow cell culture (Kojima et al. 1995), and the surface-bound 40-kDa C-terminal fragment – mesothelin. Elevated levels of soluble mesothelin have been detected in the sera of patients with mesotheliomas and ovarian and pancreatic cancers (Scholler et al. 1999; Hassan et al. 2006; Johnston et al. 2009). Studies are underway to determine if serum levels of mesothelin can serve as a marker of tumor burden or as a marker for diagnosis and follow-up of patients with mesothelin-expressing tumors. Spontaneous mesothelin-specific antibody responses have been detected in patients with mesotheliomas and ovarian cancers and less commonly in patients with pancreatic cancers (Johnston et al. 2009; Hellstrom et al. 2008; Ho et al. 2005). Although antibody responses are less common in pancreatic cancer patients, spontaneous mesothelin-specific T cell responses have been measured in approximately 50% of patients with pancreatic cancers (Johnston et al. 2009). Furthermore, early studies suggest that enhanced post-immunotherapy mesothelin-specific T cell responses may be associated with improved survival in pancreatic cancer patients receiving a whole tumor cell vaccine (Thomas et al. 2004; Laheru et al. 2008; Lutz et al. 2011). Due to the immunogenicity of mesothelin, its restricted expression in normal tissues and high expression on the surface of multiple cancers, mesothelin is considered an attractive target for immunotherapy (Hassan et al. 2004; Hassan and Ho 2008). For this reason, both T cell-mediated and antibody-mediated treatment strategies specifically targeting mesothelin are under clinical development.

Biology of the Target

The natural biological function of mesothelin is not clear. With the exception of a C-terminal GPI anchor motif, analysis of the mesothelin protein sequence shows no strong homologies to known protein functional domains (Chang and Pastan 1996). NIH 3T3 cells transfected with mesothelin are more adherent to culture dishes than non-transfected cells suggesting that mesothelin may serve as an adhesion molecule (Chang and Pastan 1996). However, mesothelin-knockout mice show no signs of anatomic, hematologic, or reproductive abnormalities, suggesting that the function of mesothelin is not essential (Bera and Pastan 2000).

Three forms of the mesothelin gene transcript have been identified encoding slightly different proteins (Hassan et al. 2004). The major variant (variant 1) encodes a 628-amino acid protein that gives rise to MPF and mesothelin. The second variant (variant 2) has a 24-base pair (bp) insert that results in an 8-amino acid insertion in the GPI-anchored mesothelin portion of the gene. The third variant (variant 3) has an 82-bp insert which results in an alternative C-terminus that lacks the GPI-anchor motif and yields a soluble form of mesothelin which has been referred to as soluble mesothelin-related protein (SMRP). Although these variant forms have been detected at the mRNA and protein level, analysis of the expressed sequence tag (EST) database and mRNA transcripts of mesothelin suggest that the major form present in both normal and tumor tissue is variant 1. In addition, the soluble form of mesothelin detected in the serum of patients with mesothelin-expressing cancers is predominantly derived from variant 1 (Hassan et al. 2004). The mechanism responsible for the release of this form of mesothelin from the cell surface has not been well characterized.

Target Assessment

Mesothelin can be measured in the serum by ELISA, on paraffin-embedded tissue by immunohistochemistry (IHC), and on cells by both flow cytometry and Western blotting. The MESOMARK assay (Beyer et al. 2007) (Fujirebio Diagnostics) is a commercially available mesothelin-specific ELISA. Since the discovery of the first mesothelin-specific monoclonal antibody (K1) in the early 1990s (Chang et al. 1992), several mesothelin-specific antibodies suitable for immunohistochemistry, flow cytometry, and Western blotting have become commercially available. Of the available antibodies, monoclonal antibodies K1 and 5B2 have been used most often for IHC analysis of tumor tissue. Although similar results have been obtained using different antibodies, some differences in staining have been observed and caution must be taken when comparing results from studies using different antibodies. The choice of antibody and the methods used for tissue preservation and antigen recovery may explain why differences in mesothelin expression on some tumors, such as lung cancer, have been observed.

Role of Target in Cancer

Mesothelin is proposed to serve as an adhesion molecule (Chang and Pastan 1996). In support of this notion, mesothelin has been shown to mediate heterotypic cell adhesion through a high-affinity interaction with the ovarian cancer surface marker MUC16/CA125 (Hassan and Ho 2008). This interaction has been implicated in the peritoneal metastasis of ovarian cancer cells since mesothelin is normally expressed on cells lining the peritoneum. In pancreatic cancer cells, increased mesothelin expression has been shown to correlate with enhanced cell proliferation and migration (Li et al. 2008). The proliferative effect was shown at least in part to be mediated

through the activation of the transcription factor STAT3 resulting in the upregulation of S phase cyclin E and faster progression through the cell cycle (Bharadwaj et al. 2008). In addition, mesothelin overexpression is associated with more aggressive tumor growth and increased rates of metastasis in mouse xenograft models of pancreatic cancer (Li et al. 2008). Collectively, the data suggest that mesothelin expression in tumors promotes tumor adhesion, proliferation, and dissemination.

The molecular mechanisms regulating mesothelin expression in tumors are not well defined. However, it has been shown that mesothelin is differentially regulated by members of the Wnt signaling pathway, a pathway involved in the development of several cancers (Hassan and Ho 2008). This may explain why mesothelin is frequently overexpressed in cancers with constitutively active Wnt signaling, such as ovarian and pancreatic cancers. Analysis of genomic DNA surrounding the mesothelin gene resulted in the identification of an enhancer element shown to play a role in regulating the transcription of mesothelin (Hucl et al. 2007). The 18-bp enhancer containing an SP1 (specificity protein 1)- like site and MCAT element was named CanScript and found to be located approximately 60-bp upstream of the transcription start site. Reporter studies showed that CanScript was selectively active in cells with aberrant overexpression of mesothelin and not cells derived from tissues having physiological expression of mesothelin, suggesting that its activity is cancer specific. CanScript-mediated transcription required transcription enhancer factor (TEF)-1; however, the availability of TEF-1 alone was not responsible for regulating its activity. Instead, an unidentified cofactor is proposed to control the cancer-specific activity of the CanScript enhancer. Identification of the unknown cofactor(s) may help uncover additional signaling pathways involved in regulating mesothelin expression in cancer cells.

(A) **Rank**: “unknown” to 10
(B) Unknown-1-2-3-4-5-6-7-8-9-10: 9

High-Level Overview

Diagnostic, Prognostic, and Predictive

Mesothelin immunostaining is not routinely used as a diagnostic marker. However, staining for mesothelin can assist in the diagnosis of some cancers that may be otherwise misdiagnosed (Ordonez 2003a). For example, mesothelin expression is frequently overexpressed in nonmucinous ovarian cancers but not in tumors with which they may be confused, such as endodermal sinus tumors or renal cell carcinomas. In addition, mesothelin immunostaining can help categorize cytologically suspicious pancreas fine needle aspirates that are otherwise difficult to interpret (McCarthy et al. 2003). Although mesothelin immunostaining lacks the specificity to distinguish between mesothelioma and lung adenocarcinoma, negative staining suggests a diagnosis other than mesothelioma (Ordonez 2003b).

Soluble mesothelin measured in serum has proven to be a useful marker in patients with mesothelioma (Scherpereel and Lee 2007; Creaney and Robinson 2009). Elevated levels of soluble mesothelin are detected in the sera of approximately 80% of patients with mesothelioma and have been shown to correlate with disease burden. The commercially available MESOMARK assay (Beyer et al. 2007) is a mesothelin-specific ELISA which has been FDA-approved for measuring mesothelin protein levels in patient sera. Although studies have shown that serum mesothelin can serve as a correlative marker of response to treatment, further validation studies prospectively incorporating correlative testing into treatment protocols to measure disease course are needed to support the routine utility of this test. In the diagnostic setting, the testing may be informative but cannot replace a histologic diagnosis. Its role in early detection is also limited. Soluble mesothelin has been investigated as a screening marker in asbestos-exposed populations at high risk of developing mesothelioma. However, as a stand-alone test, measurement of soluble mesothelin has not proven to be a sufficient screening method. Although soluble mesothelin is not routinely used to monitor ovarian or pancreatic cancer patients, studies have shown that elevated levels are detected in most patients with these diseases and therefore could potentially be useful for the diagnosis and/or follow-up of these patients (Scholler et al. 1999; Hassan et al. 2006; Johnston et al. 2009; Huang et al. 2006). However, additional studies are required in order to determine the utility of measuring serum mesothelin in patients with these diseases.

Soluble mesothelin may also be useful as an independent prognostic factor. Studies have shown that mesothelioma and ovarian cancer patients with elevated soluble mesothelin appear to have a worse prognosis than patients with normal levels (Huang et al. 2006; Cristaudo et al. 2007). As such, elevated levels of soluble mesothelin may serve as a negative prognostic factor for these patients.

Therapeutics

Given its limited expression in normal tissues, high expression in numerous human malignancies, and potential role in tumor growth and metastasis, mesothelin provides an attractive target for cancer therapy. In addition to having a favorable expression profile, mesothelin also appears to be highly immunogenic, making it an ideal candidate for targeted immunotherapy. Preclinical studies have further validated mesothelin as a potential target for immunotherapy. As a result, several immunotherapies targeting mesothelin are under clinical development. These include both antibody-based therapies targeting surface mesothelin and vaccines designed to elicit mesothelin-specific immunity (Hassan et al. 2004; Hassan and Ho 2008).

Preclinical Summary

Preclinical studies have shown that a recombinant immunotoxin consisting of an anti-mesothelin antibody variable fragment (Fv) linked to truncated *Pseudomonas* exotoxin A (SS1P) can selectively kill mesothelin-expressing human tumor cell lines and

primary tumor cell cultures. In addition, mouse xenograft studies demonstrated in vivo efficacy of SS1P treatment against mesothelin-expressing human tumors and have suggested that SS1P can act synergistically when combined with radiation or chemotherapy (Hassan et al. 2004; Hassan and Ho 2008). Although several mechanisms have been shown to be involved, the synergy appears to be primarily due to increased immunotoxin uptake and accumulation in the targeted tumor cells (Zhang et al. 2010). An unconjugated humanized mouse monoclonal antibody specific for mesothelin (MORAb-009) has also been shown to kill mesothelin-expressing tumor cell lines. The cytotoxic effect of MORAb-009 was mediated via antibody-dependent cell-mediated cytotoxicity (ADCC). Similarly, MORAb-009 was shown to synergize with chemotherapy. In addition, treatment with MORAb-009 can inhibit binding between mesothelin and MUC16/CA125 and potentially inhibit metastases (Hassan et al. 2004; Hassan and Ho 2008). Both of these agents are under clinical development.

Several vaccine strategies targeting mesothelin, including DNA vaccines, virus-like particles (VLPs), mesothelin-encoding *Listeria monocytogenes* (Lm), and whole-tumor cell vaccines, have been tested in mouse models of ovarian and pancreatic cancer (Li et al. 2008; Chang et al. 2007; Hung et al. 2007a; Brockstedt and Dubensky 2008; Leao et al. 2008). These studies have provided encouraging results suggesting that mesothelin-specific immunotherapy has potential therapeutic value. In each study, vaccination resulted in enhanced mesothelin peptide-specific $CD8^+$ T cell activity that was associated with increased in vivo antitumor activity. In addition, adoptive transfer of mesothelin peptide-specific T cells was capable of controlling the growth of preestablished intraperitoneal ovarian tumors (Hung et al. 2007b). In a few of these studies, post-vaccination mesothelin-specific antibody responses were also evaluated and shown to play a role in the antitumor response (Li et al. 2008; Chang et al. 2007). Collectively, these preclinical vaccine studies suggest that mesothelin serves as a valid tumor rejection antigen for targeted immunotherapy and that both B and T cell responses are important for the generation of protective antitumor immunity.

Clinical Summary

SS1P has been tested in two phase I clinical studies in patients with mesothelin-expressing mesotheliomas and ovarian and pancreatic cancers. In the first phase I study, SS1P was administered as a 30 min intravenous infusion. Of the 33 evaluable patients treated, 4 had minor responses, 19 had stable disease (including 2 with resolution of ascites), and 10 had progressive disease. SS1P was well tolerated with pleuritis as the dose-limiting toxicity at the highest dose level (Hassan et al. 2007). In the second phase I study, continuous infusion dosing of SS1P over 10 days showed no advantage over bolus administration, but clinical activity was again demonstrated in the form of one partial response and other clinical responses manifested by cessation of ascites, resolution of masses on positron emission tomography, and improved pain and range of motion (Kreitman et al. 2009). In light of preclinical data showing synergy between SS1P and chemotherapy, studies of SS1P in combination

with standard chemotherapy are underway in patients with lung cancer and mesothelioma.

MORAb-009 has been tested in a phase I study in a similar cohort of patients. The objectives of the study were to test the safety and tolerability of MORAb-009 as well as to monitor tumor responses. Although the final results of this study have not yet been reported, preliminary data were reported in 2007 (Armstrong et al. 2007). At the time of the report, 11 patients received MORAb-009. A subject with pancreatic cancer who progressed on gemcitabine showed stable disease by CT (CAT scan or computerized axial tomography) and a drop in CA19-9 (carbohydrate antigen 19-9). A study of MORAb-009 in combination with gemcitabine has also been completed in patients with pancreatic cancer, but not yet reported. A phase II study testing it in combination with pemetrexed and cisplatin is also underway in patients with mesothelioma.

Two vaccines targeting mesothelin have been tested in patients. The first consists of a pair of granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting allogeneic pancreatic tumor cell lines (Jaffee et al. 1998). Although this vaccine does not only target mesothelin, it is one of the targets because both vaccine cell lines were shown to express the antigen (Thomas et al. 2004). A phase I and several phase II studies testing this vaccine in pancreatic cancer patients with both resected and unresected metastatic disease have been completed (Thomas et al. 2004; Laheru et al. 2008; Lutz et al. 2011; Jaffee et al. 2001). Data from these studies have demonstrated that enhanced mesothelin-specific $CD8^{+}$ T cell responses correlate with improved disease-free survival. Although further validation studies are necessary, these findings provide support for mesothelin as a surrogate marker of response to immune-based treatment in pancreatic cancer patients. These studies have also suggested that the potency of the vaccine may be enhanced when combined with immunomodulatory doses of cyclophosphamide, a chemotherapeutic drug shown to inhibit regulatory T cells involved in promoting tumor immune tolerance (Laheru et al. 2008). Additional studies combining the vaccine with other immunomodulatory agents are underway. Mesothelin-specific T cell measures may provide a means for comparing these treatment combinations. In addition, a larger multicenter phase II study is being planned to further assess this vaccine and validate the association between enhanced mesothelin-specific T cell responses and antitumor responses. The second vaccine (CRS-207) consists of a live attenuated strain of Lm that encodes human mesothelin (Brockstedt and Dubensky 2008). A phase I study testing this vaccine in patients with mesothelioma, ovarian, pancreatic, and non-small-cell lung cancers has been completed. However, the results from this study have not yet been reported. Results from this study and other currently ongoing trials should help determine the clinical utility of mesothelin-specific immunotherapy.

Anticipated High-Impact Results

- Results from a phase II trial of SS1P in patients with pleural mesothelioma being treated with chemotherapy (cisplatin and pemetrexed) followed by immunotoxin SS1P

- Results from a phase I study testing MORAb-009
- Results from a phase I study testing CRS-207
- Results from a phase II study testing the GM-CSF-secreting pancreatic cancer vaccine in combination with immunomodulatory doses of cyclophosphamide and Erbitux
- Results from a phase I study testing the GM-CSF-secreting pancreatic cancer vaccine in combination with ipilimumab
- Results from a multicenter phase II study testing the GM-CSF-secreting pancreatic cancer vaccine

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PSA

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Abstract

Prostate-specific antigen (PSA) is a 34-kDa tissue kallikrein glycoprotein that is expressed in normal prostate tissue and prostate cancer. This androgen-regulated serine protease aids in the liquefaction of seminal coagulum to allow sperm to become more motile. Normally, PSA is secreted into the prostatic ducts; however, in prostate cancer the disordered glandular architecture causes increased amounts of PSA to diffuse into the serum. PSA measurements serve as screening and prognostic markers for prostate cancer. PSA is also the most sensitive and widely used marker of response to therapy in patients with prostate cancer. PSA has been shown to be immunogenic. It is essentially expressed only in the prostate, therefore therapeutic targeting of PSA may be beneficial. Because it is secreted and not membrane bound, antibody approaches hold no utility. However T-cell

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based approaches such as therapeutic vaccines have been developed and have shown safety and preliminary evidence of efficacy in randomized studies. A definitive phase III study of PSA-TRICOM (PROSTVAC) may have results as early as the end of 2016.

Keywords
American Society for Radiation Oncology (ASTRO) • Prostate-specific antigen (PSA) • Androgen-deprivation therapy • Assessment • Biochemical failure • Biology • Clinical studies • Preclinical models • Pretreatment • Risk stratification and prognosis • Screening tests • Therapeutics • Tumor-associated antigens (TAAs)

Target: Prostate-Specific Antigen

Prostate-specific antigen (PSA) is a 34-kDa glycoprotein that is expressed in normal prostate tissue and prostate cancer (Balk et al. 2003). PSA is expressed in very low levels in the paraurethral and perianal glands, placenta, breast (including breast cancer), and thyroid. However, except for breast cancer, these tissues do not secrete a significant amount of PSA into the serum. PSA is a member of the tissue kallikrein family located on chromosome 19q13.4. Expression of PSA is controlled by the androgen response element, as are several other genes involved with prostate cell growth. This androgen-regulated serine protease aids in the liquefaction of seminal coagulum to allow sperm to become more motile. Normally, PSA is secreted into the prostatic ducts; however, in prostate cancer the disordered glandular architecture causes increased amounts of PSA to diffuse into the serum. PSA measurements serve as screening and prognostic markers for prostate cancer. PSA is also the most sensitive and widely used marker of response to therapy in patients with prostate cancer.

Biology of the Target

The immunogenicity of PSA has been demonstrated in multiple studies. Because PSA is secreted, it is not a good target for an antibody response. However, T cells can recognize any protein made by the cell. All proteins made by cells are processed and fragments of these proteins (peptides) are bound to major histocompatibility complex (MHC) molecules which are then presented on the cell surface where they can interface with T cells specific for the given peptide. Correale et al. demonstrated that human cytotoxic T cells specific for PSA could be generated in vitro (Correale et al. 1997). McNeel et al. demonstrated that some patients with advanced prostate cancer had naturally occurring PSA-specific T-cell responses (McNeel et al. 2001). Gulley et al. demonstrated that, in patients with prostate cancer, a PSA vaccine could generate PSA-specific T cells that secreted IFN-γ and lysed PSA-expressing tumor cells in an MHC-restricted manner (Gulley et al. 2005). In a subsequent randomized

controlled clinical trial of PROSTVAC (PSA-TRICOM), a PSA-based vaccine, Kantoff et al. showed improved overall survival for patients receiving vaccine compared with placebo (25.1 months vs. 16.6 months, HR = 0.56, $P = 0.006$) (Kantoff et al. 2010). A concurrent phase II study demonstrated PROSTVAC's ability to generate PSA-specific T-cell responses, with a trend to improved overall survival for patients with the greatest increase in PSA-specific T cells ($P = 0.055$) (Gulley et al. 2010).

Target Assessment

PSA is a secreted protein detectable in the serum of virtually every prostate cancer patient, from those with early localized disease to patients with metastatic castration-dependent prostate cancer. Because the use of PSA serum assays is so widespread, target assessment for PSA is generally a serum assay, although immunohistochemistry can also be performed with anti-PSA antibodies. Two serum PSA assay kits commonly used in the United States are the Access Hybritech (Beckman Coulter) and Bayer ADVIA Centaur (Siemens) assays. There is some variation in levels between these two platforms, so guidelines call for caution when interpreting changes in PSA obtained by different assays (Arlen et al. 2008). However, each of these assays is FDA approved and highly reproducible.

When using PSA measurements to evaluate a treatment effect, it is important to note that some pharmacologic agents modulate PSA expression independent of effects on tumor growth (Dixon et al. 2001; Aragon-Ching et al. 2009).

Role of Target in the Cancer

PSA measurement is a very useful monitor of treatment response. The use of PSA as a screening test for prostate cancer is controversial.

High-Level Overview

Diagnostic, Prognostic, and Predictive

The use of PSA as a screening test for prostate cancer began in the 1980s when PSA was found to be much more sensitive than prostatic acid phosphatase in prostate cancer patients. This led to studies showing that PSA testing could identify more cancers than digital rectal examination alone. When the use of PSA screening tests became widespread in the United States in the 1990s, prostate cancer incidence increased from about 130 cases per 100,000 men per year to a peak of nearly 250 cases per 100,000 men per year, before subsiding to 160 cases per 100,000 men per year. Despite stage migration, with an increasing proportion of men diagnosed with organ-confined disease, the impact of screening on overall survival

in diagnosed patients is unclear. Two large randomized studies designed to evaluate the impact of PSA screening on overall survival are ongoing (Andriole et al. 2009; Schroder et al. 2009). A recent study suggested that men with PSA levels <4 ng/mL had a surprisingly high 15% risk of having prostate cancer, although only 15% of those men had a Gleason score of ≥7 (Thompson et al. 2004).

The percent of free PSA can help to differentiate between benign and malignant sources of the antigen. High levels of free PSA suggest a benign process, whereas low free PSA correlates more closely with prostate cancer (Walz et al. 2008; Gann et al. 2002).

Once a patient has been diagnosed with prostate cancer, absolute PSA values can provide useful information about risk stratification and prognosis. Several widely used nomograms (Prostate cancer nomograms a tool for doctors and patients; The Partin Tables; Kattan et al. 2008) utilize PSA value at diagnosis to predict outcomes for patients. For patients treated with definitive local radiation therapy, risk groups (low, intermediate, and high risk) are assigned using PSA, Gleason score, and clinical stage, with PSA groupings divided into ≤10, >10–20, and >20 ng/mL (D'Amico et al. 1998). In addition, pretreatment PSA velocity can be useful for assessing risk of prostate cancer-specific mortality (D'Amico et al. 2004, 2005).

Ideally, PSA should decline to undetectable levels within 2–3 weeks of radical prostatectomy (half-life about 2.2 days). A PSA level of ≥0.3 ng/mL following prostatectomy is suggestive of biochemical failure. Following radiation therapy, the time to PSA nadir is variable. The American Society for Radiation Oncology (ASTRO) consensus criteria for biochemical failure following radiation (with or without androgen-deprivation therapy, commonly referred to as the "Phoenix criteria") are a PSA level of 2 ng/mL above the nadir.

For patients with biochemical failure, PSA kinetics can be used to gauge the aggressiveness of the subsequent disease course (Arlen et al. 2008). A single-center retrospective analysis of 379 men with biochemical failure following radical prostatectomy (Freedland et al. 2005) demonstrated a significant difference in overall survival based on PSA doubling time. Other studies in patients with biochemical failure following radical prostatectomy or radiation also demonstrated worse outcomes for patients with more rapidly rising PSA. PSA velocity and PSA doubling time have also been shown to be predictive of time to bone metastasis in men with nonmetastatic castration-resistant prostate cancer (Arlen et al. 2008).

Finally, PSA has long been used as an efficacy marker following therapy. Three PSA Working Group publications have suggested guidelines for monitoring PSA (Arlen et al. 2008; Bubley et al. 1999; Scher and Eisenberger 2004). The depth of a PSA nadir following androgen-deprivation therapy has been associated with clinical outcome. A study in men with biochemical failure (nonmetastatic) who had a PSA doubling time of ≤6 months demonstrated that a PSA nadir of >0.2 ng/mL was associated with worse prostate cancer-specific mortality (Rodrigues et al. 2006). Another study in men with biochemical failure on androgen-deprivation therapy suggested that longer time to achieve undetectable PSA was associated with higher risk of prostate cancer-specific mortality (D'Amico et al. 2007). A large randomized intergroup study in men with metastatic prostate cancer treated with

androgen-deprivation therapy showed that achieving a PSA of $\leq$4.0 ng/mL was a strong independent predictor of overall survival (Hussain et al. 2006). Other studies have shown that PSA decline following chemotherapy is associated with improved outcome.

Therapeutics

Immunotherapies targeting PSA have been widely studied, including vaccines utilizing PSA protein, PSA peptide, dendritic cells pulsed with PSA protein or mRNA, and viral vectors expressing PSA. Of these approaches, poxviral vector vaccines such as PSA-TRICOM have undergone the most extensive preclinical and clinical testing. Clinical trials of PSA-based vaccines have recently been reviewed (Madan et al. 2006).

Preclinical Summary

To improve the immunogenicity of vaccines, investigators have experimented with altering either the T-cell-binding epitope or MHC anchor regions of peptides. Terasawa et al. demonstrated that changing an isoleucine to a leucine at position 155 on PSA (within the MHC-binding region) made the peptide bind with higher affinity to the MHC class I molecule and produced higher levels of IFN-γ in PSA-specific T cells. T-cell lines generated with this agonist epitope efficiently lysed tumor cells expressing the native PSA in an MHC-restricted manner (Terasawa et al. 2002).

Preclinical models have demonstrated the ability of poxviral vectors to break tolerance to transgenes of tumor-associated antigens (TAAs) incorporated within the vectors. Studies have also shown that adding three transgenes for T-cell costimulatory molecules (termed TRICOM) to these vectors, along with the TAA, leads to optimal immune and antitumor activity in murine models. In preclinical models, GM-CSF has been shown to be an important immune adjuvant.

Clinical Summary

Phase I clinical studies of poxviral vectors expressing PSA demonstrated safety and immune responses (Gulley et al. 2002) and led to two concurrent phase II clinical trials of PROSTVAC. A recently completed 43-center randomized, controlled, double-blind phase II clinical trial was conducted in 125 patients with metastatic castration-dependent prostate cancer (Kantoff et al. 2010). Similar to other recently reported vaccine studies, progression-free survival (the primary endpoint of the study) was not different between the arms. However, the PROSTVAC arm had a 44% reduction in death rate compared to the control arm (HR 0.56, $P = 0.006$), and median overall survival was 8.5 months longer for patients receiving vaccine

(25.1 months vs. 16.6 months for the control arm), with minimal toxicity. This is one of the largest treatment effects seen in any randomized study of metastatic castration-resistant prostate cancer.

A concurrent phase II study of PROSTVAC demonstrated a trend toward improved survival in patients who mounted the best immune response to vaccine (Gulley et al. 2010). Patients receiving vaccine lived an average of 9.2 months longer than predicted by a validated nomogram. This study also suggested that patients with longer predicted survival (based on lower tumor burden and less aggressive disease) had the greatest apparent treatment benefit, living >16.4 months longer than predicted.

Based on these studies' consistent and provocative findings of improved overall survival, a global randomized, controlled, double-blind phase III clinical trial of PROSTVAC is scheduled to opened in 2012 to confirm whether this vaccine can improve overall survival compared with placebo.

Clinical trials have also demonstrated the feasibility of combining PSA-based poxviral vaccines with standard treatment modalities. Immune responses have been induced by combining vaccine with radiation therapy (Gulley et al. 2005; Lechleider et al. 2008), chemotherapy, androgen-deprivation therapy, and an anti-CTLA-4 antibody. Further combination studies are ongoing.

Anticipated High-Impact Results

Data on time to progression from two randomized phase II clinical trials comparing standard of care vs. standard of care plus PROSTVAC vaccine are expected within 2 years. One trial is using flutamide (an oral androgen-receptor antagonist) as a second-line hormonal manipulation in patients with nonmetastatic castration-resistant prostate cancer; the other is using Quadramet (an FDA-approved bone-seeking radionuclide) in patients with castration-resistant prostate cancer metastatic to the bone who have progressed following docetaxel therapy.

Data on overall survival in a phase III clinical trial of PROSTVAC are anticipated in 2016.

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Survivin

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Mads Hald Andersen and Jürgen Becker

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Abstract

Survivin is abundantly and ubiquitously present in development, undetectable in most adult tissues, and prominently reexpressed in virtually every human cancer. Thus, one of the most significant features of survivin is its preferential expression in tumor vs. normal tissues. Even though a few normal cells do express survivin, e.g., thymocytes, CD34+ bone marrow-derived stem cells, and basal colonic epithelial cells (Altieri, Nat Rev Cancer 3:46–54, 2003), under physiological conditions, survivin is undetectable in most terminally differentiated normal tissues (Altieri et al., Lab Invest 79:1327–1333, 1999). In contrast, survivin is

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overexpressed in almost all cancers including lung, colon, breast, pancreas, stomach, liver, ovaries, and prostate cancer, as well as melanoma and hematopoietic malignancies (Ambrosini et al., J Biol Chem 273:11177–11182, 1998; Adida et al., Blood 96:1921–1925, 2000; Grossman et al., J Invest Dermatol 113:1076–1081, 1999). Data from a large analysis of human transcripts revealed survivin as the fourth most highly expressed protein in human cancer tissue compared to normal tissue (Velculescu et al., Nat Genet 23:387–388, 1999).

Keywords
Survivin • Anti-survivin T cells • Apoptosis inhibition • Apoptosis proteins • Chemotherapeutic agents • Immunogenicity • Immunohistochemistry • In cancers • In tumor vs. normal tissues • Inhibitory T-cell ligand B7-H1 • Regulation of mitosis • Standard immunohistochemistry • Synthetic peptides • Therapeutic vaccinations • Vaccination • Vs. T-cell responses

Target: Survivin

Survivin is abundantly and ubiquitously present in development, undetectable in most adult tissues, and prominently reexpressed in virtually every human cancer. Thus, one of the most significant features of survivin is its preferential expression in tumor vs. normal tissues. Even though a few normal cells do express survivin, e.g., thymocytes, CD34+ bone marrow-derived stem cells, and basal colonic epithelial cells (Altieri 2003), under physiological conditions, survivin is undetectable in most terminally differentiated normal tissues (Altieri et al. 1999). In contrast, survivin is overexpressed in almost all cancers including lung, colon, breast, pancreas, stomach, liver, ovaries, and prostate cancer, as well as melanoma and hematopoietic malignancies (Ambrosini et al. 1998; Adida et al. 2000; Grossman et al. 1999). Data from a large analysis of human transcripts revealed survivin as the fourth most highly expressed protein in human cancer tissue compared to normal tissue (Velculescu et al. 1999).

Biology of the Target

Survivin has attracted attention as a unique member of the IAP gene family with a potential dual role in apoptosis inhibition and regulation of mitosis (Altieri 2003). The role of survivin in the regulation of mitosis has recently become better understood and linked to multiple spindle microtubule functions and mitotic checkpoints (Yang et al. 2004; Li et al. 1998). Despite extensive experimental evidence in vitro, and in transgenic animals in vivo (Altieri 2003), the precise mechanism(s) by which survivin interferes with apoptosis has not been elucidated. Survivin counteracts cell death by interfering with caspase-9 processing (LaCasse et al. 1998), the upstream initiation of the intrinsic (mitochondrial) pathway of apoptosis. Furthermore, cytoprotection by suvivin is more selective than that by other IAPs and is specifically

targeted at the initiation of mitochondrial apoptosis to prevent caspase-9 activation (Salvesen and Duckett 2002).

The immunogenicity of survivin has been demonstrated in several preclinical and clinical trials. In this regard, spontaneous anti-survivin T-cell reactivity has previously been described in cancer patients suffering from breast cancer, colon cancer, lymphoma, leukemia, and melanoma (Andersen et al. 2001a, b; Reker et al. 2004a, b; Casati et al. 2003; Siegel et al. 2004). Furthermore, therapy-induced T-cell responses against survivin have been described.

Target Assessment

Survivin can be measured in tissues using standard immunohistochemistry.

Role of Target in Cancer

Rank: Unknown-1-2-3-4-5-6-7-8-9-10: 10

Survivin plays an important role in the control of apoptosis, cell division, and cell migration/metastasis. Survivin is expressed in most human cancers where presence of the protein is associated with enhanced proliferation, metastasis, poor prognosis, and decreased patient survival. Given the very broad and relatively selective expression in cancer cells, but not in normal tissue, and its importance in tumor cell biology, survivin is an attractive target for cancer diagnosis as well as treatment.

High-Level Overview

Data from a large analysis of human transcripts revealed survivin as the fourth most highly expressed protein in human cancer tissue compared to normal tissue (Velculescu et al. 1999). The extremely high expression of survivin in cancer carries prognostic and predictive implications and is consistently associated by molecular profiling with advanced disease, high grade, abbreviated survival, resistance to therapy, and accelerated recurrences.

Diagnostic, Prognostic, Predictive

It has been described that expression of survivin and the inhibitory T-cell ligand B7-H1 can be used to predict clear cell renal cell carcinoma tumor aggressiveness (Krambeck et al. 2007) Clinical exploitation of *survivin* for cancer molecular diagnosis is under way, with its inclusion as 1 of 16 genes predictive of recurrences in breast cancer (Giodini et al. 2002) and as a urine biomarker in bladder cancer (Smith et al. 2001).

Upregulation of inhibitor of apoptosis proteins play a vital role in resistance to chemotherapy and radiotherapy (Longley and Johnston 2005). Thus, overexpression of survivin in cancer cells is associated with a decreased overall survival (Islam et al. 2000; Swana et al. 1999; Kawasaki et al. 1998), an increased rate of recurrences, and a reduced apoptotic index of neoplastic cells in vivo (Altieri 2003).

Therapeutics

Strategies aiming at inhibiting the expression or function of antiapoptotic proteins have gained considerable attention (Wacheck et al. 2003; Wang et al. 2003). Thus, while survivin overexpression leads to increased resistance to apoptotic stimuli such as chemotherapeutic agents or ionizing radiation therapy, antisense-mediated silencing of survivin sensitizes the cells to these interventions (Olie et al. 2000). A parallel strategy to suppress survivin levels in tumor cells involved RNA interference (Uchida et al. 2004) or hammerhead ribozymes (Pennati et al. 2004). These reagents produced a phenotype similar to antisense and passed proof of concept with inhibition of tumor growth in xenograft models. Another strategy is the use of synthetic peptides that compete with caspases for binding to IAPs, which have been shown to sensitize tumor cell lines to apoptosis induced by cytotoxic anticancer drugs (Fulda et al. 2002). Several clinical trials using these reagents are under way.

In addition to the abovementioned means of targeting survivin, immune-mediated tumor destruction is emerging as an interesting modality to treat cancer patients. Accordingly, downregulation of survivin would severely inflict the survival capacity of tumor cells, which highlights this protein as a prime target candidate for therapeutic vaccinations against cancer, since it is not subject to immune selection, i.e., the selection of cancer cells not expressing the vaccination target. In this regard, several survivin-based vaccination trials are currently ongoing.

Preclinical Summary

In situ immunohistochemistry using multimerized HLA/survivin-peptide complexes disclosed that survivin-specific CTL could readily be detected in the tumor microenvironment both in primary tumors and metastases of melanoma patients as well as in breast cancer lesions (Andersen et al. 2001b; Reker et al. 2004a). Survivin-reactive T cells are capable of lysing HLA-matched tumor cells of different tissue origin, including breast cancer, renal cell carcinomas, colon cancer, melanoma, and multiple myeloma cell lines, as well as primary malignant cells from patients with different leukemias (Andersen et al. 2001b; Schmidt et al. 2003). Coughlin et al. used CD40-activated B cells transfected with whole tumor RNA, which induced survivin-specific T cells in vitro that lysed HLA-matched neuroblastoma cells but not autologous benign cells (Coughlin et al. 2004). Hence, survivin is

among the targeted antigens in vaccination strategies utilizing whole tumor cells as antigen source.

Clinical Summary

Supported by a favorable safety profile, the original survivin antisense oligonucleotide has completed a phase I trial in patients with advanced cancers, and a phase II trial has been announced. Another molecule, tetra-*O*-methyl nordihydroguaiaretic acid was shown to function by suppressing Sp1-dependent *survivin* gene expression, resulting in concomitant activation of mitochondrial apoptosis in tumor cells (Chang et al. 2004).

The first therapy-induced T-cell responses against survivin were described. In a compassionate use setting, heavily pretreated stage IV melanoma patients were vaccinated with an HLA-A2-restricted survivin epitope presented by autologous dendritic cells (Otto et al. 2004). These patients mounted strong T-cell responses to this epitope as measured by ELISPOT assay. Furthermore, in situ peptide/HLA-A2 multimer staining revealed the infiltration of survivin-reactive CD8+ T cells into soft tissue metastases of vaccinated patients. Notably, no vaccination-associated toxicity was observed. Hence, it is feasible to induce T-cell responses against survivin; even in late-stage tumor patients, these vaccinations are well tolerated. In that regard, Pisajev et al. recently showed that survivin-directed CTL do not affect hematopoietic colony formation of CD34+-purified progenitor cells (Pisarev et al. 2003). In addition, in a melanoma patient in complete remission following IL-2-based immunotherapy, a longitudinal examination of anti-survivin reactivity was made exceeding 7 years. Survivin-specific T-cell reactivity was found at all time points examined over the 7-year period. The data demonstrated that anti-survivin T cells may persist in the periphery for extended periods in the absence of clinical manifestation of disease as well as autoimmunity (Hadrup et al. 2006). Finally, when used in an oral DNA vaccine, the survivin-directed immune response affected both tumor cells and tumor-associated angiogenesis, eradicating pulmonary metastases without toxicity including wound healing or fertility in preclinical studies (Xiang et al. 2005).

The first example of a successful application of survivin-based vaccination in the clinical setting was recently described in a patient with pancreatic cancer (Wobser et al. 2006). This patient had a complete remission of liver metastasis under vaccination with an HLA-A2-restricted survivin peptide together with an adjuvant. Data from several ongoing phase I/II trials targeting survivin for patients with advanced cancer will provide further information toward this notion. In this regard, several survivin-based vaccination trials are currently ongoing at different institutes.

Anticipated High Impact Results

- Cancer vaccine/immunotherapy. Immune results of adjuvant/survivin-based vaccines, Becker et al. Domchek et al.

- Molecular antagonists (LY2181308, Ely Lilly and Co). Nucleic acid antisense oligonucleotide by Santaris Pharma and Enzon Pharmaceuticals
- Small molecules. YM155 by Yamanouchi Pharmaceuticals and Astellas Pharma

Combination of survivin-based therapy with cytotoxics or radiation appear to be very exciting.

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42 Telomerase-Related Proteins

Luke Wojdyla, Mark Frakes, Kymberly Harrington, Amanda Stone, and Neelu Puri

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Abstract

The ends of all eukaryotic chromosomes are protected by specialized nucleoprotein complexes called telomeres. When functional and intact, telomeres prevent end-to-end fusions, inappropriate DNA repair mechanisms, and DNA degradation. Often referred to as biological clocks, telomeres are repeatedly shortened during each replication cycle due to incomplete replication by DNA polymerases. When critically short, telomeres become dysfunctional or uncapped, losing their higher-order structures and ability to protect the chromosome, an event referred to as the "end-replication problem." This telomere instability prompts cells to enter a growth arrest state and trigger DNA damage responses (DDRs) such as cellular senescence and apoptosis. In addition, due the guanine rich properties of

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telomeric DNA, they can form intramolecular G-quadruplexes, four-stranded DNA structures that are stabilized by the stacking of guanine residues in a planar arrangement. However, the functional roles of telomeric G-quadruplexes are not understood. In cancer cells, telomere length is maintained by telomerase, a ribonucleoprotein enzyme complex with reverse transcriptase activity, which adds TTAGGG repeats to the 3' telomere end. Telomerase is comprised of two sub-units: hTERT, the catalytic component of telomerase, and hTR, an RNA template complementary to the 3' overhang. While telomerase is generally inactive in normal somatic cells, early studies demonstrated that telomerase is overexpressed in more than 85% of cancers, and its activity is believed to be a requirement for malignant cells to achieve immortality. Hence, telomerase and the telomere components which regulate it have been regarded as near-universal cancer targets and have become an active focus of cancer researchers. Currently, telomere-based targets are being tested as potential diagnostic and prognostic markers of cancers.

Keywords

DNA damage responses (DDRs) • Dyskeratosis congenital (DC) • G-quadruplex • GRN163L • Shelterin complex • Tankyrase 1 and 2 • Telomerase • Telomeres • Telomestatin • TRAP assay

Target: Telomeres, Telomerase, and Associated Proteins

The ends of all eukaryotic chromosomes are protected by specialized nucleoprotein complexes called telomeres. When functional and intact, telomeres prevent end-to-end fusions, inappropriate DNA repair mechanisms, and DNA degradation. Often referred to as biological clocks, telomeres are repeatedly shortened during each replication cycle due to incomplete replication by DNA polymerases. When critically short, telomeres become dysfunctional or uncapped, losing their higher-order structures and ability to protect the chromosome, an event referred to as the "end-replication problem." This telomere instability prompts cells to enter a growth arrest state and trigger DNA damage responses (DDRs) such as cellular senescence and apoptosis. In cancer cells, however, telomere length is maintained by telomerase, a ribonucleoprotein enzyme complex with reverse transcriptase activity, which adds TTAGGG repeats to the $3'$ telomere end (de Lange 2005). While telomerase is generally inactive in normal somatic cells, early studies demonstrated that telomerase is overexpressed in more than 85% of cancers, and its activity is believed to be a requirement for malignant cells to achieve immortality. Hence, telomerase and the telomere components which regulate it have been regarded as near-universal cancer targets and have become an active focus of cancer researchers (Shay and Keith 2008). Currently, telomere-based targets are being tested as potential diagnostic and prognostic markers of cancers (Poremba et al. 2002). In addition, several novel therapeutic agents, such as GRN163L, GV-1001, Vx-001, and GRNVAC1, which

inhibit telomerase, have recently entered numerous clinical trials (Artandi and DePinho 2010).

Biology of the Target

The 3′ ends of telomeres extend beyond their 5′ complementary strand by approximately 200 nucleotides, forming a single-stranded overhang comprised of TTAGGG repeats. The guanine-rich character of this sequence allows telomeres to adopt several higher-order folding structures, such as t-loops and G-quadruplexes, which are integral to the protective functions of the telomere (Fig. 1) (Hiyama 2009). Telomeres gradually shorten with each cell division, losing approximately 20–200 bases with each cycle. When a critically shortened length is reached, the protective structures are compromised, inducing catastrophic DNA damage responses (Neidle and Parkinson 2003).

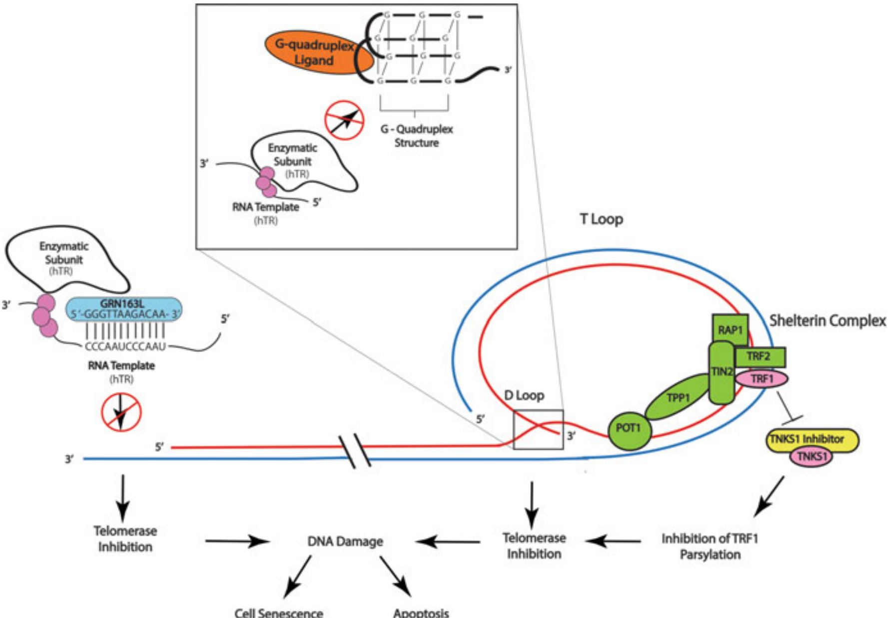

Fig. 1 Novel therapies targeting telomeres and telomerase. The single-stranded 3′ telomere overhang folds over itself and invades the double-stranded region, forming a t-loop. This process is aided by a protein complex called shelterin, which caps telomere ends and regulates telomerase. G-quadruplexes are formed in the D-loop from stabilizing hydrogen bond interactions between guanine tetrads of the 3′ overhang. Tankyrase 1 inhibitors prevent PARsylation of TRF1, thereby inhibiting telomerase from accessing the telomere. GRN163L, G-quadruplex stabilizing ligands, and tankyrase 1 inhibitors prevent telomerase from elongating the telomeres, inducing telomere attrition and subsequently activating various DDRs

The t-loop forms when the 3′ overhang folds over itself and invades the duplex region, resulting in a lariat structure which is stabilized by the protein complex shelterin. The shelterin complex is composed of six proteins (TRF1, TRF2, TIN2, POT1, TPP1, and Rap1) that bind specifically to either single-stranded or double-stranded regions of the t-loop and play intrinsic roles in mediating telomere length homeostasis. Furthermore, shelterin is integral in the formation and proper functioning of t-loops and G-quadruplexes, resisting nuclease activity and regulating telomerase (Fig. 1). Interestingly, the shortening of telomeres does not necessarily lead to senescence, but rather, it is the dissociation of shelterin proteins and subsequent unraveling of the t-loop, which in many cases is initiated by critically shortened telomeres, that results in telomere dysfunction and induction of DDRs (de Lange 2005; Hiyama 2009).

Another secondary structure that telomeres can adopt is the G-quadruplex, which is a four-stranded region of DNA stabilized by tetrads of guanine residues formed through Hoogsteen base pairing (Yang and Okamoto 2010). In telomeres, these tetrads form and stack together in a multi-tiered, planar scaffold at the D-loop, the region of the t-loop where the overhang invades the duplex region (Fig. 1) (Shalaby et al. 2013). The presence of G-quadruplexes imposes a hindrance to the various proteins involved in the replication of telomeric DNA. The activity of helicases WRN and BLM, which unwind telomeric DNA and move the replication fork, is inhibited by G-quadruplexes. POT1, a shelterin protein necessary for telomere capping and preventing chromosomal degradation, also requires the resolution of G-quadruplexes to function properly (Yang and Okamoto 2010; Shalaby et al. 2013). In addition, the structure of G-quadruplexes prevents the binding of telomerase to the telomere overhang, which in turn inhibits telomere elongation (Hiyama 2009). Hence, artificial induction of G-quadruplexes by small molecules such as telomestatin, BRACO-19, and RHPS4, in an effort to induce telomere attrition or prevent telomere replication, is being explored as a novel anticancer approach (Sampathi and Chai 2011).

Human telomerase primarily consists of two main components, hTERT, a catalytic subunit, and hTR, an RNA template whose sequence is complimentary to the telomeric TTAGGG repeat. hTERT is a reverse transcriptase which uses hTR as a template to synthesize additional TTAGGG repeats to the 3′ telomere end. In humans, telomerase activity is typically restricted to renewing tissues, such as germ cells and stem cells, and is generally absent in normal cells. While hTR is constitutively expressed in most tissue types, hTERT levels are low enough that telomere length cannot be maintained, resulting in the end-replication problem. However, in the majority of cancers, telomerase is inappropriately overexpressed and functions to maintain stable telomere length, thereby conferring immortality in malignant cells (Shay and Keith 2008).

Tankyrase 1 and 2 are poly(ADP-ribose) polymerases (PARPs) which have important roles in the regulation of the t-loop, shelterin complex, and telomerase. Tankyrase 1 adds polymers made of ADP-ribose monomers (a process called

PARsylation) to its target protein, TRF1. TRF1 helps to stabilize the t-loop by recruiting other shelterin proteins and is considered a negative regulator of telomere length by blocking the site of attachment for telomerase, thus inhibiting telomere elongation. PARsylation of TRF1 decreases its affinity for the telomere and prevents it from binding to the telomere, thereby promoting destabilization of the t-loop. This in turn can induce DDRs, caused by exposed telomeric DNA, and also provides telomerase an opportunity to lengthen the uncapped telomere overhang. The role of TRF1 as a negative regulator of telomere length corroborates with in vitro studies, demonstrating that inhibition of TRF1 by expression of a dominant-negative TRF1 results in telomere elongation, while overexpression of wild-type TRF1 induces telomere shortening (van Steensel and de Lange 1997). Although tankyrase 2 has not been studied as extensively as tankyrase 1, it is believed to have a function similar to tankyrase 1 (Riffell et al. 2012).

Target Assessment

In most tissues, hTR is expressed at low levels, and its presence does not necessarily correlate with telomerase activity. In contrast, levels of hTERT mRNA are directly related to telomerase activity, making it a more suitable cancer target than hTR. Several effective methods have been developed for the detection of hTERT mRNA, which include in situ hybridization, immunohistochemistry, reverse transcriptase-polymerase chain reaction, and immunofluorescence flow cytometry. The telomeric repeat amplification protocol (TRAP) is the standard in vitro assay used in the detection of telomerase and can provide a quantitative estimate of telomerase activity in tissues. The TRAP assay is composed of three steps which involves extension of a primer specific to hTR, PCR amplification, and detection of products (Hiyama 2009).

Analyzing the length of the G-rich telomere overhang may be indicative of overall telomere integrity, since telomere attrition induces dysfunction of the t-loop (van Steensel and de Lange 1997). Destruction of the t-loop is believed to be a significant event in the initial stages of tumorigenesis, and assays used to measure the telomere overhang could provide insight into the complex roles of telomeres in tumorigenesis. Analyzing telomere length may also be useful in evaluating patient response to anti-telomerase therapies or G-quadruplex stabilizers, both of which work by inducing t-loop attrition (Hiyama 2009). Most reliable assays used to measure telomere ends are only applicable in vitro, as they require the use of radioisotopes and gel electrophoresis which are not amenable to high-throughput analysis. However, a recently developed technique called the hybridization protection assay may be suitable for large-scale analysis of telomere overhangs in clinical samples. This method does not require the use of radioisotopes and instead uses an acridinium ester-labeled probe, which is luminescent and intercalates telomeric DNA (Hiyama 2009).

Role of the Target in Cancer

Rank: "unknown" to 10.

Unknown to 1-2-3-4-5-6-7-8-9-10: 10

Telomerase is reactivated and inappropriately expressed in greater than 85% of cancer types, and the level of its activity is higher in advanced and metastatic tumors (Shay and Keith 2008). Since its discovery almost 20 years ago, telomerase has become one of the most promising molecular targets against cancer and has had an astounding impact on our understanding of the telomere. Telomerase can be targeted directly by using agents that bind to telomerase components, or indirectly by targeting factors which mediate its activity. The formation of G-quadruplexes prevents telomerase from accessing telomeric DNA, and hence, G-quadruplex stabilizing ligands have been shown to promote telomere shortening in cancer cells. In addition, due to its ability to decrease affinity of TRF1 for the telomere, tankyrase 1 is also actively being investigated and targeted for its role in tumorigenesis (Hiyama 2009).

More research is required to further explore the consequences of long-term inhibition of telomerase, which may have toxic side effects in normal cells. Dyskeratosis congenita (DC), a disorder caused by mutations in the hTR, hTERT, and TIN2 genes, results in an approximately 50% reduction in telomerase levels, leading to bone marrow failure and early death in patients. Interestingly, patients with DC are more susceptible to cancer. Indeed, a large portion of human cancers contain very short telomeres, and mounting evidence suggests that malignant transformations occur as a result of shortened or dysfunctional telomeres in the absence of telomerase. The two-stage M1/M2 model of senescence predicts that cells enter M1 (normal replicative senescence) as a result of shortened telomeres. If further telomere attrition occurs, cells enter M2, which typically results in apoptosis. This may occur more frequently in stem cells, which have decreased levels of telomerase. If telomerase is then subsequently reactivated, transformed cells can bypass M2 crisis and establish immortality. Thus, telomerase may be required for protection against malignant transformations caused by telomere dysfunction and is likely necessary to maintain healthy populations of germline and stem cells. Despite this, patients with DC don't display extreme phenotypes until several years after birth, suggesting that prolonged anti-telomerase treatments could have minimal side effects (Hiyama 2009). In addition, in many cancers, mean telomere length is typically much shorter than in healthy cells, suggesting that most cancer cells would respond to anti-telomerase treatments more rapidly than germline or stem cells.

High-Level Overview

Diagnostic, Prognostic, and Predictive

Both telomerase activity and telomere length are currently being tested as diagnostic and prognostic markers for several cancers. In contrast to hTR, the expression of hTERT is directly related to telomerase activity and functionality. Thus, hTERT can

be used as a diagnostic and prognostic marker of cellular transformation, since levels of hTERT in tissue samples may correlate with the stages and grades of tumors (Hiyama 2009; Catarino et al. 2010). Moreover, different cancer types are highly variable in telomere length, and thus an analysis of mean telomere length in tumor tissues may predict a patient's response to anti-telomerase therapies, since malignant cells with shorter telomeres would presumably respond more rapidly to treatment (Hiyama 2009).

Therapeutics

GRN163L (Imetelstat) was the first agent used to directly inhibit telomerase by binding to hTR. GRN163L demonstrated high potential as an effective anticancer therapeutic in preclinical studies and is currently being tested in several phase I and II clinical trials. In addition, three vaccines which inhibit telomerase activity, GV100, Vx-001, and GRNVAC1, have also recently entered clinical trials. Phase I and II clinical trials using GV1001 have been initiated in patients with melanoma, NSCLC, hepatocellular carcinoma, and pancreatic cancer. In vivo studies and phase I and II clinical trials have also shown that Vx-001 is effective in decreasing tumor volume (Ruden and Puri 2013). Lastly, GRNVAC1 is currently undergoing a phase II clinical trial in patients enrolled with complete clinical remission of acute myelogenous leukemia to prolong duration of remission (Geron Corporation 2013).

Inducing the formation of G-quadruplexes can disrupt replication of the telomere, as well as lengthening by telomerase, and thus, targeting G-quadruplexes is an attractive and novel anticancer therapeutic approach (Shalaby et al. 2013). The G-quadruplex structure of DNA offers multiple recognition sites, which allows for various small ligands to bind and stabilize the structure. Currently, the most promising ligands which facilitate formation of G-quadruplexes at the telomere include telomestatin, BRACO-19, and RHPS4 (Yang and Okamoto 2010).

The primary goal of tankyrase 1-based therapies is to block telomerase from accessing telomeric DNA by preventing PARsylation of TRF1. Presently, the effects of tankyrase 1 inhibitors, such as 3′-aminobenzamide (3AB), XAV939, and JW55, on cancer and normal cells are being investigated in preclinical studies (Riffell et al. 2012; Seimiya et al. 2005).

Preclinical Summary

One of the most studied and promising anti-telomerase therapies is GRN163L, a 13-mer oligonucleotide N3′-P5′-thio-phosphoramidate with a 16 carbon palmitoyl group added to the 5′ thio-phosphate. The oligonucleotide sequence within GRN163L (5′-TAGGGTTAGACAA-3′) is complementary to the RNA sequence found in hTR and prevents the hTERT active site from interacting with hTR. As a result, GRN163L is able to completely inhibit telomere elongation (Ruden and Puri 2013). The 5′ palmitoyl group decreases the polarity and increases the lipophilic

character of the oligonucleotide, thereby improving cellular and tissue uptake (Roth et al. 2010). However, recent studies have shown that GRN163L is less potent in comparison to GRN163 (an earlier molecule which lacked the palmitoyl group), possibly because the lipophilic group sterically hinders the ability of GRN163L to bind to hTR. However, while GRN163 appeared to be more effective in vitro, in vivo studies using a monkey model demonstrated that GRN163L had enhanced cellular uptake and tissue penetration compared to GRN163 (Ruden and Puri 2013). In addition, GRN163L can freely cross the blood-brain barrier, which gives it a distinct advantage over other developing therapies.

GRN163L has been tested on a number of different cancer types, including multiple myeloma, lung, breast, bladder, glioblastoma, prostate, hepatoma, pancreatic, and Barrett's adenocarcinoma. Monotherapy using GRN163L showed inhibition of telomerase activity in all studied cancer type, and lung cancer, glioblastoma, and hepatoma tumors demonstrated a dose-dependent decrease in telomerase activity. GRN163L also induced varying degrees of tumor senescence and cell death, as well as decreased frequency of malignant phenotypes associated with aggressive tumors (increased size, invasion of peripheral tissue, metastasis, and lack of tumor differentiation) in all tumor types analyzed. GRN163L in combination with other drugs (cisplatinum, trastuzumab, Paclitaxel, doxorubicin, and ritonavir) or radiation therapy produced a synergistic inhibitory effect on tumor growth in breast cancer tumors, glioblastoma tumors, and Barrett's adenocarcinoma. In addition, GRN163L was also shown to resensitize breast tumors to radiation therapy (Roth et al. 2010).

hTERT-associated antigens have also been the target of several novel vaccines. GV1001 is an hTERT peptide-based vaccine that ultimately leads to the production of CD4+ and CD8+ T-cells. The CD4+ T-cells secrete IFN-γ and IL-2 which increase the recruitment of CD8+ T-cells and natural killer (NK) cells to the tumor site. These CD8+ T-cells and NK cells then assist with the upregulation of MHC class-I molecules. Preclinical trials demonstrated that GV1001 was an effective treatment for B-cell chronic lymphocytic leukemia strains which overexpress hTERT by inducing cell lysis. Vx-001 is a cryptic peptide-based vaccine that targets hTERT. Cryptic peptide vaccines are advantageous in that they do not undergo massive clonal deletion since their expression on the cell surface is limited. Moreover, cryptic peptides do not induce immune tolerance (Ruden and Puri 2013). Another novel vaccine under investigation is GRNVAC1, which utilizes dendritic cells to phagocytize the antigen and transport it to the lymph nodes. Once in the lymph nodes, the dendritic cells present the antigen to CD8+ T-cells, thereby activating them. These activated t-cells target tumor cells that overexpress telomerase and release signaling molecules which trigger apoptosis (Holysz et al. 2013).

Utilizing G-quadruplex-stabilizing ligands as anticancer agents is a novel approach that differs from other telomerase-inhibiting strategies, since they not only prohibit telomerase from accessing the telomere, but also rapidly induce various DDRs by disrupting telomere structures (Shalaby et al. 2013). Telomestatin, a natural macrocyclic product isolated from *Streptomyces* bacteria, has been established as one of the most potent and successful G-quadruplex stabilizers to date, due in part to its high selectivity for cancer cells. Telomestatin has a similar structure to the

G-quadruplex and has higher affinity for intramolecular G-quadruplexes than duplex DNA (Yang and Okamoto 2010). Telomestatin has been shown to inhibit telomerase activity in several preclinical studies involving multiple myeloma, neuroblastoma, myeloid leukemia, breast, pancreatic, cervical, and several other cancer cell lines (Ruden and Puri 2013). Furthermore, telomestatin may play a role in the downregulation of POT1 and complete dissociation of TRF2, resulting in disruption of telomere integrity and uncapping of telomere ends (Shalaby et al. 2013). BRACO-19 interacts with G-quadruplexes by binding within the grooves of the G-quartet, successfully inhibiting the activity of telomerase and uncapping the ends of the telomere strands while exhibiting low cytotoxicity (Yang and Okamoto 2010). In the presence of BRACO-19, breast, prostrate, uterus, and vulval cancer cells showed a decrease in telomerase expression, induction of cellular senescence, and cessation of cell growth (Shalaby et al. 2013). The stabilizing ligand RHPS4 has a similar structure to telomestatin and BRACO-19; however, its mechanism of action results in telomere dysfunction rather than telomere shortening. RHPS4 induces telomeric fusions, polynucleated cells, and telophase bridging in malignant cells (Yang and Okamoto 2010). However, since G-quadruplexes are not exclusively formed at telomeres, but instead may be prevalent throughout the chromosome, more research is needed to determine if G-quadruplex stabilizing ligands could induce deleterious side effects in normal, healthy cells (Simonsson 2001).

Inhibition of tankyrase 1 prohibits PARsylation of TRF1, thereby indirectly abrogating telomerase activity. 3AB is a nonspecific PARP inhibitor which can resensitize tumors resistant to telomerase inhibitors, such as MST-312. MST-312 is a synthetic telomerase inhibitor that causes drastic shortening of the telomere; however, its use as a monotherapy is limited due to development of resistance. Studies have shown that using 3AB, in combination with MST-312, resensitizes resistant cells to MST-312 and causes them to enter senescence (Seimiya et al. 2005). XAV939 is another PARP inhibitor that inhibits tankyrase 1 and resensitizes cancer cells resistant to telomerase inhibitors. It was also the first PARP inhibitor to show significant potency and specificity for tankyrase 1. Finally, JW55, another PARP inhibitor, has also been shown to inhibit both tankyrase 1 and tankyrase 2 and reduces tumor load and area in mice. Despite evidence showing that knockout of both tankyrase 1 and 2 in mice is lethal, no deleterious side effects of PARP inhibitors have been documented which would prohibit their development as novel anticancer therapeutics. Thus, growing evidence has demonstrated the potential of tankyrase 1 and other PARP inhibitors to be used alone or in combination therapy with telomerase inhibitors (Riffell et al. 2012).

Clinical Summary

Currently, GRN163L is being investigated in four phase II and ten phase I clinical trials in patients with pediatric solid tumors, non-small cell lung cancer (NSCLC), breast cancer, and blood ascariases. The results of the clinical trials in pediatric

glioma, ependymoma, and medulloblastoma showed a 50% decrease in telomerase activity after treatment with GRN163L. Preliminary data from the NSCLC trials did not show a statistically significant improvement in progression-free survival or overall survival; however, there was a subset of patients with shorter telomeres in their tumors that did show a significant trend toward progression-free survival and overall survival. GRN163L was also used to treat essential thrombocythemia in 18 patients, 16 of which had a complete response to the treatment. Finally, one phase II trial treated breast cancer stem cells using GRN163L in combination with paclitaxel and bevacizumab. Results from this trial have not yet been published, but a phase I trial using the same combination of drugs to treat breast cancer showed an objective response rate of 54.8% (Puri and Girard 2013). Common side effects seen in clinical trials were thrombocytopenia, myelosuppression, and hypersensitivity reactions to the drug (Ruden and Puri 2013; Puri and Girard 2013). These side effects are also seen in conventional cancer therapy, indicating the drug may affect highly proliferative somatic cell lines. Additional clinical studies are required to determine the efficacy of GRN163L, dose-limiting toxicities, and the length and rate of remission in patients.

Phase I and II clinical trials using GV1001 have been started in patients with melanoma, NSCLC, hepatocellular carcinoma, and pancreatic cancer and have demonstrated that the vaccine is a safe and relatively effective therapy. A phase II trial in patients with NSCLC demonstrated a 54% immune response after 4 weeks of treatment, which increased to 86% after 8 weeks, without any serious side effects. In vivo studies and phase I and II clinical trials have also shown that Vx-001 is effective in decreasing tumor volume. Vx-001 administered to patients with advanced stage NSCLS, breast cancer, melanoma, and cholangiocarcinoma resulted in a strong anticancer immune response, long-lasting tumor stabilization, and prolonged overall survival. In addition, these patients did not exhibit any major side effects (Ruden and Puri 2013).

Anticipated High-Impact Results

- Preclinical studies with G-quadruplex and tankyrase 1 inhibitors demonstrate efficacy in multiple cancers; however, further clinical trials need to be initiated to determine their efficacy in cancer patients.
- Telomerase inhibitors such as GRN163L have been used in phase I and phase II clinical trials in a wide range of cancers. Future studies with phase III trials are required to determine the efficacy on a larger number of patients before GRN163L can be established as an effective chemotherapeutic agent.
- GV1001, Vx-001, and GRNVAC1 vaccines are currently scheduled for additional phase II and III clinical trials. These vaccines have proved to be clinically effective with minimal side effects.

Cross-References

► DNA Repair, Overview
► P53, Immunology
► PARP

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TGF Beta Receptors

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Beverly Teicher

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Abstract

Transforming growth factor-beta 1-3 (TGFβ) are members of a large multifunctional regulatory polypeptide family that controls cellular functions including proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis, immune surveillance, and survival in many cell types. In intact canonical TGFβ signaling, the binding of a TGFβ to the TGFβ type II receptor enables the formation of a heteromeric complex between TGFβ type I and type II receptors. The type I receptor is phosphorylated by the type II receptor serine/threonine kinase. The activated type I receptor phosphorylates receptor-activated Smads that complex with Smad 4. The Smad complexes translocate into the nucleus and regulate target gene transcription through direct or indirect interaction with DNA-binding transcription factors or coactivators. Tumors are resistant to growth inhibition by TGFβ due to inactivation of the TGFβ signaling pathway or aberrant

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regulation of the cell cycle, in fact, many tumors secrete high levels of TGFβ. TGFβ knockout mice suffer from lethal multifocal inflammatory disease indicating the importance of TGFβ in maintaining immune system homeostasis. TGFβ pathway-directed therapy could reverse the immunosuppressive effects of this cytokine on the host as well as decrease extracellular matrix formation, angiogenesis, and osteolytic activity; and increase the sensitivity of the malignant cells to cytotoxic therapies and immunotherapies.

Keywords
Transforming growth factor-beta • TGFβ • GC-1008 • Anti-TGFβ • Trabedersen

Target: Transforming Growth Factor-β (TGFβ)

TGFβ is a 25-kDa disulfide-linked homodimer that can be reduced to a 12.5 kDa band (112-amino acids) on a protein gel following treatment with β-mercaptoethanol (Roberts et al. 1983). Three isoforms of TGFβ have been described; however, the number of TGFβ-related proteins now numbers 33 and forms the TGFβ superfamily (Derynck and Miyazono 2008). The TGFβ proteins are encoded by larger precursor or proproteins (390-amino acids). The proprotein is cleaved to the TGFβ monomer at a tetrabasic site by the endoprotease furin to release the active ligand. TGFβ is secreted from cells in a biologically inactive form (latent TGFβ) consisting of noncovalent association of the dimeric latency-associated peptide (LAP) protein with the dimeric mature carboxy-terminal 112 amino acid TGFβ. Latent TGFβ can be activated by a variety of stimuli resulting in dissociation of the LAP protein from mature TGFβ, thus unmasking the receptor binding epitopes. In cells with intact canonical TGFβ signaling, the binding of a TGFβ (isoform-1, −2 or −3) to the TGFβ type II receptor enables the formation of a heteromeric complex between TGFβ type I and type II receptors. The type I receptor is phosphorylated by the type II receptor serine/threonine kinase. The activated type I receptor phosphorylates selected receptor-activated Smads that then complex with Smad 4. The Smad complexes translocate into the nucleus. Activated Smad complexes regulate the transcription of target genes through direct or indirect interaction with DNA-binding transcription factors or coactivators. Activation of receptor-activated Smads can be inhibited by Smad6 or Smad7 (Teicher 2007).

Biology of the Target

TGFβ is a multifunctional regulatory protein that is a member of a large cytokine family that controls many aspects of cellular function, including proliferation, differentiation, migration, apoptosis, adhesion, angiogenesis immune surveillance, and survival (Jakowlew 2008). The action of TGFβ is dependent upon cell type, growth conditions, and other secreted protein factors. Nearly all cells both produce

and respond to TGFβ; therefore, understanding the components of the TGFβ signal transduction pathway in normal cells including the TGFβ ligands and receptors and the regulatory and inhibitory intracellular signal-transducing Smad proteins has been the subject of many investigations. TGFβ can produce varied effects that are dependent on the cell type and context of the physiological and/or pathological environment.

TGFβ knockout mice suffer from a lethal multifocal inflammatory disease indicating the importance of TGFβ in maintaining immune system homeostasis. The blockade of TGFβ signaling in T cells by transfection with a dominant negative type II receptor or in bone marrow by conditional knockout of the TGFβ type II receptor results in similar multifocal inflammatory responses.

Target Assessment

Malignant cells often secrete large amounts of TGFβ that acts on non-transformed cells present in the tumor mass as well as distal cells in the host to suppress antitumor immune responses creating an environment of immune tolerance, augmenting angiogenesis, invasion and metastasis, and increasing tumor extracellular matrix deposition. In addition, within the tumor and tumor vicinity, TGFβ may be released from the extracellular matrix or secreted by mesenchymal cells, resident leukocytes, or by monocytes and macrophages recruited to the tumor (Teicher 2007). TGFβ is a potent suppressor of the immune system. With broad activity over natural killer (NK) cells, T cells, monocytes/macrophages, and dendritic cells, TGFβ can affect the initiation and stimulation of both primary and secondary immune responses as well as suppress antitumor effector cells. TGFβ in lung and colorectal cancer patient plasma samples directly suppressed NK cell activity, a defect that could be reversed with anti-TGFβ antibodies.

Role of the Target in Cancer

TGFβ is a key player in malignant disease through its actions on host tissues and cells. Aberrant expression of TGFβ results in profound changes in the genetic stability of cells leading to alteration of both the differentiation state of the cells, altered interaction of the cells with the host environment, and the generation of therapy-resistant disease. Malignant cell resistance to TGFβ is frequently due to loss, silencing, or mutational inactivation of genes in the TGFβ signaling pathway including the type I and type II receptors and receptor-associated and common-mediator Smads. Increased TGFβ expression and production occurs in many neoplasms, including prostate, breast, pancreatic, kidney, liver, colorectal, gastric, esophageal, ovarian, cervical, bladder, myeloma, head and neck, thyroid, Kaposi's sarcoma, melanoma, and non-small cell and small cell lung cancers, and is associated with shorter patient survival. Malignant cells often secrete TGFβ that acts on non-transformed cells in the tumor mass as well as distal cells in the host producing

an environment of immune tolerance, augmenting angiogenesis, invasion and metastasis, and increasing tumor extracellular matrix deposition. Innate immune system cells contribute to high TGFβ concentrations in tumor masses. Dendritic cell subpopulations secreting TGFβ contribute to generation of regulatory T cells that actively inhibit other T cells. Elevated TGFβ is associated with advanced stage disease and may separate patients into prognostically high-risk populations.

High-Level Overview

Malignant disease grips the host influencing the behavior of cells in the vicinity of the tumor and distal cells and tissues as well. TGFβ, a secreted protein, is a key player in the malignant process. TGFβ pathway-directed therapy could reverse the immunosuppressive effects of this cytokine on the host as well as decrease extracellular matrix formation, decrease angiogenesis, decrease osteolytic activity, and increase the sensitivity of the malignant cells to cytotoxic therapies and immunotherapies.

Diagnostic, Prognostic, and Predictive

The majority of tumors from patients with advanced breast cancer as well as several other malignancies have been reported to be refractory to TGFβ-induced growth inhibition, and many produce large amounts of this cytokine (Hsu et al. 2008). In addition, elevated levels of plasma TGFβ have been associated with advanced stage disease and might separate patients into prognostically high-risk populations. It is believed that active TGFβ produced by the tumor and local stroma contributes to the progression and metastatic potential of this cancer through autocrine and paracrine effects. TGFβ is elevated in the plasma of prostate cancer patients and pancreatic cancer patients. The TGFβ levels correlate with advanced stage, metastases, and poorer clinical outcome. Increased TGFβ expression was observed in tumor cells and stroma. Plasma TGFβ is elevated in patients with renal cell cancer. TGFβ neutralization can be an effective therapy in mouse models of renal cell cancer alone or in combination regimens. TGFβ expression is increased in malignant melanoma cells but not in benign or in situ lesions. Increased TGFβ is associated with metastatic melanoma lesions and deeper invasion (worse prognosis, Clark's level 3 and higher). In transgenic models in which T cells are rendered insensitive to TGFβ, mice are able to completely eradicate B16 melanoma tumors. Anti-TGFβ enhanced tumor-specific immune response but did not completely eradicate tumors perhaps due to the incomplete TGFβ neutralization. Elevated serum TGFβ was observed in patients with myeloma and correlated with higher serum β2-microglobulin, an adverse prognostic marker. Sorted CD38 + CD45 RA-myeloma cells secrete significantly more TGFβ than peripheral blood mononuclear cells, splenic B cells, or CD40 ligand-activated B cells. TGFβ secretion by myeloma bone marrow stromal/mononuclear cells was greater than by normal bone

marrow mononuclear cells. In myeloma patients, the source of TGFβ is the malignant cells and bone marrow stromal cells. TGFβ is elevated in non-Hodgkin's lymphoma and markedly elevated in high-grade lymphomas, cutaneous T-cell lymphomas with a T regulatory phenotype, and in splenic marginal zone lymphomas presenting as myelofibrosis. Other malignant diseases are also associated with elevated TGFβ.

Therapeutics

The therapeutic approaches to the TGFβ pathway include antibodies, small molecule kinase inhibitors, and antisense. Clinical trials are underway, a small molecule inhibitor of TGFβ receptor type I kinase activity and with a fully human monoclonal antibody that neutralizes the three isoforms of TGFβ. The phosphorothioate TGFβ2-specific antisense oligodeoxynucleotide trabedersen (AP-12009) is being developed for the potential treatment of malignant gliomas via intratumoral catheter and other tumors via IV administration overexpressing TGFβ2. Pivotal phase III trials for anaplastic astrocytoma and for glioblastoma are underway as well as phase I/II trials for melanoma, colorectal cancer, and pancreatic cancer patients. LY-2382770, an anti-TGFβ antibody for the potential subcutaneous treatment of cancer, chronic renal disease, and diabetic nephropathy is under development. The small molecule kinase inhibitor LY-2157299, a TGFβ receptor I inhibitor, is in development for the potential treatment of solid tumors as are other small molecule inhibitors of TGFβ receptor 1. An intravenously infused fresolimumab (GC-1008), a monoclonal antibody against TGFβ, for the treatment of fibrotic disease and cancer has completed a phase I/II trial in cancer patients with renal cell carcinoma or malignant melanoma (Hsu et al. 2008).

Preclinical Summary

Strong evidence exists from animal models that TGFβ can promote late-stage tumor growth and metastasis. Mice heterozygous for the TGFβ1 gene have enhanced carcinogen-induced tumorigenesis. Tumors that developed retained the remaining wild-type TGFβ1 allele indicating that there was no selective pressure to abrogate TGFβ signaling. Transgenic mice expressing TGFβ1 in keratinocytes developed fewer carcinogen-induced skin papillomas. Experiments with mouse lymphoma, melanoma, and prostate carcinoma tumors support the hypothesis that inhibition of TGFβ1-mediated immunosuppression can have strong therapeutic potential in cancer. Transgenic mice expressing dominant negative TGFβ type II receptor in T cells mounted antitumor immune responses after inoculation of EL-4 lymphoma or B16-F10 melanoma cells (Pinkas and Teicher 2006). Mice reconstituted with dominant negative TGFβ type II receptor-transduced bone marrow were insensitive to TGFβ-mediated suppression of T-cell responses and survived challenge with B16-F10 melanoma or TRAMP-C2 metastatic prostate carcinoma cells better than

control mice. TGFβ1 is pro-metastatic in a mouse breast cancer model utilizing a doxycycline-inducible transgenic promoter. Transgenic expression of polyoma virus middle T oncogene (PyMT) driven by long terminal repeat-induced multifocal mammary tumors virus (MMTV) with lung metastases. Human MDA-MB-231 breast cancer cells growing in the bone microenvironment express parathyroid hormone-related peptide (PTHrP) which stimulates osteoclasts and bone resorption. Constitutively activated TGFβ type I receptor enhanced expression of PTHrP in MDA-MB-231 cells in culture and increased osteolytic bone destruction in vivo. Prostate carcinoma sublines, DU145, PC-3, and LNCaP, resistant to chemotherapy secreted more TGFβ1 into culture medium, and animals bearing xenografts of these tumor lines had increased plasma TGFβ1. TGFβ1 overexpression in subcutaneous Dunning R3327 MATLyLu rat prostate carcinoma line resulted in enhanced primary tumor growth and lung and lymph node metastasis. EMT6 mouse mammary carcinoma drug-resistant sublines were generated in vivo by treatment of tumor-bearing animals with antitumor agents. Treatment of animals bearing drug-resistant tumors with TGFβ-neutralizing antibodies restored sensitivity of the tumors to chemotherapy. Thus, TGFβ in the tumor microenvironment may contribute to drug resistance, and TGFβ antagonists may be an effective addition to treatment.

Clinical Summary

Fresolimumab (GC1008) is a human IgG4 that neutralizes TGFβ 1, 2, and 3 (Morris et al. 2008). The first clinical trial in cancer was a multicenter trial examining the safety and effectiveness of GC1008 in patients with advanced malignant melanoma or renal cell carcinoma at 1 of 6 dose levels (0.1, 0.3, 1, 3, 10 or 15 mg/kg) in a 3 + 3 design. Twenty-two patients were treated in phase I. Fresolimumab at 15 mg/kg was determined to be a safe dose. Adverse events like skin rash (including two cases of eruptive keratoacanthomas), fatigue, headache, epistaxis, gingival bleeding, and GI symptoms were all grade 1 or 2. Some patients achieved stable diseases (SD) or better.

PF-03446962 is a fully human mAb against activin receptor-like kinase-1 (ALK-1) a type I cell-surface receptor for TGFβ family ligands which is highly homologous with TGFβR1 (ALK-5). PF-03446962 produced dose-dependent antiangiogenic activity in nonclinical studies in a chimera mouse model bearing human tumors xenograft (Goff et al. 2010). A phase 1 trial in patients with solid tumors was conducted. PF-03446962 is administered IV on day 1, 29, and then q 2 weeks. Based upon 23 patients at five dose levels (0.5–4.5 mg/kg), the $t_{1/2}$ is 325 h. Treatment with PF-03446962 has been safe and is well tolerated in the first five dose levels. CE-US suggests potential vascular changes in liver metastasis, and preliminary evidence of clinical activity was also observed.

Trabedersen (AP 12009) is a TGF-β2-specific antisense oligonucleotide (Oettle et al. 2010). In a randomized and active-controlled phase IIb study in high-grade glioma patients, a clear survival benefit for trabedersen treatment over standard chemotherapy was found. Trabedersen showed high safety and tolerability.

LY2157299 is a novel small molecule inhibitor which selectively targets the kinase domain of the TGF-β receptor type I (Calvo-Aller et al. 2008). Patients with advanced/metastatic malignancies and who had exhausted all approved treatments were enrolled in this study. LY2157299 was well tolerated at 40 and 80 mg. Absorption of LY2157299 was rapid. Daily oral administration of LY2157299 was safe and well tolerated at the two dose levels, and the pharmacokinetic profile was consistent with the prediction derived from preclinical PK/PD model.

Anticipated High-Impact Results

The clinical investigation of TGFβ targeted therapies is at a very early stage.

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TLR7 and TLR8, Resiquimod, and 852A

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Abstract

The Toll-like receptors 7 and 8 as integral component of the host innate immune system are endosomally-located receptors that sense single-stranded RNAs derived from microbes or damaged cells. Engagement of these receptors triggers the host inflammatory response to eliminate and clear invading microbes or dead tissues. Harnessing the protective function of these receptors in immune cells has proven to be successful in treatment of superficial bladder and skin cancers. However, preclinical evidence suggests that aberrant activation of these TLRs in cancer cells or tumor-supporting cells can potentially accelerate cancer growth. Therefore, much work is needed to clearly delineate the role of these TLRs in different cancer, as well as each different cell-types in the tumor microenvironment, to develop a therapeutic strategies that have the highest chance of success in clinical trials.

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Aldara® • Damage-associated molecular patterns (DAMPs) • Imiquimod • Resiquimod • TLR7 • Actinic keratosis and basal cell carcinoma • Topical imiquimod • TLR8. *See* TLR7 • Toll-like receptors (TLRs) • TLR7. *See* TLR7 • Innate immunity • ssRNA • dendritic cells

Target: TLR7 and TLR8

Toll-like receptors (TLRs) are a family of phylogenetically related transmembrane innate immunity receptors that are expressed in various cell types where they collectively serve as protective sentries against invading microbes. The toll-like receptors consist of an extracellular domain characterized by leucine-rich repeats, a transmembrane domain which controls proper subcellular trafficking, and a cytoplasmic TIR (toll/interleukin-1 receptor) domain which serves to recruit adaptor protein to initiate signal transduction. To date, ten different toll-like receptors have been identified in humans. Each TLR is specialized in recognizing distinct pathogen-associated molecular patterns (PAMPs) derived from microbes, such as lipopolysaccharides, lipopeptides, DNAs, RNAs, and flagellin, to help mount innate immune response as part of the host defense mechanism. Besides these foreign antigens, the TLRs are also capable of recognizing endogenous ligands, termed damage-associated molecular patterns (DAMPs) such as heat shock proteins, hyaluronan, DNAs, and RNAs derived from dead cells in the microenvironment. Depending on the nature of the agonists and the involved TLRs, distinct sets of adaptor proteins such as MyD88, TRIF, TRAM, and Mal are engaged, which culminate in activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) transcription factors, interferon regulatory factors (IRFs), p38/mitogen-activated protein kinase (MAPK) (Chapter 86), and the c-Jun N-terminal kinase (JNK) (Chapter 82) cascades. These signaling events result in increased cellular proliferation, survival, and importantly secretion of proinflammatory cytokines, immunomodulatory molecules such as the type-1 interferons (Chapter 75), and tumor necrosis factors (TNFs) (Chapter 136) which subsequently help draw innate and adaptive immune cells to elicit local or systemic inflammatory responses (Lim and Staudt 2013). Recognition of these antigens by the TLRs in adaptive immune cells such as the T and B lymphocytes eventually triggers plasmacytic differentiation of B cells which produce protective antibodies and T_H1-weighted cellular immune response which ultimately give rise to memory cells.

TLR7 and TLR8 are both endosomally localized toll-like receptors that are specialized in sensing single-stranded RNAs (ssRNAs) (Heil et al. 2004), as well as synthetic nucleoside analogs such as the imidazoquinolines, imiquimod, or resiquimod. TLR7 is highly expressed in airway epithelial cells, spleen, placenta, and immune cells such as the macrophages, monocytes, plasmacytoid dendritic cells, and B cells, whereas TLR8 is abundantly expressed in airway epithelial cells and certain leukocytes such as the monocytes. In cells infected with bacteria or viruses,

foreign bacterial or viral RNAs are processed and released into the endolysosomes, where they are detected by TLR7 and TLR8. Stimulation of these TLRs typically leads to the abovementioned signaling events that culminate in secretion of cytotoxic mediators such as TNFs and type 1 interferons and local inflammatory response which include to help eliminate the pathogens. Pathologically, inappropriate triggering of TLR7 of B cells by self-RNAs results in autoimmune disorders such as SLE and rheumatoid arthritis (Green and Marshak-Rothstein 2011).

The protective function of the TLRs has long been harnessed to curb certain forms of human cancer. Intravesical bacillus Calmette-Guerin instillation reduces local recurrence of superficial bladder carcinoma by invoking local inflammatory response through TLR2 and TLR4. Imiquimod (R837), a potent interferon-inducing compound used topically to treat genital warts and some forms of skin cancer, is later shown to actually exert its antiviral and antitumor effects through stimulating TLR7 and, to a lesser degree, TLR8 in the surrounding immune cells such as the dendritic cells, macrophages, and B lymphocytes (Sidky et al. 1992; Hemmi et al. 2002). A more potent derivative, resiquimod (R838), is capable of activating both TLR7 and TLR8 to elicit stronger immune responses.

While TLR activation in the immune cells may result in antitumor effect, stimulation of TLRs within the tumor cells may paradoxically enhance tumorigenic growth. Many TLR members are found to be overexpressed in tumor cells, implicating that they may have pro-tumorigenic role. Overexpression of TLR7, and to a lesser extent, TLR8, has been reported in various malignancies such as multiple myeloma, CLL, NSCLC, pancreatic cancer, colorectal cancer, and esophageal squamous cell carcinoma. Expression of TLR7 is enhanced in the epithelial and stromal compartments of human and a mouse model of KRas-driven pancreatic cancer (Ochi et al. 2012). Furthermore, stimulation of TLR7 in pancreatic ductal cells greatly accelerated stromal inflammation and subsequent carcinogenesis. Conversely, blockade of TLR7 potently impaired carcinogenesis, suggesting a potential pro-tumorigenic role of TLR7 in pancreatic cancer formation (Ochi et al. 2012). In addition, the presence of MyD88, the key adaptor for TLR7 and TLR8 signaling, is essential for Ras-induced tumorigenic transformation (Coste et al. 2010; Cataisson et al. 2012), supporting tumor-intrinsic TLR signaling as a culprit in abetting oncogenic events during cancer initiation and progression.

Biology of the Target

After being synthesized at the endoplasmic reticulum, full length TLR7 and probably TLR8 are trafficked with the assistance of specialized proteins such as UNC93B1 and PRAT4A to the endolysosomes where they subsequently undergo stepwise proteolytic cleavages by asparagine endopeptidase (AEP) and the cathepsin family proteases to produce a trimmed, mature TLR that is optimized for agonist recognition (Ewald et al. 2011). The ligand-binding domain of both TLR7 and TLR8 is evolutionarily positioned within the lumen of the endolysosomes instead of

outside the plasma membrane so as to avoid inappropriate recognition of the abundant self-RNAs within the microenvironment which may otherwise trigger autoimmune responses.

The structural alteration of TLR8 following ligand binding has recently been demonstrated (Tanji et al. 2013). While unstimulated TLR8 appears to exist in dimers, ligand binding results in structural reorganization of each TLR8 monomer such that their C-termini, which include the TIR domain, were brought into closer proximity to form a docking site for other signaling adaptors such as MyD88. This event results in hierarchical assembly of multiple MyD88 proteins and the interleukin-1 receptor-associated kinases (IRAKs) which together form a macromolecular complex termed the "Myddosome" that subsequently serves as a molecular platform to engage and activate the proinflammatory signaling cascades (Lim and Staudt 2013). Utilizing the same mechanisms, stimulation of TLR7 and TLR8 in tumor-infiltrating antigen-presenting cells, such as by using synthetic imidazoquinolines, may also instigate humoral and cellular responses directed against the tumor cells.

Target Assessment

There is currently no established role of assessing TLR7 or TLR8 expression in aiding diagnosis, guiding treatment decision, or prognostication of cancer patients. In research settings, expression levels of TLR7 and TLR8 can be done using quantitative RT-PCR of the mRNAs or by protein immunohistochemistry.

Role of the Target in Cancer

The functional role of TLR7 and TLR8 in cancer treatment is multifaceted. Direct exposure of cultured cancer cell lines to imiquimod induces cellular apoptosis (Meyer et al. 2003; Schon and Schon 2004), sensitizes cancer cells to cytotoxic agents and killing by cytotoxic T lymphocytes (Shojaei et al. 2009). In mouse models, TLR7 and TLR8 agonists greatly enhance the recruitment and antigenic activation of dendritic cells, thereby enhancing T_H1-weighted cytotoxic cellular immune responses (Napolitani et al. 2005; Kastenmuller et al. 2011).

On the other hand, increasing evidence now showed that stimulation of TLR7 or TLR8 within the tumor cells may paradoxically facilitate tumorigenic growth. TLR7 signaling is essential for both initiation and progression of KRas-driven pancreatic cancer in an experimental mouse model (Ochi et al. 2012). Moreover, stimulation of TLR7 and TLR8 with synthetic ligands in lung cancer cell lines activates NF-kB signaling and promotes tumor cell survival and resistance to chemotherapeutic agents (Cherfils-Vicini et al. 2010).

High-Level Overview

Diagnostic, Prognostic, Predictive

Expression of TLR7 or TLR8 in tumor has not been shown to be associated with prognosis or response to treatment in patients.

Therapeutics

Initial studies in the late 1980s were focused primarily on its potent antiviral activity against CMV and HSV infection in animals, which was later attributed to its strong immunostimulatory effect, particularly in promoting secretion of interferon-a, TNF-a, and interleukin-6 (Reiter et al. 1994). Oral administration of imiquimod as a cytokine-inducer was soon demonstrated to also have antitumor effect against a variety of tumor types in murine xenograft models (Sidky et al. 1992), setting the stage for phase 1 clinical trials in human patients. However, at maximal tolerated dose, imiquimod as single agent did not show clinical efficacy in patients with refractory cancer (Witt et al. 1993; Savage et al. 1996). On the other hand, topical 5% imiquimod cream was found to have significant therapeutic effect in anogenital warts, with 50% or more patients cured and greater than 75% patients experiencing greater than 50% size reduction in their lesions (Edwards et al. 1998; Beutner et al. 1998). Similar results were also shown in many other studies, thereby prompting FDA approval for its use in treatment of anogenital warts. Interestingly, it was not until a few years later imiquimod was found to be a ligand for TLR7 (Hemmi et al. 2002).

The clinical efficacy of topical imiquimod was later extended to other cutaneous lesions such as actinic keratosis and basal cell carcinoma. A few randomized phase III trials showed that topical use of 5% imiquimod cream applied two or three times per week for 16 weeks resulted in 45.1–57.1% complete clearance rate, as opposed to 2.2–7.2% in vehicle-treated patients. In these studies, the median percentage reduction in target lesions was in the range of 83–86% (Lebwohl et al. 2004; Szeimies et al. 2004; Korman et al. 2005). Similar exciting findings were also shown in patients with basal cell carcinoma. Several trials showed that topical application of 5% imiquimod cream more than five times per week results in greater than 75% combined clinical and histological clearance rate in patients with superficial basal cell carcinoma (Geisse et al. 2002, 2004; Schulze et al. 2005). For patients with nodular basal cell carcinoma, daily application of 5% imiquimod cream for 6 weeks results in greater than 70% histological clearance rate (Shumack et al. 2002). In these studies, almost all patients experienced local reactions such as erythema, scrubbing, erosions, and ulceration, which were considered manageable and reversible with discontinuation of imiquimod. Other than these FDA-approved indications, successful off-label use of topical imiquimod has been

reported in eradication or control of many other skin conditions such as cutaneous T-cell lymphoma, Bowen's disease, lentigo maligna, squamous cell carcinoma, and keratoacanthoma (David et al. 2011). The use of imiquimod as immunostimulant to boost therapeutic effect of chemotherapeutic agents and radiation, as well as adjuvant to tumor vaccines, is currently being evaluated in several clinical trials (Vacchelli et al. 2012).

Resiquimod is a more potent dual TLR7 and TLR8 agonist that is currently under investigation. In a phase II clinical trial in patient with actinic keratosis, 0.01% and 0.03% topical resiquimod gel administered three times per week for 4 weeks resulted in complete clearance rates of 77.1% and 90.3%, respectively (Szeimies et al. 2008). Phase I and II clinical trials with resiquimod are ongoing for treatment of early stage cutaneous T-cell lymphoma or as immune adjuvant for peptide vaccine in patients with resected melanoma.

Despite initial setbacks in demonstrating the clinical efficacy of systemic imiquimod, TLR7 or TLR8 agonists as immunostimulants continue to hold promise as a potentially effective antitumor modality. A subcutaneously administered TLR7 agonist, 852A, has been assessed in a few phase I/II clinical trials (Dummer et al. 2008; Geller et al. 2010; Weigel et al. 2012). Although the patient numbers were small, administration of 852A was shown to be relatively well-tolerated and demonstrated some antitumor activity in patients with metastatic melanoma (Dummer et al. 2008), advanced gynecologic malignancies (Geller et al. 2010), or hematologic malignancies (Weigel et al. 2012).

Preclinical Summary

The antitumor activity of TLR7 and TLR8 agonists is derived predominantly from activation of the host innate and adaptive immune systems. Agonistic activation of TLR7 or TLR8 in antigen-presenting cells or the innate immune cells leads to secretion of proinflammatory cytokines and chemokines, which culminate in production of tumor-directed antibodies and T_H1-weighted cellular immune responses. On the other hand, increasing preclinical evidence suggests that stimulation of TLR7 in tumor cells may promote survival, proliferation, and metastasis.

Clinical Summary

Topical imiquimod (Aldara®) is a TLR7 agonist that is FDA-approved for treatment of typical, non-hyperkeratotic, non-hypertrophic actinic keratoses in immunocompetent adults, primary superficial basal cell carcinoma, and external genital and perianal warts. More potent topical and systemic TLR7/8 agonists are being tested in augmentation of cellular antitumor immune response either independently or following vaccination with cancer peptides.

Anticipated High-Impact Results

- Topical Imiquimod in Treating Patients with Grade 2/3 Cervical Intraepithelial Neoplasia (NCT00941252)
- Effect of Topical Imiquimod on Lentigo Maligna (NCT01161888)
- A Phase II Study of Imiquimod 5% Cream for the Treatment of Hemangioma in Infancy (NCT00601016)
- Transcutaneous (Topical) Peptide Immunization with NY-ESO-1b (SLLMWITQC) Peptide Using Resiquimod as an Immune Adjuvant: A Pilot Study (NCT00470379)
- Peptide Vaccine with Resiquimod as an Immune Modulator for Patients with Resected Melanoma: A Pilot Study (NCT01748747)
- Pilot Phase II, Open Label, Multicenter, Efficacy and Safety Study of 852A Administered Intravenously to Subjects with Unresectable Metastatic Cutaneous Melanoma (NCT00189332)

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TLR9

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Abstract

The Toll-like receptor 9 (TLR9) is one of the key sentries of the host innate immune system that senses DNAs derived from microbes or damaged cells. Engagement of TLR9 in immune cells triggers the host inflammatory response to eliminate and clear invading microbes or dead tissues. On this basis, ligation of TLR9 in immune cells with nucleic acid agonist has been attempted as an anti-cancer strategy in various cancer types, so far with mixed results and much room to improve. On the other hand, ample preclinical evidence also indicates that overexpression or aberrant activation of TLR9 in cancer cells may be detrimental, raising caution in utilizing TLR9 agonists in cancer treatment and need for more detailed mechanistic studies to clearly delineate the cancer- and cell-type specific role of TLR9.

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Target: TLR9

The human cells encode ten different toll-like receptors (TLRs) that collectively constitute the first line of immune defense against invading microbes. Each toll-like receptor is comprised of a bulky horseshoe-shaped extracellular domain that is highly specialized in recognizing distinct forms of ligand, a transmembrane domain which ensures proper subcellular localization of each TLR, and a cytoplasmic TIR (toll/interleukin 1 receptor) domain which recruits adaptor proteins during signal transduction (Moresco et al. 2012). Ligands of the TLRs include pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides, nucleic acids such as DNAs and RNAs, flagellin, as well as breakdown products of host cells, termed damage-associated molecular patterns (DAMPs) which include heat-shock proteins, hyaluronan, DNAs, and RNAs. Specifically, TLR9 is specialized in recognizing unmethylated double-stranded DNAs, which are found most commonly in bacteria and viruses. However, recent evidence also showed that host mitochondrial DNAs, which are also unmethylated, can activate TLR9 (Oka et al. 2012).

In humans, expression of TLR9 is restricted to plasmacytoid dendritic cells and B cells, where it functions as a sensor for unmethylated microbial DNAs and triggers the innate immune response. Importantly, TLR9 can also be stimulated using synthetic oligodeoxynucleotides containing the CpG motif (CpG ODN). Intriguingly, the use of purified bacterial DNAs or CpG ODN as immunostimulants actually preceded the discovery that they actually bind and signal through TLR9e (Hemmi et al. 2000). Specifically, CpG ODNs are able to potently activate B cells (Krieg et al. 1995), potentiate immunogenicity of tumor vaccines (Wooldridge et al. 1997), and switch on T_H1 antitumor responses (Chu et al. 1997). These valuable properties prompted frenzied research over the last two decades in trying to harness the immunostimulatory effect of CpG ODNs in anticancer treatments either as single agent or in combination with radiotherapy (Mason and Hunter 2012), chemotherapy (Holtick et al. 2011), and targeted agents (Leonard et al. 2007) or as adjuvant to cancer vaccines (Bode et al. 2011).

Biology of the Target

After being synthesized in the endoplasmic reticulum, nascent TLR9 protein is escorted to the endolysosomes by specific trafficking processes that are mediated by the UNC93B1, PRAT4A, and adaptor protein complexes (Sasai et al. 2010;

Engel and Barton 2010). Once at the endolysosomes, the N-terminal ectodomain of TLR9 is enzymatically cleaved by asparagine endopeptidase (AEP) and the cathepsins to produce a truncated TLR9 that is optimized for signal transduction (Ewald et al. 2008; Park et al. 2008). In resting state, TLR9 exists predominantly as loose homodimers. Upon agonistic activation, the TLR9 dimer assumes a tighter conformation, allowing its cytoplasmic TIR domain to recruit and form homotypic interaction with the TIR domain of adaptor protein MyD88(Ewald et al. 2011). Such TIR-TIR interaction between TLR9 and MyD88 generates a signaling platform that nucleates the IRAK kinases, TRAF6, and TAK1, resulting in the activation of the NF-kB, interferon regulatory factors (IRFs), and p38/MAPK and JNK cascades and subsequently the secretion of proinflammatory cytokines and chemokines (Lim and Staudt 2013). These inflammatory mediators trigger the activation and proliferation of antibody-producing B cells and T_H1-weighted cytotoxic T-lymphocyte responses.

Target Assessment

There is currently no established role of assessing TLR9 expression in cancer management. In research settings, expression levels of TLR9 are commonly assayed using quantitative RT-PCR of the mRNAs of tumor specimens or by protein immunohistochemistry of formalin-fixed, paraffin-embedded samples.

Role of the Target in Cancer

Synthetic CpG ODNs as an immunostimulant are widely evaluated in various kinds of cancer, either as single agent, in combination with chemotherapy, radiation, and biologics, or as vaccine adjuvant. It is widely acknowledged, based on several lines of preclinical studies, that CpG ODN directly activates plasmacytoid dendritic cells and B cells, which result in potent T_H1-weighted cytotoxic Tcell response that can be directed against tumor cells.

On the other hand, the intrinsic role of TLR9 in human cancer cells is multifaceted, with different research yielding different results. TLR9 is found to be overexpressed in B cell malignancies such as CLL (Grandjenette et al. 2007; Liang et al. 2010), diffuse large B-cell lymphomas (Huang et al. 2012), multiple myeloma, esophageal SCC (Sheyhidin et al. 2011), NSCLC (Samara et al. 2012), and breast and ovarian cancers (Berger et al. 2010). Direct stimulation of TLR9 in vitro with synthetic ODNs promotes invasion of myeloma (Jego et al. 2006; Xu et al. 2010), breast (Merrell et al. 2006) and prostate (Ilvesaro et al. 2007), lung (Ren et al. 2007, 2009), glioma (Wang et al. 2010), esophageal, gastric, and colorectal cancer cell lines (Kauppila et al. 2012), predominantly through the activation of the NF-kB cascade. On the other hand, TLR9 agonists seem to inhibit viability of cultured neuroblastoma (Brignole et al. 2010), colorectal, and pancreatic (Rosa et al. 2011) cell lines. Although these contradictory results can be explained by the

use of different cell lines, TLR9 agonists, and experimental readout, they do raise a concern of potential tumor-promoting effect of TLR9 agonists in clinical trials.

High-Level Overview

Diagnostic, Prognostic, and Predictive

Pathological analyses of tumor specimens showed that TLR9 overexpression is associated with more malignant histological features such as higher grades and proliferation indices in esophageal adenocarcinoma (Kauppila et al. 2011) and squamous cell carcinoma (Takala et al. 2011), breast cancer (Qiu et al. 2011), and prostate cancer (Vaisanen et al. 2010), as well as poorer prognosis in glioblastoma multiform (Leng et al. 2012). On the contrary, the absence of TLR9 is associated with poor prognosis in RCC (Ronkainen et al. 2011). Testing of TLR9 protein or mRNA expression has not been shown to predict response to any kind of treatment and therefore should not be ordered outside of research settings.

Therapeutics

Evasion from the immune surveillance is an acquired hallmark of cancer, with failure of host immune system to mount an antitumor response, or tolerance, being a central mechanism. The use of stimulatory TLR9 agonists, such as the stimulatory ODNs, aims at stimulating plasmacytoid dendritic cells and B cells to help mount an effective tumor-directed cellular response.

At least three major different classes of synthetic TLR9 agonists, termed CpG oligodeoxynucleotides (CpG ODNs), have been described based on the difference in structure and immune response. The class A ODNs are characterized by poly-G motifs at the 3′ and/or 5′ ends and a central phosphodiester backbone containing palindromic repeats of CpG motifs. Due to these structural characteristics, class A ODNs are poised to forming higher-ordered, multimeric structure which tend to retain them in the transferrin-positive, early endosomes where the activation of TLR9 produces exclusively type 1 interferons. The class B ODNs consist entirely of nuclease-resistant phosphothioate backbone and do not form higher-order structure, which allows them easy passage into the LAMP1-positive, late endosomes where engagement with TLR9 leads instead to strong activation of the NF-kB pathway and secretion of proinflammatory cytokines. The class C ODNs possess features in between those of class A and B and are able to stimulate both the interferon and NF-kB pathways (Krieg 2006).

Due to the strong immunostimulatory effect of class B ODNs, one of these agonists, PF-3512676 (also known as agatolimod, ODN 2006, or CPG 7909) has been extensively tested as single-agent, vaccine adjuvant or in combination with chemotherapy, radiotherapy, or targeted biologics in early phase clinical trials on multiple cancer types such as renal cell carcinoma (Thompson et al. 2009), chronic

lymphocytic leukemia (Zent et al. 2012), low-grade non-Hodgkin's lymphoma (Brody et al. 2010), cutaneous T-cell lymphoma (Kim et al. 2010), mycosis fungoides (Kim et al. 2012), basal cell carcinoma (Hofmann et al. 2008), melanoma (Pashenkov et al. 2006), and non-small cell lung cancer (Manegold et al. 2008). Most of these early trials showed that PF-3512676 has an acceptable safety profile with some evidence of efficacy. In a phase I/II trial on patients with low-grade B-cell lymphoma, local radiation to one tumor site plus intratumoral injection of PF-3512676 induced a systemic CD8 T cells that led to a 27% objective response rate at distal sites (Brody et al. 2010). The same treatment approach was later tested on patients with mycosis fungoides, who saw a 35.7% objective response rate, although these responses are less durable than patients with low-grade B-cell lymphoma (Kim et al. 2012).

The efficacy of intracerebral administration was investigated in a single-arm phase II trial on 31 patients with recurrent glioblastoma multiforme. The progression-free survival (PFS) at 6 months was 19%, which was similar to other new investigational agents. However, the authors noted a 24% 1-year survival rate of these patients compared to less than 15% in the literature (Carpentier et al. 2010).

In patients with advanced non-small cell lung cancer, two recent randomized phase III trials showed that addition of PF-3512676 to standard platinum doublets did not confer survival benefit but instead increased adverse events (Manegold et al. 2012; Hirsh et al. 2011). In one study enrolling 839 chemotherapy-naïve patients with stage IIIB or IV NSCLC, addition of PF-3512676 to cisplatin/gemcitabine, compared to cisplatin/gemcitabine, failed to improve median survival (11.0 vs. 10.7 months; $P = 0.98$) or progression-free survival (both 5.1 months) (Manegold et al. 2012). In the other study involving 828 patients with advanced stage NSCLC, addition of PF-3512676 to carboplatin/paclitaxel also did not improve the median overall survival (10.0 months vs. 9.8 months; $P = 0.56$) or progression-free survival (4.8 months vs. 4.7 months; $P = 0.79$) (Hirsh et al. 2011). In both studies, patient who received PF-3512676 experienced significantly more side effects such as hematologic adverse events and serious infections, injection-site reactions, and flu-like symptoms. The lack of efficacy and higher incidence of adverse events prompted early termination of both studies at the first interim analysis.

Another TLR9 agonist, IMO2055, was tried in combination with cetuximab compared to cetuximab alone in a phase II trial for the treatment of recurrent or metastatic squamous cell carcinoma of the head and neck. However, the manufacturer, Idera Pharmaceuticals, announced in May 2005 that the combination arm failed to meet its primary end point of improved progression-free survival.

In spite of the setbacks of TLR9 agonists in cancer clinical trials, the strong scientific evidence backing TLR9 agonists as potent immunostimulants continues to fuel the enthusiasm to develop better TLR9 agonists and strategies to augment their efficacy. Further work includes optimization of the molecular structure, rational combination with cancer vaccines, proper timing and sites of administration, and, importantly, devising methods to stimulate only the immune system while sparing the tumor cells.

Preclinical Summary

Agonistic stimulation of TLR9 in the plasmacytoid dendritic cell and B cells, and the subsequent induction of T_H1-weighted cellular response underlie the main antitumor effect of CpG ODNs. However, increasing preclinical evidence now show that inappropriate stimulation of TLR9 may paradoxically promote malignant behaviors of cancer cells, underscoring the need to further clarify the functional role of TLR9 in each cancer cell type and to strategize delivery of TLR9 agonists so as to avoid undesirable tumor-promoting effects.

Clinical Summary

To date, several trials of CpG ODNs as single agent or in combination with chemotherapy, radiation, or biologics are ongoing. Systemic administration of PF-3512676 in addition to platinum doublet did not improve overall survival and progression-free survival in patients with advanced NSCLC, whereas encouraging results were reported in several phase I/II trials on patients with other cancers such as low-grade lymphoma. While local administration of PF-3512676 by intratumoral or intracerebral injection are well tolerated, systemic administration of PF-3512676 significantly aggravates chemotherapy-induced cytopenias and predisposes patients to serious infections.

Anticipated High-Impact Results

- A Phase II Open-label Study of Subcutaneous CpG ODN (PF03512676) in Combination With Trastuzumab in Patients With Metastatic Breast Cancer (NCT00824733)
- Multicentric Randomized Phase 2. Immunotherapy With CpG ODN in Malignant Glioblastoma (NCT00190424)
- CpG7909 Injection in Melanoma (NCT00070642)
- CpG7909 in Patients With Cutaneous T-Cell Lymphoma (NCT00043420)
- NY-ESO-1 Protein With Montanide and CpG7909 as Cancer Vaccine in Several Tumors (NCT00299728)

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Transforming Growth Factor β

46

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Abstract

Transforming growth factor β (TGFβ) has recently emerged as an attractive therapeutic target in several human malignancies. While TGFβ has well-documented antiproliferative properties with respect to many benign and well-differentiated epithelial cells, TGFβ is also a potent modifier of the tumor microenvironment that can serve to promote tumor development. Though clinical strategies targeting the TGFβ pathway show promise, this approach in cancer epithelial cells harboring intact tumor-suppressive TGFβ signals may exacerbate cancer cell proliferation. Therefore, it is essential to dissect the many contributions of TGFβ to cancer development prior to utilizing TGFβ inhibitors in the clinic.

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Transforming growth factor β (TGFβ) • Tumor microenvironment (TME) • T cells • TGFβ inhibition • TGFβ receptors (TGFBR1/2) • Smad4

Target

Transforming Growth Factor β (TGFβ) is a multifunctioning cytokine that has been implicated in the development of several human malignancies. While TGFβ adversely affects the progression of many advanced cancers, it has potent tumor suppressive effects in most benign and neoplastic epithelial tissues. Therefore, while there is merit in inhibiting the TGFβ pathway clinically, careful consideration must be taken to target only its tumor promoting effects.

Biology of the Target

TGFβ is composed of three isoforms, each of which is initially synthesized as a 75-kDa homodimer known as pro-TGFβ. Pro-TGFβ is cleaved in the Golgi to form the mature 25-kDa TGFβ homodimer, which then interacts with latency-associated proteins (LAP) to form the small latent complex (SLC). In the endoplasmic reticulum, a single latent TGFβ binding protein forms a disulfide bond with the TGFβ homodimer, forming the large latent complex (LLC). The LLC is then exported to the extracellular matrix where it localizes to fibrillin-rich microfibrils, where it is stored and remains biologically unavailable until activated.

Once activated (by factors including proteases, thrombospondin 1, reactive oxygen species, and integrins), mature TGFβ is liberated from the LLC and becomes available to its target cells. Active TGFβ first binds its type 2 receptor (TGFBR2) leading to the recruitment of its type 1 receptor (TGFBR1). These receptors dimerize and autophosphorylate, activating the serine/threonine kinase activity of TGFBR1. TGFBR1 then phosphorylates SMAD2 and SMAD3, proteins that are sequestered at the intracellular membrane by the SMAD anchor for receptor activation (SARA) protein. Once phosphorylated, SMAD2 and SMAD3 dissociate from SARA to complex with SMAD4 in the cytoplasm. The SMAD2/3/4 oligomer then translocates to the nucleus binding primarily to 5'-GTCT-3' or complementary 5'-AGAC-3' sequences, known SMAD-binding elements (SBE's). Once in the nucleus, the SMADs control transcription of several target genes in a highly cell-specific manner (Fig. 1).

Additionally, TGFβ contextually interacts with several non-SMAD pathways. Such targets include p38 MAPK, p42/p44 MAPK, c-Src, m-TOR, RhoA, RAS, PI_3K/Akt, protein phosphatase 2A (PP2A)/p70s6K, and JNK MAPK. Both SMAD-dependent and SMAD-independent signaling play multiple roles in cancer development. In normal epithelial cells, SMAD-dependent TGFβ signaling induces growth arrest primarily via induction of cyclin-dependent kinase inhibitors such as $p21^{CIP11/WAF1}$. Yet SMAD-independent mechanisms also contribute to both TGFβ

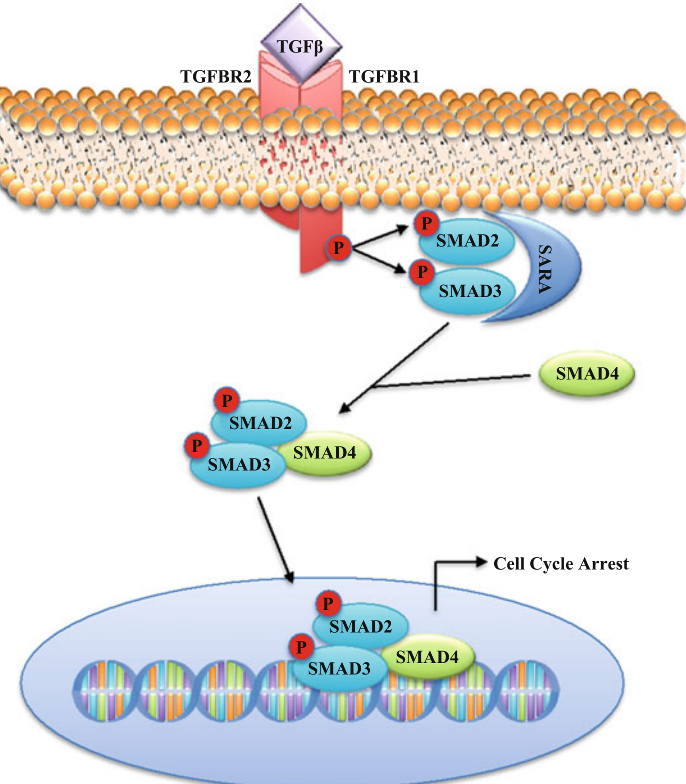

Fig. 1 Classical TGFβ signaling. The TGFβ ligand first binds its Type 2 transmembrane receptor (TGFBR2), which then recruits the Type 1 receptor (TGFBR1). Following interaction with TGFBR2, TGFBR1 phosphorylates serine/threonine residues on SMAD2 and SMAD3, allowing them to dissociate from the SMAD Anchor for Receptor Activation (SARA). This phosphorylation event allows for complexing with SMAD4 in the cytoplasm and subsequent translocation to the nucleus. Here, the SMAD2/3/4 oligomer binds DNA 5'-GTCT-3' or 5'-AGAC-3' rich sequences, culminating in growth arrest in most benign epithelial tissues

control over the cell cycle, as well as dysregulated TGFβ signaling in advanced disease, though these contributions remain unclear (Principe et al. 2014).

Role of the Target in Cancer

As discussed, in benign and neoplastic tissues, TGFβ is often considered a tumor suppressor. Through largely SMAD-dependent mechanisms, TGFβ ligands induce the upregulation of several growth inhibitory targets. These include several cyclin-dependent kinase inhibitor (CKI) proteins including p15, p16, p21, and p57. These CKIs interrupt the association between cyclins and their respective cyclin-dependent kinases, culminating in cell cycle arrest. However, some advanced tumors lose this

response and escape TGFβ control over the cell cycle. In this context, TGFβ may even begin to promote tumor progression at the level of the epithelial cell through a variety of mechanisms, the most notable being through inducing Epithelial to Mesenchymal Transition (EMT).

During the EMT process, epithelial cells begin to lose their polarity and E-cadherin expression, while acquiring mesenchymal markers such as Vimentin, Zeb1, and Snail. In addition to the morphological changes associated with EMT, post-EMT epithelial cells have enhanced migratory potential and may further dedifferentiate, eventually acquiring a stem cell-like phenotype that has been implicated in both increased self-renewal and chemoresistance (Han et al. 2005; Kim et al. 2004; Wendt et al. 2012; Zhang et al. 2009; Zavadil et al. 2004).

In epithelial cells, TGFβ has been demonstrated to repress E-cadherin through a variety of mechanisms. For example, through crosstalk with the PI_3K/Akt cascade, TGFβ signals can induce phosphorylation of α and β-catenin, which are sequestered by E-cadherin near the cell membrane. This phosphorylation event leads to conformational change, destabilizing E-cadherin complex, leading to a loss of cell-cell adhesion (Vogelmann et al. 2005).

Additionally, TGFβ can contextually induce expression of the transcription factor Snail, through both SMAD and non-SMAD pathways. Snail represses E-cadherin by binding its promoter and recruiting the Sin3A/HDAC1/HDAC2 complex. This leads to deacetylation of the E-cadherin promoter, and inhibition of mRNA transcription (Peinado et al. 2004).

In fibrous tumors such as those found in pancreatic and ovarian cancer patients, the contributions of TGFβ signaling to stromal cells can become particularly important in shaping the tumor microenvironment. While TGFβ is generally growth inhibitory to epithelial cells, it promotes the proliferation of mesenchymal cells such as fibroblasts, osteoblasts, and pancreas stellate cells. Similarly, TGFβ has been implicated in promoting both the migration and matrix deposition of these cells. Therefore, in such tumors, there may be additional merit to using inhibitors to the TGFβ pathway as an adjuvant therapy to reduce the size of the extracellular matrix in an attempt to improve the delivery of conventional therapies.

In addition to its effects on epithelial and stromal cells, TGFβ is a potent modifier of other cells in the TME, most notably of T cells. When a $CD4^+$ T cell is exposed to TGFβ, through a mechanism requiring IL2 (Zheng et al. 2007), it will begin to express the forkhead box transcription factor FoxP3. These $CD4^+FoxP3^+$ regulatory T cells (Tregs) also generally express CD25, a subunit of the high affinity IL2 receptor, and serve to suppress the cytotoxic and inflammatory function of effector T cells. Tregs secrete additional TGFβ as well as the anti-inflammatory cytokine IL10. Tregs also express CTLA4, a competitive inhibitor of CD28/B7 costimulatory interactions, which leads to anergy of other T cells in the vicinity (Tang et al. 2004). Clinically, elevated Treg populations correlate negatively with outcomes in several cancers, linking TGFβ signaling to tumor evasion of immune surveillance (Bates et al. 2006; Gobert et al. 2009).

Interestingly, TGFβ signaling also directs the proinflammatory T-helper 17 (Th17) differentiation program. While $CD4^+$ T cells can differentiate into Th17 cells in the

absence of TGFβ through a combination of IL-6, IL-23, and IL-1β, a greater number of cells will differentiate with a combination of IL-6 and TGFβ1. These Th17 cells express the RAR-related orphan receptor gamma t (RORγt) transcription factor and produce the proinflammatory cytokines IL17, IL21, and IL22. While the cytokine profile of a Treg suppresses the activity of autoreactive lymphocytes, in the cancer context, that of a Th17 cell promotes the attraction and activation of inflammatory granulocytes such as neutrophils and macrophages (Miossec and Kolls 2012).

Additional studies have examined the mechanisms through which TGFβ signaling contributes to myeloid cell function, particularly macrophages. Mice with conditional deletion of TGFBR2 in the bone marrow presented with fewer anti-inflammatory M2 macrophages and a lethal inflammatory phenotype. Furthermore, *ex vivo* studies demonstrate that TGFBR2-null bone marrow-derived macrophages have impaired M2 polarization, thereby implicating TGFβ in macrophage function (Gong et al. 2012).

TGFβ also directly affects the cytotoxic arm of the immune system by binding $CD8^+$ cytotoxic T lymphocytes (CTLs). TGFβ suppresses CTL activity and differentiation, through repression of several genes involved in an antitumor immune response including GranymeA and B, Perforin, Fas ligand, and Interferon γ (Thomas and Massague 2005). Furthermore, additional *in vivo* experiments suggest that TGFβ suppresses the activity of natural killer (NK) cells, suggesting an alternate mechanism through which TGFβ can suppress antitumor immunity (Arteaga et al. 1993).

In vivo experiments have also demonstrated that T lymphocytes with truncated TGFβ signals mount a robust antitumor immune response (Gorelik and Flavell 2001; Zhang et al. 2005, 2006; Wang et al. 2010). In light of these findings, there may be reason to consider the state of the adaptive immune system when determining patient candidacy for systemic therapies targeting the TGFβ pathway.

TGFβ is also a potent modifier of endothelial cell function, though the role of TGFβ in regulating tumor angiogenesis is also biphasic. At low levels, TGFβ appears to engage the noncanonical ALK1 pathway, leading to activation of BMP effectors SMAD1, SMAD5, and SMAD8. These proteins form a hetero-oligomer, and translocate to the nucleus to increase transcription of ID1 and matrix metalloproteinases (MMPs), resulting in increased endothelial cell proliferation and migration (Goumans et al. 2003; Lebrin et al. 2005). When an endothelial cell is exposed to high levels of TGFβ, the cell signals through the classical TGFβ cascade, activating SMAD2, SMAD3, and SMAD4 that upregulate antiangiogenic factors such as PAI-1 and Fibronectin. However, TGFβ has been demonstrated to increase the expression of vascular endothelial growth factor (VEGF) by the tumor epithelia, offering at least a partial explanation for observations suggesting that, in the clinic, levels of TGFβ correlate positively with angiogenesis in the tumor microenvironment (Goumans et al. 2003; Lebrin et al. 2005).

Given the vast and often diametrically opposed effects of TGFβ in the tumor microenvironment (Fig. 2), determining the safety and efficacy of therapies targeting the TGFβ pathway likely requires the consideration of several, nonepithelial cell types.

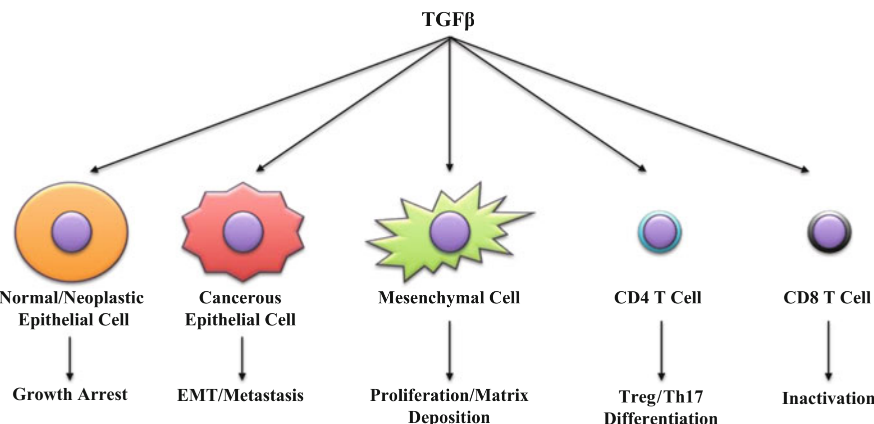

Fig. 2 The contributions of TGFβ signals to the tumor microenvironment. The effects of TGFβ signaling are highly cell specific, and precipitate multiple roles in the context of a dynamic tumor microenvironment. For example, in most benign and neoplastic epithelial tissues, TGFβ elicits a growth inhibitory response. However, in some cancerous epithelial cells, TGFβ has been shown to promote EMT and metastasis. In mesenchymal cells, such as fibroblasts and pancreas stellate cells, TGFβ promotes proliferation and secretion of matrix proteins, facilitating fibrosis and desmoplasia. In lymphoid cells, the effects of TGFβ are similarly varied. In high doses, TGFβ will convert a naïve $CD4^{+}$ T helper cell to a suppressive and anti-inflammatory regulatory T cell (T reg). Yet, when in concert with IL6, TGFβ will cause these cells to undergo differentiation in to a Th17 cell, which promotes granulocyte recruitment and inflammation

Target Assessment

TGFβ has been demonstrated to promote the progression of a variety of human cancers. Prior to targeting the TGFβ pathway clinically, serum levels of TGFβ1, 2, and 3 can be evaluated using either ELISA-based methods or via Singleplex/ Multiplex bead assays. While these approaches provide accurate quantification of the circulating ligand, without additional knowledge of downstream TGFβ signals in the tumor, this information is insufficient to determine either the safety or efficacy of therapies targeting the TGFβ pathway.

As TGFβ can both prevent and promote tumorigenesis, the degree to which TGFβ signals have been compromised in the epithelium is an important factor in predicting responsiveness to therapies targeting this pathway. To determine the status of TGFβ signaling in the primary tumor, samples can most readily be analyzed by immunohistochemistry. Tissue sections can be stained with anti-TGFβ1, 2, and 3 antibodies to qualitatively assess levels of the ligand in the tumor microenvironment. This can be combined with immunohistochemical analysis of downstream signals, such as pTGFBR1, the active form of TGFBR1. Staining for pSMAD2 and pSMAD3 may also be of key interest, though it should be mentioned that these markers are not exclusive to the TGFβ pathway, as they are also activated by other members of the TGFβ superfamily such as Activin.

Staining for SMAD4, the most commonly dysregulated target of TGFβ signals in many cancers, may also offer insight into the status of TGFβ signals *in situ*. As SMAD4 is critical for TGFβ-induced cell cycle arrest, a patient harboring loss of SMAD4 is likely insensitive to the beneficial aspects of TGFβ signaling in the tumor epithelia. Furthermore, to approximate whether TGFβ signaling is contributing to cell cycle arrest in these tissues, the described stains can be combined with those for further downstream targets of the TGFβ/SMAD axis such as p15, p16, p21, etc.

The added complexity to evaluating TGFβ signaling in patients stems from the fact that, in the dynamic tumor microenvironment, TGFβ can simultaneously function as a tumor suppressor and a tumor promoter. Therefore, as mentioned, to more accurately predict responsiveness to TGFβ-targeted therapies, more information is needed about (1) whether the tumor- suppressive effects of TGFβ are intact and (2) if TGFβ is actively prompting cancer progression through one of several possible mechanisms.

Preclinical Summary

As the effects of TGFβ signaling in tumorigenesis span so many different cell types, the unique and heterogeneous tumor microenvironments of *in vivo* models have offered insight into the potential therapeutic relevance of TGFβ signaling in many human cancers, but particularly to breast cancer.

Among the first of these experiments used athymic mice inoculated with a breast cancer cell line. These mice were then given a neutralizing antibody to TGFβ1, 2, and 3. While this antibody stimulated the proliferation of the isolated cancer cell line in culture, the same antibody inhibited the growth of tumor derived from the same cell line in mice, stimulating splenic NK cell activity (Arteaga et al. 1993). Similar results were found using a soluble, type 3 TGFβ receptor (TGFBR3), a naturally occurring glycoprotein lacking a cytoplasmic kinase domain, where nude mice inoculated with a breast cancer cell line were protected against lung metastases when treated with soluble TGFBR3 (Bandyopadhyay et al. 1999).

The effects of TGFβ inhibition were further examined using the MMTV-Polyomavirus middle T antigen (MMTV-PyV mT) transgenic model of breast tumors. Consistent with previous findings, administration of a soluble Fc: TGFBR2 fusion protein increased apoptosis in primary tumor cells, as well as reduced cell motility and invasion (Muraoka et al. 2002). When MMTV-PyV mT mice were subject to ionizing radiation to accelerate the metastatic phenotype, this response was ablated in mice given a neutralizing pan-TGFβ antibody. Similarly, ionizing radiation failed to induce lung metastases in mice with conditional loss of TGFBR2 (Biswas et al. 2007).

Furthermore, it has been recently demonstrated that pharmacological inhibition of TGFβ signaling prevents chemotherapy-induced IL-8-dependent expansion of breast cancer stem cells in mouse xenografts, increasing paclitaxel responsiveness *in vivo* and preventing tumor recurrence (Bhola et al. 2013).

While these and many other experiments clearly demonstrate the potential therapeutic benefit of TGFβ inhibition, several other *in vivo* studies seem to suggest that TGFβ functions as a classical tumor suppressor, and interruption of its downstream signals accelerates tumor formation. For instance, MMTV-PyV mT mice expressing either a dominant negative mutant or genetic deletion of TGFBR2 have accelerated tumor formation, contrasted by those expressing hyperactive TGFβ signaling through either constitutively active TGFβ1 or TGFBR1 (Forrester et al. 2005; Gorska et al. 2003; Muraoka-Cook et al. 2004, 2006).

In the pancreas, *in vivo* work is beginning to uncover the systemic contributions of TGFβ to tumor development, and its relevance as a therapeutic target. While the conditional deletion in the pancreas of the TGFβ targets TGFBR2 (Ijichi et al. 2006) and SMAD4 (Bardeesy et al. 2006; Izeradjene et al. 2007; Kojima et al. 2007) accelerates mtKRAS-induced disease, our group has previously shown that mtKRAS animals with global reduction of TGFBR1 were protected against the neoplastic phenotype, presenting with reduced lesion penetrance (Adrian et al. 2009; Zhang et al. 2006). Combined, these data appear to suggest that, while TGFβ serves as a tumor suppressor with respect to the pancreatic epithelium, the systemic contributions of TGFβ in the tumor microenvironment may override these effects. Should patients harbor loss of the beneficial aspects of TGFβ signals, yet retain its pathological effects in the tumor microenvironment, these patients may be optimal candidates for therapies targeting the TGFβ pathway.

Clinical Summary

Several therapeutic strategies have been utilized to target the TGFβ pathway clinically. Neutralizing monoclonal antibodies such as GC1008 have been used to sequester TGFβ1, 2, and 3 ligands, preventing their interaction with TGFBR2. GC1008 is currently being tested in phase I/II clinical trials for both metastatic breast cancer as well as malignant pleural mesothelioma (http://clinicaltrials.gov). Soluble forms of TGFBR2 and TGFBR3, that also serve to neutralize TGFβ ligands through ligand sequestration, are also in use (Gordon et al. 2008; Rowland-Goldsmith et al. 2001).

Other therapeutic strategies aim to inhibit the kinase activity of the TGFBRs. Several compounds have been used to this effect, such as LY2157299 (Galunisertib), a small molecule inhibitor that blocks the ATP-binding site of TGFBR1, thereby preventing its activation. LY2157299 is currently in clinical trials for many cancers including hepatocellular, glioma, and pancreatic cancer (http://clinicaltrials.gov). Small molecule inhibitors directed toward both TGFBR1 and TGFBR2 are also in use. For example, LY2109761 is a dual TGFBR1/TGFBR2 inhibitor that is undergoing preclinical testing (Melisi et al. 2008).

Antisense technology has also been used to decrease the levels of TGFβ in patients. Antisense oligonucleotides that target TGFβ1 (AP 11014) and TGFβ2 (AP 12009) effectively reduced secretion of TGFβ from cancer cells

(Schlingensiepen et al. 2004, 2006). Additionally, AP 12009 dramatically reduced levels of TGFβ2 from cancer cell lines and suppressed tumor growth *in vivo*. Early-stage clinical trials in patients with advanced melanoma, colorectal carcinoma, or pancreatic carcinoma demonstrated promising results (Oettle et al. 2009). A phase III clinical trial with AP12009 is currently underway in high-grade glioma patients (http://ClinicalTrials.gov).

Although results from current trials suggest potential promise for targeting the TGFβ pathway in humans, given the dual nature of TGFβ signaling, the challenge lies in targeting only the tumor-promoting aspect of TGFβ and minimizing the effect on the tumor-suppressive arms of TGFβ signaling. Given the dichotomy of TGFβ signaling in cancer, specific and individualized criteria must be developed to target only its detrimental effects in the clinic. To assess the state of the tumor-suppressive effects of TGFβ in individual patents, the most logical approach is to examine functional tumor biomarkers via immunohistochemistry. However, clinically, TGFβ pathway-inactivating mutations are prevalent in many cancers, and these changes may not necessarily be reflected by gross protein expression.

In cancer, the most common disruption to the TGFβ pathway occurs through loss or mutation of SMAD4 in the cancer epithelia. This phenomenon is especially frequent in pancreatic cancer. Roughly 55% of human pancreatic ductal adenocarcinoma (PDAC) patients have loss of SMAD4, with nearly 30% having homozygous deletion and the remaining 25% having loss of heterozygosity (LOH) mutations (Hahn et al. 1996). In comparison, only 2% and 4–7% of PDAC patients have mutations in TGFBR1 and TGFBR2, respectively. Therefore, criteria such as the nuclear localization of SMAD4, degree of fibrosis, and local immune responses may provide further insight into the benefit of therapies targeting the TGFβ pathway (Jaffee et al. 2002).

Mutations in the TGFβ pathway are also common to colorectal cancer patients. While studies report between 10% and 35%, (De Bosscher et al. 2004; Koyama et al. 1999; Miyaki and Kuroki 2003; Takagi et al. 1996) mutations in the TGFBRs are also prevalent, particularly in cases harboring microsatellite instability (MSI). MSI occurs in approximately 15% of colorectal cancer patients and involves defective mismatch repair functions, culminating in the accumulation of mutations in repetitive sequences. Of the affected population, 12% are caused by somatic cell hypermethylation of the MLH1 gene, the remaining 3% being associated with Lynch Syndrome (Boland and Goel 2010). While the accumulation of repetitive sequences associated with MSI are typically intronic, if they are to occur in exons such as in TGFBR2, protein expression can be affected. Patients with MSI colon cancers may therefore have compromised TGFβ signaling. Interestingly, these patients have an increase in tumor infiltrating lymphocytes, which is associated with improved survival (Deschoolmeester et al. 2011).

Several other cancers also present with severely dysregulated TGFβ signaling, or harbor frequent mutations to the TGFβ pathway (Miyaki and Kuroki 2003; Yamada et al. 1995; Nagatake et al. 1996; Schutte et al. 1996; MacGrogan et al. 1997; Yokota et al. 1997; Kim et al. 1998; Anbazhagan et al. 1999; Takakura et al. 1999; Tokunaga

Table 1 TGFβ pathway inactivating mutations are common to a several cancers. Several tumor types display perturbation to the TGFβ pathway. While loss or mutation of SMAD4 is the most common, several additional cancers present with loss or mutation of TGFBR1 and/or TGFBR2 (Miyaki and Kuroki 2003; Yamada et al. 1995; Nagatake et al. 1996; Schutte et al. 1996; MacGrogan et al. 1997; Yokota et al. 1997; Kim et al. 1998; Anbazhagan et al. 1999; Takakura et al. 1999; Tokunaga et al. 1999; Yakicier et al. 1999; Maliekal et al. 2003; Levy and Hill 2006; Wang et al. 2007; Antony et al. 2010; He et al. 2011)

	Mutation rate		
Cancer type	SMAD4	TGFBR1	TGFBR2
Pancreatic cancer	55%	2%	4–7%
Colorectal cancer	10–35%	10–20%	30%
Bladder cancer	12%	30%	44%
Prostate cancer	10%	46%	27%
Ovarian cancer	5%	27%	22%
Glioblastoma	45%		
Gastric cancer	32%		
Cervical cancer	30%		
Acute myelogenous leukemia	17%		
Liver cancer	10%		
Lung cancer	7%		

et al. 1999; Yakicier et al. 1999; Maliekal et al. 2003; Levy and Hill 2006; Wang et al. 2007; Antony et al. 2010; He et al. 2011) (Table 1). For example, in many cancers, there is a common polymorphic variant of TGFBR1 that appears to alter downstream signaling, reduce TGFBR1 expression, and confer a higher risk for tumor incidence. The 9-exon TGFBR1 gene is 56 kb and maps to 9q22.3. While TGFBR1 normally has a 9-alanine repeat at the 3' end of exon 1, this variant (TGFBR1*6A) harbors an inframe 9 bp deletion, resulting in only a 6-alanine repeat. Several other polymorphic variants with alterations to this region have been identified, including TGFBR1*10A, TGFBR1*8A, and TGFBR1*5A though these are less understood (Moore-Smith and Pasche 2011).

Recent meta-analyses have suggested that the TGFBR1*6A variant confers increased susceptibility to breast, ovarian, and colorectal cancers. Additionally, it appears that somatic cells in the tumor microenvironment can acquire TGFBR1*6A, which was observed in both epithelial and stromal cells in head and neck as well as colorectal cancer patients. Pending further study, TGFBR1*6A and similar mutations may be key in predicting the efficacy of therapies targeting the TGFβ pathway (Moore-Smith and Pasche 2011).

Anticipated High-Impact Results

- Therapeutic strategies targeting the TGFβ pathway warrant further investigation.
- Blockade of the TGFβ pathway may be highly efficacious in some patients, but not in others.

- Additional selection criteria are required to predict optimal candidates for TGFβ-inhibition therapy.
- Patients with insensitivity to growth suppressive TGFβ signals, yet harboring pathological signaling in the tumor microenvironment may have the most favorable effects to therapies targeting the TGFβ pathway.

Cross-References

▶ CD4+ T Cells
▶ CD8 T Cells
▶ NK Cells
▶ Tregs
▶ TGF Beta Receptors

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Tregs

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Abstract

Regulatory T cells (Tregs) are a subtype of T cells with immune suppressive function and play a key role in immune self-tolerance. Its immune inhibitory function has been implicated as the important mechanism of immune evasion and immune tolerance of human cancers. Tregs suppress antitumor immune responses through soluble factor-mediated as well as cell surface molecule-dependent inhibition of T cells and antigen-presenting cells. A significant increase in Treg numbers in the peripheral blood and in the tumor microenvironment has been associated with poor prognosis in various solid tumors. Better understanding of the roles of Tregs in tumor immunity has provided the rationale for the development of therapeutic modalities targeting immunosuppressive effects of Tregs. A number of therapeutic approaches have been proposed including the depletion of Treg by targeting Treg surface markers or with chemotherapeutic agents, the blockade of Treg suppressive function through inhibition of Treg receptors, and the inhibition of Treg induction and trafficking.

Keywords

Regulatory T cell (Treg) • $CD4^{+}CD25^{+}Foxp3^{+}$ • Immune suppressive function • Trafficking • CCL22/CCR4Glucocorticoid-induced tumor-necrosis-factor-receptor-related protein (GITR) • OX40 (CD134) • Daclizumab • LMB-2 • Denileukin diftitox

Regulatory T Cell (Treg)

Biology of the Target

Although the concept of suppressor T cells which inhibit immune effector function was first introduced in the 1970s, it was not until 1995 that a subpopulation of $CD4^{+}$T cells that constitutively express the interleukin-2 receptor α-chain (CD25) ($CD4^{+}CD25^{+}$) and have capacity of preventing autoimmunity were identified by Sakaguchi in a murine model. These suppressive $CD4^{+}CD25^{+}$ T cells were labeled as regulatory T cells or "Tregs." More recently, $CD4^{+}CD25^{+}$Tregs also have been shown to express the forkhead/winged helix transcription factor Foxp3 ($CD4^{+}CD25^{+}Foxp3^{+}$), a molecule that was originally felt to be an essential factor to establish a functional Treg lineage.

Subsequent studies have revealed that Tregs are a heterogeneous population with different phenotypes and have been further classified into subtypes based on their mechanism of activation and function. Naturally occurring Tregs (nTregs) are generated in the thymus through major histocompatibility complex (MHC) class II-dependent T-cell receptor (TCR) interaction and play a key role in maintaining self-tolerance. Induced Tregs (iTregs) are generated in the peripheral lymphoid tissue by conversion of naïve $CD4^{+}Foxp3^{-}$ T cells to $Foxp3^{+}$ Tregs in response to antigenic stimulation under tolerogenic conditions and contribute to the

development of an antigen-specific immunosuppressive response. iTregs can also directly inhibit the function of Teff through secretion of cytokines such as transforming growth factor-beta (TGF-β) and IL-10.

In addition, subsets of Foxp3$^-$ T cells have been found to play an important role in maintaining peripheral tolerance (Foxp3$^-$ Tregs). These cells are categorized based upon their cytokine induction profile. T-helper 3 cells (Th3) are triggered in an antigen-specific fashion but suppress in an antigen-non-specific fashion (bystander suppression) primarily through TGF-β secretion. Type 1 regulatory T cells (Tr1) arise from CD4$^+$T cells in the periphery in response to self-antigen stimulation in the presence of IL-10 and exert immunosuppressive function through the secretion of IL-10 and TGF-β.

In addition to their primary function of preventing autoimmunity by inducing and maintaining peripheral tolerance to self-antigens, Tregs have also shown to play important roles in limiting antitumor immune responses. Multiple mechanisms have been proposed for Treg-mediated suppression of antitumor responses, including soluble factor-mediated as well as cell surface molecule-dependent inhibition of T cells and antigen-presenting cells. A significant increase in Treg numbers in the peripheral blood and in the tumor microenvironment has been observed in many types of cancers and has been associated with poor prognosis. The increase in Tregs in the tumor microenvironment is mediated by various mechanisms including Treg migration into tumors (Treg trafficking) and conversion of CD4$^+$CD25$^-$T cells into Tregs. IL-10 and TGF-β have been shown to induce the differentiation of peripheral CD4$^+$CD25$^-$ precursors into functional CD4$^+$CD25$^+$iTregs through the induction of Foxp3 transcription factor. Treg trafficking is induced by chemokine gradient in the tumor microenvironment, and the expression of distinct chemokines and cognate chemokine receptors on Treg determines specific sites of Treg migration. For example, CCL22 produced by tumor cells and tumor-associated macrophages mediates the specific recruitment of CCR4$^+$Tregs into ovarian cancers, breast cancers, and Hodgkin's lymphomas, whereas CCL5 production by pancreatic cancer promotes CCR5$^+$Tregs trafficking and CXCR4/CXCL12 signals are crucial for activated CD4$^+$ Treg bone marrow trafficking.

Use of the Target in Oncology Practice

Currently, Tregs do not serve as a target in oncology practice outside the research setting.

Target Assessment

No current cell surface markers used for the identification of Tregs are exclusively restricted to Tregs to enable them to be reliably differentiated from the other T-cell subsets. The discovery of Foxp3, an essential transcription factor to establish a functional CD4$^+$CD25$^+$Treg lineage, provides the most specific and reliable marker

at present for the identification of Tregs. However, unlike in rodents, Foxp3 is also expressed transiently on activated T cells that do not exhibit inhibitory function. Although several other cell surface molecules that are important in development of Treg function have been reported as Treg-specific markers such as glucocorticoid-induced *TNF* receptor (GITR), OX40 (CD134), and CTLA-4, they are also expressed on activated T cells derived from naïve $CD4^{+}CD25^{-}$T cells. Thus, there is a need for more specific cell surface markers that can more effectively differentiate tumor-specific Tregs from activated Teffs.

Role of the Target in Cancer

Rank: “unknown” to 10
Unknown-1-2-3-4-5-6-7-8-9-10: 7

- A significant increase in Treg numbers in the peripheral blood and tumor has been observed in several types of human cancers.
- The correlation between the number of tumor-infiltrated $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs and prognosis is conflicting in different studies in different tumors (Curiel et al. 2004; Wolf et al. 2005; Hiraoka et al. 2006; Bates et al. 2006; Tzankov et al. 2008).
- However a reduced ratio of $CD8^{+}$ T cells to $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs, as well as absolute Treg numbers in tumors, correlates with poor prognosis in patients with breast cancer (Bates et al. 2006), gastric cancer (Sasada et al. 2003), ovarian cancer (Curiel et al. 2004), and colorectal cancer (CRC) (Suzuki et al. 2010).
- The accumulation of Tregs at tumor sites has been correlated with biomarkers of accelerated angiogenesis such as VEGF overexpression and increased microvessel density (Giatromanolaki et al. 2008; Gupta et al. 2007).
- Treg infiltration in tumors is correlated with a lack of responsiveness to cancer vaccine therapy.
- In the imiquimod and therapeutic human papillomavirus (HPV) vaccine trial, an increased density of Tregs in patients with vulvar intraepithelial neoplasia (VIN) was associated with poor immune response (Daayana et al. 2010).
- In a phase II TroVax (MVA-5T4) vaccine trial in patients with CRC with liver metastasis, elevated levels of Treg as a proportion of $CD4^{+}$ infiltrate in the tumor were significantly associated with poorer survival (Elkord et al. 2009).

High-Level Overview

Diagnostic, Prognostic, and Predictive

As mentioned above, several studies have reported the correlation between the Tregs in the peripheral blood or tumor in various types of cancers; however, at present Tregs are not routinely used or recommended for diagnostic, prognostic, or predictive purposes in patients with cancer.

Therapeutics

Better understanding of the roles of Tregs in tumor immunity has provided the rationale for the development of therapeutic modalities targeting immunosuppressive effects of Tregs.

