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Handbook of Antimicrobial Resistance

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Handbook of Antimicrobial Resistance

With 48 Figures and 24 Tables

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Part I

Virology

HBV Therapy and the Problem of Drug Resistance

A. J. Thompson and S. A. Locarnini

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Abstract

The current goal of management for patients with chronic hepatitis B is to achieve sustained virological suppression. This has been shown to reduce the risk of liver disease progression, including cirrhosis, hepatic failure, and hepatocellular carcinoma. Nucleos(t)ide analogue monotherapy is commonly used as first-line therapy, and most patients will require long-term antiviral therapy. As for all

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direct-acting antiviral agents, the emergence of drug resistance is an important clinical concern. In this chapter, we discuss the principles of HBV antiviral resistance and clinical pathways for preventing the selection of drug-resistant variants, as well as appropriate management strategies for antiviral treatment failure.

Keywords
Hepatitis B • Tenofovir • Entecavir • Adefovir • Lamivudine • Telbivudine • Multidrug resistance • Hepatocellular cancer (HCC)

Introduction

More than 350 million individuals are chronically infected with hepatitis B virus (HBV). Chronic hepatitis B is associated with the long-term risk of progressive liver fibrosis and cirrhosis, liver failure, and hepatocellular carcinoma. CHB is a leading cause of human mortality, and more than 500,000 deaths are directly attributable to CHB annually (Lozano et al. 2012).

The risk of complications related to chronic HBV infection is linked to persistent high-level viral replication (Iloeje et al. 2006; Chen et al. 2006). Clinical guidelines therefore recommend antiviral therapy for patients with evidence of chronic hepatitis and HBV DNA levels >2,000 IU/mL (European Association for the Study of the Liver 2012; Lok and McMahon 2009; Liaw et al. 2012). The goal of therapy is to achieve sustained virological suppression (European Association for the Study of the Liver 2012; Lok and McMahon 2009; Liaw et al. 2012) which is associated with reduced risk of liver disease progression. Nucleos(t)ide analogue (NA) therapy is the treatment of choice for most patients, due to the limited efficacy and poor tolerability of peginterferon-α. Effective NA therapy for HBV is available, and five potent NAs have been approved for the treatment of CHB. Most patients will need long-term therapy to achieve sustained virological suppression, as the risk of virological relapse is high following cessation of NA. In this context, antiviral drug resistance is a critical determinant of long-term treatment success. Drug resistance is clinically important because virological breakthrough is associated with the loss of biochemical and eventually histological therapeutic gain. In the setting of advanced liver disease, virological breakthrough may lead to hepatitis flares, hepatic decompensation, and death (Liaw et al. 2004a, b).

Drug resistance can be prevented by selecting a potent NA with a high barrier to resistance as first-line therapy. The barrier to resistance is influenced by antiviral potency, genetic barrier, viral fitness, and treatment history (cross-resistance). The two first-line agents, tenofovir (TDF) and entecavir (ETV), are both high-barrier-to-resistance drugs. Second-line agents that continue to be used in resource-limited settings have lower barriers to resistance and include adefovir (ADV), telbivudine (LdT), and lamivudine (LMV). Once drug resistance occurs, it can be treated using salvage therapy with an agent that has a complementary cross-resistance profile, as will be discussed.

Understanding the basic principles of antiviral resistance is therefore important for planning appropriate selection of first-line and salvage NA therapies for CHB. In this chapter, we present an overview of the clinical aspects and molecular virology of HBV drug resistance, with a focus on prevention, early diagnosis, and rational management strategies.

Nucleos(t)ide Analogues Used to Treat HBV Infection

There are currently five drugs that belong to the class of nucleos(t)ide analogues (NAs) that have been approved for the treatment of CHB in most parts of the world (European Association for the Study of the Liver 2012). The NAs all directly inhibit the reverse transcriptase activity of the HBV polymerase (Pol). The approved NAs include lamivudine (LMV), a synthetic deoxycytidine analogue with an unnatural L-conformation, and the related L-nucleoside, telbivudine (LdT; β-L-thymidine). A second group, the acyclic phosphonates, includes adefovir dipivoxil (ADV), a prodrug for the acyclic 2′-deoxyadenosine monophosphate analog adefovir, and the structurally similar tenofovir (TFV). A third group of agents contains a D-cyclopentane sugar moiety linked to the base guanine and is the most potent anti-HBV drug discovered to date, entecavir (ETV) (Shaw and Locarnini 2004). This structural classification of the NA is useful clinically because it does help understand and classify the patterns and pathways of NA drug resistance (Table 1).

Antiviral Drug Resistance and HBV

Molecular Virology

The replication strategy of HBV involves two key steps. The major transcriptional template of the virus is the covalently closed circular (ccc)DNA minichromosome. This nuclear reservoir is inherently stable. The first step in the replication cycle is the transcription and nuclear export of pregenomic (pg)RNA to the cytoplasm. The pgRNA is the template for synthesis of the HBV polymerase (or reverse transcriptase, Pol) and the hepatitis B core proteins which form the nucleocapsid. Following its translation, the HBV Pol mediates reverse transcription of the same pgRNA transcript from which it was synthesized, a process which occurs inside the nascent nucleocapsid to form a replicating core complex (Harrison 2006). HBV replicates at an extremely high rate and the HBV Pol is inherently error-prone, resulting in a high nucleotide substitution rate and a population of viral variants or quasispecies capable of responding rapidly to endogenous (host immune response) or exogenous (antiviral therapy) selection pressures. This pool of quasispecies provides HBV with a survival advantage by generating a population of drug escape variants.

Antiviral drug-resistant variants are defined by a reduced susceptibility to the inhibitory effect of NA and emerge following the process of random mutation with adaptive selection under the pressure of antiviral therapy (Gish et al. 2012). Two types

Table 1 Patterns and pathways of antiviral drug resistance and cross-resistance in chronic hepatitis B

Pathway	HBV Pol / rt domains	LMV	LdT	ETV	ADV	TDV
Wildtype	A – B – C – D – E	S	S	S	S	S
L-nucleosides	M204V/I	R	R*	I	S	S
	A181T/V	R	R	S	R	I***
D-cyclopentanes	L180M, M204V/I, I169T, T184**, S202**, M250I/V	R	R	R	S	S
Acyclic phosphonates	N236T	S	S	S	R	I***
	A181T/V	R	R	S	R	I***
Multi-drug resistance[Φ]	A181T/V, N236T, M250I/V	R	R	R	R	I***

* The main resistance substitution is the M204I
** T184 S/A/I/L/G/C/M, S202 C/G/I
*** Primary ADV resistance mutations have been associated with delayed kinetics of response to TDF in vivo [34]
Φ MDR has been associated with sequential monotherapy using drugs with overlapping resistance profiles

of mutations have been identified: primary resistance mutations, which are directly responsible for the associated drug resistance, and secondary or compensatory mutations. The latter occur in order to promote replication competence of resistance variants, because primary resistance mutations are typically associated with a reduction in replication fitness and competence. Compensatory mutations are important because they reduce the deleterious effects to the virus associated with acquisition of primary drug-resistant mutations (Domingo 2003) and allow the primary resistance mutations to be successfully archived in the intrahepatic cccDNA molecules.

Typically, the development of NA resistance depends on six factors: (1) magnitude and rate of virus replication, (2) fidelity of the viral polymerase, (3) selective pressure exerted by the NA (potency), (4) amount of available replication space in the liver (high ALT levels are associated with increased risk of resistance; high ALT levels are associated with hepatocyte proliferation creating replication space), (5) replication fitness of the emerging NA-resistant HBV, and (6) genetic barrier to resistance of the NA (Nafa et al. 2000; Yuen et al. 2001; Hadziyannis et al. 2006; Lai et al. 2007). Prior therapy with NAs also predicts for the development of drug resistance (Nafa et al. 2000; Yuen et al. 2001; Hadziyannis et al. 2006; Lai et al. 2007; Zoulim and Locarnini 2009).

Table 2 Definitions of antiviral resistance according to a recent expert consensus panel (REF)

Term	Definition
Primary nonresponse	Inability of nucleos(t)ide analogue (NA) treatment to reduce serum HBV DNA by $\geq 1 \log_{10}$ IU/mL after the first 6 months of treatment
Partial response	Detectable HBV DNA using a real-time PCR assay during continuous therapy
	Note that the time point for the definition of partial response has not been well defined and will vary according to a drug's potency and genetic barrier to resistance (e.g., 24 weeks, LMV/LdT; 48 weeks, ADV; >48 weeks, ETV/TDF)
Virological breakthrough	Increase in serum HBV DNA by $\geq 1 \log_{10}$ IU/mL above nadir on ≥ 2 occasions 1 month apart, in a treatment-compliant patient
Genotypic resistance	In the setting of antiviral therapy, the detection of viral populations bearing amino acid substitutions in the Pol/Rt region of the HBV genome that have been shown to confer resistance to antiviral drugs in phenotypic assays. These mutations are usually detected in patients with virological breakthrough, but they can also be present in patients with persistent viremia and no virological breakthrough
Phenotypic resistance	Decreased susceptibility of an HBV polymerase to an antiviral drug in vitro
Cross-resistance	Decreased susceptibility to more than one antiviral drug conferred by the same amino acid substitution or combination of amino acid substitutions

Clinical Definitions and Drug Resistance

Virological breakthrough is the consequence of the emergence of resistant variants and has defined as a $>1 \log_{10}$ (tenfold) increase in serum HBV DNA from nadir, in two consecutive samples taken 1 month apart, in a patient who had an initial virological response (Lok et al. 2007). Virological breakthrough is usually followed by biochemical breakthrough, with its associated risks of flare and decompensation. However, virological breakthrough may occur months and sometimes years before biochemical breakthrough; hence, early detection is possible prior to the development of clinical complications. This is particularly important in the setting of advanced liver disease. Standardized nomenclature for clinical, genotypic, and phenotypic resistance to NA therapy has been defined for CHB (Table 2).

NA and Treatment Failure

Lamivudine, LMV

Most of the literature describing the clinical consequences of resistance to NA in CHB has reported on the LMV experience. Lamivudine is a deoxycytidine analogue with an unnatural L-conformation (L-nucleoside). Lamivudine was the first NA approved and is regarded as a "first-generation" agent because of its low potency and low genetic barrier (Gish et al. 2012). The development of resistance begins with selection of mutations in the HBV Pol, followed by an increase in serum HBV DNA

levels, and then, within weeks to months, a rise in serum alanine aminotransferase (ALT) levels and progression of liver disease (Nafa et al. 2000; Yuen et al. 2001; Lai et al. 2003). The risk of increased serum ALT in the setting of LMV resistance usually correlates with the duration of detectability of the resistant variant (Lok et al. 2003). These patients also have a significant risk of ALT flare and hepatic decompensation. The association between LMV resistance and progression of liver histology and then clinical deterioration was demonstrated by a placebo-controlled randomized clinical trial in patients with advanced fibrosis (Liaw et al. 2004a).

Lamivudine resistance is associated with amino acid substitutions in the tyrosine-methionine-aspartate-aspartate (YMDD) locus of the catalytic (C) domain of the HBV Pol (Table 1). Primary resistance mutations include the rtM204V/I. Although rtM204I can occur in isolation, rtM204V has been observed only in association with secondary compensatory mutations, most commonly the rtL180M (domain B). A second primary resistance mutation for LMV is the rtA181T/V. A number of other compensatory changes have been described in other domains of the HBV Pol, including the rtL80V/I (Ogata et al. 1999), trV173L (Delaney et al. 2003), and rtT184S (Bartholomeusz et al. 2005).

The frequency of LMV resistance increases progressively during treatment at rates of 14–32 % annually (Fig. 1), exceeding 70 % after 4 years of therapy

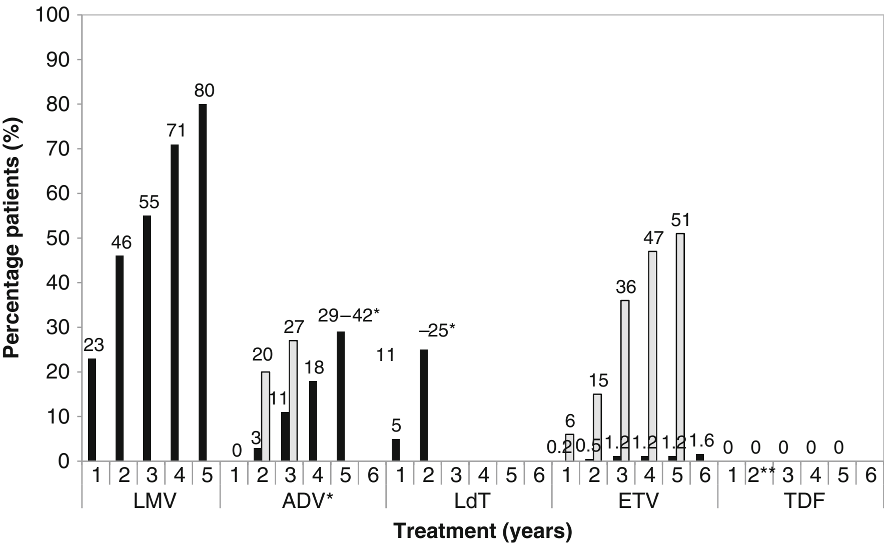

Fig. 1 Cumulative incidence of virological breakthrough due to the selection of resistant HBV variants. *Black columns* describe the prevalence of breakthrough in treatment-naïve patients. *Gray columns* describe the prevalence in lamivudine-experienced patients. *Rates in HBeAg-negative (*lower value*) and HBeAg-positive (*higher value*) patients, respectively. **For patients confirmed to be viremic at week 72+ in the registration studies, add-on emtricitabine was available at the clinician's discretion

(Lai et al. 2003). The rtM204V/I and rtA181T/V mutations both confer cross-resistance to the related L-nucleoside LdT and emtricitabine (FTC) (Table 1). The rtM204V/I does not confer cross-resistance to ADV or TDF, although the combination of M204V/I plus the L180M reduces susceptibility to ETV (Tenney et al. 2004). The rtA181T/V is also associated with resistance to ADV.

Adefovir, ADV

The kinetics of emergence of resistant variants to ADV are typically slower than was observed in the setting of LMV treatment (Fig. 1). In treatment-naïve patients, the prevalence of resistance is ~2 % at 2 years but rises progressively to 29 % after 5 years (Hadziyannis et al. 2006). Resistance to ADV emerges more rapidly in patients with prior LMV resistance (Fig. 1; Lee et al. 2006; Yeon et al. 2006; Fung et al. 2006). However, the same series of events occurs, with selection of genotypic resistant variants leading to sequential virological breakthrough, biochemical relapse/flare, and disease progression (Hadziyannis et al. 2006; Fung et al. 2005). Resistance to ADV has been associated with substitutions in the B domain (rtA181T/V) and/or the D (rtN236T) domain of HBV Pol (Angus et al. 2003; Villeneuve et al. 2003).

Only limited data are available on the clinical outcome of patients who are infected with ADV-, LdT-, ETV-, or TDF-resistant HBV, mainly because salvage treatment, usually based on in vitro cross-resistance data, has been initiated much earlier. The availability of antiviral drugs with complementary cross-resistance profiles (Table 1) has changed the management of patients with drug resistance, allowing physicians to prevent the worsening of clinical outcome resulting from the emergence of resistance.

Telbivudine, LdT

Telbivudine is cross-resistant with LMV, and the main resistance substitution is rtM204I (Table 1). Less common primary resistance mutations include the rtA181T/V and rtL229W/V. In the LdT registration studies, the prevalence of resistance to LdT increased from 4 % at 12 months to 30 % at 24 months of monotherapy (Fig. 1).

Entecavir, ETV

ETV has a high genetic barrier to resistance and requires the accumulation of multiple amino acid substitutions before drug sensitivity declines. Entecavir resistance requires the rtM204V/I plus L180M plus the selection of one of a number of signature ETV mutations: rtI169T, rtS184G, rtS202G/I, or rtM250V (Table 1). Primary resistance to ETV is uncommon in treatment-naïve individuals, being negligible in the first year and remaining low (approximately 1 %) even after 6 years of treatment (Fig. 1; Colonno et al. 2006; Tenney et al. 2009). Resistance was initially described in patients with a history of prior lamivudine resistance (Tenney et al. 2004), in whom it is far more common, reaching a prevalence of ~50 % after 4 years of treatment (Tenney et al. 2009). Entecavir is therefore not a suitable salvage therapy for patients with LMV resistance.

Tenofovir, TDF

Tenofovir disoproxil fumarate (TDF) is closely related to ADV and is also an acyclic phosphonate nucleotide analogue. Tenofovir is more potent than ADV and has a better toxicity profile, allowing higher standard dose (300 mg vs. 10 mg). To date, primary resistance mutations for TDF have not been identified. Tenofovir therefore also has a high genetic barrier to resistance. The primary resistance mutations for ADV, A181T/V and/or N236T, have been associated with small fold changes in sensitivity to TDF in vitro (<7-fold (Lok et al. 2007)). The clinical significance of this remains unclear. In three recent studies of TDF used for the treatment of patients failing ADV, continuous decline in HBV DNA was observed over time (Berg et al. 2010, 2014; Patterson et al. 2011). In one of the studies that recruited patients from the Asia-Pacific region, the presence of A181T/V and/or N236T substitutions at baseline was associated with delayed kinetics of viral decline. However, this was not seen in the European studies, where strong viral suppression was observed in the presence of preexisting LMV- or ADV-resistant mutations (Berg et al. 2010, 2014; van Bommel et al. 2010). No significant selection pressure on preexisting ADV- or LMV-resistant mutations was observed (Lavocat et al. 2013). There was no benefit of combination TDF plus FTC over TDF monotherapy following switch of therapy and no impact on long-term response (Berg et al. 2014). Further studies on whether ADV-associated substitutions reduce the antiviral response to TDF following switch of therapy are needed.

Pathways of Resistance

The primary resistance substitutions associated with drug failure for CHB are shown in Table 1. With the current five approved NAs, changes to eight codons in the HBV P ORF account for primary treatment failure. These eight substitutions can be understood based on NA chemistry and commit subsequent viral evolution to five different pathways:

- The L-nucleoside pathway (rtM204V/I). In this pathway, LMV and LdT treatment can select for rtM204V/I which predisposes to subsequent ETV resistance.
- The acyclic phosphonate pathway (rtN236T). ADV and TFV treatment can select for and/or consolidate rtN236T (van Bommel et al. 2010).
- Shared pathway (rtA181T/V). In this pathway, treatment with either L-nucleosides or acyclic phosphonates can select rtA181T/V, which occurs in about 40 % of cases of ADV failure but less than 5 % of cases of LMV failure. ADV and TFV treatment can consolidate rtA181T/V.
- The double pathway (rtA181T/V + rtN236T). In this pathway, treatment with TFV consolidates both of these variants, significantly blunting its antiviral efficacy (Patterson et al. 2011; van Bommel et al. 2010), resulting in persistent viremia (van Bommel et al. 2010).

- The D-cyclopentane-/ETV-naive resistance pathway (rtM204V/I ± rtL180M and one or more substitutions at rtI169, rtT184, rtS202, or rtM250). Three substitutions are required to be selected out on ETV, accounting for the very low resistance rates observed in NA-naïve patients (Fig. 1).
- Multidrug resistance (MDR) pathways. Monotherapy with low-potency and low-genetic-barrier NAs can promote selection for MDR strains of HBV. Multidrug-resistant HBV can also be selected out when patients are treated sequentially with drugs with overlapping resistance profiles, such as with LMV followed by ETV (Villet et al. 2007; Yim et al. 2006) or LMV followed by ADV (Liu et al. 2010; Villet et al. 2006; Brunelle et al. 2005) or ADV followed by TFV (Chang and Lai 2006; see Table 1).

Multidrug Resistance

Clonal analyses have shown that MDR usually occurs by the sequential acquisition of resistance mutations on the same viral genome; mutants that arise from this selection process may be fully resistant to multiple drugs. Studies have shown that MDR strains can arise if an "add-on" therapeutic strategy does not result in rapid viral suppression, particularly if there is sufficient replication space available for the mutants to spread (i.e., necro-inflammatory activity resulting in hepatocyte proliferation or liver graft not protected by HBIG because of the preexistence of escape mutants). These findings emphasize the need to achieve complete viral suppression during antiviral therapy: no replication (NR) = no resistance (NR). A specific single amino acid substitution may confer MDR (see Table 1). This was shown with the rtA181V/T substitutions, which are responsible not only for decreased susceptibility to the L-nucleosides LMV and LdT but also to the acyclic phosphonates ADV and TFV ((Warner and Locarnini 2008; Villet et al. 2008). This highlights the clinical usefulness of genotypic testing (drug resistance testing) in patients with treatment failure, as has been done for HIV therapy management (Clavel and Hance 2004), in order to determine the viral resistance mutation profile and thereby tailor therapy to the major viral circulating strain.

Management of Antiviral Resistance

Prevention

International guidelines recommend ETV and TDF as the best choice for first-line therapy (European Association for the Study of the Liver 2012; Lok and McMahon 2009; Liaw et al. 2012). These drugs are both potent and have a high genetic barrier to resistance. Lamivudine, LdT, and ADV should be considered as second-line choices. They will be continued to be used in resource-limited settings, however, as they are generally cheaper. It is important to emphasize the importance of compliance with patients. Patients should be tested for primary response, and we

recommend testing for a reduction in HBV DNA >1 $\log_{10}$ IU/mL at 3 months (Table 2). It is then important to continue regular virological monitoring. We recommend repeating a serum HBV DNA level at 6 months in all patients. In patients taking ETV or TDF, we then monitor serum HBV DNA levels every 6 months.

In treatment-naïve patients for whom LMV, LdT, or ADV is used as first-line therapy, we recommend testing serum HBV DNA levels every 3 months, given the higher risk of virological resistance. This also allows treatment adjustment in the setting of suboptimal or partial response (Table 2). The hepatitis B "road map" concept has been proposed for resource-limited settings (Keeffe et al. 2007). The concept is based on data showing that patients with a profound and rapid virological response during treatment with LMV, LdT, or ADV have a lower risk of resistance than patients with a delayed virological response (Yuen et al. 2001; Zollner et al. 2001; Locarnini et al. 2005). Using this algorithm, patients who start treatment with a second-line agent are monitored for virological response at 3 and 6 months. In patients with a complete response at 6 months (undetectable serum HBV DNA), it is reasonable to continue therapy with virological monitoring every 3 months. In patients with an inadequate virological response (serum HBV DNA >2,000 IU/mL at 6 months), treatment should be intensified (e.g., L-nucleoside plus ADV) or switched to a first-line agent (ETV or TDF – TDF is most suitable for patients originally taking an L-nucleoside). In patients with a partial response (0 < serum HBV DNA <2,000 IU/mL), it is reasonable to maintain the original therapy until 48 weeks. If viremia persists at week 48, then add-on/switch treatment is appropriate because of the high risk of resistance with LMV, LdT, or ADV in the setting of persistent viremia.

In the setting of ETV or TDF therapy for treatment-naïve patients, the clinical significance of a partial virological response at week 24 or week 48 is less clear. Long-term follow-up studies have shown that resistance is very uncommon and that most patients will show continued virological decline. An emerging issue is persistent low-level viremia with HBV DNA levels $<10^{2-3}$ IU/mL. In per protocol analyses, up to 5 % of NA-naïve patients remain HBV DNA positive during long-term ETV or TDF therapy using sensitive real-time PCR assays (Snow-Lampart et al. 2011; Chang et al. 2010). HBV DNA sequencing is normally not possible given the low viral load, and virological rebound has not been reported to date in compliant patients. The long-term risk of selecting resistant variants is not known. In the setting of persistent viremia beyond week 96 of therapy with ETV or TDF, it seems reasonable to recommend treatment intensification with the add-on of another drug with no cross-resistance.

Combination therapy is required to prevent the selection of resistant variants in the setting of antiviral therapy for HIV or HCV. In the setting of HBV, combination of TDF plus ETV and TDF plus emtricitabine (FTC) has recently been shown to be associated with increased potency in patients with very high viral loads ($>10^8$ IU/mL) (Lok et al. 2012; Chan et al. 2013). However, combination therapy for HBV has not been proven to prevent resistance in treatment-naïve patients and cannot be recommended as a first-line strategy.

Table 3 Treatment strategies for antiviral drug resistance in chronic hepatitis B

Resistance	Management strategy	
LMV-R or LdT-R	Preferred option	Add-on or switch to TDF
	Alternate option (TDF not available)	Add-on ADV
ADV-R	Preferred option (NA naïve prior to ADV)	Switch to TDF plus LMV/FTC/LdT or switch to ETV
	Preferred option (prior LMV exposure)	Switch to TDF + LMV/FTC/LdT
	rtA181T/V identified	Switch to TDF + ETV
ETV-R	Preferred option	Add-on or switch to TDF
	Alternate option (TDF not available)	Add-on ADV
TDF-R[a]	Preferred option	Add-on ETV
	Alternate option (no history of LMV resistance)	Switch to ETV
Multidrug[b] **resistance**	Preferred option	ETV + TDF

[a]Note – primary resistance to TDF has not been confirmed to date and therefore there is no experience (genotypic analysis is recommended)
[b]A181T/V + N236T + M250I/V

Management of Treatment Failure

The most common cause of virological breakthrough remains poor compliance. Studies have shown that up to 40 % of patients taking long-term treatment for CHB may not be fully adherent (Pol and Sogni 2010). Therefore, assessment of treatment adherence is important in all patients who experience virological rebound. Patients must be educated about the importance of good compliance for maintaining maximal suppression of HBV DNA to reduce the risk of the selection of resistant variants.

Virological breakthrough in compliant patients occurs secondary to the emergence of resistant variants and necessitates salvage therapy. The choice of salvage therapy should be based on the knowledge of cross-resistance, so that the second agent has a resistance profile that differs from the failing drug (Table 1). This is particularly important as drug-resistant variants are thought to be archived in viral cccDNA reservoirs in the liver (Zhou et al. 1999). We recommend HBV Pol sequencing to define the resistance mutation associated with virological breakthrough and guide subsequent treatment. A guide to appropriate rescue therapies is presented in Table 3. There is a theoretical advantage to using add-on combination therapy, to raise the barrier of resistance and increase potency, making the subsequent development of drug resistance less likely.

Consequences of the Pol-HBsAg Overlap

The hepatitis B virus has a unique genome with overlapping reading frames. Two potentially important consequences emerge from the fact that almost every drug-

resistant HBV has an associated change in its envelope (HBsAg), due to the overlapping nature of the HBV Pol and HBV S open reading frames.

Public Health Potential

The major public health issue related to the alteration in HBsAg antigenicity (Locarnini 1998) is the transmission of drug-resistant HBV (Kamili et al. 2009; Thibault et al. 2002) and therefore the possibility that these drug-resistant HBVs could represent a threat to the various immunization programs designed to control hepatitis B (Clements et al. 2010). These viruses have been termed antiviral drug-associated potential vaccine-escape mutants (ADAPVEMs) (Kamili et al. 2009).

The S protein of HBV, which is the main component of the envelope of the virion, carries the major target of the neutralizing antibody, the "a" determinant, and, on the genome, directly overlaps the catalytic domain of the Pol. Resistance mutations in the Pol usually result in non-synonymous changes in the S of HBsAg (Bartholomew et al. 1997; Ogura et al. 1999; Shields et al. 1999; Tipples et al. 1996), and the first study to demonstrate that these substitutions might affect HBsAg protein conformation, and more importantly its antigenicity, was carried out by Torresi et al. (2002a). These investigators showed that the common lamivudine (LMV)-resistant substitutions (rtM204V/sI195M, rtM204I/sW196S, rtM204I/sW196L, and rtV173L/sE164D plus rtL180M plus rtM204V/sI195M) resulted in reductions in the reactivity of the altered HBsAg with vaccine-induced antibody against HBsAg (anti-HBs). In addition, the converse has been shown in that changes in the S gene introduce changes in Pol that correspond with LMV-resistant compensatory-type mutations (Torresi et al. 2002b). These studies have now been independently confirmed using different in vitro models, including mammalian cell culture transfection and epitope "density" mapping (Sloan et al. 2008). The key findings are summarized in Fig. 2.

The true public health potential of these ADAPVEMs was realized when the common LMV-resistant mutation, rtV173L plus rtL180M plus rtM204V (Delaney et al. 2003), which displays the sE164D plus sI195M change in HBsAg, successfully infected hepatitis B-immunized chimpanzees that carried high titers of circulating anti-HBs pre-challenge (Kamili et al. 2009). This study also established the genetic stability of the drug-resistant ADAPVEM rtV173L plus rtL180M plus rtM204V variant in a non-immunized chimpanzee, in whom no revertants to wild type (WT) were detected over at least 6 months to a year compared with infection with the sG145R canonical vaccine-escape mutant, which quickly back-reverted to WT (Kamili et al. 2009). This latter observation reveals the important role of compensatory mutations such as rtV173L and rtL180M in "fixing the genetic archive," especially in the setting of transmission of NA resistance.

An important question is whether the emergence of ADAPVEMs poses a threat to the global hepatitis B immunization program. For a new viral species to pose a threat

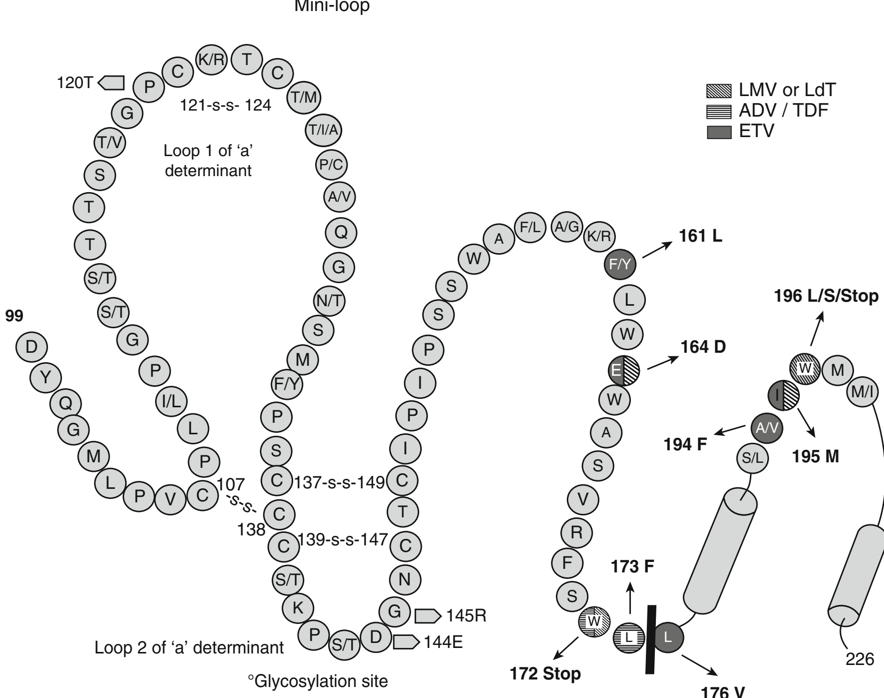

Fig. 2 The “a” determinant of HBsAg, highlighting the amino acid substitutions that have been associated with NA drug resistance due to the overlap between HBV Pol and S open reading frames

in this setting, Clements et al. have suggested that an ADAPVEM would need to possess at least four characteristics (Clements et al. 2010):

1. It needs to be a stable mutant.
2. It must have undergone sufficient changes in antigenicity such that anti-HBs generated by the current vaccine no longer neutralizes it.
3. It must be transmissible and cause infection in immunized individuals and so have the opportunity for ongoing spread.
4. It must cause disease (acute or chronic) in infected individuals.

From the studies reviewed to date, the first three have been achieved; it is not known if ADAPVEMs have the same propensity to cause disease as do current circulating strains of HBV (Clements et al. 2010), although one case of primary infection with a 3TC-resistant HBV was associated with acute hepatitis (Thibault et al. 2002). Clearly, further studies are needed to fully elucidate the clinical, pathological, and epidemiological significance of these emerging ADAPVEMs.

Molecular Pathogenesis and Oncogenic Potential

Several HBV proteins are involved in the development of HCC, transcribed from either integrated HBV DNA or the HBV genome. For example, truncated HBV surface proteins have been linked to the progression to HCC since they possess intrinsic transactivational activity, revealed by increased nuclear factor κB or activator protein 1 promoter activity (Schluter et al. 1994). It is known that NA therapy selects for point mutations in Pol that not only confer NA resistance but also result in truncated surface proteins and therefore could theoretically accelerate the progression to HCC (Lai et al. 2009; Lai and Yeh 2008; Warner and Locarnini 2008; Locarnini 1998). In particular, the point mutation that causes the rtA181T change in HBV Pol also encodes a stop codon (sW172*) in the overlapping surface proteins (Fig. 2), resulting in truncation of the last 55 amino acids of the C-terminal hydrophilic region of the HBsAg. The LMV-/LdT-resistant variant rtM204I/sW196* is another example. An extensive analysis of rtA181T/sW172* HBV in vitro has shown that it is defective in secretion of viral particles resulting in intracellular retention of surface proteins that have a dominant negative effect on WT virion secretion (Warner and Locarnini 2008). This can result in the observation of lower viral loads extracellularly and is what is often observed with the emergence of adefovir resistance (Warner and Locarnini 2008; Zoulim and Locarnini 2012). Two recent reports have now provided evidence for the involvement of HBV encoding the rtA181T/sW172* mutation in the pathogenesis of and progression to HCC (Lai et al. 2009; Lai and Yeh 2008). Analyses of HBV DNA from patients who developed HCC despite LMV therapy revealed stop codon mutations in the envelope gene in seven of eight patients compared with the control group, in which no patients developed HCC. Using expression constructs encoding the HBV surface proteins, these investigators demonstrated that surface proteins truncated at amino acids sL21, sW196, or sW172 (the last of which corresponds to the surface proteins expressed from rtA181T/sW172*) transactivated the c-myc and SV40 promoters. NIH-3T3 cells transfected with these constructs were also tumorigenic when injected into nude mice, whereas the WT full-length surface proteins were not (Warner and Locarnini 2008; Lai et al. 2009). Another common LMV/LdT resistance substitution is rtM204I/sW196* stop (Fig. 2), which is observed in up to 10 % of LMV-resistant patients and more commonly with LdT (Warner and Locarnini 2008; Yuen et al. 2007). To date, there has been no report available concerning its effects on viral replication or hepatocyte biology.

Although NA therapies significantly decrease viral load and improve patient survival in the short term (Liaw et al. 2004a) and have been shown to reduce the HCC risk by 50 % (REF), it appears that they might unexpectedly select for HBV variants that are potentially oncogenic, providing one possible mechanism for the observation that the incidence of HCC is increasing globally.

Conclusion

Current treatment for most patients with CHB involves long-term NA therapy. Antiviral drug resistance is a key factor determining the long-term outcomes. The likelihood of resistance is determined by a combination of genetic barrier, drug potency, patient adherence, treatment history, and cross-resistance. Drugs such as ETV and TDF are associated with very low rates of virological breakthrough and should be the choice for first-line therapy. Management of treatment failure requires close clinical and virological monitoring, as well as early treatment intervention with salvage antivirals according to cross-resistance profiles. Future challenges in the treatment of CHB include the development of treatment strategies that effectively inhibit HBV replication eliminating the risk of drug resistance, potentially through novel host-targeting mechanisms, as well as the development of antiviral therapies that do not select for potentially oncogenic drug-resistant HBV.

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Protease Inhibitor Resistance

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Abstract

The recent development of hepatitis C virus (HCV)–specific direct-acting antivirals (DAAs) has marked a major milestone in the treatment of chronic HCV infection, allowing for viral elimination in the majority of treated patients. The first two drugs to be approved for the treatment of HCV genotype 1 infection were the HCV NS3/4A protease inhibitors telaprevir and boceprevir. However, their administration in combination with pegylated interferon alfa and ribavirin was associated with poor tolerability despite showing improved overall efficacy. In

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addition, as in DAAs targeting other viruses, concerns were raised with regard to the selection of drug-resistant viral variants. Selection of resistance-associated variants (RAVs) allows the virus to escape from drug pressure with subsequent treatment failure. The emergence of RAVs depends on a number of drug-, host-, and virus-related factors that are reviewed here. In addition, detailed resistance profiles of approved protease inhibitors and those that are still in clinical development are also discussed.

Keywords

Hepatitis C virus • Protease inhibitor • Ribavirin • Pegylated interferon alfa • Resistance • Resistance associated variant • NS3-4A • Telaprevir • Boceprevir • Simeprevir • Paritaprevir

Introduction

For the past decade, treatment of chronic hepatitis C infection was confined to pegylated interferon alfa (PEG-IFN) and ribavirin (RBV), both of which do not directly target viral proteins. Overall, rates of viral eradication have been poor, ranging from 20 % to 80 %, depending on the disease stage, viral genotype, and polymorphisms within the host interleukin 28B gene locus (Fried et al. 2002; Lange and Zeuzem 2011; Manns et al. 2001). Achievement of a sustained virologic response (SVR) defined as HCV-RNA negativity 12 or 24 weeks after treatment cessation has been particularly difficult in patients with HCV genotype 1 which constitutes the most prevalent genotype in Europe and North America, with SVR rates in the range of 40–50 % only (Fried et al. 2002; Manns et al. 2001).

Improved knowledge of the viral life cycle that followed the development of several different cell-based culture systems as well as experimental structure models of viral proteins led to the development of small molecules that directly target the viral replication machinery (Lohmann et al. 1999). The proof of principle for these direct-acting antivirals (DAAs) was established in 2003 with the development of ciluprevir, a small molecule inhibitor of the NS3-4A serine protease that led to a significant reduction of HCV-RNA (Lamarre et al. 2003). The further development of ciluprevir, however, was halted due to animal toxicity issues (Hinrichsen et al. 2004). Therefore, it was another 8 years until regulatory approval of the first two HCV NS3-4A protease inhibitors telaprevir and boceprevir for the treatment of chronic HCV genotype 1 infection in 2011. Each of these protease inhibitors (PI) are administered in combination with pegylated interferon alfa (PEG-IFN) and ribavirin (RBV), and the respective pivotal trials have demonstrated increased cure rates by 20–30 % when compared to PEG-IFN-based dual combination therapies as well as shortened treatment duration for the majority of patients that were treatment-naïve or previous relapsers (Bacon et al. 2011; Jacobson et al. 2011; Poordad et al. 2011; Sherman et al. 2011a; Zeuzem et al. 2011). However, in the wake of the first experiments with PIs administered as monotherapy, concerns were raised regarding the development of drug resistance–associated amino acid variants (RAVs).

These concerns were based on the knowledge of the high replication rate of HCV along with the lack of proofreading activity that leads to a genetically diverse population of viral variants within an infected patient, the so-called quasispecies. The quasispecies population contains both wildtype (WT) viruses and and viral variants with every possible single- and double-nucleotide variant and subsequently every RAV generated several times each day, according to mathematical modeling (Rong et al. 2010). RAVs within the NS3-4A protease domain are often less fit in terms of replication capacity and/or virus production (Shimakami et al. 2011; Welsch et al. 2012a) and are therefore usually present in much smaller numbers within the quasispecies population compared to WT variants. However, in the presence of selective drug pressure, outgrowth of RAVs can lead to treatment failure. Rapid selection of RAVs was observed in vitro and in vivo following the administration of telaprevir and boceprevir monotherapy. Hence, viral eradication with these drugs was not achievable by monotherapy but only in triple therapy combination with PEG-IFN and RBV (Kieffer et al. 2007; Sarrazin et al. 2007a, b). A detailed knowledge of the key parameters of PI resistance development and their clinical implications for combination therapies with PEG-IFN or drugs that target other viral structures is crucial for successful HCV eradication.

Molecular Basis of HCV NS3-4A as a Drug Target

The molecular and structural properties of the NS3-4A protease as a drug target have been reviewed in detail by Welsch (Welsch 2014) and Bartenschlager (Bartenschlager et al. 2013). The nonstructural protein 3 (NS3) is a 70 kDa cleavage product (631 amino acids in length) of the HCV polyprotein that is bound to the membrane of the host cell endoplasmatic reticulum. NS3 is bifunctional, possessing a protease and a helicase domain. The carboxy-terminal two-thirds of NS3 constitute a superfamily 2 DExH/D-box RNA helicase that also shows NTPase activity. Both activities are essential for HCV replication. Development of DAAs that target the helicase has proven difficult, mainly due to structural similarities with cellular RNA helicases (Bartenschlager et al. 2013). The N-terminal ~180 amino acids of NS3, together with the cofactor NS4A, constitute a serine-type protease domain of chymotrypsin fold, NS3-4A (Morikawa et al. 2011), which is required for processing of the viral polyprotein downstream of the NS2-3 junction. NS3-4A is also considered a key contributor to viral persistence as it is involved in blocking innate immune signaling cascades by cleavage of cellular substrates such as MAVS, mitochondrial antiviral signaling protein (also known as Cardif, IPS-1, and VISA), and TRIF (toll-IL-1 receptor domain-containing adaptor inducing IFN-ß), two key adaptor molecules in the RIG-I and TLR3 viral RNA-sensing pathways and as such involved in induction of type I interferons (Li et al. 2005; Meylan et al. 2005). Direct inhibition of the NS3-4A protease does not only interfere with viral replication but may also support viral clearance by restoring the innate immune response as shown previously (Johnson et al. 2007). Besides MAVS and TRIF, NS3-4A has been found also to target several other cellular proteins.

Given its implications for the host innate immunity, the elimination threshold of an NS3-4A RAV depends not only on its drug resistance level but also its interference with such signaling cascades and consecutively the host cell IFN responsiveness. Such mechanisms are thought to play a key role in the second slope decay and late phase of viral eradication under DAA pressure by (i) complete suppression of viral replication and/or (ii) eradication of residual virus and virus-infected cells (Welsch 2014). Variants that escape such immune mechanisms likely relate to some relapses observed in recent clinical trials with PIs (Poordad et al. 2014). It is shown that a poorly fit virus can replicate for weeks within the liver of a persistently infected chimpanzee in the absence of detectable viremia even with the very sensitive TMA assay and that when the virus does mutate to a more fit phenotype, the mutations that had made it unfit are eventually no longer detectable. As a consequence, caution must be taken in inferring the absence of virologic resistance from the absence of detectable RAVs following the reappearance of virus in relapsing patients (Yi et al. 2014).

The relatively shallow active site of the NS3-4A protease has been a major challenge for the development of NS3-4A inhibitors. However, based on the observation of N-terminal product inhibition of the enzyme, potent peptidomimetic inhibitors were later developed (Summa et al. 2012).

DAAs that directly target the NS3-4A protease can be divided into three chemical classes: linear peptidomimetics that derive their potency from covalent but reversible linkage with the active site residue serine 139 of the protease, linear peptidomimetics that do not form covalent adducts with the active site, and macrocyclic inhibitors that are chemically distinct as they utilize structural constraints instead of covalent linkage. Overall, HCV PIs have a low-to-medium genetic barrier to resistance, and selection of RAVs has been observed in vitro and in vivo. Moreover, a significant overlap of resistance profiles has been observed as shown in Table 1. In addition, the structure of the NS3-4A serine protease with bound inhibitor and RAV sites is depicted in Fig. 1.

The first class of PIs comprises the two NS3-4A inhibitors telaprevir and boceprevir. Monotherapy studies of both compounds had led to a rapid and profound reduction of HCV-RNA (Sarrazin et al. 2007b; Reesink et al. 2006). However, viral rebound was observed in all patients upon treatment cessation, and viral breakthrough due to the selection of RAVs was frequently documented (Sarrazin et al. 2007a; Susser et al. 2009). Thus for the first time, the concept of antiviral drug resistance, which had not been relevant in patients receiving PEG-IFN and RBV only, had become a major issue in the context of anti-HCV therapy. Subsequent studies of telaprevir or boceprevir given in combination with PEG-IFN and RBV showed an even more pronounced decline in HCV RNA and reduced frequency of resistance-associated viral breakthrough (Sarrazin et al. 2007b; Forestier et al. 2007).

More recently, second-generation PIs including simeprevir, faldaprevir, asunaprevir, paritaprevir, and grazoprevir have been developed for therapy in combination with PEG-IFN and RBV but also as part of interferon-free all-oral direct antiviral combination therapy regimens (Pawlotsky 2014).

Table 1 Cross resistance table of mutations at amino acid positions within the HCV NS3-4A protease associated with PI resistance. Blue boxes represent first generation linear PIs, green boxes represent first-generation macrocyclic PIs and the purple boxes represent second-generation PIs

	36	54	55	80	155	156A	156B	168	170
Telaprevir (linear)	■ (blue)	■ (blue)			■ (blue)	■ (blue)	■ (blue)		
Boceprevir (linear)	■ (blue)	■ (blue)	■ (blue)		■ (blue)	■ (blue)			■ (blue)
Faldaprevir (linear)					■ (blue)			■ (blue)	■ (blue)
Simeprevir (macrocyclic)				■ (green)	■ (green)		■ (green)	■ (green)	
Asunaprevir (macrocyclic)				■ (green)	■ (green)			■ (green)	
Paritaprevir (macrocyclic)					■ (green)			■ (green)	
Grazoprevir (macrocyclic)					■ (purple)	■ (purple)		■ (purple)	
Sovaprevir (linear)			■ (purple)	■ (purple)				■ (purple) a	

36: V36A/M; 54: T54S/A; 55: V55A; 80: Q80R/K; 155: R155K/T/Q; 156A: A156S; 156B: A156T/V; 168: D168A/V/T/H; 170: A/T. [a]mutations associated with in vitro resistance only

Parameters of Resistance Development

Virus-Related Parameters

Naturally Occurring RAVs

Direct sequencing analyses revealed a number of RAVs (e.g., substitutions at positions V36, T54, V55, Q80, R155, D168, and V170) to preexist at varying frequencies in patients who had been naïve to direct antiviral treatment (Bartels et al. 2008; Kuntzen et al. 2008). The more recent utilization of next-generation sequencing (NGS) methods provided evidence that RAVs preexist within the quasispecies cloud in virtually all patients but mostly at very low frequencies (Chevaliez et al. 2011).

The clinical consequences of preexisting RAVs were further elucidated by analyzing data of patients who had not responded to PEG-IFN-based treatment regimens (prior null response or 1 log HCV RNA decline during 4 weeks of PEG-IFN/RBV). In these patients who were a priori insensitive to the PEG-IFN backbone, addition of a PI as de facto monotherapy in the setting of preexisting RAVs led to treatment failure in all cases (De Meyer et al. 2012a; Howe et al. 2013a). However, as primary PEG-IFN nonresponsiveness is rare and the probability to detect a preexisting RAV as a major variant is low (3–8 %), there was no significant impact of baseline RAVs on treatment outcome in patients who received telaprevir- or boceprevir-based triple therapies (Howe et al. 2013a; Barnard et al. 2013; Bartels et al. 2013; Hezode et al. 2014; Poordad et al. 2012; McHutchison et al. 2010).

For the second-generation protease inhibitor simeprevir, a previously unknown RAV within the NS3-4A protease domain was described that conferred

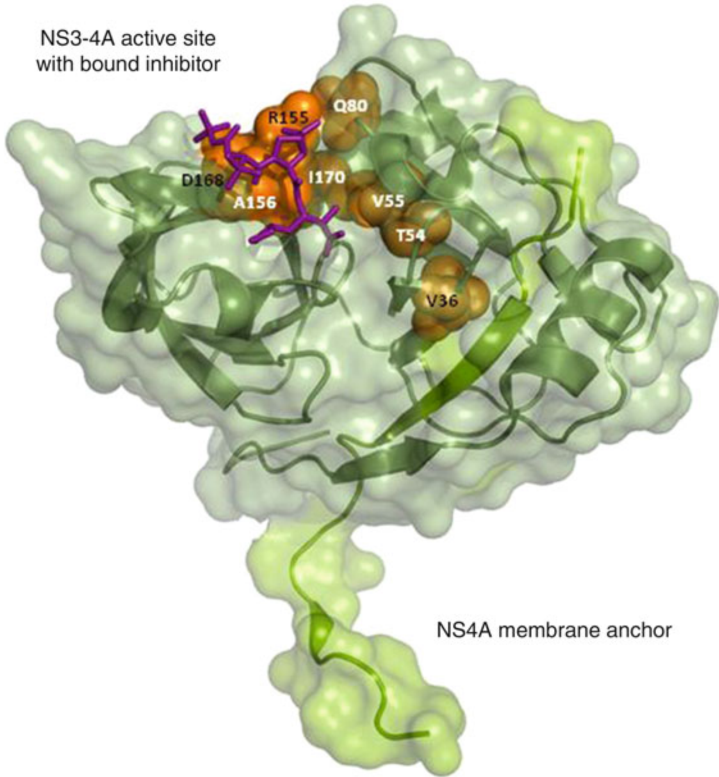

Fig. 1 Structure of the NS3-4A serine protease with bound inhibitor and resistance-associated amino acid sites. NS3-4A protease structure from Protein Databank entry 2OC8, showing the NS3 protease domain and NS4A as *dark green* and *light green* ribbon models respectively with transparent surface representation. A bound NS3-4A protease inhibitor is given as *purple* stick model. Resistance-associated sites (according to Table 1) are given as orange spheres (CPK models)

low-to-medium-level resistance in vitro (Q80K). Importantly, Q80K was confirmed to be a commonly observed RAV in untreated patients, specifically for genotype 1a, as it can be found in 19 % and 48 % of untreated HCV genotype 1a patients in Europe and the United States, respectively (Lenz et al. 2011), while it is rarely seen in genotype 1b (0.5 %). Although the resistance level for Q80K against PIs was determined to be rather low (only approximately tenfold change), its clinical consequences are all the more striking: In large phase 2 and phase 3 clinical trials, Q80K was highly associated with treatment failure in subtype 1a patients leading to response rates that were statistically not superior to the PEG-IFN/RBV control groups (Jacobson et al. 2014; Lenz et al. 2012; Manns et al. 2014c). However, the underlying molecular mechanisms remain yet to be determined.

Currently, IFN-free treatment regimens that also include NS3-4A PIs are being evaluated in phase 2–3 clinical trials. For example, simeprevir in combination with the nucleoside NS5B polymerase inhibitor sofosbuvir and a multidrug regimen

including the ritonavir-boosted PI paritraprevir in combination with the NS5A inhibitor ombitasvir and the non-nucleoside NS5B polymerase inhibitor dasabuvir are all evaluated in patients with HCV genotype 1 infection. While the clinical impact of baseline RAVs has yet to be determined for these multidrug regimens (Poordad et al. 2014; Feld et al. 2014; Zeuzem et al. 2014), some data are available for the combination of simeprevir and sofosbuvir. In a relatively small study of this drug regimen, virologic failure was observed in subtype 1a patients only. However, despite an association of Q80K with treatment failure (Q80K was noted as a baseline RAV in four out of six patients with treatment failure), the majority of patients with preexisting Q80K (>90 %) still achieved sustained virologic response (SVR) (Lawitz et al. 2014). Ongoing larger phase 3 studies will have to determine the potential impact of baseline RAVs on treatment response in patients with all-oral PI-containing DAA treatment regimens.

Genetic Barrier

The likelihood of breakthrough is not entirely dependent on the mere presence or the number of preexisting RAVs but also relates to the genetic barrier to resistance, which is defined as the number of nucleotide changes for a resistance mutation to occur (Gish et al. 2012). NS3-4A PIs have a relatively low genetic barrier to resistance with significant differences between HCV subtypes due to the nucleotide sequence pattern at respective resistance-associated amino acid residues (Kieffer et al. 2007; Sarrazin et al. 2007a). For example, HCV subtype 1b possesses a higher genetic barrier to resistance compared to subtype 1a, which is in part related to the number of mutations required for resistance development that is higher in 1b than 1a (Sarrazin et al. 2007a). For example, a resistance-associated nucleotide change at codon 155 for generation of the R155K mutation requires two nucleotide changes in subtype 1b but only one change in subtype 1a (Welsch 2014). As a consequence, RAVs develop more frequently in patients with subtype 1a who fail to achieve SVR following a PI-based treatment regimen (Bacon et al. 2011; Poordad et al. 2011). The quality of the nucleotide change may also contribute to the genetic barrier to resistance. It is shown that a mutational bias in favor of nucleotide transitions over transversions may directly affect the emergence of RAVs (Grammatikos et al. 2014; Powdrill et al. 2011).

Variant Fitness

Another important aspect is the fitness phenotype of a RAV, which has been defined as the ability to replicate in the setting of natural selection (Domingo et al. 1997). RAVs are usually less fit compared to wild-type virus. However, under selective drug pressure, the virus wild type can be rapidly suppressed whereas RAVs continue to replicate and may eventually become the dominant viral strain in a given host environment.

Compensatory Mutations

Upon discontinuation of antiviral therapy, variants that harbor resistance mutations are usually replaced by wild-type virus due to inferior variant fitness. However,

RAVs may eventually become fixed in the viral quasispecies population due to compensatory second-site mutations. The selection of such compensatory mutations may also allow for efficient replication in the presence of drug pressure. Few data exist on the underlying mechanisms and potential clinical implications of such compensatory mutations. However, complex patterns of mutations were recently described from patients treated with boceprevir or telaprevir that showed an increasing complexity of linked variant combinations in patients with viral breakthrough during therapy (Susser et al. 2012), whereas other data suggest that compensatory substitutions are not observed in patients with telaprevir treatment failure (Sullivan et al. 2011). Such second-site mutations may also explain why some RAVs persist upon treatment cessation and why RAVs that negatively impact the virus replication capacity in a cell culture model of HCV infection can dominate in untreated patients (Welsch et al. 2012b).

Drug-Related Parameters

The probability of RAVs to be selected in the presence of PI therapy depends on a number of factors related to the specific compound. One such factor is the potency of the drug, which is a function of viral susceptibility and drug exposure, where susceptibility depends on the molecular structure of the drug target site whereas the drug exposure depends on its ADME properties, absorption, distribution, metabolism, and excretion. The close interplay between drug exposure and barrier to resistance has been demonstrated for telaprevir, where trough plasma concentration levels of the drug did correlate with the emergence of RAVs and hence viral breakthrough (Sarrazin et al. 2007a). However, the specific accumulation of active drug metabolites in the liver renders plasma drug levels with only limited predictive power for antiviral efficacy of NS3-4A PIs.

Host-Related Parameters

Patient adherence is crucial for the avoidance of RAV development and associated viral breakthrough. So far, failure of DAA-based therapies was mostly restricted to post-treatment relapse whereas breakthrough during antiviral therapy was more rarely observed in clinical trials of PIs. Here, breakthrough was mostly related to poor treatment adherence. Similarly, subtherapeutic plasma trough levels of antiretroviral drugs in patients infected with the human immunodeficiency virus (HIV) were found in association with treatment failure (Gardner et al. 2009). Despite this, exposure–response analyses did not show a significant association between higher PI exposure and increased SVR rates (FDA 2011).

Another important issue is potential drug–drug interactions (DDIs) that can pose an additional threat to successful HCV eradication. As all NS3-4A PIs are metabolized to some extent by cytochrome P450 3A4 (CAP3A4), their effect on other CYP3A4 substrates that are commonly used therapeutics (e.g., antiretrovirals,

antibiotics, antimycotics, antidepressants, and immunosupressants) needs to be taken into account when starting antiviral therapy and preferably avoided during the course of antiviral treatment or the dosing of either the PI or the concomitant medication need to be adjusted.

Host factors that are relevant for immune responses and/or delivery of drugs to the target cells in the liver can be important cofounders for treatment success, i.e., host genetics such as polymorphisms within the interleukin (IL) 28B gene locus on chromosome 19. The disease stage seems to have a particular impact on treatment outcome. Patients with cirrhosis tend to achieve lower SVR rates even when treated with the most advanced PI-based all-oral drug combinations. Whether this is due to cirrhosis-related alterations in the patient's endogenous immune response and/or alterations in the pharmacokinetics/pharmacodynamics in the setting of portal hypertension requires further elucidation (Afdhal et al. 2014).

Resistance Profiles of Protease Inhibitors During Antiviral Therapy

Telaprevir

Telaprevir (VX-950; Vertex Pharmaceuticals, Cambridge, MA, USA) is a linear peptidomimetic ketoamide NS3-4A PI that forms a covalent, reversible enzyme–inhibitor complex. Telaprevir led to a median reduction of 4.4 $\log_{10}$ IU/ml and 5.49 $\log_{10}$ IU/ml HCV RNA when given as monotherapy (750 mg every 8 h) and in combination with PEG-IFN/RBV, respectively, in patients with HCV genotype 1 infection (Reesink et al. 2006; Forestier et al. 2007). Additional phase 1 and 2 studies showed that telaprevir has good antiviral activity in HCV genotype 2 but only minimal activity in patients with genotype 3 and 4 (Benhamou et al. 2013; Foster et al. 2011). Telaprevir has been approved only for the treatment of HCV genotype 1 infection.

RAVs against telaprevir were first identified at position A156 of the NS3/4A protease catalytic domain using HCV replicon cell lines (Lin et al. 2004). In vivo analysis of phase 1 trials confirmed residue 156 as a key site for telaprevir resistance (Kieffer et al. 2007; Sarrazin et al. 2007a). However, additional RAVs were also detected either as single or double mutations, such as the low-level resistance mutations V36A/M, T54A, R155K/T, and A156S that show higher fitness in terms of viral replication compared to variants with high-level resistance, such as A156V/T and V36A/M + A156V/T (Sarrazin et al. 2007a).

A comprehensive analysis of the telaprevir phase 3 trials revealed that 77 % of patients not responding to telaprevir-based triple therapy had detectable RAVs at the time of treatment failure. In patients with HCV genotype 1a infection, the most frequently detected variants were V36M and R155K or the combination of both whereas in genotype 1b patients, V36A, T54A/S, and A156S/T were mainly observed (Sullivan et al. 2013). The occurrence of RAVs was more frequently observed in HCV genotype 1a patients compared to genotype 1b but did not appear

to depend on the prior treatment status. Despite this, virologic failure occurred in 52 % of prior null responders and was associated with higher-level resistance whereas the failure rate in prior relapsers was only 1 %. In treatment-naïve patients, who represent a mixture of patients with different sensitivities to PEG-IFN/RBV, the virologic failure rate was 7 % (De Meyer et al. 2012b). Taken together, these findings suggest that the likelihood of failure to telaprevir-based triple therapy mainly depends on the responsiveness to the PEG-IFN/RBV backbone.

Follow-up investigation of telaprevir-treated patients who did not achieve SVR revealed a median time to loss of RAVs of 10.6 and 0.9 months for genotype 1a and 1b, respectively (Sullivan et al. 2013).

Long-term follow-up (median duration, 29 months) of patients ($n = 126$) treated in phase 2/3 trials with telaprevir demonstrates a loss of RAVs in 85 % of patients by using population sequencing (Fig. 2) (Sherman et al. 2011b). However, more sensitive techniques such as clonal sequencing and deep sequencing did reveal additional low-level resistant variants even at much later time points (Dierynck et al. 2013). Despite this, at baseline of retreatment with telaprevir-based triple therapy, no RAVs were detectable using deep sequencing up to 5.7 years after short-term exposure to telaprevir in phase 1 trials (Fig. 3) (Sarrazin et al. 2013). Finally, in a small study, patients exposed to telaprevir during phase 1 trials were retreated with a full course of telaprevir-based triple therapy. Here, in four patients

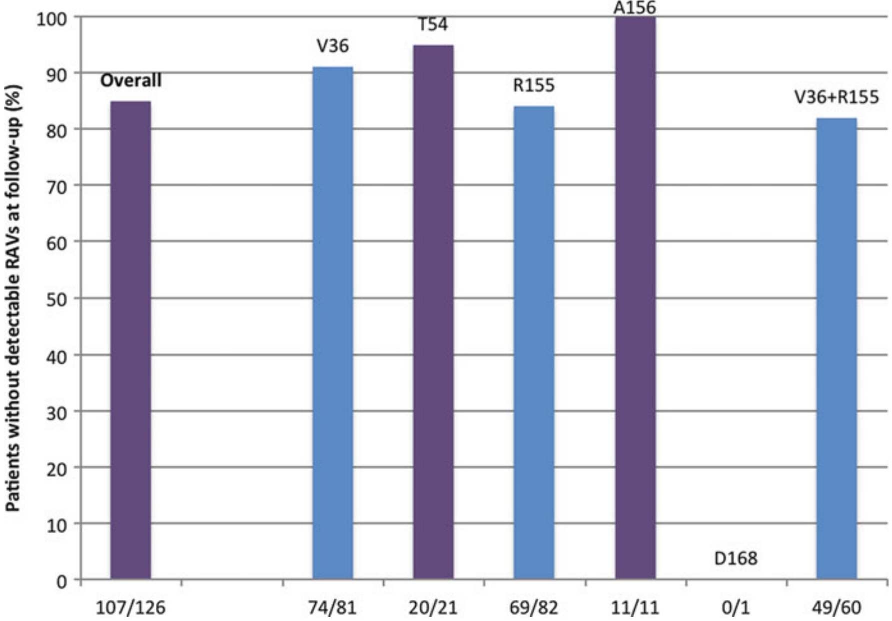

Fig. 2 Long-term follow-up of of telaprevir RAVs. The proportion of patients without detectable RAVs at a median follow-up of 29 months upon treatment failure is shown. Variant positions within the NS3-4A protease domain are shown above each column (Adapted from Sherman et al. (2011b))

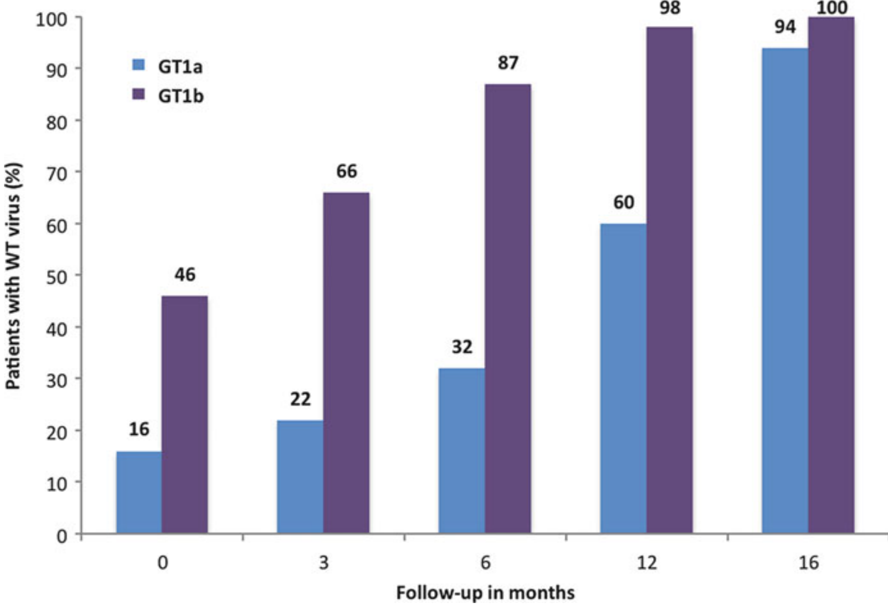

Fig. 3 RAV reversal to wild-type. Time to reversal to wild-type in months after telaprevir-based treatment in patients with HCV genotype 1a versus 1b infection (Adapted from Sullivan et al. (2013))

with treatment failure, viral variants were analyzed by means of sequential deep sequencing during the first and second telaprevir exposure. Interestingly, a heterogeneous evolution of viral isolates was observed, and no general rule for (re) selection of resistant variants after a second exposure to telaprevir could be established (Susser et al. 2015).

Boceprevir

Boceprevir (SCH 503034; Merck and Co., Whitehouse Station, NJ, USA) is a linear ketoamide PI that has led to a mean maximum reduction in HCV RNA of 1.61 and 2.88 $\log_{10}$ IU/ml after 1–2 weeks of monotherapy (400 mg three times daily) and in combination with PEG-IFN, respectively, in patients with HCV genotype 1 (Sarrazin et al. 2007b). Boceprevir has limited antiviral activity in HCV genotypes 2 and 3 (Silva et al. 2013) and was only approved for the treatment of genotype 1 infection.

Boceprevir has a largely overlapping resistance profile with telaprevir (Table 1). RAVs have been detected at positions V36, T54, R155, A156, and V170 in vitro (Tong et al. 2006) whereas additional mutations at positions Q41, F43, V55, and V158 were detected in phase 1 clinical trials (Susser et al. 2009; Vermehren et al. 2012). The mutations V36G, T54S, and R155L confer low-level resistance whereas medium-level resistance is observed for T54A, V55A, R155K, A156S, and V170A. Of note,

variant V170A has been observed more frequently during boceprevir treatment and showed higher resistance levels compared to telaprevir (Sarrazin and Zeuzem 2010). Finally, A156T is known to confer the highest resistance level to boceprevir (Susser et al. 2009).

Population sequencing analyses from patients in the phase 3 trials SPRINT-2 (Poordad et al. 2011) and RESPOND-2 (Bacon et al. 2011) with boceprevir in triple combination with PEG-IFN and RBV showed that V36M, T54S, and R155K were found more frequently with HCV genotype 1a infection whereas variants T54A, V55A, A156S, and V170A were found more frequently in genotype 1b patients. As with telaprevir, the overall frequency of RAVs was higher in genotype 1a patients. Overall, postbaseline RAVs were detectable in 53 % of non-SVR patients, showing a decline to 22.8 % within 6–14 months upon treatment discontinuation (Barnard et al. 2013). The median time for all RAVs to disappear was 1.11 (1.05–1.2) years, which is not significantly different between subtypes 1a and 1b (Howe et al. 2013b). However, when applying the more sensitive clonal sequencing approach, boceprevir-resistant variants were detectable up to 4 years following short-term boceprevir exposure in phase 1 studies (Susser et al. 2011).

As for telaprevir, a second short-term low-dose exposure to boceprevir in phase 1 studies showed a heterogeneous evolution of RAVs with reoccurrence of variants selected during a previous course of boceprevir therapy in only a minority of patients (Vermehren et al. 2012).

Simeprevir

Simeprevir (TMC435; Janssen Pharmaceutica, Beerse, Belgium) was the first macrocyclic NS3-4A PI to be approved for the treatment of HCV genotype 1 infection. Simeprevir monotherapy (200 mg once daily) led to a median maximal reduction of 3.9 $\log_{10}$ IU/ml HCV RNA after 5 days in patients with HCV genotype 1 infection. Antiviral activity was comparably high in genotypes 4 and 6, followed by genotypes 2 and 5 (2.2–2.7 $\log_{10}$ decline), whereas no antiviral activity was evident for genotype 3 (Moreno et al. 2012), and these findings correlated with preexisting RAVs that are known to reduce or abolish the binding efficiency of simeprevir to the NS3-4A protease (Lenz et al. 2013).

The main resistance loci identified in clinical trials were Q80, R155, and D168, whereas RAVs at positions F43 and A156 have been detected only in experimental studies (Lenz et al. 2010; Reesink et al. 2010). Levels of resistance range from <10-fold for Q80K to about 2,000-fold for D168V and D168I. At residue 156, the fold change ranges from 16–44 for A156G/T to 177 for A156V, depending on the specific amino acid change (Lenz et al. 2010).

Due to significantly reduced SVR rates in patients with HCV genotype 1a infection treated with simeprevir in combination with PEG-IFN/RBV in the presence of Q80K-baseline RAVs, the FDA recommended for the first time a routine resistance testing prior to antiviral treatment. Interestingly, the majority of genotype 1a patients with preexisting Q80K RAVs seem to exhibit R155K at the time of

treatment failure. Therefore, it has been concluded that a facilitated selection of additional RAVs in the presence of Q80K as opposed to Q80K alone may be responsible for treatment failure in these patients (Lenz et al. 2013, 2015). Small phase 2 studies of simeprevir as part of all-oral drug combinations consistently showed high SVR rates in patients with and without Q80K-baseline RAVs in genotype 1a patients (Lawitz et al. 2014), whereas larger phase 3 studies that elaborate the importance of Q80K for treatment failure are to be awaited. Median time until loss of Q80K was 36 and 24 months for genotype 1a and 1b, respectively. Loss of R155K in the presence of Q80K took 32 months whereas R155K alone disappeared after 64 months only (Lenz et al. 2015).

Faldaprevir

RAV selection during treatment with the linear tripeptide faldaprevir (BI201335; Boehringer Ingelheim, Ingelheim, Germany) is mainly restricted to mutations at residues 155 and 168. However, mutations at positions R155, A156, and D168 were all associated with resistance development in preclinical studies (Lagace et al. 2012). Clinically important RAVs that confer moderate (130-fold change) to high-level (up to 1,800-fold change) resistance include R155K and D168V, respectively (Berger et al. 2013). Chances of virologic failure are significantly higher in genotype 1a patients where R155K is predominantly selected (Sulkowski et al. 2013a, b). The more common Q80K variant was not associated with reduced SVR rates, and S61L was observed as a second-site mutation with D168V (Berger et al. 2014). No clinical data are available for antiviral activity of faldaprevir in genotypes 2–6. The company that developed faldaprevir recently decided not to move forward with the approval process due to the growing market of potentially more successful competitor drugs.

Asunaprevir

Asunaprevir is a linear tripeptide (BMS-650032; Bristol-Myers Squibb; New York, NY, USA) that has been approved as part of a PEG-IFN-free all-oral drug regimen that also includes the HCV NS5A replication complex inhibitor daclatasvir for the treatment of HCV genotype 1 infection in Japan. Preclinical studies identified R155K, D168G, and I170T to confer low- to moderate-level resistance against asunaprevir in genotype 1a (5- to 21-fold), whereas in genotype 1b, the main site for RAVs to occur is at position D168 with high-level asunaprevir resistance of 16- to 280-fold (McPhee et al. 2012). In clinical studies, the predominant baseline RAV was once again Q80K, which had an impact on virologic response rates in a single-ascending-dose study, but less so after administration of multiple doses of asunaprevir (McPhee et al. 2012).

In an all-oral combination therapy comprising asunaprevir and daclatasvir, 643 genotype 1b–infected patients showed preexisting RAVs in the NS3-4A protease domain in 11 % of cases. While overall SVR rates in the different study arms

were high (82–90 %), only 38 % of patients with naturally occurring RAVs achieved SVR (Kao et al. 2014; Manns et al. 2014a). Thus, RAVs had an obvious influence on virologic response in this DAA regimen with a low barrier to resistance.

Paritaprevir

Preclinical resistance data on the linear PI inhibitor paritaprevir (ABT-450; AbbVie; North Chicago, IL, USA) have not yet been disclosed, and clinical data have only been published in abstract form. The R155K and D168V RAVs were detected by population sequencing after only 3 days of dosing in most patients treated with low doses of ritonavir-boosted paritaprevir (paritaprevir/r) whereas higher doses of paritaprevir/r appeared to suppress early resistance emergence (Pilot-Matias et al. 2011). A multidrug DAA regimen comprising paritaprevir/r, the NS5A inhibitor ombitasvir, and the non-nucleoside NS5B polymerase inhibitor dasabuvir was shown to be highly effective in genotype 1a and 1b–infected patients with SVR rates of 91–99 %. The rare occurrence of treatment failure in these patients was primarily observed in subtype 1a and was typically associated with the selection of RAVs against more than one DAA. The impact of rarely observed baseline RAVs on treatment outcomes has not yet been addressed (Poordad et al. 2014; Feld et al. 2014; Zeuzem et al. 2014). As of now, the question whether multidrug combinations have the potential to reduce the impact of naturally occurring RAVs on single DAA agents remains to be determined and certainly requires further attention. No clinical (resistance) data are available for HCV genotypes 2–6.

NS3-4A Protease Inhibitor Pipeline

The structural constraints of first-generation macrocyclic inhibitors allow for high drug potency but limit their efficacy in genotypes other than HCV genotype 1 (danoprevir, vaniprevir, GS-9256, GS-9451, IDX-320) (Sarrazin et al. 2012). Thus, recent developments include compounds that are highly potent and broadly active against different HCV genotypes as well as active against variants known to confer resistance against first-generation PIs. The macrocyclic PI grazoprevir (Merck & Co, Whithouse Station, NJ, USA) is the first compound with antiviral activity against HCV strains harboring the R155K mutation (Summa et al. 2012). While clinical data suggest that R155K can be still selected during grazoprevir therapy in HCV genotype 1, the resistance level seems to be too low for virologic breakthrough to occur (Strizki et al. 2012). Due to liver toxicity at higher doses that were associated with high antiviral activity in HCV genotype 3, the further development was restricted to lower doses and genotype 1 only (Summa et al. 2012; Manns et al. 2014b).

Sovaprevir (ACH-1625; Achillion, New Haven, CT, USA) is a linear peptidomimetic PI that noncovalently binds to the active site thereby showing high potency and broad genotypic coverage in early clinical trials. While Q80K

was detected in both phase 1 and phase 2 studies, virologic response was not affected in the respective patients suggesting that drug exposure was sufficient to inhibit this variant to be selected (Fabrycki et al. 2012). Additional therapeutic trials are ongoing.

Conclusion

NS3/4A PIs represent the first class of DAAs that were approved for the treatment of chronic HCV infection. While adding a PI to the PEG-IFN-based treatment backbone has substantially increased cure rates, the selection of drug-resistant viral variants has evolved as an important limiting factor.

RAVs may be present in the viral quasispecies cloud already before treatment is started. However, while a clear association between some baseline RAVs (e.g., Q80K in simeprevir-treated patients) and treatment failure has been observed for PEG-IFN-based combination therapies, their clinical impact on the outcome of IFN-free DAA regimens is unclear to date. Parameters that determine RAV selection during antiviral therapy include genetic barriers and variant fitness phenotypes. The genetic barrier of HCV PIs is comparatively low, and combination regimes with DAAs that possess a higher genetic barrier (e.g., nucleotide polymerase inhibitors) have been more effective. However, it is the variant fitness that impacts RAV persistence most and the ability to compete with wild-type virus upon treatment cessation. Whether drug resistance will have any clinically meaningful impact on current and future DAA combination therapies remains to be determined. This will, however, require in-depth knowledge of the exact molecular escape mechanisms of each antiviral class available.

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Contribution of APOBEC3-Driven Mutagenesis to HIV Evolution and HIV Drug Resistance

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Selection of the Bibliography: An extensive review of the current literature was performed using the keywords "APOBEC3, APOBEC3G, Vif, and/or HIV drug resistance." Due to space constraints, we sometimes had to refer to reviews summarizing a given topic rather than citing original articles.

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Abstract

Sequence variation is central to the ability of HIV-1 to evade immune responses and antiretroviral therapeutics. APOBEC3 editing enzymes are potent mutagens of retroviral genomes. In humans, the APOBEC3 family comprises seven different proteins (APOBEC3A [A3A] to A3H), whose cytidine deaminase activity – if left unchecked – results in extensive mutagenesis of the HIV-1 genome. There is emerging evidence that cytidine deaminases other than A3G play an important role in restricting the spread and replication of HIV-1. APOBEC3 molecules, indeed, differ not only in catalytic activity and expression but also in susceptibility to HIV-1 Vif-mediated degradation. The interplay between these intracellular host defenses and HIV counterstrategies is discussed in this chapter with a special emphasis on viral evolution and drug resistance.

Keywords

A3G • A3H • Acquired immunodeficiency syndrome (AIDS) • Drug resistance • Hypermutations • Mutagenesis • Restriction factor • Reverse transcriptase (RT) • Viral evolution • Viral-host interaction

Introduction

The APOBEC3 family comprises seven members (A3A, A3B, A3C, A3DE, A3F, A3G, and A3H) whose genes are located in tandem on human chromosome 22 (Fig. 1, Jarmuz 2002; Wedekind et al. 2003). They constitute the most recent addition to the superfamily of cytidine deaminases (Jarmuz 2002) of which APOBEC1 and AID (activation-induced deaminase) were the first described representatives (Wedekind et al. 2003). This group of mammalian proteins with DNA-editing activity can introduce genetic modifications in retroviral genomes through cytosine deamination (Jarmuz 2002). Recent studies have shown that A3G, A3F, A3DE, and certain A3H haplotypes not only display a range of antiretroviral activities but also differ in their susceptibility to degradation by the different circulating HIV Vif variants (Albin et al. 2013; Malim 2009). Importantly, the

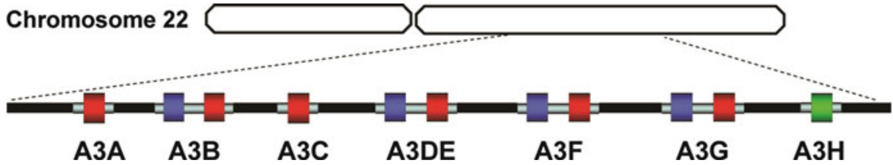


Fig. 1 Schematic representation of the human APOBEC3 locus. These proteins are composed of either one or two deaminase domains (*red*, *blue*, or *green*). A3H is identified in a different color to illustrate the fact that it has different haplotypes that differ in antiviral activity

APOBEC3 proteins are co-expressed in cell populations susceptible to HIV infection (e.g., primary human T lymphocytes and macrophages (Albin et al. 2013; Harris et al. 2003; Mangeat 2003; Koning et al. 2009)).

Cellular Cytidine Deaminases with Antiretroviral Activity

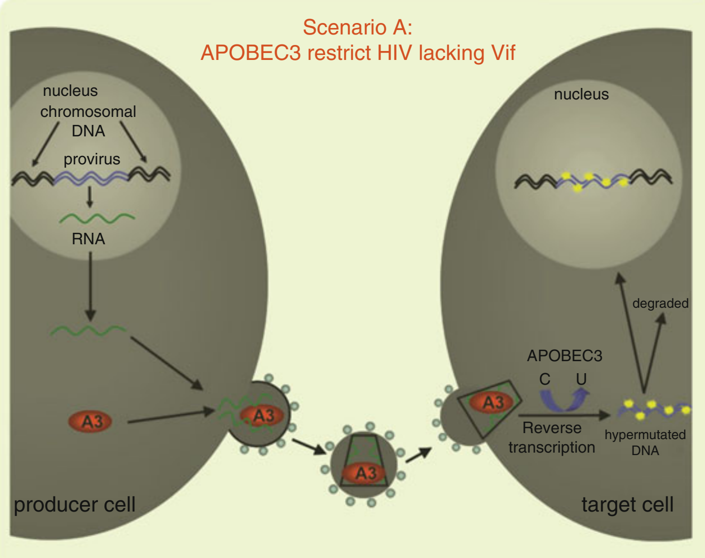
The identification of the human gene APOBEC3G (initially referred to as CEM15, abbreviated here throughout the text as A3G) as the target of the HIV Vif in 2002 (Sheehy et al. 2002) spurred a series of studies aimed at elucidating this novel host defense mechanism (see Albin and Harris 2010; Bieniasz 2004; Neuberger 2003; Refsland and Harris 2013 for reviews). Indeed, the presence of the accessory lentiviral protein Vif in "nonpermissive" (=APOBEC3 expressing) producer cells is essential for the generation of HIV particles that can productively infect other cells (e.g., T lymphocytes). Conversely, viral particles generated in the presence of A3G but in the absence of Vif yield proviruses with reduced viral infectivity due to the high frequency of G-to-A mutations (Lecossier 2003; Mariani et al. 2003).

Our current understanding of the APOBEC3 mode of action is as follows. APOBEC3 are incorporated into newly generated viral particles in the absence of Vif. Upon infection of a new cell, APOBEC3 enzymes deaminate deoxycytidine to deoxyuracil in the retroviral minus-strand cDNA generated during the reverse transcription step, which, in turn, results in guanosine-to-adenosine (G-to-A) mutations in the plus-strand cDNA (Harris et al. 2003; Mangeat 2003). Depending on the frequency and the position of these G-to-A mutations, the resulting provirus may be defective or display an attenuated phenotype (Fig. 2). In addition to this editing-dependent restriction mechanism, APOBEC3 can block HIV replication by non-editing means (Gillick et al. 2013).

All lentiviruses with the exception of equine infectious anemia virus express Vif ("viral infectivity factor"). In doing so, they have developed an efficient way to protect their genome from the deleterious effects associated with cellular cytidine deaminases. Vif reduces the level of APOBEC3 in the producer cell by targeting it for proteasomal degradation (Sheehy et al. 2002; Stopak et al. 2003; Marin 2003; Conticello et al. 2003a; Liu et al. 2004; Zennou et al. 2004). HIV Vif interacts with a complex of four cellular proteins (Cullin 5, Elongin B and C, and Rbx1 as well as APOBEC3) which leads to ubiquitination/proteasomal degradation of the deaminase (Yu et al. 2003, 2004; Yu 2004). Recently, it has been revealed that a transcription factor, CBF-beta, is essential for stabilizing HIV Vif (Zhang et al. 2012; Jager et al. 2012; Kim et al. 2013) (Fig. 3). The first crystal structure of HIV Vif was solved in 2014 in complex with the E3 ligase Cullin 5 and the transcription factor CBF-beta (Guo et al. 2014).

It was initially thought that A3G was the most potent antiretroviral deaminase, but in the past couple of years, a more nuanced picture has emerged. It is now well accepted that A3B, A3DE, A3F, and A3H, in addition to A3G, display activity against HIV variants lacking active Vif alleles (Albin et al. 2013; Sato et al. 2014; Chaipan et al. 2013; Hultquist et al. 2011).

For example, the role of A3F on HIV-1 restriction in human primary blood mononuclear cells (PBMCs) was investigated using a full-length HIV-1 NL4-3 Vif



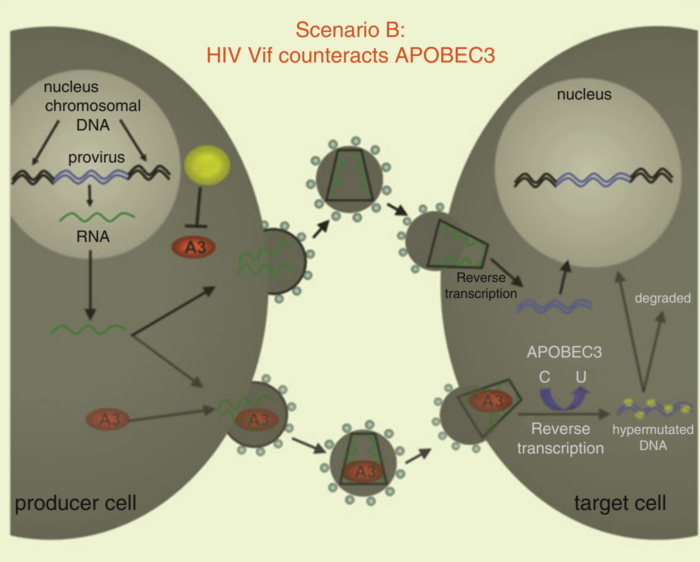


Fig. 2 The mode of action of APOBEC3 restriction is depicted. In the absence of HIV Vif, APOBEC3 proteins are incorporated into the egressing virions and deaminate the minus-strand cDNA in the next round of infection (scenario A). However, circulating HIV strains express the accessory protein Vif which excludes APOBEC3 from the virions resulting in fully infectious viral particles (scenario B)

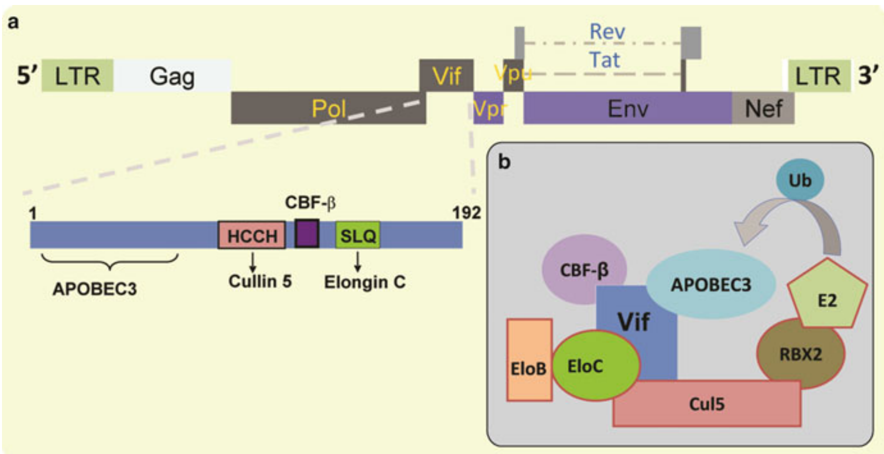


Fig. 3 The accessory protein Vif counteracts APOBEC3 by inducing their proteasomal degradation. Regions in Vif which are important for interacting with the host cell machinery as well as with the APOBEC3 protein are highlighted: (**a**) Vif binds CBF-beta and APOBEC3 and assembles a E3 ligase complex which results in the degradation of the deaminase. Abbreviations: *Ub*, Ubiquitin; *EloC*, Elongin C; *EloB*, Elongin B; *Cul5*, Cullin 5. Note that the identity of the E2 remains unknown to date

mutant (HIV W11R) that normally degrades A3G but does not target A3F (Mulder et al. 2010). This Vif mutant virus replicated as efficiently as the wild-type NL4-3 in human PBMCs suggesting a marginal contribution for A3F to HIV-1 restriction. This notion was further corroborated by the low A3F expression levels in PBMCs when compared to A3G (Mulder et al. 2010). The same mutant HIV W11R is restricted in the frequently used nonpermissive MT2 T-cell line, which expresses comparable levels of A3G and A3F. Recently, the modest activity of A3F has also been reported by others using stable cell lines (Miyagi et al. 2010).

Emerging evidence points to the importance of A3H in restricting HIV replication. A series of publications established that multiple A3H protein variants with distinct properties exist in human populations. Indeed, a cluster of single nucleotide polymorphisms (SNPs) determines protein stability, lentiviral restriction, as well as susceptibility to Vif-mediated degradation (Tan et al. 2009; OhAinle et al. 2008a, b; Harari et al. 2009; Dang et al. 2008). The Emerman laboratory reported that these destabilizing SNPs occurred twice independently during human evolution. The stable A3H variants (e.g., haplotype II) are found at high frequencies in populations of African descent (e.g., Yoruba), while they are less prevalent in European or Asian populations (OhAinle et al. 2008b). A3H is not only polymorphic in sequence but also subjected to alternative splicing (Harari et al. 2009). Evidence for HIV Vif adaptation to specific A3H haplotypes can be found in HIV-infected patients suggesting that A3H is a bona fide restriction factor (Ooms et al. 2013).

Deamination Target Site Preference of Human APOBEC3 Proteins

All APOBEC3 proteins are expressed to a varying degree in different human cell populations. Deamination by any of the APOBEC3 proteins is not specifically aimed at retroviral sequences but rather targets any single-stranded DNA. Indeed, APOBEC3-driven mutagenesis is not only apparent in retroviral genomes but also in human cancer genomes (Harris 2013; Kuong and Loeb 2013; Razzak 2013).

In contrast to this broad activity, APOBEC3 proteins display distinct target site discrimination (Albin and Harris 2010). For example, A3G favors a 5′-dCdC dinucleotide context ("GG" in the provirus), while the other deaminases with anti-HIV activity (A3DE, A3F, and A3H) favor a 5′-dTdC dinucleotide context ("GA" in the provirus). A3B has been shown to introduce mutations in both dinucleotide contexts (Albin and Harris 2010). Patient-derived proviral sequences display G-to-A mutations in both GG and GA dinucleotide contexts (Albin and Harris 2010; Fourati et al. 2014).

Vif Sequence Motifs Required for Anti-APOBEC3 Function

The HIV Vif protein promotes the ubiquitin/proteasome-dependent degradation of A3C, A3G, A3F, A3D, and A3H hapII (Albin and Harris 2010; Mehle et al. 2004). Of note, all functional domains of Vif have been mapped using Vif from subtype B molecular clones such as NL4-3, LAI or HXB2, and APOBEC3 reference proteins. Based on these analyses, the N-terminal region of Vif is important for Vif binding to A3G, A3F, and possibly other APOBEC3 proteins (Mariani et al. 2003; Mehle et al. 2004; Russell and Pathak 2007; Conticello et al. 2003b; Kao et al. 2003; Simon et al. 2005; Mehle et al. 2007; Schrofelbauer et al. 2006; Tian et al. 2006; Wichroski et al. 2005). Site-directed mutagenesis studies revealed that a number of residues located throughout the protein are essential for infectivity and viral replication in nonpermissive cells (Simon et al. 1999; Fujita et al. 2003). Mutations of the two cysteine residues (positions 114 and 133; Ma et al. 1994) as well as alanine substitutions in the conserved SLQ sequence motif (residues 144–147) abolish Vif activity. The C-terminal region of Vif contains the SLQYLAXXXX SOCS-box (residues 145–154) which is important for Vif binding to Elongin C in the E3 ubiquitin ligase complex (Yu et al. 2004; Stanley et al. 2008). Mutations of this domain abrogate Vif activity against A3G and A3F due to loss of interaction between Vif and Elongin C (Liu et al. 2004; Simon et al. 1999). This region also contains the zinc-binding HCCH motif consisting of residues H108, C114, C133, and H139, which is necessary for Vif binding to Cullin 5. By binding to Cullin 5 and Elongin C, Vif mediates polyubiquitination of APOBEC3 proteins and subsequent degradation via the 26S proteasome (Albin and Harris 2010; Paul et al. 2006; Luo et al. 2005; Mehle et al. 2006; Xiao et al. 2007a, b). In summary, the N-terminal region of Vif allows APOBEC3-specific interactions, while the C-terminal region connects to the host cell machinery.

The ability of HIV-1 Vif to "neutralize" APOBEC3-mediated HIV-1 mutagenesis varies in circulating strains. Our group found that about 20 % of naturally occurring Vif proteins are defective with some Vif proteins being selectively able to neutralize particular APOBEC3 enzymes and not others (Simon et al. 2005). Most Vif alleles from different subtypes neutralize A3G but display differences with respect to recognizing A3F and A3H haplotype (hap) II. Importantly, some subtype C and F Vif variants efficiently counteracted A3H hapII (Binka et al. 2012).

The Importance of the Vif-APOBEC3 Axis for Viral Diversity and the Shaping of the HIV Pandemic

The accessory HIV protein Vif counteracts the antiretroviral activity of some but not all APOBEC3 proteins (A3G/A3F, highly Vif sensitive; A3B/A3H, only partially Vif sensitive). HIV strains with Vif alleles unable to neutralize A3G have been identified in vivo, suggesting that complete neutralization of A3G is not necessary for the survival of HIV as a population (Mulder et al. 2008; Piantadosi et al. 2009). Indeed, the partial neutralization of APOBEC3 by Vif may be beneficial for HIV's diversity and spread. However, it remains controversial to what extent APOBEC3-driven mutagenesis contributes to viral evolution and disease control in vivo: some studies find a correlation between frequency of G-to-A mutations and viral loads (Pace et al. 2006), whereas others fail to find such association (Piantadosi et al. 2009; Gandhi et al. 2008). Work from several groups suggests that differences in Vif activity shape the phenotype of circulating viruses and facilitate emergence of drug resistance (Mulder et al. 2008; Fourati et al. 2010). For example, HIV can exploit APOBEC3 to escape from the antiretroviral drug 3TC (Mulder et al. 2008). HIV mutant viruses carrying single nucleotide Vif-inactivating mutations displayed attenuated growth in human PBMCs. In the absence of any drug selection, these infections, however, resulted in a diverse proviral population with high frequency of 3TC drug resistance-associated mutations. Moreover, wild-type HIV produced in the presence of hypermutated proviruses rapidly acquired drug resistance, likely by recombination, and thrived at 3TC concentrations that were lethal for the wild-type virus (Mulder et al. 2008). These results suggest that hypermutated, defective proviruses can shape the phenotype of circulating viruses. This observation represents a novel concept since hypermutated proviruses are generally regarded as evolutionary dead ends. The clinical and immunological relevance of deaminated long-lived proviral reservoirs will need to be reevaluated in the light of these findings. Of note, these initial observations have been reproduced and extended by other laboratories (Sadler et al. 2010; Kim et al. 2010).

Further evidence that APOBEC3 modulation drives viral evolution was provided by the examination of protease (PR), reverse transcriptase (RT), and Vif sequences of viruses derived from plasma of HIV-1-infected individuals failing antiretroviral treatment and compared them to HIV-1 from antiretroviral-naive patients (Fourati et al. 2010). In this patient cohort, a specific substitution in Vif (Vif K22H) was

positively associated with treatment failure. Indeed, RT and PR sequences derived from patients harboring Vif K22H showed a significantly higher number of G-to-A (GA and GG dinucleotide contexts) drug resistance-associated mutations. Upon infection of the nonpermissive MT2 cells, most of the K22H proviral clones encoded increased numbers of G-to-A mutations. Among these mutations, the 3TC drug resistance-associated mutation M184I in RT was detected in 25 % of the clones in the absence of any 3TC exposure. These data provide evidence that patients experiencing antiretroviral treatment failure may harbor defective Vif alleles (i.e., K22H). Such Vif variants, which may occur more frequently in certain HIV subtypes, impact virological responses to antiretroviral therapy as they lose their ability to counteract APOBEC3 proteins leading to an increased number of G-to-A mutations that facilitate the emergence of antiretroviral resistance mutations (Fourati et al. 2010; Yebra and Holguin 2011).

The Challenges Arising from HIV-1 Viral Diversity

Extensive viral diversity on both an individual and a population level is the hallmark of RNA virus infection (Moya et al. 2004). High viral replication rate, low fidelity of reverse transcription, and the ability to recombine are the viral determinants that lead to the assortment of heterogeneous HIV-1 species found in chronically infected individuals. Variations in sequence may determine changes in viral phenotype and/or fitness and allow for rapid adaptation in the face of inhibitors. Thus, the continuously evolving HIV-1 diversity poses an immense challenge to any therapeutic interventions.

Viral Diversity from a Molecular Epidemiological Perspective

From an evolutionary point of view, HIV-1 diversification takes place at two different levels: within the infected individual (intra-host) and within the entire host population (inter-host). Different forces influence these processes, and there is evidence for positive selection only within intra-host evolution.

The propensity of HIV to rapidly mutate facilitated the spread of this virus through susceptible populations. Based on phylogenetic analyses, the HIV pandemic is estimated to have originated in the first decades of the twentieth century (Korber et al. 2000). The HIV subtype 1 genome resembles most closely lentiviral sequences derived from chimpanzees in western and central Africa (Gao et al. 1999). Approximately a century after the initial cross-species transmission events, HIV has diversified into numerous clades (e.g., subtypes A-H, “main group”) as well as a number of circulating recombinant forms (CRF) that differ to some extent in their biological characteristics (e.g., co-receptor usage, anti-APOBEC3 activity of Vif alleles). Nonetheless, all HIV strains elicit a gradual depletion of CD4+ T lymphocytes leading to the clinical picture of acquired immunodeficiency syndrome (AIDS).

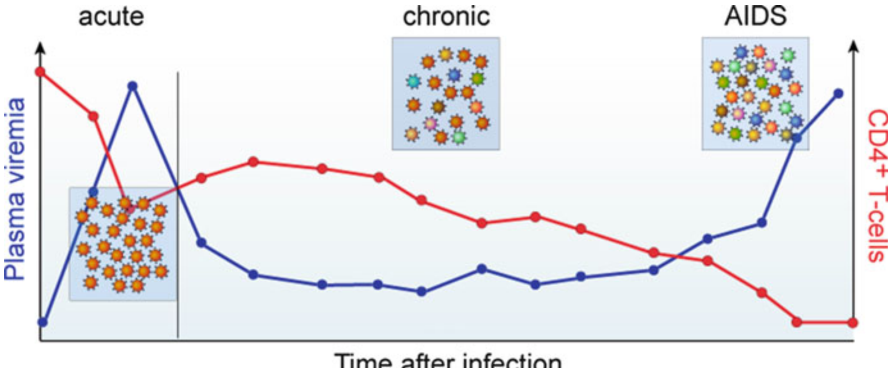


Fig. 4 Viral diversification throughout the infection is depicted. The transmitted founder virus diversifies rapidly in the new host in order to achieve optimal fitness. The interplay between APOBEC3 repertoire and HIV Vif phenotype of the transmitted HIV strain may impact AIDS disease outcome

Thus, HIV variation likely contributed to necessary viral adaptation to the human species.

HIV replication is very dynamic in nature, with the half-life of a virion being so short that half of the entire plasma virus population is replaced in less than 6 h (Simon and Ho 2003). Consequently, the total number of virions produced and released in an untreated HIV-infected person can reach on the order of $>10^{10}$ particles per day (see review by Simon and Ho 2003). The HIV virion contains two RNA copies of its genome which may differ if the producer cell was infected by more than one provirus. Since viral reverse transcription has no proofreading function, approximately 0.2–1 errors are introduced per genome in each replication cycle (Coffin 1995; Martinez et al. 1994). Consequently every single mutation at every possible position of the 9,500 nucleotide-long HIV-1 genome will arise daily (Coffin 1995). Moreover, APOBEC3-driven mutagenesis can introduce additional mutations (Simon et al. 2005). Additionally, crossover events between two genomes during the process of reverse transcription can result in the generation of recombinant forms. Recent evidence suggests that recombination takes place at a significantly higher rate than initially predicted (Levy et al. 2004). Thus, mutations – introduced by RT or APOBEC3 – and recombination fueled by a high turnover rate of virus production are the main features underlying intra-host HIV-1 variation (Fig. 4).

Viral Diversity from a Molecular Biology Perspective

HIV-1 is a complex retrovirus that encodes regulatory genes (e.g., vif, rev, nef) in addition to the gag, pol, and env genes found in all other retrovirus species (Freed and Martin 2001). Although the codon usage of retroviruses generally displays a

nucleotide composition that is skewed toward adenosine in their nucleotide composition, the HIV-1 genome reflects a remarkable preference for adenosine (A, 36 %; G, 24 %; T, 22 %; C, 18 %; Berkhout et al. 2002). Possible explanations that have been proposed to explain the bias include RT-associated preferential incorporation rate in the viral plus-strand and unbalanced intracellular nucleotide pools (Martinez et al. 1994). It has also been speculated that selection pressures could be exerted on the retroviral genome by host-specific constraints such as host restriction factors (van Hemert and Berkhout 1995; Rambaut et al. 2004).

The situation is further complicated by the phenomenon of "G-to-A hypermutation," defined as the monotonous and extensive substitution of guanosine by adenosine. Hypermutated viral genes have first been described as arising during in vitro culture of certain HIV-1 isolates (Vartanian et al. 1991). Independently of in vitro propagation, hypermutations have been described throughout the entire genome of certain viral isolates (e.g., HIV-1 group O strain Vau; Vartanian et al. 2002), resulting in a level of mutations that renders these genomes replication defective. Hypermutated HIV-1 sequences have also been amplified from PBMC of long-term nonprogressors (LTNP; Wei et al. 2004) as well as chronically infected patients (Huang et al. 1998; Janini 2001; Koulinska et al. 2003). HIV undergoes a rapid evolution as the result of the action of error-prone reverse transcriptase, fast replication turnover, and recombination (Ho et al. 1995; Jirillo et al. 1994; Wei et al. 1995). It is attractive to speculate that APOBEC3-driven mutagenesis is another mechanism for HIV-1 diversification. Viruses derived from early infections frequently encode G-to-A mutations in CTL epitopes (Wood et al. 2009) in agreement with the fact that APOBEC3s are part of the early innate immune defense system (Koning et al. 2009; Neil and Bieniasz 2009). Mutagenesis of proviral sequences has been used as a surrogate marker for past APOBEC3 activity in vivo. Endogenous expression levels of APOBEC3 and phenotypic Vif function are important elements that modify the outcome of APOBEC3 restriction. APOBEC3 expression is very variable in HIV target cells in vivo given that these proteins are constitutively expressed but are also inducible by interferons (Chen et al. 2006; Peng et al. 2006). APOBEC3 transcripts are detectable in peripheral blood mononuclear cells as well as many other human tissues (Koning et al. 2009; Cullen 2006; Refsland et al. 2010). Moreover, expression levels are cell type-dependent (Albin and Harris 2010; Refsland et al. 2010; Peng et al. 2007) and could change during cell differentiation. For example, Th1 CD4+ T cells were reported to express more A3G compared to Th2 CD4+ T-cells subsets (Vetter and D'Aquila 2009).

Taken together, hypermutations can be produced artificially in vitro by modulating the dNTP balance (Vartanian et al. 1994, 1997), but the identification of cDNA deamination by the APOBEC3 proteins suggests that intrinsic host defenses provide an additional source of in vivo mutagenesis. Both the adenosine bias of the HIV-1 genome and the occasionally observed extensive G-to-A mutations could, indeed, be interpreted as footprints of more or less successful Vif-mediated protection from intracellular deamination (Hache et al. 2006).

HIV-1 Adaptation to Antiretroviral Selection Pressure

An increasing body of evidence demonstrates that cytotoxic T-cell (CTL) escape variants as well as drug-resistant HIV are transmissible. The evolution of drug-resistant viral variants can be divided into two phases. Initially, drug-resistant viruses are selected solely based on their ability to replicate in the presence of a given pharmacological inhibitor, but typically display an impaired replication capacity in a drug-free environment. Ongoing viral evolution in the presence of suboptimal inhibitor concentrations leads, however, to the selection of variants with additional mutations that compensate for the impaired fitness of drug-resistant variants selected during the first phase. It has also been shown that an alteration of the endogenous dNTP pool can influence the emergence of drug-resistant mutants.

Is HIV Drug Resistance Mediated by G→A Mutations?

The HIV-1 genome is adenosine rich, suggesting that the protection from cytidine deamination mediated by Vif may not be complete in vivo. Moreover, the frequency of G→A mutations resulting from the antiviral activity of APOBEC3G exceeds the rate of mutations randomly generated by HIV-1 RT by almost an order of magnitude (Mariani 2003). Twenty-one percent of all drug resistance-conferring mutations in PR, RT, and envelope are due to G-to-A substitutions (Berkhout and De Ronde 2004; Jern et al. 2009). More specifically, in 6/20 (30 %) codons in protease (PR – D30N, V32I, M36I, M46I, A71T, V77I), 8/22 (33 %) codons in reverse transcriptase (RT – D67N, V75I, V106M/I, V108I, V118I, M184I, E138K, G190E), and 2/10 (20 %) codons in the heptad repeat 1 (HR1) of gp41 (G36S/D, V38M), G-to-A substitutions result in drug resistance-associated mutations. One can speculate that some Vif alleles in vivo will likely be less efficient at mediating DNA deaminase degradation, and the generation of drug resistance mutations involving G-to-A transitions may be favored in this scenario.

Not every G-to-A mutation, however, should be considered a cytidine deaminase-induced hypermutation. The latter has been shown to occur in a nucleotide context-dependent manner (GA or GG), and hypermutated HIV sequences may display a range of nucleotide transitions with up to 60 % of all guanosine being replaced by adenosine (OhAinle et al. 2008b). As mentioned previously, the intrinsic sequence preference of A3G, A3F, A3DE, or A3H leads to cytidine deamination in a dinucleotide context-dependent manner with GG, respectively, GA being the favorite target (Malim 2009). These observations may be relevant to the mutational process leading to the selection of drug-resistant mutants. It seems possible that the generation of a subset of drug resistance-associated mutations involving G-to-A mutations in a GG or GA context (e.g., the primary mutations D30N in HIV protease and M184I, E138K in HIV RT) may be favored in cytidine deaminase expressing cell populations (e.g., T lymphocytes). This would be especially true in the genetic context of a partially active Vif allele.

Resistance to the nucleoside analogue lamivudine (3TC) emerges within weeks of treatment since only a single nucleotide mutation is required to change the methionine (M) codon to valine (V) or isoleucine (I) at position 184 of RT. Residue 184 is located in the conserved YMDD motif at the polymerase active site. M184I/V mutations have been reported to alter RT processivity and reduce the viral replication rate over multiple rounds of infection although single cycle replication rates are only modestly reduced compared to that of wild-type viruses. Both M184I and M184V lead to a more than 500-fold reduced susceptibility to 3TC. Interestingly, it has been reported that mutant M184I emerges first and disappears when viral variants encoding M184V appear. These divergent kinetics have been attributed in the past to differences in replication capacity and RT polymerase function. Of note, M184I mutation results from a single G-to-A substitution, whereas a single A-to-G mutation leads to M184V. Cell culture experiments suggest that A3G-mediated mutagenesis editing facilitates emergence of M184I variants in the presence of the antiretroviral drug lamivudine (Kim et al. 2010). Moreover, HIV can exploit APOBEC3 to escape from 3TC by allowing hypermutated, defective proviruses to shape the phenotype of circulating viruses via recombination (Mulder et al. 2008).

Conclusions

All pandemic HIV strains are (multi)-APOBEC3 resistant through the expression of the accessory protein Vif. The genetic variation within HIV-1 Vif itself has the potential to determine the spread of HIV subtypes on a global level as well as HIV/AIDS disease progression on an individual level. Taken together, a better understanding of the underlying viral-host interactions will have direct implications for the prevention and treatment of HIV/AIDS disease. Additional studies aimed at dissecting the forces driving HIV drug resistance and viral evolution in the presence of APOBEC3 expression are urgently needed. This is especially relevant since future inhibitors targeting the HIV-APOBEC3 interaction will likely be administered as part of combination antiretroviral treatments.

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Resistance to Cyclophilin Inhibitors

Philippe Gallay

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Abstract

The best approach to avoid hepatitis C virus (HCV) resistance to a specific therapy is rapid and massive suppression of viral replication. This is best accomplished by combining several drugs with potent antiviral activity across multiple genotypes, with each possessing a high barrier to resistance, different mechanisms of action, and no cross-resistance. A novel class of anti-HCV agents that have shown great promise in HCV patients – the cyclophilin inhibitors (CypI) – possess such properties. CypI are host-targeting antivirals (HTAs) with a mechanism of action that differs from those of all existing direct-acting antivirals (DAAs). CypI are pan-genotypic due to their distinct mechanism of action that targets the host protein cyclophilin A (CypA), which is required for HCV replication. HCV has to develop a lengthy mutational strategy to efficiently replicate in vitro independently of the host factor CypA leading to a high genetic barrier that the virus has to cross to develop resistance to CypI. CypI mediate rapid and profound viral load suppression in patients. Very low viral breakthrough rates are associated with the CypI treatment, which result mostly from suboptimal drug exposure rather than viral resistance. The high genetic barrier and the lack of cross-resistance to DAAs make CypI attractive drug candidates to

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be part of a regimen with one or two DAAs that may constitute the backbone of a new, safe, and effective IFN-free therapy. The characteristic resistance profile of CypI offers an exceptional opportunity to cure HCV.

Keywords
Hepatitic • cyclophilin • cyclophilia inhibitas • NSSA • resistance

Abbreviations

BID	Taken twice a day
cEVR	Complete early virological response = no virus detected after 12 weeks
eRVR	Extended rapid virological response = no virus detected at week 4 and week 12
EVR	Early virological response = 2 log drop of HCV RNA after 12 weeks
QD	Taken once a day
RVR	Rapid virological response = no virus detected at week 4
SVR12	Sustained virological response = no virus detected at 12 weeks after completion of treatment
SVR24	No virus detected at 24 weeks after completion of treatment

Introduction

HCV is the major causative agent of acute and chronic liver diseases. Chronic infection is associated with higher risks of hepatocellular carcinoma and liver cirrhosis (Dienstag and McHutchison 2006). Nearly 200 million people worldwide (3 % of the population), including four to five million in the USA, are chronically infected with HCV, and four million new infections occur every year (Alter 2007; Soriano et al. 2008a; Global surveillance and control of HCV 1999; World Health Organization 2012). In the developed world, HCV accounts for 2/3 of all cases of liver cancer and transplants, and in the USA, ~12,000 people are estimated to die from HCV each year (Shepard et al. 2005; Armstrong et al. 2006). The weekly injection of pegylated IFNα together with the daily administration of the nucleoside analog ribavirin greatly enhanced the percentage of chronically HCV-infected patients able to reach a sustained antiviral response (SVR) – defined as a clearance of blood HCV RNA 24 weeks after treatment termination (Sy and Jamal 2006; Tong et al. 1997; Fried et al. 2002). However, the combination of pegylated IFNα and ribavirin has a success rate of merely ~50 % in patients with genotypes 1 and 4, often causing severe side effects as well (Cross et al. 2008; Simmonds et al. 2005; Manns et al. 2006). Not only is genotype 1 the most prevalent HCV genotype in Europe, North and South America, China, and Japan, it is also the most difficult to treat (Global Surveillance and Control of Hepatitis C 1999). There is thus an urgent need for the development of new potent pan-genotypic anti-HCV agents in order to improve, shorten, simplify, and lower the cost of HCV management.

Direct-Acting Agents. HCV treatment has been revolutionized by the development and recent FDA approval of direct-acting agents (DAAs). In contrast to the nonspecific antiviral activities of IFNα and ribavirin, DAAs target viral proteins. The binding of the DAA to its viral target neutralizes a function of the viral protein that is vital for the HCV replication cycle. The complexity of the viral cycle afforded the identification of several viral targets with DAAs such as the NS3/4A serine protease, the NS5B RNA-dependent RNA polymerase, and the NS5A protein. The NS3/4A protease inhibitors telaprevir and boceprevir were the first two DAAs to be approved by the FDA in 2011. The combination of boceprevir or telaprevir with pegylated IFNα and ribavirin significantly improved treatment efficacy to 75 % SVR in genotype 1 patients. This represented a major milestone in HCV therapy.

The persistence of the side effects associated with IFNα has lead to a novel therapeutic strategy. This approach entails an IFN-free treatment that would offer high efficacy, with manageable side effects. Three classes of DAAs could currently be part of this IFN-free regimen including second-generation NS3/4A protease inhibitors, NS5B polymerase inhibitors, and NS5A inhibitors. Recent phase II studies provided the proof of concept that combination therapy with DAAs can provide high efficacy in the absence of pegylated IFNα. Specifically, the combination of a nucleoside NS5B polymerase inhibitor and an NS5A inhibitor, with or without ribavirin, showed high SVR in treatment-naïve HCV-infected patients (Sulkowski et al. 2012; Gane et al. 2012). Moreover, the combination of an NS5A inhibitor, a protease inhibitor, and a non-nucleoside NS5B polymerase inhibitor also delivered high SVR in treatment-naïve genotype 1a-infected patients (Kowdley et al. 2012; Everson et al. 2012). These phase II studies provide the proof of concept that chronic infection can be cured using novel combinations of orally available DAAs in the absence of IFN. These regimens represent the future of HCV therapy. It is anticipated that in the coming years, the combination of existing or new DAAs without IFNα will reduce chronic infection to an easily treatable disease with the goal of cure being achieved in the vast majority of patients using simplified dosing regimens with minimal toxicity.

Host-Targeting Agents. Another promising therapeutic strategy consists of targeting host factors that are absolutely required for HCV replication, rather than viral factors (Pawlotsky 2012). Emerging host-targeting agent (HTA) candidates include inhibitors of viral entry, internal ribosome entry site-mediated viral translation, and viral RNA replication, along with viral assembly and release. Figure 1 shows the list of HTAs, which are in preclinical or clinical development. Importantly, one class of HTAs has showed great promise in HCV patients – cyclophilin inhibitors (CypI).

To date, three CypI – alisporivir (previously called DEB025), NIM-811, and SCY-635 – have demonstrated safety and efficacy in HCV-infected patients in phase I and II studies (Flisiak et al. 2007; Gallay 2009; Fischer et al. 2010; Pockros 2010; Liu 2010; von Hahn et al. 2011; Vermehren and Sarrazin 2011; Hopkins and Gallay 2012; Pawlotsky et al. 2012a). All three CypI are synthetic derivates of the immunosuppressive drug cyclosporine A (CsA) (Fig. 2). Slight chain modifications of the parental cyclic undecapeptide CsA enhance the binding affinity of alisporivir,

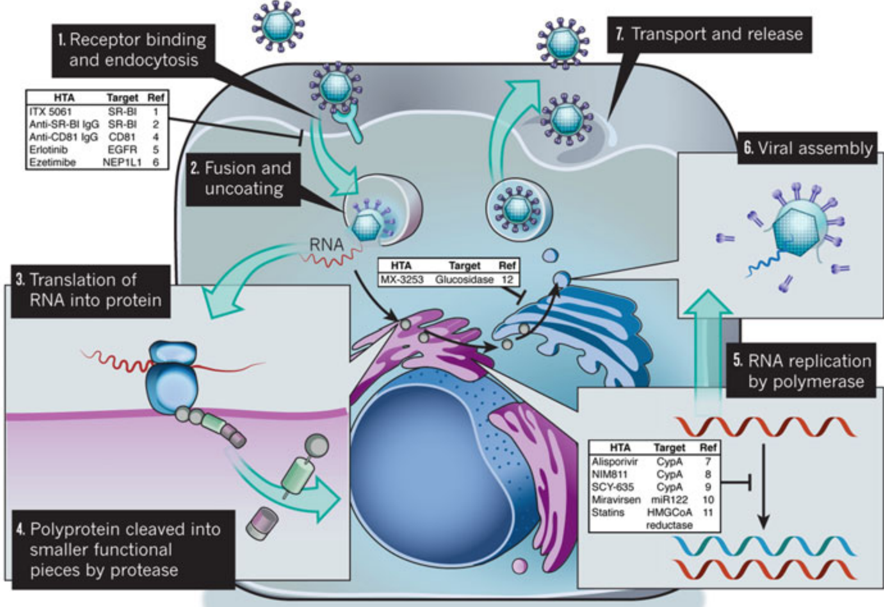


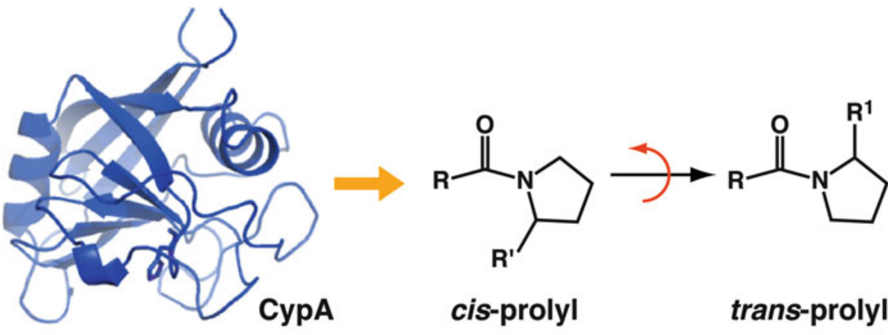
Fig. 1 Existing HTAs that interfere at various steps of the HCV replication cycle (Modified from Schlütter, *Nature*, 474 (2010))

Cyp Inhibitor:	Cyclosporin A	NIM811	Alisporivir	SCY-635
Structure:				
Anti-HCV Activity (EC_{50}):	0.3 μM	0.06 μM	0.04 μM	0.1 μM
Cyp PPIase Inhibition (Ki):	9.79 nM	2.11 nM	0.34 nM	1.8 nM
Immunosuppression Activity (IL-2 inhibition):	0.006 μg/mL	>10 μg/mL	21.5 μg/mL	13 μg/mL

Fig. 2 Antiviral, anti-PPIase, and anti-immunosuppressive activities of CypI

NIM-811, and SCY-635 to their host ligands – the cyclophilins – and abolish their binding to calcineurin, resulting in the elimination of their immunosuppressive activity (Fig. 2).

Cyclosporine A and Cyclophilins. The isolation of CsA from the fungus *Tolypocladium inflatum* in 1971 launched a new era in immunopharmacology (Borel 2002). It was the first immunosuppressive drug, which mediates specific down-immunoregulation of T cell expansion without excessive toxicity. CsA was approved in 1983 for its use to prevent graft rejection during organ transplantation



Year	Findings	References
1971	Discovery of cyclosporine A	Borel *et al.*, 2002
1983	Approval of the use cyclosporine A for organ transplantation	Flechner *et al.*, 1983
1984	Discovery of cyclophilin A as ligand for cyclosporine A	Handschumacher *et al.*, 1984
1984	Discovery of enzymes that accelerate the isomerization of peptidyl-prolyl bonds	Fischer *et al.*, 1984
1987	Classification of the peptidyl-prolyl *cis-trans* isomerases (PPIases)	Lang *et al.*, 1987
1989	Cyclophilin A is a peptidyl-prolyl *cis-trans* isomerase	Fischer *et al.*, 1989
1991	Cyclosporine A is a potent inhibitor of the peptidyl-prolyl *cis-trans* isomerase activity of cyclophilin A	Kofron *et al.*, 1991

Fig. 3 Discovery of the PPIase CypA

(Flechner 1983). The following year, cyclophilin A (CypA) was identified as the primary host cytosolic ligand of CsA (Handschumacher et al. 1984) as well as the main in vivo target for CsA in preventing tissue rejection by inhibition of the activation of T lymphocyte subsets (Borel 2002). Remarkably, the same year, a new type of enzymes, which accelerates the isomerization of peptidyl-prolyl bonds, was discovered (Fischer et al. 1984) (Fig. 3). These enzymes were classified as

peptidyl-prolyl cis-trans isomerases (PPIases) (Lang et al. 1987). The isomerization to the *cis* form is required for de novo protein folding and refolding processes following membrane trafficking. Five years later, CypA was identified as the PPIase, which is responsible for the isomerization of peptidyl-prolyl bonds (Fischer et al. 1989). By binding to the hydrophobic pocket of CypA, CsA neutralizes its isomerase activity (Kofron et al. 1991).

Cyclophilin Inhibitors and HCV Replication. The connection between HCV and cyclophilins was made by several independent labs, which demonstrated that CypI including CsA, NIM811, SCY-635, and alisporivir block HCV replication in vitro (Watashi et al. 2003; Nakagawa et al. 2004; Goto et al. 2006; Ishii et al. 2006; Ma et al. 2006; Paeshuyse et al. 2006; Houck et al. 2006). Using stable knockdown approaches, CypA was found to be the major cyclophilin member vital for HCV replication (Yang et al. 2008; Chatterji et al. 2009; Kaul et al. 2009). The isomerase activity of CypA was found to be critical to support HCV replication (Chatterji et al. 2009; Liu et al. 2009; Kaul et al. 2009). Together these findings suggested that CypI exert their antiviral effect by targeting host CypA. The existence of knockout mice (Colgan et al. 2005) and knockout human cell lines (Braaten and Luban 2001), suggest that CypA is not only optional for cell growth and survival, but that it also represents a viable target for CypI in HCV therapy.

Mechanisms of Action of Cyclophilin Inhibitors. It has been postulated that CypI block HCV replication, by binding to the hydrophobic pocket of CypA, thereby neutralizing the isomerase activity of CypA. However, the precise mechanism of antiviral action of CypI remains to be fully elucidated. Several observations suggest that the HCV NS5A protein is the main viral ligand for CypA (Hanoulle et al. 2009; Waller et al. 2010; Fernandes et al. 2010; Chatterji et al. 2010; Yang et al. 2010; Coelmont et al. 2010; Foster et al. 2011; Verdegem et al. 2011). First, under CypI pressure, HCV develops resistance mutations mostly in the NS5A gene (Fernandes et al. 2007; Hopkins et al. 2010; Kaul et al. 2009; Chatterji et al. 2010; Yang et al. 2010; Coelmont et al. 2010). Second, CypA was found to interact directly with NS5A (Hanoulle et al. 2009; Chatterji et al. 2010; Yang et al. 2010; Coelmont et al. 2010; Fernandes et al. 2010). Third, CypI including CsA, alisporivir, SCY-635, and even the non-CsA derivate sanglifehrins prevent CypA-NS5A interactions (Hanoulle et al. 2009; Chatterji et al. 2010; Yang et al. 2010; Coelmont et al. 2010; Fernandes et al. 2010; Gregory et al. 2011; Hopkins et al. 2012). Fourth, NS5A proteins derived from all genotypes tested so far (1a, 1b, 2a, 2b, and 3) bind CypA directly, suggesting that CypA-NS5A interactions are conserved among HCV genotypes (Chatterji et al. 2010), which correlates well with the fact that CypI possess a pan-genotypic anti-HCV activity in HCV-infected patients (Flisiak et al. 2007; Gallay 2012; Fischer et al. 2010; Pockros 2010; von Hahn et al. 2011; Vermehren and Sarrazin 2011; Hopkins and Gallay 2012; Pawlotsky et al. 2012). Fifth, nuclear magnetic resonance (NMR) studies showed that CypA, via its enzymatic pocket, interacts with proline residues located within domains II and III of NS5A (Hanoulle et al. 2009; Coelmont et al. 2010; Verdegem et al. 2011; Rosnoblet et al. 2012). This is in accordance with the fact that CypA possesses the ability to catalyze the *cis* to *trans* isomerization of proline-containing peptides (Fischer

et al. 1984). Altogether these data indicate that NS5A is the main viral ligand for CypA and that the abrogation of CypA-NS5A contacts by CypI is lethal to the virus.

Further work is required to determine how CypA interacting with NS5A assists HCV replication. Several findings obtained by independent labs suggest different functions for CypA in viral replication. The first finding was that CypA stimulates the RNA binding of NS5A and that the addition of CypI or the introduction of mutations in the isomerase pocket of CypA attenuates the CypA-mediated stimulation of the NS5A RNA binding (Foster et al. 2011). Thus, one can envision that the CypA-mediated enhancement of NS5A binding to the viral RNA is a critical event in HCV RNA replication. Previous work showed that NS5A, as a dimer, represents the RNA-binding-competent form of the protein (Hwang et al. 2010). NS5A, by binding to G/U-rich regions of the HCV genome, may play a key role in both HCV regulation and cellular gene expression (Huang et al. 2005; Hwang et al. 2010). The second finding was that the NS5B polymerase binds NS5A (Shirota et al. 2002) and that CypA and NS5B share a binding region in the domain II of NS5A (Rosnoblet et al. 2012). One thus can envision that CypA modulates an NS5A and/or NS5B function. For example, NS5A, by binding to NS5B, could inhibit its polymerase activity. In this scenario, CypA, by binding to NS5A, prevents its binding to NS5B, enabling the polymerase to replicate the viral genome. In this model, CypI, by blocking CypA-NS5A contacts, allows NS5A to bind to NS5B leading to inefficient RNA replication. In another example, CypA-NS5A complexes could promote either the viral RNA binding of NS5B and/or its enzymatic activity. In this scenario, the elimination of CypA-NS5A contacts by CypI blocks the NS5A-mediated activation of the NS5B polymerase activity, resulting in defective RNA replication. The action of CypA upon NS5B should be indirect since CypA does not bind to NS5B (Rosnoblet et al. 2012). Further work is required to determine which, if any, of these interesting findings represent the biologically relevant functionality of NS5A-CypA interactions.

A recent study suggested that the daily administration of a CypI (SCY-635) to HCV-infected patients causes fast increases in plasma concentrations of IFNα, λ1, λ3, 2′5′OAS-1, and neopterin (Hopkins et al. 2012). No changes were observed in either placebo-treated HCV patients or SCY-635-treated non-HCV-infected volunteers (Hopkins et al. 2012). These data suggest that in HCV-infected patients, the CypI administration and subsequent CypA neutralization reduce the plasma amounts of viruses and transiently increase the concentration of components in the IFN response. NS5A was shown to possess the ability to counter the rising IFN response that a cell develops during HCV invasion (He et al. 2006). Therefore, CypI, by preventing and disrupting CypA-NS5A interactions, may cause both a block in HCV replication and a rescue of the IFN response. It is likely that the rapid and transient increase of plasma concentration of components of the IFN response observed in SCY-635-treated HCV-infected patients originates from the block of HCV replication by the CypI and the prompt stoppage of the expression of viral proteins, which normally counter the IFN response (i.e., NS3, NS5A, core, and E2) (He et al. 2006). The SCY-635 in vivo findings remain to be confirmed for other CypI members. Indeed, it remains to be determined whether other or even all CypI also cause this

rapid and transient raise of the plasma concentrations of components of the IFN response. A recent study obtained apparent conflicting results. Specifically, a comparison analysis of the expression of IFN-stimulated genes (ISGs) between baseline and week 4 of treatment demonstrated a downregulation rather than an upregulation of the expression of ISGs upon the administration of the CypI alisporivir (Pawlotsky et al. 2012). An elevated upregulation of the expression of ISG was observed in IFN-treated patients (Pawlotsky et al. 2012), demonstrating the accuracy of the ISG analysis.

To fully elucidate the mechanisms of antiviral action of CypI, several questions remain to be answered: (a) whether the binding activity or the isomerase activity of CypA is vital for HCV replication, (b) what is the precise activity of CypA on NS5A (i.e., folding, trafficking, protein-protein interactions, etc.), (c) how does the CypA activity promote HCV replication (i.e., enhancing NS5A and/or NS5B activities), and (d) whether CypA binds viral or host proteins other than NS5A and whether these interactions are critical for HCV replication. If these questions can be addressed, our understanding of both the role of CypA in HCV replication and the mechanisms of action of CypI will be greatly improved.

Clinical Safety and Efficacy of Cyclophilin Inhibitors. Clinical trials with the CypI NIM811 and SCY-635 were restricted to exploratory phase I and II trials with small and well-defined patient populations. In contrast, the safety and efficacy profiles of alisporivir were examined using a large patient population (~1,800). Alisporivir is currently being tested for safety and efficacy in phase III studies.

NIM811: The CypI NIM811 was administered orally at total daily doses ranging from 25 to 1,600 mg in treatment-experienced and treatment-naïve genotype 1 patients ($n = 72$) (Lawitz et al. 2011). NIM811 was well tolerated in all groups; however, suppression of plasma viremia was not observed at any dose level tested as monotherapy including the maximum daily dose of 1,200 mg. One cohort of patients was added at the completion of the monotherapy to assess the antiviral activity of NIM811 when administered in combination with pegylated IFNα. Patients received placebo or NIM811 at a dose of 600 mg given twice daily. At day 14, significant reductions from baseline in viral load were reported for patients who received combination therapy (2.85 ± 1.02 $\log^{10}$ IU/mL) when compared to patients who received pegylated IFNα only (0.65 ± 0.77 $\log^{10}$ IU/mL). The development of NIM811 was discontinued due to its weaker antiviral effect compared to that of alisporivir.

SCY-635: Ascending oral doses of SCY-635 (300, 600, or 900 mg/day) were administered for 15 days in genotype 1 patients (Hopkins et al. 2012). No evidence of clinical or laboratory toxicity was identified. SCY-635 at total daily doses of 300 or 600 mg was associated with insignificant changes in viral load, whereas patients who received 900 mg/day exhibited declines in viral load (1.90 $\log^{10}$ IU/mL below baseline), demonstrating the clinical efficacy of SCY-635.

Alisporivir: Alisporivir is the most advanced CypI in clinical development. The safety and efficacy results of sequential phase I and II studies, in which alisporivir was used as part of the anti-HCV treatment are described below. A phase III study was initiated in 2012; however, no public data is currently available.

The DEB-025-103 Study: Clinical proof of concept was provided in a 15-day phase I study in HIV-1 and HCV coinfected patients, who received alisporivir (1,200 mg bid) or placebo (Flisiak et al. 2008). The maximal HCV RNA reduction from baseline for patients who received alisporivir was 3.63 log^{10} IU/mL compared to 0.73 for placebo-treated patients. The antiviral activity of alisporivir was observed irrespective of viral genotype. Several cases of reversible hyperbilirubinemia were also observed in alisporivir-treated patients.

The DEB-025-HCV-203 Study: Lower doses of alisporivir were tested in combination with pegylated IFNα in a 29-day phase II study in treatment-naive patients ($n = 90$) (Flisiak et al. 2009). In the first four arms, patients received placebo or increasing doses of alisporivir (200, 600, or 1,000 mg) in combination with pegylated IFNα. In the last arm, patients received 1,000 mg alisporivir alone. Patients received alisporivir twice daily during the first week and once daily during weeks 2 through 4. The viral load reductions for patients who received 1,000 mg alisporivir monotherapy, pegylated IFNα alone, 200 mg alisporivir plus pegylated IFNα, 600 mg alisporivir plus pegylated IFNα, and 1,000 mg alisporivir plus pegylated IFNα were 2.87, 3.56, 3.30, 5.07, and 5.09 log^{10} IU/mL, respectively.

Alisporivir was also tested in combination with pegylated IFNα and ribavirin in a 29-day phase II study in genotype 1 null responders (Nelson et al. 2009). In arm 1, patients received pegylated IFNα plus ribavirin with 400 mg alisporivir; in arm 2, patients received 400 mg alisporivir; in arm 3, patients received pegylated IFNα with 400 mg alisporivir; in arm 4, patients received pegylated IFNα and ribavirin with 800 mg alisporivir; and in arm 5, patients received pegylated IFNα and ribavirin with 400 mg alisporivir twice daily for one week and once daily until the end of the study. No decline of viral replication was observed in patients who received alisporivir alone. The antiviral activity of alisporivir combined with IFNα was similar in patients who received 800 mg qd (2.38 log^{10} IU/mL) or 400 mg bid alisporivir (1.96 log^{10} IU/mL), demonstrating that the combination of the CypI alisporivir, pegylated IFNα, and ribavirin is an attractive treatment strategy for patients who previously failed to respond to an IFN-based therapy.

The DEB-025-HCV-205 Study: *ESSENTIAL* – Larger alisporivir phase II studies were conducted. In the ESSENTIAL study (Flisiak et al. 2011), all treatment-naïve genotype 1 patients ($n = 288$) received pegylated IFNα and ribavirin. In arm 1, patients received placebo once daily for 48 weeks. In arms 2, 3, and 4, patients received 600 mg alisporivir twice daily during the first week and once daily until the end of the study. In arm 2, patients received alisporivir in combination with pegylated IFNα and ribavirin for 48 weeks. In arm 3, response-guided therapy (RGT) was used to determine the duration of treatment with alisporivir in combination with pegylated IFNα and ribavirin. In arm 3, patients who demonstrated a rapid virological response (RVR) were eligible to receive 24 weeks of treatment. Patients, who did not demonstrate an RVR, continued the treatment for 48 weeks. In arm 4, patients received alisporivir in combination with pegylated IFNα and ribavirin for 24 weeks. Alisporivir was well tolerated in all arms. The frequency of reversible hyperbilirubinemia was 1.4, 32.9, 25.4, and 41.7 % in arms 1, 2, 3, and 4, respectively.

The primary endpoint of the study was to determine the proportion of patients in each arm who achieved SVR at week 24 (SVR_{24}) using 10 IU/mL as limit detection for HCV plasma RNA. SVR_{24} were 55, 76, 69, and 53 % in arms 1 (control), 2 (48 weeks of alisporivir), 3 (24 or 48 weeks of alisporivir RGT), and 4 (24 weeks of alisporivir), respectively. Only arm 2 reached statistical significance ($p = 0.008$). End-of-treatment responses were elevated in CC IL28B patients (100 % of patients exhibited undetectable HCV plasma RNA) and were preserved at the 24-week follow-up (100 % SVR_{24}) for patients who received the originally scheduled treatment duration of 48 weeks or 24 weeks of RGT with alisporivir. End-of-treatment responses were diminished at the 24-week evaluation for CC IL28B patients who received control treatment or a planned 24-week treatment with alisporivir. The benefit of adding alisporivir to pegylated IFNα and ribavirin was evident in TT IL28B patients. SVR_{24} were 17, 33, 62, and 73 % for TT IL28B patients in arm 1, 2, 3, and 4, respectively. RVR was enhanced in all arms that contain alisporivir (arms 2–4) compared to the control arm (arm 1). The frequency of viral breakthrough was low (2.8 %) in patients who received the CypI alisporivir. In conclusion, the addition of 48 weeks of 600 mg alisporivir to pegylated IFNα and ribavirin improves SVR_{24} in treatment-naïve genotype 1 patients.

The CDEB025A2211 Study: *VITAL-1* – The goals of the phase II VITAL-1 study were to test the safety and efficacy of alisporivir administered alone, with ribavirin or with ribavirin and pegylated IFNα for 24 weeks. The addition of pegylated IFNα and ribavirin was delayed in treatment-naïve genotype 2 or 3 patients (Pawlotsky et al. 2012a). In arm 1, patients received 1,000 mg alisporivir ($n = 83$); in arm 2, patients received 600 mg alisporivir with ribavirin ($n = 84$); in arm 3, patients received 800 mg alisporivir with ribavirin ($n = 94$); in arm 4, patients received 600 mg alisporivir with pegylated IFNα ($n = 84$); and in arm 5, patients received pegylated IFNα and ribavirin ($n = 40$). All patients received 600 mg alisporivir twice daily for a week, after which patients started their randomized dose of alisporivir. Follow-up evaluations were scheduled at week 36 and 48 to measure SVR_{12} and SVR_{24}, respectively. Viral load was measured at week 4 for RVR rates and for the decision to modify the original randomized treatment. If a patient demonstrated RVR (viral load <25 IU/mL), the patient continued his original treatment. If a patient exhibited viral load >25 IU/mL, he would switch at week 6 to a treatment combining 600 mg alisporivir, pegylated IFNα, and ribavirin until the end of treatment at week 24.

Alisporivir was well tolerated in all arms. SVR_{12} rates were superior in all arms that contained alisporivir compared to the control arm (pegylated IFNα and ribavirin). SVR_{12} rates were 81 %, 83 %, 81 %, 77 %, and 58 % for arms 1, 2, 3, 4, and 5, respectively. RVR rates were 29 %, 37 %, and 42 % for arms 1, 2, and 3, respectively. Patients with RVR rates of 82 %, 93 %, and 91 % in arms 1, 2, and 3 achieved SVR_{12}, demonstrating that alisporivir as monotherapy or in combination with ribavirin is an attractive treatment strategy for treatment-naïve genotype 2 or 3 patients who demonstrated RVR at week 4. Yet, the majority of patients in all arms did not reach RVR and switched at week 6 to 600 mg alisporivir combined with pegylated IFNα and ribavirin. For these patients, SVR_{12} rates were 94 %, 92 %, and

96 % for arms 1, 2, and 3, respectively, demonstrating that the addition of pegylated IFNα and ribavirin to alisporivir by week 6 promotes viral clearance in genotype 2 or 3 patients who did not achieve RVR.

A subsequent analysis demonstrated that alisporivir improved SVR_{24} rates in all arms compared to pegylated IFNα and ribavirin (Pawlotsky et al. 2012b). SVR_{24} rates were 80 %, 85 %, 81 %, 80 %, and 58 % in arms 1, 2, 3, 4, and 5, respectively. Alisporivir treatments (arms 1–4) decreased the relapse rates compared to the control pegylated IFNα and ribavirin (arm 5): 11 %, 8 %, 6 %, 10 %, and 25 % in arms 1, 2, 3, 4, and 5, respectively. These latest results demonstrate that the combination of alisporivir and ribavirin yields high SVR_{24} rates as either IFN-free regimen or as IFN-add-on regimen in treatment-naive genotype 2 or 3 patients with rapid viral clearance, low viral breakthrough, and low relapse rates.

The CDEB025A2210 Study: *FUNDAMENTAL* – The goals of the 48-week phase II FUNDAMENTAL study were to test the safety and efficacy of alisporivir in combination with pegylated IFNα and ribavirin in genotype 1 patients with a documented history of relapse or nonresponse to prior treatment with pegylated IFNα and ribavirin (Alberti et al. 2012). In arm 1, patients received 600 mg alisporivir once daily ($n = 121$); in arm 2, patients received 800 mg alisporivir once daily ($n = 117$); in arm 3, patients received 400 mg alisporivir twice daily ($n = 109$); and in arm 4, patients received placebo once or twice daily ($n = 114$). All patients received pegylated IFNα and ribavirin. In arms 1 and 2, patients received 600 mg alisporivir twice daily for a week after which patients started their randomized dose of alisporivir. Scheduled follow-up evaluations were conducted at week 60 and 72 to measure SVR_{12} and SVR_{24}, respectively. The primary endpoint of the study was to determine at week 12 the percentage of patients who achieved complete early virologic response (cEVR) (HCV RNA <25 IU/mL). Viral load was assessed at week 4 for the determination of RVR rates.

Alisporivir was well tolerated. Alisporivir enhanced the percentage of patients who achieved cEVR with values of 46.4 %, 61.1 %, 71.3 %, and 32.7 % in arms 1, 2, 3, and 4, respectively. The 400 mg bid alisporivir administration (arm 3) yielded a superior percentage of cEVR than that of the 800 mg qd administration (arm 2). Among relapsers, alisporivir produced a higher percentage of patients who achieved cEVR, with values of 62.5, 77.6, 72.9, and 51.9 for arms 1, 2, 3, and 4, respectively. The same trend was observed for nonresponders in arms 2 (47.5 % cEVR) and 3 (70 % cEVR). The 400 mg bid alisporivir treatment was highly potent in null nonresponders (69.7 % cEVR) and partial nonresponders (68.0 % cEVR). A low viral breakthrough was observed – 3.6, 3.7, 1.8, and 2.7 % – in arms 1, 2, 3, and 4, respectively. In conclusion, the addition of the CypI alisporivir to pegylated IFNα and ribavirin is a novel and attractive choice for difficult-to-treat patients, particularly for genotype 1 patients who did not respond previously to IFN-based regimens.

A subsequent analysis was executed at week 24 and further demonstrated that the addition of alisporivir greatly improves the efficacy of the treatments (Davis et al. 2012). Specifically, alisporivir enhanced the percentage of patients who achieved cEVR with values of 48.2 %, 61.1 %, 72.5 %, and 35.5 % in arms 1, 2, 3, and 4, respectively. RVR values were 20.9 %, 25.0 %, 40.4 %, and 7.3 %, and

VR_{24} values were 64.5 %, 70.4 %, 73.4 %, and 31.8 % in arms 1, 2, 3, and 4, respectively. Alisporivir enhanced VR_{24} (70.8–79.2 %) in relapsers compared to pegylated IFNα and ribavirin (55.6 %). The 400 mg bid alisporivir treatment (arm 3) yielded not only higher VR_{24} (75.4 %) in nonresponders compared to the pegylated IFNα and ribavirin treatment (8.9 %) but also in the most difficult-to-treat null responders (70.6 %) compared to pegylated IFNα and ribavirin (5.6 %). The viral breakthrough rates were 11.8 %, 7.3 %, 1.8 %, and 4.5 % in arms 1, 2, 3, and 4, respectively. In some patients, hyperbilirubinemia was transient and reversible and not associated with liver toxicity.

HCV Resistance to Direct-Antiviral Agents. HCV replication comprises a rapid turnover rate and a lack of proofreading by the NS5B polymerase, resulting in high genetic diversity among HCV virions. These extremely variable viral quasi-species contain a mixture of viruses with numerable mutational variants. It is important to emphasize that the mutated variants are present in patients prior to the administration of antiviral therapy and may become the dominant viral population under selective drug pressure. As anticipated, quick selection of resistant variants was observed in HCV-infected patients upon administration of various DAAs including the protease and NS5A inhibitors when given as monotherapy (Wyles 2013). Thus, the development of resistance to DAAs targeting HCV can compromise successful therapy.

