

Handbook of Experimental Pharmacology 193

Constance N. Wilson

S. Jamal Mustafa

Editors

Adenosine Receptors in Health and Disease



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Adenosine Receptors in Health and Disease

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Preface

Since the first description of adenosine receptors 30 years ago, based on the valuable scientific discoveries and contributions by individuals working in the field of adenosine receptor (AR) research around the world elucidating AR molecular structure, pharmacology, and function, and the intensive efforts in chemistry to identify selective ligands for ARs, molecules that target all four AR subtypes, A₁, A_{2A}, A_{2B}, and A₃ARs have advanced to clinical trials with a recent FDA approval and an NDA submission. As contributing authors to this volume of the Handbook of Experimental Pharmacology (HEP), “Adenosine Receptors in Health and Disease”, these scientists describe the impact of their discoveries and contributions, as well as those by others, on defining the role of ARs in a number of different diseases and the advancement of this field of science and medicine. Since the inception of this area of basic science research, it has truly been an incredible experience for all of us in academia and the pharmaceutical industry to participate in and observe this captivating and fast-moving field advance from the bench to the clinic.

In the A₁AR area, A₁AR agonists have been tested in humans for the following conditions: atrial arrhythmias (Tecadenoson, Selodenoson and PJ-875); Type II diabetes (GR79236, ARA, and CVT-3619); and angina (BAY-68–4986). New partial A₁AR agonists are in development, including CVT-3619, that have the potential to provide enhanced insulin sensitivity without cardiovascular side effects and tachyphylaxis. Based on the diuretic/natriuretic and renoprotective effects of A₁ARs in the kidney, A₁AR antagonists are currently in late-stage clinical development, including KW3902 (rolofylline, Phase III), BG9928 (Adentri[®], Phase III), and SLV320 (Phase II), for acute decompensated heart failure (ADHF) with renal impairment. All three have high affinity for the human A₁AR subtype and demonstrate diuretic and renal protective effects in humans with ADHF with renal impairment. Moreover, to date, two PET ligands have been successfully tested in humans for the visualization of A₁ARs in the brain, [¹⁸F]CPFPX and [¹¹C]MPDX. The use of these PET imaging agents may provide valuable insights into sleep disorders and neurodegenerative disorders, e.g. Alzheimer’s Disease (AD).

In the A_{2A} AR area, A_{2A} AR agonists are currently in clinical trials, with one recent FDA approval and one NDA submission for the following indications: myocardial perfusion imaging (recently FDA approved LexiscanTM, regadenoson, CVT-3146; CorVue, binodenoson, MRE-0470, WRC-0470, NDA submission; apadenoson, ATL-146e), and wound healing (sonedenoson, MRE 0094). A_{2A} AR antagonists have been tested in clinical trials for Parkinson's Disease (PD), including istradefylline, KW 6002; BIIB014, V2006; and SCH 58261. Moreover, two A_{2A} AR PET ligands have been successfully tested in humans for the visualization of A_{2A} ARs in the brain, [¹¹C]TMSX and [¹¹C]KW-6002. The use of these PET imaging agents may provide valuable insights into PD, psychiatric diseases, and perhaps drug addiction.

In the A_{2B} AR area, a mixed A_{2B}/A_3 AR antagonist, QAF 805, was tested in humans with asthma and an A_{2B} AR antagonist, CVT 6883, is in clinical development for asthma and currently is in Phase I clinical trials.

In the A_3 AR area, A_3 AR agonists are in clinical trials for the following indications: rheumatoid arthritis, dry eye syndrome, psoriasis (CF 101), and liver cancer, hepatitis, and liver regeneration (CF 102).

A number of other molecules that target AR subtypes and that are at various stages of preclinical development appear to be promising drug candidates for asthma, inflammation, sepsis, ischemia-reperfusion organ injury, fibrosis, ADHD with renal impairment, PD, AD, cancer, diabetes, obesity, glaucoma, and as coronary vasodilators for myocardial imaging. Moreover, based on the growing scientific evidence supporting the role of ARs in other neurodegenerative diseases and drug abuse and addiction, it is expected that AR-based drug candidates will enter clinical trials to target these diseases. We look forward with anticipation to the advancement of these promising drug candidates towards the clinic and their approval. We expect they will significantly alter the life styles and outcomes of patients with these diseases.

It has been our pleasure to work closely with the world-renowned AR scientists who contributed to this volume of the HEP. We are extremely grateful for their invaluable contributions to this area of science and medicine, which will be realized for generations to come. In this volume of the HEP, all of us have tried to present chapters with up-to-date information about the role of ARs in health and disease and the importance of ARs as drug targets for a number of different diseases. It was our intention to present this information in such a way that those who are not as closely associated with this area of science and medicine and with different interests and backgrounds can understand and appreciate its significance. We are especially indebted to Springer for providing us the opportunity to contribute this volume of the HEP and to Susanne Dathe for her support and successfully managing this project.

Research Triangle Park, NC,
Morgantown, WV,

Constance N. Wilson
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Introduction to Adenosine Receptors as Therapeutic Targets

Kenneth A. Jacobson

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Abstract Adenosine acts as a cytoprotective modulator in response to stress to an organ or tissue. Although short-lived in the circulation, it can activate four subtypes of G protein-coupled adenosine receptors (ARs): A₁, A_{2A}, A_{2B}, and A₃. The alkylxanthines caffeine and theophylline are the prototypical antagonists of ARs, and their stimulant actions occur primarily through this mechanism. For each of the four AR subtypes, selective agonists and antagonists have been introduced and used to develop new therapeutic drug concepts. ARs are notable among the GPCR family in the number and variety of agonist therapeutic candidates that have been proposed. The selective and potent synthetic AR agonists, which are typically much longer lasting in the body than adenosine, have potential therapeutic applications based on their anti-inflammatory (A_{2A} and A₃), cardioprotective (preconditioning by A₁

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and A₃ and postconditioning by A_{2B}), cerebroprotective (A₁ and A₃), and antinociceptive (A₁) properties. Potent and selective AR antagonists display therapeutic potential as kidney protective (A₁), antifibrotic (A_{2A}), neuroprotective (A_{2A}), and antiglaucoma (A₃) agents. AR agonists for cardiac imaging and positron-emitting AR antagonists are in development for diagnostic applications. Allosteric modulators of A₁ and A₃ ARs have been described. In addition to the use of selective agonists/antagonists as pharmacological tools, mouse strains in which an AR has been genetically deleted have aided in developing novel drug concepts based on the modulation of ARs.

Keywords Adenosine receptors · G protein-coupled receptors · Purines · Nucleosides · Imaging · Allosteric modulation · Agonists · Antagonists

Abbreviations

ADHF	Acute decompensated heart failure
ADP	Adenosine diphosphate
AMP	Adenosine 5'-monophosphate
AMP579	[1 <i>S</i> -[1 α , 2 β , 3 β , 4 α (<i>S</i> [*])]]-4-[7-[[1-(3-Chlorothien-2-yl)methyl]propyl]amino]-3 <i>H</i> -imidazo[4,5- <i>b</i>]pyrid-3-yl]- <i>N</i> -ethyl 2,3-dihydroxycyclopentanecarboxamide
AR	Adenosine receptor
ATP	Adenosine triphosphate
BAY 60–6583	2-[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide
BAY 68–4986	6-Amino-2-(2-(4-chlorophenyl)thiazol-4-ylthio)-4-(4-(2-hydroxyethoxy)phenyl)-5-isocyanonicotinonitrile
BG9719	1,3-Dipropyl-8-(2-(5,6-epoxy)norbonyl)xanthine
BG9928	3-[4-(2,6-Dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1 <i>H</i> -purin-8-yl)-bicyclo[2.2.2]oct-1-yl]-propionic acid
BIIB014	3-(4-Amino-3-methylbenzyl)-7-(2-furyl)-3 <i>H</i> - [1,2,3]triazolo [4,5- <i>d</i>]pyrimidine-5-amine (V2006)
CD39	Apyrase
CD73	Ecto-5'-nucleotidase
CF101	<i>N</i> ⁶ -(3-Iodobenzyl)-5'- <i>N</i> -methylcarboxamidoadenosine (IB-MECA)
CF102	2-Chloro- <i>N</i> ⁶ -(3-iodobenzyl)-5'- <i>N</i> -methylcarboxamidoadenosine (Cl-IB-MECA)
CP-608,039	(2 <i>S</i> , 3 <i>S</i> , 4 <i>R</i> , 5 <i>R</i>)-3-Amino-5-{6-[5-chloro-2-(3-methylisoxazol-5-ylmethoxy)benzylamino]purin-9-yl}-l-4-hydroxytetrahydrofuran-2-carboxylic acid methylamide
CP-532,903	(2 <i>S</i> , 3 <i>S</i> , 4 <i>R</i> , 5 <i>R</i>)-3-Amino-5-{6-[2,5-dichlorobenzylamino]purin-9-yl}-l-4-hydroxytetrahydrofuran-2-carboxylic acid methylamide

CPFPX	8-Cyclopentyl-1-propyl-3-(3-fluoropropyl)-xanthine
CVT-3146	1-[6-Amino-9-[(2 <i>R</i> , 3 <i>R</i> , 4 <i>S</i> , 5 <i>R</i>)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]purin-2-yl]- <i>N</i> -methylpyrazole-4-carboxamide
CVT-6883	3-Ethyl-1-propyl-8-[1-(3-trifluoromethylbenzyl)-1 <i>H</i> -pyrazol-4-yl]-3,7-dihydropurine-2,6-dione
EL	Extracellular loop
ENT	Equilibrative nucleoside transporter
E-NTPDase	Ectonucleoside triphosphate diphosphohydrolase
ERK	Extracellular receptor signal-induced kinase
FK 453	(+)-(<i>R</i>)-(1-(<i>E</i>)-3-(2-Phenylpyrazolo(1,5- <i>a</i>)pyridin-3-yl)acryl)-2-piperidine ethanol
FR194921	2-(1-Methyl-4-piperidinyl)-6-(2-phenylpyrazolo[1,5- <i>a</i>]pyridin-3-yl)-3(2 <i>H</i>)-pyridazinone
GPCRs	G protein-coupled receptors
GR79236	<i>N</i> ⁶ -[(1 <i>S</i> , 2 <i>S</i>)-2-Hydroxycyclopentyl]adenosine
GRKs	G-protein-coupled receptor kinases
IL	Intracellular loop
KW3902	8-(Noradamantan-3-yl)-1,3-dipropylxanthine
KW6002	8-[(<i>E</i>)-2-(3,4-Dimethoxyphenyl)vinyl]-1,3-diethyl-7-methylpurine-2,6-dione
L-97-1	3-[2-(4-Aminophenyl)-ethyl]-8-benzyl-7-{2-ethyl-(2-hydroxyethyl)-amino}-ethyl}-1-propyl-3,7-dihydro-purine-2,6-dione
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinases
MRE0094	2-[2-(4-Chlorophenyl)ethoxy]adenosine
MRE-0470	2-[(Cyclohexylmethylene)hydrazino]adenosine (WRC-0470, binodenson)
MRS5147	(1' <i>R</i> , 2' <i>R</i> , 3' <i>S</i> , 4' <i>R</i> , 5' <i>S</i>)-4'-[2-Chloro-6-(3-bromobenzylamino)-purine]-2', 3'- <i>O</i> -dihydroxybicyclo-[3.1.0]hexane
N-0861	(±)- <i>N</i> ⁶ -Endonorboman-2-yl-9-methyladenine
NNC-21-0136	2-Chloro- <i>N</i> ⁶ -[(<i>R</i>)-[(2-benzothiazolyl)thio]-2-propyl]-adenosine
OT-7999	5- <i>N</i> -Butyl-8-(4-trifluoromethylphenyl)-3 <i>H</i> -[1,2,4]triazolo-[5, 1- <i>i</i>]purine
PET	Positron emission tomography
PI3K	Phosphoinositide-3 kinase
T-62	(2-Amino-4,5,6,7-tetrahydrobenzo[<i>b</i>]thiophen-3-yl)-(4-chlorophenyl)-methanone
SLV-320	4-[(2-Phenyl-7 <i>H</i> -pyrrolo[2,3- <i>d</i>]pyrimidin-4-yl)amino]- <i>trans</i> -cyclohexanol
SDZ WAG94	<i>N</i> ⁶ -Cyclohexyl-2'- <i>O</i> -methyl-adenosine
TM	Transmembrane helix
VER6947	2-Amino- <i>N</i> -benzyl-6-(furan-2-yl)-9 <i>H</i> -purine-9-carboxamide
VER7835	2-Amino-6-(furan-2-yl)- <i>N</i> -(thiophen-2-ylmethyl)-9 <i>H</i> -purine-9-carboxamide

V2006	see BIIB014
WRC-0571	8-(<i>N</i> -Methylisopropyl)amino- <i>N</i> ⁶ -(5'-endohydroxy- endonorboman-2-yl-9-methyladenine
ZM241385	4-2-[7-Amino-2-(2-furyl)-1,2,4-triazolo[1,5- <i>a</i>][1,3,5]triazin-5-yl- amino]ethylphenol

1 Introduction

Extracellular adenosine acts as a cytoprotective modulator, under both physiological and pathophysiological conditions, in response to stress to an organ or tissue (Fredholm et al. 2001; Haskó et al. 2008; Jacobson and Gao 2006). This protective response might take the form of increased blood supply (vasodilation or angiogenesis) (Ryzhov et al. 2008), ischemic preconditioning (in the heart, brain, or skeletal muscle) (Akaiwa et al. 2006; Cohen and Downey 2008; Liang and Jacobson 1998; Zheng et al. 2007), and/or suppression of inflammation (activation and infiltration of inflammatory cells, production of cytokines and free radicals) (Chen et al. 2006b; Martin et al. 2006; Ohta and Sitkovsky 2001). Adenosine acts on cell surface receptors that are coupled to intracellular signaling cascades. There are four subtypes of G-protein-coupled receptors (GPCRs); i.e., four distinct sequences of adenosine receptors (ARs) termed A₁, A_{2A}, A_{2B}, and A₃ (Fig. 1). The second messengers associated with the ARs are historically defined with respect to the adenylate cyclase system (Fredholm and Jacobson 2009). The A₁ and A₃ receptors inhibit the production of cyclic AMP through coupling to G_i. The A_{2A} and A_{2B} subtypes are coupled to G_s or G_o to stimulate adenylate cyclase. Furthermore, the A_{2B} subtype, which has the lowest affinity ($K_i > 1 \mu\text{M}$) of all the subtypes for native adenosine, is also coupled to G_q (Ryzhov et al. 2006). Adenosine has the highest affinity at the A₁ and A_{2A} ARs (K_i values in binding of 10–30 nM at the high affinity sites), and the affinity of adenosine at the A₃AR is intermediate (ca. 1 μM at the rat A₃AR) (Jacobson et al. 1995).

Effector mechanisms other than the adenylate cyclase and phospholipase C are associated with the stimulation of ARs. For example, adenosine action can activate phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs), and extracellular receptor signal-induced kinase (ERK) (Schulte and Fredholm 2003). The indirect regulation by adenosine of MAPKs can have effects on differentiation, proliferation, and apoptosis (Che et al. 2007; Fredholm et al. 2001; Jacobson and Gao 2006; Schulte and Fredholm 2003). Thus, the A₃AR activates Akt to inhibit apoptosis. These actions may be initiated through the β , γ subunits of the G proteins, which can also lead to the coupling of ARs to ion channels. The influx of calcium ions or the efflux of potassium ions can be induced by the activation of the A₁AR. The arrestin pathway, which has the dual role of signal transmission and downregulation of the receptor, is also activated by ARs (Klaasse et al. 2008; Penn et al. 2001). The A_{2A}AR forms a tight complex with G_s by a process described as “restricted collision coupling” (Zezula and Freissmuth 2008). The A_{2A}AR also

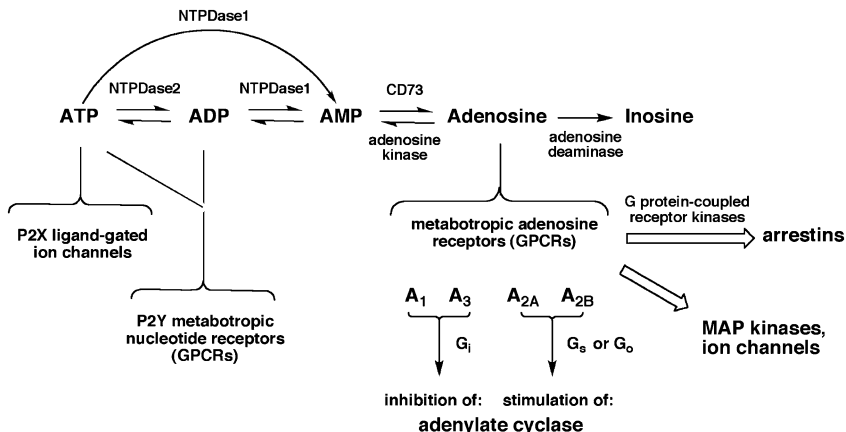


Fig. 1 Interconversion of extracellular adenine nucleotides and adenosine and their associated signaling pathways. These molecules may originate from intracellular sources. For example, adenosine may cross the plasma membrane through an equilibrative nucleoside transporter (ENT)1. The four subtypes of adenosine receptors (ARs) are grouped according to effects on adenylyl cyclase. Inosine at micromolar concentrations also activates the A₃AR. Various extracellular nucleotides activate seven subtypes of P2X receptors and eight subtypes of P2Y, which are not specified here. The ARs and P2Y receptors are G-protein-coupled receptors (GPCRs), while the P2X receptors are ionotropic receptors. The ectonucleoside triphosphate diphosphohydrolases NTPDase1 and NTPDase2 are also known as CD39 (apyrase) and CD39L1, respectively. NTPDases3 and 8 (not shown) are also involved in breakdown of extracellular nucleotides

binds to additional “accessory” proteins, such as alpha-actinin, ARNO, USP4 and translin-associated protein-X (Zezula and Freissmuth 2008).

Adenosine suppresses various cytotoxic processes, such as cytokine-induced apoptosis. In the brain, both neuronal and glial cell functions are regulated by adenosine (Björklund et al. 2008; Fredholm et al. 2005). Adenosine acts as a local modulator of the action of various other neurotransmitters, including biogenic amines and excitatory amino acids. Adenosine attenuates the release of many stimulatory neurotransmitters and can counteract the excitotoxicity associated with excessive glutamate release in the brain. Adenosine can also modulate the interaction of neurotransmitters, such as dopamine, with their own receptors. In the periphery, adenosine has been shown to attenuate excessive inflammation, to promote wound healing, and to protect tissue against ischemic damage (Chen et al. 2006a; Haskó et al. 2008). In the cardiovascular system, adenosine promotes vasodilation, vascular integrity, and angiogenesis, and also counteracts the lethal effects of prolonged ischemia on cardiac myocytes and skeletal muscle (Cohen and Downey 2008; Zheng et al. 2007).

Therapeutic applications, both in the central nervous system and in the periphery, are being explored for selective AR agonists and antagonists. A large body of medicinal chemistry has been created around the four AR subtypes, such that selective agonists and antagonists are now available for each. These ligands have been used as pharmacological probes to introduce many new drug concepts. Mouse

strains in which an AR has been genetically deleted (each of the subtypes has now been deleted) have also been useful in developing novel drug concepts based on the modulation of ARs (Fredholm et al. 2005).

Adenosine itself is short-lived in the circulation, which has allowed its clinical use in the treatment of paroxysmal supraventricular tachycardia and in radionuclide myocardial perfusion imaging (Cerqueira 2006). The many selective and potent synthetic AR agonists, which are typically much longer lasting in the body than adenosine, have been slower to enter a clinical pathway than adenosine. Recently, the first such synthetic adenosine agonist, Lexiscan (regadenoson, CV Therapeutics, Palo Alto, CA, USA), an A_{2A} AR agonist, was approved for diagnostic use (Lieu et al. 2007).

Synthetic adenosine agonists have potential therapeutic applications based on their anti-inflammatory (A_{2A} and A_3) (Haskó et al. 2008; Ohta and Sitkovsky 2001), cardioprotective (preconditioning of the ischemic heart muscle by activation of the A_1 and A_3 ARs and its postconditioning by A_{2B} AR activation) (Cohen and Downey 2008), cerebroprotective (A_1 and A_3) (Chen et al. 2006a; Knutsen et al. 1999; von Lubitz et al. 1994), and antinociceptive (A_1) (Johansson et al. 2001) properties. Potent and selective AR antagonists display therapeutic potential as kidney protective (A_1) (Gottlieb et al. 2002), antifibrotic (A_{2A}) (Che et al. 2007), neuroprotective (A_{2A}) (Yu et al. 2004), antiasthmatic (A_{2B}) (Holgate 2005), and antiglaucoma (A_3) (Yang et al. 2005) agents. A_3 AR agonists have been proposed for the treatment of a wide range of autoimmune inflammatory conditions, such as rheumatoid arthritis, inflammatory bowel diseases, psoriasis, etc. (Guzman et al. 2006; Kolachala et al. 2008; Madi et al. 2007), and also for cardiac and brain ischemia. A_1 AR agonists are useful in preclinical models of cardiac arrhythmia and ischemia and in pain. Adenosine agonists are also of interest for the treatment of sleep disorders (Porkka-Heiskanen et al. 1997). Activation of the A_{2B} AR protects against vascular injury (Yang et al. 2008).

The alkylxanthines caffeine and theophylline are the prototypical antagonists of ARs, and their stimulant actions are produced primarily through blocking the depressant actions of adenosine through the A_1 and A_{2A} ARs (Fredholm and Jacobson 2009). Prior to the work of Rall, Daly, and other pioneers in the field, the stimulant actions of the alkylxanthines were thought to occur as a result of inhibition of phosphodiesterases. It is true that caffeine inhibits phosphodiesterases and has other actions, such as stimulation of calcium release, but these non-AR-mediated actions require higher concentrations of caffeine than are typically ingested in the human diet (Fredholm and Jacobson 2009).

The nonselective AR antagonist theophylline has been in use as an antiasthmatic drug (Holgate 2005), although its use is now limited as a result of side effects on the central nervous system and the renal system. Adenosine antagonists of various selectivities remain of interest as potential drugs for treating asthma (Wilson 2008). A large number of synthetic AR antagonists that are much more potent and selective than the prototypical alkylxanthines have been introduced, although none have yet been approved for clinical use. For example, AR antagonists have been proposed for neurodegenerative diseases (such as Parkinson's disease and Alzheimer's disease) (Schwarzschild et al. 2006), although a well-advanced A_{2A} AR antagonist

KW6002 (Istradefylline) (8-[(*E*)-2-(3,4-dimethoxyphenyl)vinyl]-1,3-diethyl-7-methylpurine-2,6-dione, Kyowa Hakko Kirin Co. Ltd, Tokyo, Japan) was recently denied FDA approval for the treatment of Parkinson's disease (LeWitt et al. 2008).

2 Sources and Fate of Extracellular Adenosine

Adenosine is not a classical neurotransmitter because it is not principally produced and released vesicularly in response to neuronal firing. Most tissues in the body and cells in culture release adenosine to the extracellular medium, from where it can feed back and act as an autocoid on the ARs present locally. The basal levels of extracellular adenosine have been estimated as roughly 100 nM in the heart and 20 nM in the brain, which would only partially activate the ARs present (Fredholm et al. 2005). In the case of severe ischemic stress, the levels can rapidly rise to the micromolar range, which would cause a more intense and generalized activation of the four subtypes of ARs. Nevertheless, it is thought that the exogenous administration of highly potent and selective AR agonists in such cases of severe ischemic challenge might still provide additional benefit beyond that offered by the endogenous adenosine generated (Jacobson and Gao 2006; Yan et al. 2003).

Extracellular adenosine may arise from intracellular adenosine or from the breakdown of the adenine nucleotides, such as adenosine triphosphate (ATP), outside the cell (Fig. 1). Adenosine, which is present in a higher concentration inside than outside the cell, does not freely diffuse across the cell membrane. There are nucleoside transporters, such as the equilibrative nucleoside transporter (ENT), ENT1, which bring it to the extracellular space. Extracellular nucleotides activate their own receptors, known as P2Y metabotropic and P2X inotropic receptors (Burnstock 2008). Extracellular nucleotides may also originate from cytosolic sources, including by vesicular release exocytosis, passage through channels, and cell lysis. Ectonucleotidases break down the adenine nucleotides in stages to produce free extracellular adenosine at the terminal step (Zimmermann 2000). For example, the extracellular enzyme ectonucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1) converts ATP and adenosine diphosphate (ADP) to adenosine monophosphate (AMP). A related ectonucleotidase, E-NTPDase2, primarily hydrolyzes 5'-triphosphates to 5'-diphosphates. The final and critical step, with respect to AR activation, of conversion of AMP to adenosine is carried out by ecto-5'-nucleotidase, also known as CD73. Overexpression of CD73 has been proposed to protect organs under stress by the formation of cytoprotective adenosine (Beldi et al. 2008). The adenosine produced extracellularly is also subject to metabolic breakdown by adenosine deaminase to produce inosine or (re)phosphorylation by adenosine kinase to produce AMP. Therefore, when an organ is under stress there is a highly complex and time-dependent interplay of the activation of many receptors in the same vicinity. In addition to the direct activation of ARs by selective agonists or their blockade by selective antagonists, inhibition of the metabolic or transport pathways surrounding adenosine is also being explored for therapeutic purposes (McGaraughty et al. 2005).

3 Adenosine Receptor Structure

The ARs, as GPCRs, share the structural motif of a single polypeptide chain forming seven transmembrane helices (TMs), with the N-terminus being extracellular and the C-terminus being cytosolic (Costanzi et al. 2007). These helices, consisting of 25–30 amino acid residues each, are connected by six loops, i.e., three intracellular (IL) and three extracellular (EL) loops. The extracellular regions contain sites for posttranslational modifications, such as glycosylation. The A₁ and A₃ ARs also contain sites for palmitoylation in the C-terminal domain. The A_{2A}AR has a long C-terminal segment of more than 120 amino acid residues, which is not required for coupling to G_s, but can serve as a binding site for “accessory” proteins (Zezula and Freissmuth 2008). The sequence identity between the human A₁ and A₃ ARs is 49%, and the human A_{2A} and A_{2B} ARs are 59% identical. Particular conserved residues point to specific functions. For example, there are two characteristic His residues in TMs 6 and 7 of the A₁, A_{2A}, and A_{2B} ARs. In the A₃AR, the His residue in TM6 is lacking but another His residue has appeared at a new location in TM3. All of these His residues have been indicated by mutagenesis to be important in the recognition and/or activation function of the receptor (Costanzi et al. 2007; Kim et al. 2003).

Recently, the human A_{2A}AR joined the shortlist of GPCRs for which an X-ray crystallographic structure has been determined (Jaakola et al. 2008). The reported structure (Fig. 2) contained a bound high-affinity antagonist ligand, ZM241385 (4-2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*][1,3,5]triazin-5-yl-amino]ethylphenol), which is moderately selective for the A_{2A}AR. Prior to this dramatic step in bringing ARs into the age of structural biology, homology modeling of the ARs, based on a rhodopsin template, was the principal means of AR structural prediction and was useful in interpreting mutagenesis data. The modeling has defined two subregions within the putative agonist binding site (Costanzi et al. 2007; Kim et al. 2003). This putative binding site is located within the barrel or cleft created by five of the seven TMs (excluding TM1 and TM2), approximately one-third of the distance across the membrane from the exofacial side. The ribose moiety of adenosine binds in a hydrophilic region defined by TMs 3 and 7, and the adenine moiety binds in a largely hydrophobic region surrounded by TMs 5 and 6. Thus, the region of adenosine in the binding site is approximately the same as the position of the retinal in rhodopsin. Even the importance of the Lys residue in TM7 of rhodopsin that forms the covalent association (Schiff base) with retinal is conserved by analogy in the ARs, i.e., with a His residue that occurs at the same position (7.43) in all of the ARs. The His residue is predicted by molecular modeling to associate with the ribose moiety of adenosine. Features of the putative binding site of adenosine have been reviewed recently (Costanzi et al. 2007). Different labs have not been in agreement on the precise placement of the adenosine moiety when docked in the receptor. However, the major modeling publications in this area have zeroed in on the same limited region of the receptor structure for coordination of adenosine. One can consider the modeling approach to provide insights that are subject to refinement over time, as more is learned from mutagenesis studies and the modeling

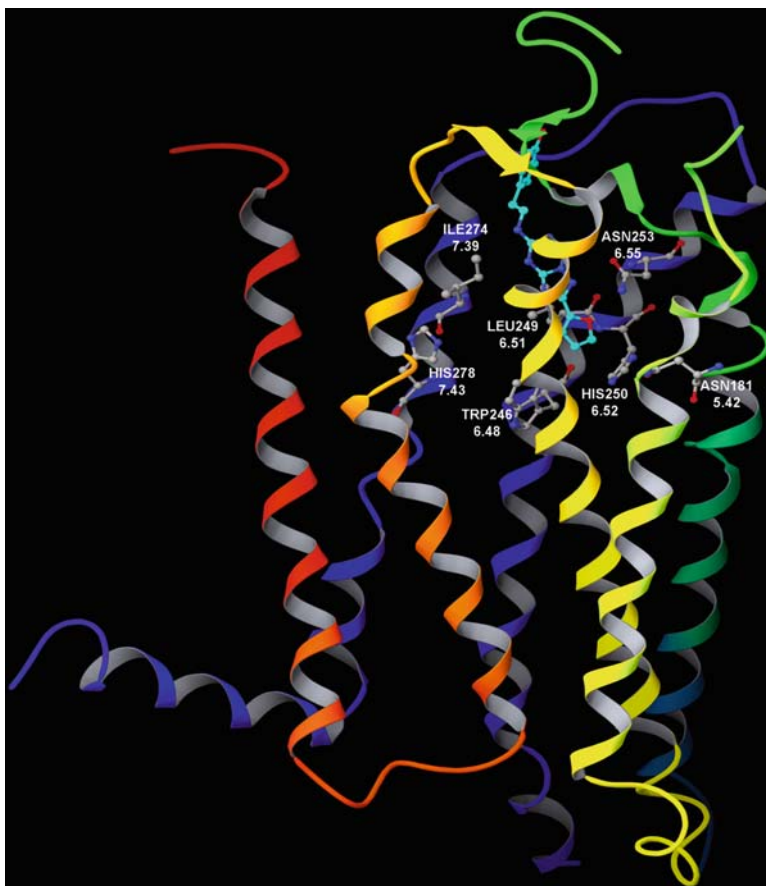


Fig. 2 X-ray crystallographic structure of the human A_{2A} adenosine receptor (AR), showing the bound antagonist ZM241385 (Jaakola et al. 2008). The structure of the A_{2A} AR is colored by region: N-terminus and transmembrane helical (TM) domain 1 in *orange*, TM2 in *ochre*, TM3 in *yellow*, TM4 in *green*, TM5 in *cyan*, TM6 in *blue*, TM7 and C-terminus in *purple*. The *p*-hydroxyphenylethyl moiety of the antagonist ligand points toward the exofacial side of the receptor

templates and computational methods are refined (Ivanov et al. 2009). Many amino acid residues predicted by molecular modeling to be involved in the coordination of antagonists by the A_{2A} AR were indeed in proximity to the bound ZM241385 in the X-ray structure, although the molecule was somewhat rotated from the orientation predicted in various docking models. These residues include Asn253 in TM6, which hydrogen bonds to the exocyclic NH of agonists and various antagonists in the AR models. The same residue was found to form a hydrogen bond with the exocyclic NH of ZM241385.

Dimerization has been proposed to occur between ARs, leading to homo- or heterodimers (Franco et al. 2006). Dimerization between ARs and other receptors has also been proposed; for example, A_1 AR/ D_1 dopamine receptor dimers

and $A_{2A}AR/D_2$ dopamine receptor dimers (Franco et al. 2006). Heterodimers of the A_1AR with either $P2Y_1$ or $P2Y_2$ nucleotide receptors or with metabotropic glutamate receptors have been detected (Prinster et al. 2005). The pharmacological properties of these heterodimers may differ dramatically from the properties of each monomer alone. For example, the $A_1AR/P2Y_1$ dimers have been characterized pharmacologically and were found to be inhibited by known nucleotide antagonists but not activated by known nucleotide agonists of the $P2Y_1$ receptor (Nakata et al. 2005). Dimers of A_{2A} adenosine/ D_2 dopamine receptors are present in striatum and display a modified pharmacology relative to each of the individual subtypes. These receptor dimers are drug development targets for Parkinson's disease (Schwarzschild et al. 2006).

4 Regulation of Adenosine Receptors

Similar to the function and regulation of other GPCRs, both activation and desensitization of the ARs occur after agonist binding. Interaction of the activated ARs with the G proteins leads to second messenger generation and classical physiological responses. Interaction of the activated ARs with G protein-coupled receptor kinases (GRKs) leads to their phosphorylation. Downregulation of ARs should be considered in both the basic pharmacological studies and with respect to the possible therapeutic application of agonists. AR responses desensitize rapidly, and this phenomenon is associated with receptor downregulation, internalization and degradation. The internalization and desensitization of ARs has been reviewed recently (Klaasse et al. 2008). Mutagenesis has been applied to analyze the molecular basis for the differences in the kinetics of the desensitization response displayed by various AR subtypes. The most rapid downregulation among the AR subtypes is generally seen with the A_3AR , due to phosphorylation by GRKs. The $A_{2A}AR$ is only slowly desensitized and internalized as a result of agonist activation.

5 Adenosine Receptor Agonists and Antagonists in Preclinical and Clinical Trials

Potent and selective AR agonists and antagonists have been synthesized for all four AR subtypes, with selective $A_{2B}AR$ agonists being the most recently reported (Baraldi et al. 2009). Some of these ligands are selective for a single AR subtype, and others have mixed selectivity for several subtypes. Thus, numerous pharmacological tools for studying the ARs are available, and some of these compounds have advanced to clinical studies (Baraldi et al. 2008; Elzein and Zablocki 2008; Giorgi and Neri 2008; Moro et al. 2006).

A general caveat in the design of selective agonists and antagonists is the frequent observation of a variation of affinity for a given compound at the same subtype in

different species. There are many examples of marked species dependence of ligand affinity at the ARs (Jacobson and Gao 2006; Yang et al. 2005). Therefore, caution must be used in generalizing the selectivity of a given compound from one species to another. In general, one must be cognizant of potential species differences for both AR agonists and antagonists.

5.1 Adenosine Receptor Agonists

Nearly all AR agonists reported are adenosine derivatives. A noteworthy exception is the class of pyridine-3,5-dicarbonitrile derivatives that fully activate ARs and that display varied selectivity at the AR subtypes (Beukers et al. 2004). One such compound is the A_{2B}AR-selective agonist BAY 60–6583 (2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide) (Cohen and Downey 2008; Eckle et al. 2007). Another AR agonist of nonnucleoside structure is BAY 68–4986 (Capadenoson), which is a selective A₁AR agonist in clinical trials for the oral treatment of stable angina pectoris (Mittendorf and Wuppertal 2008). The structure–activity relationships (SARs) of adenosine derivatives as agonists of the ARs have been thoroughly probed (Jacobson and Gao 2006; Yan et al. 2003), and representative agonists are shown in Fig. 3. In general, substitution at the N6 position with certain alkyl, cycloalkyl, and arylalkyl groups increases selectivity for the A₁AR. Substitution with an N⁶-benzyl group or substituted benzyl group increases selectivity for the A₃AR. Substitution at the 2 position, especially with ethers, secondary amines, and alkynes, often results in high selectivity for the A_{2A}AR.

All of the A₁AR agonists shown in Fig. 3 contain a characteristic N6 modification. The singly substituted A₁AR agonists NNC-21-0136 (2-chloro-N⁶-[(R)-[(2-benzothiazoly)thio]-2-propyl]-adenosine) and GR79236 (N⁶-[(1S, 2S)-2-hydroxycyclopentyl]adenosine) (Merkel et al. 1995) and the doubly substituted selodenoson have been clinical candidates. NNC-21-0136 was the result of a program to develop CNS-selective AR agonists for use in treating stroke and other neurodegenerative conditions (Knutsen et al. 1999). A₁AR agonists are of interest for use in treating cardiac arrhythmias [for which adenosine itself, under the name Adenocard (Astellas Pharma, Inc., Tokyo, Japan), is in widespread use]. The A₁AR agonist SDZ WAG94 (2'-O-methyl-N⁶-cyclohexyladenosine) was under consideration for treatment of diabetes (Ishikawa et al. 1998). The AR agonist of mixed selectivity AMP579 ([1S-[1 α , 2 β , 3 β , 4 α (S*)]]-4-[7-[[1-[(3-chlorothien-2-yl)methyl]propyl]amino]-3*H*-imidazo[4,5-*b*]pyrid-3-yl] N-ethyl-2,3-dihydroxycyclopentanecarboxamide) has cardioprotective properties (Cohen and Downey 2008). The 2-substituted A_{2A}AR agonists ATL-146e (4-{3-[6-amino-9-(5-ethylcarbamoyl)-3,4-dihydroxy-tetrahydro-furan-2-yl]-9*H*-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester), binodenoson (2-[[cyclohexylmethylene]hydrazino]adenosine, MRE-0470 or WRC-0470), and MRE0094 (2-[2-(4-chlorophenyl)ethoxy]adenosine) have been cardiovascular clinical candidates (Awad et al. 2006; Desai et al. 2005; Udelson et al. 2004). Several of the A_{2A}AR agonists shown in Fig. 3 contain the 5'-uronamide

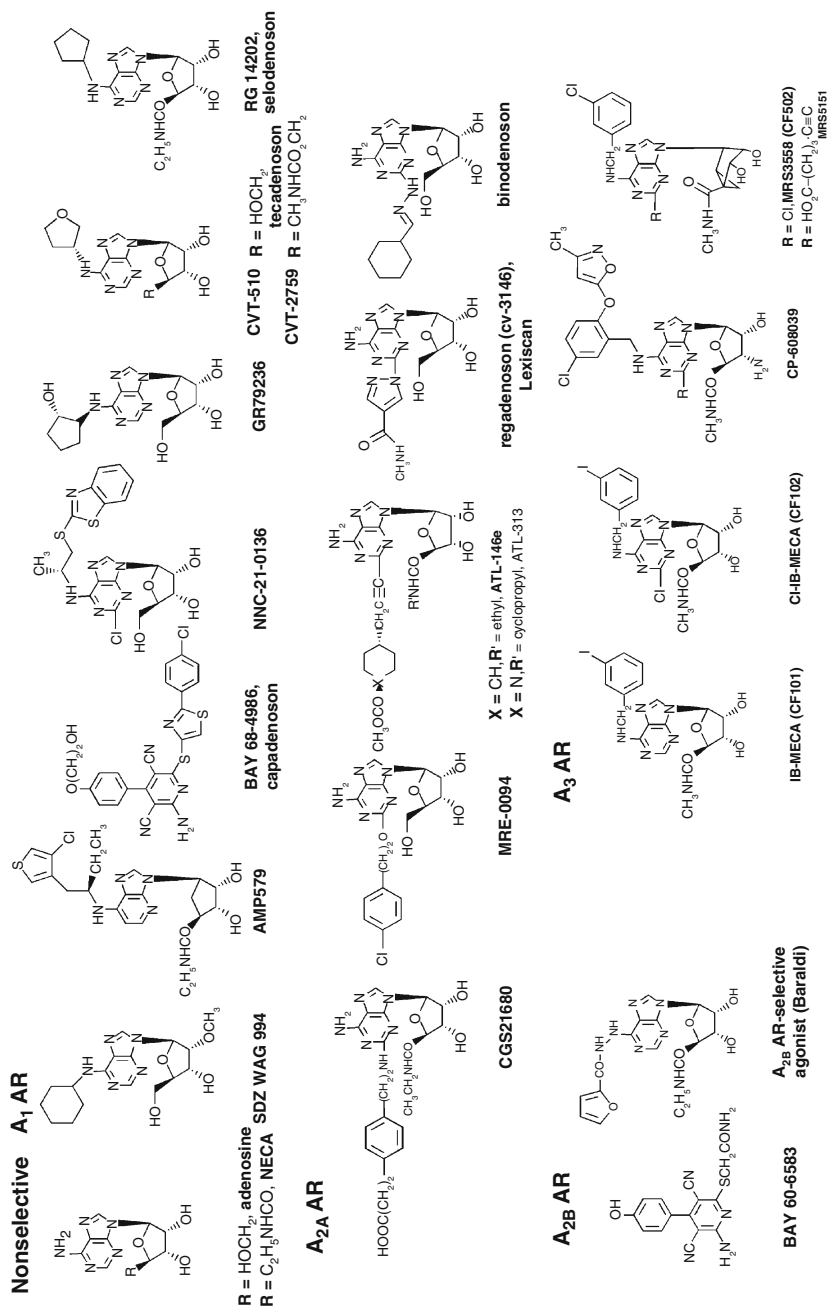


Fig. 3 Structures of selected adenosine receptor (AR) agonists. *K*₁ values in binding are available in references (Baraldi et al. 2008; Jacobson and Gao 2006; Yan et al. 2003)

modification, characteristic of NECA; others have the adenosine-like CH₂OH group. Such agonists are of interest for use as vasodilatory agents in cardiac imaging [adenosine itself, under the name Adenoscan (Astellas Pharma, Inc., Tokyo, Japan), is in use for this purpose] and in suppressing inflammation (Cerqueira 2006). CVT-3146 (1-[6-amino-9-[(2*R*, 3*R*, 4*S*, 5*R*)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]purin-2-yl]-*N*-methylpyrazole-4-carboxamide, Lexiscan, regadenoson) is already approved for diagnostic imaging (Lieu et al. 2007).

All of the A₃AR agonists shown in Fig. 3 contain the NECA-like 5'-uronamide modification and have nanomolar affinity at the receptor. CP-608,039 ((2*S*, 3*S*, 4*R*, 5*R*)-3-amino-5-{6-[5-chloro-2-(3-methylisoxazol-5-yl)methoxy]benzylamino}purin-9-yl-1-4-hydroxytetrahydrofuran-2-carboxylic acid methylamide) and its *N*⁶-(2,5-dichlorobenzyl) analog CP-532,903 ((2*S*, 3*S*, 4*R*, 5*R*)-3-amino-5-{6-[2, 5-dichlorobenzylamino]purin- 9-yl-1- 4-hydroxytetrahydrofuran-2-carboxylic acid methylamide) (Wan et al. 2008) (not shown) are selective A₃ agonists that were developed for cardioprotection. CF101 (*N*⁶-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine, IB-MECA) is being studied by Can-Fite Biopharma (Petah-Tikva, Israel) for the treatment of rheumatoid arthritis (Phase IIb), dry eye syndrome (Phase II) and psoriasis (Phase II) (<http://clinicaltrials.gov>). Can-Fite Biopharma is also developing the A₃AR agonist CF102 (2-chloro-*N*⁶-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine, CI-IB-MECA) for the treatment of liver conditions, including liver cancer, hepatitis infections and liver tissue regeneration (Bar-Yehuda et al. 2008; Madi et al. 2004). The North conformation of the ribose ring was found to be the preferred conformation at the A₃AR, which accounts for the high potency and selectivity of the rigid analog MRS3558 ((1'*S*, 2'*R*, 3'*S*, 4'*R*, 5'*S*)-4'-{2-chloro-6-[(3-chlorophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol) at the human and rat A₃ARs (Ochaion et al. 2008). The bicyclic ring constrains the ribose-like moiety in the desired conformation. The recent generation agonist in the same chemical series MRS5151 ((1'*S*, 2'*R*, 3'*S*, 4'*R*, 5'*S*)-4'-[6-(3-chlorobenzylamino)-2-(5-hydroxycarbonyl-1-pentynyl)-9-yl]-2', 3'-dihydroxybicyclo[3.1.0]hexane-1'-carboxylic acid *N*-methylamide) is designed to be A₃AR selective in at least three different species, including mouse (Melman et al. 2008a).

Recently, macromolecular conjugates (e.g., dendrimers) of chemically functionalized AR agonists were introduced as potent polyvalent activators of the receptors that are qualitatively different in pharmacological characteristics in comparison to the monomeric agonists (Kim et al. 2008; Klutz et al. 2008). The feasibility of using dendrimer conjugates to bind to AR dimers was studied using a molecular modeling approach (Ivanov and Jacobson 2008).

5.2 Adenosine Receptor Antagonists

The newer and most selective AR antagonists are more chemically diverse than the classical 1,3-dialkylxanthines, which have been used pharmacologically as

antagonists of the A₁ and A₂ ARs. A range of AR antagonists and their synthetic methods were recently reviewed (Baraldi et al. 2008; Moro et al. 2006).

Purine AR antagonists, including both xanthine and adenine derivatives, have provided a wide range of receptor subtype selectivity, depending on the substitution (Fig. 4). In general, modifications of the xanthine scaffold at the 8 position with aryl or cycloalkyl groups has led to high affinity and selectivity for the A₁AR. Highly selective xanthine antagonists of the A₁AR (e.g., the epoxide derivative BG 9719 (1,3-dipropyl-8-(2-(5,6-epoxy)norbornyl)xanthine) and the more water soluble BG9928 (3-[4-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)

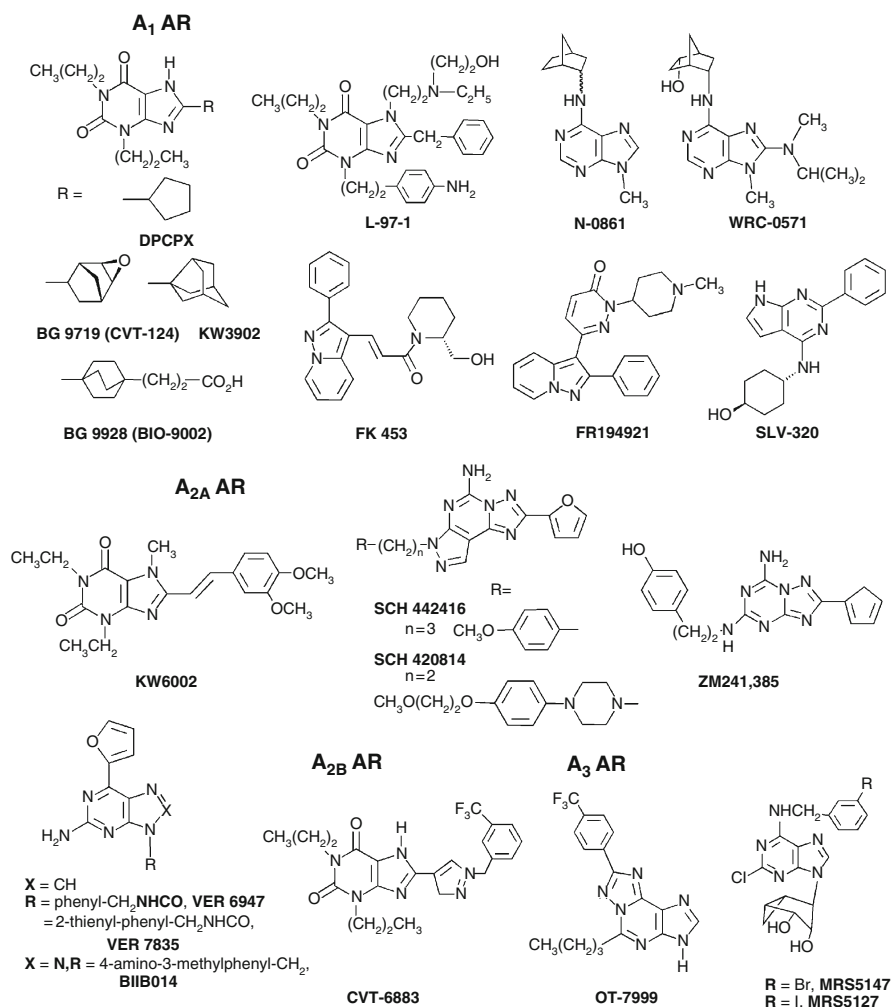


Fig. 4 Structures of selected adenosine receptor (AR) antagonists. K_i values in binding are available in references (Baraldi et al. 2008; Jacobson and Gao 2006)

-bicyclo[2.2.2]oct-1-yl]-propionic acid, Biogen Idec, Cambridge, MA, USA), as well as KW3902 (8-(noradamtan-3-yl)-1,3-dipropylxanthine, Merck and Co., Inc., Whitehouse Station, NJ, USA) have been (BG 9719) (Gottlieb et al. 2002) or are currently (BG9928 and KW3902) (Cotter et al. 2008; Ditttrich et al. 2007; Givertz et al. 2007; Greenberg et al. 2007) in clinical trials for treatment of acute decompensated heart failure (ADHF) with renal impairment. In dogs, both BG9719 and BG9928 have high affinity for both the A₁AR and A_{2B}AR (Auchampach et al. 2004) with A_{2B}/A₁ ratios of 21 and 24, respectively (Doggrell 2005). The selectivity of BG 9928 for the human A₁AR compared to the human A_{2B}AR is 12 (Kiesman et al. 2006). The 8-cyclopentyl derivative DPCPX (8-cyclopentyl-1,3-dipropylxanthine), also known as CPX, which is selective for the A₁AR in the rat with nanomolar affinity but less selective at the human AR subtypes, has been in clinical trials for cystic fibrosis through a non-AR-related mechanism (Arispe et al. 1998). The highly selective A₁AR antagonist L-97-1 (3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-[2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl]-1-propyl-3,7-dihydro-purine-2,6-dione, Endacea Inc., Research Triangle Park, NC, USA) is water soluble and in late preclinical development for the treatment of asthma (Wilson 2008). As in the cases of DPCPX, BG 9719, N-0861 ((±)-N⁶-endonorboman-2-yl-9-methyladenine), and others, a persistent problem in the development of A₁AR antagonists is low aqueous solubility, e.g., high lipophilicity, corresponding low water solubility, and low bioavailability (Hess 2001); thus, A₁AR antagonists, e.g., BG 9928 and L-97-1, with good water solubility are preferable clinical candidates. Moreover, a persistent problem in the use of xanthine derivatives as AR antagonists is their interaction at the A_{2B}AR. Modification of xanthines at the 8 position with certain aryl groups has given rise to preclinical candidates that are selective for the A_{2B}AR (e.g., CVT-6883, 3-ethyl-1-propyl-8-[1-(3-trifluoromethylbenzyl)-1H-pyrazol-4-yl]-3,7-dihydropurine-2,6-dione, CV Therapeutics, Palo Alto, CA, USA) (Mustafa et al. 2007). Use of the adenine derivatives WRC-0571 (8-(N-methylisopropyl)amino-N⁶-(5'-endohydroxy-endonorboman-2-yl-9-methyladenine) as an inverse agonist at the A₁AR provides A₁AR selective antagonism without blocking the A_{2B}AR (Martin et al. 1996). Nonxanthine antagonists of the A₁AR have also been shown to have high receptor subtype selectivity, e.g., FK453 (Terai et al. 1995) and SLV 320 (4-[(2-phenyl-7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino]-trans-cyclohexanol, Solvay Pharmaceuticals SA, Brussels, Belgium) (Hochoer et al. 2008). Moreover, various nonxanthine A₁AR antagonists have been or are currently being explored for clinical applications (Jacobson and Gao 2006). For example, SLV 320 is in clinical trials as an intravenous treatment for ADHF with renal impairment (<http://clinicaltrials.gov>).

Modification of xanthines at the 8 position with alkenes (specifically styryl groups) has led to selectivity for the A_{2A}AR. Such derivatives include the A_{2A}AR antagonist KW6002 (istradefylline), which has been in clinical trials. Some 8-styrylxanthine derivatives, such as CSC (8-(3-chlorostyryl)caffeine), have been discovered to inhibit monoamine oxidase-B, as well as the A_{2A}AR (Vlok et al. 2006). The triazolotriazine ZM241385 and the pyrazolotriazolopyrimidine

SCH 442416 (5-amino-7-(3-(4-methoxy)phenylpropyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine) are highly potent A_{2A}AR antagonists (Moresco et al. 2005; Palmer et al. 1996). ZM241385 also binds to the human A_{2B}AR with moderate affinity, and has been used as a radioligand at that subtype (Ji and Jacobson 1999). SCH 442416 displays > 23,000-fold selectivity for the human A_{2A}AR (K_i 0.048 nM) in comparison to human A₁AR and an IC₅₀ > 10 μM at the A_{2B} and A₃ ARs. A_{2A}AR antagonists, such as the xanthine KW6002 and the nonxanthines SCH 442416, VER 6947 (2-amino-*N*-benzyl-6-(furan-2-yl)-9*H*-purine-9-carboxamide), and VER 7835 (2-amino-6-(furan-2-yl)-*N*-(thiophen-2-ylmethyl)-9*H*-purine-9-carboxamide), are of interest for use in treating Parkinson's disease (Gillespie et al. 2008; LeWitt et al. 2008; Schwarzschild et al. 2006). The A_{2A}AR antagonist BIIB014 (V2006) has begun Phase II clinical trials (Biogen Idec, Cambridge, MA, USA, in partnership with Vernalis, Cambridge, UK) for Parkinson's disease (Jordan 2008).

Cyclized derivatives of xanthines, such as PSB-11 (8-ethyl-4-methyl-2-phenyl-(8*R*)-4,5,7,8-tetrahydro-1*H*imidazo[2.1-*i*]purin-5-one), are A₃AR-selective, and similar compounds have been explored by Kyowa Hakko. Selective A₃AR antagonists, such as the heterocyclic derivatives OT-7999 (5-*n*-butyl-8-(4-trifluoromethylphenyl)-3*H*-[1,2,4]triazolo-[5,1-*i*]purine), are being studied for the treatment of glaucoma (Okamura et al. 2004), and other such antagonists are under consideration for treatment of cancer, stroke, and inflammation (Gessi et al. 2008; Jacobson and Gao 2006). MRS5147 ((1'*R*, 2'*R*, 3'*S*, 4'*R*, 5'*S*)-4'-[2-chloro-6-(3-bromobenzylamino)-purine]-2', 3'-*O*-dihydroxybicyclo-[3.1.0]hexane) and its 3-iodo analog MRS5127 are highly selective A₃AR antagonists in both human and rat, based on a conformationally constrained ribose-like ring that is truncated at the 5' position (Melman et al. 2008b). No selective A₃AR antagonists have yet reached human trials. However, an antagonist of mixed A_{2B}/A₃AR selectivity in the class of 5-heterocycle-substituted aminothiazoles from Novartis (Horsham, UK), QAF 805 (Press et al. 2005), was in a Phase Ib clinical trial for the treatment of asthma. This antagonist failed to decrease sensitivity to the bronchoconstrictive effects of AMP in asthmatics (Pascoe et al. 2007).

5.3 Radioligands for *In Vivo* Imaging

With the established relevance of ARs to human disease states, it has been deemed useful to develop high-affinity imaging ligands for these receptors, for eventual diagnostic use in the CNS and in the periphery. Ligands for *in vivo* positron emission tomographic (PET) imaging of A₁, A_{2A}, and A₃ ARs have been developed. For example, the xanthine [¹⁸F]CPFPX (8-cyclopentyl-1-propyl-3-(3-fluoropropyl)-xanthine, similar in structure to DPCPX) and the nonxanthine [¹¹C]FR194921 (2-(1-methyl-4-piperidiny)-6-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl)-3(2*H*)-pyridazinone) have been developed as centrally-active PET tracers for imaging of the A₁AR in the brain (Bauer et al. 2005). The first PET

ligand for the A_{2A} AR was [7-methyl- ^{11}C]-(*E*)-8-(3,4,5-trimethoxystyryl)-1,3,7-trimethylxanthine ([^{11}C]TMSX) (Ishiwata et al. 2000). This is a caffeine analog related to the series of KW6002, introduced by the Kyowa Hakko. 5-Amino-7-(3-(4-[^{11}C]methoxy)phenylpropyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine ([^{11}C]SCH442416) has recently been explored as a PET agent in the noninvasive in vivo imaging of the human A_{2A} AR (Moresco et al. 2005). [^{11}C]SCH442416 displays an extremely high affinity at the human A_{2A} AR (K_i 0.048 nM). Recently, an A_3 AR PET ligand, [F-18]FE@SUPPY (5-(2-fluoroethyl) 2,4-diethyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate), based on a series of pyridine A_3 AR antagonists, was introduced (Wadsak et al. 2008). Several nucleoside derivatives that bind with nanomolar affinity at the A_3 AR and that contain ^{76}Br for PET imaging were recently reported, including the antagonist MRS5147 (Kiesewetter et al. 2008).

6 Allosteric Modulation of Adenosine Receptors

In addition to directly acting AR agonists and antagonists, allosteric modulators of A_1 and A_3 ARs have been introduced (Gao et al. 2005). Allosteric modulators have advantages over the directly acting (orthosteric) receptor ligands in that they would magnify the effect of the native adenosine released in response to stress at a specific site or tissue and, in theory, would not induce a biological effect in the absence of an agonist. Various allosteric enhancers of the activation of ARs by agonists are under consideration as clinical candidates. The benzoylthiophenes, represented by PD-81,723 (Fig. 5), were the first AR allosteric modulators to be identified. A structurally related benzoylthiophene derivative known as T-62 ((2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)-(4-chlorophenyl)-methanone), which acts as a selective positive enhancer of the A_1 AR, like PD-81,723 (2-amino-4,5-dimethyl-3-thienyl-[3-trifluoromethylphenyl]methanone), had progressed toward clinical trials for neuropathic pain (Li et al. 2004). LUF6000 (*N*-(3,4-dichlorophenyl)-2-cyclohexyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine) is a selective positive enhancer of the human A_3 AR (Gao et al. 2008).

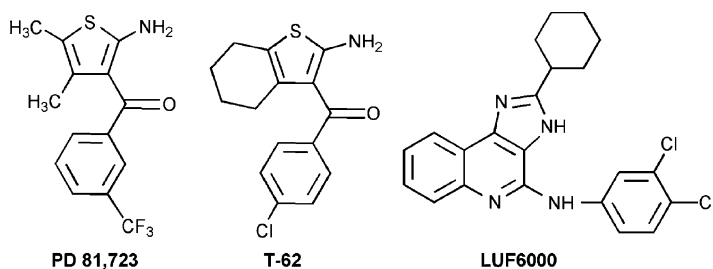


Fig. 5 Allosteric modulators of adenosine receptors (ARs)

7 Genetic Deletion of Adenosine Receptors

Deletion of each of the four AR subtypes has been carried out, and the resulting single-AR knockout (KO) mice are viable and not highly impaired in function (Fredholm et al. 2005; Yang et al. 2008). The pharmacological profile indicates that the analgesic effect of adenosine is mediated by the A₁AR, and analgesia is lost in mice in which the A₁AR has been genetically eliminated. Genetic KO of the A₁AR in mice removes the discriminative-stimulus effects but not the arousal effect of caffeine and increases anxiety and hyperalgesia. Study of A_{2A}AR KO mice reveals functional interaction between the spinal opioid receptors and peripheral ARs. A₁AR KO mice demonstrate a decreased thermal pain threshold, whereas A_{2A}AR null mice demonstrate an increased threshold to noxious heat stimulation, supporting an A₁AR-mediated inhibitory and an A_{2A}AR-mediated excitatory effect on pain transduction pathways. KO of the A_{2A}AR eliminates the arousal effect of caffeine. Genetic KO of the A_{2A}AR also suggests a link to increased anxiety and protected against damaging effects of ischemia and the striatal toxin 3-nitropropionic acid. Genetic KO of the A₃AR leads to increased neuronal damage in a model of carbon monoxide-induced brain injury. Neutrophils lacking A₃ARs show correct directionality but diminished speed of chemotaxis (Chen et al. 2006b). Although studies on A_{2B}AR KO mice have been reported (Yang et al. 2008), the importance of A_{2B}AR in the brain still awaits future investigation.

8 Conclusions

In conclusion, adenosine is released in response to organ stress or tissue damage and displays cytoprotective effects, in general, both in the brain and in the periphery. When excessive activity occurs in a given organ, adenosine acts as an endogenous quieting substance, to either reduce the energy demand or increase the energy supply to that organ. Nearly every cell type in the body expresses one or more of the AR subtypes, which indicates the central role of this feedback system in protecting organs and tissues and in tissue regeneration. Thus, a common theme to the therapeutic applications proposed for agonists is that adenosine acts as a cytoprotective modulator in response to stress to an organ or tissue.

Selective agonists and antagonists have been introduced and used to develop new therapeutic drug concepts. ARs are notable among the GPCR family in terms of the number and variety of agonist drug candidates that have been proposed. Thus, this has led to new experimental agents based on anti-inflammatory (A_{2A} and A₃), cardioprotective (preconditioning by A₁ and A₃ and postconditioning by A_{2B}), cerebroprotective (A₁ and A₃), and antinociceptive (A₁) effects. Potent and selective AR antagonists display therapeutic potential as kidney-protective (A₁), antifibrotic (A_{2A}), neuroprotective (A_{2A}), and antiglaucoma (A₃) agents. Adenosine agonists for cardiac imaging and positron-emitting adenosine antagonists are in development for diagnostic use. Allosteric modulation of A₁ and A₃ ARs has been demonstrated.

In addition to selective agonists/antagonists, mouse strains in which an AR has been genetically deleted have been useful in developing novel drug concepts based on modulation of ARs.

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A₁ Adenosine Receptor Antagonists, Agonists, and Allosteric Enhancers

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Abstract Intense efforts of many pharmaceutical companies and academicians in the A₁ adenosine receptor (AR) field have led to the discovery of clinical candidates that are antagonists, agonists, and allosteric enhancers. The A₁AR antagonists currently in clinical development are KW3902, BG9928, and SLV320. All three have high affinity for the human (h) A₁AR subtype (hA₁ K_i < 10 nM), > 200-fold selectivity over the hA_{2A} subtype, and demonstrate renal protective effects in multiple animal models of disease and pharmacologic effects in human subjects. In the A₁AR agonist area, clinical candidates have been discovered for the following conditions: atrial arrhythmias (tecadenoson, selodenoson and PJ-875); Type II diabetes and insulin sensitizing agents (GR79236, ARA, RPR-749, and CVT-3619); and angina

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(BAY 68–4986). The challenges associated with the development of any A₁AR agonist are to obtain tissue-specific effects but avoid off-target tissue side effects and A₁AR desensitization leading to tachyphylaxis. For the IV antiarrhythmic agents that act as ventricular rate control agents, a selective response can be accomplished by careful IV dosing paradigms. The treatment of type II diabetes using A₁AR agonists in the clinic has met with limited success due to cardiovascular side effects and a well-defined desensitization of full agonists in human trials (GR79236, ARA, and RPR 749). However, new partial A₁AR agonists are in development, including CVT-3619 (hA₁AR K_i = 55 nM, hA_{2A}:hA_{2B}:hA₃ > 200:1,000:20, CV Therapeutics), which have the potential to provide enhanced insulin sensitivity without cardiovascular side effects and tachyphylaxis. The nonnucleosidic A₁AR agonist BAY 68–4986 (capadenoson) represents a novel approach to angina wherein both animal studies and early human studies are promising. T-62 is an A₁AR allosteric enhancer that is currently being evaluated in clinical trials as a potential treatment for neuropathic pain. The challenges associated with developing A₁AR antagonists, agonists, or allosteric enhancers for therapeutic intervention are now well defined in humans. Significant progress has been made in identifying A₁AR antagonists for the treatment of edema associated with congestive heart failure (CHF), A₁AR agonists for the treatment of atrial arrhythmias, type II diabetes and angina, and A₁AR allosteric enhancers for the treatment of neuropathic pain.

Keywords A1 adenosine receptor agonists · A1 adenosine receptor antagonists · Acutely decompensated heart failure · Adenri · Cardiorenal syndrome · Congestive heart failure · Anti-arrhythmic agents · Tecadenoson · Selodenoson · Insulin sensitizing agents · CVT-3619 · Angina · Capadenoson · Allosteric enhancers · Neuropathic pain · Type II diabetes · BG9928 · KW3902 · SLV320

Abbreviations

ACE	Angiotensin-converting enzyme
ADHF	Acutely decompensated heart failure
ALT	Alanine aminotransferase
APD	Action potential duration
AR	Adenosine receptor
ARA	(1 <i>S</i> , 2 <i>R</i> , 3 <i>R</i>)-3-((trifluoromethoxy)methyl)-5-(6-(1-(5-(trifluoromethyl)pyridine-2-yl)pyrrolidin-3-ylamino)-9 <i>H</i> -purin-9-yl)cyclopentane-1,2-diol
ARB	Angiotensin II receptor blocker
AST	Aspartate aminotransferase
AUC	Area under the curve
(A–V) node	Atrioventricular
BG9719	(8-(3-Oxa-tricyclo[3.2.1.0 ^{2,4}]oct-6-yl)-1,3-dipropyl-3,7-dihydropurine-2,6-dione)

BG9928	(3-(4-(2,6-Dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1 <i>H</i> -purin-8-yl)bicyclo[2.2.2]octan-1-yl)propanoic acid)
cAMP	Cyclic AMP
Capadenoson	(BAY 68-4986) (2-amino-6-((2-(4-chlorophenyl)thiazol-4-yl)methylthio)-4-(4-(2-hydroxyethoxy)phenyl)pyridine-3,5-dicarbonitrile)
CHA	<i>N</i> ⁶ -Cyclohexyl adenosine
CHF	Congestive heart failure
CHO	Chinese hamster ovary
CK	Creatinine kinase
CL	Total body clearance
<i>C</i> _{max}	Maximal plasma concentration
CPA	<i>N</i> ⁶ -Cyclopentyl adenosine
CrCl	Creatinine clearance
CV	Cardiovascular
CVT-3619	(2 <i>S</i> , 3 <i>S</i> , 4 <i>R</i>)-2-((2-fluorophenylthio)methyl)-5-(6-((1 <i>R</i> , 2 <i>R</i>)-2-hydroxycyclopentylamino)-9 <i>H</i> -purin-9-yl)tetrahydrofuran-3,4-diol
DPCPX	1,3-Dipropyl-8 cyclopentylxanthine
ED ₅₀	50% Efficient dose
F (%)	% Oral bioavailability
GFR	Glomerular filtration rate
GR79236	(3 <i>R</i> , 4 <i>S</i> , 5 <i>R</i>)-2-(6-((1 <i>S</i> , 2 <i>S</i>)-2-hydroxycyclopentylamino)-9 <i>H</i> -purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol
HF	Heart failure
HSL	Hormone sensitive lipase
IP	Intraperitoneal
IPC	Ischemic preconditioning
IV	Intravenous
KW3902	(3-Noradamantyl-1,3-dipropylxanthine)
<i>L</i> -(NAME)	<i>N</i> -o-Nitro- <i>L</i> -arginine methyl ester
MRT	Mean residence time
NEFA	Nonesterified fatty acids
PVST	Paroxysmal supraventricular tachycardia
RCM	Radiocontrast media
RPF	Renal plasma flow
SAR	Structure-activity relationship
Selodenoson	(2 <i>S</i> , 3 <i>S</i> , 4 <i>R</i>)-5-(6-(Cyclopentylamino)-9 <i>H</i> -purin-9-yl)- <i>N</i> -ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide)
(S-A)	Sinoatrial
SLV320	(4-(2-Phenyl-7 <i>H</i> -pyrrolo[2,3- <i>d</i>]pyrimidin-4-ylamino)cyclohexanol)
<i>t</i> _{1/2}	Half-life
T-62	(2-Amino-4,5,6,7-tetrahydrobenzo[<i>b</i>]thiophen-3-yl)(4-chlorophenyl)methanone
Tecadenoson	(2 <i>R</i> , 3 <i>S</i> , 4 <i>R</i>)-2-(Hydroxymethyl)-5-(6-((<i>R</i>)-tetrahydrofuran-3-ylamino)-9 <i>H</i> -purin-9-yl)tetrahydrofuran-3,4-diol)

TG's	Triglycerides
T2D	Type II diabetes
UNaV	Urinary sodium excretion
UV	Urine volume
V_{ds}	Volume of distribution

1 Introduction

The A_1 adenosine receptor (AR), a member of the P_1 family of seven-transmembrane adenosine receptors, couples to G_i to decrease the secondary messenger cAMP. The P_1 family of adenosine receptors consists of the members A_1 , A_{2A} , A_{2B} , and A_3 , which have high sequence homology with many conserved residues at the active sites; however, sufficient differences are found for each active site such that selective agonists and antagonists have been generated for each receptor subtype (Akhari et al. 2006; Dhalla et al., 2003; Fredholm et al. 2001; Jacobson and Gao 2006). The major goal of this review is twofold: to highlight the structure–affinity relationships (SAR) of A_1 AR antagonists, agonists, and allosteric enhancers, and to give an overview of the A_1 AR antagonists, agonists, and allosteric enhancers currently under development for various indications.

2 A_1 Adenosine Receptor Antagonists

From the earliest reports on the physiologic effects of theophylline and adenosine to the three active clinical programs today (see Fig. 1), the study of AR ligands has a long and rich history (Baraldi et al. 2008; Jacobson and Gao 2006). There are a number of excellent reviews on A_1 AR antagonists (Baraldi et al. 2008; Hess 2001; Moro et al. 2006; van Galen et al. 1992; Yuzlenko and Kieć-Kononowicz 2006). Because these reviews discuss the historical development of this class of molecules, their structure–activity relationships (SARs), pharmacology, and therapeutic applications, a comprehensive review of A_1 AR antagonists will not be presented here. It is important to note that a number of A_1 AR antagonists have entered clinical trials; however, problems with high lipophilicity and corresponding low water solubility and bioavailability have limited their clinical development (Hess 2001). This section of our review will present brief overviews of the most advanced A_1 adenosine receptor (A_1 AR) antagonists that are promising drug candidates currently in clinical trials. The discussion will address SARs around the lead molecules, highlights of pharmacology in healthy animals and disease models, and top-line human clinical trial results.

The 1,3-dialkylxanthine core has been the mainstay of A_1 AR antagonists since the isolation of theophylline in 1886 (Kossel 1888) (Fig. 1). One hundred years passed before replacement of the methyl substituents with *n*-propyl chains and

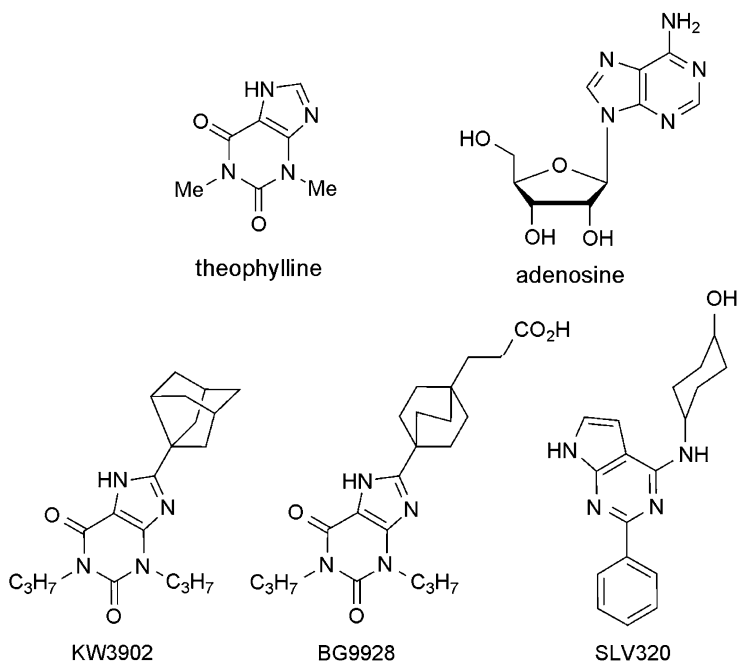


Fig. 1 Dialkylxanthine and adenine-based adenosine A₁ receptor ligands

the installation of cyclopentane at the C8 position led to the discovery of 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX), which has been used as a radioligand and pharmacologic probe for the *in vivo* effects of A₁AR antagonism in living systems (Shamim et al. 1988). Since then, significant effort has been directed toward garnering improvements in activity and selectivity on the well-optimized 1,3-dipropylxanthine, and has led to the discovery of two molecules that are in active clinical development programs: KW3902 (3-noradamantyl-1,3-dipropylxanthine) (Suzuki et al. 1992) and BG9928 (3-(4-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)bicyclo[2.2.2]octan-1-yl)propanoic acid) (Kiesman et al. 2006a). A structurally distinct nonxanthine series based upon the adenine substructure of adenosine itself has also been developed and is represented by clinical candidate SLV320 (4-(2-phenyl-7H-pyrrolo[2,3-*d*]pyrimidin-4-ylamino)cyclohexanol) (Kalk et al. 2007). All three of these A₁AR antagonists display high affinity for the A₁AR and significant selectivity over the A_{2A}AR (Table 1).

2.1 KW3902

To further characterize the hydrophobic interactions between the 8 position in the xanthine series and the A₁AR binding site, Shimada et al. (1991, 1992) investigated substitutions of the 8-cyclopentyl ring in DPCPX **1** (Table 2). Clipping the cyclopentyl ring into two ethyl groups **2** led to a loss of guinea pig (gp)A₁ affinity but

Table 1 Binding affinities for selected A₁ adenosine receptor antagonists

	<i>K_i</i> (nM) ^a					
	rA ₁	rA _{2A}	hA ₁	hA _{2A}	hA _{2B}	hA ₃
KW3902	0.2 ^b	170 ^b	8	673	296	4,390
BG9928	1.3	2,440	7	6,410	90	>10,000
SLV320	2.5 ^c	501 ^c	1 ^c	398 ^c	3980 ^c	200 ^c

^aReceptor binding experiments using cloned human receptors in CHO (hA₁, 0.3 nM ¹²⁵I-aminobenzyladenosine (¹²⁵IABA)) or HEK293-derived cell membranes (hA_{2A}, 0.7 nM ¹²⁵I-ZM241385; hA_{2B}, 0.5 nM ¹²⁵I-3-(4-aminobenzyl)-8-phenyloxyacetate-1-propyl-xanthine; and hA₃, 0.6 nM ¹²⁵I-ABA); rA₁ binding measured as inhibition of [³H]-DPCPX to rat forebrain membranes; rA_{2A} binding measured as inhibition of [³H] ZM241385 in rat striatal membranes (Kiesman et al. 2006a)

^brA₁ binding measured as inhibition of [³H]-CHA to rat forebrain membranes; rA_{2A} binding measured as inhibition of [³H] CGS21680 in rat striatal membranes (Suzuki et al. 1992)

^cReceptor binding experiments using cloned human receptors in CHO (hA₁: [³H]-DPCPX or HEK293-derived cell membranes (hA_{2A}: [³H]-CGS21680; hA_{2B}: [³H]-DPCPX; hA₃: [³H]-AB-MECA or rat cerebral cortex (rA₁: [³H]-CCPA, or striatal membranes (rA_{2A}: [³H]-CGS21680 (Kalk et al. 2007)

had no effect on rat (r)A_{2A} binding. When compared to **1**, dicyclopropyl substitution **3** showed enhanced potency and selectivity versus gpA₁ and rA_{2A} receptors, respectively, while the addition of *gem*-dimethyl substitution **4** led to diminished gpA₁ affinity but a remarkable decrease in rA_{2A} binding (>170-fold decrease). Bicyclo- and tricycloalkane systems **5**, **7**, **8** were then examined to determine if restrictions in conformational flexibility around the cyclopentyl ring in **1** effected the antagonist activity. Interestingly, the 3-noradamantyl system **8** stood out, with increases in gpA₁ affinity (*K_i* = 1.3 nM and high selectivity over the rA_{2A} receptor (890-fold; Nonanka et al. 1996). Introduction of a methylene linker between the bulky polycyclic alkane in the 8 position gave compound **9** significantly reduced gpA₁ affinity (>65-fold).

Animal Studies

The diuretic activity of KW3902 was examined in saline-loaded, conscious Wistar rats (Suzuki et al. 1992). The antagonist was orally administered in a saline suspension and the urine collected and analyzed for sodium content. Urine volume (UV) and urinary sodium excretion (UNaV) both increased in a dose-dependent manner, with maximal effects observed between 0.1 and 0.4 mg kg⁻¹ (Fig. 2).

During the development of intravenously (IV) injectable formulations for KW3902, which has a solubility in water of <1 μg mL⁻¹, Hosokawa et al. (2002) investigated the effects of a lipid emulsion and liposome formulation on the pharmacokinetics of KW3902 and its metabolite (M1-KW3902) in comparison to a 1 N NaOH–DMSO-containing formulation (Fig. 3). They reported no

Table 2 Structure–activity relationships for 8-substituted 1,3-dipropylxanthines

The chemical structure shows a xanthine core with propyl groups at the 1 and 3 positions and an R group at the 8 position. The xanthine ring is fused to an imidazole ring.

Cmpd	R	K_i (nM) ^a		
		gpA ₁	rA _{2A}	rA _{2A} /gpA ₁
1		6.4 ± 0.35 (0.49 ± 0.06) ^b rat (17 ± 6.5) ^c dog	590 ± 48 – –	92 – –
2		19 ± 1.0	570 ± 44	30
3		3.0 ± 0.21 (0.919 ± 0.04) ^b rat	430 ± 5.8 –	140 –
4		22	>100,000	>4,500
5		3.83 ± 0.32	440 ± 42	120
6		31	2,300	74
7		13 ± 2.8	5,100 ± 1,100	390
8		1.3 ± 0.12 (0.19 ± 0.042) ^b , rat 10 ± 2.6 ^c dog	380 ± 30 (170 ± 16) ^c (rat) –	290 890 (rat) –
9		880	>100,000	>110

^agpA₁ binding was carried out with *N*⁶-[³H]cyclohexyladenosine ([³H]-CHA) in guinea pig (gp) forebrain membranes and rA_{2A}, binding was carried out with *N*-[³H]ethyladenosine-5'-uronamide in the presence of 50 nM cyclopentyladenosine in rat (r) striatal membranes (Shimada et al. 1991)

^brA₁ binding measured as inhibition of [³H]-CHA to rat forebrain membranes (Shimada et al. 1992)

^crA_{2A} binding measured as inhibition of [³H] CGS21680 in rat striatal membranes and dA₁ (dog) measured with [³H]-CHA in dog forebrain membranes (Nonanka et al. 1996). All K_i measurements are given as mean ± SEM for 3–5 determinations

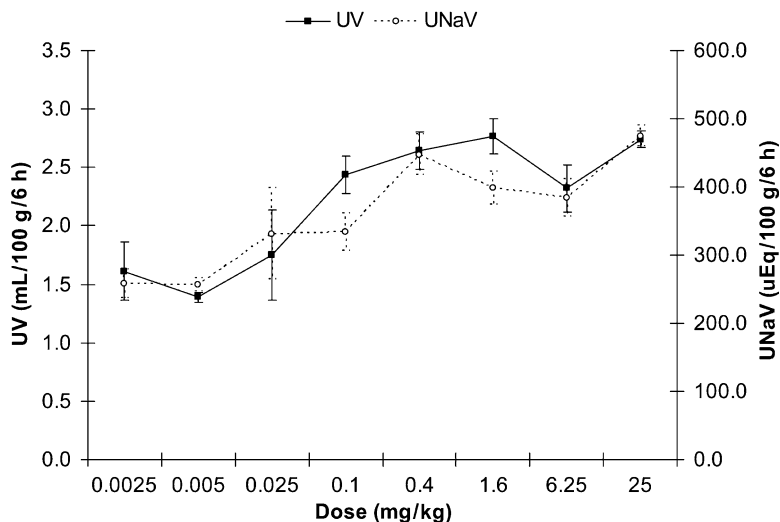


Fig. 2 Dose–response for urine volume (UV) in $\text{mL } 100 \text{ g}^{-1} 6 \text{ h}^{-1}$ (mean \pm SEM) and urinary sodium excretion (UNaV) in $\mu\text{Eq } 100 \text{ g}^{-1} 6 \text{ h}^{-1}$ (mean \pm SEM) over 6 h following oral doses of KW3902 ranging from 0.0025 to 25 mg kg^{-1} in male rats

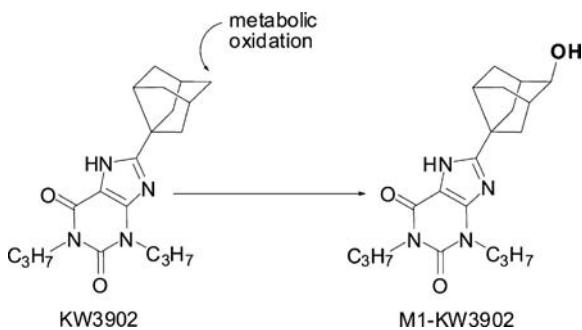


Fig. 3 Chemical structure of the metabolite of KW3902, M1-KW3902

significant differences in elimination half-life ($t_{1/2}$), area under the curve (AUC), total body clearance (CL), and mean residence time (MRT) for all of the formulations investigated. Table 3 summarizes the pharmacokinetic parameters measured for KW3902 and M1-KW3902 in the 1 N NaOH–DMSO formulation. The lipid formulation, however, did prevent the precipitation of KW3902 after IV injection, and it was suggested that this formulation may be used in further clinical studies.

The renal protective activity of KW3902 was investigated in a rat model of glycerol-induced acute renal failure (Suzuki et al. 1992). The antagonist was administered intraperitoneally (IP), and after 30 min glycerol (50% v/v in sterile saline; $0.8 \text{ mL } 100 \text{ g}^{-1}$) was injected subcutaneously. After a subsequent 24-h hold time, blood was collected and serum creatinine and urea nitrogen were determined

Table 3 Pharmacokinetic parameters of KW3902 and metabolite M1-KW3902 after IV administration of KW3902 to rats

	Free KW3902		Metabolite M1-KW3902	
	0.1 mg kg ⁻¹	1 mg kg ⁻¹	0.1 mg kg ⁻¹	1 mg kg ⁻¹
<i>t</i> _{1/2} (h)	1.0 ± 1.1	1.6 ± 0.6	4.7 ± 1.2	7.9 ± 3.9
AUC _{0-∞} (ng h ⁻¹ mL ⁻¹)	72 ± 50	561 ± 18	486 ± 113	4,916 ± 2,457
CL (L h ⁻¹ kg ⁻¹)	1.87 ± 1.11	1.79 ± 0.06	–	–
MRT (h)	1.5 ± 1.9	1.5 ± 0.3	7.3 ± 1.7	11.3 ± 5.6
<i>V</i> _{ds} (L kg ⁻¹)	1.66 ± 1.10	2.72 ± 0.51	–	–

Values represent the means ± standard deviation of three experiments.

*t*_{1/2}, Half-life; AUC, area under the curve; CL, total body clearance; MRT, mean residence time; *V*_{ds}, volume of distribution

Table 4 Renal protective activity of KW3902 in rats^a

Dose (mg kg ⁻¹ , IP)	Serum creatinine			Serum urea nitrogen (mg dL ⁻¹)		
	Concentration (mg dL ⁻¹)		% Inhibition	Concentration (mg dL ⁻¹)		% Inhibition
	Vehicle	Treated		Vehicle	Treated	
0.01	4.03 ± 0.23	1.83 ± 0.08 ^b	55	130.5 ± 7.0	45.2 ± 3.9 ^b	65
0.1	2.89 ± 0.18	1.40 ± 0.14 ^c	52	138.4 ± 9.8	48.1 ± 7.0 ^c	65
1	3.75 ± 0.43	2.00 ± 0.14 ^c	47	123.2 ± 14.2	66.4 ± 6.3 ^c	46

^aAll values are the means ± SEM; significant difference from vehicle-treated group

^b*P* < 0.01

^c*P* < 0.001

(Table 4). Typically after glycerol injection, serum creatinine and urea nitrogen increase seven- to tenfold in rats. Pretreatment with KW3902 significantly reduced (50–60%) the negative renal effects of glycerol-induced acute renal failure (Suzuki et al. 1992).

The mechanism of the protective effects of A₁AR antagonism on two additional nephrotoxic models of acute renal failure in vivo, including renal accumulation of gentamicin (Yao 2000) and radiocontrast media (RCM) (Yao et al. 2001), were examined. From these studies, it was suggested that KW3902 inhibited the action of endogenous adenosine and increased renal blood flow, which led to suppression of intrarenal accumulation of gentamicin, whereas in RCM-induced nephropathy, it prevented the drop in glomerular filtration rate (GFR); a marker for kidney function that occurs in *N*-*o*-nitro-*L*-arginine methyl ester (*L*-NAME) hypertensive rats. In the RCM model, it is unclear whether the action of the antagonist reduces RCM uptake and cellular toxicity by inhibiting sodium transport in the proximal tubule or by another mechanism.

Adenosine has been found, through its actions on A₂ARs (presumably A_{2A}ARs), to play a protective role in the ischemic preconditioning of multiple organs, including the liver (Peralta et al. 1999), heart (Forman et al. 1993), and lung (Khimenko et al. 1995). Magata (Magata et al. 2007) recently reported that blockade of the A₁AR with KW3902 attenuated hepatic ischemia-reperfusion injury in dogs. Two

groups of female beagles ($n = 6$) underwent a 2 h total hepatic vascular exclusion; one group served as the control and the other received $1 \mu\text{g kg}^{-1} \text{min}^{-1}$ of KW3902 via continuous intraportal infusion for 60 min prior to ischemia. It was noted that although peripheral IV infusion of KW3902 was effective in earlier studies (Nonaka et al. 1996), no beneficial effects were seen in the hepatic ischemia-reperfusion model by this route of administration. Thus, treatment by KW3902 via the portal vein proved beneficial in a number of outcomes in the study. The two-week survival of the control group was 16.7% versus 83.3% ($P < 0.05$) for the treated group. Serum alanine aminotransferase (ALT) levels were significantly inhibited by the A_1 AR antagonist; the control group rose rapidly to $12,625 \pm 1,010 \text{ U L}^{-1}$ after 6 h of reperfusion, while the treated animals peaked at 24 h at $2,352 \pm 452 \text{ U L}^{-1}$. In addition, the number of infiltrating neutrophils in the hepatic tissue of the KW3902 group (41.17 ± 11.01 at 60 min) was significantly lower than that of the control group (63.6 ± 23.6 at 60 min) (Fig. 4).

Treatment with KW3902 in the liver ischemia-reperfusion model prevented the bradycardia seen in the control animals just after reperfusion, significantly increased adenosine nucleotide levels in the ischemic tissues, and regulated the microcirculatory disturbances, resulting in greater hepatic tissue blood flow. It was concluded that for adenosine to protect the liver from ischemia-reperfusion injury it is necessary to block A_1 AR activation.

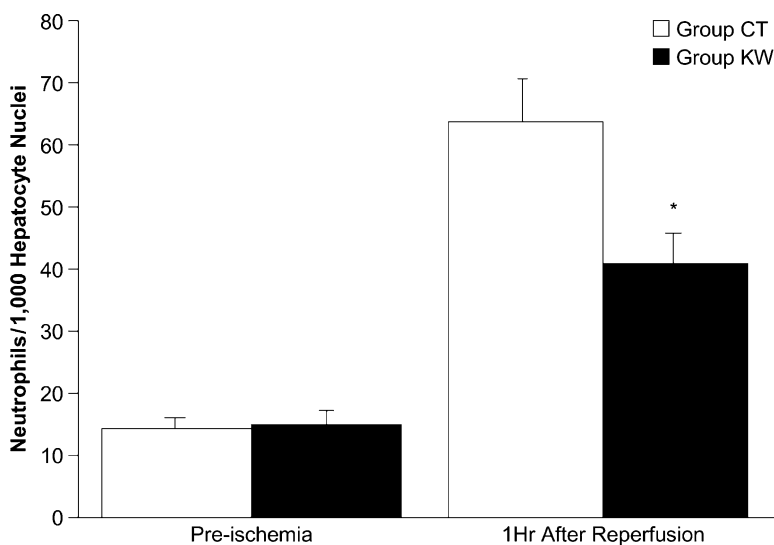


Fig. 4 The number of infiltrated neutrophils in liver tissue. Data are expressed as mean \pm SEM. Group CT, negative control ($n = 6$); group KW, treated KW3902 ($n = 6$). * $P < 0.05$ versus group CT. Reprinted with permission from Magata et al. (2007)

Clinical Studies

Building upon the earlier studies of the renal effects of another A₁AR antagonist, BG9719 (8-(3-oxa-tricyclo[3.2.1.0^{2,4}]oct-6-yl)-1,3-dipropyl-3,7-dihydropurine-2,6-dione) (Gottlieb et al. 2002), in patients with congestive heart failure (CHF), Dittrich and colleagues (Dittrich et al. 2007) examined the renal vasodilatory effects of 30 mg doses of KW3902 in patients with ambulatory heart failure (HF). The two-way crossover study followed patients with CHF with mild renal impairment (median GFR 50 mL min⁻¹) and compared IV administration of placebo or KW3902 oil emulsion followed by IV furosemide (80 mg). GFR and renal plasma flow were assessed by iothalamate and *para*-aminohippurate clearances over 8 h. After a three- to eight-day washout period, subjects crossed over to either active treatment or placebo, again followed by furosemide. Renal plasma flow (Fig. 5) and GFR increased by 48% ($P < 0.05$ vs. placebo) and 32% ($P < 0.05$ vs. placebo), respectively, over baseline for 8 h postKW3902 administration, supporting the conclusion that blockade of the A₁AR leads to vasodilation and increases in filtration rates in patients with HF with reduced kidney function. There also appeared to be a persistent positive effect on GFR (approx. 10 mL min⁻¹ increase in GFR over previous baseline) seen in the crossover patients who received KW3902 in the first dose. The pharmacokinetics of the parent compound or its metabolites could not account for the change in the baseline GFR values.

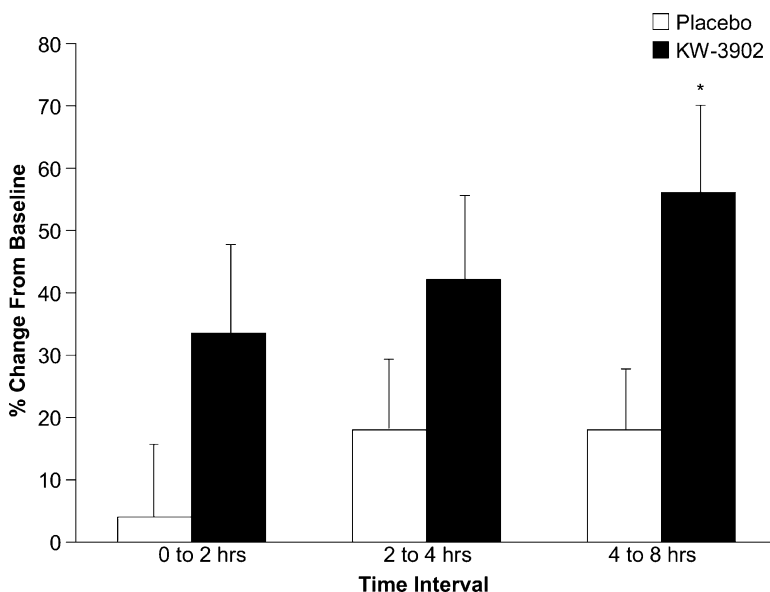


Fig. 5 Renal plasma flow (RPF). The percent change in RPF from baseline for KW-3902 and placebo in the presence of furosemide ($n = 23$). Values shown are for all subjects mean \pm SEM. P values reflect analysis of RPF percent change between KW-3902 and placebo using log RPF values (* $P < 0.05$). Reprinted with permission from Dittrich et al. (2007)

In a related clinical investigation, Givertz et al. (2007) examined the dose-dependent effects of KW3902 on diuresis and renal function in two subsets of patients with acutely decompensated heart failure (ADHF) with either renal impairment or diuretic resistance. In the first protocol, patients with volume overload and creatinine clearance (CrCl) of 20–80 mL min⁻¹ received either placebo or one of four doses (2.5, 15, 30, or 60 mg) of KW3902 as a 2 h IV infusion for up to three days of treatment. All four doses increased urine output during the 6 h period following administration (Fig. 6). There were no significant differences in systolic blood pressure or heart rate in any of the treatment groups. A transient decrease in serum creatinine was noted on day 2 of treatment for all dose levels (–0.03 to –0.08 mg dL⁻¹ for KW3902 arms vs. +0.04 mg dL⁻¹ for placebo). This effect was maintained on day 4 (or the day of discharge), except for the 60 mg dose level.

In the diuretic-resistant population, single infusions of KW3902 (10, 30, or 60 mg) were given to patients with an average baseline CrCl of 34 mL min⁻¹. Urine output increased for all dose levels (ranging from +22 to +24 mL h⁻¹), whereas the placebo arm saw a decrease in urine output (–29 mL h⁻¹). The CrCl data for this subset of patients was complex. In general, the placebo arm had decreases in CrCl over 24 h with similar trending from the 60 mg dose treatment group. However, the 10 and 30 mg doses showed increases in CrCl over the 24 h period. The inverted relationship between KW3902 dose and renal function (CrCl) at high doses bears some resemblance to the dose–response relationship seen at higher doses of the A₁AR antagonist BG9719 (Gottlieb et al. 2002). Whether these similarities are due

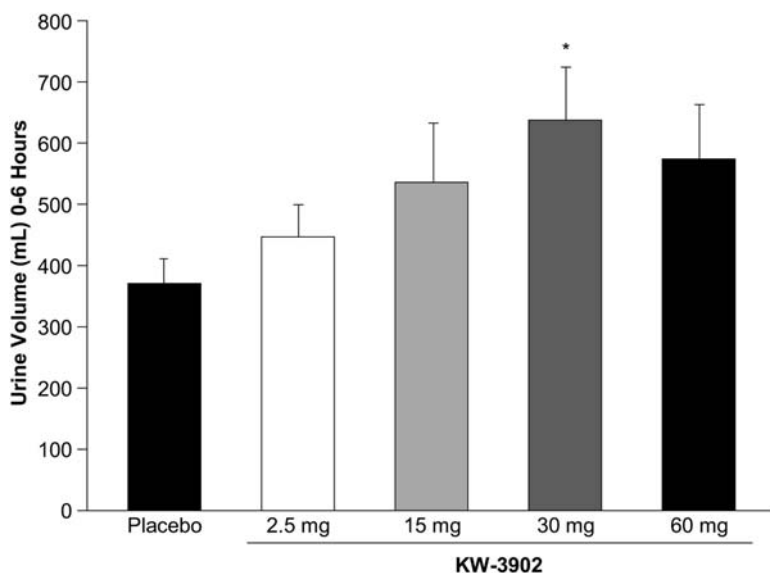


Fig. 6 Urine output in first 6 h after administration of KW3902. Cumulative urine volume (mean \pm SEM) 6 h after initiation of placebo or KW-3902 in patients with acutely decompensated heart failure (ADHF) with renal impairment (* $P = 0.02$ vs. placebo). Reprinted with permission from Givertz et al. (2007)

to cross-activity against the other adenosine receptors in the kidney at high doses or in other tissues is unclear and may require further study. Phase III clinical trials of KW3902 in patients with ADHD are currently underway, and limited releases of the data have recently appeared (Novacardia Press Release 2007).

2.2 BG9928

At the same time that formulation development began on KW3902, another selective A₁AR antagonist, BG9719, which possessed adequate pharmacologic activity, was used to demonstrate proof of concept for A₁AR antagonism in animals (Pfister et al. 1997) and humans (Gottlieb et al. 2000, 2002). However, the poor pharmaceutical properties of this molecule (low aqueous solubility and a tendency to rearrange to inactive products in both acidic and basic media) led Kiesman et al. (Kiesman et al. 2006a, b) to design more pharmaceutically acceptable antagonists by exploring the placement of polar substituents on linearly substituted 8-cycloalkyl 1,3-dipropylxanthines. For structurally related imidazolines, see Vu et al. (2006). The binding affinities of selected 8-cyclohexyl and 8-bicyclo[2.2.2]octyl xanthines are listed in Table 5.

The bicyclo[2.2.2]octyl analogs **11**, **13**, **15** had better A₁AR binding affinities than the related cyclohexyl variants **10**, **12**, **14**, and maintained similar A_{2A}AR activity. A significant improvement in receptor selectivity ($hA_{2A}/hA_1 = 161$ vs. 22) came with the replacement of the dimethylamino functional group in **15** with the carboxylic acid in **16**. Further optimization of the bridgehead chain led to the propionic acid **18**, BG9928, and single-digit nanomolar ($rA_1 = 1.3$ nM and $hA_1 = 7.4$ nM) activity and high receptor selectivities ($rA_{2A}/rA_1 = 1,880$; $hA_{2A}/hA_1 = 915$).

The functional antagonist activity of BG9928 (Kiesman et al. 2006a) was confirmed by examining the blockade or increasing doses of the compound on the inhibitory effects of *N*⁶-cyclopentyl adenosine (CPA) on the beat rates of isolated rat atria. Administration of the antagonist restored the atrial beat rates to their maxima and effectively blocked the negative chronotropic activity of CPA ($EC_{50} = 16.1 \pm 7.7$ nM). In a separate set of isolated rat atria experiments, BG9928 was found to have a pA₂ (antagonist potency) of 9.8.

Animal Studies

Single oral doses of BG9928 administered to male Sprague–Dawley rats (Fig. 7) led to increases in urine volume (UV) and sodium excretion (UNaV) with a 50% efficient dose (ED₅₀) of approximately 15 μg kg⁻¹ (Kiesman et al. 2006a; Ticho et al. 2003). The increases in urinary potassium excretion were proportional to volume increases, confirming the potassium-neutral diuresis commonly observed with A₁AR antagonists. The dose–response relationships are similar to those seen with KW3902 (Fig. 2); however, the magnitude of the pharmacodynamic effect is smaller

Table 5 Structure–activity relationships for 1,4-linearly-substituted 8-cycloalkylxanthines

Cmpd	K_i (nM) ^a or % of specific radioligand binding ^b					
	R	hA ₁	hA _{2A}	hA _{2B}	hA ₃	hA _{2A} /hA ₁
10		(31%)	(75%)	(69%)	(88%)	–
11		33	1,070	(48%)	(100%)	32
12		41	313	(18%)	(77%)	8
13		16	414	(27%)	(73%)	26
14		12	168	(16%)	(91%)	14
15		6	132	(3%)	(79%)	22
16		49	7,880	(53%)	(70%)	161
17		29 (4.0) rat	– (50%) rat	127 –	(26%) –	– (~250) rat
18		7.4 (1.3) rat	6,410 (2,440) rat	90 –	>10,000 –	915 (1,880) rat
19		(22.5) rat	(8,960) rat	–	–	(398) rat

^aReceptor binding experiments using cloned human receptors in CHO (hA₁, 0.3 nM [¹²⁵I]-aminobenzyladenosine (¹²⁵IABA) or HEK293-derived cell membranes (hA_{2A}, 0.7 nM [¹²⁵I]-ZM241385; hA_{2B}, 0.5 nM [¹²⁵I]-3-(4-aminobenzyl)-8-phenyloxyacetate-1-propyl-xanthine; and hA₃, 0.6 nM [¹²⁵I]-ABA); rA₁ binding measured as inhibition of [³H]-DPCPX to rat forebrain membranes; rA_{2A} binding measured as inhibition of [³H] ZM241385 in rat striatal membranes. All K_i values were calculated from binding curves generated from the mean of four determinations per concentration (seven antagonist concentrations), with variation in individual values of <15% (Kiesman et al. 2006a)

^bData are presented as percent of radioligand bound in the presence of target compound relative to control

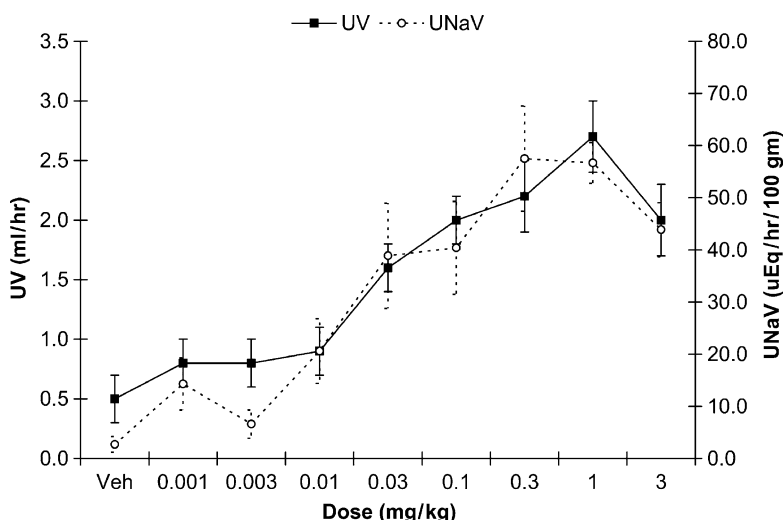


Fig. 7 Dose–response for urine volume (UV) in mL h⁻¹ (mean ± SEM) and urinary sodium excretion (UNaV) in μEq h⁻¹ 100 g⁻¹ (mean ± SEM) over 4 h following single oral doses of vehicle (0.5% carboxymethyl cellulose suspension, n = 3) or BG9928 ranging from 0.001 to 3 mg kg⁻¹ in rats (0.001 mg kg⁻¹, 0.003 mg kg⁻¹, 0.01 mg kg⁻¹, each n = 4; 0.03 mg kg⁻¹, 0.1 mg kg⁻¹, 0.3 mg kg⁻¹, each n = 5; 1.0 mg kg⁻¹, 3.0 mg kg⁻¹, each n = 3). Adapted with permission from Kiesman et al. (2006a)

Table 6 Pharmacokinetic parameters of BG9928 following single oral dose administration

	<i>F</i> (%)	<i>t</i> _{1/2} (h)	<i>CL</i> (mL min ⁻¹ kg ⁻¹)	<i>V</i> _{ds} (L kg ⁻¹)
Rat	99	3.14 ± 0.14	1.56 ± 0.26	0.32 ± 0.02
Dog	78	6.40 ± 4.0	11.8 ± 0.6	2.64 ± 1.29
Monkey	94	11.1 ± 4.2	5.82 ± 0.45	4.25 ± 0.70

n = 3 male rats, 4 male dogs, and 4 male cynomolgus monkeys

F(%), Percent bioavailability; *t*_{1/2}, half-life; *CL*, total body clearance; *V*_{ds}, volume of distribution

in the BG9928 study than in the KW3902 study because the rats in the BG9928 model were not saline-loaded prior to treatment.

The single oral-dose pharmacokinetic profile of 1 mg kg⁻¹ BG9928 was assessed in the rat, dog, and cynomolgus monkey (Table 6). Bioavailability was nearly complete in the rat and cynomolgus monkey, slightly lower in the dog, and followed the clearance difference amongst the species. Elimination half-lives were similar in the rat and dog (3–6 h), and longer in the cynomolgus monkey (11 h).

A rat model was designed to mimic the sodium-retentive state of patients with CHF (Ticho et al. 2003). Rats were given an oral dose of 100 mg kg⁻¹ furosemide and placed on a low-sodium diet for up to six days. One group (*n* = 8) then received a single IV bolus of 30 mg kg⁻¹ furosemide, while the other group (*n* = 9) received IV furosemide and 1 mg kg⁻¹ of BG9928. The results are depicted in Fig. 8.

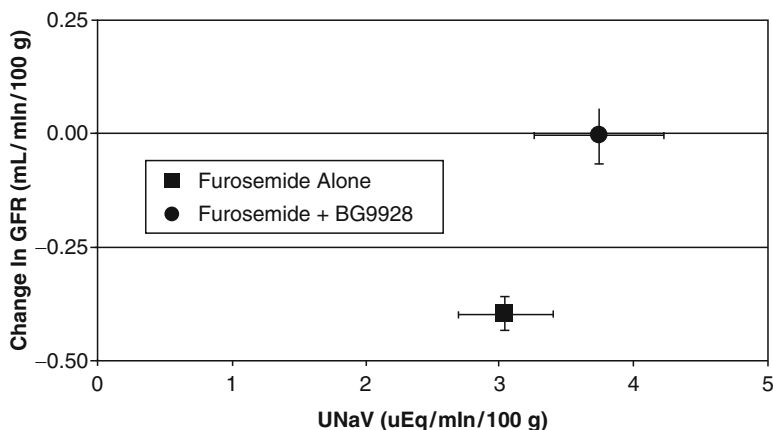


Fig. 8 Renal protective (mean \pm SEM change in glomerular filtration rate [GFR]) and natriuretic (mean \pm SEM) urinary sodium excretion (UNaV) effect of 1 mg kg^{-1} BG9928 administered IV in combination with furosemide (30 mg kg^{-1} IV) ($n = 9$, circles) compared with furosemide alone (30 mg kg^{-1} IV) ($n = 8$, squares) in low-sodium-retention rats. Reprinted with permission from Ticho et al. (2003)

Furosemide increased natriuresis and reduced GFR by approximately 50% over baseline. The addition of BG9928 not only further increased the natriuresis ($+0.71 \mu\text{Eq min}^{-1} 100 \text{ gm}^{-1}$) but also maintained the GFR, therefore preserving renal function. Similar data were presented for a Phase II proof-of-concept clinical trial for BG9719, a precursor compound to BG9928 (Gottlieb et al. 2002).

The interplay of A_1 AR antagonism and ischemic preconditioning (IPC), specifically the effects of DPCPX, BG9719, and BG9928, in an in vivo dog model of myocardial infarction was examined (Auchampach et al. 2004). The study was composed of three arms in which the dogs ($n = 6-12$) were subjected to 60 min of left anterior descending coronary artery occlusion, followed by 3 h of reperfusion. Infarct size was assessed by triphenyltetrazolium chloride staining. In protocol 1, the dogs received vehicle or 1 mg kg^{-1} of the antagonist as a pretreatment, followed by continuous infusion at $10 \mu\text{g kg}^{-1} \text{ min}^{-1}$ over the occlusion time. In protocol 2, the dogs received the same pretreatment as before but also received four 5-min occlusion/reperfusion preconditioning cycles. In protocol 3, the antagonists were not administered until 10 min prior to release of the occlusion and continued for 1 h into the reperfusion. Figure 9 summarizes the infarct size measurements across all three protocols.

Pretreatment with DPCPX or BG9928 reduced the myocardial infarct size by 51% and 49%, respectively, and none of the three antagonists blocked the protection of the myocardium afforded by the brief multiple-cycle IPC. In the most challenging experiment, it was found that treatment with either DPCPX or BG9928 just prior to reperfusion, a situation that more closely resembles clinical intervention in the course of treatment for myocardial infarction, reduced the infarct sizes by 43%

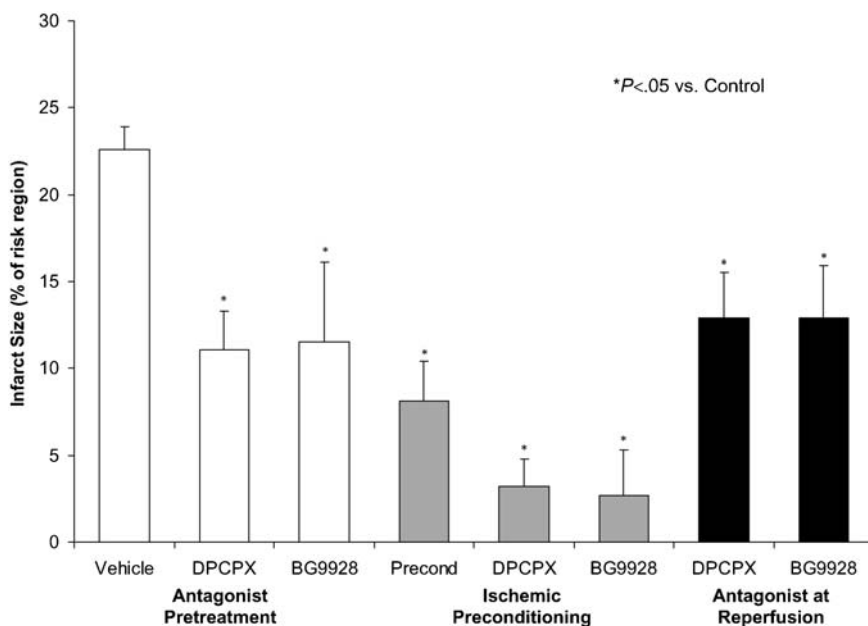


Fig. 9 Myocardial infarct size data (infarct size expressed as a percent of the area at risk) from antagonist pretreatment (protocol 1), ischemic preconditioning (protocol 2), and antagonist at reperfusion (protocol 3). $P < 0.05$ vs. control Adapted with permission from Auchampach et al. (2004)

and 45%, respectively. The study concluded that treatment with BG9928 provided cardioprotective effects that reduced infarct size and did not interfere with the protective effects of multiple-cycle IPC.

Clinical Studies

Greenberg et al. (2007) described the results of a placebo-controlled dose-escalation study designed to assess the pharmacokinetics and clinical effects of oral BG9928 in patients with HF. The study was conducted in 50 patients with HF, an ejection fraction of $\leq 40\%$ documented in the past 12 months, and who were on standard therapy including angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blocker (ARB) therapy and diuretics.

The pharmacokinetics of oral BG9928 in humans compared favorably to data from the earlier animal studies (Kiesman et al. 2006a) (Table 6). BG9928 was rapidly absorbed, with a t_{\max} of 1.5–3.1 h and similar C_{\max} , $t_{1/2}$, and clearances for days 1, 6, and 10. Steady-state AUC was reached by day 6, and the elimination half-life (14–25 h) was consistent with once-daily dosing. Patients received BG9928 (3, 15, 75, or 225 mg) or placebo orally for ten days and were evaluated for changes in sodium excretion (primary endpoint), potassium excretion, creatinine clearance,

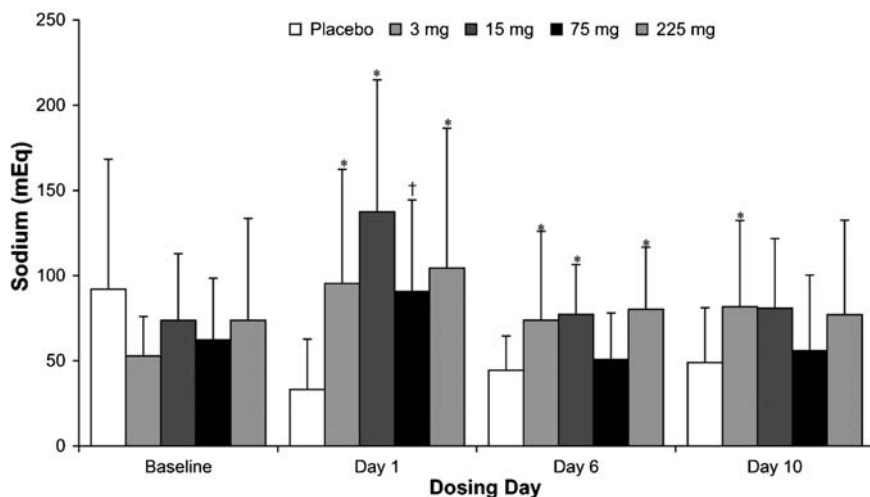


Fig. 10 Cumulative urinary sodium excretion (mEq) over the interval of 0–8 h at baseline and after placebo or BG9928 administration on days 1, 6, and 10. * $P < 0.05$ vs. placebo; † $P < 0.055$ vs. placebo; $n = 10$ per group except for 15 and 75 mg dose groups on day 10 ($n = 9$ per group). Reproduced with permission from Greenberg et al. (2007)

and body weight. In humans, BG9928 increased sodium excretion compared with placebo and maintained the natriuresis over the ten-day study period (Fig. 10) with little kaliuresis. These data followed the same trends seen in the previous rat and monkey studies (Kiesman et al. 2006a; Ticho et al. 2003) (Fig. 7).

Use of ACE inhibitors, ARBs, and diuretics in patients with HF can adversely affect renal function and depress GFR. Despite the significant increases in natriuresis, adjusted CrCl was unchanged over the study period for all of the treatment groups (Fig. 11), suggesting that BG9928 may have had a protective effect on renal function.

Patients who received daily doses of greater than 3 mg had a reduction in body weight (−0.6, −0.7, −0.5 kg) versus a net weight gain of +0.3 kg for the placebo group at the end of the study (Fig. 12).

Patients receiving BG9928 also showed favorable directional trends in other measures of clinical status, including New York Heart Association functional class (five BG9928-treated patients improved by one level); Cody edema score (mean change from day 1 to day 11: 0 for placebo and up to −0.6 for the treated groups; a negative number indicates an improvement in HF signs); and physician's global assessment. Thus, positive effects were observed for all treated groups. However, there were no significant differences in clinical status for the short duration of the trial. This is the first clinical assessment of chronic oral dosing of an A₁ AR antagonist in humans. Future studies are planned to examine clinical status and renal preservation with both oral and parenteral BG9928 in patients with HF.

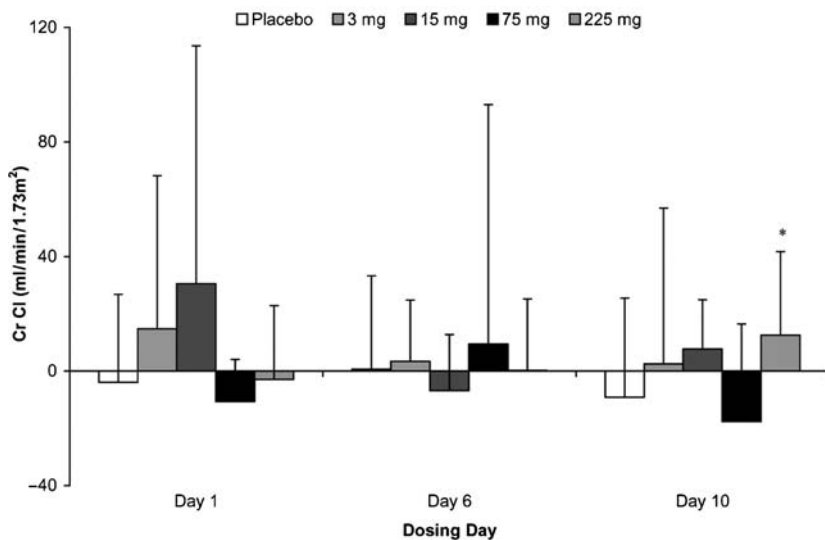


Fig. 11 Absolute change from baseline in adjusted creatinine clearance (CrCl) ($\text{ml min}^{-1} 1.73 \text{ m}^{-2}$) for the interval of 2–24 h after placebo or BG9928 administration on days 1, 6, and 10. * $P < 0.05$ vs. placebo; $n = 7$ –10 per group. Reproduced with permission from Greenberg et al. (2007)

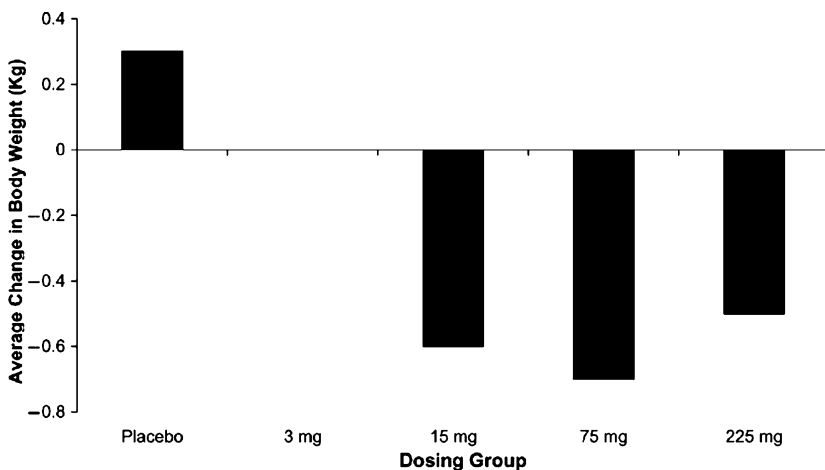


Fig. 12 Average change in body weight (kg) from baseline to day 11 after placebo or BG9928 administration. $n = 10$ per group. Reproduced with permission from Greenberg et al. (2007)

2.3 SLV320

Unlike the two xanthine-based A₁AR antagonists described in the preceding sections, SLV320 (see Fig. 1) contains an *N*⁶-substituted-7-deazapurine core (specifically a 2-phenyl-pyrrolopyrimidine) with an *N*⁶-*trans*-cyclohexanol side chain. The

Fig. 13 Structure of pyrrolopyrimidine series

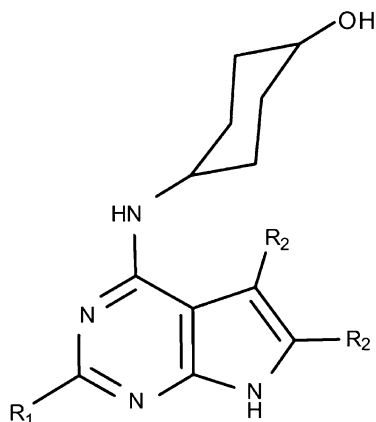


Table 7 Structure–activity relationships for pyrrolopyrimidines

Compd	R ₁	R ₂	<i>K_i</i> (nM) or % of specific radioligand binding ^a				
			hA ₁	hA _{2A}	hA _{2B}	hA ₃	hA _{2A} /hA ₁
20	Ph	Me	~22	933	138	22	42
21	4-Pyridyl	H	8.0	131	1,031	(54%)	16
22	3-Chlorophenyl	H	8.0	531	530	419	66
23	3-Fluorophenyl	H	1.8	206	802	270	114
24	Ph	H	3.7	630	2,307	630	170

^aReceptor binding experiments performed using membranes from yeast cells containing cloned human receptors (hA₁, [³H]-DPCPX) or HEK293-derived cell membranes (hA_{2A}, [³H]-CGS21680; hA_{2B}, [³H]-DPCPX; and hA₃, 0.6 nM [¹²⁵I]-AB-MECA) (Castelhana et al. 2005)

published SAR data around the pyrrolopyrimidine series (Fig. 13, Table 7) are only described within a series of patent filings (Castelhana et al. 2005). Dimethyl analog **20** had equipotent A₁ and A₃ AR affinities, a result that contrasts strikingly with those for the earlier xanthine systems (Tables 2 and 2.5). Removal of the two methyl groups (R₂ = Me) from compound **20** led to a tenfold increase in affinity for the A₁AR **24** and a significant reduction in A₃AR binding (22–630 nM) (Table 7). Substitution on the phenyl ring has small effects upon A₁AR activity and, in general, decreased selectivity versus the A_{2A}AR.

The functional antagonist activity of SLV320 was confirmed in experiments involving transient A₁AR-mediated bradycardia in anesthetized rats. Bolus injections of adenosine (100 μg kg⁻¹) lowered the heart rate in rats, and subsequent pretreatment both IV and orally with the antagonist caused a dose-dependent increase back to baseline in heart rate, with ED₅₀ values of 0.25 and 0.49 mg kg⁻¹, respectively. Similar to the results for BG9928 (Greenberg et al. 2007; Ticho et al. 2003), no significant hemodynamic effects were seen in anesthetized rats (heart rate, systolic arterial pressure, or diastolic arterial pressure) with single IV bolus doses of between 0.1 and 5 mg kg⁻¹.

In a model of chronic renal failure and myocardial fibrosis, rats were subjected to either sham operations or removal of 5/6 of their kidneys (5/6 NX animals) (Kalk

Table 8 Plasma analytes, GFR, and albumin excretion at the end of the 5/6 NX study

Parameter	Sham			5/6 NX
	Sham	+ SLV320	5/6NX	+SLV320
CK (U L ⁻¹)	481 ± 159	255 ± 31	1,267 ± 324	196 ± 64 ^d
AST (U L ⁻¹)	41 ± 2	41 ± 1	60 ± 5 ^a	35 ± 1 ^d
ALT (U L ⁻¹)	36 ± 1	31 ± 1	43 ± 1 ^a	32 ± 1 ^d
Creatinine (mg L ⁻¹)	4.7 ± 0.3	4.3 ± 0.2	7.3 ± 0.3 ^b	7.6 ± 0.42 ^b
GFR (mL min ⁻¹ 100 g ⁻¹)	0.45 ± 0.05	0.52 ± 0.02	0.32 ± 0.02 ^a	0.31 ± 0.01 ^a
Urinary albumin excretion (mg 24 h ⁻¹)	0.06 ± 0.02	0.07 ± 0.02	2.31 ± 0.39 ^b	1.08 ± 0.19 ^{b,c}

Values given as mean ± SEM

^a*P* < 0.05 versus sham

^b*P* < 0.001 versus sham

^c*P* < 0.05 versus sham

^d*P* < 0.001 versus 5/6 NX

CK, Creatinine kinase; AST, aspartate aminotransferase; ALT, serum alanine aminotransferase; GFR, glomerular filtration rate

et al. 2007). The effects of SLV320 on markers for cardiomyopathy and clinical chemistry were examined. Treatment with the A₁AR antagonist completely abolished higher creatinine kinase (CK) plasma levels as well as elevated ALT and aspartate aminotransferase (AST) in the nephrectomized animals (Table 8). The creatinine levels and GFR measurements of the 5/6 NX animals showed diminished renal function, as expected, when compared to the sham group. Treatment with SLV320 did not significantly lower creatinine or increase GFR. In addition, although albuminuria was higher in the 5/6 NX group, treatment with SLV320 led to a 50% reduction in albumin excretion and exerted beneficial effects on renal disease progression.

No significant differences in cardiac histology were seen between the arms of the study; however, immunohistochemistry uncovered a significant increase in collagen I and III in the untreated 5/6 NX group compared to the SLV320-treated group (Fig. 14). This study was the first to demonstrate that an A₁AR antagonist inhibited markers of myocardial fibrosis without changes in blood pressure. The experiments also agree with two previous studies (Amann et al. 1998a, b) that concluded that uremia promotes cardiac fibrosis independently of hypertension.

It was recently reported that the clinical development of the oral form of SLV320 was suspended and little information is available on the results of human clinical trials with the intravenous product.

3 A₁ Adenosine Receptor Agonists

Agonism at A₁ARs may provide benefit for the following disease states: paroxysmal supraventricular tachycardia (PSVT)—break the atrial arrhythmia to return to sinus rhythm (Belardinelli and Lerman 1990; Belardinelli et al. 1995; DiMarco

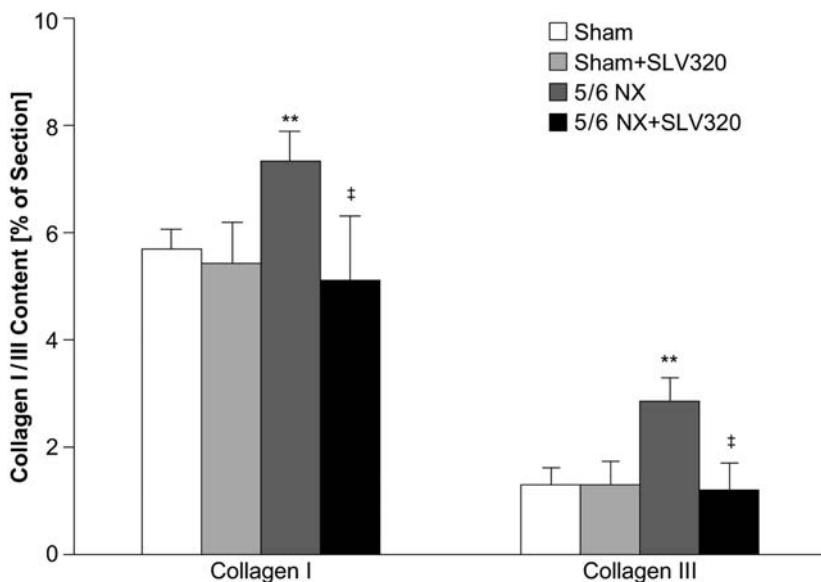


Fig. 14 Collagen I and III in rat hearts from nephrectomized rats versus normal controls. Values are given as mean \pm SEM. Unpaired *t*-test was applied to detect significant differences between the study groups. ** $P < 0.001$ vs. sham; ‡ $P < 0.001$ vs. 5/6 NX. Reprinted with permission from Kalk et al. (2007)

et al. 1985; Lerman and Belardinelli 1991), atrial fibrillation (AF)—provide ventricular rate control (Wang et al. 1996; Zablocki et al. 2004), type II diabetes (T2D)—lower nonesterified fatty acid (NEFA) levels and triglycerides (TG's), as well as enhancing insulin sensitivity (Fatholahi et al. 2006; Gardner et al. 1994; Hoffman et al. 1986; Roden et al. 1996), and angina (Liu et al. 1991; Miura and Tsuchida 1999; Mizumura et al. 1996). The A_1 AR is found in the A–V and S–A nodes, where stimulation by an A_1 AR agonist results in negative dromotropic and chronotropic effects, respectively (Belardinelli et al. 1995; Wang et al. 1996). These cardiovascular (CV) effects are often side effects of A_1 AR agonists that are being pursued for the other indications. Multiple full A_1 AR agonists, tecadenoson (2*R*, 3*S*, 4*R*)-2-(hydroxymethyl)-5-(6-((*R*)-tetrahydrofuran-3-ylamino)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol), selodenoson (2*S*, 3*S*, 4*R*)-5-(6-(cyclopentylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide) and PJ-875 are being pursued as intravenous clinical agents for the treatment of atrial arrhythmias, and the progress of these compounds will be highlighted below. In addition to the full A_1 AR agonists, CV Therapeutics has reported on orally bioavailable partial A_1 AR agonists that slow AV nodal conduction without causing third-degree AV block. For T2D, although both full and partial agonists will lower NEFA levels, a partial A_1 AR agonist has the potential to do so with fewer side effects (Song et al. 2002; Srinivas et al. 1997; Stephenson 1997; Wu et al. 2001). Partial A_1 AR agonists have the potential to

provide for a selective targeted response, avoiding CV side effects (Wu et al. 2001). Plus, partial A₁AR agonists may be able to avoid receptor desensitization due to overstimulation that can lead to tachyphylaxis. In T2D, overstimulation of hormone-sensitive lipase (HSL) due to enhanced beta-adrenergic agonism on adipocytes leads to elevated NEFA levels within T2D patients from the 0.4–0.5 mM range up to the 0.8–1.2 mM range. Elevated NEFA levels have been shown to decrease skeletal muscle uptake of glucose, lower insulin release from the pancreas, and increase glucose production in the liver (Boden et al. 2005; Dhalla et al. 2007a; Ferrannini et al. 1983; Green 1987; Itani et al. 2002; Sako and Grill 1990). Decreasing NEFA levels through A₁AR agonism has an insulin-sensitizing effect in animal models. Several full A₁AR agonists (GR79236, ARA, and RPR749) have been evaluated in animal models and in clinical trials for the treatment of T2D, and the progress and challenges of these compounds will be described. CVT-3619 (2*S*, 3*S*, 4*R*)-2-((2-fluorophenylthio)methyl)-5-(6-((1*R*, 2*R*)-2-hydroxycyclopentyl-amino)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol), a partial agonist, is in preclinical development. The SAR leading up to the discovery of this partial agonist, as well as its efficacy in animal models of T2D, will be highlighted. The last indication for A₁AR agonists that we describe in this review is the treatment of angina. One A₁AR agonist (BAY 68–4986) is currently under clinical evaluation, and the progress of this compound will be described.

3.1 Intravenous Antiarrhythmic Agents: Tecadenoson, Selodenoson, Phenylsulfide, Phenylethers, PJ-875

The first A₁AR agonists to enter clinical development with the exception of adenosine were the IV antiarrhythmic agents, tecadenoson (Ellenbogen et al. 2005; Peterman and Sanoski 2005; Prystowsky et al. 2003) and selodenoson (Bayes et al. 2003; ClinicalTrials.gov 2005) (Fig. 15). The N₆ lipophilic substituents are the key structure features that impart high affinity and selectivity for the A₁AR: N⁶-(*R*)-3-tetrahydrofuran-2-yl for tecadenoson (A₁AR K_i = 3 nM) and N⁶-cyclopentyl for selodenoson (A₁AR K_i = 6 nM). Both compounds are similar in structure to N⁶-cyclopentyl adenosine (CPA), but key structural differences are found in each molecule relative to CPA that impart beneficial pharmacological and pharmaceutical properties. For tecadenoson, the furan oxygen is favorable for imparting enhanced binding selectivity and additional solubility. For selodenoson, the 5′-*N*-ethyl carboxamide is favorable for both A₁AR and A₃ AR affinities, and it enhances oral activity. In Phase I clinical trials, both compounds were found to be safe and well tolerated at their specified IV bolus or infusion doses. With regards to efficacy, tecadenoson demonstrated favorable conversion (90%) of acute PSVT at the 300–600 μg bolus dose without significant adverse events. The A₁AR, which is highly expressed in atrial and AV nodal tissues, exerts its effects in the heart through lowering cAMP and direct activation of the inward rectifying potassium current, IK(Ado) (Belardinelli et al. 2005). In addition, A₁AR activation in the heart inhibits the

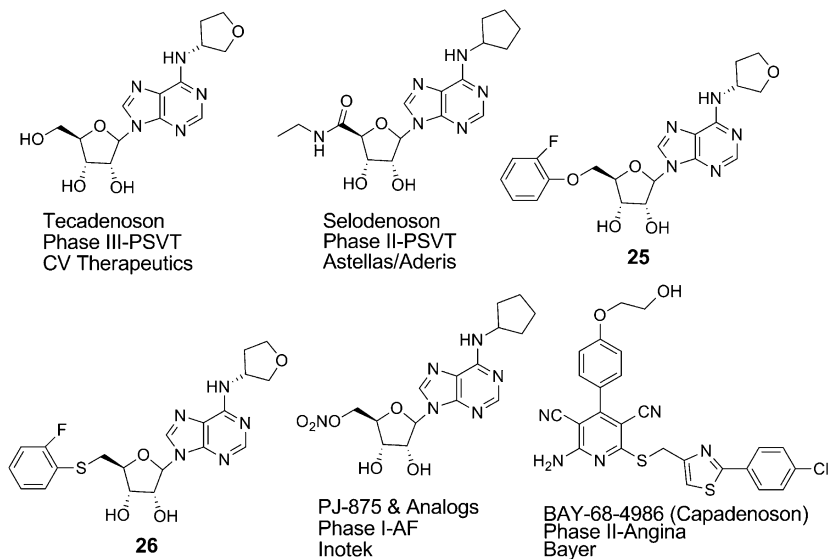


Fig. 15 Antiarrhythmic and antianginal A_1 AR agonists

catecholamine-stimulated ion currents such as pacemaker current and L-type calcium currents (Belardinelli et al. 2005). The result of A_1 AR activation in the heart is prolongation of the AV nodal refractory period, reducing sinoatrial pacemaker rate and shortening the action potential (Belardinelli et al. 2005). Because of the shortening of the atrial action potential duration (APD), it is not unexpected to have some atrial fibrillation (AF) after PSVT conversion, and this was found to be extremely low with tecadenoson (<1%), but the incidence of AF with adenosine following IV bolus is reported to be 11% and 15% in different studies (Ellenbogen et al. 2005).

Selodenoson was evaluated for the treatment of AF in a dose-ranging infusion study where it was infused for 15 min at doses of 2, 4, 6, 8, 10, and 12 $\mu\text{g kg}^{-1}$, where it provided for effective ventricular rate control in a dose-dependent manner with minimal side effects (Bayes et al. 2003). CV Therapeutics' scientists have described a number of partial A_1 AR agonists as potential oral antiarrhythmic agents that do not cause high-degree AV block at high concentrations (Morrison et al. 2004). These partial agonists were obtained by incorporating aromatic ethers and sulfides at the 5' position of the full agonist tecadenoson, a strategy that is known to decrease intrinsic activity with respect to GTP shift and induction of [^{35}S]GTP γ S binding to G-protein (Yan et al. 2003). The 5' substitution caused a significant drop in affinity for the A_1 AR when compared to tecadenoson, and the 5'-aromatic ethers had greater affinity and potency for the A_1 AR than the corresponding 5'-sulfides. Comparing the two lead molecules from both series (**25** and **26**) (Fig. 15), the 2-fluorophenyl ether analog **25** displayed higher affinity for the A_1 AR ($K_i = 12$ nM) and sixfold greater potency ($\text{EC}_{50} = 200$ nM) in slowing AV

nodal conduction than the 2-fluorophenyl sulfide **26** without causing third-degree AV block. In addition, compound **25** exhibited greater oral bioavailability (81%) relative to **26**. To our knowledge, compound **25** is the most potent partial A₁ AR agonist known to date; however, after oral administration, a small amount of the extremely potent full A₁AR agonist tecadenoson was generated; thus, compound **25** was unacceptable for further clinical development as an oral partial A₁AR agonist for chronic use. PJ-875 is a third A₁AR agonist in clinical development for AF from Inotek (DailyDrugNews.com 2008). The structure of PJ-875 has not been publicly disclosed; however, Inotek's patent application focuses on a 5'-nitrate ester of CPA with high A₁AR affinity (A₁AR K_i = 1 nM) and a 5'-nitrate ester of tecadenoson (A₁ AR K_i = 10 nM) (Jagtap et al. 2005). In Phase I clinical trials, PJ-875 did not have serious side effects, and Phase II clinical trials are planned (DailyDrugNews.com 2008).

The initial clinical trials with full A₁AR agonists in a controlled IV setting demonstrate that it may be possible to obtain antiarrhythmic properties with minimal CNS side effects. In addition, CV Therapeutics has discovered that the antiarrhythmic properties of a full A₁AR agonist, tecadenoson, can be augmented by coadministration of a subtherapeutic dose of a short-acting beta-blocker, esmolol, to achieve pronounced ventricular rate control effects in animal models (Belardinelli and Dhalla 2003). This combination approach of beta-blocker and A₁AR agonist will be interesting to watch in the clinic. Due to the pronounced CV effects at low doses of full A₁AR agonists, it is clear that a partial A₁AR agonist may be required to achieve tissue selectivity for other indications such as T2D.

3.2 *Insulin-Sensitizing Agents: GR79236, ARA, CVT-3619*

The therapeutic use of A₁ AR agonists as antilipolytic agents has been tried in the clinic; however, the CV effects mediated by the A₁AR agonists are a potential obstacle to the successful use of A₁AR agonists for this indication. A second challenge associated with the use of A₁AR agonists as antilipolytic agents is the development of acute tolerance to the antilipolytic effects due to receptor desensitization (Dhalla et al. 2007a; IJerman et al. 1995). One potential solution is to discover a partial A₁AR agonist that is capable of eliciting a greater effect in the adipocytes than in the heart (i.e., tissue selectivity). By definition, a partial agonist is a low-efficacy ligand that, in contrast to the full agonist, elicits only a submaximal biological response, and is hence less prone to receptor desensitization.