A number of therapeutic approaches have been proposed including the depletion of Treg by targeting Treg surface markers or with chemotherapeutic agents, the blockade of Treg suppressive function through inhibition of Treg receptors, and the inhibition of Treg cell induction and trafficking.

Treg Depletion

The ideal therapeutics should selectively target tumor antigen-specific and tumor-induced Tregs without affecting the polyclonal Treg population necessary for peripheral tolerance. Unfortunately, no such highly specific cell surface markers for Tregs have been established, and consequently, targeting current markers has inherent risk of pathologic autoimmune reaction and disruption of normal immune tolerance.

As previously discussed, Foxp3, although currently the most selective marker for Tregs, is not exclusively expressed on Tregs in human. Furthermore, since Foxp3 is a nuclear product that is not expressed on the cell surface, depletion of $Foxp3^+$ Tregs requires an approach that can target intracellular proteins. Thus, CD25 has been the principal target for Treg-depleting strategies. The adoptive transfer of $CD25^+$ Treg-depleted autologous T cells following conditioning chemotherapy using anti-CD25 magnetic beads (CliniMACS® CD25 Reagent System) caused reduction in circulating Tregs, although only transiently, in patients with renal cell carcinoma (RCC) and melanoma providing proof of concept for Treg depletion. Current Treg-depletion strategies mostly focus the use of monoclonal antibodies (mAbs) or ligand-directed toxins targeted to CD25 which will be further discussed below.

Some lymphodepleting chemotherapies have also been shown to preferentially deplete Tregs and enable homeostatic immune reconstitution with a more effector population. Low-dose cyclophosphamide has been shown to decrease the number of $CD4^+CD25^+$ Tregs and reduce their suppressive activity. Other chemotherapeutic agents that have shown Treg inhibition include paclitaxel, imatinib, and dasatinib.

Blockade of Treg Suppressive Function

Inhibition of immunosuppressive function of Tregs can be achieved either by enhancement of co-stimulatory pathways or blockade of inhibitory mechanisms (inhibition of immune checkpoints).

Using agonistic antibodies to bind to the co-stimulatory tumor necrosis factor (TNF) receptor family members including GITR, OX40, and 4-1BB (CD137) as therapeutic modalities to override Treg immune inhibitory effects is an area of active research. These surface receptor molecules are induced when T cells are activated and are involved in inhibition of the suppressive activity of Tregs and prolongation of Teff survival.

Toll-like receptors (TLRs) are expressed ubiquitously on a variety of mammalian cells, including Tregs, and affect specific immunity such as dendritic cell (DC) maturation. Agonist TLR2 signaling was shown to reduce Treg suppressive function. The targeting of TLR for inhibition of Treg suppressive function is reviewed in a separate chapter.

Recent development of immunotherapy involving immune checkpoints such as CTLA-4 and programmed death-1 (PD-1) has led to the approval of ipilimumab by US Food and Drug Administration, and multiple ongoing clinical trials are underway in various cancer types.

CTLA-4 is a key negative regulator of CD28-dependent T-cell activation. Two fully humanized mAbs, ipilimumab and tremelimumab, have shown clinical activity in multiple clinical trials. The details of this target and its clinical use are discussed in a separate chapter. PD-1, another T-cell inhibitory receptor, is expressed on the surface of T cells (both Teffs and Tregs) upon T-cell receptor ligation. When PD-1 binds to PD-L1 on the surface of tumor cells, the immune cell is inactivated. How this relates to Treg function remains to be sorted out. Nonetheless, recently published data of anti-PD-1 antibody and anti-PD-L1 antibody phase I trials have established that blocking the interaction between PD-1 and PD-L1 can lead to potent antitumor responses. These promising targets are also reviewed in detail in a separate chapter.

Inhibition of Trafficking and Induction of Treg

Exploiting the chemokine–chemokine receptors involved in trafficking of Tregs into the tumor is another novel strategy for inhibiting Tregs. CCL22/CCR4 axis is the most studied target. Expression of CCL22 has been correlated with higher frequencies of Foxp3^{+} Tregs in various tumor types. The blockade of CCL22 using anti-CCL22mAb significantly decreased Treg migration into tumors in an immune-deficient murine xenograft model.

Inhibiting the differentiation and expansion of tumor-associated Tregs can be potentially achieved by blocking the activity of the soluble factors involved in conversion of CD25^{-} to CD25^{+} Tregs such as IL-10 and TGF-β or by inhibition of the indoleamine-2,3-dioxygenase enzyme pathway.

Preclinical Summary

Treg Depletion

The systemic removal of Tregs enhances natural as well as vaccine-induced antitumor T-cell responses.

Daclizumab

Daclizumab (Zenapax®· F. Hoffmann-La Roche, Basel, Switzerland), a humanized IgG1-κ MAb specific to CD25, causes a marked depletion of CD25^{+}CD4^{+}Foxp3^{+} T

cells from the peripheral blood (Rech and Vonderheide 2009). It is currently used in various clinical settings including solid organ transplantation, autoimmune disorders, $CD25^+$ T-cell malignancies, and acute graft-versus-host disease (GVHD). Preliminary results of daclizumab in metastatic breast cancer demonstrated a sustained elimination of $CD25^+Foxp3^+$ T cells in the peripheral blood and effective generation of cytotoxic T cells following vaccination (Rech and Vonderheide 2009).

LMB-2

Another strategy of targeting $CD25^+$ Tregs is the use of recombinant immunotoxin. LMB-2 (anti-Tac(Fv)-PE38) is the first immunotoxin fusion protein, consisting of a single-chain Fv fragment of anti-CD25 mAb fused to a truncated form of the bacterial *Pseudomonas* exotoxin A. LMB-2 has shown promising clinical antitumor responses in patients with $CD25^+$ hematological malignancies (Kreitman et al. 1999, 2000).

Denileukin Diftitox

Denileukin diftitox (DAB389IL-2, Ontak®), a fusion protein of human IL-2 and diphtheria toxin, has been reported to reduce the number of Tregs in the peripheral blood of patients with ovarian cancer, RCC, and melanoma (Dannull et al. 2005; Mahnke et al. 2007; Rasku et al. 2008) and improve vaccine-mediated T-cell immunity in murine models (Litzinger et al. 2007). Clinical trials of Ontak combined with either vaccine or IL-2 have not shown significant benefit, although Ontak has been reported to produce tumor responses in some patients with advanced melanoma when used as a single agent (Rasku et al. 2008) [hk1].

Foxp3 Vaccine

The vaccine strategy against Foxp3 has been tested for the stimulation of Foxp3-specific cytotoxic T lymphocyte (CTL) response leading to the elimination of Tregs. Vaccination of Foxp3 mRNA-transfected DCs elicited a robust Foxp3-specific CTL response that reduced Treg numbers and enhanced antitumor immunity in a murine model (Nair et al. 2007). In addition, Foxp3 vaccination preferentially depleted $Foxp3^+$ Treg in the tumor but not in the periphery, unlike anti-CD25 mAb, potentially reducing the risk of unwanted autoimmune adverse events (Nair et al. 2007).

Blockade of Treg Suppressive Function

Agonist Anti-GITR Antibody

Targeting of GITR, a member of the TNF receptor family, with the agonistic anti-GITR mAb, DTA-1, has shown inhibitory activity against Treg function in vivo and

co-stimulatory activity of effector T cells (Teffs) and resulted in improved antitumor efficacy in murine models of CRC and melanoma (Cohen et al. 2006; Ramirez-Montagut et al. 2006). In addition, a combination of an anti-CTLA-4 mAb and anti-GITR mAb exhibited stronger antitumor effects in vitro compared with either antibody alone (Mitsui et al. 2010).

Anti-OX40 Antibody

Triggering OX40, another member of the TNF receptor family, using agonistic antibodies, has also shown to revert the immunosuppression exerted by Tregs in a model of GVHD. Agonistic anti-OX40 mAb inhibited suppressive activity of Treg and induced tumor regression in preclinical mouse models of breast cancer, CRC, and lymphoma (Kaneko et al. 2005; Piconese et al. 2008).

Clinical Summary

To date, only a few of therapeutic agents targeting Treg have been tested mostly in early phase clinical trials and a number of studies are underway.

Daclizumab

In an ongoing trial (clinicaltrials.gov, identifier: NCT00573495), a single intravenous infusion of daclizumab in patients with metastatic breast cancer resulted in a marked and prolonged elimination of $CD25^{+}Foxp3^{+}$Tregs in the peripheral blood (Rech and Vonderheide 2009). The induction of $CD8^{+}$ T-cell immunity after hTERT/survivin multipeptide vaccine has been observed despite the depletion of CD25-expressing cells when the vaccine is administered during the daclizumab-induced Treg nadir (Rech and Vonderheide 2009). Daclizumab is currently being tested in various cancer types: glioblastoma (clinicaltrials.gov, identifier: NCT00626483, NCT00626015), ovarian cancer (NCT01132014), breast cancer (NCT00573495), hematologic cancers (NCT00006350, NCT00019305, NCT00001941, NCT00001249, NCT00002681), and melanoma (NCT00847106, NCT01307618).

LMB-2

In patients with metastatic melanoma, LMB-2 followed by vaccination with melanoma antigen peptides showed a significant but transient decrease of $Foxp3^{+}CD4^{+}CD25^{+}$ Tregs; the quantity of Tregs returned to pretreatment levels within days. No immune response enhancement or objective clinical response was observed (Powell et al. 2007).

Denileukin Diftitox

Several small trials showed the potential for combining Treg cell depletion using denileukin diftitox with anticancer vaccines to enhance tumor antigen-specific immune responses.

Administration of denileukin diftitox prior to DC vaccination efficiently depleted circulating Tregs, abrogated Treg-mediated immunosuppression, and enhanced vaccine-mediated antitumor T-cell responses in RCC patients (Dannull et al. 2005). In patients with carcinoembryonic antigen (CEA)-expressing malignancies, multiple doses of denileukin diftitox showed decreased Tregs in the peripheral blood, enhancing the efficacy of the fowlpox vector rF-CEA-TRICOM vaccine (Morse et al. 2008). However, Treg reduction with denileukin diftitox in melanoma patients showed mixed results. In one study, denileukin diftitox resulted in a depletion of Treg, induction of antigen-specific $CD8^+$ T cells, and objective clinical responses in patients with metastatic melanoma (Rasku et al. 2008), whereas it failed to demonstrate significant reduction of Treg levels or antitumor efficacy in patients with advanced melanoma or renal cell carcinoma in another study (Attia et al. 2005).

Agonist Anti-GITR Antibody

Two phase I trials targeting GITR in melanoma patients have recently opened: a phase I dose escalation trial of the humanized anti-human GITR mAb and a phase I trial testing DC vaccines alone or in combination with DCs expressing GITR-L, anti-CTLA-4, or both (Schaer et al. 2012).

Anti-OX40 Antibody

A fully human IgG4 mAb against OX40, BMS 663513, showed clinical activity in locally advanced or metastatic solid tumors in multidose phase I–II study (Ascierto et al. 2010). However a subsequent phase II trial in metastatic melanoma patients was terminated due to high incidence of grade 4 hepatitis (Ascierto et al. 2010). Also clinical trials of 4-1BB agonist antibodies were put on hold after the occurrence of fatal hepatic adverse events, raising a safety concern of this approach.

Anticipated High-Impact Results

Although current clinical trials are mostly in early phases, some of the anticipated clinical trial results include:

- A couple of early stage trials are testing the effects of daclizumab in combination with therapeutic cancer vaccines in melanoma and breast cancers: daclizumab

with hTERT/survivin multipeptide vaccine in patients with metastatic breast cancer (clinicaltrials.gov, identifier: NCT00573495) and a randomized phase II study of multipeptide vaccination combined with daclizumab in patients with metastatic melanoma (clinicaltrials.gov, identifier: NCT01307618).

- The efficacy of denileukin diftitox is being evaluated in metastatic pancreatic cancer and melanoma patients: a phase II open-label, multicenter study of denileukin diftitox in patients with stage IIIC and IV melanoma (clinicaltrials.gov, identifier: NCT01127451), randomized phase II study of multipeptide vaccination with or without regulatory T-cell depletion using denileukin diftitox in patients with metastatic melanoma (clinicaltrials.gov, identifier: NCT00515528), and a pilot study, evaluating the efficacy of regulatory T-cell suppression by denileukin diftitox in metastatic pancreatic cancer (clinicaltrials.gov, identifier: NCT00726037).
- Several phase I/II studies are ongoing to evaluate mAb to OX40 as a monotherapy in advanced cancer (clinicaltrials.gov, identifier: NCT01644968) as well as in combination with other immunotherapies such as ipilimumab (clinicaltrials.gov, identifier: NCT01689870) or cyclophosphamide (clinicaltrials.gov, identifier: NCT01303705).

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Tyrosinase: Overview

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Abstract

Tyrosinase is the key enzyme of the melanin pigment production pathway in both normal melanocytes and malignant melanoma cells. The expression of tyrosinase in metastatic melanoma is more common and homogenous, as compared to other melanocyte/melanoma differentiation proteins. Therefore, several studies have indicated the clinical benefit of detecting tyrosinase in peripheral circulation and regional lymph nodes. Importantly, tyrosinase has been found to elicit cellular and humoral immune responses in patients with malignant melanoma. Accordingly, efforts have been made to develop immunotherapeutic strategies using tyrosinase peptides and/or protein. In summary, tyrosinase can provide an attractive target for the immunotherapy of malignant melanoma.

Keywords

Tyrosinase • Melanocyte • Melanoma • Immunotherapy

Target: Tyrosinase

Tyrosinase (EC 1.14.18.1) is an 80 kD melanosomal membrane-bound glycoenzyme comprising 529 amino acids. It is the principal enzyme involved in the biosynthesis of melanin pigments. Tyrosinase, encoded by the gene TYR (11q14-21, MIM 606933), is expressed in epidermal, follicular, and ocular melanocytes (Hearing 2011). Mutations of the tyrosinase gene have been primarily reported in multiple forms of albinism, an autosomal recessive disorder marked by reduced production of melanin or its absence in the skin, hair, and eyes (Tomita 1994). Additionally, tyrosinase plays a significant role in the eye developmental process (Ray et al. 2007). As a melanocyte differentiation antigen, tyrosinase is expressed almost in all primary and metastatic melanomas (Ivan and Prieto 2010; Robbins and Kawakami 1996).

Biology of the Target

Tyrosinase can be detected in peripheral blood and tissue of melanoma patients. Efforts have thus been made toward assessing the clinical relationship between tyrosinase expression and both the clinical stage of the disease and the recurrence rate or the overall survival of patients. Despite the controversial results using tyrosinase as a prognostic tumor marker, the presence of tyrosinase transcripts in patient samples correlated with clinical outcome only when sequential measurements are performed throughout the follow-up (Quaglino et al. 2004a).

Tyrosinase can elicit cellular and humoral immune responses in patients with malignant melanoma (Robbins and Kawakami 1996). Accordingly, a number of clinical trials have been conducted using tyrosinase-based immunotherapies. Among these clinically tested strategies are the adoptive transfer of tyrosinase-reactive

tumor-infiltrating lymphocytes (TILs) and vaccination with tyrosinase peptides, tyrosinase-pulsed dendritic cells (DCs), DCs transduced with tyrosinase-encoding viral vectors, or tyrosinase-encoding viral vectors (Slingluff et al. 2003, 2004; Lindsey et al. 2006; Di Nicola et al. 2004). Though these trials resulted in enhanced anti-tyrosinase immunity associated with clinical benefit only in some melanoma patients.

Target Assessment

Quantification of tyrosinase mRNA using reverse transcription-PCR (RT-PCR) in peripheral blood or regional lymph nodes of patients with malignant melanoma has been proposed as a sensitive technique for the detection of circulating melanoma cells. Data reported about the clinical value of monitoring circulating tyrosinase transcripts as a prognostic tumor marker remain controversial (Quaglino et al. 2004a). The overall sensitivity of tyrosinase detection of melanoma cells varies greatly (Glaser et al. 1997). However, this discrepancy is most often attributed to differences in sample processing, RNA extraction, amplification design protocol, and the analytical method.

Another approach is the immunohistochemical detection of tyrosinase in melanocytic lesions. This method is sensitive; however, its sensitivity decreases with increasing clinical stage, probably due to its varied levels of expression in metastatic lesions (Ivan and Prieto 2010).

Role of the Target in Cancer

Rank: “unknown” to 10

Unknown-1-2-3-4-5-6-7-8-9-10: 8

Tyrosinase was recently assigned rank 20 of 75 representative antigens based on (a) therapeutic function, (b) immunogenicity, (c) role of the antigen in oncogenicity, (d) specificity, (e) expression level and percent of antigen-positive cells, (f) stem cell expression, (g) number of patients with antigen-positive cancers, (h) number of antigenic epitopes, and (i) cellular location of antigen expression (Kawakami et al. 1996).

High-Level Overview

Diagnostic, Prognostic, and Predictive

There has been a surge in interest for the detection of circulating melanoma cells by RT-PCR as a technique for early detection of tumor progression and metastatic disease. Tyrosinase is the key enzyme of the melanin pigment production pathway in both normal melanocytes and malignant melanoma cells. Since the expression of

tyrosinase is tissue specific, the presence of metastatic cells can be elucidated by detection of tyrosinase mRNA within peripheral blood or regional lymph nodes of patients. Multiple studies have also demonstrated the clinical utility of detecting tyrosinase in peripheral circulation and regional lymph nodes.

Smith et al. were the first to use RT-PCR for the detection of tyrosinase in peripheral blood of four out of seven patients with malignant melanoma (Smith et al. 1991). They also demonstrated that all eight control subjects were negative for tyrosinase. Since then, many groups have tested the usefulness of tyrosinase detected by RT-PCR as a tumor biomarker.

Several studies have demonstrated that the determination of RT-PCR positive tyrosinase in peripheral blood substantially correlates with the clinical stage of malignant melanoma and size of the primary tumor (Mellado et al. 1996). Other studies, however, showed low positivity rates in patients with known stage IV disease (Tsao et al. 2001).

Accumulating data from many clinical trials have shown that positive tyrosinase RT-PCR results are associated with shorter progression-free and overall survival (Mellado et al. 1996, 2002). The meta-analysis conducted by Quaglino et al. concluded that RT-PCR for tyrosinase can be an effective assay, at least in stage III disease-free patients, when measurements are performed every 2 or 3 months during the follow-up (Quaglino et al. 2004b). Moreover, it has been reported that the determination of tyrosinase in blood samples obtained before, during, and after therapy can be used to monitor patients' response to treatment (Mellado et al. 2002). Though these reports were challenged by others that did not confirm the prognostic value of tyrosinase RT-PCR detection (Glaser et al. 1997).

False-positive RT-PCR results, due to the presence of nevus cells, have limited the importance of the detection of tyrosinase-positive cells within the regional lymph nodes (Calogero et al. 2000). Nonetheless, a number of clinical trials indicated that positive rates correlate with increasing tumor load, thus conferring a worse prognosis (Blaheta et al. 1998).

Given the inconsistent results from different clinical trials, the clinical significance of detection of tyrosinase RT-PCR positive cells is still debated.

Therapeutics

In the last few years, efforts have been made to develop immunotherapeutic strategies using tyrosinase peptides and/or protein. Tyrosinase is an enzyme involved in the initial stages of melanin synthesis in melanocytes and melanoma cells, and its expression in metastatic melanomas is more common and homogenous, compared with other melanocyte/melanoma differentiation proteins such as MART-1, gp100, and TRP-1 (gp75) (Chen et al. 1995). More importantly, tyrosinase can induce antibody and T-cell responses in patients with melanoma (Robbins and Kawakami 1996). Multiple MHC class I-restricted (such as HLA-A2 and HLA-A24) (Kang et al. 1995; Nishimura et al. 1999) and class II-restricted (such as HLA-DR4) (Topalian et al. 1994) epitopes, recognized by $CD8^+$ and $CD4^+$ T cells, respectively,

have been identified. Recently, it was demonstrated that tyrosinase-derived complexes occur at high levels on the surface of melanoma cells compared with other melanoma-associated antigens. Of note, this did not correlate with mRNA expression levels (Michaeli et al. 2009). Altogether, it can be considered that tyrosinase may provide an attractive target for immunotherapy of malignant melanoma.

Preclinical Summary

A number of preclinical studies indicate the potential of tyrosinase as a target for anti-melanoma immunotherapy (Roszkowski et al. 2003, 2005). Adoptive transfer of tyrosinase-specific cytotoxic T lymphocytes (CTLs) (Mullins et al. 2001) or anti-tyrosinase TCR-engineered T cells (Frankel et al. 2010), as well as vaccination with tyrosinase-pulsed DCs or tyrosinase-encoding viral vectors (Mullins et al. 2001; Drexler et al. 1999) induced memory T-cell immunity, thus imparting protection against outgrowing tumors. Using the human tyrosinase epitope-reactive, CD8-independent, high-affinity TCR isolated from tumor-infiltrating lymphocytes of a metastatic melanoma patient, we have recently developed a novel TCR transgenic mouse (named h3T) (Mehrotra et al. 2012). Adoptive transfer of the transgenic T cells from these h3T mice exhibited superior control of subcutaneously established murine melanoma and melanoma lung metastasis. This TCR is also being used in a clinical trial, as mentioned below.

Clinical Summary

The use of tyrosinase-based immunotherapeutic approaches has been explored in several clinical trials conducted on patients with metastatic melanoma. Adoptive transfer of one of the autologous tyrosinase-reactive TILs has been reported to induce complete regression of tumor (Kawakami et al. 1996). Likewise, vaccination of melanoma patients with tyrosinase vaccines resulted in detectable CTL responses, associated with sporadic tumor regressions. In a phase I trial, immunization with autologous DCs transduced with vaccinia virus encoding tyrosinase gene was safe and produced a strong T-cell response; a partial antitumor response was observed in one out of six patients (Di Nicola et al. 2004). Another phase I study employing intranodal delivery of DNA plasmid encoding tyrosinase epitopes also noted tyrosinase-specific immune responses with 16 of 26 patients alive after 12 months of follow-up (Tagawa et al. 2003). Objective clinical regressions have also been reported in two phase II trials evaluating tyrosinase-including multipeptide vaccines (Slingluff et al. 2003, 2004). Contrastingly, in a recent phase II trial, prime/boost vaccination with vaccinia and fowl pox viruses encoding tyrosinase was ineffective alone in generating clinical effect. Moreover, this vaccination regimen, when combined with IL-2, did not induce clinical responses over those resulted from treatment with IL-2 alone (Lindsey et al. 2006). Overall, a conclusion from current literature is that the clinical efficacy of tyrosinase-based immunotherapies has not been

conclusively established as yet, suggesting that applying different approaches to antigen delivery is required to enhance tyrosinase-specific immune response. However, a human tyrosinase DNA vaccine has recently been approved for the treatment of canine melanoma.

Anticipated High-Impact Results

A search for clinical trial using only tyrosinase as target gave the following results:

- Transfer of Genetically Engineered Lymphocytes in Melanoma Patients: A Phase 1 Dose Escalation Study; PI: Michael Nishimura, PhD Loyola University Medical Center. ClinicalTrials.gov Identifier: NCT01586403. This is an ongoing study testing the safety of tyrosinase-reactive, TCR-engineered T cells together with low-dose IL-2.
- Vaccination of AJCC Stage IIB, IIC, III, and IV Melanoma Patients With Human and Mouse Tyrosinase DNA Vaccines: A Phase I Trial to Assess Safety and Immune Response; PI: Jedd Wolchok, MD Memorial Sloan-Kettering Cancer Center. ClinicalTrials.gov Identifier: NCT00698100. This is a recently completed study that tested the safety of injecting human and mouse tyrosinase DNA. Antibody and $CD8^+$ T-cell responses, in addition to antitumor response, were evaluated as secondary outcomes of the trial.

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VEGF

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Susanna Ulahannan

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Abstract

VEGF is an approximately 45 kDa homodimeric glycoprotein in the VEGF family, which includes more than seven proteins. Five of the polypeptides are encoded by distinct genes in the human genome: VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D, and PGF (placenta growth factor) (Carmeliet, Oncology 69:4–10, 2005; Shibuya, Vascular permeability/vascular endothelial growth factor. In: Figg WD, Folkman J (eds) Angiogenesis. Springer, New York, pp 89–98, 2008). VEGF is considered to play a key role in regulating angiogenesis both in normal and malignant cells. VEGF-A exists in many different isoforms as a result of alternative exon splicing; the most frequent subtypes are VEGF121, VEGF165, VEGF189, and VEGF206. The shorter amino acid sequence isoform VEGF121 is soluble, in contrast to VEGF165, VEGF189, and VEGF206, which are heparin bound with varying affinity.

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VEGF121 and VEGF165, which also have the propensity to be unbound, are believed to have a central role in tumor angiogenesis (Kerbel and Ellis, Angiogenesis. In: DeVita, Hellman, Rosenberg (eds) Cancer. LWW, Philadelphia, pp 101–112, 2011).

Keywords
Bevacizumab • Neuropilin-1 • Pazopanib and axitinib • Sorafenib • Sunitinib • Vascular endothelial growth factor (VEGF) • Anti-VEGF agents • Biomarkers • Clinical studies • Endothelial functions • In cancer • Isoforms • Preclinical models • Receptors • Regulator in angiogenesis • Tyrosine kinase inhibitors (TKIs) • Vascular permeability factor (VPF) • VEGF-A gene

Target: Vascular Endothelial Growth Factor (VEGF)

VEGF is an approximately 45 kDa homodimeric glycoprotein in the VEGF family, which includes more than seven proteins. Five of the polypeptides are encoded by distinct genes in the human genome: VEGF-A (VEGF), VEGF-B, VEGF-C, VEGF-D, and PGF (placenta growth factor) (Carmeliet 2005; Shibuya 2008). VEGF is considered to play a key role in regulating angiogenesis both in normal and malignant cells. VEGF-A exists in many different isoforms as a result of alternative exon splicing; the most frequent subtypes are VEGF121, VEGF165, VEGF189, and VEGF206. The shorter amino acid sequence isoform VEGF121 is soluble, in contrast to VEGF165, VEGF189, and VEGF206, which are heparin bound with varying affinity. VEGF121 and VEGF165, which also have the propensity to be unbound, are believed to have a central role in tumor angiogenesis (Kerbel and Ellis 2011). Three VEGF receptors have been identified, VEGFR1 (Flt-1, Fms-like tyrosine kinase 1), VEGFR2 (KDR, kinase insert domain-containing receptor in humans/Flk1, fetal liver kinase 1 in mice), and VEGFR3 (Flt4). VEGFR3 has mainly been associated with lymphatic vessel growth and binds to the ligands VEGF-C and VEGF-D. VEGF-A binds to VEGFR1 and VEGFR2; both are expressed on vascular endothelial cells. The receptors have common characteristics and contain seven immunoglobulin-like domains (extra cellular ligand-binding domain), a tyrosine kinase domain, and the carboxy terminal. VEGFR2 bound to its ligand leads to receptor dimerization and triggers a cascade of signaling pathways that are believed to be essential in activating endothelial cells and promoting formation of new vessels, angiogenesis. Neuropilin-1 is a membrane protein and functions as a co-receptor, by increasing the affinity of VEGF165 for its receptor VEGFR2. The association between neuropilin-1 and VEGF165 is crucial for embryogenesis, if one of the proteins is missing; life is not sustained (Shibuya 2008, 2011).

Biology of the Target

VEGF is overexpressed in a majority of malignant tumors, and elevated VEGF has been shown repeatedly to be associated with poorer prognosis. VEGF stimulates a variety of endothelial functions inducing bone marrow mobilization of endothelial

cells, migration, proliferation, survival, and vascular permeability. VEGF has also been shown to increase synthesis of nitric oxide, and one of the main side effects seen with anti-VEGF agents is hypertension (Kerbel and Ellis 2011). Two major pathways activated by the VEGF-VEGFR complex are PLCγ (phospholipase C gamma)-PKC-MAPK cascade resulting in proliferation and P13K-Akt pathway endorsing survival (Kawamuara et al. 2008). In addition to endothelial cells, VEGF influences other cells including monocytes and neurons (Ferrara 1999, 2008). Emerging data illuminates the immunosuppressive role of VEGF, by inducing production of inhibitory cytokines (IL-10 and IL-13), modulating dendritic cells, and stimulating regulatory T cells resulting in termination of T- and B-cell-mediated immune response (Correale et al. 2011; Alfaro et al. 2009).

The VEGF-A gene on chromosome 6 consists of 8 exons and is essential for embryonic vasculogenesis; deletion of one allele is not compatible with life. In adult life, VEGF-A is important in angiogenesis associated with female menstrual cycling and reproduction. VEGF gene expression is stimulated by various signals, hypoxia being a main candidate; others include acidic microenvironment, inflammatory cytokines, growth factors, androgens, and estrogens. VEGF upregulation is also influenced by an imbalance between the activation of oncogenes and inactivation of tumor suppressor genes (Ferrara 2008; Kerbel and Ellis 2011).

Target Assessment

Many biomarkers have been evaluated but none validated in prospective randomized trials, at this time. Several biomarkers have shown promise. VEGF, basic FGF, intercellular adhesion molecule (ICAM), and E-selectin were measured and evaluated pre- and posttreatment in the E4599 trial (leading to approval in lung cancer). Low-baseline ICAM showed significant association with improved response to treatment with or without bevacizumab and also overall survival. Decreased VEGF levels correlated to tumor response but not overall survival. VEGF and VEGFR2 showed correlation with progression-free survival (PFS) in the AVADO trial (breast cancer). In contrast there are trials which have failed to find VEGF levels to be predictive. Neuropilin-1 has indicated predictive value in metastatic colorectal cancer and metastatic gastric cancer. VEGF-D has been implicated to be of importance predicting response, resistance, and disease progression. Clinically, the development of hypertension is evaluated as a surrogate marker of efficacy in patients treated with bevacizumab (Ulahannan and Brahmer 2011; Meadows and Hurvitz 2012).

Role of the Target in Cancer

Rank: “unknown” to 10.

Unknown to 1–10: 10.

High-Level Overview

Diagnostic, Prognostic, and Predictive

There are no validated serum biomarkers available on the market to predict response to anti-VEGF therapy (Meadows and Hurvitz 2012).

Therapeutics

Bevacizumab is a humanized monoclonal antibody targeting circulating VEGF-A, subsequently inhibiting the ligand from binding to VEGFR. It was the first anti-VEGF therapy approved by the FDA in 2004 for the treatment of metastatic colorectal cancer in combination with irinotecan and 5-FU, based on a phase III trial, showing an increase in overall survival (15.6 months vs. 20.3 months) with a hazard ratio (HR) of 0.65. Improvements were also seen in progression-free survival and response rate (Hurvitz et al. 2004). Bevacizumab in combination with cytotoxic chemotherapy has been approved for the treatment of a number of different solid tumors including colorectal cancer, NSCLC (non-small cell lung cancer), and renal cell cancer. Glioblastoma has FDA approval for bevacizumab as a single agent. In January 2013, bevacizumab in combination with chemotherapy (FOLFOX or FOLFIRI) was approved in metastatic colon cancer patients, who had progressed on a first-line bevacizumab-containing regimen. This was based on a randomized phase III study demonstrating statistical OS benefit (9.8 months vs. 11.2 months) with an HR of 0.81, in the arm continuing to receive bevacizumab beyond progression.

Targeting the VEGFR is another approach for anti-VEGF therapy. Small-molecule tyrosine kinase inhibitors (TKIs), compete with ATP, binding to the active site of the VEGFR and thereby inhibiting activation. Sunitinib is a TKI approved for the treatment of renal cell cancer and GIST (gastrointestinal stromal tumors). Sorafenib, another TKI, has shown clinical activity in renal cell and hepatocellular carcinoma and is FDA approved for these malignancies. Pazopanib and axitinib are TKIs, which are FDA approved for renal cell cancer. Another recently FDA-approved anti-VEGF agent is regorafenib for colorectal cancer. In contrast to bevacizumab, the mentioned TKIs are approved as single agents for their indications and have several targets. VEGF inhibition by a different mechanism is through VEGF trap, a recombinant fusion protein, which contains the VEGF-binding site and thus binds the VEGF ligand, preventing binding to VEGFR. Ziv-aflibercept is an example of a VEGF trap drug, approved in combination with irinotecan for colorectal cancer (Ulahannan and Brahmer 2011; Meadows and Hurvitz 2012; FDA website). See Table 1.

Table 1 List of FDA-approved anti-VEGF agents for cancer

Anti-VEGF agent	Class	Malignancy	Year of FDA approval
Bevacizumab	Monoclonal antibody	Colorectal cancer	2004
		NSCLC	2006
		Glioblastoma	2009
		Renal cell cancer	2009
Sorafenib	TKI	Renal cell cancer	2005
		HCC	2007
Sunitinib	TKI	Renal cell cancer	2006
		GIST	2006
Pazopanib	TKI	Renal cell cancer	2009
		Soft tissue sarcoma	2012
Axitinib	TKI	Renal cell cancer	2012
Regorafenib	TKI	Colorectal cancer	2012
Ziv-aflibercept	VEGF trap	Colorectal cancer	2012

TKI tyrosine kinase inhibitor, *NSCLC* non-small cell lung cancer, *HCC* hepatocellular carcinoma, *GIST* gastrointestinal stromal tumor

Preclinical Summary

Vascular permeability factor (VPF) was discovered in 1983 by Senger and Dvorak. A few years later, VEGF was sequenced by Ferrara, and, when compared, the two proteins turned out to be the same (Folkman 2008). VEGF is a key regulator in angiogenesis, which is a necessary process for cancer cell proliferation beyond microscopic size, further invasion, and metastasis. Tumor angiogenesis causes abnormal structure and function of the vessels; in fact they can be so altered, making them hard to identify as blood vessels. Mouse monoclonal antibodies were found to inhibit VEGF in several cancer cell lines (1993); this discovery consequently led to the development of the first anti-VEGF agent in clinic, bevacizumab (Aragon-Ching et al. 2008; McDonald 2008).

In preclinical models, it has been shown that if anti-VEGF therapy is terminated, the tumor regains neo-angiogenesis rapidly. In one tumor model, during therapy, vascular density was reduced by 60–75% but, after withdrawing the drug, returned to baseline after 7 days (McDonald 2008).

Preclinical models have found inhibition of tumor growth when combining anti-VEGF therapy with tumor vaccination but only when the anti-VEGFR2 therapy was given at 25% of the dose. The lower anti-VEGF dose was also associated with increased distribution of functional blood vessels compared to higher doses. Hence lower dose of anti-VEGF therapy in these studies were linked to normalized vasculature structure and decreased immunosuppression (Huang et al. 2012).

Clinical Summary

Angiogenesis in tumors stimulated by VEGF is associated with abnormal vessels that are poorly organized and hyperpermeable, with consequent abnormal microenvironment characterized by interstitial hypertension, hypoxia, and acidosis. Preclinical data indicate synergy when anti-VEGF agents are combined with chemotherapy. Anti-VEGF agents inhibit growth of new blood vessels, cause regression of existing vasculature, and transiently "normalize" leaky tumor vasculature, which may increase the delivery of oxygen necessary to optimize radiation therapy and transport chemotherapy to the tumor. Additional effects observed with anti-VEGF therapy are decreased amount of ascites in ovarian cancer and reduced brain tumor edema in glioblastoma. The presence of anti-VEGF agents in between cycles of cytotoxic chemotherapy may lead to sustained tumor suppression. These may be contributing factors why there seems to be a synergistic effect when VEGF inhibitors are used in conjunction with chemotherapy (Jain 2005; Folkman 2008; Kerbel and Ellis 2011). In a clinical phase II trial with an anti-VEGF agent (TKI) used to treat glioblastoma patients, advanced MRI technique indicated normalization of the vasculature upon starting treatment; when the drug was discontinued so was the effect on the vasculature. Clinically, while on the drug, there was less brain edema and decreased need of steroids (Jain et al. 2008).

Anti-VEGF agents have been fairly well tolerated compared to cytotoxic chemotherapy. Adverse effects have been postulated to be mainly class specific and secondary to its mechanism of action. The inhibition of VEGF results in decreased synthesis of nitrous oxide with subsequent increase of vascular tone, which leads to hypertension. Severe hypertension is rare (<0.1%), but hypertension requiring medical treatment seems to range from 10% to 20%. Additional less common adverse effects include arterial thromboembolic events, GI perforation, and wound healing complications (Meadows and Hurvitz 2012).

Anticipated High-Impact Results

- Validated predictive and prognostic biomarkers to individualize treatment, and select subgroups of patients who will have the greatest benefit and least toxicity from anti-VEGF treatment.
- Surrogate markers to evaluate response and resistance, and adjust drug dosage to a given anti-VEGF treatment.
- The optimal time in the course of malignancy to treat with anti-VEGF therapy
- Immunologic effects of VEGF treatment, finding the right dose and combinations, to optimize antitumor effect

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Viral-Like Proteins

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Abstract

Cancer immunotherapy is a rapidly evolving field and the search for appropriate targets to come up with robust immunotherapeutic strategies for the treatment of cancers is a continuous process. Viral-like proteins are known to have an important role in the oncogenesis and their potential as cancer therapeutic targets is yet to be completely explored. In this report we summarized different aspects of viral-like proteins such as their biology, their role in cancer and gave an insight into strategies that can be adopted to target these proteins for the treatment of cancer.

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Oncogenesis • Cancer • Immunotherapy

Target

Viral-like proteins are derivatives of endogenous retroviruses which are known to be integrated into human genome after cross-species infectious events millions of years ago. Cumulative evidence from several studies in the literature suggests that viral-like proteins participate in the malignant transformation or promote tumor growth implicating their role in the pathogenesis of cancer. Also, emerging data proves that viral-like proteins represent a source of tumor-specific antigens which makes them interesting targets for novel immunotherapeutic targets against cancer. Here, we review in detail about the role of viral-like proteins in cancer and discuss the potential strategies to target these proteins for future cancer immunotherapeutic strategies.

An Overview of Viral-Like Proteins

Based on the recent sequencing of the entire human genome, it was identified that almost half of the genome consists of transposable elements (TEs), namely, DNA transposons (2.8%, 0.3×10^6 copies) and the more abundant retroelements (42.2%, 2.7×10^6 copies) (Bannert and Kurth 2004; Deininger and Batzer 2002; van de Lagemaat et al. 2003). DNA transposons amplify without RNA intermediates, whereas retroelements require a reverse transcriptase to retrotranscribe RNA into DNA copies that will subsequently integrate into chromosomal DNA. The concept that TEs are regarded as selfish DNA or junk DNA is unclear because of the fact that TEs like all other genes, upon becoming part of our genome, are subject to natural selection and can be co-opted for the benefit of the host. Indeed, it may well turn out that TEs, in addition to other already measurable positive effects (some of which are described below), may play a major role in shaping our genome by increasing its plasticity and in the evolution of mammalian gene regulation networks (Bannert and Kurth 2006; Britten and Davidson 1971; Mikkelsen et al. 2007; Wang et al. 2007). Human endogenous retroviruses (HERVs) belong to the retroelements, which can be subdivided into those with regulatory long terminal repeats (LTRs, 8.3% of our DNA, 0.3×10^6 copies) and retroelements without LTRs (33.9%, 2.4×10^6 copies). Among the non-LTR members, short and long interspersed elements (SINEs and LINEs, respectively) are present in very high copy numbers. SINEs cannot code for proteins, whereas LINEs encode a reverse transcriptase (RT) that can be utilized by both SINEs and LINEs for retrotranspositions or for the formation of pseudogenes. It is unknown whether the LINE RT can also be used by additional retroelements like HERVs for retrotransposition. The LTR containing retroelements can be grouped into six superfamilies (Medstrand et al. 2002): Class I–III HERVs

and other superfamilies MER4, MST, and MLT. These HERVs possess limited nucleotide sequence homologies to C-, B-, or spumaretroviruses, respectively. The other superfamilies, MER4, MST, and MLT, represent ancient retrotransposons not known to be still functional in humans today. Reactivation of HERVs, especially the endogenous viruses related to a family called HERV type K (HERV-K) family, is known to be associated with different diseases including cancers such as leukemia, lymphoma, breast cancer, and melanoma (Armbruester et al. 2002; Buscher et al. 2005; Contreras-Galindo et al. 2008; Depil et al. 2002; Wang-Johanning et al. 2012). HERV-derived proteins are associated with different types of cancers. These proteins are viral-like proteins given the endogenous nature of retrovirus from which they are derived. The current knowledge on the role of viral-like proteins in cancers is rather limited, and as of yet, the data demonstrating the conclusive role of the viral-like proteins as causative agents of cancer has not been produced. But given the direct oncogenic effects associated with viral-like proteins, several of these proteins (e.g., Np9, Rec, syncytin, etc.) have been studied extensively. In addition to their direct role in cancer pathogenesis, HERVs also play an important role in the activation of proto-oncogenes such as Ras and c-Src resulting in the increase in the expression of proteins derived from these proto-oncogenes that are critical in the malignant cell transformation (Bera et al. 1998). All these endogenous retrovirus (ERV)-derived viral-like proteins and the proteins derived from the ERV-induced reactivation of proto-oncogenes will be discussed in the context of different types of cancers below.

Biology of Viral-Like Proteins

Np9 Protein

HERV-K (human endogenous retrovirus type K) type 1-encoded Np9 is a tumor-specific biomarker, but its oncogenic role remains elusive. Using reverse transcription (RT) PCR analysis of env-reading frames of HERV-K transcripts in breast cancer biopsies, germ-cell tumor samples, ovarian cancers, leukemic patients lymphocytes, and transformed cell lines, the Np9 gene was discovered (Armbruester et al. 2002). Np9 gene is derived from HERV-K type 1-specific splice donor site. So far the role of Np9 as a potent viral oncogene was well demonstrated in human leukemia. For example, it was shown that silencing of Np9 inhibits the growth of myeloid and lymphoblastic leukemic cells, whereas expression of Np9 significantly promotes the growth of leukemic cells in vitro and in vivo (Chen et al. 2013). The Np9 protein, which is a 9-kDa protein, is known to be primarily localized in the cell nucleus. It was observed that Np9 not only activates ERK, AKT, and Notch1 pathways but also upregulates β-catenin essential for survival of leukemic stem cells. In human leukemia, Np9 protein levels in blood cells in patients are substantially higher than that in normal donors (56% vs 4.5%) (Chen et al. 2013). In addition, Np9-positive samples highly express leukemia-specific pol-env polyprotein, env and transmembrane proteins, as well as viral particles. All the

abovementioned properties confirm that the viral oncogene Np9-derived protein is a critical molecular switch of multiple signaling pathways that regulate malignant cell transformation. The detailed mechanisms by which Np9 plays an important role in the transformation of normal cells into malignant cells are described below in the next section.

Rec Protein

HERV-K is a multi-copy family of endogenous retroviruses, with about 30 full-length proviral genomes per haploid genome and a high number of remnants of proviruses. These proviruses are type 1 and type 2 with a significant number of them being type 2 proviruses. Type 2 proviruses produce Rec mRNA because of the presence of proper splice signals and coding sequences within the 5′ region of their env gene (Mayer et al. 2004). The Rec protein is a 14-kDa protein that shares 87 amino acids with the env protein and an extra 18 amino acids upstream from the 3′ LTR in an open reading frame different from env protein. The Rec protein is a functional counterpart to an HIV_{REV} protein which plays an important role in exporting unspliced HIV transcript from the nucleus (Mayer et al. 2004). Rec protein interacts with the Crm1 nuclear export factor and binds to a specific RNA structure within the HERV-K RNA (Denne et al. 2007). Rec protein also interacts with promyelocytic leukemia zinc-finger (PLZF) protein. PLZF protein was first identified in patients with acute promyelocytic leukemia as a part of fusion protein containing the retinoic acid receptor alpha (RARα) as the second partner. Acute promyelocytic leukemia cells expressing the PLZF-RARα fusion protein are nonresponsive to all-trans-retinoic acid and come with a poor prognosis (Suliman et al. 2012). Functionally, PLZF is a 673-amino-acid transcriptional repressor with nine C-terminal Kruppel-like C2H2 zinc fingers and an N-terminal POZ (poxvirus and zinc-finger domain). PLZF acts primarily as a guardian of stem cell pluripotency in the hematopoietic system and the germ line. The interaction of Rec protein with PLZF protein is critical for the expression of cellular oncogenes and regulates the cell proliferation and survival (Denne et al. 2007). The tumorigenic functions of Rec protein will be discussed in the next section of this review.

Syncytin Protein

The HERV-derived syncytin has an important role in the evolution of the human placenta due to its niche function in human placentogenesis, albeit in a highly specific manner, as the placentas in different mammalian species exhibit structural discrepancies (Sorek et al. 2002). Due to its interaction with a specific receptor, known to function as a retrovirus receptor and as an amino acid transporter, and due to the stimulation of cell-cell fusion processes, the protein was designated as syncytin. The receptor for syncytin is a sodium-dependent neutral amino acid transporter, which transports alanine, serine, and cysteine, known by the acronym

ASCT2. Pronounced syncytin expression is followed by further cell differentiation and generation of the syncytium, the formation of gap junctions, and an increase in β-hCG secretion (Frendo et al. 2003). The effects of syncytin can be blocked in vitro by antibodies directed against syncytin or by the use of syncytin antisense strategies (Frendo et al. 2003; McDonald 1993). In addition to the in vitro studies, it was demonstrated recently that the syncytin locus is strongly preserved in a large cohort of individuals, including the long terminal repeat (LTR) elements involved in regulation of gene transcription (Larsson et al. 2007). Apart from these findings, syncytin orthologous loci have been identified in the genomes of great apes (Larsson et al. 2007), and beyond that, expression of syncytin has been described in the rhesus monkey endometrium where it may play a role in decidualization or receptivity (Norberg et al. 1998). The expression of syncytin receptor, ASCT2, has been demonstrated predominantly at the basal membrane of the human syncytiotrophoblast (Frendo et al. 2003; Kidwell and Lisch 1997; Mortensen et al. 2004), and the basal membrane of the syncytiotrophoblast may therefore be functioning as the interaction site between syncytin and its receptor. The observation that, during cell syncytialization, syncytin and ASCT2 correlate reversely may be partially explained by the phenomenon that retroviruses may cause a downregulation of their receptors (Stoye and Coffin 2000). This can be seen after the infection of cells by wild-type viruses of the type-D interference group which impairs neutral amino acid transport (Stoye and Coffin 2000). Apart from its important role in placentogenesis and in amino acid transport, syncytin is known to have an important role in cancer pathogenesis by allowing the fusion of cancer cells with normal cells such as endothelial cells.

Ras Proteins

The Ras oncogene family related protein has been extensively studied over the last three decades. The fundamental implication of Ras proteins in pathological processes such as cancer and in physiological processes controlling cellular proliferation, differentiation, and survival justifies the interest seen in the scientific literature, currently showing a rate of 200–300 articles published per month. The H-ras, N-ras, and K-ras oncogenes were the first human oncogenes discovered in human tumors more than 30 years ago and are the founding members of the wider Ras gene superfamily, composed of more than 150 distinct cellular members. The members of the Ras family of GTPase proteins are crucial players in many signaling networks connecting a great variety of upstream signals to an even wider set of downstream effector pathways linked to the functional control of a great assortment of cellular outcomes including cell cycle progression, growth, migration, cytoskeletal changes, apoptosis, and senescence. The cross talk between this plethora of signaling pathways and others controlled by different sets of signaling molecules created molecular networks whose balance is crucial to determine the final outcome of cellular responses in the cell (Rajalingam et al. 2007; Stites and Ravichandran 2009). The complexity of all these events controlling cell life reflects the difficult puzzle that has

to be solved when these networks are altered in pathological situations and stresses the importance of their examination to find proper therapeutic approaches able to drive the cells back to a healthy signaling balance.

Within cellular signaling networks, participation of H-Ras, N-Ras, or K-Ras proteins in the Ras-Raf-MAPK pathway has been proven essential for the control of proliferation, differentiation, and survival of eukaryotic cells. Indeed, the evolutionary relevance and importance of this pathway are underlined by the growing number of pathological conditions that have been linked to alterations in some of its components. Thus, in addition to the frequent mutation of Ras genes occurring in various types of cancer that was initially discovered about 30 years ago (Der et al. 1982; Parada et al. 1982; Santos et al. 1982), molecular alterations of many other components of the signaling pathway, such as B-Raf, EGFR, and NF-1, have been described in association with the development of a number of different types of malignancies (Cacev et al. 2005; Dhomen and Marais 2007; Khoukaz 2006; Davies et al. 2002). Such molecular alterations of these components (BRAF, EGFR, and NF-1) in inducing malignant cell transformation are either dependent or independent of Ras (Davies et al. 2002) (Amado et al. 2008).

The experimental observations that have accumulated for the last 30 years document that somatic mutations are the typical genetic lesions affecting Ras and other oncogene proteins linked to the development of sporadic human tumors. In contrast, more recent observations have uncovered the occurrence of germ line mutations in Ras and other members of the Ras-MAPK pathway that result also in constitutive activation of this pathway, although to a lesser extent than that found in tumors, and are specifically linked to the development of a number of distinct but related developmental syndromes. The first report of such type of mutations concerned the neurofibromatosis 1 (*NF1*) locus, a Ras GTPase-activating protein (RasGAP) that is the causative agent for the neurofibromatosis type 1 (Marchuk et al. 1991). Later on, germ line mutations in many other members of the Ras pathway (including the 3 Ras genes, signaling molecules as *PTPN11*, *MEK1*, and *MEK2,* and *SPRED1*; positive and negative Ras regulators as *SOS1* or *Rasa1*; or downstream effectors such as *BRAF*) have been detected in relation to various other inherited developmental syndromes including Noonan, Costello, cardiofaciocutaneous, Legius, or LEOPARD syndromes (Tartaglia et al. 2001; Tidyman and Rauen 2009; Denayer et al. 2008). Altered Ras signaling may also contribute to the development of other types of pathologies besides cancer and developmental syndromes which is beyond the scope of this review.

c-Src Proto-oncogene-Derived Proteins

The "oncogene" hypothesis of cancer was proposed based on the discovery that RNA tumor viruses could be transmitted genetically. The endogenous tumor viruses contain transforming genes or oncogenes, and the activation of these endogenous transforming viruses could cause cancer (Huebner and Todaro 1969). The discovery that Rous sarcoma virus (RSV) contained a defined gene required for transformation

opened up a new way of looking for genes that might be involved in cancer, and this eventually paved a way for the discovery of viral src gene. The cellular origin of viral src gene was confirmed by the studies which show that this particular gene was dispensable for virus replication (Golde 1970; Martin 1970; Toyoshima et al. 1970). Viral src genes capture the cellular genes instead of inserting themselves into the genome and result in the malignant transformation. This phenomenon of malignant transformation by src genes was first identified by in vitro hybridization studies (Stehelin et al. 1976). The finding that src genes sequences that are detected by in vitro hybridization studies were conserved in evolution provided evidence that they were of cellular rather than viral origin, since endogenous retroviruses are generally species-specific. The cellular nature of these sequences was confirmed when the cellular src gene was cloned and was found to be unlinked to viral sequences and to have the typical exon/intron structure of a normal cellular gene (Shalloway et al. 1981; Takeya and Hanafusa 1983). The expression and activity of proteins such as proto-oncogene c-Src, nonreceptor tyrosine kinases that are derived from these src genes, are correlated with advanced malignancy and poor prognosis in different types of cancers.

Role of Viral-Like Proteins in Cancer

Although most retroviruses tend to integrate in gene-rich regions, insertional mutagenesis has nevertheless been more often demonstrated for LINEs than for ERVs in humans and other animals (see below). Newly integrated proviral DNA possesses identical long terminal repeats at the 30- and 50-ends with primer binding sites and promoter and enhancer domains. Downstream promotion or expression enhancement of cellular genes can be a consequence of proviral chromosomal integration (Kettmann et al. 1982; Landry and Mager 2003; Landry et al. 2002). Furthermore, retroviruses, like other viruses such as herpesviruses, are capable of incorporating cellular genes, albeit at the expense of their structural genes. When retroviruses transduce cellular proto-oncogenes during reverse transcription due to template-switching of the RT, they become dependent on coinfecting wild-type viruses to provide structural proteins. Historically, much of our knowledge of oncology has been gained by the investigation of acutely transforming retroviruses carrying oncogenes (Bister and Jansen 1986). A few replication-competent retroviruses encode their own oncogenic protein, notably RSV of chickens with their src-oncogene, the tax gene of the exogenous human T-cell lymphoma virus (HTLV), or the env gene of the Jaagsiekte sheep retrovirus (Maeda et al. 2008). Oncogenic retroviral proteins stimulate cell proliferation, often by influencing signaling pathways or levels of cytokine production that leads to growth stimulation and/or immune suppression. When searching for novel human retroviruses or investigating the role of HERVs in human cancer, one has to take into account the different strategies developed by retroviruses to induce cellular proliferation and cancer and be aware that novel strains may use strategies as yet unknown. Several lines evidence such as (a) the presence of antibodies against ERV-encoded Rec

protein in patients with germ-cell tumors and (b) the propensity of transgenic mice expressing Rec for the testicular carcinoma in situ suggest the importance of endogenous retrovirus-encoded proteins, i.e., viral-like proteins in carcinogenesis.

One of the prominent proteins among these viral-like proteins is Rec. Emerging data in the literature suggests that Rec functions as an oncoprotein by derepressing cellular oncogenic transcription factors. Rec can bind and suppress a guardian of germ line stem cell pluripotency, the promyelocytic leukemia zinc-finger protein (PLZF). Another binding partner of Rec is PLZF-related testicular zinc-finger protein (TZFP) (Kaufmann et al. 2010). Rec interacts with C-terminal DNA-binding zinc-finger domain of TZFP via its N- and C-terminal. TZFP is known to act as a transcriptional repressor during specific stages of spermatogenesis, and it also known to act as a corepressor of the activated androgen receptor (AR). Rec forms a trimeric complex with TZFP and AR resulting in the relieving of the TZFP-mediated repression of AR-induced transactivation. In addition to this, results obtained from reporter assays show that Rec can result in overcoming the direct transcriptional repression by TZFP of the c-myc gene promoter, suggesting the involvement of Rec as an oncoprotein in derepressing cellular oncogenes (Denne et al. 2007; Kaufmann et al. 2010).

Np9 is a tumor-specific biomarker and its oncogenic role in different types of cancers is yet to be completely understood. In cancer cells, Np9 expression results in the upregulation of β-catenin and phospho-ERK (pERK) and cleavage of notch 1 with concomitant decrease in Numb protein. Aberrant notch 1 signaling is usually observed in cancer cells. Numb is a cell fate determinant which counteracts notch 1 signaling. Np9 proteins interact with RING-type 3 ubiquitin ligase LNX (ligand of numb protein X), and this results in targeting Numb for ubiquitin-dependent degradation ultimately leading to activation of Notch 1 signaling in cancer cells (Armbruester et al. 2002). Np9 also interacts with PLZF tumor suppressor, a transcriptional repressor which plays an important role in chromatin remodeling in cancer (Denne et al. 2007). Given that c-myc proto-oncogene is a major target of PLZF, interaction of Np9 with PLZF results in upregulation of c-myc. Np9 expression in cancer cells is also important for the maintenance of ERK1/2 signaling pathway (Chen et al. 2013). Thus, NP9 is known to simultaneously or sequentially activate multiple signaling networks essential for the survival and proliferation of cancer cells.

Syncytin has an important role in the fusion of cells. Syncytin proteins (syncytin-1 and syncytin-2) and their receptors are known to be expressed on different cancer cells and endothelial cells. Expression of syncytin and its receptor results in the fusion of cancer cells with endothelial cells. The use of syncytin-1 antisense oligonucleotide downmodulates syncytin-1 expression and is shown to inhibit the fusion of breast cancer cells with endothelial cells (Bjerregaard et al. 2006). Several studies show that cAMP and estrogen regulate syncytin expression on cells, but only cAMP results in the syncytin-mediated fusion of tumor cells and endothelial cells (Strick et al. 2007; Kudo and Boyd 2002). The inability of estrogen to induce syncytin-mediated cell fusion is attributed to the upregulation of TGFβ by estrogen. This conclusion was based on the experiments

which showed that neutralization of TGFβ results in cell fusions (Kudo and Boyd 2002). Studies by Blesa et al. also showed that syncytin expression constitutes a positive prognostic factor in breast cancer (Gasent Blesa and Candel 2009).

Ras proteins have an important role in the transformation of normal cells into malignant cells, and they are also responsible for migration and invasion of malignant cells (Giehl 2005). Using transgenic mice, in vivo function of Ras especially with regard to the complex homo- and heterotypic interaction between host and tumor cells in tumorigenesis was studied. Transgenic mice carrying Ras oncogene under the control of a viral promoter demonstrate that overexpression of oncogenic Ras perturbs cell growth and results in malignant tumor growth in several tissues and organs. Also, co-expression of c-myc synergistically enhances tumor formation which indicates that Ras and c-myc cooperate in tumorigenesis. Also, Ras-induced signaling pathways which lead to epithelial to mesenchymal transition (EMT) of cells in culture models highlight the importance of Ras proteins in the tumor formation. Most of the epithelial cells require Ras and TGF-β signaling pathways for EMT (Fensterer et al. 2004). In coordination with posttranslational enzymes like methyltransferase LCMT-1 and CAAX endoprotease Rce1, Ras induces cellular transformation. Upon farnesylation of its C-terminus, Ras interacts with plasma membrane and induces the transformation of cells (Bergo et al. 2002, 2004).

Two major pathways, i.e., the Raf/MEK/ERK pathway and PI3-K-dependent pathway, are believed to play a pivotal role in the transformation and tumorigenesis triggered by oncogenic Ras proteins (Brazil and Hemmings 2001; Downward 1998; Khosravi-Far et al. 1998). Activated Ras targets Raf to the plasma membrane, where Raf is activated and in turn phosphorylates the dual-specificity kinases MEK 1 and 2 (Herrera and Sebolt-Leopold 2002; Howe et al. 1992). MEK then phosphorylates ERK1 and 2. Phosphorylated ERK translocates into the nucleus where it interacts with different transcription factors, underlining its role in the regulation of growth factor-induced gene expression and subsequently cell proliferation, differentiation, apoptosis, or changes in cell morphology. Usually aberrant Raf/MEK/ERK signaling occurs in tumor cells. Mutated Ras proteins also lead to constitutive phosphorylation of ERK and increase in expression of vascular endothelial growth factor (VEGF) (Ross et al. 2001). This suggests that Ras contributes to malignancy through agonist-independent activation of the ERK pathway, promoting angiogenesis, invasion, and metastasis. As mentioned above, PI3-K-dependent pathway plays a critical role in tumorigenesis induced by Ras proteins. When activated by cell surface receptors directly or indirectly through Ras, PI3K regulates phospholipid metabolism and the production of phosphatidylinositol 3,4,5-triphosphate (PIP_3) at the cell membrane. PIP_3 is involved in the recruitment and activation of a wide variety of downstream targets, including the serine/threonine kinase Akt/PKB. The main effects of PI3-K/Akt activation with regard to cancer biology are the support of cell survival, cell proliferation, and cell growth (Franke et al. 2003).

Another family of oncogenic proteins which have an important role in cancer is c-Src proto-oncogene-derived proteins. Increased c-Src activity is often found in cancer. Src protein has three major domains, SH2 (for Src homology), SH3, and the kinase catalytic domain (or SH1). SH2 and SH3 both play a part in protein-protein

interactions, while the kinase catalytic domain contains the kinase active site. Src can be switched from an inactive to an active state through control of its phosphorylation state or through protein interactions (Boggon and Eck 2004). There are two major phosphorylation sites on Src: one is at Tyr416 (or Y416), the other at Tyr527. Tyr416 can be autophosphorylated, which activates Src by displacing the P-Tyr416 from the binding pocket, allowing the substrate to gain access. A more critical site is Tyr527, which can be phosphorylated and dephosphorylated by various proteins, such as CSK kinase (phosphorylates) or SHP-1 phosphorylase (dephosphorylates). Phosphorylation of Tyr527 inactivates Src through the interaction of P-Tyr527 with the SH2 domain, which effectively folds Src up into a closed, inaccessible bundle. Dephosphorylation of Tyr527 releases this bond, opening up the molecule to an active state. Protein interactions also act to regulate Src by either directly activating Src or by moving Src to sites of action. Both platelet-derived growth factor and focal adhesion kinase are able to bind to the SH2 domain, causing Src to open up into the active form.

Under normal circumstances, Src is predominantly inactive in cells, being switched on only at specific times. However, if the fine balance between phosphorylation and dephosphorylation is disrupted, changes can occur in Src activity with drastic results. Several cancers, including colon and breast cancer, have been associated with an increase in Src activity (Irby and Yeatman 2000). In fact, Src was first isolated as an oncogene, v-Src, from the transforming virus, Rous sarcoma virus. v-Src was found to lack the region of the cellular protein (c-Src) that contains Tyr527, making it continually active. In a similar fashion, c-Src can become abnormally active, either through mutations in c-Src itself or through mutations in proteins that regulate c-Src. In late stage colon cancers, mutations have been reported in the src gene that cause the loss of the region containing Tyr527, leading to Src overactivity (Frame 2002). Proteins that regulate Src have also been found at abnormal levels in cancer cells, including both those that activate and those that inactivate Src. Proteins such as PTPalpha, SHP-1, and PTP1B that activate Src by dephosphorylating Tyr527 have been detected at elevated levels in various cancer cells, including epidermal and breast carcinoma cells. Conversely, proteins such as Csk and Chk that inactivate Src by phosphorylation of Tyr527 have been detected at reduced levels in certain cancer cells. As such, proteins like Csk and Chk are considered to have a tumor-suppressing ability (Frame 2002). With its importance in cell regulation and its implication in cancer, Src, as well as other protein kinases, has become an important drug target in the battle against cancer.

Viral-Like Proteins as Therapeutic Targets in Cancer Immunotherapy

Viral-like proteins usually do not play a vital role in the normal physiology of adult tissues, but given their importance in tumor formation, development, and metastasis, they can be used as one of the prime targets for tumor therapy. Several such viral-like protein targeting therapies and their first experimental results that are reported in the

literature will be discussed in this section. Given the fact that endogenous retroviruses are the source for the viral-like proteins, the strategies such as the use of reverse transcription inhibitors which had tremendous success in HIV control among HIV-infected people can be used as one of the therapeutic strategies for inhibiting viral-like proteins (Landriscina et al. 2009; Mangiacasale et al. 2003; Sciamanna et al. 2005). Along these lines, when a reverse transcription inhibitor called Abacavir was used to reverse the expression of endogenous retrovirus sequences in prostate cancer cell lines, it showed a strong antiproliferative capacity and even triggered senescence in the cancer cells (Carlini et al. 2010). For these reverse transcription inhibitor therapies to be successful, it is critical to have the detailed knowledge about how and when retroviral restriction elements act on endogenous retroviruses. Also, direct targeting of viral-like proteins by small molecule inhibitors or via RNA interference would also be worth trying.

Passive immunotherapeutic strategies such as the use of monoclonal antibodies to target viral-like proteins are other strategies that can be used to inhibit the effect of such proteins in the transformation of normal cells into malignant cells. A monoclonal antibody (mAb) targeting endogenous retrovirus (HERV-K)-derived envelope protein was tested in in vitro and in vivo studies by Wang-Johanning and coworkers (2012). Given the fact that HERV-K expression was detected in 148 (66%) of 223 breast tumors and a higher rate of lymph node metastasis was associated with HERV-K-positive tumors, the effect of the mAbs targeting endogenous retrovirus-derived proteins was tested on breast cancer cells in in vitro experiments. Such treatments inhibited the tumor growth and induced apoptosis of breast cancer cells in vitro. Also, it resulted in an overexpression of several proteins involved in the apoptotic signaling pathways in the breast cancer cells. In the in vivo experiments, treatment of mice with these mAbs showed significantly reduced growth of xenograft tumors (Wang-Johanning et al. 2012). In principle, these mAbs targeting endogenous retrovirus-derived viral-like proteins can be used as immunotherapeutic strategies for the treatment of not only breast tumor cells but also other tumor types that are known to express such proteins. The use of passive immunotherapeutic strategies in combination with active immune therapies is another active area that can be effective in targeting viral-like proteins for the treatment of cancer.

Given the role of viral-like proteins in inducing the transformation of normal cells into malignant cells, these proteins are considered as tumor-specific antigens (TSAs). As these viral-like proteins are TSAs, the immune escape by the tumors that express these proteins is prevented because of the necessity of these proteins for the tumors in their maintenance. Also, the TSA properties of viral-like proteins suggest that the immune system should be able to mount both a cellular and humoral immune responses. All these properties make viral-like proteins ideal targets for tumor immunotherapy. Because of the existence of different types of viral-like proteins in the tumor cells, it is not an exaggeration to expect to develop a polyvalent vaccine targeting multiple epitopes of different viral-like proteins. In fact, using a combination of bioinformatics and immune-based approaches, it is possible to identify immunogenic core epitopes shared between viral-like proteins in different tumor entities to design a universal polyvalent vaccine that can be used against

multiple tumors. In recent studies, immunogenic peptides derived from viral-like proteins were reported. In their study, Mullins et al. showed that HLA-A2.1 binding peptides derived from viral-like proteins when used to stimulate peripheral T-cells in the presence of autologous antigen-presenting cells resulted in the sustained proliferation of predominantly CD8+ T cells (Mullins and Linnebacher 2012). High numbers of IFN-γ-secreting T cells were detectable after several weekly stimulations with viral protein-derived HLA-A2.1 peptides. These peptide-specific T cells were shown to kill peptide-loaded target cells as well as colorectal carcinoma cells, suggesting that the identified peptides are indeed derived from natural processing of the viral-like proteins in tumor cells. These results hint at the potentiality of active immunotherapeutic strategies targeting viral-like proteins in future studies.

Future Perspectives of Targeting Viral-Like Proteins for the Treatment of Cancer

The effectiveness of targeting viral-like proteins for the treatment of cancer in the future depends on the following points:

- Gaining complete understanding of the mechanism of viral-like proteins in the tumor cells.
- Understanding of the relationship between the tumors and the endogenous viruses that encode these proteins through the information of viral-like protein sequences.
- Obtaining the knowledge about the interaction between viral-like proteins and the host immune system and how this interaction affects the immunosuppressive mechanisms used by tumors expressing these proteins.

Such knowledge would lead to developing strategies targeting these proteins which are emerging as prominent tumor-specific antigens.

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Whole-Cell Vaccines

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Conflict of Interest
Under a licensing agreement between Aduro, Incorporated and Johns Hopkins University, the University is entitled to milestone payments and royalty on sales of the GM-CSF-secreting vaccines. The terms of these arrangements are being managed by Johns Hopkins University in accordance with its conflict of interest policies.

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Abstract

Therapeutic cancer vaccines aim to generate tumor immunity through coordinated cell- and antibody-mediated responses that ultimately result in highly specific and potent T cells capable of destroying cancers. Cancer vaccines have unique advantages compared to most other cancer therapeutics, with a high degree of specificity for tumor cells relative to normal tissues, a highly favorable side-effect profile, and the potential for a long-lasting treatment effect due to immunologic memory.

Keywords

Whole-cell vaccine • Immunotherapy • Immune response • Chemotherapy • Monoclonal antibody • Combination immunotherapy

Target: Whole-Cell Vaccines That Induce Tumor-Specific Immunity

Designing effective vaccine strategies for established cancers poses multiple challenges (Emens and Jaffee 2003). These include choosing an active vaccine platform for delivering tumor antigens; selecting the most immunogenic, tumor-specific antigens for delivery; and developing strategies for overcoming immune tolerance to enhance vaccine-induced immunity. Several vaccine platforms have been tested. These include antigen-specific peptide, protein, and plasmid DNA vaccines, bacterial or viral vectors that deliver selected tumor antigens, and unmodified or modified autologous or allogeneic whole tumor cells (which naturally deliver multiple antigens). Dendritic cells (DCs) are also a major class of whole-cell vaccine and are the most effective way to present tumor antigens to the immune system (Banchereau and Palucka 2005). The clinical efficacy of DC as a platform to deliver clinically relevant tumor antigens was established with the FDA approval of Sipuleucel-T for advanced prostate cancer (Kantoff et al. 2010). Here, DC precursors are harvested from the patient and cultured ex vivo with cytokines and growth factors to facilitate maturation into effective antigen presenting cells. These expanded and matured DCs are then loaded with tumor antigen, either by genetic modification or by direct pulsing with a target protein or peptide, and given back to the patient. Whole tumor cell vaccines, another major class of whole-cell cancer vaccines, also activate tumor immunity through DCs. In this case, the patient's own DCs take up, process, and present antigens delivered by the vaccinating tumor cells, thereby cross-priming an antigen-specific $CD4^+$ and $CD8^+$ T cell response at the vaccine site (Emens and Jaffee 2003). This avoids the process of harvesting and manipulating the patient's own DCs ex vivo.

The use of whole tumor cells as a cancer vaccine platform confers the advantage of simultaneously delivering multiple relevant tumor antigens, some of which are known and some of which are not. Autologous whole-cell vaccines are derived from

the patient's own tumor, and thus are a personalized vaccine. Despite this advantage, it is frequently difficult to obtain enough cells for manufacturing autologous whole-cell tumor vaccines in quantities that will support multiple vaccinations, especially for solid tumors. In addition, for those vaccines that are genetically modified to express an immune-modulating protein or to secrete an immune-activating cytokine, generating vaccines that express or secrete a standardized level of the immune-modulating component is not always feasible, leading to variations in vaccine potency within a given study. In addition, each personalized vaccine has to be manufactured and tested for quality separately, so the process of generating a customized vaccine for a given patient is quite expensive and time consuming. The use of allogeneic tumor cell lines circumvents many of these practical limitations of autologous vaccines. Although the opportunity to deliver unique tumor antigens generated by a specific mutation unique to the tumor of a given patient is lost, allogeneic tumor cells deliver multiple tumor antigens, of which at least half may be shared across tumors of a given histologic type. Importantly, allogeneic tumor cell lines can be more easily modified to express an immune-modulating molecule that enhances their immunogenicity and are more easily grown in large quantities to support multiple cycles of vaccination. Manufacturing large lots of allogeneic tumor cells that can be used to vaccinate many patients both ensures greater consistency in potency and overall quality and lowers the cost of vaccine production and quality testing. Early in the development of allogeneic tumor cell vaccines, concerns that human leukocyte antigen (HLA) mismatch between vaccinating cell lines and the patient might preferentially favor an immune response directed against foreign HLA molecules instead of tumor antigens were raised. However, data suggest that these molecules may function to actually enhance tumor antigen-specific immunity, thus serving as an immunologic adjuvant. Additionally, the patient's own dendritic cells take up, process, and present antigens delivered by allogeneic tumor cells, circumventing the need for an HLA match between the vaccinating cells and the patient (de Gruijl et al. 2008).

Biology of the Target: Mechanism of Action for Whole-Cell Vaccines

A seminal study systematically analyzed the ability of whole tumor cells, either unmodified or genetically modified to express different cell-surface molecules or secrete distinct immune-activating cytokines, to protect mice from a subsequent tumor challenge (Dranoff et al. 1993). This study found that whole tumor cells that secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) were most protective against a subsequent tumor challenge. The paracrine secretion of GM-CSF by vaccinating tumor cells recruits DCs to the injection site. These DCs then take up, process, and present tumor antigens delivered by the vaccinating tumor cells to $CD4^+$ and $CD8^+$ T cells. Antigenic peptides are bound to MHC Class I (in the case of $CD8^+$ T cells) or MHC Class II (in the case of $CD4^+$ T cells), and the MHC-antigen complex engages the TCR on the surface of the T cell.

The peptide: MHC-antigen-TCR delivers one signal required for T cell activation, and accessory molecules of T cell activation (CD80, CD86) deliver the second signal required for T cell activation. The strength and quality of T cell activation is fine-tuned by additional accessory molecules of activation (CD40, 4-1BB, OX-40) and inhibition (CTLA-4 and PD-1). Tumor cell killing occurs when the TCR expressed on effector $CD8^+$ T cells recognizes tumor antigens presented by MHC molecules on the surface of tumor cells. If activated efficiently, $CD8^+$ T cells can work synergistically with traditional treatments such as chemotherapy, radiation therapy, or monoclonal antibodies to kill or inhibit tumor cells (Emens et al. 2005).

Several strategies have been used to enhance priming of the immune response by DCs (Emens et al. 2012). Toll-like receptor (TLR) agonists and some chemotherapeutic agents (such as paclitaxel) can stimulate DCs directly through TLRs to upregulate costimulatory molecules, increase cytokine production, and enhance antigen processing and presentation. Tumor antigen-specific monoclonal antibodies (MAb) can bind to specific tumor antigens on the vaccine cell surface and engage DCs through the Fc portion of the MAb binding to the Fc receptor on the DC surface. Antigen presenting cells (APCs) and T cells can be suppressed by inhibitory cytokines and molecules such as transforming growth factor-β (TGFβ) and interleukin-10 (IL-10) secreted by suppressive immune cell populations like myeloid-derived suppressor cells (MDSCs) and $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs). Multiple chemotherapy agents (cyclophosphamide (CY) for example) and radiation, when used at immunomodulatory doses, can be used to inhibit these populations (Emens 2010).

Target Assessment: T Cell Immune Monitoring

For the effective clinical development of cancer vaccines, monitoring the magnitude and quality of the vaccine-induced immune response is essential. In contrast to cancer vaccines that deliver a specific antigen, whole-cell vaccines pose a challenge for immune monitoring since they deliver a battery of antigens. Identifying one of these antigens as a sentinel measure of vaccine activity is one approach for monitoring vaccine-induced immunity and correlating it with potential clinical benefit from the whole-cell vaccine. This strategy has been developed for the pancreas, prostate, and breast cancer GM-CSF-secreting vaccines by measuring immunity to mesothelin, filamin B, and HER-2, respectively (Emens 2009).

Ex vivo immune monitoring of antigen-specific T cells is key for evaluating the immunogenicity of cancer vaccines (Emens and Jaffee 2003). Currently there are three methods that are widely used to quantify cellular immune responses: the Enzyme-Linked ImmunoSpot assay (ELISpot), the Intracellular Cytokine Staining assay (ICS), and the Tetramer assay. These three different assays offer different information. The ELISpot and ICS assays may measure T cells directly out of the blood but more frequently use short-term in vitro stimulation to measure the frequency and cytokine expression profiles of T cells before and after vaccination. The ELISpot assay enumerates T cells by counting spots of captured cytokine

produced by individual T cells. The ICS assay uses flow cytometry to profile individual T cells for surface markers and the production of cytokines and measures T cells with certain characteristics as a percentage of the total. A limitation of these assays is that in vitro stimulation may not accurately reflect the biology of the starting T cells, so measuring T cells directly out of the patient is preferred to an intervening in vitro stimulation. The use of tetramers enables a more direct and specific enumeration of antigen-specific T cells. A tetramer is composed of four peptide-bound MHC molecules with a streptavidin-fluorophore component that allows detection by flow cytometry. Importantly, tetramer staining can be combined with phenotypic and functional analysis to more fully characterize antigen-specific T cells. Perhaps the most effective indicator of the ability of endogenous T cells to migrate to and recognize autologous tumor cells in vivo is by monitoring the development of delayed type hypersensitivity (DTH) to the patient's own tumor cells or to a shared antigen delivered by the whole-cell vaccine.

Role of Target in Cancer: T Cell-Dependent Tumor Immunity

Harnessing the power of the immune system – T cells – to fight cancer has two unique strengths. The first is that appropriate T cells can specifically destroy tumor cells with minimal concurrent toxicity to normal tissues. The second is that the immune system, once educated, is able to respond vigorously to a future antigenic challenge (immune memory). The recognition of tumor-associated antigens by T cells is essential for the specificity of the immune response. Some tumor antigens are easily recognized by the immune system because they are foreign. These include viral proteins expressed by virus-associated tumors, mutated proteins from oncogene mutations or fusions, and self-proteins expressed outside of their normal (immunologically privileged) site or at unusually high levels compared to normal tissues. However, tumors arise from the patient's own tissues, and they are more commonly recognized as self. Because of this, the same regulatory mechanisms that prevent autoimmune disease – the immune-mediated destruction of normal tissues – act to suppress tumor immunity. First, the process of thymic T cell selection leads to the presence of a T cell repertoire in the periphery that recognizes self and tumor antigens fairly weakly. Those autoreactive – tumor-specific – T cells that do survive thymic selection are held in check by Tregs. Those tumors that do express mutated antigens – altered self – may not effectively activate T cell immunity because they lack expression of a costimulatory molecule that can provide the second signal, or because they express an inhibitory ligand (PD-L1) that shuts down T cell activation. When a tumor-specific immune response does develop, cancer cells can further resist, avoid, or suppress the antitumor response, leading to tumor escape and malignant progression. Tumor cells themselves may become altered, through loss of tumor antigens, downregulation of MHC molecules, and loss of sensitivity to complement or cell-mediated lysis, making them a poor target for immune attack. Tregs and MDSCs accumulate within the blood and tumor microenvironment with tumor progression, and suppress immunity. Also within the tumor

microenvironment, multiple immunosuppressive cytokines are secreted by tumor or host cells (vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and interleukin-10 (IL-10), among others).

Immunotherapy aims to effectively recruit the immune system to fight cancer. A successful immunotherapy strategy will be based on specific tumor recognition, provide or enable missing or silenced immune effector function, and confer a durable treatment effect that may persist even in the absence of ongoing immunotherapy. One immunotherapy strategy induces the activation and expansion of T cells directed to well-defined tumor antigens (e.g., cancer vaccines). Another immunotherapy strategy enhances tumor immunity by specifically blocking inhibitory signaling pathways and suppressive cells in the tumor microenvironment (e.g., MAbs specific for CTLA-4, PD-1 or its ligand PDL-1). Over the last few years, single agent immunotherapies have had clinical success. The first two agents FDA-approved for clinical use were a cancer vaccine and an immune checkpoint modulator. Sipuleucel-T is a DC-based cancer vaccine that modestly improves survival in advanced prostate cancer patients (Kantoff et al. 2010). Ipilimumab is a MAb specific for CTLA-4 that improves survival both as a single agent and combined with dacarbazine in patients with advanced melanoma (Hodi et al. 2010; Robert et al. 2011). More recently, monoclonal antibodies that target the PD-1 pathway have been very successful, with response rates of 20–30% in several tumor types that include melanoma, renal cell carcinoma, and non-small cell lung cancer (Topalian et al. 2012; Brahmer et al. 2012); these have now been approved for several indications. These studies provide proof of principle that immunotherapy can make a difference for cancer patients. It is critical to remember that the ultimate target for these two major immunotherapy strategies is the T cell. Therefore, inducing highly functional T cells and providing a good environment in which they can work will be essential to the success of immunotherapy. The fact that survival gains can be modest, and that significantly fewer than half of patients respond, suggests that combination immunotherapies that simultaneously activate T cells and abrogate mechanisms of immune tolerance and suppression will be most effective. Thus, combination immunotherapies that simultaneously promote T cell activity and relieve immunosuppression have the potential to transform cancer treatment, promoting cure for many patients.

Clinical Summary: Whole-Cell Vaccine Trials

Dendritic Cell-Based Vaccine Trials: Proof of Principle for Cell-Based Tumor Vaccines

Dendritic cell-based vaccines are one major class of cell-based cancer vaccines. The first FDA-approved cell-based cancer vaccine is Sipuleucel-T. This is an autologous DC vaccine designed to enhance the T cell response to prostatic acid phosphatase. Patient-specific DCs are obtained by leukapheresis from peripheral blood mononuclear cells. These cells are exposed ex vivo to a novel recombinant protein

immunogen (PA2024), which consists of prostatic acid phosphatase (PAP) fused to human GM-CSF. These activated cells are then infused back into the patient 3 days after harvesting, with serial infusions every 2 weeks for three treatments. Sipuleucel-T was studied in three randomized trials that enrolled men with castration-resistant metastatic prostate cancer who were asymptomatic or minimally symptomatic. In the first two clinical trials D9901 and D9902A, a combined analysis of the 225 men enrolled demonstrated a statistically nonsignificant trend toward increased progression free survival (PFS) with Sipuleucel-T compared with the control (11.1 vs. 9.7 months, $p = 0.11$) (Higano et al. 2009). Although the increase in PFS – the primary endpoint – was not statistically significant, the change in overall survival (OS) – a secondary endpoint – was, with significantly longer OS in the Sipuleucel-T group compared to the control group (median 23.2 vs. 18.9 months, $p = 0.011$).

Overall survival was the primary endpoint in the phase III clinical trial, D9902B, also known as the IMPACT trial (Kantoff et al. 2010). This study enrolled 512 men with castration-resistant metastatic prostate cancer. At a median follow-up of 34 months, patients assigned to the Sipuleucel-T therapeutic vaccine had a significantly improved OS (median 25.8 vs. 21.7 months, $p = 0.03$). As in the phase II trials, PFS was not significantly prolonged (14.6 vs. 14.4 weeks, $p = 0.63$). Although Sipuleucel-T significantly prolonged OS, it rarely induced disease regression and did not have a significant impact on radiographic PFS. Furthermore, it did not induce consistent changes in the serum PSA level. Whether this is due to a delayed effect of immunotherapy or whether some other mechanism is involved is unclear. The absence of objective parameters to judge whether or not an individual patient is benefiting from vaccine therapy poses a challenge in determining when to consider Sipuleucel-T ineffective and initiate alternative treatment.

In contrast to the Sipuleucel-T study, a randomized phase III study of an autologous peptide-loaded DC vaccine compared to standard dacarbazine chemotherapy for stage IV melanoma patients failed to meet the primary end point of a difference in objective response rate (ORR) (Schadendorf et al. 2006). DCs were generated from peripheral blood mononuclear cells obtained via leukapheresis and cultured with GM-CSF and IL-4, and then matured with TNF-α, IL-1β, IL-6, and prostaglandin E2. They were then loaded with several MHC class I/II restricted peptides derived from established melanoma tumor antigens and the influenza nucleoprotein as a control. At the time of the first interim analysis, the ORR was low, with no statistically significant differences between the arms (DTIC: 5.5%, DC: 3.8%). The Data and Safety Monitoring Board therefore recommended closure of the study. No other significant differences between the 2 arms, either in OS or PFS, were found. Interestingly, subset analyses revealed that only in the DC-arm did those patients with an unimpaired general health status or HLA-A2+/HLA-B44− haplotype survive significantly longer than patients with impaired performance status or other HLA haplotypes. The study was limited by variable vaccine quality in terms of number and maturation status of the DCs obtained, the subcutaneous route of administration (felt less effective than intradermal or intranodal routes tested in other studies), and the lack of nonspecific "helper proteins" such as keyhole limpet

hemocyanin (KLH) or tetanus toxoid that had been used in previous studies to provide T cell help.

While both the optimal preparation of DCs and most active tumor antigens remains undefined, both the prostate cancer and melanoma studies described here suggest that clinical parameters that may define less aggressive cancer biology may be useful in selecting a patient population that is more likely to benefit from immunotherapy. Defining the best route of administration is also critical. In addition to improving clinical trial designs, there is a considerable effort to enhance the potency of DC-based vaccines based on our evolving understanding of DC biology. Continued optimization of DC vaccine development should translate into more potent vaccine strategies.

Whole Tumor Cell Vaccine Trials

Whole tumor cell vaccines are the other major class of cell-based cancer vaccines (de Gruijl et al. 2008). These can be autologous or allogeneic tumor cells that are unmodified, modified by haptenylation or virus infection, or modified to express or secrete various immune-modulating molecules. Early studies of unmodified, haptenylated, or virus-infected whole tumor cells provided hints of clinical and immunologic activity. Based on the seminal study of Dranoff and colleagues, the most comprehensive clinical experience with a class of whole tumor cell vaccines is with whole tumor cells engineered to secrete the cytokine GM-CSF (GVAX) (Gupta and Emens 2010). GM-CSF-modified autologous tumor cell vaccines were first evaluated in patients with advanced kidney cancer, melanoma, and prostate cancer. In the first clinical trial, 18 patients with metastatic renal cell carcinoma (RCC) were randomized and treated with autologous, irradiated unmodified RCC cells alone or autologous, irradiated RCC cells modified by retroviral gene transfer to secrete GM-CSF. Infiltrates of eosinophils developed at the DTH sites of patients who received the GM-CSF-transduced vaccine cells but not those who received unmodified vaccine cells. One vaccinated patient who developed the largest DTH conversion also displayed a partial response. Two subsequent studies administered GM-CSF-secreting autologous melanoma vaccines to metastatic melanoma patients. In both studies, DTH responses of $\geq$1 cm were observed in 100% of vaccinated patients. A trial of autologous GM-CSF-secreting tumor cells in metastatic prostate cancer patients demonstrated DTH of at least 1 cm in 5 of 6 vaccinated patients. Together, these three clinical trials showed that this type of vaccine is biologically active.

Second-generation trials tested autologous tumor cell vaccines either modified to secrete GM-CSF by adenoviral transduction or admixed with bystander cells that secrete GM-CSF. One trial of autologous GM-CSF-secreting melanoma cells in 26 patients with metastatic melanoma demonstrated DTH $\geq$ 1 cm in 68% of patients, as well as 1 complete, 1 partial, and 1 mixed response and 5 cases of stable disease. In two studies conducted in patients with metastatic non small cell carcinoma of the lung (NSCLC), DTH $\geq$ 1 cm developed in 82% of vaccinated patients in one study

and increased from 13% to 34% of patients post vaccination in the other. In the former study, the vaccine consisted of GM-CSF secreting tumor cells modified by adenoviral transduction; of 25 evaluable patients, 1 patient had a mixed response and 5 displayed stable disease. In the latter study, the vaccine consisted of autologous tumor cells admixed with GM-CSF-secreting bystander cells; clinical outcomes included 4 mixed responses and 7 cases of stable disease. In a third study of 43 patients with early-stage or metastatic NSCLC, longer survival was observed in vaccinated, advanced-stage NSCLC patients who developed serum levels of GM-CSF $\geq$ 40 ng compared to those who developed lower peak GM-CSF levels. This provides initial clinical evidence that a threshold level of GM-CSF is required for the induction of effective immunity.

Allogeneic GM-CSF-secreting vaccines were first evaluated as a single intervention in pancreatic and prostate cancer. Two studies in stages 2 and 3 pancreatic cancer patients delivered a GM-CSF-secreting pancreatic cancer cell vaccine after primary surgical therapy. The first phase I study tested four different vaccine cell dose levels in 14 patients, and demonstrated new DTH $\geq$1 cm to the patient's own pancreas tumor cells in 1 of 3 patients at 1×10^8 cells and 2 of 4 patients at 5×10^8 cells. Disease-free survival of over 10 years was demonstrated in 3 out of 14 vaccinated patients. The follow-up phase II study tested the vaccine at the highest 5×10^8 cell dose in 60 patients (Lutz et al. 2011). The overall survival rate of vaccinated patients was 26 months, compared to a historical rate of 21 months in non-vaccinated patients. An allogeneic GM-CSF-secreting breast tumor vaccine was subsequently developed and tested alone in 6 patients with metastatic breast cancer, with evidence of new vaccine-induced immunity after vaccination (Gupta and Emens 2010).

Multiple clinical trials have tested allogeneic GM-CSF-secreting vaccines in patients with prostate cancer. Two studies enrolled patients with metastatic hormone-naïve prostate cancer. One study of 19 patients demonstrated a median TTP of 9.7 months, and the other study of 21 patients revealed 1 partial response and 16 minor responses, and 14 cases of stable disease. Another study was conducted in patients with metastatic hormone-refractory prostate cancer. It enrolled 55 patients, 34 of whom had evaluable disease and 21 of whom had only a biochemical relapse (rising PSA only). This trial documented a median survival of 26.2 months in patients with evaluable disease. Two phase III randomized, controlled clinical trials (VITAL-1 and VITAL-2) tested the allogeneic GM-CSF-secreting prostate cancer vaccine. These studies enrolled 626 and 408 hormone-refractory metastatic prostate cancer patients, respectively. In the VITAL-1 trial, the vaccine alone was compared to docetaxel plus prednisone in asymptomatic patients with prostate cancer. VITAL-2 was a two-arm, randomized Phase III study of vaccine in symptomatic metastatic prostate cancer patients. One arm combined vaccine with docetaxel and the other arm employed docetaxel and prednisone. The primary endpoint of both trials was OS. In August 2008, the VITAL-2 trial was terminated after an Independent Data Monitoring Committee noted an imbalance of deaths, with 67 in the experimental arm and 47 in the control arm. Importantly, with further follow-up, this imbalance lessened to 85 and 75 deaths, respectively. All deaths were the result of disease progression and death from prostate cancer. Subsequently in October

2008, the VITAL-1 trial was terminated after futility analysis predicted a $< 30\%$ chance of meeting the primary endpoint. It is notable that chemotherapy drugs can have synergistic or antagonistic effects on immunotherapy depending on the agent, relative to both the timing of their administration, and the selected drug dose. It is likely that the dose of docetaxel used in this study inhibited a vaccine-induced immune response because docetaxel does affect white blood cell counts and was given at the time of vaccination.

The information from clinical trials gained so far informs the way forward in developing GM-CSF-secreting whole tumor cell vaccines. First, initial results of the VITAL-1 trial demonstrated that immunotherapy was better tolerated than chemotherapy, without a significant difference in clinical outcome. Second, the initial imbalance of deaths on the VITAL-2 study lessened with further follow-up, a finding consistent with an emerging appreciation that immunotherapy responses occur more slowly and later than responses to conventional cytotoxic chemotherapy. Third, it is likely that utilizing cancer vaccines earlier in the natural history of cancer, before it becomes metastatic, will be more effective. Fourth, the development of DTH responses to autologous tumor cells has been an informative measure of the immune response in small trials but is not practical to implement on a larger scale. Finally, there is an undeniable impact of conventional therapy on the vaccine-induced immune response. This was observed in the adjuvant studies of the GM-CSF-secreting pancreas vaccine and the probable negative effect of chemotherapy on vaccine activity in VITAL-2.

Vaccine Therapy Combined with Standard and Novel Cancer Drugs

Conventional cytotoxic agents, monoclonal antibodies, and novel immunomodulators like immune checkpoint blockade agents and TLR agonists may be used in conjunction with cancer vaccines to boost the antitumor response.

Chemotherapy drugs, depending on type of drug, dosage, and schedule, can inhibit or augment tumor immunity induced by cancer vaccines (Emens 2010). Vaccination after high-dose chemotherapy can reboot the immune system, skewing the T cell population towards a desired tumor specificity. Conversely, low-dose chemotherapy also has immunologic effects. Low-dose cyclophosphamide (CY) given prior to vaccination can inhibit the suppressive activity of Tregs, allowing an effective antitumor T cell response to emerge. Low-dose CY also promotes T helper type 1-mediated immunity; enhances DC activation, maturation, and cytokine secretion; and upregulates type 1 interferons to promote immunologic memory. Similarly, low doses of paclitaxel (PTX) and doxorubicin (DOX) can modulate immunity. Two clinical trials have been published that combine immune-modulating doses of chemotherapy with GM-CSF-secreting whole-cell cancer vaccines to enhance the immune response. In one study, 50 patients with metastatic pancreatic cancer were given an allogeneic GM-CSF-secreting pancreas tumor cells alone or with CY 1 day before vaccination (Laheru et al. 2008). The median survival

rates of patients who received vaccination alone and those who received vaccination with CY were 2.3 and 4.3 months, respectively. The addition of CY did not potentiate vaccine-related toxicities, and patients who received CY-modulated vaccination were more likely to develop mesothelin-specific T cell responses after to vaccination. Another study administered an allogeneic GM-CSF-secreting HER-2-expressing breast tumor cell vaccine to 28 metastatic breast cancer patients along with a range of low doses of CY 1 day prior to vaccination and DOX one week later to determine the doses of CY and DOX that optimize the immunologic response (Emens et al. 2009). The vaccine as a single agent induced HER2-specific DTH. The DTH response was maintained and the HER2-specific antibody response enhanced, when the vaccine was sequenced with doses of CY at 200 mg/m^2 1 day prior to vaccination and DOX at 35 mg/m^2 1 week after vaccination. Notably, CY doses of 250 mg/m^2 or higher abrogated vaccine-induced immunity.

Monoclonal antibodies are also able to modulate the immune system and tumor-host response in diverse ways (Ferris et al. 2010). Their Fc regions engage host immune effectors, thus facilitating tumor cell destruction via antibody-dependent cellular cytotoxicity (ADCC). They may enhance the lytic activity of antigen-specific CD8$^+$ T cells against tumor cells by promoting the processing and presentation of tumor antigens. Monoclonal antibodies also may augment immune priming when used in conjunction with a cancer vaccine by engaging the Fc receptor on phagocytes at the vaccine site and its draining lymph nodes. Finally, monoclonal antibodies may also influence the tumor microenvironment. Trastuzumab and bevacizumab decrease VEGF levels, thus inhibiting the angiogenesis required for progressive tumor growth. Consistent with these activities, HER-2-specific monoclonal antibodies can enhance immune responses and tumor-free survival after CY-modulated vaccination in the tolerant *neu*-N mouse model of breast cancer. Based on these data, ongoing clinical trials have tested CY-modulated vaccination with GM-CSF-secreting tumor cells combined with cetuximab in pancreatic cancer patients and trastuzumab in breast cancer patients. Early data from the clinical trial testing CY-modulated vaccination in the setting of standard Trastuzumab therapy for HER-2$^+$ metastatic breast cancer patients show evidence of immune activation and clinical benefit (Gupta and Emens 2010).

Combining tumor vaccines with immune checkpoint inhibitors is another highly promising strategy for altering pathways of immune tolerance and targeting the tumor microenvironment. Cell-surface molecules like CTLA-4, B7-H1, and B7-H4 control pathways that suppress tumor immunity, while others like 41BB and OX40 control regulatory pathways that amplify immunity (Pardoll 2002). Two clinical trials studied treatment with ipilimumab for metastatic melanoma or ovarian carcinoma patients previously vaccinated with autologous GM-CSF-secreting melanoma or ovarian cancer vaccines (Gupta and Emens 2010). Periodic infusions of ipilimumab stimulated extensive tumor necrosis, or the reduction/stabilization of cancer antigen-125 (CA-125) levels, suggesting that CTLA-4 blockade can unleash latent tumor immunity in patients who have been previously vaccinated. More recent trials have combined CTLA-4 blockade with GM-CSF-secreting prostate or pancreas whole-cell cancer vaccines, with a tolerable safety profile and early evidence of

possible clinical benefit (Le et al. 2013; van den Eertwegh et al. 2012; Santegoets et al. 2013). Further studies of whole-cell vaccines with checkpoint blockade strategies that target the CTLA-4 and/or the PD-1 pathways are clearly warranted.

Anticipated High-Impact Results

Several Phase II and III clinical trials are currently testing cell-based vaccines alone or with various standard and novel cancer therapies and emerging immunotherapies in various cancer types. The first is an open label, randomized phase II study evaluating the safety, vaccine-induced immunity, and clinical benefit associated with an allogeneic HER-2-expressing GM-CSF-secreting whole-cell breast cancer vaccine given with CY alone or combined with Trastuzumab to patients with stage IV HER-2-negative breast cancer (NCT000971737). This study tests whether Trastuzumab can augment immune priming at the time of vaccination to generate a higher T cell response that translates into clinical benefit in patients who would not normally benefit from Trastuzumab. Another study is evaluating the safety of a whole-cell GM-CSF-secreting pancreatic tumor vaccine (GVAX) with immune-modulating doses of CY followed by fractionated Stereotactic Body Radiation Therapy (SBRT) and FOLFIRINOX as adjuvant therapy for patients with pancreatic cancer after surgical resection (NCT01595321). Finally, recent data describing the evaluation of a prime/boost vaccination strategy that sequences mesothelin-specific vaccination using a listeria platform and the GM-CSF-secreting pancreas tumor vaccine in metastatic pancreatic cancer patients has demonstrated clear evidence of a survival benefit, and definitive trials to evaluate this strategy are being planned.

In conclusion, for optimal clinical activity whole-cell vaccines will be used in combinations with other standard cancer therapies and/or with additional innovative immunotherapies. It is clear that the future of immunotherapy lies in combination strategies. Integrating a clear understanding of the complex interplay between tumor-specific T cells and immunosuppressive pathways active within the tumor microenvironment with thoughtful clinical trial design based on the well-defined scientific and clinical principles underlying the tumor-specific immune response will ensure the future success of immune-based approaches to cancer therapy.

Cross-References

► Bacterial Vaccines
► CD4+ T Cells
► CD8 T Cells
► Dendritic Cells
► DNA Vaccines

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Part II

Angiogenesis

FGF-FGFR Signaling in Cancer

52

Moosa Mohammadi and Andrew Beenken

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Abstract

Fibroblast growth factors (FGF) have pleiotropic roles in human development and metabolism, and FGF signaling through FGF receptors (FGFRs) has been implicated in a wide range of cancers. Extensive pre-clinical and clinical studies are currently underway to elucidate the therapeutic possibilities: monoclonal antibodies, ligand traps, heparanoids, and kinase inhibitors all have potential for the treatment of FGFR-driven cancers.

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Target: FGF, FGFR

Fibroblast growth factor (FGF) signaling plays pleiotropic roles in human development and metabolism. Based on primary sequence, structural similarity, and phylogenetic analysis, the 18 human FGFs (FGF1–FGF10 and FGF16–FGF23) are grouped into five paracrine subfamilies and one endocrine subfamily. The paracrine subfamilies include the *FGF1* subfamily comprising FGF1 and 2; the *FGF7* subfamily comprising FGF3, 7, 10, and 22; the *FGF4* subfamily comprising FGF4, 5, and 6; the *FGF8* subfamily comprising FGF8, 17, and 18; and the *FGF9* subfamily comprising FGF9, 16, and 20. The endocrine-acting *FGF19* subfamily comprises FGF19, 21, and 23. The paracrine-acting FGF subfamilies play essential roles in spermatogenesis, mesoderm induction, somitogenesis, organogenesis, and pattern formation, whereas members of the FGF19 subfamily signal in an endocrine fashion to regulate major metabolic processes including glucose, lipid, cholesterol, and bile acid metabolism and serum phosphate/vitamin D homeostasis (Kuro-o 2008; Martin 1998; Ornitz 2005; Yu and White 2005).

The core homology region of FGFs (approximately 120 amino acids long) adopts a β-trefoil fold consisting of 12 antiparallel β-strands (β1–β12) that arrange into 3 sets of 4-stranded b-sheets in paracrine FGFs. Endocrine FGFs, however, lack the β11 strand and as a result have an atypical trefoil fold. The globular β-trefoil core domain is flanked by highly divergent N- and C-terminal tails. All FGFs bind heparan sulfate (HS) albeit with differing affinities. The HS binding site (HBS) in FGFs is composed of residues from the β1 to β2 loop and from the region between β10 and β12. Paracrine FGFs have substantial affinity for HS and therefore can only act locally, whereas the weak affinity of the FGF19 subfamily members allows them to avoid entrapment in the extracellular matrix (ECM) and enter blood circulation (Beenken and Mohammadi 2012; Mohammadi et al. 2005a).

FGFs carry out their diverse actions by binding and activating the FGF receptor (FGFR) subfamily of receptor tyrosine kinases encoded by four genes in humans (FGFR1–4). FGFR1–3 genes are composed of 19 exons, whereas FGFR4 gene contains 18 exons. The prototypical FGFR is composed of three extracellular immunoglobulin domains (D1–D3) connected by flexible linker sequences, a transmembrane domain, and an intracellular conserved tyrosine kinase domain. Structural studies have shown that ligand binding requires both D2 and D3 domains. Like

FGFs, FGFRs are also HS-binding proteins. HBS in FGFRs is located in D2 and is composed of basic residues that collectively localize onto one of the b-sheets of this domain. The D1 and D1–D2 linker are dispensable for ligand binding and in fact suppress FGF and HS binding affinity of the D2–D3 region. In FGFR1–3, exon 8 (known as "IIIb") and exon 9 (known as "IIIc") code for the second half of D3 and are spliced in a mutually exclusive fashion to the common exon 7 (known as "IIIa") that encodes the first half of D3. This splicing event is tissue specific and results in the expression of epithelial "b" isoforms (FGFR1b–FGFR3b) or mesenchymal "c" isoforms (FGFR1c–FGFR3c) thereby expanding the number of principal FGFRs to seven, namely, FGFR1c, FGFR1b, FGFR2c, FGFR2b, FGFR3c, FGFR3b, and FGFR4 (Beenken and Mohammadi 2009; Johnson et al. 1991).

FGF–FGFR binding specificity/promiscuity is critical in FGF signaling and is principally dictated by primary sequence differences between the 18 FGFs and the 17 principal FGFRs. Tissue-specific alternative splicing in the D3 domain of FGFR1–3 is the main mechanism in the regulation of FGF–FGFR binding specificity. Generally, paracrine FGF subfamilies also exhibit tissue-specific expression patterns and are expressed in either epithelial or mesenchymal compartments. The epithelially expressed FGFs typically show specificity for FGFRc isoforms expressed in the mesenchyme and vice versa, resulting in the establishment of an epithelial–mesenchymal signaling loop (Beenken and Mohammadi 2011). It is well documented that FGF7 and FGF10, which are expressed exclusively in the mesenchyme, specifically activate FGFR2b to mediate the epithelial–mesenchymal signaling required for the development of multiple organs and glands including lung, thyroid, pituitary, lachrymal, and salivary glands. In contrast, the members of the FGF4, FGF8, and FGF9 subfamilies are expressed in the epithelium and activate the mesenchymal FGFRc isoforms to govern patterning and morphogenesis of multiple tissues and organs, including the brain, lung, heart, kidney, eye, limb, and ear (Beenken and Mohammadi 2009). For instance, FGF8b binds FGFR1c–FGFR3c and FGFR4 but does not recognize "b" isoforms. FGF2 binds with comparable high affinity to both FGFR1c and FGFR2c but does not bind the remaining five FGFRs. FGF1 overrides the specificity barrier set by alternative splicing and binds equally well to both "b" and "c" isoforms of FGFRs. To date, crystal structures of eight FGF–FGFR complexes have been published including FGF1–FGFR1c (PDB ID: 1EVT), FGF1–FGFR2c (PDB ID: 1DJS), FGF1–FGFR3c (PDB ID: 1RY7), FGF1–FGFR2b (PDB ID: 3OJM), FGF2–FGFR1c (PDB ID: 1CVS), FGF2–FGFR2c (PDB ID: 1EV2), FGF8–FGFR2c (PDB ID: 2FDB), and FGF10–FGFR2b (PDB ID: 1NUN). Structural data show that the D3 alternative splicing alters the primary sequences of key FGF binding sites in D3 including the bC'–bE and bF–bG loops and bF and bG strands to narrow the ligand binding specificity of FGFRb isoforms to mesenchymally expressed FGFs and that of FGFRc isoforms to epithelially expressed FGFs. The structural data also show that the specificity/promiscuity profile of a given FGF is principally dictated by the primary sequence of its N-terminal region. The structural data have begun to illuminate the shared primary sequence and secondary structural elements within the N-termini of members of a given FGF subfamily that explain overlapping

FGFR binding specificity/promiscuity profile of the subfamily (Goetz and Mohammadi 2013).

A wealth of genetic studies in mice and flies and cell-based studies has established that paracrine FGF–FGFR signaling is HS dependent. Recent data show that HS controls the diffusion of paracrine FGFs and hence shapes the morphogenetic gradients in the extracellular matrix. Aside from controlling the diffusion of FGFs, HS impinges on paracrine FGF signaling through many other mechanisms as well, including coordination/stabilization of FGF–FGFR binding and dimerization, providing thermal stability and protecting against proteolytic degradation, acting as a storage reservoir for ligand, and limiting the dimensionality of FGF (Beenken and Mohammadi 2009).

HS-assisted FGF–FGFR dimerization is a key event for signal transmission across the plasma membrane by paracrine FGFs. The symmetric model of FGF–FGFR dimerization bears a 2:2:2 FGF–FGFR–HS stoichiometry in which multivalent protein–protein contacts between the two FGF–FGFR halves are the main driving force of dimerization and HS facilitates these protein–protein contacts (Mohammadi et al. 2005b). The FGFRs, located in the center of the dimer, interact directly via the membrane-proximal end of D2. The FGFs, located at either side of the centrally located FGFRs, interact with both receptors through primary and secondary receptor binding sites. On the membrane distal end of the 2:2:2 FGF–FGFR–HS symmetric dimer, the spatially separate HS binding sites of two FGFs and of two receptor D2 domains merge into one large HS-binding canyon, into which two HS oligosaccharides bind. By simultaneously engaging the HS binding sites of FGF and receptor D2 domains in the canyon, HS fortifies both the primary FGF–FGFR interface and the dimer interface that consists of both direct receptor–-receptor and secondary ligand–receptor contacts. The nonreducing end of the oligosaccharide is tucked between the two receptor D2 domains, while the reducing end interacts with HS binding site of the ligand. On average, each oligosaccharide engages in about 30 hydrogen bonds with FGF and FGFR. The binding of HS does not cause significant conformational changes to occur in either the FGF ligand or receptor. Because the endocrine-acting FGF19 subfamily members have extremely low HS affinity, HS is incapable of enhancing endocrine FGF–FGFR binding and dimerization. To overcome this deficiency, endocrine FGFs rely on α-/β-Klotho coreceptors which form binary complexes with the cognate FGFRs of endocrine FGFs to increase the affinity of FGFR for endocrine FGFs and induce FGFR dimerization (Beenken and Mohammadi 2012).

Biology of the Target

HS- or Klotho-dependent dimerization of the FGFR extracellular domains juxtaposes the cytoplasmic kinase domains allowing them to transphosphorylate each other on A-loop tyrosines. A-loop tyrosine phosphorylation elevates the intrinsic kinase activity of FGFR kinase by stabilizing the active conformation of the kinase. A-loop phosphorylation is then followed by phosphorylation on tyrosines in the

C-tail, kinase insert, and juxtamembrane regions. Among many downstream signaling pathways that FGFR kinase activation triggers are RAS–MAPK, PI hydrolysis/PKC/Ca2+, PI3K–AKT, and RAC1/CDC42 signaling pathways (Dailey et al. 2005; Eswarakumar et al. 2005).

Phosphorylation of an FGFR-invariant tyrosine (Y766 in FGFR1) at the C-tail of FGFR creates a binding site for the SH2 domain of PLCγ (also known as FRS1) and is required for PLCγ phosphorylation and activation. PLCγ recruitment serves two purposes: (i) it facilitates phosphorylation of PLCγ to increase its enzymatic activity, and (ii) it brings PLCγ to the vicinity of its substrate PIP2 in the plasma membrane. Hydrolysis of PIP2 generates two second messengers: IP3 and DAG that stimulate Ca^{2+} release from intracellular stores and PKC activation, respectively. Activated PKC then activates the MAPK pathway in a Ras-independent manner by phosphorylating and activating Raf (Schlessinger 2000).

In contrast to PLCγ, CRKL is an adaptor protein that lacks intrinsic enzymatic activity. Recruitment of CRKL to the phosphorylated tyrosine in the juxtamembrane region of FGFR1 and FGFR2 leads to translocation of associated Rac1/Cdc42 to the plasma membrane. These G-proteins act through their effector protein, PAK, to activate the MAPK pathway by phosphorylating Raf1 and Mek1, leading to changes in cytoskeletal reorganization and cell motility (Seo et al. 2009).

FRS2α is another major adaptor protein for FGFRs that, unlike PLCγ and CRKL, associates constitutively (receptor tyrosine phosphorylation independent) with the juxtamembrane region of FGFR. Phosphorylation of FRS2α by the A-loop phosphorylated (activated) FGFR generates docking sites for the SH2 domains of the adaptor protein GRB2 and the phosphatase Shp2. Grb2 is constitutively associated with SOS, Cbl, and Gab1. Since SOS is a guanine nucleotide exchange factor (GEF) for Ras, Grb2–SOS activates the RAS–MAPK pathway. Grb2–Cbl mediates FRS2 degradation, since Cbl is an E3 ubiquitin ligase. Finally, the PI3K–AKT pathway is activated by Grb2–Gab1 (Gotoh 2008).

Target Assessment

Quantitative PCR is used to measure transcripts of FGFs and FGFRs in excised tissues. Serum and urine levels of FGF can be measured using ELISA. Immunohistochemical staining is also commonly used to detect the presence of FGF and FGFR proteins in tumor tissues.

Role of the Target in Cancer

Rank: 7 – clear role in cancer but not yet a primary therapeutic target.

Uncontrolled FGF signaling can be strongly oncogenic as it can promote not only cell proliferation and migration but also neoangiogenesis, as originally shown by Klagsbrun through studies in the 1970s and 1980s on what was then known as tumor angiogenesis factor (TAF). There is ample evidence for the involvement of

deregulated FGF signaling in human cancer. FGF signaling can be deregulated through a variety of mechanisms, including receptor mutations leading to constitutive activation or loss of ligand binding specificity, transcriptional upregulation of ligands and/or receptors leading to autocrine signaling, and genetic translocations generating constitutively active FGFR fusion proteins. Aberrant FGF signaling is best known for causing craniosynostosis and dwarfism syndromes such as Apert's syndrome (AS), Pfeiffer's syndrome (PS), and achondroplasia (ACH) (Wilkie 2005). Interestingly, many of the germ line mutations in FGFRs associated with skeletal disorders also occur as somatic mutations in cancer. The FGFR2 S252W and N549K mutations that cause AS and PS, respectively, are also detected in endometrial cancers. Mutations of the analogous N546 in FGFR1 and N535 in FGFR4 are detected in glioblastomas and rhabdomyosarcomas, respectively. A mutation of FGFR2 W290C associated with PS has been found in lung carcinomas. Mutations of K650 in the A-loop of FGFR3 kinase are responsible for severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN) as well as thanatophoric dysplasia types I and II (TDI, TDII). This residue is also frequently mutated in bladder and cervical cancers and in multiple myeloma. Mutations at the K650 codon in FGFR3 leading to thanatophoric dysplasia have been identified in spermatocytic seminomas, and these mutations increase in prevalence in sperm DNA as paternal age increases. FGFR3 G380R mutation, the most common cause of achondroplasia, is also seen in bladder cancer. This mutation leads to gain of function by promoting both receptor dimerization and receptor recycling, thereby impairing efficient receptor degradation. Many of these FGFR mutations have been structurally characterized and have been shown to lead to ligand-dependent or ligand-independent gain of function by enhancing ligand–receptor affinity, overriding ligand binding specificity, or relieving FGFR kinase autoinhibiton (Beenken and Mohammadi 2011).

There is also a long list of other FGFR somatic mutations/alterations detected in cancers that do not occur in skeletal disorders. In 8p11 myeloproliferative syndrome (EMS), a hematologic cancer, FGFR1 kinase is constitutively activated by being fused to eight different dimerizing/oligomerizing domains, including the zinc finger gene ZNF198 and BCR. Interestingly, blocking the recruitment of PLCg-1 to the FGFR1 kinase fusion proteins by mutating Y766 in the PLCg-1 binding site of FGFR1 attenuates EMS, suggesting a role for PI hydrolysis/PKC/Ca2+ signaling in the progression of this cancer. A subset of glioblastomas harbor oncogenic chromosomal translocations that fuse in frame the tyrosine kinase domains of FGFR1 or FGFR3 to the transforming acidic coiled-coil (TACC) domain TACC1 or TACC3. The FGFR3–TACC3 fusion occurs in bladder cancer as well. Additionally, FGFR1 kinase domain gain-of-function mutations are seen in glioblastomas, and malignant prostate cells have elevated levels of FGFR1 expression. Oncogenic t(4:14) rearrangements of FGFR3 have been described in multiple myeloma. Translocations of FGFR3 are also seen in peripheral T-cell lymphomas. FGFR4 mutations are found in rhabdomyosarcomas and correlate with more aggressive cancer. These mutations, including V550E and V550L, promote receptor autophosphorylation and constitutive signaling (Beenken and Mohammadi 2009).

Overexpression of both FGFs and FGFRs has long been implicated in cancer. FGF1 overexpression in ovarian tumors is associated with poor survival. FGF1, 2, 6, 7, 8, and 9 are found to be overexpressed in prostate cancers. Overexpressed FGF3 is seen in breast cancers as is FGF2. Overexpressed FGF8 has been detected in 50% of in situ prostate tumors and 80% of advanced prostate cancers, and FGF8 is also overexpressed in breast cancer. Overexpression of FGF5 has been recorded in esophagus, colon, prostate, and lung cancers as well as in melanoma. FGF10 is overexpressed in breast cancers. FGF18 is overexpressed in colon cancer as is FGF19. Hepatocellular carcinomas show overexpression of FGF2, 8, 17, and 18. Decreased expression of Sprouty proteins, major cytoplasmic negative regulators of FGF signaling, is observed in breast and prostate cancers (Turner and Grose 2010).

FGFR1 overexpression is seen in ovarian cancer, bladder cancer, oral squamous carcinoma, prostate cancer, squamous cell lung cancer, small and non-small cell lung cancer, breast cancer, and rhabdomyosarcoma. In ~10% of gastric cancers, gene amplification leads to increased FGFR2 expression which correlates with a poor prognosis. FGFR2 is also overexpressed in about 10% of human endometrial carcinomas and in triple-negative breast cancers. An FGFR2 variant with a C-terminal truncation is expressed in cancer cell lines. This truncation attenuates receptor endocytosis, leading to increased levels of cell surface receptor and accompanying signaling. FGFR2 overexpression enables FGF7-dependent stimulation of gastric cancer growth. Autocrine signaling can occur when mesenchymal isoforms of FGFR are misexpressed in epithelial tissues. For instance, a switch from FGFR2b to FGFR2c in bladder cancers signals a change to a more highly invasive bladder or prostate cancer (Knights and Cook 2010).

The mechanisms by which FGF–FGFR signaling leads to cancer are being continuously explored. One interesting recent development in cancer biology has been the association of FGF signaling with the Warburg effect (Hitosugi et al. 2009). The Warburg effect describes the phenomenon that cancer cells have greater uptake of glucose compared to normal cells and preferentially engage in glycolysis, even in the presence of oxygen. Pyruvate kinase (PK) is a rate-limiting enzyme in glycolysis and catalyzes the conversion of phosphoenolpyruvate to pyruvate. Of the four isoenzymes of PK (M1, M2, L, and R), PKM2 is found mainly in malignant cells. In normal physiology, pyruvate is subsequently converted to acetyl-CoA by pyruvate dehydrogenase A1 (PDHA1) and then enters the Krebs cycle, and only under hypoxic conditions will pyruvate be converted to lactate by lactate dehydrogenase A (LDH-A). In cancer cells, however, pyruvate is converted to lactate in both hypoxic and oxidative environments. This physiology of the Warburg effect is oncogenic, possibly because it assists rapid cell division by supplying an increased amount of basic building blocks like nucleic and amino acids through the upregulation of glycolysis. Interestingly, FGFR1 is implicated in mediating the Warburg effect by numerous mechanisms, including regulating pyruvate production, preventing pyruvate from entering oxidative metabolism, and increasing the conversion of pyruvate to lactate. FGFR1 directly tyrosine phosphorylates PKM2 to inhibit its activity. Additionally, PDH kinase 1 (PDHK1), a mitochondrial Ser/Thr kinase and an inhibitor of PDHA1, is activated by FGFR1-mediated tyrosine

phosphorylation and promotes cancer cell growth. FGFR1 also tyrosine phosphorylates LDH-A, thereby increasing its activity and improving its binding to its substrate, NADH (Fan et al. 2011).

FGF signaling has also been shown to confer loss of cell polarity and increased migratory phenotypes upon cancer cells by inhibiting epithelial–mesenchymal transition (EMT). For instance, pathological FGF signaling can lead to prostate carcinogenesis via EMT. Overexpression of FGF10 in prostatic mesenchyme leads to upregulation of androgen receptor expression in the adjacent epithelium and transforms the epithelium into well-differentiated prostate adenocarcinoma. Interestingly, dominant-negative FGFR1 is able to revert the induced cancer back to normal epithelium. Inducible expression of FGFR1 also leads to development of prostate adenocarcinoma through EMT and is associated with increased Sox9 expression, a known regulator of EMT. Deactivating inducible FGFR1 signaling led to the regression of prostatic intraepithelial neoplasia and slowed progression of adenocarcinoma (Yilmaz and Christofori 2009).

The list of mechanisms by which FGF signaling contributes to tumorigenesis keeps expanding. FGF1 and FGF2 are released when tumor cells decay in the necrotic center of tumor and act as an impetus for neoangiogenesis, with melanomas being an example of this process. By implanting xenografts of prostate cancer bone metastases from humans into mice, FGF9 signaling was found to have a role in mediating the progression of bone metastases in prostate cancer. Neutralizing antibody to FGF9 reduced the size of the bone tumors that developed from the xenografts. Another FGF9 subfamily member, FGF20, was found to be necessary for maintaining the mitogenic state of β-catenin-transformed rat kidney epithelial cells, since FGF20 siRNA interfered with β-catenin-mediated growth in these cells (Beenken and Mohammadi 2009).

High-Level Overview

Diagnostic, Prognostic, Predictive

Single nucleotide polymorphisms (SNPs) in FGFR2 are associated with breast cancers carrying the BRCA2 mutation. These SNPs are postulated to increase affinity for transcription factors, causing increased FGFR2 expression. Eighty percent of superficial papillary bladder tumors harbor gain-of-function FGFR3 mutations, and thus, FGFR3 mutations are being considered as a marker for non-muscle-invasive tumors. Detection of FGFR3 mutant proteins in urine has been shown to be a marker of tumor recurrence (Miyake et al. 2010).

FGFRs are beginning to be appreciated as prognostic markers for cancer. The G388R mutation in the transmembrane domain of FGFR4 is associated with prostate cancer progression, more aggressive colon cancer, and also predicts a poor prognosis in head and neck squamous cell carcinomas and gastric cancer. The mutation has been shown to slow down receptor internalization resulting in increased cell surface expression of FGFR4 and accompanied sustained signaling. In addition, the

expression of the mutated FGFR4 induces cell migration and has also been found to confer resistance to chemotherapy. Interfering with FGFR4 signaling with an antibody resensitized cells to chemotherapy (Beenken and Mohammadi 2009).

Therapeutic

Currently, only one FGF is being used as a therapy for cancer patients. Recombinant N-terminally truncated FGF7, known as palifermin, is FDA approved for the alleviation of radiation and chemotherapy-induced mucositis in cancer patients undergoing bone marrow transplant (Spielberger et al. 2004). By administering palifermin for 3 days prior to chemotherapy and then for 3 days following hematopoietic stem cell transplant, palifermin reduced patients' use of opioids, reduced the median duration of mucositis from 9 to 6 days, and reduced the incidence of severe mucositis from 62% to 20%. The improvement in quality of life provided by this drug is significant, since some patients were enabled to continue oral feeding during their cancer therapy who otherwise would have been prevented from doing so by severe mucositis. No significant side effects from palifermin have been documented. Palifermin primarily acts by inducing increased epithelial cell proliferation. The new epithelium that is induced can persist for up to 1 week following a dose of palifermin. Other proposed mechanisms of FGF7 action include upregulating *Nrf2* that activates genes encoding antioxidant enzymes. FGF7 may also favorably impact the course of mucositis by reducing the Th1/Th2 ratio of cytokines and by reducing TNF-a and IFN-γ through its induction of IL-13.

Presently, no drugs that exclusively target FGFRs are being used in cancer therapy, but sunitinib, a broad-spectrum receptor tyrosine kinase inhibitor with coverage of FGFRs but whose activity is primarily mediated through inhibition of VEGF, PDGF, and KIT pathways, is FDA approved for treatment of GI stromal tumors, renal cell carcinomas, and pancreatic and neuroendocrine tumors. There are currently over 100 active trials evaluating the activity of sunitinib against various cancers.

Preclinical Summary

In in vitro experiments, targeting FGFR signaling has been shown to slow down the growth of multiple myeloma, bladder cancer, glioblastomas, and lung and colon cancer. Expression of a kinase-dead dominant-negative version of FGFR3c but not FGFR3b led to apoptosis in colorectal cancer cells, highlighting the specificity of FGFR signaling in carcinogenesis. The two inhibitors that have long been used in the laboratory to inhibit FGFRs for in vitro experiments, SU5402 and PD173074, have had significant issues with toxicity in vivo. Numerous new receptor kinase inhibitors are in the pipeline. For instance, ponatinib is a pan-BCR–ABL and pan-FGFR inhibitor that, in addition to having promise for the treatment of imatinib-resistant CML, is able to induce apoptosis of cells from 8p11 myeloproliferative syndrome

patients by reducing phosphorylation of FGFR1 fusion proteins and can improve survival in mice transplanted with FGFR1 fusion kinase-expressing leukemia/lymphoma cell lines (Knights and Cook 2010). AZ12908010 is a compound with FGFR1-3 selective inhibition that suppresses myeloma, urothelial, breast, and gastric cancer cell lines. LY2874455 is a pan-FGFR kinase inhibitor that functions by reversibly competing for ATP. LY2874455 inhibits FGF-induced MAPK signaling in vivo in murine heart tissue and also reduces tumor growth in xenografts of urinary tract cancer, gastric cancer, and multiple myeloma. Inhibition of FGFR2 and FRS2 phosphorylation by LY2874455 in gastric cancer xenografts underlays the reduction in tumor growth. FIIN-1, discovered at the Dana–Farber Cancer Institute, was developed by analysis of the co-crystal structure of PD173074 with FGFR1, and it is the first selective and irreversible pan-FGFR inhibitor. It functions by binding a cysteine in the ATP binding site. FIIN-1 inhibits inducible FGFR1 activation in vitro and has antiproliferative activity against a wide range of tumor cell lines. Inhibition of FGFR3 by PD173074 reduces growth of UCC. It also was able to reduce cell growth in endometrial cancer cell lines expressing FGFR2 with kinase-activating mutations (N549K, K649N) as well as induce apoptosis in HER-2-positive breast cancer cell lines.

Monoclonal antibodies are of considerable value for cancers overexpressing certain FGFRs or FGFs or for cancers harboring FGFR extracellular domain mutations. Monoclonal antibodies directed against FGF8 and FGF19 have reduced tumor growth in mouse models of prostate cancer and hepatocellular cancer, respectively. An antibody against FGF8 has induced regression of established tumors in mouse models of breast cancer. An antibody against FGF2 has inhibited tumor cell proliferation in preclinical studies of melanoma, and monoclonal antibodies against FGFR3, such as R3Mab and PRO-001, have shown antiproliferative and cytotoxic properties in mouse models of bladder cancer and MM, respectively (Qing et al. 2009).

The research for monoclonal antibodies against FGF19 is of particular interest. FGFR4 signaling is required for hepatocarcinogenesis, since transgenic FGF19 mice that develop hepatocellular carcinoma fail to do so when bred with FGFR4 knockout mice (French et al. 2012), and an anti-FGFR4 monoclonal antibody was shown to inhibit FGFR4 signaling and tumor growth in vivo. Given a direct link between FGFR4 and liver tumorigenesis, this research is proof of principle that FGFR4 is a worthwhile therapeutic target. Importantly, FGF19 is specifically overexpressed in hepatocellular carcinomas (HCCs) containing the 11q13.3 amplicon, and FGF19 mediates its effects on tumor growth via b-catenin signaling (Sawey et al. 2011). Anti-FGF19 antibody 1A6 was able to inhibit 50% of cell lines harboring the 11q13.3 amplicon, but none of the HCC cell lines lacking the amplicon, suggesting that FGF biologic therapies will have their greatest impact when carefully targeted using genetic data. FGF19 signaling has also been implicated in colon cancer, and preclinical research is underway in this field. Colon cancers with activated pregnane X receptor (PXR) have aggressive characteristics of tissue invasion, metastasis, and cell growth, and this pathophysiology is mediated by PXR's activation of the FGF19 promoter (Wang et al. 2011). Anti-FGF19 antibody inhibits the aggressive

phenotype of colon cancer seen with activated PXR. These results raise the option of targeting the FGF19–FGFR4-β-Klotho pathway to inhibit tumor growth.

Clinical Summary

Compounds that have advanced farthest in clinical trials and are closest to therapeutic use tend to also inhibit RTKs other than FGFRs and usually to an even greater degree. These drugs include brivanib, dovitinib (formerly CHIR-258), and BIBF1120 that have been promising in their ability to inhibit VEGF-independent and VEGF-dependent angiogenesis, and their eventual use in the clinic is anticipated. Even though they broadly inhibit many RTKs, the side effects from these drugs are less severe than those from FGFR-specific drugs, since the efficacious dose is lower. Combining knockdown of FGFRs with knockdown of other RTKs in combination with radiotherapy is a possible alternative to avoid the significant side effects of full FGFR inhibition. As one example of this class of drugs, dovitinib is an inhibitor of FGFR and VEGFR, and a phase I/II dose-escalation study in patients with advanced melanoma showed a reasonable safety profile, with primary side effects of nausea, fatigue, and diarrhea. Twenty-six percent of patients had stable disease after 8 weeks of treatment, and 53% continued to have progressive grade III or IV disease (Kim et al. 2011). Most notable is brivanib, an RTK inhibitor selective for VEGFR and FGFR that has been evaluated in several phase III trials. In the BRISK-FL study, it was compared against sorafenib as first-line therapy for unresectable HCC but did not meet criteria for non-inferiority as median overall survival was 9.5 months for brivanib compared to 9.9 months for sorafenib. In the AGITG CO.20 trial, brivanib was added to the anti-EGF Ab cetuximab to treat chemo-refractory colorectal cancer, but it did not significantly increase overall survival relative to cetuximab alone and also increased toxicity.

Anticipated Results

Due to the high degree of homology between ATP-binding pockets of RTK domains, compounds that preferentially inhibit one receptor subfamily tend to cross-inhibit other subfamilies as well, so the development of FGFR-specific inhibitors for clinical application has been challenging. However, there are several FGFR-specific inhibitors currently under investigation. AZD4547 is being tested in clinical trials for its efficacy against breast cancers that overexpress FGFR1 and are estrogen receptor positive, with results pending. BGJ398, another kinase inhibitor, is going to be studied in clinical trials in patients with solid tumors where FGFR1 or FGFR2 has been amplified or there is a mutated FGFR3. The main downside of FGFR-specific inhibitors is that the high doses needed for therapeutic inhibition end up leading to serious side effects, such as deregulation of calcium and phosphate metabolism and tissue calcification due to inhibition of FGF23's hormonal functions (Knights and Cook 2010).

Several heparanoids that antagonize the ability of heparan sulfate to promote FGF–FGFR binding and signaling have been investigated in clinical trials. Among the most well known is suramin, a polysulfated naphthylurea. Although phase I/II trials showed some benefit in bladder, kidney, and prostate cancers, phase III trials have failed to demonstrate a gain in survival through suramin administration. Other heparanoids such as PI-88 (muparfostat) are still being evaluated in clinical trials, but it has yet to have a dosing schedule established that avoids significant hematologic toxicity. Thalidomide is a small molecule that inhibits angiogenesis including FGF2-induced angiogenesis (Beenken and Mohammadi 2009).

One strategy to target overexpression of FGFs in certain cancers is through the use of ligand traps that sequester FGFs. FP-1039 is a ligand trap consisting of the extracellular FGFR1c domain fused to the Fc of IgG and is being used in clinical studies to examine its efficacy against advanced or recurrent cancers (clinicaltrials.gov: NCT00687505). This can enable titrating factor levels to physiologic levels rather than completely abolishing the signal. A soluble form of FGFR found in breast cancers may eventually also be used for this purpose (Ezzat et al. 2001).

All of the above approaches – including monoclonal antibodies, kinase inhibitors, heparanoids, and ligand traps – hold promise for the treatment of FGFR-driven cancers. Clinical trial results for all these potential therapeutics are highly anticipated, and the field of FGF–FGFR signaling will be further stimulated once some of these therapeutics start to be used in the clinic.

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MMPs

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Barbara Fingleton

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Abstract

Matrix metalloproteinases (MMPs) are a family of endopeptidases that have long been associated with tumor invasion and metastasis. Several small molecule inhibitors were developed in the 1980s and 1990s, but all failed in large-scale clinical trials. While these failures undoubtedly dampened enthusiasm for further consideration of MMPs as important targets in cancer, continuing research has uncovered multiple roles for these proteases in many cancers as well as other diseases. Lessons learned from the early clinical failures have informed development of more specific reagents such as antibodies targeted to particular family members. Additionally, the strong association between MMP activity and tumor progression has stirred interest in the development of MMP-activated imaging agents that may be particularly useful for assessing response to other types of cancer therapy. Tumor-associated MMP activity has also been harnessed as a

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methodology for localized activation of pro-drugs, with the intention of reducing the toxic side effects of systemic chemotherapy. Overall, although broad-spectrum inhibition of MMPs is a failed clinical approach, there are still many potentially beneficial uses of targeting MMP activity in the cancer setting.

Keywords
Protease • Metastasis • Imaging • Pro-drug

Target

The matrix metalloproteinases (MMPs) are a family of extracellular, zinc-dependent proteases that are frequently over expressed in tumors of all types, either by the tumor cells or by resident or infiltrating cells within the surrounding stroma as a response to the tumor. Of the 23 family members in the human genome, 17 are secreted proteinases and 6 are cell surface-associated, either as transmembrane or GPI-linked proteins (Lopez-Otin and Overall 2002). The MMPs are major effectors of physiological and pathological extracellular matrix and basement membrane degradation. For example, proteolytic cleavage of fibrillar collagens (i.e. Types I, II, III) is restricted to only a few enzymes, but this includes MMPs-1, -8, -13, and -14, in addition to Cathepsin K. However, MMP family members have also been demonstrated to exhibit "sheddase" activity and process a wide variety of biologically-active proteins in addition to extracellular matrix substrates, including growth factor receptors, chemokines and cytokines, adhesion molecules, and apoptotic factors (Lynch and Matrisian 2002). The consequence of MMP activity includes alterations in cellular growth, apoptosis, migration, and metabolism (Gill et al. 2010). Importantly, genetic deletion of individual family members as observed using 'knock-out' mouse technology rarely leads to a severe baseline phenotype, indicating significant redundancy both within the MMP family, as well as with a closely related family of proteinases, the ADAM [A Disintegrin And Metalloproteinase] family. An exception is the MMP14-null mouse, which demonstrates a lethal phenotype. Homozygous-null mice have severe skeletal and muscular abnormalities and die at an early age (Gill et al. 2010). In humans, a group of genetic disorders considered types of 'vanishing bone' syndromes are associated with mutations of the *MMP2* gene (Al Aqeel et al. 2000; Zankl et al. 2005, 2007). The mutations render the enzyme inactive. Phenotypically, individuals who are homozygous for the mutations have arthropathies of the metatarsals and metacarpals, nodules in the feet and palms and severe osteopenia, with other manifestations such as cardiac irregularities also possible.

Biology of the Target

Of the 23 human MMP proteins, 10 have strong evidence linking them with cancer, either as positive or negative regulators of various processes (Overall and Kleifeld 2006).

MMP1, (interstitial collagenase) is the quintessential collagen-processing enzyme. It is largely lacking in adult mouse models, thus exploration of its relationship to particular cancers is predominantly correlative. In human colorectal cancers, MMP1 was identified as a marker of aggressive disease (Murray et al. 1996). The MMP1 promoter also can contain a functional polymorphism that has been associated particularly with melanoma, but also other cancers such as colon and lung (Fingleton 2006).

MMP2 (gelatinase A) and MMP9 (gelatinase B) are considered type IV collagenases and gelatinases. Preclinical studies indicate that inhibition of either enzyme can reduce cancer-induced invasion and angiogenesis in a variety of tumor models. These are the best studied of the MMPs as they were the first to be associated with the ability of cancer cells to demonstrate a metastatic phenotype (Liotta et al. 1980). Despite having similar substrate profiles, the expression patterns are dissimilar which may explain why genetic deletion or inhibition of one gelatinase is not simply compensated by the other. MMP9 is strongly associated with inflammatory cells as it is produced at relatively high levels by neutrophils and macrophages (Egeblad and Werb 2002). MMP9 has been implicated as a major regulator of angiogenesis, largely through generation of bioactive VEGF from the extracellular matrix. It is unclear whether this is a result of direct cleavage of larger VEGF isoforms, or of matrix proteins to which they are bound (Bergers et al. 2000; Lee et al. 2005). Additionally, lymphocyte-expressed MMP9 contributes to various leukemic diseases, although this may be independent of its proteolytic activity (Redondo-Munoz et al. 2010). In melanoma, tumor cell-expressed MMP2 has been shown to act as a cancer antigen in melanoma (Godefroy et al. 2005). Recent studies have suggested that both MMP2 and MMP9 are critical contributors to the formation of a pre-metastatic niche in different murine tumor models (Erler et al. 2009; Kaplan et al. 2005). For each enzyme, the relevant cell type appears to be bone marrow-derived and recruitment of these cells to the lung allows remodeling of the environment making it suitable for tumor cells that will metastasize there. The recent finding of an exosome-directed mechanism for pre-metastatic niche development (Peinado et al. 2012) has prompted a flurry of activity in understanding the molecules that comprise exosomes. Several additional proteases including MMPs-1, -14 and -19 have been identified and shown to contribute to tumorigenic behavior (Tauro et al. 2013).

MMP14, or MT1-MMP, is a membrane-type MMP that degrades fibrillar collagen and activates MMP2, amongst other functions. In a series of preclinical studies, MMP14 was demonstrated to be both necessary and sufficient for tumor cell invasion through collagen-containing basement membrane or extracellular matrix barriers (Sabeh et al. 2004). Since MMP14 is expressed in many tumor types, it is considered an attractive candidate for targeting to limit tumor spread and thus potentially reducing the mortality associated with metastasis.

MMP7 (matrilysin) is expressed predominantly by epithelial cells, and its inhibition reduces the development of benign intestinal polyps in a mouse model (Wilson et al. 1997). Multiple non-matrix cell surface protein substrates have been identified and verified in vivo for MMP7. These include the pro-apoptotic protein

Fas ligand, the processing of which allows selection for the malignant phenotype (Fingleton et al. 2001; Vargo-Gogola et al. 2002); RANKL, which is associated with bone metastasis (Lynch et al. 2005); and E-cadherin, tumor necrosis factor (TNF)-alpha, syndecan-1 and HB-EGF (Fingleton 2006).

MMP3 (stromelysin 1) is associated with tumor invasion in mouse models of breast cancer, an effect that seems attributable to matrix degradation (Witty et al. 1995). More recently, elegant studies have suggested that this proteinase can actually be responsible for tumor initiation (Sternlicht et al. 1999), potentially through the induction of Rac1 activity and reactive oxygen species (ROS) (Radisky et al. 2005).

As indicated above, several MMPs are considered to be relevant in the process of angiogenesis (reviewed in Kessenbrock et al. 2010). Recently two high-level imaging technologies, direct contrast-enhanced magnetic resonance imaging (DCE-MRI) and 3D contrast-enhanced ultrasound, were used in a study of 183 patients with breast cancer, where they confirmed the strong correlation between levels of MMPs-2 and -9 and vascularity within tumor lesions (Jia et al. 2014). Further, fluorescence molecular tomography-microcomputed tomography (FMT-mCT) imaging using an MMP activated probe showed that the highest levels of MMP activity were in areas of highly angiogenic invasive tumors in a xenograft model of skin squamous cell carcinoma (Al Rawashdeh et al. 2014). Interestingly, treatment with the receptor kinase inhibitor sunitinib significantly reduced the MMP activity as well as the level of angiogenesis. These data suggest imaging MMP activity could be indicative of successful anti-angiogenic therapies.

In contrast to the tumor-promoting function of many MMPs, there is evidence for MMP8 (collagenase-2) and MMP12 (macrophage metalloelastase) having a protective effect against tumor progression in preclinical models of skin (Lopez-Otin et al. 2009) and lung cancer (Acuff et al. 2006), respectively. Expression of these proteinases also correlates with better prognosis in human patients with melanoma, breast cancer and several other cancers. Additional evidence exists for protective effects of MMP9, 11, and 19 in specific model systems.

Target Assessment

The expression of MMP family members in tumor tissue has been assessed by immunohistochemistry and in situ hybridization, and serum levels of several MMP family members has been assessed by ELISA (Zucker et al. 1999). Substrate zymography is a widely used method for detecting MMPs in different types of sample. In particular, the use of gelatin as the substrate is popular as a means of detecting gelatinases. Gelatinase zymography is quantitative at low concentrations in the picomolar range (Kleiner and Stetler-Stevenson 1994); however processing and storage of samples such as serum can greatly influence results (Zucker and Cao 2005). Assays that detect the activity of MMPs-2 and MMP-9 in various biological samples are commercially available (Lombard et al. 2005). As with zymography, sample storage and processing can have a profound effect on the usefulness of these

assays. Several probes have been developed and applied to the imaging of MMP activity in vivo using either optical or MR imaging approaches (Scherer et al. 2008). In addition, some investigators have re-purposed MMP inhibitors that failed to show therapeutic efficacy in clinical trials as imaging agents for positron emission tomography (PET) (auf dem Keller et al. 2010; Wagner et al. 2009; Zheng et al. 2002). Such probes may be clinically useful both for following disease progression, and also for determining which patients could benefit from therapeutic strategies that target MMPs.

Role of the Target in Cancer

Rank: unknown to 10: 6.

High Level Overview

Diagnostic, Prognostic, Predictive Biomarkers

The use of serum MMP levels as a means of early detection of cancer has been attempted but offers little clinical utility. Other bodily fluids such as urine and saliva have also been considered for specific conditions, but their usefulness has not been completely validated (Roy et al. 2009). The detection of elevated levels of several MMPs in tumor tissue has been demonstrated to have prognostic value. Generally, elevated MMP levels correlates with poor prognosis, but MMP12 levels correlates with enhanced survival in patients with colon and hepatocellular cancer (Martin and Matrisian 2007). Recent studies indicate that a change in MMP activity may be useful in predicting response to therapy (Al Rawashdeh et al. 2014) or indicating successful surgical resection of a tumor (Nguyen et al. 2010), but these concepts requires further validation.

Therapeutics

Small molecule inhibitors of MMP activity have been tested in more than a dozen Phase III clinical trials. Batimastat (British Biotech, Ltd), a broad-spectrum hydroxymate-based inhibitor, was first tested in Phase I trials in 1992. Marimastat, a more soluble version of this inhibitor, was tested in Phase III trials in advanced pancreatic, gastric, brain and lung cancers (Coussens et al. 2002). No therapeutic benefit was demonstrated, although subset analysis suggested a potential benefit for some patients in the gastric cancer trial (Fingleton 2003). A dose-limiting cytotoxicity of arthralgia was identified, and presumed to be a result of inhibition of matrix turnover in joint cartilage. Prinomastat (Agouron/Pfizer) was designed to inhibit the gelatinases but not fibrillar collagenases and tested in advanced lung and prostate cancer. Prinomastat treatment also resulted in arthralgia, and did not enhance the

efficacy of standard chemotherapy in these trials (Smylie et al. 2001; Bissett et al. 2005; Ahmann et al. 2001). Bayer Corp developed the gelatinase-selective inhibitor Tanomastat and tested it in advanced pancreatic and small cell lung cancer. Tanomastat did not show superiority to standard therapy, and in fact exhibited sufficient lack of efficacy to result in early termination of the SCLC trial (Rigas et al. 2003). Bristol Myers Squibb developed a MMP inhibitor that was selective against sheddase activity and tested it in non-small cell lung cancer trial with no evidence of therapeutic benefit. Broad spectrum and semi-selective MMP inhibitors were believed to be limited by the inhibition of both promoting- and protective MMP family members, the lack of a pharmacodynamic assay to assess modulation of tumor-associated MMP activity, and an inability to identify patients likely to respond to MMP inhibition. MMP inhibitor trials in general tested effects on patients with metastatic disease, precluding conclusions regarding effects on the prevention of metastasis. However, marimastat treatment of breast cancer patients in the adjuvant setting similarly demonstrated no therapeutic benefit. As in other trials, arthralgia-type side effects were dose-limiting (reviewed in Fingleton 2003). Given these problems, more recent iterations of MMP inhibitors have focused on increasing selectivity so as to limit effects on protective MMPs. For example, a monoclonal antibody developed against MMP14 has shown efficacy in a range of preclinical models (Devy et al. 2009). Since MMP14 appears to be rate limiting for tumors to invade and grow (Sabeh et al. 2004), this may a particularly useful anti-cancer drug. Of course, because of the way in which success is evaluated in clinical trials, such anti-invasive and/or anti-proliferative effects would have to translate into increased patient survival for the MMP14 antibody to be regarded as efficacious.