The mechanisms that determine prevalence and frequency of resistance-conferring mutations remain elusive. Several parameters control resistance to DAAs. Among them are viral, host, and pharmacokinetic factors.

Viral Parameters: As mentioned above, estimates of HCV replication in chronically infected patients suggest that viral production may be as high as 10^{10}–10^{12} virions per day. Accordingly, the clearance of free virions in plasma is exceedingly rapid yielding values of 2–3 h for the half-life of viral particles (Neumann et al. 1998). The lack of proofreading by the RNA-dependent RNA polymerase NS5B results in the accumulation of genetically distinct viral variants called quasi-species (Martell et al. 1992). Since de novo mutant variants are continuously produced, it has been proposed that in any untreated HCV-infected patient, any possible mutant variant exists (Rong et al. 2010). A recent study showed that the NS5B polymerase does not incorporate incorrect nucleotides at identical rates (Powdrill et al. 2011). Specifically, enzyme kinetic measurements revealed surprisingly elevated error rates for G:U/U:G mismatches. The observation that G:U/U:G mismatches occur at a greater frequency than all other misincorporation events correlates well with a mutational predisposition for transitions over transversions. Therefore, the probability of viral breakthrough throughout DAA therapy is contingent upon the nucleotide exchange (transition versus transversion) necessary to create a resistance-associated mutation. Thus, the nature of the nucleotide change can contribute to the genetic barrier in the development of resistance to DAAs (Powdrill et al. 2011).

In HCV-infected patients, the fitness of a mutant variant is characterized by its capacity to replicate. It is common for mutant variants that are resistant to DAAs to exhibit diminished fitness compared to wild-type virus. Wild-type viruses are the dominant viruses within the quasi-species (Sarrazin and Zeuzem 2010; Soriano

et al. 2008b), but under DAA pressure they are promptly eliminated. However, the mutant variants that contain adequate mutations to escape the effects of the drug will replicate and then represent the predominant viral population. Prolonged drug treatment then leads to the emergence of additional or secondary mutational changes, which contribute to both the fitness of the mutant virus and the resistance of the virus to the drug.

The genotype of the virus represents an additional critical parameter for DAA resistance. Since the first-generation protease inhibitors were originally designed to neutralize the protease activity of genotype 1, these DAAs exert poor antiviral activity against other genotypes (Gottwein et al. 2011). Importantly, the second-generation protease inhibitors, which are already tested for safety and efficacy in phase II and III studies, exhibit broader antiviral activities against other genotypes. Unlike the first-generation protease inhibitors, non-nucleoside polymerase inhibitors exhibit antiviral activity broadly against multiple diverse genotypes and subtypes. Nucleos(t)ide polymerase inhibitors target the highly conserved active site of the NS5B polymerase; they are potent against all genotypes (Sarrazin and Zeuzem 2010). This broad antiviral activity among genotypes is referred to as pan-genotypic antiviral activity. First-generation NS5A inhibitors also exhibited varying degrees of potency between genotypes (Fridell et al. 2010; Gao et al. 2010) likely due to the relatively high heterogeneity of the NS5A gene among genotypes, however, second-generation NS5A inhibitors (i.e., PPI-668, ACH-3102) afford pan-genotypic antiviral activity.

The subtype of the virus also contributes to the genetic barrier to resistance of DAAs. Specifically, the R155K mutation, which mediates resistance to the protease inhibitors boceprevir and telaprevir, requires only one nucleotide change in subtype 1a patients but requires two mutations in 1b patients. Since the single nucleotide exchange for the R155K mutation in 1a is created by a simple transition, whereas one of the two nucleotide exchanges for the R155K mutation in 1b is created by a transversion (Powdrill et al. 2011; Sullivan et al. 2011), R155K mutations are more often found in 1a patients treated with boceprevir or telaprevir. This is consistent with the observation that virologic failures, which are associated with HCV resistance to the DAAs boceprevir or telaprevir, are more frequently found in 1a patients than in 1b patients (Jacobson et al. 2011a; Poordad et al. 2011). It is important to emphasize that analogous transition/transversion nucleotide exchanges were also observed in HCV variants exhibiting resistance to NS5B polymerase and NS5A inhibitors (Sarrazin and Zeuzem 2010).

Because monotherapies with protease (i.e., boceprevir or telaprevir) or NS5A (i.e., BMS-790052) inhibitors resulted in the rapid (15 days) selection of resistant variants (viral breakthrough) (Sarrazin et al. 2007a, b; Fridell et al. 2011), clinicians and researchers postulated that DAA-resistant variants exist within the quasi-species prior to the beginning of the treatment. Supporting this hypothesis, resistant variants carrying NS3 mutations at specific positions (i.e., V36, T54, V55, Q80, R155, D168, and V170) that confer resistance to protease inhibitors were found at varying frequencies in untreated patients (Bartels et al. 2008; Kuntzen et al. 2008; Lenz et al. 2011; Vicenti et al. 2012). Similarly, resistant variants carrying NS5A (M28V,

Q30R/H, Q54H, Y93H) or NS5B mutations (S282T/R, C316Y/F/S, M423T/I, and V499A) that confer resistance to NS5A and non-nucleoside polymerase inhibitors, respectively, were also found to exist in untreated patients (Bartels et al. 2008; Kuntzen et al. 2008; Lenz et al. 2011; Vicenti et al. 2012). Variants, which are resistant to nucleos(t)ide polymerase inhibitors, have rarely been identified in patients (Fridell et al. 2011; Margeridon et al. 2011; Gaudieri et al. 2009; Sun et al. 2011). Novel DNA sequencing techniques, referred to as "next-generation" sequencing (NGS), provide high-speed throughput that can produce an enormous volume of sequences. The most important advantage provided by these platforms is the identification of sequence data from single DNA fragments within a library, eliminating the use of amplification techniques that can introduce spurious mutations prior to sequence acquisition. Deep sequencing by NGS techniques is being increasingly used in clinical practice to detect low abundance drug-resistant HCV variants. Although these new sequencing methodologies resulted in the detection of DAA-resistant variants with NS3, NS5A, and NS5B mutations in a majority of untreated patients (Chevaliez et al. 2011), the role of these preexisting resistant variants within quasi-species remains to be determined. Further sequencing studies will also determine whether or not these DAA-resistant variants present in quasi-species turn out to be critical in previous pegylated IFNα and ribavirin nonresponders or unfavorable CT or TT IL28 genotype patients.

Pharmacokinetic Parameters: Both the potency of the DAA and the genetic barrier to resistance define the likelihood of the emergence of a viral variant under drug selection pressure. Drug potency is characterized by the drug concentration required to inhibit 50 % or 90 % of the viral growth (IC_{50} and IC_{90}). Drug concentrations above the IC_{50} and IC_{90} are needed to inhibit drug-resistant variants. There is a direct correlation between the emergence of DAA-resistant variants (viral breakthrough) and drug exposure. The genetic barrier to resistance is characterized by an optimal transition/transversion nucleotide exchange and the number of mutations needed to develop resistance. Resistance to DAAs with a low genetic barrier to resistance necessitates only one or two amino acid substitutions and/or transitions, whereas resistance to DAAs with a high genetic barrier to resistance necessitates three or more nucleotide changes and/or transversions (Powdrill et al. 2011). DAAs with a high genetic barrier to resistance, but with a low potency, represent a therapeutic concern.

Host Parameters: Resistance emergence is highly contingent upon patient adherence. It has been clearly shown that adherence to highly active antiretroviral therapies (HAART) is correlated with suppression of detectable HIV-1 replication, reduced rates of resistance, increased survival, and improved quality of life (Kitahata et al. 1996; Kitahata et al. 2000). One thus can anticipate that a correlation between drug resistance and treatment adherence will also be observed in HCV-infected patients. Specifically, if compliance is low, plasma drug levels will drop below IC_{50} and IC_{90} levels, resulting in the emergence of resistant HCV variants. This may be particularly true for the two DAAs boceprevir and telaprevir, which exhibit fairly short half-lives. Thus, adherence to treatment as prescribed is a critical parameter to avoid drug resistance. Other

host parameters important for the emergence of drug resistance are genetic predispositions and fibrosis stage at the time of drug treatment. For example, a low fibrosis stage and the CC genotype of a single nucleotide polymorphism (rs12979860) in the IL28B gene are positive prognostic factors for high virologic response rates to DAA combination therapies (Zeuzem et al. 2012; Poordad et al. 2012; Jacobson et al. 2011b).

In Vitro HCV Resistance to Cyclophilin Inhibitors. The development of HTAs is mainly a consequence of our increasing understanding of the molecular biology of the HCV replication cycle and the interplay between the virus and the host. Targeting host factors rather than viral factors must combine a specific antiviral effect with a higher barrier to resistance and a broader genotypic activity. In contrast to viral factors, host factors are encoded by host chromosomes and therefore are not subject to the high genetic variability of the viral genome. Targeting a host factor rather than a viral factor may increase the potential for side effects such as cellular toxicity. While far less common to viral genomes, sequence variations in host genes (i.e., single nucleotide polymorphisms (SNPs)) occur and occasionally affect the development of a disease or the response to a therapy (Balagopal et al. 2010). Supporting this hypothesis, a recent study investigated the effects of non-synonymous SNPs in the CypA gene on HCV replication (von Hahn et al. 2012). Interestingly, they identified three SNPs in the CypA gene that protect cells from HCV replication. The single amino acid changes appeared to provoke rapid degradation of CypA. However, it is important to reemphasize that the existence of knockout mice (Colgan et al. 2005) and human cell lines (Braaten and Luban 2001) without evident deleterious phenotypes further suggests that CypA is a valuable target for CypI in HCV therapy. Together these findings strongly suggest that CypA is a relevant therapeutic target.

Numerous studies demonstrated that HCV variants resistant to CypI including CsA, NIM811, alisporivir, and SCY-635 can emerge under drug selection pressure in hepatoma cell in vitro systems (Fernandes et al. 2007, 2010; Robida et al. 2007; Kaul et al. 2009; Yang et al. 2010; Coelmont et al. 2010; Hopkins et al. 2012; Garcia-Rivera et al. 2012a). Importantly, the time required for CypI resistance selection is particularly lengthy (3–6 months) compared to that required for DAA resistance (2–3 weeks) including protease, NS5B polymerase, and NS5A inhibitors (Coelmont et al. 2010). The slow development of resistance to CypI represents a major benefit of using them as part of a future anti-HCV regimen.

The level of HCV resistance to CypI is relatively low compared to other DAAs such as protease, NS5B polymerase, and NS5A inhibitors (Ma et al. 2006; Coelmont et al. 2010). Specifically, the inhibition of CypI-resistant variants in vitro requires relatively low concentrations of CypI compared to that of wild-type virus (5- to 10-fold higher concentrations). In contrast, inhibition of DAA-resistant variants in vitro requires high concentrations of DAA compared to that of wild-type virus (>100-fold higher concentrations). Attempts to further increase CypI (i.e., alisporivir) concentration were unsuccessful. Prolonged exposure of HCV replicon cells resulted in viral clearance rather than the selection of increasingly resistant replicon cells (Coelmont et al. 2009).

A large number of in vitro studies investigated the resistance of HCV to various CypI (Fernandes et al. 2007; Robida et al. 2007; Kaul et al. 2009; Fernandes et al. 2010; Yang et al. 2010; Coelmont et al. 2010; Hopkins et al. 2012; Garcia-Rivera et al. 2012a). The sequencing of CypI-resistant subgenomic and genomic HCV clones showed that mutations emerged in several genes including NS3, NS5A, and NS5B (Fig. 4). Importantly, the reintroduction of the mutations into the wild-type viral genome and the subsequent examination of the sensitivity of resulting HCV mutants to CypI determined the true significance of the mutations for CypI resistance. Although early work suggested that mutations, which arose in NS5B (i.e., P540A and I432V), are important for CypI resistance (Robida et al. 2007), more recent studies indicate that only mutations in NS5A are critical for HCV resistance to CypI including CsA, alisporivir, SCY-635, sanglifehrin A, or a combination of CypI (sanglifehrin A plus NIM811 or CsA plus NIM811) (Fig. 4). This is in accordance with the fact that NS5A serves as a binding locus for CypA (Hanoulle et al. 2009; Chatterji et al. 2010; Yang et al. 2010; Coelmont et al. 2010; Fernandes et al. 2010). Note that the relevant resistance mutations occur only in the viral NS5A protein and not in the host CypA protein. Individual and combinational analyses of NS5A mutations, which emerged during CypI resistance selection, revealed that the D320E NS5A mutation frequently arose in CypI-resistant variants (Fig. 4). Specifically, the D320E mutation was identified in genotype 1b (Con1) HCV variants resistant to CsA (Goto et al. 2009), alisporivir (Coelmont et al. 2010; Garcia-Rivera et al. 2012a), sanglifehrin A (Puyang et al. 2010), sanglifehrin A plus NIM811 (Puyang et al. 2010), CsA plus NIM811 (Puyang et al. 2010), and SCY-635 (Hopkins et al. 2012). Moreover, the D320E mutation (position D316 in JFH-1) was also found in genotype 2a HCV variants which, in contrast to wild-type virus, replicate in CypA-knockdown cells (Yang et al. 2010) (Fig. 4). Importantly, the reintroduction of the D320E mutation into the wild-type genome renders the resulting subgenomic and genomic mutants either partially resistant to CypI or independent of CypA (Goto et al. 2009; Coelmont et al. 2010; Puyang et al. 2010; Yang et al. 2010; Hopkins et al. 2012; Garcia-Rivera et al. 2012a). Altogether these data strongly suggest not only that the D320E mutation is key to provide partial HCV resistance to CypI, but they also suggest that the D320E NS5A mutation partially bypasses the need for CypA.

As mentioned above, the level of HCV resistance to CypI is relatively low compared to other DAAs. Supporting this notion, the D320E NS5A mutation only reduced the HCV susceptibility to CypI by ~2- to 5-fold (Goto et al. 2009; Coelmont et al. 2010; Puyang et al. 2010; Hopkins et al. 2012; Garcia-Rivera et al. 2012a). The D320E mutation does not influence the fitness of the resistant replicons. Additional in vitro studies suggest that by itself the single D320E mutation in the domain II of NS5A does not confer high-level resistance to CypI. A combination of multiple simultaneously occurring mutations is required to render HCV more resistant to CypI (Coelmont et al. 2010; Puyang et al. 2010; Garcia-Rivera et al. 2012a). Indeed, two independent studies showed that a mutation adjacent to the D320E NS5A mutation, the Y321N mutation, is also important for CypI resistance (Fig. 4). One study showed that HCV (JFH-1) develops D320E and Y321N NS5A mutations

Cyclophilin Inhibitor	Genotype	Resistance Mutations	References
CsA	1b	5' UTR HCV IRES neo EMCV IRES NS3 NS4A NS5A NS5B 3' UTR E260G F288S V284A L307Q R360G V448A } NS5A; P538T S556G } NS5B	Fernandes *et al.*, 2007
CsA	1b	I432V P540A I432V + P540A } NS5B	Robida *et al.*, 2007
CsA	1b	Q86R I252T } NS3; ***D320E*** } NS5A	Goto *et al.*, 2009
Alisporivir	2a	Y391F } NS3; D253G L290F V464A/L } NS5A	Kaul *et al.*, 2009
CsA + NIM811	1b	Q42H S341I } NS3; L183P D333N R356Q T360P P426R } NS5A; C575G } NS5B	Puyang *et al.*, 2010
Sanglifehrin A	1b	I71V Q80R G262S } NS3; V9I M51V } NS4; L27I C446S } NS5A; W571R } NS5B	Puyang *et al.*, 2010
Sanglifehrin A + NIM811	1b	I71V Q80R G262S } NS3; V9I M51V } NS4; V278A P353S W433R C446S } NS5A; N142S W571R } NS5B	Puyang *et al.*, 2010
Alisporivir	1b	A241P } NS3; R262Q R328W ***D320E*** } NS5A	Coelmont *et al.*, 2010
shCypA	2a	***D320E*** ***Y321N*** } NS5A	Yang *et al.*, 2010
SCY-635	1b	***D320E*** ***Y321N*** } NS5A	Hopkins *et al.*, 2012
Alisporivir	1b	***D320E*** ***Y321N*** } NS5A	Garcia-Rivera *et al.*, 2012

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GT-1a        ARALPVWARPDYNPPLVETWKKPDYEPPVVHG
GT-1b        PRAMPIWARPDYNPPLLESWKDPDYVPPVVHG
GT-2a[J6]    PPALPAWARPDYNPPLVESWKRPDYQPATVAG
GT-2a[JFH]   PRALPAWARPDYNPPLVESWRRPDYQPPTVAG
GT-2b        PPALPPWARPEYNPPVIETWKRPDYEPPTVLG
GT-3         PPALPIWARPDYNPPLLDRWKAPDYVPPTVHG
GT-4         PPALPIWARPDYNPPLTETWKQQDYQAPTVHG
GT-5         PPALPVWARPGYDPPLLETWKRPDYDPPQVWG
GT-6         PPALPIWARPDYNPPLLQKWQMPGYEPPVVSG
             PPALP.WARPDYNPPL.E.WK.PDY PPTV G
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■ = DY motif (***D320E*** and ***Y321N***)

Fig. 4 In vitro development of CypI resistance mutations

(D316E and Y317N in JFH1) when cultured in CypA-knockdown cells (Yang et al. 2010). Another study showed that HCV develops a combination of D320E and Y321N NS5A mutations when cultured under the CypI alisporivir selection (Garcia-Rivera et al. 2012a). The D320 residue is conserved among HCV genotypes (GT1a, GT1b, GT2a, GT3, GT4, and GT6) except for GT2b (D320E) and GT5 (D320G), whereas the Y321 residue is highly conserved among all genotypes (Fig. 4). The D320E or Y321N mutation alone confers only partial replication in CypA-knockdown cells (Yang et al. 2010) and only a slight resistance to alisporivir (Garcia-Rivera et al. 2012a). However, the combination of the two mutations renders HCV more resistant to alisporivir in vitro and is able to robustly replicate in CypA-knockdown cells (Yang et al. 2010; Garcia-Rivera et al. 2012a). The combination of D320E and Y321N mutations renders HCV universally resistant to CypI in vitro including CsA, alisporivir, SCY-635, sanglifehrins, and sanglifehrin derivates (Garcia-Rivera et al. 2012b). Together these data not only suggest that the mutations found in the NS5A gene of CypI-resistant variants are truly responsible for the observed drug resistance, they also suggest that not a single NS5A mutation but rather the development of multiple mutations is necessary for robust CypI resistance as well as CypA independence, at least in vitro. This further suggests that CypI impose a high genetic barrier for the development of viral resistance.

The D320E and Y321 mutations that emerged for CypI resistance in vitro do not influence NS5A binding to CypA or the sensitivity of CypA-NS5A interactions to CypI (Chatterji et al. 2010; Yang et al. 2010; Fernandes et al. 2010; Garcia-Rivera et al. 2012a). This suggests that these mutations do not render CypA-NS5A interactions impervious to CypI-mediated dissociation. The D320E and Y321 mutations do not alter NS5A dimerization, NS5A binding to NS5B, or NS5A binding to RNA (). CypA enhances the binding of the domain II of NS5A to RNA, and the addition of CypI blocks the CypA effect (Foster et al. 2011). Importantly, the RNA binding of the domain II of NS5A that contains the D320E mutation was unaffected by CypA (Foster et al. 2011), suggesting that the CypI resistance mutation bypasses the need for CypA in the in vitro binding of NS5A to RNA.

Comparative NMR studies with NS5A peptides that contain either the D320 or the E320 residue revealed a shift in population between major and minor *cis-trans* conformers (Hanoulle et al. 2009). Specifically, residues preceding Pro319 in wild-type D320 peptide exist on average for 24.1 % in a minor conformation attributed to the Pro319 *cis* form. This minor conformer in D320 peptide becomes the dominant conformer in the E320 peptide, with a relative population of 70.4 % (Hanoulle et al. 2009). The CypI resistance NS5A mutations apparently relieve the CypA dependence by acquiring a conformational inversion mediated by the D320E mutation that normally is mediated by CypA. The prolyl *cis-trans* isomerization activity of CypA would thereby not be strictly required anymore, explaining the HCV resistance to CypI. The CypI resistance mutations may generate a particular conformation of NS5A that is directly or indirectly necessary for optimal HCV replication. Further work is required to unravel the precise structural origin of this conformational inversion.

The fact that HCV has to develop a lengthy mutational strategy to efficiently replicate in vitro independently of a host factor explains the high genetic barrier that the virus has to cross to develop resistance to CypI. The characteristic resistance profile of CypI offers an exceptional opportunity to cure HCV as part of a combination therapy with other antivirals such as DAAs in treatment-naive patients or as part as a rescue therapy for patients harboring resistance mutations to other classes of anti-HCV agents such as DAAs.

In Vivo HCV Resistance to Cyclophilin Inhibitors. Very low viral breakthroughs arose in CypI-treated patients such as alisporivir-treated patients (Tiongyip et al. 2011). Viral breakthrough was only observed in TT or CT IL28B allele patients in the alisporivir ESSENTIAL study (Flisiak et al. 2011). Population sequencing of HCV genomes did not identify any genotypic change consistently associated with viral breakthrough, assessed by clonal sequencing of NS5A, the putative in vivo viral target of CypA. Interestingly, the D320E mutation was seen at the time of initial viral breakthrough in one patient (Li et al. 2011). However, phenotypic assays demonstrated only a slight (~3-fold) decrease in susceptibility to alisporivir with GT1b replicons bearing D320E alone or the entire NS5A gene of the patient isolate. These data suggest that the emergence of D320E or viral resistance is not the primary cause of the viral breakthrough. Importantly, a number of mutations that confer resistance to DAAs including NS5A inhibitors were seen at baseline for patients who

achieved RVR and subsequently SVR_{24} with alisporivir (Li et al. 2011), supporting the in vitro data of lack of cross-resistance between alisporivir and DAAs. Altogether these data strongly suggest a low potential for development of resistance to CypI in treated patients.

Viral breakthrough was associated with decrease in dose or stoppage of pegylated IFNα and ribavirin or with low drug exposure. Specifically, while on full dose of the CypI alisporivir, 6/215 patients experienced viral breakthrough compared to 4/73 patients in the control arm (placebo with pegylated IFNα and ribavirin) (Li et al. 2011). No viral breakthrough occurred until week 12. In three of the six alisporivir-treated patients, viral breakthrough occurred after dose adjustment or stoppage of pegylated IFNα and ribavirin dose. Pharmacokinetic analyses demonstrated suboptimal plasma concentrations of alisporivir in two of the other three patients who exhibited viral breakthroughs.

In the alisporivir VITAL-1 study, several residue changes were recognized in the NS5A gene in patients, who underwent viral breakthrough. When certain of these mutations were introduced together in the wild-type viral genome, the resulting mutants exhibited a mild to moderate resistance to alisporivir in vitro (<17-fold increase in EC_{50}) (Li et al. 2011). These findings further suggest that, in contrast to DAAs, multiple mutations are necessary to provide substantial resistance to CypI, at least to alisporivir. Importantly, the alisporivir-resistant variants isolated from treated patients continued to be susceptible to DAAs (Li et al. 2011). Altogether these observations convincingly demonstrate that CypI offer a high barrier to the development of HCV resistance in treated patients.

The Future of Cyclophilin Inhibitors

A novel therapeutic approach is currently being tested for safety and efficacy in various clinical trials. This approach entails an IFN-free treatment that offers both high efficiency and low frequency of adverse events. This IFN-free treatment may consist of a combination of two or three DAAs, two or three HTAs including a CypI, or a combination of DAAs and HTAs. Remarkably, data generated from a rapidly growing number of phase II and III studies strongly suggests that IFN-free DAAs combination therapies represent the future for therapy for curing chronic HCV infection.

Here we briefly describe a few IFN-free regimens, which exhibited apparent successful therapeutic results. The AVIATOR study from Abbot tested a combination of the protease inhibitor ABT-450, the NS5A inhibitor ABT-267, and the NS5B polymerase inhibitor ABT-333, without pegylated IFNα and ribavirin. High SVR_{12} were observed in treatment-naive genotype 1a (83 %) and 1b (96 %) patients. The addition of ribavirin to the combination of the three DAAs yielded high SVR_{12} in null responders (89 % and 100 % for genotype 1a and 1b patients, respectively) (Kowdley et al. 2012). Based on these promising results, Abbot recently announced the design of phase III studies, which will evaluate the safety and efficacy of a 12-week regimen of the three DAAs with and without ribavirin, for the treatment of

HCV in genotype 1 non-cirrhotic, treatment-naïve and treatment-experienced patients. An additional phase III study will be conducted with ribavirin in patients with cirrhosis for 12 or 24 weeks. Another phase II study from Bristol-Myers Squibb that combined the NS5A inhibitor daclatasvir (BMS-790062), the protease inhibitor asunaprevir (BMS-650032), and the NS5B polymerase inhibitor BMS-791325, without ribavirin, yielded high SVR_{12} (94 %) in treatment-naïve genotype 1a patients without major adverse events (Everson et al. 2012). Phase III studies are anticipated to begin in 2014. The ELECTRON study from Gilead, which combined the NS5B polymerase inhibitor sofosbuvir (GS-7977), the NS5A inhibitor GS-5885, and ribavirin, yielded high SVR_{12} in treatment-naïve genotype 1 patients (Gane et al. 2012). Gilead just announced preliminary results from their FISSION study, which evaluated a 12-week course of the once-daily sofosbuvir with ribavirin in treatment-naïve genotype 2 or 3 patients. The study met its primary efficacy endpoint of non-inferiority of the sofosbuvir plus ribavirin treatment compared to control pegylated-IFNα plus ribavirin treatment, with 67 % SVR_{12} for both treatments. No major adverse events occurred. Altogether these studies provide the proof of concept that IFN-free regimens represent the future for an HCV therapy or cure.

The combination of the CypI alisporivir with ribavirin exhibited high efficiency in treatment-naïve genotype 2 and 3 patients. However, one or even two DAAs will certainly have to be added to alisporivir to yield high SVR in treatment-naïve patients infected with all genotypes, relapsers, and nonresponders (Pawlotsky et al. 2012; Alberti et al. 2012). For example, the combination of alisporivir (or another CypI) with a second-generation protease inhibitor, a NS5B polymerase inhibitor, and/or an NS5A inhibitor represents attractive IFN-free regimens. Indeed, targeting both viral and host factors critical for HCV replication should greatly improve the efficacy of the regimen, especially in difficult-to-treat patients. Combination studies of a CypI with one or two DAAs should be evaluated initially in vitro and subsequently in vivo to pinpoint the best CypI/DAA combination, which would combine synergistic and pan-genotypic properties. An appealing combination would be a CypI with an NS5A inhibitor given that CypI prevent the binding of CypA to the domain II of NS5A and that NS5A inhibitors target the domain I of NS5A (Gao et al. 2010; Lemm et al. 2010). The combination of CypI and NS5A inhibitors may be particularly effective because this combination strategy may present an unusually high genetic barrier to resistance by requiring the virus to develop several mutations simultaneously in two distinct domains of NS5A in order to escape the selection pressure of the two classes of inhibitors. Nevertheless, the in vivo efficacy of the combination of CypI and NS5A inhibitors remains to be established.

Conclusion

CypI represent a novel class of anti-HCV agents with a mechanism of action that differs from those of all existing DAAs. Because of their distinct mechanisms of action that target the host protein cyclophilin A, CypI are pan-genotypic. A very low viral breakthrough rate was associated with CypI treatments, and no consistent

genotypic change was associated with viral breakthrough. The high genetic barrier and the lack of cross-resistance to DAAs make CypI excellent drug candidates for a rescue regimen for patients who did not respond to DAAs combined with pegylated IFNα and ribavirin. More importantly, CypI, as part of a regimen with one or two DAAs (i.e., NS5A or NS5B inhibitors), may constitute the backbone of a new, safe, and effective IFN-free therapy.

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Antiviral Drug Resistance in Herpesviruses

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Abstract

The discovery of acyclovir (ACV), a nucleoside analogue, more than 30 years ago, represents a milestone in the management of herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections. The modest activity of ACV against human cytomegalovirus (HCMV) has prompted the development of another nucleoside analogue, ganciclovir (GCV), for the management of systemic and organ-specific HCMV diseases. Second-line agents such as the pyrophosphate analogue foscarnet (FOS) and the nucleotide analogue cidofovir (CDV) have

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been approved subsequently. In contrast to ACV and GCV, the latter drugs do not require an initial phosphorylation step by viral protein kinases to be converted into their active forms. Since the introduction of these antivirals, the emergence of drug-resistant mutants has been constantly reported particularly in severely immunocompromised patients such as bone marrow and solid organ transplant recipients as well as human immunodeficiency virus (HIV)-infected individuals. In this chapter, we review the characteristics of the antiviral agents currently approved for the management of HSV, VZV, and HCMV diseases, the laboratory methods for assessing drug susceptibilities, and the clinical significance of drug-resistant infections and their management.

Keywords
Herpesvirus • Herpes simplex virus • Varicella-zoster virus • Human cytomegalovirus • Antiviral drug • Resistance • Mutations • Phenotypic testing • Genotypic testing • Clinical significance • Management • Immunocompromised patient

Introduction

Herpesviridae is a large family of DNA viruses including nine different human viruses which belong to the *α-herpesvirinae* [herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV)], the *β-herpesvirinae* [human cytomegalovirus (HCMV) and human herpesviruses 6 and 7 (HHV-6 A/B and HHV-7)], and the *γ-herpesvirinae* [Epstein-Barr virus (EBV) and (HHV-8)] subfamilies. These ubiquitous viruses cause different types of pathologies which vary considerably according to the immune status of the infected individuals. They all have the ability to establish latency and to reactivate under certain circumstances. Among members of the Herpesviridae family, four of them (HSV-1, HSV-2, VZV, and HCMV) will be discussed in this chapter since they are the targets of antiviral strategies. HSV-1 and HSV-2 cause orolabial and genital infections as well as keratitis, encephalitis, and neonatal infections. VZV is the causative agent of varicella and herpes zoster. HCMV is responsible for mononucleosis-like syndromes as well as systemic and organ-specific diseases in immunocompromised patients.

Antiviral Agents for Herpesvirus Infections

All antiviral agents currently approved for the treatment of HSV, VZV, and HCMV infections ultimately target the viral DNA polymerase (Andrei et al. 2009). First-line antiviral agents for the treatment of HSV and VZV infections include acyclovir (ACV, Zovirax®, GlaxoSmithKline) and penciclovir (PCV) and their respective ester prodrugs valacyclovir (VACV, Valtrex®, GlaxoSmithKline) and famciclovir (FCV, Famvir®, Novartis). Acyclovir and PCV are deoxyguanosine analogues that must be phosphorylated by HSV- or VZV-encoded thymidine kinase (TK) and then by

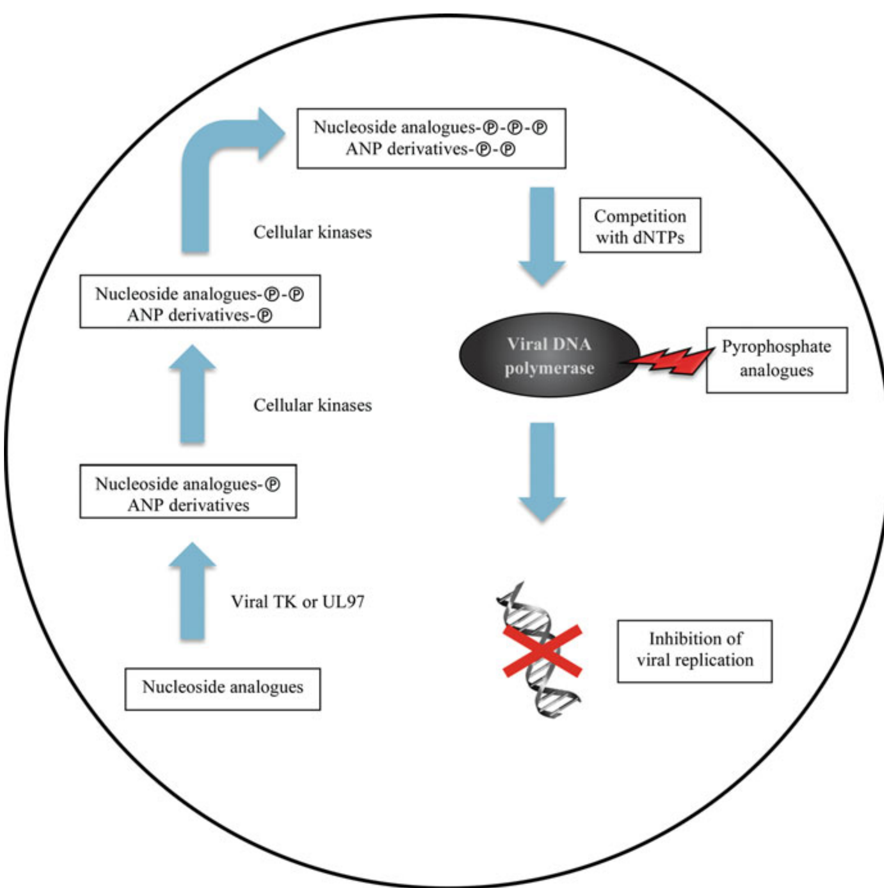

Fig. 1 Mechanisms of action of the different classes of antiviral agents. The nucleoside analogues such as acyclovir, penciclovir, and ganciclovir must be first phosphorylated by the viral thymidine kinase or UL97 protein kinase and then by cellular kinases to be converted into their active forms. The acyclic nucleoside phosphonate (*ANP*) derivatives such as cidofovir must be phosphorylated by cellular kinases only to be active. The nucleoside analogue triphosphate and the ANP derivative diphosphate compete with deoxynucleotide triphosphates (dNTPs) to inhibit the viral replication. The pyrophosphate analogues such as foscarnet directly inhibit the activity of the DNA polymerase. Key: Ⓟ represents the phosphate groups

cellular kinases to exert their antiviral activity. The triphosphate forms are competitive inhibitors of the viral DNA polymerase (Fig. 1) (Reardon and Spector 1989). Moreover, ACV triphosphate is incorporated into the replicating DNA and acts as a chain terminator of the DNA polymerase. Oral ACV, VACV, and FCV are used for short-term therapy of primary and recurrent HSV infections (particularly genital herpes), long-term suppressive therapy of recurrent genital herpes, as well as treatment of herpes zoster. The intravenous (IV) formulation of ACV is indicated for the management of severe HSV (including encephalitis and neonatal herpes) and VZV

infections. Topical formulations of ACV and PCV (Denavir®, Novartis) are used for the treatment of herpes labialis and keratitis.

Ganciclovir (GCV, Cytovene®, Roche) is the first-line antiviral drug for the prevention and treatment of HCMV diseases. Ganciclovir is a deoxyguanosine analogue that requires a first phosphorylation by the protein kinase encoded by the *UL97* gene and two subsequent phosphorylations by cellular kinases to exert its antiviral activity. Ganciclovir triphosphate acts as a potent inhibitor of the DNA polymerase encoded by the *UL54* gene by competing with deoxyguanosine triphosphate for incorporation into replicating DNA where it slows down DNA polymerization and eventually stops chain elongation (Fig. 1) (Biron et al. 1985). Ganciclovir can be given orally, intravenously, or as an intravitreal implant (Vitrasert, Chiron) for the treatment of HCMV retinitis. The poor bioavailability of GCV (~6 %) following oral administration prompted the development of its L-valyl ester prodrug, valganciclovir (VGCV, Valcyte®, Roche), which exhibits approximately 10-fold increase in oral bioavailability (Pescovitz et al. 2000). Oral VGCV and IV GCV are indicated in the treatment of established HCMV diseases in immunocompromised patients and in the prevention of symptomatic episodes, especially in transplant recipients. Two preventive strategies may be used depending on the risk for patients to develop severe HCMV diseases. The "universal prophylaxis" strategy consists of administering the antiviral to all patients after transplantation for a 3- to 6-month period, whereas the "preemptive" approach requires laboratory monitoring evidence of HCMV replication in the blood (typically monitored once weekly) based on the detection of pp65 antigen or viral DNA by real-time PCR (Boeckh et al. 2004; Piiparinen et al. 2004; Gimeno et al. 2008) before initiation of antiviral treatment.

Second-line antiviral drugs for the treatment of HCMV diseases include foscarnet (FOS, Foscavir®, AstraZeneca) and cidofovir (CDV, Vistide®, Gilead). Due to their toxicity profiles and the absence of oral formulations, they are usually reserved for patients failing or not tolerating therapy with nucleoside analogues. Foscarnet is a pyrophosphate analogue which does not require phosphorylation by viral or cellular kinases. It directly inhibits the viral DNA polymerase by binding to the pyrophosphate binding site and preventing pyrophosphate cleavage from incoming deoxynucleotide triphosphates (dNTPs), which then results in cessation of chain elongation (Fig. 1) (Oberg 1989). The IV formulation of FOS is indicated for the treatment of HCMV retinitis in individuals with the acquired immunodeficiency syndrome (AIDS) and for GCV-resistant HCMV infections in immunocompromised patients. Foscarnet may also be used in the treatment of infections caused by nucleoside analogue-resistant HSV and VZV mutants. Cidofovir is an acyclic deoxycytidine monophosphate which requires only two phosphorylations by cellular enzymes to be converted into its active form, which acts as a DNA chain terminator (Fig. 1) (Xiong et al. 1997). The IV formulation of CDV is indicated in the treatment of HCMV retinitis in AIDS patients and is occasionally also used in transplant recipients. Topical and IV formulations of CDV may be used "off label" in the treatment of ACV- and/or FOS-resistant HSV infections (Andrei et al. 2009).

Herpes Simplex Virus and Varicella-Zoster Virus Antiviral Drug Resistance

Phenotypic and Genotypic Testing for Detection of HSV and VZV Drug Resistance

Suspected HSV or VZV drug-resistant infections can be confirmed by testing the susceptibility of clinical isolates against antiviral agents in cell culture (phenotypic assays) or by the identification of specific mutations conferring drug resistance directly in clinical samples (genotypic assays).

The plaque reduction assay (PRA) is the gold standard phenotypic method to determine the susceptibility of HSV isolates to antiviral drugs and is approved as a standard protocol by the Clinical and Laboratory Standard Institute (Swierkosz et al. 2004). In this assay, cells are infected with a constant viral inoculum. The virus is then allowed to grow in the presence of serial drug dilutions for 2–3 days before fixing and staining the cells. The cytopathic effects (CPE) or viral plaques are then counted under an inverted microscope. The drug concentration that reduces the CPE by 50 % compared to controls (without antiviral) is defined as the 50 % effective concentration (EC_{50}). Breakpoint values that are widely accepted to define HSV resistance to ACV and FOS are EC_{50} equal to or greater than 9 and 330 μM, respectively (Swierkosz et al. 2004). No consensus value has been proposed for PCV. Drug resistance can also be defined by an increase in the EC_{50} value greater than three to five times that of the baseline isolate from the same patient.

The low rate of VZV isolation from vesicle samples (from 20 % to 43 %) and its slow growth in cell culture (5–6 days) limit the use of the PRA in that context (Sauerbrei et al. 1999). An increase in the EC_{50} value equal to or greater than four times that of a sensitive reference strain (e.g., the Oka strain) is generally accepted to define VZV resistance to ACV (Saint-Leger et al. 2001).

It is worth mentioning that the difficulty in obtaining an appropriate clinical specimen for cell culture, the length of time required for the propagation of the viral isolate in cultured cell lines, the subjectivity of counting plaques, and the possible selection bias introduced during the growth of heterogeneous viral populations in cell culture can all potentially limit the clinical utility of the PRA. The objectivity of the readout was improved in several phenotypic methods based on the detection of specific antigens (by ELISA, flow cytometry, or immunoperoxidase staining) or the detection of DNA (by hybridization or real-time PCR).

Genotypic testing is based on the amplification of HSV or VZV genes involved in drug resistance by PCR and the identification of specific mutations by DNA sequencing. Standard dideoxy sequencing can detect an emerging resistance mutation when it exceeds approximately 20 % of the total population. It is thus estimated that a viral load of at least 1,000 copies/ml of clinical sample is required to obtain reliable genotypic profiles (Schuurman et al. 1999). Mutations conferring resistance to nucleoside analogues occur in *UL23* (HSV) or *ORF36* (VZV) genes encoding the TKs and/or in *UL30* (HSV) or *ORF28* (VZV) genes encoding the DNA polymerases. As some degrees of inter-strain variability exist in these genes, mutations conferring

drug resistance must be discriminated from natural polymorphisms. In this respect, results of genotypic testing must be interpreted by comparison with mutations already assigned to natural polymorphism or confirmed drug resistance in the literature. Mutations with unknown significance in drug resistance must be confirmed by recombinant phenotyping which consists in the introduction of an individual mutation into the genome of a control-sensitive laboratory strain followed by the determination of the resulting drug-susceptibility phenotype (Bestman-Smith and Boivin 2003; Sergerie and Boivin 2006).

An ACV-resistant HSV or VZV mutant may exhibit a TK-deficient (lack of enzyme activity), a TK low-producer (reduced level of enzymatic activity), a TK-altered (substrate-specific enzyme which phosphorylates thymidine but not ACV and/or PCV), or a DNA polymerase-altered (altered enzyme activity) phenotype. Approximately, 95 % of HSV or VZV clinical isolates resistant to ACV possess TK-negative or TK low-producer phenotypes, whereas a minority consists of TK-altered and DNA polymerase-altered mutants (Roberts et al. 1991; Pottage and Kessler 1995; Gaudreau et al. 1998; Gilbert et al. 2002; Burrel et al. 2012; Malartre et al. 2012; Sauerbrei et al. 2012, 2013).

Both TK and DNA polymerase mutants resistant to ACV exhibit a decrease in so-called in vivo “fitness” and neurovirulence. The HSV TK is not essential for viral growth in cell culture, but this enzyme plays an important role in the pathogenesis as demonstrated in animal models (Coen et al. 1989; Efstathiou et al. 1989; Chen et al. 2004). It has been suggested that mutations arising in the TK may eliminate or markedly reduce the enzyme activity that could not fulfill the greater requirement of thymidine phosphorylation for virus replication in neurons compared to other cells (Chen et al. 1998). Indeed, TK low-producer mutants show some reduction in pathogenicity compared with wild-type strains but are generally able to reactivate (Coen 1994; Bernstein et al. 2000). In contrast, TK-deficient mutants have impaired pathogenicity, established latency in sensory ganglia with a lower efficiency than wild-type strains, and reactivate poorly. However, some TK-deficient HSV clinical isolates express ultra-low levels of enzyme activity that could be sufficient to allow reactivation (Coen 1994; Besecker et al. 2007). Moreover, phylogenetically related HSV-1 strains sensitive and resistant to ACV were shown to coexist in latently-infected trigeminal ganglia of immunocompetent individuals (van Velzen et al. 2012). Therefore, immunocompromised patients are at risk of reactivating ACV-resistant mutants that can cause infections refractory to nucleoside analogue therapy. Mutants with altered DNA polymerase activity have been less studied, but they seem to exhibit different degrees of attenuation of neurovirulence in mice (Field and Coen 1986; Pelosi et al. 1998; Andrei et al. 2007; Dambrosi et al. 2010). As the HSV DNA polymerase is essential for viral replication, mutations emerging in this enzyme must be functionally conservative. It is proposed that DNA polymerase mutants could have a lower affinity for dNTPs leading to an altered viral replication in neurons which only contain small amounts of nucleic acid precursors (Field and Coen 1986).

Role of *UL23/ORF36* and *UL30/ORF28* Gene Mutations in Conferring HSV/VZV Drug Resistance

In clinical HSV isolates, resistance to ACV is mediated in 95 % of the cases by mutations in the *UL23* gene and, in the remaining cases, by mutations in the *UL30* gene (Gaudreau et al. 1998; Gilbert et al. 2002; Morfin and Thouvenot 2003; Frobert et al. 2007; Piret and Boivin 2011, 2014). Six highly conserved domains have been identified among Herpesviridae TKs (Balasubramaniam et al. 1990). The most important regions involved in the enzyme activity are the ATP-binding site, the nucleoside-binding site, and the cysteine at codon 336 which maintains the three-dimensional structure of the active site (Evans et al. 1998). Resistance hot spots in the *UL23* gene correspond to homopolymer stretches of guanines or cytosines (Fig. 2a) (Sasadeusz et al. 1997; Gaudreau et al. 1998; Morfin et al. 2000). Approximately half (up to 80 % in a study (Burrel et al. 2013a)) of the clinical cases of ACV resistance consist in addition or deletion of nucleotides in these regions. The resulting frameshift reading introduces premature termination codons and the expression of a truncated TK polypeptide. The remaining cases of ACV resistance result from single amino acid substitutions that are usually located in the six conserved domains (especially the ATP-binding and nucleoside-binding sites) as well as at amino acid 336 of the TK (Gaudreau et al. 1998; Morfin et al. 2000; Chibo et al. 2004; Stranska et al. 2004b; Duan et al. 2009; Burrel et al. 2010; Sauerbrei et al. 2010, 2011a). Some mutations located outside these highly conserved regions may also confer resistance to ACV (Gaudreau et al. 1998; Morfin et al. 2000; Stranska et al. 2004b; Duan et al. 2009). Resistance to PCV generally maps to mutations within the *UL23* gene (Sarisky et al. 2002, 2003) and consists of 4 % single-nucleotide substitutions distributed throughout the gene and 96 % frameshift mutations (Suzutani et al. 2003).

Mutations conferring resistance to ACV, FOS, and CDV in HSV clinical isolates have been identified in the catalytic or conserved domains of the DNA polymerase (Fig. 2b) (Gilbert et al. 2002; Morfin and Thouvenot 2003; Frobert et al. 2007; Piret and Boivin 2011, 2014). The Herpesviridae DNA polymerases belong to the family of α-like DNA polymerases (Wong et al. 1988) which share regions of homology numbered I to VII. These regions correspond to the degree of conservation among these enzymes, with region I being the most conserved. Moreover, Herpesviridae DNA polymerases also contain a δ-region C, which is shared by enzymes related to eukaryotic DNA polymerases δ (Zhang et al. 1991). The HSV DNA polymerase is formed by six conserved structural domains, namely, pre-NH_2 and NH_2 domains, polymerase palm, fingers, and thumb domains, and a 3′–5′ exonuclease domain (Liu and Homa 2009). The polymerase palm domain contains regions I, II, and VII, and the thumb domain contains region V. These four regions appear to flank the catalytic site in the palm domain and contain the catalytic triad of aspartic acid residues (at positions 717, 886, and 888) that are essential for polymerase activity. Regions III and VI belong to the finger domain and may play a role in positioning the template

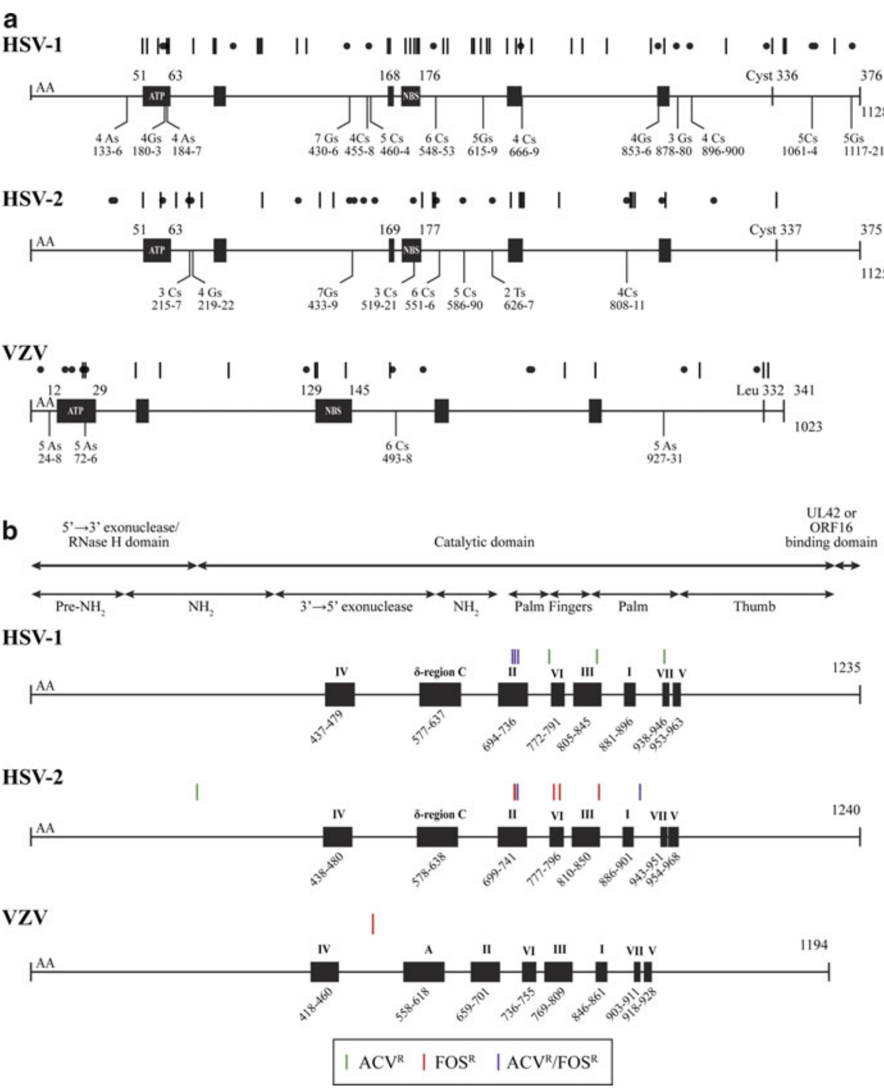

Fig. 2 Confirmed drug-resistance mutations identified in clinical HSV-1, HSV-2, and VZV isolates (laboratory-derived strains are not shown for clarity). Panel a shows mutations in the *UL23* gene of HSV-1 and HSV-2 and in the *ORF36* gene of VZV conferring resistance to acyclovir. Conserved regions among the thymidine kinase of Herpesviridae including the ATP-binding site (*ATP*) and the nucleoside-binding site (*NBS*) are represented by the black boxes. Bars (|) indicate amino acid (*AA*) substitutions, whereas dots (●) represent nucleotide additions and/or deletions. The homopolymer runs, as well as the nucleotides involved, are indicated below vertical bars. Panel b shows mutations in the *UL30* gene of HSV-1 and HSV-2 and in the *ORF28* gene of VZV conferring resistance to acyclovir (*ACV*^{R}) and/or foscarnet (*FOS*^{R}). Conserved regions among the Herpesviridae DNA polymerase are represented by the black boxes. The roman numbers (I–VII) and δ-region C corresponding to each of these regions are indicated above the boxes. Bars (|) indicate amino acid substitutions

and primer strands. The 3′–5′ exonuclease domain contains three highly conserved sequence motifs Exo I, Exo II (region IV), and Exo III (δ-region C). Single amino acid substitutions associated with ACV resistance are mostly located in regions II, III, VI, and VII of the enzyme; the greatest clusters of mutations being found in regions II and III (Schmit and Boivin 1999; Sauerbrei et al. 2011a). Only a few mutations have been described within the other conserved domains or outside such regions (Schmit and Boivin 1999). Most FOS-resistant clinical isolates contain single amino acid substitutions in conserved regions II, III, or VI and in a non-conserved region (between regions I and VII) of the DNA polymerase (Schmit and Boivin 1999; Bestman-Smith and Boivin 2002). Some of these isolates retain susceptibility or, at least, borderline levels of susceptibility to ACV and CDV (Schmit and Boivin 1999; Bestman-Smith and Boivin 2002). However, mutations within conserved regions II and VI are frequently associated with resistance to both ACV and FOS. The mutations S724N (region II) and L778M (region VI) in HSV-1, which confer cross-resistance to ACV and FOS, also cause reduced susceptibility to CDV (Bestman-Smith and Boivin 2003). Genotypic analyses of drug-sensitive HSV isolates reveal a high degree of polymorphism in the *UL23* and *UL30* genes (Frobert et al. 2008; Burrel et al. 2010; Bohn et al. 2011).

In VZV clinical isolates, resistance to ACV is mostly associated with mutations in the viral TK and, less frequently, with mutations in the viral DNA polymerase (Gilbert et al. 2002; Piret and Boivin 2014). The genome of VZV has a lower GC content (46 %) than those of HSVs (68 %) and only a few homopolymer stretches are present in the *ORF36* gene (Andrei et al. 2012). The string of six cytosines located at codon positions 493–498 within this gene emerged as a hot spot for the insertion or deletion of nucleotides involved in ACV resistance (Fig. 2a) (Boivin et al. 1994; Morfin et al. 1999; Andrei et al. 2012; van der Beek et al. 2013). In addition, non-synonymous nucleotide substitutions conferring resistance to ACV are widely dispersed in the *ORF36* gene (Sawyer et al. 1988; Talarico et al. 1993; Boivin et al. 1994; Fillet et al. 1998; Morfin et al. 1999; Saint-Leger et al. 2001; Sauerbrei et al. 2011b). However, these amino acid changes occur more frequently in the ATP-binding and nucleoside-binding sites and at amino acid 231 of the TK (Morfin et al. 1999).

A few reports have described ACV- and/or FOS-resistant clinical VZV isolates with mutations in the *ORF28* gene (Fig. 2b) (Visse et al. 1998; Kamiyama et al. 2001; Sauerbrei et al. 2011b). These amino acid substitutions are mainly found in the catalytic site and in the conserved regions of the DNA polymerase and may confer cross-resistance to ACV and FOS. The TK and DNA polymerase of VZV are highly conserved compared to those of HSVs and only very few natural polymorphisms have been identified in the *ORF36* and *ORF28* genes (Sauerbrei et al. 2011b).

Clinical Significance, Prevalence, and Risk Factors for Drug-Resistant HSV and VZV Infections

Cases of HSV infections unresponsive to treatment in immunocompetent patients are usually associated with diagnoses of recurrent genital herpes, keratitis, and

encephalitis. In general, most unresponsive cases in immunocompetent patients are not due to antiviral drug resistance. Furthermore, the rare instances of resistance in that setting are not associated with prolonged active lesions due to a functional immune system. In immunocompromised patients, infections caused by ACV-resistant isolates are associated with significant morbidity, including persistent and/or disseminated diseases refractory to antiviral therapy. Patients with AIDS can develop extensive mucocutaneous lesions usually not associated with visceral or central nervous system infections (Levin et al. 2004). A few cases of lethal disseminated visceral HSV infections due to ACV-resistant mutants have been reported in bone marrow transplant (BMT) recipients (Ljungman et al. 1990), and a case of meningoencephalitis was described in an AIDS patient (Gateley et al. 1990).

The prevalence of nucleoside analogue-resistant HSV isolates differs greatly for immunocompetent and immunocompromised patients. A low prevalence of 0.3–0.7 % was reported for HSV resistance to ACV in immunocompetent patients during extensive surveys between 1980 and 1992 (Collins and Ellis 1993). The prevalence of ACV resistance has remained constant since then ranging from 0.1 % to 0.7 % (Christophers et al. 1998; Boon et al. 2000; Bacon et al. 2002, 2003; Danve-Szatanek et al. 2004; Stranska et al. 2005). A more recent report has documented a relatively high prevalence (6.4 %) of ACV-resistant HSV-1 isolates in immunocompetent patients with herpetic keratitis (Duan et al. 2008), and some of these cases were clinically refractory to ACV therapy (Burrel et al. 2013b; James and Prichard 2013; van Velzen et al. 2013; Pan et al. 2014). The higher incidence of ACV resistance in this setting may be related to the fact that the cornea can be considered as an immune-privileged site where low immune surveillance favors the rapid selection of resistant viruses (Andrei and Snoeck 2013). Similarly, a low prevalence of 0.19–0.22 % was reported for PCV resistance in immunocompetent patients (Sarisky et al. 2003). A prevalence of less than 0.3 % was also observed in persons who used the topical formulation of PCV to treat recurrent herpes labialis (Shin et al. 2003).

Prolonged treatment with ACV, VACV, or FCV is required to prevent or to manage HSV infections in the immunocompromised host, which may result in the selection of viral isolates with reduced drug susceptibility. The prevalence of HSV infections caused by ACV-resistant isolates in these populations varies from 3.5 % to 11 % (Englund et al. 1990; Nugier et al. 1992; Christophers et al. 1998; Bacon et al. 2003; Stranska et al. 2005). The prevalence for ACV resistance ranged from 3.5 % to 7 % in human immunodeficiency virus (HIV)-positive patients (Englund et al. 1990; Reyes et al. 2003; Danve-Szatanek et al. 2004; Levin et al. 2004; Ziyaeyan et al. 2007; Lolis et al. 2008), from 2.5 % to 10 % in solid organ transplant (SOT) recipients (Christophers et al. 1998; Danve-Szatanek et al. 2004), and from 4.1 % to 10.9 % in hematopoietic stem cell transplant (HSCT) recipients (Wade et al. 1983; Chakrabarti et al. 2000; Chen et al. 2000; Morfin et al. 2000; Danve-Szatanek et al. 2004; Erard et al. 2007; Frangoul et al. 2007). An even higher frequency (36 %) of ACV resistance was also reported in the latter population (Langston et al. 2002). In one study, patients receiving either autologous or allogeneic BMT developed HSV infections at a similar rate (9.2 %), but resistance

occurred only in allogeneic transplants reaching a prevalence of 27 % (Morfin et al. 2004). The emergence of PCV-resistant HSV isolates among immunocompromised patients has been less studied but found to be 2.1 % and not associated with treatment failure in one report summarizing different clinical trials (Sarisky et al. 2003). Few reports have described the emergence of FOS-resistant HSV isolates mainly in AIDS patients failing therapy (Hwang et al. 1992; Safrin et al. 1994a, b; Chakrabarti et al. 2000; Bestman-Smith and Boivin 2002; Langston et al. 2002; Saijo et al. 2002; Danve-Szatanek et al. 2004; Stranska et al. 2004a). For instance, nine HSV strains resistant to FOS have been isolated in HIV-infected individuals for whom ACV and FOS therapy sequentially failed, but mutations conferring FOS resistance identified in the viral DNA polymerase were not associated with reduced ACV or CDV susceptibility (Schmit and Boivin 1999; Bestman-Smith and Boivin 2003).

The severity of immunosuppression and the dose and duration of ACV prophylaxis/treatment are likely important risk factors in the development of drug resistance. In the setting of T cell-depleted haploidentical transplantation, all HSV patients who received short courses of low-doses ACV for prophylaxis experienced clinical reactivation at a median $CD4^+$ T cell count of 3.5/μl (Langston et al. 2002). Lesion healing was correlated with immune recovery, but one patient had recurrent ACV-resistant HSV lesions due to a drop in the $CD4^+$ T cell count. The treatment regimen used was inadequate in this setting and drug resistance occurred less frequently when a prophylaxis with higher doses was given for longer periods. It was also reported that HSV-1 visceral infection (9.8 % versus 2.2 %) and ACV resistance (5.8 % versus 1.8 %) were more common in type-discordant [seronegative donor (D^-)/seropositive recipient (R^+)] than in type-concordant (D^+/R^+) HSCT patients, respectively (Nichols et al. 2003). However, drug-resistant HSV mutants have been isolated in some patients in the absence of known history of ACV exposure (Malvy et al. 2005; Schulte et al. 2010) and likely represent the natural rates of TK mutations.

The emergence of VZV isolates resistant to ACV has not been reported in immunocompetent individuals with primary VZV infections or herpes zoster. Cases of resistance to ACV have been described in AIDS patients, SOT and HSCT recipients, as well as hemato-oncological patients with VZV reactivations unresponsive to therapy (Talarico et al. 1993; Boivin et al. 1994; Fillet et al. 1998; Visse et al. 1998; Morfin et al. 1999). In these patients, VZV infections unresponsive to ACV therapy persist in the form of chronic skin lesions and are associated with significant morbidity and mortality due to visceral dissemination. A chronic verrucous form of VZV infections caused by ACV-resistant mutants has also been described in some of these patients (Crassard et al. 2000; Bryan et al. 2008). Two cases of immunocompromised children presenting herpes zoster due to the Oka vaccine strain and who developed chronic disseminated drug-resistant VZV infections following ACV therapy have been reported (Levin et al. 2003; Bryan et al. 2008). However, the prevalence of ACV-resistant cases in these different populations is unknown because only case reports have been published so far. In a recent study, it was reported that 27 % of hemato-oncological patients, including

HSCT recipients, with persistent VZV infections had mutations possibly associated with resistance to ACV (van der Beek et al. 2013). The isolation of PCV-resistant VZV mutants in the clinic has not been described so far possibly because this drug is not used as often as VACV. Few reports have described the emergence of VZV strains resistant to FOS in immunocompromised patients (Fillet et al. 1995; Visse et al. 1998, 1999).

Management of Infections Caused by Drug-Resistant HSV and VZV Mutants

An algorithm for the management of infections caused by drug-resistant HSV mutants is proposed in Fig. 3. The persistence of active lesions due to HSV for 7–10 days after initiation of high-dose oral ACV, VACV, or FCV therapy without appreciable decrease in size, an atypical appearance, or the emergence of satellite lesions is suggestive of treatment failure. When drug resistance is suspected, a change of therapy should be considered depending on the clinical severity of the disease. Most ACV-resistant HSV isolates harbor a mutation in the TK enzyme. Therefore, a patient failing to respond to ACV or VACV will usually not respond to FCV as there will be probably cross-resistance between the two nucleoside analogues. An initial step in case of treatment failure with oral drugs is to initiate high doses of IV ACV (10 mg/kg of body weight every 8 h adjusted for renal function). If there is no improvement after 7 days, a switch to IV FOS should be considered. Indeed, a few reports have described some efficacy of FOS therapy against ACV-resistant infections in AIDS patients (Chatis et al. 1989; Erlich et al. 1989; Safrin et al. 1990; Alvarez-McLeod et al. 1999) and BMT recipients (Verdonck et al. 1993; Reusser et al. 1996). The recommended dosage for the treatment of ACV-resistant HSV infections is 40 mg/kg every 8 h (with reduction in dose for renal dysfunction). In parallel, isolates from the lesions should be submitted for phenotypic susceptibility testing (starting with ACV and FOS and then CDV, if required) and/or genotypic assays if the patient is failing therapy. Continuous infusion of high-dose ACV (e.g., 1.5–2.0 mg/kg per hour) could also be administered as it is a well-tolerated alternative for severe ACV- or multidrug-resistant HSV infections (Engel et al. 1990; Kim et al. 2011). A switch to IV CDV (5 mg/kg once a week for 3–4 weeks) could also be considered. Cidofovir has a long intracellular half-life which makes infrequent dosing possible. Because of its nephrotoxicity, CDV is routinely administered with probenecid and requires IV hydration. Intravenous CDV has shown some efficacy in the treatment of progressive ACV- and/or FOS-resistant mucocutaneous HSV infections in immunocompromised patients (Snoeck et al. 1994; LoPresti et al. 1998; Kopp et al. 2002; Castelo-Soccio et al. 2010) but is not approved for this indication.

Topical formulations of FOS, including a 1 % cream (Javaly et al. 1999) and a 2.4 % solution (Pechere et al. 1998), were effective in the treatment of mucocutaneous HSV infections unresponsive to ACV. Topical formulations of CDV have been also used successfully for the treatment of drug-resistant mucocutaneous HSV infections

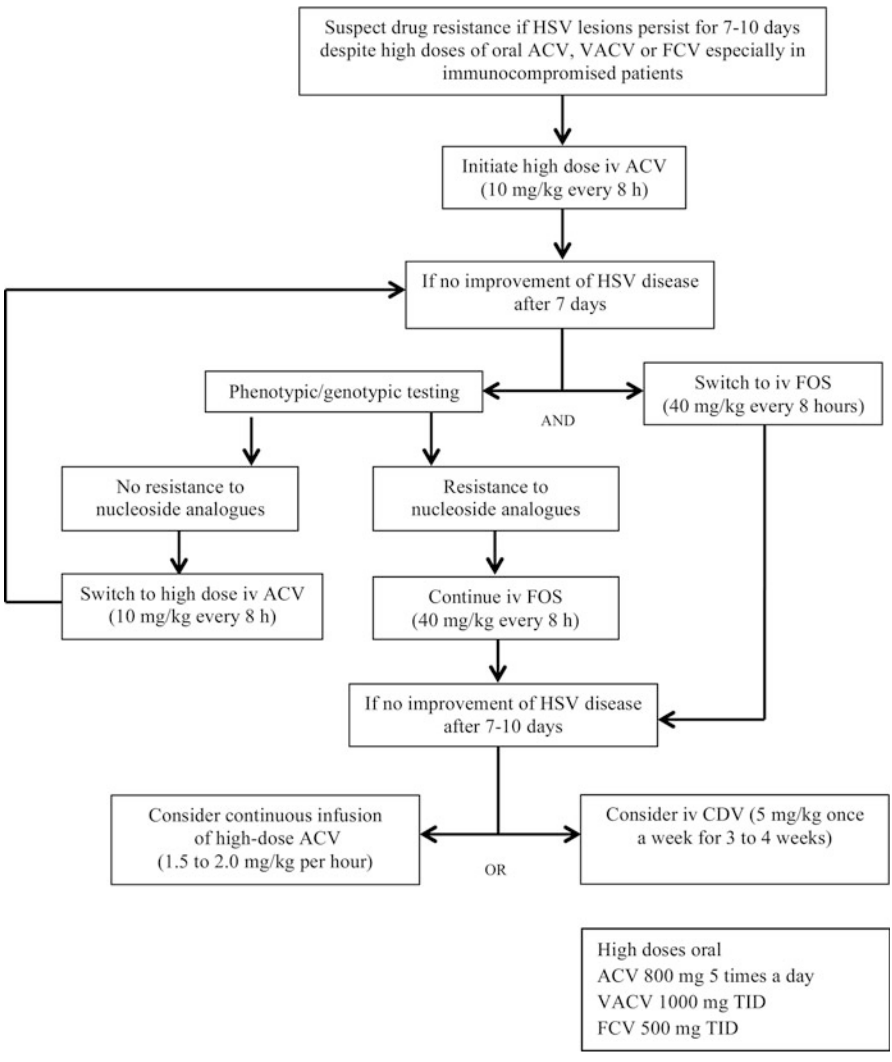

Fig. 3 Suggested algorithm for the management of suspected nucleoside analogue-resistant HSV infections. Key: *ACV* acyclovir, *VACV* valaciclovir, *FCV* famciclovir, *FOS* foscarnet, *CDV* cidofovir, *IV* intravenous

(Lalezari et al. 1997; Sacks et al. 1998; Sims et al. 2007; Evans et al. 2011). Although the use of these topical formulations could avoid the adverse effects associated with IV administration of FOS and CDV, they are not commercially available. A topical formulation containing 5 % imiquimod, an immunomodulatory drug, was effective in the treatment of recurrent and severe mucocutaneous lesions due to ACV- and FOS-resistant HSV-2 isolates in HIV-infected individuals (Lascaux et al. 2012). A 1 % topical solution of trifluorothymidine (TFT), a fluorinated

pyrimidine nucleoside analogue that inhibits thymidylate synthase, is usually administered in cases of ophthalmic herpetic infections that do not respond to ACV (Chilukuri and Rosen 2003).

The persistence of clinical signs of VZV infections for more than 10–14 days after initiation of high-dose oral ACV is suggestive of treatment failure, and it should lead to alternate therapy depending on the clinical severity of the disease (Ahmed et al. 2007). Genotypic testing of the *ORF36* gene coding for the TK protein could be performed in biopsy of mucocutaneous lesions or other body compartments when necessary (Brink et al. 2011). Foscarnet is generally used for the management of VZV infections due to suspected or confirmed ACV-resistant mutants, as described mainly in HIV-infected individuals (Safrin et al. 1991; Breton et al. 1998) and some oncology patients (Crassard et al. 2000; Levin et al. 2003; Bryan et al. 2008). The recommended IV dosage is 60 mg/kg every 8 h adjusted for renal function for at least 10 days or until complete lesion healing is observed (Ahmed et al. 2007). Clinical experience with the use of CDV in the treatment of drug-resistant VZV diseases is very limited (Schliefer et al. 1999).

Human Cytomegalovirus Antiviral Drug Resistance

Phenotypic and Genotypic Testing for Detection of HCMV Drug Resistance

The method of choice to determine HCMV drug susceptibility is the PRA, which has been standardized in a consensus protocol to decrease high inter-assay and interlaboratory variabilities (Landry et al. 2000). This assay is time-consuming (6–8 weeks) and subjective. Proposed cutoff values defining resistance to GCV, CDV, and FOS are 6, 2, and 400 μM, respectively (Drew et al. 1993; Chou 2008). An increase in the EC_{50} value greater than two- to three-fold over that of a sensitive reference strain or a baseline isolate is also a widely accepted breakpoint value (Chou 2010).

In line with their mechanisms of action, drug-resistance mutations are detected primarily in the *UL97* gene encoding the protein kinase (GCV) and/or in the *UL54* gene encoding the viral DNA polymerase (GCV, CDV, and FOS). Genotypic testing can be performed directly on clinical specimens either by restriction fragment length polymorphism (RFLP) of PCR products amplified from the *UL97* gene, melting point PCR analysis using hybridization probes specific for each mutation site in the *UL97* gene, or DNA sequencing of PCR products amplified from the *UL97* (typically codons 400–670) and/or *UL54* (typically codons 300–1,000) genes (Lurain and Chou 2010). Genotypic testing is fast (1–3 days) and objective. However, mutations conferring drug resistance must be discriminated from those associated with natural polymorphisms. Drug-resistance mutations identified in the *UL97* and *UL54* genes can be linked to resistance phenotypes by using a web-based search tool (http://www.informatik.uni-ulm.de/ni/staff/HKestler/hcmv) (Chevillotte et al. 2010).

If their significance in drug resistance is unknown, these mutations must be subjected to recombinant phenotyping (Lurain and Chou 2010; Drouot et al. 2013).

Role of *UL97* and *UL54* Gene Mutations in Conferring HCMV Drug Resistance

The great majority (>90 %) of drug-resistant HCMV clinical isolates selected from initial treatment with GCV contain one or more mutations in the UL97 kinase, whereas mutations in the UL54 DNA polymerase are less frequently encountered (Erice 1999). The catalytic domain of protein kinases consists of eleven major conserved regions numbered I to XI, with region I corresponding to the highest level of homology (Hanks et al. 1988). The ATP-binding site, the phosphate transfer domain, and the substrate-recognition site correspond to codon ranges located at positions 337–345 (region I), 453–462 (region VIB), and 574–579 (region IX), respectively. Laboratory-engineered UL97-negative HCMV mutant demonstrated a severe replicative deficiency compared to the wild-type parental strain highlighting the essential role of this enzyme in the viral replicative cycle (Prichard et al. 1999). Therefore, only a small number of mutations clustered in a relatively short genomic region of the *UL97* gene have been reported to confer resistance to GCV (Fig. 4a) (Gilbert et al. 2002; Gilbert and Boivin 2005; Lurain and Chou 2010; James and Prichard 2011; Komatsu et al. 2014). Ganciclovir-resistance mutations in the *UL97* gene consist in single-nucleotide substitutions or in-frame deletions (Gilbert et al. 2002; Gilbert and Boivin 2005). More than 80 % of GCV-resistant clinical isolates typically contain one of the seven canonical mutations (M460V/I, H520Q, C592G, A594V, L595S, and C603W) in the *UL97* gene (Lurain et al. 1994; Chou et al. 1995a, b; Wolf et al. 1995a, b). These non-synonymous mutations impair GCV phosphorylation without altering the normal kinase functions and result in EC_{50} value increases of five- to ten-fold, except for C592G which confers a three-fold increase (Gilbert and Boivin 2005; Lurain and Chou 2010). Other less frequently encountered mutations can emerge at codon 460 and between codons 590 and 607. Such mutations confer various degrees of resistance to GCV with EC_{50} increases of up to 15-fold (Chou et al. 2002). Mutation V466G, located outside typical codon ranges, confers low-grade GCV resistance (3.5-fold) and is associated with a significant replicative defect (Martin et al. 2010a). The replicative capacity of UL97 mutants has not been extensively studied. It was demonstrated that substitutions or small deletions in the *UL97* gene had no major impact on the viral replicative capacity (Emery et al. 1999; Chou and Meichsner 2000; Chou et al. 2002; Gill et al. 2009). In contrast, a virus with a deletion accounting for 70 % of the *UL97* gene (Prichard et al. 1999) and a recombinant virus with a truncated protein kinase domain (Chou et al. 2007b) had severely impaired replicative capacity. Amino acid changes associated with natural polymorphisms in the *UL97* gene are mainly clustered in two distinct regions (codons 1–249 and 427–674) (Boutolleau et al. 2011).

Ganciclovir-resistant HCMV clinical isolates with an altered DNA polymerase activity result from numerous mutations widely distributed among the different

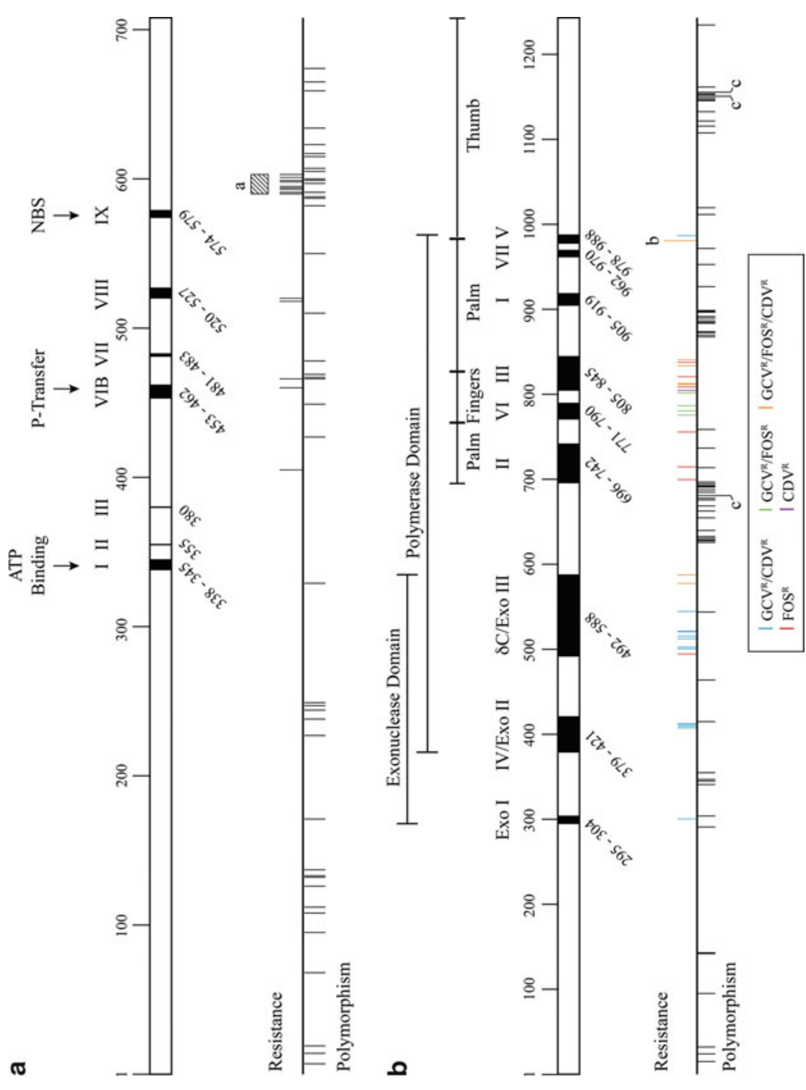

Fig. 4 (continued)

conserved domains of the enzyme, but mostly occur at codons 395–545 and 809–987 (Fig. 4b) (Gilbert et al. 2002; Gilbert and Boivin 2005; Lurain and Chou 2010; James and Prichard 2011; Komatsu et al. 2014). The catalytic domain of the UL54 DNA polymerase consists of eight conserved domains (i.e., I to VII and δ-region C) at its carboxyl-terminal portion. Moreover, a 3′–5′ exonuclease domain (containing Exo I, Exo II, and Exo III conserved motifs) maps to the N-terminal region of the polypeptide. The mechanisms of drug resistance occurring in the DNA polymerase involve either a prevention of the active drug binding to the enzyme or an alteration of the balance between exonuclease and polymerase activities which favor the removal of the incorporated drug (Hall et al. 1995; Cihlar et al. 1998b). In general, mutations in the *UL54* gene emerge after prolonged GCV exposure and increase the level of resistance conferred by mutations already present in the *UL97* gene (Lurain and Chou 2010). However, occasional reports have described mutations restricted to the *UL54* gene only after initial therapy with GCV (Boivin et al. 2005a; Hantz et al. 2010). DNA polymerase mutations that emerge under GCV therapy can confer cross-resistance to CDV and, less frequently, to FOS. Cross-resistance to GCV and CDV is associated with mutations located in the exonuclease domains (codons 301, 408–413, 501–545) and in region V (codons 981–987) of the enzyme (Sullivan et al. 1993; Chou et al. 1997, 2003, 2008; Cihlar et al. 1998a; Marfori et al. 2007; Scott et al. 2007). Resistance mutations to FOS are widely dispersed in the conserved domains of the *UL54* gene. However, clusters of mutations are mainly found in regions II, VI, and III and are associated with resistance to FOS alone, to both FOS and GCV, and to all three available antiviral agents, respectively (Baldanti et al. 1996; Chou et al. 1997, 2003, 2007a; Cihlar et al. 1998a; Weinberg et al. 2003; Scott et al. 2007). Mutations D588N (Exo III), A834P, and G841A (region III) as well as the deletion of codons 981–982 (region V) cause cross-resistance to GCV, CDV, and FOS (Chou et al. 2000; Scott et al. 2007). Regions I and VII have not been associated with drug resistance so far (Lurain and Chou 2010) probably because of their essential role in the enzyme activity. In

Fig. 4 Confirmed drug-resistance mutations identified in clinical HCMV isolates. *Panel a* shows mutations in the *UL97* gene conferring resistance to ganciclovir or associated with natural polymorphism. The ATP-binding site, the phosphate transfer (P-transfer) domain, the nucleoside-binding site (*NBS*), and some regions conserved among the protein kinase family (i.e., I, II, III, VIB, VII, VIII, and IX) are represented by the black boxes. Bars (|) indicate amino acid substitutions associated with resistance (*upper bars*) or with polymorphism (*lower bars*). [a], shaded area corresponds to the codon 590–603 region where different amino acid deletions were identified (i.e., deletions 590–593, 591–594, 591–603, 595–603, 598–601, 599–603, 600, and 601–603). *Panel b* shows mutations in the *UL54* gene conferring resistance to ganciclovir (GCV^R), foscarnet (FOS^R), and/or cidofovir (CDV^R) or associated with natural polymorphism. Conserved regions among the Herpesviridae DNA polymerase are represented by the black boxes. The roman numbers (I–VII) and δ-region C corresponding to each of these regions are indicated above the boxes. Conserved motifs (Exo I, Exo II, and Exo III) in the exonuclease domain are also indicated above the boxes. Bars (|) indicate amino acid substitutions associated with resistance (*upper bars*) or with polymorphism (*lower bars*). [b], represents amino acid deletion 981–982 that confers resistance to all three antivirals; [c], indicates amino acid deletions associated with polymorphism (i.e., deletions 681–688, 1151, and 1156)

contrast to the situation with UL97 mutants, isolates with DNA polymerase mutations conferring drug resistance usually exhibit an attenuated or slow-growth phenotype in cell culture compared to wild-type strains (Baldanti et al. 1996; Cihlar et al. 1998b). It has been suggested that these mutations could affect substrate recognition which might decrease the affinity of the viral DNA polymerase for dNTPs. Finally, the natural polymorphism is more common in the *UL54* gene than in the *UL97* gene and occurs most often at non-conserved residues (between codons 614 and 697) where little homology exists among herpesvirus DNA polymerases (Chou et al. 1999; Fillet et al. 2004). This high degree of inter-strain variability in the *UL54* gene complicates the interpretation of genotypic testing.

Clinical Significance, Prevalence, and Risk Factors for Drug-Resistant HCMV Infections

Drug-resistant HCMV isolates are associated with different clinical presentations from asymptomatic to severe organ invasive or fatal disseminated diseases. In SOT recipients, infections caused by HCMV drug-resistant isolates can manifest as asymptomatic and symptomatic viremic episodes, an earlier onset of HCMV disease, graft loss, and an increased risk of mortality (Bhorade et al. 2002).

Shortly after the introduction of GCV, the emergence of drug-resistant HCMV strains was reported particularly in untreated or poorly treated AIDS patients who developed HCMV retinitis at a high frequency (ranging from 20 % to 45 %) (Jabs 1995). Several studies in AIDS patients who had received prolonged antiviral treatment for HCMV retinitis demonstrated that the emergence of drug-resistant isolates was directly related to the duration of therapy. Indeed, no drug-resistant isolate could be recovered in AIDS patients who had received GCV therapy for less than 3 months, whereas the incidence of resistance reached 8 % after a treatment period exceeding 3 months (Drew et al. 1991). The rates of emergence of drug-resistant mutants in AIDS patients with HCMV retinitis who had received GCV for 9 months and VGCV for 12 months were found to be 27 % and 13 %, respectively (Jabs et al. 1998a; Boivin et al. 2001). The lower incidence of GCV resistance in the latter study could be due to improved AIDS therapy. Indeed, the introduction of highly active antiretroviral therapy (HAART) substantially reduced the incidence of HCMV retinitis in AIDS patients, and this was associated with a concomitant decrease in the rate of emergence of drug resistance. Another study reported a reduction in the incidence of GCV resistance from 28 % to 9 % in the pre-HAART and HAART eras, respectively (Martin et al. 2007). Patients with AIDS, especially those with $CD4^+$ T cell counts below 50 cells/μl, remain at risk of developing HCMV retinitis and eventually GCV-resistant infections even nowadays (Sugar et al. 2012).

Thereafter, the more widespread use of oral GCV (with a low bioavailability of 6 %) and the intensification of immunosuppressive regimen resulted in an increased prevalence of HCMV drug resistance in SOT recipients. The prevalence of drug resistance in transplant recipients who have received GCV therapy for more than

2 months is approximately 10 % in general and up to 30 % in high-risk patients (D^+/R^- lung transplant recipients) (Limaye et al. 2002; Lurain et al. 2002; Hantz et al. 2010). The emergence of drug resistance in R^+ patients is rarely observed except in lung transplant recipients (Lurain et al. 2002). A retrospective study that evaluated 240 SOT patients reported an incidence of GCV resistance of 2.1 % in the overall population and of 7 % in D^+/R^- patients (Limaye 2002). More specifically, drug resistance was more frequently observed among recipients of kidney-pancreas or pancreas alone (21 %) than among patients transplanted with kidney (5 %) or liver (0 %). In two US centers, phenotypic evaluation of HCMV drug resistance demonstrated incidence rates varying from less than 0.5 % to 5.6 % and from 2.2 % to 15.2 % in non-lung and lung transplant recipients, respectively (Lurain et al. 2002). It is noteworthy to mention that HCMV infections caused by GCV-resistant isolates represented 20 % of all HCMV disease that developed during the first year after transplantation in one US study (Limaye et al. 2000).

In contrast to oral GCV, VGCV is highly absorbed leading to an improved systemic exposure (about 60 %) that could limit the emergence of drug-resistant HCMV mutants. The clinical efficacy and safety profile of a daily dose of VGCV were shown to be similar to thrice-daily doses of oral GCV for the prevention of HCMV diseases in high-risk SOT recipients (Paya et al. 2004). Several studies compared the emergence of drug resistance in SOT recipients who had received one of these two prophylactic regimens. A first study investigated the emergence of drug resistance in 364 high-risk D^+/R^- patients (including liver, kidney, heart, kidney-pancreas, and liver-kidney recipients) who had received oral GCV or VGCV prophylaxis for 100 days based on *UL97* (Boivin et al. 2004) and *UL54* (Boivin et al. 2005a, b) genotypic testing. The resistance rates at the end of the prophylactic period were 0 % and 3 % in the VGCV and GCV arms, respectively. The incidence of drug resistance in lung transplant recipients who had received IV GCV (D^+/R^- patients), oral GCV (R^+ patients), or oral VGCV prophylaxis was also found to be low (Boivin et al. 2005b; Humar et al. 2005). Finally, a low incidence of drug resistance was reported in adult D^+/R^- transplant patients and pediatric liver and heart transplant recipients who had received VGCV prophylaxis (Eid et al. 2008; Martin et al. 2010b). The low frequency of drug resistance in SOT recipients receiving VGCV (compared to oral GCV) could be related to an improved GCV exposure and to a better compliance of the patients to the once daily dosing.

High-risk patients who receive VGCV prophylaxis for 100 days post transplant might still be at risk of developing late-onset HCMV disease. However, extending the prophylactic regimen may increase the risk of emergence of drug resistance. Therefore, the impact of extending VGCV prophylaxis from 100 to 200 days on the incidence of resistance was investigated in 318 D^+/R^- kidney transplant recipients based on genotypic testing (Boivin et al. 2012). The rates of drug resistance were similar (1.8 % versus 1.9 %) in patients who had received VGCV prophylaxis for 100 and 200 days suggesting that extending the prophylactic period up to 200 days did not significantly affect the incidence of GCV resistance. Of note, almost all cases of resistance occurred during VGCV prophylaxis and rarely thereafter. Prophylaxis

with VGCV for 200 days after transplantation could thus be an interesting option in high-risk kidney transplant recipients.

It is still under debate whether a prophylactic or a preemptive approach is more effective in preventing HCMV disease in high-risk transplant patients. In D^+/R^- kidney transplant recipients, both prophylaxis and preemptive therapy seem to have similar efficacy. Several studies also evaluated the effect of these treatment regimens on the emergence of drug resistance. In a first study, GCV-resistance mutations were detected in 2.2 % of the overall renal transplant recipients and, more specifically, in 12.5 % of D^+/R^- patients who had received once daily VGCV preemptive therapy (Myhre et al. 2011). Another study compared the emergence of resistance in D^+/R^- renal transplant recipients who had received VGCV prophylaxis for 3 months or VGCV preemptive therapy (Couzi et al. 2012). HCMV drug resistance was more frequent in the preemptive compared to the prophylactic group (16 % versus 3 %). The author suggested that, during preemptive therapy, patients may be exposed to suboptimal drug levels which favor an active viral replication state, thus increasing the risk of emergence of GCV resistance. Therefore, it is proposed that the use of prophylaxis may be more appropriate than the preemptive therapy in high-risk transplant recipients, although other studies are still needed to confirm this point.

Valganciclovir was shown to be noninferior to IV GCV for the treatment of established HCMV disease in SOT recipients (Asberg et al. 2007, 2009). A secondary endpoint of this trial was the evaluation of the emergence of drug resistance in 275 SOT patients (including heart, kidney, liver, and lung recipients) treated for HCMV disease with a 21-day induction dose of IV GCV or VGCV followed by VGCV maintenance dose for 49 days in both arms (Boivin et al. 2009). Probable or confirmed drug-resistance mutations were low and found to be similar for VGCV (3.6 %) and IV GCV (2.3 %) treatments. Overall, incidences of GCV drug resistance were low in kidneys (3.7 %), intermediate in livers and hearts (4.3–5.0 %), and highest in lungs (17.6 %).

Valganciclovir is also widely used for the management of HCMV disease in HSCT recipients. The incidence of drug resistance among HSCT recipients who had received preemptive therapy with GCV or VGCV was low in several studies (Gilbert et al. 2001; Nichols et al. 2001; Allice et al. 2009; Hantz et al. 2010; van der Beek et al. 2012) and cannot explain the high rate of treatment failure observed in this setting that is probably more related to the profound immunosuppression. In a recent study, a high rate of drug resistance (14.5 %) was exclusively identified in haploidentical-HSCT recipients receiving preemptive therapy with GCV (Shmueli et al. 2014).

Overall, proposed risk factors for the emergence of GCV resistance in transplant recipients include the lack of HCMV-specific immunity (typically the D^+/R^- group), lung or kidney-pancreas transplantation, high HCMV loads due to potent immunosuppressive therapy and/or suboptimal GCV levels, extended duration of therapy, and the type of antiviral regimen (i.e., treatment, prophylaxis, or preemptive therapy) (Gilbert and Boivin 2005).

As FOS and CDV constitute second-line antivirals for the treatment of HCMV diseases, only a few reports have described the emergence of resistance to these

drugs in the clinic. In one study, baseline frequencies of resistance were reported to be less than 3 % for FOS and less than 7 % for CDV in AIDS patients with HCMV retinitis (Jabs et al. 1998b). These rates reached 37 % in patients treated with FOS for 9 months and 29 % in patients treated with CDV for 3 months. Globally, resistance to FOS and CDV occurs at similar rates as those typically reported for GCV.

Management of Infections Caused by Drug-Resistant HCMV Mutants

Guidelines for the management of GCV-resistant HCMV disease have been developed following a consensus meeting organized by the Transplantation Society International CMV Consensus Group (Kotton et al. 2013) with a suggested algorithm shown in Fig. 5. Antiviral drug resistance should be suspected in case of cumulative GCV exposure for more than 6 weeks and sustained or rising viral loads (especially DNAemia levels) despite more than 2 weeks of full dose of IV GCV (5 mg/kg twice daily, adjusted for renal function). However, a rise in pp65 antigenemia or DNAemia levels during the first 2 weeks of antiviral therapy in HSCT recipients has been associated mostly with host and other transplant-related factors rather than with the emergence of drug-resistance mutations (Nichols et al. 2001). The first change to be considered could be a reduction in the immunosuppressive therapy although this may lead to organ rejection (which would require intensifying immunosuppression). As the modulation of immunosuppression is rarely sufficient to control HCMV, the use of adjunctive immunoglobulins containing HCMV antibodies could be considered, but these agents are expensive and their supply is limited. At this initial step, a clinical decision about antiviral modification is empirical but should be based on the evaluation of host risk factors (e.g., D^+/R^- recipients, lung transplant recipients) and disease severity (sight- or life-threatening disease). Full or higher doses of IV GCV (5 or 10 mg/kg twice daily, respectively, adjusted for renal function) can be administered to low-risk patients with mild disease, whereas FOS alone or combined with GCV can be initiated for high-risk patients with severe disease. Of note, clear evidence of the superiority of GCV and FOS combination over FOS alone has not yet been demonstrated (Drew 2006). As resistance mutations to GCV typically emerge in the protein kinase, *UL97* gene sequencing is first recommended. Genotypic assays are performed typically on whole-blood or plasma specimens (Lisboa et al. 2011). However, some studies have demonstrated a compartmentalization of drug-resistant HCMV strains which suggests that resistance assessment based solely on blood samples may be suboptimal in some instances (Hamprecht et al. 2003; Jeong et al. 2012). Therefore, genotypic testing of cerebrospinal fluid, bronchoalveolar lavages, or biopsy specimens could be occasionally performed in high-risk patients.

If no mutation is identified in the *UL97* gene, full dose of IV GCV (5 mg/kg twice daily, adjusted for renal function) should be continued together with an optimization of host factors. If a major UL97 mutation (more than 5-fold increase in GCV EC_{50} value) is identified, a switch to IV FOS is recommended. If a minor UL97 mutation (less than 5-fold increase in GCV EC_{50} value) is detected, IV GCV can be increased

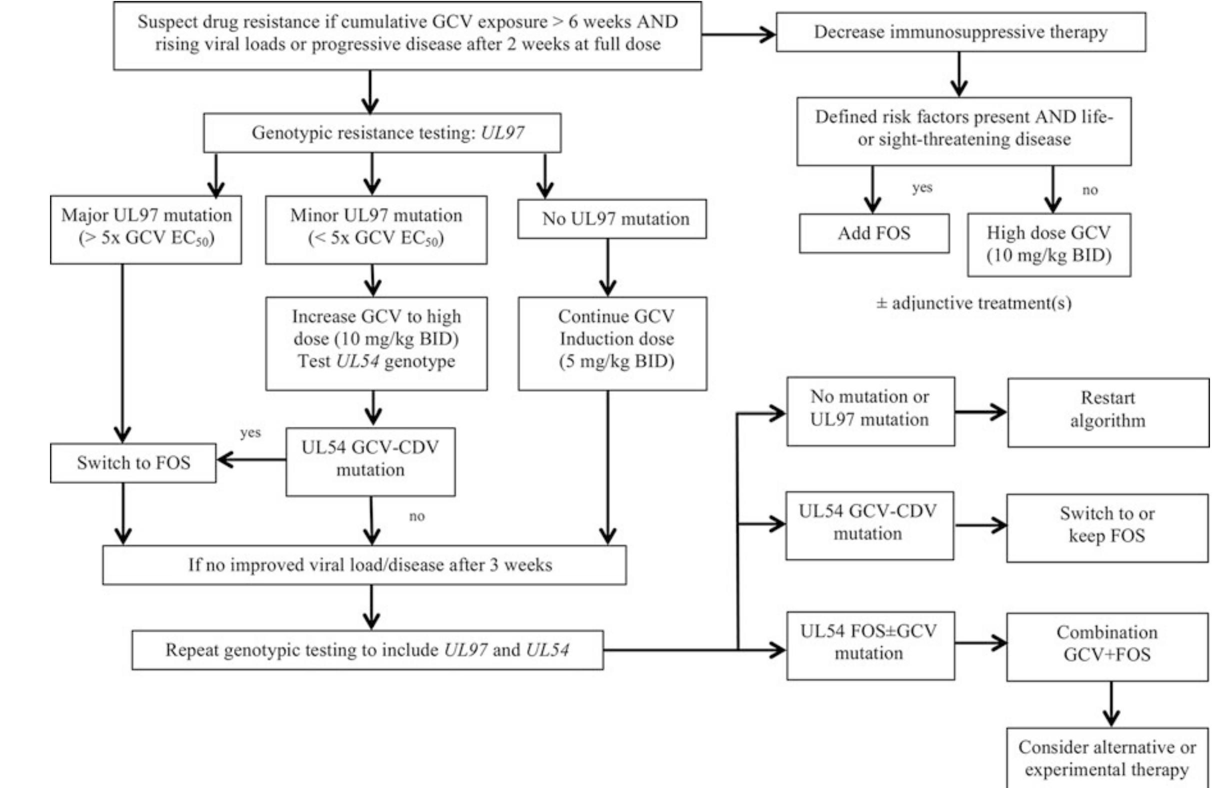

Fig. 5 Suggested algorithm for management of suspected HCMV drug resistance. Key: *GCV* ganciclovir, *FOS* foscarnet, *CDV* cidofovir, EC_{50} concentration of antiviral that reduces HCMV replication in cultured cells by 50 % compared to the control (without drug) determined in phenotypic assay (Adapted from Kotton et al. 2013)

to higher doses (10 mg/kg twice daily, adjusted for renal function), and drug-resistance mutations should be looked for in the *UL54* gene. If a mutation conferring cross-resistance to GCV and CDV is detected in the *UL54* gene, a switch to IV FOS therapy is recommended (or IV FOS should be kept).

The viral load is typically monitored once weekly by quantitative PCR during the period covering an episode of symptomatic HCMV disease. If there is no improvement in the viral load and a persistence of HCMV disease after a period of 3 weeks, genotypic testing should be repeated to assess the emergence of drug-resistance mutations in both the *UL97* and *UL54* genes. It is not recommended to switch to IV CDV for the treatment of GCV-resistant HCMV disease before obtaining the results of genotypic testing of the *UL54* gene because of the high frequency of cross-resistance between GCV and CDV. If a mutation conferring cross-resistance to GCV and CDV is detected, a switch to IV FOS is recommended (or IV FOS should be kept). If a resistance mutation to FOS is detected, a combination of high-dose IV GCV (10 mg/kg twice daily, adjusted for renal function) with IV FOS or CDV should be considered. Antiviral therapy is typically continued until viremia is no longer detectable. In case of multidrug-resistant HCMV disease, alternative or experimental therapies should also be considered (see below).

Several nonconventional interventions have been described for the treatment of multidrug-resistant HCMV diseases, although their clinical utility has not been adequately evaluated (Le Page et al. 2013). Artesunate, an antimalarial drug with activity against HCMV in vitro and in vivo (Kaptein et al. 2006), was shown to be effective in the treatment of a HSCT recipient with multidrug-resistant HCMV infections (Shapira et al. 2008). Leflunomide, an immunosuppressive agent used for rheumatoid arthritis, possesses anti-HCMV activity including against GCV-resistant isolates by acting on virion assembly (Waldman et al. 1999; Chong et al. 2006). Thus, no cross-resistance is expected with the current antiviral agents. The efficacy of leflunomide in the treatment of HCMV infection has been reported in a HSCT recipient failing to respond to all available antiviral agents (Avery et al. 2004). Leflunomide alone or in combination with standard antiviral agents has shown some efficacy in transplant recipients refractory to current therapy (Avery et al. 2010; Dunn et al. 2013; Verkaik et al. 2013). The combination of GCV and the immunosuppressive agent sirolimus for the treatment of GCV-resistant HCMV infections has led to a favorable outcome with respect to clinical status and graft rejection in kidney and kidney-pancreas recipients (Ozaki et al. 2007).

Conclusions

Drug-resistant HSV, VZV, and HCMV mutants may cause severe and chronic infections as well as increased mortality in immunocompromised patients. The number of patients with an immunocompromised status is currently increasing and the emergence of drug-resistant herpesvirus infections is not expected to fade. The development of fast and efficient methods for detecting viral mutant sequences directly in clinical specimens such as pyrosequencing (Kampmann et al. 2011) and

ultra-deep pyrosequencing (Gorzer et al. 2010; Sahoo et al. 2013) will improve the diagnosis of drug-resistant herpesvirus infections. The use of more powerful recombinant phenotyping techniques (Drouot et al. 2013) and the availability of an Internet database (Chevillotte et al. 2010) to link individual mutations to their drug-susceptibility phenotypes should result in more rational therapeutic strategies.

As all currently available antiviral agents target the viral DNA polymerase, the development of new antiherpetic compounds with different mechanisms of action retaining efficacy against nucleoside/nucleotide analogue- and FOS-resistant isolates and with adequate safety profiles is an important priority. In that regard, some promising compounds are currently in clinical trials. The orally bioavailable lipid ester prodrug of CDV (i.e., hexadecyloxypropyl-cidofovir; CMX001) could avoid the dose-limiting renal toxicity of the parent drug and provide a safe alternative for ACV- and GCV-resistant herpesviruses in immunocompromised patients (Hostetler 2010). Treatment with oral CMX001 significantly reduced the incidence of HCMV events in HSCT recipients (Marty et al. 2013). Maribavir is a competitive inhibitor of the UL97 kinase (Biron et al. 2002). Surprisingly, mutations arising after in vitro selection with this drug most often map to the *UL27* gene and, less frequently, to the *UL97* gene. Of note, mutations found in the *UL97* gene are distinct from those described in GCV-resistant strains (Chou et al. 2012) and some have been detected outside the conserved kinase domains (Chou 2008). Thus, maribavir retains activity against GCV-resistant mutants. The emergence of resistance to this drug has been reported in some cases (Strasfeld et al. 2010; Schubert et al. 2013). Recently, maribavir faced some limitations in phase III clinical studies (Marty et al. 2011) but new trials using higher doses are in progress. Letermovir targets the terminase complex of HCMV and interferes with viral DNA concatemer maturation (Lischka et al. 2010; Goldner et al. 2011). Accordingly, mutations conferring resistance to letermovir map to the *UL56* gene (Goldner et al. 2011, 2014). Successful treatment of a multidrug-resistant HCMV infection with letermovir has been reported in a lung transplant recipient (Kaul et al. 2011). The preemptive treatment of HCMV infection with letermovir in kidney transplant recipients was recently investigated in a phase IIa trial (Stoelben et al. 2014). Novel classes of antiviral agents targeting the ribonucleotide reductase, the helicase-primase complex, and the process of viral DNA encapsidation are at earlier stages of development (Greco et al. 2007).

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Quasispecies and Drug Resistance

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Abstract

Mutant spectra of viral quasispecies are complex reservoirs of genetic and phenotypic variants, including drug-resistant mutants. Here we review basic features of RNA viral quasispecies such as internal interactions within mutant spectra and the effect of population size and bottleneck events as they affect the frequency of inhibitor-escape mutants. Genetic barriers to resistance and fitness cost of specific amino acid substitutions involved in resistance are discussed, with specific examples for human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV). Prospects for new antiviral designs aimed at counteracting the adaptive potential of viral quasispecies are presented.

Keywords

Antiviral therapy • Drug resistance • Genetic barrier • Hepatitis C virus (HCV) • Human immunodeficiency virus type 1 (HIV-1) • Mutant spectrum • Replication rate • Viral fitness • Viral load • Viral quasispecies

Introduction: Relevance of Quasispecies in Virus Biology

Viral quasispecies are mutant distributions (also termed mutant spectra, clouds, or swarms) that characterize genome populations of RNA viruses and at least some DNA viruses (Fig. 1). Both clonal analyses by classic nucleotide sequencing techniques and bulk population analyses by ultra-deep sequencing have documented that mutant distributions are extremely complex with many minority mutations occurring at low frequency (1 % which is the present standard cutoff value for reliable mutant frequency determination and probably lower according to studies that achieved lower cutoff values). From all evidence, mutant spectra originate from high mutation rates in RNA (and some DNA) viruses, which have been estimated in 10^{-3} to 10^{-5} mutations introduced per nucleotide copied, together with competition and intrapopulation interactions among genomes [reviewed in (Domingo et al. 2012)]. Viral quasispecies took its name from a theory of the origin of life developed by M. Eigen, P. Schuster, and their colleagues (Eigen and Schuster 1979). Theoretical studies on quasispecies have paralleled experimental investigations with RNA viruses, reaching a considerable degree of conceptual cross-fertilization (Eigen 2013; Holland 2006; Mas et al. 2010; Ojosnegros et al. 2011).

The biological behavior of viral quasispecies is not equivalent to that of sets of identical genomes undergoing only occasional mutations for two main reasons. One is that mutant spectra constitute vast reservoirs of genetic and phenotypic variants, including, notably, drug-, antibody-, or cytotoxic T-cell (CTL)-escape mutants. The second reason is that the variant genomes which dynamically arise, persist, increase, or decrease in frequency or are eliminated (transiently or irreversibly) do not act independently. Variants can complement each other to give rise to a new phenotype (Cao et al. 2014; Shirogane et al. 2012), to trigger large evolutionary transitions such as genome segmentation (Moreno et al. 2014), or to maintain a higher average fitness of the mutant ensemble relative to its individual components (Domingo et al. 1978, 2012;

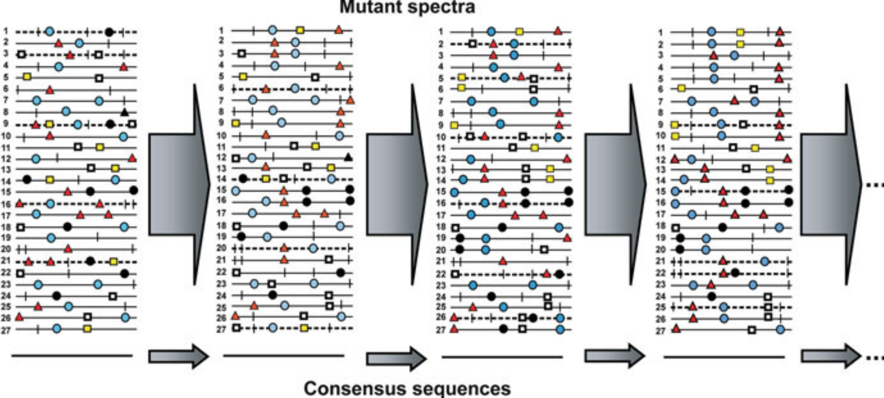

Fig. 1 Schematic representation of quasispecies evolution. The four successive populations (*lines* represent genomes and symbols on lines mutations) evolve by modification of the mutant spectrum while the consensus sequence remains invariant. In the simplified dynamics depicted here, genomes that acquire five or more mutations (genomes with discontinuous lines) do not survive. In reality, viral populations (even single replicative units in a replication complex) consist of hundreds or thousands genomes subjected to the dynamics of mutant generation, competition, and selection. (Figure reproduced from Domingo et al. (2012) with permission from ASM)

Duarte et al. 1994). A specific high-fidelity mutant of poliovirus that displayed limited mutant spectrum complexity was attenuated and could not adapt to complex environments or reach the central nervous system in a mouse model (Pfeiffer and Kirkegaard 2005a; Vignuzzi et al. 2006). However, an accompanying mutant spectrum allowed the mutant to reach its target organ *in vivo* (Vignuzzi and Andino 2010; Vignuzzi et al. 2006). Thus, mutant spectrum complexity is relevant to viral pathogenesis, and the control of replication fidelity may serve to engineer attenuated virus vaccines (Vignuzzi et al. 2008).

Quasispecies swarms can have an effect opposite to complementation: the suppression of individual viral mutant progeny which in isolation displays superior fitness than the parental quasispecies (de la Torre and Holland 1990). Theoretical quasispecies predicts that the behavior of any individual component may be modulated by the mutant spectrum that surrounds it. In one of the computer simulations, near an error threshold (preceding a second and final threshold where no genomes can be maintained), a slightly inferior mutant was strongly favored by virtue of its better mutant environment [(Swetina and Schuster 1982), reviewed in Eigen and Biebricher (1988)]. In the case of viruses, the suppressive effect of a mutant ensemble on particular variants is exerted through different biological mechanisms, derived from the biochemical reactions during genome replication and the effect of *trans*-acting proteins. Specifically, in poliovirus, four mechanisms of mutant-mediated interference were identified (Crowder and Kirkegaard 2005). Some capsid and polymerase mutants produced dominant negative phenotypes, attributed to the fact that these proteins function as oligomers. Mutations in *cis*-regulatory element (CRE) and VPg protein indicated that nonproductive priming of initiation of viral RNA replication is inhibitory. The authors confirmed that, as anticipated, a drug-

sensitive poliovirus inhibited the intracellular growth of a drug-resistant mutant (Crowder and Kirkegaard 2005). In line with these findings, a mutagenized, preextinction foot-and-mouth disease virus (FMDV) population interfered with replication of the standard virus (González-López et al. 2004). The accumulation of defective, mutated genomes in the heavily mutagenized FMDV population produced an interfering swarm, an event that was shown to participate in viral extinction by lethal mutagenesis (Grande-Pérez et al. 2005). A study with specific FMDV capsid and polymerase mutants confirmed their interfering activity on wild-type virus and showed that the mutants had to be competent in RNA replication to be inhibitory (Perales et al. 2007). This requirement is one of the factors that contribute to an advantage of sequential inhibitor-mutagen treatment over the corresponding combination, to prevent the selection of inhibitor-escape mutants and favor virus extinction (Iranzo et al. 2011; Moreno et al. 2012; Perales et al. 2009, 2012).

Modulating effects of mutant spectra have been observed also *in vivo*. Virulent poliovirus can have its phenotype suppressed by attenuated virus in the population (Chumakov et al. 1991). A growth hormone deficiency syndrome induced by lymphocytic choriomeningitis virus can be suppressed by disease-negative variants (Teng et al. 1996) [reviewed in (Domingo et al. 2012)].

Major Factors in the Generation and Dominance of Drug Resistance in Viruses

Drug-resistant mutants are those present in quasispecies that can replicate more efficiently than other components of the mutant swarm in the presence of the drug. Their selection and maintenance in a viral population is conditional upon two events which are influenced by different parameters: (i) mutant generation and (ii) the efficiency of mutant replication relative to other components of the same population. Mutant generation depends on the genetic barrier to resistance, defined as the number and types of mutations required to reach the resistance phenotype. Telaprevir resistance in hepatitis C virus (HCV) can be achieved by amino acid substitution R155K in NS3. In HCV genotype 1a, these substitutions can be attained by a single nucleotide transition (AGA → AAA). In contrast, in genotype 1b, by virtue of the R codon being CGA, the same amino acid substitution requires two mutations: a transversion and a transition (CGA → AAA). Since the probability of occurrence of two independent mutations is the product of probabilities of occurrence of the individual mutations, and transversions are usually less frequent than transitions, the HCV genetic barrier to telaprevir resistance is higher for HCV genotype 1b than 1a. There is no molecular or evolutionary reason to exclude that genetic variations that modify the genetic barrier to a drug can occur among viruses of the same genotype or among components of a mutant spectrum. Obviously, the genetic barrier will be increased when two or more amino acid substitutions (each requiring at least one mutation) are needed to reach the drug-resistance phenotype. In general terms, requirements of multiple mutations (excessive number of steps in sequence space) are what preclude viruses of surviving in some environments. This is the main reason

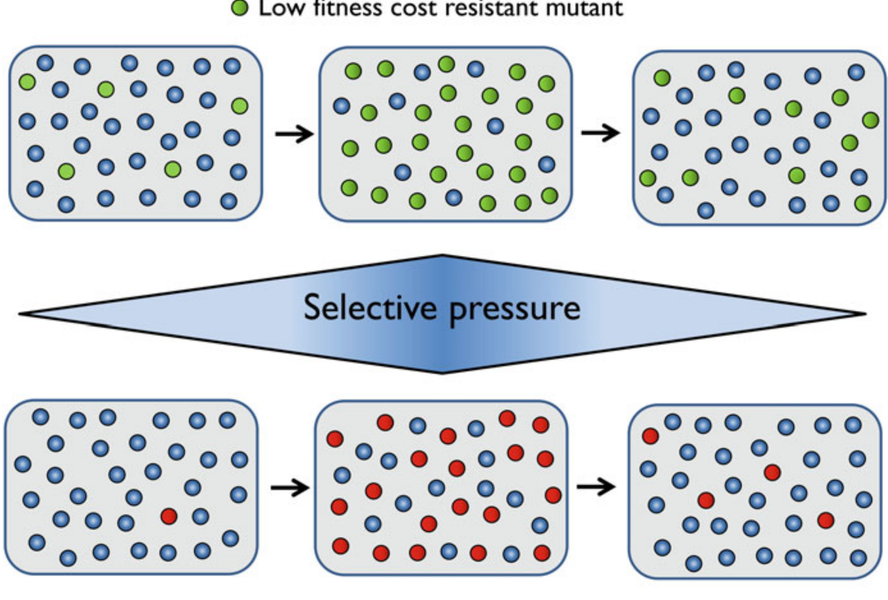

Fig. 2 Phenotypic barrier or fitness cost to overcome a selective pressure. The escape mutants that experience a low fitness cost (depicted as *green circles* in the upper three successive populations) may preexist with considerable frequency before the selective pressure is exerted; they can reach high proportions in the presence of the selective pressure and remain at elevated levels even when the selective pressure is removed (*upper right population*). The escape mutants that experience a high fitness cost (depicted as *red circles* in the bottom three successive populations) will be present at low frequency before the selective pressure is exerted; they can reach high proportions in the presence of the selective pressure and return to low levels when the selective pressure is removed (*bottom right population*)

of the advantages of combination therapies over monotherapy, with the exceptions discussed in section "Conclusions and New Prospects for Antiviral Therapy".

Once the genetic barrier has been overcome and the resistant mutant has been generated, a second barrier, termed phenotypic barrier or fitness cost, intervenes. If the relevant amino acid substitution, in addition to conferring drug resistance, impairs any step in the viral life cycle, the proportion of the mutant in the viral quasispecies will decrease. The higher the fitness cost, the lower the proportion of the mutant in the mutant spectrum. Two possible outcomes can be anticipated: either the fitness cost does not allow the mutant to become dominant or compensatory mutations (that counteract the fitness cost of the drug-resistance mutations without significantly altering the resistance level) occur that allow dominance of the resistant mutant. Fitness effects apply to viruses escaping any type of selective pressure (drugs, immune responses, tropism, host range changes, etc.). The consequence of fitness cost of a drug-resistance mutation has been schematically represented in Fig. 2, in which the frequency of the relevant escape mutant

(green circles in the upper population and red circles in the bottom population) is fitness-dependent. If the fitness cost is severe (even more than implied in the bottom panels of Fig. 2), the relevant escape mutant may not preexist in the population. With 10^{-3} to 10^{-5} mutations introduced per nucleotide copied (Domingo et al. 2012), a type of arms race is established between the occurrence of the relevant mutation and the opportunity of the genome harboring it to replicate sufficiently in the presence of the drug. These conflicting requirements may allow the virus to improve replication through compensatory mutations and evolve towards dominance or be irreversibly lost in the mutant spectrum. Studies of deep sequencing of viral populations that are confronted with a strong selective pressure [e.g., in human immunodeficiency virus type 1 (HIV-1)-infected patients treated with vicriviroc (Tsibris et al. 2009)] suggest that viral quasispecies screen multiple escape routes, and only a subset of those are successful. Drug-escape mutants are present at high frequencies in populations of many important pathogenic viruses such as HIV-1, hepatitis B virus, HCV, or influenza virus, and such mutants can dramatically lead to treatment failure. Yet, what the experimental studies on quasispecies dynamics suggest is that the observed drug-resistance mutations recorded are only a minor subset of all possible resistance mutations that would be found if fitness effects did not intervene.

To complicate matters even further, as noted in the Introduction, fitness levels are not only a property of individual viral genomes. Rather, the frequency of a given mutant can be influenced by the surrounding quasispecies. Specifically, the presence of a complex mutant spectrum can suppress a drug-resistant mutant to avoid or delay its dominance (see Introduction for references).

Effect of Variations in Population Size and Viral Load

The virus population size often varies during the course of the natural infectious cycle of viruses. It generally increases from the initial infecting dose to a viremic state, and it may decrease again if persistence or chronicity is established. During an acute infection, subsets of viruses may invade new cells, tissues, or organs, and such invasions may involve reductions of population size (bottleneck events). Since viral populations consist of mutant swarms, the viral population size that is transmitted (from host to host, organ to organ, or cell to cell) will determine the numbers and types of mutants that can continue replicating (Fig. 3). Drug-resistant mutants may be generated in an infected host subjected to therapy with the drug (e.g., the mutants depicted as yellow stars in Fig. 3). This mutant will not contribute directly to drug resistance in a recipient virus-naïve individual unless the transmitted population reaches a critical size. The term primary resistance was coined during the AIDS epidemics to denote infections by HIV-1 which harbored an antiretroviral resistance mutation selected prior to transmission.

Not only fluctuations of population size and bottleneck events are important to understand quasispecies evolution, the total population size (viral load) is also relevant. We have previously emphasized the connections between four parameters in virus survival: viral load, replication rate, genetic heterogeneity, and viral

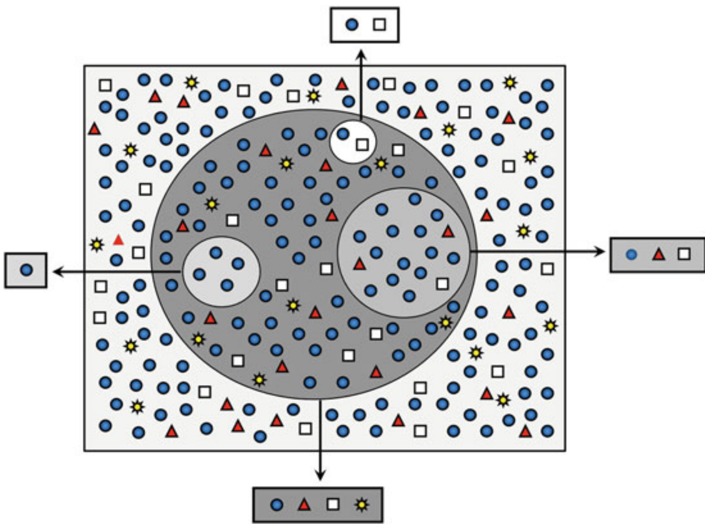

Fig. 3 **The effect of population size in a mutant repertoire**. The *large square* represents a viral quasispecies, in which four types of genomes are present. Small sample sizes will result in detection of only the highest frequency genomes (*small gray circle*) but may randomly fluctuate based on chance detection (*small white circle*). Greater diversity will be detected in larger sample sizes, represented by larger *gray circles* (Figure modified from Domingo et al. (2012) with permission from ASM)

fitness. As explained elsewhere (Domingo et al. 2012), potent replication which is a key component of fitness values tends to produce elevated viral loads. When endowed with the adequate diversity (mutant spectrum amplitude), high viral loads will contribute to adaptedness and survival. Several lines of evidence suggest that these four interconnected parameters are linked to disease progression, again emphasizing the relevance of quasispecies for viral pathogenesis (Domingo et al. 2012).

Clinical Impact of Drug-Resistant Viral Mutants

Escape mutants have been reported ever since the first controlled studies with viral populations subjected to antiviral inhibitors were performed (Eggers and Tamm 1965; Melnick et al. 1961). Many examples, both historical and current, have been periodically reviewed [Domingo (1989), Domingo et al. (2012), Richman (1996) and references therein]. Several data banks offer updated information on drug resistance of important viral pathogens.

Here we review, as specific examples, drug-resistance mutations of some clinically relevant viruses such as HIV-1 and HCV.

HIV Variability and Antiretroviral Therapy

HIV is a retrovirus that causes the acquired immunodeficiency syndrome (AIDS) in humans. Currently, there are around 34 million people worldwide infected with HIV. Although HIV-1 strains are responsible for most of the global AIDS pandemic, there are about 1–2 million people infected with HIV type 2 (HIV-2). Genetic variability is one of the hallmarks of HIV. These viruses have high mutation rates (around 10^{-4} to 10^{-5} mutations per nucleotide and replication cycle) and high recombination frequencies (reviewed in (Menéndez-Arias 2009)). This is partly due to the relatively low fidelity of the viral reverse transcriptase that like other polymerases found in RNA viruses is devoid of proofreading activity. In addition, it has been estimated that the minimum duration of the HIV life cycle *in vivo* is only 1.2 days, while the average number of virions produced per day in an infected individual is 1.03×10^{10} (Perelson et al. 1996). These characteristics of the HIV infection are responsible for the generation of complex quasispecies that facilitate the selection of strains resistant to antiretroviral drugs, as discussed in sections "Introduction: Relevance of Quasispecies in Virus Biology" to "Effect of Variations in Population Size and Viral Load".

Six years after the first clinical observation of AIDS in the United States (Centers for Disease Control [CDC] 1981), AZT (3′-azido-3′-deoxythymidine; zidovudine) became the first drug approved for treatment of HIV-1 infection (Fig. 4). AZT is a prodrug that in its triphosphate form is incorporated into the viral genome by the reverse transcriptase, while blocking DNA synthesis due to the absence of a 3′-OH in its ribose ring. For several years, AZT was administered to patients in monotherapy, leading to the selection of resistant HIV-1 strains with amino acid substitutions such as M41L, D67N, K70R, L210W, T215F or T215Y, and K219E or K219Q in the reverse transcriptase [(Larder et al. 1989); reviewed in Menéndez-Arias (2008)]. Other nucleoside analogs (e.g., didanosine, zalcitabine, stavudine, and lamivudine) were approved in the following years, and very often these drugs were prescribed alone after failure of AZT monotherapy due to the emergence of drug-resistant HIV-1. Sequential treatments facilitated the selection of multidrug-resistant viral strains due to accumulation of resistance mutations specific for each drug. On the other hand, the combined use of AZT and didanosine or zalcitabine in untreated patients facilitated the selection of multidrug-resistant HIV-1 variants containing a different set of mutations including A62V, V75I, F77L, F116Y, and Q151M (Shirasaka et al. 1995).

A remarkable breakthrough in the antiretroviral treatment was achieved in 1995 when the first HIV-1 protease inhibitor (i.e., saquinavir) was approved. Highly active antiretroviral therapy (HAART) was then introduced as a combination of two nucleoside analogs (e.g., AZT, lamivudine, etc.) and a protease inhibitor (Gulick et al. 1997; Hammer et al. 1997). By targeting two different steps in the HIV life cycle (i.e., viral genome replication and maturation), it was possible to decrease viral loads below the limits of detection and minimize the impact and emergence of drug resistance. In the following years, the approval of nonnucleoside analog inhibitors of

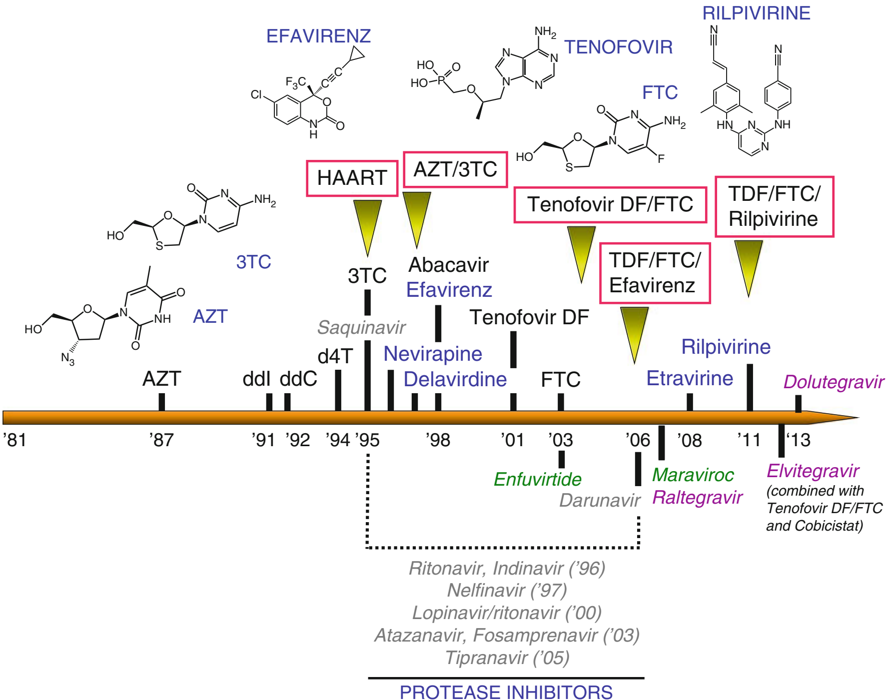

Fig. 4 Timeline for approval of antiretroviral drugs. Nucleoside reverse transcriptase inhibitors are shown in *black*, nonnucleoside reverse transcriptase inhibitors in *blue*, protease inhibitors in *gray*, integrase inhibitors in *purple*, and entry inhibitors in *green*. Abbreviations: 3TC (lamivudine), β-L-(−)-2′,3′-dideoxy-3′-thiacytidine; AZT (zidovudine), β-D-(+)-3′-azido-3′-deoxythymidine; d4T (stavudine), β-D-(+)-2′,3′-didehydro-2′,3′-dideoxythymidine; ddC (zalcitabine), β-D-(+)-2′,3-′-dideoxycytidine; ddI (didanosine), β-D-(+)-2′,3′-dideoxyinosine; FTC (emtricitabine), β-L-(−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine; HAART, highly active antiretroviral therapy; tenofovir DF, tenofovir disoproxil fumarate

HIV-1 reverse transcriptase increased the number of available HAART regimens by allowing novel drug combinations acting on different HIV targets.

Effective Combination Therapies for HIV-1 Infection

Despite their impressive success in reducing AIDS mortality, combination therapies developed in the late 1990s were still problematic due to the poor pharmacokinetic properties of HIV protease inhibitors (very high doses and a large number of pills were needed) and the low genetic barrier to resistance of nevirapine and other nonnucleoside reverse transcriptase inhibitors. In 2006, the introduction of Atripla®, a combination of two nucleoside analogs (tenofovir disoproxil fumarate and emtricitabine) and the nonnucleoside reverse transcriptase inhibitor efavirenz,

constituted a significant accomplishment as it became the standard of care for therapy-naïve patients. With one pill a day, its dosage is optimal and facilitates adherence to antiretroviral therapy. Based on the same principles, other recently approved combinations include a single tablet tenofovir disoproxil fumarate and emtricitabine either with rilpivirine (a nonnucleoside reverse transcriptase inhibitor) or with elvitegravir (an integrase inhibitor which is administered together with cobicistat). Cobicistat is an inhibitor of cytochrome P450 3A enzymes that boosts blood levels of elvitegravir.

The Molecular Basis of Drug Resistance in HIV-1

In addition to the simplification of dosing regiments, drugs used today are more potent and have longer half-lives than compounds used 15 years ago. Furthermore, current regimens have less toxicity and are more tolerable over time. At present, viral suppression using combination therapies is effective and emergence of resistance has been significantly reduced in the clinical setting. However, there are still patients infected with drug-resistant strains that were selected after successive treatments with different antiretroviral drugs or individuals that were infected with drug-resistant HIV-1 (i.e., transmitted drug resistance). In addition, natural resistance to various antiretroviral drugs has been observed in several HIV-1 clades, as well as in HIV-2 (Menéndez-Arias and Álvarez 2014). Therefore, in this scenario, compounds targeting different steps of the virus life cycle are still needed. Currently licensed drugs target (i) viral entry (e.g., maraviroc and enfuvirtide), (ii) reverse transcription (nucleoside and nonnucleoside reverse transcriptase inhibitors), (iii) integration (integrase inhibitors, such as raltegravir, elvitegravir, and dolutegravir), and (iv) viral maturation (protease inhibitors) [reviewed in (Menéndez-Arias 2013)]. A list of amino acid substitutions associated with resistance to antiretroviral drugs is given in Table 1.

Nucleoside reverse transcriptase inhibitors are the backbone of current antiretroviral therapies. Some of those drugs have a relatively low genetic barrier (section "Major Factors in the Generation and Dominance of Drug Resistance in Viruses"). For example, high-level resistance to lamivudine and emtricitabine is conferred by single mutations generating the amino acid substitutions M184I or M184V. These amino acid changes reduce the ability of the reverse transcriptase to incorporate the inhibitor relative to its natural substrates (i.e., dNTPs) [reviewed in Menéndez-Arias (2008)]. On the other hand, at least 2–3 mutations are needed to produce an AZT-resistant HIV-1 strain. The relevant thymidine analog resistance mutations (e.g., M41L, D67N, T215Y, etc.) facilitate the excision of AZT-monophosphate, stavudine-monophosphate, or tenofovir from the 3′ end of blocked DNA primers, in a reaction mediated by ATP and other pyrophosphate donors (Meyer et al. 1999; Tu et al. 2010). The same molecular mechanism operates for HIV variants having reverse transcriptases that contain a dipeptide insertion between codons 69 and 70 and thymidine analog resistance mutations such as M41L or T215Y [reviewed

Table 1 Amino acid substitutions associated with HIV-1 resistance to antiretroviral drugs

Drugs	Amino acid substitutions associated with drug resistance
Nucleoside analog reverse transcriptase inhibitors	
Zidovudine (AZT)	M41L, D67N, K70R, V118I, L210W, **T215F/Y**, K219E/Q
Didanosine (ddI)	K65R, L74V, M184V
Lamivudine (3TC)	(E44D/V118I), K65R, **M184I/V**
Stavudine (d4T)	M41L, D67N, K70R, V118I, L210W, T215F/Y, K219E/Q
Abacavir	K65R, L74V, Y115F, M184V (M41L, D67N, K70R, L210W, T215F/Y, K219E/Q)
Emtricitabine (FTC)	(K65R/Q151M), **M184I/V**
Tenofovir	**K65R**, K70E
Combinations of mutations that confer resistance to various nucleoside analogs	
	(i) M41L, D67N, K70R, L210W, T215F/Y, K219E/Q; (ii) A62V, V75I, F77L, F116Y, Q151M; (iii) insertions between codons 69–70 (i.e., T69SSS or T69SSG or T69SSA), M41L, A62V, K70R, L210W, T215F/Y
Nonnucleoside reverse transcriptase inhibitors	
Nevirapine	L100I, K101P, K103N/S, V106A/M, V108I, Y181C/I, Y188C/L/H, G190A/C/E/Q/S/T
Delavirdine	K103H/N/T, V106M, Y181C, Y188L, G190E, P236L
Efavirenz	L100I, K101P, K103H/N, V106M, V108I, Y188L, G190A/S/T, P225H, M230L
Etravirine	V90I, A98G, L100I, K101E/H/P/Q, V106I, **E138A**/G/K/**Q**/**R**/S, **V179**D/**F**/**I**/**L**, Y181C/I/V, G190A/S, F227C, M230L, T386A, E399D
Rilpivirine	V90I, K101E/P, **E138**A/G/**K**/Q/**R** (±M184I/V), V179F/I/L, Y181C/I/V, Y188L, V189I, H221Y, F227C, M230I/L
Combinations of mutations that confer cross-resistance to nevirapine, **delavirdine and efavirenz**	
	(i) K103N alone; (ii) V106M alone; (iii) Y188L alone; (iv) two or more amino acid changes of the group: L100I, V106A, Y181C/I, G190A/S, M230L, and Y318F
Protease inhibitors	
Saquinavir	L10I/R/V, **G48V**, I54L/V, A71T/V, G73S, V77I, V82A, I84V, **L90M**, and A431V [in the Gag polyprotein cleavage site p7(NC)/p1)]
Ritonavir	L10I/R/V, K20M/R, V32I, L33F, M36I, M46I/L, I54L/V, A71T/V, V77I, **V82A**/**F**/**S**/**T**, **I84V**, L90M, and A431V [in the Gag polyprotein cleavage site p7(NC)/p1)]
Indinavir	L10I/R/V, K20M/R, L24I, V32I, M36I, **M46I**/**L**, I54V, A71T/V, G73A/S, V77I, **V82A**/**F**/**S**/**T**, **I84V**, L90M, and in the Gag cleavage sites: A431V [in p7(NC)/p1)] and L449F [in p1/p6]
Nelfinavir	L10F/I, **D30N**, M36I, M46I/L, A71T/V, V77I, V82A/F/S/T, I84V, N88D/S, **L90M**, and in the Gag cleavage sites L449F and P453L [in p1/p6]
Amprenavir (fosamprenavir)	L10F/I/R/V, V32I, M46I/L, I47V, **I50V**, I54V/M, **I84V**, L90M, and Gag cleavage sites L449F and P453L [in p1/p6]

(*continued*)

Table 1 (continued)

Drugs	Amino acid substitutions associated with drug resistance
Lopinavir	L10F/I/R/V, G16E, K20I/M/R, L24I, **V32I**, L33F, E34Q, K43T, M36I/L, **M46I**/L, **I47A**/V, G48M/V, I50V, **I54L**/**V**/A/M/S/T, Q58E, I62V, L63T, A71T, G73T, T74S, **L76V**, **V82A**/F/S/T, I84V, L89I/M, L90M, and A431V [in the Gag polyprotein cleavage site p7(NC)/p1)]
Atazanavir	L10F/I/V, K20I/M/R, L24I, L33F/I/V, M36I/L/V, M46I/L, G48V, **I50L**, I54L/V, L63P, A71I/T/V, G73A/C/S/T, V82A/F/S/T, **I84V**, **N88S**, L90M
Tipranavir	L10I/S/V, I13V, K20M/R, **L33F**/**I**/**V**, E35G, M36I/L/V, K43T, M46L, I47V, I54A/M/V, Q58E, H69K, T74P, **V82L**/**T**, N83D, **I84V**, L89I/M/V, L90M
Darunavir	V11I, V32I, L33F, I47V, **I50V**, I54L/M, T74P, L76V, V82F, I84V, L89V, and in the Gag cleavage sites A431V [in p7(NC)/p1)] and S451T and R452S [in p1/p6]
Combinations that confer resistance to multiple protease inhibitors	
	L10F/I/R/V, **M46I/L**, I54L/M/V, **V82A/F/T/S**, **I84V**, **L90M**[a]
Fusion inhibitors	
Enfuvirtide	G36D/E/S, I37T/N/V, V38A/E/M, Q40H, N42T, N43D/K/S (all in gp41)
Integrase inhibitors	
Raltegravir	G140S, **Y143C**/**R**, **Q148H**/**K**/**R**, **N155H**
Elvitegravir	T66A/I/K, L74M, E92Q/V, **Q148H**/**K**/**R**, V151L, **N155H**
Dolutegravir	F121Y, E138A/K, G140A/S, Q148H, R263K
CCR5 antagonists	
Maraviroc	Resistance usually develops through the selection of viruses that use the CXCR4 (X4) coreceptor. In addition, maraviroc resistance mutations have been selected in vitro in the V2, V3, and V4 loops of gp120 (Westby et al. 2007)

For additional information, see Clotet et al. (2014), Wensing et al. (2014), and the websites of the International Antiviral Society–USA (http://www.iasusa.org) and the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu)

Major resistance mutations are shown in *bold*. Most protease inhibitors are usually prescribed in combination with a low dose of ritonavir that has a boosting effect on the protease inhibitor concentration in plasma

[a]Multiple protease inhibitor resistance can be achieved through the accumulation of four or five mutations of those indicated in the list

in (Menéndez-Arias et al. 2006)]. The substitution of Gln[151] by Met, considered as the initial step in the Q151M pathway, requires two nucleotide changes. Q151M and accompanying mutations confer resistance by reducing the viral polymerase ability to incorporate nucleoside analogs in the DNA chain.

Classical nonnucleoside reverse transcriptase inhibitors (e.g., nevirapine, delavirdine, and efavirenz) have a very low genetic barrier. Single nucleotide changes occurring at several codons in the reverse transcriptase-coding region can individually confer high-level resistance to those drugs. Interestingly, some of them (notably, K103N) confer cross-resistance to all three drugs. Next-generation inhibitors such as etravirine and rilpivirine are more potent and show a higher genetic barrier to resistance. However, E138K and other substitutions at this position are

sufficient to confer partial resistance to these drugs (Asahchop et al. 2013). Nevertheless, unlike in the case of nevirapine, delavirdine, or efavirenz, two amino acid substitutions are needed to attain high-level resistance in vitro (Azijn et al. 2010; Javanbakht et al. 2010). Rilpivirine is now substituting efavirenz in the most effective antiretroviral drug combinations.

Resistance to HIV protease inhibitors is relatively complex, since for most drugs in this class, high-level resistance involves a relatively large number of amino acid substitutions [for a recent review, see Menéndez-Arias (2013)]. Major mutations associated with resistance map within the substrate/inhibitor binding site (e.g., D30N, G48V, V82A, I84V, etc.). These amino acid changes usually have a significant impact on the viral replication capacity. Secondary mutations that are selected later during treatment increase viral fitness and usually locate out of the substrate binding site. In some cases, these amino acid substitutions have an impact on protease stability (e.g., L10I or A71V) (Chang and Torbett 2011). Further viral fitness recovery during treatment can be facilitated by mutations occurring at the viral polypeptide substrates cleaved by the HIV protease (e.g., at Gag cleavage sites NC/p1 and p1/p6). These mutations facilitate viral polyprotein processing by improving Gag susceptibility to protease cleavage.

Approved integrase inhibitors bind to the catalytic domain of the enzyme blocking its strand transfer activity. Resistance to raltegravir and elvitegravir is associated with single amino acid substitutions (usually Q148K/R/H, but also N155H). Integrase inhibitors have been recently combined with nucleoside analogs in HAART regimens. Interestingly, the latest integrase inhibitor approved for treatment (i.e., dolutegravir) shows a surprisingly high genetic barrier. In phase III clinical trials, approximately 88 % of the patients treated with dolutegravir and two nucleoside reverse transcriptase inhibitors attained viral load suppression to <50 copies of RNA/ml, without developing drug-resistance-associated mutations after 48 weeks of treatment (Raffi et al. 2013; Wainberg et al. 2013). It is possible that development of dolutegravir resistance mutations may result in viruses with greatly diminished replicative capacity, thereby constituting a major barrier towards the development of resistance.

Other drugs used in antiretroviral rescue therapy include entry inhibitors targeting either the step involving the recognition of the viral coreceptor (CCR5 antagonists) or fusion inhibitors (enfuvirtide). Enfuvirtide is a largely helical polypeptide that interferes with the packaging of HIV-1 gp41 α-helical segments required for the fusion of the viral envelope and the cell membrane. Resistance is achieved by mutations in gp41 that encode amino acid changes that disrupt interactions between α-helices in the transmembrane protein and enfuvirtide (Greenberg and Cammack 2004). On the other hand, maraviroc is a CCR5 antagonist. This drug binds to a pocket in the chemokine receptor and makes it unavailable for the HIV-1 surface glycoprotein gp120. Viral strains resistant to maraviroc may still infect the host by using other chemokine receptors (e.g., CXCR4) (Westby et al. 2006). In addition, resistance to maraviroc mediated by specific amino acid substitutions in the V3 loop of gp120 allows HIV-1 to continue using CCR5 coreceptors, even in the presence of bound maraviroc (Westby et al. 2007). This use of a drug-bound coreceptor

illustrates that viruses have multiple resources to overcome a selective pressure intended to limit their replication and that even drugs that target a cellular function are not free of the problem of selection of virus-escape mutants (see section "Conclusions and New Prospects for Antiviral Therapy").

HCV Variability and Current Therapy

HCV is a member of the *Flaviviridae* family affecting approximately 170 million individuals worldwide. HCV shows a very high variability which is mainly due to the absence of proofreading activity of the RNA polymerase and very high rate of virion production, approximately 10^{12} per infected individual per day (Neumann et al. 1998). For over ten years, the standard of care treatment for HCV infection was a combination of pegylated interferon-α (IFN-α) and ribavirin, which achieved viral eradication in 40–50 % of patients infected with HCV genotype 1 and 80 % in those infected with genotypes 2 and 3 (Quer et al. 2008; Shiffman 2008). Unfortunately, the use of IFN-α and ribavirin results in moderate to severe side effects in many patients. The approval of telaprevir and boceprevir in 2011 for the treatment of chronic HCV infection was a major breakthrough in the field of anti-HCV therapy. Therapy consisting of a protease inhibitor combined with IFN-α and ribavirin for HCV genotype 1 patients significantly increased sustained virological response (SVR) rates compared with IFN-α and ribavirin treatment alone and reduced the rate of selection of resistant variants (Bacon et al. 2011; Jacobson et al. 2011; Poordad et al. 2011; Zeuzem et al. 2011). There are currently several new compounds targeting various HCV proteins that have been or will soon be added to the arsenal of drugs available for new combination therapies, that might render possible the implementation of IFN-free regimens (deLemos and Chung 2014; Lange and Zeuzem 2013). The newest direct-acting antiviral agents (DAAs) are candidates to be included in these regimens, such as second-generation NS3, NS5A, and viral polymerase (NS5B) inhibitors. However, appropriate combinations of these inhibitors must be selected to avoid cross-resistance and overcome problems associated with low barrier of resistance to individual drugs.