GR79236 ((3*R*, 4*S*, 5*R*)-2-(6-((1*S*, 2*S*)-2-hydroxycyclopentylamino)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol) and ARA ((1*S*, 2*R*, 3*R*)-3-((trifluoromethoxy)methyl)-5-(6-(1-(5-(trifluoromethyl)pyridine-2-yl)pyrrolidin-3-ylamino)-9*H*-purin-9-yl)cyclopentane-1,2-diol) (Fig. 16), the two full A₁AR agonists, have demonstrated that A₁AR agonism can have a pronounced effect on NEFA and TG levels in both acute and chronic animal models, thus establishing the potential of this approach for the treatment of T2D (Bigot et al. 2004; Merkel et al. 1995).

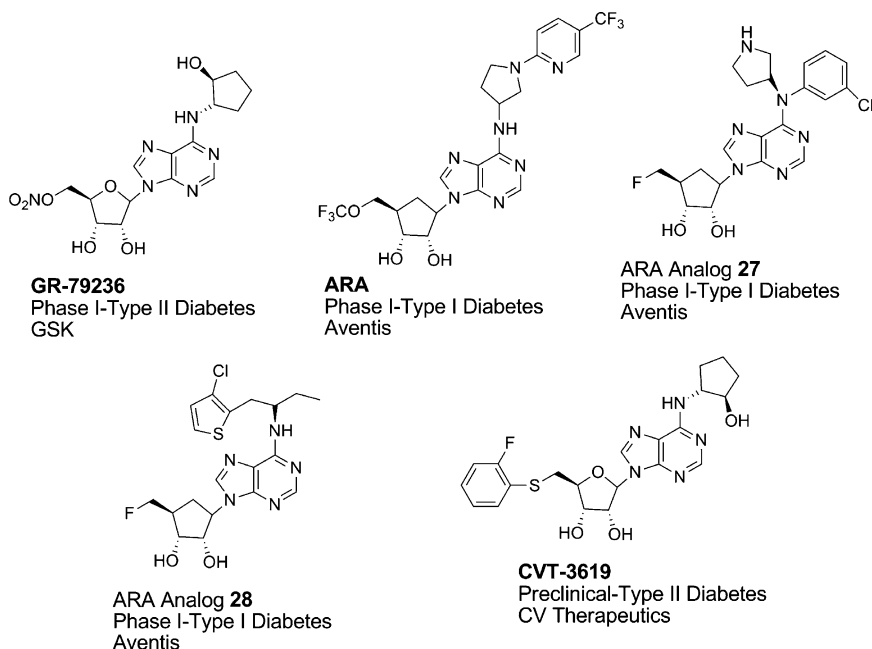


Fig. 16 Antidiabetic and insulin-sensitizing A_1AR agonists

Plus, the use of these full A_1AR agonists in clinical trials has resulted in a better understanding of the desensitization of the A_1AR and some potential limitations of using a full A_1AR agonist in a chronic setting. In early in vitro studies, GR79236 demonstrated that it inhibited catecholamine-induced lipolysis in adipocytes at low concentrations (Green et al. 1990; Qu et al. 1997; Webster et al. 1996). In addition, GR79236 was demonstrated to reduce NEFA levels by 50% in normal fasted rats (Qu et al. 1997). However, in a fructose-fed rat model of noninsulin-dependent diabetes, GR79236 (1 mg kg⁻¹ per day for eight days oral administration) did not enhance insulin sensitivity, but it did significantly lower NEFA and TGs (Webster et al. 1996). ARA, a full A_1AR agonist, has both animal data and clinical trial data supporting its effects on NEFA (Bigot et al. 2004). ARA is a C-sugar wherein the ribose oxygen is replaced by a carbon, and the ribose 5'-hydroxyl group was replaced by fluoro (as in compounds **27** and **28**) or a trifluoromethoxy group, as in ARA (Fig. 16). Compound **28** has lower affinity for the A_1AR with its unusual disubstituted N6 substituent containing an anilino moiety and a 3-pyrrolidinyl group. This is expected, since in most models of A_1AR agonist binding to the receptor, the N-H on N6 is involved in a key hydrogen-bonding interaction to the asparagine 254 side chain (IJzerman et al. 1995). ARA exhibited high affinity and selectivity for the A_1AR agonist ($K_i = 1.7$ nM and 4.5 nM in rat brain and rat adipocytes, respectively) (Zannikos et al. 2001). ARA demonstrated some tissue selectivity, being less potent (100- to 200-fold) in inducing A_1AR -mediated bradycardia than

in inducing A₁AR inhibition of lipolysis in rat and human adipocytes. This selective effect is most likely due to the high density and/or efficiency of A₁ AR coupling in adipocytes. Although ARA was effective at lowering plasma FFA when administered intravenously to fasted healthy volunteers in a Phase I clinical study, the rapid appearance of tolerance to its FFA-lowering ability was clearly evident (Zannikos et al. 2001).

These clinical findings support the need for a partial A₁AR agonist.

Partial A₁AR agonists were considered as an alternative to full agonists to avoid receptor desensitization. CVT-3619 ((2*S*, 3*S*, 4*R*)-2-((2-fluorophenylthio)methyl)-5-(6-((1*R*, 2*R*)-2-hydroxycyclopentylamino)-9*H*-purin-9-yl) tetrahydrofuran-3,4-diol) (Fig. 16), a selective partial A₁AR agonist devoid of CV effects, is being developed by CV Therapeutics as an antilipolytic agent (Dhalla et al. 2007a; Fatholai et al. 2006). This clinical candidate was obtained by further optimization of the 5'-phenylsulfide derivatives of tecadenoson (described earlier). The binding affinity of CVT-3619 for rat epididymal adipocytes was 14 nM (K_i , high affinity). CVT-3619 reduced forskolin-induced cAMP accumulation in both epididymal and inguinal adipocytes, with EC₅₀ values of 5.9 nM and 44 nM, respectively. The maximal effect of CVT-3619 at reducing cAMP levels in adipocytes was similar to that of CPA, suggesting that CVT-3619 is a full agonist with respect to reduction of cAMP. Plus, CVT-3619 reduced the forskolin-stimulated release of NEFA from both epididymal and inguinal adipocytes, with EC₅₀ values of 47 nM and 170 nM, respectively. However, CVT-3619 was found to be a partial agonist with respect to forskolin (1 μM)-stimulated NEFA release from epididymal and inguinal adipocytes, with only 42 and 58%, respectively, of CPA's effect. Most likely, the presence of a large receptor reserve and/or a higher efficacy of coupling of A₁AR in the adipocytes can explain the fact that CVT-3619 reduced the cAMP content of epididymal adipocytes with an EC₅₀ value that was lower than the K_i value from the binding assay, and the EC₅₀ value to reduce the release of NEFA was also much lower than the K_i . Furthermore, the high A₁AR receptor reserve in the adipocyte relative to the heart can explain the 1,000-fold functional selectivity of CVT-3619 to decrease epididymal adipose tissue lipolysis in the rat (EC₁₅ = 30 nM) relative to the atrial rate (both A₁AR-mediated effects) (Fatholai et al. 2006). With respect to CV side effects, CVT-3619 (10 nM–30 μM) caused only a small increase in S–H interval (6 ms) without causing second- or higher-degree AV block; however, CPA significantly prolonged the S–H interval (38 ms) and caused second- or higher-degree AV block at concentrations >30 nM. In normal, overnight-fasted awake rats, at doses of 2.5, 5 and 10 mg kg⁻¹, CVT-3619 lowered FFA by 31%, 47% and 57% from baseline, respectively (Dhalla et al. 2007b). In addition, CVT-3619 significantly reduced serum TG levels and increased insulin sensitivity in rats (Dhalla et al. 2007b). The ED₅₀ of insulin to inhibit lipolysis was potentiated fourfold by a single dose (0.5 mg kg⁻¹) of CVT-3619, suggesting that CVT-3619 increases insulin sensitivity in adipose tissue. The antilipolytic effects of CVT-3619 in rats (given twice daily) were well maintained for up to six weeks of treatment, and no tachyphylaxis or receptor desensitization were observed. Based on the above data, CVT-3619 is in preclinical development by CV Therapeutics as a partial A₁AR agonist for the potential treatment of T2D

in order to avoid CV effects and receptor desensitization. For more information on the effects of partial A₁AR agonists in diabetes and obesity, the reader is referred to Chap. 9 of this volume, “A₁ Adenosine Receptor: Role in Diabetes and Obesity” (Dhalla et al.).

3.3 Angina Agents: Capadenoson (Nonnucleoside: BAY 68–4986)

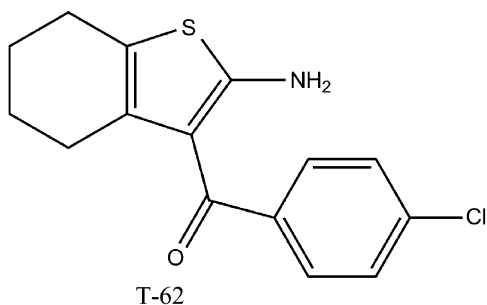
Bayer chemists were first to make a key discovery that a heterocyclic class of compounds devoid of a ribose moiety can function as agonists at the adenosine receptor, although the first compounds were nonselective (Erguden et al. 2007). IJzerman and colleagues followed this with a further elaboration of the heterocyclic class of agonists in order to introduce some receptor selectivity (Chang et al. 2005). The Bayer chemists then reported the development of a compound from this very novel class of compounds. The oral A₁AR agonist capadenoson (BAY 68–4986), 2-amino-6-((2-(4-chlorophenyl)thiazol-4-yl)methylthio)-4-(4-(2-hydroxyethoxy)phenyl)pyridine-3,5-dicarbonitrile (Fig. 15), was evaluated in a Phase II double-blinded, placebo-controlled multicenter study in patients with stable angina and coronary heart disease studying doses of 1, 2.5, 5, 10 and 20 mg. A 10 mg dose of capadenoson significantly reduced heart rate at peak exercise compared to placebo. Capadenoson is currently under going further studies and is anticipated to finish Phase III clinical trials by 2009 (Bays et al. 2007).

4 Allosteric Enhancers

4.1 Neuropathic Pain: T-62

A different approach to A₁AR agonism is to use the endogenous adenosine levels to activate the receptor coupled with an allosteric enhancer of the A₁AR. This approach has the theoretical advantage of fewer side effects, since it relies on adenosine being produced at the target tissue. In some disease states, adenosine release is a natural compensatory process to help the tissue restore balance. The A₁AR allosteric enhancer will take advantage of this local adenosine release and provide activation of a local A₁AR. The SAR of A₁AR allosteric enhancers has evolved over many years, with major contributions from IJzerman and Baraldi (Baraldi et al. 2007; Van der Klein et al. 1999). The common structural theme that has emerged is a 2-amino-3-acyl-thiophenyl core as exemplified by the lead compound in the area, T-62 (2-amino-4,5,6,7-tetrahydrobenzo[*b*]thiophen-3-yl)(4-chlorophenyl)methanone; (Fig. 17), a compound discovered by Baraldi et al. and developed by King Pharmaceuticals for neuropathic pain (Baraldi et al. 2007; Obata et al. 2003; Pan et al. 2001). T-62 demonstrated efficacy for reducing pain

Fig. 17 Allosteric A₁AR enhancer



hypersensitivity in a plantar surgical injury rat model (0.3–1 mcg intrathecal administration) in a dose-dependent manner. The dose of T-62 required for an antihyperalgesic effect was reduced by half when clonidine was coadministered, and this effect was 40% of the maximum possible effect. T-62 is under clinical evaluation in patients with postherpetic neuralgia experiencing pain. It will be interesting to see how the lead compound T-62 does in clinical trials of neuropathic pain, since it may drive further research in the area of A₁AR allosteric enhancers.

5 Conclusion

A considerable body of research over the past 20 years in the A₁AR field has resulted in the identification of clinical candidates for A₁AR antagonism, agonism, and allosteric modification. From a pharmacological perspective, the developmental path for A₁AR antagonists should theoretically be easier due to the challenges associated with developing A₁AR agonists, such as receptor desensitization and the risk of pronounced CV and CNS side effects. With two of the three active A₁AR antagonist clinical programs (KW3902 and BG9928) in Phase III human clinical trials, there is optimism in the cardiology community that an A₁AR antagonist will be available for patient use in the coming years (Dohadwala and Givertz 2008). Partial A₁AR agonism with CVT-3619, for example, may represent a way to avoid both CV and CNS side effects, which makes CVT-3619 an interesting compound to watch as it proceeds to the clinic. BAY 68–4986 opens up the A₁AR agonist field with the advent of nonribose partial agonists that possess a longer half-life for chronic agents that are no longer limited by the high polarity of the ribose ring. The A₁AR allosteric enhancer T-62 has demonstrated promising results in animal models of neuropathic pain, and is currently undergoing clinical evaluation. Based on these important scientific and clinical advances, therapeutics that target the A₁AR (A₁AR antagonists, A₁AR agonists, and allosteric enhancers) may show long-awaited clinical success in the near future.

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Recent Developments in Adenosine A_{2A} Receptor Ligands

Gloria Cristalli, Christa E. Müller, and Rosaria Volpini

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Abstract The development of potent and selective agonists and antagonists of adenosine receptors (ARs) has been a target of medicinal chemistry research for several decades, and recently the US Food and Drug Administration has approved LexiscanTM, an adenosine derivative substituted at the 2 position, for use as a pharmacologic stress agent in radionuclide myocardial perfusion imaging. Currently, some other adenosine A_{2A} receptor (A_{2A}AR) agonists and antagonists are undergoing preclinical testing and clinical trials. While agonists are potent antiinflammatory agents also showing hypotensive effects, antagonists are being developed for the treatment of Parkinson's disease.

However, since there are still major problems in this field, including side effects, low brain penetration (for the targeting of CNS diseases), short half-life, or lack of in vivo effects, the design and development of new AR ligands is a hot research topic.

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This review presents an update on the medicinal chemistry of A_{2A}AR agonists and antagonists, and stresses the strong need for more selective ligands at the human A_{2A}AR subtype, in particular in the case of agonists.

Keywords Adenosine receptor · Adenosine A_{2A} receptor · A_{2A} agonists · A_{2A} antagonists · Nucleosides · Xanthines · Adenines · Nitrogen (poly)heterocyclic compounds

Abbreviations

ADA	Adenosine deaminase
Ado	Adenosine
AK	Adenosine kinase
AR	Adenosine receptor
CCPA	2-Chloro- <i>N</i> ⁶ -cyclopentyladenosine
CHA	<i>N</i> ⁶ -Cyclohexyladenosine
CHO	Chinese hamster ovarian
CNS	Central nervous system
CPA	<i>N</i> ⁶ -Cyclopentyladenosine
HEAdo	2-(Hexyn-1-yl)adenosine
HENECA	2-Hexynyl-NECA
MECA	<i>N</i> -Methylcarboxamidoadenosine
NECA	<i>N</i> -Ethylcarboxamidoadenosine
PEAdo	2-Phenylethynyladenosine
PENECA	2-PhenylethynylNECA
PHPAdo	2-Phenylhydroxypropynyladenosine
PHPNECA	2-PhenylhydroxypropynylNECA
PIA	<i>N</i> ⁶ -(2-Phenylisopropyl)adenosine
QSAR	Quantitative structure–activity relationships

1 Adenosine A_{2A} Receptor Agonists

1.1 Adenosine

The clinical utility of adenosine (Ado, **1**, Fig. 1) was recognized late in the 1980s by Belardinelli and Pelleg, and it soon became clear that the unmodified molecule is of restricted interest as a tool for the study of adenosine receptors due to its susceptibility to extensive metabolism by a number of enzymes (Klotz 2000). In fact, the observation that the activity of exogenous Ado on the mammalian cardiovascular system is of short duration because of the rapid uptake of Ado into red blood cells and tissues (Pfleger et al. 1969), its phosphorylation by adenosine kinase

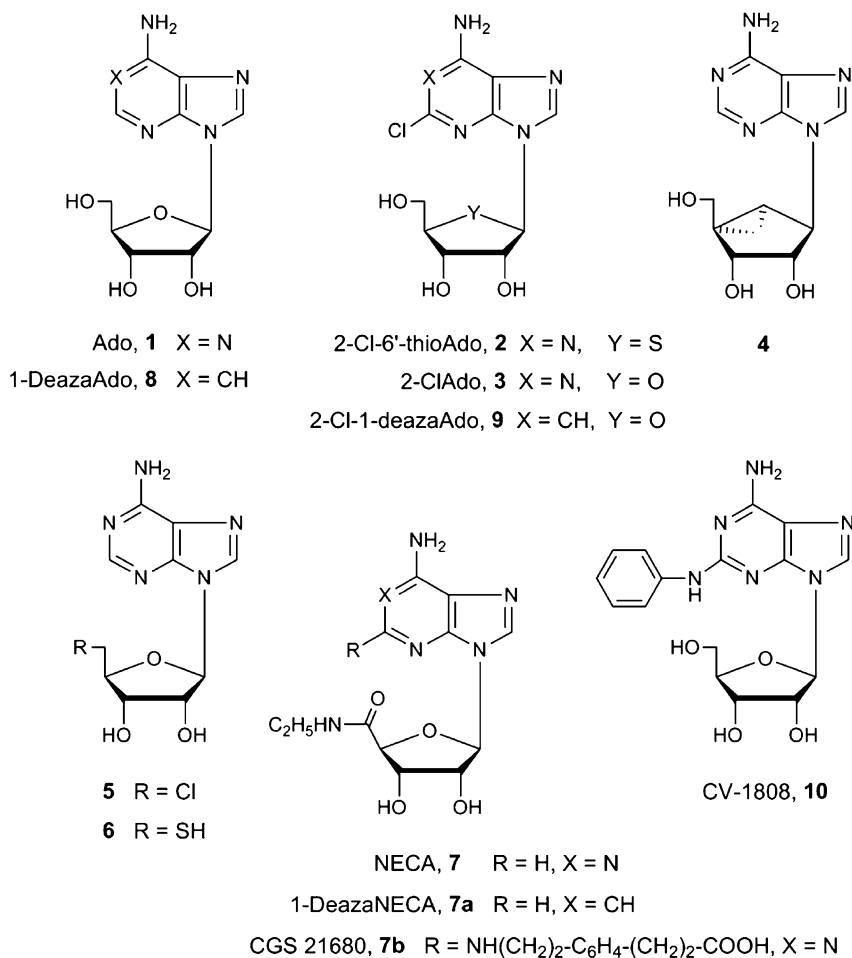


Fig. 1 A_{2A}AR agonists

(AK), and its conversion to inosine by adenosine deaminase (ADA) (Cristalli et al. 2001) led many labs to carry out several modifications of the Ado structure in order to find stable and selective ligands for the four adenosine receptor subtypes.

Almost all AR agonists known so far are derivatives of the physiological agonist Ado (Table 1). One exception is a set of substituted pyridines recently found to be agonists for human adenosine A_{2B} receptor (A_{2B}AR) (Beukers et al. 2004). Many attempts to modify the Ado structure led to the conclusion that the Ado scaffold must be conserved, although three positions in the molecule may be modified to increase affinity to specific receptor subtypes without destroying the agonistic efficacy: the 5' position of the ribose and the 2 and N⁶ positions of the purine (Cristalli et al. 2003). It must be underlined that any of these modifications render the agonists metabolically stable.

Table 1 Affinities of AR agonists in radioligand binding assays at A₁AR, A_{2A}AR, and A₃AR, and effects on adenylate cyclase activity at the A_{2B}AR

Cpd	K_i (A ₁ AR) ^a	K_i (A _{2A} AR) ^a	EC ₅₀ (A _{2B} AR) ^b	K_i (A ₃ AR) ^a
2	300 r	20 r	–	1,090 r
3	9.3 r	63 r	24,000 r	1,890 r
7	10 r	7.8 r	–	113 r
	63 r	16 r	3,100 h	10 h
	14 h	20 h	2,400 h	6.2 h
7a	51 r	580 r	16,000 h	703 r
7b	1,400 r	19 r	–	584 r
	290 h	27 h	88,800 h	67 h
8	115 r	2,900 r	–	–
9	226 r	163 r	–	2,480 r
10	400 r	100 r	–	–
11	977 r	68 r	–	–
	530 h	62 h	–	310 h
12	130 r	17 r	–	–
	221 h	9.3 h	3,490 h	54 h
13	48,000 h	270 h	>100,000 h	900 h
14	11,700 r	22 r	–	–
15	2,800 r	22 r	–	–
	1,730 h	92 h	–	83 h
16	701 r	109 r	–	–
	806 r ^c	246 r ^c	–	28 r ^c
	395 h	363 h	>100,000 h	16 h
17	98 r	2.2 r	–	–
	111 r ^c	5.2 r ^c	–	24 r ^c
	18 h	5.7 h	≈100,000 h	4.7 h
18	3.4 r	1.9 r	–	–
	3.9 r ^c	5.3 r ^c	–	0.98 r ^c
	0.67 h	7.0 h	2,400 h	3.3 h
19a	1.5 r ^c	1.0 r ^c	–	0.40 r ^c
	0.44 h	29 h	6,200 h	5.0 h
19b	2.5 r ^c	1.6 r ^c	–	24 r ^c
	0.67 h	1.8 h	920 h	1.4 h
20	332 r	14 r	–	–
21	618 r	757 r	–	–
23	0.6 r	462 r	–	–
26	356 h	1.0 h	2,780 h ^d	100 h
27	473 r	9.7 r	–	–
28	130 r	2.2 r	–	26 r
	160 r ^c	1.0 r ^c	–	18 r ^c
	60 h	6.4 h	6,100 h	2.4 h
29	2.5 r	0.9 r	–	–
	3.8 r	2.7 r	–	7.7 r
	2.7 h	3.1 h	1,100 h	0.42 h

(continued)

Table 1 (continued)

Cpd	K_i (A ₁ AR) ^a	K_i (A _{2A} AR) ^a	EC ₅₀ (A _{2B} AR) ^b	K_i (A ₃ AR) ^a
29a	5.9 r	2.6 r	–	–
	2.7 r ^c	16 r ^c	–	0.46 r ^c
	1.9 h	39 h	2,400 h	5.5 h
29b	4.0 r	0.5 r	–	–
	5.5 r ^c	1.8 r ^c	–	2.6 r ^c
	2.1 h	2.0 h	220 h	0.75 h
30	698 r	120 r	–	–
	1,000 r ^c	267 r ^c	–	768 r ^c
	560 h	620 h	>100,000 h	6.2 h
31	28 r	5.5 r	–	–
	77 h	0.2 h	–	45 h
32a	251 r	1.6 r	–	–
32b	951 r	70 r	–	–
33	>10,000 p	85 p	–	–
	380 r ^c	15 r ^c	–	46 r ^c
	189 h	24 h	>100,000 h	86 h
34	1,100 r	330 r	45,000 h	6.4 h
35	63 r	12 r	5,300 h	108 h
36	403 h	49 h	>100,000 h	16 h
37	1,700 h	720 h	>100,000 h	246 h
38	32% r	115 r	–	5,640 r
39	48% r	82 r	–	3,160 r
40	–	1,122 p	–	–
41	5,836 h	2,895 h	–	–

^aBinding data from different species: rat (*r*), human (*h*) or pig (*p*) A₁AR, A_{2A}AR, and A₃AR, expressed as K_i (nM)

^bMeasurement of receptor-stimulated adenylate cyclase activity at rat (*r*) or human (*h*) A_{2B}AR, expressed as EC₅₀ (nM)

^cUnpublished data

^dBinding data

1.2 Ribose-Modified Adenosine Derivatives

A variety of modifications of the Ado ribose ring in several positions were carried out in order to get information on the essential points of agonist activity, and possibly to obtain more active and stable compounds (Yan et al. 2003; Akkari et al. 2006). Most alterations of either the structure or the stereochemistry of the ribose resulted in a loss of receptor binding potency and possibly intrinsic activity (Siddiqi et al. 1995).

Compounds in which the furanose ring was modified have been synthesized in order to improve stability, since the glycosidic bonds of adenosine derivatives are subject to scission *in vivo*. Results have shown that the sugar moiety must be maintained as a ribose ring, but that in some cases the endocyclic oxygen ring atom can be replaced with a sulfur atom (**2**, Fig. 1) (Siddiqi et al. 1995) or a methylene

group (carbonucleoside). Comparison of 2-ClAdo (**3**) and the thio-ribose analog **2** showed a 3.2-fold higher affinity of the latter at the A_{2A}AR, whereas its adenosine A₁ receptor (A₁AR) affinity was reduced by 32-fold. In contrast, compounds **2** and **3** were of similar potency at the adenosine A₃ receptor (A₃AR) (Siddiqi et al. 1995). Carbonucleosides showed generally weak A_{2A}AR selectivity and low affinity for A₃AR. Carbocyclic modification of the agonists ribose resulted in nonglycosidic compounds that are potentially more biologically stable. The synthesis of a variety of methanocarpa analogs of Ado was reported (**4**, Fig. 1) (Jacobson et al. 2000). These compounds contain a fused cyclopropane ring that constrains the pseudo-sugar ring in either a North (*N*) or South (*S*) conformation, with the aim of defining the role of sugar puckering in stabilizing the AR-bound conformation. Such modifications lead to compounds endowed with very low A_{2A}AR affinity and high A₁AR and A₃AR selectivity.

The 2'- and 3'-hydroxy groups of the ribose moiety appear to be essential for full agonist activity (Mathot et al. 1995; Siddiqi et al. 1995; van der Wenden et al. 1995; Vittori et al. 2000), whereas the substitution of the 5'-hydroxyl group of Ado is better tolerated, although the removal of this group results in a decrease in potency (van der Wenden et al. 1995). Moreover, 5'-modified Ados are also less expected to be incorporated into DNA due to their resistance to phosphorylation by AK (IJzerman and van der Wenden 1997).

Substitution of the 5'-hydroxyl group with a chlorine or a thiol group (**5** and **6**, Fig. 1) has been observed to increase affinity for ARs (Taylor et al. 1986; van der Wenden et al. 1998). However, it has been observed that the 5'-chloro-5'-deoxy modification of N⁶-substituted Ados can increase A₁AR selectivity by reducing A₂ receptor potency (Taylor et al. 1986). A number of changes have been made to the riboses of a range of Ado analogs (Siddiqi et al. 1995). Most of the compounds with modified ribose in these studies were not substrates for ADA, and hence all were resistant to metabolism.

The introduction of an *N*-alkylcarboxamido group in position 5' was well tolerated by all AR subtypes, and produced the most active compounds, such as NECA (**7**, Fig. 1) (Prasad et al. 1980), a nonselective AR agonist. On the other hand, *N*-ethylthiocarboxamidoAdo showed a decrease in affinity compared with NECA at all AR subtypes (de Zwart et al. 1999a). In particular, the 5'-*N*-ethyluronamide group enhances receptor affinity for all AR subtypes and it leads to a further increase in the agonist activity and/or selectivity, especially if other substituents are simultaneously present at position 2 of the Ado (Prasad et al. 1980; Hutchison et al. 1990; Cristalli et al. 1995; Baraldi et al. 1998a; de Zwart et al. 1999a). Structure-activity relationships showed that the 5'-*N*-ethyl-, 5'-*N*-methyl- and 5'-*N*-cyclopropylcarboxamido substitutions give the most potent agonists (Prasad et al. 1980).

1.3 Purine-Modified Adenosine Derivatives

In general, modification of the purine scaffold results in compounds with reduced receptor binding affinity compared with the corresponding Ado analogs

(Müller and Scior 1993; IJzerman et al. 1994). In particular, 1-deazaAdo (**8**) and its *N*⁶-substituted derivatives are A₁AR selective, while the nitrogen atoms in the 3 and 7 positions are required for high affinity of Ado analogs at all subtypes (Bruns 1980; Cristalli et al. 1985; Siddiqi et al. 1995; de Zwart et al. 1998). On the other hand, 2-chloro-1-deazaAdo (**9**) showed an A_{2A}AR and A₃AR affinity similar to that of compound **3** (which is slightly A₁AR selective), and a reduced A₁AR activity, thus being slightly selective for the A_{2A}AR (Cristalli et al. 1988). Furthermore, **8** was reported to possess ADA inhibitory activity (Cristalli et al. 2001).

1.3.1 2- or *N*⁶-Substituted Adenosine Derivatives

In the last 35 years, a significant number of C2-substituted Ado derivatives were synthesized and tested for their affinities at A₁AR and A_{2A}AR, and the first Ado derivative found to have some A_{2A}AR selectivity was CV-1808 (**10**, Fig. 1) (Bruns et al. 1986). A number of substitutions were made with amine (Francis et al. 1991), hydrazine (Niiya et al. 1992a, b; Viziano et al. 1995), alkoxy (Daly et al. 1993; Matova et al. 1997), alkythio (Hasan et al. 1994; Cristalli 2000; Volpini et al. 2004), and alkynyl groups (Abiru et al. 1992, 1995; Cristalli et al. 1992; Matsuda et al. 1992; Volpini et al. 2002; Ohno et al. 2004), and the compounds with a phenylethyl (or cyclohexylethyl) group directly linked to the heteroatom (**11–15**, Fig. 2) or a triple bond (**16–18**) showed the highest A_{2A}AR affinities (Cristalli et al. 2007).

Substitutions with hydrazine led to 2-(*N*'-alkylidenehydrazino) and 2-(*N*'-aralkylidenehydrazino)Ado derivatives (Niiya et al. 1992a, b). Among these molecules, we should mention WRC-0470 (2-cyclohexylmethylidenehydrazinoAdo, also known as MRE-0470 or SHA-174 or Binodenoson, **13**) discovered at Nelson/Whitby Research and developed at Discovery Therapeutics, and now in clinical trial for myocardial perfusion imaging.

The alkynyl derivatives 2-phenylethynylAdo (PEAdo, **16**), 2-(hexyn-1-yl)Ado (HEAdo, **17**), (*R*, *S*)-2-phenylhydroxypropynylAdo ((*R*, *S*)-PHPAdo, **18**), and the corresponding diastereomers **19a** and **19b** were tested in binding studies on rat membrane A₁AR, A_{2A}AR (Cristalli et al. 1992), and A₃AR (Cristalli et al., unpublished results) and on the four human recombinant receptor subtypes, stably transfected into Chinese hamster ovarian (CHO) cells (the potency at the A_{2B}AR was measured with adenylate cyclase activity assays) (Volpini et al. 2002). All the compounds showed A_{2A}AR affinity in the low nanomolar range, and HEAdo was also shown to be slightly A_{2A}AR selective in rat membrane (A₁AR/A_{2A}AR ≈ 20 and A₃AR/A_{2A}AR ≈ 5). The phenylhydroxypropynyl derivatives are generally very potent, but are not selective at both rat and human AR subtypes. Partial and full reduction of the HEAdo triple bond led to *E*- and *Z*-alkenyl isomers **20** and **21** and 2-hexylAdo, respectively, among which the *trans* isomer **20** showed good A_{2A}AR affinity and modest selectivity (A₁AR/A_{2A}AR ≈ 24), while 2-hexylAdo proved to be inactive at both A₁AR and A_{2A}AR subtypes (Vittori et al. 1996). More recently, broad screening was carried out with the aim of characterizing the affinity and selectivity of 2-alkoxyAdo derivatives at A₃AR subtypes.

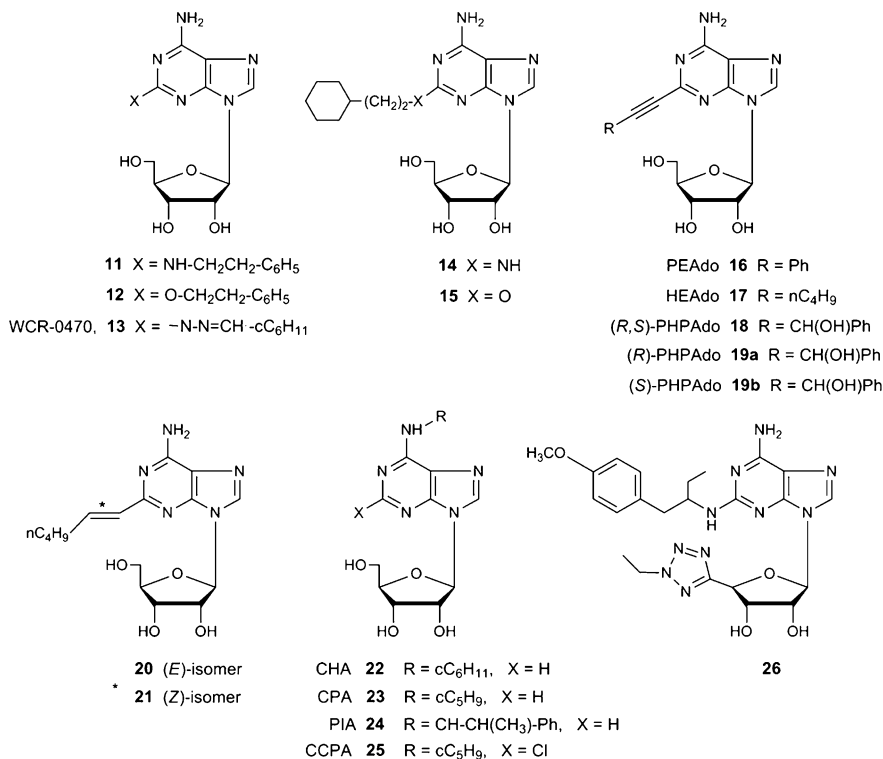


Fig. 2 A_{2A}AR agonists: various Ado derivatives

These single substitutions at the 2 position, previously found to contribute to the affinity for the rat A_{2A}AR, were also proven to be important for affinity and selectivity at the human A_{2A}AR ortholog (Gao et al. 2004).

In general, substitution of Ado at the N⁶ position (and in particular disubstitution with bulky substituents at the C2 and N⁶ positions) is detrimental to A_{2A}AR affinity (Müller and Scior 1993). In fact, the first known subtype-selective Ado derivatives modified at the N⁶ position, such as N⁶-cyclohexylAdo (CHA, **22**), N⁶-cyclopentylAdo (CPA, **23**), and N⁶-(2-phenylisopropyl)Ado (PIA, **24**) showed A₁AR selectivity (Daly 1982). Furthermore, substituents in this position were more recently shown to enhance A₃AR affinity and selectivity (Knutsen et al. 1999; Volpini et al. 2002).

In a series of 1-deaza analogs of Ados, it turned out that 2-chloro substitution in addition to an N⁶-cyclopentyl increases A₁AR selectivity (Cristalli et al. 1988). The respective modification in Ado led to the development of 2-chloro-N⁶-cyclopentylAdo (CCPA, **25**) as the most potent and selective A₁AR ligand characterized in rat brain (Lohse et al. 1988; Klotz et al. 1989).

1.4 Ribose- and Purine-Modified Adenosine Derivatives

The majority of A_{2A}AR-selective agonists are 2-substituted Ado derivatives bearing an *N*-alkylcarboxamido modification at the ribose 5' position, as in NECA (Hutchison et al. 1990; Cristalli et al. 1992, 1994b, 1995, 1996, 2003, 2007; Homma et al. 1992; Vittori et al. 1996; de Zwart et al. 1998; Müller 2000a). Also, Ado derivatives bearing bulky substituents in the C2 position and NECA derivatives with bulky substituents in the N⁶ position are not selective versus A₁AR and A₃AR. N⁶ and C2 substitution are helpful to improve A₃AR agonist activity, even if substitution at both N⁶ and C2 with large substituents led to a large drop in affinity when combined (Baraldi et al. 1998a). This effect at A_{2A}AR had been observed in a series of Ado derivatives developed as A_{2A}AR agonists (Müller and Scior 1993). QSAR (quantitative structure–activity relationship) studies on different N⁶-arylcarbonyl, 2-arylalkynyl-N⁶-arylcarbonyl, and N⁶-carboxamide Ado derivatives showed that the main determinants of the affinity at A_{2A}ARs were the bulkiness of the substituents attached at the 2 and 5' positions and the stereoselectivity of the Ado derivatives (Gonzalez et al. 2005). Moreover, the synthesis and potential human A_{2A}AR agonistic activity of Ado derivatives containing an ethyl-substituted tetrazole moiety at the 4' position of the ribose and an amino alcohol at the 2 position of the adenine core were reported (Bosch et al. 2004). The activities of these compounds were tested in radioligand binding assays using the four cloned human ARs. The compounds have also been profiled in cAMP assays using human receptors expressed on transfected CHO cells, and in functional assays using rat aorta, guinea pig aorta, and guinea pig tracheal rings. Results of these experiments show that substitution at the *para* position of the phenyl ring at the 2 side-chain by different groups greatly increases the affinity for A_{2A}AR. At the same time, the tested substituted derivatives have reduced affinity for A₁AR and A₃AR, thus greatly improving the A₁AR/A_{2A}AR and A₃AR/A_{2A}AR selectivity. Among the tested Ado derivatives, compound **26**, lacking the hydroxyl group in the side chain, was the most potent and selective in binding studies.

1.4.1 2-Substituted NECA Derivatives

The 4'-uronic acid ethyl ester analog of Ado, NECA, was reported in the early 1980s to be a potent coronary vasodilator and hypotensive (Prasad et al. 1980), and a good inhibitor of platelet aggregation induced by ADP (Cusack and Hourani 1981). However, NECA showed little or no A₂ selectivity in either functional or binding studies (Cristalli et al. 1994a, b; Klotz et al. 1999).

A series of 2-(arylalkylamino)-NECA derivatives were synthesized and evaluated for their A₁AR and A_{2A}AR binding profiles in rat brain membranes soon after the first Ado derivative with some A_{2A}AR selectivity, CV-1808 (**10**, Fig. 1), was reported. As in the case of arylalkylaminoAdos, the phenylethylamino analog of NECA **27** (Fig. 3) showed the highest rat A_{2A}AR affinity in the series and a greater than 2,000-fold separation between A₂ (coronary vasodilation) and A₁AR

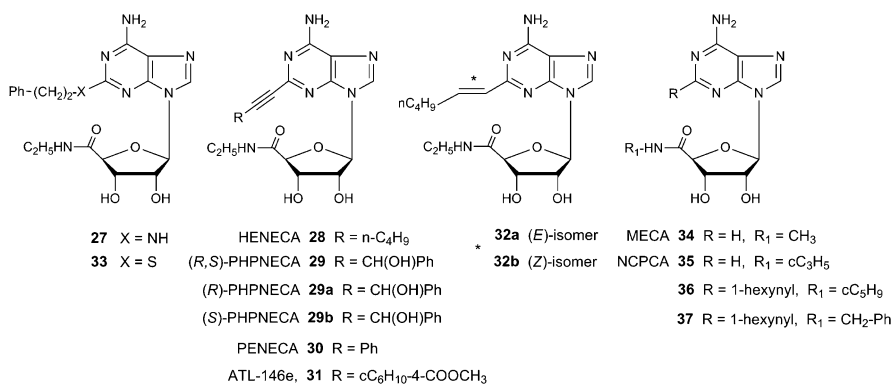


Fig. 3 A_{2A}AR agonists: NECA derivatives

(negative chronotropic effect) receptor-mediated events. Among these compounds, CGS 21680 (**7b**, Fig. 1) proved to be an A_{2A}AR-selective agonist that was 140-fold selective vs. A₁AR in a rat model (Hutchison et al. 1989). This molecule was selected for extensive biological evaluation (Hutchison et al. 1989) and tritiation for use as an A_{2A}AR-selective ligand for receptor binding (Jarvis et al. 1989). However, due to a similar affinity of CGS 21680 for A₃AR and the remarkable species variation observed for the A₁AR, with an over tenfold higher affinity of this compound for the human subtype (Klotz et al. 1998), it can no longer be considered an A_{2A}AR-selective agonist. In any case, it has been the ligand of choice to distinguish A_{2A}AR- and A_{2B}AR-mediated effects so far.

The synthesis and evaluation of 2-alkynyl derivatives of NECA, bearing from five to eight linear carbon atom chains, was driven by the same observations that led to the synthesis and testing of 2-alkynylAdos (Cristalli et al. 1992). Affinities for A₁AR and A_{2A}AR were determined in rat membranes using radioligand competition assays. All compounds showed good A₁AR and A_{2A}AR affinities (*K_i* in the nanomolar range) and moderate A_{2A}AR selectivity (Cristalli et al. 1992). Among this series of 2-substituted compounds tested at rat receptors, 2-hexynyl-NECA (HENECA, **28**, Fig. 3) exhibited 60-fold A_{2A}AR selectivity compared to the A₁AR subtype. The pharmacological profile of this compound was characterized by studies carried out by Monopoli and coworkers, using in vitro and in vivo models (Monopoli et al. 1994). In addition to the binding studies on both rat and bovine brain, which confirmed the moderate A_{2A}AR versus A₁AR selectivity, HENECA was administered intraperitoneally in conscious spontaneously hypertensive rats, and it caused a dose-dependent reduction in systolic blood pressure with minimal reflex tachycardia. It also appeared to penetrate the central nervous system, as shown by its protection against pentylentetrazole-induced convulsions in rats (Monopoli et al. 1994). In another work, administration of HENECA i.p. induced Fos-like immunoreactivity in the rat nucleus accumbens shell, lateral septal nucleus, and dorso-medial striatum, similar to that induced by atypical neuroleptics (Pinna et al. 1997).

The therapeutic potential of HENECA for the treatment of cardiovascular and psychotic diseases led to the synthesis of a series of 2-alkynyl, 2-cycloalkynyl, 2-aralkynyl, and 2-heteroaralkynyl derivatives of NECA that were tested in binding and functional assays to evaluate their potency for the A_{2A}AR compared to A₁AR (Cristalli et al. 1994b; Cristalli et al. 1995). Results showed that good A_{2A}AR affinities of the compounds were obtained with large 2-substituents containing a relatively rigid spacer, but that the affinity was reduced by introducing the bulkier naphthyl ring at the 2 position.

High agonist potency was found by introducing an α -hydroxy group into the alkynyl chain of NECA derivatives and obtaining compounds like 2-phenylhydroxypropynylNECA ((*R*, *S*)-PHPNECA, **29**), which was endowed with subnanomolar affinity in binding studies (K_i A₁AR = 2.5 nM and K_i A_{2A}AR = 0.9 nM) and was 16-fold more potent than NECA (**7**) as a platelet aggregation inhibitor. The problem with these analogs is that they also possess good A₁AR affinity, resulting in low A_{2A}AR selectivity. The diastereoisomer separation of a PHPNECA racemic mixture was accomplished obtaining compounds **29a** and **29b**. Binding tests in rat membranes showed that the (*S*)-diastereomer **29b** is about fivefold more potent and selective than the (*R*)-diastereomer **29a** as an agonist of the A_{2A}AR receptor subtype (**29b**, K_i A_{2A}AR = 0.5 nM; **29a**, K_i A_{2A}AR = 2.6 nM, Table 1) (Camaioni et al. 1997).

Things changed in the late 1990s after the cloning of the four human AR subtypes and their stable transfection into CHO cells. In fact, it was then possible to carry out comparative studies in a similar cellular background, utilizing binding studies (A₁AR, A_{2A}AR, A₃AR) or adenylate cyclase activity assays (A_{2B}AR) (Klotz et al. 1998). Transfected CHO cells were employed to screen for some nucleosides previously considered A_{2A}AR selective, and following this screening none of the prototypical AR agonists exhibited high affinity and selectivity for the human A_{2A}AR subtype. Both NECA and CGS 21680, which were available as radioligands for this subtype, demonstrated reduced affinity at the human as compared to the rat receptor, whereas HENECA (**28**) also showed high affinity at human A_{2A}AR and A₃AR, with tenfold and 25-fold selectivity versus the A₁AR subtype, respectively (K_i A₁AR = 60 nM, K_i A_{2A}AR = 6.4 nM, and K_i A₃AR = 2.4 nM). Interestingly, the potency for A_{2B}AR receptor is comparable with that of **7** (**28**: EC₅₀ A_{2B} = 6.1 μ M against **7** EC₅₀ A_{2B} = 2.4 μ M) (Cristalli et al. 1998), and it was also confirmed that **29** is a highly potent, nonselective agonist at A₁AR, A_{2A}AR, and A₃AR subtypes with a K_i in the low nanomolar range at the three subtypes. In the A_{2B}AR functional test, it was found that **29** (EC₅₀ A_{2B} = 1.1 μ M) is twofold more potent than **7**, and the (*S*)-diastereomer **29b** showed an EC₅₀ A_{2B} in the nanomolar range (EC₅₀ = 220 nM). It must be underlined that this was the first case of a NECA derivative substituted in the 2 position with a bulky group and showing good potency at the human A_{2B}AR subtype (Klotz et al. 1999; Lambertucci et al. 2003; Vittori et al. 2004). On the other hand, CGS 21680 was about 100-fold weaker than (*R*, *S*)-PHPNECA at the same subtype, with EC₅₀ A_{2B} = 89 μ M (Cristalli et al. 1998). The substituent linked to the triple bond allowed modulation of selectivity at the A₃AR, and the presence of a phenyl ring conjugated to the triple

bond was detrimental for all the subtypes with the exception of the A₃AR; for example, PENECA (**30**) showed high potency and good selectivity for the A₃AR subtype (Klotz et al. 1999; Vittori et al. 2005). Anyway, the introduction of an alkyl spacer group restored high A_{2A}AR affinity and selectivity, as in phenylpentynyl-NECA.

Another A_{2A}AR agonist, apadenoson (ATL-146e, **31**, Fig. 3), was prepared following the literature activity on alkynyl derivatives. In fact, this molecule is a NECA derivative bearing in the 2 position a propynyl-cyclohexanecarboxylic acid methyl ester group, and binding assays are reported in which the affinity to recombinant human A_{2A}AR is measured as high- and low-affinity K_i values (0.2 and 67.9 nM, respectively) (Murphree et al. 2002).

Other developments include 2-(aralkenyl)-substituted Ado and NECA derivatives (Vittori et al. 1996), and (*E*)-isomers (**32a**, Fig. 3) were 15- to 50-fold more potent at A_{2A}AR than the corresponding (*Z*)-isomers (**32b**). Alkenyl-NECA derivatives, such as (*E*)-2-hexenyl-NECA (**32a**), displayed similar potency as A_{2A}AR agonists to the corresponding alkynyl derivatives, but showed higher selectivity versus A₁AR (Vittori et al. 1996). In this series, the *N*-ethylcarboxamido modification of the ribose was critical to increasing A_{2A}AR affinity. In addition, some 2-arylalkylthio analogs of NECA were synthesized and tested in radioligand binding studies, and the 2-phenylethylthio derivative (**33**) proved to be the most potent and selective agonist at the pig and rat A_{2A}AR (Volpini et al. 2004).

In conclusion, the affinities at the human and rat A_{2A}AR are ranked as follows: PHPNECA ≥ HENECA > NECA > CGS 21680 > PENECA, even though none of these compounds are selective towards both A₁AR and A₃AR subtypes at the same time. Thus, so far, no satisfactory A_{2A}AR-selective agonists are available. In 2001, four new derivatives that are structurally similar to the 2-alkynyl derivatives of NECA that were previously reported (Cristalli et al. 2003) were evaluated by competitive binding assays employing the A_{2A}AR in rat striatal membranes and A₁AR of rat cortex. Hence, the A_{2A}AR against A₁AR selectivity was evaluated, but no A_{2A}AR against A₃AR selectivity was reported (Rieger et al. 2001). As some 2-alkynyl derivatives of NECA had been previously reported to behave as potent A₃AR agonists, affinity at this receptor should be measured before claiming selectivity for the reported compounds.

1.4.2 Ribose- and Purine-Modified NECA Derivatives

A few modifications of the ribose moiety of NECA have been reported (Jacobson et al. 1995; Volpini et al. 1998, 1999; de Zwart et al. 1999a). The ethyl group of the *N*-alkylcarboxamido function was substituted by a methyl or a cyclopropyl group, and this modification seems to be the only one that is well tolerated by the rat A_{2A}AR (see compounds **34** (MECA) and **35** in Fig. 3 and Table 1, K_i A_{2A}AR = 330 and 12 nM, respectively) (de Zwart et al. 1999a). On the other hand, replacing the same ethyl substituent in the 5' position of **28** with a cyclopentyl or benzyl group brought about a significant decrease in affinity at all of the receptor subtypes (see compounds **36** and **37** in Table 2, K_i A_{2A}AR = 49 and 720 nM, respectively)

(Volpini et al. 1999). Some deoxy and dideoxy derivatives of **34** have been described, and the general effect of these modifications is a reduced affinity at all receptor subtypes (Jacobson et al. 1995; Volpini et al. 1998). However, the removal of the 3'-hydroxy group seems to be better tolerated by the A_{2A}AR than the removal of the corresponding group in the 2' position (Cristalli et al., unpublished results).

The only purine-modified analog of NECA that has been synthesized and tested so far is 1-deazaNECA (**7a**, Fig. 1) (Cristalli et al. 1988; Siddiqi et al. 1995). As in the case of the other 1-deazaAdo analogs, the affinity of 1-deazaNECA at all ARs is reduced in comparison to that of the parent compound NECA (**7**)—in fact it is about tenfold less active than NECA—but 1-deazaNECA is clearly more active than the parent compound 1-deazaAdo (**8**) as an inhibitor of platelet aggregation and as a stimulator of cyclic AMP accumulation. However, in contrast to 2-chloro-1-deazaAdo (**9**), which was the only 1-deaza analog showing slight A_{2A}AR-selectivity, the potency of 1-deazaNECA at A₁AR, A_{2B}AR, and A₃AR is diminished by a factor of about 5, whereas that at the A_{2A}AR subtype is about 60-fold lower than that of NECA. Hence, 1-deazaNECA proved to be a moderate A_{2A}AR agonist.

1.5 Agonist Radioligands

[³H]NECA was introduced as a ligand for the A₂ receptor (K_d values of between 31 and 46 nM), but further studies demonstrated that it is a prototypical nonselective ligand (Gessi et al. 2000). It labels A₁AR, A_{2A}AR, and A₃AR with similar affinities, with a slight preference for the A₃AR subtype (Bruns et al. 1986). CGS 21680 was introduced as an A₂-selective agonist and it was also developed as a tritiated ligand (Jarvis et al. 1989), but (as reported above) this molecule is not an ideal tool for the characterization of A_{2A}ARs, particularly if differentiation from A₃AR is required. The tritiated compound displays a K_d value of 32 nM at the human A_{2A}AR and therefore shows a comparable potency to [³H]NECA (Wan et al. 1990).

1.6 Partial Agonists

Recently, a series of 2,8-disubstituted Ado derivatives were synthesized and tested. Most of these compounds appeared to have A_{2A}AR affinities in the low micromolar or nanomolar range, and also displayed reduced intrinsic activities compared to the reference agonist CGS 21680 (**7b**); hence, they behaved as partial agonists (van Tilburg et al. 2003).

The introduction of 8-alkylamino substituents led to a reduction in A_{2A}AR affinity but also to an increase in selectivity versus the A₃AR subtype. In particular, the 8-methylamino and 8-propylamino derivatives of **17** (**38** and **39**, respectively, Fig. 4)

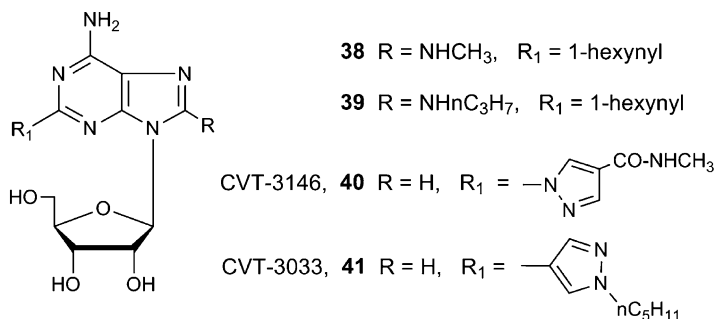


Fig. 4 A_{2A}AR partial agonists

showed K_i A_{2A}AR affinity values of 115 and 82 nM, respectively, and 49- and 26-fold selectivities for the A_{2A}AR versus the A₃AR.

Other Ado derivatives that were substituted at the 2 position with 1-pyrazolyl (Lexiscan, regadenoson, CVT-3146, **40**) or 4-pyrazolyl (CVT-3033, **41**) rings were found to be short-acting functionally selective coronary vasodilators with good potency, but they possessed low affinity for A_{2A}AR ($K_i = 1,122$ and $2,895$ nM, respectively) (Zablocki et al. 2001). One of these, Lexiscan, appears to be a weak partial agonist in stimulating cAMP accumulation in PC12 cells but a full and potent agonist in inducing coronary vasodilation, a response that has a very large A_{2A}AR reserve (Gao et al. 2001; Eggbrecht and Gossel 2006; Gordi 2006).

Very recently, the US Food and Drug Administration (FDA) has approved injected Lexiscan for use as a pharmacologic stress agent in radionuclide myocardial perfusion imaging (MPI) (CVT 2008).

2 Adenosine A_{2A} Receptor Antagonists

In the last few years, A_{2A}AR antagonists have become attractive pharmacological tools due to their potential as novel drugs for the treatment of Parkinson's disease (PD) and restless legs syndrome, Alzheimer's disease, and their antidepressive and neuroprotective activities (Impagnatiello et al. 2000; Cacciari et al. 2003; Xu et al. 2005; Jacobson and Gao 2006; Moro et al. 2006; Schapira et al. 2006; Schwarzschild et al. 2006; Cristalli et al. 2007; Dall'Igna et al. 2007; Fuxe et al. 2007; Yu et al. 2008; Salamone et al. 2008). In addition, A_{2A}AR antagonists seem to protect against cellular death induced by ischemia, and may also be active as cognition enhancers, antiallergic agents, analgesics, positive inotropics, and even for the treatment of alcoholism and alcohol and cannabis abuse (Ledent et al. 1997; Richardson et al. 1997; Monopoli et al. 1998; Brambilla et al. 2003; Pedata et al. 2005; Melani et al. 2006; Ferré et al. 2007; Thorsell et al. 2007; Bilkei-Gorzo et al. 2008; Takahashi et al. 2008). A_{2A}ARs are expressed in high density in restricted areas of

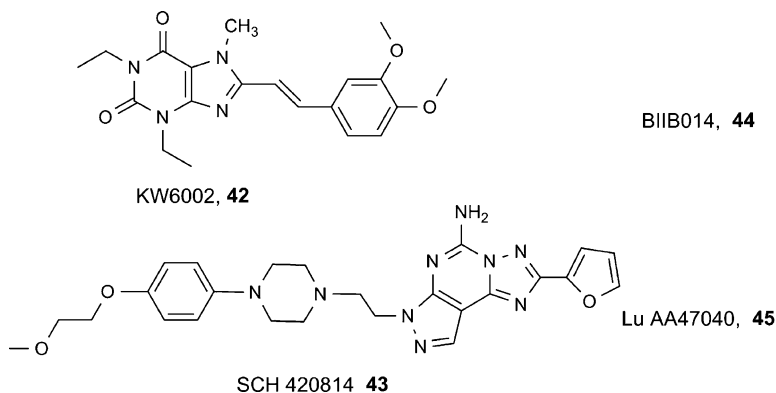


Fig. 5 A_{2A}AR antagonists in clinical trials

the brain, namely in the caudate-putamen (striatum), and there they are coexpressed with dopamine D₂ and cannabinoid CB₁ receptors (Carriba et al. 2007; Ferré et al. 2008). The restricted expression as well as the promising pharmacological potential of A_{2A}AR antagonists has led to extensive efforts to develop potent and selective A_{2A}AR antagonists (Yuzlenko and Kiec-Kononowicz 2006; Müller and Ferré 2007; Baraldi et al. 2008). Four different A_{2A}AR antagonists are currently being studied in clinical trials, istradefylline (KW-6002, **42**), preladenant (SCH-420814, **43**), BIIB014 (V2006, **44**), and Lu AA47040 (**45**). The structures of the latter two compounds have not been disclosed (Fig. 5).

Several heterocyclic classes of compounds have been studied as A_{2A}AR antagonists; these can generally be divided into xanthine and non-xanthine derivatives. The xanthine analogs represent the prototypical group of antagonists, and modifications of the xanthine scaffold resulted in a comprehensive collection of derivatives, among which several compounds showed distinct subtype selectivity. A second class of heterocyclic compounds can be envisaged as adenine-derived structures (Cacciari et al. 2003; Vu 2005; Moro et al. 2006; Müller and Ferré 2007). Very recently, other heterocyclic structures related to neither xanthine nor adenine derivatives have been described. These are based on lead structures identified by the screening of large compound libraries (Müller and Ferré 2007). The present review focuses on antagonists published in scientific articles. Thorough reviews on the patent literature have recently been published (Vu 2005; Müller and Ferré 2007).

2.1 Xanthine Derivatives

Years ago it was reported that caffeine was the “most widely consumed behaviorally active substance in the world” (Fredholm et al. 1999). In fact, the vast majority of people on our planet have enjoyed the CNS effects of the AR antagonist caffeine long before the physiological effects of Ado were discovered. Naturally occurring

xanthines like caffeine or theophylline generally have affinities at the micromolar level, with the highest affinity being at the A_{2A} AR, and this receptor subtype appears to be relevant to the activation caused by caffeine (Ledent et al. 1997; Svenningsson et al. 1997). Hence, the xanthine scaffold represented an important starting point for the development of antagonists of this family of receptors (Daly et al. 1991).

A large number of modifications at the 1, 3, 7 and 8 positions have been performed with the aim of obtaining potent and selective A_{2A} AR antagonists. The first xanthine derivative considered an A_{2A} AR antagonist was 3,7-dimethyl-1-propargylxanthine (DMPX, **46**, Fig. 6, Table 2), even though this compound proved to be poorly active (K_i rA_{2A} and hA_{2A} = 16 and 2 μ M, respectively) and moderately selective against the A_{1A} AR and A_{2B} AR subtypes (Daly et al. 1986, 1991). Nevertheless, this compound has been widely used in in vivo studies because of its good water solubility and bioavailability (Daly et al. 1986; Seale et al. 1988; Thorsell et al. 2007). Further studies on DMPX derivatives led to the 2-*O*-methyl-1-propargylxanthine derivative **47**, endowed with an affinity in the high nanomolar range (K_i A_{2A} AR = 105 nM) at the A_{2A} AR subtype and significant selectivity in comparison to the A_{1A} AR (45-fold) (Müller and Stein 1996; Müller et al. 1998a).

Starting from these observations, a program to screen various 1,3,8-substituted xanthines led to the discovery of the first very potent and selective A_{2A} AR antagonists (Erickson et al. 1991; Jacobson et al. 1993a; Nonaka et al. 1994a; Müller and Stein 1996; Müller 2000b). In particular, 3-chlorostyrylcaffeine (CSC, **48**) showed

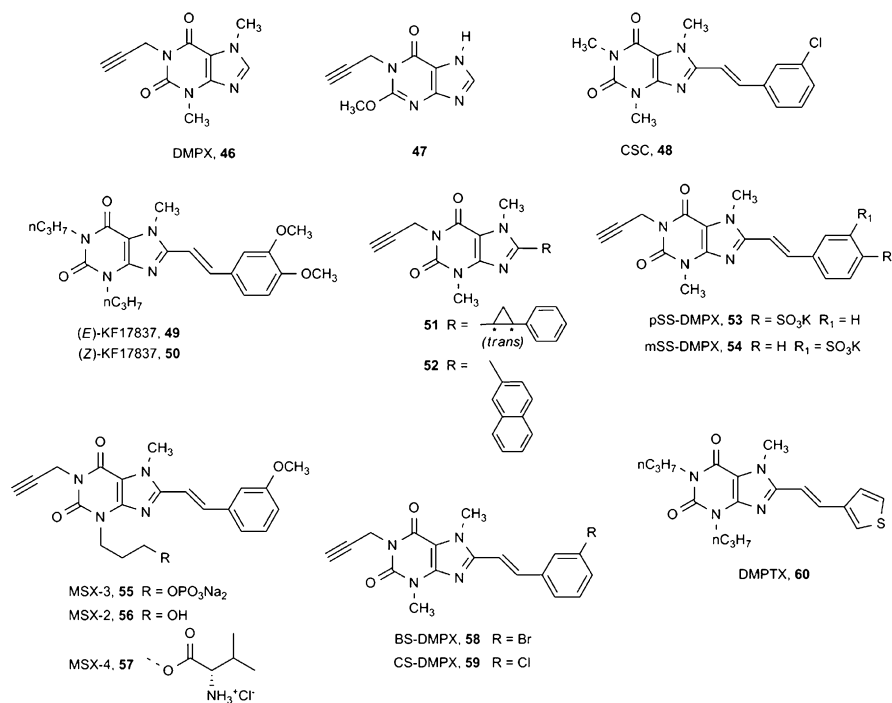


Fig. 6 A_{2A} AR antagonists: xanthines

Table 2 Affinities of AR antagonists in radioligand binding assays at A₁AR, A_{2A}AR and A₃AR. For A_{2B}AR, radioligand binding assays values are reported where available; for some compounds, values are related to the effects on adenylate cyclase activity

Cpd	K _i (A ₁ AR) ^a	K _i (A _{2A} AR) ^a	K _i (A _{2B} AR) ^a	K _i (A ₃ AR) ^a
42	580 r	13 r	–	–
	2,830 h	36 h	1,800 h	>3,000 h
43	–	2.5 r	–	–
	>1000 h	1.1 h	>1,700 h	>1,000 h
46	45,000 r	16,000 r	2,500 m	–
	–	2,000 h	4,130 h	>10,000 h
47	4,700 r	105 r	–	–
48	28,000 r	54 r	–	–
49	62 r	1 r	–	–
50	>10,000 r	860 r	–	–
51	4,600 r	1,700 r	–	–
52	980 r	380 r	–	–
53	4,900 r	240 r	–	–
	–	–	–	>100,000 h
54	8,900 r	300 r	–	–
	–	–	–	>100,000 h
56	900 r	8 r	–	–
	2,500 h	5.0 h	–	>10,000 h
58	1,200 r	8.2 r	–	–
59	1,300 r	13 r	–	–
60	561 r	19 r	–	–
61	21 r	3.3 r	–	–
	4.4 h	0.43 h	25 h	85 h
62	270 r	21 r	–	–
63	3.3 r	1.2 r	–	–
64	121 r	2.3 r	–	–
	549 h	1.1 h	>10,000 h	>10,000 h
65	504 r	2.4 r	–	–
	350 h	1.2 h	>10,000 h	>10,000 h
66	444 r	1.7 r	–	–
	–	–	–	>10,000 h
67	741 r	0.94 r	–	–
	1,111 h	1.5 h	–	>10,000 h
68	1,815 r	0.048 r	–	–
	1,111 h	0.5 h	>10,000 h	>10,000 h
69	4,927 h	4.63 h	>10,000 h	>10,000 h
70	139 h	140 h	>10,000 h	>10,000 h
71	2,160 h	0.22 h	>10,000 h	>10,000 h
72	369 h	3.8 h	>10,000 h	>10,000 h
73	15 b	6.5 b	–	–
	–	–	–	>10,000 h
74	83 h	0.8 h	–	–
75	257 r	1.8 r	–	–
	774 h	1.6 h	28 h	743 h

(continued)

Table 2 (continued)

Cpd	K_i (A_1 AR) ^a	K_i (A_{2A} AR) ^a	K_i (A_{2B} AR) ^a	K_i (A_3 AR) ^a
76	1,270 p	14 p	–	–
77	320 r	1 r	–	–
78	100 r	1.1 r	–	–
79	208 h	1.4 h	865 h	476 h
80	12.5 r	1.2 r	–	–
	7.9 m	1.6 m	–	–
	9.0 h	1.8 h	>557 h	–
81	17 h	1.1 h	112 h	1,472 h
82	170 h	1.7 h	141 h	1,931 h
83	24 h	3.7 h	380 h ^b	4,700 h
84	2,400 h	46 h	>30,000 h ^b	21,000 h
85	150 h	19 h	690 h ^b	3,100 h
86	23 h	1.7 h	569 h ^b	1,090 h
87	9.4 h	3.8 h	780 h ^b	17.6 h
88	5.8 h	2.2 h	521 h ^b	16 h
89	71.8 h	6.6 h	352 h	>10,000 h
90	–	26 r	–	–
	266 h	2.7 h	–	–
91	–	9.4 r	–	–
	60 h	0.4 h	–	–
94	–	90 r	–	–
	1,380 h	20 h	–	–

^aBinding data from different species: rat (*r*), human (*h*), pig (*p*), bovine (*b*) or mouse (*m*) A_1 AR, A_{2A} AR, A_{2B} AR, and A_3 AR, expressed as K_i (nM)

^bEffects on adenylate cyclase activity at the human (*h*) A_{2B} AR expressed as K_i (nM)

high affinity at the A_{2A} AR (54 nM) and high selectivity in comparison to the A_1 AR subtype (560-fold) (Jacobson et al. 1993a). In addition, it is a relatively potent monoaminoxidase type B (MAO-B) inhibitor, which may contribute to its pharmacological effects in models of Parkinson's disease (Petzer et al. 2003; van den Berg et al. 2007). Another compound, (*E*)-1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine ((*E*)-KF17837, **49**), proved to be potent in the nanomolar range at the A_{2A} AR subtype (1 nM) and significantly selective in comparison to the A_1 AR (62-fold) (Nonaka et al. 1994a). However, several problems have initially limited the use of these xanthine derivatives as pharmacological tools for studying the A_{2A} AR subtype, in particular their low water solubility (Jackson et al. 1993) and the rapid photoisomerization that they undergo when exposed to daylight in dilute solution (Nonaka et al. 1993; Müller et al. 1998a). It should be noted that this isomerization process does not occur when styrylxanthines are administered orally as solid substances, but the phenomenon happens very rapidly during binding studies performed in buffer solution and in the presence of light (Müller et al. 1998a). In particular, after photoisomerization, (*E*)-KF17837 becomes a stable mixture of ca. 18% (*E*) and ca. 82% (*Z*, **50**) isomers, and the binding data change (K_i A_{2A} AR = 7.9 nM, K_i A_1 AR = 390 nM) (Nonaka et al. 1993). Another

problem associated with 8-styrylxanthine derivatives is their tendency to undergo light-induced dimerization ([2 + 2]-cycloaddition reaction) in the solid state, yielding weakly active cyclobutane derivatives (Hockemeyer et al. 2004).

To overcome this photoisomerization, the styryl moiety has been replaced with different functional groups (e.g., triple bond, cyclopropyl ring, **51**, a 2-naphthyl residue, **52**) (Müller et al. 1997c), or a tricyclic constrained structure (Kiec-Kononowicz et al. 2001; Drabczynska et al. 2003, 2004, 2006, 2007). In many cases, a significant loss of affinity was observed by such modifications. Substitution of the ethenyl group with an azo structure has also been performed. The compounds obtained retained selectivity but showed only moderate affinity (Müller et al. 1997b).

Different approaches have been utilized to improve the water solubility of styrylxanthines, such as the introduction of polar groups on the phenyl ring and the preparation of phosphate or amino-acid prodrugs. The introduction of a sulfonate group on the phenyl ring of the styryl moiety at the *para*- (**53**) or *meta*- (**54**) position led to water-soluble derivatives endowed with only high nanomolar affinity at the A_{2A}AR but retaining selectivity (Müller et al. 1998b). Tricyclic styryl-substituted imidazo[2,1-*i*]purin-5-one derivatives showed enhanced water-solubility but reduced A_{2A}AR affinity and selectivity (Müller et al. 2002). The prodrug approach has been much more successful. In fact, MSX-3 (**55**), which is the phosphate prodrug of MSX-2 (3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-1-propargylxanthine, **56**), is stable and highly soluble (15 mM) in aqueous solution but readily cleaved by phosphatases to liberate MSX-2, which showed a very high affinity (rat and human A_{2A}AR $K_i = 8$ and 5 nM, respectively) and selectivity for the A_{2A}AR (Sauer et al. 2000; Hockemeyer et al. 2004). Recently, an L-valine ester prodrug of MSX-2 has been described, named MSX-4 (**57**), which shows good water solubility as a hydrochloride as well as high stability in artificial gastric fluid and at physiological pH values, but is readily cleaved by esterases (Vollmann et al. 2008). It is expected that the L-amino acid ester prodrug can be absorbed via an active transport mechanism by L-amino acid carrier proteins.

All of these studies strongly suggest that the xanthine family should be reconsidered as A_{2A}AR antagonists. In fact, the antagonist KW-6002 (istradefylline: 1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methylxanthine, **42**; human A_{2A}AR $K_i = 36$ nM) is already in Phase III clinical trials for the treatment of basal ganglia disorders such as Parkinson's disease (Knutsen and Weiss 2001; Weiss et al. 2003; Kalda et al. 2006). This compound showed a (*E*)/(*Z*) stable equilibrium ratio of 19:81 with good affinity and selectivity but most importantly a very high anticataleptic activity (0.03 mg kg⁻¹, p.o.) in a mouse haloperidol model (Shimada et al. 1997).

Further modifications of all the positions of the xanthine nucleus were introduced and investigated. For example, the bioisosteric replacement of one of the alkenyl CH groups of the 8-styryl residue with nitrogen led to more potent and selective antagonists for the A_{2A}ARs, but the compounds were highly unstable in aqueous solution because of their imine (Schiff base) structure (Müller et al. 1997b). The introduction of a propargyl or an *n*-propyl residue at the 1 position in combination with the 8-styryl group seems to increase affinity at the A_{2A}AR subtypes

while retaining the selectivity. These studies led to the discovery of two compounds, named BS-DMPX (3,7-dimethyl-1-propargyl-8-(3-bromostyryl)xanthine, **58**) and CS-DMPX (3,7-dimethyl-1-propargyl-8-(3-chlorostyryl)xanthine, **59**), which could be considered lead compounds of this series (Müller et al. 1997a). Methyl substitution at the 3 and 7 positions appears to be desirable for achieving both affinity and selectivity at A_{2A}AR subtypes (Shamim et al. 1989; Erickson et al. 1991; Del Giudice et al. 1996). However, large substituents are also tolerated at the 3 position (Massip et al. 2006). The bioisosteric replacement of the phenyl ring with a thienyl moiety led to DPMTX ((*E*)-1,3-dipropyl-7-methyl-8-[2-(3-thienyl)ethenyl]xanthine, **60**) which showed high affinity and selectivity (Del Giudice et al. 1996). Regarding the substitutions at the 8 position, it has been demonstrated that an aromatic ring attached to an ethenyl group is essential for both affinity and selectivity at the A_{2A}AR (Erickson et al. 1991; Jacobson et al. 1993b; Del Giudice et al. 1996). 8-Styryl-9-deazaxanthine derivatives were nearly as potent as the corresponding xanthine derivatives at A_{2A}ARs (Grahner et al. 1994).

2.2 Adenine Derivatives and Related Heterocyclic Compounds

Due to the initial problems with xanthine derivatives, such as poor water solubility and photoisomerization, many scientists searched for alternative heterocyclic derivatives for use as lead compounds. The first promising A_{2A}AR antagonists with a non-xanthine structure were CGS 15943 (9-chloro-2-(2-furyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine, **61**, Fig. 7) (Williams et al. 1987; Francis et al. 1988; Kim et al. 1996; Baraldi et al. 2000) and CP-66713 (4-amino-8-chloro-1-phenyl-[1,2,4]triazolo[4,3-*a*]quinoxaline, **62**) (Sarges et al. 1990), compounds that were not very A_{2A}AR selective but were important as starting points for developing new non-xanthine structures as A_{2A}AR antagonists. All of these structures are reminiscent of the nucleobase adenine, a partial structure of Ado.

A few years later, the synthesis of 8FB-PTP (5-amino-8-(4-fluorobenzyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine, **63**), a bioisoster of **61**, was reported (Gatta et al. 1993; Dionisotti et al. 1994). Here, the phenyl ring was replaced by a substituted pyrazole nucleus; this compound showed good affinity but no selectivity for A_{2A}ARs. Structure–activity relationship studies on the pyrazolo-triazolo-pyrimidine nucleus were carried out with the aim of determining the important features for high A_{2A}AR potency and selectivity, focusing on the presence of a free amino group at the 5 position and a furan ring at the triazole ring. The role of the substituents on the pyrazole ring was explored. Results showed that the substituents at the 7 and 8 positions were influential. In particular, substitutions at the 7 position gave selective compounds, whereas the same substitution at the 8 position resulted in potent but nonselective derivatives (Baraldi et al. 1994, 1996a, 2001). Furthermore, replacement of the pyrazole ring with a triazole led to affinity retention but also a complete loss of selectivity (Baraldi et al. 1996b). Recently, the pyrazole was replaced by an imidazole ring with great success (Silverman et al. 2007).

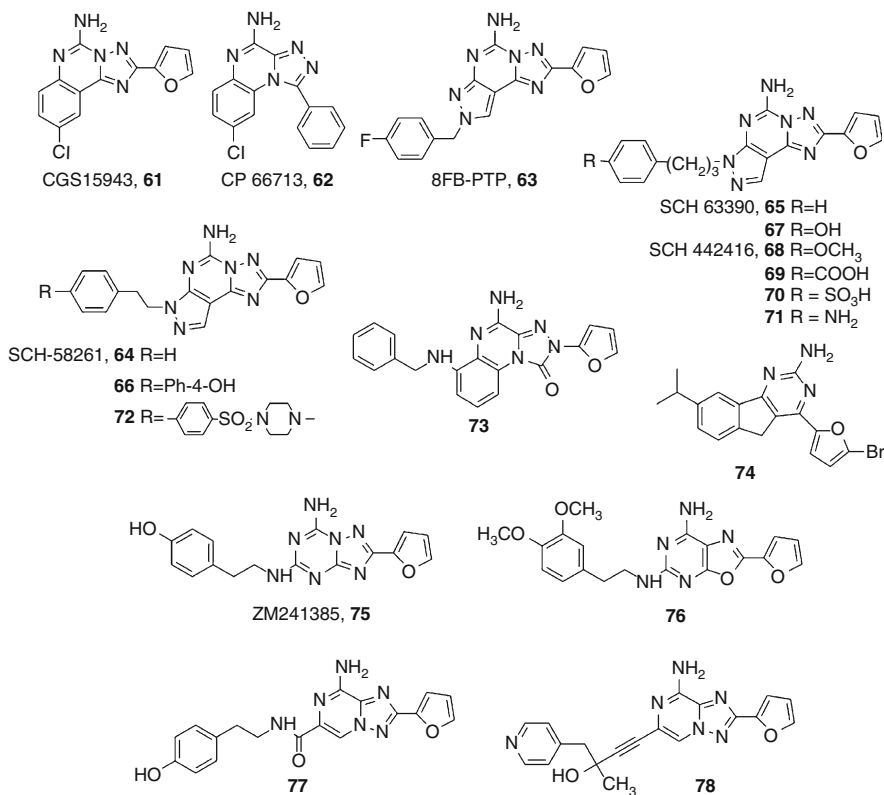


Fig. 7 A_{2A}AR antagonists: nonxanthine derivatives

Two selected compounds named SCH-58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine, **64**, Fig. 7) and SCH-63390 (5-amino-7-(3-phenylpropyl)-2-(2-furyl)pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine, **65**) proved to be very potent and selective A_{2A}AR antagonists at both rat and human receptors (Baraldi et al. 1996a, b, 1998b; Zocchi et al. 1996a).

Problems with low water solubility affected even these non-xanthine compounds, and the poor bioavailability limited their use as pharmacological tools. To improve the hydrophilicities of these derivatives, polar functions were introduced on the phenyl ring located on the side chain of the pyrazole nucleus. The presence of a hydroxyl group at the phenyl ring in the *para* positions of compounds **64** and **65** led to compounds **66** (5-amino-7-[4-(4-hydroxyphenyl)ethyl]-2-(2-furyl)pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine) and **67** (5-amino-7-[3-(4-hydroxyphenyl)propyl]-2-(2-furyl)pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine), which showed slightly enhanced hydrophilicity and also a significant increase in both affinity and selectivity (Baraldi et al. 1998b). To understand the nature of the hydrogen bond, the phenolic hydroxy group was substituted with a methoxy group (thus reducing compound hydrophilicity), leading to

SCH-442416 (5-amino-7-[3-(4-methoxyphenyl)propyl]-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine, **68**). This derivative showed an increased potency and remarkable selectivity for the A_{2A}AR, and so it has been used as a tool for PET studies in its ¹¹C-labeled form (Todde et al. 2000). The introduction of oxygen-containing groups on the phenyl ring did not confer sufficient water solubility on the derivative, so it appeared necessary to introduce different functionalities to address this problem. Several polar functions such as carboxylic (**69**) and sulfonic acid (**70**) functions were introduced for this purpose and, as expected, an increased solubility was observed, especially in the case of the sulfonate. Unfortunately, a great loss of affinity and selectivity was observed at the same time. The introduction of an amino group at the *para* position of the phenyl ring gave compound **71** ($K_i = 0.22$ nM, hA₁AR/hA_{2A}AR = 9820), which yielded the best results in terms of affinity and selectivity, without improving the water solubility. Sulfonamido derivatives seem to exhibit a good balance between solubility and affinity (**72**) (Baraldi et al. 2002). Structure–activity relationships for this group of compounds indicated that the tricyclic structure of the pyrazolo-triazolo-pyrimidine, the presence of the furan ring, the exocyclic 5-amino group, and the arylalkyl substituent on the nitrogen at the 7 position are probably crucial to their affinities and selectivities for the A_{2A}AR subtype.

A recent series of pyrazolo-triazolo-pyrimidine derivatives was obtained by modifying the phenylethyl substituent of **64** with substituted phenylpiperazinethyl groups (Neustadt et al. 2007). Introduction of fluorine atoms into the phenyl ring enhanced the affinity to subnanomolar values and the compounds displayed potent peroral activity, but their solubility still remained poor. Further introduction of ether substituents led to derivatives with high affinities and selectivities for A_{2A}ARs and improved water solubilities. In particular, one of these compounds (SCH-420814, preladenant, **43**) exhibited high affinities for both rat and human A_{2A}ARs, with K_i values of 2.5 and 1.1 nM, respectively. In addition, the compound is very selective for human A_{2A}ARs over A₁AR, A_{2B}AR, and A₃AR. Interestingly, the compound did not show significant binding against a panel of 59 unrelated receptors, enzymes, and ion channels. preladenant is now in Phase II clinical trials for dyskinesia in Parkinson's disease (Neustadt et al. 2007). Recently, the pyrazole moiety in these tricyclic derivatives was replaced by an imidazole ring, yielding 3*H*-[1,2,4]triazolo[5,1-*i*]purin-5-amine derivatives. The isomer of SCH-420814 displayed promising *in vitro* and *in vivo* profiles (Silverman et al. 2007).

The triazoloquinoxaline (Colotta et al. 1999, 2000, 2003) and the indenopyrimidine (Matasi et al. 2005) series possess promising features as A_{2A}AR antagonists. The triazoloquinoxaline nucleus seems to be very sensitive to any kind of variation and modification: alkylation of the amino group, replacement of the amino group by a carbonyl function, and substitution on the phenyl ring all reduced A_{2A}AR affinity. In this class, only compound **73** (4-amino-6-benzylamino-1,2-dihydro-2-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one) showed a favorable binding profile (Colotta et al. 1999, 2000, 2003). In contrast, the indenopyrimidine derivatives are very promising, and the derivative **74** shows affinity in the nanomolar range and good selectivity against the A₁AR subtype. It must be underlined that

binding data at A_{2B}AR and A₃AR are lacking, so it is not possible to fully assess this compound with regard to potentially being an ideal A_{2A}AR antagonist (Matasi et al. 2005). Anyway, these structures showed several problems, such as poor water solubility and (most importantly) complex and difficult synthetic accessibility.

Therefore, researchers focused their attention on simplified analogs like bicyclic systems, and the Zeneca group reported on a compound named ZM241385 (4-[2-[[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-*a*][1,3,5]triazin-5-yl]amino]ethyl]phenol, **75**), which proved to be one of the most potent A_{2A}AR antagonists ever reported, and which had a favorable water solubility (Caulkett et al. 1995; Poucher et al. 1995; de Zwart et al. 1999b; Weiss et al. 2003; Moro et al. 2006). This compound also binds with high affinity to human A_{2B}AR, and its tritiated form is actually used in radioligand binding studies for this receptor subtype (Ji and Jacobson 1999).

In the last few years, Biogen Idec Inc. has developed a large series of triazolotriazine and triazolopyrimidine analogs bearing various substituents, and a few compounds have shown high potency and selectivity for the A_{2A}AR as compared with the A₁AR (Peng et al. 2004; Vu et al. 2004a, b, c, 2005; Yang et al. 2007). However, the lack of binding data for the A_{2B}AR and A₃AR prevents any comparison of the derivatives with other fully characterized compounds. Interestingly, some of these derivatives showed good oral efficacy in a rodent catalepsy model of Parkinson's disease (Peng et al. 2004; Vu et al. 2004a, b, c, 2005).

Among synthesized isosters of the triazolotriazine nucleus, some oxazolopyrimidines (**76**) (Holschbach et al. 2006) and triazolopyrazines (**77**, **78**) should be mentioned (Dowling et al. 2005; Yao et al. 2005). All of these compounds showed good A_{2A}AR potency and selectivity against the A₁AR, but full characterization at the four AR subtypes has not been completed. Some pyrazolopyrimidines have also been reported (Chebib et al. 2000), but in all cases the affinities and/or selectivities were only moderate.

A thieno[3,2-*d*]pyrimidine, VER-6623 (**79**, Fig. 8), showed a high affinity for A_{2A}AR ($K_i = 1.4$ nM), but it also had low or poor oral bioavailability (Weiss et al. 2003; Yang et al. 2007). Very recently, a potent A₁AR and A_{2A}AR dual antagonist, 5-[5-amino-3-(4-fluorophenyl)pyrazin-2-yl]-1-isopropylpyridine-2(1*H*)-one (ASP5854, **80**), was synthesized and tested in models of Parkinson's disease and cognition (Mihara et al. 2007). The binding affinities of **80** for human A₁AR and A_{2A}AR were 9.0 and 1.8 nM, respectively. This compound also showed antagonistic action on A₁AR and A_{2A}AR agonist-induced increases in intracellular Ca²⁺ concentration, and in vivo tests showed that this molecule improves motor impairment, is neuroprotective via A_{2A}AR antagonism, and also enhances cognitive function through A₁AR antagonism.

The development of A_{2A}AR antagonists also made use of non-xanthine imidazopyrimidine (purine)-type structures, and some of these derivatives (recently reported by several groups) seem to be very promising. Some compounds, like VER-6947 (**81**) and VER-7835 (**82**), show human A_{2A}AR K_i values of around 1 nM (Weiss et al. 2003), while some 6-(2-furanyl)-9*H*-purin-2-amino derivatives

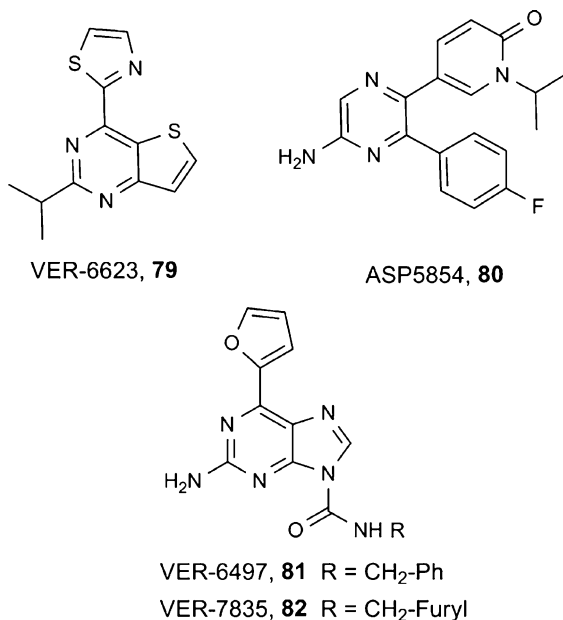


Fig. 8 A_{2A}AR antagonists: nonxanthine derivatives (2)

are endowed with A_{2A}AR affinities in the low nanomolar range and a good level of selectivity against the other receptor subtypes (Kiselgof et al. 2005).

In the late 1990s, Cristalli and coworkers reported the synthesis of a number of 9-ethylpurines bearing various substituents in the 2, 6 or 8 positions (Camaioni et al. 1998). 9-Ethyladenine showed micromolar affinities at the human A₁AR and A_{2A}AR subtypes, but the introduction of a bromine atom in the 8 position led to an enhancement of the binding affinity at all AR subtypes. Recently, rat model studies on the derivatives ANR-152 (9-ethyl-8-furyl-adenine, **83**, Fig. 9) and ANR-94 (8-ethoxy-9-ethyl-adenine, **84**) were reported. It should be noted that **83** was more potent at A_{2A}AR than at A₁AR, with poor selectivity against A₁AR, while the replacement of furan ring with an ethoxy function (**84**) (Klotz et al. 2003) led to a decrease in affinity but a significant increase in selectivity. Study results showed that both of these derivatives are able to ameliorate motor deficits in rat models of Parkinson's disease (Pinna et al. 2005).

The 2 and 8 positions of adenine were further explored through the introduction of alkynyl chains, and while the 2-alkynyl derivatives possessed good affinity and were slightly selective for the human A_{2A}AR, the affinities of the 8-alkynyl derivatives at the human A₁AR, A_{2A}AR, and A_{2B}AR proved to be lower than those of the corresponding 2-alkynyl derivatives, with improved binding data for the human A₃AR subtype (Volpini et al. 2005). The observation that the introduction at the 2 position of phenylethylamino or phenethoxy groups resulted in compounds with increased A_{2A}AR affinity (Camaioni et al. 1998) led to the synthesis of 9-ethyladenine derivatives substituted at the 2 position with phenylalkylamino

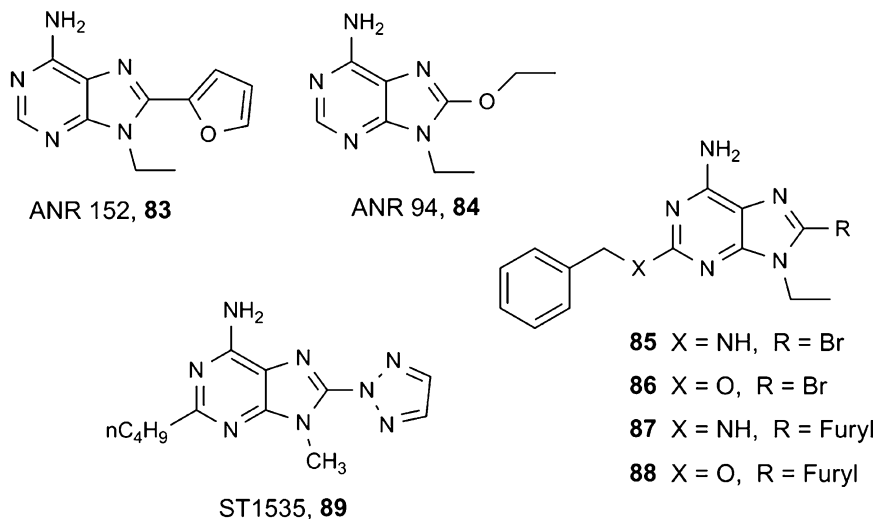


Fig. 9 A_{2A}AR antagonists: adenine derivatives

and phenylalkoxy groups and bearing a bromine atom in the 8 position (**85** and **86**, respectively) (Lambertucci et al. 2007b). This series was synthesized and tested in binding affinity assays at human ARs, and the new compounds showed good affinity and selectivity at A_{2A}AR. In particular, the introduction of a bromine atom at the 8 position increased the affinity of these compounds, leading to ligands with K_i values in the nanomolar range. Further substitution of the bromine atom of **85** and **86** with a 2-furyl group led to compounds **87** and **88** respectively, which maintained the A_{2A}AR affinity at low nanomolar levels, but with reduced selectivity versus A₁AR and A₃AR (Cristalli et al., unpublished results).

A new series of 2,6-substituted 9-propyladenines has been recently synthesized and reported (Lambertucci et al. 2007a). Results show that the introduction of bulky chains at the N⁶ position of 9-propyladenine significantly increases binding affinities at the human A₁AR and A₃AR, while the presence of a chlorine atom at the 2 position results in unequivocal effects depending on the receptor subtype and/or on the substituent present in the N⁶ position. In any case, the presence in the 2 position of a chlorine atom favors the interaction with the A_{2A}AR subtype. Among other adenine derivatives reported as A_{2A}AR antagonists, ST1535 (2-*n*-butyl-9-methyl-8-[1,2,3]triazol-2-yl-9*H*-purin-6-ylamine, **89**, Fig. 9) (Minetti et al. 2005) proved to be quite potent but barely selective against A₁AR. Nevertheless, this compound was selected for in vivo studies and was shown to induce a dose-related increase in locomotor activity.

Slee and colleagues developed a series of aminopyrimidine derivatives that were acylated at the amino group (2-amino-*N*-pyrimidin-4-yl acetamides) and showed high water solubility (Slee et al. 2008c). The lead compound **90** was optimized with regard to replacement of the metabolically problematic furan ring (Slee et al. 2008a), reducing its effects on hERG channels (Slee et al. 2008b); it showed high affinity at

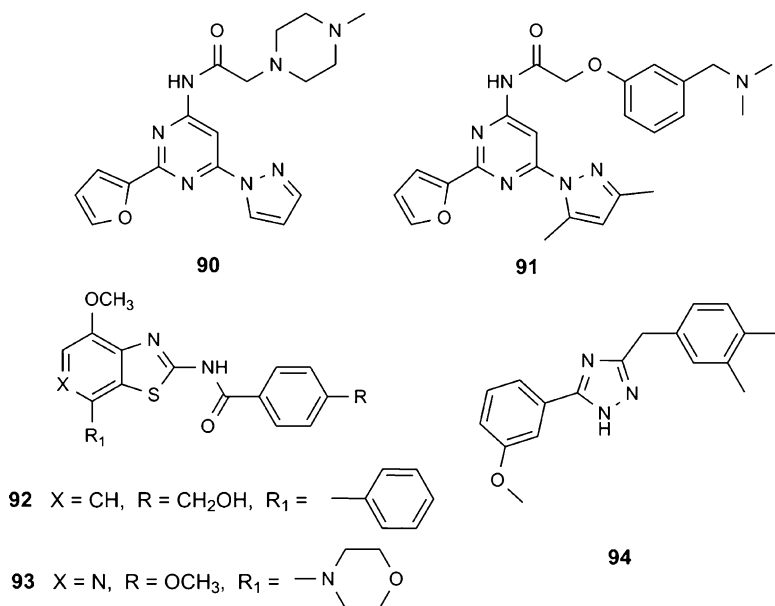


Fig. 10 Various heterocyclic compounds

both human and rodent A_{2A}ARs, as well as A_{2A}AR selectivity (Zhang et al. 2008) and efficacy in rodent catalepsy models after peroral application, yielding **91** as a new lead structure (Fig. 10).

2.3 Heterocyclic Compounds Unrelated to Adenine or Xanthine

Simplified heterocyclic compounds, such as benzothiazole (Flhor and Riemer 2006) and 1,2,4-triazole (Alanine et al. 2004) derivatives (**92–94**), have been reported by the Roche group. These derivatives have been identified by high-throughput screening of compound libraries and are structurally related to neither xanthine nor to adenine derivatives. These compounds appear to be promising new lead compounds for the development of A_{2A}AR antagonists for therapeutic applications (Müller and Ferré 2007).

2.4 Antagonist Radioligands

A number of A_{2A}AR antagonist radioligands have been developed, and again they can be divided into xanthine and non-xanthine derivatives. Among the xanthine derivatives, three biotin conjugates of 1,3-dipropyl-8-phenylxanthine were

reported in 1985 as being able to bind competitively to the ARs, but only in the absence of avidin. Results were interpreted in terms of the possible reorientation of the ligands at the receptor binding site (Jacobson et al. 1985). A few years later, a study on a radiolabeled amine-functionalized derivative of 1,3-dipropyl-8-phenylxanthine (³H]XAC) as an A₂ antagonist at human platelets was published. This molecule exhibited a K_d value at the nanomolar level, and it was reported as the first antagonist radioligand with high affinity at A_{2A}ARs (Jacobson et al. 1986; Ukena et al. 1986). In the mid 1990s, the tritiated derivative of KF17837S (the equilibrium mixture of (*E*)- and (*Z*)-KF17837 isomers) was shown to bind to rat striatal membranes in a saturable and reversible way, with K_d values at low nanomolar concentrations (Nonaka et al. 1994b). In another study, ¹¹C-labeled (*E*)-KF17837 was synthesized and tested, and it was proposed as a potential positron emission tomography (PET) radioligand for mapping the A_{2A}ARs in the heart and the brain (Ishiwata et al. 1996, 1997). Further studies on radiolabeled xanthine derivatives as A_{2A}AR radioligands were carried out by preparing and testing an ¹¹C-labeled selective A_{2A}AR antagonist, (*E*)-8-(3-chlorostyryl)-1,3-dimethyl-7-[¹¹C]methylxanthine [¹¹C]CSC). This molecule was shown to accumulate in the striatum, and PET studies on rabbits showed a fast brain uptake of [¹¹C]CSC, reaching a maximum in less than 2 min (Marian et al. 1999). Few years later, iodinated and brominated styrylxanthine derivatives labeled with ¹¹C were tested as in vivo probes (Ishiwata et al. 2000c). [7-Methyl-¹¹C]-(*E*)-3,7-dimethyl-8-(3-iodostyryl)-1-propargylxanthine ([¹¹C]IS-DMPX) and [7-methyl-¹¹C]-(*E*)-8-(3-bromostyryl)-3,7-dimethyl-1-propargylxanthine ([¹¹C]BS-DMPX) showed K_i affinities of 8.9 and 7.7 nM respectively, and high A_{2A}AR/A₁AR selectivity values. Unfortunately, biological studies proved that the two ligands were only slightly concentrated in the striatum, and that the two compounds were not suitable as in vivo ligands because of low selectivity for the striatal A_{2A}ARs and a high nonspecific binding (Ishiwata et al. 2000c). Another A_{2A}AR antagonist radioligand was prepared, [³H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine ([³H]MSX-2). This molecule showed high affinity ($K_d = 8.0$ nM) for A_{2A}AR, with saturable and reversible binding, and also a selectivity of at least two orders of magnitude versus all other AR subtypes (Müller et al. 2000). A very interesting xanthine derivative that acts as A_{2A}AR radioligand was found in [¹¹C]KF18446 ([7-methyl-¹¹C]-(*E*)-8-(3,4,5-trimethoxystyryl)-1,3,7-trimethylxanthine, also named (¹¹C)TMSX) (Ishiwata et al. 2000a, b, 2002, 2003a, b). Ex vivo autoradiography for this molecule showed a high striatal uptake and a high uptake ratio of the striatum in comparison to other brain regions; [¹¹C]KF18446 was therefore proposed as a suitable radioligand for mapping A_{2A}ARs of the brain by PET (Mishina et al. 2007). In 2001, the synthesis and the testing of [¹¹C]KW-6002 as a PET ligand was reported. This molecule showed high retention in the striatum, but it also bound to extrastriatal regions, so its potential as a PET ligand appeared to require further investigation (Hirani et al. 2001).

Among nonxanthine derivatives, in 1995 the synthesis of [¹²⁵I]-4-(2-[[7-amino-(2-furyl)[1,2,4]-triazolo[2,3-*a*][1,3,5]triazin-5-yl]amino]ethyl)phenol ([¹²⁵I]ZM241

385) and its characterization as a radioligand in A_{2A} AR-expressing membranes was reported (Palmer et al. 1995). This molecule proved to be a highly selective antagonist radioligand for studying A_{2A} ARs within some species. [3 H]ZM241385 showed A_{2A} AR affinity at subnanomolar levels (Alexander and Millns 2001; DeMet and Chicz-DeMet 2002; Kelly et al. 2004; Uustare et al. 2005) and, as reported above, it later also proved to be a high-affinity ligand for A_{2B} AR receptors, and is actually used in radioligand binding studies of this receptor subtype (Ji and Jacobson 1999). Another important A_{2A} AR antagonist radioligand was obtained with [3 H]SCH-58261, which showed a K_d value of about 1 nM (Zocchi et al. 1996b). Biological results showed that this compound directly labels striatal A_{2A} ARs in vivo, and it could be an excellent tool for studying A_{2A} AR brain distribution and its occupancy of various antagonists. Additional studies suggested that [3 H]SCH-58261 is a useful tool for autoradiography studies, and indicated that it was the first available radioligand for the characterization of the A_{2A} AR subtype in platelets (Dionisotti et al. 1996, 1997; Zocchi et al. 1996b; Fredholm et al. 1998; El Yacoubi et al. 2001).

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Recent Developments in A_{2B} Adenosine Receptor Ligands

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and Pier Giovanni Baraldi

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Abstract A selective, high-affinity A_{2B} adenosine receptor (AR) antagonist will be useful as a pharmacological tool to help determine the role of the A_{2B}AR in inflammatory diseases and angiogenic diseases. Based on early A_{2B}AR-selective ligands with nonoptimal pharmaceutical properties, such as **15** (MRS 1754: $K_i(\text{hA}_{2B}) = 2 \text{ nM}$; $K_i(\text{hA}_1) = 403 \text{ nM}$; $K_i(\text{hA}_{2A}) = 503 \text{ nM}$, and $K_i(\text{hA}_3) = 570 \text{ nM}$), several groups have discovered second-generation A_{2B}AR ligands that are suitable for development. Scientists at CV Therapeutics have discovered the selective, high-affinity A_{2B}AR antagonist **22**, a 8-(4-pyrazolyl)-xanthine derivative, (CVT-6883, $K_i(\text{hA}_{2B}) = 22 \text{ nM}$; $K_i(\text{hA}_1) = 1,940 \text{ nM}$; $K_i(\text{hA}_{2A}) = 3,280$; and $K_i(\text{hA}_3) = 1,070 \text{ nM}$). Compound **22** has demonstrated favorable pharmacokinetic (PK) properties ($T_{1/2} = 4 \text{ h}$ and $F > 35\%$ rat), and it is a functional antagonist at

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the A_{2B}AR ($K_B = 6$ nM). In a mouse model of asthma, compound **22** demonstrated a dose-dependent efficacy supporting the role of the A_{2B}AR in asthma. In two Phase I clinical trials, **22** (CVT-6883) was found to be safe, well tolerated, and suitable for once-daily dosing. Baraldi et al. have independently discovered a selective, high-affinity A_{2B}AR antagonist, **30** (MRE2029F20), 8-(5-pyrazolyl)-xanthine ($K_i(\text{hA}_{2B}) = 5.5$ nM; $K_i(\text{hA}_1) = 200$ nM; $K_i(\text{hA}_{2A}, A_3) > 1,000$), that has been selected for development in conjunction with King Pharmaceuticals. Compound **30** has been demonstrated to be a functional antagonist of the A_{2B}AR, and it has been radiolabeled for use in pharmacological studies. A third compound, **58** (LAS-38096), is a 2-aminopyrimidine derivative (discovered by the Almirall group) that has high A_{2B}AR affinity and selectivity ($K_i(\text{hA}_{2B}) = 17$ nM; $K_i(\text{hA}_1) > 1,000$ nM; $K_i(\text{hA}_{2A}) > 2,500$; and $K_i(\text{hA}_3) > 1,000$ nM), and **58** has been moved into preclinical safety testing. A fourth selective, high-affinity A_{2B}AR antagonist, **54** (OSIP339391 $K_i(\text{hA}_{2B}) = 0.5$ nM; $K_i(\text{hA}_1) = 37$ nM; $K_i(\text{hA}_{2A}) = 328$; and $K_i(\text{hA}_3) = 450$ nM) was discovered by the OSI group. The three highly selective, high-affinity A_{2B}AR antagonists that have been selected for development should prove useful in subsequent clinical trials that will establish the role of the A_{2B}ARs in various disease states.

Keywords Adenosine receptor antagonist · Asthma · CVT-6883 · MRE2029F20 · LAS-38096

Abbreviations

AR	Adenosine receptor
BALF	Bronchoalveolar lavage fluid
BSMCs	Bronchial smooth muscle cells
cAMP	Cyclic adenosine monophosphate
CGS-21680	2-[<i>p</i> -(2-Carboxyethyl)phenylethylamino]-5'- <i>N</i> -ethylcarboxamidoadenosine
CPA	<i>N</i> ⁶ -Cyclopentyladenosine
DAG	Diacylglycerol
HBECs	Human bronchial epithelial cells
HRECs	Human retinal endothelial cells
IL	Interleukin
IP3	(1,4,5)Inositol triphosphate
MCP-1	Monocyte chemotactic protein-1
NECA	5'- <i>N</i> -Ethylcarboxamidoadenosine
NIDDM	Noninsulin-dependent diabetes mellitus
NSAIDs	Nonsteroidal antiinflammatory drugs
PK	Pharmacokinetic
SAR	Structure–activity relationship
VEGF	Vascular endothelial growth factor

1 Introduction

The A_{2B} adenosine receptor (AR) is a member of the P₁ family of seven-transmembrane ARs, and it couples to G_s to increase cAMP and G_{q11} to increase (1,4,5)inositol triphosphate (IP₃)/diacylglycerol (DAG) (Fozard and McCarth 2002). The A_{2B}AR has been found to be located in smooth muscle cells of the vascular, intestinal, and bronchial tissue, chromaffin tissue, mast cells, and the brain. The goal of this review is to highlight the structure–affinity relationships (SAR) of A_{2B}AR antagonists that started with early lead compounds with nonoptimized pharmaceutical properties, which served as the genesis for second-generation compounds with high selectivity and affinity that have become development candidates. We will only briefly mention the major indications to demonstrate the potential utility of A_{2B}AR antagonists, since the potential indications of A_{2B}AR have been described in detail elsewhere (Feoktistov et al. 1998; Holgate ST 2005; Kurukulasuriya et al. 2003; Harada et al. 2001a; Hayallah et al. 2002). Although the A_{2B}AR antagonists described in this review are currently under investigation in order to fully define the role of the A_{2B}AR in disease states, early *in vitro* and *in vivo* experiments suggest that A_{2B}AR antagonists may be beneficial for the following diseases: asthma—A_{2B}AR mediates inflammatory cytokine release (Holgate ST 2005; Zhong et al. 2004, 2005, 2006); diabetes—A_{2B}AR mediates gluconeogenesis (Harada et al. 2001a, b; Kurukulasuriya et al. 2003); diabetic retinopathy (Feoktistov et al. 2004) and cancer (Zeng et al. 2003)—A_{2B}AR mediates angiogenesis (Belardinelli and Grant 2001; Feoktistov et al. 2004).

A number of studies have suggested that activation of the A_{2B}AR may play an important role in asthma. Activation of A_{2B}ARs on human bronchial smooth muscle cells (BSMCs) has been shown to induce the release of the inflammatory cytokines interleukin (IL)-6 and monocyte chemoattractant protein-1 (MCP-1) (Zhong et al. 2004), on human lung fibroblasts the release of IL-6 and differentiation of fibroblasts into myofibroblasts (Zhong et al. 2005), and on human bronchial epithelial cells (HBECs) the release of IL-19, which in turn activates human monocytes to induce the release of TNF- α , which in turn upregulates A_{2B}AR expression on HBECs (Zhong et al. 2006). Adenosine levels are elevated in the bronchoalveolar lavage fluid (BALF) of asthmatics relative to healthy volunteers (Driver et al. 1993). Moreover, when AMP is administered to asthmatics and healthy normal individuals, it provides a source of adenosine that leads to bronchoconstriction in asthmatics but not normals (Cushley et al. 1984). Furthermore, an adenosine uptake blocker, dipyrindamole, can precipitate asthma (Fozard and McCarth 2002). Therefore, the above evidence supports the notion that adenosine plays a role in asthma, and that its effects may be, at least in part, mediated through the A_{2B}AR. For more information on the role of A_{2B}ARs in asthma, the reader is referred to Chap. 11 of this volume, “Adenosine Receptors and Asthma” (Wilson et al.).

Scientists at Eisai have provided evidence that the A_{2B}AR antagonists and/or mixed A_{2B}/A₁AR antagonists may be useful in the treatment of diabetes. First, they demonstrated that the adenosine agonist analogs NECA [5'-*N*-ethylcarboxamidoadenosine] (nonselective), CPA [*N*⁶-cyclopentyladenosine] (A₁-selective) and

CGS-21680 [2-[*p*-(2-carboxyethyl)phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine] (A_{2A} -selective) stimulate glucose production from rat hepatocytes, with NECA having the most pronounced effect (Harada et al. 2001a). Then, the Eisai researchers found that their high-affinity A_{2B} AR antagonists that possess low selectivity over A_1 AR and A_{2A} AR block NECA-induced glucose production in rat hepatocytes. Eisai found in a separate study that the inhibition of glucose production was best correlated with the A_{2B} AR affinity of the compounds used in a diabetes model (Harada et al. 2001b). Specifically, a nonselective high-affinity A_{2B} AR antagonist (**52**) was found to lower plasma glucose following oral dosing (10 and 30 mg kg⁻¹ bodyweight) in a mouse model of noninsulin-dependent diabetes mellitus (NIDDM), KK-A^y mice (Harada et al. 2001b). It is clear from the above studies that adenosine likely plays a role in glucose production; however, the studies should be repeated with the highly selective, high-affinity A_{2B} AR antagonists described in this review.

Angiogenesis plays a major role in diabetic retinopathy and certain cancers. In proliferative diabetic retinopathy, it has been shown that activation of the A_{2B} AR on human retinal endothelial cells (HRECs) leads to new vessel formation that has uncontrolled growth, resulting in an increase in the permeability of the vasculature (Feoktistov et al. 2004). Support for a role of the A_{2B} AR in angiogenesis in HRECs was demonstrated when NECA caused a concentration-dependent increase in vascular endothelial growth factor (VEGF) mRNA in HRECs which was blocked by antisense oligonucleotides for the A_{2B} AR. Moreover, scientists at CV Therapeutics have suggested that labeled antibodies directed against the A_{2B} AR are potentially useful tools in detecting and possibly preventing the angiogenesis associated with gliomas, colon cancer, and solid tumors (Belardinelli and Grant 2001).

Finally, since the nonspecific AR antagonist caffeine is known to have intrinsic antinociceptive properties when used in combination with nonsteroidal anti-inflammatory drugs (NSAIDs) or opiates, Müller and coworkers investigated and discovered that A_{2B} AR antagonists possess antinociceptive effects in a hot plate test (Abo-Salem et al. 2004). In the same study, Müller et al. found that an A_{2B} AR antagonist was found to synergize with morphine for an enhanced antinociceptive effect in the same manner as caffeine.

2 A_{2B} Adenosine Receptor Antagonists

2.1 *Xanthine-Based Antagonists*

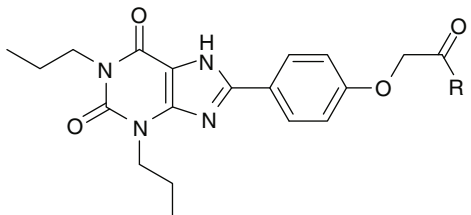
The naturally occurring alkylxanthines theophylline (**1**) and caffeine (**2**) are considered classical antagonists for the ARs. They exhibit weak affinity towards the A_{2B} AR, with no selectivity against the other ARs (Jacobson et al. 1999). Substitution of the dipropyl groups at the 1,3-methyl position of theophylline, as in 1,3-dipropyl xanthine, resulted in a 15-fold enhancement of A_{2B} AR affinity. The

introduction of hydrophobic substitution at the 8 position of the xanthine core increases affinity towards the ARs. For example, 8-cyclopentyl-1,3-dipropyl xanthine (**3**, DPCPX), a known A₁AR antagonist, displays good affinity for both A₁ ($K_i = 0.9$ nM) and A_{2B} ($K_i = 56$ nM)ARs. Introduction of aryl substitution at the 8 position of theophylline, as in 1,3-dimethyl-8-phenyl xanthine (**4**), led to good affinity for the A_{2B}AR ($K_i = 415$ nM), which is a 22-fold enhancement in affinity compared to theophylline. Further substitution of uncharged electron-donating groups like a methoxy group (**5**) or a hydroxyl group (**6**) at the *para* position of the 8-phenyl group enhances the A_{2B}AR affinity. The replacement of the 1,3-methyl groups of **4** with *n*-propyl groups, as in **7**, increased the A_{2B}AR affinity (Jacobson et al. 1999).

In an effort to develop potent and selective adenosine receptor antagonists, Jacobson et al. selected the 1,3-dipropyl-8-(*p*-hydroxyphenyl) xanthine as a lead compound to explore the effect of functionalized congeners (Kim et al. 1999). Initially, the effects of carboxylic acids, amine derivatives and amino acid conjugates at the *para* position of the phenyl group were explored, and it was observed that there was no clear preference for these groups with respect to the A_{2B}AR affinity. The neutral biotin conjugates of various chain lengths were considerably less potent than the parent amine, whereas the L-thienyl alanine derivative displayed good affinity ($K_i = 6.9$ nM) at human A_{2B}AR. The high-affinity compounds observed in the series of 8-phenylxanthine functionalized congeners were XCC [8-{4-[(carboxymethyl)oxy]phenyl}-1,3-dipropylxanthine, **8**], its hydrazide derivative (**9**), and another substituted amide derivative (**10**) (Jacobson et al. 1999) (Table 1).

The hydrazide was reacted with various mono- and dicarboxylic acids, and this structural change further enhanced selectivity, as exemplified by the dimethyl-malamide derivative (**11**, MRS1595). Further exploration of the amide derivatives

Table 1 A₁, A_{2A}, A_{2B} and A₃AR binding affinities of xanthine derivatives at human A_{2B}ARs expressed in HEK-293 cells



Compound	R	hK _i nM				A _{2B} Selectivity		
		A _{2B}	A ₁	A _{2A}	A ₃	A ₁ /A _{2B}	A _{2A} /A _{2B}	A ₃ /A _{2B}
8	OH	13	58	2,200	–	4	169	–
9	NHNH ₂	14	323	21	217	23	1	16
10	–NC(O)CH ₂ CH ₂ C(O)–	10	153	127	227	15	13	23
11 (MRS1595)	NHNdimalloyl	27	3,030	1,970	670	110	74	25
12	NH–Ph(4-CF ₃)	2.14	61	238	213	29	110	100
13 (MRS1706)	NH–Ph(4-COCH ₃)	1.4	157	112	230	110	81	170
14	NH–Ph(4-I)	2	293	5,140	1,270	140	2,400	600
15 (MRS1754)	NH–Ph(4-CN)	1.97	403	503	570	205	255	290

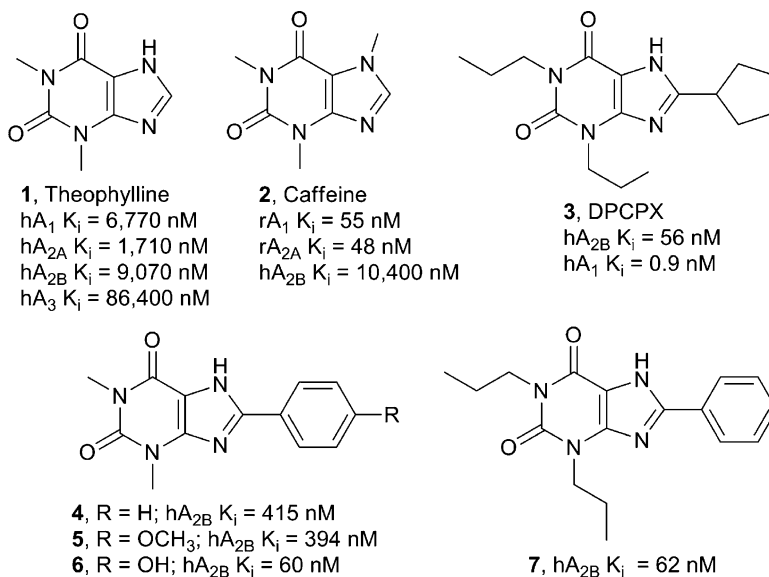


Fig. 1 1,3,7,8-Substituted xanthines

by condensing the carboxylic acid group of **8** with substituted phenylamines led to the discovery of several high-affinity and selective compounds. The anilides substituted at the *para* position with an electron-withdrawing group (**12**) showed good affinity and selectivity. Also, the *para*-acetophenone analog **13** displayed good affinity and selectivity. The *para*-halogen-substituted derivatives exhibited high affinity for the A_{2B} AR, with the *para*-iodo derivative (**14**) demonstrating the highest selectivity compared to the other halo derivatives (Kim et al. 2000). The *para*-cyano anilide derivative (**15**, MRS 1754) displayed high affinity for the A_{2B} AR ($K_i = 1.97 \text{ nM}$) and 205-, 255-, and 290-fold selectivity versus human $A_1/A_{2A}/A_3$ ARs, respectively (Kim et al. 2002) (Table 1).

The 1-alkyl-8-phenyl(cyclopentyl) xanthine derivatives were found to exhibit high affinity for A_{2B} ARs (Hayallah et al. 2002). In this study, the 1,8-disubstituted xanthine derivatives were shown to be equipotent to or more potent than 1,3,8-trisubstituted xanthines at A_{2B} ARs, but generally less potent at A_1 and A_{2A} , and much less potent at A_3 AR subtypes. 1-Propyl-8-*p*-sulfophenylxanthine (**16**, PSB1115) was the most selective compound of this family, exhibiting a K_i value of 53 nM at human A_{2B} AR. This compound is highly water soluble due to its sulfonate functional group. The 4-nitrophenylester of PSB1115 is also reported to be a potential prodrug despite its significant binding affinity for the A_1 AR subtype ($rA_{2B} = 5.4 \text{ nM}$, $rA_1 = 3.6 \text{ nM}$) (Hayallah et al. 2002). 1-Butyl-8-*p*-carboxyphenylxanthine (**17**), another polar analog bearing a carboxylate functional group, exhibited a K_i value of 24 nM for A_{2B} ARs, 49-fold selectivity versus human and 20-fold selectivity versus rat A_1 AR subtype, and greater than 150-fold selectivity versus human A_{2A} and A_3 ARs (Yan and Müller 2004) (Fig. 2).

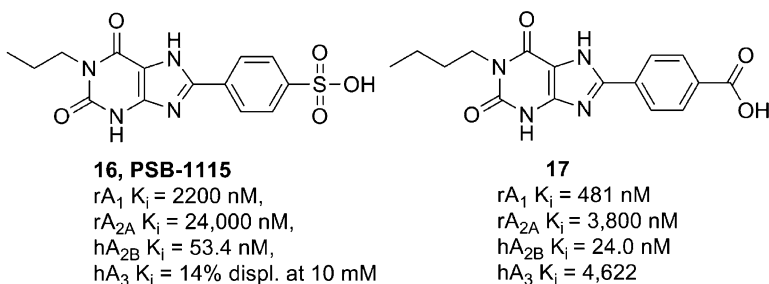
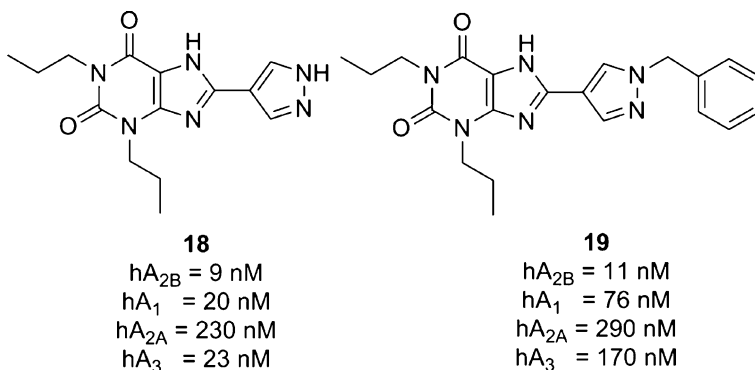


Fig. 2 1,8-Substituted xanthines

Fig. 3 8-(Pyrazol-4-yl)xanthines as A_{2B}AR antagonists

Chemists at CV Therapeutics have explored various heterocycles as bioisosteric replacements for the 8-phenyl group of 1,3-dipropyl-8-phenyl xanthines, and they observed that 1,3-dipropyl-8-(4-pyrazolyl)xanthine **18** displayed a high affinity for the A_{2B}AR (Kalla et al. 2004). Substitution of the N-1 pyrazole with a benzyl group, as in **19**, retained the A_{2B}AR affinity compared to the phenethyl and phenpropyl groups (Fig. 3). Further substitution of the phenyl ring with electron-withdrawing groups, for example CF₃ at the *meta*-position (**20**), increased the A_{2B}AR selectivity. Replacing the 1,3-dipropyl groups of the xanthine core with 1,3-dimethyl groups resulted in **21**, which has both high affinity and selectivity for the A_{2B}AR (Kalla et al. 2006). Exploration of differential substitution at the N-1 and N-3 positions of the xanthine core led to compound **22** (CVT-6883), which has good affinity for the A_{2B}AR and displayed good selectivity (Elzein et al. 2008). The introduction of monosubstitution at the N-1 position of the xanthine core, as in compound **23**, enhanced the selectivity compared to the disubstituted derivative **20** (Kalla et al. 2008). The N-3 monosubstituted derivative **24** lost the A_{2B}AR affinity, and this is in agreement with Hayallah et al.'s observation for the 8-phenyl series that 1,8-disubstituted xanthine derivatives display better A_{2B}AR affinities and selectivities than the 3,8-disubstituted xanthine derivatives (Hayallah et al. 2002). Replacing the phenyl group of **20** with different heterocycles, including 3-phenyl-

1,2,4-oxadiazoles, 5-phenyl-1,2,4-oxadiazoles and 3-phenyl-isoxazoles, resulted in compounds that display high affinity and good selectivity regardless of the substitution at the N-1 and N-3 positions on the xanthine core (**25** and **26**) (Elzein et al. 2006). The N-1 monosubstituted oxadiazole **27** and isoxazole **28** displayed high affinity and selectivity for the A_{2B} AR (Kalla et al. 2008). Compound **22** (6883) antagonized the NECA-induced cAMP accumulation in HEK- A_{2B} cells and NIH 3T3 cells (Sun et al. 2006), and compound **26** (6694) completely abolished the NECA-induced cAMP accumulation in BSMCs (Zhong et al. 2004), confirming that these compounds are antagonists for the hA_{2B} AR. In a mouse model of asthma, compound **22** demonstrated a dose-dependent blocking effect on NECA-induced increases in airway reactivity (Mustafa et al. 2007). Also, in this mouse model of allergic asthma, compound **22** significantly reduced the late allergic airway response and inflammatory cells in BALF, supporting the role of the A_{2B} ARs in asthma (Table 2).

Several heterocycles, such as pyrazole, isoxazole, pyridine and pyridazine, linked by different spacers (substituted acetamido, oxyacetamido and urea moieties) at the 8 position of the xanthine nucleus were investigated (Baraldi et al. 2004a). The synthesized compounds showed A_{2B} AR affinities in the nanomolar range and good levels of selectivity, as evaluated in radioligand binding assays at human A_1 , A_{2A} , A_{2B} , and A_3 ARs. This study allowed the identification of the derivatives 2-(3,4-dimethoxy-phenyl)-*N*-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yl]-acetamide (**29**, MRE2028F20) [K_i (hA_{2B}) = 38 nM, K_i (hA_1 , hA_{2A} , hA_3) > 1,000 nM], *N*-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide (**30**, MRE2029F20) [K_i (hA_{2B}) = 5.5 nM, K_i (hA_1) = 200 nM, (hA_{2A} , hA_3) > 1,000], and *N*-(3,4-dimethoxy-phenyl)-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]acetamide (**31**, MRE2030F20) [K_i (hA_{2B}) = 12 nM, K_i (hA_1 , hA_{2A} , hA_3) > 1,000 nM] (Fig. 4), which showed high affinity at the A_{2B} AR subtype and very good selectivity versus the other AR subtypes. The derivatives with higher affinity at human A_{2B} AR proved to be antagonists in the cyclic AMP assay, capable of inhibiting the stimulatory effect of NECA (100 nM) with IC_{50} values in the nanomolar range and a trend similar to that observed in the binding assay.

Compounds **32**, **33** (Fig. 4) bearing the isoxazole nucleus at the 8 position showed lower affinities at the A_{2B} AR than the corresponding 8-pyrazole derivatives. However, replacing the pyrazole ring with an isoxazole enhanced selectivity versus the A_1 AR. Consequently, the radiolabeled analog of compound *N*-benzo[1,3]dioxol-5-yl-2-[5-(1,3-diallyl)-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide (**30**) was prepared in the tritium-labeled form [3H] MRE2029F20, which displayed a K_d value of 1.65 ± 0.10 nM). This compound was found to be a selective, high-affinity radioligand that is useful for characterizing recombinant human A_{2B} ARs (Baraldi et al. 2004b). Very recently, the same authors also described a series of 1,3-dipropyl-8-(1-phenylacetamide-1*H*-pyrazol-3-yl)-xanthines as selective A_{2B} AR antagonists (Aghazadeh Tabrizi et al. 2008). The 4-chlorophenyl derivative **34** (Fig. 4) was found to be the most

Table 2 A₁, A_{2A}, A_{2B} and A₃AR binding affinities of 8-(4-pyrazolyl)xanthine analogs

Compound	20-24		25-28		hK _i nM			A _{2B} Selectivity			
	R ¹	R ³	R	R ³	A _{2B}	A ₁	A _{2A}	A ₃	A ₁ /A _{2B}	A _{2A} /A _{2B}	A ₃ /A _{2B}
20	Propyl	Propyl	CF ₃	Propyl	14	160	400	140	12	27	10
21	Methyl	Methyl	CF ₃	Methyl	1	990	690	1,000	990	690	1,000
22 (CVT-6883)	Propyl	Ethyl	CF ₃	Ethyl	22	1,940	3,280	1,070	88	149	48
23	Propyl	H	CF ₃	H	8	>6,000	>5,000	700	>750	>620	80
24	H	Propyl	H	Propyl	>6,000	nd	nd	nd	nd	nd	nd
25 (X = N)	Propyl	Propyl	CF ₃	Propyl	21	>6,000	>5,000	1,300	>290	>240	60
26 (X = N)	Propyl	H	Cl	H	7	>6,000	>5,000	>9,000	>850	>700	>1,280
(CVT-6694)											
27 (X = N)	Cyclopropyl methyl	H	CF ₃	H	13	>6,000	>5,000	>9,000	>460	>380	>700
28 (X = C)	Cyclopropyl methyl	H	CF ₃	H	15	>6,000	>5,000	>9,000	>400	>333	>600

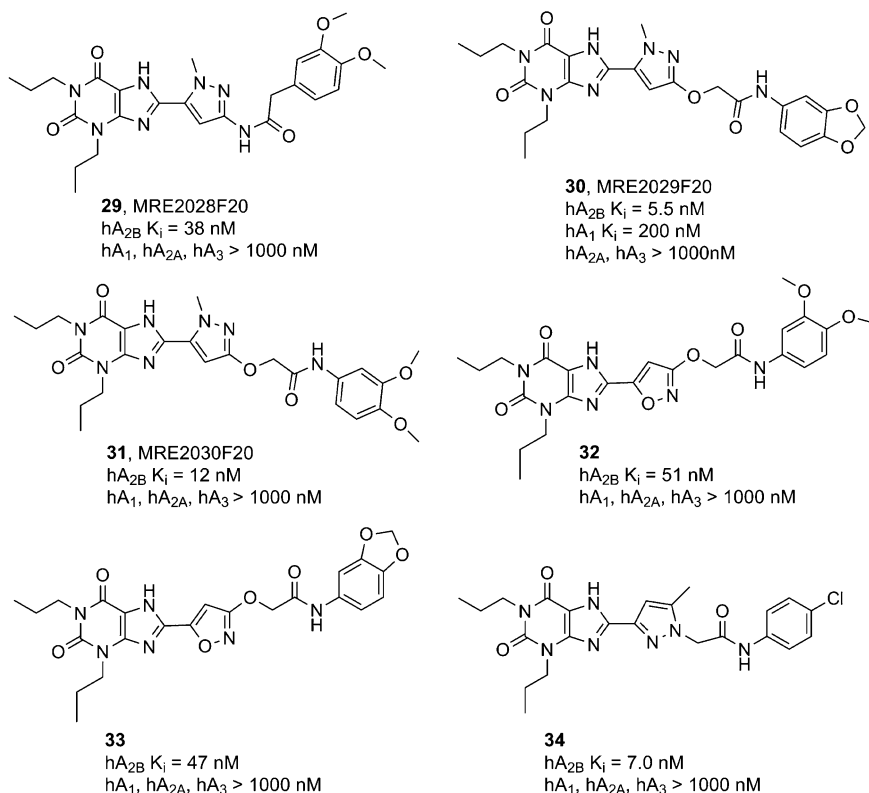


Fig. 4 1,3,8-Substituted xanthines

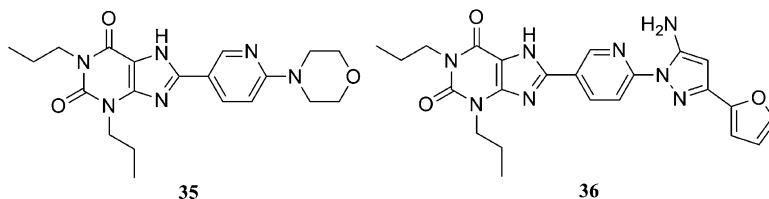


Fig. 5 Substituted 8-pyridyl xanthines as $A_{2B}AR$ antagonists

potent ($K_i A_{2B} = 7.0 \text{ nM}$) and selective compound within the series ($A_1, A_{2A}, A_3 / A_{2B} > 140$).

Scientists at Adenosine Therapeutics describe a series of 8-(3-pyridyl)xanthines that have high affinity for the $A_{2B}AR$. The morpholino derivative **35** and the pyrazolyl derivative **36** possess better than 100 nM affinity for the $A_{2B}AR$ based upon the published patent application (Wang et al. 2006). The compounds demonstrate that the six-membered pyridyl ring can serve as a favorable linker, providing high affinity for the $A_{2B}AR$ in a similar manner to the 4-pyrazolyl and 5-pyrazolyl linkers described above (Fig. 5).

2.2 Deazaxanthine-Based Antagonists

In the xanthine family, the 8-substituted-9-deaza-xanthines are reported to be antagonists with nanomolar affinities for the A_{2B}AR (Carotti et al. 2006; Stefanachi et al. 2008). The 1-, 3-, 8-, and 9-substituted-deazaxanthines of general structure **37** (Fig. 6) were prepared and evaluated for their binding affinities at the recombinant human ARs, in particular at the hA_{2B} and hA_{2A}AR subtypes. 1,3-Dimethyl-8-phenoxy-(*N-p*-halo-phenyl)-acetamido-9-deazaxanthine derivatives appeared to be the most interesting leads, with some of them, such as the compound (*N*-(4-bromo-phenyl)-2-[4-(1,3-dimethyl-2,4-dioxo-2,3,4,5-tetrahydro-1*H*-pyrrolo[3,2-*d*]pyrimidin-6-yl)-phenoxy]-acetamide (**38**), showing high hA_{2B}AR affinities and selectivity over hA_{2A} and hA₃ARs, but low selectivity over hA₁AR. Structure–affinity relationships suggested that the binding potency at the hA_{2B}AR was mainly modulated by the steric (lipophilic) properties of the substituents at positions 1 and 3 and by the electronic and lipophilic characteristics of the substituents at position 8. Electron-withdrawing groups in the *para*-position of the anilide phenyl ring increased the activity. Regarding the hA_{2B}/hA_{2A}AR selectivity,

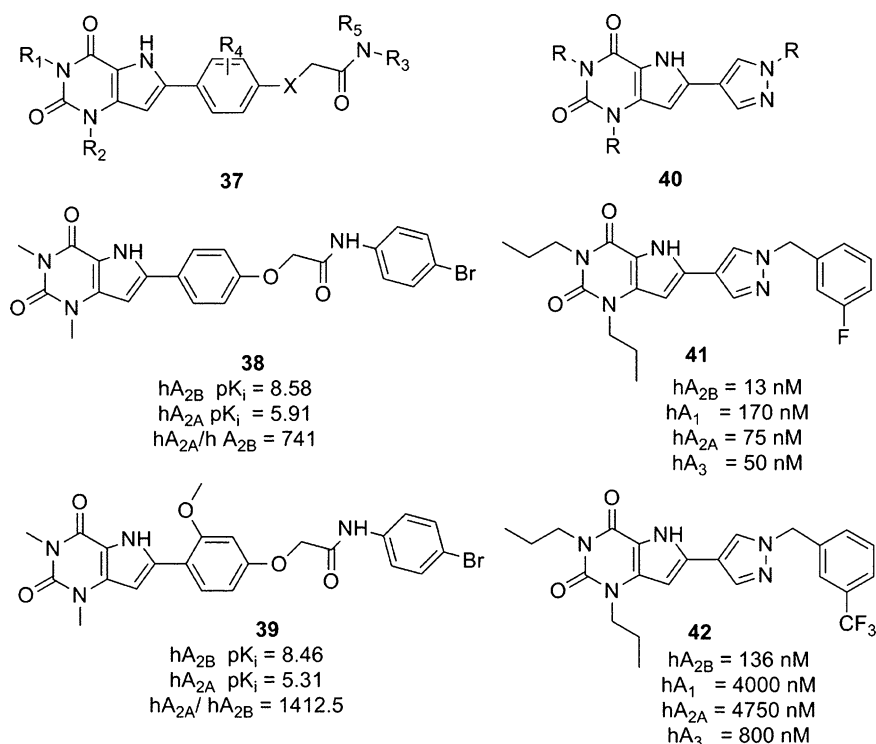


Fig. 6 General structures and binding affinities of deazaxanthine derivatives

the most interesting result came from the introduction of a methoxy substituent in the *ortho* position of the 8-phenyl ring, which led to an enhancement in selectivity for compound **39**, making it 1,412-fold more selective for the hA_{2B}AR over the hA_{2A}AR.

The 9-deaza analog **40** of the 8-(4-pyrazolyl)xanthine class was synthesized to compare its A_{2B}AR affinity and selectivity (Kalla et al. 2005). Compound **41**, a 9-deaza derivative with a *meta*-fluoro substitution on the pyrazole ring, has the same affinity as the direct xanthine analog. Compound **42**, a *meta*-CF₃ derivative and a direct analog of compound **20**, displayed a lower affinity for the A_{2B}AR but good selectivity.

A new series of 4-(1,3-dialkyl-2,4-dioxo-2,3,4,5-tetrahydro-1*H*-pyrrolo[3, 2-*d*]pyrimidin-6-yl)benzenesulfonamides (**43–46**, Table 3) are also reported to be potent A_{2B}AR antagonists (Esteve et al. 2006). In this series, the 6-(4-[(4-bromobenzyl)piperazin-1-yl]sulfonyl)phenyl)-1,3-dimethyl-1*H*-pyrrolo[3,2-*d*]pyrimidine-2,4(3*H*, 5*H*)-dione (**45**) showed a high affinity for the A_{2B}AR (IC₅₀ = 1 nM) and selectivity. The presence of metabolically stable benzenesulfonamide in this novel class of compounds improved their physicochemical properties, resulting in increased oral bioavailability.

Table 3 Deazaxanthine-benzenesulfonamides as A_{2B}AR antagonists

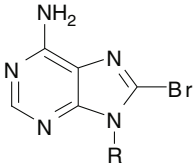
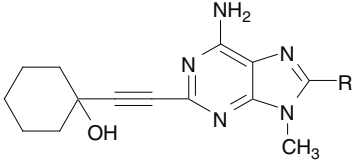
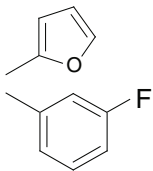
Compound	R	R ₁	R ₂	IC ₅₀ (nM)		
				hA _{2B}	hA ₁	hA ₃
43	CH ₃	CH ₃		14	150	2,085
44	CH ₃	CH ₃		16	415	3,169
45	CH ₃	CH ₃		1	183	12,260
46	<i>n</i> C ₃ H ₇	CH ₃		6	370	950

2.3 Adenine-Based Antagonists

Adenine has proven to be a useful core for the development of AR antagonists. Modifications of adenine led to various 2- and 8-substituted derivatives with moderate affinities at all four AR subtypes (Campioni et al. 1998). Generally, it appears that 2-substitution led to nonselective antagonists or to antagonists with high affinities at both A₁ and A_{2A} ARs. In the series of 8-bromo-9-alkyl-adenines **47–50** (Table 4), the presence of a propyl group at the 9 position and a bromine at the 8 position, such as in compound **49** (hA_{2B} K_i = 200 nM), increased the affinity and selectivity for the A_{2B} receptor in comparison to the parent 9-ethyladenine, with a K_i value of 0.84 μM. The experimental data show that different substituents in the 8 position result in compounds with quite different pharmacological features. The 8-phenethylamino, 8-phenethoxy, and 8-(ar)alkynyl compounds generally showed lower potency at all receptors than compound **48**. Replacement of the N9 ethyl with a methyl group retained A_{2B} AR affinity and decreased affinity at all other AR subtypes, while the N9 bulky groups led to derivatives with higher selectivity versus A_{2B} AR (Lambertucci et al. 2000; Volpini et al. 2003).

The 2-alkynyl-8-aryl-9-methyladenine derivatives were also synthesized as candidate hypoglycemic agents (Harada et al. 2001a). These analogs were eval-

Table 4 Adenine derivatives as A_{2B}AR antagonists

		R	hA _{2B}	hA ₁	hA _{2A}	hA ₃
			K _i (nM)	K _i (nM)		
47		CH ₃	720	570	120	>100,000
48		C ₂ H ₅	840	280	52	28,000
49		C ₃ H ₇	200	1,100	300	>100,000
50		cC ₅ H ₉	860	1,000	1,900	4,200
51			14	25	11	–
52			23	14	16	540

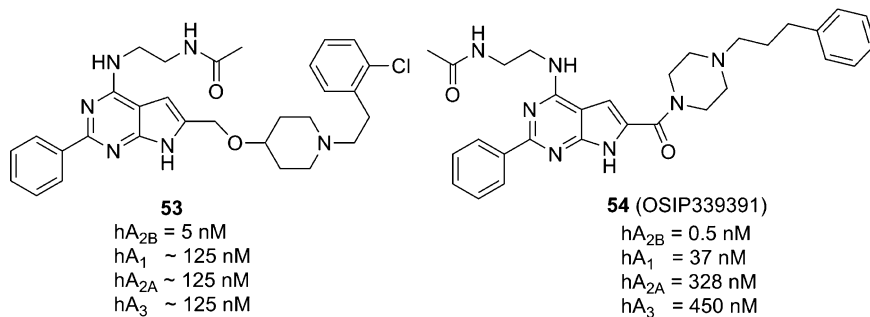


Fig. 7 Deazapurines as A_{2B} AR antagonists

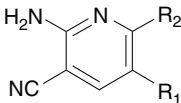
uated for inhibitory activity on *N*-ethylcarboxamidoadenosine (NECA)-induced glucose production in primary cultured rat hepatocytes. The introduction of various heteroaromatic rings and (substituted) phenyl rings at the 8 position of 9-methyladenine, and of other alkynyl groups at the 2 position, was investigated. The aromatic groups in the 8 position significantly increased the potency, and the preferred substituents at the 8 position of adenine were the 2-furyl and 3-fluorophenyl groups (**51** and **52** respectively, Table 4). Another modification at the alkynyl side chain, changes in ring size, cleavage of the ring, and removal of the hydroxyl group were all well tolerated. Compound **52** is a nonspecific adenosine antagonist, but it was hypothesized that its inhibition of hepatic glucose production via the A_{2B} AR could be at least one of the mechanisms associated with its *in vivo* activity.