Tumor-associated MMP activity has also been exploited as a means to deliver protease-activated pro-drugs to tumors (Vartak and Gemeinhart 2007). Various strategies have been used to increase circulation time and/or reduce cytotoxicity of chemotherapeutic agents including the addition of polyethylene glycol or albumin in addition to MMP-selective peptide cleavage sites (Mansour et al. 2003). Doxorubicin is the most frequently used agent for these types of studies. Since doxorubicin has a worrying dose-limiting toxicity on cardiac function, a significant improvement in tumor-to-heart ratio of drug concentration is an important goal. At least one MMP-selective pro-drug has achieved a tenfold improvement in this parameter, while also showing increased anti-tumor efficacy in a mouse model (Albright et al. 2005). Paclitaxel is another agent with a severe side-effect, neuropathy, that results from non-tumor uptake of the drug. The use of MMP-cleavable prodrug conjugates is currently being investigated as a promising method to alleviate toxicity (Huang et al. 2010). A recent study used the novel agent monomethylauristatin E combined with an MMP-cleavable domain in combination with an integrin-binding peptide to enable tumor targeting and facilitate intra-cellular delivery thus reducing systemic toxicity and increasing efficacy (Crisp et al. 2014). In another twist on the pro-drug idea, MMP inhibitors themselves have been designed as pro-drugs with an activation step required before they can function as inhibitors. Two activation strategies that have been investigated are bio-reduction in an hypoxic tumor

environment, or oxidation in an ROS-rich inflammatory environment (Daniel et al. 2011; Failes and Hambley 2007).

Pre-clinical Summary

A large body of literature supports the conclusion that inhibition of specific MMP family members in early stage cancer reduces tumor growth and progression in preclinical models (Coussens et al. 2002; Overall and Kleifeld 2006). MMP inhibition has been accomplished by genetic ablation of specific MMP genes in tumor and/or host tissues, transcriptional inhibition of tumor MMP gene expression using inhibitory DNA, RNA or ribozyme technology, overexpression of endogenous inhibitors of MMP activity, or inhibition of MMP activity using small molecule inhibitors. The broad-spectrum nature of previous small-molecule drugs meant that they could inhibit MMPs contributing to cancer progression, but also MMPs that may have beneficial anti-cancer activities. There is a small, but emerging, literature indicating that several MMPs can exhibit such anti-cancer 'protective' functions, which means that inhibition of some MMPs could actually exacerbate disease rather than treat it (Lopez-Otin et al. 2009; Martin and Matrisian 2007). Such MMPs are considered anti-targets.

Clinical Summary

The lack of efficacy, and in some cases detrimental effects, of small molecule MMP inhibitors in Phase III clinical trials has resulted in discontinuation of large clinical programs focused on MMP inhibition. Two major issues that emerged from the original studies are the development of dose-limiting toxicity, and lack of specificity resulting in inhibition of anti-target MMPs. Now, several biotechnology organizations are developing neutralizing antibodies for specific MMP family members, with encouraging preclinical results in terms of both efficacy and lack of joint-associated toxicities. The major hurdle for further clinical development of these agents is overcoming the memory of earlier failed trials. However, as has been shown with thalidomide (Zhou et al. 2013), rehabilitation is possible when there is a confluence of clinical need and preclinical evidence.

Anticipated High Impact Results

- The use of MMP inhibitors that avoid dose-limiting toxicities and protective MMPs, including neutralizing antibodies, represent compelling agents for cancer therapy. Combination of such agents with chemotherapy could provide appropriate settings in which to demonstrate efficacy. For example in a pre-clinical study with pancreatic adenocarcinoma, combination of an MMP14-targeted agent with

gemcitabine showed that targeting MMP14 could sensitize tumors to gemcitabine (Dangi-Garimella et al. 2011).

- The development of MMP-activated prodrugs has the potential to significantly increase the efficacy and reduce the toxicity of standard and novel chemotherapeutics. In this case, the MMP itself is not the target but provides a mechanism for enabling specific activation and delivery of otherwise toxic drugs directly within the environment of tumor cells.
- The development of MMP activity-based imaging probes provides an opportunity to capitalize on extensive knowledge on the prognostic and predictive value of tumor-associated MMP activity

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PDGF

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Abstract

Platelet-derived growth factor (PDGF) is a family of isoforms consisting of homodimers of A-, B-, C-, and D-polypeptide chains and the PDGF-AB heterodimer. The PDGF isoforms stimulate cell proliferation, survival and migration of fibroblasts, smooth muscle cells, pericytes, glial cells, and certain other cell types, via binding to α- and β-tyrosine kinase receptors. Activating mutations of PDGF receptors, or overproduction of PDGF-BB, has been observed in certain tumor types. PDGF has also an important role in the stimulation of cells in the stroma of solid tumors. Targeting of PDGF signaling may thus be of clinical value in tumor treatment.

Keywords

Platelet-derived growth factor (PDGF) • Anti-stromal therapy • Autophosphorylation • Immunohistochemical staining • Isoforms • Kinase

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inhibitors • Preclinical models • Selective low molecular inhibitors • Transcapillary transport in preclinical models

Target

Platelet-derived growth factor (PDGF) is a family of isoforms consisting of homodimers of homologous A-, B-, C-, and D-polypeptide chains and the PDGF-AB heterodimer (Andrae et al. 2008; Heldin and Westermark 1999). The precursors of PDGF A- and B-chains are proteolytically cleaved by furin-like proteases during secretion from the producer cell, whereas PDGF C- and D-chains are secreted as inactive precursors; N-terminal CUB domains need to be cleaved off by plasmin or tissue plasminogen activator before the PDGF-CC and -DD isoforms can bind to receptors. The C-terminals of the B-chain and a splice variant of the A-chain contain basic retention motifs, which restrict the actions of these isoforms to the local environment. The mature PDGF chains are about 100 amino acid residues long and are arranged in an antiparallel manner in the dimers.

PDGF isoforms act by binding to two structurally related tyrosine kinase receptors (Heldin and Westermark 1999). The A-, B-, and C-chains bind to the α-receptor, whereas the B- and D-chains bind to the β-receptor. Each receptor contains 5 Ig-like domains extracellularly which bind ligands and tyrosine kinase domains intracellularly which contain characteristic about 100 amino acid long inserts without homology to kinases.

PDGF isoforms and PDGF receptors have important roles during the embryonal development to regulate the formation of various mesenchymal cell types (Andrae et al. 2008). In the adult, PDGF regulates the interstitial fluid pressure of tissues and promotes wound healing.

Biology of the Target

Binding of PDGF isoforms to their receptors causes dimerization of the receptors, which brings their intracellular domains close to each other, allowing autophosphorylation in *trans* (Heldin and Westermark 1999). The autophosphorylation occurs on about ten tyrosine residues in each receptor and has two important functions: it causes a conformational change in the receptors which activates the kinases, and the phosphorylated tyrosine residues form docking sites for SH2 domain-containing signal transduction molecules. About ten different families of SH2 domain proteins are known to bind to the activated PDGF receptors, including molecules with intrinsic enzymatic activity, e.g., the tyrosine kinases of the Src family, the tyrosine phosphatase Shp2, the GTPase-activating protein for Ras (RasGAP), and phospholipase C-γ; signaling molecules that form stable complexes with enzymes, e.g., the regulatory p85 subunit of phosphatidylinositol 3′-kinase which forms a complex with the catalytic p110 subunit and the adaptor Grb2 which

forms a complex with Sos1, the nucleotide exchange molecule for Ras; or adapter molecules, like Shc, Nck, and Crk. Docking of these molecules to the PDGF receptors initiates intracellular signaling pathways leading to cell proliferation, survival, and migration.

Target Assessment

PDGF isoforms can be measured in plasma or serum or in tissues using immunohistochemistry. Since PDGF isoforms mainly act locally, and since free PDGF in the circulation is rapidly excreted via the kidneys, immunohistochemical staining of tissue sections is likely to be more informative than measurements in plasma or serum. It should also be noted that platelets contain high amounts of PDGF, so measurements in serum reflect mainly the platelet content of PDGF. PDGF and PDGF receptors can be assessed by immunohistochemical staining of tissues (Matei et al. 2006; Paulsson et al. 2009).

High-Level Overview

The gene for the PDGF B-chain has been acquired as a transforming retroviral oncogene, i.e., the *sis* oncogene of simian sarcoma virus (Doolittle et al. 1983; Waterfield et al. 1983). The mechanism of transformation was shown to involve autocrine stimulation of growth by a PDGF-like factor. This finding prompted studies to explore the possibility that PDGF is overexpressed also in human tumors. It was found that PDGF isoforms are commonly expressed in glioblastomas and sarcomas, i.e., tumors derived from cell types carrying PDGF receptors, suggesting autocrine stimulation of growth (Hermanson et al. 1992). Moreover, autocrine activation of the PDGF α-receptor has also been found to promote the progression of ovarian cancer (Matei et al. 2006). In addition, autocrine effects of different PDGF isoforms have been demonstrated in animal models of glioblastoma (Kilic et al. 2000).

PDGF has been found to be produced by several cancers that are derived from cell types that do not express PDGF receptors. Whereas the tumor cells in such cases normally do not respond directly to PDGF, cancer cells may express PDGF receptors in conjunction with epithelial-mesenchymal transition (EMT). Moreover, the PDGF produced can act on the non-tumor cells in the tumor microenvironment. Thus, PDGF has been shown to promote angiogenesis by acting on pericytes and smooth muscle cells of vessels (Hellström et al. 1999) and has also been shown to induce lymphangiogenesis (Cao et al. 2004). However, whether PDGF also can act directly on endothelial cells in vivo is still an open question. Expression of PDGF β-receptors on endothelial cells of prostate cancer metastases in the bone has been reported, whereas no expression in endothelial cells of normal tissue or tumors in soft tissue was seen (Uehara et al. 2003).

PDGF also acts on stromal fibroblasts and myofibroblasts and thereby, e.g., contributes to the increased interstitial fluid pressure (IFP) of solid tumors (Pietras et al. 2003). A possible mechanism is that PDGF stimulation promotes a contraction of stromal fibroblasts and myofibroblasts; since these cells make contacts with collagen fibers in the extracellular matrix, PDGF stimulation increases the tension in the tissue. PDGF-CC has been shown to mediate the angiogenic and tumorigenic properties of fibroblasts in tumors refractory to anti-VEGF treatment (Crawford et al. 2009). PDGF-BB promotes angiogenesis by inducing the production of erythropoietin in stromal cells, which induces endothelial cell proliferation, migration, sprouting, and tube formation, as well as extramedullary hemopoiesis, leading to increased oxygen perfusion (Xue et al. 2012). In addition, PDGF secreted by chronic lymphocytic leukemia cells has been shown to stimulate the production of VEGF by stromal cells (Ding et al. 2010).

There are examples of tumors in which the genes for PDGF or PDGF receptors have been found to be mutated (Pietras et al. 2003, and references therein). In dermatofibrosarcoma protuberans (DFSP), the gene for collagen 1A1 has been shown to be fused with the PDGF-B gene, leading to the constitutive production of a fusion protein that is processed to PDGF-BB, which then acts in a classical autocrine manner. In chronic myelomonocytic leukemia (CMML), fusions of the transcription factor Tel or rapaptin-5 with a truncated PDGF β-receptor have been found. Similarly, in hypereosinophilic syndrome (HES), fusions between FIP1L1 and a mutated form of the PDGF α-receptor have been described. In these cases, the fusion partner can dimerize or oligomerize, causing constitutive activation of the receptor kinases. Activating point mutations in the PDGF α-receptor have been described in 5–15% of patients with gastrointestinal stromal tumors (GIST), and in 5–10% of glioblastomas, the α-receptor gene is mutated or amplified (Pietras et al. 2003; Verhaak et al. 2010; Paugh et al. 2013; Ozawa et al. 2010). PDGF receptor amplification has also been observed in chordomas (Tamborini et al. 2006) and choroid plexus carcinomas (Nupponen et al. 2008).

Diagnostic, Prognostic, Predictive

Overexpression or overactivity of PDGF and/or PDGF receptors correlates with increased malignancy and poor prognosis in animal models and in patients with colorectal (Kitadai et al. 2006a, b), breast (Seymour et al. 1993), ovarian (Henriksen et al. 1993), and lung (Donnem et al. 2008) cancer.

Therapeutics

Inhibitory DNA aptamers against PDGF-BB (Sennino et al. 2007), inhibitory antibodies against the α-receptor (Loizos et al. 2005) and the β-receptor (Jayson et al. 2005), and selective low molecular weight inhibitors of the PDGF receptors kinases (Knight et al. 2010) have been used in preclinical models, as well as in

clinical trials. Examples of selective low molecular inhibitors include imatinib, sunitinib, and sorafenib. These inhibitors are not specific for PDGF receptor kinases; imatinib also inhibits the Abl and Arg kinases and the stem cell factor receptor (Kit), and sunitinib and sorafenib also inhibit vascular endothelial growth factor (VEGF) receptor and fibroblast growth factor receptor families (Knight et al. 2010). Beneficial effects of treatment of patients with CMML and HES (Apperley et al. 2002), GIST (Heinrich et al. 2003), and DFSP (McArthur et al. 2005) with imatinib have been reported, and imatinib is routinely used clinically for these indications. After resistance mechanisms have occurred by mutations in the PDGF receptor kinase domains, other kinase inhibitors such as sunitinib, sorafenib, and nilotinib have been shown to have clinical efficiency. Moreover, stable expression of siRNA directed against the unique fusion sequence of Tel-PDGF β-receptor has been shown to sensitize tumors to treatment with imatinib or the mTOR inhibitor rapamycin in preclinical models (Chen et al. 2004). In contrast, no clinical benefit has been reported from treatment of glioblastoma with imatinib (Dresemann et al. 2010).

In view of the fact that production of PDGF by tumor cells enhances stroma recruitment, targeting of PDGF and PDGF receptors in the stroma could also be beneficial in tumor treatment. Thus, PDGF receptor kinase inhibitors may synergize with anti-VEGF in antiangiogenic treatment (Bergers et al. 2003). However, other investigators have reported only modest synergism in antiangiogenic therapy (Kuhnert et al. 2008). A PDGF-B aptamer has been shown to improve proliferative retinopathies (Akiyama et al. 2006). Moreover, anti-stromal therapy using PDGF receptor kinase inhibitors has been shown to inhibit tumor growth in animal models of colorectal cancer (Kitadai et al. 2006) and cervical cancer (Pietras et al. 2008), although these observations have not been validated in clinical studies. There are indications that induction of PDGF isoforms and PDGF receptors is part of the EMT program, which correlates to increased invasiveness and metastasis of epithelial tumors. Thus, inhibition of PDGF receptor signaling has been shown to inhibit metastasis of mammary tumors (Jechlinger et al. 2006) and prostate tumors (Russell et al. 2009). Finally, PDGF inhibition reduces the interstitial fluid pressure of solid tumors, leading to enhanced drug uptake by increased transcapillary transport in preclinical models (Pietras et al. 2003). There are, however, studies which suggest that inhibition of PDGF β-receptor can cause serious side effects. Administration of an inhibitory antibody to patients with ovarian cancer caused fluid accumulation and formation of ascites necessitating early discontinuation of this dose escalation study (Jayson et al. 2005). Moreover, imatinib treatment of colorectal and pancreatic tumors grown in mice was found to increase tumor growth by decreasing the pericyte content in the environment of the tumors (McCarty et al. 2007).

Preclinical Summary

PDGF is an important mitogen for fibroblasts, smooth muscle cells, glial cells, and certain epithelial cells. Preclinical models have revealed tumorigenic and growth-promoting effects of PDGF, both directly on tumor cells through autocrine

stimulation or by mutation of PDGF receptors and indirectly via stimulation of angiogenesis and stromal fibroblasts. In several animal models of PDGF/PDGF receptor-dependent tumors, treatment by PDGF antagonists has been shown to have beneficial effects. Moreover, inhibition of PDGF signaling in non-tumor cells of the tumor tissue has been shown to inhibit angiogenesis and to lower the interstitial tumor pressure and thus improve drug uptake.

Clinical Summary

In patients with the rather uncommon tumors that are driven by PDGF or PDGF receptor overactivation, i.e., certain cases of DFSP, CMML, HES, and GIST, treatment by PDGF receptor kinase inhibitors has beneficial effects. For tumors where PDGF is not the dominant "driver" of tumor growth, targeting PDGF or PDGF receptors as a single agent has little clinical activity. It remains to be determined if targeting PDGF/PDGF receptors in the stroma compartment, along with other therapies, can improve outcomes for patients with malignancies.

Anticipated High-Impact Results

- Demonstration in patients that PDGF receptor inhibitors can improve chemotherapy of solid tumors by improving drug uptake as a consequence of lowering the tumor intracellular fluid pressure
- Demonstration in patients that PDGF antagonists synergize with VEGF antagonists in antiangiogenic treatment
- Demonstration that PDGF is an important part of the epithelial-mesenchymal transition program and that inhibition of PDGF decreases metastases of certain tumors

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TIE

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Pipsa Saharinen and Tanja Holopainen

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Abstract

The Tie receptor tyrosine kinase (RTK) family that comprises the Tie1 and Tie2 receptors forms a distinct subfamily among the mammalian RTK families. Of these endothelial tyrosine kinases, Tie2 binds to angiopoietins (Ang, Angpt), while Tie1 is an orphan receptor with no characterized ligand so far. Structurally Tie1 and Tie2 share 70% homology in their intracellular domains, and 30% in their extracellular domains, which consist of epidermal growth factor, immunoglobulin, and fibronectin type III homology domains. The expression of Tie1 and Tie2 is almost exclusively restricted to endothelial cells, and the angiopoietin-Tie system is a significant regulator of both blood and lymphatic vessel development, normal vascular homeostasis, and pathological angiogenesis in tumors.

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Ang2 expression is elevated in human cancer, and the Ang-Tie system has recently emerged as a potential novel target for anti-angiogenic tumor therapies. Currently, angiopoietin-targeting agents are tested in phase 1-3 clinical oncology trials, also in combination with VEGF-based anti-angiogenic therapies.

Keywords
Ang1 • Ang2 • Biomarker • Angpt1 • Angpt2 • Tek • Anti-angiogenic therapy • Tumor angiogenesis • Peptibody • Antibody • Angiopoietin growth factor • Angiopoietin • Angiopoietin-Tie system • Tie1 • Tie2 • Metastasis

Target

The Tie1 receptor tyrosine kinase (RTK) and the homologous Tie2 (Tek) receptor form a distinct subfamily among the 20 mammalian RTK families. Tie1 and Tie2 share approximately 70% homology in their intracellular tyrosine kinase domains and 30% homology in their extracellular domains, which consist of epidermal growth factor, immunoglobulin, and fibronectin type III homology domains. Angiopoietin growth factors (Ang1 (Angpt1), Ang2 (Angpt2), and Ang4 (Angpt4, the murine ortholog was originally termed Ang3)) are ligands for Tie2, while Tie1 is an orphan receptor with no characterized ligand. Tie1 and Tie2 are almost exclusively expressed in endothelial cells, and the angiopoietin-Tie system is a significant regulator of both blood and lymphatic vessel development, normal vascular homeostasis, and pathological angiogenesis (reviewed in Eklund and Saharinen 2013). Targeting the Ang-Tie system has recently emerged as a potential strategy for complementing current anti-angiogenic tumor therapies, and angiopoietin-targeted drugs are tested in clinical oncology trials (reviewed in Saharinen et al. 2011). Some of these investigational drugs have advanced into phase 3.

Biology of the Target

After the initial assembly of the primary vascular plexus, which requires VEGF and its receptor VEGFR-2, the Ang-Tie signaling pathway is paramount for the development of the cardiovascular and lymphatic systems at embryonic (E) days 10.5–13.5 (Fig. 1; reviewed in Eklund and Saharinen 2013). The *Tie2* gene-targeted mouse embryos have reduced numbers of endothelial cells, develop hemorrhages, and show impaired cardiac development resulting in death of the embryos by E10.5. The deletion of *Ang1* in the mouse genome resulted in a phenotype very similar to that of *Tie2*$^{-/-}$embryos with impaired cardiac development and defective remodeling of the primary vascular plexus and embryonic lethality by E12.5. In addition, endothelial cells in the *Ang1*$^{-/-}$ embryos appeared rounded and poorly associated with basement membranes. The embryonic deletion of *Ang1* specifically in cardiomyocytes reproduced much of the phenotype of the full *Ang1* knockout,

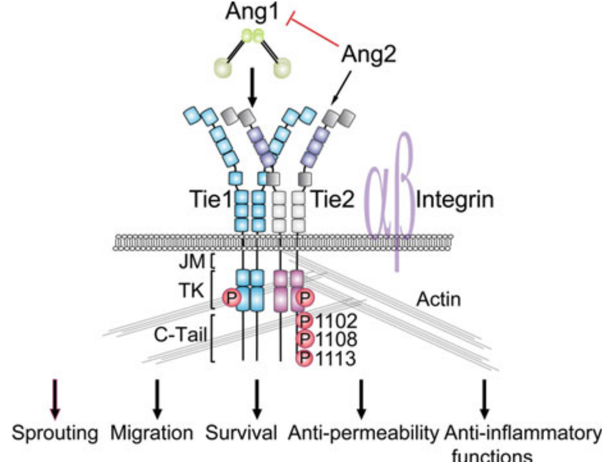

Fig. 1 The angiopoietin-Tie system. Ang1 and Ang2 bind to the Tie2 receptor tyrosine kinase on endothelial cells. Ang1 induces Tie2 activation, while Ang2 provides an antagonist or a weak agonist activity, depending on the context. The angiopoietins do not directly bind to Tie1; however, Tie1 is phosphorylated by Ang1, most likely via its interaction with Tie2. The possible signaling pathways induced by Tie1 remain to be defined but Tie1 has been found to modulate Tie2 activity. Key phosphotyrosines are shown for the carboxy-terminus of Tie2. The biological effects of Ang1 are indicated. Exogenous Ang1 has been found to inhibit cytokine-induced endothelial cell permeability and to control the inflammatory response by inhibiting leukocyte adhesion to endothelial cells, while Ang2 has been shown to promote the opposite. The Ang-Tie system has been also found to interact with integrin signaling: Ang2 may promote angiogenesis via integrin signaling in the tip cells of sprouting vessels and destablize vascular endothelium via integrin beta 1 signaling (Felcht et al. 2012; Hakanpaa et al. 2015). (The image has been modified from Brindle et al. 2006)

suggesting that impaired cardiac function results in hemodynamic problems that underly the observed vascular defects (Jeansson et al. 2011). Ang1 was also required for postnatal retinal vascularization (Lee et al. 2013) and to limit pathological tissue responses including kidney injury, wound healing and retinal neovascularization, but was dispensable during normal adult homeostasis (Jeansson et al. 2011; Lee et al. 2013).

Tie1 is an orphan receptor, with no known ligand (Partanen et al. 1992). However, Tie1 is phosphorylated in response to angiopoietins, in a complex with Tie2 (Saharinen et al. 2005). Metalloprotease-mediated shedding of the Tie1 ectodomain has been suggested to indirectly modulate Tie2 signaling (Marron et al. 2007). Recent in vivo evidence indicated that Tie1 is required for Tie2 activation by Ang1 (D'Amico et al. 2014). In addition, Tie1 was reported to regulate cell surface maintenance of Tie2, and in the absence of Tie1, Ang1-induced Tie2 activation was attenuated (Savant et al. 2015). The targeted deletion of *Tie1* in mouse embryos impaired endothelial integrity and induced lethality starting at around E13.5, and the deletion of both *Tie1* and *Tie2* resulted in a similar but more severe phenotype than that of the *Tie2*-deficient embryos (Puri et al. 1995; Savant et al. 2015). Chimeric analysis further showed that Tie1 and Tie2 were required in the microvasculature during late

embryogenesis and that *Tie1*- and *Tie2*-deficient endothelial cells were largely excluded in the adult vasculature (Partanen et al. 1996; Puri et al. 1999). Tie1 is also critical for lymphatic development; the jugular lymph sacs appear malformed in embryos with reduced Tie1 levels at around E15.5, and the embryos were swollen even earlier (D'Amico et al. 2010; Qu et al. 2010). Tie1 is dispensable during adulthood, however, conditionally targeted *Tie1* mice with reduced Tie1 levels were protected against atherosclerosis (Woo et al. 2011). Furthermore, tumor growth and vascularization were reduced in mice, where Tie1 was deleted from the endothelium (D'Amico et al. 2014).

The *Ang2*$^{-/-}$ mice survive until adulthood, or depending on the background strain, die postnatally (Gale et al. 2002). The remodeling of the lymphatic vasculature of *Ang2*$^{-/-}$ mice is defective, leading to malfunctioning lymphatic vessels with abnormally attached smooth muscle cells and accumulation of chylous ascites (Gale et al. 2002). While the blood vasculature of the *Ang2*$^{-/-}$ mice appeared normal, save for intravitreal hyaloid vessels, ectopic overexpression of Ang2 in developing embryos resulted in lethality and vessel regression, resembling *Tie2*$^{-/-}$ or *Ang1*$^{-/-}$ embryos (Maisonpierre et al. 1997). These results suggest that under certain circumstances Ang2 may act as an antagonist of Ang1. However, in the lymphatic and tumor vasculature, and in stressed endothelial cells Ang2 is reported to function as a Tie2 agonist (Daly et al. 2006; Daly et al. 2013; Thomson et al. 2014). Interestingly, the double Ang1/Ang2 deficient mice developed glaucoma due to abnormal Schlemm's canal formation, suggesting for compensating functions of Ang1 and Ang2 during this developmental process (Thomson et al. 2014).

Ang2 is expressed in endothelial cells where it is stored in Weibel-Palade bodies. Ang2 is released from endothelial cells in response to inflammatory stimuli, and its expression is also increased in hypoxia. Ang2 is highly upregulated in the tumor-associated endothelium of mouse tumors even before hypoxic induction of VEGF expression, correlating with regression of the co-opted tumor blood vessels (Holash et al. 1999). Therefore, in the absence of VEGF, Ang2 has been suggested to induce endothelial destabilization. The secondary avascular tumor that develops due to regression of the co-opted vessels is highly hypoxic, resulting in increased expression of both Ang2 and VEGF, and robust induction of neoangiogenesis ("angiogenic switch") (Fig. 2).

Angiopoietins utilize a unique Tie receptor activation mechanism, which is not used by other soluble growth factor ligands (Fig. 3; Fukuhara et al. 2008; Saharinen et al. 2008). In contacting endothelial cell junctions, angiopoietins induce homomeric Tie2 complexes that reach in trans across the cell–cell junction and preferentially activate the phosphatidyl-inositol-3 kinase (PI3K)–Akt kinase signal transduction pathway (Fukuhara et al. 2008; Saharinen et al. 2011). This pathway promotes cell survival, but Akt activation leads also to phosphorylation and activation of endothelial nitric oxide synthase (eNOS), and inhibition of the Forkhead box protein O1 (FOXO1) transcription factor that is implicated in e.g. endothelial growth regulation. Activated FOXO1, in turn, induces the expression of Ang2, which may function as an autocrine endothelial cell survival factor in stressed endothelial cells (Daly et al. 2006). However, Ang2 induces only weak Tie2

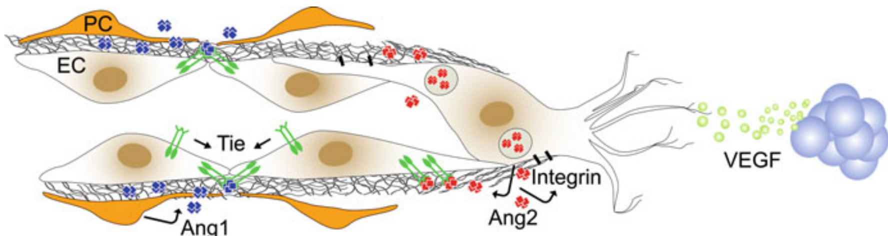

Fig. 2 A model of Ang1 and Ang2 functions in vascular sprouting. Perivascular cells, pericytes (*PC*), and vascular smooth muscle cells associate with the endothelial "stalk" cells of growing blood vessels, where Ang1 induces Tie2 activation at cell–cell junctions. This provides signals for endothelial cell survival and stability and decreases fluid leakage across the vessel wall. Endothelial tip cells express Ang2 and Tie1, whereas the levels of Tie2 are low. Ang2 may diffuse to the nearby stalk cells and decrease the Ang1-induced Tie2 signals, destabilizing the vessel structure. Hypoxia induces VEGF secretion by tumor cells, and in concert with VEGF, Ang2 may induce vessel sprouting. The angiopoietins also function by interacting with the endothelial cell integrins and pericellular matrix, possibly contributing to sprouting angiogenesis and destablization of vessel integrity. (The image has been modified from Saharinen et al. 2011)

phosphorylation, and exogenous Ang2 counteracts Ang1-induced Tie2 phosphorylation; thus, the molecular mechanisms of Ang2 action have remained incompletely understood. Tie2 also interacts with ABIN-2 (A20 binding inhibitor of nuclear factor-kappaB (NF-kappaB)), which inhibits NF-kappaB activity and may mediate some of the Ang1-induced antiapoptotic or anti-inflammatory effects. Ang1-Tie2 signaling improves endothelial barrier function via multiple mechanims involving regulation of actin cytoskeleton and VE-cadherin deposition in the cell junctions (Frye et al. 2015). In addition, Ang1 recruits the vascular endothelial protein tyrosine phosphatase (VE-PTP) to cell–cell contacts (Saharinen et al. 2008).

In mobile cells, matrix-bound angiopoietins activate Tie2 in cell-matrix contacts to induce matrix adhesion and modulate cell migration via activation of the extracellular regulated kinases (Erk) (Fukuhara et al. 2008), and phosphorylation of the adaptor protein DokR (Saharinen et al. 2008). Alpha5beta1 and alphavbeta3 integrins have been reported to interact with Ang1 and Ang2 activated Tie2 receptor, whereas Ang2 may directly interact with integrins in angiogenic tip cells (Thomas et al. 2010; Felcht et al. 2012; Hakanpaa et al. 2015) and mediate vascular destabilization particularly via beta1 integrin activation (Hakanpaa et al. 2015).

Target Assessment

Preclinical data shows that Ang2 is upregulated in angiogenic tumor vasculature, but expressed at a low level in the quiescent vasculature. Ang2 blocking inhibits tumor growth, by reducing tumor vascularization. Blocking Ang2 was also found to found to inhibit lung metastasis, lymphangiogenesis, and lymph node metastasis in

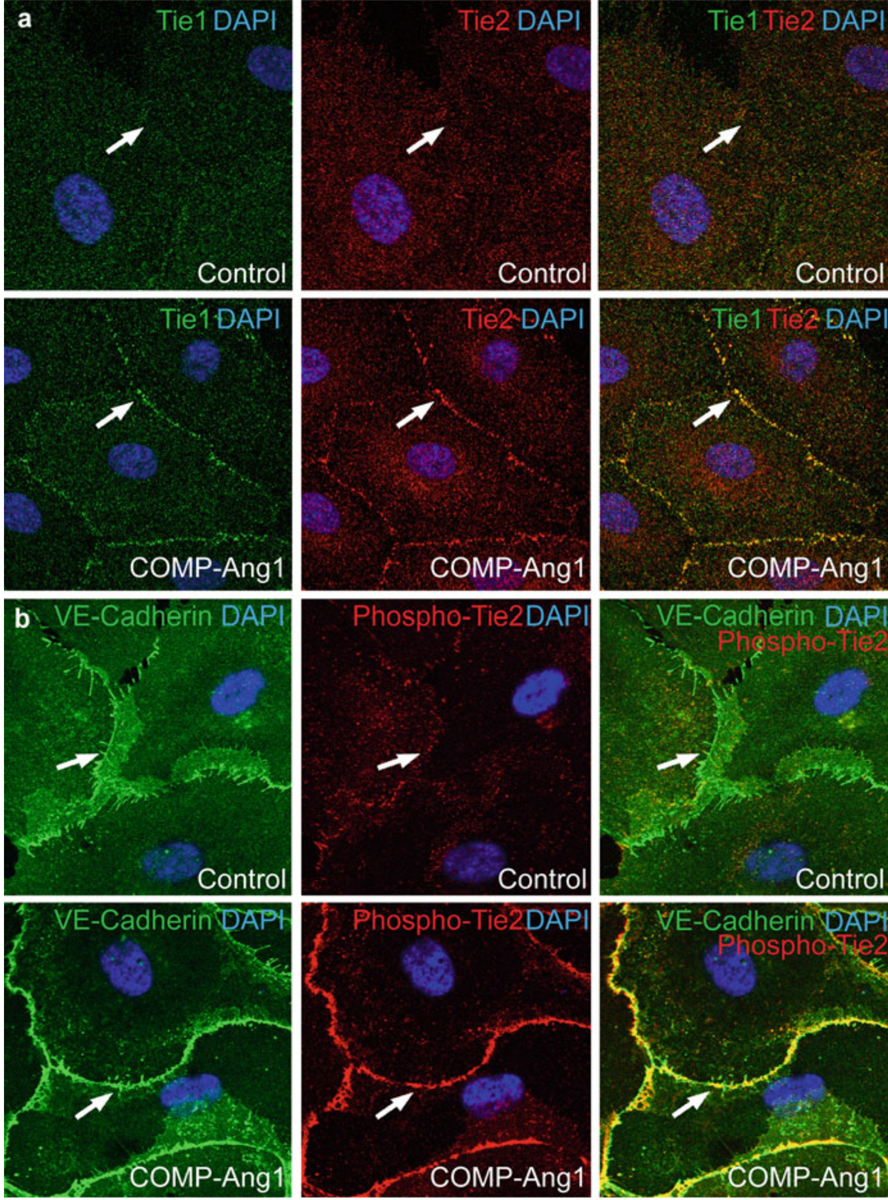

Fig. 3 Angiopoietin-induced Tie1 and Tie2 translocation to endothelial cell–cell contacts. (**a**) Primary endothelial cells were stimulated with COMP-Ang1, fixed, and stained for Tie1 (*green*) and Tie2 (*red*). (**b**) Tie2 overexpressing HUVEC were stimulated with COMP-Ang1, fixed, and stained for phosphorylated Tie2 (Phospho-Tie2, *red*) and VE-cadherin (*green*) (COMP-Ang1 (Cho et al. 2004) is a kind gift from Dr. Koh (Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea))

preclinical models (Mazzieri et al. 2011; Holopainen et al. 2012). Tie1 is a second relevant target of the angiopoietin-Tie pathway, because tumors grew less when Tie1 was deleted from adult mouse endothelium, while *Tie1* deletion did not affect the normal vasculature (D'Amico et al. 2014). In summary, Ang2 and Tie1 appear as potential novel targets for anti-angiogenic therapies; however, their functions and signaling mechanisms should be better understood for the design of optimal anti-angiogenic tumor treatment strategies.

Role of the Target in Cancer

Preclinical data supports a role for Tie1 in tumor angiogenesis. Ang2 blocking therapies are in phase 1-3 clinical trials.

High-Level Overview

Diagnostic, Prognostic, and Predictive

In human cancer patients, increased circulating plasma Ang2 or tumor mRNA levels have been reported in glioblastoma, renal cell carcinoma, colorectal cancer, breast cancer and melanoma (Sfiligoi et al. 2003; Helfrich et al. 2009; Goede et al. 2010; Wang et al. 2014; Scholz et al. 2015), and found to correlate with poor outcome. In colorectal carcinoma and renal cell carcinoma circulating Ang2 has been associated with poor response to VEGF signaling inhibitors, bevacizumab and sunitinib (Goede et al. 2010; Motzer et al. 2014) whereas Ang2 was not associated with poor outcome when Ang2 was analyzed in the vasculature of renal cell carcinoma by immunohistochemistry (Rautiola et al. 2016). The tumor endothelial cells have been reported as the primary source of Ang2 (Scholz et al. 2015; Rautiola et al. 2016).

Therapeutics

Investigational drugs blocking Ang-Tie signaling include specific ligand neutralizing peptibodies (trebananib), humanized monoclonal antibodies (nesvacumab and MEDI3617), and biscpeficic antibodies neutralizing both Ang2 and VEGF (vanucizumab). In addition, tyrosine kinase inhibitors which block, among other kinases, Tie2 kinase activity are being developed (regorafenib, altiratinib, Arry-614).

Preclinical Summary

A number of studies carried out in various preclinical murine models have shown that blocking Ang2 inhibits tumor angiogenesis and growth; for original articles see (Saharinen et al. 2011). Peptibody (Oliner et al. 2012) and antibody (Brown

et al. 2010; Daly et al. 2013) inhibitors of Ang2 have been shown to reduce tumor growth in numerous mouse models. An Ang2-specific aptamer also inhibited tumor growth in a murine colon carcinoma model (Sarraf-Yazdi et al. 2008).

In addition to inhibiting new vessel formation, Ang2 inactivation affects the vascular phenotype of tumors. The blood vessels of syngeneic and transgenic murine tumors in $Ang2^{-/-}$ mice displayed a more mature phenotype with increased numbers of pericytes and a narrower diameter than tumor vessels in wild-type mice (Nasarre et al. 2009). In a xenograft model, blocking Ang2 resulted in normalized tumor vessels with increased levels of adhesion molecules in cell–cell junctions, increased pericyte coverage, and reduced endothelial sprouting and vascular remodeling that produced smaller, more uniform vessels when compared to control antibody treated animals (Falcon et al. 2009).

Ang2 blocking agents have demonstrated enhanced antitumor effects when combined with cytotoxic drugs or anti-VEGF agents as compared to monotherapy (Brown et al. 2010; Hashizume et al. 2010; Daly et al. 2013; Kienast et al. 2013; Srivastava et al. 2014). A chimeric decoy receptor, DAAP (double anti-angiogenic protein), simultaneously capable of blocking VEGF and angiopoietin-1 and -2, effectively caused regression of the tumor vasculature and decreased metastases in implanted and spontaneous solid tumors (Koh et al. 2010). DAAP also reduced ascites formation and vascular leakage in an ovarian carcinoma model (Koh et al. 2010). In summary, the simultaneous blockade of VEGF and angiopoietins might be a relevant therapeutic strategy, which is further evaluated in ongoing clinical trials.

Blocking Ang1 has shown limited efficacy in reducing tumor growth, but has enhanced the effects of Ang2-blocking agents in some studies (Coxon et al. 2010; Falcon et al. 2009). In opposite, recombinant Ang1 protein potentiated chemotherapy-induced tumor growth inhibition in a mouse model of lung carcinoma by inducing vessel normalization that was sufficient to improve vessel perfusion (Hwang et al. 2009). Furthermore, Ang1 could be functionally important during VEGF or Ang2 inhibition, by mediating tumor vessel normalization resulting in improved endothelial cell-cell junctions and endothelial cell-smooth muscle cell association (Winkler et al. 2004; Falcon et al. 2009; Huang et al. 2009).

In contrast to Ang1, Ang2 is pro-inflammatory and has been found to sensitize endothelial cells to tumor necrosis factor-α-induced expression of endothelial cell adhesion molecules, which could promote inflammation and tumor progression. Ang2 has been also found to modulate the proangiogenic properties of Tie2-expressing macrophages (TEMs), which may contribute to tumor progression (Mazzieri et al. 2011).

Blocking Ang2 inhibited also lymphangiogenesis in human tumor xenografts in immunodeficient mice, as well as lymph node and lung metastasis (Holopainen et al. 2012). Mechanistically, the anti-Ang2 antibody induced the internalization of Ang2-Tie2, but not Ang1-Tie2 complexes from endothelial cell junctions and prevented new complexes from forming. Importantly, blocking Ang2 reduced the abnormalities of metastasis-associated lung capillaries and improved endothelial cell adherens junctions, suggesting that Ang2 modulated endothelial integrity

inhibiting tumor cell extravasation (Holopainen et al. 2012). Thus, the proangiogenic and protumorigenic effects of Ang2 appear to involve multiple mechanisms.

Tie1 is expressed in the tumor vasculature. Interestingly, Tie1 was found to regulate tumor angiogenesis and growth, as conditional Tie1 deletion in endothelial cells reduced both processes (D'Amico et al. 2014). Importantly, the normal vasculature was not affected in *Tie1*-deficient mice, while the tumor endothelial cells showed increased apoptosis. Tumor growth inhibition in *Tie1*-deficient background was as great as achieved by VEGF- or VEGFR-2 blocking antibodies in wild-type mice. Of note, Ang1/Ang2 blocking, but not VEGF-VEGFR-2 blocking, further decreased tumor growth inhibition in *Tie1*-deficient mice (D'Amico et al. 2014). Also, targeting Tie2 signaling with a soluble form of Tie2 has been demonstrated to attenuate tumor growth and angiogenesis (Lin et al. 1997, 1998).

Clinical Summary

The clinical efficacy of Ang1- and Ang2-blocking agents is being evaluated in clinical oncology trials in combination with chemotherapy, and more recently with VEGF signaling inhibitors including sunitinib, bevacizumab and ziv-aflibercept. A peptibody targeting both Ang1 and Ang2 (trebananib, AMG 386) has shown clinical activity in increasing progression-free survival of patients with ovarian cancer in a randomized, placebo-controlled phase 2 trial, where it was tested in combination with chemotherapy (Karlan et al. 2012; Monk et al. 2014). Results from a randomized phase III placebo-controlled trial of trebananib in combination with chemotherapy in recurrent ovarian cancer were recently reported, demonstrating increased progression-free survival, but not overall survival, in the trebananib arm compared to control arm (Monk et al. 2014). Trebananib was well tolerated and while edema was increased, class-specific anti-VEGF-associated adverse events were not increased.

Two humanized monoclonal anti-Ang2 antibodies, MEDI 3617 and nesvacumab (REGN910), are being tested in phase 1-2 for the treatment of various solid tumors in combination with VEGF signaling inhibitors.

AKB-9778 is a first-in-class inhibitor of the vascular endothelial protein tyrosine phosphatase (VE-PTP), which inhibits Tie2 activity by dephosphorylating and inactivating Tie2. A recent paper demonstrated that pharmacological VE-PTP inhibition normalized the structure and function of tumor vessels through Tie-2 activation, which correlated with delayed tumor growth, slower metastatic progression, and enhanced response to concomitant cytotoxic treatments (Goel et al. 2013). This compound is being tested also for the treatment of neovascular eye disease (Shen et al. 2014). Regorafenib, a multikinase inhibitor of VEGFR-2 and Tie2 is used for the treatment of metastatic colorectal cancer and advanced gastrointestinal stromal tumors.

Anticipated High-Impact Results

Like the majority of existing anticancer therapies, the efficacy of current anti-angiogenic treatments is limited due to their transient efficacy on tumor growth inhibition. Therefore, new targets and drug combinations for anti-angiogenic therapy are being searched (reviewed in Saharinen et al. 2011). Numerous preclinical studies have indicated that blocking Ang2 is a potential strategy to inhibit tumor growth and may represent one way to overcome therapy resistance associated with the use of current anti-angiogenic drugs in some tumors. However, the benefit of Ang2 targeting remains to be established, as the first phase 3 trial did not demonstrate prolonged overall survival. Preclinical models show that blocking Ang2 is more efficacious in inhibition of tumor growth than blocking Ang1. In numerous preclinical studies, the combination of Ang2 and VEGF blocking agents has provided additional tumor control benefit over monotherapy, and this is now being tested in several clinical trials in renal cell carcinoma and colorectal cancer, for example. It remains to be seen if other components of the Ang-Tie system, such as Tie1, will be become a target for drug development in the future.

Cross-References

► AKT
► Integrins, Immunology
► Rac 1
► Survivin
► VEGF Ligands

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VEGF A

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Harold Dvorak

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Abstract

VEGF-A is the founding member of the VPF/VEGF family of proteins that also includes VEGFs B, C and D as well as PlGF (placenta growth factor) and a related viral protein, VEGF-E (Dvorak, J Clin Oncol 20:4368–4380, 2002; Am J Pathol 162:1747–1757, 2003; Ferrara et al., Nat Med 9:669–676, 2003; Mukhopadhyay et al., Vascular permeability factor/vascular endothelial growth factor and its receptors: evolving paradigms in vascular biology and cell signaling. In: Aird W (ed) The endothelium: a comprehensive reference. Cambridge University Press,

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Cambridge, 2007; Bry et al., Circulation 122:1725–1733, 2010; Hagberg et al., Nature 464:917–921, 2010; Shibuya and Claesson-Welsh, Exp Cell Res 312:549–560, 2006; Veikkola and Alitalo, Semin Cancer Biol 9:211–220, 1999). VEGF-A, the subject of this chapter, has critical roles in vasculogenesis and pathological and physiological angiogenesis, acting through receptors (VEGFR-1, VEGFR-2 and neuropilin) that are expressed on vascular endothelium as well as on certain other cell types (Fig. 1) (Shibuya and Claesson-Welsh, Exp Cell Res 312:549–560, 2006; Veikkola and Alitalo, Semin Cancer Biol 9:211–220, 1999; Bielenberg et al., Exp Cell Res 312:584–593, 2006). The product of a single gene, VEGF-A is alternatively spliced to form several proteins of different lengths, properties and functions. Originally discovered as a potent vascular permeabilizing factor (VPF) (Senger et al., Science 219:983–985, 1983; Dvorak et al., J Immunol 122:166–174, 1979), VEGF-A is also an endothelial cell motogen and mitogen, profoundly alters the pattern of endothelial cell gene expression, and protects endothelial cells from apoptosis (Benjamin et al., J Clin Invest 103:159–165, 1999) and senescence (Dvorak, Am J Pathol 162:1747–1757, 2003). Recently, VEGF-A has been found to have additional critical roles in hematopoiesis and in expansion and differentiation of bone marrow endothelial cell precursors (Seandel et al., Cancer Cell 13:181–183, 2008), in maintenance of the nervous system (Ruiz de Almodovar et al., Physiol Rev 89:607–648, 2009), and in development. Mice lacking even one copy of the VEGF-A gene are embryonic lethal (Ferrara et al., Nat Med 9:669–676, 2003; Carmeliet, Nat Med 9:653–660, 2003). VEGFs C and D are essential for development of the lymphatic system (Veikkola and Alitalo, Semin Cancer Biol 9:211–220, 1999), VEGF-B has a role in the development of coronary arteries and in fatty acid metabolism (Bry et al., Circulation 122:1725–1733, 2010; Hagberg et al., Nature 464:917–921, 2010), and PlGF has important roles in pathological angiogenesis (Carmeliet, Nat Med 9:653–660, 2003; Luttun et al., Nat Med 8:831–840, 2002). VEGF-A also induces abnormal lymphangiogenesis (Nagy et al., J Exp Med 196:1497–1506, 2002).

Keywords

VEGF-A • VEGFR-1(Flt-1) • VEGFR-2 (KDR, Flk-1) • VEGFR-3 (Flt-4) • neuropilin

Target: Vascular Endothelial Growth Factor-A/Vascular Permeability Factor (VEGF-A/VPF)

VEGF-A is the founding member of the VPF/VEGF family of proteins that also includes VEGFs B, C and D as well as PlGF (placenta growth factor) and a related viral protein, VEGF-E (Dvorak 2002, 2003; Ferrara et al. 2003; Mukhopadhyay et al. 2007; Bry et al. 2010; Hagberg et al. 2010; Shibuya and Claesson-Welsh 2006; Veikkola and Alitalo 1999). VEGF-A, the subject of this chapter, has critical roles in vasculogenesis and pathological and physiological angiogenesis, acting through receptors (VEGFR-1, VEGFR-2 and neuropilin) that are expressed on vascular

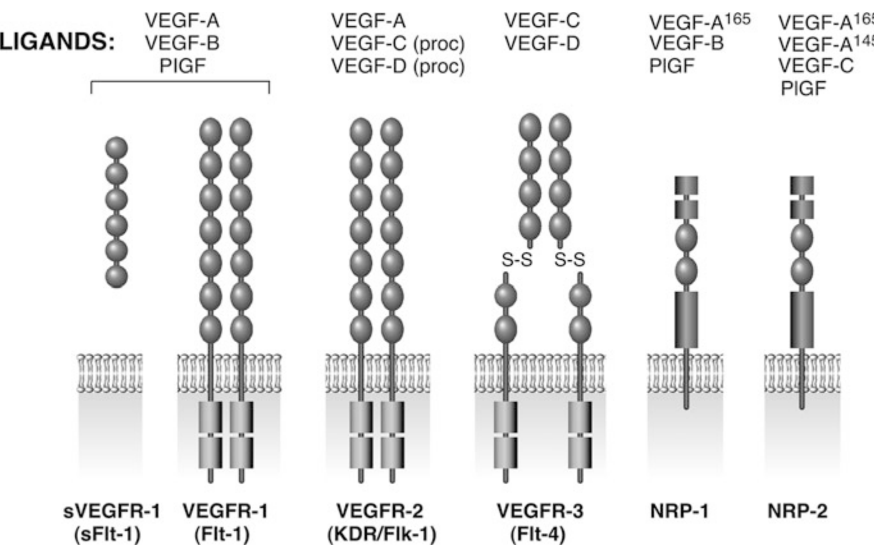

Fig. 1 Schematic diagram of VEGF receptors (Republished from Nagy et al. 2007)

endothelium as well as on certain other cell types (Fig. 1) (Shibuya and Claesson-Welsh 2006; Veikkola and Alitalo 1999; Bielenberg et al. 2006). The product of a single gene, VEGF-A is alternatively spliced to form several proteins of different lengths, properties and functions. Originally discovered as a potent vascular permeabilizing factor (VPF) (Senger et al. 1983; Dvorak et al. 1979), VEGF-A is also an endothelial cell motogen and mitogen, profoundly alters the pattern of endothelial cell gene expression, and protects endothelial cells from apoptosis (Benjamin et al. 1999) and senescence (Dvorak 2003). Recently, VEGF-A has been found to have additional critical roles in hematopoiesis and in expansion and differentiation of bone marrow endothelial cell precursors (Seandel et al. 2008), in maintenance of the nervous system (Ruiz de Almodovar et al. 2009), and in development. Mice lacking even one copy of the VEGF-A gene are embryonic lethal (Ferrara et al. 2003; Carmeliet 2003). VEGFs C and D are essential for development of the lymphatic system (Veikkola and Alitalo 1999), VEGF-B has a role in the development of coronary arteries and in fatty acid metabolism (Bry et al. 2010; Hagberg et al. 2010), and PlGF has important roles in pathological angiogenesis (Carmeliet 2003; Luttun et al. 2002). VEGF-A also induces abnormal lymphangiogenesis (Nagy et al. 2002).

VEGF-A is a highly conserved, disulfide-bonded dimeric glycoprotein of Mr ~45 kD (Dvorak 2002, 2003; Ferrara et al. 2003). It shares low but significant sequence homology with platelet-derived growth factor (PDGF), and, like PDGF, has cysteines that form integral inter- and intra-chain bonds. Crystal structure reveals that the two chains that comprise VEGF-A are arranged in anti-parallel fashion with binding sites at either end. Upon reduction, VEGF-A separates into its individual chains and loses all biological activity.

The human VEGF-A gene is located on the short arm of chromosome 6 and is differentially spliced to yield predominant isoforms which encode polypeptides of 206, 189, 165 and 121 amino acids in human cells (corresponding murine proteins are one amino acid shorter) (Ferrara 2010). Other splice variants (183, 165b, 145, etc.) have also been described (Bevan et al. 2008; Lee et al. 2005). The several major VEGF-A isoforms have distinct physical properties. VEGF-A$^{120/1}$ is acidic, freely soluble and does not bind heparin. By contrast, the 164/5 and 188/9 isoforms have increasing basic charge and bind heparin with increasing affinity; in fact, VEGF-A^{165} was originally purified on the basis of its affinity for heparin (Senger et al. 1983). Heparin, heparan sulfate, and heparinase all displace the larger VEGF-A isoforms from proteoglycan binding sites in tissues; proteases such as plasmin have a similar net effect, cleaving the C-terminal portion of bound VEGF-A to generate biologically active peptides with as few as 110 amino acids (Ferrara 2010). In a number of situations in vivo, liberation of bound VEGF-A from cells or cell matrix is the necessary trigger that initiates angiogenic activity (Ferrara 2010). The different VEGF-A isoforms have largely identical biological activities in vitro but there is increasing evidence for distinctive functions in vivo; for example, mice expressing only the 120 or 188 isoforms develop severe vascular anomalies (Stalmans et al. 2002). The effects of the different isoforms are likely based on their differential ability to bind to negatively-charged extracellular or cell surface matrix components such as heparan sulfate.

Biology of the Target

VEGF-A is essential for vascular homeostasis. It is constitutively expressed in the cells of many normal tissues and at higher levels in several types of epithelium (renal glomerular podocytes, adrenal cortex, breast and lung), in cardiac myocytes, in activated macrophages, and in endochondral bone formation (Dvorak 2003). It is also expressed normally by many cells that make steroid hormones (adrenal cortex, corpus luteum, Leydig cells) and by cells that are themselves under hormonal regulation, e.g., the cycling uterus and ovary. It is expressed at high levels in the physiological angiogenesis of ovarian follicular development and corpus luteum formation (Dvorak 2003). Circulating VEGF-A levels and VEGF-A expression correlate with estrogen receptor alpha positivity in breast cancer (Heer et al. 2001). VEGF-A is also expressed by peptide hormone-producing cells such as thyroid follicular cells, and its production in culture is up-regulated by agents such as insulin, dibutyryl cAMP, and the IgG of Graves disease (Poulaki et al. 2003). Thyroid stimulating hormone upregulates VEGF-A mRNA expression in human thyroid follicles and promotes VEGF-A secretion in several thyroid cancer cell lines.

VEGF-A is overexpressed by the vast majority of solid human and animal carcinomas, where it is thought to be the prime mover of tumor angiogenesis (Dvorak 2002, 2003; Ferrara et al. 2003). The majority of VEGF-A in solid tumors is expressed by the malignant cells themselves but tumor stroma can also synthesize VEGF-A (Yuan et al. 1996). More recently, VEGF-A has been found to be expressed in lymphomas and hematological malignancies(Medinger et al. 2010). VEGF-A is also overexpressed

in at least some premalignant lesions, e.g., precursor lesions of breast, cervix and colon cancers; furthermore, expression levels increase in parallel with malignant progression (Dvorak 2002, 2003). An association between VEGF-A expression and benign tumors is less well established, in part because the latter have been less carefully studied. However, pituitary adenomas and benign hemangiomas rarely overexpress VEGF-A, whereas uterine leiomyomas and malignant vascular tumors do so (Dvorak 2002).

VEGF-A is overexpressed in other examples of pathological angiogenesis including wound healing (e.g., in healing skin wounds, myocardial infarcts and strokes), chronic inflammation (e.g., delayed hypersensitivity, rheumatoid arthritis, psoriasis), and various retinopathies (Dvorak 2003). In all of these examples, VEGF-A is thought to be largely responsible for the accompanying angiogenesis.

VEGF-A Receptors

VEGF-A mediates its effects primarily by interacting with two high-affinity transmembrane tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1) (Shibuya and Claesson-Welsh 2006; Veikkola and Alitalo 1999). Cultured endothelial cells have ~3000 copies of VEGFR-1, a receptor that binds VEGF-A^{165} with a K_d of ~10 pM; endothelial cells have more numerous copies of VEGFR-2 which binds VEGF-A^{165} with somewhat lower affintiy (K_d of 75–125 pM). A truncated soluble form of VEGFR-1 (sFlt) that results from alternative splicing is found in serum and retains VEGF-A binding activity. sFlt has recently been implicated in preeclampsia, a serious complication of pregnancy (Young et al. 2010). A soluble form of VEGFR-2 has also been described. A third, non-kinase receptor, neuropilin (NRP-1) has been found that potentiates VEGF-A$^{164/165}$'s binding to VEGFR-2 (Bielenberg et al. 2006). Neuropilin had been known as a receptor for the semaphorin/collapsin family of neuronal guidance mediators and it is also expressed widely on non-endothelial cells. VEGF-A$^{164/5}$, B, E and PLGF bind to NRP-1 but VEGF-A$^{120/121}$ does not (Fig. 1); this inability may explain some of the abnormalities in mice engineered to express only the VEGF-A^{120} isoform. Two other members of the VPF/VEGF family (VEGF-B and both isoforms of PlGF) bind to VEGFR-1 whereas VEGFs C, D and E bind to VEGFR-2. VEGFs C and D also bind to VEGFR-3 which is found on lymphatic endothelium (Veikkola and Alitalo 1999).

Mice null for any of the three VEGF-A receptors are embryonic lethal. VEGFR-1 null mice die at day 8.5–9.0 due to vessel obstruction by an overgrowth of endothelial cells. VEGFR-2 knock-out mice die at a similar stage of development due to failure of endothelial and hematopoietic precursor cells. NRP-1 null mice also die from a failure of vascular development.

VEGFR-1 and VEGFR-2 are widely expressed on normal vascular endothelium and expression is upregulated in tumor blood vessel endothelium and in that of other examples of pathological angiogenesis induced by VEGF-A (Dvorak 2002, 2003). The mechanisms responsible for receptor overexpression are not yet fully understood but hypoxia, which stimulates VEGF-A expression (see below), may also upregulate VEGF receptor expression.

Recent reports indicate that VEGF-A receptors may also be expressed on tumor cells, raising the possibility of an autocrine loop that might stimulate tumor cell growth and migration (Masood et al. 2001; Soker et al. 1998). VEGFR-1 is additionally expressed on a population of hematopoietic stem cells and myeloid progenitors as well as on monocytes/macrophages, spermatogenic and Leydig cells, uterine smooth muscle cells and osteoclasts (Dvorak 2002). VEGFR-1 has an important role in mediating hematopoietic cell development and recruitment, particularly that of monocytes, and mediates VEGF-A induced monocyte motility. As noted above, VEGFR-1 null embryos die due to an overgrowth of endothelial cells, implying, along with data from cultured endothelial cells, that this receptor exerts a negative regulatory effect on the VEGF-A activities governed by VEGFR-2 signaling. VEGFR-2 is expressed on bone marrow endothelial cell progenitors and on megakaryocytes, uterine smooth muscle cells, and on lymphatic endothelium (Dvorak 2002; Nagy et al. 2002).

Though binding to VEGF-A with high affinity, VEGFR-1 induces only minimal stimulation of kinase activity in vascular endothelium (Shibuya and Claesson-Welsh 2006; Veikkola and Alitalo 1999); as a result, downstream signaling pathways have had to be worked out in endothelial cells that were engineered to overexpress this receptor (Mukhopadhyay et al. 2007). Much more is known about the signaling pathways initiated through VEGFR-2 (Mukhopadhyay et al. 2007; Shibuya and Claesson-Welsh 2006; Veikkola and Alitalo 1999). Upon binding to VEGFR-2, VEGF-A initiates a cascade of events that begins with receptor dimerization and autophosphorylation, followed by phosphorylation of numerous downstream proteins. G proteins have been implicated in VEGF-A signaling (Mukhopadhyay et al. 2007). Most of the biological activities mediated by VEGF-A on endothelial cells (e.g., proliferation, migration, vascular permeability, anti-apoptosis) are mediated through VEGFR-2 signaling. It has been suggested that VEGFR-1's role is to limit VEGF-A signaling by binding VEGF-A and so making it unavailable to VEGFR-2. VEGFR-2 also has an important role in the development of endothelial cell precursors present in the bone marrow and circulating in blood (Seandel et al. 2008).

Actions of VEGF-A

VEGF-A is essential for the development and organization of the vascular system (vasculogenesis) and for both physiological and pathological angiogenesis (Carmeliet 2003). VEGF-A has multiple effects on vascular endothelium that become apparent over the time frame of a few minutes to days. These include striking changes in vascular permeability, followed by changes in cell morphology and cytoskeleton, accompanied by stimulation of endothelial cell migration and division. At the molecular level, VEGF-A reprograms endothelial cell gene expression, causing increased production of a number of different proteins including the procoagulant tissue factor, fibrinolytic proteins (urokinase, tPA, PAI-1, urokinase receptor), matrix metalloproteases, the GLUT-1 glucose transporter, nitric oxide synthase, numerous mitogens, and a number of anti-apoptotic factors (e.g., bcl-2, A1, survivin, XIAP). VEGF-A also serves as an endothelial cell survival factor,

protecting endothelial cells against apoptosis and senescence (Dvorak 2002, 2003; Benjamin et al. 1999). Recently it has been shown that VEGF-A causes paracrine release of hepatotrophic molecules from sinusoidal liver endothelium, a function mediated through VEGFR-1 (LeCouter et al. 2003).

VEGF-A was originally discovered in the late 1970s because of its ability to increase the permeability of microvessels (primarily post-capillary venules) to circulating plasma and plasma proteins (Dvorak 2003; Senger et al. 1983; Dvorak et al. 1979). It is responsible for the vascular hyperpermeability characteristic of nearly all human and animal tumors. Permeability becomes evident within a minute following injection of VEGF-A protein into skin and continues for ~20 min. VEGF-A is among the most potent vascular permeabilizing agents known, acting at concentrations below 1 nM and with a potency some 50,000 times that of histamine on a molar basis (Dvorak 2003).

VEGF-A induces vascular permeability by its action on endothelial cells (primarily venular endothelial cells), the primary barrier to the extravasation of plasma proteins, but there has been debate as to the pathway that circulating macromolecules follow in traversing endothelium (Dvorak 2003; Nagy et al. 2008). The earlier view had been that vasoactive agents cause endothelial cells to pull apart, creating an inter-endothelial cell gap through which macromolecules could extravasate. Though supported by data making use of cultured endothelial cells, evidence for an exclusive inter-cellular extravasation pathway in vivo is less convincing (Dvorak 2003; Nagy et al. 2008, 2012). Recent studies have shown that macromolecules cross tumor and normal venular endothelium by means of a trans-endothelial cell pathway that involves vesiculo-vacuolar organelles (VVOs) (Dvorak 2003; Nagy et al. 2008, 2012; Dvorak et al. 1996). VEGF-A also induces endothelial fenestrations that provide an additional trans-cellular pathway for solute extravasation (Roberts and Palade 1995).

The increased microvascular permeability induced by VEGF-A leads to tissue edema, a characteristic feature of tumors, healing wounds, and other pathologies in which VEGF-A is overexpressed (Dvorak 2003). Extravascular fluid accumulation is particularly prominent in tumors growing in body cavities such as the peritoneum (ascites tumors). Plasma protein leakage has a number of consequences. One of these is activation of the clotting system via the tissue factor pathway, leading to deposition of a fibrin gel that retards clearance of edema fluid and results in locally increased interstitial tissue pressure, a characteristic feature of many solid tumors (Dvorak et al. 1979. 1985; Jain 2005). Deposited fibrin also provides a provisional stroma for endothelial cell and fibroblast migration that supports the angiogenesis and fibrogenesis necessary for generating mature stroma (desmoplasia in the case of tumors, scar formation in wound healing) (Dvorak 1986, 2003).

Effects of VEGF-A on Cells Other Than Vascular Endothelium

There is increasing interest in activities that VEGF-A exerts on non-endothelial cells that express VEGF receptors; thus, VEGF-A stimulates monocyte chemotaxis and proliferation of uterine smooth muscle (Dvorak 2003). VEGF-A also has reported effects on lymphocytes, granulocyte-macrophage progenitor cells, osteoblasts,

Schwann cells, mesangial cells and retinal pigment epithelial cells. In development, VEGF-A drives angioblasts and primitive vessels toward arterial differentiation and attracts filopodia of both endothelial cells and neurons, causing these cells to move in the direction of a VEGF-A gradient (Eichmann et al. 2005). As already noted, VEGF-A receptors have now been reported on some tumor cells, opening the possibility that VEGF-A exerts autocrine effects that enhance tumor cell motility and survival.

Regulation of VEGF-A Expression

A number of mechanisms regulate VEGF-A expression (Dvorak 2002, 2003; Semenza 2014). Hypoxia potently upregulates VEGF-A expression, both by stabilizing its mRNA and by increasing message transcription. Transcriptional regulation is mediated through hypoxia-inducible factor 1 (HIF-1), a heterodimeric protein transcription factor. One HIF-1 component, HIF-1α, is rapidly degraded under normoxic conditions by the ubiquitin pathway; however, when stabilized by hypoxia, HIF-1α dimerizes with HIF-1β, and the complex binds to and activates a hypoxia-responsive element in the VEGF-A promoter. Hypoxic regulation of VEGF-A expression is likely important in healing wounds and has been demonstrated in tumors. However, many tumors express VEGF-A constitutively at high levels even under normoxic conditions and therefore regulation is achieved by other means. For example, a number of oncogenes (src, ras) and tumor suppressor genes (p53, p73, von Hippel Lindau (vHL)) promote tumor growth, at least in part, by modulating VEGF-A expression. Numerous growth factors, cytokines and lipid mediators upregulate VEGF-A expression in different cells, including EGF, TGF-α, FGF-2, TGF-β, PDGF, keratinocyte growth factor, TNF, interleukins 1 and 6, insulin-like growth factor 1, HGF, and prostaglandins E1 and E2. These findings are likely to be important in autocrine regulation of VEGF-A expression in vivo in that many tumors that express VEGF-A also express other cytokines and their receptors (e.g., TGF-α, FGF-2, EGF).

Hormones can also regulate tumor cell expression of VEGF (see above for hormone-induced expression of VEGF in normal cells), as can a number of chemicals and proteins and processes, and by both direct and indirect means. These include thrombin, platelet aggregation, shear stress, acidosis, lysophosphatidic acid, and adenosine. On the other hand, dexamethasone downregulates or prevents cytokine- (but not hypoxia-) induced upregulation of VEGF-A expression.

New Blood Vessels Induced by VEGF-A Are Heterogeneous

Recently it has been found that mouse and human tumor blood vessels are heterogeneous and can be classified into at least six structurally and functionally distinct types (Nagy et al. 2007). Also, each of these vessel types can be induced to form by an adenoviral vector expressing VEGF-A^{164} in nude mouse tissues, allowing the

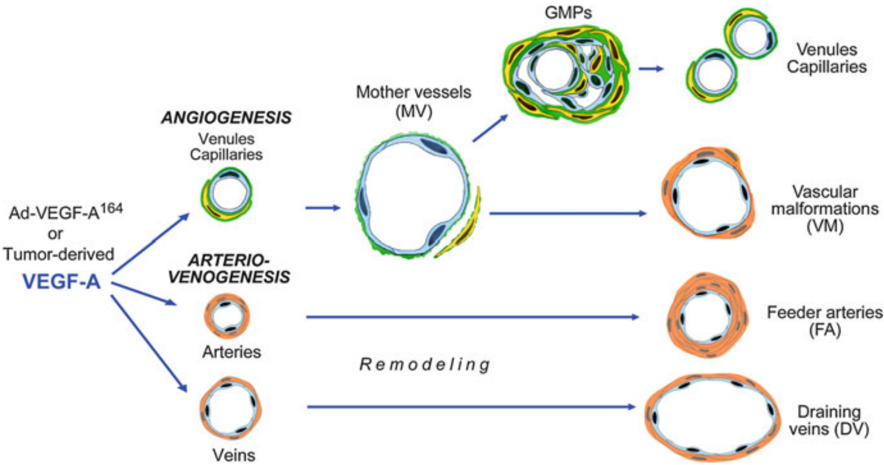

Fig. 2 Schematic diagram of the angiogenic and arterio-venogenic responses induced by VEGF

steps and mechanisms of tumor surrogate blood vessels to be elucidated in a convenient mouse model (Fig. 2) (Nagy et al. 2007, 2010).

Target Assessment

Patients with large tumor burdens and widespread metastatic disease have increased levels of circulating VEGF-A, often multiples of those found in normal individuals. Though not useful as a screening tool, increasing serum VEGF-A levels may signify increased tumor growth, recurrence, or metastatic spread in individual patients. There is debate as to whether plasma or serum levels are more meaningful because platelets sequester VEGF-A and because plasma α2 macroglobulin binds it and makes it unavailable to at least some antibodies (Dvorak 2002). Also, both megakaryocytes and leukocytes synthesize VEGF-A. Therefore, serum levels reflect not only VEGF-A of tumor origin but also that released from platelets and leukocytes, making it difficult to establish a range of normal values. VEGF-A levels are also elevated in malignant effusions and in the urine of patients with bladder cancer (Dvorak 2002). Finally, anti-VEGF-A/VEGFR therapy causes a rise in plasma VEGF-A levels for reasons and from sources that are not presently known.

Role of the Target in Cancer

VEGF-A induces the new blood vessel formation that most tumors require for growth beyond minimal size (Folkman 1971). VEGF-A, like VEGF-C, also induces lymphangiogenesis (Nagy et al. 2002; Wirzenius et al. 2007).

(A) **Rank**: 8
(B) 10

High Level Overview

VEGF-A is the single most important tumor angiogenesis factor. It also induces arterio-venogenesis, the remodeling and enlargement of arteries and veins that supply angiogenic blood vessels (Nagy et al. 2007, 2009, 2010). Therefore, neutralizing VEGF-A with antibodies/traps or its receptors with receptor tyrosine kinase inhibitors is expected to prevent the formation of new blood vessels and likely attack at least some blood vessels that have already formed. The importance of VEGF-A's role in tumor lymphangiogenesis (versus that of VEGF-C) and the importance of lymphangiogenesis induced by either VEGF isoform in tumor metastasis, are still matters of uncertainty.

Diagnostic, Prognostic, and Predictive

VEGF-A likely has no important role as a diagnostic agent. Almost all important tumors make VEGF-A and so testing for its expression in tumor tissue would not seem to offer much value (Dvorak 2002, 2003). Plasma and serum levels of VEGF-A are also unlikely to be of value as the clearance rate of VEGF-A from the blood is rapid and blood levels are complicated by VEGF-A uptake by platelets (Dvorak 2002). However, clinical studies have shown VEGF-A levels to be of prognostic significance and it's predictive value is currently being studied in clinical trials with an VEGF-antibody (http://clinicaltrials.gov/show/NCT01663727).

Therapeutics

Anti-VEGF-A antibodies (Avastin, bevacizumab, Genentech) and anti-VEGFR (tyrosine kinase inhibitors) therapies have proved to be less successful in cancer patients than in rapidly growing mouse tumors. Avastin has not proved to be useful as monotherapy for most tumor types (it may be of benefit in NETs and RCC, although it was studied with interferon for RCC) and, when accompanied by chemotherapy, it extends the life of colon cancer patients by ~4–5 months in initial studies, but has not led to such improvements in more recent studies (Saltz et al. 2008). The addition of Bevacizumab to chemotherapy in other tumor types has not increased overall survival, but emerging data in gynecologic malignancies may be an exception. There are many possible reasons for this relative lack of efficacy in patients, e.g., cancer patients are older and sicker than the young healthy mice used in laboratory experiments, humans as a species may be more susceptible

to side effects of anti-angiogenic drugs than rodents, tumor vessels may undergo "normalization" in response to therapy, and human tumor cells may begin to express other growth factors to circumvent the lack of VEGF, etc. (Jain 2008). However, another possibility is suggested by the finding that only a subset of Ad-VEGF-A^{164}-induced surrogate tumor blood vessels require VEGF-A for their maintenance, whereas others have acquired VEGF-A independence (Sitohy et al. 2012). Moreover, these VEGF-A independent vessels are long lasting and are likely to become the predominant vessel type in tumors that have been growing for any length of time. Human tumors, of course, develop over a period of many months or years prior to diagnosis and treatment. Therefore, the subset of vessels in human tumors that is dependent on VEGF-A and that is therefore vulnerable to anti-VEGF-A/VEGFR therapy may be considerably smaller than in rodent tumors that are treated shortly after transplant. A possible approach to new anti-vascular tumor therapy is to identify new targets that are selectively expressed by blood vessels that have lost their VEGF dependence (Nagy et al. 2009, 2010).

Preclinical Summary

The concept of anti-angiogenesis as an approach to tumor therapy has a long history and was brilliantly formulated by the late Judah Folkman (1971). VEGF-A has been regarded as a potential tumor vascular therapeutic target since its discovery in 1979 and its purification in 1983 (Dvorak 2002, 2003; Senger et al. 1983). Hopes for this approach were encouraged by the success that anti-VEGF-A antibodies and kinase inhibitors targeting VEGF receptors had on inhibiting the growth of many rodent tumors (Ferrara et al. 2003). Conversely, VEGF-A has been used in attempts to induce the growth of new blood vessels in ischemic tissues (e.g., myocardial infarctions, peripheral vascular disease) but there are many reasons for caution. Very recently, reduced VEGF-A has been implicated in amyotrophic lateral sclerosis and in certain neurovascular disorders (Ruiz de Almodovar et al. 2009).

Clinical Summary

As already noted, anti-VEGF and anti-VEGFR therapies have proved to be less successful in cancer patients than in rapidly growing mouse tumors. Avastin has not proved to be useful as monotherapy for most tumor types, and, when accompanied by chemotherapy, it extends the life of colon cancer patients by ~4–5 months in initial studies, but has not led to such improvements in more recent studies (Saltz et al. 2008). The addition of Bevacizumab to chemotherapy in other tumor types has not lengthened overall survival, but emerging data in gynecologic malignancies may be an exception.

Anticipated High-Impact Results

There is an ongoing effort to identify biomarkers for VEGF-targeted therapies, but efforts thus far have failed. Therefore, there are no anticipated high-impact results expected in the next few years.

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VEGF Ligands

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Abstract

Vascular endothelial growth factor (VEGF) is a family of key regulator of vasculogenesis and angiogenesis in physiology and pathology. The main clinical target and mediator of tumor angiogenesis is VEGF-A. Targeting VEGF in cancer has been a step forward in the realization of a new anticancer strategy aiming at cutting off resources to tumors instead of direct attack on tumor cells. In the present chapter we summarized the biology of VEGF as target, focusing on mechanisms leading to cancer related neoangiogenesis. We also described the most effectively developed anti-VEGF strategies for cancer treatment and possible diagnostic, prognostic, and predictive markers. Despite many improvements for cancer treatment have been achieved thanks to the adoption of VEGF as

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target, no predictive or prognostic VEGF-related biomarkers have been identified so far. Up to date no clues are available in order to identify patients for whom VEGF levels normalization might be beneficial. Mechanisms of resistance to treatment, both primary or after treatment are still under investigation.

Keywords
Vegf • Vegf receptors • Angiogenesis • Biomarker • Cancer

Target: Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a disulfide-bonded homodimeric glycoprotein of about 45 kDa. VEGF is produced by most cells in the body and stimulates VEGF receptor-expressing endothelial cells lining the vessel lumen, resulting in formation of new vessels, angiogenesis (Chung and Ferrara 2011). VEGF initiates a cascade of downstream effects via various receptors and co-receptors, some of which are exploited by tumor cells. Indeed, VEGF is a key driver of angiogenesis in physiology and pathology. VEGF is essential for vascular development during embryogenesis as demonstrated by the phenotype of VEGF knockout mice (Ferrara et al. 1996; Carmeliet et al. 1996); loss of only one VEGF allele leads to early embryogenic death due to severe developmental deficiencies. VEGF is moreover essential for vascular stability in the adult (Lee et al. 2007). VEGF was originally identified as vascular permeability factor (VPF), indicating its critical role in regulation of vascular permeability (Senger et al. 1983). VEGF receptors may be expressed by circulating endothelial progenitors, as well as non-endothelial cells such as hematopoietic and neuronal cells. In addition, certain tumor cells such as breast cancer and glioblastoma cells express VEGF receptors and respond to VEGF with increased survival (Olsson et al. 2006; Koch et al. 2011). Tumor-proximal vessels are recruited by tumor cells and by definition are not pathogenic, but do respond to subtle signals derived from the local malignant cells, such as increased VEGF availability. Targeting VEGF in cancer has been a step in realizing a new strategy of cutting off resources to tumors as opposed to a direct attack on cancer cells.

Biology of the Target

The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factors (PlGF) 1 and 2. The key clinical target and the main mediator of tumor angiogenesis is VEGF-A. Several isoforms of VEGF-A exist; they differ mainly with regard to the presence or absence of heparan sulfate (HS)- and neuropilin-binding domains. In longer isoforms (e.g., VEGF-A_{165} and VEGF-A_{189}), the HS-binding domains engage HS in the extracellular matrix (Ferrara 2010). However, shorter VEGF-A isoforms, such as VEGF-A_{121} and VEGF-A_{110} (a protease-cleaved fragment of longer VEGF-A isoforms

(Lee et al. 2005)), lack this motif and are freely soluble. Extracellular matrix-bound and soluble VEGF-A isoforms have different effects on vascular morphogenesis: soluble VEGF-A is associated with large, tortuous, unbranched vessels, whereas matrix-bound VEGF-A is associated with thinner, more branched vessels (Lee et al. 2005). Moreover, binding of long but not short VEGF-A isoforms to the VEGF co-receptor neuropilin may affect VEGF receptor intracellular trafficking and downstream signal transduction (Koch et al. 2011).

The different VEGF family members bind in a distinct pattern to three receptor tyrosine kinases (RTKs) denoted VEGF receptor-1 (VEGFR1, also known as FLT1), VEGFR2 (also known as KDR), and VEGFR3 (also known as FLT3) (Olsson et al. 2006). VEGF has also been suggested to interact with matrix receptors such as integrin alpha5 beta3 that operates via the MAPK/Erk pathway to promote cell survival (see Koch et al. (2011) and references therein). VEGFR2 is the main VEGF receptor on endothelial cells. Binding of VEGF leads to VEGFR2 dimerization, activation of its endogenous tyrosine kinase activity, and, thereby, induction of downstream signaling, following the consensus model for activation of RTKs. VEGFR2 signaling regulates endothelial cell survival, proliferation, migration, and three-dimensional arrangement to form the vascular tube. VEGFR1 is expressed on bone marrow-derived monocytes and macrophages and promotes migration of inflammatory cells to tumors. It has a high affinity for VEGF, but its kinase activity is weak. A soluble variant of VEGFR1 has been assigned a negative regulatory role by binding VEGF without transducing a biological effect. VEGFR3 is important for lymphatic formation and function but is also expressed on vascular endothelial cells during angiogenesis (Tammela et al. 2008). VEGFR2 also binds VEGF-C and VEGF-D, allowing formation of heterodimers between VEGFR2 and VEGFR3 (Nilsson et al. 2010). For details on VEGFR signal transduction and biology, see Olsson et al. (2006) and references therein.

Apart from signaling via VEGFR2 to promote endothelial function in health and disease, VEGF has a key role in hematopoiesis, in both differentiation and survival of progenitor cell (Broxmeyer et al. 1995; Bautz et al. 2000). Although VEGF acts to mobilize and recruit hematopoietic cells from the bone marrow to the blood (Grunewald et al. 2006), it also negatively affects dendritic cell maturation (Gabrilovich et al. 1996), leading to a subsequent failure to activate a T-cell response (Almand et al. 2001). It is clear that VEGF is a necessary factor in a number of cell types, evident by the promiscuity of receptor expression in endothelial cells, tumor cells, dendritic ells, T cells, monocytes, and macrophages.

Target Assessment

VEGF can be detected and quantified in serum and in tumor lysates by ELISA. It can also be detected by immunohistochemistry (IHC) and in situ hybridization (ISH) in tissues. Proximity ligation is a more sensitive, antibody-based technique for detection of VEGF in, e.g., serum (Gullberg et al. 2004). A number of excellent antibodies are available commercially; none of these detect VEGF isoforms (VEGF-A_{121}, VEGF-A_{165}, etc.) separately. A novel ELISA-based assay with enhanced affinity

for short isoforms of VEGF (VEGF-A_{110} and VEGF-A_{121}) isoforms might be more successful. As stated above, through alternative RNA splicing, several VEGF isoforms are generated, and among them the short ones are freely diffusible and do not or minimally bind to the extracellular matrix (ECM), while the longer forms bind to heparin and heparan sulfate proteoglycans. Due to differential affinity for the ECM, the detection of short VEGF isoforms in plasma could provide a more accurate and specific readout of the VEGF secreted by the tumor environment. It has been proven that VEGF-A_{110} and VEGF-A_{121} diffuse over long distances, while VEGF-A_{165} reaches distant and nearby target cells, and ECM-bound VEGF-A_{189} provides guidance cues over short ranges. Estimation of transcripts of individual VEGF isoforms can be done by traditional PCR methodology (Jayson 2011; Vernes and Meng 2015).

Role of the Target in Cancer

Rank: 6 or 7

Low oxygen tension (hypoxia) promotes expression of VEGF-A, via the transcription factor hypoxia inducible factor-1 (HIF-1). The HIF-1 pathway is not the only but probably a major mechanism for the high VEGF-A expression levels in tumors (Shweiki et al. 1992). The high and persistent VEGF-A stimulation of tumor vessels results in a characteristic tumor vessel morphology, denoted vessel “abnormalization,” that differs markedly from normal physiological vessels or vessels created in response to injury. Tumor vessels lack normal vessel hierarchy and have poor pericyte and basement membrane coating (Baluk et al. 2005). In fact, although immunostaining may show high vascular density, many tumor vessels may not have a continuous lumen, resulting in poor perfusion. The dysfunctional and leaky vasculature promotes formation of edema leading to increased interstitial pressure and impaired drug delivery (Jain et al. 2009). Still, tumor vascularization facilitates tumor establishment at an early stage of carcinogenesis after the reliance on diffusion is diminished and promotes the development from in situ lesions to locally invasive disease, as well as seeding of metastases in distant locations (Hanahan and Weinberg 2011). Moreover, by stimulation of VEGF receptors on tumor cells, VEGF-A may have an additional role in cancer by creating an autocrine loop that might contribute to progression of the cancer (Roybal et al. 2011; Hein and Graver 2013).

There is a correlation between tumor growth, hypoxia and VEGF-A production, and, in turn, tumor vascularization and distant spread of the tumor (Garcia-Donas et al. 2013). Overall, however, VEGF-A in plasma is not a safe predictive biomarker for cancer (see below), and the plasma levels of VEGF-A do not correlate with tumor stage (Jain et al. 2009). On the other hand, pretreatment plasma levels of VEGF-A have a prognostic value; high levels of VEGF-A indicate a worse prognosis, whereas low levels of the cytokine are indicative of a better prognosis.

Tumors release a spectrum of proangiogenic cytokines, driven by metabolic and acidic environmental effects as well as hypoxia. The critical role of VEGF-A in tumor angiogenesis led to the development of inhibitors such as the monoclonal anti-VEGF-A antibody bevacizumab or the recombinant fusion protein aflibercept. Other

strategies to interrupt angiogenic effects are the use of small molecule receptor tyrosine kinase (RTK) inhibitors, which inhibit the downstream effects of not only VEGF receptors but often affect a range of RTKs or the use of antibodies directed against VEGF receptor, such as ramucirumab. Antiangiogenic therapy has seen moderate effects in some cancer types, but significant advantages have been thus far, elusive, in part due to the lack of predictive biomarkers (see below).

The mechanism of action of VEGF-A and VEGF receptor inhibitors has not been fully elucidated; however, neutralizing VEGF-A alone is not effective in stopping malignant progression. As the field of angiogenesis grows with each new discovery, angiogenesis has proven to be intertwined with numerous other pathways and is highly adaptable.

Despite the benefit provided by bevacizumab-based regimens for patients, clinical resistance usually develops (Bergers and Hanahan 2008). Extensive preclinical work has suggested that alternate proangiogenic factors may modulate sensitivity to anti-VEGF therapy and allow regrowth of tumor-associated vasculature. Additional studies have implicated infiltrating monocytic cells in the angiogenic switch, recruited by cytokines derived from tumor or tumor-associated stroma.

High-Level Overview

Diagnostic, Prognostic, and Predictive

The introduction of new diagnostic, prognostic, or predictive biomarkers is an essential tool to realize personalized medicine in the future. Nowadays, a broad range of drugs targeting angiogenesis are available to treat advanced-stage cancer; however, this class of drugs are very expensive, and the clinical benefits in the unselected patient populations vary according to agent and cancer type, but benefits can be small and toxicities may be considerable. The biomarker-driven selection of the most appropriate drug for each patient is a goal that so far has been unachievable, and at the moment no approved predictive biomarker of efficacy for antiangiogenic treatments is available. It is important to consider that both patient interindividual differences and tumor heterogeneity make research in this field very challenging. The complexity and highly adaptive nature of the angiogenic process complicates the search for a single, noninvasive biomarker. Thus, many molecular, biological, and functional biomarkers of angiogenesis have been proposed and investigated, but none has been yet validated for routine clinical use (Sessa et al. 2008). It was speculated that the combination of multiple biomarkers in parallel, measured at different time points during antiangiogenic treatment, might be necessary to better understand tumor behavior during antiangiogenic therapy. Moreover, from a clinical point of view, many concerns have been raised on the identification of clear objective markers of activity for antiangiogenic therapies. In particular, it has been hypothesized that standard criteria for response assessment (such as RECIST) might be not suitable for this class of drug since they exert a cytostatic more than cytotoxic effect (Tirumani et al. 2015). Functional imaging biomarkers such as dynamic

contrast-enhanced magnetic resonance imaging (DCE-MRI) and positron emission tomography (PET) represent a promising, noninvasive approach for monitoring angiogenesis and predict responsiveness (Miller et al. 2005; de Langen et al. 2011; Ah-See et al. 2008). DCE-MRI can be used to measure properties of the microvasculature and has been found to correlate with several prognostic factors. Changes measured during treatment have been shown to correlate with clinical outcome (Junck 2011; Hylton 2006). However, no study has demonstrated the predictive role of these imaging procedures in definitive clinical trials.

VEGF-A is efficiently neutralized by the monoclonal antibody bevacizumab. Extensive translational research efforts are ongoing to identify a predictive biomarker for antiangiogenic therapeutics and in particular for bevacizumab efficacy, focused on plasma VEGF-A. VEGF-A as the target of therapeutic inhibition is the most logical biomarker. However, in initial biomarker analyses of four randomized phase III trials of bevacizumab (AVF2107g in colorectal cancer, E4599 and AVAiL in non-small-cell lung cancer, and AVOREN in renal cell carcinoma), plasma VEGF-A concentrations appeared to have prognostic more than predictive significance (Hegde et al. 2013). Indeed, high baseline plasma VEGF-A concentrations correlated with worse outcome, irrespective of the treatment administered. The assay used in biomarker analyses in these trials was a quantitative microtiter-plated VEGF-A enzyme-linked immunosorbent assay (ELISA) that recognizes all isoforms of VEGF-A with similar sensitivity.

Previous data had suggested that the soluble isoforms of VEGF-A are the most important from a clinical angiogenesis perspective (Zhang et al. 2000). Therefore, it is plausible that quantification of the pretreatment plasma concentration of soluble VEGF-A_{121} might generate a predictive biomarker enabling identification of those patients most likely to benefit from bevacizumab. Results of biomarker analyses using a VEGF-A_{121}-specific assay have recently been reported or presented from trials of bevacizumab. Biomarker results in breast cancer (AVADO and AVEREL) (Gianni et al. 2013), pancreatic cancer (AViTA), and gastric cancer (AVAGAST) suggested potential predictive value for plasma VEGF-A_{121} (Van Cutsem et al. 2012a; Miles et al. 2013). Moreover, in all trials analyzed, the pretreatment plasma concentration of VEGF-A showed also a potential prognostic (providing information about the course of the disease) effect. Patients in the control (non-bevacizumab-treated) groups who had high baseline plasma VEGF-A concentrations had shorter progression-free survival (PFS) and overall survival (OS), than patients with low concentrations.

In conclusion, plasma pretreatment VEGF-A based on the effect observed in the AViTA, AVAGAST, AVADO, and AVEREL trials might be a potential predictive biomarker for bevacizumab, and the use of isoform-specific capture antibodies may lead to a VEGF-A assay with improved predictive value. However, the predictive value of VEFG-A was not confirmed in metastatic colorectal cancer (mCRC), non-small cell lung cancer (NSCLC), and renal cell carcinoma patients (Hegde et al. 2013), thus leading to identify possible heterogeneity from different cancer types and to limit the usefulness of pretreatment levels of VEGF-A as predictive biomarker of bevacizumab activity. Finally, such results underline the

need for validation protocols and standardization of detection procedures and antibodies.

VEGFR1 has been implicated as a prognostic biomarker. However, limited data are available, and no promising drugs targeting VEGFR1 have been developed so far. VEGFR1 acts as a negative regulator of VEGF-A-mediated angiogenesis during embryonic development. It may act as a stimulator of angiogenesis when activated by its specific ligands PlGF and VEGF-B (Shibuya 2011). Tumor samples from colorectal cancer patients receiving first-line chemotherapy in the MAX study (mitomycin C, Avastin®, and Xeloda®) were tested for VEGFR1 expression. Lower expression of VEGFR1 was associated with greater bevacizumab benefit for OS, but not for PFS (Lambrechts et al. 2013).

Besides baseline levels of VEGF and its receptors, many studies analyzed the effect of single nucleotide polymorphisms (SNP) of genes involved in VEGF-related pathway. Those studies moved from a strong biologic rationale that identified genetic variability in the host DNA as possible mediator of response to antiangiogenic drugs. In E2100, the effect of five *VEGF* and two *VEGFR2* SNPs on bevacizumab outcome was evaluated in 363 patients with mBC. Results showed that mutant carriers of rs699947 and rs1570360 predicted favorable median OS for patients in the bevacizumab arm, but not in the control arm (Schneider et al. 2008). In the AVADO trial, which involved 336 mBC patients of which 231 received bevacizumab, mutant carriers of rs699947 correlated with PFS in the placebo arm (Miles et al. 2013). Those results might lead to hypothesize a possible prognostic for this SNP. In a single arm analysis of 279 mCRC patients receiving capecitabine, oxaliplatin, and bevacizumab as first-line treatment, none of the five VEGF-A SNPs analyzed were associated with PFS (Pander et al. 2011). The same set of five VEGF-A SNPs was tested (together with 29 other SNPs) in a cohort of 132 patients receiving in first-line treatment FOLFOX or XELOX and bevacizumab, without revealing any effect on patients' outcome (Gerger et al. 2011).

Subsequently, results of a meta-analysis of 158 SNPs performed on 1348 patients enrolled in five randomized phase III trials evaluating the effect of bevacizumab in advanced-stage cancer patients were presented. This experiment was by far the largest genetic study of bevacizumab-treated patients. In bevacizumab-treated patients, VEGF-A rs699946 and VEGFR2 rs11133360 were associated with improved PFS, whereas no effect was seen in placebo-treated patients, suggesting a possible predictive role for these two SNPs (Lambrechts et al. 2011). Unfortunately, in a large prospective series of mCRC treated with first-line FOLFIRI plus bevacizumab, only VEGFR2 12505758 showed an association with PFS, while no other significant associations of SNPs with outcome were observed (Loupakis et al. 2013).

Overall, although observations on the relationship between SNP in angiogenesis-related genes and outcome of bevacizumab treatment are very interesting, several questions remain open, and from a general perspective, results are inconsistent and validation of retrospective finding is always challenging. Moreover, it is not clear yet whether the effects of individual SNPs vary between tumor types and whether SNPs are sufficiently informative to assist with patient selection.

In order to catch the dynamisms of tumor and possible early mechanisms of resistance to antiangiogenic drugs, many efforts have been made in order to identify circulating level variation of VEGF family and its receptors before, during treatment, and at the time of disease progression. Interestingly, VEGF-A levels seem to increase after most antiangiogenic therapies (Willett et al. 2005; Deprimo et al. 2007; Zhu et al. 2009; Burstein et al. 2008). The transmembrane co-receptor neuropilin-1 (NRP1) binds VEGF-A and VEGFR2, of consequence for VEGFR2 internalization and signaling (Koch et al. 2011, 2014). Co-expression of NRP1 and VEGFR2 on endothelial cells promotes angiogenesis, whereas expression of NRP1 on tumor cells may retain VEGFR2 on the endothelial cell surface and thereby suppress signaling (Koch et al. 2014). NRP1 was identified as a co-receptor for the axonal guidance factor family semaphorins and has later been shown to act as a co-receptor for a growing range of growth factors such as hepatocyte growth factor and to modulatory factors such as transforming growth factor beta (Chaudhary et al. 2014). NRP1 may also independently modulate tumor cell migration and survival (Hein and Graver 2013). NRP1 increases during bevacizumab treatment (Xu et al. 2009). Moreover, NRP1 and VEGF-A blockade has been shown to additively inhibit tumor growth (Xu et al. 2009). Circulating levels of soluble VEGFR2 and VEGFR3 are decreased by tyrosine kinase inhibitors with antiangiogenic effect such as sunitinib or sorafenib, but not by bevacizumab (Deprimo et al. 2007; Zhu et al. 2009; Burstein et al. 2008; Willett et al. 2009).

Altogether, these reports suggest several biomarker candidates: circulating levels of short VEGF-A isoforms, expression of neuropilin-1 and VEGFR1 in tumors or plasma, and genetic variants in VEGF-A or its receptors (Lambrechts et al. 2013). However, due to the inconsistency of the results and the lack of validation studies, none of the abovementioned marker is routinely adopted in the clinical practice (Maru et al. 2013).

Therapeutics

Tumor cells that produce stimulatory cues such as VEGF-A recruit tumor-proximal vessels that undergo neoangiogenesis in the tumor. This improves the ability of the tumor to exploit the host; increased tumor vascularization results in better supply of nutrients and facilitates seeding of distant metastases. Targeting VEGF-A in cancer has been developed as a strategy to limit this tumor-host communication, as opposed to a direct attack on cancer cells by a systemic approach.

To date, approved therapeutic options in antiangiogenic therapy are limited to VEGF-A inhibition and receptor tyrosine kinase inhibition, both requiring, as a rule, concomitant administration of traditional cancer therapeutics (i.e., chemotherapy).

An anti-VEGF antibody, bevacizumab (Avastin®) that neutralizes VEGF by preventing its binding to VEGFR1 and VEGFR2, has been developed by Genentech, Inc. (www.gene.com) (Ferrara et al. 2004). Initial studies in animal models showed that VEGF neutralization leads to decreased vascular density and to "normalization" of remaining vessels to a less tortuous and leaky morphology. Subsequently, in these

preclinical models, vessel regression was accompanied by decreased tumor growth, although it is rare to observe actual tumor regression. Combined with standard treatment, these effects of anti-VEGF may promote a tumoristatic condition with disease stabilization also in certain cancers in humans (Ellis and Hicklin 2008). Bevacizumab is approved for the treatment of mCRC in combination with chemotherapy for first- or second-line treatment; non-squamous NSCLC with carboplatin and paclitaxel for first line; for metastatic HER-2 negative breat cancer (only in Europe), with paclitaxel for treatment of patients who have not received chemotherapy for glioblastoma, as a single agent for patients with progressive disease following prior therapy, for renal cell carcinoma in combination with interferon and for ovarian cancer in combination with chemotherapy for platinum-resistant recurrent ovarian cancer in the USA and in Europe and for first-line and platinum-sensitive recurrent patients in Europe only (http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/125085s0169lbl.pdf).

Neutralizing antibodies blocking the binding site for VEGF on the main endothelial VEGF receptor, VEGFR2, have been developed as an alternative strategy to block angiogenesis. The anti-VEGFR2 antibody ramucirumab (ImClone/Eli Lily) has been studied in phase III clinical trials for the treatment of metastatic gastric adenocarcinoma, non-small cell lung cancer, CRC, HCC, and breast cancer and has been approved in the USA for the following indications:

1. A single agent, or in combination with paclitaxel, for the treatment of patients with advanced or metastatic gastric or gastroesophageal junction adenocarcinoma after fluoropyrimidine- or platinum-containing chemotherapy
2. In combination with docetaxel for the treatment of patients with NSCLC progressed after platinum-based chemotherapy
3. In combination with FOLFIRI for the treatment of patients with mCRC after therapy with bevacizumab, oxaliplatin, and a fluoropyrimidine (http://pi.lilly.com/us/cyramza-pi.pdf) (Aprile et al. 2014)

In the phase III randomized REGARD study, ramucirumab was compared to placebo in patients with gastric adenocarcinoma and progression after first-line platinum-containing or fluoropyrimidine-containing chemotherapy. The primary endpoint was overall survival. Three hundred fifty-five patients were assigned to receive ramucirumab ($n = 238$) or placebo ($n = 117$). Median overall survival was increased by ramucirumab compared to placebo, and Tte survival benefit with ramucirumab remained unchanged after multivariable adjustment for other prognostic factors (Fuchs et al. 2014). Similarly, the random phase III RAIBOW ramucirumab trial assessed the efficacy of ramucirumab versus placebo in combination with paclitaxel in patients previously treated for advanced gastric cancer with platinum plus fluoropyrimidine with or without an anthracycline. Six hundred sixty-five patients were assigned to treatment (330 to ramucirumab plus paclitaxel and 335 to placebo plus paclitaxel). Overall survival was significantly longer in the ramucirumab plus paclitaxel group than in the placebo plus paclitaxel group, and the combination of ramucirumab with paclitaxel became a new standard second-line

treatment for patients with advanced gastric cancer (Wilke et al. 2014). The efficacy of ramucirumab in combination with docetaxel as second-line treatment for patients with metastatic NSCLC after platinum-based therapy was assessed in the randomized phase III REVEL. Among 1253 randomized patients, a benefit in terms of OS (primary objective) was demonstrated (Garon et al. 2014).

A benefit in terms of OS was also observed in mCRC patients receiving ramucirumab in combination with FOLFIRI as second-line treatment in the random phase III RAISE trial (Tabernero et al. 2015). In the mCRC scenario, aflibercept, another antiangiogenic agent, received approval in combination with FOLFIRI for the treatment of patients with metastatic CRC resistant to or progressed after an oxaliplatin-containing fluoropyrimidine-based regimen (http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/125418s000lbl.pdf). Aflibercept is a recombinant fusion protein consisting of VEGF-binding portions from the extracellular domains of human VEGFR1 and VEGFR2 that are fused to the Fc portion of the human IgG1 immunoglobulin. It binds to circulating VEGF and acts like a "VEGF trap." It thereby inhibits the activity of the vascular endothelial growth factor subtypes VEGF-A and VEGF-B, as well as to PlGF, inhibiting the growth of new blood vessels in tumor. In the phase III trial VELOUR, the combination of aflibercept to FOLFIRI was evaluated in patients with mCRC previously treated with oxaliplatin. The addition of aflibercept to FOLFIRI significantly improved overall survival progression-free survival and response rate compared to placebo plus FOLFIRI (Van Cutsem et al. 2012b).

Another strategy to suppress excess angiogenesis is the use of small molecule receptor tyrosine kinase inhibitors (RTKIs), which block the ATP-binding site in the tyrosine kinase, thereby inhibiting downstream signaling and biological responses (Zhang et al. 2009). RTKs may be activated in a ligand-independent manner. Thus, VEGFR2 may be activated, e.g., by shear stress created by the blood flow. Therefore, blocking the tyrosine kinase activity, which is a prerequisite for the biological effect, may be more efficient than blocking binding of the ligand. None of the RTKIs in use for cancer treatment today are selective, but inhibit a range of receptor tyrosine kinases. This may be of advantage since several RTKs are dysregulated in conjunction with tumor initiation and growth. On the other hand, the broad range of targets may increase the risk for toxicity. RTKIs that block the different VEGFRs as a rule also block the structurally related platelet-derived growth factor (PDGF) receptors and c-kit. Although these RTKIs may target the tumor vasculature, their effects are broad and may include inhibition also of other host-derived cells as well as tumor cells, which in certain cases express VEGFRs. Several RTKIs that block VEGFRs in addition to other receptors are approved by the FDA and EMA.

Most kinase inhibitors function by competing with the adenosine triphosphate (ATP) molecule, which establishes hydrogen bonds between the adenine ring and the amino acid residues at the binding cleft of the kinase. Small-molecule inhibitors usually target a site nearby the ATP-binding cleft, mimicking the interactions of ATP with the enzyme. Inhibitors that act by this kind of interaction, such as sunitinib, are classified as type I TKIs (Zhang et al. 2009).

Sunitinib (Sutent, Pfizer) is approved as single agent for first-line renal cell carcinoma and for certain gastrointestinal stromal tumors (GIST) after disease progression on or intolerance to imatinib mesylate and for progressive well-differentiated advanced pancreatic neuroendocrine tumors. It targets not only receptors implicated in the angiogenic signaling pathway such as VEGFR and PDGF but also stem cell growth factor receptor (SCFR) also known as c-KIT proto oncogene c-Kit, FMS-like tyrosine kinase-3 (FLT3), glial cell-line derived neurotrophic factor (GDNF) receptor known as (RET) and the receptor of macrophage-colony stimulating factor (CSF1R).

In contrast to type I TKIs, type II inhibitors, like sorafenib, have affinity to the inactive form of the protein kinase. Thus, instead of directly blocking the ATP site, they bind to a hydrophobic pocket that forms when the activation loop of the kinase acquires the DFG-out conformation. Such interaction with this allosteric site impairs conversion of the inactive to the active DFG-in conformation, thus inhibiting its kinase activity (Liu and Gray 2006). Sorafenib (Nexavar; Bayer/Onyx) blocks VEGFR, PDGFR, and the RAF serine/threonine kinases along the RAF/MEK/ERK pathway. It is approved as single agent for first-line treatment of renal cell carcinoma and hepatocellular carcinoma and for refractory to radioactive iodine-differentiated thyroid cancer.

Type III TKIs are called irreversible inhibitors, since they harbor a warhead chemical group that covalently links through a disulfide bond to specific cysteine residues in the kinase binding site. Vandetanib is a quinazoline-based inhibitor that irreversibly blocks ATP binding in VEGFR and EGFR and RET rendering these receptors inactive (Wissner et al. 2007). Vandetanib (Caprelsa, AstraZeneca) is approved for the treatment of symptomatic or progressive medullary thyroid cancer in patients with unresectable, locally advanced, or metastatic disease. The approval is based on an international multicenter randomized double-blind trial conducted in patients with unresectable locally advanced or metastatic medullary thyroid carcinoma. Patients were randomized (2:1) to receive either vandetanib, or placebo. The primary objective was met, and an improvement in PFS was observed for patients who received vandetanib compared with patients who received placebo (Wells et al. 2012).

Pazopanib (Votrient, GlaxoSmithKline) is approved as single agent for first-line renal cell carcinoma and soft tissue sarcoma. It is a multi-tyrosine kinase inhibitor of VEGFR, PDGFR, fibroblast growth factor receptor (FGFR)1 and (FGFR)3, KIT, interleukin-2 receptor-inducible T-cell kinase (Itk), leukocyte-specific protein tyrosine kinase (Lck), and transmembrane-glycoprotein receptor tyrosine kinase (c-Fms).

Regorafenib (Stivarga/Bayer) is approved as single drug in patients with refractory mCRC, based on results of the phase III CORRECT trial. Between April 30, 2010, and March 22, 2011, 760 patients were randomized to receive regorafenib ($n = 505$) or placebo ($n = 255$). The primary endpoint of OS was met at a preplanned interim analysis, and median OS was 6.4 months in the regorafenib group versus 5 months in the placebo group. Regorafenib inhibits several angiogenic and stromal kinase receptors such as VEGFR1, VEGFR2, VEGFR3, FGFR1,

PDGF-b, and Tie-2. In addition, it inhibits various oncogenic receptors (c-KIT and RET), as well as some intracellular signaling kinases (cRAF and BRAF) (Grothey et al. 2013).

Cabozantinib (Cometriq/Exelixis) is approved for the treatment of patients with progressive metastatic medullary thyroid cancer (MTC). It is a small molecule that inhibits the activity of multiple tyrosine kinases, including RET, MET, and VEGF receptor 2. The approval was based on the demonstration of improved PFS observed in an international, multicenter, randomized placebo-controlled trial enrolling 330 patients with metastatic MTC (Elisei et al. 2013). More recently, cabozantinib demonstrated its activity for second-line renal cancer in the phase III randomized METEOR trial, evaluating cabozantinib versus everolimus, a standard of care for treatment of second-line RCC. METEOR met its primary endpoint, demonstrating a statistically significant improvement in PFS for cabozantinib versus everolimus in a population of patients with metastatic RCC who experienced disease progression following treatment with at least one prior VEGF receptor tyrosine kinase inhibitor. The trial also met the secondary endpoint of improved objective response rate (Choueiri et al. 2015). In February 2016, an interim analysis of OS showed a highly statistically significant and clinically meaningful increase in OS for patients randomized to cabozantinib as compared to everolimus. In April 2016, FDA approved cabozantinib for the treatment of advanced renal cell carcinoma in patients who have received prior antiangiogenic therapy.

Axitinib (Inlyta, Pfizer) selectively inhibits VEGFR1, VEGFR2, and VEGFR3, and it is approved as single agent for second-line treatment of renal cell carcinoma based on results from the phase III AXIS 1032 trial, comparing the efficacy and safety of the drug versus sorafenib in patients who had received prior treatment. Patients treated with axitinib had a superior PFS compared to those receiving sorafenib (6.7 months vs. 4.7 months). Moreover, the objective response rates more than doubled in axitinib-treated patients when compared to the sorafenib-treated patients (Rini et al. 2011).

Lenvatinib (Lenvima, Eisai Co) is a multiple kinase inhibitor of both VEGFR2 and VEGFR3; it is indicated for treatment of progressive, radioactive iodine (RAI)-refractory differentiated thyroid cancer. In early 2016, the FDA accepted it for priority review for the treatment of unresectable advanced or metastatic renal cell carcinoma (RCC) in combination with everolimus following one prior vascular endothelial growth factor (VEGF) targeted therapy, thanks to the encouraging PFS results from a phase III trial.

Both the FDA and the EMA approved another antiangiogenic agent nintedanib, a tyrosine kinase inhibitor that targets EGFR, PDGFR, FGFR, and VEGFR as second-line therapy for NSCLC adenocarcinoma but not squamous cell carcinoma, based on results from the phase III LUME-Lung1 study that showed a significant OS benefit for nintedanib and docetaxel versus docetaxel alone in the subset of patients with adenocarcinoma. In this group of patients, median OS was 12.6 months versus 10.3 months, a 2.3-month difference, whereas there was no significant OS difference in either the overall population or those patients with squamous cell histology (Reck et al. 2014). A phase III trial currently ongoing is investigating the efficacy of

nintedanib versus placebo in chemorefractory mCRC patients (LUME-Colon1) (Lenz et al. 2015).

A considerable fraction of patients do not respond to anti-VEGF treatment, especially single-agent therapy, (thus, displaying resistance), and even among those who initially respond, nearly all patients demonstrate tumor progression at some point in their life (escape) (Bergers and Hanahan 2008). In animal models, anti-VEGF treatment has been associated with increased metastatic spread in some but not all studies (Ebos et al. 2009; Paez-Ribes et al. 2009); these observations may occur in certain cancer types or be specific to a certain mouse model. Mechanistically, resistance and escape may involve production by the tumor of other proangiogenic growth factors such as fibroblast growth factor (FGF) or hepatocyte growth factor (HGF) (Bergers and Hanahan 2008). Prolonged VEGF neutralization in animal models has been associated with morphological changes also of vasculature in healthy organs, in particular of endocrine function such as the pancreas (Kamba and McDonald 2007).

Preclinical Summary

VEGF-A is critical in essentially all aspect of vessel function during development, in the adult, and during pathological conditions. Tumors are as a rule hypoxic (reduced oxygen pressure), which stimulates VEGF-A expression. Increased VEGF-A expression promotes recruitment of vessels from the host into the tumor, followed by tumor neoangiogenesis. The vascular organization and structure of tumors differs from normal tissue. Tumor vasculature is more tortuous and chaotic, with inadequate pericyte coverage, increased breaches between endothelial cells, and alternating thick and thin basement membranes. The abnormal vascular structure leads to spatially and temporally heterogeneous blood perfusion in tumors. This heterogeneity in perfusion has multiple adverse consequences. It limits the access of blood-borne drugs and effector immune cells to poorly perfuse regions of tumors and leads to hypoxia and low extracellular pH. In physiologic angiogenesis, the effects of proangiogenic molecules, such as VEGF, are counterbalanced by endogenous antiangiogenic molecules, such as sVEGFR1 and thrombospondins. On the contrary, during tumor angiogenesis, this balance is tipped in favor of new vessel formation. However, the resulting vessels are highly abnormal both structurally and functionally. Preclinical studies have shown that anti- VEGF therapy can initiate vessel normalization. Vessel normalization is measured in the preclinical and clinical setting by decreased vessel diameter, blood volume, mean vessel density, macromolecular permeability, IFP, and edema. Vessel normalization leads to an increase in partial oxygen pressure and perivascular cell coverage in the tumor (Jain 2013; Carmeliet and Jain 2011). The concept of vessel normalization was tested and confirmed in preclinical models: it was shown that the vessels function better in response to anti-VEGF therapy, and they enhance both the delivery and effectiveness of concurrent therapies, not only chemotherapeutic agents but also immuno-agents. Subsequently adopting human glioblastoma xenografts in cranial windows, it was

found that normalization started immediately after drug administration and lasted 5–6 days. Interestingly, radiotherapy was more effective when administered during this normalization window. Moreover, the activation of Ang1/Tie2 signaling pathway was identified as responsible for recruitment of pericytes and activation of matrix metalloproteinases (MMPs) thus leading to thinning of the vascular basement membrane (Winkler et al. 2004). Preclinical studies also demonstrated that vascular normalization is dependent on the dose of anti-VEGF drug. While low doses might improve perfusion and outcome, high doses of anti-VEGF agents caused rapid vessel pruning and did not improve the outcome of concurrent therapies and even increased invasion and metastasis (Huang et al. 2012). In the vascular normalization process, anti-VEGF treatments cause a decrease in pore size of tumor vessels. In a breast cancer model in mice, it was found that VEGFR2 blockade improved the treatment benefit from small molecules such as 10-nm nab-paclitaxel but not that from 100-nm liposomal doxorubicin (Chauhan et al. 2012).

Clinical Summary

Angiogenesis is a validated target in oncology, as demonstrated through randomized trials that compare conventional therapy with or without VEGF-A pathway inhibitors.

Bevacizumab binds only VEGF-A and neutralizes all human VEGF-A isoforms and bioactive proteolytic fragments (Ferrara et al. 2004). It has been hypothesized that antiangiogenic compounds could block the formation of new blood vessels, reduce vascular permeability, promote capillary regression, stimulate vascular normalization, and restore dendritic cell function (Ferrarotto and Hoff 2013). In addition, inhibition of angiogenesis could also increase the efficacy of chemotherapy, either by decreasing the elevated interstitial pressure in tumors and improving the delivery of cytotoxic agents or by enhancing the sensitivity of tumor endothelial cells to the effects of antineoplastic therapy (Ferrarotto and Hoff 2013).

Bevacizumab has been approved by the US Food and Drug Administration (FDA) for clinical use in many human cancers. Aside from the benefit of bevacizumab monotherapy in glioblastoma and renal cell carcinoma (Junck 2011; Yang 2004), other solid cancer such as lung, and colorectal showed a greater therapeutic effect when combined with conventional chemotherapy (Sandler et al. 2006; Miller et al. 2007; Hurwitz et al. 2004). The FDA accelerated approval of the use of bevacizumab in metastatic breast cancer in 2008 was based on the results of E2100 phase III study showing that bevacizumab in combination with paclitaxel substantially improves progression-free survival of patients when compared with paclitaxel alone (Miller et al. 2007). However, the approval was revoked in 2011 (www.cancer.gov) due to the limited patient benefits, which did not outweigh the considerable risks for toxic side effects.

Bevacizumab significantly improves clinical outcomes in advanced colorectal, non-small-cell lung, renal, and ovarian cancers in terms of PFS and, in some cases, OS (Sandler et al. 2006; Miller et al. 2007; Hurwitz et al. 2004; Kubicka et al. 2013).

Subgroup analyses in these trials suggested that bevacizumab provides a significant but relatively modest benefit in almost all clinically defined subsets of patients. This resulted in a global effort, involving thousands of patients' samples, to identify the patients who are most likely to gain from bevacizumab therapy while also reducing the toxicity and costs associated with treating patients whose disease is not amenable to bevacizumab therapy (Murukesh et al. 2010; Jayson et al. 2012).

VEGF-A neutralization is clinically beneficial in first-line combinatorial treatment of patients with solid, highly vascularized tumor disease. Systemic side effects include elevated blood pressure, thromboembolic events, and hemorrhage, which may be disease specific. Predictive biomarkers for efficient anti-VEGF-A therapy have yet to be identified. By targeting VEGF-A, tumor progression can be delayed. A fraction of patients respond very well to anti-VEGF-A therapy; however, a complete response is rarely seen.

Anticipated High-Impact Results

VEGF-A neutralization has clinical benefit in combinatorial treatment in certain patients in a number of cancer diagnoses. The lack of biomarkers for anti-VEGF-A therapy has this far prevented identification of patients for whom VEGF-A neutralization is beneficial. The mechanisms of escape from responsiveness, or eventual resistance to therapy, are being unraveled.

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Part III

Growth Factors

AXL

58

Balazs Halmos and Xue-wen Liu

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Abstract

AXL is a member of the TAM family, a receptor tyrosine kinase (RTK) subfamily composed of AXL, TYRO-3, and MER. Its main ligand is the vitamin K-dependent protein named growth arrest-specific gene 6 (Gas6). AXL is abnormally activated in many cancers by protein overexpression, point mutations, and gene fusions. The Gas6-AXL axis contributes to tumor progression, invasion, metastasis, and resistance both to chemotherapeutic and targeted anticancer therapies in a wide variety of cancers. In this review, we describe the biology of

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AXL, review diverse mechanisms of activation and the established and putative roles of AXL as a biomarker and therapeutic target in cancer therapy. Pre-clinical and clinical data for anti-AXL therapies to date are also summarized.

Keywords
AXL • TAM family • Receptor tyrosine kinase (RTK) • Gas6 • Activated mechanisms • Overexpression • Point mutations • Acquired TKI resistance • Epithelial-mesenchymal transition (EMT) • Targeted inhibitors

Target: AXL

AXL is an oncogene originally isolated from chronic myelogenous leukemia cells and is a member of the TAM family, a receptor tyrosine kinase (RTK) subfamily composed of AXL, TYRO-3, and MER. The AXL gene is located on chromosome 19q13.2 and encompasses 20 exons. Structurally, AXL consists of an extracellular region composed of two immunoglobulin-like (Ig) domains and two fibronectin type III (FNIII) domains, a transmembrane domain and a cytoplasmic tyrosine kinase domain (O'Bryan et al. 1991). Its main ligand is the vitamin K-dependent protein named growth arrest-specific gene 6 (Gas6).

Biology of the Target

AXL, along with the other two members of the TAM family of RTKs, TYRO-3 and MER, is implicated in a diverse array of physiological processes, including cell proliferation, survival, cell adhesion and migration, blood clot stabilization, regulation of inflammatory cytokine release, and immune maturation. In adult tissues, AXL, TYRO-3, and MER exhibit widespread distribution with overlapping but unique expression profiles such as AXL which is expressed ubiquitously, with high levels found in the hippocampus and cerebellum, platelets, monocytes/macrophages, endothelial cells, skeletal muscle, heart, kidney, testes, and liver, as well as in different cell lines of mesenchymal, epithelial, and hematopoietic origin (Neubauer et al. 1994). TAM receptor expression is observed in embryonic tissues, but it appears nonessential for embryogenesis, as even triple-knockout mice are viable without any notable changes at birth. On the contrary, TAM receptor knockout mice in adulthood develop diverse phenotypes in different of tissues. Deficiencies in TAM signaling have been shown to play key roles in chronic inflammatory and autoimmune disease in humans, and overexpression/ectopic expression is associated with cancer progression, metastasis, and resistance to chemotherapy/targeted therapy (Lemke 2013).

AXL can be activated through several mechanisms: ligand-dependent and ligand-independent dimerization, interaction between two monomers on neighboring cells, and heterogenic dimerization with a non-TAM receptor (Korshunov 2012).

Gas6/AXL signaling plays diverse roles in numerous cellular activities (Braunger et al. 1997). Gas6 binding to the extracellular domain of AXL leads to autophosphorylation of tyrosine residues on the intracellular tyrosine kinase domain of AXL, including Y779, Y821, and Y866 followed by Gas6/AXL-induced activation of MAPK/ERK and PI3K/AKT signaling pathways (Braunger et al. 1997). TENC1 and SOCS-1 have been identified as negative regulators of AXL signaling. In particular, TENC1 can reduce cell survival, proliferation, and migration through negative regulation of AXL-mediated PI3K/AKT signaling (Hafizi et al. 2005). Soluble forms of AXL (sAXL) that are produced by proteolytic cleavage can bind to Gas6 inhibiting its cellular activation of AXL in murine and human plasma (Budagian et al. 2005).

At the transcriptional level, there are multiple mechanisms whereby the expression of AXL is regulated. Several transcription factors have been shown to upregulate AXL, such as Sp1/Sp3, myeloid zinc finger 1 (MZF1), and activator protein 1 (AP-1). In particular, AXL induction via Yes-associated protein 1 (YAP1) has been shown to play an important role in tumorigenesis and progression (Axelrod and Pienta 2014). Posttranscriptional regulation through microRNA (miR-34a and miR-199a/b) binding of the 3′ UTR of AXL has also been noted to regulate AXL expression (Mudduluru et al. 2011).

Target Assessment

AXL overexpression has been identified in multiple cancers, such as lung cancer, breast cancer, prostate cancer, etc., and has been shown to correlate with/result in invasion and metastasis (Zhang et al. 2012; Ishikawa et al. 2013; Wang et al. 2013; Paccez et al. 2013). AXL overexpression might also play an important role in resistance to chemotherapy and targeted therapies in a multitude of cancers. However, it needs to be noted that the definition of AXL overexpression is not well defined introducing potential for bias in some of these studies. As the expression of phosphorylated AXL is challenging to quantify accurately due to technical difficulties, total AXL expression level by IHC remains the main way of assessing activity of the AXL pathway and is the biomarker that is selected for most studies focused on AXL inhibition. Recently, AXL point mutations in the kinase domain were reported in 22% (5/23) of colorectal cancer cell lines (COLO205, KM12, HCT116, HCT15, and LOVO) (Donnard et al. 2014), while lower mutation frequencies (3.5%) were reported for primary colorectal tumors in the TCGA database (Cancer Genome Atlas 2012). Rare fusions of the AXL gene, such as an AXL-MBIP fusion generating a chimeric protein, carrying the AXL protein tyrosine kinase domains and a dimerization unit of MBIP, have also been identified in lung adenocarcinoma (Seo et al. 2012). Functional validation of these mutations and fusion products is largely lacking at present; however, if proven to be functional, they could serve as more robust biomarkers to define tumor subsets for AXL inhibition.

Role of the Target in Cancer

(A) **Rank**: “Unknown” to 10
(B) Unknown to 1-2-3-4-5-6-7-8-9-10: 7

AXL Overexpression in Cancers

AXL was in fact first described as a transforming gene from a chronic myeloid leukemia (CML) cell line suggestive of a role in malignancy (O’Bryan et al. 1991). Since then there have been a wide array of studies, demonstrating overexpression or ectopic expression of AXL in a multitude of cancers. AXL was found to be overexpressed in acute myeloid leukemia (AML) and also related to progression and worse overall survival (Ben-Batalla et al. 2013). AXL overexpression has been reported in a significant proportion (up to 60%) of NSCLC cell lines and primary lung tumors (Shieh et al. 2005; Wimmel et al. 2001). Both elevated mRNA and protein levels of AXL are correlated with poor prognosis and aggressive features of lung adenocarcinoma. AXL is expressed in the normal mammary gland, but overexpressed in highly invasive breast cancer cell lines (Zhang et al. 2008). Overexpression of AXL is required for the growth of mesenchymal glioblastoma stemlike cells (MES GSCs) and correlated with poor prognosis. Using AXL shRNA or pharmacologic inhibitors can result in cell death in MES GSCs (Cheng et al. 2015).

AXL in EMT

Several studies suggest a dual role for AXL in the process of epithelial-mesenchymal transition (EMT) as both a driver and effector of EMT. AXL is activated and induced by EMT through regulation of the expression of E-cadherin, N-cadherin, Slug, and Snail in breast cancer stem cells (BCSC) as well as other settings. EMT-associated upregulation of AXL can lead to autocrine interactions with Gas6 produced by endothelial cells, suggesting that the noted induction of autocrine AXL signaling might be a frequent consequence of EMT in many tumor types. On the other hand, AXL expression has also been shown to induce EMT per se, thereby regulating BCSC chemoresistance and invasion. Treatment with the AXL inhibitor MP470 reversed EMT and restored chemosensitivity in murine BCSCs (Gjerdrum et al. 2010; Asiedu et al. 2014). AXL activation consequent to EMT induction has been noted in EGFR-mutant lung cancer with acquired resistance to EGFR TKIs in vivo and in vitro, and inhibition of AXL leads to partial restoration of sensitivity to EGFR inhibition. It was also shown that vimentin expression may be required for the induction of AXL expression as silencing of vimentin decreased AXL expression levels suggestive of an amplificatory loop. All in all, AXL expression certainly is a promising biomarker of EMT, and possibly AXL inhibition could also have treatment potential for partial reversal of EMT changes (Zhang et al. 2012).

AXL Overexpression in Drug Resistance

A multitude of studies demonstrate a role for AXL in treatment resistance. In CML, AXL overexpression was shown to correlate with resistance to the ABL inhibitor, imatinib (Dufies et al. 2011). Combining AXL inhibition and chemotherapeutic drugs was noted to be more effective than chemotherapy alone in AXL-positive AML. AXL overexpression has been shown to promote cisplatin resistance through regulation of c-ABL/p73 signaling in esophageal carcinoma (Hong et al. 2013). AXL overexpression, possibly via EMT induction, has been associated with acquired resistance to the EGFR TKI, erlotinib in vitro and in vivo in EGFR-mutated lung adenocarcinomas (about 20% in primary tumors), and combined inhibition of EGFR and AXL was shown to at least partially overcome resistance in vitro identifying AXL as a promising therapeutic target to prevent or overcome resistance against EGFR TKI therapy (Zhang et al. 2012). Another pivotal study using samples from the BATTLE trial similarly showed AXL expression to be a key marker of an EMT phenotype correlating with EGFR TKI resistance. Whether AXL expression in this setting is simply a marker of EMT transition or a true driver of resistance remains to be validated. Analogous to these findings in EGFR TKI resistance, EMT transition/AXL expression has been implicated in limited studies in ALK-translocation positive lung cancers as well. Activation of AXL has also been shown to contribute to lapatinib and trastuzumab resistance in HER2-positive, ER-positive breast cancers (Liu et al. 2009). Recent studies also find a key role for AXL activation in PIK3CA-mutated or amplified head and neck squamous cell carcinomas treated with the PIK3CA inhibitor, BYL719. In this setting, AXL expression cooperates with EGFR to yield a bypass mechanism resulting in PIK3CA-independent persistent mTOR activation. Combined treatment with a PI3-kinase inhibitor along with an EGFR, AXL, or PKC alpha inhibitor successfully reverses resistance (Elkabets et al. 2015). Overexpression of MET and HGF has been described as mechanisms of resistance to EGFR-TKI therapy in vitro and in vivo. AXL/MET share numbers of structural features and play important roles in cell invasion and metastasis. Both MET and AXL are key potential targets for novel therapies in EGFR resistance and also have been shown to participate and cooperate in cell surface complexes leading to uncoupling of EGFR oncogenic activity from its tyrosine kinase function (Gusenbauer et al. 2013).

High-Level Overview

Diagnostic, Prognostic, Predictive (If Applicable)

Diagnostic

AXL appears to be an intriguing candidate target for cancer therapy, with a multitude of roles in the regulation of cell survival, proliferation, migration, adhesion, EMT, etc. While overexpression of AXL has been associated with invasiveness, metastasis, poor prognosis, and drug resistance in different cancers, there remains a strong

and urgent need for a validated tool for the objective assessment of AXL pathway activation. Mutations and genetic translocations affecting the AXL gene might provide more robust biomarkers to define tumor subsets for AXL targeting; however, these genetic abnormalities so far appear rare and their functional relevance remains ill defined. Ongoing clinical studies of AXL inhibitors incorporate a variety of techniques to assess the utility of such drugs for unique tumor subsets and results of such studies will be pivotal.

Prognostic

As AXL appears to be involved in cell proliferation, invasiveness, and metastasis as an essential regulator of the EMT process in cancer cells, it is not surprising that many studies suggest that AXL overexpression indeed has prognostic value by defining a more aggressive or resistance subset of tumors. Given the uniform use of unvalidated assays and retrospective studies in this setting, further corroboration is still needed prior to clinical implementation (Song et al. 2011).

Predictive

AXL overexpression appears to be correlated with drug resistance in several settings, and thereby assessment of AXL expression could have predictive value in this context. Clinical validation of these findings is lacking still and further corroboration to prove a clinically useful predictive role is awaited.

Therapeutics (If Applicable)

As outlined above, targeting the Gas6/AXL pathway is a very promising new avenue for cancer therapy, and effective inhibition of this pathway can be achieved pharmacologically with multiple different classes of agents, most prominently small-molecule ATP-mimetic kinase inhibitors (e.g., XL184, R428, etc.) of the AXL kinase function as well as anti-AXL antibodies (e.g., YW327.6S2). Most ATP-mimetic inhibitors are not very specific for AXL inhibition, though, and given high degree of similarity of the kinase domain, usually MET, RET, and other kinases are also blocked by most multikinase drugs. More recently, efforts have been made to develop more selective AXL inhibitors. Many ongoing early-phase clinical trials of these novel compounds, such as multikinase drugs like XL184 and XL880, have been pursued without a specific focus on AXL as a main target. However, as early-phase studies of AXL-targeted inhibitors are being completed and AXL continues to emerge as a promising target, more clinical studies are anticipated to emerge with a specific focus on AXL. Some more relevant studies are listed below.

Preclinical Studies

A variety of more or less specific small molecular tyrosine kinase inhibitors of the AXL kinase have been developed, and extensive preclinical information is available (Table 1). Other classes of molecules include anti-AXL antibodies, for example,

Table 1 List of AXL-targeted inhibitors, antibodies, and nucleotide aptamers under development adopted from a recently published review (Wu et al. 2014)

Compound	Known targets	ClinicalTrials. gov identifier	Phase of development	Sponsor	Functions in a disease indication and other preclinical research details
TP-0903	AXL		Preclinical	Tolero	Induced apoptosis in CLL B cells
DP3975	AXL		Preclinical	Deciphera Biotech	Inhibited cell migration and proliferation in mesotheliomas
NPS-1034	AXL, MET		Preclinical	NeoPharma	Newly developed drug that targets both MET and AXL in NSCLC cells with acquired resistance to gefitinib or erlotinib
LDC1267	AXL, Tyro, Mer		Preclinical		Induces NK cells to kill tumor cells in mouse metastatic breast cancer and melanoma model
NA80x1	AXL		Preclinical		Inhibits AXL phosphorylation, cell motility, and invasion in MDA-MB-435 cells
MGCD516	MET, AXL, RET, TRK, DDR2, KDR, PDGFRA, or KIT	NCT02219711	Phase 1/1b	Mirati Inc.	Inhibits AXL, MET, MER, VEGFR, PDGFR, DDR2, and TRK
BMS-777607 (ASLAN 002)	AXL, Mer, and MET	NCT01721148	Phase 1	Aslan Pharma. and Inventive Health Clinical	Selective small-molecule kinase inhibitor against AXL, Mer, and Met
S49076	AXL, MET, EGFR	ISRC TN00759419	Phase 1	Servier	Preclinical activity in colon carcinoma
LY2801653	AXL, MET, MST1R	NCT01285037	Phase 1	Eli Lilly and Co.	An orally bioavailable multikinase inhibitor against MET, AXL, MST1R
R428 (BGB324)	AXL	European Clinical trial	Phase Ia	BerGen BIO	Resensitized HN5-ER cells to erlotinib in head and neck cancer, reduced migration and invasion in melanomas, induced CLL B-cell apoptosis, reduced invasion and migration in EAC cell lines, reduced metastatic burden, and extended survival in metastatic breast cancer

(*continued*)

Table 1 (continued)

Compound	Known targets	ClinicalTrials.gov identifier	Phase of development	Sponsor	Functions in a disease indication and other preclinical research details
MP-470 (Amuvatinib)	AXL, c-KIT, PDGFR, FLT3, RET, RAD51	NCT00894894 NCT00881166 NCT01357395	Phase 1 Phase 1 Phase 2	Astex Pharma	c-Kit/AXL tyrosine kinase inhibitor investigated in stromal tumors and in breast cancer
SKI-606 (Bosutinib)	AXL, Src Kinase, Abl, TGFB, BMP	NCT00195260 NCT00319254	Phase 1 Phase 2	Pfizer	Treating HCC cells with Bosutinib decreased the AXL-specific invasiveness of HCC cell lines
MGCD 265	AXL, MET, VEGFR	NCT00697632 NCT00975767	Phase 1 and 2 Phase 1	Mirati Inc.	Phase 2 NSCLC
ASP2215	AXL, Flt3	NCT02014558	Phase 1 and 2	Astellas Pharma	A novel FLT3/AXL inhibitor: preclinical evaluation in acute myeloid leukemia
GSK1363089/ XL880 (Foretinib)	AXL, c-MET, VEGFR2	NCT02034097	Phase 2	GlaxoSmithKline	Restores lapatinib sensitivity in lapatinib-resistant breast cancer cells with AXL over expression
SGI-7079	AXL	NCT00409968	Phase 2	Astex Pharma	Decreased malignant properties in inflammatory breast cancer. Combination of SGI-7079 with erlotinib reversed erlotinib resistance in mesenchymal cell lines, xenograft model of mesenchymal NSCLC and patients
XL184 (Cabozantinib)	AXL, c-MET, VEGFR-2, c-KIT, Flt 1/3/4, Tie2, and RET	NCT01639508	Phase 2 and 3	Exelixis	Medullary thyroid cancer, brain cancer, NSCLC, and randomization discontinuation trial in various solid tumors
YW327.6S2	AXL		Preclinical		Anti-AXL monoclonal antibody
GL21.T	AXL		Preclinical		Binds to the extracellular domain of AXL to inhibit its catalytic activity in lung cancer

YW327.6S2, which has shown highly specific activity in preclinical models (Ye et al. 2010). GLT21. T is a nucleotide aptamer that binds to the extracellular domain of AXL and inhibits AXL activity in cellular models of lung cancer (Esposito et al. 2014). Another route of targeting AXL overexpression/activity might be through the inhibition of heat shock proteins, such as hsp90, which are involved in the regulation of protein trafficking (Krishnamoorthy et al. 2013).

Clinical Studies

The Gas6-AXL axis can contribute to tumor progression, invasion, metastasis, and resistance both to chemo- and targeted anticancer therapies in a wide variety of cancers, indicating that the Gas6/AXL pathway is a very promising target for cancer therapy. Many clinical trials of promising drugs, in multi-targeted or more AXL-specific tyrosine kinase inhibitors, have been recently initiated, and now biomarker-driven studies are starting to shift focus more specifically on AXL as a main target.

Anticipated High-Impact Results (Bullet Points of Anticipated Data)

- AXL is an emerging target for cancer therapy; however, there is still significant risk that some of the reported findings on AXL suggest possibly a passenger and not a key functional role in some settings, e.g., in the context of EMT. Therefore, further validation of the true functional impact of the AXL pathway in a range of settings still awaits full validation.
- Another key issue to emerge from ongoing studies, including clinical studies of AXL inhibitors, is the selection of appropriate biomarkers for patient enrichment for the development of AXL-targeted therapies for human use. Currently, IHC and AXL genetic aberrations are the lead candidates; however, the former is a less robust assay while genetic changes appear quite rare albeit existent. Responses in well-defined patient subsets in the ongoing studies will be pivotal to help our understanding of the relevance of this pathway.
- Studies specifically focusing on the acquired resistance setting will also be important to assess the development of proper biomarkers and the utility of combined inhibition to prevent the emergence of resistance.

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B-Raf

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Abstract

B-Raf is a serine threonine protein kinase downstream of RAS and as such is part of the mitogen-activated protein kinase (MAPK) signaling pathway involved in proliferation and survival of cells. B-Raf is mutated in a high percentage of cancers, 50% of melanomas arising on non-chronic UV-damaged skin and at a lower frequency in other types of melanoma, thyroid, colon, and ovarian cancers. Mutated B-Raf is an oncogenic driver in melanoma and other cancers. It is believed to be an early event since it is seen in the majority of nevi and a subset of premalignant colon polyps. Several kinase inhibitors targeting B-Raf are approved for advanced melanoma.

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Target

B-Raf is a serine threonine protein kinase and a member of the RAF family of protein kinases. It lies downstream of RAS and as such is part of the mitogen-activated protein kinase (MAPK) signaling pathway involved in proliferation and survival of cells. B-Raf, like A-Raf and C-Raf, signals through MEK and subsequently ERK, making those effector kinases also possible targets for influencing signaling of B-Raf.

Interest in B-Raf as a cancer target was initiated through the identification of BRAF mutations in a high percentage of cancers by the cancer genome project (Davies et al. 2002), where mutations were found in more than 50% of melanomas arising on non-chronic UV-damaged skin and at a lower frequency in other types of melanoma, thyroid, colon, and ovarian cancers.

Mutated BRAF is an oncogenic driver in melanoma and other cancers. It is believed to be an early event since it is seen in the majority of nevi (Pollock et al. 2003) and a subset of premalignant colon polyps (Yuen et al. 2002). Nevi will undergo oncogene-induced senescence unless additional mutations or deletions like loss of PTEN occur and allow for malignant transformation (Vredeveld et al. 2012).

Biology of the Target

The oncogenic mutation most commonly found in BRAF is an acquired gain of function mutation in the kinase domain, leading to constitutive activation and therefore multiple-fold higher kinase activity of B-Raf. In the majority of all cases in melanoma and in virtually all thyroid cancers with BRAF mutation, this is aT1796A single-base missense mutation, leading to a glutamic acid to valine substitution at position 600 (V600E) (Michaloglou et al. 2007). This and most other mutations described reside in exon 15, but in rare cases, mutations can also occur in the loop domain on exon 11 (Omholt et al. 2003). In a prospective study of 197 melanoma patients, mutated BRAF was found in 48% of cases. These were V600E in 74%, V600K in 20%, and other genotypes in 6% (Long et al. 2011). Some of these less common mutations have been described to be low kinase activity mutations reliant upon CRAF signaling (Smalley et al. 2009).

The high frequency and specificity of the BRAF V600E mutation suggest a dependency of those cancer cells on an activated MAPK pathway. This also supported by the finding that mutations in RAS, also activating the MAPK pathway,

are found in 20% of melanomas but are always mutually exclusive with BRAF V600E (Hodis et al. 2012).

Target Assessment

Second-generation B-Raf inhibitors target the V600E-mutated form with high specificity, making prescreening of patients mandatory. Vemurafenib has been approved with an RT-PCR-based companion diagnostic, the Cobas4800 (Roche) (Cheng et al.). In addition, there are reports that BRAF inhibition might actually be harmful to patients with normal BRAF. Transactivation of wild-type BRAF in melanoma tumors will lead to increased proliferation and possibly negative effects on treatment outcome (Hatzivassiliou et al. 2010; Heidorn et al. 2010; Poulikakos et al. 2010).

Role of the Target in Cancer

Rank: 9 (high).

High-Level Overview

Activating mutations in BRAF kinase are oncogenic independent of RAS signaling. They are most common in cutaneous non-chronic UV-exposed melanoma and are found at a lower frequency in colon, thyroid, and ovarian cancers.

Diagnostic, Prognostic, and Predictive

BRAF V600E is not suited as a diagnostic tool to distinguish primary melanomas from atypical nevi, since the majority of benign nevi are positive for BRAF V600E (Pollock et al. 2003; Kumar et al. 2004).

The possible application of BRAF V600E as a prognostic marker has been investigated: Although at the earlier stages of melanoma development, BRAF-mutated melanomas show a higher rate of ulceration, associated with poor outcome, compared to NRAS mutant and non-BRAF/non-NRAS mutant melanomas and an increased Breslow thickness compared to non-BRAF/non-NRAS mutant tumors, this does not translate into differences in survival outcomes based on BRAF mutation status in melanoma (Ellerhorst et al. 2011; Edlundh-Rose et al. 2006; Kaplan et al. 2011). Recently, based on single-cell analysis, it has been found that in some cases, primary melanoma lesions contain only a low percentage of cells with the BRAF V600E mutation and that BRAF mutant alleles are selected for during progression (Lin et al. 2011).

Therapeutics

Sorafenib was the first kinase inhibitor with activity against B-Raf to enter clinical trials (Hauschild et al. 2009) in the treatment of metastatic melanoma. Unfortunately, this drug failed to show a significant impact in this disease most likely because sorafenib is a broad kinase inhibitor with low activity against V600E-mutated B-Raf. Subsequently, several inhibitors have been developed specifically against the oncogenic form of B-Raf. Vemurafenib, marketed as Zelboraf, is the first FDA-approved inhibitor of BRAF V600E for treatment of late-stage V600E mutant melanoma.

Preclinical Summary

In 2008, the discovery of a potent inhibitor of the V600E-mutated form of B-Raf was reported: PLX4720 was developed specifically against the crystal structure of BRAF V600E and showed a tenfold higher specificity for the oncogenic form of B-Raf compared to wild type. Impressive activity in a panel of V600E mutant melanoma cell lines was shown in vitro and in vivo, whereas cell lines with wild-type B-Raf were not inhibited (Tsai et al. 2008).

Although significant cell death was achieved in melanoma, in thyroid cancer cells, inhibition of B-Raf V600E merely led to growth arrest, hinting at B-Raf-independent mechanisms of cell survival existing in anaplastic thyroid cancer cells (Sala et al. 2008).

Structure-guided chemistry then led to the development of a clinical candidate in a crystalline formulation, PLX4032. The pharmacological properties of PLX4032 in preclinical models of melanoma are discussed in Lee et al. (2010). Here, using a panel of melanoma cell lines with or without BRAF V600E mutation, highly significant induction of cell death in 3D spheroid and skin reconstruct models of melanoma and complete growth inhibition of xenotransplants in vivo in BRAF could be achieved V600E-mutated cell lines.

Furthermore, PLX4032 is selectively inhibiting ERK signaling in BRAF V600E mutant cells and transiently activating the MAPK pathway in BRAF wild-type cells. This property results in a broader therapeutic index (Joseph et al. 2010).

Although these results are a major breakthrough in melanoma therapy and vemurafenib has subsequently shown impressive clinical responses in melanoma patients (see below), the overwhelming majority of patients eventually develop resistance. The mechanisms leading to the intrinsic and acquired resistance phenotypes are currently explored with high priority. The current view is that multiple mechanisms can be responsible, either individually or overlapping, and a number of mechanisms have been described so far.

One is a truncated form of BRAF V600E lacking the RAS-binding domain, and thereby leading to enhanced dimerization and signaling to ERK and resistance to the BRAF inhibitor vemurafenib. This splice variant has been found in 6 of 19 patients with acquired resistance to vemurafenib (Poulikakos et al. 2011).

No additional mutations in BRAF conferring resistance to BRAF inhibitors have been described so far. On the other hand, secondary mutations in NRAS, upstream of RAF, have been found to arise after chronic treatment with BRAF inhibitor (Nazarian et al. 2010). Downstream of RAF mutation in MEK1 has also been described to be responsible for acquired resistance to BRAF inhibition (Wagle et al. 2011).

High-level expression of COT has been implicated in resistance to BRAF inhibitor, since COT provides an alternative pathway to MEK activation (Johannessen et al. 2010).

Finally, activation of alternative pathways through increases in receptor tyrosine kinases (RTKs) provide survival mechanisms for melanoma treated with BRAF inhibitors (Villanueva et al. 2010).

Since a subset of patients are intrinsically resistant to BRAF inhibition or show only very transient responses, the loss of functional PTEN observed in some melanomas has been implicated as a mechanism of intrinsic resistance through the suppression of BIM (Paraiso et al. 2011) or in conjunction with loss of Rb (Xing et al. 2012).

Overcoming these mechanisms of resistance is an important step in advancing the treatment of melanoma. One possible approach would be to target these alternative survival and proliferation pathways in combination with BRAF inhibition as shown in Villanueva et al. (2010) and Greger et al. (2012).

It has been shown that in colon cancers, rapid activation of EGFR, supporting continued proliferation, occurs after BRAF V600E inhibition with vemurafenib. This escape mechanism could be blocked by combining vemurafenib with an EGFR inhibitor and thereby sensitizing colon cancer cells to this treatment (Prahallad et al. 2012).

Clinical Summary

Vemurafenib: Results of a phase I multicenter dose-escalation trial were published in 2010 (Flaherty et al. 2010). Unprecedented results were achieved: of the 48 melanoma patients who received 240 mg or more twice daily, 77% had a clinical response. The estimated median progression-free survival of all patients treated was 7 months. The authors concluded that the majority of patients carrying the BRAF V600E mutation benefited significantly with complete or partial tumor regression.

In addition, Bollag et al. (2010) reported that 80% inhibition of ERK phosphorylation was needed to translate into clinical response. This was achieved with an amorphous formulation of the drug at an oral dose of 960 mg twice daily.

This dose was subsequently used in a phase 2 clinical trial. A total of 132 patients were treated in this study. Eight patients achieved a complete response and 62 patients a partial response for an overall response rate of 55%, and the median overall survival was 15.9 months (Sosman et al. 2012). These results independently confirmed the high response rate and response duration shown in a phase 1 trial. The

long follow-up period in our study provides critical information on long-term overall survival, not yet shown in the phase 3 trial comparing vemurafenib with dacarbazine. Nineteen targeted therapy aimed at oncogenic BRAF V600 induces responses in half of the patients and a median survival of 16 months.