Resistance to Interferon-α and to Ribavirin

The administration of exogenous IFN-α exerts antiviral effects via activation of innate immunity. It is not clear why some patients respond differently to IFN–αbased treatments, though several host factors (gender, age, ethnicity, obesity, etc.) have been implicated. Sequence polymorphisms within the IL28B locus (IFN-λ3) have been linked to variations in the virological response to IFN-α-based therapy (Ge et al. 2009). Particularly difficult cases are null responders to previous treatment with IFN-α and ribavirin, those infected by certain HCV genotypes, patients coinfected with HIV-1, or those with advanced liver fibrosis (Lange and Zeuzem 2013). The number of escape routes that a virus may use to avoid suppression by

antivirals depends on the complexity of the response exerted by the drugs. For DAAs that target specific viral proteins, resistance often depends on one or a few key amino acid substitutions. In contrast, the pluricomponent antiviral response exerted by IFN-α affects multiple cell signaling pathways, which may explain why IFN-α resistance has been linked to several HCV genes (Kozuka et al. 2012; Perales et al. 2013, 2014; Serre et al. 2013). Among other examples, the viral protease NS3/4A cleaves mitochondrial antiviral signaling (MAVS) and TIR-domain-containing adaptor-inducing interferon-β (TRIF), interrupting signal transduction via retinoic acid-inducible gene-I (RIG-I), toll-like receptor 3 (TLR3), and protein kinase R (PKR) response pathways. Additionally, HCV core protein has been linked to decreased signaling via Jak-STAT, resulting in decreased expression of interferon-stimulated genes (ISGs) (Horner and Gale 2013).

Another unsettled issue is whether resistant variants with IFN-α-specific mutations are directly responsible for treatment failure. Though sequence analysis of HCV from patients failing treatment with IFN-α and ribavirin has been performed, no consensus amino acid changes have been associated with genotype-specific IFN-α response. However, variations detected in broad regions of core, E2, and NS5A have been correlated with treatment outcome (Chayama and Hayes 2011; Enomoto et al. 1996; Pawlotsky et al. 1998). This is consistent with the results of *in vitro* studies, where HCV passaged in the presence of IFN-α has selected multiple substitutions throughout the viral genome (Perales et al. 2013, 2014; Serre et al. 2013). Interestingly, the mutations seen in patients failing therapy are not the same as those observed after *in vitro* selection in the presence of IFN-α (Kozuka et al. 2012; Perales et al. 2013, 2014).

There seems to be a strong link between enhanced fitness and IFN-α resistance *in vitro*, making it more difficult to distinguish *bona fide* IFN resistance from cell culture adaptation. Thus, the need of HCV to cope with multiple ISG proteins renders IFN-α resistance a far more complex issue than resistance to standard antiviral inhibitors that target a specific viral protein (Perales et al. 2014). In the serial passages of HCV to select IFN-α-resistant mutants, it was observed that virus that had been passaged in human hepatoma Huh-7.5 cells in the absence of IFN-α also acquired partial resistance to IFN-α (Perales et al. 2013). Further studies with the multiply passaged populations documented that the partial resistance extended to several DAAs and ribavirin, despite the virus not having been exposed to the drugs. Mutant spectrum analyses and the kinetics of progeny production by serially diluted populations and by individual clones excluded that drug resistance was associated with the presence of drug-escape mutants in the multiply passaged populations. The results established viral fitness as a multidrug-resistance factor in HCV (Sheldon et al. 2014).

The inclusion of ribavirin in combination therapies increased the rates of SVR compared with treatment using IFN-α alone, though the mechanism is not fully understood (Sostegni et al. 1998). Several antiviral mechanisms of ribavirin have been described: (i) immunomodulation and enhancement of the Th1 antiviral immune response, (ii) upregulation of genes involved in IFN signaling, (iii) inhibition of viral RNA-dependent RNA polymerases, (iv) depletion of intracellular GTP

levels, (v) inhibition of mRNA cap formation, and (vi) lethal mutagenesis. Although the precise mechanism (or combination of mechanisms) of ribavirin-mediated viral inhibition during anti-HCV therapy has not been elucidated, several lines of evidence suggest that lethal mutagenesis is involved (Asahina et al. 2005; Cuevas et al. 2009; Dietz et al. 2013; Dixit et al. 2004; Lutchman et al. 2007). The ribavirin-induced bias in the mutant spectrum (an excess of G-A and C-U transitions), which reflects the mutagenic activity of ribavirin, has been observed both *in vivo* (Dietz et al. 2013) and in cell culture (Ortega-Prieto et al. 2013). In general, resistance mutations against a mutagen are less frequent than for classical inhibitors. The first identification of a ribavirin-resistance mutation (F415Y in NS5B) in HCV was during ribavirin monotherapy in patients (Young et al. 2003). Experiments with HCV replicon containing cell lines showed that ribavirin resistance occurred by changes in the cell lines (the resistant cell lines were defective in ribavirin import) or from mutations in NS5A (G404S and E442G) (Ibarra and Pfeiffer 2009; Pfeiffer and Kirkegaard 2005b). Additionally, serial passage of a genotype 2a replicon in the presence of ribavirin resulted in reduced sensitivity to the drug, and NS5B mutation Y33H was determined to be responsible, presumably due to a decrease in replicative fitness (Hmwe et al. 2010). Passage of infectious J6/JFH1 of HCV in the presence of ribavirin yielded a resistant virus with many mutations, but the responsible mutation was not identified (Feigelstock et al. 2011).

Resistance to Directly Acting Antiviral Agents (DAAs)

The number of HCV antiviral drugs under development has increased greatly over the past few years, with many drugs now approved by the US Food and Drug Administration and more in late-phase clinical trials. These DAAs are taking a more central role in therapy, with the aim of shortening treatment duration and avoiding IFN in standard of care therapy.

Telaprevir and boceprevir were the first two DAAs to be approved for use in anti-HCV therapy. However, despite their exceptionally potent antiviral activity, use of these first-generation inhibitors of the NS3/4A protease results in the rapid selection of resistance mutations and viral “breakthrough” of monotherapy (Sarrazin et al. 2007; Susser et al. 2009). *In vitro* studies have identified many single amino acid changes associated with reduced sensitivity to protease inhibitors, indicating a low barrier to resistance that has also been evidenced in clinical trials (Lange and Zeuzem 2013; Thompson et al. 2011). The ease of crossing the resistance barrier can be partly explained by the structural characteristics of the NS3/4A protease active site. Only a few side-chain interactions are needed for the binding of inhibitors to the greatly exposed protease active site (Romano et al. 2010). There are mutations at key positions in NS3 (Arg^{155}, Ala^{156}, and Asp^{168}) that make HCV resistant to nearly all protease inhibitors (Sarrazin and Zeuzem 2010; Thompson et al. 2011; Wyles 2013). Some newer protease inhibitors, such as MK-5172, have shown increased potency against variants containing Arg^{155} mutations (Summa et al. 2012). Most of the protease inhibitors currently in use were developed to target the NS3 protease

domain of genotype 1 HCV. Due to differences in NS3 amino acid sequence, the efficacy of protease inhibitors in genotypes 2–6 is decreased (Lange et al. 2010). As mentioned in section "Major Factors in the Generation and Dominance of Drug Resistance in Viruses" above, there is also a difference in susceptibility to NS3/4A inhibitors based on HCV subtype, due to differences in nucleotide sequence at key amino acid-coding positions (McCown et al. 2009).

Daclatasvir was the first NS5A inhibitor to be used in clinical trials. NS5A inhibitors show a very potent antiviral activity across all genotypes due to conservation of targeted domains, but the barrier to resistance is relatively low, as for protease inhibitors. Substitutions at positions Met^{28}, Gln^{30}, Leu^{31}, Pro^{32}, and Tyr^{93} are frequently selected by this class of inhibitor (Gao 2013; Halfon and Sarrazin 2012; Nakamoto et al. 2014; Nettles et al. 2011). Similar to protease inhibitors, the viral genotype was found to influence the rate of resistant mutant selection, with virus from patients infected with genotype 1b being less likely to acquire resistance than virus from genotype 1a-infected patients (Fridell et al. 2011; Nettles et al. 2011).

In contrast, nucleoside analog NS5B inhibitors display high antiviral activity, broad genotype coverage, and relatively high barrier to resistance. Nucleoside inhibitors of NS5B are analogs of the polymerase substrates and bind directly to the NS5B active site. Importantly, because the active site of NS5B is highly conserved, nucleoside analogs have similar efficacy across all HCV genotypes. While amino acid substitutions resulting in weak resistance to nucleoside analog are readily selected, the resulting loss of replicative fitness limits breakthrough (McCown et al. 2008; Sarrazin and Zeuzem 2010). This contrasts with substitutions selected by NS3/4A protease and nonnucleoside inhibitors, which have greater resistance and do not profoundly affect replication capacity (Pawlotsky 2009). Sofosbuvir is an approved pyrimidine-derived nucleoside analog NS5B inhibitor that may be of great importance in future IFN-free treatment regimens. The barrier to resistance is relatively high, as only a few NS5B mutations have been confirmed to confer resistance [S282T (Sofia et al. 2010); L159F/L320F (Tong et al. 2014; Donaldson et al. 2014)].

Ongoing clinical trials with newly approved DAAs aim at finding effective, IFN-free combinations applicable to all HCV genotypes, which is challenging due to the continuing diversification of HCV in nature and the many escape routes that viruses find to combat drugs. To avoid rapid selection of cross-resistant mutant populations of HCV, combinations of inhibitors should be directed against several viral genes simultaneously (Lange and Zeuzem 2013). This concept is supported by the known sensitivity of viruses with protease inhibitor-resistance mutations to other classes of DAAs (such as NS5A, NS5B, and cyclophilin inhibitors) (Thompson et al. 2011) and the accumulated experience with treatments against HIV-1 infections (section "Effective Combination Therapies for HIV-1 Infection"). In a study where treatment-naïve HCV genotype 1a and 1b patients were treated with mericitabine, danoprevir (NS3/4A inhibitor), and ribavirin, viral breakthrough was mainly associated with NS3/4A resistance mutants while specific resistance mutations in NS5B were obtained in a single patient (Lange and Zeuzem 2013). In clinical trials with patients infected by genotype 1 HCV treated with a combination of the NS5A

inhibitor ledipasvir and sofosbuvir, high rates of SVR were achieved (>94 %), regardless of prior treatment history (Afdhal et al. 2014a, b). Combination therapy using the NS5A inhibitor daclatasvir with sofosbuvir resulted in SVR rates of 98 %, 92 %, and 89 % in patients infected by genotypes 1, 2, and 3, respectively (Sulkowski et al. 2014). Resistance mutations present in patients were limited to known NS5A resistant variants, and no sofosbuvir-specific mutations were observed. Thus, the combination of inhibitors used in IFN-free therapies must be chosen carefully in order to minimize risk of breakthrough resistance. It is difficult to anticipate to what extent a wider use of IFN-free treatments will affect HCV evolution and select for new drug-resistance mutations that will acquire epidemiological relevance (as was the case with HIV-1). It will largely depend on the administration of the new combinations to poorly responding patients, who provide environments that are prone to select for escape mutants, and also on the fitness and frequency of transmission of the newly generated mutants.

Due to the high basal mutation frequency in natural HCV populations, it may be important to evaluate the presence of preexisting resistance mutations within patient HCV quasispecies. Presence of a naturally occurring variant of genotype 1a (Q80K) has been associated with decreased SVR after simeprevir-based triple therapy (Forns et al. 2014). When resistant mutants emerge after treatment failure, it is not well known how long they remain in the population and whether they can impact future therapy. Studies using population sequencing techniques have revealed a rapid loss of detection of resistant variants (Mauss et al. 2014) although in other studies, sequencing detected resistant variants even several years after treatment with telaprevir or boceprevir (Susser et al. 2011). Furthermore, increased failure of simeprevir-based triple therapy was observed after re-treatment of patients who had developed simeprevir resistance previously during monotherapy, likely an effect of persistent resistance variants within the viral population (Lenz et al. 2012). The analysis of resistance mutations within the quasispecies both at baseline and after failure of IFN-free regimens will guide future selection of inhibitor combinations to be used.

Conclusions and New Prospects for Antiviral Therapy

High mutation rates and quasispecies dynamics confer to RNA viruses an adaptive potential that may be counteracted using five main strategies: (i) combination therapy (i.e., HAART for AIDS, as has been described in section “HIV Variability and Antiretroviral Therapy”), (ii) splitting of the treatment into a first induction regimen (to decrease the number of viral mutants and viral load) and a second maintenance regimen (to maintain a viral load sufficiently low) (von Kleist et al. 2011), (iii) targeting of cellular proteins (taking advantage of viral reliance on host cell functions) (Geller et al. 2007; Hopkins et al. 2010; Kumar et al. 2011), (iv) combined use of immunotherapy and chemotherapy (in order to stimulate a broad adaptive immune response) (Li et al. 2005; Seiler et al. 2000; Webster et al. 1986), and (v) lethal mutagenesis [Domingo et al. (2012) and references therein].

It has been proposed that the targeting of cellular functions should limit selection of viral escape mutants. This is not necessarily the case, as evidenced by selection of mutations in NS5A that were associated with resistance to cyclosporine A (Chatterji et al. 2010; Delang et al. 2011) and maraviroc-resistant HIV-1 mutants (section "The Molecular Basis of Drug Resistance in HIV-1"). Moreover, such agents may produce side effects derived from the perturbation of cellular functions. However, the ability of these compounds to simultaneously inhibit the replication of multiple virus types may increase their therapeutic potential (Pawlotsky 2014).

Lethal mutagenesis aims at extinguishing viruses by increasing mutation rates via administration of mutagenic agents. Population behavior of RNA viruses is strongly influenced by interactions among viral genomes within the mutant spectra. Thus, the generation of defective viral genomes (as a consequence of increased mutagenesis) and the collapse of the whole ensemble due to interfering interactions with the replication of the standard virus are consistent with the features of viral quasispecies (Grande-Pérez et al. 2005; Perales et al. 2007). A first clinical trial using a mutagenic nucleoside analog was conducted against HIV-1 in AIDS patients, showing that lethal mutagenesis could be effective *in vivo* (Mullins et al. 2011). Therapies involving inhibitors and mutagenic agents should consider the mechanism of action of both drugs due mainly to two reasons: (i) defective mutants (generated by the mutagen) should be replication-competent to exert their interfering activity and this is impeded in the presence of an inhibitor and (ii) due to the mutagen-induced error rate, the selection of inhibitor-escape mutants could be favored when both drugs are administered simultaneously, and this probability will increase with the viral load (Iranzo et al. 2011; Perales et al. 2009, 2012). In light of this, sequential therapies with a first phase of viral load reduction (via a combination of inhibitors) followed by a second phase of increased mutagenesis deserve further investigation. From a general perspective, such explorations of new treatment designs will become even more justified if, as can be anticipated, the new combinations fail to eradicate current and emerging pathogenic viruses worldwide.

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Substrate-Envelope-Guided Design of Drugs with a High Barrier to the Evolution of Resistance

Ayşegül Özen and Celia A. Schiffer

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Abstract

Drug resistance is a major problem in quickly evolving diseases. One way for drug resistance to emerge is through mutations in the drug target under the selective pressure of therapy. Antiviral drug targets especially have a high mutational plasticity due to the diverse genetic viral population. An ideal antiviral inhibitor should be robust against these quasispecies. Fortunately, a therapeutic target can be evolutionarily constrained by the biological function, which limits the mutational space. Taking advantage of this evolutionary constraint, the substrate-envelope hypothesis quantitatively defines the balance between natural substrate recognition and inhibitor binding and provides a framework to design robust inhibitors that retain potency against mutational ensemble of quasispecies of the target. The Substrate envelope hypothesis, based on structural studies on

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the proteases of HIV-1 and hepatitis C, provides a structural basis for the specificity of natural substrate recognition and mechanisms for the resistance mutations in the active site.

Keywords
Antiviral • HCV NS3/4A • HIV-1 protease • Crystallography • Molecular dynamics • Substrate envelope • Structure-based drug design

Introduction

Antiviral drug targets are prone to mutations under the selective pressure of drug therapies. These mutations contribute to drug resistance by reducing the activity of inhibitors while allowing the drug-resistant variant of the target to function on the native substrates. The delicate balance between inhibitor binding and substrate recognition is effectively altered by drug resistance mutations at the expense of the inhibitor. The substrate-envelope hypothesis provides the structural basis for this alteration. This review provides a general background on the evolution of drug resistance in viral proteases, specifically in human immunodeficiency viral protease and hepatitis c viral protease, NS3/4A. The general applicability of the substrate-envelope hypothesis to other systems is discussed and a framework for substrate-envelope-guided drug design is outlined to minimize the probability of drug resistance in the design of new inhibitors.

Evolution of Resistance Against Anti-HIV Drugs

Human immunodeficiency virus (HIV) is a lentivirus of the Retroviridae family that infects the human immune system and causes the acquired immunodeficiency syndrome (HIV/AIDS). HIV is a quickly evolving disease that, without effective treatment, results in serious medical, social, and economic burden. UNAIDS reports that 35.3 million people were living with HIV globally with 2.3 million new infections and 1.6 million people died from AIDS-related causes by the end of 2012 (Global report: UNAIDS report on the global AIDS epidemic 2013). HIV has two types and several clades within each type with distinct patterns of spread and progression to AIDS (Santos and Soares 2010). HIV type 1 (HIV-1) is responsible for the pandemic. Because HIV-1 cannot be cured, suppressing viral replication and maintaining viral load at low to undetectable levels have become critical goals in the field of HIV-1 research. Highly active antiretroviral therapy (HAART) has been a successful strategy in providing long, quality life for infected individuals and is the current global standard of care for HIV/AIDS patients (Palella et al. 1998; Hoggs et al. 1998). As a part of HAART, the US Food and Drug Administration (FDA) has approved more than 30 drugs that target various stages of viral replication cycle including fusion and entry, reverse transcription, and integration and proteolytic

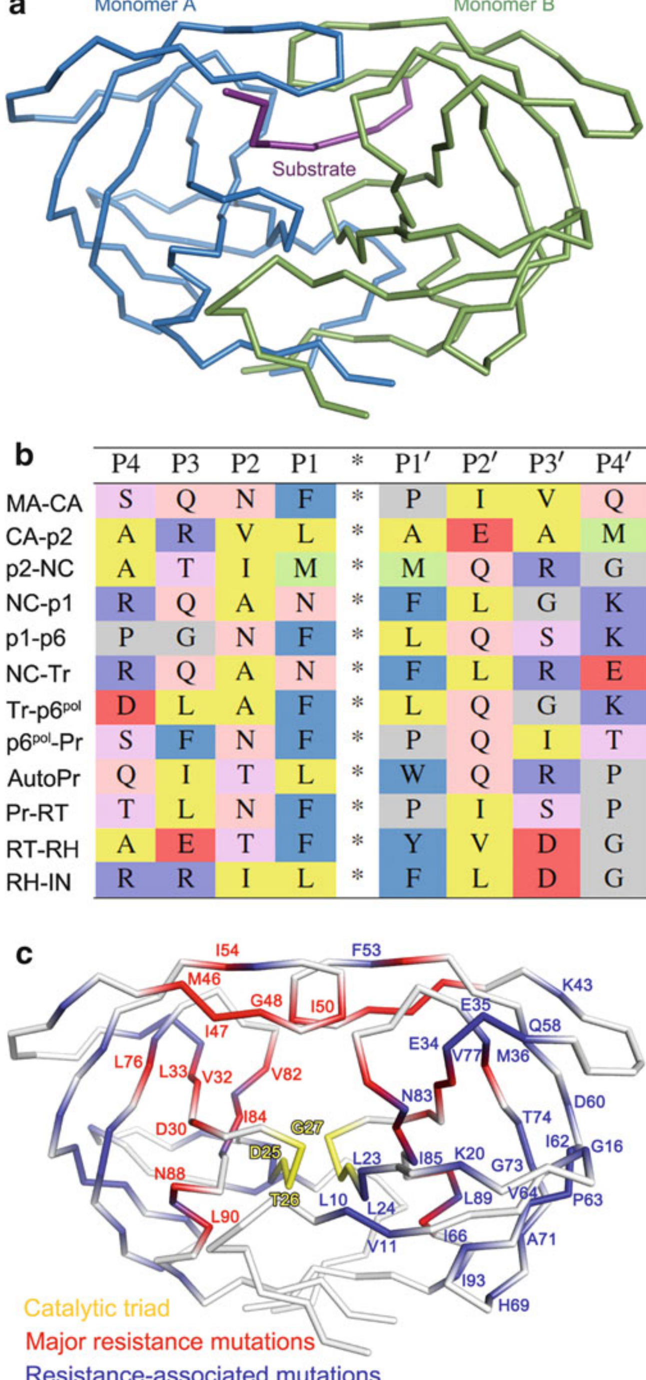
processing of viral polyproteins. However, a high frequency of random nucleotide misincorporation by the error-prone reverse transcriptase (about three mutations per virion per round of replication) and a huge reservoir of replicating virus (10^{10} infected cells in an average patient) diversify the viral population (Coffin 1995). The selective pressure of therapy, especially combined with low drug adherence, facilitates the emergence of drug resistance viral variants (Ali et al. 2010).

HIV-1 Protease: A Virally Encoded Protease as a Drug Target

Viral genome is translated as polyproteins, which are proteolytically processed by the virally encoded protease to yield functional and structural proteins. Due to this crucial role in the viral life cycle, HIV-1 protease has been a key drug target in the treatment of HIV/AIDS (Kohl et al. 1988). HIV-1 protease is an aspartyl protease and a symmetric homodimer of 99 amino acids each (Fig. 1a). Each monomer contains a flap comprising two antiparallel β-strands connected by a β-turn and situated on top of the catalytic site. Dimeric enzyme is stabilized by four antiparallel β-strands, two from each subunit, which form an interdigitated β-sheet. Substrates are hydrolyzed at the dimer interface. The active site is typically considered as the residues 25–32, 47–53, and 80–84 with each monomer contributing a catalytic triad (Asp-25/Thr-26/Gly-27). Accurate and precise processing of the viral polyproteins is critical for virion assembly and maturation; therefore, HIV-1 protease cleaves the Gag and Gag-Pol polyproteins at twelve known sites in a highly specific order. While hydrophobic residues are favored at P1/P1′ residues, between which the scissile bond is hydrolyzed, in general, the cleavage sites are nonhomologous in sequence and asymmetric in size and charge (Fig. 1b). The fact that the protease is symmetric and the cleavage sites are diverse and asymmetric has challenged complete understanding of the specificity determinants of substrate recognition using a sequence-based approach.

Development of HIV-1 PIs is regarded as a major success of structure-based rational drug design. Nine protease inhibitors (PIs) have been so far approved for clinical use: saquinavir (SQV) (Roberts et al. 1990), indinavir (IDV) (Dorsey et al. 1994), ritonavir (RTV) (Kempf et al. 1995), nelfinavir (NFV) (Kaldor et al. 1997), amprenavir (APV) (Kim et al. 1995), lopinavir (LPV) (Sham et al. 1998), atazanavir (ATV) (Robinson et al. 2000a), tipranavir (TPV) (Turner et al. 1998), and darunavir (DRV) (Fig. 1d) (De Meyer et al. 2005; Koh et al. 2003; Surleraux et al. 2005). All PIs, except for TPV, are peptidomimetics. These PIs were rationally designed to bind to the protease with the flaps of the enzyme tightly closed over the active site, mimicking the transition state between substrate binding and cleavage reaction and thereby effectively inactivating the enzyme.

As PIs are an essential component of HAART (Gulick et al. 2000, Bartless et al. 2001), drug resistance to PIs has become an issue in the failure of HAART. Mutations at almost half of the protease residues are selected in different combinations with drug treatment and some combinations confer drug resistance (Wu et al. 2003; Rhee et al. 2003) (Figure 1c). Primary mutations in the active site



	P4	P3	P2	P1	*	P1′	P2′	P3′	P4′
MA-CA	S	Q	N	F	*	P	I	V	Q
CA-p2	A	R	V	L	*	A	E	A	M
p2-NC	A	T	I	M	*	M	Q	R	G
NC-p1	R	Q	A	N	*	F	L	G	K
p1-p6	P	G	N	F	*	L	Q	S	K
NC-Tr	R	Q	A	N	*	F	L	R	E
Tr-p6pol	D	L	A	F	*	L	Q	G	K
p6pol-Pr	S	F	N	F	*	P	Q	I	T
AutoPr	Q	I	T	L	*	W	Q	R	P
Pr-RT	T	L	N	F	*	P	I	S	P
RT-RH	A	E	T	F	*	Y	V	D	G
RH-IN	R	R	I	L	*	F	L	D	G

Fig. 1 (continued)

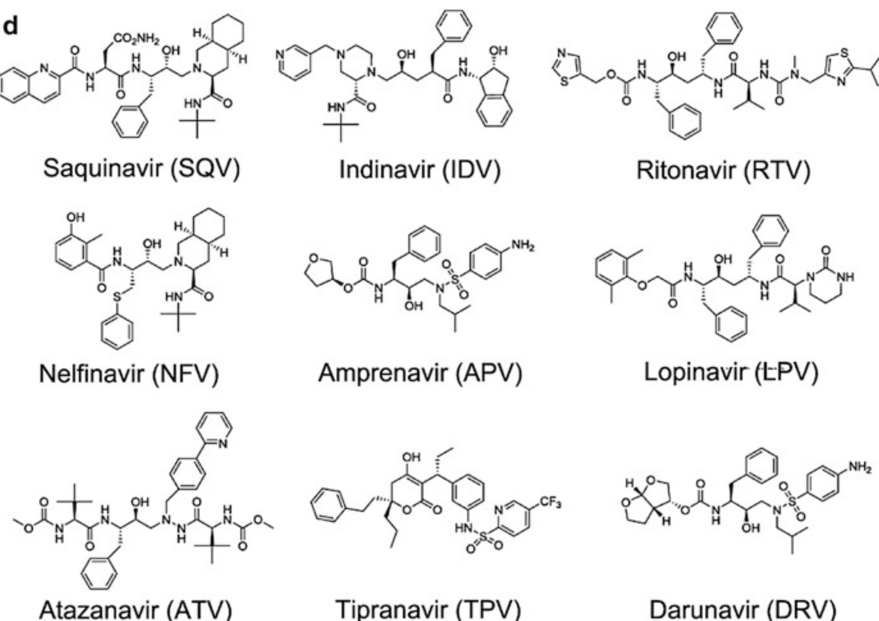


Fig. 1 (**a**) HIV-1 protease is a homodimeric aspartyl protease, shown in ribbon. Identical monomers A and B are colored in *blue* and *green*. Substrate-binding region is located at the dimer interface, and a bound-substrate peptide is colored in magenta. (**b**) HIV-1 protease recognizes 12 sites on Gag and Gag-Pol polyproteins and cleaves the scissile bond between P1 and P1' residues. (**c**) More than half of the protease gene mutates under the selective pressure of protease inhibitor involving therapies. Major drug resistance mutations and resistance-associated mutations are colored *red* and *dark blue*, respectively. Major drug resistance mutations are labeled on monomer A, while resistance-associated mutations are labeled on monomer B. The catalytic triad, at the dimeric interface, is colored *yellow*. (**d**) FDA-approved drugs targeting HIV-1 protease

reduce both protease catalytic efficiency and viral replicative capacity (Martinez-Picado et al. 2000, 1999; Croteau et al. 1997; Bleiber et al. 2001). Major PI resistance mutations occur at residues 30, 32, 33, 46, 47, 48, 50, 54, 76, 82, 84, 88, and 90, while mutations at residues 10, 11, 16, 20, 23, 24, 34, 35, 36, 43, 53, 58, 60, 62, 63, 64, 66, 69, 71, 73, 74, 77, 83, 85, 89, and 93 were reported to be selected in PI-treated patients, and some were shown to contribute to resistance (HIV Databases; Johnson et al. 2013). Among the major resistance-causing mutations, D30N, V32I, I47V/A, G48V/M, I50V/L, V82A/F/T/S/L, and I84V are located in the active site, while L33F, M46I/L, I54V/T/A/L/M, L76V, N88S/D, and L90M are non-active site mutations.

Mutations in HIV-1 protease, either within or outside the active site, can contribute to drug resistance directly by impacting inhibitor binding or indirectly in an interdependent and cooperative manner. Most primary mutations in the active site reduce binding affinity of PIs. On the contrary, some non-active site mutations are located in the hydrophobic core of the protein (13, 24, 33, 36, 62, 66, 77, 85, 90, 93) and contribute to resistance by altering the exchange dynamics of the hydrophobic

interactions within the core (Foulkes-Murzycki et al. 2007; Mittal et al. 2012). While certain primary resistance mutations are a signature of particular PIs, cross-resistance is an issue in HIV-1 PIs. For example, D30N is a nonpolymorphic NFV-selected mutation which confers phenotypic and clinical resistance to NFV (Rhee et al. 2003; Patick et al. 1998); however, I50L is selected with ATV treatment and confers high-level ATV resistance while significantly increasing susceptibility to the rest of the PIs (Colonno et al. 2004). On the contrary, I50V is selected in APV-, LPV-, and DRV-treated patients and reduces the efficacy of these PIs while increasing TPV efficacy (HIV Databases). V82A is selected primarily by IDV and LPV (Condra et al. 1996; Kantor et al. 2005). In addition to decreasing susceptibility to IDV and LPV, V82A also confers cross-resistance to ATV and NFV and is associated with decreased susceptibility to SQV and APV in combination with other mutations (Condra et al. 1996; Kempf et al. 2001). I84V is a very severe mutation that is selected by each of the available PIs and cause cross-resistance to most PIs (Rhee et al. 2003; HIV Databases). Similarly, G48V is a primary resistance mutation selected by SQV and less often IDV and LPV conferring high-level resistance to SQV, intermediate resistance to ATV, and low-level resistance to NFV, IDV, and LPV (Rhee et al. 2003; Kantor et al. 2002; Schapiro et al. 1996; Rhee et al. 2010). Mutations have been selected at either single or a combination of sites. The mechanisms by which resistance is conferred via these mutations are very complex and interdependent. Nevertheless, in addition to the accumulation of resistance mutations within the active site, mutations also develop in non-active site protease residues and within the substrate cleavage sites, predominantly at NC-p1 and p1-p6 sites, altering the susceptibility to various PIs (Zhang et al. 1997; Bally et al. 2000; Mammano et al. 1998; Maguire et al. 2002; Kolli et al. 2009). Evolution of mutations within the cleavage sites leads to not only improved viral fitness compared to the viral variants carrying protease resistance mutations (Zhang et al. 1997; Mammano et al. 1998; Doyon et al. 1996; Robinson et al. 2000b) but also often increased resistance (Kolli et al. 2009). The vast number of mutation sites in both the protease and substrates with several possibilities of amino acid substitutions at each site in combination with cross-resistance has proven drug resistance a very complex problem.

The Substrate-Envelope Hypothesis

HIV-1 protease is a structurally well-studied drug target with more than 600 entries in the Protein Databank as of December 2013 (Berman et al. 2000). A vast majority of these entries are co-crystal structures of small-molecule inhibitors with HIV-1 protease variants including drug-resistant forms. These structural studies shed light on the molecular mechanisms by which protease mutations render inhibitors less effective; however, investigating only the inhibitor complexes has not been sufficient as a rational drug design strategy to minimize the likelihood of emerging resistance mutations.

Resistance to available drugs occurs when the balance in molecular recognition is subtly altered. Drug-resistant variants of HIV-1 protease are no longer effectively blocked by the competitive inhibitors but are still active against the natural substrates efficient enough for viral survival. This observation leads to the assumption that the native function of the protease imposes an evolutionary constraint under the selective pressure of drug therapy. To discover robust drugs that can resist drug resistance, the balance between natural substrate recognition and inhibitor binding needed to be characterized at the molecular level.

The substrate-envelope hypothesis, which was established on the basis of crystallographic studies on HIV-1 protease and later shown to be valid for HCV NS3/4A protease, provides a structural explanation for the specificity determinants of natural substrate recognition and drug resistance upon primary mutations in the protease active site. According to the substrate-envelope hypothesis, the inhibitors that are better at mimicking the natural substrate-binding features are less susceptible to the rapidly emerging mutations populated upon drug treatment. In this section, crystallographic studies that lead to the substrate-envelope hypothesis are described in detail, focusing on the substrate specificity and drug resistance in HIV-1 protease. In addition, parallels in the molecular basis of resistance against HIV-1 and HCV NS3/4A protease inhibitors are highlighted, and the up-to-date evidence suggesting that mutational ensembles of NS3/4A protease can also be targeted rationally taking a substrate-envelope-based drug design approach.

Structural Basis of Substrate Specificity and Drug Resistance

HIV-1 protease, a symmetric enzyme, specifically recognizes diverse asymmetric sequences on the Gag and GagProPol (Prabu-Jeyabalan et al. 2000). Amino acid sequence alone is not the specificity determinant for asymmetric substrate recognition, but the substrates share a binding mode in an extended conformation (Prabu-Jeyabalan et al. 2002). Co-crystal structures of decameric peptides corresponding to the cleavage sites showed that HIV-1 protease recognizes a consensus shape in substrates, not necessarily a consensus sequence (Prabu-Jeyabalan et al. 2002). This consensus shape is defined by the volume adopted by the majority of the substrates within the protease active site and has been defined as the *substrate envelope* (Fig. 2a). According to the substrate-envelope hypothesis, the substrate envelope is the recognition motif for HIV-1 protease, and the cleavage sites within Gag that are able to adopt this shape are likely to be processed.

HIV-1 PIs that have been approved for clinical use are all low-molecular-weight compounds with fairly similar three-dimensional shape and electrostatic character, and they all have large, hydrophobic moieties that interact with the mainly hydrophobic S2-S2′ pockets in the binding site. In the co-crystal structures, most HIV-1 PIs adopt a very similar binding mode interacting with a common set of protease residues in the active site. The inhibitors were shown to occupy a consensus inhibitor volume within the binding site, termed the *inhibitor envelope* (Fig. 2) (King et al. 2004a). Based on the structural comparison of the inhibitor and substrate

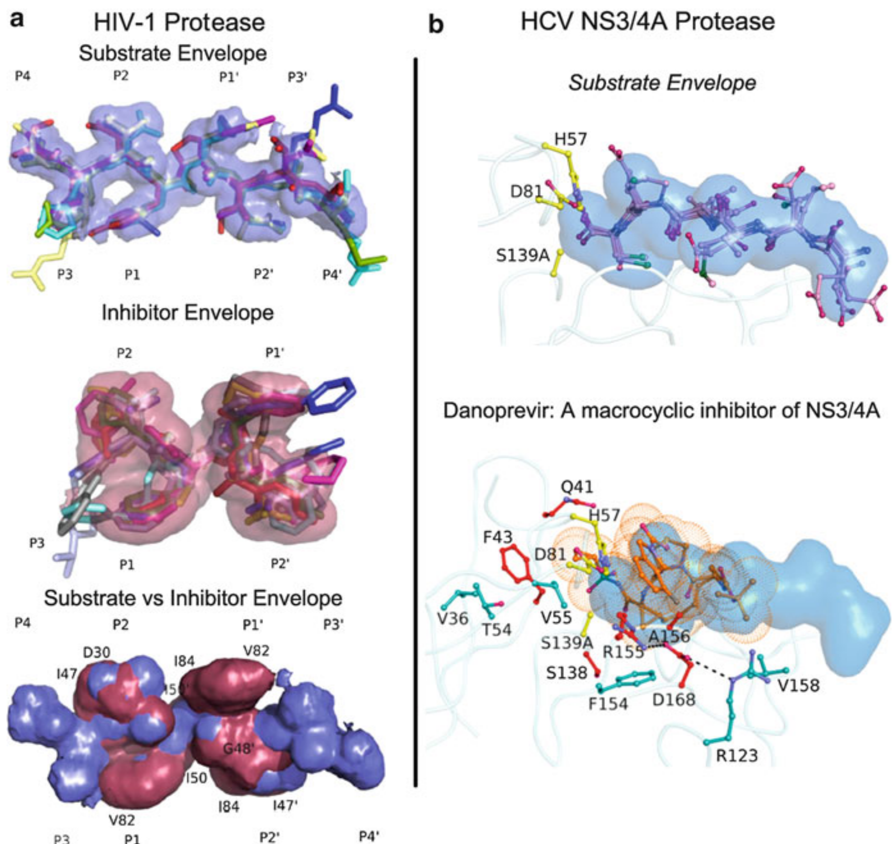


Fig. 2 (**a**) HIV-1 protease-substrate and inhibitor envelopes are colored *blue* and *red*, respectively. The two envelopes were superimposed to highlight the regions where inhibitors protrude beyond the substrate to make more extensive contacts with the protease residues that correspond to the previously known sites of drug resistance (Figure modified from King et al. (2004a)). (**b**) Hepatitis C virus NS3/4A protease-substrate envelope (*blue*) and a small-molecule inhibitor of NS3/4A protease, danoprevir, are shown in comparison along with the binding site residues (Figure modified Romano et al. (2010))

envelopes, the inhibitors were shown to protrude beyond the substrate envelope and make favorable contacts with certain protease residues of the wild-type protease. Because these protease residues interact more favorably with the inhibitors than the natural substrates, these protease residues are more important for inhibitor binding than substrate recognition. Strikingly, the protease residues contacted by inhibitors outside the substrate envelope corresponded to the previously known drug resistance mutation sites. Mutations at these sites would specifically impact inhibitor binding, while substrate recognition and cleavage would be less affected. Most sites of drug-resistant mutations in the active site do not contact the substrates, which led to the hypothesis that the inhibitors that fit well within the substrate envelope would be less

susceptible to drug resistance, because a mutation that affects inhibitor binding would simultaneously impact the recognition and processing of the majority of the substrates (King et al. 2004a). As a retrospective validation, of the currently prescribed inhibitors, the most efficacious is DRV, and although not designed using the substrate-envelope constraint, DRV fits well within this volume (King et al. 2004b; Lefebvre and Schiffer 2008).

Sequence diversification in the protease is not the only mechanism for the virus to develop resistance to PIs. Occasionally, secondary mutations in the cleavage sites are also seen in patients who have failed PI-containing regimens. Crystallographic studies coupled with molecular dynamics simulations on wild-type and coevolved substrate complexes have revealed the structural rationale for why certain cleavage sites are more susceptible to resistance than others and how the cleavage site mutations compensate for the substrate processing efficiency lost upon protease mutations. The substrate-envelope hypothesis allowed quantitative assessment of the fit of each substrate within the substrate envelope. These studies, first, showed that some substrates are less in consensus with the majority of the substrates in terms of the shape adopted within the binding site, including NC-p1 and p1-p6 cleavage sites (Ozen et al. 2011). These substrates, along with inhibitors, interact favorably with a small subset of resistance mutation sites in the protease, e.g., D30, I50, and V82 (King et al. 2004a; Ozen et al. 2011). Strikingly, the outlier substrates, NC-p1 and p1-p6, correspond to the cleavage sites at which mutations were observed in patients who failed PI-containing regimens (Kolli et al. 2009, 2006). The substrate-envelope hypothesis, based on structural evidence, suggests that these substrates protrude beyond the substrate envelope and contact the sites of drug resistance mutations in the protease, leading to impaired substrate recognition and cleavage. This results in coevolution of compensatory mutations within the protease cleavage sites but often at other positions within the cleavage site (King et al. 2004a).

Emergence of D30N/N88D mutations in the protease in a correlated manner with the L449F Gag mutation on the p1-p6 cleavage site is a good example demonstrating that the protease-substrate coevolution validates the substrate envelope as the recognition motif for HIV-1 protease. D30N, a nelfinavir-signature protease mutation, is selected with high frequency in nelfinavir-treated HIV-infected individuals. From co-crystal structures, nelfinavir is known to pick up critical interactions with D30 at one monomer of the protease, which makes nelfinavir hypersusceptible to D30N mutation. Residue 88, on the contrary, does not directly interact with nelfinavir, but the N88D mutation is thought to maintain the overall local charge in the D30N background because 88 is in close proximity of 30 (Kolli et al. 2006).

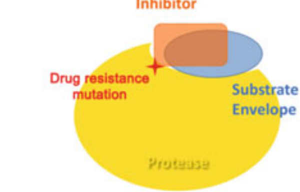
However, the resistance mechanism through L449F Gag mutation in p1-p6 cleavage site is not obvious from the nelfinavir-bound crystal structures. A complete understanding of resistance against nelfinavir requires special attention to the substrate specificity and the mechanisms by which substrate specificity is maintained by the drug-resistant virus. Evidently, p1-p6 interacts with D30 on the other monomer outside the substrate envelope (i.e., p1-p6 interacts with D30 on the other monomer more than the majority of the substrates). Therefore, D30N mutation interferes with both nelfinavir-binding and p1-p6 processing with likely minimal detrimental effects

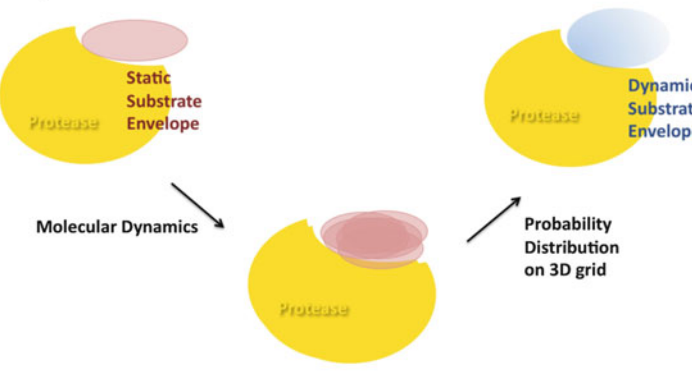
on the recognition of other substrates. Substituting the wild-type leucine with bulkier phenylalanine at Gag 449, corresponding to S1′ pocket of the binding site, compensates the loss of interactions with the D30N/N88D protease filling the substrate envelope much more efficiently (Ozen et al. 2012). As a result, drug therapy selects for the cleavage site mutations, which are able to restore the loss of fit within the substrate envelope and bring the cleavage site more in consensus with the majority of the substrates. In conclusion, coevolved mutations within the cleavage sites play a key role in the development of resistance and affect the virological response during therapy. The substrate-envelope hypothesis, in addition to specificity of the substrates, explains the development of resistance to various PIs and substrate coevolution.

Substrate-Envelope-Guided Drug Design

The substrate envelope can guide the development of robust PIs that retain potency against severely resistant HIV-1 protease variants. Based on the substrate-envelope hypothesis, the optimum strategy to minimize resistance is to design inhibitors that fit within the substrate envelope (Fig. 3). In retrospect, where the five drugs in clinical use specifically protrude outside, the substrate envelope correlates with the loss of affinity to drug-resistant proteases (Chellappan et al. 2007a). Meanwhile, DRV, the most potent of the currently prescribed inhibitors, fits well within the substrate envelope although not designed using the substrate envelope as a constraint (King et al. 2004a; Lefebvre and Schiffer 2008). Retrospective correlation of the substrate envelope with resistance mutations promoted the design of new inhibitors with substrate-envelope constraints (Nalam and Schiffer 2008; Altman et al. 2008; Nalam et al. 2010; Ali et al. 2006; Chellappan et al. 2007b).

To validate the substrate-envelope hypothesis, various groups designed new HIV-1 PIs on the hydroxyethylamine scaffold taking different approaches. Two computational methods incorporated the substrate envelope as an a priori constraint during the design stage of the inhibitors, while the third method employed a structure-activity relationship (SAR) that does not include the substrate-envelope constraint explicitly. The first computational design, based on optimized docking, resulted in two good candidates exhibiting flat affinity profiles against multidrug-resistant mutants, although the binding affinity of these candidates were in the nM range (Chellappan et al. 2007b). The second computational design systematically explored the combinatorial space for three constituent R groups on the same scaffold in two rounds of computational design, chemical synthesis, biochemical testing, and crystallographic analysis. The second round resulted in low nM–pM range compounds, the majority of which have flatter resistance profiles against a wide range of drug-resistant viral variants (Altman et al. 2008). As a negative control, the inhibitors designed with the SAR approach resulted in pM inhibitors; however, they were





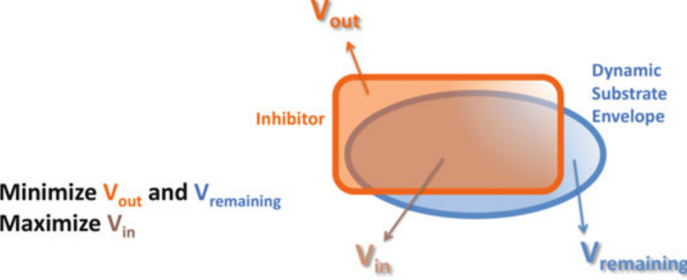


Fig. 3 Substrate-envelope-based drug design. (**a**) Most severe resistance mutations (*red*) occur at sites contacted by competitive inhibitors (*orange*) outside the substrate envelope (*blue*). (**b**) Dynamic substrate envelope can be defined as a probability distribution of the consensus substrate shape within the binding site by combining molecular dynamics simulations and three-dimensional grid-based volume calculations. (**c**) Dynamic substrate envelope can be integrated into structure-based design of robust drugs by systematically optimizing two metrics: (1) V_{out}, the probabilistic volume of an inhibitor falling outside the dynamic substrate envelope, and (2) $V_{remanining}$, the portion of the dynamic substrate envelope that is not fully occupied and, therefore, can be better utilized by an inhibitor

significantly less potent against the resistant variants (Ali et al. 2006). These studies successfully validated the substrate-envelope constraint as a robust design strategy for HIV-1 PIs with improved susceptibility to resistance and yielded several leads for potential new drugs (Nalam et al. 2010).

When the designed inhibitors effectively mimic the wild-type substrate-binding features, a larger number of mutations in the protease and cleavage sites will be needed to alter the balance between the substrate recognition and inhibitor binding in favor of substrate recognition to achieve drug resistance. Using the substrate envelope as a constraint not only improves the efficacy of the new inhibitors against the known resistant variants of HIV-1 protease but also likely minimizes the chances of potential compensatory mutations in the cleavage sites.

Generality of the Substrate-Envelope Hypothesis

Crystal structures typically capture a static image of the native state. To test the general applicability of the crystallography-based substrate envelope, the effect of substrate dynamics in the bound state was assessed by molecular dynamics simulations. In addition, drug targets other than HIV-1 protease were assessed in retrospect for the correlation of the substrate envelope with the mutational sensitivity.

Effect of Protein Dynamics on the Substrate Envelope: Dynamic Substrate Envelope

Protein dynamics is often neglected in drug design. Conformational ensembles of the native state are not readily accessible experimentally at atomistic level in a high-throughput manner. Computational methods can aid to estimate conformational dynamics at the expense of computational time. While the force field describing the molecular interactions can still benefit from improvements, the advancements in parallel computing architectures and algorithms have tremendously revolutionized the molecular dynamics field and increased the ability to simulate wider timescales and larger systems. The earlier simulations of an ~900 atom protein lasted 9 ps (McCammon et al. 1977), while protein-folding simulations as long as 1 ms (Lindorff-Larsen et al. 2011) or 50 ns simulation of an intact virion of one million atoms (Freddolino et al. 2006) can now be performed.

Taking advantage of these advancements, the substrate-envelope model was recently extended by considering the role of protein dynamics in the interactions of HIV-1 protease with its substrates. The *dynamic substrate envelope*, which was defined based on thousands of substrate conformers from molecular dynamics simulations, has turned out as a more accurate representation of protease-substrate interactions and better defined the substrate specificity for HIV-1 protease (Ozen et al. 2011). The dynamic substrate envelope, being a more realistic model, reproduced the essentials of the static substrate envelope, which was based on the crystal structures, validating the substrate envelope as a valid and realistic hypothesis but not a crystallographic artifact (Fig. 3b).

In addition, characterization of structural dynamics of a series of substrates provided insights into the interdependent nature of substrate recognition, which was not immediately evident in crystal structures. HIV-1 protease substrates all need to be recognized and processed by the same enzyme; however, the polyprotein processing is tightly regulated and premature/imprecise processing leads to noninfectious virions. Molecular dynamics studies showed that the substrates all possess common properties that allow the recognition by the protease, but also subtle differences in the interactions with the protease result in preferential recognition. The balance between the shape commonality (i.e., *consensus volume*) and sequence diversity of the substrates is maintained by interdependence within individual substrates in terms of conformational and sequence preferences. In conclusion, the interplay between the conserved and varied properties of the cleavage sites enables the preferential substrate recognition and regulation of substrate processing.

Application of the Substrate-Envelope Hypothesis to Other Drug Targets

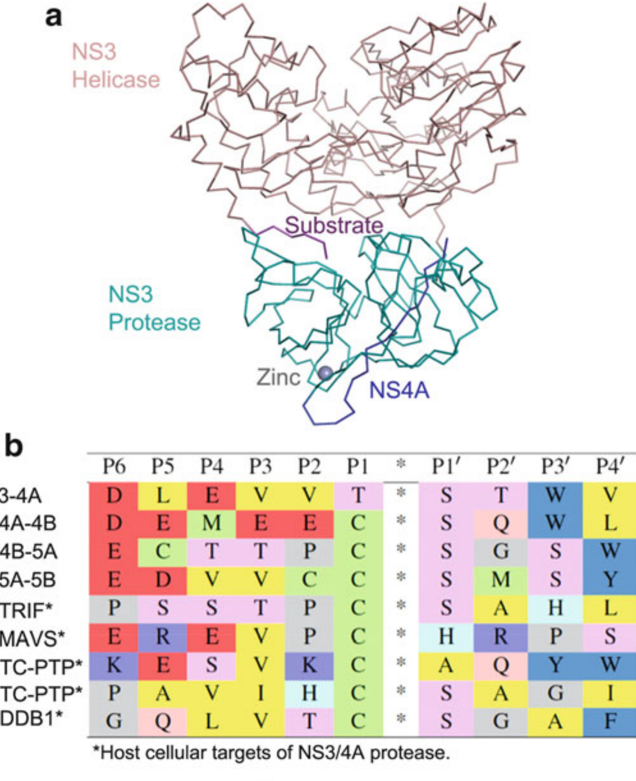
Applicability of the substrate-envelope hypothesis has been tested for five prospective drug targets from a diverse set of diseases: Abl kinase, chitinase, thymidylate synthase, dihydrofolate reductase, and neuraminidase (Kairys et al. 2009). The volume of inhibitors protruding beyond the native substrate envelope trended with average mutational sensitivity, suggesting that inhibitor design would benefit from a similar reverse engineering strategy for these enzymes. Similarly, the two reverse transcriptase inhibitors, AZT and 3TC, have elements protruding beyond the native substrate envelope formed by deoxyribonucleotides. These elements create an opportunity for the reverse transcriptase to develop resistant mutations at the deoxyribonucleotide binding site. However, tenofovir, a reverse transcriptase inhibitor designed with the substrate-envelope constraints, lacks such protrusions and is relatively effective against AZT-resistant HIV variants (Tuske et al. 2004). Finally, the substrate envelope rationalized drug resistance against hepatitis C viral serine protease NS3/4A inhibitors (Romano et al. 2010). NS3/4A is described below as an emerging candidate to target with the substrate-envelope approach.

Substrate Envelope of Hepatitis C Viral Serine Protease NS3/4A

Hepatitis C is a liver disease with significant global impact, which is also caused by an RNA virus of Flaviviridae family. The hepatitis C virus (HCV) infection can lead to liver cirrhosis and hepatocellular sarcoma and is the most common reason for liver transplants in the United States (US). The World Health Organization estimates 150 million people worldwide are infected with HCV and 3–4 million new infections coming up every year with more than 350,000 cases of death from HCV-related liver diseases (Lesage et al. 2009). Similar to HIV, HCV is also genetically highly diverse. So far, six major HCV genotypes and several subtypes within each genotype have been identified (Simmonds et al. 2005). High viral replication rate combined with the error-prone RNA-dependent RNA polymerase causes large inter-patient genetic

diversity as well as viral diversity within a single infected individual (Bukh et al. 1995a, b). Genetic heterogeneity of the virus across and within patients has greatly challenged the development of robust direct-acting antiviral agents (DAAs) that retain efficacy against multiple genotypes and drug-resistant variants of these genotypes since the discovery of HCV in 1989 as the cause of the hepatitis C (Choo et al. 1989; Kuo et al. 1989). Until 2011, the standard of care for HCV was weekly injections of pegylated interferon α combined with ribavirin (Peg-IFN/RBV), which can result in undetectable levels of HCV in 70–80 % of people with genotypes 2 and 3 but only 40–50 % of people with genotype 1 (Lesage et al. 2009). Genotype 1, the most difficult genotype to treat, is also the most common form of HCV in the US accounting for about 75–80 % of the cases (Alter et al. 1999; Blatt et al. 2000). In 2011, two DAAs, telaprevir and boceprevir, were approved by FDA for clinical use in combination with Peg-IFN/RBV for the treatment of genotype 1 patients. In addition to the problem of drug resistance, the severe side effect profile of this combination therapy amplifies the need to develop widely effective and better-tolerated DAAs.

Among the drug targets against HCV is the nonstructural protein 3 (NS3), which is a 631-amino acid bifunctional protein, with a serine protease domain located in the N-terminal one-third and an NTPase/RNA helicase domain in the C-terminal two-third (Fig. 4a). The reason for the protease and helicase domains to be physically linked is not fully understood. Although their interplay has been reported (Beran and Pyle 2008; Beran et al. 2007, 2009; Frick et al. 2004), both domains fold independently and are active in the absence of the other (Beran and Pyle 2008; Frick et al. 2004; Beran et al. 2007; Lam et al. 2003; Gallinari et al. 1998). NS3/4A protease adopts a chymotrypsin-like fold with two β-barrel domains. The catalytic triad is formed by His-57, Asp-81, and Ser-139 and is located in a cleft separating the two domains. The structure is stabilized by a Zn^{+2} ion that is coordinated by Cys-97, Cys-99, Cys-145, and His-149. The most efficient proteolytic activity of NS3 requires a cofactor NS4A, a 54-amino acid peptide that is tightly associated with the protease (Lesage et al. 2009). NS4A aids in the proper folding of NS3; the central 11 amino acids of NS4A inserts as a β-strand to the N-terminal β-barrel of NS3. The HCV genome encodes a single polyprotein of ~3,000 acids, which is processed by a series of host and viral proteases into at least 10 structural and nonstructural proteins. The viral NS3/4A hydrolyzes the polyprotein precursor at four cleavage sites (3-4A, 4A-4B, 4B-5A, 5A-5B), yielding nonstructural proteins essential for viral maturation. The first proteolytic event occurs at 3-4A junction in cis as a unimolecular reaction, while processing of the remaining junctions 4A-4B, 4B-5A, and 5A-5B occurs bimolecularly in *trans* (Bartenschlager et al. 1994). Similar to HIV, the cleavage sites of NS3/4A protease are nonhomologous except for an Asp/Glu at P6, Cys/Thr at P1, and Ser/Ala at P1′ (Fig. 4b). NS3/4A also confounds the innate immune response to viral infection by cleaving the human cellular targets TRIF and



	P6	P5	P4	P3	P2	P1	*	P1′	P2′	P3′	P4′
3-4A	D	L	E	V	V	T	*	S	T	W	V
4A-4B	D	E	M	E	E	C	*	S	Q	W	L
4B-5A	E	C	T	T	P	C	*	S	G	S	W
5A-5B	E	D	V	V	C	C	*	S	M	S	Y
TRIF*	P	S	S	T	P	C	*	S	A	H	L
MAVS*	E	R	E	V	P	C	*	H	R	P	S
TC-PTP*	K	E	S	V	K	C	*	A	Q	Y	W
TC-PTP*	P	A	V	I	H	C	*	S	A	G	I
DDB1*	G	Q	L	V	T	C	*	S	G	A	F

*Host cellular targets of NS3/4A protease.

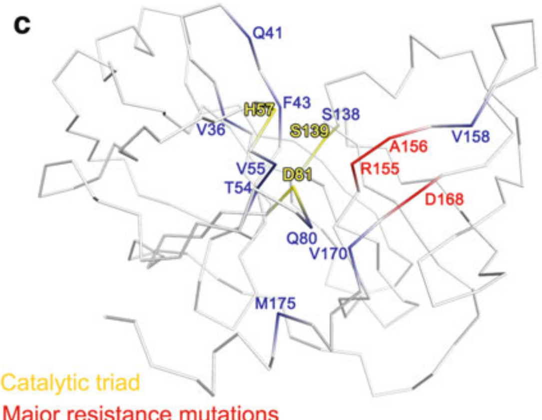


Fig. 4 (continued)

Fig. 4 (**a**) Hepatitis C viral NS3 is a bifunctional protein with helicase and protease domains, shown in ribbon. Helicase and protease are colored in *light pink* and *teal*, respectively. A cleaved substrate product is shown in *magenta*. (**b**) NS3/4A proteases recognized four sites on the ~3,000-amino acid viral polyprotein and a series of host cellular proteins. (**c**) Major drug resistance mutations and resistance-associated mutations are labeled *red* and *dark blue*, respectively. The catalytic triad is colored *yellow*. (**d**) NS3/4A protease inhibitors targeting NS3/4A protease, telaprevir and boceprevir, were approved in May 2011. Three of the macrocyclic NS3/4A inhibitors in development were shown as examples

MAVS and to block toll-like receptor three signaling and RIG-I signaling, respectively (Chen et al. 2007; Heim 2013; Li et al. 2005a, b). Cleavage of another cellular target, TC-PTP, at two separate sites enhances EGF signaling and basal Akt activity (Brenndorfer et al. 2009). Very recently, DDB1, a core subunit of the Cul4-based ubiquitin ligase complex, was reported to play a critical role in HCV replication and get cleaved by NS3/4A (Kang et al. 2013). Thus, in addition to blocking the viral maturation, effective inhibition of the proteolytic activity of the NS3/4A may also exert indirect antiviral effects, further interfering with viral replication.

The very shallow binding site of HCV NS3/4A protease has presented a big challenge to develop high-affinity and low-molecular-weight inhibitors because engineering small-molecule inhibitors to pick up tight interactions at the shallow surface was not straightforward. However, product inhibition by the N-termini of the trans-cleavage sites formed the basis for the development and optimization of peptidomimetic inhibitors of the NS3/4A protease (Steinkuhler et al. 1998; Llinas-Brunet et al. 1998; De Francesco and Migliaccio 2005). The proof of concept for antiviral efficacy was first demonstrated in 2002 with the macrocyclic inhibitor BILN-2061 (ciluprevir), which was later discontinued due to concerns about its cardiotoxicity (Lamarre et al. 2003; Hinrichsen et al. 2004; Vanwolleghem et al. 2007).

Telaprevir and boceprevir, the FDA-approved NS3/4A inhibitors, were developed by Vertex and Schering-Plough, respectively (Fig. 4d). Both telaprevir (Perni et al. 2006; Kwong et al. 2011) and boceprevir (Malcolm et al. 2006) are acyclic

ketoamide inhibitors that associate with the protease through a reversible, covalent bond with the catalytic serine (S139) as well as short-range molecular interactions with the binding site. In addition, several non-covalent inhibitors, including macrocyclic compounds, are currently at various stages of clinical development. The non-covalent acylsulfonamide inhibitors contain a macrocycle connecting either P1 and P3 groups (ITMN-191 or danoprevir (Seiwert et al. 2011)) or alternatively P2 and P4 groups (MK-5172 (Harper et al. 2012), MK-7009, or vaniprevir (Liverton et al. 2010)), reducing the entropic cost associated with binding the shallow surface on the protease. In addition to the reported resistance in replicon studies, HCV quickly evolves to confer resistance to these protease inhibitors even at early stages of clinical trials compromising their high efficacy (He et al. 2008; Kieffer et al. 2007; Lin et al. 2005; Sarrazin et al. 2007; Tong et al. 2008, 2006). Despite the subnanomolar potency, the macrocyclic inhibitors also select for drug resistance mutations in clinic. Most PIs, in clinic or development, are susceptible to a common set of protease mutations, which raises the issue of cross-resistance. However, level of susceptibility to different mutations varies with drug. For example, R155K, A156T, and D168A are three mutations that are observed in patients treated with both linear and macrocyclic inhibitors, but the macrocyclic inhibitors appear to be more susceptible to R155K than linear compounds (Fig. 4c) (Romano et al. 2012).

Limitation of the current drugs to a single genotype and their susceptibility to quickly emerging resistance mutations pushes the research for developing inhibitors with broader activity. The substrate-envelope hypothesis has aided in elucidating the mechanism by which the protease mutations confer resistance to the current inhibitors.

High-resolution co-crystal structures have been determined for the wild-type NS3/4A protease domain with the cleavage products as well as inhibitors, including telaprevir, boceprevir, simeprevir, danoprevir, MK-5172, and vaniprevir (Romano et al. 2010, 2012). In these structures, the products, despite the low sequence homology, adopted a consensus volume at P6 to P1 residues, *the substrate envelope*. Similar to HIV, the most severe resistance mutations occur at protease residues that are contacted by the inhibitors outside the substrate envelope.

Crystal structures of the resistant protease variants bound to telaprevir and three macrocyclic inhibitors in development, danoprevir, vaniprevir, and MK-5172, revealed the structural basis of the three major active site resistance mutations, R155K, A156T, and D168A (Romano et al. 2010, 2012). The protease residue 155 is contacted much more favorably by the carbamate-linked bulky isoindoline groups of vaniprevir and danoprevir outside the substrate envelope compared to telaprevir, boceprevir, and MK-5172. Therefore, a mutation at this residue renders the isoindoline-containing compounds less effective, while MK-5172 retains reasonable affinity against R155K protease since MK-5172 has an ether-linked quinoxaline group that packs against the conserved catalytic His-57.

However, the fold change in affinity against R155K protease varies with inhibitor. The wild-type Arg-155 participates in an electrostatic network of hydrogen bonds along the binding surface. This network involves residues His-57, Arg-155, Asp-168, and Arg-123. Substituting the arginine at 155, which can make two hydrogen bonds, with a lysine, which can make only one hydrogen bond, disrupts this electrostatic network and compromises the stability of the binding surface.

Although both danoprevir and vaniprevir have favorable interactions with R155, mutation has more detrimental effect on the binding affinity of vaniprevir than danoprevir because vaniprevir has a linker connecting the bulky P2 isoindoline to P4, whereas danoprevir lacks this linker. Molecular dynamics simulations, in consistent with the crystallographic temperature factors and the inhibitor conformations in multiple molecules in the asymmetric unit, suggest that the lack of this linker renders the P2 group to be locally flexible without altering the binding mode of the inhibitor core. This local flexibility likely tolerates the instabilization of the binding surface due to R155K, while vaniprevir, constrained with P2-P4 macrocycle, cannot escape from the destabilizing effects of R155K. As a result, a 4.5 fold change is observed in the loss of affinity against the R155K protease between danoprevir and vaniprevir (Ozen et al. 2013).

Although the flat binding surface of NS3/4A is difficult to target, the comparative analysis of substrates and chemically diverse small-molecule inhibitors supports that a substrate-envelope-based design approach has the potential to result in more robust novel inhibitors. Taking this approach, considering conformational dynamics is probably even more critical than HIV because even the bound compounds have unique flexibilities, which have critical implications for drug resistance.

Conclusions and Future Perspective

Drug resistance will occur anytime rapid growth and evolution exists under the selective pressure of drug treatment but the growth is not completely inhibited by the drug. This widespread problem is in everything from invasive cancers and pathogenic microbes such as bacteria, malaria, fungi, tuberculosis, and viruses. The mechanisms by which resistance can emerge include point mutations in the target protein. To overcome drug resistance, resistance should be predicted before it happens, and drugs should be designed accordingly to avoid the accurately predicted resistance mutations. To achieve this goal, target identification is critical. The enzymes with multiple substrates that cannot easily tolerate mutations and maintain function are potentially good candidates.

Crystallography is extremely informative to provide insights into the most probable molecular interactions in the native state. However, proteins are dynamic and exist in conformational ensembles even in native state. Depending on the inherent structural and dynamic properties of the drug target, ignoring protein dynamics may delay the successful discovery of novel drugs that have high potency, good selectivity, and low toxicity and are also robust against the evolution of resistance. Developing these robust drugs, experimental techniques and computational methods should be used in concert, each according to its particular strengths. Dynamic substrate envelope is a useful tool to systematically incorporate the protein dynamics and evolution into structure-based rational drug design. Substrate-envelope-guided drug design necessitates constant partnering of multiple disciplines such as chemical synthesis, thermodynamics and enzyme kinetics, crystallography, NMR, molecular modeling and dynamics simulations, deep sequencing, and virology.

The current understanding of the structure and dynamics of substrate recognition and drug resistance in HIV and HCV proteases will serve as a useful guide for the rational design of future generation drugs that remain active against diverse populations of drug targets. Combating quickly evolving diseases, all drug targets should be viewed as evolutionarily dynamic, and inhibitors should be designed as evolutionarily constrained as possible. The target is moving and robust drug design requires hitting multiple targets at a time.

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Part II

Bacteriology

The Mechanisms of Resistance to β-Lactam Antibiotics

Dustin T. King, Solmaz Sobhanifar, and Natalie C. J. Strynadka

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Abstract

Bacterial diseases have had an enormous impact on human health and continue to be a major focus in modern medicine. The most widespread class of human antibacterials is the β-lactams that target the transpeptidase enzymes, which are responsible for cross-linking the peptidoglycan cell wall. There are over 34 FDA-approved β-lactams which together constitute ~50 % of all antibiotic prescriptions worldwide (Tahlan K and Jensen SE, J Antibiot (Tokyo) 66:401–410, 2013). However, bacteria have gained resistance mechanisms to overcome all major classes of β-lactam antibiotics to date. In this chapter, we will address the major mechanisms of bacterial resistance to the β-lactams and highlight some of the recent advances in circumventing this resistance.

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Introduction

Sir Alexander Fleming's discovery of penicillin in the late 1920s initiated interest in deciphering the molecular mechanism of β-lactam action. Subsequent observations in the 1940s revealed that upon treatment with penicillin, bacteria adopt a filamentous morphology and that radioactive penicillin G localized to the membrane leading to the conclusion that β-lactams were affecting the synthesis of some key cell surface structure (Duguid 1946; Waxman and Strominger 1983). However, further target identification had to wait a further 20 years for elucidation of the peptidoglycan (PG) chemical architecture and biosynthetic pathway (for a detailed review on PG synthesis, please see (Lovering et al. 2012)). The bacterial PG is a vast glycan mesh that envelops the entire bacterial cell and imparts the rigidity necessary to define cell shape and morphogenesis as well as protect the cell from osmotic rupture (Typas et al. 2011). PG is made up of long linear polysaccharide chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid pentapeptide. The pentapeptide group (or stem peptide) in Gram-negative bacteria is typically comprised of L-alanine-γ-D-glutamate-diaminopimelate(meso-DAP)-D-alanine-D-alanine. In Gram-positive organisms, the stem peptide is typically comprised of L-alanine-γ-D-glutamate-L-lysine-D-alanine-D-alanine with a pentaglycine branch protruding from the L-lysine residue. In the mid-1960s, the stem peptide cross-linking PG transpeptidases (TPs) were identified as the lethal target of the β-lactams, and the complexity of β-lactam action was attributed to the multiple penicillin-binding proteins (PBPs) that are targeted by them (Wise and Park 1965; Tipper and Strominger 1965). The PBP TPs typically catalyze a two-step reaction in which the position 3 amino group of an acceptor strand attacks the peptide bond of the terminal D-alanine-D-alanine of a donor strand, releasing the D-alanine leaving group and forming a peptide cross-link (Sauvage et al. 2008; Macheboeuf et al. 2006). The inhibition of PBPs ultimately results in reduced PG stem peptide cross-links and deregulation of PG degradation, which causes the accumulation of sacculus defects. These localized PG defects ultimately result in the inability of the cell wall to withstand the osmotic turgor pressure of the cytoplasmic membrane resulting in outer membrane encased balloon-like structures on the surface of the bacterial cell that eventually rupture leading to cell death (Yao et al. 2012).

β-Lactams

β-Lactam-Mediated Inhibition of PG Transpeptidase

The β-lactam antibiotics act as covalent substrate analogues of the D-alanine-D-alanine portion of the acceptor stem peptide. All PBP TP domains contain three highly conserved active site sequence motifs: (i) the SXXK motif (that includes the

catalytic serine nucleophile and general base lysine), (ii) the SXN triad, and (iii) the KTG(T/S) motif (Sauvage et al. 2008). The mechanism of TP inhibition is initiated by deprotonation of the motif in catalytic S70 by the concerted general base K73 facilitating nucleophilic attack on the β-lactam amide carbonyl carbon resulting in the formation of a tetrahedral intermediate (Fig. 1a). This transiently formed intermediate is stabilized by hydrogen bonding to conserved residues in the oxyanion hole (comprised of main chain hydrogens of motifs i and iii). Subsequently, the tetrahedral intermediate collapses to expel the negatively charged nitrogen leaving group which is presumably stabilized by protonation via S120 (motif ii), thereby forming an acyl-enzyme intermediate. The stable species is resistant to hydrolysis, presumably due to steric blockage of a requisite deacylating water by the nitrogen of the former β-lactam ring (Fig. 1b; reviewed in Sauvage et al. (2008); Macheboeuf et al. (2006)).

Major Classes of β-Lactams

Since the initial discovery of benzylpenicillin, numerous other β-lactam classes have been developed, expanding our antibiotic arsenal to combat resistance. β-Lactams fall into four distinct structural classes that all have the four-membered lactam core moiety in common (penicillins, cephalosporins, carbapenems, and monobactams). Taken together, the multiple β-lactams constitute a comprehensive and structurally diverse set of compounds that display different pharmacological properties and are used for unique clinical indications.

Penicillins

The penicillins were initially derived from *Penicillium* fungi and represent the oldest "pure" antibiotic concoction used by man. The clinical testing of Fleming's purified penicillin extract in the early 1940s was met with unparalleled success and marked a seminal advancement in medical history (Keefer et al. 1943). The penicillin core consists of a five-membered thiazolidine ring fused at the 2′ and 3′ positions to the β-lactam ring (Fig. 2). Today, there are four major penicillin subclasses: (i) natural penicillins, (ii) penicillinase-resistant penicillins, (iii) aminopenicillins, and (iv) extended-spectrum penicillins (Miller 2002). The evolution of bacterial resistance to natural product penicillins stimulated a renaissance in the development of novel semisynthetic derivatives, which are made using the 6-aminopenicillanic acid (6-APA) precursor molecule (Rolinson and Geddes 2007). Although the penicillin family continues to be an important cornerstone in modern medicine, the emergence of widespread bacterial resistance has led to decreased efficacy in recent decades driving development of alternative β-lactams.

Cephalosporins

The cephalosporins (the first of which, cephalosporin C, was isolated from the fungi *Cephalosporium acremonium* in 1948) have a six-membered dihydrothiazine ring attached to the lactam core (Fig. 2). Interest in the clinical development of

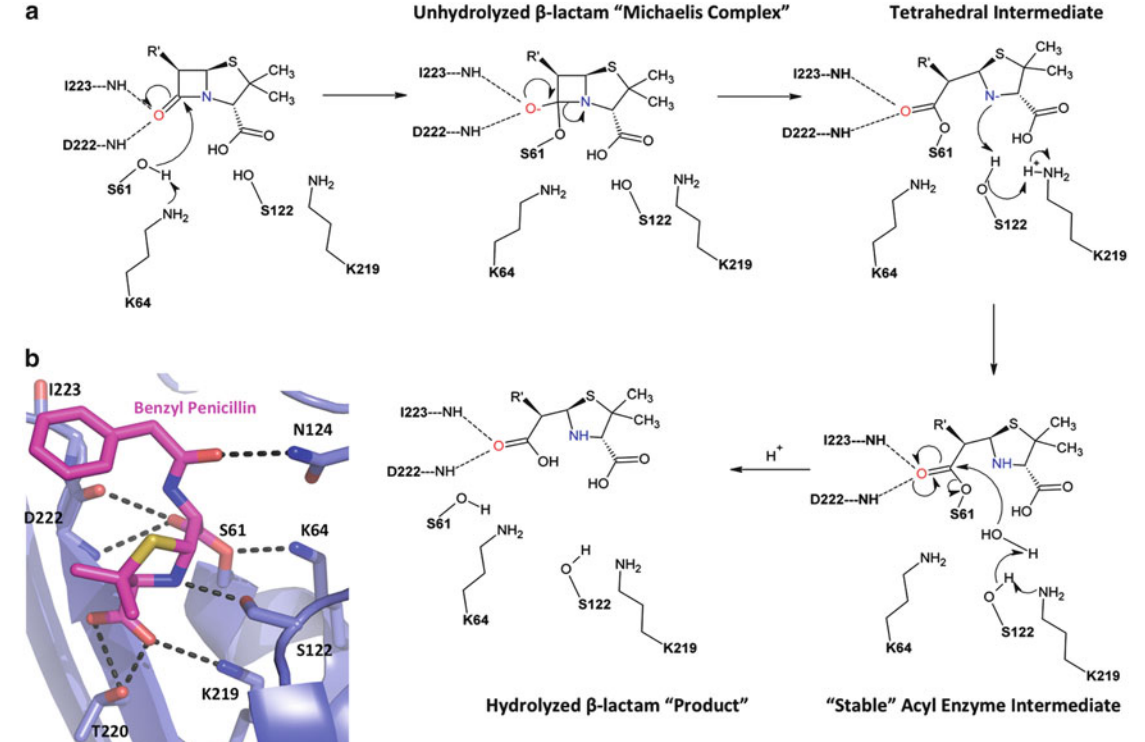

Fig. 1 β-Lactam-mediated inhibition of PBP TPase. (**a**) Mechanism of PBP-mediated β-lactam acylation and eventual hydrolysis. (**b**) Active site close-up of benzylpenicillin-bound *Thermosynechococcus elongatus* PBPA. The benzylpenicillin-bound PBPA active site (PDB ID: 2JBF) is depicted as a blue cartoon with selected active site residues shown as *blue* sticks with atoms colored by type (N, *blue*; O, *red*; S, *yellow*). The acylated benzylpenicillin is depicted as *pink* sticks with atoms colored by type. Hydrogen bonding and electrostatic interactions are shown as *black dashes*

Fig. 2 Chemical structure of β-lactam antibiotic classes that are in current clinical use

cephalosporins stemmed from their resistance to hydrolysis by penicillinases (Neu 1982). The side chains used in the development of semisynthetic penicillins were incorporated into the cephalosporin core scaffold (Page 2012). However, in contrast to the penicillins, the cephalosporin core offers an additional site of variation at the C3 position (Fig. 2), giving rise to a breadth of structural diversity. The cephalosporins are generally grouped into four distinct generations based upon several features of antimicrobial activity (reviewed in Page 2012). The most recent cephalosporins in development either display antipseudomonal activity or are effective against methicillin-resistant *Staphylococcus aureus* (MRSA) (i.e., ceftobiprole) (Bush and Macielag 2010).

Carbapenems

The carbapenems (the first of which, thienamycin, was discovered in the mid-1980s as a metabolic product of *Streptomyces cattleya* (Birnbaum et al. 1985)) have a five-membered 2,3 unsaturated system with a C1 carbon rather than sulfur 4,5 fused to the lactam core. In place of the acylamino group seen at the R1 position in penicillins and cephalosporins, the carbapenems have a hydroxyethyl side chain that is important for resisting β-lactamase-mediated hydrolysis (Fig. 2) (Maveyraud et al. 1998). Remarkably, carbapenems have overall broader antimicrobial activity than the penicillins, cephalosporins, and other β-lactam/β-lactamase inhibitor combinations (Papp-Wallace et al. 2011). A key attribute of the carbapenems is their ability to bind indiscriminately to multiple PBPs and resist hydrolysis or inhibit many β-lactamases (Queenan et al. 2009; Bonfiglio et al. 2002). Today, carbapenems are often our last line of defense against multidrug-resistant Gram-negative pathogens. However, clinically available carbapenems have low oral bioavailability and thus do not readily penetrate gastrointestinal tissues and are typically administered intravenously (Papp-Wallace et al. 2011).

Monobactams

Monobactams are predominantly synthetic monocyclic β-lactams with variable organic groups at positions C2 and C4 as well as a sulfonic acid moiety attached to the N1 nitrogen (Fig. 2). The sulfonic acid group is thought to activate the β-lactam ring assisting the acylation of transpeptidases (Finberg and Guharoy 2012). Aztreonam is currently the only clinically approved monobactam. Aztreonam binds to PBP3 of susceptible Gram-negative pathogens with high affinity, yet

displays very poor acylation of Gram-positive PBPs resulting in its inability to treat Gram-positive infections. Due to its relatively narrow spectrum of activity, aztreonam is generally used as part of antibiotic combination therapies (such as aztreonam-vancomycin) (Ellis-Grosse et al. 2005). However, there is substantial interest in developing new monobactams given that they are stable to the emerging metallo-β-lactamase (MBL) enzymes (King and Strynadka 2012).

β-Lactam Resistance

Major Resistance Mechanisms

Even before penicillin was commercialized in the early 1940s, penicillin-resistant β-lactamase expressing strains of *E. coli* were identified (Abraham and Chain 1988). The identification of β-lactam resistance led to the development of extended-spectrum antibiotics such as ceftazidime, cefotaxime, and the carbapenems as well as β-lactam-based serine β-lactamase inhibitors such as tazobactam, sulbactam, and clavulanic acid (Page 2000). However, extensive use of these compounds both in medicine and in the agricultural industry has placed a tremendous selective pressure on bacteria, such that currently no single β-lactam is free from resistance. It is now commonplace for individual bacteria to have multiple different resistance genes that function in concert to confer extended-spectrum resistance. The three main mechanisms of bacterial resistance to the β-lactam antibiotics are (i) enzymatic degradation by β-lactamases, (ii) target modification of the PBPs resulting in a lack of β-lactam binding, and (iii) regulation of β-lactam entry and efflux.

Enzymatic Degradation

The single most prominent mechanism of bacterial resistance to the β-lactams is the expression of hydrolytic enzymes called β-lactamases. These enzymes specifically recognize and hydrolyze the four-membered β-lactam ring leading to an inactivated product that is no longer effective at inhibiting TPs. Most frequently, resistance is conferred by mutation of preexisting β-lactamase enzymes resulting in an enhanced spectrum or targeted specificity of their hydrolytic properties against the various β-lactam classes listed above. Many β-lactamases are encoded on mobile genetic elements leading to increased transmission and spread such that it is now commonplace to find bacterial strains harboring as many as eight different β-lactamases each tailored to inactivate a unique subset of antibiotics (Bush 2013). β-Lactamases themselves are typically grouped into four distinct classes based upon DNA sequence similarity (molecular classes A–D). Molecular classes A, C, and D evolved from TPs and utilize an active site serine to initiate bond hydrolysis and are thereby referred to as serine β-lactamases (SBLs). In contrast, the unique molecular class B enzymes are metallo-β-lactamases (MBLs) that use active site zinc ions to coordinate a nucleophilic hydroxide to mediate ring opening. The class B enzymes are further categorized into

the subclasses B1, B2, and B3 based upon DNA sequence similarities. Collectively, these enzymes are capable of hydrolyzing every clinically available β-lactam.

Class A (SBLs)

The class A penicillinase TEM (or RTEM) was the first clinically relevant plasmid-encoded β-lactamase identified in Gram-negative bacteria (*E. coli* and *Salmonella enterica*) in the early 1960s (reviewed in Bush 2012). By the late 1970s and early 1980s, broad-spectrum TEM and SHV were the most common plasmid-encoded β-lactamases in Gram-negative isolates. Their apparent abundance and location on mobile genetic elements provided a rich environment for the evolution of these enzymes in response to the introduction of new β-lactams (Turner 2005). Class A extended-spectrum β-lactamases (ESBLs) of the TEM, SHV, and CTX-M families are currently among the most clinically significant β-lactamases and have evolved to not only hydrolyze the penicillins but also broad-spectrum cephalosporins and monobactams (Bush 2012). Today, the CTX-M family of class A β-lactamase is the most prominent set of ESBL enzymes globally and has the ability to readily hydrolyze extended-spectrum cephalosporins such as cefotaxime (Delmas et al. 2010). KPC-2 is the most frequently reported class A carbapenemase to date and has been found as the causative agent in numerous carbapenem-resistant outbreaks worldwide (Nordmann et al. 2009).

The active site of class A β-lactamases contains four distinct motifs that are important for substrate binding and catalysis: (i) S70XXK, (ii) S130XN, (iii) K234-T/SG, and (iv) the Ω loop (Fig. 3a). The general mechanism of class A β-lactamase hydrolysis begins with the activation of S70 by deprotonation. There are currently two proposed mechanisms for S70 activation: (i) K73 acts as a general base to deprotonate the catalytic S70 (Swaren et al. 1995; Strynadka et al. 1992), and (ii) E166 activates a water molecule which subsequently deprotonates S70 (Hermann et al. 2005). Once activated, S70 attacks the β-lactam amide bond resulting in the formation of a tetrahedral intermediate that is stabilized by the oxyanion hole of the enzyme (Strynadka et al. 1996). Subsequently, the tetrahedral intermediate breaks down to expel the N4 nitrogen leaving group, which is subsequently protonated by S130 resulting in the formation of the transient acyl-enzyme intermediate. K73 is thought to shuttle a proton to S130 for leaving group protonation during this process (Hermann et al. 2005). Deacylation is generally thought to proceed through activation of a nucleophilic water molecule by E166, resulting in the hydrolysis of the acyl bond with concomitant back donation of a proton, likely through a concerted shuttle via K73, to the catalytic S70 and release of the de-activated product from the active site.

Class B (MBLs)

The first discovered class B enzyme was the *Bacillus cereus* metallo-β-lactamase (MBL) BcII in 1966 by Sabath and Abraham (Sabath and Abraham 1966). By 1989, only four MBL enzymes had been discovered, and each appeared to be chromosomally encoded and species specific. For the following two decades, the MBLs were seen as interesting, yet clinically insignificant. However, in 1991, the discovery of plasmid-encoded IMP-1 from *Pseudomonas aeruginosa* in Japan launched a

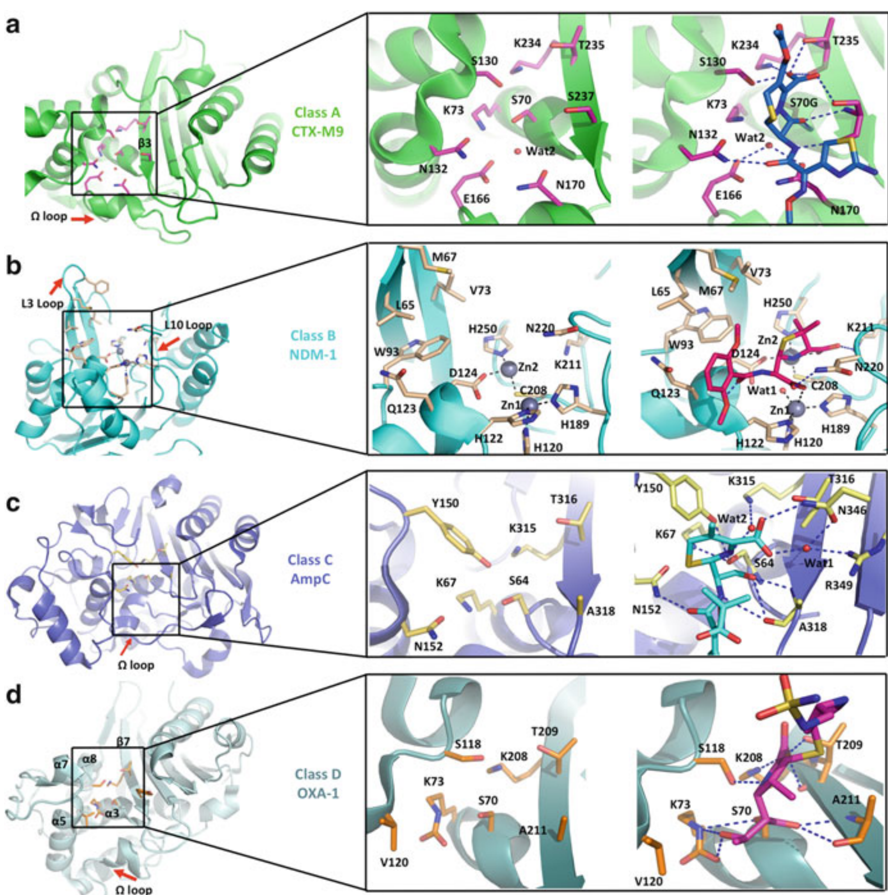

Fig. 3 Structural comparison of β-lactamase enzymes. (**a**) Overall structure and active site close-up of the class A β-lactamase CTX-M9 (PDB ID: 3HLW). The CTX-M9 protein is depicted as a *green* cartoon with selected active site residues shown as *pink* sticks. Acylated cefotaxime is depicted as *blue* sticks. (**b**) Overall structure and active site close-up of the class B1 β-lactamase NDM-1 (PDB ID: 4EY2). The NDM-1 protein is depicted as a *cyan* cartoon with selected active site residues shown as *beige* sticks with atoms colored by type. Hydrolyzed methicillin is depicted as *pink* sticks. (**c**) Overall structure and active site close-up of the class C β-lactamase AmpC (PDB ID: 1IEL). The AmpC protein is depicted in *blue* cartoon representation with selected active site residues shown as *gold* sticks with atoms colored by type. Acylated ceftazidime is shown as *cyan* sticks. (**d**) Overall structure and active site close-up of the class D β-lactamase OXA-1 (PDB ID: 3ISG). The OXA-1 protein is depicted in *dark teal* cartoon representation with selected active site residues shown as *orange* sticks with atoms colored by type. Acylated doripenem is shown as *pink* sticks. In (**a**–**d**), a close-up of the apo (*left*) and acylated (*right*) enzyme is depicted. In all panels, hydrogen bonding and electrostatic interactions are shown as *blue dashes*, and all non-carbon ligand and residue atoms are colored by type

renaissance in the discovery and characterization of new MBLs (Bebrone 2007). MBL-mediated resistance in nosocomial infections has gained traction in many multidrug-resistant Gram-negative pathogens including *P. aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Bacteroides fragilis*, and *Aeromonas hydrophila* (Bebrone 2007). Today, MBLs are predominantly plasmid encoded as part of mobile genetic cassettes, which facilitates their transmission throughout microbial populations (Walsh et al. 2005). Furthermore, MBLs are known for their promiscuous nature and ability to hydrolyze nearly all β-lactams with the exception of the monobactams. Furthermore, MBLs constitute the most molecularly diverse class of carbapenemases (Walsh 2010). Recent years have seen the development of MBLs such as the New Delhi metallo-β-lactamase that can confer enteric pathogens such as *E. coli* and *K. pneumoniae* with nearly complete resistance to all β-lactams including the carbapenems (Yong et al. 2009). Additionally, bacteria co-expressing SBLs and MBLs are often capable of hydrolyzing the clinically relevant monobactam aztreonam (Nordmann et al. 2011). Despite vast research efforts, and due in part to the lack of a covalently bound adduct during hydrolysis, the development of a clinically useful MBL inhibitor is yet to materialize.

Despite having divergent sequences, the MBL enzymes have a remarkably conserved fold, which is characterized by an internal β-sandwich flanked on its outer face by five solvent-exposed α-helices (Fig. 3b). The zinc-containing active site is localized to one face of the β-sandwich in a broad, yet shallow groove (King and Strynadka 2012). Although MBLs are generally homovalent zinc-dependent hydrolases, several have nevertheless been found to bind cobalt and cadmium in addition to zinc, with varying degrees of hydrolytic efficiency (de Seny et al. 2001). The MBLs are either monovalent or divalent depending upon the particular enzyme subclass being considered. The B1 and B3 enzymes utilize a divalent zinc center to mediate hydrolysis, while the B2 MBLs are monovalent enzymes that are inhibited by the presence of a second active site zinc ion and display high specificity for carbapenem hydrolysis (Bebrone et al. 2009; Hernandez Valladares et al. 1997).

For the subclass B1 and B3 MBLs (for which the mechanism has been more extensively studied), the β-lactam carbonyl is coordinated by Zn1 in the precatalytic complex. The C3 carboxylate of the substrate interacts electrostatically with the conserved K224 (Fig. 3b). Binding by the electropositive zinc ions maintains the bridging catalytic hydroxide at a measured pKa of 5–6 (Wang et al. 1999). Nucleophilic attack by this hydroxide on the activated carbonyl results in the formation of a tetrahedral intermediate, which is stabilized by a predicted oxyanion hole (consisting of Zn1 and potentially the amide side chain nitrogen of N220). The tetrahedral intermediate then breaks down to expel the negatively charged nitrogen, which is proposed to be protonated by bulk solvent (Page and Badarau 2008). The product is subsequently released from the active site, and the nucleophilic hydroxide is reloaded between the zinc ions for another round of catalysis. For a more complete analysis of the MBL catalytic mechanism, please refer to the following reviews (Page and Badarau 2008; Xu et al. 2006).

Class C (SBLs)

The class C β-lactamases or AmpC enzymes originally evolved to hydrolyze cephalosporin antibiotics. However, today many of these enzymes show high catalytic efficiency toward the penicillins. These enzymes are typically chromosomally encoded carbapenemases that are often under inducible expression. However, several class C enzymes have now been found localized on high copy number mobile plasmids (Philippon et al. 2002). The class C enzymes are predominantly found in Gram-negative organisms such as *E. coli* and *K. pneumoniae* (Fenollar-Ferrer et al. 2008). Typically, AmpC enzymes are nonsusceptible to the clinically approved β-lactamase inhibitors; however, some remain susceptible to sulbactam and tazobactam (Jacoby 2009).

The four active site motifs that define the class C enzymes are (i) the S64XXK, (ii) Y150AN, (iii) K314TG, and (iv) the Ω loop. The Ω loop occupies a unique position when compared to the class A enzymes, leaving room for more bulky cephalosporin β-lactam side chains (Fig. 3c) (Jacoby 2009). The general mechanism of catalysis for AmpC β-lactamases is assumed to be quite similar to the class A enzymes. However, the unique Y150 (motif ii) is ideally positioned to act as a potential proton donor to the β-lactam nitrogen leaving group following acylation, and it is generally accepted that this residue has a vital role in catalysis for the class C enzymes (Fenollar-Ferrer et al. 2008).

Class D (SBLs)

The class D β-lactamases are the most structurally divergent of the SBL subclasses, and DNA sequence similarity to the class A and C enzymes is restricted to distinct active site regions. These β-lactamases are predominantly known as OXA enzymes, which are named due to their ability to hydrolyze oxacillin (Majiduddin et al. 2002). The class D genes are typically plasmid encoded and are often localized to gene cassettes in integron regions. Similar to class A enzymes, the class D β-lactamases were originally identified as penicillinases, which have subsequently evolved the ability to hydrolyze a specific subset of cephalosporins and carbapenems (Majiduddin et al. 2002). Recently, the carbapenem-hydrolyzing OXA-48 enzyme has gained attention due to its broad specificity and large clinical prevalence (Poirel et al. 2012). Several class D SBLs have been found to exist as a dimer in solution, and the dimer-monomer equilibrium appears to be an important factor governing the kinetics of β-lactam hydrolysis (Paetzel et al. 2000; Vercheval et al. 2010). It is disquieting that this emerging class of enzymes cannot be efficiently inhibited by any of the clinically approved β-lactamase inhibitors and is developing hydrolytic activity toward the heralded carbapenems (Szarecka et al. 2011).

Class D enzymes have a truncated sequence between helices α3 and α5, as well as between α8 and strand β7, resulting in a dramatically larger active site cleft (Fig. 3d). As for other SBLs, the class D enzymes contain four key active site sequence motifs, (i) S67XXK, (ii) S115XXV, (iii) the K205-T/S-G motif, and (iv) the Ω loop (Paetzel et al. 2000). When compared to the class A enzymes, the Ω loop is more compact and further from the active site core, which results in a more open substrate-binding

cleft. In addition, the (motif i) K70 is *N*-carboxylated to a varying extent depending on the particular OXA enzyme in question (Majiduddin et al. 2002). As in other SBLs, S67 (motif i) acts as a nucleophile to attack the β-lactam amide. However, it is thought that the role of carboxylation is to increase the basicity of K70 so as to serve as a more potent base to abstract a proton directly from S67 (Docquier et al. 2009). Furthermore, it is thought that the carboxylated K70 is positioned ideally to activate the deacylating water during hydrolysis (Golemi et al. 2001). The class D active site has significant hydrophobic character in proximity to K70, an environment that likely favors the free base form of lysine, promoting its carboxylation in the presence of carbon dioxide.

β-Lactamase Inhibitors

The identification of plasmid-borne TEM β-lactamase in gonococci in the late 1960s stimulated pharmaceutical companies to focus on the development of TEM-stable β-lactams and to discover inhibitors that could be used as part of a combination therapy to potentiate the activity of previously ineffective β-lactams. These efforts resulted in the development of β-lactam-based β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) (Payne et al. 1994). Initially, these compounds provided excellent antimicrobial properties against common Gram-negative pathogens harboring TEM-1 and SHV-1 type β-lactamases as well as penicillinases from the Gram-positive *S. aureus*. However, almost immediately after their introduction into clinical practice, bacteria began to produce new β-lactamase variants that were immune to inhibition by these compounds. Currently, there are several SBL inhibitors, both β-lactam and non-β-lactam, in clinical development. As this topic is beyond the scope of this chapter, we refer you to recent reviews on SBL inhibitors in current clinical development and MBL inhibitors in preclinical development (King and Strynadka 2012; Buynak 2013).

Target Modification

A common method by which bacteria avoid the action of antibiotics is via alteration of the intended target. Indeed, this mechanism is so successful that it can be found for every class of antibiotic, regardless of mechanism. These target alterations generally occur as a result of genetic mutations upon selective pressures in the presence of antibiotics. In other cases, however, modified targets may be acquired by way of genetic exchange. There are many examples of target modification among the PBPs from several bacterial types (reviewed in Lambert 2005). One of the best studied examples is the *S. aureus* PBP2a, responsible for high-level resistance in MRSA (Fig. 4). The encoding gene of PBP2a, mecA, resides on a large mobile genetic element called the staphylococcal chromosomal cassette (SCCmec), believed to have been acquired by horizontal transfer from a coagulase-negative staphylococcus species (reviewed in Shore and Coleman 2013). PBP2a is a high molecular weight class B D,D-transpeptidase that catalyzes the formation of cross-bridges in bacterial PG and is effectively resistant to most β-lactams at clinical concentrations. Together,

with the transglycosylase activities of other PBPs, it is capable of maintaining the biosynthesis of PG and securing bacterial survival in the presence of β-lactams (Kim et al. 2012; Pinho et al. 2001). Kinetic studies performed with several β-lactams (including penicillins, cephalosporins, and carbapenems) suggest that a combination of decreased noncovalent binding of these drugs to the active site, as well as reduced subsequent acylation may be responsible for the lower sensitivity of PBP2a (Fuda et al. 2004). The structure of PBP2a furthermore suggests that poor acylation may result from a β-strand alteration, resulting in a distorted active site, which must undergo energetically unfavorable conformational changes for acylation to occur (Lim and Strynadka 2002). It is also suggested that an allosteric mechanism involving binding of nascent PG may participate in rearrangements that facilitate preferential active site binding to substrates rather than inhibitors (Fuda et al. 2005; Otero et al. 2013), allowing MRSA to survive during antibiotic treatment. Modified PBPs are however not always acquired, but in many cases are derived from recombination or mutation events. For example, the PBP1a and PBP2× of *Streptococcus pneumoniae* (Pernot et al. 2004; Dowson et al. 1994) and the PBP2 of *Neisseria meningitidis* (Bowler et al. 1994) possess mosaic structures derived from recombination of PBP genes with those acquired from closely related species. Mutation-derived low-affinity PBPs are also encountered in the *Helicobacter pylori* PBP1 (Okamoto et al. 2002), the *Neisseria gonorrhoeae* PBP2 (Brannigan et al. 1990) and PBP1 (Ropp et al. 2002), and the *Haemophilus influenzae* PBP3 (Dabernat et al. 2002). Enterococci on the other hand possess intrinsically low-affinity PBPs. Interestingly, the crystal structure of the *Enterococcus faecium* PBP5fm reveals a loop residing on the edge of the catalytic cavity that confers increased rigidity and restricted access to the active site, a feature observed in other low-affinity PBPs such as the *S. aureus* PBP2a (Sauvage et al. 2002).

Signaling Pathways Involved in β-Lactam Sensing

Bacteria have developed intricate systems of sensing the presence of β-lactams in their surroundings and responding by regulating the expression of β-lactamases and β-lactam-resistant PBPs. Indeed, both Gram-negative and Gram-positive bacteria sense and orchestrate a response to β-lactam antibiotics in a process intimately linked to the PG recycling pathway.

Sensor-/Transducer-Mediated Regulation of β-lactam Resistance

Although biologically distinct, the expression of β-lactamases and nonsensitive PBPs (i.e., PBP2a) is regulated in a very similar fashion. In staphylococci and certain Gram-positive species such as *Bacillus licheniformis*, β-lactamase expression is controlled by the concerted action of the repressor BlaI and the sensor/transducer protein BlaR1. The genes encoding β-lactamase (*blaZ*), BlaI (*blaI*), and BlaR1 (*blaR1*) are organized on a single divergon, with the expression of all three under the control of the BlaI repressor, occupying a palindromic promoter region (Fig. 4). On exposure, BlaR1 acts as a sentinel by covalently binding the β-lactam antibiotic,

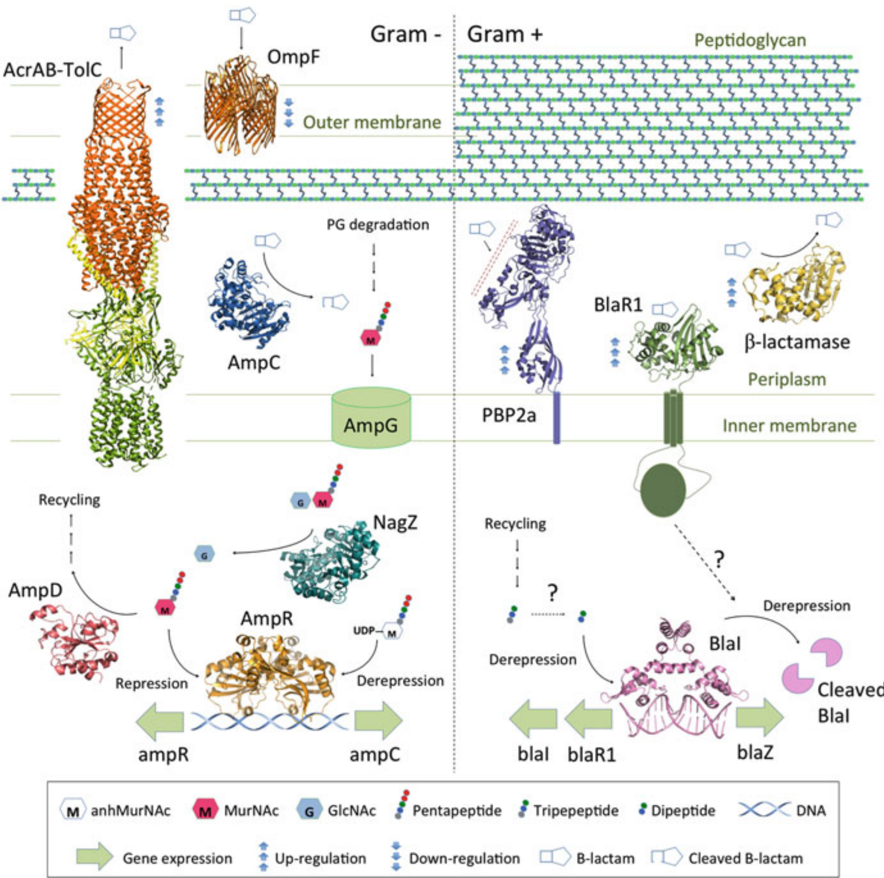

Fig. 4 Major mechanisms involved in β-lactam resistance. Reduced porin entry (e.g., OmpF, PDB ID: 2ZFG), increased efflux (e.g., AcrAB-TolC, PDB ID: 2F1M, 1OYE, 1EK9), β-lactam hydrolysis (e.g., AmpC, PDB ID: IKE4; β-lactamase, PDB ID: 1BLC), target modification (e.g., PBP2a, PDB ID: 1MWU), sensor-transducer signaling (e.g., BlaR1 sensor domain, PDB ID: 1XA7; BlaI, PDB ID: 1XSD), and murein recycling (AmpG; NagZ, PDB ID: 1TR9; AmpD, PDB ID: 2Y28; AmpR, PDB ID: 3KOS; BlaR1 sensor domain, PDB ID: 1XA7; BlaI, PDB ID: 1XSD) all play a part in resistance to β-lactams. Porin and efflux pump regulation and modification play important roles in Gram-negative resistance, whereas Gram-positive bacteria that lack an outer membrane rely heavily on β-lactamase-mediated β-lactam inactivation and PBP target modification

the ultimate consequence of which is the release and cleavage of the BlaI repressor (Hackbarth and Chambers 1993). The BlaR1 signal/transducer is a bi-modular protein consisting of an extracellular sensor domain linked via a transmembrane four-helix bundle to a cytoplasmic protease domain. The sensor domain of BlaR1 (BlaRs) shares the greatest structural homology and mechanistic similarity with the class D β-lactamases (Figs. 3d and 4; Birck et al. 2004; Wilke et al. 2004). Upon contact with β-lactams, BlaRs is irreversibly acylated at its active site serine,

concurrent with cleavage of the β-lactam ring. Much like the class D β-lactamases, this acylation event is proposed to be mediated by an adjacent *N*-carboxylated lysine that deprotonates the nucleophilic serine (Golemi et al. 2001). However, unlike the class D β-lactamases, the carboxylated lysine of BlaR1 is proposed to be unstable and is rapidly decarboxylated following serine acylation, such that it is no longer able to activate a water molecule for subsequent deacylation (Cha and Mobashery 2007). In this manner, the stability of the carboxylated lysine may have been decisive in the evolution of the respective β-lactamase vs. β-lactam sensor functions. The cytosolic protease domain of BlaR1 (BlaRp) harbors the signature HEXXH and EXXXD motifs common to the gluzincin family of zinc metalloproteases and is believed to be in a zymogenic state until activated by auto-cleavage (Zhang et al. 2001). This cleavage event is believed to be initiated by the β-lactam acylation of BlaRs, resulting in conformational changes in the transmembrane bundle that carry the signal across the bilayer (Zhang et al. 2001). Although the ultimate result of BlaRp activation is the cleavage of BlaI, it is not yet certain whether BlaI is the direct substrate of BlaRp or the end result of a proteolytic relay (Llarrull and Mobashery 2012; Amoroso et al. 2012).

Interestingly, the *mec* operon of MRSA strains has analogous molecular players to that of bla operon, yet is involved in the regulation of PBP2a expression. Here, genes encoding the signal/transducer MecR1 (mecR1), PBP2a (mecA), and the transcriptional repressor MecI (mecI) are similarly organized on a single divergon under control of a repressor (either MecI or BlaI, as is discussed below). The MecR sensor domain (MecRs) displays a similar overall fold to BlaRs and likewise forms acylated β-lactam intermediates via the active site serine (Marrero et al. 2006). MecR1 also possesses a four transmembrane helical bundle as well as a cytoplasmic metalloprotease domain. Although most of the data concerning the mechanism of signal transduction described above has been obtained from the bla pathway, the many parallels in the mec system suggest strongly that it also adopts a similar induction mechanism. Indeed, in *Macrococcus caseolyticus*, a bacteria whose genus is closely related to that of staphylococci, the mec divergon takes a peculiar form: instead of the mecA-mecR1-mecI present in SCCmec, the divergon is composed of blaZ-mecA-mecR1-mecI. This structure is considered the ancestral form of the mec gene complex that is thought to have been generated by the integration of the mecA gene into the bla divergon, blaZ-blaR1-blaI (Baba et al. 2009; Tsubakishita et al. 2010). Indeed, BlaI and MecI have been shown to be interchangeable as repressors of both the bla and mec divergons (McKinney et al. 2001), an observation in agreement with their similar overall structures and active site composition. BlaR1 and MecR1 are, however, only specific for their cognate repressors and display distinct kinetics, where BlaR1 induces β-lactamase in a matter of minutes in contrast to MecR1 which requires hours for PBP2a induction (McKinney et al. 2001). MecI is a stronger repressor of mecA as compared to BlaI (McKinney et al. 2001), while MecR1 is much less efficient than BlaR1 in sensing β-lactams (Cha et al. 2007). It is perhaps for this reason that certain highly resistant MRSA strains have evolved to rely on the efficient bla system for the expression of PBP2a rather than the mec

machinery which in these strains is defective or altogether absent (Archer et al. 1994; Ryffel et al. 1992; Milheirico et al. 2011).

PG Recycling and Regulation of β-Lactam Resistance

In Gram-negative bacteria, radiolabelling experiments have revealed that a surprisingly small amount of labeled PG is lost per generation and is instead recycled and reincorporated into new PG(Goodell 1985). We are now beginning to understand the importance of PG recycling to β-lactam resistance, where fluctuations in the cytoplasmic pool of broken-down PG fragments allow bacteria to gage the state of the cell wall, alerting them to the possible presence of β-lactams. In *E. coli*, the major recycling pathway involves the cytoplasmic transport of anhydro-muropeptides. These PG fragments are generated by the lytic transglycosylases and require the permease AmpG for delivery to the cytoplasm (Jacobs et al. 1994), where they are further hydrolyzed by the glucosaminidase NagZ to anhydro-MurNAc peptides and subsequently by the amidase AmpD to precursors for recycling (Fig. 4) (reviewed in Johnson et al. 2012). In the presence of β-lactams, there is an increase in the cytoplasmic pool of anhydro-MurNAc peptides that compete for binding to the transcriptional activator, AmpR. This leads to the induction of the β-lactamase, AmpC, and the subsequent breakdown of the antibiotic. Although the recycling pathway in Gram-positive bacteria is far less understood, it appears that these bacteria rely on lysozyme-like muramidases rather than lytic transglycosylases to produce MurNAc-containing muropeptides. These peptides and their breakdown products are postulated to be transported to the cytoplasm via conserved ABC transporters or the phosphotransferase system for recycling (Reith and Mayer 2011). Recently, it has been demonstrated that in *B. Licheniformis*, a cell-wall-derived dipeptide fragment, γ-D-Glu-m-DAP, is capable of binding the BlaI (MecI) repressor, leading to its dissociation from the bla operon and subsequent induction of the β-lactamase, BlaP (Amoroso et al. 2012). Although the exact pathway for the generation of the dipeptide has not yet been discerned, this finding draws an interesting parallel between recycling and antibiotic resistance in Gram-positive and Gram-negative bacteria.

Regulation of β-Lactam Entry and Efflux

β-Lactams are among the few drugs effective against both Gram-positive and Gram-negative strains, facilitated by the accessibility of the PBP targets that reside on the outer leaflet of the cytoplasmic membrane. Nevertheless, some Gram negatives such as *P. aeruginosa* are notorious for their resistance to β-lactams and other drugs, using cell wall modifications and changes to outer membrane architecture to create a near-impenetrable barrier. The two most well-understood mechanisms that regulate this drug-resistance phenomenon at the Gram-negative outer membrane are the restricted entry of drugs via the alteration or loss of porins and their active expulsion via efflux pumps.

Porin-Mediated Resistance

Porins are the most abundant proteins in the outer membrane of bacteria and act as selective permeable barriers that facilitate the uptake of nutrients and confer protection against harmful compounds. These water-filled channels act as molecular sieves that allow hydrophilic molecules below a specific exclusion limit to enter as determined by the channel diameter. High-resolution structures of porins have revealed a large similarity in architecture, with small variations in loop topology and surface charge. Porins lack a classic hydrophobic region and instead consist of transmembrane antiparallel β-strands with alternating hydrophobic and hydrophilic residues that, respectively, line the membrane- and water-exposed surfaces of the β-barrel. In general, the higher strand number β-barrel porins act as channels, often in a stable trimeric form, with those consisting of 18 β-strands often classified as substrate-specific porins and the 16 β-strand variants acting as general porins with typically less stringent substrate specificity. In Gram negatives, general porins are believed to be the primary route of entry for many antibiotics including small hydrophilic drugs such as β-lactams, tetracycline, chloramphenicol, and fluoroquinolones (reviewed in Galdiero et al. 2013). With pore exclusion limits close to the size of many antibiotics, general porins can act to promote or limit the diffusion rate of these compounds. Dependent on the bacterial species, the number and type of porins can also dictate the degree of susceptibility and intrinsic resistance of the organism (pseudomonas is a prominent example). Bacteria can limit porin-mediated drug entry either by adaptive or mutational means. In the first instance, porin expression can be modified upon antibiotic exposure by responsive regulatory elements (Farra et al. 2008). In the second instance, disruptive mutations in the porin-encoding gene can lead to either loss or defect in the porin, and mutations in the promoter region or regulatory elements can likewise decrease porin levels (Doumith et al. 2009). For example, the loss of OmpF (Harder et al. 1981) or mutations in OmpC around the point of pore "constriction" (Lou et al. 2011) is involved in β-lactam resistance in *E. coli*, and resistance to carbapenems in *P. aeruginosa* has been linked to a combination of pore-modulating loop mutations or complete loss of the general porin OprD (Huang and Hancock 1996), as well as regulatory mutations involving the MexT transcriptional repressor (Ochs et al. 1999) (reviewed in Fernandez and Hancock 2012).

Antibiotic Efflux

Bacterial efflux pumps may be categorized as primary or secondary transporters, the first type being driven by ATP hydrolysis and the second by proton motive force. These pumps are further categorized into five major superfamilies: the major facilitator superfamily (MFS), the ATP-binding cassette superfamily (ABC), the small multidrug-resistance family (SMR), the resistance-nodulation-cell division superfamily (RND), and the multi-antimicrobial extrusion protein family (MATE). Of these, only ABC family members are primary transporters, all others being secondary transporters using proton (MFS, RND, SMR, MATE) or sodium (MATE) antiport (Brown et al. 1999). Efflux pumps are often capable of recognizing multiple substrates, since affinity tends to be based on physiochemical characteristics such as

hydrophobicity, aromaticity, or charge rather than a distinct structural chemistry. This can account for the prevalence of multidrug-resistance (MDR) efflux pumps, found in all five major superfamilies, which can capture and expel many structurally diverse antibiotics, in addition to nonantibiotic compounds (reviewed in Lewis (1994), Lomovskaya et al. (2007)). In Gram-positive bacteria, MSF efflux systems, such as the *S. aureus* NorA (Deng et al. 2012), are mainly responsible for MDR efflux. In Gram-negative bacteria, the MDR phenotype is largely conferred by RND efflux systems such as MexAB-OprM and AcrAB-TolC, contributing to the intrinsic resistance of *P. aeruginosa* and *E. coli*, respectively, to β-lactams and other antibiotics.

RND pump complexes are assembled as tripartite membrane machineries, composed of the RND pump located in the cytoplasmic membrane and two accessory proteins: a periplasmic adaptor protein of the membrane fusion protein (MFP) family and an outer membrane channel belonging to the outer membrane factor (OMF) family. As such, this complex is able to expel substrates across the entire cellular envelope and out of the cell. Interestingly, it has been found that dianionic β-lactams such as carbenicillin which are not able to cross into the cytoplasm are nevertheless expelled from the cell, suggesting that the RND complex is able to capture these substrates from the periplasm or from the periplasmic-cytoplasm interface (Li et al. 1994a; Nikaido and Takatsuka 2009). Although no structure currently exists for the intact tripartite complex, the atomic structures of each of the three pump components are available along with proposed composite models (Symmons et al. 2009; Su et al. 2011). The RND pump is typically trimeric with a total of 12 transmembrane helices and two large periplasmic loops (Murakami et al. 2002). The pump appears to function in a rotatory manner driven by alternate protonation of individual subunits in a process that results in substrate capture and subsequent release (Pos 2009; Murakami et al. 2006; Seeger et al. 2006). The periplasmic MFP adaptor protein is proposed to stabilize weak interactions between the RND and OMF and consists of an extended β-barrel connected by a lipoyl domain to a long periplasmic α-helical hairpin (Higgins et al. 2004; Mikolosko et al. 2006). The OMF channel protein consists of a trimeric arrangement that collectively forms a 12-stranded β-barrel inserted into the outer membrane, attached to an extended ~100 Å coiled-coil α-helical domain that protrudes into the periplasm (Koronakis et al. 2000), providing an iris-like mechanism to regulate access of small-molecule substrates (Andersen et al. 2002; Bavro et al. 2008) (Fig. 4). These observations are based on a wealth of structural information, reviewed in Hinchliffe et al. (2013).

In Gram-negative bacteria, MDR efflux pumps can form a near-impenetrable barrier against antimicrobial agents. Certain species of Gram-negative bacteria such *P. aeruginosa* and *Acinetobacter* spp. are notorious for efflux-mediated β-lactam resistance. For example, in *P. aeruginosa*, upregulation of the MexAB-OprM (RND) combined with the organisms low outer membrane permeability can contribute to increased resistance to penicillins and cephalosporins (Li et al. 1994b), and the overexpression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* can contribute to a higher level of resistance to most β-lactams including carbapenems (Heritier et al. 2005). It has also been demonstrated that AcrAB-TolC

(RND) efflux plays a key role in β-lactam uptake and susceptibility in *H. influenzae* (Kaczmarek et al. 2004) and *K. pneumoniae* (Pages et al. 2009).

Future Perspectives

Antibiotic resistance is a constant threat that must be challenged with the same innovation and ingenuity by which it emerges. This ongoing struggle will likely rely on various combined strategies, some of which are touched upon here. Given that porin loss or downregulation is an effective way of limiting antibiotic entry, clever attempts are being made to avoid this form of resistance by taking advantage of other bacterial uptake machinery. For example, as many bacteria are heavily reliant upon iron for their survival, they secrete iron-chelating molecules known as siderophores that upon iron binding are transported back into the bacteria (reviewed in Saha et al. 2012). By fusing β-lactam and other drugs to siderophores, it is possible to bypass the need for porins to cross the bacterial outer membrane (reviewed in Mollmann et al. 2009). A promising example of this is the siderophore sulfactam BAL30072, currently in phase 1 clinical study (Landman et al. 2014). Similarly, inhibition of bacterial efflux systems is an appealing anti-resistance strategy, although past efforts with promising drugs have often faced in vivo toxicity related to cross inhibition of efflux pumps in human cells. However, certain phenothiazines, some of which are previously approved antipsychotic drugs, have recently been used for the successful efflux-targeted treatment of extremely drug-resistant mycobacterium tuberculosis (Abbate et al. 2011; Amaral and Molnar 2012; Amaral et al. 2008). Another approach is the development of new β-lactam and β-lactamase inhibitor combinations. Indeed, several examples of these are in phase 3 clinical trials, including ceftaroline-avibactam (Castanheira et al. 2014), ceftazidime-avibactam (Keepers et al. 2014), and ceftolozane-tazobactam (Sader et al. 2014). In addition, synergistic combinations of β-lactams and glycopeptides in overcoming resistance in strains such as VRSA have proven useful both in vitro and in animal models and may provide a viable option for patient treatment (Fox et al. 2006; McConeghy et al. 2013). Other non-β-lactam-related strategies are also being explored in the fight against resistance, including the use of the peptide antibiotics, vaccination, and phage therapy, to name a few (reviewed in Jovetic et al. 2010).

Conclusion

Fleming's fortuitous and triumphant discovery of the "miracle drug," penicillin, was shortly followed by the sobering observation that the improper use of the drug led to rapid bacterial resistance in the clinic and community. Indeed, resistance is a natural consequence of general antibiotic use against microorganisms that are exquisitely designed to persist against threats to their survival. As it is imperative to keep pace with continuing resistance by way of new antibiotic design, it is likewise increasingly necessary to understand resistance processes in order to expose strengths and

weaknesses that may be exploited. It is furthermore of the utmost importance that we use our already present antibiotic artillery wisely and conscientiously, particularly with regard to the precious β-lactams that arguably distinguish themselves as the most valuable drugs in human history.

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Antibiotic Resistance and Tolerance in Bacterial Biofilms

Geoffrey McKay and Dao Nguyen

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Abstract

Bacteria can grow as multicellular communities called biofilms, and this sessile lifestyle is distinct from planktonic growth. While microbial biofilms are ubiquitous in the natural and industrial environment, their importance in human infections has only been fully recognized in the past few decades. Biofilm-associated bacteria typically cause subacute and chronic infections. Many bacterial pathogens, such as *Staphylococcus aureus,* readily form biofilms, and *Pseudomonas aeruginosa,* which causes chronic airway infections in patients with cystic fibrosis, is an important model organism for biofilm studies. They are clinically significant due to their persistence despite sustained antimicrobial treatments and adequate host defenses. Biofilm bacteria are highly resistant to a wide range of antimicrobial compounds and disinfectants, and the mechanisms underlying this resistance are likely multifactorial. This chapter will review the cellular processes and pathways implicated in antibiotic resistance and tolerance of bacterial biofilms.

Keywords

Biofilms • Antibiotic • Resistance • Tolerance • Infections • Pseudomonas aeruginosa

Introduction

Bacteria can grow as multicellular communities called biofilms, and this sessile lifestyle is distinct from planktonic growth. While microbial biofilms are ubiquitous in the natural and industrial environment, their importance in human infections has only been fully recognized in the past few decades (Potera 1999; Costerton et al. 1999; Parsek and Singh 2003). Biofilm-associated bacteria cause many subacute and chronic infections, ranging from periodontitis to chronic wound infections, endocarditis, chronic otitis media, osteomyelitis, recurrent urinary tract infection, medical device and indwelling catheter infections, and cystic fibrosis-associated airway infections. They are clinically significant due to their persistence despite sustained antimicrobial treatments and adequate host defenses. Many bacterial pathogens, such as *Staphylococcus aureus,* readily form biofilms, and *Pseudomonas aeruginosa,* which causes chronic airway infections in patients with cystic fibrosis, is an important model organism for biofilm studies. Biofilms are also often polymicrobial communities composed of multiple bacterial species.

Biofilms: Multicellular Communities Distinct from Unicellular Planktonic Organisms

Biofilms are multicellular aggregates, in which cells are encased in an extracellular polymeric matrix. In many natural and *in vivo* conditions, as well as in laboratory models, biofilm aggregates are adherent to a surface. For example, biofilms can grow attached to indwelling medical devices or dental surfaces. Biofilm aggregates can also be suspended at the air-liquid interface or within a semisolid environment, such as in the viscous sputum of cystic fibrosis patients (Ude et al. 2006; Alhede et al. 2011; Staudinger et al. 2014). Biofilm structures allow bacteria to grow at high-cell density and in close proximity, in contrast to planktonic bacteria growing as free-living individual cells in a liquid environment. The biofilm environment facilitates cell-cell communication (Parsek and Greenberg 2000) and horizontal transfer of virulence traits (Hausner and Wuertz 1999). In polymicrobial communities, the close proximity also promotes interspecies communication, metabolic and functional cooperation, or competition between different species. These complex interactions have implications on disease pathogenesis, host-pathogen relationships, as well as resistance to antibiotics and host defense mechanisms.

Biofilms differ significantly from planktonic bacteria on many levels. Global transcriptomic or proteomic studies have shown wide-ranging changes, with hundreds of genes and as much as 50 % of the proteome being differentially expressed during biofilm growth (Whiteley et al. 2001; Beenken et al. 2004; Folsom et al. 2010; Dotsch et al. 2012; Waite et al. 2005; Southey-Pillig et al. 2005; Sauer et al. 2002). It is important to note that laboratory biofilm models vary significantly, from continuous flow systems, 96-well plates, to colonies on agar surfaces or membranes, and such diverse experimental conditions have generated conflicting results on the differences between planktonic and biofilm cells (Mikkelsen et al. 2007). Although there are no hallmark gene or cellular processes that define biofilm bacteria, they typically have reduced motility, have increased production of extracellular polysaccharides, and can express specific protective factors not present during planktonic growth (Whiteley et al. 2001; Sauer et al. 2002).

In many bacterial species, the transition to biofilm growth is also associated with the downregulation of acute virulence factors, and this contributes to the persistence and chronic nature of biofilm infections. Biofilm bacteria are less invasive, causing less tissue destruction, and expression of key ligands recognized by the immune system, such as flagella, is repressed (Whiteley et al. 2001). Many studies have described the transition from planktonic to biofilm growth as an ordered process, starting with surface attachment, leading to microcolony formation and maturation, and followed by dispersal of biofilm cells back to a planktonic state (Sauer et al. 2002; O'Toole et al. 2000). While some experimental systems support the notion that biofilm formation is the result of such a structured process, this is likely not necessary nor universal across different bacterial species and conditions (Alhede et al. 2011; Staudinger et al. 2014; Sriramulu et al. 2005). Much research has also investigated the role of cell signaling in biofilm formation. Quorum sensing is a cell-cell signaling system in many gram-negative and

gram-positive bacteria that controls cell density-dependent gene expression via the secretion and detection of chemical signals that accumulate as cell density increases. While the chemical signals and genes under quorum sensing control differ between different bacterial species, quorum sensing allows bacteria to respond and coordinate their gene expression in response to population density (Waters and Bassler 2005). In *P. aeruginosa*, quorum sensing can alter biofilm formation through multiple different pathways: swarming (a form of surface motility), production of rhamnolipids (a surfactant that influences biofilm structures), LecA (a carbohydrate-binding lectin involved in surface adhesion), or the exopolysaccharide Psl (Shrout et al. 2011). In recent years, the second messenger cyclic-di-GMP (c-di-GMP) has emerged as a conserved signal that controls the switch between motile planktonic states and sessile biofilm formation in several bacterial species (Romling et al. 2013).

Biofilms: A Sheltered Lifestyle that Confers High Resistance and Tolerance to Antibiotics

Biofilm growth confers many advantages to bacteria, allowing them to better survive and adapt to diverse environmental stresses encountered in the natural and host environment. A hallmark of biofilm bacteria is their remarkable resistance and tolerance to a wide range of biocides, antibiotics, and host defenses, which play a key role in their persistence during chronic infections. Multiple mechanisms are at work, some regulated through biofilm-specific mechanisms, while others are physiological adaptions to the biofilm microenvironment.

Antibiotic resistance typically refers to an inheritable trait that allows bacteria to replicate at elevated concentrations of antibiotics and is defined by increased minimal inhibitory concentrations (MIC) of antibiotics. Such resistance mechanisms may be intrinsic or acquired and include antibiotic-modifying enzymes, genetic mutations of target molecules, low membrane permeability, or drug efflux pumps. While the term biofilm “resistance” is widely used in the literature, this term can be misleading as it often refers to tolerance (also variably called non-inherited resistance, drug indifference, or persistence), a phenotypic characteristic where bacteria are refractory to antibiotic or biocide killing in the absence of inheritable resistance mechanisms or genetic mutations. Distinct from resistant bacteria, tolerant ones remain viable but do not grow at elevated concentrations of antibiotics. This phenotypic tolerance is also reversible when biofilm bacteria resume a planktonic state (Anwar et al. 1989). This chapter will be focused on the mechanisms involved in both antibiotic resistance and tolerance of biofilms. While the concepts of resistance and tolerance are distinct, the contribution of specific mechanisms to one or the other is not always well defined, and many studies do not make this distinction. For this review, the term tolerance will be used whenever the studies specifically defined and addressed this phenotype.

Biofilm-Specific Mechanisms of Resistance

Matrix and Diffusion Barriers

Biofilm bacteria are embedded in a self-produced extracellular matrix (also termed extracellular polymeric substances or EPS) typically composed of extracellular polysaccharides, DNA, and proteins. The matrix can represent up to 90 % of the biofilm biomass, and its exact composition, while often poorly characterized, can vary considerably dependent on the strains, species, and growth conditions (such as nutrient source and shear forces) (Zogaj et al. 2001; Branda et al. 2005). The EPS provides mechanical structure to multicellular aggregates and promotes physical aggregation and surface adhesion. Due to its physicochemical properties, the EPS acts as a molecular sieve that can confer a protective barrier against some antimicrobial compounds, including disinfectants and antibiotics. While the diffusion barrier and limited penetration of antimicrobials were originally thought to play a primary role in biofilm resistance, more recent research suggests that its contribution to the overall biofilm resistance is more limited.

Extracellular Polymeric Substances (EPS)

Exopolysaccharides are a major component of the EPS in several bacterial species, such as *Bacillus subtilis, P. aeruginosa*, and *S. aureus* (Branda et al. 2005). These polymers are composed of different polysaccharides, from sucrose-derived glucans and fructans, cellulose, or mixtures of neutral and charged heteropolysaccharides. Their chemical composition and substituents determine the physicochemical properties of the EPS. Most known exopolysaccharides are polyanionic, but polycationic exopolysaccharides such as b-1,6-linked *N*-acetylglucosamine are found in staphylococcal biofilms, for example (Gotz 2002). The exopolysaccharide composition varies considerably from one species to another and even between strains of the same species.

Exopolysaccharides have been identified in biofilms from natural environments, laboratory experimental systems, as well as biofilms associated with human infections. Mutants lacking components of the EPS produce biofilms with altered morphology or reduced surface adhesion. While exopolysaccharides clearly have a role in the adhesion and structure of biofilms, their role in antibiotic resistance is still poorly defined. Exopolysaccharides may sequester charged molecules or indirectly alter the EPS structure. Across several studies comparing biofilm resistance to different antibiotics, the positively charged aminoglycosides appear to be most susceptible to the sequestering effect of exopolysaccharides.

Pseudomonas aeruginosa produces at least three chemically distinct polymers for its EPS: alginate, Pel, and Psl. Alginate is a polymer of uronic acids and among the best-studied exopolysaccharides. While alginate is not required for biofilm formation (Wozniak et al. 2003) and is not produced by all *P. aeruginosa* strains, it is

overproduced by mucoid *P. aeruginosa* variants isolated from cystic fibrosis patients. In several laboratory biofilm models, alginate-overproducing strains formed biofilms more tolerant to aminoglycoside treatment than wild-type strains (Alkawash et al. 2006), and alginate can inhibit diffusion of aminoglycosides (Gordon et al. 1988), suggesting that alginate has a protective role against aminoglycosides. Studies with Pel-deficient mutants or Pel-overexpressing strains show a limited role in biofilm resistance to aminoglycosides and aztreonam, but the contribution of Pel is likely strain-specific and most significant in the Psl-negative strain PA14 (Colvin et al. 2011; Khan et al. 2010; Yu et al. 2012). Psl may also be involved in resistance to detergents, as Psl-deficient mutants form biofilms susceptible to polysorbate-80, while Psl overproduction confers resistance (Zegans et al. 2012). The mechanism of this resistance remains unknown.

Extracellular Proteins

Various extracellular proteins can accumulate within the biofilm EPS. Many enzymes are involved in the degradation of EPS constituents, while others nonenzymatic proteins are involved in EPS stability and structure, such as the carbohydrate-binding proteins (lectins) (Lynch et al. 2007; Diggle et al. 2006). Periplasmic beta-lactamases may be released in the EPS following cell death or by membrane vesicles, but their contribution to biofilm resistance to beta-lactams remains unclear (Ciofu et al. 2000).

Extracellular DNA

Extracellular DNA (eDNA) is passively or actively released by bacteria, and can be a major constituent of the EPS in certain bacterial species. eDNA primarily provides a structural role and enhances aggregation and stability of biofilms. Interestingly, exogenous eDNA can induce resistance to cationic antimicrobial peptides and aminoglycosides in *P. aeruginosa* by sequestering cations and inducing PhoPQ/PmrAB-mediated LPS modifications (Mulcahy et al. 2008). However, it remains unclear whether the eDNA in the biofilm EPS is sufficient to induce the PhoPQ/PmrAB systems (Pamp et al. 2008).

Diffusion of Antimicrobial Compounds

The biofilm EPS contains a mixture of charged or hydrophobic polymers that can sequester or limit the penetration of molecules. Although the protective barrier function of the EPS was initially proposed as a major mechanism of biofilm resistance, many studies now suggest that this diffusion limitation only plays a minor role. Biofilms grown on dialysis membranes retarded the diffusion of piperacillin, a weakly anionic beta-lactam, while high Ca^{2+} concentrations further

reduced its diffusion (Hoyle et al. 1992). *Pseudomonas aeruginosa* biofilms sequestered and delayed the diffusion of tobramycin (a positively charged aminoglycoside) but not ciprofloxacin (a neutral fluoroquinolone) (Walters et al. 2003; Shigeta et al. 1997; Yasuda et al. 1993; Beloin et al. 2004). Kumon et al. examined the diffusion of antimicrobials through alginate: only aminoglycosides and polypeptides showed limited diffusion, while beta-lactams, fluoroquinolones, and macrolides did not (Kumon et al. 1994). Biocides such as hypochlorite also do now show any significant limitation in diffusion (Stewart et al. 2001). Taken together, these studies show that the diffusion rate and chemical structure of specific compounds do not correlate with their efficacy against biofilms. Among antibiotics, only aminoglycosides show a degree of limited penetration, and this likely does not fully account for the high tolerance of biofilms to these compounds (Vrany et al. 1997; Stewart 1996).

Other Biofilm-Specific Mechanisms

Liao et al. reported on BrlR, a Mer-like transcriptional regulator involved in both antibiotic tolerance and resistance to multiple antibiotics in *P. aeruginosa*. Inactivation of *brlR* reduced biofilm tolerance without altering susceptibility in planktonic bacteria (Liao and Sauer 2012). This is likely due to its high expression during biofilm but not planktonic growth. While the mechanism of BrlR is still unknown, MerR family members can activate the expression of multidrug efflux pumps. Since *brlR* is induced under oxygen-limiting conditions (Trunk et al. 2010), Liao et al. speculate that such conditions may lead to high *brlR* expression in biofilms.

Other examples of biofilm-specific mechanisms of antibiotic resistance include periplasmic glucans. In *P. aeruginosa*, *ndvB* encodes a glucosyltransferase required for the synthesis of periplasmic glucans, which is preferentially expressed during biofilm growth and may contribute to biofilm resistance by sequestering antibiotics (Mah et al. 2003).

Physiologic Heterogeneity

In contrast to well-mixed planktonic bacterial cultures, biofilms are bacterial aggregates where subpopulations encounter diverse microenvironments, leading to a physiologically heterogeneous population (Fig. 1). This heterogeneity is therefore a central characteristic of biofilm communities that confers antibiotic tolerance to biofilm bacteria through multiple different mechanisms (Stewart and Franklin 2008).

The heterogeneous physical environment, and the nutrient consumption and metabolism by spatially distinct bacterial subpopulations lead to gradients in nutrient and oxygen availability within biofilm aggregates (Stewart and Franklin 2008). The microenvironment within biofilms also changes over time, as the biofilm structure and surrounding physicochemical environment change during biofilm development and maturation. Different bacterial subpopulations therefore respond to their local

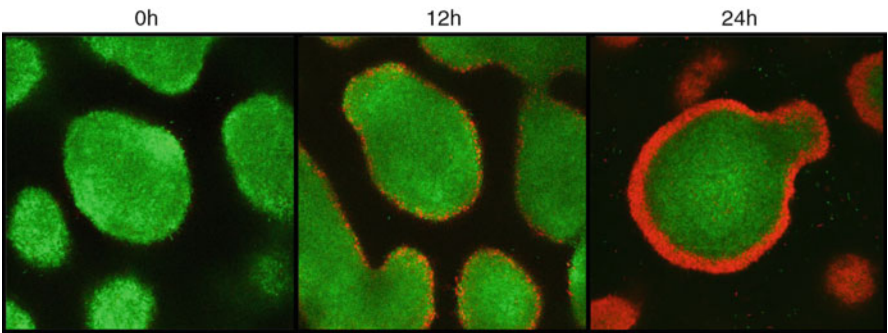

Fig. 1 Confocal microscopy images of *P. aeruginosa* biofilms grown in flow cell chambers. Mature biofilms are challenged with tobramycin for 0–24 h: bacterial killing occurs only in a subpopulation of biofilm cells located in the "outer layers" of biofilm aggregates. Viable bacteria are labeled with GFP (*green*) and dead cells are stained with propidium iodine (*red*) (Images were kindly provided by Pradeep Singh (U. Washington))

environments and express distinct biological activities, metabolic pathways, and stress responses. For example, biofilm bacteria may respond to microaerophilic or anaerobic conditions, pH stress, or nutrient limitation. Concentrations of solutes such as nitrate (Schramm et al. 1996), hydrogen peroxide (Lu et al. 1998), chlorine (De Beer et al. 1994a), or oxygen (Borriello et al. 2004) have been determined experimentally. This creates gradients in growth rate and metabolic activity and induces stress responses.

Several studies using reporter fusions (e.g., GFP, LacZ, alkaline phosphatase), dyes staining for nucleic acids or cell membrane permeability clearly demonstrated the physiological heterogeneity of biofilm communities (Stewart and Franklin 2008; Serra et al. 2013). Transcriptomic studies also support the importance of such environmental conditions on biofilm cells' physiology (Beenken et al. 2004; Folsom et al. 2010; Dotsch et al. 2012). However, it is worth noting that these global approaches, which typically analyze the bacterial population as an average, are not well suited to capture the heterogeneity present within biofilms (An and Parsek 2007). The total RNA abundance as well as the amount of specific RNA sequences can vary by over 100-fold in different biofilm subpopulations when measured by laser capture microdissection microscopy combined with qRT-PCR (Lenz et al. 2008; Pérez-Osorio et al. 2010).

Metabolic and Growth Rate

It has been long reported that the ability of antibiotics (such as β-lactams) to kill bacteria was linked to bacterial growth rate. Using chemostat systems where the rate of bacterial replication is controlled by the influx of nutrients, the killing rate of *Escherichia coli* by beta-lactam antibiotics was proportional to the bacterial generation time (Tuomanen et al. 1986; Cozens et al. 1986; Evans et al. 1991;

Brown et al. 1988). Thus, nongrowing cells are generally tolerant to antibiotics. Using an IPTG-inducible GFP reporter as an indicator of metabolic activity, Kim et al. sorted biofilm cells by flow cytometry based on their GFP fluorescence. By challenging the bright (active) and dim (dormant) cells with antibiotics or biocides, they determined that active cells were slightly more tolerant to tobramycin and silver ions but not ciprofloxacin (Kim et al. 2009). These results suggest that metabolic activity may determine tolerance to certain antibiotics (such as aminoglycosides) but not others (such as fluoroquinolones). The latter observation is not surprising, with others observing that fluoroquinolones are effective at killing non-replicating or stationary phase cells (Dalhoff et al. 1995; Zeiler 1985).

It has been frequently suggested that biofilm growth leads to steep gradients in growth rate and metabolic activity, and slow-growing or metabolically inactive subpopulations are thus more tolerant to antibiotic killing. For example, using an *rrnBP1*-GFP reporter where the growth rate-regulated *rrnBP1* promoter is fused to a short half-life GFP reporter, Sternberg et al. monitored the rates of rRNA synthesis in *E. coli* biofilms (Sternberg et al. 1999). With confocal microscopy imaging, they detected subpopulations in the center of biofilm aggregates showing reduced metabolic rate, while those in the outer layers of biofilm aggregates had high metabolic rate. Similar spatial patterns were observed by several others (Walters et al. 2003; Werner et al. 2004). While the use of GFP-based reporters provides elegant visualization of live biofilm cells over time and space without disrupting biofilm growth and structures, GFP does not mature under anaerobic conditions. These fluorescent reporters can thus pose a limitation in inferring metabolic or growth rate in the presence of oxygen gradients and anaerobic microenvironments within biofilms (as described in the next section).

Using laser capture microdissection microscopy combined with qRT-PCR, Lenz et al. and Perez-Osorio et al. demonstrated that mRNA levels for individual genes vary significantly across different biofilm sections in *P. aeruginosa*, using both drip flow biofilm and colony biofilm models (Lenz et al. 2008; Pérez-Osorio et al. 2010). These included housekeeping genes (*acpP*), quorum sensing (*rhlR*), and quorum sensing-regulated genes (*aprA, phzA1*) and the stationary phase sigma factor *rpoS*. Furthermore, Lenz et al. showed that the 16S rRNA to rDNA ratio varied across different biofilm layers, with the inner bacterial subpopulations showing rRNA/rDNA ratio similar to stationary phase cells and the outer layer subpopulations showing a higher rRNA/rDNA ratio closer to exponential phase cells (Lenz et al. 2008).

Oxygen Gradients

The oxygen gradient is primarily determined by the rate of consumption by the layers of cells first exposed to oxygen but also by the overall balance of diffusion. The role of oxygen availability in determining the local physiological responses has been well documented (Folsom et al. 2010; Xu et al. 1998). Significant oxygen gradients can be measured by microelectrodes in laboratory-grown biofilm structures

formed by aerobic or facultative anaerobic bacteria (Walters et al. 2003; Borriello et al. 2004; de Beer et al. 1994b), with oxygen concentrations declining within the depths of biofilm structures. The presence of anaerobic microenvironments explains the growth of strict anaerobes within aerated biofilms, such as dental plaques (Kolenbrander 2000). Several transcriptomic studies show activation of genes involved in anaerobic metabolism during biofilm growth (Folsom et al. 2010; Phillips et al. 2012; Patell et al. 2010). Furthermore, the *P. aeruginosa* terminal oxidases required for growth under microaerophilic conditions are also required for optimal biofilm growth (Alvarez-Ortega and Harwood 2007). Xu et al. also used alkaline phosphatase activity as a physiological indicator of biofilm bacteria in phosphate-limiting conditions. They demonstrated a gradient of alkaline phosphatase activity, primarily determined by oxygen concentration (Xu et al. 1998).

Oxygen availability to biofilm subpopulations correlates with their antibiotic tolerance (Walters et al. 2003; Borriello et al. 2004; Werner et al. 2004; Tresse et al. 1995). Borriello et al. showed that bacterial subpopulations growing in oxygen replete biofilm regions were more readily killed by several classes of antibiotics compared to subpopulations growing in oxygen-limited regions (Borriello et al. 2004). Similarly, Werner showed that antibiotic-induced cell damage (filamentation, cell vacuolization, and lysis) were predominantly at the air-biofilm interface, within regions of high protein synthetic activity (as measured by IPTG-inducible GFP) (Werner et al. 2004). Antibiotic killing was also reduced when the biofilms were transferred to anaerobic conditions, an observation also reported by others (Field et al. 2005; Hill et al. 2005). Collectively, these studies suggest that oxygen-limited subpopulations exist within biofilms, and these are more tolerant to antibiotics. Whether the tolerance is directly due to oxygen depletion or anaerobic conditions per se, or whether it is caused by an indirect reduction in metabolic/growth rate, is still unclear. In the case of *P. aeruginosa*, a facultative anaerobe, growth stops under anaerobic conditions in the absence of an alternate electron acceptor (such as nitrate) or fermentation substrate (such as arginine). Borriello et al. thus investigated whether stimulating bacterial anaerobic metabolism could restore antibiotic susceptibility (Borriello et al. 2006). They showed that arginine and nitrate supplementation enhanced ciprofloxacin and tobramycin killing of *P. aeruginosa* biofilms grown under anaerobic conditions but not aerobic conditions. These findings support the idea that oxygen limitation confers a degree of antibiotic tolerance due to its effect on growth and metabolic rate.