Scientists at OSI Pharmaceuticals have discovered several very high affinity A_{2B} AR antagonists, **53** and **54** (OSIP339391), that are based on a deaza-adenine scaffold (Castelhano et al. 2003) (Fig. 7). Compound **54** has extremely high affinity for the A_{2B} AR, making it suitable for use as a radiolabeled ligand for competitive binding assays, and it possesses moderate selectivity over hA_1 , hA_{2A} and hA_3 ARs (>70-fold). The tritium-labeled **54** was prepared from the acetylene precursor, affording a compound with radiolabeled OSIP339391 that had a selectivity of greater than 70-fold for A_{2B} ARs over other human AR subtypes (Stewart et al. 2004). The radiolabel was introduced by hydrogenation of the acetylenic precursor, affording a compound with high specific activity. These compounds should possess good water solubilities, since they incorporate a basic piperidine or piperazine moiety that should be protonated at physiological pH. It would be interesting to know whether these compounds are metabolically stable and whether they have favorable PK properties, but this information has not been reported.

2.4 2-Aminopyridine-Based Antagonists

2-Aminopyridine derivatives that are selective A_{2B} AR antagonists have been reported (Harada et al. 2004). The core structure of this class of compounds is

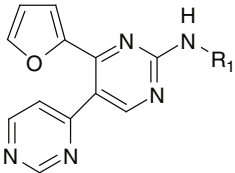
Table 5 2-Aminopyridine derivatives as A_{2B}AR antagonists

			hA ₁	hA _{2A}	hA _{2B} IC ₅₀ (nM)
	R ₁	R ₂	K _i (nM)		
55	4-Pyridine	2-Furyl	990	23	2.7
56	4-Pyridine	3-Fluoro-phenyl	66	22	3.7
57	1-Ethyl-6-oxo-3-pyridine	2-Furyl	400	7	6.5

a 2-aminopyridine that presents a cyano group at the 3 position together with substituents at the 4 and 5 positions (**55–57**). Few data were presented, but analog **55** (6-amino-2-(2-furyl)-3,4'-bipyridine-5-carbonitrile; Table 5) showed at least ninefold selectivity and good affinity at A_{2B}AR. Apparently, the introduction of a furyl group at the 2 position and a pyridine group at the 4 position (**55**) introduced some selectivity with respect to the A₁AR and A_{2A}AR, respectively.

2.5 Bipyrimidine-Based Antagonists

2-Amino-substituted pyrimidines have been identified as suitable templates for the construction of adenosine A_{2B}AR antagonists (Vidal et al. 2007c). Several compounds of this novel series of *N*-heteroaryl-4'-(furyl)-4,5'-bipyrimidin-2'-amines (Table 6) were very selective over other ARs and had a low nanomolar affinity at the A_{2B}AR. The introduction of unsubstituted nitrogen-containing heterocycles in R₁, such as pyridine, pyrimidine or pyrazine, yielded compounds **58–67**. Among these, the 3-pyridyl derivative **58** (LAS-38096) was found to show high potency and selectivity. Substitution by a methoxy group (**61** and **62**) led to a drop in potency while retaining good selectivity. Compounds **63** and **64** were investigated to evaluate the effect of substitution of the nitrogen atoms of the 3-pyridyl or the 3-pyrimidinyl rings with a hydrogen bond acceptor. The cyano derivative **63** showed lower affinity but an increase in selectivity versus A_{2A}AR. The *N*-oxide **64** had a twofold decrease in affinity compared to that of the corresponding reduced analog **58**. The introduction of a pyridone moiety yielded compound **65**, which was found to be one of the most potent and selective compounds within the series. Alkylation at the pyridine nitrogen had a slightly detrimental effect on A_{2B}AR potency for the more lipophilic analog **67**. LAS-38096, which represents the lead for this series, was capable of inhibiting A_{2B}AR-mediated NECA-dependent increases in intracellular cAMP, with IC₅₀ values of 321 nM and 349 nM in cells expressing human and mouse ARs, respectively; it also displayed a favorable PK profile in preclinical species. The efficacy of compound **58** was evaluated in vivo in an allergic mouse model, and the mice showed significantly less bronchial

Table 6 2-Amino substituted pyrimidines as A_{2B}AR antagonists


	R ₁	K _i (nM)			
		hA _{2B}	hA _{2A}	hA ₁	hA ₃
58 (LAS-38096)	3-Pyridyl	17	>2, 500	>1, 000	>1, 000
59	3-Pyrimidinyl	24	>2, 500	>10, 000	>1, 000
60	2-Pyrazinyl	116	>2, 500	>10, 000	>1, 000
61	6-Methoxypyridin-3-yl	115	>2, 500	>10, 000	>1, 000
62	2-Methoxypyrimidin-5-yl	39	>2, 500	>10, 000	>1, 000
63	5-Cyano-pyridin-3-yl	69	>2, 500	>10, 000	>1, 000
64	1-Oxido-pyridin-3-yl	34	>2, 500	>10, 000	>1, 000
65	6-Oxo-1,6-dihydropyridin-3-yl	16	>2, 500	>10, 000	>1, 000
66	1-Methyl-6-oxo-1,6-dihydropyridin-3-yl	28	>2, 500	>10, 000	>1, 000
67	1-Cyanopropylmethyl-6-oxo-1,6-dihydropyridin-3-yl	119	>2, 500	>10, 000	>1, 000

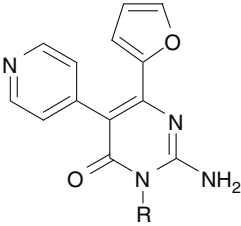
hyperresponsiveness, mucus production, and a slight decrease in eosinophil infiltration and Th2 cytokine levels (Aparici et al. 2006).

2.6 Pyrimidone-Based Antagonists

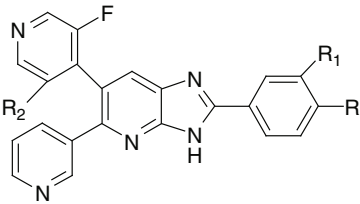
A series of compounds with a pyrimidine-4-(3*H*)-one core structure has been reported as antagonists for the A_{2B}AR (Harada et al. 2003). However, few data were reported, and data on the A₃AR are lacking. The most representative compound, 2-amino-6-(2-furyl)-3-methyl-5-(4-pyridyl)pyrimidin-4-(3*H*)-one (**69**), derived by introducing a methyl group at the 3 position of the unsubstituted analog **68**, gave K_i values of 966 nM and 493 nM, respectively, against A₁ and A_{2A}ARs in binding assays. It also inhibited NECA-stimulated cAMP production in A_{2B}-transfected CHO-K1 cells, with an IC₅₀ value of 71 nM (Table 7).

2.7 Imidazopyridine-Based Antagonists

The imidazopyridine nucleus was recently identified in a patent as a core structure in a new series of A_{2B}AR antagonists (Vidal et al. 2005). This patent presented few data, and data on the A₁, A_{2A} and A₃ARs are lacking. Several compounds (**70–75**) of this new class had low nanomolar (<10 nM) affinities for the A_{2B}AR (Table 8).

Table 7 Pyrimidone derivatives as A_{2B}AR antagonists


	R	hA _{2B} IC ₅₀ (nM)	hA ₁ K _i (nM)	hA _{2A} K _i (nM)
68	H	256	1108	345
69	CH ₃	71	966	493

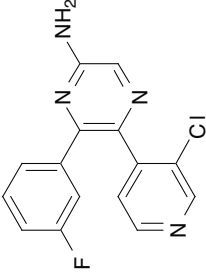
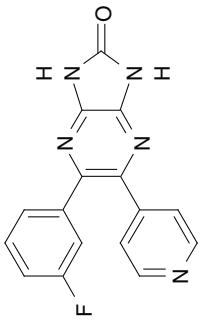
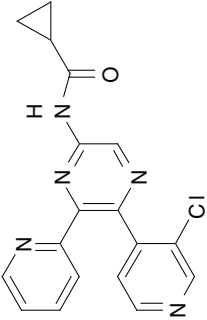
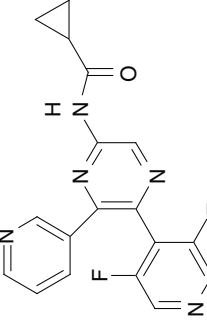
Table 8 Imidazopyridines as A_{2B}AR antagonists


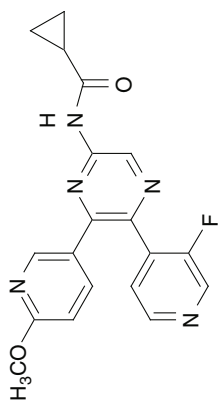
	R	R ₁	R ₂	hA _{2B} K _i (nM)
70	OCH ₃	H	H	0.8
71	N(CH ₃) ₂	H	H	1.7
72	COOH	H	H	8
73	CH ₃	F	H	2.2
74	H	F	H	2.8
75	F	H	F	2.8

2.8 Pyrazine-Based Antagonists

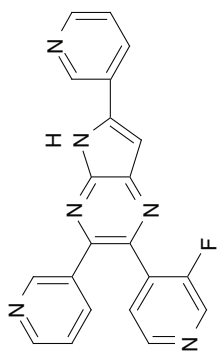
Scientists at Almirall Pharmaceuticals have found that pyrazine derivatives are novel potent antagonists of A_{2B}ARs (Vidal et al. 2007a, b). Table 9 shows the binding activities for human A_{2B}AR of some of these compounds (**76–83**). In this patent, affinity data for other AR subtypes are lacking. Generally, these 2-aminopyrazines present the pyridine nucleus at the 5 and 6 positions. Apparently, the introduction of a furyl group at the 6 position of the pyrazine ring (**79**) was tolerated by A_{2B}ARs. The imidazopyrazine **80** also showed high affinity at A_{2B}AR. The lead compound of this new series is 2-(3-fluoropyridin-4-yl)-3,6-di(3-pyridyl)-5H-pyrrolo[2,3-*b*]pyrazine, **83** (K_i = 0.9 nM).

Table 9 Pyrazine derivatives as A_{2B}AR antagonists

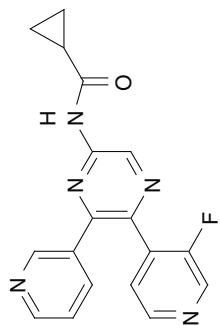
Compound	Chemical structure	hA _{2B} K _i (nM)	Compound	Chemical structure	hA _{2B} K _i (nM)
76		4	80		4
77		16	81		3



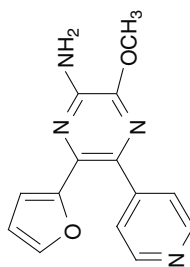
26



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78



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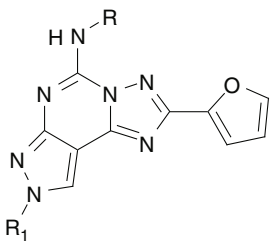
83

0.9

2.9 Pyrazolo-Triazolo-Pyrimidine-Based Antagonists

Pyrazolo[4,3-*e*]1,2,4-triazolo-[1,5-*c*]pyrimidine derivatives were initially investigated for the development of selective A₃AR antagonists (Baraldi et al. 2000, 2002). From preliminary studies, it has been demonstrated that the *N*⁵-unsubstituted derivatives show different binding profiles according to the substitution position (N7 or N8) on the pyrazole nucleus. The N7 derivatives showed high affinity for the human A_{2A}ARs but did not bind to the human A_{2B}AR subtype (range > 1 μM). The *N*⁸-substituted derivatives (derivatives with phenethyl or *iso*-pentyl groups, such as in compounds **84** and **85**, Table 10) displayed affinity in the nanomolar range to human A_{2B}AR, but no selectivity versus the A₁ and A_{2A}AR subtypes was observed. In parallel studies on human A₃AR antagonists, it was observed that the introduction of a phenylacetyl group at the N5 position (**86**) produces an increase in affinity at the A₃AR. In fact, a combination of an aryl acetyl moiety at the N5 position and a phenylethyl or phenylpropyl group at the N8 position led to compounds **87** and **88**, which were found to be nonselective AR antagonists. The introduction of an alkyl-carbamoyl moiety at the N5 position yielded **89**, which is also a nonselective AR antagonist (Baraldi et al. 2001). The introduction of a α -naphthoyl chain at the N5 position instead of a phenyl group (**90**) was tolerated by the A_{2B}AR but not the other AR subtypes, resulting in the most selective A_{2B}AR antagonist of this series (Pastorin et al. 2003).

Table 10 Pyrazolo[4,3-*e*]1,2,4-triazolo-[1,5-*c*]pyrimidines as A_{2B}AR antagonists



	R	R ₁	hA ₁ hA _{2B} hA _{2A} hA ₃			
			K _i (nM)			
84	H	(CH ₂) ₂ Ph	1	5	0.31	2,030
85	H	Iso-pentyl	2	9	0.8	700
86	COCH ₂ Ph	Methyl	702	165	423	0.81
87	COCH ₂ Ph	(CH ₂) ₂ Ph	120	35	60	45
88	COCH ₂ Ph	(CH ₂) ₃ Ph	75	40	60	121
89	CO(CH ₂) ₃ NH ₃	(CH ₂) ₂ Ph	1.6	27	54	65
90	α -Naphthoyl	(CH ₂) ₃ Ph	1, 100	20	800	300

3 Conclusion

The challenge to obtain second-generation, selective, high-affinity A_{2B}AR antagonists has been met, as exemplified by the xanthines 8-(4-(*N*-1-benzylpyrazolyl))xanthine **22** (A_{2B}AR $K_i = 1$ nM, CV Therapeutics) and 8-(5-pyrazolyl)xanthine **30** (A_{2B}AR $K_i = 5.5$ nM, Baraldi et al. and King Pharmaceuticals) and the pyrimidine 2-(amino-5-pyrimidinyl)pyrimidine **58** (A_{2B}AR $K_i = 17$ nM, Almira Prodesfarma). These compounds should prove useful as tools to define the role of the A_{2B}AR in various disease states, including asthma, diabetes, cancer, and management of inflammatory pain in clinical trials in the near future.

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Medicinal Chemistry of the A₃ Adenosine Receptor: Agonists, Antagonists, and Receptor Engineering

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Abstract A₃ adenosine receptor (A₃AR) ligands have been modified to optimize their interaction with the A₃AR. Most of these modifications have been made to the N⁶ and C2 positions of adenine as well as the ribose moiety, and using a combination of these substitutions leads to the most efficacious, selective, and potent ligands. A₃AR agonists such as IB-MECA and Cl-IB-MECA are now advancing into Phase II clinical trials for treatments targeting diseases such as cancer, arthritis, and psoriasis. Also, a wide number of compounds exerting high potency and selectivity in antagonizing the human (h)A₃AR have been discovered. These molecules are generally characterized by a notable structural diversity, taking into account that aromatic nitrogen-containing monocyclic (thiazoles and thiadiazoles), bicyclic

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(isoquinoline, quinoxalines, (aza)adenines), tricyclic systems (pyrazoloquinolines, triazoloquinoxalines, pyrazolotriazolopyrimidines, triazolopurines, tricyclic xanthines) and nucleoside derivatives have been identified as potent and selective A₃AR antagonists. Probably due to the “enigmatic” physiological role of A₃AR, whose activation may produce opposite effects (for example, concerning tissue protection in inflammatory and cancer cells) and may produce effects that are species dependent, only a few molecules have reached preclinical investigation. Indeed, the most advanced A₃AR antagonists remain in preclinical testing. Among the antagonists described above, compound OT-7999 is expected to enter clinical trials for the treatment of glaucoma, while several thiazole derivatives are in development as antiallergic, antiasthmatic and/or antiinflammatory drugs.

Keywords A₃ adenosine receptor · A₃ adenosine receptor agonist · A₃ adenosine receptor antagonist · Purines · Structure activity relationship · Nucleoside · G protein-coupled receptor · Neoeceptor

Abbreviations

ADME	Absorption, distribution, metabolism, and excretion
AR	Adenosine receptor
b	Bovine
cAMP	Cyclic adenosine monophosphate
CHO cells	Chinese hamster ovary cells
Cl-IB-MECA	2-Chloro- <i>N</i> ⁶ -(3-iodobenzyl)-5'- <i>N</i> -methylcarboxamido-adenosine
CoMFA	Comparative molecular field analysis
CVT-3146	1-{9-[(4 <i>S</i> , 2 <i>R</i> , 3 <i>R</i> , 5 <i>R</i>)-3,4-Dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-6-aminopurin-2-yl}pyrazol-4-yl)- <i>N</i> -methylcarboxamide
DBXRM	7-β-D-Ribofuronamide
DHP	1,4-Dihydropyridine
Et	Ethyl
GPCR	G-protein-coupled receptor
h	Human
HEK293 cells	Human embryonic kidney 293 cells
I-AB-MECA	<i>N</i> ⁶ -(4-Amino-3-iodobenzyl)-5'- <i>N</i> -methylcarboxamido-adenosine
IB-MECA	<i>N</i> ⁶ -(3-Iodobenzyl)-5'- <i>N</i> -methylcarboxamido-adenosine
KF-26777	2-(4-Bromophenyl)-7,8-dihydro-4-propyl-1 <i>H</i> -imidazo[2,1- <i>i</i>]purin-5(4 <i>H</i>)-one
LJ-529	2-Chloro- <i>N</i> ⁶ -(3-iodobenzyl)-4'-thioadenosine-5'-methyluronamide
LJ-1251	(2 <i>R</i> , 3 <i>R</i> , 4 <i>S</i>)-2-(2-Chloro-6-(3-iodobenzylamino)-9 <i>H</i> -purin-9-yl)tetrahydrothiophene-3,4-diol

LJ-1416	(2 <i>R</i> , 3 <i>R</i> , 4 <i>S</i>)-2-(2-Chloro-6-(3-chlorobenzylamino)-9 <i>H</i> -purin-9-yl)tetrahydrothiophene-3,4-diol
LUF6000	<i>N</i> -(3,4-Dichloro-phenyl)-2-cyclohexyl-1 <i>H</i> -imidazo[4,5- <i>c</i>]quinolin-4-amine
Me	Methyl
MRE-3005-F20	5- <i>N</i> -(4-Methoxyphenylcarbamoyl)amino-8-ethyl-2-(2-furyl)pyrazolo[4,3- <i>e</i>]-1,2,4-triazolo[1,5- <i>c</i>]pyrimidine
MRE-3008-F20	5- <i>N</i> -(4-Methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3- <i>e</i>]-1,2,4-triazolo[1,5- <i>c</i>]pyrimidine
MRS1191	1,4-Dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl 5-(phenylmethyl) ester
MRS1220	<i>N</i> -[9-Chloro-2-(2-furanyl)[1,2,4]triazolo[1,5- <i>c</i>]quinazolin-5-yl]benzeneacetamide
MRS1292	(2 <i>R</i> , 3 <i>R</i> , 4 <i>S</i> , 5 <i>S</i>)-2-[<i>N</i> ⁶ -3-Iodobenzyl]adenos-9'-yl]-7-aza-1-oxa-6-oxospiro[4.4]-nonan-4,5-diol
MRS1523	5-Propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate
MRS3558	(1' <i>S</i> , 2' <i>R</i> , 3' <i>S</i> , 4' <i>R</i> , 5' <i>S</i>)-4-{2-Chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo-[3.1.0]-hexane-2,3-diol
MRS3777	2-(Phenylloxy)- <i>N</i> ⁶ -cyclohexyladenine
MRS5127	(1' <i>R</i> , 2' <i>R</i> , 3' <i>S</i> , 4' <i>R</i> , 5' <i>S</i>)-4'-[2-chloro-6-(3-iodobenzylamino)-purine]-2', 3'- <i>O</i> -dihydroxybicyclo-[3.1.0]hexane
MRS5147	(1' <i>R</i> , 2' <i>R</i> , 3' <i>S</i> , 4' <i>R</i> , 5' <i>S</i>)-4'-[2-chloro-6-(3-bromobenzylamino)-purine]-2', 3'- <i>O</i> -dihydroxybicyclo-[3.1.0]hexane
MRS5151	(1' <i>S</i> , 2' <i>R</i> , 3' <i>S</i> , 4' <i>S</i> , 5' <i>S</i>)-4'-[6-(3-chlorobenzylamino)-2-(5-hydroxycarbonyl-1-pentynyl)-9-yl]-2', 3'-dihydroxybicyclo[3.1.0]hexane-1'-carboxylic acid <i>N</i> -methylamide
NECA	adenosine 5'- <i>N</i> -ethyluronamide
OT-7999	5- <i>n</i> -Butyl-8-(4-trifluoromethylphenyl)-3 <i>H</i> -[1,2,4]triazolo-[5,1- <i>i</i>]purine
Pr	Propyl
PSB-10	8-Ethyl-1,4,7,8-tetrahydro-4-methyl-2-(2,3,5-trichlorophenyl)-5 <i>H</i> -imidazo[2,1- <i>i</i>]purin-5-one
PSB-11	(<i>R</i>)-4-Methyl-8-ethyl-2-phenyl-4,5,7,8-tetrahydro-1 <i>H</i> -imidazo[2,1- <i>i</i>]purin-5-one
QSAR	Quantitative structure–activity relationships
r	Rat
SARs	Structure–activity relationships
TM	Transmembrane domain
VUF 5574	<i>N</i> -(2-Methoxyphenyl)- <i>N</i> '-(2-(3-pyridyl)quinazolin-4-yl)urea
VUF 8504	4-Methoxy- <i>N</i> -(3-(2-pyridinyl)-1-isoquinolinyl)benzamide

1 Introduction

The four subtypes of adenosine receptors (ARs), designated A₁, A_{2A}, A_{2B}, and A₃, are all seven-transmembrane spanning (7TM) receptors that couple to G proteins. The A₃AR inhibits adenylate cyclase through coupling to G_i. A₃AR activation may lead to an activation of the phospholipase C pathway through the β , γ subunit. The A₃AR is found at a high receptor density in the lungs, liver, and in immune cells such as neutrophils and macrophages, as well as at lower densities in the heart and brain (Fredholm et al. 2001) The A₃AR is expressed in neurons in the brain (Lopes et al. 2003; Yaar et al. 2002).

ARs in general, and the A₃AR in particular, are involved in many of the body's cytoprotective functions. Recently, agents that act at the A₃AR have been targeted for pharmaceutical development based on their anti-inflammatory, anticancer, and cardioprotective effects. For example, activation of the cardiac A₃AR preconditions cardiac myocytes against ischemic damage (Strickler et al. 1996; Tracey et al. 2003) and protects against apoptosis. Selective A₃AR agonists have been shown to protect cardiac muscle in various ischemic models and are protective against the cardiotoxic effects of the anticancer drug doxorubicin (Shneyvavis et al. 2001). A₃AR antagonists are of interest as potential antiglaucoma agents (Yang et al. 2005) and as anticancer agents (Gessi et al. 2008).

Agonist ligands for the ARs, including the A₃AR, are almost exclusively nucleoside derivatives. The search for antagonists of the A₃AR in the early 1990s initially encountered an unanticipated difficulty: the lack of an obvious lead structure. Previously, efforts to develop antagonist ligands for the A₁ and A_{2A} ARs focused on xanthine derivatives. However, at the A₃AR, the prototypical AR antagonists (i.e., the xanthines) are typically much weaker in binding than at the other AR subtypes. This observation stimulated the screening of structurally diverse heterocyclic molecules as potential antagonists (Moro et al. 2006). Chemically diverse leads were discovered in this process that were subsequently optimized to achieve high antagonist selectivity for the A₃AR.

While the A₃AR may be activated by orthosteric agonists that are competitive with adenosine, the action of nucleosides at this receptor may also be enhanced by allosteric modulators. Several heterocyclic classes of positive allosteric modulators of the A₃AR, including 1*H*-imidazo-[4,5-*c*]quinolines such as LUF6000 (*N*-(3,4-dichloro-phenyl)-2-cyclohexyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine) and pyridinylisoquinolines, have been reported (Gao et al. 2005; Göblyös et al. 2006).

The structure–activity relationships (SARs) of nucleoside derivatives in binding to the A₃AR and other ARs have been extensively studied, leading to the development of both selective agonists and, more recently, antagonists. Most of the useful modifications of adenosine **1** (Fig. 1) to achieve high A₃AR affinity and selectivity have been made at the N⁶ or C2 positions of adenine or on the ribose group of adenosine. The systematic probing of SAR of both adenosine derivatives and nonpurine antagonists is frequently guided by molecular modeling (Kim et al. 2003), in which the receptor protein is modeled based on structural homology to the light receptor, rhodopsin. The effects of substitution at various

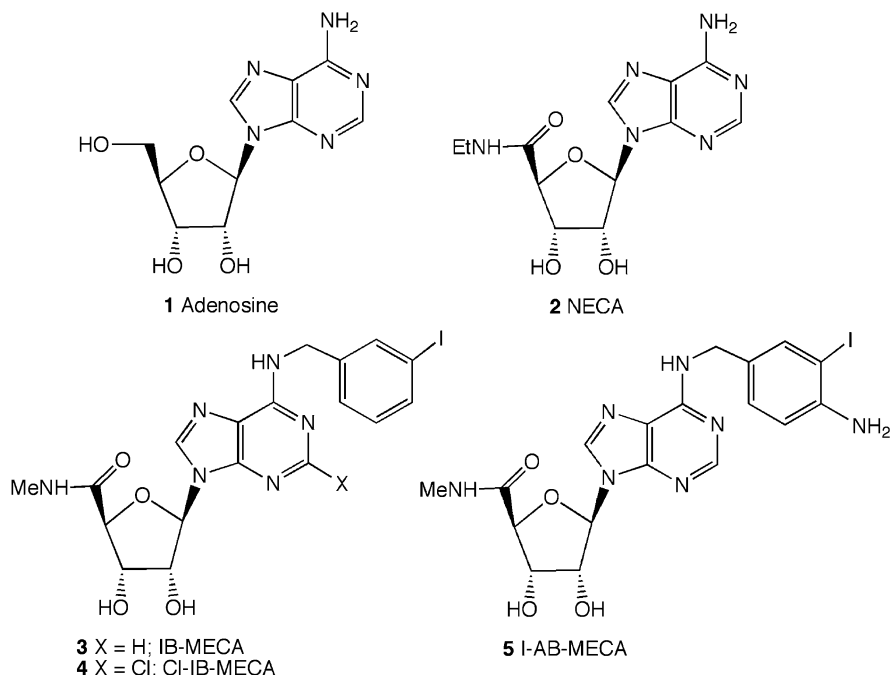


Fig. 1 Structures of adenosine and widely used agonist probes of the A₃AR

sites (i.e., on the nucleobase and ribose moiety) on both the affinity and relative efficacy of nucleoside derivatives at the A₃AR have been extensively probed (Gao et al. 2003, 2004). The approach initially taken to identify agonists for the newly cloned A₃AR was to screen known AR ligands in binding assays. The agonist NECA **2** (adenosine 5'-*N*-ethyluronamide) was found to be highly potent but nonselective for this receptor (Zhou et al. 1992). The structural features that promoted A₃AR potency were combined, leading to the first selective A₃ agonist, IB-MECA (*N*⁶-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine), developed in 1993 at the National Institutes of Health (Jacobson et al. 1993). This potent A₃AR agonist IB-MECA **3** and its more selective 2-chloro analog, Cl-IB-MECA **4** (2-chloro-*N*⁶-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine), are used widely as pharmacological tools. A related derivative **5** is widely used as an iodinated radioligand for the A₃AR. IB-MECA and Cl-IB-MECA have entered clinical trials for the treatment of rheumatoid arthritis and cancer (Baharav et al. 2005; Ohana et al. 2001).

One problem encountered in refining selective A₃AR ligands into pharmaceutically useful agents has been the species dependence of binding. This difference in affinity reflects the difference in sequence between the rodent and the human receptors, with only a 74% sequence identity between the rat (r) and human (h) A₃ARs (Fredholm et al. 2001). The species-dependence of A₃AR affinity is particularly

pronounced for agonists that contain small alkyl N^6 substituents and for various heterocyclic antagonists, both of which are more potent in binding to the human than to the rat A_3AR (Yang et al. 2005). The first report of a cloned receptor sequence to be later identified as an A_3AR was that of the rat (Meyerhof et al. 1991; Zhou et al. 1992), and this species was initially used for screening purposes. Nevertheless, many of the nucleoside analogs that were shown to be rat A_3AR agonists, including CI-IB-MECA and IB-MECA, were later found to be moderately selective for the human A_3AR after it was cloned (Jacobson and Gao, 2006; Salvatore et al. 1993).

The ligand recognition within the putative binding site of the ARs has also been probed through extensive mutagenesis to confirm the predictions concerning ligand recognition made using molecular modeling (Kim et al. 2003). The hydrophobic environment surrounding the purine ring of AR agonists, as found in the putative $A_{2A}AR$ model, is defined mainly by residues of TM5 and TM6 (Kim et al. 2003). This region is very similar to the putative binding region of hydrophobic heterocyclic (e.g., triazolopyrimidine) antagonists. An exocyclic amino group is common to both adenosine agonists and to typical heterocyclic antagonists, and this amine is generally required to donate a hydrogen bond to the receptor protein. Amino acid residues involved in the ligand recognition in the putative A_{2A} and A_3 AR binding sites have been reviewed (Kim et al. 2003).

2 A_3AR Agonists

The subtype selectivity of adenosine derivatives as AR agonists has been probed extensively, principally through modification of the N^6 -amine moiety (where large hydrophobic groups tend to produce A_1AR and A_3AR selectivity, Table 1) and the C2 position (where large hydrophobic groups tend to produce $A_{2A}AR$ selectivity, but have also been shown to enhance A_3AR selectivity, Table 2). The ribose moiety is less amenable than the adenine moiety to the addition of steric bulk, although substitution of the 5'-CH₂OH moiety with certain amides, ethers, or other hydrophilic groups has resulted in enhancement of A_3AR selectivity.

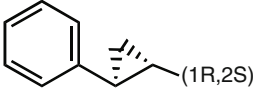

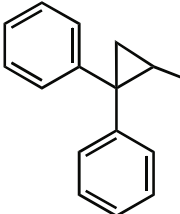
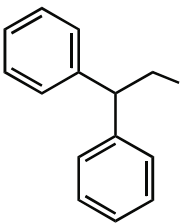
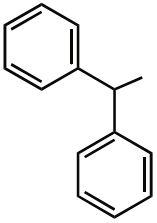
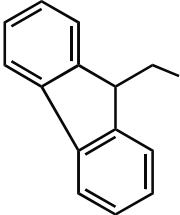
The binding of a nucleoside to the A_3AR and its activation of the receptor are separate processes that appear to have distinct structural requirements. There is no general correlation between the affinity of a given nucleoside derivative in binding to the A_3AR and its ability to fully vs. partially activate the receptor (Table 1). Specific functionality on the nucleoside structure that lowers efficacy relative to that of a full agonist (e.g., NECA) has been identified. For example, N^6 -benzyl and certain 2-position substituents on the adenine moiety reduce the relative efficacy at the A_3AR . 2-Chloro alone does not reduce A_3AR efficacy, but, in combination with a substituted N^6 -benzyl moiety, it leads to a further reduction (Gao et al. 2002). Other N^6 substitutions have been studied using the same criteria. For example, the relative efficacy of N^6 -(2-phenylethyl) derivatives is extremely sensitive to substitution of the phenyl ring and the β -methylene carbon (Tchilibon et al. 2004).

Table 1 Binding affinities of monosubstituted adenosine derivatives (*N*⁶-substituted) at the human A₃AR expressed in CHO cells and at A₁ and A_{2A} ARs, and maximal A₃AR agonist effect

Compound	Substitution R ¹ =	p <i>K</i> _i at A ₁ AR ^a	p <i>K</i> _i at A _{2A} AR ^a	p <i>K</i> _i at A ₃ AR ^a	%Activation, A ₃ AR ^a
6	CH ₃	7.22 ^b	<5 ^b	8.03	96
7	CH ₂ CH ₃	8.31 ^b	5.05 ^b	8.34	102
8	OCH ₃	6.65 ^b	<5 ^b	7.55	107
9		9.15 ^b	5.76 ^b	8.19	100
10		9.35 ^b	6.34 ^b	7.14	97
11		9.05 ^b	6.28 ^b	7.14	76
12		8.48 ^b	6.18 ^b	7.83	102
13		6.76 ^b	6.55 ^b	7.38	55
14		7.35	<5 ^b	8.36	80
15		7.89, 7.89 ^b	7.17, 7.17 ^b	8.68, 6.62 ^b	84
16		6.91	5.60	9.06	101
17		6.98	5.60	8.72	101

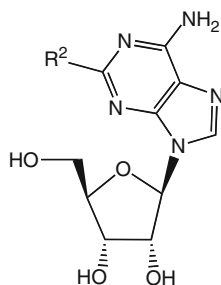
(continued)

Table 1 (continued)

Compound	Substitution R ¹ =	p <i>K</i> _i at A ₁ AR ^a	p <i>K</i> _i at A _{2A} AR ^a	p <i>K</i> _i at A ₃ AR ^a	%Activation, A ₃ AR ^a
18	 (1R,2S)	7.82 ^b	5.52 ^b	9.20	100
19	 (1S,2R)	7.93 ^b	5.60 ^b	7.62	87
20		8.15	6.24	7.04	100
21		7.30	6.29	8.41	0
22		6.31	<5	5.48	87
23		7.85	6.84	9.04	99

^aA₃AR binding experiments were performed with membranes prepared from adherent CHO cells stably transfected with cDNA encoding the human A₃AR, using as radioligand [¹²⁵I]N⁶-(4-amino-3-iodobenzyl)adenosine-5'-N-methyluronamide ([¹²⁵I]I-AB-MECA; 2000 Ci/mmol) at a final concentration of 0.5 nM, in Tris-HCl buffer (50 mM, pH 8.0) containing 10 mM MgCl₂, 1 mM EDTA. Nonspecific binding was determined using 10 μM Cl-IB-MECA. The mixtures were incubated at 25°C for 60 min. Maximal A₃AR agonist effect is the inhibition of forskolin-stimulated adenylate cyclase at 10 μM using a reference value for Cl-IB-MECA of 100%. (Gao et al. 2003; Tchilibon et al. 2004).

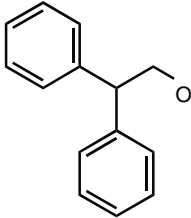
^bIn rat brain (Gao et al. 2003; Tchilibon et al. 2004).

Table 2 Binding affinities of monosubstituted adenosine derivatives (2-ether-substituted) at the human A₃AR expressed in CHO cells and at A₁ and A_{2A} ARs, and maximal A₃AR agonist effect

Compound	Substitution R ¹ =	pK _i at A ₁ AR ^a	pK _i at A _{2A} AR ^a	pK _i at A ₃ AR ^a	%Activation, A ₃ AR ^a
29	Cl	8.12	6.20	7.06	100
30		5.29	7.36	6.44	32
31		6.19	6.23	6.93	17
32		6.66	8.03	7.27	71
33		5.43	6.23	5.71	ND
34		6.28	7.21	6.51	72
35		6.66	7.75	6.85	1
36		6.54	7.19	6.98	91
37		5.32	7.57	6.76	0

(continued)

Table 2 (continued)

Compound	Substitution R ¹ =	p <i>K</i> _i at A ₁ AR ^a	p <i>K</i> _i at A _{2A} AR ^a	p <i>K</i> _i at A ₃ AR ^a	%Activation, A ₃ AR ^a
38		<5	6.51	7.27	0

^aA₃AR binding experiments were performed with membranes prepared from adherent CHO cells stably transfected with cDNA encoding the human A₃AR, using as radioligand [¹²⁵I]N⁶-(4-amino-3-iodobenzyl)adenosine-5'-*N*-methyluronamide ([¹²⁵I]I-AB-MECA; 2000 Ci/mmol) at a final concentration of 0.5 nM, in Tris-HCl buffer (50 mM, pH 8.0) containing 10 mM MgCl₂, 1 mM EDTA. Nonspecific binding was determined using 10 μM Cl-IB-MECA. The mixtures were incubated at 25°C for 60 min. ND, not determined. Maximal A₃AR agonist effect is inhibition of forskolin-stimulated adenylate cyclase at 10 μM using a reference value for Cl-IB-MECA of 100% (Gao et al. 2004)

Modifications of the ribose moiety have also been explored for effects on both A₃AR binding affinity and efficacy (Gao et al. 2004; van Tilburg et al. 2002). SAR studies also indicate that flexibility in the ribose 5' region is a prerequisite for A₃AR activation, in concert with a proposed required rotation of TM6 (Kim et al. 2006). Thus, with proper manipulation of groups at the N⁶ and/or ribose moieties, a high-affinity agonist may be converted into a selective A₃AR antagonist. Conversely, agonist function may be maintained fully with proper derivatization of the ribose moiety. A flexible 5'-uronamide moiety is particularly well suited to maintaining efficacy, and even overcomes the reduction of efficacy induced by various adenine substituents at the N⁶ and C2 positions.

2.1 Substitution of the Adenine Moiety of Adenine Nucleosides

2.1.1 N⁶ Position

Multiple studies have been undertaken to optimize the N⁶ position of adenosine in order to design selective A₃AR agonists (Table 1, 6–23). Addition of small groups such as methyl (6) and oxymethyl (8) to the N⁶ amine gave at least a tenfold increase in potency over adenosine and increased the selectivity of the ligand for the human A₃AR over other human ARs (Volpini et al. 2007). However, increasing the alkyl chain length to an ethyl group (7) increased the affinity of the ligand for both the A₃ and A₁ ARs, thus, decreasing the selectivity (Gao et al. 2003). Larger alkyl chains were not well tolerated at the N⁶ position, and increased branching of the chain caused a decrease in A₃AR affinity and efficacy. Various cycloalkyl groups were

also appended to the N⁶-amino group. Adding an N⁶-cyclobutyl (**9**) or cyclopentyl (**10**) ring resulted in agonists that had greater affinity to the A₁AR than the A₃AR (Gao et al. 2003) but were full agonists at the A₃AR. Analogs bearing larger N⁶-cycloalkyl rings such as **11** were only partially efficacious as A₃AR agonists. When a benzyl ring was attached to the N⁶ amine (**13**), the compound was three- to four-fold selective in binding to the human A₃AR in comparison to the A₁ and A_{2A} ARs, but only displayed a 55% relative efficacy at the A₃AR. N⁶-Phenyladenosine (**12**) was fully efficacious as an A₃AR agonist. N⁶-(2-Phenylethyl)adenosine (**15**) was the most potent in binding to the A₃AR among a series of arylalkyl-substituted homologs. However, the N⁶-benzyl and the N⁶-phenyl substituents provided greater selectivity than 2-phenylethyl for the A₃AR. Generally, halogen substitution at the 3 position of the N⁶-benzyl ring caused an increase in A₃AR affinity and selectivity. For example, N⁶-(3-chlorobenzyl)adenosine (**14**) showed a tenfold selectivity for the A₃AR and a nanomolar affinity. Halogen substitution at other positions of the ring frequently decreased the A₃AR affinity.

Addition of certain larger N⁶ substituents also increased the potency and affinity of the ligands at the A₃AR. For instance, N⁶-(*trans*-2-phenylcyclopropyl)adenosine (**16**) was a full agonist with high selectivity and a subnanomolar potency (Gao et al. 2003). Further variations on this substituent were prepared, and the importance of conformational factors in the relative efficacy was demonstrated. The addition of one bond to bridge the phenyl rings could change an antagonist into an agonist. Thus, while N⁶-(2,2-diphenylethyl)adenosine (**21**) was an antagonist at the A₃AR, adding a bond between the phenyl groups to create N⁶-(9-fluorenylmethyl)adenosine (**23**) restored the efficacy. This compound also had a subnanomolar A₃AR affinity but was less selective than N⁶-(*trans*-2-phenylcyclopropyl)adenosine (Tchilibon et al. 2004). The most selective compound of the series was N⁶-(*trans*-2-(3-trifluoromethyl)phenyl)-1-cyclopropyl adenosine (**17**), which had a 100-fold selectivity at the A₃AR in comparison to the A₁AR.

Various labs have combined sterically bulky N⁶ groups with a 5'-uronamide moiety on the ribose group to make potent, selective A₃AR agonists. The first A₃AR-selective compounds combined a 5'-*N*-alkyluronamide with an N⁶-benzyl group (Gallo-Rodriguez et al. 1994; Jacobson et al. 1993; van Galen et al. 1994). One of the most common A₃ agonists, Cl-IB-MECA (Fig. 1), has an N⁶-iodobenzyl group, a 2-chloro group, and a 5'-methylcarboxamido group. This compound has a K_i of 0.33 nM at the rat A₃AR, but K_i values of only 2,500 and 1,400 nM at rat A₁ and A_{2A} ARs, respectively (Kim et al. 1994a). At the human ARs, the binding affinities of Cl-IB-MECA are (nM): A₁AR 220, A_{2A}AR 5400, and A₃AR 1.4. Thus, Cl-IB-MECA is more selective for the rat A₃AR than the human A₃AR (Melman et al. 2008a). The ¹²⁵I form of I-AB-MECA (N⁶-(4-amino-3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine, **5**, Fig. 1) is commonly used as a high-affinity radioligand for characterizing binding to the A₃AR of various species.

Baraldi et al. (1998) prepared a series of N⁶-substituted-aminosulfonylphenyl derivatives of NECA (e.g., compound **24**). Among these compounds, the most favorable substituents of the sulfonamido group for increasing affinity at the A₃AR were small alkyl groups, such as ethyl or allyl moieties, and disubstitution of the

sulfonamido group. The A₃AR selectivity was increased by the addition of a saturated heterocyclic ring, such as piperidine or morpholine, to the sulfonamido moiety.

Finally, A₃ selective fluorescent probes have also been made by attaching 7-nitrobenzofurazan fluorophores to NECA derivatives using an alkyl spacer (e.g., compound **25**). These compounds displayed 500-fold selectivity at the A₃AR and bound in the low nanomolar range (Cordeaux et al. 2008).

2.1.2 Adenine 2 Position

Many modifications at the 2 position of adenosine (Table 2, **29–38**) tend to increase A_{2A}AR potency, but some additions have been found to contribute to A₃AR selectivity. Adding a simple 2-chloro group (**29**) increased the A₃AR affinity in comparison to adenosine, but it also significantly increased the potency at the A₁AR (Gao et al. 2004). Generally, 2-ether modifications decreased A₃AR affinity, with certain exceptions. For example, adding a 2-*i*-pentyloxy moiety increased A₃AR affinity threefold, and the compound was slightly selective. 2-Benzyloxy substitution (**31**) decreased the efficacy to 17% of the full agonist CI-IB-MECA. 2-Phenylethoxy substitution (**32**) often increased affinity at both the A₃ and A_{2A}ARs, but many such analogs displayed a decreased efficacy as A₃AR agonists. Other 2-ethers, such as 2-(2,2-diphenylethoxy)adenosine (**38**), were A₃AR antagonists, in curious parallel to the effect of the same group when placed at the N⁶ position (Tchilibon et al. 2004).

Many other substitutions at the 2 position of adenosine were combined with previously introduced substitutions at the N⁶ position of adenosine. For instance, adding a 2-cyano group to N⁶-(3-iodobenzyl)adenosine created an A₃AR antagonist, but when the 2-cyano group was added to N⁶-methyladenosine, the compound was a full agonist that was 30-fold selective for the human A₃AR in comparison to the A₁AR (Ohno et al. 2004). However, when other small modifications were made at the 2 position of N⁶-methyladenosine, such as an amino or a trifluoromethyl group, there was a decrease in selectivity and affinity toward the A₃AR. Elzein et al. (2004) synthesized a series of 2-pyrazolyl-N⁶-substituted adenosine derivatives that were very potent and selective for the A₃AR. Cosyn et al. (2006b) found that several 2-triazol-1-yl substitutions of N⁶-methyladenosine increased affinity at the A₃AR. However, in order to maintain efficacy, a 5'-ethyluronamide was necessary. 9-(5-Ethylcarbamoyl-β-D-ribofuranosyl)-N⁶-methyl-2-(4-pyridin-2-yl-1,2,3-triazol-1-yl)adenine **26** (LC257, Fig. 2) was a full agonist with a K_i of 1.8 nM at the A₃AR and a minimum of 900-fold selectivity over other ARs.

Additions at the 2 position of NECA often increased potency and/or selectivity. For instance, 2-(3-hydroxy-3-phenyl)propyn-1-yl-NECA **27** (PHPNECA) (Fig. 2) exhibited a subnanomolar affinity at the A₃ receptor (Volpini et al. 2002). Also, Zhu et al. (2006) made a series of N⁶-ethyl-2-alkynyl-NECA derivatives which had sub- to low nanomolar affinities and were very selective in comparison to the A_{2A} and A_{2B}ARs, with some selectivity over the A₁AR. The most potent compound in that series (**28**) had a (*p*-(methoxy)phenyl)alkynyl substituent at the 2 position.

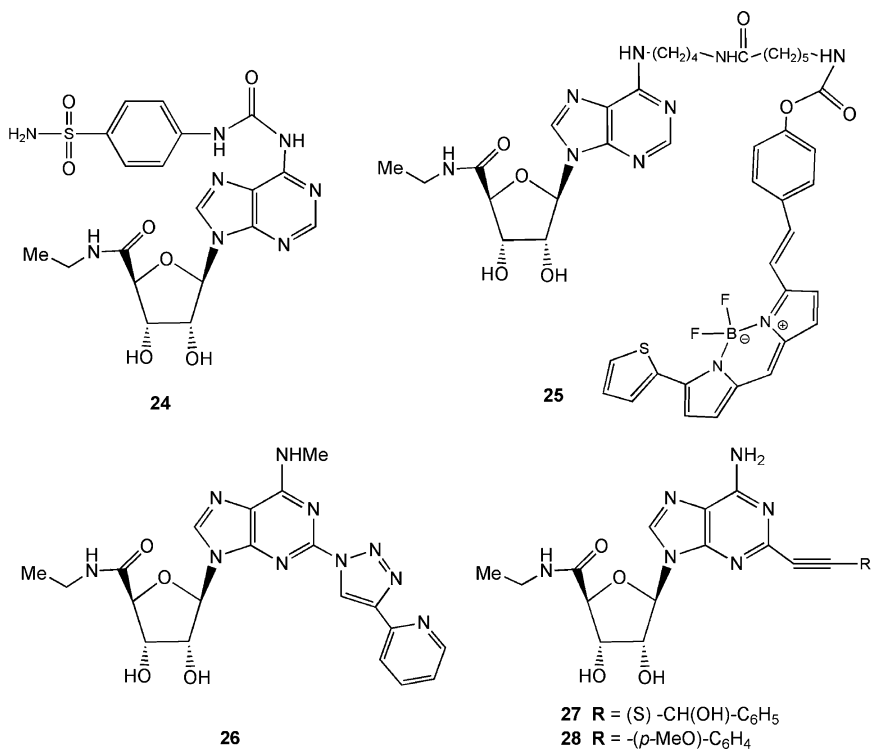


Fig. 2 Structures of a novel, multiply substituted A₃AR agonists

2.2 Ribose Modifications

2.2.1 Modification of Ribose Hydroxyl Groups

Many modifications have been made to the ribose ring. As mentioned above, the 5'-N-alkyluronamide modification has been particularly fruitful. Gallo-Rodriguez et al. (1994) initially found that adding a 5'-N-methyluronamide group to N⁶-benzyl derivatives increased the binding affinity at all three ARs examined and resulted in several of the compounds gaining selectivity for the A₃AR. They also found that adding a 5'-N-ethyluronamide more than doubled the potency of several N⁶-benzyl derivatives of adenosine. Other modifications at the ribose 5' position, such as alkylthioethers (van Tilburg et al. 2002) have been found to modulate affinity and efficacy at the A₃AR.

Both the 2'- and the 3'- hydroxyl groups contribute to the binding process, since replacing either of these groups in Cl-IB-MECA with a fluoro group caused a significant drop in both affinity and efficacy (Gao et al. 2004). A less drastic decrease in binding and efficacy was seen when the 3'-hydroxyl of the adenosine analogs was replaced with an amino group (DeNinno et al. 2003). When a methylene spacer

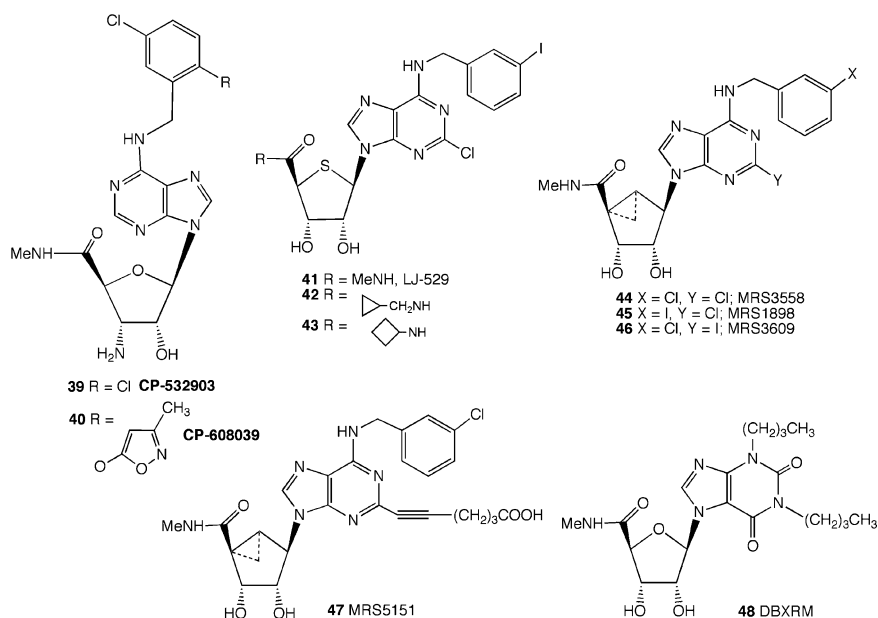


Fig. 3 Structures of ribose ring-modified selective A₃AR agonist probes

was added between the 3'-amino and ribose groups, there was a total loss of affinity (Van Rompaey et al. 2005). Also, 3'-deoxy-3'-acetyl amino analogs were weak at the A₃AR. However, DeNinno et al. (2003, 2006) found that the 3'-amino substitution was tolerated and gave high selectivity when the 5' and N⁶ positions of adenosine were also appropriately modified in compounds **39** and **40** (Fig. 3). Replacement of the 3'-hydroxyl with an azido group generally abolished A₃AR activation. The 2'-hydroxyl group appeared to be more important than the 3'-hydroxyl group, because when it was replaced with the fluoro group there was no binding or activation of the A₃AR (Gao et al. 2004).

2.2.2 Modification of the Pentose Ring

4'-Thio derivatives were usually equipotent or slightly more potent at ARs than their oxygen equivalents (Jeong et al. 2006a). Many 4'-thio derivatives of adenosine have been found to be full agonists. For example, LJ-529 **41** (2-chloro-*N*⁶-(3-iodobenzyl)-4'-thioadenosine-5'-methyluronamide) (Fig. 3) is a highly potent ligand ($K_i = 0.38$ nM against [¹²⁵I]-AB-MECA binding to the human A₃AR expressed in CHO cells). In the same 4'-thio-modified series, a wide variety of ribose 5'-alkyluronamides have shown that there is tolerance for groups larger than *N*-ethyl (Jeong et al. 2006a). For example, compounds **42** and **43** were full agonists with K_i values of 3.6 and 18 nM at the hA₃AR, respectively. The nature of the *N*-alkyl or *N*-arylalkyl group can modulate affinity and efficacy at the A₃AR (Jeong et al. 2008).

However, when the thio modification was combined with shifting the adenine moiety of Cl-IB-MECA from the 1' to the 4' position of the ribose ring, the compound was curiously transformed into a potent antagonist (Gao et al. 2004).

Ring-constrained nucleosides have been used to define conformational preferences at the A₃AR. Medicinal chemists frequently utilize the approach of conformationally constraining otherwise flexible molecules to probe the “active” conformation(s) and to increase ligand affinity by overcoming the energy barriers needed to attain this preferred conformation. Nucleoside analogs containing novel rigid ring systems in place of the ribose ring have been explored as ligands for the ARs. The focus on conformational factors of the ribose or ribose-like moiety allows the introduction of general modifications that lead to enhanced potency and selectivity at certain subtypes of these receptors. One ring system selected for this purpose is the methanocarba (bicyclo[3.1.0]hexane) ring system, which has been incorporated in either of two isomeric forms that adopt either a North (N) or South (S) envelope conformation (Jacobson et al. 2000; Marquez et al. 1996). These ribose modifications were combined with known enhancing modifications at other positions on the molecule to explore the resulting SARs. (N)-Methanocarba-adenosine was favored in binding at the A₃AR by 150-fold over the (S) conformation and by 2.5-fold over adenosine. Doubly modified nucleoside derivatives containing the (N)-methanocarba ring system have confirmed that this conformation of the pseudoribose ring is highly preferred over the (S) conformation for agonists at the A₃AR in general.

Introducing an (N)-methanocarba modification to adenosine 5'-ethyluronamide increased the human A₃AR binding affinity by sixfold. This modification also demonstrated that the ring oxygen is not required for binding or activation of the receptor (Lee et al. 2001).

Highly selective ring-constrained agonists of the A₃AR have been designed and synthesized based on the (N)-methanocarba ring system (Fig. 3). This led to the introduction of MRS3558 **44** ((1'*R*,2'*R*,3'*S*,4'*R*,5'*S*)-4-{2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo-[3.1.0]-hexane-2,3-diol) as a full agonist with subnanomolar potency at the A₃AR and its congeners (e.g., **45** and **46**) as full agonists with nanomolar potency at the A₃AR (Tchilibon et al. 2005). The SAR of MRS3558 and related congeners as A₃AR agonists (Melman et al. 2008a) was recently explored in detail. The utility of MRS3558 in treating lung injury was shown in a model of ischemia reperfusion lung injury (Matot et al. 2006). In this series of (N)-methanocarba nucleosides, a 5'-uronamide moiety is needed in order to achieve full efficacy at the A₃AR. The corresponding 5'-alcohol is an antagonist of the A₃AR. The 5'-uronamide moiety overcomes the loss of efficacy associated with substitution of the N⁶ and ribose ring moieties. Thus, in the (N)-methanocarba series, as in the ribose series, a freely rotating 5'-uronamide that is able to make and break multiple hydrogen bonds provides a necessary degree of flexibility during the receptor activation step.

2.3 *Nonadenine Nucleosides and Nonnucleosides as A₃AR Agonists*

Occasionally, nonadenine nucleotides are also found to activate the A₃AR. For instance, xanthines such as caffeine are generally found to act as antagonists, but *N*-methyl-1,3-dibutylxanthine 7-β-D-ribofuronamide **48** (DBXRM) acted as a moderately selective A₃AR agonist (Kim et al. 1994b).

A series of atypical, nonnucleoside agonist ligands that activated various ARs were reported (Chang et al. 2005). In addition to compounds in this family of pyridine-3,5-dicarbonitriles that were selective agonists of the A₁AR, various members of this series substantially activated the A₃AR.

2.4 *Further Optimization of A₃AR Agonists Using Multiple Modifications*

Interestingly, certain modifications (such as a 5'-alkylamide or an *N*⁶-methyl group) can restore efficacy to previously modified compounds. For instance, adding a 2-chloro group to *N*⁶-cyclopentyladenosine creates an A₃AR antagonist (Gao et al. 2002), but activation is restored by the 5'-methylcarboxamide and 4'-thio substitutions. This is particularly interesting since 4'-thioadenosine is also an A₃AR antagonist, and 2-chloro-4'-thioadenosine is only a partial agonist (Jeong et al. 2006b).

A series of (N)-methanocarba-2,*N*⁶-disubstituted adenine nucleosides were made by Tchilibon et al. (2004), who found that adding the (N)-methanocarba, 2-chloro, and 5'-methyluronamido groups significantly improved the selectivity and efficacy of several compounds. For instance, *N*⁶-(2,2-diphenylethyl)adenosine was an A₃AR antagonist with 12-fold and 130-fold selectivity over A₁ and A_{2A} ARs, respectively. However, by adding the above substitutions, the compound became a full agonist with a *K*_i of 0.69 nM and a selectivity of close to 2,000-fold over A₁ and A_{2A} AR (Tchilibon et al. 2004). Also, the 2-cyano derivative of *N*⁶-methyl adenosine was a full agonist whereas the 2-cyano derivative of *N*⁶-(2-phenylcyclopropyl) adenosine was an A₃AR antagonist (Ohno et al. 2004).

Adding several substitutions may also improve selectivity for the A₃AR. Adding an *N*⁶-methyl group and 2-chloro group to 4'-thioadenosine-5'-methyluronamide created a compound with a *K*_i of 0.28 nM and a nearly 5,000-fold selectivity for the A₃AR (Jeong et al. 2006a). A series of these compounds was made by varying the *N*⁶ and 5' groups. While none of these derivatives could match the potency and selectivity of the original compound, it was found that 4'-thioadenosine derivatives were often more potent than their oxy counterparts. The most potent compound was 9-(3-amino-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-2-amino-*N*⁶-methylpurine. Another highly substituted yet extremely potent *N*⁶-methyl derivative is 2-chloro-*N*⁶-methyl-4'-thioadenosine-5'-methyluronamide,

which has a K_i of 0.28 nM (Jeong et al. 2006a). N^6 -Methylation also seems to improve human A₃AR selectivity, as N^6 -methyl-2-(2-phenylethyl)-adenosine is much more selective than 2-(2-phenylethyl)-NECA (Volpini et al. 2002). While large 2-position substitutions are not always tolerated, (2*R*,3*S*,4*R*)-tetrahydro-2-(hydroxymethyl)-5-(6-(methylamino)-2-(4-pyridin-2-yl)-1*H*-pyrazol-1-yl)-9*H*-purin-9-yl) furan-3,4-diol had a K_i of 2 nM and was extremely selective (Van Rompaey et al. 2005).

Recently, new potent and A₃-selective N^6 ,2-disubstituted adenosine derivatives have been reported. Volpini et al. (2007) made a series of N^6 -methoxy-2-alkyladenosine derivatives, of which N^6 -methoxy-2-*p*-acetylphenylethylMECA was the most potent and selective. This compound had a K_i of 2.5 nM at the human A₃AR and selectivities of 21,000 and 4,200 against A₁ and A_{2A} ARs, respectively. Recently, a series of water-soluble A₃AR agonists were synthesized (DeNinno et al. 2006). Of these compounds, (2*S*,3*S*,4*R*,5*R*)-3-amino-5-{6-[5-chloro-2-(2-oxo-2-piperazin-1-yl-ethoxy)-benzylamino]-purin-9-yl}-4-hydroxy-tetrahydro-furan-2-carboxylic acid methylamide was the most potent/selective derivative, with a K_i of 10 nM. Van Rompaey et al. (2005) found that adding additional substitutions to 3-amino-3-deoxyadenosine increased the potency, but these compounds were only partial agonists. 9-[3-Amino-3-deoxy-5-(methylcarbamoyl)-β-D-ribofuranosyl]- N^6 -(5-chloro-2-methoxybenzyl)adenine had a K_i of 27 nM and was extremely selective for the A₃AR, but had an efficacy of only 51%. Cosyn et al. (2006a) made a series of 3'-amino-3'-deoxy congeners that were highly selective for the A₃AR.

The selectivity at the mouse A₃AR of analogs containing the (N)-methanocarba ring system was reduced due to an increased tolerance of this ring system at the mouse A₁AR (Melman et al. 2008a). Substitution of the 2-chloro atom with iodo or hydrophobic alkynyl groups tended to increase the A₃AR selectivity (up to 430-fold) in mouse and preserve it in human. Extended and chemically functionalized alkynyl chains attached at the C2 position of the purine moiety preserved A₃AR selectivity more effectively than similar chains attached at the 3 position of the N^6 -benzyl group. For example, the carboxylic acid congener MRS5151 **47** (Fig. 3) is a highly potent agonist (K_i 2.38 nM at hA₃AR) and is selective in binding at human (6,260-fold) and mouse (431-fold) A₃ARs in comparison to A₁ARs in the same species.

3 A₃AR Antagonists

Initial attempts at obtaining potent and highly selective A₃AR antagonists focused on wide pharmacological screening of different heterocyclic compounds (Jacobson et al. 1995; Ji et al. 1996; Siddiqi et al. 1995). One of the first nonxanthine heterocyclic derivatives (Fig. 4) found to be selective for the human A₃AR (K_i 0.65 nM) was MRS1220 (*N*-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-yl]benzeneacetamide) **49**, which was based on appropriate acylation of the exocyclic amino group of this class of known AR antagonists (Kim et al.

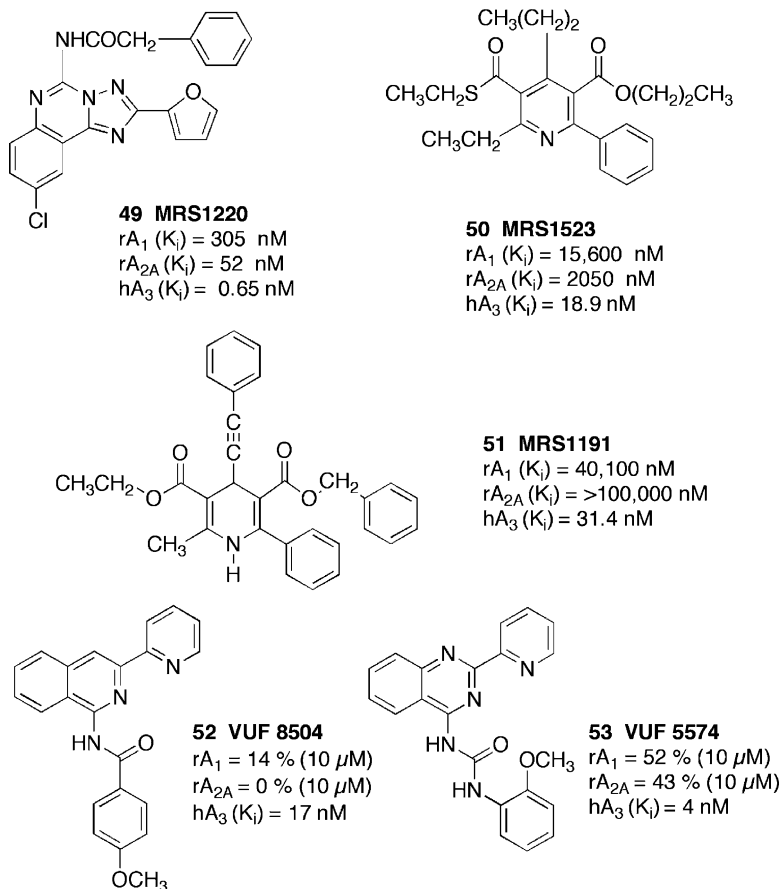


Fig. 4 Structures of heterocyclic derivatives that are widely used as selective human A_3AR antagonists

1996). During subsequent evaluations, different classes of nonxanthine nitrogen-containing molecules were identified as potent A_3AR antagonists: flavonoids, 1,4-dihydropyridines and pyridines, triazoloquinazolines, isoquinolines and quinoxalines (Baraldi et al. 2003a; Müller et al. 2003). The 1,4-dihydropyridine (DHP) derivative MRS1191 (1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl 5-(phenylmethyl) ester) **51** was structurally optimized for binding to the A_3AR (K_i 31 nM) from library screening that identified various DHP calcium channel blockers as weak A_3AR antagonists (Jacobson et al. 1997). The pyridine derivative MRS1523 (5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate) **50** was the first heterocyclic A_3AR antagonist to display considerable potency and selectivity for the rat A_3AR (K_i 113 nM), as well as the human (18.9 nM) and mouse A_3AR (Li et al. 1998). In this section, the recent advancements in this field have been summarized, with particular attention paid to the most important reports of the last five years.

3.1 Recent Developments in Nonpurine Heterocycles

3.1.1 Thiazole and Thiadiazole

IJzerman and coworkers investigated a series of 3-(2-pyridinyl)-isoquinoline derivatives for their affinity at the A₃AR (Van Muijlwijk-Koezen et al. 2000). The effect of an additional nitrogen atom was valued by synthesizing bioisosteric quinazoline derivatives. The compounds VUF 8504 (4-methoxy-*N*-(3-(2-pyridinyl)-1-isoquinolinyl)benzamide, **52**) and VUF 5574, (*N*-(2-methoxyphenyl)-*N'*-(2-(3-pyridyl)quinazolin-4-yl)urea, **53**) (Fig. 4) display considerable A₃AR affinity and appreciable selectivity versus A₁ and A_{2A} AR subtypes.

The bicyclic system of isoquinoline and quinazoline has been replaced by several monocyclic rings (Van Muijlwijk-Koezen et al. 2001). Some thiazole and thiadiazole derivatives were shown to be most promising candidates for the identification of new A₃AR ligands.

The derivative *N*-[3-(4-methoxy-phenyl)-[1,2,4]thiadiazol-5-yl]-acetamide (**54**, Fig. 5) has been claimed to be the most potent A₃AR antagonist of the series, exhibiting a *K_i* value of 0.79 nM at hA₃AR and antagonistic properties in a cAMP functional assay (Jung et al. 2004). A series of potent and selective A₃AR antagonists have been obtained via an optimization study of compound **55** that revealed that a 5-(pyridine-4-yl) moiety on the 2-aminothiazole ring was optimal for enhanced receptor potency and selectivity (Press et al. 2004). Of particular note, *N*-[4-(3,4,5-trimethoxyphenyl)-5-pyridin-4-ylthiazol-2-yl]-acetamide **56** showed subnanomolar affinity at the human A₃AR as a competitive antagonist of [¹²⁵I]-AB-MECA, binding with 1,000-fold selectivity versus the other ARs.

Binding affinity data on thiazole and thiadiazole derivatives at the hA₃AR have been subjected to QSAR analysis (Bhattacharya et al. 2005). This study disclosed the importance of the molecular electrostatic potential surface (Wang-Ford charges)

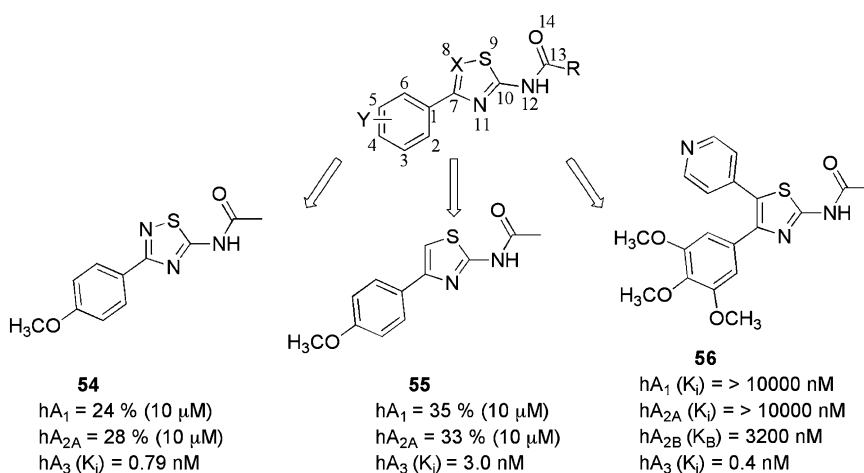


Fig. 5 Thiazole and thiadiazole derivatives as human A₃AR antagonists

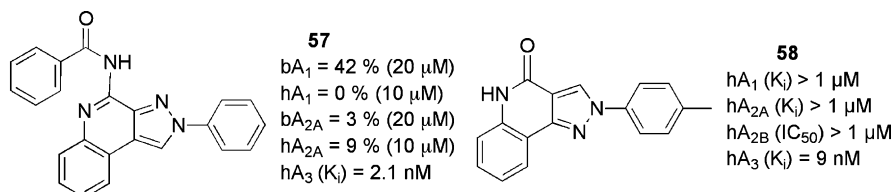


Fig. 6 Pyrazoloquinoline derivatives as human A_3AR antagonists

in relation to atoms C2, C5, C7, X8 and S9 (Fig. 5), the last two playing the most important roles. Furthermore, the A_3AR binding affinity increases with decreasing lipophilicity of the compounds and in the presence of short alkyl chains—methyl (Me) or ethyl (Et)—at the R position.

3.1.2 Pyrazoloquinolines

The binding affinities at bovine A_1 and A_{2A} ARs and at human cloned A_3AR s of some 2-arylpyrazolo[3,4-*c*]quinolin-4-ones along with their corresponding 4-amines and 4-substituted-amino derivatives were reported by Colotta et al. (2000). The 4-benzoylamido derivative **57** (Fig. 6) displayed one of the best binding profiles of the series of A_3AR antagonists. The same group recently reported an extension of the SAR study of this class of compounds (Colotta et al. 2007) which highlighted that bulky and lipophilic acyl-amino groups at the 4 position seemed able to promote hA_3AR potency and selectivity. Selected compounds of these series were tested in an in vitro rat model of cerebral ischemia and prevented the irreversible failure of synaptic activity induced by oxygen and glucose deficiency in the hippocampus, thus confirming that potent and selective A_3AR antagonists may substantially increase the tissue resistance to ischemic damage.

The synthesis and the affinity profile at ARs of a series of 2-phenyl-2,5-dihydro-pyrazolo[4,3-*c*]quinolin-4-ones, conceived as structural isomers of the parent 2-arylpyrazolo[3,4-*c*]quinoline derivatives, have also been reported (Baraldi et al. 2005a). Some of the synthesized compounds showed A_3AR affinities in the nanomolar range and good selectivities, as evaluated in radioligand binding assays at hARs. In particular, substitution at the 4 position of the 2-phenyl ring with methyl, methoxy, or chlorine and the presence of a 4-oxo functionality gave good activity and selectivity (**58**).

3.1.3 Triazoloquinoxalines

Triazolo[4,3-*a*]quinoxalines

Interesting studies performed in the last decade by Colotta and coworkers highlighted that the 1,2,4-triazolo[4,3-*a*]quinoxalin-1-one moiety is an attractive

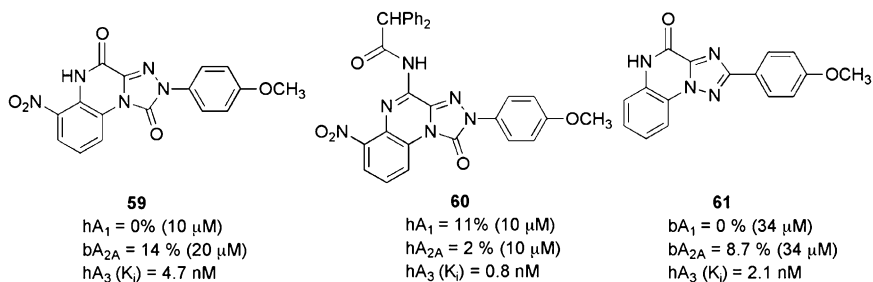


Fig. 7 Triazoloquinoxaline derivatives as A₃AR antagonists

scaffold for obtaining potent and selective hA₃AR antagonists (Colotta et al. 2004; Lenzi et al. 2006). Intensive efforts in the chemical synthesis of compounds based on the systematic substitution of the 2, 4 and 6 positions of the tricyclic template, along with molecular modeling investigations performed to rationalize the experimental SAR findings, led to the identification of optimal structural requirements for A₃AR affinity and selectivity. In particular, the introduction into the triazoloquinoxaline moiety of a 4-oxo (**59**) or a 4-*N*-amido (**60**, Fig. 7) function affords selective and/or potent A₃AR antagonists, indicating that a C=O group (either extranuclear or nuclear) is necessary for A₃AR affinity. This suggested that the probable engagement of this site of the molecule is a hydrogen bond with the A₃AR binding site. Hindering and lipophilic acyl-amino moieties at the 4 position showed enhanced A₃AR affinity (**60**). Substitution of the 4 position of the 2-phenyl ring with a methoxy or a nitro group and 6-nitro substitution, as well as the combination of these substituents, afforded nanomolar A₃AR affinity and better A₃AR selectivity. 1-Oxo, 6-nitro, and 4-amino groups have been proposed to be involved in hydrogen bonds that anchor the antagonists to the binding site.

Triazolo[1,5-*a*]quinoxalines

Some 2-aryl-8-chloro-1,2,4-triazolo[1,5-*a*]quinoxaline derivatives have been synthesized and tested in radioligand binding assays at bovine (b) A₁ and bA_{2A}ARs and at hA₁ and hA₃ARs (Catarzi et al. 2005a, b). The SAR of these compounds are in agreement with those of previously reported for 2-aryl-1,2,4-triazolo[4,3-*a*]quinoxalines and 2-arylpyrazolo[3,4/4,3-*c*]quinolines, thus suggesting a similar AR-binding mode. These studies provided some interesting compounds; among them, 2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one (**61**, Fig. 7) is the most potent and selective hA₃AR antagonist of this series.

3.1.4 Pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines

The first example of an AR antagonist containing the pyrazolo-triazolo-pyrimidine scaffold (Cacciari et al. 2007) was reported by Gatta and coworkers (Gatta et al. 1993).

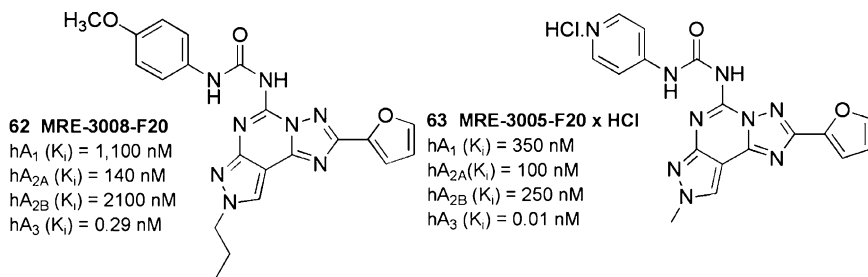


Fig. 8 A_3AR antagonists based on a pyrazolo-triazolo-pyrimidine scaffold

A wide number of compounds (MRE series) originated from the structure–activity optimization work based on systematic substitution at the C2, C5, C9, N7, and N8 positions (Baraldi et al. 2002a; 2003b; 2006). The N^7 -substituted derivatives were found to bind principally to the $hA_{2A}AR$ (Baraldi et al. 2002b), while the most potent and selective hA_3AR antagonists in this series were derived from the combination of a small alkyl chain at the N^8 -pyrazole position with a (substituted)phenylcarbamoyl chain at the N5 position (Baraldi et al. 2003a). The compound designated MRE-3008-F20, (5-*N*-(4-methoxyphenylcarbamoyl)amino-8-propyl-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine, **62**) (Fig. 8), one of several high-affinity antagonists of this series, is a highly potent A_3AR ligand ($K_i = 0.29 \text{ nM}$ against [125 I]I–AB–MECA binding to human AR receptors expressed in HEK293 cells) with good selectivity over the other hARs. It showed antagonist activity in a functional assay blocking the effect of IB–MECA on cAMP production in CHO cells with an IC_{50} value of 4.5 nM. [3 H]MRE 3008-F20 shows a K_d value of $0.82 \pm 0.08 \text{ nM}$ and a B_{max} value of $297 \pm 28 \text{ fmol mg}^{-1} \text{ protein}$ (Varani et al. 2000).

An important problem with the pyrazolo-triazolo-pyrimidine series was the low water solubilities typically observed, which could limit their use as pharmacological and diagnostic ligands. The bioisosteric replacement of the phenyl ring of the 5-phenylcarbamoyl moiety with a 4-pyridyl moiety (Maconi et al. 2002) provided high water solubility while enhancing hA_3AR affinity. Compound MRE-3005-F20, (5-*N*-(4-methoxyphenylcarbamoyl)amino-8-ethyl-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine, **63**) and the corresponding HCl salt, which showed very high affinities and good selectivities at the hA_3 receptor subtype, with K_i values in the picomolar range (40 and 10 pM, respectively), can be considered ideal candidates for pharmacological and clinical investigations of the hA_3AR subtype. Receptor modeling ascribed this increase in affinity, compared to neutral arylcarbamate derivatives, to strong electrostatic interactions between the pyridinium moiety and the side chain carbonyl oxygen atoms of Asn274 and Asn278, both located on TM7. Additional studies suggested that involvement of the residue Tyr254 in a hydrogen bond with the pyridyl ring was responsible for both enhanced receptor affinity and selectivity (Tafi et al. 2006). The replacement of the N^5 -pyridine moiety with several N^5 -heteroaryl rings produced a general loss of affinity and selectivity at the hA_3AR (Pastorin et al. 2006).

In order to rationally design and synthesize hA₃AR antagonists with improved binding and/or absorption, distribution, metabolism, and excretion (ADME) profiles, and as suitable clinical candidates, different molecular modeling investigations have been carried out in the last years. Particular attention has been paid to the pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine family, the most potent class of A₃AR antagonists ever reported (Tafi et al. 2006). A combined target-based (high-throughput molecular docking) and ligand-based (CoMFA) (comparative molecular field analysis) drug design approach has recently been performed by Moro and coworkers (Moro et al. 2005), which defined a novel “Y-shaped” binding motif for pyrazolo-triazolo-pyrimidines and rationally delineated some key ligand–receptor interactions for this class of molecules as follows: (1) steric control around the 3 and 4 positions of the *N*⁵-phenyl ring justifies the decrease in affinity of 3- or 4-substituted-phenyl derivatives; (2) an important π – π interaction takes place between the 2-furyl ring and two phenylalanine residues of the binding site; (3) a hydrophobic pocket, bordered by two hydrophilic amino acids, surrounds the N8 interaction area; and (4) strong hydrogen bonding is possible between a residue of Asn and the N4 of the triazolo ring.

3.1.5 Various Heterocycles

In the last few years, other classes of heterocyclic compounds have been identified as A₃AR antagonists, but large structural dissimilarities meant that none of these could be classified into particular family groups. The quinoxaline derivative **64** (Fig. 9) deserves to be mentioned here, not only because of its good binding profile as an A₃AR antagonist, but also (especially) due to the novelty of the strategy applied to its design, which was based on a 3D database-searching approach (Novellino et al. 2005). There is increasing evidence of the importance of 2D/3D database searching as a valuable tool to discover novel lead compounds for the A₃AR and for other G-protein-coupled receptors (GPCRs) (Costanzi et al. 2008).

The structural manipulation of a series of phenyltriazolobenzotriazinones, previously described as ligands at the central benzodiazepine receptor, led Da Settimo

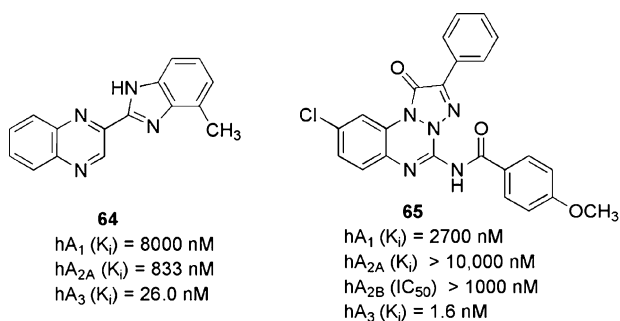


Fig. 9 A₃AR antagonists based on quinoxaline and triazolobenzotriazinone scaffolds

and coworkers to the identification of a series of aminophenyltriazolobenzotriazinones. Among these, compound **65**, a result of a systematic lead optimization, stands out for its remarkable potency and selectivity at the A₃AR (K_i values at the A₁, A_{2A}, A₃ARs of 2,700 > 10,000, 1.6 nM, respectively, and IC₅₀ value from cAMP assay at the A_{2B} > 1,000 nM) (Da Settimo et al. 2007). Interestingly, the triazolobenzotriazinone nucleus is isomeric with that of the triazoloquinoxalinone series described above (compounds **59–61**, Fig. 7).

3.2 Purine Derivatives

3.2.1 Adenines

The first class of A₃AR-selective antagonists with a bicyclic structure strictly correlated to the adenine nucleus was claimed in 2005 by Biagi and coworkers (Biagi et al. 2005). The authors described the synthesis of a series of *N*⁶-ureidosubstituted-2-phenyl-9-benzyl-8-azaadenines whose adenine-like structure was responsible for the antagonist activity and whose phenylcarbamoyl group ensures selectivity at the A₃AR. The structure–activity relationship studies were performed based on the systematic optimization of substituents at the 2, 6 and 9 positions of the bicyclic scaffold, and led to the desired enhancement of A₁/A₃ selectivity (compound **66**, Fig. 10).

Basing on the finding that the known differentiation agent “reversine” (2-(4-morpholinoanilino)-*N*⁶-cyclohexyladenine) exerted a moderate antagonist activity at the hA₃AR (K_i value of 0.66 μM), Jacobson and coworkers developed a series of reversine analogs, focusing their attention on the substitution pattern at the 2 and *N*⁶ positions of the adenine scaffold (Perreira et al. 2005). One of most interesting compounds in terms of hA₃AR affinity and selectivity, MRS3777, (2-(phenyloxy)-*N*⁶-cyclohexyladenine, **67**), combines the *N*⁶-cyclohexyl moiety of reversine with a 2-phenyloxy group. A few derivatives tested in binding assays to the rat A₃AR seemed to reflect the species dependence of the affinity typical of most known nonnucleoside A₃AR antagonists, and were shown to be inactive at 10 μM in this species.

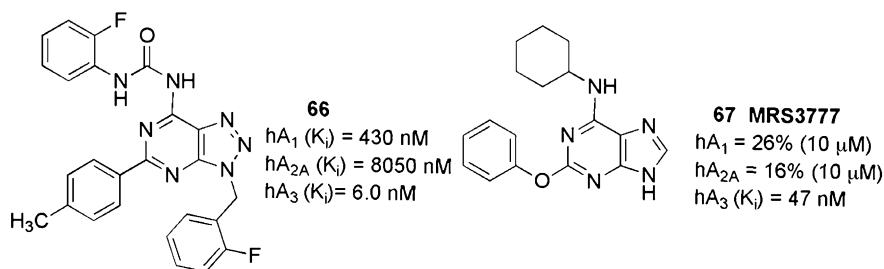


Fig. 10 A₃AR antagonists based on nonnucleoside adenine scaffolds

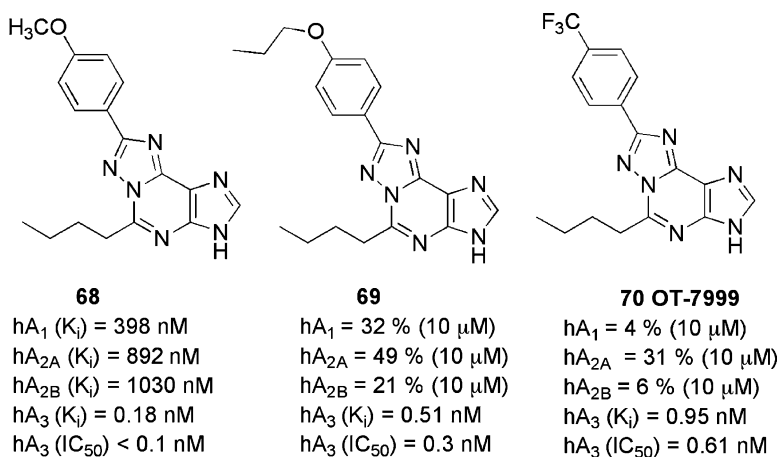


Fig. 11 A₃AR antagonists based on triazolopurine scaffolds

3.2.2 Triazolopurines

Okamura et al. (Okamura et al. 2002, 2004a) recently reported the study of a new series of 1,2,4-triazolo[5,1-*i*]purines. This research group highlighted the structural similarity between the new class of compounds and the triazoloquinazoline derivatives and consequently evaluated the corresponding A₃AR affinities. These investigations led to potent and selective hA₃AR ligands, the most potent of which are reported in Fig. 11 (68,69). In particular, 5-*n*-butyl-8-(4-*n*-propoxyphenyl)-3*H*-[1,2,4]triazolo[5,1-*i*]purine (69) exhibited the best selectivity profile of this series (affinity ratios vs. other AR subtypes > 19,600). Compound (70), 5-*n*-butyl-8-(4-trifluoromethylphenyl)-3*H*-[1,2,4]triazolo-[5,1-*i*]purine (OT-7999), significantly reduced intraocular pressure in cynomolgus monkeys at 2–4 h following topical application (500 mcg) (Okamura et al. 2004b).

3.2.3 Tricyclic Xanthines

Natural antagonists for ARs such as caffeine and theophylline show, in general, low affinity for the A₃AR subtype (Baraldi et al. 2003a; van Galen et al. 1994). In a recent study, the approach based on the ring annelation of xanthine derivatives for the development of AR antagonists was considered in depth (Drabczyńska et al. 2003).

Some pyrido[2,1-*f*]purine-2,4-dione derivatives, which could be considered tricyclic xanthine derivatives, have been reported to exert subnanomolar affinity at the hA₃AR (Priego et al. 2002). The most potent compound of this recent series is the 1-benzyl-3-propyl-1*H*, 3*H*-pyrido[2,1-*f*]purine-2,4-dione derivative (71, Fig. 12), which presents a K_i value of 4.0 ± 0.3 nM at hA₃AR. The replacement of the benzyl nucleus at the 1 position with a methyl moiety caused dramatic losses of

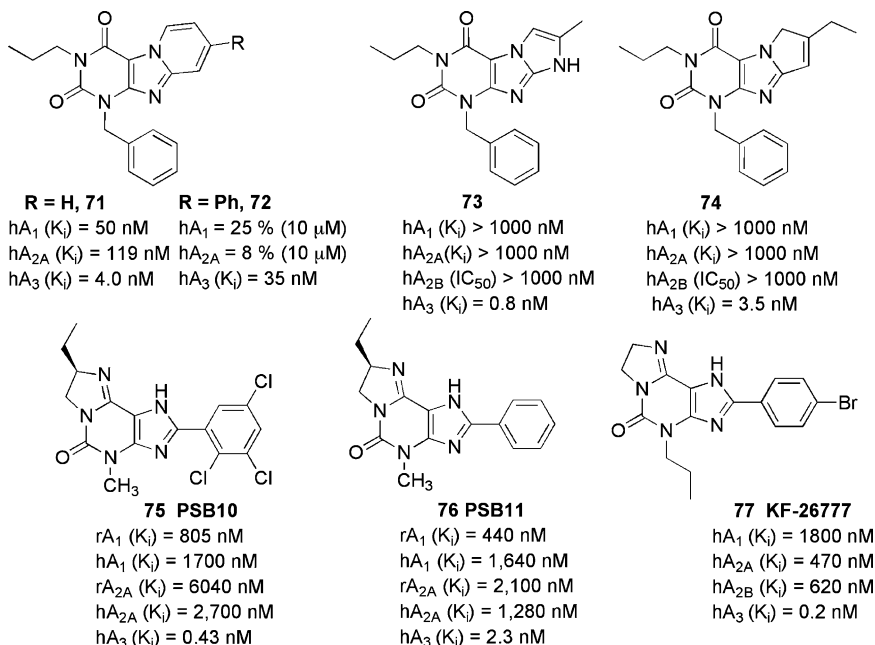


Fig. 12 A_3 AR antagonists based on tricyclic xanthine scaffolds

both affinity and selectivity. The effect of the replacement of the pyrido[2,1-*f*]purine-2,4-dione core with different five-membered heterocycles was examined. In particular, the synthesis and the SAR profile at the ARs of a series of 1-benzyl-3-propyl-7-aryl/alkyl-1*H*,6*H*-pyrrolo[2,1-*f*]purine-2,4-dione and 1-benzyl-3-propyl-7-aryl/alkyl-1*H*,8*H*-imidazo[2,1-*f*]purine-2,4-dione derivatives were recently reported (Baraldi et al. 2005b). Among the examined tricycles, the imidazo[2,1-*f*]purine-2,4-dione derivatives were two- to tenfold more potent than the corresponding pyrrolo[2,1-*f*]purine-2,4-dione derivatives. The best results were obtained with the introduction of small alkyl chains at the 7 position (1-benzyl-7-methyl-3-propyl-1*H*,8*H*-imidazo[2,1-*f*]purine-2,4-dione **73**, 1-benzyl-7-ethyl-3-propyl-1*H*,6*H*-pyrrolo[2,1-*f*]purine-2,4-dione **74**, Fig. 12). Compound **73** shows a subnanomolar affinity towards the target A_3 AR, with noteworthy selectivity with respect to the other AR subtypes (K_i (hA_3) = 0.8 nM, K_i (hA_1/hA_3) = 3, 163, K_i (hA_{2A}/hA_3) > 6,250, IC_{50} (hA_{2B})/ K_i (hA_3) = 2,570).

The synthesis and biological evaluation of a series of fused xanthine derivatives was investigated by Müller and coworkers (Müller et al. 2002a). In particular, the (*R*)-4-methyl-8-ethyl-2-phenyl-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-one (PSB-11 (**76**), Fig. 12) exhibited a K_i value of 2.3 nM for the A_3 AR and good selectivity vs. all other AR subtypes. The radiolabeled derivative of this compound ($[^3\text{H}]$ PSB-11) exhibited a K_d value of 4.9 nM and a B_{max} value of 3,500 fmol mg^{-1} of protein in human A_3 AR binding in transfected CHO cells (Müller et al. 2002b). An important innovation of such a series, in comparison with

xanthines, is a significant increase in water solubility due to the introduction of a basic nitrogen atom, which can be protonated in physiological conditions. Compound PSB-10, bearing a 2,3,5-trichlorophenyl moiety at the 2 position, showed inverse agonist activity in binding studies in CHO cells expressing recombinant hA₃ARs (IC₅₀ = 4 nM) (Ozola et al. 2003). The 2-(4-bromophenyl)-derivative named KF-26777 (**77**) with subnanomolar affinity at the hA₃AR ($K_i = 0.2$ nM) and high selectivity over A₁, A_{2A} and A_{2B} ARs (9,000-, 23,500-, 31,000-fold, respectively) was considered a potential lead molecule for development for the treatment of brain ischemia and inflammatory diseases such as asthma (Saki et al. 2002).

3.3 Nucleoside-Derived A₃AR Antagonists

Based on the observation that the relative efficacy of purine nucleosides depends on structural features (see Sect. 2), new subtype-selective nucleoside antagonists of the A₃AR have been designed. One of the first such antagonists was the rigid spiroactam MRS1292 (**78**) (Fig. 13, (2*R*,3*R*,4*S*,5*S*)-2-[*N*⁶-3-iodobenzyl]adenosine-9'-yl]-7-aza-1-oxa-6-oxospiro[4.4]nonan-4,5-diol) (Gao et al. 2002), which binds potently and selectively to the rat and human A₃ARs but does not activate these receptors, and thus acts as an antagonist.

Modeling/mutagenesis of ARs has focused on distinct residues related to ligand binding and the relative efficacy of adenosine derivatives, and on a conserved Trp residue (6.48) which is involved in the activation process (termed a “rotamer switch,” Shi et al. 2002). Docking studies of agonists suggest that the activation pathway of the A₃AR involves a characteristic anticlockwise rotation of this residue, as viewed from the exofacial side (Kim et al. 2006). The docking of MRS1292 (**78**) to the A₃AR model is not accompanied by rotation of this residue, as occurs with nucleoside agonists, consistent with its action as an antagonist (Kim et al. 2006). Moreover, the affinity and selectivity of MRS1292 occurs across species, unlike most other heterocyclic antagonists for the A₃AR reported. This allows its use in nonprimate (e.g., murine) experimental animals used as clinical models. For example, MRS1292 applied directly to the eye in mouse has been shown to be effective in reducing intraocular pressure, which may be predictive of its utility as an antiglaucoma agent (Yang et al. 2005).

The removal of the ability of the 5'-*N*-alkyluronamide to donate a hydrogen bond was found to convert agonists into selective antagonists (Gao et al. 2006a). In both the 4'-oxo and the 4'-thio series, *N*-methylation of an *N*-methylamide (i.e., to form a dimethylamide) resulted in potent and selective A₃AR antagonists. Recently, nucleosides that are truncated at the 4' position were found to act as A₃AR antagonists. For example, (2*R*,3*R*,4*S*)-2-(2-chloro-6-(3-chlorobenzylamino)-9*H*-purin-9-yl)tetrahydrothiophene-3,4-diol (LJ-1416, **80**) and (2*R*,3*R*,4*S*)-2-(2-chloro-6-(3-iodobenzylamino)-9*H*-purin-9-yl)tetrahydrothiophene-3,4-diol (LJ-1251, **81**) (Fig. 13) (Jeong et al. 2007) displayed K_i values of 1.66 and 4.16 nM, respectively, at the human A₃AR,

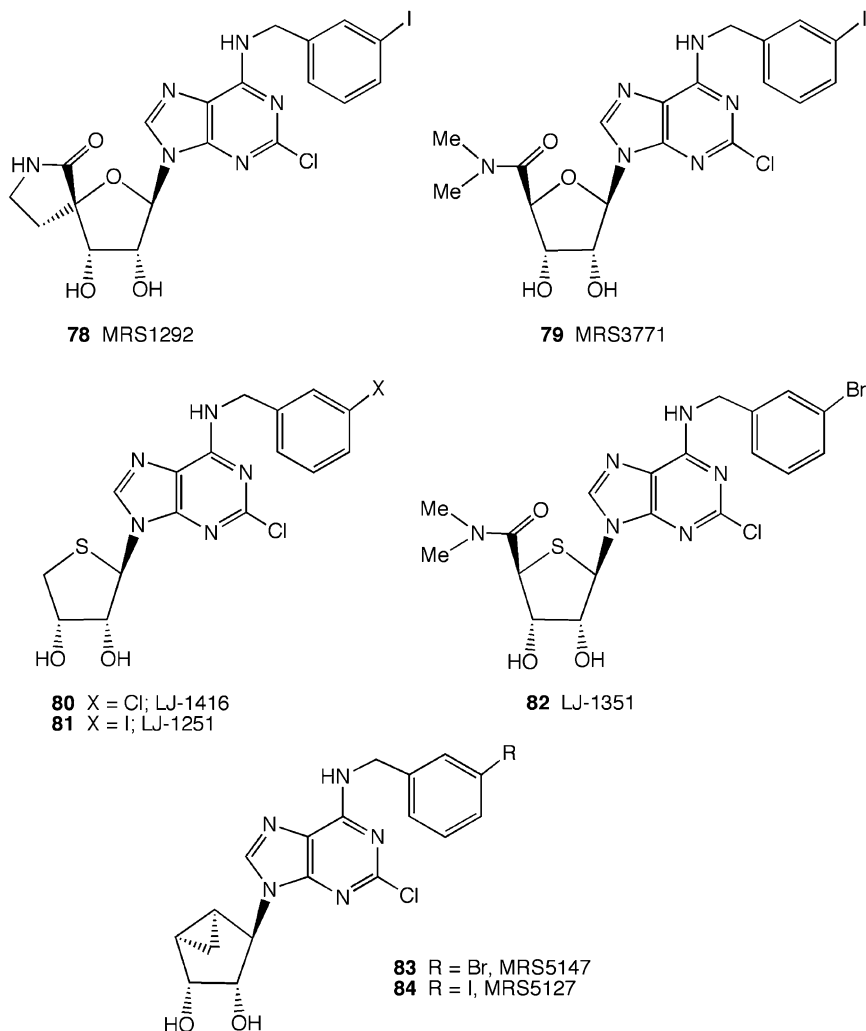


Fig. 13 A₃AR antagonists based on nucleoside scaffolds

with > 600-fold selectivity in comparison to the A₁AR. LJ-1251 was shown to have neuroprotective properties in an ischemia model in the rat hippocampus (Pugliese et al. 2007). Truncation at the 4' position of A₃AR agonist in the (N)-methanocarba series produces potent and selective A₃AR antagonists (Melman et al. 2008b), such as the 3-bromo derivative 1'*R*, 2'*R*, 3'*S*, 4'*R*, 5'*S*)-4'-[2-chloro-6-(3-bromobenzylamino)-purine]-2', 3'-*O*-dihydroxybicyclo-[3.1.0]hexane (MRS5147) (**83**, Fig. 13) (2,900-fold selective for hA₃ vs. hA₁AR) or its 3-iodo analog, MRS5127 (**84**) (2,400-fold selective for hA₃ vs. hA₁AR). MRS5127 (**84**) displayed a *K_B* (Schild constant) value of 8.9 nM as an antagonist of the human A₃AR in a functional assay.

4 Engineering of the A₃AR to Avoid Side Effects of Conventional Synthetic Agonists

Although selective agonists of several of the ARs have been known for years, their use as pharmaceutical agents has been impeded by undesirable side effects of exogenously administered adenosine derivatives. In spite of the clinically useful protective properties of adenosine agonists observed in experimental animals, such as protection against ischemic damage and suppression of excessive inflammation, none of the selective synthetic agonists have yet been approved for human therapeutic use. The A_{2A}AR-selective agonist Lexiscan (regadenoson, CV Therapeutics, Palo Alto, CA, USA) (CVT-3146, 1-{9-[(4*S*, 2*R*, 3*R*, 5*R*)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-6-aminopurin-2-yl}pyrazol-4-yl)-*N*-methylcarboxamide) was recently approved for cardiac imaging in patients. The only other adenosine agonist currently in clinical use is adenosine itself, for the treatment of supraventricular tachycardia and as an aid in cardiac imaging.

Since ARs are widespread in the body, in order to overcome inherent nonselectivity of activating the native ARs using synthetic agonists, we have introduced the concept of neoreceptors, by which the putative ligand binding site of a 7TM receptor is re-engineered for activation by synthetic agonists (neoligands) that are built to have a structural complementarity. This is a molecular modeling approach to receptor engineering by which a mutant receptor (neoreceptor) is designed for selective activation by a novel synthetic ligand (neoligand) at concentrations that do not activate the native receptor. An amino acid residue of the receptor and a functional group of the ligand moiety thought to be in close proximity can be modified in a complementary fashion so that the two groups exhibit a novel mode of interaction (e.g., reversing the polarity in a salt bridge or introducing unique hydrogen-bonding sites). If a stabilizing interaction exists between these two groups, an increase in affinity is expected at the mutant receptor relative to the native receptor. This strategy is intended for eventual use in gene therapy and may also be useful in mechanistic elucidation, using neoreceptor–neoligand pairs that are pharmacologically orthogonal with respect to the native species. Neoreceptors have so far been applied successfully to A_{2A} and A₃ ARs (Gao et al. 2006b; Jacobson et al. 2001, 2005). Compounds **85–87** (Fig. 14) were found to interact selectively with the H272E mutant hA₃AR. All three compounds activated this neoreceptor.

5 Conclusions

A₃AR ligands have been modified to optimize their interaction with the A₃AR. Most of these modifications have been made to the N⁶ and 2 positions of adenine as well as the ribose moiety, and using a combination of these substitutions leads to the most efficacious, selective, and potent ligands. A₃AR agonists such as IB–MECA and Cl–IB–MECA are now advancing into Phase II clinical trials for treatments targeting diseases such as cancer, arthritis, and psoriasis.

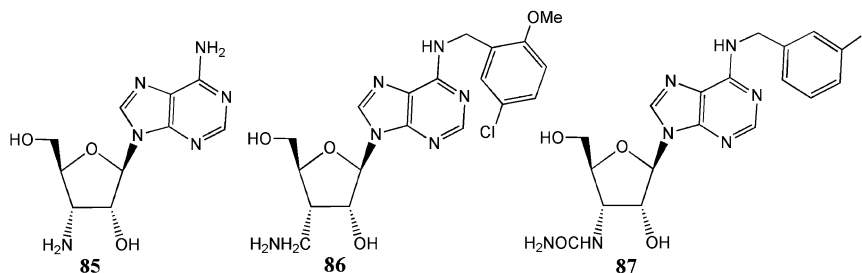


Fig. 14 Compounds that interact selectively with the H272E mutant hA₃AR neoreceptor

Also, a wide number of compounds exerting high potency and selectivity in antagonizing the hA₃AR have been discovered. These molecules are generally characterized by a notable structural diversity, taking into account that aromatic nitrogen-containing monocyclic (thiazoles and thiadiazoles), bicyclic (isoquinoline, quinoxalines, (aza)adenines), tricyclic systems (pyrazoloquinolines, triazoloquinoxalines, pyrazolotriazolopyrimidines, triazolopurines, tricyclic xanthines) and nucleoside derivatives have been identified as potent and selective A₃AR antagonists. Probably due to the “enigmatic” physiological role of A₃AR, whose activation may produce opposite effects (for example, concerning tissue protection in inflammatory and cancer cells) and may produce effects that are species dependent, only a few molecules have reached preclinical investigation. Indeed, the most advanced A₃AR antagonists remain in preclinical biological testing. Among the antagonists described above, compound OT-7999 is expected to enter clinical trials for the treatment of glaucoma, while several thiazole derivatives are in development as anti-allergic, antiasthmatic and/or anti-inflammatory drugs.

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Adenosine Receptors and the Heart: Role in Regulation of Coronary Blood Flow and Cardiac Electrophysiology

S. Jamal Mustafa, R. Ray Morrison, Bunyen Teng, and Amir Pelleg

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Abstract Adenosine is an autacoid that plays a critical role in regulating cardiac function, including heart rate, contractility, and coronary flow. In this chapter, current knowledge of the functions and mechanisms of action of coronary flow regulation and electrophysiology will be discussed. Currently, there are four known

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adenosine receptor (AR) subtypes, namely A₁, A_{2A}, A_{2B}, and A₃. All four subtypes are known to regulate coronary flow. In general, A_{2A}AR is the predominant receptor subtype responsible for coronary blood flow regulation, which dilates coronary arteries in both an endothelial-dependent and -independent manner. The roles of other ARs and their mechanisms of action will also be discussed. The increasing popularity of gene-modified models with targeted deletion or overexpression of a single AR subtype has helped to elucidate the roles of each receptor subtype. Combining pharmacologic tools with targeted gene deletion of individual AR subtypes has proven invaluable for discriminating the vascular effects unique to the activation of each AR subtype.

Adenosine exerts its cardiac electrophysiologic effects mainly through the activation of A₁AR. This receptor mediates direct as well as indirect effects of adenosine (i.e., anti-β-adrenergic effects). In supraventricular tissues (atrial myocytes, sinoatrial node and atrioventricular node), adenosine exerts both direct and indirect effects, while it exerts only indirect effects in the ventricle. Adenosine exerts a negative chronotropic effect by suppressing the automaticity of cardiac pacemakers, and a negative dromotropic effect through inhibition of AV-nodal conduction. These effects of adenosine constitute the rationale for its use as a diagnostic and therapeutic agent. In recent years, efforts have been made to develop A₁R-selective agonists as drug candidates that do not induce vasodilation, which is considered an undesirable effect in the clinical setting.

Keywords A₁ adenosine receptor · A_{2A} adenosine receptor · A_{2B} adenosine receptor · A₃ adenosine receptor · Endothelium · Coronary artery · Smooth muscle · Adenosine receptor knockout · Phospholipase C · MAPK · Adenosine receptor agonist · Adenosine receptor antagonist · Sinus node · AV node · Cardiac electrophysiology · PSVT · Anti-beta adrenergic action

Abbreviations

AC	Adenylate cyclase
AH	Atrial to His bundle activation time (representative of AV-nodal conduction time)
AR	Adenosine receptor
ATP	Adenosine 5'-triphosphate
AV	Atrioventricular
AVN	AV-nodal
CCPA	2-Chloro- <i>N</i> ⁶ -cyclopentyl-adenosine
CF	Coronary flow
CGS-21680	2-[<i>p</i> -(2-carboxyethyl)]-phenylethyl-amino-5'- <i>N</i> -ethylcarboxamidoadenosine
CGS-22492	2-[(2-Cyclohexylethyl)amino]-adenosine
Cox-I	Cyclooxygenase I

CPA	<i>N</i> ⁶ -Cyclopentyladenosine
DAD	Delayed afterdepolarizations
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine
DPMA	<i>N</i> ⁶ -[2-(3,5-Dimethoxyphenyl)-2-(2-methoxyphenyl) ethyl adenosine
ECG	Electrocardiogram
ERK	Extracellular regulated kinase
HV	His bundle to ventricular activation time
HUT	Head-up tilt table test
<i>I</i> _{Ca}	Inward calcium current
<i>I</i> _{CaL}	Inward L-type Ca ²⁺ current
<i>I</i> _{Cl}	Chloride current
<i>I</i> _f	Hyperpolarization-activated current (“funny” current)
<i>I</i> _{KAdo,Ach}	Outward potassium current
<i>I</i> _{K,ATP}	ATP-dependent outward potassium current
<i>I</i> _{Ti}	Transient inward current
JNK	Jun <i>N</i> -terminal kinase
KO	Knockout
L-NMA	<i>N</i> ^G -Methyl-L-arginine
LAD	Left anterior descending artery
LQTS	Long QT interval syndrome
MAPK	Mitogen-activated protein kinase
NECA	Adenosine-5'- <i>N</i> -ethylcarboxamide
NO	Nitric oxide
PDBu	Phorbol 12,13-dibutyrate
PI ₃ -kinase	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
PKA	Protein kinase A
PKB (Akt)	Protein kinase B
PKC	Protein kinase C
PR	P wave to R wave interval on the ECG
PSVT	Paroxysmal supraventricular tachycardia
QT	Q wave–T wave interval in the ECG
QTc	Corrected QT interval
RR	R wave–R wave interval in the ECG
SN	Sinus node
SR	Sarcoplasmic reticulum
SSS	Sick sinus syndrome
SVT	Supraventricular tachycardia
VF	Ventricular fibrillation
VT	Ventricular tachycardia

1 General Background: The Adenosine Hypothesis

The heart is an astounding organ, capable of pumping over 8,000 liters of blood through the efficient operation of $\sim 100,000$ heartbeats per day. To place this in perspective, the total volume of blood ejected by the heart in a single day weighs over nine tons, and over one's lifetime the volume of blood pumped by the heart could fill the Empire State Building! The energy required to perform work of this magnitude is almost exclusively derived from aerobic oxidation of various substrates in the form of adenosine 5'-triphosphate (ATP) (Ingwall 2007; Knaapen et al. 2007). Cycling through up to 30 times its own weight in ATP per day (Ingwall and Weiss 2004; Neubauer 2007), the heart consumes more oxygen than any other organ (Taegtmeier et al. 2005). Yet, because the myocardial ATP content is relatively small ($4\text{--}6\ \mu\text{mol g}^{-1}$) compared to the rapid basal rate of ATP expenditure ($30\ \mu\text{mol g}^{-1}$ per minute), it is absolutely crucial that ATP production, and therefore oxygen supply, is closely matched across a broad range of cardiac work loads (Deussen et al. 2006). This inextricable link between myocardial function and metabolic demand is the basis of the "adenosine hypothesis" for the metabolic regulation of coronary flow (Berne 1963; Gerlach and Deuticke 1966).

Using an anesthetized open-chest working dog heart model, Berne demonstrated that myocardial hypoxia results in coronary venous efflux of adenine nucleotides, and that adenosine induces coronary dilation (Berne 1963). Together, these findings led to the following hypothesis:

Reduction in myocardial oxygen tension by hypoxemia, decreased coronary blood flow, or increased oxygen utilization by the myocardial cell leads to the breakdown of adenine nucleotides to adenosine. The adenosine diffuses out of the cell and reaches the coronary arterioles via the interstitial fluid and produces arteriolar dilation. The resultant increase in coronary blood flow elevates myocardial oxygen tension, thereby reducing the rate of degradation of adenine nucleotides, and decreases the interstitial fluid concentration of adenosine by washout and enzymatic destruction. This feedback mechanism serves to adjust coronary blood flow to meet the new metabolic requirements and a new steady state is achieved. (Berne 1963)

Soon afterward, it was demonstrated that adenosine levels increase almost three-fold within as little as 5 s of myocardial ischemia in vivo (Olsson 1970), and the incremental increase in coronary flow correlates highly with this rapid release of endogenous adenosine (Rubio et al. 1974). In the 45 years since the adenosine hypothesis was proposed, extensive investigation has established that adenosine serves as both a "sensor" of imbalances in energetic supply and demand and as a local metabolic regulator of coronary flow (Berne 1980; Deussen et al. 2006; Hori and Kitakaze 1991; Morrison et al. 2007; Tune et al. 2004). Although other effectors confer built-in redundancy for control of the coronary circulation (nitric oxide, ATP-sensitive potassium channels, acidosis, carbon dioxide, pO₂, etc.; Deussen et al. 2006), it is clear that under conditions of impaired oxygen supply-to-demand ratio, rapid local production of adenosine leads to marked coronary dilation.

2 Adenosine and Coronary Regulation

Adenosine is an autacoid that plays a critical role in regulating coronary circulation. Adenosine is produced by the action of ecto-5'-nucleotidase on extracellular ATP released from the parenchymal tissue (including endothelium). Extracellular adenosine interacts with specific cell-surface receptors located on the smooth muscle and endothelial cells of the coronary artery to produce relaxation. Currently, there are four known adenosine receptor (AR) subtypes, namely A₁, A_{2A}, A_{2B}, and A₃. Although all four AR subtypes are found in coronary smooth muscle cells, only A_{2A}AR and A_{2B}AR have been shown to be present on coronary endothelial cells (Olanrewaju et al. 2002, 2000). Recently, A₃AR has been localized on endothelial cells in mouse aorta, leading to contraction of smooth muscle through cyclooxygenase I (Cox-I) (Ansari et al. 2007).

To date, pharmacological interventions using adenosine or its analogs are mostly directed toward adenosine-mediated effects on the cardiovascular system, such as the treatment of supraventricular arrhythmia, pharmacological stress myocardial perfusion imaging, congestive heart failure, controlling blood pressure, attenuating reperfusion injury following regional myocardial infarction, reducing infarct size, reducing incidence of arrhythmias, and improving postischemic cardiac function (Geraets and Kienzle 1992; Neubauer 2007; Peart and Headrick 2007; Smits et al. 1998). In the coronary circulation, A_{2A}AR plays a pivotal role in controlling vasodilation, while other receptors play a lesser role (Frobert et al. 2006; Hodgson et al. 2007; Morrison et al. 2002; Talukder et al. 2003). For instance, A_{2B}AR also mediates coronary vasodilation, while both A₁AR and A₃AR have been found to negatively modulate coronary vasodilation induced by A_{2A}AR and/or A_{2B}AR activation (Morrison et al. 2002; Talukder et al. 2002a; Tawfik et al. 2006). However, the significance of A₁, A_{2B}, and A₃ARs in coronary flow regulation remains to be fully elucidated.

The distribution of ARs along the branches of coronary arteries also varies. In the porcine heart, expression of A₁ and A_{2A}AR proteins has been documented in the left anterior descending artery (LAD), but only A_{2A}ARs are expressed coronary arterioles (Hein et al. 2001). Another study found that A₁, A_{2A}, and A_{2B}ARs are also expressed in coronary arterioles and venules (Wang et al. 2005). Functional studies in A_{2A}AR knockout (KO) mice suggested that A_{2B}AR may be more important in regulating larger coronary arteries (e.g., the LAD) than previously thought (Teng et al. 2008).

3 Endothelium-Dependent and Endothelium-Independent Regulation

It has been suggested that both A_{2A}AR and A_{2B}AR mediate hyperpolarization of smooth muscle and nitric oxide (NO) release from coronary artery endothelium (Hasan et al. 2000; Olanrewaju et al. 2002; Watts et al. 1998). Cell culture

studies have demonstrated the involvement of $A_{2A}AR$ - and $A_{2B}AR$ -mediated NO release in porcine and human coronary endothelial cells (Li et al. 1998; Olanrewaju and Mustafa 2000). However, very few functional studies demonstrated that NO release is responsible for $A_{2A}AR$ - or $A_{2B}AR$ -mediated coronary vasodilation. Inhibition of NO synthase has been found to limit basal coronary flow (CF) in various species (Flood et al. 2002; Zatta and Headrick 2005). It has been shown in porcine coronary arterial rings that N^G -methyl-L-arginine (L-NMA, 30 μ M), an NO synthase inhibitor, attenuated the relaxations of endothelium-intact but not endothelium-denuded rings induced by adenosine-5'- N -ethylcarboxamide (NECA), a nonselective adenosine agonist, and 2-[p -(2-carboxyethyl)]phenylethylamino-5'- N -ethylcarboxamidoadenosine (CGS-21680), a selective $A_{2A}AR$ agonist (Abebe et al. 1995). It has been speculated that endogenously released adenosine and prostanoids induce NO- and/or K_{ATP} channel-dependent vasodilation and thereby modulate basal coronary tone (Flood et al. 2002; Hein et al. 2001; Talukder et al. 2002b; Zatta and Headrick 2005). Using two different NO synthase inhibitors in isolated hearts from wild-type and $A_{2A}AR$ KO mice, it was found that $A_{2A}AR$ plays a significant role in background NO release, thus affecting basal coronary tone (Teng et al. 2008). The role of $A_{2B}AR$ in NO release remains to be determined, however.

The ARs responsible for endothelial-independent relaxation of coronary artery smooth muscle have not been conclusively determined; however, both $A_{2A}AR$ and $A_{2B}AR$ have been implicated (Morrison et al. 2002; Talukder et al. 2003, 2002b). A study with denuded porcine coronary arteries clearly demonstrated that $A_{2A}AR$ plays a predominant role in endothelial-independent vasodilation, while $A_{2B}AR$ may play a minor role (Teng et al. 2005).

4 Baseline Coronary Flow Control

It has been shown that both $A_{2A}AR$ and $A_{2B}AR$ mediate endogenous and exogenous adenosine-induced dilation of mouse coronary arteries (Morrison et al. 2002; Talukder et al. 2003). $A_{2A}AR$ activation also contributes significantly to basal NO release and basal tone in coronary circulation (Flood et al. 2002; Teng et al. 2008; Zatta and Headrick 2005).

The cardiovascular effects of $A_{2B}AR$ activation are similar to those mediated by $A_{2A}AR$; however, the affinity of adenosine to the latter is lower (Feoktistov and Biaggioni 1997; Hack and Christie 2003; Schulte and Fredholm 2003). The role of $A_{2A}AR$ in basal vascular tone remains to be determined. However, it has been speculated that under pathological conditions such as ischemia, $A_{2B}AR$ may be upregulated to compensate for the downregulation of $A_{2A}AR$ -mediated responses. Indeed, an upregulation of $A_{2B}AR$ gene expression has been found in ischemic mouse hearts (Ashton et al. 2003; Morrison et al. 2007). A more recent study has also demonstrated upregulation of $A_{2B}AR$ in coronary arteries of $A_{2A}AR$ gene KO mice, suggesting that $A_{2B}AR$ provides a supportive role to the predominantly A_{2A} -mediated control of the coronary circulation (Teng et al. 2008).

5 Second-Messenger Systems

It has been well recognized that A_1 and A_3 ARs are coupled to $G_i/G_o/G_q$ proteins and inhibit the activity of adenylate cyclase (AC), while A_{2A} and A_{2B} ARs are coupled to G_s and activate AC, leading to cyclic adenosine 5'-monophosphate (cAMP) accumulation and subsequent activation of protein kinase A (PKA) (Fredholm et al. 2000). Indeed, in coronary arteries, where A_{2A} AR is predominant, A_{2A} AR-induced vasodilation is mediated mainly by the cAMP-dependent pathway (Hussain and Mustafa 1993; Rezik and Mustafa 2003). However, other second-messenger systems, such as phosphatidylinositol 3-kinase, tyrosine kinase and phospholipase C (PLC), may also be activated by ARs (Ansari et al. 2008; Peart and Headrick 2007; Tawfik et al. 2005), but their roles in mediating the effects of adenosine on the coronary vasculature have not been clearly defined. In addition, crosstalk between the cAMP/PKA pathway and the PLC/PKC pathway has also been reported (Germack and Dickenson 2004). Currently, the tangled web of these two second-messenger systems has garnered the most attention in studies of AR mechanism of action in cardiovascular tissue.

5.1 cAMP-MAPK

Following the activation of G_s protein by A_{2A} AR and A_{2B} AR, various second messenger signaling pathways including mitogen-activated protein kinases (MAPK) are initiated. The signal transduction pathway from G-protein-coupled receptors to MAPK is not fully understood, and may vary in different cell types (Fredholm et al. 2000). There are three well-characterized MAPKs: extracellular regulated kinase (ERK), or p42/44, p38, and jun *N*-terminal kinase (JNK). They seem to play a role in ischemic preconditioning, postconditioning (Morrison et al. 2007), smooth muscle cell growth, vascular smooth muscle migration, and vascular contraction (Haq et al. 1998; Kalyankrishna and Malik 2003; Wilden et al. 1998). Adenosine is reported to stimulate all MAPKs in the perfused rat heart (Haq et al. 1998). Agonist binding to A_{2A} AR can result in both activation and inhibition of ERK phosphorylation, depending on the type of cell expressing these receptors, and so can the second messenger pathway controlled by A_{2A} AR (Fredholm et al. 2000). A_{2B} AR is the only subtype capable of activating all three types of MAPKs (ERK1/2, p38, and JNK). It has also been shown that the same concentration of NECA and adenosine induces ERK1/2 phosphorylation to a greater extent than cAMP production (Fredholm et al. 2000). The involvement of p38 MAPK in adenosine-induced vasodilation has been recently reported (Teng et al. 2005); however, the role of MAPKs in the regulation of vascular tone requires more complete characterization.

There are a few reports linking ARs to p38 MAPK that provide clues as to which mediators are involved in the activation of p38 MAPK. A recent report demonstrated that cAMP inhibits p38 MAPK activation in endothelial cells derived from human umbilical vein (Rahman et al. 2004). In contrast, PKA was found to activate p38

MAPK in macrophages (Chio et al. 2004). Furthermore, the signaling pathways both up- and downstream of the p38 MAPK pathway are diverse, which may explain why p38 can be activated and create crosstalk among various stimuli (Eckle et al. 2007; Ono and Han 2000). For instance, it has been reported that p38 MAPK plays a significant role in angiotensin II-induced contraction (Meloche et al. 2000; Watts et al. 1998), while others have found that p38 MAPK is involved in adenosine-induced vasodilation (Teng et al. 2005). It is also possible that different p38 MAPK subtypes (p38 α , p38 β , and p38 γ) are responsible for signaling via different pathways. Further investigation is needed to clarify the relationship between ARs and MAPKs vis-à-vis coronary regulation.

5.2 PLC–PKC

By virtue of differential coupling to either G_s (A_{2A} and A_{2B}ARs) or G_i proteins (A₁AR and A₃AR), along with variable tissue distribution of AR subtypes, adenosine elicits both relaxation (A_{2A}- and A_{2B}-mediated) and constriction (A₁- and A₃-mediated) in the peripheral and coronary vasculature. While this is discussed in further detail below with regard to coronary regulation, recent evidence supports a role for the phospholipase C (PLC)–protein kinase C (PKC) system in A₁AR-mediated contraction of aortic vascular smooth muscle (Tawfik et al. 2005). Specifically, isolated aortic rings from wild-type and A₁AR-KO mice were treated with adenosine, NECA, a nonselective AR agonist or 2-chloro-N⁶-cyclopentyl-adenosine (CCPA), an A₁AR selective agonist, demonstrating uniform contractile responses in the 100 nM to 1 μ M range in wild-type aortas only. Adenosine-induced vasoconstriction was not observed in aortas from A₁AR knockout mice with either nonselective (adenosine, NECA) or A₁-selective (CCPA) agonists, and the contractile response in wild-type aortas was eliminated by an A₁AR-selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). CCPA-mediated contraction in wild-type aortic rings was also eliminated by the PLC inhibitor U-73122, indicating a role for the PLC–PKC pathway in adenosine-mediated vasoconstriction (Tawfik et al. 2005). Other studies have shown that A₁AR enhances PKC expression in porcine coronary arteries (Marala and Mustafa 1995a, b, c). Moreover, a PKC inhibitor, GO-6893, was able to inhibit ENBA-induced contraction in mouse aorta (Ansari et al. 2008). Taken together, these findings suggest that the PLC–PKC pathway has a major role in A₁AR-mediated vascular tone (i.e., contraction of coronary arteries and the aorta).

5.3 Other Second Messengers

Phosphatidylinositol 3-kinase (PI₃ kinase) activates protein kinase B (PKB, also known as Akt), which phosphorylates and activates a cyclic nucleotide

phosphodiesterase, 3B. Increases in cyclic nucleotide concentrations inhibit agonist-induced contraction of vascular smooth muscle. A PI₃-kinase inhibitor, LY 294002, has been shown to inhibit KCl, phorbol 12,13-dibutyrate (PDBu), and serotonin-induced contraction in bovine carotid artery smooth muscle strips, suggesting that the PI₃-kinase pathway plays a role in vascular smooth muscle tone (Komalavilas et al. 2001). A recent study provided the first evidence that A_{2B}AR-mediated cAMP formation activates ERK1/2 via a pathway dependent on PI₃ kinase, tyrosine kinases and Rap1 in CHO cells (Schulte and Fredholm 2002).

Tyrosine kinase and PKC are also found to trigger the MAPK system (Fredholm et al. 2000; Lowes et al. 2002; Robinson and Dickenson 2001; Yang et al. 2000; Zhao et al. 2001). Studies in A₁AR-induced delayed preconditioning in rabbits have suggested an important role for tyrosine kinase and PKC. These studies also speculate that the p38 MAPK/Hsp27 pathway may be a distal effector of this protection (Dana et al. 2000).

5.4 K⁺ Channels

All four major types of K⁺ channels (K_{ATP}, K_v, K_{IR} and K_{Ca}) are present in both coronary endothelial and smooth muscle cells (Frieden et al. 1999; Glavind-Kristensen et al. 2004; Kim et al. 2003; Li et al. 1999; Liu et al. 2001; Quayle et al. 1997; Rogers et al. 2007; Sun Park et al. 2006). However, the involvement of K⁺ channels in adenosine-induced responses remains unclear. Activation of A_{2B}AR activates K_{ATP} channels in human and guinea pig coronary arteries independent of NO (Kemp and Cocks 1999; Mutafova-Yambolieva and Keef 1997; Niiya et al. 1994). K_{ATP} channels have been shown to mediate A_{2A}AR-induced vasodilation in systemic artery circulation and afferent arterioles of rat kidney and porcine coronary arterioles (Bryan and Marshall 1999; Hein et al. 2001; Tang et al. 1999). A study of cultured porcine coronary endothelial cells demonstrated the involvement of both K_{ATP} and K_{Ca} in A_{2A} and A_{2B}AR-mediated hyperpolarization (Olanrewaju et al. 2002), which also leads to NO release (Olanrewaju and Mustafa 2000). Another study also suggested that activation of A₁AR on endothelial cells leads to K_{ATP} channel opening and subsequent Ca²⁺ influx, and an increase in [Ca²⁺]_i, which may lead to direct activation of eNOS via the Ca²⁺-calmodulin pathway and NO release (Ray and Marshall 2006). In guinea pig coronary artery smooth muscle cells, the A_{2A}AR selective agonists CGS-21680 and N⁶-[2-(3,5-dimethoxyphenyl)-2-(2-methoxyphenyl) ethyl] adenosine (DPMA) failed to induce hyperpolarization, while the nonselective agonist NECA induced glibenclamide-sensitive hyperpolarization, suggesting that A_{2B}AR may be the only AR subtype involved in K_{ATP}-induced hyperpolarization in coronary smooth muscle (Mutafova-Yambolieva and Keef 1997). Further studies using patch clamp techniques will be valuable in clarifying the role of K⁺ channels in adenosine-mediated vasoregulation.

6 Insight from Adenosine Receptor Gene-Modified Models

Most of what is known about adenosine-mediated coronary regulation is derived from pharmacologic studies using a broad spectrum of experimental models (Abebe et al. 1994; Belardinelli et al. 1998; Berne 1980; Deussen et al. 2006; Flood et al. 2002; Hasan et al. 2000; Hori and Kitakaze 1991; Makujina et al. 1992; Mustafa and Abebe 1996). Applying an ever-expanding collection of highly selective AR analogs (both agonists and antagonists) in such a variety of models has confirmed that the A_{2A} AR is the predominant subtype mediating adenosine-induced coronary vasodilation (Belardinelli et al. 1998; Shryock et al. 1998). However, the pharmacologic approach is limited by the selectivity of the ligands and/or potency, and frequently results in only indirect evidence that activation of other AR subtypes modifies adenosine-mediated coronary responses (Kemp and Cocks 1999; Makujina et al. 1992; Talukder et al. 2002b). The advantage of gene-modified models with targeted deletion or overexpression of a single AR subtype has allowed a more complete evaluation of adenosine-mediated responses than previously possible through agonist/antagonist studies alone. The cardiovascular phenotypes of several AR KO/overexpression models are reviewed by Ashton et al. (2007); Table 1 summarizes available data on the vascular phenotypes of A_1 , A_{2A} , A_{2B} , and A_3 AR KO models.

6.1 A_{2A} AR KO Mouse

The A_{2A} AR KO mouse model was developed and characterized by Ledent et al. 1997. Although the model was developed primarily as a tool for Parkinson's disease research, A_{2A} knockout mice were noted to be hypertensive, suggesting a direct vascular/cardiovascular effect of this targeted deletion. Subsequent studies combined the specificity of A_{2A} AR deletion with the traditional pharmacologic approach to demonstrate that although basal coronary flow was unchanged by A_{2A} AR deletion (Morrison et al. 2002, 2007; Talukder et al. 2003), adenosine-induced coronary dilation was significantly impaired in isolated hearts (Morrison et al. 2002; Talukder et al. 2003) and in isolated coronary arteries (Teng et al. 2008). During recovery from global ischemia, isolated A_{2A} AR KO hearts also demonstrated reduced coronary flow compared to wild-type littermate controls (Morrison et al. 2007). Together, these studies indicate that A_{2A} AR plays a primary role in murine coronary regulation. Importantly, the observation that a nonselective adenosine analog, NECA, induced coronary dilation in hearts lacking A_{2A} ARs documented for the first time that other AR subtypes modulate adenosine-induced coronary regulation (Morrison et al. 2002). This NECA-induced coronary dilation in A_{2A} knockout hearts was attenuated by alloxazine, a putatively selective A_{2B} AR antagonist, indicating that A_{2B} ARs act in concert with A_{2A} ARs to elicit murine coronary dilation (Morrison et al. 2002; Talukder et al. 2003).

Table 1 Vascular phenotype of AR KO mice

Gene deletion	Experimental model (refs.)	Vascular phenotype (refs.)
A ₁ AR KO	<ul style="list-style-type: none"> Isolated aortic rings (Tawfik et al. 2005) Isolated hearts (Morrison et al. 2006; Reichelt et al. 2005; Salloum et al. 2007; Tawfik et al. 2006) Postischemic isolated hearts (Morrison et al. 2006; Salloum et al. 2007) 	<ul style="list-style-type: none"> Enhanced basal coronary flow (Morrison et al. 2006; Tawfik et al. 2006) Unchanged basal coronary flow (Reichelt et al. 2005; Salloum et al. 2007) Enhanced adenosinergic dilation (Tawfik et al. 2005; Tawfik et al. 2006) Unchanged adenosinergic dilation (Reichelt et al. 2005) Reduced postischemic coronary flow (Morrison et al. 2006) Unchanged postischemic coronary flow (Salloum et al. 2007)
A _{2A} AR KO	<ul style="list-style-type: none"> In vivo tail cuff pressure (Ledent et al. 1997) Isolated hearts (Morrison et al. 2002, 2007; Talukder et al. 2003) Isolated coronary arteries (Teng et al. 2008) 	<ul style="list-style-type: none"> Hypertension (Ledent et al. 1997) Unchanged basal coronary flow (Morrison et al. 2002, 2007; Talukder et al. 2003) Impaired adenosinergic coronary dilation (Morrison et al. 2002, 2007; Talukder et al. 2003; Teng et al. 2008) Reduced postischemic coronary flow (Morrison et al. 2007)
A _{2B} AR KO	<ul style="list-style-type: none"> In vivo tail-cuff pressure (Yang et al. 2006; Hua et al. 2007) Isolated hearts (unpublished observations from Mustafa's group) 	<ul style="list-style-type: none"> Normal basal blood pressure (Yang et al. 2006; Hua et al. 2007) Unchanged basal coronary flow (unpublished observations from Mustafa's group) Unchanged postischemic coronary flow (unpublished observations from Morrison's group)
A ₃ AR KO	<ul style="list-style-type: none"> In vivo tail-cuff pressure (Ge et al. 2006; Zhao et al. 2000) Isolated hearts (Cerniway et al. 2001; Ge et al. 2006; Harrison et al. 2002; Talukder et al. 2002a) Postischemic isolated hearts (Cerniway et al. 2001; Harrison et al. 2002) Isolated aortic rings (Ansari et al. 2007) 	<ul style="list-style-type: none"> Normal basal blood pressure (Ge et al. 2006; Zhao et al. 2000) Enhanced adenosinergic hypotension (Zhao et al. 2000) Enhanced adenosinergic coronary dilation (Talukder et al. 2002a) Unchanged basal coronary flow (Cerniway et al. 2001; Ge et al. 2006; Harrison et al. 2002; Talukder et al. 2002a) Unchanged postischemic coronary flow (Cerniway et al. 2001; Harrison et al. 2002) Reduced A₃-mediated vasoconstriction (Ansari et al. 2007)

6.2 *A_{2B}AR KO Mouse*

Limited data exist regarding the vascular phenotype of *A_{2B} KO* mice, as this is the latest of the AR KO models to be developed. Most recent reports on studies with *A_{2B} KO* mice demonstrate a critical role for the *A_{2B}AR* in protecting against excessive vascular adhesion and injury (Yang et al. 2008, 2006), hypoxia-induced vascular leak (Eckle et al. 2008), and infarct size associated with regional ischemia-reperfusion (Eckle et al. 2007). In two distinct *A_{2B}AR KO* *in vivo* models, tail-cuff measurements showed no differences in resting blood pressure (Hua et al. 2007; Yang et al. 2006). The effects of *A_{2B}AR* deletion on coronary flow are not yet reported. However, using the *A_{2B} KO* model recently characterized by Hua et al. (2007), preliminary data have indicated that targeted deletion of *A_{2B}ARs* has no effect on either basal or postischemic coronary flow compared to wild-type hearts (Morrison et al., unpublished observations). While prior studies have shown that *A_{2B}ARs* plays a role in adenosine-induced coronary dilation (Morrison et al. 2002), it is not clear whether the absence of *A_{2B}ARs* would result in the attenuation of this effect.

6.3 *A₁AR and A₃AR KO Mouse*

Based on data obtained in earlier pharmacologic studies, along with more recent data from studies using *A_{2A}* and *A_{2B}AR* knockout mice, it can be concluded that adenosine-induced coronary dilation is predominantly mediated by *A_{2A}AR* and to a smaller extent by *A_{2B}AR*. As noted above, the vasodilatory effect of both *A_{2A}* and *A_{2B}AR* activation is largely due to their coupling to *G_s* proteins and the resultant activation of AC and production of cAMP (Hussain and Mustafa 1993; Rezik and Mustafa 2003). Since both *A₁AR* and *A₃AR* are *G_i* coupled, and their activation attenuates cAMP production by AC inhibition, it is not unreasonable to expect that *A₁AR* and/or *A₃AR* activation would negatively modulate *A_{2A}AR*- and *A_{2B}AR*-mediated vasodilation. Early evidence of *A₁AR*-mediated coronary vasoconstriction is derived from pharmacologic studies (Hussain and Mustafa 1995), but more recently this phenomenon has been confirmed in both coronary (Tawfik et al. 2006) and aortic (Tawfik et al. 2005) vasculature using *A₁AR KO* mouse. Similarly, early attempts to clarify the relative roles of each AR subtype in aortic and coronary vasoregulation using pharmacologic agents (Talukder et al. 2002b) have been followed by direct and convincing evidence from *A₃AR KO* mice demonstrating that *A₃* activation causes vasoconstriction in both the coronary (Talukder et al. 2003) and aortic vasculatures (Ansari et al. 2007). Thus, while activation of *A_{2A}* and *A_{2B}ARs* leads to coronary dilation, *A₁* and *A₃AR* activation negatively modulates this effect through vasoconstriction.

Using functional studies with even more selective and potent pharmacologic ligands, targeted gene deletion of individual AR subtypes has proven invaluable for discriminating the vascular effects unique to the activation of each AR subtype.

Coming full circle, it seems fitting that information gained from these models is now being harnessed to improve our pharmacologic approach to both diagnostic and therapeutic interventions in the clinical management of heart disease.

7 Clinical Application of Selective A_{2A}AR Agonists for the Detection of Coronary Artery Disease

Adenosine (Adenoscan) has been used as a pharmacological stress agent in conjunction with radionuclide myocardial perfusion imaging (MPI) in patients unable to undergo adequate exercise stress. Dipyridamole, an adenosine uptake blocker, was also used for this purpose for several years prior to the approval of Adenoscan by the US Food and Drug Administration (Cerqueira 2006). Due to frequent side effects (e.g., bronchospasm, AV nodal conduction block) of these two agents, there was a need for better selective drugs for myocardial stress testing. In the late 1980s to early 1990s (Abebe et al. 1994; Mustafa and Askar 1985), it was discovered that adenosine-induced vasodilation of coronary arteries of several species, including humans, was mediated predominately by A_{2A}AR (Ramagopal et al. 1988; Shryock et al. 1998). This discovery led to the development of more selective agonists for the A_{2A}AR subtype, including the Ciba–Geigy (Novartis) compound CGS-21680 (Francis et al. 1991; Hutchison et al. 1989). CGS-21680 was later discovered to be a very selective agonist for the A_{2A}AR subtype in a number of species, including humans (Abebe et al. 1994; Makujina et al. 1992). It was shown that CGS-21680 and another A_{2A}-selective compound from Ciba–Geigy (2-[(2-cyclohexylethyl)amino]-adenosine, CGS-22492) produced significant relaxation in isolated human coronary arteries from organ donors (Makujina et al. 1992). However, it was also discovered that the nonselective analog NECA produced greater relaxation than the A_{2A}-selective CGS-21680 and CGS-22492, suggesting that there was another AR subtype (possibly A_{2B}AR) causing this additional relaxation (Makujina et al. 1992).