Before completion of this phase 2 trial, a phase 3 trial was initiated with the first patient dosed in August 2010. In a phase 3 randomized clinical trial, comparing vemurafenib with dacarbazine in 675 patients with previously untreated, metastatic melanoma with the BRAF V600E mutation vemurafenib produced improved rates of overall and progression-free survival in patients with previously untreated melanoma with the BRAF V600E mutation (Chapman et al. 2011). The most commonly reported adverse events were arthralgia, rash, a photosensitivity reaction, fatigue, and alopecia. Development of cutaneous squamous cell carcinoma or keratoacanthoma was reported in 34 patients (26%). These were found to have frequent RAS mutations consistent with paradoxical activation of MAPK signaling by vemurafenib in BRAF V600E wild-type cells (Su et al. 2012).

Vemurafenib was FDA approved in August 2011 for the treatment on BRAF V600E-positive metastatic melanoma.

In the wake of the unprecedented effectiveness of vemurafenib and the dismal response rates in the dacarbazine control arm, the ethicality of parallel design studies in a disease-like metastatic melanoma with no effective standard therapy and a high mortality rate was discussed extensively (Miller and Joffe 2011). Therefore, after review of the interim analysis in the vemurafenib phase 3 study, crossover after progression on dacarbazine was allowed into the vemurafenib group. Such a cross-over design was then inherent for the randomized phase 3 study of dabrafenib, a compound also targeting BRAF V600E.

Dabrafenib, GSK2118436, was tested in a phase 1/2 accelerated dose-escalation trial, and although no maximum tolerated dose was recorded, the phase 2 dose was set on the basis on safety, PK, and response data at 150 mg twice daily. The most common adverse events were SCC, fatigue, and pyrexia. This study included all VAL600 mutations of BRAF, and responses were reported in 69% of patients treated; the response rates were higher in patients with the V600E mutation only. Forty-seven percent of responders were still on treatment 6 months after start of treatment. Interestingly, this study included a cohort of patients with previously untreated brain metastasis, and nine out ten patients treated had reductions in size of their brain lesions. Some antitumor activity was also observed in 28 patients, including solid tumors other than melanoma harboring Val600 BRAF mutations (Falchook et al. 2012).

The efficacy of dabrafenib was then assessed in a randomized open-label phase 3 trial in patients with BRAF V600E-mutated metastatic melanoma. Patients received dabrafenib 150 mg twice daily or dacarbazine 1,000 mg/m2 every 3 weeks. The ratio between the two groups was 3:1, and patients were allowed to cross over into the dabrafenib arm after progression on dacarbazine. One hundred eighty-seven patients received dabrafenib with a median progression-free survival of 5.1 months at the data cutoff date, compared to 63 patients receiving dacarbazine

with a median progression-free survival of 2.7 months. The most common adverse events in the dabrafenib group were skin-related toxic effects, fever, fatigue, arthralgia, and headache. Grade 3–4 adverse events were uncommon. In conclusion, dabrafenib significantly improved progression-free survival compared with dacarbazine (Hauschild et al.).

Mutations in RAS have been found in cutaneous SCC arising in dabrafenib-treated patients comparable to reports for vemurafenib-treated patients (Anforth et al. 2012). Trametinib, GSK1120212, a small-molecule kinase inhibitor targeting MEK, the downstream effector caspase of B-Raf, showed single-agent activity in a phase III clinical trial for patients with metastatic melanoma harboring the V600E mutation improving overall survival compared to chemotherapy. Rash, diarrhea, and peripheral edema were the most common toxic effects in the trametinib-treated patients (Flaherty et al. 2012). In a phase 1/2 clinical trial, combining both dabrafenib and trametinib at full dose, both the rash seen with trametinib alone and the cutaneous SCC arising with dabrafenib alone were greatly reduced. The clinical activity seen with this combination was also encouraging and is currently assessed in randomized clinical trials (Weber et al. 2012).

In addition to dabrafenib which is in late-stage clinical development, other BRAF V600E-targeting small-molecule compounds, including RAF265 (ClinicalTrials.gov Identifier, NCT00304525) and LGX818 (ClinicalTrials.gov Identifier, NCT01436656), are currently evaluated in early clinical trials.

Anticipated High-Impact Results

Multiple combination therapies of BRAF inhibitors with small-molecule kinase inhibitors within the MAPK pathway (dabrafenib + trametinib) as well as other pathways such as PI3K (vemurafenib + BKM120, trametinib + GSK2126458) are in clinical trials, and first results are anticipated in a few months.

It has been shown that BRAF inhibitor treatment does not impact negatively on immune responses (Hong et al. 2012), and a combination trial of vemurafenib with ipilimumab is ongoing (ClinicalTrials.gov Identifier, NCT01400451). Due to the almost mirrorlike response profiles of both drugs, and their completely different mechanisms, the results are highly anticipated.

Adjuvant trials of vemurafenib are planned and might provide improved survival over currently available adjuvant therapies for melanoma.

CNS metastasis of melanoma is associated with the highest mortality in this disease, and currently systemic treatment options are very limited. Dabrafenib has shown promising results in a phase 1/2 trial in a small cohort of patients with brain metastasis. Therefore, a larger population was investigated in a phase 2 trial for patients with previously treated or untreated brain metastasis of melanoma. Results showing high clinical activity were presented at ASCO 2012 (Kirkwood et al. 2012).

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CKIT

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Abstract

KIT is a type III receptor tyrosine kinase encoded by a gene locus on the long arm of chromosome 4. It is closely related to Fms-like tyrosine kinase 3 (FLT3), platelet-derived growth factor receptor alpha and beta (PDGFRα, PDGFRβ), and colony-stimulating factor 1 receptor (CSF1R). Depending on its degree of glycosylation, the molecular mass of KIT is 140–160 kD. KIT is normally expressed on the surface of hematopoietic stem and progenitor cells, mast cells, melanocytes, germ cells, and interstitial cells of Cajal. There are both transmembrane and soluble forms of KIT; however, the transmembrane form is believed to be biologically active, while the role of soluble KIT is poorly understood. The ligand for KIT is stem cell factor (SCF), also known as steel factor or mast cell growth factor. Both soluble and membrane-bound forms of SCF exist, resulting from alternative splicing of exon 6 (Broudy, Blood 90:1345–1364, 1997; Heinrich

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ABL • BCR-ABL • JAK/STAT • KIT • MAP kinase • MetaGIST study • PI3-K • Platelet-derived growth factor receptor alpha (PDGFRα) • Platelet-derived growth factor receptor alpha (PDGFRα) • Stem cell factor (SCF)

Target: KIT (Formerly c-Kit)

KIT is a type III receptor tyrosine kinase encoded by a gene locus on the long arm of chromosome 4. It is closely related to Fms-like tyrosine kinase 3 (FLT3), platelet-derived growth factor receptor alpha and beta (PDGFRα, PDGFRβ), and colony-stimulating factor 1 receptor (CSF1R). Depending on its degree of glycosylation, the molecular mass of KIT is 140–160 kD. KIT is normally expressed on the surface of hematopoietic stem and progenitor cells, mast cells, melanocytes, germ cells, and interstitial cells of Cajal. There are both transmembrane and soluble forms of KIT; however, the transmembrane form is believed to be biologically active, while the role of soluble KIT is poorly understood. The ligand for KIT is stem cell factor (SCF), also known as steel factor or mast cell growth factor. Both soluble and membrane-bound forms of SCF exist, resulting from alternative splicing of exon 6 (Broudy 1997; Heinrich et al. 2002; Lennartsson and Ronnstrand 2006).

Biology of the Target

KIT is critical for hematopoiesis, the development and migration of melanocytes, the development of the gonads, gut peristalsis, and the survival and function of mast cells. In mice, KIT is the gene product of the white spotting locus (*W*), and SCF is encoded by the steel locus (*Sl*). It was observed that loss of function mutations at these two locations results in similar phenotypes – bone marrow failure/anemia, white spotting of the fur, loss of mast cells, abnormal peristalsis (decrease in the interstitial cell of Cajal), and sterility. Complete or near-complete loss of KIT expression is embryonic lethal. These observations prompted studies which suggested that SCF is the cognate ligand of KIT (Dexter and Moore 1977).

KIT becomes activated when stem cell factor (SCF) binds to the KIT extracellular domain. SCF is expressed by cells that make up the microenvironment of KIT-expressing cells including epithelial cells, endothelial cells, fibroblasts, Sertoli cells, etc. Binding of SCF leads to receptor dimerization, kinase activation, KIT autophosphorylation, and activation of downstream signaling pathways including the PI3-K, MAP kinase, and JAK/STAT pathways. Signaling via KIT promotes cell growth, survival, and proliferation. However, certain mutations in *KIT* lead to its constitutive activation in the absence of SCF. These activating mutations have been

linked to acute myeloid leukemia (AML), mast cell tumors, melanoma, seminoma, and gastrointestinal stromal tumors (GIST) (Lennartsson and Ronnstrand 2006; Lennartsson et al. 2005).

Activating KIT mutations can be located in the intracellular or extracellular domains. Extracellular mutations are typically located in exons 8 and 9. *KIT* exon 8 mutations are associated with AML, and these mutations are believed to induce hypersensitivity to SCF, rather than constitutive activation in the absence of SCF. Exon 9 mutations are found in approximately 10% of GIST patients. The activation mechanism of these mutations is being investigated and may be related to KIT dimerization or conformational changes; however, these mutations do cause constitutive activation in the absence of SCF. Intracellular mutations are most commonly associated with exons 11 and 17. Mutations in exon 11 are found in approximately 70% of GIST patients. These mutations occur in the juxtamembrane domain and prevent this autoinhibitory region from locking the kinase in “off” position in the absence of SCF. D816V, a mutation in exon 17, is associated with mast cell neoplasms, leukemia, and seminoma. This mutation is located in the activation loop and stabilizes the kinase activation loop in the active conformation, promoting spontaneous kinase activity.

Target Assessment

KIT protein expression is readily assessed in fixed tissue using immunohistochemistry (fixed tissue) or flow cytometry (blood, bone marrow) (Craig and Foon 2008; Turner and Goldsmith 2009; Miettinen and Lasota 2005; Rubin and Heinrich 2015). As noted below, measurement of KIT expression has some diagnostic utility. More importantly, detection of the presence or absence of *KIT* mutations is predictive of response of GIST, melanoma, and mast cell neoplasms to KIT kinase inhibitors.

Role of the Target in Cancer

Rank: 10

High-Level Overview

A number of KIT kinase inhibitors have been approved by the FDA. Three of these inhibitors, imatinib, sunitinib, and regorafenib, have specific FDA-approved indications for treatment of GIST (Blay 2010; Overton and Heinrich 2014). In addition, imatinib is FDA approved for treatment of adult patients with aggressive systemic mastocytosis without the D816V KIT mutation or with unknown *KIT* mutational status (Piccaluga et al. 2007). Multiple phase 2 trials have shown efficacy of imatinib and nilotinib in *KIT*-mutant melanoma, though no KIT inhibitors have been FDA approved for treatment of this disease (Hodi et al. 2013; Guo et al. 2011; Carvajal et al. 2011, 2015; Lee et al. 2015a).

Diagnostic, Prognostic, and Predictive

Over the past decade, immunohistochemistry for detection of KIT protein (CD117 antigen) has helped standardize the diagnosis of GIST (Rubin and Heinrich 2015). GIST is the most common spindle cell neoplasm (sarcoma) of the GI tract, but morphologically it can be difficult or impossible to distinguish from smooth muscle tumors, schwannomas, desmoids tumors (aggressive fibromatosis), and metastatic melanoma. Indeed, until the application of KIT immunohistochemistry to the pathologic classification of these lesions, GIST was not even recognized as a separate pathologic entity and these tumors were classified as either benign or malignant smooth muscle tumors (Turner and Goldsmith 2009; Miettinen and Lasota 2005). As noted below, the use of KIT kinase inhibitors has revolutionized the treatment of GIST – making the accurate diagnosis of GIST even more critical. KIT immunohistochemistry can also be used in the diagnosis of melanoma, AML, mast cell neoplasms, and germ cell tumors (Turner and Goldsmith 2009). In addition, KIT is a useful marker for flow cytometric identification of bone marrow blast cells and classification of cases of myelodysplastic syndrome and AML (Craig and Foon 2008).

The presence and type of *KIT* mutation found in primary GIST has been shown to have prognostic value in retrospective population studies (Andersson et al. 2006; Hou et al. 2009). Similar results have been shown in the placebo arm of a double blind, randomized study of placebo versus 1 year of adjuvant imatinib following curative intent resection of primary GIST. Notably, the presence of a *KIT* exon 11 in frame deletion mutation was associated with a much higher risk of recurrence than seen in tumors with other *KIT* genotypes (HR 3.45, $p = 0.024$ compared with wild-type tumors) (Turner and Goldsmith 2009; Miettinen and Lasota 2005; Corless et al. 2010). *KIT* mutation status also influences the effectiveness of adjuvant imatinib given after curative intent surgery, with patients whose GIST harbor *KIT* exon 11 mutations deriving the greatest benefit from 3 years of adjuvant imatinib compared with 1 year of adjuvant imatinib (Joensuu et al. 2016). *KIT* mutation data is now being incorporated into risk stratification algorithms to help predict the risk of recurrence after surgery and to help guide decision making concerning the use of adjuvant imatinib in GIST (Joensuu et al. 2015).

Translational studies utilizing tumor samples from large clinical studies have identified tumor genotype as a strong predictor of clinical benefit for patients with metastatic GIST treated with imatinib. Specifically, patients with *KIT* exon 11-mutant GIST (approximately 70% of GIST) have the highest rates of objective response, progression-free survival (PFS), and overall survival (OS) compared with patients whose tumors had no kinase mutations (wild-type GIST, approximately 10–15% of GIST) or GIST with somatic *KIT* exon 9 mutations ($\sim$10% of patients). In this SWOG/NCIC study, PFS was 24.7 months for *KIT* exon 11-mutant tumors versus 16.7 months for wild-type GIST and 12.8 months for patient with *KIT* exon 9-mutant tumors. In terms of the effect on overall survival, *KIT* exon 11-mutant GIST patients had a median OS of 60 months versus 38.4 months for wild-type GIST patients and 49 months for *KIT* exon 9-mutant GIST patients (Heinrich et al. 2003, 2008a; Debiec-Rychter et al. 2004, 2006).

The effect of tumor genotype and imatinib dose on clinical outcomes was also analyzed in the MetaGIST study (400 versus 800 mg dosing for metastatic GIST). Within patients with *KIT* exon 9-mutant GIST, PFS was significantly longer for patients treated with the high-dose arm ($P = 0.017$). For patients whose tumor had genotypes other than *KIT* exon 9 mutation, no difference in clinical outcomes was observed between treatment arms. In terms of OS, there was a trend toward a survival advantage for patients with *KIT* exon 9-mutant GIST treated with high-dose therapy ($p = 0.15$) (2010). Tumor genotyping is recommended in oncology professional guidelines to help optimize care of patients with newly diagnosed metastatic GIST (von Mehren et al. 2012, 2014). In addition, *KIT* mutation status has recently been incorporated into a prognostic nomogram for patient with metastatic GIST treated with first-line imatinib (Lee et al. 2015b).

Besides its impact on response to imatinib, *KIT* mutation status also influences clinical outcome in patients treated with sunitinib as second-line therapy for metastatic GIST. In contrast to the experience with first-line imatinib, patients with *KIT* exon 9-mutant or wild-type GIST are predicted to have better outcomes with sunitinib treatment compared with patients with *KIT* exon 11-mutant GIST (Heinrich et al. 2008b; Reichardt et al. 2016).

Therapeutics

To date, all of the FDA-approved anti-KIT therapeutics are small molecule tyrosine kinase inhibitors. Currently, there are numerous agents with KIT inhibitory activity that are FDA approved for treatment of one or more human malignancies, including imatinib, sunitinib, regorafenib, nilotinib, dasatinib, sorafenib, pazopanib, and ponatinib. However, only imatinib (GIST, mastocytosis), sunitinib (GIST), and regorafenib (GIST) are FDA approved for treatment of *KIT*-mutant disease.

Preclinical Summary

A large body of evidence has established KIT as therapeutic target, and subsequent research has studied the efficacy of various tyrosine kinase inhibitors in blocking its activity in vivo and in vitro. Research in 2000 by Ma et al. showed efficacy of a small group of indolinones against KIT (Ma et al. 2000). They also reinforced a direct link between KIT function and mast cell survival. In the same year, Heinrich et al. and Buchdunger et al. investigated the use of imatinib (formerly STI-571) as a KIT inhibitor. Imatinib was found to selectively inhibit KIT tyrosine kinase activity as well as inhibit the activation of downstream effector proteins. They also found that imatinib was more potent against certain activating KIT mutations than against WT KIT and concluded that the clinical profile of imatinib should be expanded to include KIT (in addition to its known targets: ABL, BCR-ABL, and PDGFRA and PDGFRB) (Buchdunger et al. 2000; Heinrich et al. 2000).

In this same time frame, Tuveson et al. developed a GIST tumor cell line – GIST882 – harboring an activating mutation in the KIT tyrosine kinase I domain. Incubation of this cell line with imatinib led to decreased proliferation and increased apoptosis supporting a role of KIT in GIST pathology and the therapeutic potential for imatinib in GIST patients (Tuveson et al. 2001). Later, in 2003, Abrams et al. evaluated the activity of sunitinib (formerly SU11248) against KIT in a small cell lung cancer model (Abrams et al. 2003). Treatment with sunitinib inhibited KIT tyrosine phosphorylation and cellular proliferation. The results of this study suggested a clinical potential for sunitinib in the treatment of tumors with activating KIT mutations.

KIT mutations are found in the vast majority of human mast cell neoplasms. In particular, the KIT D816V mutation is found in >90% of cases. Pre-clinical studies of mastocystosis cell lines and/or patient samples have shown that KIT inhibition by kinase inhibitors reduces proliferation and induces apoptosis of cells. Unfortunately, the D816V mutation is resistant to most of the available kinase inhibitors. However, these studies do indicate that KIT is a compelling target in mastocytosis and have spurred efforts to develop inhibitors with activity against the D816V mutation (Gotlib et al. 2005; Schittenhelm et al. 2006; Shah et al. 2006).

More recently, *KIT* mutations have been found in a subset of human melanoma. In particular, these mutations are more common in acral or mucosal melanomas. In vitro studies of *KIT*-mutant melanoma cell lines have demonstrated that KIT inhibitors can exert an anti-proliferative and pro-apoptotic effect on these cells (Beadling et al. 2008; Jiang et al. 2008).

All of these studies and many more have established KIT as a therapeutic target in cancers driven by the hyperactivation of KIT and demonstrated the efficacy of specific tyrosine kinase inhibitors in controlling cell growth resulting from KIT hyperactivity.

Clinical Summary

Prior to 2000, there was no active medical treatment for metastatic GIST (DeMatteo et al. 2002). However, the introduction of small molecule tyrosine kinase inhibitors (TKIs) has revolutionized the treatment of GIST. Currently, there are three FDA-approved treatments for advanced GIST: imatinib for front-line treatment, sunitinib for second-line treatment, and regorafenib for third-line treatment. A number of other TKIs have been tested in phase 2 studies for treatment of GIST in the fourth-line or later clinical setting. Overall, the use of KIT inhibitors has changed the prognosis for patients with metastatic GIST, with median survival increasing from an estimated 1–1.5 years to the current 6–8 years (Barrios et al. 2015). Notably, resistance to KIT inhibitors in *KIT*-mutant GIST is typically associated with the development of secondary *KIT* mutations that confer drug resistance (Heinrich et al. 2006, 2008b; Corless et al. 2011; Gramza et al. 2009). Developing new inhibitors to prevent or overcome secondary mutations is a major focus of ongoing GIST research (Blay 2010).

In addition to GIST, some therapeutic progress has been made in treating mast cell neoplasms with KIT kinase inhibitors. Currently, available KIT kinase inhibitors have reduced potency against the KIT D816V mutation associated with mast cell neoplasms (Verstovsek et al. 2008; Vega-Ruiz et al. 2009). However, it is anticipated that development of novel KIT inhibitors that are active against the D816V mutation will be clinically effective for treating mast cell neoplasms. Currently, imatinib is FDA approved for treatment of aggressive mastocytosis lacking the D816V mutation or with an unknown *KIT* genotype.

Clinical studies using KIT inhibitors to treat unselected cases of malignant melanoma have been disappointing (Wyman et al. 2006). However, KIT kinase inhibitors have shown strong activity against *KIT*-mutant melanoma (Hodi et al. 2008). Three phase 2 trials for imatinib and two for nilotinib have shown promising responses in *KIT*-mutant melanoma, specifically in mucosal, acral, and chronically sun-damaged subtypes (Hodi et al. 2013; Guo et al. 2011; Carvajal et al. 2011, 2015; Lee et al. 2015a). The disease control rate for patients with *KIT* mutations treated with imatinib was 77% (including partial response of 54%). To date, none of the tested KIT inhibitors has been approved by any national health authority agency for treatment of *KIT*-mutant melanoma.

Anticipated High-Impact Results

- Final reports from ongoing phase 2 and phase 3 studies of imatinib, sunitinib, or nilotinib for treatment of *KIT*-mutant melanoma
- Final analysis of the impact of *KIT* mutations on the clinical efficacy of adjuvant imatinib after resection of primary GIST
- Planned phase 1–2 studies of mechanistically novel KIT inhibitors with activity against D816V and other mutations that are resistant to current inhibitors

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DNA Repair, Overview

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is a lethal disease with a five-year survival rate of approximately 5%. Any hope for long-term survival hinges on tumor resectability; however, less than 15% of PDA cases are operable. One of the differentiating factors of PDA is its ability to form micrometastases early in tumor growth, preventing surgery from being wholly effective in treating the cancer (Kelly KJ, Wong J, Gladdy R, Moore-Dalal K, Woo Y, Gonen M, et al., Ann Surg Oncol 16(12):3333–9, 2009). Moreover, there have been few advances in the treatment of metastatic disease in recent years. Newer cytotoxic treatments such as gemcitabine/nab-paclitaxel and FOLFIRINOX have made only marginal improvements in the quality of life and overall survival. Because of the overall poor prognosis and paucity of treatment options, there is a strong need for the development of new therapeutic targets and treatment modalities. Multiple recent studies have demonstrated the dysregulation of DNA repair mechanisms in PDA cells (Helleday T, Mol Oncol 5(4):387–93, 2011). Herein, we use PDA as a model

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tumor system in which to provide a thorough review of DNA repair mechanisms involved in cancer and how they may be targeted to generate novel therapeutics and potential cancer treatments (Table 1). We will review the exciting notion that although DNA repair defects may set the stage for tumorigenesis (prognostic marker), it also may be an Achilles heel of cancer cells that can be targeted (predictive marker). Specifically, targeting the DNA repair pathway in combination with traditional DNA damaging chemotherapeutics may lead to synergy and hopefully to better patient outcomes.

Keywords
DNA repair defects and cancer • DNA-protein kinase catalytic subunits • Double-strand break damage • *ELAVL1* • Fanconi anemia • Homologous repair • Human antigen R • Immunohistochemistry • Ku proteins • miRNAs • Non-homologous end joining • Nucleotide excision repair • Poly (ADP-ribose) polymerase (PARP) inhibition • Synthetic lethality

Introduction to the Target (the DNA Repair Pathways)

The ability of normal cells to repair DNA damage is of utmost importance. On a daily basis, cells are bombarded by both intrinsic and extrinsic stimuli, such as radiation or chemical exposure, which lead to DNA damage as well as replication infidelity. Upon recognition of DNA damage, cells orchestrate a coordinated response leading to cell cycle arrest and subsequent activation of DNA damage repair machinery. This sequence of events protects normal cells from irreparable damage and cell death as well as from transforming into cancerous cells (Alberts 2002). One particular type of DNA damage is the double-strand break (DSBs); this type of damage is particularly destructive as it can cause DNA defects such as deletions, translocations, improper fusions, and cell death if not properly repaired (Aplan 2006). To prevent these deleterious results, cells use a variety of pathways to repair damage related to DSBs including base excision repair, nucleotide excision repair, and double-stranded break repair (DSBR). Each of these repair systems can be compromised in cells, leading to DNA damage accumulation and potentially heritable disease and cancer (Hoeijmakers 2001). By dissecting the pathways involved and identifying key players in this process, we may be able to develop novel treatments in patients predisposed to DNA repair defects, either by heritable transmission or by *de novo* mutation.

In normal cells, DSBs are prevented by a coordinated set of DNA damage repair proteins. Initially smaller lesions such as single-strand nicks may be formed. It is the goal of this machinery to repair these smaller DNA damage events, so that more deleterious DSBs can be avoided (Kelly et al. 2009). If, however, these systems allow DSBs to occur, the cell has two main pathways to repair the defect: homologous recombination (HR) and nonhomologous end joining (NHEJ). These are complementary pathways utilizing different machinery to accomplish the same

goal, namely, restoration of DNA integrity. These two pathways will be dissected in depth in the following sections.

Biology of the Target (Including Preclinical and Clinical Utility)

Homologous repair. When DSBs occur in normal cells, HR systems are vital in maintaining the integrity of DNA. When a cell recognizes DSBs, the 5′ ends near the break undergo resection in which nucleases excise the overhangs in the break; after resection is completed, the 3′ overhang invades the corresponding unbroken chromosome to rebuild using the matching sequence. At this stage, the DSBs can be repaired by two separate pathways: the double-strand break repair (DSBR) pathway or the synthesis-dependent strand annealing (SDSA) pathway (Mimitou and Symington 2009). The DSBR pathway is common in DNA repair in normal cell function, and the SDSA pathway is common during cell replication such as meiosis and mitosis. As this review is concerned with impairments in normal cellular function leading to transformative potential, the DSBR pathway is more relevant in terms of tumorigenesis (Helleday et al. 2007; McMahill et al. 2007).

There are multiple dysregulations of the HR pathway which lead to a multiplicity of diseases including tumor formation and premature aging. For example, mutations in the helicase RecQ, important in the HR pathway, can lead to conditions like Bloom’s syndrome, Werner’s syndrome, or Rothmund-Thomson Syndrome (Ellis et al. 1995); it is important to mention that these syndromes are associated with conditions like adult progeria or sun sensitivity in addition to increasing cancer risk (Epstein et al. 1966; Wang et al. 2001; German 1997) (see Table 1).

The most well-studied examples of HR pathway dysfunction come from the *BRCA1* and *BRCA2* genes (Powell and Kachnic 2003). Loss of the BRCA1 or BRCA2 protein function, typically by point mutation, and an accompanying event, such as loss of heterozygosity, cause a loss of the HR pathway as a means to DNA repair in the affected cell. It is believed that this loss of HR function allows mutations to accumulate in affected cells. Thus, these patients are at increased risk of many tumor types including, breast, ovarian, prostate, melanoma, and PDA. Understanding of the pathway led researchers to believe that tailored therapy, in the form of Poly (ADP-ribose) polymerase (PARP) inhibition, could generate tumor specific toxicity in BRCA mutant patients. These inhibitors work through a process known as *synthetic lethality*. PARP proteins function by repairing single-strand “nicks” in DNA. Inhibition of this pathway leads to the accumulation of DSBs which are normally repaired by BRCA proteins (Kelly et al. 2009). Patients with inherited BRCA mutations generally do so in a heterozygous manner and ultimately do not manifest with tumor formation until there is a loss of heterozygosity (i.e., loss or mutation of the other allele). In this scenario, normal cells harboring a single copy of BRCA proteins are fully capable of repairing the subsequent DSB; however, the affected tumor cells without a functional copy of BRCA proteins accumulate DSBs ultimately leading to their demise (Helleday 2011). Therefore, the concept of

Table 1 Description of the relationship between DNA repair-related gene defects and cancer

Disease	Associated gene	Symptoms	High cancer risks
Mutations in homologous repair			
Bloom's syndrome	BLM (DNA helicase protein) (Ellis et al. 1995)	Genomic instability, immunodefiency, hypogonadism (German 1993; Sanz and German 1993)	High risk for cancer in general, no specific type (Sanz and German 1993; German 1993)
Werner's syndrome	WRN (DNA helicase protein) (Sugimoto et al. 2004)	Growth retardation, premature aging, heart disease, sun sensitivity (Hasty et al. 2003; Navarro et al. 2006)	Skin cancers (particularly malignant melanomas), soft-tissue sarcomas, thyroid and liver cancer, and non-Hodgkins lymphomas (Sugimoto et al. 2004; Monnat 2010)
Rothmund-Thomson syndrome	ReqQ4 (DNA helicase protein) (Larizza et al. 2006)	Sun sensitivity, cataracts, growth defects in rapidly dividing cell lines (hair and gastrointestinal systems) (Wang et al. 2001)	Osteosarcomas (Wang et al. 2001)
BRCA 1 mutation	BRCA1 (tumor suppressor protein) (Duncan et al. 1998)		Breast cancer, ovarian cancer, fallopian tube cancer, and prostate cancer (Friedenson 2007)
BRCA 2 mutation	BRCA2 (tumor suppressor protein) (Duncan et al. 1998)		Breast cancer, ovarian cancer, fallopian tube cancer, prostate cancer, pancreatic cancer, and skin cancer (particularly malignant melanomas) (Friedenson 2007; Liede et al. 2004)
Ataxia-telangiectasia	Ataxia-telangiectasia Mutated gene (serine/threonine protein kinase) (Savitsky et al. 1995)	Causes immunodeficiency and can cause difficulty in movement and coordination (Crawford et al. 2000; Cabana et al. 1998; Nowak-Wegrzyn et al. 2004)	Leukemia, lymphoma, and breast cancer (Reiman et al. 2011)
Nijmegen breakage syndrome	NBS1 (repairs double-strand DNA breaks) (Iijima et al. 2004)	Microcephaly, stunted growth, distinct facial features, immunodeficiency, and sensitivity to radiation (Dutrannoy et al. 2010)	Lymphoid malignancies (Dutrannoy et al. 2010)

(*continued*)

Table 1 (continued)

Disease	Associated gene	Symptoms	High cancer risks
MRE11A	MRE11A (repairs DSBs in microhomology-mediated end joining) (Petrini et al. 1995)	Overexpressed in cancer cells and mutations in this pathway allow other mutations to easily develop (Yuan et al. 2012; Sharma et al. 2015)	
Fanconi anemia	FANCA, FANCB, FANCC, FANCDl, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN (DNA cross-link repair) (Kutler and Auerbach 2004)	Short stature, pale complexion, frequent infections caused by weakened immune system, bone marrow failure (Sirak et al. 2015)	Acute myeloid leukemia, liver cancers, and other solid tumors (Spinella et al. 2015)
Lynch syndrome	hMSH2, hMSH6, hMLH1, hPMS2 (tumor suppressor genes) (Fishel et al. 1994; Papadopoulos et al. 1994; Miyaki et al. 1997; Nicolaides et al. 1994)		Colorectal, endometrial, ovarian, pancreatic, stomach, upper urinary tract, small intestine, hepatobiliary tract, brain, and skin cancers (Lynch and Smyrk 1996)
Mutations in nucleotide excision repair			
Xeroderma pigmentosum	XPA, XPB, XPC, XPD, XPE, XPF, XPG, XPV (proteins essential NER pathway) (van Steeg and Kraemer 1999)	Sun sensitivity, stunted mental growth, progeria-like symptoms, microcephaly (Patton and Valdez 1991)	Skin and CNS cancer (Patton and Valdez 1991)
MUTYH	MUTYH (DNA glycosylase that acts under oxidative damage) (Tenesa et al. 2006)		Colon cancer (Tenesa et al. 2006)
Mutations in nonhomologous end joining			
LIG4 syndrome	Lig4 (DNA ligase) (Ben-Omran et al. 2005)	Severely compromised immune system, microcephaly, and cellular radiosensitivity (Ben-Omran et al. 2005)	Multiple myeloma (Ben-Omran et al. 2005)
XLF-SCID	XLF (nonhomologous end joining factor) (Li et al. 2007)	Premature aging of hematopoietic stem cells, severely compromised immune system, microcephaly, and cellular radiosensitivity (Li et al. 2007)	

synthetic lethality in this case could be a very powerful approach to define a therapeutic window in these BRCA-mutated patients (Lord and Ashworth 2008). For example, the drug olaparib was recently approved by the FDA for the treatment of ovarian cancer and multiple PARP inhibitors are currently in clinical trials for PDA.

Other targeted therapies have been developed for those with HR deficiencies. Specifically, patients with Fanconi anemia (FA), a deficiency in one of several proteins in the HR machinery (Tercyak et al. 2001), are at a higher risk for developing acute myelogenous leukemia, as well as other cancers (Carrasco et al. 1998). However, the loss of specific DNA repair mechanisms makes them vulnerable to DNA damaging agents such as DNA cross-linking agents like mitomycin C or a platinum-based chemotherapeutic agent (Sasaki and Tonomura 1973). Pancreatic cancer patients with defects in the HR pathway (e.g., BRCA2 and FA pathways) are currently being evaluated in clinical trials for PARP inhibitor-based therapies (Pishvaian et al. 2012).

Nonhomologous End Joining. As there are numerous causes for DSBs, cells have adapted to have multiple repair mechanisms. Another such pathway is the nonhomologous end joining. This pathway is spearheaded by the DNA-protein kinase catalytic subunits (DNA-PKcs) which are critical to the overall function of DNA repair. This group of proteins functions by opening up the broken ends of the DSBs and act as a scaffold for the attachment of other repair factors such as XRCC4, DNA ligase IV, Artemis, XLF, and aprataxin (Lieber 2010). In a DNA-PKcs knockout mouse model, mice demonstrated chromosomal instability and premature aging, indicating that normal function of the NHEJ pathway is necessary for regular maintenance of DNA (Difilippantonio et al. 2000). One major regulator of DNA-PKcs is PIM kinases. Upon recognition of DNA damage, PIM Kinases are upregulated. This ultimately leads to activation of DNA-PKcs and repair of DSBs (Hsu et al. 2012). Our lab has recently demonstrated a role for the RNA-binding protein HuR. In response to cellular insults, HuR translocates from the nucleus where it regulates a variety of RNAs into the cytoplasm, suggesting that HuR is an indirect regulator of DNA-PKcs efficiency (Lal et al. 2014; Blanco et al. in press, Oncogene, see "Posttranscriptional Regulation of the Targets" section below).

A second major component of the NHEJ pathway are Ku proteins. These proteins bind to DNA-PKcs and recruit nucleases, polymerases, and ligases to the site of DNA damage (Lieber 2008). Ku protein knockout mice are shown to be smaller than normal and appear to undergo premature aging, a sign of genomic damage accumulation (Featherstone and Jackson 1999).

While the inhibition of both the Ku and DNA-PKcs pathways offer therapeutic targets, DNA-PKcs inhibition is currently the only NHEJ component to be targeted in clinical trials (clinical trials: NCT02516813 and NCT02316197). There are multiple mechanisms with which to target DNA-PKcs. These include interfering with PI3K and mTOR pathways, inhibition of Pim1 or HuR (see below section) (Toulany et al. 2008; Lal et al. 2014; Mukherjee et al. 2012), and through the interference of the ATM pathway (Chen et al. 2007). All of these pathways have traits associated with tumor formation including cell survival, growth, proliferation,

cell migration, DNA repair, and angiogenesis (Manning and Cantley 2007; Lee and Paull 2007). The inhibition of DNA-PKcs causes a dramatic decrease in the efficiency of NHEJ, leading to fractionation of chromosomes and cell death (Davidson et al. 2013).

Posttranscriptional Regulation of the Targets

As DNA damage and repair are both common in cellular life, it follows that the pathways of DNA repair are tightly regulated. In particular, the posttranscriptional regulation of DNA repair machinery significantly impacts the expression of the DNA repair pathway. Because DNA damage also affects the stability of mRNA, systems must be in place so that in the event of DNA damage, cells are able to mount a systematic response and synthesize proteins to repair the damage (McKay 2014). Since the process of preserving the integrity of protein function in cells is vital, there are multiple pathways in place to regulate the integrity of the mRNA cargo, with micro-RNAs (miRNAs) and RNA binding proteins (RBPs) being particularly important (Friedman et al. 2009; Stefl et al. 2005).

miRNAs are a class of RNAs that are usually small, around 22 nucleotides, (Lund and Dahlberg 2006) noncoding segments of the genome, which are highly conserved throughout the evolutionary process (Peterson et al. 2009; Nozawa et al. 2010). miRNAs normally function as a way to downregulate the expression of their mRNA targets by binding to the 3′ UTR, promoting cleavage of the mRNA (Wang et al. 2004; Kawasaki and Taira 2004), but miRNAs can also function as a way of upregulating expression of their target genes, through both direct and indirect pathways (Vasudevan 2012). In the tumorigenic process, miRNAs tend to downregulate the proteins involved in DNA repair systems and cell cycle checkpoints, allowing cells to accumulate DNA damage. In particular, hypoxia, seen in almost all solid tumors, causes an upregulation of miR-210 and miR-373 through the upregulation of the hypoxia inducible factor-1α (HIF-1α); these miRNAs decrease expression of proteins important in HR and nucleotide excision repair processes (Crosby et al. 2009). miRNAs are a useful, fast-acting system for cells being able to manipulate proteins as they are able to affect protein synthesis both directly and indirectly in response to both intra and extracellular stimuli. As we expand upon our knowledge of multiple miRNAs and their targets (Londin et al. 2015), undoubtedly we better understand how this molecular mechanism regulates DNA repair pathways.

RBPs are proteins that play a vital role in the process of posttranscriptional gene regulation. These proteins serve many roles throughout the processing of fully functional mRNA transcripts. In the context of DNA repair, the most important function of RBPs is mRNA stabilization (Hogan et al. 2008; Glisovic et al. 2008). One protein of particular importance in posttranscriptional mRNA activity is the protein human antigen R (HuR), also known as *ELAVL1*. This ubiquitously expressed protein shows particular importance in tumor biology (Wang et al. 2013), where HuR stabilizes mRNA cargo when stress (hypoxia, glucose

deprivation, chemotherapeutic treatment) causes the translocation of HuR from the nucleus to the cytoplasm (Lal et al. 2014; Burkhart et al. 2013; Costantino et al. 2009; Hostetter et al. 2008). Indeed, HuR is activated and undergoes translocation from the nucleus to the cytoplasm where it is able to affect mRNA stability and subsequent protein expression. Immunohistochemistry (IHC) experiments have demonstrated the importance of HuR translocation in cancer cells. HuR resides in the nucleus under normal cellular conditions. Upon tumorigenic conversion or stressful environments such as the tumor microenvironment, IHC demonstrates HuR is translocated and more abundant to the cytoplasm as compared to normal tissue controls (Lal et al. 2014; Abdelmohsen and Gorospe 2010). While HuR is typically associated with the stabilization of genes concerning cell survival and treatment resistance (Hostetter et al. 2008; Costantino et al. 2009), it also plays an important role regulating mRNAs in DNA repair pathways through direct as well as indirect mechanisms. Related to this review, interesting target mRNAs include HIF-1α (Sakuma et al. 2008), PIM-1 (Al-Ahmadi et al. 2013), PARG (Chand et al. data unpublished), WEE1 (Lal et al. 2014), and BRCA genes (Heinonen et al. 2007).

miRNAs and RBPs have been shown to be involved in normal cell biology as well as the tumorigenic process. By understanding these molecules' roles in cancer biology, there are significant amounts of knowledge and potential targets that could lead to better therapeutics and biomarkers.

Target Assessment and the Role of This Target in Cancer

Among different ethnic groups, DNA repair pathway defects are more common, as certain mutations have become a larger percent of the gene pool of particular groups caused by the founder effect. These mutations have been characterized in terms of name, gene, symptoms, and associated cancer risk in Table 1. This demonstrates how targetable systems exist and validates the notion that defects in these pathways most likely set the stage for tumorigenesis. Note: Perhaps a future review will be able to confidently add a column to this table that reads, "Successful Targeted Therapies," based on prospective clinical trials.

Role of the target in cancer. In relation to one of the most difficult cancers to treat, pancreatic cancer, the discovery of DNA repair pathways being mutated and disrupted is very exciting. First, within the familial pancreatic cancer setting (i.e., a patient who has at least two first degree relatives with pancreatic cancer) may have an inherited link or marker for likelihood of presenting with this disease. In this instance, these mutations (such as a germline *BRCA2* mutation) may be detected by a simple blood test (sequencing) and this target/marker can be used as a **prognostic marker**. Second, these mutations (identified currently by next generation sequencing, NGS) can provide a **predictive marker** for DNA damaging agents and PARP inhibitor-based trials. As far as targeted therapy is concerned these list of mutations (Table 1) would be **ranked as a 10 out of 10**, until we develop better targeted therapeutics. Similarly, since we have no validated early detection markers to date,

these mutations in high-risk individuals (with a family history) are considered a **top ranked marker** (**#10**).

High Level Overview (Clinical Utility and Future Use)

While advances have been made in the area of targeted therapies in many tumor types, this is not the case in PDA. In terms of current chemotherapeutic options, the addition of DNA damage repair inhibitors could, and has in the case of PARP inhibitors, add synergistic gains to current treatments. Alternately, the development of inhibitors that target key regulatory pathways, like HuR, PIM, PI3Kinase, or mTOR, could provide a mechanism to knockout several pathways at once leading to a potent abrogation of DNA damage machinery (a term we coined, global synthetic lethality) (Jimbo et al., 2015, Oncotarget).

Novel approaches to treating patients in a personalized manner use genetic and proteomic data from primary tumors or metastatic lesions to tailor therapies specifically targeted towards pathways in the patients tumor (e.g., a DNA repair pathway). This hypothesis is currently being tested in the MATCH clinical trial being run by the NCI and more specifically in a RAN grant (Pancreatic Cancer Action Network, Thomas Jefferson University and Georgetown University). In these trials, patients' tumors undergo molecular profiling and are matched with approved and investigational therapeutics targeted against the genetic mutations present (ClinicalTrial.gov identifier: NCT02465060). Specific to the pancreas, the PanCAN grant will use NGS as well as phosphoproteomics, and ex-vivo modeling to determine the best, personalized second-line therapy for PDA patients.

The clinical applications of DNA damage and its cellular response provide a new avenue to generate novel anticancer compounds and therapeutic strategies (e.g., synthetic lethality). By understanding the underlying pathways, our hope is that we will have both preventative measures and targeted, effective curative treatments for those with mutations in pathways that repair DNA damage.

Anticipated High-Impact Results

- DNA repair proteins are dysregulated in cancer (e.g., pancreatic cancer).
- When disrupted these proteins can shut down active DNA repair pathways which can: (1) set the stage for tumorigenesis and (2) be used to target an Achilles heel of a developed cancer.
- In both aforementioned instances, a DNA repair gene mutation can represent a powerful prognostic and predictive marker for cancer patients.

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EGFR, Growth Factors

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Manisha Bhutani and Helen Gharwan

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Abstract

Epidermal growth factor receptor (EGFR) (also known as HER1 or ErbB1) is a 170-kd transmembrane glycoprotein in the ErbB family of receptor tyrosine kinases (TKs) that also includes HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Stanley Cohen was awarded the Nobel Prize in 1986 for the discovery of EGFR and its ligand EGF. EGFR is encoded by the proto-oncogene *c-erbB-1 or EGFR*, located on the short arm of chromosome 7 (7p11.2). Structurally, EGFR contains (i) a cysteine-rich, extracellular N-terminal ligand binding

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domain and a dimerization arm, (ii) a hydrophobic transmembrane domain, and (iii) an intracellular cytoplasmic C-terminal TK domain with several phosphorylation sites. While intracellular TK domain is highly conserved, the variable extracellular ligand binding domain enables binding to different ligands.

Keywords
Cetuximab • Epidermal growth factor receptor (EGFR) • Antagonists • Biomarkers • Cetuximab and panitumumab • Clinical trials • Ectodomain mutations • Gefitinib and erlotinib • Immunostaining • Kinase domain mutations • Pharmacologic and genetic inhibitors • Vaccination approaches • ErbB1 • Panitumumab

Target: Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor (EGFR) (also known as HER1 or ErbB1) is a 170-kd transmembrane glycoprotein in the ErbB family of receptor tyrosine kinases (TKs) that also includes HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Stanley Cohen was awarded the Nobel Prize in 1986 for the discovery of EGFR and its ligand EGF. EGFR is encoded by the proto-oncogene *c-erbB-1 or EGFR*, located on the short arm of chromosome 7 (7p11.2). Structurally, EGFR contains (i) a cysteine-rich, extracellular N-terminal ligand binding domain and a dimerization arm, (ii) a hydrophobic transmembrane domain, and (iii) an intracellular cytoplasmic C-terminal TK domain with several phosphorylation sites. While intracellular TK domain is highly conserved, the variable extracellular ligand binding domain enables binding to different ligands. At least 13 different ligands have been identified that can bind to EGFR; EGF, transforming growth factor alpha, amphiregulin, and epiregulin are receptor specific (Cantley et al. 2011). Cognate ligand binding induces a structural change that favors dimerization with a same (homodimer) or a different (heterodimer) member of the family. When dimerized, the TK domains are activated and phosphorylate key tyrosine residues, which lock the kinase into a high-activity conformation, stimulating phosphorylation of other sites on the receptor, as well as recruitment of scaffolding and signaling proteins (Stern 2008). This then triggers a cascade of pathways that are important for tumor growth, proliferation, survival, angiogenesis, metastasis, and a decrease in apoptosis. The main pathways downstream of EGFR activation include those mediated by Ras-Raf-MEK, Pi3K-AKT-mTOR, and JAK2-STAT3 (Yarden and Sliwkowski 2001).

Biology of the Target

EGFR plays a crucial role in maturation and development of various tissues of epithelial, mesenchymal, and neural origin. Deregulation of EGFR signaling is implicated in carcinogenesis, maintenance of a malignant phenotype, and cancer

progression. EGFR is frequently overexpressed in lung cancers and colorectal cancers. In addition, EGFR is expressed in a variety of other tumors, including gliomas and carcinomas of the head and neck, pancreas, breast, ovary, bladder, and kidney. EGFR is predominantly present on the cell surface. On ligand binding, it is internalized into the endosome. Besides the conventional role of EGFR as a membrane-associated receptor TK, recent reports have shown a nuclear localization of EGFR, wherein it acts as a transcription factor (Lin et al. 2001), which suggests the possibility of a plethora of genes being directly transactivated by EGFR, rather than just through a signal transduction cascade. EGFR, besides interacting with other ErbBs, engages in cross talk with other receptors, for example, G protein-coupled receptors, src-mediated activation of matrix metalloproteinases, c-Met, and IGF1 receptor (Hynes and Lane 2005). EGFR plays an important role not only in tumor cells but also in the tumor microenvironment, for example, it is expressed in endothelial cells and is implicated in tumor angiogenesis. Several possible mechanisms account for aberrant expression and functioning of EGFR in cancers, one of which is increased gene copy number, as seen in a proportion of gliomas, lung cancers, and breast cancers. Alternatively autocrine stimulation, by which tumor produces its own ligand, can lead to EGFR expression (Cantley et al. 2011), as occurs frequently in cancers of lung, head and neck, and gastrointestinal tract. However, the most important mechanism underlying EGFR dysregulation is mutations causing intrinsic alterations of the receptor.

Two important types of *EGFR* mutations are described: (i) kinase domain mutations as seen in non-small cell lung cancer (NSCLC) and (ii) ligand binding domain mutations, best characterized in glioblastomas. Notably, activating EGFR mutations in kinase domain are observed in approximately 10% of North American and European populations and 30–50% of Asian populations, with higher *EGFR* mutation frequency in never smokers, women, and non-mucinous cancers (Kaufman et al. 2011). In general, kinase domain mutations have been classified into three types (Shigematsu et al. 2005). Class I mutations include short in-frame deletions that result in the loss of four to six amino acids (E746 to S752) encoded by exon 19. Class II mutations are single-nucleotide substitutions that may occur throughout exons 18–21. Class III mutations are in-frame duplications and/or insertions that occur mostly in exon 20. The most common activating *EGFR* mutations result in an arginine for leucine substitution at amino acid 858 (L858R) in exon 21 and in-frame deletions around the conserved LREA motif of exon 19 (residues 747–750) (Lynch et al. 2004; Paez et al. 2004). Together, these two classes of mutations account for approximately 85% of *EGFR* mutations in lung cancer. These mutations are oncogenic and result in prolonged activation of the receptor and downstream signaling through phosphorylated AKT, in the absence of ligand stimulation of the extracellular domain. Biochemical studies indicate that these mutants preferentially bind to drugs like gefitinib and erlotinib over ATP. Kinase domain *EGFR* mutations can also be found in patients with other cancer types, albeit at a lower frequency, and unlike NSCLC do not define a subset of patients that can be treated more effectively by specific targeted therapy. In glioblastoma, the most commonly expressed mutant

form is Δ*EGFR* (also named EGFRvIII), which is generated by an in-frame genomic deletion of 801 bp from exons 2–7 of the coding region of *EGFR* (Ekstrand et al. 1992). This produces a truncated receptor lacking a portion of the extracellular ligand binding domain causing the receptor to be constitutively active in the absence of ligand.

Target Assessment

EGFR-activating mutations represent a critical determinant for proper therapy selection in lung cancer. The American Society of Clinical Oncology (ASCO) recommends that patients with advanced NSCLC who are being considered for first-line therapy with an EGFR tyrosine kinase inhibitor (TKI) should have their tumors tested for *EGFR* mutations (Keedy et al. 2011). The National Comprehensive Cancer Network (NCCN) recommends testing *EGFR* mutation status for select lung cancer patients with adenocarcinoma or adenocarcinoma component, large cell, and NSCLC not otherwise specified. *EGFR* mutation testing is not generally recommended in patients with squamous cell lung cancers unless they are never smokers or a small nonrepresentative biopsy specimen (not surgical resection) was used to assess histology. A variety of molecular testing methods have been developed for assessing *EGFR* mutation status, which include immunostaining of the EGFR protein, copy number changes, and DNA mutation analysis. Neither EGFR protein expression by immunostaining nor gene copy number by fluorescence in situ hybridization (FISH) is a reliable biomarker for the presence of *EGFR* mutation. DNA mutational analysis is the preferred method. Direct sequencing of polymerase chain reaction (PCR)-amplified genomic DNA corresponding to exons 18–21 or just testing the exons 19 and 21 is a reasonable approach (Shaw et al. 2011). Testing should be performed only by laboratories with demonstrated proficiency. Analysis is typically done on a tumor tissue, obtained by biopsy or by surgical resection, that has been formalin fixed, paraffin embedded, or snap frozen. A number of alternative methods to detect somatic *EGFR* mutation have been used, many with improved sensitivity and turnaround times, such as denaturing high-performance liquid chromatography (dHPLC) and high-resolution melting analysis (HRMA) and massively parallel sequencing. There is still no standardized test that has been approved by the US Food and Drug Administration for *EGFR* mutation analysis.

Role of the Target in Cancer

Rank: “unknown” to 10 unknown to-1-2-3-4-5-6-7-8-9-10: 10.

High-Level Overview

Diagnostic, Prognostic, Predictive

Knowledge of *EGFR* mutation status is most predictive in lung cancer and not as well described in other cancers. A number of prospective trials have now shown that *EGFR* mutational status is the best predictor of a major clinical response and progression-free survival (PFS), independent of other clinical parameters, when choosing EGFR-targeted therapy for NSCLC patients. Most importantly, *EGFR* mutations (mostly exon 19 deletions and L858R point mutations) are associated with a clinical benefit from gefitinib and erlotinib. In early phase III trials, these drugs were tested in unselected patients with NSCLC and showed less than 10% responses with short PFS rates. After the discovery of *EGFR* mutations, several prospective single-arm first-line studies enrolling only patients with *EGFR*-mutant tumors reported unprecedented response rates (73–91%) and prolonged PFS (7.7–13.3 months). Thereafter, several large prospective phase III first-line trials directly compared an EGFR TKI versus platinum doublet chemotherapy in patients with NSCLC harboring EGFR mutations. These trials strongly confirmed the benefit of gefitinib or erlotinib versus chemotherapy in *EGFR*-mutant lung cancer (Hirsch et al. 2013). While most of these studies were conducted on Asian populations, the European Tarceva (erlotinib) versus chemotherapy (EURTAC) study was conducted in a white population (Rosell et al. 2012). These results suggest that although the incidence of activating *EGFR* mutations is less in whites, there are no significant differences in treatment outcomes between patients with *EGFR* mutation of different ethnicity. Other *EGFR* mutations (e.g., T790M and exon 20 insertion) have been associated with acquired resistance to TKIs.

EGFR copy number changes also have some predictive value, but it is not clear whether it is just a surrogate of *EGFR* mutational status, or it reflects true gene amplification, which typically closely correlates with gene mutation. The *EGFR* gene copy number with balanced polysomy by FISH is seen in a high proportion of cancer cells – in approximately 25–40% of patients with NSCLC, HNSCC, or CRC. The predictive role of increased *EGFR* copy numbers has been evaluated in patients with these cancers, although the results have been conflicting, with some trials showing higher response rates and longer survival in FISH-positive tumors who were treated with EGFR-targeted therapies than patients receiving placebo or chemotherapy, but others showing no such correlation (reviewed in Ciardiello and Tortora 2008). Positive EGFR protein expression, as determined by immunohistochemistry, has also not proven to be a clinically effective predictor of responsiveness. Although initial approval of cetuximab and panitumumab in mCRC was limited to patients with high EGFR expression by immunohistochemistry, there is now clear evidence that patients can benefit from anti-EGFR monoclonal antibodies for mCRC or TKIs for other solid tumors even in the absence of EGFR expression (Amado et al. 2008; Douillard et al. 2010).

The *KRAS* mutations at codon 12 or 13 are predictive of lack of response to EGFR-targeted therapies, especially in mCRC (Benvenuti et al. 2007). *KRAS* testing is mandatory before proceeding with the decision to initiate EGFR-targeted therapy. Only patients whose tumors have the wild-type (normal) *KRAS* genes should receive treatment with the EGFR inhibitors cetuximab and panitumumab in mCRC.

There is not sufficient evidence from the published studies, to determine whether *EGFR* mutation is a prognostic biomarker of better survival, independent of TKI treatment. Reports regarding its prognostic significance in various cancer types have been conflicting. Some studies found positive correlations among high levels of EGFR, tumor invasiveness, and poorer survival, whereas others showed no correlation between EGFR expression and survival (Ciardiello and Tortora 2008).

Therapeutics

Potential therapeutic strategies that specifically target either the intracellular or extracellular segment of the EGFR/EGFRvIII and its family members include anti-receptor antibodies, small-molecular-weight TKI, receptor-ligand conjugates, receptor-immunoconjugates, dominant-negative receptor constructs, and antisense oligonucleotides, all of which are capable of blocking EGFR/EGFRvIII function (Panousis et al. 2005). Two distinct classes of EGFR antagonists, i.e., small-molecule TKIs and monoclonal antibodies (mAbs), have been approved by the Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) for the treatment of metastatic NSCLC, metastatic colorectal cancer (mCRC), head and neck squamous cell cancer (HNSCC), and pancreatic cancer.

Gefitinib and erlotinib are reversible small-molecule TKIs that block the ATP pocket located in the intracellular catalytic domain of the receptor, thus inhibiting EGFR phosphorylation and its downstream cascade. Two second-generation irreversible EGFR TKIs, afatinib and dacomitinib, are in late-stage clinical development. The second-generation irreversible EGFR TKIs theoretically have a higher affinity for the EGFR kinase domain, target other HER2 and HER4, and have modest in vitro activity against the T790M gatekeeper mutation that render the first-generation reversible EGFR TKIs ineffective. Gefitinib and erlotinib were the first targeted agents to demonstrate significantly improved responses and outcomes in patients with advanced NSCLC, first as second- and third-line therapy, and later, in the first-line and maintenance settings (Schrump et al. 2011). The FDA approved gefitinib through a new accelerated process in May 2003 as monotherapy for the treatment of patients with locally advanced or metastatic NSCLC progressed after prior platinum-based and docetaxel chemotherapies. After three large, prospective studies (INTACT 1, INTACT 2, and ISEL) showed no improvement in overall survival, the original FDA approval was modified in 2005, limiting the indication to cancer patients who, in the opinion of their treating physician, are currently benefiting or have previously benefited from gefitinib treatment. In July 2009, the EMEA granted approval for the use of gefitinib in any line of therapy for patients

with NSCLC who carry activating *EGFR* mutations. Erlotinib was originally approved in 2004 as monotherapy for the treatment of NSCLC patients who did not respond to at least one prior chemotherapy. In November 2005, erlotinib was approved in combination with gemcitabine for advanced pancreatic cancer patients who have not received previous chemotherapy.

Cetuximab and panitumumab belong to the class of anti-EGFR mAbs that are specifically designed against the extracellular region of EGFR, and create a ligand competitive inhibition, thus preventing receptor dimerization. Cetuximab is a mouse-human chimeric antibody, while panitumumab is a fully humanized antibody. Other mAbs, including nimotuzumab, zalutumumab, matuzumab, and necitumumab, are still in clinical development (Yarden and Pines 2012). Cetuximab has exhibited promising antitumor activity in clinical trials as monotherapy and when used in combination with chemotherapy and/or radiation, particularly in the settings of mCRC and HNSCC. In 2004, the FDA approved cetuximab for use in combination with irinotecan for the treatment of patients with EGFR-expressing mCRC refractory to irinotecan-based chemotherapy. In addition, cetuximab was approved for use as a single agent in patients with mCRC who have failed in oxaliplatin- or irinotecan-based therapy and who are intolerant to irinotecan. Cetuximab has been studied in combination with FOLFOX and FOLFIRI in mCRC. Combining chemotherapy with cetuximab improved efficacy in the first-line or second-line mCRC settings. Cetuximab is indicated for the treatment of patients with EGFR-expressing, *KRAS* wild-type mCRC in combination with FOLFIRI for first-line treatment. In 2006, the FDA approved the use of cetuximab in combination with radiation for the treatment of locoregionally advanced HNSCC. In addition, cetuximab was approved as a single agent for the treatment of patients with recurrent or metastatic HNSCC for whom platinum-based therapy had failed. Panitumumab has exhibited promising antitumor activity in several clinical trials and in 2006 gained FDA approval for the treatment of patients with EGFR-expressing mCRC with disease progression following chemotherapy regimens containing fluoropyrimidine, oxaliplatin, and irinotecan. While there is evidence that immunohistochemical EGFR receptor testing does not predict response to either cetuximab or panitumumab, assessment for EGFR expression is required for use in colorectal cancer according to FDA guidelines, but not in head and neck cancer. Approximately 75% of patients with mCRC have an EGFR-expressing tumor and are therefore considered eligible for treatment with cetuximab or panitumumab, according to FDA guidelines.

Preclinical Summary

Despite the dramatic efficacy of EGFR TKIs, acquired resistance to EGFR TKI is inevitable. Much of the preclinical work has led to unraveling of mechanisms that trigger innate and acquired resistance to EGFR TKI. Multiple groups have modeled acquired resistance in vitro using *EGFR*-mutant NSCLC cell lines and increasing levels of TKI exposure. The resultant TKI-resistant cells harbor T790M and/or *MET*

amplification, validating this approach as a useful in vitro tool for the study of clinically relevant acquired resistance mechanisms. Other biomarkers of resistance have been identified, such as exon 19 mutations (i.e., D761Y and L747S), AXL activation, and signaling through the HER2/neu protein – either through upregulation of protein production or overexpression of the gene. Pharmacologic and genetic inhibitors of activated MAPK, AKT, and NFkB pathways have shown to increase sensitivity to EGFR TKI in several models of EGFR-mutant lung cancers. These observations thereby provide a strong rationale to develop therapeutic strategies designed to circumvent these molecular mechanisms. Ectodomain mutations have been suggested to favor inactive-like conformation of the EGFR that seems more compatible with binding type II inhibitors such as lapatinib or neratinib. These drugs have shown reasonable inhibitory effects on glioblastoma cell lines expressing extracellular missense mutations. Additionally, vaccination approaches targeting the EGFRvIII mutant featuring a tumor-specific antigen have shown promising results that warrant larger controlled clinical trials. A number of additional EGFR-targeting kinase inhibitors are also in the preclinical stage and early clinical development, for example, third-generation WZ4002, which has an anilinopyrimidine core that fits better into the ATP pocket of EGFR T790M compared to quinazoline core of first- or second-generation EGFR TKIs.

Clinical Summary

EGFR is a clinically established target for the treatment of various cancers because of its crucial role in regulating cellular proliferation and carcinogenesis. Several monoclonal antibodies and small-molecule EGFR TKIs have been tested in phase III trials and are now in clinical use. *EGFR*-mutant lung cancer is a validated unique subset, with its own clinical features and natural history that serves as a paradigm for oncogene-driven solid tumor that can be effectively treated with EGFR-targeted therapy. Several large prospective phase III first-line trials directly comparing an EGFR TKI against platinum doublet chemotherapy in patients with EGFR-mutant NSCLC have strongly confirmed the benefit of gefitinib or erlotinib. *EGFR* mutational status has demonstrated significant predictive value in the selection of patients for EGFR TKI therapy. As a consequence, the analysis of tumor biopsy samples for *EGFR* mutations in patients with NSCLC has been introduced as a routine diagnostic test in some centers. Despite dramatic responses, eventually all patients treated with reversible EGFR TKI develop resistance. Second-generation irreversible EGFR TKIs including canertinib, neratinib, afatinib, and dacomitinib are being developed and tested in clinical trials to potentially overcome resistance encountered with the use of first-generation inhibitors. Advances in the understanding of EGFR signaling in mCRC have led to the development of mAbs including cetuximab and panitumumab that has helped improve the median survival of patients with this cancer. Approximately 27–43% of mCRC patients harbor *KRAS* gene mutations in

their tumors. The identification of mutated *KRAS* status as a predictive marker for lack of response to EGFR-targeted mAbs in mCRC has perhaps had the greatest impact on patient management. This means that mCRC patients unlikely to benefit from a targeted therapy could be identified ahead of treatment. Data support the routine use of *KRAS* mutational analysis in mCRC patients being considered for EGFR-targeted therapies. On the other hand, *KRAS* mutation status has not shown to be predictive of response to EGFR-targeted therapies in other cancers, implicating that not all *KRAS* mutations are equal. In contrast to the experience in lung cancer with *EGFR* mutations, TKIs appear relatively ineffective for GBM expressing a constitutively activated and immunogenic mutation EGFRvIII. The unique amino acid sequence of EGFRvIII is immunogenic and has been well validated as a target for cancer immunotherapy. Such vaccination approaches targeting the EGFRvIII mutant have shown promising results in phase II trial and have led to initiation of phase III clinical trials.

Anticipated High-Impact Results

- Identification of potential predictive biomarkers that might form the basis of companion diagnostics for both small-molecule inhibitors and mAbs, in various cancer types
- Genotype-driven trials of rationally targeted therapies for patients with acquired TKI resistance
- Hypothesis-driven preclinical studies testing novel combinations of anti-EGFR therapies with other targeted therapies or chemotherapy
- Comprehensive mutational profiling in smaller samples or even in circulating tumor cells or cell free DNA
- Platforms using multiplex PCR to identify potentially actionable molecular targets in cancer
- Clinical development of third-generation TKI

Cross-References

► AKT
► DNA Vaccines
► EGFR, Growth Factors
► HER2/neu
► HER3
► Jak2/Stat5a/b Pathway in Prostate Cancer
► K-Ras
► NF-κB

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HER3

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Abstract

ErbB3/HER3 belongs to the family of ErbB receptors, which comprises four transmembrane receptors that can bind more than ten different ligands. The ErbB3 receptor forms heterodimers with the other ErbB receptor family members, which is the necessary step to activate downstream prosurvival signaling. While ErbB3 signaling plays an important role in embryonic development, it has also been implicated in the development of cancer and in mediating resistance to anti-cancer treatments. In this chapter, we summarize the state of anti-ErbB3 therapies currently in clinical development including the emerging clinical data on HRG mRNA as a potential prognostic and predictive biomarker.

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Biology of ErbB3/HER3

ErbB3 belongs to the ErbB family of type I receptor tyrosine kinases (RTKs). The ErbB network plays an important role in development and is often dysregulated in cancer (Yarden and Sliwkowski 2001). The family comprises four structurally related transmembrane receptors: EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. All receptors except for HER2 interact with multiple ligands that can be divided into three groups: (1) ligands that bind to EGFR, such as epidermal growth factor (EGF), amphiregulin (AR), and transforming growth factor-α (TGF-α); (2) ligands that bind to EGFR and ErbB4, such as betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR); and (3) ligands that bind to ErbB3 and/or ErbB4, such as heregulins 1 and 2 [HRG1 and HRG2, also known as Neu differentiation factors (NDFs) or neuregulins (NRGs)], which bind to both ErbB3 and ErbB4, and HRG3 and HRG4, which bind only to ErbB4/HER4 (Burden and Yarden 1997).

Heregulins (HRGs) were first identified independently by several groups as factors that activate ErbB2 (Holmes et al. 1992; Peles et al. 1993) and stimulate Schwann cell proliferation (Lemke and Brockes 1984; Falls 2003). They fall into a large family of EGF-like signaling molecules that are involved in cell-cell communication during development as well as in the adult. They are primarily expressed in the nervous system, heart, mammary glands, intestine, and kidneys. Upon binding to ErbB3, heregulins activate intracellular signaling cascades that induce a variety of cellular responses, including proliferation, migration, differentiation, survival, and apoptosis (Fig. 1; Citri et al. 2003; Olayioye et al. 2000). Dysregulation of HRG ligands and their receptors has been implicated in many human cancers, including breast, ovarian, and lung (Breuleux 2007).

ErbB receptors do not act in isolation. Instead, they form homo- or heterodimers with distinct binding affinities for their more than 10 different ligands. Dimerization of ErbB3 with EGFR, HER2, or ErbB4 is enhanced by a conformational change induced by ligand binding to the extracellular domain of ErbB3. It is a necessary step for receptor phosphorylation and the subsequent recruitment of intracellular signaling molecules. All four ErbB receptors have an extracellular ligand-binding domain and an intracellular tyrosine kinase domain, with a C-terminal tail that has multiple tyrosine residues that are capable of recruiting downstream signaling molecules when phosphorylated. Unlike other ErbB receptors, ErbB3 was long assumed to be “kinase dead” and therefore considered to need an active heterodimerization partner to become phosphorylated and initiate signaling. More recently, however,

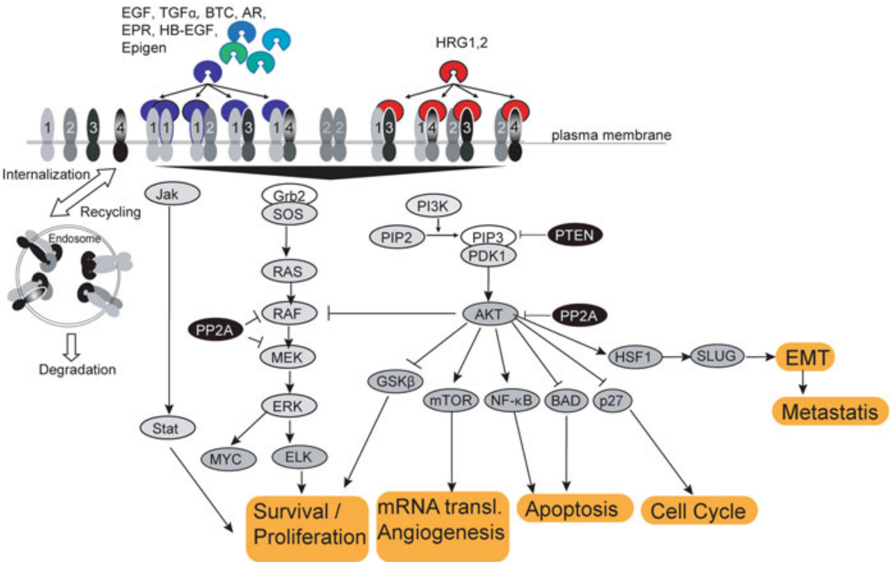

Fig. 1 Simplified ErbB signal transduction network. Phosphorylation of the C-terminal tail of ErbB3 leads to recruitment of adapter proteins and subsequent activation of the PI3K and MAPK and Jak/Stat cascades. Activation of PI3K leads to phosphorylation of membrane phosphoinositides, producing PIP3, which in turn recruits the PH domain-containing proteins PDK1 and Akt. Membrane-bound Akt is phosphorylated and activated by PDK1. Activation of ErbB3 also induces binding of the Grb2-SOS complex, which in turn enables the formation of Ras-GTP from Ras-GDP, thereby activating the MAPK cascade. *transl.* translation

ErbB3 has been found to exhibit weak residual kinase activity and is therefore capable of autophosphorylation, enabling downstream signaling in situations where the kinase activity of its dimerization partners has been attenuated by kinase inhibitors (Telesco et al. 2011). Interestingly, of all the ErbB homo- and heterodimers, the HER2/ErbB3 dimer is the most mitogenic (Pinkas-Kramarski et al. 1996) and transforming (Alimandi et al. 1995; Wallasch et al. 1995; Holbro et al. 2003). It has very high affinity for HRG and potently activates a variety of downstream signaling pathways, including the pro-proliferative mitogen-activated protein kinase (MAPK) pathway, the pro-survival phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, and the Janus kinase (Jak)/signal transducer and activator of transcription (Stat) pathways. Moreover, in *ERBB2*-amplified cancer cells, heterodimerization of HER2 with ErbB3 can activate downstream signaling even in the absence of ligand. Heterodimerization with or without transactivation of ErbB3 is not limited to ErbB family members (Huang et al. 2010). ErbB3 can also be activated in a ligand-independent manner by the receptor tyrosine kinase Met in *MET*-amplified cell lines (Engelman et al. 2007). Similarly, ErbB3 can be activated by FGFR2 in *FGFR2*-amplified cell lines (Kunii et al. 2008).

The C-terminal tail of ErbB3 features 14 tyrosine residues, 10 of which can be phosphorylated and 6 of which bind with high affinity to the Src homology 2 (SH2)

domains of PI3K (Jones et al. 2006). Upon phosphorylation of these sites, ErbB3 recruits PI3K, which potently activates downstream signaling through Akt (Soltoff et al. 1994). Mutations in PI3K do not preclude further ligand-mediated activation of the ErbB3 pathway if cancer cells express sufficient levels of ErbB3 (Yarar et al. 2015). The extent to which ErbB3 activates PI3K sets it apart from the other ErbB receptors and highlights its importance as an oncology target. Most studies of ErbB3 have focused on its activation of the MAPK and PI3K/Akt pathways, which activate a plethora of cellular substrates involved in diverse biological and pathological processes, including proliferation, cell cycle progression, and tumor metastasis (Fig. 1).

Role of ErbB3 in Development

ErbB family receptors are expressed in cells of epithelial, mesenchymal, and neuronal lineages and play an important role in development (Britsch 2007). The phenotype induced by a lack of ErbB3 in the development of mouse mammary glands was more pronounced than the knockout of any other ErbB receptor, highlighting the significance of ErbB3 in mammary development (Balko et al. 2012). Gene knockout studies have also uncovered the importance of ErbB3 in the development and maintenance of various integrative body systems, including the cardiovascular and nervous systems (Casalini et al. 2004). ErbB3 knockout (KO) mice die on day 13.5 of embryonic development due to pronounced heart defects, aberrant valve formation, and vasculature abnormalities (Erickson et al. 1997). Heregulins were shown to promote neuronal migration and differentiation and regulate the selective expression of neurotransmitter receptors in neurons and at the neuromuscular junction. They also regulate glial commitment, proliferation, survival, and differentiation.

Role of ErbB3 in Cancer

Because the gene for ErbB3 is not frequently mutated or amplified in cancer, it was not initially recognized as a therapeutic target. More recently, however, it has been well established that ErbB3 plays a critical role both in tumor development and in mediating resistance to chemo- and targeted therapy.

The essential role of ErbB3 in tumor development has been demonstrated in *ERBB2*-amplified breast cancers as well as in *Apc^{Min}* models of colon cancer (Lee et al. 2009; Morrison et al. 2013). Intestine-specific *ERBB3* deletion does not affect proliferation, apoptosis, or signal transduction in normal intestinal epithelium, but makes the intestinal epithelium more susceptible to injury. Intestine-specific *ERBB3* deletion, however, vastly decreases the penetrance of *Apc^{Min}* colon tumors due to reduced PI3K/Akt pathway activity and subsequent tumor-specific increase in apoptosis. In breast cancer, HER2/ErbB3 heterodimers drive mitogenic signaling in the mammary epithelium, and genetic ablation of *ERBB3* in mammary glands inhibits

premalignant HER2-induced hyperplasia and vastly decreases the penetrance of both palpable tumors and ductal carcinoma in situ (Atlas et al. 2003; Vaught et al. 2012). ErbB3 is also required for maintenance of the malignant phenotype of HER2-overexpressing mammary tumors with ablation of *ERBB3* leading to decreased PI3K and MAPK signaling pathway activity (Cook et al. 2011). In luminal breast cancers, ErbB3 was shown to drive the growth and survival of estrogen receptor (ER)-positive breast cancer cells (Morrison et al. 2013). Interestingly, ErbB3 activation by HRG also induces phosphorylation of the estrogen receptor, leading to estrogen-independent activation of ER (Pietras et al. 1995; Osborne and Schiff 2003).

More recently, somatic mutations in *ERBB3* have been discovered in a variety of cancers (Jaiswal et al. 2013). *ERBB3* is mutated in ~11% of colon and gastric cancers and in 1% of non-small cell lung cancers. Recurring mutations are mostly in the extracellular domain of ErbB3 and promote oncogenic signaling in the presence of kinase-active HER2. Some of the mutations are oncogenic in the absence of HRG, but are further stimulated by ligand.

Next to the mechanistic studies and discovery of mutations elucidating the role of ErbB3 in cancer, high levels of ErbB3 have been linked to poor prognosis in multiple solid tumor types, including pancreatic, gastric, breast, skin, lung, and ovarian cancers (Ocana et al. 2013; Hirakawa et al. 2011; Berghoff et al. 2014; Richards 2010; Hayashi et al. 2008; Tanner et al. 2006).

Role of ErbB3 in Drug Resistance and the Potential Role of anti-HER3 therapies as "Resensitizers"

The role of ErbB3 in drug resistance has been described for multiple types of therapeutic modalities. Three major resistance mechanisms have been observed: (i) increased levels of ErbB3 by enhanced *ERBB3* transcription, (ii) increased HRG autocrine signaling, and (iii) ligand-independent activation of ErbB3 by other RTKs such as HER2 and Met.

First, cancers driven by EGFR or HER2 use ErbB3 signaling to mediate resistance to ErbB inhibitors. Akt activation inhibits the nuclear localization of FOXO and *ERBB3* transcription. Therefore, the enhanced transcription of *ERBB3* and partial rescue of PI3K/Akt signaling activity is frequently observed as a compensatory mechanism in response to PI3K/Akt pathway inhibition (Chandarlapaty et al. 2011). By this mechanism, ErbB-targeted therapies such as lapatinib and gefitinib increase ErbB3 signaling, thereby attenuating their efficacy (Sergina et al. 2007; Garrett et al. 2011). The combination of an anti-EGFR agent with an anti-ErbB3 inhibitor has been shown to effectively inhibit the MAPK and PI3K signaling cascade activated by EGFR/HER2 and ErbB3, respectively. Additive or even synergistic responses have been observed across indications and in different preclinical models when an EGFR and an ErbB3 inhibitor are combined (Jiang et al. 2014; Schoeberl et al. 2010a; Tao et al. 2014; Kawakami et al. 2014; Huang et al. 2013). Based on these preclinical findings, combined inhibition of EGFR and

ErbB3 has been tested clinically in several Phase 1 and Phase 2 trials. For example, the small molecule tyrosine kinase inhibitor (TKI) erlotinib has been evaluated in non-small cell lung cancer (NSCLC) in combination with Merrimack's anti-ErbB3 antibody seribantumab (MM-121) (Sequist et al. 2014) and in combination with Daiichi's anti-ErbB3 antibody patritumab (Mendell et al. 2015). In both of these trials, progression-free survival benefit was observed when the anti-ErbB3 antibody was added to erlotinib, but only in the subset of patients expressing the ErbB3 ligand HRG. This concept has now been advanced to an ongoing Phase 2/3 study in which the combination of erlotinib and patritumab is being evaluated in patients with locally advanced or metastatic NSCLC (NCT02134015). Genentech has also developed a cross-reactive antibody, MEHD7945A, that inhibits both EGFR and ErbB3 and has tested this agent in Phase 2 studies in squamous cell carcinoma of the head and neck (Penuel et al. 2015). Although no advantage was observed relative to the anti-EGFR antibody cetuximab, (Eli Lilly), biomarker selection was not employed in these studies. Several Phase 1 studies have also been conducted examining combined inhibition of EGFR and ErbB3 (Jiang et al. 2014; Papadopoulos et al. 2014), and Merrimack is currently evaluating safety and preliminary efficacy of MM-151, an ultrapotent mixture of three anti-EGFR antibodies, in combination with seribantumab in patients selected for expression of HRG (NCT02387216).

Second, ErbB3 is also a resistance mechanism if other kinases or RTKs besides EGFR or HER2 are inhibited. For example, upon inhibition of either PI3K or Akt, increased levels of phospho-ErbB3 are observed in triple negative breast cancer cell lines (Tao et al. 2014). The combination of a PI3K or Akt inhibitor with either MEHD7945A or ErbB3 knockdown results in decreased cell proliferation compared with inhibition of the PI3K/Akt pathway alone, both in vitro and in mouse xenograft models as shown by Tao and colleagues. Similarly, dual blockade of ErbB3 with either PI3K or Akt is a potentially effective treatment approach in HER2-overexpressing cancers (Garrett et al. 2013). The combination of an anti-ErbB3 antibody that inhibits HER2/ErbB3 dimers and a p110α-specific inhibitor in the absence of a direct HER2 antagonist is active in a trastuzumab-resistant xenograft model. Based on these findings, a Phase 1 study is currently under way evaluating the safety and tolerability of LJM716 (a fully human anti-ErbB3 antibody; Novartis), BYL719 (a PI3Kα-specific small molecule inhibitor; Novartis), and trastuzumab (a humanized anti-HER2 antibody; Genentech) in patients with metastatic HER2+ breast cancer (NCT02167854). Furthermore, activation of ErbB3 by HRG blunts the effects of anti-IGF-1R treatment and appears to be the dominant resistance mechanism for IGF-1R inhibitors (Desbois-Mouthon et al. 2009; Fitzgerald et al. 2014). Finally, BRAF or MEK inhibition in V600 mutant BRAF-driven cancer cells is reduced if ErbB3 is active. In this case, potent activation of the PI3K/Akt pathway appears to compensate for strong inhibition of the MAPK pathway (Fattore et al. 2013).

Third, ErbB3 signaling mediates resistance to antihormonal therapies. In hormone receptor-positive breast cancer cells, proliferation is strongly driven by either the estrogen receptor (ER) or the progesterone receptor (PR). These receptors can be activated directly by their respective hormones (estrogen or progesterone), or they

can be activated in a hormone-independent fashion by ligand-driven RTK signaling (Musgrove and Sutherland 2009). When hormone-dependent ER/PR signaling is blocked by antihormonal therapy, one way in which cells overcome this inhibition is through activation of HRG-driven ErbB3 signaling (Hutcheson et al. 2011; Curley et al. 2015). For example, the antiestrogen receptor therapeutic fulvestrant induces transcriptional upregulation of *ERBB3*, and ErbB3 activation in breast cancer cells increases proliferation (Morrison et al. 2013; Hutcheson et al. 2011). Similarly, heregulin mediates resistance to the aromatase inhibitor letrozole in a mouse xenograft model of ER+ breast cancer (Curley et al. 2015). ErbB3 may also modulate responses to androgen withdrawal in prostate cancers (Mellinghoff et al. 2004).

Fourth, ErbB3 signaling has been implicated as a resistance mechanism to chemotherapy. The chemotherapeutic drug doxorubicin was shown to induce activation of the ErbB3/PI3K/Akt cascade in ovarian cancer cells, and inhibition of ErbB3 in this context significantly increased apoptosis (Bezler et al. 2012). Similarly, ErbB3 inhibition has been shown to enhance the magnitude and duration of chemotherapy in multiple NSCLC in vivo models (Hegde et al. 2013). This hypothesis has also been tested clinically. In a randomized Phase 2 trial conducted in women with platinum-resistant/platinum-refractory advanced ovarian cancer, seribantumab was evaluated in combination with paclitaxel relative to paclitaxel alone. The addition of seribantumab extended paclitaxel progression-free survival, but only in the subset of patients (38%) whose tumors showed expression of heregulin and low levels of HER2 (Wang et al. 2013; Macbeath et al. 2014).

Anti-ErbB3 Therapies in Clinical Development

A broad collection of antibodies targeting human ErbB3 has been advanced into clinical development in the past several years (Figs. 2 and 3) (Aurisicchio et al. 2012). In this section we will review their properties and status of clinical development. Whereas the majority of these agents target ErbB3 alone, some have been engineered to bind not only ErbB3 but additional co-receptors as well (bispecific antibodies). All of the monoclonal antibodies block ligand binding to ErbB3, with exception of LJM716, which locks ErbB3 in its inactive conformation. In addition, they each induce receptor turnover, albeit to different extents. Patritumab and seribantumab are currently the furthest along in clinical development and provide the largest set of clinical data to date.

As ErbB3 is rarely a key driver of tumor growth, but rather plays an important role in mediating resistance to therapy, clinical development of ErbB3 inhibitors has largely focused on combining them with standard-of-care therapies in an effort to either reverse or delay resistance. To date, ErbB3 inhibitors have generally not elicited partial or complete responses as single agents, but instead have produced prolonged disease stabilizations in Phase 1 and prolonged progression-free survival or overall survival when combined with standard therapy, relative to standard therapy alone.

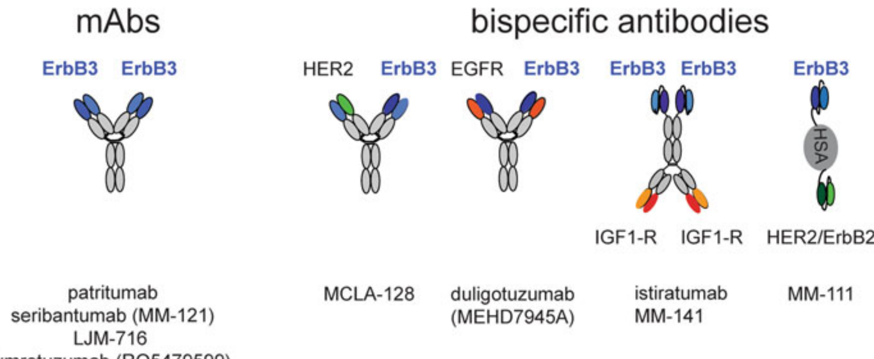

Fig. 2 Graphical representation of monoclonal antibodies (mAbs) and bispecific antibodies in clinical development as of 2015

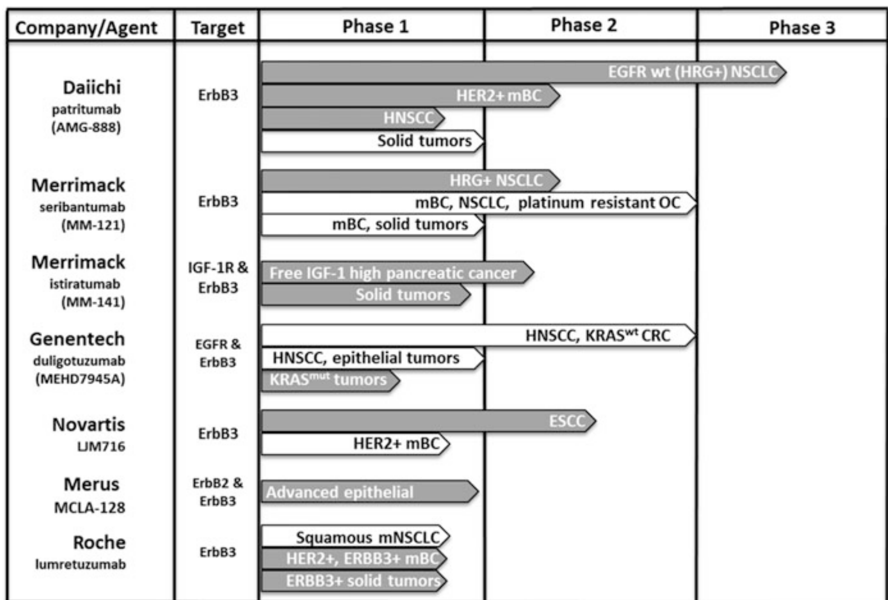

Fig. 3 ErbB3-/HER3-targeting molecules in clinical development. *Horizontal gray bars* represent trials that are currently ongoing in the specified indications, while *horizontal white bars* represent trials that are inactive or have been completed. *NSCLC* non-small cell lung cancer, *mBC* metastatic breast cancer, *HNSCC* head and neck squamous cell carcinoma, *OC* ovarian cancer, *KRAS^{wt} CRC* KRAS wild-type colorectal cancer, *KRAS^{mut} CRC* KRAS mutated colorectal cancer, *ESCC* esophageal squamous cell carcinoma

The first ErbB3 inhibitor to enter clinical development was seribantumab (MM-121; Merrimack Pharmaceuticals). It is a fully human monoclonal IgG2 antibody, designed to inhibit heregulin-driven ErbB3 signaling based on systems-level insights from computational modeling of the ErbB network (Schoeberl et al. 2009). As an IgG2 molecule, it has limited capacity to induce antibody-dependent cellular cytotoxicity (ADCC) and is therefore a pure signaling inhibitor, although it also induces receptor internalization and degradation. Preclinically, seribantumab potently inhibits ErbB3-induced PI3K/Akt signaling and demonstrates single-agent antitumor activity in mouse xenograft models of lung, breast, renal, head and neck, prostate, and ovarian cancer (Schoeberl et al. 2010b; Sheng et al. 2010; Wang et al. 2013; Curley et al. 2015). In a first-in-human Phase 1 trial (NCT00734305), seribantumab extended did not induce partial or complete responses as a single agent, but appeared to induce prolonged stable disease in a subset of patients. It was generally well tolerated and was shown to combine safely with a variety of agents, including antihormonal therapies (exemestane; NCT01151046), targeted therapies (erlotinib, cetuximab, XL147; NCT00994123, NCT01451632, NCT01436565), and chemotherapies (paclitaxel, irinotecan, gemcitabine, carboplatin, pemetrexed, cabazitaxel; NCT01209195, NCT01451632, NCT01447225). To test the hypothesis that ErbB3 inhibition can either delay or reverse resistance to therapy, seribantumab was evaluated in three randomized Phase 2 trials of metastatic cancer: in combination with paclitaxel versus paclitaxel alone in platinum-resistant/platinum-refractory ovarian cancer (NCT01447706), in combination with exemestane versus exemestane alone in ER/PR+ HER2- breast cancer (NCT01151046), and in combination with erlotinib versus erlotinib alone in EGFR wild-type non-small cell lung cancer (NSCLC; NCT00994123). Although seribantumab did not extend progression-free survival (PFS) in the unselected patient populations in these trials, a subset of patients was found in all three settings, defined largely by tumoral expression of heregulin, that derived benefit from the addition of seribantumab to standard therapy. Consistent with the Phase 1 trials, seribantumab was generally well tolerated, with the most common adverse events being diarrhea, nausea, fatigue, and anemia (Denlinger et al. 2013). Most events, however, were mild to moderate in severity.

Similar to seribantumab, patritumab (Daiichi Sankyo) is a fully human anti-ErbB3 monoclonal antibody. Unlike seribantumab, it is an IgG1 antibody and so, in principle, capable of eliciting ADCC. Preclinically, patritumab demonstrated single-agent antitumor activity in xenograft models of pancreatic cancer, NSCLC, and colorectal cancer and activity in combination with panitumumab (an anti-EGFR monoclonal antibody; Amgen) in xenograft models of head and neck squamous cell carcinoma (HNSCC) (Freeman et al. 2009, 2011). In first-in-human Phase 1 studies (NCT00730470), patritumab was well tolerated and showed preliminary evidence of disease stabilization and tumor reduction in some patients (Lorusso et al. 2013). Patritumab has also been evaluated in the Phase 1 setting in combination with paclitaxel and trastuzumab in women with HER2+ metastatic breast and in combination with cetuximab and platinum-containing chemotherapy in patients with HNSCC (NCT02350712). Similar to seribantumab, patritumab was also evaluated

in the Phase 2 setting in NSCLC, comparing erlotinib plus patritumab with erlotinib plus placebo (NCT01211483). A preplanned retrospective analysis showed that the subset of patients with above-median expression of heregulin in their tumors benefitted from the addition of patritumab (Mendell et al. 2015). Based on these results, Daiichi Sankyo has recently initiated a Phase 2/3 trial, mimicking the previous Phase 2 trial, but with the Phase 3 portion in patients prospectively selected for high heregulin expression (HER3-Lung, NCT02134015).

In contrast to seribantumab and patritumab, LJM716 (Novartis) is a monoclonal anti-ErbB3 antibody that does not directly compete with heregulin for binding to ErbB3. Instead, LJM716 locks ErbB3 in an inactive conformation, rendering it unable to heterodimerize with other ErbB receptors such as EGFR and HER2. Treatment with LJM716 is therefore able to inhibit ErbB3 signaling and downstream effector activation in both heregulin-dependent and heregulin-independent *HER2*-amplified cell lines in vitro (Garner et al. 2013). To date, LJM716 has advanced through initial Phase 1 testing as a single agent (NCT01598077) and, based on preclinical data, is currently being evaluated in the Phase 1 setting in combination with trastuzumab and the PI3K inhibitor BYL719 in patients with metastatic HER2+ breast cancer (NCT02167854). It is also being evaluated in a randomized Phase 2 trial in patients with previously treated esophageal squamous cell carcinoma (ESCC), comparing LJM716 plus BYL719 with single-agent chemotherapy (taxane or irinotecan).

Finally, lumretuzumab (RO5479599; Roche) is an ErbB3-targeting antibody that has a glycoengineered Fc region, which increases its capacity to engage immune effector cells as compared with traditional IgG1 or IgG2 antibodies (Meneses-Lorente et al. 2015). A Phase 1 study of RO5479599 alone or in combination with cetuximab or erlotinib is currently ongoing in patients with ErbB3-positive solid tumors (NCT01482377).

In addition to these monospecific anti-ErbB3 antibodies, several bispecific molecules have also been developed that co-target ErbB3 and another RTK. These molecules have been developed in an effort either to increase the potency of ErbB3 inhibition or inhibit compensatory resistance mechanisms that arise as a consequence of drug treatment. The hypothesis is that patients with tumors that exhibit inherent or acquired dependence on more than one RTK signaling pathway may derive more benefit from treatment with a bispecific antibody. In this section, we will introduce two types of bispecific antibodies: those like MM-111 (Merrimack) and MCLA-128 (Merus) that target the HER2/ErbB3 heterodimeric complex and those like MM-141 (Merrimack) and duligotuzumab (MEHD7545A; Genentech) that target ErbB3 and another closely interconnected receptor.

It has long been established that, of the ten possible ErbB homo- and heterodimers, the HER2/HER3 dimer is the most transforming (Alimandi et al. 1995; Wallasch et al. 1995) and mitogenic (Pinkas-Kramarski et al. 1996). It is therefore a compelling strategy to engineer antibodies that specifically target this receptor complex. To this end, MM-111 is a bispecific antibody, built from two single chain Fv molecules that target HER2 and ErbB3, linked together by modified human serum albumin (McDonagh et al. 2012; Fig. 2). MM-111 is designed to dock

onto HER2 in *HER2*-amplified cells, thereby constraining it to the cell surface and dramatically increasing its relative affinity for ErbB3. In this way, MM-111 is able to compete with heregulin for binding to ErbB3 in *HER2*-amplified cells more effectively than monospecific anti-ErbB3 antibodies like seribantumab or patritumab (Onsum et al. 2012). In preclinical models, MM-111 is most active when administered in combination with HER2-targeting agents like trastuzumab or lapatinib (Kirouac et al. 2013). In Phase 1 studies, MM-111 was shown to be well tolerated alone or in combination with trastuzumab and various chemotherapeutic agents (NCT01304784, NCT01097460). MM-111 was also evaluated in a Phase 2 trial of patients with HER2+ carcinomas of the distal esophagus, gastroesophageal junction, and stomach, comparing MM-111 in combination with trastuzumab and paclitaxel with trastuzumab and paclitaxel alone (NCT01774851). This trial, however, was stopped early for safety reasons.

Like MM-111, MCLA-128 (Merus) is a bispecific antibody targeting the HER2/HER3 complex. It is an effector function-enhanced IgG1 bispecific antibody that blocks ligand-dependent ErbB3 signaling. In preclinical studies in breast cancer cell lines, MCLA-128 was more active at inhibiting HRG-driven growth than either of its bivalent parental antibody components and potently inhibited ErbB3 and Akt phosphorylation (Geuijen et al. 2014, 2015). MCLA-128 was also more active than lapatinib in mouse xenograft models of trastuzumab-resistant JIMT-1 breast tumors. In HER2-high breast cancer cell lines, MCLA-128 exhibited ADCC activity equivalent to trastuzumab, but showed significantly increased ADCC activity in HER2-low cell lines. MCLA-128 is currently being evaluated for safety, tolerability, and antitumor activity in a European multicenter dose escalation study.

Bispecific antibodies have also been developed that target ErbB3 and other receptors whose network is integrally connected with that of ErbB3. Duligotuzumab (MEHD9945A) falls into this category, although it is not bispecific in the same way as MM-111 and MCLA-128. Instead, duligotuzumab is designed to recognize both ErbB3 and EGFR, targeting an epitope that is shared between these two receptors. Because duligotuzumab is a bivalent IgG1 antibody, it can, in principle, bind simultaneously to ErbB3 and EGFR, with each of its Fab arms recognizing a different molecule. As an IgG1, it can also induce ADCC (Schaefer et al. 2011). As discussed above, cancers driven by EGFR frequently use ErbB3 signaling to mediate resistance to EGFR inhibitors. In preclinical studies, single-agent treatment with duligotuzumab inhibited ligand-dependent growth and cell cycle progression of cancer cell lines that are resistant to EGFR-targeted antibodies. In principle, inhibition of ErbB3 could either delay the onset of resistance to EGFR inhibitors in the EGFR-naïve setting or resensitize tumors to EGFR inhibition in the EGFR-refractory setting. Focusing on the former, duligotuzumab was compared to cetuximab in two different randomized Phase 2 trials: one in combination with FOLFIRI in KRAS wild-type metastatic colorectal cancer (NCT01652482) (Hill et al. 2015) and a second as monotherapy in recurrent/metastatic SCCHN (NCT01577173) (Penuel et al. 2015). In both trials, duligotuzumab failed to demonstrate a PFS advantage over cetuximab, and the frequency of serious adverse

events was higher in patients treated with duligotuzumab relative to cetuximab. At this point, however, it is unclear whether combined ErbB3 and EGFR inhibition is of benefit in at least a subpopulation.

The other bispecific antibody of note is istiratumab (MM-141; Merrimack). It is a tetravalent bispecific IgG1 antibody that co-targets IGF-1R and ErbB3 (Fig. 2). The design of istiratumab is based on the observation that, when IGF-1R signaling is blocked, Akt-mediated repression of FOXO is relieved, causing immediate upregulation of IGF-1R, HER2, and ErbB3 (Chandarlapaty et al. 2011). ErbB3 signaling is therefore an adaptive response – hardwired into the IGF-1R network – that compensates for IGF-1R blockade. Istiratumab is a second-generation IGF-1R inhibitor, designed to simultaneously block IGF-1R and compensatory signaling through ErbB3. In preclinical models, istiratumab demonstrated improved activity relative to a combination of its components, consistent with the presence of ErbB3/IGF-1R heterodimers, but also probably reflective of the efficiency with which this tetravalent molecule downregulates cell surface expression of its targets (Fitzgerald et al. 2014). Istiratumab was well tolerated in Phase 1 studies, alone and in combination with a variety of targeted and chemotherapeutic agents (NCT01733004) (Isakoff et al. 2014). It is currently being evaluated in a randomized, Phase 2 trial in frontline metastatic pancreatic cancer, comparing the combination of gemcitabine, nab-paclitaxel, and istiratumab with gemcitabine, nab-paclitaxel, and placebo in patients selected for high serum levels of free IGF-1 (NCT02399137).

In addition to these mono- and bispecific antibodies, several other ErbB3-targeting antibodies are currently being evaluated in Phase 1 clinical trials. They include REGN1400 (Regeneron), GSK-2849330 (GlaxoSmithKline), KTN-3379 (Kolltan), and AV-203 (AVEO Pharmaceuticals). Finally, rhErbB3-f is a recombinant human ErbB3 receptor fragment under development by Zensun as a therapeutic vaccine for the treatment of breast cancer associated with overexpression of ErbB3.

Diagnostic Strategies for Anti-ErbB3 Therapies

Like all targeted therapies, ErbB3 inhibitors are only expected to provide clinical benefit in the subpopulation of patients in which the target pathway is active. As such, it is in the best interests of patients that these inhibitors be co-developed with a companion diagnostic. In addition, clinical trials that include patients that do not benefit from the drug are larger, are more expensive, take longer, and are more likely to miss their primary end points. The benefit of using biomarkers to guide therapeutic decisions is well appreciated in breast cancer, where expression levels of estrogen receptor, progesterone receptor, and HER2 are routinely assessed.

Early on in the development of anti-ErbB3 therapies, attempts were made to identify biomarkers preclinically. Sensitivity analysis of a mechanistic model of ErbB signaling was used to identify five potential biomarkers of ErbB3 pathway activation: HRG, BTC, EGFR, HER2, and ErbB3 (Schoeberl et al. 2010a). Using a broad collection of mouse xenograft models representing both responders and non-responders to seribantumab, a supervised learning model (support vector machines)

was trained to predict response based on the quantitative measurement of these five biomarkers (Schoeberl et al. 2010a). Notably, the most predictive of these five biomarkers was HRG. These findings were translated into the clinical development of seribantumab, where measurement of all five biomarkers was prespecified and then analyzed retrospectively in three randomized Phase 2 clinical trials in metastatic cancer: one in hormone receptor-positive, HER2-negative breast cancer (with exemestane) (Higgins et al. 2014), one in NSCLC (with erlotinib) (Sequist et al. 2014), and one in platinum-resistant/platinum-refractory ovarian cancer (with paclitaxel) (Liu et al. 2014). Quantitative fluorescence-based immunohistochemistry (qIHC) was used to visualize and quantify the protein levels of the three receptors, RNA in situ hybridization (RNA-ISH) was used to visualize and quantify the mRNA levels of the two ligands, and RT-qPCR was used to measure the mRNA levels of all five biomarkers in homogenized tissue (Macbeath et al. 2014). Interestingly, all three trials gave similar results: the most predictive biomarker in all three settings was HRG mRNA. First, control arm patients with high levels of HRG mRNA progressed more rapidly on standard therapy than patients with low HRG mRNA, suggesting that tumors in which HRG is expressed respond poorly to therapy. Second, the subpopulation of patients in each trial with high HRG mRNA, which ranged from 38% to 54% depending on the indication, exhibited increased PFS on the experimental arm (seribantumab plus standard therapy) relative to the control arm (standard therapy alone). Importantly, no significant increase in PFS was observed in the unselected patient populations in all three trials, emphasizing the need for biomarker-based patient selection. Based on these results, seribantumab is currently being evaluated in combination with chemotherapy in a randomized Phase 2 study in NSCLC in which patients are prospectively selected based on HRG expression (NCT02387216).

A second observation from these trials is that seribantumab benefit was localized to patients with relatively low levels of HER2 (<126,000 receptors per cell). This underscores the different biology encountered in the HER2-high versus HER2-low setting and the need for creative approaches targeting the HER2/HER3 heterodimer. To this end, pertuzumab (Perjeta; Genentech) is a HER2-directed antibody that blocks HER2/HER3 dimerization and is currently approved for the treatment of HER2-positive breast cancer in combination with trastuzumab and docetaxel (Traynor 2012).

Interestingly, the biomarker findings in the seribantumab Phase 2 trials are remarkably consistent with those from the HERALD study of patritumab. In the Phase 1b/2 HERALD study, patritumab in combination with erlotinib was compared with placebo plus erlotinib in EGFR inhibitor-naïve advanced NSCLC. In this trial, RT-qPCR was used to measure the levels of HRG mRNA in tumors (Mendell et al. 2015). As in the seribantumab NSCLC study, no significant benefit was observed in the unselected patient population, but the subpopulation with above-median levels of HRG mRNA showed extended PFS in the experimental arm relative to the control arm. HRG mRNA was also found to correlate with rapid progression on the control arm, again suggesting that ErbB3 signaling mediates insensitivity to therapy – in this case anti-EGFR therapy. Based on these results, a Phase 3 study has now been initiated (HER3-Lung, NCT02134015), comparing

patritumab plus erlotinib with placebo plus erlotinib in EGFR wild-type, locally advanced, or metastatic NSCLC. In Part A of this study, patients will be enrolled regardless of HRG mRNA levels and a predefined analysis used to set a cut point for patient selection. Then, in Part B of this study, only patients with HRG mRNA levels above this cut point will be enrolled.

The biomarker findings detailed above are further supported by several epidemiological studies. For example, Shames and colleagues analyzed HRG and ErbB3 mRNA levels in more than 750 tumors of diverse origin, including over 150 primary and recurrent HNSCC tissue samples (Shames et al. 2013). They found that high HRG expression is associated with activation of ErbB3 in HNSCC (as assessed by ErbB3 phosphorylation) and that HRG expression is significantly higher in recurrent HNSCC specimens compared to patient-matched and unmatched therapy-naïve specimens. These findings suggest that HRG expression may be both predictive of response to ErbB3 inhibitors and prognostic for recurrence of HNSCC. Similarly, Qian and colleagues assessed the prognostic value of HRG mRNA and ErbB receptor protein levels in 96 patients with oropharyngeal squamous cell carcinoma (OPSCC) (Qian et al. 2015). Consistent with previous findings, HRG mRNA and ErbB3 protein levels were found to independently correlate with poor overall survival (OS), with the stronger effect coming from HRG.

In summary, HRG mRNA has now emerged as the leading biomarker for predicting benefit from anti-ErbB3 therapies. The fact that four independent studies in three types of cancer run by two different companies find HRG to be a potential biomarker for benefit from ErbB3 inhibition suggests that HRG-mediated activation of ErbB3 may be a broad phenomenon and that ErbB3 inhibition may be part of a general strategy to restore sensitivity to anticancer agents.

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IGF 1 and IGF 2

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Douglas Yee

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Abstract

Insulin-like growth factor-1 (IGF-1) and IGF-2 are circulating peptide growth factor hormones important in normal growth and development. Their biological effects are mediated by specific cell surface receptors, the type I IGF receptor (IGF1R) and the insulin receptor. In cancer, preclinical and epidemiological evidence support a role for these growth factors in regulating cancer risk and tumor biology. Thus, neutralization of these growth factors could play a role in cancer prevention and therapy.

Keywords

Growth Factor • Insulin-like growth factors

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Target: Insulin-Like Growth Factor I and II (IGF-I and IGF-II)

IGF-I and IGF-II are polypeptide hormones important in normal growth and development (Baker et al. 1993). As their name implies, they are homologous in structure to insulin (Bell et al. 1984; Jansen et al. 1983). Like insulin, both hormones interact with specific heterotetrameric transmembrane tyrosine kinase receptors. IGF-I has the highest affinity for the type I IGF receptor (IGF1R). IGF-II can activate this receptor but also has high affinity for the fetal form of the insulin receptor (insulin receptor A, IRA) (Frasca et al. 1999).

Biology of the Target

IGF-I expression is increased during puberty by action of growth hormone on the liver (Mauras 2001). Once produced, IGF-I circulates bound to a high-affinity binding protein (IGF binding protein-3) and is further stabilized by the "acid labile subunit" in a ternary complex (Martin and Baxter 2011; Domene et al. 2009). High levels of serum IGF-I are found in adult life, but most of it is bound in the ternary complex and not available for receptor binding. During periods of stress, IGF binding protein-specific protease can cleave the binding protein and release IGF-I to interact with receptors. Thus, adults have a substantial reservoir of IGF-I in the circulation. IGF-I can also be found in the bone matrix in complex with other IGF binding proteins (Govoni et al. 2005).

IGF-II has an important function in fetal growth. In mice, IGF-II is necessary for normal fetal development. In rodents, IGF-II levels drop shortly after birth. However, humans maintain high levels of IGF-II during adult life and its function is not understood.

IGF1R activation requires binding by either IGF-I or IGF-II to signal. Thus, ligand production and receptor binding are necessary for activation of this receptor system.

Target Assessment

IGF-I and IGF-II can be measured by commercially available ELISA assays (Frystyk et al. 2010). "Free" IGF-I (unbound to IGF binding proteins) has been reported to predict outcome in some clinical trials of anti-IGF1R drugs. This assay is not commercially available.

Role of the Target in Cancer

Rank: 5

High-Level Overview

Diagnostic, Prognostic, and Predictive

The level of IGF-I and insulin is associated with increased cancer risk (Vigneri et al. 2009; Renehan et al. 2004). In preliminary reports, levels of free IGF-I are associated with benefit from IGF1R inhibition by the monoclonal antibody figitumumab in non-small cell lung cancer trials (Gualberto et al. 2009).

Therapeutics

Since hepatic IGF-I is under the control of growth hormone, disruption of growth hormone action could be a cancer therapy. This possibility is supported by the clinical observation that humans with mutation in growth hormone receptor are unable to respond to growth hormone, have low levels of IGF-I and IGF-II, and rarely, if ever, get cancer. Strategies to disrupt growth hormone releasing hormone or growth hormone receptor have been described (Schally et al. 2008; Divisova et al. 2006). A polyethylene glycol-conjugated mutant of growth hormone is available for treatment of growth hormone access (acromegaly). This drug, pegvisomant, has been evaluated in normal subjects (Yin et al. 2007). However, its development in cancer was discontinued by the manufacturer.

Monoclonal antibodies that bind both IGF-I and IGF-II have been described (Dransfield et al. 2010; Goya et al. 2004). A ligand-binding antibody is currently in phase I clinical trial (NCT00816361).

Preclinical Summary

Substantial preclinical data in cell culture and mouse model systems have shown that IGF-I is a potent mitogen for cancer cells. Expression of IGF-I can accelerate tumorigenesis in mouse model systems (Kleinberg et al. 2008). IGF-II is an imprinted gene. Loss of imprinting allowing biallelic expression has been linked to cancer development and progression (Hu et al. 2011; Kaneda et al. 2007).

Clinical Summary

Humans who lack the ability to produce IGF-I do not get cancer (Steuerman et al. 2011; Guevara-Aguirre et al. 2011). Studies examining lowering of IGF-I and IGF-II levels by neutralizing antibodies have not yet been reported.

Anticipated High Impact Results

Publication of phase I and II results from IGF-I and IGF-II neutralizing antibody clinical trials.

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Jak2/Stat5a/b Pathway in Prostate Cancer

Marjha Nevalainen and Shilpa Gupta

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Abstract

Stat5a and Stat5b are two highly homologous proteins (94-kDa for Stat5a and 92-kDa for Stat5b), and their genes map to the human chromosome 17 (bands q11-1 to q22) (Lin et al., J Biol Chem 271:10738–10744, 1996). Besides Stat5a and Stat5b, there are five other members in the Stat family of transcription factors (Stat1, Stat2, Stat3, Stat4, and Stat6) which mediate diverse biological processes, including cell growth, differentiation, and survival. Stat5a was first discovered as a mammary gland factor (MGF) which mediates the effects of prolactin (Prl) in mice (Wakao et al., J Biol Chem 267:16365–16370, 1992). The Stat5b was encoded by a separate gene and was identified later in mouse mammary gland

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Keywords
MgcRacGAP • Signal transducer and activator of transcription 5a and 5b (Stat5a/b) • Androgen receptor • Functional domains in protein structure • High vs. low grade • Homo-/heterodimers • In prostate cancer • Jak2/Stat5a/b signaling pathway activation • Janus kinases • Mammary gland factor (MGF) • MgcRacGAP • Preclinical studies • Prognostic marker • Therapeutics • Tyrosine residues Y694 and Y699

Stat5a and Stat5b are two highly homologous proteins (94-kDa for Stat5a and 92-kDa for Stat5b), and their genes map to the human chromosome 17 (bands q11-1 to q22) (Lin et al. 1996). Besides Stat5a and Stat5b, there are five other members in the Stat family of transcription factors (Stat1, Stat2, Stat3, Stat4, and Stat6) which mediate diverse biological processes, including cell growth, differentiation, and survival. Stat5a was first discovered as a mammary gland factor (MGF) which mediates the effects of prolactin (Prl) in mice (Wakao et al. 1992). The Stat5b was encoded by a separate gene and was identified later in mouse mammary gland (Lin et al. 1996; Liu et al. 1995). The major difference in the sequences between Stat5a and Stat5b reside in their C-termini, where there are 20 amino acids unique to Stat5a and 8 amino acids specific to Stat5b. Stat5a/b, like other members in the Stat family, shares six functional domains in protein structure: the N-terminal domain (NTD, aa 1–126), coiled-coil domain (CCD, aa 138–330), central DNA-binding domain (DBD, aa 332–583), linker domain (LD, aa 475–592), Src homology 2 domain (SH2, aa 593–670), and transcriptional activation domain (TAD, Stat5a, aa 722–794; Stat5b, aa 727–787) in the C-terminus (Schindler and Darnell 1995). Stat5a/b exists in the cytoplasm in a latent, transcriptionally inactive form. Stat5a/b is activated by phosphorylation of the specific tyrosine residue in the C-terminus (Y694 for Stat5a and Y699 for Stat5b) (Liu et al. 1995). Phosphorylated Stat5a/b forms homo- or heterodimers, translocates into nucleus, binds to the Stat response element of DNA, and regulates gene transcription. Stat5 proteins can be activated in response to a wide variety of cytokines and growth factors, such as prolactin (Prl) (Gouilleux et al. 1994), interleukin-2 (IL-2), IL-3, IL-5, granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-7, IL-9, IL-15, erythropoietin (EPO), thrombopoietin (TPO), epidermal growth factor (EGF), growth hormone (GH), insulin, and platelet-derived growth factor (PDGF).

Biology of Stat5a/b

Stat5a/b are cytoplasmic transcription factors, and their activation require phosphorylation of specific tyrosine residues typically by receptor-associated cytoplasmic Janus kinases (Jaks) (Hennighausen and Robinson 2008). The Jak family consists of four members (Jak1, Jak2, Jak3, and Tyk2), among which Jak2 is the primary kinase to activate Stat5a/b (Gouilleux et al. 1994). While Jak3 is preferentially expressed in

hematopoietic tissues and lymphocyte precursor cells, the other three Jak family members (Jak1, Jak2, and Tyk2) show relatively ubiquitous expression in mammalian cells.

This review mainly focuses on the Prl/PrlR/Jak2/Stat5a/b pathway in prostate cancer. Human Prl is not only a pituitary-secreted hormone but also expressed locally in both breast and prostate cancer (Tan and Nevalainen 2008). Prl belongs to the cytokine family whose members utilize single-transmembrane domain receptors (PrlR) with associated tyrosine kinases. Both Prl and PrlR are expressed in prostate epithelial cells (Nevalainen et al. 1997). Prl binding brings two PrlR together and initiates a change in the conformation of the signal transducing portions of the PrlR. This change results in a closer approximation of the receptor-associated Jak2 proteins, allowing Jak2 self-phosphorylation and subsequent phosphorylation of specific tyrosine residues in the PrlR. Stat5a/b is recruited to the PrlR by the interaction between the SH2 domain of Stat5a/b and the phosphorylated tyrosine residues of the receptor, followed by rapid phosphorylation of a conserved tyrosine residue in the C-terminus of Stat5a/b by Jak2. The phosphorylation of tyrosine residues Y694 and Y699 activates Stat5a and Stat5b, respectively, which leads to their homo- or heterodimerization through a phosphotyrosine peptide–SH2 domain interaction (Chen et al. 1998). Active Stat5a/b dimers translocate from the cytoplasm into the nucleus, where they bind to the consensus DNA sequence containing the motif TTCNNNGAA to regulate transcription of target genes (Horvath et al. 1995). The glycine residue at position 433 in Stat5b and a glutamic residue at a similar position in Stat5a confer distinct DNA-binding specificities (Boucheron et al. 1998). Moreover, their cell type-specific expression and/or the interactions of their divergent C-terminus with different co-regulators may contribute to the nonredundant functions of Stat5a and Stat5b. The phosphorylation of Stat5 on serine residues by other protein kinases may provide additional signaling pathways to regulate the primary activating stimulus (Yamashita et al. 1998, 2001; Decker and Kovarik 2000). The exact molecular mechanisms underlying the nuclear import or export of Stat5a/b remain largely unclear. In the absence of cytokine activation, nuclear and cytoplasmic pools of unphosphorylated Stat5a/b proteins may traffic freely at high exchange rates. While non-phosphorylated Stat5a/b proteins shuttle between the cytoplasm and nucleus, the nuclear translocation of Stat5a/b dimers has been proposed to be an energy-dependent active process, and the coiled-coil domain contributes to the nuclear transport of latent and activated Stat5a. MgcRacGAP, a chaperone protein, may form a shuttling complex by binding to phosphorylated Stat5a/b dimer and help the transport between the nucleus and cytoplasm (Kawashima et al. 2009).

Target Assessment

Role of Stat5a/b in Prostate Cancer

Rank: 8

Stat5a/b is involved in prostate cancer growth and progression. Stat5a/b is active in human prostate cancer cells but not in adjacent normal prostate acini

(Ahonen et al. 2003). Stat5a/b mediates the effects of Prl, which is a powerful mitogen and survival factor for prostate epithelium (Nevalainen et al. 1993, 1997; Li et al. 2004). Stat5a/b critically regulates viability of human prostate cancer cells in vitro (Ahonen et al. 2003; Dagvadorj et al. 2007, 2008; Gu et al. 2010a). Inhibition of Stat5a/b by various methodological approaches causes rapid apoptosis of human prostate cancer cells (Dagvadorj et al. 2007, 2008). Specifically, Stat5a/b inhibition by antisense oligonucleotides or siRNA induces rapid and massive apoptotic cell death, and adenoviral expression of DNStat5a/b inhibits clonogenic survival of LNCaP, CWR22Rv, and DU145 cell lines (Dagvadorj et al. 2008). Stat5a/b had a preferential role over Stat3 in promoting prostate cancer cell viability and tumor growth in vitro and in vivo, while the effect of Stat3 inhibition were only minor (Gu et al. 2010b). Importantly, inhibition of Stat5a/b blocked both incidence and growth of subcutaneous and orthotopic human prostate xenograft tumors in nude mice (Dagvadorj et al. 2008; Gu et al. 2010b). Stat5a/b promotion of prostate cancer cell viability involves regulation of Bcl-X_L and cyclin D1 expression (Dagvadorj et al. 2008) among other genes. In addition, Stat5a/b inhibition also induced the expression of Kruppel-like factor 4, which is known to repress cyclin D1 and mediate growth suppression of various cell types.

High-Level Overview

Active nuclear Stat5a/b expression in prostate cancer is associated with a loss of differentiation. Stat5a/b is significantly more frequently active and nuclear in high-grade human prostate cancers as compared to intermediate- or low-grade prostate cancers (Tan and Nevalainen 2008; Li et al. 2004, 2005). Stat5a/b activation in primary prostate cancer predicted early disease recurrence and shorter progression-free survival after radical prostatectomy (Li et al. 2005). Importantly, in intermediate Gleason grade prostate cancers, active Stat5a/b is as well an independent prognostic marker of early disease recurrence and was associated with progressive disease (Li et al. 2005). In addition, Stat5a/b was more frequently active and nuclear in primary prostate cancers treated with hormone therapy compared to those who did not receive androgen deprivation (Tan et al. 2008). Moreover, Stat5a/b was active in 95% of castration-resistant clinical human prostate cancers. Active Stat5a/b signaling pathway increased transcriptional activity of androgen receptor and vice versa, thus suggesting that Stat5a/b may contribute to the development of castration-resistant prostate cancer (Tan et al. 2008). Active Stat5a/b was shown to induce metastatic progression of human prostate cancer cells by 11-fold in in vivo prostate cancer experimental metastases assay (Gu et al. 2010a), suggesting that it could serve as a potential therapeutic protein in disseminated prostate cancer. Stat5a/b induced migration and invasion of prostate cancer cells and heterotypic adhesion to endothelial cells and suppressed cell surface E-cadherin expression.

Diagnostic, Prognostic, and Predictive

As discussed above, active Stat5a/b is found more frequently in high-grade human prostate cancers (Tan and Nevalainen 2008; Li et al. 2004, 2005) and predicts early disease recurrence and shorter progression-free survival after radical prostatectomy (Li et al. 2005). Even in intermediate Gleason grade prostate cancers, active Stat5a/b is an independent prognostic marker of early disease recurrence and progression (Li et al. 2005). Stat5a/b may contribute to the development of castration-resistant prostate cancers and is active in majority of such cancers (Tan et al. 2008). Active Stat5a/b also induced metastatic behavior of human prostate cancer cells in vitro and in vivo (Gu et al. 2010a). In addition to being a prognostic marker, active Stat5a/b may serve as a predictive marker to select patients whose prostate cancer is more likely to progress earlier and offer individualized treatment to such patients.

Therapeutics

The Jak2/Stat5a/b signaling pathway provides various molecular targets at different levels for developing rational targeted therapeutics for prostate cancer. This pathway can be inhibited through several approaches leading to inhibition of Stat5a/b: First, PrlR activation can be blocked by PrlR antagonists, such as S179D-hPrl (Xu et al. 2001) and the specific human Prl antagonist Del1-9G129R-hPrl (Dagvadorj et al. 2007; Llovera et al. 2000). Second, the Jak2 kinase can be directly targeted by various small-molecule inhibitors which are currently in active development for myeloproliferative disorders, leukemias, and solid tumors (Pardanani 2008). These Jak2 inhibitors can potentially be utilized to block the Jak2/Stat5a/b pathway in those prostate cancers in which Jak2 is responsible for Stat5a/b activation. AZD1480 is one such small-molecule Jak2 inhibitor with promising preclinical activity (Hedvat et al. 2009). Third, direct blocking of the SH2 domain of Stat5a/b could potentially result in the specific inhibition of both Stat5a/b dimerization and recruitment to an activated receptor (such as PrlR) for its activation (Liao et al. 2010).

Preclinical Summary

Stat5a/b is critical for prostate cancer cell survival and growth, and there is preclinical data suggesting the role of the Jak2/Stat5 signaling pathway in the progression of organ-confined prostate cancer to castration-resistant and/or disseminated disease (Ahonen et al. 2003; Dagvadorj et al. 2007, 2008; Kazansky et al. 2003).

Clinical Summary

The Jak2/Stat5a/b signaling pathway activation in clinical prostate cancer is associated with high-grade prostate cancer. Active Stat5a/b in primary prostate tumors predicts early disease recurrence and progression in both high-grade as well as intermediate-grade prostate cancers. Nuclear active Stat5a/b expression is increased in castration-resistant and disseminated prostate cancers. Various inhibitors of the Jak2/Stat5a/b signaling pathway are in preclinical testing including small-molecule Jak2 and Stat5a/b inhibitors, and phase I trials are underway to evaluate the efficacy in clinical prostate cancers.

Anticipated High-Impact Results

Currently, there are no effective pharmacological therapies for castration-resistant prostate cancer, and there is an urgent need for better pharmacological therapies. One such potential therapeutic target is the Jak2/Stat5a/b signaling pathway. In addition, Stat5a/b may also serve as a predictive marker to identify patients whose prostate cancer is likely to respond to Stat5-inhibition therapy. Nuclear Stat5a/b may also serve as a prognostic factor for identification of prostate cancers that are likely to progress aggressively to metastatic disease. In conclusion, the Jak2-Stat5a/b signaling pathway may provide critical tools for the development of personalized medicine for prostate cancer patients.

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66 JNK Signaling in Diseases

Francois X. Claret and Terry Shackleford

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Abstract

The c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases are intracellular protein kinases that play a key central role in the transduction of extracellular signals to potentiate cellular responses. JNKs are tightly regulated and are essential for regulating many physiological processes, including cell proliferation, differentiation, proliferation, death, and survival and inflammation. Thus, JNK disregulation contributes to the development of several different diseases, such as type 2 diabetes, obesity, inflammation, neurodegenerative disorders, and cancer. The present review summarizes the recent findings regarding the distinct

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roles of the three JNK members: JNK1, JNK2, and JNK3. JNK1 and JNK2 are ubiquitously expressed and have both redundant and opposing functions. The mounting evidence for the role of JNK activation in the development of cancer and other diseases has spurred interest in JNK inhibitors as a therapeutic approach for diseases. A strong understanding of the tissue-specific roles of the three JNK members, in combination with therapeutics that have high on-target specificity, will be the key to the successful therapeutic inhibition of JNK. In this chapter, we summarize the evidence that demonstrates the importance of JNK in the development of cancer and other diseases and review the current advances and challenges in the development of JNK inhibitors.

Keywords
c-Jun • JNK • SAPK • MAPK • SP600125

Introduction

The mitogen-activated protein kinase (MAPK) signaling pathway is an evolutionary conserved, intracellular signaling pathway that responds to various extracellular stimuli and is essential in regulating fundamental cellular processes including cell growth, cell differentiation, cell proliferation, inflammation, apoptosis, survival, and stress response. There are four major groups of MAPK signaling modules: the p38 Map kinase family (α/β/γ/δ), the extracellular signal-regulated kinase (Erk) family (Erk1/Erk2), Erk5, and the Jun N-terminal kinase (JNK) family (JNK1–3). These cascades are very tightly regulated, and their dysregulation often cause diseases, such as cancer, diabetes, inflammation, as well as developmental and neurological disorders. JNK proteins, also known as stress-activated protein kinases (SAPKs), share a threonine-proline-tyrosine (TPY) motif within their activation loop. They are recognized as key regulators of many cellular events, including cell proliferation, differentiation, and cell death (Chang and Karin 2001; Davis 2000).

JNKs are serine/threonine protein kinases that can be activated by a variety of stimuli, including environmental stress (such as ultraviolet or ionizing radiation and osmotic shock), inflammatory cytokines, and growth factors. There are three *JNKs*: *JNK1* and *JNK2* are ubiquitously expressed, while *JNK3* is expressed primarily in the brain, heart, and the testis (Kyriakis et al. 1994; Yang et al. 1997). Due to their differential expression distribution, it is thought that *JNK3* controls different functions than *JNK1* and *JNK2*, which may have redundant functions. Each *JNK* gene is alternatively spliced, and there are at least 10 different isoforms of JNK mRNAs overall (four JNK1, four JNK2, and two JNK3 splice variants) (Gupta et al. 1996). These isoforms further increase the diversity of JNK proteins. *JNK1* is a major *JNK* that is significantly activated by most known JNK inducers and is responsible for most known JNK functions, such as stimulation of expression and activation of the nuclear oncogene c-Jun which is involve in regulation of cell death, inflammation, and tumorigenesis (Sabapathy et al. 2004). JNK2 has also been implicated in tumorigenesis through the activation of oncogenic signaling. Further, mice who do

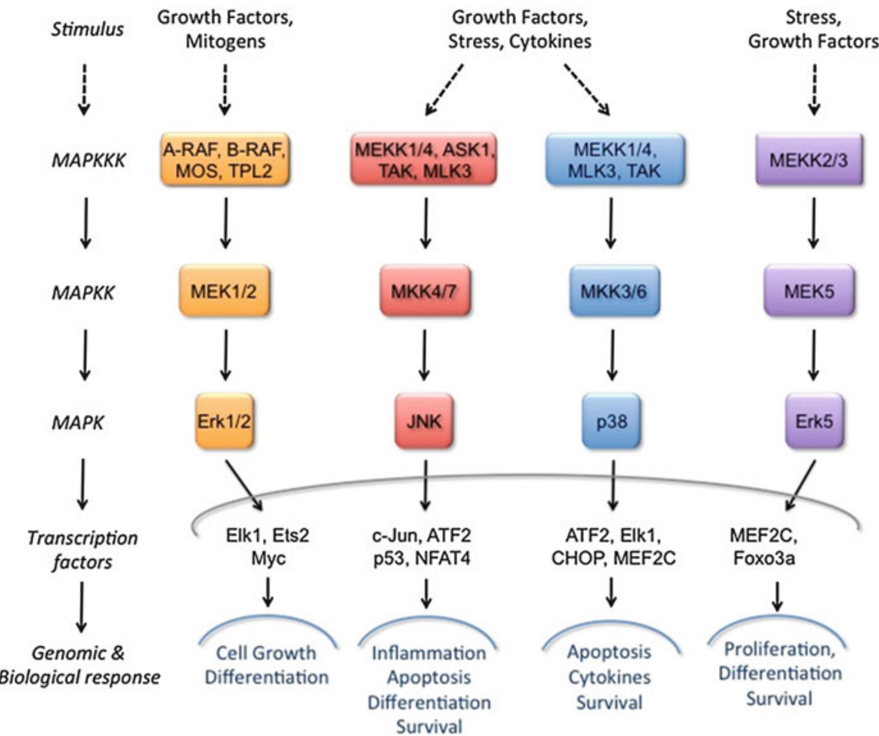

Fig. 1 Mammalian MAP kinase signaling pathways

not express JNK2 were less likely to develop skin papillomas following treatment with a tumor promoting agent, suggesting the importance of JNK2 in the tumor promotion process (Chang and Karin 2001).

JNK activation is carried out by sequential protein phosphorylation via the MAPK cascade, i.e., MAP3K → MAP2K → MAPK (Fig. 1). JNKs are activated by upstream MKK4 (JNKK1) and MKK7 (JNKK2) kinases. JNK activation by extracellular stimuli, such as stress or cytokines, leads to phosphorylation of several transcription factors and cellular substrates – including c-Jun transcription factor (Hibi et al. 1993; Derijard et al. 1994) – that are implicated in cell survival and proliferation, insulin receptor signaling, and mRNA stabilization (Karin and Gallagher 2005; Weston and Davis 2007; Minden and Karin 1997; Liu and Lin 2005). Activation of the JNK pathway can result in sustained cell proliferation and survival in response to cytokine stimulation. JNK pathway has also been shown to mediate cell death through induction of apoptosis in response to cellular stress. The functions of JNKs are complex and can exert antagonist effects on cell proliferation and survival which depend on cell-type-specific difference, type of extracellular stimuli, duration of activation, and cross-talk between other signaling pathways (e.g., p38 MAPK). It has been postulated that cancer cells modulate JNK signaling

to stimulate proliferation, survival, and invasion, whereas JNK signaling in other types of cells does not.

JNK pathway has been shown to be upregulated in the pathogenesis of several diseases, including: type-2 diabetes, obesity, cancer (including head and neck, gastric, retinoblastoma, breast, ovarian and colorectal cancers, and melanoma), atherosclerosis, stroke, Alzheimer's disease, and Parkinson's disease. Studies from knockout mouse models indicate that loss of *JNK* function improve the overall disease, suggesting a causal role of JNK in disease pathology. Thus, JNKs represent valuable targets in the development of novel therapies (Manning and Davis 2003).

The JNKs are essential mediators of cell stress responses following their implication as regulators of proapoptotic death signaling events. This link between JNK activation and cell death, as revealed by studies using JNK gene knockout and/or inhibitors, has contributed to the development of programs to identify JNK inhibitors that prevent cell death, particularly the neuronal death that can underlie both acute and chronic neurodegenerative diseases and the death of pancreatic β-cells that exacerbates the poor control over circulating glucose levels in type-2 diabetes. While a focus has been on ATP-competitive JNK inhibitors, a cell-permeable JNK inhibitory peptide has shown in vivo efficacy. Therefore, JNK inhibitors, whether they are ATP competitive or ATP noncompetitive in their actions, may become novel therapeutic approaches for diseases such as stroke, chronic neurodegeneration, diabetes, and malignant diseases.

Biology of the Target

JNKs have been studied in many solid cancers and are potential targets for therapy (Manning and Davis 2003). Several studies point to the significant role of JNK activation in the development of cancer. JNK activation was found to be required for the cellular transformation and tumorigenesis induced by the well-known oncogene Ras (Dhillon et al. 2007; Wagner and Nebreda 2009). JNK1 activation has been described in a number of tumor cell lines and cancers, and increased phosphorylation of JNK1 has been reported to correlate with increased proliferation in a number of tumor types, including non-small cell lung cancer, hepatocellular carcinoma, prostate cancer, ovarian cancer, and papillary thyroid carcinoma (Wagner and Nebreda 2009; Hui et al. 2008). Similarly, activation of JNK2 was detected in a large number of human squamous cell carcinomas, and inhibition of JNK2 impaired tumorigenesis of these cancer cells (Sabapathy et al. 2004; Hochedlinger et al. 2002). The remaining family member, JNK3, is only expressed in the brain, heart, and testes. Loss-of-function mutations of JNK3 were detected in brain tumors, where it is thought to act as a tumor suppressor gene. The JNK/c-Jun pathway was found to act as a negative regulator of the p53 tumor suppressor gene and supports the oncogenic role of activated JNK1 in tumor models (Das et al. 2007). Further, AKT-mediated activation of JNK due to loss of the well-known tumor suppressor gene PTEN was demonstrated in prostate cancer specimens from patients and in prostate cancer cell lines (Vivanco et al. 2007). Because PTEN is the second most

commonly mutated tumor suppressor gene, it is clear that changes in signaling as a result of PTEN loss, such as activation of JNK, play an important role in the development of cancer. Also, the leukemogenic oncogene BCR-ABL can lead to activation of JNK1, and JNK signaling through c-Jun contributes to the development of proliferative diseases (Hess et al. 2002).

JNKs phosphorylate their cognate and noncognate substrates, which include c-Jun, JunD, ATF2, c-Fos, p53, c-Myc, p53, FoxO4, STATs, IRS-1, Itch, 14-3-3, histone H3, and other proteins. However, the evidence also implies that JNK1, rather than JNK2 or JNK3, is the key JNK family kinase responsible for the phosphorylation of c-Jun on serines 63 and 73 in response to UV irradiation and other stress stimuli (Kyriakis et al. 1994; Derijard et al. 1994; Smeal et al. 1991). In myoblast cells, JNK1, but not JNK2, mediates TNFα-induced cell proliferation by inhibiting myoblast cell differentiation and promoting the generation of inflammatory cytokines such as interleukin-6 (IL-6) and leukemia inhibitory factor. In addition, the importance of JNK1 over JNK2 was shown in the pathogenesis of several human diseases, including diabetes, lung fibrosis, and cancer (Liu 2007). Furthermore, gene knockout studies in mice revealed that JNK1 is the most important JNK family kinase for the proliferation of CD8+ T cells and for neural development.

JNK is often dysregulated in cancer; however, JNK signaling can have different effects and can act as a tumor suppressor or promoter in different tissues. Because several findings highlight the role of JNK in the development of cancer, JNK has been proposed to be an important therapeutic target for some tumor types. In fact, inhibition of JNK results in reduced oncogenic transformation in some tumor cell types.

Target Assessment

JNK phosphorylation status, which correlates with activation, and JNK level can be assessed by immunohistochemical staining of tissue specimens and by ELISA methods. The feasibility of this staining has previously been demonstrated and supported by Western blot analysis. Evaluation of JNK or activated JNK is not yet routine in clinical practice; however, the assay itself is a gold standard for the measurement of proteins and phosphorylated proteins for a number of other targets.

High-Level Overview

The stress-activated protein kinases, JNKs, are members of the MAPK group of signaling proteins whose kinase activity mediates activation of downstream signaling affecting various cellular processes including cell proliferation, survival, apoptosis, and differentiation. Aberrant activation of JNKs can lead to the progression of cancer through these signaling mechanisms which make it an attractive therapeutic target. Investigation into the exact functions of the various JNK isoforms has been focused on determining the role each plays in the progression of cancer. To date,

there is still conflicting data regarding whether a certain isoform contributes or inhibits cancer progression and is also apparent that these proteins have varying functions depending on the cell type in which it is expressed. Therefore, careful consideration to isoform specificity is necessary for the development and testing of inhibitors of JNK to alleviate the possibility of undesired side effects. However, there remains great promise that JNK inhibition can indeed be an effective therapeutic target, and investigations of new agents are currently being tested.

Several studies suggest that JNK1 plays a critical role in the malignant transformation and in tumorigenesis. Recent finding suggest that *JNK1* deficiency in mice decreased their susceptibility to a BCR-ABL-induced lymphoma. Also, in UV-induced tumorigenesis, activation of JNK1 is essential for cell transformation and proliferation in response to the oncogenic Ras signal. In rhabdomyosarcoma, the most common soft tissue sarcoma of childhood, JNK1 silencing, but not of JNK2, suppressed the growth of these tumor cells, thus indicating JNK1 function as proproliferative, whereas JNK2 might be proapoptotic.

Similarly, JNK1 activation had also been demonstrated as a key factor for the chemical carcinogen-induced hepatocellular carcinoma (HCC). HCC is the third leading cause of cancer death worldwide. JNK1 deficiency (but not JNK2 deficiency) has been shown to significantly decrease susceptibility to diethylnitrosamine (DEN)-induced HCC formation (Hui et al. 2008; Sakurai et al. 2006). Animal models of gastric cancer also demonstrated that JNK1 contributes to the development of gastric tumors induced by the chemical carcinogen *N*-methyl-*N*-nitrosourea. Conclusive studies showing importance of JNK activation in the initiation and progression of human HCC were demonstrated only recently by two independent groups using human HCC tissue samples who reported that JNK1, rather than JNK2, is overactivated in more than 50% of the human HCC samples (Hui et al. 2008). Impaired cell proliferation and tumor formation following JNK1 knockdown are causes of reduced expression of MYC oncogene- and cyclin-dependent kinase inhibitor, p21. Several studies further revealed that greater JNK1 activation was associated both with a poorer prognosis in patients and with overexpression of several hepatic stem cell or progenitor cell markers. HCC mouse models with genetic disruption of *JNK1* locus significantly reduced the number and size of HCCs that were induced by DEN. JNK1 is an important determinant for HCC development in the presence of a hepatocyte-specific deficiency of IKKβ or IKKγ, which are the key subunits of the IKK kinase complex for NF-κB signaling in mice.

Diagnostic, Prognostic, and Predictive

Obesity has become an increasing health problem in the United States and around the world and increases the risk of a number of diseases including cancer in several organ sites (Bianchini et al. 2002). Obesity can cause chronic inflammation that results in increased cytokine production, including tumor necrosis factor alpha

(TNF-a) in response to the associated stress. Increased activation of JNK has been observed in obesity models (Hirosumi et al. 2002). Obesity also results in increased factors secreted from adipocytes including the cytokine leptin, which controls body weight homeostasis by regulating food intake and energy expenditure. This increased secretion of leptin has been linked to increased cellular proliferation and activation of JNK in cancers such as colon cancer and androgen-independent prostate cancer (Onuma et al. 2003). Conversely, inhibition of JNK was sufficient to inhibit leptin stimulation of androgen-independent prostate cancer cell proliferation. These studies suggest that JNK is a key to obesity-related development of cancer and is a potentially important therapeutic target. Conversely, a mechanism whereby leptin-stimulated activation of JNK, STAT3, and Akt in androgen-independent cells provided a mechanism for obesity-mediated growth of cancer. The coactivation of JNK with STAT3 and Akt has been postulated to be predictive and diagnostic for androgen-independent progression of prostate cancer (Miyazaki et al. 2008).

Therapeutics

Activation of JNK has been detected in a number of cancer types including ovarian, prostate, glioma, osteosarcoma, and squamous cell carcinoma (Vivas-Mejia et al. 2010). Development of kinase inhibitors has proven successful in other cancer types. Development of therapeutics to target JNK that use both peptide inhibitors and small molecules is actively being pursued by a number of pharmaceutical companies and universities (Bogoyevitch et al. 2010). Molecules being studied include antisense oligonucleotides, inhibitory peptides, derived from the JNK-interacting protein (JIP) (e.g., BI-78D), and chemical inhibitors of JNK. SP600125 and AS601245 are competitive inhibitors of the ATP-binding site of the kinase and for this reason show only moderate specificity. Serono's (Geneva, Switzerland) lead JNK-inhibiting compound AS601245 has neuroprotective properties and also could protect neurites and preserve memory after cerebral ischemia in various experimental setting, after global ischemia. Celgene's (Summit, New Jersey, USA) JNK inhibitor SP600125 is widely employed in vitro and in vivo. Inhibition of JNKs by SP600125 in adult animals has been reported to decrease amyloid beta-peptide production in Alzheimer's disease and attenuate vasospasms in a model of experimental subarachnoid hemorrhage. Several small molecule and peptide inhibitors of JNK are under current clinical investigation. Celgene has completed a phase I study (myeloid leukemia) on their compound CC-401 and is performing preclinical studies on CC-359 (ischemia/reperfusion damage) and CC-930 (fibrotic diseases). Both compounds are derivates of SP600125, the first publicly available ATP-competitive small-molecule antagonist. Xigen Pharmaceuticals (Lausanne, Switzerland) currently is conducting a phase I study with a cell-penetrating JNK inhibitor peptide, XG-102, in elderly patients who have recently had a stroke. These JNK inhibitors

inhibit cell proliferation in many human cancer cells by blocking cell-cycle progression and inducing apoptosis. Additionally, downstream signaling molecules that are activated by the JNK pathway offer additional potential targets for therapy.

Careful consideration however needs to be followed with the use of JNK inhibitors. As kinases are often highly conserved, inhibitors developed for the JNK family members will have to specifically target the intended JNK isoform because targeting of the other family members could result in undesirable side effects or even possibly tumor promotion. Increased knowledge of JNK structural features has allowed for the development of JNK isoform-selective inhibitors such as was seen with the aminopyrazole inhibitor which shows selectivity of JNK3 over JNK1. Another challenge is ensuring that JNK inhibition does not also inhibit the other MAPK family members such as ERK and p38MAPKs. Also, these signaling pathways often have redundant signaling molecules, and the possibility of redundancy where one molecule could compensate for the loss of another needs to be realized. Therefore, detailed characterization of the mechanism of action of these inhibitors needs to be fully realized before they are subsequently tested in human trials.

Combinations of JNK inhibitors and other therapies are also of interest. Evidence of sensitization to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-induced cell-cycle arrest was observed in hepatocellular carcinoma cells. Overall, however, as the field of JNK inhibitors is advancing, it is anticipated that several targeted therapies with new drugs will be successfully applied and used in the clinic in the near future.

Preclinical Summary

Preclinical studies have provided strong evidence of the involvement of JNK signaling in tumorigenesis and have highlighted differences in JNK function in different tumor types. Studies in mouse models with knockout of different JNK proteins have demonstrated that JNK can have pro-oncogenic or antioncogenic functions depending on the cell type and stage in cancer development (Wagner and Nebreda 2009). The JNK1 knockout mouse had reduced incidence of liver and gastric cancer following induction but increased susceptibility to DMBA- and TPA-induced skin cancer. The JNK2 knockout mouse had reduced incidence of skin cancer following DMBA and TPA treatment; the incidence of liver cancer occurrence was not altered. The results from the JNK3 knockout mice did not provide conclusive evidence of an effect on tumorigenesis.

Pharmacologic inhibition of JNK1 by an inhibitory peptide reduced the incidence of HCC, and JNK1 was suggested to be a potential new therapeutic target for HCC (Chen et al. 2009). The inhibitor, SP600125 was effective at inhibiting head and neck squamous cell carcinoma in vitro, and the JNK1 isoform was found to be the driving factor for promoting these tumors (Gross et al. 2007). Treatment with a newly developed inhibitor of JNK1 and c-Kit, WBZ_4, had antitumor efficacy in vivo in an ovarian cancer model (Vivas-Mejia et al. 2010).

Clinical Summary

Because JNKs play a key role in the progression of cancer through control of proliferation, differentiation, survival, and migration, a number of JNK inhibitors have been investigated in the clinic. A new generation of JNK inhibitors promises improved efficacy with the possibility of fewer off-target effects, particularly if the targeting of protein substrate docking domains provides a higher degree of specificity than previously achieved with ATP-competitive inhibitors. Given the complexity of the JNK signaling pathway and the fact that the impact of JNK signaling depends on the specific JNK family member and the type of cell, the targeting molecule will need to be specific for a particular target and appropriate for the tumor type in question to avoid any unwanted side effects.

There are several JNK inhibitors in development for the treatment of cancer and other conditions, such as inflammatory diseases, Crohn's disease, and Alzheimer's disease. The inhibitors currently under development include peptide inhibitors and small molecules. While the inhibitor SP-600125 has shown promise in preliminary studies, it is not specific for a particular JNK family member and may have unwanted side effects in the clinic. The Celgene JNK inhibitor CC-401 has been studied in a completed phase I/II trial and patients with acute myelogenous leukemia. Celgene's next JNK inhibitor, CC-930, is in preclinical development, advancing toward clinical testing. While these studies are ongoing, it is anticipated that several therapies targeting JNK will soon be proposed for use in the clinic.