Stress Responses

During biofilm growth, several stress responses are activated, which in turn can regulate cellular pathways that confer antibiotic resistance and tolerance. Such stress responses may be induced by the environmental cues that trigger the switch from planktonic to biofilm lifestyles or by the microenvironment created by biofilm growth. Interestingly, nonoptimal growth conditions can promote biofilm formation in many bacterial species. For example, biofilm formation is increased during growth

in defined medium compared to rich medium (Castonguay et al. 2006; Wijman et al. 2007), and growth at reduced temperatures enhances surface adhesion and EPS production (Wijman et al. 2007; Perry et al. 2004; Sommerfeldt et al. 2009). Such stress may be environmental cues that promote cellular processes involved in both formation of biofilm structures as well as mechanisms of antibiotic resistance or tolerance.

Environmental and Nutrient Cues Affect Antibiotic Tolerance

Environmental and nutrient conditions have a significant impact on antibiotic tolerance and adaptive resistance, and biofilm growth creates nutrient gradients, with spatially distinct biofilm subpopulations experiencing nutrient limitation. While nutrient limitation may slow growth, it also induces specific stress responses that play an important protective role in antibiotic tolerance (Poole 2012). For example, cells in the central regions of *P. aeruginosa* biofilm aggregates are sufficiently limited for iron that expression of siderophores are induced as part of iron starvation responses (Kaneko et al. 2007; Banin et al. 2005). Iron limitation induces multidrug tolerance through several mechanisms. Importantly, intracellular iron homeostasis modulates the oxidative stress pathways that contribute to antibiotic killing (Kohanski et al. 2007; Frawley et al. 2013; Vinella et al. 2005; Yeom et al. 2010). Mg^{2+}, Ca^{2+}, and phosphate limitation also promote resistance to cationic antimicrobial peptides, and eDNA within the EPS matrix may chelate and limit Mg^{2+} and Ca^{2+} cations, as described in the section on eDNA (Macfarlane et al. 1999; Groisman et al. 1997). Finally, the carbon sources and transition can also affect antibiotic tolerance and the formation of persisters (Allison et al. 2011; Amato et al. 2013), suggesting that the nutrient environment within biofilms is an important determinant of antibiotic tolerance.

Stringent Response

The stringent response is a conserved bacterial stress response induced by amino acid, carbon, iron, and other nutrient limitation, as well as environmental stressors such as osmotic or temperature shifts (Cashel et al. 1996; Braeken et al. 2006). In response to stress, synthesis and accumulation of the signal (p)ppGpp (guanosine 5′-(tri) diphosphate, 3′-diphosphate), in cooperation with the action of DksA, trigger a widespread reorganization of cellular metabolism, macromolecule biosynthesis, and other protective cellular processes allowing stress adaptation and survival (Potrykus and Cashel 2008). (p)ppGpp likely promotes multidrug tolerance through multiple different mechanisms, from regulation of toxin-antitoxin modules and persister formation (Maisonneuve et al. 2011; Aizenman et al. 1996; Korch et al. 2003) to modulation of oxidative stress pathways (Khakimova et al. 2013; Nguyen et al. 2011). As a master regulator of the adaptive responses to nutrient and environmental stress, (p)ppGpp likely plays a central role in biofilm tolerance.

Nguyen et al. showed that *P. aeruginosa* and *E. coli* mutants lacking the (p)ppGpp synthetases RelA and SpoT, and thus unable to produce any (p)ppGpp, were profoundly susceptible to killing from multiple different classes of antibiotics, including quinolones, aminoglycosides, and antimicrobial peptides (Nguyen et al. 2011).

Oxidative Stress

Several observations support the idea that biofilms are an oxidative-stress-inducing environment. Oxidative DNA damage is increased and is associated with the increased genetic mutability in several species (Boles and Singh 2008; Ryder et al. 2012; Allegrucci and Sauer 2007) (Arce Miranda et al. 2011). Although discrepant results have been reported, several transcriptomic and proteomic studies reveal an upregulated response to oxidative stress and expression of antioxidant defenses (Resch et al. 2005; Patrauchan et al. 2007; van Alen et al. 2010; Kim et al. 2006; Tremoulet et al. 2002). This oxidative stress-rich environment can promote antibiotic resistance and tolerance to biofilm cells through several mechanisms. The increased genetic mutability promotes the emergence and selection of antibiotic-resistant mutants in biofilm bacterial populations (see section "Genetic Mutations and Horizontal Gene Transfer"). Oxidative stress induces multidrug efflux pumps (Chen et al. 2008; Fraud and Poole 2011; Miller and Sulavik 1996), and enhanced antioxidant defenses also contribute to antibiotic tolerance (Kohanski et al. 2007; Khakimova et al. 2013; Seneviratne et al. 2012). Finally, the SOS response and expression of *recA*, triggered by DNA damage, are also activated in *E. coli* biofilms and confer tolerance to quinolones (Beloin et al. 2004; Bernier et al. 2013; Dorr et al. 2010).

Envelope Stress and Other Stress Responses

Biofilm growth is associated with the activation of CpxA/CpxR stress responses (Beloin et al. 2004), a two-component regulatory system in *E. coli* that is triggered by various signals related to cell envelope stress (Dorel et al. 2006) and can mediate antibiotic resistance (Raivio et al. 2013). The role of the Cpx system in *E. coli* and AmgRS, a functionally similar system in *P. aeruginosa*, is discussed in the section "Two-Component Systems (TCS)."

RpoS (or σ^{38}), the stationary phase alternative sigma factor in γ- and β-proteobacteria (including *E. coli* and *P. aeruginosa*), controls many genes involved in a general stress response (Battesti et al. 2011; Potvin et al. 2008) and contributes to antibiotic tolerance (Hansen et al. 2008; Kayama et al. 2009). Since expression of RpoS or RpoS-regulated genes is induced in biofilms, this response may also contribute to biofilm antibiotic tolerance (Xu et al. 2001; Ito et al. 2009).

Persisters

Persisters are a subpopulation of bacteria that survive bactericidal antibiotics yet are genetically identical to susceptible bacteria. They show phenotypic multidrug tolerance and are often considered “dormant.” Such cells replicate slowly and are metabolically inactive (Balaban et al. 2004; Shah et al. 2006; Wood et al. 2013). Persisters represent a very small fraction of exponentially growing planktonic cultures (approximately one in one million) but become more abundant in stationary phase planktonic cultures (Keren et al. 2004). Biofilm bacteria are also highly tolerant to multiple classes of antibiotics, and it has been proposed that biofilm growth gives rise to a greater fraction of multidrug tolerant persister cells (Wood et al. 2013; Lewis 2008).

What molecular pathways are involved in the persister phenotype and whether all persister cells are alike remain actively debated. Multiple mechanisms have been implicated in persister formation and have been extensively reviewed recently (Wood et al. 2013; Gerdes and Maisonneuve 2012; Balaban 2011; Kint et al. 2012; Cohen et al. 2013; Yamaguchi and Inouye 2011; Lewis 2010). A primary model for persister formation involves expression of toxin-antitoxin (TA) modules. TA systems are functionally redundant, with *E. coli* encoding at least 15 TA modules and *M. tuberculosis* encoding 88, for example. They typically include a stable toxin protein that disrupts an essential cellular process and a labile antitoxin (RNA or protein) that prevents toxicity. Degradation of antitoxins or overexpression of toxins in excess of their corresponding antitoxins induces a state of dormancy. Several studies have shown that persister formation and activation of TA systems require the stringent response alarmone (p)ppGpp (Maisonneuve et al. 2011; Aizenman et al. 1996; Korch et al. 2003). In particular, the Lon protease, which plays a major role in inactivating type II antitoxins in *E. coli*, is activated by (p)ppGpp levels (Maisonneuve et al. 2013). Persister formation may also be under stochastic control (Balaban et al. 2004; Fasani and Savageau 2013; Rotem et al. 2010), induced by the SOS response (Dorr et al. 2009), by specific carbon metabolism (Allison et al. 2011; Amato et al. 2013) or in response to a specific environmental cue (Vega et al. 2012).

Most studies on persisters have been done in planktonic bacteria. Whether persisters in biofilms arise from similar molecular mechanisms remains to be determined. Harrison et al. reported that deletion of the toxin-encoding gene *yafQ* reduced multidrug tolerance in *E. coli* biofilms but not stationary phase planktonic bacteria, and *yafQ* overexpression increases tolerance (Harrison et al. 2009). The persister phenotype presents many similarities with tolerant biofilm cells; it is thus compelling to infer that both tolerant populations share common mechanisms of tolerance. However, tolerant subpopulations in biofilms typically display distinct non-stochastic spatial patterns, in keeping with the physiological heterogeneity that arises from metabolic and stress gradients within biofilms. Such nutrient or environmental cues may signal or promote persister formation, including through activation of TA systems.

Cyclic Di-GMP (c-di-GMP)

C-di-GMP has emerged in recent years as an important intracellular secondary messenger in a wide range of bacteria, synthesized by GGDEF domain-containing diguanylate cyclases and hydrolyzed by EAL or HD-GYP domain-containing phosphodiesterases. C-di-GMP is implicated in biofilm formation, motility, virulence, and other cellular processes. Several recent reviews on c-di-GMP signaling and its function in bacteria were recently published (Romling et al. 2013; Romling 2012; Mills et al. 2011).

C-di-GMP plays a key role in the transition from the motile planktonic to sessile adherent biofilm lifestyle: through transcriptional, translational, and posttranslational mechanisms, c-di-GMP regulates multiple cellular functions required for biofilm growth. C-di-GMP downregulates bacterial motility, from flagellar (swimming and swarming) to type IV pilus surface (twitching) motility, thus promoting the initial transition from planktonic to biofilm growth and the detachment from biofilms back to planktonic growth. Furthermore, c-di-GMP can also regulate all components of the biofilm matrix, in particular EPS production, thus promoting bacterial surface adherence and biofilm formation.

Bacterial variants with increased c-di-GMP levels (such as small colony variants (SCV) described below) are associated with enhanced biofilm formation, surface adherence, and cell-cell aggregation. While such variants are associated with increased biofilm resistance, this is likely through indirect c-di-GMP-dependent regulation of EPS production (Starkey et al. 2009; Drenkard and Ausubel 2002), peptidoglycan cross-linking, and autolysin production (Luo and Helmann 2012).

Colony Variants

Small colony variants (SCV) are phenotypic variants that arise during biofilm growth and chronic infections, such as abscesses and cystic fibrosis airway infections. They can be observed in multiple bacterial species, including *S. aureus, P. aeruginosa, Streptococcus pneumonia, Salmonella enterica,* and *Vibrio cholerae* (Starkey et al. 2009; Proctor et al. 2006; Haussler et al. 2003; von Gotz et al. 2004; Allegrucci and Sauer 2008; Yildiz and Schoolnik 1999). They share a common colony morphology associated with slow growth and increased auto-aggregation that is attributed to the overexpression of exopolysaccharides (Starkey et al. 2009; Singh et al. 2010; Laham et al. 2007). SCV have an enhanced ability to form biofilms, can be more resistant to antibiotics, and are formed through several different genetic pathways (Proctor et al. 2006).

In *P. aeruginosa*, a subset of SCV have a rugose (or wrinkly) appearance, and mutations leading to increased intracellular levels of c-di-GMP levels are identified from rugose SCV (RSCV) isolated from both laboratory biofilms, as well as clinical isolates from cystic fibrosis patients (Starkey et al. 2009; Drenkard and Ausubel 2002; Meissner et al. 2007; Mikkelsen et al. 2009). Interestingly, environmental signals regulate the emergence of resistant RSCV, as their frequency increases

during growth in the presence of NaCl, lower temperature, or minimal media (Drenkard and Ausubel 2002). SCV in *S. aureus* have also been extensively studied: different metabolic alterations in SCV cause their slow growth, and many SCV are deficient in electron transport (menadione or hemin auxotrophs) (Proctor et al. 2006). The latter likely is the cause of aminoglycoside resistance due to the loss of the proton motor force. *S. aureus* SCV overexpress the polysaccharide intercellular adhesin (PIA) (Singh et al. 2010).

While some of these variants share similar colony morphologies (e.g., SCV), the underlying genetic and molecular mechanisms are clearly varied. Similarly, the association between the SCV phenotype with increased antibiotic resistance during biofilm growth depends on the underlying molecular pathways involved.

Genetic Mutations and Horizontal Gene Transfer

Several studies have shown that biofilm growth leads to increased genetic diversification and horizontal gene transfer compared to planktonic growth, thus promoting the emergence of inheritable antibiotic resistance. Under antibiotic selective pressures, populations of antibiotic-resistant variants can expand (Molin and Tolker-Nielsen 2003; Driffield et al. 2008; Conibear et al. 2009; Król et al. 2011).

Genetic variants readily emerge during biofilm growth in different bacterial species (Boles and Singh 2008; Allegrucci and Sauer 2008; Boles et al. 2004; Koh et al. 2007), through genetic mutations or rearrangements (Drenkard and Ausubel 2002; McEllistrem et al. 2007). Genetic variants with altered colony morphology are readily recognized, with many sharing a common small and/or rugose colony morphology associated with increased EPS production (see section "SCV"). Antibiotic-resistant variants have been described in *P. aeruginosa* (Drenkard and Ausubel 2002; Boles et al. 2004) and *S. aureus* (Garcia et al. 2013) that overproduce EPS, while others may harbor other mutations unrelated to EPS production. For example, Boles et al. reported that the genetic diversification in biofilms not only led to many colony morphology variants but also a ~1,000 fold increase in the number of gentamicin-resistant bacteria (Boles et al. 2004).

Several studies have shown that the mutation frequency is greater in biofilm compared to planktonic bacteria (Ryder et al. 2012; Driffield et al. 2008; Conibear et al. 2009). Genetic mutations may be due to DNA damage and *recA*-mediated DNA repair from oxidative stress generated during biofilm growth (Boles and Singh 2008). However, genetic variants can represent more than 10 % of a biofilm population after only a few days of laboratory biofilm growth (Boles et al. 2004). Mutations, even a high frequency, are therefore unlikely to account for all variants observed, and variant subpopulations are likely enriched in the biofilm environment. For example, SCV overexpressing exopolysaccharides have enhanced surface adhesion and self-aggregate (Zogaj et al. 2001). Boles et al. proposed that this genetic diversity provided greater community stress resistance and sustainability through the "insurance effect," a benefit that is clear under antibiotic stress (Boles et al. 2004). Chromosomal rearrangements can also lead to genetic variants.

Adaptive Resistance Mechanisms

Adaptive resistance is induced by the antibiotics themselves or various environmental signals. The mechanisms presented below are typically not specific to biofilm growth and can also confer resistance to planktonic bacteria under certain conditions.

Efflux Pumps

Drug efflux is an important mechanism of antibiotic resistance, particularly in gram-negative bacteria. Species such as *P. aeruginosa* have a large number of multicomponent efflux pumps that can export many substrates into the extracellular environment. Among the different families of efflux pumps, resistance-nodulation division (RND) efflux systems are particularly relevant to antibiotic resistance because of their ability to extrude a wide array of compounds, including aminoglycosides, beta-lactams, fluoroquinolones, macrolides, and tetracyclines (Gotz 2002). The expression of these efflux pumps is highly regulated and typically low without inducing conditions.

While the contribution of efflux pumps to antibiotic resistance in planktonic cells is well established, their contribution to biofilm antibiotic resistance remains equivocal. For example, expression of *P. aeruginosa* multidrug efflux systems is low during biofilm growth (Folsom et al. 2010; De Kievit et al. 2001). Inactivation or overexpression of the MexAB-OprM and MexCD-OprJ systems has no or limited impact on biofilm antibiotic resistance, and the MexEF-OprN and MexXY systems do not appear to contribute to biofilm antibiotic resistance either (De Kievit et al. 2001; Brooun et al. 2000). However, MexCD-OprJ is induced by the antimicrobial peptide colistin, and MexCD-OprJ mutant biofilms are more susceptible to colistin during biofilm growth (Chiang et al. 2012). Gillis et al. reported that MexCD-OprJ is induced in biofilms by the macrolide azithromycin, and mutant biofilms lacking both MexCD-OprJ and MexAB-OprM are susceptible to azithromycin (Gillis et al. 2005). Interestingly, the expression of several RND efflux systems is modulated by oxygen concentration, raising the intriguing possibility that reduced oxygen levels within biofilms may induce drug efflux (Schaible et al. 2012).

Zhang et al. described a novel efflux system (PA1874-1877) in *P. aeruginosa* strain PA14 that contributes to biofilm-specific resistance toward tobramycin, gentamicin, and ciprofloxacin. The PA1874-1877 genes are more highly expressed in biofilms than in planktonic bacteria and encode a putative RND efflux and ABC transporter system that confer increased antibiotic resistance to biofilms but not planktonic bacteria (Zhang and Mah 2008). Liao et al. also recently identified a MerR-like transcriptional regulator BrlR only expressed in biofilms. BrlR appears to contribute to biofilm tolerance to aminoglycosides and colistin, partly by inducing the expression of the MexAB-OprM and MexEF-OprM efflux pumps (Liao et al. 2013) and by repressing *phoPQ* expression (Chambers and Sauer 2013).

In *E. coli*, AcrAB-TolC, an RND family efflux pump, is the most common efflux system and can export a wide range of antibiotics. Although its expression can be

upregulated during biofilm growth, this does not correlate well with increased antibiotic resistance (Maira-Litran et al. 2000). On the other hand, many other genes encoding efflux pumps are upregulated in *E. coli* biofilms, and efflux pump inhibitors enhance tetracycline susceptibility of *E. coli* and *Klebsiella pneumoniae* biofilms (Kvist et al. 2008).

Determining the contribution of specific efflux systems is challenging because of their redundant functions and heterogeneous expression within biofilm structures (Pamp et al. 2008; Chiang et al. 2012). Furthermore, expression of the efflux systems can be induced by poorly characterized conditions or substrates. However, in most studies, drug efflux likely does not account for the profound antibiotic resistance of biofilms.

β-Lactamases

ampC is a chromosomally encoded β-lactamase, and it's expression and activity can be activity can be induced in mature biofilms, as well as biofilm subpopulations treated with beta-lactams (Bowler et al. 2012; Bagge et al. 2004). While this can lead to adaptive beta-lactam resistance, this is also unlikely to explain the high resistance of biofilm cells to this class of antibiotics, observed without prior beta-lactam exposure.

Two-Component Systems (TCS)

Two-component systems (TCS) are key signaling systems in bacteria, allowing them to sense their environment and elicit an appropriate response. They are typically made of a sensor kinase and a response regulator, although many are hybrid kinases or orphan response regulators. While many TCS contribute to biofilm formation, as well as antibiotic resistance in planktonic bacteria, their direct implication in antibiotic resistance of biofilms is less well characterized.

The CpxAR system in *E. coli* is a TCS that primarily responds to cell envelope stress, such as accumulation of misfolded proteins. This system controls many targets, including genes involved in motility and adhesion and transcription of several drug exporter genes and can confer resistance to aminoglycosides and antimicrobial peptides (Hirakawa et al. 2003). Cpx-regulated genes are induced at initial attachment and during biofilm growth in *E. coli* (Beloin et al. 2004; Dorel et al. 1999; Otto and Silhavy 2002), suggesting that the Cpx system may contribute to antibiotic resistance in biofilms. Interestingly, the AmgRS TCS in *P. aeruginosa*, although closest to the *E. coli* OmpR/EnvZ system by sequence homology, share several functional similarities with the *E. coli* Cpx system. It controls a conserved cell envelope stress response and nearly half of its target genes are homologous to Cpx-controlled genes (Lee et al. 2009). Lee et al. reported that mutations in *amgRS* increase tobramycin killing of tolerant biofilms and improved outcomes in lethal murine infections (Lee et al. 2009).

The PhoPQ and PmrAB TCS present in several gram-negative bacterial species (including *Salmonella* spp., *E. coli*, and *P. aeruginosa*) are activated by conditions limited for divalent cations (Mg^{2+} and Ca^{2+}) and confer resistance to cationic antimicrobial peptides (CAP) and aminoglycoside (Macfarlane et al. 1999; McPhee et al. 2003). Both TCS induce the *pmr(arn)BCADTEF-pmrE* operon responsible for amino-arabinose modification of lipopolysaccharide (LPS) molecules within the outer membrane. Such LPS modifications alter the charge interactions at the cell surface, leading to CAP and aminoglycoside resistance in planktonic bacteria. Whether these pathways contribute significantly to resistance in biofilm bacteria remains to be determined. In *P. aeruginosa*, the *pmr* genes are required for colistin (a CAP) tolerance in flow chamber-grown biofilms, but *pmr* expression in biofilms is low and only induced by colistin treatment (Pamp et al. 2008). Chambers et al. recently characterized BrlR, a transcriptional regulator that represses the PhoPQ system during biofilm growth, thus suppressing CAP resistance (Chambers and Sauer 2013).

Zhang et al. recently identified a putative TCS in *P. aeruginosa* encoded by PA0756-0757 that confers aminoglycoside resistance and is highly expressed in biofilms (Zhang et al. 2013). The unusual PrpAB TCS in *P. aeruginosa* is associated with enhanced biofilm formation when activated but reduced antibiotic resistance and attenuated virulence (de Bentzmann et al. 2012). Both these systems contribute to biofilm resistance through yet uncharacterized mechanisms.

Conclusion

Biofilm infections pose a great medical challenge, as conventional antimicrobial therapies do not effectively eradicate such bacterial infections. Our evolving understanding of the mechanisms of antibiotic resistance and tolerance in biofilm bacteria reveals multiple contributing cellular processes and pathways. Whether these mechanisms can be targeted to effectively overcome biofilms multidrug resistance and tolerance remains to be seen. Such strategies will undoubtedly have a major impact in our therapeutic arsenal against devastating infections such as the chronic *P. aeruginosa* airway infections of cystic fibrosis patients.

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Strategies for Circumventing Bacterial Resistance Mechanisms

Jed F. Fisher, Jarrod W. Johnson, and Shahriar Mobashery

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Abstract

The future practices for the control of bacterial infections are uncertain. The intransigent infection is no longer found just among the immune compromised but is now found both in and out the boundaries of the hospital. Preserving the efficacy of the antibacterials we have, in order to secure the time needed to discover and develop new antibacterials, will require abrupt change: in the way antibacterials are dispensed and disposed, in the criteria used to measure clinical safety and efficacy, in the financial incentives for antibacterial development, and in the understanding of the molecular mechanisms governing the relationship between the antibacterial and the bacterium. This review examines this

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relationship from the particular perspective of the eventual need to circumvent resistance mechanisms in order to reclaim the lifesaving value of the antibacterial chemical.

Keywords
Antibiotic • Cell Wall • Cytoskeleton • Natural Products • Resistome • Synergy

Introduction

The parlous confluence of expanding bacterial resistance and contracting investment in antibacterial discovery will be well recognized by the readers of this essay (Lewis 2013; Spellberg et al. 2013; Howard et al. 2014; Singh 2014). The reasons underlying this confluence – found at the microbiological, medical, and societal levels – are equally well understood (O'Connell et al. 2013). These reasons include, at the microbiological level, naiveté of the extraordinary breadth (McArthur et al. 2013; Modi et al. 2013) and resilience of the intrinsic resistome (Cox and Wright 2013; Hede 2014) of bacteria and the ease with which this resistome is shared (Carattoli 2013; Otto 2013a; Perry and Wright 2013; Rodriguez-Rojas et al. 2013); at the medical level, failures of antibiotic stewardship (Leuthner and Doern 2013; Munoz-Price and Quinn 2013; Laxminarayan 2014; Livermore 2014); and at the societal level, willful ignorance of the ecological interplay between antibiotics and animals of all species (Allen et al. 2013; Finley et al. 2013; Stanton 2013; Wellington et al. 2013) and of the magnitude of the financial investment required to ameliorate this confluence (Shlaes et al. 2013; Schäberle and Hack 2014). Moreover, sustaining this amelioration will require simultaneous change at *each* of these three levels (Paphitou 2013; Metz and Shlaes 2014). While the resistome is ancient, the profligate exploitation of the antibiotics has accelerated resistance emergence and galvanized its distribution (Galán et al. 2013). While it is uncertain whether an event short of a pandemic will realize this sea change, the scientific and medical objectives for preserving successful antibacterial chemotherapy in the face of a seeming universe of resistance mechanisms are obvious. These objectives include the discovery of new antibacterials (Gammon 2014), the reincarnation of old antibacterials, the optimization of multi-agent chemotherapy by mechanistic correlation of interdependent targets, a better understanding of the control mechanisms for the expression of resistance mechanisms, a better understanding of the bactericidal mechanisms used by clinically successful antibacterials, and improved clinical trial design (Shlaes and Spellberg 2012; Dalhoff et al. 2014). The exploitation of these discoveries, combined with a recognition of the importance of the clinical practices that ensure antibacterial efficacy (Bogan and Marchaim 2013; Ciccolini et al. 2013), can preserve the antibiotic miracle (Bartlett et al. 2013; Davies et al. 2013).

Nonetheless, the magnitude of the challenge of continually intercepting the moving landscape of antibacterial resistance, by correlation of strategy to objectives, cannot be underestimated (Lewis 2013). At the most fundamental biological levels, we simply do not understand the antibacterial substance. The abundance of

antibacterial natural products – the antibiotics – gave rise to the belief that their sole purpose was as offensive weapons. This belief is now understood to be a gross oversimplification (Davies 2013, 2014). It is a trivial matter to demonstrate in vitro mechanistic synergy between two antibacterials. Sustaining this synergy in clinical practice, where it is necessary to align both the respective mechanisms and pharmacokinetics of the drugs, is much more difficult (Paul and Leibovici 2013). Yet we must try (Sun et al. 2013). This essay addresses five perspectives on the relationship between antibiotic discovery and the future chemotherapy of resistant bacteria:

- Will the discovery of new antibacterials contribute to overcoming bacterial resistance?
- Will the discovery of new targets contribute to overcoming bacterial resistance?
- Will understanding how bacteria recognize the presence of antibacterials identify new anti-resistance strategies?
- Will understanding the bactericidal mechanisms of antibacterials identify new anti-resistance strategies?
- How will these answers shape the antibiotic future in the face of the inexorable expansion of the resistome?

We discuss here the current strategies that address these questions and illustrate the extraordinary breadth of the current scientific efforts toward antibacterial discovery in the face of the emergence of extensively resistant bacterial pathogens.

Will the Discovery of New Antibacterials Contribute to Overcoming Bacterial Resistance?

In the immediate decades following the discoveries of the sulfonamides, penicillins, and the aminoglycosides, innumerable antibacterial substances were discovered, primarily from Nature (the antibiotics). Although few of these had the requisite pairing of efficacy and safety, the ease with which this universe of antibacterials was found sustained the belief that Nature held a seemingly inexhaustible trove of antibacterial structures. While successive generations of antibacterials have followed based upon these first structures, this optimism has faded in the face of the experimental difficulties of dereplication (is the biological activity the result of an already known structure?) and validation (Berdy 2012). Indeed, while the past decades have yielded new antibacterial classes of synthetic origin (such as the oxazolidinones) and of semisynthetic origin (such as the fluoroquinolones and carbapenems), few new antibiotics (these new antibiotics are exemplified by daptomycin (**1**), pleuromutilin, and fidaxomicin (**2**)) have progressed to the clinic. While the question as to whether new antibiotic structures will be found in Nature and new antibacterials found by synthesis was once provocative, the new structures, the new combinations of structure, and the new targets that have emerged recently provide guarded optimism for the future (Butler et al. 2013; Chopra 2013; Pucci and Bush 2013; Walsh and Wencewicz 2014) (Fig. 1).

Fig. 1 A selection of recently discovered antibacterials of natural origin

New Natural Products as Antibacterials

The argument that we are entering a new era for the discovery of biologically active natural products is indisputable (Brown et al. 2014). It is not simply that our interrogation now includes both terrestrial and marine organisms (Gerwick and Moore 2012; Gammon 2014) but, additionally, the recognition that our anthropomorphic interest in antibiotics belies the vastly more complex roles these structures have for communication within their ecological niches (Davies 2013). A direct implication from this understanding that the coculture of organisms can profoundly alter secondary metabolite expression is proven (Bertrand et al. 2013; Rateb et al. 2013; Kalghatgi et al. 2013; Hopwood 2013). The vast realm of the so-called "uncultured" bacteria may open for exploration, as we further understand their chemical means of communication (Nikitushkin et al. 2013; Wilson and Piel 2013). Advances in structure analysis (Seger et al. 2013; El-Elimat et al. 2013), including the ability to directly interrogate the presence of secondary metabolites by imaging mass spectrometry (Watrous et al. 2012; Graupner et al. 2012), have diminished dereplication as a barrier to discovery. Finally, numerous advances in the understanding of the genomic organization used for secondary metabolite

biosynthesis (Andersen et al. 2013; Wright 2014), coupled to an understanding of the genetic control of biosynthesis by small molecules (Ahmed et al. 2013), by pathway metabolic modeling (Breitling et al. 2013), and by development of new bacterial vectors (Ongley et al. 2013; Komatsu et al. 2013), anticipate a future ability to attain (by manipulation of starting material, enzymes, and vectors) unprecedented antibacterial structure (Baltz 2014; Craney et al. 2013; Walsh et al. 2013; Zakeri and Lu 2013; Thaker et al. 2013; Thaker and Wright 2014).

This opinion implies that disclosures of potentially transformative natural product antibacterials are evident already (Bologa et al. 2013; Kirst 2013). Among the most recently registered antibiotics are daptomycin (**1**) (Cubicin®, a lipopeptide with a membrane-based mechanism) (Pogliano et al. 2012), retapamulin (**10**) (Altabax®/ Altargo®, a pleuromutilin that inhibits the 23S rRNA interaction with the 50S subunit of the ribosome with topical Gram-positive activity) (Novak 2011), and fidaxomicin (**2**) (Dificid®, a macrolide that inhibits the RNA polymerase of Gram-positive bacteria, approved as a narrow-spectrum agent against *C. difficile*) (Sears et al. 2013). The seven parenteral Gram-negative antibacterials (Bush 2012; Boucher et al. 2013) in late-stage clinical evaluation include four β-lactam/β-lactamase inhibitor pairs, plazomicin (**4**) (an aminoglycoside also with Gram-positive activity) (Becker and Cooper 2013), brilacidin (**3**) (an antimicrobial peptidomimetic also having Gram-positive activity), and eravacycline (**5**) (a "fluorocycline" tetracycline derivative and also having Gram-positive activity) (Xiao et al. 2012; Wright et al. 2014). An outstanding characteristic of this list is the explicit role of the evasion of resistance mechanisms in their design (the β-lactam pairs, eravacycline). As was also true of tigecycline (the preceding generation tetracycline), eravacycline was selected on the basis of potency, safety, and the ability to overcome both efflux and ribosomal protection resistance mechanisms (Clark et al. 2012). The other end of the development pipeline – the discovery of structurally unprecedented biological structure – is illustrated with the structures of anthracimycin (**9**), orthoformimycin (**8**), psammaplysin (**6**), and flavomannin (**7**). Anthracimycin is a marine natural product with exceptional potency as an RNA/DNA synthesis inhibitor, especially Gram-positive activity against *Bacillus anthracis*: MIC 0.3 μg mL^{-1} (Jang et al. 2013). Orthoformimycin (Maffioli et al. 2013) is a moderate inhibitor of Gram-positive protein synthesis as a result of binding to the 50S ribosomal subunit, thus blocking elongation. It was identified by the dual screening of microbial fermentation extracts to identify inhibitors of bacterial, but not yeast, protein synthesis. The psammaplysins exemplify a previously known class of marine natural products but now identified as having the remarkable mechanism of inhibiting DNA partitioning into the daughter cell of Gram-positive bacteria (Ramsey et al. 2013). Screening of an extract of *Talaromyces wortmannii* and endophytic fungus of the plant *Aloe vera* identified the flavomannins, atropisomeric dihydroanthracenones with a similar mechanism and equally unknown target (Bara et al. 2013). Antibacterials that are present in small quantities in these extracts but show promising biological activity can be enriched significantly (in the identical fashion as was done decades ago for other antibiotics) by optimization of the fermentation medium. This ability, most recently demonstrated for the platensimycin/platencin class

(Aluotto et al. 2013), enables the further synthetic transformations required to impart clinical potency. While the likelihood that any of these four natural products will progress to the clinic is remote, they illustrate the emerging criteria now used to identify captivating antibiotic structure: evaluation of novel microbial sources and/or the use of sophisticated screening methodologies to discover unprecedented mechanism and target (Thaker et al. 2013).

New Synthetics as Antibacterials

The interrogation of industrial chemicals for compounds with potential biological activity was foundational in the mid-nineteenth century to the creation of the synthetic dye industry, and the interrogation of synthetic dyes was foundational decades later to the discovery of the sulfonamide (sulfa) class of antibacterials. The emergence of an entire breadth of new technologies to address both new antibacterial targets and reassess old targets has led to new horizons for antibacterial discovery. Even with respect to sulfonamide antibacterials, new discoveries have been made with respect to both resistance mutation(s) of the target and to off-target mechanisms. Crystallographic examination of the molecular target of sulfonamides, the enzyme dihydropteroate synthase, allowed cross-correlation of the resistance mutations seen within this enzyme to both the catalytic mechanism and sulfonamide binding (Yun et al. 2012). The probable basis for the CNS side effects of certain antibacterial (and other) sulfonamides was identified as competitive inhibition within the tetrahydrobiopterin biosynthetic pathway (Haruki et al. 2013). Whether access to these protein structures will enable the redesign of a sulfonamide to simultaneously evade resistance mutation and eliminate off-target binding is uncertain. Rather, it is the simple demonstration that it is now possible to conceptualize structural change within a (seemingly aged) antibacterial class based on structure-based design (Agarwal and Fishwick 2010).

Among the most intensively studied targets for the identification of novel antibacterial structure is the bacterial cytoskeleton (Celler et al. 2013; Pilhofer and Jensen 2013; Ojima et al. 2014). The dramatic increase in the understanding of the bacterial cytoskeleton as a control mechanism for coordinating bacterial cell growth has identified new antibacterial opportunity (Ma and Ma 2012; Pinho et al. 2013; Wilson and Gitai 2013). A Gram-negative bacterium cannot merely organize the assembly of its cell-wall polymer, but it must coordinate this assembly with *inter alia* the duplication and translocation of its genome (Sass and Brötz-Oesterhelt 2013), the synthesis of its inner membrane, the export of proteins to the periplasm and outer membrane, the assembly of the inner leaflet of the outer membrane, and the export of the lipopolysaccharide as the outer leaflet of its outer membrane. Among the key contributors to this coordination are the central cell-division mediator (tubulin-like) protein FtsZ (Reimold et al. 2013) and the membrane-adhering (actin-like) protein MreB (Celler et al. 2013). Notwithstanding the challenge of confirming and identifying the precise molecular mechanisms of compounds that bind to these proteins (Anderson et al. 2012; Foss et al. 2013), compounds that disrupt this coordination

are antibacterial and/or are synergistic with other antibacterials. The most prominent inhibitors with respect to binding to FtsZ are PC190723 (**15**) and related structures (Anderson et al. 2012; Elsen et al. 2012; Matsui et al. 2012). An *N*-dimethylaminomethyl prodrug derivative of PC190723 (TXY436) shows improved solubility and has oral efficacy in mice infected with MSSA and MRSA (Kaul et al. 2013). One of the best compounds in this series has potent antistaphylococcal activity (MIC 0.12 μg mL^{-1}) in vitro, oral activity in infection models, and an acceptable frequency of FtsZ point mutation resistance selection (4.1×10^{-9}) given the evidence for impaired in vitro fitness accompanying the point mutation (Stokes et al. 2013). PC190723 also acts synergistically with β-lactam antibiotics against *S. aureus*, possibly as a result of mislocalization of FtsZ with concomitant disruption of its binding partner (and target of the β-lactams), the PBP bifunctional enzyme(s) (Tan et al. 2012). A no less significant observation is an even lower resistance frequency (1.6×10^{-9}) for the PC190723–β-lactam pairing. PC190723 and other cytoskeleton-interacting structures are shown in Fig. 2.

The potential of structural biology-enabled screening for the serendipitous discovery of new antibiotic leads is vast. A high-throughput screening of drug-like structures against the MipZ protein identified one compound ("divin," **18**) as a weak inhibitor; subsequent analysis showed neither MipZ nor FtsZ was its true target. The target is unidentified. Divin is bacteriostatic against several Gram-negative bacteria (MIC 1–50 μg mL^{-1}) as a result of its ability to disrupt the assembly of division proteins (including for the cell wall) and to block compartmentalization of the cytoplasm (Eun et al. 2013). A virtual screening effort against the structure of the AgrA response regulator identified an FDA-approved compound, the nonsteroidal anti-inflammatory drug diflunisal, with micromolar anti-virulence (but not antibacterial) activity against MRSA as a result of its inhibition of the production of α-hemolysin and phenol-soluble modulins (Khodaverdian et al. 2013). As a third example, the structure of the *P. aeruginosa* multidrug efflux transporter inhibited by a pyridopyrimidine compound sets the opportunity for successful structure-based design of a "universal" efflux inhibitor (Nakashima et al. 2013). As antibacterial targets are identified, a phalanx of physical and virtual methods is available to interrogate the target.

Will the Discovery of New Targets Contribute to Overcoming Bacterial Resistance?

The correct choice of the therapeutic target is essential (Bugg 2014). Given the smaller genome of the bacteria, an early presumption of the post-genomic era was that the identification of the essential bacterial targets would in turn identify exploratory antibacterials. A reappraisal of the Gram-positive *B. subtilis* genome identified 261 genes (259 proteins, only six without assigned function, and two functional RNAs) as essential (Commichau et al. 2013). Extensive bioinformatic efforts, such as by genome cross-correlation, have further generalized our understanding of the essential bacterial genome (Duffield et al. 2010; McArthur et al. 2013). Nonetheless,

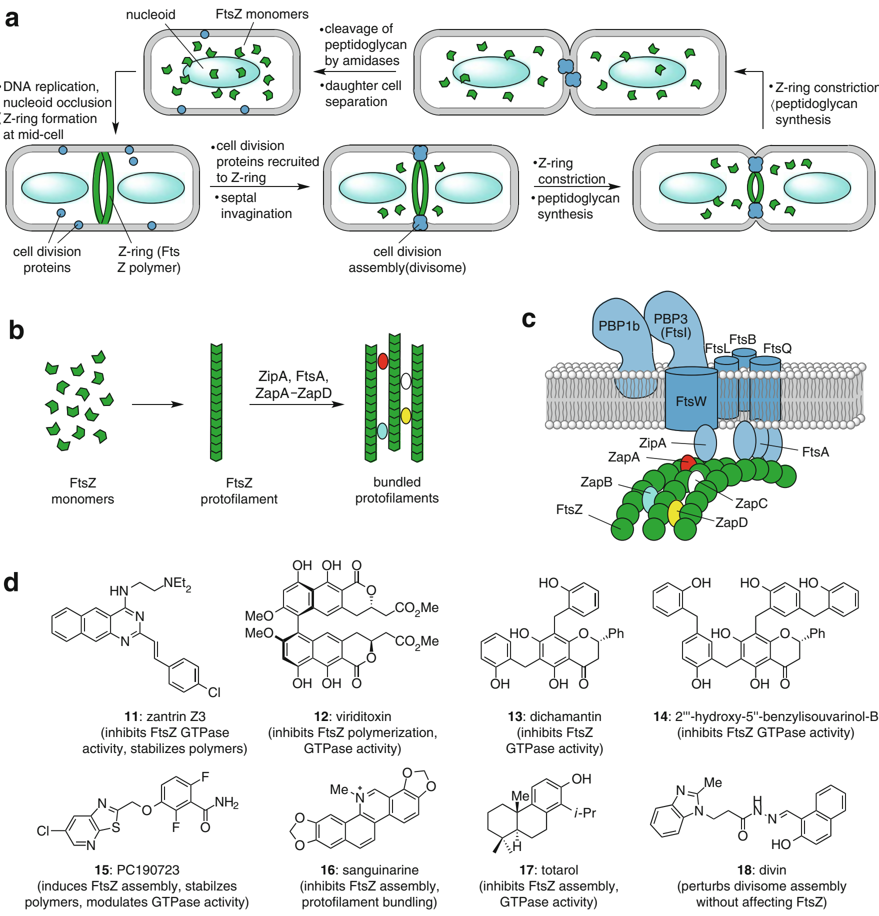

Fig. 2 Bacterial cell division, divisome assembly, and cell-division inhibitors. (**a**) Selected steps in *E. coli* cell division, including nucleoid occlusion, followed by Z-ring formation, recruitment of cell-division assembly proteins, Z-ring contraction, and daughter cell separation. (**b**) Polymerization of FtsZ into protofilaments and bundling of the protofilaments for Z-ring assembly. (**c**) A simplified representation of interactions of Z-ring proteins with selected membrane-bound proteins of *E. coli*. For recent reviews of cell division and divisome assembly, see (Egan and Vollmer 2013; Huang et al. 2013; Pinho et al. 2013). (**d**) Selected cell-division inhibitors. For recent reviews, see Sass and Brötz-Oesterhelt (2013), Schaffner-Barbero et al. (2012)

the limitation of a singular focus on essentiality with respect to successful antibacterial discovery was revealed and candidly discussed by Payne et al. in the context of successive failed high-throughput antibacterial target screens (Payne et al. 2007). The lessons from this collective failure – the surprising genetic diversity of bacteria, the mutational ease of resistance generation, the extraordinary versatility of bacteria to adapt to metabolic challenge, the unusual chemical space for antibacterial structure, and the synthetic challenge in entering that space – have been transformative to current thinking on bacterial targeting (Roemer and Boone

2013). Notwithstanding these transformations in thinking, empirical exploration of chemical structure to identify new targets has not lost value, as exemplified by the most recently approved addition to *Mycobacterium tuberculosis* chemotherapy, bedaquiline (Sirturo®). The identification of its target as the bacterial ATP synthase was foundational to further exploration of both its structural class and the target with respect to other Gram-positive pathogens (Balemans et al. 2012). Likewise, one of the most promising new exploratory classes of drugs against *M. tuberculosis*, the imidazopyridines, was discovered by the serendipity of classic antibacterial screening (Moraski et al. 2013; Pethe et al. 2013). The imidazopyridines also block ATP synthesis, although at a different target (the cytochrome bc1 complex).

And while structure-based design will indeed contribute to the discovery of new antibacterials – as discussed below and as exemplified by the opportunities implicit in the Aeropath compilation of *Pseudomonas aeruginosa* target structures (Moynie et al. 2013) – complementary new approaches have emerged to address the central relationship of validating targets with respect to resistance-compromised chemical structure. Proteomic analysis of the resistance response will contribute to an understanding of the overall metabolic adjustment (Lima et al. 2013) and the identification of critical protein interaction networks (Zoraghi and Reiner 2013). Mass spectrometric analysis eventually will demonstrate clinical value for bacterial diagnosis (Mitsuma et al. 2013) and for the detection of resistance mechanisms, as exemplified by the shotgun proteomic identification of a β-barrel outer membrane-located carbapenem-resistance protein (of unknown function) in *Acinetobacter baumannii* (Chang et al. 2013). Underlying many of these efforts is the emerging realization that future chemotherapy of multidrug-resistant bacteria will demand antibacterials either with multi-target activities (East and Silver 2013; Yan et al. 2013) or synergistic mechanisms of action (Lázár et al. 2013; Roemer and Boone 2013; Worthington and Melander 2013a).

The inevitable transition from single-drug treatment of bacterial infection to multidrug treatment – as is already the case for *Mycobacterium tuberculosis* – will not be guided by empirical experimentation. While the enormously successful pairing of β-lactamase inhibitors with β-lactams is the obvious example of the facile selection of synergistic targets (Worthington and Melander 2013b), strategies for the experimental validation of equally promising synergistic pairs are emerging (Roemer et al. 2012; Roemer and Boone 2013; Lázár et al. 2013; Zoraghi and Reiner 2013). We noted the synergistic pairing with β-lactams (targeting the PBP enzymes) with FtsZ antagonists against Gram-positive bacteria. The possibility of synergy with proven antibacterials will certainly be used to prioritize the additional FtsZ antagonists that emerge from screening against both Gram-positive (Foss et al. 2013; Ruiz-Avila et al. 2013) and Gram-negative (Keffer et al. 2013) bacteria.

Small-molecule screening of drug-like structures may now be done using antibacterial synergy as the focus. Using a small (20,000 compounds) library to identify compounds showing synergy with β-lactams (potentiation agents) against methicillin-resistant *S. aureus* (MRSA) identified an inhibitor of MurG (Huber et al. 2009), an enzyme that catalyzes the second glycosyltransferase step in the biosynthesis of the pivotal cell-wall precursor, Lipid II (Mann et al. 2013).

The primary mechanism for this synergy was depletion of Lipid II as a result of MurG inhibition, resulting in the inability of the essential penicillin-binding protein enzyme (and target of the β-lactam) to correctly localize to the division septum. Perhaps not surprisingly, the enzymatic transformations that follow MurG and complete Lipid II biosynthesis also show promise as targets identifying potentiation agents against MRSA (Roemer et al. 2013). An inhibitor specific for one of these *Fem* (*f*actor *e*ssential for *m*ethicillin resistance) enzymes (cyslabdan, **25**) has been already identified and demonstrates β-lactam synergy (Koyama et al. 2012). Moreover, the first crystal structure of a Fem enzyme suggests an unexpected mechanism (Fonvielle et al. 2013) that opens the possibility of both structure- and mechanism-based design. Lastly, glycosylation of the cell-surface teichoic acids of *S. aureus* [is critical for antibacterial resistance, but not for] viability (Bertsche et al. 2013; Brown et al. 2012b; Pasquina et al. 2013) function. The relationship between the integrity of the cell-surface teichoic acids to resistance is substantiated by the identification of a series of inhibitors addressing different events in their biosynthesis, including a synthetic compound (**24**) with in vitro and in vivo antibiotic activity as a result of its inhibition of LtaS (lipoteichoic acid polymerase) (Richter et al. 2013); a registered drug (ticlopidine, **22**) discovered to also act as an inhibitor of TarO (first enzyme of teichoic acid synthesis) and that is synergistic with β-lactams (Farha et al. 2013a); and a synthetic inhibitor (targocil, **20**) of the TarG teichoic acid transporter that also synergizes with β-lactams (Lee et al. 2010; Campbell et al. 2012; Wang et al. 2013). Moreover, blocking the biosynthesis of the wall teichoic acids may suppress horizontal gene transfer in chronic polymicrobial infections (Winstel et al. 2013). Exemplification of the correlation of new structures to classic Gram-positive targets, as well as emerging Gram-positive targets, is given in Fig. 3.

At the same conceptual level as for teichoic acid structure integrity for Gram-positives (Sewell and Brown 2014), there is an identical relationship between integrity and resistance for many antibacterials with respect to the lipopolysaccharide (LPS) leaflet of the outer membrane of Gram-negative bacteria. This relationship is exemplified by the discrete structural alterations that are made to the LPS as a resistance mechanism against polymyxin (Hankins et al. 2012; Fernández et al. 2013; Wanty et al. 2013) and colistin (Pelletier et al. 2013). Given the importance of the integrity of the LPS leaflet, interference with LPS biosynthesis and transport has been identified as a promising strategy to attenuate resistance (Srinivas et al. 2010; Werneburg et al. 2012; Sherman et al. 2013). High-throughput screening identified a lead compound (**32**) as an inhibitor of the ATPase component of the multienzyme complex (IC_{50} 120 μM, compared to 20 μM against the LptB ATPase alone) with antibacterial activity (MIC 12.5 μM) against outer membrane-permeabilized (but not wild type) *E. coli*. This result substantiates the expectation that a more potent compound would potentiate clinically proven antibacterials, as a result of its ability to compromise outer membrane permeability (Sherman et al. 2013).

Clinical validation of in vitro promise is arduous. The seemingly subtle changes that occur with respect to transcription following antibiotic challenge

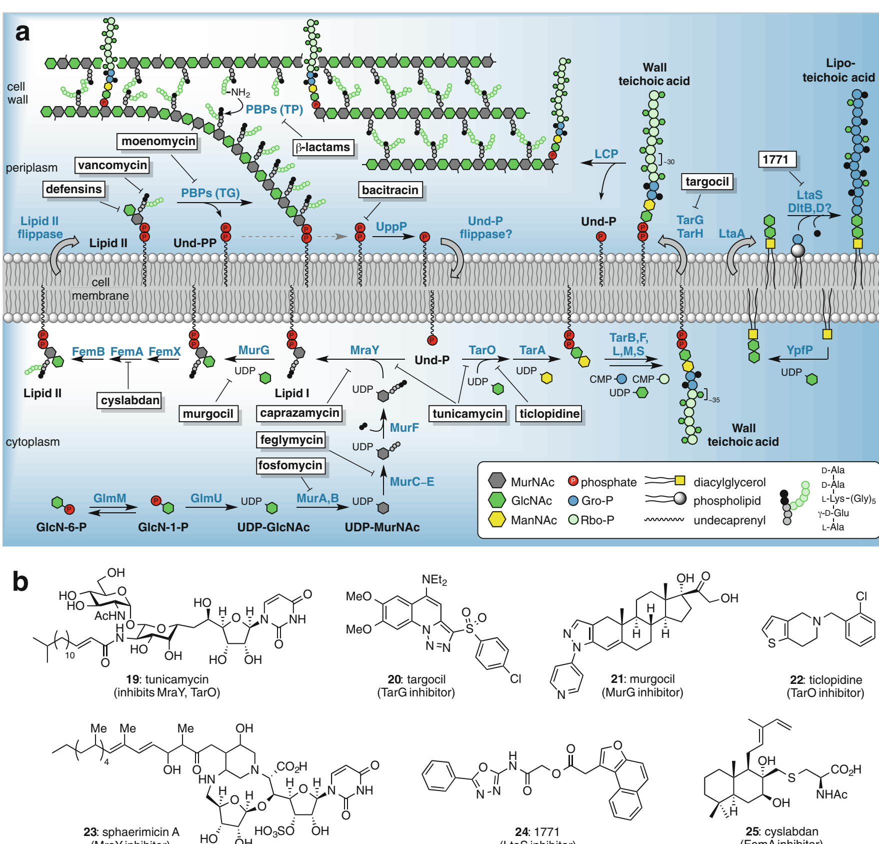

Fig. 3 (**a**) Cell-wall biosynthesis and teichoic acid biosynthesis in *S. aureus*. For recent reviews, see Barreteau et al. (2008), Brown et al. (2013), Pasquina et al. (2013), Sewell and Brown (2014). (**b**) A selection of cell-wall-targeting inhibitors and antibiotics active against Gram-positive bacteria (GlcNAc *N*-acetylglucosamine, MurNAc *N*-acetylmuramic acid, PBP penicillin-binding protein, TG transglycosylase, TP transpeptidase, Und undecaprenyl, Gro glycerol phosphate, Rbo ribitol phosphate)

(Van Oudenhove et al. 2012; Abranches et al. 2013) can be interpreted either as encouraging (even small alterations in activity can reduce fitness) or daunting (numerous compensatory pathways). The remarkable (and not yet understood) “seesaw” effect observed in MRSA – wherein β-lactam susceptibility increases as glycopeptide and lipopeptide susceptibility decreases, suggesting subtle structural interplay between their proximal cell-wall targets (Werth et al. 2013a) – cautions against the expectation that the identification of synergistic target pairs is straightforward. Nonetheless, increasingly sophisticated analytic methodologies have proven that such pairs exist and that the time-proven medicinal chemistry strategies can be expected to deliver on this promise. Indeed, the judicious pairing with vancomycin of a β-lactam with advantageous activity against MRSA

(the cephalosporin ceftaroline) shows in vitro synergy (Werth et al. 2013b). A key aspect of future clinical chemotherapy of resistant bacteria will be chemotherapeutic synergy, as identified and confirmed by biochemical, proteomic, and genomic analyses.

Will an Understanding of How Bacteria Recognize the Presence of Antibacterials Identify New Anti-resistance Strategies?

The increasing prevalence of antibiotic-resistant bacteria might be interpreted as evidence that bacterial fitness is not compromised by resistance mechanisms. This interpretation is facile. Rather, it is that bacteria have a remarkable ability to reorganize their metabolic networks so as to accommodate the resistance mechanism(s) at the cost of a reduced ecological range (Händel et al. 2013). While there are numerous examples of the constitutive expression of powerful resistance mechanisms (such as β-lactamase expression by *M. tuberculosis*) (Tremblay et al. 2010), the expression of many resistance mechanisms is regulated. The depth of these mysteries surrounding such regulation is superbly exemplified by vancomycin, an antibiotic with a decades-long history. Although its molecular mechanism – formation of a stable non-covalent complex with the cell-wall peptidoglycan – is long known, in bacteria where vancomycin resistance is induced (such as *S. aureus*) the structural relationship of complex formation to resistance expression is unknown. The study of this relationship in *Streptomyces coelicolor* implicates direct binding of the peptidoglycan–vancomycin complex to the VanS histidine kinase component of the VanRS two-component regulatory system (Koteva et al. 2010; Kwun et al. 2013). This two-component system has no human counterpart, and ensuing structural change to the VanR response regulator is profound (Leonard et al. 2013). A ligand capable of exploiting this difference in structure so as to prevent VraR dimerization would compromise the ability to respond to the presence of vancomycin. This conclusion presumes, however, that regulation of the resistance mechanism is advantageous with respect to fitness. Notwithstanding the complexity of the vancomycin resistance pathway (seven genes organized into two operons), in the enterococci the evolutionary pressure exerted by vancomycin exposure has abolished the fitness cost of maintaining this pathway (Foucault et al. 2010). However, fitness cost is not the only basis used by bacteria to compel regulation of the resistance pathway. Sustaining resistance mechanism against one antibacterial may simultaneously sensitize to another antibacterial. This "seesaw" effect has been observed in vitro between two pairs of cell-wall-targeting antibacterials against resistant staphylococci: daptomycin and β-lactams (Dhand et al. 2011; Vignaroli et al. 2011; Mehta et al. 2012) and vancomycin and β-lactams (Werth et al. 2013a). Should these conclusions be sustained in vivo, the use of such antibacterial pairs may improve efficacy while decreasing the likelihood of transmitting resistance mechanisms.

While the value of β-lactams is increasingly endangered due to resistance (McKenna 2013; Watkins and Bonomo 2013), many Gram-positive and

Gram-negative bacteria use distinct sentinel pathways to initiate a β-lactam resistance response. Neither signal transduction pathway is well understood. The sentinel protein that initiates the β-lactam resistance response in MRSA (Llarrull and Mobashery 2012) detects β-lactams by acylation of a serine residue by the β-lactam, an event with extraordinary identity to the first half-reaction in class D serine β-lactamase turnover (Kumarasiri et al. 2012). Although the penultimate event leading to the β-lactam resistance of MRSA is proteolytic inactivation of a repressor, the intermediate events of signal transduction – between the initial acylation and this proteolysis – are uncertain (Arede et al. 2013). A possible messenger is a co-activator derived from perturbed peptidoglycan recycling (Amoroso et al. 2012). This possibility is especially intriguing as perturbed peptidoglycan recycling (Cavallari et al. 2013; Johnson et al. 2013) – possibly as a result of the selective inactivation of low-molecular-mass penicillin-binding proteins (Moya et al. 2012) – is central to the induction of β-lactamase expression in many Gram-negative bacteria.

The control of β-lactamase expression is instructive. These enzymes exhibit high catalytic efficiency with respect to many β-lactam substrates, and the co-substrate used to detoxify the β-lactam – water – is independent of all other metabolic pathways within the bacterium. Yet for certain β-lactamases there appears to be a permissive correlation with particular bacteria, implying a fitness cost underlying the control of their expression (Fernández et al. 2012). The biochemical basis for the much more complex transition from planktonic bacteria to the biofilm, as regulated by quorum-sensing pathways and correlated to virulence (Beceiro et al. 2013), has revealed strategies – chemical (Morkunas et al. 2012; Stacy et al. 2012, 2013; Imperi et al. 2013b; Melamed Yerushalmi et al. 2013; Saroj and Rather 2013), biochemical (Chatterjee et al. 2013), and microbiological (Gupta et al. 2013) – to interfere with this transition (Blackledge et al. 2013; Hirakawa and Tomita 2013; Zhu and Kaufmann 2013). While the determination of the optimal structure and target for intervention is still uncertain (and is likely different for each bacterium), whole-genome analysis of the bacterial resistome (McArthur et al. 2013) is a credible strategy to identify lineage in outbreaks (Harris et al. 2013; Otto 2013b; Reuter et al. 2013; Holt et al. 2013); to identify resistance–evolution pathways (Abranches et al. 2013; Kamen Ek and Gur-Bertok 2013; Méhi et al. 2013); to correlate biochemical adaptation to resistance (as exemplified for daptomycin (Kelesidis et al. 2013; Peleg et al. 2012; Song et al. 2013; Tran et al. 2013); and to validate targets (Wang et al. 2013). Exemplification of the correlation of new structures to old Gram-negative targets, as well as emerging Gram-negative targets, is given in Fig. 4.

Bacteria recognize and respond to antibiotics and to each other. These responses may be relatively simple (such as lipid remodeling as a resistance mechanism to daptomycin) or complex (such as caused by activation of cell-wall stress stimulons). Within each of these responses are critical nexuses, and disrupting these connections will be future strategies to combat resistance.

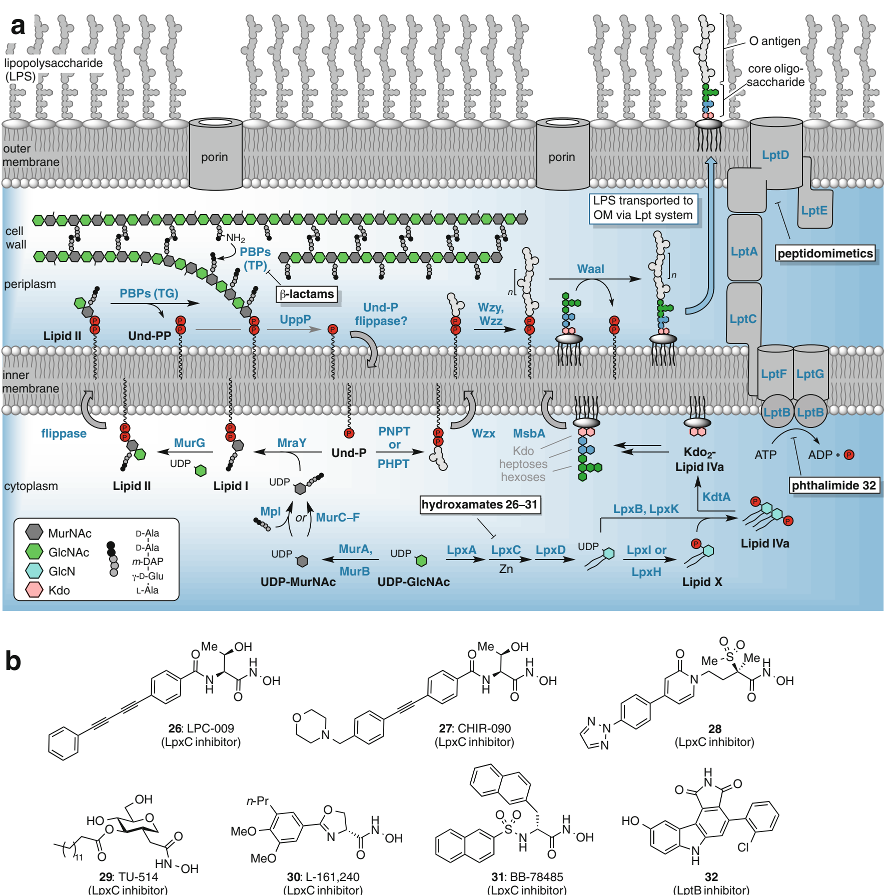

Fig. 4 (**a**) Cell-wall biosynthesis, *O*-antigen biosynthesis, and lipopolysaccharide (*LPS*) biosynthesis and transport in *E. coli*. For recent reviews, see Greenfield and Whitfield (2012), Zhang et al. (2013). (**b**) A selection of LpxC inhibitors (Jackman et al. 2000; Brown et al. 2012a; Montgomery et al. 2012; Warmus et al. 2012) and inhibitors of LptB (Sherman et al. 2013). For peptidomimetic inhibitors of LptD, see Srinivas et al. (2010)

Will Understanding the Bactericidal Mechanisms of Antibacterials Identify New Anti-resistance Strategies?

A parallel opportunity to combat resistance by identifying critical connectivity exists for the bacterial responses subsequent to encounter with the antibacterial. While there is certainly a mechanistic correlation between the engagement of the target by the antibacterial and the ensuing loss of bacterial viability, it is now more than ever evident that the mechanistic relationship between these two events is not linear (Kohanski et al. 2010). Knowledge of the antibacterial activity of silver ions is ancient: but at a molecular level, how should we interpret the ability of silver ions to

potentiate (both in vitro and in murine pharmacological assays of infection) a variety of antibiotics against both Gram-negative and Gram-positive bacteria (Morones-Ramirez et al. 2013)? One possible explanation for this synergy is cooperative induction of oxidative stress as the key bactericidal event (Brynildsen et al. 2013; Vatansever et al. 2013). The induction by β-lactams of the RpoS regulon (so as to facilitate mutagenesis) (Gutierrez et al. 2013) and the toxicity of antibacterials to mitochondria are consistent with this hypothesis (Kalghatgi et al. 2013). Other experimental data, however, are inconsistent (Liu and Imlay 2013; Keren et al. 2013; Ezraty et al. 2013) and emphasize the extraordinary challenges both of experimental design and interpretation necessary to decipher tightly integrated response pathways (Mahoney and Silhavy 2013; Manoil 2013).

Oxidative stress is not the only source of connectivity loci directly relevant to resistance. Bacteria control pathways by kinase/phosphatase signaling and the breadth of phosphorylation control – whether histidine or serine/threonine/tyrosine – encompass resistance responses (Worthington et al. 2013; Wilke and Carlson 2013; Hall et al. 2013). The two-component histidine kinases have no eukaryotic counterpart and thus present opportunity as targets for structure-based inhibitor design (Velikova et al. 2013). Conversely, given the diversity of structures already perfected for the control of eukaryotic kinases, the attractiveness of a strategy that re-optimizes these structures as inhibitors of bacterial kinases – as exemplified by the phosphotransferases used to detoxify aminoglycosides (Stogios et al. 2013) – is obvious.

A third connection is recognition of the relationship of the proton-motive force to antibacterial resistance (Farha et al. 2013b; Lázár et al. 2013). This force is, of course, necessary for bacterial vitality: it contributes directly, for example, to the correct localization of an essential high-molecular-mass penicillin-binding protein in *B. subtilis* as shown by the collapse of this force following exposure of the bacterium to the lantibiotic, nisin (Lages et al. 2013). And while the collapse of this force is intimately connected to the cellular damage arising from oxidative stress (Ezraty et al. 2013), the enzymes that sustain the proton-motive force represent independent targets for antibacterial discovery. For example, exposure of bacteria to sub-MIC concentrations of the OAK antimicrobial peptide sufficiently depolarizes its membrane with compromise of antibacterial efflux, thus potentiating the efficacy of erythromycin in a murine infection model of MDR *E. coli* (Zaknoon et al. 2012; Goldberg et al. 2013). A similar potentiation is demonstrated for *S. aureus* (Kaneti et al. 2013). Exploiting the emerging capabilities of high-resolution microscopy for bacterial cytological profiling, Nonejuie et al. identified the mechanism of a Gram-positive active spirotetronate as the collapse of the proton-motive force (Nonejuie et al. 2013). Indeed, this discovery demonstrates the value of emerging technologies for finding solutions to the resistance conundrum: here, a simple and one-step assay that rapidly identifies the antibacterial mechanism.

A final emerging theme for new strategies to combat antibacterial resistance is invigoration of bacterial persisters – slow-growing or hibernating bacterial cells that show tolerance to the presence of antibiotics (Gerdes and Ingmer 2013) – so as to abolish their tolerance. For example, application of a depsipeptide derivative to an

in vitro culture of *S. aureus* persisters activates the ClpP protease of these bacteria. When combined with a second antibacterial (rifampicin), the persisters are killed (Conlon et al. 2013). Moreover, this combination eradicates, in a murine infection model, a biofilm infection of *S. aureus* (Conlon et al. 2013). As suggested by these authors, ClpP may not be unique as a target capable of "corruption" by specific agents, with ensuing sensitization of the bacterial persister. A second enzyme target, NAD synthetase, is a target for the eradication of *M. tuberculosis* persisters (Kim et al. 2013). The emerging abilities of whole-genome bacterial analysis to identify resistance mutations (Reuter et al. 2013) and of proteomics to identify resistance responses (Conlon et al. 2013; Chang et al. 2013; Lima et al. 2013) will enable target (s) validation with respect to collateral sensitivity networks for multi-antibiotic therapy (Roemer and Boone 2013) or multi-antibiotic therapeutic cycling (Imamovic and Sommer 2013).

Conclusion: The Antibiotic Future in the Face of an Inexorable Expansion of the Resistome

These examples emphasize the depth of the resources available to discover new structures and to implement new strategies to combat bacterial resistance. The evolution of the resistance pathways, following the clinical introduction of antibiotics, is recognized as the result of the evolutionary refinement by bacteria of *inter alia* their protein structures, their genomes, and their metabolic pathways (Derewacz et al. 2013). These refinements are interdependent, and identifying and exploiting the key nexuses that are the foundation of this interdependence will be critical to the antibacterial future (Roemer et al. 2012; Roemer and Boone 2013; East and Silver 2013; Lewis 2013; Zlitni et al. 2013). The success of β-lactam/β-lactamase therapy – measured both by three decades of clinical practice (Drawz and Bonomo 2010; Hasan et al. 2013) and the current antibacterial pipeline (Butler et al. 2013; Pucci and Bush 2013; Shlaes 2013) – proves the value of this strategy. The failure of efflux inhibitors to attain clinical impact (to date) proves the challenge of the strategy. Adding to this challenge are the increased expectations for the clinically efficacious antibacterial. The antibacterial expectation is no longer measured with respect to a Gram-positive and Gram-negative divide, but with respect to an emerging array of pathogenic species, each with its own unique resistance capabilities (Boucher et al. 2013; Master et al. 2013; Pendleton et al. 2013). Moreover, the new antibacterial may now be expected to retain efficacy against intracellular bacteria (Miskinyte and Gordo 2013) and bacterial persisters (Lewis 2013; Bald and Koul 2013; Cohen et al. 2013; Maisonneuve and Gerdes 2014) and to preferentially target virulent strains (Beceiro et al. 2013) while preserving the nonpathogenic microbiome (Riley et al. 2013). As we transition from populations at particular risk of bacterial infection – such as the transplant recipient (Bodro et al. 2013), the immunocompromised (Bow 2013), and the critically ill (Cohen 2013) – to the entire populations at risk, such may result from the entry of pathogenic bacteria into

the food supply (Le Hello et al. 2013), neither new structures nor new technologies may alone suffice to provide the antibacterial future. We will need to aggressively evaluate empirical opportunities, such as pursuing repurposed compounds that act to sensitize (Thorsing et al. 2013) or to attenuate quorum sensing (Imperi et al. 2013b; O'Loughlin et al. 2013), virulence (Imperi et al. 2013a; Long et al. 2013), and efflux (Jiang et al. 2013). Reassessing old discovery strategies as well as implementing new strategies will be required (Shapiro 2013; Tegos and Hamblin 2013). While the scientific paths to move forward against bacterial resistance are largely evident, the resources needed to engage the breadth of these paths are not, nor, as emphasized in the introduction, is the realization that engaging these paths is necessary but not sufficient. The question is not what needs to be done but rather whether a pandemic is the only circumstance that will galvanize an antibiotic future.

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Drug-Resistant Tuberculosis

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Abstract

Tuberculosis, an airborne infectious disease caused by the organism *Mycobacterium tuberculosis*, has been a leading cause of death for centuries and remains a major cause of morbidity and mortality in many parts of the world. In 2012, 8.6 million people became sick with tuberculosis, and 1.3 million died from this curable disease (World Health Organization 2013a). While the majority of cases are caused by strains susceptible to all antituberculosis antibiotics, drug resistance is a major concern that carries the potential to reverse decades of progress in tuberculosis control. This chapter examines the global epidemiology of drug-resistant tuberculosis and the drivers for development of drug resistance and its spread within populations. We explore the reasons for the complex nexus between drug resistance and access to health care, including case studies of countries whose epidemics have taken different trajectories. We examine the important implications of drug-resistant tuberculosis for health systems in both the developed world and low- and middle-income countries. Finally, we discuss the elements needed to control the spread of drug-resistant tuberculosis.

Keywords

Tuberculosis • MDR-TB • XDR-TB • nosocomial tuberculosis • health systems

Introduction

Tuberculosis, an airborne infectious disease caused by the bacterium *Mycobacterium tuberculosis*, has been a leading cause of death for centuries and remains a major cause of morbidity and mortality in many parts of the world. In 2012, 8.6 million people became sick with tuberculosis, and 1.3 million died from this curable disease (World Health Organization 2013a). While the majority of cases are caused by strains susceptible to all antituberculosis antibiotics, drug resistance is a major concern that carries the potential to reverse decades of progress in tuberculosis control. This chapter examines the global epidemiology of drug-resistant tuberculosis and the drivers for the development of drug resistance and its spread within populations. We explore the reasons for the complex nexus between drug resistance and access to health care, including case studies of countries whose epidemics have taken different trajectories. We examine the important implications of drug-resistant tuberculosis for health systems in both high-income and low/middle-income countries. Finally, we discuss the elements needed to control the spread of drug-resistant tuberculosis.

Background and Global Epidemiology of Drug-Resistant Tuberculosis

A Brief History of Tuberculosis Treatment and Drug Resistance

Prior to the era of antibiotic treatment for tuberculosis, patients remained contagious for years, few were cured, and case fatality approached 100 % (Rieder and International Union Against Tuberculosis and Lung Disease 1999). The advent of medications capable of curing the disease was a major breakthrough and hastened the fall in tuberculosis incidence and mortality in many industrialized nations (Armstrong et al. 1999; Murray 2004).

In the initial clinical trials of tuberculosis treatment, it became evident that *Mycobacterium tuberculosis* rapidly acquires resistance in patients treated with a single antibiotic (Crofton and Mitchison 1948; Medical Research Council Streptomycin in Tuberculosis Trials Committee 1948; Daniels and Hill 1952; Knox et al. 1952). Studies from this era also suggested that poor adherence to treatment increased the risk of drug-resistant tuberculosis (Crofton 1959; Simpson 1959) and that treatment outcomes were worse among patients infected with resistant organisms (Daniels and Hill 1952). In 1960, the International Union Against Tuberculosis published results from the first global survey of tuberculosis drug resistance. The survey, which pooled data from patients admitted to sanitoria or hospitals in Europe, North America, Brazil, and India, found 42 % of previously treated patients, and 6.5 % of previously untreated patients, had strains resistant to at least one of the three antituberculosis drugs in use at the time (Crofton 1960).

The rising prevalence of drug-resistant tuberculosis among previously untreated cases prompted the use of regimens utilizing three drugs. This ensured that patients with undiagnosed resistance to one drug would receive treatment with at least two medications to which the strain remained susceptible (Bell and Brown 1960).

Standard treatment of drug-susceptible tuberculosis lasted 1–2 years until the 1970s, when several clinical trials led to the establishment of "short-course chemotherapy" (SCC) regimens, which were able to cure the disease in 6–8 months with low rates of relapse (East African/British Medical Research Council 1973a, b; Fox et al. 1999). Despite the advent of effective, shorter treatment regimens, tuberculosis continued to afflict millions and remained the leading cause of death due to a single infectious agent (Kochi 1999; Snider and La Montagne 1994). The emergence of the HIV epidemic in the 1980s further strained tuberculosis control programs in many parts of the world. To improve tuberculosis control and increase the number of affected persons being treated, a new global strategy was developed and advocated by the World Health Organization (WHO) – "DOTS," an acronym for Directly Observed Treatment Short-course (Kochi 1999; Snider and La Montagne 1994). DOTS was intended to build the capacity to diagnose and treat drug-susceptible tuberculosis, while using direct observation of pill ingestion to ensure adequate adherence and prevent the emergence of resistance.

In the early 1990s, a series of deadly outbreaks of highly drug-resistant tuberculosis occurred in the United States. As data became available, it was soon clear that

resistance posed a threat to tuberculosis control in many parts of the world (Centers for Disease Control and Prevention 1999; Coninx et al. 1999; Farmer et al. 1998; Kim et al. 1997; Kimerling et al. 1999a; Pablos-Mendez et al. 1998). Today, drug-resistant tuberculosis is found in every country and is recognized as a global problem that needs to be addressed urgently.

Definitions and Clinical Implications of Drug-Resistant Tuberculosis

Antituberculosis antibiotics are often broadly grouped into two categories: first-line drugs and second-line drugs. Orally administered first-line drugs are isoniazid, rifampin, pyrazinamide, and ethambutol. Streptomycin is an injectable first-line drug. All other antituberculosis drugs are considered second line, although, as shown in Box 1, these second-line drugs are further categorized into four categories. This categorization is useful when creating regimens to treat drug-resistant tuberculosis (World Health Organization 2011a; World Health Organization and Stop TB Department 2008).

All cases of tuberculosis, regardless of resistance or susceptibility, can be categorized as either “new” or “previously treated.” New cases have never been treated for tuberculosis in the past (or have been treated for less than 1 month), whereas previously treated cases are those that have previously received more than 1 month of treatment (World Health Organization 2013b).

Antibiotic resistance is broadly divided into two forms, according to the host in which the resistance-conferring mutations arose. *Primary drug resistance* occurs when the bacilli were already resistant to antibiotics at the time they were transmitted from another patient. This is seen in new cases. *Acquired drug resistance* (also called “secondary drug resistance”) occurs when new drug resistance arises in an individual during the course of their infection (Dye 2009). This is seen in previously treated cases. Further, it is possible for a strain resistant to some drugs to be transmitted to an individual who then acquires resistance to additional antibiotics during treatment.

Categorization of drug-resistant tuberculosis is also based upon the number and type of medications to which the organism is resistant, as these characteristics impact treatment and outcomes (Box 2).

Among the different types of drug-resistant tuberculosis, the categories of multidrug-resistant (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) have the greatest impact on treatment and outcomes. MDR- and XDR-TB are resistant to the most potent categories of antituberculosis medications, and their treatment requires more drugs (at least 5) and lasts much longer (at least 20 months for MDR-TB, at least 2 years for XDR-TB). Their treatment is also more challenging because it necessitates utilization of injectable antibiotics as well as second-line oral agents which are more expensive, more commonly associated with adverse events, and less effective than first-line drugs. The average cure rate for MDR-TB in the published literature has been approximately 60 % and mortality during treatment 15 % (Ahuja et al. 2012), although, with adequate resources and timely diagnosis, it is possible to achieve cure rates of >80 % (Mitnick et al. 2003). Cure rates for XDR-TB are 25–30 % – similar to spontaneous cure rates seen in the pre-antibiotic era (Falzon et al. 2012).

Because MDR- and XDR-TB have the greatest impact on treatment and outcomes, we focus on these forms when describing the global epidemiology of drug-resistant tuberculosis.

Further reading on the diagnosis and treatment of tuberculosis and drug-resistant tuberculosis can be found at:

http://www.who.int/tb/publications/tb_treatmentguidelines/en/

http://www.who.int/tb/challenges/mdr/programmatic_guidelines_for_mdrtb/en/

Box 1: Groups of Antituberculosis Medications†

First-line agents	
Group 1. First-line oral agents	Isoniazid, rifampin, ethambutol, pyrazinamide
Second-line agents	
Group 2. Injectable antituberculosis drugs[a]	Kanamycin, amikacin, capreomycin
Group 3. Fluoroquinolones	Levofloxacin, moxifloxacin, gatifloxacin
Group 4. Second-line oral agents	Ethionamide, prothionamide, cycloserine, PAS
Group 5. Agents of unclear efficacy against drug-resistant tuberculosis	Clofazimine, linezolid, amoxicillin-clavulanate, thioacetazone, imipenem-cilastatin, clarithromycin, high-dose isoniazid

†Two new second-line antituberculosis medications, bedaquiline and delamanid, have yet to be classified and are not included in this table.

[a]Streptomycin, an injectable, is not a second-line medication as it is not used to treat multidrug-resistant tuberculosis (see Box 2)

Box 2: Categorization of Tuberculosis Drug Resistance

Resistance pattern	***Definition***
Pan-susceptible	Susceptibility to all antituberculosis medications
Mono-resistant	Resistance to one antituberculosis medication
	For example, "isoniazid mono-resistant" organisms are resistant to isoniazid and susceptible to other antituberculosis medications
Polydrug-resistant (PDR)	Resistance to at least two tuberculosis medications, excluding organisms resistant to both isoniazid *and* rifampin
Multidrug-resistant (MDR)	Resistance to at least isoniazid *and* rifampin
Extensively drug-resistant (XDR)	MDR strains that are also resistant to fluoroquinolones *and* at least one second-line injectable agent

Global Epidemiology of Drug-Resistant Tuberculosis

Isoniazid mono-resistance is of epidemiologic importance as it is common in many parts of the world and increases the risk of relapse and of developing MDR-TB (Menzies et al. 2009). A recent study described the global prevalence, distribution, and trends in isoniazid-resistant tuberculosis between 1994 and 2007 (Jenkins et al. 2011). The global average prevalence of isoniazid-resistant tuberculosis among new and previously treated cases was estimated to be 13.9 % and 29.0 %, respectively. These estimates were calculated excluding data from Eastern Europe, where prevalence was much higher (33.5 % among new and 61.4 % among previously treated cases). The highest rates among new cases were reported in some countries of the former Soviet Union, China, Vietnam, the Dominican Republic, and parts of India.

In 2012, MDR-TB represented 3.6 % of new and 20.2 % of previously treated tuberculosis cases (World Health Organization 2013a). Thus, among tuberculosis notified to the WHO, it is estimated that 170,000 new and 140,000 previously treated cases had MDR-TB. While MDR-TB caused 5 % of the world's incident tuberculosis cases in 2012, it was responsible for 13 % of tuberculosis deaths (World Health Organization 2013a). XDR-TB accounts for 9.6 % of all MDR-TB and has been reported in 92 countries (World Health Organization 2013a).

Worldwide summary statistics do not adequately describe the epidemiology of drug-resistant tuberculosis, a disease whose global burden is unequally distributed (Fig. 1). "High-burden countries" are those that have reported at least 4,000 cases of

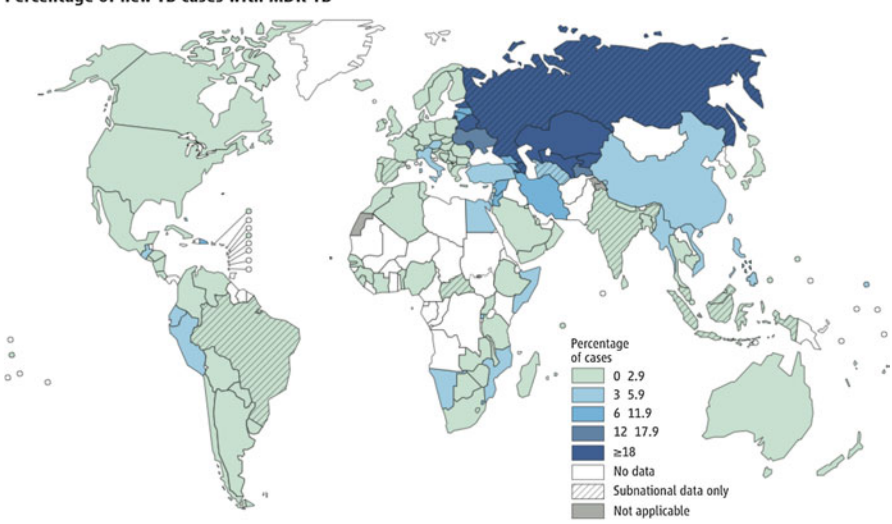

Fig. 1 Proportion of new tuberculosis cases with MDR-TB. With permission from the World Health Organization (http://www.who.int/tb/publications/global_report/en/) (World Health Organization 2013a)

MDR-TB or where at least 10 % of new cases have MDR-TB (World Health Organization 2010a). Over 50 % of the world's MDR cases are found in three countries: China, India, and Russia (Zignol et al. 2012a). In China, 10 % of tuberculosis patients have MDR-TB, and over half of these are cases of primary drug resistance, caused by transmission of resistant strains (World Health Organization 2010a; Zhao et al. 2009; Zhao et al. 2012). In India, where only subnational data on the prevalence of drug resistance have been gathered, the WHO estimates that 100,000 cases of MDR-TB emerge annually (World Health Organization 2010b). In Russia, 23 % of new and 49 % of previously treated cases have MDR-TB; however, certain regions have a higher prevalence than others. For example, the highest proportion of MDR-TB among new cases in the world – 41.9 % – has been found in the Russian Federation's Yamalo-Nenets Autonomous Area (World Health Organization 2013b), and in the Ulyanovsk oblast, over 70 % of all tuberculosis cases have MDR-TB (World Health Organization 2013b). The elevated prevalence of MDR-TB in Russia is part of a larger epidemic involving several Eastern European and Central Asian countries.

Unfortunately, drug resistance surveillance data are lacking for most of Africa, a region that accounts for over one-fifth of the world's tuberculosis cases and 80 % of those occurring among people living with HIV (World Health Organization 2013b). Available data suggest MDR-TB accounts for a smaller proportion of tuberculosis in Africa than other parts of the world (World Health Organization 2013b); however, because tuberculosis is highly prevalent in this region, the absolute number of MDR-TB cases could be high enough to strain resources (Zignol et al. 2012b). In South Africa, the prevalence of drug-resistant tuberculosis has risen rapidly – MDR-TB accounted for 3.1 % of all tuberculosis cases in 2002 and for 9.6 % in 2008 (Streicher et al. 2012). The majority of MDR-TB in South Africa is seen in new cases of tuberculosis, and it has been estimated that 80 % of MDR-TB in this country is caused by transmission of resistant organisms (Streicher et al. 2012).

The Pathogenesis of Drug-Resistant Tuberculosis

The Biological Basis of Drug Resistance

Antibiotic resistance arises when the mutation of mycobacterial genes confers bacilli with a survival advantage that enables them to withstand antituberculosis antibiotics. If mycobacteria are exposed to inadequate concentrations of an antibiotic, those that are resistant to that medication become dominant (David 1970). These bacteria then replicate and can be transmitted to others. Many different genetic changes can confer resistance to any given drug. For example, studies using whole genomic sequencing have demonstrated that resistance to the drug isoniazid may be caused by hundreds of possible genetic changes (Hazbon et al. 2006; Almeida Da Silva and Palomino 2011).

In some cases, genetic changes that confer resistance may be associated with a fitness cost to the bacteria that renders them potentially less pathogenic or transmissible. However, it has been suggested that over time the increased survival and transmission of fittest isolates will lead them to become dominant (Sun et al. 2012). Relative strain fitness is considered one of the most important factors in mathematical modeling of the dissemination of drug resistance in populations, as it is thought to influence the transmissibility of the strain and hence its epidemiological impact (Almeida Da Silva and Palomino 2011; Borrell and Gagneux 2011).

Another important mycobacterial factor in the development of drug resistance is the very slow replication rate of *M. tuberculosis*. In contrast to most bacterial pathogens, which replicate in minutes to hours, the doubling time of *M. tuberculosis* is about 24 h. As a result, effective therapeutic drug levels must be maintained for long periods in order to eradicate all viable mycobacteria (David 1970).

The rate at which bacilli develop drug-resistant mutations may also be related to the mycobacterial strain. There is some evidence from clinical (Cox et al. 2007) and in vitro (Ford et al. 2013) studies showing that the prevalent Beijing strains of *M. tuberculosis* may acquire drug resistance more rapidly than others, although this has been disputed. All *Mycobacterium tuberculosis* strains originated from an original ancestor, in the distant past. Over time, selective pressures have caused genetic differences to emerge, creating strain diversity. The “Beijing” family of strains (also known as the W family of strains) comprises one dominant branch of the phylogenetic tree (Borrell and Gagneux 2011). This particular strain represents about 50 % of strains in Asia and more than 13 % of all strains worldwide (Parwati et al. 2010).

At a population level, the incidence of drug-resistant disease is determined by the complex interplay between (a) the rate at which resistant isolates are generated, (b) the effect these resistance-conferring genetic changes have upon infectiousness and transmissibility of the pathogen, (c) the susceptibility of the exposed population to tuberculosis, and (d) the selective pressures generated by antibiotic use (Dye 2009). Consequently, there has been considerable clinical and research interest in developing strategies to address each aspect.

In the following section, we examine the epidemiological factors affecting acquired and primary drug resistance and present several case studies to illustrate their impact.

Epidemiological Factors Associated with Acquired Drug Resistance

Nonadherence with Therapy

Standard treatment for drug-susceptible tuberculosis involves taking multiple antibiotics at least 3 times per week for a minimum of 6 months (World Health Organization and Stop TB Department 2010). These patients are generally

prescribed four antibiotics during the initial 2-month "intensive phase," followed by two drugs in the 4-month "continuation phase." The duration and complexity of this therapy is a substantial barrier to adherence. Furthermore, common side effects of tuberculosis medications such as nausea, anorexia, and rash often deter patients from continuing treatment. Interrupted or inadequate drug therapy can lead to subtherapeutic drug levels and give rise to drug resistance.

Past tuberculosis treatment is a strong predictor of drug resistance (Dalton et al. 2012). A frequently cited mechanism by which treatment leads to drug resistance is noncompliance with medications, with irregular treatment leading to subtherapeutic levels and the selection of resistant isolates (David 1970). However, the data to support this theory is limited and largely confined to retrospective studies (Pablos-Mendez et al. 1997; Gelmanova et al. 2007). A study in San Francisco found that noncompliance was 20 times (95 % CI 1.7–234) more likely to be associated with acquired drug resistance (Bradford et al. 1996). The study showed that side effects of therapy may have contributed to the nonadherence, with gastrointestinal symptoms of therapy increasing the odds of acquired resistance by 11.5 times (95 % CI 1.2–107). A widely reported epidemic of drug-resistant tuberculosis in New York City in the early 1990s found nonadherent cases took almost four times as long to achieve sputum culture conversion (254 vs. 64 days, $p < 0.00001$) – suggesting that interrupted treatment was also considerably less effective. However, there was no overall association between acquired resistance and noncompliance with therapy (Pablos-Mendez et al. 1997).

Inappropriate Drug Regimens

The selection of drug regimens containing an inadequate number of antituberculosis medications to which the bacteria are susceptible substantially increases the likelihood of acquired drug resistance. The success of standard therapy may be reduced markedly if tuberculosis is caused by a mycobacterial strain that is already resistant to one or more antibiotics. A review of two studies that characterized drug resistance before treatment of non-MDR-TB showed that the relative risk of developing MDR-TB was 29 (3.8–226.7) times increased among individuals who were given inappropriate treatment, as defined by drug susceptibility (van der Werf et al. 2012). Another review of the effect of standardized treatments found the odds of acquiring drug resistance were between five- and tenfold higher if disease was caused by a resistant strain compared to a pan-sensitive strain (Menzies et al. 2009). This study also found that the number of drugs to which strains were susceptible was inversely related to the likelihood of acquired drug resistance.

Despite the availability of evidence-based guidelines for managing drug resistance, the reality "on the ground" is often very different. In many countries with limited access to diagnostics or optimal tuberculosis treatment, the haphazard prescription of drugs to patients failing treatment creates the preconditions for acquired drug resistance. The use of substandard drug regimens was an important

factor driving the high rates of drug resistance that emerged in the former Soviet Union in the 1990s (Drobniewski et al. 1996a).

Case Study: Drug Resistance in the Former Soviet Union

The rapid rise in MDR-TB incidence after the collapse of the former Soviet Union illustrates how the disease can become prevalent in a population. In the two decades until 1990, Russia had achieved a 3.7 % annual decline in tuberculosis notifications. After a sudden economic downturn with the end of the Soviet Union, tuberculosis notifications rose by 7.5 % per year (Shilova and Dye 2001). As early as 1996, the incidence of MDR-TB among new cases in prisons had risen to 20 % (Kimerling et al. 1999b). The emergence of drug resistance was largely driven by the widespread use of suboptimal regimens. A study among prisoners in the mid-1990s found that 79 % of patients with tuberculosis had experienced treatment interruption (Kimerling et al. 1999b). Inappropriate first-line treatment for patients with past tuberculosis histories and inadequate re-treatment regimens that added a single additional drug to failing regimens (the WHO "Category 2" regimens) also contributed to the rise of MDR-TB. There was also evidence that prisons became reservoirs of MDR-TB and XDR-TB, and drug-resistant tuberculosis spread from there to the general population (Drobniewski et al. 1996b). As a result, in 2012, MDR-TB comprised 23 % of all newly diagnosed tuberculosis cases in Russia (World Health Organization 2013a). An interesting contrast with the Russian experience was Latvia and Estonia, which maintained their functioning tuberculosis control programs and saw a decline in the incidence of MDR-TB during the same time period (Dye 2009). The contrast between the settings emphasizes the importance of the public health response to tuberculosis in the control of drug-resistant tuberculosis.

Suboptimal Drug Levels and Poor Drug Quality

Even when appropriate antibiotics are prescribed and patients are compliant, treatment still may be ineffective. Poor drug quality is a major problem for tuberculosis control in many countries. Problems with manufacturing standards, storage of medications, or expiry of drugs may all contribute to suboptimal drug levels. One study of drug quality in hospitals and pharmacies in six low- and middle-income countries found that 10 % of all samples contained inadequate quantities of the active tuberculosis drugs (Laserson et al. 2001). Interestingly, fixed dose combinations were 1.7 times more likely to be associated with low drug quantities than individually administered tablets.

Physiological differences between individuals may also contribute to diminished drug effectiveness. There is some evidence that HIV may reduce drug absorption, leading to higher treatment failure rates and acquired drug resistance (Andrews et al. 2007). This finding was supported by a study of tuberculosis pharmacokinetics, which also found a substantial proportion of individuals without other comorbidities

had subtherapeutic drug levels despite being compliant with therapy (Babalik et al. 2011).

Case Study of Factors Contributing to Drug Resistance: India

India, which is home to over 20 % of all MDR-TB cases (World Health Organization 2013a), illustrates how weakness in tuberculosis control creates the environment where drug-resistant tuberculosis can readily emerge. There are several factors that may explain why drug resistance has become an important problem in India. Firstly, weaknesses in drug prescribing may create a setting where drug resistance can emerge. The diagnosis and treatment of tuberculosis in India is highly decentralized, with more than half of all tuberculosis patients receiving care through poorly regulated private providers (Prasad 2010). One study from Mumbai illustrated the problems with treatment practices in the private sector. Among 106 health-care workers, 63 different regimens were recommended, of which only six were considered adequate (Udwadia et al. 2010). Surprisingly, the survey showed no improvement in prescribing practices compared to a similar one 20 years earlier. Such variable prescription practices are likely to give rise to ineffective treatment, treatment failure, and acquired drug resistance. Poor-quality drugs may contribute to substandard regimens, with reports of low quality of medications, poor drug storage, and drug supply chain problems (Prasad 2010; Bhaumik 2013). Thirdly, diagnostic delay may also contribute to prolonged periods of transmission of disease, with one study finding that patients saw 6–9 doctors before receiving standardized therapy (Prasad 2010). Finally, access to appropriate second-line treatment in India remains low – with less than 25 % of patients with MDR-TB receiving appropriate treatment (World Health Organization 2013a). In summary, recognized weaknesses in the Indian health-care system, particularly in the private sector, have created many of the preconditions for the development of drug-resistant tuberculosis. However, limitations in routine disease reporting have made it difficult to quantify the epidemiological impact of these trends.

Epidemiological Drivers of Primary Drug Resistance

Delays in Diagnosis and Effective Treatment

In many high-tuberculosis-burden settings, the most widely used diagnostic test for tuberculosis is "smear microscopy" wherein sputum, after special staining, is examined under a microscope for the presence of tuberculosis bacilli. While it is inexpensive, smear microscopy is unable to determine whether drug resistance is present. Traditional methods for determining drug resistance can take several months, involving sputum culture and then culture-based (phenotypic) drug susceptibility testing (DST). There can be long delays in the diagnosis of MDR-TB, particularly if sputum culture is initiated only after a patient fails first-line therapy. Newer, more

rapid methods of DST use PCR-based technologies to identify the presence of *M. tuberculosis* and resistance-conferring DNA sequences with a turnaround time of hours to days, substantially decreasing time to diagnosis (Boehme et al. 2010). However, it is unclear whether availability of these tests translates to meaningful reductions in the time to treatment initiation.

The risk of transmission of tuberculosis to exposed contacts is influenced by the duration and closeness of exposure to the index patient and environmental factors such as ventilation (Kenyon et al. 1996; Lienhardt et al. 2003; Escombe et al. 2007). Transmission of drug-resistant tuberculosis is most pronounced in settings where there are lengthy delays in the diagnosis and appropriate treatment of patients with disease. Diagnostic delay is common for patients with drug-resistant disease, in light of the patient and health system factors described above.

Diagnosis of drug resistance – the necessary precursor to effective therapy – is particularly challenging in settings where access to confirmatory testing is limited. In many countries where there is limited access to laboratory-based diagnostics, a diagnosis of "clinical" MDR-TB is routinely made after a patient fails empirical therapy with first-line drugs, which can take 5 months (World Health Organization and Stop TB Department 2010), and often only when patients fail empiric therapy with a re-treatment regimen (which also utilize first-line medications). Even in settings where DST is routinely available, there may be delays of 12 weeks or more until drug susceptibility results are available. Even after diagnosis and commencement of appropriate second-line drug therapy, patients may remain infectious for weeks to months, until they no longer secrete viable bacilli (Ahuja et al. 2012). During this time, whether patients are managed at home or in hospital, they can transmit drug-resistant disease to many others before they are cured.

During the prolonged infectious period, strategies to reduce transmission for which there is some evidence include standard infection control procedures such as isolation, ventilation, and other environmental controls and use of personal protective devices (Jensen et al. 2005). Administrative controls also play a critical role in avoiding unnecessary exposure. However, some infection control strategies may be practically difficult or expensive to implement routinely in the overstretched health-care systems of resource-limited settings. Therefore, transmission of MDR-TB and XDR-TB is likely to be substantial in settings where health systems are weak or overstretched (Gandhi et al. 2006).

Unfortunately, the vast majority of patients with drug-resistant tuberculosis are never diagnosed nor treated, because diagnostic capacity is very limited in most countries where this disease is common. Although the WHO recommends DST for all tuberculosis patients at increased risk of drug resistance, in 2012, only 9 % of previously treated cases underwent DST (World Health Organization 2013a). In 2012, in India and China – home to over half of the world's MDR-TB cases – only 26 % and 5 % of MDR-TB patients were detected and reported (World Health

Organization 2013a). Left undiagnosed and untreated, most people with drug-resistant tuberculosis are likely to transmit the disease to others and die.

Environmental Risk Factors for Transmission

The environment in which an infectious patient resides affects the likelihood of transmission and the populations who are likely to have been exposed. Household contacts in particular are a high-risk group, owing to the duration and proximity of their exposure to the patient (Becerra et al. 2011; Fox et al. 2013). The risk of transmission is also high in health-care settings, prisons, and other congregate settings (Nodieva et al. 2010). A widely reported outbreak of XDR-TB in Tugela Ferry, Kwazulu-Natal Province, in South Africa tragically illustrated the problem of nosocomial transmission (transmission of infection within health-care settings). The authors found that 39 % of all tuberculosis patients had MDR-TB and 6.3 % were infected with XDR-TB. Subsequent epidemiological investigation showed that 67 % of the XDR-TB patients had been admitted to hospital within the preceding 2 years before diagnosis, and none had other history of close contact with tuberculosis (Gandhi et al. 2006). Further support for the hypothesis of transmission of resistant strains was given by genotypic testing, which found that 85 % of isolates were genetically similar. This suggested that a dominant strain of XDR-TB had spread throughout the province (Moodley et al. 2011). Importantly, 80 % of patients in the Tugela Ferry outbreak were infected with HIV. Not only did HIV coinfection contribute to the high mortality rates, but HIV also has the effect of "telescoping" outbreaks, by reducing the time for infected individuals to develop the disease and hence the time to further propagation of the infection.

Challenges in Measuring the Impact of Drug-Resistant Tuberculosis

An accurate assessment of the global impact of drug-resistant tuberculosis is extremely challenging. Estimates of disease incidence in many high-burden countries are extrapolated from very limited data and rely upon numerous assumptions such as the representativeness of the population sampling and the correct classification of cases. Given this, the estimates of the global annual incidence of MDR-TB are imprecise, with the 95 % confidence limits ranging from 300,000 to 600,000 new cases (World Health Organization 2013a). In its 2013 report, the WHO was unable to obtain any data on drug resistance from 30 % of its member states (Fig. 1). Even among the 136 countries for which data is available, many estimates rely upon subnational surveys that may not accurately reflect the national situation (Cohen 2013).

There is also a considerable gap between the predicted number of incident cases and the number that are actually reported, making it difficult to verify the estimates.

The settings with the greatest case-detection gaps are the Western Pacific and Southeast Asian regions of the WHO, where only 6 % and 21 % of incident cases of MDR-TB, respectively, were diagnosed in 2012 (World Health Organization 2013a).

Furthermore, only 48 % of patients that were reported to WHO as diagnosed in 2010 had successfully completed treatment 2 years later (World Health Organization 2013a). In summary, there remain significant limitations in the data available about the incidence of MDR-TB, particularly in many of the highest-burden settings.

The Health System Impacts of Drug-Resistant Tuberculosis

Treatment of MDR-TB and XDR-TB is both complex and costly, necessitating at least 20 months of expensive multidrug therapy to achieve optimal outcomes (World Health Organization 2011a). While the cost of sputum smear to diagnose new tuberculosis is less than $2, the costs to diagnose MDR-TB are considerably greater. Culture and phenotypic DST to make the diagnosis of MDR-TB cost at least $20, and rapid PCR-based tests cost at least $10. Treatment regimens for MDR-TB typically cost at least $2,000, compared to a $20 course of standard therapy for drug-susceptible disease (Oxlade et al. 2012). Given these high costs, the WHO estimates that treating MDR-TB consumes over 20 % of the eight billion dollars that low- and middle-income countries require for treating tuberculosis, despite representing less than 6 % of disease (World Health Organization 2013a). Expensive second-line medications can comprise 20–50 % of all treatment costs despite being off-patent. Clearly, this has major financial implications for both patients and tuberculosis control programs.

A major determinant of costs relates to the manner in which tuberculosis care is provided. In one study, participants in MDR-TB treatment studies in Russia and Estonia were found to have lengthy inpatient hospital stays. This hospital-based approach more than doubled the cost compared to similar programs in Peru and the Philippines (Fitzpatrick and Floyd 2012). To address this issue, WHO guidelines recommend an ambulatory model of care for MDR-TB where possible (World Health Organization 2011a; Falzon et al. 2011).

The timing of diagnostic testing also affects the cost of MDR-TB treatment. A modeling study showed that in a country with a moderate incidence of MDR-TB (2.1 % of incident cases), a strategy of rapid DST (such as GeneXpert) at the beginning of active tuberculosis treatment was more cost-effective than a strategy of waiting until treatment failure before diagnosing MDR-TB and starting MDR-TB therapy (Oxlade et al. 2012). While parameters may vary between epidemiological contexts, the study illustrates the importance of MDR-TB management policies that match the local epidemiological and socioeconomic circumstances.

Case Study: The Cost of MDR-TB Control in the United States

The high cost of controlling drug-resistant tuberculosis is demonstrated by the experience in the early 1990s in New York City. Between 1979 and 1991, the number of incident cases of tuberculosis rose from 1,530 to 3,673 (Landesman 1993). During the same period, there was a 130 % increase in drug-resistant disease, with the proportion of MDR-TB in previously untreated cases rising from 3 % to 7 % (Frieden et al. 1993). The MDR outbreak predominantly affected the homeless, people of low socioeconomic status, people living with HIV, and intravenous drug users (Frieden et al. 1993). Prior treatment was the strongest predictor of MDR disease, with only 54 % of patients with all forms of tuberculosis completing treatment (Landesman 1993).

The rise of MDR-TB coincided with a period of sustained funding cuts to New York's public health infrastructure, characterized by a lack of coordination between tuberculosis services and poor infection control (Sterling 2006). At its nadir, owing to chronic underinvestment by government, only six nurses were responsible for community-based supervised treatment, despite there being over 3,000 incident cases of tuberculosis each year (Brudney and Dobkin 1991). By the time the problem was finally addressed in the early 1990s, the number of patients in New York had tripled in the previous 15 years, and the incidence of MDR-TB among previously untreated patients had increased from 10 % in 1983 to 23 % in 1991 (Frieden et al. 1995). Further, the mortality among MDR-TB patients approached 80 %. As the scale of the problem became apparent and funding was restored, the epidemic was rapidly contained. However, by the end of the decade, the overall cost of reestablishing tuberculosis control was estimated to be over one billion dollars (Coker 1998).

This outbreak in New York demonstrates the substantial financial and human costs of neglecting tuberculosis control and the importance of maintaining a sustained and effective public health response in order to prevent drug-resistant tuberculosis.

Addressing Drug-Resistant Tuberculosis: What Will It Take?

Scaling Up the Response

Very few patients who develop drug-resistant tuberculosis are able to access timely and appropriate therapy. A major scale-up of the existing repertoire of tuberculosis diagnostics, therapies, and public health strategies could substantially reduce MDR-TB. For example, modeling analyses suggest the WHO's "Consolidated

Action Plan to Prevent and Combat MDR and XDR Tuberculosis in the European Region" – which is based on the utilization of existing diagnostics and therapies – would place 127,000 MDR-TB patients on treatment, save 120,000 lives, and could prevent the emergence of 250,000 MDR and 13,000 XDR cases (World Health Organization and Regional Office for Europe 2011). Another modeling study suggested that scaling up diagnostic capacity in South Africa, in order to perform DST in 37 % of new and 85 % of previously treated cases, could prevent the emergence of 7,759 cases of MDR-TB and lower MDR-TB mortality by 50 % over a 10-year period (Dowdy et al. 2008).

Inadequate funding for MDR-TB control is an important obstacle to scale up. In 2010, funding for MDR-TB control in high-burden countries was only 3 % of what was needed (World Health Organization 2010b). While funding has since increased, the gaps remain substantial: in 2013, funding for MDR-TB management in low- and middle-income countries fell $84 million short of the required amount (World Health Organization 2012).

The high cost of medications to treat MDR-TB is another obstacle to scale up. A major global initiative, the Global Drug Facility, was developed to lower costs and facilitate procurement and distribution of second-line medications to WHO-approved MDR-TB treatment programs (Kim et al. 2003). While the program has successfully driven down prices of second-line drugs, MDR-TB treatment regimens continue to be costly (at least $1500 for a full course) (Global Drug Facility Stop TB Partnership). Drug costs for XDR-TB treatment may exceed tens of thousands of dollars (Pooran et al. 2013).

While scaling up of existing approaches for the diagnosis and treatment of MDR-TB could have a major effect, the impact of greater investment could be improved by innovations in MDR-TB diagnosis or treatment.

Advances in Diagnostics for MDR-TB

The advent of nucleic acid amplification tests (NAATs) has marked a major leap forward in our ability to diagnose MDR-TB (Boehme et al. 2010; Steingart et al. 2013). With these assays, drug-resistant tuberculosis can be diagnosed in a few hours, rather than weeks. Cartridge-based NAATs, such as the GeneXpert MTB/RIF assay, have the added advantage of not requiring the stringent biosafety levels needed for other DST methods (including non-cartridge PCR-based assays), which means they can be used in district- and sub-district-level facilities. A cost and affordability analysis suggests that widespread use of the Xpert assay could *lower* the cost of diagnosing MDR-TB in high-burden countries, compared to scale-up of conventional DST (Pantoja et al. 2013). The rapid result time may also reduce time to initiation of MDR-TB treatment, although this has not yet been demonstrated in the published literature. By June 2012, the Xpert MTB/RIF assay had been implemented in 67 low- and middle-income countries (Weyer et al. 2013). While

this progress is promising, advances in diagnostics need to be paralleled with advances in the availability of effective treatment.

Advances in the Treatment of MDR-TB

At the time of writing, two new antituberculosis drugs have been approved by regulatory agencies: bedaquiline and delamanid. As noted in a review by Brigden and colleagues, while the development of new drugs is important, it is also essential to develop new drug combinations (i.e., treatment regimens) for MDR- and XDR-TB that are shorter in duration, less toxic, and yet more effective (Brigden et al. 2014). In a cohort of 206 MDR-TB patients in Bangladesh, a 9-month regimen using seven drugs in the intensive phase cured 88 % of patients, with a default rate of only 5.8 % (recall, the WHO recommendation is at least 20 months of treatment with a minimum of five intensive phase medications) (Van Deun et al. 2010). While these results are promising, this was an observational study rather than a randomized controlled trial; hence, it is unclear whether the high rates of good outcomes were truly attributable to the treatment regimen utilized and not due to characteristics of the patients, *M. tuberculosis* strain, or study setting. The STREAM study, a multicenter, randomized trial taking place in South Africa, Vietnam, and Ethiopia, is under way to determine whether these results are attributable to the treatment regimen and can be achieved in other settings.

Strengthening Infection Control

Innovation is also needed to curb nosocomial transmission of drug-resistant tuberculosis. In certain high-burden settings, such transmission is thought to be contributing to MDR-TB in new cases (Nardell and Dharmadhikari 2010; Basu et al. 2011) and also among patients hospitalized while being treated for drug-susceptible tuberculosis (Zhao et al. 2012; Gelmanova et al. 2007). With MDR-TB, it can be challenging to implement measures needed to prevent nosocomial transmission (World Health Organization 2009). In many parts of the world, tuberculosis patients share living quarters while they are hospitalized. If patients with drug-resistant tuberculosis are not separated from those with drug-susceptible strains, the latter will be at risk of infection by resistant strains. The risk of transmission will be particularly elevated if patients with drug-resistant tuberculosis are not being treated with effective second-line regimens, a situation that could arise when resistance is undiagnosed and a patient only receives first-line medications. Box 3 describes an innovative protocol that incorporates the use of rapid molecular diagnostics to reduce the likelihood of nosocomial transmission of MDR-TB.

Box 3: The FAST Strategy

Molecular-based diagnostics that rapidly diagnose resistance could play an important role in curbing nosocomial transmission of drug-resistant tuberculosis. One infection control strategy that incorporates these tests is called FAST, which stands for **F**inding MDR tuberculosis **A**ctively, **S**eparating safely, **T**reating effectively. In this strategy, a molecular-based test is used to screen all tuberculosis suspects presenting to hospital, and all hospitalized tuberculosis patients, for drug-resistant tuberculosis. The strategy should lower nosocomial MDR-TB transmission because patients with resistant strains will be rapidly identified, separated, and started on the second-line regimens needed to render them noncontagious. The strategy is being implemented in Bangladesh and in parts of the Russian Federation (http://tbcare2.org/resources/infection-control).

Expanding the MDR-TB Research Agenda

The development of new diagnostics to reduce time to diagnosis, and new medications and treatment regimens to reduce the length and complexity of therapy, will facilitate the scale-up of global drug-resistant tuberculosis control (World Health Organization 2011b). However, tuberculosis research and development has been chronically underfunded, in part because the disease is uncommon in most high-income countries (Addington et al. 1977; Bloom and Murray 1992; O'Brien and Nunn 2001). As a result, drug development has been slow: after rifampin was approved in 1971, it took 41 years for another new class of drug designed for tuberculosis treatment to be developed and approved for use (while fluoroquinolone antibiotics are also highly effective drugs for tuberculosis, they were developed for other indications) (Cohen 2013). Because so few drugs have been developed, the treatment of drug-resistant tuberculosis now relies mostly on old and less effective medications, most of which were discovered prior to the 1960s and whose use in drug-susceptible disease was largely abandoned due to poor tolerability and efficacy. Additional new antibiotics to treat tuberculosis are now in the drug development pipeline, and investment in research for tuberculosis has improved since the early 2000s. However, progress remains precarious, with funding declining by $30.4 million (USD) between 2011 and 2012 (Frick and Jiminez-Levi 2013).

Conclusion

The hurdles to effectively prevent, diagnose, and treat MDR- and XDR-TB are daunting. Yet the global response to the HIV epidemic, which has seen millions of people placed on lifelong HIV therapy in some of the world's most poverty-stricken areas, suggests that complex public health problems can be addressed

with sustained political will and resource allocation. The Stop TB Partnership has outlined an ambitious goal of tuberculosis elimination by the year 2050 (World Health Organization and Stop TB Partnership 2010) and has highlighted MDR-TB as an urgent priority. Greater resources have been committed to tuberculosis control from international donors, new rapid diagnostic tests are now available, and new drugs have recently been developed. These gains have been underpinned by a growing political commitment at a national and international level. There is cause for cautious optimism. Lessons learned in the MDR outbreaks of New York City in the 1990s, and a successful national program for treatment of MDR-TB in Peru, show that treatment success is attainable in a variety of settings. Despite the development of new diagnostic technologies and promising treatment regimens, these alone will not control the drug resistance epidemic without strategies to improve access to health care by the poorest communities, among whom tuberculosis is most prevalent.

Get Involved: The MSF Access Campaign

Grassroots campaigns can help convince governments, pharmaceutical companies, and international donor agencies to enact policies and provide funding needed to increase health-care access in poor parts of the world. Médecins Sans Frontières (MSF) is an international humanitarian organization that won the Nobel Peace Prize in 1999 for providing medical relief to populations living in poverty-stricken and war-torn areas. In 1999, MSF started the "Access Campaign" whose purpose is "to push for access to, and the development of life-saving and life prolonging medicines, diagnostic tests and vaccines for patients in MSF programmes and beyond." Ensuring access to effective diagnostics and treatment for drug-resistant tuberculosis is one of the issues the Access Campaign has been tackling. To get involved in the campaign for drug-resistant tuberculosis, visit http://msfaccess.org/TBmanifesto/

To learn more about the Access Campaign in general, visit http://msfaccess.org/

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Part III

Parasitology

The Biochemistry of Quinoline Antimalarial Drug Resistance

Paul S. Callaghan and Paul D. Roepe

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Abstract

Mutations in the *Plasmodium falciparum* chloroquine-resistance transporter (PfCRT) have been shown to be central to the molecular mechanism of quinoline antimalarial drug resistance. However, additional facets to resistance biochemistry are emerging, and it is now clear that multiple quinoline drug resistance phenotypes exist in different regions of the globe. Different public health policies and drug use histories across the globe, along with natural genetic drift, have created this diversity, such that there are now dozens of distinct chloroquine-resistant (CQR) strains of *P. falciparum*. Some of these can be described in detail, but information is incomplete. This leads to some degree of continued uncertainty on how best to proceed in controlling malaria in some regions. This issue is even

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more critical for controlling chloroquine-resistant *P. vivax*, about which even less is known. This review summarizes key features of quinoline antimalarial drug resistance in *P. falciparum* malaria and suggests concepts relevant for "staying ahead of the resistance curve."

Keywords
Malaria • *Plasmodium falciparum* • PfCRT • Chloroquine resistance • Quinoline multidrug resistance

List of Abbreviations

ABC	ATP-binding cassette
ACT	Artemisinin combination therapy
ATP	Adenosine triphosphate
CQ	Chloroquine
CQR	CQ resistant (resistance)
CQS	CQ sensitive
DV	Digestive vacuole
FPIX	Ferriprotoporphyrin IX
Hb	Hemoglobin
HF	Halofantrine
Hz	Hemozoin
iRBC	Red blood cell infected with *P. falciparum*
ISOV	Inside–out yeast plasma membrane vesicle
MDR	Multidrug resistant (resistance)
MQ	Mefloquine
pfcrt/PfCRT	*Plasmodium falciparum* chloroquine-resistance transporter (*gene*/PROTEIN)
PfMDR1	*P. falciparum* multidrug resistance protein 1
PfNHE	*P. falciparum* Na^+/H^+ exchanger
pvs	Parasitophorous vacuolar space
QD	Quinidine
QN	Quinine
QNR	Quinine resistance
QTL	Quantitative trait loci
RBC	Red blood cell
VPL	Verapamil

Introduction

Five *Plasmodia* spp. infect humans, and these cause distinct malarias that are distinguished by different pathophysiology and rates of mortality. These unicellular eukaryotic parasites belonging to the phylum Apicomplexa exist in the body as multiple highly differentiated forms. Mixed infections with multiple strains and multiple species can occur, and the pathophysiology of malaria in pre-immune

versus naïve children, adults, and pregnant women differs. The overall point is that malaria is actually a spectrum of diseases with a variety of effects on different human populations, which presents many unique challenges in controlling the disease. The emergence and spread of drug-resistant strains of *P. falciparum* and *P. vivax* have further complicated treatment and threaten the lives of millions annually.

The life cycle of malarial parasites is complex, involving two hosts (*Anopheles* mosquitoes and humans) or, for *P. knowlesi*, three (transmission of this species is zoonotic via macaques). *P. falciparum* and *P. vivax* infections are the most common, with the former causing most mortality. *P. falciparum* sporozoites injected into the skin during an *Anopheles* blood meal quickly migrate to the liver, invade hepatocytes, and are then released back into the blood stream approximately 2 weeks later as large clusters of merozoites called merosomes. The individual merozoites then rapidly invade red blood cells (RBC). Once in the erythrocyte, the parasite proceeds through ring, trophozoite, and schizont stages of development before lysing the RBC within 48 h and emerging as $\geq$ 8 new merozoites. These then reinvade fresh RBC. Most clinical symptoms of malaria are a consequence of the RBC cycle, and most antimalarial drugs act against the RBC stages. See Bogitsch et al. (2005) for a detailed discussion of the parasite life cycle.

Quinoline Drugs, Emergence of Resistance, and Drug–Heme Interactions

Multiple effective classes of antimalarial drugs exist including the quinolines (4-amino-quinoline, 8-amino-quinoline, and quinoline methanols), the reactive endoperoxides (artemisinins), and antifolates such as pyrimethamine (typically administered in combination with sulfadoxine) that poison pyrimidine biosynthesis or utilization. Quinoline-based drugs (Fig. 1) have long been used in the battle against malaria, beginning with quinine [QN], originally extracted from the leaves of the cinchona tree. Upon the synthesis of chloroquine (CQ) and other 4-aminoquinolines during World War II (see Kauer et al. (2010) for a recent review), efficacious, cost-effective antimalarial drug therapy became readily available worldwide. Resulting widespread use of CQ led to the emergence of CQ-resistant (CQR) *P. falciparum* parasites, which was aided by prophylactic use and population-based dosing directed towards global eradication (Foley and Tilley 1998). Today, the majority of *P. falciparum* infections in S.E. Asia are CQR, and >50 % are CQR in many African countries, but pockets of CQ-sensitive (CQS) *P. falciparum* malaria still exist in South America and elsewhere. Importantly, quinoline antimalarials (QN), amodiaquine (AQ) (first synthesized at Parke-Davis in the late 1940s Burckhalter et al. 1948), and mefloquine (MQ), despite prolonged use, are still effective against most CQR parasites, and AQ and MQ are also important partners in approved artemisinin-based combination therapies (ACTs) (Schlagenhauf et al. 2010). Quinoline-quinoline and quinoline-non-quinoline combinations (Bell 2005), as well as quinoline-resistance reversal strategies (Peyton 2012), are additional components of ongoing development of new therapy.

Fig. 1 Common antimalarial drugs

In recent decades, as CQR strains have both spread around the globe and continued to evolve, new geographically distinct CQR strains with unique genotypes and phenotypes have appeared. Resistance to the antifolate drugs is also now widespread, and delayed clearance of parasites is now seen in some patients treated with artemisinin-based drugs (Cheeseman et al. 2012; Takala-Harrison et al. 2013) which may be an early sign of emerging resistance to this class of compounds as well. To use current drugs more effectively, and to develop new therapies, molecular details of antimalarial drug resistance phenomena must be elucidated.

Detailed biochemical and molecular analyses of drug resistance is complicated, but conceptually, drug resistance phenomena are quite simple. A drug must interact with one or more molecular targets to exert its effect, and so resistance to that drug is due to either (or both) disruption of that interaction or to altered signal transduction propagated from the drug–target interaction that would normally promote growth arrest or cell death. Disruption of drug–target interactions seen in drug resistance phenomena typically fall into one of three categories: increased enzymatic degradation of the drug, mutation or altered expression of the drug target, or altered cellular

transport of the drug. In some cases, particularly in examples of "multidrug" resistance, more than one mechanism may be relevant. There are many examples of altered signal transduction related to drug resistance, with the best understood being disrupted apoptotic signal transduction (disrupted induction of programmed cell death) in multidrug-resistant tumor cells.

In the case of *P. falciparum* resistance to CQ and related quinoline antimalarial drugs, there is no known enzymatic degradation of drug that reduces drug–target interactions. As described in more detail below, the principal target for quinoline drugs is believed to be ferriprotoporphyrin IX (FPIX) heme released upon the host red blood cell hemoglobin (Hb) catabolism. Thus, the drug target cannot be mutated by the drug-resistant parasite because it is synthesized by the host. This leaves altered cellular transport of quinoline drugs as the likely pathway to disrupting drug–target interactions. However, as more has been learned about how free FPIX is processed by the parasite, it may also be possible that target accessibility is reduced in interesting novel ways, as briefly described below, which would then also disrupt drug–target interactions.

Regarding signal transduction related to parasite growth or death, very little is currently known about how quinoline drugs might affect that signaling, or how that signaling might be altered in drug-resistant parasites. Being a single-celled micro-organism without clear caspases and other key apoptosis effectors encoded within its genome, *P. falciparum* does not appear to express a typical apoptosis pathway (Sinai and Roepe 2012), and our understanding of cell cycle regulation for the parasite is limited (Doerig et al. 2002; Halbert et al. 2010). Thus, changes in the signal transduction relevant for cell cycle regulation or cell death have not yet been inspected in any detail for drug-resistant *P. falciparum* malaria, although one very recent paper suggests that autophagy signaling may be related to parasite cell death (Gaviria et al. 2013). Progress in understanding signal transduction and other biology relevant for drug resistance in the related pathogen *P. vivax* is even more limited, but just as crucial (Shanks 2012; Douglas et al. 2012).

To analyze how drug–target interactions might be perturbed in drug resistance, the drug target must be understood in molecular terms. Quinoline antimalarials have long been thought to target FPIX heme within the digestive vacuole (DV) of the parasite, which is released upon Hb digestion during the trophozoite stage of the intraerythrocytic cycle (Banerjee et al. 2002; Gamboa de Domínguez and Rosenthal 1996; Elliot et al. 2008). The parasite must digest most Hb found in the RBC cytosol, both to provide room for very rapid trophozoite growth and to obtain necessary amino acids. FPIX is toxic in its free state (Fitch et al. 1983), and due to the lack of a heme oxygenase pathway, the malarial parasite must sequester FPIX as nontoxic crystalline hemozoin (Hz). At cytostatic dosages, quinoline drugs slow the production of Hz, presumably by interacting with uncrystallized Hz precursors, growing Hz crystal faces, or both. This inhibition of Hz presumably leads to the buildup of free heme which is then believed to inhibit Hb-degrading proteases (Vander Jagt et al. 1987) leading to growth arrest. Precisely how quinoline drugs target heme to

inhibit Hz formation in vivo is not fully known nor is it understood whether different quinoline antimalarials inhibit Hz via similar or different pathways. Different pathways are likely since some CQR parasites remain sensitive to related quinoline drugs such as QN, AQ, and MQ (Fig. 1), whereas others do not.

Hz is a crystal of heme dimers, and the unit cell is a unique heme structure, with the ferric iron of each FPIX coordinated to a carboxyl side chain of an adjacent moiety. These "head-to-tail" dimers are stabilized in the crystal lattice via hydrogen bonding (Pagola et al. 2000; Bohle et al. 2012). Current evidence strongly supports catalysis of Hz formation by lipid in vivo (Jackson et al. 2004; Pisciotta et al. 2007; Gorka et al. 2013b), and certain lipids are known to efficiently catalyze Hz crystal growth in vitro (Jackson et al. 2004; Pisciotta et al. 2007; Egan et al. 2006). Structures for a number of quinoline drug-FPIX heme structures have recently been solved, and these are reviewed elsewhere (Gorka et al. 2013a).

As a diprotic weak base with pK_a of 8.4 and 10.2, CQ exists as neutral, singly, or doubly charged compound under biological conditions, and these different drug species have different reactivity towards multiple chemical forms of free heme (i.e., monomers vs dimers in either aqueous or lipid phase, see Gorka et al. 2013a). In vitro, some drug–heme species aggregate and fall out of solution, generating amorphous drug–heme aggregates that then reestablish aqueous equilibria between heme species not complexed with drug. Others prefer to partition into lipid as 1:1 drug–heme complexes (Alumasa et al. 2010; Casabianca et al. 2008). This drug–heme chemistry likely competes with heme-to-hemozoin conversion; however, quantification of drug–heme aggregation or lipid partitioning phenomena within the parasite has not yet been done. Factors that reduce efficiency of quinoline drug–FPIX heme binding will alter DV retention of drug as well as the rate of Hz formation and could therefore contribute to resistance in multiple ways.

Genetic Basis of CQR

CQR is both spreading and continuing to genetically evolve via ongoing selective pressure. As briefly summarized in the next section, it was initially thought that ATP-binding cassette (ABC) protein drug pumps for CQ and other antimalarials must exist in drug-resistant *P. falciparum* and that these would be similar to the drug pump proposed for tumor cells (HsMDR1, or Pgp) believed by many investigators to directly translocate vinblastine, doxorubicin, and other antitumor drugs out of drug-resistant tumor cells. However, subsequent work showed that genotypes of some drug-resistant *P. falciparum* do not necessarily include mutation or increased expression of *pfmdr* genes (Wellems et al. 1990; Barnes et al. 1992) and that other genetic events must therefore be important. Similar to *P. falciparum* multidrug resistance (PfMDR) protein versus malarial MDR, the precise role of HsMDR1 protein in tumor MDR has been questioned (e.g., Roepe et al. 1996) as clinical pathology data have not correlated HsMDR1 overexpression with clinically relevant drug resistance as strongly as initially suspected.

Initial Genetic Studies of CQR: CG2 Versus Na^+/H^+ Exchange

More detailed genetic definition of CQR begins with cloning of *pfcg2*, which was suggested to be a resistance determinant based on quantitative trait loci (QTL) analysis of the progeny of a CQS × CQR parasite cross (Su et al. 1997). Subsequently, Lanzer and coworkers concluded that mutated PfCG2 protein was a dysregulated Na^+/H^+ exchanger that also pumped CQ out of parasites (Sanchez et al. 1998; Wünsch et al. 1998). Wellems and colleagues questioned this (Wellems et al. 1998). Subcellular localization of PfCG2 reveals it resides in vesicle-like structures near the parasitophorous vacuolar space (pvs) and the DV (Cooper et al. 2005) but is not localized within the plasma or pv membranes of the parasite as originally envisioned (Sanchez et al. 1998). A follow-up study (Bray et al. 1999) questioned Na^+ dependency for CQR phenomena, arguing against a strong role for Na^+/H^+ exchange in CQ transport or CQR. More recently, QTL analysis, availability of the *P. falciparum* genome, and novel single-cell imaging of Na^+/H^+ exchange in a series of drug resistant progeny suggested that altered Na^+/H^+ might be related to QN resistance (QNR), but not to CQR, and that the relevant exchanger is not PfCG2, but *Plasmodium falciparum* Na^+/H^+ exchanger (PfNHE) (Ferdig et al. 2004; Bennett et al. 2007).

Wellems and colleagues found that mutant PfCG2 did not confer CQR in and of itself (Fidock et al. 2000a). Attention thus focused on another gene found within the same 36 kbp fragment that harbored *pfcg2*, namely, *pfcrt* (Fidock et al. 2000b). Results described in this and additional papers (Sidhu et al. 2002; Cooper et al. 2002) show that mutations in the PfCRT protein are the ultimate determinant of CQR (and of some degree of resistance to other drugs) in *P. falciparum* malaria (see "PfCRT," below). While PfCRT mutations play a dominant role, importantly, PfMDR1 protein appears to modulate cross-resistance patterns in interesting ways.

The Elusive Role of PfMDR1

Early studies of CQR showed that drug resistance was associated with decreased drug accumulation (Krogstad et al. 1987) that was reversed by the ion channel blocker verapamil (VPL). Similar phenomena had been seen in drug-resistant tumor cells; thus, early on Wirth and colleagues screened *P. falciparum* for *Hsmdr1* homologues and identified *Pfmdr1* and *Pfmdr2* (Wilson et al. 1989). Another group found *Pfmdr1* to be upregulated in some CQR *P. falciparum* (Foote et al. 1989). But subsequent experiments (Barnes et al. 1992) showed that *Pfmdr1* overexpression did not correlate with CQR. This was not entirely surprising since Wellems et al. had earlier shown that CQR did not segregate with the *Pfmdr1* chr 5 locus in progeny from a CQS × CQR genetic cross (Wellems et al. 1990).

On the other hand, polymorphisms in *pfmdr1* were also associated with CQR early on (Foote et al. 1990). While CQS isolates had identical *pfmdr1* sequences, there were five changes in CQR isolates. In strains K1 and ITG2, N86Y was the only change. CQR strain 7G8 had four mutations: Y184F, S1034C, N1042D, and

D1246Y. The 184F mutation was postulated as not likely involved in CQR since it was also found in CQS strains. Thus, the *pfmdr1* overexpression hypothesis was revised to suggest that CQR strains expressed mutant *pfmdr1* but did not necessarily overexpress wild-type *pfmdr* (Foote et al. 1990).

Subsequently, when MQR *P. falciparum* were selected to higher levels of MQR, *pfmdr1* was found to be amplified (Cowman et al. 1994), and a very interesting inverse relationship between resistance to MQ and CQ was observed in the series of strains. Also, halofantrine (HF) and QN resistance increased with increasing *pfmdr1*, whereas AQ resistance did not (Cowman et al. 1994). However, when CQR strain K1 was selected versus HF, it did not result in MQR or amplification of *pfmdr1* (Ritchie et al. 1996). In another study, which used allelic exchange of *pfmdr* to probe these questions, incorporation of *pfmdr1* 7G8 polymorphisms into a CQS strain not previously exposed to drug had no effect on CQR, but incorporating wild-type *pfmdr1* into a CQR strain expressing mutant *pfmdr1* did decrease the level of resistance by half (Reed et al. 2000). Also, the CQS strains expressing mutant *pfmdr1* alleles showed some mild QNR and altered sensitivity to MQ and HF. Variations on this theme have also been described by Fidock and colleagues (Sidhu et al. 2005). Essentially, these data suggest that the *pfmdr1* effects measured by Reed et al. may be strain specific, and they bring us to our current understanding (Roepe 2009). It seems unlikely that mutations in *pfmdr1* confer CQR in and of themselves, but they can provide an important modulatory effect in some strains and isolates (Price et al. 2004; Dorsey et al. 2001; Patel et al. 2010). Interestingly, a recent report shows that PfMDR1 binds a high-affinity CQ photoaffinity analogue, suggesting that the protein does indeed react with quinoline drugs in some fashion (Pleeter et al. 2010), but the significance of this binding remains to be elucidated.

PfCRT

As mentioned, work by Wellems and colleagues showed that *pfmdr1* was unlikely to cause CQR since the relevant region of chr 5 harboring *pfmdr1* did not segregate with the CQR phenotype in a genetic cross (Wellems et al. 1990). Another key paper suggested that the CQR locus resided within the *cg2* gene on chr 7 (Su et al. 1997), but this paper also showed that one CQS strain (Sudan 106/1) carried CQR-associated *cg2* yet was nonetheless CQS. The 36 kbp chr 7 locus that segregated with CQR was thus reexamined, and a previously unrecognized gene, now known as *pfcrt,* was found (Fidock et al. 2000b). Mutations in *pfcrt* are the central determinant for *P. falciparum* CQR. The 13 exons of *pfcrt* span 3.1 kbp and encode a 424 amino acid, 48.6 kDa protein. Mutant-*crt* alleles found in CQR parasites contain a number of point mutations that confer multiple amino acid substitutions, with the pattern of mutations depending on the region of the globe from which the CQR parasite originates (Table 1). CQR arose (and continues to evolve) independently in at least five locations – S.E. Asia (which then spread to Africa), Papua New Guinea,

Peru, Colombia, and the Philippines (Wooton et al. 2002; Chen et al. 2003). CQR parasites from S.E. Asia and Africa carry 7–8 point mutations, whereas South American CQR strains carry 5. Novel patterns continue to be discovered, including new alleles recently identified in the Philippines (Chen et al. 2003), Cambodia (Durrand et al. 2004), Columbia (Echeverry et al. 2007) China (Yang et al. 2007), and Thailand (Chaijarkoenkul et al. 2011). Based on these mutations, it appears that at least four amino acid substitutions are required for conversion to CQR, with a change at codon 76 always required. It is not completely understood why South American CQR strains segregate into two groups with distinct mutations, but a likely explanation is variable AQ selective pressure (Sá et al. 2009). The pattern of PfCRT mutations thus provides identification of the likely geographic origin of a CQR isolate. The number of mutations apparently required for conversion to CQR explains two riddles, namely, why CQR took so long to appear on a large scale and why it had historically been impossible to create CQR strains from CQS in the laboratory via drug selection pressure.

Thus, over the past 10 years, it has become clear that a number of distinct *pfcrt* alleles encoding unique PfCRT isoforms exist (see Table 1). These have presumably arisen for two reasons: (1) different antimalarial drug use in various regions of the globe has provided different selective pressure for the persistence of PfCRT mutations in these regions and (2) the patterns of mutations may provide different "fitness" advantages, some of which could be more specific to one region versus another. Ongoing efforts to sequence entire genomes of multiple *P. falciparum* strains and isolates will help further explain this ongoing parasite evolution (e.g., Wooton et al. 2002; Volkman et al. 2007). *To date, a full molecular understanding of the relative resistance-conferring function of the different PfCRT isoforms known to exist is yet to be elucidated.*

Similar to PfMDR1, PfCRT protein is localized to the DV membrane (Cooper et al. 2002) and is a polytopic integral membrane protein that performs some type of transport function (see Roepe 2011; Ecker et al. 2012 for recent reviews). Most functional hypotheses for PfCRT involve either ion or drug transport or both, since CQR parasites accumulate less antimalarial drug versus time relative to CQS (see below) and quinoline antimalarial drugs are hydrophobic weak bases. In fact, CQ and related drugs are dibasic, and the DV is known to be quite acidic. So, passive concentration of CQ within the DV (where FPIX heme CQ target is found) is dependent upon the square of the net pH gradient and will be 10^5–10^6-fold by the predictions of weak-base partitioning theory. A repercussion is that very subtle changes in DV pH will have quite significant consequences for drug sequestration. Regulation of DV pH is not fully understood, but it includes a V type H^+ ATPase that hydrolyzes cytosolic ATP to pump H^+ into the DV. Interestingly, small changes in DV pH and volume caused by mutation of PfCRT have been measured in some studies (Roepe 2011; Gligorijevic et al. 2006). These can affect drug partitioning, FPIX heme to Hz biomineralization, and the chemistry of Hz inhibition by drug (see Gorka et al. (2013a) for a more extensive discussion).

Table 1 All known PfCRT isoforms. Amino acid substitutions relative to wild type are shown in *green*. Where literature shows more than one measured IC50 for a strain harboring the PfCRT isoform, the high and low values are reported

	*Pf*CRT Amino Acid Positions																												
Origin	Clone/Isolate	39	72	74	75	76	77	97	123	144	148	152	160	163	194	198	205	220	251	271	275	277	326	3270	333	334	350	352	356	371	IC_{50} (Low, High)
Honduras	HB3	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	12.3, 33.9
Netherlands	3D7	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	5.8, 30.4
PNG	D10	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	6.0, 40.7
Kenya	K39	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	3.9, 43.2
Thailand	T2/C6	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	9.7, 29.5
S. Africa	Fab9	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	9.7, 27.5
Sierra Leone	SL/D6	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	7.8, 25.9
Kenya	M24	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	5.8, 23.4
Sudan	REN	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	5.8, 16.2
Liberia	LF4/1	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	7.8, 15.0
Malaysia	Camp/A1	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	7.8, 12.5
Mali	BC5	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	11.6
Mali	M5	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	21.0
Haiti	Haiti	S	C	M	N	K	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	N	I	T	S	C	Q	I	R	5.8, 16.2
Sudan	106/I	S	C	I	E	K	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	37.8, 15
Thailand	Dd2	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	404.1, 48
Thailand	Thai16	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	436.0
Thailand	Thai19	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	874.1
Thailand	TM284	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	155.1, 363.3
Thailand	C2A	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	301.5
Vietnam	V1/S	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	186.1, 659.1
Cambodia	JCK	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	521.3
Sudan	102/1	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	164.8, 431.6
SEA	D5	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	353.6
Thailand	TM93-C1088	S	C	I	E	T	I	L		A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	1200.2
Thailand	FCB	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	135.7, 492.2
Thailand	K1	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	75.22, 320.0
Uganda	PAR	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	124.1
Gambia	FCR-3	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	200.0
Kenya	KMWII	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	77.5
South Africa	RB8	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	116.3, 152.5
South Africa	RB20	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	232.6
Ghana	9020	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	468.4
Ghana	9013	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	305.5
SEA	P31	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	454.6
Sudan	124/8	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	439.3
Sudan	128/4	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	87.2, 379.4
Mali	M2	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	116.3, 320.0
SEA	ItG2F6	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	124.1, 128.0
Gambia	M97	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	517.7
Sao Tome	Cai	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	T	S	C	Q	I	I	96.9
Thailand	TM6	S	C	I	E	T	I	H	R	A	L	T	L	S	I	E	A	S	F	E	P	N	S	I	T	S	C	Q	I	I	111, 167.0
Thailand	BC7	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	-	T	S	C	Q	T	I	54.0
Thailand	J9	P	C	I	E	A	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	D	S	I	T	S	C	Q	T	I	123.0
Thailand	KS28	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	-	E	P	N	S	I	T	S	C	Q	T	I	74.0
Thailand	BC22	S	C	I	E	T	I	H	H	A	L	T	L	S	I	K	T	S	F	E	P	N	S	I	T	S	C	Q	T	I	42.0

Brazil	7G8	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	34, 220
Peru	PC26	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	689.4
Brazil	DIV17	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	441.3
Brazil	DIV14	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	430.0
Brazil	ECP	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	287.7
Brazil	PAD	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	269.1
Brazil	ICS	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	347.1
PNG	PNG3	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	242.9
PNG	PNG2	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	225.9
Brazil	DIV30	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	217.4
PNG	PNG13	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	198.4
Solomon	PNG4	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	A	F	Q	P	N	D	I	T	S	C	Q	L	R	250.5
Ghana	GB4	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	N	I	T	S	C	Q	I	I	89.8, 144.8
Mali	S35CQ	S	C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	N	I	T	S	C	Q	I	I	229.5
Cambodia	742		C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	N	I	T	S	C	Q	I	I	466.7
Cambodia	766		C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	N	I	T	S	C	Q	I	I	53.9
Cambodia	783		C	I	E	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	N	I	T	S	C	Q	T	I	133.7
Cambodia	738		C	I	D	T	I	H	H	A	I	T	L	S	T	E	T	S	F	E	P	N	N	I	S	S	C	Q	I	R	156.8
Cambodia	734		C	I	D	T	I	H	H	F	I	T	L	S	T	E	T	S	F	E	P	N	N	I	S	S	C	Q	I	R	169.4
Cambodia	613		C	I	D	T	I	H	H	F	I	T	L	S	T	E	T	S	F	E	P	N	N	I	S	S	C	Q	I	R	33.6
Phillipines	PH1	S	C	M	N	T	I	H	H	T	L	T	Y	S	I	E	T	A	F	Q	P	N	D	I	T	S	C	Q	I	R	95.0
Phillipines	PH2		S	M	N	T	I	H		T	L	T	Y	S	I	E	T	A	F	Q	P	N	D	I	T	S	C	Q	I	R	N.D.
Ecuador	Ecu1110	S	C	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	90.0, 156.4
Peru	PC17	S	C	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	C	Q	L	R	373.8
Colombia	Jav	S	C	M	E	T	I	Q	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	N	I	T	S	C	Q	I	T	137.3, 305.9
Colombia	TU741	S	C	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	N	C	Q	L	R	N.D.
Colombia	TA7519	S	C	M	E	T	I	Q	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	N	I	T	S	C	Q	I	T	N.D.
Colombia	TA6182	S	C	M	E	T	I	Q	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	S	I	T	S	C	Q	I	I	N.D.
China	Isolate B	S	C	I	D	T	I	H		Y	L	T	L	S	I	E	T	A	F	E	P	N	N	I	T	S	C	Q	I	R	N.D.
China	Isolate C	S	C	I	D	T	I	H		Y	L	T	L	S	I	E	T	A	F	E	P	N	N	I	T	S	C	Q	I	I	N.D.
China	Isolate D	S	C	I	E	T	I	H		Y	L	T	L	S	I	E	T	A	F	E	P	N	N	I	T	S	C	Q	I	R	N.D.
China	Isolate E	S	C	I	E	T	I	H		A	L	T	L	S	I	E	T	S	F	E	P	N	N	I	T	S	C	Q	I	R	N.D.
Indo. Papau	2300	S	C	I	K	T	I	H	H	A	L	T	L	S	I	E	T	S	F	E	P	N	S	I	I	S	C	Q	I	I	N.D.
French Guiana	H209	S	S	M	N	T	I	H	H	A	L	T	L	S	I	E	T	S	F	Q	P	N	D	I	T	S	R	Q	L	R	35

Altered Drug Transport Observed in CQR *P. falciparum*

Coy Fitch first observed reduced accumulation of CQ into iRBC infected with drug-resistant parasites (Fitch 1969), and decreased retention of CQ for CQR parasites was subsequently reported in zero-trans efflux experiments (Krogstad et al. 1987). This was termed “increased efflux” and quantified as the percent preloaded ^{3}H-CQ remaining versus time after dilution into a drug-free medium. Efflux was hypothesized to be 40–50 times faster for CQR parasites (Krogstad et al. 1987); however, no molar quantification of transport (moles-free CQ per parasite per unit time) was possible. Soon thereafter, as described above several papers suggested that CQR was due to outward pumping of CQ by a *P. falciparum* homologue of human P-glycoprotein (HsMDR1 or Pgp), named PfMDR1, and encoded by the *pfmdr1* gene on Pf chr 5 (Wilson et al. 1989; Foote et al. 1990). However, at about the same time, Wellems and colleagues reported that the determinant for CQR identified in a Mendelian cross resided on Pf chr 7, not chr 5 (Wellems et al. 1990), which led to the search for other genes involved in CQR and the subsequent identification of *pfcrt* 10 years later (Fidock et al. 2000b) (see “Genetic Basis of CQR” above).