Since ARs were not cloned at the time of the study described above, and little was known about A_{2B}ARs, the possibility was left open that another AR subtype contributes to relaxation of human coronary arteries. It was not until the availability of A_{2A}AR KO mouse that an unequivocal demonstration of the role that the A_{2B}AR plays in the regulation of coronary flow became possible (Morrison et al. 2002). Using A_{2A}AR KO mice, Mustafa and his coworkers showed that NECA increased coronary flow, whereas CGS-21680 did not have an effect (Morrison et al. 2002). Moreover, it has recently been reported that there is a compensatory upregulation of the A_{2B}AR receptor in A_{2A}AR KO mice (Teng et al. 2008), further lending support to the theory that most likely the A_{2B}AR is responsible for NECA-induced vasorelaxation of coronary arteries in the A_{2A}AR KO mouse model and perhaps in human coronary arteries. Validation of the role of A_{2B}ARs in functional responses in coronary arteries will be determined in A_{2B}AR KO mouse hearts, as the A_{2B}AR KO mouse model has just recently become available. This is an area of active investigation in Mustafa's group.

These data strongly suggest that, in addition to the A_{2A} AR, the A_{2B} AR also plays a role in the regulation of coronary flow in humans and animals. Therefore, complete dilation of the coronary vascular bed to determine coronary reserve in patients with suspected coronary artery disease may require the use of a combination of A_{2A} AR and A_{2B} AR agonists. These observations are supported by a recent report (Nitenberg et al. 2007) showing that intracoronary infusion of adenosine (60 μ g) elicits a lower hyperemic response than postocclusion hyperemia (30 s). These authors concluded that the use of an adenosine infusion represents a potential source of error in determination of coronary reserve, and may result in an underestimation of the physiological significance of coronary stenosis. It is true that adenosine, being the natural endogenous nonselective AR agonist, will activate all four AR subtypes, including A_{2A} and A_{2B} . However, adenosine is a less potent and nonselective agonist that will also activate A_1 and A_3 ARs, causing a reduction in coronary flow (Talukder et al. 2002a; Tawfik et al. 2006), which serves to counter the increase in flow due to A_{2A} and A_{2B} AR activation.

If A_{2B} ARs play an important role in human coronary vasodilation, as suggested by earlier studies (Makujina et al. 1992), then re-evaluating the sole use of A_{2A} AR-selective agonists in myocardial perfusion stress testing may be warranted. It is possible that adjunctive use of selective A_{2B} AR agonists in concert with currently employed highly selective A_{2A} agonists may lead to a more complete evaluation of both coronary artery disease and coronary reserve. This becomes important in light of the fact that two A_{2A} -selective AR agonists are in Phase III clinical trials (binodenoson, MRE-0470/WRC-0470, Aderis Pharmaceuticals; apadenoson, ATL-146e, Adenosine Therapeutics) and another, LexiscanTM (regadenoson, CVT-3146, CV Therapeutics), has recently received FDA approval for use in pharmacological stress myocardial perfusion imaging. Newly available and highly selective A_{2B} AR analogs are beginning to advance our understanding of the role of A_{2B} ARs in the heart, and it is plausible to envision their adjunctive use for coronary dilation in this clinical setting.

8 Cardiac Electrophysiology of Adenosine: Recent Developments

8.1 Introduction

This section focuses on several aspects of the cardiac electrophysiology of adenosine and gives an update on clinical applications of second-generation AR ligands. For a broader discussion of the cardiac electrophysiology of adenosine, the reader is referred to several previously published reviews (Belardinelli et al. 1995; Dhalla et al. 2003; Pelleg and Belardinelli 1993; Pelleg et al. 2002; Shen and Kurachi 1995; Zablocki et al. 2004).

Adenosine is a ubiquitous adenine nucleoside found in every cell of the human body; it is released into the extracellular space under physiologic and pathophysiologic conditions. The actions of extracellular adenosine are mediated by four subtypes of AR coupled to G proteins: A_1 , A_{2A} , A_{2B} and A_3 . In the heart, the electrophysiologic effects of adenosine are mediated mainly by A_1 AR. The latter receptor mediates the direct effects as well as the indirect effects; i.e., the anti- β -adrenergic effects of adenosine (Dobson et al. 1987; Schrader et al. 1977).

8.2 Basic Aspects

8.2.1 Negative Chronotropic Action

Adenosine suppresses the activity of cardiac pacemakers including the sinus node (SN), atrio-ventricular (AV) junction, and His-Purkinje system; an inverse relationship between pacemaker hierarchy and sensitivity to adenosine was observed. Specifically, the following sensitivity cascade has been observed: Purkinje fibers > His bundle > AV junction > SN (for references, see Pelleg et al. 1990a). This action is mediated by A_1 AR and the activation of a potassium outward current ($I_{KAdo,Ach}$), as well as the suppression of inward calcium current (I_{Ca}) and the hyperpolarization-activated current (“funny” current) (I_f) (Belardinelli et al. 1988; Zaza et al. 1996). Since norepinephrine shifts the activation curve of I_f to the right (DiFrancesco and Borer 2007) and enhances I_{Ca} , the antiadrenergic action of adenosine can also play an important role in its negative chronotropic effects. Data obtained in vitro were interpreted to suggest that the suppression of I_f is more relevant than the activation of $I_{KAdoACh}$ to the modulation of SN automaticity by adenosine (Zaza et al. 1996). However, data obtained in vivo suggest that I_f plays a larger role in the pacemaker activity of His-Purkinje fibers vs. SN (Pelleg et al. 1990a). Specifically, in dogs with complete AV nodal conduction block where SN and ventricular pacemakers were operating concurrently but independently, adenosine suppressed the activities of both pacemakers in a dose-dependent manner through the activation of A_1 AR; however, in the presence of isoproterenol, the dose-response to adenosine in the SN and in the ventricular pacemaker shifted to the left and right, respectively (Pelleg et al. 1990a). Thus, the accentuation of the adenosine’s action in the SN seemed to be the result of its suppression of isoproterenol-enhanced I_f and I_{Ca} (indirect, anti- β -adrenergic action), which was added to its induced $I_{KAdoACh}$ (i.e., direct action), while in the ventricular pacemakers, the action of adenosine was mediated mainly by its suppression of I_f , an action which was attenuated in the presence of isoproterenol due the rightward shift of the I_f activation curve induced by the catecholamine (DiFrancesco and Borer 2007). This interpretation agrees well with the maximal diastolic potentials of approximately -65 mV and -90 mV in the SN and His-Purkinje pacemaker cells, respectively, as well as the I_f activation curve, which indicates fractional activation

(i.e., activated channel probability of 0.33) and full activation (i.e., activated channel probability of 1.0) of this current at membrane potentials of -65 mV and -90 mV, respectively (DiFrancesco and Borer 2007). The limited yet significant role of I_f in the pacemaking mechanism in the SN is indicated by the fact that CsCl and ZD7288, which are known blockers of I_f , slowed but did not arrest spontaneous pacemaking in SN cells (Denyer and Brown 1990; Sanders et al. 2006). Further support for this interpretation was given by the fact that in this canine model, quinidine, which suppresses acetylcholine-induced $I_{KAdoACh}$, and probably also adenosine-induced $I_{KAdoACh}$, attenuated the negative chronotropic action of adenosine in the SN but not in ventricular pacemakers (Pelleg et al. 1990a). Thus, data obtained in isolated single cells in vitro should be extrapolated to the in vivo setting with great caution; the lack of electrotonic interactions and constitutive neural input (among other factors) in commonly used in vitro models may affect this process.

Overexpression of A_1AR was associated with (i) a 20-fold increase in the potency of 2-chloroadenosine in slowing heart rate and a 35% reduction in maximal heart rate induced by β -adrenoceptor stimulation (Headrick et al. 2000), (ii) a reduced positive chronotropic response to exercise, and (iii) little effect on the resting heart rate (Kirchhof et al. 2003). Interestingly, overexpression of A_3AR was associated with depressed heart rate preferentially at rest (Fabritz et al. 2004). These data give further support to the notion that A_1AR mediates the negative chronotropic action and anti- β -adrenergic effects of adenosine. The role of A_3AR , if any, in the cardiac electrophysiology of adenosine remains to be determined.

8.2.2 Negative Dromotropic Action

The negative dromotropic action of adenosine is manifested in the prolongation of the PR and AH intervals as well as complete AV nodal (AVN) conduction block. Adenosine does not alter the HV interval; therefore, its dromotropic action is mainly due to its effects on the AVN. The seminal work of Belardinelli et al. (see (Belardinelli et al. 1987) elucidated the mechanisms of action of adenosine on the AVN; their major findings were: (i) adenosine mediates hypoxia/ischemia-induced AVN conduction block; (ii) adenosine hyperpolarizes cell membrane potential, shortens action potential duration, slows the recovery of I_{Ca} , and prolongs postrepolarization refractoriness in isolated single AVN cells; (iii) these actions of adenosine are mediated by A_1AR , and; (iv) the degree of amplification of A_1AR occupancy as determined by the negative dromotropic response to adenosine is relatively minimal, indicating “tight” coupling between receptor occupancy and its physiologic outcome (Belardinelli et al. 1981; Clemo and Belardinelli 1986; Clemo et al. 1987; Dennis et al. 1992). A subsequent study confirmed that A_1AR and a pertussis toxin-sensitive G protein mediate the AVN conduction block associated with global myocardial ischemia in vivo (Xu et al. 1993).

8.2.3 Adenosine's Effects on Atrial and Ventricular Myocardium

In the atria, adenosine exerts direct and indirect anti- β -adrenergic effects. The activation of $I_{K_{ACh}}$ in atrial myocytes, which is mediated by A_1 AR and pertussis toxin-sensitive G protein, results in shortened action potential duration and refractoriness (Pelleg et al. 1996), thereby facilitating re-entry. Indeed, a common side effect of adenosine is the induction of transient atrial fibrillation (Pelleg et al. 2002). Recently, Hove-Madsen et al. (Hove-Madsen et al. 2006) have demonstrated that A_{2A} AR is expressed in the human right atrium and distributed in a banded pattern along the Z lines, overlapping with the ryanodine receptor. In this study, an A_{2A} AR-selective agonist did not affect the L-type inward Ca^{2+} current (I_{CaL}) amplitude, but it did increase spontaneous calcium release from the sarcoplasmic reticulum (SR) and reduce the fast time constant for I_{Ca} inactivation (Hove-Madsen et al. 2006). These data were interpreted to suggest that activation of the A_{2A} AR stimulates the ryanodine receptor itself (Hove-Madsen et al. 2006).

In general, adenosine does not directly affect ventricular myocytes; although direct activation by adenosine of the ATP-dependent potassium outward current ($I_{K, ATP}$) in isolated rat ventricular myocytes has been proposed (Kirsch et al. 1990), subsequent studies in vitro and in vivo failed to support this hypothesis (Song et al. 2002; Xu et al. 1994). Adenosine exerts pronounced anti- β -adrenergic effects in the ventricular myocardium, which are mediated by A_1 AR and reduced intracellular levels of cAMP (Belardinelli and Isenberg 1983). Adenosine attenuates the catecholamine-dependent increase in inward L-type Ca^{2+} current (I_{CaL}), the delayed rectifier potassium current and chloride current (I_{Cl}). In addition, adenosine attenuates I_{CaL} - and transient inward current (I_{Ti})-dependent afterdepolarizations and triggered activity (Song et al. 1992). Interestingly, adenosine terminated episodes of ventricular tachycardia (VT) and abolished the delayed afterdepolarizations (DAD) associated with digoxin toxicity in the perfused guinea-pig heart in vitro and guinea-pig and canine hearts in vivo (Fogaça and Leal-Cardoso 1985; Xu et al. 1995). Because catecholamines play a mechanistic role in digoxin-induced DAD and triggered activity, it was concluded that this antiarrhythmic effect of adenosine was mediated by its anti- β -adrenergic action (Xu et al. 1995). Indeed, several studies have indicated that adenosine can exert an antiarrhythmic effect in the setting of other catecholamine/cAMP-dependent ventricular tachycardias (see below).

8.3 Clinical Aspects

8.3.1 Supraventricular Tachycardias

The seminal work of Belardinelli et al. in the late 1970s and early 1980s led in 1989 to the introduction of adenosine as an effective and safe antiarrhythmic drug for the acute termination of paroxysmal supraventricular tachycardia (PSVT) involving the AVN (Adenocard) (DiMarco et al. 1983; for reviews, see Pelleg and

Kutalek 1997; Pelleg et al. 2002). The rationale for the use of adenosine as an antiarrhythmic drug in this setting is derived from its potent suppression of AVN conduction; the latter breaks or slows down re-entrant circuits involving the AVN. However, it has also led to several “off label” uses of adenosine as a diagnostic drug, including the differential diagnosis of broad QRS complex tachycardia (i.e., SVT with aberrant ventricular conduction vs. VT), and assessment of accessory AV pathway ablation (Conti et al. 1995; Keim et al. 1992).

In recent years, a second generation of adenosine receptor-related drug candidates has been developed (Hutchinson and Scammells 2004). For example, tecadenoson (CVT-510; CV Therapeutics, Inc.) is a novel selective A₁AR agonist that is being evaluated as a drug candidate for the acute suppression of PSVT (Cheung and Lerman 2003; Peterman and Sanoski 2005). Clinical trials have shown that the drug effectively terminates PSVT without the side effects caused by the activation of ARs other than the A₁AR, which is associated with the use of adenosine in this setting.

Focal atrial tachycardias are a group of SVTs characterized by the concentric spread of a wave of depolarization from a specific localized source, the mechanism of which includes abnormal automaticity, triggered activity and microreentry (Lindsay 2007). The response to programmed atrial stimulation as well as several pharmacologic agents including adenosine has been used to differentiate these mechanisms. Regarding adenosine, data obtained in recent years support the hypothesis that adenosine-induced suppression or termination of a focal atrial tachycardia is indicative of a microreentry mechanism rather than abnormal automaticity or triggered activity (Iwai et al. 2002; Markowitz et al. 2007, 1999).

8.3.2 Ventricular Tachycardia/Fibrillation

Due to its anti- β -adrenergic actions in the ventricular myocardium, adenosine can affect catecholamine-dependent ventricular arrhythmias. In an early study, adenosine terminated sustained, exercise-triggered VT in four patients with structurally normal hearts (Lerman et al. 1986). Observations in this study have led to the hypothesis that the mechanism of the adenosine-sensitive VT is cAMP-mediated triggered activity (Lerman et al. 1986). Data obtained in subsequent studies in similar patients have supported this hypothesis and indicated that the action of adenosine is mediated by A₁AR (Lerman 1993). Idiopathic repetitive nonsustained monomorphic VT, which is characterized by frequent ectopic beats and salvos of VT, and typically occurs at rest, can also be sensitive to adenosine (Lerman et al. 1995). Among the idiopathic VT, the right outflow tract VT, which is the most common form (and presents as repetitive monomorphic VT or exercise-induced VT) and the left outflow tract VT (Nogami 2002) are both adenosine sensitive (Iwai et al. 2006; Lerman et al. 1997). Thus, the responsiveness to adenosine suggests that the mechanism of these tachycardias is probably cAMP-mediated triggered activity (Lerman et al. 2000).

In a swine model of prolonged ventricular fibrillation (VF), a selective A₁AR antagonist accelerated the deterioration in the VF waveform; this finding was

interpreted to suggest that endogenous adenosine exerts cardioprotective effects during sudden cardiac arrest associated with VF (Mader et al. 2006). However, data obtained in human subjects raise doubts regarding the use of an A₁AR antagonist in this setting. Specifically, because endogenous adenosine (which accumulates during hypoxia and ischemia) may perpetuate asystole, the use of aminophylline, a nonselective AR antagonist, in the setting of cardiac arrest has been proposed as an acute pharmacologic intervention to improve resuscitation outcome (Viskin et al. 1993). However, subsequent studies have shown that aminophylline offers no benefits in this situation (Hayward et al. 2007).

8.4 Adenosine as a Diagnostic Tool

Several diagnostic applications of adenosine, in addition to the diagnosis of broad QRS complex tachycardia mentioned above, have been proposed. Viskin et al. (Viskin et al. 2006) have shown that by provoking transient bradycardia followed by sinus tachycardia, adenosine challenge induces changes in QT interval that could be useful in distinguishing patients with long QT syndrome (LQTS) from healthy subjects. Specifically, adenosine challenge resulted in dissimilar responses in patients with LQTS and healthy subjects; the largest difference was recorded during maximal bradycardia, where the difference between the mean QT and QT_c values of the two groups was 121 ms (vs. a 59 ms difference at baseline) and 125 ms (vs. a 55 ms difference at baseline), respectively (Viskin et al. 2006). These observations by Viskin et al. (2006) explain the several cases of adenosine-induced polymorphic ventricular tachycardia (i.e., Torsade-de-Pointe; see review by Pelleg et al. 2002).

Several studies have indicated that adenosine can also identify patients with sick sinus syndrome (SSS). For example, Fragakis et al. (2007) found that, when a cut-off value of 525 ms for sinus recovery time (i.e., the time elapsed from sinus arrest until the emergence of the first sinus beat) was used as an indicator of sinus node dysfunction, sinus node recovery time (corrected for baseline rate) had 74% and a specificity of 100% for diagnosis of SSS, while the recovery time following adenosine had a sensitivity of 94 and a specificity of 84%, respectively (Fragakis et al. 2007). Earlier studies reported similar values; i.e., 80% sensitivity and 97% specificity (Burnett et al. 1999), and 67% and 100%, respectively (Resh et al. 1992).

Adenosine has been used in the diagnosis of patients with neurally mediated syncope; i.e., vasovagal syncope and syncope of unknown origin. Based on its sympathomimetic action (direct via activation of chemoreceptors and indirect via the baroreflex), adenosine has been proposed as an adjuvant provocative agent in the protocol of head-up tilt table test (HUT) (Mittal et al. 2004; Shen et al. 1996). The fact that adenosine plasma levels (Carrega et al. 2007; Saadjian et al. 2002) and the number of A_{2A}AR, which were upregulated (Carrega et al. 2007), were higher in patients with a positive HUT was interpreted to suggest that endogenous adenosine mediates syncope in a specific cohort of syncopal patients. However, prolonged adenosine induced AV block in conjunction with HUT in patients with unexplained syncope failed to predict recurrent syncopal episodes (Cheung et al. 2004).

Unfortunately, many studies as well as reviews of those mentioned above (and other similar studies) have treated the cardiovascular effects of adenosine and ATP as being identical. While ATP mimics adenosine due to its rapid degradation to the nucleoside by ectoenzymes, the reverse does not hold; specifically, before its degradation, ATP triggers a cardio-cardiac central vagal reflex mediated by the activation of P2X_{2/3} receptors localized on vagal sensory nerve terminals in the left ventricle; adenosine is devoid of this action (Xu et al. 2005). Thus, the negative chronotropic and dromotropic actions of ATP are mediated by adenosine and the vagus nerve (Pelleg et al. 1997); a mechanism found in cat, dog and man, but not in rodents (Pelleg et al. 1990b). Brignole et al. (Brignole et al. 2003) and Flammang et al. (2006) used bolus intravenous injections of ATP as a diagnostic tool in patients with syncope of unknown cause (for references, see Parry et al. 2006). The former group has used a maximal RR interval >6 s while the latter group has used complete AVN block duration >10 s as an indication of a positive test. In view of the fact the both ATP and adenosine suppress ventricular escape rhythms (Lerman et al. 1988; Pelleg et al. 1986), it is difficult to interpret the RR interval data as the rate of ATP degradation, and hemodynamic factors can directly affect this parameter. Using the RR interval >10 s criterion, it seems that ATP can identify a cohort of elderly patients in whom the mechanism of syncope is bradycardia and who may benefit from pacemaker therapy (see Flammang and AMS Investigators 2006; Flammang et al. 2005; Parry et al. 2006). In these patients, the bradycardia can be due to SN dysfunction, AVN dysfunction, abnormal vagal input to the heart, and any combination of these three causes.

8.5 Future Prospects

In view of the current efforts by the pharmaceutical industry, one can expect the introduction of AR ligands as new drugs for the treatment and diagnosis of cardiac arrhythmias in the near future. This would constitute a quantum step forward in the harnessing of adenosine signal transduction for the benefit of patients.

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Adenosine Receptors and Reperfusion Injury of the Heart

John P. Headrick and Robert D. Lasley

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Abstract Adenosine, a catabolite of ATP, exerts numerous effects in the heart, including modulation of the cardiac response to stress, such as that which occurs during myocardial ischemia and reperfusion. Over the past 20 years, substantial evidence has accumulated that adenosine, administered either prior to ischemia or during reperfusion, reduces both reversible and irreversible myocardial injury. The latter effect results in a reduction of both necrosis or myocardial infarction (MI) and apoptosis. These effects appear to be mediated via the activation of one or more G-protein-coupled receptors (GPCRs), referred to as A₁, A_{2A}, A_{2B} and A₃ adenosine receptor (AR) subtypes. Experimental studies in different species and models suggest that activation of the A₁ or A₃ARs prior to ischemia is cardioprotective. Further experimental studies reveal that the administration of A_{2A}AR agonists during reperfusion can also reduce MI, and recent reports suggest that A_{2B}ARs may also play an important role in modulating myocardial reperfusion injury. Despite convincing

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experimental evidence for AR-mediated cardioprotection, there have been only a limited number of clinical trials examining the beneficial effects of adenosine or adenosine-based therapeutics in humans, and the results of these studies have been equivocal. This review summarizes our current knowledge of AR-mediated cardioprotection, and the roles of the four known ARs in experimental models of ischemia-reperfusion. The chapter concludes with an examination of the clinical trials to date assessing the safety and efficacy of adenosine as a cardioprotective agent during coronary thrombolysis in humans.

Keywords Adenosine receptor subtypes · Cardioprotection · Ischemia · Myocardial infarction · Reperfusion · Signaling

Abbreviations

AR	Adenosine receptor
CCPA	2 Chloro- <i>N</i> ⁶ -cyclopentyladenosine
CHF	Congestive heart failure
CSC	8-(13-Chlorostyryl) caffeine
FMLP	Formyl-Met-Leu-Phe
GPCR	G-protein-coupled receptor
I/R	Ischemia-reperfusion
iNOS	Inducible nitric oxide synthase
IPC	Ischemic preconditioning
KO	Knockout
MI	Myocardial infarction
mito K _{ATP}	Mitochondrial ATP-sensitive K ⁺ channels
MPTP	Mitochondrial permeability transition pore
NECA	5'- <i>N</i> -Ethyl-carboxamidoadenosine
p38-MAPK	p38 Mitogen-activated protein kinase
PC	Preconditioning
PIA	<i>N</i> ⁶ -1-(Phenyl-2 <i>R</i> -isopropyl)adenosine
PKC	Protein kinase C
PTCA	Percutaneous transluminal coronary angioplasty
ROS	Reactive O ₂ species
SPECT	Single-photon emission computed tomography
STEMI	ST-segment elevation myocardial infarction
SR	Sarcoplasmic reticulum

1 Introduction

Since extent of myocardial cell death is the primary determinant of outcome from planned or unplanned cardiac ischemia, protective strategies to limit this damage during ischemia-reperfusion (I/R) are highly sought after. It is now clear that a num-

ber of GPCR families can activate cytoprotective responses. These receptors, including the adenosine, opioid and bradykinin families, may act not only as acute “retaliatory” systems mediating immediate responses to injurious stimuli, but function as sensors of low-level stress to initiate a signaling cascade culminating in the expression of more prolonged protected phenotypes. These adaptive or hormesis responses predate mammals, and offer potential as targets for therapeutic cardioprotection.

The AR family, composed of A₁, A_{2A}, A_{2B}, and A₃ subtypes, has been implicated in both acute protection and adaptive preconditioning (PC) responses. Not only does preischemic activation of ARs generate potent protection, but significant evidence indicates that this receptor class also mediates powerful cardioprotection when targeted during the reperfusion phase. This brief review focuses on temporal properties of AR-mediated cardioprotection (prior to, during, after ischemia), their contributions to PC responses, and their relevance to the protection of human myocardium.

2 Cardioprotection with Tonic A₁AR Agonism: A₁AR Overexpression

Given early evidence of cardioprotection in response to adenosine and (subsequently) selective A₁AR agonism, the A₁AR subtype seemed an obvious target for manipulating myocardial ischemic tolerance. To test the hypothesis that A₁AR density (rather than endogenous [ligand]) limits the resistance of the heart to I/R, Matherne and colleagues developed a cardiac-specific A₁AR overexpression model. The model employed a construct containing the rat A₁AR gene under the control of a mutated α -myosin heavy chain promoter (Matherne et al. 1997), with extent of A₁AR expression varying across the lines generated (with up to 100-fold overexpression of coupled A₁ARs). The resulting phenotype was characterized by modest bradycardia, conduction disturbances, and a small increase in heart mass in some lines (Matherne et al. 1997; Gauthier et al. 1998; Kirchof et al. 2003). Initial studies of I/R revealed profound reductions in cell death and contractile dysfunction compared with wild-type hearts (Matherne et al. 1997; Headrick et al. 1998; Morrison et al. 2000). Tolerance to hypoxic challenge (Cerniway et al. 2002), and long-term cold storage of hearts (Crawford et al. 2005) were also improved. Cardioprotection was evident in isolated tissue preparations (Matherne et al. 1997; Headrick et al. 1998) and in vivo (Yang et al. 2002). These outcomes were consistent with protective effects of artificially enhanced A₁ (and A₃) expression in isolated myocytes (Dougherty et al. 1998). Differing components of cardiac protection were apparent, with reduced necrosis and infarction (Matherne et al. 1997; Morrison et al. 2000; Yang et al. 2002), inhibition of apoptosis (Regan et al. 2003; Crawford et al. 2005), enhancement of bioenergetic state during ischemia (Headrick et al. 1998), and selective modulation of contractile injury: A₁AR overexpression consistently reduces diastolic (and not systolic) dysfunction during I/R (Matherne et al. 1997; Reichelt et al. 2007). The latter suggests that A₁ARs selectively target processes underlying diastolic contracture (e.g., Ca²⁺ handling, myofibrillar function).

While the signaling basis of cardioprotection with A₁AR overexpression remains to be established, analysis to date implicates players common to protective signaling in wild-type tissue, including mitochondrial ATP-sensitive K⁺ (mito K_{ATP}) channels and inducible nitric oxide synthase (iNOS) (Headrick et al. 2000; Nayeem et al. 2003). Curiously, mito K_{ATP} channels (or 5-hydroxydecanoate-sensitive targets) were not implicated in protection against hypoxia (Cerniway et al. 2002). Other work supports a role for p38 mitogen-activated protein kinase (p38-MAPK)-dependent signaling, though this remains to be more fully tested (Jones et al. 1999). Sarcoplasmic reticulum (SR) Ca²⁺ handling is impaired (Zucchi et al. 2002), which could contribute to specific aspects of associated cardioprotection. Another interesting outcome with A₁AR overexpression is restoration of ischemic resistance in aged hearts: aging may limit the capacity of hearts to withstand damage during I/R (Willems et al. 2005), and this effect was reversed by A₁AR overexpression in mice (Headrick et al. 2003b), in parallel with restoration of adenosine responsiveness.

In terms of PC responses, overexpression of A₁ARs mimics the benefit with this stimulus, actually surpassing the degree of protection with ischemic PC (IPC) (Morrison et al. 2000). Protection with A₁AR overexpression is also nonadditive with IPC, suggesting a commonality of signaling/end-effectors and/or maximally effective protection with A₁AR overexpression. However, the latter is inconsistent with reports that acute application of adenosine (Peart et al. 2002) or A₁AR agonist (Nayeem et al. 2003) can augment the protection with A₁AR overexpression.

Overexpression of A₁ARs in cardiac cells did confirm the hypothesis that normal levels of A₁AR expression in wild-type hearts do appear to limit the extent of cardioprotection possible, and thus the heart's intrinsic resistance to I/R (Matherne et al. 1997). Nonetheless, pharmacologically activating A₁ARs does provide benefit in wild-type hearts (see Sect. 2.1.1 below), demonstrating that normally expressed A₁ARs can be targeted to achieve further cardioprotection. This may reflect additional effects of transient AR agonism (and induction of a short-lived PC state), as opposed to the longer-lived effects of tonic A₁AR activity in transgenic hearts.

3 Cardioprotection via Preischemic AR Activation: A Role in PC Responses

Since its discovery by Murry and colleagues (Murry et al. 1986), the molecular basis of IPC has been the subject of intense investigation. An ultimate goal is translation to the clinical setting, enabling activation of similar protection in cardiac patients. Through a simplified scheme, we can examine the roles of ARs in PC responses from the viewpoint of the initial "trigger" phase and the subsequent "mediation" phase.

The initial and rather crude ischemic trigger of PC is now known to involve the release and actions of several GPCR ligands (including opioids, bradykinin, and adenosine). A "threshold" model for triggering PC has evolved, in which summation

of multiple GPCR stimuli is required to activate delayed protection (Goto et al. 1995; Baba et al. 2005). The response may involve not only summation of GPCR triggers but also downstream kinase signaling (Vahlhaus et al. 1998). The kinase cascades involved in PC have been elaborated over recent years, and are currently thought to converge on modulation of mitochondrial effectors, including K_{ATP} channels and the mitochondrial permeability transition pore (MPTP) (Murphy 2004; Hausenloy and Yellon 2007; Liem et al. 2008). Nonetheless, there remains considerable disagreement regarding the roles of different signaling components, and putative end-effectors, in AR-mediated protection and PC. As the focus of this review is on AR involvement in cardioprotection, and since the signaling basis of PC responses has been very well addressed in recent reviews (Murphy 2004; Downey et al. 2007; Hausenloy and Yellon 2007), interested readers are directed to these for further details.

3.1 Adenosine as a Preischemic Trigger of PC

It should be clarified that true PC describes a delayed protective state persisting in the absence of the initial stimulus. Many studies refer to “preconditioning” effects when assessing preischemic receptor or pathway activation. However, application of receptor agonists up to induction of ischemia (with no intervening washout) will modify the same targets during ischemia and possibly early reperfusion. This is an inherent limitation to *in vivo* studies, since exogenously applied AR agonists (or antagonists) may be slowly removed and thus exert potentially long-lasting effects beyond the desired “window.” Thus, while discussion of the effects of preischemic AR activation (or antagonism) can be informative in terms of roles of ARs in PC responses, these experimental scenarios do not simulate PC *per se*.

In seeking a released factor capable of transducing protection with PC, adenosine seemed a likely candidate: adenosine release increases rapidly in response to different conditions of stress (Headrick et al. 2003a); the interstitial concentrations achieved are sufficient to activate one or more AR subtypes (Van Wylen 1994; Lasley et al. 1995a; Headrick 1996; Harrison et al. 1998); rapid transport and catabolism ensures a brief extracellular half-life and localized signaling; and exogenous AR agonists appear to induce similar protective states.

3.1.1 AR-Triggered Pharmacological PC

In early work Liu et al. showed that preischemic treatment with adenosine or N^6 -1-(phenyl-2*R*-isopropyl) adenosine (PIA) mimicked the protective effects of PC in rabbit myocardium (Liu et al. 1991). Subsequent studies confirmed protection via preischemic A_1 AR agonism in different models and species (Lasley and Mentzer 1992; Thornton et al. 1992; Liu and Downey 1992; Tsuchida et al. 1993; Strickler et al. 1996; Carr et al. 1997; Liang and Jacobson 1998; de Jonge and de Jong

1999; de Jonge et al. 2002; Germack et al. 2004; Germack and Dickenson 2005). Toombs and colleagues not only showed that preischemic adenosine limited infarct size (Toombs et al. 1992), but further showed that activation of 8- ρ -sulfophenyltheophylline-sensitive ARs (likely A₁ and/or A₂ARs) during the ischemic period itself was required for protection.

Preischemic activation of the A₃AR subtype can also generate cardiac protection. Strickler et al. (1996) presented some of the first evidence that A₃AR activation prior to ischemia could confer protection against ischemia-like insult in myocytes (of avian origin), while Tracey and colleagues acquired evidence for A₃AR-triggered protection in rabbit hearts (Tracey et al. 1997). Other groups confirmed A₃AR-mediated protection in multiple models (Strickler et al. 1996; Carr et al. 1997; Liang and Jacobson 1998; de Jonge et al. 2002; Maddock et al. 2002; Germack et al. 2004; Germack and Dickenson 2005; Wan et al. 2008). Indeed, Liang and Jacobson (1998) found that the A₃AR induced a more sustained state of protection than the A₁AR when activated prior to ischemia.

In contrast to PC-like effects of A₁AR or A₃AR agonism, preischemic activation of A_{2A}ARs or A_{2B}ARs is generally ineffective in limiting myocardial injury during subsequent I/R (Thornton et al. 1992; Lasley and Mentzer 1992; Maddock et al. 2002; Germack and Dickenson 2005). Studies with the natural agonist adenosine yield mixed results, likely due to rapid uptake and catabolism of extracellular adenosine, complications of potent hemodynamic actions of the endogenous agonist, and the impact of mixed AR activation on different cell types.

3.1.2 ARs as Intrinsic Triggers of IPC

Studies demonstrating PC-like responses to preischemic AR activation provided support for AR involvement in IPC. To more directly test for a role of AR activation in triggering nonpharmacological forms of PC, AR antagonists or adenosine deaminase have been added, often in both trigger and mediation phases, to limit any contributions from ARs. A number of these studies independently provided no evidence for essential roles for ARs in PC (Liu and Downey 1992; Lasley et al. 1993; Hendrikx et al. 1993; Bugge and Ytrehus 1995; Lasley et al. 1995b), leading to premature elimination of this class of GPCRs as contributing to PC (Cave et al. 1993; Li and Kloner 1993). In the context of protective thresholds and contributions of multiple stimuli, a more accurate conclusion may be that the roles of ARs in triggering/mediating PC are redundant, with other concomitant stimuli (e.g., endogenous opioids and bradykinin) being able to compensate and surpass the signaling threshold required for protection.

On the other hand, considerable evidence supporting essential AR involvement in PC has been reported. Studies employing different AR antagonists or adenosine deaminase supported roles in rabbit (Liu et al. 1991; Tsuchida et al. 1992; Thornton et al. 1993; Urabe et al. 1993; Weinbrenner et al. 1997) rat (Headrick 1996; de Jonge and de Jong 1999; de Jonge et al. 2001; Tani et al. 1998), dog (Auchampach and Gross 1993; Hoshida et al. 1994), and pig (Schulz et al. 1995;

Vogt et al. 1996; Louttit et al. 1999). Early studies of PC responses in human myocardium also supported involvement of endogenous adenosine, likely via A₁ARs (Walker et al. 1995; Tomai et al. 1996).

Reasons for differing outcomes with AR blockade in varied models of PC are not clear. Evidence has been presented for substantial species differences in adenosine handling and receptor activation (Headrick 1996), which might dictate differing roles for adenosine and certainly contribute to differing abilities of competitive AR antagonists to limit these responses. Moreover, the affinity and selectivity of AR ligands varies across species, and in the event of poor solubility, bioavailability may limit the effects of a ligand. Furthermore, the relative contributions of adenosine and ARs in triggering PC may be species dependent, with a greater and essential contribution in rodent myocardium. Nonetheless, evidence for essential AR involvement has been reported in large animal models (Auchampach and Gross 1993; Hoshida et al. 1994; Schulz et al. 1995; Vogt et al. 1996; Louttit et al. 1999) and in human tissue (Walker et al. 1995; Tomai et al. 1996; Ikonomidis et al. 1997). Responses may be model specific, in part, since some aspects of I/R injury are dependent upon blood components and activation of pathways for inflammation, while others are intrinsic to the myocardial cells themselves (and these cell-dependent responses may also vary across species). Thus, injury and counteracting protective processes may differ between *ex vivo* or blood-free models and the *in situ* myocardium. Finally, differences reported with the use of AR antagonists in PC studies may be related to the nature and duration of the PC stimulus (see below), which may influence the contribution of ARs to protection.

In terms of the identity of the ARs implicated in triggering PC, initial work supported the involvement of A₁ARs (Liu et al. 1991; Tsuchida et al. 1992; Auchampach and Gross 1993). However, subsequent studies (Armstrong and Ganote 1994, 1995; Liu et al. 1994; Wang et al. 1997) demonstrated that partially selective A₃AR antagonism also impaired the protective efficacy of PC. Liang and colleagues documented A₁AR and A₃AR involvement in PC responses in chick cardiomyocytes (Strickler et al. 1996; Liang and Jacobson 1998), while Wang et al. (1997) reported additive contributions from A₁AR and A₃ARs to optimize PC in rabbit myocytes. Although other studies initially supported A₃AR involvement in IPC in intact rabbit myocardium (Tracey et al. 1997), this group subsequently presented evidence of a quantitatively more critical role for A₁AR vs. A₃AR (Hill et al. 1998). More recent studies confirm that endogenous adenosine contributes to IPC via A₁AR and/or A₃AR activation, though the contribution of ARs may be dependent upon the nature and duration of the PC stimulus, being less important with shorter periods of triggering ischemia (Liem et al. 2001, 2008). This is consistent with earlier observations of Schulz et al. in pigs (1995).

Ultimately, preservation of AR-dependent protection in human myocardial tissue is of key importance. Walker and colleagues provided some of the first support for mediation of PC by ARs in human myocardium (Walker et al. 1995). Cleveland et al. (1996, 1997) subsequently confirmed AR-mediated PC responses in human myocardial tissue. Carr et al. (1997) further established that A₁ARs and A₃ARs trigger PC in human atrial muscle, while Ikonomidis et al. (1997) demonstrated AR

dependence of PC in human pediatric myocytes. Thus, AR-mediation of PC is relevant to human myocardium. Indeed, an early study by Tomai et al. (1996) supported A₁AR-dependent PC in patients undergoing coronary angioplasty. Furthermore, the importance of ARs in determining resistance to myocardial ischemia is supported by associations between AR polymorphisms, specifically for A₁ and A₃ARs, and infarct size in patients with ischemic cardiomyopathy (Tang et al. 2007).

3.1.3 Evidence from Gene-Modified Models

Essential contributions of ARs to PC are borne out by recent gene manipulation studies. Analysis of A₃AR gene knockout (KO) in mice revealed no impact on induction of IPC (Guo et al. 2001), apparently negating an essential role for this AR subtype. However, A₁AR KO eliminates protection with both IPC (Lankford et al. 2006) and remote PC triggered by cerebral ischemia (Schulte et al. 2004). Moreover, ecto-5'-nucleotidase deletion also eliminates protection with IPC, supporting an essential role for endogenous adenosine generated at the cell surface (Eckle et al. 2007). This latter study also confirmed an essential role for ARs in IPC, although their data differed in implicating only the A_{2B}AR. The basis of this discrepancy is not clear, but may, in part, be model related (in vivo vs. in vitro). This latter observation is, however, consistent with recent data from the laboratory of Downey and colleagues, who reported evidence for protein kinase C (PKC) dependent sensitization of A_{2B}ARs during the trigger or ischemic phases and their role in protection during the subsequent reperfusion phase (Kuno et al. 2007).

Of course, a limitation inherent to gene deletion (or overexpression) is an inability to distinguish events temporally. Since gene deletion eliminates the actions of targeted ARs at all time points, it is unclear from such work when the receptors are involved. For example, A₁ARs or A₃ARs may trigger protection with IPC prior to or during ischemia, while recent evidence implicates a role for A_{2B}AR in mediating the protection with PC during the reperfusion phase (Kuno et al. 2007). This A_{2B}AR-mediated protection during reperfusion could depend to some extent upon A₁AR and/or A₃AR activation of PKC prior to or during ischemia. Such complex responses are not amenable to interrogation by gene manipulation.

3.2 AR Activity During Ischemia

Cardioprotective effects of PC and preischemic GPCR activation were initially thought to manifest primarily during ischemia itself (Cohen et al. 2000). Preischemic AR agonism (or A₁AR overexpression) modifies substrate and energy metabolism, H⁺ and Ca²⁺ accumulation, and contracture development during the ischemic episode (Lasley et al. 1990; Fralix et al. 1993; Lasley and Mentzer 1993; Headrick 1996). Similarly, there is evidence of specific protective actions of adenosine and A₁ARs during ischemia versus reperfusion (Peart and Headrick 2000;

Peart et al. 2003). IPC also modifies ischemic events relevant to tissue protection (de Jonge and de Jong 1999), reducing purine moiety accumulation and washout (Van Wylen 1994; Lasley et al. 1995a; Harrison et al. 1998; de Jonge et al. 2002) and ionic perturbations (Fralix et al. 1993). Such observations are consistent with the idea that modulation of injury during ischemia itself contributes to overall protection and improved postischemic outcome. This is supported by early work of Thornton et al. (1993), who showed that protection with IPC is mediated, at least in part, via intrinsic activation of A₁ARs during the subsequent ischemic insult. Studies such as that of Stambaugh et al. (1997) also show that AR activation throughout the period of ischemia/hypoxia is beneficial.

While a majority of studies across differing species support beneficial actions of either exogenously or intrinsically activated ARs during myocardial ischemia, there are a small number of reports of improved outcomes with AR antagonists applied prior to ischemia *in vivo* (and thus reflecting possible blockade of ARs prior to, during, or following ischemia). Neely et al. (1996) initially documented infarct limitation with three different A₁AR antagonists, DPCPX (1,3 dipropyl-8-cyclopentylxanthine), XAC (xanthine amine congener) and bamiphylline, in a feline regional myocardial infarct model. To rule out that the possibility that these A₁AR antagonists were producing their effects via a nonspecific intracellular action (i.e., inhibition of intracellular enzymes, e.g., phosphodiesterases), Forman and colleagues (2000) reported that another (albeit poorly selective) A₁AR antagonist, DPSPX (1,3-dipropyl-8-*p*-sulfophenylxanthine), which is negatively charged and thus does not accumulate in intracellular spaces because of its high water solubility, also reduced infarct size in dogs. Because DPSPX significantly reduced FMLP (formyl-Met-Leu-Phe)-induced chemoattraction of human neutrophils, the authors of this study suggested that this A₁AR antagonist produced sustained myocardial protection in dogs by reducing inflammation. However, DPSPX is also known to interact with the A_{2B}AR (Feoktistov and Biaggioni 1997), and at the doses applied in this study, to block A₂-dependent coronary dilation (Forman et al. 2000). A later detailed study by Auchampach et al. (2004) described the effect of three different A₁AR antagonists, DPCPX, BG 9928 (1,3-dipropyl-8-[1-(4-propionate)-bicyclo-[2,2,2]octyl])xanthine and BG 9719 (1,3-dipropyl-8-[2-(5,6-epoxynorbornyl) xanthine), of varying specificities in a regional myocardial infarct model *in vivo* in dogs. A₁AR antagonists could limit infarct size in dog hearts, though only with those agents (DPCPX and BG 9928) that also antagonized A_{2A}AR-mediated coronary dilation and possessed appropriate affinities for A_{2B}ARs, raising the possibility of actions at multiple AR subtypes. An alternative explanation by the authors of this study was that differences in the pharmacokinetic and pharmacodynamic properties of BG 9719 may have limited the *in vivo* potency of this A₁AR antagonist in these studies. They additionally showed that the A₁AR antagonists DPCPX and BG 9928 were equally protective when applied just prior to reperfusion or throughout ischemia-reperfusion, suggesting a primarily postischemic mode of action.

The basis of these mixed observations remains to be determined, though they do raise the possibility of opposing effects of ARs through cell-specific responses. For example, A₁AR activity may augment chemotaxis and neutrophil-dependent injury,

whereas the same receptor limits injury in cardiomyocytes. A number of studies confirm a lack of any infarct-sparing effects of nonselective or subtype-specific AR antagonists *in vivo* in multiple species (Toombs et al. 1992; Tsuchida et al. 1992; Auchampach and Gross 1993; Thornton et al. 1993; Zhao et al. 1993; Hoshida et al. 1994; Baba et al. 2005; Kin et al. 2005; Lasley et al. 2007). However, with the exception of the study by Zhao et al. (1993), the antagonists used in these studies were administered as single doses and not as continuous infusions or multiple doses to achieve a steady state plasma concentration of the AR antagonist, as was done by Neely et al., Forman et al., and Auchampach et al.. Moreover, problems with the selectivity of AR antagonists for specific AR subtypes, particularly during *in vivo* studies, limit their interpretation with respect to the definitive roles of the four AR subtypes in the setting of acute myocardial ischemia-reperfusion injury.

4 Reperfusion Injury and ARs in Experimental Studies

Although reperfusion is necessary to salvage ischemic myocardium, the process of restoring blood flow also contributes to the total injury observed in ischemic-reperfused myocardium. Reperfusion injury is caused by intracellular calcium overload and oxidative stress induced by the formation of reactive O₂ species (ROS) in the presence of decreased cellular redox state. Reperfusion injury in intact animals and in humans following myocardial ischemia durations of >15 min produces irreversible injury that is also associated with a general inflammatory process including the release of numerous cytokines, adhesion and infiltration of neutrophils across the damaged coronary endothelium, platelet aggregation, and activation of the complement cascade (Ambrosio and Tritto 1999; Park and Lucchesi 1999; Verma et al. 2002).

Similar to the beneficial protective effects of AR agonists discussed in the first sections of this chapter, there is now convincing evidence that the activation of ARs during reperfusion is cardioprotective in animal models. However, in contrast to reports nearly 20 years old documenting the cardioprotective effects of adenosine treatment prior to ischemia, initial studies on the effects of treatment with adenosine after reperfusion were much more controversial. Two initial reports in canine models indicated that intracoronary and intravenous adenosine infusions for the first 1–2.5 h of reperfusion after 90 min coronary occlusions significantly reduced infarct size after 24 and 72 h reperfusion, respectively (Olafsson et al. 1987; Pitarys et al. 1991). In both of these studies, the ischemic myocardium from animals treated with adenosine exhibited significantly less neutrophil accumulation and erythrocyte plugging of capillaries. These observations are consistent with adenosine's ability to inhibit both neutrophil adherence to endothelium (Cronstein et al. 1992) and platelet aggregation (Söderbäck et al. 1991). Several subsequent reports were, however, unable to reproduce these positive findings (Homeister et al. 1990; Goto et al. 1991; Vander Heide and Reimer 1996). Negative results with adenosine treatment following reperfusion may be due to the use of inadequate doses, which must be high enough to overcome its rapid uptake and metabolism by red blood cells and endothelial cells.

However, high concentrations of adenosine can be associated with severe hypotension, reflex tachycardia, and coronary steal. These side effects will likely limit the use of adenosine as a cardioprotective agent in humans.

4.1 Effects of the A_{2A}AR During Reperfusion

Despite the contradictory reports regarding the beneficial effects of adenosine as a reperfusion treatment, there have been an increasing number of reports that reperfusion treatments with infusions of certain AR agonists are cardioprotective. Such studies support the hypothesis that the cardioprotective effects of adenosine are mediated primarily via activation of one or more AR subtypes. The majority of such studies indicate that the infusion of adenosine A_{2A}AR agonists during reperfusion reduces myocardial infarct size. It appears that the first such study was conducted by Norton et al. (1992), who reported that the A_{2A}AR agonist CGS21680 (4-[2-[[6-Amino-9-(*N*-ethyl-*b*-D-ribofuranuronamidoyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid), infused during reperfusion *in vivo*, significantly reduced myocardial infarct size measured after 48 h of reperfusion in rabbits in the absence of hypotension. Subsequent studies have reproduced similar infarct size-reducing effects of reperfusion A_{2A}AR stimulation in dogs, pigs, rats, and mice (Schlack et al. 1993; Zhao et al. 1996; Jordan et al. 1997; Budde et al. 2000; Lasley et al. 2001; Boucher et al. 2005; Yang et al. 2005, 2006).

Although there is a significant expression of A_{2A}ARs on vascular cells (vascular smooth muscle and endothelial cells), and activation of this receptor is associated with coronary vasodilatation, the beneficial effects of reperfusion A_{2A}AR agonists are independent of increased coronary blood flow and can be achieved without systemic hypotension. The prevailing current hypothesis for the beneficial A_{2A}AR effects during reperfusion are related to its anti-inflammatory properties, such as inhibition of neutrophil production of ROS and adherence to endothelium (Visser et al. 2000; Sullivan et al. 2001). Recent studies in mice further suggest that this A_{2A}AR-mediated reperfusion protection is due to effects on bone marrow-derived cells, more specifically to CD4⁺ T-helper lymphocytes (Toufektsian et al. 2006).

However, two additional studies conducted in intact animal models of myocardial stunning indicate that reperfusion treatment with A_{2A}AR agonists can exert beneficial effects in the absence of severe inflammation and myocardial necrosis. In porcine regionally stunned myocardium, an intracoronary infusion of the A_{2A}AR agonist CGS21680, initiated after 2 h reperfusion following 15 min coronary occlusion, significantly increased regional preload-recruitable stroke work and stroke work area, both of which are load-insensitive parameters of cardiac contractility. This effect, which appeared to be independent of increased coronary blood flow, occurred in stunned (i.e., no infarction was detected), but not normal, myocardium (Lasley et al. 2001). The fact that the A_{2A}AR agonist exerted its beneficial effects 2 h after reperfusion suggests that the improvement in regional contractility is likely to have been independent of a reduction in myocardial reperfusion injury, but rather

may have been a true positive inotropic effect. Using another myocardial stunning model in dogs, Glover et al. (2007) observed that the A_{2A} AR agonist ATL-146e, given just prior and during reperfusion following multiple brief (5 min) coronary occlusions, improved reperfusion wall thickening in the absence of any increase in coronary blood flow. Infusion of ATL-146e had no effect on regional function in normally perfused myocardium. Whether these beneficial effects of reperfusion A_{2A} AR stimulation in the absence of necrosis are due to a direct effect on the myocardium remains to be determined.

Although the evidence implicating the anti-inflammatory effects of postischemic A_{2A} AR activation in the setting of myocardial infarction is compelling, the above two studies in stunned myocardium indicate that A_{2A} AR activation may also protect the reperfused heart via mechanisms independent of neutrophils and inflammatory processes, as well as increased coronary blood flow. There are several reports that A_{2A} ARs are expressed in porcine, human, and rat ventricular myocytes (Marala and Mustafa 1998; Kilpatrick et al. 2002), which raises the possibility that the beneficial effect of A_{2A} AR agonists during reperfusion may also be due to direct effects on the cardiac myocyte. There have been numerous studies over the past 15 years investigating the effects of A_{2A} AR agonists on cardiac myocyte physiology, but these reports have yielded conflicting findings (Shryock et al. 1993; Stein et al. 1994; Xu et al. 1996, 2005; Boknik et al. 1997; Woodiwiss et al. 1999; Hleihel et al. 2006; Hove-Madsen et al. 2006). The majority of these reports indicate that A_{2A} AR activation alone exerts little, if any, direct effects on normal cardiac ventricular myocytes. However, it is possible that during myocardial ischemia, when endogenous adenosine levels increase and multiple AR subtypes are activated, cardiomyocyte A_{2A} AR may modulate the cardioprotective effects of adenosine.

There remain several interesting and incomplete aspects to our understanding of the cardioprotective effects of reperfusion AR agonist treatment. Although A_{2A} AR agonists administered during reperfusion have been shown to be cardioprotective in intact animals, the administration of A_{2A} AR antagonists does not exacerbate myocardial injury or infarct size in normal animals (Kin et al. 2005; Reid et al. 2005; Lasley et al. 2007). However, there is evidence that the A_{2A} AR does participate in the cardioprotective effect of ischemic postconditioning. Ischemic postconditioning is the phenomenon by which brief interruptions in coronary flow during the initial minutes of reperfusion following a prolonged occlusion reduce myocardial infarct size. This phenomenon is thus somewhat analogous to ischemic preconditioning, which was described earlier. The AR antagonist ZM241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol), which exhibits some selectivity for the A_{2A} AR subtype, has been shown to block ischemic postconditioning *in vivo* in rat hearts and in isolated perfused mouse hearts (Kin et al. 2005). A more recent report indicated that ischemic postconditioning could not be induced in mouse hearts from A_{2A} AR KO mice (Morrison et al. 2007). These findings indicate that stimulation of A_{2A} ARs plays a pivotal role in reducing myocardial reperfusion injury. Observations in isolated buffer perfused hearts in these latter two reports further support the hypothesis that this protective effect is mediated, at least in part, by the cardiomyocyte A_{2A} AR.

As described above, there are now numerous reports indicating that the infusion of A_{2A}AR agonists during reperfusion is cardioprotective. Although the administration of A_{2A}AR agonists prior to ischemia does not reduce myocardial ischemia-reperfusion injury, there is increasing evidence that A_{2A}ARs may modulate the protective effects of A₁AR stimulation. Reid et al. (2005) and Lasley et al. (2007) reported that the A_{2A}AR antagonist ZM241385 blocked the infarct reducing effects of preischemic treatments with three different AR agonists—AMP579 (1*S*-[1*a*, 2*b*, 3*b*, 4*a*(*S*^{*})]-4-[[2-(3-chloro-2-thienyl)-1-methylpropyl]amino]-3*H*-imidazo[4,5-*b*]pyridyl-3-yl]cyclopentane carboxamide), 2 chloro-*N*⁶-cyclopentyladenosine (CCPA), 5'-*N*-ethyl-carboxamidoadenosine (NECA)—in two different studies. The A_{2A}AR antagonist did not alter the A₁AR-induced bradycardia with these agonists, indicating that the A₁AR was not blocked; however, the ability of ZM241385 to block the protection by these AR agonists was comparable to that achieved with the A₁AR antagonist DPCPX. Preliminary observations in one of these studies suggested that the A_{2A}AR antagonist partially blunted the effects of AMP579 on preischemic mitogen-activated protein kinase (MAPK) signaling (Reid et al. 2005). These findings regarding the effects of A_{2A}AR antagonists on A₁AR cardioprotection are supported by an increasing number of reports of interactions between AR subtypes, including the formation of heterodimers (Karcz-Kubicha et al. 2003; O'Kane and Stone 1998; Lopes et al. 1999, 2002; Nakata et al. 2005).

There is also evidence that the beneficial effects of reperfusion AR agonist treatments may involve interactions among AR subtypes. In the isolated perfused rabbit heart, a reperfusion infusion (500 nM) of the AR agonist AMP579, which has a high affinity for both A₁ and A_{2A}ARs (Smits et al. 1998), reduced infarct size—an effect that was blocked by 8-(13-chlorostyryl) caffeine (CSC), which exhibits some selectivity for A_{2A}ARs, but not by the A₁AR antagonist DPCPX (Xu et al. 2001). The beneficial effect of AMP579 was mimicked by the nonselective agonist NECA at a dose (100 nM) activating both A₁ and A_{2A}ARs, but not by the A_{2A}AR agonist CGS21680 (50 nM). Kis et al. (2003) reported similar findings in the intact rabbit, where an infusion of AMP579 during reperfusion reduced infarct size, and this effect was blocked by the A_{2A}AR antagonist ZM241385 but not mimicked by the same dose of the A_{2A}AR agonist CGS21680. It is not clear why these studies did not observe protection with the A_{2A}AR agonist alone, when numerous other studies have reported such protection; however, these findings support a role for the A_{2A}AR in reduction of myocardial injury. Since ZM241385 has some affinity for A_{2B}ARs, it is also possible that the effects of this agent could be due to antagonism of this receptor subtype (Hasan et al. 2000).

4.2 Effects of A₁ and A₃ARs During Reperfusion

To date, the primary emphasis on AR reduction of reperfusion injury has focused on the role of the A_{2A}AR. However, given that there are four AR subtypes, all of

which appear to be expressed in the heart, it is possible that one or more of these other AR subtypes may modulate reperfusion injury. The one exception to this hypothesis is the A₁AR. Although, as described in the first section of this chapter, there is significant evidence that A₁AR agonists administered prior to ischemia are protective, it is clear that A₁AR agonists administered during reperfusion are not protective (Thornton et al. 1992; Baxter et al. 2000). There is evidence that A₃AR activation during reperfusion may be cardioprotective, as studies in isolated hearts and intact animals indicate that the A₃AR agonists IBMECA (1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-N-methyl-b-D-ribofuranuronamide) and Cl-IBMECA (1-[2-chloro-6-[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-b-D-ribofuranuronamide), administered during reperfusion, reduce myocardial infarct size (Maddock et al. 2002; Auchampach et al. 2003; Park et al. 2006). In two of these studies, the effects of the A₃AR agonists were blocked by A₃AR antagonists (Maddock et al. 2002; Park et al. 2006). Interestingly, in the former study (Maddock et al. 2002) the reperfusion A₃AR agonist protection was also blocked by the A_{2A}AR antagonist CSC. Finally, Kin et al. (2005) observed that postconditioning could be blocked by an A₃AR antagonist. Thus, in contrast to the A₁AR, activation of the A₃AR either prior to ischemia or during reperfusion appears to be cardioprotective.

4.3 Emerging Roles for the A_{2B} AR During Reperfusion

With respect to the fourth AR subtype, only now are a limited number of studies supporting a role for the A_{2B}AR in modulating myocardial reperfusion injury appearing. Investigations of this receptor in the heart have been hindered by the fact that there are no radioligand binding studies defining A_{2B}AR receptor density or affinity in mammalian myocardium or cardiomyocytes. The role of this receptor has also been hindered by the lack of studies with well-characterized, selective A_{2B}AR agonists and antagonists. To date there are four pharmacological studies providing some evidence for the involvement of A_{2B}ARs, although the results are conflicting. Auchampach et al. (2004) reported that reperfusion treatments with DPCPX and BG 9928, but not BG 9719, all of which are selective A₁AR antagonists, reduced infarct size in dogs by ~40%. These effects were compared to radioligand binding studies performed with recombinant canine ARs expressed in HEK cells, and blockade of canine A₁ (heart rate) and A_{2A}AR (coronary conductance) effects. Based on these observations, the authors concluded that DPCPX and BG 9928 may exert their infarct-reducing effects by blocking A_{2B}ARs; however, they could not discount the possibility that DPCPX and BG 9928 reduced infarct size by blocking A₁ARs.

Three additional studies in rabbit heart models of ischemia/reperfusion concluded that A_{2B}AR activation, rather than inhibition, contributes to reperfusion cardioprotection (Solenkova et al. 2006; Phillip et al. 2006; Kuno et al. 2007). In the first of these studies, the infarct-reducing effect of IPC was blocked by the A_{2B}AR antagonist, MRS1754 (*N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,

3-dipropyl-1*H*-purin-8-yl]phenoxy]-acetamide), but not an A_{2A}AR antagonist, CSC, administered at the onset of reperfusion. Subsequently, Phillip et al. (2006) reported that the cardioprotective effect of NECA administration at reperfusion (i.e., pharmacological postconditioning) in intact rabbits was blocked by MRS1754. Interestingly, a previous report from this same laboratory concluded that the reperfusion protection induced by NECA was due to A_{2A}AR activation (Xu et al. 2001). More recently, Kuno et al. (2007) demonstrated that a novel A_{2B}AR agonist, BAY 60–6583, administered during reperfusion, is protective. Given the apparent expression of multiple AR subtypes in the heart and their possible interactions, as well as the lack of selectivity for many of the commonly used AR agonists and antagonists, studies in AR KO mice will likely be needed to address the question of the A_{2B}AR, as well as the definitive roles of other AR subtypes. Interestingly, the results of a recent study by Eckle et al. (2007) indicated that in vivo IPC was ablated in A_{2B}AR KO mice, but not in mice lacking A₁, A_{2A} or A₃ receptors.

5 Reperfusion Injury and ARs in Human Myocardium

Despite all of the experimental evidence to date indicating the cardioprotective effects of adenosine and AR agonists, there have been very few studies examining the beneficial effects of these agents in humans in the setting of myocardial ischemia-reperfusion and thrombolysis. The initial such report was the acute myocardial infarction study of adenosine (AMISTAD) trial conducted between December 1994 and July 1997, the results of which were published in 1999 (Mahaffey et al. 1999). This was an open-label, placebo-controlled, randomized study to determine the safety and efficacy of adenosine as an adjunct to thrombolytic therapy in the treatment of acute myocardial infarction (MI). The effect of an intravenous infusion of adenosine (70 μg kg⁻¹ min⁻¹) for 3 h was compared to a placebo infusion in patients treated with thrombolysis within 6 h of the onset of an MI. After modification for slow enrollment, 197 patients were included, with the primary end-point being myocardial infarct size, as determined by Tc-99m sestamibi single-photon emission computed tomography (SPECT) imaging 5–7 days after enrollment. The results indicated that there was a 33% relative reduction in infarct size in patients that received adenosine ($p = 0.03$). Patients with an anterior MI exhibited a 67% relative reduction in infarct size, whereas there was no beneficial effect in patients with a nonanterior MI. Patients receiving adenosine, particularly those with nonanterior MI, experienced more bradycardia, heart block, hypotension and ventricular arrhythmias (Mahaffey et al. 1999).

There is a significant amount of preclinical data on the efficacy of AR agonists in reducing myocardial reperfusion injury, and these studies are clearly more consistently positive than the often contradictory findings with adenosine. Despite this wealth of information, today there remains only one documented clinical trial examining the effects of an AR agonist in the setting of clinical myocardial ischemia-reperfusion injury, the ADMIRE (AMP579 Delivery for Myocardial Infarction

REduction) study. This was a double-blind, multicenter, placebo-controlled trial of 311 patients undergoing primary percutaneous transluminal coronary angioplasty (PTCA) after acute ST-segment elevation MI (Kopecky et al. 2003). Patients were randomly assigned to placebo or to one of three different doses of AMP579 (15, 30 or 60 $\mu\text{g kg}^{-1}$) continuously infused over 6 h. This AR agonist, which has a high affinity for both A_1 and A_{2A} ARs, has been shown to reduce experimental myocardial ischemia-reperfusion in multiple species when administered both prior to ischemia or during reperfusion (Merkel et al. 1998; McVey et al. 1999; Meng et al. 2000; Xu et al. 2001; Kis et al. 2003; Kristo et al. 2004). The primary end-point was final myocardial infarct size measured by technetium Tc-99m sestamibi scanning at 120–216 h after PTCA. Secondary end-points included myocardial salvage and salvage index at the same time interval (in a subset of patients), left ventricular ejection fraction, duration of hospitalization, heart failure at 4–6 weeks, and cardiac events at four weeks and six months. Results indicated that there was no difference in final infarct size or in any of the secondary end-points. There was a trend towards increased myocardial salvage in patients with anterior MI. The authors of this study concluded that, based on the pharmacokinetic data, the maximal dose used in this trial was comparable to the lowest dose proven effective in animal studies.

The promising results of AMISTAD I led to a second trial (AMISTAD II) to determine the effects of adenosine infusion on clinical outcomes and infarct size in ST-segment elevation myocardial infarction (STEMI) patients undergoing reperfusion therapy (Ross et al. 2005). A total of 2,118 patients receiving thrombolysis or primary angioplasty were randomized to a 3 h infusion of either adenosine (50 or 70 $\mu\text{g kg}^{-1} \text{min}^{-1}$) or placebo. The primary end-point was new congestive heart failure (CHF) beginning >24 h after randomization, or the first rehospitalization for CHF, or death from any cause within six months. Infarct size was measured in a subset of 243 patients by Tc-99m sestamibi tomography. There was no effect of either adenosine dose on primary end-points, although patients receiving the higher dose (70 $\mu\text{g kg}^{-1} \text{min}^{-1}$) exhibited a median infarct size (11%) that was significantly lower ($p = 0.023$) than that of the placebo group (median infarct size 23%). It was concluded that a larger clinical trial was warranted to determine whether the decreased infarct size observed with adenosine was associated with enhanced long-term outcome. A post hoc subanalysis of these data indicated that patients receiving the adenosine infusion within 3 h of the onset of symptoms exhibited significantly reduced mortality at one and six months, and event-free survival was enhanced compared to patients treated with placebo (Kloner et al 2006).

Given all of the experimental evidence supporting the cardioprotective effects of AR agonists administered either prior to ischemia or during reperfusion, there clearly needs to more research and development into the synthesis, screening, and testing of potent, selective AR agonists. Basic scientists must also utilize consistent experimental models to determine the specific contributions of the multiple AR subtypes and their mechanisms of action. Because animal efficacy studies do not always translate to human efficacy, preclinical models with high relevance to humans and that closely simulate the human condition should be designed. Finally, clinical trials must be better designed along the lines of the information learned from the multitude of preclinical studies and clinical studies performed to date.

6 Impact of Age and Disease

Ischemic heart disease occurs predominantly in the elderly population (affecting up to 50% of those over 65), and can be associated with multiple underlying disease states, including atherosclerosis, hyperlipidemia, hypertension, and diabetes. From a clinical perspective, it is thus essential that protective strategies derived from research into PC or other protective modalities are effective across age groups and in diseased hearts. Unfortunately, aging limits or even abrogates protection with PC (Abete et al. 1996; Fenton et al. 2000; Schulman et al. 2001), AR activation (Gao et al. 2000; Schulman et al. 2001; Headrick et al. 2003b; Willems et al. 2005), and other GPCR stimuli (Peart et al. 2007). Newly discovered postconditioning is also impaired (Przyklenk et al. 2008). These age-dependent failures may stem from ineffective activation of key components of downstream signaling cascades (Peart et al. 2007; Przyklenk et al. 2008). On the other hand, age-related failure of AR-dependent protection is not universally observed. For example, Kristo et al. (2005) found no age-related changes in functional AR sensitivity, and augmentation of the infarct-sparing actions of adenosine. Thus, adenosine's role in aged hearts as well as the efficacy of cardioprotection in these hearts by targeting ARs with adenosine or AR agonists are questions that remain open.

Disease states underlying or contributing to ischemic disorders (when intrinsic protective responses such as PC are more important) can also impair these responses. For example, Ghosh et al. (2001) showed failure of PC in diabetic human myocardium, which may also reflect abnormalities in distal signaling cascades. In terms of AR responses, Donato et al. (2007) showed not only involvement of A₁ARs (and the mito K_{ATP} channel) in ischemic PC in normal hearts, but confirmed the ability of this stimulus to limit ischemic injury in hypercholesterolemic hearts. Moreover, A₁ and A₃ AR-triggered PC responses appear to be preserved in hypertrophic myocardium (Hochhauser et al. 2007). Thus, the few studies to date do support the preservation of AR-mediated protection in animal models of some relevant disease states. Whether this extends to patients suffering from chronic forms of cardiovascular disease remains to be established. It is worth considering that combined effects of age and disease may well underlie the rather modest benefit obtained with adenosine in clinical trials (AMISTAD I and II) versus the profound protective responses observed in the laboratory.

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Adenosine Receptors and Inflammation

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Abstract Extracellular adenosine is produced in a coordinated manner from cells following cellular challenge or tissue injury. Once produced, it serves as an autocrine- and paracrine-signaling molecule through its interactions with seven-membrane-spanning G-protein-coupled adenosine receptors. These signaling pathways have widespread physiological and pathophysiological functions. Immune

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cells express adenosine receptors and respond to adenosine or adenosine agonists in diverse manners. Extensive *in vitro* and *in vivo* studies have identified potent anti-inflammatory functions for all of the adenosine receptors on many different inflammatory cells and in various inflammatory disease processes. In addition, specific proinflammatory functions have also been ascribed to adenosine receptor activation. The potent effects of adenosine signaling on the regulation of inflammation suggest that targeting specific adenosine receptor activation or inactivation using selective agonists and antagonists could have important therapeutic implications in numerous diseases. This review is designed to summarize the current status of adenosine receptor signaling in various inflammatory cells and in models of inflammation, with an emphasis on the advancement of adenosine-based therapeutics to treat inflammatory disorders.

Keywords Adenosine · Adenosine Receptor · G-Protein Coupled Receptor · Inflammation

Abbreviations

A ₁ AR	Adenosine A ₁ receptor
A _{2A} AR	Adenosine A _{2A} receptor
A _{2B} AR	Adenosine A _{2B} receptor
A ₃ AR	Adenosine A ₃ receptor
AC	Adenylate cyclase
ADA	Adenosine deaminase
AR	Adenosine receptor
CD26	Dipeptidyl peptidase
CD39	Ectonucleoside triphosphate diphosphohydrolase
CD73	Ecto-5'-nucleotidase
CLP	Cecal ligation and puncture
CNTs	Concentrative nucleoside transporters
COPD	Chronic obstructive pulmonary disease
DC	Dendritic cell
ERK	Extracellular signal-related protein kinase
fMLP	Formyl methionyl-leucyl-phenylalanine
GRK	G-protein-coupled receptor kinase
HIF	Hypoxia-inducible factor
HMVECs	Human microvascular endothelial cells
HPAECs	Human pulmonary artery endothelial cells
HSP	Heat shock protein
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
IL	Interleukin

imDC	Immature dendritic cell
KO	Knockout
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NF- κ B	Nuclear factor kappa B
NK	Natural killer
NR	Normothermic recirculation
PAF	Platelet-activating factor
PBMCs	Peripheral blood mononuclear cells
PDCs	Plasmacytoid dendritic cells
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis toxin
TCR	T-cell receptor
TNF- α	Tumor necrosis factor alpha
Tregs	Regulatory T cells
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

1 Introduction

Adenosine is an endogenous signaling molecule that engages cell surface adenosine receptors to regulate numerous physiological and pathological processes (Fredholm et al. 2001). Extracellular adenosine is produced in excess in response to cellular stress, largely from the breakdown of released adenine nucleotides. Substantial evidence demonstrates that adenosine is an important signaling molecule and adenosine receptors are important molecular targets in the pathophysiology of inflammation. All inflammatory cells express adenosine receptors, and research into the consequences of adenosine receptor activation has identified numerous avenues for adenosine-based therapeutic intervention. Indeed, adenosine-based approaches are currently being developed for the treatment of various disorders where inflammatory modulation is a key component (reviewed in Jacobson and Gao 2006). This chapter was designed to review the contribution of adenosine and adenosine receptors to the regulation of key inflammatory and immune responses.

1.1 Adenosine Production and Metabolism

Regulation of extracellular adenosine levels is orchestrated by the actions of proteins that regulate adenosine production, metabolism and transport across the plasma

membrane. The release and catabolism of adenine nucleotides to adenosine is believed to be the major route of adenosine production following cellular stress or injury. Possible routes of ATP release include the constitutive release of ATP through vesicle fusion with the plasma membrane, and programmed release through membrane channels such as the ATP binding cassette family of membrane transporters, including the cystic fibrosis transmembrane conductance regulator (Reisin et al. 1994) and multiple drug resistance channels (Roman et al. 2001), connexin hemichannels (Cotrina et al. 1998), maxi-ion channels (Bell et al. 2003), stretch-activated channels (Braunstein et al. 2001) and voltage-dependent anion channels (Okada et al. 2004). A number of different cell types are sources of adenine nucleotides, including platelets, neurons, and endothelial cells. In addition, inflammatory cells such as mast cells (Marquardt et al. 1984), neutrophils (Madara et al. 1993) and eosinophils (Resnick et al. 1993) are able to release adenine nucleotides and adenosine into the local environment.

Extracellular ATP is rapidly dephosphorylated by ectonucleoside triphosphate diphosphohydrolases such as CD39 to form ADP and AMP (Kaczmarek et al. 1996), and extracellular AMP is dephosphorylated to adenosine by the 5'-nucleotidase CD73 (Resta et al. 1998). CD39 and CD73 are widely expressed on the surface of cells and are essential for the production of adenosine following cellular stress or injury (Thompson et al. 2004; Volmer et al. 2006). Recent findings demonstrate that CD73 and CD39 are novel markers on regulatory T cells (Tregs), where they serve to convert extracellular adenine nucleotides to adenosine, which in turn promotes immunosuppressive activities (Kobie et al. 2006; Deaglio et al. 2007). This process is an example of the concerted role of extracellular adenosine production and signaling in the regulation of inflammatory processes. Adenosine is also generated inside cells by either the dephosphorylation of AMP by cytosolic nucleotidases (Sala-Newby et al. 1999) or the hydrolysis of *S*-adenosylhomocysteine (Hermes et al. 2005). Alterations in cellular metabolic load or methylation reactions that utilize *S*-adenosylmethionine as a methyl donor can lead to increased intracellular adenosine levels and subsequent release.

Adenosine is transported across the plasma membrane by both facilitated and cotransport mechanisms. The facilitated nucleoside transporters, known as the equilibrative nucleoside transporters, are bidirectional transporters (Baldwin et al. 2004). They are widely distributed in mammalian tissues, and play a major role in transporting adenosine in and out of the cell. Adenosine transported across the cell membrane also occurs through concentrative nucleoside transporters (CNTs), which are Na⁺-dependent concentrative transporters (Gray et al. 2004). The tissue distributions of the CNTs vary, with CNT1 localized primarily to epithelial cells, while CNT2 and CNT3 are more widely distributed.

Finally, adenosine is metabolized by one of two pathways. It can be phosphorylated to form AMP intracellularly by the enzyme adenosine kinase (Spychala et al. 1996), or it can be deaminated to inosine by adenosine deaminase (ADA) (Blackburn and Kellems 1996). ADA is a predominantly cytosolic enzyme. However, it is also found outside the cell as a component of plasma. In humans,

ADA can complex with the cell surface protein CD26 (Hashikawa et al. 2004). This interaction may play an important role in localizing adenosine metabolism to certain regions of the cell surface to impact adenosine signaling. These enzymes, together with rapid cellular uptake, serve to regulate the levels of intra- and extracellular adenosine. In homeostatic situations, adenosine levels range from 10 to 200 nM, whereas extracellular adenosine levels can be elevated to 10–100 μ M in hypoxic or stressed tissue environments (Fredholm 2007). The concerted production and metabolism of adenosine is an important mechanism that contributes to the ability of this signaling molecule to regulate aspects of immunobiology and tissue homeostasis.

1.2 Adenosine Receptors

Adenosine exerts its effects by interacting with receptors located on the cell surface. Four adenosine receptor subtypes, A₁, A_{2A}, A_{2B} and A₃, have been defined by pharmacological and molecular biological approaches (Fredholm et al. 2001). These receptors belong to the superfamily of G-protein-coupled receptors and are characterized by seven-transmembrane-spanning α -helical domains with an extracellular amine terminus and a cytoplasmic carboxy terminus. Receptor subtypes are distinguished based on their affinity for adenosine, pharmacological profiles, G-protein coupling and signaling pathways, and genetic sequence. The physiological effects of adenosine are mediated by intracellular signaling processes that are specific to the receptor subtype and the type of cell. The adenosine A₁ receptor (A₁AR) is coupled to the pertussis toxin (PTX)-sensitive inhibitory G proteins (G_i) or G_o. Activation of the A₁AR can lead to the activation a number of effector systems, including adenylylate cyclase (AC), phospholipase A₂, phospholipase C (PLC), potassium channels, calcium channels, and guanylate cyclase (Akbar et al. 1994; Olah and Stiles 2000; Fredholm et al. 2001). The primary changes in second messengers associated with A₁AR activation are decreased production of cAMP or increased Ca²⁺, depending on the effector system. Like the A₁AR, the adenosine A₃ receptor (A₃AR) is coupled to the PTX-sensitive G_i protein and also to G_q (Fredholm et al. 2001). Activation of the A₃AR results in an inhibition of AC (leading to decreased cAMP) or stimulation of PLC and phospholipase D (Gessi et al. 2008). The adenosine A_{2A} receptor (A_{2A}AR) and adenosine A_{2B} receptor (A_{2B}AR) share a relatively high homology and are coupled to G_s (Fredholm et al. 2001), leading to increased levels of cAMP. In addition, the A_{2B}AR has been shown to couple to G_q (Feoktistov et al. 2002), thereby regulating intracellular Ca²⁺ levels. In general, the A₁AR, A_{2A}AR and A₃AR subtypes have high affinity for adenosine, while the A_{2B}AR has a lower affinity (Fredholm 2007).

2 Adenosine Receptors on Immune Cells

2.1 Neutrophils

Neutrophils are the most abundant leukocyte and represent the body's first line of defense in response to a pathogenic challenge; they are the predominant leukocyte involved in acute inflammation (Burg and Pillinger 2001; Edwards 1994; Witko-Sarsat et al. 2000). All four adenosine receptor subtypes are expressed on neutrophils (Bours et al. 2006; Marone et al. 1992; Fortin et al. 2006; Fredholm 2007). At submicromolar adenosine concentrations, A₁AR activation on human neutrophils produces a proinflammatory response by promoting chemotaxis and adherence to the endothelium (Bours et al. 2006; Cronstein et al. 1990, 1992; Forman et al. 2000; Rose et al. 1988). A₁AR-mediated chemotaxis in neutrophils is disrupted by PTX, an agent that inhibits the function of G_i-linked receptors, and requires an intact microtubule system (Cronstein et al. 1990, 1992).

Activation of A_{2A}AR and A_{2B}ARs on neutrophils is anti-inflammatory. High concentrations (micromolar) of adenosine inhibit neutrophil adhesion to endothelial cells by activating A_{2A}AR and A_{2B}ARs on neutrophils (Bours et al. 2006; Eltzschig et al. 2004; Sullivan et al. 2004; Thiel et al. 1996; Wakai et al. 2001). In human neutrophils, A_{2A}AR activation inhibits the formation of reactive oxygen species (Cronstein et al. 1983, 1990; Salmon and Cronstein 1990). In addition, A_{2A}AR activation inhibits the adherence of *N*-formyl methionyl-leucyl-phenylalanine (fMLP)-activated neutrophils to endothelium (Cronstein et al. 1992) and downregulates Mac-1 (Wollner et al. 1993), β_2 -integrin (Thiel et al. 1996; Zalavary and Bengtsson 1998), and L-selectin (Thiel et al. 1996). Activation of the A_{2A}AR also downregulates the activity of other endothelial cell surface proteins, including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) (McPherson et al. 2001), alpha 4/beta 1 integrin VLA4 (Sullivan et al. 2004), and platelet cell adhesion molecule (Cassada et al. 2002). Activation of A_{2A}ARs on activated human neutrophils produces an anti-inflammatory effect by decreasing the formation of the proinflammatory cytokine tumor necrosis factor alpha (TNF- α) (Harada et al. 2000, Thiel and Chouker 1995), chemokines such as macrophage inflammatory protein (MIP)-1 α /CCL3, MIP-1 β /CCL4, MIP-2 α /CXCL2, and MIP-3 α /CCL20 (McColl et al. 2006), and leukotriene LTB₄ (Flamand et al. 2000, 2002; Grenier et al. 2003; Krump et al. 1996, 1997; Krump and Borgeat 1999; Surette et al. 1999), and platelet activating factor (PAF) (Flamand et al. 2006). Other important immunoregulatory effects mediated by the A_{2A}AR include the inhibition of Fc gamma (Fc γ) receptor-mediated neutrophil phagocytosis and inhibition of degranulation (Bours et al. 2006; Cronstein et al. 1983; Harada et al. 2000; Salmon and Cronstein 1990; Sullivan et al. 1999; Visser et al. 2000; Zalavary et al. 1994, Zalavary and Bengtsson 1998). Activation of the A_{2B}AR inhibits neutrophil extravasation across human umbilical vein endothelial cell (HUVEC) monolayers and inhibits the release of vascular endothelial growth factor (VEGF) (Wakai et al. 2001).

Conflicting reports suggest that activation of A₃ARs on neutrophils may produce proinflammatory or anti-inflammatory effects. Studies with A₃AR knockout mice suggest that the A₃AR promotes recruitment of neutrophils to lungs during sepsis (Inoue et al. 2008). Moreover, A₃ARs play an important role in the migration of human neutrophils in response to chemoattractant molecules released by microbes (Chen et al. 2006). In isolated human neutrophils, extracellular adenosine (1–1,000 nM) induces a redistribution of A₃ARs to the neutrophil's leading edge, the portion of the membrane closest to the chemoattractant stimulus (Chen et al. 2006). In addition, selective A₃AR antagonists inhibit fMLP-mediated chemotaxis in human neutrophils (Chen et al. 2006). In other studies, activation of A₃ARs on human neutrophils has been shown to counteract inflammation by inhibiting degranulation and oxidative burst (Bouma et al. 1997; Fishman and Bar-Yehuda 2003; Gessi et al. 2002).

2.2 *Monocytes and Macrophages*

Monocytes and macrophages are a heterogeneous group of mononuclear cells that present an early line of innate immune defense. They represent a primary source of inflammatory modulators and are highly adaptable with a phenotype that can change rapidly in response to the local environment of the inflamed tissue (Hasko et al. 2007; Rutherford et al. 1993). Macrophages also serve an important role in terminating the inflammatory process, which is critical for preventing excessive tissue injury (Duffield 2003; Gilroy et al. 2004; Hasko et al. 2007; Wells et al. 2005; Willoughby et al. 2000). All four adenosine receptors are expressed on monocytes and macrophages, although expression levels differ markedly throughout the maturation and differentiation process (Eppell et al. 1989; Thiele et al. 2004). In quiescent monocytes, adenosine receptor expression is low and is increased following activation by inflammatory stimuli. It is hypothesized that the temporal changes in the expression of adenosine receptor subtypes play an important role in the resolution of inflammation. In human monocytes, A₁AR activation produces a proinflammatory effect whereas A_{2A}AR activation produces an anti-inflammatory effect. A key function of the A₁AR is a rapid enhancement of the activity of the Fcγ receptor (Salmon et al. 1993). Activation of A_{2A}ARs limits inflammatory reactions by inhibiting phagocytosis in monocytes (Salmon et al. 1993) and macrophages (Eppell et al. 1989), decreasing the production of reactive oxygen species (Thiele et al. 2004), and altering cytokine release. In addition, A₃AR activation inhibits fMLP-triggered respiratory burst in human monocytes (Broussas et al. 1999).

Monocytes and macrophages are a primary source of TNF-α, a proinflammatory cytokine involved in the pathophysiology of a number of chronic inflammatory diseases. Early studies suggested that activation of the A_{2A}AR suppresses production of TNF-α in human monocytes activated by bacterial lipopolysaccharide (LPS) (Le Vraux et al. 1993). In primary cultures of human monocytes activated by LPS (Zhang et al. 2005) and LPS-stimulated mouse macrophages (Ezeamuzie and Khan

2007), activation of the A_{2A}AR attenuated the release of TNF- α , whereas activation of the A₁AR and A₃AR subtypes had no effect on the formation of TNF- α (Zhang et al. 2005). Similar results were obtained in studies with primary cultures of mouse peritoneal macrophages, in which activation of the A_{2A}AR inhibited LPS-induced TNF- α release, while activation of the A₃AR had no effect (Kreckler et al. 2006). In other studies, activation of the A₃AR was shown to inhibit LPS-induced TNF- α release in vitro in the RAW 264.7 murine leukemia macrophage line (Haskó et al. 1996; Martin et al. 2006), U937 human leukemic macrophage cell line (Sajjadi et al. 1996), murine J774.1 macrophages (Bowlin et al. 1997; McWhinney et al. 1996) and in vivo in endotoxemic mice (Haskó et al. 1996). In the RAW 264.7 macrophage line, the inhibitory effects of A₃ARs were mediated by a mechanism involving Ca²⁺-dependent activation of nuclear factor-kappa B (NF- κ B) (Martin et al. 2006).

Interleukin (IL)-12. IL-12 is a proinflammatory cytokine that is produced in response to certain bacterial and parasitic infections. IL-12 activates naïve T lymphocytes to mount a T helper 1 response. The production of IL-12 is modulated by adenosine and ARs (Haskó et al. 1998, 2000, 2007; Le Vraux et al. 1993; Link et al. 2000). Pharmacological studies (Haskó et al. 2000; Le Vraux et al. 1993; Link et al. 2000) and studies with A_{2A}AR knockout (KO) mice (Haskó et al. 2000) have demonstrated that A_{2A}AR activation downregulates IL-12 production, thereby producing an anti-inflammatory response. In human peripheral monocytes, A_{2A}AR activation decreases IL-12 and IL-12p40 (Link et al. 2000). The effects of the A_{2A}AR on IL-12 production are strongly influenced by the presence of proinflammatory cytokines (Khoa et al. 2001). In THP-1 monocytic cells, TNF- α and IL-1 enhanced A_{2A}AR-mediated inhibition of IL-12 production, whereas interferon (IFN)- γ attenuated A_{2A}AR-mediated inhibition of IL-12 production (Khoa et al. 2001). The effects of TNF- α and IL-1 were associated with an upregulation of A_{2A}ARs, while IFN- γ effects were associated with downregulation of A_{2A}ARs.

Activation of the A₃AR negatively regulates the synthesis of IL-12 in murine RAW 264.7 macrophages (Szabo et al. 1998), human monocytes (la Sala et al. 2005), and mice treated with LPS (Haskó et al. 1998). The A₃AR-mediated effects appear to be mediated through the phosphatidylinositol 3-kinase signaling pathway (la Sala et al. 2005). Taken together, these studies suggest an anti-inflammatory role for the A₃AR via negative regulation of IL-12.

IL-10. IL-10 is an anti-inflammatory cytokine (Kotenko 2002; Moore et al. 1993, Mosmann 1994; Haskó et al. 2007) that functions by inhibiting the secretion of proinflammatory cytokines, including TNF- α and IL-12 (Moore et al. 2001). IL-10 is produced by T helper 2 cells, monocytes, and macrophages (Moore et al. 2001). Following the induction of proinflammatory cytokines, IL-10 regulates the termination of inflammatory processes. Both the A_{2A}AR and A_{2B}AR subtypes have been implicated in the stimulation of IL-10 production in monocytes and macrophages (Haskó et al. 1996, 2000, 2007; Khoa et al. 2001; Link et al. 2000; Nemeth et al. 2005).

Other cytokines, chemokines, and adhesion molecules. Treatment of peripheral blood mononuclear cells (PBMCs) with IL-18, a proinflammatory cytokine released by T cells and dendritic cells, results in increased TNF- α , IL-12, IFN- γ release,

and increased expression of ICAM-1 (Takahashi et al. 2003). In PBMCs, adenosine inhibited the IL-18-induced release of TNF- α , IL-12, and IFN- γ , and expression of ICAM-1. This inhibitory effect was mimicked by an A_{2A}AR agonist and blocked by A_{2A}AR antagonism (Takahashi et al. 2007a). Moreover, the A_{2A}AR-mediated anti-inflammatory effects on the IL-18-induced production of TNF- α , IL-12, IFN- γ , and ICAM-1 were reversed by an A₁AR agonist and an A₃AR agonist. The results of these studies suggest that the anti-inflammatory effect of adenosine on human PBMCs activated by IL-18 occurs by activation of the A_{2A}AR; however, an A₁AR proinflammatory effect predominates when the A_{2A}AR is saturated with agonist. Thus, the net effect of adenosine on PBMCs activated by IL-18 is a function of the activation of multiple adenosine receptor subtypes, including an anti-inflammatory effect via A_{2A}ARs and proinflammatory effects via A₁AR and A₃ARs (Takahashi et al. 2007a).

With respect to activation of A_{2B}AR and A₃ARs on monocytes and macrophages, in both in vivo and in vitro studies, activation of the A_{2B}AR induces the release of the proinflammatory cytokine IL-6 from macrophages (Ryzhov et al. 2008a), and activation of the A₃AR inhibits the production of MIP- α in LPS-stimulated RAW 264.7 macrophages (Szabo et al. 1998) and inhibits tissue factor expression in LPS-stimulated human macrophages (Broussas et al. 2002). In human monocytes, A₁AR activation induces the release of VEGF (Clark et al. 2007).

2.3 Dendritic Cells

Dendritic cells (DCs) are highly specialized antigen-presenting cells that play an important role in the initiation and regulation of immune responses by migrating to sites of injury and infection, processing antigens, and activating naive T cells (Banchereau and Steinman 1998; Macagno et al. 2007). Immature DCs (imDCs) undergo a maturation process following exposure to proinflammatory signals, including pathogens, LPS, TNF- α , IL-1, and IL-6 (Banchereau and Steinman 1998). The maturation process results in decreased phagocytic activity and increased expression of membrane major histocompatibility complex (MHC), CD54, CD80, CD83, and CD86. Mature DCs release a number of cytokines, including TNF- α , IL-12 and IL-10. IL-12 is a major contributor to the differentiation of Th1 cells. In human blood, DCs are classified as the CD1c⁺ DCs and the CD123⁺ DCs (Shortman and Liu 2002). CD123⁺ DCs, also known as plasmacytoid DCs (PDCs), are located in blood and secrete IFN- γ (Siegal et al. 1999). In addition, PDCs are powerful regulators of T-cell responses (Gilliet and Liu 2002; Kadowaki et al. 2000).

Adenosine receptors are differentially expressed on human DCs (Fossetta et al. 2003; Hofer et al. 2003; Panther et al. 2001, 2003; Schnurr et al. 2004). Immature, undifferentiated human DCs express mRNAs for the A₁AR, A_{2A}AR and A₃AR but not for the A_{2B}AR (Fossetta et al. 2003; Hofer et al. 2003; Panther et al. 2001; Schnurr et al. 2004). Activation of the A₁AR and A₃AR subtypes in undifferentiated DCs induces chemotaxis and mobilization of intracellular Ca²⁺, while activation of

the A_{2A}AR subtype has no effect (Panther et al. 2001; Fossetta et al. 2003). Activation of the A_{2A}AR, but not A₁AR and A₃ARs, in imDCs is linked to increased cell surface expression of CD80, CD86, human leukocyte antigen-DR, and MHC-I (Panther et al. 2003). Activation of A₁ARs in resting DCs suppresses vesicular MHC class I cross-presentation by a G_i-mediated pathway (Chen et al. 2008).

Following treatment with LPS to induce differentiation and maturation, human DCs primarily express the A_{2A}AR (Fossetta et al. 2003; Panther et al. 2001). Activation of the A_{2A}AR increases AC activity and inhibits production of the proinflammatory cytokine IL-12, thereby reducing the ability of the DC to promote the differentiation of T cells to the Th-1 phenotype, and stimulates the production of the anti-inflammatory cytokine IL-10 (Banchereau and Steinman 1998; Panther et al. 2001, 2003).

In immature PDCs, adenosine acting via the A₁AR promotes the migration of PDCs to the site of infection. As PDCs differentiate and mature, the expression of the A₁AR is downregulated, corresponding to a decrease in migratory capability. In mature PDCs, the A_{2A}AR is the predominant subtype and A_{2A}AR activation decreases the production of IL-6, IL-12 and IFN- α (Schnurr et al. 2004). Moreover, IL-3-induced maturation of human PDCs results in a downregulation of A₁ARs and an upregulation of A_{2A}ARs (Schnurr et al. 2004). The mouse DC line XS-106 expresses functional adenosine A_{2A}AR and A₃ARs (Dickenson et al. 2003). A_{2A}AR activation increases cAMP levels and p42/p44 mitogen-activated protein kinase (MAPK) phosphorylation, whereas activation of the A₃AR inhibits cAMP accumulation and increases in p42/p44 MAPK phosphorylation. Functionally, the activation of both subtypes produces a partial inhibition of LPS-induced release of TNF- α .

2.4 Lymphocytes

Lymphocytes are critically involved in adaptive immunity (Alam and Gorska 2003; Larosa and Orange 2008). Adenosine regulates multiple physiologic processes and inflammatory actions on lymphocytes (Bours et al. 2006; Marone et al. 1986, 1992; Priebe et al. 1988, 1990a, b, c). In early studies, it was demonstrated in mixed human lymphocytes that R-PIA (*N*⁶-*R*-phenylisopropyladenosine) and low concentrations of adenosine (1–100 nM) inhibit cAMP accumulation in human lymphocytes via an A₁AR mechanism, while high concentrations of adenosine (100 nM–100 μ M) stimulate cAMP via an A_{2A}AR mechanism (Marone et al. 1986, 1992).

CD4⁺ and CD8⁺ T lymphocytes express A_{2A}AR, A_{2B}AR, and A₃ARs (Gessi et al. 2004, 2005; Huang et al. 1997; Hoskin et al. 2008; Koshiba et al. 1997, 1999; Mirabet et al. 1997). In activated human CD4⁺ and CD8⁺ T lymphocytes, A_{2B}AR expression is increased and A_{2B}AR activation is linked to decreased IL-2 production (Mirabet et al. 1999). Activation of human CD4⁺ T lymphocytes with phytohemagglutinin results in increases in A₃AR mRNA and protein levels that are accompanied by increased agonist potency (Gessi et al. 2004).

A number of studies suggest that A_{2A} AR engagement on $CD4^+$ T lymphocytes results in anti-inflammatory effects. In mouse $CD4^+$ T lymphocytes, A_{2A} AR engagement inhibits T-cell receptor (TCR)-mediated production of IFN- γ (Lappas et al. 2005). TCR activation results in A_{2A} AR mRNA upregulation, which functions as an anti-inflammatory mechanism for limiting T-cell activation and subsequent macrophage activation in inflamed tissues (Lappas et al. 2005). In vitro and in vivo studies suggest that the A_{2A} ARs selectively inhibit TCR-activated T cells, thereby inhibiting lymphocyte inflammatory activity (Apasov et al. 1995, 2000; Erdmann et al. 2005; Huang et al. 1997; Lappas et al. 2005). Activation of the A_{2A} AR on $CD4^+$ T lymphocytes prevents myocardial ischemia-reperfusion injury by inhibiting the accumulation and activation of $CD4^+$ T cells in the reperfused heart (Yang et al. 2006b). Moreover, an anti-inflammatory role in chronic inflammation was demonstrated for the A_{2A} AR in an in vivo murine model of inflammatory bowel disease, where activation of the A_{2A} AR attenuated the production of IFN- γ , TNF- α , and IL-4 in mesenteric T lymphocytes in a rabbit model of colitis (Odashima et al. 2005).

In a mixed lymphocyte reaction of human PBMCs and lymphocytes, adenosine-induced inhibition of IL-18-induced increases in IL-12, IFN- γ , ICAM-1, and lymphocyte proliferation was blocked by an A_{2A} AR antagonist, ZM-241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol), was enhanced by an A_1 AR antagonist, DPCPX (8-cyclopentyl-1,3-dipropylxanthine), and an A_3 AR antagonist, MRS1220 (*N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-yl]benzene acetamide), and was not affected by an A_{2B} AR antagonist (Takahashi et al. 2007b). Moreover, the anti-inflammatory effect of an A_{2A} AR agonist, CGS 21680 (2-(*p*-(2-carnonylethyl) phenylethylamino)-5-*N*-ethylcarboxamido adenosine) on IL-18-induced increases in IL-12, IFN- γ , ICAM-1, and lymphocyte proliferation were reversed by A_1 AR and A_3 AR agonists. These results suggest that the anti-inflammatory effects of adenosine in a mixed lymphocyte reaction are mediated by A_{2A} ARs; however, an A_1 AR proinflammatory effect predominates when the A_{2A} AR is saturated with agonist. As such, the net effect of adenosine on a mixed lymphocyte reaction activated by IL-18 is a function of activation of multiple adenosine receptor subtypes, including an anti-inflammatory effect via A_{2A} ARs and proinflammatory effects via A_1 AR and A_3 ARs (Takahashi et al. 2007b).

In primary cultures of B lymphocytes, activation of B-cell antigen receptors results in the activation of NF- κ B pathways (Minguet et al. 2005). Adenosine inhibits the NF- κ B pathway by a mechanism related to increased cAMP levels and activation of protein kinase A. This study suggests that adenosine-mediated signals represent an important step in mediating the activation of B lymphocytes.

In activated human and mouse natural killer (NK) cells, adenosine inhibited the production of cytokines and chemokines (Raskovalova et al. 2005, 2006). In in vitro studies with lymphocytes derived from mouse spleen, A_1 AR activation increased NK cell activity while A_{2A} AR activation decreased NK cell activity (Priebe et al. 1990a). In mouse LAK cells, the adenosine agonist CADO (2-chloroadenosine) inhibited the cytotoxic activity and attenuated the production of IFN- γ , granulocyte

macrophage colony-stimulating factor, TNF- α , and MIP-1 α (Lokshin et al. 2006). Taken together, these results suggest that elevated adenosine levels in tumors may inhibit the tumoricidal effects of activated NK cells (Raskovalova et al. 2005; Lokshin et al. 2006). In addition, recent studies have shown that adenosine exhibits anti-inflammatory activities by engaging A_{2A}ARs on regulatory cells (Deaglio et al. 2007).

2.5 Mast Cells

Mast cells are important effector cells of allergic diseases such as asthma (Shimizu and Schwartz 1997). They can be stimulated to release mediators that have both immediate and chronic effects on airway constriction and inflammation. Adenosine can impact both the degranulation of mast cells and the production of inflammatory mediators. Rodent and human mast cells express the A_{2A}AR, A_{2B}AR and A₃AR (Feoktistov and Biaggioni 1995; Salvatore et al. 2000; Zhong et al. 2003b; Ryzhov et al. 2008b). Engagement of the A₃AR on rodent mast cells mediates degranulation in a manner that involves phosphoinositide 3-kinase (PI3K) activation and increase in intracellular Ca²⁺ (Salvatore et al. 2000; Zhong et al. 2003b). With regards to humans, it is not clear which adenosine receptor mediates the degranulation of mast cells, particularly in the airways. However, emerging evidence suggests that the A_{2B}AR mediates the production and release of proinflammatory mediators such as IL-8, IL-4 and IL-13 from both mouse and human mast cells (Feoktistov and Biaggioni 1995; Ryzhov et al. 2004, 2008b). The role of A₃AR and A_{2B}AR contributions to mast cell degranulation and the production of mediators are areas of active research that will aid in the development of adenosine-based therapeutics for diseases such as asthma, where mast cells play an important role.

2.6 Eosinophils

Eosinophils are involved in the pathophysiology of allergic diseases, including asthma (Frigas and Gleich 1986; Frigas et al. 1991; Gleich et al. 1983). During airway inflammation, eosinophils infiltrate tissues and release inflammatory mediators, including leukotrienes, reactive oxygen species, and granular proteins such as major basic protein. Activation of the A₁AR on human eosinophils enhances O₂⁻ release (Ezeamuzie and Philips 1999), whereas activation of A₃ARs on human eosinophils elevates intracellular Ca²⁺ (Kohno et al. 1996), inhibits PAF-induced chemotaxis (Knight et al. 1997; Walker et al. 1997), inhibits C5a-induced degranulation (Ezeamuzie and Philips 1999, 2001), and inhibits C5a-induced O₂⁻ release (Ezeamuzie et al. 1999). Eosinophils isolated from the lungs of patients with airway inflammation have higher levels of A₃AR mRNA compared to controls (Walker et al. 1997). In contrast to these findings, adenosine and A₃AR engagement has been shown to have proinflammatory effects on mouse (Young et al. 2004) and

guinea pig eosinophils (Walker 1996). Together, these studies have led to the suggestion that selective A_3 AR ligands may be useful therapies for the treatment of eosinophil-dependent inflammatory disorders such as asthma.

2.7 Endothelial Cells

Under normal physiological conditions, the endothelium provides several important regulatory and protective functions by serving as a physical barrier with both anti-coagulant and anti-inflammatory properties (Hordijk 2006; Mehta and Malik 2006; Sands and Palmer 2005). An initiating event in inflammation is the recruitment and adhesion of leukocytes to the vascular endothelium and changes in endothelial permeability that permit the passage of leukocytes out of the vasculature and into the site of infection or tissue damage.

Adenosine receptors are expressed heterogeneously on endothelial cells, with the predominant subtypes generally being A_{2A} AR and A_{2B} AR (Deguchi et al. 1998; Feoktistov et al. 2002, 2004; Iwamoto et al. 1994; Khoa et al. 2003; Lennon et al. 1998; Olanrewaju et al. 2000; Sexl et al. 1997). In cell culture, endothelial cells derived from different sources have unique expression patterns of adenosine receptor subtypes (Feoktistov et al. 2002, 2004; Khoa et al. 2003). For example, mRNA levels of the A_{2A} AR are approximately tenfold greater than mRNA levels for the A_{2B} AR in HUVECs, whereas mRNA expression of the A_{2B} AR is approximately fourfold greater than A_{2A} AR mRNA expression levels in human microvascular endothelial cells (HMVECs) (Feoktistov et al. 2002). In endothelial cells, activation of the A_{2A} AR inhibits the expression of VCAM-1 (Zernecke et al. 2006), E-selectin (Bouma et al. 1996; Hasko and Cronstein 2004), and tissue factor (Deguchi et al. 1998). Furthermore, activation of the A_{2A} AR (Sullivan et al. 1999) and A_{2B} AR (Eltzschig et al. 2003; Lennon et al. 1998; Yang et al. 2006a) is associated with decreased permeability of the vascular endothelium. These studies suggest that the A_{2A} AR and A_{2B} AR on endothelial cells play an important role in the prevention and mitigation of the inflammatory process. As opposed to these anti-inflammatory effects, activation of A_1 ARs on human pulmonary artery endothelial cells (HPAECs) induces the release of thromboxane A_2 and IL-6, substances that are cytotoxic to endothelial cells and increase endothelial permeability (Wilson and Batra 2002). A_1 AR antagonists prevented endothelial adhesion and digestion of the endothelial plasmalemma of alveolar capillaries by granulocytes, as well as the diapedesis of neutrophils toward the alveolar lumen in endotoxin-induced acute lung injury (Neely et al. 1997). In addition, activation of A_1 ARs and A_3 ARs on stimulated HUVECs results in an upregulation and downregulation of tissue factor expression, respectively, representing a potential mechanism for regulating the procoagulant activity of vascular endothelial cells in vivo by adenosine receptors (Deguchi et al. 1998).

3 Regulation of Adenosine Receptor Expression in Inflammatory Environments

Adenosine receptor expression is under dynamic regulation during various forms of physiological and pathophysiological stress, including hypoxia/ischemia and inflammation. For example, a distinct time-dependent alteration in adenosine receptor levels was observed in primary HMVECs subjected to hypoxic culture conditions (Eltzschig et al. 2003). After 12 h, hypoxia induced a selective upregulation of the A_{2B}AR, while at later time points (18 and 24 h), expression levels of the A₁AR and A_{2A}AR were downregulated and expression levels of the A₃AR were unchanged. This example demonstrates how a single stimulus can lead to complex alterations in adenosine receptor subtype expression.

Expression of the A₁AR is upregulated under conditions of stress. Numerous studies have demonstrated that stress-induced upregulation of the A₁AR involves increased transcriptional regulation by NF- κ B, including *in vitro* oxidative stress (Nie et al. 1998), *in vivo* oxidative stress (Ford et al. 1997), *in vivo* cerebral ischemia (Lai et al. 2005), *in vitro* hyperosmotic stress (Pingle et al. 2004), *in vivo* exposure to LPS (Jhaveri et al. 2007), cardiac dysfunction induced by TNF- α overexpression (Funakoshi et al. 2007), and sleep deprivation stress (Basheer et al. 2007). Studies with genetically modified mice lacking the p50 subunit of NF- κ B underscore the role of NF- κ B in regulating A₁AR expression under basal conditions and pathogenic conditions (Jhaveri et al. 2007). LPS, an activator of NF- κ B, increases A₁AR expression levels in the cortices of wild-type but not NF- κ B p50 KO mice. In addition, expression of the A₁AR is upregulated in the bronchial epithelium and bronchial smooth muscle of asthmatics (Brown et al. 2008).

In a number of different cell types, the expression of the A_{2A}AR increases following exposure to proinflammatory conditions (Thiel et al. 2003). Following exposure to proinflammatory cytokines, including TNF- α and IL-1 β , the expression and functional activity of the A_{2A}AR increases in cultured human monocytic THP-1 cells (Khoa et al. 2001), HMVECs (Nguyen et al. 2003), isolated human neutrophils (Fortin et al. 2006), and A549 human lung epithelial cells (Morello et al. 2006). In A549 cells, the upregulation of A_{2A}AR expression is regulated by NF- κ B (Morello et al. 2006). Conversely, IFN- γ downregulates A_{2A}AR expression in THP-1 cells (Khoa et al. 2001) and HMVECs (Nguyen et al. 2003). Following exposure to LPS, mRNA levels for the A_{2B}AR and A₃AR were slightly upregulated in primary mouse intraperitoneal macrophage and WEHI-3 cells (Murphree et al. 2005); however, A_{2A}AR mRNA levels increased dramatically. The increased transcription of A_{2A}AR mRNA in the mouse intraperitoneal macrophages occurred via an NF- κ B pathway. In WEHI-3 cells, the LPS-induced upregulation of A_{2A}AR mRNA was accompanied by an increase in cell surface A_{2A}AR expression and increased A_{2A}AR agonist-mediated cAMP production. Functionally, A_{2A}AR agonists inhibited TNF- α production with greater potency in the LPS-treated mouse intraperitoneal macrophages as compared to untreated control cells. These findings demonstrate the role of inflammatory stimuli in the upregulation of A_{2A}AR signaling.

In models of inflammatory bowel disease, characterized by altered levels of proinflammatory cytokines and local tissue hypoxia (Taylor and Colgan 2007), the expression of A₁AR, A₃AR (Sundaram et al. 2003) and A_{2B}ARs (Kolachala et al. 2005a) are altered. In a rabbit model of chronic ileitis, transcription of the A₁AR and A₃AR is upregulated in the ileum (Sundaram et al. 2003). The A_{2B}AR is upregulated in intestinal epithelia of a mouse model of colitis and in human intestinal epithelial mucosa during active colitis (Kolachala et al. 2005a). In T84 human colonic mucosal epithelial cells, TNF- α increases A_{2B}AR mRNA and protein levels (Kolachala et al. 2005a). IFN- γ inhibits A_{2B}AR-mediated effects without changing protein expression or A_{2B}AR membrane recruitment (Kolachala et al. 2005b). Thus, in inflammatory conditions of the bowel, the regulation of the low-affinity A_{2B}AR occurs via direct effects on receptor expression and indirect effects on signal transduction pathways (Kolachala et al. 2005a, b).

Expression of the A_{2B}AR is upregulated during hypoxic and ischemic conditions (Linden 2001; Eltzschig et al. 2003; Zhong et al. 2005). In primary cultures of human lung fibroblasts, hypoxia induces an increase in A_{2B}AR expression levels (Zhong et al. 2005). Moreover, activation of the A_{2B}AR acts synergistically with hypoxia to increase the release of IL-6 from fibroblasts and promotes differentiation to myofibroblasts, suggesting that the upregulation of the A_{2B}AR may be relevant to chronic lung inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) (for more information on the role of the A_{2B}AR in asthma, see Chap. 11 of this volume, "Adenosine Receptors and Asthma," by Wilson et al.).

Hypoxia also selectively increases the expression of the A_{2B}AR in HMVEC and T84 cells (Kong et al. 2006). The A_{2B}AR promoter contains a functional binding site for hypoxia-inducible factor (HIF)-1 α , a transcriptional regulator that is important for adaptive responses to hypoxia. Disruption of this element blocks hypoxia-induced A_{2B}AR upregulation, and hypoxia-induced A_{2B}AR expression is directly proportional to HIF-1 α activity (Kong et al. 2006). In an in vivo mouse model of colitis, a disorder characterized by increased HIF-1 α , A_{2B}AR expression in colon endothelial tissue was increased (Kong et al. 2006). Moreover, HIF-1 α KO mice have decreased A_{2B}AR levels in intestinal epithelia. Thus, HIF-1 α directly regulates the expression of the A_{2B}AR by modulating gene transcription.

In an in vivo mouse model of LPS-induced peritonitis, a distinct time course for the differential expression of adenosine receptor subtypes is observed (Rogachev et al. 2006). In mouse mesothelial cells, the early stages of peritonitis are characterized by an induction of the A₁AR, with a peak in receptor protein at 12 h and a return to baseline by 24 h. During this phase of peritonitis, activation of the A₁AR is proinflammatory and results in the recruitment and extravasation of leukocytes, with the peak in A₁AR expression correlating with peak leukocyte counts. The A_{2A}AR protein reached a plateau between 12 and 24 h, and the expression of the A_{2B}AR reached a peak after 48 h in mesothelial cells. Functionally, the A_{2A}AR reduced TNF- α and IL-6 levels and decreased leukocyte accumulation. A similar adenosine receptor upregulation profile and time course was observed for the A_{2A}AR and A_{2B}ARs in mouse peritoneal neutrophils (Rogachev et al. 2006). In addition, the effect of proinflammatory cytokines on adenosine receptors was evaluated in human

primary peritoneal mesothelial cells (Rogachev et al. 2006). Following exposure to IL-1 and TNF- α , early proinflammatory cytokines, mRNA and protein expression levels of A_{2A}AR and A_{2B}ARs were upregulated. IFN- γ , secreted later during the course of peritonitis, decreased A_{2A}AR levels but increased A_{2B}AR expression levels in human primary peritoneal mesothelial cells. These results suggest that the acute phase of the peritoneal infection involves a proinflammatory A₁AR response and increased release of proinflammatory cytokines, which upregulate the expression of the anti-inflammatory A_{2A}AR and A_{2B}ARs (Rogachev et al. 2006). These findings further demonstrate the intricate regulation of adenosine receptor expression in specific inflammatory environments (Rogachev et al. 2006).

3.1 Adenosine Receptor Desensitization

Desensitization, a mechanism by which a cell attenuates its response to prolonged agonist stimulation, has been studied for all four adenosine receptor subtypes and is driven by a number of factors, including receptor subtype, compartmentalization and scaffolding, and the complement of intracellular proteins involved in the desensitization and signaling process (Klaasse et al. 2008). While both the A₁AR and A₃AR are G_i-coupled receptors, their desensitization responses to agonist stimulation are very different. The cloned rat A₃AR desensitizes rapidly via the phosphorylation of serine and threonine residues on the intracellular carboxy terminus (Palmer et al. 1995a, 1996; Palmer and Stiles 2000). In contrast, the cloned human A₁AR is not phosphorylated in response to agonist and only becomes desensitized after prolonged agonist exposure (Ferguson et al. 2000, 2002).

A number of *in vitro* studies with cultured cells have demonstrated agonist-mediated A_{2A}AR desensitization, including Chinese hamster ovary cells (Palmer et al. 1994), rat pheochromocytoma PC-12 cells (Chang et al. 1997), NG108-15 mouse neuroblastoma \times rat glioma hybrid cells (Mundell and Kelly 1998; Mundell et al. 1998), rat aortic vascular smooth muscle cells (Anand-Srivastava et al. 1989), and bovine aortic endothelial cells (Luty et al. 1989). In addition, A_{2A}AR desensitization has been demonstrated in native tissue, including rat brain (Barraco et al. 1996) and porcine coronary artery (Makujina and Mustafa 1993). Desensitization of both the A_{2A}AR and A_{2B}AR is mediated by the G-protein-coupled receptor kinase (GRK) 2 isozyme (Mundell et al. 1998). In human astroglial cells, chronic treatment with TNF- α increases the functional responsiveness of A_{2B}ARs (Trincavelli et al. 2004); however, short-term treatment with TNF- α causes A_{2B}AR phosphorylation, impaired A_{2B}AR-G protein coupling, and reduced cAMP production (Trincavelli et al. 2008).

In addition to direct effects on expression level, adenosine receptor signaling can be modified by indirect changes in intracellular signal transduction components. In HMVEC₁ cells treated with TNF- α and IL-1 β , there is an increase in A_{2A}AR activity related to receptor upregulation and increased levels of the G protein β 4 isoform (Nguyen et al. 2003). Moreover, TNF- α prevents A_{2A}AR desensitization in human

monocytoid THP-1 cells by blocking the translocation of GRK2 and β -arrestin to the cell membrane, which together with TNF- α stimulation results in upregulation of the A_{2A}AR (Khoa et al. 2001) and enhanced A_{2A}AR activity (Khoa et al. 2006). In T84 human colonic mucosal epithelial cells treated with IFN- γ , a reduction in A_{2B}AR signaling occurs in response to a downregulation of AC isoforms 5 and 7 without affecting A_{2B}AR expression levels or membrane recruitment (Kolachala et al. 2005b). In human astrocytoma ADF cells, TNF- α increased A_{2B}AR functional responses and receptor G-protein coupling without altering expression levels. This increased functional response was mediated by attenuating agonist-mediated phosphorylation and desensitization of the A_{2B}AR (Trincavelli et al. 2004).

Thus, desensitization is an important phenomenon that contributes to the net effect of adenosine signaling on specific cell types involved in inflammation and to the development of agonists as therapeutic agents, since the potential for tolerance/tachyphylaxis as an unwanted effect could limit their efficacy with chronic use.

4 Adenosine Receptor Contributions to the Regulation of Inflammation

4.1 *A₁AR and Inflammatory Responses*

4.1.1 Historical Perspective

A seminal study published in 1983 by Cronstein and colleagues demonstrated that the A₁AR mediates proinflammatory events and the A_{2A}AR mediates antiinflammatory effects in isolated human neutrophils (Cronstein et al. 1983). In recent years, the role of the A₁AR in inflammation has been extensively studied using a number of approaches, including selective agonists and antagonists, monoclonal antibodies, selective antisense molecules, and genetically modified animals (Bours et al. 2006; Hasko and Cronstein 2004; Salmon et al. 1993; Sun et al. 2005). These studies have contributed to the delineation of the role of A₁AR in inflammation.

4.1.2 Proinflammatory Effects

Activation of the A₁ARs produces proinflammatory effects in a number of different tissues and cell types. On human neutrophils, A₁AR activation induces neutrophil chemotaxis, adherence to endothelial cells, and Fc γ receptor-mediated phagocytosis and O₂⁻ generation (Cronstein et al. 1990, 1992; Forman et al. 2000; Salmon and Cronstein 1990). In cultured human monocytes, the A₁AR enhances Fc γ receptor-mediated phagocytosis (Salmon et al. 1993; Salmon and Cronstein 1990), and promotes multinucleated giant cell formation on synovial fluid mononuclear phagocytes of patients with rheumatoid arthritis (Salmon et al. 1993; Merrill et al.

1997). Furthermore, A₁AR activation induces VEGF release from human monocytes (Clark et al. 2007). In human PBMCs, A₁AR antagonist enhanced and A₁AR agonist reversed the anti-inflammatory effects of adenosine mediated by A_{2A}ARs on expression of ICAM-1 and production of IFN- γ , IL-12 and TNF- α in the presence of IL-18 (Takahashi et al. 2007a). In addition, A₁AR antagonism enhanced and A₁AR agonism reversed the anti-inflammatory effects of adenosine mediated by A_{2A}ARs on expression of ICAM-1 and production of IL-12 and IFN- γ and lymphocyte proliferation during a human mixed lymphocyte reaction (Takahashi et al. 2007b). These findings suggest that activation of the A₁AR on a number of different inflammatory cells results in proinflammatory effects.

A proinflammatory role for the A₁AR has also been demonstrated in *in vivo* studies in a number of different species and disease states, including a rat model of pancreatitis (Satoh et al. 2000), ischemia-reperfusion injury of the lung (Neely and Keith 1995), ischemia-reperfusion injury of the heart in cats (Neely et al. 1996), dogs (Auchampach et al. 2004; Forman et al. 2000), and rats (Katori et al. 1999), ischemia-reperfusion injury of the liver in dogs (Magata et al. 2007) and pigs (Net et al. 2005), and endotoxin-induced lung injury in cats (Neely et al. 1997). Moreover, in an allergic model of asthma, L-97-1 (3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl-1-propyl-3,7-dihydro-purine-2,6-dione), a selective A₁AR antagonist reduced airway inflammation following allergen challenge, specifically reducing the number of eosinophils, neutrophils and lymphocytes in the airways (Nadeem et al. 2006). Recently, Ponnoth et al. have shown a proinflammatory role for A₁AR in vascular inflammation using a mouse model of allergic asthma (Ponnoth et al. 2008).

In rat models of acute pancreatitis induced with cerulein or taurocholate, the pancreas showed morphological changes that included interstitial edema and leukocyte infiltration (Satoh et al. 2000). Intraperitoneal administration of CCPA (2-chloro-*N*⁶-cyclopentyladenosine), a selective A₁AR agonist, produced similar dose- and time-dependent effects on leukocyte infiltration and interstitial edema in pancreatic tissue, while A_{2A}AR and A₃AR agonists had no effect (Satoh et al. 2000). The proinflammatory histopathological effects produced by CCPA in this model were attenuated by FK-838 (6-oxo-3-(2-phenylpyrazolo[1,5- α]pyridin-3-yl)-1(6*H*)-pyridazinebutanoic acid), an A₁AR-selective antagonist. These results suggest that activation of the A₁AR may play an important role in the tissue damage observed in acute pancreatitis.

Whole animal studies in models of ischemia-reperfusion in the lungs (Neely and Keith 1995), heart (Auchampach et al. 2004; Forman et al. 2000; Neely et al. 1996), and liver (Magata et al. 2007; Net et al. 2005) demonstrated that the A₁AR plays a proinflammatory role in these systems. In a feline model of ischemia-reperfusion injury of the lung, infusion of the A₁AR antagonists XAC (xanthine amine congener) or DPCPX reduced the percentage of injured alveoli (Neely and Keith 1995). In addition, DPCPX prevented endothelial damage, as well as margination and adhesion of neutrophils to pulmonary endothelial cells. Moreover, in a feline regional cardiac infarct model, pretreatment with the A₁AR antagonists DPCPX, bamiphylline, and XAC prevented ischemia-reperfusion injury; in other

words, reduced infarct size (Neely et al. 1996). Similarly, in the canine model of myocardial ischemia-reperfusion, pre- and posttreatment with the A₁AR antagonist DPSPX (1,3-dipropyl-8-*p*-sulfophenylxanthine) decreased the area of cardiac necrosis and improved regional ventricular function (Forman et al. 2000). Based on studies with isolated human neutrophils demonstrating that DPSPX and DPCPX blocked fMLP-induced chemoattraction, it was hypothesized that the cardioprotective effect of the A₁AR antagonist DPSPX in the canine model was due to inhibition of neutrophil chemoattraction (Forman et al. 2000). For more information on A₁ARs and ischemia-reperfusion injury of the heart, please refer to Chap. 7 of this volume, "Adenosine Receptors and Reperfusion Injury of the Heart," by Headrick and Lasley.

The role of the A₁AR in hepatic ischemia-reperfusion injury was studied in a model using total hepatic vascular exclusion in beagles (Magata et al. 2007) and in a normothermic recirculation (NR) model of liver transplantation in pigs (Net et al. 2005). In the canine model, pretreatment with KW3902 (8-(noradamantan-3-yl)-1,3-dipropylxanthine), an A₁AR antagonist, significantly increased survival following hepatic ischemia-reperfusion (Magata et al. 2007). Moreover, histopathological examination of liver tissue revealed that pretreatment with KW3902 preserved hepatic architecture and decreased the infiltration of neutrophils into hepatic tissue. In the porcine model, NR following warm ischemia reversed the injury associated with liver transplantation and increased five-day survival. This protective effect of NR was simulated by the preadministration of adenine (Net et al. 2005). Blockade of the A₁AR with DPCPX during NR further protected the liver. Taken together, these studies suggest that the A₁AR plays a proinflammatory role in hepatic ischemia-reperfusion injury.

In an in vivo feline model of LPS-induced lung injury, blockade of the A₁AR prevents acute lung injury (Neely et al. 1997). In this model, an intralobar arterial infusion of LPS produced dose-dependent lung injury characterized by perivascular and peribronchial edema and hemorrhage, margination of neutrophils along the venular endothelium, thickened alveolar septae, alveolar infiltration of neutrophils and macrophages, alveolar edema, and alveolar hemorrhagic necrosis. In this study, lungs from animals treated with the A₁AR antagonists DPCPX or bamiphylline could not be distinguished from controls, suggesting that LPS-induced pulmonary injury involves activation of the A₁AR (Neely et al. 1997). To further evaluate A₁AR function in LPS-induced lung injury, LPS from various Gram-negative bacterial sources were evaluated using cultured cells derived from HPAECs (Wilson and Batra 2002). LPSs from *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* bind directly to the A₁AR. Additional studies with HPAECs demonstrated that the CCPA- and LPS-induced release of IL-6 and thromboxane A₂, cytotoxic substances that increase permeability of the endothelium, was blocked by DPCPX. Together, these studies suggest that activation of the A₁AR on pulmonary artery endothelial cells by LPS during sepsis directly contributes to the pathology of acute lung injury (Neely et al. 1997; Wilson and Batra 2002).

To demonstrate the efficacy of an A₁AR antagonist as an antiendotoxin, antiseptic adjunctive therapy in combination with antibiotics in sepsis, the A₁AR antagonist

L-97-1 has been tested in a model of polymicrobial sepsis and endotoxemia (rat cecal ligation and puncture, CLP). Administration of L-97-1 as an intravenous therapy postCLP improved the seven-day survival in a dose-dependent manner (30–40% survival) as compared to untreated CLP controls (17% survival) or antibiotics alone (23% survival) (Wilson et al. 2006). In combination with antibiotics, L-97-1 increased survival to 50–70% in a dose-dependent manner. Improvement in seven-day survival was statistically significant for L-97-1 versus CLP, as well as for L-97-1 versus antibiotics. Moreover, L-97-1 plus antibiotics had a significant trend towards increased survival time based on the dose of L-97-1. Furthermore, efficacy for the A₁AR antagonist L-97-1 has been demonstrated in a bioterrorism animal model of pneumonic plague (Wilson, Endace, Inc., unpublished data). In this model, rats are infected via intratracheal administration with *Yersinia pestis*, a Gram-negative bacterium that releases endotoxin, a major virulence factor for *Y. pestis*. In these studies, L-97-1 plus antibiotics (ciprofloxacin) improves six-day survival and lung injury scores versus antibiotics alone in 72 h delay treatment groups. During sepsis, the expression of A₁ARs is upregulated (Rogachev et al. 2006), and furthermore, LPS upregulates A₁AR expression (Jhaveri et al. 2007). These studies, taken together with the findings that A₁AR antagonists block LPS-induced acute lung injury and improve survival in both a CLP and a Gram-negative sepsis model induced by *Y. pestis*, suggest that the A₁AR is an important target in sepsis, and that A₁AR antagonists may represent an attractive class of compounds for development as anti-sepsis drugs.

Collectively, *in vitro* studies in inflammatory cells and *in vivo* studies in animal models suggest that the A₁AR is an important target in inflammation, and that A₁AR antagonists may be efficacious as anti-inflammatory drugs. Several biotechnology, biopharmaceutical, and pharmaceutical companies have engaged in developing A₁AR antagonists for different medical conditions. Phase I/II/III clinical trials demonstrate that A₁AR antagonists as a class of drugs appear to be safe in humans. For example, Aderis Pharmaceuticals (formerly Discovery Therapeutics) developed an A₁AR antagonist, N-0861 (*N*⁶-endonorboman-2-yl-9-methyladenine), for the treatment of bradyarrhythmias (Bertolet et al. 1996). This compound was in Phase I/IIa clinical trials until it was put on clinical hold due to solubility problems. The high volume of diluent required to administer the drug intravenously, not the safety of N-0861, prevented further clinical development of this molecule. CV Therapeutics (Palo Alto, CA, USA) licensed CVT 124 to Biogen as BG-9719 (ENX) (1,3-dipropyl-8-[2-(5,6-epoxynorbonyl)]xanthine) for the treatment of congestive heart failure with renal impairment (Gottlieb et al. 2002). In clinical trials, both N-0861 and BG-9719 were well tolerated. However, problems with solubility, bioavailability, and formulation prevented the further clinical development of these A₁AR antagonists (Bertolet et al. 1996; Gottlieb et al. 2002; Doggrell 2005). Biogen (Biogen Idec, Cambridge, MA, USA) is developing another A₁AR antagonist, Adentri (BG 9928; 1,3-dipropyl-8-[1-(4-propionate)-bicyclo-[2,2,2]octyl]xanthine) for chronic congestive heart failure and renal impairment. This molecule is safe and is in Phase III clinical trials (Doggrell 2005; Greenberg et al. 2007; Press release Biogen, August 21, 2008). Two other A₁AR antagonists, SLV320 (Solvay)

and KW-3902 (rolofylline) (previously NovaCardia, now Merck) are in Phase II and Phase III clinical trials for congestive heart failure with renal impairment, respectively, and are well tolerated (Givertz et al. 2007; Dittrich et al. 2007; <http://www.clinicaltrials.gov>).

4.1.3 Anti-inflammatory Effects

Studies with genetically modified mice suggest that the A₁AR also has context-dependent anti-inflammatory functions (Sun et al. 2005; Joo et al. 2007; Lee and Emala 2000; Lee et al. 2004a, b, 2007). A₁AR function was evaluated in adenosine deaminase (ADA) knockout mice by generating ADA/A₁AR double-knockout mice. ADA knockout mice exhibit increased levels of adenosine and increased levels of the A₁AR transcript, which was most predominant in activated alveolar macrophages (Sun et al. 2005). These animals developed pulmonary inflammation, characterized by an increase in macrophages, eosinophils, fibrosis, and airway hyperreactivity. Pulmonary inflammation was exacerbated in mice lacking both ADA and the A₁AR (Sun et al. 2005). The lungs of ADA/A₁AR double-knockout mice were characterized by higher levels of cytokines, including IL-4 and IL-13, and chemokines such as eotaxin 2 and thymus- and activation-regulated chemokine. Interestingly, lung adenosine levels in ADA/A₁AR double-knockout mice were approximately 200% higher than those found in ADA-deficient mice (Sun et al. 2005). Furthermore, an anti-inflammatory role of the A₁AR was demonstrated in A₁AR knockout mice with experimental allergic encephalomyelitis, an *in vivo* model of multiple sclerosis (Tsutsui et al. 2004). A₁AR knockout mice exhibited severe demyelination and axonal injury, enhanced activation of macrophages and microglial cells, increased transcription of proinflammatory cytokines, and decreased transcription of anti-inflammatory cytokines.

The anti-inflammatory role associated with the A₁AR has been extensively studied in models of renal ischemia-reperfusion in mice and rats and in cultured renal tubule cells (Joo et al. 2007; Lee and Emala 2000; Lee et al. 2004a, 2007). Initially, the anti-inflammatory role for the A₁AR was described in a rat model of renal ischemia-reperfusion, where preconditioning, adenosine, and an A₁AR agonist, R PIA, produced a protective effect improving renal function and morphology (Lee and Emala 2000). Interestingly, an A₃AR agonist, IB-MECA (1-deoxy-1-(6-((3-iodophenyl)methyl)amino-9*H*-purin-9-yl) - *N*-methyl-D-ribofuranuronamide), worsened and an A₃AR antagonist, MRS 1191 (3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-dihydropyridine-3,5-dicarboxylate), improved renal function in this model. The protective effect of the A₃AR antagonist was greater than that of adenosine. In these studies, DPCPX blocked the protective effect of adenosine but not that of preconditioning (Lee and Emala 2000). Subsequently, a protective effect for CCPA, an A₁AR agonist, was demonstrated in a mouse model of renal ischemia-reperfusion (Lee et al. 2004b). In these studies, DPCPX worsened renal function and increased expression of inflammatory markers, necrosis and apoptosis, and blocked the protective effect of CCPA. Next, the protective effect of

the A₁AR in renal ischemia-reperfusion was studied in A₁AR knockout mice (Lee et al. 2004a). In these studies, A₁AR knockout mice showed worsened renal function and histology compared to the wild-type controls. Moreover, DPCPX increased markers of renal inflammation while CCPA reduced markers of renal inflammation. Interestingly, the A₃AR antagonist MRS 1191 improved renal function in A₁AR knockout mice with an efficacy similar to that produced by CCPA in wild-type mice (Lee et al. 2004a). Collectively, these studies suggest that both A₁AR and A₃ARs play an important role in ischemia-reperfusion injury in the kidney of rats and mice.

In mice, the mechanism of renal protection was found to consist of an acute and a delayed phase. Renal protection involved A₁AR-mediated phosphorylation of ERK MAPK and Akt, which are involved in the upregulation of cytoprotective genes (Joo et al. 2007). Activation of the A₁AR also resulted in increased phosphorylation of heat shock protein (HSP) 27 (Joo et al. 2007; Lee et al. 2007), a molecular chaperone involved in the cytoprotection of cellular proteins through the prevention of denaturation and aggregation under conditions of oxidative stress (Joo et al. 2007). In contrast, A₁AR knockout mice had decreased levels of basal HSP27 (Lee et al. 2007). Specific inhibitors of HSP synthesis blocked the A₁AR-mediated renal protection in A₁AR wild-type mice. Inhibition of G_i proteins with PTX blocked both the early phase and the late phase protective effects mediated by the A₁AR. The early phase of the A₁AR-mediated antiinflammatory effect was blocked with chelerythrine, a protein kinase C (PKC) inhibitor. The early and delayed phases of renal protection were blocked by deletion of PI3K gamma and inhibition of Akt, but not inhibition of ERK.

The role of A₁AR activation has also been studied in an immortalized porcine renal tubule cell line (LLC-PK1 cells) overexpressing the human A₁AR, and in primary cultures of renal proximal tubule cells from A₁AR knockout mice (Lee et al. 2007). In the LLC-PK1 cells, overexpression of the A₁AR was associated with increased basal expressions of total and phosphorylated HSP27, reportedly due to A₁AR-mediated stimulation of p38 and MAPK. Renal epithelial cells overexpressing the A₁AR showed decreased peroxide-induced necrosis and TNF- α -induced apoptosis, which was blocked by selective blockade of the A₁AR. In contrast, primary cultures of proximal tubule cells from A₁AR knockout mice showed increased levels of necrosis and apoptosis. Taken together, these studies suggest that A₁AR activation exerts a protective preconditioning effect in renal ischemia-reperfusion by modulating the inflammatory response and tissue necrosis, and that this process may involve HSP27.

Furthermore, studies with A₁AR knockout and wild-type mice suggest that activation of the A₁AR protects against sepsis (Gallos et al. 2005). Following CLP, mortality was increased in both the A₁AR knockout mice and in wild-type mice treated with DPCPX to antagonize A₁ARs. In addition, A₁AR knockout mice had increased levels of TNF- α , suggesting that the A₁AR modulates TNF- α production during sepsis. Finally, renal tissue in the A₁AR knockout mice exhibited increased levels of neutrophils, ICAM-1, and proinflammatory cytokines, indicating a higher degree of renal dysfunction induced by sepsis. These results suggest that the A₁AR attenuates the inflammatory response and diminishes the hyperacute inflammatory response characteristic of sepsis.

The differences in the studies suggesting both a proinflammatory role for the A₁AR in the pancreas, lung, heart, and liver as well as an anti-inflammatory role in the lung and kidney may be due to differences in the models (i.e., genetically modified mice and cell lines overexpressing adenosine receptors versus other models). For example, in the A₁AR knockout models and cell lines, other proteins such as the A_{2B}AR and A₃ARs may be responsible for the protective effects of adenosine described. There is substantial evidence suggesting that the protective effect of preconditioning is mediated by PTX-sensitive G_i-coupled proteins, including the A₁AR and A₃ARs. The studies that described a protective effect of both A₁AR and A₃ARs in the kidney are consistent with what is reported in the literature for other species. Therefore, the protective effects of adenosine and the selective A₁AR agonists R-PIA and CCPA in studies of renal ischemia-reperfusion are not surprising. The protective effect of overexpression of the A₁AR in LLC-PK1 cells is also not surprising, for the same reasons.

The deleterious effects of DPCPX on the renal function and histology of the kidney in the rat and mouse is surprising in light of the protective effect of a number of A₁AR antagonists in different models of inflammation. DPCPX is at best tenfold selective for A₁ARs versus A_{2B}ARs (Fredholm et al. 2001). In the *in vivo* renal ischemia-reperfusion studies and sepsis studies in mice, it is possible that DPCPX may be blocking the anti-inflammatory effects of the G_s-coupled A_{2B}AR (Yang et al. 2006a). Although another highly selective A₁AR antagonist, FSCPX 8-cyclopentyl-3-[3-[[4-(fluorosulfonyl)benzoyl]oxy]propyl]-1-propylxanthine, reversed the resistance to cell death in LLC-PK1 cells produced by overexpressing the A₁AR, FSCPX was used in a high concentration (20 μM), and a dose-response relationship for the blocking effects of FSCPX was not demonstrated. In other models of inflammation, anti-inflammatory effects for highly selective A₁AR antagonists, including BG-9928 in ischemia-reperfusion injury of the heart in dogs (Auchampach et al. 2004), FK-838 in pancreatitis in the rat (Satoh et al. 2000), FK-352 ([*R*]-1-[(*E*)-3-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl) acryloyl]piperidin-2-yl acetic acid) in ischemia-reperfusion injury of the heart in rats (Katori et al. 1999), and L-97-1 in allergic asthma in the rabbit (Nadeem et al. 2006), were demonstrated.

Differences in the studies described above suggesting both a proinflammatory and an anti-inflammatory role for the A₁AR may be due to differences in (i) the A₁AR-activated signaling pathway that results in tissue injury (i.e., proinflammatory pathway) versus that for protection (i.e., anti-inflammatory pathway), and which pathway predominates as a function of species and the stage/progression of injury; (ii) predominant inflammatory cell type as a function of species; in other words, the role of the macrophage where the transcript for the A₁AR was high in mice (Sun et al. 2005) versus the predominant role of the neutrophil in acute inflammation in other species; (iii) expression of A₁ARs on endothelial cells and different inflammatory cells, possibly a function of species differences; (iv) intracellular signaling and desensitization mechanisms as a function of species or cell/tissue/organ; and (v) density of homo- and heterodimers of adenosine receptors and their functional properties as a function of the cell type, organ, and species. For example, there is evidence from *in vitro* studies with native neural tissue as well as *in vivo*

studies to suggest that adenosine receptors, including the A₁AR, can form homo- and heterodimers that have unique pharmacological profiles and functional effects, including altered ligand affinity, G-protein coupling, and desensitization characteristics (Ciruela et al. 2006; Ferre et al. 2008; Franco et al. 2006; Fuxe et al. 1998; Nakata et al. 2004). To date, adenosine receptor dimerization has not been studied in cells from the immune system, but it is likely that this phenomenon is relevant in inflammatory processes. These considerations are of particular significance given that inflammatory cells undergo significant phenotype changes (e.g., adenosine receptor expression levels, altered cytokine profiles, altered cell surface protein levels) that are unique to various physiological and pathophysiological challenges. Finally, differences in the phenotypes of genetically modified animals and cells are very complex and are not yet completely understood. It is possible that genetically manipulated animals and cells exhibit compensatory expression or functions of other proteins that alter the phenotype of cells and organs in a manner that is not fully appreciated at this time.