Anticipated High-Impact Results

Recently, JNKs have become a novel therapeutic target, and SP600125, an anthrapyrazolone inhibitor of JNK, has been used for the treatment of autoimmune and neurodegenerative diseases and ovarian cancer. With improved small molecule inhibitors of JNK being released commercially, we anticipate that the number of studies evaluating JNK function in both health and disease will continue to increase.

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K-Ras

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Abstract

Mutational activations of KRas is one of the most common oncogenic events in human cancers and a heavily pursued target in therapeutic development for decades. Mutant KRas protein engages a host of signaling cascades that culminate in uncontrolled cell proliferation, enhanced survival, and set the stage for acquisition of further genetic events that propel cancer cells towards more malignant phenotypes. Direct targeting of KRas protein with inhibitors that disrupt its maturation and proper trafficking has not been successful in clinic. Instead, much attention is now focused on targeting the effector cascades that mutant KRas utilizes to exert its oncogenic feats, which include the PI3K/AKT/mTOR and Raf/MEK/ERK cascades. Numerous inhibitors targeting these pathways have been developed and are being tested in clinic. However, durable

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clinical success will depend on identifying effective combinations with tolerable side effects and strategies to overcome resistance mechanisms.

Keywords
Epidermal growth factor receptors (EGFRs) • Farnesyl thiosalicylic acid • FOLFIRI • Kirsten rat sarcoma viral oncogene homolog (KRas) • Anti-EGFR monoclonal antibodies • Assessment • Cetuximab • Clinical trials • Effector • In human cancer • mFOLFOX6 • Mutations • Ras GTPase activity • SOS • Therapeutics • Tyrosine kinase inhibitor • Ras GTPase-activating proteins • Ras guanine nucleotide exchange factor (RasGEF) proteins • Salirasib • Tipifarnib • Vemurafenib

Target: KRas

The three human Ras genes (HRas, NRas, and KRas) encode four different proteins, HRas (Chapter 57), NRas Chapter 95), and two splice variants of KRas (***K*irsten *ra*t *s*arcoma viral oncogene homolog**), KRas4A and KRas4B (Barbacid 1987). All Ras proteins are about 21 kDa in size and expressed ubiquitously in all tissues. Despite high similarity in amino acid sequence, each Ras isoform possesses distinct cellular functions. Targeted deletion of KRas in mice resulted in embryonic lethality (Johnson et al. 1997; Koera et al. 1997), whereas deletion of NRas and HRas individually or in combination did not result in any detectable developmental or growth defect (Ise et al. 2000; Umanoff et al. 1995; Esteban et al. 2001). Nonetheless, a shared key function of the Ras proteins is that they are the universal signaling switch that relays extracellular growth signals into the cell.

Once synthesized, the Ras protein undergoes sequential posttranslational modifications at its carboxyl-terminus which eventually tether it to the cytoplasmic side of the cell membrane. At resting state, Ras is bound to guanosine diphosphate (GDP). Following mitogenic stimulation of transmembrane growth factor receptors such as the epidermal growth factor receptors (EGFRs) (Chapters 42 and 43), the cytoplasmic domain of these receptors recruits a series of signaling molecules which include the RasGEF (Ras guanine nucleotide exchange factor) proteins such as *S*on *O*f *S*evenless (SOS). When brought to the inner side of the plasma membrane where the Ras proteins reside, the RasGEFs are able to catalyze an exchange of Ras-bound GDP for GTP, causing GTP-bound Ras to assume an "active" conformation that is capable of recruiting and activating other molecules, termed the effectors. In normal cells, activation of Ras is quickly quenched when the bound GTP is hydrolyzed to GDP by the intrinsic Ras GTPase activity, a process that is also facilitated by RasGAPs (Ras GTPase-activating proteins). Such tightly regulated mechanism of Ras activation/inactivation is extremely important in determining the magnitude and duration of mitogenic signaling to achieve the appropriate physiologic outcome.

On the other hand, oncogenic mutations of Ras, which almost always result from missense mutations involving the codons 12,13, and 61, abolish its intrinsic GTPase

activity and catalytic action by RasGAP, thereby rendering the mutant Ras proteins permanently GTP bound and active. Such alteration results in sustained, stimuli-independent activation of normally transient effector signaling events, which, in collaboration with other deleterious genetic changes, collectively transform normal cells into tumorigenic state.

About one third of all human cancers harbor activating mutations of KRas. These include 85–90% of pancreatic adenocarcinoma, 35–40% of colorectal adenocarcinoma, and 25–30% of lung adenocarcinoma (Bos et al. 1987). Activating mutations of NRas (Chapter 95) are found in about 15% of all human cancer, with higher incidence in melanoma, thyroid carcinomas, and hematologic and lymphoid malignancies. Mutations of HRas (Chapter 57) are much less frequent and seen predominantly in uroepithelial carcinomas, follicular thyroid carcinoma, and sarcomas.

Biology of the Target

Upon ligand stimulation, multiple conserved tyrosine residues within the cytoplasmic domain of growth factor receptors become phosphorylated and serve as docking sites for recruitment of other signaling mediators including SOS, which is a RasGEF. Recruitment of SOS leads to GTP exchange and subsequently activation of adjacent KRas, which in turn engages and activates a suite of downstream effectors to regulate various cellular functions such as proliferation, survival, metabolism, cytoskeletal rearrangement, and secretion of cytokines. Among the best studied effector cascades include the Raf-MEK-ERK (Chapters 20, 47, and 88), PI3K-Akt-mTOR (Chapters 109, 4, and 93), and the RalGEF-Ral pathways (Cox and Der 2002). In KRas-mutated cancer cells, these effector pathways are constitutively engaged and activated, leading to uncontrolled cellular proliferation, increased survival, dysregulated energy metabolism, and other transformed phenotypes (Shields et al. 2000). In support of the central role of KRas in cellular signal transductions, mutations of KRas are mutually exclusive with those of EGFRs (Chapters 42 and 43) or downstream effectors such as B-Raf (Chapter 20) or PI3K/Akt (Chapters 109 and 4) kinases. Concordantly, patients with KRas-mutated colorectal cancer do not respond to anti-EGFR therapies.

Target Assessment

Mutational analysis of KRas status can be done using fresh, frozen, or formalin-fixed paraffin-embedded tumor samples by allele-specific PCR or direct Sanger sequencing methods. To ensure reliability, the testing should only be performed in laboratories that are certified under the clinical laboratory improvement amendments of 1988 (CLIA-88). In 2012, the US FDA approved the therascreen® KRAS RGQ PCR Kit as the first genetic screen for KRas mutations as part of the pretreatment workup to guide anti-EGFR therapy in patients with metastatic colorectal cancer.

Role of the Target in Cancer

Mutational activations of the Ras genes are one of the most common and heavily studied oncogenic event in human cancer. Longitudinal molecular analysis of precancerous and cancer samples in colorectal and pancreatic cancer shows that mutations of KRas are acquired and retained since early stage of tumorigenesis, supporting its role as a bona fide driver oncogene in human cancer. In parallel, decades of scientific studies using human cancer cell lines and transgenic mouse models established that mutant KRas are responsible for tumor initiation, maintenance, progression, as well as resistance to radiation and chemotherapy.

High-Level Overview

Diagnostic, Prognostic, Predictive

In colorectal cancer, the prognostic value of KRas mutations was largely controversial prior to the introduction of anti-EGFR monoclonal antibodies. By analyzing the tumor samples of 3,459 colorectal cancer patients, the RASCAL II (the Kirsten RAS in-colorectal-cancer collaborative group) study showed that of all different KRas mutations, only the one in which glycine at codon 12 is mutated to valine (KRas G12V) is associated with poorer overall survival ($P = 0.008$, HR 1.29) (Andreyev et al. 2001). However, most of other studies did not find KRas status to be predictive of response to standard chemotherapy in colorectal cancer (Loriot et al. 2009).

In 2009, a large randomized clinical trial (CRYSTAL) involving 1,198 patients with metastatic colorectal cancer showed that addition of cetuximab to FOLFIRI significantly improved progression-free survival (hazard ratio 0.68, 95% CI, 0.50–0.94) and overall response rate in patients with wild-type (hazard ratio 0.68, 95% CI, 0.50–0.94), but not mutant, KRas (Van Cutsem et al. 2009). However, cetuximab did not seem to significantly improve overall survival of patients with wild-type KRas. Improved progression-free survival but not overall survival has also been shown with combinations of FOLFOX plus cetuximab (OPUS trial) or FOLFOX plus panitumumab (PRIME trial) in metastatic colorectal cancer patients with wild-type KRas. Keeping in line with the resistance of KRas-mutant colorectal cancer to cetuximab, a few retrospective studies later showed that patients with KRas mutations at codon 12 or 13 actually had poorer prognosis when treated with cetuximab. As such, the FDA later restricted the indication for cetuximab and panitumumab to metastatic colorectal cancer patients with documented wild-type KRas genotype. Due to the established benefits of anti-EGFR monoclonal antibodies in selected patient populations, current NCCN guidelines strongly recommend pretreatment KRas genotyping of tumor tissues from either the primary or metastatic sites in all patients with metastatic colorectal cancer.

In a recent randomized study, addition of cetuximab to systemic chemotherapy such as FOLFIRI or mFOLFOX6 in patients with KRas wild-type colorectal cancer with limited liver metastasis significantly improved tumor response and

subsequent R0 resection of metastatic liver lesion (25.7% vs. chemotherapy alone arm 7.4%; $P < 0.01$) and 3-year overall survival (41% vs. chemotherapy alone 18%; $P = 0.013$). In patients who received cetuximab plus chemotherapy, those who underwent resection of liver lesion had significantly longer median survival than those who did not (46.4 vs. non-resected 25.7 months; $P < 0.01$) (Ye et al. 2013).

The survival benefit of cetuximab is limited only to metastatic, KRas wild-type colorectal cancer. In a large randomized phase III trial enrolling 2,686 patients with stage IIIB, resected colorectal cancer, addition of cetuximab to adjuvant chemotherapy mFOLFOX6 did not improve disease-free survival but instead led to increased toxicity especially in patients aged above 70 years (Alberts et al. 2012).

In non-small cell lung cancer, the prognostic value of KRas mutations remains largely controversial, with different studies showing contradictory results. Overall, the presence of KRas mutations has not been convincingly shown to influence response to standard chemotherapy, time to progression, and overall survival (Loriot et al. 2009). However, NSCLC patients with KRas mutations showed poorer clinical outcome when treated with standard chemotherapy plus erlotinib, a tyrosine kinase inhibitor (TKI) that suppresses EGFR (Chapters 42 and 43) signaling, indicating intrinsic resistance of KRas-mutant NSCLC to TKIs (Eberhard et al. 2005). Similarly, NSCLC patients with EGFR (Chapters 42 and 43) mutations or overexpression, but not KRas mutations, derive survival benefit from maintenance therapy with erlotinib following standard chemotherapy (Brugger et al. 2011; Cappuzzo et al. 2010). These findings resonate the established signaling paradigm where KRas acts downstream of EGFR (Chapters 42 and 43) and therefore activating mutation of KRas renders signaling input from upstream EGFR (Chapters 42 and 43) dispensable. Contrary to the limited benefit of erlotinib, cetuximab appears to have benefit in NSCLC patients with mutant KRas. In two large phase III studies (BMS099 and FLEX), addition of cetuximab to first-line carboplatin/taxanes or cisplatin/vinorelbine in patients with advanced-stage NSCLC significantly improved overall survival, regardless of KRas status, although it is noteworthy that patients with EGFR mutations derived the most survival benefit from addition of cetuximab (Khambata-Ford et al. 2010; O'Byrne et al. 2011). Several targeted agents against mutant KRas-driven NSCLC are currently being evaluated in clinical trials. In a randomized, placebo-controlled phase II trial that enrolled only KRas-mutant NSCLCs, addition of selumetinib, an inhibitor that targets MEK1/MEK2 (Chapter 88) kinase, to docetaxel as a second-line therapy significantly improved median progression-free survival (5.3 months vs. 2.1 months (95% CI 1.4–3.7) in the placebo group, HR for progression 0.58, 80% CI 0.42–0.79; one-sided $p = 0.014$). The response rate was 37% in the selumetinib group as opposed to 0% in the placebo group (Janne et al. 2013). However, it remains unclear whether patients with wild-type KRas would also benefit from the same combination. Due to the lack of convincing data to support KRas genotyping in guiding currently available therapeutic regimens, routine testing of KRas mutational status in NSCLC is not recommended.

Contrary to colorectal cancer and NSCLC, EGFR (Chapters 42 and 43) is required for oncogenic KRas signaling in pancreatic cancer. In a large double-blind, randomized, placebo-controlled phase III trial involving 569 patients, addition of erlotinib to gemcitabine in patients with unresectable or metastatic pancreatic adenocarcinoma conferred a small but statistically significant improvement in overall 1-year survival (23% vs. 17%; $P = 0.023$) as well as progression-free survival (Moore et al. 2007). Further analysis from this study showed that the presence of KRas mutations did not preclude the beneficial effect of erlotinib (da Cunha Santos et al. 2010), indicating distinct signaling interplay between EGFR and oncogenic KRas signaling in pancreatic as opposed to colorectal cancer. The unique requirement of EGFR for oncogenic KRas signaling in pancreatic cancer was later experimentally supported by elegant studies using murine and pancreatic cancer cell line models (Navas et al. 2012; Ardito et al. 2012).

Therapeutics

In the last three decades, the pursuit of anti-Ras therapeutics is the one of intense clinical interest but, to date, largely unsuccessful. The initial endeavors focused on directly inhibiting the proper localization of Ras proteins. As a prerequisite for membrane association, all Ras proteins undergo farnesylation, a three-step covalent modification involving farnesyltransferase (FTase), Ras-converting enzyme 1 (Rce1), and isoprenylcysteine carboxyl methyltransferase (ICMT), which culminate in covalent addition of a C15 farnesyl isoprenoid lipid at their C-terminal CAAX motif. On this basis, inhibitors against these enzymes have been developed to specifically target Ras-mutant cancer. Among these, tipifarnib is an imidazole-containing heterocyclic FTI that is best studied in clinical trials. In a large randomized phase III trial enrolling 688 patients with advanced-stage pancreatic cancer, addition of tipifarnib to gemcitabine as a first-line systemic therapy failed to improved overall and progression-free survival compared to gemcitabine alone (Van Cutsem et al. 2004). In another randomized, placebo-controlled, phase III trial on patients with refractory, advanced colorectal cancer, single-agent tipifarnib did not improve overall survival compared to best supportive care (Rao et al. 2004). In stage IIb–IIIc breast cancer, addition of tipifarnib to neoadjuvant doxorubicin and cyclophosphamide resulted in pathological complete response rate of 25% compared to 10–15% based on historical data of doxorubicin and cyclophosphamide (Sparano et al. 2009). However, it was unclear whether addition of tipifarnib resulted in improved R0 resection or survival in these patients. In postmenopausal patients with advanced breast cancer who progressed after tamoxifen, addition of tipifarnib to letrozole did not improve objective response rate, time to progression, or survival (Johnston et al. 2008). In a phase III, multicenter, open-label study, tipifarnib as a first-line therapy in elderly patients with newly diagnosed acute myeloid leukemia did not improve overall survival compared to best supportive care which included the use of hydroxyurea (Harousseau et al. 2009).

The clinical failure of FTIs is now known to result from the fact that NRas and KRas, the two most commonly mutated Ras isoforms, can undergo alternative prenylation that is catalyzed by geranylgeranyltransferase type I (GGTI) to become membrane bound and therefore escape the toxicity of FTIs (Baines et al. 2012). Currently one geranylgeranyltransferase inhibitor, GGTI-2418, has entered phase I clinical trial in 2009. Inhibitors against other enzymes in farnesylation such as Rce1 and ICMT have been reported in preclinical studies, but to date none has entered clinical trials, presumably due to the low probability of clinical efficacy as the FTIs. Salirasib (or farnesyl thiosalicylic acid), which was designed to mimic the farnesylated cysteine moiety of Ras thereby competitively displacing all Ras proteins from being attached to the inner membrane, farnesyl-binding docking sites, showed no discernable clinical efficacy in KRas-mutant NSCLC patients (Riely et al. 2011).

An alternative approach to inhibit KRas is by targeting its signaling effectors. This approach has now proven to be equally challenging because activated KRas is known to activate several effectors, thereby rendering blockade of a single pathway insufficient to curb KRas-driven tumors. Several inhibitors against key kinases such as B-Raf (Chapter 20), MEK (Chapter 88), PI3K (Chapter 109), Akt (Chapter 4), or mTOR (Chapter 93) are currently being evaluated in clinical trials as single agents or in combination with chemotherapy, but so far none by itself has demonstrated specificity or remarkable efficacy against KRas-driven tumors (Baines et al. 2012). Paradoxically, the use of B-Raf (Chapter 20) inhibitor such as vemurafenib on Ras-mutated cancer may activate other Raf (Chapter 20) isoforms such as C-Raf to hyperstimulate the MAPK pathway and instead augment tumorigenic growth (Hatzivassiliou et al. 2010; Poulikakos et al. 2010; Heidorn et al. 2010). Therefore, simultaneous blockade of multiple key Ras effector pathways with combined use of inhibitors will most likely be the next logical approach in therapeutic targeting of KRas-driven cancers.

Preclinical Summary

Mutations of KRas are one of the most common oncogenic events in human cancer. Despite decades of intensive research, development of effective anti-Ras therapeutics is impeded by the multitude of complex signaling network driven by KRas and potential toxicity due to the shared biochemical properties of mutant KRas with a wide array of other essential GTPases. Newer strategies such as KRas-mutant specific small-interfering oligonucleotides or vaccines are being developed and evaluated. Genome-wide, synthetic lethal RNAi screen has been conducted to identify essential molecular partners of mutant KRas that can be explored as new therapeutic targets (Luo et al. 2009; Barbie et al. 2009).

Clinical Summary

To date, no effective anti-Ras therapy is available, although many novel agents and strategies are being developed in the laboratory and evaluated in early phase clinical trials. Testing of KRas mutational status should be routinely done in colorectal cancer patients in whom treatment with anti-EGFR monoclonal antibodies is being considered, as patients with mutated KRas will not benefit from these antibodies. In NSCLC, KRas genotype likely predicts resistance to TKIs but not anti-EGFR monoclonal antibodies, but larger studies are required to confirm these findings. In pancreatic cancer, inhibition of EGFR (Chapters 42 and 43) signaling with erlotinib interferes with KRas signaling and confers a small survival benefit.

Anticipated High-Impact Results

- MEK Inhibitor MSC1936369B Plus FOLFIRI in Second Line K-Ras Mutated Metastatic Colorectal Cancer (NCT01085331)
- A Dose Finding and Phase II Study of Selumetinib (AZD6244) in Combination with Irinotecan, in 2nd Line Patients with K-ras or B-raf Mutation Positive Advanced or Metastatic Colorectal Cancer (NCT01116271)
- Vaccine Therapy With Tumor Specific Mutated Ras Peptides and IL-2 or GM-CSF for Adult Patients With Solid Tumors (NCT00019331)

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MET

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Abstract

The receptor tyrosine kinase MET activates numerous cellular signaling pathways after binding with its ligand, hepatocyte growth factor (HGF). MET is involved in a wide range of biological processes and is critical for tissue homeostasis under physiological conditions. MET is also a known oncogene that is abnormally activated in many human cancers by mutation, protein overexpression or amplification. Furthermore, MET is implicated as a common mechanism of resistance to targeted therapies such as EGFR inhibitors. In this review, we describe the

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biology of MET, the mechanisms by which it becomes an oncogenic driver, its role as a target in cancer medicine, and emerging biomarkers to select patients for MET-targeted therapy. Pre-clinical and clinical data for anti-MET therapies to date are then summarized.

Keywords
Acquired TKI resistance • Biomarker • Cell scatter • Crizotinib • Gene amplification • Hepatocyte growth factor (HGF) • Hereditary papillary renal cell carcinoma • Mesenchymal-epithelial transition (MET) receptor • Oncogene • Tivantinib

Target Overview: MET

The mesenchymal-epithelial transition (MET) receptor is a receptor tyrosine kinase (RTK) encoded by the c-MET proto-oncogene located on chromosome 7q21–31. It is the prototypic member of a small family of RTKs that share structural and sequence homology, including recepteur d'origine nantais (RON) and the avian protein Sea (Huff et al. 1993; Ronsin et al. 1993). MET is a single-pass heterodimer with a molecular mass of 190 kDa that consists of an extracellular α-subunit and a transmembrane β-subunit with intracellular catalytic activity (Maroun and Rowlands 2014; Trusolino et al. 2010). It exists in both membrane bound and soluble forms and is widely expressed by the epithelial cells of many organs during both embryogenesis and adulthood (Organ and Tsao 2011). MET can also be found on endothelial cells, neurons, melanocytes, hematopoietic precursors, B-cells, and antigen-presenting dendritic cells (Organ and Tsao 2011; Beilmann et al. 1997; van der Voort et al. 1997).

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is the high-affinity ligand of MET. A circulating plasminogen-like zymogen, single-chain pro-HGF is secreted primarily by mesenchymal cells and converted into a functional heterodimer by extracellular proteases. Biologically competent HGF then acts in a paracrine fashion on MET-expressing epithelium, resulting in receptor dimerization, transphosphorylation of catalytic tyrosine residues, and recruitment of signaling effectors and scaffolding proteins. Ultimately, the MET kinase is activated, after which numerous downstream signal transduction pathways are turned on, including the mitogen-activated protein kinase (MAPK) cascade, the PI3K-Akt axis, the signal transducer and activator of transcription (STAT) pathway, and the IκBα-NF-κB transcription factor complex (Trusolino et al. 2010; Sonnenberg et al. 1993). The end result of this highly complex physiologic process is the stimulated transcription of various genes that encode mitogenic and antiapoptotic regulatory proteins. Of note, MET interacts directly with other important oncogenic signaling pathways – a phenomenon known as "biological crosstalk." For example, cells that coexpress MET and the epidermal growth factor receptor (EGFR) are capable of ligand-independent MET activation through EGFR.

In nonneoplastic tissues, MET is under tightly controlled negative regulation through a variety of mechanisms, such as extracellular shedding of the receptor, as well as internalization via endocytosis followed by ubiquitin-mediated degradation (Foveau et al. 2009; Galvani et al. 1995). This process is regulated by the ubiquitin ligase c-Cbl, which is recruited to the juxtamembrane domain and heavily phosphorylated after MET activation. c-Cbl is also a known proto-oncogene – if mutated, the negative regulation of MET (or other RTKs) may become disrupted via loss of the ubiquitin tagging that is necessary for appropriate lysosomal degradation of the receptor (Peschard et al. 2001).

Biology of the Target

MET function is critically important in both embryonic and adult life. MET/HGF signaling results in a complex phenotype that is essential for tissue remodeling, morphogenic differentiation, and cell scattering during normal development. For example, skeletal muscle progenitor cells are dependent on the MET axis for long-range migration during embryogenesis – muscle groups derived from these cells are completely absent in MET or HGF gene knockout mice (Bladt et al. 1995). Hepatocytes and placental trophoblasts are also dependent on MET kinase activity for survival and proliferation. Mouse embryos with homozygous mutant (−/−) MET or HGF demonstrate significantly reduced liver size, deranged placental development, and death in utero (Schmidt et al. 1995; Uehara et al. 1995). After birth, MET continues to play a key role in cell proliferation, survival, motility, and cell scattering. Hepatic, renal, and myocardial regeneration after cellular injury appears to be reliant upon MET activation. This is supported by multiple studies that have demonstrated elevated circulating HGF levels and/or increased MET expression in the setting of acute or chronic tissue damage (Kawaida et al. 1994; Nakamura et al. 2000; Tsubouchi et al. 1991). In a cirrhotic animal model, hepatocellular hypoxia was shown to dramatically decrease HGF and MET expression, suggesting that decreased MET signaling may play a role in cirrhotic liver regeneration failure (Corpechot et al. 2002). Impairment of normal repair pathways is thus a potential concern in the development of MET inhibitors for cancer therapy. MET has been implicated in numerous other biological processes including angiogenesis (via induction of pro-angiogenic cytokines and VEGF) and regulation of immune function (B-cell homing, monocyte activation, and suppression of antigen presentation by dendritic cells) (Beilmann et al. 1997; Benkhoucha et al. 2010; Galimi et al. 2001; Okunishi et al. 2005).

Deregulation of MET signaling is an important mediator of tumorigenesis and cancer progression. Numerous derangements in the c-MET gene have been identified in a wide variety of tumor types – lesions that may result in prolonged or constitutive activation of the MET receptor. Translocations, gene amplification, and various activating mutations have all been described; overexpression of the MET protein in the absence of a genetic disturbance has also been reported.

c-MET was first recognized as an oncogene in 1984, in a carcinogen-treated human osteosarcoma cell line, where a chromosomal rearrangement fused the MET tyrosine kinase domain to a translocated promoter region (TPR), resulting in constitutive dimerization (Cooper et al. 1984). A definitive link between aberrant MET activation and human malignancy was established in the late 1990s, with the discovery of activating mutations in hereditary and sporadic papillary renal cell carcinoma (PRCC). Germline mutations are in fact present in 100% of hereditary cases, and in a large series of sporadic PRCC, 17 of 129 cancers (13%) carried a somatic mutation (Choueiri et al. 2013; Schmidt et al. 1999). Since then, numerous unique point mutations in the MET gene sequence have been cataloged, many of which occur outside the kinase domain but retain the potential to be activating. In thoracic malignancies and melanoma, direct sequencing analysis has demonstrated that missense mutations are clustered primarily in the extracellular Sema domain (HGF binding site) and the cytoplasmic juxtamembrane domain (Ma et al. 2008). MET activation is also known to occur by exon 14 skipping secondary to various splice site (or splicing factor) mutations (Comprehensive molecular profiling of lung adenocarcinoma 2014). A recent analysis of 38,028 cancer patients undergoing expanded mutation testing identified 221 cases with exon 14 MET mutations (0.6%), with 126 distinct variants across different tumor histologies – the highest frequency was confirmed in non-small cell lung cancers, in particular lung adenocarcinoma (3%) (Frampton et al. 2015).

c-MET gene amplification with resultant constitutive RTK activation has been reported in numerous human cancers, including NSCLC, gastric and esophageal carcinomas, and medulloblastomas (Maroun and Rowlands 2014; Organ and Tsao 2011). In a series of 489 consecutive gastroesophageal cancers that were genetically screened, 10 (2%) harbored MET amplifications; these tumors were generally high-grade, advanced adenocarcinomas (Lennerz et al. 2011). A different study demonstrated polysomy (increased gene copy number) in 16% (61 out of 381) of gastric carcinoma cases (Lee et al. 2012). In an investigation of 106 surgically resected NSCLCs from a tyrosine kinase inhibitor (TKI)-naïve cohort, MET was amplified in 22 cases (21%), although more recent estimates are on the order of 5–7% (Beau-Faller et al. 2008). Interestingly, gene amplification was detected in a majority of liver metastases from a colon cancer series, despite being present in only 10% of the primary colonic lesions (Di Renzo et al. 1995). These data require validation but may indicate that MET amplification is a late genetic event, conferring further selective advantage for a tumor to acquire metastatic potential.

Increased protein expression in the absence of gene amplification (i.e., due to transcriptional upregulation) is frequently reported in human cancers, including thyroid, colorectal, ovarian, pancreatic, lung, and breast carcinomas (Comoglio et al. 2008). Hypoxia-mediated activation of the c-MET promoter by hypoxia inducible factor 1a (HIF-1α), a transcription factor regulated by intracellular oxygen concentrations, has been shown to increase c-MET transcription both in vitro and in vivo (Pennacchietti et al. 2003; Kitajima et al. 2008). Increased ligand expression is also known to cause MET overstimulation. HGF is ubiquitously expressed throughout the body, and increased levels have been found in the reactive stroma

of tumors, as well as in the plasma of cancer patients (Matsumoto and Nakamura 2006). This may support the notion of inappropriate paracrine feedback loops stimulating the dissemination of cancer cells. Despite these findings, MET/HGF overexpression studies have been subject to significant bias, and increased protein expression remains unvalidated as an oncogenic effector or biomarker.

Lastly, there is a growing body of evidence to suggest that MET is overexpressed and activated in cancer stem cells (CSCs) – the highly clonogenic subset of tumor cells responsible for cancer initiation, invasion, and ultimate metastasis. During embryogenesis, undifferentiated progenitor cells undergo a transient mesenchymal-epithelial transition (MET) for appropriate cell migration and organ morphogenesis. CSCs appear to hijack this rudimentary genetic program as they acquire metastatic potential; multiple cell models have demonstrated that HGF-induced cell scattering is a biologic equivalent of embryonic MET (Boccaccio and Comoglio 2006).

Target Assessment

The role of MET assessment in the evaluation of newly diagnosed malignancies is currently evolving. Upfront analysis for activating mutations is clearly important in papillary RCC. Beyond this, MET evaluation is now expanding into other tumor types, often in the context of next-generation sequencing (NGS). Timing of the analysis (i.e., at diagnosis versus at time of disease progression) and techniques used varies widely between institutions.

Both MET and HGF protein expression can be measured with immunohistochemical (IHC) techniques, using a number of different commercially available antibodies. Gene amplification is typically assessed and quantified by fluorescence in situ hybridization (FISH) or NGS. MET mRNA expression can be quantified with reverse transcription polymerase chain reaction (RT-PCR), though this is rarely done in the clinical setting. Mutational analysis is performed via direct DNA sequencing or with high-throughput techniques. Biomarker studies have indicated that elevated levels of MET protein and mRNA expression in tumor tissue do not necessarily correlate with MET gene amplification (based on negative FISH results in the same specimen) (Janjigian et al. 2011). This highlights the salient point that protein overexpression and gene amplification are non-synonymous derangements.

It is important to note that there is currently no consensus in terms of scoring criteria for MET IHC assays, which remain largely unstandardized. The development of a validated biomarker for aberrant MET signaling is a crucial area of ongoing clinical research, as accurate, reliable, and reproducible diagnostic testing is necessary in order to select patients who will be most likely to respond to MET-targeted agents. There are numerous challenges in this regard, including limited availability of archival tumor tissue, the need for repeat/multiple tumor biopsies (pre-/posttreatment), and intratumoral molecular heterogeneity – the presence of distinct tumor cell subpopulations that display differing molecular signatures

within the same cancer. This phenomenon was demonstrated in a glioblastoma dataset analysis, where independent focal amplification of two or more RTKs was observed (including EGFR, platelet-derived growth factor receptor alpha (PDGFRA), and MET) (Szerlip et al. 2012). The recent emergence of next-generation sequencing has allowed for rapid identification of relevant genetic alterations in cancer – these techniques will hopefully pave the way toward consistent and efficient MET genotyping.

Role of the Target in Cancer

Rank: 9

Diagnostic, Prognostic, and Predictive (as Applicable)

Diagnostic

As mentioned above, germline missense mutations in the kinase domain of the MET gene are found in all cases of hereditary papillary renal cell carcinoma, a genetically distinct familial malignancy (Schmidt et al. 1997). These specific mutations are pathognomonic for the disease, and the MET oncogene is sometimes referred to as the HPRCC gene.

Prognostic

Numerous studies have correlated increased MET signaling with poor prognosis and decreased survival in a variety of tumor types – most of these findings require further validation to ascertain their true clinical meaning. In a retrospective study of 447 surgically resected NSCLC patients, MET FISH analysis was performed in 435 cases. High gene copy number was observed in 48 patients (11.1%) and was associated with advanced age ($P = .01$), higher grade histology ($P = .016$), and shorter overall survival ($P = .005$). Multivariate analysis confirmed a significant reduction in risk of death for MET-negative patients (HR 0.66, $P = .04$) (Cappuzzo et al. 2009). In an ovarian carcinoma cohort, MET protein was overexpressed by IHC in 15 of 138 patients (11%). Median overall survival for those with high MET levels was 17 months versus 32 months for those with normal MET expression ($P = .0001$) (Sawada et al. 2007). In a tissue microarray based on 324 patients with node-negative breast carcinoma, high level of MET expression was a statistically significant prognostic marker, conferring shortened survival compared to the remainder of the cohort ($P = .0035$, relative risk 2.04). Lastly, increased MET mRNA copy number is significantly associated with depth of tumor invasion and nodal metastases in colorectal adenocarcinomas (Takeuchi et al. 2003). Gene amplification is noted in only 2% of primary colon cancers but in as many as 18% of liver metastases

($P < .01$); among patients treated with hepatic metastaectomy, a trend toward poorer 3-year survival was observed in the MET-amplified subset ($P = .07$) (Zeng et al. 2008).

Predictive

MET plays an important mechanistic role in the development of resistance to approved targeted therapies, such as EGFR, vascular endothelial growth factor receptor (VEGFR), human epidermal growth factor receptor 2 (HER2), and BRAF inhibitors – aberrant MET expression may ultimately have utility as a means of predicting primary or acquired resistance to such agents. This phenomenon has been best described in NSCLCs that harbor activating mutations in the EGFR gene. Despite excellent initial responses to EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib or gefitinib, patients with EGFR-mutated disease will eventually develop acquired resistance to these agents. While this is predominantly mediated by the secondary T790M "gatekeeper" mutation, a smaller subset will manifest resistance via activation of alternate pathways. Analysis of repeat tumor biopsies at the time of disease progression has demonstrated MET upregulation in anywhere from 5% to 20% of cases (Bean et al. 2007; Sequist et al. 2011a). Transactivation of HER3 by MET (receptor crosstalk) with subsequent downstream PI3K/Akt signaling effectively bypasses the EGFR blockade. Preclinical data support the notion that small molecule MET inhibitors can overcome erlotinib resistance in lung cancer cell lines and tumor xenograft models providing a strong rationale to explore the use of anti-MET agents in this patient population (Tang et al. 2008). Furthermore, pre-existing clonal selection of MET amplification has been demonstrated in subpopulations of EGFR-mutated lung cancer cells (prior to EGFR-directed therapy), which may support the notion of up-front dual MET/EGFR blockade (Turke et al. 2010). More recently, MET-mediated resistance to EGFR inhibition has been described in colorectal carcinoma, where gene amplification was associated with resistance to the anti-EGFR monoclonal antibodies cetuximab and panitumumab (Bardelli et al. 2013).

Therapeutics (Overview)

Inhibition of the MET axis has been pursued as a rational therapeutic strategy since as early as 2000. Interest in MET as a target has risen substantially in the last few years, with a significant increase in registered clinical trials for MET-directed agents. The majority of these are phase I/II trials evaluating the safety and efficacy of over 20 different anti-MET compounds, several of which have now entered phase III development (Furlan et al. 2014). Agents that target the MET axis include both monoclonal antibodies (that block either the receptor or the ligand) and small molecule tyrosine kinase inhibitors (ATP mimetics that compete for the kinase domain binding site – with the exception of tivantinib, a unique non-ATP

competitive inhibitor). There are currently no agents that have been FDA approved specifically as anti-MET therapies.

Preclinical Summary

There is ample preclinical evidence to justify the translation of MET-targeted therapies from bench to bedside – much of this work has been aimed at defining drug-responsive subsets, in an effort to ultimately design clinical trials directed at susceptible patients. PHA-665752, a small molecule inhibitor of the MET kinase, was identified in the early 2000s and continues to be of prominent use in basic research. With over 50-fold selectivity for MET compared to other kinases, PHA-665752 was shown to potently inhibit both HGF-stimulated and constitutive MET phosphorylation in cellular studies, with resultant inhibition of downstream signaling and MET-driven phenotypes. In GTL-16, a MET-amplified gastric carcinoma cell line, PHA-665752 completely inhibited colony growth and cell proliferation, inducing apoptosis with or without HGF present (Christensen et al. 2003). Of note, morphologic studies demonstrated an induction of cell differentiation after treatment. Additional work in gastric cancer continued to yield promising results, both in vitro and in vivo. PHA-665752 induced massive apoptosis in five of five gastric tumor cell lines with amplified wild-type MET but in 0 of 12 non-amplified cell lines ($P = .00016$) (Smolen et al. 2006). In a GTL-15 xenograft model, treatment resulted in sustained MET inhibition for up to 12 h after a single dose. Indeed, repeated administration of the agent successfully inhibited tumor growth in a dose-dependent fashion, demonstrating potent in vivo cytoreductive activity (Christensen et al. 2003). Inhibitory effects on growth/proliferation have been reported in other cell lines to varying degrees, including NCI-H441 (papillary lung adenocarcinoma with constitutive MET activity) and BxPC-3 (pancreatic adenocarcinoma with moderate levels of MET expression); changes in cell morphology, however, were only observed with GTL-16 cells.

Another important arena of preclinical work has been the study of TKI resistance in EGFR-mutated lung cancer, which ultimately elucidated MET amplification as a significant resistance mechanism. In a series of elegant experiments published in 2007, an EGFR exon 19-mutated NSCLC cell line (HCC827) was exposed to increasing concentrations of gefitinib over a period of 6 months. Subclones of gefitinib-resistant cells (HCC827-GR) were isolated from the parental line – persistent phosphorylation of ERBB3 and Akt (downstream of EGFR) in the presence of gefitinib was demonstrated. Whole genome copy number analysis and mRNA expression profiling were then performed in both the resistant and parental cell lines. This revealed a striking focal amplification at the MET gene locus, found only in the GR line – by quantitative PCR, a five- to tenfold amplification was confirmed, with no MET mutations detected on sequence analysis (Engelman et al. 2007). HCC827-GR cells demonstrated resistance to gefitinib alone, as well as to PHA-665752 alone. Combined treatment, however, yielded significant growth inhibition and apoptosis, successfully suppressing ERBB3 and Akt phosphorylation.

These studies support increased MET signaling as a mechanism of EGFR TKI resistance and provide a strong rationale for clinical investigation of combined EGFR/MET blockade.

Clinical Summary

Despite over a decade of research, efforts to develop MET-targeted cancer therapies have been met with considerable challenges. Several recent failures are worth noting, as they provide insight into past mistakes and future drug development. Onartuzumab (MetMAb) is a recombinant, humanized monoclonal antibody directed against MET, with a monovalent design that blocks HGF binding but does not cause receptor dimerization. This agent has been investigated in combination with erlotinib for advanced, refractory NSCLC. In a randomized phase II trial comparing MetMAb/erlotinib versus erlotinib alone, there was no progression-free survival (PFS) or overall survival (OS) benefit in the intent-to-treat population; however, both PFS (HR, .53; $P = .04$) and OS (HR, .37; $P = .002$) were improved in a subset analysis of patients who were MET positive by IHC (Spigel et al. 2013). This led to the METLung trial – a randomized, multicenter, placebo-controlled phase III study that enrolled 499 previously treated MET IHC-positive patients with stage IIIb/IV NSCLC. The trial was closed early due to futility, as the addition of onartuzumab to erlotinib did not improve OS, PFS, or response rates (Spigel et al. 2014). This highlights the importance of biomarker validation – MET expression by IHC can be detected in nearly 50% of NSCLCs, but this does not necessarily mean that these tumors are MET activated.

Rilotumumab is a fully humanized monoclonal antibody against HGF. It was assessed in combination with cytotoxic chemotherapy in a phase I/II trial of patients with advanced gastric or gastroesophageal (GE) junction adenocarcinoma, where it appeared to be promising. PFS was 5.7 months with rilotumumab plus chemotherapy versus 4.2 months with chemo alone (HR 0.60). OS was also improved in the combination arm, 10.6 versus 8.9 months (HR 0.70) (Davidenko et al. 2012). Subsequent biomarker analysis suggested that high MET expression by IHC may predict clinical benefit in this group (Oliner et al. 2012). These data led to initiation of RILOMET-1 and RILOMET-2, a pair of phase III randomized placebo-controlled trials investigating rilotumumab plus ECX (or CX) as first-line therapy for advanced G/GEJ carcinoma (Cunningham et al. 2013). An interim safety analysis in November 2014 revealed an increased number of deaths in the treatment arm, resulting in closure of both studies.

Tivantinib is a novel, non-ATP competitive TKI that selectively inhibits MET by stabilizing the receptor in its inactive conformation. In a randomized phase II study of 167 patients with advanced, previously treated NSCLC, erlotinib plus tivantinib (ET) was compared to erlotinib plus placebo (EP). The study's primary endpoint (PFS) was not met, albeit in a highly unselected ITT population. Median PFS was 3.8 months with ET versus 2.3 months with EP (HR 0.87, $P = .24$). Post hoc subset analyses demonstrated clinical benefit in specific groups, including patients with

increased MET gene copy number by FISH, EGFR wild-type status, KRAS mutations, and non-squamous histology (Sequist et al. 2011b). Based on these findings, tivantinib was further investigated in the randomized phase III MARQUEE trial, which aimed to enrich the study population for "MET positivity" by only enrolling non-squamous NSCLCs. Roughly 1000 patients were recruited, but a preplanned interim analysis in October 2012 indicated that the primary endpoint of OS would not be met, again leading to early discontinuation. Tivantinib remains of interest in hepatocellular carcinoma (HCC), where it has shown promising activity in patients who have progressed on (or are unable to tolerate) sorafenib. In a subgroup of advanced HCC patients with high MET expression (by IHC), second-line tivantinib improved OS compared to placebo in a randomized phase II trial (7.2 vs. 3.8 months; HR 0.38, $P = 0.01$) (Santoro et al. 2013). A phase III study investigating tivantinib in preselected MET-high patients is currently open for enrollment (NCT01755767).

Despite the disappointments described above, an increased understanding of MET biology has led to promising drug development strategies moving forward. Foretinib is an oral multikinase inhibitor with potent anti-MET activity that has had some success in patients with advanced PRCC. In a single-arm phase II study (the largest multicenter PRCC trial to date), foretinib therapy yielded an objective response rate of 13.5% and a median PFS of 9.3 months, with a toxicity profile that was comparable to other multikinase inhibitors. Although this objective response rate (ORR) did not meet the study's predefined rate for efficacy (25%), the data still compare favorably with frontline agents such as sunitinib or sorafenib, which generally have poor efficacy in non-clear cell histologic subtypes. The presence of a germline MET mutation was in fact highly predictive of a response to foretinib (with five of ten patients responding) (Choueiri et al. 2013).

Crizotinib is a small molecule TKI that inhibits anaplastic lymphoma kinase (ALK), ROS1, and MET. Preliminary results from a phase I study investigating the safety and efficacy of crizotinib in advanced, c-MET-amplified NSCLC were reported at the 2014 American Society of Clinical Oncology Annual Meeting. Of 12 evaluable patients, four showed a partial response to the drug, with a median duration of response of 35 weeks (Camidge et al. 2014). While this suggests that crizotinib does have antitumor activity in this population, further study is necessary and accrual of additional c-MET-amplified patients is ongoing. The previously described unique MET variant characterized by exon 14 skipping has recently been identified in a high fraction of pulmonary sarcomatoid carcinomas (8 of 36 cases, 22%). This particular alteration was also confirmed in two cell lines that displayed marked MET inhibition on exposure to crizotinib, and several patients with this mutation have recently demonstrated a rapid clinical and radiographic partial response to therapy (Paik et al. 2015; Liu et al. in press). These exciting findings potentially identify a subset of carcinomas that may be uniquely sensitive to MET pathway disruption.

MGCD516 is an orally available, potent small molecule inhibitor of a closely related spectrum of tyrosine kinases including MET, AXL, VEGFR, and PDGFR. The compound is currently in phase I/Ib development for patients with advanced solid tumor malignancies (NCT02219711). In addition to dose/regimen exploration

and safety analysis, expansion cohorts will be organized by molecular biomarker, including MET amplification, exon 14 skipping, and other mutations. If an objective disease response is observed with a particular defined marker, additional patients may then be enrolled in that cohort. This strategy will hopefully identify and enrich for a biomarker that confers sensitivity to the agent. AMG 337, a highly selective MET kinase inhibitor, has demonstrated efficacy against MET-amplified GI cancers in early phase trials; however further development is currently on hold. Multiple other promising drugs targeting the MET axis are in various stages of development, and further study of these compounds is eagerly anticipated.

Anticipated High Impact Results

- Correlative studies that will identify optimized biomarkers that can accurately predict sensitivity to MET-targeted agents.
- Completion of ongoing clinical trials from which clinically impactful MET inhibitors for biomarker-selected patients will rapidly emerge (e.g., crizotinib, cabozantinib, MGCD516).
- New studies exploring combination therapy with MET inhibitors in the setting of acquired resistance to other targeted agents.
- Additional basic and clinical research to explore a more expanded utility of MET inhibition (such as blockade of cancer stem cell signaling).

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NEDD9

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Joy Little and Erica Golemis

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Abstract

Neural Precursor Cell Expressed Developmentally Downregulated 9 (NEDD9; also known as HEF1 and CAS-L) is a multi-domain protein that acts as a scaffold to promote the interactions of proteins involved in cell migration, attachment, and cilia formation, among other processes. While NEDD9 has multiple binding partners, Focal Adhesion Kinase (FAK), SRC, and Aurora-A kinase are the most well-studied in regard to their contributions to oncogenesis. NEDD9 has no catalytic activity, but accumulating evidence supports NEDD9 as a potential biomarker for metastatic cancers.

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Neural precursor cell expressed developmentally downregulated 9 (NEDD9) • Crk-associated substrate-related protein (CAS-L) • Human enhancer of filamentation 1 (HEF1) • Aurora-A • Biomarker • Cancer • Metastasis • Oncogene • Preclinical studies • SRC and FAK • Crk-associated substrate (CAS) family • Integrin-mediated attachment • Receptor tyrosine kinase signaling • Tumor cell adhesion • Tumor cell migration • Centrosome • Cilia

Target

Neural precursor cell expressed developmentally downregulated 9 (NEDD9; also known as HEF1 and CAS-L) is a scaffolding protein that regulates key cellular processes such as adhesion, migration, division, and survival. Increased expression of NEDD9 has been associated with enhancing the metastatic phenotypes of various cancer cell types including glioblastoma, breast, lung, melanoma, leukemia, and advanced stage head and neck squamous cell carcinoma (HNSCC) (reviewed in Tikhmyanova et al. 2010). Moreover, NEDD9 loss or siRNA-mediated knockdown can sensitize tumor cells to specific chemotherapeutics, implying NEDD9 has a role in tumor drug resistance (Astsaturov et al. 2010; Singh et al. 2010).

Biology of the Target

NEDD9 is one member of the Crk-associated substrate (CAS) family of proteins: the other three CAS proteins are BCAR1/p130Cas, EFS/SIN, and HEPL/CASS4. There are significant connections between NEDD9 and regulation of cellular functions altered in cancer including adhesion, chemotaxis, cell survival, cell cycle, migration, and invasion. Therefore, it is logical that NEDD9 is associated with progression and metastasis of several tumor types. Further, NEDD9 is a binding partner and activator of various known oncogenes including FAK and SRC, which directly influence migration and adhesion (Law et al. 1996; Minegishi et al. 1996; Astier et al. 1997). NEDD9 is controlled in tumor cell lines through gene amplification, transcriptional upregulation, and hyperphosphorylation, as well as changes in stability (Tikhmyanova et al. 2010).

Two groups independently described NEDD9 in 1996. The first descriptions of the complete protein designated it human enhancer of filamentation 1 (HEF1), based on its isolation in a screen of human genes inducing filamentous growth of yeast (Law et al. 1996), and Crk-associated substrate-related protein, lymphocyte type (CAS-L), as a hyperphosphorylated protein in T lymphocytes after integrin-b1 stimulation (Minegishi et al. 1996). It was subsequently recognized the 3′ UTR of HEF1/CAS-L had homology to an expressed sequence tag, *NEDD9*, previously reported as downregulated in the early embryonic murine brain, causing NEDD9 to be assigned as the official gene name (Kumar et al. 1992). NEDD9 localizes to

chromosome 6p25-p24 in humans and chromosome 13 A3.3 in mice. NEDD9 levels vary by tissue, cell type, and cell growth conditions. Normal human fetal brain, adult kidney, lung, and tissue rich with immature lymphoid cells have the highest levels of NEDD9 mRNA and protein (Kumar et al. 1992; Law et al. 1996; Minegishi et al. 1996).

Various dynamic stimuli can regulate NEDD9 abundance and phosphorylation (reviewed extensively in Tikhmyanova et al. 2010). While NEDD9 sequence analysis predicts a molecular weight of 93 kDa, NEDD9 protein migrates as a doublet at 105 kDa and 115 kDa (Law et al. 1996, 1998). The higher molecular weight band reflects the fact that this population of NEDD9 is highly phosphorylated. NEDD9 phosphorylation, binding to associated proteins, and localization is dependent on various factors including protein association and cell cycle phase (Law et al. 1998).

NEDD9 has multiple domains that dictate partner binding and have conserved structure homology with other CAS family members. These domains include a SH3 (Src-homology) domain that confers binding to FAK (Law et al. 1996), a substrate domain to bind SH2-containing proteins (Law et al. 1996), a serine-rich domain (Singh et al. 2008), and a carboxy-terminal domain important for adhesion (Law et al. 1996). The validated partners of NEDD9 are plentiful but the primary defined oncogenic partners are SRC, focal adhesion kinase (FAK), and Aurora-A kinase.

Direct binding and phosphorylation of NEDD9 by SRC and FAK is a critical component of driving the integrin-mediated attachment and adhesion processes (Law et al. 1996; Minegishi et al. 1996; Natarajan et al. 2006). NEDD9 positively regulates migratory signals driven through FAK and SRC (Tikhmyanova et al. 2010). NEDD9 is also involved in cell cycle regulation through its interaction with Aurora-A kinase (Pugacheva and Golemis 2005). Aurora-A activation at mitotic entry requires NEDD9 binding; in addition, activated Aurora-A phosphorylates NEDD9, likely so that NEDD9 is able to take part in cellular re-spreading processes at focal adhesions during cytokinesis (Pugacheva and Golemis 2005). Furthermore, NEDD9 is required for the role of Aurora-A in ciliary resorption (Pugacheva et al. 2007). Cilia represent sites of action for important cancer-regulatory signaling, and NEDD9 has the potential to broadly influence cancer cell-signaling processes through this function.

NEDD9 has no catalytic activity and is intracellular rather than cell-surface exposed, limiting its potential as a target for development of either small molecule or antibody-based therapeutic agents. However, its partnership with and regulation of key oncogenes such as Aurora-A, SRC, and FAK, as well as its implication in tumor initiation and metastasis, make NEDD9 an important target to investigate for clinical biomarker use in invasive or metastatic disease.

Target Assessment

Total NEDD9 levels can be measured by immunohistochemistry on the tissue. Activation-associated phosphorylation status of NEDD9 is conveniently assessed by western blot of tissues or purified cells. Two NEDD9 antibodies are widely

commercially available for western blotting and immunohistochemical staining. The 14A11 NEDD9 antibody, corresponding to aa 82-398, primarily recognizes NEDD9 at the centrosomes of mitotic cells (Pugacheva and Golemis 2005). The 2G9 antibody recognizes the full-length forms of NEDD9 (Pugacheva and Golemis 2005). Additionally, NEDD9 is often upregulated at the gene or mRNA level in aggressive tumors, thereby making RT-PCR detection a readily viable method (Tikhmyanova et al. 2010).

Role of the Target in Cancer

Rank: Unknown to 1-2-3-4-5-6-7-8-9: 6-7

High-Level Overview

There are extensive studies linking elevated NEDD9 expression to aggressive behavior of multiple tumor types. These studies include in vitro cell culture experiments, mouse models of tumorigenesis and metastasis, and analysis of expression data from patient tumor samples. There is evidence that genetic ablation of NEDD9 limits tumor initiation, and depletion of NEDD9 can make cancer cells more sensitive to drug treatment. Given the rationale for NEDD9 as a biomarker and the capabilities to detect NEDD9 expression at the protein and mRNA levels, NEDD9 is well placed for future use as a biomarker for aggressive and metastatic disease.

Diagnostic, Prognostic, Predictive

The primary tumor cell types that have associated perturbation of NEDD9 function and signaling include breast, melanoma, lung, T-cell leukemia, and glioblastoma (Tikhmyanova et al. 2010). Increased NEDD9 expression has also been linked to metastatic colorectal cancer and HNSCC (Kim et al. 2010; Lucas et al. 2010). *NEDD9* levels are upregulated in 34% of lung adenocarcinomas (Ji et al. 2007) and in glioblastoma cells; FAK or PDGF-dependent activation of NEDD9 results in enhanced migration and metastasis (Natarajan et al. 2006). Hyperphosphorylated *NEDD9* is typical in T-cell leukemias, particularly, leukemias resulting from human T-lymphotropic virus type 1 (HTLV-1) infection where NEDD9 binds viral Tax protein to prevent Tax-dependent activation of NF-kB (Iwata et al. 2005). In addition, BCR-ABL phosphorylates NEDD9 to activate cell migration in CML and AML (Tikhmyanova et al. 2010). Hypoxia significantly upregulates NEDD9 expression in colorectal cancer cell lines, and NEDD9 levels are high in hypoxic regions of human colorectal cancer (Kim et al. 2010).

High levels of NEDD9 were implicated as enhancing melanoma metastasis of mouse models driven by Ras and Met through the use of comparative genome hybridization (CGH) (Kim et al. 2006). This study indicates that NEDD9 could

enhance motility of primary melanocytes, but is insufficient for transformation. However, interactions between NEDD9 and FAK enhance the metastasis of melanocytes transformed with either H-RAS^{V12G} or B-RAF^{V600E}oncogenes, and 35% of human metastatic samples have elevated NEDD9 (Kim et al. 2006). This data is consistent with studies indicating that 57% of human metastatic melanoma tissues show gain of the chromosome region 6p24 that contains NEDD9 (Moore et al. 2008). Additionally, an analysis of the growth and metastasis of human melanoma cell line xenografts shows that NEDD9 expression is high in all metastatic cell lines, but overexpression is insufficient to induce metastasis on its own (Rozenberg et al. 2010). The sum of these data makes a compelling case for the role of NEDD9 driving melanoma metastasis.

At present, the evidence for the precise role of NEDD9 in breast cancer is conflicting. Multiple in vitro experiments indicate that NEDD9 significantly promotes invasion of MCF7 breast adenocarcinoma cells, possibly through its induction of ErbB2 and matrix metalloproteases (Fashena et al. 2002). Additionally, heregulin-stimulated MCF7 cells utilize NEDD9 at mRNA and protein levels (Nagashima et al. 2008). However, two separate groups have indicated conflicting data on the role of NEDD9 in migration and metastasis. The gene signature of TGF-b-stimulated MDA-MD-231 breast cancer cell metastasis to the lung indicates a threefold downregulation of *NEDD9* (Minn et al. 2005). In addition, MCF-10A cells have been reported to be less migratory after siRNA-mediated downregulation of *NEDD9* (Simpson et al. 2008). It is possible that the levels of NEDD9 are controlled as a rheostat at the cellular level and tumors must be able to manipulate NEDD9 levels within the microenvironment.

Recent work has shown that *Nedd9* is extremely important for early stages of mammary tumor formation (Izumchenko et al. 2009; Little et al. 2014). Nedd9 null mice crossed to MMTV-polyomavirus middle T antigen (PyVT) animals exhibit a significant delay in tumor formation, size, and total burden (Izumchenko et al. 2009). More recent work crossing *Nedd9*$^{-/-}$mice to the MMTV-*neu* (HER2, ErbB2) model of mammary tumor development has revealed an even more striking necessity for *Nedd9* in mammary tumor formation (*unpublished data)*. In fact, a recent screen to identify genes that promote drug resistance to EGFR-targeted therapies, such as erlotinib, reveals NEDD9 as a powerful controller of cellular survival upon drug treatment (Astsaturov et al. 2010). To date, NEDD9 has no known catalytic function, but acts as a scaffold for various oncogenic cellular partners described. As targeted therapeutics against NEDD9's oncogenic partners Aurora-A and SRC move through clinical trials, it will become important to determine how NEDD9 status affects sensitivity to such agents.

Therapeutics

NEDD9 has no catalytic activity and is intracellular, so is not obviously druggable. However, it is possible to develop inhibitors to disrupt NEDD9 interactions with oncogenic partners (Tikhmyanova et al. 2010). It is also possible that targeting the

most important signaling kinase partners to NEDD9 can yield a potential way to target activity without directly drugging NEDD9 (Astsaturov et al. 2010).

Preclinical Summary

The most promising tumor types for which NEDD9 may be used as a biomarker are melanoma, HNSCC, and breast cancer. More work needs to be done investigating (1) NEDD9 in human breast tumors and (2) the effect of NEDD9 status on sensitivity to oncogenic therapeutics. Key references include the characterization by Kim et al. of NEDD9's role in Ras- or Met-driven melanomas (Kim et al. 2006), the report of the *Nedd9* knockout (Seo et al. 2005), and the description of the phenotypes resulting when the *Nedd9* knockout mouse is crossed to the MMTV-PyVT model (Izumchenko et al. 2009).

Clinical Summary

There are no active or current protocols for the use of NEDD9 as a biomarker for aggressive or metastatic disease and no therapeutic inhibitors of NEDD9. However, there is accumulating data demonstrating NEDD9 as a biomarker for metastatic disease (Kim et al. 2006; Lucas et al. 2010) and documenting the ability for (1) inhibitors of NEDD9-interactors to sensitize tumor cells to death induced by EGFR-targeted therapy (Astsaturov et al. 2010) and (2) for mammary tumor cells to have differential sensitivities to cancer therapeutics based on the presence or loss of *NEDD9* (Singh et al. 2010). Therefore, NEDD9 represents a viable and likely biomarker.

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N-Ras

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Abstract

RAS (first identified as being responsible for the transforming activity in rat sarcoma viruses) proteins are a large family of low molecular weight guanosine triphosphate (GTP)-binding proteins that localize to the plasma membrane. Within this superfamily, the RAS proteins primarily regulate growth, whereas the closely related Rho proteins regulate the actin cytoskeleton (Sahai and Marshall, Nat Rev Cancer 2:133–142, 2002; Cully and Downward, Cell 133:1292–1292, 2008; Downward, Nat Rev Cancer 3:11–22, 2003). Three of the RAS family members, NRAS, HRAS (Harvey rat sarcoma virus), and KRAS

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(Kirsten rat sarcoma virus), are often mutated in human cancers, and >20% of all tumors harbor activating mutations in one of their RAS genes (Downward, Nat Rev Cancer 3:11–22, 2003). HRAS and NRAS are not required for normal embryonic development, where KRAS knockout is embryonically lethal (Downward, Nat Rev Cancer 3:11–22, 2003).

Keywords
Farnesyltransferase inhibitors (FTIs) • Harvey rat sarcoma virus (HRAS) • Kirsten rat sarcoma virus (KRAS) • MEK inhibition • NRAS assessment • Farnesyltransferase inhibitors (FTIs) • In cancer • MEK inhibition • MEK inhibitors • Melanoma oncogenes • Mutations in • PLX4032 • Rapamycin analog inhibitors • Serine/threonine kinase RAF • Tipifarnib • PLX4032 • Rat sarcoma viruses (RAS) • HRAS • KRAS • NRAS (*See* NRAS) • Phosphoinositide 3-kinase (PI3K)/AKT pathway • Receptor tyrosine kinase (RTK)-induced activation

Target: NRAS

RAS (first identified as being responsible for the transforming activity in rat sarcoma viruses) proteins are a large family of low molecular weight guanosine triphosphate (GTP)-binding proteins that localize to the plasma membrane. Within this superfamily, the RAS proteins primarily regulate growth, whereas the closely related Rho proteins regulate the actin cytoskeleton (Sahai and Marshall 2002; Cully and Downward 2008; Downward 2003). Three of the RAS family members, NRAS, HRAS (Harvey rat sarcoma virus), and KRAS (Kirsten rat sarcoma virus), are often mutated in human cancers, and >20% of all tumors harbor activating mutations in one of their RAS genes (Downward 2003). HRAS and NRAS are not required for normal embryonic development, where KRAS knockout is embryonically lethal (Downward 2003).

The first activating oncogenic mutation to be reported in melanoma was in *NRAS* (Padua et al. 1984, 1985). Mutations in *NRAS* have since been identified in 15–20% of all melanomas and are most commonly the result of a point mutation leading to the substitution of leucine to glutamine at position 61 (Brose et al. 2002; Davies et al. 2002). *NRAS* mutations have also been reported at positions 12 and 13 (Lin et al. 2008). Other tumors known to have mutations in *NRAS* include liver cancers, thyroid carcinomas, and seminomas. Mechanistically, the acquisition of point mutations in *NRAS* leads to impairment of GTPase activity, so that the GTP-bound NRAS is more abundant than GDP-bound NRAS leading to the dysregulation of intracellular signaling.

In its GTP-bound state, RAS binds to and activates a number of effector enzymes involved in proliferation. The best characterized of these is the serine/threonine kinase RAF (named after its ability to induce “rapidly growing fibrosarcomas” in mice), which constitutes a three-membered family consisting of ARAF, BRAF, and CRAF (also called Raf-1) (Dhomen and Marais 2007). Most of the oncogenic activity of RAF is mediated through activation of the mitogen-activated protein kinase (MAPK) cascade, which regulates the cell cycle entry through control of cyclin D1 expression

(Sahai and Marshall 2002). Pharmacologic inhibitors of RAF kinases are emerging, but the potent and selective inhibitors of CRAF have not been developed, making it difficult to describe the sensitivity of *NRAS*-mutant cancer models. However, highly selective MEK inhibitors (with greatest potency against MEK1 and MEK2) have been in development for a decade. In vitro, panels of *NRAS*-mutant melanoma are variably sensitive to MEK inhibition with such agents, with inhibition of proliferation rather than induction of apoptosis being the most reproducible finding. While it remains possible that different pharmacologic inhibitors (such as CRAF or ERK inhibitors) could reveal greater vulnerability in *NRAS*-mutant cancers, it is also possible that other RAS effector pathways are critical contributors to the transforming properties of *NRAS*. RAS is also known to activate the phosphoinositide 3-kinase (PI3K)/AKT pathway, which contributes to tumor progression via the modulation of growth and survival of transformed cells (Sahai and Marshall 2002). In addition to MAPK and PI3K/AKT, mutated *NRAS* can also activate other intracellular signaling pathways important for malignant transformation. In particular, recent studies have demonstrated the importance of Ral guanine nucleotide exchange factors (Ral-GEFs) in the anchorage-independent growth observed following the *NRAS*-mediated melanocytes transformation (Mishra et al. 2010). The relative importance of each RAS effector pathway in driving the malignant phenotype has not been determined. The available evidence with MEK inhibitors suggests that there will be heterogeneity in this regard even within the subset of *NRAS*-mutated tumors.

Biology of the Target

The presence of an activating *NRAS* mutation in a melanoma sample can be used as a criterion to exclude patients from a BRAF inhibitor clinical trial (Flaherty et al. 2009). Currently, the detection of mutated *NRAS* in melanoma samples is not routinely used for either diagnostic or prognostic purposes, largely because there is no treatment strategy that has been developed that directly or indirectly targets NRAS. However, it is likely that as the personalization of melanoma therapy continues, mutational profiling for a spectrum of melanoma oncogenes (*BRAF*, *NRAS*, *c-Kit* (the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) could become commonplace as unique therapeutic strategies are developed for each.

Target Assessment

The presence of *NRAS* mutations can be measured by extracting tumor DNA using punch biopsy or fine needle aspirate followed by sequencing of exons 12, 13, and 61 of the gene using PCR amplification and mutational sequencing. Such a method is standard in the assessment of KRAS mutations in colorectal cancer, as a means for excluding patients from cetuximab therapy. As there is no therapy related to NRAS mutation status in cancer, NRAS mutation testing has not been formally developed for regulatory approval.

Role of the Target in Cancer

Rank: “unknown” to 10
Unknown to 1-2-3-4-5-6-7-8-9-10:7.

High-Level Overview

Diagnostic, Prognostic, and Predictive

There is emerging evidence that melanomas can be grouped according to their initiating oncogenic events (Smalley et al. 2009). A recent pathological study where melanomas were subdivided according to *BRAF*/*NRAS* mutational status showed *BRAF*-mutated melanomas have distinct morphological features (Viros et al. 2008). It was found that *BRAF*-mutated melanomas had an increased tendency to upward migration and nest formation and gave rise to larger, rounded, and more pigmented tumor cells. In contrast, *NRAS*-mutated melanomas were not found to exhibit these morphological and phenotypic characteristics (Viros et al. 2008). However, there is emerging evidence that *NRAS*-mutant primary melanomas may pose a higher metastasis as they tend to be more deeply invasive at the time of initial diagnosis than *BRAF*-mutated melanomas and have a higher mitotic rate, on average (McArthur et al. 2010). At this juncture, no *NRAS*-specific therapies exist and there is little evidence to suggest that *NRAS*-mutated melanomas follow a different clinical course to *BRAF*-mutated melanomas.

Therapeutics

NRAS is a small GTPase and thus a difficult target for conventional drug discovery (Downward 2003). Farnesyltransferase inhibitors (FTIs), a class of drugs that prevent the membrane localization (and thereby activation) of small G proteins, were originally developed as agents to target oncogenic RAS signaling (Konstantinopoulos et al. 2007) by inhibiting a key posttranslational modification that permits membrane localization of all RAS isoforms. Membrane localization is essential in order for RAS to complex with RAS effector proteins, so inhibition of farnesylation appeared to deplete the pool of RAS available to drive downstream signaling. Of course, all RAS isoforms are farnesylated, so this type of intervention would not be expected to be selective for NRAS. Furthermore, a vast array of intracellular proteins are farnesylated, so FTIs could not exert a selective effect on RAS signaling. Despite being evaluated in many cancer context, these compounds have shown little single-agent activity, even in colorectal carcinoma where ~40% of the tumors have activating mutations in *KRAS* (Konstantinopoulos et al. 2007). In several clinical trials, assessment of target inhibition was assessed with serial tumor biopsies, and in such cases, only modest inhibition of RAS signaling has been observed when these agents are administered at the maximum-tolerated doses. These results leave open the possibility that the effect of FTIs on non-RAS proteins that require farnesylation

was responsible for dose-limiting toxicities and limited the ability to deliver a more effective anti-RAS dose.

FTIs have never been evaluated in a clinical trial of melanoma patients selected for their *NRAS* status, one of potential proof-of-concept populations for this approach. Only a small phase II trial was undertaken in genetically unselected metastatic melanoma patients in which NRAS mutation status was not tested even in retrospect (Gajewski et al. 2006). However, given the concerns raised above, this class of agents may not be the best test of the therapeutic value of NRAS blockade.

Instead, attention has now turned to pathways that are downstream of Ras activation that are more tractable to pharmacological intervention. There is now good preclinical evidence that simultaneous blockade of the MEK and PI3K pathways leads to the regression of RAS-driven tumors in animal models (Hoeflich et al. 2009; Engelman et al. 2008) and would potentially serve some portion of the subpopulation of NRAS-mutated cancers that are not susceptible to single-agent MEK inhibition. Other preclinical studies have shown that dual inhibition of BRAF and CRAF or BRAF and PI3K (using shRNA knockdown) was effective at reducing the growth and survival of *NRAS*-mutated human melanoma xenografts (Jaiswal et al. 2009). The possibility of pharmacologically targeting RAF signaling as an approach for NRAS-mutant melanoma has not been thoroughly explored.

A selective RAF inhibitor, PLX4032, has demonstrated remarkable efficacy in BRAF-mutant melanoma (Flaherty et al. 2010). This agent appears capable of inhibiting both BRAF and CRAF and nearly equal potencies in isolated kinase assays (Bollag et al. 2010). However, it has been described that BRAF and CRAF form homodimers and heterodimers in their activated state (Rushworth et al. 2006). An activated RAF complex contains one inactivated and one activated protomer. PLX4032 and RAF inhibitors with similar selectivity appear to inhibit one protomer in the BRAF/CRAF heterodimer or CRAF/CRAF homodimer and facilitate signaling through CRAF (Poulikakos et al. 2010; Heidorn et al. 2010). This effect appears to be irrelevant in BRAF-mutated cancers in which BRAF activation of MEK is the dominant signaling process. But, in NRAS-mutant melanoma, this effect appears to hyperstimulate signaling through RAF to ERK. These observations raise the theoretical possibility that a RAF inhibitor that disrupts or prevents dimer formation could fully inhibit RAF signaling and have therapeutic potential in NRAS-mutant cancers. While MEK inhibitors are plausible agents for the purpose of inhibiting MAP kinase signaling, as discussed above, CRAF appears to have MEK-independent effects that may be important in cancer pathophysiology (Hood et al. 2002).

It is also possible that MEK inhibition is a suboptimal strategy for blockade of NRAS signaling due to feedback regulation that occurs as consequence of pharmacologic inhibition. Under normal physiologic conditions, activated RAS, leading to ERK activation, will cause the upregulation of the negative regulator of RAF signaling, sprouty, and the dual-specific phosphatases that inactivate MEK (Pratilas et al. 2009). MEK inhibition downregulates these feedback mechanisms, resulting in increased RAF and MEK activation. While ERK inhibition is maintained in the presence of a MEK inhibitor, the upstream activation of RAF could provide an

escape mechanism through non-MEK-dependent mechanisms alluded to above (Hood et al. 2002). ERK is not regulated by these same feedback mechanisms, making ERK inhibition a potential point of intervention with which one would not observe the upstream activation of RAF and MEK. This concept is likely to be tested clinical in the near future.

Preclinical Summary

A role for *RAS* mutations in melanoma initiation has been confirmed in animal models, where the introduction of mutated *HRAS* or *NRAS* (Q61K) leads to melanoma in transgenic mice lacking expression of the CDK inhibitor $p16^{INK4A}$ (Chin et al. 1997; Ackermann et al. 2005). Melanomas harboring activating *NRAS* mutations are different from melanomas with *BRAF* mutations in that they rely upon CRAF to induce their MAPK pathway activity (Dumaz et al. 2006). In normal melanocytes, receptor tyrosine kinase (RTK)-induced activation of RAS leads to the stimulation of both BRAF and CRAF (Dumaz et al. 2006). Under these conditions, activation of the MAPK pathway only proceeds via BRAF, as constitutive protein kinase A (PKA) activity leads to the phosphorylation and inactivation of CRAF. In melanomas with *NRAS* mutations, the cyclic AMP/PKA system is deregulated, so that PKA no longer suppresses CRAF, allowing CRAF-mediated MAPK activation to occur (Dumaz et al. 2006). Thus, as noted previously, RAF inhibitors that are able to block both BRAF and CRAF activity are of interest in NRAS-mutated cancers.

Clinical Summary

One FTI, tipifarnib, was evaluated in a single-agent, single-arm phase II trial among patients with metastatic melanoma (Gajewski et al. 2006). The lack of response among the first 14 patients enrolled led to planned closure of the trial. These patients were unselected with regard to NRAS mutation status. Given the 15–20% prevalence of NRAS mutations across all types of melanoma, it is probable that only one or two patients in the trial harbored an NRAS mutation. Another phase II trial was conducted combining tipifarnib and sorafenib in genetically unselected patients, with the hypothesis that such a combination would antagonize two distinct points in the MAP kinase pathway. This regimen was associated minimal clinical activity and was abandoned after the first stage of accrual (Margolin et al. 2010).

Combinations of MEK inhibitors with PI3K, Akt, or mTOR inhibitors are anticipated to be effective for a subset of NRAS-mutant melanomas. Several potent and selective inhibitors are MEK have emerged from phase I clinical trials. The most active of them, GSK2110212, is associated with tumor regression in a subset of patients with BRAF wild-type melanoma, nearly half of whom would be expected to have NRAS mutations (Infante et al. 2010). But RAF or ERK inhibitors could

ultimately prove to be a superior foundation upon which to build a combination RAS-effector blocking strategy in the future.

Potent and selective PI3K, Akt, and mTOR kinase inhibitors have yet to be evaluated in cancers harboring NRAS mutations. Rapamycin analog inhibitors are increasingly abundant, and one such agent has been tested as a single agent in a small cohort of metastatic melanoma, but prospective or retrospective genetic characterization of patients' tumors was not performed. There are concerns that rapamycin analogs might be suboptimal in cancers driven by oncogenic RAS due to the feedback upregulation of Akt signaling that has been documented preclinically (Werzowa et al. 2009). So, inhibitors of PI3K, Akt, or mTOR kinase (which would inhibit TORC1 and TORC2 signaling and not result in Akt activation) are of interest in NRAS-mutant cancers. However, little is known about the ideal point of intervention among the PI3K and Akt isoforms in the setting of NRAS mutations. So, preclinical and clinical investigations are proceeding with a variety of isoform-selective PI3K inhibitors, pan-PI3K inhibitors, dual PI3K/mTOR kinase inhibitors, and a smaller number of Akt and selective mTOR kinase inhibitors. From preclinical investigations with these agents in NRAS-mutant models, we may gain further insight into the key RAS effector molecules in the PI3K pathway and refine the selection of an agent for use as a single agent or in combination with optimal RAF-MEK-ERK blockade.

Anticipated High-Impact Results

The first combination regimen of a MEK inhibitor (AZD6244) with an inhibitor of a component of the PI3K pathway (MK-2206 targeting Akt) will be ready for phase II evaluation in 2011 (NCT01021748). This represents the first of what is anticipated to be a growing constellation of combination regimens targeting RAF-ERK and PI3K pathway signaling in NRAS-mutant cancers, with metastatic melanoma being the clinical context for evaluation given the prevalence of NRAS mutations in this disease and absence of effective systemic therapies currently for these patients.

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P38

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Hyuk-Jin Cha and Albert J. Fornace Jr.

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Abstract

Following stimulation of the relevant upstream receptors, MAPKs mediate intracellular signaling through sequential phosphorylation of substrates containing the consensus sequence Ser/Thr Pro, once the corresponding upstream MAPK kinases (MKKs) are activated. Eventually, the serial phosphorylation results in the phosphorylation and consequent activation of various transcription factors and induces a set of gene response(s) depending on the extracellular stimuli. p38 MAPK belongs to the family of MAPKs, which also includes extracellular signal-regulated kinase (ERKs) and c-Jun N-terminal kinases (JNKs), which transduce the extracellular signals to achieve adequate gene responses depending on the

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stimuli (Lewis et al. 1998). The mammalian p38 consists of four isoforms (α, β, γ, and δ), which differ in their expression pattern and signaling pathways. p38α and β are ubiquitously expressed, whereas the remaining p38 isoforms are expressed in a more tissue-specific manner. p38 can be activated by a wide range of stresses and has major roles in inflammatory and immune signaling. Like some other stress signaling proteins, p38 has been shown to have tumor suppressor features in normal cells, but in some tumor cells pro-oncogenic-like signaling may be unmasked.

Keywords

Chemotherapy agents • Circulating tumor cells (CTCs) • Disseminating tumor cells (DTCs) • Dual specificity phosphatases (DUSP) • Estrogen Receptor a (ERa) • Extracellular signal-regulated kinase (ERK) • Oncogenic-induced senescence (OIS) • p38 Mitogen activated protein kinase (MAPK) • Tamoxifen

Target: p38 Mitogen-Activated Protein Kinase (MAPK) α (*MAPK14*)

Following stimulation of the relevant upstream receptors, MAPKs mediate intracellular signaling through sequential phosphorylation of substrates containing the consensus sequence Ser/Thr-Pro, once the corresponding upstream MAPK kinases (MKKs) are activated. Eventually, the serial phosphorylation results in the phosphorylation and consequent activation of various transcription factors and induces a set of gene response(s) depending on the extracellular stimuli. p38 MAPK belongs to the family of MAPKs, which also includes extracellular signal-regulated kinase (ERKs) and c-Jun N-terminal kinases (JNKs), which transduce the extracellular signals to achieve adequate gene responses depending on the stimuli (Lewis et al. 1998). The mammalian p38 consists of four isoforms (α, β, γ, and δ), which differ in their expression pattern and signaling pathways. p38α and β are ubiquitously expressed, whereas the remaining p38 isoforms are expressed in a more tissue-specific manner.

The ERK pathway predominantly governs growth factor-mediated proliferation signals, while p38 and JNK primarily mediate stress or inflammatory responses following various cytokine or genotoxic stimuli (Lewis et al. 1998). Similar to the other MAPKs, p38 is activated by MAPK-specific upstream signaling events, referred to as the "MAPK signaling cascade." MKK6 and MKK3 are upstream kinases that specifically activate p38 through direct phosphorylation of threonine and tyrosine residues (TXY motif, which is conserved throughout all MAPKs) within the activating T loop (Lewis et al. 1998). Alternately, p38 MAPK can be activated in MKK3/6-independent manner, either by Zap70-dependent alternative tyrosine phosphorylation (Salvador et al. 2005) or TAB1-dependent autophosphorylation (Ge et al. 2002). p38 activation has been shown to be involved in inflammatory responses, cell death, cell differentiation, and cell cycle arrest, which have been intensively reviewed elsewhere (Hui et al. 2007). In mice, p38α knockout is embryonic lethal due to a defect in placental development (Adams et al. 2000) or erythropoiesis (Tamura et al. 2000). In

cellular models, p38 has proven to be an important signaling mediator in inflammatory responses (Kumar et al. 2003), stress responses, and tumor suppression (Bulavin and Fornace; Jr 2004). The excessive mitogenic stimulation induced by the action of oncoprotein(s), e.g., Ras mutation triggers sequential activation of ERK and p38. Activation of p38 by oncogenic stress leads to the induction of cellular senescence (also frequently referred as oncogenic-induced senescence (OIS), to protect cells from oncogenic transformation (Lee et al. 2011). Induction of p16Ink4a, a cyclin-dependent kinase inhibitor (CDKI), by p38 activation under oncogenic stress is responsible for OIS (Kwong et al. 2009). Activated p38, in turn, further inhibits mitogenic or survival signals by negatively regulating MEK/ERK activity (Li et al. 2003). Such inhibition of MEK/ERK signaling following p38 activation is a result of the direct activation of protein phosphatase 2A (PP2A), a phosphatase responsible for dephosphorylating active MEK/ERK (Liu and Hofmann 2004).

Biology of the Target

Reflecting on the role of p38 as a tumor suppressor, abrogation of p38 under oncogenic stimuli is often necessary during tumorigenesis. Thereby, significant suppression of p38 protein expression and activity are observed in human lung cancer (Ventura et al. 2007) and hepatocellular carcinoma (Iyoda et al. 2003). Consistently, in an animal model, the conditional deletion of p38 in the lung and colon was sufficient to favor oncogene-induced (Ventura et al. 2007) or spontaneous tumorigenesis (Wakeman et al. 2012), respectively. An inactive mutation of p38 has not yet been identified in cancer; hence, an alternative mechanism(s) to weaken p38 activity under oncogenic challenges must be present in cancers. Unlike other MAPKs, where the active phosphorylation is eliminated by the family of dual-specificity phosphatases (DUSP), p38 is dephosphorylated by both the DUSPs and by Wip1 phosphatase (abbreviation for initial description of this protein: wild-type p53-induced phosphatase), which is a member of the protein phosphatase C family (Takekawa et al. 2000). Thus, the premature loss of p38 activity following Wip1 expression is considered an important mechanism in the abrogation of p38/16Ink4a-dependent tumor suppression (Bulavin et al. 2004). In breast cancer patients, Wip1 expression bears an inverse correlation with the active phosphorylation of p38 or with p16Ink4a expression (Yu et al. 2007a). These results suggest that gene amplification of Wip1 favors tumorigenesis by functioning as a negative regulator of p38 activity. This hypothesis is corroborated by the fact that DUSP26 preferably dephosphorylates p38 and inhibits p38-dependent tumor surveillance (Yu et al. 2007b).

Target Assessment

Although the inhibition of p38 expression or activity was reported in human lung cancer (Ventura et al. 2007), hepatocellular carcinoma (Iyoda et al. 2003), and a number of solid cancers (Liao and Hung 2003), the active form of p38 is also

observed in lung cancers (Lee et al. 2011), prostate cancer (Uzgare et al. 2003), thyroid cancer (Pomerance et al. 2006), and in tamoxifen-resistant breast cancers (Gutierrez et al. 2005). Therefore, it would be an overgeneralization to ascribe p38 expression level or activity for cancer therapy assessment. It is worth noting that DNA damage checkpoint proteins such as ATM, Chk2, p53, and γ-H2AX, which can function as important tumor suppressors, are also found to be active in precancerous lesions (Gorgoulis et al. 2005). In the case of p38, cancer-promoting properties may well be unmasked in tumor cells where key tumor suppressor pathways, e.g., p53, have been inactivated. Therefore, rather than evaluating the role of p38 in cancer, assessing the role of the negative regulators of p38 (such as Wip1 or DUSP26) as oncogenes to abrogate p38-dependent tumor suppression,has presented a superior correlation with cancer. For example, gene amplification of the protein phosphatase 1D magnesium-dependent gene *PPM1D*, which encodes the Wip1 protein, was identified in breast adenocarcinoma, ovarian adenocarcinoma, neuroblastoma, medulloblastoma, gastric carcinoma, and pancreatic adenocarcinoma (Lu et al. 2008). DUSP26 is located in chromosome 8p12, where gene amplification is frequently observed in a several cancers, including breast, urinary bladder, and lung (Simon et al. 2001; Theillet et al. 1993), as well as in thyroid cancer (Yu et al. 2007b) and neuroblastoma (Shang et al. 2010).

Role of the Target in Cancer

Rank: “unknown” to 10

Unknown to 1-2-3-4-5-6-7-8-9-10: 8

Diagnostic, Prognostic, Predictive

Although p38 functions as an important tumor suppressor, high levels of active p38 expression in cancer were used to determine the status of the disease. Several studies have utilized this role of p38; the presence of active p38 has been reported to be a potential diagnostic marker for tamoxifen-resistant breast cancer (Massarweh et al. 2008), while high expression of active p38 can be used to determine malignancy in follicular thyroid carcinoma (FTA) but not in follicular adenoma (FA) (Pomerance et al. 2006). In line with this, the gene expression of Wip1 and DUSP26, negative regulators of p38, is significantly amplified in various types of cancer, thus serving a diagnostic or prognostic role (Lambros et al. 2010; Yu et al. 2007a). For prognostic purpose, Wip1 expression showed a significant correlation with the overall survival of patients suffering from lung cancer (Satoh et al. 2011) but not breast cancer (Lambros et al. 2010; Yu et al. 2007a). In case of DUSP26, the prognostic significance in cancer survival remains undetermined.

Therapeutics

The induction of cell death by various conventional DNA- or microtubule-damaging chemotherapeutics in cancer is markedly attenuated following inhibition of p38 activity; therefore, concomitant p38 activation with conventional chemotherapy might achieve a synergistic effect in cancer treatment (Olson and Hallahan 2004). Thus, the development of a specific activator of p38 but not other MAPKs would be an important approach to augment the chemotherapeutic activity of conventional anticancer agents. However, no specific activator of p38 has been developed so far due to close structural similarity among the various MAPKs. Thereby, instead of directly targeting p38 using a chemical approach, targeting the aforementioned negative regulators of p38, such as Wip1 and DUSP26 which are closely associated with cancer incidence or patient survival, has been suggested as a more rational drug development approach (Lu et al. 2008; Nunes-Xavier et al. 2011).

Several groups have attempted to develop and characterize specific inhibitors of Wip1. The cyclic phosphopeptides, which were designed based on the conserved sequence of Wip1, demonstrated a potent inhibitory effect on Wip1 phosphatase activity (Yamaguchi et al. 2006). Further optimization of the substrate-mimetic phosphopeptides of the Wip1 inhibitor was performed to develop 50-fold higher potent inhibition, with increased specificity for Wip1 over other PP1A phosphatases (Hayashi et al. 2011). Soon after the development of the cyclic phosphopeptides, a drug-like selective inhibitor of Wip1 was reported on the basis of the molecular scaffold of the cyclic phosphopeptide (Bang et al. 2008). Additionally, a number of groups reported the discovery of various Wip1 chemical inhibitors using a chemical library screen. The first 14 Wip1 chemical inhibitors were developed following the screening of 1990 compounds at the National Institute of Health (NIH), the most potent of which increased p38 activity and demonstrated an antiproliferative effect, not only in Wip1-amplified breast cancer cells but also in mammary tumors in a mouse xenograft tumor model (Belova et al. 2005). The use of more intense high-throughput screening with 65,500 chemical compounds resulted in the identification of one specific, potent, and cell-permeable compound that could selectively induce cell death in Wip1-expressing breast cancer cells in a p38-dependent manner, since co-inhibition of p38 was shown to rescue cell death by a Wip1 inhibitor (Rayter et al. 2008). Treatment of ovarian cancer cells, which express high levels of Wip1, with the same compound resulted in selective cell death in a p38-dependent manner; additionally, the cells remained viable following treatment with the compound following Wip1 depletion, suggesting its higher selectivity to Wip1 (Tan et al. 2009). The chemical compound used in the aforementioned study is widely used for the selective inhibition of Wip1, being the only commercially available Wip1 inhibitor to date (Chock et al. 2010; Tan et al. 2009). However, an unexpected off-target effect of the inhibitor toward JNK has been demonstrated in skin keratinocytes (Lee et al. 2014).

Independent of this chemical compound, another novel potent Wip1 inhibitor with a different chemical structure was developed through a chemical library

screening and demonstrated an antiproliferative effect on a Wip1-expressing breast cancer cell line (Yagi et al. 2012).

In a similar fashion, inhibitors of DUSP are currently being developed (Nunes-Xavier et al. 2011). However, unlike the Wip1 inhibitor, which can induce spontaneous cell death of Wip1-expressing cancer cells in a p38-dependent manner, the DUSP inhibitors have been suggested as adjuvant therapeutics in cancer treatment (Tsujita et al. 2005). Of note, a SHP1/2 protein tyrosine phosphatase (PTP) inhibitor was found effective against DUSP26, thereby promoting p38 activation (Song et al. 2009). Nevertheless, specific inhibitor(s) of DUSP26 for promoting spontaneous p38-dependent cell death are yet to be developed.

Preclinical Summary

As the pro-inflammatory effect of p38 has been well-characterized suggesting that p38 is an effective target of inflammatory diseases (Kumar et al. 2003), preclinical tests of specific p38 inhibitors have particularly focused on inflammatory diseases (Hope et al. 2009). Considering the role of p38 as a tumor suppressor, long-term systemic treatment of inflammatory diseases with p38 inhibitors is concerning because of interference with p38-dependent tumor protective mechanisms (Coulthard et al. 2009). Unlike the active preclinical trial of p38 in inflammatory diseases, a preclinical approach targeting p38 has not been sufficiently explored. Chemotherapy agents can significantly attenuate cancer cell death by inhibiting p38; therefore, p38 activation using specific activators or by activating the upstream kinases during chemotherapy has been suggested to be an effective cancer therapeutic approach (Olson and Hallahan 2004). However, in the absence of specific p38 activators, a majority of the preclinical approaches have been directed to the p38 phosphatases such as Wip1 or DUSP26.

However, the therapeutic strategy to target p38 in cancer cannot be simplified to the activation of p38 or inhibition of its negative regulators. To the contrary, the positive role of p38 activity in growth (Chiacchiera et al. 2009), survival (Comes et al. 2007), metastasis (Kumar et al. 2010), angiogenesis (Yoshizuka et al. 2012), and drug resistance (Milone et al. 2013) of certain types of cancer have been recently demonstrated. The direct phosphorylation of estrogen receptor a (ERa) by activated p38 following estrogen stimulation retains nuclear localization of ERa and also promotes protein interaction with its co-activators (Lee and Bai 2002).

Thus, ER-dependent cell growth (Frigo et al. 2006), survival (Antoon et al. 2012), and tamoxifen resistance (Gutierrez et al. 2005) in ER-overexpressing breast and endometrial cancers are even more susceptible to p38 inhibition. These studies corroborate the results of a preclinical study conducted by Lilly USA, demonstrating the anticancer activity of a novel and potent p38 inhibitor, LY2228820, in recurrent ovarian cancer (Campbell et al. 2011).

Clinical Summary

Although the effect of p38 inhibitors on inflammatory diseases has been extensively investigated through clinical studies in asthma and rheumatoid arthritis, clinical trials utilizing pharmacological approaches to target p38 in cancer have not yet been actively undertaken. Eli Lilly and Company has performed the first clinical trial targeting p38 in cancer. A phase I clinical study examining the effect of the oral administration of LY2228820, a selective and potent p38 inhibitor that demonstrated anticancer and anti-angiogenic activity in vitro and in vivo (Tate et al. 2013; Zhao et al. 2009), has been conducted in advanced cancer patients; however the outcome of the trial is yet to be determined (Goetz et al. 2012). Additionally, Eli Lilly is in the process of conducting phase I and II clinical studies with LY2228820 for recurrent ovarian cancer and a phase I clinical study with LY3007113, another potent p38 inhibitor for advanced cancer (http://clinicaltrials.gov).

Anticipated High-Impact Results

- Determining the role of p38 in cancer dormancy. The balance between p38 and ERK1/2 has been shown to be critical for converting disseminating tumor cells (DTCs) into circulating tumor cells (CTCs) in the bone marrow. Activation of p38 in DTCs is important to maintain them in the quiescent stage in the bone marrow (Aguirre-Ghiso et al. 2003).
- Determining the role of p38 in metabolic reprogramming (DeBerardinis et al. 2008) following oncogenic challenges (Ying et al. 2012).
- Determining the role of p38 in "cancer stemness," which is acquired by cancer dedifferentiation (Medema 2013). Epithelial mesenchymal transition (EMT), which is closely associated with the acquisition of "cancer stemness," is negatively regulated by p38, while the expression of Wip1, a p38 negative regulator, has been found to play an important role in "cancer stemness" in breast cancer (Pandolfi et al. 2013) and melanoma (Pandolfi et al. 2013).

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Rac 1

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Jonathan Chernoff

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Abstract

The Rac1 small GTPase is a key regulator of actomyosin structure and dynamics and plays a pivotal role in a variety of cellular processes including cell morphology, gene transcription, cell cycle progression, and cell adhesion. Because Rac1 is required for transformation by activated forms of Ras, and, when mutated, is itself a driver of malignant melanoma and perhaps other cancers, key components of the Rac1 signaling apparatus are attracting interest as potential therapeutic targets. While Rac1 itself has proven challenging to target directly, several Rac1 effector proteins, including p21-activated kinases and phosphatidyl inositol-3 kinase beta, show promise as therapeutic targets in Rac1-dependent cancer cells.

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Keywords
Cancer • Signal Transduction • Small GTPases • Transformation • Oncogenes • Driver mutations

Target: Ras-Related C3 Botulinum Toxin Substrate-1 (Rac1)

Rac1 is a 21,000-Da protein belonging to the Ras superfamily of small GTP-binding proteins. Members of this superfamily regulate a diverse array of cellular events, including cell proliferation, apoptosis, cytoskeletal reorganization, and cell motility.

Small GTPases such as Rac1 are downstream effectors of most receptor tyrosine kinases (RTKs) and are coupled to multiple intracellular signaling cascades. In most cases, activation of small GTPases is induced by ligand binding to a RTK. Rac1 is associated with the plasma membrane by virtue of C-terminal lipid modifications. At the plasma membrane, Rac1 cycles between active GTP-bound and inactive GDP-bound states. In its active state, Rac1 binds to a variety of effector proteins 25 to regulate cellular responses. The Rac1-effector interaction catalyzes the activation of effector proteins through recruitment to a particular cellular localization and/or induction of conformational changes within the effector proteins.

Biology of the Target

Due to the difficulty in assessing Rac1 GTP levels, Rac1 activity measurement in clinical specimens is not widely used in cancer patients. However, as a research tool, Rac1 activity has been assessed in a number of patient-derived cancer tissues and cell lines. Interestingly, Schnelzer et al. have shown that Rac1 mRNA and protein levels are frequently elevated in breast cancer tissue samples. In addition, breast tumor cells from patients with recurrent disease had Rac1 expression at the plasma membrane, suggesting the activation of Rac1. In some breast cancers, an alternately spliced form of Rac1, termed Rac1b, is expressed (Schnelzer 2000). This isoform of Rac1 shows fast-cycling kinetics of GTP/GDP exchange, a property associated with elevated biological activity. Rac1 plays an essential role in heregulin ß1 mitogenic signaling (Yang et al. 2006) and contributes to trastuzumab resistance of breast cancer cells. Thus, Rac1 may represent a potential therapeutic target for the treatment of trastuzumab-resistant breast cancer (Dokmanovic et al. 2009).

Recent genomic sequencing efforts have revealed a causative role for Rac1 in malignant melanoma. Following mutations in genes that encode Braf and Nras, the Rac1 P29S amino acid change represents the next most frequently observed protein-coding hot-spot mutation in this disease (Krauthammer et al. 2012; Hodis et al. 2012; Davis et al. 2013). Expression of the Rac1 P29S protein is associated with melanocyte proliferation, and melanoma cells bearing this mutation are insensitive to Braf inhibitors such as vemurafenib and dabrafenib (Krauthammer et al. 2012;

Watson et al. 2014) and also may evade immune surveillance due to enhanced expression of PD-L1 (Vu et al. 2015). Activating mutations in *RAC1* are of special interest, as small molecule inhibitors for the Rac effector p21-activated kinase (Pak) are in late-stage clinical development and might be of therapeutic benefit in this setting (Ong et al., 2013; Rudolph et al. 2015).

Target Assessment

The presence of mutations in the *RAC1* gene that alter amino acid residue 29 indicates constitutive activation of the Rac1 protein. Biochemically, Rac1 activity can be detected using ELISAs based on "pulldown assays" that employ the p21-binding domains from p21-activated kinase. Rac1 activity can also be measured indirectly via its activating effects on downstream proteins such as p21-activated kinases, using phospho-specific antibodies. This latter procedure is suitable for archived clinical (paraffin) specimens. There are no FDA-approved tests for measuring Rac1 activity.

Role of Target in Cancer

As noted above, activating *RAC1* mutations have recently been found as driver events in 5–9% of malignant melanoma. Such mutations have also been described, albeit at very low frequencies, in breast cancer and in aerodigestive cancers (http://cancer.sanger.ac.uk/cosmic/mutation/overview?id=125734). The mechanisms by which activated Rac1 promotes cancer initiation and/or progression are unknown, but likely involve Rac1 effector proteins such as the Group A Paks and possibly PI3K-ß (Fritsch et al. 2013; Radu et al. 2014; Thorpe et al. 2015). As Rac1 has key roles in cell motility and invasiveness, it is also likely that Rac1 overactivation contributes to metastatic spread of cancer.

High-Level Overview

Rac1 is a member of the Ras superfamily of small guanosine triphosphatases (GTPases) that act as molecular switches to control cytoskeletal rearrangements and cell growth. Rac1 can be artificially activated by point mutations that impede its ability to cleave GTP or that cause it to cycle rapidly between GDP- and GTP-loaded states. Recently activating "fast-cycling" point mutations of Rac1 have been found in human tumors. Such mutations are most commonly found in UV-associated malignant melanoma, appearing in about 5% of these tumors.

Predictive: (NA Diagnostic, Prognostic)

Therapeutics

Rac1 has not yet been targeted by any clinically approved drugs. Small GTPases have so far proved recalcitrant to direct inhibitors, though drugs that interfere with posttranslational modifications, such as farnesylation and geranylgeranylation, may prove useful. In addition, several small molecule inhibitors have been reported that interfere with Rac1 activation. These compounds are not in clinical use, however. As with Ras, it may be simpler to impair the downstream signaling apparatus of Rac1 than it will be to block Rac1 itself.

Preclinical Summary

Cell-based and mouse models suggest that inhibition of Rac or of some of its effectors could be an effective means of impeding oncogenesis. Given the role of Rac1 in invasiveness and motility, blockade of Rac1 function might be particularly germane in the setting of metastasis.

Clinical Summary

Rac1 plays a vital role in mediated signals from RPTKs. In addition to mutational activation, Rac1 represents an important node downstream of RPTKs and mediates signals from such receptors to the cytoskeleton and nucleus. Interfering with Rac1 activation or with its downstream effectors might be an effective route to impairing oncogenic stimuli (Fritz et al. 2006; Qui et al. 1995; Sun et al. 2007). Small molecule inhibitors are becoming increasingly available that target Rac1 activation or the activation of Rac1 effectors. One such compound, Pfizers' PF3758309 (Murray et al. 2010), an inhibitor of Paks, was used in phase 1 trials, but later withdrawn due to poor bioavailability. Other Pak small molecule inhibitors have also been described that might have better pharmacologic properties.

Anticipated High-Impact Results

- Further demonstrations that Rac1 and/or its effectors are critical to oncogenesis for a nonmelanoma human tumors
- Development of new, clinically usable inhibitors of Rac1 and/or its effectors

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Type I Insulin-Like Growth Factor Receptor 73

Douglas Yee

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Abstract

The insulin-like growth factor (IGF) system has been implicated in many times of cancer. Upon ligand binding, the type I IGF receptor (IGF1R) transmembrane tyrosine kinase receptor is activated and serves to activate multiple downstream signaling targets. Multiple drugs have been developed to disrupt the function of this receptor, but to date the clinical trial results have been disappointing.

Keywords

Ewing's sarcoma • Type I insulin-like growth factor receptor (IGF1R) • IGF-II • Clinical trials • Fibroblast cells • Functional IGF1R • Breast cancer • Monoclonal antibodies • mRNA expression and protein levels • Multianalyte molecular techniques • ras/raf/MEK/MAPK and PI3K/Akt/mTORC1 signaling cascades • TKIs • Tyrosine kinase inhibitors (TKIs)

Type I insulin-like growth factor receptor (IGF1R) is a heterotetrameric transmembrane tyrosine kinase receptor highly homologous in structure and sequence to the insulin receptor. The two chains are transcribed from a single gene and spliced into a

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covalently linked tetrameric structure. The extracellular alpha subunit is ~130 kDa, while the transmembrane/intracellular domain is ~95 kDa (Steele-Perkins et al. 1988). IGF1R is widely expressed in all human tissues except the liver and T lymphocytes. In addition, most cancer cells express IGF1R including breast, prostate, colorectal, lung, myeloma, and sarcoma (Sachdev and Yee 2007).

Biology of the target: By binding its cognate ligands (IGF-I and IGF-II), the receptor's beta chains catalytic domains are activated to phosphorylate-specific tyrosine residues on the opposing beta subunit. Activation of this receptor in the absence of ligand is not well characterized in physiologic or pathophysiologic conditions. Once phosphorylated, these tyrosines serve as a docking sight for adaptor proteins which link the initial ligand binding event to multiple downstream signaling cascades. Among the most prominent pathways are the ras/raf/MEK/MAPK and the PI3K/Akt/mTORC1 signaling cascades. Functional IGF1R is critical for growth; humans with mutation in the receptor have reduced linear skeletal growth. The IGF1R system is highly conserved and homologous signaling pathways exist in C. elegans, drosophila, and fish. In these systems, IGF1R activation is critical for normal growth, while defects in this pathway enhance the lifespan of the organism (Kaletsky and Murphy 2010). Hybrid receptors, composed of one chain of the insulin receptor and one chain of the IGF1R, also exist and have high affinity for the IGF ligands (Treadway et al. 1991; Soos et al. 1990). Insulin can also bind IGF1R and hybrid receptors, but at a much lower affinity.

In addition to expression of the receptor, levels of serum ligand have been linked to risk of cancer development and poor outcome for patients with cancer (Renehan et al. 2004).

Target assessment: IGF1R gene copy number, mRNA expression, and protein levels measured by immunohistochemistry or radioimmunoassay have been reported in several tumors (Dziadziuszko et al. 2010). The ability to measure receptor confirmation (homodimers vs. heterodimers) has been described (Avnet et al. 2009), but has not been widely utilized in clinical specimens.

Role of the Target in Cancer

Rank: 9

Diagnostic, Prognostic, Predictive: The level of IGF-I and insulin are associated with increased cancer risk (Renehan et al. 2004; Vigneri et al. 2009). In pancreas cancer, higher circulating ligand levels of IGF-I and IGF-II identified patients who received the most benefit from an anti-IGF1R antibody (ganitumab) and gemcitabine (McCaffery et al. 2013).

In breast cancer, levels of IGF1R expression have been associated with favorable outcome (Papa et al. 1993), in a manner analogous to expression of estrogen receptor alpha. However, these data are controversial as other groups have suggested that IGF1R activation correlates with poor prognosis (Law et al. 2008). Differences in technique as well as the measurement of total versus phosphorylated IGF1R could account for this discrepancy.

Levels of IGF1R have been associated with sensitivity of breast cancer cell lines to anti-IGF1R therapies in vitro (Litzenburger et al. 2009). In addition to IGF1R, insulin receptor may also play a role in determining benefit from inhibiting this pathway (Avnet et al. 2009), and studies quantifying all the relevant receptors have been limited. Because IGF1R signaling is complex, multianalyte molecular techniques have been described to identify "IGF-activated" gene signatures (Creighton et al. 2008) or as tools to predict benefit from anti-IGF1R therapies (Pitts et al. 2010). These types of multianalyte tools will need to be validated in clinical trials for their predictive value.

Therapeutics: Disruption of IGF1R function could occur at multiple levels: ligand neutralization, inhibition of ligand binding to the receptor by monoclonal antibodies, disruption of receptor tyrosine kinase activity, or inhibition of key downstream signaling pathways. All of these strategies have been tested.

Monoclonal antibodies that bind both IGF-I and IGF-II have been described (Dransfield et al. 2010; Goya et al. 2004). Clinical trials using ligand binding antibodies have been reported (Iguchi et al. 2015; Friedbichler et al. 2014).

The bulk of anti-IGF1R therapies are directed at the receptor. Multiple monoclonal antibodies have been produced. While there are differences between the immunoglobulin backbones, the binding epitopes, and the nature of the antibody (fully human vs. humanized), the preclinical data suggest that all of the antibodies downregulate receptor levels. By binding the receptor, causing internalization, and targeting the receptor to endosomal degradation, the antibodies effectively decrease the number of receptors on the cell surface (Zhang et al. 2009; Sachdev et al. 2003). Results of the phase I studies have been reported for several of the monoclonal antibodies (Tolcher et al. 2009; Lacy et al. 2008; Haluska et al. 2007; Scartozzi et al. 2010; McKian and Haluska 2009). Elevated growth hormone, IGF-I, insulin, and glucose levels have been seen with antibody treatment consistent with the disruption of the negative feedback between IGF-I and growth hormone; receptor blockade leads to increased pituitary release of growth hormone. Growth hormone excess has been associated with insulin resistance, thus accounting for the hyperinsulinemia (Vijayakumar et al. 2010).

To date, single agent activity has been demonstrated in phase I and II studies. Most prominent are responses against Ewing's sarcoma (Tap et al. 2012), a tumor that expresses IGF-I in an autocrine fashion (Yee et al. 1990). Single agent activity has also been reported in prostate cancer (Graff et al. 2015). Unfortunately, essentially all of the phase III studies have not shown benefit for addition of a IGF1R moAb (Iams and Lovly 2015). The moAbs induce counter-regulatory endocrine responses, most notably hyperinsulinemia, and this may account for the lack of benefit when combining these agents with conventional endocrine or chemotherapy (Yee 2015). Since insulin receptor signals to identical pathways as IGF1R, the unintended effect of hyperinsulinemia may account for the lack of benefit in phase III reports. A remaining phase II randomized trial in Ewing's sarcoma is still underway.

IGF1R tyrosine kinase inhibitors (TKIs) have also been evaluated in clinical trials. These drugs are not specific for IGF1R and also inhibit insulin receptor

(Carboni et al. 2009; Ji et al. 2007). Linsitinib, a TKI, showed activity in phase I trials including an apparent pathological complete response for a patient with melanoma (Puzanov et al. 2014). A randomized placebo control trial in adrenocortical cancer showed no benefit of continuous twice daily dosing (Fassnacht et al. 2015). It is notable that in the phase I study of intermittent dosing, responses were seen in adrenal cortical carcinoma (Jones et al. 2014).

Preclinical Summary

Multiple studies performed in cell culture and in animal model systems have shown that tumor cells are dependent on the expression of IGF1R. Using fibroblast cells from mice with gene deletion of IGF1R, it was shown that the presence of the receptor was necessary for response to growth factors and transformation by oncogenes (Coppola et al. 1994; Valentinis et al. 1994). Development of monoclonal antibodies directed against IGF1R (Arteaga et al. 1989) and small molecule inhibitors of IGF1R tyrosine kinase activity (Garcia-Echeverria et al. 2004) further demonstrated the potential to disrupt signaling through this pathway.

Clinical Summary

As mentioned above, a number of clinical trials have suggested single agent activity in a variety of disease. These responses are rare, but some seemed to be durable. The disappointing results in phase III trials likely have many reasons. Most trials were performed without biomarker selection which makes it difficult to discern a rare responding group of patients. In addition, the upregulation of insulin levels also could counteract the effects of IGF1R blockade by activating a companion pathway. While IGF1R is still potentially an important target, better methods to disrupt this signaling pathway will be necessary to translate the epidemiologic and preclinical data into a therapeutic advance.

Anticipated High Impact Results

- Publication of remaining phase II and III clinical trials of monoclonal antibodies in the neoadjuvant therapy of breast cancer (NCT01042379) and Ewing's sarcoma (NCT02306161).
- Further report of ligand neutralization clinical trials.
- Validation of predictive biomarker profiling.

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Part IV

Apoptosis

74 Anti-apoptotic Bcl-2

Stanley R. Frankel and Dow-Chung Chi

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Abstract
The B-cell lymphoma/leukemia 2 (BCL-2) family represents a group of proteins involved in the regulation of the intrinsic (mitochondrial) apoptotic pathway. Approximately 25 members of the BCL-2 family have been grouped into three

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categories based on structure and function: (1) anti- apoptotic, (2) pro-apoptotic, and (3) BH3 only proteins. This chapter will focus on a family of five anti-apoptotic proteins: (1) BCL-2, (2) BCL-xL, (3) BCL-w, (4) MCL-1, and (5) A1. The decision for a cell to undergo apoptosis is regulated by the BCL-2 family of proteins. Inhibition of anti-apoptotic BCL-2 proteins is an active area of research and represents a novel pathway in cancer therapeutics. BH3 profiling is a functional assay that measures the degree to which a cancer cell is primed to undergo apoptosis by investigating how the cells evade apoptosis by three possible mechanisms: (1) inability of activator BH3-only proteins (Bim/Bid) to function, (2) loss of pro-apoptotic BCL-2 proteins (Bax/Bak), and (3) sequestration of activator BH3 proteins by anti-apoptotic BCL-2 proteins. Approaches to target BCL-2 include RNA antisense molecules (oblimersen, SPC2996); small-molecule inhibitors (venetoclax, obatoclax, ABT-737, navitoclax, AT-101); and stabilized alpha helix of BCL-2 (SAHB) peptides. These compounds are under various stages of clinical investigation in both solid and hematologic malignancies, both as single agents and in combination with other cytotoxic regimens

Keywords

Anti-apoptotic BCL-2 proteins • Apoptosis • Augmerosen • B-cell lymphoma/leukemia 2 (BCL-2) • BH3 profiling • BHS mimetics • A1 • ABT-199 • ABT-737 • AT-101 • BCL-w • BCL-xL • Gossypol derivatives • Maritoclax • Mcl-1 • Mitochondrial outer membrane permeabilization (MOMP) • Navitoclax • Obatoclax mesylate • Oblimersen sodium • Programmed cell death • SPC2996 • Stabilized alpha helix of BCL-2 domains • TW37 • BCL-w protein • Genasense® • Inhibition of anti-apoptotic BCL-2 proteins • Sabutoclax • Stabilized alpha helix of BCL-2 domains (SAHBs) • Venetoclax

The B-cell lymphoma/leukemia 2 (BCL-2) family represents a group of proteins involved in the regulation of the intrinsic (mitochondrial) apoptotic pathway. The prototypical *BCL-2* was cloned in the 1980s from the chromosomal breakpoint of follicular lymphoma cell lines carrying a translocation t(14:18). This translocation of the *BCL-2* gene on 18q21 to a heavy chain immunoglobulin region on 14q32 results in deregulated expression of *BCL-2*. The identification of this gene demonstrated for the first time the association between defects in programmed cell death and cancer (Tsujimoto et al. 1984). Approximately 25 members of the BCL-2 family have been identified and grouped into three categories based on structure and function: (1) anti-apoptotic, (2) pro-apoptotic, and (3) BH3 only proteins. The scope of this chapter will focus on a family of five anti-apoptotic proteins: (1) BCL-2, (2) BCL extra long (BCL-xL, also known as BCL-2L1), (3) BCL-2-like protein 2 (BCL-w, also known as BCL-2L2), (4) myeloid cell leukemia 1 (MCL-1), and (5) BCL-2-related gene expression in fetal liver (A1, also known as BFL-1 or BCL-2A1). This group shares similar structure and function and will be collectively referred to as anti-apoptotic BCL-2 proteins.

The physiologic and pathologic roles of the anti-apoptotic BCL-2 proteins must be considered in the context of their structure and complex interactions with other members of the BCL-2 family. These other members include the pro-apoptotic BCL-2 proteins (Bax, Bak, Bok) and the BH3-only proteins (Bim, Bid, Bad, Bik, Noxa, Puma, Bmf, Hrk) and are discussed in ▶ Chap. 75, "BH3-Only Mimetics." Pro-apoptotic Bax and Bak are the main effectors of mitochondrial outer membrane permeabilization (MOMP). BH3-only proteins can be subdivided further into activating BH3 (Bim and Bid) which activate Bax and Bak and sensitize BH3 (Bad, Bik, Noxa, Puma) which inhibit anti-apoptotic BCL-2 proteins, thereby lowering the apoptotic threshold. This division is proposed by the "direct model of activation" of Bax/Bak. An alternative "indirect model of activation" purports that all BH3 proteins act on anti-apoptotic BCL-2 proteins, thereby blocking their ability to inhibit Bax/Bak (Letai 2008).

The structure of the anti-apoptotic BCL-2 proteins consists of four conserved BCL-2 homology (BH) domains and a transmembrane anchor domain. BH1, BH2, and BH3 form a hydrophobic pocket that binds the BH3 component of pro-apoptotic BCL-2 proteins and BH3 only proteins (see Fig. 1). These proteins also have five amphipathic alpha-helices surrounding two central hydrophobic alpha-helices, suggesting the ability to dimerize with itself or pro-apoptotic BCL-2 proteins.

Cell death can occur through necrosis, autophagy, mitotic catastrophe, and apoptosis. The ability to escape cell death is a hallmark of cancer. Apoptosis, also called programmed cell death, is an important pathway that efficiently catabolizes defective or senescent cells and is a normal physiologic function of many organ

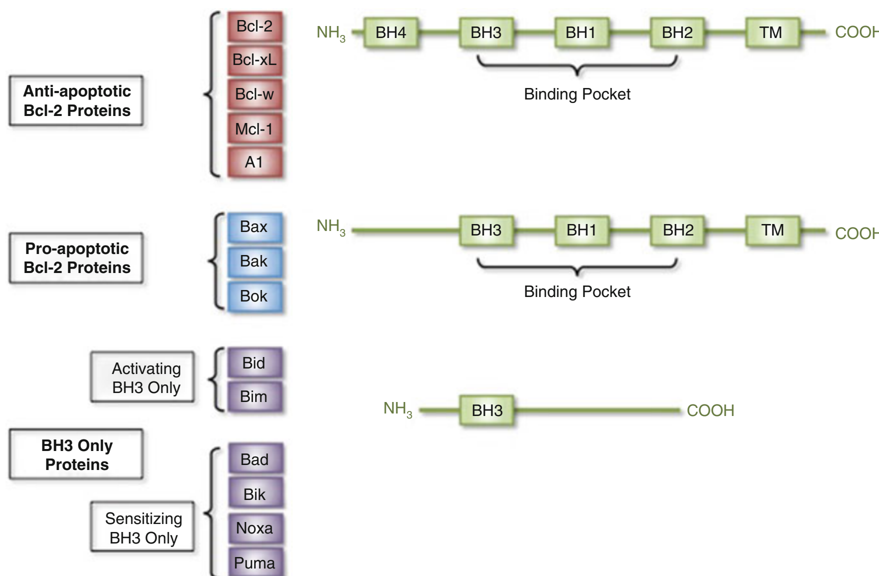

Fig. 1 Structural homology of the BCL-2 family. *TM* transmembrane domain

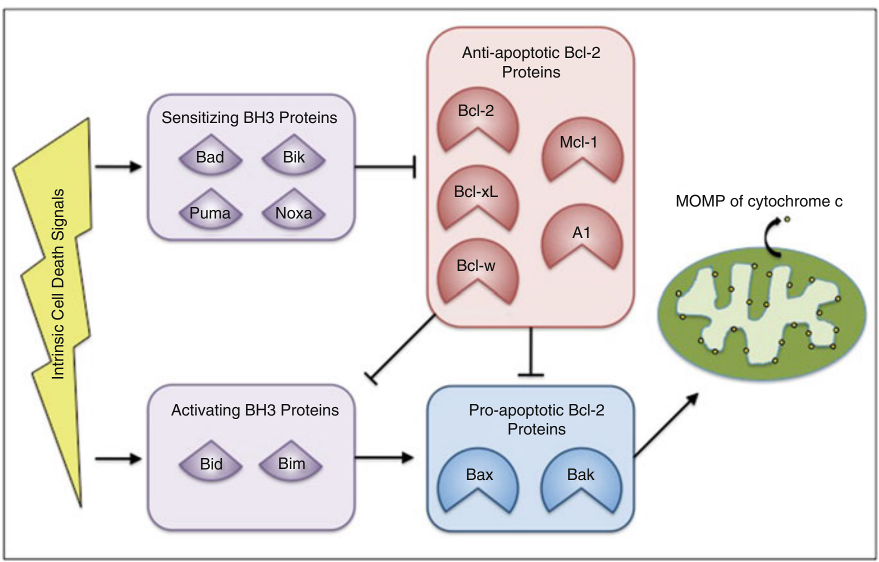

Fig. 2 Functional relationships of the BCL-2 family

systems. Triggers of apoptosis include DNA damage, growth factor withdrawal, and oncogene activation (see Fig. 2). These signals activate both classes of BH3-only proteins. Activator BH3 proteins (Bid and Bim) bind to pro-apoptotic BCL-2 proteins, Bax or Bak, facilitating their homodimerization and thus forming an intermembrane channel for cytochrome c, calcium ions, Smac/Diablo, endonuclease G, apoptosis-inducing factors, and other mediators of apoptosis to be released from the mitochondria into the cytosol. This process is called mitochondrial outer membrane permeabilization (MOMP) and is considered to be the committing step of apoptosis. Cytosolic cytochrome c interacts with apoptotic protease-activating factor 1 (APAF1) and recruits caspase 9, forming the apoptosome, and thereby activates the caspase cascade, the final common pathway leading to programmed cell death. This decision to undergo apoptosis is regulated by the BCL-2 family of proteins. Anti-apoptotic BCL-2 proteins sequester activator BH3 proteins (Bid and Bim), thereby inhibiting their ability to activate the pro-apoptotic BCL-2 proteins (Bax and Bak). They also form heterodimers with monomeric pro-apoptotic BCL-2 proteins, thereby inhibiting their ability to self-dimerize and initiate MOMP.

Apoptosis is believed to be the primary mechanism by which cytotoxic chemotherapy kills cancer cells. Cancer cells escape these critical controls of programmed cell death by increasing the ratio of anti-apoptotic to pro-apoptotic proteins through overexpression of anti-apoptotic BCL-2 proteins, thereby increasing the apoptotic threshold. A blunted response to normal apoptotic signals is thought to be one mechanism of resistance to cytotoxic chemotherapy.

Biology of the Target

There are five primary members of the anti-apoptotic BCL-2 protein group. BCL-2 is the prototype for this group and is the most widely studied in cancer. Most compounds in clinical development, therefore, target this protein. The other members of the anti-apoptotic BCL-2 protein family have been identified through sequence homology in different cell lines and share similar structure and function.

BCL-2

The *BCL-2* gene is widely expressed during fetal development. In adults, it is mainly expressed in the peripheral nervous system, the thymus, and the immune system. It plays a role in maintaining the survival of memory B cells in lymph nodes. *Bcl-2* knockout mice are smaller, have earlier mortality, and are characterized by polycystic kidney disease, hair hypopigmentation, distortion of the small intestines, and failure to develop an immune system. Double knockout *Bcl-2*$^{-/-}$ *Bim*$^{-/-}$ restore these degenerative defects. From these experiments, it is thought that Bcl-2 plays a role in the maintenance of melanocytes in hair follicles, osteogenesis, and clonal expansion of myocytes. Within the cell, BCL-2 proteins are located on the outer membrane of mitochondria, endoplasmic reticulum (ER), and nuclear envelope. While the role of BCL-2 in non-mitochondrial organelles is under investigation, there is evidence that ER-associated BCL-2 also play a role in the apoptosis pathway by controlling the amount of calcium ions in the cytosol and by sequestration of excess BCL-2 proteins in the cytosol (Tsujimoto et al. 1984; Kirkin et al. 2004).

BCL-xL

BCL-xL is the long splice variant of *BCL-x* and has anti-apoptotic activity, whereas the short splice variant (BCL-xS) has pro-apoptotic activity. *Bcl-x* is expressed mainly in the brain, kidney, and thymus. *Bcl-x* knockout mice display embryonic lethality with loss of fetal erythroid progenitors, loss of neuronal proliferation, decreased male germ cells, and decreased immature thymocytes, hepatocytes, and platelets. *Bcl-x*$^{+/-}$ males displayed reduced fertility that was restored with concomitant loss of *Bim*. Double knockout *Bcl-x*$^{-/-}$ *Bim*$^{-/-}$ restored erythroid progenitors, but still displayed embryonic lethality due to persistent defects in neurologic development (Strasser et al. 2011). Within the cell, its inactive form is found in the cytosol which, upon activation, is inserted into the mitochondrial membrane. BCL-xL overexpression has been observed in hepatocellular carcinoma (HCC), renal cell carcinoma (RCC), and pancreatic cancer. In human clinical trials, BCL-xL inhibition has led to dose-limiting thrombocytopenia.

BCL-w

BCL-w protein is mainly found in myeloid and lymphoid cells and the epithelium (Kirkin et al. 2004). *Bcl-w* knockout mice display defects in spermatogenesis and small intestine epithelial cells. It also has been associated with the progression colonic adenoma to adenocarcinoma as well as with the development of gastric cancer.

MCL-1

MCL-1 was discovered in ML-1 human myeloid leukemia cell lines, and its expression occurs in early monocyte development. MCL-1 differs from its other anti-apoptotic proteins in structure and binding partners. Its binding groove is more electropositive and binds with high affinity Noxa and less with Bad. Its expression is induced by a variety of cytokines and signaling pathways including PI3K/AKT, Stat-3, and p38/MAPK pathways. It has two splice variants, MCL-1_L and MCL-1_S, which have opposing effects on apoptosis. MCL-1_S appears to have a pro-apoptotic effect. MCL-1 has a short half-life (2–3 h) due to its rapid degradation through the proteasome pathway (Quinn et al. 2011). *MCL-1* knockout mice display preimplantation lethality. Conditional knockout mice show defects in the development of hematopoietic stem cells, hepatocytes, and neurons. In cancer, MCL-1 overexpression has been observed in melanoma, head and neck cancer, HCC, pancreatic cancer, myeloma, and non-Hodgkin's lymphoma (NHL). Overexpression of MCL-1 has also been observed in cancer cell lines that have developed resistance to BCL-2-, BCL-x-, and BCL-w-specific inhibitors (Perciavalle and Opferman 2013).

A1 (Bfl-1, BCL-2A2)

A1 was isolated from human fetal liver cells and identified by sequence homology. It is the least well-understood member. In vitro studies provide conflicting data, but suggest that A1 preferentially binds to Bak rather than Bax. A1 has similar affinity for Bid, Bim, and Puma as MCL-1. A1 also has been found to bind to Beclin-1, suggesting a role in autophagy. In humans, A1 is a highly regulated nuclear factor κB (NF-κB) target gene. It is mainly expressed in the hematopoietic system, where it facilitates survival of selected leukocytes subsets and inflammation. It is regulated by the ubiquitin/proteasome pathway although no E3-ligase for A1 has been identified. Its expression has also been found in the normal human lung, small intestine, testis, and smooth muscle tissues. Expression studies have mainly examined mRNA levels. There is limited data on protein expression levels due to lack of commercially available A1-specific antibodies. However, A1 is overexpressed in a variety of cancer cells, including hematologic malignancies and solid tumors, and may contribute to tumor progression. Overexpression has also been associated with

chemoresistance to cytotoxic chemotherapy and BCL-2 inhibitors in cell lines and mouse models (Tsujimoto et al. 1984; Vogler 2012).

Anti-apoptotic BCL-2 proteins also inhibit entry into the cell cycle and thus have counteractive antiproliferative effect that is structurally separate from its anti-apoptotic function. Mutational analysis shows that point mutations in the BH4 domain can eliminate this cell cycle inhibitory effect without affecting the apoptotic function. In *BCL-2* transgenic mice, mitogen-stimulated lymphocytes enter the cell cycle at a slower rate when compared to wild-type mice. In a breast cancer mouse model, BCL-2 expression delays tumor development. The mechanism for this antiproliferative effect is unclear, but may involve interaction with various cell cycle regulators such as proliferating cell nuclear antigen (PCNA), cell cycle inhibitor P27, G1 cyclin-dependent kinases, and Cdk2 (Kirkin et al. 2004; Letai 2008; Danial et al. 2010).

Target Assessment

Inhibition of anti-apoptotic BCL-2 proteins is an active area of drug development and represents a novel pathway in cancer therapeutics. As detailed above, *BCL-2* is overexpressed in tumor tissues, but knockout of this gene is not lethal in mouse models. Overexpression of *BCL-2* was first discovered in follicular lymphoma cell lines through a chromosomal translocation t(14;18). Immunohistochemical (IHC) studies have demonstrated abnormal expression of BCL-2 in tumor tissue samples, and overexpression usually is associated with poor clinical outcomes. For example, breast, colon, and ovarian cancer tissue samples have demonstrated an increase in BCL-2 and MCL-1 protein levels by IHC. In CNS malignancies, increasing BCL-2, BCL-xL and MCL-1 levels were associated with progression of disease. IHC studies in prostate cancer samples showed elevated levels of BCL-2, BCL-xL, and MCL-1. Analysis of BCL-2 subtypes has identified that MCL-1 levels are also significantly elevated and associated with resistance to selective inhibition of the BCL-2 subtype (Tsujimoto et al. 1984; Davids and Letai 2012). Gene expression studies using quantitative PCR of mRNA suggest that leukemia and lymphoma cell lines overexpress BCL-2, whereas tumor cell lines for lung, prostate, breast, ovarian, renal, and CNS malignancies overexpress MCL-1 to the higher levels than BCL-2 (Kirkin et al. 2004; Placzek et al. 2010). BCL-xL was found to be overexpressed in myeloma cells and CML. BCL-w overexpression was found in gastric and colorectal cancer and did not have a role in hematologic malignancies (Kirkin et al. 2004; Strasser et al. 2011). Although less well studied, A1 has been found to be overexpressed in both hematologic (ALL and CLL) and solid (stomach, colon, and breast cancers) malignancies (Kirkin et al. 2004; Vogler 2012). Expression of the individual members of the anti-apoptotic BCL-2 protein group is variable among different tumor types and will need to be assessed in the clinical development of selective and nonselective inhibitors to these proteins.

The mechanism of overexpression of anti-apoptotic BCL-2 proteins in cancer is also variable among tumor types. Chromosomal translocation t(14;18)(q32;q21) can

be measured by fluorescence in situ hybridization (FISH). Hypomethylation of the promoter region has also been identified in other hematologic malignancies. Gene amplification has also been studied in lymphoma samples (Iqbal et al. 2011; Quinn et al. 2011). Finally, a large-scale analysis of somatic copy number alterations in over 3,000 tumor samples identified *MCL-1* and *BCL-w* as novel genes shown that were amplified in lung and breast cancer samples (Beroukhim et al. 2010).

BCL-2 gene expression levels have been measured in prognostic tests for breast cancer. Oncotype Dx (Genomic Health) evaluates the expression levels of a panel of 23 genes, including *BCL-2*, by RT-PCR from formalin-fixed tissue samples. MammaPrint (Agendia) is another breast cancer prognostic test that uses microarrays to analyze expression levels of 70 genes including *BCL-2* from fresh frozen or formalin-fixed breast tissue samples.

Role of the Target in Cancer

(a) **Rank**: 6
(b) While *BCL-2* expression does not drive abnormal cellular proliferation, its abnormal activity permits cancer cells to proliferate and escape normal programmed cell death. The block of apoptosis by increased BCL-2 expression and activity can also contribute to chemoresistance of cancer cells.

High-Level Overview

Diagnostic, Prognostic, and Predictive

Despite demonstration of *BCL-2* overexpression in multiple cancer types, its utility as a diagnostic, prognostic, or predictive biomarkers has been limited. BCL-2 overexpression is a biomarker of poor outcome in the germinal center B cell (GCB) but not in activated B-cell (ABC) subtype of DLBCL in patients treated with R-CHOP chemoimmunotherapy (Iqbal et al. 2011). Two commercially available prognostic tests used to predict breast cancer recurrence (Oncotype DX and MammaPrint) measure *BCL-2* expression as part of a panel of genes in an individual tissue sample. High BCL-2 expression in breast cancer has been validated as an independent positive prognostic biomarker and shown to be inversely correlated with risk of recurrence (Dawson et al. 2010). This suggests that breast cancers with BCL-2 overexpression may have a less aggressive phenotype than those with other genetic drivers. Additionally, Letai et al. are investigating BH3 profiling as a predictive biomarker to determine sensitivity to conventional chemotherapy as well as BCL-2 inhibition by drug candidates. BH3 profiling is a functional assay that measures the degree to which a cancer cell is primed to undergo apoptosis (Davids and Letai 2012). It investigates how the cells evade apoptosis by three

possible mechanisms: (1) inability of activator BH3-only proteins (Bim/Bid) to function, (2) loss of pro-apoptotic BCL-2 proteins (Bax/Bak), and (3) sequestration of activator BH3 proteins by anti-apoptotic BCL-2 proteins. This test requires extraction of mitochondria from fresh frozen tissue in order to preserve native mitochondrial activity. This assay is currently a research tool, but may have potential as a predictive biomarker with further clinical validation.

Therapeutics

Two general approaches have been taken to target BCL-2: RNA antisense molecules (oblimersen, SPC2996) and small-molecule inhibitors (obatoclax, ABT-737, navitoclax, ABT-199, AT-101). Clinical development to date has not led to a commercially available drug. A third approach in preclinical development is stabilized alpha helix of BCL-2 (SAHB) peptides to trigger BH3-only activation and inhibit BCL-2 anti-apoptotic proteins.

Preclinical Summary

Two main approaches to targeting anti-apoptotic BCL-2 proteins have been inhibition of the protein function by BH3 mimetics and the reduction of protein levels by reduction of gene expression through antisense molecules or increased degradation of the protein through the ubiquitin/proteasome pathway. Further, inhibition of CDK pathway is also associated with decrease in MCL-1 expression (Quinn et al. 2011).

The Abbott family of BCL-2 small-molecule inhibitors (ABT-737, ABT-263, ABT-199) are the first compounds designed to bind with high affinity and selectivity to BCL-2 and with variable affinity to BCL-xL and BCL-2. Preclinical studies of ABT-737 demonstrate high affinity for BCL-2, BCL-xL, and BCL 2 with $K_i < 1$ nM and low affinity for MCL-1 and A1 with $K_i > 1$ mM. Mammalian yeast two-hybrid systems and confocal light microscopy demonstrate binding to the hydrophobic pocket of these proteins, thereby disrupting protein-protein interactions. ABT-737 shows single-agent activity in cell lines and xenograft mouse models for small-cell lung cancer and several lymphoid malignancies, such as CLL and follicular lymphoma, while, in other tumor types, it does not show any activity on tumor growth or destruction. Synergy with various cytotoxic chemotherapies has been observed, however, with decreases in EC_{50} by two- to fourfold with etoposide, cisplatin, paclitaxel, doxorubicin, and radiation therapy (Oltersdorf et al. 2005). Most of the preclinical work for subsequent generations of BCL-2 inhibitors (ABT-263 and ABT-199) has been based on the data from ABT-737.

Antisense oligonucleotides directed toward the *BCL-2* mRNA transcript have demonstrated reduction in BCL-2 protein levels, antineoplastic effects, and synergy with cytotoxic chemotherapy. In melanoma cell lines, the administration of antisense oligonucleotides led to a loss of mRNA within 24 h and a 35–61% decrease in

BCL-2 protein levels in 48 h. Xenograft mouse models of melanoma demonstrated a decrease in tumor weight after administration of oblimersen. Western blot analysis showed a 66–72% reduction in *BCL-2* gene product. When co-administered with dacarbazine, antisense compounds prevented the development of measurable tumor in the same mouse model. Increased levels of apoptosis were by TUNEL assay (Jansen et al. 1998).

TW37 (University of Michigan) is a small-molecule inhibitor of BCL-2, Bcl-xL, and MCL-1, synthesized based on a benzenesulfonyl derivative. Preclinical studies have demonstrated activity in a variety of cancer cell lines including those for lymphoma, Kaposi's sarcoma, breast cancer, pancreatic cancer, head and neck cancer, and melanoma. In prostate cancer cell lines, TW37 inhibits cell growth and induces apoptosis. In xenograft models for prostate cancer, the compound inhibits tumor growth alone and in combination with docetaxel (Bajwa et al. 2012). There are no clinical studies of this compound planned.

Several gossypol derivatives have been patented as potential cancer therapeutics: gossypolic acid, gossypolonic acid, apogossypol, and apogossypolone. Gossypolic acid and gossypolonic acid have demonstrated inability to cross the cell membrane due to negative charge in physiological conditions (Bajwa et al. 2012). Sabutoclax (BI-97C1, Oncothyreon, Sanford-Burnham Medical Research Institute) is an apogossypol derivative that binds to BCL-2, MCL-1, BCL-xL, and A1. It has demonstrated activity in B-cell lymphoma cell lines and prostate cancer xenograft mouse models (Quinn et al. 2011). No human trials have been registered to date.

Other potential BCL-2 inhibitors have been identified through screening of compound libraries and are being evaluated in preclinical studies. Maritoclax (marinopyrrole A, Penn State University) is a naturally occurring compound with specificity for MCL-1. It is under preclinical evaluation in multiple cancers with resistance to small-molecule BCL-2 inhibition (Doi et al. 2012). The benzoyl urea derivates (Walter and Eliza Hall Institute of Medical Research) have shown affinity to BCL-w, BCL-xL, and MCL-1 and have activity in follicular cell lymphoma, DLBCL, nasopharyngeal carcinoma, and HCC. Isoxazolidine analogues (Infinity Pharmaceuticals) have affinity for BCL-2 and BCL-xL with activity in lymphoma and pancreatic cancer. Other classes of compounds under preclinical and clinical evaluation are BH3 mimetics (BH3-M6, HA 14-1, antimycin A), CDK inhibitors (flavopiridol, sorafenib, SNS-032), and deubiquitinase inhibitor (WP1130) (Quinn et al. 2011; Vogler 2012).

The development of A1-specific inhibitors has been difficult due to structural similarities of the hydrophobic binding groove among its family members. However, screening of compound libraries has identified gambogic acid and *N*-aryl maleimides as potential selective A1 inhibitors (Vogler 2012).

One approach likely to enter the clinic is the use of stabilized alpha helix of BCL-2 domains (SAHBs), peptides designed to bind and inhibit BCL-2 anti-apoptotic family members. A hydrocarbon-stapled peptide modeled after the BIM BH3 helix broadly targeted the anti-apoptotic BCL-2 proteins with high affinity, blocked inhibitory anti-apoptotic interactions, directly triggered pro-apoptotic activity, and induced dose-responsive and BH3 sequence-specific

cell death of hematologic cancer cells in vitro and in mouse xenografts (LaBelle et al. 2012).

Clinical Summary

Inhibition of anti-apoptotic BCL-2 proteins has focused on two modalities: (1) attenuating gene expression through antisense nucleic acid molecules and (2) preventing ligand binding with small-molecule antagonists. While there are no FDA-approved agents, several compounds are under various stages of clinical investigation in both solid and hematologic malignancies, both as single agents and in combination with other cytotoxic regimens (see Table 1). Two antisense compounds have targeted the *BCL-2* transcript and have had limited success in clinical trials. Future development of these compounds is uncertain. Early small-molecule inhibitors have primarily targeted BCL-2, BCL-xL, and BCL-w and have had mixed results in clinical trials. One explanation for these results may be the lack of activity against MCL-1 and A1. Follow-up studies have shown a compensatory upregulation of these two proteins in the presence of BCL-2 inhibition (Davids and Letai 2012). This compensatory effect has been purported to be the mechanism of resistance to BCL-2 inhibition and is the basis for the development of newer MCL-1 inhibitors. Further, recent studies of tissue samples and cancer cell lines illustrate the importance of these other subtypes in several types of cancer. Another reason for lack of efficacy of BCL-2 inhibition in human studies may be related to the specific defect in the apoptotic blockade of individual tumors. BH3 profiling of various tumors has demonstrated that decreased activity of pro-apoptotic BCL-2 proteins (Bax/Bak) can also lead to loss of apoptotic ability in cancer cells. Inhibition of the anti-apoptotic BCL-2 proteins in this scenario would not be effective in inducing apoptosis in cancer cells.

Oblimersen sodium (Genasense®, augmerosen, G3139, Genta) is an 18-base pair antisense DNA molecule to the first 6 codons of the *BCL-2* mRNA transcript. This DNA/RNA duplex triggers RNAse H destruction of the *BCL-2* transcript, leading to decreased levels of BCL-2 protein. This compound has been investigated in at least 45 clinical trials both as monotherapy or in combination with chemotherapy in hematologic and solid malignancies. Four indications were studied in phase III trials: CLL, melanoma, myeloma, and AML. A randomized phase III trial (NCT0024440) of oblimersen in combination with fludarabine and cyclophosphamide in 241 relapsed/refractory chronic lymphocytic leukemia (CLL) patients demonstrated a clinical benefit in complete response or nodular partial response (17% versus 7%, $p = 0.025$). A 5-year post-study analysis did not demonstrate a difference in overall survival in an intention to treat analysis (HR 0.87, $p = 0.34$). Subgroup analysis of 40% of patients who had a complete or partial response demonstrated a 5-year overall survival benefit (HR 0.60, $p = 0.038$) (O'Brien et al. 2009). Although statistically positive, the FDA did not grant approval due to the modest clinical benefit demonstrating progression-free survival benefit, but no overall survival benefit and requested a second phase III trial. The Oblimersen Melanoma Study Group conducted a randomized phase III trial of oblimersen in

Table 1 List of BCL-2 inhibitors in clinical development (Source: www.clinicaltrials.gov)

Study drug	Latest phase	Cancer type	Description
Venetoclax (ABT-199)	P1	CLL, NHL	Third-generation small-molecule inhibitor of BCL-2 and BCL-w with less affinity for BCL-xL. Compound is in P1 trials as single agent in relapsed/refractory CLL and NHL and also in combination with bendamustine/rituximab in relapse/refractory NHL
Navitoclax (ABT-263)	P2	CLL, NHL, SCLC, solid tumors	Second-generation small-molecule inhibitor BCL-2, BCL-W, and BCL-xL with greater oral bioavailability. Compound has been evaluated in multiple tumor types. P2 trials in relapsed/refractory CLL in combination with fludarabine/cyclophosphamide/rituximab; bendamustine/rituximab. It has also been evaluated as first-line therapy in CLL patients in combination with dose-intensive rituximab. P2 studies in relapsed refractory NHL in combination with rituximab and bendamustine/rituximab. In P1 studies in various solid tumors in combination with docetaxel, paclitaxel, gemcitabine, erlotinib, irinotecan, and etoposide/cisplatin. Further development of this compound is unclear
ABT-737	n/a	n/a	First-generation small-molecule inhibitor to BCL-2, BCL-W, BCL-xL in preclinical trials. Clinical development on hold in favor of second-generation compounds
AT-101	P2	CLL, NHL, various solid tumors	Synthetic (-) enantiomer of gossypol as pan-BCL-2 antagonist. P2 studies in relapse/refractory CLL in combination with lenalidomide and with rituximab. P2 studies in untreated NHL in combination with rituximab and relapsed/refractory NHL as a single agent. P2 trials in combination with chemotherapy in head and neck cancer, GBM, NSCLC, SCLC, GEJ, prostate, adrenal
Obatoclax	P3	AML, CLL, NHL, HL, MM, NSCLC, SCLC	Small-molecule inhibitor to BCL-2, BCL-W, BCL-xL, MCL-1, AF-1. Compound has been evaluated in P1 and 2 trials in SCLC, AML, CLL, MDS, myelofibrosis, MM, NHL, and HL. A Phase III trial in SCLC has been put on hold by the company

(*continued*)

Table 1 (continued)

Study drug	Latest phase	Cancer type	Description
Oblimersen	P3	CLL, MM, SCLC. Also in NHL, AML, and other various solid tumors	RNA antisense molecule to BCL-2 family of proteins. Compound has been evaluated in multiple Phase III trials in CLL, MM, and melanoma with mixed results. It has been evaluated in several Phase II trials in AML, ALL, NHL, GIST, breast cancer, HCC, gastric cancer, and Merkel cell carcinoma. Further development of this compound has been terminated by the company
SPC2996	P1/2	CLL	Antisense nucleic acid molecule to BCL-2. Phase I/II as monotherapy in CLL
TW37	Preclinical	n/a	Small-molecule inhibitor of BCL-2
Maritoclax	Preclinical	n/a	Selective MCL-1 inhibitor

combination dacarbazine versus dacarbazine alone in 771 treatment-naïve Stage III and IV melanoma patients. Significant improvement in all secondary endpoints, including overall response and progression-free survival (PFS), was observed in the oblimersen-treated group, but the primary end point of intention to treat analysis overall survival (OS) did not achieve statistical significance (median 9.0 v 7.8 months, $p = 0.077$) (Bedikian et al. 2006). Further subgroup analysis showed that the OS benefit was driven by patients with low to normal LDH. A second phase III similarly designed trial (NCT00518895, AGENDA) was conducted to evaluate whether the addition of oblimersen to dacarbazine could confirm the OS benefit that was previously observed in patients with low to normal LDH. The median survival was 13.5 months in the combination group and 13.1 months in the dacarbazine monotherapy group ($p = 0.73$). There was also no difference in rate of durable response (major objective response persisting for at least 6 months; 10.8% and 7.6%, respectively; $p = 0.32$). A randomized, phase III trial (NCT00017602) of 224 patients with advanced refractory/relapsed multiple myeloma evaluated oblimersen with dexamethasone versus dexamethasone alone. The primary outcome was time to tumor progress and was not achieved (94 versus 108 days, respectively; $p = 0.26$) (Chanan-Khan et al. 2009). In an interim futility analysis of a phase III trial (NCT00085124, CALGB 10201) of oblimersen in combination with induction chemotherapy with cytarabine and daunorubicin in 503 patients over 60 years old failed to demonstrate a benefit in complete response (48% versus 52%, $p = 0.75$) or 1 year overall survival (estimated 36% versus 40%) (Marcucci et al. 2007). The trial was subsequently terminated early due to lack of benefit.

Clinical development of oblimersen in other malignancies has had mixed results. A CALGB 30103 phase II study (NCT00042978) of oblimersen in combination with carboplatin and etoposide in 56 treatment-naïve extensive stage small-cell lung cancer (SCLC) patients failed to demonstrate a benefit in overall response rate or

overall survival (Rudin et al. 2008). An EORTC phase II study (NCT00085228) of oblimersen in combination with docetaxel in 111 patients with metastatic castrate-resistant prostate cancer also failed to demonstrate a benefit in biochemical or objective response (Sternberg et al. 2009). Based on the mixed results on efficacy in several therapeutic areas, further clinical development of oblimersen has been terminated by the company (SEC Form 10-K, 12/31/11).

SPC2996 (Santaris Pharma) is an antisense nucleic acid molecule against the BCL-2 transcript. A phase I/II clinical trial (NCT 0285103) of SPC2996 as monotherapy in 25 patients with relapsed/refractory CLL demonstrated a decrease in leukocyte count (Tilly et al. 2007). Further clinical development of this compound has been put on hold.

Obatoclax mesylate (GX15-070MS, Teva Pharmaceutical Industry) is small-molecule pan-BCL-2 antagonist with broad but modest affinity for BCL-2, BCL-xL, BCL-2, MCL-1, and A1. Although the compound has demonstrated activity in SCLC in preclinical studies, several phase II-II trials have not been successful in demonstrating a response. An open-labeled, single-arm, phase II-II study (NCT00521144) of obatoclax in combination with topotecan in nine patients with relapsed SCLC demonstrates stable disease in 56% of patients but did not observe any partial or complete response. This response did not surpass historical response rates of topotecan monotherapy (Paik et al. 2011). Another randomized phase II-II study (NCT00682981) of obatoclax in combination with carboplatin and etoposide in 165 refractory extensive-stage SCLC patients demonstrated a trend in favor of obatoclax arm with a benefit in objective response rate, progression-free survival, 12-month survival, or overall survival (Langer et al. 2011). A phase III-III trial (NCT01563601) was planned, but is currently placed on hold. Obatoclax has also been evaluated in other phase II-II trials in non-small cell lung cancer, AML, MDS, myelofibrosis, multiple myeloma, NHL, and HL with mixed results. While obatoclax does demonstrate binding in the hydrophobic pocket, there is evidence that other targets are also affected, thereby calling into the question the drug's true mechanism of action (Davids and Letai 2012). Further, patients in clinical trials do not develop thrombocytopenia, a proposed marker of BCL-xL inhibition. The future clinical development of obatoclax is uncertain.