Many cell-based drug influx and/or efflux studies have been reported for CQS versus CQR parasites (e.g., Geary et al. 1986; Bray et al. 1992; Roepe 2011). These were performed using iRBC populations or detergent-extracted parasites and various filtration or oil layer centrifugation approaches. Non-saturable drug accumulation was often calculated (e.g., see Hawley et al. 1998), and the different protocols quickly generated a variety of data. Consensus was nonetheless eventually reached, namely, at low external [CQ] (1–50 nM) CQR parasites typically accumulate 2–10-fold less CQ in similar time relative to CQS. Depending on calculated non-saturable accumulation subtracted from these data, some studies (Bray et al. 1992) hypothesize that saturable uptake differs by as much as 100–1,000 fold for CQR versus CQS parasites; however, this conclusion rests on mathematical modeling assumptions. Measured differences in net CQ accumulation are typically 2–10 fold (see Roepe 2011 for more detailed review).

A proposed faster rate of drug efflux back out of the iRBC was one popular explanation for reduced iRBC drug accumulation early on (Krogstad et al. 1987; Martin et al. 1987), and initially the PfMDR1 protein (see section “The Elusive Role of PfMDR1”) was thought to mediate the hypothesized increased efflux. After identification of PfCRT (see section “The Elusive Role of PfMDR1”), it was proposed that PfCRT, not PfMDR1, was responsible for the putative increased cellular drug efflux. Several interpretations were offered: (1) PfCRT-mediated outward pumping of drug from CQR parasites, (2) PfCRT-mediated drug counterflow (exchange) in the presence of appropriate drug gradients, (3) altered binding to intracellular targets caused by PfCRT mutations promoted decreased drug retention (increased efflux) in zero-trans efflux experiments.

However, both PfCRT and PfMDR1 proteins reside in a subcellular organellar membrane (the DV membrane), with three additional membranes between it and the outside of the iRBC. How one transporter at the DV membrane (via whatever thermodynamic mechanism one chooses to invoke) could kinetically compete with

the fast passive influx of drug across these other membranes to result in net movement of drug out of the entire iRBC remains unclear. This leads to the proposal that PfCRT facilitates downhill leak of charged CQ from the DV to the cytosol (Zhang et al. 2004). Thus, PfCRT would not "pump" CQ from the iRBC per se but would promote decreased accumulation over time by lowering time-dependent CQ binding to intra-DV targets (e.g., FPIX heme, discussed above).

Interestingly, with one noted exception (Geary et al. 1986), all cell-based transport during this period was assayed at 1–10 nM levels of CQ. However, these concentrations are approximately 100–1,000 times below peak plasma [CQ] in malaria patients. In situ autoradiography of trophozoites showed that accumulated ^{3}H-CQ localized nearly exclusively to the DV of the mid-stage trophozoite (Sullivan et al. 1996), but these experiments were done at even lower external levels of drug (pM). Nonetheless, when abundant PfCRT was found expressed in the DV membrane, nearly all evidence seemed to point in favor of a straightforward interpretation of the Fitch hypothesis (Fitch 1969) and a DV membrane drug pump or drug channel explanation for CQR. Only recently have resistance phenomena related to higher (cytocidal) levels of CQ been investigated and evaluated versus drug transport phenomena (see below "New Insights: Cytostatic Versus Cytocidal Resistance").

An additional caveat for interpreting altered drug accumulation in iRBC is that some studies have reported altered DV pH and volume for CQR parasites (reviewed in Roepe 2011). It seems likely that traffic of endogenous DV osmolytes (ions and/or small molecule metabolites) is perturbed upon mutation of wild-type PfCRT to CQR isoforms found in CQR parasites (Roepe 2011). Altered osmolyte traffic perturbs regulation of important biochemical characteristics of the DV (e.g., pH, volume, ionic composition) that have direct effects on the efficiency of quinoline drug–FPIX heme interactions, and hence on net drug accumulation versus time (see Gorka et al. 2013a).

Vesicle Studies

From studies with whole cells, it was not entirely clear whether reduced accumulation of drug versus time for iRBC harboring CQR parasites was due to transporter-mediated efflux from the DV, altered binding of drug to FPIX heme targets caused by perturbations in DV physiology, or some combination. In most cases, analysis of drug transport with vesicles or proteoliposomes reduces complexity in interpretation. Initial vesicle-based studies of hypothesized PfCRT drug transport function used plasma membranes from yeast and first tested for direct binding of ^{3}H-CQ to PfCRT (Zhang et al. 2004). Scatchard analysis indicated a single-drug binding site in PfCRT, and, surprisingly, that CQS and CQR isoforms of PfCRT have similar affinity for CQ (K_d = 435 and 385 nM, respectively). A recent follow-up study of CQ binding used covalent attachment of a perfluoroazido-tagged CQ probe to quantify CQ probe binding versus other quinoline antimalarials and to further define the drug binding site in PfCRT, which is predicted to be disposed towards the DV side of the DV membrane (Lekostaj et al. 2008). Satisfyingly, this binding site can

also easily place the quinoline ring of CQ near mutations in PfCRT isoforms that are known to modulate response to drugs (Fidock et al. 2000b; Cooper et al. 2002). It is clear at this point that both wild-type (CQS) and mutant (CQR) isoforms of PfCRT bind CQ at a single-drug binding site, that related quinoline drugs such as MQ and QN compete with CQ for binding to this site, and that chemoreversal agents such as VPL inhibit CQ binding only for some PfCRT isoforms (Lekostaj et al. 2008).

The paper showing direct binding of ^{3}H-CQ (Zhang et al. 2004) also examined ^{3}H-CQ efflux from inside–out plasma membrane vesicles (ISOV) via flow dialysis techniques and concluded that one CQR isoform of PfCRT mediated downhill passive efflux of ^{3}H-CQ faster than that observed for control ISOV or ISOV harboring CQS PfCRT. This was the first direct biochemical evidence in support of CQ transport by PfCRT. Subsequently, another paper applying a similar approach with vesicles made from *D. discoideum* reached similar conclusions after observing that vesicles harboring mutant PfCRT accumulated less CQ than those harboring wild-type PfCRT (Naude et al. 2005). In this study as well as an earlier yeast vesicle study (Zhang et al. 2002), additional evidence for ion or osmolyte transport via PfCRT was also obtained. A more recent *D. discoideum* vesicle study supports the notion that CQ transport is likely driven by electrochemical potential (Papakrivos et al. 2012).

Analysis of CQ Transport Using Proteoliposomes and Oocytes

Injection of oocytes with modified *pfcrt* mRNA followed by measuring ^{3}H-CQ accumulation into individual eggs (Martin et al. 2009) and purification of recombinant PfCRT from yeast followed by reconstitution into proteoliposomes (PLs) and analysis of fluorescently tagged CQ efflux from these PLs (Paguio et al. 2009) have both recently been pursued to test conclusions regarding PfCRT-mediated drug transport (see Roepe 2011 for more detailed review). Both the PL and oocyte approaches provide the best evidence for direct CQ transport by PfCRT; however, there are important differences in interpretation between the two studies. One is quantification of apparent turnover (mole drug/mol transporter(s)), and the other is whether both CQS and CQR isoforms are capable of drug transport. The paper reporting CQ transport in oocytes (Martin et al. 2009) does not calculate explicit turnover, presumably because the transport measured in this study is quite slow and does not plateau. However, initial rates are calculated and expressed as pmol CQ/oocyte/h. Assuming site density of PfCRT is within the range reported for many other transporters and channels expressed in oocytes (68), then these data convert to 0.002–0.02 CQ molecules/PfCRT(s) (CQS isoform) and 0.009–0.09 CQ molecules/PfCRT(s) (CQR isoform) at 300 nM external ^{3}H-CQ. This estimated turnover is 1–2 orders of magnitude lower than that measured with fluorescent drug probe and purified protein reconstituted into PLs (Paguio et al. 2009) and does not appear sufficient to account for reduced CQ accumulation in the parasite DV for CQR parasites (Cabrera et al. 2009; Roepe 2011).

The PL experiments are done with preparations wherein efflux of free CQ probe from an acidified PL interior can be measured instead of influx into the oocyte from a neutral egg perfusate. Experiments with these PLs and the fluorescent CQ analogue yield turnovers that are much higher than those computed from the oocyte data and that are also found to be highly dependent on the magnitude of ΔpH and $\Delta\Psi$ (Paguio et al. 2009). This is expected for a DV transporter since high electrochemical driving forces exist across the DV membrane. At 5 μM NBD-CQ, turnover numbers were determined to be 0.8 NBD-CQ molecules/PfCRT(s) in the presence of a 1 unit ΔpH and 0 mV $\Delta\Psi$, 1.6 NBD-CQ molecules/PfCRT(s) in the presence of a 2 unit ΔpH and 0 mV $\Delta\Psi$, and 3.4 NBD-CQ molecules/PfCRT(s) in the presence of a 2 unit ΔpH and ~120 mV $\Delta\Psi^{+}$ (Paguio et al. 2009).

Data from the two approaches probably differs for several reasons. First, oocyte plasma membranes have low electrochemical driving force that cannot be conveniently manipulated, whereas driving force can be both increased significantly and conveniently manipulated for the PLs. Also in the PL experiments, the only modification to the amino acid sequence of PfCRT is a hexa-His tag added at the C-terminus, but in oocytes, four putative lysosomal-/endosomal-targeting motifs were removed in PfCRT by replacing 15 residues (a.a. # 17, 20, 22, 23, 26, 27, 47, 48, 50, 51, 409, 412, 414, 421, 422) with alanine (Martin et al. 2009). It is possible that, along with very different electrochemical driving force, these extensive modifications to the PfCRT primary sequence affect catalytic efficiency of CQ transport.

A second key difference in comparing these studies is the relative transport measured for CQS versus CQR isoforms of PfCRT. In the PL study, small differences in CQ transport by the two isoforms are noted when transport is measured at the same ΔpH and $\Delta\Psi$. In contrast, the oocyte system does not show statistically significant CQ transport above background for the CQS isoform of PfCRT. Levels of transporter are difficult to quantify for the oocyte system, and western blot data that directly compares CQS to CQR PfCRT isoform expression in the oocytes is not shown in this study (Martin et al. 2009). Also, as mentioned, the extensive N-terminal modifications that are necessary for effective oocyte expression of PfCRT could be compromising activity, as could different lipid composition in the different membrane systems.

Most recently, Baro et al. (2011) engineered galactose-inducible expression of PfCRT in metabolically active growing yeast. Since the majority of PfCRT protein in this system is expressed at the plasma membrane and since the topology of the protein (cytosolic domains remain cytosolic, intra-DV domains are disposed outside the cell) as well as DV membrane bioenergetics (high delta pH acid outside) are preserved relative to DV-disposed PfCRT, function of PfCRT can be analyzed by plating or growing the live yeast versus various external [CQ]. In these experiments, both CQS and CQR isoforms of PfCRT are found to transport CQ, but the CQR isoforms transport at higher efficiency and with a stronger dependence on membrane potential (Baro et al. 2011), consistent with observations made with purified PfCRT reconstituted into proteoliposomes (Paguio et al. 2009). A recent review (Roepe 2011) discusses other factors that could explain the disagreements between oocyte versus yeast and

proteoliposome data; regardless, the important point is that considerable evidence now exists in support of direct transport of CQ by PfCRT driven by membrane potential. There is not yet complete agreement on whether this transport is more "channel-like" versus "carrier-like" (see also Papakrivos et al. 2012), thus additional studies with purified protein will be needed to refine the remaining hypotheses.

Most recently, (Baro et al. 2013) the inducible yeast expression system has been used to quantify differences in CQ transport by multiple CQR-associated PfCRT isoforms. Surprisingly, plots of CQ transport efficiency versus CQ IC_{50} for the strains in which the PfCRT isoforms are found reveal very poor correlation. A simple interpretation is that mutant PfCRT alone does not explain the degree of CQR.

New Insights: Cytostatic Versus Cytocidal Resistance

The above summary presents a satisfying model for PfCRT-mediated CQR further modulated by PfMDR1 and perhaps other factors. However, this assessment of CQR phenomena is based upon an incomplete definition of CQ pharmacology. With one exception (Paguio et al. 2011), for decades, all quantification of CQR has been via computing a ratio in CQ IC_{50} for CQR versus CQS strains or isolates. IC_{50} are determined from long-term growth inhibition assays wherein live parasites are grown for 1–3 iRBC cycles in the constant presence of CQ. These IC_{50} are in the 10^1–10^2 nM range (depending on the strain) and are relatively easy to obtain, including in high-throughput fashion with live cells (Smilkstein et al. 2004; Bennett et al. 2004a). Growth inhibition of parasites is highly relevant to the development of antimalarial therapy, because a good antimalarial drug should prevent increases in parasitemia and recrudescence. But it is also true that when CQ is administered to a malaria patient, the plasma concentration of the drug is typically >1 μM (not 10–100 nM), for at least the first 6–12 h. The most important initial effect of CQ therapy is significant reduction of parasitemia from 10^{12} to 10^{11} parasites to $\leq 10^9$, within hours. Meaning, successful clinical administration of CQ kills many parasites, it does not merely prevent their growth. A patient infected with CQR *P. falciparum* does not show this dramatic drop in parasitemia due to parasite death from micromolar CQ dose. Meaning, clinically relevant CQR can also be defined via an elevated LD_{50} ("**L**ethal **D**ose"). LD_{50} (defined as survival after bolus dose of CQ, see Paguio et al. 2011; Cabrera et al. 2009) have only been reported for two laboratory strains of *P. falciparum*. Many more such studies obviously need to be done. In one recent study, when drug accumulation is analyzed for intact iRBC using LD_{50} levels of drug (not IC_{50} levels), reduction in DV accumulation is not found for the CQR parasites relative to CQS (Cabrera et al. 2009). In fact, this study reports that CQR parasites can accumulate **more** toxic CQ relative to CQS and still exhibit resistance to drug-induced cell death. It is of course not uncommon for antimicrobial or anticancer drugs to show both growth inhibitory (cytostatic) and cell killing (cytocidal) effects. When they do, cytocidal activity often (but not always) requires higher levels of drug or longer exposure to drug. It is also not

uncommon for targets that are relevant for cytostatic functions of a drug to differ from those that are relevant for cytocidal. It is critical then to point out that, with two exceptions (Geary et al. 1986; Cabrera et al. 2009), nearly all detailed CQ transport analyses for CQR versus CQS parasites, vesicles, or oocytes that have been used to develop models for CQR and PfCRT function have been done at sub IC_{50} levels of drug (typically, 1–50 nM). This has led to logical explanations for CQR that are relevant for resistance to the cytostatic functions of CQ ("CQR^{CS}") but that, at least initially, do not appear to be completely relevant for resistance to the cytocidal functions of CQ ("CQR^{CC}," Cabrera et al. 2009). To fully elucidate CQR, and in the design of additional antimalarial chemotherapy, both facets are critical, and more work is required to understand the latter.

At LD_{50} doses of the drug, [CQ] in the DV will be very far above K_d for PfCRT, suggesting any direct PfCRT-mediated CQ transport would be overwhelmed by increased passive diffusion of the drug. Assuming therefore that targets for CQ static versus cidal effects differ (see also Gorka et al. 2013b), then those relevant for the latter might exist outside the DV, in the cytosol, in the nucleus, or in some other organelle. A search for these possible targets, better definition of LD_{50} versus IC_{50} for a number of drugs, and continued analysis of the differences between strains harboring various PfCRT and PfMDR1 isoforms are important topics for future research. These points are likely also relevant for defining what is apparently a non-mutated CRT mechanism for CQR in *P. vivax* malaria (Nomura et al. 2001; Baro et al. 2011).

Most recently, at least some of the additional biochemistry relevant for CQR^{CC} has been revealed (Gaviria et al. 2013). By performing LD_{50}-directed QTL for progeny of the HB3 (CQS) × Dd2 (CQR) cross (instead of IC_{50}-directed; Fidock et al. 2000b), it was found that CQS^{CS} and CQR^{CC} are genetically distinct, and additional chromosomal loci were found to be inherited in an LD_{50}-dependent fashion. Analysis of these loci suggests biological processes that could be altered in the development of resistance to the cytocidal effects of CQ. One process in particular, similar to autophagy, was found to be dysregulated in CQR parasites via analysis of PfATG8 protein distribution upon exposure to LD_{50} levels of CQ (Gaviria et al. 2013).

Conclusion

Resistance to CQ and other common antimalarials has historically been quantified by ratioing IC_{50} that quantify drug cytostatic activity. Drug transport experiments at IC_{50} dosages, genetics, and molecular pharmacology of drug–heme interactions have generated a molecular model for cytostatic CQR wherein mutations in PfCRT cause increased, electrochemically downhill, leak of CQ (and possibly other quinoline drugs) from the DV. This model is strongly supported by recent experiments with purified PfCRT protein that both define a single CQ-binding site and that show membrane potential-driven transport by PfCRT. Some conflicting interpretations regarding the efficiency of drug transport by different PfCRT

isoforms exist based on other recent experiments with oocytes, but overall these experiments also support PfCRT-mediated CQ transport. Questions that remain to be elucidated include defining relative affinities of related quinoline drugs for PfCRT isoforms and determining the efficiency with which they might be transported. Also, resistance to the cytocidal action of CQ does not appear to require decreased cellular accumulation of CQ. The precise role that PfCRT and other transport proteins (PfMDR1, PfNHE) play in this phenomenon remains to be explored.

The Fitch/Macomber/Sprinz hypothesis (Fitch 1969; Macomber and Spintz 1967) states that the DV is the principal site of CQ accumulation because heme released from Hb catabolism within the DV is its principal molecular target. Simply moving CQ from the DV faster than it passively diffuses back inward from the cytosol would then decrease binding to heme target that is continuously delivered as Hb is digested, and thereby cause CQR. In addition, the dynamics of CQ^{2+} versus CQ^{+} versus CQ binding to different chemical forms of heme (monomer vs μ-oxo dimer vs head-to-tail dimer) might be different in CQS versus CQR parasites, further contributing to decreased retention of CQ. In support of both models, several studies have defined both covalent and non-covalent heme–drug complexes for CQ and related drugs that are influenced by CQ protonation (Gorka et al. 2013a). Decreased pH and volume-dependent CQ-heme adduct accumulation within the DV of CQR parasites may be part of the explanation for elevated IC_{50} in these CQR parasites. However, the early observations of Geary and Ginsburg (Geary et al. 1986), along with recent distinction between IC_{50} versus LD_{50} phenomena (Paguio et al. 2011; Gaviria et al. 2013), continue to hint that the mechanism of clinically relevant cytocidal CQR is likely multifaceted. Perhaps related to this, interestingly, mutations in the *P. vivax* orthologue PvCRT apparently do not cause CQR in *P. vivax* malaria (Nomura et al. 2001). We suggest the additional layers to CQR in *P. falciparum*, which likely represent biochemistry relevant to raising CQ LD_{50} (but not necessarily IC_{50}), will provide important clues for the mechanism of CQR in *P. vivax* malaria.

Lastly, for both *P. falciparum* and *P. vivax* malaria, parasite CQR can be overcome with new chemotherapy that can be perfected by screening versus CQR strains (e.g., Peyton 2012). It has been hoped that detailed knowledge of CQR would expedite second-tier drug development versus malaria, and this hope is now beginning to be realized. A key component to new antimalarial chemotherapy is (and will continue to be) the additive or synergistic effects of two or more drugs given in combination. We suggest more detailed quantification of IC_{50} versus LD_{50} synergies is an important new avenue to explore in antimalarial drug discovery (Gorka et al. 2013c).

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Drug Resistance in *Leishmania*

Danielle Légaré and Marc Ouellette

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Abstract

Protozoan parasites of the genus *Leishmania* cause a wide range of diseases affecting 12 million people worldwide with 1.5–2 million new cases each year. With no vaccine available yet, the control of these parasites relies solely on chemotherapy. Low-cost antimony-derived compounds remain the primary antileishmanial treatment in most developing countries. Increasing drug resistance towards these molecules has forced the use of alternative therapies in highly endemic areas including amphotericin B, paromomycin, and miltefosine. This chapter is presenting our current understanding of the mode of action and underlying resistance mechanisms of the few therapeutic drugs used against *Leishmania*.

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Keywords
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Abbreviations

AMB	Amphotericin B
CL	Cutaneous leishmaniasis
DCL	Diffuse cutaneous leishmaniasis
GSH	Glutathione
MCL	Mucocutaneous leishmaniasis
MIL	Miltefosine
PKDL	Post-kala-azar dermal leishmaniasis
PM	Paromomycin
SAG	Sodium antimony gluconate
Sb^{III}	Trivalent antimonials
Sb^{V}	Pentavalent antimonials
SILAC	Stable isotope labeling by amino acids in cell culture
TSH	Trypanothione
VL	Visceral leishmaniasis

Introduction

With 12 million people infected in 98 countries on 5 continents and close to 350 million people at risk of infection globally, human leishmaniasis is considered by the World Health Organization as one of the top ten threatening infective conditions worldwide (WHO 2010). In fact, it corresponds to the second-biggest parasitic killer after malaria with an annual death rate of up to 50,000 people and an estimated 2 million disability-adjusted life years (DALYs) lost (Alvar et al. 2012; King and Bertino 2008). Each year, ~1.5–2 million new cases are estimated to occur, although this is probably an underestimate because the disease is reportable only in approximately half of the countries in which it is known to occur. Furthermore, many *Leishmania* infections are asymptomatic, subclinical, or misdiagnosed. Leishmaniasis affects the poorest of the poor, particularly in Africa, South and Central America, Asia, the Mediterranean basin, and the Middle East. Protozoa of the genus *Leishmania* are passed on to humans and animals (mainly small rodents and dogs which act as reservoirs) by female *Phlebotomus* spp. or *Lutzomyia* sand flies that need blood for their eggs. Other forms of transmission such as via intravenous needles shared by drug users (Cruz et al. 2002), transfusion, transplant, or placental contaminations have been reported (Antinori et al. 2008; Grogl et al. 1993). Once injected, promastigote parasites are engulfed by macrophages in the dermis and throughout the reticuloendothelial system (mainly macrophages) where they transform into obligatory intracellular amastigotes within acidified phagosomes. In this

sanctuary, after a short period of latency of weeks to few months that may extend to many years, the parasite starts to proliferate. This multiplication will cause a systemic infection of neighboring macrophages that will propagate the disease and result in a large diversity of pathologies. To date, 21 *Leishmania* species pathogenic for humans have been recognized, traditionally divided between Old World (Africa, Asia, Europe) and New World (Americas) parasites, although the taxonomy of the *Leishmania* species is still in debate (Boite et al. 2012). Depending of the species and the host immune status, the clinical manifestations of leishmaniasis range from limited cutaneous (CL) lesions to disseminating visceral disease (VL) also called "kala-azar" which translates to "black disease" because of the skin pigmentation (Alvar et al. 2012; Kaye and Scott 2011). In more than 90 % of patients, CL undergoes self-cure within 3 to 18 months, even though it may develop into a more serious and complex clinical disease such as mucocutaneous (MCL, also known as espundia) or diffuse cutaneous leishmaniasis (DCL). In contrast to CL, VL is uniformly fatal within 2 years if untreated. Even with treatment, VL may result in case-fatality rates of 10–20 % (Alvar et al. 2012). After recovery from a VL, some patients experience dermal leishmaniasis referred to as "post-kala-azar" (PKDL) characterized by indurated nodules or depigmented papules frequently scattered on exposed parts of the body, mainly the face and neck. PKDL patients are extremely difficult to treat, and because these skin lesions are highly infectious to sand flies (e.g., lesions contain many parasites), this particular clinical manifestation is considered as a significant contributor to the spread of VL, particularly in Sudan and India where, respectively, 50 % and 1 to 3 % of the VL patients will develop this insidious cutaneous form (Zijlstra et al. 1995, 2000, 2003). Interestingly, host genetics may have a role in susceptibility to VL and PKDL since studies have reported that some tribes are more vulnerable to VL and PKDL than others (Bucheton et al. 2003; Farouk et al. 2010; Mohamed et al. 2003, 2004; Saha et al. 2007; Salih et al. 2007).

Another rising concern and major threat to the control of VL is its intersection with HIV-1 infection. To date, HIV-*Leishmania* coinfections are reported from more than 35 countries with Ethiopia having by far the highest prevalence of HIV/VL worldwide (e.g., 15–30 % of VL cases) (Orsini et al. 2012). HIV-infected people are particularly vulnerable to VL; the HIV condition increases the risk of developing an active VL by between 100 and 2,320 times, while VL accelerates HIV replication and progression to AIDS (Alvar et al. 2008; Bentwich 2003; Bernier et al. 1995; Mock et al. 2012; Wolday et al. 1999). Polyinfection also exists with *Mycobacterium tuberculosis* which represents an even greater public health challenge (Delobel et al. 2003; Mohamed et al. (2004) Rathnayake et al. 2010; Wang et al. 1999; el-Safi et al. 2004). There is as yet no effective vaccine for prevention of any form of leishmaniasis, although significant effort is being invested (Costa et al. 2011; Kobets et al. 2012; Palatnik-de-Sousa 2008). Leishmanization, e.g., inoculating people with live virulent parasites to cause a local limited lesion and provide protection, has been traditionally used for CL but is not recommended as a control measure. Current limited treatment options are based on chemotherapy but remain

unsatisfactory for reasons such as toxic side effects, high cost, route of administration and duration of treatment, need for monitoring and hospitalization, and the increasing incidence of drug-resistant parasite strains.

Current Therapies for Leishmaniasis

Chemotherapy has always been a critical issue in the management of leishmaniasis. Excellent up-to-date reviews have been published recently on the current pharmacotherapy deployed against the parasite *Leishmania*, and readers are referred to them for a more exhaustive description on this topic (Kobets et al. 2012; Singh et al. 2012; Sundar and Chakravarty 2013; Zucca et al. 2013). Only a brief overview of the main therapeutic options available will be presented. The reality is that access to antileishmanial medicines is not always easy in every endemic country, that all forms are difficult to treat, and that current therapies for leishmaniasis are far from ideal. The VL, MCL, DCL, and PKDL forms are principally treated by intravenous drugs, whereas CL is generally treated with local drug injections or topical ointments according to country-specific drug regimens (Table 1). The injectable pentavalent antimonials (Sb^V) have been the cornerstone of antileishmanial chemotherapy for more than 70 years and still remain the first pharmacotherapeutic choice for all clinical forms worldwide, except in Bihar state, India, where resistance has reached such high proportions (~65 % refractory cases) that the use of these compounds is now limited. Two branded antimonials are available, the sodium stibogluconate (SSG, Pentostam®) and meglumine antimoniate (Glucantime®). Second-line drugs include the polyene antibiotic amphotericin B and its various deoxycholate (Fungizone®) or liposomal (AmBisome®) formulations, the hexadecylphosphocholine miltefosine (Impavido®) which is the first orally administrated antileishmanial drug, the diamidine or pentamidine isethionate (Lomidine®, Pentacarinat®), and the aminosidine antibiotic paromomycin sulfate. Also, the primaquine analogue sitamaquine would potentially represent the second promising antileishmanial oral drug if late-stage clinical trials are successful. Preliminary results suggest however that sitamaquine will need to be given in combined therapies because of resistance issues as well as adverse effects observed in clinical trials (Loiseau et al. 2011). Few other drugs are currently in development.

A number of alternative treatments for CL have been developed in the hope to decrease the toxic effects of chemotherapeutic injectable drugs and the difficulty to administer them. Since *Leishmania* amastigotes are heat sensitive (39 °C or higher), thermotherapy has revealed to be a good option for CL treatment. This treatment implies local applications of the device (e.g., ThermoMed® or other radiofrequency heat devices), maintained at 50 °C, to the infected skin sores. The equipment however is very expensive and thus remains difficult to access in endemic countries. Other alternative CL treatments include cryotherapy using liquid nitrogen, CO_2 laser, photodynamic therapy, curettage, and surgical excision either alone or in combination with intralesional antimony (Table 1). These treatment options are however labor intensive and not suitable for multiple or complicated lesions.

Table 1 Leishmaniasis treatments[a]

Antileishmanial compound	Syndrome	Adult regimen	Comments
Current standard chemotherapeutics			
Pentavalent antimony (Pentostam®)	CL VL and MCL PKDL	IL injections at 20 mg/kg/day for 20 days or up to healing IV or IM at 20 mg/kg/day for 28–30 days IM at 20 mg/kg/day for 4–5 months	Antimonial compounds were used against *Leishmania* as early as 1912 and are still active against all species. For pregnant woman, Sb^V should not be used. Increasing resistance in India, Sudan, France, and parts of South America. About 90 % efficacy in US patients. GlaxoSmithKline has never applied for FDA approval so Pentostam® is not licensed for US commercial use and has to be obtained from CDC drug services. Toxicities include myalgias, arthralgias, abdominal symptoms, liver enzyme elevation, pancreatic and cardiac toxicity, and sudden death. Mortality during treatment is high (up to 20 %) in HIV-*Leishmania*-coinfected patients
Pentamidine isethionate (Pentacarinat®)	All	Intravenous or intramuscular 2–4 mg/kg/day for up to 15 days	A diamidine drug active mainly against CL and VL, when other drugs are not available. It may require up to 4 months of treatment. Pentamidine is now mostly used against African trypanosomiasis and patients with HIV and *Leishmania* coinfections
Pentamidine mesylate (Lomidine®)			Pentamidine mesylate is no longer available in certain countries including France. It may cause hypotension and later insulin-dependent diabetes mellitus
Amphotericin B deoxycholate	All	Intravenous 1–5 mg/kg/day, alternate days for 3–4 weeks	In the early 2000s, amphotericin B became the first-line treatment of VL in Bihar, India, because of antimony resistance. Amphotericin B causes secondary effects such as renal disturbances, anemia, fever,

(*continued*)

Table 1 (continued)

Antileishmanial compound	Syndrome	Adult regimen	Comments
			malaise, hypokalemia, and renal failure. These can be reduced by adequate hydration and infusion over several hours
Liposomal amphotericin B (Amphomul®, AmBisome®, Amphotec®, Abelcet®)	All	Intravenous 5–20 mg/kg/day for 3–4 weeks	The most active antileishmanial agent in use and the only US FDA-approved agent against VL. AmBisome® is however very expensive, being almost 3,000 times higher than the sodium stibogluconate price and ~900 times more than that of paromomycin in India. A preferentially low price has been secured however by the MSF and WHO. Toxicities include nephrotoxicity, electrolyte disturbances, fever, and rigors. AmBisome® seems less toxic than other amphotericin B lipid formulations. AmBisome® is a complex of amphotericin B and distearoylphosphatidylgycerol bound to a liposome of cholesterol and phosphatidylcholine. Abelcet® is a lipid complex and Amphocil® is a colloidal dispersion
Miltefosine (Impavido®)	VL and CL	Oral 100–150 mg daily for 28 days	Miltefosine, also called hexadecylphosphocholine, is a phospholipid analogue originally developed as an anticancer drug. Its effectiveness is species and local dependent. Does not work well against *L. braziliensis*, *L. guyanensis*, and *L. mexicana*. Contraindicated in pregnancy and requires effective contraception during and for 3 months after therapy. May cause vomiting, gastrointestinal disturbance, and diarrhea

(*continued*)

Table 1 (continued)

Antileishmanial compound	Syndrome	Adult regimen	Comments
Paromomycin sulfate	CL and VL	Intravenous or intramuscular 11–15 mg/kg daily for 21 days	Also known as aminosidine. Aminoglycoside antibiotic produced by *Streptomyces rimosus*. Often used in combination with miltefosine. Highest activity was against *L. major* and *L. tropica* while of the New World species *L. panamensis* was most, and *L. mexicana* was least sensitive. *L. donovani* species showed variable sensitivity
Paromomycin sulfate (15 %) ointment	CL	Apply twice daily for 20–30 days	Combined therapy with intralesional Sb^V (weekly or alternate-day injections at multiple doses). Commercially available in Israel. Randomized trials are showing only modest efficiency against *L. major*
Allopurinol	CL	Oral 300–600 mg/day for 4–6 weeks	Allopurinol is phosphorylated by the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and incorporated into nucleic acid, thus leading to selective death of *Leishmania* parasites (Fish et al. 1985). Parasites of the Old World seem more susceptible, especially *L. major*. Works better in combination with an azole (e.g., itraconazole). Adverse effects include rash, itching, fever, eosinophilia, hepatic granulomas, nephritis, vasculitis, and exfoliative dermatitis
Rifampicin	CL	Oral 200 mg/day for 6 weeks	
Fluconazole (Triflucan® or generic)	CL	Oral 200 mg daily for 6 weeks	Imidazole compound with high skin concentration. Seems effective against *L. major* but remains untested for most species of *Leishmania*. Fluconazole, itraconazole, and ketoconazole inhibit a key enzyme of the ergosterol

(*continued*)

Table 1 (continued)

Antileishmanial compound	Syndrome	Adult regimen	Comments
			biosynthesis pathway, the 14-α-demethylase
Itraconazole	CL	Oral 200 mg twice daily for 28 days	Imidazole compound. Better tolerated than ketoconazole but may be less effective than ketoconazole, at least against the *vianna* subgenus. Works well for *L. major*
Ketoconazole (Nizoral®)	CL	Oral 600 mg/day for 28 days	Imidazole compound. Ketoconazole has modest activity against *L. mexicana* and *L. (V.) panamensis* infections. Its usefulness against *L. major* is still unclear. Does not work for *L. tropica*, *L. aethiopica*, and *L. braziliensis*. Fluconazole seems less toxic and better than ketoconazole
Azithromycin			Macrolide antibiotic that concentrates in macrophages. Seems active against *L. major* in mice model
Dapsone	CL	Oral 2 x 100 mg/day for 6 weeks	
Recombinant interferon gamma (*Imukin*®, Torental®, other immunomodulators)		Subcutaneous or intramuscular	Adjunct therapy, often in combination with miltefosine and paromomycin. Pentoxifylline under the brand name Torental® is an inhibitor of tumor necrosis factor-α
Immunotherapy	CL	Intramuscular. Three doses of killed *L. amazonensis* preparation plus BCG	In South America, particularly in Venezuela, immunostimulation by injections of heat-killed promastigotes plus BCG is used in the treatment of CL due to *L (v.) braziliensis*. This approach promotes healing. BCG vaccination may cause adverse reactions
Preclinical stages leads or compounds tested in academia (only few example are given)			
Fexinidazole	VL	Oral formulation	Fexinidazole is already in phase 1 clinical trials for African sleeping sickness, but it seems also active against *Leishmania* (Wyllie

(*continued*)

Table 1 (continued)

Antileishmanial compound	Syndrome	Adult regimen	Comments
			et al. 2012) with cure rate of 98 % in mice model
Lipid-based amphotericin B (iCo's formulation)	VL	Oral formulation	Developed by Dr. Kishor M. Wasan at the University of British Columbia, Canada, with collaborators. This formulation is stable at the temperatures of WHO Climatic Zones 3 and 4 (30–43 °C) and seems effective against systemic fungal infections and drug-resistant VL. In 2010, iCo's formulation for treating VL received "orphan drug" designation by the FDA, an important recognition of its promise
Imiquimod	CL	Topical formulation	Immunomodulatory agent initially developed as an antiviral drug recommended for facial CL lesions. Seems effective in combination with antimony compounds (Khalili et al. 2011). Imiquimod induces the production of cytokines and nitric oxide in macrophages at the site of application
ER-119884 and E5700			Two quinuclidine derivatives active against the leishmanial squalene synthase enzyme (SQS) in the sterol biosynthetic pathway. These compounds have been tested against *L. amazonensis* (Fernandes Rodrigues et al. 2008)
Bisphosphonates			Bisphosphates inhibit the isoprenoid pathway in *Leishmania* that is catalyzed by the enzyme farnesyl diphosphate synthase (FPPS)
Sitamaquine (WR 6026)	VL	Oral 1–3 mg/kg/day for 28 days	8-Aminoquinoline in development in combination with miltefosine. Abdominal complaints of discomfort, pain, vomiting, diarrhea, and headache are the commonest side effects. Nephrotoxicity

(*continued*)

Table 1 (continued)

Antileishmanial compound	Syndrome	Adult regimen	Comments
			may occur. Tafenoquine is another 8-aminoquinoline in advanced stage of development that will also be administrated orally. Tafenoquine is active against both Sb^V-resistant and Sb^V-sensitive *L. donovani* strains (Yardley et al. 2010)
Nelfinavir			Highly active against strains of *L. braziliensis*, *L. donovani*, and *L. chagasi* with inhibition rates over 90 % (Santos et al. 2013)
Several adenosine analogues			Specific inhibitors for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Aronov et al. 1999)
Trypanothione reductase (TR) inhibitors			Several compounds that inhibit TR have been identified including polyamine derivatives, tricyclics, and aminodiphenyl sulfides (Venkatesan and Dubey 2012). One of these is auranofin, a gold-containing drug already in clinical use as an antiarthritic agent (Ilari et al. 2012)
Inhibitors of topoisomerases			
PTR1 and DHFR-TS inhibitors			PTR1 and DHFR-TS are key in folate metabolism in *Leishmania*. Specific inhibitors are being developed against these proteins (Corona et al. 2012; Rajasekaran and Chen 2012)
Other treatments			
Phototherapy (Omnilux PDT®, Photo Therapeutics Inc)	CL	~100 J/cm^2/treatment at 635 nm in the presence of photosensitizing agents for several weeks	The production of highly reactive oxygen species (ROS) will inhibit and even kill by photolysis the amastigote parasite
Cryotherapy	CL	Freeze/thaw cycles of 15–20 s each using	In combination with Sb^V (intralesional). Effective against *L. tropica* and *L. major*

(*continued*)

Table 1 (continued)

Antileishmanial compound	Syndrome	Adult regimen	Comments
		liquid nitrogen, treatment of 1–2 weeks	CL uncomplicated lesions, e. g., dryer and smaller (<1 cm) lesions
Thermotherapy (ThermoMed®)	CL	Radiofrequency waves with 50 °C for 30 s intralesional	Parasites are mostly killed by heat

[a]Only the main leishmaniasis treatments and some promising compounds are presented in this table. *Leishmania* species are known to present intrinsic variation in drug sensitivity to almost all antileishmanial agents. *IV* intravenous, *IM* intramuscular, *IL* intralesional, *CL* cutaneous leishmaniasis, *MCL* mucocutaneous leishmaniasis, *DCL* diffuse cutaneous leishmaniasis, *PKDL* post-kala-azar dermal leishmaniasis, *VL* visceral leishmaniasis, *MSF* Médecins Sans Frontières, *WHO* World Health Organization

Mechanisms of Drug Resistance

Leishmaniasis is treatable by chemotherapy, but resistance can be developed experimentally for all drugs, suggesting that clinical resistance is just a question of time. The next section will describe the ingenious mechanism(s) of resistance evolved by *Leishmania* parasites to survive the toxic effects of drugs. Emphasis will be put on the main therapeutic options currently used in endemic countries. The mode of action and the underlying resistance mechanisms either demonstrated in vitro, in vivo, or in clinical isolates will be presented when available.

Pentavalent Antimonials

Pentavalent antimonials (Sb^V) are the classical first-line treatment against leishmaniasis and the only drugs that are barely affordable in most endemic countries. These drugs are toxic and poorly tolerated with the consequence that a significant proportion of the patients do not complete their full course regimen. This situation appears to be the principal reason behind the selection and emergence of antimony-resistant parasite strains. Apart low compliance, the use of counterfeit medicines with none optimal curative efficacy was also incriminated recently in further promoting the emergence of antimonial resistance in endemic countries (Cockburn et al. 2005; Mackey and Liang 2011). Another interesting hypothesis is that arsenic contamination (arsenic is a metal related to antimony) in drinking water in Bihar has also contributed to the selection of resistant mutants (Ait-Oudhia et al. 2011; Perry et al. 2011).

Antimonials mediate part of their antileishmanial activity via the generation of oxidative stress which leads to the disruption of the synthesis of macromolecules in the parasite cell. To survive, the parasite therefore must control this oxidative assault and this is achieved by a complex molecular response. After nearly 20 years of

in vitro studies punctuated with few reports describing work in clinical isolates, a global model for antimonial resistance in *Leishmania* has been proposed (Fig. 1). It is generally accepted that all pentavalent antimonials are prodrugs that require biological reduction to their trivalent form (Sb^{III}) in order to acquire antileishmanial activity (Shaked-Mishan et al. 2001). Antimony reduction apparently may occur in both the host cell and in parasites. The reduced trivalent form in macrophages enters the parasite cell through the aquaglyceroporin AQP1 localized at the parasite surface (Gourbal et al. 2004). It has been shown that a lower activity of AQP1 by gene deletion of reduced expression resulted in Sb^{III} increased resistance (Gourbal et al. 2004; Marquis et al. 2005). Recently, a deletion of the AQP1 gene was observed in in vitro antimony-resistant parasites through a telomeric gene deletion (Mukherjee et al. 2013a). Sb^{V} enters into the parasite via a protein that is different than AQP1. It has been hypothesized that this transporter may either recognize phosphate or a sugar moiety shared with gluconate (Brochu et al. 2003). Increased intracellular levels of the antioxidant molecule trypanothione (TSH, the most abundant thiol in trypanosomatids) have been observed in antimony-resistant parasites, an event usually related to the overexpression of rate-limiting enzymes involved in the synthesis of glutathione (gamma-glutamylcysteine synthetase, γ-GCS) and polyamines (ornithine decarboxylase, ODC) (Fig. 1). Another resistance protein implicates the ABC transporter MRPA (ABCC3 alias PgpA) which confers resistance by sequestering drug-trypanothione conjugates within an intracellular organelle near the flagellar pocket, where the antimonial target(s) is probably absent (Legare et al. 2001). It is thought that the sequestered drugs are then expelled from the parasite through exocytosis occurring at the flagellar pocket (Fig. 1). Finally, a protein localized at the parasite cell surface was reported to be responsible for the active efflux of TSH conjugated-antimonial compounds outside the parasite (Dey et al. 1996) (Fig. 1). One possible candidate would be the recently characterized ABCI4 protein (Manzano et al. 2013). Part of the in vitro antimonial resistance model presented in Fig. 1 was recently confirmed in natural antimony-resistant *Leishmania* clinical isolates recovered from patients unresponsive to sodium antimony gluconate (SAG). Indeed, an active role for MRPA, γ-GCS, and ODC in resistance was confirmed (Mukherjee et al. 2007; Singh et al. 2010), which tends to corroborate the resistance model proposed for in vitro-resistant strains. Nonetheless, since different paths lead to resistance in *Leishmania*, alternative mechanisms other than those described here may also operate in field isolates (Downing et al. 2011; Singh 2006; Vanaerschot et al. 2012). Recently, it has been discovered that *Leishmania* influences cell functions of its host cell via glycans deployed at its cell surface (Mukherjee et al. 2013b). Indeed, particular glycans at the parasite cell surface outwit the immune system of the host and permit to resist to the toxic effect of antimonial drugs by making the human host cell expelling antimony drugs through the ATP-binding cassette (ABC) transporter MDR1 localized at the macrophage cell surface (Fig. 1, left insert). Thus, antimony resistance in *Leishmania* is multifactorial with contributions of several independent events, leading to parasite resistance.

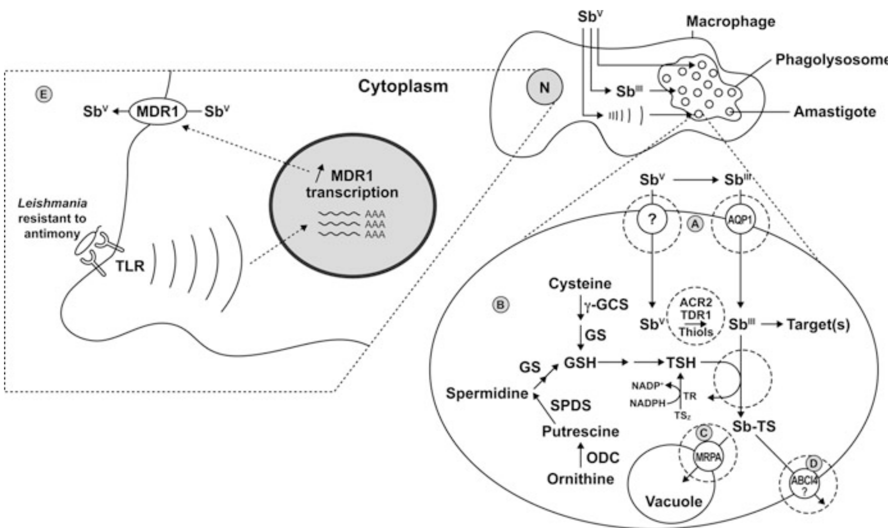

Fig. 1 Resistance mechanisms to antimonials in *Leishmania*. Resistance to antimonials involves modulation of influx in the parasite cell **(a)**, thiol and antimony metabolisms **(b)**, drug sequestration through MRPA **(c)**, drug efflux potentially by the ABC transporter ABCI4 **(d)** at the parasite cell surface, and overproduction of host MDR1 at the macrophage cell surface **(e)**. The drug Sb^{V} is converted to its reduced form (Sb^{III}, the toxic form of the metal) in the macrophage and/or in the parasite cell. Sb^{V} and Sb^{III} enter *Leishmania* through two different transporters, a still uncharacterized transporter (depicted by a question mark at the parasite cell surface) and AQP1, respectively. The intracellular Sb^{V} is potentially converted to Sb^{III} by two parasite enzymes (ACR2 and TDR1) or by reduced thiols. Once reduced, Sb^{III} is detoxified by its conjugation with trypanothione (TSH). Trypanothione is maintained in its reduced form by the enzyme trypanothione reductase (TR) which uses NADPH. In antimony-resistant parasites, the trypanothione biosynthesis pathway is activated which leads to an increase in the concentration of intracellular THS. The Sb^{III}-TS conjugate is either effluxed out of the cell through an efflux pump (potentially ABCI4) or is sequestered in a vacuole by the activity of the ABC transporter MRPA. It is thought that these vacuoles are then exocytosed at the flagellar pocket. Particular glycans at the parasite cell surface are responsible of the MDR1 modulation at the macrophage plasma membrane. The interaction of a unique terminal sugar at the surface of Sb^{V}-resistant parasites with host TLR2 will result in the activation of a series of transcriptional factors and IL10 production by the macrophage that will ultimately drive the overexpression of MDR1 transcripts in the macrophage nucleus. Translation of MDR1 transcripts will lead to an overproduction of MDR1 proteins at the host plasma membrane, resulting in an increased efflux of antimony outside the macrophage, leading to resistance. *AQP1* aquaglyceroporin 1 transporter, *GSH* glutathione, *TSH* reduced trypanothione, *TS_2* oxidized trypanothione, *Sb^{V}* pentavalent antimony, *Sb^{II}*, trivalent antimony, *Sb-TS* trypanothione-antimony conjugate, *MRPA* multidrug resistance protein A alias PgpA. *?* still uncharacterized transporter for Sb^{V}, *ACR2* antimony reductase, *TDR1* thiol-dependent reductase, *ABCI4* an ABC transporter that may act as an efflux pump, *N* nucleus, *MDR1* multidrug resistance protein 1, *γ-GCS* gamma-glutamylcysteine synthase, *ODC* ornithine decarboxylase, *SPDS* spermidine synthase, *GS* glutathione synthetase, *TR* trypanothione reductase, *TLR* Toll-like receptor

Amphotericin B

Amphotericin B (AMB) is a polyene antibiotic originally extracted from the filamentous bacterium *Streptomyces nodosus* (Lemke et al. 2005; Trejo and Bennett 1963) and first developed as an antifungal drug. AMB is the current secondary treatment of choice against leishmaniasis and the best treatment against antimonial refractive leishmaniasis in highly endemic regions such as Bihar state in India. It is currently available as a plain AMB solution, as cholesteryl sulfate or lipid complexes, and as liposomal formulations (Table 1). AMB is a highly effective antileishmanial drug, but major drawbacks and side effects limit however its widespread use. Indeed, AMB is nephrotoxic and is only available in injectable formulations requiring several hours' administration schedules by infusion and close monitoring. A study aiming at testing the efficacy and safety of one single infusion of AMB over only a 2 h infusion is currently in trials (Sundar and Chakravarty 2013). Similarly, AMB-lipid formulations have been developed to improve tolerability for patients and to increase plasma concentration for a better efficacy, but the cost of these formulations is highly prohibitive. Oral and safer formulations of AMB for the treatment of leishmaniasis are being developed. Positive preclinical data are suggesting an absence of kidney toxicity and a dramatic knockdown of parasitic VL infections with greater than 99 % eradication of parasitic infection at the tested dosages (Wasan et al. 2009, 2010).

The mechanism of action of AMB is based on the binding of the AmB molecule to ergosterol, the predominant sterol in the membranes of *Leishmania* parasites. It produces an aggregate that creates a transmembrane channel (e.g., aqueous pores in the lipid bilayers), allowing the cytoplasmic contents to leak out (especially K^+), probably accelerating cell death (Brajtburg et al. 1990; Ramos et al. 1996; Urbina et al. 1987). Another mechanism by which AMB could affect the parasite is its autooxidation and subsequent formation of free radicals which are highly toxic to *Leishmania*. Thus, both ion movement and oxidative effects induced by AMB may lead to parasite cell death, and any molecular change that interferes with these processes may lead to resistance. The level of sensitivity to AMB is species dependent and depends on the variation in the ergosterol content in membranes (Escobar et al. 2002). Resistance in in vitro-generated *Leishmania* promastigotes was shown to be caused by a significant change in plasma membrane sterols, with ergosterol being replaced by a precursor, cholesta-5,7,24-trien-3β-ol (Mbongo et al. 1998) (Fig. 2a). This change is apparently due to a loss of function of the s-adenosyl-L-methionine-C24-Δ-sterol methyltransferase (SCMT) that impaired C24 transmethylation (Fig. 2a). In addition, AMB uptake was decreased in an in vitro-generated *L. donovani* AMB-resistant cell line, and efflux, most likely due to the overexpression/deregulation of an ABC transporter (MDR1?), was increased (Fig. 2b) (Mbongo et al. 1998). Deep understanding of the putative role of ABC proteins in AMB resistance in *Leishmania* needs to be formally addressed however since it remains possible that the decrease in uptake and/or increase in efflux in this in vitro-generated AMB-resistant mutant may also be due to a weak affinity of AMB for such modified membranes in resistant parasites.

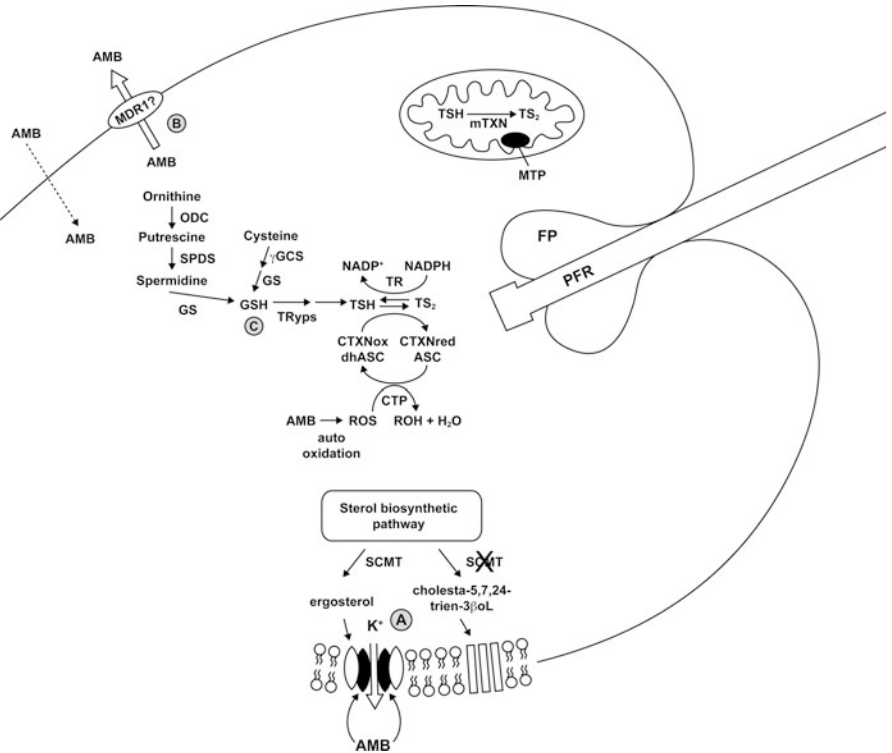

Fig. 2 **Resistance mechanisms to amphotericin B in *Leishmania***. Multiple mechanisms conferring AMB resistance have been reported in in vitro-generated mutants, with some recently detected in field isolates. These include an altered membrane sterol profile **(a)**; efflux, possibly by the parasite ABC transporter MDR1 (Mbongo et al. 1998; Purkait et al. 2012), although this remains to be formally addressed **(b)**; and detoxification of the AMB-mediated reactive oxygen species (ROS) through the trypanothione/tryparedoxin cascade **(c)**. AMB binds to ergosterol of sensitive *Leishmania* parasites, causing membrane depolarization and leakage of ion K^+, leading to cell death. In AMB-resistant parasites, AMB binding to membrane ergosterol is impaired due to an altered membrane sterol profile caused by a loss of function of the enzyme *S*-adenosyl-L-methionine: C24-D-sterol methyltransferase (SCMT) **(a)**. Although the plasma membrane composition is altered in resistant parasites, it still remains possible that a low amount of AMB manages to enter the parasite cells (schematized by a *dotted line* in figure crossing the parasite plasma membrane). Part of the small amount of AMB that manages to be taken up into the resistant parasites is thought to be rapidly effluxed out by a plasma membrane ABC transporter (possibly MDR1), although this assumption remains to be formally addressed **(b)**. The remaining intracellular AMB auto-oxidizes and will lead to the production of ROS. The toxic effects of ROS may be nullified by the parasite trypanothione/tryparedoxin cascade **(c)**. Two enzymes, the cytoplasmic tryparedoxin peroxidase (CTP) and the mitochondrial tryparedoxin peroxidase (MTP), involved in the terminal steps of the trypanothione/tryparedoxin cascade may cleave the ROS, producing water and alcohols (ROH) **(c)**. *GSH* glutathione, *TSH* reduced trypanothione, TS_2 oxidized trypanothione, *MDR1* multidrug resistance protein 1, *γ-GCS* gamma-glutamylcysteine synthase, *ODC* ornithine decarboxylase, *SPDS* spermidine synthase, *GS* glutathione synthetase, *Tryps* trypanothione synthase, *TR* trypanothione reductase, $cTXN_{ox}$ oxidized cytosolic tryparedoxin, $cTXN_{red}$ reduced cytosolic tryparedoxin, *mTXN* mitochondrial tryparedoxin, *dhASC* dehydroascorbate, *ASC* ascorbate, *FP* flagellar pocket, *PFR* paraflagellar rod. This schematic is adapted from Purkait et al. (2012)

To date, only few cases of AMB clinical resistance were reported (Purkait et al. 2012; Srivastava et al. 2011). The analysis of one clinical isolate of *L. donovani* has shown that similar resistance mechanisms previously observed in in vitro AMB-resistant mutants are also operating in clinical isolates (Purkait et al. 2012). Indeed, the resistant clinical strain had an altered membrane composition (with the cholesta-5,7,24-trien-3β-ol substitution, Fig. 2a), resulting in a higher fluidity of the plasma membrane compared to sensitive clinical strains. A decrease in AMB uptake as well as an increase in the expression level of MDR1 (Fig. 2b) at the parasite cell surface was also found in the resistant clinical strain, suggesting an increase in efflux of AMB. The tryparedoxin cascade was also upregulated in resistant parasites as well as a more reduced intracellular thiol level (Fig. 2c). It was suggested that this increase in reduced thiol in resistant isolates may help in better scavenging of reactive oxygen species (ROS) produced by AMB (Purkait et al. 2012), although this assumption needs further investigation. It must be stressed however that resistance to AMB must be rare. A study has shown that HIV patients with several relapses with the same strains were treated with several courses of AMB and the parasites never became resistant (Lachaud et al. 2009).

Paromomycin

The aminoglycoside *paromomycin* (PM) has been available in India since 2006. It seems highly effective in this endemic country, but its efficacy in Africa seems however lower. Since there is a concern on the development of clinical resistance, the longevity of PM would be better preserved in combination regimens than as a single agent therapy. Recently, a quantitative SILAC-based proteomic analysis of an in vitro-generated PM-resistant strain has brought some light on the mode of action of PM and the underlying resistance mechanisms (Chawla et al. 2011). The uptake of PM is thought to proceed by endocytosis, a process facilitated by the binding of PM to a number of parasite surface proteins (Fig. 3a). Two of them were the paraflagellar rod (PFR) proteins 1D and 2C that are only found at the flagellum of the parasite. Another protein interacting with PM is prohibitin that is also found at the surface of the flagellar and the aflagellar poles. Apparently, these PM-interacting proteins may help the drug to be taken up by the parasite through endocytosis (Fig. 3a). Another protein that interacts with PM is the putative P-type H^+ ATPase (Fig. 3b). Its localization in *Leishmania* is unclear, but in the parasite *Trypanosoma cruzi*, the orthologous protein is localized in vacuolar compartments and is probably involved in the acidification of these intracellular vacuoles (Vieira et al. 2005). It is thus suspected that this protein plays a similar role in *Leishmania* parasites and that the potential interaction of PM with this putative vacuolar pump promotes its endocytosis/sequestration process or helps to keep the drug inside vacuoles. Interestingly, the resistant strain had a higher number of vesicular vacuoles and an increase in a number of proteins involved in vesicular trafficking compared to the parental sensitive strain. This suggests that PM could affect vesicle-mediated trafficking. The quantitative proteomic analysis also revealed an upregulation of ribosomal

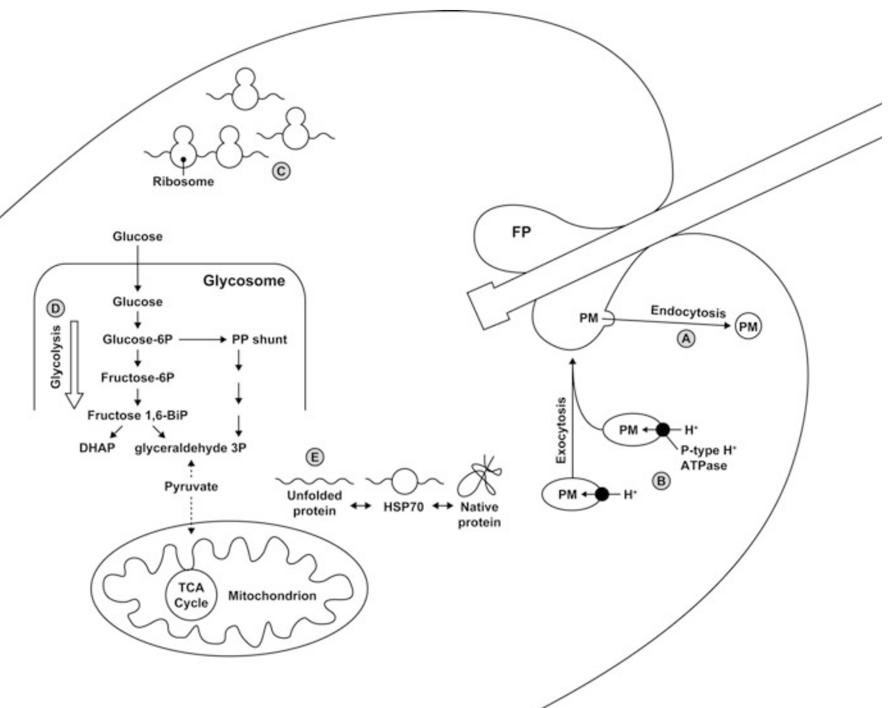

Fig. 3 Proposed resistance mechanisms to paromomycin in *Leishmania*. **(a)** The drug paromomycin (PM) is probably taken up by the parasite by endocytosis **(a)**. PM is then sequestered into vacuoles. The pump vacuole ATPase is upregulated and the number of vacuoles is increased in resistant parasites **(b)**. A number of ribosomal protein subunits are upregulated in PM-resistant parasites increasing the overall protein synthesis **(c)**. The glycolytic pathway is also upregulated in resistant parasites **(d)**. The chaperone proteins are found upregulated in resistant cells as a result of the stress caused by PM, and these proteins are also involved in protein turnover **(e)**. FP, flagellar pocket. This schematic was inspired from Chawla et al. (2011)

proteins, suggesting that PM also affects translation by targeting these ribosomal proteins (Chawla et al. 2011) (Fig. 3c), although the exact molecular mechanistic remains to be elucidated (Fernandez et al. 2011). Elevated levels of glycolytic enzymes were also detected in the PM-resistant strain, indicating that the resistant strain heavily relies on glycolysis (either aerobic or anaerobic) for its energy requirement (Fig. 3d). An increase in the basal levels of a number of stress protein parts of the HSP70 family was also observed in resistant parasites when compared to the parental sensitive strain (Fig. 3e). All together these results are suggesting that once PM is internalized by endocytosis, a process facilitated by a number of parasite cell surface proteins, it is probably sequestered in vacuole compartments that are most likely exocytosed at the flagellar pocket, thus conferring resistance. The sequestering hypothesis remains however to be formally addressed. Thus, modulation of translation rate, interaction with vesicle-mediated trafficking, an increase in energetic metabolism through glycolysis, and an effective protection of important

key players in resistance and survival by chaperone/stress-related proteins are all important features of PM resistance in *Leishmania*.

Miltefosine

Miltefosine (MIL, under the brand name Impavido®) is an alkylphosphocholine initially developed as an anticancer drug. It is the latest antileishmanial agent to reach the market and currently the only oral treatment commercially available, although oral formulations of AMB (as described above) and sitamaquine (a primaquine analogue) are currently under development. MIL is a well-tolerated and effective oral treatment of VL with only minor side effects. It is also highly effective in antimony-resistant cases but cannot be given to women of childbearing age due to a substantial risk of birth defects or fetal malformation. This antileishmanial agent has been licensed in India and Nepal for at least 5 years now, but its use is not under strict control. In addition, since MIL has a long half-life (between 150 and 200 h) (Bryceson 2001), the emergence of resistance remains a concern for public health authorities in these countries. Consequently, after only 5 years, the failure rate for MIL has increased in certain endemic regions (Rijal et al. 2013; Sundar et al. 2012). Recent data would suggest that treatment failure is not associated with resistant parasites, however (Rijal et al. 2013).

Drug uptake is a prerequisite for MIL activity against *Leishmania* which is reputed to act principally by perturbing the metabolism of lipids and especially phospholipids (Imbert et al. 2012). Inhibition of cytochrome c oxidase activity and mitochondrial depolarization resulting to an apoptosis/PCD-like death have also been reported (Luque-Ortega and Rivas 2007). Since experimental resistance to MIL is very easily achieved (Perez-Victoria et al. 2006b) and only cross-resistance (and not primary resistance) to MIL has been observed in clinical isolates from VL patients resistant to Sb^V (Kumar et al. 2009; Vergnes et al. 2007), MIL resistance mechanisms have been only studied in in vitro-generated resistant strains to date. A common feature in all MIL-resistant lines is a decreased drug accumulation. This is achieved by a decrease in uptake (Fig. 4a) and/or an increase in efflux (Fig. 4b). The MIL uptake machinery is composed of 2 proteins, the miltefosine transporter MT (a member of the P4-ATPase subfamily) and its specific beta subunit ROS3 (reviewed in Perez-Victoria et al. 2006b). Both are essential for MIL uptake at the parasite cell surface, and any mutations inactivating or decreasing the expression of any of these 2 components render the parasite cells highly resistant to MIL (Perez-Victoria et al. 2003, 2006a). Interestingly, the resistance phenotype seems stable in in vitro macrophages infected with MT-null mutant and MT-mutated parasites (Seifert et al. 2007), suggesting that what is observed in MIL-resistant promastigotes might be applied to intracellular amastigotes. Moreover, several mutations in MT can lead to resistance, and within a resistant population, they may be several different MT genotypes, leading to resistance (Coelho et al. 2012).

Decreasing MIL uptake (Fig. 4a) seems to be the easiest way for *Leishmania* to develop high levels of MIL resistance, but if a certain amount of MIL manages to

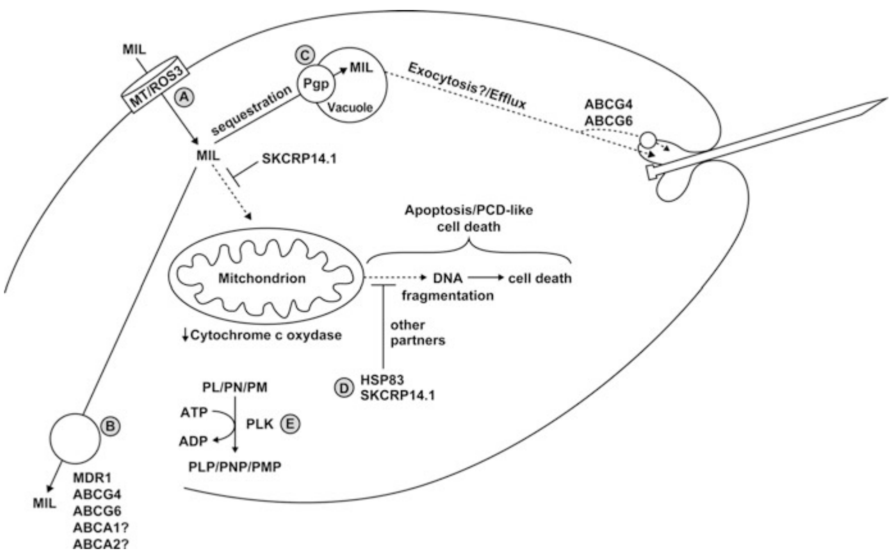

Fig. 4 **Resistance mechanisms to miltefosine in *Leishmania***. Miltefosine interacts either directly or indirectly with the mitochondrion, eventually leading to its depolarization. Point mutations in the miltefosine translocation machinery MT/ROS3 have been detected in resistant mutants **(a)**. Changes in the expression level of a number of ABC transporters involved either in the sequestration or efflux (MDR1/PGP, ABCG4, ABCG6) of miltefosine out of the parasite cells have been incriminated in the resistance phenotype **(b** and **c)**. Other factors such as the HSP83 and the calpain-like protein SKCRP14.1 may help to confer miltefosine resistance by interfering with the parasite apoptosis/programmed cell-like death pathway (PCD) **(d)**. The SKCRP14.1 protein also protects against miltefosine-induced PCD by preventing depolarization of the mitochondrion. The enzyme pyridoxal kinase (PLK) catalyzes the addition of phosphate from ATP to the 5′ alcohol group of pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PM) to form, respectively, pyridoxal-5-phosphate (PLP), pyridoxine-5-phosphate (PNP), and pyridoxamine-5-phosphate (PMP) **(e)**. Mutations in PLK were discovered in a number of miltefosine-resistant mutants selected in vitro, and transfection of the WT PLK version in mutants has shown that this enzyme plays a role in miltefosine resistance

enter the parasite cells, an increase in efflux (Fig. 4b) or in sequestration (Fig. 4c) may also lead to in vitro MIL resistance. The parasite protein MDR1, a P-glycoprotein-like transporter part of the *Leishmania* ABC family, was the first molecule shown to be involved in in vitro MIL resistance (Perez-Victoria et al. 2001). This plasma membrane transporter pumps xenobiotic drugs including MIL out of the parasite, thereby decreasing their intracellular concentration. Two members of the ABCG subfamily were also reported to be involved in MIL resistance in *Leishmania*, namely, ABCG4 and ABCG6, whose localization is mainly to the parasite plasma membrane and flagellar pocket (BoseDasgupta et al. 2008; Castanys-Munoz et al. 2007, 2008) (Fig. 4). Interestingly, both genes were also shown to be involved in sitamaquine resistance (Castanys-Munoz et al. 2007, 2008) due to rapid efflux at the plasma membrane. The physiological roles of ABCG4 and ABCG6 remain to be established, but these two

half-transporters were reported to be involved in phospholipid trafficking at the plasma membrane (trans-bilayer lipid movement) as they were shown to reduce the accumulation of short-chain phospholipid analogues when overexpressed (Castanys-Munoz et al. 2008). Since ABCG4 and ABCG6 show similar spectra of activity (i.e., similar substrates) and both proteins are half ABC transporters co-localizing mainly at the plasma membrane, it was speculated that these two ABC proteins may not only homodimerize but may heterodimerize together to confer MIL resistance or to extend the substrate specificity of each individual homodimer (Castanys-Munoz et al. 2008). Along with MDR1, ABCG4, and ABCG6, it is not excluded that other ABC proteins (ABCA1, ABCA2) might also contribute to MIL resistance (Fig. 4b). Finally, other proteins involved in experimental MIL resistance in *Leishmania* include the heat shock protein HSP83 and a calpain-like protein (SKCRP14.1) that both could protect against programmed cell death (PCD) induced by MIL (Vergnes et al. 2007) (Fig. 4d). Finally, the sequencing of the genome of miltefosine-resistant mutants pinpointed a mutation in the pyridoxal kinase (*PLK*) gene (Fig. 4e), and the role of this gene in MIL resistance was confirmed by gene transfection experiments (Coelho et al. 2012).

Resistance Reversal Agents

The concept of using a compound able to reverse resistance in refractory cases of leishmaniasis due to parasite-acquired resistance was inspired from what has been achieved in tumor cells. Multidrug resistance (MDR) is a main impediment to successful anticancer chemotherapy and is mediated, for instance, by the P-glycoprotein (MDR1/ABCB1/Pgp) and/or the multidrug resistance protein 1 (MRP1/ABCC1), two ATP-binding cassette (ABC) transporters, that either efflux or sequester drugs. Classical MDR was later associated with other ABC proteins including MDR2/ABCB4, cMOAT/MRP2/ABCC2, BCRP/ABCG2, and more recently MRP7/ABCC10 (Sun et al. 2013). In an attempt to overcome MDR in tumor cells, specific Pgp/MRP inhibitors have been developed with the rationale that blocking the action of these pumps will result in an increased net accumulation of drugs and thus a greater clinical efficacy of chemotherapeutic agents in tumors overexpressing these pumps. Unfortunately, after more than four decades of intensive efforts, there is no MDR reversal drug that has yet been approved by the US Food and Drug Administration (FDA). Nonetheless, third-generation lead compounds with less toxic effects and better pharmacokinetic interactions are currently being tested in advanced clinical studies (Kelly et al. 2012; Robey et al. 2008). Similar to tumor cells, reversal agents were assayed in *Leishmania* cell lines resistant to various therapeutic agents (reviewed in Pradines 2013). It has been shown that sitamaquine reversed miltefosine resistance in an MDR *L. tropica* line that overexpressed MDR1 (Perez-Victoria et al. 2011). The same study demonstrated that sitamaquine was also able to modulate antimony resistance mediated by MRPA/ABCC3, another key player in experimental and clinical antimony resistance in this parasite. If sitamaquine pass clinical trials with success, combination therapy with

miltefosine or antimony may represent a promising strategy to avoid the appearance of resistance mediated by ABC proteins in *Leishmania*. Recently, new natural or synthetic sesquiterpenes, flavonoids, acridone carboxamide derivatives (zosuquidar and elacridar), or phenothiazines were shown to revert the resistance phenotype in *Leishmania* to sodium stibogluconate and miltefosine by modulating intracellular drug concentrations (reviewed in Pradines 2013). These compounds may also represent promising modulators of Pgp-/MRP-mediated resistance in *Leishmania*.

Innovative Strategies to Combat Drug Resistance

The WHO has enlisted leishmaniasis to be eliminated in the Indian continent by 2015, which is in less than two years (Gupta et al. 2013). As stated before, the unavailability of a vaccine in clinical use and the widespread emergence of resistance in India to the most effective and affordable drugs against leishmaniasis, pentavalent antimonials, constitute major obstacles in achieving this goal. A number of therapeutic options are in the pipeline however (Table 1), but the large pharmas are not necessarily prioritizing research in neglected diseases including leishmaniasis. Innovative ways coming from academia-industry partnerships are thus more than needed to tackle this deadly parasite and to control the emergence of resistance to the limited number of drugs still active against the parasite.

Studies of in vitro resistance mechanisms in the 1980s and 1990s have shown that one frequent road to achieve resistance in *Leishmania* is gene amplification (e.g., amplification of target or efflux pump genes). These studies demonstrated that amplified DNAs can be either circular or linear with their formation mediated through homologous recombination between direct or inverted repeats of ~400–1000 bp in length. Homologous recombination (HR) between directly repeated homologous sequences leads to circular amplicons, whereas the annealing of inverted homologous repeats leads to either circular or linear amplicons. No further mechanistic insights related to gene amplification in *Leishmania* have emerged in the last decade except for the important observation that gene amplification was also detected in several clinical isolates resistant to chemotherapeutic drugs (Mittal et al. 2007; Singh et al. 2003). Resistance through point mutations (e.g., in drug importer genes such as in the miltefosine transporter MT/ROS3) has also been described but seems rare in the field. Recently, loss of function associated with drug resistance was found to be mediated by gene deletions that also occurred by HR between directly repeated sequences (Mukherjee et al. 2013a). Since *Leishmania* lacks transcriptional control at initiation, there is apparently no easy way for this pathogen to increase (or decrease) the expression of one specific gene except by gene amplification (or deletion). Thus, one can speculate that any molecules or compounds interfering with gene amplification events or HR in *Leishmania* may represent a valuable and innovative strategy to limit the development of resistance in this parasite. We are pursuing this exciting working hypothesis in the hope to develop more effective medicines against *Leishmania*. This type of molecules

might be used in combination with current drugs to circumvent or prevent the emergence of drug resistance.

Apart targeting amplification and/or deletion molecular events for limiting the development of resistance in the parasite *Leishmania*, we are also evaluating the peculiar mode of gene expression in *Leishmania* as a valuable therapeutic target and innovative strategy to limit the development of drug resistance in this parasite. More precisely, we are looking at genome signatures or nucleic acid 3D structures encoding signals potentially involved in replication or translation control in the parasite. It is well known that *Leishmania* displays unique features regarding genome organization (no cluster of functional genes) and control of gene expression. Indeed, its ~8,300 genes (of which only ~40 % have a putative assigned function) are organized into unidirectional gene clusters comprising up to 100 functionally unrelated genes that are co-transcribed in the absence of typical polII promoters into long polycistronic primary transcripts. Individual mRNAs are produced from polycistronic molecules by two posttranscriptional RNA-processing reactions, namely, *trans*-splicing and 3′-cleavage/polyadenylation, to generate mature monocistronic mRNAs. Thus, the regulation of gene expression in *Leishmania* occurs almost exclusively at the posttranscriptional level, and sequences within 3′-untranslated regions have been shown to play a key role in controlling either the stability of mRNAs or the efficiency of their translation (reviewed in Requena 2011). In our quest of understanding the regulation of gene/protein expression in *Leishmania*, we have recently discovered numerous G-DNA structures (G4) spread between genes and in telomeres of the parasite's genome (unpublished results). In mammals, there is now compelling evidence for functions of some G4 motifs in essential processes including initiation of DNA replication, telomere maintenance, regulated recombination events, control of gene expression, and genetic and epigenetic instability (Maizels and Gray 2013). Since the control of gene expression in *Leishmania* appears to be posttranscriptional, our hypothesis is that G-structures present in mature monocistronic mRNAs will, upon binding or release of specific factors/proteins, respectively block or allow the translation of mRNAs by ribosomes. This hypothesis may be tested with the newly established "ribosome profiling" methodology (Ingolia et al. 2012) which correlates ribosome occupancy on mRNAs with relative mRNA translation efficiencies. The design of small-molecule compounds able to interact with specific G4-structures and block the translation of mRNA encoding essential proteins or proteins involved in resistance pathways may lead to new alternatives in the treatment and control of leishmaniasis.

Conclusion

Antimicrobial resistance is one of the key health challenges of the twenty-first century. Globally, the threats of resistance are increasing at an alarming rate. Resistance in *Leishmania* parasites has been slow to emerge but is now encountered

more frequently in endemic areas including northeastern India where the incidence of kala-azar is highest. With the limited armamentarium of drugs in clinical use, we need to preserve the efficacy of the ones still active against this deadly parasite. Combination therapy, better compliance of patients, good practices in drug supply, and hopefully the development of effective reversal agents should expand the lifespan of existing medicines and slow the spread of drug resistance, but the development of novel antileishmanial medicines remains a priority. The plant kingdom and anticancer drugs are providing an impressive series of medicinal compounds with antileishmanial activities that still need to be tested in clinical trials. The licensing of an effective vaccine for human usage along with more effective strategies for point-of-care diagnostic will be also important milestones that will impact the control and management of leishmaniasis. Concerted efforts are thus required from the whole scientific community in partnership with the pharmaceutical industry.

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Part IV

Mycology

Microevolution of Antifungal Drug Resistance

Leah E. Cowen

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Abstract

The evolution of drug resistance in fungal pathogens threatens the utility of the limited number of antifungal drugs available to treat life-threatening invasive fungal infections. Fungal infections are on the rise with the increasing populations of individuals with impaired immune function who are most vulnerable to the opportunistic pathogens. Most antifungal drugs target the distinct composition of

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the fungal cell membrane (azoles and polyenes) or the fungal cell wall (echinocandins). Mortality rates associated with invasive fungal infections often exceed 50 % even with current treatment options, demanding new strategies to minimize the evolution of resistance and reverse it once it has evolved. This chapter focuses on the evolution of drug resistance in the leading human fungal pathogens, including species of *Candida*, *Aspergillus*, and *Cryptococcus*. The emphasis is on the microevolution of drug resistance that occurs on timescales relevant to experimental populations and human hosts receiving treatment. The relevant agents of selection are introduced, followed by a discussion of the diversity in drug resistance phenotypes, the scope of adaptive mechanisms, the fitness consequences of drug resistance, and strategies to thwart the evolution of drug resistance.

Keywords

Antifungal drug resistance • Fungal pathogen • Azole • Echinocandin • Stress response • Hsp-90 • Fitness • Experimental evolution • Microevolution • *Candida* • *Cryptococcus* • *Aspergillus*

Introduction

The emergence of drug resistance provides one of the most poignant examples of microevolution with a severe impact on human health. It is a ubiquitous process in nature, as microbes produce a dazzling array of small molecules that exert selection for resistance in neighboring microbial communities (Allen et al. 2010; Wright 2007, 2012). The rate at which resistance evolves has been accelerated by the widespread use of antimicrobial agents in medicine and agriculture, which selects for organisms capable of survival and reproduction despite drug exposure (Anderson 2005; Davies and Davies 2010). As a consequence, the rapid emergence of drug resistance in diverse pathogen populations now threatens the utility of many of the drugs that we critically depend on. The annual economic cost of the evolution of drug resistance is staggering, and in the United States alone, it is in excess of $33 billion to cover treatment of patients with drug-resistant infections, to manage resistant plant pathogens, and to compensate for crop loss to resistant pests (Palumbi 2001).

The evolution of drug resistance is an acute problem in the context of fungal pathogens. Fungal pathogens are eukaryotes and closely related to their human hosts, which limits the number of drug targets that can be selectively inhibited in the pathogen (Cowen 2008). Consequently, there are a limited number of clinically useful antifungal drugs to treat the increasing incidence of fungal infections worldwide. The global impact of fungal pathogens on human health remains largely unappreciated. They can cause life-threatening invasive infections in immunocompromised individuals, as well as in healthy hosts (Pfaller and Diekema 2004, 2010). The incidence of fungal bloodstream infections has increased by 207 % in recent decades, along with the growing population of individuals with impaired immune function, such as those undergoing immunosuppressive therapy for transplants or chemotherapy for cancer, as well as those infected with HIV (Martin et al. 2003;

Pfaller and Diekema 2007). Despite the latest therapeutic options, invasive fungal infections are associated with mortality rates in excess of 50 %, and fungal pathogens kill as many people as tuberculosis or malaria worldwide (Brown et al. 2012a, b). The majority of all deaths due to fungal infection are due to species of *Candida*, *Aspergillus*, and *Cryptococcus*.

Here, we focus on the evolution of drug resistance in the leading fungal pathogens of humans. We explore the microevolution of drug resistance as it operates on timescales that can be observed in experimental populations or in human hosts undergoing treatment. We introduce the relevant agents of selection, diversity in drug resistance phenotypes, adaptive mechanisms, fitness consequences, and strategies to thwart the evolution of drug resistance.

Agents of Selection

Antifungal drug targets must be sufficiently divergent between fungal pathogens and their human hosts to enable selective inhibition in the pathogen, thereby minimizing host toxicity. The majority of clinically relevant antifungal drugs exploit the distinct plasma membrane composition of fungi and target ergosterol or its biosynthesis (Fig. 1). Ergosterol is analogous to cholesterol in mammals, and serves to modulate membrane integrity and fluidity, and the function of enzymes; anchored in the membrane (Ostrosky-Zeichner et al. 2010; Shapiro et al. 2011). The fungal cell wall provides other attractive antifungal drug targets (Fig. 1), as it is not conserved in mammals. Fungal cell walls are rigid structures composed of covalent linkages of (1,3)-β-D-glucans with (1,6)-β-D-glucans and chitin (Netea et al. 2008; Tada et al. 2013). Here, we focus on the antifungal drugs currently employed to treat the most prevalent fungal infections.

Azoles

The azoles have been the most broadly and extensively used class of antifungal drug in recent decades. They include both imidazoles and triazoles and are five-membered, nitrogen-containing, heterocyclic compounds that inhibit ergosterol biosynthesis (Cowen and Steinbach 2008; Odds et al. 2003). Imidazoles are restricted to topical formulations due to toxicity and bioavailability issues, while there are currently four triazoles available in oral or injection formats: fluconazole, itraconazole, voriconazole, and posaconazole, each with distinct pharmacokinetic properties. Isavuconazole is an additional triazole in clinical development. The azoles enter the fungal cell by facilitated diffusion (Mansfield et al. 2010) and inhibit the ergosterol biosynthetic enzyme lanosterol demethylase (a cytochrome P450), encoded by *ERG11* in *Candida* and *Cryptococcus* and by *cyp51A* and *cyp51B* in *Aspergillus*. In addition to blocking ergosterol biosynthesis, azoles cause accumulation of 14 α-methyl-3,6-diol, a toxic sterol produced by the Δ-5,6-desaturase encoded by *ERG3* (Shapiro et al. 2011). Azoles are effective against diverse fungi, including *Candida*, *Cryptococcus*, and

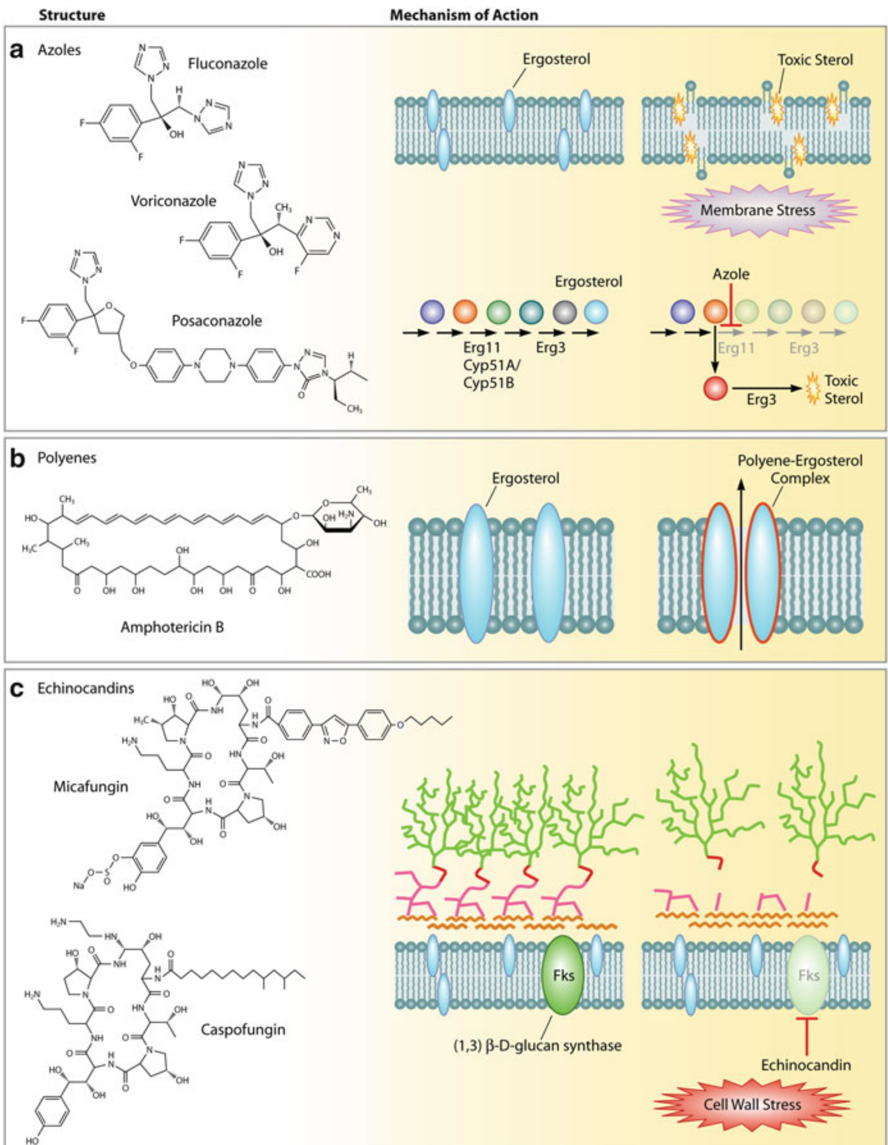

Fig. 1 Antifungal drugs and their targets. (**a**) The azoles inhibit the ergosterol biosynthesis enzyme lanosterol demethylase, which is encoded by *ERG11* in *Candida albicans* and *Cryptococcus neoformans* or by *cyp51A* and *cyp51B* in *Aspergillus fumigatus*, thereby blocking ergosterol production and leading to the accumulation of a toxic sterol produced by Erg3. (**b**) The polyenes bind to ergosterol creating drug-lipid complexes that intercalate into fungal cell membranes to form a membrane-spanning channel that causes leakage of cellular ions, destruction of the proton gradient, and ultimately osmotic cellular lysis. (**c**) The echinocandins inhibit (1,3)-β-D-glucan synthase, which is encoded by *FKS1* in *C. albicans*, *C. neoformans*, and *A. fumigatus* and by *FKS1* and *FKS2* in *Candida glabrata* and *Saccharomyces cerevisiae* (Reproduced by permission from Copyright © American Society for Microbiology [Microbiology and Molecular Biology Reviews, 75, 2011, 213–67 and ► 10.1128/MMBR.00045-10] (Shapiro et al. 2011))

Aspergillus species, as well as dimorphic fungi and dermatophytes. Posaconazole also has efficacy against zygomycetes (Alastruey-Izquierdo et al. 2009). The azoles typically exert fungistatic activity against yeasts, such as *Candida* species, and fungicidal activity against molds, such as *Aspergillus*. The fungistatic activity against yeasts generates strong selection on the surviving population that facilitates the evolution of azole resistance in the laboratory and in the clinic.

Polyenes

The polyenes have been in clinical use for over 50 years. These amphipathic drugs have both hydrophobic and hydrophilic moieties and exert fungicidal activity by binding to ergosterol to form drug-lipid complexes that intercalate into the cell membrane (Shapiro et al. 2011; Odds et al. 2003; Gruszecki et al. 2003). The resulting pores that span the membrane cause leakage of ions and destruction of the proton gradient. Nystatin is a commonly used polyene for superficial infections, and amphotericin B is a polyene that is effective against systemic fungal infections caused by species of *Candida*, *Cryptococcus*, and *Aspergillus*. The major limitation of polyenes is host toxicity, including renal dysfunction, which is likely a consequence of structural similarities between ergosterol and cholesterol (Fanos and Cataldi 2000). Toxicity can be mitigated by lipid-complexed formulations that can enhance fungal selectivity (Cifani et al. 2012). Polyene resistance has emerged in clinical cases, although it is not widespread, perhaps due to fitness costs of resistance mutations (Vincent et al. 2013).

Echinocandins

The echinocandins are the only new class of antifungal drug to enter clinical use in recent decades. There are currently three echinocandins on the market: caspofungin, micafungin, and anidulafungin. These large lipopeptide molecules are noncompetitive inhibitors of the fungal cell wall biosynthetic enzyme (1,3)-β-D-glucan synthase, leading to loss of cell wall integrity (Denning 2003; Turner et al. 2006). The echinocandins typically exert fungicidal activity against yeasts such as *Candida* species and fungistatic activity against molds such as *Aspergillus* species. Notably, echinocandins are not effective against *Cryptococcus neoformans* (Denning 2003). The echinocandins have little host toxicity and are the drug of choice for treatment of azole-resistant invasive fungal infections. Despite their relatively short history of clinical use, resistance has already begun to emerge in the clinic.

Other Antifungal Drugs

There are several additional antifungal drugs currently in clinical use or in development (Ostrosky-Zeichner et al. 2010; Pitman et al. 2011). The long-standing antifungal drug 5-flucytosine inhibits fungal nucleic acid biosynthesis; its efficacy is

restricted by fungistatic activity and by the rapid emergence of resistance, and thus it is typically only prescribed in combinations with other antifungal drugs. Antifungals in development include sordarins and nikkomycin Z. Sordarins are semisynthetic natural products that inhibit protein biosynthesis by targeting fungal elongation factor 2. Nikkomycin Z impairs cell wall biosynthesis by competitively inhibiting chitin synthases.

Diversity in Drug Resistance

The emergence of antifungal drug resistance can manifest in a multitude of different forms. Antifungal drug resistance itself can be defined in distinct ways in clinical and laboratory contexts. In the clinical context, drug resistance is the persistence or progression of infection despite appropriate antifungal treatment (White et al. 1998). In the laboratory context, drug resistance is a continuous trait that is quantified using susceptibility assays, in which fungal growth is measured across a series of drug concentrations (Shapiro et al. 2011). The minimum inhibitory concentration (MIC) is the drug concentration that inhibits fungal growth by a defined amount, most often either 50 % or 90 %. There is extensive variation in response to antifungal drugs between fungal species, as well as between strains of the same species and even between cells of a single strain (Hill et al. 2012). Fungi can differ in their inherent capacity to survive antifungal drug exposure, referred to as basal tolerance when the drug has activity against the relevant fungal species (Shapiro et al. 2011). Tolerance can facilitate the evolution of drug resistance by enabling surviving cells to respond to selection and acquire resistance mutations. Here we focus on variation in antifungal drug resistance.

Perhaps most fascinating from the perspective of microevolution of antifungal drug resistance is variation within a population. In contrast to the largely stable diversity in resistance phenotypes observed between strains and species, variation within a population of cells is often more transient in nature. One intriguing example of variation in azole resistance in *Cryptococcus* is heteroresistance, which is the emergence of both azole-resistant and azole-susceptible cells from a susceptible progenitor (Sionov et al. 2009). In this case, resistance increases incrementally and is lost with passage in the absence of drug. The resistant cells tend to be disomic for chromosome 1, which harbors genes important for resistance such as those encoding the target of the azoles, *ERG11*, and an azole efflux transporter, *AFR1* (Sionov et al. 2010). The phenomenon of heteroresistance has also been reported for *Candida albicans* (Marr et al. 2001).

Another source of diversity in drug resistance within a population is heterogeneous resistance, which is a broad term for a phenomenon in fungi and bacteria that involves a susceptible population giving rise to resistant cells at a frequency of ~10^{-1} – 10^{-4}; the distinguishing feature is that the resistant cells reproducibly generate the same distribution of resistance phenotypes among their progeny as the original susceptible parent (White and Oliver 2004). Such heterogeneous resistance has been observed in *Candida*, as has the related phenomenon of

high-frequency azole resistance in which some strains give rise to azole-resistant progeny at a frequency that is higher than the mutation rate (White and Oliver 2004). High-frequency azole resistance is associated with stable resistance phenotypes, which may be a result of altered mitochondrial function.

Diversity in drug resistance among genotypically identical cells is also prevalent in the clinic and in nature in the context of biofilms. Fungal biofilms are surface-associated communities that initiate upon adherence to specific surfaces such as plastics, catheters, and other medical devices (Blankenship and Mitchell 2006). Biofilms are surrounded by an extracellular matrix and are characterized by a level of antifungal drug resistance that vastly exceeds what is observed in their planktonic counterparts. Species of *Candida*, *Cryptococcus*, and *Aspergillus* all form drug-resistant biofilms that present a major clinical challenge (Ramage et al. 2009). There are numerous resistance mechanisms that operate in biofilms (d'Enfert 2006), but one of the most striking is the emergence of persister cells. Persister cells are phenotypic variants that can tolerate high drug concentrations (LaFleur et al. 2006). They arise from biofilms, which offer protection from the full impact of antifungal drugs and host immune response. While bacterial persisters are quiescent, the state of metabolic activity of fungal persisters remains unknown.

Adaptive Mechanisms

Antifungal drug resistance can evolve by numerous mechanisms including mutation or overexpression of the drug target, increased drug efflux, and activation of cellular stress responses (Fig. 2). High-level drug resistance observed in clinical isolates is often a consequence of the stepwise acquisition of multiple mechanisms, such as those discussed below.

Alteration of the Drug Target

A prevalent mechanism of resistance to diverse drugs is mutation of the drug target that impairs drug binding. In the context of azoles, numerous mutations in genes encoding the drug target (*ERG11* in *Candida* and *Cryptococcus* and *cyp51A* and *cyp51B* in *Aspergillus*) have been identified in mutational "hot spots" that cause amino acid substitutions in or adjacent to the active site (Alcazar-Fuoli et al. 2011; MacCallum et al. 2010; Pfaller 2012; Sionov et al. 2012; Lamb et al. 1997). These mutations are thought to reduce the azole binding affinity of the target lanosterol demethylase, thereby causing increased azole resistance. Increased dosage of the mutant allele is associated with elevated resistance, as is observed with loss of heterozygosity (White 1997a). In the context of echinocandins, the best characterized mechanism of resistance is mutations in hot spots of the genes *FKS1* and *FKS2*, which encode the catalytic subunit of the target glucan synthase (Perlin 2007). These mutations reduce sensitivity of the enzyme to inhibition by echinocandins

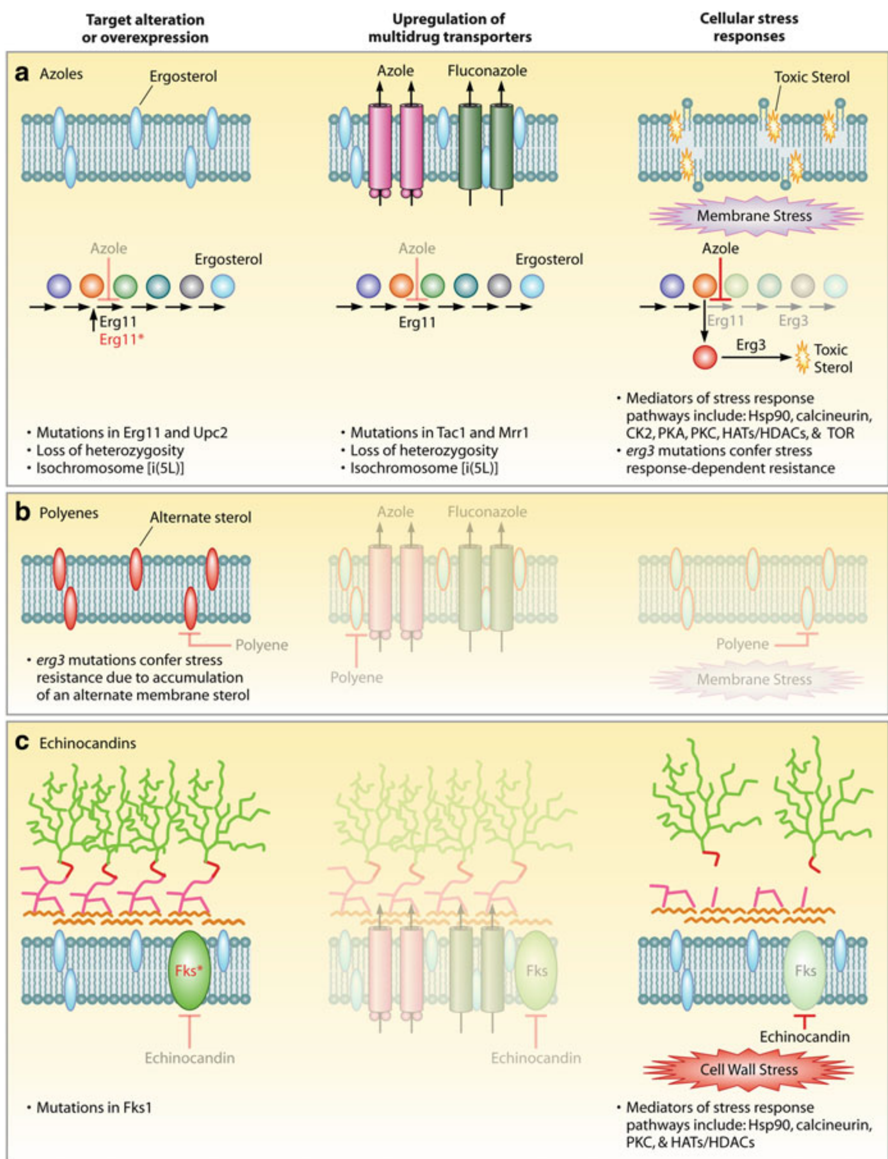

Fig. 2 Drug resistance mechanisms of *C. albicans*. (**a**) Resistance to azoles can arise by multiple mechanisms including overexpression or alteration of the drug target Erg11 and overexpression of the multidrug efflux transporters Cdr1, Cdr2, or Mdr1 or by modulation of cellular stress responses. (**b**) Resistance to polyenes can be caused by loss of function of Erg3, which blocks the production of ergosterol, thereby inhibiting the formation of the drug-lipid complex that causes osmotic cellular lysis. Drug transporters do not have a major impact on polyene resistance, and stress responses have not been identified as key resistance determinants. (**c**) Resistance to echinocandins is most often due to mutations in two hot-spot regions of *FKS1*. Drug transporters do have a major impact on resistance, although cellular stress responses do. Bright images indicate important mechanisms of

(Garcia-Effron et al. 2009), and their phenotypic effect is amplified with increased dosage of the resistance alleles (Niimi et al. 2010).

Overexpression of the Drug Target

Elevated resistance can also result from increased drug target expression. This has been studied extensively in *C. albicans*, where overexpression of *ERG11* can be attributed to gain-of-function mutations in the transcription factor encoded by *UPC*2, which controls expression of ergosterol biosynthesis genes (Dunkel et al. 2008a; Hoot et al. 2011). Mutations in the C-terminal region *UPC2* are associated with azole resistance, which is amplified with increased dosage of the mutant allele (Dunkel et al. 2008a; Hoot et al. 2011; Heilmann et al. 2010). In *Candida glabrata* there are two *UPC2* homologs, which influence azole susceptibility (Nagi et al. 2011). In many other Ascomycetes and in Basidiomycetes such as *C. neoformans*, transcriptional control of ergosterol biosynthesis genes is regulated by an SREBP-like transcription factor Sre1 (Bien and Espenshade 2010), which has functional homology to the mammalian cholesterol regulatory transcription factor SREBP. In *Aspergillus fumigatus*, SrbA is the SREBP homolog that controls sterol biosynthesis (Willger et al. 2008). SrbA influences cellular responses to azoles, as do genes implicated in SrbA regulation (Willger et al. 2008, 2012). The SREBP-like proteins of *C. neoformans* and *A. fumigatus* influence not only sterol biosynthesis but are also implicated in virulence in animal models.

Increased Drug Efflux

A pervasive mechanism of drug resistance is overexpression of drug efflux transporters leading to reduced intracellular drug concentration. In terms of antifungal drugs, increased efflux has the greatest impact on resistance to azoles (Cannon et al. 2009; Morschhauser 2010). In *C. albicans*, the ATP-dependent transporters Cdr1 and Cdr2 and the major facilitator Mdr1 are key for azole efflux. Transcriptional upregulation of *CDR1* and *CDR2* can be achieved by gain-of-function mutations in the transcription factor encoded by *TAC1*; such mutations are often homozygous in azole-resistant clinical isolates (Coste et al. 2006, 2004). Activation of *CDR1* transcription is also influenced by the transcription factor Ndt80 (Wang et al. 2006), while *MDR1* expression is controlled by the transcription factor Mrr1 (Dunkel et al. 2008b). Considerable advances have been made in understanding the

Fig. 2 (continued) resistance, while dimmed images are those mechanisms that have little impact on resistance to the relevant drug class (Reproduced by permission from Copyright © American Society for Microbiology [Microbiology and Molecular Biology Reviews, 75, 2011, 213–67 and ▸ 10.1128/MMBR.00045-10] (Shapiro et al. 2011))

circuitry through which transcriptional control of drug efflux transporters is achieved in *C. albicans* (Mogavero et al. 2011; Sasse et al. 2012a; Schubert et al. 2011; Dhamgaye et al. 2012; Shukla et al. 2011).

Increased efflux of antifungal drugs is also a prevalent resistance mechanism in other fungi. In *C. neoformans*, reduced azole susceptibility can be attributed to overexpression of the efflux transporters encoded by *AFR1* and *MDR1* (Heilmann et al. 2010). In *A. fumigatus* overexpression of *atrF*, which encodes an ABC transporter, is associated with azole resistance (Sionov et al. 2010). Reduced intracellular azole accumulation may also arise from reduced drug import, as azoles enter fungal cells by facilitated diffusion and some azole-resistant clinical isolates have reduced azole import relative to the majority of susceptible isolates (Mansfield et al. 2010).

Genomic Plasticity

The emergence of antifungal drug resistance is associated with multiple genomic alterations, likely due to the resulting increase in dosage of relevant resistance determinants. *C. albicans* azole-resistant clinical isolates have an elevated incidence of aneuploidies, the most common of which involves chromosome 5. Recombination events at a breakpoint in repetitive sequences flanking the centromere generate an isochromosome with two left arms of chromosome 5, i5L (Selmecki et al. 2006). Azole resistance due to i5L is attributable to duplication of *ERG11* and *TAC1*, which are located on the left arm of chromosome 5 (Selmecki et al. 2008). Azole resistance in *C. neoformans* can also arise by duplication of chromosome 1, where the drug target gene *ERG11* and drug transporter gene *AFR1* reside (Sionov et al. 2010).

Activation of Cellular Stress Responses

Cellular stress response pathways have a profound impact on the evolution of antifungal drug resistance. Diverse signaling pathways are crucial for sensing and responding to the stress exerted by antifungal drug exposure (Cowen and Steinbach 2008; Cannon et al. 2007). One of the key examples of a global regulator of cellular stress responses required for basal tolerance and resistance to antifungal drugs is the molecular chaperone Hsp90. Inhibition of Hsp90 abrogates resistance to azoles and echinocandins in diverse fungi and can block the evolution of drug resistance (Cowen et al. 2006, 2009; Cowen and Lindquist 2005; Singh et al. 2009; Singh-Babak et al. 2012). The molecular details have been studied most extensively in *C. albicans*, where Hsp90 regulates drug resistance by stabilizing signal transducers including the protein phosphatase calcineurin and the terminal mitogen-activated protein kinase in the Pkc1-mediated cell wall integrity pathway, Mkc1 (Fig. 3) (Singh et al. 2009; LaFayette et al. 2010). Inhibition of Hsp90, calcineurin, or Pkc1 reduces drug resistance of *C. albicans* that evolved resistance in a human host (Singh et al. 2009; LaFayette et al. 2010; Cruz et al. 2002). Hsp90 function is

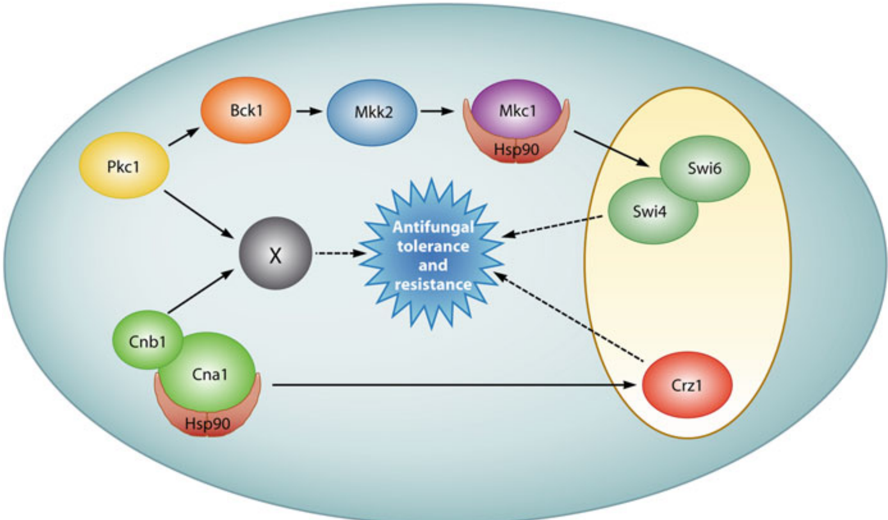

Fig. 3 Hsp90's role in *C. albicans* drug resistance. Schematic of the mechanisms by which Hsp90 regulates basal tolerance and resistance to antifungal drugs that target the cell membrane and the cell wall. Hsp90 physically interacts with and confers stability to the catalytic subunit of calcineurin (Cna1), thereby supporting stress responses mediated by calcineurin and its downstream effector protein Crz1 as well as through an additional target. Stress induced by antifungal drug exposure also activates signaling through the Pkc1 cell wall integrity pathway. Hsp90 stabilizes the terminal kinase Mkc1. Pkc1 also influences antifungal drug resistance and tolerance through a distinct pathway in common with calcineurin (Adapted by permission from Copyright © American Society for Microbiology [Microbiology and Molecular Biology Reviews, 75, 2011, 213–67 and ► 10.1128/MMBR.00045-10] (Shapiro et al. 2011))

itself regulated by acetylation such that hyperacetylation of Hsp90 compromises its function, thereby impairing the evolution of drug resistance and reversing resistance that had already evolved (Robbins et al. 2012). Targeting these cellular stress response pathways enhances antifungal efficacy in diverse models of infection including biofilm and systemic infections and attenuates virulence (Cowen et al. 2009; Singh et al. 2009; Singh-Babak et al. 2012; LaFayette et al. 2010; Robbins et al. 2011, 2012; Shapiro et al. 2009; Uppuluri et al. 2008; Sanglard et al. 2003; Steinbach et al. 2006, 2007a; Chen et al. 2011, 2012; Reedy et al. 2010). These signaling pathways are critical components of the cellular circuitry regulating the emergence and maintenance of drug resistance.

Evolution of Drug Resistance in the Human Host

The human host provides the most clinically relevant context to study microevolutionary processes by examining fungal specimens isolated from an infected individual over the course of treatment. Antifungal drug resistance can arise in a patient due to replacement of the susceptible strain with a resistant strain or species

(White et al. 1998). Resistance can also evolve as mutations accumulate in a susceptible population under drug selection, as is the focus of this section.

The evolution of drug resistance in the human host is most often accompanied by a stepwise increase in resistance due to the acquisition of multiple resistance mutations. A classic example is with a set of 17 *C. albicans* isolates collected from an HIV-infected patient over 2 years of azole treatment for recurrent oropharyngeal candidiasis (White 1997b). The progressive increase in drug resistance was accompanied by increased expression of the efflux transporter gene *MDR1*, mutations in the drug target gene *ERG11*, a mutation in *UPC2* causing transcriptional upregulation of *ERG11*, and ultimately a *TAC1* mutation leading to transcriptional upregulation of the drug efflux transporter genes *CDR1* and *CDR2* (White 1997a, b; Hoot et al. 2011; White et al. 1997). The first global analysis of mutations that accompany the evolution of antifungal drug resistance in a human host focused on a series of 7 *C. glabrata* isolates recovered over 10 months from a patient with Crohn's disease who received multiple rounds of echinocandin treatment for recurrent bloodstream candidemia (Singh-Babak et al. 2012). Whole genome sequencing revealed a mutation in the drug target gene *FKS2* that coincided with the largest increase in resistance, as well as 8 additional non-synonymous mutations (Fig. 4). A mutation in *CDC6*, whose product contributes to DNA replication initiation, was acquired at an earlier time point and was associated with a small increase in resistance (Singh-Babak et al. 2012). With advances in sequencing technologies, it is now feasible to sequence fungal genomes on a much larger scale to refine our appreciation of the dynamics of the evolution of antifungal drug resistance in the human host.

Experimental Evolution of Drug Resistance

A powerful complement to studying the evolution of drug resistance in the human host is the analysis of experimental populations that evolved under controlled laboratory conditions. This approach can overcome some of the limitations inherent to the analysis of clinical isolates, including the inability to control population parameters such as the genotype of the initial strain, the number of generations, the effective population size, and the strength of selection. Experimental evolution typically involves a population initiated from a single progenitor that is propagated for numerous generations, such that the evolution of new traits can be monitored and evolutionary intermediates can be archived (Elena and Lenski 2003). As a model for the evolution of drug resistance, this approach enables high levels of replication and fine-scale analysis of factors influencing the evolution of drug resistance. Despite the fact that experimental evolution does not capture complexities of evolution in the host where organisms face microenvironments, nutrient limitation, spatial structure, and competition with other pathogens, there is often a concordance of resistance mechanisms that evolve in the host and those that evolve in the test tube (Cowen et al. 2000; MacLean et al. 2010; Scully and Bidochka 2005).

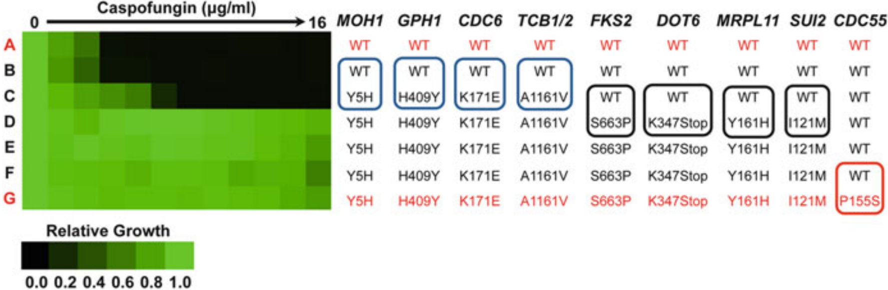

Fig. 4 The evolution of echinocandin resistance in *C. glabrata* clinical isolates is accompanied by multiple non-synonymous mutations. Isolates are arranged in the order they were recovered from the patient, with isolate A recovered prior to treatment and isolate G recovered after multiple rounds of treatment with the echinocandin caspofungin. Resistance increases in a stepwise manner. The nine non-synonymous mutations identified in isolate G compared to isolate A using whole genome sequencing were mapped in isolates B to F. Mutations in *MOH1*, *GPH1*, *CDC6*, and *TCB1/2* were identified between isolates B and C, accompanying a small increase in resistance (*blue outline*). Mutations in *DOT6*, *MRPL11*, *FKS2*, and *SUI2* were identified between isolates C and D, accompanying a sharp increase in echinocandin resistance (*black outline*). A mutation in *CDC55* was identified between isolates F and G (*red outline*) (Reproduced by permission from PLoS Pathogens, 8(5), 2012, e1002718. doi:10.1371/journal.ppat.1002718 (Singh-Babak et al. 2012))

The majority of experimental evolution studies of antifungal drug resistance have been performed with *C. albicans*. Evolutionary dynamics were monitored in replicate *C. albicans* populations propagated for 330 generations in the presence of fluconazole (Cowen et al. 2000). The populations evolved cross-resistance to multiple azoles following distinct trajectories with different expression levels of four resistance determinants: *ERG11*, *CDR1*, *CDR2*, and *MDR1*. Global analyses revealed three distinct gene expression profiles among the evolved lineages, profiles that were also observed among clinical isolates (Cowen et al. 2002). Aneuploidy was prevalent in the populations evolved with fluconazole, evidenced by the rapid emergence of i5L (Selmecki et al. 2006). Population dynamics can differ considerably in mouse models from test tubes, with increased genotypic and phenotypic variation in mouse-evolved strains (Forche et al. 2009), suggesting that infection models may provide an attractive system experimental evolution with more commonalities to the human host.

There have been a limited number of studies focused on experimental evolution of drug resistance in other human fungal pathogens. Replicate experimental populations of *A. fumigatus* evolved resistance to itraconazole by distinct mechanisms including overexpression of efflux pumps and mutations in the drug targets encoded by *cyp51A* and *cyp51B* (da Silva Ferreira et al. 2004). Multiple resistance mutations accumulated within populations, as is often the case with clinical isolates. Experimental evolution of *C. neoformans* in the presence of increasing concentrations of arsenite yielded highly resistant strains with amplification of a subtelomeric region that includes the arsenite efflux transporter encoded by *ARR3* to over 50 copies, accounting for up to ~1 % of the whole genome (Chow et al. 2012).

The model yeast *Saccharomyces cerevisiae* has provided a tractable system to study the mechanisms underpinning the evolution of antifungal drug resistance. In one study, the mode of selection was determined to have a profound impact on the mechanisms by which resistance emerges (Anderson et al. 2003). *S. cerevisiae* populations exposed to a single high dose of fluconazole evolved resistance by loss-of-function mutations in the ergosterol biosynthetic gene *ERG3*, while those propagated for 400 generations in the presence of increasing concentrations of azole acquired mutations in *PDR1* and *PDR3*, which encode transcription factors that control the expression of multidrug efflux transporters (Anderson et al. 2003). There is antagonism between the resistance mechanisms favored by the different selection regimes, as hybrids with both resistance determinants have impaired growth in the presence of fluconazole (Anderson et al. 2006). Experimental evolution studies with *S. cerevisiae* have also identified factors that enable the evolution of drug resistance and the impact of ploidy on adaptation. For example, deletion of *PDR16*, which is involved in lipid metabolism, caused extinction during selection with azoles (Anderson et al. 2009). Further, different ploidies were found to favor the emergence of distinct resistance mechanisms; recessive, loss-of-function mutations in *ERG3* were recovered in haploids, while mutations in *PDR1* and *PDR3* were favored in diploids as a result of the larger mutational target with twice the number of genes as haploids (Anderson et al. 2004).

Fitness Consequences of Drug Resistance

The ultimate fate of a drug-resistant pathogen is contingent on its fitness relative to its drug-sensitive counterparts. If resistance mutations are associated with a fitness cost that reduces growth and reproduction in the absence of drug, then discontinuing the use of the drug may allow susceptible isolates to outcompete those that are resistant. If there is no cost of resistance or if any cost is mitigated by the accumulation of compensatory mutations, then the resistant population will likely persist.

The fitness effects of resistance mutations in human fungal pathogens have been studied most extensively in *C. albicans*. Fitness of experimental populations that evolved azole resistance was measured by monitoring growth rates and by competing the evolved strains against their progenitor, in the presence and absence of azole. Some populations demonstrated no cost of resistance, while for others that had a fitness disadvantage in the absence of drug at earlier points, this cost was ameliorated with further evolution (Cowen et al. 2001). In some of these populations, the isochromosome i5L was associated with improved fitness both in the presence and absence of the drug (Selmecki et al. 2009). In contrast to populations that evolved azole resistance in response to drug selection, introduction of specific resistance mutations individually or in combination without the opportunity for compensatory evolution can be costly. This was investigated by introducing activating mutations in the *C. albicans* transcription factors genes *MRR1*, *TAC1*, and *UPC2* that control the expression of azole resistance determinants, individually and in combinations, and monitoring the impact on azole resistance and fitness (Sasse et al. 2012b). The multiple resistance mutations resulted in

an incremental increase in azole resistance that was associated with reduced fitness in the absence of drug in vitro as well as in a mammalian host (Sasse et al. 2012b). Together, this suggests that compensatory mutations are key for reducing the cost of azole resistance that may be associated with resistance mechanisms such as constitutive changes in gene expression.

Recent work has identified the premier compensatory mutation that mitigates the cost of antifungal drug resistance. The most well-characterized echinocandin resistance mechanism is amino acid substitutions in the target (1,3)-β-D-glucan synthase that reduce the sensitivity of the enzyme to echinocandins (Garcia-Effron et al. 2009). These substitutions also reduce catalytic capacity of the enzyme even in the absence of echinocandin, imparting a cost of resistance (Ben-Ami and Kontoyiannis 2012). In a *C. glabrata* lineage that evolved echinocandin resistance in a human host, the acquisition of resistance by mutation of the drug target gene was associated with a fitness cost that was ameliorated with further evolution in the host (Singh-Babak et al. 2012). Whole genome sequencing identified a mutation in *CDC55*, which encodes the regulatory subunit of protein phosphatase 2A, that conferred a fitness increase evident in vitro and in the host (Singh-Babak et al. 2012). Cdc55 is involved in meiotic spindle assembly, mitotic exit, pseudohyphal growth, and chromosome disjunction, though the mechanism by which it mitigates the cost of echinocandin resistance remains unknown. Other studies with *C. glabrata* have established that drug resistance mechanisms may not always be costly. For example, gain-of-function mutations in *C. glabrata* Pdr1 cause azole resistance due to upregulation of multidrug transporters and are also associated with increased virulence (Ferrari et al. 2011). Given that a cost to antifungal drug resistance is not ubiquitous and that compensatory evolution can readily overcome any cost of resistance, it is likely that once resistance has evolved it will remain a persistent problem.

Drug Combinations to Thwart the Evolution of Drug Resistance

The evolution of drug resistance far outpaces the development of new antifungal drugs, demanding new strategies to block the evolution of drug resistance and render existing antifungal drugs more effective. Drug combinations have emerged as a powerful therapeutic strategy to extend the life of current antimicrobial agents (Torella et al. 2010). They can impair the emergence of drug resistance by more effectively eliminating pathogen populations and by necessitating multiple mutations to confer resistance to both drugs simultaneously (zur Wiesch et al. 2011). Drug combinations can also enhance the efficacy of either agent and can create cidal combinations with static agents. Combination therapy is fundamental for the treatment of HIV (Martin et al. 2003; Pfaller and Diekema 2007; Brown et al. 2012a), and it is the recommended therapeutic strategy for tuberculosis and malaria (Brown et al. 2012b; Ostrosky-Zeichner et al. 2010).

Combination therapies have been less well explored in the clinic for fungal pathogens. One clinically relevant example of combination therapy for fungal

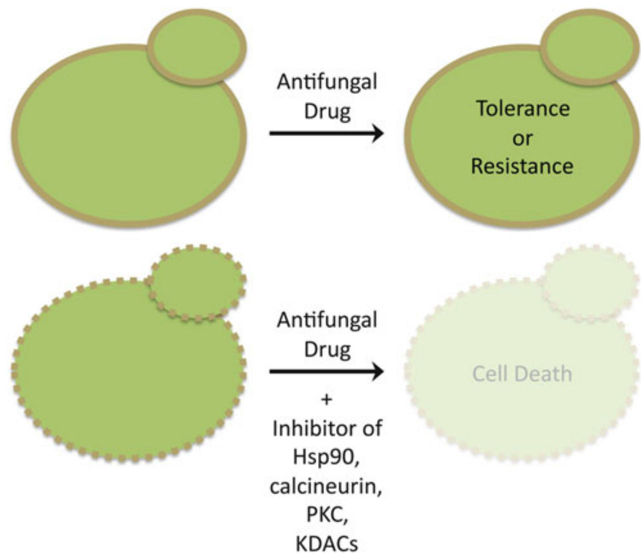

Fig. 5 Inhibition of cellular stress response regulators reduces basal tolerance and resistance to antifungal drugs such as azoles and echinocandins, transforms fungistatic agents to a fungicidal combination, and impairs the evolution of drug resistance. Examples of relevant inhibitors include those that target Hsp90, calcineurin, protein kinase C (PKC), and lysine deacetylases (KDACs)

infections is the treatment of patients with cryptococcal meningitis with the combination of 5-flucytosine and amphotericin B (Johnson and Perfect 2010). The rapid emergence of resistance to 5-flucytosine precludes its use as a single agent, though it has efficacy in combination with polyenes or azoles (White et al. 1998).

Targeting regulators of cellular stress responses has emerged as a promising strategy to enhance the efficacy of azoles and echinocandins and to minimize the emergence of resistance to these agents (Fig. 5). Multiple cellular signaling pathways are required for fungal pathogens to mount crucial responses to the cellular stress exerted by exposure to antifungal drugs. One of the most promising examples of a global regulator of cellular stress responses with broad antifungal potential is the molecular chaperone Hsp90. Hsp90 enables the evolution of drug resistance by stabilizing regulators of cellular stress responses and thereby allows for basal tolerance as well as the phenotypic effects of resistance mutations (Cowen 2008, 2009). In *S. cerevisiae* and *C. albicans*, compromise of Hsp90 function impairs the evolution of resistance to azoles and abrogates azole resistance that had already been acquired by diverse mutations (Cowen et al. 2006; Cowen and Lindquist 2005). Inhibiting *C. albicans* Hsp90 function also impairs biofilm growth, blocks biofilm dispersal, and abolishes biofilm azole resistance (Robbins et al. 2011). In *C. albicans*, *C. glabrata*, and *A. fumigatus*, Hsp90 inhibition also abrogates echinocandin resistance acquired by diverse mutations and renders echinocandins fungicidal against azole-resistant *A. fumigatus* strains (Cowen and Lindquist 2005; Cowen et al. 2009; Singh et al. 2009; Singh-Babak et al. 2012; Lamoth et al. 2012).

Hsp90 compromise also reduces echinocandin resistance of *A. fumigatus* biofilms (Robbins et al. 2011).

The therapeutic promise of targeting Hsp90 is corroborated by multiple infection models. Inhibiting Hsp90 with molecules that are well tolerated in humans and in clinical development for cancer transforms the therapeutic efficacy of azoles against *C. albicans*, rescuing infections that are otherwise lethal in the invertebrate *Galleria mellonella* (Cowen et al. 2009). Although these Hsp90 inhibitors are generally well tolerated in mice, their therapeutic utility is compromised by toxicity observed in the context of acute fungal infections (Cowen et al. 2009). Genetic reduction of *C. albicans* Hsp90 enhances the efficacy of azoles and echinocandins in a murine model of disseminated candidiasis, providing further proof-of-principle that Hsp90 is an attractive target for combination therapy (Cowen et al. 2009; Singh et al. 2009). Notably, Hsp90 inhibitors in clinical development transform azoles from largely ineffective to highly efficacious in a rat model of central venous catheter infection, where the infection and drug delivery remain more localized (Robbins et al. 2011). Current efforts focus on development of fungal-selective Hsp90 inhibitors for the treatment of systemic fungal infections.

Many of the other regulators of cellular stress responses that show promise for thwarting drug resistance are integral components of the Hsp90 chaperone network. The best example is the Hsp90 client protein calcineurin. Hsp90 physically interacts with and stabilizes the catalytic subunit of the protein phosphatase calcineurin; consequently, depletion of Hsp90 leads to depletion of calcineurin, thereby blocking calcineurin-mediated stress responses required for survival during the stress induced by antifungal drug exposure (Singh et al. 2009). Inhibition of calcineurin with agents used widely in the clinic as immunosuppressants, tacrolimus (FK506) or cyclosporin A, enhances the efficacy of azoles and echinocandins against diverse fungal pathogens and creates fungicidal combinations (Singh et al. 2009; Cruz et al. 2002; Sanglard et al. 2003; Lamoth et al. 2012; Onyewu et al. 2003; Steinbach et al. 2007b). Calcineurin inhibitors also act synergistically with azoles against fungal biofilms both in vitro and in vivo (Uppuluri et al. 2008). As with Hsp90 inhibitors, current efforts focus on identifying fungal-selective calcineurin inhibitors that lack the immunosuppressive effects on the host. PKC signaling intersects with calcineurin signaling and provides another downstream effector of Hsp90 with a key role in drug resistance (LaFayette et al. 2010). Upstream regulators of Hsp90 function also serve to modulate the evolution and maintenance of antifungal drug resistance with broad therapeutic potential, as is the case with lysine deacetylases (Robbins et al. 2012). Additional regulators that may be independent of the Hsp90 chaperone network have also emerged as promising targets to block drug resistance (Wurtele et al. 2010). Chemical and genomic screens promise to reveal many diverse molecules and genes that control antifungal drug resistance, offering possibility for therapeutic exploitation (LaFayette et al. 2010; Spitzer et al. 2011; Epp et al. 2010; Zhang et al. 2007).

While drug combinations should theoretically minimize the evolution of drug resistance, there has been a paucity of experimental studies that address this directly. The impact of drug combinations on the evolution of resistance has recently been

explored with experimental populations of *S. cerevisiae* and *C. albicans* (Hill et al. 2013). Populations that were resistant to azoles due to loss of function of Erg3 were evolved with a combination of an azole and an inhibitor of Hsp90 or calcineurin, in order to recapitulate a clinical context where inhibitors of Hsp90 or calcineurin could be administered in combination with an azole to render azole-resistant pathogenes responsive to treatment. Of 290 lineages initiated, the majority went extinct, consistent with the inherent challenge of evolving resistance to drug combinations (Hill et al. 2013). Genome sequencing and genetic analyses revealed diverse resistance mutations including mutations in genes encoding: the target of the inhibitor of Hsp90 (Hsp90) or calcineurin (Fpr1, the immunophilin that binds to FK506 to form the protein-drug complex that inhibits calcineurin); a transcriptional activator of drug efflux pumps, Pdr1; a regulator of sphingolipid biosynthesis, Lcb1; the catalytic subunit of calcineurin; and a repressor of ergosterol biosynthesis genes, Mot3 (Hill et al. 2013). Numerous mutations conferred resistance to the inhibitor of Hsp90 or calcineurin, while others rendered azole resistance independent of calcineurin. Extensive aneuploidy was also identified in several of the *C. albicans* lineages (Hill et al. 2013). This approach integrating experimental evolution and genome-scale analyses provides a framework for predicting and preventing the evolution of antifungal drug resistance.

Conclusion

The evolution of drug resistance is inevitable, but there are effective strategies to delay this ubiquitous process. While resistance to drug combinations can evolve by multiple mechanisms, there is limited evidence in the clinic. For example, ~40 % of transplant recipients suffer from invasive fungal infections, including those that receive a calcineurin inhibitor as an immunosuppressant; however, resistance to calcineurin inhibitors has not been observed in fungal pathogens recovered from patients receiving this immunosuppressive therapy (Blankenship et al. 2005; Reedy et al. 2006). Consistent with this, target-based resistance to Hsp90 inhibitors has yet to emerge in Hsp90 inhibitor clinical trials. That resistance has not been observed in the clinic suggests that these mutations may be associated with a fitness cost in conditions relevant to the human host. Given that there may be ample possibility for compensatory evolution to ameliorate the fitness costs, there is strong impetus to invest in the discovery of a multitude of strategies to improve clinical outcome for patients with invasive fungal infections.

Approaching antifungal drug resistance as an evolutionary problem has great potential to improve our capacity to predict and prevent it (Antonovics et al. 2007). Many of the paradigms for the evolution of drug resistance in fungal pathogens of humans also pertain to the evolution of fungicide resistance in fungal pathogens of plants, which have a staggering impact on agriculture. In fact, the processes may be intimately related. Drug resistance can be selected for with the prophylactic administration of antifungal drugs for high-risk patients and with the agricultural deployment of fungicides, and there is potential for pathogens to be transmitted between

these environments (Verweij et al. 2009; Mortensen et al. 2010). This is illustrated by the finding that environmental isolates of azole-resistant *A. fumigatus* show greater genetic similarity to azole-resistant clinical isolates than to those that are susceptible, suggesting that patients are being colonized with isolates that acquired azole resistance in the field (Snelders et al. 2009). In the broader context, it is clear that many parallels exist in the evolution of resistance to diverse agents by fungi, bacteria, protozoan parasites, insects, and even mammalian cancer cells. An interdisciplinary approach is poised to accelerate our understanding of the principles and mechanisms governing the evolution of drug resistance, which should ultimately manifest in novel and effective strategies to keep pace with the rapid evolution of resistance across the kingdoms.

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The Role of Biofilm Matrix in Mediating Antifungal Resistance

Jeniel E. Nett and David Andes

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Abstract

Fungal pathogens can adhere to medical devices and other surfaces, forming resilient biofilms. One of the hallmark features of biofilm formation is the production of a polymeric extracellular matrix which encases the cells within the biofilm. This protective covering has been linked to a mulit-drug resistant phenotype for a variety of fungi, including *Candida* spp. and *Aspergillus* spp.

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Here we describe environmental and genetic factors governing manufacture of the fungal biofilm matrix. We also highlight key matrix components, including β-1,3 glucan and extracellular DNA, which have been specifically shown to be instrumental for production the biofilm drug-resistant phenotype.

Keywords
Candida • Aspergillus • Biofilm • Matrix • Glucan • DNA • Antifungal • Drug resistance

Introduction

Candida albicans, the most common hospital-acquired fungal pathogen, frequently grows as a community of adherent cells encased in an extracellular matrix (Donlan 2001a). The majority of *Candida* infections are now thought to involve growth in this biofilm lifestyle, including virtually all infections associated with medical devices, such as vascular and urinary catheters (Douglas 2002; Kojic and Darouiche 2004). The susceptibility of medical devices to infection has been increasingly appreciated. There are estimated to be more than 45 million medical devices implanted in patients in the USA per year, and at least 50 % of all nosocomial infections are associated with these devices (Raad et al. 1992; Groeger et al. 1993; Kralovicova et al. 1997; Richards et al. 1999; Schmitt et al. 1990). The consequences of device infections can be disastrous, including life-threatening infection and device malfunction necessitating device removal (Donlan 2001b). Successful treatment of foreign-body infections requires device removal in most instances (Anaissie et al. 1998; Lecciones et al. 1992; Rex et al. 1995; Andes et al. 2012). These infections are extremely difficult to cure without removal of the medical device, in part due to the drug resistance associated with biofilm growth.

The ability to live as a fungal biofilm was first described for *Candida*, and the most common pathogenic species, including *C. albicans*, C. *dubliniensis*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis*, have now been shown to cause biofilm infections (Shin et al. 2002). As *C. albicans* is the most frequently encountered fungal biofilm infection, this species has been used as a model pathogen for many investigations regarding biofilm pathogenesis and drug resistance. However, more recent findings suggest that numerous medically important fungi may also adopt a biofilm lifestyle, including filamentous fungi (*Aspergillus*, *Zygomycetes*), *Pneumocystis*, and other yeasts (*Blastoschizomyces*, *Saccharomyces*, *Malassezia*, *Trichosporon*, and *Cryptococcus*) (Ramage et al. 2012; Loussert et al. 2010; Seidler et al. 2008; Davis et al. 2002; Singh et al. 2011; D'Antonio et al. 2004; Reynolds and Fink 2001; Cannizzo et al. 2007; Di Bonaventura et al. 2006; Walsh et al. 1986). These fungi have been shown to exhibit properties similar to those described for *Candida* biofilms, including the ability to adhere to medical devices and an increased tolerance of antifungal therapy (Ramage et al. 2009). As much of our knowledge of fungal biofilms and drug resistance was learned from investigations of *Candida*, it will be the focus of this chapter.

Candida biofilms have characteristic architecture and phenotypic traits distinct from free-floating or planktonic cells (Chandra et al. 2001; Costerton et al. 1999; O'Toole 2003; Ramage et al. 2001). Perhaps the most clinically relevant biofilm-specific property is the development of profound drug resistance, allowing biofilm cells to withstand antifungal concentrations up to 1,000-fold higher than those required to inhibit planktonic cells (Chandra et al. 2001; O'Toole 2003; Hawser and Douglas 1994; Mah et al. 2003). This characteristic of biofilms makes them extremely difficult, if not impossible to control in the medical setting. *Candida* biofilms have been shown to be resistant to various antifungals, including amphotericin B, triazoles, echinocandins, and flucytosine, the most commonly used drugs for treatment of *Candida* infections (Chandra et al. 2001; Hawser and Douglas 1994; Baillie and Douglas 1998; Lewis et al. 2002; Mukherjee et al. 2003; Ramage et al. 2002a). Similarly, both *Aspergillus* and *Cryptococcus* biofilms have shown to be less susceptible to multiple drug classes (Seidler et al. 2008; Martinez and Casadevall 2006; Mowat et al. 2008; Beauvais et al. 2007).

Key investigations have explored the mechanisms underlying biofilm-associated drug resistance (Mukherjee et al. 2003; Ramage et al. 2002b; Kumamoto 2005; Khot et al. 2006; LaFleur et al. 2006; Al-Fattani and Douglas 2006; Mitchell et al. 2013). These studies suggest that multiple factors contribute to development of this resilient phenotype. Furthermore, the influence of the individual mechanisms varies throughout the phases of biofilm development and is specific to the antifungal drug class. Factors contributing to *Candida* biofilm resistance include an increase in efflux pump activity, alterations in cell membrane ergosterol content, development of resistant "persister cells," activation of stress responses, an increase in cell density, and the presence of the extracellular matrix (Mukherjee et al. 2003; Ramage et al. 2002b; Kumamoto 2005; Khot et al. 2006; LaFleur et al. 2006; Perumal et al. 2007; Uppuluri et al. 2008; Robbins et al. 2011). Likewise, the *Aspergillus* biofilm phenotype has been linked to increased efflux pump activity and production of an extracellular matrix (Mowat et al. 2008; Rajendran et al. 2013; Bugli et al. 2013). Manufacture of biofilm matrix, a defining biofilm property, is one of the most influential resistance mechanisms, promoting resistance to multiple drug classes for *Candida* biofilms formed by a variety of species (Al-Fattani and Douglas 2006; Mitchell et al. 2013; Hawser et al. 1998; Nett et al. 2007, 2010a; Taff et al. 2012; Martins et al. 2012). The focus of this chapter is the role of extracellular matrix in resistance to antifungals during biofilm growth. The following sections describe pioneering investigations that examined the content of biofilm matrix and established its role in drug resistance. More recent studies have discovered key matrix components, identified a mechanism of resistance, and characterized several factors governing this process.

Extracellular Matrix of Fungal Biofilms

Production of an extracellular matrix is one of the distinguishing characteristics of both eukaryotic and prokaryotic biofilms (O'Toole 2003; Hawser et al. 1998; Donlan and Costerton 2002). This material is critical for biofilm formation, providing the

scaffold for surface adhesion and cellular aggregation to maintain the biofilm architecture (Flemming and Wingender 2010). Extracellular matrix has also been shown to assist with retention of water and sorption of nutrients. In addition, the material may be degraded during nutrient-limiting conditions by microbial produced enzymes, providing a carbon or nitrogen source (Flemming and Wingender 2010). One of the most medically important attributes of the matrix is the ability to provide protection from environmental insults, including host defenses and antimicrobial therapies (Donlan 2001b; Costerton et al. 1999).

Candida Biofilm Matrix

Candida frequently causes disease by forming biofilm on the surface of medical devices and has become the most commonly used model for study of fungal biofilms. Hawser et al. initially described the presence of matrix material for *C. albicans* biofilms (Hawser et al. 1998). The extent and character of matrix production was found to vary with environmental conditions. Compared to statically grown biofilms, those formed under rotary shaking conditions produced a denser, canopy-like, extracellular material when imaged by scanning electron microscopy. Also, *C. albicans* biofilms appear to produce less matrix under hypoxic conditions (Stichternoth and Ernst 2009). The clinical relevance of this material is evident by its presence on the catheters of patients with *Candida* biofilm (Paulitsch et al. 2009). Animal models of device-associated infections also reveal abundant biofilm matrix material and provide a means to study the function of this material in vivo (Fig. 1) (Andes et al. 2004; Nett et al. 2010b; Johnson et al. 2012). In vivo, the matrix is postulated to contain both the microbial-derived components identified in vitro and host proteins, such as plasma and salivary proteins that adsorb to the surface of medical devices (Nett and Andes 2006).

The Douglas group has performed several key investigations to examine the content of *Candida* biofilm matrix, including the description of a technique to extract and isolate the matrix of in vitro biofilms (Baillie and Douglas 2000). Quantitative analysis of the *C. albicans* matrix material removed by sonication showed a composition of carbohydrate (41 %, including 15.9 % glucose), protein (5.2 %), and hexosamine (3.4 %). As a complementary method to explore the matrix content, the Douglas group also exposed biofilms to various enzymatic treatments and measured cellular detachment (Al-Fattani and Douglas 2006). This study suggested that protein, chitin, DNA, and β-1,3 glucan are present in the extracellular matrix and contribute to surface adhesion or cellular cohesion (Table 1). Further studies have corroborated these findings and have linked two of these components, β-1,3 glucan and DNA, to the biofilm-specific drug-resistant phenotype, as described in the following section "*Candida* Biofilm Matrix and Drug Resistance" (Table 2) (Nett et al. 2007; Martins et al. 2010, 2012).

To examine the protein content of *C. albicans* biofilm matrix, the Lopez-Ribot group employed 2-D PAGE followed by mass spectrometry (Thomas et al. 2006).