4.2 *A_{2A}AR and Inflammatory Responses*

4.2.1 Historical Perspective

Numerous investigations in cellular and animal model systems have provided evidence that A_{2A}AR signaling pathways are active in limiting inflammation and tissue injury (Hasko and Cronstein 2004; Linden 2005; Sitkovsky and Ohta 2005; Hasko and Pacher 2008). Some of the earliest observations that A_{2A}AR signaling is anti-inflammatory came from Cronstein and colleagues, who demonstrated that engagement of the A_{2A}AR could inhibit elicited superoxide formation from neutrophils (Cronstein et al. 1983). Expression of the A_{2A}AR has subsequently been found on most inflammatory cells (Sitkovsky et al. 2004), where it has numerous anti-inflammatory properties, including inhibiting T-cell activation (Huang et al. 1997; Erdmann et al. 2005) and limiting the production of inflammatory mediators such as IL-12, TNF- α and INF γ (Hasko et al. 2000; Pinhal-Enfield et al. 2003; Lappas et al. 2005). Ohta and colleagues performed a series of studies in vivo using A_{2A}AR knockout mice to demonstrate that this receptor plays an important role in limiting the degree of inflammatory mediator production and tissue injury in response to challenges with concanavalin A or endotoxin (Ohta and Sitkovsky 2001). Subthreshold doses of these agents that caused minimal responses in wild-type mice led to extensive inflammatory mediator production, tissue damage and death in A_{2A}AR knockout mice. Thus, adenosine signaling through the A_{2A}AR appears to serve as a critical endogenous regulator of tissue inflammation and damage. Given that hypoxia and subsequent adenosine generation is likely an acute response to numerous injuries, this pathway is likely to have important and widespread implications in dictating the balance of tissue injury and repair.

4.2.2 Anti-inflammatory Effects

Substantial lines of evidence suggest that the A_{2A} AR is the major adenosine receptor mediating the anti-inflammatory properties of adenosine (Hasko and Pacher 2008). The ability of A_{2A} AR activation to suppress Th1 cytokine and chemokine expression by immune cells is likely the dominant mechanism involved. For example, A_{2A} AR activation can attenuate IL-12, $INF\gamma$ and $TNF-\alpha$ production from important immunomodulatory cells such as monocytes (Hasko et al. 2000), dendritic cells (Panther et al. 2003) and T cells (Lappas et al. 2005). The ability to diminish the production of such cardinal inflammatory molecules likely contributes to the decreased inflammation and tissue damage due to effector cell activation that is often seen with A_{2A} AR activation. However, there is also evidence that A_{2A} AR activation can prevent effector cell activities such as neutrophil migration (Cronstein et al. 1992) and oxidative burst (Cronstein et al. 1983). Collectively, these anti-inflammatory properties of the A_{2A} AR represent a sensitive and widespread mechanism for the immunoregulation of tissue injury and repair.

Findings in disease-relevant animal models suggest that A_{2A} AR activation on immune cells is beneficial in environments associated with acute inflammation and hypoxia. A_{2A} AR agonists have remarkable anti-inflammatory and tissue-protective effects in models of ischemic liver damage (Day et al. 2004), myocardial injury (Lasley et al. 2001; Glover et al. 2005), spinal cord injury (Reece et al. 2004), renal injury (Day et al. 2003), inflammatory bowel disease (Naganuma et al. 2006), and lung transplantation (Ross et al. 1999). Many of these models involve postischemic environments and suggest that A_{2A} AR activation (on various immune cells) limits or inhibits the degree of inflammation and subsequent tissue damage. Activation of the A_{2A} AR has also been shown to play an important role in the promotion of wound healing and angiogenesis (Montesinos et al. 2002), and the A_{2A} AR and A_3 AR are responsible for the anti-inflammatory actions of methotrexate in the treatment of inflammatory arthritis (Montesinos et al. 2003). Collectively, these studies suggest that activation of the A_{2A} AR has a significant impact on stemming inflammation and tissue damage in a number of disease-relevant models, suggesting that there may be numerous clinical benefits from the use of A_{2A} AR-activating compounds.

Recent studies have utilized bone marrow transplantation approaches together with gene knockout and selective A_{2A} AR agonist treatments to identify populations of immune cells that contribute to the anti-inflammatory properties of this receptor in disease models. In a model of ischemia-reperfusion liver injury, activation of the A_{2A} AR with the selective agonist ATL146e (4-(3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl)-cyclohexanecarboxylic acid methyl ester) was associated with decreased inflammation, and the liver was protected from damage brought about by reperfusion following ischemia (Day et al. 2004). When A_{2A} AR knockout mice were subjected to the same insult, the effectiveness of ATL146e was lost. Moreover, A_{2A} AR knockout mice exhibited increased liver damage, suggesting that endogenous adenosine is involved in the tissue protection seen. Subsequent studies using

bone marrow transplantation approaches and A_{2A}AR knockout mice suggested that it was A_{2A}AR on bone marrow-derived cells that conferred A_{2A}AR agonist protection. Subsequent studies identified CD1d-activated NK T cells as being the critical cells mediating the protective effects of A_{2A}AR agonist treatment in this model, where A_{2A}AR engagement reduced the production of IFN- γ from NK T-cells in association with blocking liver reperfusion injury (Lappas et al. 2006). Bone marrow transplantation studies using A_{2A}AR knockout mice were also used to demonstrate that the protective effect of A_{2A}AR agonist in a model of renal ischemia-reperfusion injury was due to A_{2A}AR activation on marrow-derived cells (Day et al. 2003). Although the exact cell type has not been identified, there is evidence to suggest that it is a cell type other than macrophages, which have been shown to be important in mediating the protective effects of A_{2A}AR agonism in a model of diabetic nephropathy (Awad et al. 2006). Similar approaches demonstrate that A_{2A}AR expression on bone marrow-derived cells is responsible for A_{2A}AR agonist anti-inflammatory and tissue-protective effects in models of myocardial infarction (Yang et al. 2006b), acute lung injury (Reutershan et al. 2007), and spinal cord compression injury (Li et al. 2006).

Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis are associated with severe tissue inflammation and damage. A_{2A}AR activation has anti-inflammatory and tissue protective properties in several studies investigating inflammation in the gastrointestinal tract (Odashima et al. 2005; Cavalcante et al. 2006; Naganuma et al. 2006). A_{2A}AR knockout mice are more sensitive to experimental colitis, and treatment with the A_{2A}AR agonist ATL 146e is associated with decreased leukocyte infiltration, inflammatory mediator production and necrosis in a model of inflammatory bowel disease (Odashima et al. 2005). CD25+ CD4+ Tregs play an important role in regulating inflammatory responses, including those associated with inflammatory bowel disease (Izcue et al. 2006). Recent studies have identified A_{2A}ARs on Tregs as playing an important role in regulating inflammation in inflammatory bowel disease (Naganuma et al. 2006). Tregs isolated from wild-type mice and transferred to immunodeficient mice together with colitis-inducing CD4+ T cells were able to confer protection from the development of colitis, whereas Tregs isolated from A_{2A}AR knockout mice were not. These studies highlight the importance of A_{2A}AR signaling as an anti-inflammatory pathway in inflammatory bowel disease.

Given that endogenous adenosine acting through the A_{2A}AR appears to be a potent regulator of inflammation and tissue injury, it stands to reason that mechanisms must exist to tightly regulate adenosine's actions during the natural course of the inflammatory response. This could occur at multiple levels, including the regulation of adenosine production and the availability of effective receptor signaling pathways. A recent study by Deaglio and colleagues provided new and interesting information on the mechanisms of adenosine generation and immunoregulation by Tregs (Deaglio et al. 2007). Extracellular adenosine is generated from the dephosphorylation of extracellular nucleotides (Zimmermann 2000). ATP and ADP are converted to AMP by the ectonucleoside triphosphate diphosphohydrolase CD39. AMP is dephosphorylated to adenosine by the ectonucleotidase CD73. Both of

these enzymes play critical roles in producing extracellular adenosine (Deaglio et al. 2007). A newly recognized feature of Tregs is that they express a unique combination of both CD39 and CD73 together with the forkhead transcription factor Foxp-3 (Deaglio et al. 2007). These findings provide an important new signature for defining Tregs, but more importantly they demonstrate that the production of adenosine through this cascade on the surface of Tregs is important to the A_{2A}AR-mediated immunosuppressive effects of these cells (Deaglio et al. 2007). These findings provide an elegant example of how the coordinate regulation of adenosine production and signaling can impact the immune response.

Anti-inflammatory properties of A_{2A}AR signaling have also been noted in animal models of inflammatory lung disease. In a model of LPS-induced lung injury, treatment with the A_{2A}AR agonist ATL202 was associated with decreased recruitment of neutrophils to the lung, together with reduced cytokine levels and pulmonary edema (Reutershan et al. 2007). There was enhanced neutrophil recruitment in A_{2A}AR knockout mice treated with LPS and bone marrow transplantation, as well as tissue-specific A_{2A}AR deletion studies suggested that A_{2A}AR expression on leukocytes was important in the anti-inflammatory effects seen. In addition to models of acute lung injury, anti-inflammatory effects of A_{2A}AR signaling have been noted in allergic lung inflammation. Treatment of allergic rats with the A_{2A}AR agonist resulted in diminished pulmonary inflammation (Fozard et al. 2002). Similar findings were seen in a mouse model of allergic lung inflammation (Bonneau et al. 2006). A_{2A}AR knockout allergic mice have also been shown to have higher lung inflammation as compared to A_{2A}AR wild-type mice upon allergen challenge (Nadeem et al. 2007). Finally, in a recent study on the ADA knockout model of adenosine-dependent lung inflammation and damage, genetic removal of the A_{2A}AR led to enhanced pulmonary inflammation, mucus production and alveolar airway destruction (Mohsenin et al. 2007), further implicating A_{2A}AR signaling pathways as important anti-inflammatory networks in the lung. These findings suggest that A_{2A}AR agonism may be beneficial in the treatment of diseases such as asthma; however, recent investigations into this in humans have been inconclusive (Luijk et al. 2008).

4.2.3 Detrimental Aspects of A_{2A}AR Engagement

It is becoming increasingly clear that the suppression of various T-cell functions is a major mechanism by which A_{2A}AR signaling limits tissue inflammation and damage in response to acute injury. Whereas this has obvious benefits in protecting tissues and promoting repair, there are examples where this paradigm is detrimental. A recent study by Ohta and colleagues demonstrated that A_{2A}AR-mediated immunosuppressive activities serve to protect cancer cells from the activities of antitumor T cells, and thus promote the survival and growth of tumors (Ohta et al. 2006). It was shown that the hypoxic environment of certain tumors promotes adenosine formation, and that treatment of wild-type mice with A_{2A}AR antagonists can decrease tumor size. Moreover, injection of A_{2A}AR knockout mice with cancer cells was associated with remarkable decreases in tumor size and animal survival

relative to what was seen in wild-type mice injected with the same cancer cells. This effect appears to be mediated largely by CD8⁺ T cells and the production of INF γ and TNF- α . These findings suggest that the well-characterized anti-inflammatory properties of A_{2A}AR signaling may actually serve to protect certain tumors from the body's attempt to eliminate them. This raises several attractive avenues for novel cancer therapies. The use of A_{2A}AR antagonists or strategies to lower adenosine levels in tumors may prove beneficial in allowing the immune system to attack cancer cells. In addition, dampening the anti-inflammatory effects of A_{2A}AR signaling in certain tumors with the development of targeted antitumor T cells with A_{2A}AR gene deletion may improve strategies for cancer immunotherapy.

Another area where A_{2A}AR receptor signaling has received substantial attention as a potential target for therapeutic intervention is neurodegenerative disorders. Adenosine levels markedly increase in the brain in response to hypoxic, traumatic, and inflammatory insults (Pedata et al. 2001). Interestingly, engagement of the A_{2A}AR in brain injury models appears to have both protective and detrimental effects. Akin to what is seen in other organ systems (see above), activation of the A_{2A}AR has been shown to reduce brain damage in kainate-induced hippocampal injury and hemorrhagic brain injury (Jones et al. 1998; Mayne et al. 2001). In contrast, A_{2A}AR antagonists have been shown to attenuate ischemic brain injury (Monopoli et al. 1998) and neurotoxicity induced by kainate and quinolinate (Jones et al. 1998; Popoli et al. 2002). In addition, A_{2A}AR antagonists are protective in neurotoxic models of Parkinson's disease (Ikeda et al. 2002). The contribution of A_{2A}AR signaling to the promotion of neuronal injury has been validated genetically. A_{2A}AR knockout mice were found to have smaller infarct volumes and neural behavioral deficit scores following ischemic brain injury than wild-type mice (Yu et al. 2004). The A_{2A}AR is expressed on many cell types in the brain, including neuronal components such as striatal neurons and glial cells (Svenningsson et al. 1999), endothelial cells and various bone marrow-derived cells such as neutrophils, macrophages and dendritic cells (Hasko and Cronstein 2004). Engagement of A_{2A}ARs on different cells during different types or even stages of injury may be responsible for the destructive or protective effects of this receptor in the injured brain. A series of experiments using bone marrow transplantation of cells from A_{2A}AR knockout mice and tissue-specific knockout of the A_{2A}AR in neuronal cells has recently provided evidence that it is the expression of the A_{2A}AR on bone marrow-derived cells that is responsible for the detrimental effects of A_{2A}AR signaling in ischemic brain injury and 3-nitropropionic acid-induced striatal damage (Yu et al. 2004; Huang et al. 2006). The mechanisms underlying potential protective effects of A_{2A}AR are not clear. However, the observation that both protective and detrimental effects have been noted in different types of brain injury suggests that multiple sites of action for injury-induced adenosine production and A_{2A}AR signaling must be understood before A_{2A}AR agonists and antagonists can be effectively utilized in the treatment of neurodegenerative disorders. For more information on A_{2A}ARs in neuroprotection and neurodegenerative diseases, please refer to other chapters in this volume, including Chap. 16, "Adenosine Receptors and the Central Nervous System" (by Sebastião and Ribeiro), Chap. 17, "Adenosine Receptors and Neurological

Disease: Neuroprotection and Neurodegeneration” (by Stone et al.), and Chap. 18, “Adenosine A_{2A} Receptors and Parkinson’s Disease” (by Morelli et al.).

Thus, studies in cellular models and preclinical investigations in animal models suggest that A_{2A} AR agonists will be useful in the treatment of many diseases where inflammation is a detrimental component, while A_{2A} AR antagonists may be beneficial in the treatment of neurological disorders such as Parkinson’s disease. As clinical trials advance, it will become evident whether these preclinical observations translate into beneficial effects in humans (Schwarzschild et al. 2006; Gao and Jacobson 2007).

4.3 A_{2B} AR and Inflammatory Responses

4.3.1 Historical Perspective

Initial identification of the A_2 ARs was based on the ability of this class of receptor to activate AC (Londos et al. 1980). Both high-affinity and low-affinity A_2 AR subtypes were described (Bruns et al. 1986), and it was not until the successful molecular cloning of these receptors in the early 1990s that it became clear that the high-affinity A_2 AR was the A_{2A} AR and the low-affinity A_2 AR was the A_{2B} AR (Pierce et al. 1992; Rivkees and Reppert 1992). Subsequently, studies in HMC-1 cells have shown that the A_{2B} AR can not only couple AC through G_s (Feoktistov and Biaggioni 1997), but that it also interacts with G_q to activate PLC (Feoktistov et al. 1999). However, the majority of in vitro studies in normal human cell lines, including human airway epithelial and bronchial smooth muscle cells as well as lung fibroblasts, and in vivo studies in mice suggest that the primary signaling pathway for the A_{2B} AR is via AC, to produce an increase in intracellular cAMP (see below). The A_{2B} AR is expressed on most inflammatory cells, and its expression is induced in hypoxic and inflammatory environments (Xaus et al. 1999a; Eltzschig et al. 2003). Furthermore, as with the other adenosine receptors, both anti- and proinflammatory activities have been associated with A_{2B} AR activation. However, the observation that the A_{2B} AR has a relatively low affinity for adenosine has led to the notion that A_{2B} AR activation may serve important functions in pathological situations where adenosine production is increased (Fredholm 2007). Deciphering the contributions of A_{2B} AR signaling in models of tissue inflammation and tissue injury is an active area of research with exciting possibilities for novel adenosine-based therapeutics.

4.3.2 Anti-inflammatory Effects

Perhaps the best-characterized anti-inflammatory actions associated with the A_{2B} AR are its ability to inhibit monocyte and macrophage functions. Along these

lines, IFN- γ can upregulate the A_{2B}AR expression on macrophages as part of a proposed mechanism for macrophage deactivation (Xaus et al. 1999a). Consistent with this, A_{2B}AR activation can inhibit the production or release of proinflammatory cytokines such as TNF- α and IL-1 β from macrophages or monocytes, and it can inhibit macrophage proliferation following inflammatory stimulation (Xaus et al. 1999b; Sipka et al. 2005; Kreckler et al. 2006). In addition, A_{2B}AR activation can increase the production of IL-10 from macrophages (Nemeth et al. 2005), a process that can be considered anti-inflammatory. As with the A_{2A}AR, these anti-inflammatory effects on macrophages likely stem from the ability of the A_{2B}AR to couple to adenylate cyclase to increase cAMP levels.

Recent observations in A_{2B}AR knockout mice provide compelling evidence that this receptor is associated with anti-inflammatory events *in vivo*. Yang and colleagues published on the initial characterization of A_{2B}AR knockout mice and demonstrated that these mice show evidence of increased inflammation at baseline, in that levels of cytokines such as TNF- α and IL-6 were elevated in naïve A_{2B}AR knockout mice, while IL-10 levels were elevated (Yang et al. 2006a). Exposure of A_{2B}AR knockout mice to LPS resulted in the exaggerated production of TNF- α and IL-6, further suggesting an anti-inflammatory role for the A_{2B}AR *in vivo*. These findings were surprising given data suggesting that A_{2B}AR signaling is proinflammatory *in vitro* and *in vivo* (see below). Furthermore, these studies suggest a role for physiological A_{2B}AR activation, which is paradoxical considering the notion that A_{2B}AR signaling is only activated during pathological situations when adenosine levels are high, and that it is a low-affinity receptor. Interestingly, in A_{2B}AR knockout mice there was evidence of increased expression of vascular adhesion molecules that mediate inflammation (Yang et al. 2006a), a finding that was associated with the A_{2B}AR-dependent production of cytokines from macrophages. These findings are consistent with the anti-inflammatory properties previously attributed to this receptor on macrophages.

A second manuscript by Hua and colleagues using independently generated A_{2B}AR knockout mice demonstrated enhanced mast-cell activation in the absence of the A_{2B}AR (Hua et al. 2007). These studies showed a reduction in basal levels of cAMP in mast cells isolated from A_{2B}AR knockout mice, suggesting that A_{2B}AR engagement may play a role in regulating mast-cell activation at baseline. Furthermore, experiments in this study demonstrated that mice lacking the A_{2B}AR exhibit increased sensitivity to IgE-mediated anaphylaxis, suggesting that this receptor may limit the magnitude of antigen-driven responses on mast cells. These findings are somewhat paradoxical given the touted proinflammatory functions of the A_{2B}AR receptor on HMC-1 cells (Feoktistov et al. 1998). A recent manuscript by Ryzhov and colleagues probed this further by examining adenosine-dependent effects on mast cells isolated from A_{2B}AR knockout mice (Ryzhov et al. 2008b). These studies confirmed the anti-inflammatory effects of A_{2B}AR signaling on mast cells and demonstrated that the A_{2B}AR is necessary for antigen-induced proinflammatory cytokine production from these cells. Thus, the A_{2B}AR seems to play both anti- and proinflammatory functions on mast cells.

A recent study by Eckle and colleagues demonstrated increased vascular leakage in $A_{2B}AR$ knockout mice exposed to hypoxia (Eckle et al. 2008). In addition, there was an increase in neutrophil influx into tissues of $A_{2B}AR$ knockout mice exposed to hypoxia. Bone marrow transplantation studies suggested that this enhanced neutrophilia was, in part, due to $A_{2B}AR$ expression on bone marrow-derived cells. The ability of the $A_{2B}AR$ to regulate vascular leak represents a potentially major anti-inflammatory role for this receptor. Moreover, the anti-inflammatory effects of $A_{2B}AR$ s are not surprising for a G_s -coupled receptor that increases intracellular cAMP, and are similar to those produced by other agents that increase cAMP, in other words $A_{2A}AR$ agonists and phosphodiesterase IV inhibitors.

4.3.3 Proinflammatory Effects

Numerous studies have demonstrated a proinflammatory function for the $A_{2B}AR$, largely through its regulation of proinflammatory cytokine and chemokine production. A prominent example of this is the promotion of IL-6 release from a number of cells, including intestinal (Sitaraman et al. 2001) and airway epithelial cells (Sun et al. 2008), macrophages (Ritchie et al. 1997; Ryzhov et al. 2008a), pulmonary fibroblasts (Zhong et al. 2005), bronchial smooth muscle cells (Zhong et al. 2003a), astrocytes (Schwaninger et al. 1997) and cardiomyocytes (Wagner et al. 1999). In addition, $A_{2B}AR$ activation can promote the release of IL-8 from HMC-1 cells (Feoktistov and Biaggioni 1995), IL-4 and IL-13 (Ryzhov et al. 2004, 2008b) from HMC-1 cells and murine bone marrow-derived mast cells, and the release of IL-19 from airway epithelial cells (Zhong et al. 2006) and MCP-1 from bronchial smooth muscle cells (Zhong et al. 2003a). In addition, $A_{2B}AR$ activation can stimulate the production of VEGF (Feoktistov et al. 2003; Ryzhov et al. 2008b), which can be considered proinflammatory in certain disease states. The $A_{2B}AR$ driven production of these proinflammatory molecules has been attributed to both G_s and G_q pathways (Feoktistov et al. 1999; Sitaraman et al. 2001).

$A_{2B}AR$ expression has been shown to be increased in the gastrointestinal track during inflammatory bowel disease and colitis (Hosokawa et al. 1999). Evidence in animal models of inflammatory bowel disease demonstrates that $A_{2B}AR$ activation can stimulate the release of IL-6, an important proinflammatory cytokine in inflammatory bowel disease, from the apical surface of the colonic epithelium (Sitaraman et al. 2001). This increased IL-6 secretion is proposed to promote the degranulation of neutrophils and contribute to disease progression. In support of this model, emerging work from the Sitaraman laboratory suggests that treatment of animal models of inflammatory bowel disease with the $A_{2B}AR$ antagonist ATL 801 inhibits IL-6 production and is associated with an improvement in clinical and histological signs in these models (Kolachala et al. 2008a, b). Collectively, these findings suggest that $A_{2B}AR$ signaling may play a role in the progression of inflammatory bowel disease, and so an $A_{2B}AR$ antagonists may have therapeutic benefit in related conditions.

As mentioned earlier, engagement of the A_{2B}AR on HMC-1 cells appears to have predominantly proinflammatory functions (Feoktistov et al. 1998); however, recent studies in A_{2B}AR knockout mice have revealed that this receptor plays an anti-inflammatory function in bone marrow-derived mast cells (Hua et al. 2007). Understanding the complexity of A_{2B}AR signaling in this cell type may relate to the distinct inflammatory functions of this cell type. Following antigen priming and stimulation, mast cells undergo a degranulation process where preformed mediators are released (Shimizu and Schwartz 1997). This process is part of an acute response to antigen that mediates important processes such as bronchoconstriction in airways. In addition to this acute response, mast cells are stimulated to produce and release cytokines and chemokines. This inflammatory response is part of a more chronic or late-stage response that can promote additional tissue inflammation and injury. The recent work from Ryzhov and colleagues demonstrates that engagement of the A_{2B}AR on mouse mast cells does not contribute to adenosine's ability to promote mast cell degranulation; however, the A_{2B}AR does contribute to the production of IL-13 and VEGF (Ryzhov et al. 2008b). Thus, adenosine-mediated degranulation is likely mediated by the A₃AR in rodents, while the A_{2B}AR regulates mediator production. It is important to note that significant species differences have been noted in relation to adenosine's effects on mast cells, and relatively little is known about the contribution of the A_{2B}AR in human mast cells. Continued efforts to define the functions of adenosine receptors on mast cells in specific disease environments such as asthma will be critical to the development of adenosine-based therapeutics targeting mast-cell effector activities.

The apparent low affinity of adenosine for the A_{2B}AR suggests that this receptor may have important roles in pathological environments where adenosine levels are elevated (Fredholm 2007). Consistent with this, work in the ADA-deficient model of adenosine-dependent lung inflammation and injury has demonstrated proinflammatory features of A_{2B}AR signaling (Sun et al. 2006). In this model, mice that lack ADA exhibit progressive increases in lung adenosine concentrations in association with progressive pulmonary inflammation and tissue remodeling (Blackburn et al. 2000; Chunn et al. 2005). Noted features include the accumulation of activated alveolar macrophages that produce numerous inflammatory mediators, including IL-6, CXCL1, TGF- β 1 and osteopontin (Sun et al. 2006). Production of these mediators is associated with alveolar airway destruction, mucus cell metaplasia and pulmonary fibrosis. A_{2B}AR expression is elevated in the lungs of ADA-deficient mice, and treatment of these mice with the selective A_{2B}AR antagonist CVT-6883 resulted in decreased production of proinflammatory mediators from macrophages, which was associated with decreased alveolar airway enlargement and pulmonary fibrosis (Sun et al. 2006). Similarly, a recent study using a mouse model of ragweed sensitization and challenge in the lung revealed that A_{2B}AR antagonism with CVT-6883 (3-ethyl-1-propyl-8-[1-(3-trifluoromethylbenzyl)-1*H*-pyrazol-4-yl]-3,7-dihydropurine-2,6-dione) was associated with decreased airway inflammation and airway hyperreactivity (Mustafa et al. 2007). These studies demonstrate that A_{2B}AR signaling plays a proinflammatory role in the lung, and suggest that A_{2B}AR antagonists may prove beneficial in the treatment of lung disorders such as asthma, COPD, and pulmonary fibrosis.

4.4 *A₃AR and Inflammatory Responses*

4.4.1 Historical Perspective

The A₃AR was first identified through molecular cloning from a rat testis cDNA library based on 40% sequence homology with the canine A₁AR and A_{2A}AR (Meyerhof et al. 1991). A₁AR, A_{2A}AR and A_{2B}AR are antagonized by methylxanthines, such as caffeine, theophylline and enprofylline, while the A₃AR is relatively xanthine insensitive, which may have been a reason for its relatively late discovery. In 1992, Zhou et al. cloned the A₃AR from rat striatum, expressed the protein in Chinese hamster ovary cells, and showed that A₃AR engagement leads to inhibition of AC (Zhou et al. 1992). Later it was shown that inhibition of AC is achieved through activation of the pertussis toxin-sensitive Gia_{2,3} protein (Palmer et al. 1995b). Ligand binding can also result in activation of PLC through G_{q/11} or the βγ subunits, leading to increased release of Ca²⁺ (Abbracchio et al. 1995). More recent studies revealed several additional intracellular pathways that can be accessed by the A₃AR in different cell types to promote tissue-specific functions (for a review, see Gessi et al. 2008). Homologs of the A₃AR gene have been cloned from several species, and only 74% sequence homology was found between the genes from rat and human or sheep, while there is 85% homology between human and sheep. The recently cloned and characterized equine A₃AR gene shows a high degree of sequence homology to the human and sheep genes, but has a different pharmacological profile (Brandon et al. 2006). These species differences make it possible to design highly selective ligands for the human A₃AR, but the disadvantage is that these ligands cannot be adequately tested in rodent models. In an effort to circumvent this problem, Yamano and his colleagues created an A₃AR-humanized mouse by replacing the mouse A₃AR gene with the human gene (Yamano et al. 2006). When bone marrow-derived mast cells from the A₃AR-humanized mice were treated with an A₃AR agonist, an elevation of intracellular Ca²⁺ concentration was observed, and this increase could be completely antagonized by a human-selective A₃AR antagonist. However, the A₃AR agonist did not potentiate antigen-dependent degranulation, probably because the agonist-stimulated human A₃AR could not activate the phosphorylation of either ERK 1/2 or protein kinase B due to uncoupling of the receptor from G proteins (Yamano et al. 2006). To overcome the uncoupling, the group generated A₃AR functionally humanized mice by replacing the mouse A₃AR gene with a chimeric human/mouse sequence in which the intracellular regions of the human receptor were substituted for the corresponding regions of the mouse A₃AR. Activation of the chimeric A₃AR led to intracellular Ca²⁺ elevation and activation of the PI3Kγ-signaling pathway, which are equivalent to the actions induced by the A₃AR in wild-type mice. The human A₃AR antagonist had the same binding affinities for this chimeric receptor as for the human A₃AR, and completely antagonized the potentiation of antigen-dependent mast-cell degranulation. These studies provided the first direct evidence that the uncoupling of mouse G protein(s) to the human A₃AR is due to a sequence difference in the intracellular regions of

the receptor protein critical for G-protein recognition/coupling. It is expected that the A₃AR functionally humanized mice can be employed for pharmacological evaluations of the human A₃AR antagonists (Yamano et al. 2006).

4.4.2 Anti-inflammatory Effects

The A₃AR has been shown to suppress LPS-induced TNF- α production in vitro from various macrophage cell lines (Le Vraux et al. 1993; McWhinney et al. 1996; Sajjadi et al. 1996; Martin et al. 2006) and microglial cells (Lee et al. 2006b), where, depending on the cell type, different signal transduction pathways are responsible for the inhibition. This inhibitory effect was also assessed in vivo by treating wild-type and A₃AR knockout mice with the A₃AR agonist 2-Cl-IB-MECA (2-chloro-*N*⁶-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide) following LPS challenge, resulting in decreased TNF- α production that was more pronounced in wild-type mice (Salvatore et al. 2000). In peritoneal macrophages isolated from A₃AR knockout or wild-type mice, treatment with IB-MECA reduced TNF- α release to the same extent (Kreckler et al. 2006). Both of these in vivo studies demonstrate that A_{2A}AR activation inhibits the production of TNF- α regardless of the presence of A₃AR. Recent studies with human monocytes implicate both the A_{2A}AR and A_{2B}AR in the regulation of LPS-induced TNF- α production (Zhang et al. 2005; Hasko et al. 2007). In human neutrophils, both the A₃AR and the A_{2A}AR are involved in the reduction of O₂⁻ generation (Bouma et al. 1997; Gessi et al. 2002), and the A₃AR also promotes neutrophil migration (Chen et al. 2006), thus performing both anti- and proinflammatory actions, respectively.

4.4.3 A₃AR in Disease Progression and Potential Agonist Therapies

A₃AR agonists can exert significant protective effects in animal models of arthritis. In a collagen-induced arthritis model, A₃AR activation inhibited CCL3 (MIP-1 α) production (Szabo et al. 1998), while in autoimmune arthritis models, suppression of TNF- α production was found (Baharav et al. 2005). Moreover, inhibition of proinflammatory cytokine production was achieved by inhibiting the PI3K NF- κ B signaling pathway in adjuvant-induced arthritis (Madi et al. 2007). Methotrexate, a therapeutic agent that is widely used to treat arthritis, exerts its anti-inflammatory effect through adenosine, and was shown to upregulate the expression of A₃AR on peripheral blood mononuclear cells both in rats with adjuvant-induced arthritis and in patients with rheumatoid arthritis (Ochaion et al. 2006). Concomitant treatment with IB-MECA and methotrexate resulted in additive anti-inflammatory effects in the adjuvant-induced arthritis animal model. IB-MECA (CF-101) has been tested in Phase I and Phase IIa clinical trials, where it was found to be safe, well tolerated, and shows evidence of an anti-inflammatory effects in patients with rheumatoid arthritis (Silverman et al. 2008). IB-MECA was also found to be protective in other mouse models of inflammatory diseases. In endotoxemic mice, pretreatment

with IB-MECA decreased mortality by reducing IL-12 and IFN- γ production independently of IL-10 production (Hasko et al. 1998). A similar effect was observed in different mouse models of colitis (Mabley et al. 2003) and septic peritonitis (Lee et al. 2006a), where IB-MECA treatment decreased the expression of proinflammatory cytokines, mainly TNF- α , while in A₃AR knockout mice inflammation was heightened. The A₃AR has been reported to have a protective role *in vivo* in lung injury following ischemia and reperfusion (Matot et al. 2006). Recently, in the bleomycin-induced lung injury model, significantly enhanced inflammatory cell recruitment was observed in the lungs of A₃AR knockout mice due to elevated expression of the chemokines CXCL-1, CCL11 (eotaxin-1) and GM-CSF (Morschl et al. 2008). These observations suggest that A₃AR agonists may represent a new family of orally bioavailable drugs in the treatment of inflammatory diseases (Bar-Yehuda et al. 2007). For more information on A₃ARs and inflammation, please refer to Chap. 10 of this volume, “A₃ Adenosine Receptor: Pharmacology and Role in Disease,” by Borea et al.

4.4.4 Proinflammatory Effects

Early studies in mast cells indicated that A₃AR activation leads to increased inflammation by inducing the release of mediators and the potentiation of antigen-dependent degranulation (Ramkumar et al. 1993; Fozard et al. 1996; Reeves et al. 1997). A₃AR activation can enhance the degranulation of mast cells isolated from mouse lung through elevations of intracellular Ca²⁺ mediated by the coupling of G_i to PI3K (Zhong et al. 2003b). In contrast, in canine mast cells (Auchampach et al. 1997), degranulation was mediated by the A_{2B}AR instead of the A₃AR. The specific adenosine receptors involved in the degranulation of human mast cells are not known.

A₃AR activation in sensitized guinea pigs resulted in increased inflammatory cell recruitment to the lung (Spruntulis and Broadley 2001). In rat mast cells, activation of the A₃AR inhibited apoptosis through protein kinase B phosphorylation (Gao et al. 2001), and this enhanced survival may contribute to inflammatory cell expansion in inflamed tissues.

A₃AR mRNA expression is elevated in transbronchial biopsy samples from asthma and COPD patients, where expression is localized to infiltrating eosinophils rather than mast cells (Walker et al. 1997). In ADA-deficient mice, which exhibit adenosine-mediated lung disease, genetic removal of the A₃AR or treatment with an A₃AR antagonist, MRS-1523 (3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate), prevented airway eosinophilia and decreased mucus production, suggesting that A₃AR signaling contributes to the regulation of features of chronic lung disease (Young et al. 2004). Additional *ex vivo* studies with mouse eosinophils confirmed the results of *in vitro* observations with human eosinophils, where A₃AR activation suppressed eosinophil chemotaxis (Ezeamuzie and Philips 1999). These observations suggest that the diminished eosinophilia in the ADA/A₃AR double-knockout mice or in the antagonist-treated ADA-deficient

mice in Young's study is not a direct effect on the eosinophils. Indirect regulators may be cytokines and chemokines that are known to be involved in eosinophil recruitment, but these were not affected by removal or inhibition of the A₃AR in ADA-deficient mice, suggesting that other mediators, such as proteases, extracellular matrix proteins and cell adhesion molecules, may be responsible (Young et al. 2004).

In addition to regulating chemotaxis, the A₃AR is also important for eosinophil activation and degranulation, although results are contradictory. For example, A₃AR activation decreased degranulation and O₂⁻ production in human eosinophils isolated from blood (Ezeamuzie and Philips 1999; Ezeamuzie 2001), while there was no degranulation in the absence of the A₃AR assessed by eosinophil peroxidase release in the bronchoalveolar lavage fluid in bleomycin-challenged mice (Morschl et al. 2008). Moreover, the treatment of human eosinophils with Cl-IB-MECA (a selective A₃AR agonist) elevated intracellular Ca²⁺ levels, suggesting the presence of PLC-coupled A₃AR and supporting the role of A₃AR in eosinophil degranulation and chemotaxis, which are both Ca²⁺-driven events (Kohno et al. 1996).

4.4.5 Potential Use of Antagonist in the Treatment of Inflammation

Early observations showing an A₃AR-mediated enhancement of antigen-dependent degranulation of mast cells in mice and bone marrow-derived cell lines (Reeves et al. 1997; Salvatore et al. 2000) suggested that selective A₃AR antagonists may have therapeutic potential as antiasthmatic agents. A compound with dual antagonist properties for both the A_{2B}AR and the A₃AR, QAF 805 (Novartis), is under development as an antiasthma drug (Press et al. 2005). However, this mixed A_{2B}/A₃ AR antagonist has now entered human clinical trials and has failed to increase the PC₂₀ for AMP versus placebo in 24 AMP-sensitive asthmatics in a placebo-controlled, double-blind, randomized, two-way crossover Phase Ib clinical trial (Pascoe et al. 2007). Moreover, researchers at GlaxoSmithKline developed a compound with dual A_{2A}AR agonist and A₃AR antagonist effects that was able to inhibit both the production of reactive oxygen species and degranulation from human eosinophils and neutrophils *in vitro*, but provided very little clinical benefit when used in a clinical study for the treatment of allergic rhinitis (Bevan et al. 2007; Rimmer et al. 2007).

Although several potent and selective antagonists of the human A₃AR have been identified, they show extremely low binding affinity for the rodent A₃AR (typically 1,000 times lower), and since rodent models are used for the pharmacological evaluation of new therapeutic agents, this poses a serious drawback. The humanized A₃AR chimera mice (Yamano et al. 2006) may prove useful in overcoming this problem; however, further *in vivo* studies are needed to confirm that these mice can be utilized to test human A₃AR-selective compounds.

The A₃AR exerts both pro- and anti-inflammatory effects on different cell types and cell functions, but how these cells interact and influence each other in their microenvironment is still not known. Methods to examine A₃AR protein expression

in situ are not sensitive, and it is possible that cells with low surface expression of A₃AR may be important in the regulation of inflammation in a manner that has not yet been appreciated.

Most cells express multiple types of adenosine receptors, and their actions can be overlapping or opposing, which may be an important mechanism to keep cell function in balance. It might prove beneficial to design analogs with dual or multiple affinities towards different types of adenosine receptors in order to influence various inflammatory actions at the same time. There are some reports of ligands that have A_{2B}AR and A₃AR antagonist or A_{2A}AR agonist and A₃AR antagonist properties that were designed to treat allergic airway diseases and that show promise in cell culture experiments, but further studies need to be performed with animal models of inflammation and in humans.

5 Conclusions

Orchestrated responses of cells to injury are essential for survival. As part of the body's ability to respond and recover from infection and injury, inflammatory processes help to limit infection and promote pathways for wound healing and the establishment of homeostasis. During various injurious situations, cells are placed under stress and must adapt to survive during the resolution of injury. Adenosine production and signaling has emerged as a major mechanism whereby cells respond to injury and regulate inflammation. There are precise mechanisms for regulating the production of extracellular adenosine at the cell surface, and there are now numerous studies demonstrating that this process helps to set in place pathways that can limit detrimental inflammatory processes while promoting beneficial inflammatory processes and promoting wound healing. The extensive research into the contributions of individual adenosine receptors on various immune cells, which has been extensively reviewed here, suggests that selective adenosine receptor agonists and antagonists may prove useful in regulating the immune response and hence the treatment of various injuries or diseases states. However, work from genetically modified mice and the use of selective adenosine receptor ligands in vivo have shown us that the path forward for the use of adenosine-based therapeutics will present many challenges. The engagement of all of the adenosine receptors has potent anti-inflammatory and tissue protective features in many situations. However, demonstrated proinflammatory and tissue destructive properties can also be ascribed to each of the adenosine receptors. Though this seems paradoxical, it may, in general terms, highlight the importance of adenosine signaling in regulating the balance between tissue injury and repair. For example, stimulation of anti-inflammatory adenosine receptor pathways will likely serve to stem inflammatory processes associated with numerous infections and challenges, as well as to promote wound-healing features such as angiogenesis and matrix deposition; however, overstimulation of such wound healing processes may actually promote disease. In addition, activation of anti-inflammatory pathways, such as the

downregulation IL-12 and upregulation of IL-10, may bias the tissue environment toward Th2-like inflammation, which may present exacerbations of inflammation in certain environments. Thus, therapeutic approaches must take into account numerous factors, including the stage of the disease, the immunological and pathological processes involved, and the duration of treatment. Finally, the numerous observations that adenosine receptor expression increases in inflammatory environments suggest that we must learn more about receptor number or availability on the cell surface during specific inflammatory insults. Despite these challenges, it is clear that selective adenosine receptor engagement can regulate many of the features of inflammation, and with time and continued research, adequate approaches will be developed for the treatment of human disease with adenosine-based approaches.

In this regard, it is clear that adenosine receptors are important molecular targets for adenosine-based therapeutics for the entire spectrum from inflammation to immune suppression. Approaches utilizing adenosine receptor-based therapeutics will be dependent on the role of the adenosine receptors in mechanisms of disease in humans, the timing of treatment with respect to the therapeutic window and the stage/progression of injury, and the duration and monitoring of treatment for both beneficial effects and adverse events. A number of adenosine receptor-based ligands with good safety profiles and high selectivity are available for testing in humans. Preclinical efficacy in animal models does not always translate into human efficacy. The development of preclinical model systems with relevance to the human condition of inflammation is essential for successful drug discovery. Only by testing adenosine receptor-based ligands that are safe and selective for the adenosine receptor subtypes in humans will we understand the role of these receptors in human conditions of inflammation, which will allow for the successful development of human therapeutics towards these important molecular targets.

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A₁ Adenosine Receptor: Role in Diabetes and Obesity

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Abstract Adenosine mediates its diverse effects via four subtypes (A₁, A_{2A}, A_{2B} and A₃) of G-protein-coupled receptors. The A₁ adenosine receptor (A₁AR) subtype is the most extensively studied and is well characterized in various organ systems. The A₁ARs are highly expressed in adipose tissue, and endogenous adenosine has been shown to tonically activate adipose tissue A₁ARs. Activation of the A₁ARs in adipocytes reduces adenylate cyclase and cAMP content and causes inhibition of lipolysis. The role of A₁ARs in lipolysis has been well characterized by using several selective A₁AR agonists as well as A₁AR knockout mice. However, the contribution of A₁ARs to the regulation of lipolysis in pathological conditions like insulin resistance, diabetes and dyslipidemia, where free fatty acids (FFA) play

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an important role, has not been well characterized. Pharmacological agents that reduce the release of FFA from adipose tissue and thus the availability of circulating FFA have the potential to be useful for insulin resistance and hyperlipidemia. Toward this goal, several selective and efficacious agonists of the A₁ARs are now available, and some have entered early-phase clinical trials; however, none have received regulatory approval yet. Here we review the existing knowledge on the role of A₁ARs in insulin resistance, diabetes and obesity, and the progress made in the development of A₁AR agonists as antilipolytic agents, including the challenges associated with this approach.

Keywords A₁ Adenosine Receptor · Antilipolytic · Insulin Resistance · Diabetes · Obesity

Abbreviations

A ₁ AR	A ₁ Adenosine receptor
A _{2A} AR	A _{2A} Adenosine receptor
A _{2B} AR	A _{2B} Adenosine receptor
AR	Adenosine receptor
ATGL	Adipose triglyceride lipase
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CCPA	2-Chloro- <i>N</i> ⁶ -cyclopentyladenosine
FFA	Free fatty acids
GPCR	G-protein-coupled receptor
HPIA	Hydroxyphenylisopropyl adenosine
HSL	Hormone-sensitive lipase
KO	Knockout
NFκB	Nuclear factor kappa beta
PIA	Phenylisopropyladenosine
PI3	Phosphoinositide 3
PKA	Protein kinase A
PKC	Protein kinase C
R-PIA	<i>N</i> ⁶ -(<i>R</i>)-Phenylisopropyladenosine
SPA	<i>N</i> ⁶ -(<i>p</i> -Sulfophenyl)adenosine
TG	Triglycerides
VLDL-TG	Very low density lipoprotein triglyceride

1 Introduction

In 1961, Dole demonstrated that adenosine and some adenosine metabolites inhibited the breakdown of triglycerides (TG) to FFA in isolated rat epididymal fat pads (Dole 1961). Subsequent studies indicated that the initial event in hormone-induced

lipolysis was a rapid rise in intracellular cyclic adenosine monophosphate (cAMP) content (Butcher et al. 1965; Fain and Malbon 1979; Fain et al. 1972). Interestingly, lipolysis remained elevated for some time, and then began to decrease despite the continued presence of excess stimulatory agents (Butcher et al. 1965). Investigations into this phenomenon revealed that lipolysis could be restored by the addition of fresh incubation medium, suggesting that a component of the medium was limiting lipolysis, or that an inhibitor of lipolysis was being generated by the cells (Ho and Sutherland 1971; Schwabe et al. 1973). Schwabe et al. demonstrated that the inhibitory effect on lipolysis could be minimized by the addition of adenosine deaminase, as well as by reducing the number of incubated cells (Schwabe et al. 1973; Schwabe and Ebert 1974). Finally, Fain et al. showed that adenosine and adenosine analogs inhibited adenylate cyclase and antagonized the stimulation of cAMP by catecholamines (Fain et al. 1972), thereby inhibiting catecholamine-induced lipolysis in adipocytes. Later work established that inhibition of lipolysis by adenosine is mediated by activation of the A₁ adenosine receptor (A₁AR), and that this receptor was potentially an important regulator of lipolysis, fatty acid storage and tissue partitioning of fat (Fain et al. 1972; Johansson et al. 2007b; Schwabe et al. 1974). The focus of this review is on the role of A₁AR in the regulation of lipolysis in adipose tissue and its consequences for insulin resistance, diabetes and dyslipidemia. The potential of A₁AR agonists as therapeutically useful antilipolytic agents is also discussed.

Adenosine, an endogenous nucleoside, mediates its pharmacological actions via four distinct G-protein-coupled receptors (GPCR), classified as A₁, A_{2A}, A_{2B} and A₃ adenosine receptors (ARs). The role of A₁ARs in mammalian physiology and pharmacology has been well established (Dhalla et al. 2003; Fredholm and Sollevi 1986; Fredholm et al. 2001; Jacobson et al. 1996). The primary actions of adenosine mediated via A₁ARs include decreases in; heart rate and atrial contractility, release of neurotransmitters, lipolysis and renal function (for reviews see Belardinelli et al. 1989; Fredholm and Sollevi 1986; Gao and Jacobson 2007; Linden 1991; Press et al. 2007). A₁ARs mediate the inhibitory effects of adenosine and are primarily coupled to pertussis toxin-sensitive inhibitory guanine nucleotide-binding (G_i and G_o) proteins (Munshi et al. 1991). These G proteins regulate adenylate cyclases, kinases and ion channels (e.g., potassium) that play crucial roles in various cellular functions (Belardinelli et al. 1989; Brechler et al. 1990; Okajima et al. 1989). As depicted in Fig. 1, activation of A₁ARs in adipocytes causes inhibition of adenylate cyclase activity, reduction of cyclic AMP formation, inhibition of protein kinase A (PKA), and inhibition of lipolysis (Dobson 1978; Fain and Malbon 1979; Fain et al. 1972; Londos and Wolff 1977; Schrader et al. 1977). Although a direct inhibition of hormone-sensitive lipase (HSL) by A₁AR agonists has not been demonstrated, because of the well-established role of HSL and more recently adipose triglyceride lipase (ATGL) in lipolysis, it is assumed that inhibition of lipolysis by adenosine and its analogs is due to the activation of A₁ARs, resulting in the inhibition of HSL and/or ATGL.

The means by which activation of A₁ARs causes inhibition of adenylate cyclase and reduction in cAMP have been well characterized. A₁AR agonists inhibit

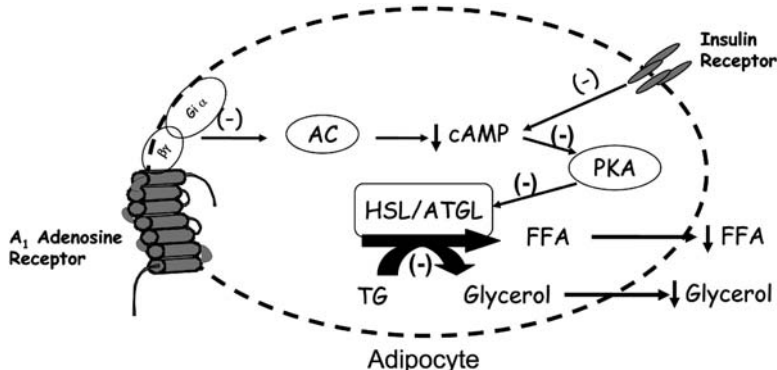


Fig. 1 Schematic representation of mechanisms by which A₁ adenosine receptors (A₁ARs) mediate antilipolytic effects in adipocytes. Activation of A₁ARs causes inhibition of adenylate cyclase (AC) activity via G_i (inhibitory GTP-binding protein), reduction of cyclic AMP (cAMP) formation, and inhibition of protein kinase A (PKA), leading to a reduction of hormone-sensitive lipase (HSL) and/or adipose triglyceride lipase (ATGL) activity which results in inhibition of the breakdown of triglycerides (TG) to free fatty acids (FFA)

the activation of adenylate cyclase activity caused by stimulatory agents (e.g., catecholamines), but have minimal effect in the absence of such agents (Dobson 1983; Dobson et al. 1986; Schrader et al. 1977). The affinity of adenosine for the adipocyte A₁ARs is estimated to be in the low micromolar range and is similar to that seen in cardiac myocytes (Liang et al. 2002; Srinivas et al. 1997). Uncoupling of the receptor from G protein with pertussis toxin (which causes ADP ribosylation of the α -subunit of G_i) attenuates the inhibition by adenosine of adenylate cyclase activity (Moreno et al. 1983), as well as the antilipolytic effects mediated by A₁AR activation. Hence, it has been proposed that A₁ARs and G_i proteins are intimately coupled in adipocytes, and that one is not likely to be affected independently of each other, suggesting that inactivation of G proteins cannot be overcome by activating a greater number of adenosine receptors (Liang et al. 2002). Functional uncoupling of A₁ARs from its effectors leads to de novo synthesis of A₁ARs by nuclear factor kappa beta (NF- κ B) and protein kinase C (PKC) activation (Jajoo et al. 2006). Each A₁AR appears to activate a certain number of G protein molecules (more than one), and amplification of G protein activation appears to be independent of total number of receptors (Baker et al. 2000). Thus, it has been suggested that the A₁AR–G protein activation ratio may be a better measure of cell responsiveness to agonists than the independent quantification of receptors and G proteins (Baker et al. 2000).

In addition to direct antilipolytic effects, adenosine and its analogs have been shown to modulate insulin action and insulin sensitivity in muscle and adipose tissue (Budohoski et al. 1984; Rolband et al. 1990) which is suggested to be mediated via A₁ARs. For instance, a partial A₁ agonist, CVT-3619 (2-{6-[(1*R*,2*R*)-2-hydroxycyclopentyl]amino}purin-9-yl){4*S*,5*S*,2*R*,3*R*}-5-[(2-fluorophenylthio)methyl]oxolane-3,4-diol) decreased the EC₅₀ for insulin to inhibit lipolysis in vivo by fourfold, suggesting that CVT-3619 increases insulin sensitivity

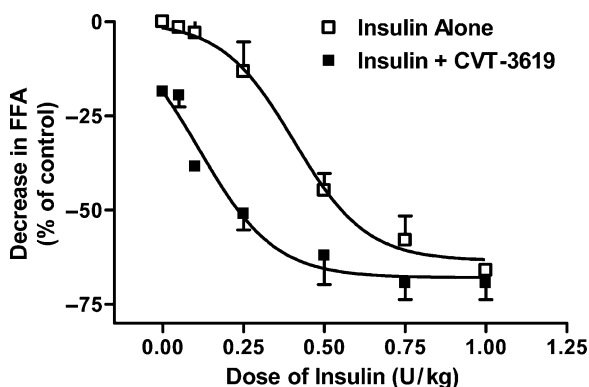


Fig. 2 CVT-3619 potentiates the effect of insulin to reduce FFA levels. Shown are the dose-response curves for the effect of insulin in reducing FFA in the absence and presence of CVT-3619 (0.5 mg kg^{-1}) in awake rats. Both insulin and CVT-3619 were given via i.p. injection. Each data point is the mean \pm SEM of the maximal (peak effect) percent decrease in FFA levels from baseline for 3–5 rats. The doses of insulin that cause a 50% decrease (ED_{50}) in FFA level in the absence and presence of CVT-3619 were 0.4 (0.3916–0.4208, 95% CI) and 0.1 (0.0935–0.133) U kg^{-1} , respectively

in adipose tissue (Fig. 2). Phenylisopropyladenosine (PIA), a full A₁AR agonist, potentiates the insulin-induced activation of phosphoinositide 3-kinase (PI3K), a second messenger for insulin actions, in rat adipocytes (Takasuga et al. 1999). In summary, the role of adenosine in the regulation of lipolysis and insulin homeostasis is well established, and these effects are mediated by the A₁AR.

2 A₁AR Expression in Adipose Tissue

A₁ARs are highly expressed in adipose tissue (Dhalla et al. 2003; Trost and Schwabe 1981; Ukena et al. 1984b), and are sensitive to eliciting a functional response with low affinity ligands due to highly efficient coupling (Liang et al. 2002). A₁AR density (B_{max}) is reported to be comparable in adipose tissue from rat (690 fm mg^{-1} protein) and humans ($360\text{--}1,800 \text{ fm mg}^{-1}$ protein) (Green et al. 1989; Liang et al. 2002), although lower numbers ($72\text{--}95 \text{ fm mg}^{-1}$) for human adipose tissue have also been reported (Larrouy et al. 1991). It should be noted that the results of the binding studies depend on the ligand used to determine receptor density and, in general (as expected), agonist radioligands give lower numbers than that obtained by using antagonist radioligands (Kollias-Baker et al. 1997; Larrouy et al. 1991; Leung et al. 1990). Gene expression and functional studies using rat epididymal adipocytes and mouse Ob17 cells demonstrated that A₁ARs are expressed and functionally active in differentiated adipocytes (Borglum et al. 1996; Vassaux et al. 1993). In contrast, A₂AR expression and functional activity is generally decreased following differentiation (Borglum et al. 1996; Vassaux et al. 1993). Human

and rat A₁ARs are 90% homologous (Tatsis-Kotsidis and Erlanger 1999). In studies using human primary adipocyte membranes, the human A₁AR protein was found to have a slightly higher molecular weight than rat and a lower affinity for hydroxyphenylisopropyl adenosine (HPIA) (Green et al. 1989). While multiple A₁AR mRNA (messenger ribonucleic acids) transcripts have been identified, the common variant in human adipocytes is identical to the one characterized in human brain (Tatsis-Kotsidis and Erlanger 1999). Taken together, the available data suggests that human and rodent A₁ARs are structurally and functionally similar. The discrepancies regarding the A₁AR density in human adipose tissue remain to be resolved.

There is some evidence that the number of A₁ARs differs between fat depots. White adipose tissue from rats has a much higher affinity and binding capacity for the A₁AR agonist PIA than brown adipose tissue (Saggerson and Jamal 1990). Functionally, these differences result in white adipose tissue lipolysis being three-fold more sensitive to inhibition by PIA than brown adipose tissue (Saggerson and Jamal 1990). Adenosine receptor gene expression and A₁AR-mediated inhibition of lipolysis in epididymal and inguinal adipose tissue of normal rats were not different (Fatholahi et al. 2006). A₁AR protein expression was fourfold higher and B_{max} was twofold higher in subcutaneous adipose tissue than visceral adipose tissue isolated from women with body mass index (BMI) > 42 kg m⁻² (Barakat et al. 2006), even though mRNA expression was similar in both fat depots. Higher receptor numbers in subcutaneous tissue could mean that the A₁AR plays a larger role in regulating subcutaneous adipose fat storage than visceral fat storage; however, functional data to support this possibility is lacking. Finally, because it appears that measurement of gene expression may not accurately reflect the A₁AR number, determination of receptor number using either radiological binding studies or protein expression may be necessary to quantitate the number of receptors in various fat depots.

3 Adipocyte A₁AR Function and Regulation in Disease Models

A₁AR level (protein expression or receptor number) and activity in adipose tissue in models of insulin resistance, diabetes and obesity has not been systematically characterized. There are relatively few studies describing changes at the A₁AR expression and function in insulin resistance (Vannucci et al. 1989) and obesity (Kartinen et al. 1994). More specifically, it has been reported that A₁AR signaling is more active in adipocytes from obese Zucker rats (Berkich et al. 1995), resulting in increased sensitivity to inhibition by A₁AR agonists (Vannucci et al. 1989). A₁AR-mediated inhibition of adenylate cyclase was increased in adipocytes from Zucker fatty rats (Vannucci et al. 1989). Tonic activity of A₁ARs on lipolysis has also been shown to be higher in obese rats (LaNoue and Martin 1994). Kartinen et al., using adipocytes (large and small) from the same fat depot, showed that the large fat cells were more responsive to the inhibition of forskolin-stimulated adenylate cyclase activity by PIA than the small cells (Kartinen et al. 1991). It has been suggested that the changes in the receptor-effector complex on adipocytes could

influence the effectiveness and tissue selectivity of adenosine and its analogs. This notion is supported by data from in vivo studies showing that the A₁AR agonist, ARA, ([1*S*,2*R*,3*R*,5*R*]-3-methoxymethyl-5-[6-(1-[5-trifluoromethyl-pyridin-2-yl]pyrrolidin-3-[*S*]-ylamino)-purin-9-yl]cyclopentane-1,2-diol) is more potent in inhibiting tissue lipolysis in Zucker fatty rats (which are not diabetic) as compared to Wistar rats, and is equally efficacious/effective in lowering plasma FFA concentrations (Schoelch et al. 2004). Recently, it was reported that A₁AR gene expression was similar in normal (SD) and diabetic ZDF rats (although cell surface A₁AR number was not measured), and that inhibition of lipolysis by a partial A₁AR agonist is not different in SD and ZDF rats (Dhalla et al. 2008).

On the other hand, it has been reported that concentration of inhibitory protein (G_{i1}) is lower in fat cells isolated from obese subjects as compared to lean subjects, and that the decrease is negatively correlated with BMI (Kaartinen et al. 1994). Furthermore, it has been shown that *N*⁶-(*p*-sulphophenyl) adenosine (SPA), a selective A₁AR agonist, is less potent in diabetic ZDF rats as compared to control rats, resulting in a short-lasting antilipolytic effect (van Schaick et al. 1998b). Because no difference in the pharmacokinetics of SPA between control and diabetic rats was observed, it was suggested that metabolic alterations in diabetic ZDF rats might be associated with an altered sensitivity to A₁AR agonists.

Overall, the available data suggest that A₁AR-mediated responses may be more sensitive to ligands in adipose tissue isolated from animal models of obesity. Thus, it has been proposed that that inhibition of lipolysis due to excessive activity of A₁AR may lead to obesity (Barakat et al. 2006); however, there are not enough data to support this hypothesis. On the contrary, data from studies with in vivo pharmacological modulators of A₁AR function and adipose tissue specific A₁AR transgenic mice show that activation of A₁ARs in models of insulin resistance and obesity results in improvement in insulin sensitivity with no significant effect on weight gain (Dhalla et al. 2007b; Dong et al. 2001; Schoelch et al. 2004). Overexpression of A₁ARs in adipose tissue protects mice from diet-induced insulin resistance (Dong et al. 2001), whereas the number of A₁ARs in adipocytes from obese individuals has been reported to be decreased (Kaartinen et al. 1991). Similarly, HSL-deficient mice are also resistant to weight gain on a high-fat diet (Harada et al. 2003; Osuga et al. 2000). No significant changes in weight gain were observed up to four months in A₁AR knockout (KO) mice as compared to wild-type mice (Johansson et al. 2007a), suggesting that weight gain is not associated with A₁AR activity. In summary, the preponderance of the published data suggests that the inhibition of adipocyte lipolysis may simply result in a redistribution of fat to adipose tissue rather than an accumulation of fat and weight gain.

A simple comparison of A₁AR expression in obese vs. lean adipocytes from human and animal models does not provide insight into how A₁ARs modulate circulating FFA concentrations, insulin sensitivity or type 2 diabetes. To understand the regulation of lipolysis through the A₁ARs, studies correlating A₁AR expression and function to disease end-points, like circulating FFA, insulin and glucose homeostasis, body-weight changes and whole body fat distribution, are needed. This can be achieved by using selective ligands (agonists and antagonists) of the A₁AR, as discussed below.

4 Inhibition of Lipolysis: A Therapeutic Approach

Under normal conditions, FFAs released from the adipose tissue due to lipolysis are an important source of fuel for many tissues. However, when the effect of insulin in inhibiting FFA release from adipose tissue is reduced (adipose tissue insulin resistance), chronic increases in circulating FFA concentrations occur (Reaven 1995). In addition to aggravating muscle insulin resistance, increases in plasma FFA concentrations have other adverse metabolic effects that play an important role in the pathogenesis of type 2 diabetes (Boden 2001; Fruhbeck and Gomez-Ambrosi 2002; Fruhbeck et al. 2001; Wyne 2003). Included in the consequences of chronically elevated FFA concentrations are increased deposition of TG in tissues such as skeletal muscle, liver, pancreas and heart; contributing to the defects in insulin stimulation of muscle glucose uptake and glucose-stimulated insulin secretion that characterize patients with type 2 diabetes (Boden et al. 2005; Itani et al. 2002; Roden et al. 1996; Sako and Grill 1990).

Thus, pharmacological inhibition of lipolysis to lower plasma FFA would seem to be an attractive therapeutic approach for the management of insulin resistance and diabetes (Bays et al. 2004; Boden 2001, 2002; Jensen 2006; Langin 2006; Large and Arner 1998; Reaven 1995). Because FFAs are continually being mobilized from adipose tissue via lipolysis, inhibition of release of FFA can be expected to affect various metabolic processes. Despite overwhelming evidence of a role of elevated FFA in insulin resistance and diabetes, very few inhibitors of lipolysis are available for either experimental or clinical use. Nicotinic acid and its analog acipimox are the only well-characterized antilipolytic agents that are currently used for treatment of dyslipidemia (Carlson 2005; Vega et al. 2005). Their therapeutic usefulness is limited because the initial decrease in plasma FFA levels is followed by a rebound effect that leads to transient increases in FFA and insulin resistance (Poynten et al. 2003). In addition, nicotinic acid has an unfavorable side-effect profile, and it has been suggested that it may not be an appropriate drug to use in the treatment of diabetic patients as it can increase plasma glucose levels (Garg and Grundy 1990; Grundy et al. 2002; McKenny et al. 1994; Poynten et al. 2003). Lowering circulating FFA levels by inhibiting adipose tissue lipolysis by A₁AR agonists can potentially fulfill an unmet need for novel antilipolytic agents in the treatment of pathological conditions where FFA are elevated. In this context, activation of A₁ARs has been shown to result in lowering plasma glucose levels in streptozotocin-induced diabetic rats (Cheng et al. 2000; Nemeth et al. 2007; Reaven et al. 1988). Dipyridamole, which increases endogenous adenosine levels, also lowers plasma glucose levels in a dose-dependent manner (Cheng et al. 2000). In addition, because FFA flux to the liver is an important modulator of very low density lipoprotein-triglyceride (VLDL-TG) synthesis and secretion, lowering plasma FFA concentrations by inhibiting lipolysis reduces the supply of FFA to the liver, thereby decreasing hepatic VLDL-TG production and circulating plasma TG concentrations. Therefore, antilipolytic effects of A₁AR agonists may be beneficial in a variety of conditions wherein plasma FFA and TG concentrations are elevated, including insulin resistance, diabetes and dyslipidemia.

4.1 A₁AR Agonists

A large number of selective and potent A₁AR agonists and antagonists have been synthesized over the last three decades for research and therapeutic purposes (Ashton et al. 2008; Cappellacci et al. 2008; Klotz et al. 1989; Klotz 2000; Morrison et al. 2004; Muller 2001; Palle et al. 2004). Ligands with high (several hundredfold) selectivity for the A₁AR versus one or two of the other subtypes of ARs have been synthesized and characterized; however, ligands with high selectivity for the A₁AR versus all three other subtypes of ARs have not been described to date (Klotz 2000). Several agonists of the A₁AR have been developed as potential antilipolytic agents, and a few of them have entered early-phase clinical trials (Cox et al. 1997; Dhalla et al. 2007a; Fraser et al. 2003; Fredholm et al. 2001; Hoffman et al. 1986b; Ishikawa et al. 1998; Jacobson et al. 1992; Klotz 2000; Leblanc and Soucy 1994; Press et al. 2007; Schoelch et al. 2004; Shah et al. 2004; Strong et al. 1993; Van der Graaf et al. 1999; van Schaick et al. 1998b; Wagner et al. 1995; Zannikos et al. 2001). However, none has received regulatory approval. In the following section we summarize the salient features of the few selected A₁AR ligands that have been reported to have antilipolytic effects.

SDZ WAG-994 (*N*-cyclohexyl-2'-*O*-methyladenosine) is an orally bioavailable, selective A₁AR agonist and is one of the first compounds developed as a potential therapeutic agent based on A₁AR agonism. In isolated adipocytes, SDZ WAG-994 inhibited lipolysis and increased insulin-dependent glucose uptake with an EC₅₀ = 8 nM (Foley et al. 1997; Wagner et al. 1995). Single doses of SDZ WAG-994 given to normal rats caused dose-dependent decreases in serum FFA, glucose and insulin levels, with maximal reductions of up to 68% (Ishikawa et al. 1998). The hypoglycemic effect of SDZ WAG-994 was abolished when FFA levels were kept constant by infusion of lipids, suggesting that glucose lowering by SDZ WAG-994 was due to FFA reduction (Foley et al. 1997). Although SDZ WAG-994 has beneficial metabolic effects in various animal models, the antilipolytic effects could not be separated from cardiovascular effects. For example, a 66% reduction in FFA with a dose of 3 mg kg⁻¹ was accompanied by a 73% reduction in heart rate and 50% reduction in mean arterial pressure in rats (Cox et al. 1997).

GR79236 (*N*-[(1*S*,2*S*)-2-hydroxycyclopentyl]-adenosine) is a selective and very potent A₁AR agonist (Strong et al. 1993). GR79236 inhibited lipolysis in isolated adipocytes and was shown to reduce plasma FFA and glucose levels in normal rats (Gardner et al. 1994; Merkel et al. 1995; Strong et al. 1993). Rats fed a high fructose diet also showed improved glucose tolerance; however, a significant reduction of blood pressure following GR79236 treatment was observed (Qu et al. 1997). In a rat model of diabetic ketoacidosis, GR79236 reduced plasma FFA but did not affect blood glucose levels. The lack of effect on glucose was proposed to be a result of stimulation of gastric emptying and enhanced absorption of stomach contents (Thompson et al. 1994). However, in addition to its potent antilipolytic effects, GR79236 also caused hypotension and bradycardia in conscious rats (Merkel et al. 1995).

ARA is an AR agonist with high affinity and selectivity for A_1 ARs (Schoelch et al. 2004). ARA treatment significantly lowered dialysate glycerol levels in subcutaneous and visceral adipose tissue and gastrocnemius muscle, as measured by the tissue microdialysis technique in Wistar and Zucker fatty rats. ARA treatment caused significant reductions in plasma FFA, glycerol and TGs and an improvement in insulin sensitivity in Zucker fatty rats. The effects of ARA on heart rate and blood pressure were not reported in this study (Schoelch et al. 2004).

CVT-3619 is a selective and partial agonist of the A_1 AR (Fatholahi et al. 2006). The binding affinity of CVT-3619 for A_1 AR is 113 nM. The K_i values for CVT-3619 to bind to A_{2A} , A_{2B} and A_3 ARs are $>5,000$ nM, suggesting that CVT-3619 has very low affinity for these receptor subtypes (Fatholahi et al. 2006). The antilipolytic effects of CVT-3619, *in vitro* and *in vivo*, have been well characterized (Dhalla et al. 2007b; Fatholahi et al. 2006). CVT-3619 reduces forskolin-induced increase in cAMP and FFA levels in epididymal adipocytes. The IC_{50} values for CVT-3619 for reducing cAMP levels and FFA release are 6 and 47 nM, respectively (Fatholahi et al. 2006). CVT-3619 lowers circulating FFA and triglyceride (TG) levels (20–60%) in a dose-dependent manner in awake rats at doses (1–10 mg kg^{-1}) that do not have any significant effect on heart rate or blood pressure. In a two-week high fat diet-induced model of insulin resistance in rats, pretreatment with CVT-3619 prevented the development of insulin resistance (Dhalla et al. 2007a).

Tecadenoson (6-(*N*-3'-(*R*)-tetrahydrofuran-2-ylamino)-purine riboside), a potent and selective A_1 AR agonist, has been shown to lower FFA levels in rats in a dose-dependent manner (Fraser et al. 2003). Although tecadenoson was not designed to be an antilipolytic agent, infusion of tecadenoson also reversibly reduced elevated FFA levels in a pilot Phase I study in patients (data on file at CV Therapeutics). Interestingly, the antilipolytic effect of tecadenoson was observed at doses that did not affect heart rate or P–R interval (AV nodal conduction). This result is consistent with the presence of a high receptor density and greater receptor reserve in adipocytes as compared to the heart (Liang et al. 2002) (see Sect. 6.1).

Pharmacokinetics and pharmacodynamics of another AR agonist, referred to as ARA, which has high affinity for A_1 ARs and A_2 adenosine receptors (A_{2A} ARs), were determined in a Phase I clinical study with two parallel groups of 13 healthy males following administration of a single 6 h intravenous infusion of ARA or placebo (Zannikos et al. 2001). ARA was found to have high clearance (Cl: 0.79 L h^{-1} kg^{-1}), with a modest volume of distribution (V_{ss} : 0.91 L kg^{-1}) and short half-life ($t_{1/2}$: approximately 1 h). The reduction in circulating levels of FFA by ARA was related to its plasma concentrations using a modified E_{max} (maximal effective concentration)-based tolerance model, and the EC_{50} value was 17.0 ng mL^{-1} . The results of this study led to the suggestion that the use of A_1 AR agonists as antilipolytic drugs may be limited due to the potential development of tolerance or desensitization, and that a period free from the agonist may be required before the response of FFA returns to baseline conditions. It should be noted that this agonist, which was given as a continuous IV infusion for 6 h, is not selective for A_1 ARs and also has high affinity for A_{2A} ARs (Zannikos et al. 2001). Activation of A_{2A} ARs would increase FFA levels indirectly by causing sympathetic stimulation (Dhalla et al. 2006), and therefore will counteract the effect mediated by A_1 ARs.

RPR749 and its methylated metabolite RPR772 are reported to be orally active and selective A₁AR agonists that inhibit lipolysis and lower plasma FFA and TG levels in various animal models (Shah et al. 2004); however, preclinical reports on these compounds have not been described. The pharmacokinetics and pharmacodynamics (effect on FFA) of RPR749 were evaluated in humans in a double-blind, placebo-controlled, parallel-group, randomized Phase I study with a single oral dose of up to 200 mg (Shah et al. 2004). Six parallel groups of eight healthy men (six active and two placebo/group) were enrolled in the study. Plasma samples were collected for up to 72 h postdose. RPR749 was safe and well tolerated as a single oral dose up to 200 mg. Plasma concentrations of RPR749 were approximately 30-fold higher than the mean RPR772 plasma concentrations. The mean terminal half-lives of RPR749 and RPR772 were similar (approximately 16.4 h). Serum FFA concentrations decreased (between 25 and 70%) in all treatment groups, with the maximal decrease in the 200 mg dose group. However, significant decreases in FFA concentrations were also observed in the placebo group. RPR749 seems to have pharmacological properties that may be beneficial in treating insulin resistance and hyperlipidemia; however, further development of this compound has been discontinued for reasons that are not publicly disclosed.

In summary, data in the literature suggests that A₁AR agonists are a viable approach to lowering FFA by inhibiting adipose tissue lipolysis; however, a number of hurdles need to be overcome (as described in Sect. 5) before this class of molecules can be successfully developed as antilipolytic drugs.

4.2 A₁AR Antagonists

Interestingly, an antagonist of the A₁AR, BW-1433 (1,3-dipropyl-8-[*p*-(carboxy-ethynyl) phenyl]xanthine), has also been reported to improve glucose tolerance in Zucker rats after a six-week treatment (Xu et al. 1998). This is contrast to the many studies showing that glucose tolerance improves in response to treatment with A₁AR agonists (Dhalla et al. 2007a; Hoffman et al. 1986b; Schoelch et al. 2004; van Schaick et al. 1998b). Given that agonists of A₁AR inhibit lipolysis and lower circulating FFA, one would expect an increase in FFA levels with administration of an A₁AR antagonist, which will lead to a worsening of insulin resistance. The paradoxical findings of Xu et al. 1998 can be explained based on the observation that treatment with BW-1433 actually resulted in a very small increase in FFA levels. Furthermore, the increase in FFA was transient and disappeared after seven days. On the other hand, one-week treatment with BW-1433 resulted in a selective increase in the number of A₁ARs in the adipose tissue. It has been reported that endogenous adenosine tonically inhibits adipose tissue A₁ARs (Liang et al. 2002). Therefore, improvement in glucose tolerance with BW-1433 may be due to the inhibitory effects of endogenous adenosine acting on a higher background of A₁ARs upregulated by chronic treatment with BW-1433 (Xu et al. 1998). In this regard, overexpression of A₁ARs in mice has also been shown to improve glucose tolerance

in a model of diet-induced insulin resistance and obesity (Dong et al. 2001). Furthermore, BW-1433 is a nonselective antagonist of the AR subtypes, and has been reported to have higher affinity for A_{2B} adenosine receptors (A_{2B}ARs) (US patent no. 6060481). Thus, the antidiabetic effects of BW-1433 may not solely be due to A₁AR antagonism, as suggested (Xu et al. 1998).

5 Challenges for the Development of A₁AR Agonists as Therapeutic Agents

In general, the challenges for the development of A₁AR agonists as therapeutic agents are similar to those for other GPCR agonists. They include at least (a) selectivity, (b) receptor downregulation, and (c) receptor desensitization. The development of A₁AR agonists as antilipolytic agents has been further limited by the concurrent side effects induced by the activation of A₁ARs in other organs such as heart, kidney and brain (Belardinelli et al. 1989; Wu et al. 2001). The following section describes the specific challenges and hurdles that need to be overcome for the successful development of A₁AR ligands as antilipolytic agents.

5.1 Receptor Density and Distribution

A₁ARs are widely expressed in both the central nervous system and peripheral tissues, as shown by radioligand binding (Gould et al. 1997; Kollias-Baker et al. 1995; Ukena et al. 1984a), in situ hybridization (Reppert et al. 1991; Schiffmann et al. 1990), immunohistochemistry (Rivkees 1995), mRNA expression (Dixon et al. 1996; Tatsis-Kotsidis and Erlanger 1999; Shen et al. 2005) and PET (positron emission tomography) scanning (Meyer et al. 2003). Under normal physiological conditions, A₁ARs are found at their highest density in the brain (cortex, cerebellum and hippocampus), followed by adipose tissue. Moderate to high densities of A₁ARs appear to be present on specialized cells of the thyroid, spinal cord, eye, adrenal gland, kidney, and sinoatrial and atrioventricular nodal tissues of the heart. In most other tissues, including the cardiac ventricles, lung, pancreas, liver and GI tract, the expression of A₁ARs is low (Lohse et al. 1984; Dixon et al. 1996). The expression of A₁ARs is increased under conditions of oxidative stress, ischemia, inflammation and diabetes (Funakoshi et al. 2007; Grden et al. 2007; Lai et al. 2005; Liu et al. 2003; Nie et al. 1998; Pawelczyk et al. 2005; Rogachev et al. 2006).

Receptor density and coupling efficiency of receptor activation to response (i.e., receptor reserve) influence and determine the responses to receptor agonists in various organs in the intact animal; in general, an agonist is more potent or efficacious where the receptor number is high (Strange 2008). Thus, although adipose tissue has the second highest receptor density of A₁ARs, ubiquitous expression of A₁ARs is one of the biggest challenges to the development of antilipolytic agents without

eliciting unwanted side effects. However, the high density of receptors efficiently coupled to a functional response enables the adipocytes to respond with high sensitivity even to low-affinity ligands such as adenosine, which can inhibit lipolysis at concentrations as low as 1–2 nM (Liang et al. 2002). Thus, differences in receptor reserve and coupling efficiency in various organs can be exploited to achieve functional selectivity, as described in Sect. 6.1.

5.2 Receptor Desensitization

Desensitization of the response or tachyphylaxis is a potential problem when considering a receptor agonist for long-term therapeutic use. It has been suggested that the A₁AR desensitizes slowly (hours to days) (Baker et al. 2000; Green et al. 1990; Hoffman et al. 1986a; Parsons and Stiles 1987), which was attributed to the finding that A₁AR does not undergo phosphorylation upon short-term agonist treatment (Gao et al. 1999; Palmer et al. 1996). However, rapid acute tolerance to the FFA-lowering effect of the AR agonist, ARA, has been reported following a continuous intravenous infusion of the agonist to healthy volunteers (Zannikos et al. 2001). Whether this was due to desensitization of the FFA-lowering effect of the agonist on A₁ARs or due to stimulation of A_{2A} receptors because of lack of selectivity of this agonist is not known.

Mechanism(s) underlying desensitization of A₁ARs include changes in the following: (a) receptor number and/or affinity; (b) receptor to G-protein-coupling efficiency, and; (c) quantity of G proteins (Kenakin 1984). Desensitization of adipocyte responses to AR agonists is accompanied by downregulation of A₁ARs, loss of high-affinity receptor sites, and decrease in alpha subunit of G_i protein (Green 1987; Green et al. 1990; Jajoo et al. 2006; Parsons and Stiles 1987). The changes in G proteins (an increase in G_{αs}; decreases in G_{α1} and G_{α2}) have been suggested to be at the protein level, as no differences were detected in their respective transcripts (Longabaugh et al. 1989). Using an A₁–A₃ chimeric receptor, it was demonstrated that A₁ARs lack the necessary molecular determinants for desensitization in response to short-term agonist treatment (Palmer et al. 1996). Chronic exposure to agonists has been shown to induce phosphorylation, clustering and desensitization of A₁ARs (Ciruela et al. 1997). Recent findings indicate that A₁ARs aggregate with adenosine deaminase on the cell surface and then translocate either into or out of caveolae upon treatment with an agonist (Gines et al. 2001; Lasley et al. 2000), suggesting another mechanism for regulation of A₁AR signaling. Regardless of the underlying mechanism, receptor desensitization is a potential problem for the development of A₁AR agonists as therapeutic agents for chronic use. Desensitization of the antilipolytic effect of A₁ARs has been shown to occur with prolonged and continuous exposure to high concentrations of full A₁AR agonists in *in vitro* and *in vivo* studies (Green 1987; Hoffman et al. 1986a; Zannikos et al. 2001). Prolonged incubation of isolated adipocytes with the A₁AR agonist

PIA resulted in downregulation of A_1 ARs (Green 1987). Adipocytes isolated from epididymal fat pads of rats treated with PIA for six days were insensitive to the acute inhibitory effects of PIA on lipolysis (Hoffman et al. 1986a).

It should be noted that the abovementioned studies on receptor desensitization have been conducted using full agonists (e.g., N^6 -(*R*)-phenylisopropyladenosine (R-PIA)), which are more likely to cause desensitization. It has been suggested that partial agonists of G-protein-coupled receptors may cause less receptor desensitization than full agonists (Kovoor et al. 1998; Vachon et al. 1987). Consistent with this notion was the finding that both the magnitude and the duration of the FFA-lowering effect of the partial A_1 AR agonist CVT-3619 were similar for at least three consecutive (1 h apart) injections, suggesting that the effect of this agonist does not undergo acute desensitization; i.e., tachyphylaxis (Dhalla et al. 2007a). However, to our knowledge, desensitization upon continuous infusion of partial agonists has not been investigated.

On the other hand, A_1 ARs can be upregulated by chronic exposure to AR antagonists (Murray 1982; Szot et al. 1987; Xu et al. 1998). Upregulation of the receptors is also accompanied by an increase in the functional response to the agonist (Szot et al. 1987; Wu et al. 1989). Chronic exposure to the AR antagonists theophylline and caffeine resulted in an increase in the total number of A_1 ARs, an enhanced coupling of the receptors to G_i protein, and an increased quantity of $G_{\alpha i}$ protein (Ramkumar et al. 1988; Wu et al. 1989). Treatment with BW-1433, an antagonist of A_1 ARs, in insulin-resistant rats led to an increase in A_1 AR number and an improvement in glucose tolerance (Xu et al. 1998), suggesting that an increase in A_1 AR number in adipose tissue is beneficial; perhaps by increasing the tonic effects of adenosine on lipolysis due to the increase in receptor density (Liang et al. 2002). Transgenic mice overexpressing A_1 ARs have been shown to be protected from high fat diet-induced obesity and glucose intolerance (Dong et al. 2001). The proposed beneficial effects of coffee consumption on insulin resistance may also be a result of an increase in A_1 ARs by caffeine, an unselective and nonspecific AR antagonist.

6 Possible Solutions to the Challenges Involved in Developing A_1 AR Agonists as Antilipolytic Agents

The unwanted effects of A_1 AR agonists can theoretically be minimized, and functional selectivity achieved with these compounds, as a consequence of differential receptor reserve for the A_1 AR-mediated responses in nonadipose (e.g., cardiac) and adipose tissue (Fraser et al. 2003; Liang et al. 2002; Wu et al. 2001). Furthermore, by using partial agonists (low-efficacy agonists), the differences in receptor reserve between various tissues (e.g., cardiac vs. adipose) are sufficient to achieve functional selectivity of drug action (Dhalla et al. 2007a; van Schaick et al. 1998a), as discussed below.

6.1 Receptor Reserve

Receptor reserve is a term used to indicate that maximal response to a given agonist in a given cell or tissue can be obtained by activation of less than 100% of the receptors. It is thus an indicator of both the number of receptors that mediate a response and the efficiency (amplification) of receptor-to-response coupling. Because receptor reserve is dependent on the intrinsic activity of the agonist, each value of receptor reserve must specify the agonist, response and tissue for which the measurement is pertinent. When receptor reserve is present, the agonist concentration–response curve lies to the left of the concentration–receptor occupancy curve, and hence the K_A value (concentration of agonist that occupies 50% of receptors) is greater than the EC_{50} value (concentration of agonist that causes a half-maximal response). That is, the potency of the agonist is greater than its affinity when receptor reserve is present, whereas the potency and affinity of an agonist are equal when receptor reserve is absent. The ratio between K_A and EC_{50} is referred to as the pharmacological shift ratio (PSR), and is used to quantify the extent of receptor reserve (Ruffolo 1982). Receptor reserves for A₁AR-mediated responses in cardiac and adipose tissues have been measured by using a specific, selective and irreversible A₁AR antagonist (Baker et al. 2000; Liang et al. 2002; Morey et al. 1998; Srinivas et al. 1997). As stated above, the extent of A₁AR reserve is unique for each agonist, response, and tissue such that the receptor reserve for the A₁AR agonist 2-chloro-*N*⁶-cyclopentyladenosine (CCPA) is higher than that for R-PIA for reducing the cAMP content of adipocytes (Liang et al. 2002). The EC_{50} for adenosine to reduce lipolysis in human adipocytes has been reported to be 6 nM, suggesting that receptor reserve of A₁ARs is also present in human adipocytes (Kather 1988). Results of these studies provide much of the rationale for the use of partial agonists (discussed below) for antilipolytic effects. In summary, significant differences in agonist receptor reserve among tissues (and/or responses) can thus be exploited to selectively elicit responses in tissues with the highest receptor reserves. Greater selectivity is more likely to be achieved with partial agonists (discussed below) than with full agonists (Dhalla et al. 2007a; van Schaick et al. 1998a), because the partial agonist is expected to elicit a robust response only in tissues with relatively high receptor reserve, whereas the full agonist will elicit a robust response wherever A₁ARs are present.

6.2 Partial Agonists

Partial agonists are useful for achieving drug selectivity for the target tissue and minimizing toxicity caused by the activation of the same class of receptors in nontarget tissues (de Ligt and IJzerman 2002). In contrast to a full agonist, a partial agonist is a low-efficacy receptor ligand that elicits a submaximal response, even when all ($\geq 95\%$) available receptors are occupied (Stephenson 1997; Wu et al. 2001). Therefore, a partial agonist is less effective than a full agonist in evoking a response in a

tissue(s) with low amplification of the signal transduction pathway (Kenakin et al. 1992), and will elicit fewer responses in the intact animal than a full agonist. Partial agonists have pharmacological features that offer several advantages over a full agonist for therapeutic purposes, as described below.

Studies of structure–activity relationships for adenosine derivatives have led to the discovery of a few selective A_1 partial agonists (Cristalli et al. 1988; Lorenzen et al. 1997; Mathot et al. 1995; Muller 2001; Palle et al. 2004; van Tilburg et al. 2001), which have been synthesized by substitution at the 2′-, 3′- and 5′-hydroxyl groups of the ribose moiety of adenosine (de Ligt and IJzerman 2002; Klotz 2000; Morrison et al. 2004; Poulsen and Quinn 1998; van der Wenden et al. 1995). A few studies reporting the antilipolytic and cardiac effects of partial A_1 AR agonists have appeared (Dhalla et al. 2007a; Fatholahi et al. 2006; Srinivas et al. 1997; van Schaick et al. 1998a; Wu et al. 2001). However, few partial agonists with demonstrated high affinity and selectivity for the A_1 AR have been described in the literature. Therefore, for the discussion of partial agonists in the following section, we will focus on the antilipolytic effects of CVT-3619, a recently discovered novel partial A_1 AR agonist (Dhalla et al. 2007a; Fatholahi et al. 2006).

6.2.1 Organ and Response Selectivity

In adipose tissue, a partial agonist can provoke a maximal functional response at concentrations that do not affect the heart rate because receptor density is high and receptor reserve is large in adipocytes, whereas it is low in the heart. For example, CVT-3619 causes minimal or no A_1 AR-mediated slowing of heart rate (Fatholahi et al. 2006), indicating that it is a partial agonist for this cardiac response. In contrast, CVT-3619 is a full agonist to decrease cAMP content in rat adipocytes and decrease plasma levels of FFA (Dhalla et al. 2007a; Fatholahi et al. 2006). As shown in Fig. 3, while the full agonist CPA decreases lipolysis and markedly reduces

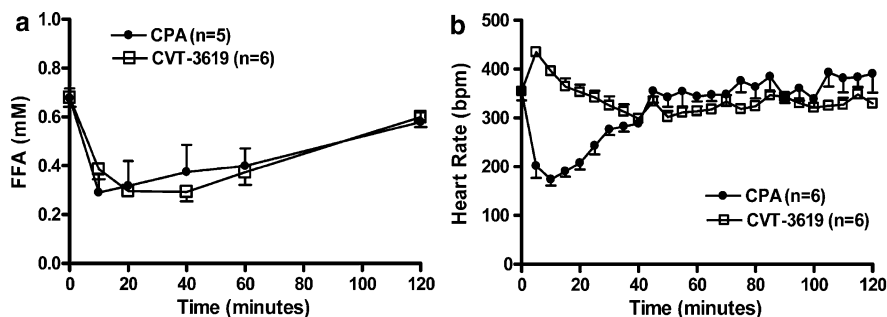


Fig. 3 a–b Comparison of the **a** antilipolytic effect and **b** cardiac effect of CVT-3619 to that of full agonist CPA. For FFA measurements, animals were fasted overnight and were treated with CVT-3619 (10 mg kg^{-1} , p.o.) or CPA ($20 \mu\text{g kg}^{-1}$, i.p.). Effect of CVT-3619 and CPA on heart rate was determined by telemetry in awake rats at the same doses. Data are presented as the mean \pm SEM derived from the number of animals indicated in the parentheses after each group

heart rate, the partial agonist CVT-3619 reduces plasma FFA concentration to similar extent without causing bradycardia. Administration of another partial A₁ agonist, CVT-2759 ($\{[(5\text{-}[6\text{-}[(3R)\text{oxolan-3-yl]amino}]purin-9\text{-yl})(3S,2R,4R)\text{-}3,4\text{-dihydroxyoxolan-2-yl]}\text{-methoxy}\}\text{-}N\text{-methylcarboxamide}$), to the intact rat caused a greater response in adipocytes (decrease of lipolysis) than in the heart (decrease of heart rate) (Liang et al. 2002).

6.2.2 Less Receptor Desensitization than Full Agonist

It has been suggested that partial agonists of G-protein-coupled receptors cause less receptor desensitization than full agonists (Kovoor et al. 1998; Vachon et al. 1987). The FFA lowering effect of the A₁AR partial agonist CVT-3619 was found to be devoid of acute desensitization (Dhalla et al. 2007a). The acute antilipolytic effect of CVT-3619, given twice daily, was maintained up to six weeks of treatment (unpublished data). However, it remains to be determined whether the antilipolytic effect of CVT-3619 is sustained over long-term use (months) or continuous infusion. Thus, additional work with new partial agonists of the A₁AR is needed to confirm the hypothesis that partial agonists are less prone to cause desensitization and the unwanted therapeutic effect of tolerance/tachyphylaxis.

6.2.3 Functions as an Antagonist of a Full Agonist

Partial agonists may also be considered ligands that display both agonistic and antagonistic effects. When both a full agonist and partial agonist are present, the partial agonist acts as a competitive antagonist, competing with the full agonist for receptor occupancy and producing a net decrease in the receptor activation observed with the full agonist alone. A partial agonist will displace the concentration–response curve of a full agonist to the left, just as an antagonist would (Stephenson 1997). This has also been shown to be the case for the A₁AR agonists. For example, CVT-2759 was shown to cause a leftward shift of the adenosine concentration–response curve to slow AV nodal conduction in guinea pig isolated heart (Wu et al. 2001). Interestingly, it is unlikely that a partial A₁AR will reduce diuresis, an effect expected from a full A₁AR agonist, because it will antagonize the antidiuretic effect of endogenous adenosine (a full agonist).

7 Conclusions

A₁AR agonists are potent inhibitors of adipose tissue lipolysis and have the potential to be developed as clinically useful antilipolytic agents. Because of the ubiquitous presence of these receptors, achieving organ and response selectivity is most important for developing A₁AR agonists as successful antilipolytic agents, and this may

be possible with partial agonists. Additional studies are needed to clearly understand the role of A₁AR in the regulation of lipolysis in various pathological conditions where lipolysis may be dysfunctional and the expression of A₁ARs is changed (e.g., during oxidative stress and inflammation).

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A₃ Adenosine Receptor: Pharmacology and Role in Disease

P.A. Borea, S. Gessi, S. Bar-Yehuda, and P. Fishman

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Abstract The study of the A₃ adenosine receptor (A₃AR) represents a rapidly growing and intense area of research in the adenosine field. The present chapter will provide an overview of the expression patterns, molecular pharmacology and functional role of this A₃AR subtype under pathophysiological conditions. Through studies utilizing selective A₃AR agonists and antagonists, or A₃AR knockout mice, it is now clear that this receptor plays a critical role in the modulation of ischemic diseases as well as in inflammatory and autoimmune pathologies. Therefore, the potential therapeutic use of agonists and antagonists will also be described. The discussion will principally address the use of such compounds in the treatment of brain and heart ischemia, asthma, sepsis and glaucoma. The final part concentrates on the molecular basis of A₃ARs in autoimmune diseases such as rheumatoid arthritis, and includes a description of clinical trials with the selective agonist CF101. Based on this chapter, it is evident that continued research to discover agonists and antagonists for the A₃AR subtype is warranted.

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Keywords A₃ Adenosine receptor · Gene and tissue localization · Ischemic conditions · Inflammation · Autoimmune diseases

Abbreviations

$\Delta\psi$	Mitochondrial membrane potential
A ₁ ARA ₁	Adenosine receptor
A _{2A} ARA _{2A}	Adenosine receptor
A _{2B} ARA _{2B}	Adenosine receptor
A ₃ AR ^{-/-}	Functional deletions of the A ₃ AR
A ₃ ARA ₃	Adenosine receptor
AC	Adenylyl cyclase
ACR	American College of Rheumatology
ADA	Adenosine deaminase
ADA ^{-/-}	Adenosine deaminase deficient
AIA	Adjuvant-induced arthritis
AICAR	Aminoimidazole carboxamide ribonucleotide
AR	Adenosine receptor
Ca ²⁺	Calcium
cAMP	Cyclic adenosine monophosphate
CHO – hA ₃	Chinese hamster ovary cells transfected with human A ₃ AR
Cl–IB–MECA	2-Chloro- <i>N</i> ⁶ -(3-iodobenzyl)- <i>N</i> -methyl-5'-carbamoyladenosine
CNS	Central nervous system
ConA	Concanavalin A
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CP-532,903	<i>N</i> ⁶ -(2,5-Dichlorobenzyl)-3-aminoadenosine-5- <i>N</i> -methylcarboxamide
DAG	1,2-Diacylglycerol
DMARDs	Disease-modifying antirheumatic drugs
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
ERK1/2	Extracellular signal-regulated kinases
GPCR	G-protein-coupled receptor
GRKs	G-protein-coupled receptor kinases
GSK-3 β	Glycogen synthase kinase
HIF-1 α	Hypoxia-inducible factor 1 α
IB–MECA	<i>N</i> ⁶ -(3-Iodobenzyl)-adenosine-5'- <i>N</i> -methylcarboxamide
I κ B	Inhibitor of κ B
IKK	I κ B kinase
IL	Interleukin
IPC	Ischemic preconditioning
IP ₃	Inositol triphosphate
JNK	Jun N-terminal kinase

K _{ATP}	ATP-sensitive potassium
KO	Knockout
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MEK	Mitogen-activated protein kinase kinase
Mito	Mitochondrial
MTX	Methotrexate
mPTP	Mitochondrial permeability transition pore
MRS 1191	3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate
MRS 1523	5-Propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate
NF-κB	Nuclear factor kappa B
NOS	Nitric oxide synthase
OGD	Oxygen and glucose deprivation
p38	Stress-activated protein kinase with molecular weight 38 kDa
PBMC	Peripheral blood mononuclear cells
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB/Akt	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PTX	Pertussis toxin
RA	Rheumatoid arthritis
RBL	Rat basophilic leukemia
STAT3	Signal transducer and activator of transcription 3
<i>t</i> _{1/2}	Half-life
TNF-α	Tumor necrosis factor alpha

1 Cloning, Distribution and Gene Structure of the A₃ Adenosine Receptor (A₃AR)

The A₃AR is the last member of the adenosine family of G-protein-coupled receptors (GPCR) to have been cloned. It was originally isolated as an orphan receptor from rat testis and designated *tgpcr1*, and it has 40% sequence homology with canine A₁ and A_{2A} adenosine receptor (AR) subtypes (Meyerhof et al. 1991). Subsequently, an identical clone was obtained from rat striatum, initially named R226, and functionally expressed in Chinese hamster ovary (CHO) cells (Zhou et al. 1992). Homologs of the rat striatal A₃AR have been identified and cloned from sheep hypophysial pars tuberalis and from human striatum and heart (Linden et al. 1993; Sajjadi and Firestein 1993; Salvatore et al. 1993). Recently, an equine A₃AR

was cloned and pharmacologically characterized, and revealed a high degree of sequence similarity with that of human and sheep A₃AR transcripts (Brandon et al. 2006). Whilst the cDNA sequences of A₁, A_{2A} and A_{2B} ARs are highly conserved between rat and human, interspecies differences in A₃AR structure are large, with the rat A₃AR showing only 72% sequence homology with that of sheep and human. This led to different pharmacological profiles for the species homologs, especially in relation to antagonist binding (Jacobson and Gao 2006).

Interspecies differences have also been found in the peripheral expression of A₃AR mRNA. In particular, the tissue distribution of the human A₃AR transcript was found to be more similar to the sheep than to the rat homolog. In the rat, it has a very narrow distribution, being expressed mainly in the testes, lung, kidneys, heart and brain; in the sheep, the A₃AR transcript is expressed in lung, spleen, pars tuberalis and pineal gland; in the human it is highly expressed in lung and liver and at a moderate level in heart, kidney, placenta and brain (Dixon et al. 1996; Linden 1994; Rivkees 1994; Salvatore et al. 1993; Zhou et al. 1992). The presence of A₃AR protein has been evaluated through radioligand binding and immunoassays in primary cells, tissues and cell lines of differing origins. The first cell line that was demonstrated to have high levels of endogenous A₃AR was the rat mast cell line RBL-2H3, where binding experiments detected a density of about 1 pmol mg⁻¹ of protein (Olah et al. 1994; Ramkumar et al. 1993). Low levels of A₃AR binding sites have been observed in the mouse, rat, gerbil and rabbit brain (Jacobson et al. 1993; Ji et al. 1994). No direct evidence of the presence of A₃AR has been obtained in cardiomyocytes (Peart and Headrick 2007), even though several functional studies reported that it was responsible for cardioprotection (Cross et al. 2002; Headrick and Peart 2005; Shneyvays et al. 1998, 2001; Tracey et al. 1997; Xu et al. 2006). Recently, functional A₃ARs have been detected on mice aorta, mediating contraction through a cyclooxygenase (COX)-dependent mechanism (Ansari et al. 2007). Importantly, the A₃AR was found at high levels in a variety of primary cells involved in inflammatory responses, including human eosinophils (Khono et al. 1996a), neutrophils (Bouma et al. 1997; Chen et al. 2006a; Gessi et al. 2002), monocytes (Broussas et al. 1999, 2002; Thiele et al. 2004), macrophages (McWhinney et al. 1996; Szabo et al. 1998), dendritic cells (Dickenson et al. 2003; Fossetta et al. 2003; Hofer et al. 2003; Panther et al. 2001) and lymphocytes (Gessi et al. 2004a). Finally, very high expression of A₃AR protein was observed in a variety of cancer cell lines (Gessi et al. 2001, 2007; Merighi et al. 2001, 2003; Suh et al. 2001) and in cancer tissues, suggesting a role for this subtype as a tumor marker (Gessi et al. 2004b; Madi et al. 2004).

The A₃AR receptor coding region was found to be divided into two exons separated by a single intron of about 2.2 kb. The upstream sequence does not contain a TATA-like motif, but it has a CCAAT sequence and consensus binding sites for SP1, NF-IL6, GATA1 and GATA3 transcription factors (Murrison et al. 1996). The involvement of the latter factors in transcriptional control of this gene would be consistent with a role of the receptor in immune function. Bioinformatics studies revealed the presence of nuclear factor kappa B (NF-κB) in the A₃AR promoter, demonstrating the role of this transcription factor in determining A₃AR expression level (Bar-Yehuda et al. 2007).

The A₃AR has been mapped on human chromosome 1 (Atkinson et al. 1997) and consists of 318 amino acid residues. The A₃AR subtype is a GPCR characterized by its C-terminal portion facing the intracellular compartment and seven transmembrane-spanning domains. Differently to other adenosine receptors, the C-terminal region presents multiple serine and threonine residues, which may serve as potential sites of phosphorylation that are important for receptor desensitization upon agonist application (Palmer et al. 1995a, b).

2 A₃ Adenosine Receptor (A₃AR) Signal Transduction

The first second-messenger systems found to be associated with A₃AR activation were adenylyl cyclase (AC) activity, which is inhibited, and phospholipase C (PLC), which is stimulated, through G_i and G_q protein coupling, respectively (Abbracchio et al. 1995; Ramkumar et al. 1993). Activation of PLC is responsible for inositol triphosphate (IP₃) and intracellular calcium (Ca²⁺) elevation in a variety of cellular models. Initially, A₃AR agonist-induced effects on Ca²⁺ mobilization were observed in HL-60 cells, in human eosinophils, and in cardiomyocytes, where a high micromolar EC₅₀ value was shown by 2-chloro-*N*⁶-(3-iodobenzyl)-*N*-methyl-5'-carbamoyladenine (CI-IB-MECA), making it difficult to reconcile this functional effect with its high affinity in binding and cyclic adenosine monophosphate (cAMP) inhibition assays (Kohnno et al. 1996a, b; Shneyvays et al. 2000). Other studies reporting similar results followed (Gessi et al. 2001, 2002; Merighi et al. 2001; Reshkin et al. 2000; Shneyvays et al. 2004), suggesting the possibility that a GPCR might have different potencies in different signaling pathways in the same cellular system (Schulte and Fredholm 2000; Fredholm et al. 2000). Recently, through a transgenic mammalian animal model that expresses apoaequorin, allowing intracellular Ca²⁺ concentrations to be measured in living organisms, functional expression of A₃AR in pancreatic cells was observed and CI-IB-MECA was effective in increasing calcium at the micromolar level (Yamano et al. 2007).

On the other hand, there have also been studies showing that this A₃AR agonist has nanomolar affinity in calcium mobilization studies (Fossetta et al. 2003), suggesting that this pathway may be differentially activated by A₃ARs depending on the cellular system investigated. Recently, a role for A₃AR activation in the reduction of calcium increase induced by P2X₇ receptors in retinal ganglion cells has also been reported, shifting the balance of purinergic action from that of death to the preservation of life (Zhang et al. 2006).

In addition, other intracellular pathways have been described as being linked with A₃AR activation. Starting from the pioneering work by Schulte and Fredholm, who reported the coupling of all adenosine receptors with mitogen-activated protein kinases (MAPKs), a plethora of studies has followed showing the modulation of these kinases by A₃AR in different recombinant and native cell lines (Schulte and Fredholm 2000). A₃AR signaling in CHO cells expressing human A₃AR (CHO – hA₃) triggers the stimulation of extracellular signal-regulated

kinases (ERK1/2) through $\beta\gamma$ release from pertussis toxin (PTX)-sensitive G proteins, phosphoinositide 3-kinase (PI3K), Ras and mitogen-activated protein kinase kinase (MEK) (Schulte and Fredholm 2002). Functional A_3AR activating ERK1/2 has also been described in microglia cells, where a biphasic, partly G_i -protein-dependent influence on the phosphorylation of the ERK1/2 has been found (Hammarberg et al. 2003). In colon cancer cells, after adenosine deaminase (ADA) treatment, A_3AR activation stimulates cell proliferation through ERK1/2 activation (Gessi et al. 2007), whilst in melanoma cells it stimulates PI3K-dependent phosphorylation of protein kinase B (PKB/Akt), leading to the reduction of basal levels of ERK1/2 phosphorylation (Merighi et al. 2005a). MAPK kinase activation is also responsible for adenosine-mediated hypoxia-inducible factor- α (HIF1- α) stimulation in melanoma, colon carcinoma and glioblastoma cells (Merighi et al. 2005b, 2006, 2007a). An active MAPK signaling pathway appears to be essential for A_3AR phosphorylation, desensitization and internalization (Trincavelli et al. 2002a). ERK1/2 are also involved in cardiac hypertrophy and can play a protective role in ischemic myocardium. Interestingly, A_3AR activation in rat cardiomyocytes has been demonstrated to increase ERK1/2 phosphorylation by involving $G_{i/o}$ proteins, protein kinase C (PKC), and tyrosine kinase-dependent pathways (Germack and Dickenson 2004). An ERK-dependent signal has been also reported in the protective effects of A_3AR activation in lung injury following *in vivo* reperfusion (Matot et al. 2006). Another important pathway triggered by adenosine via A_3AR is that of PI3K/Akt. There is evidence that A_3AR activation mediates phosphorylation of PKB/Akt, protecting rat basophilic leukemia (RBL)-2H3 mast cells from apoptosis through the $\beta\gamma$ subunits of G_i proteins and PI3K- β (Gao et al. 2001). In contrast, it has been reported that A_3AR activation is able to decrease the levels of PKA, a downstream effector of cAMP, and of the phosphorylated form of PKB/Akt in melanoma cells. It is well known that protein kinase A (PKA) and PKB/Akt phosphorylate and inactivate glycogen synthase kinase 3 β (GSK-3 β), a serine/threonine kinase that acts as a key element in the Wnt signaling pathway. In its active form, GSK-3 β suppresses mammalian cell proliferation (Fishman et al. 2002a, b, 2004). This implies the deregulation of the Wnt signaling pathway, which is generally active during embryogenesis and tumorigenesis in order to increase cell cycle progression and cell proliferation (Fishman et al. 2002b). Support for this mechanistic pathway comes from the work of Chung et al. (2006), who demonstrated the deregulation of the Wnt pathway in LJ-529 breast cancer cells. A central role of PI3K has been demonstrated for A_3AR -induced p38 and ERK1/2 stimulation in CHO-h A_3 and in immortalized N13 microglial cells (Hammarberg et al. 2003). Furthermore, it was found that serine 727 phosphorylation of signal transducer and activator of transcription 3 (STAT3) is a possible downstream target of A_3AR -mediated ERK1/2 activation (Hammarberg et al. 2004). Modulation of these pathways is relevant, as it may represent the molecular basis for the apoptotic-modulating effects of the A_3AR . Activation of PI3K-Akt-phospho-BAD by A_3AR has been observed recently in glioblastoma cells, leading to cell survival in hypoxic conditions (Merighi et al. 2007b). Contrasting results have been obtained on PI3K modulation related to cytokine production by A_3AR s. For example, in BV2 mouse

microglial cells, A₃AR stimulation inhibited LPS-induced PI3K/Akt activation, leading to the inhibition of tumor necrosis factor alpha (TNF- α) and the NF- κ B pathway (Lee et al. 2006b). However, in human monocytes, N⁶-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA) activated the PI3K/Akt signaling pathway and induced the phosphorylation of the MAPK p38, ERK, and Jun N-terminal kinase (JNK), thus leading to a reduction in interleukin (IL)-12 production (Haskó et al. 1998; la Sala et al. 2005). The PI-3K/PKB and MEK1/ERK1/2 pathways, which are involved in cell survival, have been linked with preconditioning effects induced by A₃AR activation in cardiomyocytes from newborn rats during hypoxia/reoxygenation (Germack and Dickenson 2005). Cardioprotection at reperfusion has been observed after A₃AR activation of the PI3K/Akt pathway, leading to a reduction of GSK-3 β and mitochondrial permeability transition pore opening (mPTP) (Park et al. 2006). In the heart, A₃AR mediates cardioprotective effects through ATP-sensitive potassium (K_{ATP}) channel activation. Moreover, it is coupled to the activation of RhoA and the subsequent stimulation of phospholipase D (PLD), which in turn mediates the protection of cardiac myocytes from ischemia (Lee et al. 2001; Mozzicato et al. 2004; Parsons et al. 2000).

2.1 A₃ Adenosine Receptor (A₃AR) Desensitization

The initial characterization of the A₃AR expressed in RBL-2H3 rat mast cells demonstrated that agonist-stimulated calcium mobilization is subject to a rapid, homologous desensitization that is apparent after only a few minutes of agonist exposure (Ali et al. 1990). This phenomenon in GPCR-coupled receptors is typically triggered by receptor phosphorylation induced by either second-messenger-activated kinases or G-protein-coupled receptor kinases (GRKs). In the case of A₃AR, it has been demonstrated that desensitization of the rat subtype after 10 min of agonist exposure is associated with rapid phosphorylation on serine and threonine residues by a GRK2 kinase (Palmer et al. 1995a). This was related to a reduction of 30–40% in the number of high-affinity binding sites and to a functional receptor desensitization, as manifested by an eightfold increase in the IC₅₀ value of IB-MECA-mediated inhibition of cAMP levels. It has been reported that under conditions in which the A₃AR undergoes agonist-dependent phosphorylation and desensitization, the A₁ adenosine receptor (A₁AR) was not affected. Indeed, the A₃R contains multiple serine and threonine residues in the region of the C-terminal tail that are important for phosphorylation by GRK2. Therefore, a chimeric A₁–A₃AR obtained by introducing the extreme C-terminal 14-amino acid segment of the A₃AR into the A₁AR, expressed in CHO cells, undergoes rapid desensitization, suggesting that the C-terminal domain of the A₃AR is the site for phosphorylation by GRK2, 3 and 5-kinases, with the last being less important (Palmer et al. 1996). It has been also demonstrated that in response to short-term agonist exposure, A₃ARs internalize profoundly and rapidly ($t_{1/2}$ = 10 min) over a time frame that follows the onset of receptor phosphorylation, in contrast to the A₁AR, which internalized quite

slowly ($t_{1/2} = 90$ min). A nonphosphorylated A₃AR mutant failed to internalize over a 60 min time course, suggesting that receptor phosphorylation was essential for rapid A₃AR internalization to occur. In addition, fusion onto the A₁AR of the A₃AR C-terminal domain containing the sites for phosphorylation by GRKs conferred rapid agonist-induced internalization kinetics ($t_{1/2} = 10$ min) on the resulting chimeric AR; this suggests that GRK-stimulated phosphorylation of threonine residues within the C-terminal domain of the A₃AR is obligatory to observe rapid agonist-mediated internalization of the receptor (Ferguson et al. 2000). In particular, the amino acid residues in the C-terminus responsible for rapid desensitization were Thr(307), Thr(318), and Thr(319). Individually mutating each residue demonstrated that Thr(318) and Thr(319) are the major sites of phosphorylation. Phosphorylation at Thr(318) appeared to be necessary to observe phosphorylation at Thr(319), but not vice versa. In addition, the mutation of two predicted palmitoylation-site cysteine residues proximal to the regulatory domain resulted in the appearance of an agonist-independent basal phosphorylation. Therefore, GRK-mediated phosphorylation of the C-terminal tail of the A₃AR in situ appears to follow a sequential mechanism, perhaps involving receptor depalmitoylation, with phosphorylation at Thr(318) being particularly important (Palmer et al. 2000).

The agonist-induced internalization of the human A₃ARs in CHO-hA₃ cells and the relationship between internalization, desensitization and resensitization have been investigated. Agonist-induced internalization of A₃ adenosine receptors was directly demonstrated by immunogold electron microscopy, which revealed the localization of these receptors in plasma membranes and intracellular vesicles. Moreover, short-term exposure of these cells to the agonist caused rapid desensitization, as tested in AC assays. Subsequent removal of the agonist led to restoration of the receptor function and recycling of the receptors to the cell surface. Blockade of internalization and recycling demonstrated that internalization did not affect signal desensitization, whereas recycling of internalized receptors was implicated in the signal resensitization (Trincavelli et al. 2000). These mechanisms have been also evaluated on native A₃ARs in human astrocytoma cells. Short-term exposure to the agonist Cl-IB-MECA caused rapid receptor desensitization, within 15 min. Agonist-induced desensitization was accompanied by receptor internalization. A₃AR internalized with rapid kinetics, within 30 min, after cell exposure to Cl-IB-MECA. After desensitization, the removal of agonist led to the restoration of A₃AR functioning through receptor recycling to the cell surface within 120 min (Trincavelli et al. 2002a). The involvement of ERK 1 and 2 in A₃AR phosphorylation has been demonstrated. A₃AR mediated the activation of ERK 1/2 with typical transient monophasic kinetics within 5 min. The activation was not affected by hypertonic sucrose cell pretreatment, suggesting that this effect occurred independently of receptor internalization. The exposure of cells to the MEK inhibitor PD98059 prevented MAPK activation and inhibited homologous A₃AR desensitization and internalization, impairing agonist-mediated receptor phosphorylation. PD98059 inhibited the membrane translocation of GRK2, which is involved in A₃AR homologous phosphorylation, suggesting that the MAPK cascade is involved in A₃AR regulation by a feedback mechanism which controls GRK2 activity and

probably involves direct receptor phosphorylation (Trincavelli et al. 2002b). Receptor activation, internalization and recycling events have also been described in B16F10 murine melanoma cells, where they play an important role in turning on/off receptor-mediated signal transduction pathways. It has been observed that melanoma cells highly express A₃AR on the cell surface, which is rapidly internalized to the cytosol and “sorted” to the endosomes for recycling and to the lysosomes for degradation. Receptor distribution in the lysosomes was consistent with the downregulation of receptor protein expression and was followed by mRNA and protein resynthesis. Receptor binding experiments reveal a reduction in receptor density after 15 and 60 min, and a full recovery after 24 h (Madi et al. 2003). In an *in vivo* prostate cancer model, chronic treatment of the tumor-bearing rats with IB-MECA resulted in receptor downregulation shortly after treatment. Interestingly, full recovery of the A₃AR was noted after 24 h, demonstrating the continuing presence of the receptor upon chronic agonist treatment (Fishman et al. 2003).

As for the effect of prolonged agonist exposure of CHO cells expressing a recombinant rat A₃AR, it has been shown that this induces a desensitization of receptor function that is associated with the downregulation of specific G protein subunits (Palmer et al. 1995b). Given the structural and pharmacological differences displayed by rat and human A₃ARs, it has been reported that in CHO_hA₃ cells the prolonged agonist exposure results in not only a receptor density decrease and functional desensitization but that it also induces a sensitization of the stimulatory pathway of AC by increasing its activity by 1.5- to 2.5-fold. This sensitization was not a consequence of the downregulation of G_i proteins induced by agonist treatment, and was not associated with sustained or transient increases in the expression of Gs. Moreover, it was not due to the synthesis of new proteins, because cycloheximide treatment failed to inhibit sensitization. Instead, the inability of the sensitization process to alter the forskolin-stimulated AC activity in the presence of manganese chloride, which uncouples AC from G-protein regulation, suggested that prolonged A₃AR activation increased the coupling efficiency between Gs and AC catalytic units (Palmer et al. 1997). This phenomenon might provide a molecular basis for the observation that for many of the effects mediated by adenosine receptors, acute and chronic agonist treatment often produce opposite effects. A marked downregulation of A₃ARs following prolonged agonist exposure (1–24 h) has been observed also by Trincavelli and colleagues (2002a). After downregulation, the recovery of receptor functioning was slow (24 h) and associated with the restoration of receptor levels close to control values.

3 A₃ Adenosine Receptor (A₃AR) and Ischemic Brain Disease

Despite low levels of A₃AR message in the central nervous system (CNS), one of the first effects observed following intraperitoneal injection of an A₃AR agonist (IB-MECA) was depression of locomotor activity in mice (Jacobson et al. 1993). However, contrasting results on how A₃AR activation might influence neuronal

activity in rat brain in both normoxic and hypoxic conditions have been reported, making it difficult to understand whether an A₃AR agonist or antagonist would be better to treat cerebral ischemia.

It has been reported that in the CA1 region of the rat hippocampus, A₃AR had no direct effect on synaptically evoked excitatory responses, whilst it induced heterologous desensitization of A₁ARs, thus limiting adenosine-mediated cerebroprotection (Dunwiddie et al. 1997). Moreover, in the CA3 area of immature rat hippocampal slices, it has been observed that CI-IB-MECA facilitates epileptiform discharges, suggesting that activation of A₃ARs following a rise in endogenous adenosine facilitates excitation, thus again limiting the known inhibitory and neuroprotective effects of adenosine in immature brain (Laudadio and Psarropoulou 2004).

Other studies suggested that A₃AR activation in cortical neurons mediated a depression of synaptic transmission by inhibiting glutamate release additionally to and independently from the A₁ARs, thus providing neuroprotection (Brand et al. 2001; Lewerenz et al. 2003; Lopes et al. 2003). These contrasting actions may lead to both protective or deleterious effects during ischemia, when adenosine concentrations rise to levels that activate the A₃AR subtype. In particular, it has been reported that chronic preischemic treatment with the agonist IB-MECA before forebrain ischemia in gerbils induces a significant protection of neurons and a reduction in the subsequent mortality, whilst acute administration of the drug results in a pronounced worsening of neuronal damage and postischemic mortality (von Lubitz et al. 1994). Accordingly, for the acute effect, cell death induction was also observed in cell cultures of rat cerebellar granule neurons where high concentrations of CI-IB-MECA induced lactate dehydrogenase release, neuronal cell death and glutamate-mediated neurotoxicity (Sei et al. 1997).

Destructive and protective actions of A₃AR stimulation have also been demonstrated in astroglial cells, where CI-IB-MECA at nanomolar doses was responsible for "trophic effects" related to the reorganization of the actin cytoskeleton, whilst it was a mediator of apoptosis in the micromolar range (Abbracchio et al. 1997, 2001; Appel et al. 2001; Di Iorio et al. 2002). It has been suggested that astrocyte death induced during severe metabolic stress by A₃AR activation may isolate the worst-affected tissue by physically excising cells from sites of irreversible injury (von Lubitz 1999). This may help to shift energetic resources to less severely injured tissue (the penumbra) and increase the chance of survival for the penumbra (von Lubitz 1999). It was later demonstrated that desensitization/downregulation of the A₃AR may be the basis of cytoprotection, suggesting a role for this receptor in induction of cell death (Trincavelli et al. 2002a). The effect of A₃AR activation has been investigated during "preconditioning," a phenomenon consisting of exposure to brief periods of hypoxia or ischemia that result in increased tolerance to severe ischemic insults (Dawson and Dawson 2000; Ishida et al. 1997). Pugliese et al. (2003) demonstrated that blocking A₃AR during preconditioning episodes improved the recovery of field excitatory postsynaptic potential, and this suggests that the stimulation of A₃ARs by endogenous adenosine may be one of the negative stimuli that eventually leads to the irreversible loss of neurotransmission during prolonged ischemic episodes. Later, this group reported that A₃AR antagonism prevented or

delayed the appearance of anoxic depolarisation induced by prolonged (7 min) oxygen and glucose deprivation (OGD) episodes, and exerted a protective effect on neurotransmission, supporting the evidence that A₃AR stimulation is deleterious during prolonged ischemia (Pugliese et al. 2006). Interestingly, the same results in terms of protection were obtained after the extended application of an A₃AR agonist, suggesting that A₃ARs undergo rapid desensitization following exposure to exogenous agonist (Pugliese et al. 2007). In contrast, when the A₃AR agonist was applied for a short time, so that it was unable to cause receptor desensitization, A₃AR activation was responsible for a depression of synaptic activity, like that obtained after A₁AR activation. This is in agreement with previous data concerning A₃AR-mediated inhibition of excitatory neurotransmission in rat cortical neurons (Hentschel et al. 2003). Therefore, it has been suggested that prolonged ischemic conditions could be crucial in switching the effects of A₃AR stimulation from protective to dangerous by counteracting the inhibitory effects of A₁AR on excitatory neurotransmission or potentiation of excitotoxic glutamate effects. On this basis, it has been speculated that A₃AR stimulation by adenosine released during brief periods of ischemia might exert A₁AR-like protective effects on neurotransmission. Prolonged periods of ischemia are able to change the A₃AR-mediated effects from protective to dangerous (Pugliese et al. 2007).

The potential neuroprotective actions of the A₃AR have been further demonstrated using mice with functional deletions of the A₃AR (A₃AR^{-/-}). The A₃AR^{-/-} mice reveal a number of CNS functions where the A₃ARs play a role, including nociception, locomotion, behavioral depression and neuroprotection. Pharmacologic or genetic suppression of A₃AR function enhances some aspects of motor function and suppresses pain processing at supraspinal levels. In response to repeated episodes of hypoxia, A₃AR^{-/-} mice show an increase in neurodegeneration, suggesting the possible use of A₃AR agonists in the treatment of ischemic, degenerative conditions of the CNS (Fedorova et al. 2003). Other authors found that the purine inosine exerted protective effects in stroke animals, in terms of reduced bradykinesia and cerebral infarction induced by middle cerebral artery occlusion, and suggest that they were mediated by A₃AR activation (Shen et al. 2005). Accordingly, in cortical culture, Cl-IB-MECA pretreatment antagonized the hypoxia-mediated decrease in cell viability. Animals subjected to focal cerebral ischemia and treated with Cl-IB-MECA showed increased locomotor activity and decreased cerebral infarction. In these animals, Cl-IB-MECA also reduced the density of TUNEL labeling in the lesioned cortex. Furthermore, in A₃AR^{-/-} mice, an increase in cerebral infarction was found compared with the A₃AR wild-type controls, suggesting that A₃ARs are tonically activated during ischemia. Additionally, intracerebroventricular pretreatment with Cl-IB-MECA decreased the size of infarction in the wild-type controls, but not in the A₃AR^{-/-} animals, suggesting that Cl-IB-MECA induced protection through the A₃ARs (Chen et al. 2006b).

Different evidence suggests that some of the neuroprotection induced by A₃AR derives from its modulation of the brain immune system (Daré et al. 2007; Haskó et al. 2005). A₃AR stimulation induces the synthesis of neuroprotective chemokine ligand 2 (Wittendorp et al. 2004). Moreover, in lipopolysaccharide (LPS)-treated

BV2 microglial cells, A₃AR activation suppresses TNF- α production by inhibiting PI3K/Akt and NF- κ B activation, suggesting that selective ligands of this receptor may have therapeutic potential for the modulation and possible treatment of brain inflammation (Lee et al. 2006b).

It has long been known that adenosine plays an important role in ischemia, and abundant evidence indicates that it is an endogenous neuroprotective agent. Apart from the well-established protective role exerted by adenosine through A₁AR activation, a lot of work has been carried out to shed light on the effects exerted through A₃AR stimulation. Even though the role of this AR subtype in neuroprotection has been enigmatic for a long time, new data from *in vitro* and *in vivo* A₃AR^{-/-} mice models suggest a neuroprotective role. It can also be speculated that apparently contrasting results concerning protective effects induced through A₃AR block may be explained by the very fast internalization and desensitization of the A₃AR, making agonist exposure therapeutically equivalent to antagonist occupancy of the receptor.

4 A₃ Adenosine Receptor (A₃AR) and Ischemic Heart Disease

One of the most important topics in the area of A₃AR-targeted therapy is the protective role of this adenosine receptor subtype in cardiac ischemia. To date, several studies have pointed to the evidence that the A₃AR is a key player in adenosine-induced cardioprotection during and following ischemia-reperfusion (Headrick and Peart 2005). Following the discovery of ischemic preconditioning (IPC) as a mechanism to reduce infarct size (Murry et al. 1986), and the identification of adenosine as one of the mediators of this phenomenon, a lot of work has been done that attributes A₁AR with a major role in adenosine-mediated effects. Liu et al. (1994) found that the A₁AR antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) was not able to abolish the anti-infarct effect induced by IPC in rabbit, thus suggesting the possible involvement of another adenosine subtype that they pharmacologically identified as the A₃AR (Liu et al. 1994). Furthermore, it was demonstrated in rabbit that IB-MECA reproduced IPC, suggesting the involvement of A₃AR subtype modulation, and there was also a lack of efficacy in reducing IPC-induced cardioprotection by A₁AR-selective antagonists in dog models (Auchampach et al. 1997a, 2004). In terms of the timing of cardioprotection, some reports have indicated that preischemic A₃AR agonism is effective and necessary, while others suggested that protection occurs postischemia, and still others have found that A₃AR agonism is able to trigger an anti-infarct response with either pre- or postischemic treatment (Auchampach et al. 2003). Pretreatment with an A₃AR agonist is responsible for cardioprotection, and may be classified into classic or early preconditioning, in which adenosine treatment occurs for 5 min, before exposure to ischemia (Armstrong and Ganote 1994; Tracey et al. 1997, 1998; Wang et al. 1997), and delayed or late preconditioning, in which adenosine treatment occurs 24 h before the induction of ischemia (Takano et al. 2001; Zhao et al. 2002). The mechanism involved in these effects (shared with the A₁AR subtype) was

shown to be the activation of PKC and the regulation of mitochondrial K_{ATP} channels (Auchampach et al. 1997a; Thourani et al. 1999). However, in avian cells it has been reported that the signaling pathways activated by the A₁ARs and A₃ARs are distinct and involve selective coupling to PLC and PLD/RhoA, respectively (Lee et al. 2001; Mozzicato et al. 2004). Recently, by studying the cardioprotective profile of the A₃AR agonist N⁶-(2,5-dichlorobenzyl)-3-aminoadenosine-5-N-methylcarboxamide (CP-532,903) in an isolated mouse heart model of global ischemia and reperfusion and an *in vivo* mouse model of infarction, it has been found that A₃AR activation provides ischemic protection by facilitating the opening of the sarcolemmal isoform of the K_{ATP} channel (Wan et al. 2008). In addition, roles for MAPK and Akt/PI3 kinase have been documented for early preconditioning (Germack and Dickenson 2004, 2005), whilst for late preconditioning the involvement of NF-κB, synthesis of inducible nitric oxide synthase (NOS) and mitochondrial K_{ATP} channels has been suggested (Zhao et al. 2002). This was not recognized by Takano et al., who reported that an NOS-dependent pathway was implicated in the effect mediated through A₁AR, but not in A₃AR activation (Takano et al. 2001). In any case, late preconditioning is more relevant than early preconditioning due to its sustained duration and the possibility of maintaining patients in a protracted, preconditioned, defensive state.

The cardioprotective effects of A₃ARs were also detected in A₃AR-overexpressing mice, where infarct size was lower than in wild-type mice after *in vivo* regional ischemia and reperfusion (Black et al. 2002). In these animals, A₃ARs overexpression decreased basal heart rate and contractility, preserved ischemic ATP, and decreased postischemic dysfunction (Cross et al. 2002). Recent evidence obtained by using pharmacological agents and genetic methods suggest that Cl-IB-MECA protects against myocardial ischemia/reperfusion injury in mice via A₃AR activation. These conclusions were suggested by experiments with a selective A₃AR antagonist and by evaluating the A₃AR agonist effects on A₃AR knockout (KO) mice. Interestingly, in this paper, by using congenic (C57BL/6) A₃AR KO mice, the deletion of the A₃AR gene itself has no effect on ischemic tolerance, suggesting that the previous contradictory results from the same and other groups (Cerniway et al. 2001; Guo et al. 2001; Harrison et al. 2002) can probably be explained by differences in the genetic backgrounds of the mice rather than specific deletion of the A₃AR gene. Interestingly, additional studies using wild-type mice treated with compound 48/80 (a condensation product of *p*-methoxyphenethyl methylamine with formaldehyde) to deplete mast cell contents exclude the possibility that Cl-IB-MECA exerts a cardioprotective effect by releasing mediators from mast cells (Ge et al. 2006) and support the idea that therapeutic strategies focusing on the A₃AR subtype are a novel and useful approach to protecting the ischemic myocardium. However, an important question arises from these data. Preconditioning obtained through adenosine receptor modulation may have clinical relevance (for example in cardiac surgery), but pretreatment is rarely permitted during acute myocardial infarction. For this reason, it would be more useful to achieve a protective effect from ischemia-reperfusion injury when the drug is administered postischemia or during reperfusion. Literature data indicate that A₃AR agonism is able to protect the

heart when given after the onset of ischemia or during reperfusion, suggesting its role in the treatment of acute myocardial infarction. In particular, Vinten-Johansen's group has reported that A_3AR agonist administration at reperfusion protects isolated rabbit hearts by reducing neutrophil activation (Jordan et al. 1999). After that, other studies also demonstrated a cardioprotective effect after A_3AR activation upon reperfusion in rat (Maddock et al. 2002), guinea pig (Maddock et al. 2003), and dog (Auchampach et al. 2003) hearts. As for the molecular mechanism involved in this effect, it has been reported that the opening of mPTP plays a crucial role in myocardial ischemia/reperfusion injury and that blockade of the pore opening is cardioprotective (Suleiman et al. 2001; Weiss et al. 2003). Interestingly, the inhibition of mPTP opening through the activation of PI3K/Akt and the consequent inhibition of glycogen synthase kinase after the activation of A_3AR have been reported (Park et al. 2006).

Despite the fact that the bulk of literature has reported the efficacy of adenosine in triggering cardioprotection, clinical trials to test adenosine as an adjunct to reperfusion therapy in patients with acute myocardial infarction have revealed controversial results (Mahaffey et al. 1999; Ross et al. 2005).

It has been commented that these discrepancies may be due to the age differences between animals used for experimental work and patients tested in clinical trials. In particular, experimental work has been done in healthy young adult animals, whilst heart disease is a typical pathology of the elderly population (Cohen and Downey 2008; Peart and Headrick 2007). Following this proposal, Ashton et al. (2003) reported reduced A_3AR and increased A_{2B} adenosine receptor ($A_{2B}AR$) mRNA levels with aging, similar to what happens during ischemia in young hearts (Jenner and Rose'meyer 2006). Additionally, a reduction in A_1AR has been observed during ischemia in aged hearts. Although it is just a hypothesis, decreased A_1AR and A_3AR expression might be responsible for the puzzling results mentioned above. Therefore, it is possible that differences in the modulation of adenosine receptor subtypes occur during aging and, due to the differences and simultaneous involvement of all AR subtypes in cardioprotection (Philipp et al. 2006; Solenkova et al. 2006), it is possible that a better understanding of their interplay and age dependence will provide insights into the treatment of ischemic injuries in the myocardium.

5 A_3AR Adenosine Receptor (A_3AR) and Inflammatory Diseases

The role of A_3AR in inflammatory diseases is currently controversial, and both anti- and proinflammatory effects have been attributed to its activation. One of the first therapeutic applications that was hypothesized for A_3AR antagonists was the treatment of asthma. In fact, it was reported that in rodents, A_3AR activation was responsible for mast cell degranulation (Fozard et al. 1996; Ramkumar et al. 1993; Shepherd et al. 1996). This was confirmed by Salvatore et al., who showed that the potentiating effect of Cl-IB-MECA on antigen-dependent mast cell degranulation disappeared in A_3AR KO mice, and that the inhibition of LPS-induced

TNF- α production was lower in mice lacking the A₃AR subtype (Salvatore et al. 2000). The involvement of A₃ARs in mast cell degranulation was further confirmed in murine lung mast cells, where it was dependent on intracellular Ca²⁺ elevations through G_i and PI3K coupling (Zhong et al. 2003). In addition, it has been reported that A₃AR mRNA was higher in lung tissue of patients with airway inflammation, and that A₃AR activation mediates rapid inflammatory cell influx into the lungs of sensitized guinea pigs (Spruntulis and Broadley 2001; Walker et al. 1997). Furthermore A₃AR activation in RBL-2H3 mast cells was found to inhibit the apoptosis of inflammatory cells expressing A₃ARs in inflamed tissues, thus allowing inflammatory cell expansion (Gao et al. 2001). However, in contrast with these findings, it has been demonstrated that human and canine mast cell degranulation is mediated by A_{2B}ARs instead of A₃ARs (Auchampach et al. 1997b; Feoktistov and Biaggioni 1995; Ryzhov et al. 2004). This discrepancy reflects the low overall coidentity of human and rat at the aminoacid level of A₃AR, and questions the role of the A₃AR as a target for asthma therapy. Another discrepant result that questions the involvement of A₃ARs in asthma is the recent finding that in the lung parenchymal strip from brown Norway rats, where contraction in response to adenosine is mast cell-mediated, the receptor involved shows similarities to the A₃AR, but Cl-IB-MECA is a high-affinity antagonist and 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate (MRS 1523) and 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(\pm)-dihydropyridine-3,5-dicarboxylate (MRS 1191) are inactive at concentrations that substantially exceed their affinities for the rat A₃AR, suggesting a puzzling A₃AR pharmacological picture in brown Norway rats (Wolber and Fozard 2005). However the idea of using an A₃AR antagonist in the treatment of asthma seems to be sustained by data obtained in other cells involved in this pathology. In fact, a high expression of A₃AR transcript levels has been found in eosinophilic infiltrates of the lungs of patients affected by asthma and chronic obstructive pulmonary disease (COPD) (Walker et al. 1997). Interestingly, similar findings were seen in the lungs of adenosine deaminase-deficient (ADA^{-/-}) mice that showed adenosine-mediated lung disease. Treatment of ADA^{-/-} mice with MRS 1523, a selective A₃AR antagonist, prevented airway eosinophilia and mucus production. Similar results were obtained in the lungs of ADA/A₃AR double KO mice, suggesting that A₃AR signaling plays an important role in regulating chronic lung disease, and that A₃AR antagonism may be useful for reducing eosinophilia (Young et al. 2004). These results are in contrast with experiments performed in human eosinophils *ex vivo*, where chemotaxis, degranulation and superoxide anion production were reduced by A₃AR activation (Ezeamuzie and Philips 1999). This discrepancy was later attributed to the *ex vivo* nature of the chemotaxis experiments performed, suggesting that the diminished airway eosinophilia seen in the lungs of ADA^{-/-} mice following the disruption of A₃AR is not a cell-autonomous effect of eosinophils, but may be due to the modulation of key regulatory molecules from other cells that express A₃ARs and that affect eosinophil migration (Young et al. 2004). However the clinical relevance of the A₃AR subtype in the pathogenesis of asthma remains a conundrum, and differences in pharmacology between the A₃AR subtypes

from different species make it difficult to determine whether an A₃AR agonist or antagonist could provide a better treatment for asthma. A novel A_{2A} adenosine receptor (A_{2A}AR) agonist/A₃AR antagonist used in a randomized, double-blind, placebo-controlled study for the treatment of allergic rhinitis demonstrated limited clinical benefits in both the early- and late-phase responses to intranasal allergen challenge, even though it reduced the release of some mediators after allergen challenge (Rimmer et al. 2007). However, as correctly pointed out by the authors, the study had a variety of defects. As an example, the dose of the drug was limited by the narrow therapeutic index, due to side effects like tachycardia, raising the possibility that higher doses of new compounds with fewer side effects might be more efficacious. Therefore, it is possible that future studies targeting a different receptor (perhaps the A_{2B}AR) or using dual A₃AR/A_{2B}AR antagonists will be more successful (Press et al. 2005).