Abbott Laboratories has developed a family of small-molecule inhibitors of the various members of the anti-apoptotic BCL-2 protein family with greater affinity and selectivity compared to previous compounds in development. The company has partnered with Genentech/Roche to develop some of these compounds.

ABT-737 (Abbott Laboratories) is a selective small-molecule inhibitor of BCL-2, BCL-xL, and BCL-W. It has less binding affinity to MCL-1 and Bfl-1, thereby mimicking the binding pattern of the BH3 protein, Bad. Preclinical studies have demonstrated a Bax- and Bak-dependent cell killing with a direct effect on the mitochondria. Animal studies also have demonstrated a dose-dependent thrombocytopenia (Davids and Letai 2012). Further clinical development has been deferred in favor of next-generation compounds.

Navitoclax (ABT-263, Abbott Laboratories) is derivative of ABT-737 with greater oral bioavailability and similar selectivity for BCL-2, BCL-xL, and

BCL-W. It has been evaluated in 19 clinical trials as monotherapy and in combination in several malignancies, including NSCLC, SCLC, CLL, and NHL. A phase II study (NCT00445198) of navitoclax monotherapy in 39 patients with refractory SCLC showed limited efficacy as a single agent. Partial response was observed in one patient, and stable disease observed in nine patients. Median PFS was 1.5 months and median OS was 3.2 months. This same study identified several potential biomarkers that warrant further investigation including pro-gastrin releasing peptide levels which had a strong association with tumor BCL-2 copy number. Other biomarkers of interest include cytokeratin 19 fragment antigen 21-1, neuron-specific enolase, and circulating tumor cell number (Rudin et al. 2012). A phase 1 study (NCT00481091) that evaluated safety of navitoclax monotherapy in relapsed/refractory CLL suggests activity with 19 out of 21 patients exhibiting >50% reduction in baseline lymphocytosis (Roberts et al. 2012). Further clinical development of ABT-263 in hematologic malignancies has been put on hold in favor ABT-199, although an open-label extension study (NCT00788684) remains active.

Ventoclax (ABT-199, Abbott Laboratories) is a selective small-molecule inhibitor of BCL-2 with high affinity to BCL-2 and BCL-W but lacks affinity binding to BCL-xL. This selectivity reduces drug-related thrombocytopenia and is expected to improve tolerability in hematologic malignancies. There are two ongoing phase 1 clinical trials underway, evaluating safety and pharmacokinetics in combination with bendamustine and rituximab for relapsed, refractory NHL (NCT01594229) and in relapsed, refractory CLL (NCT01328626). Preliminary clinical data showed that a single dose of ABT-199 in three patients with refractory CLL induced rapid tumor lysis within 24 h administration, requiring dose reduction in the phase 1 trial (Souers et al. 2013).

AT-101 (Ascenta Therapeutics) is a small-molecule, pan-BCL-2 inhibitor with affinity for BCL-2, BCL-W, BCL-xL, MCL-1, and AF-1. It also has demonstrated indirect activity against p53-independent upregulator of Noxa and Puma. Structurally, it is a synthesized (-) enantiomer of gossypol, a natural phenol derived from the cotton plant that has demonstrated pro-apoptotic activity in cell models. It has been investigated in several phase II-II clinical trials for glioblastoma multiforme (GBM), prostate cancer, head and neck cancer, lung cancer (NSCLC and SCLC), as well as other hematologic or solid malignancies. In metastatic prostate cancer, phase II trials have been conducted in combination with docetaxel (NCT00571675), in combination with androgen deprivation therapy (NCT00666666), and as monotherapy (NCT00286806). A phase II trial (NCT00540722) evaluated AT-101 in 56 patients with recurrent or progressive glioblastoma multiforme. Preliminary data from the trial shows 16% of patient demonstrated stable disease as best response (Fiveash et al. 2009). Other active clinical trials include a phase II trial in extensive stage SCLC in combination with cisplatin and etoposide (NCT00544596), a phase II trial in combination with chemotherapy in nonoperative laryngeal carcinoma (NCT01633541), and a phase II trial in combination with docetaxel in head and neck cancer (NCT01285635). Results from these trials are pending completion.

Anticipated High-Impact Results

2014: anticipated completion of P1 trial of ABT-199 in CLL.
2015: anticipated completion of P1 trial of ABT-199 in NHL.

Cross-References

- ► BH3-Only Mimetics
- ► Survivin
- ► X-Linked IAP

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BH3-Only Mimetics

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Abstract

BH3 mimetics are a class of drugs designed to tip the balance of pro- and anti-apoptotic factors within a cell to favor apoptosis. They bind within a specific groove of anti-apoptotic Bcl-2 family survival factors in a similar manner to native proteins within the cell that regulate these factors. This primes the cell for apoptosis. Several small molecule BH3 mimetics are currently undergoing evaluation in clinical trials.

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Target: Pro-survival Proteins via the Administration of BH3-Only Mimetics

The Bcl-2 family of proteins are intracellular factors important in regulating cellular apoptosis. All members of the family contain at least one BH (for "Bcl-2 homology") domain. Anti-apoptotic Bcl-2 family members (such as Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and A1) consist of multiple BH domains (BH1–BH4) and a conserved C-terminal membrane domain that allows anchoring at the outer mitochondrial membrane or the endoplasmic reticulum. These anti-apoptotic globular proteins contain a hydrophobic pocket where pro-apoptotic factors bind with high affinity, inhibiting their ability to initiate apoptosis. The pro-apoptotic Bcl-2 proteins Bax and Bak have a similar structure to the anti-apoptotic Bcl-2 proteins (Elkholi et al. 2011), but initiate apoptosis following activation by appropriate intracellular signals. When activated, they homo-oligomerize to form pores in the outer mitochondrial membrane, releasing mitochondrial factors into the cytosol and initiating activation of the caspase enzymes that orchestrate cell destruction. Cells lacking both Bax and Bak are almost entirely resistant to apoptosis. The balance of anti-apoptotic and free pro-apoptotic Bcl-2 family proteins regulates entrance into the death cycle (Happo et al. 2012).

A third subclass of the Bcl-2 family, the BH3-only proteins (Bad, Bid, Bim, Bmf, Hark, Noxa, Puma), responds to cellular stressors and extracellular death signals by altering the balance between the pro- and anti-apoptotic factors, directing the cell toward apoptosis (Lomonosova and Chinnadurai 2008). All BH3-only proteins contain just a single BH domain, called BH3. The BH3 domain alone is both necessary and sufficient for the apoptotic function of these proteins (Chen et al. 2005). BH3 consists of 9–16 amino acids and forms an amphipathic α-helix capable of interacting with Bcl-2 family members of both the anti- and pro-apoptotic subclasses. Some BH3-only proteins called activators (Bid, Bim) can interact directly with pro-apoptotic Bcl-2 family members to promote apoptosis. Others, called sensitizers, bind to anti-apoptotic Bcl-2 family members at the same hydrophobic groove as the pro-apoptotic factors, reducing the number of binding sites available to sequester activated pro-apoptotic factors, favoring progress down the death pathway (Khosravi-Far and White 2008). Some BH3-only proteins are capable of binding and thereby inhibiting the anti-apoptotic function of all anti-apoptotic

proteins in the Bcl-2 family (Bid, Bim, and Puma), while others are selective for particular factors (Lomonosova and Chinnadurai 2008).

BH3-only proteins respond to a wide variety of stressors and are closely regulated at both the transcriptional level and by posttranslational modifications. Under unstressed conditions, many BH3-only proteins are barely detectable, but are then rapidly produced in response to physiologic and pathologic stressors. For example, multiple BH3-only proteins are rapidly upregulated in response to chemotherapy administration and are important factors in mediating cancer cell death under these conditions (Lomonosova and Chinnadurai 2008). Growth factor withdrawal causes upregulation of Bim by the Forkhead transcription factor Foxo3a. Puma and Noxa are transcriptionally regulated by p53 and respond to genotoxic stress. In the posttranslational setting, intracellular sequestration away from the mitochondria, phosphorylation, and protein cleavage are also used to regulate activity of these proteins. Bid is activated through the caspase cascade by extracellular death signals. Bad is phosphorylated in response to cytokine and growth factor signaling, resulting in its sequestration to binding proteins in the cytosol. Bim is sequestered to the microtubule-associated dynein motor complex and released with loss of cell adhesion (Elkholi et al. 2011). Each BH3-only protein is uniquely able to transduce the apoptotic signal in response to specific cellular stressors.

Biology of the Target

Bcl-2 was initially identified as a gene frequently translocated in follicular lymphoma. Relocation of *bcl-2* from its usual position on chromosome 18 to the q31:32 region of chromosome 14, called t(14,18):(q32;q21), places transcriptional regulation of this anti-apoptotic factor under the control of the immunoglobulin heavy chain promoter resulting in profound overexpression. Cells with these augmented levels of Bcl-2 have no change in their proliferation rate, but do not die when exposed to growth factor restriction (Kelly and Strasser 2011). When tested in vivo, translocation of the *bcl-2* gene to the heavy chain promoter region in mice caused a profound B-cell lymphocytosis. Adding this genetic abnormality to mice already overexpressing the *myc* oncogene accelerated tumor formation; all of these mice die from their tumors by 7 weeks of age, compared to just 40% with the *myc* translocation alone. Up to this point, all oncogenes previously discovered functioned by increasing cell growth. Bcl-2 was the first gene found to promote oncogenesis by inhibiting cell death. High levels of Bcl-2 are also found in diffuse large B-cell lymphoma and mantle cell lymphoma as well as lung, brain, and breast cancers. These cancers do not contain the t(14,18) translocation and overexpression is typically attributed to hypomethylation of the promoter. In chronic lymphocytic leukemia, mutational loss of microRNAs that control the level of Bcl-2 results in overexpression of the anti-apoptotic factor (DeVita et al. 2011). Overexpression of other anti-apoptotic Bcl-2 family members has also been found in cancer specimens. Bcl-xL overexpression is common in multiple myeloma. Mcl-1 overexpression is

seen in acute myelogenous leukemia, multiple myeloma, and cholangiocarcinoma (Kelly and Strasser 2011).

Given their role as inhibitors of anti-apoptotic Bcl-2 proteins (like Bcl-2, itself), BH3-only proteins were anticipated to be strong tumor suppressors; however, no consistent evidence has emerged to support this hypothesis. Mouse knockouts of each BH3-only family member have been created and show no developmental phenotype. Later in life, Bad knockout mice do form B-cell lymphoma, and Bid knockout mice develop myeloid hyperplasia which ultimately transforms to leukemia; however, the late onset of these tumors suggests tumorigenesis requires additional mutations accumulated through the aging process (Lomonosova and Chinnadurai 2008). The minimal effect of individual BH3-only protein knockout is attributed to the high physiologic redundancy between BH3-only family members.

Target Assessment

During diagnostic work-up, evaluation of tumor Bcl-2 expression level by immunohistochemistry is part of the standard of care for patients with a suspected B-cell malignancy. Assessment of tumor cells for the presence or absence of the t(14:18) translocation of the *bcl-2* gene by RT-PCR, FISH, and/or cytogenetic studies is also recommended as part of the standard of care work-up for many B-cell malignancies including follicular, marginal zone, mantle cell, and diffuse large B-cell lymphomas (DeVita et al. 2011).

There are no commercial clinically validated assays available to assess levels of BH3-only proteins. Testing for BH3-only proteins is not currently recommended for any cancer type.

Role of the Target in Cancer

Rank: “unknown” to 10 unknown to-1-2-3-4-5-6-7-8-9-10: 7

High-Level Overview

Diagnostic, Prognostic, and Predictive

Bcl-2 expression level is not a consistent predictor of prognosis in cancer (Davids and Letai 2012). CLL and ALL have the highest levels of Bcl-2 of any cancers, but are exquisitely chemosensitive. Conversely, overexpression of Bcl-2 has been seen in the majority of SCLC (75–95%) (DeVita et al. 2011) and is associated with decreased sensitivity to chemotherapy.

There is no evidence that levels of BH3-only proteins are prognostic or predictive of outcome in the large majority of cancers, although some isolated associations have been reported. Levels of some BH3-only factors may be independent predictors

of survival and of response to 5-FU-based chemotherapy in colon cancer (Sinicrope et al. 2008a, b). The expression level of Bim is predictive for response to prednisone in childhood ALL (Jiang et al. 2011). Breast cancer cells expressing Bad are more sensitive to taxanes than those lacking Bad expression (Craik et al. 2010). Interestingly, levels of BH3-only proteins taken in composite have been predicted to correlate with overall survival in glioblastoma multiforme, although expression level of any individual protein is non-correlative (Cartron et al. 2012). Studies in many other cancer types have shown no such association. This is consistent with the hypothesis that there is considerable redundancy of function among BH3-only proteins.

Therapeutics

BH3-only mimetics are small molecules or peptide agents designed to bind within the hydrophobic groove of anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-xL, and Mcl-1), mimicking the interaction of native BH3-only proteins. Occupation of these binding sites by a mimetic results in increased levels of free pro-apoptotic Bax and Bak, theoretically lowering the cellular threshold for apoptosis rendering the cell more susceptible to killing by cytotoxic agents. Cells with high levels of Bcl-2-like proteins (as is seen in many cancers and particularly in CLL and SCLC) are frequently “primed” with a large population of bound and sequestered pro-apoptotic Bax and Bak ready to be released upon administration of a BH3-only mimetic, which can result in spontaneous induction of apoptosis with just the single agent (Davids and Letai 2012). Currently, there are no FDA-approved BH3-only mimetics, but many are under active development.

Attempts have been made to create a peptidic BH3-only therapeutic, but multiple hurdles were encountered in this process. Studies have shown that the entire BH3 domain with intact α-helical structure is required for activity. Unfortunately, these peptides are poorly soluble in aqueous solution, fold poorly if solubilized, and cannot pass the cell membrane without the attachment of internalization tags (Khosravi-Far and White 2008). Modification of BH3 peptides by “hydrocarbon stapling” resulted in stabilization of the α-helix with more favorable solubility and permeability properties and increased half-life in vivo. In vivo apoptotic activity was also demonstrated with some of these “stapled” molecules (Walensky et al. 2004); however, none of these compounds have been tested in clinical trials.

Gossypol is a natural product derived from cotton seed pigments. The (-) enantiomer was developed into a therapeutic called AT-101, which binds Bcl-2, Bcl-xL, and Mcl-1 with moderate affinity. Administration of this compound in multiple cell lines has shown to cause release of mitochondrial contents into the cytosol, triggering apoptosis. In a phase I study in prostate cancer patients, dose-limiting toxicity was grade 3 small bowel obstruction (Liu et al. 2009). Sufficient activity was seen that the compound was advanced into phase II trials in combination with docetaxel, but results of this study are not yet available. A phase I trial also showed activity in breast cancer, but a phase I/II study for small-cell lung cancer in combination with

topotecan was negative (Heist et al. 2010). Results of early stage clinical trials of gossypol both alone and in combination with chemotherapeutic or targeted agents for additional cancers including CLL, esophageal cancer, small-cell lung cancer, squamous head and neck cancers, and adrenocortical cancer are ongoing (Hartman and Czyz 2012).

Obatoclax (GX015-070) is a small-molecule inhibitor of Bcl-2, Bcl-xL, and Mcl-1 at low micromolar concentrations. The prodigiosin parent molecule was identified by high-throughput screen of a natural products library and then chemically optimized to produce obatoclax. Obatoclax has been shown to kill lung cancer cells at low-micromolar IC50, but produces strong synergistic response with numerous chemotherapeutic agents as well as gefitinib, lapatinib, and bortezomib in vitro. The compound is considered to have clinically relevant Bcl-2-family-independent activity because (1) binding affinity for anti-apoptotic molecules is quite low, (2) double knockout of Bak and Bax does not inhibit cell killing by the compound, and (3) it has been demonstrated to cause cell cycle arrest (Lessene et al. 2008). In a phase I clinical trial of heavily pretreated patients with CLL, dose-limiting toxicity was transient adverse CNS toxicity during infusion (O'Brien et al. 2009). A phase II trial of the single agent in patients with relapsed or refractory Hodgkin lymphoma was stopped early due to lack of response (Oki et al. 2012). The compound has also been tested in combination for small-cell lung cancer. A phase I trial in combination with carboplatin and etoposide in chemotherapy-naive patients established safety for this regimen and produced an 88% response rate (Chiappori et al. 2012), but no increased efficacy was seen in phase II testing (Davids and Letai 2012). Similar lack of efficacy was seen in combination with topotecan in relapsed patients (Paik et al. 2011). Combination trials are ongoing in CLL, non-Hodgkin lymphoma, mantle cell lymphoma, and follicular lymphoma (Hartman and Czyz 2012).

ABT-737 and its oral equivalent ABT-263 (navitoclax) were discovered by directed screen for small-molecule-binding partners of the Bcl-xL hydrophobic groove. Both bind to Bcl-2 and Bcl-xL with IC50s in the nanomolar range, but have little affinity for Mcl-1. Consequently, cells with high levels of Mcl-1 tend to be resistant to these compounds in vitro. All preclinical studies support a mechanism of action consistent with perturbation of the pro- and anti-apoptotic balance of Bcl-2 family proteins. In preclinical studies and in clinical trials, ABT-737 and ABT-263 cause dose-dependent thrombocytopenia within hours of administration through direct killing of circulating platelets. Prolonged administration results in a compensatory upregulation of platelet production that can partially abrogate this toxicity. Consequently, administration is begun at smaller doses and then ramped up to allow time for the compensatory response; however, this adverse effect remains a barrier to clinical use particularly in patients with platelet production already suppressed by their malignancy or by prior treatment regimens (Davids and Letai 2012). Navitoclax has shown significant activity as a single agent in both phase I and phase II studies of CLL (Roberts et al. 2012). In combination with rituximab/fludarabine/cyclophosphamide or rituximab/bendamustine, an overall response rate of 81% was seen in patients with relapsed or refractory disease, with some patients achieving complete remission (Davids and Letai 2012).

Success has been more limited in solid tumors. A partial response and several patients with stable disease were reported in single agent phase I trial in SCLC, but limited activity was again seen in phase II trial (Rudin et al. 2012). Results of combination trials in solid tumors have yet to be reported.

Preclinical Summary

Active investigation continues to identify a molecular pattern that could predict which cancers are best "primed" to die by perturbations in Bcl-2 family proteins. It is hypothesized that treatment with chemotherapy or targeted agents could synergize with BH3 mimetics to produce a dramatic effect, but this has not yet been seen in vivo. Further research is needed to identify which antitumor agents are best suited for this role (Cragg et al. 2009).

ABT-263 (navitoclax) shows promise in the early stage clinical trials conducted so far; however, tumors expressing high levels of Mcl-1 show resistance in preclinical work. Modeled after the native Bad protein, ABT-263 does not bind Mcl-1. Research is ongoing to develop a molecule capable of interacting with all anti-apoptotic Bcl-2 family members at the high-affinity ABT-263 partners with Bcl-2 and Bcl-xL (Lessene et al. 2008).

Clinical Summary

Multiple BH3-only mimetics are currently undergoing testing in clinical trials and have shown limited activity as single agents. Results of combination trials for the most specific agent, ABT-263, are not yet available; however, the use of this compound in the clinic is limited by on-target thrombocytopenia. Recently, ABT-263 has been reengineered to decrease affinity for Bcl-xL (implicated in the adverse effect on circulating platelets) and increase affinity for Bcl-2. The new compound, ABT-199, shows decreased toxicity to platelets with no loss of activity against CLL cells in vitro. In first-in-human studies in CLL patients, a single dose of ABT-199 reduced tumor burden by 95% within 24 h of administration without causing significant thrombocytopenia (Souers et al. 2013). There are currently multiple open phase I trials testing ABT-199 in combination with therapy for CLL and non-Hodgkin lymphoma (http://www.clinicaltrials.gov).

Anticipated High-Impact Results

Results of clinical trials with ABT-199.

Results of clinical trials with ABT-263 in combination with other agents.

Cross-References

► Anti-apoptotic Bcl-2
► Caspase
► P53, Immunology

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Caspase

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Anne Noonan

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Abstract

Caspases are essential components of the apoptosis or programmed cell death pathway. There is an intrinsic and an extrinsic apoptosis pathway. Dysregulation of apoptosis is a fundamental component of some tumors. At least 15 caspases have been identified. Mutations and polymorphisms of caspase genes have been described in several solid tumors, and these mutations may prevent damaged cells from being destroyed and may facilitate development of metastases. Protein and RNA levels of caspases can be measured but there are currently no FDA-approved tests for the measurement of caspases in either tumor tissue or blood. Restoration of apoptosis in tumor cells is a priority for cancer drug development. Drugs directly and indirectly targeting caspases are currently in development and

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some are in clinical trials. Second mitochondrial-derived activator of caspases (SMAC) mimetics such as birinapant and AEG35156 target the inhibitors of caspases (inhibitors of apoptosis proteins [IAPs]) and are currently in clinical trials for solid tumors and hematological malignancies. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) agonists stimulate the extrinsic apoptosis pathway to activate caspases and induce apoptosis. Caspase-3 imaging agents are also in development.

Keywords
Caspases • AEG35156 • Effector • Epigenetic modification • Extrinsic and intrinsic apoptosis pathways • Genetic polymorphisms • Imaging agents • Inducible caspase-9 • Inhibitors of apoptosis proteins • Mapatumumab • Preclinical studies • Promega • Role in cancer • Genetic polymorphisms • Inhibitors of apoptosis proteins (IAPs) • Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) • X-linked inhibitor of apoptosis proteins (XIAP)

Target

Caspases are aspartate-specific cysteine proteases that are the principle effectors of the apoptotic response. Fifteen caspases have been characterized. They range from 30 to 50 kD Dorsey 2008. Caspases contain an N-terminal domain, a large subunit and a small subunit. Caspases play a role in cell survival, differentiation, inflammation, cancer, and embryonic development. During programmed cell death (apoptosis), initiator (apical) caspase-2, caspase-8, caspase-9, and caspase-10 are the first players in the caspase cascade and contain a caspase activator and recruitment domain (CARD). Effector (executioner) caspases, such as caspase-3 and caspase-6, are at the end of the apoptotic cascade Lodish 2012. These caspases possess a death effector domain (DED) that is critical in caspase signal transduction. Caspases work as homodimers, with one domain of each stabilizing the active site of the other. All caspases reside intracellularly as procaspases that must be cleaved to become active. Caspases are capable of cleaving over 400 substrates Vaculova and Zhivotovsky (2008). Caspases participate in the extrinsic (receptor-mediated) apoptosis pathway or the intrinsic (mitochondrial) apoptosis pathway (see Fig. 1). Activated caspases target specific intracellular proteins, such as cytoskeletal and nuclear laminar proteins, whose cleavage leads to destruction of the cell. Mutations in killer genes may prevent damaged cells from initiating apoptosis Green 2011. Disruption of apoptosis allows damaged cells, which may carry somatic mutations to survive, thus increasing the potential for tumor development and metastases. Functional mutations and polymorphism of caspase genes have been described in several solid tumors. There is accumulating evidence to suggest that caspases may act as tumor suppressors; however this has not been confirmed Dorsey 2008.

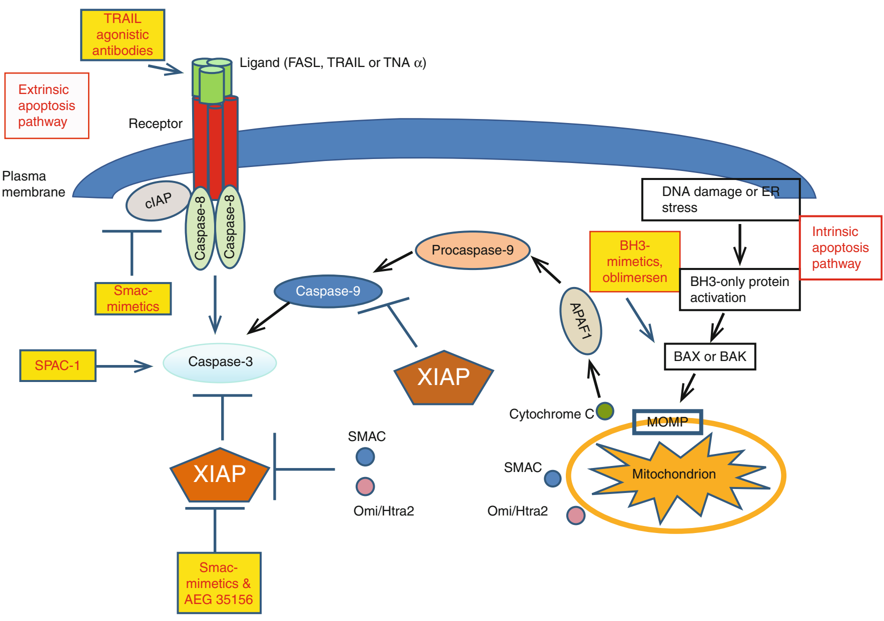

Fig. 1 Overview of the extrinsic and intrinsic apoptosis pathways and drugs targeting the components of the apoptosis pathway resulting in activation of caspases

Biology of the Target

There is currently no approved test or evidence to support measuring serum levels of caspases as a tumor marker. Several studies are examining the role of immunohistochemical assessment of caspases on tumor tissues as prognostic and predictive makers.

Caspases may be targeted therapeutically either directly or indirectly for cancer therapy. Drugs directly activating caspases have been investigated in clinical trials but have been limited by toxicity. A novel approach is the use of drugs which target the inhibitors of caspases, known as the inhibitors of apoptosis proteins (IAPs). Such drugs include SMAC mimetics which simulate the endogenous protein SMAC (second mitochondrial-derived activator of caspases) by degrading the IAPs. AEG35156 is an antisense oligonucleotide that inhibits XIAP mRNA expression, thus reducing the levels of XIAP. XIAP directly binds to and inhibits the effector caspase-3. Inducible caspase-9 is being investigated in hematologic stem cell transplant to reduce the risk graft-versus-host disease. BH3 mimetics indirectly activate caspases by triggering the intrinsic apoptosis pathway. BH3 mimetics bind to and antagonize the anti-apoptotic BCL2 proteins thus releasing the pro-apoptotic

proteins Bax and Bak allowing alteration of the mitochondrial membrane and unleashing apoptogenic mitochondrial proteins such as cytochrome *c*, SMAC/ Diablo, and AIF that cause activation of caspases (see ▶ Chaps. 75, "BH3-Only Mimetics," and Pro-apoptotic BCL-2 Proteins for further details). Studies are also exploring the role of caspase imaging agents to monitor the response to therapies inducing apoptosis.

Target Assessment

There are currently no clinical guidelines on the use of caspase levels in cancer diagnosis, follow-up, or monitoring of response to treatment. There is no FDA-approved test for the measurement of caspases in either tumor tissue or serum. In the research setting, caspase-3, caspase-7, caspase-8, and caspase-9 have been measured in serum using Caspase-Glo® assay (Promega), but this test has not been validated for clinical utility. Caspase protein expression can be measured in tumor tissue using immunohistochemistry and in tumor protein lysates using western blot, ELISA, and reverse phase protein array. RNA levels of caspases can also be measured.

Role of the Target in Cancer

Rank: 7

High-Level Overview

Diagnostic, Prognostic, and Predictive

Measurement of caspase levels or activity is currently not a routine test in the diagnosis of or follow-up of cancer. Measurement of circulating caspases using Caspase-Glo® 3/7 assay has been investigated in a few small studies but was not sufficiently specific or sensitive to detect cancer or distinguish between benign and malignant neoplasms.

Genetic polymorphisms of key caspases have been associated with certain cancers. Yin et al. (2010) published a meta-analysis of 23 publications with a total of 55,174 cancer cases and 59,336 controls from 55 individual studies, in which several potentially functional polymorphisms in caspase-8 were assessed; D302H CC and CG variant genotypes were associated with significantly reduced overall risk of cancers, suggesting that these polymorphisms are potential biomarkers for cancer risk. Frameshift, nonsense, and missense mutations have been described in caspase-8 in invasive colorectal cancer. A meta-analysis comparing Chinese and Caucasian populations showed that two polymorphisms, CASP8-652 6Ndel and D302H, were associated with reduced risk of breast cancer (Sergentanis and Economopoulos

2010). CASP8 D30H was associated with an increased risk of non-Hodgkin's lymphoma in an Egyptian population (Arnaout et al. 2012). Caspase-8 mutations have also been reported in colorectal, gastric, and hepatocellular carcinomas. A Korean study of 397 patients with curatively resected colorectal cancer assessed the prognostic significance of ten single nucleotide polymorphisms in CASP3, CASP6, CASP7, CASP8, and CASP10 genes; no association was observed between SNPs and either progression-free or overall survival in this cohort (Choi et al. 2012). A similar study in 411 Korean patients with early stage NSCLC showed that two polymorphisms in CASP7 genes were significantly associated with prognosis (Yoo et al. 2009). A meta-analysis by Xu et al. (2012) explored caspase-9 polymorphisms and found that in Caucasians the caspase-9 polymorphism rs4645978 conferred a reduced overall cancer risk; carriers of this polymorphism also had a reduced risk of prostate cancer. The A allele of caspase-9 polymorphism rs105276 might be a protective factor for cancer, especially among Asians, while rs4645981 conferred increase susceptibility to lung cancer in Asians. Yan et al. (2012) published a meta-analysis which suggested that the rs13006529*T polymorphism might be a risk factor for cancer susceptibility, especially for breast cancer. Somatic mutations are common in caspase-7 in hematologic malignancies and colon, esophageal, and head and neck cancers and in caspase-10 in gastric cancer.

Epigenetic modification of caspases may inhibit their activity. Caspase-8 is silenced (by either gene deletion or promoter methylation) in several pediatric tumors such as neuroblastoma, rhabdomyosarcoma, medulloblastoma, and retinoblastoma. Compared to normal tissue, reduced expression levels of caspases-1, caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, and caspase-10 have been noted in tumor tissue. Although it may appear counterintuitive that some caspase mutations which decrease apoptotic ability can confer protective effects against tumors, they may facilitate a more potent antitumor immune response as a consequence of reduced apoptosis of tumor-infiltrating lymphocytes.

Several research studies have measured the cleavage of caspase-3, caspase-7, and caspase-8 in cancer cell lines or tumor specimens to confirm apoptosis following cancer drug treatments. As such cleavage of caspases may be a surrogate biomarker for treatment effect when correlated with response on CT scan. Confirmatory studies are ongoing in several clinical trials which require biopsies of tumor tissue before and after treatment. Correlation with survival is also being explored.

Caspase-3 imaging agents are currently being developed. The agents are activated upon cleavage of caspase-3 in tumor. The aim is to demonstrate apoptosis in tumor abrogating the need for tissue biopsy.

Therapeutics

Dysregulation of apoptosis is a problem in numerous cancers. The targeting of the caspase cascade has been a topic of interest for several years. Caspases are present in both normal and tumor tissue. The role of caspases as tumor markers is being investigated, but measurement of caspases in either blood or tumor tissue is not

yet a standard test in cancer. The apoptotic activity of caspases may be reduced in cancer due to an increase in the inhibitors of caspases and the inhibitors of apoptosis proteins (IAPs), such as X-linked inhibitor of apoptosis proteins (XIAP), which play an important role in the extrinsic apoptosis pathway. Inhibition of the intrinsic apoptosis pathway may impede the caspase cascade due to overexpression of anti-apoptotic proteins such as BCL-2. Restoration of the apoptotic machinery and death inducing function of caspases has been achieved through novel mechanisms in in vitro and in vivo models. Several approved cancer-directed therapies indirectly activate caspases resulting in apoptosis of tumor cells. More specific caspase-targeted therapies are in preclinical and clinical development. These either directly activate caspases or indirectly activate them by removing the inhibitors of caspases. Other therapies trigger the extrinsic apoptosis pathway by binding to death receptors.

Preclinical Summary

Extensive preclinical work has been performed to define the mechanisms by which the caspase cascade is inhibited. Several direct activators of caspases are in preclinical development but have not yet entered the clinical arena. S-PAC-1 activates procaspase-3 to caspase-3 and has been studied in a phase I trial of spontaneous lymphoma in pet dogs with good safety profile in this model Lucas 2011. Elucidation of the mechanisms of relief of inhibition of caspases by removal of IAPs led to the development of molecules targeting mRNA expression of IAPs, such as the antisense oligonucleotide AEG35156 which targets XIAP. The development of drugs simulating the effect of the endogenous destroyer of IAPs, second mitochondrial-derived activators of caspases (SMAC), has produced several SMAC mimetics, many of which are in clinical development. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binds to the pro-apoptotic TRAIL receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), and stimulates the extrinsic apoptosis pathway, activating caspase-8 and caspase-10, which then cleave and activate effector caspase-3. Recombinant human TRAIL and TRAIL agonists have produced promising results in the preclinical setting and have entered clinical trials. Mechanisms of stimulating the intrinsic apoptosis pathway include targeting the anti-apoptotic BCL-2 proteins using, for example, antisense oligonucleotides such as oblimersen, O'Brien 2009 or BH3 mimetics such as navitoclax or obatoclax, which relieve the inhibition on Bak and Bax allowing alteration of the mitochondrial membrane potential; release of cytochrome C, SMAC, and AIF; and activation of caspases (see ▶ Chaps. 75, "BH3-Only Mimetics," and Pro-apoptotic BCL-2 Proteins for further details). Preclinical studies of drugs targeting BCL-2 proteins have been promising and some have entered clinical trials. See Fig. 1 for overview of apoptosis pathways and drugs targeting apoptosis.

Clinical Summary

Inducible caspase-9 is being investigated in two phase I trials in hematologic stem cell transplant to reduce the risk graft-versus-host disease. Allodepleted T cells are transduced with an inducible form of caspase-9 which is activated when AP1903 is administered triggering apoptosis of the graft T cells which cause graft-versus-host disease. This concept of inducible caspases may have future applications in solid tumors.

AEG35156 is an antisense oligonucleotide targeting XIAP (an inhibitor of caspases) which is being tested in phase I and II trials, including phase Ib trials in combination with cytotoxic agents. In a randomized phase II trial, the addition of AEG35156 to acute myeloid leukemia induction regimen did not improve remission rates in primary refractory AML (Schimmer et al. 2011). The development of AEG35156 continues. Two SMAC mimetics, Brininapant (TL32711) and AT406, are currently being tested in phase I and II trials in hematologic malignancies and solid tumors with results pending.

Mapatumumab, a fully human monoclonal antibody targeting TRAIL R1 receptor which activates the extrinsic apoptosis pathway, is being tested in phase I and II trials. To date, it has been well tolerated and showed promise in follicular lymphoma (Mom et al. 2009). Results are still pending on many of the trials looking at the combination of mapatumumab and cytotoxic agents, sorafenib, and radiotherapy. A phase I trial of tigatuzumab, a murine monoclonal agonistic antibody to death receptor 5 (TRAIL R2), was well tolerated and showed promising results (Forero-Torres et al. 2010). A phase II trial of tigatuzumab and Abraxane (nab-paclitaxel) is ongoing. Dulanermin (recombinant human TRAIL) was well tolerated in phase Ia and Ib trials. The addition of dulanermin to paclitaxel and carboplatin and paclitaxel, carboplatin, and bevacizumab did not improve outcomes in unselected patients with previously untreated advanced or recurrent NSCLC (Soria et al. 2011). Two studies of dulanermin in metastatic colorectal cancer are ongoing.

Anticipated High-Impact Results

CASPALLO trial of allodepleted T cells transduced with inducible caspase-9 suicide gene

Phase 1 trial of caspase-3 imaging agents

Phase I and II trials of the SMAC mimetics AT-406 and birinapant as single agents and in combination with cytotoxic agents

Phase I trial of the SMAC mimetic GDC-0917

Phase II trials of mapatumumab in combination with cytotoxic agents, sorafenib, and radiotherapy

Phase I and II trials of tigatuzumab and dulanermin in combination with cytotoxic agents and targeted therapies

Cross-References

▶ BH3-Only Mimetics
▶ X-Linked IAP

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DR4 and DR5

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Michael Krainer and Ahmed El-Gazzar

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Abstract

TRAIL has two intact functional receptors, termed as TRAIL receptors 1 and 2. TRAIL receptor 1 (TRAILR1) is also known as DR4, TNFRSF10A, APO2, TR10A_HUMAN, was identified in 1997 (Pan et al., Science 276:111–113, 1997), and possesses 468 amino acids (accession number U90875). TRAIL receptor 2 (TRAILR2) is also known as TNFRSF10B, DR5, KILLER, TRICK2, was also identified in 1997, and has two isoforms resulting from

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alternative splicing. TRAILR2a and TRAILR2b are encoded by 412 and 441 amino acids, respectively (accession numbers AF018657 and AF018658) (Screaton et al., Curr Biol 7:693–696, 1997; Wu et al., Nat Genet 17:141–143, 1997). The extracellular and intracellular domains for TRAILR2 have 58% and 65% similarity to TRAILR1 (Screaton et al., Curr Biol 7:693–696, 1997).

Keywords
APO2 • Death-inducing signaling complex (DISC) • DR4 • DR5 • KILLER • TNFRSF10A • TNFRSF10B • TR10A_HUMAN • TRAIL receptor 1 (TRAILR1) • TRAIL receptor 2 (TRAILR2) • TRICK2

Target: TRAILR1 and TRAILR2 Death Receptors

TRAIL has two intact functional receptors, termed as TRAIL receptors 1 and 2. TRAIL receptor 1 (TRAILR1) is also known as DR4, TNFRSF10A, APO2, TR10A_HUMAN, was identified in 1997 (Pan et al. 1997), and possesses 468 amino acids (accession number U90875). TRAIL receptor 2 (TRAILR2) is also known as TNFRSF10B, DR5, KILLER, TRICK2, was also identified in 1997, and has two isoforms resulting from alternative splicing. TRAILR2a and TRAILR2b are encoded by 412 and 441 amino acids, respectively (accession numbers AF018657 and AF018658) (Screaton et al. 1997; Wu et al. 1997). The extracellular and intracellular domains for TRAILR2 have 58% and 65% similarity to TRAILR1 (Screaton et al. 1997). TRAILR1 and TRAILR2 mRNAs were found to be distributed in almost all tissues including the spleen, thymus, prostate, testis, ovary, small intestine, heart, lung, liver, and peripheral blood leukocytes. TRAILR2 expression was particularly high in the peripheral blood lymphocytes, pancreas, and heart (Screaton et al. 1997). Interestingly, the genes encoding TRAILR1 and TRAILR2 are tightly clustered on human chromosome 8p21-22, a region frequently deleted in cancer. Notably, in mice it has been shown that there is only one functional death-inducing receptor homologous to human TRAILR2 (mTRAIL-R2/mDR5).

Biology of the Target

Apoptotic signaling through death receptors is regulated by the recruitment of receptors into lipid rafts, where receptors, signaling enzymes, and adaptor proteins assemble into a complex. Ligation of TRAILR1/2 leads to their localization in lipid rafts, resulting in assembly of the DISC and activation of the intracellular apoptotic machinery. Induction of TRAILR1/2 redistribution and clustering into lipid rafts is essential for mediating apoptosis signals. In TRAIL-resistant cells, TRAILR1/2 remain localized in non-lipid rafts and are associated with the inhibitor protein cellular (FLICE)-like inhibitory protein (c-FLIP) after stimulation with TRAIL. On

the other hand, the histone deacetylase inhibitor (HDACi) depsipeptide and polyphenol resveratrol induce the distribution of TRAILR1/2 in the lipid raft leading to an increase of apoptosis and inhibition of tumor development.

At the transcriptional level, there are various transcriptional factors that tightly regulate expression of TRAILR1 and TRAILR2. The TRAILR1 promoter encodes an activator protein 1 (AP-1) binding site, which was shown to be important for promoter activation upon AP-1 activator phorbol 12-myristate 13-acetate (TPA) treatment (Guan et al. 2002). Moreover, TRAILR1 promoter encodes a p53-responsive element, and functional p53 was shown to be important for expression of TRAILR1 (Guan et al. 2001). TRAILR2 is known to be a transcriptional target for p53, since, similar to TRAILR1, the TRAILR2 gene encodes a p53-responsive element in the first intronic region in which p53 binds and enhances expression of TRAIL2 (Sheikh et al. 1998). Nevertheless, TRAILR2 expression may also be regulated in a p53-independent manner; it has been shown that the treatment of various cancer cells, harboring mutated p53, with carboplatin or interferon gamma and glucocorticoids increases TRAILR2 expression independent from the p53 status of the treated cells (El-Gazzar et al. 2010; Meng and El-Deiry 2001). It was suggested that this upregulation may be mediated by STAT1. Moreover, there are several transcriptional factors found to regulate TRAILR2 expression including NFkB (Shetty et al. 2005), Myc (Wang et al. 2004), CCAAT/enhancer-binding protein homologous protein (CHOP) (Sun et al. 2007), and SP1 (Kim et al. 2004).

Posttranslational modification of TRAILR1 and TRAILR2 has been shown to be important in inducing the intracellular apoptotic machinery. O-glycosylation of TRAILR1 and TRAILR2 is essential for inducing ligand-mediated receptor clustering and subsequent DISC formation and caspase-8 activation. Small interfering RNA (siRNA)-mediated downregulation of genes encodes enzymes carryout O-glycosylation (GALTNT14 or FUT16) suppressed TRAIL-mediated apoptosis (Wagner et al. 2007).

Target Assessment

TRAILR1/2 are characterized by an extracellular cysteine-rich domain and an intracellular death domain giving them the ability to trigger the assembly of the death-inducing signaling complex (DISC) upon ligand stimulation which initiates the apoptotic machinery. Trimerization of TRAILR1 and TRAILR2 by TRAIL on the surface of target cells leads to recruitment of adaptor molecule Fas-associated death domain protein (FADD), which in turn leads to recruitment and activation of caspase-8. In certain cell types, type I activation of caspase-8 is sufficient for subsequent activation of the effector caspase-3 to execute cellular apoptosis (extrinsic pathway). In other cell types, type II, amplification occurs through the mitochondrial pathway (intrinsic pathway), which is initiated by cleavage of Bid by caspase-8. The truncated Bid (tBid) translocates to the mitochondria and leads to Bax and

Bak-mediated release of cytochrome-c (cyt-c) and Smac/DIABLO from mitochondria. The released cyt-c binds to Apaf-1 to activate caspase-9, which in turn activates caspase-3. The Smac/DIABLO promotes caspase-3 activation by preventing IAPs from attenuating caspases.

Role of the Target in Cancer

The TRAIL-TRAILRs system has been proposed to regulate tumor onset and development. Genetic and epigenetic mutations in functional TRAIL receptors have been observed in several cancers. Mutation in TRAILR1 was detected in lung cancer and head and neck cancer (Fisher et al. 2001). Hypermethylation of the TRAILR1 promoter could be found in 27.7% of ovarian cancer patients (Horak et al. 2005). Mutations in the intracellular domain, a region that mediates the intracellular signaling, of TRAILR2 were found in 10.6% of non-small cell lung cancer (NSCLC) (Lee et al. 1999). Somatic mutations in TRAILR1 and 2 were found in 6.8% of non-Hodgkin's lymphoma (NHL) cases (Lee et al. 2001). Moreover, mutations in the intracellular domain of TRAILR1 and 2 were identified in patients with metastatic breast cancer (Shin et al. 2001). In the light of recent data from in vitro and in vivo studies, upregulation of TRAILR1 or TRAILR2 expression has a clear effect on enhancing the sensitivity of cancer cells to apoptosis (El-Gazzar et al. 2010; Kurbanov et al. 2007). Interestingly, it has been suggested that apoptotic signaling through TRAILR2 may be more potent than through TRAILR1 in cancer cells that express both receptors (Kelley et al. 2005). Different studies have shown that TRAILR2 is more efficiently activated by secondary cross-linked trimers of soluble TRAIL than by non-cross-linked molecules, whereas TRAILR1 is stimulated with the same efficiency by cross-linked and non-cross-linked TRAIL (Kelley et al. 2005).

TRAIL and its functional receptors have been shown to be key effectors in mediating host immune surveillance against cancer progression, and loss of function of TRAILR1 and TRAILR2 may confer resistance to TRAIL-induced apoptosis.

Overall, at this stage, human TRAILR1 and TRAILR2 play a fundamental role in the development of various cancers and therefore are promising targets for cancer therapy. The ability of the agonistic molecules targeting these death receptors to induce apoptosis in cancer cells has become attractive candidates for anticancer treatment and is currently being tested in clinical trials. Accordingly, we rank TRAILR1 and TRAILR2 eight on a scale of 1 “unknown” to 10 “known.”

High-Level Overview

Diagnostic, Prognostic, Predictive

Several recent studies have demonstrated the correlation between TRAILR1 and TRAILR2 expression and development of different cancers. In a clinical study of 376 stage III colon cancer patients treated with adjuvant chemotherapy, TRAILR1

expression was found to be associated with worse disease-free and overall survival (van Geelen et al. 2006). In other study, 90 breast cancer patients with invasive ductal carcinoma showed that TRAILR1 expression positively correlates with the tumor grade (Sanlioglu et al. 2007). In colorectal cancer study of 82 patients, TRAILR2 expression was found to be decreased with increased colorectal cancer stage (Perraud et al. 2011).

Therapeutics

So far, a number of therapeutic strategies involving agonistic antibodies to the TRAILR1 and TRAILR2 have been developed. It has been reported that agonistic antihuman TRAILR1 or TRAILR2 monoclonal antibodies (mABS) exhibited potent tumoricidal activities against human tumor xenografts in nude or SCID mice without apparent toxicity. These agonistic antibodies may be more effective than the ligand at eradicating tumors for several reasons: first the fact that there is a prolonged half-life time in vivo when compared to the recombinant proteins. In human, the half time of agonistic antibody in serum is about 15–20 days, whereas the recombinant soluble TRAIL in serum is only 20–30 min. Second, agonistic antibodies possess an Fc domain, which can recruit and activate FcR-expressing immune cells like NK cells, macrophages, and dendritic cells. Accordingly, administration of either TRAILR1 or 2 agonistic antibody kills TRAIL-sensitive tumor cells as well as induces specific T cells that eliminate the TRAIL-resistant cells. Induction of T cells produces also memory T cells, providing an ideal environment for a long-term protection from tumor recurrence. Third, the decoy receptors, which have been implicated in modulating response to TRAIL, are not targeted by these agonistic antibodies. On the other hand, agonistic antibodies may for the same reason be more toxic to normal tissue because decoy receptors have also been proposed to protect normal tissue cells from apoptosis mediated by TRAIL.

Preclinical Summary

To date, several agonistic antibodies have been reported to exhibit a notable degree of apoptosis, growth inhibition, and cytotoxicity in a broad range of human cancer cell lines and tumor xenografts. Of these approaches, HGS-ETR1 (mapatumumab; developed by Human Genome Sciences), a fully human agonistic mAB targeting TRAILR1; HGS-ETR2 (lexatumumab) and HGS-TR2J (developed by Human Genome Sciences), a fully human agonistic mAB targeting TRAILR2; AMG 655 (developed by Amgen), a fully human agonistic mAB targeting TRAILR2; Apomab (developed by Genentech), a fully human mAB targeting TRAILR2; LBY135 (developed by Novartis), a chimeric agonistic mAB targeting TRAILR2; TRA-8 (Ichikawa et al. 2001), mouse mAB targeting TRAILR2 are in development. Further encouraging results were obtained in recent years by developing AD5-10, antihuman agonistic mAB against TRAILR2 that has exhibited a clear tumoricidal

activity in various cancer mouse models (El-Gazzar et al. 2010; Guo et al. 2005). In contrast to Apomab, AD5-10 is characterized by its unique binding site that does not compete with TRAIL for binding to TRAILR2 and has no toxic effect on human normal hepatocytes (Guo et al. 2005).

Interestingly, numerous reports have noted more favorable interactions following treatment of tumors with a combination of TRAILR1/2 agonistic antibodies and distinct classes of pharmaceutical and cellular anticancer agents. For example, combination of AD5-10 with carboplatin eradicates ovarian tumors in xenograft mouse model (El-Gazzar et al. 2010). HGS-ETR1 has been demonstrated to augment apoptosis in vitro in combination with cisplatin, camptothecin, topotecan, and doxorubicin.

Clinical Summary

Clinical trials have been started in patients using agonistic antibodies targeting TRAILR1 and TRAILR2. In this approach, TRAILR1 human agonistic antibody (HGS-ETR1) and TRAILR2 human agonistic antibodies (HGS-ETR2, HGS-TR2J, CS-1008, Apomab, and AMG 6555) in addition to chimeric (LBY135) antibody against TRAILR2 are in phase I/II clinical trials (Table 1). Overall, data from these clinical trials indicate antitumor activity against a range of different tumors as both monotherapy and in combination with different anticancer agents. These early clinical findings support the safety of these agonistic antibodies, and further studies are required alone and in combination with pharmaceutical and cellular anticancer agents. All in all, agonistic antibodies targeting TRAILR1 and TRAILR2 seem to be a promising regimen for future cancer therapy.

Anticipated High-Impact Results

See Table 1

Table 1 Summary of clinical trials of existing agonistic TRAILR1- and TRAILR2-specific antibodies. This table is adapted and updated from several tables contained in references (Duiker et al. 2006, 2009; Elrod and Sun 2008; Johnstone et al. 2008; Mahalingam et al. 2009)

Molecule tested	Targeted receptor	Clinical trials	N	Tumor type	Status	Comment
HGS-ETR1 (mapatumumab)	TRAILR1	Phase I	49	Solid tumors	Completed	39% of patients showed stable disease
		Phase I	24	Solid tumors	Completed	33% of patients showed stable disease
		Phase I	15	Solid and NHL	Ongoing	
		Phase II	40	NHL	Completed	7.5% of patients showed response and 27% stable disease
		Phase II	38	Relapsed or refractory colorectal cancer	Completed	32% of patients showed stable disease
		Phase II	32	NSCLC	Completed	29% of patients showed stable disease
HGS-ETR1 in combination with paclitaxel and carboplatin	TRAILR1	Phase I	28	Solid tumors	Completed	14% of patients showed partial response
HGS-ETR2 (lexatumumab)	TRAILR2	Phase I	31	Solid tumors	Completed	32.3% of patients showed stable disease
		Phase I	37	Solid tumors	Completed	29.7% of patients showed stable disease
HGS-ETR2 in combination with gemcitabine, pemetrexed, doxorubicin, or FOLFIRI	TRAILR2	Phase Ib	41	Solid tumor and hematological malignancies	Completed	Patients showed partial response
HGS-TR2J	TRAILR2	Phase I		Not reported	Ongoing	
CS-1008 (TRA-8)	TRAILR2	Phase I	17	Solid tumors or lymphomas	Completed	41.1% of patients showed stable disease
CS-1008 in combination with chemotherapy	TRAILR2			TRAILR2-positive epithelial tumors	Ongoing	
Apomab	TRAILR2	Phase I	26	Solid tumors	Completed	3.8% of patients showed stable disease
		Phase II		Soft tissue sarcomas	Ongoing	
Apomab in combination with different anticancer drugs	TRAILR2	Phase I/II		NHL, NSCLC, colorectal cancer	Ongoing	

(*continued*)

Table 1 (continued)

Molecule tested	Targeted receptor	Clinical trials	N	Tumor type	Status	Comment
AMG 655	TRAILR2	Phase I	16	Solid tumors	Completed	6% of patients showed partial response, and 25% of patients showed stable disease
AMG 655 in combination with panitumumab	TRAILR2 and EGFR	Phase Ib/II	15	Solid tumors	Ongoing	
AMG 655 in combination with gemcitabine	TRAILR2	Phase II	13	Metastatic pancreatic cancer	Ongoing	Preliminary data indicated that 23% of patients showed partial response and 46% stable disease
LBY135	TRAILR2	Phase I	32	Solid tumors	Completed	43.7% of patients showed a minor response
LBY135 in combination with capecitabine	TRAILR2	Phase I	24	Solid tumors	Completed	8.3% of patients showed partial response

N number of patients, *NSCLC* non-small cell lung cancer, *NHL* non-Hodgkin's lymphoma

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FLIP

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Abstract

FLIP (FLICE-Inhibitory Protein), also known as c-FLIP, Casper, Cash, CLARP, I-Flice, Flame-1, MRIT or usurpin is a cytosolic protein that can be expressed as three isoforms. cFLIPs are considered as the most potent inhibitors of caspase-8, an initiator caspase required for apoptosis triggering by the extrinsic pathway. Owing to their structural homology with caspase-8, c-FLIPs can be recruited to macromolecular complexes arising from the stimulation of membrane-bound receptors such as members of the TNF or Toll-like receptor superfamilly. Since they are often overexpressed in cancer cells, cFLIPs are considered as interesting targets for cancer therapy. Recent advances in our understanding of cFLIPs expression or stability through post-translational modifications and/or signaling pathways are discussed here to provide ground for future targeting in oncology

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Keywords
APO2L • Cancer • Caspase-8 • CD95 • CFLAR • Death-receptor • DR4 • DR5 • Fas • FLIP • HDAC • Histone deacetylase • NF-kB • Phosphorylation • Proteasome • Receptor • ROS • Targeting • TLR3 • TNF • Toll-like receptor • TRAIL • TRAIL-R1 • TRAIL-R2 • Ubiquitination

FLIP (FLICE-inhibitory protein) is the main inhibitor of caspase-8, the most important initiator caspase activated by death domain-containing proapoptotic receptors of the Tumor Necrosis Factor (TNF) superfamily (Shirley and Micheau 2013). This caspase-8 inhibitor was first identified in the late 1990s from viruses and coined v-FLIP (Thome et al. 1997). Its mammalian cellular orthologue was then identified simultaneously by several independent groups as c-FLIP (Irmler et al. 1997), Casper (Shu et al. 1997), Cash (Goltsev et al. 1997), CLARP (Inohara et al. 1997), I-Flice (Hu et al. 1997), Flame-1 (Srinivasula et al. 1997), MRIT (Han et al. 1997), or usurpin (Rasper et al. 1998). FLIP variants are encoded by c-FLAR, a gene located in chromosome 2q33.1, in close proximity with caspase-8 and caspase-10. c-FLAR contains 14 exons and is transcribed into 13 splice variants, of which three have been demonstrated to be expressed in mammalian cells, c-FLIP long (c-FLIP$_L$), c-FLIP short (c-FLIP$_S$), and c-FLIP Raji (c-FLIP$_R$), respectively. The long isoform of FLIP is a 55 kDa protein, structurally similar to procaspase-8, constituted of two N-terminal death effector domains (DED) and a C-terminal caspase-like domain devoid of the catalytic cysteine residue which confers the proteolytic activity of caspases (Irmler et al. 1997). c-FLIP$_S$ (26 kDa) and c-FLIP$_R$ (24 kDa) also contain two N-terminal DEDs, but these shorter isoforms differ from c-FLIP$_L$ owing to a shorter carboxy terminus, displaying a stretch of residues that play an important role in their ubiquitylation and degradation (Chang et al. 2006; Morlé et al. 2015).

Biology of the Target

Owing to their ability to be recruited with caspase-8, within caspase-8-related macromolecular complexes, and to inhibit both caspase-8 oligomerization and release of its active cleaved products in the cytosol, c-FLIP isoforms are mainly associated with inhibition of apoptosis induced by death receptors (DR; Irmler et al. 1997; Feoktistova et al. 2011; Majkut et al. 2014; Micheau and Tschopp 2003). Like caspase-8, c-FLIP variants are recruited within these complexes through homotypic interactions involving their own DED and the DED of the adaptor protein FADD. Recruitment of c-FLIP to FADD appears to be slightly different than recruitment of caspase-8. Contrary to FADD, which harbors a single DED, c-FLIP and caspase-8 contain two DEDs, and a recent study suggested that while FADD likely preferentially recruits c-FLIP through its DED2, recruitment of caspase-8 on the other hand preferentially occurs via its first DED (Majkut et al. 2014). Beyond their ability to regulate DR-induced apoptosis, growing body of evidence suggests

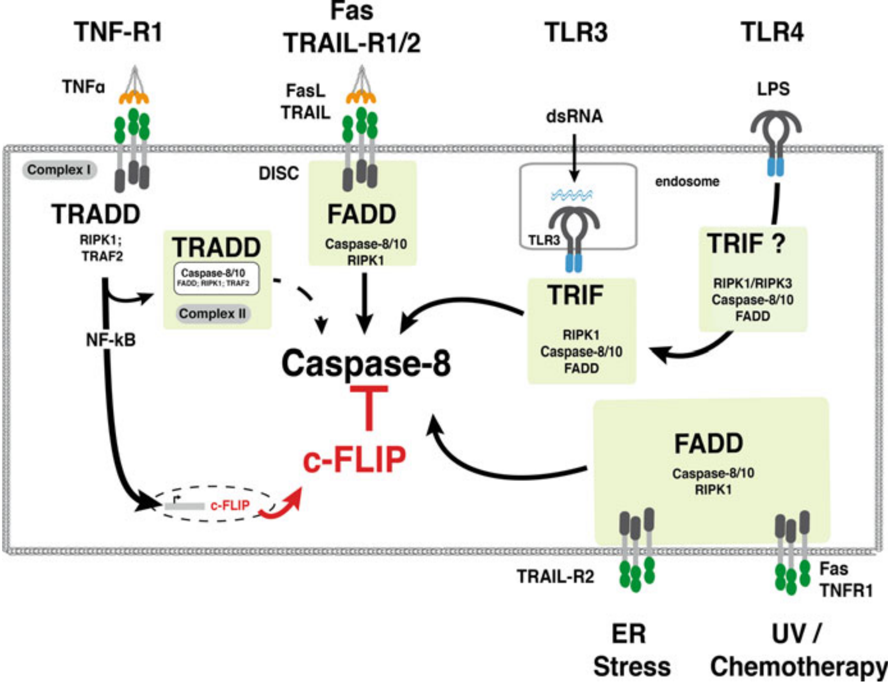

Fig. 1 Caspase-8-related macromolecular complexes regulated by c-FLIP (see text for details)

that other nonconventional proapoptotic macromolecular complexes may be negatively regulated by c-FLIP variants (Fig. 1). The best example has recently been provided with TLR3, which has been demonstrated to recruit FADD and caspase-8 through RIPK1 and TRIF, upon cognate ligand binding (Estornes et al. 2012). This caspase-8 recruiting platform is thought to be assembled through homotypic protein interactions involving the RHIM domains of TRIF and RIPK1 and the death domains of RIPK1 and FADD, allowing subsequent recruitment and activation of caspase-8, through death effector domain interactions. Like the death-inducing signaling complex (DISC) or TNFRI-complex II, this atypical complex is highly susceptible to negative regulation by c-FLIP (Estornes et al. 2012). Another caspase-8 proapoptotic macromolecular related complex is also known to contribute to TLR4-mediated cell death (Lawlor et al. 2015). Yet contrary to TLR3 (Estornes et al. 2012), direct recruitment of caspase-8 through RIPK1 to TLR4 has still not been demonstrated. Whatsoever, since caspase-8 appears to play a prominent role in TLR4-mediated apoptosis (Weng et al. 2014; Iordanov et al. 2005), c-FLIP isoforms are also likely to regulate TLR4 proapoptotic signaling activity. Apoptosis induced by endoplasmic reticulum (ER) stress or UPR (unfolded protein response) may also be targeted by c-FLIP proteins, since this biological response has recently been shown to converge to TRAIL-R2 and to involve receptor aggregation-induced caspase-8 activation, independent of TRAIL (Lu et al. 2014). Similarly, more than

a decade ago, UV radiations and chemotherapeutic drugs were shown to trigger apoptosis, at least in part, through Fas, TRAIL-R2, or TNFR1 in a ligand-independent manner (Micheau et al. 1999; Aragane et al. 1998; Sheikh et al. 1998; Su et al. 2011). Consistent with these findings, both v-FLIP and c-FLIP were shown to be able to inhibit chemotherapy-induced apoptosis (Micheau et al. 1999; Su et al. 2011; Longley et al. 2006; Rogers et al. 2007), further extending current interest to design selective c-FLIP inhibitors.

Expression of c-FLIP isoforms, at the transcriptional level, is tightly regulated by a large number of transcription factors including FOXO3, E2F1, NF-κB, p53, or c-MYC (for more details see Shirley and Micheau 2013). NF-κB is, so far, considered as the most important positive regulator of c-FLIP. Its activation, alone, is sufficient to inhibit TNFα-induced apoptosis, but also albeit to modulate apoptosis induced by Fas ligand or TRAIL (Micheau and Tschopp 2003; Micheau et al. 2002; Travert et al. 2008). On the other hand, repression of c-FLIP expression by c-MYC has been demonstrated to play a prominent role for TRAIL sensitivity (Ricci et al. 2004). At the post-transcriptional level, FLIP isoforms are short-lived proteins, whose expression is highly susceptible to metabolic inhibitors, such as actinomycin D (Griffith et al. 1998), cycloheximide (Mori et al. 2005), or 5-fluorouracil (Galligan et al. 2005). Downregulation of c-FLIP expression has also been shown to occur upon reactive oxygen species (ROS) production (Wilkie-Grantham et al. 2013) or after inhibition of histone deacetylases (HDACs) (Riley et al. 2013), through ubiquitin-mediated proteasomal degradation. Regulation of c-FLIP ubiquitination and degradation has been associated, so far, with three lysine residues, K167, K192, and K195. Ubiquitination of c-FLIP on these lysine residues is tightly controlled through phosphorylation or protein/protein binding (Fig. 2). Likewise, ROS-generating compounds, such as menadione, were demonstrated to induce c-FLIP$_L$ degradation through phosphorylation of threonine 166 and subsequent ubiquitination of lysine 167 (Wilkie-Grantham et al. 2013). Mutation of either corresponding phosphorylation or ubiquitination sites abolished c-FLIP$_L$ degradation induced by ROS. Noteworthy, both threonine 166 and lysine 167 are located within the second death effector domain (DED) of c-FLIP, a region recently suggested to be required for FADD binding, as well as binding to Ku70 (Kerr et al. 2012), a subunit of Ku, a protein involved in nonhomologous end joining (NHEJ) and DNA double-strand break (DSB) repair pathway. Interestingly enough, it was found that HDAC inhibitors disrupt c-FLIP/Ku70 interaction, through Ku70 acetylation, targeting c-FLIP for degradation by the proteasome (Kerr et al. 2012). While these results clearly demonstrate that Ku70 binding protects c-FLIP from ubiquitin-mediated degradation, the molecular mechanisms governing this protection remain unknown.

Lysines 192 and 195 have also been demonstrated to contribute to ubiquitin-mediated c-FLIP degradation. Ubiquitination of these lysines is regulated by phosphorylation. In particular, phosphorylation of serine 193 by PKC was shown to stabilize c-FLIP$_S$ and c-FLIP$_R$, preventing their degradation by the proteasome, through inhibition of K192 and K195 ubiquitination (Kaunisto et al. 2009).

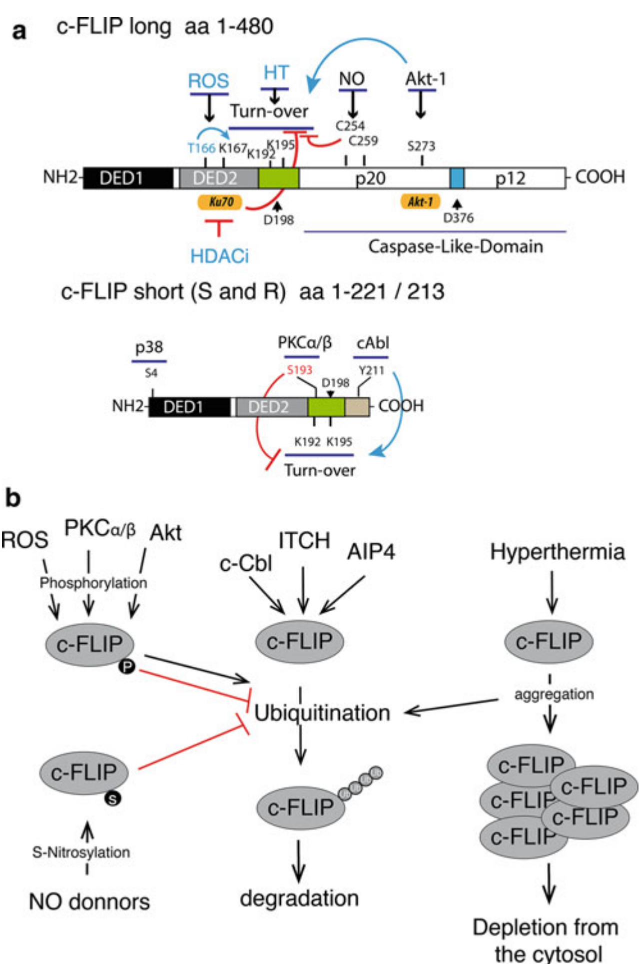

Fig. 2 (**a**) Post-translational modifications regulating c-FLIP protein stability (See text for details). (**b**) Summary of the four main modifications having an impact on c-FLIP function. Ubiquitination of c-FLIP by E3 ubiquitin ligases can lead to its degradation by the proteasome. Ubiquitination of c-FLIP and thus its degradation can be induced or repressed by phosphorylation events as well as S-nitrosylation. Inhibition of c-FLIP function can also be induced by hyperthermia through aggregation (see text for details)

Intriguingly, although phosphorylation of serine 193 also happened in c-FLIP$_L$, and despite the fact that it also compromised c-FLIP$_L$ ubiquitination, it failed to protect this variant from degradation by the proteasome, in the presence of cycloheximide, suggesting that the stability of c-FLIP variants may be regulated differentially. Accordingly, it has been demonstrated that selective c-FLIP$_S$ ubiquitination by the E3 ubiquitin ligase CBL (Fig. 2b) induced c-FLIP$_S$ degradation in mouse macrophages during mycobacteria-mediated apoptosis, due to the phosphorylation of

serine 4 and tyrosine 211 by p38 and the tyrosine kinase ABL, respectively (Kundu et al. 2009). Selective degradation of mouse c-FLIP$_S$ induced by the E3 ubiquitin ligase CBL required phosphorylation of tyrosine 211, which is absent in mouse c-FLIP$_L$. The E3 ubiquitin ligase Itch on the other hand, owing to its ability to interact with c-FLIP$_L$ caspase-like domain, selectively triggered c-FLIP$_L$ ubiquitination and degradation by the proteasome in mouse macrophages stimulated with concanavalin A (Chang et al. 2006). Like CBL, ubiquitination of c-FLIP$_L$ by Itch required JNK activation. The human orthologue of Itch, atropin-interacting protein 4 (AIP4), on the other hand, has been described to control c-FLIPS stability. AIP4 E3 ubiquitin ligase activity, in glioblastoma cells, appeared to be negatively regulated by Akt. Inhibition of AIP4 in these cells decreased c-FLIP$_S$ ubiquitination, increasing thus its stability and cellular resistance to apoptosis (Panner et al. 2009). In primary human macrophages, activation of Akt on the other hand induced selective ubiquitination of c-FLIP$_L$ and degradation by the proteasome through phosphorylation of serine 273 (Shi et al. 2009). Although the E3 ubiquitin ligase involved in this case has not been identified, the authors demonstrated that Itch was not required for c-FLIP$_L$ ubiquitination.

Regulation of c-FLIP isoform stability by the post-translational modifications described here is likely to be the tip of the iceberg, since additional post-translational modifications, including S-nitrosylation or aggregation, are also likely to regulate c-FLIP antiapoptotic function. Likewise, S-nitrosylation of c-FLIP$_L$ on cysteines 254 and 259, induced by nitric oxide donors (NO), was demonstrated to stabilize c-FLIP$_L$ expression (Fig. 2a), by inhibiting c-FLIP$_L$ ubiquitination, and to impair FasL-induced cell death (Chanvorachote et al. 2005). Conversely, albeit degradation of c-FLIP$_L$ through ubiquitination of lysine 195 during hyperthermia has been suggested to contribute to restoration of TRAIL-induced cell death (Song et al. 2013), recent evidence indicates that restoration of this signaling pathway during hyperthermia is primarily due to mere heat-induced c-FLIP aggregation and loss of solubility (Fig. 2b), leading to inhibition of c-FLIP recruitment within TRAIL DISC (Morlé et al. 2015).

Target Assessment

Despite the fact that c-FLIP variants are the most important inhibitors of apoptosis induced by death receptors, none of the clinical trials aiming at assessing the therapeutic potential of recombinant TRAIL (APO2L) or its derivatives have examined their expression levels in tumor biopsies obtained from included patients (Micheau et al. 2013). Assessment of their expression by IHC together with cognate TRAIL receptors would certainly be required to stratify patient's response, as preclinical studies clearly demonstrate that elevated expression levels of c-FLIP and TRAIL-R4 abolish efficacy of TRAIL and its derivatives, even when these compounds are combined with chemotherapy (Morizot et al. 2011).

Role of the Target in Cancer

Rank: 10

Targeting c-FLIP for cancer therapy has emerged as a therapeutic option not only because c-FLIP variants play a central regulatory role during death receptors or toll-like receptors apoptotic signaling but also because their expression is elevated in tumor tissues including colorectal carcinoma (Ullenhag et al. 2007), cervical carcinoma (Wang et al. 2007), Burkitt's lymphoma (Valnet-Rabier et al. 2005), lymphoma (Valente et al. 2006), acute myeloid leukemia (McLornan et al. 2013), and urothelial carcinoma (Korkolopoulou et al. 2004). Moreover, because NF-κB is often associated with proinflammatory signaling within tumor microenvironment, sustained c-FLIP expression in primary tumors is likely to contribute to resistance to apoptosis induced by death receptors or chemotherapy. Likewise, CD40 through its ability to induce NF-κB upregulation was shown to regulate c-FLIP expression and to inhibit TRAIL-induced cell death in follicular lymphoma cells (Travert et al. 2008). Some reports also suggest that c-FLIP may contribute to chemoresistance (Micheau et al. 1999; Su et al. 2011; Longley et al. 2006). Its ability to impair apoptosis induced by death receptors makes c-FLIP a key player for cancer cells to escape tumor immune surveillance (Djerbi et al. 1999).

High-Level Overview

Diagnostic, Prognostic, Predictive

Detection of c-FLIP expression levels in patient tumor biopsies has, so far, mostly been performed from formalin-fixed tissues embedded in paraffin by immunohistochemistry (McLornan et al. 2010), but flow cytometric analysis has also been proposed to measure c-FLIP expression levels in ovarian carcinoma effusions (Dong et al. 2011). Irrespective of the method, robust and selective anti-c-FLIP antibodies are required to obtain reproducible assessment of c-FLIP expression levels from patient samples. An effort should be made to develop better anti-c-FLIP antibodies, since most commercial antibodies remain poorly validated (Bucur et al. 2013). From the studies published, so far, albeit some inconsistencies might exist as regards c-FLIP staining specificity, elevated c-FLIP expression is increasingly being associated with poor patient outcome (Riley et al. 2013; Djerbi et al. 1999; Rao-Bindal et al. 2013; Schmid et al. 2013; Zheng et al. 2014).

Therapeutics

A large number of therapeutic strategies have been developed to target c-FLIP in cancer therapy. So far, these approaches, with the exception of two studies using antisense phosphorothioate oligonucleotides (Logan et al. 2010) or hyperthermia

(Morlé et al. 2015), have mostly targeted c-FLIP indirectly, taking advantage of the short half-life of these variants. Our inability to develop selective c-FLIP inhibitors is likely to be due to our misunderstanding of the fine-tuning of the homotypic interactions governing recruitment of c-FLIP or caspase-8 to FADD, which is likely to be explained by the high structural homology of their DEDs. Second, unlike these initiator caspases, c-FLIP proteins are devoid of enzymatic activity. Their antiapoptotic function, which relies mostly on protein/protein interactions, is thus difficult to target. Moreover, like caspase-8, caspase-10, or FADD, c-FLIP variants are recruited to proapoptotic platforms, such as those induced by ligands of the TNF superfamily or TLR3. Henceforth, selective c-FLIP inhibitors should in principle be able to inhibit c-FLIP recruitment, while preserving recruitment of initiator caspases to the adaptor protein FADD, within these complexes. Yet this task remains highly challenging. Comprehension of recruitment modalities of caspase-8, FADD, and c-FLIP to these macromolecular platforms may help design selective inhibitors in the near future (Majkut et al. 2014; Dickens et al. 2012).

Preclinical Summary

Preclinical studies clearly demonstrate that downregulation of c-FLIP variants by a large variety of compounds through transcriptional or post-transcriptional regulatory events, both in vitro and in vivo, restores sensitivity to apoptosis induced by death receptors (Shirley and Micheau 2013; Morlé et al. 2015; Wilkie-Grantham et al. 2013; Kerr et al. 2012; Shi et al. 2009; Song et al. 2013; Logan et al. 2010). Since these approaches may also extend to apoptosis induced by engagement of TLR3 or TLR4, selective c-FLIP inhibitors could represent lead candidates for cancer therapy.

Clinical Summary

Although c-FLIP variants are critical regulators of apoptosis induced by death receptors and despite the fact that they have been discovered nearly two decades ago, selective c-FLIP targeting for cancer therapy in the clinic is still awaited. From a diagnostic or prognostic point of view, analysis of their expression level by IHC would certainly be useful to stratify patients who may benefit from therapies based on TRAIL derivatives or chemotherapies, whose efficacy has been demonstrated to be reduced in tumor cells expressing high levels of c-FLIP. Reagents allowing evaluation of c-FLIP expression status by IHC are already at reach. Their use in the clinic could be of value as a prognostic biomarker not only for therapies aiming at using TRAIL or its derivatives but also for conventional chemotherapy or therapies targeting TLR3 and TLR4.

Anticipated High-Impact Results

- Identifying selective c-FLIP inhibitors (FLIPi) should be useful for cancer therapy.
- Combined therapies associating FLIPi and TRAIL, TRAIL derivatives, TLR3 or TLR4 ligands, chemotherapy, or radiotherapy should result in significant antitumor efficacy.

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MLH1

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Timothy Kinsella and Kara Lynne Leonard

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Abstract

MLH1 along with other mismatch repair (MMR) genes play a critical role in postreplicative MMR during DNA replication. Hereditary nonpolyposis colon cancer (HNPCC) is associated with mutations in MLH1 and other MMR genes. Mutations in MLH1 are associated with the microsatellite instability (MSI) seen in colon cancers.

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Mismatch Repair • MLH1 • MSH2 • PMS2

Target

MutL homolog 1 (*MLH1*) is a human DNA repair gene located on the short arm of chromosome 3 at position 21.3 (3p21.3). Its homolog in the *E coli* genome, MutL, is involved in DNA repair in the prokaryote. MLH1 is required for mismatch repair during mitosis and meiosis as detailed below.

MutS homolog 2 (*MSH2*) is also a human DNA repair gene. MSH2 is located on chromosome 2 at position (2p21). Together with MSH6, MSH2 forms the heterodimer MutSα, which binds DNA mismatches and initiates DNA repair as detailed below.

PMS2 post-meiotic segregation increased 2 (*PMS2*) is also a DNA repair gene that interacts with *MLH1* and *MSH2* in the mismatch repair process. PMS2 is located on the short arm of chromosome 7 at position 22.2. PMS2 joins with MLH1 to form a protein complex involved in mismatch repair.

MutS homolog 6 (*MSH6*) is another DNA repair gene involved in the mismatch repair process. The *MSH6* gene is located on the short arm of chromosome 2 at position 16. Together with MSH2, MSH6 forms the heterodimer MutSα.

Biology of the Target

MLH1, *MSH2*, and *PMS2* play a critical role in postreplicative mismatch repair (MMR) of erroneous insertions, deletions, and substitutions occurring during DNA replication. The MLH1 protein, which exists as part of one of three heterodimers (MLH1 heterodimerizes with PMS2 to form the MutLα complex, with PMS1 to form MutLβ, and with MLH3 to form MutLγ), has been referred to as the "molecular matchmaker" owing to its role in the recognition of replication mismatches and in the coordination of downstream repair of those mismatches.

The mismatch repair process is initiated by MSH2 (along with MSH6, in MutSα) as part of the MutS (α or β) heterodimer, which recognizes DNA mismatches and insertion-deletion loops and then recruits MutLα. MutLα in turn heterodimerizes with other proteins to bind and hydrolyze ATP, bind DNA, and modulate DNA repair (Modrich 2006). DNA binding by MutLα proteins may occur at both the MLH1 and PMS2 binding sites. Once bound, the MutLα complex translocates along DNA in both directions searching for postreplicative errors ("sliding clamp" model). MutLα then contributes to the correction of single base mismatches and short insertion and deletion loops. Ultimately, exonucleases (principally EXO1) are recruited to help remove the erroneously replicated bases and DNA polymerases (and ligases) are recruited to resynthesize DNA (Fig. 1).

Additionally, *MLH1* plays an integral role of the mismatch repair of chemo-therapeutic agent-induced DNA damage (Jiricny 2006; Kinsella 2009).

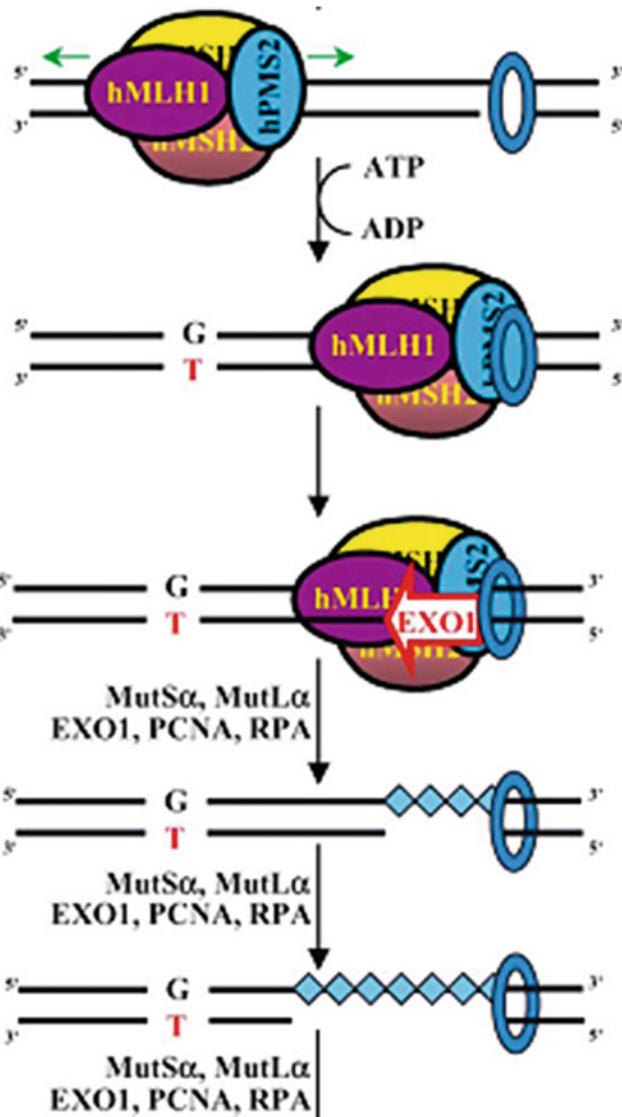

Fig. 1 Postreplicative mismatch repair (Used with permission from Stojic et al. 2004)

Chemotherapeutic agents that induce damage susceptible to repair by the MMR pathway include: methylating agents such as the nitrosoureas and temozolamide; thiopurines including 6-thioguanine, 6-mecaptopurine and azathioprine; platinum analogs such as cis- and carbo-platin; and the fluoropyrimidines (both 5-FU and FUdR). Mismatch repair proteins such as *MLH1* also participate in recognition of DNA base damage such as 8-oxyguinine-thymine mispairs induced by both

low-dose and high-dose rate ionizing radiation (Colussi et al. 2002; Macpherson et al. 2005; Yan et al. 2001, 2009).

As explained by the "futile cycling" model, in cells with functioning MMR, the addition of chemotherapeutic agent-induced and ionizing radiation-induced base damage modifications prompts *MLH1* and *MSH2*, in close conjunction with DNA polymerases, to initiate multiple subsequent cycles of MMR which ultimately induce a prolonged G2 cell cycle arrest and/or cell death via the initial accumulation of DNA single stranded breaks (SSBs). Consequently, MMR-deficient human cancers demonstrate chemotherapy drug and/or ionizing radiation resistance (damage tolerance) to these types of cancer treatments (Hewish et al. 2010). However, chemotherapy drugs such as mitomycin C, oxaliplatin, and topoisomerase inhibitors (camptothecin and its derivative irinotecan) as well as radiosensitizing agents such as Iododeoxyuridine (IUdR) can be used to "target" MMR-deficient cancers (Hewish et al. 2010; Martin et al. 2010b; Berry et al. 2000; Berry and Kinsella 2001).

Target Assessment

Currently, testing of colorectal cancers for mismatch repair deficiency is performed directly by immunohistochemical (IHC) identification of mutated MLH1, PMS2, MSH2, and MSH6 protein expression. Such testing has a sensitivity and specificity of 85–90% (Palomaki et al. 2009). Such tests identify mutated MLH1, MSH2, **PMS2**, **and MLH6** protein but do not distinguish between germline and sporadic mutations. Reduced (or absent) MLH1, **MSH2**, **PMS2**, **or MLH6** protein expression by IHC does not distinguish between genetic (germline) and epigenetic (principally promoter methylation of *MLH1*) mutation. Simultaneous mutations in the *BRAF* (V600E) gene have been identified in the majority (70%) of sporadic mutations of *MLH1*. Direct assessment of *BRAF* mutations may help to distinguish between germline and sporadic mutations, as the common somatic V600E mutation in the *BRAF* gene is never found in Lynch Syndrome (Palomaki et al. 2009).

Role of the Target in Cancer

MLH1

Rank: 9

The role of *MLH1* in cancer is well established. Mutations in *MLH1* are responsible for a major subset of Hereditary Nonpolyposis Colorectal Cancer (HNPCC) syndrome, also known as Lynch Syndrome (de la Chapelle and Hampel 2010). Carriers of mutations in *MLH1* (as well as in *MSH2*, *MSH6*, or *PMS2*) with Lynch syndrome are at a high risk of developing colorectal cancer and endometrial cancer as well as a relatively increased risk of developing gastric cancer, cancers of the small intestine and biliary tract, ovarian cancer, upper urinary tract cancers, and cancers of the sebaceous glands. *MLH1* mutations account for 30–35% of documented cases of HNPCC and are transmitted in an autosomal dominant fashion.

Somatic mutations of *MLH1*, typically by hypermethylation of the *MLH1* promoter, have been implicated in approximately 12–15% of sporadic colon cancers usually associated with a microsatellite instability-high (MSI-H) phenotype (Bettstetter et al. 2007). Similar somatic mutations of *MLH1* by promoter methylation are seen in many common solid tumors of gastrointestinal, genitourinary, and gynecologic origin.

MSH2

Rank: 8

Mutations in MSH2 are the second most common cause of HNPCC/Lynch syndrome.

Human MSH2 maps to human chromosome 2p22-21 near a locus implicated in hereditary nonpolyposis colon cancer (HNPCC). In *E. coli*, expression of hMSH2 in E. coli causes a dominant mutator phenotype, suggesting that hMSH2, interferes with the normal bacterial mismatch repair pathway (Fishel et al. 1993).

Mutations in the *MSH2* gene have also been associated with a variant of Lynch syndrome called Muir-Torre syndrome. Patients with Muir-Torre syndrome are at increased risk of developing colon cancer and skin tumors including sebaceous adenomas and carcinomas and keratocanthomas.

PMS2

Rank: 6

Mutations in the *PMS2* gene are associated with about 2% of cases of familial lynch syndrome. Mutations in the PMS2 gene have also been associated with a variant of Lynch syndrome called Turcot syndrome. Patient with Turcot syndrome are at increased risk of developing colon cancers and glioblastoma.

High Level Overview

Diagnostic, Prognostic, Predictive

In 1997, the National Cancer Institute workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome developed the Bethesda guidelines to identify patients with colorectal cancer who should undergo genetic testing (Rodriguez-Bigas et al. 1997). These guidelines, again updated in 2004, ascertained that the tumors of colon cancer patients younger than 50 years of age, with synchronous or metachronous colon or other HNPCC-related cancers, with colorectal cancers of MSI-high histology younger than 50 years of age, with colorectal cancer in one or more first degree relatives younger than 60 years of age, or with colorectal cancer diagnosed in two or more first or second degree relatives, should be tested for MSI (Umar et al. 2004).

Identification of genetic or epigenetic alterations in MMR genes, including *MLH1*, typically begins with identification of microsatellite instability (MSI) by PCR techniques and/or IHC staining for protein expression in a pathologic sample. **IHC is also used to detect alterations in MSH2, PMS2, and MSH6 protein expression**. Microsatellites are genomic regions in which short DNA sequences or single mononucleotides are repeated. During DNA replication, these repetitions lead to misalignment between the DNA template and the strand being replicated resulting in a daughter strand that is either shorter or longer than its parent, and thus considered "unstable." The intact MMR process usually effectively repairs such microsatellite instability. When MMR genes are mutated, unstable microsatellites are not as efficiently repaired and may accumulate. Using the Bethesda five-marker panel consisting of three dinucleotide repeats and two mononucleotide repeats, a sensitivity and specificity of up to 90% are obtained for detecting MSI by PCR. A methylation-specific PCR method is also a relatively simple assay to test for *MLH1* promoter methylation (Herman et al. 1998). Currently IHC is replacing these PCR approaches as a screening method for MMR deficiency as it is cheaper and more convenient with comparable sensitivity and specificity (Palomaki et al. 2009). Additionally, IHC directly confirms which MMR gene is likely to be mutated (Boland et al. 2008). Mutations in MMR genes can be directly identified by DNA sequencing.

Prognosis

Although not found in all reports, a meta-analysis of available data through 2005 showed that surgically resected MSI (mismatch repair-deficient) cancers have a better prognosis than microsatellite stable (MSS) colorectal cancers (Popat et al. 2005). Interestingly, MSI colorectal cancers are more commonly proximal in location (nearly 75% proximal to the splenic flexure), poorly differentiated, and associated with excessive tumor-infiltrating lymphocytes and mucin (French et al. 2008). However, sporadic MSI colorectal cancers with V600E mutation in *BRAF* have a worse prognosis (Ogino et al. 2009; Tol et al. 2009). Additionally, MSI colorectal cancers associated with chromosomal instability and aneuploidy also have a poor prognosis (Walther et al. 2008). As such, the prognostic effect of MSI is eliminated in the setting of associated chromosomal instability and/or ploidy.

Response to Therapeutics

Perhaps paradoxically, tumors with mutations in MMR display resistance to several different classes of base modifying chemotherapy agents and to ionizing radiation as mentioned previously. With germline or somatic mutations in *MLH1* and *MSH2*, futile cycling does not occur, and cells with deficient MMR treated with chemotherapy, radiation therapy, and fluropyrimidine- or platinum-based chemoradiotherapy do not undergo a G2 cell cycle arrest nor cell death via apoptosis or autophagy. Conversely, damage induced by halogenated pyrimidines, such as IUdR, exploit MMR deficiencies, rendering cells with mutated *MLH1* and *MSH2* comparatively radiosensitive.

A very important finding regarding the prognosis of MMR deficient colorectal cancers is the questionable response of these tumors to 5U based chemotherapy. The Popat et al. meta-analysis found no benefit to the addition of adjuvant 5U based chemotherapy in Stage II and Stage III colorectal cancers. The lack of benefit to chemotherapy in patients with MMR deficient colorectal cancers was replicated in subsequent studies (Jover et al. 2009; Sargent et al. 2010; Bertagnolli et al. 2011, and Hutchins et al. 2011). Conversely, the National Surgical Adjuvant Breast and Bowel Project (NSABP) performed a retrospective review of the colorectal cancer trials carried out between 1977 and 1990 which revealed no such relationship between MMR deficiency and lack of benefit of chemotherapy (Kim et al. 2007). Moreover, a meta-analysis examining the effect of 5FU based chemotherapy in patients with metastatic colon cancer also did not reveal a relationship between MMR deficiency and lack of benefit of chemotherapy (Des Guetz et al. 2009). The relationship between MMR deficiency and benefit of 5FU based chemotherapy is currently being investigated by the U.S. Intergroup Trial (E5202) in patients with stage II colon cancers.

Hutchins et al. performed an analysis on patients treated on the Quick and Simple and Reliable (QUASAR) Trial which randomized patients with Stage II colon cancer to observation vs. adjuvant chemotherapy with 5FU and leucovorin. They found that those with microsatellite instability and the BRAF V600E mutation had a worse prognosis both with and without the use of chemotherapy.

Pre-clinical Summary

Exploitation of mismatch repair-deficient cell sensitivity to IUdR has been investigated in the laboratory (Gurkan et al. 2007a, b). IUdR (a halogenated thymidine analog) is sequentially phosphorylated and competes with thymidine (dTTP) for DNA incorporation. One of the principal IUdR generated mispairs G:IU is efficiently repaired by MMR. Consequently, MMR- deficient tumors do not recognize these IUdR-generated mispairs and retain higher levels of IUdR than cells with normal mismatch repair capabilities. This preferential retention of IUdR is further exploited when MMR-deficient cells are irradiated and exhibit increased radiosensitivity.

Other recent pre-clinical studies have suggested other therapeutic strategies to target sporadic MMR- (*MLH1* or *MSH2*) deficient cancers (Hewish et al. 2010; Kinsella 2009). First, standard chemotherapy drugs including oxaliplatin, topoisomerase I inhibitors (such as camptothecin and its derivative irinotecan) and mitomycin C have shown enhanced cytotoxicity in MMR deficient vs. proficient cancer cells. Additionally, the combination of topoisomerase I inhibitors and thymidine has shown marked enhanced cytotoxicity in MMR-deficient cancer cells associated with an additional frameshift mutation of *MRE11A* (Bolderson et al. 2004). Second, since MMR genes function as tumor suppressors, synthetic lethal approaches have been proposed as targeted therapy for MMR-deficient cancers including inhibitors of DNA polymerases (Martin et al. 2010). Third, since MMR- (*MLH1* or *MSH2*) deficient cancer also acquire gain-of-function mutations in oncogenes such as in

the PIK3-AKT-mTOR pathway, specific small molecule inhibitors of this pathway should demonstrate enhanced cytotoxicity in selected sporadic MMR-deficient cancers (Vilar et al. 2009).

Additional Clinical Results

In the clinical setting, the majority of current investigations are focused on diagnosis of mismatch repair deficiency. Testing is performed regularly on tumor specimens of patients who meet at least one of the Bethesda criteria. Multiple studies have confirmed that the Bethesda guidelines constitute a cost effective and reliable means to identify patients who are at risk of having HNPCC due to *MLH1* or *MSH2*. Many series have carefully studied the genomes of families diagnosed with Lynch Syndrome. To date, however, clinical results examining therapeutics to target MMR deficiency are limited. Listed below are proposals for future investigations of this kind.

Anticipated High-Impact Results

A phase I/II clinical trial has been proposed using IUdR or its oral prodrug IPdR concurrently with radiation therapy in the treatment of MMR-deficient gastrointestinal and gynecologic cancers (Kinsella 2009). Beyond this, combination treatment with IUdR and methoxyamine delivered with radiation therapy has been proposed to co-exploit MMR and base excision repair (BER). Finally, radiation therapy given concurrently with a three drug combination of IUdR, methoxyamine, and temazolamide has been proposed.

Cross-References

▸ AKT
▸ DNA Vaccines
▸ DNA Repair, Overview

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80 NF-κB

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Abstract

Nuclear factor kappa B (NFκB) is a group of structurally related transcription factors, including RelA (p65), RelB, c-Rel, NF-κB1 (p50 and precursor p105), and NF-κB2 (p52 and precursor p100) (Youn et al., Nutr Cancer 61:847–854, 2009). They form homodimers and heterodimers with different combinations when binding to their consensus DNA elements to regulate gene transcription

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at the promoters and enhancers. NFκB is active in many cellular processes and plays a key role in regulating innate and adaptive immune response, inflammation, proliferation, and cell death. Aberrant activation of NFκB and the signaling pathways that regulate its activity contributes to the carcinogenesis in most cancer sites and can be linked to tumor resistance to chemotherapy and radiotherapy (Baud and Karin, Nat Rev 8:33–40, 2009).

Keywords
Bortezomib • Casein kinase II • IkB kinase (IKK) complex • Non-steroidal anti-inflammatory drugs (NSAIDs) • Nuclear Factor Kappa B (NFκB) • Primary mediastinal B-cell lymphoma (PMBL) • Selective estrogen receptor modulators (SERMS)

Target: Nuclear Factor Kappa B (NFκB)

Nuclear factor kappa B (NFκB) is a group of structurally related transcription factors, including RelA (p65), RelB, c-Rel, NF-κB1 (p50 and precursor p105), and NF-κB2 (p52 and precursor p100) (Youn et al. 2009). They form homodimers and heterodimers with different combinations when binding to their consensus DNA elements to regulate gene transcription at the promoters and enhancers. NFκB is active in many cellular processes and plays a key role in regulating innate and adaptive immune response, inflammation, proliferation, and cell death. Aberrant activation of NFκB and the signaling pathways that regulate its activity contributes to the carcinogenesis in most cancer sites and can be linked to tumor resistance to chemotherapy and radiotherapy (Baud and Karin 2009).

Biology of NF-κB

NFκB consists of p65:p50 and RelB:p52 heterodimers which differentially lead to the activation of canonical and noncanonical NF-κB pathways. IκBα is a major inhibitor of NF-κB that binds to Rel proteins in the cytoplasm and masks the nuclear translocation signal of NF-κB components, thereby blocking NF-κB translocation to the nucleus. The IκB kinase (IKK) complex containing IKKα, IKKβ, and IKKγ (NF-κB essential modifier: NEMO) is a major activator for canonical NF-κB signaling (Ghosh and Karin 2002). IKK specifically phosphorylates serine 32 and 36 of IκBα. The phosphorylation induces IκBα protein degradation through the S26 proteasome ubiquitination machinery, allowing the freed NF-κB to move to the nucleus and function as a transcription factor. IKKα and IKKβ contain a kinase domain, a leucine zipper (LZ), and a helix-loop-helix (HLH) motif and form homodimers and heterodimers through their motifs. The two are highly conserved serine/threonine kinases and share many kinase substrates, although IKKβ is more active in phosphorylating IκBα than IKKα. IKKγ is a regulatory subunit. In addition, IKKα phosphorylates the C-terminal region of p100 to induce p100 processing and

generate p52 (Dejardin et al. 2002; Senftleben et al. 2001). Sequentially, RelB:p52 heterodimers translocate from the cytoplasm to the nucleus. The two major NF-κB pathways highly integrate as well (Basak et al. 2007; Saccani et al. 2003).

Role of NF-κB in Cancer

Rank on a 0–10 scale: 8/9.
NF-κB regulates the expression of many genes encoding proteins involved in immune and inflammatory responses, cell death, cell-cycle regulation, cell proliferation, and cell migration. Deregulated NF-κB activity has an important impact on tumor development. NF-κB's ability to regulate genes that inhibit apoptosis and necrosis promotes cell survival of tumor cells. Resistance to chemo- and radiation therapy has also been linked to an increase in NF-κB activity (Baud and Karin 2009). NF-κB is one of the most important drivers of tumorigenesis in primary multiple myeloma cells, and inhibition of NF-κB results in a decrease in the expression of known anti-apoptotic NF-κB target genes. In solid tumors constitutively active NFκB has been linked to breast, cervical, prostate, renal, lung, colon, liver, pancreatic, esophageal, gastric, laryngeal, thyroid, parathyroid, bladder, and ovarian cancers, melanoma, cylindroma, squamous cell carcinoma (skin, head, and neck), oral carcinoma, endometrial carcinoma, retinoblastoma, and astrocytoma/glioblastoma (Baud and Karin 2009). Recently, Meylan et al. have shown elevated NF-κB activity is linked to activated RAS mutation and p53 loss in lung cancers (Meylan et al. 2009). Inhibition of NF-κB signaling in vivo resulted in significant reduction in tumor development providing support for the development of NF-κB inhibitory drugs as targeted therapies for the treatment of patients with defined mutations in Kras and p53.

Microenvironmental inflammation is important for tumor development, particularly in colon, lung, and breast cancers. IKKβ, required for NF-κB activation, is a critical regulator of inflammatory cytokine production (Karin and Greten 2005), and it links NF-κB activity to chronic inflammation. Repetitive exposure to tobacco smoke promotes tumor development both in carcinogen-treated mice and in transgenic mice undergoing sporadic Kras mutation in lung epithelial cells. NF-κB activity and IKKβ-/NF-κB-dependent production of cytokines IL-6 and TNFα are elevated in induced lung carcinomas (Vallabhapurapu and Karin 2009). IKKβ ablation in myeloid cells abrogates enhanced pneumocyte proliferation and reduces lung carcinogenesis. Similarly, deletion of IKKβ in myeloid cells reduced tumorigenesis in a mouse model of colitis-associated colon cancer (Greten et al. 2004).

NF-κB Assessment

Assessing aberrant NFκB activity and the consequences of therapeutically targeting NFκB is challenging as multiple combinations of NFκB dimers and activated pathways require confirmation of specificity of the compound. Gene expression

profiling has been used to distinguish NFκB-dependent activated B-cell-like (ABC) and primary mediastinal B-cell lymphoma (PMBL) diffuse large B-cell lymphoma (DLBCL) from germinal center B-cell-like (GCB) DLBCL (Takahashi et al. 2010). In solid tumors, biopsies are required to assess activation of NFκB proteins by immunohistochemistry or expression of specific NFκB target genes by QPCR. Target gene assessment, however, may be cancer site specific. In colorectal cancer, MSX1, CXCL1, THBS2, CCK5, and TNC have been evaluated by both gene expression and immunohistochemistry (Horst et al. 2009). Gene expression of a different set of markers, BCL2, BLC-X_L, cIAP1 and cIAP2, and TRAF1 and TRAF2, was used to measure the effects of bortezomib and 5-fluorouracil or radiation therapy for the treatment of locally advanced or metastatic rectal cancer (O'Neil et al. 2010). A detailed review of methods for detection of NFκB activity in cancer cells is described in Mauro et al. (2009). Whether these methods can be extended to the clinic will have to be evaluated.

High-Level Overview

Diagnostic, Prognostic, and Predictive

Diagnosing is limited to gene profiling and assessment of the activation of NFκB proteins. Diagnosis is most often made by analysis of tissue from biopsies. Elevated NFκB activity, especially in chemo- or radioresistant cancers, is considered a poor prognostic marker. For multiple myeloma and activated B-cell lymphoma, diagnosis of NFκB dependency is predictive of a positive response to the proteasome inhibitor bortezomib (Staudt and Dave 2005). In colorectal adenocarcinomas, T2, T3, and T4, immunohistochemical detection of (a) proteins that mark the tumor cells in the invading front in contrast to the tumor center and (b) proteins that distinguish stromal cells in the invading front (Horst et al. 2009) are diagnostic for NFκB-dependent cancer. For head and neck squamous cell carcinoma specimens, inhibitor of differentiation 1 (Id1) is overexpressed along with activated p65 and NFκB target gene survivin, contributing to apoptosis resistance (Lin et al. 2010). Clinical trials targeting NFκB in solid tumors have not been forthcoming for assigning a predictive value (Russo et al. 2010).

Therapeutics: Cancer Prevention

Natural products and synthetic antiinflammatory agents that target NFκB are being investigated as safe and inexpensive methods for cancer prevention, particularly in medium to high-risk individuals. Nonsteroidal antiinflammatory drugs (NSAIDs) such as aspirin, selective COX-2 inhibitors, and sulindac can inhibit NFκB activity by targeting multiple points in the pathway. The use of aspirin for preventing cancer

is best documented for colon cancer. Randomized trials show that long-term use of aspirin can prevent colorectal carcinogenesis (Chan and Giovannucci 2010). The high dose required for protection against colon cancer may result in unwanted side effects such as gastric ulcers. Clinical trials also showed that the selective COX-2 inhibitors, celecoxib and rofecoxib, prevented adenoma recurrence (Chan and Giovannucci 2010). Unfortunately, there was an increased risk of cardiovascular events in patients with a history of atherosclerotic heart disease. In addition to the effects of NSAIDs on COX-2, a downstream target of NFκB, these agents may also regulate NFκB activity by sequestering RelA in the cytoplasm or by inhibiting IKK activity. Collectively, the data suggest that NSAIDs that are targeting NFκB and COX-2 could be potential chemoprevention agents against lung, prostate, and esophageal cancers in addition to colon cancer (Brown et al. 2008).

Many natural products affect NFκB activity, either directly or indirectly, and due to their low toxicity, they are good candidates for chemoprevention. Curcumin is a polyphenol derived from turmeric (*Curuma longa*). In the laboratory, curcumin inhibits NFκB activity in ovarian, breast, head and neck, lung, and prostate cancer cell lines (Brown et al. 2008). In the clinic, curcumin is well tolerated (Sharma et al. 2004) and has some biological activity in phase II pancreatic cancer trials (Dhillon et al. 2008). Resveratrol is a polyphenol derived from red grapes and berries. Like curcumin, a significant amount of in vitro data has shown that resveratrol inhibits the growth of multiple cancer cell lines including the breast, prostate, thyroid, head and neck, ovarian, and cervical. Resveratrol appears to regulate cancer cell growth by inhibition of IKK and suppression of NFκB activity (Brown et al. 2008). Resveratrol also inhibits IKK activity in animal models of colitis (Zikri et al. 2009). Data from in vivo preclinical trials shows that resveratrol can prevent tumor growth or carcinogenesis in several cancer sites including the breast, skin, prostate, gastrointestinal, and lung (Bishayee 2009). Clinical trials to date, which are mostly risk assessment studies, show that resveratrol-rich products may be beneficial for cancer prevention. Ongoing clinical trials with pure resveratrol will provide toxicity and efficacy dosage for this chemopreventive agent (Bishayee 2009). A third chemopreventive agent that regulates NFκB activity is epigallocatechin 3-gallate (EGCG), the major polyphenol found in green tea. In animal studies, green tea polyphenols in the drinking water resulted in a delay of primary tumor incidence and tumor burden in a mouse model of prostate cancer that correlated with a substantial reduction in NFκB activity. The data from human studies suggest that green tea polyphenols may provide greater efficacy for preventing prostate cancer than for treating cancer patients (Khan et al. 2009). There are multiple ongoing clinical trials to access the effects of green tea on prostate, lung, bladder, esophageal, breast, and head and neck cancer (see www.clinicaltrials.gov for an updated list of trials). Two other natural products known to inhibit NFκB activity, dietary isothiocyanates, from watercress, and sulforaphanes, from crucifiers (Cheung and Kong 2010), are also being evaluated in clinical trials against various cancers (www.clinicaltrials.com). Other natural products known to block NFκB activity include catechins, silymarin, caffeic acid

phenethyl ester (CAPE), sanguinarine, anethole, emodin, piceatannol, capsaicin, ursolic acid, betulinic acid, flavopiridol, oleandrin (Dorai and Aggarwal 2004), parthenolide, kambekaurin (Brown et al. 2008), and freeze-dried black raspberries (Huang et al. 2002).

Therapeutics: Cancer Treatment

Activated or aberrant regulation of NFκB has been detected not only in lymphoid cancer but in many solid tumors as well (Karin 2009). Often NFκB activation is the result of activating one or more of the upstream components of the NFκB signaling pathway, all of which are possible targets for therapeutic intervention. The primary targets include the IKKs, the 26s proteasome, CK2, and PPAR-γ (Brown et al. 2008). IKKβ is the most active target of the IKK complex and many inhibitors of IKKβ have been developed; however, long-term inhibition of IKKβ may impair the immune system (Youn et al. 2009).

Bortezomib, which targets the ubiquitin-proteasome pathway, inhibits proteasome degradation of IκB, thereby inhibiting NFκB. While bortezomib has been approved to treat multiple myeloma because it delays progression of the disease, it is not clear if NFκB is the only target of bortezomib (Staudt and Dave 2005). For solid tumors, phase II trials with bortezomib showed low to no objective responses for metastatic renal, neuroendocrine, NSCLC, metastatic colorectal, metastatic melanoma, sarcomas, metastatic breast, SCLC, metastatic urothelial, and castration resistant metastatic prostate cancer (Russo et al. 2010). Preclinical data predicted that combinations with bortezomib would sensitize tumors to chemo- or radiotherapy. Unfortunately, in clinical trials, combining bortezomib with cytotoxic drugs including docetaxel, carboplatin, or paclitaxel against hormone refractory prostate, advanced breast, metastatic gastrointestinal cancer, or advanced NSCLC proved to be no more effective than using bortezomib alone (Russo et al. 2010).

The results from current clinical trials have provided new insights into the role of NFκB in cancer and are generating guidelines for new compounds in the pipeline. New drugs in preclinical studies target multiple components of the NFκB pathway. IKK inhibitors include BMS-345541, which targets both IKKα and IKKβ; BAY 11-7085, an irreversible inhibitor of IκBα phosphorylation; MLN120B; and PS-1145. MLN4924 targets the proteasome by inhibiting neddylation of βTrCP. Selective estrogen receptor modulators (SERMS) regulate estrogen receptor ligand activation of NFκB. Peroxisome proliferator-activated receptor (PPAR), a transcription factor that regulates proliferation and inflammation, can be targeted by NSAIDs and antidiabetic agents such as thiazolidinediones (TZDs). Casein kinase II, (CK2), a kinase with multiple substrates in the NFκB pathway, can be targeted by dimethylamino-4,5,6,7-tetrabromo-benzimidazole (DMAT) and by apigenin, a natural plant flavone (Brown et al. 2008; Staudt and Dave 2005). An extensive list of other natural products that affect NFκB activity can be found in Luqman and Pezzuto (2010). Antimalarial quinacrine inhibits both basal and activated NFκB and doing so restores tumor suppressor p53 activity (Gurova et al. 2005).

Clinical Summary

Proteasome inhibitor bortezomib has shown the most promise in treating patients with multiple myeloma and is in clinical trials in combinations with other drugs for solid tumors (Russo et al. 2010). However, more studies are required before bortezomib will be approved for treatment of solid tumors. Similarly, more information is needed for inhibitors of IKK, and caution needs to be taken with long-term use of NFκB inhibitors for prevention. Complications due to suppression of the immune response and their effects on inflammation (Karin 2009) need to be avoided. On the other hand, targeting NFκB for cancer prevention is more promising than for treatment, possibly because the pre-cancer cells have not become addicted to elevated NFκB activity and because carcinogenesis is driven by microenvironment-associated inflammation which can be attenuated by these compounds (Grivennikov et al. 2010).

Preclinical Summary

Because NFκB is over activated in many cancers, it remains an attractive target for cancer therapy. Preclinical studies need to incorporate what has been learned from clinical trials. These studies need to monitor the effects of NFκB inhibition on the immune system and on inflammation. Also more focus is needed on downstream targets of NFκB, including IL-6 and STAT3 (Karin 2009). Finally, biomarkers of efficacy and of off-target effects need to be identified and incorporated in all trials.

Anticipated High-Impact Results

- Targeting NFκB activity in the microenvironment
- Targeting NFκB activity in the neoplastic, especially preinvasive cells
- Targeting signaling events downstream of activated NFκB
- Identifying biomarkers of safety and efficacy
- Targeting NFκB for cancer prevention may offer higher impact than for cancer treatment

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PARP

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Miranda J. Patterson, Yvette Drew and Nicola J. Curtin

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Abstract

PARP enzymes synthese poly(ADP-ribose) (PAR) using nicotinamide adenine dinucleotide (NAD^{+}) as a substrate. PARP-1 is the most extensively studied of a family of PARP enzymes. It is a highly abundant nuclear protein that is activated by DNA breaks and facilitates their repair. PARP inhibitors (PARPis), originally designed to enhance the activity of DNA damaging chemo- and radiotherapy can exploit defects in homologous recombination DNA repair (HRR) by a process termed synthetic lethality. This potential for tumour-selective non-toxic therapy with PARPi has proved the impetus to progress the development of these compounds further.

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Keywords

Poly(ADP-ribose) polymerase • PARP • Poly(ADP-ribose) PAR • DNA single strand breaks • SSB • DNA double strand breaks • DSB • Base excision repair • BER • Homologous recombination repair • HRR • Temozolomide • Topoisomerase I • Camptothecin • Topotecan • Ionising radiation • Chemosensitisation • Radiosensitisation • BRCA1, BRCA2, Rad51

Target: Poly(ADP-ribose) Polymerase (PARP)

PARP enzymes synthese poly(ADP-ribose) (PAR) using nicotinamide adenine dinucleotide (NAD^+) as a substrate. The first PARP enzyme was discovered in the 1960s, and subsequently a superfamily consisting of 17 PARP enzymes identified by sequence homology to the original PARP, PARP-1, have been identified. However, only six of these proteins are true polymerases: PARP1, PARP2, PARP3, VPARP, Tankyrase 1, Tankyrase 2 (Schreiber et al. 2006). PARP-1 is the most extensively studied; it is a highly abundant nuclear protein that contains three zinc finger domains: two (F1 and F2) are located in the amino terminal DNA-binding domain (DBD) (Domain A), and the third (F3) is located in Domain C between a nuclear localization sequence (NLS) (Domain B) and a BRCT domain (Domain D). The central automodification domain separates the DBD from the carboxy-terminal catalytic domain (CAT) (Domain F) (Fig. 1).

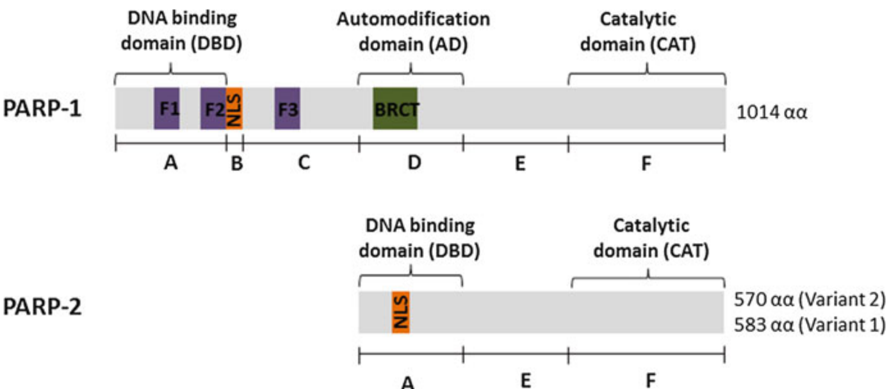

Fig. 1 Architecture of human PARP-l and PARP-2. PARP-1 is a 1014 amino acid (*αα*) protein consisting of six domains. (**a**–**f**) An N-terminal DNA-binding domain (*DBD*) is located in domain (**a**) and contains two zinc fingers (F1 and F2). Adjacent to this is, in domain (**b**), a nuclear localization sequence (*NLS*). A third zinc finger (F3) is located in domain (**c**). A central automodification domain is located in domain (**d**) and contains the BRCT domain. A catalytic domain is found in the C-terminal in domain (**f**). PARP-2 is a 570–583 αα protein consisting of three domains (**a**, **e,** and **f**). Domain A contains a putative N-terminal DNA-binding domain (*DBD*) with a putative NLS. A putative C-terminal catalytic domain (*CAT*) is located in domain (**f**)

PARP-1 is constitutively expressed, but binding to single- or double-strand DNA breaks (SSBs or DSBs) causes a conformational change resulting in a 100–400-fold increase in activity (Altmeyer et al. 2009). The zinc fingers are involved in both DNA-binding and activation of the enzyme, with F2 having the greatest affinity for DNA; F1 participates in binding and activation and F3 in activation. The CAT domain of PARP-1 catalyzes the cleavage of NAD^+ releasing nicotinamide and catalyzing the formation of linear or branched chains of PAR covalently attached to proteins (Fig. 2). PARP-1 covalently PARylates itself (automodification) and other nuclear proteins in the vicinity of the break, in particular histones (heteromodification). The polymers promote loosening of the chromatin to facilitate repair (Althaus et al. 1994). PAR chains are rapidly degraded by the action of poly (ADP-ribose) glycohydrolase (PARG) (Davidovic et al. 2001). PARP-1 activation facilitates the repair of DNA breaks (see section "Biology of Target"), but when damage is excessive, caspase cleavage of PARP-1 helps preserve NAD^+ and ATP pools to allow apoptosis to occur.

PARP-2 shares the greatest sequence homology with PARP-1 and has a similar, overlapping function in DNA repair. Interestingly, while mice with individual knockout of *PARP-1* or *PARP-2* are viable, the knockout of both genes is embryonically lethal (Menissier de Murcia et al. 2003). PARP-3 has recently been shown to

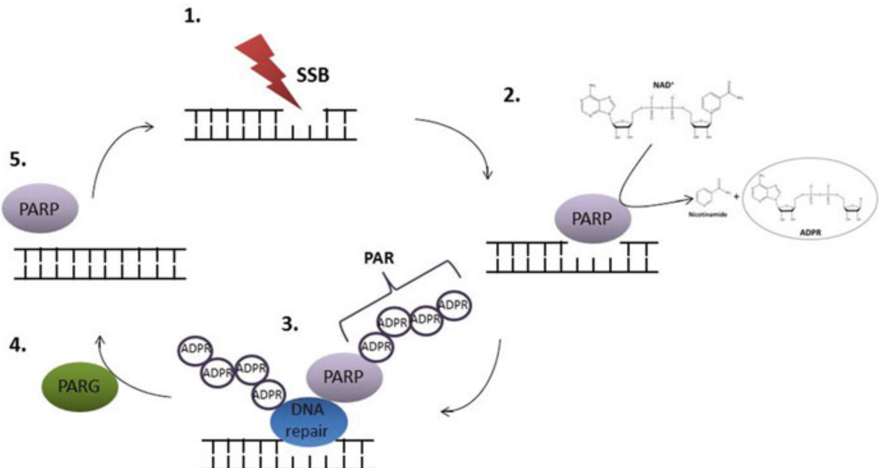

Fig. 2 Schematic of the cycle of PARP-l/2 activation in single strand DNA breaks (SSB). *1* DNA damage results in SSB. *2* SSB cause increased activation of PARP which catalyzes the cleavage of NAD+ to nicotinamide and ADP-ribosyl moities (*ADPR*). *3* PARP forms linear or branched polymers of ADPR (*PAR*) on acceptor proteins; this is either automodification of PARP itself or heteromodification of other proteins involved in the repair of SSB. *4* PAR is degraded by the action of poly(ADP-ribose) glycohydrolase (PARG). *5* Removal of PAR returns PARP to its basally active form

play a role in DNA repair, and tankyrases 1 and 2 are involved with telomere maintenance (Schreiber et al. 2006).

Biology of Target

PARP-1 plays a critical role in the maintenance of genomic stability, cell death, energy metabolism, and transcriptional regulation. The main function of PARP-1, and PARP-2, appears to be in sensing and repairing DNA breaks. It is estimated that between 10,000 and 40,000 base lesions and SSB, and around 50 highly cytotoxic DSBs, accumulate per cell/day. SSBs are repaired by the base-excision DNA repair (BER) pathway (also called single-strand break repair SSBR) in which PARP-1 plays a pivotal role. PAR formation is essential for the recruitment of the BER scaffold protein XRCC1 (El-Khamisy et al. 2003) and the other BER components, DNA polymerases and ligases. Numerous proteins involved in DNA maintenance and cell cycle control also bind non-covalently to PAR via PAR-binding motifs (Gagne et al. 2008).

PARP-1 has also been implicated in the repair of DSB by homologous recombination repair (HRR) and nonhomologous end-joining (NHEJ) pathways. For example, PARP-1 is required for the recruitment of MRE11 and NBS, sensors associated with signaling to HRR and NHEJ, at sites of DSBs (Haince et al. 2008). Additionally, PARP-1 may participate in NHEJ or a backup NHEJ pathway via interactions with the Ku proteins and DNA-PK, key players in NHEJ to facilitate repair of DSB (Audebert et al. 2004; Spagnolo et al. 2012).

Role of the Target in Cancer

Rank: 4–6

PARP-1 may have an indirect role in tumor progression as a transcriptional co-activator of NFκB, a stress-inducible transcription complex that enhances cell survival (Hassa and Hottiger 2002; Veuger et al. 2009). PARP-1 may also regulate the nuclear accumulation and activity of the tumor suppressor gene p53 (reviewed in Wesierska-Gadek et al. 2005). However, the role of PARP-1 in DNA repair is likely to have the most important implications for cancer.

Genomic instability is an enabling characteristic of cancer (Hanahan and Weinberg 2011). PARP-1 promotes genome maintenance limiting genomic instability; PARP-1 deficient mice are susceptible to induced and age-dependent spontaneous tumor formation, particularly when also carrying p53 deletions (Tong et al. 2001). In humans a single-nucleotide polymorphism (SNP) in *parp-1* (T2444C), conferring the amino acid substitution V762A in the catalytic domain and reducing PARP-1 activity, has been linked with risk of developing some cancers (Lockett et al. 2004; Zhang et al. 2005).

Dysregulation in DNA repair is common in cancer, with the impairment of one pathway being compensated by increased dependence on complementary pathways that may be upregulated as a consequence. Loss of PARP-1 activity leads to a

hyper-recombinogenic phenotype (Lindahl et al. 1995) suggesting that BER and HRR were complementary DNA repair pathways. HRR defects are relatively common in cancer (Cerbinskaite et al. 2012), the best characterized being mutations in the breast and ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, which are also associated with prostate, pancreatic, and some other cancers. This led to investigations of PARP inhibitors (PARPi) in HRR-defective cells, with the exciting observation that PARPi alone kill BRCA mutant and other HRR-defective cells at concentrations that are nontoxic to HRR functional cells (Bryant et al. 2005; Farmer et al. 2005; McCabe et al. 2006). This phenomenon, where loss of two pathways (BER and HRR) together causes cell death when loss of either pathway alone is compatible with viability, is known as synthetic lethality. The mechanism is thought to be that persistence of endogenous DNA SSB when BER is impaired by PARP inhibition leads to stalled replication forks and single-ended DSB during S-phase. Under normal circumstances, these would be resolved by HRR, but in HRR-defective cells, they persist or are repaired by an error-prone pathway, resulting in cell death (Fig. 3). Interestingly, in HRR dysfunctional cells, synthetic lethality

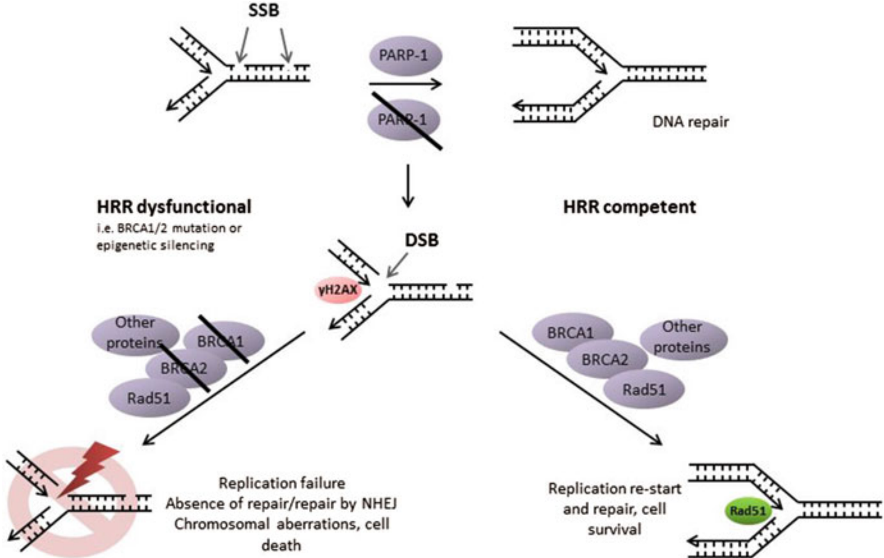

Fig. 3 Synthetic lethality of PARP inhibition in homologous recombination repair (HRR)-defective cell PARP-l is involved in the repair of single-strand DNA breaks (*SSBs*). If PARP-l function is impaired or inhibited, the SSB is unrepaired leading to the formation of double-strand DNA breaks (*DSBs*), which may be visualized by the formation of γH2AX foci at the lesion. In HRR competent cells (i.e., noncancer cells), DBS are repaired by the HRR machinery; HRR repair can be visualized by formation of Rad 51 foci at the repair site. Some cancer cells are HRR dysfunctional due to mutations in HRR proteins; classically, this is associated with mutation of BRCAI/2, but mutations in other HRR proteins (i.e., ATR/ATM, Mrell, Rad 50/51/52/54, Nbsl, RPA, ERCCI, lCRCC3, FANCD2) may also lead to HRR dysfunction. In these cells, DSBs are not repaired efficiently which ultimately leads to cell death

appears to be dependent on a functional NHEJ pathway; deletion or inactivation of 53BP1 or DNA-PKcs restores HRR function resulting in PARPi resistance in BRCA1, BRCA2, or ATM mutant cells (Bouwman et al. 2010; Bunting et al. 2010; Patel et al. 2011). Hyperactivation of PARP has been reported in BRCA2-deficient cells (Gottipati et al. 2010), but PARP activity is higher in tumors compared to the surrounding tissues implying its possible role in cancer growth and survival rather than HRR status (reviewed in Virag and Szabo 2002).

Many anticancer cytotoxic agents act by damaging DNA and an upregulated repair pathway can cause therapeutic resistance. PARP-1 and PARP-2 promote the repair of DNA damage induced by therapeutic DNA-methylating agents (e.g., temozolomide (TMZ) and dacarbazine (DTIC)), topoisomerase I poisons (e.g., topotecan and irinotecan), and ionizing radiation (IR), and genetic inactivation of PARP-1 and PARP-2 increases sensitivity to these agents.

DNA-methylating agents methylate nucleobases at the N^7 and O^6 positions of guanine and the N^3 position of adenine. N^7-Methylguanine and N^3-adenine can be rapidly repaired by the BER pathway. Genetic deletion or depletion of PARP-1 or PARP-2, and PARP inhibition, leads to hypersensitivity to DNA-methylating agents (Durkacz et al. 1980; de Murcia et al. 1997). *Ionizing radiation* (*IR*) kills cells mostly by the generation of reactive-oxidating species (ROS) that cause base damage, SSBs and DSBs. PARP-1 and PARP-2 participate in the repair of these lesions, and PARP-1 and PARP-2 knockout mice, and cells derived from these animals, are hypersensitive to IR (de Murcia et al. 1997; Menissier de Murcia et al. 2003). Additionally, inhibition of PARP leads to radiosensitization of human cancer cell lines (Ben-Hur et al. 1985). *Topoisomerase I* (topo I) forms reversible covalent complexes with DNA and catalyzes the formation of transient DNA SSBs. Topo I poisons stabilize the DNA-topo I complex, leading to persistent SSBs that ultimately lead to stalled replication forks. Topo I poison treatment stimulates PARP activity (Bowman et al. 2001), and PARP-1 is able to interact with topo I and repair topo I-associated SSBs (Malanga and Althaus 2005). PARP-1 knockout mice and PARP-1-deleted mouse embryonic fibroblasts are hypersensitive to topo I poisons (Burkle et al. 2000; Smith et al. 2005).

Target Assessment

Mutations in PARP-1 have not been reported to be associated with the development of cancer, with the exception of sporadic reports on the T2444C SNP. However, increased PARP activity is observed in tumors (reviewed in Virag and Szabo 2002) suggesting dependence on the enzyme and that PARP inhibition may have therapeutic benefit in cancer patients.

The very promising application of PARPi is as a monotherapy, working by a principal of synthetic lethality in HRR-defective tumors. Since HRR defects are associated with tumors rather than normal tissues (with the exception of Fanconi's anemia patients), this is likely to be a tumor-specific therapy. Screening for BRCA1/2 mutation may identify cancer patients who may benefit from PARPi monotherapy.

However, BRCA dysfunction can arise without mutation in spontaneous cancer (reviewed in Turner et al. 2004), and epigenetic silencing of BRCA1 function also leads to PARPi hypersensitivity (Drew et al. 2011b). Moreover, HRR is a multi-protein process such that mutations in other key components confer HRR dysfunction and PARPi sensitivity. The challenge in the field is to develop biomarkers that will identify HRR dysfunctional tumors likely to respond to PARPi therapy. Gene expression profiling has been used to identify a BRCA-like phenotype in ovarian cancer (Konstantinopoulos et al. 2010). Alternatively, evidence of gross genomic instability identified by array comparative genomic hybridization (CGH) may reflect HRR dysfunction (Vollebergh et al. 2012). Another promising approach is to assay HRR function biochemically ex vivo using RAD51 focus formation (a necessary step in HRR downstream of BRCA1, BRCA2, and the most commonly mutated HRR genes) as an indication of ongoing HRR. This approach has been used to identify HRR function in AML, ovarian cancer ascites cells, and breast cancer biopsies (Gaymes et al. 2009; Willers et al. 2009; Mukhopadhyay et al. 2010) and also in FFPE breast cancer biopsies (Graeser et al. 2010). Importantly, AML, MDS, and ovarian cancer ascites cells with reduced ability to form Rad51 foci also display hypersensitivity to PARP inhibition (Gaymes et al. 2009; Mukhopadhyay et al. 2010). Interestingly, the ovarian study found that 50% of samples were HRR defective compared to the 10–15% which would have been identified if screened for BRCA mutations, highlighting the need for biomarkers of HRR function rather than reliance on BRCA mutation screening (Mukhopadhyay et al. 2010).

Therapeutics

PARPi represent an exciting new class of anticancer drugs. The first inhibitors, developed more than 30 years ago, were the three-substituted benzamides (e.g., 3-aminobenzamide, 3AB) that are simple analogues of nicotinamide, which is the by-product of the PARP reaction and itself a weak PARP inhibitor (Purnell and Whish 1980). The proposal that PARPi may be useful to augment the efficacy of DNA-damaging therapeutics was mooted in 1980 following the discovery that 3AB inhibited DNA repair and increased the cytotoxicity of a DNA-methylating agent (Durkacz et al. 1980). More potent PARPi have been developed employing different strategies such as structure activity relationships (SAR), analogue by catalogue, and structure-based drug design (see review Curtin 2005). Most PARPi compounds contain a nicotinamide pharmacocophore, are competitive inhibitors with respect to NAD+ (Fig. 4), and are generally active against both PARP-1 and PARP-2. BSI-201 (4-iodo-3-nitrobenzamide) is proposed to disrupt binding of the zinc fingers of PARP-1 to DNA (Buki et al. 1991), but the PARP inhibitory activity of BSI-201 has now been put into serious question (Patel et al. 2012). The first PARPi to enter clinical trials for cancer patients was AG-014699 (now called rucaparib) in 2003, and since then a further eight PARPi compounds have entered clinical trials. Applications of the PARPi in clinical trial are either as a monotherapy or in a combination therapy.

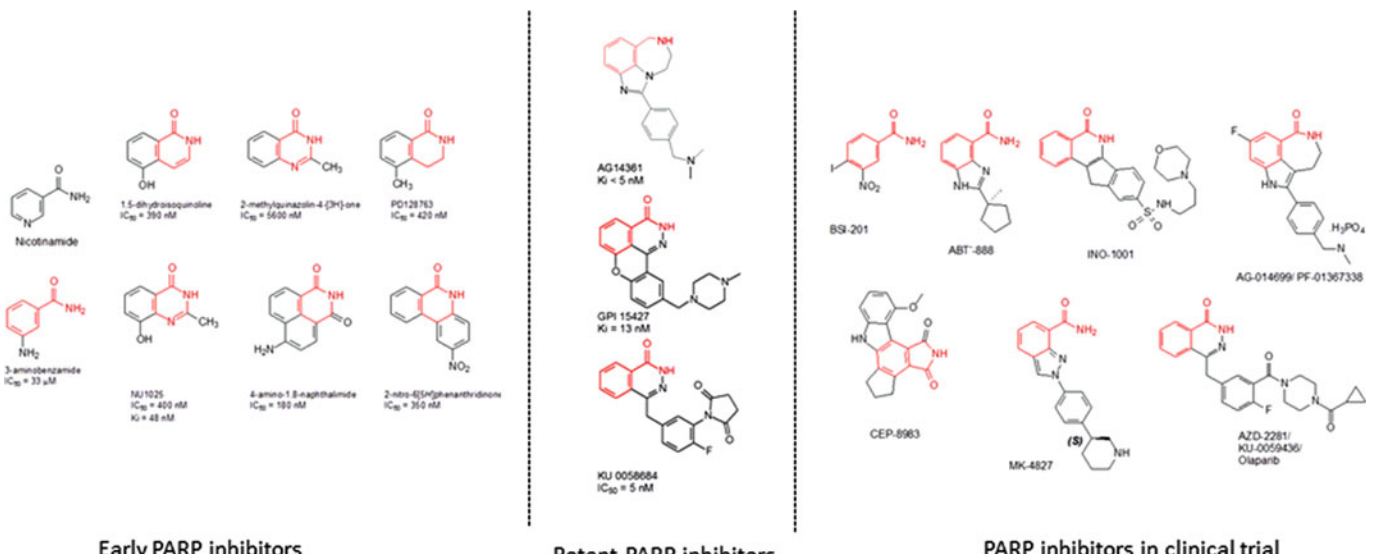

Fig. 4 Structures of PARP inhibitor compounds. PARP inhibitors have been developed around a nicotinamide pharmacocophore (*red*). The earliest PARP inhibitors developed were low potency (examples seen on *left*); this was followed with the development of more potent PARP inhibitors (examples seen in *middle*). Structures of the PARP inhibitor compounds which have been taken into clinical trial are shown on the *right*

Combination Therapies

A wealth of data supports the fact that PARP inhibitors chemopotentiate the antitumor effects of DNA-methylating agents such as methyl methanesulfonate (MMS), TMZ, and DTIC. TMZ is used clinically in the treatment of brain tumors and melanoma and DTIC is used for melanoma. Inactivation of PARP potentiates the effects of TMZ by inhibiting repair of SSB generated by the excision of methylpurines (Villano et al. 2009). Recently it was shown that the PARPi, ABT-888 (veliparib), enhanced TMZ cytotoxicity preferentially during S-phase, indicating that an accumulation of replication-associated DSBs were largely responsible for cell death (Liu et al. 2008b). Defects in DNA mismatch repair (MMR), which are common in colon and ovarian cancer, both sporadic and those associated with HNPCC or Lynch syndrome, confer resistance to TMZ (Friedman et al. 1998). Several independent studies, including studies in tumor-bearing mice, have shown that a variety of PARPi (3AB, PD128763, NU1025, INO-1001, AG14361, and ABT-888) cause the greatest chemopotentiation of TMZ in a MMR-defective background and may restore TMZ sensitivity (Wedge et al. 1996; Tentori et al. 1999; Curtin et al. 2004; Cheng et al. 2005; Horton et al. 2009).

Preclinical studies demonstrate exciting results using the combination of PARPi with TMZ. NU1025 and TMZ co-treatment increased the survival of mice with brain lymphomas (Tentori et al. 2002). Several other studies with various PARPi (CEP-6800, GPI 15427, AG14361, rucaparib (AG-014699)) have shown preclinical activity with TMZ in a variety of cancer models, including pediatric tumors (Miknyoczki et al. 2003; Tentori et al. 2003; Daniel et al. 2009, 2010). However, it was the observation that AG14361 and AG-014699 (rucaparib) in combination with TMZ caused the complete regression of SW620 cell line xenografts (Calabrese et al. 2004; Thomas et al. 2007) that prompted the first clinical trial of rucaparib in combination with TMZ in 2003.

In this first in human PARPi phase I trial of rucaparib in combination with TMZ, a test dose of single agent rucaparib was given 1 week prior to the combination to allow safety, pharmacokinetic (PK), and pharmacodynamic (PD) evaluation before it was combined with TMZ. The study was driven by a pharmacodynamic endpoint, which was to establish a PARP inhibitory dose (PID) of rucaparib, before attempting to evaluate the maximum tolerated dose (MTD) of the combination. The PID was established at 12 mg/m^2, with sustained PARP inhibition observed in PBMCs and tumor biopsies. No dose-limiting toxicities (DLTs) of single agent rucaparib were reported, and the DLT of the combination was myelosuppression (a recognized normal tissue DLT of TMZ) (Plummer et al. 2008). However, this combination in a phase II study in metastatic melanoma caused enhanced TMZ-induced myelosuppression, necessitating a 25% dose reduction of TMZ. Importantly, the study reported an increase in the response rate and median time to progression compared to historical reports of TMZ alone (Plummer et al. 2006). Phase I investigations of other PARPi (INO-101, CEP 9722, olaparib, ABT-888) in combination with TMZ or dacarbazine have been undertaken to establish the MTD, but these studies have generally been too preliminary or too small to comment on the efficacy (Bedikian

et al. 2009; Isakoff et al. 2010; Khan et al. 2011; Campone et al. 2012). DLTs have largely been myelosuppression and elevated transaminases. In a phase I trial of metronomic cyclophosphamide in combination with ABT-888(veliparib), the MTD was not reached and activity was reported in only a small number of *BRCA*-mutated ovarian and triple negative breast cancers (Kummar et al. 2010).

Topoisomerase I (topo I) poisons are used in the treatment of a variety of cancers; topotecan is used to treat small-cell lung cancer and ovarian and cervical cancer and irinotecan in the treatment of colorectal cancer. Both are clinical derivatives of the parent compound camptothecin. Several studies have shown that PARPi are able to potentiate the cytotoxicity of topo I poisons. One of the first showed that the PARPi NU1025 increased camptothecin induced DNA strand breaks and cytotoxicity to a similar extent (Bowman et al. 2001); additionally NU1025 and NU1085 enhanced the toxicity of topotecan by up to fivefold in a panel of human lung, ovarian, colon, and breast cancer cell lines (Delaney et al. 2000). The PARPi, CEP-6800, potentiated irinotecan in a colon carcinoma cell line (Miknyoczki et al. 2003), and AG14361 enhanced topotecan-induced growth inhibition in human colorectal and non-small cell lung carcinoma cells (Calabrese et al. 2004). This effect is PARP dependent, rather an off-target effect, as demonstrated by the selective potentiation of PARP-1 wild-type cells over PARP-1 null cells (Smith et al. 2005).

Investigations in tumor-bearing mice also demonstrate PARPi chemosensitization of topo I poisons. Coadministration of CEP-6800 increased irinotecan-induced growth inhibition of HT29 human colon carcinoma xenografts by 60% (Miknyoczki et al. 2003), but the PARPi GPI 15427 did not increase the activity of irinotecan in the same model (Tentori et al. 2006). The preclinical lead PARPi, AG14361, significantly increased the antitumor activity of irinotecan in a human colorectal cancer (LoVo) xenograft model (Calabrese et al. 2004), and the clinical PARPiAG-014699 (rucaparib) enhanced topotecan-induced tumor growth delay in a neuroblastoma model (Daniel et al. 2009).

Clinically, the data reported so far have focused on determining the MTD and proof of mechanism. In a phase I study of ABT-888 (veliparib) with topotecan, myelosuppression was observed. Further preclinical studies informed a revised schedule, and the MTD was established as topotecan 0.6 mg/m^2/d with ABT-888 10 mg twice daily on days 1–5 of a 21-day schedule, with some disease stabilization. In this study, PARP activity was reduced in both tumor and PBMCs, and importantly, increased DNA breaks were detected in circulating tumor cells and PBMCs with the combination (Kummar et al. 2011). In a study of irinotecan in combination with ABT-888, DLTs were diarrhea and neutropenia with a MTD of 100 mg/m^2 irinotecan (LoRusso et al. 2011). A phase I study of olaparib and topotecan saw DLTs of neutropenia and thrombocytopenia at doses of topotecan 1 mg/m^2/daily for 3 days and olaparib 100 mg twice daily, so further dose levels were not explored (Samol et al. 2011).

Conflicting literature is available about the potential ability and efficacy of PARPi to potentiate the cytotoxicity of platinum agents such as carboplatin and cisplatin. These agents induce inter- and intrastrand cross-links in DNA, which are repaired by nuclear excision repair (NER) and HRR and are used in the treatment of a variety of

tumor types but most commonly ovarian, lung, testicular, and GI cancers. PARP-1 has been reported to bind to, and be activated by, cisplatin-induced DNA damage (Burkle et al. 1993; Guggenheim et al. 2008). However, PARP-1-deleted cells are not reported to be sensitive to platinum agents, and the ability of PARPi to sensitize cells to cisplatin appears to be cell line and compound dependent (Bernges and Zeller 1996; Guggenheim et al. 2008). Evidence indicates that PARPi are preferentially effective with platinum therapy in HRR-defective cells, for example, the PARPi, AZD2281 (olaparib), selectively sensitized BRCA2-defective cell lines but not BRCA2-proficient cells to platinum therapy (Evers et al. 2008).

Several in vivo studies have investigated combination therapy with PARPi and platinum agents with evidence suggesting a positive effect in HRR-defective tumors. The PARPi, ABT-888 (veliparib), in combination with platinum drugs caused regression of BRCA1- and 2-deficient MX-1 xenografts (Donawho et al. 2007). Moreover, treatment of genetically engineered mouse models of hereditary BRCA1-associated breast cancer with the PARPi olaparib (AZD2281) alongside cisplatin or carboplatin inhibited tumor growth and increased their recurrence-free survival, although this treatment did not eradicate tumors (Rottenberg et al. 2008). Similarly, mice bearing BRCA2 mutant Capan1 xenografts treated with AG-014699 (rucaparib) displayed enhanced carboplatin-induced tumor growth delay (Drew et al. 2011b). Platinum chemopotentiation by PARPi appears to be more apparent in the in vivo setting based on reports that the PARPi CEP-3000 and BGP-15 enhanced cisplatin-induced tumor xenograft growth delay but failed to enhance cisplatin toxicity in corresponding cell line models (Racz et al. 2002; Miknyoczki et al. 2003).

The National Cancer Institute (NCI) sponsored a clinical study of the combination of olaparib with cisplatin and gemcitabine but reported DLT of myelosuppression at the first dose level explored. Investigators were subsequently forced to de-escalate to establish tolerable PARP inhibitory doses of olaparib with gemcitabine at 400 mg/m^2 and cisplatin at 40 mg/m^2. The study was undertaken in *non*-heavily pretreated patients (Giaccone et al. 2010).

Radiotherapy is used at some stage in the treatment of around 50% of cancer patients; this and the fact that radiotherapy induces DNA damage that is repaired by PARP-dependent processes suggested that it would be a good candidate for combination therapy with PARPi. Indeed, early studies demonstrate radiosensitization of human tumor cell lines by the PARPi 3AB (Ben-Hur et al. 1985). Additional studies have demonstrated radiosensitization by a variety of PARPi (ANI, NU1025, AZD2281, E7016) in multiple cell line models with dose-enhancement ratios of 1.3–1.7 (Schlicker et al. 1999; Bowman et al. 2001; Brock et al. 2004; Dungey et al. 2008; Russo et al. 2009). It is proposed that the mechanism by which PARPi increases IR sensitivity is by inhibiting the repair of SSBs that convert to DSBs upon collision with replication forks in S-phase (Saleh-Gohari et al. 2005), which can be visualized by the persistence of IR-induced γH2AX foci following PARPi treatment (AZD2281 and E7016) (Dungey et al. 2008; Russo et al. 2009). In some studies, PARPi selectively radiosensitize actively replicating S-phase cells (Banasik et al. 1992), but in others, PARPi sensitization of growth-arrested cells has been

demonstrated, for example, the PARPis PD128763, NU1025, and AG14361 inhibited recovery from potentially lethal IR doses in a variety of growth-arrested human and mammalian cell lines (Bowman et al. 1998; Calabrese et al. 2004). These observations have clinical importance as growth-arrested hypoxic radio-resistant cells can repopulate the tumor after radiotherapy and are a major contributing factor to failure of radiotherapy treatment (Liu et al. 2008a).

In vivo radiosensitization with PARPi has been demonstrated with several compounds. In the first study, PD128763 enhanced the x-ray-induced growth inhibition of SCC7 xenografts in mice by threefold (Sebolt-Leopold and Scavone 1992). Subsequent studies demonstrate that AG14361 significantly increased the antitumor activity of x-rays in colon cancer xenografts (Calabrese et al. 2004); GPI15427 significantly enhanced the irradiation-induced growth inhibition in HNSCC xenografts (Khan et al. 2010); ABT-888 increased the antitumor radiotherapy effects in human colon, lung, and prostate cancer xenografts (Albert et al. 2007; Donawho et al. 2007; Barreto-Andrade et al. 2011); MK-4827 radiosensitized human lung and triple negative human breast carcinoma xenografts (Wang et al. 2012); and olaparib (AZD2281) in combination with radiotherapy significantly increased radiotherapy-induced tumor regression of Calu-6 non-small cell lung carcinoma xenografts (Senra et al. 2011). In vivo studies have also highlighted that the PARPi E7016 can enhance the combination treatment of temozolomide plus irradiation, slowing tumor growth by an additional 6 days in human glioma xenografts (Russo et al. 2009).