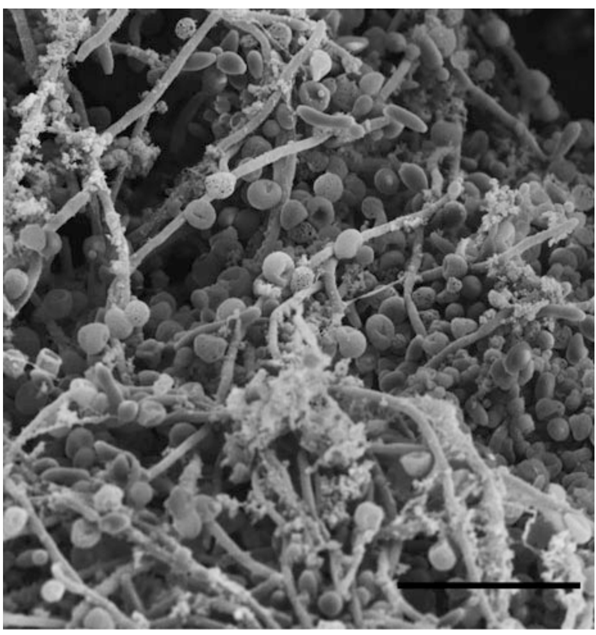

Fig. 1 Extracellular matrix of an in vivo *Candida* biofilm SEM and model

Table 1 Components of fungal biofilm matrix

C. albicans	*A. fumigatus*
Glucose	Galactomannan
Protein	Galactosaminogalactan
Hexosamine	α-1,3 glucan
DNA	Monosaccharides
β-1,3 glucan	Polyols
	Melanin
	Protein

Table 2 *C. albicans* and *A. fumigatus* matrix components influencing antifungal drug resistance

C. albicans	*A. fumigatus*	
β-1,3 glucan		
Fluconazole		
Amphotericin B		
Flucytosine		
DNA	DNA	
Amphotericin B	Amphotericin	
	C. albicans	*A. fumigatus*
β-1,3 glucan	Fluconazole	
	Amphotericin B	
	Flucytosine	
DNA	Amphotericin B	Amphotericin B

Surprisingly, the proteins found in the biofilm matrix were very similar to those identified in the supernatants of non-biofilm cultures. This suggests that in vitro, the proteins that are normally secreted during non-biofilm growth become incorporated into the extracellular matrix during biofilm growth. Several of the identified proteins had previously been shown to be part of the *C. albicans* secretome, including Cht3p, Mp65p, and Mp58p. However, others were not predicted secretory proteins. The role of the individual matrix proteins in biofilm function is not well understood.

Interestingly, the matrix content appears to vary significantly among *Candida* species (Al-Fattani and Douglas 2006; Silva et al. 2009). For example, compared to *C. albicans*, *C. tropicalis* biofilms were found to have a higher hexosamine content (27 %) and were not disrupted by DNase treatment (Al-Fattani and Douglas 2006). Also, the matrix composition is dependent on environmental and media conditions. The matrix of *C. albicans* biofilms formed in RPMI has a nearly a 1,000×-fold higher concentration of DNA when compared to YNB conditions (Martins et al. 2010). Although the concentration of individual matrix components varies among these conditions, *Candida* biofilms uniformly remain resistant to antifungals when adopting a biofilm lifestyle.

Aspergillus Biofilm Matrix

Although *Candida* has been the most commonly studied fungal biofilm pathogen, the role of *Aspergillus* biofilms in clinical disease has become increasingly recognized (Loussert et al. 2010; Ramage et al. 2009). Like *Candida*, *Aspergillus* biofilms have been linked to infection of medical devices (Ramage et al. 2011). However, biofilm growth has also been associated with the most common *Aspergillus* infections, including invasive pulmonary aspergillosis and aspergilloma (Loussert et al. 2010). As a clinical niche, the lung varies greatly from the environments for most *Candida* biofilm infections. Here, fungal biofilms proliferate under static, aerial conditions. The ability of the most common *Aspergillus* sp., *A. fumigatus*, to form a multicellular community with a surrounding extracellular matrix material has been demonstrated both in vitro and in vivo (Loussert et al. 2010; Beauvais et al. 2007). For this organism, biofilm formation and matrix production are greatest under static, aerial conditions. Ultrastructure analysis shows that these biofilms are composed of hyphae coalescing into a three-dimensional structure covered in dense extracellular material with embedded air channels (Beauvais et al. 2007).

Beauvais et al. analyzed the extracellular matrix of in vitro *A. fumigatus* biofilms and found this material to be composed of galactomannan, α-1,3 glucan, monosaccharides, polyols, melanin, and protein (Table 1) (Beauvais et al. 2007). Using immunolabeling, they were able to show galactomannan throughout the cell wall and matrix. In contrast, the labeling of α-1,3 glucan was the highest in the amorphous extracellular matrix material near the hyphal surface. Only a small portion of the matrix contained protein (2 %). Identified proteins included several major secreted antigens, as well as a group of hydrophobins. These hydrophobic surface active proteins, commonly associated with aerial growth, are presumed to play a role in

cell-cell adhesion required for mycelial colony formation during biofilm growth. Recent analysis has also shown that DNA accumulates in the matrix of *A. fumigatus* biofilms in vitro (Rajendran et al. 2013; Shopova et al. 2013). This extracellular DNA is identical to genomic DNA and is proposed to be released to the matrix following autolysis (Rajendran et al. 2013). The role of matrix DNA in *Aspergillus* biofilm resistance will be discussed in the following section "*Aspergillus* Biofilm Matrix and Drug Resistance."

By studying the resected aspergillomas of several patients, Loussert et al. shed light on the in vivo composition of *A. fumigatus* biofilms (Loussert et al. 2010). Similar to in vitro biofilms, galactomannan was identified throughout the fungal cell wall and extracellular matrix of in vivo biofilms upon immunolabeling. Also, α-1,3 glucan was found in the matrix material located closest to the cell wall surface. Compared to in vitro biofilms, in vivo biofilms appeared to have a higher concentration of the recently identified cell wall polysaccharide galactosaminogalactan. The presence of melanin in the biofilm matrix was confirmed in vivo, while antigenic proteins were not as apparent in vivo. When comparing the aspergillomas of patients with a murine model of invasive pulmonary aspergillosis, many similarities were noted, including the presence of both matrix galactomannan and galactosaminogalactan (Loussert et al. 2010). However, unlike in vitro biofilms and the aspergilloma biofilm, the extracellular matrix of the pulmonary aspergillosis biofilms did not appear to have a high concentration of α-1,3 glucan.

Candida Biofilm Matrix and Drug Resistance

Linking Matrix to Biofilm Drug Resistance

Several investigations have examined the contribution of the biofilm extracellular matrix to the drug-resistant phenotype associated with the *Candida* biofilm lifestyle (Al-Fattani and Douglas 2004, 2006; Baillie and Douglas 2000; Samaranayake et al. 2005). These studies have used various experimental designs to test the hypothesis that matrix may prevent access of antifungals to the cells embedded in the biofilm, postulating that the material may either slow the rate of drug transport or specifically bind antifungals extracellularly. Together, these investigations show that an intact matrix is one of most influential factors promoting the drug resistance of biofilms formed by various *Candida* species.

The Douglas group performed several of the initial studies exploring *Candida* biofilm matrix and drug resistance (Al-Fattani and Douglas 2004, 2006; Baillie and Douglas 2000). They took advantage of the variable matrix observed under flow and static conditions to determine its impact on drug resistance. *C. albicans* biofilms formed under laminar flow produced an extensive extracellular matrix as observed by scanning electron microscopy (Al-Fattani and Douglas 2006). Compared to biofilms with minimal matrix following growth in static conditions, the dense matrix biofilms were significantly more resistant to amphotericin B. This suggests that the character of the matrix may influence its ability to impede antifungals. However, the

Douglas group also examined the drug susceptibility of biofilms produced under shaking conditions. Although these biofilms produced more matrix than those grown under static conditions, a difference in drug susceptibility was not detected (Baillie and Douglas 2000).

To correlate matrix production with biofilm drug resistance, Samaranayake et al. utilized an antifungal diffusion apparatus (Samaranayake et al. 2005). Biofilms were grown on filter disks, and the penetration of antifungal through the biofilm to a fresh filter was measured by a zone-of-inhibition assay. *C. albicans* biofilms inhibited the penetration of all antifungals tested, including fluconazole, amphotericin B, and flucytosine. *C. parapsilosis* and *C. krusei* biofilms were also found to impede amphotericin B, but flucytosine and fluconazole penetration was higher than for *C. albicans* biofilms. Findings from this investigation support a role for matrix in biofilm antifungal resistance. However, the finding that antifungal concentrations exceeding the amount needed to kill planktonic cells traversed the biofilm questions the importance of the results. One study limitation was the use of a filter substrate which is anticipated to have properties distinct from common device substrates, such as vascular catheters or dentures. Unlike the filter, these confluent surfaces support an adjacent basal layer of cells deep within the biofilm. It is quite possible that antifungal penetration to these cells would be even less than that measured for the filter biofilms in the study.

Matrix Glucan and Drug Sequestration

Several key observations led to the discovery of a drug sequestration activity for the *Candida* biofilm matrix. The Andes group added isolated *Candida* biofilm matrix to planktonic MIC assays and found that this material was able to provide a degree of antifungal resistance to the non-biofilm cells (Nett et al. 2007). To test the hypothesis that matrix was interacting with an antifungal, preventing drug penetration into the cells, radiolabeled fluconazole was tracked through biofilm. The vast majority of the drug was found in association with matrix, suggesting drug sequestration. To explore the contribution of individual matrix components, the biofilm matrix was exposed to enzymes targeting the constituents. Degradation of matrix β-1,3 glucan impaired the biofilm-associated resistance mechanism, indicating a role for this matrix component in protecting biofilms from antifungal drugs. The role for matrix β-1,3 glucan in biofilm drug resistance during clinical infection was supported by the synergistic action of glucanase and fluconazole on rat venous catheters infected with *C. albicans* (Nett et al. 2007).

While investigating the impact of drug treatment on *Candida* biofilm transcription, Vediyappan et al. noted a very interesting finding (Vediyappan et al. 2010). Upon exposure to amphotericin B, biofilms rapidly converted to the yellow color of the drug. After DMSO extraction of biofilms, active amphotericin B was recovered. A similar drug binding pattern was observed for several preparations of β-1,3 glucan, suggesting that this component of the matrix may be binding amphotericin B as well, trapping the antifungal and preventing its activity on cells within the biofilm.

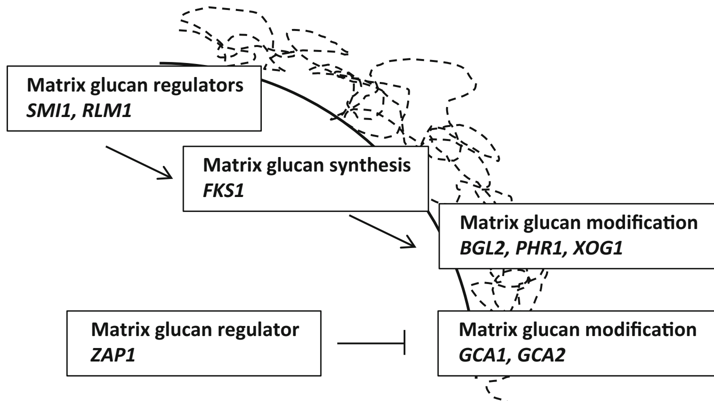

Fig. 2 Genetic regulation of *C. albicans* biofilm matrix productions

Linking matrix β-1,3 glucan to resistance with a second antifungal in a distinct drug class points to a multidrug mechanism specific to the biofilm lifestyle. A similar resistance mechanism has also been shown for flucytosine (Nett et al. 2010a). Furthermore, this mechanism does not appear to be unique to *C. albicans*. Biofilms formed by other common *Candida* species, including *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*, also have been shown to produce matrix β-1,3 glucan that sequesters antifungal and enhances drug resistance (Mitchell et al. 2013).

Matrix Glucan Production and Modification

Uncovering the genetic basis underlying matrix antifungal sequestration has been of great interest. As β-1,3 glucan was found to be the matrix component most closely linked to biofilm drug resistance, gene products capable of synthesizing and modifying this polysaccharide were ideal suspects (Fig. 2). In *C. albicans*, *FKS1* encodes a glucan synthase responsible for manufacture of cell wall β-1,3 glucan during planktonic growth. By employing a set of genetic mutants, Nett et al. linked this gene to the production of matrix β-1,3 glucan, matrix sequestration of antifungal, and multidrug resistance (Nett et al. 2010a, c). The finding was described both in vitro and in a rat venous catheter model of biofilm infection. The enhanced drug resistance related to *FKS1* appears to be specific to the biofilm lifestyle. During biofilm growth, *C. albicans* mutants with *FKS1* disruption were significantly more susceptible to fluconazole while this mutation did not impact planktonic susceptibility.

To delineate the process by which glucan is modified and delivered to the extracellular biofilm matrix, Taff et al. employed a candidate gene approach, examining a set of mutants based on transcriptional profiling of *C. albicans* in a rat venous catheter biofilm (Taff et al. 2012). They identified a role for two predicted glucan transferases (Bgl2p and Phr1p) and an exo-glucanase (Xog1p) in both delivery of biofilm matrix and the development of the resistance to antifungals during biofilm

growth. Mutants with disruption of one of these genes produced significantly less matrix β-1,3 glucan, ultimately resulting in disruption of matrix function. During biofilm growth, the mutants were approximately 50–70 % more susceptible to azole drugs compared to the parent strain both in vitro and in a rat venous catheter biofilm model. The isolated matrix of the mutant strains exhibited decreased capacity for antifungal sequestration. The gene products appear to have partially redundant functions as the phenotype was augmented in strains with two of the three genes deleted. Furthermore, studies support a role for Bgl2p, Phr1p, and Xog1p downstream of Fks1p, the primary glucan synthase for biofilm matrix β-1,3 glucan. These gene products are proposed to cooperate in modification of matrix glucan for development of functional matrix capable of sequestering antifungal drugs and promoting biofilm antifungal drug resistance.

Regulation of Matrix Glucan

Several pathways have now been shown to be involved in regulating production of *Candida* biofilm matrix and drug resistance. Nobile et al. discovered a critical negative regulator of *C. albicans* biofilm matrix glucan production both in vitro and in vivo (Nobile et al. 2009). In a screen of transcription factor mutants, the *zap1−/−* mutant was noted to have a glistening appearance that correlated with abundant matrix glucan. Through transcription profiling and chromatin immunoprecipitation, several groups of target genes were identified. First, Zap1p was found to repress transcription of two glucoamylases (*GCA1* and *GCA2*). These enzymes are postulated to have a positive role in matrix production, likely by hydrolyzing insoluble carbohydrates for release into the matrix. However, their specific role has not been further identified. A second group of Zap1p targets includes three alcohol dehydrogenases (*ADH5*, *CSH1*, and *LFD6*). These gene products are proposed to participate in production of alcohols, influencing biofilm formation and matrix glucan production through quorum signal pathways. However, further investigation is needed to establish this link. Interestingly, the enzymes required for matrix glucan modification do not appear to be under Zap1p control (Taff et al. 2012). Transcription of *XOG1*, *PHR1*, and *BGL2* was not altered in the *zap1−/−* mutant, suggesting that Zap1 governs matrix production through an independent pathway.

The Cowen group discovered heat shock protein, Hsp90p, as novel regulator *of C. albicans* biofilm matrix production and drug resistance (Robbins et al. 2011). Disruption of this pathway increased the effectiveness of triazole drugs both in vitro and in a rat venous catheter model. Unlike planktonic conditions, this pathway was not modulated via calcineurin or Mkc1 pathways. The finding that inhibition of Hsp90p activity led to decreased β-1,3 glucan in the matrix of *C. albicans* biofilms suggests that Hsp90p is a key player in the production of drug-sequestering matrix. How Hsp90p specifically modulates this activity is unknown, but it is hypothesized to stabilize one or more of the proteins involved in production and regulation of matrix production, such as Fks1p or Zap1p.

A search for additional regulators of biofilm matrix-associated drug resistance identified downstream components of the yeast PKC pathway (Nett et al. 2011). *C. albicans* mutants with disruption of either *SMI1* or *RLM1* were deficient in both matrix glucan production and susceptibility to azole drugs. These factors are thought to govern matrix-associated drug resistance through *FKS1*, as overexpression of this glucan synthase gene restored the biofilm-associated drug-resistant phenotype. Surprisingly, upstream PKC pathway components do not appear to be involved in regulation of biofilm matrix production and drug resistance. This suggests that matrix-associated drug resistance and cell wall integrity are networked, but that the biofilm drug resistance is triggered through a pathway distinct from the PKC pathway.

Matrix Extracellular DNA and Drug Resistance

Extracellular DNA is one of the *Candida* biofilm matrix components critical for biofilm integrity and maintenance (Martins et al. 2010). Degradation of matrix DNA not only destroys the biofilm architecture, but the addition of exogenous DNA promotes biofilm growth (Martins et al. 2010). To determine the contribution of matrix DNA to biofilm drug resistance, Martins et al. treated *C. albicans* biofilms with DNase and measured the impact on drug susceptibility (Martins et al. 2012). Degradation of extracellular DNA significantly improved the activity of amphotericin B against *Candida* biofilms. However, biofilms treated with DNase remained resistant to both fluconazole and caspofungin. This suggests that the mechanism involves a specific DNA-amphotericin B interaction. An alternative possibility is that the matrix DNA may interact with a variety of antifungals, but other resistance processes in play maintain biofilm resistance upon DNA degradation. The mechanism underlying how matrix DNA contributes to *Candida* biofilm resistance remains unknown. It is also a mystery if the biofilm resistance phenotypes linked to matrix glucan and matrix DNA are intertwined.

Aspergillus Biofilm Matrix and Drug Resistance

Considerably less is known about the process of *Aspergillus* biofilm matrix production and how it impacts drug susceptibility during biofilm growth. Based upon findings from bacterial and *Candida* biofilm investigations, the matrix of *Aspergillus* biofilms is similarly thought to contribute to antimicrobial resistance, perhaps through impairing drug diffusion to the fungal cells, either by slow transit or by binding the drugs (Beauvais et al. 2007; Rajendran et al. 2013; Bugli et al. 2013). As the extracellular matrix content increases as *A. fumigatus* biofilms mature, this material is thought to play a role in drug resistance during the later phases of biofilm growth (Beauvais et al. 2007).

One recently recognized matrix component shown to impact drug resistance is extracellular DNA. Rajendran et al. showed that the content of DNA in the matrix

increased as in vitro *A. fumigatus* biofilms matured from 8 to 48 h (Rajendran et al. 2013). Using RAPD, they were able to confirm that this extracellular DNA was identical to genomic DNA. The finding that this DNA release was linked to chitinase activity suggests autolysis as the underlying process. The matrix DNA of *A. fumigatus* biofilms appears to be important for both biofilm structural integrity and resistance to antifungals. Degradation of this material by DNase decreases the biomass of the biofilm and renders biofilms more susceptible to both amphotericin B and the echinocandin, caspofungin. Interestingly, exogenous DNA also promotes structural integrity and increases biofilm matrix carbohydrate production, suggesting a potential role for host DNA during in vivo *Aspergillus* biofilm formation (Rajendran et al. 2013; Shopova et al. 2013).

Much remains unknown about *Aspergillus* biofilm drug resistance and how the various components of the biofilm matrix influence this phenotype. An intriguing study by Bugli et al. found that administration of alginate lyase increased in vitro *A. fumigatus* biofilm susceptibility to amphotericin B (Bugli et al. 2013). This enzyme degrades uronic acid-containing carbohydrates and is anticipated to be acting on the extracellular matrix carbohydrates of *Aspergillus* biofilms. This study suggests that a previously unidentified matrix carbohydrate is contributing to antifungal drug resistance during biofilm growth. Further investigation is of interest to characterize this material and its specific role in resistance to antifungal therapy.

Conclusions

The most common nosocomial fungal pathogens produce disease by growing as drug-resistant, multicellular, biofilm communities. Production of an extracellular matrix, one of the distinguishing biofilm traits, is not only critical for adhesion and structural support but confers resistance to antimicrobials (Fig. 3). In *Candida*, the presence of β-1,3 glucan has been linked to the ability of the matrix material to sequester antifungals and prevent them from reaching their cellular targets. For both *Candida* and *Aspergillus*, extracellular DNA in the biofilm matrix has been shown to be instrumental in maintaining the biofilm drug resistance. Whether the matrix carbohydrates and DNA cooperate for this process remains unknown. Several key regulators of *C. albicans* biofilm matrix and drug resistance have been identified, while regulation of this pathway in *Aspergillus* has been relatively unexplored.

With the increasing prevalence of immunosuppressed patients with invasive fungal infections, further understanding of this drug resistance mechanism is a logical step to identification of novel drug targets and therapeutic strategies. However, delivery of novel compounds targeting biofilms cells may prove a challenge, as the matrix often impedes or sequesters drugs extracellularly. Potential strategies to overcome this phenomenon include identification of matrix-degrading compounds, development of matrix-permeable compounds, and combination therapy targeting both the matrix and biofilm cells.

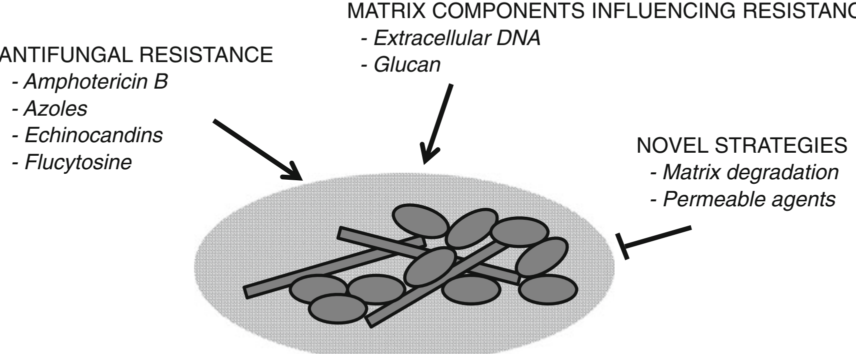

Fig. 3 Overview of fungal biofilm matrix and drug resistance

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Use of Novel Tools to Probe Drug Resistance in Fungi

Yanan Zhao and David S. Perlin

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Abstract

Antifungal drug resistance threatens therapeutic effectiveness and needs to be diagnosed in a timely manner. Currently, recognition of antifungal resistance still relies on culture-based susceptibility testing. Yet, antifungal susceptibility testing is not routinely performed and often comes too late to influence a timely decision on patient management. With the quantum leap of molecular technology and accrued insights on basic fungal cell biology and antifungal drug resistance mechanisms, some novel molecular techniques are now available to provide a faster and more accurate assessment of both primary and secondary resistance than classical methodologies. Validated targets for echinocandin resistance in *Candida* spp. and triazole resistance in *Aspergillus fumigatus* and *Candida* spp. are particularly well suited for molecular detection. Yet, implementation of a

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molecular diagnosis for drug resistance into the clinical settings requires validation in well-designed clinical trials, as well as improved methods for highly efficient primary sample preparation.

Keywords
Invasive fungal infections • Molecular diagnosis • Species identification • Susceptibility • Triazole • Echinocandin • Antifungal drug resistance • Primary resistance • Secondary resistance • *Candida* spp. • *Aspergillus* spp. • Efflux pumps • *CYP51A* • 1,3-β-D-glucan synthase • *FKS*

Introduction

Opportunistic fungal infections are widespread in immunosuppressed individuals and are a growing concern for the management of such patients. In the past two decades, the frequency of invasive fungal infections (IFIs) has risen (Pfaller and Diekema 2010) and the high mortality rate poses challenges for clinicians. The treatment options for IFIs are limited since there are relatively few chemical classes and targets represented by existing antifungal drugs. Triazole and/or echinocandin antifungal drugs are widely used as first-line antifungal therapy, depending on the pathogen, disease, and host status. Antifungal agents are often prescribed for prophylaxis and empiric or preemptive therapy for patients at risk of developing IFIs. Both facts have raised concern about the emergence of antifungal drug resistance. Antifungal resistance is generally classified as either primary (present before exposure to antifungals) or secondary (develops after exposure to antifungals). Primary resistance is observed as a shift toward colonization with inherently less susceptible organisms, while secondary resistance involves the emergence of cell-specific resistance mechanisms in normally susceptible strains. In both cases, antifungal-resistant strains threaten therapeutic effectiveness and need to be diagnosed in a timely manner. Delays in the administration of appropriate therapy beyond 12 h can increase the apparent mortality for *Candida* bloodstream infections by threefold (Morrell et al. 2005).

Currently, recognition of antifungal resistance still relies on culture-based susceptibility testing. Antifungal susceptibility is normally measured by broth microdilution using the minimum inhibitory concentration (MIC) or disk diffusion assays in accordance with guidelines of the Clinical Laboratory Standards Institute (CLSI) standard and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Definitive Document (2008a, b; CLSI 2008a, b). Yet, antifungal susceptibility testing is not routinely performed and requires 48–72 h following identification, which often comes too late to influence a timely decision on patient management. In the meantime, with the quantum leap of molecular technology and accrued insights on basic fungal cell biology and antifungal drug mechanisms, some novel and robust molecular techniques are now available to provide a faster and more accurate assessment of both primary and secondary resistance than classical methodologies.

Primary Resistance Assessment by Molecular Methods

As primary resistance occurs due to inherently less susceptible fungal species, a rapid and accurate identification of fungal pathogen to the species level provides a workable inference of drug susceptibility that guides treatment choices. For example, serious infection due to *C. krusei* would not be expected to be treated effectively with triazoles like fluconazole or itraconazole because of the well-known intrinsic resistance. Using culture-based identification, the gold standard of IFI diagnosis, such information is available in 48 ~ 72 h after blood draw. However, in the case of *Candida* infections, the mortality rate increases steeply from 11.1 % if antifungal treatment is initiated within 12 h following the first culture-positive blood draw to 33.1 % at 48 h (Morrell et al. 2005). Whereas it seems formidable to fit culture-based identification into this narrow time window, molecular methods offer a promising alternative to facilitate rapid and accurate diagnosis. In principle, a primary sample like blood or tissue specimen can be analyzed in a matter of hours to generate species-specific information, which predominantly involves amplification-detection platforms, including polymerase chain reaction (PCR) (DNA amplification) and nucleic acid sequence-based amplification (RNA amplification)-based assays. Various molecular tools, in particular, post-amplification reporting methods like allele-specific molecular beacon (MB) technology, DNA sequencing, and melt curve analysis have been incorporated with these amplification techniques for species identification (Al-Wathiqi et al. 2013; Loeffler et al. 2000a; Park et al. 2000). Of note, some new high-throughput technologies seem more attractive, such as Luminex xMAP technology, which utilizes microbeads and specific capture probe hybridization to identify up to 100 different target sequences in a single reaction vessel (Loeffler et al. 2000b). The assay permits rapid identification of a broad spectrum of fungal pathogens, including 10 fungal genera and 29 fungal species, covering both commonly occurring and emerging fungi (Landlinger et al. 2009). PCR/electrospray ionization mass spectrometry (PCR/ESI-MS) is another robust tool, which is capable of identifying nearly all known human pathogens, including previously unknown or unculturable organisms directly from primary clinical samples such as blood or respiratory specimen (Ecker et al. 2010; Wolk et al. 2012). By identifying minute quantities and mixtures of nucleic acids without the need for a detection probe, PCR/ESI-MS offers a critical advantage of discovering unusual or unculturable pathogens and mixed infections. This was evidenced by a recent study finding *Aspergillus terreus* in culture-negative bronchoalveolar lavage (BAL) fluid from a myelogenous leukemia patient who was on empiric amphotericin B therapy (Modi et al. 2012).

Molecular Diagnosis of Acquired Triazole Resistance

Triazole antifungal drugs (fluconazole, voriconazole, itraconazole, and posaconazole) have been in clinical use since early 1980s. Acquired azole resistance was rare during the first decade of clinical use, but it became a significant problem in

the 1990s when more and more azole-resistant *Candida* spp. isolates were reported in patients with compromised immune systems (Heald et al. 1996; Rautemaa et al. 2007; Rex et al. 1995). Later, a global antifungal surveillance study involving more than 140,000 *Candida* spp. isolates collected over the period of 1997–2005 showed that the overall resistance to fluconazole and voriconazole was 6.2 % and 3.1 %, respectively (Pfaller et al. 2007). Similarly, it is increasingly recognized that *Aspergillus fumigatus* has been rapidly acquiring resistance to triazole agents (Verweij et al. 2007), the first-line therapy for all forms of aspergillosis and the only orally active group of antifungal drugs within the limited therapeutic options (Walsh et al. 2008). The frequency of azole resistance in clinical *A. fumigatus* isolates by patient was 0 % in 2002 and 2003, significantly increased to 17 % in 2007, 14 % in 2008, and 20 % in 2009 at a referral center for chronic aspergillosis in Manchester, United Kingdom (Bueid et al. 2010; Howard et al. 2009). Given the fact that *Candida* spp. are the most common overall invasive fungal infection, and *Aspergillus* infections are the most predominant invasive mold infection, and collectively they account for 90 % of all nosocomial fungal infections (Fridkin and Jarvis 1996), triazole resistance is a serious issue. Going forward, effective management of invasive fungal infections requires rapid diagnosis of both the infecting organism and associated drug resistance, as well as discovery of new drugs with novel mechanisms of action.

The target of triazole drugs is lanosterol 14α-demethylase, a key enzyme in ergosterol biosynthesis. The action of triazole agents on this target enzyme results in depletion of ergosterol, which disrupts the structure and alters the functional properties of the fungal plasma membrane leading to inhibition of fungal growth. Triazole resistance is well characterized and several principal mechanisms have been described in *Candida* species (MacCallum et al. 2010; Perlin 2009; Verweij et al. 2009a) (Table 1). One prominent mechanism is the acquisition of point mutations in the gene encoding for the target enzyme (*ERG11*), resulting in an altered drug-binding domain with reduced affinity for or incapacity to bind azoles. Another mechanism related is the overexpression or upregulation of the target enzyme. The third prominent mechanism involves upregulation of a variety of multidrug efflux pumps including the ATP-binding cassette (ABC) transporters encoded by *CDR* genes (*CDR1* and *CDR2*) and major facilitator superfamily (MFS) encoded by *MDR* genes (*MDR1*). Upregulation of the *CDR* gene-encoded efflux pumps appears to confer resistance to multiple triazole drugs, while induction of that encoded by *MDR* genes are more closely associated with fluconazole resistance (Pfaller 2012). The increased mRNA level of these genes are further observed to be associated with alterations on global transcriptional regulators such as *TAC1*, *PDR1*, and *UPC2* (Coste et al. 2006; Flowers et al. 2012; Nagi et al. 2011; Oliver et al. 2007; Sanglard and Odds 2002; Vermitsky and Edlind 2004). Another mechanism of azole resistance in *Candida* species is revealed by the observation that growth inhibition of *Candida* cells can be circumvented by the accumulation of 14α-methylfecosterol if cells are deficient in sterol $\Delta^{5,6}$-desaturase, encoded by *ERG3* (Kelly et al. 1997; Martel et al. 2010). Last, chromosomal aneuploidy

Table 1 Summary of potential targets for acquired triazole and echinocandin resistance

	Molecular mechanism	Organism	Genetic target	References	Suitable detection methods
Triazoles	1. Target site mutations	*C. albicans*	*ERG11*	MacCallum et al. 2010; Morio et al. 2010; White et al. 2002	DNA sequencing; real-time PCR with allele-specific molecular beacon probe or melting curve analysis
		C. glabrata	*ERG11*	Hull et al. 2012; Sanguinetti et al. 2005	
		A. fumigatus	*CYP51A*	Bueid et al. 2010; Howard et al. 2009; Verweij et al. 2009a	
	2. Overexpression of target site	*C. albicans*	*ERG11*	Pancholi et al. 2004; Perea et al. 2001	Quantitative RT-PCR; microarray
		C. glabrata	*ERG11*	Henry et al. 2000	
		A. fumigatus	*CYP51A*	Arendrup et al. 2010	
	3. Drug efflux pumps				
	ABC	*C. albicans*	*CDR1, CDR2*	Chau et al. 2004; Holmes et al. 2008; Karababa et al. 2004	Quantitative RT-PCR; microarray
		C. glabrata	*CDR1, CDR2, SNQ2*	Sanguinetti et al. 2005	
		A. fumigatus	*MDR1, MDR4, CDR1B*	Ferreira et al. 2005; Fraczek et al. 2013; Nascimento et al. 2003; Slaven et al. 2002	
	MFS	*C. albicans*	*MDR1*	Chau et al. 2004; Karababa et al. 2004	
		A. fumigatus	*MDR3*	Ferreira et al. 2005; Nascimento et al. 2003	
	4. Transcription factors				
	ABC	*C. albicans*	*TAC1, CZF1*	Coste et al. 2006; Dhamgaye et al. 2012	Microarray; DNA sequencing; real-time PCR; RNA sequencing

(*continued*)

Table 1 (continued)

	Molecular mechanism	Organism	Genetic target	References	Suitable detection methods
		C. glabrata	*PDR1*	Ferrari et al. 2011	
	MFS	*C. albicans*	*MRR1, CAP1*	Mogavero et al. 2011	
	ERG	*C. albicans*	*UPC2*	Flowers et al. 2012; Oliver et al. 2007; Schubert et al. 2011	
	5. Accumulation of alternative sterols	*C. albicans*	*ERG*	Kelly et al. 1997; Morio et al. 2012	DNA sequencing
		C. glabrata	*ERG3*	Geber et al. 1995	
	6. Chromosomal aneuploidy	*C. albicans*	Chromosome 5	Selmecki et al. 2006	Comparative genome hybridization
		C. glabrata	Chromosome D, E, F, M	Polakova et al. 2009	
Echinocandins	Target site mutations	*C. albicans*	*FKS1*	Park et al. 2005	DNA sequencing; real-time PCR
		C. glabrata	*FKS1, FKS2*	Garcia-Effron et al. 2010; Katiyar et al. 2006	
		A. fumigatus	*FKS1*	Gardiner et al. 2005; Lewis et al. 2011; Rocha et al. 2007	

which presents as chromosome missegregation has been linked to azole resistance in *C. albicans* by comparative genome hybridization analysis (Selmecki et al. 2006).

With increased insights into these resistance mechanisms, molecular detection of azole resistance in *Candida* species seems feasible under certain circumstances. In particular, resistance due to drug target gene mutations can be easily detected by DNA sequencing (Sanguinetti et al. 2005), allele-specific real-time molecular probes (Park and Perlin 2005), LightCycler melt curve analysis (Loeffler et al. 2000a), or DNA microarray technology (Yan et al. 2008). However, these target gene mutations do not seem to be the dominant mechanism of azole resistance in *Candida* in the clinic. Instead, prominent resistance arises from overexpression of the sterol pathway genes and upregulation of efflux pumps (Perea et al. 2001; White et al. 2002). Quantitative reverse transcription PCR (RT-PCR) has been the mainstream of expression profiling in assessing such overexpression and upregulation associated with secondary triazole resistance (Gygax et al. 2008; Kofla and Ruhnke 2007; Park and Perlin 2005). However, measurement of gene expression levels requires cell cultures grown in the presence/absence of drug, and primary specimens are not suitable for such assessment. A quantitative correlation must be made that links an overall level of expression with a resistance phenotype to establish a threshold level of overexpression (Park and Perlin 2005). Such measurements can be complicated when resistance is a product of multiple mechanisms operating in tandem. This problem can be effectively overcome by evaluating gain-of-function mutations in transcription factors like *CgPDR1*, *TAC1*, *MRR1*, *UPC2*, and *CAP1*, as predictive markers for upregulation of efflux pumps. These mutations can be directly targeted without cell culture by real-time PCR, high-throughput sequencing, or microarray analysis for rapid identification (Perlin 2009). Nevertheless, the variety of resistance mechanisms prominent in *Candida* spp. can make direct molecular detection of triazole resistance difficult to interpret. Clinical studies are needed to relate such markers to existing gold-standard culture-based technology to assess their relative importance for therapeutic response. It remains to be seen whether profiling a single target mechanism among a multitude of potentially operative mechanisms in a single organism is sufficient to assess the azole resistance phenotype.

In contrast, triazole resistance in *A. fumigatus* appears more limited and predominantly involves mutations in the gene (*CYP51A*) encoding the protein sterol 14-α-demethylase. Mutational hot spots confirmed to cause resistance have been characterized at Gly54, Met220, Leu98, Gly138, and Gly448; and other mutations in *CYP51A* have been reported (Bueid et al. 2010; Howard et al. 2009; Verweij et al. 2009a). In the Netherlands, most resistance is due to tandem mutations in Leu98 and the promoter region of *CYP51A*, which arise as a consequence of azole use in agriculture (Snelders et al. 2009; Verweij et al. 2009b). This specific resistance mechanism has been detected in many countries in Europe and Asia (Arikan-Akdagli 2012; Chowdhary et al. 2012), although it has not been observed in patients that acquired resistance during therapy. Overexpression of ABC and MFS drug transporters has been described more recently in 15–20 % of isolates, as they confer resistance to itraconazole, voriconazole, and posaconazole (Bowyer et al. 2012; Fraczek et al. 2013). Most recently, high-level expression of the alternative gene

CYP51B was observed in two clinical azole-resistant strains which did not carry mutations in *CYP51A* (Buied et al. 2013). Finally, in a small percentage of isolates, the mechanism of triazole resistance is unclear and may be novel.

Modification of *CYP51A* remains the most dominant mechanism, and a limited number of mutations have been proven to confer resistance, which makes it ideal as a detection target for the development of real-time PCR assays to assess triazole resistance in *A. fumigatus* (Balashov et al. 2005; Garcia-Effron et al. 2008; Klaassen et al. 2010). An early comprehensive molecular diagnostic assay combined allele-specific molecular beacon technology and associations between specific *CYP51A* mutations and different triazole drug resistance to distinguish azole-resistant *A. fumigatus* from susceptible strains. Furthermore, it provided possible therapeutic options depending on the mutation detected, as some mutations confer limited resistance to the triazole class of drugs (Garcia-Effron et al. 2008). In this assay, strains that harbor mutations associated with azole resistance in the first tier of screening enter the next tier which was composed of multiple MB probes to pinpoint specific mutations as well as to reveal the relationship with different triazole antifungal drugs. In another study, a novel mixed-format real-time PCR assay was used to detect the most frequent mutations occurred at codons G54, L98, G138, and M220 and the existence of tandem repeat (TR) in the *CYP51A* promoter region (Klaassen et al. 2010). This assay mixed fluorescence resonance energy transfer (FRET) probes, melting curve analysis, and asymmetric PCR together and successfully identified four triazole-resistant strains from a random collection of 209 clinical isolates of *A. fumigatus*. Success of these molecular detections has shed light on faster diagnosis of azole resistance in *Aspergillus* and holds the promise of resolving infections with cryptic azole-resistant *Aspergillus* where culture isolation is not feasible or available.

Molecular Diagnosis of Resistance in Unculturable Cryptic Infections

Aspergilli are frequently not cultured from primary samples obtained from chronic pulmonary aspergillosis (CPA) patients, and molecular methods hold promise for the rapid detection of triazole resistance. Yet fungal burdens are usually very low and sample preparation is often inefficient. This makes it difficult to directly detect azole resistance in single-copy genes like *CYP51A* from the primary samples using standard real-time PCR assays. To enhance sensitivity, nested PCR with subsequent sequencing of the entire *CYP51A* gene was adopted by Denning et al. to investigate the triazole resistance in bronchoalveolar lavage (BAL) and sputum samples from patients with chronic fungal diseases (Denning et al. 2011). The high-frequency triazole resistance found in unculturable *A. fumigatus* in this study indicated that nested PCR/sequencing approach is a useful tool for rapid detection of triazole-resistant *Aspergillus* from the primary clinical samples. This method was also successfully applied on BAL fluids collected from patients with aspergillosis (Zhao et al. 2013). The study showed 4.3 % azole resistance rate in cultured *Aspergillus* isolates, but 14.8 % *CYP51A* PCR-positive samples were found to

have mutations resulting in amino acid substitutions, including two confirmed azole resistance-associated mutations M220V and P216L. Both cases highlight the potential of detecting cryptic resistant *A. fumigatus* with the aid of molecular diagnostic tools.

Molecular Detection of Acquired Echinocandin Resistance

Echinocandin-class antifungals target the fungal cell wall by inhibiting the synthesis of β-1,3-D-glucan, a critical cell wall component of most pathogenic fungi. It has been over a decade since caspofungin was approved for clinical use as the first echinocandin, followed by micafungin and anidulafungin. Echinocandins are highly effective against a wide range of *Candida* spp., including azole-resistant strains and biofilms (Bowman et al. 2002; Denning 2003; Ferreira et al. 2009; Morrison 2006; Wiederhold and Lewis 2003), and are increasingly being used as first-line therapy in many hospitals to treat mucosal and invasive forms of candidiasis (Kartsonis et al. 2002; Pappas et al. 2007). On a limited basis, echinocandins are also used to treat invasive aspergillosis, as they are fungicidal against actively growing hyphal tips but less effective against nongrowing subapical cells (Bowman et al. 2002). In general, resistance to echinocandin drugs remains low, but reports of clinical failure due to echinocandin-resistant *Candida* spp. are rising with the expanding drug use (Arendrup et al. 2009; Castanheira et al. 2010; Pfaller et al. 2011, 2012; Zimbeck et al. 2010), and it was reported to be >13 % isolates at one high-risk center (Alexander et al. 2013).

Just like other antifungals, detection of echinocandin resistance still largely relies on in vitro antifungal susceptibility testing. The CLSI has defined laboratory standards to determine MICs for wild-type (WT) susceptible strains and clinical breakpoints (CBP) for echinocandin drugs (Pfaller et al. 2011). It is now well recognized that amino acid substitutions in the catalytic subunits (Fks1p and/or Fks2p) of the 1,3-β-D-glucan synthase confer reduced echinocandin susceptibility and are associated with clinical failure (Perlin 2007) (Table 1). However, the degree of MIC elevation and reduced sensitivity of glucan synthase (the kinetic parameters 50 % inhibitory concentration IC_{50}/Ki) to various echinocandins can vary by 50 to several 1,000-fold relative to those for the WT, depending on the specific amino acid substitution (Garcia-Effron et al. 2009a, b). With numerous studies on clinical isolates with echinocandin resistance, the dominant point mutations have been localized in two highly conserved "hot spot" regions of *FKS1* of all *Candida* spp. (Park et al. 2005) or both *FKS1* and *FKS2* of *C. glabrata* (Garcia-Effron et al. 2010; Katiyar et al. 2006). The limited spectrum of mutations conferring resistance is ideal for detection by molecular diagnostic tools. One of a few pioneer studies assessed caspofungin resistance in *C. albicans* by developing a multiplex real-time PCR assay with molecular beacons targeting a range of prominent *FKS1* mutations (Balashov et al. 2006). It must be pointed out that the three amino acid alterations occurred at codon Ser645 (S645P, S645Y, S645F) targeted by this multiplex assay along with alterations at codon Phe641 (F641S and F641L) are indeed the most prominent

amino acid changes in *C. albicans*, which can lead to 2–3 logs shifts in the enzyme sensitivity to drug (Garcia-Effron et al. 2009b; Park et al. 2005). These mutations not only account for nearly 80 % of the *FKS1*-mediated resistance in our database ($n >$ 180) but also have stronger phenotypes associated with resistance and much higher IC_{50}/Ki values than mutations occurred at other positions within hot spots (Perlin 2011).

Now that kinetic inhibition studies of glucan synthase have revealed a strong correlation between specific *FKS* mutations, elevated MICs, and pharmacodynamic behavior in animal models (Arendrup et al. 2012; Garcia-Effron et al. 2009a, b; Howard et al. 2011; Slater et al. 2011; Wiederhold et al. 2011), ranking *FKS* mutations based on enzyme sensitivity to drug seems to be a reasonable way to predict resistant phenotypes and, more importantly, to help guide appropriate antifungal treatment. In general, the greater the ability of an *FKS* mutation to confer reduced drug sensitivity on glucan synthase, the more pronounced the resistance observed. In *C. albicans*, the rank order of relative resistance conferred from Fks1p amino acid substitutions has been suggested as: S645, F641 >> L642, T643, L644, R647, D648 > P649 (Perlin 2011). This is consistent with what was observed by pharmacodynamic (PD) study and drug dose escalation experiment on animal models, where conventional dosing of echinocandins cannot elicit an antifungal response in animals infected with S645 and F641 *C. albicans* mutants (Slater et al. 2011; Wiederhold et al. 2011). Similarly, in *C. glabrata*, amino acid substitutions at S663 in Fks2p, which is equivalent to S645 in Fks1p in *C. albicans*, cause the most prominent resistance observed in clinical isolates resulting in high MICs and remarkably reduced glucan synthase sensitivity to all three echinocandin drugs (Garcia-Effron et al. 2009a). Other mutations occurred at F659 in Fks1p and S629, F625, and D632 in Fks2p in *C. glabrata* also had similar rank order of resistance comparable to that in *C. albicans* (Perlin 2011). The key aspect of such ranking is to reflect the pattern of antifungal response in resistance caused by different *FKS* mutations and to further assist finding the most effective therapeutic options. In fact, a recent study demonstrated the superiority of detection of *FKS* mutations to MICs in predicting echinocandin therapeutic responses among patients with invasive candidiasis (Shields et al. 2012). In the meantime, another study, from pharmacodynamic perspective, revealed a dose–response relationship between *FKS* mutations and echinocandin drug exposure (Lepak et al. 2012).

Although the limited spectrum and genetic clustering of *FKS* mutations conferring echinocandin resistance for the major *Candida* species seems amenable to real-time PCR techniques, there are only limited published studies that applied these techniques to characterization of clinical echinocandin-resistant isolates (Balashov et al. 2006; Ben-Ami et al. 2011). Until now, no study has reported direct detection of echinocandin resistance using molecular methods from primary clinical samples such as tissue specimen or blood.

As aforementioned, echinocandin drugs have a largely fungistatic effect against filamentous fungi such as *Aspergillus* spp. The deficiency of having complete growth inhibition has resulted in an alternative susceptibility endpoint testing method known as the minimum effective concentration (MEC) to determine the

activity of echinocandins against filamentous fungi. The MEC is defined as the lowest drug concentration at which short, stubby, highly branched hyphae are observed (Arikan et al. 2001; Espinel-Ingroff 2003; Imhof et al. 2003). MEC testing demonstrated that echinocandin susceptibility profiles are varying in different *Aspergillus* species (Antachopoulos et al. 2008; Imhof et al. 2003). Overall, *A. fumigatus*, *A. flavus*, and *A. terreus* have comparable susceptibilities to all three echinocandins, but *A. niger* seems more susceptible to caspofungin than *A. fumigatus*. Much less is known about echinocandin resistance in *Aspergillus*, although the upregulated expression level of *FKS1* gene was observed in *A. fumigatus* clinical isolate with the reduced susceptibility to caspofungin (Arendrup et al. 2008) and in vitro studies with manipulated laboratory strains indicate that modification of Fks1p confers high-level resistance (Gardiner et al. 2005; Rocha et al. 2007). To date, only a few clinical isolates associated with echinocandin treatment failures have been investigated so far. Limited experience along with insufficient insight of molecular mechanism has made molecular detection of echinocandin resistance in *Aspergillus* spp. a challenging task.

Challenges and Future Perspectives

At the present time, culture-based methods still dominate diagnosis of fungal infections and related antifungal resistance. Yet, molecular diagnostics have the potential to transform the modern clinical microbiology laboratory by providing rapid identification of infecting organisms while profiling the presence of inherently resistant species or acquired genetic mechanisms that alter susceptibility to antifungal drugs. However, molecular diagnosis of antifungal drug resistance must overcome a few hurdles to open up the door for routine clinical use. A key to the successful application of molecular technology for antifungal resistance is a strong association between genetic mechanisms, in vitro reduced susceptibility, and clinical outcome. Fully elucidating genetic mechanisms of various antifungal drugs in different fungal species is a prerequisite to utilize novel molecular tools to probe drug resistance. In addition, a systematic evaluation relationship between specific molecular mechanisms of resistance and clinical outcome in well-designed clinical trials is critical to establish the value of molecular diagnosis in the clinical settings. Another challenge is the inefficient sample preparation, which must be improved and automated. The difficulties of efficient extraction from the primary samples exist in two aspects: first, the low circulating levels of pathogens or naked DNA for most IFIs, especially invasive aspergillosis, and, second, the relatively large amount of human DNA compared to target fungal DNA present in the extracts that may either inhibit downstream detection or cross-react with fungal primers/probes to cause false-positive results. Considerations to address the unfavorable pathogen-human DNA ratio have involved novel technologies for pathogen DNA enrichment. One uses selective lysis of blood cells to reduce the human DNA background in an extracted blood sample (Molzym). The manual version of this sample preparation is known as MolYsis, and the automated platform is named as SelectNA^{TM}.

The MolYsis method showed good performance with whole blood from patients with candidemia (Wellinghausen et al. 2009), but there was no head-to-head study to measure the exact benefit in terms of extraction efficiency of fungal DNA from using this particular method. Another technology called LOOXSTER® (SIRS-Lab) utilizes the methylation differences between bacterial/fungal DNA to enrich pathogen DNA in the clinical sample by affinity chromatography, but the performance on antifungal resistance has not been evaluated. Clearly, novel and practical approaches and massive evaluation in clinical settings of these methods are urgently needed for sample preparation. The full potential of molecular diagnosis of both primary and secondary antifungal resistance will not be fully reached until a phenomenal advance in specimen preparation is achieved.

Conclusion

In summary, molecular diagnostic platforms are ideal for rapid detection of drug resistance in fungal pathogens. However, more comprehensive and deeper insights into genetic mechanisms of antifungal resistance, extensive validation in well-designed clinical trials, as well as innovative methods for highly efficient and selective fungal nucleic acid extraction from primary samples are needed for the implementation of molecular diagnosis of drug resistance into the clinical settings serving as the guidance of antifungal therapy.

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Azole Resistance in *Aspergillus fumigatus*: Mechanisms, Route of Resistance Selection, and Clinical Implications

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Abstract

The medical triazoles, itraconazole, voriconazole, and posaconazole, are the most widely used drugs for the management of infections caused by the saprophytic mold *Aspergillus fumigatus*. However, acquired azole resistance in *A. fumigatus* is an emerging problem that compromises the clinical efficacy of azole antifungals. Several mutations in the cyp51A gene of *A. fumigatus* affect the activity of

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all mold-active antifungal azoles. These mutations result in the complete loss of activity of a specific azole and are commonly associated with cross-resistance to other azoles. While azole resistance may emerge during antifungal therapy of individual azole-treated patients, selection of resistance can also occur in the environment. The selection for azole resistance within the environment poses an emerging global threat as mutations associated with environmental resistance have now been detected with increasing frequency in multiple European countries, Asia, the Middle East, and Africa.

Keywords

Aspergillus fumigatus • Azole- resistance • Epidemiology • Clinical implications

Introduction

Aspergillus spp. are cosmopolitan filamentous fungi often found in soil, where they thrive as saprophytes, but occasionally they infect living hosts including plants, insects, and mammals (Pitt 1994; Heitman 2011). Aspergillosis is an umbrella term coined by Hinson, Moon, and Plummer in 1952, encompassing a range of conditions from localized to fatal disseminated infections caused by fungi belonging to the genus *Aspergillus*.

In humans, *Aspergillus fumigatus* is the most common and life-threatening airborne fungal pathogen, especially among immunocompromised hosts (Kwon-Chung and Sugui 2013). *A. fumigatus* was first described as a pathogen by Fresenius in 1863 when it was isolated from the bronchi and alveoli of a great bustard (*Otis tarda*) (Fresenius 1863). It has been recognized for most of this century as a pathogen, capable of invading the human lungs, brain, paranasal sinuses, eyes, pharynx, skin, and open wounds, but most commonly this has been observed in immunocompromised individuals (Latge 1999; Meersseman et al. 2004; Denning 1998; Segal and Romani 2009; Patterson and Strek 2010; DeLone et al. 1999; Gefter 1992; Germaud and Tuchais 1995; Galimberti et al. 1998; Garrett et al. 1999). The potential of *Aspergillus* spp. to cause severe disease in humans was recognized by Young and colleagues in 1966 when a series of 98 patients was described with invasive aspergillosis (Young et al. 1970).

Depending on the immunological status of the host, inhalation of *A. fumigatus* spores (conidia) into the lungs can cause multiple diseases (Latge 1999; Greub and Bille 1998; Henriet et al. 2013; Gallin and Zarember 2007). Healthy hosts are able to ward off infections, so that severe illness usually results only from massive or long-term exposure (Pitt 1994; Arne et al. 2011). It can cause acute and subacute invasive disease in immunocompromised patients and chronic pulmonary aspergillosis (CPA) and aspergilloma in immunocompetent patients with underlying lung disease (Smith and Denning 2011). In CPA, *Aspergillus* gradually destroys lung tissue, resulting in the formation and expansion of cavities as well as the formation of fungal balls (aspergilloma) within these cavities (Smith and Denning 2011; Saraceno et al. 1997; Soubani and Chandrasekar 2002).

Notably, the population at risk for invasive aspergillosis (IA) is expanding due to recent advances in human medicine including patients on steroids and chemotherapy treatment resulting in severe neutropenia, stem cell and solid organ transplantation, and advances in the development of immunosuppressive and myeloablative therapies for autoimmune and neoplastic disease, later stages of AIDS, and hereditary immunodeficiencies such as chronic granulomatous disease (Brown et al. 2012; Patterson 2005). Approximately 300,000 people are estimated to develop IA annually, 1.5–10 % of the millions of highly immunocompromised patients at risk worldwide (Brown et al. 2012). The global burden of CPA has recently been estimated at three million patients (Brown et al. 2012; Denning et al. 2011).

Phylogeny and Subgeneric Taxonomy of *Aspergillus* spp.

Polyphasic taxonomy has had a major impact on species concepts in the genus *Aspergillus* (Peterson et al. 2008; Peterson 2008), as shown in Fig. 1. The genus has been subdivided into 22 distinct sections, of which *Fumigati, Circumdati, Terrei, Nidulantes, Ornati, Warcupi, Candidi, Restricti, Usti, Flavipedes*, and *Versicolores* contain clinically relevant species (Peterson et al. 2008). Although there are more than 200 known species in the genus, only a small number is associated with infection. Among them, *A. fumigatus* (subgenus *Fumigati*, section *Fumigati*), *A. flavus* (subgenus *Circumdati*, section *Flavi*), and *A. niger* (subgenus *Circumdati*, section *Nigri*) are the most frequently encountered species (Greub and Bille 1998; Peterson et al. 2008; de Hoog et al. 2009). Others, such as *A. terreus* (subgenus *Terrei*, section *Terrei*), *A. versicolor* (subgenus *Nidulantes*, section *Versicolores*), and *A. nidulans* (subgenus *Nidulantes*, section *Nidulantes*), are occasionally isolated from clinical specimens (Balajee 2009). The impact of new taxonomy of *Aspergillus* spp. on antifungal susceptibility profiles will be discussed below.

Treatment of *A. fumigatus* Diseases in Humans

Triazole antifungals play an important role in the management of *Aspergillus* diseases (Herbrecht et al. 2002; Walsh et al. 2008). These agents exert their antifungal activity by blocking the demethylation of lanosterol, thereby inhibiting the synthesis of ergosterol, a key lipid in the cell membrane of fungi. They have an expanded spectrum with fungicidal activity against a wide spectrum of molds as well as enhanced activity against *Candida* spp. and other yeasts (Denning 1998). Three triazole compounds (itraconazole, voriconazole, and posaconazole) have been clinically licensed for and are currently in use for the prevention and treatment of invasive aspergillosis (EMA 2012a, b). A fourth triazole, isavuconazole, is currently in phase III clinical development for treatment of aspergillosis and expected to be licensed in the near future (Seyedmousavi et al. 2015). Itraconazole, voriconazole, posaconazole, and isavuconazole have been shown to be fungicidal against *Aspergillus* spp. (Mohr et al. 2008; Guinea et al. 2008; Pfaller et al. 2002). Itraconazole is

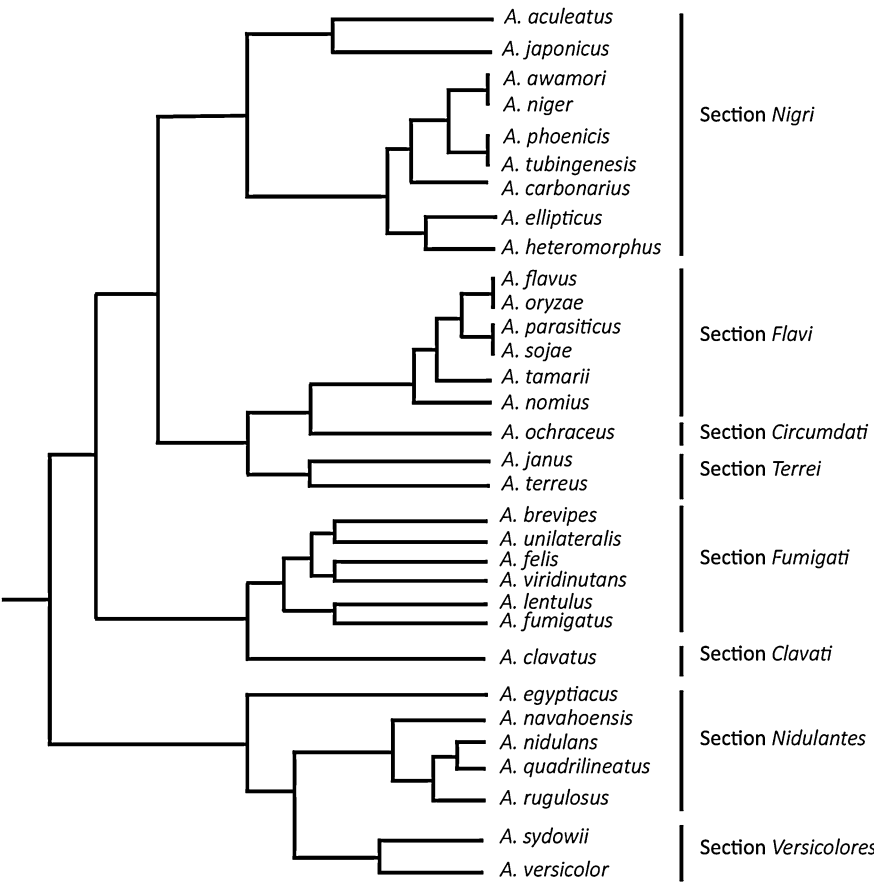

Fig. 1 The phylogenetic relationships of ten gene regions of *Aspergillus* species

commonly used for the treatment of chronic pulmonary disease (EMA 2012a, b), while voriconazole is the treatment of choice for IA (EMA 2012b). Posaconazole is licensed for prophylaxis in patients receiving remission induction chemotherapy for acute myelogenous leukemia (AML) or myelodysplastic syndromes (MDS) and in hematopoietic stem cell transplant (HSCT) recipients who are undergoing high-dose immunosuppressive therapy for graft-versus-host disease. It is also licensed in some countries for salvage therapy of IA in patients with disease that is refractory to amphotericin B or itraconazole or in patients who are intolerant of these agents (Herbrecht et al. 2002; Walsh et al. 2008; Cornely et al. 2007; Ullmann et al. 2007).

Besides azoles, only amphotericin B and the echinocandins (caspofungin, micafungin, and anidulafungin) have demonstrated clinical activity against *Aspergillus* diseases. None of these agents however have been directly compared with azoles in randomized controlled clinical trials, and they are therefore considered as alternative agents for the therapy of IA (Walsh et al. 2008).

Table 1 Examples of intrinsic resistance against antifungals in *fumigatus* and non-*fumigatus* *Aspergillus* species

	A.fumigatus	*A.lentulus*	*A.nidulans*	*A.quadrilineata*	*A.terreus*	*A.calidoustus*	*A.tubigenesis*	*A.niger*
AmB	Green	Yellow	Yellow	Green	Red	Yellow	Yellow	Green
ITC	Green	Red	Green	Green	Green	Red	Yellow	Green
VRC	Green	Red	Green	Green	Green	Red	Yellow	Green
POS	Green	Yellow	Green	Green	Green	Red	Yellow	Green
Ecan	Yellow	Yellow	Yellow	Red	Yellow	Yellow	Yellow	Yellow

AmB amphotericin B, *ITC* itraconazole, *VRC* voriconazole, *POS* posaconazole, *Ecan* echinocandins. *Green*, sensitive; *yellow*, intermediate susceptibility; *red*, no sensitivity

Intrinsic Versus Acquired Azole Resistance in *A. fumigatus*

Although *Aspergillus* spp. are generally susceptible to the medical triazoles, intrinsic and acquired resistance has been documented. In general there are two types of resistance: microbiological versus clinical. Microbiological resistance relates to an in vitro susceptibility test, which indicates that the activity of a certain drug against the pathogen is low or absent and corresponds with a high probability of treatment failure. In vitro resistance can be primary (intrinsic) or secondary (acquired). Primary resistance occurs naturally, without prior exposure to the drug. Secondary resistance is generated following exposure to an antifungal and may be associated with altered gene expression or with the acquisition of mutations (Diekema et al. 2009; Verweij et al. 2009a). Clinical resistance, however, occurs when a patient fails to respond to antimicrobial therapy despite the administration of an antifungal. Clinical resistance may indicate microbiological resistance of the pathogen but can also be a consequence of other factors such as drug pharmacokinetics or the immune status of the host (Diekema et al. 2009).

Recent changes in the taxonomy of *Aspergillus* spp. have had major implications on our understanding of intrinsic drug susceptibility profiles (Van Der Linden et al. 2011a). New sibling species of *A. fumigatus* exhibit in vitro susceptibility profiles that differ significantly from that of *A. fumigatus*. While acquired azole resistance is an emerging problem in *A. fumigatus* (Verweij et al. 2007, 2009b), some other *Aspergillus* spp. are intrinsically more resistant to specific classes of antifungal agents (Table 1). Minimum inhibitory concentrations (MICs) of amphotericin B and azoles for some of the non-*fumigatus Aspergillus* spp. are elevated compared to *A. fumigatus* (Van Der Linden et al. 2011a). The MICs of *A. flavus* clinical isolates to amphotericin B are consistently twofold dilution steps higher than those of *A. fumigatus* (Gomez-Lopez et al. 2003). Using Clinical Laboratory Standards Institute (CLSI) methodology (CLSI 2008), *A. nidulans* was shown to have MIC values of 1–2 mg/L of amphotericin B, which is

higher than commonly observed with *A. fumigatus* (van der Linden et al. 2013). In the section *Usti*, the azoles are not active against *A.calidoustus* with MICs of ≥8 mg/L, and the other classes of antifungal drugs also appear less active as compared with their activity against *A. fumigatus*. For example, the MICs of amphotericin B were found to range from 1 to 2 mg/L, which is relatively high (Varga et al. 2008). Resistance of *A. terreus* to amphotericin B is well recognized (Lass-Florl et al. 2009). The black aspergilli, *Aspergillus* section *Nigri*, exhibit three different azole susceptibility patterns: low azole MICs, high MICs, and a less common paradoxical effect. However, the classification of these fungi by azole MICs does not match with their species classification making it difficult to determine if these alterations in drug susceptibility represent intrinsic or acquired properties of these molds (Alcazar-Fuoli et al. 2009).

Azole Resistance Phenotypes in *A. fumigatus*

Antifungal drug resistance is normally quantified by MIC determination. Both the CLSI and European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST) have developed and standardized phenotypic methods that enable the reliable and reproducible determination of the MIC for conidia-forming molds such as *Aspergillus* spp. (CLSI 2008; Subcommittee on Antifungal Susceptibility Testing of the EECfAST 2008). The MIC represents the lowest drug concentration resulting either a significant reduction or complete lack of fungal growth (European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility T 2008). Interpretation of resistance from the results of MIC testing requires the application of breakpoints. There are currently three sets of breakpoints and epidemiological cutoff values available. The first was published in 2009 by Verweij et al. based on clinical experience and the available knowledge at that time (Verweij et al. 2009b). More recently, the CLSI (Espinel-Ingroff et al. 2010) and the EUCAST-AFST (Arendrup et al. 2012a; Hope et al. 2013) have published guidelines in which strains with MICs <2 mg/L are considered susceptible for itraconazole and voriconazole, while those >2 mg/L are considered resistant; while for posaconazole strains with an MIC ≤0.25 are considered susceptible, those with an MIC >0.5 mg/L are considered resistant.

Azole-Resistant Genotypes in *A. fumigatus*

Several mechanisms of acquired resistance have been described in *Aspergillus* spp. Azole resistance has most commonly been associated with alterations in cyp51A, which represents the target enzyme of the azoles (Verweij et al. 2007). The corresponding phenotype depends on the particular mutation and may affect the activity of multiple triazoles. The most frequently characterized polymorphisms associated with resistance are found at codons 54, 98, 138, 220, and 448, although other single nucleotide polymorphisms (SNPs) have been reported (Verweij et al. 2007; Snelders et al. 2008; Mellado et al. 2004, 2007; Chen et al. 2005;

Howard et al. 2006). In addition to coding SNPs, other mutations have been described in azole-resistant *A. fumigatus* isolates which increase cyp51A expression (Verweij et al. 2007; Snelders et al. 2008; Mellado et al. 2004, 2007). At the present time, three common genetic variants associated with resistance to azoles have been described: a 34 base pair tandem repeat combined with a L98H substitution in the Cyp51A gene (TR_{34}/L98H) (Mellado et al. 2007), a 53 bp tandem repeat without substitutions in the Cyp51A gene (TR_{53}) (Camps et al. 2012a), and recently a 46 bp tandem repeat with two substitutions in the cyp51A gene (TR_{46}/Y121F/T289A)(van der Linden et al. 2013).

Notably, there are several studies indicating that mutations unrelated to Cyp51A might be associated with azole resistance in *Aspergillus* spp. Buied et al. reported that Cyp51B overexpression was associated with azole resistance mechanism in *A. fumigatus* (Buied et al. 2013). Other researchers demonstrated that the changes in a drug efflux pump of *Aspergillus* spp. can also contribute to the emergence of microbiological resistance. Overexpression of the *cdr1B* efflux transporter genes (Fraczek et al. 2013), modifications in AfuMDR1 and AfuMDR2 genes (da Silva Ferreira et al. 2004), and changes in expression of AfuMDR3 and AfuMDR4 (da Silva Ferreira et al. 2004; Nascimento et al. 2003) were all linked to high-level azole resistance in *A. fumigatus*. Similarly, Krishan-Natesan et al. showed that overexpression of ATP-binding cassette transporters and changes in major facilitator superfamily class efflux pumps contribute to voriconazole resistance in *A. flavus* (Natesan et al. 2013). Camps et al. reported a novel resistance mechanism, consisting of a mutation in the CCAAT-binding transcription factor complex subunit HapE (Camps et al. 2012b). A substitution was found in P88L within the exonic region of HapE gene resulting in an azole-resistant phenotype. Unlike cyp51A-mediated resistance mechanisms, HapE was associated with a fitness cost (Arendrup et al. 2010). Finally, as is the case for *A. fumigatus*, azole resistance in other species of *Aspergillus*, such as *A. flavus* (Liu et al. 2012) and *A. terreus* (Arendrup et al. 2012b), may be also caused by alterations and overexpression of the azole target 14α-demethylase (Buied et al. 2013). Collectively these observations indicate that acquired azole resistance is a clinical challenge that is not restricted to *A. fumigatus*, and multiple mechanisms may contribute to this resistance.

Routes of Azole Resistance Development: Clinical Versus Environmental

In *A. fumigatus* two routes of resistance selection have been reported. First, resistance may develop in patients treated with azole antifungal agents. Azole resistance has been reported in patients with chronic cavitating *Aspergillus* diseases, such as aspergilloma, that received long-term azole therapy (Howard et al. 2009). In these patients the initial infection was caused by an azole-susceptible isolate, but through therapy azole-resistant isolates were cultured. Azole resistance in isolates from these patients has been associated with point mutations only. Until recently, this mode of

resistance development was believed to account for the majority of cases of acquired resistance.

A second route of selection for acquired resistance has recently been suggested. The exposure of *A. fumigatus* to azole 14α-demethylase inhibitors (DMIs) in the environment (Verweij et al. 2009a; Snelders et al. 2008, 2009, 2012), although still controversial, has been linked to the emergence of azole resistance (Enserink 2009). Azole fungicides inhibit fungal Cyp51A activity and are used abundantly for crop protection and material preservation. *A. fumigatus*, which is a saprophytic fungus, is thus hypothesized to develop azole resistance in response to environmental exposure to azole fungicides (Verweij et al. 2009a). In support of this hypothesis, five DMI fungicides were identified with in vitro activity against *A. fumigatus* and with molecule structures that are highly similar to that of the medical triazoles (Verweij et al. 2009a; Snelders et al. 2012).

Molecular Basis of Environmental Resistance

The first strains that were reported to be associated with environmental acquisition of resistance were found to contain TR_{34}/L98H in the Cyp51A gene (Snelders et al. 2008). Duplication of the 34 bp sequence in the promoter region was found to significantly increase the expression of Cyp51A gene; however it was not sufficient for the full azole-resistant phenotype. Similarly isolates harboring the L89H mutation only were not fully azole resistant. Introduction of both mutations into the same strain was required to reproduce the multi-azole-resistant phenotype (Mellado et al. 2007).

More recently a second set of mutations associated with presumed environmental acquisition of resistance was described (TR_{46}/Y121F/T289A) (van der Linden et al. 2013; Chowdhary et al. 2014; Vermeulen et al. 2012). TR_{46}/Y121F/T289A conferred high resistance to voriconazole and was associated with treatment failure in patients with IA. Interestingly, both sets of mutations consist of a combination of genomic changes that include a tandem repeat in the promotor region of CYP51a in addition to point mutations within the gene itself. The TR_{46}/Y121F/T289A resistance mechanism included three genomic changes, and it appears unlikely that these would have evolved independently during azole therapy in all the reported cases (van der Linden et al. 2013).

Epidemiology of Azole Resistance in *A. fumigatus*

Acquired triazole resistance among *Aspergillus* spp. is an emerging phenomenon (Pfaller et al. 2008, 2011; Verweij et al. 2002) that has now been reported across multiple continents (Snelders et al. 2008; Howard et al. 2009; Mellado et al. 2013; Lockhart et al. 2011; Rath et al. 2012; Seyedmousavi et al. 2013a; van der Linden et al. 2011b; Chowdhary et al. 2012a; Tashiro et al. 2012). A number of studies report that azole resistance in *A. fumigatus* has increased in recent years, with both

intrinsic and acquired resistances having been documented in different regions and patient groups. Therefore, knowledge of the local epidemiology of azole-resistant *Aspergillus* diseases is important with respect to the development of management strategies.

The overall incidence of azole resistance has been increasing. Reports from the Netherlands and Manchester, United Kingdom, display an alarming increase of azole resistance in *A. fumigatus* since 1998 (Fig. 2; Verweij et al. 2009b). The widespread increase of azole resistance in Manchester is primarily related to long-term azole treatment in patients (Howard et al. 2009). In Manchester, the first published case of itraconazole resistance in *A. fumigatus* appeared in 1997 (the isolate originated from the late 1980s) (Denning et al. 1997), then in 2000 epidemiological surveys showed a 2 % prevalence of itraconazole resistance (Moore et al. 2000), and in 2007 the percentage of patients with an azole-resistant *A. fumigatus* increased up to 15 % (Verweij et al. 2007; Pfaller et al. 2008).

In the Netherlands azole resistance increased dramatically from 2.5 % in 2000, to 4.9 % in 2002, to 6.6 % in 2004, to 10 % in 2009 (van der Linden et al. 2011b). TR_{34}/L98H first emerged in clinical *A. fumigatus* isolates from the Netherlands in 1998 and is now endemic in Dutch hospitals (van der Linden et al. 2015). This change in epidemiology is of major importance as >90 % of Dutch clinical azole-resistant isolates are believed to share this molecular mechanism of resistance (Verweij et al. 2009a; Snelders et al. 2008). Both invasive and noninvasive aspergillosis

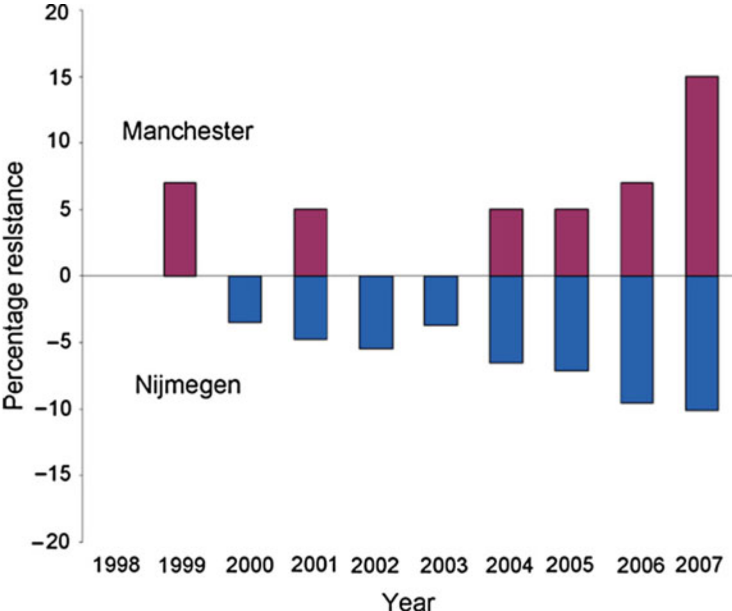

Fig. 2 The percentage of patients with azole-resistant *A. fumigatus* strains in Manchester (United Kingdom) and Nijmegen (the Netherlands) between 1998 and 2007 (Adapted from Verweij et al. (2009b))

infections due to TR_{34}/L98H have been reported in azole-treated as well as in azole-naive patients (Snelders et al. 2008; van der Linden et al. 2011b). Importantly, the geographic area where TR_{34}/L98H strains have been reported coincides with the region with the most intensive use of fungicides, suggesting that an environmental source is very likely (Snelders et al. 2008, 2009). Azole resistance, due to the TR_{34}/L98H resistance mechanism, has also been reported in clinical *A. fumigatus* isolates from other European countries and more recently from China and India (Snelders et al. 2008; Howard et al. 2009; Mellado et al. 2013; Lockhart et al. 2011; Rath et al. 2012; Seyedmousavi et al. 2013a; Chowdhary et al. 2012a, b; Burgel et al. 2012; Morio et al. 2012; Mortensen et al. 2010; Lagrou et al. 2008). Genotyping studies indicate that in Europe, TR_{34}/L98H isolates represent offspring of a common ancestor (Camps et al. 2012c) and could have developed locally, possibly in the Netherlands, and then subsequently spread across countries through wind-dispersed conidia or ascospores.

Similar findings have been observed with the newly emerging TR_{46}/Y121F/T289A mutation which is spreading rapidly in Dutch hospitals (van der Linden et al. 2013; Chowdhary et al. 2014). Strains bearing this TR_{46}/Y121F/T289A mutation have been recovered from epidemiologically unrelated patients, most of whom were azole naive, as well as from the environment. Furthermore, genetic typing of isolates bearing these mutations showed clustering of these strains in separate clades from wild-type (azole-susceptible) isolates.

As with strains bearing the TR_{34}/L98H mutation, the rapid geographical migration of TR_{46}/Y121F/T289A strains in Dutch hospitals indicates that this resistance mechanism will likely spread more widely. Indeed, a lethal case of azole-resistant invasive aspergillosis due to TR_{46}/Y121F/T289A was recently reported in a patient from Belgium, suggesting that this strain has already spread beyond the borders of the Netherlands (Vermeulen et al. 2012). The presence of TR_{46}/Y121F/T289A mutation has also been reported in environmental *A. fumigatus* strains in India, which were also cross-resistant to commonly used azole fungicides (Chowdhary et al. 2013, 2014). From a global perspective, fungicide use is second highest in the Asia-Pacific regions (24 %), preceded only by Western Europe (37 %) (Christen Rune Stensvold et al. 2012).

Clinical Implications of Azole Resistance and Impact of Underlying Diseases

The clinical implications of azole resistance depend on the route of resistance selection (Table 2). The patient profiles are very different with patients with chronic lung diseases, cavitary pulmonary lesions, and chronic azole therapy at risk for resistance selection through patient therapy. However, azole-resistant aspergillosis can occur in *any* patient if infected through the environmental route, causing any kind of *Aspergillus* disease, including ABPA and IA. For the environmental route, there are no clear clinical risk factors other than living in an area where environmental azole resistance is found. Both environmental sampling of soil or ambient air

Table 2 Implications for the patient and environmental routes of azole resistance selection in *A. fumigatus*

Patient route	Environmental route
Chronic *Aspergillus* diseases (usually with chronic lung disease)	All *Aspergillus* diseases including IA
Cavitary lesion	No specific lesion
(Previous) azole therapy in all patients	(Previous) azole therapy in one third of patients
High diversity of mutations; both Cyp51A mediated and others	Low diversity of azole resistance mutations associated with the Cyp51A gene
Cyp51A point mutations	Tandem repeat
Both azole-susceptible and azole-resistant phenotypes simultaneously present	Both azole-susceptible and azole-resistant phenotypes simultaneously present
High genetic diversity between azole-resistant isolates from unrelated patients	Low genetic diversity between azole-resistant isolates from unrelated patients
A. fumigatus colonies may show an abnormal phenotype, sporulation, and growth rate	No apparent fitness cost

for the presence of azole-resistant *A. fumigatus* and testing collection of clinical isolates will help to determine if azole resistance is an issue in the patient population. Previous studies indicate that at least 70 isolates need to be tested in order to detect azole-resistant isolates (van der Linden et al. 2011b).

There are currently no controlled trials that have evaluated the effect of azole resistance on the probability of treatment success. However, case series of patients with azole-resistant chronic *Aspergillus* diseases and azole-resistant IA have found that the recovery of an azole-resistant isolate is associated with a higher probability of azole treatment failure (Snelders et al. 2008; Howard et al. 2009; Mellado et al. 2013; Rath et al. 2012; Hodiamont et al. 2009; van der Linden et al. 2009; van Leer-Buter et al. 2007; Warris et al. 2002; Hamprecht et al. 2012).

In patients receiving chronic azole therapy, a wide range of mutations was found in azole-resistant *Aspergillus* spp. isolates (Howard et al. 2009). Consistent with these findings, cross-resistance among the azoles varied between isolates. Of 34 itraconazole-resistant isolates studied, 65 % (Soubani and Chandrasekar 2002) were cross-resistant to voriconazole and 74 % (Denning et al. 2011) were cross-resistant to posaconazole, likely reflecting the similarity in structure between itraconazole and posaconazole. Thirteen of 14 evaluable patients had prior azole exposure; eight infections failed therapy, and five failed to improve (Howard et al. 2009).

Two case series of patients with environmentally acquired azole-resistant IA were reported from the Netherlands (van der Linden et al. 2011b, 2013). In the first study, seven of eight (88 %) patients with proven or probable, culture-positive IA due to *A. fumigatus* harboring the TR_{34}/L98H resistance mechanism died at 12 weeks. Voriconazole was the initial treatment choice in most patients. In a second study the emergence of a voriconazole resistance associated with the TR_{46}/Y121F/T289A mutation was reported (van der Linden et al. 2013). At 12 weeks after recovery of

the TR_{46}/Y121F/T289A isolate, four of eight patients with IA had died and two patients had persisting infection. In addition, a number of single cases have been described harboring TR_{34}/L98H (Snelders et al. 2008; Howard et al. 2009; Mellado et al. 2013; Rath et al. 2012; Hodiamont et al. 2009; van der Linden et al. 2009; van Leer-Buter et al. 2007; Warris et al. 2002; Hamprecht et al. 2012) or TR_{46}/Y121F/T289A mutations (Vermeulen et al. 2012). In all cases, patients with infection due to an azole-resistant isolate failed to respond to azole therapy. Primary invasive infections due to resistant *A. fumigatus* isolates have been reported involving the lung (Verweij et al. 2007; Howard et al. 2009), bone (Hodiamont et al. 2009), and brain (Howard et al. 2009; van der Linden et al. 2009) as well as from respiratory isolates in allergic bronchopulmonary aspergillosis (Howard et al. 2006). Importantly, there is no apparent risk of spread of azole-resistant isolates to other patients, consistent with the observation that *A. fumigatus* does not sporulate in the human host during invasive infection.

These data notwithstanding, it must be recognized that there are numerous other factors that can impact treatment success in *Aspergillus* infection. Patients with refractory underlying malignancy are prone to failure of therapy, even if the infection is caused by an azole-susceptible isolate. Azole exposure might have been insufficient in patients failing therapy, and as most patients were culture positive, treatment might have been initiated relatively late in the course of the infection. Furthermore, azole-resistant infection might occur predominantly in patients in poor clinical condition, compared to wild-type isolates.

In the absence of robust clinical evidence, experimental models of *Aspergillus* infection may help us to understand the implications of azole resistance on treatment efficacy. In animal models of IA, the MIC has been found to be a powerful predictor of the efficacy of voriconazole and posaconazole (Mavridou et al. 2010a; Howard et al. 2011; Seyedmousavi et al. 2014). There was a clear association between the MIC and efficacy in the animal model, with increasing MIC corresponding with decreasing efficacy (Mavridou et al. 2010a, b; Seyedmousavi et al. 2014). The results of combination therapy also suggested that a combination of voriconazole or posaconazole with an echinocandin may be effective (Seyedmousavi et al. 2013b, c; Lepak et al. 2013). However, the synergistic interaction may be lost when an azole-resistant isolate (voriconazole MIC, 4 mg/L) is the infectious agent.

These results highlight a significant clinical problem. With the increased use of non-culture diagnostics and imaging studies for the early detection of IA, the isolation of *A. fumigatus* by culture is relatively uncommon. As voriconazole resistance rates increase (van der Linden et al. 2013; Snelders et al. 2008), the choice of empiric therapy in culture-negative patients is difficult. While preclinical studies suggest that the efficacy of liposomal amphotericin B (L-AmB) is preserved in azole-resistant infections (Seyedmousavi et al. 2013d), some post-marketing studies suggest that L-AmB may be less active than voriconazole against *Aspergillus* (Nivoix et al. 2008; Baddley et al. 2010; Denning and Bowyer 2013). Balancing the benefits of voriconazole therapy against the chance of a resistant isolate requires up-to-date knowledge of local resistance rates, which is often not available to clinicians.

Concluding Remarks

Azole-resistant *Aspergillus* infection is commonly associated with treatment failure. Although azole resistance may emerge during antifungal therapy of individual azole-treated patients, selection of resistance may also occur in the environment. The evolving epidemiology of strains bearing TR_{34}/L98H and TR_{46}/Y121F/T289A mutations indicates that these resistance mechanisms will be increasingly observed in European Union member states and outside Europe. Given the prominent role of azoles in the management of *Aspergillus* diseases, successful management of azole-resistant *Aspergillus* diseases in patients with infection caused by *A. fumigatus* is a challenge and suggests a need to critically examine our use of azole-type antifungals in agriculture.

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The Ins and Outs of Azole Antifungal Drug Resistance: Molecular Mechanisms of Transport

Martin Zavrel, Brooke D. Esquivel, and Theodore C. White

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Abstract

Azoles are a major class of antifungal drugs commonly used to treat pathogenic fungi. Azole antifungals are relatively inexpensive, share similar chemical structures, and are effective against most fungal species. Azoles target a crucial enzyme in the ergosterol biosynthesis pathway whose inhibition leads to reduced

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fungal growth. Azole treatment, combined with the host's immune system, results in the elimination of the fungus from the host. Since azoles are fungistatic instead of fungicidal, their prolonged use and abuse often results in the development of resistance, which is a serious clinical problem in antifungal therapy. The main mechanisms by which fungi become resistant to azoles are increased efflux of the drug from the fungal cell, and modifications in the sterol biosynthesis pathway, especially in the azole target enzyme. In general, all known fungal pathogens share these two basic types of resistance mechanisms, although the specific efflux pumps or mutations in the sterol pathway may be unique for each fungus. This chapter summarizes the development of azole resistance in the major human fungal pathogen, *Candida albicans*, and compares these mechanisms to those in other fungal pathogens. Resistance to other non-azole antifungal drugs is also discussed.

Keywords
Azoles • Antifungal drug resistance • Drug efflux • Membrane transporters • Pathogenic fungi

Introduction

The frequency of patients with immune deficiency has grown dramatically in the last three decades due to the AIDS epidemic, increased organ and bone marrow transplants, as well as aggressive cancer chemotherapy. All of these conditions compromise the immune system of the host and have led to an increased emergence of opportunistic fungal pathogen infections as a result of these natural or artificially induced immune deficiencies. Not surprisingly, there has also been a dramatic increase in the use of antifungals to treat these infections. As a result of this increased antifungal use, strains resistant to each of the classes of antifungals are emerging. In medicine, the most widely used antifungals are polyenes, azoles, allylamines, echinocandins, and 5-flucytosine. The most common antifungals and their targets are summarized in Fig. 1.

Antifungal drug resistance is difficult to define. Its threshold is set up individually for each drug, organism, and site of infection, respectively. Antifungal drug resistance can be categorized as intrinsic or acquired. Intrinsic resistance is an inherited characteristic of a species or strain that causes antifungal drug therapy to fail. The fungal species and strain will clearly determine which drugs are effective. The main human fungal pathogens and the effectiveness of antifungals to each are presented in Table 1. Acquired resistance, on the other hand, occurs when a previously susceptible isolate develops a resistant phenotype, usually as a result of prolonged treatment with antifungals.

While resistance is a clinical problem, the "90–60" rule states that susceptible isolates respond to appropriate therapy approximately 90 % of the time, while resistant isolates respond to therapy about 60 % of the time, despite their drug resistance (Rex and Pfaller 2002). The 90–60 rule applies to most microbes, including fungal and bacterial pathogens.

There are several factors that affect treatment outcome of a fungal infection, including a variety of characteristics associated with the host, the drug, and the

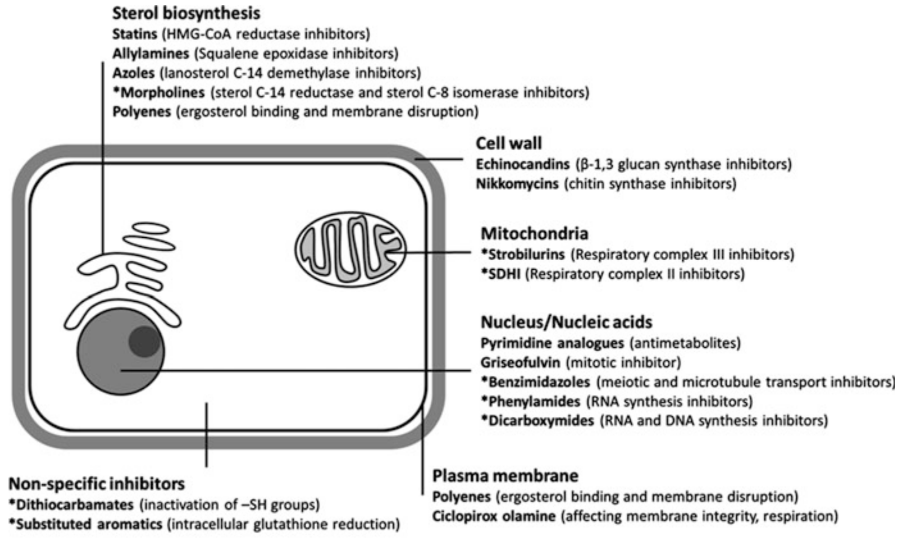

Fig. 1 Antifungal drugs and their site of action

fungus. Generally, antifungal drugs work alongside the host immune system to control the infection. Infections in immunocompromised patients are generally more recalcitrant to treatment because of the lost additive effect from the host immune system. The infection location (systemic, skin, oral mucosa, vaginal mucosa, eye, brain, etc.) is also an important factor in drug resistance, as some infection sites may be less accessible to drug therapy.

Finally, the cell type or morphological stage of the fungi can alter drug efficacy. Most antifungals are effective only against actively growing fungi, while dormant stages with minimal metabolic activity are usually not responsive to drug. Most fungi exist as various cell types or morphologies, including yeast stages (blastospores), pseudohyphae, hyphae, chlamydospores, and conidiospores, each of which can have a specific susceptibility to antifungal drugs. In some *Candida albicans* strains, yeast forms can further switch between additional phenotypes, including white and opaque. Besides phenotypic switching, some fungi also exist in different serotypes. These phenotypic switches or different serotypes are usually distinguished by different cell surface markers and display alterations in their transcriptional profiles, often effecting genes involved in antifungal resistance.

In general, the organism's susceptibility to an antifungal is expressed as a minimal inhibitory concentration (MIC). The MIC can be presented as MIC_{50} or MIC_{80}, which is drug concentration leading to 50 % or 80 % reduction in fungal growth, respectively. Protocols for drug susceptibility testing in different organisms have been standardized by the Clinical and Laboratory Standards Institute (CLSI) and by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Both organizations define the specific growth media and conditions for testing of each organism.

Table 1 Antifungal spectrum of activity against common fungi

Organism	Antifungal agent								
	AmB[a,b]	Flu[c]	Itr[c]	Vor[c]	Pos[c]	Anid[d]	Casp[d]	Mica[d]	5-FC
***Aspergillus* species**	+	–	+	+	+	+	+	+	–
A. flavus	±	–	+	+	+	+	+	+	–
A. fumigatus	+	–	+	+	+	+	+	+	–
A. niger	+	–	±	+	+	+	+	+	–
A. terreus	–	–	+	+	+	+	+	+	–
***Candida* species**	+	+	+	+	+	+	+	+	+
C. albicans	+	+	+	+	+	+	+	+	+
C. glabrata	+	±	±	+	+	+	+	+	+
C. krusei	+	–	±	+	+	+	+	+	±
C. lusitaniae	–	+	+	+	+	+	+	+	+
C. parapsilosis	+	+	+	+	+	±	±	±	+
C. tropicalis	+	+	+	+	+	+	+	+	+
Cryptococcus neoformans	+	+	+	+	+	–	–	–	+
***Coccidioides* species**	+	+	+	+	+	±[e]	±[e]	±[e]	–
Blastomyces	+	+	+	+	+	±[e]	±[e]	±[e]	–
***Histoplasma* species**	+	+	+	+	+	±[e]	±[e]	±[e]	–
***Fusarium* species**	±	–	–	+	+	–	–	–	–
Scedosporium apiospermum	±	–	±	+	+	–	–	–	–
Scedosporium prolificans	–	–	–	±	±	–	–	–	–
Zygomycetes	±	–	–	–	+	–	–	–	–

AmB amphotericin B, Flu fluconazole, Itr itraconazole, Vor voriconazole, Pos posaconazole, Anid anidulafungin, Casp caspofungin, Mica micafungin, 5-FC 5-fluorocytosine
[a]Polyenes
[b]Includes lipid formulations
[c]Azoles
[d]Echinocandins
[e]*In vitro* data show that the echinocandins (specifically micafungin) may have variable activity against the dimorphic fungi, depending on whether they are in the mycelial or yeastlike form. Reproduced from "Dodds Ashley et al., Pharmacology of systemic antifungal agents, 2006, Clin. Infect. Dis. 43, pp. S28–S39" with permission from Oxford University Press (Dodds Ashley et al. 2006)

Azole Antifungal Drugs

Ergosterol is a fungal-specific sterol that is homologous to cholesterol and similar sterols found in the membranes of other eukaryotic cells. Ergosterol is an essential component of fungal plasma membranes and its metabolism is tightly regulated by

fungal cells. Alterations in ergosterol levels significantly affect the susceptibility of fungal cells to a variety of environmental stresses. Although similar to plant and animal sterol biosynthesis pathways, ergosterol biosynthesis is unique to fungi, and so its metabolism is a prime target of antifungal therapy with minimal negative effects on the animal or plant host. Currently, there are four classes of drugs that target enzymes in the sterol biosynthesis pathway (Fig. 1), including statins (HMG-CoA reductase inhibitors; used in mammalian cells), allylamines (squalene epoxidase inhibitors), azoles (lanosterol 14α-demethylase inhibitors), and morpholines (sterol C-14 reductase and sterol C-8 isomerase inhibitors; only used agriculturally). One additional class of drugs, polyenes, directly targets ergosterol in fungal membranes and negatively affects the native membrane structure.

Azole antifungals, such as fluconazole, are one of the most commonly used drugs to treat fungal infections. Azoles are fungistatic drugs, which do not kill the cells, and are thus more likely to allow cells to develop resistance. Azole antifungals are a class of organic compounds consisting of a five-membered nitrogen-containing heterocyclic ring (imidazole or triazole), and a halogenated benzene ring. These two prerequisites are crucial for their antifungal activity, as well as their successful import into the fungal cells (Mansfield et al. 2010; Esquivel et al. 2015). Apart from having a nitrogen-containing ring and a halogenated benzene ring, each azole antifungal has a distinctive chemical structure that dictates its pharmacological properties.

Once in the fungal cell, azoles target and inhibit the endoplasmic reticulum (ER)-located cytochrome P450-dependent enzyme, lanosterol 14α-demethylase (*ERG11* in *C. albicans*; see Table 2). At the moment there are numerous azoles on the market, both for medical and agricultural use. The medical azoles are highly specific to the fungal enzyme and generally not effective at inhibiting the human homolog. This is important because these drugs are capable of entering both the host mammalian cells as well as the fungal pathogen cells (Campoli et al. 2013). In contrast, agricultural azoles have less selectivity for the fungal lanosterol 14α-demethylase over its human homolog, which has led to concerns over agricultural azole side effects on humans and animals (Verweij et al. 2009; Warrilow et al. 2013).

Azole Resistance

Many fungal species display intrinsic resistance to azoles, making azole treatment ineffective against such fungi. Their mechanisms of resistance are probably the same as those acquired in other fungal species (see below), but in this case they are naturally occurring. These fungi include *Candida krusei*, most strains of *Candida glabrata*, *Fusarium* species, and the Zygomycetes. Both *C. krusei* and *C. glabrata* are increasing in frequency in oral and systemic candidiasis in patient populations that use azole drugs for treatment or prophylaxis. There are also clear differences in azole susceptibility among strains within one species, due to randomly occurring genetic differences.

Table 2 Genes encoding proteins involved in azole resistance

Organism	*Ca*	*Cg*	*Sc*	*Af*	*Cn*
Lanosterol 14α-demethylase	*ERG11*	*ERG11*	*ERG11*	*cyp51A*	*ERG11*
				cyp51B	
Sterol metabolism regulators	*UPC2*	*UPC2A*	*UPC2*	*srbA*	*SRE1*
		UPC2B	*ECM22*		
MFS transporters	*MDR1*	*FLR1*	*QDR2*	*mdr3* (?)	
	FLU1	*QDR2*	*FLR1* (?)		
Regulators of MFS transporters	*MRR1*	*YAP1*	*GCN4*		
		PDR1	*YAP1* (?)		
ABC transporters	*CDR1*	*CDR1*	*PDR5*	*abcC*	*AFR1*
	CDR2	*PDH1*		*mdr1*	
		SNQ2		*atrF* (?)	
				mdr4 (?)	
Regulators of ABC transporters	*TAC1*	*PDR1*	*PDR1*		
			PDR3		

Ca Candida albicans, *Cg Candida glabrata*, *Sc Saccharomyces cerevisiae*, *Af Aspergillus fumigatus, Cn Cryptococcus neoformans*

Acquired azole resistance was rare in the 1980s, when azoles were primarily used to treat patients with chronic mucocutaneous candidiasis. However, with AIDS epidemic in the 1990s, azole resistance in *C. albicans* became a significant problem as oral candidiasis occurred in over 90 % of all HIV-positive individuals. In recent years, highly active antiretroviral therapy, which restores the patient's immune response, is reducing the frequency of most opportunistic fungal infections and also the need for azole prophylaxis. In addition to *Candida* species, acquired azole resistance has been detected in isolates of *Cryptococcus neoformans* from AIDS patients on prophylactic azole therapy to prevent the recurrence of cryptococcal meningitis and in isolates of *Aspergillus fumigatus* from patients who have received regular treatment with itraconazole or voriconazole. Azoles are also used in surgical wards to prevent systemic candidiasis and as nonprescription drugs to treat fungal skin infections, including athlete's foot. The use of azoles in the environment to treat and prevent fungal pathogens of plant crops is a concern. It has been correlated with an increasing number of agricultural azole-resistant *A. fumigatus* isolates occurring in azole-naïve human patients, and unfortunately these isolates are also resistant to medical azoles such as itraconazole, posaconazole, and voriconazole.

In fungal pathogens naturally susceptible to azoles, there are several mechanisms of azole resistance that can develop as a result of azole treatment (Fig. 2). These mechanisms include (1) reduced azole import; (2) increased azole efflux; (3) import of sterols from host, replacing endogenous ergosterol biosynthesis; (4) increased expression of lanosterol 14α-demethylase, the azole target enzyme; (5) mutation of lanosterol 14α-demethylase, altering its azole affinity; (6) alteration of the ergosterol biosynthetic pathway; and (7) additional nonspecific metabolic adjustments. There are additional possible drug resistance mechanisms that have not yet been observed,

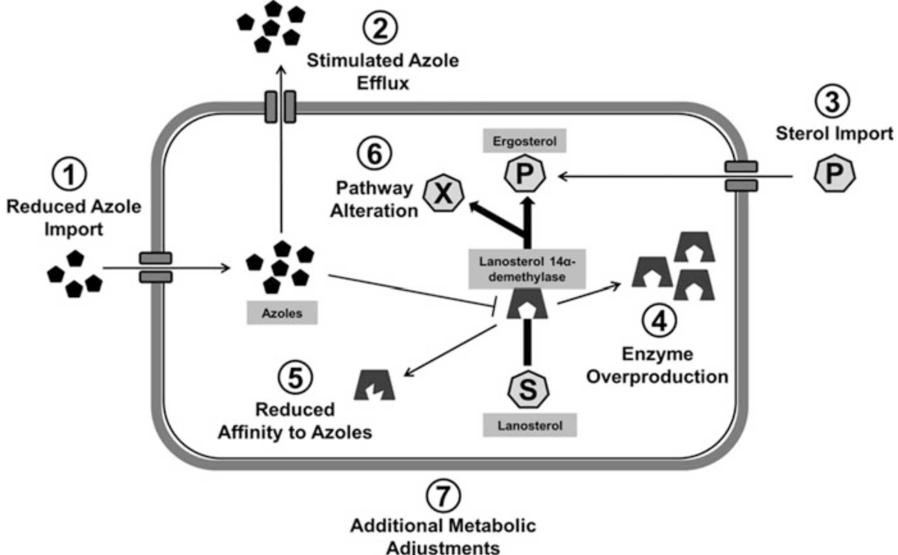

Fig. 2 Potential and actual azole resistance mechanisms in fungi. Actual mechanisms include *2*, *3*, *4*, *5*, *6*, and *7*. A potential mechanism is represented by 1

including intracellular drug sequestration into vesicles/vacuole or azole degradation. Table 2 lists the gene homologs involved in these mechanisms of azole resistance in the most prevalent fungal pathogens and in the model organism *Saccharomyces cerevisiae*.

Resistance in *C. albicans* clinical isolates is not usually the result of a single alteration. The resistance is gradually developed through a series of independent steps. Each acquired mutation decreases azole susceptibility and increases the fitness of the cell under the drug-selective pressure. As a result, this newly acquired allele allows the corresponding clone to outgrow the rest of the population, becoming the new major strain. A series of such steps result in the development of a highly azole-resistant phenotype, where each alteration only partially contributes to the resulting phenotype. This has been well documented in a series of sequential isolates from a single patient during azole treatment (White et al. 1998). All the acquired resistance mechanisms are described in detail in the latter parts of this chapter, but they include mutation and overexpression of the azole target enzyme and efflux of the drug from the fungal cells with two types of efflux pumps. The timing and the order in which each of these changes occurred are unlikely to be critical to the development of resistance, but the sum of each of these alterations results in the resistant phenotype.

Molecular analyses of antifungal drug resistance have focused mainly on *C. albicans*, which is at the moment the best understood, but have included studies in *C. glabrata*, *C. neoformans*, and *A. fumigatus*. This chapter will concentrate

mainly on the mechanisms identified in *C. albicans*, with the discussion of mechanisms identified in other fungi when applicable.

Azole Import

Azole accumulation within a fungal cell is the result of a balance among drug import, retention, and efflux. Azole drug accumulation has been studied primarily in *C. albicans* but has also been described for *S. cerevisiae*, *C. glabrata*, *C. krusei*, *C. neoformans*, and *A. fumigatus* ((Mansfield et al. 2010; Denning et al. 1997; Manavathu et al. 1999; Tsai et al. 2004) and our unpublished results Esquivel et al. 2015). In general, all fungal species susceptible to azoles are expected to import these drugs.

Based on the information obtained from studies on *C. albicans*, azole import into fungi is independent of ATP or pH. It follows a concentration gradient, is saturable, and proceeds via facilitated diffusion through a yet unknown transporter. All analyzed azoles seem to utilize the same import mechanism. The drug import depends strictly on the chemical structure of azoles, requiring both a halogenated benzene ring and an imidazole or triazole ring in one molecule. Omitting either one results in the failure of the molecule to be imported ((Mansfield et al. 2010) and our unpublished results). Additional structures, as well as molecule size, seem rather insignificant. In *C. albicans*, conditions that favor the maximum rate of import include an optimal temperature of 30 °C, cells harvested from exponential phase of growth, and cells grown anaerobically. Hyphae also display a higher import rate than yeast cells.

So far, no natural environmental compounds have been identified that utilize the same import mechanism and thus compete with azoles for import. Thus, the origin and evolutional importance of the import mechanism are unclear. Due to the failure of genetic screens to identify the transporter, it is likely to be either essential for cell viability or part of a multimember family of transporters with overlapping function. Import is specific for fungi, since bacteria (*Escherichia coli*) are incapable of fluconazole import (Mansfield et al. 2010). Import into mammalian cells has been reported as well, with azoles accumulating in their cellular membranes (Campoli et al. 2013).

Drug import into the fungal cell by facilitated diffusion is a mechanism that might be manipulated by the fungal cell to confer drug resistance and may play a role in azole-resistant isolates. However, direct evidence for this is lacking. Clinical isolates of *C. albicans* and other species show high variability in the rate of azole import, as well as different end points for saturation with azoles. Although reduced azole import can be involved in azole resistance, the reduced import levels cannot always be correlated with the individual strain's azole susceptibilities ((Mansfield et al. 2010) and our unpublished results).

Experiments in *A. fumigatus* using itraconazole also showed that drug import is saturable and time and concentration dependent (Manavathu et al. 1999). The correlation between increased itraconazole resistance and its decreased intracellular

accumulation has been reported (Denning et al. 1997; Manavathu et al. 1999); however, this was most likely due to increased azole efflux, since the cells were not depleted of an energy source.

Finally, in *C. glabrata*, reduced activity of squalene epoxidase (Erg1; another enzyme in the ergosterol biosynthesis pathway) results in the disruption of ergosterol biosynthesis and stimulates azole import. Even import of exogenous ergosterol in this strain does not revert the phenotype (Tsai et al. 2004). Thus, this phenomenon cannot be attributed to lower ergosterol content in membranes. Upc2 is the transcription factor that regulates sterol metabolism in yeasts, and the *UPC2* null mutants in *C. albicans*, *C. glabrata*, and *S. cerevisiae* all have decreased ergosterol content, but do not display significant changes in azole import from their respective parents (our unpublished work).

As mentioned, the concentration of azoles within the cells is saturable. This saturation level can be partially correlated with the level of the azole binding target, Erg11 (our unpublished work). Also, the levels of *A. fumigatus* lanosterol 14-α-demethylase (*cyp51A and cyp51B*, two homologs of *C. albicans ERG11* (Mellado et al. 2001)) and resulting ergosterol levels both seem to partially correlate with intracellular itraconazole concentration (Denning et al. 1997). Since mutations in *cyp51A* seem to be the major resistance mechanism in *A. fumigatus* (discussed later), the reduced affinity of this enzyme to azoles may partially result in the observed lower azole levels in the azole-resistant cells (Denning et al. 1997; Manavathu et al. 1999). Also in the case of *C. glabrata*, the reduced Erg1 activity might result in the upregulation of ergosterol biosynthesis pathway, including *ERG11*, thus resulting in higher azole retention (Tsai et al. 2004).

ERG11 Mutation

In *C. albicans*, once the azoles have been imported, they target the ER-located enzyme Erg11, which is an essential enzyme for ergosterol biosynthesis. Erg11 is a P450-dependent enzyme containing a heme moiety in its active site. The azoles bind to the heme iron through an unhindered nitrogen, thus inhibiting the Erg11-catalyzed enzymatic reaction. The second nitrogen of the azoles interacts directly with the apoprotein. The position of the second nitrogen is thought to modulate the affinity of different azoles (Hitchcock 1991). The inhibited Erg11 fails to remove methyl groups at the 14α-carbon of ergosterol precursors, resulting in an accumulation of 14α-methyl sterols, which are further processed into toxic intermediates by sterol C5-desaturase (Erg3) (Odds et al. 2003). These intermediates affect the fluidity and function of the fungal plasma membrane, resulting in higher susceptibility to environmental stress, including host immune system defense mechanisms.

One way of developing azole resistance is mutation of *ERG11* that decreases its affinity for azoles. Depending on the location of the mutation and the amino acid change, the reduced susceptibility can be specific for all azoles or only a subset of them. Gene conversion/mitotic recombination in diploid *C. albicans* can also occur, increasing the azole resistance even further (see below). Permanent *ERG11*

upregulation is the second way of gaining azole resistance through *ERG11*. This could be mediated through mutations in transcription factors such as *UPC2*, through mutations in *ERG11* promoter, or through *ERG11* gene amplification.

The Erg11 amino acid structure is crucial for azole susceptibility or resistance in different fungal species. *ERG11* in intrinsically azole-resistant fungi have lower affinity to azoles, as shown, for example, in *C. krusei* (Venkateswarlu et al. 1997). In azole-susceptible fungi, mutation of *ERG11* can result in a high level of resistance. A list of different Erg11 amino acid exchanges in *C. albicans* has been provided by numerous studies; some of them have summarized these changes (Morschhauser 2002; Marichal et al. 1999). Unique amino acid substitutions in *ERG11* resulting in an azole-resistant phenotype have been reported from all over the world. In general, the amino acid (AA) substitutions can be found all over the enzyme, but they are enriched in three regions, called hotspots. These regions include AA 105–165, which is exposed to the substrate access channel, AA 266–287, which forms the substrate entry site, and AA 405–488, which forms the active site and docks the cytochrome (Marichal et al. 1999).

In *A. fumigatus*, mutations have rarely been detected in *cyp51B* and have rarely been shown to be related to azole resistance (Snelders et al. 2011). On the other hand, mutations identified in *cyp51A* correspond with azole resistance (Diaz-Guerra et al. 2003; Howard et al. 2006; Mellado et al. 2004). The most common mutations are reported in AA positions G54, L98, and M220 (Howard and Arendrup 2011). *cyp51A* resistance mutations also include tandem repeats in the promoter, increasing *cyp51* expression. This is often combined with mutations in the *cyp51A* coding region itself (most common is a mutation termed "TR/L98H" with 36-base tandem repeat in the promoter and an L98H substitution in Cyp51A) (Verweij et al. 2009; Warrilow et al. 2013; Camps et al. 2012a; Snelders et al. 2008, 2012; Mellado et al. 2007). Mutations in *cyp51A* are often correlated with the use of agricultural azoles. Tebuconazole, a common agricultural azole, has been shown to induce a tandem repeat mutation in the *cyp51A* promoter (Snelders et al. 2012).

ERG11 mutation has also been shown to cause azole resistance in *C. neoformans* (Rodero et al. 2003), although further analyses are warranted.

ERG11 Overexpression

Another way of gaining resistance through *ERG11* is by its overexpression, which results in an increased production of the encoded enzyme. Increased amounts of enzyme require increased amounts of drug for the same level of inhibition. Normally, as a response to azole treatment (or other ergosterol biosynthesis inhibitors), *ERG11* expression is increased above normal in many *Candida* species (Henry et al. 2000). This upregulation often includes other *ERG* genes in the pathway. In certain cases, the upregulation of the *ERG11* or the whole pathway can be permanent, leading to the resistant phenotype. The permanent upregulation in *C. albicans* can happen basically by two mechanisms: (1) duplication of chromosome 5, increasing the

copy number of the *ERG11* gene located on it (see below), and (2) a gain-of-function (GOF) mutation in Upc2 (Table 2).

GOF mutations in Upc2 lead to the overexpression of many ergosterol biosynthesis genes, including the already mentioned *ERG11* (MacPherson et al. 2005; Silver et al. 2004). Upc2 is a Zn_2–Cys_6 transcriptional factor and central regulator of the ergosterol biosynthesis pathway in *C. albicans*. In the model organism, *S. cerevisiae*, constitutively active mutants of the two paralogs Upc2 (G888D) and Ecm22 (G790D) only induce sterol uptake under aerobic conditions (Crowley et al. 1998; Davies et al. 2005; Lewis et al. 1988; Shianna et al. 2001). However, in *C. albicans*, Upc2 constitutively active mutants in the C-terminus (Y642F, A643V/T, A646W, and G648D/S) or additional areas (G304R and W478C) increase resistance to the azoles by direct *ERG11* upregulation (Dunkel et al. 2008a; Flowers et al. 2012; Heilmann et al. 2010; Hoot et al. 2011). Upc2 forms dimers (our unpublished data) and these GOF mutations can express their full potential in diploid *C. albicans* only when found in the homozygous state (Heilmann et al. 2010; Coste et al. 2004, 2007; Dunkel et al. 2008b; Schubert et al. 2011). Homozygous GOF Upc2 (G648D) stimulates *ERG11* promoter expression approximately fourfold resulting in a fourfold increase in MIC (Schubert et al. 2011; Sasse et al. 2011).

Distantly related fungi (e.g., *Aspergillus* and *Cryptococcus*) are missing Upc2 homologs but have genes that regulate ergosterol biosynthesis that are functional homologs of the *s*terol *r*egulatory *e*lement-*b*inding *p*rotein (SREBP) of higher eukaryotes. The genes are *srbA* in *Aspergillus* and *SRE1* in *Cryptococcus*. These proteins belong to helix-loop-helix transcriptional factors, are structurally unrelated to Upc2, and also have a different mechanism of activation. No GOF mutations have been identified in these proteins to date. However, the *A. fumigatus srbA* deletion results in azole hypersusceptibility similar to *upc2* mutants in *Candida* (Willger et al. 2008). Recently, increased expression of *Aspergillus cyp51A* has been linked to a P88L mutation in the unrelated transcription factor HapE (Camps et al. 2012b), which may interact with SrbA. Finally, *ERG11* overexpression has been shown to affect azole susceptibility in *C. glabrata* (Marichal et al. 1997) and *C. neoformans* (Lamb et al. 1995), but the mechanisms for this overexpression have not yet been identified.

Alterations in Ergosterol Biosynthesis

Besides Erg11, alterations in other enzymes involved in ergosterol biosynthesis can affect azole susceptibility. One of these is the inactivation of the enzyme sterol C5-desaturase, encoded by *ERG3*, which introduces a double bond in sterol substrate molecules between carbons C5 and C6. Normally, after Erg11 inhibition, Erg3 converts nontoxic 14α-methylated sterol intermediates (mostly 14-α-methylfecosterol) into the toxic 14α-methylergosta-8,24(28)-dien-3β,6α-diol. Thus, *ERG3* inactivation in *C. albicans* undergoing azole treatment allows the cells to bypass the synthesis of toxic sterols and leads to azole resistance (Watson et al. 1989; Kelly et al. 1995, 1997). However, this type of resistance is rather

uncommon in clinical isolates, probably due to the fact that the inactive *ERG3* allele has to be homozygous to manifest the reduced azole susceptibility (Miyazaki et al. 2006) and the inactive *ERG3* alleles in *C. albicans* lead to the loss of filamentation and attenuated virulence (Chau et al. 2005). Interestingly, azole treatment is still effective against *ERG3* mutants in vivo (Miyazaki et al. 2006). Surprisingly, the *ERG3* mutation does not result in azole resistance in *C. glabrata* (Geber et al. 1995), although in its closely related *S. cerevisiae* it does (Watson et al. 1989). Studies in *A. fumigatus* (deletion of *erg3A* and *erg3B*) also demonstrated that neither of the two genes is implicated in azole resistance (Alcazar-Fuoli et al. 2006).

In *S. cerevisiae*, the loss of function of *ERG6* (sterol C24-methyltransferase) or *ERG28* (a scaffold protein mediating the interaction between Erg26, Erg27, and possibly Erg6) has been shown to decrease azole susceptibility (Anderson et al. 2003). However, the *ERG6* mutation has no such effect in either *C. albicans* (Jensen-Pergakes et al. 1998) or *C. glabrata* (Vandeputte et al. 2008). The *ERG28* deletion in these species has not been reported to date.

As illustrated above, the *ERG* gene deletion phenotypes work very differently in different fungal species. This suggests high plasticity of the ergosterol biosynthesis pathway and its regulation among different fungi. Thus, it is highly probable that mutations in other ergosterol biosynthesis enzymes will be isolated from azole-resistant fungal pathogens in the future.

Azole Efflux

In *C. albicans*, there are several transporters involved in azole efflux – the major ones are Cdr1, Cdr2, and Mdr1 and minor ones are Pdr16 and Flu1. The upregulation of *CDR1* and *CDR2* (*Candida* *d*rug *r*esistance) is the most frequent azole resistance mechanism in *C. albicans* (Perea et al. 2001). Pdr16 (*p*leiotropic *d*rug *r*esistance), which is induced together with Cdr1 and Cdr2, is also involved in azole resistance, although indirectly – it acts as phosphatidylinositol transfer protein (Saidane et al. 2006). While Pdr16, Cdr1, and Cdr2 belong to the class of ABC (ATP-binding cassette) transporters using ATP for transport, Mdr1 (*m*ulti*d*rug *r*esistance) and Flu1 (*flu*conazole resistance) are representatives of the major facilitator superfamily (MFS), which use a membrane proton (H^+) gradient for drug/H^+ antiport. Interestingly, *C. albicans* Mdr1 is specific only for fluconazole and not for other azoles (Sanglard et al. 1995). Although Flu1 has been shown to be involved in fluconazole resistance (Calabrese et al. 2000), it is not considered a major azole transporter. And although there are other ABC and MSF transporters in *C. albicans* genome, none of them have been shown to date to participate in azole resistance.

All the above mentioned transporters are naturally expressed in response to toxic substances in the environment and are associated with efflux of small hydrophobic or lipophilic molecules. In general, the efflux pump genes *CDR1*, *CDR2*, *PDR16*, *MDR1*, and *FLU1* are not induced in response to azole drugs, but they do respond to steroids, fluphenazine, benomyl, and stress inducers including oxidizing agents.

Mrr1 and Tac1 are two transcriptional activators in *C. albicans* whose GOF mutations affect azole resistance through the upregulation of the abovementioned efflux pumps. Mrr1 and Tac1 both belong to Zn_2–Cys_6 type of zinc cluster transcription factors, similar to Upc2. In clinically resistant *C. albicans* isolates, the mutations in Mrr1 and Tac1 are often combined with each other and with the already mentioned GOF Upc2 version. The azole resistance in these strains is significantly higher than it is in strains containing a single GOF mutation in one of these three transcriptional factors (Sasse et al. 2012). Additional transcription factors that are known to regulate the expression of *CDR1* and *CDR2* include Ndt80, Fcr1, and Fcr3, but to date, none of these has been shown to be able to induce azole resistance *in vivo*. The *C. albicans MDR1* gene is also transcriptionally regulated by the transcription factors Cap1 and Mcm1, but these factors have not been linked to azole resistance in clinical isolates either. Only Cap1 has been shown to be able to increase azole resistance *in vitro*, when its cysteine-rich C-terminus is truncated (Alarco and Raymond 1999).

GOF mutations (P683S, P683H) in Mrr1 promote the overexpression of *MDR1* (Dunkel et al. 2008b; Morschhauser et al. 2007; Schubert et al. 2008). Similar mutations were also identified in the *C. dubliniensis* Mrr1 homolog, proving that GOF mutations affect the activity of Mrr1 similarly in both closely related species (Schubert et al. 2008). Upc2 (mentioned above) is also capable of binding to the *MDR1* promoter and stimulating its expression, although the effect of GOF Upc2 is minimal, with roughly twofold *MDR1* induction (Schubert et al. 2011). GOF Upc2 might be further involved in increased azole efflux by inducing a not yet characterized efflux pump *CDR11* (Flowers et al. 2012).

Tac1 is perhaps the most important transcription factor involved in azole resistance of clinical isolates. It is the transcriptional activator of *CDR1*, *CDR2*, and *PDR16*, which binds to the *d*rug *r*esponse *e*lement (DRE) sequence in the promoters of these genes (Coste et al. 2004; de Micheli et al. 2002). Interestingly, the *TAC1* gene is located on the left arm of chromosome 5, 15 kb from mating-type locus (MTL) in the area where *ERG11* is also located. Constitutively active alleles of *TAC1* contain GOF mutations resulting in amino acid substitutions T225A, V736A, N972D, N977D, G980E, and G980W or deletions ΔM677 and Δ962-969. T225A appears to be located in the transcriptional inhibitory domain and the other mutations in the transcriptional activation domain. Each mutation causes increased expression of many responsive genes including the already mentioned *CDR1*, *CDR2*, and *PDR16* (Coste et al. 2006, 2007, 2009; Znaidi et al. 2007). As in the case of *MDR1*, Upc2 also seems to slightly induce *CDR1* expression (Znaidi et al. 2008). In addition to transcriptional regulation, recent studies have demonstrated increased mRNA stability and increased transcriptional initiation for the *CDR1* gene in resistant isolates, possibly the result of altered sequences in the 3′ untranslated region of the mRNA (Manoharlal et al. 2008).

The ABC transporters Cdr1 and Cdr2, whose expression is controlled by Tac1, are more efficient fluconazole efflux pumps than the major facilitator Mdr1 (Lamping et al. 2007). However, strains with the hyperactive Mrr1 are slightly more resistant to fluconazole than strains with the hyperactive Tac1, pointing out the significant

contribution of additional Mrr1 target genes to fluconazole resistance (Schubert et al. 2011; Sasse et al. 2012). The combination of both transcription factors has only a minor additive effect (Sasse et al. 2012). Like GOF Upc2, both GOF Mrr1 and GOF Tac1 increase their effect with homozygosity, selecting for the loss of heterozygosity under azole-selective pressure (Dunkel et al. 2008b; Sasse et al. 2011; Morschhauser et al. 2007). In the presence of homozygous GOF Mrr1 (P683S), the *MDR1* promoter displays approximately 50-fold induction. Homozygous GOF Tac1 (G980E) stimulates the *CDR1* promoter approximately 8-fold and *CDR2* promoter 10-fold. The increase in MICs for homozygous GOF alleles is 8-fold for *TAC1* and 16-fold for *MRR1* (Schubert et al. 2011; Sasse et al. 2011).

Although intrinsically highly azole resistant, an increase in azole resistance in *C. glabrata*, based on the ABC drug efflux pumps Cdr1, Pdh1, and Snq2, has been described (Torelli et al. 2008; Tsai et al. 2006; Sanglard et al. 2001). Members of the MFS transporter family Flr1 (Chen et al. 2007) and Qdr2 (Costa et al. 2013) are also involved in azole efflux in *C. glabrata*. GOF mutations in the transcription factor Pdr1 induce the overexpression of the ABC efflux pumps Cdr1, Snq2, and Pdh1 (Torelli et al. 2008; Tsai et al. 2006; Ferrari et al. 2009) and may also overexpress the MFS transporter Qdr2, since the *QDR2* gene has been shown to be induced through Pdr1 in the presence of azoles (Costa et al. 2013). GOF mutations in the transcription factor Pdr1 in *C. glabrata* are another proof of induced azole export through mutation of an efflux pump transcription factor. Interestingly, also mitochondrial loss in *C. glabrata* is connected to increased azole resistance through the upregulation of *CDR1*, *PDH1*, and possibly other genes (Sanglard et al. 2001). The loss of mitochondria has been reported even in clinical isolates (Bouchara et al. 2000), despite their reduced virulence (Brun et al. 2005). An effect of mitochondrial loss on increased azole resistance has not been reported for any other organism yet.

Compared to the other studied species, the fungal pathogen *A. fumigatus* contains unusually high number of genes encoding ABC (close to 50) and MFS (close to 300) transporters, which are mostly uncharacterized to date (Nierman et al. 2005). Although several ABC and MFS transporters have been proposed to be involved in azole resistance (Slaven et al. 2002), only *abcC*/*cdr1B* and *mdr1* have been shown to play a direct role in azole efflux (Fraczek et al. 2013). In contrast to *C. albicans*, and similar to *C. glabrata*, some ABC and MFS transporters are induced in the presence of azoles (Fraczek et al. 2013; da Silva Ferreira et al. 2006). Many transporters are also induced in biofilms (Rajendran et al. 2011), possibly playing similar role in azole resistance similar to azole transporters in *C. albicans* (see below).

Although less studied, *C. neoformans* also connects azole efflux to the acquired azole resistance (Joseph-Horne et al. 1995). To date, only the ABC transporter Afr1 has been shown to be capable of fluconazole efflux (Posteraro et al. 2003), although mutants in this transporter regulation have never been reported in clinical isolates. However, *AFR1* duplication, caused by chromosome 1 disomy, leads to an increase in azole resistance (Sionov et al. 2010).

Finally, in the intrinsically highly azole-resistant *C. krusei*, azole export can also be involved in increased resistance. This organism contains the main azole transporter Abc1 that is induced in the presence of azoles (Lamping et al. 2009).

Sterol Import

Sterol import is a potential azole resistance mechanism described relatively recently. To date, this phenomenon is related only to two fungal species – the model organism *S. cerevisiae* (Andreasen and Stier 1953) and the closely related pathogen *C. glabrata* (Zavrel et al. 2013; Bard et al. 2005; Nakayama et al. 2007; Nagi et al. 2013). Although *C. albicans* is also capable of sterol import, the rate is insufficient to replace the endogenous ergosterol biosynthesis (Zavrel et al. 2013). *S. cerevisiae* and *C. glabrata* are the only two described species able to import extracellular sterols in quantities sufficient to replace endogenous ergosterol biosynthesis in the presence of azoles. The difference from other fungi is the presence of *AUS1*/*PDR11* ABC type of sterol importers (Nakayama et al. 2007; Wilcox et al. 2002), which have not been identified in any other fungal species. In *S. cerevisiae*, sterols are imported exclusively anaerobically (Andreasen and Stier 1953) or in strains that mimic anaerobic conditions because of heme biosynthesis defects (*HEM1* mutations) or containing GOF Upc2/Ecm22 allele (Crowley et al. 1998; Lewis et al. 1985). Like *S. cerevisiae*, *C. glabrata* also imports sterols anaerobically and in mutants with defective *HEM1* (Bard et al. 2005). Additionally, sterol import is observed in mutants in early steps of ergosterol biosynthesis (*ERG1*, squalene epoxidase, or *ERG7*, lanosterol synthase) (Bard et al. 2005). *C. glabrata* also imports small amounts of sterols aerobically, and this can be greatly stimulated in the presence of blood serum together with a block in ergosterol biosynthesis caused by azoles (Zavrel et al. 2013; Nagi et al. 2013). Thus, an azole-induced block in ergosterol biosynthesis can be compensated *in vivo* by sterol import from its host. Similarly, a defect in the ergosterol biosynthesis pathway can also trigger sterol import in host.

Finally, sterol import has also been reported for *A. fumigatus* (Xiong et al. 2005). The presence of cholesterol or blood serum in the growth medium, and the subsequent cholesterol uptake, reduces the susceptibility to itraconazole and voriconazole. However, compared to *C. glabrata*, the rescue is only partial, suggesting that the rate of sterol import is insufficient for complete replacement of endogenous ergosterol biosynthesis. The sterol transporter in *A. fumigatus* has not been identified yet, but there is no direct homolog of the *AUS1*/*PDR11* type of sterol transporter in its genome.

Gene Conversions and Aneuploidy

The azole resistance mechanisms described above can be facilitated by chromosomal recombination and/or duplication of resistant alleles, which can involve either whole chromosome or segmental aneuploidy. Because *C. albicans* is diploid, the GOF alleles of transcription factors *TAC1*, *MRR1*, and *UPC2*, as well as an azole-resistant allele of *ERG11*, can be present either in a heterozygous or homozygous state. All of the mentioned transcription factors are likely to form dimers, a common characteristic of zinc cluster proteins. The loss of heterozygosity (LOH), through allele gene conversion, resulting in the presence of GOF transcription factor alleles or mutated

ERG11 alleles in two identical copies, contributes to azole resistance (Heilmann et al. 2010; Coste et al. 2004, 2006, 2007; Dunkel et al. 2008b; Schubert et al. 2011; Selmecki et al. 2006).

Azole resistance can be increased also through isochromosome 5 L [i(5 L)] formation. This results in an artificial chromosome containing two left arms of chromosome 5, where both *TAC1* and *ERG11* genes are located (Coste et al. 2007). Such aneuploidy is not well tolerated; it is unstable; and it can be easily lost in the absence of drugs, resulting in partial loss of the drug resistance. The mechanism by which these isochromosomes are created is not known in detail, but it has been suggested that they are created by recombination at an inverted repeat in the centrosome region of the chromosome (Coste et al. 2007; Selmecki et al. 2006). There is indirect evidence that chromosome 5 duplication and subsequent loss may occur relatively often during stepwise acquisition of azole resistance. Besides *ERG11* and *TAC1*, the MTL mating locus is also localized at chromosome 5. Compared to azole-susceptible strains, there is an increase in MTL locus homozygosity among azole-resistant isolates (Rustad et al. 2002). This might be indicative of frequent duplication of "azole-resistant" chromosome 5, leading to trisomy, and subsequent loss of the "susceptible" third chromosome, reverting the chromosome 5 back to disomy.

An additional possibility for decreased azole susceptibility is in vivo in-host mating of two strains with opposite mating types, both carrying different alleles contributing to decreased azole susceptibility. As described about a decade ago, *C. albicans* is able to mate; however, instead of sporulation, the tetraploid cells revert back to diploids by continuous chromosome loss (Bennett and Johnson 2003). This is more theoretical option for gaining azole resistance and it has not yet been reported.

Chromosome duplication has also been reported in *C. glabrata*, increasing the copy number of efflux pump encoding genes (Polakova et al. 2009). A fluconazole-resistant clinical isolate was found to contain an additional minichromosome, consisting of a duplicated portion of chromosome F, where ABC transporter gene, *CDR2*, is located. This minichromosome was also stable in the presence of fluconazole but again was frequently lost in drug-free medium. *ERG11* gene amplification through chromosome duplication has also been identified in this organism, although this isolate demonstrated other resistance mechanisms (Marichal et al. 1997). Again, the duplicated chromosome was unstable and was lost in the absence of azoles.

In *C. neoformans*, chromosome duplication is a very common azole resistance mechanism, known as heteroresistance. It is caused by disomy of chromosome 1, sometimes in combination with other chromosomes (Sionov et al. 2010). Chromosome 1 contains both known genes involved in resistance, *ERG11* and *AFR1*. This phenomenon is strain dependent and the aneuploidy is induced in the presence of fluconazole and does not seem to be spontaneous due to its unusually high frequency. However, the chromosome 1 disomy has a growth disadvantage and is reduced in virulence in animal models. Again, without fluconazole-selective pressure, the additional chromosomes are lost like in other organisms.

Biofilms

Another mechanism of resistance is the formation of a biofilm, the predominant mode of growth of many microorganisms in nature. The presence of foreign objects, including catheters, dentures, and artificial heart valves, can contribute to a recalcitrant infection, as the fungus can attach to and invade these objects, creating a source of constant inoculation and protection from drug therapy.

Candida infections primarily begin with adherence and colonization on a biotic or an artificial host surface. This process leads to the formation of biofilms – surface-attached communities encased in a matrix of secreted polymeric substances. The matrix encased cells forming the biofilm display unique characteristics over their planktonic or free-floating counterparts. In many *Candida* species, drug resistance is a common characteristic of biofilms (Lewis et al. 2002). In general, these biofilms require 5–8 times higher drug concentrations for a similar reduction in metabolic activity compared to their planktonic cells, and their MICs can differ up to four orders of magnitude (Hawser and Douglas 1995). Mechanisms that explain this higher antifungal resistance include the presence of extracellular matrix, a robust architecture with reduced drug diffusion, decreased cellular metabolic activity, the existence of "persistent cells," altered gene expression including the overexpression of efflux pumps and stress genes, and finally higher anti-oxidative capacities (Lopez-Ribot 2005; Nobile and Mitchell 2006; Ramage et al. 2002). Efflux pump activity plays a contributory role in biofilm azole resistance but is not the principal mechanism. *C. albicans* biofilms formed by *MDR* and *CDR* deletions still display the resistant phenotype despite high susceptibility of planktonic cells to fluconazole (Ramage et al. 2002). Recent evidence suggests that the extracellular matrix material in the biofilm, partially formed by glucans, can act as a chelator for antifungal drugs, so that the cell is not exposed to high drug concentrations (Nett et al. 2007). The glucan is produced by β-1,3-glucan synthase, Fks1, and its reduced activity results in increased biofilm azole susceptibility (Nett et al. 2010).

A. fumigatus and *C. glabrata* also form biofilms. *A. fumigatus* seems to exploit the same resistance mechanisms as *C. albicans*, including the overexpression of numerous transporters (Rajendran et al. 2011). However, compared to *C. albicans* and *A. fumigatus* biofilms, formed mostly by hyphae, *C. glabrata* biofilms are exclusively composed of yeast cells. These *C. glabrata* biofilms are also highly antifungal resistant compared to the planktonic cells, and this may be partially caused by an induced stress response (Seneviratne et al. 2009).

Heat Shock Proteins

Cellular stress conditions, including drug treatment, high temperatures, starvation, mutations, and oxidative stress, can negatively affect intracellular homeostasis and cause protein unfolding and aggregation leading to cell death. *H*eat *s*hock *p*roteins (HSPs) are dramatically upregulated during times of stress to act in part as chaperones to regulate the folding, transport, and degradation of many important proteins

(Cowen and Lindquist 2005; Lamoth et al. 2013; Mayer et al. 2013). *C. albicans* contains multiple HSPs including Hsp104, Hsp90, Hsp78, Hsp70, and Hsp60, which all function in the heat shock response either with unique functions or in response to specific stressors (Mayer et al. 2013). Hsp90 is the major HSP in *C. albicans* and in other fungi that is involved in drug resistance and virulence among its other functions (Cowen and Lindquist 2005; Mayer et al. 2013).

The main role of Hsp90 in drug resistance is to buffer the negative effects induced by drug treatment including the buildup of toxic protein aggregates. Its role is to unfold, refold, or stabilize proteins damaged in response to the stresses. Hsp90 activation following azole treatment allows cells to adapt to the resulting stress, including altered sterol biosynthesis and membrane composition.

Hsp90 is also critical for adaptation to newly acquired resistance mechanisms. In conditions of high cellular stress when the amount of damaged proteins outnumber the available Hsp90s, the *absence* of Hsp90 regulation can also benefit the cell by allowing protein mutations to go unchecked, such as those mutations that alter sterol biosynthesis and membrane composition. The exposure of a variety of new mutations offers the stressed cell a new repertoire of selectable variants for overcoming the stress agent and developing resistance.

Hsp90 is crucial for toleration of *ERG3* mutations as an azole-resistant mechanism (Cowen and Lindquist 2005). In cells, Hsp90 functions in drug resistance through the calcineurin pathway, which is known to be implicated in virulence of several pathogenic fungi and also enables fungal cells to tolerate ergosterol biosynthesis inhibitors (Cowen and Lindquist 2005). Exposure of cells to stress leads to elevated cytosolic Ca^{2+} concentrations and activation of calcineurin, a protein phosphatase whose primary function in yeast is to dephosphorylate and activate the transcription factor Crz1p. Calcineurin itself is an Hsp90 substrate and Hsp90 stabilizes its catalytic domain. Screens in *C. albicans* have also identified another gene involved in calcineurin signaling, *CKA2*, encoding casein kinase II (Bruno and Mitchell 2005). Recently, a new link between calcineurin signaling and iron homeostasis has been reported (Hameed et al. 2011). Iron deprivation results in reduced expression of the catalytic subunit of calcineurin, *CMP1*, plus several of the *ERG* genes, disrupting ergosterol biosynthesis.

cAMP signaling also plays a role in azole susceptibility. Deletion of enzymes involved in cAMP synthesis results in increased susceptibility to azoles and other sterol biosynthesis inhibitors (Jain et al. 2003). Cyclic AMP-dependent protein kinase A (PKA) in *S. cerevisiae* phosphorylates and negatively regulates Crz1 activity by inhibiting its nuclear import (Kafadar and Cyert 2004). Otherwise, PKA regulates the general stress response in yeast and coordinates this response with nutrient availability. In contrast, calcineurin regulates the cellular response to a restricted set of environmental insults.

Not surprisingly, Hsp90 inactivation leads to increased susceptibility to environmental stress including exposure to azoles. In general, a compromised Hsp90 or calcineurin transforms the fungistatic effect of many antifungals to fungicidal. Thus, HSP response in the evolution of fungal drug resistance has broad therapeutic implications and so has become a promising drug target (see below).

Resistance to Other Drugs

The mechanisms of resistance to the other three clinically used classes of drugs (echinocandins, polyenes, and 5-fluorocytosine) are limited. *C. albicans* strains can develop resistance to echinocandins (caspofungin, micafungin, and anidulafungin) due to mutations in the target enzyme β-l,3-glucan synthase (Gcsl/Fks1). This enzyme produces β-1,3-glucan and is essential for cell wall synthesis and fungal viability (Perlin 2007). There are two other Gscl homologs in *C. albicans*, Gsl1 and Gs12/Fks2, however they cannot completely compensate for Gscl loss of function. Mutations in the plasma membrane (PM)-localized Gsc1 enzyme usually occur in two hotspots (AA regions 641–649 and 1345–1365) and reduce the enzyme's affinity for the echinocandins. The drugs bind to Gsc1/Fks1 from the extracellular side of the PM and reduce its enzymatic activity. The same mechanism of resistance is employed in *C. glabrata* (Garcia-Effron et al. 2009) and *A. fumigatus* (Arendrup et al. 2009). However, in *A. fumigatus*, resistance due to the *FKS1* overexpression has also been reported (Arendrup et al. 2008). *C. albicans* cells respond to echinocandin treatment with an increase in chitin levels, and strains with higher chitin content in the cell wall (including those naturally occurring) are less echinocandin susceptible (Plaine et al. 2008; Lee et al. 2012). In some *C. albicans* isolates, the "paradoxical effect" is observed, where cell growth is stimulated at concentrations of echinocandin significantly above the MIC (Stevens et al. 2004). This is most likely due to the induction of cell wall stress response, resulting in increased chitin levels (Stevens et al. 2006).

Resistance to polyenes, like amphotericin B or nystatin, is usually associated with lowered levels of ergosterol in the plasma membrane (Dick et al. 1980). Polyenes bind to the ergosterol in fungal membranes resulting in pores that affect the membrane integrity and lead to the loss of the membrane potential. The molecular mechanisms of resistance to polyenes are poorly understood; however, resistance is associated with alterations in *ERG* genes (Sanglard et al. 2003) as well as an increase in resistance to oxidative stress (Sokol-Anderson et al. 1986).

The last medically important drug, 5-fluorocytosine (flucytosine; 5-FC), is metabolized via the pyrimidine salvage pathway, where it acts as a substrate with the subsequent production of toxic nucleotides and disruption of DNA and protein synthesis. After being actively transported into the cell by the permease Fcy2, 5-FC is converted to 5-fluoro-uridylate (5-FU) with the help of the enzymes cytosine deaminase (Fca1) and uracil phosphoribosyltransferase (Fur1). 5-FU is then processed and incorporated into RNA. 5-FU by-products also inhibit the enzyme thymidylate synthase (Cdc21) and thus DNA synthesis by decreasing the available nucleotide pool. 5-FC resistance mechanisms include mutation of enzymes, Fur1 (R101C) and Fca1 (G28D) (Hope et al. 2004). Both mutant alleles must be in homozygous state. These mutations most likely lead to reduced activity or even complete inactivation of the enzymes, since both genes are nonessential. In *Candida lusitaniae*, deletion of the cytosine permease Fcy2, as well as deletions of Fur1 and Fca1, leads to increased 5-FC resistance (Chapeland-Leclerc et al. 2005). However, the effect of the inactivation of *C. albicans* Fcy2 permease has not been reported to date.

Drug Combinations and Perspectives

In recent years, multiple drugs have been reported to have a synergistic effect with azoles, which provides new hope for combined therapy that will be less likely to result in rapid drug resistance development. One treatment combination would be to use antifungal drugs from different drug classes/families. For example, the composition and genetic regulation of *Candida* biofilm matrix are a promising target for the development of treatment of medical device-associated infections. The synergistic effect of azoles and echinocandins would be expected to negatively affect biofilms that were previously unresponsive to a single-drug treatment alone (Nett et al. 2010).

Calcineurin and Hsp90 inhibitors are another group of drugs displaying a synergistic effect with azoles and other antifungals. Since the cell wall integrity is connected to calcineurin signaling, there is a synergistic effect of echinocandins with HSP90/calcineurin inhibitors creating a fungicidal combination (Singh et al. 2009). There are several drugs with an inhibitory effect on calcineurin signaling. First, tacrolimus (FK506) forms a complex with peptidyl-prolyl cis-trans isomerase, Fpr1, a binding partner of calcineurin (Kissinger et al. 1995). Another drug, cyclosporine A, binds to another calcineurin partner – the peptidyl-prolyl cis-trans isomerase Cpr1 (Etzkorn et al. 1994). There are also two direct Hsp90 inhibitors, geldanamycin and radicicol, both docking into Hsp90's nucleotide-binding pocket (Schulte et al. 1998; Stebbins et al. 1997). Although these drugs exhibit their own direct antifungal activity (Lamoth et al. 2013), they also significantly increase in vitro susceptibility of *Candida* and *Aspergillus* species to azoles and other antifungals (Lamoth et al. 2013; Cruz et al. 2002; Onyewu et al. 2003). These drug combinations induce a better response in drug-resistant pathogens, turning fungistatic drugs to fungicidal and delaying or disrupting the acquisition of new resistance mechanisms. Their potential is also in the sensitization of biofilms to other antifungals (Shinde et al. 2012). Moreover, tacrolimus has been shown to work directly as a *C. albicans CDR1* inhibitor (Schuetzer-Muehlbauer et al. 2003).