Recently, it has been demonstrated that A₃AR activation decreases mortality and renal and hepatic injury in murine septic peritonitis (Lee et al. 2006a). Higher levels of endogenous TNF- α were observed in A₃AR KO mice after sepsis induction in comparison to wild-type animals, and IB-MECA significantly reduced mortality in mice lacking the A₁AR or A_{2A}AR but not the A₃AR, demonstrating the specificity of the A₃AR agonist in activating A₃AR subtype and mediating protection against sepsis-induced mortality (Lee et al. 2006a). A similar mortality reduction associated with a decrease in interleukin (IL)-12 and interferon-gamma production induced by A₃AR activation was previously observed in endotoxemic mice (Haskó et al. 1998). In addition, other investigators reported reduced inflammation and increased survival following A₃AR activation in two murine models of colitis (Mabley et al. 2003). Furthermore, a protective role for A₃AR in lung injury following *in vivo* reperfusion has been reported. Rivo et al. demonstrated that in a spontaneously breathing cat model, IB-MECA given both before ischemia-reperfusion or during reperfusion conferred powerful protection against reperfusion lung injury, which was associated with decreased apoptosis (Rivo et al. 2004a). This effect was found to be mediated by a NOS-independent pathway and involved the opening of K_{ATP} channels (Rivo et al. 2004b). The signaling pathway linked to this effect was further identified in the A₃AR-induced upregulation of phosphorylated ERK (Matot et al. 2006). Furthermore, a reduction in the recruitment of neutrophils to the lungs after sepsis was found to be mediated by A₃AR and P2Y₂ receptors, suggesting that targeting these receptors might be useful to control acute lung tissue injury in sepsis (Inoue et al. 2008). Recently, a role for A₃AR activation has also been reported in the protection of skeletal muscle from ischemia and reperfusion injury. Because the use of an A₃AR agonist is not associated with cardiac or hemodynamic depression, the A₃AR represents a potential therapeutic target because of its ability to ameliorate skeletal muscle injury (Zheng et al. 2007). In contrast, it has been demonstrated that A₃AR activation exacerbates renal dysfunction, and mice lacking A₃ARs show better renal function following renal ischemia reperfusion injury (Lee and Emala 2000; Lee et al. 2003). Expression of A₃ARs is upregulated in ocular ischemic diseases and in conditions associated with oxidative stress. The A₃AR-selective agonist IB-MECA did not affect intraocular pressure in A₃AR^{-/-} mice,

but raised it in A₃AR^{+/+} mice (Avila et al. 2002). The use of a cross-species A₃AR antagonist in the mouse reduced intraocular pressure (Yang et al. 2005). Activation of A₃AR leads to the regulation of chloride channels in nonpigmented ciliary epithelial cells, suggesting that A₃AR agonists would increase aqueous humor secretion and thereby intraocular pressure *in vivo*, whilst A₃AR antagonists may represent a specific approach for treating ocular hypertension (Mitchell et al. 1999; Okamura et al. 2004; Schlotzer-Schrehardt et al. 2005). Unfortunately, there are currently no A₃AR antagonists in clinical phases. However, in light of the plethora of biological effects attributed to A₃ARs, substantial efforts in medicinal chemistry have been directed towards developing antagonists for the A₃AR subtype. As a result, a number of molecules are in biological testing as therapeutic agents for asthma and COPD, glaucoma, stroke, cardiac hypoxia and cerebral ischemia (Baraldi et al. 2008; Press et al. 2007).

5.1 A₃ Adenosine Receptor (A₃AR) and Autoimmune Inflammatory Diseases

During the last decade, new immunotherapy approaches have been introduced for the treatment of autoimmune diseases. Anti TNF- α monoclonal antibody drugs are now widely used since they are one of the most effective classes of biological drugs. This treatment has remarkable effects on several autoimmune diseases, including rheumatoid arthritis (RA), Crohn's disease, psoriasis, ankylosing spondylitis, and others (Codoro and Feldman 2007; Danese et al. 2007; McLeod et al. 2007; Rigopoulos et al. 2008; Tilg et al. 2007; Toussirot et al. 2007; Valesini et al. 2007). Anti TNF- α drugs are considered disease-modifying antirheumatic drugs (DMARDs) which modulate the pathophysiology of autoimmune diseases, but at the same time these drugs may interfere with host defense and disease pathology, resulting in severe adverse events (Desai et al. 2006; Hansen et al. 2007; Mader and Keystone 2007).

Recent findings indicate that the inhibition of TNF- α by adenosine is mediated via the A₃AR (Lee et al. 2006b; Levy et al. 2006). Selective agonists to A₃AR such as IB-MECA, CI-IB-MECA and MRS3558 inhibit TNF- α production both *in vitro* and *in vivo* (Baharav et al. 2005; Fishman et al. 2006; Hasko et al. 1996; Lee et al. 2006a, b; Martin et al. 2006; Ochaion et al. 2008; Rath-Wolfson et al. 2006). It was further shown that A₃AR is overexpressed in inflammatory tissues derived from adjuvant-induced arthritis (AIA) experimental models. Interestingly, A₃AR overexpression was also found in the peripheral blood mononuclear cells (PBMCs) of the arthritic animals, reflecting receptor status in the remote inflammatory organs (Bar Yehuda et al. 2007; Fishman et al. 2006; Ochaion et al. 2006; Rath-Wolfson et al. 2006). These findings are in agreement with data obtained from patients with colorectal cancer, who demonstrated that elevated expression of A₃ARs in this cancer is reflected in PBMCs (Gessi et al. 2004a).

The ability of A₃AR agonists to inhibit TNF- α and the upregulation of the receptor in inflammatory cells led to the development of the concept that A₃AR may be a specific target to combat inflammation.

In this section, data from *in vivo* experiments demonstrating the anti-inflammatory effect of A₃AR agonists and the molecular mechanisms involved will be presented. In addition, results from a human clinical study in RA patients showing the ability of IB-MECA to improve signs and symptoms of arthritis, as well as the safety of the drug will be presented. The utilization of A₃AR as a biological predictive marker to be analyzed prior to treatment with the agonist will be discussed.

IB-MECA, CI-IB-MECA and MRS3558 act as potent anti-inflammatory agents in experimental animal models of various inflammatory diseases. IB-MECA was tested in three experimental models that imitate Crohn's disease, including a rat chronic model of 2,4,6-trinitrobenzene sulfonic acid-induced colitis, dextran sodium sulfate-induced colitis and spontaneous colitis found in IL-10 gene-deficient mice. Treatment with IB-MECA (1.5 mg kg⁻¹ b.i.d., 1 or 3 mg kg⁻¹ per day, accordingly) protected against colitis (Guzman et al. 2006; Mabley et al. 2003).

Studies were performed to explore the mechanisms by which A₃AR agonists produce their anti-inflammatory effect. The effects of IB-MECA, MRS3558 and in some experiments CI-IB-MECA on the development of arthritis in experimental animal models were extensively studied. The agonists suppressed the clinical and pathological manifestations of arthritis in the mouse collagen-induced arthritis model, in the rat AIA model, and in the rat tropomyosin-induced arthritis model (Baharav et al. 2005; Fishman et al. 2006; Ochaion et al. 2008; Rath-Wolfson et al. 2006). The mechanism of action entailed direct effects of the A₃AR agonists on cells from synovial tissue and paw, which included deregulation of the NF- κ B signaling pathway manifested by downregulation of PI3K, PKB/Akt, I κ B kinase (IKK) and inhibitor of κ B (I κ B), resulting in decreased expression levels of TNF- α and apoptosis of inflammatory cells (Baharav et al. 2005; Fishman et al. 2006; Ochaion et al. 2008; Rath-Wolfson et al. 2006). IB-MECA affected T-cell-mediated responses by inhibiting T regulatory cell proliferation and adoptive transfer in AIA rats upon treatment of the donors with the agonist (Bar Yehuda et al. 2007). MRS3558 was also able to induce a dose-dependent inhibitive effect on the proliferation of fibroblast-like synoviocytes cultured from synovial fluids from RA patients via the same mechanism (Ochaion et al. 2008).

An important finding that supported the selection of IB-MECA as a drug candidate for the treatment of RA was its efficacy in enhancing the anti-inflammatory effect of methotrexate (MTX) (Ochaion et al. 2006). The latter is the most widely used DMARD, and it is the "gold standard" therapy, which other systemic medications are compared (Weinblatt et al. 1985). It was suggested by Montesinos et al. (2003) that the anti-inflammatory effect of MTX is mediated by adenosine, produced in the cells upon metabolism of MTX. When MTX is taken up by cells, it is converted to long-lived polyglutamates known to inhibit the activity of aminoimidazole carboxamide ribonucleotide (AICAR) transformylase, thereby leading to an

increase in the cellular level of AICAR. AICAR inhibits adenosine degradation, resulting in its accumulation in the extracellular fluid (Baggott et al. 1999; Chan et al. 2002; Laghi Pasini et al. 1997). Adenosine has been reported to exhibit a number of anti-inflammatory effects. It was further shown that the anti-inflammatory effect of MTX is mediated via A_{2A}ARs and the A₃ARs (Cronstein et al. 1994; Montesinos et al. 2003). Thus, it seems that the enhanced anti-inflammatory effects of IB-MECA and MTX are mediated via the A₃AR. This hypothesis was confirmed in a study where the combined treatment of AIA rats with IB-MECA and MTX resulted in an additive anti-inflammatory effect. Mechanistic studies revealed that MTX induced upregulation of the A₃AR in inflammatory cells from AIA rats, making the cells more susceptible to treatment with IB-MECA. It was further found that A₃AR is overexpressed in PBMCs of RA patients treated with MTX, suggesting that combined treatment with A₃AR and MTX in RA patients may be beneficial (Ochaion et al. 2006).

The above preclinical data, demonstrating the marked anti-inflammatory effect of IB-MECA (designated as CF101), prompted the initiation of a clinical development program to look at the safety and efficacy of CF101 for the treatment of RA. In a Phase I study conducted in healthy subjects, CF101 was given orally, and its plasma half-life ($t_{1/2}$) was 8 h. Single oral doses of CF101 of 1 and 5 mg were well tolerated, whereas at 10 mg, CF101 was associated with adverse events including asymptomatic sinus tachycardia and mild elevations of systolic blood pressure. These events are presumed to represent effects on cardiovascular ARs, which are most likely to be A_{2A}AR-mediated at the plasma concentrations for the 10 mg dose. In a subsequent trial of twice-daily repeat-dose testing of CF101, 4 mg every 12 h, the schedule was found to be well tolerated in male volunteers, with an adverse event profile comparable to placebo (van Troostenburg et al. 2004).

The safety and efficacy of CF101 was studied in a Phase IIa study in RA patients. The trial was a multicenter (ten sites), randomized, double-blind, parallel-group study and included 74 patients with active RA who failed between one and four DMARDs, excluding the biologic drugs. CF101 was administered at doses of 0.1, 1.0 and 4.0 mg twice-daily, orally for 12 weeks. The primary efficacy end-point was American College of Rheumatology (ACR) 20 (ACR response outcome score for 20% improvement in a number of different measurements) at week 12. CF101 reduced disease activity, showing maximal response at 1 mg, with somewhat lower responses at 0.1 and 4 mg. At week 12, there were 60, 36, and 12% of the patients receiving CF101 1 mg who achieved ACR 20, 50, and 70 responses, respectively. The respective mean percentage reduction in the number of tender and swollen joints was ~80% in all dose groups. CF101 was well tolerated with no dose-limiting side effects. During this study, blood was withdrawn from patients at baseline (prior to drug administration) and at week 12. A statistically significant direct correlation was found between A₃AR overexpression at baseline and ACR 50 at week 12, demonstrating that A₃AR may be a predictive biomarker. Overall it was concluded that CF101 showed a clinical response in this Phase IIa study without dose-limiting side effects in patients with active RA. A₃AR levels may be a predictive surrogate marker

of response to this therapy (Silverman et al. 2008). More clinical studies are underway to explore the effect of A₃AR agonists in RA and additional autoimmune inflammatory diseases.

6 Conclusion

Knowledge of the structure and function of the A₃AR has evolved dramatically in the last decade, and now this subtype, which originally appeared to be quite enigmatic in terms of its effects, has started to reveal its secrets. A synopsis of A₃AR-regulated pathways and functions is provided in Fig. 1. It appears evident that a plethora of biological functions have been attributed to the A₃AR in ischemic and inflammatory pathologies, and substantial efforts in medicinal chemistry have been directed at developing agonists and antagonists that target this AR subtype.

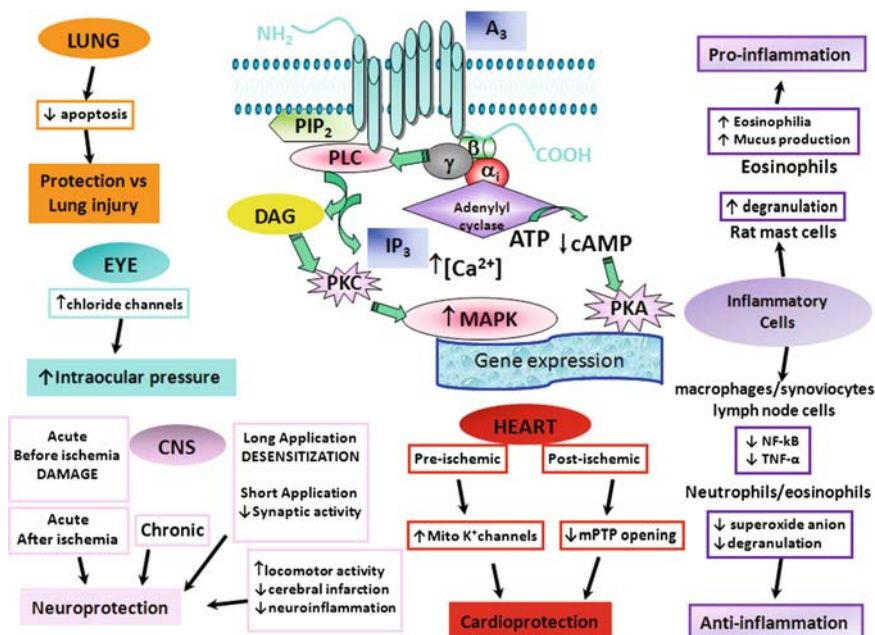


Fig. 1 Synopsis of the A₃ adenosine receptor (A₃AR)-regulated pathways and functions. Activation of the A₃AR results in the modulation of different intracellular pathways. Classically, this adenosine receptor subtype is coupled to the inhibition of adenylyl cyclase, leading to a reduction in cAMP levels. In addition, it may activate phospholipase C (PLC), through Gβγ subunits, inducing an increase in intracellular calcium and activation of protein kinase C (PKC). Recently, it has also been demonstrated that it is coupled to mitogen-activated protein kinases (MAPKs), suggesting its involvement in cell growth, survival, death and differentiation. Activation of the A₃AR subtype induces protective effects in the CNS, heart and lung, and both pro- and antiinflammatory effects in peripheral blood cells

As a result, there are currently A₃AR agonists in clinical phases for several autoimmune diseases, such as RA. Unfortunately, there are no A₃AR antagonists currently in clinical development, but a number of molecules are in biological testing as therapeutic agents for asthma and COPD, glaucoma, and stroke, which are waiting to enter the clinical arena. This is only the starting point of more expensive and challenging work, and it is likely that, with the availability of both selective ligands and animal models, several roles of the A₃AR that are currently ambiguous will be clearer in the near future. This will allow the chemistry and pharmacology of the A₃AR to be utilized clinically with the development of selective molecules for this important target that may improve the outcomes of patients with a number of diseases.

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Adenosine Receptors and Asthma

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Abstract The pathophysiological processes underlying respiratory diseases like asthma are complex, resulting in an overwhelming choice of potential targets for the novel treatment of this disease. Despite this complexity, asthmatic subjects are uniquely sensitive to a range of substances like adenosine, thought to act indirectly to evoke changes in respiratory mechanics and in the underlying pathology, and thereby to offer novel insights into the pathophysiology of this disease. Adenosine is of particular interest because this substance is produced endogenously by many cells during hypoxia, stress, allergic stimulation, and exercise. Extracellular adenosine can be measured in significant concentrations within the airways; can be shown to activate adenosine receptor (AR) subtypes on lung resident cells and migrating inflammatory cells, thereby altering their function, and could therefore play a significant role in this disease. Many preclinical in vitro and in vivo studies have documented the roles of the various AR subtypes in regulating cell function and

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how they might have a beneficial impact in disease models. Agonists and antagonists of some of these receptor subtypes have been developed and have progressed to clinical studies in order to evaluate their potential as novel antiasthma drugs. In this chapter, we will highlight the roles of adenosine and AR subtypes in many of the characteristic features of asthma: airway obstruction, inflammation, bronchial hyperresponsiveness and remodeling. We will also discuss the merit of targeting each receptor subtype in the development of novel antiasthma drugs.

Keywords Adenosine · Adenosine receptors · Asthma · Bronchial hyperresponsiveness · Airway smooth muscle · Airway remodeling · Airway inflammation

Abbreviations

AC	Adenylate cyclase
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
AK	Adenosine kinase
AMP	Adenosine monophosphate
AR	Adenosine receptor
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness
BMMC	Bone marrow-derived mast cell
CFTR	Cystic fibrosis transmembrane conductance regulator
CPA	Cycloptyadenosine
CXCR4	Chemokine receptor 4
cyto-5'-NT	Cytosolic form of nucleotidase
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine
EAR	Early asthmatic response
ecto-5'-NT	Ecto-5'-nucleotidase
FEV1	Forced expiratory volume in 1 s
fMLP	Formyl-Met-Leu-Phe
HBEC	Human bronchial epithelial cell
HPRT	Hypoxanthine phosphoribosyltransferase
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IL	Interleukin
IMP	Inosine monophosphate
iNOS	Inducible nitric oxide synthase
LABA	Long-acting beta-adrenoceptor agonist
LAR	Late asthmatic response
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1

NADPH	Nicotinamide adenine dinucleotide phosphate
NF- κ B	Nuclear factor kappa B
NTPDase	Nucleoside triphosphate diphosphohydrolase
PC	Provocative concentration
PEFR	Peak expiratory flow rate
PDE	Phosphodiesterase
PG	Prostaglandin
PLC	Phospholipase C
PNC	Purine nucleotide cycle
PNP	Purine nucleoside phosphorylase
POC	Proof of concept
RASON	Respiratory antisense oligonucleotide
RT-PCR	Reverse transcriptase polymerase chain reaction
SAHH	S-Adenosylhomocysteine hydrolase
TNF	Tumor necrosis factor
VEGF	Vascular endothelium growth factor

1 Adenosine: An Important Signaling Molecule in Asthma

Asthma is a lung disease characterized by airway hyperresponsiveness and inflammation. The pathogenesis of asthma involves the release of a broad array of mediators such as cysteinyl leukotrienes, histamine and cytokines from various cell types, leading to bronchoconstriction, proinflammatory effects, chemoattraction of leukocytes, and airway remodeling (Busse and Lemanske 2001). A number of clinical features distinguish asthmatic subjects from other respiratory diseases and may be considered characteristic of this phenotype (Avital et al. 1995). These include an exacerbation of disease following exposure to beta-adrenoceptor antagonists (Bond et al. 2007), an impairment in the ability to bronchodilate following deep inspiration (Slats et al. 2007), and their bronchoconstrictor sensitivity to a wide range of innocuous stimuli (Cockcroft and Davis 2006; Van Schoor et al. 2002). Various mechanisms have been proposed to account for this bronchial hyperresponsiveness (BHR) phenomenon, and these include increased airway smooth muscle function (An et al. 2007; Gil and Lauzon 2007), altered airway epithelial cell function (Holgate 2007), and the recruitment and activation of numerous inflammatory cells, including dendritic cells, T lymphocytes and eosinophils (Beier et al. 2007; Hammad and Lambrecht 2007; Jacobsen et al. 2007; Kallinich et al. 2007; Lloyd and Robinson 2007; Rosenberg et al. 2007), whose cell-derived products trigger a cascade of events within the lung that lead to airway epithelial cell damage, increased bronchial smooth muscle contractility and airway remodeling.

Asthmatic subjects bronchoconstrict in response to a number of physiological stimuli, such as exercise, distilled water, cold air and hypertonic saline, to which healthy subjects are refractory. Similarly, acidification, pollutants like sulfur dioxide, and chemical substances including adenosine, bradykinin and neuropeptides

evoke bronchoconstriction in asthmatics but have little if any effect in nondiseased individuals. These agents are commonly referred to as indirect-acting stimuli, since they do not appear to mediate bronchoconstriction by the direct activation of airway smooth muscle. They are thought to elicit bronchospasm by activating a number of different cell types, including mast cells, vascular smooth muscle cells, vascular endothelial cells, and/or airway nerves (Spina and Page 1996, 2002; Van Schoor et al. 2000). It is therefore of interest that asthmatic subjects are sensitive to such stimuli whilst healthy subjects are invariably unresponsive to these agents (Van Schoor et al. 2000). This suggests that the mechanisms by which these stimuli provoke bronchoconstriction are upregulated in asthma and are characteristic of this phenotype.

Furthermore, airway inflammation appears to be correlated better with BHR to indirect stimuli like adenosine (van den Berge et al. 2001), bradykinin (Polosa et al. 1998; Roisman et al. 1996) and hypertonic saline (Sont et al. 1993) than it is to more direct-acting stimuli like methacholine. Similarly, during an exacerbation of BHR following the deliberate exposure of an asthmatic subject to an environmental allergen (e.g., house dust mite), there is a preferential increase in BHR to an indirect-acting stimulus like bradykinin in contrast to methacholine (Berman et al. 1995). On the other hand, a number of pharmacological drugs used to treat asthma, including nedocromil sodium and ipratropium bromide, suppress airway responsiveness to these indirect-acting stimuli, suggesting the likely involvement of neural reflexes (Van Schoor et al. 2000). Furthermore, it is now recognized that glucocorticosteroids preferentially suppress BHR to adenosine (Ketchell et al. 2002; van den Berge et al. 2001) and bradykinin (Reynolds et al. 2002) compared with methacholine.

It is common for clinicians to use stimuli like methacholine and histamine as provocative inhalation challenge agents to induce bronchoconstriction because these agents are relatively convenient to use. However, whilst there is a separation in airway responsiveness to these agents between asthmatic subjects and healthy individuals, there is also a considerable degree of overlap, and it has been suggested that airway responses to these agents may not be sensitive indicators of the asthma phenotype (Avital et al. 1995; O'Connor et al. 1999). In contrast, asthmatic subjects invariably bronchoconstrict in response to the indirect-acting stimuli described earlier, which provoke little if any response in otherwise healthy individuals or in subjects with other respiratory diseases (Avital et al. 1995; Van Schoor et al. 2000).

A growing body of evidence has emerged in support of the purine nucleoside adenosine in the pathogenic mechanisms of asthma (Spicuzza et al. 2006). This body of evidence is supported by the following reported findings. (a) In asthmatics adenosine levels are elevated in bronchoalveolar lavage (BAL) fluid (Driver et al. 1993), in the circulation following allergen inhalation (Mann et al. 1986a), and in exhaled breath condensate in patients with asthma (Csoma et al. 2005). (b) Adenosine given by inhalation causes a dose-dependent bronchoconstriction in subjects with asthma (Cushley et al. 1983; Polosa 2002; Rorke and Holgate 2002). (c) Inhalational challenge with adenosine monophosphate (AMP), which is metabolized locally by the ectonucleotidase 5'-nucleotidase to adenosine, increases the release of leukotrienes and other bronchoconstrictive mediators in asthmatics (Bucchioni et al. 2004). (d) Adenosine enhances mast-cell allergen-dependent activation (Polosa et al. 1995);

(e) Treatment with dipyridamole, a blocker of adenosine reuptake, significantly enhances the bronchoconstrictor response to inhaled adenosine in subjects with asthma (Crimi et al. 1988). (f) The sensitivity of airways to adenosine and AMP more closely reflects an inflammatory process and the phenotype for allergic asthma than the sensitivity of airways to other known inhalational bronchoprovocative agents, such as methacholine and histamine (de Meer et al. 2002; Holgate 2002; Spicuzza et al. 2003; van den Berge et al. 2001).

1.1 Adenosine Metabolism

The physiological effects of adenosine in asthma via its stimulation of cell-surface adenosine receptors (ARs) and subsequent downstream signaling pathways are a function of the local concentration of adenosine. Adenosine concentrations in unstressed cells and tissue are below 1 μM (estimates 10–100 nM); however, in metabolically stressed inflamed or ischemic tissues, adenosine levels may rise to 100 μM (Fredholm 2007; Hasko and Cronstein 2004). Lower concentrations of adenosine (10–100 nM) activate the high-affinity A_1 , A_{2A} , and A_3 ARs and high adenosine concentrations (10 μM) stimulate low-affinity A_{2B} ARs (Fredholm 2007). Factors that determine the net effect of adenosine on specific cell and tissue function are AR expression and coupling to intracellular signaling pathways, all of which are tightly regulated in different tissues and cells.

The local adenosine concentration at its receptor subtypes is determined by several processes, which include extracellular and intracellular adenosine generation, adenosine release from cells, cellular reuptake and metabolism (Fig. 1). These processes are closely intertwined and strictly regulated. For, example, under the hypoxic and inflammatory conditions encountered in asthmatic airways, the increased intracellular dephosphorylation of adenosine 5'-triphosphate (ATP) to adenosine by the cytosolic metabolic enzyme 5'-nucleotidase may be accompanied by a suppression of the activity of the salvage enzyme adenosine kinase, which prevents the rephosphorylation of adenosine to AMP (Deussen 2000). These processes lead to high adenosine concentrations inside the cell and the release of adenosine from the dephosphorylation of AMP into the extracellular space through nucleoside transporters (Hyde et al. 2001; Pastor-Anglada et al. 2001).

The other major pathway that contributes to high extracellular adenosine concentrations during metabolic stress is release of adenine nucleotides (ATP, adenosine diphosphate (ADP), and AMP) from inflammatory and injured cells. This is followed by extracellular degradation to adenosine by a cascade of ectonucleotidases, which include CD39 (nucleoside triphosphate diphosphohydrolase (NTPDase)) and CD73 (5'-ectonucleotidase) (Eltzschig et al. 2004; Kaczmarek et al. 1996; Resta et al. 1998; Thompson et al. 2004; Zimmermann 1999). Adenosine accumulation is limited by its catabolism to inosine by adenosine deaminase. Inosine is finally degraded to the stable end-product uric acid (Hasko et al. 2000, 2004). Mechanisms of nucleotide release and metabolism, or adenosine release and metabolism, as well

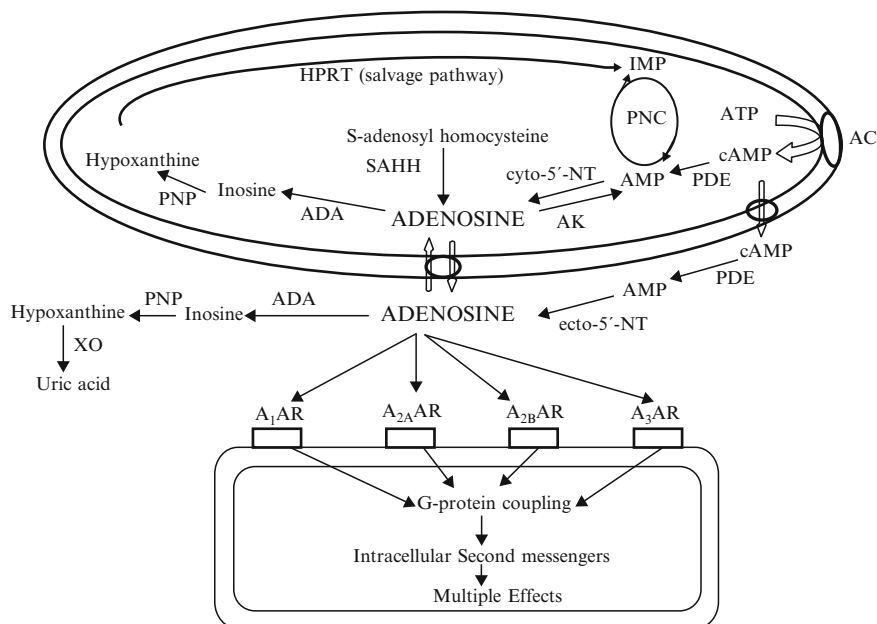


Fig. 1 Metabolism of adenosine. Adenosine is generated mainly by two enzymatic systems: intra/extracellularly localized nucleotidases and cytoplasmic *S*-adenosylhomocysteine hydrolase (SAHH). In response to hypoxia/cellular damage or other stressful/inflammatory stimuli, ATP is rapidly dephosphorylated by combined effects of adenylate cyclase (AC), phosphodiesterases (PDE) and nucleotidases to form intra/extracellular adenosine. Ecto-5'-nucleotidase (ecto-5'-NT) is one such enzyme that plays an important role in regulating local adenosine production for receptor signaling. Extracellular adenosine can interact with adenosine receptors (AR) that are coupled to heterotrimeric G proteins, which, in turn, couple AR activation to various effector molecules that can regulate second-messenger systems to influence cell and tissue function. Adenosine can also be deaminated to inosine by adenosine deaminase (ADA) that can exist intra- or extracellularly, or it can be transported into and out of the cells via membrane-associated nucleoside transporters. Intracellular adenosine is generated from the dephosphorylation of AMP by a cytosolic form of nucleotidase (cyto-5'-NT) or the hydrolysis of *S*-adenosylhomocysteine by SAHH. Adenosine can also be phosphorylated back to AMP by adenosine kinase (AK). AMP can also be directly deaminated to inosine monophosphate (IMP) by AMP deaminase. The reaction of phosphorylation predominates when adenosine occurs at a low physiological concentration ($<1 \mu\text{M}$), whereas ADA is activated at higher concentrations of the substrate ($>10 \mu\text{M}$). Hypoxanthine is formed after the removal of ribose from inosine by the actions of purine nucleoside phosphorylase (PNP). PNP has only negligible activity towards adenosine and degrades mainly inosine. Hypoxanthine can be salvaged back to IMP by hypoxanthine phosphoribosyltransferase (HPRT), which is again converted to AMP through the purine nucleotide cycle (PNC). Hypoxanthine can also enter the xanthine oxidase (XO) pathway to form xanthine and uric acid sequentially as byproducts

as transport mechanisms that account for the increased adenosine levels in exhaled breath condensate after exercise (Csoma et al. 2005), in the circulation following allergen inhalation (Mann et al. 1986a), and in BAL fluid (BAL adenosine concentration of $2.55 \pm 0.50 \mu\text{M}$ in asthmatics versus $0.72 \pm 0.16 \mu\text{M}$ in normals) (Driver et al. 1993) in human asthmatics, are yet to be determined.

There are several important cell types that are sources of extracellular adenosine. Neutrophils and endothelial cells release large amounts of adenosine at sites of metabolic distress, inflammation and infection (Cronstein et al. 1983; Gunther and Herring 1991; Madara et al. 1993; Rounds et al. 1994). Activated leukocytes are a major source of extracellular adenosine (Mann et al. 1986b). ADP released by platelets can be a significant source of adenosine after dephosphorylation (Marcus et al. 1995). Under conditions of stress including infection, activated macrophages can also serve as a major source of extracellular adenosine via ATP metabolism. Bacterial lipopolysaccharide (LPS) augments the release of ATP from macrophages (Sperlagh et al. 1998). Moreover, T-helper lymphocytes may be an important source of extracellular ATP. The presence of ecto-ATPase and antigen-triggered accumulation of extracellular ATP from T-helper cells has been reported (Apasov et al. 1995). In addition to inflammatory cells, airway epithelial cells and other structural cells in the lung may be important sources of high levels of adenosine in the airways of human asthmatics (Cohn et al. 2004).

1.2 Adenosine-Induced Bronchoconstriction, Airway Inflammation, and Airway Remodeling

In asthmatics, adenosine produces bronchoconstriction, inflammation, and airway plasma exudation, which lead to airway obstruction. Moreover, by acting on ARs, adenosine induces the release of inflammatory mediators that are important in the pathogenesis of airway remodeling in asthmatics. In both humans and animals, adenosine induces increases in BHR in asthmatics but not normal subjects, both in vivo following inhalation (Ali et al. 1994a; Cushley et al. 1983; Dahlen et al. 1983) and in vitro in small airways (Ali et al. 1994b; Bjorck et al. 1992). Adenosine produces bronchoconstriction in airways by directly acting on ARs in bronchial smooth muscle cells or indirectly by inducing the release of preformed and newly formed mediators from mast cells, and by acting on ARs on airway afferent sensory nerve endings (Hua et al. 2007a; Keir et al. 2006; Livingston et al. 2004; Polosa 2002). Multiple mechanisms may be involved in adenosine-induced bronchoconstriction; for example, the effects of adenosine in asthmatic subjects are sensitive to muscarinic receptor antagonists, suggesting that adenosine mediates obstruction indirectly (Crimi et al. 1992; Mann et al. 1985; Polosa et al. 1991), which would be consistent with the preclinical evidence that adenosine can activate afferent nerves in vivo (Hua et al. 2007a; Keir et al. 2006). However, since muscarinic antagonists do not completely abolish bronchoconstriction in response to adenosine, it is plausible to conclude that the “atropine-resistant” component of this response is mediated by direct activation of airway smooth muscle (Brown et al. 2008; Ethier and Madison 2006) and/or indirectly via mediators released from other cell types expressing these receptors.

Adenosine exposure through inhalation increases enhanced pause (Penh), a measure of airway resistance, in allergen-sensitized and -challenged mice (Fan

and Mustafa 2002). This increase in enhanced pause due to adenosine was reversed by theophylline with methacholine-mediated enhanced pause being unaffected, suggesting the involvement of ARs (Fan and Mustafa 2002). This finding that adenosine-induced bronchoconstriction is mediated by ARs is supported by an earlier study in a rabbit model of allergic asthma, where adenosine-induced bronchoconstriction was blocked by theophylline (Ali et al. 1992). Following inhalation and its local metabolism to adenosine in the airway, AMP induced bronchoconstriction is attenuated by potent cyclooxygenase inhibitors, H₁ receptor and leukotriene receptor antagonists, suggesting that adenosine induces the release of prostaglandins, histamine and leukotrienes in the airways of asthmatics (Phillips and Holgate 1989; Rorke et al. 2002; Rutgers et al. 1999). Another study has shown that inhalation challenge with adenosine, but not methacholine, produces mild airway plasma exudation (Belda et al. 2005). Collectively, these effects of adenosine on airway nerves, contraction of bronchial smooth muscle, release of mast cell mediators, and airway edema produce airflow obstruction.

Adenosine produces inflammation in airways in allergic animals and humans. Animals with increased adenosine concentrations in the lung (adenosine deaminase (ADA)-deficient mice) develop severe pulmonary inflammation, with airway accumulation of eosinophils and activated macrophages, mast cell degranulation, and mucus metaplasia in the airways—features similar to that found in asthmatic bronchi (Blackburn et al. 2000; Chunn et al. 2001). Treatment of these mice with exogenous ADA to reduce adenosine concentrations results in the reversal of these asthmatic features (Chunn et al. 2001). In a mouse model of allergic asthma, inhalation of adenosine has also been shown to cause airway inflammation, as evidenced by an increased release of proinflammatory mediators from eosinophils and mast cells (Fan and Mustafa 2002, 2006; Oldenburg and Mustafa 2005; Tilley et al. 2003). Moreover, in human asthmatics, an inhalational challenge with AMP produced an increase in eosinophils and neutrophils in the sputum (Manrique et al. 2008; van den Berge et al. 2004).

Adenosine-mediated inflammation is not limited to the lung; it also reaches the systemic circulation. In a recent report in a mouse model of asthma activities of eosinophilic peroxidase, myeloperoxidase and beta-hexosaminidase were increased not only in the lung but also in the systemic circulation of allergic mice exposed to adenosine aerosol (Fan and Mustafa 2006). In human asthmatics, adenosine aerosol increases the release of neutrophil chemotactic factor in serum (Driver et al. 1991). Moreover, in a recent study it was demonstrated that adenosine-induced effects on urinary 9 α , 11 β -prostaglandin (PG) F₂ levels (a sensitive biomarker of mast cell degranulation) were enhanced during repeated low-dose allergen challenge in allergic asthmatics (Ihre et al. 2006). These earlier findings in asthmatics were confirmed by a recent study showing an increase in plasma 9 α , 11 β -PGF₂ levels after adenosine challenge in asthmatics (Bochenek et al. 2008). These studies suggest that following inhalation, adenosine enhances the release of systemic inflammatory mediators from sensitized inflammatory cells. Thus, following inhalation, adenosine not only produces inflammation in the airways of asthmatics but it also induces a systemic inflammatory response that would, in turn, amplify the inflammation locally in the airways of asthmatics.

Adenosine in the lung may also be involved in the airway remodeling process (Cohn et al. 2004). Pathogenic hallmarks of airway remodeling are mucous gland hyperplasia, subepithelial fibrosis, hypertrophy of bronchial smooth muscle, and angiogenesis (Cohn et al. 2004; Jarjour and Kelly 2002). In a recent report, substantial angiogenesis in the tracheas of ADA-deficient mice were seen in association with high levels of adenosine (Mohsenin et al. 2007). ADA replacement enzyme therapy in these mice resulted in a lowering of adenosine levels and reversal of tracheal angiogenesis. Moreover, in lung alveolar epithelial cells and lung fibroblasts, adenosine caused an induction of fibronectin (a matrix glycoprotein highly expressed in injured tissues that has been implicated in wound healing) mRNA and protein expression in a dose- and time-dependent manner (Roman et al. 2006). Furthermore, there appears to be a connection of IL-13 levels to high adenosine levels, ADA activity and airway remodeling (Blackburn et al. 2003). Studies in CC10 IL-13 Tg mice showed that IL-13 induced high levels of adenosine, inflammation, lung collagen content and subepithelial airway fibrosis and reduced ADA activity in the lung. ADA therapy administered to these mice decreased adenosine levels, inflammation, and subepithelial airway fibrosis (Blackburn et al. 2003). Moreover, in ADA-deficient mice, IL-13 was strongly induced. These findings suggest that IL-13 and adenosine stimulate one another to amplify the pathway that contributes to airway inflammation, fibrosis, and remodeling. Similar findings were also seen in the lungs of mice overexpressing the Th2 cytokine IL-4 (Ma et al. 2006).

2 Adenosine Receptors in Asthma

Collectively, the studies presented above suggest a strong role for adenosine not only in the bronchoconstriction of allergic airways but also in the progression and amplification of airway inflammation and airway remodeling. The effects of adenosine as an important signaling molecule in asthma may depend not only on the bioavailability of the nucleoside but also on the expression, density, and affinity of ARs, which are known to be finely modulated by physiological and/or pathological conditions, signaling mechanisms, the local metabolism of adenosine, and the predominant inflammatory cell types in the asthma model, which may be species specific (Chunn et al. 2001; Fan et al. 2003; Sun et al. 2005; Zhong et al. 2006).

Adenosine produces its effects in asthmatics by acting on membrane-bound extracellular ARs on target cells. Four subtypes of ARs (namely A₁, A_{2A}, A_{2B}, and A₃) have been cloned in humans, are expressed in the lung, and are all targets for drug development for human asthma (Polosa 2002; Rorke and Holgate 2002). These receptors are heptaspanning-transmembrane G-protein-coupled receptors. Three of the AR subtypes (A₁, A_{2A}, and A_{2B}) demonstrate 80–95% sequence homology across a wide evolutionary range of species (Fredholm et al. 2001). In contrast, the A₃ARs demonstrate significant species variation. Signal transduction by the ARs varies; not only among the subtypes but also for a particular subtype between different cell sources (Fredholm et al. 2001). A₁ARs were originally characterized

Table 1 Characteristics and pharmacology of adenosine receptors

	A ₁ AR	A _{2A} AR	A _{2B} AR	A ₃ AR
Agonists	CPA, CCPA, CHA, S-ENBA	CGS 21680, ATL146e, CV-1808, CVT-3146, MRE0740; MRE0094	BAY 60-6583	IB-MECA (CF 101), 2-CI-IB-MECA (CF102), MRS3558 (CF502)
Antagonists	DPCPX, FSPCPX, N-0861, BG-9719, BG-9928, WRC-0571; KW 3902, L-97-1, SLV320, EPI-2010	ZM 241385, KW 6002, SCH 58261, SCH 442416, CSC	IPDX, MRS1754, MRS1706, CVT-6883, CVT-5440	MRS1220, MRS1191, MRS1523, VUF 5574
Transduction mechanisms	G _{i/o} , ↓ cAMP; ↑K ⁺ , ↓Ca ²⁺ channels, PLA ₂ ; G _{α16} NF-κB, PLC, PKC, ↑IP3/DAG	G _{s/olf} , ↑cAMP	G _s /↑cAMP; G _q /PLC, ↑IP3/DAG	G _i /↓cAMP; ↑Ca ²⁺ , ERK1/2, G _q /PLC, ↑IP3/DAG; PLD

References: Beukers et al. (2006); Baraldi et al. (2000); Fredholm (2007); Fredholm et al. (2001); Gao and Jacobson (2007); Gessi et al. (2008); Hess (2001); Liu and Wong (2004); Moro et al. (2006); Yuzlenko and Kiec-Kononowicz (2006)

as being coupled to pertussis-toxin-sensitive G_i-coupled signal transduction pathways, but in some cells they are directly associated with, and act through, ion channels. The A₂AR subtypes (A_{2A} and A_{2B}) are typically coupled to G_s-linked signal transduction pathways. In some cells, A₁AR receptor-mediated inhibition and A_{2A}AR-mediated stimulation of adenylate cyclase may coexist and their functions may be counterregulatory (Fredholm et al. 2001). A summary of the AR subtypes, their signal transduction mechanisms, and selective agonists and antagonists is presented in Table 1.

Adenosine receptors have been described on a number of different cell types that are important in the pathophysiology of asthma, including dendritic, antigen-presenting cells, human airway epithelial and bronchial smooth muscle cells, lymphocytes, mast cells, eosinophils, neutrophils, macrophages, fibroblasts and endothelial cells (Thiel et al. 2003; Young et al. 2006; Wilson 2008). Activation of ARs on these different cell types is responsible for inducing the release of mediators and cytokines, leading to BHR, inflammation, edema, and airway remodeling. Activation of ARs on afferent sensory airway nerves contributes to BHR in asthma (Hua et al. 2007a). The contributions of the different AR subtypes to the pathophysiology of asthma will be discussed in the following sections and are presented in Fig. 2. In this review, the pathophysiological role of each AR and its signaling in asthma is discussed. Furthermore, the targeting of ARs with selective agonists or antagonists as therapeutic strategies in the treatment of asthma is also discussed and is presented in Table 2.

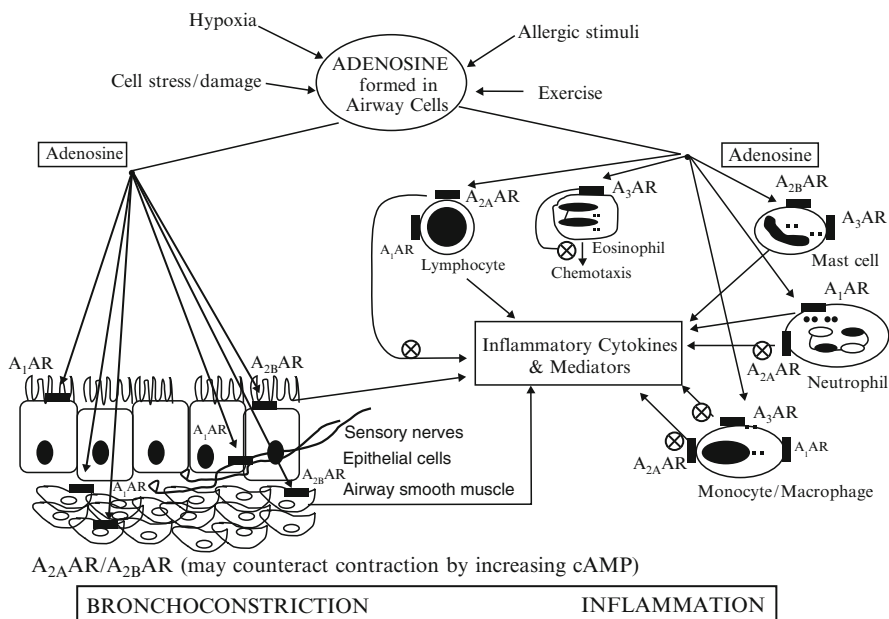


Fig. 2 Adenosine receptors and pathophysiology of asthma. By acting on adenosine receptors (ARs), A_1 , A_{2A} , A_{2B} , and A_3 ARs, adenosine released under conditions of cellular stress as seen in asthmatic airways produces bronchoconstriction and inflammation. The net effect of adenosine on ARs will depend on the relative expression of these receptors on different cell types in asthmatic airways, and is concentration-dependent, as adenosine frequently exhibits opposing effects through the activation of AR subtypes expressed on the same cells coupled to different G proteins and signaling pathways. By acting on A_1 ARs on bronchial smooth muscle cells and afferent sensory airway nerves, adenosine produces bronchoconstriction. By acting on A_1 ARs on inflammatory leukocytes such as neutrophils, monocytes, macrophages, and lymphocytes, adenosine produces proinflammatory effects. Activation of A_{2A} ARs on the inflammatory cells suppresses the release of proinflammatory cytokines and mediators. Activation of A_{2A} ARs coupled to G_s and adenylate cyclase may also lead to bronchial smooth muscle relaxation via the cAMP–PKA (cyclic adenosine monophosphate–protein kinase A) pathway. Activation of A_{2B} ARs coupled to G_s and adenylate cyclase induce cytokine release from human bronchial epithelial and smooth muscle cells. Activation of A_{2B} ARs on murine bone marrow-derived mast cells (BMMCs) regulates the release of cytokines. The effect of adenosine on A_3 ARs is species dependent. In mice, rats, and guinea pigs, activation of A_3 ARs by adenosine produces bronchoconstriction, airway inflammation, mast cell degranulation, and mucus hyperplasia. In humans, activation of A_3 ARs by adenosine produces anti-inflammatory effects, inhibition of chemotaxis and degranulation of eosinophils and cytokine release from monocytes. *Circled times* denote inhibition

2.1 A_1 Adenosine Receptors and Asthma

Until relatively recent times, the A_1 AR received little attention as an important target in human asthma. However, a number of reports have demonstrated that expression of the A_1 AR is upregulated in the airways of both animal models of allergic airway inflammation and human asthmatic subjects. Moreover, it is now appreciated

Table 2 Comparison of different potential therapeutic approaches targeting adenosine receptors in asthma

	A ₁ AR antagonists	A _{2A} AR agonists	A _{2B} AR antagonists	A ₃ AR agonists	A ₃ AR antagonists
Potential effects	Inhibition of bronchoconstriction, mucus hypersecretion, and inflammation	Bronchodilation and inhibition of inflammation	Inhibition of bronchoconstriction, inflammation, and airway remodeling	Inhibition of inflammation	Inhibit bronchoconstriction, inflammation, mucus hyperplasia
Disadvantages	No safety concerns reported to date for A ₁ AR antagonists in humans	CV side effects; tachyphylaxis; immune suppression	Reduce airway hydration; bronchoconstriction; inflammation	Tachyphylaxis; immune suppression	Inflammation
Latest developments in asthma	L-97-1 (Preclinical); EPI-2010 (Phase II; discontinued, no additional effect with ICSs)	GW328267X (Phase II; discontinued due to CV side effects)	CVT 6883 (Phase I); QAF 805 (Phase Ib)		QAF 805 (Phase Ib)
Pharmaceutical company involved in AR drug discovery	Epigenesis Pharmaceuticals; Endacea, Inc.; Biogen Idec; Merck; Solvay Pharmaceuticals; OSI Pharmaceuticals, Inc.	Glaxo Group Ltd; Pfizer; Novartis	CV Therapeutics; Novartis	Can-Fite Biopharma	Novartis

AR, Adenosine receptor; CV, cardiovascular; ICSs, inhaled corticosteroids
Adapted with permission from Wilson (2008)

that various functions relevant to asthma have also been associated with activation of the A₁AR, including bronchoconstriction, leukocyte activation and inflammation, BHR, and mucus secretion.

A pivotal study generated convincing evidence that A₁ARs could well play a significant role in the pathophysiology of asthma. The authors demonstrated that airway obstruction in response to aerosol administration of adenosine and allergen was inhibited in a rabbit model of allergic airway inflammation following treatment with antisense oligonucleotides as well as antagonist to this receptor (Ali et al. 1994a, b; Nyce and Metzger 1997). These data suggested that the A₁AR not only directly mediates bronchoconstriction following administration of exogenous adenosine, but that endogenous adenosine is an important component of the allergic response.

Although important species differences have been observed with regards to the expression and function(s) of the four AR subtypes, there is evidence that supports similar observations of an increased expression of the A₁AR and of A₁AR-induced bronchoconstriction in human asthmatic subjects. Firstly, it was demonstrated that adenosine-induced contraction of isolated bronchial tissue *in vitro* was greater in tissues obtained from asthmatic subjects than healthy subjects, and that this contraction could be significantly inhibited following preincubation with a selective A₁AR antagonist (Bjorck et al. 1992). Furthermore, it has been very recently demonstrated for the first time that expression of the A₁AR is increased in bronchial biopsies obtained from steroid-naïve mildly asthmatic subjects when compared with healthy subjects (Brown et al. 2008). This increased expression of the A₁AR appeared to be predominantly located in the airway epithelium and smooth muscle regions of the tissue, the latter observation thus correlating with the preclinical findings in the rabbit model of allergic asthma. In support of this, it has been demonstrated that activation of the A₁AR on human airway smooth muscle cells *in vitro* results in an increase in intracellular calcium mobilization, which could potentially mediate airway smooth muscle contraction (Ethier and Madison 2006). The finding of increased expression of A₁ARs in the airways and increased sensitivity of the airways to adenosine could well be of clinical significance. In asthmatics, the level of adenosine in plasma and exhaled breath condensate is increased following allergen or exercise challenge (Csoma et al. 2005; Mann et al. 1986a; Vizi et al. 2002) and therefore could lead to the activation of A₁ARs, thereby contributing toward airway obstruction during an acute exacerbation of asthma.

The report that the expression of A₁ARs is increased in bronchial biopsies of asthmatics is confirmed by the findings from another laboratory. In a preliminary study of a small number of human subjects, gene expression for A₁ARs is increased approximately 200% in bronchial tissue from small airways obtained from asthmatics ($n = 3$) versus normal subjects ($n = 3$) (Nadeem and Mustafa, unpublished data, West Virginia University). In these studies, expression of A_{2A}ARs is decreased while there is little to no change in the expression of A_{2B}ARs and A₃ARs in bronchial tissue from small airways in asthmatics versus normal subjects. The results of these studies were determined with the use of RT-PCR and confirmed with the use of western blots, with the exception of the A_{2B}AR, which was not tested in western blot studies.

A number of other studies using experimental animals have implicated a role for A₁ARs in mediating airway obstruction to adenosine. For example, the A₁AR agonist cyclopentyladenosine (CPA) selectively induces airway obstruction only in sensitized guinea pigs (Keir et al. 2006) and allergic rabbits (Ali et al. 1994a; el-Hashim et al. 1996). Further studies with the allergic rabbit model demonstrated that CPA also induced bronchoconstriction and stimulated IP₃ generation in airway smooth muscle (Abebe and Mustafa 1998). Allergic rabbits treated with the selective A₁AR antagonist L-97-1 ([3-(2-(4-aminophenyl)-ethyl)-8-benzyl-7-(2-ethyl-(2-hydroxy-ethyl)-amino)-ethyl)-1-propyl-3,7-dihydro-purine-2,6-dione]) provided bronchoprotection against inhaled adenosine (Obiefuna et al. 2005). However, atypical (Hannon et al. 2002) and adenosine A₁, A_{2B} and A₃ ARs (Fan et al. 2003; Hua et al. 2007a) have been suggested to mediate airway obstruction in response to adenosine in the brown Norway rat and mouse, respectively, underlying important species and strain differences.

Expression of the A₁AR has also been identified on a number of inflammatory cells. In general, these effects appear to be proinflammatory in nature. Activation of the A₁AR on human eosinophils, for example, promotes superoxide release (Ezeamuzie and Philips 1999). Furthermore, the A₁AR also mediates the respiratory burst in neutrophils (Salmon and Cronstein 1990), in addition to chemotaxis (Cronstein et al. 1990) and their adherence to endothelial cells (Cronstein et al. 1992). Furthermore, adenosine has been shown to promote monocyte phagocytosis (Salmon et al. 1993) and chemotaxis of immature dendritic cells (Panther et al. 2001), in addition to increasing the release of cytotoxic substances from endothelial cells that increase endothelial cell permeability (Wilson and Batra 2002) via the A₁AR.

The effects of adenosine upon inflammatory cells have been determined largely from *in vitro* experiments, and it should be noted that these effects are concentration dependent, as adenosine frequently exhibits opposing effects through the activation of other AR subtypes expressed on the same cells, since they are coupled to different G proteins. Thus, the relative expression of these receptors on inflammatory cells resident in asthmatic airways and the overall cellular effect of adenosine at the concentration present remain to be determined. It is likely, however, that the pattern of cellular expression for ARs changes following exposure to adenosine, since experimental evidence shows that an increased extracellular level of adenosine somewhat unusually appears to promote AR signaling. This was unequivocally demonstrated in mice partially deficient in ADA that consequently have high levels of adenosine in the lung (Chunn et al. 2001). Besides the severe pulmonary inflammation typical of this phenotype, these mice exhibited an increased transcript level for the A₁, A_{2B} and A₃ ARs.

In light of the many studies demonstrating the proinflammatory action attributed to activation of the A₁ARs, it is perhaps surprising that a preclinical study has purported to document an anti-inflammatory effect of A₁AR signaling (Sun et al. 2005). Adenosine deaminase is a ubiquitous enzyme responsible for the inactivation of adenosine, and mice deficient in this protein demonstrate profound

pulmonary injury, the presence of elevated levels of macrophages, and increased mucus production. These indices of tissue damage were exacerbated in ADA double-knockout mice also deficient in the expression of A₁AR, thereby implicating the loss of an anti-inflammatory pathway mediated by this receptor (Sun et al. 2005). However, the relevance of this model to human asthma or chronic obstructive pulmonary disease is debatable, since two of the principal cell types observed in these diseases, namely eosinophils and neutrophils, respectively, are present in such small numbers (<1.7%). In contrast to these findings, the A₁AR antagonist L-97-1 inhibited the recruitment of eosinophils and neutrophils to the airways of allergic rabbits challenged with house dust mite antigen (Nadeem et al. 2006).

Very few studies have specifically addressed the question of whether activation of A₁ARs is important in the development of BHR. Animal models of allergic inflammation are characterized by increased sensitivity to inhaled histamine, and interference in A₁AR signaling following either treatment with an antisense against this receptor (Nyce and Metzger 1997) or the use of a selective antagonist (Nadeem et al. 2006; Obiefuna et al. 2005) provided some degree of protection against the development of BHR. One can only speculate as to the mechanism by which adenosine, released within the inflammatory milieu of the airways, causes BHR via an A₁AR-dependent mechanism. Activation of these receptors on inflammatory cells including mast cells, eosinophils, dendritic cells, and lymphocytes could stimulate the release of other inflammatory mediators that, in turn, increase the sensitivity of the airways. Alternatively, adenosine might stimulate C fibers, thereby lowering the threshold for the activation of afferent input into the nucleus tractus solitarius, and thus facilitating reflex activation of parasympathetic nerves (Chuaychoo et al. 2006; Hong et al. 1998).

The mechanism(s) by which adenosine mediates airway obstruction *in vivo* in animal models may constitute indirect components. For example, adenosine activates pulmonary C fibers in the rat (Hong et al. 1998) and in the guinea pig (Chuaychoo et al. 2006; Lee et al. 2004), and cholinergic neural pathways in conscious mice (Hua et al. 2007a) via an A₁AR-dependent mechanism. Moreover, the effect of activation of A₁ARs by a selective A₁AR agonist, CPA, was specific for nodose but not jugular ganglion-derived C fibers (Chuaychoo et al. 2006). The consequence of activating these nerves following the endogenous release of adenosine during an inflammatory response may be airway obstruction, a phenomenon that was abolished in guinea pigs chronically treated with capsaicin in order to chemically inactivate C fibers (Keir et al. 2006). Reflex activation of parasympathetic nerves was further implicated, since vagotomy or treatment with the muscarinic antagonist atropine attenuated bronchospasm induced by CPA (Keir et al. 2006). Moreover, in mice, an adenosine-induced increase in airway resistance was abolished in A₁AR knockout mice and following vagotomy in wild type mice, but not in A_{2A}, A_{2B}, or A₃ AR knockout mice (Hua et al. 2007a). In conscious mice, the adenosine-induced increase in airway resistance was significantly reduced by the selective A₁AR antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) as well as atropine and bupivacaine, suggesting that the adenosine-induced bronchoconstriction was via the activation of A₁ARs on the cholinergic neural pathway. Similarly,

the cholinergic-dependent reflex activation of tracheal smooth muscle in situ in response to CPA was mediated by the activation of A₁AR (Reynolds et al. 2008).

Finally, in addition to its effects on bronchoconstriction, leukocyte activation and inflammation, and BHR, the A₁AR may play an important role in mucus secretion and airway remodeling of human asthma. It has been shown that adenosine is able to induce mucus secretion via activation of the A₁AR in the canine trachea in vivo (Johnson and McNee 1985), which has now been confirmed in human bronchial epithelial cells in vitro, where activation of the A₁AR was shown to increase the expression of the *MUC2* mucin gene (McNamara et al. 2004). Thus, it could be speculated that the reported increased expression of A₁AR on asthmatic bronchial epithelium (Brown et al. 2008) promotes adenosine-induced mucin secretion, although the extent to which adenosine contributes to the overall mucus hypersecretion in asthma clearly remains to be determined. Further studies will hopefully precisely define the functional effects of the A₁AR expressed in human asthmatic epithelium. With respect to a potential role of A₁ARs in airway remodeling, recent reports, albeit not pertaining to the lung per se, suggest that activation of A₁ARs may play an important role in angiogenesis and fibrosis, cardinal features of airway remodeling in human asthma (Clark et al. 2007; Cohn et al. 2004; Kalk et al. 2007). For example, activation of A₁ARs on human monocytes induces the release of vascular endothelial growth factor (VEGF) (Clark et al. 2007), and an A₁AR antagonist with high affinity and high selectivity for the human A₁AR, SLV320, significantly reduced levels of collagen I and III in an animal model of myocardial fibrosis (Kalk et al. 2007).

Validation of the A₁AR as an important target for human asthma is supported by positive proof of concept (POC) results in patients with asthma for EPI-2010, an antisense (“knockout”) compound that is a respiratory antisense oligonucleotide (RASON) for the human A₁AR, in a small clinical trial conducted by EpiGenesis Pharmaceuticals (Cranbury, NJ, USA). EpiGenesis reported that a single dose of EPI-2010 reduced the need for bronchodilator drugs to control asthma symptoms concomitant with a reduction in symptom scores, an effect that was statistically and clinically significant and lasted for one week following a single dose (Ball et al. 2003). However, disappointing results in a Phase II clinical trial with EPI-2010 administered to patients who were taking inhaled corticosteroids (ICSs) were reported (Langley et al. 2005). In this Phase II clinical trial, 146 patients with persistent airway obstruction (forced expiratory volume in 1 S (FEV₁) 74.5% predicted, $\geq 12\%$ reversibility) and currently receiving ICSs were administered EPI 2010 (1, 3, or 9 mg) via nebulizer once or twice weekly for 29 days. In this clinical study there was no significant change in the FEV₁ after 29 days of treatment compared to baseline. It was concluded that EPI-2010 showed no additional therapeutic effect in patients currently receiving ICSs. Patients with a stable FEV₁ of 74.5% predicted have mild/moderate asthma, depending on the frequency of symptoms and magnitude of variability in the peak expiratory flow rate (PEFR). In patients with mild/moderate asthma treated with ICSs, the FEV₁ may be 90–100% of the predicted value when measured between exacerbations and without provocation. Thus, the FEV₁ is not

a sensitive measure of asthma severity per se, vis-à-vis acute changes in airway function reflected by PEFV variability in ICS-treated patients with mild/moderate asthma. The lack of efficacy for EPI-2010 in this Phase II clinical trial (i.e., that EPI-2010 showed no additional therapeutic effect in patients taking ICSs) was not surprising.

Because of these effects of activation of A_1 ARs on different cell types to produce bronchoconstriction, inflammation, mucous gland hyperplasia, angiogenesis, and fibrosis, all of which are important in the pathophysiology of human asthma, an A_1 AR antagonist, L-97-1 (Endaceca, Inc.), is in development as a once-daily, oral treatment for human asthma. L-97-1 is a water-soluble, small-molecule A_1 AR antagonist with high affinity and high selectivity for the human A_1 AR (Obiefuna et al. 2005). In an animal model of allergic asthma, L-97-1 blocks allergic airway responses, BHR to histamine, and airway inflammation (Nadeem et al. 2006; Obiefuna et al. 2005). A number of A_1 AR antagonists have been or currently are in clinical trials for a number of different medical indications and, as a class, appear to be safe and well tolerated in humans (Barrett 1996; Bertolet et al. 1996; Dittrich et al. 2007; Doggrell 2005; Gaspardone et al. 1993; Givertz et al. 2007; Gottlieb et al. 2002; Greenberg et al. 2007).

2.2 A_{2A} Adenosine Receptors and Asthma

A_{2A} AR signaling in the pathophysiology of asthma may be critical considering the fact that A_{2A} ARs are present on most of the inflammatory cells (including neutrophils, mast cells, macrophages, eosinophils, platelets, and T cells; Lappas et al. 2005; Thiel et al. 2003). Activation of A_{2A} AR on these cell types is almost universally inhibitory, and therefore could modulate inflammatory events in the airways. The anti-inflammatory effects of activation of A_{2A} AR on these cell types include inhibition of chemotaxis, elastase release, phagocytosis, oxidative stress, adherence of neutrophils to endothelial cells, mast cell degranulation, and the release of proinflammatory cytokines (Lappas et al. 2005; Nadeem et al. 2007).

There are a multitude of mechanisms by which an agonist, acting through A_{2A} ARs, could suppress inflammation in asthmatic airways. In human neutrophils, stimulation of A_{2A} AR reduces neutrophil adherence to the endothelium, inhibits formyl-Met-Leu-Phe (fMLP)-induced oxidative burst, and inhibits superoxide anion generation (Visser et al. 2000). In monocytes and macrophages, activation of A_{2A} ARs inhibits LPS-induced tumor necrosis factor (TNF)- α expression (Bshesh et al. 2002). A_{2A} AR-deficient allergic mice have increased oxidative stress in the lung as well as the airway smooth muscle after ragweed/ovalbumin allergen challenge as compared to their wild type. This oxidative stress is caused by activation of inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase signaling due to A_{2A} AR deficiency (Nadeem et al. 2007, 2008). Moreover, genetic removal of the A_{2A} AR from ADA-deficient mice results in enhanced inflammation (composed largely of macrophages and neutrophils, mucin

production in the bronchial airways, and angiogenesis) relative to that seen in the lungs of ADA-deficient mice with the A_{2A}AR, suggesting a protective role of this receptor in pulmonary inflammation when adenosine levels are high (Mohsenin et al. 2007). A_{2A}AR-mediated suppression of inflammation is mainly thought to be mediated by activation of protein kinase A and cyclic AMP response element-binding protein (Allen-Gipson et al. 2005; Bshesh et al. 2002), and inhibition of nuclear factor kappa B (NF-κB) signaling (Bshesh et al. 2002; Lukashev et al. 2004; Nadeem et al. 2007).

Strong anti-inflammatory properties for A_{2A}AR have been shown in an inflammatory disease model using A_{2A}AR gene-deficient mice (Lukashev et al. 2004; Nadeem et al. 2007). Consistent with this, in rat and mouse animal models of allergic asthma, the selective A_{2A}AR agonist CGS 21680 (2-*p*-(2-carboxyethyl) phenethylamino-5'-*N*-ethylcarboxoamido adenosine) significantly reduced the number of inflammatory cells in the BAL fluid during allergen-induced airway inflammation (Bonneau et al. 2006; Fozard et al. 2002). However, in these rodent animal models of allergic asthma, this selective A_{2A}AR agonist reduced airway inflammation but not BHR. Moreover, in an A_{2A}AR-deficient allergic mouse model, not only was airway inflammation enhanced but BHR was too (Nadeem et al. 2007). The discrepancy in A_{2A}AR-deficient allergic mice on airway reactivity versus the earlier report wherein airway reactivity was not reduced with the A_{2A}AR agonist, CGS 21680, in allergic mice is not apparent and may be due to differences in strains of mice.

Recently, the effects of a new A_{2A}AR agonist, GW328267X, in human asthmatics was reported (Luijk et al. 2008). In this study, treatment with GW328267X delivered as an inhalational treatment did not protect against the late asthmatic response (LAR), expressed as the decline in FEV₁ after allergen challenge, or the accompanying increase in airway inflammation (Luijk et al. 2008). However, in an earlier study, GW328267X partially inhibited the early asthmatic response (EAR) and LAR after nasal allergen challenge in patients with allergic rhinitis (Rimmer et al. 2007). There may be several possible explanations for the observed discrepancies between these two human studies. First, this A_{2A}AR agonist is not entirely selective for the A_{2A}AR; it also exhibits some inhibitory effect on A₃AR (Luijk et al. 2008). It is possible that inhibition of the A₃AR by GW328267X blocked the anti-inflammatory effects of A₃AR activation by adenosine, since it is reported that activation of the A₃AR in humans produces anti-inflammatory effects, including inhibition of migration of human eosinophils and inhibition of oxidative burst, degranulation and release of inflammatory cytokines in human neutrophils, monocytes, and macrophages (Fishman and Bar-Yehuda 2003). Thus, the inhibitory effect of GW328267X on A₃ARs may have counteracted possible beneficial effects of A_{2A}AR activation. Secondly, it is possible that the dose of the GW328267X (inhaled dose, 25 µg twice daily) was subtherapeutic. It was previously determined that higher doses of GW328267X caused cardiovascular side effects (reduction in blood pressure and increase in heart rate) following inhalational delivery (Luijk et al. 2008).

As mentioned above, even following inhalational delivery in small doses, the cardiovascular side effects of A_{2A}AR agonists may limit their clinical development. Moreover, tachyphylaxis and immune suppression may limit the clinical efficacy and safety of A_{2A}AR agonists as antiasthma drugs. For example, with the chronic administration of A_{2A}AR agonists, tachyphylaxis to the bronchodilator and anti-inflammatory effects may occur via the desensitization of G_s-coupled intracellular signaling pathways (Sullivan 2003). This potential effect of A_{2A}AR agonists was evident with the chronic administration of CGS-21680 over a two-week period wherein tachyphylaxis to the blood pressure lowering effect was reported and prevented the development of this A_{2A}AR agonist as an antihypertensive agent (Webb et al. 1993). Furthermore, because A_{2A}AR agonists act via G_s to stimulate adenylate cyclase, they may be associated with an increased risk of sudden death in asthmatics in a similar fashion to that of long-acting β₂-agonists (LABAs) (Salpeter et al. 2004). Moreover, activation of A_{2A}ARs produces neovascularization (angiogenesis) (Cronstein 2006; Montesinos et al. 1997; Montesinos et al. 2006). Because of this effect of A_{2A}ARs on neovascularization/angiogenesis, an A_{2A}AR agonist, MRE-0094, is in Phase II clinical trials as a treatment for wound healing in diabetic foot ulcers (Aderis Pharmaceuticals). However, angiogenesis is a cardinal feature of airway remodeling of human asthma, and despite the report that activation of A_{2A}ARs promotes wound healing in bronchial epithelial cells (Allen-Gipson et al. 2005), the effect of A_{2A}AR agonists on angiogenesis may limit their development as antiasthma drugs.

Further to these clinical considerations for the development of A_{2A}AR agonists as antiasthma drugs, others include their potential to produce antitumor effects and immune suppression (Ohta et al. 2006; Sullivan 2003). Because A_{2A}AR agonists block oxidative and nonoxidative activity of neutrophils, cause functional repression and/or apoptosis of lymphocytes, and inhibit the release of (interleukin) IL-12, which promotes bacterial clearance in infection, these agents may cause immune suppression and predispose to infection (Sullivan 2003). Moreover, in an adenosine-rich tumor microenvironment, activation of A_{2A}ARs produces inhibition of antitumor T cells (Ohta et al. 2006). In mice, genetic deletion of the A_{2A}AR or the use of A_{2A}AR antagonists improved inhibition of tumor growth, destruction of metastasis and prevention of neovascularization by antitumor T cells. Despite what should be advantageous effects from the activation of A_{2A}ARs (i.e., bronchodilation and anti-inflammatory effects), the potential side effects of hypotension and tachycardia may limit the use of A_{2A}AR agonists as acute rescue antiasthma drugs, and the potential side effects of tachyphylaxis and immune suppression as well as the angiogenesis and antitumor effects produced by A_{2A}AR agonists may limit the use of these molecules as chronic maintenance antiasthma drugs.

2.3 A_{2B} Adenosine Receptors and Asthma

Studies in animals both in vitro and in vivo and human cell lines in vitro have suggested that A_{2B}ARs may play an important role in mediating airway reactivity,

inflammation, and remodeling in asthma. A_{2B} ARs are coupled via both G_s and G_q proteins to intracellular signaling pathways, which results in the release of cytokines and other mediators that are important in the pathophysiology of human asthma (Feoktistov et al. 1999; Zhong et al. 2004). In a human mast cell line (HMC-1), by coupling primarily to G_q , activation of A_{2B} ARs by adenosine induces the release of inflammatory cytokines such as IL-4, IL-8 and IL-13 which, in turn, can induce immunoglobulin E (IgE) synthesis by B lymphocytes (Feoktistov et al. 1999; Ryzhov et al. 2004). Moreover, in these HMC-1 cells, the selective A_{2B} AR antagonists IPDX (3-isobutyl-8-pyrrolidinoxanthine) and MRS 1754 ([*N*-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)-phenoxy]acetamide]) inhibited activation of HMC-1 cells induced by NECA (5'-*N*-ethylcarboxamido adenosine), a stable analog of adenosine (Feoktistov et al. 2001). Since HMC-1 cells are derived from a highly malignant, undifferentiated human mastocytoma cancer, the relevance of these findings in this human mast cell line to that in IgE immunologically sensitized human mast cells in allergic asthma is unknown. In the allergic response, antigens bind and crosslink IgE molecules bound to the functional high-affinity receptor for IgE, FcεRI, on mast cells to induce degranulation and the release of a broad spectrum of proinflammatory mediators (Nilsson et al. 1994; Xiang et al. 2001). HMC-1 cells do not express FcεRI (Nilsson et al. 1994). For this reason the reference to HMC-1 cells as human mast cells in allergic conditions, including human asthma, is misleading.

The presence of A_{2B} ARs on IgE immunologically sensitized human mast cells has not been reported. However, bone marrow-derived mast cells (BMMCs) from mice do express FcεRI (Hua et al. 2007b). Moreover, as opposed to the HMC-1 cells, A_{2B} ARs on murine BMMCs (Hua et al. 2007b), as well as human bronchial epithelial and smooth muscle cells and fibroblasts (Zhong et al. 2003, 2004, 2005), are coupled to G_s and adenylate cyclase, as compared to HMC-1 cells, where they are primarily coupled to G_q and phospholipase C (PLC) (Feoktistov and Biaggioni 1995). Furthermore, as opposed to the HMC-1 cell line, mast cell activation is enhanced in mice deficient for the A_{2B} AR (Hua et al. 2007b). The authors of this study suggested that in mice lacking this G_s -coupled receptor, BMMCs expressing FcεRI have reduced levels of cyclic AMP and an excess of intracellular calcium via store-operated calcium channels following antigen activation, thereby increasing their sensitivity to antigen-mediated degranulation. In addition, these A_{2B} AR-deficient mice display an increased sensitivity to IgE-mediated tachyphylaxis. In a recent study, genetic ablation of the A_{2B} AR had no effect on A_3 AR-dependent potentiation of antigen-induced degranulation in mouse BMMCs, but abrogated A_{2B} AR-induced release of IL-13 and VEGF. The authors of this study suggest that in the mouse the A_3 AR regulates mast cell degranulation, whereas the A_{2B} AR regulates mediator release, e.g., IL-13 and VEGF (Ryzhov et al. 2008a).

As mentioned above, as opposed to that seen in HMC-1 cells, by coupling to G_s and adenylate cyclase, adenosine activation of A_{2B} AR increases the release of inflammatory cytokines from human bronchial epithelial cells (HBECs) (Zhong et al. 2006), human bronchial smooth muscle cells (Zhong et al. 2004) and human fibroblasts (Zhong et al. 2005). In human bronchial smooth muscle cells, activation

of A_{2B}ARs induces the release of IL-6 and the chemokine monocyte chemoattractant protein 1 (MCP-1) (Zhong et al. 2004). In HBECs, activation of A_{2B}ARs induces the release of IL-19, which in turn induces the release of TNF- α from monocytes, which in turn upregulates the expression of A_{2B}ARs on HBECs (Zhong et al. 2006). In human lung fibroblasts, activation of A_{2B}ARs induces the release of IL-6, which, in the presence of hypoxia, synergistically induced the differentiation of lung fibroblasts into myofibroblasts (Zhong et al. 2005). These effects of activation of A_{2B}ARs by NECA, a stable analog of adenosine, in human bronchial smooth muscle cells (Zhong et al. 2004) and HBECs (Zhong et al. 2006) are blocked by selective antagonists of the A_{2B}AR. Furthermore, in a recent study, genetic ablation of A_{2B}AR abrogated NECA-induced increases in IL-6 release from mouse peritoneal macrophages *ex vivo* and dramatically reduced the ability of NECA to increase IL-6 plasma levels *in vivo* (Ryzhov et al. 2008b). Moreover, stimulation of the A_{2B}AR on isolated mouse BMMCs can directly promote the production and secretion of IL-13 and VEGF (Ryzhov et al. 2008a). Taken together, these studies indicate that stimulation of A_{2B}AR is coupled to the release of proinflammatory cytokines, and may play an important role in airway remodeling of asthma.

Although the importance of these *in vitro* studies to support the role of the A_{2B}AR *in vivo* in humans with asthma remains to be determined, studies in animal models of allergic asthma support the role of this AR in asthma. In ragweed-sensitized allergic mice, airway challenge with adenosine increased bronchoconstrictor responses and amplified the pulmonary inflammatory response to an allergen challenge (Fan and Mustafa 2002, 2006). This increase in bronchoconstrictor responses and airway inflammation to adenosine was blocked by theophylline and attenuated by a specific antagonist of the A_{2B}AR, which suggests, in part, a role for the A_{2B}AR (Fan and Mustafa 2002; Fan et al. 2003; Mustafa et al. 2007). Moreover, in this allergic mouse model of asthma, adenosine-induced increases in β -hexosaminidase activity (a mast cell marker) were decreased by pretreatment with theophylline (Fan and Mustafa 2006). Furthermore, in another study involving the use of this allergic mouse model of asthma from this same group, aerosolized NECA- and AMP-elicited concentration-dependent increases in Penh were significantly attenuated by CVT-6883, an A_{2B}AR antagonist (Mustafa et al. 2007). In this study, an allergen challenge-induced increase in LAR was inhibited by CVT-6883, and the increase in the number of inflammatory cells in BAL fluid was also inhibited by CVT-6883 or theophylline.

These findings, that the A_{2B}AR antagonist CVT-6883 reduces inflammation in the lung in an animal model of allergic asthma, were demonstrated in another animal model of lung inflammation with a phenotype similar to allergic asthma, albeit not an allergic asthma animal model, ADA-deficient mice (Sun et al. 2006). As previously stated, ADA-deficient mice develop pulmonary inflammation, fibrosis, and enlargement of alveolar airspaces. In CVT-6883-treated ADA-deficient mice there was less pulmonary inflammation, fibrosis, and alveolar airspace enlargement (Sun et al. 2006). Moreover, in ADA-deficient mice, A_{2B}AR antagonism with CVT-6883 significantly reduced elevations in proinflammatory cytokines and chemokines as well as mediators of fibrosis and airway destruction (Sun et al. 2006). These findings

in these animal models suggest that $A_{2B}AR$ signaling influences pathways critical for airway reactivity and inflammation.

As opposed to these reports suggesting that activation of $A_{2B}AR$ s play an important role in bronchoconstriction and airway inflammation in allergic asthma, recent reports suggest that activation of $A_{2B}AR$ s may produce bronchorelaxant and anti-inflammatory effects. In a recent study in guinea pigs, NECA evoked relaxing responses of isolated tracheal preparations precontracted with histamine in normal and sensitized animals, and this effect was reversed by the $A_{2B}AR$ antagonist MRS 1706 (Breschi et al. 2007). Moreover, *in vitro* desensitization with 100 μM NECA markedly reduced the relaxing effect of NECA, raising the possibility that higher adenosine levels in the lung might desensitize this receptor to cause bronchorelaxation (Breschi et al. 2007). Furthermore, activation of $A_{2B}AR$ s may produce anti-inflammatory effects. In $A_{2B}AR$ knockout/reporter gene-knockin mice, there was low-grade baseline inflammation, augmented release of proinflammatory cytokines (including TNF- α and IL-6), as well as leukocyte adhesion to the vasculature (Yang et al. 2006). This finding that TNF- α levels are increased in $A_{2B}AR$ knockout mice was confirmed by a more recent report by the same group (Yang et al. 2008). In a femoral artery injury model that resembles restenosis following angioplasty, $A_{2B}AR$ knockout mice had higher levels of TNF- α , an upregulator of chemokine receptor 4 (CXCR4), and proliferation of vascular smooth muscle cells (Yang et al. 2008).

It is possible that the bronchorelaxant and anti-inflammatory effects of $A_{2B}AR$ s described above may be due to an increase in intracellular cyclic AMP levels following activation of $A_{2B}AR$ s. It is well known that an increase in intracellular cyclic AMP produces relaxation of bronchial smooth muscle and bronchodilation, suppresses inflammation, and prevents changes in endothelial cells that lead to an increase in endothelial permeability. Given these effects of intracellular cyclic AMP, it is unclear why an approach to the treatment of asthma would be to block these salutary effects of a receptor coupled via G_s to adenylate cyclase (i.e., the $A_{2B}AR$). It is now reported that the use of $A_{2B}AR$ antagonists may increase endothelial permeability (Lennon et al. 1998). Moreover, in human airway epithelial cells via coupling to G_s and adenylate cyclase, $A_{2B}AR$ s play an important role in control of the cystic fibrosis transmembrane conductance regulator (CFTR)-operated Cl^- channel (Clancy et al. 1999; Huang et al. 2001). Because of the importance of this Cl^- channel in airway hydration, the use of $A_{2B}AR$ antagonists may induce a cystic fibrosis-like phenotype associated with an increased viscosity of mucus in humans, and may therefore limit their development as antiasthma drugs. Thus, although it appears that $A_{2B}AR$ s may play an important role in airway remodeling of human asthma, because of their effect on the CFTR-operated Cl^- channel in human airway epithelial cells and airway hydration, the safety of $A_{2B}AR$ antagonists in human asthmatics remains to be determined. Moreover, the efficacy of $A_{2B}AR$ antagonists may depend on the relative contribution of this G_s -coupled receptor to adenylate cyclase and increases in intracellular cyclic AMP to produce bronchodilation and anti-inflammatory effects.

With respect to the therapeutic approach to the A_{2B}AR as a target in human asthma, based on the reports that activation of A_{2B}ARs in HBECs (Zhong et al. 2006), human bronchial smooth muscle cells (Zhong et al. 2004) and human lung fibroblasts (Zhong et al. 2005) induces the release of mediators important in the pathophysiology of airway remodeling of human asthma, as well as the efficacy of the A_{2B}AR antagonist CVT 6883 in an acceptable animal model of allergic asthma (Mustafa et al. 2007), CVT 6883 has entered Phase I clinical trials as an antiasthma drug (CV Therapeutics, Inc.). Moreover, a combined A_{2B}/A₃ AR antagonist, QAF 805 (Novartis), has been tested in humans as an antiasthma drug. This mixed A_{2B}/A₃ AR antagonist failed to increase the provocative concentration (PC)₂₀ for AMP (concentration of AMP required to reduce the FEV₁ by 20%) versus placebo in 24 AMP-sensitive asthmatics in a placebo-controlled, double-blind, randomized, two-way crossover Phase Ib clinical trial (Pascoe et al. 2007). The results of the clinical trials with CVT 6883 and other selective A_{2B}AR antagonists should more clearly define the role of A_{2B}ARs in human asthma, and are eagerly awaited.

2.4 A₃ Adenosine Receptors and Asthma

The functional relevance of the A₃AR in the pathogenesis of asthma is a matter of debate, primarily due to species differences. In humans, A₃ARs have been identified on eosinophils, neutrophils, and monocytes; however, they have not been identified on mast cells (Gessi et al. 2008; Walker et al. 1997). In rats and mice, A₃ARs play an important role in adenosine-induced mast cell degranulation, bronchoconstriction, eosinophilia, and mucus production; however, they exhibit poor sensitivity to methylxanthines (Fan et al. 2003; Ramkumar et al. 1993; Tilley et al. 2003; Young et al. 2006). In an A₃AR knockout mouse model, a selective A₃AR agonist, IB-MECA (*N*⁶-(3-iodobenzyl)-adenosine-5'-*N*-methylcarboxamide) delivered via a nebulizer had no effect on lung mast cell degranulation compared to wild-type mice (Zhong et al. 2003). In murine primary lung mast cells, activation of A₃ARs induced mast cell histamine release in association with increases in intracellular calcium mediated through G_i and phosphoinositide 3-kinase signaling pathways (Zhong et al. 2003). Furthermore, in ADA-deficient mice, A₃ARs appear to be important in endogenous adenosine-induced lung mast cell degranulation in the absence of antigen stimulation (Zhong et al. 2003). Moreover, in ADA-deficient mice, an increase in eosinophils and mucus production were reversed by a selective A₃AR antagonist, suggesting an important role for A₃ARs in mediating the lung eosinophilia and mucus hyperplasia in this animal model (Young et al. 2004). Further to these studies, in a murine model of allergic asthma it appears that A₃ARs play an important role in adenosine-induced bronchoconstriction (Fan et al. 2003). Finally, in allergen-sensitized guinea pigs, an A₃AR antagonist, MRS-1220, significantly inhibited 5'-AMP-induced migration of eosinophils and macrophages into the airways (Spruntulis and Broadley 2001). Taken together, these studies suggest

that A₃ARs play an important role in adenosine-induced mast cell degranulation as well as eosinophilia, mucus hyperplasia, and bronchoconstriction in mice and guinea pigs, and would support the approach to asthma with an A₃AR antagonist.

In humans, expression of A₃ARs is elevated in lung biopsies of patients with asthma, and is mostly localized on eosinophils where activation by adenosine via this receptor inhibits chemotaxis (Walker et al. 1997). This initial report describing this anti-inflammatory effect of activation of A₃ARs on human eosinophils was reproduced, and the studies were expanded by the same group to show that the activation of A₃ARs produced a dose-dependent inhibition in the chemotaxis of human eosinophils to platelet-activating factor, RANTES, and leukotriene B₄, and this effect was completely reversed by selective A₃AR antagonists (Knight et al. 1997). Moreover, following these reports, another group reported that A₃ARs on human eosinophils mediate inhibition of both degranulation and superoxide anion release, and that therapeutic concentrations of theophylline inhibit the human eosinophil partly by acting as an A₃AR agonist, thus contributing to the mechanism of the anti-inflammatory action of this drug in vivo (Ezeamuzie 2001; Ezeamuzie and Philips 1999). However, another group studied IB-MECA-induced effects on free radical generation in eosinophils of asthmatics and reported that stimulation of A₃ARs does not appear to be a prime mechanism for free radical generation by human peripheral blood eosinophils (Reeves et al. 2000). Taken together, these studies in humans suggest that an A₃AR agonist should be considered as a therapeutic option for the treatment of human asthma, as opposed to the studies in mice, rats, and guinea pigs that suggest that the A₃AR target to treat asthma should be approached with an A₃AR antagonist.

Based on the reports in animals that activation of A₃ARs produce mast cell degranulation, bronchoconstriction, eosinophilia and mucus hyperplasia, and that activation of A_{2B}ARs on human mast cells may play an important role in human asthma, a combined A_{2B}/A₃ AR antagonist QAF 805 (Novartis) is under development as an antiasthma drug (Press et al. 2005; Pascoe et al. 2007). However, as mentioned above, this mixed A_{2B}/A₃ AR antagonist has now entered human clinical trials and it failed to increase the PC₂₀ for AMP versus placebo in 24 AMP-sensitive asthmatics in a placebo-controlled, double-blind, randomized, two-way crossover study Phase Ib clinical trial (Pascoe et al. 2007). With respect to the use of A₃AR agonists as antiasthma drugs, the use of this new class of drugs for this therapeutic indication may be limited by hypotension, tolerance/tachyphylaxis, and immune suppression (Gessi et al. 2008). Because of the anti-inflammatory and specifically the anti-TNF- α effects of the activation of A₃ARs on human monocytes, an A₃AR agonist, CF-101, is in Phase IIb clinical trials for the treatment of rheumatoid arthritis (Can-Fite Biopharma). It is reported that CF-101 has an acceptable safety and tolerability profile in humans (van Troostenburg et al. 2004). In this report, bronchospasm was not reported as a side effect of CF-101; however, the patients in this study were taking another anti-inflammatory immune suppressant, methotrexate, and CF-101 has not been tested in humans with asthma. Thus, the safety and efficacy of A₃AR agonists as antiasthma drugs are yet to be determined in humans.

3 Conclusions and Future Directions

It is now well accepted that adenosine is an important signaling molecule in the pathogenesis of human asthma, and all AR subtypes are important targets for anti-asthma drug development for humans. A number of AR molecules with good safety profiles and selectivity are now available for testing in humans, in order to determine the role of ARs in human asthma and the therapeutic approach to these AR targets that will produce safe and effective antiasthma therapeutics. Because many patients with asthma are either not controlled or are noncompliant with current antiasthma therapies, a shift in focus towards new mechanisms and novel targets (e.g., adenosine signaling and AR targets) is necessary to discover new classes of drugs that are safe and effective that will not only control the symptoms of asthma, but interrupt the disease of airway remodeling and the progressive loss of lung function, and thus improve not only the quality of life, but the outcome for the patient with asthma.

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Adenosine Receptors, Cystic Fibrosis, and Airway Hydration

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Abstract Adenosine (Ado) regulates diverse cellular functions in the lung through its local production, release, metabolism, and subsequent stimulation of G-protein-coupled P1 purinergic receptors. The A_{2B} adenosine receptor (A_{2B}AR) is the predominant P1 purinergic receptor isoform expressed in surface airway epithelia, and Ado is an important regulator of airway surface liquid (ASL) volume through its activation of the cystic fibrosis transmembrane conductance regulator (CFTR). Through a delicate balance between sodium (Na⁺) absorption and chloride (Cl⁻) secretion, the ASL volume is optimized to promote ciliary activity and mucociliary clearance, effectively removing inhaled particulates. When CFTR is dysfunctional, the Ado/A_{2B}AR regulatory system fails to optimize the ASL volume, leading to its depletion and interruption of mucociliary clearance. In cystic fibrosis (CF), loss of CFTR function and resultant mucus stasis leaves the lower airways susceptible to mucus obstruction, chronic bacterial infection, relentless inflammation, and eventually panbronchiectasis. Adenosine triphosphate (ATP) also regulates transepithelial Cl⁻ conductance, but through a separate system that relies on stimulation of P2Y₂ purinergic receptors, mobilization of intracellular calcium, and activation of calcium-activated chloride channels (CaCCs). These pathways remain functional in CF, and may serve a protective role in the disease. In this chapter, we will review

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our current understanding of how Ado and related nucleotides regulate CFTR and Cl^- conductance in the human airway, including the regulation of additional intracellular and extracellular signaling pathways that provide important links between ion transport and inflammation relevant to the disease.

Keywords Adenosine receptors · Cystic fibrosis · Adenosine · Airway hydration · P1 purinergic receptors

Abbreviations

Ach	Acetylcholine
ADA	Adenosine deaminase
Ado	Adenosine
AKAP	A kinase anchoring protein
AMP	Adenosine monophosphate
ASL	Airway surface liquid
ATP	Adenosine triphosphate
A ₁ AR	A ₁ adenosine receptor
A _{2A} AR	A ₂ adenosine receptor
A _{2B} AR	A _{2B} adenosine receptor
A ₃ AR	A ₃ adenosine receptor
CaCC	Calcium-activated chloride channel
Ca ⁺⁺	Calcium
cAMP	Cyclic adenosine monophosphate
CF	Cystic fibrosis
CFF	Cystic Fibrosis Foundation
CFTR	Cystic fibrosis transmembrane conductance regulator
Cl ⁻	Chloride
COPD	Chronic obstructive pulmonary disease
EBP50	Ezrin-binding protein 50
ENaC	Epithelial sodium channel
EP4	E4 prostaglandin receptor
FEV ₁	Forced expiratory volume in one second
GI	Gastrointestinal
GPCR	G-protein-coupled receptor
GSH	Glutathione
HBEC	Human bronchial epithelial cell
HCO ₃	Bicarbonate
ICAM-1	Intracellular adhesion molecule-1
IL-4	Interlukin-4
IL-5	Interlukin-5
IL-13	Interlukin-13
Iso	Isoproterenol

K ⁺	Potassium
MMP-9	Matrix metalloproteinase-9
MMP-12	Matrix metalloproteinase-12
MCC	Mucociliary clearance
MCP	Monocyte chemoattractant protein
Na ⁺	Sodium
NBD-1	Nucleotide-binding domain 1
NBD-2	Nucleotide-binding domain 2
NPD	Nasal potential difference
ORCC	Outwardly rectified chloride channel
PDZ	Postsynaptic density protein 95, disk-large tumor suppressor protein, <i>Zonula occludens</i> 1
PGE ₂	Prostaglandin E ₂
PKA	Protein kinase A
PS	Pancreatic sufficient
R domain	Regulatory domain
RT-PCR	Reverse transcriptase polymerase chain reaction
SCN ⁻	Thiocyanate
TIMP-1	Tissue inhibitor of metalloproteinase 1
TNF- α	Tumor necrosis factor α
UTP	Uridine triphosphate
VIP	Vasoactive intestinal peptide

1 Cystic Fibrosis and Airway Ion Transport

CF is a serious and life-threatening disease that affects approximately 30,000 US citizens and >70,000 people worldwide (CFF 2005; Pilewski and Frizzell 1999; Rowe et al. 2005). It is caused by autosomal recessive mutations in the gene encoding CFTR. The protein product is a membrane-localized traffic ATPase that functions as a Cl⁻ channel (Anderson et al. 1991), and it is also a regulator of many ion transport pathways (including nonCFTR Cl⁻ channels, Na⁺, bicarbonate, ATP, glutathione (GSH), and potentially other small molecules (Hudson 2001; Moskwa et al. 2007; Park et al. 2002; Rowe et al. 2005; Schweibert et al. 1999). CFTR is expressed in many tissues, including the airways, the gastrointestinal (GI) tract and the hepatobiliary tree, the pancreatic ducts, the sweat glands, and in the male reproductive tract. In the airways, CFTR is expressed at high levels in submucosal gland ducts, and to a lesser extent at the apical cell membrane of the surface pseudostratified epithelium of medium and large airways, and in the distal epithelium of the small airways (Engelhardt et al. 1992, 1994).

Central to our understanding of CF pathophysiology is an appreciation of the role played by CFTR in regulating submucosal gland and airway surface liquid composition (Choi et al. 2007; Joo et al. 2002, 2004, 2006; Wine and Joo 2004; Wu et al. 2007). Much of the work describing defects in glandular function in CF have been

recently reported by Wine and colleagues, examining gland activity in strips of airway tissue removed from CF patients undergoing lung transplantation. Submucosal gland secretory activity is regulated by neurogenic pathways [acetylcholine (Ach) and vasoactive intestinal peptide (VIP)] and also by local paracrine regulatory factors, which together hydrate the glandular secretions and promote their release upon gland stimulation. The released glandular contents provide a significant volume to the airway surface that is rich in a variety of antimicrobial peptides and factors. The glands therefore contribute to both the innate defense system through the release of antimicrobial molecules and through contributions to hydration of the airway. In CF, loss of CFTR function leads to thickening of glandular secretions and plugging of gland ducts. This can be seen pathologically as enlargement and hypertrophy of glands that are filled with retained secretions, demonstrating ductal dilation and hyperplasia of glandular acini. The glands fail to secrete in response to VIP, but retain Ach-stimulated secretion and release of abnormal glandular contents. In this model, glandular dysfunction is a primary cause of CF lung disease, initiating the obstructive, infectious, and inflammatory consequences of CFTR dysfunction (Choi et al. 2007; Wine and Joo 2004).

On the apical airway surface, epithelial cells are covered with numerous cilia that are bathed by a watery fluid compartment known as the airway surface liquid or ASL (Boucher 2007a; Matsui et al. 1998a; Rowe et al. 2005). The cilia beat rapidly within this compartment in a coordinated fashion across the epithelial sheet, and this arrangement allows swift and unimpeded ciliary activity that promotes cephalad movement of the overlying mucus blanket. This mucus or gel layer is constructed from hydrated mucins (MUC5AC and 5B) and also serves as a volume reservoir for the ASL. Trapped particulates are rapidly cleared from the airway surface, and this is accomplished prior to the activation of secondary host defense mechanisms. The end result is the continuous clearance of pathogens and the avoidance of unnecessary activation of host responses that could potentially injure the airway.

The composition of the ASL is determined in large part by the activity of epithelial ion transporters present in the airway cells, and these systems have been carefully characterized by researchers at the University of North Carolina using polarized mature airway epithelial monolayers isolated from human subjects. In the prevailing model of airway epithelial ion transport (see Fig. 1), a sodium (Na^+) absorptive phenotype is produced by the epithelial sodium channel (ENaC), which is a protease-regulated channel that absorbs Na^+ across the airway epithelium. There is complimentary passive Cl^- flow through CFTR (and potentially other CFTR-dependent Cl^- transport pathways) from the luminal compartment, with accompanying H_2O transport through the paracellular pathway and potentially aquaporins (Boucher 2007a, b; Donaldson et al. 2006). In addition to CFTR-dependent Cl^- transport, there is a CFTR-independent pathway that is regulated by cell calcium (Ca^{++}) that activates CaCCs (Boucher 2007a, b). These processes result in a “dry” luminal compartment, and provide a rational explanation for the ability of the large airways to avoid flooding in the face of the surface airway reduction that occurs as fluid moves from the smallest bronchioles to the mainstem bronchi. Loss of CFTR activity manifests as a loss of cAMP-dependent Cl^-

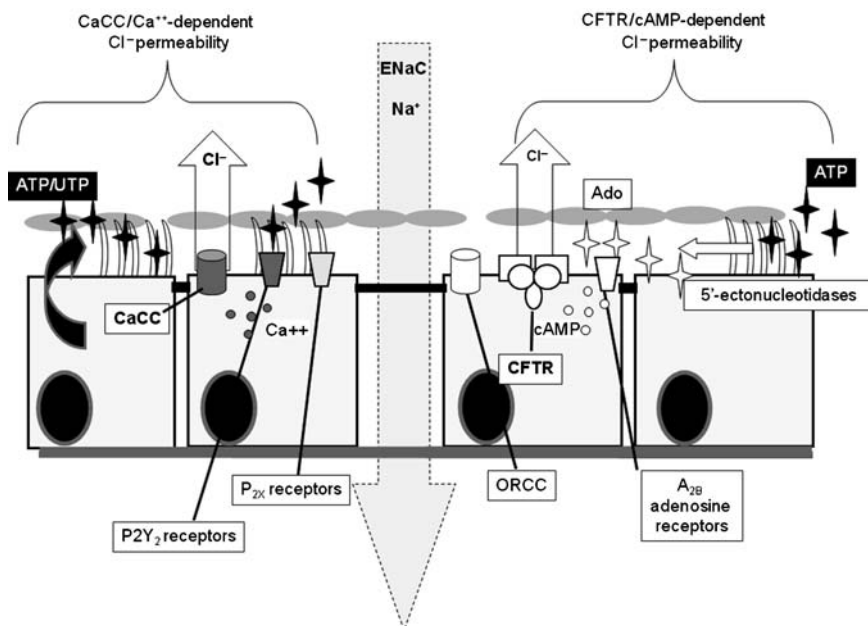


Fig. 1 Model of adenosine triphosphate (ATP) and adenosine (Ado)-regulated chloride (Cl⁻) transport in airway cells. Na⁺ absorption through epithelial sodium channels (ENaC) generally dominates transcellular ion flow, maintaining a dry luminal surface. On the *left*, ATP and uridine triphosphate (UTP) stimulate P_{2Y2} receptors to mobilize intracellular calcium, and ATP stimulates P_{2X} calcium receptor channels, inducing an influx of extracellular calcium. Increased intracellular Ca⁺⁺ stimulates calcium-activated Cl⁻ channels (CaCCs), leading to transcellular Cl⁻ secretion. On the *right*, Ado nucleotides are dephosphorylated by 5'-ectonucleotidases on the cell surface, leading to the production of Ado. Ado can bind to A_{2B} adenosine receptors (A_{2B}ARs), stimulating local cAMP production and activation of CFTR. CFTR transports select anions such as Cl⁻ and bicarbonate (HCO₃⁻) and thiocyanate (SCN⁻). Activated CFTR also facilitates the release of ATP, which can positively couple to outwardly rectified Cl⁻ channels (ORCCs). The balance between Na⁺ and Cl⁻ transport sets the airway surface liquid (ASL) volume, which is disturbed (reduced) in CF. For clarity, basolateral ion transport pathways and other apical Cl⁻ transport pathways are not included

transport across airway epithelia, and heightened Na⁺ absorption through ENaC. The nature of the increased Na⁺ absorption is increased ENaC activity, which is proposed to be secondary to the loss of direct negative regulation provided by intact CFTR, and protease activation of ENaC (Stutts et al. 1995; Tarran et al. 2006b). As Na⁺ is absorbed across the airway, complimentary Cl⁻ transport occurs through a paracellular route, leading to a hyperpolarized surface epithelium and water depletion. Interestingly, the Ca⁺⁺ regulated Cl⁻ transport pathway remains intact in the CF airway (and is upregulated relative to the nonCF phenotype). Despite this, the end result of CFTR dysfunction is a reduction in the ASL volume, which impedes normal ciliary beating (Boucher 2007a, b; Matsui et al. 1998a, b). In this model of CF lung disease, the interruption of mucus clearance by ASL volume depletion and

ciliary collapse is the first in a series of cascading steps, including mucus obstruction of the airways, intermittent and subsequently chronic bacterial infection, and severe, sustained inflammation, that lead to irreversible bronchiectasis and eventual respiratory failure. Supportive care for CF continues to advance steadily, with the median life expectancy of persons with CF now exceeding 37 years (CFF 2006). Unfortunately, CF lung disease remains devastating for many patients, with the majority of patients who die from CF succumbing in early adulthood.

2 Regulation of Airway Cl^- Transport by Adenosine and Related Nucleotides

Figure 1 provides a simplified model of ion transport pathways in the surface airway epithelium, and of how Ado nucleotides on the epithelial surface regulate anion transport and mucociliary clearance. At least two pathways are operative; one dominated by Ca^{++} and CaCCs, and the other dominated by cAMP and CFTR. On the left, ATP-stimulated functions are highlighted, including (1) ATP regulation of ciliary beat frequency, (2) stimulation of P2Y_2 receptors, and (3) stimulation of P2X receptor channels (Alfahel et al. 1996; Bennett et al. 1996; Knowles et al. 1991; Korngreen and Priel 1996; Olivier et al. 1996; Zhang and Sanderson 2003; Zsembery et al. 2004). Recent reports by Tarran, Boucher and colleagues have led to a clearer view of how purinergic receptors regulate Cl^- transport, based on studies completed in their established primary human airway epithelial cultures (Button et al. 2007; Tarran et al. 2006a, b). In this model, shear stress produced by homeostatic processes such as the breathing cycle or coughing leads to the release of nucleotides from the airway epithelium. ATP can bind to P2Y_2 receptors on the epithelial surface, stimulating Ca^{++} release and Cl^- transport via CaCCs. New therapies in development that are designed to overcome defects in CF Cl^- transport exploit this signaling pathway (e.g., denufosal) and restore airway Cl^- conductance (Bye and Elkins 2007; Deterding et al. 2007). Early-phase studies have demonstrated that treated subjects exhibit improved lung function (forced expiratory volume in 1 s, or FEV_1) compared with placebo-treated controls following short-term exposure (28 days). Further studies will be needed to clarify that the short-term benefits of P2 receptor stimulation on lung function are durable over longer treatment periods, particularly since there is potential for off-target effects of nebulized nucleotides on other pulmonary P2Y receptors that may not be beneficial (Brunschweiler and Muller 2006). In addition to P2Y_2 receptors, extracellular ATP can stimulate P2X receptors on the luminal airway surface that function as ATP-regulated Ca^{++} entry channels, raising cell Ca^{++} , activating CaCCs and subsequent Cl^- conductance (Zsembery et al. 2003). Recent studies by Zsembery and colleagues indicate that zinc is an important cofactor to maximize P2X channel activity, which is further enhanced under low $[\text{Na}^+]$ conditions (Zsembery et al. 2004). Stimulation of Ca^{++} -dependent, CFTR-independent Cl^- transport typically produces a relatively short-lived spike in Cl^- conductance in vitro, likely due to the short

half-life of ATP on the epithelial surface and the relatively short-lived effects of nucleotides on cellular Ca^{++} levels. As noted previously, Cl^- transport through this pathway is typically enhanced in CF patients and tissues relative to normal controls, which may be due to expansion of the ER and Ca^{++} storage, serving a protective function in the absence of CFTR activity (discussed in more detail below) (Knowles et al. 1991; Paradiso et al. 2001; Ribeiro et al. 2005a, b, 2006; Tarran et al. 2002).

On the right (Fig. 1), Ado-dependent Cl^- transport is highlighted, focusing on Ado and $\text{A}_{2\text{B}}\text{AR}$ -regulation of CFTR. In current models of ion transport, released ATP can be dephosphorylated to Ado and stimulate P1 purinergic receptors (Boucher 2007a, b; Hirsh et al. 2007; Lazarowski et al. 2004). In the absence of airway infection or stress, baseline Ado levels on the airway surface are determined by a number of interrelated factors, including the production and release of Ado and related nucleotides by the surface epithelia, uptake by concentrative nucleoside transporters 2 and 3, and breakdown of Ado to inosine by ADA1 (Hirsh et al. 2007). The predominate P1 receptor found in airway cells is the $\text{A}_{2\text{B}}\text{AR}$, which couples to G_s and activates adenylyl cyclase, raising local cAMP concentrations and stimulating cAMP-dependent protein kinase A (PKA) (Cobb et al. 2002; Cobb and Clancy 2003; Hentchel-Franks et al. 2004; Huang et al. 2001; Li et al. 2006; Rollins et al. 2008; Tarran et al. 2001). PKA then phosphorylates the regulatory (R) domain of CFTR, activating the CFTR Cl^- channel (Cheng et al. 1991; Rowe et al. 2005). As a member of the ATP binding cassette protein family, CFTR also binds and hydrolyzes ATP through nucleotide binding domain 1 and 2 (NBD-1 and NBD-2) dimerization, an important step in the gating of Cl^- channel activity (Mense et al. 2006; Vergani et al. 2005a, b). Once activated, CFTR can positively couple to the outwardly rectified Cl^- channel (ORCC) through an ATP release process that is also sponsored by CFTR (Schwiebert et al. 1995; Schwiebert 1999). CFTR has also been implicated in the transport of other small molecules, including bicarbonate (HCO_3^-), GSH, and thiocyanate, each of which has described relationships to the pathogenesis of CF lung disease. The end result of CFTR activation is amplification of the CFTR Cl^- conductance signal and enhanced epithelial Cl^- secretion, promoting hydration and volume expansion of the ASL and luminal compartment. Work by Tarran and colleagues provide an elegant view of ASL volume regulation that is dependent on fluctuations in airway Ado concentrations. When the ASL volume falls, the relative Ado concentration rises (from its low baseline value to levels $>1 \mu\text{M}$), promoting Ado binding to the low-affinity $\text{A}_{2\text{B}}\text{AR}$ and activation of CFTR (Tarran et al. 2006b). When the ASL volume increases, Ado concentrations fall, reducing $\text{A}_{2\text{B}}\text{AR}$ and CFTR activity and Cl^- transport. Thus, expansion and retraction of the ASL may be self-regulated by Ado concentrations that fluctuate, with secondary effects on $\text{A}_{2\text{B}}\text{AR}$ and CFTR activity.

Figure 2 compares expression of the four P1 purinergic receptors (via real-time RT-PCR), demonstrating a relative expression profile of $\text{A}_{2\text{B}}\text{ARs} \gg \text{A}_{2\text{A}} > \text{A}_1 \sim \text{A}_3$ ARs in human bronchial epithelial cells (HBECs) [primary normal HBECs and immortalized CFBE41o- cells: $\Delta\text{F508}/\Delta\text{F508}$ genetic background]. The majority of published studies examining Ado regulation of CFTR and transepithelial Cl^- secretion implicate $\text{A}_{2\text{B}}\text{ARs}$ as the primary P1 receptor responsible for CFTR

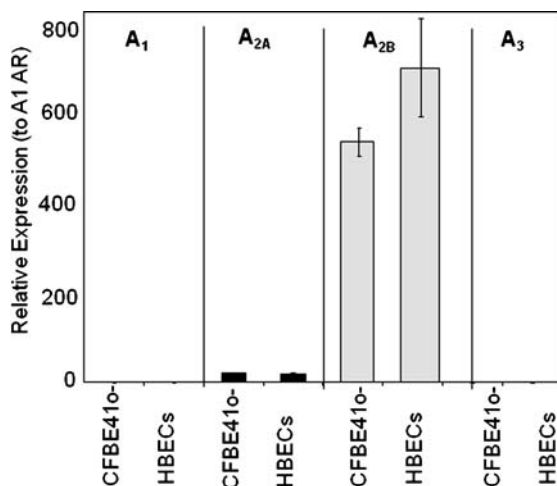


Fig. 2 P1 receptor expression in primary human bronchial epithelial cells (HBECs) and CFBE41o- cells. Isoform transcripts are compared to A₁ adenosine receptor (A₁AR) levels for the four known P1 purinergic receptors. In both cell types, A_{2B}AR expression dominates over A₁, A_{2A} and A₃ adenosine receptor (AR) transcripts (**p* < 0.01). Methods: a TaqMan One Step RT-PCR protocol (Applied Biosystems, Foster City, CA, USA) was used to quantify P1 purinergic receptor mRNA transcripts using “Assays on Demand” Gene Expression Products, coupled with the ABI Prism 7500 sequence detection system (Applied Biosystems). Briefly, total RNA was isolated using the Qiagen RNeasy mini kit according to the manufacturer’s instructions. To prevent possible DNA contamination, the samples were pretreated with RNase-free DNase (Qiagen, Valencia, CA, USA). Sequence-specific primers and probes for human P1 receptors and 18S rRNA were purchased from Assays on Demand (ABI, Foster City, CA, USA). TaqMan One Step PCR Master Mix Reagents Kit (ABI) was used for reverse transcriptase and PCR. The reaction volume was 25 μ l, including 12.5 μ l of 2 \times Master Mix without UNG, 0.625 μ l of 40 \times MultiScribe and RNase Inhibitor Mix, 1.25 μ l of 20 \times target primer and probe, 5.625 μ l of nuclease-free water (Ambion, Austin, TX, USA), and 5 μ l of RNA sample. The reaction plates were covered with an optical cap and centrifuged briefly to remove bubbles. The thermocycler conditions were as follows: Stage 1: 48°C for 30 min; Stage 2: 95°C for 10 min; Stage 3: 95°C for 15 s, repeat 40 cycles, 60°C for 1 min. All experiments were run in triplicate on at least two separate days. Six experiments per condition were performed. The absolute value of the slope of log input amount vs. Δ Ct was >0.1, implying that the efficiencies of AR isoform and 18S rRNA amplification were not equal. Therefore, the relative quantification of transcript levels (compared with endogenous 18S rRNA) was performed using the standard curve method (Li et al. 2006)

activation (Bebok et al. 2005; Clancy et al. 1999; Cobb et al. 2002; Cobb and Clancy 2003; Huang et al. 2001; Li et al. 2006; Tarran et al. 2005, 2006a, b). It is difficult to completely exclude participation of A_{2A}ARs (which also typically signal through G_s, adenylyl cyclase, cAMP and PKA), as low-level CFTR activation in monolayers can be seen at Ado concentrations that may be below the threshold to stimulate A_{2B}ARs. A recent report noted A_{2B}AR transcript levels that were approximately threefold higher than that of A₁ and A_{2A} receptor transcripts in laser-dissected specimens of human lower airway tissue (Rollins et al. 2008). The relative lack of A_{2B}AR-specific blockers also complicates the evaluation of P1 receptor regulation of CFTR.

Elegant studies completed by Huang and Stutts provided evidence for a tightly compartmentalized signaling complex at the airway epithelial surface comprising several proteins, including the A_{2B}AR, adenylate cyclase, A kinase anchoring proteins (AKAPs), PKAII, and CFTR (Huang et al. 2000, 2001). Their work indicated that all of the components necessary to activate CFTR through A_{2B}ARs were available within the boundary of a micropipette tip, and that signaling did not extend beyond this border to other transporters along the cell membrane. Their findings indicate that the coupling between CFTR and A_{2B}ARs is extremely efficient, as A_{2B}ARs can activate regionally localized CFTR without detectable effects on total cell cAMP. This regional regulation of CFTR by A_{2B}ARs underlies the importance of this signaling molecule in CFTR control and airway hydration in the upper and lower airways. This unique and highly compartmentalized signaling is also highlighted in Fig. 3, where cell cAMP levels produced by Ado are far lower than those produced by isoproterenol (Fig. 3a; Iso, a β_2 adrenergic receptor agonist), using concentrations that fully stimulate CFTR and Cl⁻ transport. Ado activates Cl⁻ transport across polarized airway epithelial cells when added to either the apical or basolateral compartment (Fig. 3b), which is sensitive to the relatively selective A_{2B}AR receptor blocker alloxazine. The results confirm that Ado stimulates Cl⁻ transport via A_{2B}ARs present on both the apical and basolateral membrane, and suggest that Ado may also directly regulate basolateral transporters (e.g., K⁺ channels) that promote transepithelial Cl⁻ transport.

Using the nasal potential difference (NPD) measurement (an established bioelectric measure of Na⁺ and CFTR-dependent Cl⁻ transport in the airway (Knowles et al. 1995; Rowe et al. 2007; Standaert et al. 2004)), our laboratory has confirmed that Ado activates CFTR-dependent Cl⁻ transport in the airways of human subjects, with robust responses seen in nonCF subjects that are absent in CF patients with severe, nonfunctional CFTR mutations (Hentchel-Franks et al. 2004). The response to 10 μ M Ado exceeds that produced by 10 μ M Iso (an agonist commonly used to detect CFTR activity in vivo), but the two agonists do not demonstrate additivity. Complementary NPD studies were recently completed in human subjects without CF and reported by Tarran and Rollins, in which Ado-stimulated Cl⁻ transport was inhibited by perfusion with caffeine, a known blocker of adenosine receptors (Rollins et al. 2008). These findings indicate that Ado activates predominantly CFTR-dependent and not CFTR-independent Cl⁻ transport in vivo through stimulation of P1 receptors. Similar Cl⁻ transport results have also been demonstrated in NPDs completed in *Cftr*⁺ mice, while Ado failed to activate Cl⁻ transport in *Cftr*^{-/-} littermates (Cobb et al. 2002). We have also examined the capacity of Ado to stimulate Cl⁻ transport in patients with partial function CFTR mutations, mild disease with surface localized CFTR mutations, and/or nonclassic CF. Figure 4 summarizes NPD results obtained in six subjects with positive sweat tests (>60 mM) and clinical findings of CFTR dysfunction. Three subjects had two identified CFTR mutations, and three had one identified CFTR-causing mutation (demographic and relevant clinical information for these study subjects is summarized in Fig. 4a). Each subject underwent three standard NPD measurements using one of three agonist conditions in a random fashion (Fig. 4b; 10 μ M adenosine

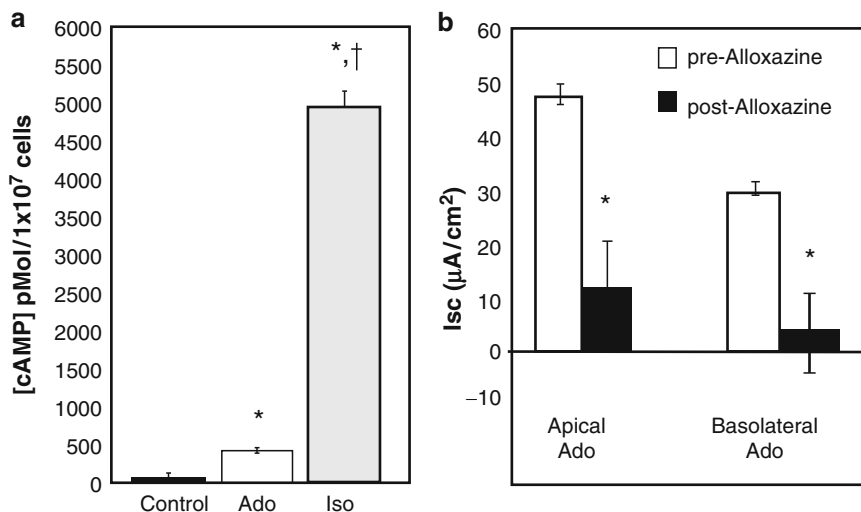


Fig. 3 a–b cAMP and Cl^- transport produced by Ado in airway cells. **a** Calu-3 cells were grown on impermeable supports and stimulated with agonists for 10 min prior to lysis and cAMP measurement by ELISA (Cayman Chemicals, Minneapolis, MN, USA). Cells were stimulated with 10 μM adenosine (Ado) or isoproterenol (Iso) (+100 μM papaverine, a nonspecific PDE inhibitor); * $p < 0.001$ compared with control, † $p < 0.001$ compared with Ado alone ($n = 4$ experiments per condition). **b** Calu-3 cells grown as polarized monolayers were studied in Ussing chambers under voltage clamp conditions. Ado (10 μM) stimulated short circuit current (I_{sc}) when added to either the apical or basolateral compartment that was sensitive to pretreatment with the $\text{A}_{2\text{B}}$ AR blocker alloxazine (20 μM). $n = 6$ experiments studied per condition. Methods: Calu-3 cells (immortalized human serous glandular cells) expressing wtCFTR were seeded on Costar 0.4 μm permeable supports (Bethesda, MD, USA; 5×10^5 cells per filter, 6.5 mm diameter) after coating with fibronectin. Cells were grown to confluence and then placed at an air/liquid interface (48 h) and mounted in modified Ussing chambers (Jim's Instruments, Iowa City, IA, USA), and initially bathed on both sides with identical Ringers solutions containing (in mM) 115 NaCl, 25 NaHCO_3 , 2.4 KH_2PO_4 , 1.24 K_2HPO_4 , 1.2 CaCl_2 , 1.2 MgCl_2 , 10 D-glucose (pH 7.4). Bath solutions were vigorously stirred and gassed with 95% O_2 : 5% CO_2 . Solutions and chambers were maintained at 37°C. Short-circuit current (I_{sc}) measurements were obtained by using an epithelial voltage clamp (University of Iowa Bioengineering, Iowa City, IA, USA). A 3 mV pulse of duration 1 s was imposed every 100 s to monitor resistance, which was calculated using Ohm's law. To measure stimulated I_{sc} , the mucosal bathing solution was changed to a low- Cl^- solution containing (in mM) 1.2 NaCl, 115 Na gluconate, and all other components as above plus 100 μM amiloride. Ado (10 μM) in the presence or absence of alloxazine (20 μM) was added to the apical or basolateral solutions as indicated, and I_{sc} was measured ($\mu\text{A}/\text{cm}^2$; minimum 15 min of observation at each concentration) (Bebok et al. 2005)

(Ado), 10 μM isoproterenol (Iso), or 10 μM Ado and 10 μM Iso included in the fourth perfusate). As expected, the mean polarizing response to Iso (representative of CFTR-dependent Cl^- transport) was minimal, consistent with CFTR dysfunction (as predicted based on genotype, sweat Cl^- values, and/or clinical symptoms). In contrast, inclusion of Ado in the final perfusate produced a polarizing response distinct from that seen with Iso alone. These results, coupled with the previously

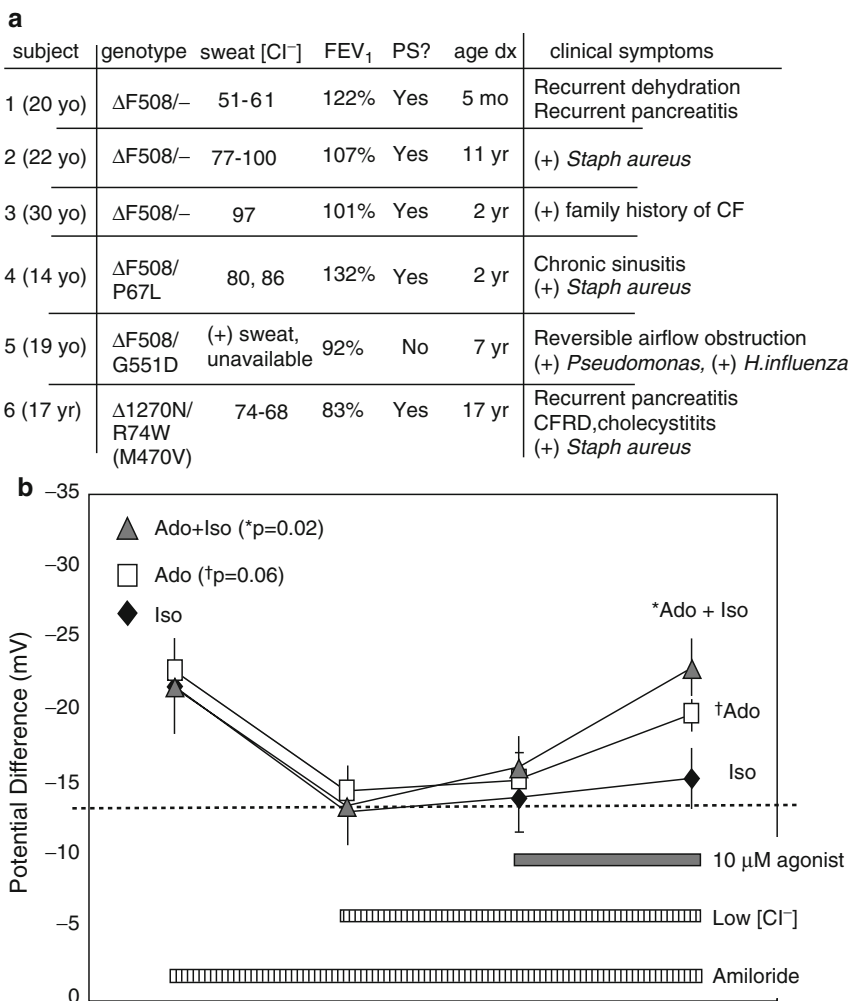


Fig. 4 a–b Ado-stimulated Cl⁻ transport by nasal potential difference (NPD) in subjects with partial CFTR function and/or mild disease manifestations. In **a**, clinical information on the six subjects is summarized. All subjects had mild lung disease based on forced expiratory volume in 1 s (FEV₁), a relatively mild phenotype based on age at diagnosis, 5:6 were pancreatic sufficient (PS), and all demonstrated elevated sweat Cl⁻ values. In **b**, NPD values for the six subjects studied under the three conditions are compared. All subjects underwent three NPDs with varying CFTR agonists in the fourth perfusate (isoproterenol (Iso): 10 μM; adenosine (Ado): 10 μM; or Ado combined with Iso: 10 μM each). Inclusion of Ado in the perfusate produced enhanced Cl⁻ transport compared with Iso alone (*p < 0.01, †p = 0.06). Methods: for these studies, we used a NPD protocol based on that published by Knowles and colleagues (Knowles et al. 1995), and previously described by our laboratory. Subjects underwent three NPD measurements over a two-week period, including 1 min perfusion with Ringers, 3 min perfusion with Ringers + amiloride (100 μM, to block ENaC), 3 min perfusion with low [Cl⁻] solution and amiloride (solution matching Ringers' except that [gluconate] = 115 mM and [Cl⁻] = 6 mM), and 3 min perfusion with low [Cl⁻] solution and amiloride and 10 μM isoproterenol (Iso), 10 μM Ado, or 10 μM Ado and 10 μM Iso. NPD values after the completion of the perfusion conditions are shown using methods as previously described (Hentchel-Franks et al. 2004)

published work from our laboratory and that by Stutts, confirm that CFTR activation by A_{2B} ARs is highly efficient and effective relative to β_2 adrenergic receptor stimulation. The results also raise questions regarding the sensitivity of current NPD protocols to detect partial function CFTR activity (Rowe et al. 2007), and how intracellular signaling pathways connect CFTR activation to A_{2B} ARs. The mechanism appears to be independent of protein:protein interactions mediated by the postsynaptic density protein 95, disk-large tumor suppressor protein, *Zonula occludens* 1 (PDZ) motif binding to ezrin binding protein 50 (EBP50), which has been shown to be operative in β_2 adrenergic receptor regulation of CFTR (Naren et al. 2003).

It is clear that an important function of Ado and related nucleotides in the airway is to regulate transepithelial ion transport and ASL volume, ciliary function, and coordinated mucociliary clearance. Indeed, recent work has demonstrated that Ado is a vital regulator of the minute-to-minute ASL depth, maintaining a volume that optimizes ciliary function via regulation of CFTR. Loss of CFTR function results in a decrease in the ASL volume secondary to unopposed/excessive Na^+ (and fluid) absorption via ENaC. Failure of this system is demonstrated in cystic fibrosis, where the absence of CFTR activity removes the positive contributions of Ado to airway anion and fluid homeostasis. A current model of cystic fibrosis offered by Boucher and colleagues implicates retention of transepithelial Cl^- and fluid flow via ATP, $P2Y_2$ and CaCC signaling that serves to maintain the ASL volume, MCC and cough clearance of lower airway secretions in the absence of CFTR activity (Boucher 2007a; Tarran et al. 2005). Unfortunately, this system eventually falters (possibly as a result of recurrent viral and/or bacterial infections) with the development of the hallmarks of CF airway disease.

3 Repercussions of Altered Adenosine Levels in the Airway

Ado and related nucleotides are ubiquitous signaling molecules that are regulators of a variety of pulmonary and airway processes, including ion transport, bronchial tone, mucus production, and inflammatory signaling. Ado and related nucleotides on the epithelial surface can have phosphate groups added to or removed from their 5' end by surface kinases and 5'-ectonucleotidases (CD73), and the dominant effect is dependent upon the cell type, the balance of kinases/nucleotidases expressed, the nucleotide transport mechanisms at play, the activities of extracellular enzymes such as *ADA1* and 2, and nucleoside transporters (concentrative and equilibrative) (Hirsh et al. 2007; Lazarowski et al. 2004). Paracellular transport of nucleosides does not appear to be of high importance under conditions where the airway epithelial integrity remains intact. In human airways, a dominant pathway of nucleotide metabolism includes the removal of phosphate groups in excess of phosphorylation, leading to the relative production of Ado on the airway surface (Hirsh et al. 2007). These processes generally maintain a short $t_{1/2}$ life of Ado in the ASL, and measurable levels are dependent on continuous and fluctuating production of Ado, nucleotide release and metabolism, and cellular uptake. Nucleotide release

is a normal part of active homeostatic mechanisms that continuously function in the airway, including shear stress produced by the breathing cycle, cough, diffusion down chemical gradients from the intracellular to the extracellular compartment, and nucleotide release following stimulation of intracellular signaling pathways (Donaldson et al. 2000; Tarran et al. 2005, 2006a). Under conditions of stress, Ado and related nucleotides can accumulate to high levels on airway epithelia. Additionally, dead and apoptotic leukocytes, bacteria, and other microorganisms in the airway lumen are potential sources of surface nucleotides, contributing to levels that can be measured in excess of 50 μM in expectorated sputum (from CF patients) *ex vivo* (Li et al. 2006).

The four P1 purinergic receptors are all members of the G-protein-coupled receptor (GPCR) protein superfamily, but differ from one another in several defining features, including G-protein coupling, Ado affinity, and regulation of cell signaling (Cobb and Clancy 2003). For a more detailed discussion of P1 receptor structure, function and pharmacology, the reader is directed to excellent reviews within this publication. A_1 ARs traditionally couple to G_i and inhibit cAMP production, while A_{2A} (high-affinity) and A_{2B} (low-affinity) ARs frequently couple to G_s and raise cell cAMP. The more recently identified A_3 adenosine receptor also inhibits cAMP production and has been linked to a variety of signaling pathways. Recent studies have demonstrated a more diverse signaling repertoire for A_1 , A_{2A} and A_{2B} ARs, and the sum balance of Ado effects *in vivo* are at times difficult to predict (Caruso et al. 2006; Cobb and Clancy 2003; Polosa and Holgate 2006; Russo et al. 2006; Spicuzza et al. 2006). For example, A_1 ARs have a role in Ado-induced bronchoconstriction and proinflammatory processes in animal models, and stimulation of MUC2 expression [a highly insoluble mucin that has been implicated in asthma pathology (McNamara et al. 2004)]. Stimulation of A_{2A} ARs expressed in leukocytes tends to activate a number of anti-inflammatory signaling pathways, while stimulation of A_{2B} ARs tends to favor proinflammatory mediator release, which is independent of its regulation of CFTR and Cl^- conductance (Spicuzza et al. 2006). A_3 AR stimulation has been linked to several proinflammatory processes, including mast cell degranulation, airway responsiveness, and mucus production in animal models. In addition to airway epithelial cells, all P1 receptor isoforms are expressed at varying levels in resident macrophages and granulocytes of the lung, particularly in neutrophils, eosinophils and mast cells. Moreover, nebulized AMP has been shown to be a sensitive and specific agonist for the detection of allergic-based asthma in human subjects, producing reversible airway obstruction specifically in asthmatics but not in subjects with other chronic airway disorders, including chronic obstructive pulmonary disease (COPD) and cystic fibrosis (Caruso et al. 2006; Feoktistov et al. 1998; Polosa et al. 1995; Polosa and Holgate 1997, 2006; Polosa 2002; Russo et al. 2006; Spicuzza et al. 2006). For more detailed discussions regarding ARs, inflammation, and asthma, please refer to separate chapters in this volume, Chap. 8, "Adenosine Receptors and Inflammation" (by Blackburn et al.), and Chap. 11, "Adenosine Receptors and Asthma" (by Wilson et al.).

Ado has been reported to have both pro- and anti-inflammatory signaling properties, and is a significant paracrine regulator of inflammatory processes. An important

series of studies recently reported by Blackburn and colleagues have demonstrated that mice deficient in adenosine deaminase (ADA) are prone to Ado accumulation in many organs, including the lungs (Blackburn et al. 2003; Blackburn 2003; Blackburn and Kellems 2005; Chunn et al. 2001). Elevations in pulmonary Ado levels produced a dramatic proinflammatory phenotype, with influx of neutrophils, and elevations of a variety of inflammatory chemokines, cytokines, and proteases including monocyte chemoattractant proteins (MCP-1, 2, 5), eotaxine, intracellular adhesion molecule 1 (ICAM-1), IL-4, IL-5, IL-13, TNF- α , matrix metalloproteinases 9 and 12, and tissue inhibitor of metalloproteinase 1 (TIMP-1). In addition, extensive mucus production and bronchial plugging in response to Ado can further alter the airway structure. Subsequent studies implicated the A_{2B}AR as the primary receptor subtype responsible for the stimulation of many of these proinflammatory pathways, and highlighted the potential damaging effects of chronic Ado elevation in the lung (Sun et al. 2006). Treatment of ADA-deficient mice with A₃AR antagonists resulted in decrease in mucus production, suggesting that A₃AR signaling plays an important role in the development of Ado-stimulated mucus metaplasia (Young et al. 2004). In this animal model, the end result was chronic pulmonary injury and eventual fibrosis, with increased mortality seen in partially ADA-deficient mice by several months of age. These effects could be ameliorated by cotreatment with ADA, implicating Ado as the causative signaling molecule in this process.

Recently published work from our laboratory also implicates PLA₂ signaling following A_{2B}AR stimulation in airway epithelial cells, leading to the release of arachidonic acid and the accumulation of PGE₂ on the airway surface following stimulation with Ado or exposure to hypoxic conditions (Li et al. 2006). Both arachidonic acid and PGE₂ are effective stimuli of CFTR and transepithelial Cl⁻ transport in airway epithelia, and the EP4 receptor has recently been proposed to mediate CFTR-dependent halide transport produced by isoprostanes in Calu-3 cells (Cowley 2003; Joy and Cowley 2005, 2008; Li et al. 2006). Arachidonic acid is also a potent blocker of CFTR Cl⁻ channels from the cytoplasmic surface (Li et al. 2006; Linsdell 2000). These reports highlight the complex interrelationships between Ado signaling, ion transport and inflammation, and that Ado-stimulated mediators often serve several roles in the host response.

As evidence suggests that excessive Ado levels in the lung have a proinflammatory phenotype, one approach to reducing airway inflammation in conditions where Ado levels are elevated (asthma, COPD) could include inhibition of P1 receptor function. The A_{2B}AR is a logical target based on the work reported by Blackburn and others, and it is known to be expressed in granulocytes that contribute to airway pathology. A recent report by Rollins examined the impact of A_{2B}AR inhibition on ASL volume of human airway cells (Rollins et al. 2008). The results suggest that inhibition of A_{2B}AR activity in airway epithelial cells can lead to depletion of the ASL, producing a phenotype reminiscent of CF. Thus, the development of agents to target Ado-mediated inflammation in lung diseases may be complicated by off-target effects of receptor antagonists on airway hydration and innate defense.

4 Conclusions

ATP and Ado regulate distinct but interrelated Cl^- transport pathways that play important roles in the pathogenesis of cystic fibrosis. Results from several investigators support a model in which Ado is a central molecule in the disease, providing direct and indirect regulation to CFTR, the ASL volume, and inflammation. Evidence suggests that Ado serves a vital role in innate defense as a primary regulator of CFTR and thus a secondary regulator of mucociliary clearance. High levels of Ado appear to be causative of lower airway pathology, but targeting the $\text{A}_{2\text{B}}\text{AR}$ pathway to reduce inflammation is complicated by potential deleterious effects on mucociliary clearance. Continuing to clarify the relative roles of P1 receptor subtypes in airway homeostasis and pathologic responses should provide logical approaches to modulate these pathways to understand and potentially treat a variety of pulmonary diseases.

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Adenosine Receptors in Wound Healing, Fibrosis and Angiogenesis

Igor Feoktistov, Italo Biaggioni, and Bruce N. Cronstein

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Abstract Wound healing and tissue repair are critical processes, and adenosine, released from injured or ischemic tissues, plays an important role in promoting wound healing and tissue repair. Recent studies in genetically manipulated mice demonstrate that adenosine receptors are required for appropriate granulation tissue formation and in adequate wound healing. A_{2A} and A_{2B} adenosine receptors stimulate both of the critical functions in granulation tissue formation (i.e., new matrix production and angiogenesis), and the A₁ adenosine receptor (AR) may also contribute to new vessel formation. The effects of adenosine acting on these receptors is both direct and indirect, as AR activation suppresses antiangiogenic factor production by endothelial cells, promotes endothelial cell proliferation, and stimulates angiogenic factor production by endothelial cells and other cells present in

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the wound. Similarly, adenosine, acting at its receptors, stimulates collagen matrix formation directly. Like many other biological processes, AR-mediated promotion of tissue repair is critical for appropriate wound healing but may also contribute to pathogenic processes. Excessive tissue repair can lead to problems such as scarring and organ fibrosis and adenosine, and its receptors play a role in pathologic fibrosis as well. Here we review the evidence for the involvement of adenosine and its receptors in wound healing, tissue repair and fibrosis.

Keywords Adenosine receptors · Wound healing · Fibrosis · Angiogenesis · Neovascularization

Abbreviations

ADA	Adenosine deaminase
AR	Adenosine receptor
bFGF	Basic fibroblast growth factor
IFN	Interferon
IL	Interleukin
MMP	Matrix metalloproteinase
NECA	Adenosine 5'- <i>N</i> -ethyluronamide
TGF	Transforming growth factor
TNF- α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

1 Introduction

Tissue repair is an essential homeostatic mechanism that involves a series of coordinated and overlapping phases: inflammation, neovascularization, new tissue generation, and tissue reorganization. In acute inflammation, tissue damage is followed by resolution, whereas in chronic inflammation, damage and repair continue concurrently. Inflammatory cells neutralize invading pathogens, remove waste and debris, and promote restoration of normal function, either through resolution or repair. Inflammation also promotes angiogenesis and vasculogenesis, the formation of new blood vessels, which in turn may enhance the recruitment of inflammatory cells and the subsequent laying down of extracellular matrix to repair tissue damage. Although usually beneficial to the organism, inflammation may lead to tissue damage, resulting in escalation of chronic inflammation. Furthermore, aberrant or inadequate repair can lead to excessive and poorly ordered matrix deposition and fibrosis, which affects normal tissue architecture and can ultimately disable the proper functioning of organs. Like matrix generation, overly exuberant vessel formation may lead to medical problems as well, and diabetic retinopathy and macular degeneration are examples of this phenomenon.

Extracellular accumulation of adenosine in response to tissue damage is an important event in the control of all aspects of tissue repair. The nature of adenosine's action depends on the magnitude of changes in extracellular adenosine concentrations as well as on the identity and expression levels of each adenosine receptor subtype on individual cell types. The role of adenosine in the regulation of inflammation is extensively covered in other chapters of this book. In this chapter, we will discuss the roles of specific adenosine receptors in the regulation of neovascularization and fibrosis in different organs and tissues.

2 Role of Adenosine in Neovascularization

Accumulating evidence indicates that adenosine is an important regulator of neovascularization, including angiogenesis and vasculogenesis. Stimulation of new blood vessel formation by adenosine was demonstrated in the chick chorioallantoic membrane and embryo (Adair et al. 1989; Dusseau et al. 1986; Dusseau and Hutchins 1988), the mouse retina (Afzal et al. 2003; Mino et al. 2001), and the optical tectum of *Xenopus leavis* tadpoles (Jen and Rovainen 1994). Adenosine reportedly modulates a number of steps involved in angiogenesis, including endothelial cell proliferation (Dubey et al. 2002; Ethier et al. 1993; Grant et al. 1999, 2001; Meininger et al. 1988; Meininger and Granger 1990; Van Daele et al. 1992), migration (Dubey et al. 2002; Grant et al. 2001; Luty et al. 1998; Meininger et al. 1988; Teuscher and Weidlich 1985), and tube formation (Grant et al. 2001; Luty et al. 1998). Adenosine has been also suggested to play an important role in adult vasculogenesis by directing the homing of endothelial progenitor cells to the site of tissue injury (Montesinos et al. 2004; Ryzhov et al. 2008b).

Adenosine has direct mitogenic effects on vascular cells that may contribute to angiogenesis (Ethier and Dobson Jr. 1997; Meininger et al. 1988; Sexl et al. 1995; Van Daele et al. 1992). However, the main proangiogenic actions of adenosine have been attributed to its ability to regulate the production of pro- and antiangiogenic substances. Adenosine modulates the release of angiogenic factors from various cells and tissues (Feoktistov et al. 2003, 2004; Gu et al. 1999, 2000; Hashimoto et al. 1994; Leibovich et al. 2002; Olah and Roudabush 2000; Pueyo et al. 1998; Takagi et al. 1996; Wakai et al. 2001; Zeng et al. 2003), thus regulating capillary growth in a paracrine fashion. In addition, adenosine can modulate release of angiogenic factors from endothelial cells (Desai et al. 2005; Feoktistov et al. 2002; Fischer et al. 1995, 1997; Grant et al. 1999; Khoa et al. 2003; Takagi et al. 1996), which may regulate capillary growth in an autocrine fashion.