These data support a role for combining radiotherapy and PARPi in patients with cancer and clinical trials are finally underway (www.clinicaltrials.gov) with results eagerly awaited. One study has reported interim results of a phase I dose escalation trial of ABT-888 (veliparib) in combination with whole-brain radiotherapy (37.5 Gy in 15 fractions or 30 Gy in 10 fractions) in patients with brain metastasis from advanced solid tumors. The study showed that up to 200 mg veliparib twice daily was well tolerated with radiotherapy and further dose escalation is planned (Mehta et al. 2012). The further appeal of combining PARPi with radiotherapy is that the toxicities seen with the chemotherapy combinations may be avoided as the treatment is targeted.

Monotherapy

Arguably the most exciting use of PARPi is as a single agent based on the pioneering studies demonstrating that HRR-defective cells and tumor xenografts were hypersensitive to PARPi alone (Bryant et al. 2005; Farmer et al. 2005). These initial studies demonstrated that cells lacking BRCA2, XRCC2, and XRCC3 or which had BRCA2-depleted by siRNA were hypersensitive to a panel of PARPi (3AB, NU1025, and AG14361) (Bryant et al. 2005). Additionally, BRCA1- and BRCA2-defective mouse embryonic stem cells were sensitive to the PARPi KU0058948 (Farmer et al. 2005). The use of PARPi in this synthetic lethal manner exploits the molecular pathology of cancer cells so that they are selectively targeted, as normal cells will have functional HRR and will be able to repair any PARPi-induced damage.

In vivo studies have shown that PARPi treatment specifically causes growth inhibition of BRCA mutant xenografts; for example, AG14361 inhibited the growth of BRCA2-deficient V-C8 xenografts but had no effect on the growth of BRCA2 corrected V-C8 + B2 xenografts (Bryant et al. 2005). Additionally the PARPi KU0058684 showed inhibition of tumor growth of BRCA2-deficient but not wild-type embryonic stem cell teratocarcinomas (Farmer et al. 2005). Rucaparib (AG-014699) reduced the growth of xenografts with epigenetically silenced *BRCA1* as well as with BRCA1/2 mutations (Drew et al. 2011b). In this study, doses of AG-014699 in excess of 10 mg/kg/day for extended periods were tolerated. This is in contrast to the chemosensitization studies where the MTD of the same PARPi was only 1 mg/kg/day for 5 days in combination with TMZ (Thomas et al. 2007).

Recent work has identified a potential further use of PARPi as a monotherapy in ETS gene fusion-positive cancer. ETS genes are driving mutations which can be found in a number of cancers including Ewing's sarcoma, acute myeloid leukemia, and prostate cancer. Brenner and colleagues showed that the product of the ETS gene fusion in prostate cancer, *TMPRESS*:*ERG*, interacts with PARP-1 and that PARP-1 is required for ETS-mediated gene transcription and cell invasion. Importantly, *TMPRESS*:*ERG* was found to induce DNA damage which was enhanced with PARPi treatment (Brenner et al. 2011). The efficacy of PARPi in ETS gene fusion-positive xenografts has also been investigated; olaparib (AZD2281) treatment caused tumor growth inhibition of ETS-positive VCap and PC3-ERG tumors but not in ETS-negative 22RV1, DU145, or PC3 tumors (Brenner et al. 2011).

Clinical trials investigating PARPi as single agents in *BRCA*-mutated cancers commenced in 2005. The first to report was the pivotal phase I study of the oral PARPi olaparib (Fong et al. 2009). Olaparib was well tolerated in all patients, including those with germ line BRCA mutations. DLTs were myelosuppression and central nervous system side effects with the MTD of 400 mg olaparib twice daily. PARP inhibition was confirmed in surrogate and tumor tissue, and antitumor activity (defined as radiologic or tumor marker response or disease stabilization $\geq$4 months) was reported in 12 (63%) of the 19 evaluable *BRCA1* and *2* mutation carriers, including patients with breast, ovarian, and prostate cancer. No responses were observed in non-*BRCA* mutation carriers. The study incorporated an expansion phase, which focused specifically on patients with BRCA1/2 mutations, and in a total of 50 ovarian cancer patients, a clinical benefit rate of 46% was observed (Fong et al. 2010). 400 or 100 mg of olaparib twice daily on a 28-day cycle was subsequently taken forward in separate phase II *BRCA*-mutated breast and ovarian cancer studies. In the breast cancer study, there was an objective response rate (ORR) of 41% (11/27) and progression-free survival (PFS) of 5.7 months in the 400 mg dose group ($n = 27$). Response rate was lower (22%) in the 100 mg group ($n = 27$) (Tutt et al. 2010). In the ovarian cancer study, 33 patients with recurrent epithelial ovarian cancer and primary peritoneal or fallopian tube carcinoma treated with 400 mg dose had an ORR of 33% (11/33), and again ORR was lower (13%) in the 100 mg group ($n = 24$) suggesting that the degree of PARP enzyme inhibition may be important (Audeh et al. 2010). The toxicity profile in both studies was acceptable with the most

common toxicities being grade 1 or 2: nausea, vomiting, fatigue, and anemia (ovarian study only).

In addition to olaparib, a number of other PARP inhibitors are currently being investigated in patients with germ line *BRCA* mutations. Preliminary results of some of these studies have been presented. A phase I study of the PARPi, MK-4827, in patients with advanced solid tumors enriched for *BRCA*-mutated cancers reported a partial response rate of 20% (12/60) and established a MTD of 300 mg daily with continuous dosing (Schelman et al. 2011). Interim results of the phase II trial investigating the single agent activity of the PARP inhibitor rucaparib in patients with *BRCA*-mutated breast and/or ovarian cancer were also presented at ASCO 2011 and reported a clinical benefit rate (CBR) of 34% (Drew et al. 2011a).

PARP Inhibitors as Single Agents in the Wider Cancer Population

The preclinical data discussed previously in this chapter suggests that there is a wider role for PARP inhibitors in the treatment of cancer. Clinical studies are now underway investigating the efficacy of PARPi in non-germ line *BRCA*-mutated cancers, in particular high-grade serous ovarian cancers (HGSOC) and triple negative breast cancer (TNBC).

In a four-arm phase II correlative study recruiting (1) HGSOC patients with BRCA mutations, (2) HGSOC patients with unknown BRCA status, (3) BRCA- mutated breast cancer, and (4) TNBC patients with unknown BRCA status, patients received continuous olaparib dosing at 400 mg twice daily (Gelmon et al. 2011). A response rate of 24%, as assessed by the Response Evaluation Criteria in Solid Tumors (RECIST), was reported in the patients with non-germ line BRCA-mutated HGSOC and 41% in the confirmed BRCA mutation ovarian cancer patients. Responses were seen in both the platinum-sensitive and platinum-resistant patients. It should be noted that this is the *first* study to show single agent PARP inhibitor activity in non-germ line BRCA-mutated cancers, indicating that sporadic HGSOC could be targeted with PARP inhibitors. Interestingly, no responses were observed in the two breast cancer arms of the study which conflicts the data from Tutt et al. showing that BRCA-mutated breast cancer is sensitive to olaparib (Tutt et al. 2010).

The role of PARP inhibitors as maintenance therapy in HGSOC is currently being investigated, with interim results of two phase II studies reported. Preliminary results from patients with platinum-sensitive HGSOC randomized on a 1:1 basis to olaparib 400 mg twice daily or placebo until disease progression showed a significant benefit in PFS (8.4 vs. 4.8 months; $P < 0.00001$) favoring the maintenance olaparib (Ledermann et al. 2011; Oza et al. 2012).

Triple negative breast cancers (TNBC), by definition, lack ER, PR, and HER2/neu expression and make up around 15% of all breast cancers, share molecular features of BRCA1 breast cancers, and therefore may be associated with defective HRR. However, at present, there is no convincing evidence of *single* agent PARPi activity in TNBC. Initial phase I and II studies showing remarkable activity of BSI-201 (iniparib)

in combination with carboplatin and gemcitabine in TNBC have been confounded by the finding that BSI-201 does not inhibit PARP activity (Patel et al. 2012) and therefore does not contribute to the evaluation of PARPi as a class.

Preclinical Summary

There is abundant data showing that PARPi, from the earliest benzamide analogues to the inhibitors selected for clinical evaluation, increase the persistence of DNA damage induced by DNA-methylating agents, IR, and topoisomerase I poisons; this is accompanied by an increase in the cytotoxicity of these agents. Tumor xenograft studies in mice also show that PARP inhibitors increase the efficacy of IR, topoisomerase I poisons, and, in particular, DNA-methylating agents, usually TMZ. Furthermore, PARPi can restore TMZ sensitivity in MMR-defective cells and xenografts. PARPi cause a cell line-dependent variable potentiation of cisplatin, which appears to be influenced by the HRR status of the cell. However the in vivo activity of cisplatin and PARPi combinations may not be HRR dependent and may even be active even when no chemosensitization has been detected in vitro.

However, the most exciting finding was that PARPi alone were selectively cytotoxic to cells and xenografts lacking HRR function for reasons including, but not exclusively limited to, *BRCA1* or *BRCA2* mutation. This has been demonstrated with multiple models and a variety of PARPi confirming that it is not specific to the inhibitor used nor the particular model. Importantly, PARPi were not toxic to cells or mice with heterozygous *BRCA* mutations indicating that they would be selectively toxic to the (homozygous mutant/deleted) tumor without undue toxicity to the (heterozygous) patient. What is very clear from the preclinical studies is that different doses and schedules are needed for monotherapy and combination therapy. For monotherapy, high doses and prolonged treatment schedules are well tolerated and are required to convert endogenous DNA damage into lethal events in a HHR-defective background for antitumor activity. In contrast, these doses and schedules are highly toxic in combination with chemotherapy, and much lower doses and shorter exposure periods are needed for chemosensitization.

Clinical Summary

In 2003, in Newcastle upon Tyne, UK, the first PARPi, rucaparib, was given to a cancer patient. The last 9 years have seen a major leap forward in the development of these novel agents with now at least nine inhibitors in various stages of clinical trial development. PARPi development pipelines are pursuing two therapeutic applications: (1) PARPis as potentiators of chemotherapy or radiotherapy and (2) PARPis as

single agents, selectively killing cells with inherited or acquired defects in DNA repair pathways. Results of the PARP inhibitor chemotherapy combination studies have highlighted that toxicity; in particular, increased myelosuppression is a limiting factor and may stifle the clinical application. Some of this toxicity may be due to the use of doses determined as safe when administered as single agents, which as the preclinical data show are toxic in combination. Radiotherapy combinations may be more promising and we await the results of the planned early studies. The most exciting development in the recent history of PARP inhibitors is their single agent activity in germ line *BRCA*-mutated cancers and more recently their role in the treatment of high-grade serous ovarian cancers. The assumption is that these HGSOC harbor defective HRR rendering them sensitive to PARPi. The possibility that many other cancers also have such HRR defects is an exciting one as this may be exploited by PARPi if biomarkers can be developed to identify them.

Anticipated High-Impact Results

- In 2005, two reports from two independent groups using two different PARPi demonstrated the synthetic lethality of PARPi in cells and tumors defective in BRCA1 or BRCA2 (Bryant et al. 2005; Farmer et al. 2005).
- These exciting findings were rapidly translated into clinical studies of single agent olaparib in patients with BRCA mutation-associated breast, ovarian, and pancreatic cancer, with very encouraging response data and minimal toxicity in early clinical trials (Fong et al. 2009, 2010).
- Subsequent laboratory studies demonstrate that the synthetic lethality of PARPi is not restricted to cells and xenografts with BRCA mutations but that inactivation of other components of HRR, or epigenetic silencing of BRCA1, also render them sensitive to PARPi in preclinical studies (McCabe et al. 2006; Drew et al. 2011b).
- Investigation of HRR function in primary cultures of human HGSOC demonstrates that around 50% are defective (Mukhopadhyay et al. 2010), and responses were seen in a similar proportion of HGSOC patients treated with olaparib in a phase II trial (Gelmon et al. 2011).
- These examples are the first demonstration of the exploitation of synthetic lethality in human cancer. It is anticipated that future proof of principle phase III clinical trials will provide further validation of these proof-of-concept phase I and II trials.

Cross-References

► BRCA1 and 2
► NF-κB
► P53, Immunology

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ROS

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Abstract

Cancer cells have increased generation of reactive oxygen species (ROS). A moderate increase in ROS can promote cell proliferation and differentiation, but excessive amounts of ROS cause oxidative damage to cancer cells. Intrinsic oxidative stress of cancer cells suggest that compared to normal cells, the malignant cells may be more dependent on the antioxidant system and more vulnerable to further oxidative stress induced by exogenous ROS-generating agents or compounds that inhibit the antioxidant system. The difference in redox status between normal and cancer cells provides an important biochemical basis of therapeutic selectivity in cancer treatment. Therefore, targeting ROS in

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cancer cells with redox-modulating strategies alone or combined with conventional chemotherapy or radiotherapy is an attractive new approach that may have therapeutic selectivity and overcome drug resistance.

Keywords
Mitochondrial respiratory chain • Nicotine adenine dinucleotide phosphate (NADPH) oxidases • Oxidative stress biomarkers • Reactive oxygen species (ROS) • 2-Methoxyestradiol and tetrathiomolybdate • Definition • Enzymatic antioxidant defenses • Functions • In cancer cells • Indicator of cancer risk • Mitochondrial respiratory chain • NADPH oxidase • NADPH oxidases • Non-enzymatic antioxidants • Oxidative stress biomarkers • Preclinical studies • Stress in cancer cells • Therapeutic selectivity • Upregulated ROS-scavenging systems

Reactive oxygen species (ROS) are broadly defined as oxygen-containing chemical species with reactive chemical properties. Compared with normal cells, many types of cancer cells have increased levels of ROS (Szatrowski and Nathan 1991). There are two types of ROS, those of free radicals such as superoxide and hydroxyl radicals, which contain one or more unpaired electron(s), and non-radical ROS such as hydrogen peroxide, which does not have unpaired electron(s) but is chemically reactive and can be converted to radical ROS. In biological systems, ROS are constantly generated through both enzyme-catalyzed and non-enzyme reactions. ROS derived from oxygen represent the most important class of ROS in living system. Molecular oxygen has a unique electronic configuration. The addition of one electron to oxygen forms superoxide anion radicals, which is considered the primary ROS that can further interact with molecules to generate other ROS. Mitochondrial respiratory chain is a major ROS-generating site. In aerobic life, the mitochondrial electron-transport chain is the main source of ATP. ROS are generated during the production of ATP in mitochondria. Electron leakage from the mitochondrial respiratory chain during the electron-transport steps of ATP production can react with molecular oxygen, resulting in the formation of superoxide, which can subsequently be converted by superoxide dismutase (SOD) to generate hydrogen peroxide, from which further hydroxyl radicals are generated in a reaction catalyzed by Fe^{2+} or Cu^{2+} ions (Richter et al. 1995). Another important cellular source of ROS is nicotine adenine dinucleotide phosphate (NADPH) oxidases. NADPH oxidases were originally considered as enzymes expressed only in phagocytic cells. Recent evidence indicates that there is a family of NADPH oxidases expressed in cancer cells and serves as an important source of ROS (Valko et al. 2007). Studies in phagocytic cells indicate that the enzyme complex consists of two membrane-bound components, $gp91^{phox}$ and $p22^{phox}$, which comprise cytochrome b558. On stimulation $p47^{phox}$ is phosphorylated, and the cytosolic components involving $p67^{phox}$, $p40^{phox}$, and the small G-coupled proteins, Rac and Rap1A, translocate to the membrane, where they associate with cytochrome b558 to assemble the active oxidase, which transfers electrons from the substrate to oxygen forming ROS. In addition, ROS can also be produced by peroxisomes, which are known to produce hydrogen peroxide. Catalase

in peroxisomes prevents accumulation of this toxic compound, thus maintaining a delicate balance. However, dysfunctional peroxisomes have downregulated catalase; hydrogen peroxide releases into the cytosol contributing to oxidative stress.

Biology of the Target

ROS functions as a double-edged sword. While a moderate increase in ROS can promote cell proliferation and differentiation (Schafer and Buettner 2001), excessive amounts of ROS cause oxidative damage to lipids, proteins, and DNA, inhibiting their normal function. The delicate balance between beneficial and harmful effects of ROS is very important and is achieved by redox regulation, which protects cells from oxidative stress and maintains cellular redox homeostasis. To overcome the potential toxicity of ROS, cells have developed a series of enzymatic and non-enzymatic systems to counteract these highly dangerous insults and control the redox status. Enzymatic antioxidant defenses include superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). Non-enzymatic antioxidants involve glutathione (GSH), thioredoxin (TRX), ascorbic acid (vitamin C), α-tocopherol (vitamin E), and other antioxidants. GSH is the most abundant nonprotein sulfhydryl-containing molecule with an intracellular concentration of 1–10 mM; the electrophilic properties of GSH enable it to react with hydrogen peroxide nonenzymatically or, by the action of GPX, yield the oxidized form GSSG (Arrick and Nathan 1984).

Increased ROS stress in cancer cells seems to correlate with the aggressiveness of tumors and poor prognosis (Kumar et al. 2008). Compelling evidence suggests that elevated ROS in cancer cells may play a pivotal role in the acquisition of the hallmarks of cancer. ROS can promote immortalization and transformation, cell proliferation and mitogenic signaling, cell survival and disruption of cell-death signaling, epithelial-mesenchymal transition, metastasis, and angiogenesis. On the other hand, when the increased ROS reaches the toxic threshold, it may cause the death of cancer cells, thus limiting cancer progression (Kumar et al. 2008). Increased ROS generation in cancer cells may trigger a redox adaptation response, leading to an upregulation of antioxidant capacity to maintain the ROS levels below the toxic threshold (Irmak et al. 2003). The redox adaptation further promotes cancer development as well as drug resistance.

Target Assessment

Direct measurement of ROS in vivo is difficult because the half-lives of ROS are usually short. In clinic, ROS stress levels can be indirectly detected by measuring "oxidative stress biomarkers" using special techniques. Oxidative stress biomarkers can be classified into two categories: (1) Formation of ROS-modified biomolecules. These include 8-hydroxy-2′-deoxyguanosine (8-OHdG) (a marker of oxidative DNA damage), malondialdehyde-lysine (a marker of protein oxidative modification), pentosidine (a marker of glyco-oxidation), and BOM (bilirubin oxidative

metabolites). (2) Consumption or induction of enzymes or antioxidants. These include superoxide dismutase, catalase, glutathione peroxidase, glutathione, cysteine, and selenium (Oberley and Oberley 1997). These biomarkers are often determined in samples of body fluids or breath condensate by chromatography, enzymatic reaction, or ELISA (Patel et al. 2007).

Role of the Target in Cancer

Rank for cancer relevance: 10

Compared with normal cells, cancer cells have intrinsic oxidative stress. Studies have revealed several intrinsic and extrinsic mechanisms to cause oxidative stress during cancer development (Trachootham et al. 2009). The intrinsic factors to cause increased ROS in cancer cells include loss of functional p53; activation of oncogenes such as Ras, Bcr-Abl, and c-Myc; aberrant metabolism; and ROS-generating and ROS-scavenging system dysfunction. P53 plays an important role to guard genome by sensing and removing oxidative damage to nuclear and mitochondrial DNA and maintaining genetic stability. As a transcription factor, p53 also regulates redox-relevant gene expression in cancer cells (Horn and Vousden 2007). Thus, p53 mutation would cause redox imbalance and aggressive tumor growth. Moreover, mitochondrial DNA mutations are correlated with increased ROS in cancer cells. It has been shown that mutated mitochondrial DNA impairs electron transfer, thus leading to leakage of electrons and further ROS generation in mitochondria (Brandon et al. 2006). Moreover, a large amount of ROS can also be generated through the activation of membrane-bound NADPH oxidases (Irani et al. 1997). The extrinsic factors that affect intracellular redox homeostasis include abnormal small molecule metabolism, inflammatory cytokines, and a hypoxic condition in the cancer cell microenvironment. For example, among the three precursor amino acids (glutamate, cysteine, glycine) for GSH synthesis, cysteine is chemically unstable and exists at a lower concentration than glutamate and glycine and thus is a rate-limiting substrate for GSH synthesis. Cysteine is a conditionally essential amino acid that can be synthesized from methionine only in certain tissues via the transsulfuration pathway. Therefore, the uptake of cysteine by ubiquitously expressed ASC transporter is an important determinant of GSH status in cells. Since cysteine is present in blood predominantly in the oxidized form cystine, uptake of cysteine in most cells occurs in the form of cystine by a transporter known as Xc- (Zhang and Huang 2011). A recent study reports a biochemical mechanism by which bone marrow stromal cells modulate the redox status of chronic lymphocytic leukemia (CLL) cells and promote cellular survival and drug resistance (Zhang et al. 2012). CLL cells from patients exhibit limited ability to transport cystine for glutathione (GSH) synthesis due to low expression of the Xc- transporter, while stromal cells effectively import cystine and convert it to cysteine, which is released into the microenvironment for uptake by CLL cells to enhance GSH synthesis, which protects leukemia cells from oxidative stress and drug-induced cytotoxicity.

The dual functions of ROS seem evident. A moderate increase in ROS can promote cell proliferation and differentiation, which correlates with the aggressiveness of tumors and poor prognosis (Schafer and Buettner 2001). However, excessive amounts of ROS cause oxidative damage to lipids, proteins, and DNA (Perry et al. 2000), leading to the death of cancer cells and limiting cancer progression. In order to adapt to survive under increased oxidative stress, cancer cells have acquired adaptive mechanisms to counteract the toxic effects of increased ROS and to activate cell-survival pathways. For example, oncogenic Ras-transformed cells exhibit increased ROS as well as higher levels of antioxidants that serve as a key mechanism to evade ROS-mediated apoptosis (Trachootham et al. 2006). The reliance of Ras-transformed cells on antioxidants makes it more sensitive to depletion of cellular antioxidant, leading to ROS accumulation and cell death. Moreover, redox adaptation is crucial not only in cancer development but also in drug resistance. For example, severe accumulation of ROS in cancer cells induces elevation of glutathione (GSH) – the most abundant antioxidant in cells. High levels of glutathione (GSH) have been found in many tumors including lung cancer, breast cancer, colon cancer, melanoma, and leukemia (Estrela et al. 2006). Besides removing endogenous free radical, increased GSH levels also largely affect the efficacy and interactions of a variety of antineoplastic interventions. The mechanisms that contribute to GSH and GSH-dependent reaction-mediated drug resistance include: (1) defense against oxidative stress produced by ROS-generating drugs, (2) drug inactivation and alterations in drug transport, (3) increased repair and tolerance of DNA damage, and (4) apoptosis inhibition (Estrela et al. 2006). Recent reports also suggest that GSH inhibits apoptosis through other mechanisms. For example, posttranslational modifications of proteins through glutathionylation are critical for regulation of apoptosis in cancer cells (Trachootham et al. 2008). GSH may promote lymphoid cell survival through maintaining intracellular ionic homeostasis (Franco et al. 2008). Therefore, upregulated GSH as an adaptive response to increased ROS greatly affiliates cancer progression. In addition to upregulate antioxidant in cancer cells, persistent ROS stress may also activate redox-sensitive transcription factors including NF-kB, leading to the elevation of survival factors such as MCL-1 and BCL-2 and inhibition of cell death factors. All these events confer an increased capacity to tolerate high ROS level and maintain cellular viability. Thus, aberrant metabolisms together with the activation of cell-survival pathways contribute to survival and drug resistance in cancer cells.

High-Level Overview

ROS is a group of highly reactive molecules including radical ROS (superoxide, nitric oxide, and hydroxyl radical) and non-radical ROS (hydrogen peroxide, ozone, peroxynitrate, and hydroxide). ROS is generated from mitochondrial electron-transport chain and tightly regulated enzymes, such as NADPH oxidase complexes. ROS exhibits vital role in aerobic organisms to regulate cell growth and differentiation and involves physiological roles in cellular response in defense against

infectious agents, in the function of a number of cellular signaling pathways, and the induction of a mitogenic response. However, overproduction of ROS results in oxidative stress, a deleterious process that can be an important mediator of damage to lipid, membrane, proteins, and DNA. To prevent the harmful effect of ROS and maintain the redox balance, cells regulate ROS levels by expressing enzymatic and non-enzymatic systems including GPX, SOD, CAT, and GSH or acquiring precursor of antioxidant from the microenvironment.

Cancer cells exhibit increased ROS generation due to oncogene activation, antioxidant deficit, aberrant metabolism, or mitochondrial dysfunction. Increased ROS levels induce oxidative DNA damage and gene mutation or deletion, cause loss of functional p53, and decrease DNA response capacity, which promotes genome instability that further increases ROS levels in cancer cells. On one hand, oxidative stress in cancer cells may promote cell proliferation, senescence, evasion, angiogenesis, and metastasis. On the other hand, increased ROS levels may trigger redox adaptation that promotes cell survival through increasing the expression of survival factors and the capacity of DNA repair. Furthermore, upregulated ROS-scavenging systems during redox adaptation in cancer cells would alter drug metabolism and thus render drug resistance. These phenomena highlight the crucial role of ROS stress in tumor development and drug resistance. Targeting ROS alterations in cancer cells with redox-modulating strategies is a feasible therapeutic approach that may have therapeutic selectivity and overcome drug resistance.

Diagnostic, Prognostic, and Predictive

Elevated oxidative stress has been observed in many cancer cell types. For example, leukemia cells freshly isolated from blood samples from patients with chronic lymphocytic leukemia or hairy cell leukemia showed increased ROS production compared with normal lymphocytes (Zhou et al. 2003). In solid tumors such as oral squamous cell carcinoma, lung cancer, and colorectal cancer, studies have shown increased levels of oxidative damage products and aberrant level of ROS-scavenging enzymes and antioxidants in clinical tumor specimens and plasma compared to normal controls (Patel et al. 2007). Therefore, oxidative stress might be a useful indicator of cancer risk in healthy people, and high levels of basal ROS in cancer cells might predict poor clinical outcomes.

Therapeutics

Therapeutic selectivity is essential in cancer treatment. Based on the different redox status in normal and cancer cells, ROS-mediated mechanism has been proposed. Intrinsic oxidative stress contributes to cancer development and progression. However, when the increase of ROS reaches the toxic threshold, it may trigger cell death. Normal cells maintain redox homeostasis by controlling the balance between ROS generation and elimination by ROS-scavenging systems to prevent the ROS level

from reaching the cell-death threshold. Compared with normal cells, malignant cells seem to have higher levels of endogenous oxidative stress in culture and in vivo (Szatrowski and Nathan 1991). Intrinsic oxidative stress of cancer cells suggests that, compared to normal cells, the malignant cells may be more dependent on the antioxidant systems and more vulnerable to further oxidative stress, which could be induced by exogenous ROS-generating agents or compounds that inhibit the antioxidant systems. The difference in redox status between normal and cancer cells provides an important biochemical basis of therapeutic selectivity in cancer treatment (Pelicano et al. 2003). Exogenous agents that increase ROS generation can induce significant accumulation of ROS in cancer cells, leading to oxidative damage and selective cell death (Trachootham et al. 2009). The therapeutic agents that directly promote ROS generation include arsenic trioxide (As_2O_3), daunorubicin, doxorubicin, β-lapachone (ARQ 501), and elesclomol (STA-4783). Those compounds enhance ROS levels with various mechanisms. For example, As_2O_3 impairs the function of respiratory chain to increase the production of ROS (Pelicano et al. 2003). Doxorubicin may react with flavoprotein reductases in the presence of reduced NADPH and generate ROS in the presence of molecular oxygen. It may also mediate ROS production through intracellular chelation of iron (Vasquez-Vivar et al. 1997). Another strategy to induce ROS generation is suppressing cellular antioxidant systems, such as GSH and thioredoxin systems. This type of agents includes buthionine sulfoximine (BSO), imexon, phenylethyl isothiocyanate (PEITC), mangafodipir, 2-methoxyestradiol, and tetrathiomolybdate (ATN-224). BSO is an inhibitor of gamma-glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis, and decreases intracellular GSH level through inhibiting GSH synthesis (Maeda et al. 2004). GSH depletion can also be achieved by PEITC (Trachootham et al. 2006) and imexon (Dragovich et al. 2007) through conjugating with GSH and transporting it out of cells (Trachootham et al. 2006, 2008). Furthermore, a new finding suggests that leukemia cells exhibit a low ability to directly use extracellular cystine for GSH synthesis and are dependent on stromal cells to take up cystine by transporter Xc- and convert cystine to cysteine for GSH synthesis in leukemia cells (Zhang et al. 2012). Therefore, the inhibitors of cystine transporter Xc-, such as (S)-4-carboxyphenylglycine (S-4-CPG) and sulfasalazine (SSZ), may render leukemia cells starve for GSH precursor cysteine through inhibiting cystine uptake and cysteine secretion of stromal cells, and thus cause GSH depletion in leukemia cells and enhance sensitivity to traditional chemotherapeutic agents fludarabine and oxaliplatin (Zhang et al. 2012). Overcoming drug resistance of leukemia cells in the stromal environment by abolishing the GSH protective mechanism has been proven both in vitro and in vivo (Zhang et al. 2012). Further evaluation of the redox intervention strategy in preclinical and clinical settings is important for the development of effective therapy to overcome drug resistance in vivo.

Owing to the presence of redox adaptation mechanisms, a combination of ROS-generating agents with compounds that are capable of abrogating cellular antioxidant systems is likely to have an additive or synergistic effect against cancer cells. This might be particularly useful in cancer cells that have adapted to stress and

become resistant and anticancer agents. Moreover, the combination strategy might enable to achieve the goal of killing cancer cells selectively due to the intrinsic oxidative stress in the malignant cells, where the toxic ROS threshold may be easily reached due to the higher ROS outputs (Trachootham et al. 2009).

Preclinical Summary

Extensive preclinical work has been done in using ROS-mediated cancer cell death as a therapeutic strategy. Exploiting the vulnerability of cancer cells with intrinsic oxidative stress to further ROS insults by ROS-modulating agents to preferentially kill the malignant cells has been shown to be feasible in experimental systems. The selective strategy is based on the redox difference between normal cells and cancer cells. One such example is to target the key antioxidant GSH by PEITC. Recent studies demonstrated that rapid depletion of GSH by the naturally produced PEITC can preferentially kill the Ras-transformed ovarian cells and primary leukemia cells from patients (Trachootham et al. 2006, 2008). Another report has shown that interrupting the redox interaction between CLL cells and the bone marrow stromal cells by either PEITC or cystine transporter inhibitor S-4-CPG or SSZ could circumvent microenvironment-mediated drug resistance in CLL cells (Zhang et al. 2012). This report provides a biochemical basis for developing new therapeutic strategy to overcome cancer cell drug resistance in vivo. Moreover, redox adaptation in some cancer cells, especially those in advanced disease stages, provides a mechanism of resistance to anticancer agents (Lenehan et al. 1995; Zhou et al. 2005); preclinical studies have shown that agents that disable the redox adaptive mechanisms in combination with ROS-generating agents have a more-than-additive effect (Zhou et al. 2003).

Clinical Summary

Based on the rationale of targeting ROS in cancer cells, several ROS modulation agents are currently in clinical trials. ROS-generating compound β-lapachone (ARQ 501) is now in phase I/II studies in tumors overexpressing NQO1 (Bey et al. 2007). 2-Methoxyestradiol and tetrathiomolybdate (ATN-224) can cause significant accumulation of ROS in cancer cells and are in phase II studies in prostate, ovary, brain, and renal tumors and melanoma, myeloma, and prostate and breast carcinomas, respectively (Trachootham et al. 2009). Redox-based drug combination strategies have also been tested in clinical trials. A multicenter phase II trial of the ROS-generating agent triapine and gemcitabine in advanced non-small cell lung cancer has been completed (Ma et al. 2008). Combinations of arsenic trioxide and ascorbic acid-mediated GSH depletion were shown to be clinically effective in the treatment of relapsed or refractory multiple myeloma (Bahlis et al. 2002). Phase I/II studies of buthionine sulfoximine in combination with As_2O_3 or melphalan, imexon

with docetaxel, and gemcitabine are ongoing (Maeda et al. 2004; Dragovich et al. 2007).

Anticipated High-Impact Results

- Targeting ROS in cancer cells with redox-modulating strategies is a feasible therapeutic approach that may have therapeutic selectivity and can overcome drug resistance.
- Combinations of ROS-generating agents and drugs that inhibit ROS elimination could be a potent strategy to promote ROS accumulation in cancer cells to effectively kill the malignant cells.
- The use of agents to abrogate redox adaptation in combination with conventional chemotherapy or radiotherapy is an attractive new approach to improving therapeutic outcomes.

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X-Linked IAP

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Anne Noonan

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Abstract

X-linked inhibitor of apoptosis protein (XIAP) is a member of the family of inhibitor of apoptosis proteins and part of the programmed cell death (apoptosis) pathway. Also known as BIRC4 (baculovirus IAP repeat-containing 4), XIAP resides in the cytoplasm. It acts as an inhibitor of caspases by directly binding to and inhibiting caspases 3, 7, and 9 and thus may inhibit both the intrinsic and extrinsic apoptosis pathways. Protein and RNA levels of XIAP can be measured but there are currently no FDA-approved tests for the measurement of XIAP in

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either tumor tissue or blood. XIAP is overexpressed in numerous malignancies. XIAP is currently being investigated as a predictive and prognostic tool in cancer and as a marker of resistance to cancer therapies. Restoration of apoptosis is an important priority for cancer drug development, thus targeting of XIAP is of critical significance. Second mitochondrial-derived activator of caspases (SMAC)-mimetics such as LCL-161 and antisense oligonucleotides such as AEG35156 inhibit XIAP and are being investigated in therapeutic clinical trials.

Keywords

AT-406 • Baculovirus IAP repeat-containing 4 (BIRC4). *See* X-linked inhibitor of apoptosis protein (XIAP) • Birinapant • HGS-1029 • LCL-161 • Smac mimetics • X-linked inhibitor of apoptosis protein (XIAP) • AEG35156 • Assessment • Chemoresistance • Diagnostic marker • Immunohistochemistry • In cancer • Preclinical studies • Response to chemotherapy • Smac mimetics • Therapeutics • XLP2 • Zinc-binding domain • X-linked lymphoproliferative disease 2 (XLP2)

Target: X-Linked Inhibitor of Apoptosis (XIAP)

X-linked inhibitor of apoptosis protein (XIAP) is a member of the family of inhibitors of apoptosis. Members of this family of inhibitors of apoptosis proteins share a baculovirus IAP repeat, which is necessary for their anti-apoptosis function. XIAP is also called BIRC4 (baculovirus IAP repeat-containing 4). The XIAP gene is located on Xq25 which encodes a 56,685 Da protein. XIAP is located in the cytoplasm. XIAP participates in a variety of cellular processes which include but are not limited to apoptosis. XIAP consists of three BIR domains and a ubiquitin-binding domain, and XIAP functions as an E3 ubiquitin ligase via its RING domain, thus catalyzing the ubiquitination of its substrate proteins. XIAP acts as an inhibitor of caspases by directly binding to and inhibiting caspases 3, 7, and 9. XIAP may potently inhibit both the intrinsic and extrinsic apoptosis pathways by restraining both initiator and effector caspases. XIAP has a zinc-binding domain that can bind directly to caspases and inhibit their protease activity. XIAP is itself inhibited by second mitochondrial-derived activator of caspases/direct IAP-binding protein with low pI (SMAC/DIABLO) and Omi/Htra2. SMAC and Omi are mitochondrial proteins which are released after mitochondrial outer membrane permeabilization when the intrinsic apoptosis cascade is triggered. SMAC and Omi thus release the caspases from blockade by XIAP so that apoptosis may proceed. Thus, inhibition of XIAP represents a therapeutic option in cancer cells.

mRNA levels of XIAP have been noted in all fetal and adult tissues except in peripheral blood leucocytes. Immunohistochemistry can be used to measure levels in individual cells and tissues. XIAP is overexpressed in numerous malignancies including acute myeloid leukemia, lung cancer, renal cell carcinoma, Her-2 overexpressing inflammatory breast cancer, mesothelioma, melanoma, ovarian cancer, cervical cancer, head and neck cancer, and hepatocellular carcinoma.

Biology of the Target

XIAP is involved in apoptosis, cell migration, development, and cancer invasion. There is some evidence that higher levels of expression of XIAP are associated with more aggressive phenotype and thus XIAP may function as a tumor marker. XIAP overexpression in cancer cells has been shown to be associated with chemotherapy and radiotherapy resistance and thus may act as a biomarker of resistance to certain cancer therapies. There is insufficient evidence to date to recommend using XIAP in either the diagnosis of or follow-up of treatment for cancers, although its role as a tumor marker is promising. Several studies are exploring the value of XIAP as a marker of more poorly differentiated disease and as a prognostic marker.

XIAP is itself a target for cancer therapeutics. Early phase trials have explored the role of XIAP antisense oligonucleotide AEG35156 which targets XIAP mRNA thus lowering XIAP levels and therefore also lowering the apoptotic threshold of cancer cells (LaCasse et al. 2012). Smac mimetics are small molecule peptidomimetics which degrade IAPs such as XIAP, thus releasing caspases for apoptosis to proceed. The results of these trials have not been fully reported, but early results suggest that reduction of IAP levels may be associated with tumor responses (www.clinicaltrials.gov). Although other members of the IAP family have been targeted by vaccines, XIAP to date has not.

Target Assessment

There is no standard approved clinical test for the measurement of XIAP. Several studies have reported measurement by immunohistochemistry, western blot for protein measurement, or measurement of mRNA levels in tumor tissue. In addition, exome sequencing to detect alterations in XIAP gene has been reported. Measurement of XIAP levels currently is not a routine test in the diagnosis of or follow-up of treatment for cancer.

Role of the Target in Cancer

Rank: "unknown" to 10

Unknown to 1-2-3-4-5-6-7-8-9-10:7.

High-Level Overview

Diagnostic, Prognostic, Predictive

X-linked lymphoproliferative disease 2 (XLP2) is a rare disease due to mutation in XIAP resulting in reduction in or elimination of XIAP protein. The disease is characterized by hemophagocytic lymphohistiocytosis, splenomegaly, hypogammaglobulinemia, and colitis ± liver disease. Lymphoma is not a feature of XLP2, unlike in XLP due to mutations in SH2D1A.

Studies examining XIAP as a prognostic or predictive marker have yielded inconsistent results across tumor types. Currently, there is insufficient evidence to recommend using XIAP in either the diagnosis of or follow-up of treatment for cancers, although it may be a potential tumor marker. In one Korean study (Kang et al. 2008), XIAP polymorphisms were not associated with increased or decreased susceptibility to lung cancer. Measurement of XIAP by immunohistochemistry or levels in blood is not yet a standard test in clinical oncology practice. The role of XIAP as a diagnostic marker is currently being explored. Li et al. (2012) measured XIAP mRNA levels in pleural fluid of patients with benign (56 patients) and malignant (42 patients) pleural effusions. XIAP mRNA levels were significantly higher in malignant compared to benign pleural effusions ($p = 0.0002$). The role of XIAP as a predictive and prognostic tool and as a marker of resistance to cancer therapies is currently being investigated in several cancer types. Several small studies in ovarian, cervical, and breast cancers and head and neck squamous cell cancer have shown that higher expression of XIAP was associated with more poorly differentiated cancers and invasiveness. Mizutani et al. (2007) used western blot to measure protein level of XIAP in lysates of 109 surgical specimens of renal cell cancer and 109 matched normal kidney tissue specimens. The mean level of XIAP expression was higher in tumor tissue compared to matched normal kidney tissue. Higher levels of XIAP were seen in stage III/IV tumors compared to stage I/II, and survival was worse for patients with high compared to low levels of XIAP. Xiang et al. (2009) used RT-PCR and immunohistochemistry to measure XIAP levels in specimens from patients with newly diagnosed stage I–IV colorectal cancer and found that the group with higher XIAP levels had lower disease-free and overall survival rates. Tamm et al. (2004) reported that high expression levels of XIAP correlated with poor overall survival in childhood de novo acute myeloid leukemia. Using immunohistochemistry, Emmanuel et al. (2008) demonstrated that XIAP is detectable nearly three times more frequently in thick compared to thin melanomas, suggesting that higher levels of XIAP are associated with increasing melanoma thickness and tumor progression.

XIAP as a tumor marker and measure of response to chemotherapy has been examined in a study by Wu et al. (2008) of serial head and neck squamous cell carcinoma fine needle aspirate samples. Immunohistochemical measurement of XIAP levels in cytology samples was found to be feasible and may be a useful tool for measuring response to apoptosis-inducing therapies, although confirmatory studies are needed.

XIAP may be a marker of chemoresistance (Kashkar 2010). In studies in which XIAP levels were reduced by either RNA interference, antisense oligonucleotides, or with Smac mimetics, synergism and increased cell death were observed when chemotherapeutic agents were added in vitro, in xenograft models and in clinical phase I/II trials.

There are currently no guidelines from ASCO or NCCN related to the use of XIAP in cancer diagnosis or follow-up.

Therapeutics

XIAP has been a target for cancer therapy for a number of years. IAPs were first discovered and characterized in 1993. The primary aim of therapeutics has been relief of the binding of XIAP to caspases, usually by reducing the amount of XIAP present in tumor cells. XIAP is overexpressed in numerous malignancies including acute myeloid leukemia, lung cancer, renal cell carcinoma, Her-2 overexpressing inflammatory breast cancer, mesothelioma, melanoma, ovarian cancer, cervical cancer, head and neck cancer, and hepatocellular carcinoma.

Preclinical Summary

Numerous preclinical studies in solid tumors and hematological malignancies have demonstrated a role for inhibition of XIAP as a therapeutic mechanism. XIAP inhibits apoptosis by directly binding to caspases 3, 7, and 9. One mechanism of inhibition of XIAP is with Smac mimetics or IAP antagonists which are peptidomimetics which simulate the effect of the mitochondrial protein SMAC. Smac mimetics can remove IAPs by triggering autoubiquitination and proteasomal degradation of IAPs (Green et al. 2011). Degradation of XIAP frees the caspases so that apoptosis may proceed. In addition, Smac mimetics stimulate autocrine TNFα (Tumor necrosis factor) secretion resulting in activation of the extrinsic apoptosis pathway. Increased levels of pro-inflammatory cytokines TNFα and TRAIL have been reported in the tumor microenvironment. Since XIAP protects cancer cells from the cytotoxic effects of these cytokines, reduction in the amount of XIAP may facilitate TNFα- and TRAIL-mediated apoptosis. Several preclinical studies using both cell lines and xenograft models have demonstrated target inhibition and induction of apoptosis by monovalent and bivalent Smac mimetics. The induction of apoptosis appears to be specific for tumor cells and spares normal tissues. Another approach to targeting XIAP is by the use of antisense oligonucleotides, for example, AEG35156, which inhibit XIAP mRNA expression and thus prevent XIAP protein translation. AEG35156 has shown promising results in preclinical models and has been studied in clinical trials (LaCasse et al. 2012).

Clinical Summary

To date there are no approved XIAP targeted agents. There are currently at least five Smac mimetics in early phase clinical trials with results pending (www.clinicaltrials.gov). AT-406 is an oral monovalent Smac mimetic being tested in a phase I dose escalation trial in solid tumors and lymphomas. HGS-1029 is a bivalent intravenous Smac mimetic currently in phase I trial in solid tumors. LCL-161 is an oral monovalent Smac mimetic which is being tested in a phase I trial of solid tumors. In addition there is a phase Ib trial of LCL-161 with weekly paclitaxel in breast and

ovarian cancer and a randomized phase II trial of neoadjuvant LCL-161 with weekly paclitaxel in triple negative breast cancer. Five trials of birinapant (TL32711), a bivalent intravenous Smac mimetic, are currently ongoing including a dose escalation trial of single agent birinapant and a dose escalation chemotherapy combination study in solid tumors and lymphomas, a phase I study of birinapant and gemcitabine in advanced solid tumors, a phase I/II single agent study in elderly patients with acute myelogenous leukemia, and a phase II single agent trial in relapsed platinum refractory and resistant ovarian cancer. A phase I trial of the oral monovalent Smac mimetic GDC-0197 in refractory solid tumors and lymphomas has been completed, and results are pending.

AEG35156 is a second generation antisense oligonucleotide (ASO) that targets the X-linked inhibitor of apoptosis mRNA. Several phase I/II trials of AEG35156 have been performed in metastatic solid tumors and hematologic malignancies, as both a single agent and in combination with different chemotherapies. Peripheral neuropathy and transaminitis were the most significant side effects requiring adjustment of the protocols. An acceptable safety profile was demonstrated with some evidence of anticancer activity and anticipated pharmacodynamic effects. Further development is anticipated.

Anticipated High-Impact Results

- Several phase I dose escalation trials of single agent Smac mimetics or Smac mimetics in combination with chemotherapies are currently ongoing with results pending. These drugs include birinapant, HGS 1029, AT-406, LCL-161, and GDC-0917.
- Ongoing phase II trials include birinapant for relapsed platinum refractory and resistant ovarian cancer, phase II trial of LCL-161 in breast and ovarian cancer, and LCL-161 in combination with paclitaxel in neoadjuvant treatment of triple negative breast cancer.

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Part V

Cell Cycle

APC 84

Joanna Louise Groden, William Hankey, and Kenechi Ebede

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Abstract

The *APC* gene is located at chr5q21 and is expressed in many tissues throughout the human body. In the colorectal epithelium in particular, *APC* functions as a critical suppressor of cancer initiation. Individuals who inherit inactivating mutations in one allele of the *APC* gene exhibit familial adenomatous polyposis (FAP) coli, an autosomal dominant syndrome characterized by the formation of a variety of benign lesions, particularly numerous adenomatous polyps of the colorectal epithelium.

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In the absence of preventative surgery to remove the source of these precancerous adenomas, FAP patients are highly susceptible to the development of colorectal cancer at an early age. Adenomas from FAP patients exhibit somatic mutations in the second allele of *APC*. Sporadically occurring colorectal adenomas in the general population frequently harbor biallelic *APC* mutations as well. The APC protein protects against adenoma formation in the colorectal epithelium at least in part by negatively regulating the canonical WNT signaling pathway. APC loss activates canonical WNT signaling, which coordinates changes in gene expression that promote proliferation over differentiation and cell survival over apoptosis. Ongoing research is focused on improving the accuracy of genetic screens for *APC* mutations, determining the extent to which colorectal cancers with *APC* mutations can be effectively treated with agents that downregulate canonical WNT signaling and testing the value of *APC* promoter hypermethylation as a diagnostic, prognostic, or predictive marker for other forms of cancer.

Keywords
APC • β-catenin • Canonical WNT signaling • Transcription • Colon • Colorectal • Cancer • CRC • Adenoma • Polyp • Polyposis • Familial • FAP • Tumor suppressor

APC is a 312-kilodalton protein expressed in numerous tissues throughout the human body. It is encoded by the *APC* gene located at chr5q21 (Senda et al. 2007). This locus was identified as the site of a tumor suppressor gene based on the observation of a deletion spanning this region in the germline of a patient affected by an autosomal dominant syndrome of colorectal cancer susceptibility known as familial adenomatous polyposis (FAP) coli (Heinen 2010) and inactivating mutations in a large open reading frame in other FAP patients (Senda et al. 2007). FAP is characterized by predisposition to various benign lesions, particularly numerous adenomas of the colorectal epithelium (Senda et al. 2007), some of which progress to colorectal cancer (CRC) at an early age in the absence of preventative surgery. The APC protein protects against tumor formation at least in part by negatively regulating the canonical WNT signaling pathway (Senda et al. 2007). APC interacts with glycogen synthase kinase 3β (GSK-3β) (Senda et al. 2007), Axin (Senda et al. 2007), and several other kinases and phosphatases (McCartney and Nathke 2008) to form a cytoplasmic complex with the transcriptional cofactor β-catenin (Senda et al. 2007). This complex (Fig. 1) promotes the sequential phosphorylation, ubiquitination, and proteolytic degradation of β-catenin (Senda et al. 2007). Activation of canonical WNT signaling is transduced through the disruption of this complex, accumulation of β-catenin in the cytoplasm, interaction with TCF family transcription factors, and translocation to the nucleus, with subsequent changes in gene expression (Senda et al. 2007). In the colorectal epithelium, the activation of canonical WNT signaling coordinates gene expression changes that promote proliferation over differentiation (McCartney and Nathke 2008) and cell survival over apoptosis (McCartney and Nathke 2008).

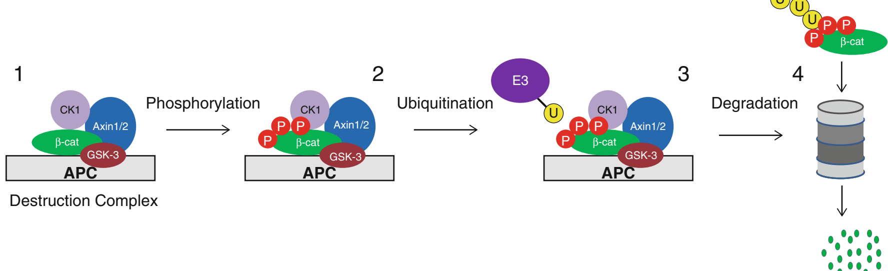

Fig. 1 APC is an essential component of the cytoplasmic destruction complex (1) which regulates negatively cellular β-catenin levels. This complex promotes the phosphorylation (2), ubiquitination (3), and proteasomal degradation (4) of β-catenin, a transcriptional cofactor required for canonical WNT signaling (Van der Auwera et al. 2008; Henrique et al. 2007; Hubers et al. 2012; Berrada et al. 2012; Half et al. 2009; Pack et al. 2013; Matuschek et al. 2010; Lynch and de la Chapelle 2003; Spigelman et al. 1989; Aretz et al. 2004; Lynch et al. 2007; Nielsen et al. 2007; Davila et al. 2006)

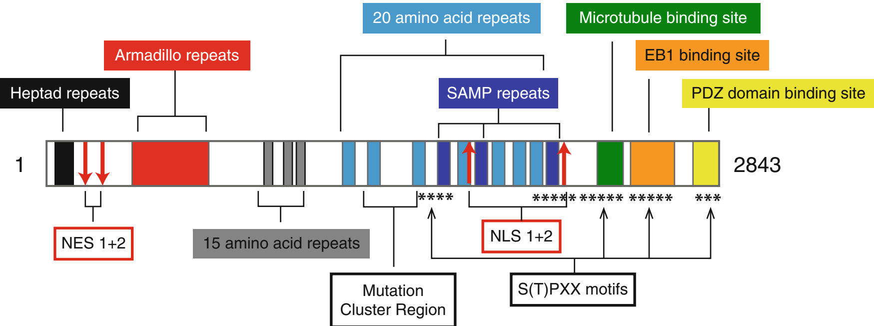

Fig. 2 The APC protein consists of 2843 amino acids whose major structural features include heptad repeats which mediate its dimerization (*black*) (Aziz et al. 2006), two nuclear export sequences (*down arrows*) [46], two nuclear localization sequences (*up arrows*) [45], clusters of S (T)PXX motifs (indicated by *asterisks*) which interact with A/T-rich DNA [50], armadillo repeats (*red*), and binding sites for microtubules (*green*) (Voronkov and Krauss 2013; Morton et al. 2011), the microtubule-associated protein EB1 (*orange*) (Anastas and Moon 2013), and PDZ domain-containing proteins (*yellow*). Interaction with Axins 1 and 2 is mediated by SAMP repeats (*dark blue*) (Henrique et al. 2007), while interaction with β-catenin is mediated by 15-amino acid repeats (*gray*) and 20-amino acid repeats (*light blue*) (Tonelli et al. 2000; Giardiello et al. 1993). Truncating mutations are observed most frequently in a central mutation cluster region, abolishing the Axin1/2 binding sites as well as the inducible interaction with β-catenin [68]

APC is an intrinsically unstructured scaffolding protein (Fig. 2) whose central region interacts with β-catenin constitutively through three repeats of 15 amino acids (Senda et al. 2007) and inducibly upon phosphorylation of seven additional repeats of 20 amino acids (McCartney and Nathke 2008) by GSK-3β (McCartney and Nathke 2008) and casein kinase I (CKI) (McCartney and Nathke 2008). This inducible interaction is required for APC to regulate negatively β-catenin levels

(Senda et al. 2007). The interaction of APC with Axin is mediated by three serine-alanine-methionine-proline (SAMP) repeats also located near the center of the protein (Senda et al. 2007). Additional domains in both its N- and C-terminal regions enable APC to interact with microtubules (Senda et al. 2007) and microtubule-associated proteins (Senda et al. 2007). These interactions reflect diverse functions of APC in cytoskeletal reorganization (McCartney and Nathke 2008) and cell adhesion (Senda et al. 2007) and in the control of apoptosis, cell division, and genomic stability (McCartney and Nathke 2008). APC contains multiple nuclear localization (Senda et al. 2007) and nuclear export sequences (Senda et al. 2007) which enable it to mediate the export of β-catenin to the cytoplasm (Senda et al. 2007) and to interact with chromatin (Senda et al. 2007). The C-terminus of APC contains clusters of S (T) PXX motifs that mediate binding to A/T-rich regions of DNA (Senda et al. 2007) and negative regulation of DNA replication (Lui et al. 2012). APC protein has been recently implicated in regulating the cellular response to replication stress (Lui et al. 2012) and base excision repair (McCartney and Nathke 2008). Finally, APC facilitates apoptosis (McCartney and Nathke 2008), both through transcriptional (McCartney and Nathke 2008) and transcription-independent mechanisms (Senda et al. 2007). The transcription-independent pro-apoptotic function is performed by an N-terminal fragment of APC following cleavage by caspase 8 (Lui et al. 2012). Thus, APC performs diverse functions at several different subcellular locations in order to suppress the development of CRC and other cancers.

Biology of the Target

Germline mutations in a single allele of *APC* are inherited by patients with familial adenomatous polyposis (FAP) coli and result in the production of a truncated APC protein (Senda et al. 2007) lacking most, if not all, normal functions. The extent of the inherited APC truncation influences the frequency of colorectal adenomas and extracolonic tumors in these patients, as well as the age of CRC onset (Heinen 2010). Truncated APC proteins also carry out acquired dominant functions that promote colorectal tumorigenesis (Lui et al. 2012) but that are likely of secondary importance to the loss of normal APC function. Adenomas arising in the colorectal epithelia of FAP patients exhibit inactivating somatic mutations in the second allele of *APC* (Heinen 2010).

The acquisition of biallelic *APC* mutations is an initiating event in the development of sporadic and familial colorectal tumors (Heinen 2010). *APC* mutations are observed in 50–80% of CRCs, many of which exhibit inactivation of both alleles (McCartney and Nathke 2008; Giles et al. 2003). More than 60% of *APC* mutations in CRC are found within a mutation cluster region located in Exon 15, resulting in the expression of truncated APC proteins of 1286–1513 amino acids or approximately the N-terminal half of the full-length protein (McCartney and Nathke 2008). Truncated proteins generally lack some or all of the 20-amino acid repeats required to regulate β-catenin levels, as well as the SAMP domains required to interact with

Axin. The consequences of APC loss are best understood in the context of CRC, where *APC* mutations drive tumor development by causing the accumulation of nuclear β-catenin and constitutive activation of canonical WNT signaling (McCartney and Nathke 2008), which in turn promote proliferation over differentiation (McCartney and Nathke 2008) and evasion of apoptosis (McCartney and Nathke 2008). The simultaneous loss of transcription-independent functions of APC additionally causes defects in mechanisms of apoptosis, cytoskeletal function, and control of cell division (McCartney and Nathke 2008).

Inactivating *APC* mutations occur less frequently outside of CRC but have been observed in tumors of the stomach (Giles et al. 2003), mouth (Uesugi et al. 2005), liver (Giles et al. 2003), pancreas (particularly solid-pseudopapillary tumors) (Giles et al. 2003), prostate (Giles et al. 2003), and breast (18%) (Virmani et al. 2001). Loss of APC function also occurs through epigenetic silencing, primarily due to promoter hypermethylation. Hypermethylation of the *APC* promoter region has been observed in carcinomas of the mouth (30%) (Uesugi et al. 2005), esophagus (15%) (Esteller et al. 2000), stomach (Esteller et al. 2000), liver (33%) (Esteller et al. 2000), pancreas (33%) (Esteller et al. 2000), bladder (10%) (Esteller et al. 2000), breast (45%) (Virmani et al. 2001; Van der Auwera et al. 2008), prostate (Henrique et al. 2007), lung (30–46%) (Virmani et al. 2001), colon and rectum (18%) (Esteller et al. 2000), and kidney (8%) (Esteller et al. 2000). In some cases, including bladder, prostate, lung, and breast cancers, the detection of *APC* mutations or epigenetic silencing is of diagnostic (Hubers et al. 2012; Berrada et al. 2012) and/or prognostic significance (Van der Auwera et al. 2008; Henrique et al. 2007). A variety of cancers exhibit constitutively activated canonical WNT signaling through mechanisms other than APC loss, including activating mutations in the *CTNNB1* gene encoding β-catenin (Giles et al. 2003).

Target Assessment

Genetic testing to assess *APC* germline status is indicated following either a clinical diagnosis of FAP based on a phenotype of polyp number or age of onset or the identification of an inherited *APC* mutation in a family member. *APC* mutation can be detected in peripheral blood lymphocytes by direct end-to-end sequencing of the *APC* gene, or less commonly by the protein truncation test (PTT), which detects a truncated APC protein synthesized in vitro from *APC* mRNA (Half et al. 2009). A genotype-phenotype correlation has been observed linking *APC* mutations in certain regions with an attenuated FAP (AFAP) variant in which affected individuals typically exhibit fewer than 100 colorectal polyps and later onset of disease (Heinen 2010). Identifying the germline *APC* mutation is useful for distinguishing AFAP and FAP (Half et al. 2009); however, these two phenotypes are more often clinically distinguished by polyp number. AFAP also differs from typical FAP in its lower associated risk of rectal polyps and rectal cancer (Half et al. 2009). Surgical recommendations for FAP and AFAP, respectively, are total proctocolectomy with ileal pouch-anal anastomosis (IPAA) and the less-radical colectomy with ileorectal

anastomosis (IRA) (Half et al. 2009). In addition to surgical resection and continued endoscopic screening, medications for FAP individuals to reduce polyp formation in the remaining large bowel epithelium include NSAIDs such as sulindac, aspirin, and celecoxib (Half et al. 2009).

Genetic testing to assess *APC* status is a critical step in FAP diagnosis, but the diagnostic and prognostic application of *APC* status in the context of various sporadic cancers remains in the developmental stages. The status of the *APC* gene in sporadic CRC is not generally tested because *APC* mutations do not seem to be strong indicators of prognosis in this context. In other tumor types where loss of APC function occurs more commonly through epigenetic rather than genetic mechanisms, disease-specific *APC* promoter hypermethylation in tumor tissue, urine (bladder cancer) (Berrada et al. 2012), sputum (lung cancer) (Hubers et al. 2012), and serum (colorectal cancer and breast cancer) (Pack et al. 2013; Matuschek et al. 2010), has been studied as a potential diagnostic marker (Hubers et al. 2012; Berrada et al. 2012) or marker of tumor progression, prognosis, or subtype (Van der Auwera et al. 2008; Henrique et al. 2007; Matuschek et al. 2010). Many of these studies have examined the methylation status of the *APC* promoter in the context of a larger panel of hypermethylated promoters. Several studies have shown some promise for prognosis in cancers of the breast, prostate, lung, liver, and bladder but have yet to be fully characterized as tools to advance clinical practice.

Role of the Target in Cancer

Rank: 10 (colorectal).

High-Level Overview

Diagnostic, Prognostic, and Predictive

Mutation of the *APC* gene causes familial adenomatous polyposis (FAP) coli which is characterized by more than 100 colorectal adenomas and often accompanied by extracolonic manifestations. Those affected carry a 100% lifetime risk of developing colorectal cancer by the age of 50 without prophylactic colectomy (Lynch and de la Chapelle 2003). Colorectal cancer attributable to FAP accounts for 1% of all CRC cases. In 60% of those with FAP, adenomas also develop in the duodenum (Spigelman et al. 1989). Due to the early onset of colorectal cancer in this patient population compared to the general population, the diagnosis of FAP leads to screening guidelines with consistent screening by colonoscopy and invariable planning for eventual surgical removal of the colon.

Current genetic testing guidelines call for evaluation of first-degree relatives of those with confirmed *APC* mutations or a clinical diagnosis of FAP. In 15–20% of adolescent FAP patients, a *de novo* mutation is discovered (Aretz et al. 2004). Commercial genetic testing for *APC* gene mutation has a sensitivity of 80%

(Lynch et al. 2007). For patients that show clinical signs of disease without an identifiable *APC* mutation, genetic testing for a biallelic mutation of *MUTYH* is indicated (Nielsen et al. 2007). This mutation leads to a phenotype that is similar to an attenuated FAP mutation.

Endoscopic screening of patients with FAP significantly reduces mortality attributable to colorectal cancer. Based on the available data on screening endoscopy, the American Society of Gastrointestinal Endoscopy recommends annual flexible sigmoidoscopy or colonoscopy for patients with a diagnosis of FAP starting between the ages of 10 and 12. For patients with an attenuated phenotype, endoscopic screening should start in their late teens to early twenties (Davila et al. 2006).

APC promoter hypermethylation indicates a poor prognosis in cancers such as prostate (Henrique et al. 2007) and breast (Matuschek et al. 2010) and defines a disease subtype in breast (Van der Auwera et al. 2008) and colorectal cancer (Fu et al. 2009). Further study of these correlations may lead to the emergence of *APC* promoter methylation as a predictive marker in certain contexts. In the context of CRC, loss of APC is associated with increased resistance to microtubule-stabilizing drugs such as Taxol or paclitaxel and increased sensitivity to the microtubule-destabilizing agent vinorelbine (Klotz et al. 2012). *APC* status in CRC has also been suggested to predict the effectiveness of COX-2 inhibitors, as COX-2 expression is activated by canonical WNT signaling (Giles et al. 2003).

Therapeutics

Due to the near certain development of colorectal cancer in the FAP patient population, prophylactic colectomy is recommended by the age of 20 (Davila et al. 2006). The pathologic staging of disease after surgery determines the need for adjuvant chemotherapy (Edge and Compton 2010). The determination of the proper surgical procedure between total proctocolectomy with ileal pouch-anal anastomosis (IPAA) and total abdominal colectomy with ileorectal anastomosis (IRA) requires an in-depth assessment of the patient polyp burden and location. IRA is preferred for patients with (1) low rectal polyp burden (<20 polyps), (2) small rectal lesions (<3 cm in diameter), and/or (3) an attenuated FAP phenotype (da Luz Moreira et al. 2009). The remaining rectal tissue imparts a greater lifetime risk of rectal cancer (Vasen et al. 2001). IPAA reduces the risk of rectal cancer by the near-complete removal of the rectal mucosa and is preferred to IRA. However, IPAA is more technically demanding than IRA, and the need for pelvic dissection has been associated with higher morbidity (Aziz et al. 2006). For female patients, recent studies have demonstrated reduced fertility and sexual function in women after IPAA compared to IRA (Olsen et al. 2003). Due to the risk of recurrent colorectal cancer in the rectal stump, ileal pouch, and anorectal cuff, scheduled lower endoscopy is recommended after surgery regardless of the procedure performed (Church and Simmang 2003).

The role of pharmaceutical agents in primary chemoprevention is unclear, although many drugs have shown efficacy as adjunct therapy for FAP patients

after surgery (Tonelli et al. 2000). Sulindac, a nonsteroidal anti-inflammatory drug, effectively reduces the size and number of rectal polyps (Giardiello et al. 1993). Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, also has shown benefit as adjunct therapy for lower gastrointestinal polyp, although the use of COX-2 inhibitors is controversial due to reported increases in cardiac events (Steinbach et al. 2000). In a recent randomized controlled clinical trial, eicosapentaenoic acid, an omega-3 fatty acid metabolite, significantly reduced polyp number by 22% and polyp size by 29% (West et al. 2010).

Targeted therapeutic strategies for APC-deficient cancers have developed in response to the observation that APC-deficient CRC cells undergo cell cycle arrest and differentiation or apoptosis upon repression of canonical WNT signaling or restoration of APC function (Senda et al. 2007). Inhibitors of canonical WNT signaling have long been studied as potential therapeutic agents for APC-deficient CRC, particularly targeting components of the pathway that lie downstream of APC. Recent studies have identified small molecules that disrupt TCF/β-catenin transcription factor complexes (Voronkov and Krauss 2013) or block the effects of transcriptional target genes activated by canonical WNT signaling, particularly the critical c-Myc transcription factor (Morton et al. 2011). Components of the canonical WNT signaling pathway acting upstream of APC have also been targeted with some success in preclinical studies of APC-deficient CRC (Voronkov and Krauss 2013; Anastas and Moon 2013). β-catenin degradation has been stimulated either through a small molecule that partially restores cytoplasmic destruction complex function despite the absence of APC (Chen et al. 2009) or by alternative therapeutic mechanisms independent of the canonical WNT signaling pathway (Voronkov and Krauss 2013). Other pathways such as Vitamin D signaling and inhibition of the tankyrase enzyme downregulate canonical WNT signaling in CRC through mechanisms not yet fully understood (Voronkov and Krauss 2013; Anastas and Moon 2013). Despite the efficacy of these emerging WNT inhibitors in preclinical studies, substantial toxicity in humans is anticipated in some cases, due to the importance of the canonical WNT signaling pathway for stem-cell maintenance.

Novel therapeutic strategies have emerged recently to restore APC function in cancers with *APC* mutations through gene therapy (Macnab et al. 2011) or premature termination codon read-through (Zilberberg et al. 2010). In cancers with epigenetic silencing of *APC* such as breast and lung carcinomas, treatment with DNA demethylating agents such as decitabine inhibits canonical WNT signaling effectively *in vitro* (Virmani et al. 2001). MicroRNAs 135a and 135b, which downregulate APC at the mRNA level in a subset of CRCs, have shown early promise as therapeutic targets as well (Holleman et al. 2011). Finally, small interfering RNAs targeting mutant but not wild-type *APC* mRNA have the potential to silence the expression of truncated APC proteins and reduce proliferation *in vitro* (Chandra et al. 2012). Targeted therapeutics restoring APC function may act synergistically with established chemotherapeutic or chemopreventive agents such as NSAIDs to reverse CRC phenotypes *in vitro* and *in vivo* (Giles et al. 2003).

Preclinical Summary

APC loss occurs in the majority of CRCs, predominantly through genetic mutation (Esteller et al. 2000; Fu et al. 2009) but is not strongly predictive of prognosis. APC loss is also a common feature in other cancer types but occurs predominantly through promoter hypermethylation in these contexts (Esteller et al. 2000) and in some cases is associated with a poor-prognosis subset of disease (Van der Auwera et al. 2008; Henrique et al. 2007; Matuschek et al. 2010; Fu et al. 2009). *APC* promoter methylation status has shown promise in preclinical studies as a diagnostic or prognostic marker, often as part of a larger panel of gene promoters (Matuschek et al. 2010).

Targeted therapeutic strategies for APC-deficient cancers have focused either on targeting components of the canonical WNT signaling pathway downstream of APC (Voronkov and Krauss 2013; Morton et al. 2011) or on promoting β-catenin degradation through WNT-dependent (Chen et al. 2009) or WNT-independent mechanisms (Voronkov and Krauss 2013). Substantial toxicity is anticipated, yet some natural products found in the human diet may have the potential to promote β-catenin degradation through as yet-undefined mechanisms (Anastas and Moon 2013). Other strategies to restore APC function to cancer cells with *APC* mutations remain in the early stages of preclinical study (Macnab et al. 2011; Zilberberg et al. 2010).

Clinical Summary

The discovery of the role of *APC* gene mutation in the initiation of colorectal cancer has led to a greater understanding of the disease. Mutations of *APC* currently do not have a significant role in treatment algorithms for sporadic colon cancer, but its function in the canonical WNT signaling pathway portends a greater role in the future as more breakthroughs are achieved. For now, in the clinical realm, understanding the *APC* gene directly affects those patients afflicted with FAP. Those patients who receive genetic testing benefit by consistent screening and timely prophylactic surgery and by the diagnosis of at-risk family members. Current clinical research is aimed toward improving the accuracy of genetic screens and providing less-invasive screening methods. Although surgery remains the primary treatment modality, many clinical studies search for effective drugs to use in primary chemoprevention. Understanding the molecular mechanisms subsequent to *APC* mutation that underpin its effect on cancer initiation is an important way to generate new diagnostic options and therapeutic interventions for those patients with familial and sporadic colorectal cancer.

Anticipated High-Impact Results

- Determining the extent to which CRCs with mutations in *APC* can be safely and effectively treated by inhibitors of canonical WNT signaling, WNT-independent downregulation of β-catenin, *APC* gene therapy, or reagents promoting read-through of premature stop codons in the *APC* transcript

- Determining the extent to which CRCs and other cancers with *APC* promoter hypermethylation can be safely and effectively treated by demethylating agents, WNT-independent downregulation of β-catenin, or inhibitors of canonical WNT signaling
- Determining the value of *APC* promoter hypermethylation detectable in the serum as a diagnostic, prognostic, and/or predictive marker for therapeutic decision-making in the contexts of CRC and breast cancer
- Determining the value of *APC* promoter hypermethylation detectable in sputum as a diagnostic, prognostic, and/or predictive marker for therapeutic decision-making in lung cancer
- Determining whether *APC* promoter hypermethylation detectable in urine will be valuable as a diagnostic, prognostic, and/or predictive marker for therapeutic decision-making in bladder cancer
- Determining the extent to which *APC* status predicts CRC response to emerging therapeutic interventions

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AR, Overview

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Travis Van der Steen, Lucy J. Schmidt, and Donald J. Tindall

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Abstract

The androgen receptor (AR) is a nuclear hormone receptor that plays a key role in the development of the prostate and progression of prostate cancer (PCa). AR is the initial target for treatment in hormone dependent PCa, where inhibition of AR activity results in a decrease in tumor volume and increased survival. However, PCa often returns as castration-resistant prostate cancer (CRPC) due to several factors including AR overexpression, reactivation of the full-length receptor (AR-FL), and expression of constitutively activate truncated AR-variants (AR-Vs) that lack the ligand binding domain. Upon reactivation of AR-FL and expression of AR-Vs, traditional methods of treatment targeting the ligand binding domain are

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ineffective. Targeting the evolutionarily conserved N-terminal domain, DNA binding domain (DBD) or using transcription factors that interact with AR will be key in developing new treatments for PCa.

Keywords
Androgen receptor (AR) • Prostate cancer (PCa) • Androgen receptor variants (AR-Vs) • Castration-resistant prostate cancer (CRPC) • Phosphorylation • Reactivation of AR • Androgen deprivation therapy (ADT) • Androgen response element (ARE) • DNA-binding domain (DBD)

Target: Androgen Receptor

The androgen receptor (AR) is a member of the steroid hormone nuclear receptor superfamily; other family members consist of the estrogen (ER), progesterone (PR), mineralocorticoid (MR), and glucocorticoid (GR) receptors. AR plays a vital role in sexual development and in the development of the prostate by regulating cellular proliferation, survival, and apoptosis. The AR gene is located on chromosome Xq11-12 and is transcribed and then translated into a 919 amino acid protein. Full-length AR (AR-FL) is comprised of eight exons that encode four distinct regions of the protein. The N-terminal domain (NTD) contains an activation function motif (AF1) and is highly unstructured. Within the AF1 motif, there are two transcriptional activation units, termed TAU1 and TAU2, that modulate AR transcriptional activity. Adjacent to the NTD is the DNA-binding domain (DBD) that contains two zinc fingers; the first zinc finger binds DNA and the second facilitates dimerization with a second AR monomer. A short hinge region links the DBD to the C-terminal ligand-binding domain (LBD). The LBD encompasses a second transcriptional activation function (AF2) region. Upon ligand binding, AR undergoes a conformational change, exposing functional residues that are critical for dimerization of AR and transcriptional activation. A number of amino acids in AR that are posttranslationally modified are important for regulating transcriptional activity, protein stability, cellular localization, and cellular growth (Bennett et al. 2010; Van der Steen et al. 2013).

Biology of Androgen Receptor

The androgen receptor is shuttled between the cytoplasm and nucleus, depending upon its activation status. Inactive AR resides in the cytoplasm, bound to chaperone and heat shock proteins (HSP90, HSP70, and HSP56) that facilitate folding, impede nuclear localization, and increase stability of the of the nascent inactive receptor. Testosterone, which is one of the ligands for AR, is produced in the Leydig cells of the testis in response to hormonal signals from the hypothalamus and pituitary gland. Testosterone circulates in the serum bound tightly to sex hormone binding globulin

and loosely to albumin. After testosterone enters the cell, it is converted to the more potent androgen 5-alpha-dihydrotestosterone (DHT) by 5-alpha-reductase enzymes. The affinity of DHT for AR is 2–3 times greater than that of testosterone. DHT binds to the inactive monomeric AR LDB resulting in a conformational change of AR, release from HSPs, dimerization, and translocation to the nucleus (Vander Griend and Isaacs 2009; Mohler et al 2011).

Nuclear AR acts as a potent transcription factor. The first zinc finger binds to the major groove of DNA to a sequence known as an androgen response element (ARE) that is located within androgen-regulated genes. The full ARE motif is a 15-base pair sequence that consists of two hexameric half-sites, 5′-AGAACA-3′, arranged as a palindromic repeat with a three-base pair spacer sequence, 5′-AGAACA-XXX-ACAAGA-3′, where X is any nucleotide. Additionally, AR can also bind to a half-ARE, where only one of the two hexameric sites is encoded in the DNA. These response elements are located within the enhancer and promoter regions of androgen-regulated genes and are critical for transcriptional activation or silencing of genes (Agoulnik and Weigel 2009; Sahu et al. 2014). More than 300 cofactors can interact with AR depending upon cell type, gene, and stage of the cell cycle (Agoulnik and Weigel 2009; Nuclear Receptor Signaling Atlas 2014). Other transcription machinery such as histone acetyltransferase, histone methyltransferase, and DNA polymerase are recruited to change the conformation of chromatin into an active motif. Activated full-length AR (AR-FL) stimulates cell proliferation via the production and secretion of many growth factors such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), and insulin-like growth factor (IGF), cyclin-dependent kinases (cdks), and cyclins and by the recruitment of replication machinery and checkpoint proteins to ensure proper replication of DNA prior to cell division (Balk and Knudsen 2008; Vander Griend and Isaacs 2009).

Recently, AR splice variants (AR-Vs) have been identified in prostate cancer cell lines and patient tumor samples. Most AR-Vs contain the NTD and DBD, but have a truncated C-terminus. Due to the absence of the LBD, AR-Vs are regulated by the two TAU domains (TAU1 and TAU2) located within the AF1 region in the N-terminus and are constitutively active. To date, more than 20 AR-Vs have been identified, and their role in prostate cancer is still being investigated (Lonergan and Tindall 2013). It has been speculated that AR-Vs arise from androgen deprivation or antiandrogen treatments, which are the current mainstay treatments for prostate cancer (PCa). There are two hypotheses for their function, the first being that the full-length and variant receptors regulate the same genes, implying that androgen-regulated genes are controlled by both the constitutively active variants and the activated full-length receptor. An alternative hypothesis is that AR-Vs regulate a unique subset of genes. This would suggest that AR-FL and AR-Vs do not regulate the same genes and that AR-Vs may regulate a distinct set of genes in PCa. The coexistence of AR-FL and AR-Vs within the same cells results in the potential for the formation of several different dimers. As with the ligand-activated AR-FL dimer, AR-Vs contain the first and second zinc fingers needed for dimerization and DNA binding. Therefore, AR-Vs can form homodimers with the same AR-V and can potentially form heterodimers between different AR-Vs or AR-Vs and the full-

length receptor. However, the extent of the formation of heterodimers is unknown, and their function is still under investigation.

High-Level Overview of Androgen Receptor

Most of what is known about the role of AR in cellular proliferation is based on AR-FL studies. Several protein-protein interactions that occur with AR during cell cycle progression require the binding of androgen to the LBD. This induces conformational change, presumably to expose new binding sites or allow for modifications such as phosphorylation, methylation, SUMOylation, ubiquitination, and acetylation to various AR residues (Balk and Knudsen 2008). Due to the importance of the LBD for protein-protein interaction, it is still unknown how, or if, the AR-Vs that lack this domain are able to regulate the cell cycle in a similar manner when interactions occur within the LBD. Additionally, prostate cells that express truncated AR-Vs, but not AR-FL, are able to proliferate, suggesting that AR-Vs are able to regulate cellular proliferation in the absence of AR-FL. Endogenous interactions with the NTD of AR-FL are found to occur in AR-Vs, which could regulate the cell cycle.

AR regulates several genes that are key for cell cycle progression. Androgen stimulation promotes mTOR-dependent accumulation of cyclins D1 and D3, which are required for progression through the G1/S transition of the cell cycle. The accumulation of cyclin D1 results in phosphorylation of retinoblastoma (RB) that allows for expression of cell cycle genes. Cyclin D1 binds to the N-terminus of AR and disrupts the N-terminal-LBD interaction of AR, thereby limiting AR transactivation and binding to DNA. As a result of cyclin D1 binding to AR, cellular proliferation decreases due to insufficient AR activity. In the absence of androgens, cdk 4/6-cyclin D and cdk 2-cyclin E complexes needed for G1/S transition are mostly inactive. AR-cdk 6 interaction occurs at the chromatin level, suggesting that cdk 6 is part of the AR transcriptional complex that assembles at AREs. AR-cdk 6 interaction is independent of cyclin D. Cell division cycle 25 (Cdc25) is a dual function phosphatase and is a coactivator of AR along with other steroid receptor family members (ER, PR, and GR). Cdc25 mediates the activation of cdk1, which allows for progression through the cell cycle (Balk and Knudsen 2008; Ruscetti and Wu 2013; Kokontis et al. 2014).

Phosphorylation of AR at serine 81 (AR-S81) regulates cellular translocation of AR to the nucleus, protein stability, recruitment to specific AREs, and overall transcriptional activity. Several cdks phosphorylate AR-S81 including cdk 1, cdk 5, and cdk 9. Phosphorylation at AR serine 308 by cdk11-cyclin D3 is delayed upon AR activation and thus acts as an inhibitory site. Alternatively, cyclin E binds to AR at amino acids 419–556 and enhances AR activity. Cyclin H and cdk7 regulate AR by interacting with the N-terminal domain of AR. Cdk7 phosphorylates AR serine 515 allowing for polyubiquitination of AR, resulting in degradation of AR protein (Van der Steen et al. 2013).

Androgen Receptor Assessment

Expression of AR in the laboratory is determined largely by western blotting where antibodies are available to detect and delineate between AR-FL and AR-Vs based on molecular weight. In clinical samples, it is possible to examine expression of AR-FL by immunostaining, but there are currently no reliable antibodies that can be used to detect individual AR-Vs. To monitor AR activity in cells in culture, luciferase assays are used with constructs comprised of AR-regulated gene promoters attached to the luciferase gene allowing for detection of AR activity. Additionally, measuring mRNA expression changes of AR-regulated genes as a result of treatment with activators/inhibitors can determine AR activity. In patients, in vivo AR activity is measured by monitoring serum PSA levels, where increased PSA levels may indicate prostatitis, benign prostate hyperplasia (BPH), or prostate cancer. Androgen deprivation therapy (ADT) reduces PSA levels initially, but often PCa recurs in the form of CRPC; recurrence is initially determined by an increase in serum PSA levels (Gupta et al. 2011). There are no current clinical assessments to delineate between expression of AR-FL or AR-Vs in serum samples.

Role of Androgen Receptor in Cancer

Prostate Cancer Target AR

Rank: 10

The discovery that the AR plays a pivotal role in proliferation and apoptosis in the prostate was made over 70 years ago by Charles Huggins and Clarence Hodges. Their work set the stage for the investigation and study of AR in normal and cancerous prostate as a key transcription factor and as a therapeutic target for treatment of PCa. Removal of androgens results in the reduction of prostate tumor volume suggesting that the androgen-AR signaling axis plays a critical role in regulating cellular proliferation and apoptosis (Balk and Knudsen 2008). Limiting the amount of androgens available to activate AR results in decreased tumor burden, but in castration recurrent prostate cancer (CRPC), the AR is reactivated at insignificant levels of androgen or is independent of androgens. In CRPC, reactivated AR allows for proliferation resulting in an increase in tumor size. An increase in kinase activity resulting in increased phosphorylation yields an increase in AR activity and cell proliferation. The recent identification of AR splice variants that lack the LBD and are constitutively active may play a critical role in CRPC (Lonergan and Tindall 2013; Ware et al. 2014). AR-Vs arise as a result of androgen deprivation therapy (ADT) and antiandrogen treatment. Currently, there is great interest in how these variants arise, how they are regulated, if variants regulate the same genes as the full-length receptor, and if all AR-Vs are similar in their function in PCa.

Reactivation of AR is known to occur through several additional mechanisms including (1) AR mutations, (2) AR overexpression, (3) ligand-independent

activation, and (4) increased AR protein stability. Mutations to the AR gene are found in approximately 10% of CRPC. Most of these mutations occur in the C-terminus, which results in decreased ligand specificity. These mutations allow for other steroids to bind to the LBD of AR such as adrenal androgens, glucocorticoids, and progesterone and even antiandrogens (The Androgen Receptor Gene Mutation Database World Wide Web Server 2014). Overexpression of AR is observed in approximately 35% of CRPCs after ADT. The increase in AR mRNA expression may occur as a result of either AR gene amplification, which often occurs in cancers, or increased transcriptional expression of the AR gene (Bennett et al. 2010). The third possible mechanism is ligand-independent activation of AR. Noncanonical activation of AR can occur as a result of cytokines and growth factor signaling that activates downstream kinases. For example, interleukin 6 (IL-6) expression increases during progression to CRPC and can transactivate AR. IL-6 signaling through the MAPK pathway can regulate p300, an acetyltransferase, which is a coregulator of AR. The loss of the tumor suppressor PTEN, which is a potent regulator of the PI3K/AKT/mTOR pathway, is often observed in CRPC. Kinases in both of these pathways phosphorylate and regulate AR activity. In CRPC, degradation of AR protein is approximately half of that in androgen-dependent PCa. The increased half-life of AR may result in enhanced AR activity (Ruscetti and Wu 2013; Vander Steen et al. 2013).

Preclinical Summary

The increase of AR-Vs in CRPC and the reactivation of the AR-FL have resulted in the focus of treatment to shift from the CTD of AR toward the NTD of AR, which is conserved between most AR-Vs and AR-FL. One hypothesis is that AR-Vs arise as a result of the current treatment for PCa leading to development of CRPC. Identifying inhibitors of the NTD of AR have the potential to limit the activity of not only the full-length AR but also AR-Vs. Since AR-Vs are present in CRPC, in theory, this would allow for a single type of treatment that would target all forms of AR.

One potential treatment directed toward the NTD of AR is small molecule inhibitor EPI-001. EPI-001 interacts with the AF1 region, which is the regulatory region for AR-Vs, and inhibits protein-protein interactions. The loss of these interactions results in decreased recruitment of both AR-FL and AR^{v567es} to AREs, thereby limiting expression of AR target genes. Treatment of cells that express either AR-FL and/or AR-Vs with EPI-002, an analog of EPI-001, results in a decrease in growth of CRPC xenografts expressing either AR-FL or AR^{v567es} (Myung et al. 2013). These studies have demonstrated the potential to target AR-Vs and limit cell cycle progression; however, additional studies using other AR-Vs are needed.

A second region that is highly conserved between AR-FL and AR-Vs is the DNA-binding domain. Inhibiting AR interaction with chromatin by targeting its DBD is the focus of further research. One small molecule inhibitor, pyrvinium pamoate, that is a noncompetitive inhibitor directed toward the AR DBD has

therapeutic potential (Dalal et al. 2014). Xenografts treated with pyrvinium pamoate result in inhibition of AR-V constitutive activity and cellular growth. Other molecules that are derivatives of morpholine have been shown to bind to a pocket in the DBD and disrupt AR-chromatin interactions (Lu et al. 2015). Through inhibition of these interactions, there is therapeutic potential to treat all forms of AR that bind DNA.

Other potential means of inhibiting AR activity include targeting heat shock proteins, which bind AR in the cytoplasm and affect protein stability. Through inhibition of HSP90, there is improper folding and destabilization of AR protein. However, two variants (AR-V7 and AR^{v567es}) have exhibited resistance to HSP90 inhibitors due to the lack of interaction of AR-Vs with HSP90 (Azad et al 2015).

Clinical Summary

Currently, limiting the activity of AR is used for treatment of advanced PCa. Impeding AR activity is obtained through (1) removal of the prostate, (2) chemical/surgical castration, (3) treatment with antiandrogens, and (4) use of an AR antagonist. Initially, androgen deprivation therapy (ADT) is used to reduce androgen levels in the body and ultimately leads to suppression of AR activity. The decrease in androgen synthesis does not cure prostate cancer, but is used if PCa has metastasized and cannot be contained by surgery or radiation. ADT is also used upon recurrence of PCa to the refractory state but in these cases only results in a remission of 2–3 years from the time of initiation of ADT.

To decrease testosterone, men with PCa may undergo surgical castration (orchiectomy) to remove the testes where most of the androgens are produced. A second option that is used is treatment with luteinizing hormone-releasing hormone (LHRH) analogs, also known as chemical or medical castration, to lower androgen levels, similar to orchiectomy. Due to the loss of testicular androgens, some prostate cells synthesize their own androgens to activate AR. CYP17 is a key enzyme for the biosynthesis of androgens and is increased in CRPC when compared to primary prostate tumors of untreated men. Abiraterone is often given as a treatment to inhibit CYP17 activity in cells making their own androgens (Mohler et al 2011; Stein et al. 2014).

Inhibition of AR is also achieved using antiandrogens, which bind to AR and prevent androgens from binding and activating AR. Antiandrogens include flutamide, bicalutamide, and nilutamide as a daily treatment. Antiandrogens are usually given in conjunction with ADT treatment to inactivate AR. Use of these treatments results in decreased cell proliferation. Enzalutamide (MDV3100) is a newer antiandrogen that binds to AR, thereby inhibiting its interaction with endogenous androgens. The use of enzalutamide as a therapeutic for CRPC resulted in a decrease in PSA levels in 50% of men and was found to result in an increased life span. Current clinical studies are being conducted to determine the effectiveness of enzalutamide versus bicalutamide in men with CRPC who have progressed while receiving LHRH therapy or orchiectomy (Suzman and Antonarakis 2014).

Current treatments for advanced PCa all target the LBD of AR, either through treatment with antiandrogens or ADT. Once PCa progresses to CRPC, the AR-FL can be activated at castrate levels of androgens by a number of mechanisms, and in many cases there is a rise in the expression of AR-Vs. AR-Vs lack any regulation by androgens due to the truncation of the C-terminal domain and are unresponsive toward these methods of treatment. New treatments that focus on the NTD and DBD of AR are being developed but have yet to be approved for clinical use.

Anticipated High-Impact Results

- Determine mechanistically how AR-Vs arise in prostate cancer and if they contribute to the development of CRPC.
- Delineate pathways allowing for constitutive activity of AR-Vs.
- Develop methods for detecting the presence of AR-Vs in PCa patient samples.
- Determine if there is a difference between AR-FL and AR-V gene targets that allow for the proliferation of cells to be uncontrolled due to the constitutive activity of AR-Vs.
- Development of novel therapeutic agents that are directed toward the N-terminal domain of AR allowing for targeting of both the full-length and variant forms of AR.

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BRCA1 and 2

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Eliot M. Rosen

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Abstract

BRCA1 and BRCA2 were identified by linkage to familial predisposition to breast or ovarian cancer. Both genes confer an autosomal dominant predisposition to breast cancer, and BRCA2 additionally predisposes to ovarian cancer. Women who carry high-risk BRCA1 mutant alleles have a 50–85% chance of developing breast cancer by age 70 years. Differences in cancer risk estimates appear to be due to the extent of family history, with a strong family history (e.g., four or more affected members) of bilateral breast cancer, family history of both breast and ovarian cancers, and early onset of breast cancer (before age 40) conferring a

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higher risk of breast cancer development. BRCA1 and BRCA2 loss predispose to a variety of other cancers at lower frequencies. Synthetic lethality studies have identified BRCA1 and BRCA2 mutations as conferring therapeutic sensitivity to PARP inhibitors. In addition, these mutations confer sensitivity to platinum salts.

Keywords
BRCA1 • BRCA2 • Homology-directed DNA repair (HDR) • Homologous recombination • BRCT (BRCA1 C-terminal domain) • RAD51

Targets: BRCA1 and BRCA2

Biology of the Target

BRCA1

The breast cancer susceptibility gene 1 (*BRCA1*) was identified by positional cloning based on its linkage to breast-ovarian cancer families (Miki et al. 1994). The *BRCA1* gene is located on human chromosome 17q21 (and mouse chromosome 11), contains 24 exons (2 of which are noncoding and 22 coding), and encodes a 1863-amino acid protein with an apparent molecular mass (M_r) of 220 kDa. BRCA1 contains an N-terminal RING domain and a C-terminal acidic transcriptional activation domain that contains two BRCT (BRCA1 C-terminal) domains. The BRCT domain(s) is found in DNA repair and cell cycle regulatory proteins and serves as a phosphoprotein binding motif (Rodriguez and Songyang 2008). The BRCA1 RING domain was found to interact with another RING-containing protein (BRCA1-associated RING domain protein 1 (BARD1)) to mediate an enzymatic function as an E3 ubiquitin ligase, the only known enzymatic activity of BRCA1. The role of the E3 ubiquitin ligase activity of BRCA1 in mediating tumor suppression remains controversial (see below). Since its discovery, BRCA1 has been found to participate in the regulation of various cellular processes, including the DNA damage response, transcription, cell cycle progression, and apoptosis (reviewed in Rosen 2013).

Clues to the function of BRCA1 in DNA repair were the findings that: (1) BRCA1 colocalizes with RAD51, a DNA recombinase and the homolog of the bacterial RecA protein, in nuclear foci during S-phase and (2) BRCA1 becomes hyper-phosphorylated and colocalizes with RAD51, BRCA2, and BARD1 (BRCA1-associated RING domain protein 1) in repair-related foci following exposure of cells to DNA-damaging agents (Scully et al. 1997). Subsequently, BRCA1 has been found to be essential for homology-directed DNA repair (HDR) (also called homologous recombination (HR), an error-free pathway for the repair of DNA double-strand breaks and interstrand DNA cross-links) (Moynahan et al. 1999). Since HDR requires a sister chromatid as a template for repair DNA synthesis, it can only occur during mid-late S-phase or G2. In contrast, nonhomologous end joining (NHEJ), an error-prone mechanism for DNA repair of DNA double-strand

breaks (DSBs), occurs preferentially during G1 but can occur during any phase of the cell cycle. In addition to participating in the process of HDR, BRCA1 (along with CtIP) helps to push cells into the HDR pathway, while the proteins phospho-53BP1 and RIP1 push cells into the NHEJ pathway (reviewed in Rosen 2013).

The precise function of BRCA1 in HDR is not certain, but it appears that the BRCA1 BRCT domains form at least three mutually exclusive complexes with different sets of proteins that mediate various functions. Complex A includes phospho-ABRAXIS, RAP80, and BRCC36 and may function in regulation of DNA end resection, de-ubiquitination, and G2/M checkpoint control (reviewed in Rosen 2013). Complex B contains phospho-BACH1 and appears to function in maintaining orderly progression through S-phase and bypassing of stalled replication forks. Complex C contains phospho-CtIP and functions in DNA end resection in HDR and G2/M checkpoint control. The role of BRCA1 in HDR but not its activity as a ubiquitin ligase appears to be required for its tumor suppressor activity since mice harboring a *Brca1* mutation that disrupts the BRCT function develop cancer in three different models, whereas mice with a *Brca1* mutation that selectively disrupts the Brca1 E3 ubiquitin ligase function (I26A) failed to develop tumors (reviewed in Rosen 2013). However, this view has been challenged by a recent study suggesting that silencing of satellite DNA within heterochromatin by BRCA1, which requires its ubiquitination of histone H2A, is necessary for tumor suppression (Zhu et al. 2011).

BRCA1 regulates a variety of transcription pathways although BRCA1 is not itself a sequence-specific DNA-binding transcription factor. The usual mechanism is that BRCA1 binds directly to various transcription factors (e.g., p53, estrogen receptor, progesterone receptor, androgen receptor, STAT1, c-Myc, NF-κB, OCT1, and others), while other portions of the BRCA1 molecule make contact with components of the basal transcription machinery (RNA polymerase II holoenzyme) (e.g., RNA helicase A) and/or with components of chromatin remodeling complexes (e.g., BRG1, a component of the SWI/SNF complex) (reviewed in Rosen et al. 2006). Here, BRCA1 functions as a transcriptional co-regulator that may either stimulate (e.g., p53, androgen receptor, OCT1) or inhibit (e.g., estrogen receptor, progesterone receptor, c-Myc) the transcriptional activity. Thus, some BRCA1 functions are linked to the regulation of transcription, although which of these contribute to tumor suppression remains unclear to date. Interestingly, the ability of BRCA1 to inhibit estrogen receptor and progesterone receptor could, in part, explain why *BRCA1* mutation carriers have a specific predilection to develop breast cancer, which is not explained by its generic function in the DNA damage response.

Mice with a harboring a homozygous *Brca1*-null mutation died by embryonic day 7.5–8.5 due to widespread proliferative defect associated with overexpression of the cell cycle inhibitor protein p21 (Hakem et al. 1997; Ludwig et al. 1997). It was suggested that the proliferative defect (associated with senescence and apoptosis) was due to activation of p53 due to accumulation of chromosomal abnormalities caused by the absence of functional Brca1. Mice homozygous for a *Brca2* mutation died of a similar proliferative failure 1 day later (i.e., embryonic day 8.5–9.5). These findings suggest that even though some Brca1 and Brca2 functions may overlap (i.e.,

they may function in the same molecular pathway(s)), *Brca2* cannot substitute for *Brca1* nor can *Brca1* substitute for *Brca2* (see below). Consistent with the above ideas, deletion of p53 or its transcriptional target p21 each extended the lifespan of Brca1-null embryos for several days, although none survived to birth. And consistent with the idea that Brca1-deficient cells accumulate chromosomal abnormalities, cultured *Brca1*-deficient mouse embryonic fibroblasts, mammary cancers from mice with a mammary-targeted homozygous deletion of *Brca1* exon 11 (the largest exon of *Brca1*), and human *BRCA1* mutant breast cancers showed a consistent pattern of aneuploidy, centrosomal abnormalities, and a large number of chromosomal aberrations (reviewed in Rosen et al. 2005).

BRCA2

The breast cancer susceptibility gene *BRCA2* was identified by positional cloning based on its linkage to breast-ovarian cancer families in the mid-1990s (Wooster et al. 1995; Tavtigian et al. 1996). The *BRCA2* gene is located on human chromosome 13q13 (mouse chromosome 5), consists of 27 exons (like *BRCA1*, the largest of which is exon 11), and codes for a 3418-amino acid nuclear protein with an apparent molecular mass of about 350 kDa. Like BRCA1, BRCA2 is widely expressed in fetal and adult tissues, and its expression appears to be regulated coordinately with BRCA1 during mammary epithelial cell growth and differentiation, suggesting that BRCA1 and BRCA2 may function in overlapping pathways in the breast (Rajan et al. 1996). BRCA1 and BRCA2 expression also appear to be similarly regulated in various mouse tissues. However, there is little or no structural similarity between BRCA1 and BRCA2, aside from the fact that both proteins have an unusually large number of charged amino acids, with about 25% of the residues in BRCA2 being either basic or acidic. Like BRCA1, BRCA2 follows the classical Knudson two-hit model for a tumor suppressor gene in which the germ-line inheritance pattern is autosomal dominant but the tumors almost invariably exhibit deletion of the wild-type allele. Also like *BRCA1*, *BRCA2* mutations are rarely found in sporadic breast or ovarian cancers, consistent with a two-hit model for *BRCA2*-related oncogenesis. And like BRCA1, its main function appears to be as a caretaker in the maintenance of genomic integrity due in part to its role in the repair of DSBs by HDR (see below).

Like BRCA1, BRCA2-deficient cancers show aneuploidy and a pattern of chromosomal aberrations, suggesting that BRCA2 functions to maintain chromosomal stability. And similar to BRCA1, BRCA2-deficient cells are unusually sensitive to ionizing radiation. A clue to BRCA2 function was the finding that BRCA2 in complex with DSS1 (deleted in split hand-split foot 1) bound to single-stranded DNA through a helix-turn-helix (HTH) domain. In the same study, it was also shown that the HTH domain could also bind to double-stranded DNA and that BRCA2 stimulates RAD51-mediated recombination in vitro (Yang et al. 2002). BRCA2 contains eight BRC domains, motifs that are similar to the oligomerization motif present in RAD51. These findings suggested that BRCA2 could stimulate the formation of RAD51 filaments on single-stranded DNA, an observation that was verified experimentally (Pellegrini et al. 2002). And they suggested a role for

BRCA1 (like BRCA1) in homology-directed DNA repair of DSBs and explained previous findings that *BRCA2*-deficient cells are very sensitive to ionizing radiation. Consistent with these observations, BRCA2 levels increase during S-phase and BRCA2 translocates to sites of repair DNA synthesis in response to DNA damage (see above).

Finally, BRCA2 was shown to be required for HDR (as is BRCA1) but is not required for NHEJ (Xia et al. 2001). Although BRCA1 and BRCA2 each function in HDR, their roles in this pathway are different, and one cannot substitute for the other. Thus BRCA1 is involved in the regulation of resection of the broken ends of DNA (i.e., regulation of the nucleolytic conversion of double-strand to 3′ single-strand DNA) and is also necessary for the DNA damage-responsive S-phase and G2/M checkpoints. BRCA2, on the other hand, is involved in the assembly of RAD51 filaments on the single-strand DNA and their replacement of RPA (replication protein A), and BRCA2 participates in stabilizing RAD51 filaments by inhibiting ATP hydrolysis (Jensen et al. 2010). BRCA2 does not appear to participate in DNA damage-responsive checkpoints nor does it participate in the initial choice of the repair pathway (HDR vs. NHEJ), as does BRCA1.

In addition to DSBs, BRCA2 also participates in the repair of interstrand DNA cross-links (ICLs), one stage of which involves the formation of DSBs. Fanconi anemia (FA) is a syndrome characterized by bone marrow failure, short stature and other congenital abnormalities, a high incidence of cancer (leukemia and solid tumors), and chromosomal instability. FA cells are very sensitive to agents that cause ICLs. BRCA2 is identical to FANCD1 (FA complementation group D1), a component of the FA complex of proteins. The FA clinical syndrome requires biallelic mutations of BRCA2/FANCD1 (Howlett et al. 2002; Alter et al. 2007). The mutations were located in a highly conserved region of the BRCA2 protein, and the clinical syndrome appeared to be particularly severe, occurring at an early age of onset.

BRCA2, like BRCA1, also participates in the regulation of transcription, but BRCA2 has been studied to a much smaller extent than BRCA1 in this regard. Thus, a conserved region within the N-terminus of BRCA2 shows homology to the activation domain of the transcription factor JUN. Shin and Verma (2003) showed that wild-type BRCA2, but not a tumor-related mutant BRCA2, synergized with the nuclear receptor coactivator GRIP1 to increase transcriptional activation by the androgen receptor (AR). BRCA2 associated with both AR and GRIP1 and cooperated with the histone acetyltransferase P/CAF and BRCA1 to increase AR-dependent transactivation. The authors concluded that BRCA2 may function, in part, to regulate AR signaling, which has been implicated in male breast cancer (see below).

Target Assessment

Assessment of BRCA1 and BRCA2 is achieved through genetic testing: PCR-direct sequencing, large genetic rearrangement (LGR) testing (BRACAnalysis Rearrangement Test or “BART™”), and next-generation sequencing (NGS). The

largest BRCA1/BRCA2 testing laboratory in the United States is the Myriad Genetics Laboratory. Cancer-associated mutations of the BRCA genes may be exonic or intronic or may occur within the regulatory region of the gene. A small percentage of such mutations occur in the form of LGRs. Hence, LGR testing is recommended along with standard sequencing analysis in some candidates whose history meets certain criteria.

Role of the Target in Cancer

Rank: 9

The *BRCA1* and *BRCA2* genes follow the classical Knudson model of tumor suppressor genes. In this model, a woman is born with a single germ-line mutant *BRCA* allele (first "hit"), and the other (wild-type) allele is lost or mutated within the tumor (second "hit"). Thus the inheritance pattern is autosomal dominant with a high penetrance, but both *BRCA* alleles must be inactivated for a tumor to develop (i.e., a recessive molecular pattern). Mutations of *BRCA1* and *BRCA2* lead predominantly to breast and ovarian cancers, but see below for other tumor types linked to *BRCA* mutations. The *BRCA* genes are classified as caretaker genes that play a role in the maintenance of genomic integrity. Thus *BRCA* mutant cells show higher levels of chromosomal abnormalities (e.g., aneuploidy, centrosome amplification, and chromosome rearrangements) than the corresponding cell types with wild-type *BRCA* genes.

High-Level Overview

Diagnostic, Prognostic, Predictive

BRCA1

Most high-risk *BRCA1* mutations are insertions (e.g., 5682insC) or deletions (e.g., 185delAG) that affect the open reading frame and result in protein truncation, although point mutations within the RING domain or the C-terminal transcriptional activation domain that disrupt BRCA1 function are known. However, there are a number of women who undergo BRCA1 testing, the results of which are variants of unknown clinical significance (VUS). These are mostly missense mutations that result in amino acid substitutions, although there are also intronic mutations and in-frame insertions and deletions. There is no certain method of establishing the significance of these VUS, but determining the effect of the variant on BRCA1 protein function and evaluating sequence conservation across species are proposed methods of predicting significance (Millot et al. 2012).

About 5–10% of human breast cancers are hereditary in origin, while 90–95% are sporadic. Of the hereditary breast cancer cases, 50–80% are accounted for by *BRCA1* or *BRCA2* mutations, while 20–50% are non-*BRCA1*/*BRCA2*. Women who carry high-risk *BRCA1* mutant alleles have a 50–85% chance of developing breast cancer

by age 70 (Rosen et al. 2005). An overview of recent series of patients suggests an average of about a 60% risk of breast cancer by age 70 and a 60% risk of ovarian cancer by age 75 in BRCA1 mutation carriers (Foulkes 2014). Differences in cancer risk estimates appear to be due to the extent of family history, with a strong family history (e.g., four or more affected members, bilateral breast cancer, family history of both breast and ovarian cancers, and early onset of breast cancer (before age 40)) conferring a higher risk of breast cancer development. So far, no common high penetrance gene (a "*BRCA3*") has been identified to explain the high fraction of hereditary breast cancers (20–50%) that have neither *BRCA1* nor *BRCA2* mutations. However, a low percentage of hereditary breast cancers can be explained by rare mutations in a set of genes including: *p53* (Li-Fraumeni syndrome), *PTEN* (Cowden syndrome), androgen receptor, *LKB1/STK11* (Peutz-Jeghers syndrome), *CHEK2*, *CHD1*, *PALB2*, *BRIP1*, *ATM*, and others (Larsen et al. 2014). It should be noted that BRCA1 may play a role in some sporadic (non-hereditary) breast cancers since 30–40% of sporadic cancers exhibit absent or greatly reduced levels of BRCA1 protein (Wilson et al. 1999). Some of these tumors exhibit low expression of BRCA1 due to epigenetic silencing (i.e., methylation of the *BRCA1* gene promoter).

Gayther et al. (1995) noted a genotype-phenotype correlation for germ-line *BRCA1* mutations in breast and ovarian carcinoma families. Specifically, the location of the mutation within the *BRCA1* gene appeared to be correlated with the ratio of breast/ovarian cancers in the family. Thus, mutations occurring within the 3′ region of the gene had a lower proportion of ovarian cancer than mutations within the 5′ region of the gene, which resulted in a mixture of breast and ovarian cancer. The crossover point corresponded to about amino acid 1440 in the BRCA1 protein. These findings are consistent with a model in which there is a domain(s) that mediates ovarian cancer suppression within the C-terminal portion of the protein but not at the extreme C-terminus, while the entire BRCA1 protein is required to suppress breast cancer development. However, this idea does not explain the finding that 5′ mutations within the regulatory region of the *BRCA1* gene (15% of the *BRCA1* mutations studied), which usually resulted in the absence of BRCA1 expression, yielded only breast cancer.

There have been a number of studies, with confusing and conflicting results, to determine the incidence of other cancer types than breast and ovarian cancers that occur in *BRCA1* mutation carriers. In one large cohort study including 699 BRCA1 families, several additional cancer types were identified (Thompson et al. 2002). Thus, *BRCA1* mutation carriers were found to have a higher risk for development of pancreatic cancer, cervical cancer, cancer of the uterine body, and prostate cancer in male mutation carriers less than 65 years of age. Thus, in women with *BRCA1* mutations, there may be a slightly increased incidence of other gynecologic cancers in addition to that of the breast and ovary. Interestingly, breast, cervical, and uterine cancers are all estrogen-driven cancer types at some time during their development.

Breast cancers that occur in *BRCA1* mutation carriers differ in pathological and immunophenotypic characteristics from sporadic breast cancers (Larsen et al. 2014). For example, a higher proportion of *BRCA1* tumors than sporadic cancers are medullary carcinomas. These are high-grade poorly differentiated tumors with extensive

lymphocytic infiltration, a low incidence of lymph node involvement, and a generally good prognosis. Most *BRCA1* cancers exhibit the "triple-negative" phenotype (estrogen receptor negative, progesterone receptor negative, and HER2 negative), and these often exhibit a high nuclear grade, whereas a minority (15% or so) of sporadic cancers are triple negative. A very high proportion of *BRCA1* cancers (>80%) exhibit p53 mutations, as compared to about 20–25% of sporadic cancers. The latter finding suggests that mutation of p53 is an obligate component of the molecular pathogenesis of *BRCA1*-related cancers, possibly because wild-type p53, if present, would tend to push cells with chromosomal aberrations into senescence or apoptosis.

BRCA2

In four recent series, the risk of breast cancer in *BRCA2* mutation carriers varied from 28% to 55%. The low estimate of 28% occurred in a series of Ashkenazi Jews, many of whom have the founder mutation 6174delT, which has a relatively low penetrance. The penetrance of *BRCA2* for non-Ashkenazi Jews is 50%, which is closer to that for *BRCA1* (60%) (Foulkes 2014). The risk of ovarian cancer for BRCA2 mutation carriers (*ca*. 20%) is significantly lower than that for *BRCA1* carriers (60%). Gayther and colleagues (1997) noted a genotype-phenotype correlation for mutations of *BRCA2* in breast-ovarian cancer families, just as they had noted earlier for *BRCA1* (see above). As for *BRCA1*, most *BRCA2* mutations were of the frameshift type, leading to premature protein truncation. And as for *BRCA1*, Gayther et al. (1997) noted a significant genotype-phenotype correlation, whereby mutations with the highest risk of ovarian cancer relative to breast cancer occurred within a region of approximately 3.3 kb in exon 11 (the largest exon on *BRCA2*), the so-called ovarian cancer cluster region (OCCR).

BRCA2 mutation carriers also appear to be at somewhat of an increased risk for cancer types other than breast and ovary, as are *BRCA1* mutation carriers. These include cancers of the pancreas and prostate, the latter usually occurring in men under age 65 (see Foulkes 2014). There is anecdotal evidence to suggest that *BRCA2* carriers with pancreas carcinoma may be particularly sensitive to DNA-damaging agents, including *cis*-platinum (a DNA cross-linking agent) and camptothecin (a DNA topoisomerase I inhibitor). BRCA2 carriers may also be at increased risk for other gastrointestinal cancers and for melanoma, but the data are not as solid as they are for cancers of the pancreas and prostate. Interestingly, while *BRCA1* carriers are at increased risk for female breast cancer predominantly, *BRCA2* carriers are at increased risk for both male and female breast cancers.

We had previously described the immunophenotype of *BRCA1*-related breast cancers, which differs from that of sporadic breast cancers. The immunophenotype of *BRCA2*-related breast cancers differs from that of *BRCA1*-related breast cancer. Thus, while most *BRCA2* cancers are invasive ductal cancers (IDCs), there is an excess of invasive lobular cancers and tubular carcinomas (see Larsen et al. 2014). *BRCA2* cancers are more frequently grade 2/3 than *BRCA1* cancers, which are often high grade (grade 3/3). With regard to immunochemical markers, *BRCA2* cancers are more similar to sporadic cancers than are *BRCA1* cancers. For example, 78% of *BRCA2* cancers are ER positive consistent with 70% of sporadic cancers being ER

positive. The percentage of triple-negative *BRCA2* tumors (16%) is similar to that for sporadic cancers (15–20%) and less than that of *BRCA1* tumors (>50%). However, both *BRCA1* and *BRCA2* tumors exhibit a lower proportion of *HER2* amplification than do sporadic tumors. Finally, with regard to targeted therapy, we have mentioned the synthetic lethal combination of PARP inhibitors and *BRCA1* or *BRCA2* mutant tumors which is showing promise as monotherapy for advanced breast and ovarian cancers and is under investigation in the adjuvant setting (Farmer et al. 2005; reviewed in Rosen and Pishvaian 2014). The same rationale exists for studying the use of platinum salts for *BRCA2* mutant cancers as exists for *BRCA1* mutant cancers.

Therapeutics

Tamoxifen use is associated with a reduction in the risk of contralateral breast cancer risk for *BRCA1* and *BRCA2* mutation carriers. Options for primary prevention of cancer in BRCA1 and *BRCA2* mutation carriers include bilateral mastectomy, bilateral salpingo-oophorectomy, and tamoxifen. However, it remains to be validated in large studies that tamoxifen can prevent *BRCA* mutation-related breast cancer.

An advance in the development of targeted therapy for *BRCA1* and *BRCA2* mutant cancers was the finding that these mutant cancers are exquisitely sensitive to poly(ADP-ribose)polymerase (PARP) inhibitors (Farmer et al. 2005; reviewed in Rosen and Pishvaian 2014). This observation is an example of "synthetic lethality," a concept in which two gene mutations (*BRCA* and *PARP* in this case) combine to cause cell death, whereas neither mutation alone is lethal. The enzyme PARP is a component of the base excision repair pathway and accumulates at sites of single-strand DNA breaks (SSBs). During DNA replication, unrepaired SSBs can become DSBs due to collapsed replication forks. Such DSBs would normally be repaired by HDR, which is defective in *BRCA1* and *BRCA2* mutant cells. These results gave rise to the idea to use PARP inhibitors to treat *BRCA1/2* mutant cancers. Early clinical trials showed high objective response rates (≥40%, including some CRs (complete remissions)) in patients with advanced cancers. PARP inhibitors are now being studied as adjuvant treatments along with conventional chemotherapy. Finally, although it cannot be considered "targeted" therapy per se, *BRCA* mutant tumor cells appear to be very sensitive to platinum salts, which are DNA cross-linking agents. These agents cause the formation of DSBs and so they may synergize with mutations of genes involved in repair of DSBs. In fact, patients with *BRCA* mutant tumors have shown high response rates to platinum salts.

Preclinical Summary

BRCA1 is a tumor suppressor gene for breast and ovarian cancer located on human chromosome 17q21. *BRCA2* is a tumor suppressor gene located on human chromosome 13q13. These genes encode large-sized proteins of 1863 and 3418 amino acids, respectively. The main function of these genes is in the repair of DNA double-strand

breaks by homology-directed repair (also called homologous recombination). These genes exhibit different functions within HDR, with BRCA1 regulating the 5′ end resection at the DSB and BRCA2 regulating RAD51-mediated DNA recombination. Both genes are required for the DNA damage-activated S-phase and G2 cell cycle checkpoints. Both are caretaker genes involved in the maintenance of chromosomal stability. Both BRCA1 and BRCA2 are also transcriptional co-regulators. They are no sequence-specific DNA-binding transcription factors, but they bind to other transcription factors and positively or negatively regulate their activity. In addition, BRCA1 also functions as an E3 ubiquitin ligase, and BRCA2 participates in the Fanconi anemia DNA repair pathway. Finally, both Brca1 and Brca2 are required for early embryonic cell proliferation in mice, but they each exhibit some nonoverlapping functions in this regard, since Brca1 cannot replace Brca2 and Brca2 cannot replace Brca1 in mice harboring homozygous deletions of one or the other gene.

Clinical Summary

BRCA1 mutations confer about a 60% risk of breast cancer and a 60% risk of ovarian cancer, while *BRCA2* mutations confer about a 50% and a 20% risk of breast and ovarian cancers, respectively. *BRCA* mutations are diagnosed by genetic testing. Other tumor types associated with *BRCA* mutations are BRCA1 (cervical cancer, endometrial cancer, and pancreatic cancer) and BRCA2 (prostate and pancreatic cancers). Most *BRCA* mutations are of the insertion/deletion type resulting in protein truncation. Ashkenazi Jews (from Eastern Europe) exhibit several common founder mutations of the *BRCA* genes including 185delAG (*BRCA1*, 1% incidence), 5382insC (*BRCA1*, 0.3% incidence), and 6174 delT (*BRCA2*, 1% incidence). *BRCA1*- and *BRCA2*-related breast cancers exhibit higher levels of chromosomal abnormalities (e.g., aneuploidy, centrosome amplification, and chromosomal rearrangements) than sporadic breast cancers. BRCA1-related breast cancers exhibit a characteristic pathology and immunophenotype, with most tumors having p53 mutations and being of high grade and of the triple-negative variety (i.e., negative for estrogen receptor, progesterone receptor, and HER2 amplification). On the other hand, the immunophenotype of BRCA2-related breast cancers is similar to that of sporadic breast cancers. Advanced *BRCA* mutant breast cancers appear to be very sensitive to platinum salts and to targeted therapy using poly(ADP-ribose) polymerase (PARP) inhibitors.

Anticipated High-Impact Results

1. Large-scale primary prevention studies of BRCA mutation carriers using tamoxifen
2. Optimization of use of PARP inhibitors and cytotoxic chemotherapy for usage in patients with *BRCA*-related breast and ovarian cancers or cancers with other types of defects in homology-directed repair

3. Efficacy of PARP inhibitors in combination with chemotherapy as adjuvant treatment for earlier-stage *BRCA*-related breast cancers

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Cell Cycle-Related Kinases

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Abstract

Cyclin proteins are regulatory subunits of a holoenzyme, which together with CDKs, phosphorylate key substrates to modulate cellular physiology and drive cell cycle progression. At least 13 human CDKs have been identified that have different functions that contribute to the appropriate coordination of cell cycle transitions, as well as other processes (e.g. transcription). For example, cyclin D1 and assembled CDK complexes phosphorylate and inactivate the retinoblastoma protein and thereby promote DNA synthesis (Arnold and Papanikolaou J Clin Oncol 23(18):4215–24, 2005). Cyclin D1 kinases also phosphorylate and inactivate the NRF1 transcription factor, thereby inhibiting mitochondrial biogenesis

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Physiological stresses and mitogenic signaling pathways converge on the activity of cyclin CDK complexes to control cell cycle transitions. For example, multiple cyclin CDK complexes are targets of cell cycle checkpoints controlling G_1/S and G_2/M transitions, thereby protecting the cell from erroneous DNA replication, ensuring the integrity and precision of cellular division (Pestell. Am J Pathol 183(1):3–9, 2013). Conversely, mitogen-mediated passage through the restriction point in G_1 is controlled by the cyclin families, the cyclin D family (D1, D2, and D3), and the cyclin E family (E1 and E2). Due to the central role of CDKs in controlling proliferation, they represent important targets for cancer therapy.

Keywords
Cyclin D1 • Cyclin proteins • Cyclin-dependent kinases (CDKs)

Biology of the Target

The cyclin D1 gene encodes a rate limiting step in the G_1/S phase of the cell cycle and is required for oncogenesis in the mouse (Fu et al. 2004). Inactivation of cyclin D1 abundance, historically first through antisense and subsequently through gene deletion in mice or siRNA, demonstrated the requirement for cyclin D1 in tumorigenesis (Lee et al. 2000; Yu et al. 2001). MMTV-ErbB2-induced mammary tumorigenesis requires cyclin D1 (Lee et al. 2000; Yu et al. 2001), as does APC-induced gastrointestinal tumorigenesis (Hulit et al. 2004). The cyclin D1 gene is induced by mitogenic and oncogenic signals including activating mutations of RAS (Albanese et al. 1995), Notch (Stahl et al. 2006), Stat 3 (Bromberg et al. 1999), ErbB2 (Lee et al. 2000), and Src (Lee et al. 1999). The cyclin D1 gene is overexpressed in a variety of human malignancies, including breast cancer, lung cancer, and GI malignancies. In addition to encoding the holoenzyme regulatory subunit, cyclin D1 functions to regulate a variety of transcription factors and thereby gene expression (Fu et al. 2004). Cyclin D1 functions in the context of local chromatin to regulate gene expression by targeting a subset of gene promoters (Reutens et al. 2001; McMahon et al. 1999).

Target Assessment

Cyclin D1 protein abundance can be measured by immunohistochemical staining in tumors of patient samples, or by Western blot, and mRNA gene expression can be assessed by Northern bot, QT-PCR, and microarray. Amplification or translocation of the cyclin D1 gene can be measured by fluorescence in situ hybridization. A serum measurement is currently not available.

Role of the Target in Cancer

Rank: 9

Expression of cyclin D1 is increased in a variety of malignancies including breast cancer, lung cancer, and mantle cell lymphoma (Arnold and Papanikolaou 2005). In many tumors, commensurate with the induction of cyclin D1 abundance, Cdk activity is increased, providing the rationale for Cdk inhibitors. In some tumors, however, cyclin D1 is amplified or overexpressed without a correlative increase in proliferative indices. Such clinical findings have given rise to consideration of Cdk-independent functions of cyclin D1 (Pestell 2013) that may promote tumorigenesis, including the induction of chromosomal instability (Casimiro et al. 2012, 2015a, b). A variety of cyclin-dependent kinase inhibitors have been developed and are in clinical trials.

High Level Overview

Over the last two decades, there is substantial corroborating evidence that cyclin D1 overexpression drives many types of human solid tumors and lymphomas (Fu et al. 2004). The identification of cyclin D1 misexpression is found in half of breast cancers, and the pattern of its expression in disease progression is indicative of an early involvement in breast cancer. One area of controversy had been in prostate cancer. Early experiments evidenced a growth-promoting function of cyclin D1 in prostate (Chen et al. 1998). Surprisingly a subsequent publication suggested cyclin D1 overexpression in tissue culture reduced cell proliferation. Subsequent studies demonstrated the pro-proliferative and growth functions of cyclin D1 in the prostate in vitro and in vivo. Evidence suggests that cyclin D1 is also a key determinant in prostate cancer progression based on murine models (Ding et al. 2011; Casimiro et al. 2007; Ju et al. 2014). Cyclin D1–regulated gene expression predicts risk of biochemical recurrence (BCR) in human prostate cancer samples. A gene expression signature of cyclin D1 activity indicated that the majority of genes induced by cyclin D1 are more expressed in the high-risk prostate cancer cohort (Ju et al. 2014). Cyclin D1–repressed genes also correlated with poor outcome by BCR. A similar analysis evaluated the association of genes regulated by cyclin D1 and AR signaling. Genes up- or down-regulated by cyclin D1 or DHT were identified and evaluated for association with BCR as described above. A majority of the genes induced by cyclin D1 are also repressed by DHT and visa versa for repressed by cyclin D1 and DHT induced, which is also associated with risk of BCR. Cyclin D1 gene deletion reduced prostate cellular proliferation in vivo and cyclin D1 inactivation reduced prostate cancer cell line growth in vitro and in vivo (Chen et al. 1998; Ju et al. 2014). Collectively, these findings suggest that cyclin D1 drives cellular proliferation in the prostate and that a signature of genes regulated by endogenous cyclin D1 correlates with poor patient prognosis, which provides a rational basis for using CDK inhibitors in prostate cancer.

Preclinical. AT7519, a multiple Cdk inhibitor, has shown in preclinical studies antiproliferative effects in solid and hematological malignancies (Squires

et al. 2009). SHC727965 (dinaciclib) is a second-generation cdk inhibitor, which has shown activity in preclinical studies of pancreatic cancer, osteosarcoma, melanoma, and leukemia cells. Novartis has not released any clinical data on a number of phase I, II, and III trials for ribociclib (LEE011) (described below); however, they have released early preclinical data showing LEE011 is active in neuroblastoma cell lines and xenograft models (Rader et al. 2013). Similarly, Eli Lily have developed a CDK4/6-specific inhibitor, abemaciclib (LY2853219). It shows potent cell cycle arrest and inhibits tumor growth in vivo xenograph models (Gelbert et al. 2014). Abemaciclib has entered into clinical trial (see below).

Clinical Therapeutics

The cyclin-dependent kinases have been targeted by a number of pharmaceutical strategies and are reviewed extensively elsewhere (Sridhar et al. 2006; Knights and Pestell 2005; Lapenna and Giordano 2009; Asghar et al. 2015). The first generation of CDK inhibitors included flavopiridol. The broad range Cdk inhibitors mainly block the ATP-binding pocket. Flavopiridol is a semisynthetic flavonoid with a broad spectrum of inhibitory activity against CDKs (CDK1, CDK2, CDK4, CDK6, CDK7, and CDK9). The dose schedule of flavopiridol has been optimized and was evaluated in a number of clinical trials. Flavopiridol used as a single agent has effectively induced cytoreduction in refractory and relapsed acute leukemias; however, clinical responses were transient (Blum et al. 2010). Flavopiridol elicited a number of dose-limiting toxicities that are likely independent of the effect on cell cycle control and are currently believed to be due to the inhibition of transcriptional processes dependent on CDK7/9 (Newcomb 2004).

Extensive effort has subsequently been placed on developing specific CDK-inhibitory compounds that have improved pharmacodynamic and clinical properties relative to flavopiridol. In particular the CDK inhibitors roscovitine (seliciclib-CYC202) has been extensively studied in preclinical and clinical trials (Lapenna and Giordano 2009; Senderowicz 2003). Like flavopiridol this drug targets multiple CDK complexes (CDK1, CDK2, CDK5, CDK7, and CDK9). In preclinical studies, treatment with roscovitine induces cell cycle arrest or cell death and has been shown to cooperate with specific chemotherapy regimens (Appleyard et al. 2009; Abaza et al. 2008; Coley et al. 2007). In a recently published phase 1 clinical trial, the dose-limiting toxicity and maximum tolerated dose were defined (Le Tourneau et al. 2010). Minimal responses were observed in this trial of advanced disease. Currently, roscovitine is in phase 2 clinical trials for nonsmall cell lung cancer and nasopharyngeal cancer and hematological tumors.

The second-generation cdk inhibitor SHC727965 (dinaciclib) is being assessed in a phase III study (NCT01580228), evaluating its efficacy in refractory CLL.

In parallel with the clinical development of broad range CDK inhibitors, there has been extensive effort to define highly specific CDK inhibitors. Among those that would target cyclin D1-associated kinase activity, the most established is palbociclib (Pfizer-PD0332991). Numerous preclinical studies have demonstrated that this agent

is highly specific and induces a pure cytostatic response (Rivadeneira et al. 2010; Fry et al. 2004; Dean et al. 2010). This response is directly associated with suppression of CDK4/6 activity, and there are no indications of off-target activity. Phase I clinical trials are complete, demonstrating modest dose-limiting toxicity, mainly neutropenia. From the phase I trial, there was effective tumor stasis reported in malignant teratoma over a period of approximately 2 years, suggesting that for specific diseases CDK4/6 inhibition represents a key means to delay disease progression (Vaughn et al. 2009). Interestingly, palbociclib has efficacy in combination therapies (Michaud et al. 2010), although the mechanism of cooperativity remains poorly understood. A randomized combination phase 2 clinical trial of palbociclib together with an aromatase inhibitor, letrozole (PALOMA-1 trial), demonstrated a doubling of patient progression-free survival (PFS) (20 months) compared to letrozole alone (10 months) (Finn et al. 2015). Due to these findings, the FDA accelerated approval for palbociclib (IMBRANCE®) on February of this year, to be given, along with letrozole, to ER+ Her^{-} postmenopausal patients as a first-line treatment option. Recently, the results of a phase 3 (PALOMA-3) multicenter trial involving 140 global sites and 521 patients comparing palbociclib plus fulvestrant compared to fulvestrant alone in postmenopausal women with HR^{+}, Her^{-} metastatic breast cancer whose disease had progressed during or after endocrine therapy 26030518. The compelling results demonstrate that palbociclib plus fulvestrant more than doubled PFS (median PFS, 9.2 vs. 3.8 months) (Turner et al. 2015). Just as recent, similar agents from Novartis (ribociclib-LEE011) and Eli lily (abemaciclib-LY2853219) are in clinical development (Asghar et al. 2015). For ribociclib, multiple phase I, II, and III studies are underway for early and advanced/metastatic breast cancer and solid tumors (norvatisoncology.com), for example, a phase Ib/II study of ribociclib in patients with advanced ER+ breast cancer, a phase Ib/II study of ribociclib in postmenopausal women with HER2^{-} locally recurrent or advanced metastatic breast cancer, a phase Ib/II study of ribociclib in postmenopausal women with ER^{+}, HER2^{-} locally advanced or metastatic breast cancer, a phase III study of ribociclib combined with letrozole for postmenopausal women with HER2^{-} advanced breast cancer who have not received therapy (obtained from Clinicaltrials.gov). For abemaciclib multiple phase I, II, and III trials are underway for advanced/metastatic breast cancer, nonsmall cell lung cancer, and hematologic cancers (lillyoncologypipeline.com). The newest CDK4/6 inhibitors to enter early phase trials are produced by G1 Therapeutics Inc. and called G1T28 and G1T38. G1T28 has been tested in phase 1, healthy individuals for pharmokinetics and safety studies (https://ClinicalTrials.gov/show/NCT02243150 and Roberts et al., Phase 1A trial to evaluate safety and biologic activity in the bone marrow of G1T28, a CDK4/6 inhibitor; submitted for publication), and two phase 1b/1a studies have been started in small cell lung cancer as a potential agent to reduce chemotherapy-induced myelosuppression (https://ClinicalTrials.gov/show/NCT02499770; https://ClinicalTrials.gov/show/NCT02514447; Bisi et al. 2016). G1T38 has been found to inhibit the growth of clinically relevant models of advanced castrate-resistant prostate cancer (Stice et al. 2015). The outcome of these studies will be of great interest in determining the utility of targeting cyclin D1-associated kinase activity in the clinic.

Anticipated High Impact Results

Deregulation of the cyclin-dependent kinases (CDKs) results in uncontrolled cellular proliferation, genomic instability, and chromosomal instability that contribute to tumor development and pathogenesis (Kastan 2005; Kops et al. 2005). Inhibition of CDK activity does represent a key approach to limit tumorigenic proliferation and disease progression. Importantly, given the substantial cross talk between CDK activity and the response to chromosome damage, there are important opportunities for combining CDK-inhibitory compounds with conventional chemotherapy. Additionally molecular therapies targeting cell proliferation, genomic instability, and chromosomal instability represent complementary therapeutic opportunities that could represent important combinatorial targets (Malumbres and Barbacid 2009).

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ER

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Elizabeth E. Sweeney and V. Craig Jordan

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Abstract

The estrogen receptor (ER) is the most valuable target in cancer therapeutics. The ER signal transduction pathway controls breast and endometrial tumor cell replication. Members of the group of medicines called Selective ER Modulators such as tamoxifen or raloxifene are antiestrogenic in the breast tumor but can build bone in postmenopausal women. Tamoxifen is used ubiquitously to treat all stages of breast cancer and to prevent breast cancer in high-risk women. Raloxifene is used to prevent breast cancer in high-risk postmenopausal women as to treat osteoporosis and prevent breast cancer at the same time.

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Keywords

Antihormone therapy • Estrogen receptor (ER) • Assessment • Decision network • ERα • ERβ • Hormone receptor • Long-term antihormone therapy • Luminal A and B intrinsic subtypes • Nuclear receptor coregulator recruitment • Oophorectomy • Paradox • Prognosis • Raloxifene • Role in breast cancer • SERMs • Therapeutics • Selective serotonin reuptake inhibitors (SSRIs) • Tamoxifen

Target

Two forms of the estrogen receptor (ER) exist, ERα and ERβ; they are encoded by different genes, on chromosomes 6 and 14, respectively, and have different primary structures (Deroo and Korach 2006; Couse and Korach 1999). Seventy percent of breast tumors are ERα-positive, making the ER an effective although imperfect target for breast cancer therapy. The ERα target is correlated with breast tumor responsiveness to antihormone therapy. ERβ has no defined role in breast cancer; however, ERβ could modulate physiological responses around the body and would be considered a target for selective estrogen receptor modulators (SERMs).

Biology of the Target

The ER was discovered through the injection of [^{3}H] estradiol into immature rats, followed by analysis of radioactivity in specific tissues. It was found that the uterus and vagina, estrogen target tissues, bound and retained the [^{3}H] estradiol, while organs such as the kidney and liver, estrogen nontarget tissues, washed out the radioactive marker (Jensen and Jordan 2003). This suggested that a receptor may be present in the target tissues, allowing the ligand to induce estrogen-associated cellular function and activity. X-ray crystallography was subsequently used to visualize ligand binding to the purified ER ligand-binding domain (Jensen and Jordan 2003). These findings sparked further research on the receptor function and its role in normal biology and disease.

Belonging to the nuclear receptor superfamily, the ER resides in the cytoplasm and nucleus to bind and retain estrogens that have diffused through the cell from the bloodstream (Jordan 2009). It comprises five regions: the activating region, DNA-binding domain, hinge domain, ligand-binding domain, and the C-terminus region (Fig. 1). Within ER target tissues, such as the breast, uterus, vagina, and bone, the inactive ER is bound to heat shock proteins that dissociate once the ligand binds, allowing an active conformational change to occur, depending on the nature of the ligand (Couse and Korach 1999). A planar steroidal estrogen or estrogenic molecule permits the “jaws” (helix 12) of the receptor to close, while antagonistic or antiestrogenic molecules bind the receptor but prevent sealing of the ligand within the receptor.

Upon ligand binding, the receptor-ligand complex binds to estrogen response elements (EREs) in the promoter region of estrogen-responsive genes. Coregulators

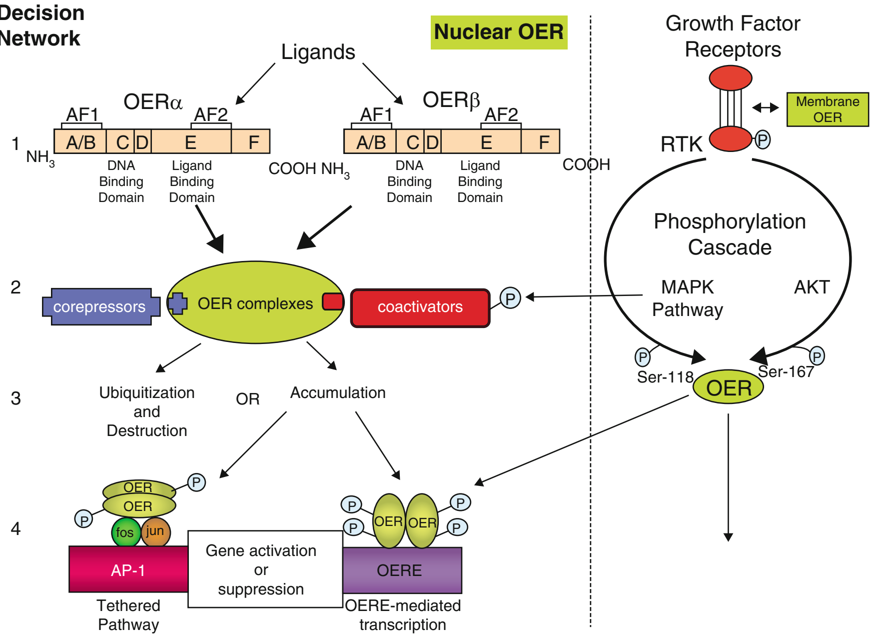

Fig. 1 The potential decision network in estrogen target tissues that program a ligand-receptor complex to activate estrogenic or antiestrogenic responses. There are two distinct estrogen receptors (*ERs*) (α and β) that are differentially distributed throughout the body. The shape of the ligand can change the shape of the receptor complex. This in turn preprograms the complex to bind either a coactivator or corepressor protein to enhance the intrinsic activity of the complex for estrogenic responses or reduces intrinsic activity for antiestrogenic responses, respectively. The final decision point is to activate or suppress genes directly at DNA estrogen response elements (EREs) or tether the AP-1 sites to increase gene transcription. Overall, a tissue can modify the decision network through cell surface receptor tyrosine kinases (RTKs) enhancing the phosphorylation cascade. This in turn can increase phosphorylation of coactivators and/or the ER. The balance of decision outcomes modulates the response of a particular tissue (Reprinted from V. Craig Jordan (2006), with permission from John Wiley and Sons)

are recruited to the ER complex, depending on its conformation, to modulate gene activation. Tissues possess unique levels of the 258 known nuclear receptor coregulators (Jordan and O'Malley 2007). Ligands of various shapes can bind to the ER, which program the complex to form different conformations. This modifies the ability of coactivators or corepressors to bind to the ER complex. Thus, the gradient of ER conformations programmed by the ligand determines estrogenic or antiestrogenic responses (Jordan 2008). In the extremes, estradiol will recruit coactivators; tamoxifen, an antiestrogen, will recruit corepressors. Nuclear receptor coregulator recruitment is considered the rate-limiting factor of transcription in mammals, making the arrival of coactivators and/or corepressors at the DNA an integral step in the regulation or control of ER activity (Jordan and O'Malley 2007). EREs can also influence ER conformation, thereby causing varied recruitment of

coregulators and therefore gene function (Jordan and O'Malley 2007). Figure 1 summarizes the activation of the ER through its signal transduction pathway (Jordan 2006). Estrogen target genes, of which the estrogen-ER complex can induce transcription, include myc, fos, and jun (Van Slooten et al. 2001). SERMs, such as tamoxifen and raloxifene, can switch on and off estrogen or antiestrogen action at target sites around the body, but this is probably achieved by integrating ERα and ERβ signal transduction in the target tissues.

Target Assessment

Historically, the ER was measured by extracting soluble ER from frozen or fresh breast tumors and measuring the specific binding of tritium-labeled estradiol. Breast tumors contain a wide range of ER from zero to over 1,000 femtomoles/mg protein. Using this method, tumors were classified as ER-positive (>10 femtomoles/mg protein) or ER-negative (<10 femtomoles/mg protein). A clinical trial database was developed in the 1980s and 1990s that correlates ER status with patient response to tamoxifen therapy. Overall, a 30% reduction in mortality, with a 50% decrease in recurrence, was observed in ER-positive patients when given 5 years of tamoxifen as adjuvant therapy. In ER-negative tumors, no benefit was demonstrated.

Currently, fluorescence-linked monoclonal antibodies are used to guide physicians with immunohistochemistry to evaluate the ER target. Clinically, when a patient presents with breast cancer, a biopsy of the breast tissue is taken, observed under a microscope, and the concentration of stained cells is estimated for the presence of the ER.

Role of the Target in Cancer

Rank: 8 (breast cancer)

High-Level Overview

The ER is the first successful major target for cancer therapy (Jordan 2007), but its role in cancer treatment began as a marker in a diagnostic test to predict whether endocrine ablation, such as oophorectomy, would be of value to the patient (McGuire 1973). The assay indicated whether the patient's tumor was ER-positive, therefore likely responsive to estrogen withdrawal (Deroo and Korach 2006; Jensen and Jordan 2003; Jordan 2009). ER-negative breast cancer tumors do not respond to endocrine ablation because there is no ER present by which cellular functions can be modulated (McGuire 1973).

Because the ER has the capacity to be modulated, SERMs such as raloxifene and tamoxifen were recognized to be useful in the clinic for targeted ER agonism and antagonism. By targeting the ER with drugs like tamoxifen, breast cancer patients

gain a survival advantage, thus demonstrating the efficacy of ER as a major therapeutic target in the breast tumor.

Diagnostic, Prognostic, Predictive

Five intrinsic types of breast cancer have been identified using gene array analysis: luminal A, luminal B, Human Epidermal growth factor Receptor (HER)2-enriched, claudin-low, and basal-like breast tumors (Prat and Perou 2011). The four clinical subtypes used by clinicians to classify disease are hormone receptor (HR)-positive/HER2-negative, HR-positive/HER2-positive, HR-negative/HER2-positive, and HR-negative/HER2-negative (Prat and Perou 2011). HR comprises both the ER and the progesterone receptor (PR) in its definition, making the determination of ER status in breast tumors essential for accurately diagnosing cancer phenotype.

ER-positive tumors are generally composed of luminal A and luminal B intrinsic subtypes. Luminal A disease offers a good prognosis and is sensitive to endocrine therapy, while luminal B disease offers a poorer prognosis and is less sensitive to endocrine therapy, although it generates greater benefit from chemotherapy (Prat and Perou 2011).

ER-negative tumors can be HER2-enriched, but are most frequently basal-like and claudin-low (Prat and Perou 2011). These phenotypes present poor prognoses and do not respond to endocrine therapy. Overall, ER-positive tumors give patients longer relapse-free survival and overall survival than the other clinical subtypes, with ER-positive luminal A disease representing the best prognosis (Prat and Perou 2011).

Therapeutics

Many medicines have been proposed and developed based on blocking ER action in breast cancer cells. SERMs catalyze the tissue-specific modulation of the ER (Jordan 2004). SERMs bind to the ER, causing a conformational change in the receptor, thereby influencing what coregulators are recruited to the DNA. SERMs are neither purely antagonists or agonists, but a mixed complex generating partial agonism and partial antagonism when ER-bound. The conformation of the SERM-receptor complex has mixed affinity for coactivators and corepressors; consequently, concentrations of the coregulators in the physical context of the receptor are of critical importance in determining gene function (Jordan and O'Malley 2007). Tissue-specific SERM actions are not only regulated by coregulators, but also on the response elements to which the ligand-bound ER complex binds (Deroo and Korach 2006), and other factors including receptor isoform subtypes, ERE DNA sequences, and the turnover of the ER complex (Jordan and O'Malley 2007) (Fig. 1). Figure 2 details an ideal SERM (Jordan 2004). SERM effects can be modulated through a combination of ERα and ERβ within the target tissues. In the breast, SERMs are antiestrogenic. In bone, they weakly exhibit estrogenicity, maintaining bone density.

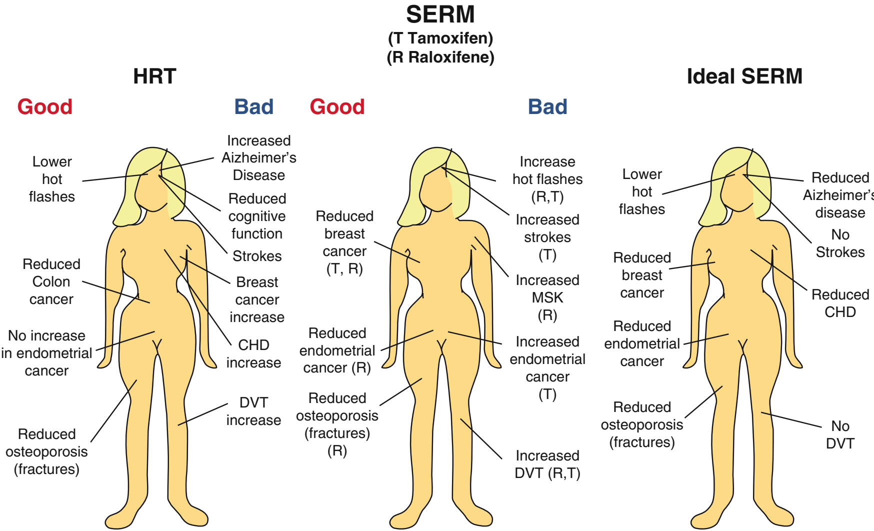

Fig. 2 Progress toward an ideal SERM. The overall good and bad aspects of administering hormone replacement therapy (*HRT*) to postmenopausal women compared with the observed site-specific actions of the selective estrogen receptor modulators (*SERMs*) tamoxifen and raloxifene. The known beneficial or negative actions of SERMs have opened the door for drug discovery to create ideal or targeted SERMs to either improve quality of life or prevent diseases associated with aging in women (Reprinted from V. Craig Jordan (2004), with permission from Elsevier)

This biological property of tamoxifen and raloxifene creates a benefit for long-term use in postmenopausal women. Raloxifene can be used long term for osteoporosis while preventing breast cancer simultaneously. Endometrial cancer is ERα-positive, but tamoxifen will increase the risk of endometrial cancer. That is, tamoxifen is estrogen-like in the uterus, whereas raloxifene is not.