Depletion of iron, an important part of heme present in Erg11 and Erg25 enzymes, also has a negative effect on calcineurin signaling, as mentioned above. Recently, doxycycline and to a lesser extent tetracycline have been shown to act synergistically with azoles in *C. albicans* in vitro through iron chelation (Fiori and Van Dijck 2012). The involvement of calcineurin signaling is likely, since the cyclins shift the azole effect from fungistatic to fungicidal and since the combination of azoles and doxycycline does not allow cells to develop resistance to this combination. Another proof of synergism between azole and iron chelation is lactoferrin, a transferrin present in milk and in body secretory fluids and part of innate immunity (Kobayashi et al. 2011).

Another group of drugs that might be considered for combined therapy are efflux pump inhibitors. Several have been identified to date. Two inhibitors, clorgyline (monoamine oxidase A inhibitor) and ebselen (antioxidant under investigation for several medical uses), were described to inhibit several efflux pumps including the *C. albicans* pumps Cdr1 and Cdr2, the *C. glabrata* pump Cdr1, and the *C. krusei* pump Abc1 (Holmes et al. 2012). Clorgyline is also active against and shows better potency with the *C. albicans* pump Mdr1. It displays synergistic effect with azoles

against azole-resistant strains of both *C. albicans* and *C. glabrata* in vitro. On the other hand, ebselen is known to have antifungal activity by inhibition of Pma1, the main plasma membrane H^+-ATPase (Billack et al. 2009). Other potent ABC pump inhibitors are the milbemycins (antiparasitic agents of macrolide type) (Lamping et al. 2007), unnarmicins (bacterial cyclic peptides) (Tanabe et al. 2007), tetrandrine (anti-inflammatory calcium channel blocker) (Zhang et al. 2009), enniatins (cyclic depsipeptides) (Hiraga et al. 2005), and ofloxacin and grepafloxacin (both synthetic fluoroquinolone antibiotics) (Sasaki et al. 2000). Ibuprofen also displays synergistic effect with azoles against azole-resistant strains of *C. albicans* (Ricardo et al. 2009). Although ibuprofen's mechanism of action has not been proven directly, it is suspected to be another ABC pump inhibitor. Another drug showing synergy with the azoles is amiodarone (an antiarrhythmic drug) (Guo et al. 2008). Although its mode of action is unknown, it is suggested to either affect cellular Ca^{2+} membrane transport, consistent with its antiarrhythmic use, or to inhibit ABC transporters. Lastly, several analogs of cerulenin (an inhibitor of fungal sterol synthesis and fatty acid synthesis) have been described to block the *C. albicans* Mdr1 major facilitator (Diwischek et al. 2009).

Several of the above drugs are already marketed for medical use. Therefore, clinical trials are needed to show efficacy, but not to ascertain their safety in humans. Synergy *in vivo* is not guaranteed due to the limits in physiological concentrations and possible alterations in metabolism. Several of the efflux pump inhibitors are used in traditional medicine and clearly warrant clinical trials for safety and efficacy. It is clear that as we learn about mechanisms of resistance in the fungi, we are also identifying strategies to interfere with these mechanisms.

Conclusion

Prolonged exposure of human fungal pathogens to azoles and other classes of antifungals often results in the development of resistance to these drugs. Every organism contains numerous pathways that can contribute to drug resistance. Azole resistance is best understood in the human fungal pathogen *C. albicans*, where the upregulation of the three major transporters responsible for azole efflux, Cdr1, Cd2, and Mdr1, is the most common resistance mechanism. This is usually accomplished by the acquisition of a GOF mutation in transcription factors Mrr1 and Tac1 that regulate these pumps, resulting in pump overexpression. A second major mechanism of resistance involves alterations in the ergosterol biosynthesis pathway. This includes the overexpression of *ERG11*, altering the affinity of Erg11 for azoles or inactivating the *ERG3* gene, which is responsible for synthesis of toxic intermediates after azole inhibition of Erg11. Further increases in resistance can be accomplished by copying the resistant allele into the susceptible allele in a diploid fungus, either by gene conversion or gene duplication.. All the above-described mechanisms can be combined, resulting in strains completely nonresponsive to therapeutic doses of azoles. Other fungi share identical mechanisms of azole resistance, although *C. glabrata* and *A. fumigatus* appear to be capable of exogenous sterol import, which can eliminate the importance of endogenous ergosterol

biosynthesis. This fact might lead to an alteration of the conditions for antifungal susceptibility testing in the future.

New antifungal treatments in the near future may require the combination of existing antifungal drugs with the development of new compounds. Numerous drugs approved for other clinical uses display antifungal activity, often acting as efflux inhibitors. Combined therapy with two drugs, attacking two different targets in the cell, has a lower probability of developing resistance to both drugs than the probability of developing resistance to a single drug. One major target of this strategy is the HSP90/calcineurin pathway. The inactivation of this pathway results in the inability of fungal cells to deal with some of the newly acquired mutations involved in azole resistance. Specificity of these drugs for fungal cell targets is important because the current HSP90/calcineurin-targeting drugs can act as immunosuppressants in humans. Therefore, their use in a human host and subsequent immune suppression often results in the acquisition of a fungal infection.

To prevent the development of antifungal drug resistance in the future, it is crucial to identify new fungal drug targets and to create drugs that are fungicidal. One of these new targets might be cell wall assembly and cell wall remodeling enzymes. These drugs work on the exterior of the cell, thus omitting the need for the drug to be imported into the fungal cells. This is similar to the bacterial transpeptidases for β-lactam antibiotics that also work outside of the cell. The future of antifungal drug development lies in using the information we currently have to develop therapies that inhibit or kill the fungal cells while minimizing the possibility of drug resistance.

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Part V

HIV

Transmission of HIV-1 Drug Resistance

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Abstract

Infection with an HIV strain harboring drug resistance-related mutations is referred to as transmitted drug resistance (TDR) or primary resistance. As transmitted drug resistance increases the risk of virological failure, current guidelines recommend to perform drug resistance testing at baseline in all newly diagnosed individuals to guide the choice of antiretroviral therapy.

The prevalence of TDR varies among regions, risk groups, and drug classes due to different exposure to antiretroviral therapy (ART), risk behavior, and access to therapy. Of concern in developed countries is the rising prevalence of mutations associated with NNRTI resistance, a drug class frequently used in first-line therapy which has a low genetic barrier for development of resistance. In resource-limited settings (RLS), rollout of ART with limited virological monitoring frequently results in the risk of prolonged virological failure with selection and accumulation of drug resistance mutations and subsequent transmission of drug resistance. Most surveys in RLS showed still low to moderate prevalence of TDR, but greater coverage of ART is associated with a higher prevalence of TDR. Also in RLS the rise in prevalence of TDR is mostly driven by NNRTI resistance, which is of particular concern as this drug class constitutes the foundation of current first-line ART regimens and prophylaxis for prevention of mother-to-child transmission.

Introduction

In the 1980s, soon after the introduction of the first antiretroviral drug zidovudine, the emergence of viral variants harboring mutations associated with decreased susceptibility to zidovudine was reported. In 1996, the development of viral load assays provided a tool for clinicians to monitor the efficacy of antiretroviral therapy (ART), and it became clear that the presence of drug resistance mutations was associated with therapy failure (Katzenstein and Holodniy 1995; Lorenzi et al. 1999).

The first case of transmission of drug resistance was described by Erice et al. (1993). A young homosexual man presented with a primary HIV-1 infection. Genotypic analysis of the virus before the start of zidovudine therapy showed the presence of a mutation at position 215 in reverse transcriptase, which is associated with decreased susceptibility to zidovudine. Phenotypic analysis confirmed the decreased susceptibility of the virus. The authors suggested that he acquired a zidovudine-resistant virus from a sexual partner who was receiving zidovudine and they already inferred that the possibility of horizontal transmission of resistance may have a great impact on the clinical approach to newly infected persons (Erice et al. 1993). More case reports followed that showed transmission of resistance via various routes such as injection drug use and vertical transmission (de Ronde et al. 1996) and transmission of resistance to other drugs than zidovudine, including multidrug resistance (Hecht et al. 1998).

Since these first case reports, many have studied the transmission of HIV drug resistance. In this chapter, we will discuss the current knowledge on the different aspects of transmitted drug resistance, including evolution, prevalence, and clinical significance.

Development of Drug Resistance and Viral Dynamics

HIV is an RNA virus, a retrovirus, characterized by the presence of a viral enzyme called reverse transcriptase. By reverse transcription of the viral RNA, this enzyme will form a DNA double helix, which subsequently is transversed to the cell nucleus and integrated into the host cell's genome by another viral enzyme: integrase. Transcription of proviral DNA results in the formation of new viral RNA and viral proteins that together will form a new viral particle. After budding and release of the viral particle, precursor polyproteins are cleaved by the viral enzyme protease, which results in the maturation of the particle to fully infectious virus. HIV replicates billions of times per day, but reverse transcriptase is lacking proofreading activity, thus introducing errors in each replication cycle (Perelson et al. 1996). As a result of these random mutations, a large number of slightly genetically distinct viral variants are circulating within a particular host, named quasispecies (Eigen 1993). However, mutations may affect the replicative capacity and thereby the fitness of the virus. In the absence of antiretroviral drugs, wild-type virus is considered the most fit variant and dominates the quasispecies.

Antiretroviral drugs that are currently available interfere with the different viral enzymes: reverse transcriptase (nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs)), protease (protease inhibitors (PIs)), and integrase (integrase inhibitors (INIs)). Entry inhibitors interfere at the level of binding and fusion of the viral membrane with the host cell membrane. It is important that ART sufficiently suppresses replication of the virus, because continued viral replication in the presence of antiretroviral drugs will often lead to the selection of viral variants with resistance mutations. In the presence of antiretroviral drugs, these resistance mutations provide a replication advantage, which will result in fixation of this mutation in the population. The drug-resistant variant will become the dominant variant in the quasispecies, since replication of wild-type virus is suppressed by the antiretroviral drugs. When ART is interrupted, the resistant variants suddenly no longer have a replication advantage but instead have an impaired fitness compared to wild-type virus. Over time, wild-type virus that is archived as proviral DNA in infected cells will emerge again and become the dominant variant (Fig. 1).

Resistance mutations appear in the quasispecies by random error. These mutations can become fixed and dominate the quasispecies in case of suboptimal treatment or incomplete adherence to therapy. This is called acquired or secondary resistance. Certain resistance mutations will only become fixed in the viral population in the presence of selective drug pressure, but other resistance mutations can be present as natural polymorphism and occur commonly in the absence of selective

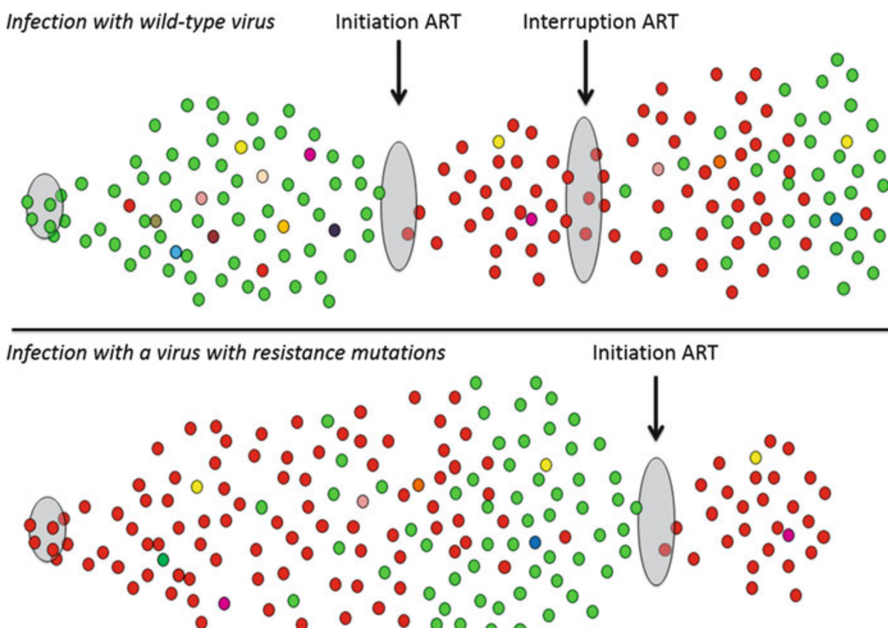

Fig. 1 Viral dynamics in acquired drug resistance (*top*) or transmitted drug resistance (*bottom*). *ART* antiretroviral therapy; *green variants* represent wild-type virus, *other colored variants* represent virus with mutations of which *red* variants represent virus with mutations that cause resistance to ART. Acquired drug resistance: after infection with wild-type virus, various variants with mutations emerge (quasispecies), but wild-type virus is the dominant variant. When ART is initiated but replication is not sufficiently suppressed, selection of variants with resistance mutations may occur, causing failure of therapy. When ART is interrupted, wild-type virus that is archived in proviral DNA will emerge and become the dominant variant again. Transmitted drug resistance: after infection with a virus with resistance mutations, reversion to wild type may occur over time. When ART is initiated, the initially transmitted variant with resistance mutations that is archived in proviral DNA may emerge and become the dominant variant, causing therapy failure

drug pressure. These resistance-associated natural polymorphisms only have a minor effect on susceptibility or only display an effect in a particular background. Resistance mutations can also be present in the dominant variant because the patient was initially infected with a drug-resistant strain, which is called primary resistance or transmitted drug resistance.

Evolution of Transmitted Drug Resistance

As described previously, in patients with acquired drug-resistant virus that emerged during therapy, a rapid reappearance of archived drug-susceptible virus has been observed at the time of treatment interruption as wild-type virus has a higher fitness than the resistant variants in absence of drug pressure. In contrast, in patients infected with a virus with resistance mutations, wild-type virus is usually not

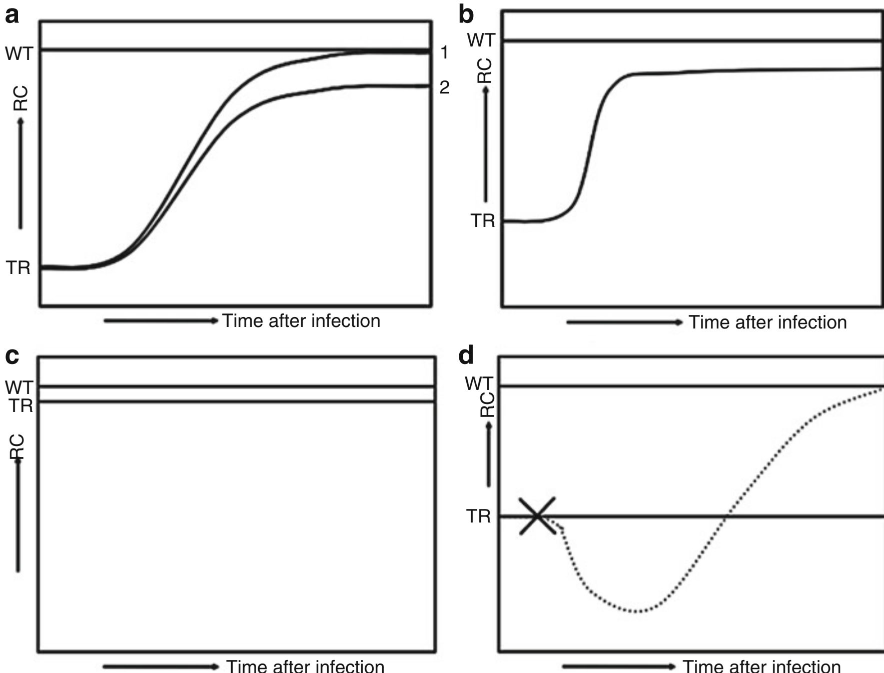

Fig. 2 Evolution of transmitted drug resistance. (**a**) Evolution to wild type. After transmission, the RC (y-axis) of the transmitted drug-resistant variant (*TR*) is lower than that of wild type (*WT*). Due to complete (1) or incomplete (2) reversion of the drug resistance mutation, the RC is restored or improved. (**b**) Selection of atypical mutations. After transmission of a drug-resistant HIV variant, atypical amino acids that are neither wild type nor intermediated may be selected, leading to improved RC. (**c**) Persistence because of a minimal reduction in RC. If the RC of the resistant variant (almost) equals the RC of wild type, persistence may occur for a considerable time. (**d**) Reversion is blocked by compensatory fixation. Due to compensatory mutations, multiple mutations are required for full reversion. The first mutation would decrease the RC, so reversion is blocked (Adapted from Pingen et al. JAC (2011))

co-transmitted and therefore not archived in latently infected cells. In the absence of drug pressure in the new host, transmitted viruses with resistance mutations may either (partly) revert to wild type, evolve to other variants, or persist in the viral population, depending on their relative fitness in the new environment. A recent review distinguished the particular pathways of evolution (Pingen et al. 2011) (Fig. 2).

Major drug resistance mutations may lower the replicative capacity of HIV. In the absence of drug pressure in a newly infected host, a virus with resistance mutations can revert rapidly to wild type if this has a substantial fitness benefit. A well-known example is the NRTI resistance mutation M184V/I, which has a significant negative effect on the replicative capacity. In a cohort of patients with acute HIV infection, the longitudinal follow-up of baseline mutations showed a markedly faster replacement of M184V/I than NNRTI mutations which only have a minor effect on replicative

capacity (Jain et al. 2011). Since reversion from M184V/I to wild-type virus requires only one nucleotide change, a virus containing only M184V/I can rapidly revert to wild type with improved fitness.

Some resistance mutations require more than one mutation to revert to wild type, such as mutation T215Y/F in RT. Partial reversion to wild type results in intermediates (T215S/N/I). These intermediate variants have a replicative capacity similar to wild type and therefore tend to persist in the viral population. At position 215 in RT, also evolution toward atypical variants is often observed (T215C/D/L/V/G). A novel amino acid may be selected that is neither the wild-type amino acid nor an intermediate toward wild type. Due to the improved fitness compared to the originally transmitted variant, these atypical variants may persist for a long time. Both intermediate and atypical variants are called 215 revertants. Follow-up of ART-naïve patients showed that 215 revertants, in contrast to resistance mutation 215Y/F, were highly stable and are therefore often detected in treatment-naïve patients (Castro et al. 2013).

For some resistance mutations, it is known that they have a limited effect on the replicative capacity. There is no or only limited replicative advantage for wild-type virus; thus, the mutations may persist for years. Examples are NNRTI-resistance mutations, particularly K103N, and NRTI-resistance mutations L210W and K219R/G/Q/E (Little et al. 2008; Collins et al. 2004).

Persistence can also occur if reversion to wild type is not possible due to compensatory fixation by secondary mutations. This particular mechanism is seen if a primary resistance pattern is accompanied by one or more secondary (compensatory) mutations. These additional mutations have little or no contribution to the level of resistance, but (partly) compensate for the reduction in replicative capacity (Huigen et al. 2006). This process occurs in the drug-exposed host during therapy before the virus is transmitted, but has also been shown in vitro after transmission in absence of drug pressure. The pathway of reversion to wild type after the selection of compensatory mutations in the new host will include an initial decrease in fitness and therefore this pathway is blocked. As a result, viruses with drug resistance mutations may persist after transmission despite a lower replicative capacity than wild type.

First Insights in Prevalence of Transmitted Drug Resistance

After the first case reports of transmitted drug resistance, further analyses were performed to gain insight in the magnitude of this issue. The first studies on the prevalence of transmitted drug resistance were retrospectively performed in small cohorts of patients with primary HIV infection. During this time, patients were treated with mono- or dual therapy with thymidine analogues, which is now known to not sufficiently suppress the replication of HIV. A disturbing observation was the increasing prevalence of the resistance mutation at codon 215 in RT over time, from 1.4 % in 1988–1991 to 10.4 % in 1993–1994 (Mayers et al. 1995).

The increasing prevalence of transmitted resistance raised serious clinical and public health concerns and demonstrated the need to conduct epidemiologic surveys

to monitor transmitted resistance. In the years when HAART (highly active antiretroviral therapy, a combination therapy with at least three drugs to sufficiently suppress HIV replication) became the standard of care, a study in a cohort of newly infected individuals, mostly homosexual men, from American urban areas showed a prevalence of 16.3 % (13/80), with multidrug resistance in three individuals (Boden et al. 1999). A Swiss study reported a prevalence of transmitted resistance of 11 % (9/82). The majority was infected with a virus harboring mutations associated with resistance to zidovudine. They observed an unexpected low prevalence of transmission of the M184V mutation, which causes high-level resistance to lamivudine and is frequently detected in patients failing on therapy that includes this compound (Yerly et al. 1999). The authors suggested that this supports the lower fitness of HIV-1 variants harboring the M184V mutation that had been shown in vitro (Schuurman et al. 1995). In 2002, two studies that only included patients with primary HIV infection showed that transmitted drug resistance was also increasing among recently infected patients (Little et al. 2002; Grant et al. 2002). One of the studies that was done in 10 American cities showed detection of genotypic resistance increasing to 22.7 % (Little et al. 2002). Both studies also reported a longer time to virological suppression after start of therapy in patients with transmitted drug resistance. This resulted in the first suggestions that clinicians could no longer rely on empirical treatment guidelines and that resistance testing should have a role in guiding antiretroviral therapy.

Guidelines on Drug Resistance

Due to the presumed lower replicative capacity of certain resistant viral variants, it was initially uncertain for how long the transmitted resistance mutations could be detected in the absence of drug pressure in the newly infected host. Therefore, guidelines first recommended drug resistance testing for subjects who are recently infected (Hirsch et al. 2003; Vandamme et al. 2004). A landmark study among more than 2000 individuals from 19 European countries showed that 10.4 % of patients who had never been exposed to ART carried HIV with $\geq$1 drug resistance mutation (Wensing et al. 2005a). A significant difference in prevalence was observed between recently infected patients (13.5 %) and patients infected for more than 1 year (8.7 %). This could either be due to a lower exposure to drug-resistant virus in the past or it is caused by reversion to wild-type viruses over time. Nevertheless, it was shown that drug resistance mutations could still be detected among a considerable percentage of chronically infected patients. As more evidence became available that variants with resistance mutations can persist, guidelines were updated and genotype testing was recommended for all newly diagnosed HIV patients at the moment of diagnosis (Hirsch et al. 2008). The routine use of baseline genotyping also allows large-scale surveillance of transmitted drug resistance.

In 1997, the International AIDS Society-USA assembled an independent panel of experts to review the data on antiretroviral drug resistance. They provided a first list summarizing common mutations selected by PIs, NRTIs, and NNRTIs

(Hirsch et al. 1998), which has been frequently updated since then with the latest published update in 2014 (Wensing et al. 2014). Periodically updates are also available online (www.iasusa.org). The current list includes 22 NRTI-resistance mutations, 34 NNRTI-resistance mutations, 75 PI-resistance mutations, 10 enfuvirtide resistance mutations, and 12 INI resistance mutations. The overview is designed to help clinicians identifying key mutations associated with viral resistance to antiretroviral drugs and to guide clinical decisions regarding antiretroviral therapy.

As previously explained, polymorphisms can occur in the absence of selective drug pressure. However for some polymorphisms it is known that they contribute to resistance to antiretroviral drugs. Since the IAS-USA list is used for clinical purposes, these particular polymorphisms are included in this list. But these polymorphic mutations should not be taken into account when one wants to monitor transmission of resistance, because presence of these polymorphisms before start of therapy is not an indication of drug pressure in a (previous) host. Including resistance-related polymorphisms in surveillance monitoring could lead to falsely elevated estimates of transmitted resistance (Wensing and Boucher 2003).

Many countries have implemented surveillance programs to monitor transmission of drug resistance. Study groups used different criteria to define transmitted drug resistance, resulting in widely varying estimates. In 2007, the WHO developed a consensus list of surveillance drug resistance mutations (SDRMs) (Bennett et al. 2009). A standard list of mutations would also allow comparison of prevalence of transmitted drug resistance from different times and regions. Four criteria were used to identify surveillance drug resistance mutations: (1) SDRMs should be recognized as causing or contributing to drug resistance, (2) mutations should be non-polymorphic and should not occur at highly polymorphic positions, (3) the mutation list had to be applicable to the eight most common HIV-1 subtypes, and (4) the list should be parsimonious, excluding mutations resulting exceedingly rarely from drug pressure. It is not a simple task to provide a list of mutations that are specific for transmitted drug resistance, since certain mutations associated with resistance in subtype B occur as polymorphisms in other subtypes. The list is subject of ongoing discussion and was last updated in 2009. It differs from the IAS-USA list in terms of number, position, and mutations and currently includes 93 mutations: 34 NRTI-resistance mutations at 15 RT positions, 19 NNRTI-resistance mutations at 10 RT positions, and 40 PI-resistance mutations at 18 protease positions (Fig. 3) (Bennett et al. 2009).

Current Trends

The prevalence of transmission of drug resistance varies among regions, risk groups, and drug classes, due to different exposure to ART, the use of diverse definitions of drug resistance mutations, varying sampling times after infection, and different risk behavior and access to therapy among risk groups.

NRTI		NNRTI		PI	
M41	L	L100	I	L23	I
K65	R	K101	E, P	L24	I
D67	N, G, E	K103	N, S	D30	N
T69	D, Ins	V106	M, A	V32	I
K70	R, E	V179	F	M46	I, L
L74	V, I	Y181	C, I, V	I47	V, A
V75	M, T, A, S	Y188	L, H, C	G48	V, M
F77	L	G190	A, S, E	I50	V, L
Y115	F	P225	H	F53	L, Y
F116	Y	M230	L	I54	V, L, M, A, T, S
Q151	M			G73	S, T, C, A
M184	V, I			L76	V
L210	W			V82	A, T, F, S, C, M, L
T215	Y, F, I, S, C, D, V, E			N83	D
K219	Q, E, N, R			I84	V, A, C
				I85	V
				N88	D, S
				L90	M

Fig. 3 Surveillance drug resistance mutations (SDRM) list of the WHO (Adapted from Bennett et al., PLoS One (2009))

Western Countries

In Europe, the overall prevalence of transmission of drug resistance seems to be stabilizing just below 10 % (Vercauteren et al. 2009; Hofstra et al. 2013a). A European surveillance cohort that includes newly diagnosed patients from 26 countries reported an overall prevalence of TDR of 9.2 % in 2008–2010. Mutations associated with resistance to NRTIs were observed most frequently (5.1 %), followed by resistance to NNRTIs (3.7 %). Regarding the different drug classes, no significant trends over time were observed (Hofstra et al. 2013a).

In the USA and Canada, an increasing prevalence of transmitted NNRTI resistance is observed. In a cohort of recently infected MSM in New York, enrolled from 1995 to 2010, the overall prevalence of resistance was 14.3 %. Over time NRTI resistance declined, but the prevalence of the NNRTI mutation K103N increased from 1.9 % to 8.0 % in 2010 (Castor et al. 2012). A cohort in San Francisco that included patients with acute HIV infection, mostly MSM, showed a rise in TDR from 2003 to 2007 to levels up to 24 %. Although this upward trend did not continue in 2008–2009, TDR remained substantial at 15 % (Jain et al. 2010). Convenience

sampling of more than 18,000 newly diagnosed individuals of all risk groups in 2007–2010 from 10 surveillance areas in the USA showed an overall prevalence of 16.2 % transmitted drug resistance. The majority of transmitted drug resistance was due to mutations conferring drug resistance to a single drug class. Over time, NNRTI resistance significantly increased from 7.1 % in 2007 to 8.6 % in 2010, with K103N as the most frequent detected mutation (Kim et al. 2013). In a multi-site cohort of treatment-naïve individuals from Canada studied from 2002 to 2009, the overall prevalence was 13.6 %. An increase over time was observed which was driven by an increase in NRTI- and NNRTI-resistance mutations (Burchell et al. 2012).

This is likely due to the increased use of NNRTIs in first-line regimens. NNRTIs have a good efficacy and come with a low pill burden, but they have a low genetic barrier to resistance. A single amino acid change is sufficient to cause high-level resistance to most commonly used NNRTIs. These mutations (e.g., K103N) can be easily selected in patients failing on NNRTI-based regimens and can persist after transmission to a new host due to their limited effect on replicative capacity of the virus.

Transmission of PI resistance remains low in Europe (2.3 % in 2008–2010) (Hofstra et al. 2013a). In the different cohorts in the USA and Canada, prevalence of PI resistance varied between 2.7 % and 4.5 %. This is probably due to the preference of NNRTIs over PIs in first-line regimens. Furthermore, current PIs which are boosted with ritonavir have a high genetic barrier to resistance, rarely resulting in selection of PI-related resistance in protease in treated individuals.

In general, higher prevalence of transmitted drug resistance was reported in subtype B virus than in non-B viruses (Wensing et al. 2005a; Frentz et al. 2011). The question is whether this difference in prevalence can be attributed to the lack of exposure to drugs in countries with a high prevalence of non-B HIV or whether this is a result of specific viral characteristics. Furthermore, MSM infected with subtype B virus were more likely to be infected with drug-resistant HIV than other patients (Vercauteren et al. 2009). Risk behavior may play a role, although MSM may also be more exposed to antiretroviral drugs compared to patients from other risk groups.

Resource-Limited Setting

Sub-Saharan Africa has the largest number of people living with HIV/AIDS, an estimated 22.9 million people in 2010. This number is still increasing due to expanded access to antiretroviral therapy and care, currently reaching nearly 50 % of those in immediate need of therapy (World Health Organization et al. 2011). At the end of 2011, more than eight million people were receiving antiretroviral therapy in low- and middle-income countries, a dramatic 26-fold increase from December 2003 (World Health Organization 2012).

A consequence of ART scale-up is the possibility of treatment failure with the selection of drug-resistant HIV-1 variants (acquired drug resistance) and subsequent spread (transmitted drug resistance). In Europe and North America, the initial treatment with non-potent mono- and dual therapies resulted in high levels of

acquired resistance and subsequent high levels of transmitted resistance. Due to increased potency of ART, virological monitoring, and baseline resistance testing to guide first-line therapy, the level of acquired resistance decreased and transmitted resistance stabilized. However, in resource-limited settings, both history and conditions of HIV treatment have been very different, making it difficult to predict trends for transmitted drug resistance in the developing world.

Antiretroviral drugs were initially introduced into African countries through preventing mother-to-child transmission (PMTCT) programs. Zidovudine was the first drug used in 1994, followed by nevirapine, lamivudine, efavirenz, tenofovir, and lopinavir. Single-dose usage of nevirapine resulted in a high prevalence of acquired nevirapine resistance in both mothers and infants. The public health approach of the WHO for the recent scale-up of ART is based on simplified treatment protocols, including standard first-line NNRTI-based regimens and second-line boosted PI-based regimens. A recognized limitation of NNRTI-based regimens is their relatively lower genetic barrier to resistance when compared to boosted PI regimens (World Health Organization 2012). Suboptimal regimens, such as the peripartum use of single-dose nevirapine in PMTCT, drug interactions (e.g., concomitant use of tuberculostatic therapy), inappropriate prescribing practices, and poor adherence can further increase the risk of acquiring drug resistance. Programmatic factors such as limited human resources, inadequate infrastructure, and weak supply management systems can negatively influence treatment adherence and retention in care and can result in drug stockouts (Hamers et al. 2013). A WHO survey showed that in 2004–2009, a large proportion of ART programs in 2107 clinics in more than 50 countries did not perform optimally regarding patient adherence, retention in care, and drug supply continuity, raising concern on potential increasing drug resistance (World Health Organization 2012). Abrupt interruption of combination therapy is of particular concern for selection of NNRTI resistance, since the longer plasma half-life of efavirenz results in functional efavirenz monotherapy with a low genetic barrier for resistance.

Plasma viral load testing is not generally available to monitor the efficacy of therapy and detect therapy failure. Instead, monitoring is often based on clinical staging and CD4 cell counts. However, it has been shown that virological failure precedes the WHO-defined immunological and clinical failure, often with detection of NNRTI resistance. Rapid accumulation of drug resistance (mostly NRTI resistance) occurred when ART was continued despite virological failure, resulting in the loss of treatment options (Barth et al. 2012). A randomized controlled trial at seven primary health clinics in rural Kenya showed that 6 monthly viral load testing of patients taking ART reduced the risk of virological failure at 18 months of follow-up with 46 % (Sawe et al. 2013). New guidelines of the WHO advise viral load testing at 6 months after initiating therapy and every 12 months thereafter (World Health Organization 2013).

Baseline resistance testing is not readily available and is mainly performed within studies and WHO surveys to monitor transmitted drug resistance. The WHO survey method is intended to classify transmitted resistance as low (<5 %), moderate (5–15 %), or high (>15 %) and is not meant as representative surveillance for the entire country.

In 2004–2010, 72 WHO surveys have been conducted in 26 countries, of which 52 (72.2 %) had a low prevalence classification to all drug classes (World Health Organization 2012). Twenty surveys showed moderate levels of transmitted drug resistance. Main detected mutations included NNRTI-resistance mutations (K103N, Y181C) and M184V and to a lesser extent TAMs (thymidine analogue mutations). Pooled analysis indicated that the estimated prevalence of transmitted HIV drug resistance to NNRTIs increased between 2004 and 2010, particularly in the African region. The data suggested that greater coverage of ART was associated with a higher prevalence of TDR, particularly to NNRTIs (Fig. 4) (World Health Organization 2012). The PharmAccess African Studies to Evaluate Resistance Monitoring (PASER-M) cohort study among 2,436 antiretroviral-naïve individuals in 11 geographic areas in Kenya, Nigeria, South Africa, Uganda, Zambia, and Zimbabwe reported an overall baseline prevalence of drug resistance of 5.6 %. They estimated a 38 % increase of the average rate of resistance per year since ART rollout (Hamers et al. 2011).

In Asia, ART is also being rapidly scaled up, based on a similar public health approach as seen in sub-Saharan Africa. Several surveys have been done, but generally the studies have small sample sizes. Furthermore, generalizability is limited because the methodology is often unstandardized and based on convenience sampling that is often performed in urban populations or only in a particular risk group. Despite these caveats, most of the performed surveys report a low prevalence of TDR (Trotter et al. 2013; Yu et al. 2011). The TREAT Asia Studies to Evaluate Resistance Monitoring (TASER-M) program studied prevalence of transmitted drug resistance in 1,340 ART-naïve patients from 11 sites in Thailand, Malaysia, Hong Kong, Philippines, and Indonesia from 2007 to 2010 (Kiertiburanakul et al. 2013). With use of the WHO surveillance list for TDR, they reported a prevalence of 4.0 %. The TASER-S (Surveillance) program included 458 recently infected patients from four sites and reported a slightly higher prevalence of 6.1 % (Kiertiburanakul et al. 2013). A recent review of available studies in naïve patients from Asia that also used the WHO surveillance list for TDR reported an overall prevalence of 7.6 % (Stadeli and Richman 2013). Regarding the different drug classes, resistance mutations related to NRTIs (4.3 %) and NNRTIs (3.8 %) were observed most frequently. Only 0.3 % had resistance mutations associated with PIs. Compared to patients in Africa, Asian patients were more likely to harbor a virus with resistance mutations. In some Asian countries such as Thailand and Vietnam, ART has been available since the mid-1990s and included the use of suboptimal dual nucleoside regimens which may have resulted in higher levels of acquired drug resistance and subsequent transmitted drug resistance.

Recently a comprehensive global assessment of published studies and WHO surveys was performed on HIV-1 drug resistance in untreated patients, including 26,102 persons in 42 countries (Gupta et al. 2012). East Africa had the highest estimated rate of increase at 29 % per year since rollout, with estimated resistance prevalence at 8 years after rollout of 7.4 %. An annual increase of 14 % was estimated in Southern Africa. For West and Central Africa, Latin America, and Caribbean, no such increase was found. For Asia, no consistent trend could be observed due to the small amount of studies that were mainly done in Thailand, China, India, and Vietnam and due to the large differences among these countries.

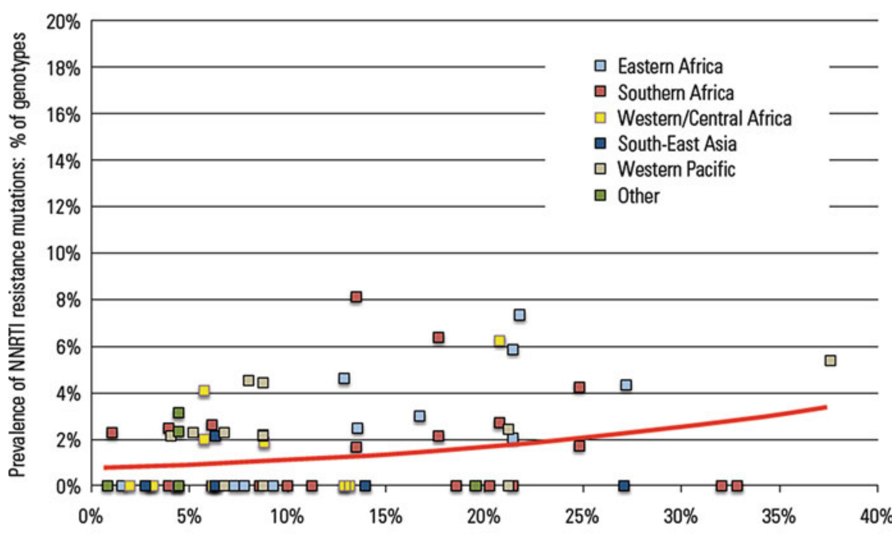

Fig. 4 Relationship between transmitted resistance mutations to NNRTI drugs and antiretroviral therapy coverage (Adapted from World Health Organization, *The HIV drug resistance report – 2012* (World Health Organization 2012))

Particularly NNRTI resistance increased substantially in east and southern Africa. However, a limitation of the meta-analysis was that the data included in the analyses were not likely to represent national prevalences, since most studies included urban populations, where ART coverage and pre-rollout ART use is likely to be higher than in rural areas.

In summary, cumulated data suggest a rise in TDR in east and southern Africa after ART scale-up. The rise is mostly driven by NNRTI resistance, which is of particular concern, as this drug class constitutes the foundation of current first-line ART regimens and prophylaxis for prevention of mother-to-child transmission. It highlights the need for virological monitoring of therapy failure and the importance of establishing surveillance of transmitted drug resistance. However, despite the rise in transmitted drug resistance, the current standard first- and second-line regimens are still shown to be effective in the majority of patients in resource-limited settings (Barth et al. 2010; Schoffelen et al. 2013; Siripassorn et al. 2010).

Molecular Epidemiology: Sources of Transmitted Drug Resistance

The availability of baseline resistance data of newly diagnosed HIV patients allows studying phylogenetic relationships. A large percentage of patients with recent HIV infection are part of transmission clusters, indicating that patients in early

stage of infection play an important role in the spread of HIV (Brenner et al. 2007, 2008). Longitudinal studies among HIV serodiscordant couples in the Ugandan Rakai cohort also showed that the rate of HIV transmission was highest during early-stage infection (Wawer et al. 2005). In the early stage of infection, HIV RNA plasma levels are high, and the majority of patients is still unaware of their infection and therefore may not have yet adapted their risk behavior. A Swiss study showed that almost half of the transmitted drug resistance was linked to transmission clusters that included only newly diagnosed individuals (Yerly et al. 2009). For a long time, it had been assumed that transmitted drug resistance reflects direct infection from drug-experienced individuals, but these results demonstrated that newly diagnosed untreated individuals are also a source of onward transmission of drug resistance.

Onward transmission of resistance by treatment-naïve individuals may represent a limit to the decline in transmitted drug resistance. In Europe, a stable prevalence is observed, in particular for NRTI-resistance mutations. Thymidine analogue mutations (TAMs), such as M41L and T215Y/F, are usually selected in patients failing on regimens that include zidovudine or stavudine. Despite the decreased use of these compounds in first-line regimens, M41L and T215 revertants are among the most frequent observed mutations in case of transmitted drug resistance, most likely due to onward transmission by naïve patients. This phenomenon is also proposed as an explanation for the substantial level of transmitted drug resistance (15 %) in acutely infected patients in San Francisco, besides the increasing NNRTI resistance (Jain et al. 2010). Phylogenetic analysis of more than 2500 HIV-infected MSM in Montreal (Canada) revealed between 2002 and 2009 an episodic expansion of sub-epidemics with NNRTI-resistant virus among therapy-naïve individuals (Brenner et al. 2012).

Cluster analyses in the UK and the Netherlands have shown that drug-resistant viruses can circulate for a prolonged time among therapy-naïve patients, up to 8 years (Hue et al. 2009; Hofstra et al. 2013b). In the cluster observed in the Netherlands, it concerned a virus harboring a partly reverted resistance pattern. In clinical practice when such a reverted pattern is detected in naïve patients, the possibility that reversion occurred in these patients and initial infection was established by a fully resistant virus is usually taken into account. Concerns for more extensive resistance present in the quasispecies then result in the choice of more complex initial regimens with a higher genetic barrier, increased pill burden, more frequent toxicity, and elevated costs. However, the prolonged onward transmission of this particular strain made it more likely that reversion occurred in previous hosts in the transmission chain (Hofstra et al. 2013b). Mutation patterns that are frequently observed are single mutations or combinations of partly reverted TAMs. These patterns have little effect on replicative capacity which could be one of the reasons these patterns are transmitted by and circulating among therapy-naïve individuals. In this case, fear of hidden extensive resistance may not be necessary and one can initiate standard first-line therapy. The use of phylogeny to identify clusters may provide insight into the risk of more extensive resistance patterns in the quasispecies and prevent excessive use of antivirals.

Transmissibility of Resistant Virus

As was already noted in the early reports on transmitted drug resistance, there is a discrepancy in resistant profiles observed in drug-experienced patients failing treatment and those detected in treatment-naïve patients. This has been attributed to the well-known fitness costs of certain resistance mutations, which have not only been observed in vitro but also in vivo, reflected by a lower pre-therapy viral load in patients with the M184V mutation (Harrison et al. 2010). Plasma HIV RNA levels are one of the correlates of risk of transmission (Quinn et al. 2000). It has been hypothesized that drug-resistant variants may also have reduced transmission capacity.

Clinical studies evaluated this issue by comparing the percentage of acquired drug resistance among potential transmitters to the actual detected percentage of transmitted drug resistance among acutely HIV-infected individuals in the same region. Both studies observed lower transmission of resistance than could be expected based on the prevalence of acquired drug resistance and suggested that this could be due to a lower transmissibility of resistant variants (Leigh Brown et al. 2003; Yerly et al. 2004). However, a possible confounder is the risk behavior of the potential transmitters. Patients who failed on therapy may be more ill and therefore more likely to have reduced sexual activity and/or may have adapted their risk behavior to their HIV-positive status. Furthermore, these studies may underestimate the prevalence of transmitted drug resistance due to reversion of resistance mutations in untreated individuals. A study that used a more sensitive method to detect resistant variants showed that the M184V mutation is transmitted at higher rates than currently detected in clinical practice because its presence wanes over time (Wainberg et al. 2011).

Two recent studies did show diminished transmission efficiency relative to wild-type virus. In macaques, efficient systemic infection with SHIV162P3 mutants (M184V and K65R) after rectal and vaginal exposure required an increase in the virus inocula compared to wild-type SHIV162P3 (Cong et al. 2011). In a skin model, drug-resistant variants with a low replicative capacity (M184V/I/T) were less able to infect primary Langerhans cells compared to resistant viruses with a higher replicative capacity (K103N) or wild-type virus. Subsequent transmission to target cells was also decreased in these resistant variants (Pingen et al. 2014). This issue needs further study in the future.

Clinical Outcome of Transmitted Drug Resistance

In 2002 the first studies showed that patients infected with a virus with drug resistance mutations needed a longer period of time after initiation of treatment to achieve virological suppression while the time to virological failure was shorter, compared to patients infected with wild-type virus (Little et al. 2002; Grant et al. 2002). Virological failure was not only observed earlier, but also more frequently in case of transmitted drug resistance (Violin et al. 2004; Kuritzkes

et al. 2008). Chronically infected patients with primary resistance from a German cohort that received a first-line regimen based on the results of resistance testing reported a similar efficacy of first-line HAART as patients infected with wild type, which suggested that resistance test guidance for selection of first-line regimen could improve therapy outcome (Oette et al. 2006). This was recently confirmed in a retrospective study that included over 10,000 patients who initiated combination therapy after 1998 (Wittkop et al. 2011). Of patients with TDR who received a regimen that was not fully active against the virus, 15.1 % experienced virological failure at 12 months. In contrast, patients infected with wild-type virus or patients with TDR who received a regimen that was still fully active against the virus experienced virological failure at much lower rates (4.2 % and 4.7 %, respectively). Patients with low-level or intermediate resistance to at least one drug had a 2.2-fold higher risk for virological failure, while patients with high-level resistance to at least one drug had a 6.3 times higher risk (Fig. 5). A recent study among more than 1,600 patients from 13 European centers who are receiving an efavirenz (NNRTI)-based regimen as first-line regimen also showed that intermediate baseline resistance to one drug significantly increases the risk of virological failure (Swartz et al. 2015).

It is clear that transmitted drug resistance influences therapy outcome, but whether it also impacts the natural course of the infection remains subject of debate. A faster CD4 cell decline in the first year of infection in patients with transmitted drug resistance was observed. However, a negative impact on the longer term was not demonstrated (Pillay et al. 2006). An alarming case report described a patient who was recently infected with a viral variant of HIV resistant to multiple classes of antiretroviral but who progressed to symptomatic AIDS in 4–20 months (Markowitz et al. 2005). The probable source patient and his partner experienced a relatively indolent clinical course, suggesting that possibly other factors than the virologic properties (such as genetic, immunological, or behavioral factors) may have contributed to the rapid clinical decline described in the case report (Blick et al. 2007). Comparison of patients infected with resistant virus vs. wild-type virus showed no significant difference in progression to AIDS in the first year (Wensing et al. 2005b).

Minority HIV Variants

The high mutation rate of HIV allows quick adaptation to new environments, where the viral variant with the highest fitness will dominate the quasispecies. When wild-type virus is the dominant variant, resistance mutations can still be present in minority variants. Genotypic testing in clinical practice is performed with population (Sanger) sequencing, which is limited to the detection of viral variants that constitute 10–20 % of the total virus population in a sample (Larder et al. 1993). To detect minority variants, more sensitive methods have been developed, utilizing two main approaches: point mutations assays and newer sequencing technologies.

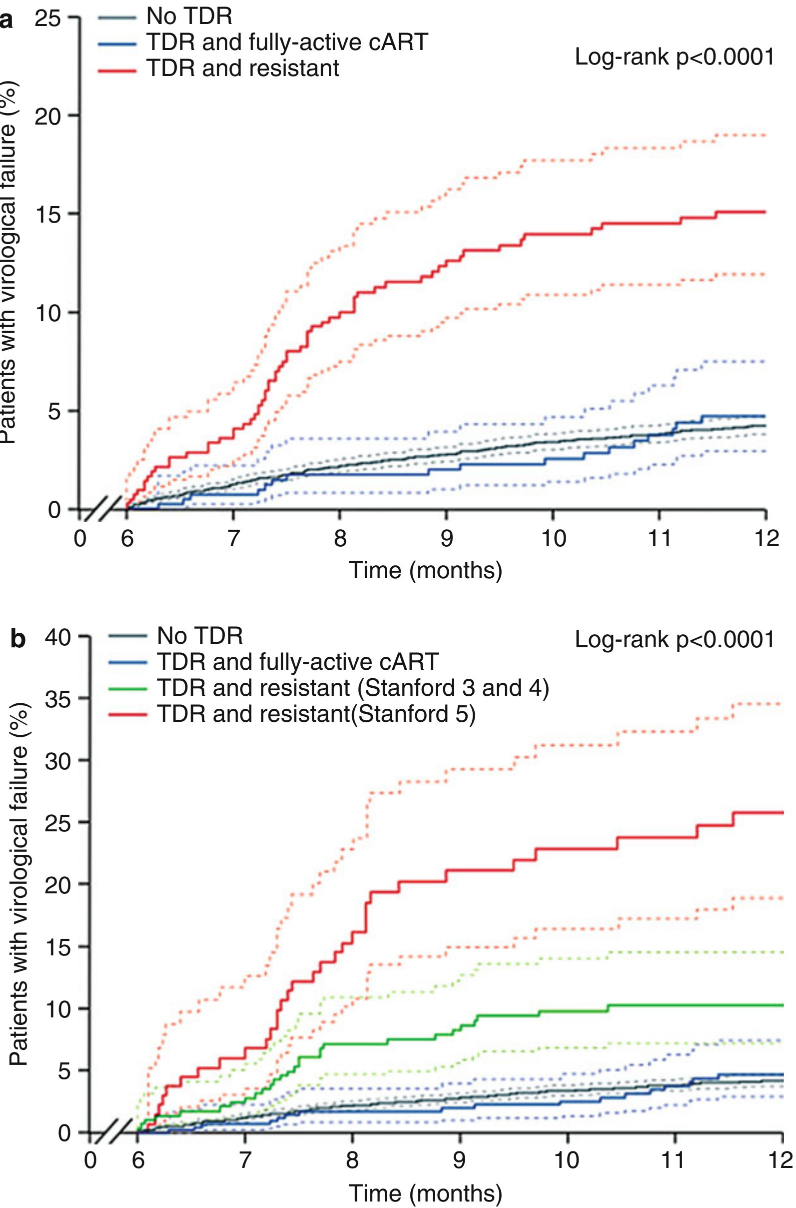

Fig. 5 Kaplan-Meier estimates of the proportion of patients with virological failure. (**a**) Risk of virological failure according to patient groups. (**b**) Risk of virological failure in patients with intermediate- and high-level resistance. *cART* combination antiretroviral therapy. *TDR* transmitted drug resistance. *Dotted lines* = 95 % CI (Adapted from Wittkop et al. (2011))

Point mutation assays such as allele-specific polymerase chain reaction (AS-PCR) are generally highly sensitive and specific for detecting a selected minority drug resistance mutation, but are limited to the detection of only a single point mutation at a time. In contrast, newer sequencing methods including single-genome sequencing (SGS) and ultra-deep pyrosequencing (UDPS) permit analyses of the entire sequence context but are more labor intensive and costly than point mutation assays. Allele-specific PCR can detect minorities at frequencies as low as 0.01 %, but may generate false positive results at the lower level of detection. Therefore, for each mutation one needs to assess the individual cutoff of detection. This is not necessary for the newer sequencing methods, which can detect viral variants at frequencies <1 %. For both approaches, the tested sample must have a large enough viral population size to ensure representative results on the minority variants present (Gianella and Richman 2010).

Using these more sensitive methods, minority resistant variants have been detected in acutely infected individuals that were not detected by population sequencing (Metzner et al. 2005; Toni et al. 2009). Possibly current surveillance studies with use of population sequencing are underestimating transmission of drug resistance. It had been shown that persisting minority resistant variants in NNRTI-experienced patients contributed to failure of an NNRTI regimen (Halvas et al. 2010). Whether the presence of minority resistant variants in therapy-naïve patients may affect the response to antiretroviral treatment is an essential question that remains subject of debate.

To date, several studies have been performed to determine the prevalence of baseline minority resistance mutations and their influence on rates of virological failure. Minority drug resistance mutations were detected in patients with wild-type virus by standard population sequencing (Paredes et al. 2010; Johnson et al. 2008). With the use of AS-PCR that could detect minority variants with K103N at levels as low as 0.003 % and Y181C at 0.03 %, one study detected minority variants in up to 40 % of patients (Paredes et al. 2010). Most studies showed that minority NNRTI- and NRTI-resistant variants significantly increased the risk of failure, particularly for NNRTI-based regimens, but not all could demonstrate such an effect. The studies have been performed on small cohorts, usually less than 100 patients. Therefore, a pooled analysis of the data of 10 studies was performed, including 1,263 patients, which showed that the presence of drug-resistant minority variants was associated with more than twice the risk of virological failure in patients receiving an initial NNRTI-based regimen, after controlling for adherence, race, baseline CD4 cell count, and baseline HIV RNA load (Li et al. 2011).

Not all patients with presence of drug-resistant minority variants experienced virological failure. It was shown that the presence of minority variants above 1 % conferred a higher risk of failure compared to minority variants present at less than 1 %. To explain this observation, the authors proposed a theory that minorities can arise from different sources: mutations present at frequencies >1 % may represent transmitted drug resistance that over time has been replaced by wild-type revertants, whereas mutations at lower frequencies could be the result of de novo mutations resulting from random error or laboratory artifacts (Li et al. 2011).

A previous study proposed to distinguish between proportion and magnitude of drug-resistant variants in the population. By multiplying the proportion of virus harboring resistance mutation K103N in RT and the viral load, Goodman et al. showed that the number of resistant copies correlated better with the rate of failure than the proportion of drug-resistant variants (Goodman et al. 2011). It has been frequently shown that viral load correlates with failure. In some clinical trials, particular regimens were less successful in patients with higher viral loads, which could be due to a higher load of resistant variants. The pooled analysis also showed an increasing risk of failure when the number of resistant copies increased (Li et al. 2011).

Resistance testing after the occurrence of virological failure in the patients of the pooled analysis showed that the presence of NNRTI-resistant minority variants was associated with an increased risk of NNRTI resistance detected at virological failure. However, the emerged resistance at failure frequently differed from those detected as minority variants at baseline. The researchers proposed several explanations for this unexpected finding: minority variants could predispose for the development of additional resistance mutations or more fit resistant variants have been selected before failure was detected. They also suggest the possibility that there could have been other undetected resistance mutations that eventually became the dominant species (Li et al. 2013).

The question is whether results of these sensitive assays can guide clinical decisions on treatment in the future. More research is necessary to establish a threshold and understand at what point a minority resistant variant may become clinically relevant.

Transmitted Drug Resistance to Other Classes

Maraviroc is an antagonist of CC chemokine receptor 5 (CCR5), one of the coreceptors used by the virus for cell binding and is the only antiretroviral drug aiming at a human instead of viral target. Resistance to maraviroc is not fully understood. There is yet no consensus on specific signature mutations that are associated with resistance of CCR5-tropic viruses to maraviroc. Resistance may also evolve from a tropism switch resulting in the use of the CXCR4 coreceptor, which also occurs without maraviroc pressure. As such no data can be presented on transmitted resistance to maraviroc.

Enfuvirtide is a fusion inhibitor that is not often used in clinical practice because it needs to be injected twice daily. In contrast to maraviroc, for enfuvirtide several signature mutations in the first heptad repeat (HR1) region of the gp41 envelope gene are described (Wensing et al. 2014). In 2007 the first two cases of transmitted drug resistance to enfuvirtide were reported in a cohort of 53 newly infected patients (Peuchant et al. 2007). Surveillance studies usually include only the *pol* region; therefore, no extensive baseline reports for transmitted resistance to enfuvirtide are available.

Integrase inhibitors (INIs) are a potent option for treatment of HIV and are becoming more in use in first-line treatment. Both raltegravir and elvitegravir are highly efficacious in first-line ART with small numbers of failure in clinical trials. However, both have a relatively low genetic barrier to resistance and cross-resistance between the two drugs precludes their sequential use. Dolutegravir does retain activity against most viruses that are resistant to raltegravir and elvitegravir (Geretti et al. 2012). With the increasing use in clinical practice, acquired resistance to INIs and subsequent transmitted drug resistance may emerge. The first two cases of transmitted drug resistance to INIs were reported in 2011 (Boyd et al. 2011; Young et al. 2011). Both cases were antiretroviral-naïve patients with multidrug resistance. In a cohort of 79 antiretroviral-naïve patients who were recently infected in a time when INIs were in use, no transmitted INI drug resistance was observed (Cossarini et al. 2011). It is too early to perform INI resistance testing routinely, but the two case reports show that INI resistance testing currently has additional value in patients that present with multiclass transmitted drug resistance. More surveys will be done in the future to determine at which point in time the prevalence of integrase mutations among treatment-naïve patients rises to a level that warrants routine baseline resistance testing for INI resistance.

Conclusion

Transmitted drug resistance remains an issue of concern. Due to onward transmission of drug resistance by therapy-naïve individuals that is observed in developed countries and the rise in acquired drug resistance and subsequent transmitted drug resistance after ART rollout in resource-limited settings, it is unlikely that the prevalence of transmitted drug resistance will decrease in the near future. In particular, the rising resistance to NNRTIs, a drug class that is frequently used as first line – in both developed countries and resource-limited settings – warrants continued surveillance. As this drug class has a low genetic barrier to development of resistance, possibly even minority resistant variants may have an impact on clinical outcome. Future research with the use of more sensitive assays is necessary to establish a threshold for clinically relevant resistant variants. In addition, future surveillance efforts will include *integrase* resistance testing, to monitor transmitted drug resistance to integrase inhibitors, a drug class that will be increasingly used in first-line therapy.

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HIV-1 Drug Resistance in Pre-exposure Prophylaxis Trials

Teri Liegler and Robert Grant

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Abstract

When used with other proven strategies for prevention of HIV-1 acquisition, oral and topical preexposure prophylaxis (PrEP) has been shown to be effective in multiple randomized, placebo-controlled clinical trials throughout the world. Preexposure prophylaxis trials have included over 20,000 men and women at

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risk for HIV infection through sexual or intravenous exposure. A consistent finding is that drug exposure is essential for PrEP efficacy. In PrEP users with breakthrough infection, selection of drug-resistant virus is a possible outcome, presenting a unique sequence of events and outcomes compared with therapeutic use of antiretroviral drugs. Study findings have indicated that drug resistance selected by PrEP occurs rarely, except in cases where PrEP is initiated in very early infection, prior to seroconversion, and detectable only with nucleic acid tests. In this review, we discuss the factors associated with PrEP which may contribute to drug resistance and summarize the frequency and characteristics of HIV-1 drug resistance reported to date from global clinical trials. A theoretical framework of the causes and consequences of drug resistance in PrEP is considered as a basis of the real-life outcomes and challenges in implementing PrEP.

Keywords
Preexposure prophylaxis • PrEP • HIV-1 • Antiretrovirals • Drug resistance

Introduction

The concept of chemoprophylaxis, or using antimicrobial agents in uninfected humans to prevent infection, is a widely used and successful strategy for prevention of infection with endemic microbes such as malaria (Breman and Brandling-Bennett 2011). One of the great successes in HIV prevention is providing antiretroviral therapy (ART) perinatally to infected pregnant women to block mother-to-child transmission (MTCT) (Connor et al. 1994). Now with the availability of a large number of potent antiviral drugs, coupled with reduced toxicities and convenient dosing formulations (Gandhi and Gandhi 2014), the benefits of using oral or topical ART as preexposure prophylaxis (PrEP) may outweigh risks of prolonged drug exposure in healthy, uninfected people. As a result, using preexposure prophylaxis (PrEP) to prevent HIV infection in at-risk individuals has moved from the conceptual realm (Youle and Wainberg 2003a, b) to phase II/III safety and efficacy trials and now to initial implementation in demonstration projects and clinical practice.

However, as was revealed in early single-dose treatment strategies for MTCT prevention, suboptimal exposure to ART can result in PrEP failure and selection for drug-resistant variants in the infected infants (Arrive et al. 2007; Eshleman and Jackson 2002; Eshleman et al. 2001; Johnson et al. 2005; Micek et al. 2010). Due to the incidence and nature of drug resistance in this setting, concern over the use of PrEP for sexual transmission has been raised (Cohen and Baden 2012; Hurt et al. 2011; Liu et al. 2006). While the clinical impact and treatment options of viruses harboring drug-resistance mutations acquired by suboptimal ART initiated after established infection are well known and may be relevant to breakthrough infections during PrEP, the potential impact of PrEP-selected drug resistance at the population level is less clear. The benefits (infections averted) versus risks (drug resistance) with PrEP use have been modeled with significantly differing outcomes and interpretations based on input variables and assumptions (Abbas et al. 2011;

Baggaley et al. 2011; Dolling et al. 2012). In addition, in antiretroviral drug-experienced populations, the prevalence of circulating strains with drug resistance to PrEP agent(s) may impair the efficacy of PrEP. Now, with accumulating results from initial global randomized PrEP efficacy trials, the benefits and risks of PrEP use for HIV acquisition can undergo evidence-based assessment, allowing an in-depth understanding of the nature and frequency of PrEP-associated drug resistance, a critical step toward optimizing its use as a prevention strategy in all at-risk populations.

PrEP Efficacy for Prevention of HIV Sexual Transmission: Summary of Results from Randomized Controlled Trials

There are now multiple reports from randomized double-blind placebo-controlled clinical trials spanning four continents and over 20,000 individuals testing the safety and efficacy of oral and topical PrEP coupled with other proven prevention strategies [reviewed in (Baeten and Celum 2013; Celum and Baeten 2012)]. Topical PrEP as 1 % tenofovir (TVF) vaginal gel used pre- and postcoitally was first shown to be effective in preventing HIV transmission by sexual exposure in African women (Abdool Karim et al. 2010). Daily oral dosing of tenofovir disoproxil fumarate (TDF, the oral prodrug of TFV) used alone or co-formulated with emtricitabine (FTC) proved efficacious in preventing sexual transmission in men who have sex with men and transgender women (MSM/TGW) from South America, South Africa, Thailand, and the United States in the iPrEx study (Grant et al. 2010), in serodiscordant African male and female partners in the Partners PrEP study (Baeten et al. 2012), and in African men and women in the TDF2 study (Thigpen et al. 2012). Finally, the Bangkok tenofovir study demonstrated that daily oral TDF dosing was associated with a 48.9 % reduction in HIV infections in injecting drug users randomized to taking TDF compared with placebo (Choopanya et al. 2013). A consistent finding from these studies showing a reduction in HIV-1 acquisition ranging from 42 % to 73 % in participants randomized to the PrEP arms is that PrEP efficacy is directly associated with drug exposure. In nested, case-control studies within each of these trials, the overall relative infection risk reduction further increased to over 90 % in participants with measurable plasma or cellular drug levels.

Not all PrEP trials with similar designs have shown reduced infections in the active drug arms when compared to placebo. The FEM-PrEP study, which enrolled African women, was stopped early due to futility where a similar infection frequency occurred in participants randomized to oral FTC/TDF and placebo (Van Damme et al. 2012). And in the multi-arm VOICE trial, a statistically indistinguishable number of infections occurred in women randomized to either 1 % TFV vaginal gel, daily oral TDF or FTC/TDF, or placebo (Marrazzo et al. 2013). The basis of differences in efficacy outcomes between these two studies and those demonstrating protection against HIV acquisition is an active area on investigation. One key factor is product adherence, determined directly by antiretroviral drug level measurements in the blood plasma and cells. Overall, women randomized to the active arms in the

FEM-PrEP and VOICE trials had insufficient product use to measure efficacy. While it is evident that distinct PrEP modalities will need to be tailored to particular at-risk populations and their circumstances contributing to HIV-1 transmission and that tenofovir-based PrEP regimens can effectively block HIV acquisition when used regularly and in combination with other prevention methods, a setting of incomplete adherence coupled with exposure risk potentially increases the chances of infection and selection for PrEP-associated resistance.

Selection and Expansion of Drug-Resistant HIV in Response to Suboptimal Antiretroviral Therapy

There are now nearly three decades of experience with therapeutic antiretroviral agents designed to target multiple stages in the HIV-1 life cycle (Arts and Hazuda 2012). Regular use of combination therapy can provide durable virologic suppression within an individual to levels below that detected by standard clinical viral load assays and can have a favorable impact in lowering the community viral load or the aggregate level within a defined geographical region (Das et al. 2010; Montaner et al. 2010). But although the available arsenal of antiretroviral drugs shows continued improvement in potency, pharmacodynamics, formulation, and toxicities, the generation and selection of drug-resistant variants continue to be a barrier to durable suppression, especially in developing countries with limited regimen choice and lack of regular virologic monitoring (Hamers et al. 2013; Sigaloff et al. 2011).

In individuals with existing infection and ongoing viral replication, sustained use of non-suppressive therapy or intermittent use of suppressive therapy will quickly promote selection and expansion of drug-resistant variants. Even during subsequent virologic suppression following a regimen change, drug-resistant variants remain archived in target cells as proviral DNA, potentially limiting future therapeutic options. Distinct outcomes of ART use following established infection within an individual are shown schematically in Fig. 1a–c to highlight the dynamic makeup of quasispecies that may arise during treatment failure. The same ART used as PrEP can also select for drug-resistant variants when breakthrough infections occur or when PrEP is initiated with unrecognized infection (Fig. 1d–f), underscoring the importance of careful virologic monitoring before and during PrEP use. The outcomes schematized in Fig. 1 have been observed in many of the PrEP trials where regular serologic testing was performed.

The development of resistance to any particular drug is driven by the high error rate in HIV-1 reverse transcriptase. With a mutation frequency of approximately 4×10^{-5} per target base per replication cycle and a nearly 10 kb genome size, there is roughly one mutation produced per replication cycle (Mansky 1996; Mansky and Temin 1995). Coupled with an estimated 10^{10} virions produced per day (Ho et al. 1995; Wei et al. 1995; Perelson et al. 1996), the fixation of a new, randomly generated mutation under targeted selection can be rapid, as notably illustrated by M184V selection after suboptimal lamivudine monotherapy (Wainberg et al. 1995; Larder et al. 1995; Schuurman et al. 1995). While mutations conferring reduced

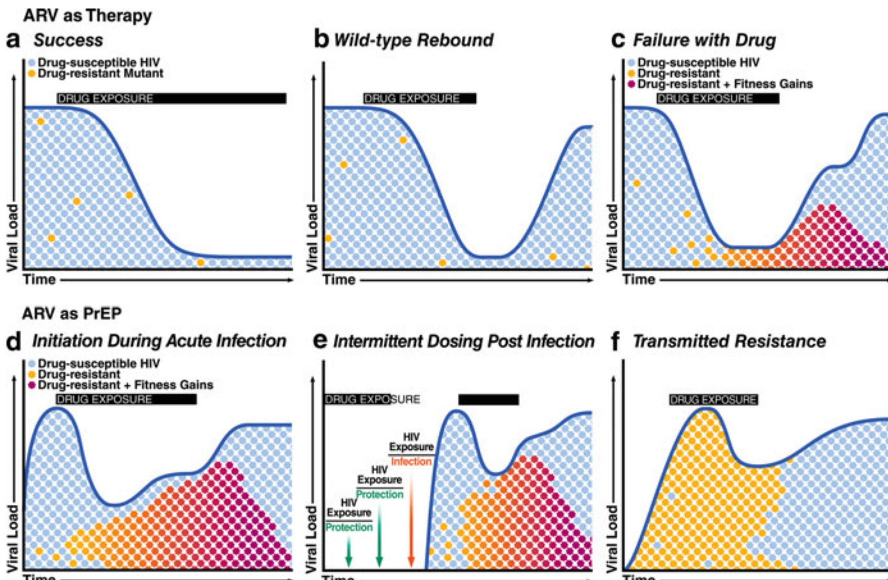

Fig. 1 **Antiretroviral (ARV) drug exposure and the emergence of drug-resistant variants when used as therapy versus PrEP.** The frequency of emergent drug-resistant variants and relative abundance within the viral quasispecies during distinct treatment modalities are influenced by factors such as the genetic barrier in establishing the codon(s) conferring resistance, the drug activity within a given target of viral replication, and the replication capacity in a particular environment (fitness). The *top panel* (**a**–**c**) is a schematic of possible outcomes in which ARVs are administered therapeutically, after incident infection. (a) Through random mutation, drug-resistant variants are generated sporadically in individuals with ongoing replication but remain at residual levels in settings of successful therapeutic ARV. (**b**) Treatment interruption leads to rapid virologic rebound of the more highly fit, wild-type species. (**c**) Drug-resistant variants may be selected in settings of non-suppressive therapy where continued exposure with ongoing replication may select for increasingly more fit viruses (*darker symbols*). If drug exposure is removed, residual archived wild-type virus will typically outgrow the drug-resistant species and predominate. The *lower panel* (**d**–**f**) shows possible outcomes when ARVs are inadvertently administered as PrEP in a setting of unrecognized infection. (**d**) When administered as PrEP in a setting of unrecognized infection, inadvertent postexposure initiation of ARV may be ineffective for durable suppression, selecting for minor variant drug-resistant species that may expand and evolve with fitness gains due to continued exposure. Following treatment interruption, archived wild-type virus outgrows. (**e**) Intermittent dosing with temporal lapses of protective drug exposure risks selection and outgrowth of drug-resistant variants during periods of continued ARV exposure. (**f**) Acquisition of transmitted or primary drug resistance to PrEP regimens will result in PrEP failure. Reversion by back mutation to wild-type, drug-susceptible virus can occur after discontinuation of PrEP, followed by eventual outgrowth of drug-susceptible variants if gains in fitness occur. Residual drug-resistant variants remain archived as proviruses in the cellular reservoir and may influence future treatment outcomes

susceptibility to ART often have impaired fitness in the absence of selection compared with wild-type, drug-susceptible strains, continued replication under selection can further select for additional compensatory mutations conferring fitness gains in the host's viral population (Condra et al. 1995; Cote et al. 2001; Gatanaga et al. 2002; Molla et al. 1996; Zhang et al. 1997), altered tropism, and virulence

(Coffin 1995; Kuritzkes 1996; Milich et al. 1993; Nijhuis et al. 2001) as shown in Fig. 1c. Following transmission of monophyletic or a limited number of polyphyletic founder viruses (Keele et al. 2008), the rapid expansion, high mutation and recombination frequencies (Onafuwa-Nuga and Telesnitsky 2009), multiple host, and therapeutic selection pressures can collectively promote the creation of complex viral quasispecies within an individual. Sensitive diagnostic assays that can quantify drug-resistant variants present at a minor proportion of the population within an individual have revealed mutations conferring drug resistance in ART-naïve individuals at residual levels ($\approx$1 %) within the viral quasispecies (Johnson et al. 2007, 2008; Liu et al. 2011; Metzner et al. 2011; Simen et al. 2009; Havlir et al. 1996). Preexisting low-level or minor variant drug resistance in treatment-naïve individuals can affect treatment outcomes, especially with particular NRTI- and NNRTI-selected mutations (Simen et al. 2009; Johnson et al. 2008; Havlir et al. 1996; Metzner et al. 2009; Li et al. 2011).

An alternative source of drug-resistant HIV-1 is that transmitted from a treatment-experienced partner, also known as primary resistance. Individual mutations conferring drug resistance can be detected in upwards of 20 % of the circulating strains in geographical areas that have access to ARV, changing with regional exposure levels and predominate treatment regimens over time (Chaix et al. 2009; Grant et al. 2002; Hamers et al. 2011; Jain et al. 2010; Little et al. 2002; Yerly et al. 2007; Wheeler et al. 2010). Such levels of circulating resistance within a population have driven national treatment guidelines to include baseline, pretreatment genotyping. How transmitted resistance might impact PrEP efficacy is an area of interest, especially where ARV included in PrEP regimens are also a component of first- and second-line therapies. Interestingly, numerous outcome predictions based on modeling the impact of the spread of drug resistance result in disparate scenarios [reviewed in (Baggaley et al. 2011)].

When assessing the role of PrEP agents in contributing to the selection and expansion of drug-resistant viruses, it is important to consider drug resistance in the context of drug exposure within the infection window to aid in differentiating transmitted from acquired (drug-selected) resistance. While transmitted resistance can be unequivocally confirmed by phylogenetic mapping of the source and index virus within the partnership, the presence of drug-resistance mutations associated with any particular PrEP regimen in the absence of drug exposure is highly likely to originate from transmitted strain(s) and not selected de novo by PrEP. As the frequency, nature, and origin (e.g., whether PrEP selected or transmitted) of drug-resistance findings accumulate from randomized clinical trials and demonstration projects, the impact of circulating resistance on PrEP efficacy can be directly assessed.

Detecting Drug Resistance in PrEP Studies

HIV-1 drug resistance in clinical practice is primarily measured and interpreted through two distinct but complementary approaches: (1) genotype testing, which includes direct sequencing of the HIV-1 drug target reading frames, usually *pol*, and

Table 1 Drug resistance assays used in phase II/III PrEP trials

	Genotype	Phenotype	Ultrasensitive
CAPRISA 004	In-house	None	AS-PCR
iPrEx	TRUGENE	PhenoSense	AS-PCR UDS[a]
Partners PrEP	ViroSeq, In-house	None	UDS
TDF2	In-house	None	AS-PCR
FEM-PrEP	TRUGENE	PhenoSense	AS-PCR UDS
Bangkok tenofovir study	TRUGENE	None	None

[a]*UDS* Ultradeep sequencing

(2) phenotypic susceptibility testing, which involves determining the concentration of a given compound necessary to inhibit viral growth in vitro [reviewed in (Tang and Shafer 2012)]. Both approaches measure the bulk population of viruses within an individual and, as a result, are insensitive to viral species carrying drug-resistance mutations below a given threshold (e.g., ≈20 % for population genotyping). As a research tool, multiple methods have been used to detect and quantify minor variant drug resistance within a population but below that detected by clinical tests, including allele-specific PCR-based assays that differentiate single-base changes conferring resistance, probe-based ligation assays, clonal sequencing, and highly parallel ultradeep sequencing [reviewed in (Gianella and Richman 2010)]. Ideally, genotype, phenotype, and ultrasensitive detection methods would be used together for monitoring drug resistance in PrEP failures as each approach can provide unique insights into the extent and nature of drug resistance. However, due to the high clinical diagnostic value, global accessibility, standardization of interpretation, and relatively low cost, drug-resistance genotyping is the primary diagnostic tool for drug-resistance monitoring in PrEP clinical trials (Table 1).

Designing PrEP Regimens to Minimize Drug Resistance

When designing effective regimens for PrEP, a number of factors are taken into consideration (Anderson et al. 2011; Derdelinckx et al. 2006; Fernandez-Montero et al. 2012; Garcia-Lerma et al. 2008; Amico 2012). Ideally, these include selecting compounds that target pre-integration events in the viral life cycle, demonstrate high antiviral activity and extended half-life in target tissues, exhibit synergies in activity and mutation impact if used in combination, and posses a high genetic barrier to resistance, which is the combined components that contribute to the generation of the specified resistance mutation and maintenance of the viral species in the population (Luber 2005). For these reasons, coupled with relatively favorable toxicity profiles, flexible formulations, efficacy in preventing transmission in nonhuman primate models under conditions that mimic sexual transmission in humans (Garcia-Lerma et al. 2008; Van Rompay et al. 2006; Subbarao et al. 2006; Radzio et al. 2012), and extensive history of therapeutic use, two nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) have been used in the completed clinical trials to date.

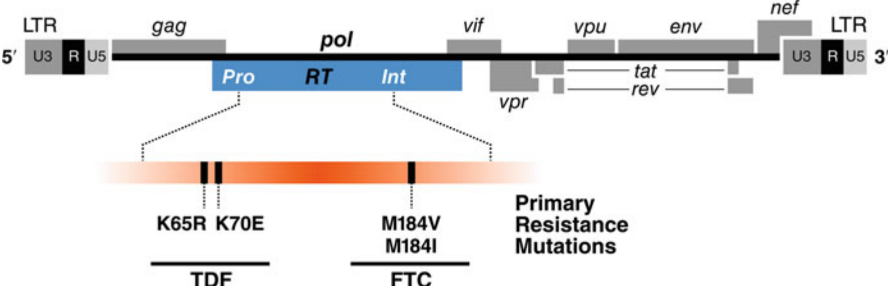

Fig. 2 Drug-resistance mutations in HIV-1 reverse transcriptase selected by FTC/TDF PrEP. Two primary drug-resistance mutations in HIV-1 reverse transcriptase are selected by each of tenofovir (TFV/TDF) and emtricitabine (FTC). TFV-associated codon changes are K65R (Lys to Arg) and K70E (Lys to Glu) and FTC-associated codon changes are M184I or V (Met to Ile or Val). Each mutation confers reduced susceptibility in vitro and in vivo. A single-base nucleotide change in RT codon A62V (Ala to Val) or S68G (Ser to Gly) does not directly confer changes in susceptibility to TFV but is a compensatory mutation associated with TFV exposure and partially restores viral replication capacity impairment conferred by K65R

Tenofovir (TFV), formulated as either a 1 % topical vaginal gel or as the orally available prodrug tenofovir disoproxil fumarate (TDF), has been administered as PrEP alone or together with emtricitabine (FTC). The co-formulated FTC/TDF oral pill TRUVADA™ is cleared by the US Food and Drug Administration for use as prevention in uninfected adults at high risk of HIV acquisition through sexual exposure. Both compounds act at pre-integration steps by terminating the nascent DNA chains in RNA-dependent and DNA-dependent DNA synthesis during the viral life cycle (Arts and Hazuda 2012).

The viral mutations associated with reduced susceptibility to TFV/TDF and related drugs are K65R and K70E (Margot et al. 2006a; Miller et al. 1999; Wainberg et al. 1999; Gallant et al. 2004) and to FTC are M184V and M184I (M184V/I) (Margot et al. 2006b), where the first amino acid listed for a given codon in RT represents the wild-type, drug-susceptible form and the second represents the mutant, drug-resistant form (Fig. 2). Additional RT mutations A62V and S68G associated with TDF exposure are considered compensatory mutations that improve viral replication capacity of poorly fit K65R mutants (Margot et al. 2006b; Svarovskaia et al. 2008). Although K65R and M184V/I are generated by a single-base substitution and thus may arise frequently in the course of HIV replication, viral species with these mutations demonstrate significantly reduced replication capacity and fitness in vitro and in vivo in the absence of selection (Yerly et al. 2007; Wheeler et al. 2010; Margot et al. 2006a; Petrella and Wainberg 2002; Miller et al. 2002; White et al. 2002; Frankel et al. 2007) thus conferring a relatively high barrier to resistance. Additionally, the presence of M184V causes increased sensitivity to TDF (Miller et al. 1999; Whitcomb et al. 2003; Deval et al. 2004), a synergy that is often taken advantage of in clinical practice (Wainberg and Gotte 2000). Finally, these ARVs provide a strong pharmacological barrier for sexual transmission. Emtricitabine concentrations are significantly higher in vaginal secretions compared

to that measured in blood after single oral dosing, while TDF-DP (the active intracellular form) is up to 100× higher in the colorectal mucosa compared with vaginal and cervical tissues following a single dose (Anderson et al. 2011; Kwara et al. 2008; Patterson et al. 2011).

Genotypic, Phenotypic, and Minor Variant Drug Resistance in PrEP Trials

To date there are six completed phase III, randomized, placebo-controlled PrEP trials reporting drug-resistance results. The overall study design for monitoring HIV-1 infection status and drug resistance was similar across studies. HIV status at screening, entry, and post-randomization visits was assessed by serologic monitoring. Blood plasma, cells, or other tissue samples were typically collected and archived for retrospective measurements of HIV-1 nucleic acid and/or drug levels and, when collected at sufficient frequency, were used to establish the infection window and drug exposure levels. Upon receiving a positive rapid test result post-randomization, study drug was discontinued and confirmatory serotesting and/or RNA testing performed. In the iPrEx, Partners PrEP and TDF2 studies, participants with unrecognized, acute infection (RNA positive, seronegative) at entry were retrospectively identified. In confirmed seropositives, blood plasma collected at or proximal to the initial seropositive visit was tested for drug resistance by standard genotyping. In some studies, additional diagnostics were performed including drug-resistance phenotype and allele-specific PCR and/or deep sequencing for ultrasensitive detection of minor variant drug resistance (Table 1). In the iPrEx and FEM-PrEP studies, longitudinal sampling and testing was performed to monitor drug resistance over time in participants with FTC/TDF-associated resistance at seroconversion and randomized to the active drug arm (Grant et al. in press; Liegler et al. 2014).

The drug-resistance mutations and frequencies reported from the CAPRISA 004 (Abdool Karim et al. 2010; Wei et al. 2014), the iPrEx (Grant et al. 2010; Liegler et al. 2014), Partners PrEP (Baeten et al. 2012; Lehman et al. in press), TDF2 (Thigpen et al. 2012), FEM-PrEP (Van Damme et al. 2012; Grant et al. in press), and the Bangkok tenofovir (Choopanya et al. 2013) trials are summarized in Table 2, categorized by participants' timing of infection (pre-randomization vs incident) and randomization arm. Overall, in participants with incident (on-study post-randomization) infection, the frequency of TFV/TDF- or FTC-associated drug resistance was low, including those randomized to the PrEP arms with measurable drug levels near the infection window. Of the 142 seroconverters with incident infections and in the PrEP arms of the CAPRISA 004, iPrEx, Partners PrEP, TDF2, and Bangkok tenofovir studies, none showed genotypic or phenotypic drug resistance associated with the PrEP regimens used at or near the seroconversion visit. In contrast, four of 33 (12 %) women on the oral FTC/TDF arm in the FEM-PrEP study showed genotypic and phenotypic resistance to FTC (M184V/I) at the seroconversion visit (Van Damme et al. 2012; Grant et al. in press). Tenofovir resistance was not observed, and two showed phenotypic hypersusceptibility to this drug. Two of

Table 2 Genotypic drug-resistance testing results reported from PrEP trials

			Infected at entry				Incident infection			
			Study drug		Placebo		Study drug		Placebo	
Study	Subjects randomized	Agent	Resist/ Tot Tested	PrEP-associated mutations	Resist/ Tot Tested	PrEP-associated mutations	Resist/ Tot Tested	PrEP-associated mutations	Resist/ Tot Tested	PrEP-associated mutations
CAPRISA 004	1,085 women	1 % TFV gel coitally dependent use	0/1	none	0/9[a]	none	0/38	none	0/60	none
iPrEx	2,499 MSM and transgender women	FTC/TDF daily oral use	2/2	M184V selected[b] M184I unknown[c]	1/8	M184V transmitted	0/48	none	0/83	none
Partners PrEP	4,758 male/ female couples	TDF daily oral use	1/5	K65R selected[d]	0/6	none	0/15	none	0/51	none
		FTC/TDF daily oral use	1/3	M184V selected[e]			0/12	none		
TDF2	1,219 men and women	FTC/TDF daily oral use	1/1	K65R M184V A62V selected[f]	0/2	none	0/9	none	0/24	none
FEM-PrEP	2,120 women	FTC/TDF daily oral use	0/1	none	0/1	none	4/33	1 ea M184I selected[g] 3 ea M184V 2 transmitted 1 selected[h]	1/35	M184V transmitted
Bangkok tenofovir study	2,413 men and women (IVDU)	TDF daily oral use	none	none	0/2	none	0/15	none	0/32	none

[a]Two participants randomized to placebo were deemed ineligible for study.
[b]Mutation not found at enrollment. FTC-selected resistance
[c]Unable to genotype sample (VL = 48 cps/mL). Presence of mutation at enrollment unknown
[d]Mutation not found at enrollment. TDF-selected resistance
[e]Mutation not found at enrollment. FTC-selected resistance
[f]Mutations not found at enrollment. FTC- and TDF-selected resistance
[g]FTC detected in plasma during infection window
[h]FTC detected in plasma during infection window

these participants, 1 with M184I and 1 with M184V, had moderate- and high-study drug levels at seroconversion, respectively, implicating selection by the PrEP regimen. However, seroconversion occurred within 4 (M184V) and 8 (M184I) weeks of study entry, leaving open the possibility that infection was incubating prior to PrEP initiation – a situation with increased frequencies of emergent drug resistance.

In PrEP studies reporting ultrasensitive testing for minor variant drug resistance in seroconverters performed by AS-PCR and/or deep sequencing, background mutation frequencies (that observed in WT viruses in the absence of drug selection) were established for each individual assay and were typically $\leq$1 %. While minor variant drug resistance was observed above background levels from seroconverters in both placebo and active drug arms, examples seen in subjects randomized to the PrEP arms and therefore potentially PrEP selected are highlighted here.

In CAPRISA 004 (Wei et al. 2014) and TDF2 (Thigpen et al. 2012), AS-PCR measurements in blood plasma and vaginal swabs (CAPRISA 004) near the seroconversion visit showed no evidence of minor variant resistance to TFV. Seven of 27 (26 %) women in the CAPRISA 004 TFV gel arm had measurable TFV in vaginal fluids. However the majority had insignificant or undetectable TFV levels indicating the absence of drug selection pressure.

In the iPrEx and FEM-PrEP studies, minor variant DR in blood plasma from participants randomized to the FTC/TDF arms was observed, however infrequent and at very low proportions within the population measured by AS-PCR and 454 deep sequencing (Grant et al. in press; Liegler et al. 2011). In iPrEx, one seroconverter's virus had M184I detected at 0.53 % of the plasma viral population by AS-PCR but below background by 454 sequencing This subject had detectable but low drug levels in blood plasma and cells, opening the possibility of selection by PrEP but without significant outgrowth within the population. Similarly, one FEM-PrEP seroconverter showed M184I at 0.66 % of the population but at background levels by 454 sequencing. Study drug was not detected in this woman near the seroconversion window, suggesting spurious detection of drug-resistance mutations near the background cutoff level, rather than PrEP-selected resistance.

Blood plasma samples at the seroconversion and proximal follow-up visits from subjects in the Partners PrEP study (oral FTC/TDF, TDF alone, placebo) were analyzed for minor variant drug resistance by 454 deep sequencing (Lehman et al. in press). Of those in the oral FTC/TDF arm, a virus from 1 subject showed M184V at 16 % of the viral population (SC visit), decreasing to 1.7 % 4 weeks later, without detectable study drug. Viruses from two other participants with detectable drug showed minor variant resistance mutations: 1 with M184V at 1.9 % from the post-seroconversion visit and another with M184V (at 7.7 %), M184I (at 5.4 %), and K65R (at 1.2 %) in the seroconversion visit sample. This rare example of K65R in incident infections may reflect the significantly impaired fitness or replication capacity conferred by K65R, especially when in combination with M184V (Miller et al. 1999, 2002; Margot et al. 2006b; Petrella and Wainberg 2002; White et al. 2002; Frankel et al. 2007), and/or insufficient drug exposure, as PrEP was discontinued at the first evidence of seroconversion. In the oral TDF arm, 1 of 30 participants showed M184I at a low level (2.5 %), a mutation that is not selected

by TDF. This mutation was detected as a minor variant in the placebo arm of Partners PrEP, FEM-PrEP, and iPrEx participants, possibly maintained at low levels by APOBEC3G-induced G-to-A hypermutation (Neogi et al. 2013).

Development of Elevated Drug-Resistance Frequencies When Initiating PrEP During Acute, Seronegative Infection

A striking finding from these studies is the relatively high frequency of PrEP-associated drug-resistance mutations seen in subjects who inadvertently initiated PrEP with unrecognized (RNA positive, seronegative) infection at randomization when compared to that seen in study participants with incident infections. Of the 13 participants with unknown acute infection initiating PrEP at randomization, five in the combined iPrEx, Partners PrEP, and TDF2 studies showed genotypic resistance to FTC (M184V) or TDF (K65R) at their initial seroconversion visit. In four of these participants, the virus at enrollment did not carry these resistance mutations, favoring selection by PrEP regimens during the initial 4 weeks of use. The additional subject from the iPrEx study with M184I had a low viral load at entry that was insufficient for a genotype (48 copies/mL), so it cannot be unequivocally determined whether the mutation conferring resistance to FTC was transmitted or selected. Of note, one subject from the TDF2 study developed multidrug resistance in a stepwise manner over time where M184V was detected at the first visit after study entry, followed by additional TDF-associated mutations K65R and A62V at the second visit 6 months later. Although enrollment of participants with unrecognized acute infection who went on to develop PrEP-selected resistance was rare among all in PrEP studies, the relative frequency of generating resistance in this subset of participants was high (5/13, 38 %) and possibly avoidable with HIV-1 RNA testing prior to PrEP initiation and delaying PrEP in those with symptoms consistent with acute viral infections.

In the absence of continued selection by PrEP, the FTC-associated resistance in blood plasma virions declines to residual levels over time, through outgrowth of the more highly fit WT variant generated through back mutation, or presents at very low levels under PrEP selection. Participants in both the iPrEx and FEM-PrEP trials with FTC resistance mutations M184V/I were followed longitudinally after stopping PrEP for up to nearly 18 months, and blood plasma samples were assayed for the relative proportion of coexisting drug-resistant and susceptible variants by sensitive allele-specific PCR and deep sequencing assays (Grant et al. in press; Liegler et al. 2012, 2014) and unpublished data). In all cases analyzed from both the placebo and control arms ($n = 7$), the drug-resistant variants proportionally decreased from 95 % to 100 % at seroconversion to residual levels (<0.5 %) in the blood plasma over time. Although most demonstrated a more prolonged time course for complete reversion (median 9 months), one participant showed complete reversion and overgrowth at the RT codon 184 from Ile to Met within 4 weeks of discontinuing study drug. These results are consistent with the time course of transmitted M184V

reversion over time in ARV-naïve subjects (Liegler et al. 2011; Jain et al. 2011) and highlight the value of baseline resistance testing as early in infection as possible.

Distinct Patterns of Drug Resistance in PrEP: What Is Driving It?

The frequencies and nature of PrEP-associated drug resistance fall into distinct patterns that are likely outcomes of multiple diverse factors including the temporal sequela of exposure to drug and infectious virus, the pharmacodynamics of the individual compounds and formulations in diverse anatomical target sites under changing physiologic states, and the genetic barrier to resistance specific for each PrEP regimen and other factors. The interplay between the drug activity, viral resistance barriers, and how these factors might affect the relative risk of infection and frequency of resistance is schematized in Fig. 3. The distinct scenarios diagramed in panels A to D reflect various outcomes noted with use of chemoprophylaxis and HIV infection. Panel A represents effective PrEP where infection occurs only with very low drug exposure and where the overall genetic barrier to resistance is sufficiently high to prevent its emergence. This scenario reflects WT

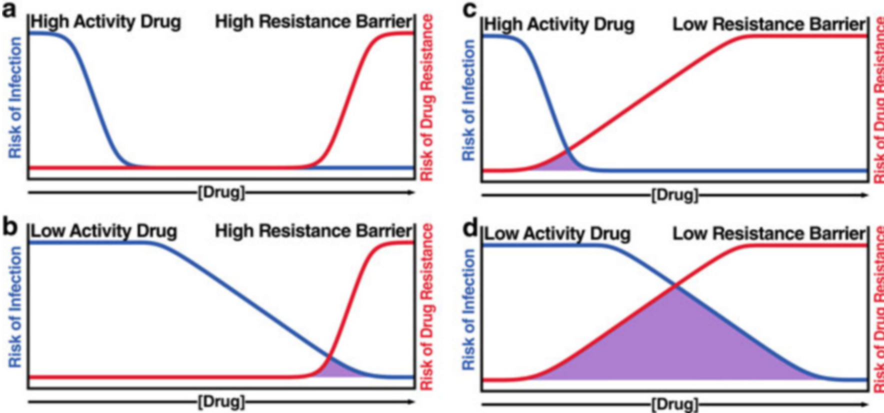

Fig. 3 Schematic of the interplay between pharmacologic and virologic factors that influence the risk of infection and drug resistance in a PrEP setting. Panels **a to d** represent theoretical schematics of the relative frequency of generating drug-resistant HIV-1 (*shaded area* at curve intersections) in settings of breakthrough infection during PrEP use. The relative risk of infection (*blue line*) is plotted against the relative risk of emergent drug resistance (*red line*) with increasing drug concentration at the anatomical and subcellular target of entry. (**a**) In a setting of high drug activity and a high barrier to resistance, the infection window occurs with insufficient drug levels to select for resistance. (**b**–**c**) Drug resistance can occur, although infrequently with either low drug activity or a low barrier to resistance where drug levels are suboptimal, allowing viral replication, but sufficiently high to select for drug resistance. (**d**). Increased frequency of drug resistance may occur in a setting of both low drug activity and low resistance barrier, such as that resulting from single-dose nevirapine treatment given to pregnant women to prior to delivery to prevent mother-to-child transmission

infection seen in the majority of seroconverters in PrEP studies with low or undetectable drug levels. Panels B and C may reflect the infrequent cases of resistance seen in PrEP where local concentrations of the drug may be insufficient to block infection and/or create a sufficient barrier to resistance. Distinct tissue-specific pharmacodynamics for FTC and TFV may uncouple the combined synergy in target tissues such as the cervicovaginal or colorectal mucosa (Thompson et al. 2013), leading to the observed predominance of FTC-selected mutations M184V/I in FTC/TDF oral PrEP. Finally, panel D represents settings where drug resistance is high, such as that observed with limited dosing monotherapy for prevention of MTCT. A deeper understanding of the factors that influence ARV activity and emergence of resistance in target tissues of viral entry and dissemination is critical for designing more effective PrEP regimens, formulations, and dosing strategies.

Progress Toward Next-Generation PrEP

Favorable results from initial randomized placebo-controlled PrEP efficacy trials and the US Food and Drug Administration's first label approval for an antiretroviral compound to be used as chemoprophylaxis for prevention of sexual HIV-1 transmission have led to demonstration projects worldwide where PrEP efficacy is tested in open-label, clinical settings. Ongoing demonstration projects include daily oral FTC/TDF PrEP [reviewed in (Baeten et al. 2013)], allowing direct comparisons to the PrEP efficacy trials. Comprehensive monitoring for drug resistance and drug exposure in seroconverters from these studies should yield additional insights into the overall impact of PrEP use and drug resistance. There is, however, room for overall improvement in strategies for optimizing PrEP and monitoring virologic, behavioral, toxicity, and other outcomes.

Additional compounds and formulations with improved penetration in target tissues, innovative dosing and delivery strategies, and additional viral targets are needed to further increase PrEP efficacy with expanded use while maintaining low toxicity and high genetic resistance barriers [reviewed in (Abraham and Gulick 2012)]. The ÉCLAIR study is a phase IIa safety and tolerability study evaluating the injectable long-acting investigational integrase inhibitor GSK-744 LA in uninfected men. Promising results were reported using a long-lasting nanoparticle formulation of the HIV-1 integrase inhibitor dolutegravir, with successful protection against rectal SHIV challenges (Andrews et al. 2013). Other long-lasting nanoparticle ARV formulations intended for periodic injections and targeting multiple HIV-1 pol enzymes are in various stages of investigation in small animal models measuring pharmacokinetic profiles in target tissues and cells (Puligujja et al. 2013; Martin et al. 2013).

There are multiple ongoing trials testing oral tenofovir-based PrEP dosing strategies and drug combinations to reduce pill burden and minimize overall drug exposure but maintain effective exposure for situational risk. Intermittent PrEP (pre- and postexposure) use has been shown to be efficacious in reducing SHIV

infection through rectal exposure in macaques (Garcia-Lerma et al. 2010). In this study of multiple dosing strategies, none of the breakthrough infections showed evidence of drug resistance. The ANRS IPERGAY study includes MSM and "on demand" oral FTC/TDF, taken at the time of sexual exposure. The HIV Prevention Trials Network (HPTN) 067 study ADAPT, enrolling MSM/TGW and women who have sex with men (WSM), is a behavioral study with a 1:1:1 randomization of three arms using oral FTC/TDF with either daily dosing, time-driven dosing, or event-driven dosing. The NEXT-PrEP (HPTN 069/ACTG 5305) study is a four-arm phase II safety and tolerability trial investigating combinations of oral daily FTC, TDF, and the HIV-1 entry inhibitor maraviroc (MVC). Drug concentration measurements in all study participants and drug resistance testing in seroconverters in these various studies will aid in determining the oral dosing formulation and timing needed to prevent infection while minimizing exposure for reduced toxicity. This relationship was estimated using drug level measurements in blood and levels of protection from HIV acquisition in the iPrEx trial combined with defined intermittent and daily dosing strategies in the STRAND study (Anderson et al. 2012). While this serves as an important basis for determining the most effective and least harmful dosing strategy, further evaluations within these and other trials are necessary to further optimize the next-generation PrEP for diverse user needs.

Conclusions

The proven efficacy of PrEP in preventing HIV acquisition in clinical trial settings is one of the celebrated successes in HIV prevention research and brings cautious optimism for continued success with more widespread use. One clear message from PrEP trials is that successful PrEP requires drug uptake. The risk of infection increases with suboptimal PrEP use, as does the potential drug resistance. Despite a range of efficacies and adherence levels reported, drug resistance selected by PrEP was largely seen in subjects initiating PrEP during acute, unrecognized infection. Monitoring for acute viral symptomatology and the presence of HIV nucleic acids may be useful diagnostic tools at PrEP initiation. Additionally, using combination regimens and drug formulations with increased potency at PrEP initiation may minimize this occurrence. In incident infections, the occurrence of drug resistance, even as minor variants, was infrequent in participants with measurable drug levels indicating exposure. However, there are limitations in interpreting these findings – in all PrEP trials, study drug was discontinued at the first evidence of infection, thus limiting drug exposure that may generate resistance with longer duration. Guidelines for PrEP use in clinical practice indicate monitoring for infection with PrEP at a minimum of every 12 weeks (Centers for Disease Control and Prevention 2011, 2012), less frequent than the monthly monitoring in clinical trials.

Continued rigorous assessment of drug resistance in breakthrough infections while using PrEP is necessary with expanded use in clinical settings and as other compounds, formulations, dosing strategies, and novel drugs are tested and implemented.

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HIV-1 Entry Inhibitor Resistance

Victor G. Kramer and Mark A. Wainberg

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Abstract

Purpose of review: This review discusses resistance to HIV entry inhibitors with a focus on currently approved drugs and future uses.

Recent findings: The HIV entry inhibitor class is unique among HIV antiretrovirals as it encompasses drugs that target the different stages of the HIV entry cascade. There are currently 2 FDA-approved drugs in this class, the chemokine receptor-5 (CCR5) antagonist maraviroc (MVC) and the fusion inhibitor enfuvirtide (T-20). Attachment inhibitors are still under development, with

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multiple candidates in various states of development. Coreceptor antagonists are the most widely studied because patients who lack CCR5 are protected from HIV infection. CXCR4-antagonist development has proceeded more cautiously, due to limited antiviral effect in clinical trials. Fusion inhibitor development is advancing slowly, with the majority of research focusing on orally available small-molecule inhibitors. Resistance to these subclasses manifests in a complex manner and does not conform to the resistance paradigm of other antiretroviral classes.

Summary: This review will describe this heterogeneous class of antiretrovirals and the unique challenges and opportunities that they present in the pursuit of improving options for treatment.

Keywords
HIV • entry inhibitors • resistance • antiretrovirals • tropism

Key Points

1. Entry inhibitors are effective antiviral agents against HIV.
2. Two entry inhibitors are currently approved for treatment.
3. Entry inhibitors in development target alternative steps of the entry cascade that include CD4 and CXCR4.
4. Resistance to current entry inhibitors represents a unique challenge unseen with other antiretroviral classes.
5. The limitations of entry inhibitors are still being investigated and have implications for treatment that extend beyond antiviral activity.

Introduction

The development of highly active antiretroviral therapy (HAART) for HIV has led to a marked decrease in morbidity and mortality. Drug candidates for HAART regimens come from the following classes: reverse transcriptase inhibitors, protease inhibitors, integrase strand-transfer inhibitors, and entry inhibitors. A typical regimen includes two reverse transcriptase inhibitors and either a non-nucleoside reverse transcription inhibitor or a protease inhibitor. A number of concerns arise as a result of lifelong HAART, including development of drug resistance, long-term toxicity, and unfavorable drug-drug reactions. It is therefore vital to continue to develop and refine new drug classes in an effort to broaden the treatment options that are available. This review focuses on currently approved and candidate entry inhibitors and the development of resistance to said inhibitors.

HIV enters host target cells through a complex, stepwise process that begins with virus envelope subunit gp120 binding to the CD4 receptor on the cell surface (reviewed in Kuritzkes 2009, Tilton and Doms 2010). The gp41 and gp120 subunits

are the result of furin cleavage of gp160, which is the major product of the HIV *env* gene, in the Golgi apparatus (Moulard and Decroly 2000). After cleavage, noncovalently associated trimers that are the result of their cleavage are transported to the cell membrane where new virions incorporate them onto their surfaces via budding (Freed and Martin 1995). CD4 D1 domain binding to a conserved site on gp120 causes the latter to undergo a conformational shift that allows it to bind to one of two chemokine coreceptors at the cell surface, CCR5 or CXCR4. These chemokine coreceptors are G-protein coupled receptors that possess an extracellular N-terminus, intracellular C-terminus, and 6 loops – equally divided into intracellular and extracellular loops. These receptors are responsible for lymphocyte chemotaxis and activation and are expressed on T cells, monocytes, macrophages, and dendritic cells. The conformational shift involves V1/V2 loop changes as well as exposure of the V3 loop (Chen et al. 2005; Kwong et al. 1998; Huang et al. 2005). The specific coreceptor that gp120 interacts with is determined by the amino acid composition of the V3 loop (De Jong et al. 1992; Shioda et al. 1991, 1992). Viruses that interact with CXCR4 are positively charged at amino acids 11, 24, and 25 on the V3 loop. Once gp120 binds to a coreceptor, it induces further conformational change that allows the gp41 fusion peptide to insert itself into the cell membrane. This precipitates the formation of a six-helix bundle comprised of six heptad repeat domains, three of which are termed HR1 and three others that are termed HR2 (Delwart et al. 1990; Chambers et al. 1990; Gallaher et al. 1989). The bundle is formed when the C-terminal HR2 region binds to the N-terminal HR1 region in an opposing orientation. The formation of this bundle brings the virus and cell membrane into close proximity and culminates in fusion, allowing the viral capsid to enter the cytoplasm and begin reverse transcription (Figs. 1 and 2).

Each step of the entry cascade has potential targets for inhibition. Thus, unlike other antiretroviral classes, the entry inhibitor class consists of a number of agents with diverse antiviral mechanisms that act at disparate points in the viral entry cascade. These steps include: CD4 binding, coreceptor binding, and membrane fusion. The first step in the cascade, CD4 binding, is not targeted by any currently approved agent. Although previous candidates were shown to inhibit HIV entry in tissue culture, this was not reflected in patients (Daar et al. 1990; Schooley et al. 1990). The blockage of the CD4 receptor carries an inherent risk of immunological disruption, as this receptor is essential in processes involving antigen recognition. Soluble CD4 (sCD4) was developed as a promising early candidate following identification of the role of CD4 in entry. Despite promising results in vitro, sCD4 was ineffective in patients in clinical trials, and subinhibitory concentrations of sCD4 were found to enhance infection (Sullivan et al. 1998). Candidate compounds still under development in this subclass include BMS-663068 (Nettles et al. 2012), a drug that binds to gp120 and prevents attachment to CD4. Earlier generations of this drug (BMS-488043) required high doses for antiviral activity (Hanna et al. 2011). BMS-663068 has now been shown to lower HIV-1 viral load by at least 1 $\log_{10}$ during 8 days of monotherapy (Nettles et al. 2012). These compounds induce a conformational change in gp120 that render it unable to bind to CD4 (Ho et al. 2006).

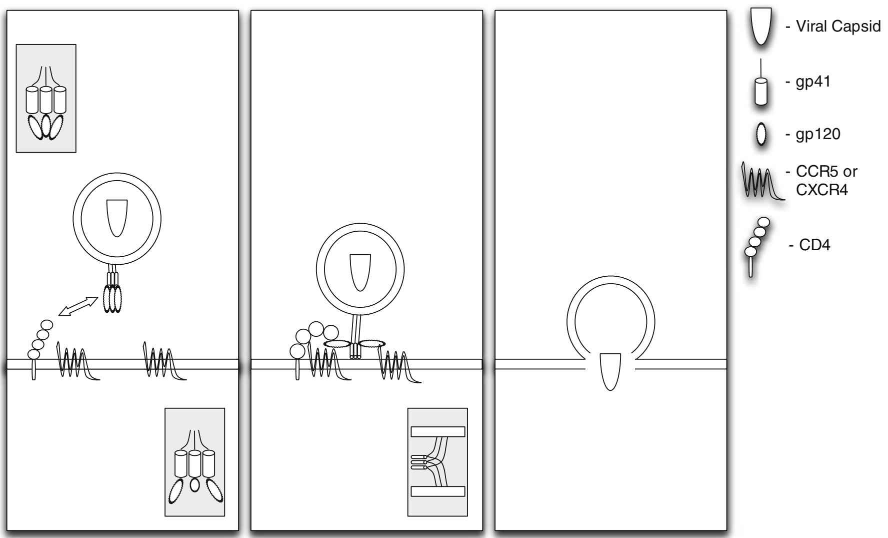

Fig. 1 Diagram of HIV-1 entry. *Left panel*: gp120/gp41 trimer on the virus surface attaches to CD4 on the surface of the cell membrane. *Left panel, insert; top left*: Native gp120/gp41 trimer, unbound to CD4. *Left panel, insert; bottom right*: gp120 conformation bound to CD4. *Middle panel*: Interaction between gp120 and coreceptor, post-CD4 attachment. gp120 binds to the N-terminus and extracellular loop 2 of the coreceptor. This allows the gp41 fusion peptide to insert into the cell membrane. *Middle panel, insert; bottom right*: Formation of the six-helix bundle following interaction of the HR1 and HR2 domains of gp41, post-fusion peptide insertion. This brings together viral and cellular membranes for fusion. *Right panel*: Fusion of viral and cellular membranes creates a pore that viral capsid uses to enter the target cell

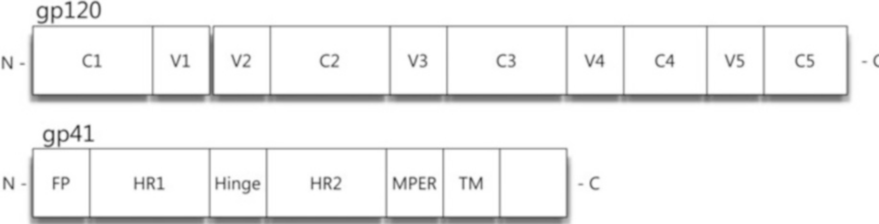

Fig. 2 Schematic representation of HIV gp120 and gp41 envelope proteins on the virion surface. *Top*: gp120 contains five conserved domains (C1–C5) and five variable domains (V1–V5). *Bottom*: gp41 contains a fusion peptide (FP), heptad repeat region 1 (HR1), heptad repeat region 2 (HR2), and the hinge region between both the membrane proximal external region (MPER) and the transmembrane anchor (TM)

A post-attachment inhibitor, the monoclonal antibody ibalizumab, has been developed that targets the D2 domain of the CD4 receptor (Burkly et al. 1992). It blocks gp120 conformational change following the gp120-CD4 interaction. Used as a single dose, it has shown antiviral activity with HIV viral load reductions up to

1.5 $\log_{10}$ when given together with optimized background therapy (OBT) (Kuritzkes et al. 2004). Phase IIb trials demonstrated that weekly or biweekly doses led to viral load reductions of up to 1.7 $\log_{10}$ together with OBT (Jacobson et al. 2009). Ibalizumab is currently in phase I subcutaneous administration dose-escalation trials for subcutaneous administration in HIV-negative individuals (www.clinicaltrials.gov, NCT01292174). CD4 inhibitors are currently the only subclass of entry inhibition without drugs for HIV therapy.

CCR5 coreceptor inhibitors are the best researched entry inhibitor subclass. This is due to a variety of factors such as (1) the existence of individuals who do not express CCR5 due to a homozygous gene variant (CCR5 delta32/delta32). These individuals possess a high level of resistance to HIV-1 R5-tropic infection (Dean et al. 1996; Liu et al. 1996; Samson et al. 1996). Heterozygotes for this deletion experienced slower disease progression (Dean et al. 1996; Samson et al. 1996; Huang et al. 1996; Michael et al. 1997; Rappaport et al. 1997). (2) A lack of CCR5 has not led to major detrimental effects. Interestingly, CCR5 homozygotes show increased susceptibility to West Nile virus and tick-borne encephalitis infections (Kindberg et al. 2008; Lim et al. 2008). (3) Recent reports described several potential benefits of CCR5 antagonists that extend beyond antiviral effect such as a reduction in inflammation (Funderburg et al. 2010; Schroder et al. 2007), although not on progression of rheumatoid arthritis (Fleishaker et al. 2012). CCR5 blockade has also led to an increase in levels of CD4 T cells, though it is unknown whether these regenerated T cells are extant cells that have gone uninfected or a *de novo* population of other replicative cells (Funderburg et al. 2010). Finally, one HIV-positive patient who had his viremia controlled by HAART subsequently developed acute myeloid leukemia and received a hematopoietic stem cell transplant from a homozygous CCR5 delta32/delta32 donor (Hütter et al. 2009). HAART was stopped before transplantation and, after 5 years, his viremia has not returned (Allers et al. 2011; Symons et al. 2012). This has been attributed to the fact that the donor was a homozygous CCR5 delta32/delta32 donor. The success of this patient as proof of concept of HIV cure, notwithstanding the impracticality of hematopoietic stem cell transplant solely for HIV treatment, is among the reasons that CCR5-blockade research is so important.

CXCR4 is the alternative coreceptor that a virus may use to enter cells in conjunction with CD4. It is unknown what the long-term effects of CXCR4 blockade are, though there are concerns that it may have serious ramifications due to the fact that cases of CXCR4-receptor augmentation in humans often result in serious immunodeficiency (WHIM syndrome) (Hernandez et al. 2003; Liu et al. 2012) and CXCR4 knockout in mice led to abnormalities in embryonic development (Tachibana et al. 1998; Zou et al. 1998; Ma et al. 1998; Nagasawa et al. 1996). CXCR4 functions as a receptor of SDF-1 and does not appear to be as redundant, as CCR5. But, homozygotes possessing mutant ligand SDF1-3′A also displayed delayed progression to AIDS (Winkler et al. 1998). Promising CXCR4 inhibitors such as AMD3100 have demonstrated antiviral activity in vitro but did not exhibit the same antiviral efficacy in vivo (Hendrix et al. 2004; Donzella et al. 1998). This may be attributed to the fact that X4-tropic strains of HIV are rarely found on their

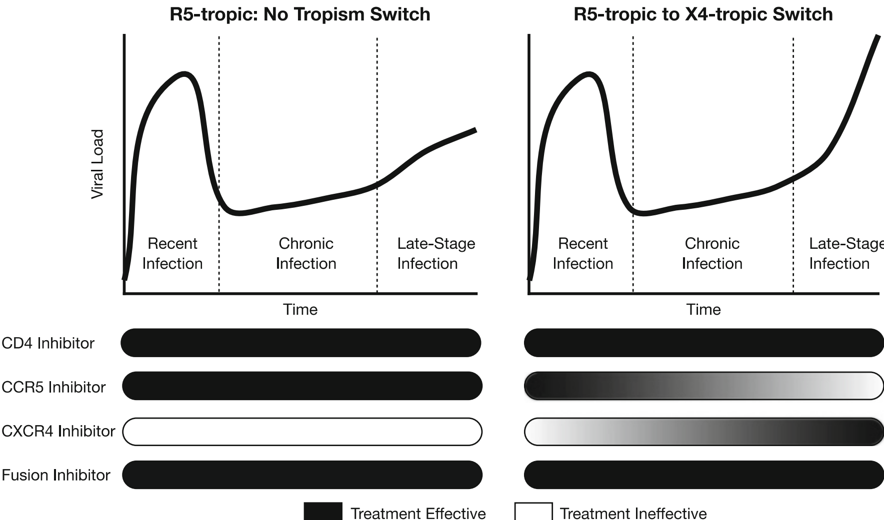

Fig. 3 Relative viral load through three stages of HIV-1 disease course and corresponding efficacy of entry inhibitor subclasses. *Left*: Patients infected with a CCR5-tropic virus that does not undergo tropism switch. CD4, CCR5, and fusion inhibitors are effective for treatment; CXCR4 inhibitors are not. *Right*: Patients infected with a CCR5-tropic virus that switches tropism to CXCR4, characterized by a pronounced increase in viral load at late-stage disease. CD4 and fusion inhibitors are effective throughout disease course. The efficacy of CCR5 inhibitors decreases with a concomitant rise in CXCR4 tropism during chronic and late-stage infection; CXCR4 inhibitors are more effective during these stages

own in vivo; they generally appear in the presence of R5-tropic virus, even in late-stage disease (Moyle et al. 2005; Wilkin et al. 2007; Brumme et al. 2005; Regoes et al. 2005) (Fig. 3). Although CXCR4 blockade may completely inhibit the X4-tropic component of the viral quasispecies, R5-tropic replication may continue unabated in the presence of CXCR4 antagonists. AMD3100, initially developed as an HIV antiretroviral, is no longer being developed for HIV therapy but is now being used as a hematopoietic stem cell mobilizer (plerixafor) in order to increase stem cell harvests before transplantation. Additionally, AMD3100 is now undergoing clinical trials for treatment of WHIM syndrome as it ablates hyper-CXCR4 signaling following binding to SDF-1 (Liu et al. 2012; McDermott et al. 2011a, b).

Entry Inhibitors Currently Approved for Treatment

Maraviroc

Maraviroc is a CCR5 entry inhibitor that prevents R5-tropic virus from infecting a target cell. It is currently the only CCR5 inhibitor approved for HIV-1 treatment by the US Food and Drug Administration and other regulatory agencies for both

first-line and second-line therapies (USFDA 2009). It is not yet licensed for first-line therapy in Europe (Society EAC. Guidelines 2011). Since a majority of new transmissions are R5-tropic (Pope and Haase 2003), it is reasonable that MVC could have its greatest benefit early in treatment (Fig. 3). X4-tropic variants are more likely to develop over time, rendering the use of maraviroc inappropriate (Brumme et al. 2005; Regoes et al. 2005; Schuitemaker et al. 1992; Saag et al. 2009).

MVC is unique among currently approved antiretrovirals in that it exerts its antiviral properties by binding to a cellular target rather than a viral one. It binds at a hydrophobic transmembrane pocket on extracellular loop 2 (ECL2) that is distinct from the gp120 binding site and induces a conformational change that renders the receptor unusable for entry (Dorr et al. 2005; Garcia-Perez et al. 2011). This allosteric inhibition prevents CCR5 ligand (MIP-1a, MIP-1b, and RANTES) signaling, although there does not appear to be any sequelae associated with such interference (Dorr et al. 2005).

MVC, as a cellular inhibitor, may have effects beyond its ostensible antiviral activity. The results of the MOTIVATE-1 and MOTIVATE-2 trials showed that treatment-experienced patients who received MVC with OBT had increased CD4+ T-cell counts versus placebo groups (Fatkenheuer et al. 2008; Gulick et al. 2008). This increase in CD4+ T-cell count was observed in both patients with solely R5-tropic virus and also in patients harboring X4 and dual/mixed population virus at failure. It is possible that the increase in CD4+ T cells results from anti-inflammatory effects that are a natural consequence of CCR5-blockade, since naive cells may no longer be recruited to sites of inflammation. The precise mechanisms responsible for the increased CD4+ cell count are still under investigation.

MVC appears to limit graft versus host disease (GVHD) in patients receiving allogeneic stem cell transplants. Phase I and II trials revealed decreased incidence of GVHD in patients taking a 33-day course of combination maraviroc, tacrolimus, and methotrexate (23.6 %, grade II to IV disease; 5.9 %, grade III or IV) compared to patients taking only tacrolimus and methotrexate (38.5 %, grade II to IV disease; 21.9 %, grade III or IV) (Reshef et al. 2012). This is attributed to MVC-mediated inhibition of CCR5 internalization and lymphocyte recruitment.

CCR5 was recently implicated as the receptor required for *Staphylococcus aureus*-produced leukotoxin ED toxicity (Alonzo et al. 2013). Leukotoxin ED is one of four pore-forming toxins produced by the bacteria and is responsible for targeted killing of macrophages, dendritic cells, and effector memory T cells and can serve as an immune evasion strategy by this bacterial pathogen. MVC, along with natural ligands and a monoclonal antibody to ECL-2, reduced the interaction between leukotoxin and CCR5, whereas antibody 3A9, specific for the CCR5 N-terminus, did not affect the interaction. Although MVC treatment had no effect on leukotoxin ED-mediated killing of neutrophils and monocytes, CCR5 antagonists may potentially represent complementary therapeutic strategies in the treatment of *S. aureus* infections.

CCR5 delta32/delta32 homozygotes appear to have more favorable outcomes in rheumatoid arthritis (Prahalad 2006; Wheeler et al. 2007). Indeed, it had been hypothesized that CCR5 blockade might mediate rheumatoid arthritis and maraviroc

was originally developed with antiarthritic use in mind. However, maraviroc did not demonstrate any beneficial effects when used to treat rheumatoid arthritis in conjunction with methotrexate (Fleishaker et al. 2012).

Enfuvirtide (T-20)

Fusion inhibitors exert their antiviral effect during the last stage of viral entry through inhibition of virus and cell membrane fusion. Enfuvirtide (T-20) is the only approved representative of the fusion inhibitor subclass of HIV entry inhibitors. It was the first entry inhibitor approved for therapy and is indicated for inclusion in salvage regimens in treatment-experienced patients despite requiring subcutaneous administration. Two randomized phase III trials (TOROs 1 and 2) showed reduced viral loads to <400 copies/ml in a greater percentage of patients than a placebo arm after 48 weeks (Lalezari et al. 2003a; Lazzarin et al. 2003; Nelson et al. 2005). A 96-week follow-up demonstrated viral loads below 400 copies/ml in 26.5 % of patients, with 17.5 % achieving viral loads <50 copies/ml (Reynes et al. 2007).

T-20 is a 36-amino-acid synthetic peptide that mimics the HR2 region of gp41. T-20 prevents formation of the six-helix bundle required to bring viral and cell membranes into close proximity by competitively binding to HR1 (Wild et al. 1993). It has demonstrated antiviral efficacy across all viral subtypes and tropisms in vitro and in vivo (Kilby et al. 1998, 2002; Lalezari et al. 2003b; Derdeyn et al. 2000). As such, it has a broader antiviral range than coreceptor inhibitors. However, the need for twice-daily subcutaneous injection as a consequence of a short plasma half-life (~4 h) has meant that the use of T-20 is limited (Zhang et al. 2002; Makinson and Reynes 2009).

Other Entry Inhibitors in Development

CCR5 Inhibitors

The recent case of the Berlin patient has renewed interest in development of entry inhibitors as a potent antiretroviral class. CCR5 entry inhibitors currently in development include an anti-CCR5 antibody PRO-140, which binds to an extracellular region of ECL2 on CCR5 (Olson et al. 1999). PRO-140 has yielded declines in viral load of up to 1.83 $\log_{10}$ in single-dose studies (Jacobson et al. 2008). It has also shown favorable results in dose-escalation studies, in which 0.99 to 1.65 $\log_{10}$ viral load reductions were witnessed after weekly (162 mg, 324 mg) or biweekly (324 mg) subcutaneous administration (Jacobson et al. 2010). There was no detectable rebound in viremia between doses. Phase IIb studies using PRO-140 as an adjunct to oral antiretroviral treatment in treatment-experienced patients are ongoing (www.clinicaltrials.gov; NCT01272258).

Cenicriviroc (CVC) (TBR-652) is a small-molecule CCR5 inhibitor that has shown promise in phase IIb dose-finding studies. It demonstrated a favorable tolerability profile and potent antiviral efficacy with a reduction of up to 1.8 $\log_{10}$ viral load copies in a phase IIb study (Lalezari et al. 2011; Marier et al. 2011). Treatment-experienced patients who received 10-day monotherapy with escalating doses of drug displayed dose-dependent reductions in viral load. The plasma half-life of CVC is 35–40 h and is not influenced by cytochrome P450. As such, it is suitable for once-daily dosing. Interestingly, CVC may also possess anti-inflammatory effects by virtue of its ability to antagonize CCR2 (Baba et al. 2005). Levels of the CCR2 ligand macrophage chemoattractant protein 1 (MCP-1) were increased in patients receiving CVC monotherapy, also suggesting CCR2 antagonism (Lalezari et al. 2011). The MCP-1/ CCR2 pathway has been implicated in a number of inflammatory diseases, and CVC that can simultaneously antagonize CCR2 and CCR5 bears further investigation in HIV-1-infected patients. Phase IIb studies of CVC plus two nucleoside reverse transcriptase inhibitors (NRTIs) versus efavirenz plus two NRTIs in treatment-naïve patients are ongoing (www.clinicaltrials.gov; NCT01338883).

Fusion Inhibitors

Fusion inhibitor development has proceeded with the goal of achieving oral availability or at least a reduction in the numbers of injections required for dosing. As mentioned, enfuvirtide (T-20) must be injected subcutaneously twice daily, leading to painful treatment-site reactions. The pursuit of oral bioavailability has led to successive generations of inhibitors; the third-generation compounds T-2635 (Eggink et al. 2011) and sifuvirtide (He et al. 2008) show potent antiviral efficacy in vitro and have a longer half-life than T-20 as a result of stabilization between HR1 and HR2 mimetics (reviewed in Eggink et al. 2010). This stability was a product of ion pair interactions that increased α-helix stability in these monomeric peptides that, in turn, stabilized the six-helix bundle. As a result, sifuvirtide has a higher potency and longer half-life than T-20 (26 h). Prolongation of half-life is also being pursued for T-20 with novel strategies that include the use of antibodies attached to T-20. The serum half-life of this conjugate was 72 h in mice and antiviral potency was increased (Chang et al. 2012).

CXCR4 Inhibitors

AMD070 is a derivative of the CXCR4 inhibitor AMD3100. It has antiviral potency in vivo and in vitro and has completed dose-escalation phase I studies (Moyle et al. 2009; Stone et al. 2007); oral bioavailability is currently being improved (Skerlj et al. 2010, 2011). Phase IIa and IIb safety and antiviral activity trials have been completed and results are expected soon (www.clinicaltrials.gov; NCT00089466, NCT00063804).

Resistance to Current Entry Inhibitors

There are concerns and limitations unique to entry inhibitors since resistance to entry inhibitors, particularly chemokine receptors, is unlike resistance to other antiretroviral agents. Entry inhibitors act either directly or indirectly against the viral envelope, one of HIV's most mutable proteins. Therefore, in addition to development of resistance, baseline sensitivities of patient viruses must also be taken into consideration (Tilton et al. 2010; Yu et al. 2011; Covens et al. 2009; Leung et al. 2010; Araujo et al. 2012).

Susceptibility to entry inhibitors can vary widely among different patient isolates, up to 1000-fold in cases. This difference is the result of envelope diversity at baseline. The envelope diversity of HIV results from a number of factors, including level of glycosylation, structural plasticity, continuous immune pressure, incomplete processing, and availability of target cells.

Resistance to each entry inhibitor subclass is unique and develops differently for each inhibitor. Resistance to the BMS attachment inhibitor compounds has involved mutations in gp41 and gp120, particularly in the CD4 binding site. Resistance to BMS-378806 which was discontinued following phase II clinical studies developed in vitro in gp41 (I595F, K655E) (Lin et al. 2003; Zhou et al. 2010). This was in addition to mutations in the CD4 binding site. However, the presence of these mutations did not correlate completely with in vivo resistance, revealing the complexities involved. One mutation that appeared in patients and in in vitro selections was M426L in the CD4 binding site (Zhou et al. 2011). This mutation also conferred resistance to BMS-626529 (Charpentier et al. 2012; Soulie et al. 2013). Although the appearance of M426L was common to both these drugs, two distinct mechanisms of resistance may be at work, as BMS-378806 is thought to prevent gp120-induced gp41 exposure, while BMS-626529 prevents gp120-CD4 interactions through induced gp120 conformational change. The M426L polymorphism was observed primarily in attachment-inhibitor-naïve patients harboring subtypes D and CRF_02 and did not frequently appear in subtype B. Virus tropism was evenly distributed among patient viruses harboring this polymorphism. Synergy between this inhibitor and all other antiretroviral classes was observed in vitro (Zhou et al. 2011). Resistance to ibalizumab has shown dependence on the loss of potential N-linked glycosylation sites in the V5 loop of gp120, although the molecular mechanism of resistance is still undefined (Pace et al. 2013; Toma et al. 2011).

Ibalizumab-resistant viruses demonstrated higher levels of infectivity versus wild type and sensitivity to soluble CD4. However, the resistant viruses did not regain wild-type infectivity in the presence of fully inhibitory concentrations of antibody (Toma et al. 2011). This implies that a possible mechanism of resistance may involve the ability of an ibalizumab-resistant virus to use antibody-bound CD4 in entry as a result of increased efficiency of CD4 usage, an enhancement of CD4-induced conformational change, or a combination of the two.

In regard to coreceptor inhibitors, resistance can occur in one of two ways. The first mechanism of resistance is a tropism switch that is defined as the emergence of X4-tropic strains from a predominantly R5-tropic virus population. In natural

infections, tropism switches occur in ~50 % of patients and are usually accompanied by disease progression and a steep decline in CD4 T cells (Scarlatti et al. 1997; Connor et al. 1997; Berger et al. 1999). Whether this tropism switch is the cause or consequence of advanced disease progression is still the subject of debate. In consideration of conflicting reports showing antiviral activity against dual/mixed (D/M) tropic viruses (Saag et al. 2009; Ceccherini-Silberstein et al. 2011; Perez-Olmeda et al. 2012; Symons et al. 2011), maraviroc and other CCR5 inhibitors can only be administered following a tropism test that indicates a purely R5-tropic virus population. Whether the emergence of detectable X4-tropic strains represents R5-tropic envelope evolution or an outgrowth of preexisting X4-tropic strains is a question that requires further investigation (Savkovic et al. 2012; Pastore et al. 2004; Fiser et al. 2010; Trkola et al. 2002). In vitro selection studies performed in PBMCs have demonstrated that tropism switch does not occur and that the inhibitor-resistant strains can still use CCR5 for entry. This was shown regardless of which entry inhibitor or CCR5-tropic virus was used.

In the MOTIVATE trials, 50 % of patients who failed MVC-containing regimens harbored X4/dual-tropic virus as opposed to the control group, for which only 6 % of patients failing treatment exhibited altered tropism (Fatkenheuer et al. 2008; Gulick et al. 2008). The emergence of X4-tropic virus was also observed in the MERIT trial in which a tropism test was used to screen out patients possessing X4 or dual-tropic virus at baseline. 31 % of patients who failed MVC-containing regimens harbored X4-tropic virus and 14 % harbored CCR5-tropic virus resistant to MVC (Cooper et al. 2010). A number of patients in clinical trials that failed on MVC treatment were found to harbor CXCR4-tropic virus at baseline. In a study of patients failing MVC treatment, 30 % of patients who experienced virologic failure harbored CXCR4. Of these, patients who exhibited X4-tropic virus at baseline displayed an outgrowth of these viruses (Recordon-Pinson et al. 2013). In MVC-treated patients in whom CXCR4 virus appeared, cessation of CCR5-inhibitor therapy resulted in a return of CCR5-tropic virus predominance suggesting that CCR5 variants may be more replication fit than CXCR4 variants (Gulick et al. 2007; Lalezari et al. 2005; Westby et al. 2006). It remains to be seen whether subtype-specific differences play a role in the development of this resistance pathway, as particular subtypes such as subtype C are far less prone to tropism switch during the course of infection (Cecilia et al. 2000; Coetzer et al. 2011).

Resistance to CCR5 coreceptor inhibitors can also occur if the virus is able to use drug-bound CCR5 for entry. This has notably been demonstrated in vitro. Different CCR5 antagonists have shown different resistance profiles in vitro, complicating the characterization of R5 resistance in general. Variations in envelope between different isolates, as well as the particular CCR5 antagonist used, have led to different manifestations of resistance with little overlap. MVC-resistant virus has been shown to use drug-bound receptor through a noncompetitive mechanism where an increase in drug concentration has no effect on entry. This has also been shown for attachment inhibitors (Nowicka-Sans et al. 2012). Evidence of a competitive mechanism has also been shown in vitro (Ratcliff et al. 2013), whereby an increase in IC50 has characterized a resistant virus that is still inhibited by high amounts of MVC.

Furthermore, coreceptor inhibitor mutations that appear in one HIV strain may not necessarily confer resistance when introduced into another (Henrich et al. 2010; Kuhmann et al. 2004; Marozsan et al. 2005; McNicholas et al. 2010, 2011; Tsibris et al. 2008).

Viral efficiency in utilizing CD4 and CCR5 appears to play a role in development of resistance (Fig. 4). A recent study compared the MVC-resistant viruses of two different patients. One of these was characterized as having strong resistance as evidenced by a low maximal percent inhibition (Roche et al. 2013). The other had weak resistance characterized by a high maximal percent inhibition. These viruses exhibited disparate Env mutations, but both showed a high reliance on sulfated tyrosine residues on the CCR5 N-terminus. The virus characterized by weak resistance could not efficiently engage maraviroc-bound receptors; the strongly resistant virus could efficiently use the maraviroc-bound receptor. Strains that utilize CCR5 efficiently appear to be more prone to developing resistance to CCR5 antagonists (Roche et al. 2011a), and resistance to CCR5 inhibitors may be related to baseline viral use of drug-bound coreceptor (Roche et al. 2011b). Interestingly, viral entry in the presence of CCR5-inhibitor vicriviroc may partially depend on the presence of the drug (Putcharoen et al. 2012). This is in contrast to what had been shown for aplaviroc, a CCR5 inhibitor that is no longer being clinically pursued (Pfaff et al. 2010).

Viral strains resistant to CVC have been generated that harbor changes in the V3 loop. Cenicriviroc selected T306K and Q309E in tissue culture in addition to changes in C2 (K221N), C4 (M424T), and gp41 (V766A, I769) (Baba et al. 2007). It is important to note that no one amino acid was considered sufficient for resistance to cenicriviroc. Rather, the accumulation of amino acid changes at various locations was required. Resistant viruses to other small-molecule CCR5 inhibitors have followed a similar pattern whereby the V3 loop mutations occurred most frequently though but were not the only Env mutations that were observed.

Pure X4-tropic viral populations in patients are rare, and it is unknown whether an X4 blockade might lead to resistance. One study showed that treatment of PBMCs with AMD3100 would lead to the emergence of R5 tropism in clinical isolates (Harrison et al. 2008; Armand-Ugon et al. 2003; Este et al. 1999). However, the clonal X4 virus NL4-3 did not exhibit a tropism switch when used in in vitro selections with the CXCR4 inhibitors SDF-1α and T134 (Kanbara et al. 2001; Schols et al. 1998). It is unclear what consequences may arise from resistance to CXCR4 blockade and what the impact on late-stage HIV pathogenesis might be.

Resistance to T-20 is more straightforward and does involve mutations within the HR1 region of gp41 that allows preferential binding to occur to HR2 instead of enfuvirtide (Marcelin et al. 2004; Carmona et al. 2005). These mutations localize between amino acids 36 and 45 in the N-terminal region. Common mutations include G36D, V38M, N42D, and N43D/Q. Combinations of these and other mutations in HR1 can select for high-level resistance to T-20. There is no cross-resistance between T-20 and other antiretrovirals (Greenberg and Cammack 2004).

These resistance mutations decrease the efficiency of the fusion process, causing T-20-resistant strains to be more sensitive to neutralizing antibodies

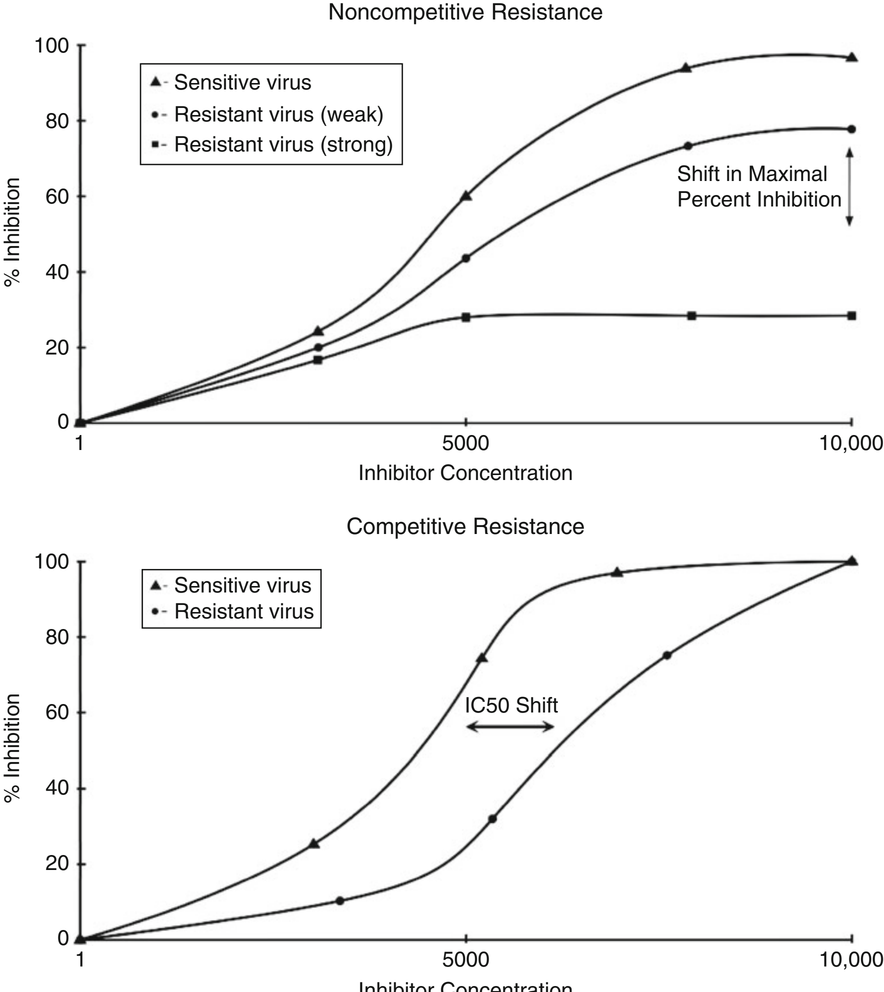

Fig. 4 Representations of noncompetitive and competitive resistance to entry inhibitors. *Top*: Noncompetitive entry inhibition allows for resistant virus to utilize drug-bound receptor. The virus does not compete with drug in receptor binding. The difference between sensitive, weakly, and strongly resistant viruses can be compared by maximal percent inhibition, defined as the percent inhibition of infection in overwhelmingly inhibitory concentrations of drug. The difference between weakly and strongly resistant viruses is a result of their efficiency in drug-bound receptor utilization. *Bottom*: Competitive entry inhibition occurs when the virus competes with drug for binding to the receptor. The difference between sensitive and resistant viruses is represented by a fold change in IC50 values. Resistant virus outcompetes the drug for binding to the receptor, but increasing amounts of drug will produce full inhibition of entry

(Reeves et al. 2005). The notion that a less efficient process of entry allows more time for a virion to be bound by neutralizing antibodies (Platt et al. 2012) may be one of the mechanisms involved and may also play a role in antiviral synergy among different entry inhibitors. The accumulation of varying mutations to different entry inhibitors may lead to a severe fitness cost to the virus. N125D in HR2 can compensate for the loss of fitness as a result of the resistance mutations Q40H and Q56R. S138A is another compensatory mutation that can restore entry efficiency following selection of N43D (Izumi et al. 2009; Ray et al. 2009; Xu et al. 2005). Two mechanisms may account for restoration of entry efficiency. First, compensatory mutations in HR2 can restore its affinity for HR1 (Baldwin and Berkhout 2008). Second, mutations in both gp41 and stem loop 3 of the Rev-responsive element can result in an E57A mutation that is associated with an increase in viremia in patients harboring T-20-resistant viruses (Svicher et al. 2008) that is in contrast to results of other studies in which CD4 increases were observed in patients harboring T-20-resistant isolates (Deeks et al. 2007; Melby et al. 2007; Soria et al. 2008).

Limitations of Current Entry Inhibitors

The results of recent clinical trials involving maraviroc as part of an antiretroviral regimen for treatment-naïve patients showed that a greater percentage of patients in the maraviroc treatment group harbored viral loads between 50 and 400 copies/ml compared with patients taking either an RT inhibitor or an integrated strand-transfer inhibitor (INSTI) together with optimized background therapy (Cooper et al. 2010; Sierra-Madero et al. 2010; Mills et al. 2012). With more laboratories switching to viral load assays with a limit of 20 copies/ml, the entry inhibitor class may not appear to be as potent as other classes. Furthermore, antiretrovirals of various classes may have differing effects on the overall rate of viral load decline. Integrase inhibitors, for example, led to a much steeper decline in viral load than reverse transcriptase and protease inhibitors, by virtue of their method of action (Donahue et al. 2010; Markowitz et al. 2007; Grinsztejn et al. 2007; Sedaghat et al. 2008). Clinical trials have empirically shown that entry inhibitors do not exhibit a faster rate of decline on viral loads compared to other classes (Mills et al. 2012). This may be attributed to a number of factors: (1) inherent antiretroviral activity (Sedaghat et al. 2008), (2) increased CD4 trafficking (Gulick et al. 2008), or (3) redistribution of repelled virus (Kramer et al. 2012). The clinical implications of a slightly elevated viral load (>50 copies/ml) as a result of entry inhibitor suppression bear further investigation.

Conclusions

Targeting HIV entry has yielded an antiretroviral drug class with two representatives approved for treatment. The complexity of the entry process has given rise to a number of candidates, each with novel antiviral mechanisms, which are currently progressing in the clinic. The diversity of the class demands consideration of a

number of issues: antiviral efficacy, treatment indications, development of resistance, and the importance of viral tropism. The need for further study to precisely designate the role of this class in HIV treatment is warranted by the potential that this class represents in both early treatment/prophylaxis and functional cure.

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HIV-1 Resistance to Reverse Transcriptase Inhibitors

Grant Schauer and Nicolas Sluis-Cremer

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Abstract

Purpose of Review: This review discusses the mutations and mechanisms associated with HIV-1 resistance to nucleoside reverse transcriptase (RT) inhibitors (NRTIs) and nonnucleoside RT inhibitors (NNRTIs).

Recent Findings: First-line antiretroviral therapy (ART) for the treatment of HIV-1 infection typically includes two NRTIs in combination with an NNRTI or a protease inhibitor. NRTIs and NNRTIs are also routinely used in second-line and salvage ART therapies. HIV-1 resistance to all of the FDA-approved NRTIs and NNRTIs has been documented. An understanding of the mutations associated with RT inhibitor (RTI) resistance, the antagonistic or complementary interactions between RTI-resistance mutations, and the mechanisms of HIV-1 resistance to RTIs is of critical importance for the development and formulation of effective ART therapies. Of concern, there has been a significant increase in circulating and transmitted NNRTI drug resistance in resource-limited settings due to the extensive use of NNRTIs in prevention and treatment strategies for HIV-1 infection. Despite this increase in NNRTI drug resistance, the diarylpyrimidine NNRTIs, dapivirine, etravirine, and rilpivirine, will be increasingly used in resource-limited settings. As such, there is a continued need to monitor and understand NNRTI resistance, particularly in sub-Saharan Africa where non-subtype B HIV-1 predominates.

Summary: This review describes HIV-1 resistance to NRTIs and NNRTIs.