All four adenosine receptor (AR) subtypes have been implicated in the regulation of neovascularization. In a similar manner to our early observation that the stimulation of A₁ARs on neutrophils increased their adherence to vascular endothelium (Cronstein et al. 1992), we have recently demonstrated that A₁ARs located on embryonic endothelial progenitor cells promote their adhesion to cardiac microvascular endothelial cells, suggesting an important role of this receptor subtype

in vasculogenesis (Ryzhov et al. 2008b). A₁ARs have been also reported to upregulate vascular endothelial growth factor (VEGF) production from monocytes, thus promoting angiogenesis in an in vitro model (Clark et al. 2007). Among all of the AR subtypes, A₁ARs have the highest affinity to adenosine (Fredholm et al. 2001). It is possible, therefore, that engagement of the high-affinity A₁ARs is especially important for circulating cells moving toward a gradient of adenosine concentrations generated by tissue injury and/or hypoxia, whereas the lower-affinity A₂ARs are more important for the regulation of cells located in the vicinity of the injured or ischemic loci, where concentrations of adenosine are the highest.

Indeed, both A₂AR subtypes, A_{2A} and A_{2B}ARs, have been implicated in regulation of angiogenesis and vasculogenesis. Depending on tissue or cell studied, either one of these receptor subtypes can take the lead and play a dominant role in the regulation of angiogenic factors. For example, A_{2B}ARs upregulate the proangiogenic factors VEGF, basic fibroblast growth factor (bFGF), insulin-like factor 1, and IL-8 in human microvascular endothelial cells (Feoktistov et al. 2002; Grant et al. 1999). Conversely, A_{2A}ARs were reported to upregulate VEGF in macrophages (Leibovich et al. 2002; Pinhal-Enfield et al. 2003). However, A_{2B}ARs may also contribute to regulation of VEGF in these cells, since genetic deletion of A_{2B}ARs significantly decreased adenosine-dependent secretion of VEGF in mouse peritoneal macrophages (our unpublished observations). In addition, the stimulation of A₃ARs in mast cells and some tumors can result in the upregulation of certain proangiogenic factors, complementing the actions of adenosine mediated via A_{2B}ARs (Feoktistov et al. 2003; Merighi et al. 2005, 2007). Thus, the contribution of adenosine to the regulation of neovascularization can be dictated by the expression profile of AR subtypes and by the intracellular machinery to which they are coupled in specific cell types. Furthermore, the expressions of AR subtypes and their functions are subject to dynamic regulation by conditions present during inflammation, such as hypoxia and cytokine exposure (Bshesh et al. 2002; Eltzschig et al. 2003; Feoktistov et al. 2004; Khoa et al. 2003). Because the A_{2B}AR promoter contains a functional binding site for hypoxia-inducible factor (Kong et al. 2006), the onset of hypoxia strongly induces A_{2B}AR expression. Hypoxia-induced upregulation of A_{2B}ARs has been reported in human tumor cells (Zeng et al. 2003), rat hippocampus (Zhou et al. 2004), and human dermal microvascular endothelial cells (Eltzschig et al. 2003). This may have important functional implications for regulation of angiogenesis. For example, in human bronchial smooth muscle cells and human umbilical vein endothelial cells, adenosine does not stimulate VEGF secretion under normoxic conditions, but hypoxia increases expression of A_{2B}ARs, which are then able to stimulate VEGF release (Feoktistov et al. 2004). Similarly, treatment of human dermal microvascular endothelial cells with interferon (IFN)- γ increases A_{2B}AR expression but decreases A_{2A}AR levels. In contrast, other proinflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor alpha (TNF- α) increase both A_{2A} and A_{2B} AR expression and function (Khoa et al. 2003). Because the expression and function of adenosine receptor subtypes may differ depending on the tissue and the nature of the tissue injury, we will next examine the role of AR subtypes in specific organs and pathological states.

2.1 Regulation of Neovascularization in the Skin

We have previously reported (Montesinos et al. 2002) that mice with genetically disrupted A_{2A} ARs form significantly fewer microvessels in healing wounds and in response to mechanical trauma by the formation of an air pouch (Montesinos et al. 2002). Furthermore, application of an A_{2A} AR agonist to wounds increases microvessel formation from both pre-existing endothelial cells and bone marrow-derived endothelial progenitors as compared to vehicle-treated mice, observations that provide the first in vivo evidence that A_{2A} AR occupancy promotes angiogenesis and vasculogenesis (Montesinos et al. 2002, 2004). Further studies indicate that the angiogenic effects of A_{2A} AR occupancy are mediated both directly on endothelial cells (increased endothelial cell migration and microvascular endothelial cell VEGF production; Khoa et al. 2003; Montesinos et al. 1997) and indirectly via promotion of VEGF production by macrophages (Leibovich et al. 2002). Desai and colleagues (Desai et al. 2005) have also reported evidence to indicate that A_{2A} AR occupancy suppresses the production of thrombospondin I, a potent inhibitor of angiogenesis, and this inhibition is responsible for enhanced vascular tube formation in vitro. Thus, there is growing evidence that A_{2A} ARs play an important role in skin neovascularization, and particularly during wound healing.

2.2 Regulation of Neovascularization in the Heart and Skeletal Muscles

Many studies have demonstrated that chronic elevation of tissue adenosine concentrations induced by the adenosine reuptake blocker dipyridamole (Adolfsson et al. 1981, 1982; Adolfsson 1986a, b; Belardinelli et al. 2001; Mall et al. 1987; Mattfeldt and Mall 1983; Symons et al. 1993; Tornling et al. 1978, 1980a, b; Tornling 1982a, b; Torry et al. 1992), or long-term administration of adenosine and its analogs (Hudlicka et al. 1986; Wothe et al. 2002; Ziada et al. 1984), promotes capillary proliferation in the heart and skeletal muscles. Antagonism of ARs with caffeine abrogated VEGF upregulation in skeletal muscles induced by local injection of adenosine 5'-*N*-ethyluronamide (NECA) into the mouse hind limb and produced a 46% reduction in neovascularization in a mouse ischemic hind limb model (Ryzhov et al. 2007). In the isolated heart model, adenosine but not selective A_{2A} or A_3 AR agonists increased retention of embryonic endothelial progenitors to microvascular endothelium, suggesting that A_1 and A_{2B} ARs may play an important role in the initial phase of vasculogenesis, promoting homing of endothelial progenitor cells to the site of ischemic injury (Ryzhov et al. 2008b). Indeed, endothelial progenitor cells and cardiac microvascular endothelial cells preferentially express functional A_1 and A_{2B} ARs, respectively, and both subtypes are involved in the regulation of the adhesion of endothelial progenitors to microvascular endothelial cells in the heart. Moreover, the interaction between P-selectin and its ligand PSGL-1 plays

an important role in these process, and stimulation of A_{2B} ARs in cardiac microvascular endothelial cells induces rapid cell surface expression of P-selectin (Ryzhov et al. 2008b). These findings suggested a role for A_1 and A_{2B} ARs in myocardial vasculogenesis, and provided a rationale for the potential use of adenosine to stimulate engraftment in cell-based therapies.

2.3 Regulation of Neovascularization in the Lung

Angiogenesis is a feature of chronic lung diseases such as asthma and pulmonary fibrosis. Studies in adenosine deaminase (ADA)-deficient mice, characterized by elevated lung tissue levels of adenosine, strongly suggest a causal association between adenosine and an inflammatory phenotype (Blackburn et al. 2000; Blackburn 2003). These mice exhibit a lung phenotype with features of lung inflammation, bronchial hyperresponsiveness, enhanced mucus secretion, increased IgE synthesis, and elevated levels of proinflammatory cytokines and angiogenic factors that could be reversed by lowering adenosine levels with exogenous ADA (Blackburn et al. 2000). In particular, levels of the angiogenic chemokine CXCL1 (mouse functional homolog of human IL-8) are significantly elevated in an adenosine-dependent manner in the lungs of ADA-deficient mice, leading to substantial angiogenesis in the tracheas (Mohsenin et al. 2007a). The A_{2B} AR subtype appears to play an important role in this model, because pharmacological inhibition of A_{2B} ARs significantly reduced elevations in proinflammatory cytokines as well as mediators of airway remodeling induced by high adenosine levels in the lungs of ADA-deficient mice (Sun et al. 2006). In contrast, genetic removal of the A_{2A} AR enhances pulmonary inflammation, mucin production, and angiogenesis in ADA-deficient mice (Mohsenin et al. 2007b).

2.4 Regulation of Neovascularization in Tumors

Metabolically active solid tumors grow rapidly and routinely experience severe hypoxia and necrosis, which causes adenine nucleotide degradation and adenosine release. Expression of A_{2B} ARs was documented in various cancerous cells (Feoktistov and Biaggioni 1993, 1995; Panjehpour et al. 2005; Phelps et al. 2006; Rodrigues et al. 2007; Zeng et al. 2003), and analysis of gene expression in primary human tumors uncovered overexpression of A_{2B} ARs, suggesting their potential role in cancer biology (Li et al. 2005). Studies from different laboratories demonstrate that stimulation of A_{2B} ARs in cancer cell lines upregulates the production of angiogenic factors, suggesting that tumor A_{2B} ARs may promote neovascularization (Feoktistov et al. 2003; Merighi et al. 2007; Zeng et al. 2003). A_3 ARs expressed in some tumor cell lines may also complement these A_{2B} AR-mediated effects by upregulating other proangiogenic factors (Feoktistov et al. 2003; Merighi et al. 2005,

2007). In addition, host tumor-infiltrating immune cells can also play an important role in tumor angiogenesis, since Lewis lung carcinoma isografts in A_{2B} AR knockout mice contained lower VEGF levels and exhibited lower vessel density compared to tumors grafted in wild-type mice (Ryzhov et al. 2008a). Furthermore, treatment with A_{2A}/A_{2B} AR antagonists inhibited neovascularization of CL8-1 melanoma in mice (Ohta et al. 2006). Thus, there is growing evidence that adenosine acting via A_{2B} and possibly A_3 or A_{2A} ARs can promote tumor neovascularization. Involvement of different AR subtypes in the regulation of neovascularization is not surprising due to the multifaceted mechanism of blood vessel development.

3 Role of Adenosine in Fibrosis

3.1 A_{2A} Adenosine Receptor Agonists Promote Wound Healing

Recent reports indicate that topical application of an A_{2A} AR agonist increases the rate at which wounds close (Montesinos et al. 1997). That A_{2A} ARs were involved in this pharmacologic effect was demonstrated by the observation that a specific A_{2A} AR antagonist, but not antagonists at other ARs, reversed the effect of the selective A_{2A} AR agonist CGS21680 on wound healing. Treatment of wounds with this AR agonist promoted fibroblast migration in vitro, and in the AR agonist-treated mice there was an increase in matrix and fibroblast infiltration into the wounds (Montesinos et al. 1997). More recent studies demonstrate that a more highly selective A_{2A} AR agonist, sonedenoson, is a more potent promoter of wound healing than recombinant platelet derived growth factor (becaplermin) (Victor-Vega et al. 2002). The role of A_{2A} ARs in the promotion of wound healing was more fully confirmed by the observation that a selective A_{2A} AR agonist promotes wound healing in wild-type but not A_{2A} AR knockout mice (Montesinos et al. 2002; Victor-Vega et al. 2002). In these studies, there was a marked increase in the number of blood vessels in the healing wounds of wild-type mice treated with the A_{2A} AR agonist as compared to untreated controls. Absence of A_{2A} ARs was associated with disorganized granulation tissue although re-epithelialization was not delayed in the knockout mice. In contrast to this study, Sun and colleagues observed that N^6 -cyclopentyladenosine, a relatively selective A_1 AR agonist, promotes wound healing (Sun et al. 1999). In this study, there was no confirmation that the high concentrations of the agonist used were indeed selective for A_1 ARs or whether the phenomenon could be mediated by A_{2A} ARs. These findings indicate that A_{2A} ARs stimulate wound healing by modulating inflammatory cell, endothelial cell and fibroblast functions that promote wound healing. A topical A_{2A} AR agonist, sonedenoson, is currently undergoing testing in Phase II clinical trials for the treatment of diabetic foot ulcers.

3.2 *A_{2A} Adenosine Receptor Occupancy Stimulates Fibroblast Matrix Production*

Replacement of the collagenous matrix of the skin and other tissues is an integral part of wound healing. Once the debris and destroyed matrix at the site of injury are eliminated, fibroblasts lay down a new matrix. This matrix may be remodeled over a longer period of time and the wound develops the characteristic appearance of a scar. A_{2A}AR occupancy stimulates fibroblasts to synthesize type I and III collagen at an increased level, similar to that induced by the growth factor transforming growth factor (TGF)- β , and downregulates matrix metalloproteinase (MMP) 9 but not MMP2 (Chan et al. 2006a).

The observation that adenosine, acting at A_{2A}ARs, stimulates the formation of matrix suggests the possibility that adenosine A_{2A}ARs play a role in fibrosing conditions and scarring, a hypothesis confirmed by *in vivo* experiments. Animals lacking A_{2A}ARs or treated with an A_{2A}AR antagonist were protected from developing diffuse dermal fibrosis in response to bleomycin (Chan et al. 2006a). The role of A_{2A}ARs in fibrosis in tissues outside of the skin is less clear. Prior studies have demonstrated that A_{2B}ARs regulate production of collagen in pulmonary and cardiac fibroblasts (Chen et al. 2004; Dubey et al. 2000, 2001), but other studies have demonstrated that A_{2A}ARs regulate collagen I and III production by hepatic stellate cells (Che et al. 2007), the fibroblasts of the liver, and A_{2A}AR knockout mice are protected from developing hepatic fibrosis following treatment with either CCl₄ or thioacetamide (Chan et al. 2006b). These observations help to explain the protection against death from liver disease provided by coffee drinking (Corrao et al. 1994, 2001; Gallus et al. 2002; Klatsky et al. 1993, 2006; Klatsky and Armstrong 1992; Ruhl et al. 2005; Sharp et al. 1999; Tverdal and Skurtveit 2003), since caffeine is a relatively weak and nonselective AR antagonist which offers some protection (although not complete) from the development of hepatic fibrosis in murine models (Chan et al. 2006b).

In a murine model of diffuse dermal fibrosis resembling scleroderma, we have also found that A_{2A}ARs play a central role in the development of fibrosis. A_{2A}ARs are present on human dermal fibroblasts and, when occupied, regulate collagen production by these cells (Chan et al. 2006a). Mice treated with subcutaneous bleomycin develop diffuse dermal fibrosis and we found that both A_{2A}AR knockout mice and mice treated with a selective A_{2A}AR antagonist were protected from the development of bleomycin-induced dermal fibrosis (Chan et al. 2006a). These results are consistent with the hypothesis that A_{2A}ARs play a role in organ and tissue fibrosis and that blockade or elimination of these receptors can prevent fibrosis.

Recently published indirect evidence provides further support for a role for adenosine and its receptors in dermal fibrosis. Imiquimod is an immune modulator that promotes a shift from Th2- to Th1-type immune responses (reviewed in Schon and Schon 2007) by mechanisms that have not been fully evaluated. Studies in inflammatory cells indicate that imiquimod, at pharmacologically relevant concentrations, is an A_{2A}AR antagonist, and that this may account for its

immunological effects (Schon et al. 2006). Imiquimod, applied topically, has been used to treat morphea, a skin disease characterized by localized fibrosis, and its use has been advocated for the treatment of Dupuytren's contracture, another fibrosing disease (Dytoc et al. 2005; Man and Dytoc 2004; Namazi 2006; Schon et al. 2006). While intriguing (and supporting the clinical relevance of this work), we do realize the anecdotal nature of these reports.

3.3 A_{2B} Adenosine Receptor Occupancy Regulates Fibroblast Collagen Production and Fibrosis

As described above, a number of recent studies have demonstrated that cardiac and pulmonary fibroblasts express A_{2B}ARs that regulate their production of collagen (Chen et al. 2004; Dubey et al. 1997, 1998; Zhong et al. 2005). Stimulation of A_{2B}ARs in cardiac fibroblasts inhibited their proliferation, protein synthesis and collagen production (Chen et al. 2004; Dubey et al. 1997, 1998). Furthermore, it has been demonstrated in vivo that long-term stimulation of A_{2B}ARs after myocardial infarction prevents cardiac remodeling (Wakeno et al. 2006). In contrast, studies in ADA-deficient mice indicate that these animals develop pulmonary inflammation and pulmonary fibrosis that appear to be mediated by A_{2B}ARs (Sun et al. 2006), thus suggesting a role for A_{2B}ARs in pulmonary fibrosis. Based on these studies and the results described above, it is reasonable to conclude that adenosine can either inhibit (heart) or stimulate (skin, liver, lungs) fibrosis, and that adenosine-regulated fibrosis is mediated by different receptors depending on which organ is studied (skin and liver vs. heart and lungs).

3.4 A₁ Adenosine Receptors Play a Role in Cardiac and Vascular Fibrosis

Recently, Kalk and coworkers reported that SLV320 (Solvay Pharmaceuticals), a highly selective A₁AR antagonist, reduced myocardial fibrosis in a model of uremic cardiomyopathy (Kalk et al. 2007). In this model, partially (5/6) nephrectomized rats were treated with SLV320 or vehicle and myocardial fibrosis was markedly reduced, as was albuminuria, without any change in blood pressure or other factors that might have accounted for the change. Another problem associated with fibrosis and abnormal "wound" healing that may be mediated by A₁ARs is intimal hyperplasia and stenosis following stent placement, and recent studies suggest that an A₁AR antagonist diminishes both intimal hyperplasia and smooth muscle proliferation in a model of stent stenosis (Edwards et al. 2008) Thus, A₁ARs may also play a role in fibrosis, although their role seems to be confined to the cardiovascular system.

4 Conclusion

Adenosine and its receptors play important roles in both matrix production and neovascularization, processes that are critical for wound healing and tissue repair. Moreover, adenosine and its receptors play a direct role in stimulating fibrosis in the skin, lungs and liver, but inhibiting fibrosis in the heart. Adenosine and its receptors may also play an important role in physiologic and pathologic angiogenesis. Targeting of ARs to promote wound healing and neovascularization of ischemic tissues or to diminish pathologic fibrosis and angiogenesis is currently underway.

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Adenosine Receptors and Cancer

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Abstract The A₁, A_{2A}, A_{2B} and A₃ G-protein-coupled cell surface adenosine receptors (ARs) are found to be upregulated in various tumor cells. Activation of the receptors by specific ligands, agonists or antagonists, modulates tumor growth via a range of signaling pathways. The A₁AR was found to play a role in preventing the development of glioblastomas. This antitumor effect of the A₁AR is mediated via tumor-associated microglial cells. Activation of the A_{2A}AR results in inhibition of the immune response to tumors via suppression of T regulatory cell function and inhibition of natural killer cell cytotoxicity and tumor-specific CD4⁺/CD8⁺ activity. Therefore, it is suggested that pharmacological inhibition of A_{2A}AR activation

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by specific antagonists may enhance immunotherapeutics in cancer therapy. Activation of the A_{2B}AR plays a role in the development of tumors via upregulation of the expression levels of angiogenic factors in microvascular endothelial cells. In contrast, it was evident that activation of A_{2B}AR results in inhibition of ERK1/2 phosphorylation and MAP kinase activity, which are involved in tumor cell growth signals. Finally, A₃AR was found to be highly expressed in tumor cells and tissues while low expression levels were noted in normal cells or adjacent tissue. Receptor expression in the tumor tissues was directly correlated to disease severity. The high receptor expression in the tumors was attributed to overexpression of NF-κB, known to act as an A₃AR transcription factor. Interestingly, high A₃AR expression levels were found in peripheral blood mononuclear cells (PBMCs) derived from tumor-bearing animals and cancer patients, reflecting receptor status in the tumors. A₃AR agonists were found to induce tumor growth inhibition, both *in vitro* and *in vivo*, via modulation of the Wnt and the NF-κB signaling pathways. Taken together, A₃ARs that are abundantly expressed in tumor cells may be targeted by specific A₃AR agonists, leading to tumor growth inhibition. The unique characteristics of these A₃AR agonists make them attractive as drug candidates.

Keywords A₁ adenosine receptor · A_{2A} adenosine receptor · A_{2B} adenosine receptor · A₃ adenosine receptor · Expression · Tumor growth · Agonists · Antagonists

Abbreviations

A ₁ AR	A ₁ adenosine receptor
A _{2A} AR	A _{2A} adenosine receptor
A _{2B} AR	A _{2B} adenosine receptor
A ₃ AR	A ₃ adenosine receptor
APCs	Antigen-presenting cells
AR	Adenosine receptor
bFGF	Basic fibroblast growth factor
CCPA	2-Chloro- <i>N</i> ⁶ -cyclopentyladenosine
CD39	Cluster of differentiation 39
CD73	Cluster of differentiation 73
GGAP	Cancer Genome Anatomy Project
CGS21680	2- <i>p</i> -(2-Carboxyethyl)phenethylamino-5'- <i>N</i> -ethylcarboxamidoadenosine 1680
CHO	Chinese hamster ovary cells
Cl-IB-MECA	2-Chloro- <i>N</i> ⁶ -3-iodobenzyladenosine-5'- <i>N</i> -methyluronamide
CNS	Central nervous system
CPA	<i>N</i> ⁶ -Cyclopentyladenosine
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CTLs	Cytotoxic T lymphocytes

DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
EGF	Epidermal growth factor
Epac	Exchange protein activated by cAMP
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
G-CSF	Granulocyte colony stimulating factor
GPCR	G-protein-coupled receptor
GSK-3 β	Glycogen synthase kinase 3 β
HA	Hyaluronan
HCC	Hepatocellular carcinoma
HIF-1	Hypoxia-inducible factor 1
HMG1b	High mobility group 1b
HUGO	Human Genome Organization
IB-MECA	Methyl 1-[<i>N</i> ⁶ -(3-iodobenzyl)-adenin-9-yl]- β -D-ribofuronamid
IKK	I κ B kinase
IL	Interleukin
Lef/Tcf	Lymphoid enhancer factor/T-cell factor
MAP	Mitogen-activated protein
MMP	Metalloproteinase
MRS1191	3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(\pm)-dihydropyridine-3,5-dicarboxylate
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan thiazolyl
NECA	Adenosine-5'- <i>N</i> -ethyluronamide
NF- κ B	Nuclear factor kappa B
NK	Natural killers
PAMPs	Pathogen-associated molecular patterns
PARP	Poly(ADP-ribose) polymerase
PBMCs	Peripheral blood mononuclear cells
PDTC	Pyrrolidine dithiocarbamate
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKB/Akt	Protein kinase B/Akt
PLC	Phospholipase C
PLD	Phospholipase D
TCR	T-cell receptor
TGF- β	Transforming growth factor β
thio-Cl-IB-MECA	2-Chloro- <i>N</i> ⁶ -(3-iodobenzyl)-4'-thioadenosine-5'- <i>N</i> -methyluronamide
TNF- α	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
Wt	Wild type

1 Introduction

During the last decade different approaches to treating cancer have been developed based mainly on specific targets that are mostly expressed in tumor but not in normal cells. Furthermore, it is now recognized that individualizing therapy for patients being treated with anticancer agents is an important goal, leading to the prediction of agents that will be efficacious. Adenosine is a purine nucleoside found within the interstitial fluid of tumors at concentrations that are able to modulate tumor growth by interacting with four G-protein-coupled adenosine receptor (AR) subtypes, designated A₁, A_{2A}, A_{2B} and A₃. Selective agonists and antagonists are now available for all four AR subtypes, enabling the examination of these ligands as immunomodulators and anticancer agents. Interestingly, AR levels in various tumor cells are upregulated, a finding which may suggest that the specific AR may serve as a biological marker and as a target for specific ligands leading to cell growth inhibition.

In this chapter, we will present the role played by each of the ARs in mediating tumor growth. Since immune cells such as lymphocytes, macrophages and natural killer (NK) cells were also found to express ARs, their ability to act as cytotoxic cells against tumor cells or to be involved in the antitumor process will be discussed as well. Based on these studies, possible drug candidates (anticancer agents that target ARs) will be presented.

2 A₁ Adenosine Receptor

The A₁AR is a G-protein-coupled receptor that mediates many of the physiological effects of adenosine in the brain. The binding of agonists to A₁AR induces inhibition of adenylate cyclase, leading to a decrease in intracellular cAMP levels or stimulation of phospholipase C (PLC). The A₁AR has a high affinity for adenosine and has been implicated in both pro- and anti-inflammatory aspects of disease processes. On the one hand, A₁AR signaling can promote neutrophil (Salmon and Cronstein 1990) and monocyte activation (Merrill et al. 1997; Salmon et al. 1993); on the other hand, A₁AR signaling is involved in anti-inflammatory and protective pathways in neuroinflammation and injury (Tsutsui et al. 2004), and in cardiac and renal injury (Liao et al. 2003; Lee et al. 2004a, b). Adenosine-mediated anti-inflammatory effects have been studied extensively in macrophages and macrophage cell lines. Adenosine inhibits the production of several proinflammatory cytokines (TNF- α , IL-6, and IL-8) by LPS-stimulated macrophages and enhances the release of the anti-inflammatory cytokine IL-10 (Hasko et al. 1996; Le Moine et al. 1996; Sajjadi et al. 1996). Recent studies suggest an anti-inflammatory role for chronic A₁AR activation by high levels of adenosine in the lung, a surprising and important finding in light of the fact that A₁AR antagonists are being investigated as a potential treatment for asthma (Sun et al. 2005). In the CNS, the A₁AR is highly expressed on microglia/macrophages and neurons (Johnston et al. 2001). In the latter, A₁AR is coupled to activation of K⁺ channels (Trussell and Jackson 1985) and

inhibition of Ca^{2+} channels (MacDonald et al. 1986), both of which are mechanisms that attenuate neuronal excitability, thereby reducing excitotoxicity, and so adenosine can act as a neuroprotective factor. Since A_1ARs are expressed throughout the brain (Dunwiddie 1985), adenosine has the potential to be involved in different brain pathologies. Although A_1ARs may play an important role in some physiological functions in the brain (e.g., sleep), A_1AR -deficient mice show no obvious abnormal behavior, levels of alertness, or appearance of focal neurological deficits, such as seizures (Synowitz et al. 2006). However, upon exposure to pathophysiological conditions like hypoxia, A_1AR -deficient mice show more neuronal damage and have a lower survival rate (Johansson et al. 2001). It was therefore concluded that, in the brain, A_1ARs are primarily important in mediating effects of adenosine during pathophysiological conditions (Gimenez-Llort et al. 2002; Johansson et al. 2001).

It has recently been reported that the deletion of functional ARs, specifically A_1AR , results in an increase in brain tumor growth, specifically glioblastoma tumor growth (Synowitz et al. 2006). This implies that adenosine acting via A_1AR impairs glioblastoma growth. In the context of glioblastoma, A_1ARs are prominently expressed by the tumor cells and those microglial cells associated with the glioblastoma tumor cells. In an experimental approach using an A_1AR -deficient mouse as a tumor host, the importance of the microglial cells for mediating the A_1AR anti-cancer effect is highlighted (Synowitz et al. 2006). In these studies, A_1AR -deficient mice and their wild-type littermate controls are inoculated with GI261 tumor cells; thus, with this approach, the A_1AR is deleted in host cells but not tumor cells. In the control wild-type littermates the microglial cells accumulated at the tumor site, and this accumulation was even more pronounced in the A_1AR -deficient mice. However, tumor volume was significantly greater in A_1AR -deficient mice, suggesting that the microglial cells are the cellular candidates for inhibiting tumor growth. The importance of microglial A_1AR is further supported by a brain slice model where inhibition of tumor growth is only observed in the presence of microglial cells. To test the functional effect of A_1AR activity on glioblastoma growth, an organotypical brain slice model was employed where glioblastoma cells could be injected and ARs could be stimulated or inhibited (Synowitz et al. 2006). Brain slices (250 μm thick) were cultured for four days and 10^4 GFP-labeled GI261 tumor cells were injected (suspended in 0.1 μL) into the tissue. The tumor size was evaluated by measuring the area occupied by the fluorescently labeled GI261 cells. In these studies, adenosine and an A_1AR agonist, N^6 -cyclopentyladenosine (CPA) significantly decreased tumor size. To determine if this effect of adenosine or activation of A_1ARs depends on the presence of microglia, endogenous microglia were selectively depleted from cultured organotypical brain slices by a 24 h treatment with clodronate-filled liposomes without affecting other cell types (e.g., neurons, oligodendrocytes, and astrocytes). As reported previously, activated microglia supported glioblastoma tumor growth, resulting in significantly smaller tumors in microglia-depleted slices compared with control slices. This serves as an internal control and thus confirms the observation that the presence of microglial cells per se is tumor promoting (Markovic et al. 2005). There was no significant change in the population of astrocytes or neural progenitor cells. The latter is of particular interest,

since it was recently reported that neural progenitor cells are attracted to tumors or to gliomas and attenuate tumor growth (Glass et al. 2005). In these organotypical brain slice studies, tumor cells were injected three days after liposome treatment, and the size of the tumor bulk was evaluated with and without microglia. In these studies, activation of A₁ARs with adenosine or CPA resulted in a larger tumor size in brain slices devoid of microglia. Moreover, as expected, the tumor size was greater in brain slices from A₁AR-deficient mice versus their littermate wild-type controls. Furthermore, in these studies, adenosine or CPA had no effect on tumor growth in brain slices from A₁AR-deficient mice. Taken together, the *in vivo* studies in A₁AR-deficient mice and *in vitro* studies in organotypical brain slices suggest that CPA and adenosine specifically act on A₁ARs on microglial cells to reduce tumor size.

The presence of ARs has been previously reported on astrocytoma cells (Prinz and Hanisch 1999) using an A₁AR-specific ligand. The presence of ARs on microglia is well established, and some functional implications of their activation have become apparent (Burnstock 2006; Farber and Kettenmann 2006). Cultured rat microglial cells express A_{2A}ARs, since the specific A_{2A}AR agonist CGS21680 triggers the expression of K⁺ channels that are linked to microglial activation (Kust et al. 1999). In contrast, A_{2A}AR stimulation in rat microglia triggers the expression of nerve growth factor and its release, thereby exerting a neuroprotective effect (Heese et al. 1997). Moreover, cyclooxygenase-2 expression in rat microglia is induced by A_{2A}ARs, resulting in the release of prostaglandin (Fiebich et al. 1996). Hammarberg et al. provided evidence for functional A₃ARs in mouse microglial cells while A₁ARs were not detected in this study (Hammarberg et al. 2003). However, other studies, based on immunocytochemical data, indicate that microglial cells express A₁ARs and that the presence of tumor cells upregulates the expression of A₁ARs in microglia (Synowitz et al. 2006). Moreover, the results of these studies indicate that loss of A₁AR leads to an increase of tumor size associated with microglia, which may be due to infiltration and/or proliferation.

The potential source of extracellular adenosine in the brain is most likely ATP, which is released from presynaptic and postsynaptic terminals of neurons and also from glial cells (Fields and Burnstock 2006). In the extracellular space, adenosine is generated from ATP after dephosphorylation by specific ectoenzymes (e.g., cluster of differentiation 39 (CD39) and cluster of differentiation 73 (CD73)). These ectoenzymes represent a highly organized enzymatic cascade for the regulation of nucleotide-mediated signaling. They control the rate of nucleotide (ATP) degradation and nucleoside (adenosine) formation (Farber et al. 2008; Plesner 1995). Microglial cells express specific ectonucleotidase isoforms, CD39 and CD73, which are not expressed by any other cell type in the brain. Due to this specific expression, both molecules served as microglia-specific markers long before their functional importance was recognized (Braun et al. 2000; Schnitzer 1989; Schoen et al. 1992).

The role of adenosine in microglial proliferation remains controversial. One study reports that adenosine stimulates the proliferation of microglial cells through a mechanism that involves the simultaneous stimulation of A₁ and A₂ ARs (Gebicke-Haerter et al. 1996). By contrast, adenosine has been reported to inhibit the

proliferation of microglial cells; i.e., phorbol 12-myristate 13-acetate-stimulated microglial proliferation is reduced following treatment with an A₁AR agonist (Si et al. 1996). Moreover, stimulation of the A₁AR can also cause microglial apoptosis (Ogata and Schubert 1996). Adenosine levels in the extracellular fluid are lower in human glioblastoma tissue than in control tissue, namely 1.5 and 3 μ M, respectively. These values were obtained from human glioblastomas of high-grade malignancy and measured by brain microdialysis coupled to high-performance liquid chromatography (Bianchi et al. 2004). Whether this rather small difference causes the accumulation of microglia close to tumors is speculative.

Recent studies support the idea that ARs and specifically the A₁AR are good targets for drug development in several diseases that affect the CNS (Fredholm et al. 2005). A₁AR deficiency aggravates experimental allergic encephalomyelitis (Tsutsui et al. 2004), and it has been repeatedly shown that adenosine can protect tissues against the negative consequences of hypoxia or ischemia (Fredholm 1997), mainly by acting on the A₁AR. Hence, survival after a hypoxic challenge may be reduced if A₁ARs are absent or blocked (Johansson et al. 2001). The tissue-protective effect of A₁AR has been implicated in experimental paradigms using A₁AR-deficient mice. In a model of renal ischemia and reperfusion injury, A₁AR-deficient mice exhibited an increase in production of proinflammatory mediators and showed an increase in renal injury (Lee et al. 2004a, b). Similarly, in a model of experimental allergic encephalomyelitis, A₁AR deficiency led to increased neuroinflammation and demyelination and also augmented axonal injury. Both studies concluded that A₁AR serves anti-inflammatory functions that regulate subsequent tissue damage. Furthermore, metalloproteinase (MMP) 9 and MMP-12 are significantly elevated in A₁AR-deficient mice (Tsutsui et al. 2004). Indeed, MMPs play an important role in glioblastoma progression and, as was recently demonstrated, the expression of MMPs by microglia has an impact on tumor growth (Markovic et al. 2005). Matrix degradation by MMPs is an important prerequisite for glioblastoma invasion (Rao 2003). A₁AR activation on microglia/macrophages inhibits not only the production of cytokines like interleukin-1 β but also matrix MMPs like MMP-12 (Tsutsui et al. 2004). MMP-12, also known as macrophage elastase, is an MMP that is produced by activated macrophages and preferentially degrades elastin (Werb and Gordon 1975). Hence, inhibition of microglial MMP-12 secretion via activation of A₁AR could explain the glioblastoma growth inhibition observed in the studies described above. Moreover, the lack of inhibition of MMP-12 by A₁ARs on microglia may explain why there is enhanced accumulation of microglia at the tumor sites in A₁AR-deficient mice along with their tumor-promoting effects (i.e., associated increased tumor size). Adenosine does not appear to directly regulate MMP-12 expression in microglia/macrophages since direct stimulation of cultured macrophages with AR agonists did not induce expression of MMP-12 (Sun et al. 2005). It is therefore likely that the removal of A₁AR signaling leads to enhanced production of mediators in the CNS, which then leads to enhanced MMP-12 production. A likely candidate for this is interleukin (IL)-13, since IL-13 has been shown to be involved in the production of MMP-12 in other model systems (Lanone et al. 2002).

The results from the studies described above suggest that the A₁AR plays an antitumorigenic role mediated by microglial cells in the development of glioblastomas. Further research into the mechanisms of how the pathways of A₁AR signaling modulate glioblastoma development may ultimately lead to treatments to reduce the progression of this disease.

3 A_{2A} Adenosine Receptor

3.1 The A_{2A}AR: Protector of Host Tissue, Protector of Tumors

The seminal observations of Ohta and Sitkovsky (2001) clearly established a role for the A_{2A}AR in protecting host tissue from destruction by overexuberant immune responses. Considering that the tumor microenvironment contains relatively high levels of extracellular adenosine, data is emerging to support the hypothesis that tumor-derived adenosine is one mechanism by which tumors evade immune destruction (Blay et al. 1997; Ohta et al. 2006). In this section, we will discuss the role of adenosine in thwarting antitumor immunity and the potential pharmacologic interventions on the horizon that may serve to overcome this hurdle to immunotherapy.

3.2 Tumors Evade the Immune System by Inhibiting Immune Cell Function

The ability of the immune system to specifically recognize antigen makes it a potentially powerful tool in terms of developing modalities to treat cancer. However, in spite of many recent advances in understanding of and ability to identify tumor antigens, immunotherapy is clearly yet to live up to its full potential. In part, this is because tumors evade immune destruction by inhibiting tumor-specific immune cells (Pardoll 2002). For example, while a particular tumor may express a very unique and readily recognized tumor antigen, if this antigen is presented by resting or nonprofessional antigen-presenting cells (APCs), T-cell receptor (TCR) recognition will not lead to the destruction of the tumor but rather the inactivation of the tumor-specific T cell.

In this context, it is not the inability of T cells to recognize the tumor that is hampering cancer immunotherapy, but rather a lack of antigen-induced immune activation. That is, tumors readily express and T cells readily recognize tumor antigens (Overwijk and Restifo 2001). The problem is that T-cell recognition of the tumor does not lead to tumor destruction but rather to T-cell tolerance. In this regard, the tumor microenvironment is fraught with humors and cells that facilitate the ability of tumors to evade immune destruction (Drake et al. 2006). For example, the cytokines IL-10 and transforming growth factor β (TGF- β) in the tumor microenvironment

can both directly inhibit T-cell function as well as promote the induction of regulatory T cells and tolerogenic APCs. Likewise, tumors can express coinhibitory ligands such as B7–H1 and B7–H4. These in turn engage molecules on the surfaces of T cells such as PD-1 that serve to inhibit T-cell function. In this context, it is becoming clear why tumor vaccines have failed to live up to their potential so far (Pardoll 2002). Vaccine regimens which have focused on trying to enhance tumor-specific T cells by utilizing viral vectors, DNA vaccines, cytokine-secreting cells and antigen-pulsed dendritic cells have all shown promise in animal models and even some clinical trials. Put simply, in spite of the ability of such approaches to generate activated tumor antigen-specific T cells, the efficacy of such cells is thwarted by the multiple immunologic checkpoints exploited by the tumor. With this in mind, current immunotherapeutic strategies are focused on blocking these checkpoints. In this regard, blocking antibodies against cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 (a negative regulator of T-cell activation) has shown great promise in a number of animal models (Egen et al. 2002). Likewise, blocking anti-PD-1 antibodies are also currently being tested in order to enhance tumor immunotherapy (Blank and Mackensen 2007).

3.3 The A_{2A}AR Negatively Regulates Immune Responses

The ability of adenosine to inhibit immune function has been known for some time (Linden 2001). However, in light of the fact that there are four known AR subtypes, the critical, nonredundant role of the A_{2A}AR in mediating adenosine-induced anti-inflammatory responses was somewhat surprising. In a series of experiments, Sitkovsky's group demonstrated that normally nonlethal, self-limiting inflammation in wild-type (Wt) mice led to excessive inflammation and death in A_{2A}AR-null mice (Ohta and Sitkovsky 2001). These observations and additional studies led to a model whereby tissue damage resulting from inflammation leads to the release of extracellular adenosine, which then acts to quell the inflammatory response by acting on bone marrow-derived immune cells. Indeed, A_{2A}AR signaling on immune cells such as macrophages, T cells and dendritic cells has been shown to limit effector cell function (Erdmann et al. 2005; Huang et al. 1997; Khoa et al. 2001; Lappas et al. 2005; Naganuma et al. 2006; Panther et al. 2001; Schnurr et al. 2004). The existence of this negative feedback loop has led Sitkovsky to propose that, from an immunologic prospective, adenosine should be viewed as a metabokine that acts as an inhibitory second signal (Sitkovsky and Ohta 2005). For example, during an infection, pathogen-associated molecular patterns (PAMPs) along with host-derived uric acid, high mobility group (HMG1b) and hyaluronan (HA) would promote activating "danger signals" (Scheibner et al. 2006; Shi et al. 2003; Williams and Ireland 2008). As the inflammation progresses, the pathogen will be eliminated and the concentration of the potent immune-activating PAMPs will markedly decrease. In this setting, the inhibitory affects of adenosine released by damaged tissue will dominate to protect the tissue from further destruction by overacting immune responses.

Adenosine acting via the A_{2A}AR has the ability to influence inflammation by inhibiting proinflammatory cytokine secretion, C2 activation, macrophage-mediated phagocytosis and superoxide production (Sullivan 2003). Likewise, A_{2A}AR activation has profound effects on the adaptive immune response. A_{2A}AR activation inhibits both CD4⁺ and CD8⁺ T-cell function (Erdmann et al. 2005; Lappas et al. 2005; Naganuma et al. 2006; Seigny et al. 2007; Zarek et al. 2008). Interestingly, A_{2A}AR activation on T cells seems to selectively inhibit proinflammatory cytokine expression while sparing anti-inflammatory cytokine expression (Naganuma et al. 2006). In addition, antigen activation in the presence of A_{2A}AR agonists can promote T-cell tolerance in the form of anergy (Zarek et al. 2008). Likewise, A_{2A}AR engagement can prevent the development of IL-17 producing cells and promote the development of Foxp3⁺ and LAG-3⁺ regulatory T-cells. Along these lines, it has been shown that adenosine acting via the A_{2A}AR might partially mediate the suppressive function of regulatory T cells by engaging the A_{2A}ARs on the suppressed cells (Deaglio et al. 2007). It was found that the ectoenzymes CD39 and CD73 appear to be more specific markers for Foxp3⁺ regulatory cells than CD25 (Deaglio et al. 2007). Further data supporting the role of adenosine acting via the A_{2A}AR in facilitating regulatory T-cell function has also been demonstrated in a colitis model of autoimmunity. In these studies, CD45RB^{low} or CD25⁺ T cells derived from A_{2A}AR-null mice were unable to regulate CD45RB^{high} cells and prevent disease (Naganuma et al. 2006). Furthermore, the CD45RB^{high} cells from A_{2A}AR-null mice were not inhibited by regulatory T cells, even when they were derived from wild-type mice (Naganuma et al. 2006). Thus, with regard to the adaptive immune response, the A_{2A}AR protects the host from excessive tissue destruction by not only acutely inhibiting T-cell function but also promoting the development of regulatory T cells.

3.4 Adenosine Protects Tumors from Immune Destruction

Tumors are very adept at usurping negative regulatory mechanisms of the immune system in order to evade antitumor responses. As mentioned above, the tumor microenvironment is replete with inhibitory cytokines, inhibitory ligands and regulatory T cells (Drake et al. 2006). Considering that A_{2A}AR activation is a potent inhibitor of adaptive immune responses, it is not surprising that tumor-derived adenosine has been implicated in blocking antitumor immunity. Indeed, the tumor microenvironment has been shown to contain relatively high concentrations of adenosine (Blay et al. 1997). In part, this is due to the hypoxic nature of the tumor microenvironment (Lukashev et al. 2007). Hypoxia regulates the levels of adenosine by inhibiting enzymes involved in the destruction of adenosine and simultaneously increasing the activity of enzymes charged with the generation of adenosine.

Hoskin and colleagues were one of the first groups to propose that adenosine within the microenvironment of solid tumors might inhibit T-cell function (Hoskin et al. 1994). Their initial studies demonstrated that adenosine could inhibit natural

killer (NK) cell function as well as the ability of cytotoxic T cells to adhere to tumor cell targets (MacKenzie et al. 1994; Williams et al. 1997). Subsequently, this group went on to formally demonstrate that the extracellular fluid of tumors contains concentrations of adenosine that are sufficient to inhibit lymphocyte activation (Blay et al. 1997). This observation has since been confirmed by others (Ohta et al. 2006). Note that the initial studies by the Hoskin's group did not implicate the $A_{2A}AR$ as playing a critical role in the inhibition of antitumor immune function. However, more recently it has been shown that adenosine can inhibit NK cell and IL-2/NKp46-activated NK cells specifically via the $A_{2A}AR$ (Raskovalova et al. 2006). These studies showed that $A_{2A}AR$ -specific agonists inhibit the cytotoxicity of NK cells as well as their ability to elaborate cytokines. Interestingly, by employing various protein kinase A (PKA) inhibitors it was suggested that the ability of $A_{2A}AR$ activation to inhibit these functions is mediated downstream via PKA-I but not PKA-II. It has subsequently been shown that $A_{2A}AR$ -specific agonists could also inhibit both tumor-specific CD4+ and CD8+ T cells (Raskovalova et al. 2007). In these studies, similar to the NK cell studies, $A_{2A}AR$ -specific agonists inhibited the ability of human antimelanoma-specific cytotoxic T lymphocytes (CTLs) and human anti-melanoma-specific CD4+ T cells with regard to their ability to kill tumor cells and elaborate cytokines and chemokines in response to tumor cells. Biochemically, it was found that molecules that activated PKA-I but not PKA-II mimicked the affects of $A_{2A}AR$ activation on T-cell function. The $A_{2A}AR$ -mediated inhibition, in turn, was blocked by Rp-8-Br-cAMPS, which antagonizes the binding of cAMP to the regulatory subunit of PKA-I. Alternatively, inhibitors of the PKA catalytic subunit did not mitigate the inhibitory affects of $A_{2A}AR$ activation.

As discussed, tumors evade host responses by acutely inhibiting immune function and promoting tolerance. Considering that $A_{2A}AR$ activation inhibits immune responses by suppressing immune activation and promoting tolerance, the following question arises: does tumor-derived adenosine play this role *in vivo*? Initial studies addressing this question suggest that the answer is yes (Ohta et al. 2006). $A_{2A}AR$ -null mice have been shown to more readily reject melanoma and lymphoma tumor challenge. In addition, treating mice with $A_{2A}AR$ antagonists (including caffeine) led to increased tumor rejection by CD8+ T cells. These findings have been confirmed by another group that has also been able to demonstrate the ability of $A_{2A}AR$ -null mice to more readily reject tumors and respond more robustly to tumor vaccines (Powell et al., unpublished data). In particular, the data from these studies suggest that genetic deletion of the $A_{2A}AR$ leads to more robust initial responses to vaccines. There are a number of important implications of these *in vivo* findings. First, the fact that genetic deletion of the $A_{2A}AR$ markedly enhances antitumor responses suggests that adenosine plays an important role in mediating tumor evasion of the immune system. Second, adenosine appears to block both the generation and effector phases of antitumor responses. Third, and perhaps most importantly, these findings support a role for pharmacologic inhibition of $A_{2A}AR$ activation as a means of enhancing immunotherapy.

3.5 *A_{2A}AR Antagonism as a Means of Enhancing Immunotherapy*

Adenosine acting via the A_{2A}AR has been shown to inhibit dendritic cell function, T-cell activation and differentiation, and T-cell effector function (Sitkovsky et al. 2004). Additionally, the A_{2A}AR has been implicated in selectively enhancing anti-inflammatory cytokines, promoting the upregulation of PD-1 and CTLA-4, promoting the generation of LAG-3 and Foxp3+ regulatory T cells, and mediating the inhibition of regulatory T cells (Naganuma et al. 2006; Sevigny et al. 2007; Zarek et al. 2008). All of these immunosuppressive properties have also been identified as mechanisms by which tumors evade host responses. Initial *in vivo* studies demonstrating that genetically and pharmacologically inhibiting the A_{2A}AR leads to robust antitumor responses suggest that adenosine is at least partially responsible for promoting these tumor defense mechanisms (Ohta et al. 2006). As such, the addition of A_{2A}AR antagonists to cancer immunotherapeutic protocols represents an exciting approach to enhancing tumor immunotherapy. Interestingly, the safety of such compounds has already been shown in trials employing A_{2A}AR antagonists for the treatment of Parkinson's disease (Jenner 2005).

Chemotherapy and radiation therapy result in the release of copious amounts of tumor antigen. However, this form of tissue destruction can also result in increases in extracellular adenosine. Therefore, the concomitant administration of A_{2A}AR antagonists during chemotherapy or radiation therapy might actually lead to the expansion of tumor-specific T cells, while at the same time preventing the induction of tumor-specific regulatory T cells. In terms of combining A_{2A}AR antagonists with tumor vaccines, we believe that there are two time points that are relevant. First, administration of antagonists during the perivaccination period might serve to enhance the generation of tumor-specific effector memory cells. This would be accomplished by both enhancing the activity of the antigen-presenting cells (e.g., dendritic cells), as well as blocking adenosine-mediated negative feedback on the T cells themselves. Second, the continued administration of A_{2A}AR antagonists will enhance the effector function of these cells and potentially block the upregulation of regulatory T cells. Finally, perhaps the most effective use of A_{2A}AR antagonists will be in combination with not only vaccines but also other checkpoint blockers. For example, blocking PD-1 engagement as well as the A_{2A}AR will perhaps mitigate the ability of tumors to turn off tumor-specific effector T cells.

4 *A_{2B} Adenosine Receptors*

The A_{2B} adenosine receptor (A_{2B}AR) is found in many different cell types and requires higher concentrations of adenosine for activation than the A₁, A_{2A}, and A₃ AR subtypes (Fredholm et al. 2001). Thus, unlike the other AR subtypes, the A_{2B}AR is not stimulated by physiological levels of adenosine, but may therefore play an

important role in pathophysiological conditions associated with massive adenosine release. Such conditions occur in ischemia or in tumors where hypoxia is commonly observed (Illes et al. 2000; Merighi et al. 2003). Although potent and selective tools are scarce for the A_{2B} AR subtype, it has become increasingly clear in recent years that this AR subtype regulates a number of functions (e.g., vascular tone, cytokine release, and angiogenesis; Volpini et al. 2003). A_{2B} ARs may also play a role in cancer, based on a number of observations. Gaining an understanding of the exact mechanisms by which adenosine regulates the growth and proliferation of tumor cells via this AR subtype could potentially lead to a target for novel therapies or at least for cotherapies for cancer. In the following sections, potential mechanisms suggesting that A_{2B} AR might be involved in tumor development and progression are discussed.

One of the pivotal mechanisms for tumor growth is angiogenesis, a process that is highly regulated by an array of angiogenic factors and is triggered by adenosine under various circumstances that are associated with hypoxia. Although the A_3 AR subtype is involved in the release of angiogenic factors, in some cases the A_{2B} AR also seems to be responsible for the release of a certain subset of cytokines (Feoktistov et al. 2003; Merighi et al. 2007). A_{2B} ARs are expressed in human microvascular endothelial cells, where they play a role in the regulation of the expression of angiogenic factors like vascular endothelial growth factor (VEGF), IL-8, and basic fibroblast growth factor (bFGF) (Feoktistov et al. 2002). Moreover, in HMC-1 cells derived from a highly malignant, undifferentiated human mastocytoma cancer, activation of A_{2B} ARs induces the release of IL-8 and VEGF, and the activation of A_3 ARs induces angiopoietin 2 expression (Feoktistov et al. 2003). However, capillary formation induced by HMC-1 media was maximal when both HMC-1 A_{2B} ARs and A_3 ARs were activated. Activation of A_{2B} ARs alone was less effective, suggesting a cooperation between A_{2B} ARs and A_3 ARs on HMC-1 cells to produce angiogenesis. Furthermore, Merighi et al. demonstrated in HT29 human colon cancer cells that adenosine increases IL-8 expression via stimulation of A_{2B} ARs, while the stimulation of A_3 AR caused an increase in VEGF (Merighi et al. 2007). In the glioblastoma cell line U87MG, a similar A_{2B} AR-mediated increase of IL-8 was observed (Zeng et al. 2003). In addition, it was shown that hypoxia caused an upregulation of A_{2B} ARs in these tumor cells. As these findings point to a crucial role for A_{2B} ARs in mediating the effects of adenosine on angiogenesis, blockade of A_{2B} ARs may limit tumor growth by limiting the oxygen supply.

There are numerous reports of a potential role of adenosine and ARs in breast cancer (Barry and Lind 2000; Madi et al. 2004; Panjehpour et al. 2005; Spychala et al. 2004). Although AR agonists acting through A_3 ARs were shown to possess antitumor activity in breast cancer, it turned out (at least in some cases) that these effects were receptor independent (Chung et al. 2006; Lu et al. 2003). The very high concentrations of IB-MECA required for growth inhibition in some studies (Panjehpour and Karami-Tehrani 2004) may lend further support to the notion of A_3 AR-independent effects.

A most striking observation was that the estrogen receptor-positive MCF-7 cells appeared to be devoid of any detectable amount of ARs, whereas the

estrogen receptor-negative MDA-MB-231 cells express very high levels of A_{2B}ARs (Panjehpour et al. 2005). Both binding and functional experiments showed that other AR subtypes were not present in detectable levels in these tumor cells. Stimulation with the nonselective AR agonist 5'-(*N*-ethylcarboxamido)adenosine (NECA) resulted in the activation of adenylate cyclase, whereas 10 μM 2-*p*-(2-carboxyethyl)phenethylamino-5'-(*N*-ethylcarboxamido)adenosine (CGS21680; which, at this concentration, activates all but the A_{2B}AR subtype) had no effect. Moreover, there was no A₁AR or A₃AR receptor-mediated inhibition of adenylate cyclase, confirming the exclusive presence of A_{2B}ARs as a functionally relevant AR subtype in MDA-MB-231 cells (Panjehpour et al. 2005).

In addition to the classical adenylate cyclase activation, A_{2B}ARs also mediate a Ca²⁺ signal (Feoktistov et al. 1994; Linden et al. 1999; Mirabet et al. 1997). A similar Ca²⁺ signal was detected in MDA-MB-231 cells, most likely as a result of the activation of G_q (Panjehpour et al. 2005). With the use of selective agonists and antagonists for A₁AR, A_{2A}AR, and A₃ARs, a pharmacological profile identical to the one found for the adenylate cyclase response was demonstrated for the Ca²⁺ signal in these cells, again suggesting an A_{2B}AR as the sole AR subtype in these cells.

The mitogen-activated protein (MAP) kinase pathways are critically important in the regulation of cell proliferation and differentiation (Raman et al. 2007). There are numerous extracellular signals feeding into these cascades, including input via GPCRs (Goldsmith and Dhanasekaran 2007). All four subtypes of ARs were shown to mediate extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in transfected CHO cells (Graham et al. 2001; Schulte and Fredholm 2000). MAP kinase signaling and hence cell proliferation might be amenable to manipulation through specific ARs in tumor cells. Such a possibility seems to be particularly attractive in a situation where one AR subtype is highly expressed, as is the case for A_{2B}ARs in MDA-MB-231 cells. As mentioned above, A_{2B}ARs are stimulated only by pathophysiological high concentrations of adenosine (Fredholm et al. 2001). Thus, selective blockade or stimulation of this AR subtype may not interfere with the numerous important physiological functions of adenosine mediated via other AR subtypes.

MDA-MB-231 cells show a very high basal ERK 1/2 phosphorylation, indicative of constitutively active growth signals (Bieber et al. 2008). This basal activity seems to be maximal, as stimulation of the MAP kinase pathway (e.g., with epidermal growth factor, EGF) does not cause a further increase in ERK phosphorylation. The nonselective AR agonist NECA, on the other hand, causes a time-dependent decrease in ERK 1/2 phosphorylation, whereas CGS 21680 shows no inhibitory effect. As described above, functional and binding studies suggest that only A_{2B}ARs are present in MDA-MB-231 cells. Therefore, it seems that this AR subtype is responsible for the unusual inhibitory signal on ERK 1/2 phosphorylation. Moreover, antagonists like 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) block this response, confirming the identity of the AR subtype as the A_{2B}AR mediating the inhibition of ERK 1/2 phosphorylation (Bieber et al. 2008).

The exact pathway leading to A_{2B}AR-mediated inhibition is not fully understood at this point. Both the Ca²⁺ signal detected following A_{2B}AR stimulation in

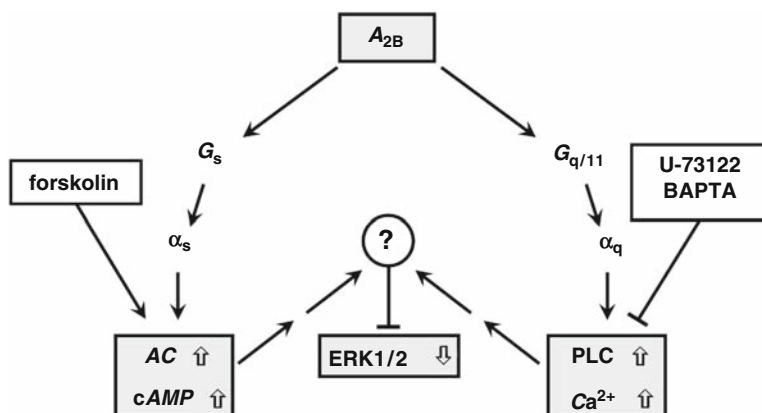


Fig. 1 Possible pathways leading to inhibition of extracellular signal regulated kinase (ERK)1/2 phosphorylation by A_{2B} adenosine receptors (A_{2B} ARs). Stimulation of adenylate cyclase (AC) via the G_s pathway results in inhibition of mitogen-activated protein kinase (MAPK) activity. Forskolin mimics this inhibition, confirming a role of cAMP. Alternatively, G_{q/11} may be activated by A_{2B} AR stimulation, resulting in an increased activity of phospholipase C (PLC) and in intracellular Ca²⁺ signal. The PLC inhibitor U-73122 and the Ca²⁺ chelator BAPTA (applied as the cell-penetrating ester BAPTA-AM) both abolish A_{2B} AR-mediated inhibition of MAPK, providing evidence for a second pathway leading to the inhibition of ERK 1/2 phosphorylation. Both the G_s- and the G_{q/11}-mediated signals are linked to MAPK inhibition via currently unknown pathways

MDA-MB-231 cells (Panjehpour et al. 2005) and PLC activation are sufficient, as their blockade abolishes the inhibition of ERK 1/2 phosphorylation. On the other hand, forskolin stimulation mimics the effect of NECA, suggesting that cAMP may also play a role. Several inhibitors of PKA have no effect on NECA-induced inhibition of ERK 1/2 phosphorylation. Similarly without effect are activators of PKA and exchange protein activated by cAMP (Epac), making these effectors unlikely to be targets involved in mediating the inhibitory A_{2B}AR signal on MAP kinase activity. Figure 1 summarizes the current knowledge of potential pathways leading to A_{2B}AR-mediated inhibition of ERK 1/2 phosphorylation in MDA-MB-231 cells.

Although it was shown that A_{2B}ARs convey a stimulatory signal into MAP kinase pathways in transfected CHO cells (Schulte and Fredholm 2000), an inhibitory input was found in MDA-MB-231 cells. A few studies describe such an uncommon antiproliferative GPCR-mediated signal in glomerular mesangial cells (Haneda et al. 1996) and in vascular smooth muscle cells (Dubey et al. 2000). The high expression levels of A_{2B}ARs in an estrogen-negative breast cancer cell line together with a link to an antiproliferative signaling pathway make this AR subtype a potentially interesting target for tumor treatment, perhaps in combination with drugs interfering with downstream effectors in MAP kinase signaling pathways (Dhillon et al. 2007).

There is an increasing amount of data confirming that A_{2B}ARs play an important role in mediating the effects of adenosine on tumor growth and progression. The effects which are most interesting for a potential anticancer treatment based on A_{2B}ARs as a target are inhibition of angiogenesis and inhibition of ERK 1/2

phosphorylation. The dilemma is, however, that inhibition of angiogenesis requires the use of $A_{2B}AR$ antagonists, whereas inhibition of growth signaling via the MAP kinase pathway might be achieved through treatment with $A_{2B}AR$ agonists. The relative importance of these effects needs to be investigated using *in vivo* models before therapeutic suggestions can arise. It may eventually turn out that both agonists and antagonists will provide useful options for treatment in combination with other therapeutic measures if used at different stages of the disease and its treatment.

5 A_3 Adenosine Receptor

A_3AR belongs to the family of seven-transmembrane-domain GPCRs. The human A_3AR has been cloned and expressed and its adenosine agonist binding specificities characterized. The A_3AR was found to be most abundantly expressed in human lung and liver, with low amounts observed in the brain (Sajjadi and Firestein 1993). Low levels of expression were also observed in testes and heart. No expression was found in spleen or kidney. This expression profile differed from those for the A_1AR , $A_{2A}AR$ and $A_{2B}AR$, which are expressed in variable levels in brain, heart, lung and kidney but not in liver tissues (Salvatore et al. 1993). Ligand structure–activity studies have identified selective agonists, partial agonists and antagonists for ARs (Cristalli et al. 2003; Muller 2003; Volpini et al. 2003; Zablocki et al. 2004). For the human and rat A_3AR , potent and selective agonists as well as selective A_3AR antagonists (e.g., PSB-10, PSB-11, MRE-3005F20 and MRS-1334) have been identified (Muller 2003). Site-directed mutagenesis and molecular modeling studies have also been performed that provide detailed information about the physical properties of ligand binding sites and the process of receptor activation (Gao et al. 2002; Muller 2003). Because of their selective tissue distribution and the development of specific A_3AR agonists and antagonists for them, A_3AR s have recently attracted considerable interest as novel drug targets.

Agonists to the A_3AR exert a differential effect on normal and tumor cells. In normal cells, the agonists induce the production of growth factors via induction of the NF- κ B signaling pathway. In contrast, in tumor cells, the agonists induce apoptosis and tumor growth inhibition via deregulation of the NF- κ B and the Wnt signaling pathways. This will be further detailed in Sect. 5.4.1 of this chapter.

Moreover, A_3AR agonists showed efficacy as cardioprotective, cerebroprotective, anti-inflammatory and immunosuppressive agents (Bar-Yehuda et al. 2007; Chen et al. 2006; Xu et al. 2006). For additional information on the pharmacology of the A_3AR and its role in disease, the reader is referred to Chap. 10, “ A_3 Adenosine Receptor: Pharmacology and Role in Disease” (by Borea et al.), in this volume.

In this manuscript, the activity of A_3AR ligands as anticancer and chemoprotective agents will be presented. In addition, various aspects of A_3AR -targeted therapy, mainly in solid tumor malignancies such as melanoma, prostate, colon and

hepatocellular carcinoma (HCC), will be discussed. Signal transduction pathways involved with A₃AR targeting utilizing highly selective A₃AR agonists and antagonists will be presented.

A significant part of the review is dedicated to the therapeutic effect of A₃AR agonists based on the concept that these compounds target mainly malignant cells that highly express A₃ARs without damaging normal body cells that barely express the receptor.

5.1 Overexpression of the A₃AR in Tumor Versus Normal Adjacent Tissues

Earlier studies revealed A₃AR expression in tumor cell lines including astrocytoma, HL-60 leukemia, B16-F10 and A378 melanoma, human Jurkat T-cell lymphoma, and murine pineal tumor cells, whereas low expression was described in most normal tissues (Auchampach et al. 1997; Gessi et al. 2002; Madi et al. 2003; Merighi et al. 2001; Suh et al. 2001; Trincavelli et al. 2002).

In more recent studies, a comparison between A₃AR expression in tumor vs. adjacent and relevant normal tissues supported the assumption that the receptor is upregulated in different types of malignancies. Recently, A₃AR in solid tumors was analyzed, leading to robust findings showing overexpression of the A₃AR in tumor tissues vs. low expression in the adjacent normal tissues. Furthermore, there is substantial evidence showing that A₃AR expression level is directly correlated to disease severity (Gessi et al. 2004; Madi et al. 2004).

In a comparative study, Morello et al. showed that primary thyroid cancer tissues express high levels of A₃ARs, as determined by immunohistochemistry analysis, whereas normal thyroid tissue samples do not express A₃ARs (Morello et al. 2007). Gessi et al. looked at the receptor binding values (K_d and B_{max}) of the A₃AR ligand [³H]MRE 3008F20 in colon carcinoma tissue samples from 73 patients, and found an increased binding value in comparison to adjacent, remote and healthy colon mucosa (Gessi et al. 2004). Interestingly, they found that large adenomas showed increased binding versus small adenomas, which had affinity and density values that were very similar to those of the mucosa of healthy subjects. An additional important result of this study was that the high receptor binding values (K_d and B_{max}) were reflected in the peripheral blood lymphocytes and neutrophils of the patients with colon carcinoma. Upon tumor resection, the A₃AR binding value (K_d and B_{max}) returned to that of the healthy subjects, suggesting that the receptor may also serve as a biological marker (Gessi et al. 2004). Similar data were reported by Madi et al. showing higher A₃AR protein and mRNA expression levels in colon and breast carcinomas vs. adjacent non-neoplastic tissue or normal tissue (Madi et al. 2004). Further analysis revealed that the lymph node metastasis expressed even more A₃AR mRNA levels than the primary tumors, supporting the notion that A₃AR levels may reflect the status of tumor progression (Madi et al. 2004).

Madi et al. also reported that in human melanoma, colon, breast, small-cell lung, and pancreatic carcinoma tissues, A₃AR mRNA was upregulated compared to adjacent non-neoplastic tissue and normal tissue derived from healthy subjects (Madi et al. 2004). Moreover, computational analysis using different database sources supported the biological analysis that A₃AR is overexpressed in tumor tissues (Madi et al. 2004). A 2.3-fold increase in the expression of A₃AR in human colon adenoma versus normal colon tissue using microarray analysis (Princeton University database) was found. A search in the Cancer Genome Anatomy Project (CGAP); SAGE (website: <http://cgap.nci.nih.gov/SAGE>; Virtual Northern Legend) based on serial analysis of gene expression revealed that A₃AR was abundant in brain, kidney, lung, germ cells, placenta and retina, but that brain, lung, and pancreatic tumors expressed more A₃AR in the malignant than the normal non-cancerous tissues from the same organs of the same patients. A search of the Expression Viewer (Human Genome Organization (HUGO) Gene Nomenclature Committee/CleanEX) based on expressed sequence tags revealed that the relative expression of A₃AR was 1.6-fold higher in all of the cancer tissues compared with normal tissues (Madi et al. 2004).

In a recent study, Bar-Yehuda et al. showed that A₃AR mRNA expression is upregulated in HCC tissues in comparison to adjacent normal tissues (Bar-Yehuda et al. 2008). Remarkably, upregulation of A₃AR was also noted in peripheral blood mononuclear cells (PBMCs) derived from the HCC patients compared to healthy subjects. These results further show that A₃AR in PBMCs reflect receptor status in the remote tumor tissue (Bar-Yehuda et al. 2008). Moreover, the high expression level of the A₃AR was directly correlated to overexpression of NF- κ B, a transcription factor for the A₃AR.

It is well established that G_i-protein-coupled receptors are internalized to early endosomes upon agonist binding (Bunemann et al. 1999; Claing et al. 2002). Early endosomes serve as the major site of receptor recycling, whereas the late endosomes are involved with the delivery of the internalized receptor to the lysosomes (Bunemann et al. 1999; Claing et al. 2002). Former studies have shown that chronic exposure of the A₃AR to the specific agonist methyl-1-[N⁶-(3-iodobenzyl)-adenin-9-yl]- β -D-ribofuronamid (IB-MECA) resulted in receptor internalization/externalization in B16-F10 melanoma cells (Madi et al. 2003). It was also demonstrated that in experimental animal xenograft models of colon and prostate carcinoma, chronic treatment with IB-MECA (designated CF101) induced receptor downregulation shortly after agonist administration. Interestingly, 24 h after treatment there was no tachyphylaxis and the A₃AR was fully expressed, showing that the target is not downregulated upon chronic treatment with the agonist (Fishman et al. 2003, 2004).

The data showing a direct correlation in A₃AR expression between tumor tissue and PBMCs suggest that receptor expression in the PBMCs mirrors receptor status in the tumor tissue. It is possible that TNF- α upregulation induces an increase in the expression level and activity of NF- κ B, a transcription factor for A₃ARs (Madi et al. 2004). This assumption is supported by the following finding. Upon treatment with 2-chloro-N⁶-3-iodobenzyladenosine-5'-N-methyluronamide (Cl-IB-MECA; designated CF102), the expression levels of TNF- α and NF- κ B were decreased,

resulting in a downregulation of A₃AR expression in both PBMCs and the tumor tissue (Bar-Yehuda et al. 2008). Similar data were reported by Gessi et al., showing that A₃AR is upregulated in both colon carcinoma tissue and PBMCs of patients with colon carcinoma. This group further demonstrated that the expression levels of A₃AR were downregulated in the PBMCs upon tumor removal (Gessi et al. 2004).

Taken together, the findings described above that show A₃AR overexpression in different tumor cell types provide the rationale that this receptor may be utilized as a specific target to treat cancer.

5.2 *In Vitro* Studies

The A₃AR plays an important role in regulating normal and tumor cell growth. Cell response to a given A₃AR agonist is determined by a plethora of factors, including agonist concentration and affinity, receptor density, interaction between different ARs expressed on the cell surface, cell type, and the cell microenvironment.

5.2.1 Effect of Low-Concentration A₃AR Agonists on Tumor Cell Growth

The effects of A₃AR agonists, mainly IB-MECA and CI-IB-MECA, on the proliferation of various tumor cells have been extensively tested. The rationale for using low concentrations of these two A₃AR agonists was based on their high affinity and selectivity at the A₃AR (approximately three orders of magnitude more than at the other ARs) (Fishman et al. 2007; Jeong et al. 2004; Joshi and Jacobson 2005). Moreover, Phase I clinical studies in healthy subjects, testing of IB-MECA (designated CF101) showed that the maximal tolerated dose of the drug was 5 mg kg⁻¹. At this dose, the plasma concentration was 40 ng ml⁻¹, which correlates with a concentration of 20 nM (van Troostenburg et al. 2004). This value correlates nicely with the affinity of IB-MECA to the mouse/rat/human A₃AR, exclusively activating this AR subtype, not any other AR subtype. Based on these data, IB-MECA and CI-IB-MECA were tested both *in vitro* and *in vivo* at low concentrations and dosages, respectively. Remarkably, at this low concentration range these agonists induced a differential effect on tumor and normal cell proliferation.

Inhibition of the growth of tumor cells, including rat Nb2-11C and mouse Yac-1 lymphoma, K-562 leukemia, B16-F10 melanoma, MCA sarcoma, human LN-Cap and PC3 prostate carcinoma, MIA-PaCa pancreatic carcinoma and HCT-116 colon carcinoma, was found. The agonists induced a cytostatic effect towards the tumor cells, as manifested by a decrease in ³[H]thymidine incorporation and cell cycle arrest at the G₀/G₁ phase (Bar-Yehuda et al. 2001; Fishman et al. 2000a, 2001, 2002a, b, 2003; Merimsky et al. 2003; Ohana et al. 2003). This effect was abolished by A₃AR antagonists (Madi et al. 2003), demonstrating that the response was A₃AR mediated. IB-MECA enhanced the cytotoxic effect of chemotherapy when tested in 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan thiazolyl (MTT) and colony

formation assays. A combined treatment of 5-fluorouracil plus IB-MECA yielded higher growth inhibition of HCT-116 human colon carcinoma cells in comparison to the chemotherapy alone (Bar-Yehuda et al. 2005).

At the same time, CI-IB-MECA stimulated the proliferation of bone marrow cells (Fishman et al. 2001). Interestingly, both IB-MECA and CI-IB-MECA up-regulated the production of granulocyte colony stimulating factor (G-CSF), known to act as a differentiation factor of neutrophils (Brandt et al. 1988). This novel activity mediated the stimulatory effect on bone marrow cell growth and prompted the examination of IB-MECA and CI-IB-MECA as myeloprotective agents that prevent neutropenia upon treatment with chemotherapeutic agents (Bar-Yehuda et al. 2002, Fishman et al. 2000b, 2001, 2002b, 2003).

As opposed to the results of the studies described above, demonstrating an inhibition of tumor cell lines by A₃AR agonists, in a set of experiments conducted by Gessi et al., low-concentration (100 nM) CI-IB-MECA stimulated the proliferation of some cancer cell lines such as Caco-2, DLD1, and HT29 human colon carcinoma cell line (Gessi et al. 2007). In addition, the same group showed that under hypoxic conditions, CI-IB-MECA induced upregulation of hypoxia-inducible factor 1 (HIF-1) alpha and VEGF in HT-29 human colon carcinoma cells, A375 human melanoma cells, and A172 and U87MG glioblastoma cell lines. This effect could be blocked with the A₃AR antagonist (MRE3008F20) or by siRNA silencing (Merighi et al. 2005b, 2006, 2007). Moreover, Abbracchio et al. showed that CI-IB-MECA modulates cytoskeleton reorganization, increases expression of Rho, and induces the intracellular distribution of the antiapoptotic protein Bcl-xL in ADF human astrocytoma cells (Abbracchio et al. 1997, 2001). Thus, A₃AR agonists can on the one hand induce the inhibition of tumor cell growth via cell cycle arrest, and on the other hand stimulate the proliferation of tumor cells, depending on cell type and culture conditions.

5.2.2 Effect of High-Concentration A₃AR Agonists on Tumor Cell Growth

The effect of high-concentration A₃AR agonists on tumor cell growth was an inhibitory one that was either A₃AR dependent or independent. CI-IB-MECA at a concentration of 10 μM inhibited the growth of A375 human melanoma cells by inducing cell cycle arrest in the G₀/G₁ phase. This effect was blocked by an A₃AR antagonist, demonstrating the role of A₃AR activation in this response (Merighi et al. 2005a). Moreover, IB-MECA at high concentration (30–60 μM) produced cell growth inhibition in both ERα-positive MCF-7 cells and in ERα-negative MDAMB468 human breast carcinoma cells. In both cell types, the introduction of an A₃AR antagonist, MRS1220, blocked the effect of this A₃AR agonist (Panjehpour and Karami-Tehrani 2004, 2007).

The A₃AR agonist 2-chloro-*N*⁶-(3-iodobenzyl)-4'-thioadenosine-5'-*N*-methyluronamide (thio-CI-IB-MECA) has high affinity and specificity for the human A₃AR. The introduction of μM concentrations of this agonist to HL-60 human

leukemia cell cultures resulted in apoptosis, as manifested by DNA fragmentation and poly(ADP-ribose) polymerase (PARP) cleavage (Lee et al. 2005).

Interestingly, an additional compound that inhibits the growth of tumor cells via A_3AR is cordycepin (3'-deoxyadenosine), an active ingredient of *Cordyceps sinensis*, a parasitic fungus used in traditional Chinese medicine (Nakamura et al. 2006). This molecule, at μM concentrations, induced a remarkable inhibitory effect on the growth of murine B16–BL6 melanoma and of Lewis lung carcinoma tumor cells. This inhibitory effect was abolished by the A_3AR antagonist 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(\pm)-dihydropyridine-3,5-dicarboxylate MRS1191 (Nakamura et al. 2006).

In contrast, IB–MECA and Cl–IB–MECA at μM concentrations inhibit the growth of various tumor cell lines (including NPA papillary thyroid carcinoma, HL-60 leukemia cells and U-937 lymphoma cells) in an A_3AR -independent mechanism (Kim et al. 2002; Morello et al. 2007). This inhibitory effect was characterized by apoptosis and was not abolished by antagonism or knockdown of the A_3AR . Based on these results, it was concluded that IB–MECA or Cl–IB–MECA at high concentrations can induce tumor cell death through receptor-independent mechanisms, perhaps via active transport into the cells through the nucleoside transporters (Kim et al. 2002; Merighi et al. 2002; Morello et al. 2007). Moreover, in MCF-7 human breast cancer cells, 100 μM of IB–MECA markedly reduced cell number and inhibited colony formation (Lu et al. 2003). These cancer cells do not express A_3AR s, overexpression of A_3AR did not lower the concentrations of IB–MECA needed to induce the inhibition of cell proliferation, and the introduction of MRS1191 (an A_3AR antagonist) did not abolish the IB–MECA inhibitory effect, suggesting that A_3AR was not involved in the cell growth inhibition of these human breast cancer cells. In these studies, an explanation for this inhibitory effect by IB–MECA may be related to its ability to reduce the expression level of estrogen receptor (ER) alpha, which plays a role in different signaling pathways leading to the transcription of genes responsible for G_1 –S cell cycle progression (Lu et al. 2003). The effects of the various A_3AR agonists at low and high concentrations on tumor cell growth in *in vitro* studies are summarized in Table 1.

5.3 *In Vivo* Studies

In this part of the review, *in vivo* studies showing the efficacy of A_3AR agonists in various tumor-bearing animals will be presented, supporting the utilization of A_3AR as a target to treat cancer. In all experimental models, the A_3AR agonists were administered orally due to their stability and bioavailability profile. The dose used in these studies was calculated based on the affinity data, resulting in exclusive activation of the A_3AR . The studies included syngeneic, xenograft, orthotopic and metastatic experimental animal models utilizing IB–MECA and Cl–IB–MECA as the therapeutic agents.

Table 1 Effects of A₃AR agonists at low and high concentrations on tumor cell growth in *in vitro* studies

Drug	Low/high A ₃ AR concentrations	A ₃ AR agonist	Tumor cell type	A ₃ AR-related	Effect	Suggested mechanism of action	References
IB-MECA	Low (1–100 nM)		Murine NB2–11C lymphoma	Yes	Growth inhibition	Deregulation of the Wnt signaling pathway	Fishman et al. (2000a, 2002a, 2003) Madi et al. (2003)
			Murine B16–F10 melanoma			Cell cycle arrest at the G ₀ /G ₁ phase	
			Human PC3 prostate carcinoma				
CI-IB-MECA	Low (100 nM)		Human Caco2 colon carcinoma	Yes	Cell proliferation	Upregulation of HIF-1alpha and VEGF	Gessi et al. (2007)
			Human DLD1 colon carcinoma			Reorganization of cytoskeleton	Merighi et al. (2005b, 2006, 2007)
			Human HT29 colon carcinoma*			Increased expression of Rho	Abbracchio et al. (1997, 2001)
			Human A375 melanoma*			Induction of intracellular distribution of Bcl-xL	

Thio- Cl-IB-MECA	Low (10nM)	Human A172 and U87MG glioblastoma* Human ADF astrocytoma Human HL-60 promyelocytic leukemia	Not determined	Growth inhibition	Deregulation of the Wnt signaling pathway	Lee et al. (2005)
IB-MECA	High (30-60 μM)	Human ERα-positive MCF-7 breast carcinoma Human ERα-negative MDAMB468 breast carcinoma	Yes	Growth inhibition	Inhibition of anchorage-dependent cell growth	Panjehpour and Karami-Tehrani (2004, 2007)
Cl-IB-MECA	High (10 μM)	Human A375 melanoma	Yes	Growth inhibition	Cell cycle arrest in the G ₀ /G ₁ phase	Merighi et al. (2005a)
Thio- Cl-IB-MECA	High (25-50 μM)	Human HL-60 promyelocytic leukemia	Not determined	Growth inhibition	Downregulation of cyclin D1 and <i>c-myc</i> protein expression Cell cycle arrest in the G ₀ /G ₁ phase Induction of apoptosis	Lee et al. (2005)

(continued)

Table 1 (continued)

Drug	Low/high A ₃ AR agonist concentrations	Tumor cell type	A ₃ AR-related?	Effect	Suggested mechanism of action	References
Cordycepin (3'-deoxyadenosine)	High (25–50 μM)	Mouse B16-BL6 melanoma Mouse Lewis lung carcinoma	Yes	Growth inhibition	Not determined	Nakamura et al. (2006)
IB-MECA	High (100 μM)	Human MCF-7 breast carcinoma	No	Growth inhibition	Downregulation estrogen receptor expression level Inhibition of colony formation	Lu et al. (2003)
CI-IB-MECA	High (10, > 30 μM)	Human HL-60 promyelocytic leukemia Human MOLT-4 leukemia Human NPA papillary thyroid carcinoma	No	Growth inhibition	Dephosphorylation of ERK 1/2 Inhibition of cell growth blocking the G ₁ cell cycle phase Induction of apoptosis	Kim et al. (2002)

5.3.1 Melanoma

Oral administration of 10–100 $\mu\text{g kg}^{-1}$ IB-MECA and CI-IB-MECA once or twice daily inhibited the growth of primary B16-F10 murine melanoma tumors in syngeneic models (Madi et al. 2003). Moreover, in an artificial metastatic model, IB-MECA inhibited the development of B16-F10 murine melanoma lung metastases (Bar-Yehuda et al. 2001; Fishman et al. 2001, 2002b). The specificity of the response was demonstrated by the administration of an A₃AR antagonist that reversed the effect of the agonist (Madi et al. 2003).

Furthermore, IB-MECA or CI-IB-MECA in combination with the chemotherapeutic agent cyclophosphamide induced an additive antitumor effect on the development of B16-F10 melanoma lung metastatic foci (Fishman et al. 2001, 2002b).

5.3.2 Colon Carcinoma

Oral administration of 10–100 $\mu\text{g kg}^{-1}$ IB-MECA once or twice daily inhibited the growth of primary CT-26 colon tumors (Ohana et al. 2003). Furthermore, in xenograft models, IB-MECA inhibited the development of HCT-116 human colon carcinoma in nude mice (Ohana et al. 2003). In these studies, the combined treatment of IB-MECA and 5-fluorouracil resulted in an enhanced antitumor effect. IB-MECA was also efficacious in inhibiting liver metastases of CT-26 colon carcinoma cells inoculated in the spleen. (Bar-Yehuda et al. 2005; Fishman et al. 2002b, 2004; Ohana et al. 2003).

5.3.3 Prostate Carcinoma

IB-MECA inhibited the development of PC3 human prostate carcinoma in nude mice. Additionally, IB-MECA increased the cytotoxic index of Taxol in PC3 prostate carcinoma-bearing mice (Fishman et al. 2002b, 2003).

5.3.4 Hepatocellular Carcinoma

Recent studies showed that A₃AR is overexpressed in tumor tissues and in PBMCs of N1S1 HCC tumor-bearing Sprague-Dawley rats (Bar-Yehuda et al. 2008). For these studies, an orthotopic rat model was established in which a subxiphoid laparotomy was performed and N1S1 cells were injected into the right hepatic lobe. Treatments with CI-IB-MECA at doses of 1, 50, 100, 500 and 1,000 $\mu\text{g kg}^{-1}$ three times daily were initiated on day 3 after tumor inoculation and continued until day 15. CI-IB-MECA treatment exerted a bell-shaped, dose-dependent inhibitory effect on tumor growth with a maximal effect at a dose of 100 $\mu\text{g kg}^{-1}$ (Bar-Yehuda et al. 2008).

5.3.5 Potentiation of Natural Killer Cell Activity

IB-MECA and CI-IB-MECA also upregulate serum levels of IL-12 and potentiate NK cell activity (Harish et al. 2003). In mice, CI-IB-MECA increased serum levels of IL-12 and potentiated the activity of NK cells (Harish et al. 2003). This effect of CI-IB-MECA on NK cell activity was seen in adoptive transfer experiments utilizing melanoma-bearing mice where marked inhibition in the development of lung metastatic foci was observed in the mice engrafted with splenocytes derived from CI-IB-MECA treated mice. Similar results were observed in HCT-116 human colon carcinoma-bearing nude mice treated with $10 \mu\text{g kg}^{-1}$ IB-MECA (Ohana et al. 2003).

5.3.6 Chemoprotective Effect

IB-MECA and CI-IB-MECA act also as chemoprotective agents. With cyclophosphamide treatment of B16-F10 melanoma-bearing mice or 5-fluorouracil treatment of HCT-116 human colon carcinoma-bearing nude mice, a marked decline in white blood cells and neutrophil counts occurs (Bar-Yehuda et al. 2002; Fishman et al. 2000b, 2001, 2002a, b, 2003). Administration of the A_3AR agonist restored the number of white blood cells and the percentage of neutrophils to their normal values. This was attributed to the ability of IB-MECA to induce the production of G-CSF (Bar-Yehuda et al. 2002; Fishman et al. 2000b, 2001, 2002a, b, 2003; Hofer et al. 2006, 2007)

Overall, the unique characteristics of the A_3AR agonists—they are orally bioavailable, exert their effects at low doses, enhance the effects of cytotoxic agents, and at the same time act as myeloprotective agents—together with their potential cardio- and neuroprotective activities suggest that this class of compounds may produce attractive clinical candidates as anticancer drugs.

5.4 Mechanisms of Action for the Anticancer Activity of the A_3AR

Adenosine receptors operate through distinct biochemical signaling mechanisms. The A_1 and A_3AR subtypes control most, if not all, of their cellular responses via pertussis toxin-sensitive G proteins of the G_i and G_o family. The A_3AR triggers G_i -protein activation, induces an intracellular signaling cascade that increases intracellular calcium concentrations, activates PLC and phospholipase D (PLD) as well as the production of intracellular second-messenger systems, which in turn, leads to related cellular responses such as cell proliferation or tumor cell apoptosis (Abbracchio et al. 1995; Murthy and Makhoulouf 1995; Olah and Stiles 1995; Olah et al. 1995).

Activation of the A₃AR inhibits adenylate cyclase activity, thereby leading to a decrease in the level of the second messenger, cAMP. The latter modulates the level and activity of protein kinase A (PKA) that phosphorylates downstream elements of the MAPK and protein kinase B (PKB)/Akt (PKB/Akt) signaling pathways (Poulsen and Quinn 1998; Seino and Shibasaki 2005; Zhao et al. 2000). In addition, it was reported that PKA phosphorylates PKB/Akt directly, thereby mediating its activity (Fang et al. 2000). Both PKA and PKB/Akt regulate the NF- κ B signaling pathway by phosphorylating and activating the downstream kinase I κ B kinase (IKK), which phosphorylates I κ B, thereby sorting it to degradation via the ubiquitin system. As a result, NF- κ B is released from its complex with I κ B and translocates to the nucleus to induce the transcription of genes such as cyclin D1 and *c-Myc* that control cell cycle progression (Karin and Ben-Neriah 2000; Li et al. 1999).

Taken together, since the activation of A₃AR induces the inhibition of adenylate cyclase and reduces the level of intracellular cAMP, the downstream elements PKA and PKB/Akt are not activated and so do not phosphorylate IKK. This leads to the reduced activity and expression levels of the NF- κ B, resulting in tumor cell cycle arrest and tumor growth inhibition.

5.4.1 Direct Effect of A₃AR Agonists on Tumor Cells: Deregulation of the NF- κ B and Wnt Signaling Pathways

In melanoma, colon, prostate and hepatocellular carcinoma cell lines, treatment with IB-MECA or CI-IB-MECA produced a decrease in PKA and PKB/Akt expression (Bar-Yehuda et al. 2008; Fishman et al. 2002a, b, 2003, 2004). As a result, the phosphorylation of IKK was inhibited, leading to the accumulation of I κ B/NF- κ B complex in the cytoplasm. This resulted in the downregulation of *c-myc* and cyclin D1 expression levels (Fig. 2) (Bar-Yehuda et al. 2008; Fishman et al. 2003, 2004).

Further studies showed that the Wnt signaling pathway is also involved in the anticancer activity mediated via the A₃AR. The rationale to investigate this pathway came from data showing that PKA and PKB/Akt phosphorylate and inactivate glycogen synthase kinase 3 β (GSK-3 β) (Cross et al. 1995; Fang et al. 2000). GSK-3 β is a serine/threonine kinase that acts as a key element in the Wnt signaling pathway, which is known to play a pivotal role in dictating cell fate during embryogenesis and tumorigenesis (Peifer and Polakis 2000). GSK-3 β phosphorylates the cytoplasmic protein β -catenin, which is sorted for degradation by the ubiquitin system. Upon phosphorylation, GSK-3 β loses its ability to phosphorylate β -catenin, resulting in the accumulation of the latter in the cytoplasm and its subsequent translocation to the nucleus, where it associates with lymphoid enhancer factor/T-cell factor (Lef/Tcf) to induce the transcription of genes responsible for cell cycle progression, like *c-myc* and cyclin D1 (Fig. 2) (Ferkey and Kimelman 2000; Morin 1999; Novak and Dedhar 1999).

An inability of GSK-3 β to phosphorylate β -catenin has been demonstrated in various malignancies, including colon carcinoma, melanoma and HCC (Bonvini et al. 1999; Cui et al. 2003; Robbins et al. 1996)

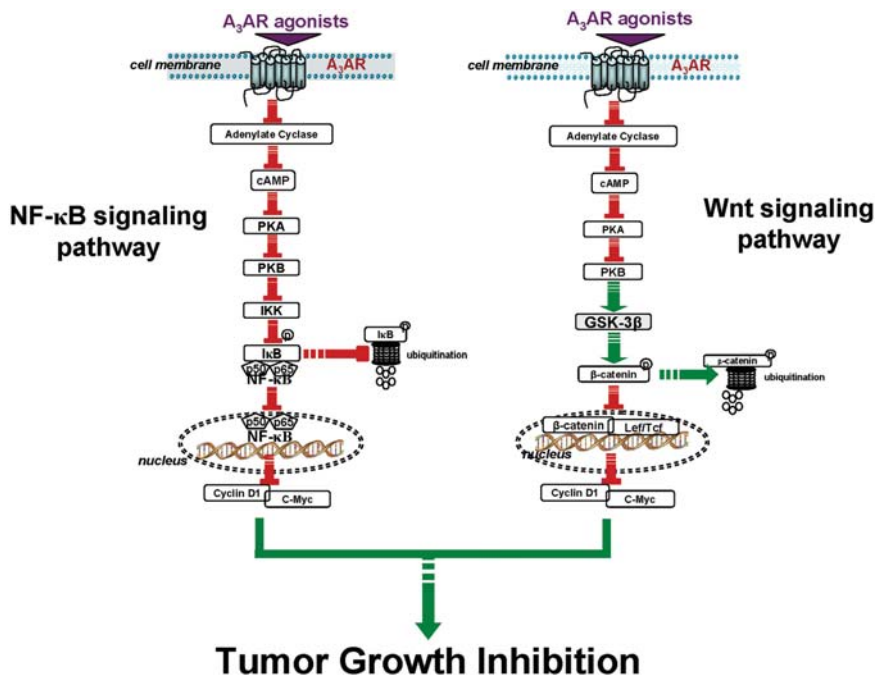


Fig. 2 Anticancer effect of A_3 adenosine receptor (A_3AR) agonists entails deregulation of the nuclear factor kappa B (NF- κ B) and the Wnt signaling pathways. Activation of the A_3AR in tumor cells with specific agonists inhibits the activity of adenylate cyclase, inducing a decline in the level of cAMP, leading to decreased levels of protein kinase A (PKA) and its substrate protein kinase B (PKB)/Akt. Consequently, this leads to a downregulation in the expression levels of signal proteins that play a role in the NF- κ B (I κ B kinase (IKK) and I κ B) and the Wnt (glycogen synthase kinase-3 β (GSK-3 β) and β -catenin) signaling pathways. As a result, the levels of *c*-Myc and cyclin D1, known to play a crucial role in cell cycle progression, are decreased. This chain of events leads to tumor growth inhibition

Treatment of B16-F10 melanoma, HCT-116 human colon carcinoma cells and PC-3 human prostate carcinoma cells *in vitro* with IB-MECA decreased PKA and PKB/Akt expression levels, resulting in the upregulation of GSK-3 β and the subsequent phosphorylation and ubiquitination of β -catenin (Fishman et al. 2002a, 2003; Madi et al. 2003). In these studies, downregulation of cyclin D1 and *c*-myc expression levels, as well as tumor cell growth suppression, were observed (Fishman et al. 2002a, 2003; Madi et al. 2003). Moreover, the group of Lee et al. further reported that a highly specific A_3AR agonist, thio-Cl-IB-MECA, induced apoptosis of HL-60 promyelocytic leukemia cells and lung cancer cells via deregulation of the Wnt signaling pathway. The levels of β -catenin, phosphorylated forms of GSK3- β and Akt were downregulated upon treatment with thio-Cl-IB-MECA (10 nM) in a time-dependent manner (Kim et al. 2008; Lee et al. 2005).

Additional evidence to support the *in vitro* mechanistic pathways presented above came from the analysis of tumor tissues excised from melanoma, prostate, colon and HCC tumor-bearing animals treated with IB-MECA or Cl-IB-MECA (Bar-Yehuda et al. 2008; Fishman et al. 2003, 2004; Madi et al. 2003).

Both the NF- κ B and Wnt signal transduction pathways were deregulated upon treatment with the A₃AR agonists, demonstrating a definitive molecular mechanism. Remarkably, Cl-IB-MECA induced marked apoptosis of tumor cells in the N1S1 HCC-bearing rats (Bar-Yehuda et al. 2008; Fishman et al. 2003, 2004; Madi et al. 2003).

In these studies, apoptosis of tumor cells was seen in the tunnel assay, and increases in the expression levels of the proapoptotic proteins Bad, BAX and caspase 3 were observed as well (Bar-Yehuda et al. 2008; Fishman et al. 2003, 2004; Madi et al. 2003).

5.4.2 A₃AR Agonists as Myeloprotective Agents

Some chemotherapeutic agents are known to induce myelosuppression, as manifested by a decline in the number of white blood cells (especially neutrophils), making patients susceptible to infections and sepsis. G-CSF is a hematopoietic growth factor produced by endothelium, macrophages, and a number of other immune cells, and its synthesis is induced by activation of the transcription factor NF- κ B. It stimulates the proliferation and differentiation of white blood cells. A recombinant form of G-CSF has become a standard supportive therapy for cancer patients to accelerate recovery from neutropenia after chemotherapy (Brandt et al. 1988; Rusthoven et al. 1998). In mice, IB-MECA induces G-CSF production and increases white blood cell and neutrophil counts in naïve and chemotherapy-treated animals (Bar-Yehuda et al. 2002). The myelostimulative effect of IB-MECA was also evidenced by high levels of G-CSF in bone marrow cells, splenocytes, and serum derived from IB-MECA-treated mice. Moreover, in splenocytes derived from IB-MECA-treated mice, increased expression levels of phosphoinositide 3-kinase (PI3K), known to play a role in the regulation of cell survival and proliferation (Gao et al. 2001), was noted. Consequently, the expression levels of PKB/Akt, IKK and NF- κ B were enhanced, resulting in G-CSF upregulation (Fig. 3).

The role of the A₃AR and PI3K-NF- κ B pathway in the production of G-CSF was further confirmed by treating the mice with pertussis toxin, a G_i-protein inactivator that interferes with the coupling of the receptor to the G_i protein. Splenocytes derived from mice that were treated with IB-MECA and pertussis toxin did not upregulate NF- κ B levels. Moreover, the NF- κ B inhibitor pyrrolidine dithiocarbamate (PDTC), known to suppress the release of I κ B from the latent cytoplasmic form of NF- κ B, counteracted the effect of IB-MECA and prevented the increase in NF- κ B expression levels (Bar-Yehuda et al. 2002).

Taken together with the studies described in Sect. 5.4.1 above, these studies suggest that activation of the A₃AR by specific agonists induces differential effects on normal and tumor cells to produce modulations of definitive signal transduction

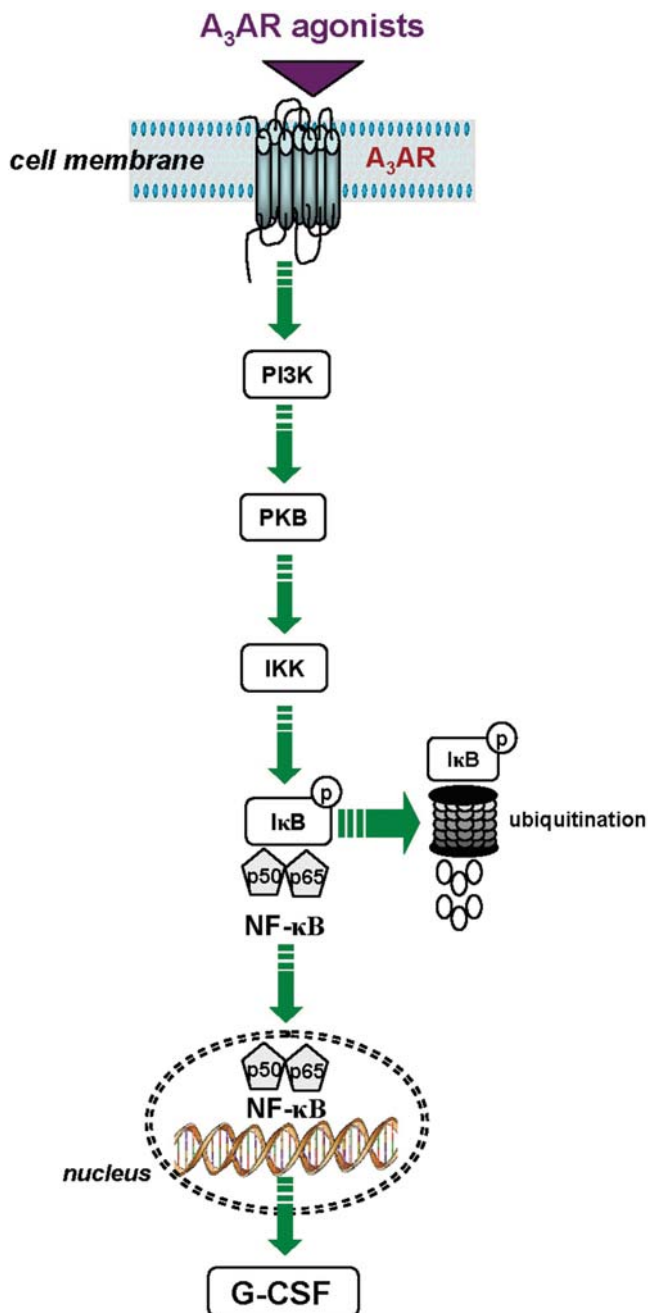


Fig. 3 Agonists for the A_3 adenosine receptor (A_3AR) induce granulocyte colony stimulating factor (G-CSF) production via nuclear factor kappa B (NF- κ B). Activation of A_3AR in splenocytes induces upregulation of phosphoinositide 3-kinases (PI3K) and its downstream target protein kinase B (PKB)/Akt. The latter activates I κ B kinase (IKK), which is responsible for the phosphorylation and ubiquitination of I κ B. As a result, NF- κ B translocates to the nucleus, where it induces the transcription of G-CSF

pathways that control cell growth regulatory mechanisms in the case of tumor cells and growth factor production in the case of normal hematopoietic cells (e.g., bone marrow cells and splenocytes).

6 Anticancer Activity of A₃AR Antagonists

A very interesting area of application of A₃AR ligands concerns cancer therapies. The possibility that the A₃AR plays an important role in the development of cancer has aroused considerable interest in recent years (Fishman et al. 2002b; Gessi et al. 2008; Merighi et al. 2003). The A₃AR subtype has been described in the regulation of the cell cycle, and both pro- and antiapoptotic effects have been reported, depending on the level of receptor activation (Gao et al. 2001; Gessi et al. 2007; Jacobson 1998; Merighi et al. 2005a; Yao et al. 1997). However, based on the studies presented above, it is important to note that A₃AR receptor activation appears to be involved in the inhibition of tumor growth both *in vitro* and *in vivo*.

Based on the relationships between tumors, hypoxia and adenosine concentrations, there are reports describing the potential utility of A₃AR antagonists for cancer treatment. Growing evidence from experimental and clinical studies points to the fundamental pathophysiological role of hypoxia in solid tumors. Hypoxia is the result of an imbalance between oxygen supply and consumption. Clinical investigations carried out over the last 15 years have clearly shown that the prevalence of hypoxic tissue areas is a characteristic pathophysiological feature of solid tumors. As the oxygen concentration decreases with increasing distance from the capillary, cell proliferation rates and drug concentrations both decrease. These two factors lead to resistance to anticancer drugs; firstly, because the majority of anticancer drugs are only effective against rapidly proliferating cells; secondly, because adequate levels of chemotherapy drugs have to reach the tumor cells from the blood vessels. Hypoxia inhibits enzymes that are involved in the breakdown of adenosine and increases the activities of those responsible for generating adenosine, thereby resulting in an increase in extracellular and intracellular adenosine. The elevated adenosine levels in response to hypoxia are not exclusive to tumor tissues, but, in this context, the increase in adenosine is localized to the tumor microenvironment, since the surrounding tissue is normally oxygenated (Blay et al. 1997). To survive under hypoxic conditions, tumor cells run numerous adaptive mechanisms, such as glycolysis, glucose uptake, and survival factor upregulation (Hockel and Vaupel 2001). Hypoxia-inducible factor (HIF) 1 is the most important factor involved in the cellular response to hypoxia (Semenza 2003). It is a heterodimer composed of an inducibly expressed HIF-1 α subunit and a constitutively expressed HIF-1 β subunit (Epstein et al. 2001). HIF-1 α and HIF-1 β mRNAs are constantly expressed under normoxic and hypoxic conditions (Wiener et al. 1996). However, during normoxia, HIF-1 α is rapidly degraded by the ubiquitin proteasome system, whereas exposure to hypoxic conditions prevents its degradation (Minchenko et al. 2002; Semenza 2000). HIF-1 α expression and activity are also regulated by the PI3K and MAPK

signal transduction pathways (Semenza 2002; Zhong et al. 2000). A growing body of evidence indicates that HIF-1 α contributes to tumor progression and metastasis (Hopfl et al. 2004; Welsh and Powis 2003). Immunohistochemical analyses have shown that HIF-1 α is present in higher levels in human tumors than in normal tissues (Zhong et al. 1999), and the levels of HIF-1 α activity in cells correlate with the tumorigenicity and angiogenesis in nude mice (Carmeliet et al. 1998). Tumor cells lacking HIF-1 α expression are markedly impaired in their growth and vascularization (Jiang et al. 1997; Kung et al. 2000; Maxwell et al. 1997). Therefore, since HIF-1 α expression and activity appear central to tumor growth and progression, HIF-1 α inhibition becomes an appropriate approach to treating cancer (Kung et al. 2000; Ratcliffe et al. 2000; Semenza 2003). Hypoxia creates conditions that, on the one hand, are conducive to the accumulation of extracellular adenosine, and on the other hand stabilize hypoxia-inducible factors, such as HIF-1 α (Fredholm 2003; Hockel and Vaupel 2001; Linden 2001; Minchenko et al. 2002; Semenza 2000; Sitkovsky et al. 2004). In particular, the correlation between AR stimulation and HIF-1 α expression modulation in hypoxia has recently been investigated. It has been reported that adenosine increases HIF-1 α protein accumulation in response to hypoxia in a dose- and time-dependent manner in human melanoma, glioblastoma and colon carcinoma through the involvement of the cell surface A₃AR (Merighi et al. 2005b, 2006, 2007). The signaling pathway involved in A₃AR-mediated accumulation of HIF-1 α in hypoxia involves MAPKinase activity (Merighi et al. 2005b, 2006, 2007). It is well established that HIF-1 α plays a major role in VEGF expression and angiogenesis. Furthermore, there is strong evidence that adenosine released from hypoxic tissues is an important player in driving the angiogenesis, by enhancing vascular growth through various mechanisms including the release of different factors, with VEGF being one of the most relevant (Adair 2005). A role for A_{2B}ARs in angiogenesis through an HIF-1 α -independent intracellular pathway has been observed in human endothelial and smooth muscle cells (Feoktistov et al. 2004), but involvement of HIF-1 α with the A₃AR has been demonstrated in different cancer cell lines (Merighi et al. 2005b, 2006, 2007). In particular, activation of the A₃AR subtype in glioblastoma and colon carcinoma cells stimulates VEGF expression in an HIF-1 α -dependent manner (Merighi et al. 2006, 2007). In addition, A₃AR activation results in increased expression of another angiogenic factor, angiopoietin 2, in melanoma cells and HMC-1 cells derived from a highly malignant, undifferentiated human mastocytoma cancer (Feoktistov et al. 2003; Merighi et al. 2005b). This may be relevant because the effect of adenosine on new capillary formation is potentiated by the concomitant stimulation of A_{2B}ARs and A₃ARs acting on VEGF and angiopoietin 2 levels, respectively (Feoktistov et al. 2003). Recent studies indicate that pharmacologic inhibition of HIF-1 α and particularly of HIF-regulated genes, which are important for cancer cell survival, may be more advantageous than HIF-gene-inactivation therapeutic approaches (Mabjeesh et al. 2003; Merighi et al. 2005b; Sitkovsky et al. 2004). In this regard, by blocking hypoxia-induced increases in HIF-1 α , angiopoietin 2 and VEGF protein expression in the tumor microenvironment, A₃AR antagonists may represent a novel approach to the treatment of cancer.

7 Summary and Conclusions

Adenosine, the natural ligand of the four AR subtypes, affects all of these receptors under neoplastic conditions due to its mass accumulation in the tumor microenvironment. Its role in maintaining pro- and anticancer effects via each of its receptor subtypes was extensively reviewed in this chapter. Based on the studies presented in this review, it appears that all the AR subtypes are possible targets for the development of novel approaches to the treatment of cancer.

The antitumorogenic role of A₁AR in cancer was mainly studied in A₁AR-deficient mice, demonstrating that activation of the A₁AR on microglia inhibits the growth of glioblastomas.

Based on a number of reports, it has been suggested that the A_{2A}AR blocks antitumor immunity. In the tumor environment of hypoxia and high adenosine levels, activation of A_{2A}ARs leads to T-cell tolerance, inhibition of effector immune cells (including T cells, CTLs, NK cells, dendritic cells, and macrophages), an increase in regulatory T cells, and a decrease in proinflammatory cytokines, all of which thwart antitumor immunity and thus encourage tumor growth. Importantly, A_{2A}AR-null mice have been shown to more readily reject melanoma and lymphoma tumor challenge and to also respond to vaccines. Moreover, treating mice with A_{2A}AR antagonists (including caffeine) leads to increased tumor rejection by CD8⁺ T cells. For all these reasons, it was suggested that the addition of A_{2A}AR antagonists to cancer immunotherapeutic protocols may enhance tumor immunotherapy. Interestingly, the safety of such compounds has already been shown in trials employing A_{2A}AR antagonists for the treatment of Parkinson's disease.

The role of the A_{2B}AR in cancer is not clear. On the one hand, under conditions of hypoxia and high adenosine levels in the tumor microenvironment, activation of A_{2B}ARs leads to the release of angiogenic factors that promote tumor growth, suggesting that the use of A_{2B}AR antagonists may represent a novel approach to the treatment of cancer. On the other hand, the activation of A_{2B}ARs exclusively expressed on the surface of breast cancer cell line MDA-MB-231 cells exerts an inhibitory signal mediated via the inhibition of ERK 1/2 phosphorylation, suggesting that A_{2B}AR agonists may produce anticancer effects. The resolution of this dilemma will initially come from testing selective ligands for the A_{2B}AR in *in vitro* and *in vivo* studies in various cancer cell lines and tumor-bearing animals, and then, depending on the results of these studies, perhaps in humans with cancer.

The unique characteristics of the A₃ARs that are highly expressed in tumor cells suggest that this receptor subtype is an attractive target to combat cancer. Targeting the A₃AR with synthetic agonists results in cell cycle arrest and apoptosis towards different cancer cells both *in vitro* and *in vivo*. Preclinical and Phase I studies show that these agonists are safe and well tolerated in humans and thus may be considered possible therapeutic agents for certain neoplasmas such as HCC, where a significant apoptotic effect was demonstrated. However, by blocking hypoxia-induced increases in HIF-1 α , angiopoietin 2 and VEGF protein expression in the tumor microenvironment, A₃AR antagonists may represent a novel approach for the treatment of cancer.

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Adenosine Receptors and the Kidney

Volker Vallon and Hartmut Osswald

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Abstract The autacoid, adenosine, is present in the normoxic kidney and generated in the cytosol as well as at extracellular sites. The rate of adenosine formation is enhanced when the rate of ATP hydrolysis prevails over the rate of ATP synthesis during increased tubular transport work or during oxygen deficiency. Extracellular adenosine acts on adenosine receptor subtypes (A_1 , A_{2A} , A_{2B} , and A_3) in the cell membranes to affect vascular and tubular functions. Adenosine lowers glomerular filtration rate by constricting afferent arterioles, especially in superficial nephrons, and thus lowers the salt load and transport work of the kidney consistent with the concept of metabolic control of organ function. In contrast, it leads to vasodilation in the deep cortex and the semihypoxic medulla, and exerts differential effects on NaCl transport along the tubular and collecting duct system. These vascular and tubular effects point to a prominent role of adenosine and its receptors in the intrarenal metabolic regulation of kidney function, and, together with its role in inflammatory processes, form the basis for potential therapeutic approaches in radiocontrast media-induced acute renal failure, ischemia reperfusion injury, and in patients with cardiorenal failure.

Keywords Adenosine receptors · Kidney · Tubuloglomerular feedback · Renin · Fluid and electrolyte transport · Metabolic control · Acute renal failure · Acute kidney injury · Radiocontrast media · Ischemia reperfusion injury · Heart failure

Abbreviations

AA	Afferent arteriole
ADO	Adenosine
ARF	Acute renal failure
A_x AR	Adenosine receptor subtype x
B	Bowman's capsule
BG9719	1,3-Dipropyl-8-[2-(5,6-epoxynorbornyl)] xanthine
BG9928	1,3-Dipropyl-8-[1-(4-propionate)-bicyclo-[2,2,2]octyl)]xanthine
BM	Basement membrane
BS	Bowman's space
cAMP	Cyclic adenosine monophosphate
CD39	Ecto-nucleoside triphosphate diphosphohydrolase-1
CD73	Ecto-5'-nucleotidase
CGS21680	2-[<i>p</i> -(2-Carboxyethyl)phenethylamino]-5'- <i>N</i> -ethylcarboxamido adenosine
CVT-124	<i>S</i> -Enantiomer of 1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)] 1xanthine
DMPX	3,7-Dimethyl-1-propargylxanthine
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine
DPSPX	1,3-Dipropyl-8-sulfophenylxanthine
DWH 146e	4-(3-(6-Amino-9-(5-ethylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9 <i>H</i> -purin-2-yl)prop-2-ynyl)cyclohexanecarboxylic acid methyl ester

EA	Efferent arteriole
EGM	Extraglomerular mesangium
ENTPDase	Ectonucleoside triphosphate diphosphohydrolase
FK-453	(+)-(R)-[(E)-3-(2-Phenylpyrazolo[1,5-a]pyridin-3-yl)acryloyl]-2-piperidine ethanol
FK-838	6-Oxo-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)-1(6H)-pyridazinebutanoic 'acid
GFR	Glomerular filtration rate
HSP27	Heat-shock protein 27
IMCD	Inner medullary collecting duct
KW-3902	8-(Noradamantan-3-yl)-1,3 dipropylxanthine
MBF	Medullary blood flow
MC	Mesangium cells
mTAL	Medullary thick ascending limb
NHE	Na ⁺ – H ⁺ exchanger
NKCC2	Na ⁺ – K ⁺ – 2Cl ⁻ cotransporter
NO	Nitric oxide
NY ₂ HA	New York Heart Association
pO ₂	Partial oxygen pressure
PT	Proximal tubule
SNGFR	Single nephron glomerular filtration rate
TAL	Thick ascending limb
TGF	Tubuloglomerular feedback
T _{Na}	Transport of sodium
VSMC	Vascular smooth muscle cells

1 Introduction

Adenosine is a tissue hormone that is locally generated in many organs and that binds to cell surface receptors to mediate various aspects of organ function. Many of these effects revolve around a role of adenosine in metabolic control of organ function, including local matching of blood flow with energy consumption. According to this concept, the interstitial concentration of adenosine rises when cells are in negative energy balance. Adenosine locally activates vasodilatory adenosine A₂ receptor (A₂AR) and adjusts blood flow to meet demand. The role of adenosine in the kidney is analogous but, as a consequence of the specific renal structural organization and function, more complicated than its role in other organs. We will first describe the differential effects of adenosine on the renal cortical and medullary vascular structures, and its role in tubuloglomerular feedback (TGF), the regulation of renin secretion and in transport processes in the tubular and collecting duct system. These issues are subsequently discussed with regard to a potential role of adenosine receptors as new potential targets in the treatment of patients with radiocontrast media-induced acute renal failure, ischemia-reperfusion injury, and in

patients with acute decompensated heart failure or cardiorenal failure. Please see recent reviews on the expression of adenosine receptors in the kidney and the role of adenosine in kidney function in general (Vallon et al. 2006), and in acute renal failure (Osswald and Vallon 2009) and fluid retention in particular (Welch 2002; Modlinger and Welch 2003; Vallon et al. 2008).

2 Vascular Effects of Adenosine in Kidney Cortex and Medulla

In contrast to other organs, blood flow into the cortex of the kidney generates, via the formation of an ultrafiltrate, the metabolic burden for tubular electrolyte transport and thus the demand for energy. Hence, to recover from negative energy balance in the kidney, a mechanism is required that lowers glomerular filtration rate (GFR) or the ratio between glomerular filtration rate and cortical renal blood flow. In comparison, blood flow in the renal medulla is nutritive. It derives from the postglomerular circulation of deep nephrons, and due to the way the kidney concentrates the urine, blood flow and O₂ supply are low in this area, although active NaCl reabsorption in the medullary thick ascending limb is essential for function. With regard to metabolic control, this requires a *vasodilator* to prevent hypoxic injury in the renal medulla. As outlined in the following, adenosine is a vasodilator in the renal medulla but induces cortical vasoconstriction and lowers GFR.

2.1 Activation of A₁AR Lowers Glomerular Filtration Rate

Healthy volunteers responded to an intravenous infusion or direct application of adenosine into the renal artery with a reduction in GFR of 15–25% while blood pressure and renal blood flow were unchanged (Edlund and Sollevi 1993; Edlund et al. 1994; Balakrishnan et al. 1996). Adenosine infusion into the renal artery of rats or dogs reduced single-nephron GFR (SNGFR) in superficial nephrons to a larger extent than whole-kidney GFR, indicating that deep-cortical vasodilation (see below) counteracts superficial vasoconstriction (Osswald et al. 1978a, b; Haas and Osswald 1981). Adenosine lowers SNGFR in superficial nephrons due to afferent arteriolar vasoconstriction (Osswald et al. 1978b; Haas and Osswald 1981) (Fig. 1). Direct videometric assessment of pre- and postglomerular arteries using the “split-hydronephrotic” rat kidney technique revealed adenosine-induced constriction of afferent arterioles via high-affinity A₁AR and dilation via activation of both high-affinity A_{2A}AR and low-affinity A_{2B}AR (Tang et al. 1999). Whereas activation of A₁AR led to the constriction of mainly afferent arterioles near the glomerulus, A₂AR activation lead to the dilation of mainly postglomerular arteries (Holz and Steinhausen 1987; Dietrich and Steinhausen 1993; Gabriels et al. 2000). A₁AR-mediated afferent arteriolar constriction involves a pertussis toxin-sensitive G_i protein and subsequent activation of phospholipase C, presumably through βγ

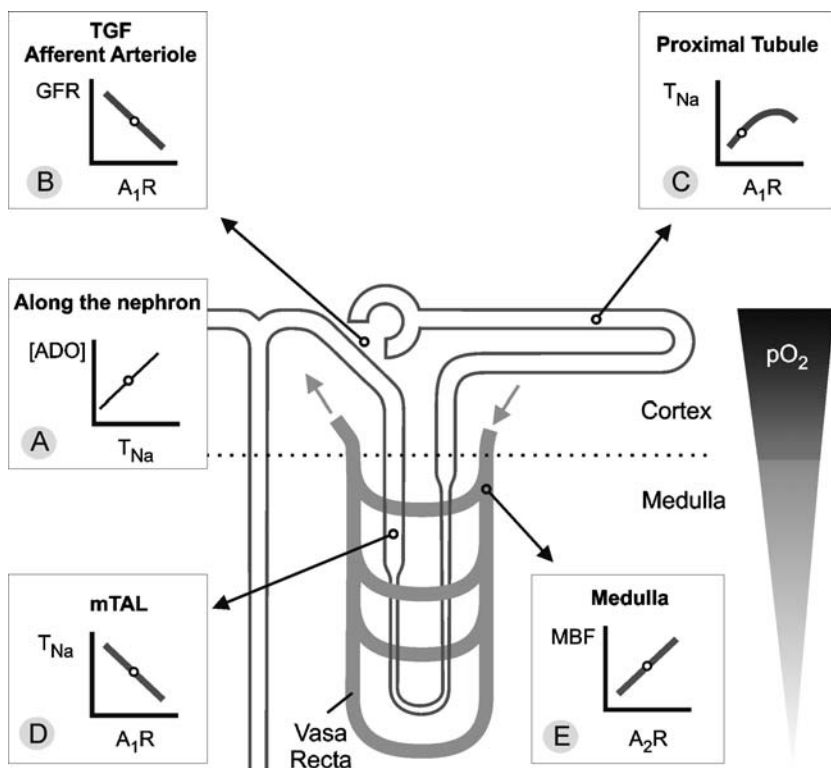


Fig. 1 a–e Control of renal hemodynamics and transport by adenosine (ADO). The *line plots* illustrate the relationships between the given parameters. *Small circles* on these lines indicate ambient physiological conditions. In general, the medulla is at greater risk for hypoxic damage than the cortex due to a lower partial oxygen pressure (pO_2). **a** In every nephron segment, an increase in reabsorption or transport of sodium (T_{Na}) increases extracellular ADO. **b** ADO via A_1AR mediates tubuloglomerular feedback (TGF) and constricts the afferent arteriole to lower GFR. **c** In the proximal tubule, ADO via A_1AR stimulates T_{Na} and thus lowers the Na^+ load to segments residing in the semihypoxic medulla. **d** In contrast, ADO via A_1AR inhibits T_{Na} in the medulla, including medullary thick ascending limb (mTAL). **e** In addition, ADO via A_2AR enhances medullary blood flow (MBF), which increases O_2 delivery and further limits O_2 -consuming transport in the medulla (adapted from Vallon et al. 2006)

subunits released from $G_{\alpha i}$ (Hansen et al. 2003b). $A_{2A}AR$ -mediated renal vasodilation may involve activation of ATP-regulated potassium channels (Tang et al. 1999) and endothelial nitric oxide synthase (Hansen et al. 2005).

Oral application of the A_1AR antagonist (+)-(R)-[(E)-3-(2-phenylpyrazolo [1,5-a]pyridin-3-yl)acryloyl]-2-piperidine ethanol (FK-453) to healthy male subjects increased GFR by $\sim 20\%$ without significantly altering effective renal plasma flow or mean arterial blood pressure (Balakrishnan et al. 1993), providing evidence that *endogenous* adenosine elicits a tonic suppression of GFR through the activation of A_1AR . Consistent with a prominent role of adenosine in the regulation of afferent

arteriolar tone, autoregulation of renal blood flow and glomerular filtration rate (i.e., their constancy in spite of changes in renal perfusion pressure) is dependent upon the activation of A₁AR (Hashimoto et al. 2006).

2.2 Factors Modulating Adenosine-Induced Cortical Vasoconstriction

Suppression of the renin–angiotensin system by dietary salt or pharmacological means reduces or blocks the renal vasoconstrictive action of adenosine (Osswald et al. 1975, 1982; Spielman and Osswald 1979; Arend et al. 1985; Macias-Nunez et al. 1985; Dietrich et al. 1991; Dietrich and Steinhausen 1993). In contrast, activation of the renin–angiotensin system potentiates adenosine-induced vasoconstriction and lowering of GFR (Osswald et al. 1975, 1978a, 1982). Further studies identified a mutual dependency and cooperation of adenosine and angiotensin II in producing afferent arteriolar constriction (Weihprecht et al. 1994; Traynor et al. 1998; Hansen et al. 2003a). Adenosine enhances angiotensin II-induced constriction of afferent arterioles by receptor-dependent and -independent pathways. The latter involves adenosine uptake and intracellular effects that increase the calcium sensitivity by phosphorylating the myosin light chain (Lai et al. 2006; Patzak et al. 2007). Moreover, inhibiting the synthesis of vasodilators like nitric oxide (NO) (Barrett and Droppleman 1993; Pflueger et al. 1999b) or prostaglandins (Spielman and Osswald 1978; Pflueger et al. 1999a) increases the sensitivity of the kidney to adenosine-induced vasoconstriction. The outlined interactions can be of clinical relevance.

2.3 Activation of A₂AR Induces Medullary Vasodilation

Intrarenal adenosine infusion in rats initially induces vasoconstriction in all cortical zones; this is followed by persistent superficial cortical vasoconstriction but *deep cortical* vasodilation (Macias-Nunez et al. 1983; Miyamoto et al. 1988). While A₁AR-mediated afferent arteriolar constriction dominates in *superficial* nephrons, *deep cortical* glomeruli, which supply the blood flow to the renal medulla, can respond to adenosine with A₂AR-mediated vasodilation (Inscho et al. 1991; Weihprecht et al. 1992; Inscho 1996; Yaoita et al. 1999; Nishiyama et al. 2001). In accordance, renal interstitial infusion in rats of the A₂AR agonist 2-[*p*-(2-carboxyethyl)phenethylamino]-5'-*N*-ethylcarboxamido adenosine (CGS-21680) increased medullary blood flow (Agmon et al. 1993), whereas intramedullary infusion of the selective A₂AR antagonist 3,7-dimethyl-1-propargylxanthine (DMPX) (but not the A₁AR antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX)) decreased medullary blood flow (Zou et al. 1999). This indicates that *endogenous* adenosine dilates medullary vessels and sustains medullary blood flow via activation of A₂AR (Fig. 1).

2.4 Adenosine is a Mediator of Tubuloglomerular Feedback via Activation of A₁AR

The mammalian kidney has a rather high GFR (~180 l per day in humans). About 99% of the filtered fluid and NaCl are subsequently reabsorbed along the tubular and collecting duct system, such that urinary excretion closely matches intake. As a result, GFR is a significant determinant of renal transport work, and GFR and reabsorption have to be closely coordinated to avoid renal loss or retention of fluid and NaCl. The tubuloglomerular feedback (TGF) is a mechanism that helps to coordinate GFR with the tubular transport activity or capacity. In this mechanism, specialized tubular cells, the macula densa, sense the tubular NaCl load at the end of the thick ascending limb (TAL; where about 85% of the filtered NaCl has been reabsorbed), and induce a change in afferent arteriolar tone such that an inverse relationship is established between the tubular NaCl load and SNGFR of the same nephron. This way, the TGF stabilizes the NaCl load to further distal segments, where the fine regulation of NaCl and fluid balance takes place under *systemic* neurohumoral control.

The TGF response, in other words an inverse change in SNGFR or glomerular capillary pressure in response to changes in the NaCl concentration at the macula densa, is inhibited by unselective adenosine receptor blockers like theophylline or 1,3-dipropyl-8-sulfophenylxanthine (DPSPX) (Schnermann et al. 1977; Osswald et al. 1980; Franco et al. 1989), as well as by selective A₁AR antagonists like DPCPX, 8-(noradamantan-3-yl)-1,3-dipropylxanthine (KW-3902, rolofylline), CVT-124 (the *S*-enantiomer of the highly selective racemic A₁AR antagonist 1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)] xanthine), or 6-oxo-3-(2-phenylpyrazolo [1,5-*a*]pyridin-3-yl)-1(6*H*)-pyridazinebutanoic acid (FK838) (Franco et al. 1989; Schnermann et al. 1990; Kawabata et al. 1998; Wilcox et al. 1999; Thomson et al. 2000; Ren et al. 2002a). Mice with gene knockout for A₁AR lack the TGF response (Brown et al. 2001; Sun et al. 2001; Vallon et al. 2004), and have an impaired ability to stabilize the Na⁺ delivery to the distal tubule (Vallon et al. 2004). Most importantly, an intact TGF response requires local concentrations of adenosine to fluctuate depending on the NaCl concentration in the tubular fluid at the macula densa, indicating that adenosine serves as a *mediator* of TGF (Thomson et al. 2000).

In 1980, Osswald and colleagues proposed that adenosine may be a mediator of TGF. Figure 2 illustrates a current model. Changes in luminal concentrations of Na⁺, K⁺, and Cl⁻ alter NaCl uptake by macula densa cells via the furosemide-sensitive Na-K-2Cl cotransporter in the luminal membrane. This triggers basolateral ATP release (Bell et al. 2003; Komlosi et al. 2004) as well as transport-dependent hydrolysis by basolateral Na⁺-K⁺-ATPase (Lorenz et al. 2006) of ATP to AMP. Plasma membrane-bound ectonucleoside triphosphate diphosphohydrolase 1 (CD39) converts ATP and ADP to AMP (Oppermann et al. 2008) and ecto-5'-nucleotidase (CD73) converts extracellular AMP to adenosine (Thomson et al. 2000; Castrop et al. 2004; Ren et al. 2004; Huang et al. 2006). Part of the extracellular adenosine involved in the TGF response is generated independent of

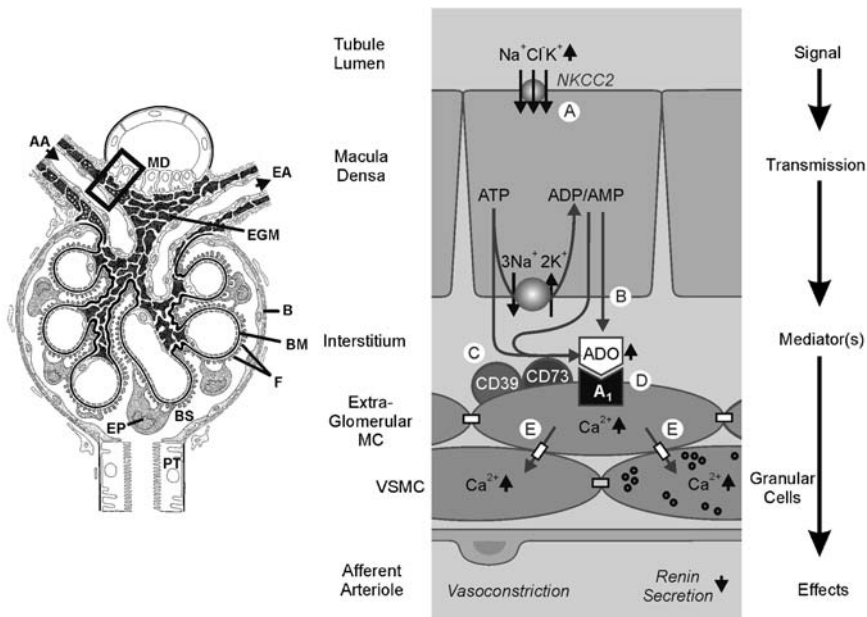


Fig. 2 a–e Adenosine is a mediator of the tubuloglomerular feedback: a proposed mechanism. *Left panel:* schematic drawing illustrating the macula densa (MD) segment at the vascular pole with the afferent arteriole (AA) entering and the efferent arteriole (EA) leaving the glomerulus; extraglomerular mesangium (EGM); glomerular basement membrane (BM); epithelial podocytes (EP) with foot processes (F); Bowman’s capsule (B) and space (BS), respectively; proximal tubule (PT). (Adapted from Kriz, Nonnenmacher and Kaissling). *Right panel:* schematic enlargement of area in rectangle. An increase in concentration-dependent uptake of Na⁺, K⁺ and Cl⁻ via the furosemide-sensitive Na⁺ – K⁺ – 2Cl⁻ cotransporter (NKCC2) **a** leads to transport-related, intra- and/or extracellular generation of adenosine (ADO) **b, c**. Extracellular ADO activates A₁AR, triggering an increase in cytosolic Ca²⁺ in extraglomerular mesangium cells (MC) **d**. The intensive coupling between extraglomerular MC, granular renin-containing cells, and vascular smooth muscle cells (VSMC) of the afferent arteriole by gap junctions allows propagation of the increased Ca²⁺ signal **e**, resulting in afferent arteriolar vasoconstriction and inhibition of renin release (adapted from Vallon et al. 2006)

ecto-5'-nucleotidase and may reflect direct adenosine release from macula densa cells (Huang et al. 2006). Extracellular adenosine binds to A₁AR at the surface of extraglomerular mesangial cells (Olivera et al. 1989; Weaver and Reppert 1992; Toya et al. 1993; Smith et al. 2001) and increases cytosolic Ca²⁺ concentrations (Olivera et al. 1989). Gap junctions between extraglomerular mesangial cells and smooth muscle cells of glomerular arterioles can transmit intracellular Ca²⁺ transients to these target structures, inducing afferent arteriolar constriction (Iijima et al. 1991; Ren et al. 2002b). Potential candidates for the formation of gap junctions in the juxtaglomerular apparatus include connexins 37, 40, and 43 (Wagner et al. 2007; Takenaka et al. 2008a, b).

3 Activation of A₁AR Inhibits Renin Secretion

Tagawa and Vander reported in 1970 that adenosine infusion into the renal artery of salt-depleted dogs inhibited the renal secretion of renin into the venous blood (Tagawa and Vander 1970). This was confirmed in various species including humans (Osswald et al. 1978b; Edlund et al. 1994). Most notably, a single application of the A₁AR antagonist FK-453 increased plasma renin concentrations in humans (Balakrishnan et al. 1993), indicating a tonic inhibition of renin secretion by A₁AR activation. In accordance, knockout mice for A₁AR have increased renal mRNA expression and content of renin (Schweda et al. 2003) as well as greater plasma renin activity (Brown et al. 2001; Rieg et al. 2007) compared with wild-type mice.

Jackson and coworkers proposed an extracellular cyclic adenosine monophosphate (cAMP)-adenosine pathway in the control of renin release: the increase in intracellular cAMP in renin-secreting cells causes efflux of cAMP, the latter being converted to adenosine in the extracellular space. The generated adenosine, by acting on A₁AR on the renin-secreting cells, then acts as a negative-feedback control on renin release (Jackson and Raghvendra 2004). In addition, *high* NaCl concentrations in the tubular lumen enhance adenosine generation in a macula densa-dependent way, and the adenosine generated inhibits renin release via activation of A₁AR (Itoh et al. 1985; Weihprecht et al. 1990; Lorenz et al. 1993; Kim et al. 2006) (Fig. 2). In contrast to A₁AR stimulation, activation of A₂AR can increase renin secretion (Churchill and Churchill 1985; Churchill and Bidani 1987). The latter may have contributed to the observation that the unselective adenosine receptor antagonist caffeine reduced plasma renin concentration in mice lacking A₁AR (Rieg et al. 2007).

4 Differential Effects of Adenosine on Fluid and Electrolyte Transport

In addition to its effects on renal blood flow, GFR, and renin release, adenosine induces *direct* effects on fluid and electrolyte transport along the tubular and collecting duct system.

4.1 Activation of A₁AR Increases Reabsorption in the Proximal Tubule

Endogenously formed adenosine can *stimulate* proximal tubular reabsorption of fluid, Na⁺, HCO₃⁻, and phosphate by activation of A₁AR (Takeda et al. 1993; Cai et al. 1994, 1995; Tang and Zhou 2003). Importantly, systemic application of selective A₁AR antagonists (such as CVT-124, DPCPX, KW-3902, or FK-453) elicits diuresis and natriuresis predominantly by inhibiting reabsorption in the proximal tubule in rats and humans (Mizumoto and Karasawa 1993; Balakrishnan et al. 1993;

van-Buren et al. 1993; Knight et al. 1993b; Wilcox et al. 1999; Miracle et al. 2007), indicating a tonic stimulation of proximal tubular reabsorption via A_1AR activation (Fig. 1). As a consequence, selective A_1AR antagonists are being developed as eukaliuretic natriuretics in Na^+ -retaining states such as heart failure (see below). A_1AR -mediated increases in proximal tubular reabsorption may involve increases of intracellular Ca^{2+} (Di Sole et al. 2003), reductions of intracellular cAMP levels (Kost Jr et al. 2000), and activation of the Na^+-H^+ exchanger (NHE3) (Di Sole et al. 2003).

Similar to selective A_1AR blockade, systemic application or consumption of the unselective adenosine receptor antagonist theophylline or caffeine induces natriuretic and diuretic responses. These responses to theophylline and caffeine are absent in mice lacking A_1AR , strongly suggesting that A_1AR blockade mediates the natriuresis and diuresis in response to these compounds (Rieg et al. 2005).

4.2 Activation of A_1AR Inhibits Reabsorption in Medullary Thick Ascending Limb

In contrast to the proximal tubule, adenosine via activation of A_1AR inhibits $NaCl$ reabsorption in medullary TAL (Torikai 1987; Burnatowska-Hledin and Spielman 1991; Beach and Good 1992). Medullary TAL is a site of adenosine release, and adenosine release in this segment is transport dependent (Beach et al. 1991; Baudouin-Legros et al. 1995) and enhances significantly during hypoxic conditions (Beach et al. 1991). Studies using pharmacological inhibition (Zou et al. 1999) or gene knockout (Vallon et al. 2004) are consistent with a tonic inhibition of Na^+ reabsorption in medullary TAL by A_1AR activation (Fig. 1). This is relevant since the renal medulla has a low partial oxygen pressure (Brezis and Rosen 1995). The described inhibitory effects of adenosine on transport work together with its A_2AR -mediated renal medullary vasodilation (see above) may serve to maintain metabolic balance in the renal medulla.

4.3 Effects of Adenosine on Transport in Distal Convolution and Cortical Collecting Duct

In general, natriuretics that act proximal to the aldosterone-sensitive distal nephron stimulate K^+ secretion in the latter segment and thus increase renal K^+ excretion. The natriuretic but eukaliuretic effect of A_1AR inhibitors suggests an additional site of action in the aldosterone-sensitive distal nephron, but the exact site of action and the involved mechanisms are unclear.

A_1AR activation can stimulate Mg^{2+} and Ca^{2+} uptake in the cortical collecting duct in vitro (Hoenderop et al. 1998, 1999; Kang et al. 2001), but the clinical relevance (e.g., during pharmacological inhibition of A_1AR) is not known.

4.4 Activation of A₁AR Counteracts Vasopressin Effects in Inner Medullary Collecting Duct

Extracellular adenosine feedback can inhibit vasopressin-induced cAMP-mediated stimulation of Na⁺ and fluid reabsorption in the inner medullary collecting duct (IMCD) (Yagil 1990; Yagil et al. 1994; Rieg et al. 2008) and decrease vasopressin-stimulated electrogenic Cl⁻ secretion through the activation of A₁AR (Moyer et al. 1995). Vasopressin-induced adenosine may derive from the extracellular cAMP-adenosine pathway (Jackson et al. 2003) or follow the cellular release and breakdown of ATP (Vallon 2008). Studies on water transport in knockout mice indicate efficient compensation by other pathways in the absence of A₁AR, including upregulation of ATP-sensitive P2Y₂ receptors (Rieg et al. 2008).