To enhance the value of ERα target in breast cancer, it was been proposed to use a therapeutic approach that has no estrogen-like side effects. In postmenopausal women, estrogen is synthesized through the transformation of steroids, beginning with cholesterol stored in stromal fat and adipose tissue. Enzymes are required for each step of the biosynthesis; cholesterol desmolase converts cholesterol to progestogens, C17-20 lyase converts progestogens to androgens, and aromatase converts androgens, finally, into estrogens (Miller 2006). Aromatase inhibitors block the aromatase enzyme either competitively or as suicide inhibitors. This prevents the putative transformation of androgen to estrogen, therefore blocking estrogen production (Deroo and Korach 2006). These compounds are used to treat postmenopausal patients but are antiestrogenic everywhere in the body. Similarly, the pure antiestrogen fulvestrant has no estrogen-like activity as it binds to the ER and causes its rapid destruction. Without the ER in breast tumors, there is no estrogen-stimulated growth. ER-negative tumors proliferate through ER-independent mechanisms and are unresponsive to anti-ER therapy.

Preclinical Summary

The physiologic role of the ER has been interrogated by knock-out animals. In the therapeutic arena, it is still the main effective target for the treatment of breast cancer. Initial preclinical animal studies investigated responses to endocrine ablation therapy in ER knock-out mice (Couse and Korach 1999). These studies began to illustrate actions requiring genomic ER function. Other preclinical studies illustrated the correlation between breast tumorigenesis and duration of lifetime estrogen exposure (Couse and Korach 1999).

In the laboratory presently, investigations focus on mechanisms of response and resistance. Most successful responses to antihormone therapy occur in ER-positive/PR-positive patients. In ER-positive/PR-negative patients with increased growth factor levels, the response mechanism of action is unknown.

Two forms of antihormone resistance can arise in breast cancer tumors. The first is intrinsic to the tumor; antihormone therapy produces no effect in ER-positive/PR-negative tumors, probably due to cross talk with growth factors. Further, some ER-positive breast cancer cells are intrinsically resistant to tamoxifen, perhaps dependent on the presence or absence of other receptors, such as PR or HER-2/neu, or high levels of the coactivator SRC3 (Jordan and O'Malley 2007).

Acquired resistance, on the other hand, develops after ER-positive/PR-positive tumors are treated long term with tamoxifen or aromatase inhibitors and is considered to be a major concern that limits the effectiveness of long-term antihormone therapy. Athymic (immune-deficient) mouse studies show that ER-positive/PR-positive tumors treated at length with tamoxifen will eventually grow when treated with either estradiol or tamoxifen. Long-term SERM therapy induces a profound change in the signal transduction of breast cancer cells from estrogen-stimulated growth to SERM-stimulated growth (Jordan 2008). After extended antihormone therapy for many years, estrogen, once a breast tumor growth enhancer, remarkably becomes an apoptotic trigger. This clinical and laboratory observation is seemingly counterintuitive, since it is established that oophorectomy can prevent tumors and estrogen can enhance tumor growth in the laboratory (Jordan 2004).

This "estrogen paradox" is under intense investigation in the laboratory to facilitate effective translation to clinical practice (Jordan 2008). It had been established in 1944 that high-dose estrogen therapy could cause regression of some breast tumors in postmenopausal patients, a then perplexing paradox (Haddow et al. 1944). This pioneering use of high-dose estrogen, the first clinical therapy to treat any cancer, could not be explained at the time but now supports the principle behind the "estrogen paradox." Subsequent studies using ER assays in the 1970s established that only ER-positive tumors regressed in response to estrogen therapy. In normal physiological premenopausal breast cancer environment, the ligand-bound ER promotes tumor growth. When this environment is deprived of estrogen for a prolonged period of time, whether it be through the use of SERMs or decades after menopause, estrogen deprivation resistance develops, and estrogen eventually triggers cellular apoptosis in the long-term surviving estrogen-deprived tumor cells

(Jordan 2008). Preclinical laboratory investigation continues to focus on elucidation of acquired SERM resistance and estrogen-induced apoptosis.

Clinical Summary

Antihormone therapy is the treatment most relevant to the ER. ER-positive breast cancer accounts for about 70% of all breast tumors (Masood 1992). Studies show that estrogen causes growth and proliferation of ER-positive breast cancer cells. Tamoxifen, a SERM, acts as an antagonist of the ER in breast tissue, allowing it to block estrogenic action in breast cancer cells, therefore providing effective therapy. Tamoxifen exhibits estrogen-like agonist action in the bone and the uterus (Deroo and Korach 2006). Nevertheless, tamoxifen has had widespread and pioneering success, saving hundreds of thousands of lives by treating breast cancer and becoming the pioneering medicine for the prevention of any cancer (Jordan and O'Malley 2007). Long-term adjuvant therapy targets the breast ER specifically, and tamoxifen is the first drug approved to successfully treat high-risk pre- and postmenopausal patients. Tamoxifen was also found to inhibit the formation of contralateral primary breast cancer. Unfortunately, the SERM effect of tamoxifen is evidenced by a small but significant increase in the incidence of endometrial cancer in postmenopausal women. This is an estrogen-like effect in the uterus which limits its use as a chemopreventive for breast cancer in postmenopausal women at high risk.

Selective serotonin reuptake inhibitors (SSRIs) are used to lessen menopausal symptoms such as hot flashes that can occur during treatment with tamoxifen. However, paroxetine and fluoxetine, two SSRIs, block tamoxifen's conversion to its active metabolite, endoxifen, thereby nullifying the drug. Fortunately, venlafaxine, a serotonin-norepinephrine reuptake inhibitor (SNRI), does not block CYP2D6 from metabolizing tamoxifen to endoxifen and can be taken simultaneously with tamoxifen to prevent hot flashes (Jordan 2009).

Raloxifene, previously known as keoxifene or LY126758, is another SERM structurally similar to tamoxifen. Raloxifene is not an ER-mediated breast cancer therapy but is used to treat and prevent osteoporosis with the prevention of breast cancer as a beneficial side effect (Cummings et al. 1999). This is SERM action (Jordan 2009). Additionally, raloxifene is available with FDA approval to reduce breast cancer incidence in postmenopausal women at risk for developing the disease (Vogel et al. 2010). Raloxifene does not increase the incidence of endometrial cancer. A new SERM, lasofoxifene, is 100× more potent than raloxifene for the treatment and prevention of osteoporosis. Its beneficial side effects are a reduction of strokes, breast cancer, endometrial cancer, and coronary heart disease (Cummings et al. 2010).

Patients with ER-positive breast cancer respond effectively to treatment with SERMs and aromatase inhibitors; these therapies are used routinely in the clinic. Fulvestrant is a pure antiestrogen that enhances ER protein destruction and is used as a second-line therapy after acquired resistance occurs with tamoxifen or aromatase inhibitors in metastatic breast cancer (Jordan 2009).

Long-term treatment of ER-positive breast cancer patients with tamoxifen is the standard of care for premenopausal women. Alternatively, the majority of postmenopausal patients receive aromatase inhibitors instead of tamoxifen since it causes fewer side effects while still preventing estrogenic action (Jordan 2008).

Anticipated High-Impact Results

- SERMs producing a 50% decrease in the development of breast cancer
- Thirty percent decrease for mortality extending for more than 15 years after tamoxifen treatment cessation
- Multifunctional medicine to decrease risk of breast cancer, coronary artery disease, and osteoporosis

The application of the ER as a cancer therapeutic target continues to offer promise in laboratory science and for the benefit of patients worldwide. Past scientific discoveries involving ER modulation have laid the foundation for other hormonal receptors and their applicable cancer therapy and/or prevention. The defining principles drawn from ER targeting in breast cancers are already being applied to the androgen receptor and the treatment of prostate cancer (Chen et al. 2005). In the future, the therapeutic targeting of the hormone receptor superfamily will have profound impact on cancer medicine. Investigation continues in this field to optimally exploit the expressed biology in breast tumors.

Cross-References

► AR, Overview
► HER2/neu
► PR

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Histone Deacetylases (HDAC) 89

Keith R. Unger, Mira Jung, and Anatoly Dritschilo

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Abstract

Histone deacetylases (HDACs) are important epigenetic regulators of gene expression and aberrant HDAC activity is observed in many cancers. Numerous HDAC inhibitors have been assessed in preclinical and clinical studies. HDAC inhibitors have been tested as monotherapy and as combination therapy with chemotherapy, other targeted agents, or with radiation therapy. HDAC inhibitors have been successfully used for treatment of selected hematologic malignancies; future research will attempt to identify treatment strategies for solid tumors.

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Target

The balance of activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) is important in the higher-order chromatin organization. Increased acetylation of histones leads to changes in chromatin accessibility for key cellular proteins to specific target sites. HATs acetylate lysine groups at the amino terminal tails of nuclear histones to neutralize positive charges on the histones, yielding a more open, transcriptionally active chromatin structure. In contrast, the HDACs deacetylate and suppress transcription. In this model, inhibitors of HDACs bias the balance toward a more acetylated state. Such a shift in the relative activities of these enzymes may change chromatin conformation and gene expression necessary for cell growth, DNA repair, replication, cell cycle checkpoint activation and tumor suppression.

Biology of Target

Human histone deacetylases can be divided into four classes based on structure, sequence homology, and domain organization (De Ruijter et al. 2003). Class I consists of HDACs 1, 2, 3, 8, and 11, albeit a recent report puts HDAC 11 into a new class, class IV, based on a phylogenetic analysis. Class I HDACs are nuclear and play roles in cell proliferation and apoptosis. Class II includes HDACs 4, 5, 6, 7, 9, and 10. Class III enzymes, include the SIRTs (sirtuins), and are NAD-dependent deacetylases (Gregoretti et al. 2004) while other classes are ATP-dependent.

Non-histone proteins are also subject to acetylation and deacetylation by HATs and HDACs, respectively. Key targets include tubulin, p53, and Ku; playing roles in cell cycle regulation and response to DNA damage. HDACs also play a key function in transcriptional regulation. HDACs function as components of large multi-protein complexes that bind to promoters and repress transcription. Class II compounds target HDACs shuttling between the nucleus and the cytoplasm. Both classes of HDACs have conserved deacetylase core domains of approximately 400 amino acids and zinc binding sites (Gregoretti et al. 2004). It is the core domain that presents the principal target for design of inhibitory small molecules.

Target Assessment

Identifying isoform-specific HDAC inhibitors has proven problematic due to the close homology among catalytic sites. Since HDACs are often complexed with other proteins, purified recombinant HDAC protein are not always representative of the

active state. These difficulties have limited comprehensive HDAC inhibitor specificity profiling. The expression levels of HDACs and histones are readily measured by PCR and Western immunoblotting techniques. In clinical studies, hyperacetylation of histones H2B, H3, and H4 has been used as a surrogate for HDAC activity following drug treatment (Ramalingam et al. 2010). The level of histone acetylation can be measured from tumor biopsies or from circulating lymphocytes. The measurement of histone acetylation status from peripheral blood is a particularly attractive non-invasive option for use in clinical trials.

Role of Target in Cancer

Rank: 9
Four HDAC inhibitors have been approved to date by the US Food and Drug Administration (FDA). Vorinostat was approved in 2006 for use in patients with cutaneous T-cell lymphoma (CTCL). In 2009, romidepsin was approved for patients with CTCL and peripheral T-cell lymphoma (PTCL). Belinostat was approved in 2014 for use in PTCL. Most recently in 2015, panobinostat was approved for use in previously treated multiple myeloma when given in combination with dexamethasone and bortezomib. Additional clinical trials of HDAC inhibitors are ongoing to define the full role of this target in cancer therapy.

High Level Overview

Diagnostic, Prognostic, Predictive

Early evidence suggests that altered HDAC expression and histone modifications may provide prognostic and predictive information in cancer patients. Aberrant expression of HDAC isomers has been reported in a number of solid tumors including, breast cancer, prostate cancer, gastric cancer, and colon cancer. Differential expression of HDACs has been associated with outcomes in childhood acute lymphoblastic leukemia and relapsed acute lymphocytic leukemia. Histone modification has also been correlated with known prognostic factors and clinical outcomes in breast cancer. Future study is necessary to further evaluate the role of HDACs and histones as potential biomarkers for cancer diagnosis and outcomes.

Therapeutics

HDAC inhibitors are a promising class of drugs with potential for clinical applications as monotherapy or in combination with other drugs and as radiosensitizers. HDAC inhibitors enhance tumor chemosensitivity and radiosensitivity in preclinical studies in a wide variety of solid tumors, and may serve as radioprotectants of normal

tissues. Emerging evidence suggests that HDAC inhibitors modify the cellular response to DNA damage from radiation. In recent clinical trials, HDAC inhibitors have been generally well tolerated given as a single modality or in combination with chemotherapy or radiation therapy. Several active clinical trials are investigating systemic and radiation therapy with various HDAC inhibitors for the treatment of solid tumors.

Preclinical Summary

A large number of HDAC inhibitors have been synthesized or derived from natural sources and can be divided into classes based on their chemical structures. Sodium butyrate, an early HDAC inhibitor was initially investigated as a differentiating agent, without full understanding its molecular mechanisms. The full recognition of the potential for HDAC inhibitors was advanced with the discovery and development of the hydroxamic acid inhibitors, which include trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA, vorinostat). Hydroxamic acid based compounds are active at nanomolar concentrations and have proven to be relatively non-toxic in clinical trials since normal cells are relatively resistant to HDAC inhibitor induced cell-death.

HDAC inhibitors primarily lead to cell-death through induction of apoptosis by intrinsic and extrinsic pathways via transcription dependent and transcription independent mechanisms. Valproic acid has been shown to upregulate TNF family ligands and receptors leading to extrinsic apoptosis in leukemic cells but not in normal cells (Insinga et al. 2005). Suberic bishydroxamate induces apoptosis by upregulation of pro-apoptotic encoding genes (Bim, Bax, Bak) and by decreased expression of anti-apoptotic proteins (Bcl-2, Bcl-X_L, Mcl-1, X-linked inhibitor of apoptosis) (Zhang et al. 2004b). Additionally, HDAC's can block tumor angiogenesis by inhibition of pro-angiogenic factors, including hypoxia inducible factor-1a (HIF-1α). Vorinostat has been shown to reduce HIF-1α translation through HDAC9 silencing (Hutt et al. 2014).

Recent reports have shown that specific HDACs isoforms interact with DNA damage and repair pathway proteins. ATM kinase is considered a primary regulator of responses to DNA double-strand breaks (DSBs) and activates a number of downstream effectors, including H2AX, MDC1/NFBD1, 53BP1, Brcal, and MRN (Mre11, Rad50 and Nbs1). Kim et al. demonstrated that the histone deacteylase HDAC1 interacts with ATM, and that cells with mutated ATM did not show an increase in histone deactylase activity following irradiation (Kim et al. 1999). Bakkenist et al. have reported that ATM also can be activated by trichostatin (TSA) by a process that involves chromatin changes in the absence of DNA breaks (Bakkenist and Kastan 2003). Furthermore, recent studies have demonstrated that chromatin condensation promotes ATM activation of upstream DNA damage response signaling in a break-independent manner and is required for fully activating DNA damage repair as an integral step in the damage response (Burgess et al. 2014).

Therefore, dynamic changes in chromatin structure occur during DNA damage response and repair processes.

The class I isoforms, HDAC1 and HDAC2 have been implicated in the promotion of non-homologous end-joining, a critical cellular DNA damage response and repair process (Miller et al. 2010). HDAC4 has been found to be an important component of the DNA repair pathway, interacting with 53BP1 at sites of DNA DSBs (Kao et al. 2003). Geng et al. found that HDAC inhibitor treatment leads HDAC4 confinement to the cytoplasm, indicating an effect on HDAC nuclear localization (Geng et al. 2006). HDAC inhibitors have also been shown to modulate the gene expression of a number of DNA repair proteins, including Ku70, Ku86, and DNA-dependent protein kinase catalytic subunit (Munshi et al. 2005). The FDA-approved HDAC inhibitor, Vorinostat, has been shown to suppress the DNA DSB repair protein, Rad50 and Mre11 in cancer cells, but not in normal cells, supporting observations of differential effects on cancer cells as compared to normal cells (Lee et al. 2010).

In vitro and vivo preclinical studies have demonstrated the ability of HDAC inhibitors to enhance the effectiveness of ionizing radiation using a variety of human cancer cell lines (Fig. 1). The benzamide-based HDAC inhibitor, MS-275, in combination with radiation enhances tumor growth delay in mice bearing human prostate carcinoma xenografts (Camphausen et al. 2004). Folkvord et al. demonstrated that SAHA treatment with fractionated radiation resulted in tumor growth delay in colorectal carcinoma in an in vivo model (Folkvord et al. 2009).

HDAC inhibitors are hypothesized to act through a variety mechanisms to cause radiosensitization in cancer cells (Fig. 2). In response to DNA damage, signal transduction pathways are activated to regulate cell cycle arrest, repair, differentiation, apoptosis, and transcription. Such responses are a complex feature of the cellular radiation phenotype, and their effectiveness determines cell survival or death. Following radiation-induced double-strand DNA breaks (DSBs), the histone H2AX becomes rapidly phosphorylated to form γ-H2AX. γ-H2AX foci have been shown to be a sensitive marker of DSBs, and clonogenic survival after radiation has been associated with dispersal of γ-H2AX foci (MacPhail et al. 2003). A number of HDAC inhibitors prolong the expression of radiation-induced γ-H2AX foci, suggesting that HDAC inhibitors may decrease the rate of DSB repair or chromatin structural changes may cause increased numbers of DSBs (Munshi et al. 2005).

The responses of cells to ionizing radiation may be viewed as a complex phenotype involving various signal transduction pathways associated with cell cycle regulation, DNA repair and regulation of apoptosis. HDAC inhibitors cause cell cycle arrest which may contribute to the observed radiation sensitizing properties. TSA treatment has been shown to result in G1 and G2/M arrest, both relatively radiosensitive phases of the cell cycle (Zhang et al. 2004a).

HDAC inhibitors have been shown to selectively increase the radiosensitivity of tumor cells over normal cells, an important property necessary for clinical application. Munshi et al. showed that SAHA treatment did not result in increased radiosensitivity in normal fibroblasts (Munshi et al. 2005). The selectivity of HDAC

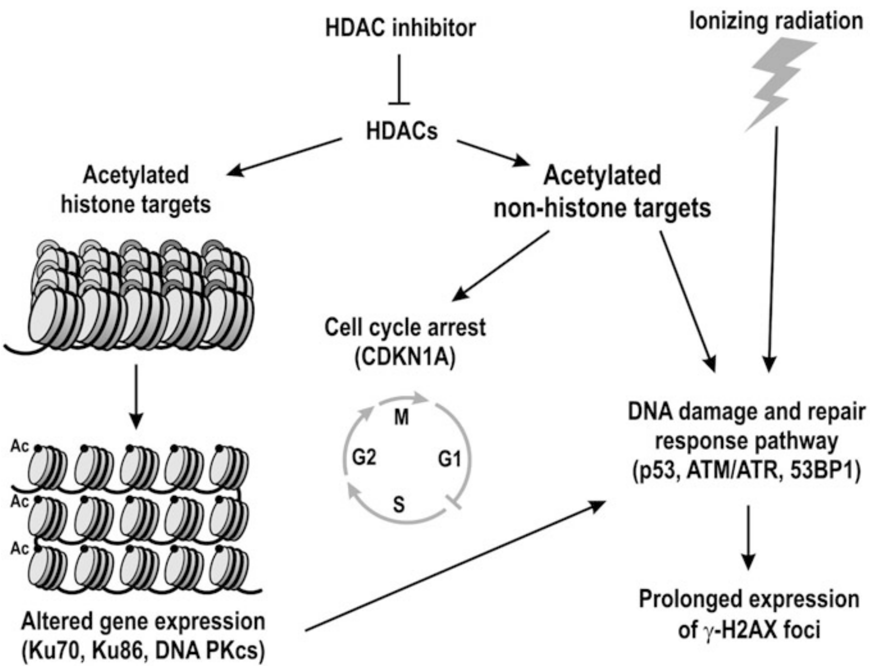

Fig. 1 Proposed mechanism of radiosensitization with targeting of histone deacetylases (*HDACs*). DNA double-strand breaks (*DSBs*) breaks from ionizing radiation results in activation of the DNA damage response and repair pathways. Prolonged expression of γ-H2AX foci, correlating with sites of DNA DSBs has been observed following irradiation and HDAC inhibitor treatment. HDACs act on a number of non-histone targets, including the DNA damage response pathways as well as the cell cycle regulation pathways. HDAC inhibitors also cause relaxation of the chromatin structure, leading to transcription changes in DNA damage response pathway proteins

inhibitors may be a result of HDAC overexpression observed in a number tumors including prostate, colon, and breast carcinomas (Bolden et al. 2006). Additionally, HDAC inhibitors may have radioprotectant properties in normal tissue. Pretreatment with TSA and SAHA decreased the lethal effect of total body irradiation in a mouse model (Brown et al. 2008). Topical application of HDAC inhibitors has also been shown to reduce skin fibrosis associated with cutaneous radiation syndrome (Chung et al. 2004).

Clinical Summary

Multiple clinical trials have been reported using HDAC inhibitors as a single agent or in combination with chemotherapy for the treatment of hematologic and solid malignancies. In addition, there are numerous active clinical studies involving HDAC inhibitors for a variety of malignancies (Table 1). HDAC inhibitors are generally well tolerated; the side effects are reversible with drug cessation and

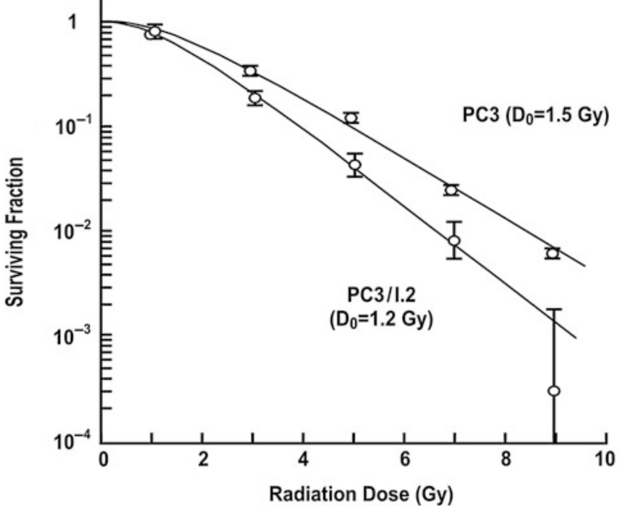

Fig. 2 Radiosensitization in vitro with a hydroxamate histone deacetylase (*HDAC*) inhibitor. Dose response curves of a human prostate cancer cell line (PC-3), with and without the hydroxamate HDAC inhibitor, I.2, shows a decrease in the slope of the terminal portion of the cell survival curve (D_0) with the addition of the drug

Table 1 Summary of selected active clinical trials with histone deacetylase (HDAC) inhibitors in combination with other drugs

HDAC inhibitor	Drug (s) in combination	Treatment Indication	Phase
Vorinostat	Thalidomide, lenalidomide, bortezomib	Newly diagnosed multiple myeloma	III
Entinostat	Exemestane	Recurrent hormone receptor positive breast cancer	III
Valproic acid	Gemcitabine, bevacizumab, docetaxel	Metastatic sarcoma	II
Panobinostat	None	Relapsed or refractory Non-Hodgkin lymphoma	II
Vorinostat	Cytarabine and daunorubicin or idarubicin	Untreated acute myeloid leukemia	III
Vorinostat	Temsirolimus	Metastatic prostate cancer	I

primarily include fatigue, nausea, dehydration, diarrhea, prolonged QT, thrombocytopenia, lymphopenia, and neutropenia.

HDAC inhibitors have been approved for use in hematologic malignancies, including CTCL, PTCL, and multiple myeloma. A phase II study of vorinostat was conducted in 74 patients with persistent, progressive, or recurrent mycosis fungoides or Sezary syndrome CTCL after at least two prior systemic therapies. The objective response rate was 29.7% and the median time to progression of

4.9 months. Most adverse events were grade 2 or lower and included diarrhea, fatigue, and nausea (Olsen et al. 2007). Single-agent romidepsin demonstrated an objective response rate of 25%, including a 15% complete response rate, in a phase II trial in patients with relapsed or refractory PTCL. Activity was seen in all major subtypes of PTCL and regardless of prior therapies (Coiffier et al. 2012).

The pan-histone deacetylase inhibitor, panobinostat, has demonstrated clinical activity when combined with the proteosome inhibitor, bortezomib. It is hypothesized that the dual inhibition of the aggresome pathway by HDAC inhibition and the proteasome pathway by bortezomib has synergistic effects in multiple myeloma (Hideshima et al. 2005). The PANORAMA-1 study was a large, double-blind, placebo-controlled phase III trial which enrolled 768 patients with relapsed or refractory multiple myeloma (San-Miguel et al. 2014). Panobinostat in combination with bortezomib and dexamethasone resulted in a significantly improved progression-free survival over the standard regimen of bortezomib and dexamethasone alone (11.99 vs. 8.08 months; hazard ratio 0.63; $p < 0.0001$). The overall response rate did not differ between the two arms, which may indicate that panobinostat did not overcome resistance to bortezomib but enhanced the responses to bortezomib. Grade 3 or 4 diarrhea occurred in 26% of patients as compared to 8% in the control arm. This study led to the approval of panobinostat in combination with bortezomib and dexamethasone by the FDA for treatment of patients with multiple myeloma after at least two prior regimens, marking the first HDAC inhibitor approved as part of combination therapy.

As a single agent, HDAC inhibitors have demonstrated limited anticancer activity in the treatment of solid malignancies, leading primarily to disease stabilization. In a phase II trial, 66 patients with recurrent glioblastoma multiforme were treated with up to two cycles of vorinostat, resulting in 9 of 52 patients being progression free at 6 months. Twenty-six percent of patients developed grade 3 or higher nonhematologic and hematologic toxicity (Galanis et al. 2009). The combination of HDAC inhibitors with chemotherapy has also been studied in solid tumors with encouraging results. In a randomized phase II trial in 94 patients with locally advanced or metastatic non-small cell lung and treated with carboplatin and paclitaxel, the addition of vorinostat resulted in increased response rates. There was a trend toward improved progression-free survival and overall survival (Ramalingam et al. 2010).

To date there is one published clinical trial and several ongoing studies of HDAC inhibitors combined with radiation therapy for solid tumors (Ree et al. 2010; National Cancer Institute). In a phase I dose escalation study, 16 patients were treated with palliative pelvic radiation therapy to 30 Gy and vorinostat given orally. Vorinostat was given at dose levels of 100, 200, 300, and 400 mg. Two of six patients developed dose limiting toxicity at 400 mg (grade 3 diarrhea, anorexia, fatigue, and hematologic toxicity); therefore the maximum tolerated dose was 300 mg. Tumor biopsies taken during treatment demonstrated hyperacetylation of histone H3 and H4 2.5 h after vorinostat dosing. Table 2 shows selected active clinical trials involving HDAC inhibitors with radiation therapy for the treatment of solid tumors.

Table 2 Summary of selected clinical trials of the combination of histone deacetylase inhibitors and radiation therapy

Drug (s)	Treatment Indication	Radiation Fractionation	Phase (comment)	Status
Vorinostat	Palliation of pelvic tumors	Conventional fractionation	I	(Ree et al. 2010)
Vorinostat	Brain metastasis	Conventional fractionation	I	In progress
Vorinostat	Pediatric pontine glioma	Conventional fractionation	I/II	In progress
Vorinostat	Metastatic non-small cell lung cancer to the brain	Large fraction radiation therapy	I	In progress
Vorinostat	Metastatic non-small cell lung cancer	Conventional fractionation	I	In progress
Vorinostat + infusional fluorouracil	Locally advanced pancreatic cancer	Conventional fractionation	I/II	In progress
Vorinostat + capecitabine	Nonmetastatic pancreatic cancer	Conventional fractionation	I	In progress
Vorinostat or temozolomide or bevacizumab	Pediatric high grade glioma	Conventional fractionation	II/III	In progress
Valproic acid + Temodar	Glioblastoma	Conventional fractionation	II	In progress
Valproic acid	Pediatric high grade glioma	Conventional fractionation	II	In progress

Anticipated High-Impact Results

- Early and late phase clinical trials assessing HDAC inhibitors as combination therapy with chemotherapeutic and biotherapeutic agents as well as with radiation therapy in solid and hematologic malignancies
- Preclinical and clinical applications of novel isoform-specific HDAC inhibitors
- Exploration of radioprotective properties of HDAC inhibitors
- Further research into the mechanisms and complex pathways involved in the observed properties of HDAC inhibitors

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Methylation

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Abstract

Alterations of DNA methylation patterns and other epigenetic changes, such as covalent histone posttranscriptional modifications (methylation, acetylation, phosphorylation, ubiquitination, sumoylation), chromatin-remodeling processes, and more, are well-known phenomena observed in various disease processes including cancer. In fact, global DNA hypomethylation and promoter hypermethylation are promising biomarkers for cancer.

Keywords

Azacytidine • Bladder cancer • Breast cancer-associated gene 1 (*BRCA1*) • CpG island methylation phenotype (CIMP) • Decitabine • Formalin-fixed paraffin-

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embedded (FFPE) tissue • Glioblastoma • Histone demethylases (HDMs) • Histone methyltransferases (HMTs) • Methylation • MethyLight method • Mixed lineage leukemia (*MLL*) gene • *MLH1* • S-adenosylmethionine (SAM) • *SLC19A1* gene

Target: Methylation

Alterations of DNA methylation patterns and other epigenetic changes, such as covalent histone posttranscriptional modifications (methylation, acetylation, phosphorylation, ubiquitination, sumoylation), chromatin-remodeling processes, and more, are well-known phenomena observed in various disease processes including cancer. In fact, global DNA hypomethylation and promoter hypermethylation are promising biomarkers for cancer.

Biology of the Target

Methylation of DNA(Miranda and Jones 2007; Bhutani et al. 2011) occurs as a natural biochemical process during the development of eukaryotes with a genome size larger than approximately 5×10^8 base pairs. Methylation irreversibly alters gene expression as cells undergo mitosis and differentiation. Examples of such methylation-induced alterations of gene expression are X-chromosome inactivation and genomic imprinting. Methylation also functions to silence noncoding DNA (introns, repetitive sequences, and transposable elements) and RNA sequences; in addition, methylated sequences can bind various proteins leading to modifications of surrounding chromatin.

DNA methyltransferases facilitate the transfer of methyl groups (CH_3) from the methyl donor S-adenosylmethionine (SAM) to position 5 of the pyrimidine ring of cytosines in CpG dinucleotide sequences (cytosine – phosphodiester bond – guanine) (typically in somatic cells) and/or to non-CpG nucleotides, such as CpA dinucleotides (cytosine – phosphodiester bond – adenine) or CpNpG sequences (cytosine – phosphodiester bond – any nucleotide – phosphodiester bond – guanine) of DNA (more frequently seen in embryonic stem cells and plants but rare in somatic mammalian and in human tissues). CpG dinucleotides in humans are located mainly in CpG-rich areas of the genome, so-called CpG islands, and in regions possessing large repetitive sequences. Of note, CpG islands (usually 1–4 kb in size) are found in approximately two thirds of human gene promoters and in the first exon of many genes. They are defined as regions that have a G + C content of >50%, a minimum size of 200 bp, and an observed-to-expected CpG ratio greater than 60%.

It is not yet fully elucidated which sequences cause DNA methyltransferases to target genomic regions for methylation. We do know that CpG islands of various

gene promoters differ in their propensity to become methylated. Among the five mammalian DNA methyltransferase isoforms, three are catalytically active: DNMT1, DNMT3A, and DNMT3B. DNMT1's main role is to maintain genomic methylation patterns with DNA replication. It is therefore specific for hemi-methylated DNA. However, DNMT3A and DNMT3B act primarily as de novo methyltransferases, thus targeting both hemi- and unmethylated DNA. In mammals, 60–90% of the CpG dinucleotides can be methylated. However, in course of evolution, a great part of methylated cytosine residues spontaneously deaminate to thymidine (mCpG → TpG), resulting in a significantly lower percentage of CpG dinucleotides than expected in the human genome when compared to other mammals.

Although the mechanisms of methylation are fairly well understood and have been described extensively in the literature, this is not the case for demethylation. Removal of methyl groups from the genome has been proposed to occur either passively in course of mitosis (e.g., by inhibition of DNMT1) or actively by enzymes such as the ten-eleven translocation (TET) proteins 1, 2, and 3. TET proteins convert methylated cytosine via several oxidation steps to 5-carboxylcytosine. In addition, DNA repair enzymes could play a role as they remove and restore unmodified cytosine.

Target Assessment

Based on the extensive changes in DNA methylation patterns in cancer, profiling DNA methylation could be a suitable approach to aid in diagnosis and prognosis of cancers. It can also serve to gather information on treatment responses, cancer recurrence, and/or development of metastases, thereby utilizing a cancer cell's DNA methylome as biomarker.

Several promising techniques for clinical application of methylation analysis have been developed, and various primarily hypermethylated DNA signatures with diagnostic potential for different types of cancer have been identified (Toraño et al. 2012; Heyn and Esteller 2012; Shanmuganathan et al. 2013; Laird 2010).

The principle is to use bisulfite-treated DNA to convert unmethylated cytosine into uracil, while leaving methylated cytosine unchanged. Subsequently, DNA amplification and sequencing determines whether or not thymidine has been incorporated for uracil or unmethylated cytosine for each methylated cytosine.

When methylation-specific primers that overlap CpG dinucleotides are used for the PCR, only methylated alleles are amplified. These can be visualized with simple gel electrophoresis without the need for further sequencing. With the MethyLight method, which is based on such methylation-specific PCRs, in addition, quantitative analysis is possible, using real-time PCR.

If non-methylation-specific primers (that do not overlap CpG dinucleotides) are chosen, methylated and non-methylated alleles are amplified equally. Therefore, subsequently, the amplified product must be subject to sequencing or other types

of analysis, such as matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Applying such "sequence-specific" methodologies led to the identification of hypermethylation of the *GSTP1* gene as a good biomarker for prostate cancer with a sensitivity of 82% and a specificity of 95%. This marker, which is currently used in additional/optional tests of formalin-fixed paraffin-embedded (FFPE) tissue samples when prostate cancer is suspected, but not unequivocally diagnosed, could potentially be used in the future to analyze easily collectible clinical specimen, such as blood or urine. Combining *GSTP1* methylation tests with analysis of various other genes that have a high incidence of methylation in prostate cancer ("prostate cancer signature"), such as *APC or RASSF1A*, was shown to further increase the cancer's detection rate with improved sensitivity and specificity.

Similar good results are available assessing hypermethylation of several genes to diagnose, for example (Heichman and Warren 2012):

(a) Bladder cancer at various stages: For instance, the combination of *GDF15/NAG-1*, *TMEFF2*, *and VIM* analysis in urine can distinguish bladder cancer from healthy bladder tissue with 94% sensitivity and 100% specificity.
(b) Glioblastoma: Analysis of the *MGMT* promoter region can lead to diagnosis of the disease with 95% sensitivity and 60% specificity.

For other cancer types, such as colorectal cancer (*SEPT9*, *TFPI2*, etc.), non-small cell lung cancer (*MGMT*, *CDKN2A GSTP1*), or cancers of the head and neck (*MGMT*, *CDKN2A*, *DAPK1*), the sensitivities and specificities of biomarkers that can potentially be used routinely range roughly between 60% and 90%, respectively.

So far, only a few of these DNA methylation tests have found their way into clinical use, such as for the promoter of *MGMT* (test is performed on FFPE tissue samples) to predict treatment responses of glioblastomas when treated with alkylating agents, *SEPT9* in plasma to screen for colorectal cancer (sensitivity 90%, specificity 88%), or *SHOX2* as adjunct to diagnose lung cancer in bronchial lavage samples (sensitivity 78%, specificity 96%). However, several research groups are working on advancing our knowledge on the methylation profile of various cancer types applying both established and novel technologies and techniques (Fernandez et al. 2012).

While hypermethylated DNA can be detected independent of the level of expression with the above-explained techniques, analyzing global DNA hypomethylation is more problematic, as approaches for a potential use in clinical practice are still too expensive and neither well defined nor standardized. Examples are among others whole-genome bisulfite sequencing, which has been used to obtain information about the DNA methylome of breast and colorectal cancers, and DNA methylation assays. The latter technology is more cost effective and enables determination of genome-wide DNA methylation profiles in a large number of samples, albeit at the expense of resolution.

High-Level Overview

Background

The genome of cancer cells is characterized by global DNA hypomethylation and aberrant side-specific hypermethylation of CpG islands in promoter regions. Hypomethylation affects primarily noncoding sequences, such as introns, repetitive sequences, or transposable DNA elements. Whether it causes cancers to develop or is a consequence of cancerogenesis has been debated in the literature. Regardless, it is accepted that DNA hypomethylation results in increased genomic instability and chromosomal rearrangements/alterations. The consequences can be manifold. They include among others the possibility of activating previously inactivated genes that possess growth-promoting abilities and are important in the development of various types of cancers, such as the activation of melanoma-associated antigen in melanomas, *MAPSIN* in gastric cancer, or *S-100* in colon cancer. DNA hypomethylation can moreover lead to loss/reversal of imprinted genes, such as *IGF2* in Wilms' tumors, hence bringing to expression previously suppressed genes.

Promoter hypermethylation accounts for silencing of tumor suppressor genes, such as breast cancer-associated gene 1 (*BRCA1*), retinoblastoma gene (*RB*), von Hippel-Lindau (*VHL*) tumor suppressor gene, or cyclin-dependent kinase inhibitor 2A (*CDKN2A*), thus facilitating the initiation and further progression of cancer. It is not yet known how these genes are targeted for methylation.

Promoter hypermethylation can enable cancer progression also through silencing of transcription factors and their potential downstream antineoplastic target genes. Examples are the epigenetically silenced transcription factor genes *GATA-4* and *GATA-5* in colorectal and gastric cancer and the silenced *RUNX3* gene in esophageal squamous cell cancer. Hypermethylation of promoters can furthermore repress cell cycle regulatory genes (*p15*, *p16*, *RB*), as well as genes involved in differentiation, apoptosis (*caspases*, *p14*, *DAPK*), and DNA repair (*MLH1*, *BRCA1*, etc.), thereby preventing proper growth and maturation of healthy cells and salvage of genetic lesions that are accumulated within cells.

Genomic instability can furthermore be caused by mutations affecting either the enzymes involved in DNA (de)methylation or their genomic coding regions, hence setting the stage for the development of cancer.

In addition, lysine and arginine residues of histone proteins can undergo methylation or acetylation. Depending on the amino acid affected and the type of the covalent modification, transcription of genes can be either activated or repressed.

The enzymes catalyzing and regulating the changes of histone methylation patterns are histone methyltransferases (HMTs) and histone demethylases (HDMs). They interact with each other and with other enzymes involved in regulating modifications of DNA, thus impacting chromatin stability and structure.

In addition, HMTs and HDMs can affect DNMT protein stability and influence DNA methylation patterns and levels through effects on intracellular turnover of DNMTs. But, DNMTs, as well, can exert effects on histone-modifying enzymes and influence both the degree and extent of gene silencing and chromatin condensation.

Finally, translocation of genes encoding HMTs to other chromosomal regions can result in malignant transformation of affected cells. This is exemplified in the translocation of the mixed lineage leukemia (*MLL*) gene, which in its SET domain encodes a protein that methylates lysine 4 on H3. Translocations of the gene result in the formation of various types of fusion genes, all of which but one known exception are associated with the loss of the SET domain, and hence HMT activity. The consequence of such *MLL* rearrangements is the transformation of hematopoietic cells into leukemia stem cells, as seen in 10% of cases of acute leukemia in humans.

Diagnostic, Prognostic, Predictive

The CpG methylation status of cells can be used to determine whether or not a cell has undergone malignant transformation. Currently, this is accomplished primarily by analyzing tissue samples, which are obtained through needle or surgical biopsies or even more extensive surgical procedures. However, efforts are ongoing to expand diagnostic options by using bodily fluids (blood, urine, stool, or other specimens) that can be collected easily, independent of risk factors, such as patient's age or medications (e.g., anticoagulants), and through less invasive approaches. For more details, please see section "Target Assessment."

As for prognostication, methylation of several genes has been linked with either good or poor prognosis. In solid tumors, unfavorable outcome has been described, e. g., for breast cancers with methylated *BRCA1* promoter, early-stage non-small cell lung cancers with methylated *DAPK* promoters, or bladder cancers with either methylated *CDKN2A*, *RASFF1A*, or *myopodin* promoters. Furthermore, the presence of the so-called CpG island methylation phenotype (CIMP), which denotes the concurrent methylation of multiple genes in cancer cells, has been associated with worse prognosis and increased risk of metastases in, e.g., colon, hepatocellular, ovarian cancers, or other malignancies.

Improved survival has been reported for, e.g., diffuse large B-cell lymphomas with methylation of the *MGMT* promoter or acute myelogenous leukemias with methylated estrogen receptors.

While these are only a few examples of gene promoters, whose methylation status has been reported to have predictive potential, more of such information is to be expected in future as knowledge in this field advances.

Information on the CpG methylation status of various gene promoters in cancer cells can furthermore aid in predicting response to treatment. This is best exemplified by the *MGMT* gene promoter, where hypermethylation predicts good response to alkylating agents (e.g., carmustine) and temozolomide. Examples of other genes with promoters whose methylation status predicts response to treatment are *MLH1* in the treatment of ovarian cancer with cisplatin, *BRCA1* when using PARP inhibitors to treat breast and ovarian cancer, or *WRN* in the treatment of colorectal cancer with irinotecan. These genes all code for proteins involved in DNA repair. Methylation of their promoters results in gene inactivation and impairs the cell's ability to repair DNA. Thus, hypermethylation of these promoters often predicts favorable clinical response.

On the contrary, the *SLC19A1* gene belongs to the class of transporter genes. Its gene product is involved in the transport of folate compounds into mammalian cells. Individuals with primary CNS lymphomas, who have hypermethylated *SLC19A1* promoters, have lower intracellular levels of folate and are therefore resistant to treatment with the antifolate methotrexate. Similar findings are reported for the *UGT1A1* promoter and the effect of irinotecan treatment in colon cancer.

Finally, promising results were described for the predictive potential of the *GSTP1* promoter methylation status in the treatment outcome of breast cancer in response to doxorubicin.

Therapeutics

Since the discovery of epigenetic modifications as crucial events for initiation and progression of cancer and the realization that these modifications are reversible, much effort has been put into developing drugs that can be used to interfere with the genome alterations and restore the epigenetic profile of cancer cells.

Four epigenetic drugs have been approved for clinical application by the FDA, two of them affecting genome methylation (azacytidine and decitabine), while two interfere with histone deacetylation (vorinostat and romidepsin).

Azacytidine (or 5-azacytidine, 5-AZA) and its deoxy derivative decitabine (or 5-aza-2′-deoxycytidine) are both DNMT inhibitors, which were approved for the treatment of myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML) in 2004 and 2006, respectively. Azacytidine was also approved for treatment of chronic myelomonocytic leukemia. The rationale behind using these drugs in MDS is the hypermethylation of the cyclin-dependent kinase 4 inhibitor B (*CDKN2B*) gene promoter (aka multiple tumor suppressor-2 or p15INK4B) in MDS. This gene encodes a protein that acts as a regulator for cell growth and inhibits the progression of cells in the G1 phase of the cell cycle. The G1 phase is known as a growth phase during which synthetic activities for proteins required during the cell cycle's synthesis phase are resumed. Silencing of the gene through hypermethylation allows cells to advance rapidly from the G1 phase to mitosis, resulting in uninhibited cell divisions as characteristic for malignancies.

While azacytidine can be incorporated into both RNA and DNA with a preference of RNA over DNA, decitabine is incorporated only in DNA. The interactions of these agents with DNA cause irreversible inhibition of DNMT enzymatic activities, including inhibition of de novo DNA methylation, and result in hypomethylation of genomic DNA.

Treatment of MDS with azacytidine resulted in a 60% response rate seen in a multicenter, phase III randomized trial (CALGB 9221) as compared to a 5% response rate with best supportive care (Silverman et al. 2002). Seven percent of patients achieved complete responses, 16% partial responses, and 37% hematologic improvements. Median time to progression to AML or death was also significantly improved with azacytidine (21 vs. 12 months; $p = 0.007$). However, the overall survival was not different between the two groups (20 months in the azacytidine

group vs. 12 months with best supportive care; $p = 0.1$). The subsequently performed AZA-001 trial (Fenaux et al. 2009) investigated survival and reported improved overall survival in MDS patients treated with azacytidine compared to best supportive care or low-dose cytarabine (21.1 vs. 11.5 months; $p = 0.0045$ and 24.5 vs. 15.3 months; $p = 0.0006$, respectively) but no statistically significant difference when compared with patients treated with intensive chemotherapy per investigator's choice (25.1 vs. 15.7 months; $p = 0.51$). The overall response rate (complete or partial responses) was 29% with azacytidine vs. 12% with conventional care regimens. Improvement of overall survival was independent of the type of response achieved (complete or partial response or hematologic improvement). The 1-year survival rate for patients treated with azacytidine was 68.2% compared to 55.6% for those who received conventional care ($p = 0.015$), while the 2-year survival rate with azacytidine was 50.8% vs. 26.2% ($p < 0.0001$) with conventional care. The results of the AZA-001 study were particularly good for patients, who had the cytogenetic abnormalities of 7q deletions, but for patients who progressed on the epigenetic drug, the overall survival rate was not superior to the rates achieved with conventional care (15.7 vs. 21.1 months; $p = 0.51$).

Decitabine, which has been described to inhibit DNMTs 10× more effectively than its prodrug azacytidine, led to significantly higher overall response rates (17% vs. 0%; $p < 0.001$) and hematologic improvement (13% vs. 7%; $p < 0.001$) than supportive care in patients with either de novo or secondary MDS(Kantarjian et al. 2006). In this randomized phase III study, which led to the approval of the drug for clinical application, the rates of overall improvement (complete, partial, hematologic improvement) were 30% with the epigenetic drug vs. 7% with supportive care. In addition, time to progression to AML or death was longer on the decitabine arm (12.1 vs. 7.8 months; $p = 0.16$ by the log-rank test), and the responses were durable (median 10.3 months; range 4.1–13.9 months). All patients with partial or complete response with decitabine were independent for red blood cell and platelet transfusions during the time of their response. Their median time to the development of AML or to death was almost twice as long as for nonresponders (17.5 vs. 9.8 months; $p = 0.01$).

Both drugs were considered safe for clinical use with an overall well-manageable toxicity profile.

Preclinical Summary

There is considerable interest among scientists to discover and develop new epigenetic drugs that can be applied in the future to effectively treat cancer patients and/or to expand the indication of currently available drugs (e.g., to ovarian and prostate cancer or melanoma), either as single agents or in combination with chemotherapy or other anticancer agents, including other epigenetic drugs.

Much preclinical work is focused on exploring different types of DNMT inhibitors with potentially improved and expanded activity profiles and less cytotoxicities. Among the agents under investigation is zebularine [1-(β-D-ribofuranosyl)-1,2

dihydro-pyrimidine-2-one], a first-generation cytidine analog, like azacytidine and decitabine (Zhou et al. 2002). However, zebularine, contrary to the other two analogs and to cytidine, lacks a 5-nitrogen and the amino group in position 4 of the pyrimidine ring, which is required to activate the atom in position 5 (= carbon in cytidine and nitrogen in azacytidine and decitabine) and thus for methylation. Therefore, upon zebularine's interaction with DNMTs, a reversible covalent intermediate is formed on the 6-position, as opposed to the irreversible complex generated after interaction of azacytidine or decitabine with DNMTs, resulting in the stabilization of the DNA/DNMT bond and significantly decreased dissociation rate, thus keeping the enzyme trapped in the interaction and causing its inhibition. Zebularine has been tested so far in vitro and in animal experiments to evaluate its effects on leukemia (AML), lymphoma, and ovarian cancer cells, but it has not yet been developed for clinical application.

Inhibitors of HMTs and HDMs have been studied not only as single agents but in attempts to increase efficacy, also in combination with HDAC inhibitors, various chemotherapy regimens, and/or other agents, such as sirtuin inhibitors and inhibitors of aurora kinases.

Many challenges have yet to be overcome, among them finding the best sequence for administration of these drugs, as it is still unclear how they may interfere with each other.

Clinical Summary

Cancer is a disease condition that is associated with genetic lesions and epigenetic modifications. During oncogenesis and malignant cellular transformation, DNA undergoes significant changes in methylation, resulting in both activation and silencing of genes. Particularly genes involved in cell cycle regulation, cell growth, and differentiation are affected leading to uncontrolled expansion of cancerous cells.

In recent years, new methods have enabled researchers to analyze DNA methylation profiles of various types of cancer cells. In some instances, methylation patterns were found, which allow prognostication of the disease course and prediction about possible treatment responses, while in other instances, no such patterns could yet be identified.

From a clinical perspective, the main interests lie on expanding the present pool of cancer-specific DNA methylation biomarkers and on the development of agents to effectively reverse the abnormal methylation patterns as seen in cancer.

Anticipated High-Impact Results

- Advancing the development of (high-throughput) technologies to study epigenetic processes
- Applying those novel technologies to advance knowledge on the methylation profile of various types of cancer

- Identifying and defining cancer type-specific methylated DNA biomarkers
- Developing DNA methylation tests that can be utilized for screening, diagnosis, prognostication, prediction, and/or monitoring of treatment response
- Developing DNA methylation tests that can be used to diagnose cancer in biologic fluids (plasma, serum urine, ejaculate, etc.) and/or stool
- Expanding basic research in epigenomics
- Advancing the development of epigenetic drugs for cancer treatment
- Advancing knowledge on how to best administer epigenetic drugs (dose, route, duration, sequence when given in combination with other drugs, etc.) by performing clinical trials

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PR

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Abstract

The action of the ovarian hormone, progesterone, is mediated through its nuclear receptor, progesterone receptor (PR). While PR is expressed in a variety of human tissues, progesterone plays a critical role in female reproduction, particularly in the normal development of the breast and the endometrium. Expression of PR is also important in breast and endometrial cancer, with the presence or absence of PR being a critical indicator of prognosis and therapeutic options in those patients. This chapter summarizes the current status of knowledge regarding the biology of PR, its role in the diagnosis and prognosis of cancer, and potential therapeutic applications in the clinic.

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PRA • PRB • Progesterone receptor (PR) • Clinical trials • DNA and carboxyl-terminal ligand-binding domains • Immunohistochemical methods • Intervening endocrine therapy • Isoforms • Mifepristone • Predictive marker • Reverse transcription-polymerase chain reaction (RT-PCR) • Role in cancer • Therapeutics • Transcription activation function • Progesterone response elements (PREs)

Target

The progesterone receptor (PR) mediates progesterone action, which plays a pivotal role in normal female reproduction. PR proteins are expressed in a variety of human tissues, including the uterus, the mammary gland, the ovaries, and the brain. PR is a member of a large family of ligand-activated nuclear transcription factors and is expressed as two distinct isoforms, PRA and PRB. Both these isoforms are transcribed from distinct promoters on a single gene residing on chromosome 11q22-q23 (Rousseau-Merck et al. 1987). PRA and PRB are detected with molecular masses of approximately 81 kDa and 115 kDa, respectively, and are identical in sequence except that the shorter form, PRA, lacks 164 amino acids at the N-terminus (Kastner et al. 1990). PR comprises a central DNA-binding domain and a carboxyl-terminal ligand-binding domain. In addition, the receptor contains a number of activation (AF) and inhibitory (IF) function elements which enhance and repress transcriptional activation of PR by association of these regions with transcriptional coregulators. The region of the protein that is unique to PRB contains a transcription activation function, AF3, in addition to AF1 and AF2, which are common to PRA (Sartorius et al. 1994). The tissue-specific distribution of PR varies greatly, from positive expression in virtually every cell in the uterus, both the epithelial and stromal (Press et al. 1988), to being only expressed in a small subset of cells in the breast (20–30% of luminal mammary epithelial cells, and some progenitor-like cells) (Clarke et al. 1997; Hilton et al. 2015).

Biology of the Target

The mechanisms by which PR regulates hormone-responsive target genes are complex. Newly transcribed cytoplasmic PR is assembled in an inactive multi-protein chaperone complex which dissociates upon ligand binding and receptor activation. Binding of progesterone to PR induces a conformational change leading to dissociation of chaperones, dimerization, binding to specific progesterone response elements (PREs) in the promoters of target genes, and recruitment of specific coactivators and general transcription factors, resulting in modulation of transcription of those genes. These ligand-dependent active transcription units can

be visualized as discrete nuclear aggregates, or foci, as opposed to the diffuse, fine granular nuclear distribution of PR in unstimulated cells (Arnett-Mansfield et al. 2007). The protein products from PR target genes mediate a diverse array of cellular activities, including cell proliferation, transcription, lipid metabolism, and membrane-associated signal transduction, indicating an extensive range of potential progesterone-mediated effects. This wide range of progesterone function is illustrated in PR knockout mouse models, which display pleiotropic reproductive abnormalities, including an inability to ovulate, uterine hyperplasia and inflammation, and severely limited lobuloalveolar development in the mammary gland (Lydon et al. 1995). PR also mediates progesterone action involving nonreproductive functions, for example, PR is widely distributed throughout parts of the brain, including the hippocampus, cortex, hypothalamus, and cerebellum, where it plays a role in cognition, mood, and myelination, among others (Brinton et al. 2008). In addition, PR also plays an important role in nonreproductive tissues such as the cardiovascular system, the central nervous system, and bone maintenance, highlighting the widespread role of this hormone in normal physiology (Conneely et al. 2003).

Because there are two PR isoforms, there is the potential for three molecular species (PRB homodimers, PRA homodimers, and PRA-PRB heterodimers) to exist concurrently and to contribute to the complexity of PR action. In humans, the majority of PR-positive (PR+) cells co-express PRA and PRB at equivalent levels, suggesting that both proteins are required to mediate physiologically relevant progesterone signaling (Mote et al. 1999, 2002). Despite this, there is some evidence for differential hormonal regulation of the two PR isoforms in the glandular epithelial cells of the endometrium. During the secretory phase of the menstrual cycle, when high circulating levels of progesterone are associated with decreased PR expression, PRA was preferentially decreased, resulting in a distinct predominance of PRB in these cells at this time (Mote et al. 1999). There is also increasing evidence that PRA and PRB are functionally unique and that PRB acts mostly as a transcriptional activator, while PRA can act as a transdominant inhibitor of PRB in situations where PRA has little or no trans-activational activity. Moreover, PRA can regulate the transcriptional activity of other nuclear receptors such as glucocorticoid, mineralocorticoid, androgen, and estrogen receptors (Tung et al. 1993; Vegeto et al. 1993).

Target Assessment

PR status is routinely assessed in all newly diagnosed invasive breast tumors and in breast cancer recurrences, using immunohistochemical methods, to determine patient eligibility for adjuvant hormonal therapy, and it has been recommended that tumors be considered PR+ if there are at least 1% positive nuclei present (Hammond et al. 2010). More recently, PR status has begun to be measured quantitatively by reverse transcription-polymerase chain reaction (RT-PCR) using

commercially developed assays, such as Oncotype DX, a diagnostic multigene expression assay (Genomic Health Inc., Redwood City, CA).

Role of the Target in Cancer

Rank: 8
While essential in normal breast development, progesterone has long been implicated in breast carcinogenesis. For example, ablation of PR in mice dramatically reduces mammary carcinogenesis (Lydon et al. 1999). In humans, removal of the ovaries reduces breast cancer risk by more than 50%, implicating the ovarian hormones in breast tumorigenesis (Trichopoulos et al. 1972). In addition, cumulative exposure to the cycling levels of estrogen and progesterone throughout a woman's reproductive life significantly influences the lifetime risk of developing breast cancer, and women exposed to progestin-containing hormone replacement therapy (HRT) have an increased breast cancer risk compared to women taking estrogen alone, or no HRT at all (Beral et al. 2011; Chlebowski et al. 2013). It has been hypothesized that the progestin component of HRT can reactivate stem-like properties in breast cancer cells and can promote the growth of pre-existing undetectable lesions by increasing the proliferation of stem and progenitor cells (Horwitz and Sartorius 2008). Conversely, the strong protective factor against breast cancer provided from a first full-term pregnancy occurring at an early age can be recapitulated in rodents by low-dose treatments of estrogen and progesterone (Sivaraman and Medina 2002). Therefore, depending on the time of administration, formulation, and dosage used, progesterone signaling mediated by PR can either promote or provide protection against breast cancer. As a result, inappropriate exposure to progesterone or its analogues, and their proliferative effects, could result in increased susceptibility to tumor initiation.

In contrast to the balanced expression of PRA and PRB in normal human tissues, progression of breast and endometrial tissues from normal to malignancy is frequently accompanied by changes in the balance of PR isoform expression. This alteration in isoform ratio, which usually is an excess of PRA, progressively increased from normal to early lesions, through to invasive cancers (Mote et al. 2002). Moreover, there is a highly significant increase in inter-cell heterogeneity of PRA/PRB expression associated with breast lesion progression to malignancy (Mote et al. 2002), suggestive of a breakdown in the mechanism which ensures concordant PRA/PRB expression. Similar to hyperplastic lesions of the breast, there is increased predominance of one PR isoform in hyperplastic areas of the endometrium, suggesting that lack of coordinated PRA/PRB expression is an early event in tumor initiation (Arnett-Mansfield et al. 2001). Furthermore, in endometrial cancers, one PR isoform is frequently completely lost, particularly in those of higher histological grade (Arnett-Mansfield et al. 2001). Disrupted expression of PRA and PRB also occurs in the normal breasts of women with a high risk of developing breast cancer due to germline mutations in BRCA1 or BRCA2 (Mote et al. 2004). Tissue from these mutation carriers differs from normal breast tissue as it displays reduced

levels of total PR, with a striking lack of PRB isoform expression, resulting in PRA predominance (Mote et al. 2004). Finally, transgenic mice which express excess PRA display aberrant mammary development, including exhibiting ductal hyperplasia, a disorganized basement membrane, and decreased cell-cell adhesion, features commonly associated with neoplasia (Shyamala et al. 1998). Thus an imbalance in the relative levels of PRA and PRB is likely to result in aberrant progesterone signaling in hormone-dependent tissues and may contribute to an altered hormonal milieu able to facilitate subsequent events in the development of cancer.

The existence of altered PR signaling in cancers is further supported by the distribution of PR. While PR foci are detected in both normal human tissues and in cancers, they differ in that PR foci are ligand independent and significantly larger in cancers, compared with normal tissue (Arnett-Mansfield et al. 2004). In addition, PR isoform composition in foci can be aberrant in cancers. In endometrial cancer, foci are associated with clinical grade, and in contrast to PRB, PRA is seldom observed in foci (Arnett-Mansfield et al. 2004). This irregular formation of PR foci in cancers suggests that PR forms complexes with different and/or larger numbers of co-regulatory proteins in these tissues, potentially leading to functionally different PR foci in normal and malignant cells, and is a physical demonstration of aberrant progesterone signaling.

High-Level Overview

Diagnostic, Prognostic, and Predictive

PR content has been established as an important predictive marker and indicator of response to therapy in a number of different tumor types. PR is frequently expressed in meningiomas, and this expression is associated with a good prognosis, and low or absent PR expression has been associated with a high risk of recurrence (Strik et al. 2002). PR can also predict survival in endometrial cancers, with high concentrations found in low-grade endometrial tumors, as well as a significant reduction in these levels associated with increasing stage and grade (Fukuda et al. 1998). Similarly, PR positivity has been shown to correlate with improved patient survival in ovarian cancers (Hempling et al. 1998).

Like the estrogen receptor (ER), PR is an important predictive marker for response to endocrine therapy in breast cancer patients (Purdie et al. 2014), and co-expression of PR provides additional predictive information to ER status in the metastatic and adjuvant setting (Bardou et al. 2003; Cancello et al. 2013; Salmen et al. 2014). PR has long been known to distinguish ER-positive tumors into two prognostically different groups (Horwitz and McGuire 1975), and PR expression in ER-positive tumors has been shown to correlate with higher survival rates in patients receiving tamoxifen (Liu et al. 2010), thus PR may be used as a prognostic factor in this group of patients. In addition, elevated PR levels have been demonstrated to better define luminal A tumors (Prat et al. 2013), and to independently correlate with

longer time to treatment failure and longer overall survival (Ravdin et al. 1992). Approximately 60% of breast tumors express PR, and since PR is an estrogen responsive gene, PR positivity indicates not only ER being present, but also being functional (Horwitz and McGuire 1975). While approximately 60% of patients have breast tumors that co-express ER and PR and have the best prognosis, a small proportion of tumors (<5%) are ER−/PR+, yet still respond more favorably to hormonal therapies than ER−/PR− tumors (Osborne et al. 2005). These underline the importance of PR as an independent marker for breast cancer development and treatment, and not merely a marker of ER function.

While uncommon for ER, the levels of PR frequently decrease dramatically following intervening endocrine therapy, and the resulting ER+/PR− secondary tumors display more aggressive characteristics, compared with tumors retaining PR expression (Balleine et al. 1999). Reports from the ATAC (Arimidex, Tamoxifen, Alone, or in Combination) adjuvant trial have shown that twice as many patients with ER+/PR− tumors suffered recurrences, compared with those with ER+/PR+ tumors (Dowsett et al. 2008), providing further support that PR expression is associated with a favorable prognosis. In contrast, unequal levels of each PR can impact on treatment response, and an overabundance of PRA in breast tumors has been associated with resistance to tamoxifen and poorer clinical outcomes (Osborne et al. 2005; Mote et al. 2015).

Therapeutics

With growing experimental as well as clinical evidence, the PR pathway is emerging as a major driver of breast cancer risk, and so blocking this pathway, either alone or in combination with other treatments, has arisen as a logical and feasible therapeutic option in certain cancers. While commonly used in the medical termination of pregnancy and for contraceptive purposes, PR antagonists have many other potential clinical purposes, including a variety of gynecological conditions, and in the treatment of tumors. Even non-steroid-dependent tumors can contain steroid receptors, and for this reason, PR antagonists may be used in the treatment of several cancer types, including breast, ovarian, prostate, and endometrial cancer, as well as meningiomas, gliomas, and leiomyosarcomas (Spitz and Chwalisz 2000). The first PR antagonist, mifepristone (or RU-486), was discovered in the early 1980s, and since then numerous other PR antagonists have been used and tested in experimental and clinical applications. To date, their therapeutic use has been limited, as the first compounds that were developed had shown some adverse side effects, for example, liver toxicity (Robertson et al. 1999), and thus they have not been suitable for long-term applications. However, these compounds remain a promising new tool for cancer therapy, and the development of safe and effective PR antagonists for the inhibition of tumor growth continues to be investigated.

Preclinical Summary

Hundreds of compounds designed to target the PR pathway have been synthesized, including pure agonists, such as progesterone and progestins, as well as PR antagonists, for example, onapristone and ZK-230211, and selective PR modulators, which display mixed agonist–antagonist properties. Extensive work has been carried out using this myriad of compounds in cell line studies and animal models, with the aim to test the safety and efficacy of potential therapeutics. Experiments using PR antagonists have demonstrated their ability to strongly inhibit tumor growth in a panel of hormone-dependent rodent mammary tumor models in vivo and in human breast cancer cell lines *in vitro* (Michna et al. 1992). They have also been demonstrated to inhibit tumor growth additively when used in combination with tamoxifen, suggesting a potential clinical benefit of adding PR antagonists to antiestrogen therapy in breast cancer patients (Möller et al. 2008). In addition, mifepristone has also been reported to display antitumor activity in ovarian cancer cells, in human prostate cancer cells in mice, and in meningioma cells *in vitro* (Dueñas-González et al. 2008). Finally, tumor growth inhibition in mouse mammary tumor models has been achieved by using synthetic antisense oligonucleotides against PRA and PRB (Lamb et al. 2005).

Clinical Summary

Several small clinical trials have demonstrated the efficacy of PR antagonists, for example, mifepristone and asoprisnil, in the treatment of women with uterine fibroids and endometriosis (Möller et al. 2008; Benagiano et al. 2014), and considerable evidence exists supporting the potential of pure PR antagonists in cancer therapy. Despite this, they have so far been of limited use in the adjuvant setting and have predominantly been utilized in the treatment of advanced stages of disease. A number of early phase II clinical studies have been carried out which examined the effects of mifepristone and onapristone in metastatic breast cancer. Mifepristone had a modest effect, with partial responses being observed in about 10% of PR+ breast cancer patients who had received no prior therapy (Perrault et al. 1996). Mifepristone has also displayed activity against ovarian cancer in patients who have recurrent tumors that have developed resistance to the chemotherapeutic drugs, cisplatin and paclitaxel (Rocereto et al. 2000). The use of onapristone, a more selective PR antagonist, gave a better response in breast cancer patients, with an overall tumor remission rate of 67%, a response rate comparable to tamoxifen (Robertson et al. 1999). This confirmed that PR antagonists can induce tumor responses in human breast cancer patients; however, some patients developed transient liver function impairment, and so its clinical development was terminated (Robertson et al. 1999). While the data supporting

the use of these PR antagonists as a single agent in breast cancer treatment has so far been underwhelming, there is reason to investigate their effect in combination with antiestrogen or aromatase inhibitor therapy on the basis of preclinical studies, and the future development of new generation PR antagonists is warranted.

Anticipated High-Impact Results

- Mechanism of PR action and genome-wide identification of PR binding sites and target genes
- Development of PR antagonists for use as adjuvant therapies

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Retinoids

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Ethan Dmitrovsky and Michael Spinella

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Abstract

Retinoids are natural and synthetic derivatives of vitamin A. The anticancer properties of retinoids have been extensively investigated in the preclinical, epidemiological and clinical settings. The primary targets of the retinoids are retinoid receptors. Retinoid receptors are ligand-activated transcription factors, members of the steroid nuclear receptor family. Retinoid receptors are deregulated in a number of malignant diseases including the fusion of RARα with PML in acute promyelocytic leukemia (APL) and the repression of RARβ in lung and head and neck cancers. In some cases, pharmacologic doses of retinoids can overcome these cancer specific defects in retinoid signaling. High-doses of retinoids induce differentiation and apoptosis of tumor cells, which is related to their physiologic effects in regulating embryonic development and maturation during hematopoiesis and in diverse epithelial tissues. Retinoid receptors have

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been cancer therapy targets for more than 30 years, and there are a number of FDA-approved retinoid-based therapies, the most impactful is the standard of care of all-trans-retinoic acid induced differentiation therapy of APL. Clinical evaluation of retinoids, especially newer synthetic retinoids in combination with molecular targeted and epigenetic therapy remains an active area of investigation in oncology.

Keywords
retinoids • retinoic acid receptors • retinoid x retinoids • promyelocytic leukemia

Target: Retinoids and Retinoid Receptors (RARs, RXRs)

Retinoid receptors are ligand-activated transcription factors and members of the steroid nuclear receptor family (Germain et al. 2006a, b). Retinoid receptors mediate the biologic effects of retinoids, a class of small lipophilic molecules derived from dietary vitamin A (Sporn et al. 1994). Retinoids are required for embryonic development and in adult life for vision, growth, immune function, reproduction, and homeostasis of diverse tissues (Sporn et al. 1994; Duester 2008). The retinoid receptors are comprised of several critical functional domains including those responsible for transactivation, DNA binding, and ligand binding. Two classes of retinoid receptors exist, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Six distinct genes encode three RAR subtypes [RARα (NR1B1), RARβ (NR1B2), and RARγ (NR1B3)] and three RXR subtypes [RXRα (NR2B1), RXRβ (NR2B2), and RXRγ (NR2B3)] and different isoforms of these retinoid receptors exist. The main physiologic ligand for RARs is all-*trans* retinoic acid (ATRA), and 9-*cis* retinoic acid is a bifunctional ligand that activates RARs and RXRs. Structural differences in the ligand-binding pocket of the retinoid receptors have made possible the design of synthetic ligands specific for RXRs and each RAR subtype. Several synthetic RAR antagonists also exist.

Biology of the Target

Retinoid receptors function as heterodimers of RXR/RAR or as RXR homodimers. RXR also forms heterodimers with several other members of the nuclear receptor superfamily including the vitamin D receptor (VDR), the peroxisome proliferator-activated receptor (PPAR), and the liver x receptor (LXR) (Germain et al. 2006a, b). ATRA regulates gene expression via binding of ATRA-bound RXR/RAR heterodimers to consensus retinoic acid response elements (RAREs) in the proximal promoter region of RAR target genes. An important aspect of the pharmacology of RXR receptors is the concept of permissive and nonpermissive RXR heterodimers (Rochette-Egly and Germain 2009). Permissive heterodimers can be activated by either RXR ligands or the binding partner ligand. Examples include RXR

heterodimers with PPAR, LXR, farnesoid X receptor (FXR), and pregnane X receptor (PXR). Nonpermissive RXR heterodimers are only activated by the binding partner ligand, although RXR ligands can further enhance activation. Examples include RXR heterodimers with RAR, VDR, and thyroid receptor (TR).

Retinoid receptors regulate gene transcription by ligand-dependent recruitment of transcriptional coregulators (O'Malley et al. 2008). In the absence of ligand, retinoid receptors recruit corepressors such as silencing mediator of RAR and TR (SMRT) and nuclear receptor corepressor (NCoR). Transcriptional repression occurs through further recruitment of histone deacetylases (HDACs), DNA methyltransferases (DNMTs), and other factors that maintain chromatin in a repressive state. Upon ligand binding, a conformation change occurs in the retinoid receptors that results in the release of corepressors and the recruitment of coactivators such as steroid receptor coactivators 1, 2, and 3 (SRC1-3), CREB-binding protein (CBP), and p300. These factors are associated with histone acetyltransferase activity.

Direct target genes of retinoid receptors have been identified including genes involved in retinoid signaling such as RARβ, CRBPII, CRABPII, and RIP140 (Spinella et al. 2003; Tang and Gudas 2011). Other classic RAR target genes include genes involved in differentiation and development such as the homeobox (Hox) genes. In addition, numerous microarray-based studies have confirmed the long-held belief that retinoids target hundreds of genes in a context-dependent manner thereby mediating pleiotropic effects on growth and differentiation-signaling pathways.

A strong rationale exists for the use of retinoids in cancer therapy and chemoprevention based on preclinical, epidemiological, and clinical findings (Lippman and Lotan 2000; Freemantle et al. 2003). Retinoids alone and in combination with other agents can prevent or inhibit progression of cancer in carcinogen-induced and spontaneous transgenic tumor models (Moon et al. 1994). There is also evidence for an inverse relationship between cancer incidence at specific sites and serum vitamin A levels. Retinoids have been shown to have activity alone and in combination with other classes of antineoplastics for the treatment of overt malignancy and premalignancy and for the prevention of certain second cancers in high-risk patients (Freemantle et al. 2003). One of the most successful clinical examples of retinoid activity is that of ATRA in the differentiation therapy of a rare but lethal leukemia, acute promyelocytic leukemia (APL) (Nowak et al. 2009).

Target Assessment

ATRA treatment for APL is FDA approved, based at least partly on the ability of ATRA to induce terminal differentiation of this leukemia. The etiology of APL is tightly linked to the reciprocal t(15;17) rearrangement resulting in the fusion product PML-RARα. A PCR-based test and other genetic tests are available for the detection of PML-RARα. These are commonly used for initial APL diagnosis and to evaluate response to therapy and to monitor for minimal residual disease in APL.

Molecular studies on cell lines and several mouse studies established that PML-RARα plays an etiologic role in leukemogenesis by acting in a dominant-negative

manner to inhibit wild-type RARα activity during granulocytic maturation (Nowak et al. 2009). This is due to transcriptional repression mediated by stable association of PML-RARα with HDAC-containing corepressor complexes that are resistant to physiologic levels of ATRA. However, therapeutic levels of ATRA dissociate the corepressor complexes, restoring normal activation function to PML-RARα. In contrast, PLZF-RARα-expressing APL is resistant to pharmacologic levels of ATRA presumably due to tighter binding of PLZF to corepressor complexes, despite therapeutic levels of ATRA (Petrie et al. 2009).

RARβ is a putative tumor suppressor gene that is frequently silenced in solid tumors and reexpressed after retinoid treatment, in some cases when combined with HDAC or DNA methylation inhibitors. RARβ expression is used experimentally as a biomarker in retinoid-based clinical trials by assessing its levels in cancers by a variety of RNA and immunological-based techniques, although no clinically approved, standardized test yet exists. Silencing of RARβ may be a mechanism that accounts for resistance to retinoid therapy in cancer therapy or prevention (Klass and Shin 2007).

Role of the Target in Cancer

Rank: “unknown” to 10

Unknown to 1-2-3-4-5-6-7-8-9-10:10

High-Level Overview

Diagnostic, Prognostic, Predictive

Ligands for RARs are used clinically for the treatment and prevention of certain cancers, and in specific cases, the expression of RARs is used as a diagnostic or prognostic marker. Investigations as to the role of RARs as a cancer target are ongoing in both the in vitro, preclinical, and clinical settings. There is intense interest to uncover the molecular mechanisms of retinoid antitumor effects.

The test for the presence of the PML-RARα fusion product is diagnostic for APL. After therapy, absence of a positive test is an indication of a complete remission, and presence of a positive test is indicative of relapse or ATRA resistance. Other tests are available for the rare alternative RARα fusion products in APL involving PLZF, NuMA, NPM, and STAT5B (Petrie et al. 2009).

There is a critical need for prognostic and predictive tumor biomarkers during therapy and prevention trials involving retinoids. Promising biomarkers include RARβ, cyclin D1, cyclin E, and Ki-67 (Freemantle et al. 2007; Klass and Shin 2007). Serum levels of ATRA and other retinoids are often monitored during therapy, as failure to sustain long-term retinoid levels is a common mechanism observed during clinical resistance (Freemantle et al. 2003). This has been associated with induction of specific cytochrome P450 enzymes, which degrade ATRA. The

value of measuring intra-tumor retinoid levels has also been recently evaluated (Dragnev et al. 2007). Other mechanisms of resistance to ATRA and other retinoid agonists include mutations in the ligand-binding domain of PML-RARα, sequestration of retinoids by induction of retinoid-binding proteins, alterations in coregulator activity, and repression of RARβ and other retinoid receptors through promoter methylation (Freemantle et al. 2003).

Therapeutics

ATRA (tretinoin) is FDA approved for the treatment of APL (FAB M3). ATRA-based therapy has dramatically changed the course of APL from an often lethal disease to one that is now cured in the majority of cases. The combination of ATRA followed by anthracycline therapy results in long-term remissions in greater than 70% of APL cases (Grimwade et al. 2010).

The synthetic RXR ligand, bexarotene (Targretin), is FDA approved for the treatment of cutaneous T-cell lymphoma (Lansigan and Foss 2010). In clinical trials, bexarotene was found to induce a response in 50% of patients with refractory cutaneous T-cell lymphoma with minimal toxicity. The retinoid, 9-*cis* retinoic acid (alitretinoin) is FDA approved for the topical treatment of Kaposi's sarcoma. Alitretinoin gel is effective in patients with cutaneous Kaposi's sarcoma who have never received treatment or have failed or are refractory to previous treatments. Alitretinoin gel produces objective responses and disappearance of some lesions in patients with AIDS-related Kaposi's sarcoma, reduces the rate of progression, increases the time to progression and provides durable responses (Dezube et al. 2004). The natural retinoid, 13-*cis* retinoic acid (isotretinoin), is successfully used to treat minimal residual disease in high-risk neuroblastoma after induction and consolidation therapies (Matthay et al. 2009). A large randomized trial has shown that the nonclassical retinoid 4-hydroxy(phenyl)retinamide (fenretinide) induced a significant breast cancer risk reduction in premenopausal women aged 40 years or younger (Veronesi et al. 2006).

Early phase II and small-scale randomized trials reported that high-dose retinoids successfully treated preneoplastic diseases including oral leukoplakia, cervical dysplasia, and xeroderma pigmentosum (Lippman et al. 1995). Similarly, small-scale retinoid studies have reported reduced occurrence of second malignancies in the liver and certain aerodigestive tract cancers (Lippman et al. 1995). Beneficial effects were also reported in treating juvenile chronic myelogenous leukemia, mycosis fungoides, squamous cell cancers of the skin and cervix, and advanced renal cancer, as reviewed (Freemantle et al. 2003). However, recent phase III trials have not shown overall benefits and even harmful effects in several of these settings (especially in smokers), which may be due to dose reductions from toxicity, reversibility, and emergence of resistance, among other mechanisms (Goodman et al. 2004). Subset analyses are underway to determine whether more defined disease groups benefit from retinoid therapy.

Several natural and synthetic retinoids are currently being explored in the clinic with innovative trial designs as single agents or in combinations with other agents for the prevention and treatment of several malignancies. There are several ongoing trials with rexinoids such as targretin that is specific for RXRs (Tanaka and De Luca 2009). Rexinoids are less toxic than naturally occurring retinoids, perhaps since they only activate RXR homodimers and permissive RXR heterodimers. Several clinical studies are evaluating whether combining retinoids with epigenetic agents including the DNA methylation inhibitor, decitabine, and HDAC inhibitors will be effective in treating non-APL forms of AML as well as other malignancies (Soriano et al. 2007; Stapnes et al. 2009). Several studies are assessing the benefits of combining retinoids with molecular targeted therapies, for example, epidermal growth factor receptor-tyrosine kinase inhibitors to combat lung cancer (Dragnev et al. 2005).

Preclinical Summary

Cell line studies have been instrumental in defining mechanisms of retinoid action and are uncovering retinoid target genes of biologic and therapeutic importance. Extensive preclinical work has established the anticancer properties of specific retinoids in rodent models. These include studies of chemical-induced carcinogenesis in rodent models and spontaneous carcinogenesis in transgenic models. Retinoids have been shown to have in vivo activity as both a preventive and therapeutic agent, alone and in combination with other agents. Ongoing and future preclinical studies are vital to define optimal scheduling, dosing, and how best to combine retinoids with conventional cytotoxic or newer, molecular targeted therapies.

Clinical Summary

Retinoid receptors have been cancer therapy targets for more than 30 years, and there are successful examples of clinical activity. Retinoids induce differentiation and apoptosis of tumor cells. ATRA treatment of APL is a validated and successful example of differentiation therapy and has altered the clinical management of this leukemia. Differentiation and to a lesser extent apoptotic tumor responses are the basis for the use of retinoids as anticancer agents. Numerous clinical trials have been completed or are ongoing. However, clinically active doses of retinoids in many settings result in toxicities, and therapeutic effects are often reversible and subject to the development of resistance. Newer, synthetic retinoids with less clinical toxicity are currently being evaluated along with a shift from combining retinoids with classic cytotoxic therapy to combining retinoids with molecular targeted and epigenetic therapy.

Anticipated High-Impact Results

- Treatment and prevention studies using newer synthetic retinoids, especially rexinoids
- Treatment and prevention studies using novel combinations of retinoids with other agents especially epigenetic and molecular targeting agents
- Uncovering new drug targets based on further understanding of retinoid downstream targets in cancer

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Topoisomerase 1

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Yves Pommier

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Abstract

Humans have two type IB topoisomerases (Top1 and Top1mt), and TOP1 is one of the six vertebrate topoisomerase genes (TOP1, TOP1MT, TOP2A, TOP2B, TOP3A and TOP3B). Topoisomerase activity is essential for transcription and a replication as DNA is an extremely long polymer (up to 2 meters in the nucleus of human cell) that readily forms loops, supercoils, knots and catenanes. Top1 autoantibodies are commonly found in the blood of patients with autoimmune diseases (Scleroderma pigmentosum). Top1 is generally overexpressed in cancer cells and the target of widely used camptothecin derivatives. Novel indenoisoquinoline Top1 inhibitors and targeted delivery Top1 inhibitors are in clinical trials.

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Target: DNA Topoisomerase I (Top1)

TOP1 is one of the six topoisomerase genes present in human cells (Pommier et al. 2010; Pommier 2012). DNA topoisomerases are enzymes that enable topological changes in the DNA three-dimensional structure. Their activity is essential for transcription and replication as DNA is an extremely long polymer (up to 2 m in the nucleus of a human cell) that readily forms loops, supercoils, knots, and catenanes. Humans have two type IB topoisomerases (Top1 and Top1mt), two type II topoisomerases (Top2α and Top2β), and two type IA topoisomerases (Top3α and Top3β). Type I enzymes cleave and religate one strand of the DNA duplex to change DNA topology (reversible DNA nicking-closing that translates into DNA untwisting activity), whereas type II topoisomerases cleave both strands in concert (reversible DNA double-strand breaking that enables DNA duplex passing activity).

Biology of the Target (Top1 and Top1mt)

Top1 activity was discovered by Champoux and Dulbecco in 1972 as the human DNA untwisting enzyme (Champoux and Dulbecco 1972), soon after the discovery that DNA was under torsional strain (i.e., supercoiled). The gene was cloned 15 years later (D’Arpa et al. 1988), and a second Top1 gene (TOP1mt) was discovered another 13 years later in the nuclear genome of all vertebrates, whose product is specific for mitochondrial DNA (Zhang et al. 2001). Thus, both Top1 enzymes divide their tasks: Top1 for the nuclear genome and Top1mt for the mitochondrial genome. Top1 and Top1mt probably arose by evolutionary duplication of a common ancestral TOP1 gene still found in invertebrates (including primitive chordates, plants, and yeast). Top1 and Top1mt are highly efficient enzymes that relax DNA supercoils in the absence of energy cofactor, metal catalyst, and even at ice temperature. The DNA nicking-closing is fast (thousands of rounds per minute) (Seol et al. 2012).

However, Top1 can be trapped while it forms DNA cleavage complexes. Topoisomerase I cleavage complexes (Top1cc) are the target of highly effective anticancer drugs (see below). They are also stabilized by endogenous base alterations (abasic sites, mismatches, oxidative base damage) and carcinogenic DNA adducts (Pommier 2006, 2009). Moreover, high-level Top1cc form in cells that undergo programmed cell death (apoptosis) (Sordet et al. 2004).

Target Assessment

Top1 and Top1mt can be detected by gene expression and protein levels using Western blotting, Elisa assays, and immunofluorescence microscopy (Pfister et al. 2009). Top1 is concentrated in the cell nucleus with the highest levels in nucleoli, whereas Top1mt is concentrated in mitochondria.

Role of the Target in Cancer

The target should be ranked 1 (one) based on the fact that it is validated for at least four clinically approved drugs (topotecan, irinotecan, belotecan, and onivyle) and several drugs are in clinical trials with novel active groups (indenoisoquinolines) and targeted delivery).

Top1 is the target of anticancer topoisomerase-targeted drugs that convert the enzyme into a cellular poison. Therefore, the higher the Top1 levels, the greater the sensitivity of the cell to anticancer Top1 poison (Burgess et al. 2008; Miao et al. 2007). Elevation of Top1 levels in colon cancers has been proposed as a determinant of sensitivity of colon cancers to camptothecin derivatives (Giovanella et al. 1989). High levels of Top1 might be a useful predictor of response to Top1cc-targeted drugs (Pfister et al. 2009).

Top1 can act as an efficient DNA recombinase (Pommier et al. 1995) and has recently been shown to generate DNA mutations (short deletions and insertions) (Kim et al. 2011) that could be carcinogenic.

High-Level Overview

Diagnostic, Prognostic, and Predictive

Top1 autoantibodies are commonly found in the blood of patients with autoimmune diseases (Scleroderma pigmentosum). The Top1 autoantibodies were discovered as diagnostic and potentially prognostic markers (Scl-70 antibodies) in scleroderma patients before Top1 was recognized as their antigen.

Top1 is an essential and highly conserved gene across species. Disease-causing mutations have not been reported. Based on murine knockout experiments, Top1mt is not an essential gene (Douarre et al. 2012). However, it is important for proper mitochondrial functions (Douarre et al. 2012; Khiati et al. 2014, 2015), and TOP1mt mutations have begun to be reported in patients with mitochondrial diseases (Wang et al. 2011).

Therapeutics

The selective targeting of Top1 by the plant alkaloid, camptothecin, was a serendipitous discovery when it was tested as a candidate Top2 inhibitor (Hsiang et al. 1985; Wall and Wani 1995). Three camptothecin derivatives are presently approved (topotecan, irinotecan, and belotecan) for the treatment of colon, ovarian, lung, and pediatric cancers. Because of the clinical anticancer activity of camptothecins but also because of their limitations (chemical instability, limited cellular accumulation due to drug efflux pumps, and dose-limiting bone marrow suppression and intestinal toxicity), non-camptothecin Top1 inhibitors are in clinical development, the indenoisoquinolines LMP400 (indotecan), LMP776 (indimitecan), and LMP744 (Pommier 2006, 2009; Pommier et al. 2010). Several targeted-delivery Top1 inhibitors are in clinical trials. One of them was recently approved (Onivyle 2015).

Preclinical Summary

Top1 and Top1mt are two critical genes for efficient DNA metabolism (DNA replication and transcription) (Douarre et al. 2012; Khiati et al. 2014, 2015; Miao et al. 2007). Top1 is the target of commonly used anticancer drugs derived from the plant alkaloid camptothecins.

Clinical Summary

Top1 antibodies (Slc-70) are used for the diagnosis of autoimmune diseases. Generally high Top1 level in tumors is one of the determinants of tumor response. Top1 mutations are rare in tumor samples and are not involved in lack of response to camptothecin drugs. Top1mt mutations need to be considered for patients with mitochondrial diseases that are not explained by mutations in mitochondrial genes or nuclear genes that encode mitochondrial DNA-processing enzymes. Top1 antibodies (Slc-70) are used for the diagnosis of autoimmune diseases.

Anticipated High-Impact Results

Top1 inhibitors are part of the current anticancer armamentarium. Novel non-camptothecin inhibitors are in clinical trials with Top1 levels determination serving to monitor therapy.

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Topoisomerase 2

94

Bruce C. Baguley

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Abstract

Topoisomerase IIα and IIβ, with molecular weights of 170 K and 180 K, respectively, are homodimeric enzymes that catalyze the passage of one double-stranded DNA helix through another. Together with topoisomerase I, these enzymes are able to relieve the torsional stress in chromatin caused by DNA helix unwinding during RNA and DNA synthesis. Although catalytic inhibitors of topoisomerase IIα and IIβ are known, drugs that poison the action of these enzymes, leading to failure of DNA religation and subsequent DNA double-strand breakage, are more significant in terms of cancer therapy. Clinical topoisomerase II poisons bind to the enzyme DNA complex with doxorubicin and epirubicin binding mainly to DNA, etoposide mainly to the enzyme, and

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amsacrine binding to both. Selectivity for individual tumor types depends on the activity of inward and outward drug transporters, of which P-glycoprotein is an example. In addition to transport, cellular responses to DNA breakage and immunological responses to dying cells also contribute to antitumor activity. Further understanding of the multiple factors that influence cellular uptake and retention of drug, as well as of tumor and host tissue responses, is important for the development of future drugs in this class.

Keywords
Topoisomerase • DNA binding • Antitumor • Doxorubicin • Daunorubicin • Etoposide • Amsacrine

Target

Topoisomerases IIα and IIβ are homodimeric enzymes with molecular weights of 170 K and 180 K, respectively. They catalyze an essential cellular function: that of passing of one double-stranded DNA helix through another at a high rate and in an error-free fashion. The corresponding genes, *TOP2A* and *TOP2B*, are located on chromosomes 17 and 3, respectively, and show similar gene structure. The name topoisomerase comes from the mathematical branch of topology, which deals with changes of shape associated with deformation but not with rupture or fusion. Studies with closed circular duplex DNA demonstrated that different forms of DNA could exist with the same primary sequence but with a different number of helical turns; these were termed topoisomers, and enzymes that catalyzed the transition between different topoisomers were called topoisomerases. Topoisomerases IIα and IIβ can be compared with topoisomerase I the other main mammalian topoisomerase and also with a third minor class (topoisomerase IIIA/B) (Wang 2002). Topoisomerases I and IIIA/B allow the rotation of a single strand of the DNA double helix through another while topoisomerase II catalyzes the passage of one double strand of the DNA double helix through second double strand.

The molecular action of topoisomerase IIα/β is summarized in Fig. 1. The initial DNA breakage is accompanied by the transfer of the 3′-phosphate end of the broken DNA to a protein tyrosine hydroxyl group on the enzyme active site; the remaining free DNA 5′-end is therefore potentially free to rotate. Because topoisomerase II is a homodimeric enzyme, the monomers catalyze breakage on both complementary DNA strands, with the two breaks staggered by four nucleotides from each other. The energy for the reversible transfer of a DNA end to the tyrosine hydroxyl group is provided by the supercoiling stress, but the energy for strand passage associated with topoisomerase II action requires ATP. Each of the two topoisomerase II monomers has three main structural domains (Vavrova and Simunek 2012). The N-terminal domain is the most conserved among species, contains an ATPase, and is called the N-gate. The second domain binds to the DNA strand that is destined to be broken

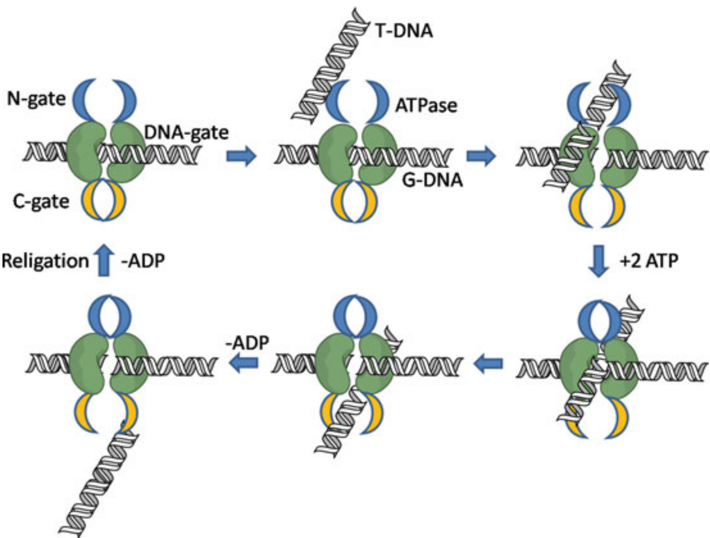

Fig. 1 The topoisomerase IIα/β catalytic cycle. The first step in the cycle is the binding of the topoisomerase II dimer to double-stranded DNA, followed by the opening of the N-gate to admit the second DNA strand (T-DNA); this is accompanied by ATP hydrolysis. The next step is the binding of ATP (one molecule to each monomer) to the middle domain, causing a conformational change in the enzyme and locking the T-DNA in the C-gate. This conformational change also results in the transfer of a DNA end to a tyrosine hydroxyl group on each of the monomers, leaving the other DNA end free and creating a channel between the two enzyme monomers. A further conformational change allows the T-DNA to be translocated through the channel but constrained in the enzyme by the C-gate; this step is driven by ATP hydrolysis to ADP. The final step involves a further conformational change that transfers the DNA ends from enzyme tyrosine links back to the corresponding free DNA ends. This restores the original DNA sequence but potentially alters the topology, as well as releasing ADP from the DNA-gate, opening the C-gate and releasing the T-DNA

(G-DNA in Fig. 1) and is called the DNA-gate. The third N-terminal domain is called the C-gate.

Biology of the Target

Chromatin in the mammalian chromosome is organized in a series of loops or domains, each anchored to a proteinaceous core (Gasser et al. 1986), and the anchoring of each chromatin loop to the core means that DNA is topologically constrained. Both RNA and DNA synthesis are associated with localized DNA unwinding, and these processes are completely dependent on a mechanism to relieve both positive and negative supercoiling. All topoisomerases can relieve such topological stress, and individual cells may vary in which individual enzymes make the

biggest contribution. However, the cellular topoisomerase IIα makes a major contribution since it is associated with chromosome domains and is cell cycle regulated with increased activity in S and G_2 phases. In addition, this enzyme becomes phosphorylated in G_2 phase, further increasing its activity. Following completion of S phase, cells enter G_2 phase where the DNA of the two sister chromosomes are progressively separated from each other in preparation for mitosis and cell division. Separation of chromosomes absolutely requires the DNA double-strand passing activity of topoisomerase IIα/IIβ.

Target Assessment

As shown in Fig. 1, the molecular action of topoisomerase II involves a series of steps, often termed the catalytic cycle (Vavrova and Simunek 2012). The main aim of topoisomerase II-directed anticancer therapy is to subvert this highly delicate action in order either to induce cell death or to prevent progress through the cell division cycle. Drugs that inhibit the religation step are often referred to as topoisomerase II poisons, while drugs that inhibit other steps in the catalytic cycle are usually referred to as catalytic inhibitors. Catalytic inhibitors include ICRF-187 (dexrazoxane), merbarone, novobiocin, and coumermycin (Fortune and Osheroff 1998); it is likely that all DNA-binding agents can interfere with this cycle, since they potentially interfere with the binding of topoisomerase II to DNA. However catalytic inhibitors have not found extensive use clinically, and the focus of this review will be on topoisomerase II poisons.

Topoisomerase II poisons act by distorting the structure of either the enzyme or the DNA (or both) so that the religation step (Fig. 1), which is critically dependent on the geometric relationship between the free end of the G-DNA and the phosphotyrosine-linked strand of the G-DNA, is prevented. Failure of this step appears to affect the stability of the DNA-topoisomerase complex, particularly in the presence of topological (unwinding) stress, so that the topoisomerase II monomers, each attached to one DNA end, dissociate from each other to create a double-stranded DNA break. Because the generation of double-stranded DNA breaks resembles that of ionizing radiation, topoisomerase II poisons are sometimes referred to as radiomimetic agents. There are three main classes of clinical topoisomerase II poisons, examples of which are shown in Fig. 2. The first class includes doxorubicin, epirubicin, and daunorubicin; these drugs comprise a DNA-intercalating tetracyclic chromophore attached to a positively charged (amino sugar) side chain, which lies in the minor groove of the double helix. Drug intercalation between base pairs of the DNA partially unwinds it, preventing ligation. The second class comprises drugs such as etoposide and teniposide, which bind predominantly to the enzyme, causing an allosteric change that again prevents the alignment of the DNA free end with the phosphotyrosine-bound end and thus prevents ligation. The third class of drug is

Fig. 2 Examples of topoisomerase II poisons: doxorubicin, etoposide, and amsacrine

typified by amsacrine; here the acridine moiety intercalates into DNA, but the aniline moiety, which lies in the DNA minor groove, also makes productive contact with topoisomerase II in a ternary complex and distorts both DNA and enzyme, again preventing religation.

Role of the Target in Cancer

Rank: 10

Topoisomerase IIα is often expressed in higher amounts in cancer cells as a consequence of an increased demand for DNA topological changes during DNA replication. Increased topoisomerase II expression may also reflect gene amplification. The frequency of induction of double-stranded DNA breaks by a topoisomerase II poison is related to the number of active enzymes and possibly to the degree of local topological stress, which influences the separation of the topoisomerase II monomers. Topoisomerase II therefore differs from many other chemotherapeutic targets in that overexpression is associated the greater susceptibility to therapeutically active drugs. The amounts and subcellular locations of topoisomerase IIα and IIβ can be measured with specific antibodies, and overexpression of topoisomerase IIα has been reported in cancer tissue (Di Leo et al. 2011). However, there is unfortunately no clear relationship between the cellular amount of topoisomerase II enzymes and the susceptibility of cells to topoisomerase II poisons.

Preclinical Summary

Historical Development

Topoisomerase II-directed drugs have a long history that has its origins in the development of antibiotics for the treatment of bacterial infections. It was reasoned that, if bacteria could produce compounds such as streptomycin that killed other bacteria, they might also produce compounds that could kill cancer cells. Actinomycins were isolated in the 1940s (from actinobacteria), and following demonstration of the broad cytotoxic properties of actinomycin D, clinical studies were carried out; 13 children with inoperable cancer were treated with actinomycin D with or without radiotherapy. Four regressions were reported, leading to subsequent widespread use in pediatric cancer. The molecular target of actinomycin D was completely unknown at this stage, but studies in the 1960s showed it to bind very strongly to DNA and that it intercalated between adjacent DNA base pairs. A further important advance was made with the discovery of further products of the Streptomyces family, the anthracyclines. Daunorubicin (daunomycin) was found to have particular activity against leukemia, while doxorubicin (Adriamycin) proved to have highly significant activity against breast cancer (Arcamone 1985). Both drugs, as well as related antibiotics, were also found to bind to DNA by intercalation. Another early avenue of research uncovered a completely different kind of drug, a plant product from the root of the mandrake, which had long been known for its medicinal properties. One component, podophyllotoxin, had been characterized as a mitotic poison, but unexpectedly, related compounds, called epipodophyllotoxins, were found to have antitumor activity; unlike podophyllotoxin they did not bind to tubulin, and they also did not intercalate into DNA (Hainsworth and Greco 1995). Studies of using natural products were complemented by synthetic approaches, giving rise to the clinical agents amsacrine (Arlin 1983) and mitoxantrone (Smith 1983). The way in which these structurally diverse compounds were functionally related to each other had to wait until 1984, when all were found to target the same enzyme, topoisomerase IIα/β (Nelson et al. 1984).

Subsequent studies demonstrated the activity as topoisomerase II poisons of many more antibiotics, particularly analogs of doxorubicin, with a number of these being advanced to clinical trial. They also led to synthesis of hundreds of synthetic compounds with experimental activity, and some of which were also advanced to clinical trial (Denny et al. 1983). The most common of these had structural similarities to doxorubicin in that they comprised a DNA-intercalating chromophore (usually bicyclic, tricyclic, or tetracyclic) attached to a positively charged side chain. However, not all molecules of this general structure were active, and the ability to inhibit the topoisomerase II religation step efficiently appears to have a complex number of steric and kinetic criteria. Early preclinical studies to discover new topoisomerase II poisons generally utilized murine tumors such as the L1210 and P388 leukemias, the Lewis lung carcinoma, and the B16 melanoma, complemented in some cases by human tumor xenografts, as screening strategies. Studies on natural products were complemented by those with synthetic drugs,

particularly those developed in analog programs. Compounds identified as having high experimental activity were subjected to further study of pharmacology and metabolism prior to clinical trial (Wadler et al. 1986).

Drug Transport

Another area of great preclinical interest is the relationship between drug structure and antitumor activity. Many factors contribute to the overall efficacy of topoisomerase II poisons as cytotoxic agents, and some of the steps are shown in Fig. 3. The drug firstly has to reach the target cells, which in the case of solid tumors requires drug movement from the blood through multiple layers of cells. Some drugs, such as doxorubicin and mitoxantrone, are taken up relatively slowly and may require specific transporters to reach the cytoplasm, while others such as amsacrine are taken up rapidly. Evidence has been provided from the analysis of the US National Cancer Institute 60-cell line panel that in vitro sensitivity to the drugs doxorubicin and mitoxantrone is significantly related to the expression of the OCTN1 (SLC22A4) inward cation transporter (Okabe et al. 2008), emphasizing the potential importance of inward transporters to sensitivity and selectivity.

Topoisomerase II poisons are often substrates of drug transporters such as P-glycoprotein (Pgp; ABCB1), multidrug resistance protein 1 (MRP1, ABCC1), and breast cancer resistance protein (BCRP, ABCG2). In some cells these proteins

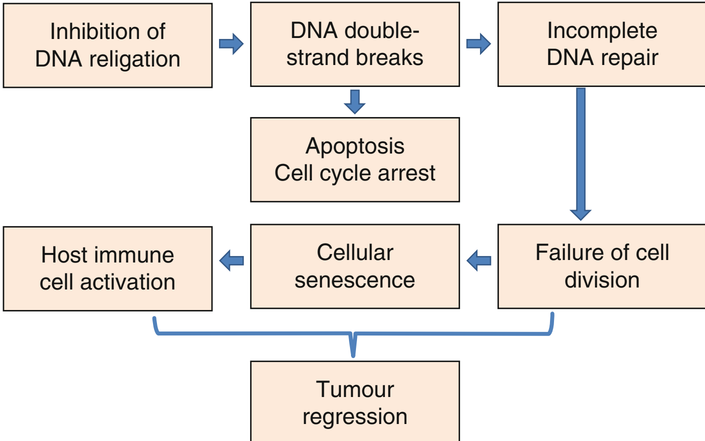

Fig. 3 Steps in the cellular action of topoisomerase II poisons. Interference of the DNA religation step in the topoisomerase II catalytic cycle leads to DNA damage including DNA double-strand breaks. Cellular responses to these breaks increase the frequency of three processes: cell death (apoptosis), cell cycle arrest, and DNA repair. It appears that incomplete DNA repair leads to a secondary effect which is manifested near the end of the cell cycle and often called mitotic catastrophe, again causing cell cycle arrest and senescence. Cellular changes in these tumor cells appear to result in activation of several host immune responses, which are thought to contribute to elimination of surviving tumor cells

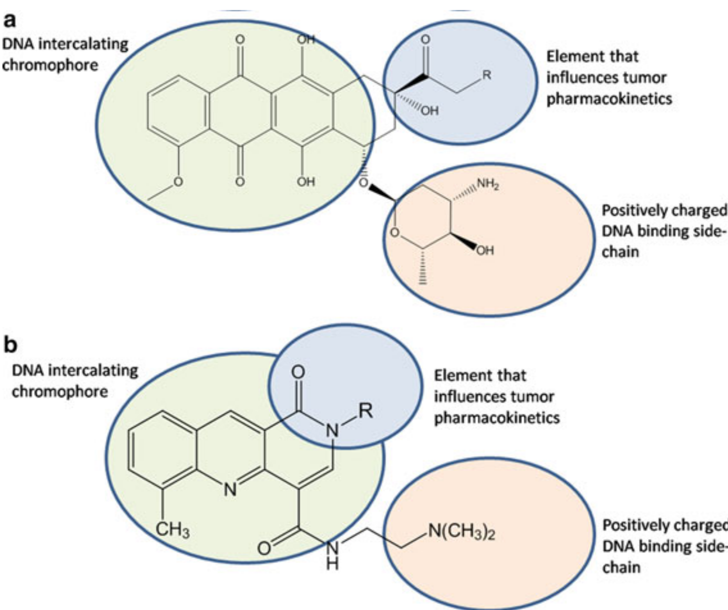

Fig. 4 Molecular features of topoisomerase II poisons that may modify tumor tissue pharmacokinetics. Two pairs of DNA-binding anticancer drugs are shown; both comprise a DNA-intercalating chromophoreDNA intercalating chromophore (*green* shading) attached to a positively charged side chain (*brown* shading) that interacts with the phosphodiester backbone of the DNA and stabilizes drug binding. In the case of the established anthracycline drugs, (**a**) the structure of doxorubicin and daunorubicin differ in a key area of the *gray*-shaded zone (R = OH and R = H, respectively). The two drugs differ in both tumor pharmacokinetics Lukka et al. 2012 and clinical antitumor activity (Arcamone 1985). In the case of SN 28049 and SN 28101, two experimental benzonaphthyridine-based experimental anticancer drugs, (**b**) the structures also differ in the *gray*-shaded zone (R = CH_3 and R = H, respectively). These differ substantially in tumor pharmacokinetics (Martini et al. 1977)

act primarily to expel drug from the cell, while in others the drug is sequestered in cytoplasmic vesicles, some of which lead to drug expulsion by exocytosis. In a solid tumor microenvironment, a drug that is released from one cell may be taken up by a neighboring cell, and tumor tissue pharmacokinetics may therefore play a major role in the overall efficacy of individual drugs. The alteration of tumor tissue pharmacokinetics by small differences in structure can be illustrated by comparison of the structures shown in Fig. 4. In the first example, doxorubicin and daunorubicin, which differ only by the presence of a hydroxyl group attached to the chromophore, showed large differences in tumor pharmacokinetics using the Lewis lung murine adenocarcinoma. Mice were treated with similar drug doses (20 mg/kg), and tumor tissue concentrations over the period 0–24 h were measured (Martini et al. 1977). The tumor AUC (area under the concentration-time curve) was calculated from this published data to be approximately threefold higher for doxorubicin than for daunorubicin, indicating that doxorubicin provided a much higher tumor tissue exposure

than doxorubicin, although providing similar plasma drug concentrations. These differences were also reflected in antitumor activity (Martini et al. 1977). In the second example, the tumor pharmacokinetics of two benzonaphthyridine derivatives, each known to have activity as topoisomerase II poisons and differing only in the presence of a methyl group on the chromophore, were compared using the colon 38 murine adenocarcinoma. Here the differences were even more dramatic; following administration of the same drug dose, the tumor tissue AUC for the methyl derivative was more than 80-fold higher than that of the corresponding drug lacking the methyl group, despite similar plasma AUC values (Lukka et al. 2012). The difference in AUC values reflected a longer tumor tissue half-life, which was more than three times as long as the half-lives for normal tissues. The mechanisms involved in this higher tumor retention are not yet understood but appear to involve specific sequestration of drug in cytoplasmic vesicles.

Cellular Responses

Once a topoisomerase II poison has entered the cell nucleus, it can destabilize the topoisomerase II-DNA complex and induce DNA double-stranded breaks, as described earlier in this chapter. DNA damage leads to a complex cellular repair response involving a number of proteins, and the efficiency of this response will vary with different cells, presenting a further basis for antitumor selectivity. A complex of proteins including MRE11, NBS1, RAD50, and ATM (ataxia-telangiectasia mutated) kinase form around the break site (Stracker and Petrini 2011), and ATM kinase has a variety of targets including H2AX, a variant of the H2A histone, that comprises 10–15% of the total H2A histone. The chromatin surrounding the breakpoint is thus modified by extensive γ-phosphorylation of H2AX and provides a useful biomarker for DNA damage. ATM kinase also phosphorylates CHK2, a checkpoint kinase that phosphorylates a number of proteins, including the transcription factor p53, the repair protein BRCA1, and the protein phosphatases cdc25A and cdc25C. The breakpoint also attracts the repair enzyme poly(ADP-ribose) phosphorylase-1 (PARP-1), which adds poly(ADP-ribose) groups to a number of proteins and modulates cellular responses.

While cellular responses to DNA double-strand breaks can lead to cell cycle delays and, in extreme cases, apoptosis, the main response is DNA repair and eventual resumption of progress through the cell division cycle. Repair of DNA double-strand breaks involves both PARP-1 and BRCA1 and occurs by two main mechanisms, nonhomologous end joining and homologous recombination, each of which is susceptible to error. Through processes not yet fully understood, it is likely that entry into mitosis with unrepaired DNA lesions leads to a process often termed mitotic catastrophe, which is associated with failure of cell division, induction of cellular senescence, induction of autophagy, and induction of cell death. Individual differences in the efficacy of double-strand DNA repair and cell death induction will contribute to the selectivity of topoisomerase II poisons.

An important late effect of topoisomerase II poisoning is the induction of host immune responses (Fig. 3). Cells may release the protein HMGB1, which activates TLR4 (toll-like receptor 4) on immune cells (Apetoh et al. 2007). Furthermore, an endoplasmic stress response may be triggered in which calreticulin, a protein normally associated with the endoplasmic reticulum, is translocated to the plasma membrane and facilitates recognition by dendritic cells and stimulation of host immunity. Reduced host immune responses could be a major reason for treatment failure.

Toxicity

Topoisomerase II poisons act not only on proliferating tumor cells but also on normal cells. DNA damage induced by topoisomerase II poisoning leads to the induction of apoptosis of the hematopoietic cells in the bone marrow and epithelial cells in the gut, giving rise to the well-known toxicities of granulopenia and gut toxicity. On a longer time scale, DNA double-stranded breaks induced by topoisomerase II poisons can generate mutations and contribute to the development of tumors, particularly leukemias (Baguley and Ferguson 1998). In addition to these effects, which are related to the target of action, some topoisomerase II poisons may have two types of off-target effects that can affect heart function.

The first relates to the capacity of some compounds, particularly those containing quinone groups, to undergo redox cycling; one-electron reduction of the anthracycline by NADH produces a semiquinone free radical, which subsequently reacts with molecular oxygen to produce superoxide of free radicals (Doroshow and Davies 1983). Free radicals can damage mitochondrial membranes and potentially compromise mitochondrial function. Heart muscle, because of high mitochondrial activity, has a high susceptibility to such redox cycling activity, and extended administration of anthracycline derivatives has been found to cause accumulation of pathological changes leading to congestive heart failure. Considerable efforts have been made in the anthracycline series to minimize such cumulative chronic cardiotoxicity.

The second effect involves prolongation of the so-called Qt interval of the heart; such prolongation can potentially lead to fatal arrhythmias. The target here is a voltage-gated potassium channel which is specified by hERG (human ether-a-go-go-related gene) and which is essential for repolarization of heart muscle. hERG toxicity can be estimated in vitro using patch-clamp methods and several topoisomerase II poisons, including amsacrine, that have been shown to be positive in this assay (Baguley 2012). In keeping with this experimental observation, amsacrine was found in clinical trials to induce potentially fatal ventricular arrhythmias in hypokalemic patients, although these could be avoided by normalizing serum potassium concentrations prior to treatment (McLaughlin et al. 1983). Design of topoisomerase II poisons that minimize cumulative cardiotoxicity may lead to drugs with higher lipophilicity and consequently greater susceptibility to the induction of hERG toxicity. However, it may be possible to design topoisomerase II poisons that minimize both types of toxicity (Baguley 2012).

Clinical Summary

Drugs targeting topoisomerase II have an established place in cancer treatment, and it is notable that many of the available drugs have been used for several decades. Doxorubicin and epirubicin have formed the basis of most combination chemotherapy regimens in breast cancer and have application in a wide variety of solid tumors including pediatric cancers. Cumulative cardiotoxicity (congestive heart failure) is a serious concern, particularly for younger patients, and the risk of induction of secondary leukemia is also a consideration. Many different anthracycline derivatives have been tested in clinical trials with the aim of reducing chronic cardiotoxicity, and in addition, anthracycline formulations such as pegylated liposomal doxorubicin (Caelyx or Doxil) have been used to minimize cardiotoxicity. Such liposome formulations have a prolonged circulation time and may accumulate preferentially in the tissues because of increased microvascular permeability. Cardiotoxicity remains a significant problem for combination therapy of anthracycline-based drugs with other drugs, such as trastuzumab, that have a cardiotoxicity profile.

Anthracyclines also have a role in the treatment of lymphoma; doxorubicin in combination with rituximab, cyclophosphamide, vincristine, and prednisone (R-CHOP) is the standard of care for some lymphomas. Daunorubicin has an important role in the treatment of acute leukemias and is commonly used in first-line treatment; cardiotoxicity is also a consideration for this drug. Amsacrine has proven efficacy in acute myeloblastic leukemia, lacks the chronic toxicity of daunorubicin, and any hERG toxicity can be managed by normalizing blood potassium concentrations. Etoposide has the advantage of not showing chronic cardiotoxicity or hERG toxicity but has a narrower spectrum of antitumor activity; it has an established role in several malignancies, showing single-agent activity in non-Hodgkin's lymphoma, Kaposi's sarcoma, and testicular cancer. In combination with cisplatin or carboplatin, it is used in the treatment of small cell lung cancer.

A surprising feature of the topoisomerase II poisons as a class of clinical anticancer drugs is that despite huge advances in our ability to generate new structural variants and to analyze multiple features of gene expression and intracellular signaling, most of the drugs in clinical use were developed before 1980. Why has it been so difficult to develop analogs with a quantum improvement in clinical activity? A possible reason is that while it is comparatively easy to design a drug that has activity as a topoisomerase II poison, it is difficult to design drugs with optimal pharmacological features such as selective uptake/retention by tumor tissue and minimal host toxicity. Recently, more attention has been paid to "targeted" anticancer agents, where the presence of a biochemical target can be measured by protein or DNA sequence, than to cytotoxic drugs. However, topoisomerase II drugs may be targeted to cell-specific transport mechanisms, as well as to defects in DNA repair mechanisms. If these could be identified using biopsies of tumor material, they would greatly increase the utility of this class of agents.

Anticipated High-Impact Results

- The demonstration of new drugs that have the broad spectrum of clinical activity of doxorubicin in the absence of any cardiotoxicity.
- The development of new drugs that are selectively retained in tumor tissue, particularly if the reasons for such selectivity, such as susceptibility to specific transport mechanisms, could be delineated.
- The development of predictive biomarkers for cellular response that could be applied to individual patients in clinical studies.
- The demonstration in cancer patients that the immunostimulatory effects of topoisomerase II poisons lead to tumor response, on analogy with what has been demonstrated in mice.

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VDR

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Abstract

Vitamin D is a steroid hormone which binds to the vitamin D receptor (VDR) and has been implicated in carcinogenesis. Pre-clinical data on the VDR demonstrates a direct correlation between the vitamin and cell growth, differentiation, and apoptosis. Through nuclear transcription as well as cytoplasmic pathway induction, binding of the active form of vitamin D, calcitriol, to the VDR has been shown to mechanistically affect multiple carcinogenic cell lines. Mouse models have been further utilized to demonstrate the in vivo effects of calcitriol and the VDR on tumorogenesis. While pre-clinical data supporting the potential of calcitriol as a cancer therapeutic agent abounds, evidence from clinical trials remains sparse. Most studies thus far do not clearly demonstrate a correlation between intermittent doses of calcitriol and decreased rates of development or

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recurrence of malignancy. However, few large clinical trials have been performed, and some of the smaller studies that have been done demonstrate decreases in cancer markers, fewer rates of progression, and rarely complete remissions. The inconsistent results suggest that our understanding of the potential anti-tumor effects of vitamin D is limited, and further clinical investigation is necessary.

Keywords
Vitamin D • Vitamin D receptor • Retinoid X receptor (RXR) • Calcitriol • 25-hydroxyvitamin D • Cholecalciferol(Vitamin D3) • Ergocalciferol (Vitamin D2) • Preclinical studies • Cancer cell lines • Mouse models of carcinogenesis • Clinical trials • Cancer risk • Cancer therapy

Target

Vitamin D is a steroid hormone that must be modified within the body to produce its functional forms (Eitenmiller et al. 2008). It can exist as two metabolites which are produced via different mechanisms: as vitamin D_3 (cholecalciferol), which is metabolized in the skin by the action of ultraviolet (UV) light on 7-dehydrocholesterol (a cholesterol product which is synthesized in the skin), or as vitamin D_2 (ergocalciferol), a molecule obtained via plant-based diet. Foods of animal origin rich in vitamin D include egg yolk, dairy fat, liver, and oily fish (Webb 2011). Once produced or ingested (Fig. 1), cholecalciferol/ergocalciferol is then transferred

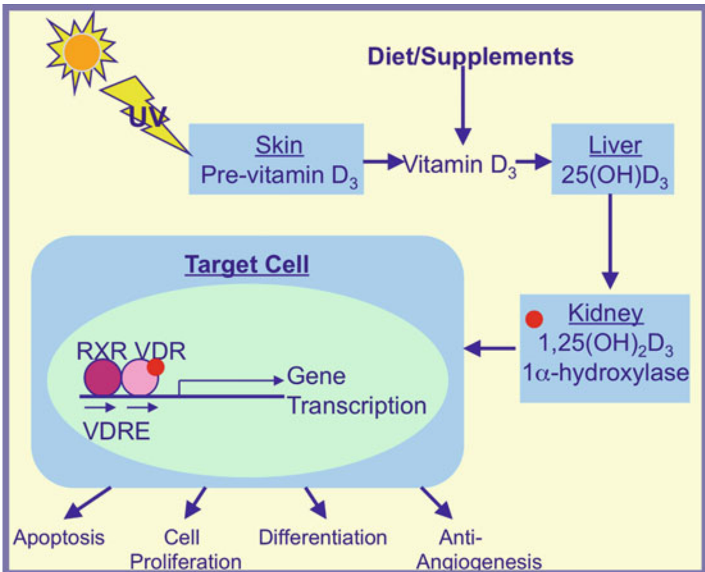

Fig. 1 Vitamin D metabolism

bound to vitamin D-binding protein (DBP) within the bloodstream to the liver, where it is hydroxylated by mitochondrial and microsomal 24-hydroxylase (encoded by *CYP24A1*) to 25-hydroxyvitamin D [25(OH)D]. This product is taken to the kidneys, where it is hydroxylated by mitochondrial 1a-hydroxylase (encoded by *CYP27B1*) into calcitriol or 1,25-dihydroxyvitamin D, 1,25$(OH)_2$D, the hormonally active form of vitamin D (Zittermann 2011). The active molecule and 25(OH)D are further hydroxylated, creating two less active metabolites, 24,25$(OH)_2$D and 1a,24,25 $(OH)_2$D, which are then excreted primarily in the feces. While 1a-hydroxylase was previously thought to exist solely in the kidney, it is now known to exist normally in multiple other tissues, including the breast, prostate, and colon, allowing for intracellular production of calcitriol.

The vitamin D receptor (VDR) is a ligand-dependent transcription factor that is part of a nuclear hormone receptor family of transcription receptors, which include thyroid hormone receptor and other steroid receptors. The gene is located on chromosome 12q, and the receptor itself is composed of a two zinc finger structure with a characteristic DNA-binding domain and a carboxy-terminal ligand-binding domain (Carlberg and Dunlop 2006). Unlike other receptors in its class, the VDR does not contain an activation domain on its amino terminus, but rather on its carboxy terminus. When unbound to its ligand, calcitriol [1,25$(OH)_2$D], the receptor is distributed predominately within the nucleus, but also in smaller quantities cytoplasmically and membrane bound (Thorne and Campbell 2011). The VDR dimerizes with the retinoid X receptor (RXR), causing a conformational change that allows the heterodimer to translocate into the nucleus. In the nucleus, the heterodimer is then able to bind to vitamin D-response elements (VDRE) in promoter regions, allowing for transcriptional regulation of target genes (Zittermann 2011). When unbound to calcitriol, the VDR/RXR heterodimer will bind to DNA at VDRE and recruit corepressor proteins and histone deacetylases that promote chromatin compaction, resulting in silencing of target genes. When ligand-bound receptors enter the nucleus, corepressors are switched for co-activators, histone acetyltransferases and chromatin-remodeling complexes, which result in decompaction, thereby allowing for transcription.

Biology of the Target

The VDR has long been well understood to be involved in calcium homeostasis via regulation of calcium in the gut and kidney as well as via bone mineralization. More recently, the receptor's actions have been expanded, with prolific activities that affect organs outside of the gut, kidney, and bone. In fact, almost 3% of the human genome is thought to be either directly or indirectly regulated by vitamin D (Zittermann 2011).

The VDR is able to affect the cell via transcriptional regulation, as well as direct activation of intracellular metabolic pathways via binding to the membrane-bound VDR (Zittermann 2011). The latter is caused via transcription-independent modulation of the activity of membrane ion channels and cytosolic kinases, phosphatases,

and phospholipases intracellularly (Larriba and Munoz 2010). Ultimately, the VDR has been implicated in utilizing three specific mechanisms to regulate growth and prevent neoplasia: cell cycle arrest, apoptosis, and promotion of differentiation.

The VDR inhibits cell proliferation via p21 and p27, two proteins which act on G0/G1 cell cycle arrest. The receptor modulates p21 via direct binding to the gene's VDRE, whereas it induces p27 via activation of various transcription factors and protein stabilization mechanisms (Larriba and Munoz 2010). G1 cell cycle arrest is also affected by the VDR via direct induction of GADD45-alpha, a regulator of NF-kB and a protein involved in G1 arrest and cell death (Thorne and Campbell 2011).

VDR has also been linked to regulation of cellular apoptosis. In breast, colon, prostate, and myeloma cell lines, vitamin D upregulated BAX and BAK (proapoptotic proteins) and downregulated BCL2 and BCL-XL (antiapoptotic proteins). By affecting these molecules, the VDR activates apoptosis and cellular death independent from the p53 pathway, allowing for activation of tumor cell death in cell lines with p53 mutations. While the VDR can function independently of p53, it also is involved directly in the p53 pathway, as the receptor is both transcriptionally upregulated by p53 and also has overlapping transcriptional targets. Via the p53 pathway, VDR has been found to be able to detect DNA damage and facilitate DNA repair, preventing mutations and promoting appropriate development (Thorne and Campbell 2011).

The role of VDR in differentiation has been studied extensively since the 1980s. The VDR has been found to instill mitotic restraints, thereby facilitating differentiation in hematopoietic and cancer cells lines (Thorne and Campbell 2011). Specific pathways affected include both epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 (IGF1), causing inhibition of the mitogen-activating protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways, thus affecting differentiation (Deeb and Trump 2007). By promoting differentiation, the cell facilitates normal development and prevents progression to malignant neoplasms.

Vitamin D was first analyzed as a potential anticancer agent in in vitro studies in the 1970s, when Rubin and Levij looked at suppression of vitamin D2 and D3 in hamster carcinoma. Since that time, it has been studied extensively in multiple cancer types, including breast, prostate, lung, ovarian, pancreatic, bladder, and squamous cell carcinoma. Common cell lines used include MCF-7 and SUM159 cells in breast cancer, OVCAR3 ovarian cell lines, LNCaP prostate cells lines, and CaCo-2 and SW480 colon cancer cell lines. Most studies used calcitriol as the agent with which cells were treated, bathing cells continuously in the drug for 24–72 h; however, some researchers used various vitamin D analogues and kept the cells bathed for weeks at a time. Ultimately, researchers evaluated the drug's role in these cell lines' in vivo proliferation, differentiation, angiogenesis, and apoptotic events (Beer et al. 2005).

Interestingly, studies of these cell lines demonstrate that malignant cells have certain physiologic changes, which decrease their susceptibility to calcitriol. Malignant cells have decreased intracellular levels of *CYP27B1* expression and activity as

compared with normal cells, which decreases intracellular calcitriol production. Furthermore, there is increased breakdown of calcitriol in tumor cells, causing resistance to the antitumor effects of vitamin D (Larriba and Munoz 2010). With decreased levels of calcitriol, more VDR remains in its deactivated conformation, bound to corepressors and incapable of activating transcription. Not only are the quantities of cellular calcitriol affected in malignant cells, but the VDR itself can become altered and restricted to the nucleus, decreasing its binding to the predominately cytoplasmic calcitriol (Thorne and Campbell 2011). Colon, breast, and lung cancers have all demonstrated downregulation of expression of VDR when compared to normal cell lines, while well-differentiated cancers show comparably more VDR expression as measured by immunohistochemistry when compared to their poorly differentiated counterparts (Larriba and Munoz 2010).

Animal models have long been used to study the role of vitamin D in cancer therapeutics. Researchers have created transgenic VDR knockout mice to fully analyze the effect of the VDR on cell growth and development (Welsh 2004). VDR-deficient mice are often born with profound disruption in their calcium homeostasis, as the VDR is critical to murine embryonic development. These mice are born with changes to their duodenal calcium absorption and bone mineralization. Even when supplemental calcium is administered, further phenotypic symptoms present themselves such as growth retardation, uterine hypoplasia, impaired ovarian folliculogenesis/reproductive dysfunction, alopecia, and others (Thorne and Campbell 2011). When the breast tissue of VDR mice was analyzed, it was noted to have accelerated growth and branching morphogenesis during pubertal development, as well as increased growth in response to exogenous estrogen and progesterone (Welsh 2004).

When exposed to chemical carcinogens such as DMBA, VDR knockout mice are more prone to carcinogen-induced skin proliferation and skin tumors compared to wild-type mice. The VDR knockout mice also demonstrate greater rates of carcinogen-induced preneoplastic mammary lesions, compared with the wild-type control mice, and increased proliferation and oxidative stress in the colon, resulting in higher rates of spontaneous colon cancer (Welsh 2004).

Other mouse models used to study vitamin D and carcinogenesis include feeding studies in mouse xenografts. For instance, cultured MCF-7 human breast cancer cells and PC-3 human prostate cancer cells were injected into nude mice fed a control diet with or without oral or injected calcitriol. The study found that both oral and IV calcitriol inhibited growth of prostate and breast tumors in nude mice (Swami et al. 2012).

In order to further tease out vitamin D's therapeutic potential, researchers have studied the effects of using calcitriol in combination with other chemotherapeutic agents. Many have found that it acts to potentiate certain therapies such as platinum analogues, taxanes, and DNA-intercalating agents. One study by Ma et al. looking at the use of vitamin D and cisplatin in combination in a squamous cell carcinoma (SCC) model found that calcitriol sensitizes SCC cells to cisplatin's growth inhibition and that pretreatment with calcitriol resulted in enhanced effects of the cisplatin in certain cell lines (resulting in induction of apoptosis, an effect not normally seen

with cisplatin when used alone) (Ma et al. 2008). Similarly, a study by Chaundry et al. determined that pretreatment of breast cancer cells in vitro with a vitamin D analog enhanced the effects of adriamycin as well as irradiation on tumor cells by causing apoptosis, reducing clonogenic survival, and decreasing viable cell numbers (Chaudhry et al. 2001). Paclitaxel and calcitriol combinations have been studied in SCC and prostate cancer cell lines, demonstrating yet again the enhanced therapeutic abilities of taxanes when combined with calcitriol (Trump et al. 2010). Interestingly, many of these antineoplastic agents have been found to inadvertently increase vitamin D levels by decreasing the stability of *CYP24A1* mRNA (encoding the deactivating enzyme, 24-hydroxylase), demonstrating a method by which these agents work synergistically with vitamin D (Larriba and Munoz 2010).

Target Assessment

The VDR itself is not routinely assessed in the clinical setting. In research, it can be measured via flow cytometry of specific cell cytoplasms or via measurement of VDR mRNA levels. In clinical settings, serum levels of 25(OH)D and 1,25 $(OH)_2D$ are measured to determine vitamin D status. 25(OH)D is the metabolite most often quantified due to its long half-life, which allows it to exist within the serum in concentrations which are three orders of magnitude higher than the serum concentrations of 1,25$(OH)_2D$ (Eitenmiller et al. 2008). 25(OH)D was first measured using a binding protein assay, in which vitamin D-binding protein (DBP) was used to measure circulating levels of 25(OH)D. A similar method was later developed to measure 1,25$(OH)_2D$ once it was discovered to be the biologically active form. The problem with these methods was that the DBP was relatively nonspecific as it bound to other vitamin D metabolites, causing overestimation of the levels of vitamin D by 10–20%. In 1985, a radioimmunoassay was developed to measure 25(OH)D; however, it too measured other metabolites and had a similar level of inaccuracy. In the last few years, a more specific radioimmunoassay (RIA) has been developed to measure 25(OH)D with 100% specificity. Other methods used to measure 25(OH)D include high-performance liquid chromatography (HPLC) and mass spectroscopy (Holick 2009).

1,25$(OH)_2D$ itself is rarely, if ever, measured. This is for a number of reasons, including its short half-life of 4–6 h and its low serum levels. Furthermore, there is concern that it is less accurate over all vitamin D status (despite being the metabolically active form), as levels of 1,25$(OH)_2D$ are very sensitive to parathyroid hormone (PTH), allowing for acute changes in PTH to cause false elevations in 1,25$(OH)_2D$ that do not reflect chronic status. Furthermore, serum levels are unable to assess specific levels of 1,25$(OH)_2D$ in tissues and cannot therefore evaluate the vitamin's autocrine or paracrine effects (Holick 2009).

Role of the Target in Cancer

Rank: "unknown" to 10.
Unknown to-1-2-3-4-5-6-7-8-9-10: 4.

High Level Overview

Diagnostic, Prognostic, and Predictive

Ecological studies have suggested a potential link between increasing rates of certain cancers and geographic latitude, allowing researchers to postulate that sunlight (possibly through increased endogenous production of vitamin D) may in fact decrease rates of malignancy (Webb 2011). As early as the 1930s, Peller found that US navy personnel had increased incidence of skin cancer but fewer events of non-skin cancer than the general populace. In the 1940s, evidence was published demonstrating an inverse relationship between UV irradiation and certain malignancies.

There is also observational data linking levels of serum 25(OH)D levels with cancer risk. A meta-analysis of prospective studies of 25(OH)D level and colorectal cancer risk demonstrated a lower risk of colorectal cancer among those with higher serum 25(OH)D levels. Studies related to breast and prostate cancer show less of a clear inverse relationship, but do suggest a potential link. For instance, in the Nurses' Health Study, women with the highest levels of serum 25(OH)D had an RR of 0.73 of developing breast cancer. In the Physicians' Health Study, participants with low 25(OH)D were twice as likely to develop aggressive prostate cancer compared to those with high 25(OH)D (with high and low levels of vitamin D being calculated based on batch, season, and cohort specific quartiles). Unfortunately, these findings are far from uniform, as the Prostate, Lung, Colorectal, and Ovarian Cancer Screening (PLCO) Trial found no association between 25(OH)D and an increased risk of aggressive prostate or breast cancer (Giovannucci 2011).

Researchers have expanded on this concept, looking not only at the role of sunlight in the risk of malignancy but also on its role in cancer survival. For instance, studies show that patients with melanoma counterintuitively demonstrate improved survival with increased exposure to sunlight post-diagnosis. Results for other malignancies remain inconclusive (Reichrath 2011). In terms of mortality benefits and recurrence, studies have shown an inverse relationship between levels of vitamin D and mortality. One large study conducted in Norway from tumor registry data from 1964 to 1992 determined that the lowest risk of cancer death, once diagnosed, occurred in those diagnosed in the seasons associated with the highest levels of vitamin D, summer or fall. The study therefore determined that high levels of vitamin D at the time of diagnosis and during treatment resulted in improved survival

in breast cancer, colon cancer, and prostate cancer (Robsahm et al. 2004). More recent studies have associated higher circulating 25(OH)D levels with improved survival for breast cancer (Freedman et al. 2008; Goodwin et al. 2009) and colorectal cancer (Ng et al. 2008). Another study by Zhou et al. looked specifically at survival rates in 447 patients with non-small cell lung cancer and demonstrated increased survival in patients with higher levels of serum vitamin D, particularly among those with advanced disease (Zhou et al. 2007).

Therapeutics

In general, clinical data regarding the use of calcitriol or vitamin D analogs as therapeutic agents for cancer have demonstrated inconsistent results. The first clinical trials looking at treatment involving calcitriol were performed in the 1980s on patients with myelodysplasia and acute leukemia. Unfortunately, these studies demonstrated few benefits, in part because they were limited by side effects: 20–30% of patients on calcitriol developed severe hypercalcemia (Deeb et al. 2007). To address this issue of hypercalcemia, investigators tried changing the dosing schedule of calcitriol or using non-hypercalcemic synthetic vitamin D analogs. Instead of administering daily oral calcitriol (continuous dosing), Trump et al. evaluated the toxicity associated with tri-weekly administration (intermittent dosing), arguing that not only was this dose associated with fewer side effects, but it was also more consistent with the dosing performed during in vitro studies. In a 4-week analysis, participants experienced only limited toxicity with intermittent dosing. While the dosing regimen was therefore viewed as a success, the drug formulation used in the study was determined to be suboptimal, as the pharmacokinetics were not linear (dose escalation did not directly relate to systemic levels), making it impractical to apply the medication pharmacologically (Trump et al. 2010).

In terms of vitamin D analogs, Beer et al. used an oral formulation called DN-101 (Ascentar; Novocea, Inc), which demonstrated more linear pharmacokinetics and less hypercalcemia compared to calcitriol (Beer et al. 2007a). Another agent, Calcijex (Abbott Pharmaceuticals), can be administered intravenously at high doses with linear pharmacokinetics. Like the other calcitriol agents, the dose-limiting toxicity for Calcijex is hypercalcemia. EB1089 (Seocalcitol), inecalcitol, and paricalcitol have all been studied as possible synthetic substitutes for calcitriol therapy. Pilot studies show promise for these analogs, as they demonstrate fewer side effects, particularly hypercalcemia (Trump et al. 2010). These studies are summarized in Table 1.

A number of relatively small clinical trials have been developed, exploring the potential for calcitriol as a therapeutic agent either alone or in combination with other chemotherapeutics. Table 1 summarizes these trials, including tumor types, calcitriol formulations, and clinical endpoints. Unfortunately, these studies are limited by their small sample sizes and inconsistent dosing, and results are equivocal. One early study by Osborn et al. looked at the effects of oral calcitriol in 14 patients with metastatic castration-resistant prostate cancer. None of the patients showed a clinical response, but

Table 1 Clinical trials of vitamin D for cancer treatment

First author	Tumor type	N	Vitamin D formulation	Dose	Duration	Study endpoints	Results
Osborn 1995	Castration-resistant prostate cancer	14	Oral calcitriol	0.5 µg daily, increasing to 1.5 µg daily	Continued until disease progression vs. toxicity	Clinical response, PSA	Decreased PSA level
Gross 1998	Asymptomatic, postsurgical/ radiation therapy prostate adenocarcinoma with rising PSA after initial drop	7	Oral calcitriol	0.5 µg daily, increasing to 2.5 µg daily	6–15 months	Recurrence	6/7 had drop in rate of PSA rise
Rustin 1996	Ovarian cancer in asymptomatic women post-chemotherapy who had rising CA-125 levels	22	Calcitriol and isotretinoin therapy	0.5 µg daily, increasing to 4 µg daily	Continued until disease progression vs. toxicity	CA-125 level, tumor regression slopes	No change in CA-125 level
Dalhoff 2003	Inoperable hepatocellular carcinoma	56	Seocalcitol	10 µg daily, increased every 2 weeks until hypercalcemia	Up to 1 year (longer in complete responders)	Tumor response	2 patients with complete tumor response, 12 with stable disease, 19 with progression of disease, and 23 not assessed; 2/2 death or disease progression prior to CT follow-up
Trump 2006	Castration-resistant prostate cancer	43	High-dose dexamethasone with	8 µg TID × 1 month, then 10 µg TID ×	3 months	PSA and clinical status	No clinical soft tissue disease response, but 19% of patients

(*continued*)

Table 1 (continued)

First author	Tumor type	N	Vitamin D formulation	Dose	Duration	Study endpoints	Results
			intermittent oral calcitriol	1 month, then 12 μg TID			demonstrated a >50% decrease in their PSA levels
Chadha 2010	Castration-resistant prostate cancer	18	IV calcitriol with dexamethasone	74 μg over 1 h	Median doses administered were 13.5	Clinical or PSA response	No clinical effect or effect on PSA
Beer 2005 (ASCENT trial)	Castration-resistant prostate cancer	250	DN-101 combined with docetaxel (pulse administration)	45 μg weekly × 3 weeks out of every 4 weeks	Disease progression or toxicity	Primary: PSA reduction >50% Secondary: tumor response in measurable disease, progression-free and overall survival	Primary: 58% of DN-101 patients with PSA response in 6 months compared to 49% of placebo patients
Beer 2007	Castration-resistant prostate cancer	37	DN-101, administered weekly	Cohorts of 3–10 patients were treated at doses of 15, 30, 45, 60, and 75 μg; 45 determined to be MTD		To determine MTD of DN-101	45 μg determined to be MTD
Scher 2011	Metastatic castration-resistant prostate cancer	953	DN-101 combined with docetaxel and dexamethasone	45 μg weekly × 3 weeks out of every 4 weeks	Mean follow-up 11.7 months, study halted early due to increased deaths in treatment arm	Overall survival	Increased deaths in DN-101 arm, trial halted (note: arms given different docetaxel/dexamethasone doses)

MTD maximum tolerated dose

two patients had decreased levels of serum prostate-specific antigen (PSA), suggesting a possible subclinical effect and encouraging further research (Osborn et al. 1995). A trial by Gross et al. the following year expanded on this finding, looking specifically at prostate adenocarcinoma recurrence. Seven asymptomatic patients with early-stage prostate who completed definitive surgery or radiation therapy and had a PSA recurrence were enrolled. The men were given oral calcitriol for 6–15 months, with serial PSA measurements. Ultimately, six of the seven demonstrated a significant drop in the rate of PSA increase while on treatment, suggesting that calcitriol may help to prevent prostate cancer progression (Gross et al. 1998).

Further studies that expanded on this evidence and looked at tumor markers for other malignancies were less promising. Rustin et al. looked at the effects of calcitriol and isotretinoin therapy on ovarian cancer in asymptomatic women post-chemotherapy, who had rising CA-125 levels. Unlike in Gross's trial, there was no effect on the tumor marker level with administration of calcitriol (Rustin et al. 1996). A promising phase II multicenter study looked at the use of Seocalcitol in treating patients with inoperable hepatocellular carcinoma (HCC). Patients were monitored using serum markers and CT scans for progression of disease. Two patients demonstrated complete response, 12 patients had stable disease, 19 patients progressed, and 23 were unevaluable. The response rate was demonstrated to be 3.5%, with most of the benefit seen with disease stabilization. Despite the low response rate, the complete response in two participants led investigators to recommend Seocalcitol as a potential adjuvant therapy for HCC (Dalhoff et al. 2003).

There have been two large randomized controlled trials looking at vitamin D for cancer therapeutics, the ASCENT trials I and II. The ASCENT (androgen-independent prostate cancer study of calcitriol-enhancing taxotere) trial was one of the largest clinical trials looking at the effects of calcitriol in combination with a chemotherapeutic agent. The primary goal of this double-blind, randomized, placebo-controlled study was to evaluate the efficacy of high-dose pulse administration of calcitriol, DN-101, combined with docetaxel as measured by PSA reduction of $>50\%$. Secondary objectives included tumor response in measurable disease and progression-free and overall survival. The trial looked at chemotherapy-naïve patients, administering calcitriol versus placebo by mouth followed by docetaxel. The results showed that 58% of patients on DN-101 demonstrated a PSA response at 6 months compared to 49% on placebo ($p = 0.16$). Although not statistically significant, some of the secondary objectives including overall survival showed compelling results, allowing investigators to pursue a second follow-up study (ASCENT-2) (Beer et al. 2007b). This trial looked at the efficacy and safety of docetaxel plus DN-101 when compared to docetaxel plus prednisone in 953 men with metastatic castration-resistant prostate cancer. The results demonstrated more deaths in those given DN-101; however, results remained controversial as the dosing of docetaxel used in both arms was different, with the dosing regimen used in the control arm having previously been shown to be more efficacious (Scher et al. 2011). Researchers continue to perform trials to assess the therapeutic benefits of calcitriol when used synergistically with other chemotherapeutic agents. Many of the current ongoing trials are summarized in Table 2.

Table 2 Ongoing clinical trials of vitamin D for cancer treatment

Primary investigator(s)	Vitamin D formulation	Combination agent(s)	Tumor type	Phase	ClinicalTrials.gov
Trump, DL	Oral calcitriol	Ketoconazole and hydrocortisone	Advanced or recurrent prostate cancer	I/II	NCT00536991
Dy, GK	Oral calcitriol	Cisplatin and gemcitabine	Inoperable advanced solid tumors	I	NCT01093092
Kuzel, TM	Oral calcitriol	Temozolomide	Metastatic stage IV melanoma	I/II	NCT00301067
Vajarabhongsa, B	Oral calcitriol	5-Fluorouracil, mitomycin C and leucovorin	Advanced intrahepatic cholangiocarcinoma	II	NCT01039181
Johnson, RH	Oral calcitriol	Celecoxib or None	Mouth neoplasms	I/II	NCT00953849
Brinkhuizen, T; Mosterd, K	Topical calcitriol (Silkis)	Diclofenac or None	Basal cell carcinoma	III	NCT01358045
Slaton, JW	Cholecalciferol	Genistein	Early stage prostate cancer	II	NCT01325311
Ramnath, N	IV calcitriol	Cisplatin and docetaxel	Non-small cell lung cancer	I/II	NCT00794547

Guidelines on the use of vitamin D supplementation for cancer risk reduction and therapy are sparse. In 2008, the WHO/IARC came out with a limited statement regarding the epidemiologic effects of vitamin D in risk reduction of specific malignancies. The statement cited the following: (i) consistent epidemiological evidence for an inverse association between 25(OH)D and colorectal cancer/colorectal adenomas; (ii) suggested epidemiological evidence for an inverse association between 25(OH)D and breast cancer; (iii) there is insufficient evidence linking vitamin D with other types of cancer; (iv) there is a need for increased randomized controlled trials. No mention was made to the therapeutic effects of vitamin D, as clinical evidence is still inconclusive (Zittermann 2011).

Preclinical Summary

Preclinical data on the VDR thus far demonstrates a direct correlation between the vitamin and cell growth, differentiation, and apoptosis. Through nuclear transcription as well as cytoplasmic pathway induction, calcitriol ligand binding to the VDR has been shown to mechanistically affect multiple carcinogenic cell lines. Mouse models have been further utilized to demonstrate the in vivo effects of calcitriol and the VDR on tumorogenesis. While evidence from clinical trials remains sparse, preclinical data supporting the potential of calcitriol as a cancer therapeutic agent abounds.

Clinical Summary

Most studies thus far do not clearly demonstrate a correlation between intermittent doses of calcitriol and decreased rates of development or recurrence of malignancy; however, few large clinical trials have been performed, and some of the smaller studies that have been done demonstrate decreases in cancer markers, fewer rates of progression, and rarely complete remissions. The inconsistent results suggest that our understanding of the potential antitumor effects of vitamin D is limited and further clinical investigation is necessary.

Anticipated High-Impact Results

Role of vitamin D supplementation to reduce cancer incidence in healthy individuals or prevent recurrence among cancer survivors

Clinical relevance of circulating levels of 25(OH)D on cancer risk and prognosis

Studying combinations of vitamin D supplements or synthetic vitamin D analogs with cancer therapeutic agents (i.e., potential synergy with hormonal agents)

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