Keywords

HIV • Reverse transcriptase • Nannucleosicle • Efavirens • Neviapine • Rilpivirine • Etravirine

Key Points

1. NRTIs and NNRTIs form the backbone of most first-line ART.
2. Thirteen RTIs (eight NRTIs and five NNRTIs) have been approved for treatment, although only ten of these are routinely used.
3. RTI therapy selects for viruses that have mutations in HIV-1 RT.
4. NRTI-associated resistance mutations can be broadly categorized into two groups depending on whether they confer resistance via a discrimination of excision phenotype.

5. HIV-1 resistance to NNRTIs correlates directly with mutations of one or more RT residues in the NNRTI-binding pocket.
6. Interactions between different NRTI and NNRTI-resistance mutations can be complementary or antagonistic.
7. There has been a significant increase in NNRTI drug resistance in resource-limited settings that could impact future prevention and treatment strategies.

Introduction

HIV-1 reverse transcriptase (RT) catalyzes the conversion of the viral single-stranded (+)RNA into double-stranded DNA. The enzyme is multifunctional and possesses both a DNA polymerase activity that can use either RNA or DNA as a template and a ribonuclease H (RNase H) activity that degrades the RNA strand in RNA/DNA duplexes. HIV-1 RT is composed of a 560-residue 66 kDa subunit (p66) and a p66-derived 440-residue 51 kDa (p51) subunit. The p66 subunit contains three domains, namely, DNA polymerase (residues 1–318), connection (residues 319–426), and RNase H (residues 427–560) (Kohlstaedt et al. 1992). In comparison, p51 comprises only the polymerase and connection domains. Each p66/p51 RT molecule has one binding cleft for the template/primer (T/P) nucleic acid substrate, one DNA polymerization active site, and one RNase H active site. Both the polymerase and RNase H active sites reside in p66; although p51 is identical in amino acid sequence to p66, the polymerase active site in this subunit is not functional (Kohlstaedt et al. 1992; Wang et al. 1994).

RT Inhibitors (RTIs)

The US Food and Drug Administration (FDA) has approved 13 RTIs for the treatment of HIV-1 infection, although only 11 of these are currently used (see below). These inhibitors, all of which bind at or near to the DNA polymerase active site of the enzyme, can be classified into two distinct groups: (1) the nucleoside and nucleotide RT inhibitors (NRTIs) and (2) the nonnucleoside RT inhibitors (NNRTIs).

The NRTIs are analogs of naturally occurring dNTPs that lack a 3′-hydroxyl group on the ribose sugar/pseudosugar. They were the first drugs used to treat HIV-1 infection, and they remain integral components of nearly all antiretroviral (ART) regimens. To exhibit antiviral activity, NRTIs must be metabolically converted by host-cell kinases to their corresponding triphosphate forms (NRTI-TP). The NRTI-TP inhibit HIV-1 RT DNA synthesis by acting as chain terminators of DNA synthesis (Goody et al. 1991). Eight NRTIs have been approved for clinical use, namely, 3′-azido-3′-deoxythymidine (zidovudine, AZT); 2′,3′-dideoxyinosine (didanosine, ddI); 2′,3′-dideoxycytidine (zalcitabine, ddC); (−)-β-2′,3′-dideoxy-3′-thiacytidine (lamivudine, 3TC); 2′-deoxy-2′,3′-didehydrothymidine (stavudine, d4T); (1*S*,4*R*)-4-[2-amino-6-(cyclopropyl-amino)-9H-purin-9-yl]-2-cyclopentene-1-

Fig. 1 Chemical structures of FDA-approved NRTIs

methanol succinate (abacavir, ABC); (*R*)-9-(2-phosphonylmethoxypropyl) adenine (TFV, tenofovir); and 5-fluoro-1-[(*2R*,*5S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (emtricitabine, FTC) (Fig. 1). ddC is less potent than the other NRTIs, has inconvenient dosing schedules, and is associated with serious adverse events. For these reasons, it is now rarely used to treat HIV-1 infection. Similarly, the World Health Organization advocated that d4T should be phased out of use because of its long-term, irreversible side effects. However, d4T is still used in first-line therapy in developing countries due to its low cost and widespread availability.

The NNRTIs are a group of amphiphilic compounds that bind to a hydrophobic pocket in HIV-1 RT that is proximal to but distinct from the polymerase active site (Kohlstaedt et al. 1992; Sluis-Cremer et al. 2004). NNRTI are allosteric inhibitors of HIV-1 RT DNA polymerization reactions (Sluis-Cremer et al. 2004). FDA-approved NNRTIs include 11-cyclopropyl-4-methyl-5,11-dihydro-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one (nevirapine; NVP); *N*-[2-({4-[3-(propan-2-ylamino)pyridin-2-yl]piperazin-1-yl}carbonyl)-1*H*-indol-5-yl]methanesulfonamide (delavirdine; DEL); (4*S*)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dihydro-1*H*-3,1-benzoxazin-2-one (efavirenz; EFV), 4-[6-Amino-5-bromo-2-[(4-cyanophenyl)amino]pyrimidin-4-yl]oxy-3,5-dimethylbenzonitrile (etravirine; ETV); and 4-{[4-({4-[(*E*)-2-cyanovinyl]-2,6-dimethylphenyl}amino)pyrimidin-2-yl]amino}benzonitrile (rilpivirine; RIL). The efficacy of delavirdine is lower than that of the other NNRTIs, especially EFV, and it also has an inconvenient dosing schedule. These factors have led the US Department of Health and Human Services (DHHS) to recommend against its use as part of initial therapy (Fig. 2).

Fig. 2 Chemical structures of FDA-approved NNRTIs

HIV-1 Drug Resistance

Although combination therapies that contain two or more RTI have profoundly reduced morbidity and mortality from HIV-1 infection, their long-term efficacy is limited by the selection of drug-resistant variants of HIV-1. Antiviral drug resistance is defined by the presence of viral mutations that reduce drug susceptibility compared with the drug susceptibility of wild-type (WT) viruses. HIV-1 drug resistance within an individual arises from the genetic variability of the virus population and selection of subpopulations of resistant variants with therapy (Chen et al. 2004). HIV-1 genetic variability arises from the inability of HIV-1 RT to proofread nucleotide sequences during replication, the high rate of HIV-1 replication, the accumulation of proviral variants during the course of HIV-1 infection, and the genetic recombination when viruses of different sequence infect the same cell (Sluis-Cremer et al. 2004; Chen et al. 2004). As a consequence of these mechanisms, innumerable genetically distinct variants (termed quasispecies) evolve within an individual in the years following infection. Whether or not drug resistance develops depends on the extent to which virus replication continues during therapy, the ease of acquisition of a particular mutation (or set of mutations), and the effect of drug-resistance mutations on drug susceptibility and viral fitness (Chen et al. 2004). In general, RTI therapy selects for viruses that have mutations in RT. From a clinical perspective, the development of drug-resistant HIV-1 limits future treatment options by effectively decreasing the number of available drugs that remain active against the resistant virus. As a consequence, more complicated drug regimens are required that involve intense dosing schedules and greater risk of severe side effects due

to drug toxicity. These factors often contribute to incomplete adherence to the drug regimen and lower therapeutic efficacy.

HIV-1 Resistance to NRTIs

NRTI-associated resistance mutations can be broadly categorized into two groups depending on their phenotypic mechanism of resistance (Sluis-Cremer et al. 2000a; Selmi et al. 2003; Deval et al. 2004a). The mutations K65R, K70E, L74V, Q151M (in complex with A62V, V75I, F77L, and F116Y), and M184V increase the selectivity of RT for incorporation of natural dNTP substrate versus the NRTI-TP (Selmi et al. 2001; Deval et al. 2002, 2004b, c; Feng and Anderson 1998; Sluis-Cremer et al. 2007). This resistance mechanism has been termed NRTI-TP discrimination. In comparison, the mutations M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E are typically referred to as thymidine analog mutations (TAMs). These mutations augment the ability of HIV-1 RT to excise a chain-terminating NRTI monophosphate (NRTI-MP) from a prematurely terminated DNA chain (Arion et al. 1998; Meyer et al. 1998). This resistance mechanism has been termed NRTI-MP excision. Each of these mechanisms is described in more detail below. The locations of the mutations associated with the NRTI-TP discrimination and NRTI-MP excision phenotypes relative to the DNA polymerase active site of HIV-1 RT are shown in Fig. 3.

Drug-Resistance Mutations that Increase NRTI-TP Discrimination

This mechanism involves the acquisition of one or more resistance mutations in RT that improve the enzyme's ability to discriminate between the natural dNTP substrate and the NRTI-TP. Resistance by this mechanism is typically associated with decreased catalytic efficiency of NRTI-TP incorporation. NRTI-TP (and dNTP) catalytic efficiency is driven by two kinetic parameters: (i) the affinity of the nucleotide for the RT polymerase active site (K_d) and (ii) the maximum rate of nucleotide incorporation (k_{pol}), both of which can be determined using pre-steady-state kinetic analyses (Reardon 1992). In general, NRTI-TP discrimination is achieved by the resistance mutation affecting only one of these kinetic parameters, as described below.

K65R

The K65R mutation in HIV-1 RT decreases susceptibility to all FDA-approved NRTI, with the exception of AZT (Parikh et al. 2005). Residue K65 resides in the β3-β4 loop in the "fingers" subdomain of the 66 kDa subunit of HIV-1 RT, and in the crystal structure of the ternary HIV-1 RT-template/primer (T/P)-dNTP complex, the ε-amino group of K65 interacts with the γ-phosphate of the bound dNTP substrate (Huang et al. 1998). Pre-steady-state kinetic analyses have demonstrated that K65R

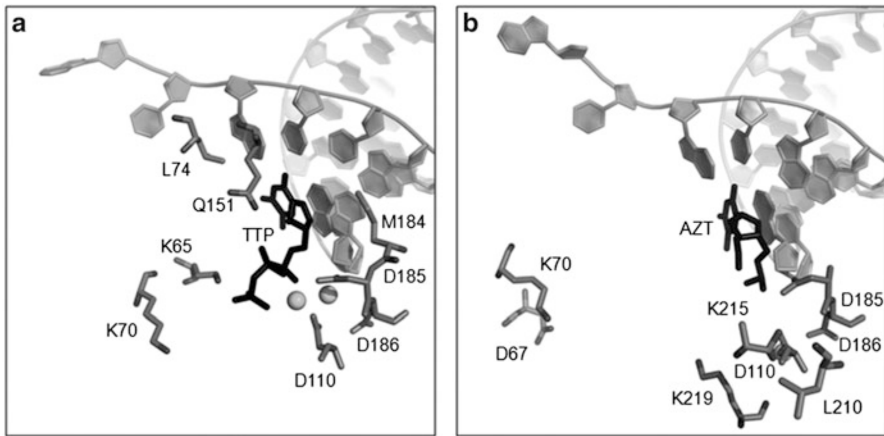

Fig. 3 Location of amino acid residues associated with the NRTI-TP discrimination (**a**) and NRTI-MP excision (**b**) resistance phenotypes

confers resistance to ddATP (active metabolite of ddI), 3TCTP, carbovir-TP (CBVTP, active metabolite of ABC), and TFV-diphosphate (DP) by selectively reducing k_{pol} without affecting K_d (Selmi et al. 2001; Deval et al. 2004b; Sluis-Cremer et al. 2007). However, for ddCTP, the resistance involves both reduction in k_{pol} and increase in K_d (Selmi et al. 2001). Structural studies suggest that the K65R mutation in HIV-1 RT distorts optimal positioning of the NRTI-TP in the active site, which decreases the catalytic efficiency of incorporation (Selmi et al. 2001; Sluis-Cremer et al. 2000b; Das et al. 2009).

K70E

The K70E mutation was initially selected in vitro with the NRTI adefovir (Cherrington et al. 1996). However, it has become more prevalent in clinical samples since the introduction of tenofovir and was recently reported in 10 % of antiretroviral-naïve subjects receiving the triple NRTI combination of tenofovir, ABC, and 3TC (Kagan et al. 2007). We demonstrated that K70E confers resistance to TFV-DP, CBVTP, and 3TCTP through a discrimination mechanism involving reduction in k_{pol} with little effect on K_d (Sluis-Cremer et al. 2007).

L74V

The L74V mutation was originally identified as causing ddI resistance but has also been associated with ABC resistance (Winters et al. 1994; Miller et al. 2000). Pre-steady-state kinetic experiments have demonstrated that the L74V mutation confers resistance to

ddATP by decreasing k_{pol} without impacting K_d. Molecular modeling suggests that the L74V mutation leads to the loss of a stabilizing interaction between the nucleotide base of the incoming nucleotide and the side chain of L74 (Deval et al. 2004c). This may induce a rotation of the base (7° for ddATP compared with dATP), which indirectly affects the positioning of the phosphates and catalytic efficiency (Deval et al. 2004c).

Q151M Complex

The Q151M complex consists of a cluster of mutations in HIV-1 RT that includes the Q151M mutation plus four additional mutations: A62V, V75I, F77L, and F116Y (Ueno et al. 1995; Matsumi et al. 2003). The Q151M mutation generally occurs first before the acquisition of the other mutations (Ueno et al. 1995; Matsumi et al. 2003). Although rare (~1 % prevalence among resistance databases), the Q151M complex is most often selected by regimens containing d4T and ddI (Balotta et al. 2000). The mechanism of resistance mediated by Q151M and the Q151M complex is a selective reduction in the catalytic rate constant (k_{pol}) for incorporation of NRTI-TP (Deval et al. 2002).

M184I/V

The M184I/V mutation in HIV-1 RT causes high-level (>100-fold) resistance to 3TC and FTC resistance (Schinazi et al. 1993; Faraj et al. 1994). However, this mutation also confers resistance to ABC, ddC, and ddI (Winters et al. 1997; Miller et al. 2000; Hammond et al. 2005). Pre-steady-state kinetic analyses have demonstrated that M184V exerts a profound effect on the K_d for 3TCTP, without impacting k_{pol} (Deval et al. 2004b; Feng and Anderson 1998). M184 forms part of the highly conserved YMDD motif, and crystal structures of 3TC-resistant M184I RT, obtained in the presence or absence of a nucleic acid substrate, suggest that steric hindrance between the oxathiolane ring of 3TCTP and the side chain of the β-branched amino acids (Val or Ile) at position 184 reduces inhibitor binding thus increasing K_d (Gao et al. 2000).

Drug-Resistance Mutations Affecting NRTI-MP Excision

For the excision mechanism of NRTI resistance, the mutant HIV-1 RT does not discriminate between the natural dNTP substrate and the NRTI-TP at the nucleotide incorporation step (Kerr and Anderson 1997). Instead, RT containing excision-enhancing mutations shows an increased capacity to unblock NRTI-MP terminated primers in the presence of physiological concentrations of ATP (Meyer et al. 1998). Resistance mutations associated with the excision mechanism include thymidine analog mutations (TAMs) and T69S insertion mutations. These mutations are described below.

TAMs

AZT resistance is associated with multiple mutations in RT including M41L, D67N, K70R, L210W, T215F/Y, and K219E/Q (Larder and Kemp 1998; Kellam et al. 1992; Harrigan et al. 1996; Hooker et al. 1996). Because each of these mutations has also been selected with d4T therapy, they have been termed thymidine-analog mutations or TAMs (Ross et al. 2001). The presence of four or more TAMs in HIV-1 RT typically results in >100-fold resistance to AZT, fivefold to sevenfold reduced susceptibility to ABC, and twofold to fivefold reduced susceptibility to d4T, ddI, and TFV (Whitcomb et al. 2003). TAMs also reduce susceptibility to 3TC, but via a discrimination mechanism rather than an excision mechanism (Parikh et al. 2006, 2007). The efficiency of the NRTI-MP excision reaction by RT containing TAMs depends on the chain-terminating NRTI-MP residing in the nucleotide-binding site (N-site) of the RT active site. Under physiological conditions, the binding of the next-correct dNTP can drive the terminating nucleotide into the primer-binding site (P-site) resulting in the formation of a dead-end complex (DEC) (Meyer et al. 1998). Biochemical studies have proposed several mechanisms by which TAMs can increase the efficiency of RT to excise NRTI-MP from chain-terminated primers. These mechanisms include (i) an increase in the binding affinity of ATP for RT (Meyer et al. 1998), (ii) an increase in the kinetic rate of ATP-mediated NRTI-MP excision (Ray et al. 2003), (iii) a decrease in sensitivity of RT to DEC formation (Meyer et al. 1998), and (iv) a shift in the translocation equilibrium of the primer terminus between the N- and P-sites such that the N-site is favored (Marchand and Gotte 2003). While it has been suggested that the 3′-azido group of the AZTMP-terminated primer is the primary structural determinant for the excision phenotype, we recently showed that the potency of 3′-azido-2′,3′-dideoxyadenosine (3′-azido-ddA) and 3′-azido-2′,3-′-dideoxyguanosine (3′-azdio-ddG) is retained against AZT-resistant virus (Sluis-Cremer et al. 2005). This indicates that the nucleoside base has an important influence on the efficiency of excision resulting from TAMs.

T69S Insertions

HIV-1 RT containing dipeptide insertions (typically Ser-Ser, Ser-Gly, or Ser-Ala) between codons 69 and 70, together with the amino acid substitutions T69S, T215Y, and other TAMs, has been identified in heavily NRTI-experienced patients, albeit at low prevalence (0.5–2.7 %) (Winters and Merigan 2005). In phenotypic assays, viral isolates containing insertion mutations in RT demonstrate high-level resistance to AZT and moderate levels of resistance to other NRTI, such as d4T, ddC, ddI, ABC, and tenofovir. In combination with TAMs (in particular T215Y), the dipeptide insertions in HIV-1 RT confer enhanced ATP-dependent phosphorolytic activity that facilitates removal of terminating AZTMP, d4TMP, ddAMP, or tenofovir, even when relatively high levels of dNTPs are present in the reaction (Meyer et al. 2003; Boyer et al. 2002; Mas et al. 2000).

HIV-1 Resistance to NNRTIs

Typically, HIV-1 resistance to NNRTIs correlates directly with mutations of one or more RT residues in the NNRTI-binding pocket. Mutations associated with resistance to NVP and EFV include L100I, K101E/P, K103N/S, V106A/M, Y181C/I/V, Y188C/L/H, G190A/E/S, and M230L (Stanford University HIV Drug Resistance Database: http://hivdb.stanford.edu/). Although ETV and RIL have been reported to have higher in vitro genetic barriers to resistance than EFV or NVP (Andries et al. 2004; Azijn et al. 2010), 17 mutations in HIV-1 RT have been associated with decreased virologic response to ETV (V90I, A98G, L100I, K101E/H/P, V106I, E138A, V179D/F/T, Y181C/I/V, G190A/S, and M230L) (Vingerhoets et al. 2010) and 15 mutations with decreased virologic response to RIL (K101E/P, E138A/G/K/Q/R, V179L, Y181C/I/V, H221Y, F227C, and M230I/L) (Anta et al. 2013). The locations of the mutations associated with NVP, EFV, RIL, and ETV resistance are shown in Fig. 4. In general, these NNRTI-resistance mutations can affect inhibitor binding in a number of ways. (1) They can remove one or more favorable interactions between the inhibitor and NNRTI-binding pocket. For example, the Y181C mutation eliminates π-stacking interactions between this residue and the aromatic ring of the NNRTI pharmacophore (Ren et al. 2001). (2) They can introduce steric barriers to NNRTI binding. For example, the G190E mutation introduces a bulky side chain which may prevent NNRTI binding by sterically interfering with functional groups, such as the cyclopropyl ring of NVP (Huang et al. 2003; Yap et al. 2007). (3) The mutations may introduce or eliminate inter-residue contacts in the NNRTI-binding pocket, which interfere with the ability of other residues in the pocket to fold down over the NNRTI (Sluis-Cremer et al. 2004).

RTI-Resistance Mutations in the Connection Domain of HIV-1 RT

While genotypic analysis of HIV-1 isolates in infected patients is usually restricted to residues 1–250 of the RT, recent reports indicate that several residues in the connection domain of RT can also modulate NRTI and NNRTI resistance. Mutations in the connection domain of RT with potential clinical relevance include N348I, A360V, and A367S.

N348I

The N348I mutation in HIV-1 RT confers both NRTI (AZT) and NNRTI resistance (Yap et al. 2007; Hachiya et al. 2008; von Wyl et al. 2010; Sluis-Cremer et al. 2010). N348I can appear early in therapy and is found to be highly associated with TAMs, M184V/I, and the NNRTI-resistance mutations K103N, Y181C/I, and G190A/S (Yap et al. 2007). N348I was also found to be significantly associated with therapies that contained AZT and NVP (Yap et al. 2007). Yap et al. showed that N348I reduces the rate of RNA template degradation by RT in either a wild-type background or in

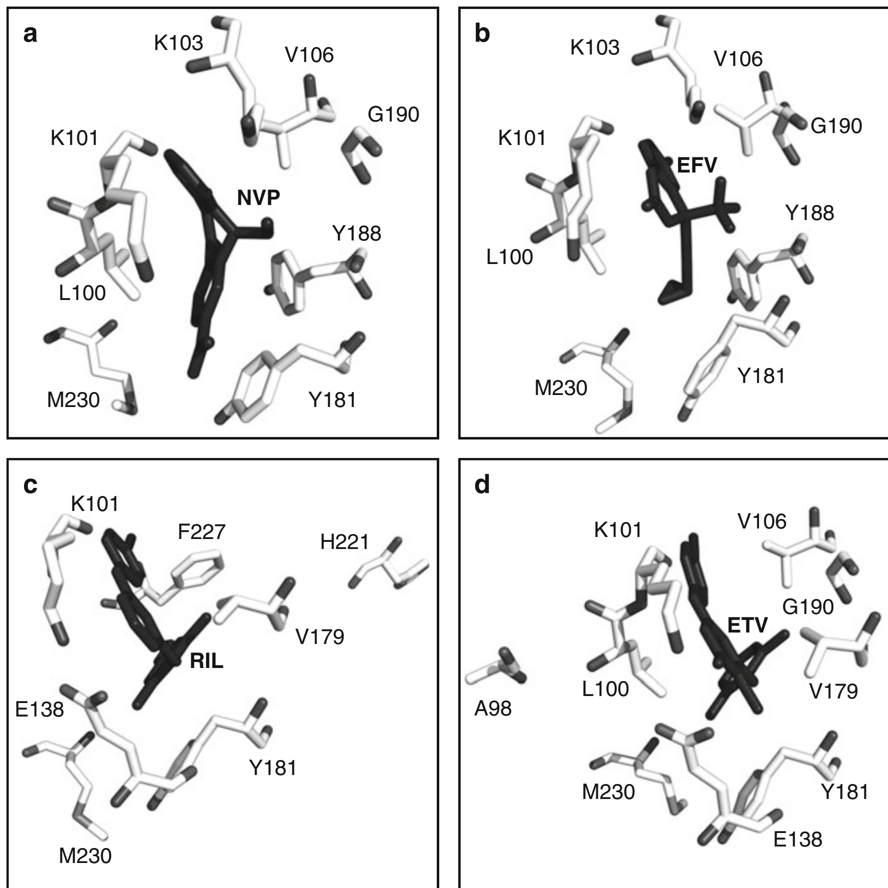

Fig. 4 Location of amino acid residues associated with NVP (**a**), EFV (**b**), RIL (**c**), and ETV (**d**) resistance

the presence of TAMs (Yap et al. 2007). This property would facilitate the excision of AZT-MP by giving RT more time to excise the blocking nucleoside analog from the terminated primer. However, it has been shown that N348I could also modulate the excision activity of the RT by an RNase H-independent mechanism, since this mutation could increase the processivity of HIV-1 RT in the absence or in the presence of TAMs (Ehteshami et al. 2008; Schuckmann et al. 2010). In regard to NNRTI resistance, three different mechanisms have been proposed to explain the resistant phenotype conferred by N348I. These include: (i) N348I directly decreases NNRTI binding affinity to RT (Schuckmann et al. 2010), (ii) N348I decreases RT RNase H activity (Radzio and Sluis-Cremer 2011; Nikolenko et al. 2010), and (iii) N348I impacts the orientation of RT relative to the T/P substrate, a property that appears to be critical for polypurine tract removal during plus strand DNA synthesis (Biondi et al. 2010).

A360V

The A360V in the connection domain of HIV-1 RT is also highly associated with TAMs (Ehteshami et al. 2008). Importantly, Brehm et al. showed that the A360V mutation was selected in HIV-infected individuals who received AZT monotherapy and contributed to AZT resistance (Brehm et al. 2012). Like N348I, the A360V mutation reduces the rate of RNA template degradation by WT and TAM containing RT, therefore providing more time for the enzyme to excise AZT-MP from the terminated primer (Brehm et al. 2012).

A376S

Paredes et al. reported that preexisting A376S was associated with an increased risk of virologic failure to NVP (relative hazard (RH) = 10.4; 95 % confidence interval (CI), 2.0–54.7), but did not affect EFV outcome the same way (RH = 0.5; 95 % CI, 0.1–2.2) ($p = 0.013$) (Paredes et al. 2011). A376S confers selective low-level NVP resistance in vitro (Paredes et al. 2011). Interestingly, Gupta et al. reported that virologic responses to an ETV-containing regimen were slightly diminished when A376S was present (Gupta et al. 2011).

Interactions Between RTI-Resistance Mutations

Antagonistic Interactions

Several NRTI (e.g., K65R, K70E, L74V, and M184V) and NNRTI mutations (e.g., Y181C) reverse HIV-1 resistance to AZT when added to a genetic background containing TAMs (Sluis-Cremer et al. 2007; Parikh et al. 2006; Miranda et al. 2005; Gotte et al. 2000; Larder 1992). It has also been shown that TAMs can antagonize the phenotypic effects of K65R, decreasing resistance to TFV, ABC, ddI, and d4T (Parikh et al. 2006). Biochemical studies have demonstrated that K65R, L74V, and M184V significantly reduce the ability of RT containing TAMs to excise NRTI-MP (Sluis-Cremer et al. 2007; Parikh et al. 2007; Miranda et al. 2005; Gotte et al. 2000). By contrast, TAMs decrease the extent to which RT containing K65R can discriminate against D-nucleotide analogs, but not L-nucleotide analogs (such as 3TC-TP), by partially restoring the maximum rate of NRTI-TP incorporation (Parikh et al. 2007). Despite this antagonism, multidrug-resistant HIV-1 can still develop, although the current literature suggests that this may require the accumulation of several additional mutations. For example, substitutions at RT codons 44, 118, 207, 208, 333, and 348 have been associated with increased AZT resistance in viruses that carry both TAMs and M184V (von Wyl et al. 2010; Girouard et al. 2003; Zelina et al. 2008; Radzio et al. 2010).

Complementary Interactions

In the phase III ECHO and THRIVE clinical trials which compared the efficacy of RIL versus EFV in treatment-naïve HIV-1-infected individuals, the most frequent mutation combination that emerged in the RIL virologic failures was E138K + M184I (Rimsky et al. 2013; Molina et al. 2013; Cohen et al. 2013). Typically, the M184I mutation emerges first during FTC or 3TC containing ART regimens but is rapidly replaced by the M184V mutation because viruses carrying M184V are fitter than the M184I mutants. In this case, it was found that the E138K mutation compensated for the poor replicative capacity of M184I (Hu and Kuritzkes 2011; Xu et al. 2011). Indeed, the replicative capacity of E138K/M184I HIV-1 was comparable to that of the WT virus in the absence of drug and was found to be significantly greater than that of the E138K and E138K/M184V mutants in the presence of ETV (Hu and Kuritzkes 2011). Kinetic analyses have demonstrated that E138K compensates for a deficit in dNTP usage that is inherent to the M184I HIV-1 RT (Xu et al. 2011).

Subtype Differences in HIV-1 Resistance to RTIs

The majority of research into HIV-1 drug resistance has focused on subtype B viruses, yet non-subtype B strains are responsible for 90 % of global infections. Importantly, there is increasing evidence of subtype differences in RTI drug resistance, as described below.

V106M

Subtype C viruses harbor GTG (valine) at codon 106 in HIV-1 RT, whereas subtype B harbors GTA (valine). The GTG polymorphism facilitates the emergence of subtype C virus with the V106M mutation (GTG to ATG) that confers resistance to all NNRTIs (Brenner et al. 2003).

E138A

Recent data from our group shows that a glutamic acid to alanine substitution at codon 138 in RT occurs significantly more frequently in subtype C than B sequences in both treatment-naïve and RT inhibitor-experienced HIV-1-infected individuals (Sluis-Cremer et al. 2013). E138A has been clinically associated with virologic failure of regimens that contain ETV or RIL.

Silent Mutations at Codons 64, 65, and 66 in Subtype C HIV-1 RT

Subtype C viruses harbor AAA (lysine), AAG (lysine), and AAG (lysine) at codons 64, 65, and 66 or HIV-1 RT, respectively. In contrast, all other HIV-1 subtypes harbor

AAG (lysine), AAA (lysine), and AAA (lysine) at the same codons. There is recent clinical evidence demonstrating frequent and early emergence of K65R on TNF-based first-line ART regimens in South Africa (Sunpath et al. 2012; Theys et al. 2013). In this regard, Mark Wainberg's group has shown that the difference in selection of K65R between subtypes B and C is related to the template nucleotide sequence and preferential pausing of reverse transcription at the homopolymeric stretch of adenine bases at codons 64, 65, and 66 of RT (Coutsinos et al. 2010; Invernizzi et al. 2009).

The Threat of Transmitted NNRTI Resistance in Resource-Limited Settings

The past decade has seen an enormous global scale-up of ART. In 2011, more than eight million people were receiving ART in low- and middle-income countries, which was 26 times higher than the number in 2003 (Together we will end AIDS). Although this widespread distribution of ART has dramatically reduced HIV/AIDs-related mortality, current data suggests that up to 24 % of individuals receiving first-line ART in sub-Saharan Africa experience virologic failure within 12 months of initiation of therapy (Barth et al. 2010). Between 53 % and 90 % of these have viruses with clinically important HIV-1 drug-resistance mutations (Gupta et al. 2009; Hamers et al. 2012; Hosseinipour et al. 2009). As such, antiretroviral drug resistance is one of the main threats to the global control of HIV-1.

NVP or EFV in combination with 2 NRTIs forms the foundation of most first-line ART regimens in resource-limited settings. NVP has also been routinely used in regimens for prevention mother-to-child transmission. Due to the extensive use of NNRTIs, there has been a significant increase in NNRTI drug resistance in regions of sub-Saharan Africa (Gupta et al. 2012). The prevalence of NNRTI mutations has increased by 36 % per year in east Africa and by 23 % per year in southern Africa since the inception of ART rollout (Gupta et al. 2012). There has also been a significant increase in transmitted NNRTI resistance in resource-limited settings (Gupta et al. 2012). The spread of NNRTI resistance threatens the success of prevention and first-line and salvage ART therapies.

This threat is of even of greater significance when one considers that the diarylpyrimidine (DAPY) NNRTIs, dapivirine (TMC120), ETV, and RIL, will be increasingly used for the treatment and prevention of HIV-1 infection in resource-limited settings. Many sub-Saharan African countries already have access to ETV, which has been approved for the treatment of HIV infection in ART-experienced individuals. RIL, which has been co-formulated with FTC and TFV, is pending approval as a first-line ART regimen in sub-Saharan Africa. A long-acting RIL formulation is in development as a preexposure prophylaxis agent for use in resource-limited settings (Baert et al. 2009; van't Klooster et al. 2010). Finally, the ASPIRE study is currently assessing whether TMC120 can safely prevent HIV infection when continuously released in the vagina from a silicone ring replaced once a month. Given the escalating frequency of NNRTI-resistant variants present in

ART-naïve and ART-experienced HIV-infected individuals in sub-Saharan Africa, as well as the potential for bidirectional cross-resistance between first-generation NNRTIs (NVP and EFV) and the DAPY analogs, there is a continued need to monitor and understand NNRTI drug resistance in resource-limited settings.

Conclusions

RTIs are routinely used in first-line, second-line, and salvage ART therapies. HIV-1 resistance to all of the FDA-approved RTIs has been documented. In general, there is extensive cross-resistance among the NRTIs, and separately the NNRTIs, although in some instances antagonistic interactions occur. Of concern, there has been a significant increase in circulating and transmitted NNRTI drug resistance in resource-limited settings due to the extensive use of NNRTIs in prevention and treatment strategies for HIV-1 infection. Despite this increase in NNRTI drug resistance, the diarylpyrimidine NNRTIs, dapivirine, etravirine, and rilpivirine, will be increasingly used in resource-limited settings. As such, there is a continued need to monitor and understand NNRTI resistance, particularly in sub-Saharan Africa where non-subtype B HIV-1 predominates.

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HIV Drug Resistance in Mothers and Infants Following Use of Antiretrovirals to Prevent Mother-to-Child Transmission

Scott Olson, Quy Ton, and Lisa Frenkel

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Abstract

In the United States and other resource-rich nations, combination antiretroviral therapy (**ART**) that suppresses viral replication below the limits of detection in the plasma is standard of care for prevention of mother-to-child transmission of HIV (**PMTCT**). In many resource-limited communities, financial and infrastructure limitations preclude ART for pregnancy. Instead, abbreviated PMTCT regimens of short-term antiretrovirals (**ARV**) (mono- or dual therapy) that do not suppress viral replication to undetectable levels are recommended. While these less costly approaches achieve significant decreases in the rates of MTCT, selection of HIV drug resistance (**HIV-DR**) has been detected in both mothers and infected infants. This chapter reviews prominent studies that provide insight into HIV-DR related to use of ARV for PMTCT and discusses how recent findings and therapeutic advances have led to policy changes and new directions in this developing field.

Keywords

HIV • Mother-to-child-transmission • Zidovudine • Nevirapine • Drug-resistance

Introduction

Single-dose nevirapine (**sdNVP**) given to the mother at onset of labor and to the infant after birth has been the most common PMTCT regimen used in resource-limited settings and has been part of the World Health Organization (**WHO**) PMTCT recommendations since 2001 (World-Health-Organization 2001). Significant HIV-DR occurs in mothers and in HIV-infected infants treated with sdNVP (Eshleman et al. 2001, 2005a, b, c, 2006; Flys et al. 2005, 2006, 2007a; Palmer et al. 2006; Dross et al. 2010; Micek et al. 2010; Farr et al. 2010) and is associated with an increased risk of virologic failure (defined as non-suppression of HIV replication) when women or infants are treated with non-nucleoside reverse transcriptase (**NNRTI**)-based ART (Jourdain et al. 2004, 2010; Lockman et al. 2007, 2010; Coovadia et al. 2010; Palumbo et al. 2010; Moorthy et al. 2011). However, the effects of drug resistance from sdNVP appears to fade over time (Eshleman et al. 2001; Palmer et al. 2006; Johnson et al. 2005; Loubser et al. 2006; Flys et al. 2007b), with no significant increased risk of virologic failure detected in mothers when NNRTI-based ART is initiated >12–18 months after exposure to sdNVP (Lockman et al. 2010).

NVP resistance is particularly concerning because it confers cross-resistance to efavirenz (**EFV**), another NNRTI, and either NVP or EFV is part of the first-line regimens used globally to treat HIV-infected adults, children, and some infants. The detrimental effect of NVP resistance on the outcome of NNRTI-based ART led the WHO to modify the recommended ARV for PMTCT in 2010 (World-Health-Organization 2010). Currently, sdNVP is only recommended when ART is not

prescribed and zidovudine (**ZDV** or **AZT**) is given to the pregnant mother for <4 weeks prior to the onset of labor. The challenges to safe and effective PMTCT include the lack of human capacity, inadequate infrastructure, and insufficient financial backing needed to enact the WHO guidelines. Additionally, under the current WHO guidelines (World-Health-Organization 2010; Moorthy et al. 2009; Fogel et al. 2011a), development of ARV resistance in breastfeeding infants occurs with both recommended regimens: NVP monotherapy prophylaxis of nursing infants (World-Health-Organization 2010; Moorthy et al. 2009; Fogel et al. 2011a) and ART use by breastfeeding mothers (Zeh et al. 2011; Fogel et al. 2011b).

Mechanisms Leading to the Transmission, Selection, and Decay of HIV-DR from sdNVP

Dynamics of HIV-DR

The selection of HIV drug resistance in PMTCT is influenced by the pharmacokinetic properties of the antiretrovirals administered and the genetic barrier posed by a single or a combination of drugs. NVP has a long half-life (~60 h in adults and ~45 h in infants) (Mirochnick et al. 2001; Cressey et al. 2005) and a low "genetic barrier to resistance," with any of several single-base-pair mutations conferring high-level resistance (Richman et al. 1994; Kantor et al. 2001). Lamivudine (**3TC**) and ZDV, nucleoside reverse transcriptase inhibitors (**NRTI**) commonly used in both PMTCT and in first-line ART regimens, have shorter half-lives. 3TC has a low genetic barrier to resistance, with a single-base change conferring high-level resistance, while two or three mutations are required to confer high-level resistance to ZDV (Boucher et al. 1992). Single-base changes conferring resistance to NNRTI and 3TC are estimated to occur on a daily basis due to properties of the viral DNA polymerase (Coffin 1995). However, the likelihood of multiple resistance mutations occurring spontaneously in one virion prior to ARV treatment is exceedingly low (Perelson et al. 1997); therefore, resistance to ZDV and ART occurs by the sequential accumulation of mutations while receiving therapy if there is HIV replication. The selection of ZDV resistance was uncommon when administered for ~10 weeks to mothers for PMTCT (Jourdain et al. 2004; Shapiro et al. 2010), consistent with its moderate genetic barrier to selection of resistance. Based on these findings, ZDV monotherapy is one of two WHO-recommended regimens for pregnant women with CD4 cells counts >350/uL for PMTCT (World-Health-Organization 2010). However, as discussed below, mutants are selected in pregnant women given ZDV for longer periods of time. Because NVP remains detectible in maternal plasma for 1–3 weeks after sdNVP, the administration of a short-course "tail" of ZDV +/− 3TC postpartum decreases viral replication and increases the genetic barrier to resistance, which limits the selection of NVP resistance. Therefore, ARV tails are recommended

to reduce resistance when sdNVP is used for PMTCT (World-Health-Organization 2006, 2010).

Individuals administered with mono- or dual-ARV regimens and those who experience virologic failure during ART often experience selection of mutants. These drug-resistant variants infect cells, including populations of long-lived cells that constitute the latent reservoir of viruses. Generally, virions with selected HIV-DR have decreased replication capacity compared to wild-type viruses, and in the absence of selective ARV pressure, the mutants decrease in frequency over time, largely replaced by wild-type virus (Deeks et al. 2001). However, if replication-competent mutants persist in the viral reservoir, these variants rapidly reemerge when an ARV is recycled or an ARV with cross-resistance is administered. The reservoir of mutants selected by sdNVP persist for less time compared to mutants selected by failing ART, presumably because a longer period of selection archives a greater number of viable ARV-resistant viruses in long-lived cells. In mothers and infected infants, NVP-resistant viruses in plasma decay to undetectable levels within several months by consensus sequencing, and generally within 6–12 months using more sensitive assays (Eshleman et al. 2001; Palmer et al. 2006; Johnson et al. 2005; Loubser et al. 2006; Flys et al. 2007b; Wagner et al. 2010). When NVP-based ART is started after a sufficient interval (~12 months), resistant variants generally do not reemerge (Lockman et al. 2007, 2010; Palumbo et al. 2010). However, a threshold concentration of NVP resistance or a time interval between sdNVP and initiation of NVP-based ART that can predict virologic failure of NVP-based ART has not been definitively established.

In contrast to HIV-DR selected by sdNVP, persons directly infected with a drug-resistant virus, commonly called transmitted drug-resistant HIV (**TDR**), have HIV-DR that persists over many years, even without selective drug pressure (Little et al. 1999, 2008). This is likely because acute infection corresponds to the time that viruses infect a large number of susceptible cells, and a proportion of these become quiescent and part of the latent HIV reservoir (Schacker et al. 2000). Also, TDR variants generally have a high replication capacity. In Africa, variable rates of TDR have been observed, ranging from 3 % to 19 %, with significant increases in resistance to NNRTI medications occurring progressively since access to ART has improved (Price et al. 2011; Bennett et al. 2008a, b; Jordan et al. 2008; Maphalala et al. 2008; Myatt and Bennett 2008; Nguyen et al. 2008; Pillay et al. 2008; Shafer et al. 2008; Somi et al. 2008; Gupta et al. 2012). In resource-limited communities, screening tests for drug resistance are not used prior to administering ARV for PMTCT or when initiating ART, as is routine in resource-rich communities. Thus, increasing rates of TDR are of concern for PMTCT as well as general ART programs. The WHO recommends the surveillance of ARV-naïve individuals to monitor rates of TDR and at specific thresholds to strengthen voluntary counseling and testing and adherence programs and address weaknesses in the supply of ARV and other identified programmatic insufficiencies (Bennett et al. 2008a, b).

History of Antiretrovirals Used for PMTCT in Resource-Rich Communities and Associated Resistance

Zidovudine Monotherapy

In 1994, ZDV monotherapy was shown to reduce MTCT by 67.5 % in a randomized placebo-controlled trial conducted in infants given formula in place of breast milk (Pediatric Clinical Trials Group Protocol [PACTG 076]) (Connor and Mofenson 1995). ZDV and other mono- and dual-ARV therapies generally do not suppress viral replication to undetectable levels and therefore can select drug-resistant forms. In spite of a moderate genetic barrier to resistance, ZDV resistance has been detected during pregnancy (Eastman et al. 1998; Frenkel et al. 2006) and has been transmitted to infants (Frenkel et al. 1995).

In an observational study of US pregnant women receiving ZDV for their medical care or PMTCT, ZDV resistance was detected in the blood of 16 of 48 (33 %) women using a sensitive oligonucleotide ligation assay (**OLA**) (Frenkel et al. 2006). ZDV resistance was not associated with low maternal CD4, high HIV-1 RNA levels in the plasma, or with the duration of antiretroviral therapy. The median length of ZDV treatment in women with and without ZDV mutants in the blood was 21.4 and 21.0 weeks, respectively. In contrast, larger studies conducted in mid- or low-resource communities with shorter durations of ZDV, and use of less sensitive assays, have reported lower rates of ZDV resistance (Jourdain et al. 2004; Shapiro et al. 2006).

More recently, among Tanzanian women who received antenatal ZDV (median duration 53 days), sdNVP, and ZDV/3TC postpartum for 1 week, at time of delivery, ZDV resistance was detected in 10 % using a sensitive allele-specific PCR (**AS-PCR**) (Hauser et al. 2012). Over a 16-week follow-up period, however, ZDV resistance was detected in 22 %, suggesting that postpartum ARV tails administered following sdNVP to diminish the emergence of NVP-resistant virus may continue to exert selective pressure for ZDV resistance mutations.

The observations of resistance in a substantial proportion of US (Frenkel et al. 2006) and Tanzanian (Hauser et al. 2012) women with >350 CD4 cells/uL after ZDV argue for studies to evaluate the effect of ZDV monotherapy on subsequent maternal ART, as mutations selected by ZDV monotherapy may be archived in long-lived cells, potentially diminishing the efficacy of subsequent maternal ART containing ZDV or other NRTI.

Combination Antiretroviral Therapy (ART)

In 1996, combination ART revolutionized the treatment of HIV infection (Hammer et al. 1996). Among women taking ART to treat their HIV infection, a lower rate of MTCT was observed compared to those taking ZDV monotherapy (Cooper et al. 2002). Use of ART was associated with diminished rates of transmission to

non-breastfeeding infants (1–2 %) in North America and Europe (Lindegren et al. 1999; World-Health-Organization 2009).

ART during gestation became standard of care for PMTCT in resource-rich settings. HIV-infected pregnant women with high CD4 cell counts often elect to stop ART postpartum. When viral replication is suppressed by a ritonavir-boosted protease inhibitor (PI)-based ART regimen, HIV-DR mutations generally are not selected during the elimination of the ARV. However, women treated with less potent regimens, for example, with nelfinavir-based ART, or who have been nonadherent can select HIV-DR during or upon the cessation of ART (Ellis et al. 2011). While studies that use consensus sequencing show overall low rates of resistance following either NNRTI- or PI-based ART for PMTCT (Souda et al. 2013), those using more sensitive assays, such as OLA or AS-PCR, reveal selection of resistant variants below the limit of detection of consensus sequencing (Lehman et al. 2009; Perez et al. 2008; Paredes et al. 2010). The clinical importance of low-frequency variants has not been fully investigated, though there is growing evidence that they may negatively impact the efficacy of later ART (Metzner et al. 2009; Li et al. 2011; Stekler et al. 2011).

Several studies have compared rates of resistance that develop following ART versus mono- or dual-drug combinations for PMTCT. In Kenya ART (ZDV/3TC/NVP) started at 34 weeks' gestation and continued through 6 months of breastfeeding was associated with lower rates of resistance compared to antenatal ZDV plus sdNVP at 3 months post-ARV by AS-PCR (18 % vs. 75 %, $p = 0.007$) (Lehman et al. 2009). In the Kesho Bora study, ART (ZDV/3TC/LVP/rt) started at 34 weeks' gestation and continued through breastfeeding selected less resistance compared to antenatal ZDV plus sdNVP (1.4 % vs. 24 %) (The Kesho Bora Group 2012). Importantly, however, none of the subjects in the ZDV/sdNVP arm who received postpartum ARV tails developed resistance mutations.

History of Antiretrovirals Used for PMTCT in Resource-Limited Communities and Associated Resistance

The Rise of Single-Dose Nevirapine (sdNVP)

In contrast to resource-rich communities, women in resource-limited communities had virtually no PMTCT services in the 1990s. Insufficient infrastructure and a lack of funds and political will resulted in millions of HIV-infected children, including over one million infants in sub-Saharan Africa – the region with the greatest number of HIV-infected women of childbearing age.

In response to these challenges, shortened, simplified, and inexpensive PMTCT regimens were explored. In 1999, the HIVNET 012 trial demonstrated that one oral dose of nevirapine given to mothers during labor and one dose administered to breastfeeding infants shortly after birth reduced MTCT by 50 % at 6 weeks of age and by 38 % at 18 months of age (Jackson et al. 2003). sdNVP does not require refrigeration and thus can be taken by a woman at home when she begins labor.

Given its feasibility and low cost, sdNVP was included in WHO recommendations beginning in 2001 (World-Health-Organization 2001) and quickly became the most common regimen for PMTCT in resource-limited settings. While access to PMTCT programs has improved in recent years, many women receive suboptimal care. A recent random sampling of African sites with PMTCT services found that only 51 % of HIV-exposed infants received NVP (Stringer et al. 2010a).

Unfortunately, sdNVP selects resistant viruses at high rates in both mothers and infected infants: 10–75 % of mothers (Flys et al. 2005; Eshleman et al. 2005a, d; Micek et al. 2010; Johnson et al. 2005; Arrive et al. 2007) and 4–87 % of children (Eshleman et al. 2005b; Micek et al. 2010; Lockman 2008; Lockman and McIntyre 2007; Martinson et al. 2007a). The prevalence of NVP resistance after sdNVP varies across HIV subtypes, with higher rates in subtypes C and D compared to subtype A (Eshleman et al. 2005a; Flys et al. 2006), and detection is increased when evaluated using sensitive assays. A meta-analysis reported a pooled prevalence estimate of 35.7 % among women exposed to sdNVP by consensus sequencing and 62.4 % by sensitive research assays (Arrive et al. 2007).

ARV "Tails" to Prevent Selection of NVP-Resistant HIV

To reduce resistance following sdNVP, a strategy that gives short courses of postpartum ARV, so-called "tails," to suppress viral replication and increase the genetic barrier to resistance has been investigated across many studies. These ARV tails vary in costs and feasibility, but all effectively reduce selection of NVP resistance. Postpartum tails were incorporated into WHO recommendations in 2006 (World-Health-Organization 2006) and continue to be recommended for women receiving sdNVP (World-Health-Organization 2010).

Among women receiving sdNVP, the TOPS study found that 4 or 7 days of postpartum ZDV/3TC significantly diminished NVP resistance compared to untreated controls as detected by consensus sequencing: 11.7 %, 7.3 %, and 59.2 %, respectively ($p < 0.0001$) (McIntyre et al. 2009). Similarly, the BAN trial found that a 7-day tail of ZDV/3TC significantly reduced NVP resistance in mothers assessed by consensus sequencing and AS-PCR (10 % vs. 64 %, $p < 0.0001$) (Farr et al. 2010). More recently, a longer, 21-day ARV tail of either ZDV/3TC, TDF/FTC, or lopinavir/rt (**LPV/rt**) reduced resistance more than a 7-day tail of the same medications by AS-PCR (5 % vs. 18 %, $p = 0.019$), though rates were similar by consensus sequencing (McMahon et al. 2013).

Among mothers receiving antenatal ZDV +/−3TC and sdNVP in labor, the administration of ZDV/3TC for 3-days postpartum decreased NVP resistance compared to historical controls (Chaix et al. 2006). A randomized study administering a single dose of TDF/FTC during labor markedly reduced NVP resistance compared to controls by consensus sequencing (Chi et al. 2007) and by OLA (Chi et al. 2009). A two-dose TDF/FTC tail was similarly effective (Arrive et al. 2009).

In Thailand, where antenatal ZDV was standard of care and hepatitis B is prevalent, tails composed of ZDV/didanosine (**ddI**) +/− lopinavir/rt were

investigated because the cessation of 3TC allows HBV to replicate, which can lead to a clinical flare of hepatitis B (Altfeld et al. 1998; Bessesen et al. 1999). One study detected no NVP resistance among women receiving a 1-month tail of ddI/ZDV, which was significantly less compared to historical controls (0 % vs. 10.4 %, respectively, by consensus sequencing and 1.8 % vs. 18.9 % by OLA, $p = <0.001$ for both analyses) (Lallemant and Jourdain 2010). A second study of tails comprised of 1 week of ddI/ZDV/LPV/rt, 1 month of ddI/ZDV, and 1 month of ddI/ZDV/LPV/rt similarly detected no NVP resistance in any group by consensus sequencing and 1.8 %, 7.1 %, and 5.3 % by OLA, respectively, which compared favorably to untreated historical controls with 13.4 % by sequencing and 29.4 % by OLA ($p < 0.001$ for each study arm vs. comparison group) (Van Dyke et al. 2012).

Reuse of sdNVP in Subsequent Pregnancies: HIV Transmission and Maternal/Infant Resistance Risks

Studies of the reuse of sdNVP with subsequent pregnancies have generally shown similar efficacies for PMTCT and similar rates of NVP resistance. Following reuse of sdNVP at median intervals of 21–32 months (Martinson et al. 2007b, 2009; McConnell et al. 2007; Walter et al. 2008), the proportion with NVP resistance mutations after the first compared to subsequent sdNVP did not differ at 6 weeks (Kuhn et al. 2006; Flys et al. 2008), 6 months (Flys et al. 2008), and 12 months (Flys et al. 2008) by consensus sequencing (Kuhn et al. 2006; Flys et al. 2008) or by more sensitive assays (Flys et al. 2008). Similarly, in infected infants the proportion of NVP resistance mutations was similar at 6 weeks (Flys et al. 2008). These data on reuse of sdNVP in subsequent pregnancies indicate that NVP resistance does not reemerge in the mother postpartum at a rate that increases the transmission of drug-resistant HIV via breastfeeding and furthermore suggests that the reservoir of NNRTI mutants following sdNVP is relatively short-lived.

Effects of sdNVP on Efficacy of Subsequent NNRTI-Based ART in Women

Evidence of the negative impact of sdNVP on subsequent NNRTI-based ART began to accumulate when postpartum women in resource-limited settings gained access to ART in the mid-2000s. A subset of Thai women who received antenatal ZDV and were randomized to receive sdNVP or placebo during labor (Lallemant et al. 2004) were first reported to have increased rates of virologic failure when subsequently administered with NVP-based ART to treat their HIV disease (Jourdain et al. 2004). Fewer sdNVP-exposed women had viral suppression (HIV-1 RNA levels <50 copies/mL) compared to placebo-treated women (49 % vs. 68 %, respectively, $p = 0.03$). Consensus sequencing of maternal virus 1–2 months postpartum was not associated with virologic failure; however, detection of NVP resistance in DNA

isolated from blood cells collected just prior to the initiation of ART by OLA was linked with failure (Jourdain et al. 2010).

The Mashi trial, among women with or without a history of sdNVP who subsequently took NNRTI-based ART, observed increased rates of virologic failure (18 % vs. 5 %, $p = 0.002$) (Lockman et al. 2007). sdNVP was associated with virologic failure among women who initiated ART within 6 months of sdNVP (42 % vs. 0 %, $p < 0.001$), but not among women who initiated NVP-ART 6 months or more after sdNVP exposure (12 % vs. 7.8 %, $p = 0.39$). These results suggest that drug resistance selected by sdNVP decays over time, which is consistent with multiple studies showing that NVP resistance mutations fade from detection over time (Eshleman et al. 2001; Palmer et al. 2006; Johnson et al. 2005; Loubser et al. 2006; Flys et al. 2007b; Wagner et al. 2010). In pre-ART specimens, NVP resistance mutations K103N and Y181C were detected by AS-PCR in 65 % of subjects at concentrations of 0.1–4.1 % (Rowley et al. 2010). NVP resistance of >0.19 % was associated with virologic failure (86 % of 7 subjects with failure vs. 32 % of 19 without failure; OR = 13, 95 % CI 1.27–133); however, the input of viral templates for PCR amplification was not reported, which likely influenced the nominal concentration determined to be clinically predictive.

The decay of mutants selected by sdNVP to clinically insignificant concentrations has been confirmed in multiple studies. In an observational study in South Africa, an interval of 18–36 months between sdNVP and the initiation of NNRTI-based ART revealed rates of suppression of viral replication comparable to women not exposed to sdNVP (Coovadia et al. 2009). Similarly, long intervals were associated with increased viral suppression in Zambian women (Kuhn et al. 2009). Treatment failure (defined as virologic failure, death, loss to follow-up, or discontinuation of NNRTI-based ART for any reason before 48 weeks) was greater among women who initiated NVP-based ART at an interval of <6 months (41 %, $p = 0.001$) or 7–12 months (37 %, $p = 0.04$), but not if >12 months compared to sdNVP-unexposed women in Zambia, Kenya, and Thailand (Stringer et al. 2010b).

The multi-country OCTANE trial demonstrated the superiority of LPV/rt- compared to NVP-based ART among sdNVP-exposed women but not among NVP-unexposed women (Lockman et al. 2010). Again, the effect of sdNVP appeared to decrease as the interval between sdNVP exposure and ART initiation increased. NVP resistance was detected prior to ART by consensus sequencing in 15 (13 %) women in the NVP group and 18 (15 %) women in the LPV/rt group. Among those with NVP resistance, 73 % in the NVP group compared to only 6 % in the LPV/rt group died or had virologic failure ($p = 0.006$). Analysis of the OCTANE participants revealed that at the time of starting ART with an NNRTI regimen, those women with either K103N or Y181C at a concentration >1 % of the plasma HIV population by AS-PCR testing were at increased risk of virologic failure or death (HR 2.93, 95 % CI 1.27–6.75) (Boltz et al. 2011). This finding supports the assertion that minority variant resistant virus can impact the response to ART and highlights the potential impact of sensitive resistance assays for women who used sdNVP.

Importantly, use of two inexpensive HIV-DR assays, AS-PCR in the OCTANE (Boltz et al. 2011) and OLA in the PHPT-2 trials (Jourdain et al. 2010), detected

NVP resistance associated with virologic failure. These data suggest that testing for HIV-DR prior to ART initiation by point mutation assays may be useful in women after sdNVP to guide the choice of an appropriate ART regimen. While ARV testing is available to a limited degree for clinical care and research studies in Africa and Asia, studies are needed to clarify the clinical relevance of mutations at single versus multiple NVP resistance codons or in virus in plasma versus cells and whether there are specific threshold concentrations of mutants that predict the outcome of NNRTI-based ART.

Effects of sdNVP on HIV-DR in Infants and Subsequent NNRTI-Based ART

In HIV-infected infants exposed to sdNVP, the timing of perinatal HIV acquisition determines the size of the NVP-resistant viral reservoir and whether it persists. A study of Mozambican infants revealed three distinct dynamic patterns of NVP resistance (Micek et al. 2010). Most frequent were infants whose HIV infection had been established in utero prior to administration of sdNVP. Among these infants, mutants were selected to concentrations approaching 100 %, followed by rapid decay by 4–6 months of age. Among a smaller group of infants, who appeared in the midst of acute HIV infection at the time of birth, NVP resistance was selected when their viral population was large enough to contain spontaneously generated mutants prior to administration of sdNVP. These mutants expanded rapidly to high concentrations and apparently entered long-lived cells, as these mutants persisted over time. Among infants infected peri- or postpartum, approximately 30 % acquired pure populations of NVP-resistant virus, which also persisted over time. Given that NVP resistance was not detected in maternal blood or milk at delivery, NVP-resistant variants were most likely transmitted through breastfeeding.

Not surprisingly, following prophylaxis with sdNVP, the treatment of infants with NVP-based ART showed diminished rates of virologic efficacy in the Mashi trial (76.9 % vs. 9.1 %, respectively, $p < 0.001$) (Lockman et al. 2007). NVP resistance was evaluated in specimens collected from 33 infants prior to ART initiation (MacLeod et al. 2010). Consensus sequencing identified NVP resistance in only 11 % infants compared to 39 % infants by AS-PCR. Testing of pre-ART blood plasma and cell samples at a median age of 6.5 months detected NVP resistance in 9 of 16 infants experiencing virologic failure compared to 4 of 17 infants without virologic failure (risk ratio 2.4, CI 0.94–7.8, $p = 0.08$).

A randomized trial (P1060) of NVP- versus LPV/rt-based ART in infants following sdNVP was conducted across multiple African countries (Palumbo et al. 2010). After 24 weeks of ART, treatment failure was greater among infants randomized to NVP- compared to LPV/rt-based ART (39.6 % vs. 21.7 %, respectively, $p = 0.02$). Among those randomized to NVP-ART, the detection of NVP resistance mutations in the pre-ART specimen by consensus sequencing was predictive of treatment failure (83 % with vs. 36 % without detection of NVP resistance failed, $p = 0.02$).

One study of sdNVP-treated infants aimed to capitalize on the decay of NVP resistance by using a novel ART strategy (NEVEREST Study) (Coovadia et al. 2010). All infants initiated LPV/rt-based ART, and those with suppression of viral replication to $\leq$400 copies/mL for $\geq$3 months (median = 10) were then randomized to either continue LPV/rt- or switch to NVP-based ART. The switch occurred at a median of 19 months of age (range 9–43), which in most women given sdNVP is a sufficiently long interval from treatment with sdNVP for NVP resistance to decay to clinically insignificant concentrations (Lockman et al. 2007). However, virologic failure (plasma HIV RNA >1,000 copies/mL) was greater in infants who switched compared to those who continued PI-based ART (20 % vs. 2 %, respectively, $p < 0.001$). Children switched to NVP-ART who had NNRTI mutations detectible by consensus sequencing at switch experienced viral failure at a greater rate compared to those without mutations (10/19 vs. 7/51; $p < 0.02$) (Kuhn et al. 2012).

It is possible that this significant difference in failure rates among NEVEREST infants who switched to NVP-based ART was due to fundamental differences in the reservoir of NVP-resistant virus of infants compared to adult women. While most adult women have rapid decay of NVP resistance, as previously mentioned, a large and persistent reservoir of NVP-resistant viruses is established in a subset of infants following sdNVP, including infants with acute infection at birth (Micek et al. 2010) and who acquired HIV via breastfeeding (Dross et al. 2010). Thus, the NEVEREST infants with virologic failure after the switch to NVP-based ART likely include infants with long-lived reservoirs of ARV-resistant HIV (Little et al. 2008). Indeed, pre-ART consensus (Kuhn et al. 2012) and pyrosequencing (Moorthy et al. 2009) detected high concentrations of NVP mutants in those with virologic failure. The findings that NVP resistance does not predictably decay in infants and that testing of infants for NVP resistance predicts virologic response to NVP-based ART (Palumbo et al. 2010; Moorthy et al. 2011) suggest that in low-resource communities studies are needed to evaluate the feasibility and cost-effectiveness of pre-ART drug resistance testing compared to alternative strategies, such as viral load monitoring during ART.

HIV-DR Associated with Maternal ART or Infant NVP Prophylaxis During Breastfeeding

In resource-limited settings, breastfeeding offers infants protection from disease and death compared to artificial infant formulas, which require mixing with potentially unsanitary water (Nduati et al. 2000; Arvelo et al. 2010; Kunz et al. 2009; Kuhn et al. 2008). Strategies to protect infants from HIV infection while providing them with the beneficial effects of breast milk include two approaches that have been examined in multiple studies: first, administration of ARV prophylaxis directly to infants and, second, maternal ART to reduce HIV load in breast milk. NVP prophylaxis of various durations has been examined in breastfeeding infants (Petra-Study-Team 2002; Moodley et al. 2003; SWEN-Study-Team 2008; Kumwenda et al. 2008;

Coovadia et al. 2011). Across these studies the efficacy of prophylaxis appears to increase with the coverage of the breastfeeding period. Maternal ART was shown to reduce rates of MTCT compared to short-course ZDV plus sdNVP during pregnancy by the Kesho Bora study (de Vincenzi 2011) and during breastfeeding by the Kesho Bora and BAN studies (Chasela et al. 2010).

ARV-resistant virus can be selected by ARV given to the infants as prophylaxis or ARV transferred to the infant in breast milk. In addition, primary infection with drug-resistant virus can occur in the postnatal period by exposure to ARV-resistant virus in breast milk (Dross et al. 2010; Lidstrom et al. 2010). Following sdNVP, rates of NVP resistance in breast milk range from 40 % to 65 %, persist for up to 8 months, and can differ from concurrent plasma viruses (Lee et al. 2005; Kassaye et al. 2007; Hudelson et al. 2010; Gantt et al. 2012).

In the Stopping Infection from Mother-to-child via Breastfeeding in Africa (SIMBA) study, infants were given either daily lamivudine or nevirapine after birth until 1 month after cessation of breastfeeding, while their mothers had received zidovudine and didanosine from 36 weeks' gestation until 1 week postpartum (Giuliano et al. 2006). The HIV-infected infants who received prophylaxis prior to confirmation of diagnosis frequently selected drug-resistant variants, including 12/13 (92.3 %) of those on NVP for a median of 45 days and 9/13 (69.2 %) on 3TC for a median of 14 days.

Multiple studies of daily NVP prophylaxis administered to infants include a randomized controlled trial of 6 weeks of extended-dose nevirapine (SWEN) (SWEN-Study-Team 2008). A higher prevalence of NVP resistance was detected in infants randomized to continue NVP compared to those who only received sdNVP by population sequencing (92 % of 12 % vs. 38 % of 29 infants, $p = 0.002$), which persisted as a majority population through 6 months (Moorthy et al. 2009). In the Post Exposure Prophylaxis of Infants (PEPI) trial in Malawi, infants who had received sdNVP and a week of ZDV after birth were randomized to no additional intervention, daily NVP for 14 weeks, or daily NVP/ZDV for 14 weeks (Kumwenda et al. 2008). While the continuation of NVP and NVP/ZDV conferred similar rates of protection from MTCT, infants with in utero HIV infection had lower rates of NVP resistance if assigned to NVP/ZDV compared to NVP (54.5 % vs. 85.7 %, $p = 0.007$) (Lidstrom et al. 2010). However, the protective effect of ZDV on NVP resistance was lost if infants continued to take ARV beyond 6 weeks of age (83.3 % vs. 87.5 %, $p = 1.0$). In a similar randomized trial (HPTN/IMPAACT 046) of NVP prophylaxis for 6 weeks versus 6 months, NVP was selected for resistant variants in infants infected after 6 weeks of age (75 % in the NVP arm vs. 6 % in the placebo arm at 6 months of age; $p = 0.001$) (Fogel et al. 2013).

ARV concentrations in breast milk of mothers receiving ART appear variable, in part due to sparse sampling. 3TC is concentrated in breast milk with levels 3–5 times higher than in maternal plasma; ZDV concentrations are similar or slightly lower compared to maternal plasma; NVP concentrations are 60–75 % that of plasma; and protease inhibitors penetrate poorly into breast milk (Mirochnick et al. 2009). Studies evaluating ARV levels in the plasma of breastfeeding infants have demonstrated

NVP and 3TC at biologically active levels, while ZDV is typically at lower concentrations (Shapiro et al. 2005), (Mirochnick et al. 2009).

Several studies have evaluated HIV-DR in the HIV-infected infants of mothers receiving ART. HIV-infected PEPI infants whose mothers initiated NVP-based ART during their first year of life had HIV-DR to multiple drugs detected in 30 % ($n = 37$) in association with earlier initiation of postpartum maternal ART and exclusive breastfeeding at initiation of ART (Fogel et al. 2011b). Comparisons of maternal and infant patterns of HIV-DR mutations suggested MTCT of virus resistant to multiple ARV in a subset of infants.

The KiBS study assessed the safety and efficacy of maternal ART (ZDV/3TC plus either NVP or nelfinavir) beginning at 34–36 weeks' gestation and continuing through to 6 months of breastfeeding (Zeh et al. 2011). The cumulative HIV transmission rates assessed at infants' birth, 6 weeks, and 6, 12, and 24 months of age were 2.5 %, 4.2 %, 5.0 %, 5.7 %, and 7.0 %, respectively. A secondary analysis of HIV-DR in 32 HIV-infected infants by consensus sequencing found that the rate of HIV-DR increased over time: 30 % of infants at 6 weeks, 63 % at 14 weeks, and 67 % at 6 months had HIV-DR (Zeh et al. 2011). In contrast to the infants in the PEPI study, only one mother-infant pair had similar patterns of HIV-DR, suggesting that HIV-DR was principally the result of selective pressure from ARV in mothers' breast milk rather than transmitted directly from the mother. Importantly, HIV-infected infants in these studies were not started immediately on LPV/rt-based ART as currently recommended, which would likely preclude the selection of HIV-DR variants (but may incur additional ARV toxicities).

To date, the efficacy of concomitant maternal ART and infant NVP prophylaxis during breastfeeding for PMTCT has not been systematically studied. However, the mothers of a subset of infants participating in HPTN/IMPAACT 046 of NVP prophylaxis initiated ART, thus exposing the infants to both NVP prophylaxis and ARV via maternal milk (Coovadia et al. 2011). All four infants whose mothers started NNRTI-based ART developed NVP resistance, while only 14 % of the infants whose mothers did not start ART developed resistance (Fogel et al. 2011b). Cost and potential NVP toxicity to the infant from the combined prophylactic NVP plus ARV ingested in maternal breast milk may limit this approach.

Modifications of WHO Recommendations for PMTCT to Reduce Use of sdNVP and Increase Maternal ART

The results of the multiple studies described above indicate that the use of sdNVP for PMTCT can negatively impact the outcome of subsequent NNRTI-based ART for mothers and infected infants. As the time interval between sdNVP and the initiation of maternal or infant ART increases, the effect of ARV resistance on treatment outcome diminishes, presumably due to decay of NVP-resistant viruses. However, a threshold time interval, after which NVP resistance is no longer clinically significant, has not been clearly defined, particularly in infected infants. Given that maternal baseline CD4 and viral load (Micek et al. 2012) and HIV-1 subtype affect

selection of NVP resistance, this time interval may vary across individuals and geographic regions. Furthermore, in a subset of infected infants, HIV-resistant mutants do not appear to decay but rather persists in the viral reservoir (Micek et al. 2010).

These issues, combined with the growing infrastructure to administer ART to adults, bring into question the balance between providing simplified regimens to reach the greatest number of women and providing more effective therapies that minimize both MTCT and HIV-DR. In response, the WHO PMTCT recommendations changed in two stages. In 2006, ZDV in late gestation was added to sdNVP to further reduce MTCT and postpartum combination ARV tails were recommended to reduce selection of NVP resistance (World-Health-Organization 2006). In 2010, in response to the growing evidence of the negative effect of NVP resistance on the efficacy of maternal and infant ART, the WHO recommended ART or ZDV monotherapy for women with >350 CD4/uL, with sdNVP given only if mothers received fewer than 4 weeks of ART or ZDV (World-Health-Organization 2010). The rationale for continued use of ZDV monotherapy in women with high CD4 cell counts included the high efficacy of ZDV monotherapy in PMTCT (Shapiro et al. 2006), lower cost of ZDV compared to ART, avoidance of the rare but fatal hepatic toxicity associated with NVP-based ART in women with CD4 > 250 cells/uL, and past studies showing that ZDV resistance is rarely selected in women with relatively high CD4 counts who took ~10 weeks of ZDV (Jourdain et al. 2004; Shapiro et al. 2010). As infrastructure has improved, some low-resource communities have adopted ART for PMTCT regardless of a woman's immune (CD4) status (Chimbwandira et al. 2013). However, given the poor access to care in some, especially rural communities (Stringer et al. 2010a), the use of sdNVP may continue for some women.

A WHO update in 2012 discusses the use of ART for PMTCT with continuation of lifelong treatment of the woman, regardless of her CD4 count or clinical status. This strategy, termed "Option B+," was adopted in Malawi and was associated with an increase in PMTCT administration due to the integration of antenatal and HIV care (Chimbwandira et al. 2013). Option B or B+ is gaining support in other resource-poor nations. Some experts express concerns about the higher cost of ART, potential adverse reactions, and lapses in adherence that could select HIV-DR and oppose the blanket recommendation of B+ (Coutsoudis et al. 2013a, b). Few studies evaluate ARV resistance in association with long-term ART in communities without routine testing for HIV-DR.

Conclusions: Future Directions

Since ~1996, ART has nearly eliminated MTCT in North America and Europe. During the past decade, multiple studies have shown that simplified mono- and dual-ARV strategies generally are less effective for PMTCT or have a negative impact on EFV- or NVP-based ART subsequently administered to treat HIV disease in mothers and infants. Given these findings and the increasing access to ART around the world,

a number of research and policy experts promote universal ART for pregnant and breastfeeding HIV-infected women to eliminate MTCT (Becquet et al. 2010). Others question the ethics of continuing to conduct research using suboptimal PMTCT regimens given the superiority of ART regimens over suboptimal short-course mono-, dual-, or triple-ARV regimens (Schouten et al. 2013; Goosby 2013; Ammann 2009). While the WHO recommendations have shown a continuing trend away from short-course mono- or dual-ARV, with the 2010 WHO Guidelines avoiding the use of sdNVP when possible (World-Health-Organization 2010), new 2013 Guidelines will likely recommend ART exclusively.

While modeling suggested that HIV-DR prevalence would remain low in Africa (Blower et al. 2001), recent surveillance detected substantial rates of TDR in some communities (Price et al. 2011; Gupta et al. 2012). Continued increases in TDR could undermine PMTCT programs (Geretti 2007; Shet et al. 2006). Expansion of PMTCT could increase the prevalence of HIV-DR viruses and further fuel TDR. Testing for NNRTI resistance before the initiation of ART in communities with high rates of TDR could allow clinicians to select PI-based regimens for affected individuals, which modeling suggests is cost effective in the United States (Sax et al. 2005). Pre-ART testing for HIV-DR should lead to better rates of suppression of viral replication in treated individuals and help prevent the spread of HIV infection by diminishing MTCT and reducing heterosexual transmission from women to their serodiscordant sexual partners (Cohen et al. 2011). Research directed at optimization of operational aspects and cost-effectiveness of testing for HIV-DR prior to ART is needed as ART services expand to allow recognition of virologic failure and better tailoring of post-failure treatment.

The evolution of PMTCT interventions in resource-limited communities during the past decade demonstrates the challenges faced by public health officials who worked to balance the limited infrastructure and resources with effective prevention of MTCT and HIV-DR. As access to ART continues to increase, there is potential to further decrease MTCT. Importantly, both monitoring of HIV-DR and programmatic investments to limit the spread of ARV-resistant variants will be needed to maximize the gains antiretrovirals can deliver to PMTCT.

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HIV Protease Inhibitor Resistance

Annemarie M. J. Wensing, Axel Fun, and Monique Nijhuis

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Abstract
HIV protease is pivotal in the viral replication cycle and directs the formation of mature infectious virus particles. The development of highly specific HIV protease inhibitors (PIs), based on thorough understanding of the structure of HIV protease and its substrate, serves as a prime example of structure-based drug design. The introduction of first-generation PIs marked the start of combination

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antiretroviral therapy. However, low bioavailability, high pill burden, and toxicity ultimately reduced adherence and limited long-term viral inhibition. Therapy failure was often associated with multiple protease inhibitor resistance mutations, both in the viral protease and its substrate (HIV gag protein), displaying a broad spectrum of resistance mechanisms. Unfortunately, selection of protease inhibitor resistance mutations often resulted in cross-resistance to other PIs.

Therefore, second-generation approaches were imperative. Coadministration of a cytochrome P-450 3A4 inhibitor greatly improved the plasma concentration of PIs in the patient. A second advance was the development of PIs that were efficacious against first-generation PI-resistant HIV. Both approaches increased the number of protease mutations required by the virus to develop clinically relevant resistance, thereby raising the genetic barrier towards PI resistance. These improvements greatly contributed to the success of PI-based therapy.

Keywords
Antiretroviral therapy protease inhibitors • Evolution • HIV • Mechanisms of resistance • Protease • Resistance

Introduction into HIV Evolution and Selection of Resistance

HIV has an intrinsic high mutation rate, which is a feature that is common to all RNA viruses. The underlying biochemical mechanism explaining the high mutation rate is the low fidelity of viral RNA polymerase and reverse transcriptase both lacking 3′–5′exonuclease activity, which is a proofreading mechanism that normally amends incorrect base pairs. The estimated mutation rate of HIV-1 is 3.4×10^{-5} per base pair per replication cycle (Mansky and Temin 1995), which, when multiplied with the size of the HIV-1 genome of about 10 kb, translates to approximately one third of all newly generated virus particles containing a nucleotide change in their genome. The high mutation frequency and recombination capacity of the viral reverse transcriptase enzyme in combination with the high turnover rate of HIV in an untreated, infected individual results in the generation of large numbers of genetically distinct viruses, also referred to as a viral quasispecies (Domingo et al. 1996). Within this quasispecies, the most frequent individual variant (wild type) is surrounded by diverse but closely related mutant genomes. The number of mutants is orders of magnitude higher than the number of wild-type viruses. It is therefore predicted that any single genome selected at random from the population is likely to have a mutation relative to wild type, rendering it less fit. Fitness is defined as the replicative adaptability of an organism in its environment. Upon environmental changes (e.g., antiretroviral therapy), the viral population is subject to evolutionary pressure, and its genetic flexibility may allow selection of a mutant virus that has an increased fitness in the new environment (drug-resistant variant). Within an HIV-infected individual, viral fitness is largely reflected by the size of the viral population.

Early estimates suggested that the viral population within an infected individual was virtually infinite and therefore evolution deterministic and antiretroviral resistance inevitable (Coffin 1995). Later it was recognized that the number of HIV variants that produce infectious progeny (i.e., the effective population size) is finite and can be relatively small (Brown 1997; Brown and Richman 1997; Nijhuis et al. 1998; Balagam et al. 2011). This would allow more stochastic viral evolution, although it was also argued that the effective population size is currently underestimated (Kouyos et al. 2006). The majority of mutations in newly produced viral particles result in noninfectious virus. In addition, limited target cell availability, clearance of potentially infectious particles by the host immune system, and epigenetic silencing of infected cells further reduce the effective population size. This relatively small effective population size suggests a population in which only single or double mutants as compared to wild type are present. This model is supported by the observation that current cART (combination antiretroviral therapy) is capable of (fully) inhibiting viral replication, which is unlikely if more genetically diverse variants are present at baseline.

The genetic barrier of an antiretroviral compound is usually defined by the number of resistance mutations conferring virological failure. However, other factors have to be taken into consideration, such as impact of the mutations on viral replication, drug resistance, and recognition by the host immune system. As such the "genetic" barrier towards resistance can also be viewed as the kinetic obstacle for the generation of genetic changes required to overcome selective pressure (Götte 2012).

HIV Protease: Function and Structure

HIV is released from the cell membrane as a noninfectious particle also called the immature virus. During or shortly after the assembled virus particles are released from the infected cells, it undergoes a dramatic structural rearrangement. The transition of the amorphous, noninfectious particle into the mature, infectious virus that is characterized by its election-dense conical core is called maturation (Fig. 1). This transition is triggered by the proteolytic cleavage of the Gag and GagPol precursor polyproteins by the viral enzyme protease (Fig. 1).

HIV protease is a homodimeric aspartic protease that cleaves the Gag polyprotein into six structural viral proteins: matrix (p17, MA), capsid (p24, CA), and nucleocapsid (p7, NC); the p6 protein; and the two spacer peptides p2 (SP1) and p1 (SP2) (Fig. 1a). The GagPol polyprotein is generated through a −1 ribosomal frameshift event that occurs at a 5–10 % frequency (Jacks et al. 1988). This GagPol polyprotein encodes MA, CA, p2, NC, the transframe protein (TFP) and the virally encoded enzymes protease (PR), reverse transcriptase (p66, RT-RH), including its two subunits RT (p51, RT) and RNaseH (p15, RH), and integrase. HIV protease cleaves the unfolded linker regions between the individual folded domains of the encoded Gag and GagPol proteins. The substrate specificity of HIV protease is rather complex; the enzyme recognizes the asymmetric shape of the substrates rather than a specific amino

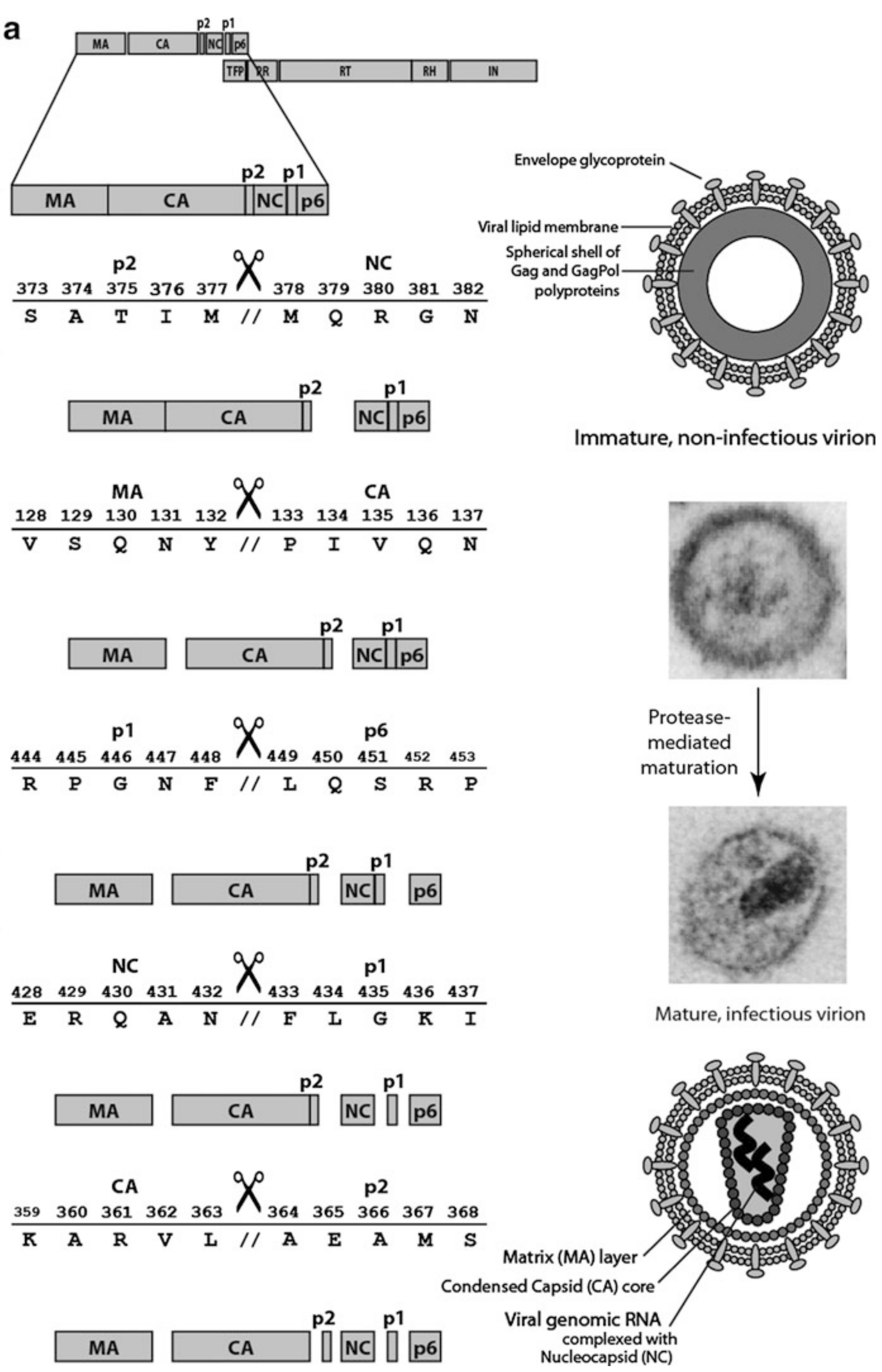

Fig. 1 (continued)

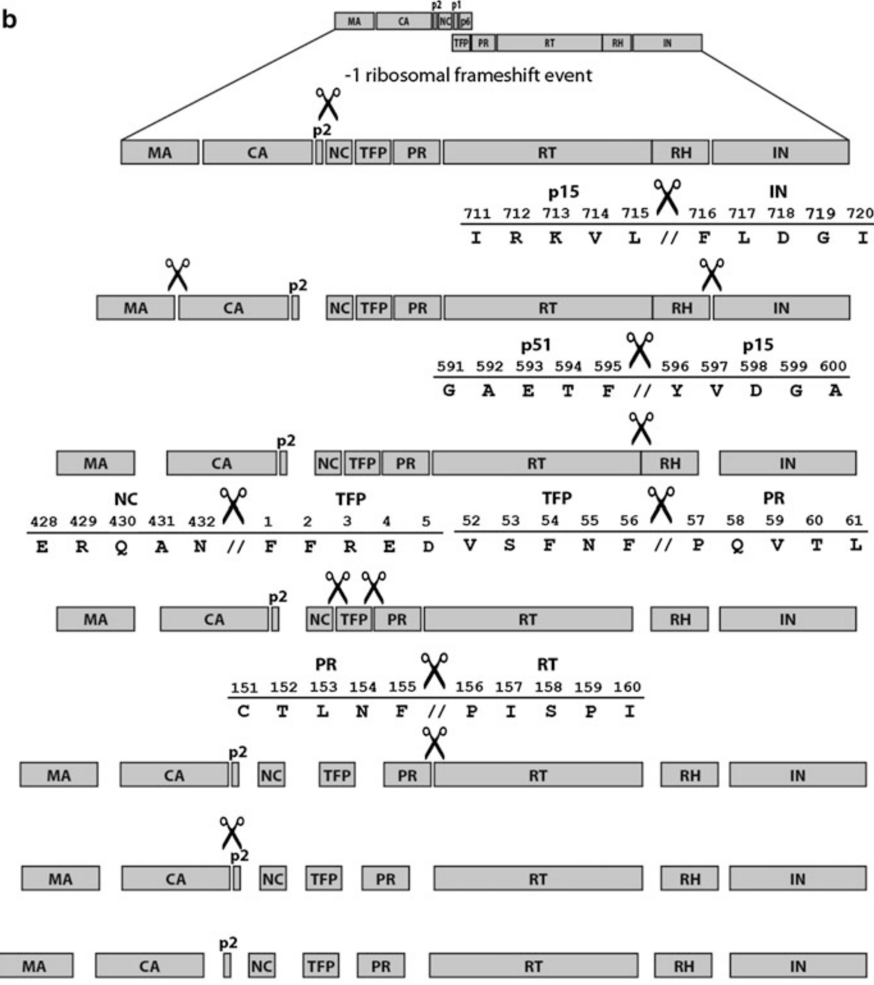

Fig. 1 HIV particle maturation. (**a**) Proteolytic processing of the Gag precursor protein. On the left the five individual cleavage steps are shown with their corresponding amino acid sequences. On the right the transition from the immature noninfectious particle (*top panels*) to infectious virion with its characteristic electron-dense conical is depicted. (**b**) Processing of the GagPol polyprotein that occurs after a −1 frameshift event in the 3′ end of Gag. The amino acid sequences of the cleavage sites are given. The scissors indicate where the viral protease cleaves the precursor GagPol polyprotein

acid sequence (Prabu-Jeyabalan et al. 2002). There is a slight preference for aromatic amino acids and proline at the P1, P1′ positions and large and hydrophobic amino acids at the P2–P2′ position (Fig. 1). There is a discrepancy between the relaxed sequence specificity of the protease enzyme regarding cleavage of peptide substrates in vitro and the very strict requirements for the orderly, precise processing of the Gag and GagPol precursor proteins during virus maturation. The peptides forming the

cleavage sites (CS) have a superimposable secondary structure, yielding the so-called substrate "envelope" which fits within the substrate-binding pocket of the viral protease. However, there are a few subtle differences in the way the amino acid side chains protrude from the "envelope." It is thought that these differences in structure play a central role in the highly ordered stepwise process of viral maturation in which all the individual cleavages occur at different rates (Fig. 1a, b; Lee et al. 2012a; Pettit et al. 1994, 2005; Kräusslich et al. 1989; Wiegers et al. 1998; Erickson-Viitanen et al. 1989). First, the scissile bond between p2 and NC (MA-CA-p2↓NC-p1-p6) is cleaved, followed by separation of MA from CA-p2 (MA↓CA-p2) and NC-p1 from p6 (NC-p1↓p6). Finally, the two small spacer peptides p2 (CA↓p2) and p1 (NC↓p1) are removed in the rate-limiting cleavage steps. In case the −1 ribosomal frameshift occurs and the GagPol protein is synthesized, the viral protease also cleaves the Pol-encoded enzymes into its functional units. Similar to cleavage of Gag, the processing cascade starts by cleaving p2/NC(MA-CA-p2↓NC-TFP-PR-RT-RH-IN), followed by MA/CA (MA↓-CA-p2), and releasing the integrase protein at RH/IN (NC-TFP-PR-RT-RH↓IN). Subsequently, the RNaseH domain is removed from RT (NC-TFP-PR-RT↓RH). The following step is excision of the transframe protein from the GagPol junction: NC/TFP(NC↓-TFP-PR-RT) and TFP/PR(TFP↓PR-RT). Finally the mature PR and RT enzymes are released (PR↓RT). Exactly when p2 is removed from capsid, (CA↓p2) in these final stages of processing is not clear but is likely to be one of the last cleavages (Pettit et al. 2005). As indicated above, the exact factors determining the ordered processing of these substrates are not identified, although cleavage appears to be predominantly regulated by the amino acids that are in close proximity to the actual protease cleavage site (positions p4–p3′). However, it is demonstrated that also the context surrounding the processing sites (Lee et al. 2012b) including the p4′and p5′ positions (Nijhuis et al. 2007; Dam et al. 2009) plays a role in determining the cleavage rate of a subset of processing sites.

HIV protease is an aspartic protease and is a symmetrically assembled homodimer consisting of two identical subunits of 99 amino acids (Navia et al. 1989; Wlodawer et al. 1989). Both subunits contribute catalytic residues to the active site (aspartic acid at codon 25). The substrate-binding pocket is at the center of the homodimer and interacts with the substrate sequences in the Gag and GagPol proteins. HIV protease is itself embedded in the GagPol protein, and the mechanism by which the viral protease becomes activated is not yet fully understood. It is known, however, that the viral protease is responsible for its own release from the precursor polyprotein (autoprocessing). Since protease is only active as a dimer, it is thought that autoprocessing is initiated when two protease domains which are still embedded in the GagPol precursor dimerize. Recently, it was shown that autoprocessing at the N-terminus of protease mediates stable dimer formation essential for catalytic activity, leading to the formation of infectious virus. An antiparallel β-sheet interface formed by the four N- and C-terminal residues of each subunit is important for the dimer stability (Agniswamy et al. 2012). The initial cleavage is a transient, intramolecular event, and the low occupancy of the embedded dimer configuration can explain its low enzymatic activity compared to the fully matured protease enzyme (Tang et al. 2008).

HIV Protease Inhibitors and Their Mechanism of Action

The development of HIV protease inhibitors (PIs) is often regarded as a textbook example of structure-based rational drug design. Currently, there are nine PIs approved for clinical use: saquinavir, ritonavir, indinavir, nelfinavir, (fos) amprenavir, lopinavir, atazanavir, tipranavir, and darunavir (Fig. 2). Of these, atazanavir, lopinavir, and darunavir are most frequently used in current clinical practice. Most PIs are prescribed with a concomitant low dose of ritonavir, which is also a cytochrome P450-3A4 inhibitor and thereby improves bioavailability and half-life of the PIs (Kempf et al. 1997). Except for tipranavir, all PIs are competitive peptidomimetic inhibitors, mimicking the natural substrate of the viral protease. These inhibitors contain a hydroxyethylene core, which prohibits cleavage of the inhibitor by the HIV-1 protease (Fig. 2; Kempf et al. 1995; Sham et al. 1998; Craig et al. 1991; Koh et al. 2003; Partaledis et al. 1995; Patick et al. 1996; Robinson et al. 2000; Vacca et al. 1994). Tipranavir contains dihydropyrone ring as a central scaffold instead of a peptidomimetichydroxyethylene core (Fig. 2; Turner et al. 1998).

Inhibition of the initial GagPol processing steps which involves self-cleavage of the embedded HIV protease from the GagPolpolyprotein (autoprocessing) would prevent viral maturation at the earliest stage and therefore be an ideal drug target. However, all PIs have been developed to bind the active site of the mature protease dimer instead of the precursor protein. It was shown that the embedded HIV protease dimer is 10,000 fold less susceptible to a protease inhibitor (ritonavir) than the mature protease dimer (Pettit et al. 2004). Recently, two groups demonstrated independently and using different assays that of all approved PIs, darunavir and tipranavir are the most potent inhibitors of autoprocessing (Davis et al. 2012; Louis et al. 2011). However, both inhibitors are still three orders of magnitude less active against the embedded dimer as compared to the mature viral protease.

As stated above, the HIV protease inhibitors have been designed as competitive inhibitors with a high affinity for the substrate-binding region of the active viral protease dimer. Analysis of inhibitor-protease complexes revealed that tightly binding inhibitors “lock” into the protease active site (Nalam et al. 2010). Furthermore, it was demonstrated that PIs that fit within the substrate envelope, regardless of their affinity, are more active against drug-resistant protease variants than inhibitors that protrude beyond the substrate envelope (Nalam et al. 2010). Tipranavir and darunavir exert an additional mechanism of action by impeding dimerization of the viral protease (Koh et al. 2007). This dual mechanism of action may explain their high genetic barrier towards resistance, although this has not yet been fully elucidated.

Despite its critical role in HIV infectivity, it was until recently unclear where in the virus life cycle inhibition of virus maturation becomes most manifest. It was known that virus maturation was important for early post-entry steps including reverse transcription (Kawamura et al. 1997). Recently, it was shown that inhibition of viral maturation by PIs not only blocks reverse transcription but also post-reverse transcription steps and viral entry (Rabi et al. 2013). This observation is in line with

Fig. 2 Chemical structures of the nine PIs currently approved for clinical use. All protease inhibitors are peptidomimetic inhibitors and have a hydroxyethylene core (*dashed boxes*), except tripanavir which contains a dihydropyrone ring (*dashed box*) as a central scaffold

earlier findings that HIV-1 Gag mutants impairing processing are defective in viral entry (Wyma et al. 2004; Murakami et al. 2004; Davis et al. 2006). It was shown that approximately half of the inhibitory effect of PIs becomes manifest at the entry step, most likely reflecting interactions between the unprocessed Gag and the cytoplasmic tail of the viral envelope protein (Rabi et al. 2013). Understanding the mechanisms responsible for the high antiviral potency of PIs and the different steps in the viral life

cycle affected by these PIs is essential and also provides insight in the different mechanisms of protease inhibitor resistance.

Mechanisms of HIV Protease Resistance

The International AIDS Society-USA group yearly reviews data on HIV-1 drug resistance to maintain an updated list of mutations associated with PI resistance (Table 1). Currently, major PI resistance mutations have been identified at 15 different protease codons, and an additional 21 protease codons have been included in the update to contain minor mutations (Fig. 3; Johnson et al. 2013). In addition to protease-based PI resistance, also several alternative protease resistance mechanisms have been suggested of which some may provide an explanation for PI therapy failure in the absence of HIV protease mutations.

Protease-Based PI Resistance

Development of PI resistance usually has a biphasic signature reflecting the initial selection of amino acid changes in or near the substrate-binding cleft of the viral protease, e.g., at codons 30, 50, 82, or 84 (Fig. 3). It has been shown that these resistance mutations are mainly selected at those positions where the PIs protrude beyond the substrate-binding envelope, are in direct contact with inhibitor, and result in an overall enlargement of the catalytic region (Nalam et al. 2010; Kolli et al. 2006; Prabu-Jeyabalan et al. 2006). The affinity for the natural substrates (Gag, GagPol) is also slightly altered, often reducing viral replication (Croteau et al. 1997; Gulnik et al. 1995; Nijhuis et al. 1999; Mammano et al. 2000; Mahalingam et al. 1999). These resistance mutations, which are initially selected and reduce the susceptibility to PIs, are called primary or "major" resistance mutations (Tables 1 and 2; Fig. 3; Johnson et al. 2013). In a second step, compensatory or "minor" mutations emerge, e.g., at codons 20, 36, and 71 which by themselves do not have a substantial effect on drug resistance but improve resistance and/or replication of viruses containing major mutations (Table 1; Fig. 3; Johnson et al. 2013; Nijhuis et al. 1999; Mammano et al. 1998, 2000). These amino acid changes can be observed in the viral protease as well as in the substrate (Dam et al. 2009; Nijhuis et al. 1999; Mammano et al. 1998, 2000; Maguire et al. 2002; Borman et al. 1996; Doyon et al. 1996; Zhang et al. 1997; Prado et al. 2002; Kozísek et al. 2012; Kolli et al. 2009; Shibata et al. 2011). The gag substrate changes are mainly observed in the NC/p1 and p1/p6 CS and are thought to increase the affinity of the drug-resistant viral protease, which has an altered substrate-binding pocket, for its substrate.

Occasionally during PI therapy, amino acid insertions ranging from 1 to 6 amino acids are selected at various sites in the viral protease sequence (Kim et al. 2001; Grantz Sasková et al. 2013; Amiel et al. 2011; Winters and Merigan 2005; Winters et al. 2005; Kozísek et al. 2008; Jordan et al. 2009). The insertions may also appear as polymorphism and can be considered as minor mutations since they modestly

Table 1 Mutations in the HIV protease gene associated with resistance to protease inhibitors atazanavir, darunavir, fosamprenavir, indinavir and lopinavir

Atazanavir +/- ritonavir																								
Wild type	L	G	K	L	V	L	E	M	M	G	I	F	I	D	I	I	A	G	V	I	I	N	L	I
Position	10	16	20	24	32	33	34	36	46	48	**50**	53	54	60	62	64	71	73	82	**84**	85	**88**	90	93
Mutations	I F V C	E	R M I T V	I	I	I F V	Q	I L V	I L	V	L	L Y	L V M T A	E	V	L M V	V I T L	C S T A	A T F I	V	V	S	M	L M

Darunavir/ ritonavir										
Wild type	V	V	L	I	I	I	T	L	I	L
Position	11	32	33	**47**	**50**	**54**	74	**76**	**84**	89
Mutations	I	I	F	V	V	M L	P	V	V	V

Fosamprenavir/ ritonavir											
Wild type	L	V	M	I	I	I	G	L	V	I	L
Position	10	32	46	47	**50**	54	73	76	82	**84**	90
Mutations	F I R V	I	I L	V	V	L V M	S	V	A F S T	V	M

Indinavir/ ritonavir														
Wild type	L	K	L	V	M	M	I	A	G	L	V	V	I	L
Position	10	20	24	32	36	**46**	54	71	73	76	77	**82**	**84**	90
Mutations	I R V	M R	I	I	I	I L	V	V T	S A	V	I	A F T	V	M

Lopinavir/ ritonavir																	
Wild type	L	K	L	V	L	M	I	I	F	I	L	A	G	L	V	I	L
Position	10	20	24	**32**	33	46	**47**	50	53	54	63	71	73	**76**	**82**	84	90
Mutations	F I R V	M R	I	I	F	I L	V A	V	L	V L A M T S	P	V T	S	V	A F T S	V	M

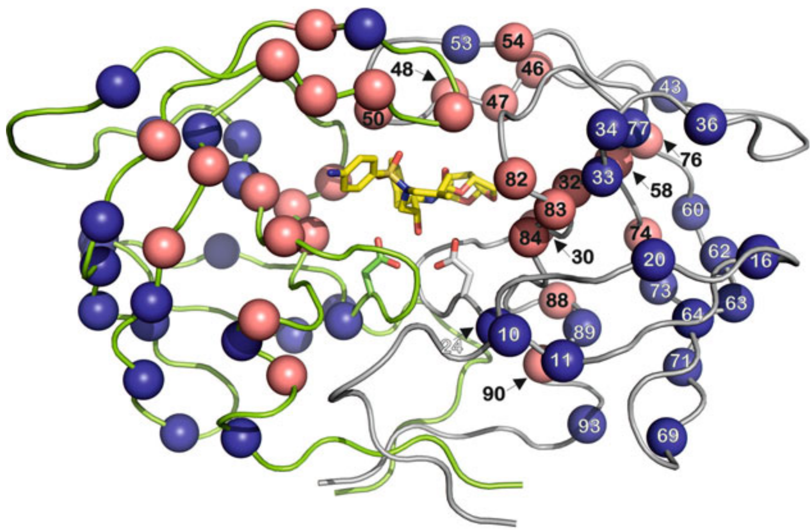

Fig. 3 Three-dimensional structure of the HIV protease dimer. The numbers indicate the amino acids that are associated with PI resistance. In *red* are major (primary) resistance mutations and in *blue* the minor (secondary) resistance mutations. Only one side of the dimer is numbered (*grey* backbone), the corresponding amino acids on the other chain (*green* backbone) are colored but not numbered. The active site aspartates and darunavir bound to the substrate envelope are represented in sticks

improve viral replication (Kim et al. 2001; Kozísek et al. 2008) and only contribute to PI resistance in combination with other mutations either in the PR or in Gag (Kim et al. 2001). Presence of these insertions is positively correlated with protease resistance mutations conferring reduced susceptibility to the contemporary PIs atazanavir, lopinavir, amprenavir, and tipranavir (Kozísek et al. 2008).

Alternative PI-Resistance Mechanisms

Several alternative mechanisms of protease inhibitor resistance have been postulated (Fig. 4).

1. *Improved packaging of viral enzymes*. Duplications of the PTAP motif (Pro-Thre-Ala-Pro) in p6 Gag were identified in patients treated with antiretroviral compounds, and it was proposed that these duplications mediate PI resistance by improved packaging of the viral enzymes (Peters et al. 2001). However, different results were obtained in cross-sectional analyses demonstrating either increased rates (Martins et al. 2011) or comparable rates (Gallego et al. 2003) of PTAP duplications in isolates from antiretroviral therapy-experienced patients versus drug-naïve patients. Furthermore, no effect of p6 Gag insertions was observed on time to virological failure or immunological failure (Brumme et al. 2003),

Table 2 Mutations in the HIV protease gene associated with resistance to protease inhibitors nelfinavir, saquinavir and tipranavir

Nelfinavir										
L	D	M	M	A	V	V	I	N	L
10	**30**	36	46	71	77	82	84	88	**90**
F I	N	I	I L	V T	I	A F T S	V	D S	M

Saquinavir/ritonavir										
L	L	G	I	I	A	G	V	V	I	L
10	24	**48**	54	62	71	73	77	82	84	**90**
I R V	I	V	V L	V	V T	S	I	A F T S	V	M

Tipranavir/ritonavir													
L	L	M	K	M	I	I	Q	H	T	V	N	I	L
10	33	36	43	46	**47**	54	**58**	69	**74**	**82**	**83**	**84**	89
V	F	I L V	T	L	V	A M V	E	K R	P	L T	D	V	I M V

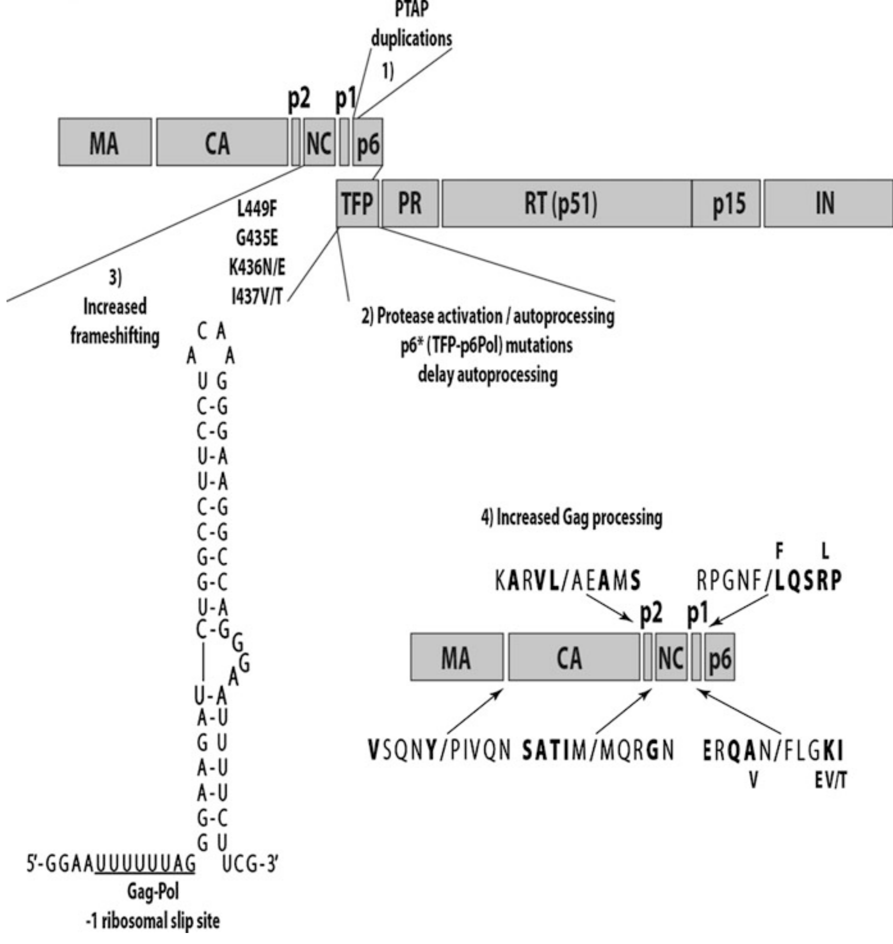

Fig. 4 Proposed alternative mechanisms for protease inhibitor resistance. (*1*) Duplications of the PTAP motif in p6 of Gag. (*2*) Modification of protease activation/autoprocessing. (*3*) Increased GagPol frameshifting. (*4*) Enhanced processing of the Gag substrate. Amino acids associated with protease inhibitor resistance are marked in *bold* and known resistance mutations are indicated

suggesting that these insertions may not be exclusively related to drug resistance but rather reflect (natural) polymorphisms.

2. *Altering protease activation/autoprocessing.* This alternative protease resistance mechanism was proposed based on the observation that mutations in p6* (TFP + p6pol; Figs. 1b and 4) in patient-derived viral isolates can delay Gag autoprocessing and as such decrease PI susceptibility (Whitehurst et al. 2003). The p6* region and especially p6* cleavage is essential for complete activation of the protease and subsequent processing of the viral precursor polyproteins (Ludwig et al. 2008; Paulus et al. 1999, 2004; Tessmer and Kräusslich 1998).

Further research is warranted to investigate the role of mutations in p6* in delayed protease activation and decreased PI susceptibility.

3. *Increased GagPol frameshifting.* Over 15 years ago, an L449F amino acid change in the Gag p1/p6 cleavage site was observed in protease-resistant isolates, and it was suggested that the reduced susceptibility was related to an increased frequency of Gag-Pol ribosomal frameshifting, thereby increasing the levels of the viral enzymes (Doyon et al. 1998). More recent analyses demonstrated that this particular substitution along with other changes in the NC-p1-p6 region of Gag (G435E, K436N, K436N/E, I437V/T) has no effect or only a very modest effect on RNA structure and frameshift efficiency and is therefore unlikely to affect protease susceptibility through this mechanism (Nijhuis et al. 2007; Knops et al. 2012; Girnary et al. 2007).
4. *Enhanced Gag substrate cleavage.* A clear association has been identified between the use of PIs, selection of mutations in protease, and the concurrent substitutions in the viral Gag protein and especially in the protease cleavage sites (Mammano et al. 1998; Zhang et al. 1997; Kolli et al. 2009). Over a decade ago, it was shown that substitutions L449F and P453L in the p1/p6 cleavage site, which do not affect drug susceptibility on their own, reduce PI susceptibility in combination with primary protease mutation I50V (Maguire et al. 2002; Prado et al. 2002). Since then, many Gag cleavage site substitutions have been identified that increase PI resistance in the background of protease mutations, indicating the interactions between the viral protease and its substrate to overcome drug pressure (Fun et al. 2012; Clavel and Mammano 2010).

Interestingly, several mutations in the NC/p1 cleavage site have been identified (A431V, K436E, and/or I437V/T) that confer PI resistance without any mutations in protease (Nijhuis et al. 2007). The Gag-mediated PI resistance was found to be the result of an increased Gag processing (Nijhuis et al. 2007; van Maarseveen et al. 2012). Also emergence of resistance to GS-8374, a potent HIV PI with a unique diethyl-phosphonate moiety, involved a combination of substrate mutations without typical resistance mutations in the viral protease (Stray et al. 2013). Analysis of viral particles indicated that these substrate mutations rendered Gag more susceptible to protease-mediated cleavage in the presence of GS-8374. These data demonstrate that substrate substitutions not only function as compensatory mutations or reduce PI susceptibility in the background of resistance mutations in the viral protease but also represent and alternative PI-resistance mechanism.

Besides mutations in the cleavages sites, mutations outside the Gag cleavage sites have been identified in in vitro selection experiments with different PIs (Stray et al. 2013; Gatanaga et al. 2002) and in patient-derived virus isolates (Parry et al. 2011). These non-cleavage site mutations decrease the potency of the protease inhibitors several fold. The mechanism for the Gag non-cleavage site-mediated resistance is currently not understood.

Several studies evaluated the natural variation within Gag and its cleavage sites (Kolli et al. 2009; Côté et al. 2001; de Oliveira et al. 2003; Verheyen et al. 2009, 2010; Larrouy et al. 2010; Bally et al. 2000; Lambert-Niclot et al. 2012) and suggest

that the variation in HIV-1 non-B subtypes is greater than in subtype B (de Oliveira et al. 2003; Verheyen et al. 2009; Larrouy et al. 2011a). The level of conservation differs dramatically between the different CS as within CS (Fig. 1). The p2/NC CS is the most variable of the 5 Gag CS, followed by p1/p6, NC/p1, CA/p2, and finally MA/CA, which is the most conserved CS in subtype B isolates.

Virological failure during boosted-protease inhibitor first-line protease triple combination is usually not associated with the detection of resistance mutations in the viral protease. Thus, alternative protease resistance pathways/mechanisms are being investigated. Substitutions in all gag CS have been described during PI exposure, and amino acid changes in MA/CA (codon 128), NC/p1 (codons 431, 436, and 437), and p1/p6 (codons 449, 452, and 453) are observed most frequently and have been shown to reduce PI susceptibility (Fun et al. 2012).

Further research is needed to investigate if these alternative gag-based PI drug resistance mechanisms play a significant role during boosted-PI therapy failure. In individual cases, gag mutations were shown to be relevant, e. g., that first-line LPV/r failure was associated with the initial selection of the A431V mutation in Gag followed by the 46I and 76V substitution in the viral protease (Nijhuis et al. 2009). Furthermore, it was shown that pre-therapy mutations in the gag CS sequences were significantly associated with virological outcome of a first-line LPV/r single drug regimen in the Monark Trial (Ghosn et al. 2011) and may impact virological response in naïve patients receiving a combination of two protease inhibitors (Larrouy et al. 2010, 2011b). However, other studies have indicated that Gag CS mutations did not significantly contribute to PI-resistance development and virological failure in patients on a (simplified) boosted-PI regimen (McKinnon et al. 2011; Lillemark et al. 2011).

Recently, it was shown that inhibition of viral maturation by PIs also blocks viral entry (Rabi et al. 2013). Approximately half of the inhibitory potential of the inhibitors becomes manifest at this particular step in the viral life cycle, most likely reflecting the interaction between HIV gag and the cytoplasmic tail of the viral envelope protein. The authors studied patients on a PI-based regimen who had detectable viremia and no major PI-resistance mutations in the viral protease and showed that env sequences may contribute to PI failure in a subset of these patients (Rabi et al. 2013). This may provide an explanation for PI therapy failure without the detection of mutations in the viral protease, the target gene of the drugs.

Twenty Years of Protease Inhibitor-Based Therapy

The introduction of cART greatly reduced HIV-associated morbidity and mortality (Palella et al. 1998). Despite this huge achievement, first-generation PI-based cART was characterized by low bioavailability, high pill burden, toxicity, a low genetic barrier to resistance development, and extensive cross-resistance impairing long-term efficacy.

The first protease inhibitor to be licensed was saquinavir in 1995 (Fig. 2). As monotherapy, the drug had failed to establish sustained antiviral efficacy, and

selection of resistance characterized by mutations 48V and/or 90M in the protease-coding gene was frequently detected (Jacobsen et al. 1996; Noble and Faulds 1996; Kitchen et al. 1995; Table 1; Fig. 3). Combination of saquinavir with two NRTIs in the ACTG-229 trial resulted in a greater reduction in plasma viral RNA level compared to dual NRTI regimens at 24 weeks. However, efficacy markers tended to return to pretreatment values at 48 weeks (Collier et al. 1996; zidovudine, and zalcitabine. AIDS Clinical Trials Group). Resistance to saquinavir was observed less frequently compared to monotherapy but still occurred in about a quarter of patients using a saquinavir-based triple combination regimen (Jacobsen et al. 1996).

As a result of limited absorption and extensive first-pass metabolism by the hepatic cytochrome P450 3A system, very low oral bioavailability of saquinavir was observed with the initial hard gel formulation (HGC). In order to achieve more adequate absorption, saquinavir had to be taken three times daily with a high-fat meal. To address this problem, saquinavir was later reformulated as a soft-gel capsule (SGC) which provided better systemic exposure.

Ritonavir, approved by the FDA in 1996, was the first protease inhibitor licensed for treatment of HIV infection in the European Union (Fig. 2). Its prolonged absorption phase and half-life permitted the use of a twice-daily dosing schedule (Danner et al. 1995). When used as monotherapy, partial loss of antiviral efficacy was seen 3–4 months following initial impressive decrease in HIV-RNA plasma levels (Danner et al. 1995; Markowitz et al. 1995). Monotherapy of ritonavir was associated with accumulation of resistance characterized by signature mutations at amino acid positions 46, 54, 82, and 84 in the viral protease (Fig. 3; Schmit et al. 1996; Molla et al. 1996). Combination therapy consisting of ritonavir and two NRTIs resulted in significant increase in CD4 cell counts and >2log declines in plasma HIV-RNA levels in observational studies (Mathez et al. 1997; Notermans et al. 1998). In patients with advanced disease, addition of ritonavir to double nucleoside therapy prolonged survival compared to placebo. Unfortunately, the PI was poorly tolerated and use of full dose ritonavir in clinical practice was gradually abandoned.

The other protease inhibitor that became licensed in 1996 was indinavir (Fig. 2). Indinavir, when used as monotherapy, also caused extensive declines in HIV-RNA plasma levels. But over time antiviral efficacy diminished and HIV-RNA almost returned to baseline values at 24 weeks (Stein et al. 1996). Multiple drug resistance-related mutations in the protease-coding region were commonly detected at positions 46, 82, and 84, inducing cross resistance to other PIs (Table 1; Fig. 3; Drusano et al. 1998; Condra et al. 1996). A historical breakthrough was observed when indinavir was combined with zidovudine and lamivudine in patients with prior zidovudine exposure. This triple combination not only dramatically reduced HIV-RNA plasma levels, to below the limit of quantification (at that time <400 copies HIV-RNA/mL) in the majority of patients (Condra et al. 1996; Gulick et al. 1997), but also significantly slowed the progression of HIV disease and mortality (ACTG-320 trial) (Hammer et al. 1997). As a result, triple PI-based combination therapy was implemented as standard of care for treatment of HIV infection in resource-rich areas all over the world. In the long-term, success of

indinavir was hampered due to strict intake recommendations and renal toxicity. The drug had to be dosed three times daily preferably on an empty stomach, and a high intake of fluid was needed to prevent indinavir-associated renal toxicity (Plosker and Noble 1999).

Nelfinavir was the fourth protease inhibitor that was registered (Fig. 2). In patients previously exposed to antiretroviral therapy, nelfinavir-based triple combination therapy suppressed HIV-RNA at 28 weeks to below 400 copies/mL in 72 % of patients compared with only 17 % in the control arm of NRTIs and placebo (Gartland and Group 2001). Subsequently, comparable levels of HIV-RNA suppression were observed with a twice-daily dosing regimen which was formally approved in 1999 (Marzolini et al. 2001). Resistance to nelfinavir appeared to be initially driven by the specific protease resistance mutation 30 N, which does not lead to PI cross resistance, followed by changes at amino acid position 88, which also mildly affects susceptibility to several other PIs (Table 1; Fig. 3). In addition, nelfinavir resistance could be observed in the presence of extensive mutational patterns in the viral protease selected by earlier used PIs (Atkinson et al. 2000; Pellegrin et al. 2002; Martinez-Picado et al. 1999). Diarrhea and loose stool frequently limited nelfinavir tolerability.

Second-Generation Protease Inhibitor Therapy; Boosting of Protease Inhibitors

A major advance in the use of protease inhibitors came when it was recognized that ritonavir reduces the metabolism of concomitantly administered PIs through hepatic and intestinal cytochrome P-450 3A4 inhibition, leading to dramatic improvement of bioavailability and half-life of PIs. The first combination used in clinical trials was saquinavir and ritonavir both in therapeutic doses. This combination rapidly pushed HIV-RNA below 200 copies/mL in 80 % of treated individuals (Cameron et al. 1999). Tolerability of a considerable dose of ritonavir remained, however, a concern. The approach really took off when it was found that lower, nontherapeutic doses of ritonavir (100 mg) were sufficient to enhance the pharmacokinetics of coadministrated PIs allowing twice-daily dosing (van Heeswijk et al. 2001).

Boosting of the HGC formulation of saquinavir achieved similar improvement of systemic exposure compared with boosting saquinavir SGC, resulting in reintroduction of the HGC formulation (Kurowski et al. 2003; Cardiello et al. 2003). Boosting of indinavir was not broadly implemented because of high plasma peak levels of the PI leading to enhanced renal toxicity (Voigt et al. 2002; Boyd et al. 2006). Boosting did not greatly enhance the bioavailability of nelfinavir. This limited the efficacy of nelfinavir compared to newly approved PIs and reduced its role for use in pregnancy only until other drugs were found to be a safe and more efficient alternative for this indication as well.

Amprenavir was approved for twice-daily dosing in 1999 (Fig. 2). The combination of amprenavir with two NRTIs was compared to the two NRTIs plus placebo. Although high efficacy of amprenavir versus placebo (89 % vs. 60 % HIV-RNA

<400 copies/mL) was observed at 48 weeks, the intention to treat analysis showed disappointing results with less than 30 % suppressed HIV-RNA in the amprenavir arm (Haubrich et al. 1999). It appeared that many patients prematurely discontinued amprenavir, most likely because of the exceptional pill burden. Furthermore, suboptimal drug concentrations regularly resulting in weak activity of the PI and frequent selection of resistance (Arvieux and Tribut 2005; Sadler et al. 2001). Resistance in the protease-coding region appeared to be somewhat different from the earlier registered PIs and involving key mutations at positions I50V, I54L/M, and V32I + I47V and less commonly I84V (Table 1; Fig. 3; Paulsen et al. 2003).

Introduction of the prodrug fosamprenavir led to improved plasma concentrations and lower pill burden. This formulation showed higher efficacy when combined with two NRTIs compared to nelfinavir-based cART in antiretroviral-naïve individuals in the NEAT trial (Rodriguez-French et al. 2004). Subsequent boosting with ritonavir further improved the efficacy of fosamprenavir-based cART, resulting in suppression of HIV-RNA (<400 copies/mL) in 73 % of naïve individuals at 48 weeks in the KLEAN-trial (Eron et al. 2006). Diarrhea and elevation of fasting cholesterol and triglyceride levels were the most frequent observed adverse events (Eron et al. 2006). There are only limited data available on the efficacy of boosted amprenavir in PI-experienced individuals, but in the CONTEXT trial, viral suppression (<50 copies/mL) in about 50 % of individuals was observed (Arvieux and Tribut 2005; Quercia et al. 2005). Later on, once-daily use of a high dose of boosted fosamprenavir (1,400/100 mg) for combination therapy in therapy-naïve individuals was approved.

Lopinavir (Fig. 2) was the first and thus far only PI co-formulated with a low-dose ritonavir as Kaletra. Lopinavir capsules received approval in 2000 as a twice-daily regimen. Subsequently, the capsules were replaced by pills that were better tolerated and did not require dietary restrictions or refrigeration (Schrader et al. 2008). Efficacy of lopinavir/ritonavir as initial therapy was compared in a randomized placebo controlled study with nelfinavir (three times a day) as comparator with an NRTI backbone in both arms. At 48 weeks, 75 % of individuals on the lopinavir-based regimen had suppressed plasma HIV-RNA (<400 copies/mL) compared to only 63 % in the control arm (Walmsley et al. 2002). Remarkably, no genotypic or phenotypic resistance to lopinavir was observed over 96 weeks in the small group of patients that experienced virological failure (Kempf et al. 2004). Development of resistance during first-line lopinavir-based cART has remained extremely rare but may occur by selection of protease mutations 32I, 47A, and 46I or L33F, I54V, and V82A or combinations of L76V, M46I, and V82A in protease and A431V in gag (Table 1; Figs. 3 and 4) (Nijhuis et al. 2009; Conradie et al. 2004; Friend et al. 2004).

In 2005, once-daily lopinavir was approved based on comparable efficacy with twice-daily lopinavir-based cART. However, the lower Ctrough concentrations observed with once-daily dosing limited registration to antiretroviral-naïve individuals (Johnson et al. 2006a). Lopinavir was the first boosted PI compared head-to-head with an NNRTI as initial therapy. In ACTG study 5142, 48 % of lopinavir-ritonavir recipients versus 61 % of efavirenz recipients (both combined with two NRTIS) maintained plasma HIV-1 RNA at <50 copies/mL through week 96.

Although superior viral suppression was observed in the efavirenz arm, double-class resistance was more frequently detected in this arm. In addition, a significantly better CD4 response was obtained with lopinavir/ritonavir compared with efavirenz (Riddler et al. 2008). Investigations in previously PI-exposed patients demonstrated that the high genetic barrier of lopinavir can be compromised by accumulation of mutations during prior PI-based regimens (Mo et al. 2005). Acquisition of mutations at codons 82, 54, and 46 and less commonly L33F, I50V, and V32I + I47V/A was observed (Table 1; Fig. 3). In PI-experienced patients, lopinavir-/ritonavir-based combination therapy showed superior virological efficacy compared to other at that time available boosted and non-boosted PI-based regimens (Oldfield and Plosker 2006).

The approval of atazanavir in 2003, the first PI that was immediately approved for once-daily dosing, further simplified boosted PI-based combination therapy (Fig. 2). Atazanavir raises plasma bilirubin levels in almost all treated individuals by inhibiting UDP glucuronyltransferase. The bilirubin elevation does generally not result in clinically relevant symptoms. When used unboosted, the drug has only limited effect on fasting cholesterol and triglyceride levels. Unboosted once-daily atazanavir showed equal immunological and superior virological efficacy when compared with nelfinavir twice daily with two NRTIs in antiretroviral-naïve patients (64 % vs. 53 % HIV-RNA suppression <400 copies/mL) (Murphy et al. 2003). Disappointing virological results were obtained in a head-to-head comparison of efavirenz and atazanavir both combined with two NRTIs as first-line regimens. Although plasma HIV-RNA was suppressed below 400 copies/mL in 70 % and 64 % of the atazanavir and efavirenz arms respectively, much lower rates of suppression (32 % and 37 %) were observed in both arms using the more stringent <50 copies/mL criterion (Squires et al. 2004). Soon after the trial, it became apparent that nonstandard collection tubes were possibly responsible for the unexpected low efficacy in both arms (Giordano et al. 2006). Better results were obtained in the Castle study in which once-daily boosted atazanavir was demonstrated as being not inferior to twice-daily lopinavir-ritonavir for the treatment of antiretroviral-naïve patients in the background of a fixed-dose NRTI combination. In both arms, high levels of suppression were observed (78 % vs. 76 % <50 copies/mL) and an increase in CD4 cell counts of more than 200 cells/mm^3 (Molina et al. 2008).

Subsequently, it was recognized that a high genetic barrier to resistance was not limited to lopinavir-ritonavir but could be reached through boosting other PIs. Comparable efficacy and only rarely selection of resistance were demonstrated in several trials comparing lopinavir-ritonavir with either twice-daily boosted fosamprenavir or twice-daily saquinavir in antiretroviral-naive patients (Eron et al. 2006; Molina et al. 2008; Johnson et al. 2006b). Moreover, the use of boosted saquinavir or atazanavir also resulted in better tolerability with lower lipid profiles and less diarrhea compared with lopinavir-ritonavir (Molina et al. 2008; Johnson et al. 2006b; Walmsley et al. 2009).

In PI-experienced individuals, boosted atazanavir with two NRTI proved to be virologically and immunologically non-inferior to twice-daily lopinavir-ritonavir regimens; however, this did not hold up for unboosted atazanavir. Unboosted

atazanavir was therefore not approved for use in this patient group (Johnson et al. 2005; Cohen et al. 2005). Selection of resistance in treatment-naive patients experiencing therapy failure during unboosted atazanavir-based regimens was characterized by the I50L mutation in protease which reduced susceptibility to atazanavir, but increased susceptibility to other PIs (Table 1; Fig. 3; Colonno et al. 2004). In contrast, in treatment-experienced patients and patients on boosted therapy, other mutations conferring PI cross-resistance are generally observed including I84V, L90M, A71V/T, N88S/D, and M46I (Table 1; Fig. 3; Pellegrin et al. 2006).

Tipranavir was approved in 2005 (Fig. 2) for the treatment of highly experienced patients with resistance to multiple PIs. Tipranavir is a non-peptidomimetic protease inhibitor and as such less potent than the peptidomimetic PI as described above. Instead of a peptidomimetichydroxyethylene core, tipranavir contains a dihydropyrone ring as a central scaffold (Fig. 2; Turner et al. 1998). Tipranavir is a potent inducer of its own metabolism through induction of the cytochrome P450 expression, which results in a need for double-dose ritonavir boosting. Tipranavir showed superior efficacy in salvage relative to the comparator protease inhibitor (CPI), both with optimized background in heavily pretreated patients with extensive PI resistance in the RESIST trials. At 48 weeks, significantly more patients achieved and maintained treatment response in the boosted tipranavir arm than in the CPI-ritonavir arm (plasma HIV-RNA $<$400 copies/mL: 33.6 % vs. 15.3 % and $<$50 copies/mL: 22.8 % vs. 10.2 %). However, tipranavir appeared not to be superior to lopinavir in lopinavir-naïve individuals. Moreover, gastrointestinal system disorders, liver-related toxicity, and elevated lipid profiles were more frequently reported in the boosted tipranavir arm (Gathe et al. 2006; Cahn et al. 2006; Hicks et al. 2006).

During tipranavir in vitro selection experiments, major PI-resistance mutations were observed: V32I, I54V, V82L, and I84V (Table 1; Fig. 3; Doyon et al. 2005). After failure of tipranavir-based cART, mutations at positions 82 and 84 have been reported most frequently (Table 1; Fig. 3; Naeger and Struble 2007). Based on the results in the RESIST study, a weighted genotypic susceptibility list has been reported including a larger number of mutations, T74P, I47V, V82L/T, Q58E, N83D as the strongest predictors of reduced efficacy and I54A/M/V, I84V, M36I, K43T, L10V, and M46L as weaker predictors, whereas L24I, I50L/V, I54L, and L76V were predictors of virological response (Fig. 3; Schapiro et al. 2010). Even though the virological benefits of tipranavir were evident, significant risk of hepatotoxicity, high pill burden, and availability of new treatment options restricted wide uptake.

One such new treatment option was darunavir, a ninth protease inhibitor approved in 2006 (Fig. 2). Although this PI was especially designed to inhibit drug-resistant strains, its powerful antiviral potency and limited adverse events profile rapidly expanded use of the drug into earlier lines of therapy. In the Artemis trial, once-daily darunavir/ritonavir was compared to once- or twice-daily lopinavir-ritonavir with a fixed-dose NRTI backbone in antiretroviral-naïve patients. At week 96, significantly more patients in the darunavir (79 %) than the lopinavir arm (71 %) had a plasma HIV-RNA less than 50 copies/ml. Median CD4 cell count increase from

baseline was circa 180 cells/mm^3. Darunavir had a more favorable gastrointestinal and lipid profile compared to lopinavir (Mills et al. 2009). In treatment-experienced patients, the dose-finding Power 1 and 2 studies evaluated the efficacy and safety of boosted darunavir with that of currently approved PIs combined with an optimized background therapy. 96-week efficacy and safety data of both trials confirmed the recommended the 600/100 mg twice-daily dose as the preferred option for pretreated individuals (Arastéh et al. 2009). In a combined analysis of both trials, 39 % of patients receiving boosted darunavir regimens achieved plasma HIV-RNA <50 copies/mL compared to 9 % with comparator PI-based regimens (Arastéh et al. 2009). In the Titan study, twice-daily boosted darunavir was compared to twice-daily lopinavir-ritonavir both with optimized backbone. At 48 weeks, significantly more patients receiving darunavir achieved a plasma HIV-RNA load of <50 copies/mL. CD4 cell count increases were similar in both treatment groups (Madruga et al. 2007). Fewer patients with virologic failure in the darunavir arm than in the lopinavir arm developed resistance (De Meyer et al. 2009).

In vitro selection of darunavir-resistant HIV-1 appears to be slower and less frequent than with other PIs, probably reflecting the particularly strong binding of darunavir to the HIV protease, resulting in a higher intrinsic genetic barrier than observed with the other boosted PIs (De Meyer et al. 2005; King et al. 2004). This makes it very difficult for the PI-naïve virus to escape via the traditional protease-based route and alternative gag substrate-based resistance may be selected (Fig. 4).

Pooled resistance data from the Power trials who initiated boosted darunavir 600/100 mg in PI-experienced patients was used to establish a list of resistance mutations associated with a diminished response to darunavir (de Meyer et al. 2008). The list was updated using data from more trials with therapy-experienced patients, including the following mutations in the protease-coding region: V11I, V32I, L33F, I47V, I50V, I54L/M, T74P, L76V, I84V, and L89V (Table 1; Fig. 3). The presence of more than three darunavir-listed mutations was associated with a median darunavir FC >10 and a diminished virological response (De Meyer et al. 2009). Of interest, some of the darunavir mutations are associated with improved response to tipranavir, which might give additional options for future salvage therapy, if needed at all.

Double Boosting Protease Inhibitor-Based Therapy

Preceding the recent approval of several new antiretroviral compounds, physicians experienced difficulties building an effective regimen for a group of heavily therapy-experienced patients with extensive drug resistance. In this setting of limited therapeutic options, the use of double-boosted PI was explored to gain possible synergistic or added antiviral activity of both drugs and to increase the genetic barrier to PI resistance. Although no large randomized trials assessing the clinical efficacy of double-boosted-PI regimens were reported, several comparative and cohort studies suggested potential utility of combinations such as lopinavir/ritonavir with either saquinavir or atazanavir, or atazanavir plus low-dose ritonavir with saquinavir all in combination with two NRTIs as a backbone (Smith et al. 2005; Gilliam et al. 2006;

Ribera et al. 2006; Manosuthi et al. 2008; von Hentig et al. 2007; Petersen et al. 2007; Stebbing et al. 2009).

A high pill burden, increased risk for toxicity, and lower efficacy compared to standard of care regimens did not make double-boosted PIs an appealing option beyond salvage therapy (Ulbricht et al. 2011). The combination of darunavir with drugs from new classes has resulted in impressive efficacy in heavily pretreated patients discarding the use of double-boosted PIs as salvage therapy in resource-rich settings.

Protease Inhibitor: Mono or Dual Therapy

Although NRTIs had been the cornerstone of cART, the finding that originally to PIs attributed lipoatrophy was mainly induced by these NRTIs fuelled a search for alternative regimens. Moreover, the profound efficacy of boosted PI-based cART and the high genetic barrier to resistance questioned the paradigm of a three-drug regimen. Combined with the challenge of lifelong adherence, high costs of triple therapy, and the risk for selection of multidrug resistance, these considerations led to reevaluation of the concept of mono or dual therapy in several trials.

A dual NRTI-sparing PI-NNRTI combination of lopinavir/r and efavirenz demonstrated comparable efficacy to efavirenz and lopinavir-based cART but extensive elevation of triglyceride levels and a trend towards more NNRTI resistance upon therapy failure (Riddler et al. 2008). More elaborated selection of resistance was also reported with the use of NRTI-sparing regimens of either lopinavir or boosted indinavir with an NNRTI in the ANRS-121 trial (Soulié et al. 2009). The results from these two important trials clearly limited the role for future initiatives using NNRTI/PI combinations as NRTI-sparing regimens. Several trials addressed efficacy of NRTI-sparing regimens using boosted PIs and the integrase inhibitor raltegravir. In the randomized Spartan trial, which was not powered for statistical comparison of efficacy, twice-daily unboosted atazanavir plus raltegravir was compared to once-daily atazanavir plus two NRTIs in therapy naive patients (Kozal et al. 2012). At 24 weeks of treatment, 74.6 % of patients in the dual arm had confirmed virological response, compared with 63.3 % in the standard of care arm. However, patients in the dual arm frequently suffered from grade 4 hyperbilirubinemia and occasionally selected resistance to raltegravir. As a result, this strategy has not been broadly implemented. In an uncontrolled trial, first-line therapy of boosted darunavir plus raltegravir was well tolerated, but virologic failure and selection of integrase resistance mutations were common, particularly in patients with baseline viral load more than 100,000 copies/mL (Taiwo et al. 2011). The same dual combination appeared to be non-inferior to a standard three-drug combination of boosted darunavir with two NRTIs in the randomized controlled NEAT001/ANRS143 trial. A very high percentage of patients received virological control in both arms (93 % in the three-drug arm vs. 89 % in the dual arm). Of note selection of resistance to raltegravir was occasionally observed in the dual arm, while no resistance was detected in the comparator arm (Raffi et al. 84LB, CROI 2014).

Recently a multicenter, randomized, double-blind comparative trial of boosted darunavir plus the CCR5-blocker maraviroc versus the standard of boosted darunavir with two NRTIs in antiretroviral-naïve HIV-infected patients with CCR5 tropic HIV was terminated after preliminary review of week 48 data. Reason of termination was significant inferior efficacy of the dual arm as compared to the comparator arm (http://clinicaltrials.gov/ct2/show/NCT01345630).

A systematic review of monotherapy on the available clinical trials with lopinavir and non-comparative atazanavir monotherapy studies found an absolute risk difference of 10.3 % of failure compared to standard PI-based cART (Bierman et al. 2009). In antiretroviral-naïve patients initiating PI-monotherapy, low-level viremia and development of resistance was also more frequently observed compared to standard of care 3-drug-based regimens (Bierman et al. 2009). However, no significant difference in short-term efficacy was observed in individuals starting monotherapy after induction with full suppressive cART (Bierman et al. 2009). Furthermore, among individuals who successfully controlled HIV after restarting nucleosides, failure rates did not significantly differ between monotherapy groups and standard-regimen groups (Bierman et al. 2009).

A potential explanation for the increased risk of failure with lopinavir monotherapy without induction phase may be insufficient viral suppression by boosted PIs in all body compartments. A lower genetic barrier to resistance for monotherapy than originally foreseen may provide an alternative explanation for increased failure as just two mutations can be sufficient for viral breakthrough during (mono)therapy with lopinavir-ritonavir (Nijhuis et al. 2009; Delaugerre et al. 2009). However, the reported combination of protease mutations M46I plus L76V is not frequently observed in cases of lopinavir-based therapy failure. This indicates that the genetic barrier to resistance is not simply a calculated sum of the two mutations but also includes the selective advantage of these particular mutations in the viral quasispecies.

Maintenance monotherapy with once-daily boosted darunavir, a PI for which it is generally accepted that it has a very high genetic barrier, was performed in two relatively large clinical trials. In the Monet trial, HIV RNA during monotherapy was non-inferior to a three-drug regimen at 48 weeks (84.3 % vs. 85.3 % HIV-RNA <50 copies/mL) (Arribas et al. 2012). At 96 and 144 weeks, non-inferiority was still observed in an ITT analysis considering switches not as failure. However, non-inferiority was not maintained in a TLOVR (time to loss of virological response) switch equals failure analysis (Arribas et al. 2012). Also in the MONOI-ANRS trial, non-inferiority was observed at 48 weeks; however, in the ITT analysis, the rate of success was 87.5 % in the monotherapy arm and 92 % in the three-drug arm. Longer-term data at 96 weeks showed high efficacy rate of darunavir (85 %); nevertheless, there was a consistent small difference favoring the three-drug combination (Katlama et al. 2010). In both studies, patients failing on darunavir/r monotherapy had virtually no emergence of darunavir resistance mutations (Arribas et al. 2012; Katlama et al. 2010).

The Influence of Genetic Diversity on Protease Inhibitor Efficacy and Selection of Resistance

Initial drug design and data on efficacy and selection of drug resistance have focused on subtype B infections. Among subtypes the difference in nucleotide sequence in the protease-coding *pol* gene is approximately 10–15 %, leading to distinct variation at amino acid level. The genetic differences may influence baseline susceptibility of PIs, the genetic barrier for selection of PI drug resistance and mutational pathways (Martinez-Cajas et al. 2009).

In vitro decreased susceptibility to PIs was reported in a limited number of CRF02_AG isolates from therapy-naïve individuals (Fleury et al. 2006; Kinomoto et al. 2005). Caution is warranted by interpretation of these results since deletion clones and phenotypic resistance cutoffs applied are based on subtype B virus backbones. Additional reports on small numbers of non-B HIV-1 isolates with decreased baseline susceptibility or hypersusceptibility for PIs in vitro have been published. Slight differences in protease inhibitor efficiency based on conformational differences with subtype B leading to a more flexible enzyme in several non-B subtypes may explain at least partially these results (Huang et al. 2014). In the absence of clinical trials specifically addressing differences in susceptibility, no obvious differences in susceptibility of non-B isolates compared to subtype B isolates have been observed in clinical practice (Martinez-Cajas et al. 2009; Frater 2002).

Wild-type sequences at several resistance-related codons differ between non-B subtypes and subtype B (van de Vijver et al. 2006). These differences may influence viral replication capacity, the genetic barrier, or specific pathways to resistance (Lisovsky et al. 2010). Examples are the minor mutations 10 V and 36I, which are generally present in non-B viruses and which are included in the tipranavir resistance score (Table 1; Fig. 3; Schapiro et al. 2010; van de Vijver et al. 2006). Diversity in nucleotide sequence may also lead to differential selection of PI-resistant variants on position 82 (van de Vijver et al. 2006; Abecasis et al. 2006).

In addition, the frequency of selection of specific mutational patterns may differ among subtypes. For instance, in subtype C and other non-B subtypes, selection of resistance to nelfinavir preferably occurs via PI cross-resistance pathways including mutation L90M, and to a lesser extent via the, in subtype B most frequently observed, nelfinavir-specific pathway with mutation D30N (Cane et al. 2001; Grossman et al. 2004).

Finally, alternative resistance pathways may be selected among non-B subtypes. This is most extensively described for a PI-resistance pathway that includes positions 89 and 90 in non-B subtypes. In non-B subtypes such as subtype C, F, G, and CRF01_AE, M89 is the consensus amino acid, compared to L89 in subtype B. The M89 polymorphism present in non-B subtypes may lead to the selection of the M89T mutational pathway conferring reduced susceptibility to atazanavir, lopinavir, and nelfinavir (Martinez-Cajas et al. 2012). Acquisition of the mutations M89I and L90M results in decreased susceptibility to nelfinavir in these subtypes (Abecasis et al. 2005). Additional presence of mutation 71T or 74S has been correlated with high levels of resistance to nelfinavir in subtype G viruses (Gonzalez et al. 2008).

The Use of Boosted PIs in Resource-Limited Settings

cART became available in resource-limited settings all over the world after adoption of the Doha-declaration in 2001, enabling countries to circumvent patent rights for essential medicines. Two years later, the World Health Organization (WHO) launched their "3 by 5" initiative as a global target for provision of antiretroviral therapy to three million people by the end of 2005 in resource-limited settings. Even though this goal was not met, the initiative led to massive programmatic rollout of NNRTI-based first-line therapy. By the end of 2013, nearly ten million people were on cART worldwide. For second-line therapy in resource-limited settings, WHO advises a PI-based regimen with lopinavir-ritonavir plus two NRTIs. The important advantage of this choice is that no refrigeration of ritonavir capsules is required as ritonavir is co-formulated with lopinavir tablets. Furthermore, a PI-based regimen is likely to demonstrate still viral efficacy even if the NRTI backbone is compromised by first-line therapy. At present there are only anecdotal data available regarding the efficacy of second-line lopinavir-ritonavir-based regimens in resource-limited settings. One small study showed accumulation of protease mutations among patients with long-term failure on second-line PI-based cART in Nigeria, but more extensive results are needed before firm conclusion on the extent of selection of PI-related resistance can be drawn (Rawizza et al. 2013).

Conclusion

HIV protease plays a crucial role in the viral life cycle, and the introduction of protease inhibitors marked the start of combination antiretroviral therapy. Initially, therapy failure was often associated with the selection of multiple drug resistance mutations, a broad range of resistance mechanisms, and cross-resistance to other PIs. Fortunately, the genetic barrier towards PI resistance could be raised which greatly contributed to the current success of PI-based therapy. Still there are concerns regarding use of PIs that require compelling attention, with respect to toxicity and the potential for development of resistance especially if used as maintenance therapy or if used in resource-limited setting with less frequent monitoring.

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