5 Adenosine and Metabolic Control of Kidney Function

The above outlined functions of adenosine can be integrated into the concept of metabolic control of renal function (Fig. 1). Adenosine-induced vasoconstriction via A₁AR activation is predominant in the outer cortex by increasing the resistance of afferent arterioles, which lowers GFR and thus renal transport work. Under physiological conditions, adenosine-induced afferent arteriolar constriction primarily derives from tonic activation of the TGF, for which adenosine acts as a mediator. Adenosine via A₁AR tonically stimulates NaCl reabsorption in the cortical proximal tubule, which is a tubular segment with a relatively high basal oxygen supply, thereby limiting the NaCl load to downstream medullary segments. In the deep cortex and medulla, adenosine induces vasodilation via A₂AR activation, which is associated with an increase of medullary blood flow and thus increased medullary oxygenation. Moreover, adenosine inhibits NaCl reabsorption in medullary TAL and IMCD (i.e., nephron segments with relatively low oxygen delivery). In addition, the A₂AR-mediated rise in medullary blood flow lowers medullary transport activity by washing out the high osmolality in the medullary interstitium (Zou et al. 1999). In accordance, interstitial infusion of adenosine in rat kidney decreased partial pressure of O₂ in the cortex but increased it in the medulla, consistent with an important regulatory and protective role of adenosine in renal medullary O₂ balance (Dinour and Brezis 1991).

6 Adenosine and Acute Renal Failure

The renal effects of adenosine fit into the concepts of acute renal failure (ARF) in as much as adenosine is an *intrarenal* metabolite that accumulates in the kidney during renal ischemia and that can lower GFR. In addition, ischemia or nephrotoxins can

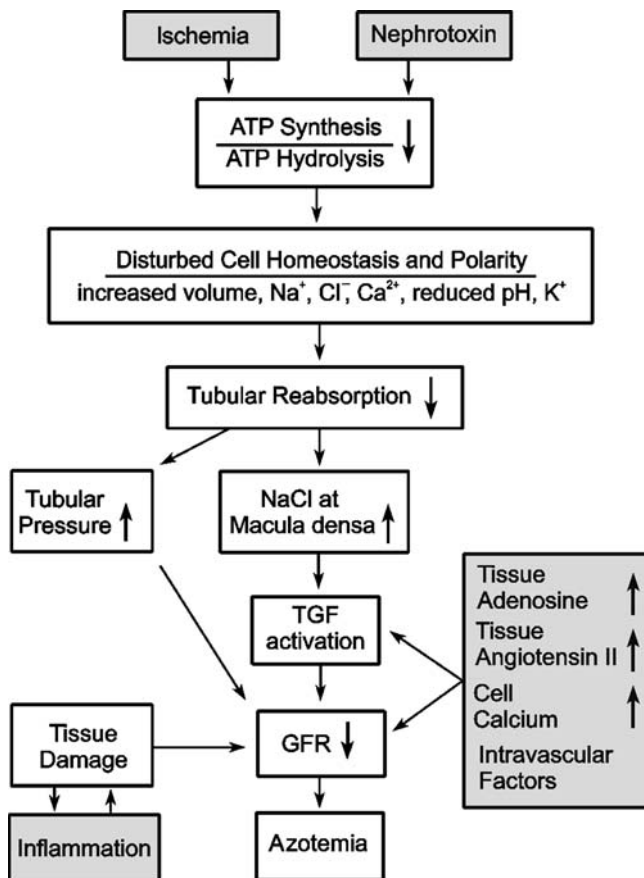


Fig. 3 Schematic illustration of intrarenal mechanisms in acute renal failure. See text for further explanation (adapted from Osswald and Vallon 2009)

inhibit renal transport activity, with the resulting increase in the NaCl concentration at the macula densa further lowering GFR (Fig. 3). Moreover, experimental models of ARF can be associated with increased expression of A₁AR in glomeruli, which may contribute to depressed GFR (Smith et al. 2000). Thus, inhibition of adenosine vasoconstrictor actions in the kidney could be beneficial in conditions of ARF. On the other hand, the ARF-associated reduction in GFR and thus in tubular NaCl load may, to some extent, protect the tubular system—especially the medulla—from hypoxic injury, and the body from excess NaCl loss. Moreover, adenosine can induce direct cytoprotective effects in renal cells. Therefore, inhibition of adenosine receptors in ARF could be a two-sided sword. In the following we discuss the role of adenosine in ARF induced by radiocontrast media and ischemia-reperfusion, respectively.

6.1 Radiocontrast Media-Induced Acute Renal Failure: Theophylline and A₁AR Antagonists Induce Protective Effects

Application of radiocontrast media to humans can lead to an impairment of renal function, including a fall in GFR. Concomitant volume and NaCl depletion increases the severity and can result in ARF. Unselective or A₁AR-selective antagonists can prevent renal impairment induced by radiocontrast media, as shown in dogs (Arend et al. 1987), rats (Erley et al. 1997), mice (Lee et al. 2006), and, most importantly, in humans (Erley et al. 1994; Katholi et al. 1995; Kolonko et al. 1998; Kapoor et al. 2002; Huber et al. 2002, 2003). In accordance, mice lacking A₁AR preserved kidney function better, and had lesser renal cortical vacuolization and enhanced survival 24 h after radiocontrast media treatment compared with wild-type mice (Lee et al. 2006). In comparison, dipyridamole, which increases extracellular adenosine concentrations, augmented the severity of renal impairment in response to radiocontrast media in dogs (Arend et al. 1987) and humans (Katholi et al. 1995). Two studies indicated that the unselective adenosine receptor antagonist theophylline is as effective as saline hydration at preventing ARF in response to contrast media, but the benefits of the two maneuvers are not additive (Abizaid et al. 1999; Erley et al. 1999). Thus, use of theophylline can be beneficial in patients where sufficient hydration may be impossible or in patients with a concomitant decrease in renal blood flow (e.g., congestive heart failure or chronic renal insufficiency (Erley et al. 1999; Huber et al. 2002)). A recent meta-analysis of clinical trials concluded that theophylline may reduce the incidence of radiocontrast media-induced nephropathy, and recommended a large, well-designed trial to more adequately assess the role of theophylline in this condition (Bagshaw and Ghali 2005). Notably, unselective or A₁AR-selective antagonists can also prevent renal impairment in response to other nephrotoxic substances (Table 1).

6.2 Ischemia-Reperfusion Injury

Ischemia-reperfusion injury plays a major role in delayed graft function and long-term changes after kidney transplantation. It has become evident that the cellular and molecular mechanisms that operate during ischemia and reperfusion resemble an acute inflammatory response (Gueler et al. 2004). To what extent the acute cellular alterations persist and affect organ function later on remains unclear.

In the kidney, extracellular adenosine derives to a large extent from the extracellular breakdown of ATP and ADP to AMP and adenosine via ectonucleoside triphosphate diphosphohydrolases (ENTPDases) and CD73 (Grenz et al. 2007a, b). Using knockout mouse models for these ectoenzymes, Grenz et al. showed that CD39-dependent nucleotide phosphohydrolysis as well as CD73-dependent adenosine formation serve to protect against renal ischemia-reperfusion injury and to

Table 1 Adenosine receptor antagonists improve renal function in various models of nephrotoxic acute renal failure (ARF)

Models of ARF	Species	Adenosine antagonist	References	
Glycerol Injection	Rat	Theophylline	Bidani and Churchill (1983); Bidani et al. (1987)	
		8-Phenyl-theophylline	Bowmer et al. (1986); Yates et al. (1987) Kellett et al. (1989); Panjehshahin et al. (1992)	
		DPCPX		
		FK-453	Ishikawa et al. (1993)	
		KW-3902	Suzuki et al. (1992)	
Uranyl nitrate	Rat	Theophylline	Osswald et al. (1979)	
Cisplatin	Rat	Theophylline	Heidemann et al. (1989)	
	Human	DPCPX	Knight et al. (1991)	
		KW-3902	Nagashima et al. (1995)	
		Theophylline	Benoehr et al. (2005)	
Contrast media	Dog	Theophylline	Arend et al. (1987)	
	Human	Theophylline	Erley et al. (1994); Katholi et al. (1995); Kolonko et al. (1998); Kapoor et al. (2002); Huber et al. (2002, 2003)	
	Rat	DPCPX, KW3902	Erley et al. (1997); Yao et al. (2001)	
Endotoxin	Rat	DPCPX	Knight et al. (1993a)	
Amphotericin B	Rat	Theophylline	Heidemann et al. (1983)	
	Dog	Theophylline	Gerken et al. (1983)	
Gentamicin	Rat	KW-3902	Yao et al. (1994)	

8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), (+)-(R)-[(E)-3-(2-phenylpyrazolo[1,5-a]pyridin-3-yl)acryloyl]-2-piperidine ethanol (FK-453) and 8-(noradamantan-3-yl)-1,3 dipropylxanthine (KW-3902) are A₁ AR-selective antagonists. Adapted from Vallon et al. (2006)

increase the ischemia tolerance of the kidney. In addition, the authors presented evidence that treatment with apyrase or soluble 5'-nucleotidase to increase extracellular adenosine concentrations could serve as potential novel pharmacological approaches to renal diseases precipitated by limited oxygen availability (Grenz et al. 2007a, b).

6.2.1 Theophylline Induces Protective Effects

Different animal studies assessed the effect of a *single* application of the unselective adenosine receptor antagonist theophylline in ischemia-reperfusion injury. Animals were pretreated with theophylline or it was given at day 5 after the renal ischemic/hypoxemic event. Pretreatment with a single dose of theophylline in rats attenuated the reduction in renal blood flow and GFR observed during the initiation phase of postischemic ARF as determined 1 h after releasing a 30 or 45 min occlusion of the renal artery (Lin et al. 1986). Similar results were obtained with

theophylline in the rabbit (Gouyon and Guignard 1988). In rats subjected to 60 min occlusion of the left renal artery, theophylline given i.v. 20 min before the release of the renal artery clamp in doses which antagonize the renal actions of adenosine in vivo improved the recovery of renal function after ischemic injury by increasing urinary flow rate, GFR (measured by inulin clearance), and histology, as assessed by morphometric quantification of tubular damage, tubular obstruction and pathologic alteration of glomeruli at 3 h after initiating reperfusion (Osswald et al. 1979; Helmlinger 1979). In contrast, pretreating rats prior to renal artery occlusion for 30 min with dipyridamole, which increases extracellular adenosine concentrations, intensified the fall in renal blood flow and GFR determined about 1 h after releasing the clamp, and this impairment was blocked by theophylline (Lin et al. 1987).

Notably, single-dose pretreatment of rats with theophylline during a 30 min renal artery occlusion lead to increased renal blood flow and GFR during the maintenance phase of ARF after five days, indicating that the effects of theophylline in the acute phase affected the outcome in the maintenance phase (Lin et al. 1988). Similarly, a single dose of theophylline, given early after birth in asphyxiated full-term infants, has beneficial effects in reducing the renal involvement and fall in GFR as determined over the first five days (Bakr 2005). Finally, acute theophylline treatment given at five days after ischemia acutely increases renal blood flow and GFR in previously untreated rats, indicating that adenosine contributes to the suppression of renal blood flow and GFR in the maintenance phase of ischemia-reperfusion injury (Lin et al. 1988). These data provide strong evidence that pretreatment with theophylline can exert beneficial effects in the initiation and maintenance phase of ischemia-reperfusion injury.

6.2.2 Adenosine Induces Protective Effects via A₁AR and A₂AR

Similar to theophylline, systemic intravenous infusion of adenosine (1.75 mg kg⁻¹ min⁻¹ × 10 min, intravenously) 2 min before a 45 min ischemic insult protected renal function against ischemia and reperfusion injury, as indicated by lower blood urea nitrogen and creatinine and improved renal morphology after 24 h of reperfusion. The effects of adenosine were proposed to be mediated by A₁AR (Lee and Emala 2000), involve G_{i/o} proteins and protein kinase C activation (Lee and Emala 2001a), and include a reduction in inflammation, necrosis, and apoptosis (Lee et al. 2004a). Direct cytoprotective effects of endogenous A₁AR activation in renal proximal tubules involve modulation of heat-shock protein (HSP)27 due to A₁AR-mediated enhancement of p38 and AP2 mitogen-activated protein kinase activities (Lee et al. 2007). In comparison, mice lacking A₁AR exhibited significantly higher plasma creatinines and worsened renal histology compared with wild-type mice at 24 h after renal ischemia for 30 min (Lee et al. 2004b). Similarly, wild-type mice pretreated with an A₁AR antagonist or agonist demonstrated worsened or improved renal function, respectively, after ischemia-reperfusion that was associated with increased or reduced markers of renal inflammation, respectively (Lee et al. 2004b) (Fig. 3). More recent work indicated that A₁AR activation produces not only acute

but also delayed renal protection; i.e., pretreatment with a selective A₁AR agonist 24 h before renal ischemia was also protective against renal ischemia-reperfusion injury. Furthermore, the study showed that acute protection from A₁AR activation is dependent on protein kinase C and Akt activation, whereas the delayed protection is dependent on Akt activation and induction of HSP27 (Joo et al. 2007).

Continuous application in the reperfusion period of 4-(3-(6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9H-purin-2-yl)prop-2-ynyl)cyclohexanecarboxylic acid methyl ester (DWH-146e), a selective A_{2A}AR agonist, protected kidneys from ischemia-reperfusion injury, as evidenced by a lower rise in serum creatinine and blood urea nitrogen following 24 and 48 h of reperfusion. Histological examination revealed widespread tubular epithelial necrosis and vascular congestion in the outer medulla of vehicle-treated rats. These lesions were significantly reduced in DWH-146e-treated animals (Okusa et al. 1999). Similarly, systemic adenosine given after 45 min of renal ischemia but before reperfusion protected renal function, as indicated by lower rises in creatinine and less histologically evident renal tubular damage. Pharmacological maneuvers indicated that these effects of adenosine were mediated by A_{2A}AR activation (Lee and Emala 2001b). Whereas A_{2A}AR activation could improve medullary hypoxia, other studies suggested that protection from renal ischemia-reperfusion injury by A₂AR agonists or endogenous adenosine requires activation of A₂AR expressed on bone marrow-derived cells (Day et al. 2003). Activation of A_{2A}AR on macrophages was also shown to inhibit inflammation in a rat model of glomerulonephritis (Garcia et al. 2008). Moreover, activation of A_{2B}AR in the renal vasculature contributes to the increased ischemia tolerance produced by the procedure of renal ischemic preconditioning (Grenz et al. 2008).

Finally, A₃AR stimulation in rats deteriorated renal ischemia-reperfusion injury, whereas inhibition of A₃AR protected renal function as efficiently as preconditioning (Lee and Emala 2000). In accordance, mice lacking A₃AR presented significant renal protection, functionally and morphologically, from ischemic or myoglobinuric renal failure (Lee et al. 2003). The mechanisms of these A₃AR-mediated effects are not understood at present.

In summary, beneficial effects on GFR and renal morphology beyond 3–24 h of reperfusion after ischemia can be induced by (1) pretreatment with the unselective adenosine receptor antagonist theophylline, (2) pretreatment or treatment immediately before reperfusion with adenosine, (3) pretreatment with A₁AR agonists, (4) treatment immediately before or during reperfusion with A_{2A}AR agonists, (5) treatment with A_{2B}AR agonists, and (6) deficiency of A₃AR. In comparison, the outcome is worsened by (1) pretreatment with A₁AR antagonists or deficiency of A₁AR, (2) pretreatment with A_{2B}AR antagonists or deficiency of A_{2B}AR, and (3) pretreatment with A₃AR agonists. The findings appear contradictory because theophylline can inhibit both A₁AR and A_{2A}AR, and possibly acts as an agonist at A₃AR (Ezeamuzie 2001). Further studies are necessary to resolve this issue, which may relate to the nature of adenosine being a double-edged sword in ARF, and the situation being further complicated by the role of adenosine in inflammatory responses.

7 A₁AR Antagonists in the Treatment of Cardiorenal Failure

Concomitant renal dysfunction is one of the strongest risk factors for mortality in ambulatory heart failure patients (Dries et al. 2000; Hillege et al. 2000; Mahon et al. 2002). In patients hospitalized for decompensated heart failure, worsening of renal function further predicts an adverse outcome (Forman et al. 2004). Intravenous loop diuretics are the mainstay of therapy for patients with both systemic volume overload and acute pulmonary edema decompensated heart failure. Treatment, however, may be complicated by diuretic resistance and/or worsening of renal function, indicating the need for alternative approaches.

Volume overload heart failure in dogs increases myocardial adenosine release (Newman et al. 1984), and circulating levels of adenosine can be increased in patients with chronic heart failure (~200 vs 60 nM) (Funaya et al. 1997) (Fig. 4). Whether this increases circulating adenosine to an extent that affects afferent arteriolar tone and thus GFR is unclear. Nonetheless, the renal vasculature in heart failure patients can be sensitized to the GFR-lowering effects of adenosine by the associated activation of the renin–angiotensin system and/or impairment of the local formation of NO (endothelial dysfunction) or prostaglandins (see above and Fig. 4). In addition, impaired renal perfusion and hypoxia enhance adenosine formation within the kidney (Nishiyama et al. 1999). As a consequence, the normally homeostatic adenosine system may become maladaptive and overshoots with regard to the downregulation of GFR in patients with heart failure. Fluid retention is further potentiated by stimulation of NaCl and fluid reabsorption in the proximal tubule, a mechanism also mediated by A₁AR activation (see above and Fig. 4). Based on this concept, pharmacological blockade of A₁AR could improve kidney function and fluid retention in heart failure. Since adenosine (through the activation of A₁AR) mediates TGF, the expected TGF-induced reduction in GFR in response to inhibition of proximal reabsorption by A₁AR antagonists should be blunted. In accordance, a study in rats showed that A₁AR antagonism with KW-3902 prevented the GFR-lowering effect of the proximal diuretic benzolamide, a carbonic anhydrase inhibitor (Miracle et al. 2007).

7.1 Animal Studies

Lucas et al. used a pig model of systolic dysfunction and induction of chronic heart failure by pacer-induced tachycardia. They observed that *acute* application of the selective A₁AR antagonist 1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)xanthine (BG9719) (CVT-124) increased creatinine clearance and urinary flow rate and sodium excretion. This was associated with lower pulmonary capillary wedge pressure and pulmonary vascular resistance in the absence of significant changes in mean arterial blood pressure, heart rate or cardiac output compared with vehicle control (Lucas Jr et al. 2002). Similar effects were described by Jackson et al. in aged, lean SHHF/Mcc-fa(cp) rats, a rodent model of hypertensive dilated cardiomyopathy

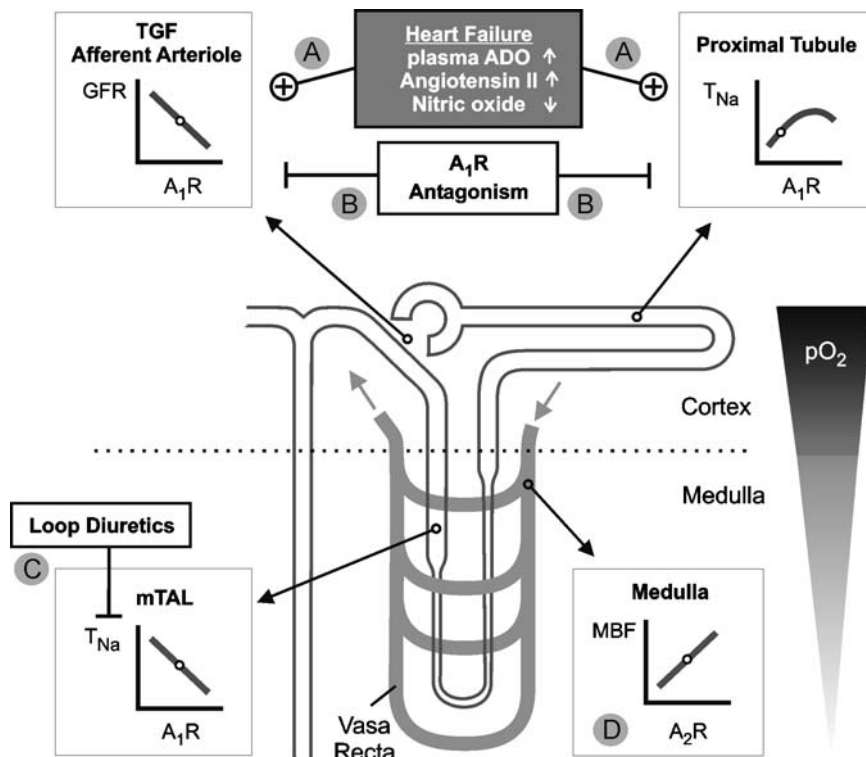


Fig. 4 a–d Basis for a therapeutic effect of A_1 AR antagonism in heart failure. The basic effects of adenosine on renal functions are outlined in the legend to Fig. 1. **a** Heart failure can be associated with increased plasma concentrations of adenosine (ADO) and angiotensin II, and endothelial dysfunction can impair nitric oxide (NO) formation, all of which can enhance the A_1 AR-mediated lowering of GFR and may, in addition, stimulate proximal reabsorption. **b** A_1 AR antagonism induces natriuresis and diuresis by inhibiting proximal reabsorption and preserving or increasing GFR. **c** A_1 AR antagonism can enhance sodium transport (T_{Na}) in semihypoxic medullary thick ascending limb (mTAL). This is prevented by coadministration of loop diuretics, and diuresis and natriuresis are potentiated. **d** A_2 AR-mediated medullary vasodilation is preserved (adapted from Vallon et al. 2008)

in response to the same compound (Jackson et al. 2001). The rats were pretreated for 72 h before experiments with the loop diuretic furosemide to mimic the clinical setting of chronic diuretic therapy, and were given 1% NaCl as drinking water to reduce dehydration/sodium depletion. Acute application of BG9719 increased GFR and urinary fluid and sodium excretion. In comparison, acute application of furosemide decreased renal blood flow and GFR and increased fractional potassium excretion. Neither drug altered afterload or left ventricular systolic function ($+dP/dt$ (max)); however, furosemide, but not BG9719, decreased preload and attenuated diastolic function (decreased $-dP/dt$ (max), increased tau). Thus, in the setting of left ventricular dysfunction, chronic salt loading and prior loop diuretic

treatment, selective A₁AR antagonists are effective diuretic/natriuretic agents that do not induce potassium loss and have a favorable renal hemodynamic/cardiac performance profile (Jackson et al. 2001).

7.2 Human Studies

Gottlieb et al. compared the *acute* effects of furosemide and BG9719 on renal function in 12 patients categorized as New York Heart Association (NYHA) functional classes II, III or IV (Gottlieb et al. 2000). Both BG9719 and furosemide increased sodium excretion compared with placebo. However, only furosemide lowered GFR. Subsequently, Gottlieb et al. compared BG9719 and furosemide in 63 patients categorized as NYHA functional classes II, III or IV, which despite receiving standard therapy, including furosemide (at least 80 mg daily) and angiotensin-converting enzyme inhibitors, remained edematous (Gottlieb et al. 2002). Patients received 7 h infusions of placebo or BG9719 to yield serum concentrations of 0.1, 0.75, or 2.5 $\mu\text{g ml}^{-1}$. BG9719 tripled urine output without lowering GFR or inducing kaliuresis. In comparison, furosemide increased urine output eightfold and increased potassium excretion while reducing GFR. Notably, when BG9719 was given with furosemide, GFR remained unaltered compared with placebo and sodium excretion increased further. These results indicate that A₁AR antagonism can preserve renal function while simultaneously promoting natriuresis during *acute* treatment of heart failure (Gottlieb et al. 2002).

Similar results were more recently reported in studies using the A₁AR antagonist KW-3902 in patients with congestive heart failure and impaired renal function (Dittrich et al. 2007; Givertz et al. 2007). Dittrich et al. assessed baseline GFR and renal plasma flow 3 h before and over 8 h following the intravenous administration of furosemide along with KW-3902 (30 mg) or placebo. After a washout period of 3–8 days (median six days), the crossover portion of the study was performed. KW-3902 increased GFR by 32% and renal plasma flow by 48% compared with placebo. Notably, subjects who initially received KW-3902 had a statistically significant 10 ml min⁻¹ increase in GFR when they returned for the crossover phase compared with the previous baseline. Thus, the increase in GFR persisted for several days longer than predicted by pharmacokinetics. These findings suggest that KW-3902 reset the complex network that determines kidney function in these patients, and provided first evidence for potential longer-term benefits of using A₁AR antagonists (Dittrich et al. 2007). Greenberg et al. assessed the effects of the selective A₁AR antagonist 1,3-dipropyl-8-[1-(4-propionate)-bicyclo-[2,2,2]octyl]xanthine (BG9928) given orally for ten days to 50 patients with heart failure and left ventricular systolic dysfunction who were receiving standard therapy (Greenberg et al. 2007). BG9928 (3–225 mg per day) increased sodium excretion without causing kaliuresis or reducing GFR. Notably, these effects were maintained over the ten-day period. BG9928 at doses of 15, 75, or 225 mg also reduced body weight at the end of the study compared with placebo (Greenberg et al. 2007).

In summary, the above described acute and short-term studies employing A₁AR antagonists in patients with heart failure yielded promising results. Since A₁AR blockade may increase transport in the semihypoxic medullary TAL, combining A₁AR antagonists with furosemide may potentiate natriuresis while helping to prevent transport-induced medullary hypoxia (Fig. 4). Whereas the presented animal and human studies were *acute* or *short-term* treatments, it remains to be determined whether longer-term application of A₁AR antagonism has beneficial effects. These studies should also reveal whether a clinically relevant effect of A₁AR blockade on renin release occurs. Consideration should also be given to the evidence that A₁AR activation is potentially important for protection in response to ischemia of the kidney (see above) and the heart (Cohen and Downey 2008). Apart from these issues, A₁AR blockade is unique in inducing natriuresis without potassium loss and lowering renal vascular resistance independent of all other organs. With regard to preserving renal function, this is an advantage over all vasodilator heart failure therapies that have been tried so far.

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Adenosine Receptors and the Central Nervous System

Ana M. Sebastião and Joaquim A. Ribeiro

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Abstract The adenosine receptors (ARs) in the nervous system act as a kind of “go-between” to regulate the release of neurotransmitters (this includes all known neurotransmitters) and the action of neuromodulators (e.g., neuropeptides, neurotrophic factors). Receptor–receptor interactions and AR–transporter interplay

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occur as part of the adenosine's attempt to control synaptic transmission. A_{2A}ARs are more abundant in the striatum and A₁ARs in the hippocampus, but both receptors interfere with the efficiency and plasticity-regulated synaptic transmission in most brain areas. The omnipresence of adenosine and A_{2A} and A₁ ARs in all nervous system cells (neurons and glia), together with the intensive release of adenosine following insults, makes adenosine a kind of "maestro" of the tripartite synapse in the homeostatic coordination of the brain function. Under physiological conditions, both A_{2A} and A₁ ARs play an important role in sleep and arousal, cognition, memory and learning, whereas under pathological conditions (e.g., Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, stroke, epilepsy, drug addiction, pain, schizophrenia, depression), ARs operate a time/circumstance window where in some circumstances A₁AR agonists may predominate as early neuroprotectors, and in other circumstances A_{2A}AR antagonists may alter the outcomes of some of the pathological deficiencies. In some circumstances, and depending on the therapeutic window, the use of A_{2A}AR agonists may be initially beneficial; however, at later time points, the use of A_{2A}AR antagonists proved beneficial in several pathologies. Since selective ligands for A₁ and A_{2A} ARs are now entering clinical trials, the time has come to determine the role of these receptors in neurological and psychiatric diseases and identify therapies that will alter the outcomes of these diseases, therefore providing a hopeful future for the patients who suffer from these diseases.

Keywords Adenosine receptors · A1 adenosine receptor · A2A adenosine receptor · Central nervous system · Receptor cross-talk · G protein coupled receptors · Neurotrophic factor receptors · Ionotropic receptors · Receptor dimmers · Caffeine · Drug addiction · Neurodegenerative diseases · Pain · Ischemia · Hypoxia · Adenosine levels

Abbreviations

AC	Adenylate cyclase
ACh	Acetylcholine
ADO	Adenosine
ADP	Adenosine 5'-diphosphate
AK	Adenosine kinase
AMP	Adenosine 5'-monophosphate
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AOPCP	α , β -Methylene ADP
Ap5A	Diadenosine pentaphosphate
AR	Adenosine receptor
ATP	Adenosine 5'-triphosphate
BDNF	Brain-derived neurotrophic factor
BRET	Bioluminescence resonance energy transfer
CamK	Calmodulin-dependent kinase

cAMP	Cyclic adenosine 5'-monophosphate
CB	Cannabinoid
CGRP	Calcitonin gene-related peptide
DA	Dopamine
DARPP	Dopamine- and cAMP-regulated phosphoprotein
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine
ENT	Equilibrative nucleoside transporter
ERK	Extracellular signal-regulated kinase
GABA	γ -Aminobutyric acid
GAT	GABA transporter
GLU	Glutamate
GDNF	Glial cell line-derived neurotrophic factor
GPCRs	G-protein-coupled receptors
HEK cells	Human embryonic kidney cells
HFS	High-frequency stimulation
IL-6	Interleukin 6
IP3	Inositol triphosphate
i.v.	Intravenous
KO	Knockout
LFS	Low-frequency stimulation
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor (mGlu1–8 refer to mGluR subtypes)
NAc	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NBTI	Nitrobenzylthioinosine
NGF	Nerve growth factor
NMDA	<i>N</i> -Methyl-D-aspartate
NT	Neurotransmitter
NTR	Neurotransmitter receptor
NPY	Neuropeptide Y
NR	NMDA receptor subunit
NT-3	Neurotrophin 3
NTDase	Ecto-5'-nucleotidase
NTPDase	Ectonucleoside triphosphate diphosphohydrolase
PDE	Phosphodiesterase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis toxin
REM	Rapid eye movement
Trk receptors	Tropomyosin-related kinase receptors
VIP	Vasoactive intestinal peptide

1 Introduction

Before we go into the scope of this review, we would like to stress that we are starting with some areas of high general complexity: (a) the *nervous system*, the most complex biological system in the human body; (b) *adenosine*, which is ubiquitously present in all cells, with receptors distributed throughout all brain areas; any imbalance of such a widespread system is expected to lead to neurological diseases; (c) *caffeine*, an antagonist of all subtypes of ARs and the most widely consumed psychostimulant drug; moreover, chronic or acute intake of caffeine may affect ARs in different and even opposite ways; (d) finally, ARs or adenosine-related molecules are potential therapeutic targets for neurologic diseases, but this role can be *multifactorial*, with different receptors involved, different time windows of action, age-related changes, etc.

Many publications are now appearing that are devoted to research in animal models or humans that is directed towards many nervous system pathologies and towards novel therapeutic approaches based on adenosine and ARs. We have therefore chosen to focus the present review on the insights gained from recent studies related to the subtle way that ARs regulate other receptors and transporters for neurotransmitters and neuromodulators, and on the pathophysiological implications of this regulation. We believe that further advances in the therapeutic potential of adenosine-related drugs require a deeper understanding of the above mentioned complexities in the context of fine-tuning modulation by adenosine. This might be accomplished at the receptor–receptor level or through several receptors in sequence and/or in parallel and/or via the transducing system cascade. What occurs inside the cells with the transducing system's variability and crosstalk may also occur at the extracellular membrane level via receptor–receptor interaction and formation of heteromers.

The AR field in neuroscience started with an apparent paradox reported by Sattin and Rall (1970): the ability of adenosine to increase cyclic adenosine 5'-monophosphate (cAMP) in the brain was prevented by theophylline, which at the time was known only as a phosphodiesterase (PDE) inhibitor. This was the starting point for the hypothesis that adenosine was acting through a membrane receptor antagonized by theophylline, but several years elapsed before the birth of the first nomenclature of purinergic receptors, proposed by Burnstock (1976). The field for the identification of different subtypes of ARs was then opened, and a further breakthrough was attained by the end of the 1970s by van Calcar et al. (1979), who first proposed ARs in brain cells as A₁ (inhibitory) and A₂ (stimulatory). At that time, as for most receptors, the AR classification relied upon pharmacological criteria and transducing pathways and the ability to stimulate or inhibit adenylate cyclase. AR cloning possibilities had to wait until the beginning of the 1990s. The first AR to be cloned was the A₁ AR from brain tissue (Mahan et al. 1991). All four G-protein-coupled ARs (A₁, A_{2A} and A_{2B}, and A₃) have been cloned. Major advances have been made in the pharmacological tools available for all of them, as reviewed in great detail in the first five chapters of this book.

Animal research in the last two to three decades firmly established that ARs are involved in several pathophysiological conditions, and that manipulation of their degree of activation might prove therapeutically useful. Therefore, the time is now ripe for studies in humans. In fact, the number of adenosine-related research reports in humans is increasing. From the summary in Table 1, it is clear that the highest incidence of adenosine-related research in humans is related to sleep and Parkinson's disease. This is certainly due to the great advances made in basic research in these fields, which have allowed the clear identification of the role of A_{2A} ARs in Parkinson's disease as well as that of adenosine in sleep and epilepsy. The identification of caffeine and theophylline as AR antagonists, together with the empirical knowledge at the time that xanthine-rich beverages such as coffee and tea affect sleep also boosted the interest in adenosine-related research into human sleep. Objective-oriented adenosine-related research in epileptic humans is still scarce, but one retrospective (Miura and Kimura 2000) and one case report study (Bahls et al. 1991) clearly identified an increased risk of seizures in patients taking theophylline as a bronchodilator. Interestingly, and highlighting the frequent gap between basic and clinical research, neither of those two reports mentioned the putative scientific grounds for the increased risk of seizures induced by theophylline: its ability to antagonize ARs. At the time, adenosine had already been recognized as an anticonvulsant, with the pioneering report being published as early as 1984 (Barraco et al. 1984) and the first review highlighting the subject appearing in the 1980s (Chin 1989).

In this review, we will pay particular attention to the implications of AR function in neuropathophysiological conditions, but before we do this, we will briefly provide an overview of the state of the art on how adenosine acts as a neuromodulator, the distribution of ARs in the brain, and their ability to interact with other receptors to harmoniously fine-tune neuronal activity.

2 Adenosine as a Ubiquitous Neuromodulator

While ATP may function as a neurotransmitter in some brain areas (Burnstock 2007; Edwards et al. 1992), adenosine is neither stored nor released as a classical neurotransmitter since it does not accumulate in synaptic vesicles, and is released from the cytoplasm into the extracellular space through a nucleoside transporter. The adenosine transporters also mediate adenosine reuptake, the direction of the transport being dependent upon the concentration gradient on both sides of the membrane (Gu et al. 1995). Since it is not exocytotically released, adenosine behaves as an extracellular signaling molecule that influences synaptic transmission without itself being a neurotransmitter. Using G-protein-coupled mechanisms, that not only lead to changes in second-messenger levels but also to the modulation of ion channels (such as calcium and potassium channels), adenosine modulates neuronal activity—presynaptically by inhibiting or facilitating transmitter release, postsynaptically by affecting the actions of other neurotransmitters, and nonsynaptically by

Table 1 AR research relating to the human central nervous system

Target	Comment/reference
Cognition	Caffeine facilitates information processing and motor output in healthy subjects. Dixit et al. (2006) Caffeine appears to reduce cognitive decline in women without dementia. Ritchie et al. (2007)
Sleep	Decrease in sleep efficiency and of total sleep in healthy subjects by preingestion (16 h) of caffeine. Landolt et al. (1995a, b) Attenuation of sleep propensity in healthy subjects. Landolt et al. (2004) Insomnia patients with greater sensitivity to awakening caffeine actions. Salín-Pascual et al. (2006) Involvement of adenosine in individual variations in sleep deprivation sensitivity. Rétey et al. (2006) Variations in A_{2A} receptor gene associated with objective and subjective responses to caffeine in relation to sleep. Rétey et al. (2007) Prolonged wakefulness induces A_1 receptor upregulation in cortical and subcortical brain regions. Elmenhorst et al. (2007)
Epilepsy	An increased risk of seizures after theophylline or caffeine intake. Bahls et al. (1991), Kaufman and Sachdeo (2003), Miura and Kimura (2000), Mortelmans et al. (2008) <i>Increase</i> (Angelatou et al. 1993) or <i>decrease</i> (Glass et al. 1996) in A_1 receptor density in different post-mortem brain areas of epileptic subjects
Parkinson's disease	Decrease in A_{2A} mRNA levels in post-mortem caudate and putamen, and increase in A_{2A} mRNA in the substantia nigra in Parkinson's disease patients. Hurley et al. (2000) Polymorphism of A_{2A} receptors did not confer susceptibility to Parkinson's disease in a Chinese population sample. Hong et al. (2005) Caffeine improved the "total akinesia" type of gait freezing in Parkinson's disease patients. Kitagawa et al. (2007) Caffeine administered before levodopa may improve its pharmacokinetics in some parkinsonian patients. Deleu et al. (2006) Significant association between higher caffeine intake and lower incidence of Parkinson's disease. Ross et al. (2000)
Alzheimer's disease	Caffeine intake inversely associated with risk of Alzheimer's disease. Maia and de Mendonça (2002), Ritchie et al. (2007) Co-localization of A_1 receptor and β -amyloid or tau and increase in A_{2A} receptor expression in post-mortem cerebral cortex and hippocampus of Alzheimer's disease patients. Angulo et al. (2003) Upregulation of A_1 and A_{2A} receptor expression and function in post-mortem cerebral cortex of Alzheimer's disease patients. Albasanz et al. (2008)
Pick's disease	Upregulation of A_1 and A_{2A} receptor expression and function in post-mortem frontal lobe of Pick's disease patients. Albasanz et al. (2006, 2007).
Pain	Beneficial effects of adenosine (i.v.) in 2 patients with neuropathic pain. Sollevi et al. (1995) Intrathecal adenosine reduces allodynia in patients with neuropathic pain, but has a side effect of backache. Eisenach et al. (2003)

(continued)

Table 1 (continued)

Target	Comment/reference
	Reduction of secondary hyperalgesia by adenosine in human models of cutaneous inflammatory pain. Sjölund et al. (1999)
	Local opioid receptor stimulation in the spinal cord of humans induces the release of adenosine. Eisenach et al. (2004)
	Theophylline improves esophageal chest pain (a randomized, placebo-controlled study), possibly by altering adenosine-mediated nociception. Rao et al. (2007)
Anxiety	A _{2A} receptor gene polymorphism associated with increases in anxiety in healthy volunteers. Alsene et al. (2003)
	A _{2A} receptor gene polymorphism associated with increases in anxiety response to amphetamine in healthy volunteers. Hohoff et al. (2005)
Panic disorder	Panic disorder patients with increased sensitivity to one cup of coffee. Boulenger et al. (1984)
	Anxiogenic and panic-inducing effects of caffeine in a double-blind study. Klein et al. (1991)
	Adenosine A ₁ receptor supersensitivity, a probable compensatory process. DeMet et al. (1989)
Panic disorder and anxiety	A _{2A} receptor gene polymorphism associated with anxiety or panic disorder in Occidental but not Asiatic populations. Alsene et al. (2003), Deckert et al. (1998), Hamilton et al. (2004), Lam et al. (2005)
Schizophrenia	Increase in A _{2A} receptor density in post-mortem striatum of schizophrenic patients, a consequence of typical antipsychotic medication. Deckert et al. (2003)
	Polymorphism of A _{2A} receptors is not related to the pathogenesis of schizophrenia in a Chinese population sample. Hong et al. (2005)
Bipolar disorders	Typical, but not atypical, antipsychotics induce an upregulation of A _{2A} receptors assessed in platelets of patients with bipolar disorders. Martini et al. (2006)
Dependence behavior	Polymorphism of the A _{2A} receptor gene related to caffeine consumption in healthy volunteers. Cornelis et al. (2007)
Ventilation dyspnea and apnea	Adenosine produces hyperventilation and dyspnea in humans resulting from a direct activation of the carotid body. Uematsu et al. (2000), Watt et al. (1987)
	Caffeine and theophylline are effective in the treatment of apnea in premature and newborn infants. Aranda and Turmen (1979), Bairam et al. (1987), Uauy et al. (1975)
Miscellaneous (A ₂ /D ₂ interaction)	A ₂ /D ₂ interaction in post-mortem human striatal brain sections. Díaz-Cabiale et al. (2001)
	A ₂ /D ₂ dimers in human platelets. Martini et al. (2006)
Miscellaneous (receptor localization)	Distribution of A ₁ and A _{2A} receptors in post-mortem human brain. James et al. (1992), Svenningsson et al. (1997)
	Mapping A _{2A} receptors in the human brain by PET. Ishiwata et al. (2005), Mishina et al. (2007)
	Mapping A ₁ receptors in the human brain by PET. Elmenhorst et al. (2007), Fukumitsu et al. (2003, 2005)

hyperpolarizing or depolarizing neurones. Curiously, the discovery of the presynaptic inhibitory action of adenosine, which we now know occurs through A₁AR, had the same paradoxical starting point as the identification of theophylline as a putative AR antagonist; that is, adenosine was used at the neuromuscular junction in an attempt to increase cAMP in motor nerve terminals and, in contrast with the expected excitatory effect, adenosine markedly inhibited neurotransmitter release (Ginsborg and Hirst 1971). ATP, released together with acetylcholine (ACh) (Silinsky 1975), mimicked the adenosine effect (Ribeiro and Walker 1973), decreasing both the synchronous and the asynchronous release of acetylcholine, with the maximum effect being about 50%. This presynaptic inhibitory action of ATP results from its extracellular hydrolysis into adenosine (Ribeiro and Sebastião 1987). Interestingly, high-affinity ARs positively coupled to adenylate cyclase do enhance neurotransmitter release, and this was also first identified in cholinergic synapses almost simultaneously in the central nervous system (Brown et al. 1990) and at the cholinergic nerve terminals of the neuromuscular junction (Correia-de-Sá et al. 1991). In the latter case, due to the reduced complexity of the model, it was possible to clearly demonstrate for the first time that both A₁ and A_{2A}ARs are present at the same nerve terminal (Correia-de-Sá et al. 1991). Adenosine research at cholinergic motor nerve endings to some degree anticipated and inspired the studies at central excitatory glutamatergic synapses, where adenosine decreases both synchronous and asynchronous transmitter release (Lupica et al. 1992; Prince and Stevens 1992).

The past few years have brought new insights into our understanding of the role of the tripartite synapse and gliotransmission in neurological diseases (Halassa et al. 2007). Adenosine and ATP are also among the most relevant players in neuron–glia communication (Fields and Burnstock 2006). ATP has a dual role since it acts upon its own receptors, mostly of the P2Y subtype, which are abundant in astrocytes and are relevant to calcium signaling; ATP is also a substrate of ectonucleotidases leading to adenosine formation, which then operates its own receptors. The adenosine system is critically involved in modulating glial cell functions, namely glycogen metabolism, glutamate transporters, astrogliosis and astrocyte swelling (Daré et al. 2007). ARs on oligodendrocytes regulate white matter development and myelination (Daré et al. 2007; Fields and Burnstock 2006).

3 Manipulation of Endogenous Levels of Adenosine and its Neuromodulation

By using highly selective A₁AR antagonists such as 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), it is possible to unmask a tonic A₁AR-mediated adenosinergic tonus. A similar strategy can be used towards endogenous A_{2A}AR activation, using selective A_{2A}AR antagonists such as SCH-58621 or ZM-241385. Simultaneous removal of tonic adenosinergic influences over all receptor types has been achieved through the use of adenosine deaminase, an enzyme used in isolated preparations that does not penetrate cell membranes and that deaminates adenosine into inosine,

usually an inactive ligand for ARs. In studies with this enzyme, an appropriate control with an adenosine deaminase inhibitor together with adenosine deaminase (Sebastião and Ribeiro 1985) will allow actions of the enzyme not related to its enzymatic activity to be determined. The ability of inosine to influence the biological signal in the assay has to be directly tested since inosine can activate ARs, namely those of the A₃AR subtype, in some circumstances (Jin et al. 1997). Whenever aiming to evaluate endogenous adenosine actions in a mirror-like way (i.e., by using selective AR antagonists or extracellular adenosine removal), one must also bear in mind that irreversible AR-mediated actions will not be apparent. In these cases, further receptor activation by exogenously added ligands may be the only way to demonstrate a role for adenosine, providing that the system is not already fully saturated with endogenous adenosine actions.

Inhibition of the cascade of ectoenzymes that metabolize ATP to adenosine can be used whenever attempting to discriminate between ATP- and AR-mediated actions. Due to the multiplicity and redundancy of the ectoenzymes involved in the process (Zimmermann 2006), it has been difficult or almost impossible to fully inhibit extracellular ATP breakdown into adenosine. Ectonucleoside triphosphate diphosphohydrolase (NTPDase; EC 3.6.1.5), previously identified as ecto-ATPase, ecto-ATPDase or CD39, is the ectonucleotidase mainly responsible for the sequential hydrolysis of β - and γ -phosphates of tri- and diphosphonucleosides. Its inhibition by ARL-67156 has proven useful to enhance extracellular ATP actions and/or to avoid its breakdown into adenosine (Sperlágh et al. 2007). For a discussion on the therapeutic potential of NTPDase inhibition, see Gendron et al. (2002). Inhibition of adenosine formation from released adenine nucleotides can also be partially achieved through the use of an ecto-5'-nucleotidase inhibitor, α , β -methylene ADP (AOPCP). By comparing the action of adenosine deaminase with that of AOPCP, it was possible to estimate that the formation of adenosine from adenine nucleotides and the release of adenosine as such contribute in nearly equal amounts to the pool of endogenous adenosine that presynaptically inhibits acetylcholine release from motor nerve terminals at the neuromuscular junction (Ribeiro and Sebastião 1987), as well as in the hippocampus (Cunha et al. 1994c). However, this relationship may differ in other brain areas and according to neuronal activity and the regional distribution of ecto-5'-nucleotidase. For example, in cholinergic nerve terminals of the hippocampus, there is significant activity from ecto-5'-nucleotidase, whereas this is not the case in cerebral cortical cholinergic nerve terminals (Cunha et al. 1992). High-frequency neuronal firing favors ATP release (Cunha et al. 1996a) as well as activation of adenosine A_{2A}AR, and this has been shown to occur both in a peripheral nervous system preparation, the neuromuscular junction (Correia-de-Sá et al. 1996), and in a central nervous system preparation, the hippocampus (Almeida et al. 2003). At the neuromuscular junction, however, ecto-AMP deaminase shunts the pathway for adenosine formation, thus reducing its ability to activate A_{2A}AR (Magalhães-Cardoso et al. 2003).

An enhancement of extracellular adenosine levels can be achieved by inhibiting intracellular enzymes that are responsible for keeping intracellular adenosine concentrations low, such as the adenosine kinase (AK) that phosphorylates adenosine

into AMP. Inhibition of this enzyme selectively amplifies extracellular adenosine concentrations at cell and tissue sites where adenosine release occurs. AK can be inhibited with iodotubercidin, which markedly enhances extracellular adenosine levels and causes an inhibition of synaptic transmission (Diógenes et al. 2004) at sites where A₁ARs are operative (e.g., hippocampus). The therapeutic antiepileptic potential of AK inhibition or of its underexpression in implanted cells has been highlighted recently (Li et al. 2007).

Manipulation of adenosine transporter activity with inhibitors such as dipyrindamole or nitrobenzylthioinosine (NBTI) has also proven to be a useful approach, but one has to keep in mind that, due to the equilibrative nature of adenosine transporters in the brain cells, adenosine transport inhibition can either enhance or reduce extracellular adenosine levels according to the gradient of concentration of adenosine across the cell membrane, as well as according to the proportion of extracellular adenosine that is formed from the catabolism of released adenine nucleotides.

A still less explored way to increase extracellular adenosine levels is deep brain stimulation, and a very interesting report on this subject appeared recently (Bekar et al. 2008). Deep brain stimulation is used empirically to treat tremor and other movement disorders (Yu and Neimat 2008) as well as psychiatric diseases, including obsessive–compulsive disorders and depression (Larson 2008). As Bekar et al. (2008) clearly showed, deep brain stimulation is associated with a marked increase in the release of ATP from thalamic nuclei, resulting in accumulation of its catabolic product, adenosine. ATP, which is released in a nonexocytotic way, probably from astrocytes or other glial cells, is therefore crucial in adenosine accumulation following deep brain stimulation, leading to A₁AR-mediated inhibition of synaptic transmission in the thalamus (Bekar et al. 2008), in a way that is probably similar to the inhibition of synaptic transmission induced by ATP in the hippocampus, which requires localized extracellular catabolism by ectonucleotidases and channeling to A₁ARs (Cunha et al. 1998). Infusion of A₁AR agonists directly into the thalamus reduces tremor, whereas A₁AR-null mice show involuntary movements and seizures at stimulation intensities below the therapeutic level, suggesting that depression of synaptic transmission in the thalamus controls the spread of excitability and reduces side effects of deep brain stimulation (Bekar et al. 2008). Depression of synaptic transmission due to deep brain stimulation mimics in several aspects the depression of synaptic transmission caused by hypoxia, which is neuroprotective and can also be reversed by A₁AR antagonists (Sebastião et al. 2001). Hypoxia is, indeed, another highly efficient way to increase extracellular adenosine levels (Fowler 1993; Frenguelli et al. 2003), but in this case adenosine is mostly released as such (Latini and Pedata 2001), while deep brain stimulation appears to predominantly induce ATP release (Bekar et al. 2008).

A schematic representation of the different pathways involved in the control of extracellular adenosine concentrations, as well as the relevance of neuronal firing frequency to A₁AR vs. A₂AR activation by extracellularly formed adenosine, is depicted in Fig. 1.

High frequency stimulation (HFS) favours ATP release and activation of A_{2A} AR which facilitate ADO uptake through ENT

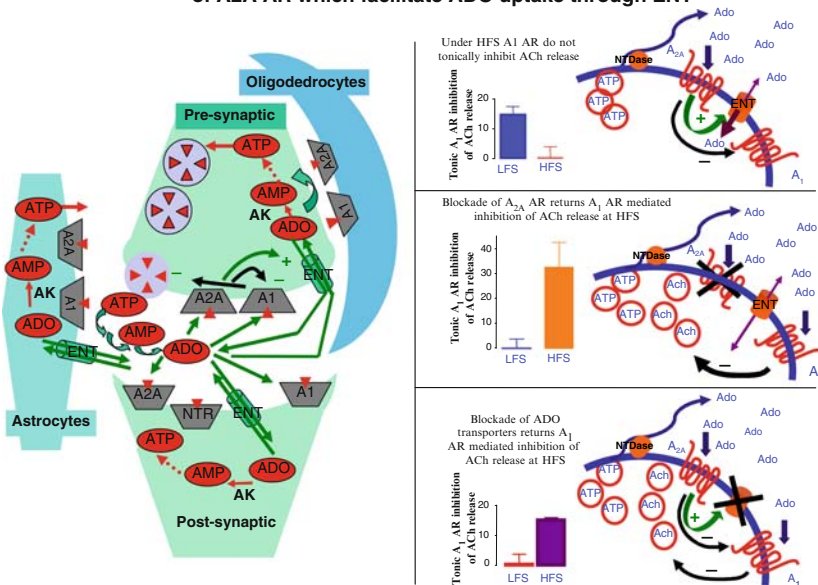


Fig. 1 Main pathways that control extracellular adenosine (ADO) concentrations, and their relationships to the activation of A₁ or A_{2A} adenosine receptors (ARs) under low or high neuronal firing rates induced experimentally by low- or high-frequency stimulation (LFS or HFS). Adenosine can be formed from extracellular catabolism of ATP by a cascade of ectoenzymes, the ecto-5'-nucleotidases (*NTDase*), or can be released as such through an equilibrative nucleoside transporter (*ENT*). Intracellularly the key enzyme influencing ADO concentration is adenosine kinase (*AK*), which is present in most cell types, including neurons and glia. The intracellular pathways for ADO metabolism into ATP are not depicted in oligodendrocytes for the sake of clarity. A₁ and A_{2A} receptors are present pre- and postsynaptically as well as in astrocytes and glia. At nerve terminals, A₁ARs decrease neurotransmitter (*NT*) release, thus reducing the availability to activate postsynaptic NT receptors (*NTR*). A_{2A} receptors have been shown to inhibit (*minus symbol*) A₁ receptor functioning in nerve terminals. A₁/A_{2A} receptor interactions might also occur in other cell types, namely in astrocytes (*see text*), but they are not represented for the sake of clarity. A_{2A}ARs are preferentially activated at high-frequency neuronal firing, which favors ATP release and adenosine formed from extracellular ATP catabolism. A_{2A}ARs enhance (*plus symbol*) adenosine transport through ENT, which in the case of HFS is in the inward direction, decreasing the availability of ADO for A₁ARs, the main consequence of which is a lower tonic inhibition of neurotransmitter release. Data shown in the *left panels* are adapted from data published by Pinto-Duarte et al. (2005), who reported the influence of the firing rate upon the tonic inhibition of acetylcholine (*ACh*) release from the CA3 area of hippocampal slices, and how it is related to the ability of A_{2A} receptors to enhance ENT activity at hippocampal nerve terminals. See text for further references

4 Distribution of ARs in the Central Nervous System and the Effect of Aging

Neuromodulation by adenosine is exerted through the activation of high-affinity A₁ and A_{2A} ARs, which are probably of physiological importance, and of low-affinity A_{2B}ARs, which may be relevant in pathological conditions. The A₃AR is

a high-affinity receptor in humans, but has a low density in most tissues. These four ARs are also known as P1 purinoceptors, from the P1 (adenosine-sensitive)/P2 (ATP-sensitive) nomenclature (Burnstock 1976). They belong to the G-protein-coupled receptor (GPCR) family and all have been cloned and characterized from several mammalian species including humans (Fredholm et al. 2001).

The adenosine A_1 AR is highly expressed in brain cortex, cerebellum, hippocampus, and dorsal horn of spinal cord (Ribeiro et al. 2003). The A_{2A} AR is highly expressed in the striatopallidal γ -aminobutyric acid (GABA)ergic neurones and olfactory bulb, and for a long time it was assumed that this receptor was circumscribed to these brain areas. The first evidence that the A_{2A} AR could influence neuronal communication outside the striatum or olfactory bulb was reported in 1992 using hippocampal slices (Sebastião and Ribeiro 1992). This was followed by evidence that A_{2A} AR mRNA and protein are expressed in the hippocampus (Cunha et al. 1994a). The initial scepticism was broken (Sebastião and Ribeiro 1996), and it is now widely recognized that A_{2A} ARs are expressed in several brain regions albeit in lower levels than in the striatum. A_{2B} ARs are expressed in low levels in the brain (Dixon et al. 1996), and the level of expression for the A_3 AR is apparently moderate in the human cerebellum and hippocampus and low in most other areas of the brain (Fredholm et al. 2001) (Fig. 2).

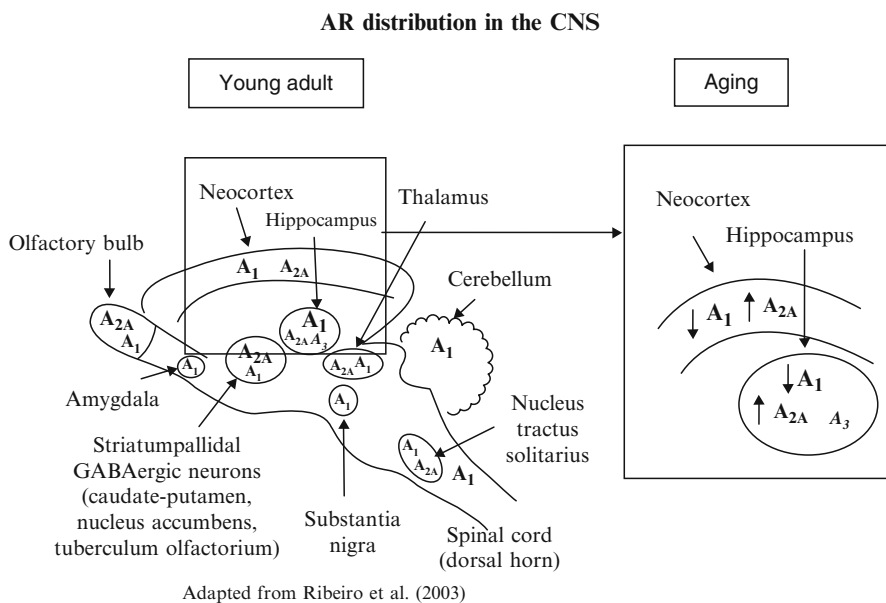


Fig. 2 Schematic representation of the distribution of adenosine receptors (ARs) in the different brain areas. The *inset* illustrates the reported changes in AR density in the forebrain (hippocampus and cortex). In aged rats, the density and functioning of A_{2A} ARs is increased (*upward arrow*) in the hippocampus and cortex, whereas the density and functioning of A_1 ARs is decreased (*downward arrow*). No information, so far, is available for age-related changes in A_3 AR density upon aging. See text for references

The relative densities of A_1 and A_{2A} ARs in subregions of the same brain area may differ. For instance, with respect to the modulation of acetylcholine in the hippocampus, there is a preponderance of A_1 AR-mediated modulation by endogenous adenosine in both the CA1 and CA3 areas, but the CA3 has a relatively higher influence of A_{2A} ARs than the CA1 (Cunha et al. 1994b). Whenever two receptors coexist, one may ask about their relative importance (i.e., the hierarchy of one receptor with respect to the other). This may change with neuronal activity, age, and even with other molecules that are in the vicinity of the site of action and that may be relevant for the production or inactivation of the ligand. High-frequency neuronal firing favours ATP release (Cunha et al. 1996a), and adenosine formed from released adenine nucleotides seems to prefer A_{2A} AR activation (Cunha et al. 1996b), which may be due to the geographical distribution of ecto-5-nucleotidases and A_{2A} ARs. A_{2A} AR activation activates adenosine transport, which in the case of high neuronal activity and ATP release is in the inward direction (Fig. 1). This induces a decrease in extracellular adenosine levels and a reduced ability of A_1 ARs to be activated by endogenous extracellular adenosine (Pinto-Duarte et al. 2005). By themselves, A_{2A} ARs are able to attenuate A_1 AR activation (Cunha et al. 1994a), which may further contribute to a decreased activity of A_1 ARs under high-frequency neuronal firing. The ability of A_1 ARs to inhibit synaptic transmission is attenuated by protein kinase C (PKC) activation (Sebastião and Ribeiro 1990), and a similar mechanism appears to be involved in the A_{2A} AR-mediated attenuation of A_1 AR responses (Lopes et al. 1999a).

Aging also decreases the ability of A_1 ARs to inhibit neuronal activity (Sebastião et al. 2000a). This may be a function of an age-related decrease in the density of A_1 ARs in the brain, which has been shown in both mice (Pagonopoulou and Angelatou 1992) and humans (Meyer et al. 2007). Low A_1 AR receptor density and function, however, can be compensated for by higher levels of extracellular adenosine, which keep tonic inhibition high in aged animals (Bauman et al. 1992). While comparing changes in A_1 AR density in the cerebral cortex, hippocampus and striatum, it was concluded that the most affected area was the cerebral cortex, followed by the hippocampus (Fig. 2), whereas the density of A_1 ARs in the striatum was little affected by aging in rats (Cunha et al. 1995). A_1 AR density in the cerebellum is also poorly affected by aging (Pagonopoulou and Angelatou 1992).

In contrast to A_1 ARs, there is a significant increase in the density of A_{2A} ARs in the cortex (Cunha et al. 1995) and hippocampus (Diogenes et al. 2007) of aged rats, which correlates with their enhanced ability to facilitate glutamatergic synaptic transmission (Rebola et al. 2003) and acetylcholine release (Lopes et al. 1999b) in the hippocampus (Fig. 2). In the striatum there is a tendency for a decrease in A_{2A} AR density in aged rats (Cunha et al. 1995), and within the striatum, age may influence the A_{2A} ARs in glutamatergic, dopaminergic or GABAergic nerve terminals in different ways (Corsi et al. 1999, 2000). Taken together, these findings clearly show that there are age-related shifts in the A_1 AR inhibitory/ A_{2A} AR excitatory balance, and that this shift may be different in different areas of the brain, with the trend for the forebrain being towards an increase in A_{2A} AR-mediated influences and a decrease in A_1 AR density. Due to the A_{2A} / A_1 AR interactions

(see Sect. 5.1.5), an increase in $A_{2A}AR$ density may itself reduce A_1AR tonus. Due to the influence of $A_{2A}ARs$ on other receptors (see Sect. 5), the change in the $A_{2A}AR$ influence upon aging may markedly affect the action of other modulators. Indeed, the nonmonotonous age-related changes in the ability of brain-derived neurotrophic factor (BDNF) to influence synaptic transmission in the hippocampus are related to both a decrease in the density of tropomyosin-related kinase receptors (Trk) for BDNF (TrkB receptors) and an increase in the density of $A_{2A}ARs$, which allow TrkB receptor-mediated actions in the aged hippocampus (Diogenes et al. 2007).

5 Adenosine as a Modulator of Other Neuromodulators

Besides its direct pre- and postsynaptic actions on neurones, adenosine is rich in nuances of priming, triggering and braking the action of several neurotransmitters and neuromodulators. Because adenosine acts in such a subtle fashion, it was proposed as a fine tuner. In this way, adenosine is a partner in a very sophisticated interplay between its own receptors and receptors for other neurotransmitters and/or neuromodulators. Several possibilities exist for this interplay, either at the transducing system level (Sebastião and Ribeiro 2000) or as a consequence of receptor–receptor heteromerization (Ferré et al. 2007a), greatly expanding the number of possible receptor combinations to modulate cell signalling.

5.1 Interactions with G-Protein-Coupled Receptors

Besides the well known A_{2A}/D_2 dopamine interaction in the striatum (Ferré et al. 1991), which has been explored intensively due to the implication of this receptor interaction for Parkinson's disease and other basal ganglia dysfunctions (Fuxe et al. 2007; Morelli et al. 2007), adenosine, mostly through activation of $A_{2A}ARs$, is also able to influence the functioning of other GPCRs (Fig. 3a). A brief overview of the influence of adenosine on these receptors will follow.

5.1.1 Dopamine Receptors

A first hint at the ability of $A_{2A}ARs$ to interact with dopamine D_2 receptors came from binding studies showing that activation of $A_{2A}ARs$ decreases the affinity of dopamine D_2 receptors in rat striatal membranes (Ferré et al. 1991). The possibility that this $A_{2A} - D_2$ receptor interaction is crucial to the behavioral effects of adenosine agonists and antagonists (like caffeine) was immediately highlighted (Ferré et al. 1991) and soon tested (Svenningsson et al. 1995). The functional consequences of $A_{2A}AR$ and D_2 receptor agonists upon dopamine and GABA release (Ferré et al. 1994; Mayfield et al. 1996) in the basal ganglia became evident soon thereafter.

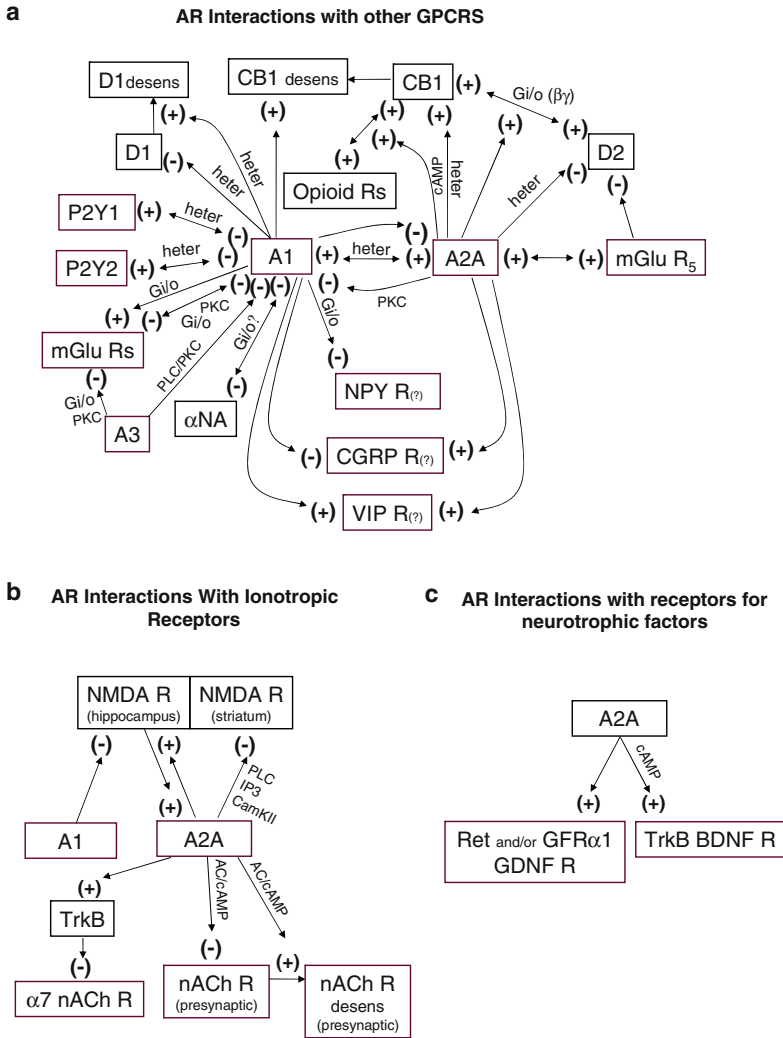


Fig. 3 a–c Interactions between adenosine receptors and receptors for other neurotransmitters. The known interactions with other G-protein-coupled receptors (GPCRS) **a**, with ionotropic receptors **b**, and with receptors for neurotrophic factors **c** are illustrated, where a *plus symbol* represents a facilitation or triggering of the action, or synergy between receptors, or the facilitation of desensitization (*desens*), and a *minus symbol* represents an inhibition, or an occlusion of the action, or less than additive effects. Whenever the mechanisms involved in the interaction have been evaluated, they are indicated close to the *arrow*. G-protein sharing is indicated by the name of the G protein close to the *arrow*. Whenever receptor heteromerization (*heter*) has been shown to occur, it is also indicated close to the *arrow*. An absence of knowledge about the receptor subtype is indicated by a *question mark* close to the receptor name. See text for references. Other abbreviations: αNA , α receptor for noradrenaline; *BDNF*, brain-derived neurotrophic factor; *CB*, cannabinoid; *CB₁*, cannabinoid receptor type 1; *CGRP*, calcitonin gene-related peptide; *D*, dopamine receptor; *GFR α 1* and *Ret*: neurotrophic factors for GDNF; *GDNF*, glial cell line-derived neurotrophic factor; *mGluRs*, metabotropic glutamate receptor; *nAChR*: nicotinic acetylcholine receptor; *NMDAR*: *N*-methyl-D-aspartate receptor; *NPY*, neuropeptide Y; *VIP*, vasoactive intestinal peptide; *P2Y*, ATP receptor; *TrkB*, tropomyosin-related kinase receptor type B

Since this time, interest in the adenosine/D₂ interaction has continued to increase, extending to psychiatric and neurologic fields such as drug addiction, schizophrenia and Parkinson's disease, and has been the subject of many reviews by groups that have been involved in this subject since its origin (Ferré et al. 2007b). For more information on A_{2A}ARs and Parkinson's disease, please refer to Chap. 18, "Adenosine A_{2A} Receptors and Parkinson's Disease" (by Morelli et al.), in this volume.

A₁ARs and D₁ receptors also interact in the basal ganglia (Ferré et al. 1996), an interaction that has implications for the control of GABA release at the substantia nigra (Florán et al. 2002) and nucleus accumbens (Mayfield et al. 1999), as well as dopamine release in the striatum (O'Neill et al. 2007). Furthermore, A₁AR activation has been shown to facilitate D₁ receptor desensitization (Le Crom et al. 2002). D₁/A₁ receptor heteromerization may play a role in D₁ receptor desensitization mechanisms and be a molecular basis for the antagonistic modulation of A₁AR over D₁ receptor signaling (Ginés et al. 2000).

5.1.2 Neuropeptides

By activating A_{2A}ARs, adenosine tonically potentiates a facilitatory action of the neuropeptide calcitonin gene-related peptide (CGRP) on neurotransmitter release from motor nerve terminals (Correia-de-Sá and Ribeiro 1994a). The ability of CGRP to facilitate synaptic transmission in the CA1 area of the hippocampus is also under tight control by adenosine, with tonic A₁AR activation by endogenous adenosine "braking" the action of CGRP, and the A_{2A}ARs triggering this action (Sebastião et al. 2000b). This A₁AR-mediated inhibition of the action of CGRP, together with the A₁AR-induced inhibition of CGRP release (Carruthers et al. 2001), can be related to pain inhibition by adenosine (see Sect. 7). Indeed, CGRP is a potent vasodilator released from activated trigeminal sensory nerves that dilates intracranial blood vessels and transmits vascular nociception, and is implicated in the genesis of vascular pain such as migraine. Hence, inhibition of trigeminal CGRP release and CGRP receptor blockade have been proposed as promising antimigraine strategies (Goadsby 2008).

The facilitatory action of vasoactive intestinal peptide (VIP) on ACh release from motor nerve endings is prevented by A_{2A}AR blockade or by the removal of extracellular adenosine with adenosine deaminase, indicating that the activation of these A_{2A}ARs, attained with high-frequency motor neuron firing, is necessary to trigger the facilitatory action of VIP (Correia-de-Sá et al. 2001). VIP enhances synaptic transmission at the CA1 area of the hippocampus by enhancing GABA release from GABAergic neurones that make synapses with other interneurones, therefore reducing GABAergic inhibition into pyramidal glutamatergic neurones (Cunha-Reis et al. 2004, 2005). This action of VIP is dependent on both A₁ and A_{2A} AR activation by endogenous adenosine (Cunha-Reis et al. 2007, 2008). Interestingly, the finding that VIP-induced modulation of GABA release from hippocampal nerve terminals is under the control of adenosine A₁ARs constituted the first evidence of a role of A₁ receptors in hippocampal GABAergic terminals. This is an example of

a situation where A₁ARs per se may not affect neurotransmitter release, just like GABA in the hippocampus (Lambert and Teyler 1991; Yoon and Rothman 1991), but instead influence the actions of other modulators of GABA release.

Neuropeptide Y (NPY) agonists inhibit presynaptic calcium influx through N- and P/Q-type calcium channels and inhibit glutamate release at the CA3–CA1 synapses of rat hippocampus, an action that is fully occluded by coactivation of adenosine A₁ARs (Qian et al. 1997). Interestingly, the inhibitory action of the GABA_B agonist baclofen was not fully occluded by AR activation, indicating partially shared pathways between G-protein-coupled NPY, adenosine and GABA receptors. In PC12 cells, exocytosis of NPY-containing vesicles is facilitated by A_{2A}AR activation (Mori et al. 2004), but this does not occur in nerve endings from the rat mesenteric artery, where ARs affect noradrenaline but not NPY release (Donoso et al. 2006).

In cultured primary hippocampal neurones, agonists of delta-opioid receptors and of cannabinoid (CB) receptors of the CB₁ subtype act synergistically to activate protein kinase A (PKA) signaling through Gi-β/γ dimers, and this synergy requires A_{2A}AR activation (Yao et al. 2003). CB₁ agonists also act synergistically with μ opioid receptors in primary nucleus accumbens/striatal neurones, and again this synergy requires adenosine A_{2A}ARs (Yao et al. 2006). Interestingly, A_{2A}AR blockade eliminates heroin-seeking behavior in addicted rats (Yao et al. 2006), suggesting that A_{2A}AR antagonists may be effective therapeutic agents in the management of abstinent heroin addicts (see Sect. 9).

5.1.3 Metabotropic Glutamate Receptors

Activation of metabotropic glutamate receptors (mGluR) with 1*S*, 3*R*-ACPD potentiates cAMP responses mediated by several receptors that are positively coupled to adenylate cyclase, namely A₂ARs and VIP and β-adrenergic receptors (Alexander et al. 1992; Winder and Conn 1993). mGluRs also influence A₁AR functioning in neurones, and this seems to involve PKC activity. In fact, PKC activity is required for the attenuation of the inhibitory effect of A₁AR activation on synaptic transmission at the hippocampus by agonists of group I mGluRs (mGlu1, mGlu5) which are coupled to phospholipase C, as well as by agonists of group III mGluRs (mGlu4, mGlu6, mGlu7, mGlu8), which are usually negatively coupled to cAMP (de Mendonça and Ribeiro 1997a). Agonists of group I mGluRs also attenuate GABA_B-mediated inhibition of synaptic transmission, a process that involves PKC activity (Shahraki and Stone 2003). In addition, activation of PKC by phorbol esters or activation of PKC-coupled mGluRs suppresses the inhibitory action of A₁AR agonists on glutamate release from cerebrocortical synaptosomes (Budd and Nichols 1995).

The inhibitory effects of an A₁AR agonist and of an agonist of group II mGluRs (mGlu2, mGlu3) on glutamate release or cAMP formation was less than additive (Di Iorio et al. 1996), suggesting that the presynaptic A₁ and group II mGluRs are reciprocally occlusive, probably by sharing a pertussis toxin (PTX)-sensitive, PKC-regulated G protein (Zhang and Schmidt 1999).

Activation of A₃AR leads, through a PKC-dependent process, to a marked attenuation of the presynaptic inhibitory functions of cAMP-coupled mGluRs (groups II and III) at the CA1 area of the hippocampus (Macek et al. 1998). Again, the action of PKC and probably also that of A₃ARs on mGluRs might result from an inhibition of the coupling of mGluRs with G proteins, because PKC activation inhibits the increased [³⁵S]GTPγS binding induced by mGluR agonists (Macek et al. 1998). Thus, the actions of A₁ or A₃ ARs and those of mGluRs in neurones are mutually occlusive, through a process probably involving the crosstalk of transducing systems or the sharing of G proteins, as proposed several years ago to explain the mutual occlusion between presynaptic adenosine A₁ and α₂-adrenergic receptors (Limberger et al. 1988).

In contrast, in astrocytes, activation of A₁AR enhances the intracellular calcium response induced by mGluRs (Ogata et al. 1994), a process that involves a PTX-sensitive G protein (Cormier et al. 2001; Tom and Roberts 1999). Adenosine-induced calcium response in astrocytes requires A₁/A₂ AR cooperation, and is synergistic with mGluR response, leading to enhancement of cAMP levels (Ogata et al. 1996).

With respect to the interaction between A_{2A}AR and mGluR, A_{2A}AR agonists act synergistically with group I mGluR agonists to modulate dopamine D₂ receptors in the rat striatum, decreasing the affinity state of these receptors (Ferré et al. 1999). Furthermore, A_{2A}ARs act synergistically with mGlu5 receptors to increase dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) phosphorylation, so that blockade of one of the receptors is enough to prevent phosphorylation induced by activation of the other receptor (Nishi et al. 2003). A_{2A}ARs and mGlu5 receptors are also co-localized presynaptically, namely at striatal glutamatergic terminals, where they facilitate glutamate release in a synergistic manner (Pintor et al. 2000; Rodrigues et al. 2005). Prevention of mGlu5 receptors and A_{2A}AR synergy at the pre- and the postsynaptic level will, therefore, eventually lead to decreased glutamate release with consequent reduced excitotoxicity, together with a facilitation of D₂ dopaminergic receptor functioning, and this is the rationale for the use of antagonists of these receptors as antiparkinsonian drugs (see also Chap. 18, "Adenosine A_{2A} Receptors and Parkinson's Disease," by Morelli et al., in this volume). Indeed simultaneous blockade of A_{2A} and mGlu5 receptors showed high efficacy in reversing parkinsonian deficits in rodents (Coccarello et al. 2004; Kachroo et al. 2005). Combined antagonism of mGlu5 receptors and A_{2A}ARs also efficiently reduced alcohol self-administration and alcohol-seeking in rats (Adams et al. 2008), further reinforcing the importance of the mGlu5 and A_{2A}AR interaction in the mesolimbic and basal ganglia areas.

5.1.4 Cannabinoid Receptors

The high density of adenosine A_{2A}ARs in the basal ganglia, together with the profound motor-depressant effects of cannabinoids (CBs), prompted interest in investigating a putative crosstalk between A_{2A}ARs and CB₁ receptors in this brain

area. CB₁ receptor signaling in a human neuroblastoma cell line is dependent on A_{2A}AR activation, and blockade of A_{2A}ARs counteracts the motor-depressant effects produced by CB₁ receptor activation in vivo (Carriba et al. 2007). Interestingly, the motor-depressant effects produced by CB₁ receptor activation are attenuated by genetic inactivation of the DARPP-32 (Andersson et al. 2005), which is abundantly expressed in the medium spiny neurons of the striatum and is crucially involved in the striatal actions of cAMP-coupled receptors (Greengard 2001), as in the case of A_{2A}ARs. Molecular interactions between striatal A_{2A}ARs and CB₁ receptors at the striatum may also exist since CB₁ and A_{2A}ARs form heteromeric complexes once transfected to human embryonic kidney cells, HEK-293T (Carriba et al. 2007).

A_{2A}AR activation is required for the synergistic actions between CB₁ receptors and μ opioid receptors in nucleus accumbens (NAc)/striatal neurons (Yao et al. 2006), as well as for the synergistic actions that occur between CB₁ agonists and D₂ agonists (Yao et al. 2003). Synergy between G_i-coupled receptors, such as CB₁ and D₂ receptors, with respect to the facilitation of cAMP-mediated signaling involves β - γ dimers of G_i proteins, and these are also required for the interplay with A_{2A}ARs (Yao et al. 2003). The implications of these multiple interactions as they pertain to therapies for drug addiction will be discussed below (see Sect. 9.4).

A₁ARs also appear to be involved in motor impairment by CBs, and this may occur at the cerebellum, since the incoordination induced by CB₁ agonists is attenuated by intracerebellar injection of an A₁AR-selective antagonist (DeSanty and Dar 2001). A reciprocal ability to heterologously desensitize CB₁ and A₁AR responses through prolonged agonist exposure has also been reported (Kouznetsova et al. 2002; Selley et al. 2004).

5.1.5 A₁, A_{2A} and A₃ARs

The existence of A₁ – A_{2A} AR heteromers has been demonstrated and complicates the overall picture for adenosine as a neuromodulator and the role of ARs in neurotransmission. Co-immunoprecipitation and bioluminescence resonance energy transfer (BRET) techniques have shown the existence of A₁ – A_{2A} AR heteromers in co-transfected HEK cells, as well as the existence of an intermolecular crosstalk, and radioligand-binding techniques have allowed the identification of an intramembrane receptor–receptor interaction in the A₁ – A_{2A} receptor heteromer (Ciruela et al. 2006). According to Ferré et al. (2007a), the A₁–A_{2A} receptor heteromer may provide a “concentration-dependent switch” mechanism by which low and high concentrations of synaptic adenosine produce the opposite effects on glutamate release. Thus, a weak input might cause stimulation of the receptor with the highest affinity in the A₁/A_{2A} heteromer, while a strong input might cause additional stimulation of the other receptor, with crosstalk between both receptors that may allow a response that is different from the summation of both of them. However, as discussed in point 3 above, other factors such as the topographical arrangement of ectoenzymes, transporters and receptors as well as the neuronal firing frequency may also influence the A₁ versus A_{2A}AR-mediated actions at each synapse where both receptors co-localize.

With respect to crosstalk between A_1 and A_{2A} ARs, this was clearly documented with data obtained from experiments at the hippocampus, where activation of A_{2A} ARs attenuates the ability of A_1 AR agonists to inhibit excitability and synaptic transmission (Cunha et al. 1994a; O'Kane and Stone 1998). An A_{2A} AR-mediated decrease in A_1 AR binding was also shown to occur in hippocampal (Lopes et al. 1999a) and striatal (Dixon et al. 1997) synaptosomes. A_{2A} AR-induced inhibition of A_1 AR binding does not occur in membrane fragments, which indicates that the cross talk between A_1 and A_{2A} receptors involves a diffusible second messenger. The A_{2A}/A_1 AR crosstalk might be related to PKC, rather than to the classical A_{2A} AR second messenger, the adenylate cyclase-cAMP-PKA pathway, because the interactions between A_{2A} and A_1 ARs are prevented by PKC inhibitors but not by PKA inhibitors (Dixon et al. 1997; Lopes et al. 1999a). PKC activators, such as phorbol esters, mimic the ability of A_{2A} receptor agonists to decrease A_1 AR binding (Lopes et al. 1999a). Thus, with respect to their ability to inhibit A_1 AR-mediated responses, A_{2A} ARs appear to behave similarly to the phospholipase C-coupled metabotropic glutamate and muscarinic receptors (Worley et al. 1987); i.e., they operate through a phosphoinositide-PKC-dependent pathway. Activation of PKC inhibits presynaptic A_1 ARs on motor nerve terminals without affecting the affinity of competitive receptor antagonists (Sebastião and Ribeiro 1990), suggesting that the target of PKC is not the receptor ligand-binding domain, but probably a locus related to G-protein coupling, the G protein itself, or both.

Besides the A_{2A}/A_1 AR interaction, which can be observed using either BRET, radioligand binding, or functional studies with selective agonists for both receptors, there are other ways through which A_{2A} AR activation can also induce a decrease in A_1 AR tone. A_{2A} ARs enhance adenosine transport through equilibrative nucleoside transporter (ENT)s, with a consequent reduction in the availability of endogenous adenosine to tonically activate A_1 ARs (Pinto-Duarte et al. 2005). As occurs with A_{2A} AR-mediated inhibition of A_1 AR binding (Lopes et al. 1999a), the A_{2A} AR-induced enhancement of ENT activity is lost upon the inhibition of PKC but not PKA activity, suggesting the involvement of the phospholipase C (PLC) pathway rather than the adenylate cyclase/cAMP one (Pinto-Duarte et al. 2005).

While evaluating the evoked release of acetylcholine at different frequencies of stimulation from hippocampal slices, it becomes clear that the A_{2A} AR-mediated enhancement of ENT activity plays a pivotal role in adjusting adenosine neuromodulation to different physiological needs (Pinto-Duarte et al. 2005). Thus, at high-frequency neuronal firing, there is a predominant release of ATP and a predominant formation of adenosine from released ATP (Cunha et al. 1996b). Therefore, the extracellular adenosine concentration exceeds the intracellular one and the gradient of adenosine concentration across the plasma membrane will direct ENT to take up adenosine. Since A_{2A} ARs are concomitantly activated, the A_{2A} AR-induced enhancement of ENT activity leads to an enhancement of the removal of adenosine from the synaptic cleft, leading to a reduced tonic A_1 - AR-mediated inhibition of hippocampal acetylcholine release at high-frequency firing rates (Pinto-Duarte et al. 2005). This A_{2A} AR-mediated inhibition of tonic inhibitory adenosinergic tone may add to the A_{2A} AR inhibition of A_1 AR activation (see above), thus efficiently

reinforcing the enhanced firing rate of cholinergic afferents into the hippocampus, which are known to play a key role in the control of cognitive processes such as attention and memory (Hasselmo and Giocomo 2006).

Other interactions of A_1 and A_2 ARs include the influence of A_1 ARs on A_{2A} AR activity, where desensitization of striatal A_1 ARs is accompanied by a time-dependent amplification of A_2 – AR-mediated stimulation of adenylate cyclase (Abbracchio et al. 1992). Moreover, presynaptic interactions between A_1 and A_{2A} ARs were clearly observed at motor nerve terminals where A_1 AR inhibitory responses are enhanced in the presence of A_2 AR antagonists, and A_{2A} AR excitatory responses are increased in the presence of A_1 AR antagonists (Correia de Sá et al. 1996). However, in contrast to what occurs in neurones, positive cooperation between A_1 and A_2 ARs, which also requires concomitant activation of metabotropic glutamate receptors (groups I and II), was observed in cultured astrocytes (Ogata et al. 1996).

With respect to A_3 ARs and the interaction of A_3 ARs with other ARs, A_3 AR activation attenuates the synaptic inhibitory actions of adenosine in the CA1 area of the hippocampus (Dunwiddie et al. 1997). Because adenosine A_3 ARs might couple to phospholipase C, and phospholipase C-coupled receptors are able to inhibit A_1 – AR-mediated responses (see above), it is possible that this A_3 – A_1 AR-mediated interaction involves this transduction pathway, in a similar manner to that described in relation to the A_3 – AR-mediated inhibition of metabotropic receptor functioning (Macek et al. 1998).

5.1.6 P2 Purinoceptors

Although ATP and adenosine operate distinct families of receptors and although they may play very distinct roles in the CNS—adenosine being exclusively a neuromodulator and ATP behaving as a neurotransmitter, neuromodulator, or co-modulator—interactions between receptors for these two “family related” molecules have been reported. $P2Y_1$ receptors and A_1 ARs can form heteromeric complexes and display a high degree of colocalization in the brain (Yoshioka et al. 2002). $P2Y_1$ receptors and A_1 ARs are colocalized at glutamatergic synapses and surrounding astrocytes, and $P2Y_1$ receptor stimulation impairs the A_1 AR coupling to the G protein probably by inducing heterologous desensitization (Tonazzini et al. 2008), whereas the stimulation of A_1 ARs increases the functional responsiveness of $P2Y_1$ receptors (Tonazzini et al. 2007). Similar findings were found in relation to the crosstalk between A_1 ARs and $P2Y_2$ receptors, where oligomerization of A_1 ARs and $P2Y_2$ receptors generates a complex in which the simultaneous activation of the two receptors induces a structural alteration that interferes with signaling via $G_{i/o}$ but enhances signaling via $G_{q/11}$ (Suzuki et al. 2006).

The presynaptic facilitatory dinucleotide receptor is also under the control of ARs colocalized at the same nerve terminals. Thus, the apparent affinity of diadenosine pentaphosphate (Ap5A) for its receptor in hippocampal nerve terminals is increased up to the low nanomolar range by coactivation of A_1 or A_{2A} ARs, whereas it is

decreased towards the high micromolar range when A₃ARs are coactivated (Díaz-Hernández et al. 2002). P2 purinoceptor activation by endogenous ATP may also inhibit dinucleotide receptor functioning (Díaz-Hernández et al. 2000).

5.2 Interaction with Ionotropic Receptors

ARs can interact with ionotropic receptors (Fig. 3b), with putative implications for neuroprotection, plasticity and learning, as it is the case for AMPA and NMDA glutamate receptors as well as nicotinic acetylcholine receptors (nAChRs). A brief overview of the published data follows.

5.2.1 Modulation of NMDA and AMPA Receptors by A₁ and A₂ ARs

In isolated rat hippocampal neurones (de Mendonça et al. 1995), as well as in bipolar retinal cells (Costenla et al. 1999), A₁AR activation inhibits *N*-methyl-D-aspartate (NMDA) receptor-mediated currents. Interestingly, the inhibitory postsynaptic action of A₁AR agonists is observed at very low concentrations, compatible with a tonic inhibitory action of adenosine. Accordingly, selective A₁AR antagonism enhances the NMDA component of excitatory postsynaptic currents in CA1 hippocampal neurones, probably due to the recruitment of previously silent NMDA receptors at synapses (Klishin et al. 1995). Through a postsynaptic action, endogenous adenosine also inhibits voltage- and NMDA receptor-sensitive dendritic spikes in the CA1 area of the hippocampus (Li and Henry 2000). Because of the important role played by NMDA receptors in synaptic plasticity phenomena, as well as in neuronal injury after prolonged stimulation or depolarizing conditions, it is conceivable that the ability of A₁ARs to inhibit NMDA receptor-mediated currents together with the well-known A₁AR-mediated inhibition of glutamate release are the basis for the A₁-AR-mediated inhibition of synaptic plasticity phenomena such as long-term potentiation (LTP) and long-term depression (LTD) at CA3/CA1 excitatory synapses of the hippocampus (de Mendonça and Ribeiro 1997b). These two A₁AR-mediated actions also contribute to A₁-AR-mediated neuroprotective actions during hypoxia (Sebastião et al. 2001) and to stopping epileptiform firing in CA1 pyramidal cells (Li and Henry 2000).

On medium spiny neurones at the striatum, A_{2A}AR activation inhibits (rather than facilitates) the conductance of NMDA receptor channels by a mechanism involving the phospholipase C/inositol (1,4,5)-triphosphate/calmodulin and calmodulin kinase II pathway (Wirkner et al. 2000). In Mg²⁺-free conditions, and therefore in conditions where NMDA receptors are not blocked, A_{2A}AR activation postsynaptically inhibits the NMDA receptors in a subpopulation of striatal neurones; however, if the NMDA receptors are blocked by Mg²⁺, the predominant A_{2A}AR-mediated action is a presynaptic inhibition of GABA release (Wirkner et al. 2004). Whether the A_{2A}AR-mediated inhibition of NMDA receptors in the

striatum explains the unexpected protective influence of A_{2A}AR agonists towards NMDA-induced excitotoxicity (Popoli et al. 2004; Tebano et al. 2004) remains to be evaluated.

Interactions between A_{2A}ARs and ionotropic glutamate receptors with implications for synaptic plasticity have been reported. LTP of synaptic transmission between CA3 and CA1 hippocampal areas of the hippocampus involves a post-synaptic facilitation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) currents, a well-known process that requires previous activation of NMDA receptors and involves both pre- (enhanced glutamate release) and post- (depolarization-induced relief of NMDA receptor blockade by Mg²⁺) synaptic mechanisms. Interestingly, A_{2A}AR activation induces a form of LTP in the CA1 area that is NMDA receptor independent (Kessey and Mogul 1997). In contrast, A_{2A}ARs localized postsynaptically at synapses between mossy fibers and CA3 pyramidal cells are essential for a form of LTP of NMDA currents, sparing AMPA currents (Rebola et al. 2008). Considering that CA3/CA1 LTP is predominantly NMDA receptor dependent, and that LTP at mossy fibers/CA3 synapses is predominantly presynaptic and NMDA receptor independent, it appears that A_{2A}ARs are particularly devoted to unmasking nonpredominant forms of plasticity and therefore fine-tuning the networking and information flow within the hippocampus.

NMDA receptor activation suppresses neuronal sensitivity to adenosine in the hippocampus, and this interaction appears to result from an increase in the excitatory action of adenosine A_{2A}ARs rather than a depression of A₁AR function (Nikbakht and Stone 2001).

Direct actions of purines upon NMDA receptor subunits (NR) may also occur. Thus, ATP, probably by directly binding to the glutamate-binding pocket of the NR2B subunit and not to ARs or ATP purinoceptors, can inhibit NMDA receptors and attenuate NMDA-mediated neurotoxicity (Ortinou et al. 2003).

5.2.2 Nicotinic Acetylcholine Receptors

Endogenous adenosine, by activating A_{2A}ARs coupled to the adenylate cyclase/cAMP transduction pathway, tonically downregulates nAChR-mediated control of [3H]-ACh release at either the skeletal neuromuscular junction (Correia-de-Sá and Ribeiro 1994b) or myenteric plexus (Duarte-Araújo et al. 2004). Furthermore, at the skeletal neuromuscular junction, A_{2A}ARs enhance nicotinic receptor desensitization due to prolonged agonist exposure (Correia-de-Sá and Ribeiro 1994b).

The homopentameric α -7 subtype of nAChR is particularly relevant to brain functioning due to its high calcium permeability. By supplying calcium signals, these receptors influence several calcium-dependent events, including transmitter release and plasticity (Gray et al. 1996; Ji et al. 2001), and so several pathways must converge on their regulation. Adenosine, through A_{2A}AR and BDNF, through TrkB receptors, exert double control over α -7-nicotinic currents at GABAergic interneurons in the hippocampus, since blockade of A_{2A}ARs abolishes the BDNF-induced

current inhibition (Fernandes et al. 2008). Since postsynaptic $\alpha 7$ nAChR-mediated inputs to GABAergic interneurons regulate inhibition within the hippocampus, $A_{2A}AR$, by allowing the inhibition of cholinergic currents by BDNF, may temporarily relieve GABAergic inhibition and therefore facilitate plasticity phenomena.

5.3 Interaction with Receptors for Neurotrophic Factors

Trk receptors belong to a third class of membrane receptors which, by themselves, possess catalytic activity involving autophosphorylation in tyrosine residues as a consequence of ligand binding, triggering a subsequent chain of phosphorylations that leads to the activation of several cascades involved in the regulation of cell death, survival and differentiation. Examples of this class of receptors are the receptors for neurotrophins, such as TrkA for nerve growth factor (NGF), TrkB for BDNF, TrkC for neurotrophin 3 (NT-3), and receptors for other neurotrophic factors, such as GFR $\alpha 1$ and Ret for GDNF. In spite of the structural differences between the GPCRs and receptor kinases, ARs, in particular $A_{2A}AR$, can tightly interact with receptors for neurotrophic factors, namely with receptors for BDNF and GDNF (Fig. 3c), which may have several implications for neurodegenerative diseases, as discussed below.

It has been known for several years that presynaptic depolarization (Boulanger and Poo 1999a)—which is known to increase extracellular adenosine levels, as well as enhancement of intracellular cAMP (Boulanger and Poo 1999b)—the most frequent $A_{2A}AR$ transducing pathway, trigger synaptic actions of BDNF. On the other hand, $A_{2A}AR$ s are known to transactivate TrkB receptors in the absence of the neurotrophin (Lee and Chao 2001). This transactivation requires long-term incubation with GPCR agonists and receptor internalization (Rajagopal et al. 2004), and it is not yet clear whether it operates the same mechanism as the more recently identified ability of $A_{2A}AR$ s to trigger synaptic and promote survival actions of neurotrophic factors. Indeed, it has recently been recognized that adenosine $A_{2A}AR$ activation is a crucial prerequisite for the functioning of neurotrophic receptors at synapses. This has been shown for the facilitatory actions of BDNF on synaptic transmission (Diógenes et al. 2004; Tebano et al. 2008) and on LTP (Fontinha et al. 2008) at the CA1 area of the hippocampus, as well as for the action of GDNF at striatal dopaminergic nerve endings (Gomes et al. 2006). $A_{2A}AR$ s and TrkB BDNF receptors can coexist in the same nerve ending since the facilitatory action of $A_{2A}AR$ s upon TrkB-mediated BDNF action is also visible at the neuromuscular junction (Pousinha et al. 2006), a single nerve ending synapse model.

The ability of BDNF to facilitate synaptic transmission is dependent on the age of the animals (Diógenes et al. 2007), and this may be related to the degree of activation of $A_{2A}AR$ s by endogenous adenosine at different ages. Thus, in infant animals (i.e., immediately after weaning), in order to trigger a BDNF facilitatory action it is necessary to increase the extracellular levels of adenosine, either by inhibiting AK, through a brief depolarization (Diógenes et al. 2004; Pousinha et al. 2006), or by inducing high-frequency neuronal firing, such as those inducing LTP

(Fontinha et al. 2008); in all cases the actions of BDNF are lost by blocking A_{2A}ARs with selective antagonists. In adult animals, BDNF per se can facilitate synaptic transmission through TrkB receptor activation, but this effect is also fully lost with blockade of A_{2A}ARs (Diógenes et al. 2007) or in A_{2A}AR knockout (KO) mice (Tebano et al. 2008). Nicotinic α 7 cholinergic currents in GABAergic hippocampal neurons are inhibited by BDNF, and this also requires coactivation of adenosine A_{2A}ARs (Fernandes et al. 2008). Inhibition of GABA transporters (GAT) of the predominant neural subtype, GAT1, by BDNF does not fully depend upon coactivation of A_{2A}ARs since it is not abolished by A_{2A}AR blockade; however, A_{2A}AR activation can facilitate this BDNF action (Vaz et al. 2008).

Whether the ability of A_{2A}ARs to protect retinal neurones against glutamate-induced excitotoxicity (Ferreira and Paes-de-Carvalho 2001) is due to its ability to facilitate actions of neurotrophic factors, as has been shown to occur in relation to A_{2A}AR-mediated neuroprotection of motor neurones (Wiese et al. 2007), requires further investigation. It is worth noting that while Wiese et al. (2007) reported a TrkB-mediated enhancement in the survival of injured facial motor neurons in vivo, TrkB receptor activation by BDNF may render spinal cord-cultured motor neurons more vulnerable to insult (Mojsilovic-Petrovic et al. 2006). Interestingly enough, in both cases, activation of A_{2A}ARs by endogenous adenosine was required, since A_{2A}AR antagonism prevented both the favorable (Wiese et al. 2007) and the deleterious (Mojsilovic-Petrovic et al. 2006) TrkB-mediated actions.

Activation of A_{2A}ARs enhances NGF-induced neurite outgrowth in PC12 cells and rescues NGF-induced neurite outgrowth impaired by blockade of the mitogen-activated protein kinase (MAPK) cascade, an action that requires PKA activation (Cheng et al. 2002). Furthermore, activation of A_{2A}ARs through Trk-dependent and phosphatidylinositol 3-kinase/Akt mechanisms promoted PC12 cell survival after NGF withdrawal (Lee and Chao 2001). A similar A_{2A}AR-mediated neuroprotection mechanism has been shown to occur in hippocampal neurones after BDNF withdrawal (Lee and Chao 2001). In contrast to A_{2A} receptors, which usually promote the actions of neurotrophic factors, A_{2A}ARs inhibit neurite outgrowth of cultured dorsal root ganglion neurons in both the absence and the presence of NGF (Thevananther et al. 2001).

Besides interactions at the neurotrophin receptor level, AR activation may also induce the release of neurotrophic factors. Thus, the expression and/or release of NGF are enhanced by the activation of A_{2A}ARs in microglia (Heese et al. 1997) and by the activation of A₁ARs in astrocytes (Ciccarelli et al. 1999). A_{2B}ARs in astrocytes are also able to enhance GDNF expression (Yamagata et al. 2007). A_{2A}ARs are required for normal BDNF levels in the whole hippocampus (Tebano et al. 2008).

Interactions among purinergic, growth factor and cytokine signaling are also highly relevant in nonpathologic brain functioning, namely in the regulation of neuronal and glial maturation as well as development. In neuronal-dependent glial maturation, both ATP purinoceptors and adenosine ARs are involved (Fields and Burnstock 2006). The extracellular adenosine levels attained during high-frequency neuronal firing are sufficient to stimulate ARs in oligodendrocyte ancestor cells,

inhibiting their proliferation and stimulating their differentiation into myelinating oligodendrocytes (Stevens et al. 2002), but unfortunately the nature of the AR involved was not identified in this work. In premyelinating Schwann cells, A_{2A}ARs activate phosphorylation of extracellular signal-regulated kinases (ERKs), namely ERK1/2, and inhibit Schwann cell proliferation without arresting differentiation (Stevens et al. 2004).

Decreases in the levels and/or actions of neurotrophic factors have been implicated in the pathophysiological mechanisms of many diseases of the nervous system, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, diabetic neuropathies, amyotrophic lateral sclerosis, and even depression, making the use of naturally occurring neurotrophic factors a very promising approach to the treatment of these disorders (Schulte-Herbrüggen et al. 2007). However, the pharmacological administration of neurotrophic factors *in vivo* has not been easy so far because these molecules are unable to cross the blood–brain barrier, making invasive application strategies like intracerebroventricular infusion necessary. The evidence that A_{2A}ARs trigger or facilitate actions of neurotrophins upon synaptic strength and neuronal survival has led to a new therapeutic strategy: the use of adenosine A_{2A}AR agonists that cross the blood–brain barrier to potentiate neurotrophic actions in the brain.

However, we should particularly mention epilepsy, where neurotrophic factors have been considered both harmful, being causal mediators in the development of acquired epileptic syndromes, and also eventually useful in treating epilepsy-associated damage (Scharfman and Hen 2007; Simonato et al. 2006). On top of this controversy, we can add discrepant findings of both anticonvulsive (Huber et al. 2002) and proconvulsive (Zeraati et al. 2006) A_{2A}AR-mediated actions, with the proconvulsive actions being the more expected due to the usually excitatory nature of these receptors.

Finally, the crosstalk between A_{2A}ARs and receptors for neurotrophins also points to the need for caution about therapies with A_{2A}AR antagonists in neurodegenerative diseases, as has been proposed for Parkinson's disease to ameliorate L-DOPA-induced dyskinesias. For more information on A_{2A}ARs and Parkinson's disease, please refer to Chap. 18, "A_{2A} Adenosine Receptors and Parkinson's Disease" (by Morelli et al.), in this volume. Indeed, the identification of postsynaptic A_{2A}/D₂ receptor interactions in the striatum, together with the findings that A_{2A}AR antagonists are neuroprotective in Parkinson's disease models (Chase et al. 2003) and increase dopamine synthesis from L-DOPA (Golembiowska and Dziubina 2004), led to the proposed use of A_{2A}AR antagonists in Parkinson's disease. On the other hand, neurotrophic factors, in particular GDNF, may be a potential therapeutic approach in the management of Parkinson's disease (Love et al. 2005; Patel et al. 2005). Enhancing GDNF actions via A_{2A}AR agonists (Gomes et al. 2006) may also be of high therapeutic interest. In any case, the finding that the actions of GDNF on dopamine release in the striatum are prevented by A_{2A}AR antagonism (Gomes et al. 2006) points to the need for further studies on the consequences of long-term therapy with A_{2A} receptor blockers in neurodegenerative diseases where

neurotrophic factors may play a beneficial role. One issue that should be explored in the future is the optimal time window for combined beneficial effects of neurotrophic factors and A_{2A}AR agonists/antagonists. Perhaps A_{2A}AR antagonists may be advantageous in the late stages of neurodegenerative diseases; however, in the early stages, where an enhancement of neurotrophic factors is highly desirable, A_{2A}AR antagonists should be avoided and A_{2A}AR agonists should perhaps be considered, in order to allow neurotrophic influences. A schematic representation of what has been reported so far on the interactions of ARs and neurotrophin receptors and on neurotrophin release, as well as the implications of these interactions at the hippocampus and striatum in relation to Alzheimer’s and Parkinson’s diseases, is illustrated in Fig. 4.

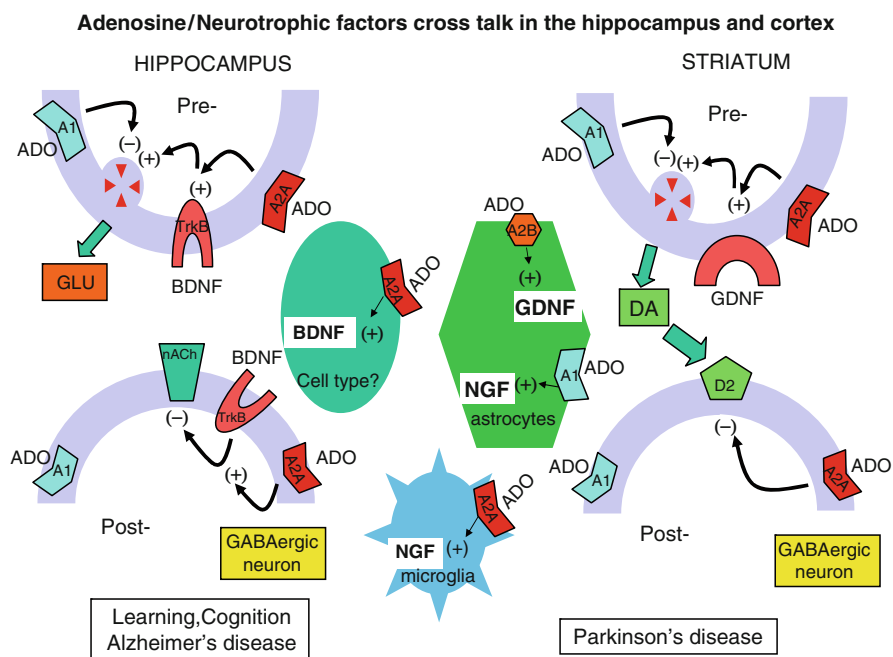


Fig. 4 Schematic representation of what has been reported regarding the interaction between adenosine receptors (ARs) and neurotrophic factor receptors, as well as the influence of ARs on neurotrophic factor synthesis or release, focusing on brain areas with implications for learning, cognition and Alzheimer’s disease (hippocampus) or Parkinson’s disease (striatum). A *plus symbol* denotes facilitation and a *minus symbol* denotes inhibition of receptor functioning or neurotrophic factor synthesis or release. The positive influence of A_{2A}ARs upon brain-derived neurotrophic factor (BDNF) levels was studied in slices, so the cell type cannot be identified. See text for references. Other abbreviations: ADO, adenosine; D, dopamine; DA, dopamine; GDNF, glial cell line-derived neurotrophic factor; GLU, glutamate; nAChR, nicotinic acetylcholine receptor; NGF, nerve growth factor; TrkB, tropomyosin-related kinase receptor type B

6 Hypoxia and Ischemia

6.1 *Adenosine and Control of Synaptic Transmission During Hypoxia*

A very intimate relationship between hypoxia/ischemia and adenosine is well established. This relationship has been the subject of extensive reviews (de Mendonça et al. 2000), and the implications of this relationship for neuroprotection are discussed in Chap. 17, “Adenosine Receptors and Neurological Disease: Neuroprotection and Neurodegeneration” (by Stone et al.), in this volume. Thus, we will focus on hypoxia and the synaptic actions of adenosine.

High amounts of adenosine, and perhaps surprisingly, of ATP are released into the synaptic cleft during a hypoxic/ischemic insult (Freguelli et al. 2007), leading to A₁AR activation and profound inhibition of synaptic transmission (Fowler 1993). This A₁AR-mediated inhibition promotes recovery after the insult, since blockade of A₁ARs reduces inhibition of synaptic transmission but also impairs recovery after reoxygenation (Sebastião et al. 2001). Similar observations have been made using A₁AR KO mice (Johansson et al. 2001) or through focal deletion of the presynaptic A₁ARs (Arrigoni et al. 2005). The facilitation of recovery of synaptic transmission after a hypoxic insult involves both presynaptic inhibition of glutamate release and subsequent reduction of NMDA receptor activation during the hypoxic episode (Sebastião et al. 2001). In other words, the neuroprotection induced by adenosine operates two well-known synaptic actions of A₁ARs that also occur under normoxic conditions and are of particular relevance in the case of hypoxia: a decrease of neurotransmitter release via inhibition of presynaptic calcium entry through the blocking of calcium channels (Ribeiro et al. 1979), and postsynaptically inhibiting calcium entry via inhibition of NMDA receptors (de Mendonça et al. 1995).

The mammalian brain can adapt to injurious insults such as cerebral ischemia to promote cell survival in the face of subsequent injury, a phenomenon known as ischemic preconditioning (Gidday 2006). Adenosine, through A₁AR, is responsible for the protective actions of ischemic preconditioning in the hippocampus; A_{2A}ARs are not involved in this process, whereas A₃AR activation is harmful to ischemic preconditioning, impairing recovery (Pugliese et al. 2003). However, hypoxia leads to a rapid (<90 min) homologous desensitization of A₁AR-mediated inhibition of synaptic transmission that is likely due to an internalization of A₁ARs in nerve terminals (Coelho et al. 2006). This may alleviate A₁AR-mediated functional disconnection of GABAergic neurones (Congar et al. 1995; Lucchi et al. 1996), allowing sequential time windows for a protective role of adenosine and GABA during hypoxia (Sebastião et al. 1996). Changes in the activity of adenosine-producing enzymes also occur during hypoxic/ischemic episodes. This is the case for AK, which is downregulated (Pignataro et al. 2008), and for the enzyme chain-hydrolyzing extracellular ATP, which is upregulated (Braun et al. 1998), with both processes leading to more intense extracellular adenosine production and contributing to its neuroprotection.

6.2 Adenosine and Control of Ventilation

The partial pressure of oxygen in the blood is sensed by the carotid body located at the bifurcation of the carotid artery. Low levels of oxygen in the arterial blood activate the carotid body and ventilation is subsequently enhanced. Since the early 1980s, when the first description of the excitatory effects of adenosine on carotid body chemoreceptor activity appeared (McQueen and Ribeiro 1981), this nucleoside has emerged as a key molecule in the regulation of chemosensory activity and ventilation (Lahiri et al. 2007). Adenosine enhances carotid body chemosensory activity either *in vivo* (McQueen and Ribeiro 1986) or *in vitro* (Runold et al. 1990), as well as ventilation (Monteiro and Ribeiro 1987). This action of adenosine is mediated by A_{2B}ARs in sensory terminals and A_{2A}ARs at carotid body cells, which are activated by its endogenous release as a consequence of a decrease in the partial pressure of oxygen around those cells (Conde et al. 2006; McQueen and Ribeiro 1986). A_{2A}AR mRNA, but not A₁AR mRNA, is expressed in type I carotid body cells, and these receptors modulate Ca²⁺ homeostasis during hypoxia (Kobayashi et al. 2000a).

Upon denervation of the carotid bodies, AR agonists depress ventilation by activating A_{2A}ARs in the CNS (Koos and Chau 1998). Adenosine also modulates cardiorespiratory control through presynaptic actions in the nucleus tractus solitarius, where it modulates transmitter release (Spyer and Thomas 2000).

In humans, intravenous (*i.v.*) injection of adenosine produces hyperventilation and dyspnea resulting from direct activation of the carotid body (Watt et al. 1987). However, some secondary effects, including heat sensation, flushed face, dyspnea and chest discomfort in humans, have been reported after *i.v.* adenosine infusion (Uematsu et al. 2000). Adenosine enhances the ventilatory response to hypoxia but not to hypercapnia (Maxwell et al. 1986), which argues against a major contribution from the central chemosensory centers, where adenosine increases the sensitivity to hypercapnia (Phillis 2004), suggesting a major role for peripheral sensors in the ventilatory response to adenosine in humans (Lahiri et al. 2007).

The usefulness of the carotid body in maintaining oxygen homeostasis is magnified by its plasticity, which to a large extent is due to changes in gene expression (Lahiri et al. 2007). The contribution of purines to the control of carotid body activity may also be developmentally regulated. For example, the A_{2A}AR and D₂ dopaminergic receptors are differentially expressed in glomus cells during development, with greater relative expression of mRNA message for the A_{2A}AR found in earlier stages, and for the D₂ receptors in the adult animal (Gauda et al. 2000). A_{2B}ARs in the carotid body are slightly downregulated within 24 h exposure to moderate (10% O₂) hypoxia (Ganfornina et al. 2005), whereas A_{2A}ARs are upregulated by chronic hypoxia, at least in PC12 cells (Seta and Millhorn 2004). As occurs in brief hypoxic/ischemic episodes (see Sect. 6.1), chronic hypoxia decreases the expression of AK, adenosine deaminase and the adenosine transporter, while it increases the expression of ecto-5'-nucleotidase (Kobayashi et al. 2000b). All of these hypoxia-induced changes in the expression of ARs and the enzymes involved in the control of extracellular adenosine levels may contribute to a protective adaptation to hypoxia.

6.2.1 Adenosine and Respiration in the Newborn

The inhibitory effect of CNS A_{2A} AR activation on respiratory drive is more evident early in life, and is mediated via GABAergic inputs to the inspiratory timing neural circuitry (Mayer et al. 2006). Blockade of these receptors is probably the mechanism by which xanthine therapy alleviates apnea in prematures (Aranda and Turmen 1979; Bairam et al. 1987; Uauy et al. 1975). Indeed, blockade of A_{2A} ARs blunts the respiratory roll-off response to hypoxia in newborn lambs (Koos et al. 2005). Xanthine therapy in the newborn may, however, increase the risk of seizures (see Table 1).

7 Role of ARs in Pain

Pain can have multiple causes and origins, and therefore the ability of adenosine to influence pain also has multiple sites of action and diverse mechanisms. Activation of A_1 AR in the spinal cord produces antinociceptive properties in acute nociceptive, inflammatory and neuropathic pain tests (Sawynok 2007; Sawynok and Liu 2003). In humans, the first evidence for antinociceptive actions of adenosine was detected during adenosine infusion (i.v.), which had beneficial effects in two patients with neuropathic pain (Sollevi et al. 1995). A few years later, the same group showed that adenosine can also reduce secondary hyperalgesia in two human models of cutaneous inflammatory pain (Sjölund et al. 1999). Although peripheral A_{2A} AR activation can exacerbate pain responses (Sawynok 1998), its anti-inflammatory action may also contribute to decreasing inflammatory pain. As a consequence, A_1 AR agonists have entered clinical trials for neuropathic pain, whereas A_{2A} AR agonists are entering clinical trials as anti-inflammatory agents (Gao and Jacobson 2007). There is also growing interest in the use of allosteric enhancers of A_1 AR activation due to the putative tissue selectivity of A_1 ARs. Allosteric modulation of adenosine A_1 ARs reduces allodynia, and this has been shown to occur not only after intrathecal injection but also after systemic administration (Pan et al. 2001).

The pain-relieving effect of activating A_1 ARs at the level of the spinal cord is related to their ability to presynaptically inhibit excitatory transmission to neurons of the substantia gelatinosa (Lao et al. 2001). The inhibition of NMDA receptors by adenosine (see Sect. 5.2.1) probably also occurs at the level of the spinal cord (DeLander and Wahl 1988) and contributes to a reduction of central sensitization and plasticity mechanisms involved in chronic pain. In contrast, adenosine A_{2A} AR activation sensitizes peripheral afferent fibers that project to the spinal cord, enhancing nociception (Hussey et al. 2007). Accordingly, mice lacking the A_{2A} AR have reduced responses to thermal nociceptive stimuli (Ledent et al. 1997), whereas mice lacking the A_1 AR show increased nociceptive response (Wu et al. 2005)

The peripheral administration of adenosine in humans produces pain responses resembling those generated under ischemic conditions (Sawynok 1998). This pain-initiating effects of adenosine are augmented by substance P (Gaspardone et al.

1994) and nicotine (Sylvén et al. 1990), and are usually a limiting factor in the use of adenosine-related compounds for the control of chronic pain. Activation of A₃ARs produces pain due to the release of histamine and 5-hydroxytryptamine from mast cells and subsequent actions on the sensory nerve terminal (Sawynok 1998). However, in spite of these algogenic consequences of peripheral administration of adenosine, its net action on pain processing is inhibitory, since enhancers of extracellular adenosine levels have antinociceptive action (see below).

Due to the simultaneous A₁AR-mediated antinociceptive and A_{2A}AR-mediated anti-inflammatory actions of adenosine, there has been increasing interest in the development of drugs that, by influencing extracellular adenosine levels, could have analgesic actions. Successful examples include inhibitors of AK (see Sect. 3), whose spinally-mediated antinociceptive properties were noted over a decade ago (Keil and DeLander 1992). Most likely due to the anti-inflammatory actions of adenosine, AK inhibitors administered orally are more effective at reducing inflammatory pain than neuropathic or acute pain (Jarvis et al. 2002). By comparing the antinociceptive and anti-inflammatory properties of AK inhibitors administered at the ipsilateral or contralateral sides of the injury, it was concluded that much of the anti-inflammatory action is locally mediated, whereas the antinociceptive action is systemically mediated, exerted predominantly at the level of the spinal dorsal horn (Poon and Sawynok 1999). Indeed, AK inhibitors are able to reduce the increased *c-Fos* expression in the spinal dorsal horn induced by peripheral injection of an inflammatory (carrageenan) substance (Poon and Sawynok 1999).

Antidepressants are widely used in the treatment of neuropathic pain, but their analgesic efficacy seems to occur irrespective of mood-altering effects, and may involve an increase in extracellular adenosine levels. This has been shown after either acute (Esser and Sawynok 2000) or chronic (Esser et al. 2001) amitriptyline administration in rat models of neuropathic pain. Similarly, endogenous adenosine seems to be involved in the antiallodynic action of amitriptyline in a rat model of painful diabetic neuropathy (Ulugol et al. 2002). As pointed out by Esser and Sawynok (2000), the manipulation of endogenous adenosine by amitriptyline, while important, is unlikely to be the sole mechanism underlying its ability to reduce pain, but the attenuation of its effect by modest doses of caffeine (within those levels easily attained in humans after two cups of strong coffee) raise the possibility that dietary caffeine consumption may influence the efficacy of amitriptyline in alleviating neuropathic pain in humans.

Increases in adenosine levels may contribute to the analgesic action of opioids. An increase in adenosine levels in the cerebrospinal fluid has been detected in humans following intrathecal administration of morphine (Eisenach et al. 2004). It is of interest that in neuropathic rats the release of adenosine induced by morphine is reduced (Sandner-Kiesling et al. 2001), which may explain a decreased efficacy and potency of opioids in the treatment of neuropathic pain. Moreover, modifications in the expression of several types of opioid receptors were recently detected in mice lacking the A_{2A}AR gene (Bailey et al. 2002), suggestive of a functional interplay between A_{2A}AR and opioid receptors with respect to pain modulation.

A critical review of the applications of adenosine and ATP in pain control, summarizing most of the human studies, suggests a high potential for adenosine compounds to alleviate pain (Hayashida et al. 2005). This review suggests that the doses, the routes and the timing of administration together with the tissue penetration of the drugs must be taken into consideration, and that there is a need for more basic research to clarify several points. Caffeine, via its antagonistic actions on ARs, can modulate pain; however, as recently discussed (Shapiro 2007), the type of effect (e.g., generation or alleviation of headache) depends on the site of action as well as the dosage and timing of exposure. Both A_{2A} and A_{2B} ARs are probably involved in the interaction between paracetamol and caffeine in pain control. Blockade of A_{2B} ARs causes an enhancement of the action of paracetamol in tail immersion and hot-plate tests in mice, and blockade of A_{2A} ARs produces an antinociceptive effect, even in the absence of paracetamol (Godfrey et al. 2006). Moreover, theophylline ameliorates chest pain in patients with a hypersensitive esophagus, possibly by altering adenosine-mediated nociception (Rao et al. 2007).

As a potent vasodilator, CGRP, which is released by the trigeminocerebrovascular system, plays a key role in the pathophysiology of migraine headache; antagonism of CGRP has been suggested as a promising new approach for the treatment of this condition (Goadsby 2008). Another approach to blocking the trigeminovascular system and CGRP to treat migraine headache may include the use of A_1 AR agonists. Activation of A_1 ARs inhibits trigeminovascular activation by acting on the trigeminal nucleus and by inhibiting the release of CGRP in the cranial circulation, with this second action being attributable to activation of A_1 ARs on peripheral terminals of the trigeminal nerve (Goadsby et al. 2002). Tonic activation of A_1 ARs may also prevent the facilitatory actions of CGRP, as has been shown to occur in the hippocampus (Sebastião et al. 2000b). Interestingly, A_{2A} AR activation facilitates the actions of CGRP (Correia-de-Sá and Ribeiro 1994a; Sebastião et al. 2000a, b), but the relevance of these observations to an approach for the treatment of migraine headache (i.e., with A_2 AR antagonists) remains to be established.

8 Caffeine and ARs

Ever since the delights of tea were first discovered by Emperor Shen Nung in 2737 BC, methylxanthines, including caffeine, have been widely consumed by humans all over the world. The broad caffeine intake associated with common beverages, together with the impact of xanthines on biomedical research, prompted many studies that have focused on specific caffeine effects rather than using it as a tool to antagonize ARs (Daly 2007; Ferré 2008). Indeed, as a pharmacological tool, caffeine is no longer very useful, because its affinity for ARs is low and its selectivity towards the different ARs is also very poor. It is interesting to note that the first proposal for the existence of an A_3 AR was based upon pharmacological characteristics, namely high affinity for agonists and xanthine sensitivity (Ribeiro and Sebastião 1986). Cloning and cellular expression of the rat A_3 AR (Zhou et al. 1992) challenged these criteria,

since the rat A_3 receptor is xanthine insensitive and has low agonist affinity. Cloning and expression of the human A_3 AR (Salvatore et al. 1993) reversed the situation again, since the human A_3 AR is xanthine sensitive and a high-affinity receptor for A_3 AR ligands. For more information on the affinity of the human A_3 AR for A_3 AR ligands, the reader is referred to Chap. 5, “Medicinal Chemistry of the A_3 Adenosine Receptor: Agonists, Antagonists, and Receptor Engineering” (Jacobson et al.), in this volume.

However, xanthines such as caffeine have other biological actions besides AR antagonism. They inhibit PDEs (PDE4, PDE1, PDE5), promote calcium release from intracellular stores, and interfere with $GABA_A$ receptors (Daly 2007). Caffeine analogs can be developed to target any of these mechanisms rather than ARs, and this may be explored therapeutically (Daly 2007), but in the case of caffeine, the effects seen at the low doses taken in during normal human consumption are mostly due to AR antagonism (Fredholm et al. 1999). Due to its ability to antagonize ARs, to cross the blood–brain barrier, and also due to the low risk of intake, caffeine has therapeutic potential in central nervous system dysfunctions (e.g., Alzheimer’s disease and Parkinson’s disease). Adverse effects of caffeine may include anxiety, hypertension, drug interactions, and withdrawal symptoms (Daly 2007). In human volunteers, caffeine improves cognition; however, it also affects sleep (see Table 1). Moreover, a relationship between adenosine A_{2A} ARs and genetic variability in caffeine metabolism associated with habitual caffeine consumption has been proposed (Cornelis et al. 2007), which provides a biological basis for caffeine consumption. In this study, persons with the *ADORA2A TT* genotype were significantly more likely to consume less caffeine than carriers of the *C* allele.

The therapeutic or adverse effects of caffeine are quite different depending on whether it is administered chronically or acutely. For example, chronic caffeine intake, which increases plasma concentrations of adenosine (Conlay et al. 1997), may be neuroprotective. This contrasts with the consequences of acutely antagonizing A_1 ARs (de Mendonça et al. 2000). Chronic AR antagonism with caffeine may also influence cognition and motor activity in a way that resembles the acute effects of AR agonists (Jacobson et al. 1996). Such opposite actions of chronic versus acute treatment not only have important implications for the development of xanthine-based compounds as therapeutic agents but also constitute a frequent confounding parameter in research. Upregulation of A_1 ARs after chronic AR antagonism with xanthines does occur, but A_{2A} AR levels apparently do not change; in addition, there are changes in the levels of receptors for neurotransmitters with chronic administration of xanthines, namely a marked decrease in β -adrenergic receptors and an increase in 5-HT and $GABA_A$ receptors (Jacobson et al. 1996). The increased expression of A_1 ARs in response to chronic antagonism of ARs by caffeine, as compared with A_{2A} ARs, may lead to a shift in the A_1/A_{2A} AR balance after prolonged caffeine intake (Ferré 2008). Moreover, chronic caffeine treatment leads to modifications in the function of the A_1R – $A_{2A}R$ heteromer and this may, in part, be the scientific basis for the strong tolerance to the psychomotor effects of chronic caffeine (Ciruela et al. 2006).

Furthermore, alteration of astrocytogenesis via A_{2A} AR blockade during brain development raises the possibility that postnatal caffeine treatment could have long-term negative consequences on brain function, and should perhaps be avoided in breast-feeding mothers (Desfrere et al. 2007)

8.1 Influence on Brain Function and Dysfunction

8.1.1 Sleep

One of the main reasons for drinking a cup of strong coffee is to repel sleep. Most studies on ARs and sleep regulation in humans rely upon consequences of caffeine ingestion by human volunteers (see Table 1), and it is now widely accepted that caffeine prolongs wakefulness by interfering with the key role of adenosine upon sleep homeostasis (Landolt 2008). In an innovative review of the role of adenosine upon sleep regulation, Porkka-Heiskanen et al. (2002) proposed adenosine as a sleeping factor and hypothesized that adenosine functions in a similar way to neuroprotection against energy depletion. In the critical arousal area (basal forebrain), extracellular adenosine levels start to rise in response to prolonged neuronal activity during wakeful periods. This increase leads to a decrease in neuronal activity, and sleep is induced before the energy balance in the whole brain is affected. Microdialysis measurements performed in freely moving cats showed an increase in the concentrations of adenosine during spontaneous wakefulness, and adenosine transport inhibitors mimicked the sleep-wakefulness profile that occurs after prolonged wakefulness (Porkka-Heiskanen et al. 1997). In contrast, AR antagonists like caffeine increase wakefulness (see Table 1). Prolonged wakefulness induces signs of energy depletion in the brain, which induces an increase in sleep (Benington and Heller 1995). Molecular imaging provided evidence for an A_1 receptor upregulation in cortical and subcortical brain regions after prolonged wakefulness in humans (Elmenhorst et al. 2007). Adenosinergic mechanisms contribute to individual differences associated with sleep deprivation sensitivity in humans (Rétey et al. 2006); furthermore, a genetic variation in the adenosine A_{2A} AR gene may contribute to individual sensitivity to the effects of caffeine on sleep (Rétey et al. 2007, see Table 1).

It is well documented that A_1 ARs are involved in sleep regulation through the inhibition of ascending cholinergic neurons of the basal forebrain (Basheer et al. 2004). However, more recent studies, which include experiments with A_{2A} and A_1 AR KO mice, indicate that A_{2A} ARs (most probably localized in the ventrolateral preoptic area of the hypothalamus) also play a crucial role in the sleep-promoting effects of adenosine and the arousal-enhancing effects of caffeine (Huang et al. 2005). These studies suggest that A_{2A} AR antagonists may represent a novel approach as potential treatments for narcolepsy and other sleep-related disorders (Ferré et al. 2007b). Adenosine A_{2A} ARs in the pontine reticular formation promote acetylcholine release, rapid eye movement (REM) and non-REM sleep in mice. This effect on non-REM sleep is probably due to A_{2A} AR-induced enhancement of GABAergic

inhibition of arousal promoting neurons (Coleman et al. 2006). In addition to its effect in the basal forebrain, adenosine exerts its sleep-promoting effect in the lateral hypothalamus by A₁AR-mediated inhibition of hypocretin/orexin neurons (Liu and Gao 2007; Thakkar et al. 2008).

In conclusion, the two high-affinity ARs, the A₁ and the A_{2A} ARs, affect multiple mechanisms in several brain areas involved in the regulation of sleep and arousal. Therefore, the influences of caffeine upon sleep, felt by many humans and recently also documented in controlled studies in healthy volunteers (see Table 1), can be attributed to both A₁ and A_{2A} AR blockade. As discussed above (see Sect. 8), chronic caffeine consumption may alter AR function and the A₁/A_{2A} AR balance and consequently the influence of both ARs upon sleep.

8.1.2 Epilepsy

There are several clinical reports on caffeine or theophylline intake and seizure susceptibility (Kaufman and Sachdeo 2003; Mortelmans et al. 2008), but surprisingly, no mention is made of the main cause of seizure induction by these drugs, AR antagonism.

Indeed, after the initial observation that adenosine has an anticonvulsant action (Barraco et al. 1984), the therapeutic potential of adenosine-related compounds in epilepsy was immediately pointed out (Dragunow et al. 1985), and it is now widely accepted that adenosine is an endogenous anticonvulsant, an action mediated by inhibitory A₁ARs that restrain excessive neuronal activity. Other ARs are, however, involved in seizure control, though their roles are most frequently related to exacerbating seizures. An influence of A₃ and A₂ ARs in GABA_A receptor stability has been suggested recently (Roseti et al. 2008), based on the observation that A₃ or A_{2B} AR antagonists reduce rundown of GABA_A currents. A_{2A}ARs, by promoting neuronal excitability, may also increase seizure susceptibility. Indeed, A_{2A}AR KO mice are less sensitive to pentylenetetrazol-induced seizures (El Yacoubi et al. 2008).

It has been shown that A₁AR activation by locally released adenosine is an efficient way to keep an epileptic focus localized (Fedele et al. 2006). Therefore, attention is now focused on the development of biocompatible materials for adenosine-releasing intrahippocampal implants (Wilz et al. 2008). In line with the evidence for the antiepileptic role of A₁ARs, A₁AR KO mice are more susceptible to seizures and develop lethal status epilepticus after experimental traumatic brain injury (Kochanek et al. 2006). There are, however, limitations on the use of A₁AR agonists as anticonvulsant drugs due to their pronounced peripheral side effects, like cardiac asystole as well as central side effects like sedation (Dunwiddie 1999). One possibility would be the use of partial agonists, which are more likely to display tissue selectivity. An N⁶,C8-disubstituted adenosine derivative with low efficacy towards A₁AR activation in whole brain membranes but with high efficacy as an inhibitor of hippocampal synaptic transmission was identified (Lorenzen et al. 1997). Another approach that has been more intensely explored is with the use of compounds that increase the extracellular concentrations of adenosine. This has been

attempted with AK inhibitors, which showed beneficial effects in animal models of epilepsy and an improved preclinical therapeutic index over direct-acting AR agonists (McGarraughty et al. 2005). An even more refined approach would be local reconstitution of the inhibitory adenosinergic tone by intracerebral implantation of cells engineered to release adenosine, and this has been done using AK-deficient cells (Güttinger et al. 2005). The reverse also holds true, since transgenic mice overexpressing AK in the brain have increased seizure susceptibility (Fedele et al. 2005). Furthermore, intrahippocampal implants of AK-deficient stem cell-derived neural precursors suppress kindling epileptogenesis (Li et al. 2007). The above evidence suggests that adenosine-augmenting cell and gene therapies may lead to improved treatment options for patients suffering from intractable epilepsy (Boison 2007).

AK is mostly expressed in astrocytes (Studer et al. 2006), and overexpression of AK after seizures, with consequent reduced adenosine inhibitory tone, contributes to seizure aggravation (Fedele et al. 2005). However, release of interleukin-6 (IL-6) from astrocytes induces an upregulation of A₁ARs in both astrocytes (Biber et al. 2001) and neurons (Biber et al. 2007). This leads to an amplification of A₁AR function, enhances the responses to readily released adenosine, enables neuronal rescue from glutamate-induced death, and protects animals from chemically induced convulsing seizures (Biber et al. 2007). Indeed, IL-6 KO mice are more susceptible to seizures and lack the well-known seizure-induced upregulation of A₁ARs (Biber et al. 2007).

Seizure-induced release of neurotrophic factors, such as BDNF, may have beneficial and aggravating actions upon epilepsy, with the beneficial ones being mostly related to promotion of cell survival and the deleterious ones being related to excessive cell proliferation and neuronal sprouting (Simonato et al. 2006). Adenosine, through A_{2A}AR activation, triggers and facilitates BDNF actions in neurons (Diógenes et al. 2004; Fontinha et al. 2008, see Sect. 5.3 above), but the relevance of this interplay for epilepsy remains to be explored. This may be of particular relevance whenever designing therapies that lead to enhanced extracellular adenosine levels, since besides A₁ARs, A_{2A}ARs can also be activated.

8.1.3 Cognition, Learning, and Memory

Endogenous adenosine, through A₁ARs, inhibits long-term synaptic plasticity phenomena such as LTP (de Mendonça and Ribeiro 1994), LTD, and depotentiation (de Mendonça et al. 1997c). In accordance, A₁AR antagonists have been proposed for the treatment of memory disorders (Stone et al. 1995). Cognitive effects of caffeine are mostly due to its ability to antagonize adenosine A₁ARs in the hippocampus and cortex, the brain areas mostly involved in cognition, but as already discussed in detail (see Fredholm et al. 1999), positive actions of caffeine on information processing and performance may also be attributed to improvements in behavioral routines, arousal enhancement and sensorimotor gating. This interpretation was supported by the observation that the AR antagonist theophylline enhances spatial memory performance only during the light period, which is the time of

sleepiness in rats (Hauber and Bareiss 2001). Independently of the processes caffeine or theophylline use to improve cognition, there is little doubt that the beneficial effects most of us feel after a few cups of coffee or tea are due to the actions of these psychoactive substances upon ARs. Recent evidence that blockade of A_1 receptors improves cognition came from a study using a mixed A_1/A_{2A} receptor antagonist, ASP5854 (Mihara et al. 2007). This orally active drug could reverse scopolamine-induced memory deficits in rats, whereas a specific adenosine A_{2A} AR antagonist, KW-6002, did not. Reduced A_{2A} AR activation may also be relevant for cognitive improvements, since A_{2A} AR KO mice have improved spatial recognition memory (Wang et al. 2006). Accordingly, overexpression of A_{2A} ARs leads to memory deficits (Giménez-Llort et al. 2007).

8.1.4 Alzheimer's Disease

There is the possibility that chronic intake of caffeine during one's lifetime might protect from cognitive decline associated with aging. Elderly women who drank relatively large amounts of coffee over their lifetimes gave better performances in memory and other cognitive tests than nondrinkers (Johnson-Kozlow et al. 2002). A case-control study was specifically designed to evaluate whether chronic intake of caffeine might be related to a lower risk of Alzheimer's disease (Maia and de Mendonça 2002), the most common form of dementia. Levels of caffeine consumption in the 20 years that preceded the diagnosis in patients were compared with those taken by age- and sex-matched controls with no signs of cognitive impairment. Data analysis showed that caffeine intake was inversely associated with the risk of Alzheimer's disease and that this association was not explained by several possible confounding variables related to habits and medical disorders (Maia and de Mendonça 2002). This was confirmed in a larger-scale study (4,197 women and 2,820 men) with similar objectives, showing that the psychostimulant properties of caffeine appear to reduce cognitive decline in aged women without dementia (Ritchie et al. 2007).

Long-term protective effects of dietary caffeine intake were also shown in a controlled longitudinal study involving a transgenic murine model of Alzheimer's disease. Caffeine was added to the drinking water of mice between four and nine months of age, with behavioral testing done during the final six weeks of treatment; the results revealed that moderate daily intake of caffeine may delay or reduce the risk of cognitive impairment in these mice (Arendash et al. 2006). Amnesia can be induced experimentally in mice by central administration of β -amyloid peptides, a process that involves cholinergic dysfunction (Maurice et al. 1996). Acute i.v. administration of caffeine or A_{2A} AR antagonists afforded protection against β -amyloid-induced amnesia (Dall'Igna et al. 2007). These acute effects of A_{2A} AR blockade are somewhat unexpected, because A_{2A} ARs are known to facilitate cholinergic function (namely in the hippocampus; Cunha et al. 1994b), and therefore either adenosine A_{2A} AR agonists or A_1 AR antagonists (to prevent A_1 AR-mediated inhibition of acetylcholine release) were expected to be cognitive enhancers. Indeed, the

most widely used drugs in Alzheimer's disease are directed towards an increase in cholinergic function by inhibiting acetylcholinesterase (Doody et al. 2001). These apparent discrepancies point towards the need for more basic research to understand the biological basis and the potential benefits of the emerging adenosine-based therapies for Alzheimer's disease.

8.1.5 Anxiety

The inhibitory action of A_1 ARs on the nervous system, together with the identification of crosstalk mechanisms between benzodiazepines and ARs (Boulenger et al. 1982) and transporters (Bender et al. 1980), soon suggested that adenosine could mediate the anxiolytic action of several centrally active drugs (Phillis and Wu 1982). The possibility that drugs that facilitate A_1 AR-mediated actions could be effective for anxiety was supported by the observations that A_1 AR agonists have anxiolytic actions in rodents (Florio et al. 1998; Jain et al. 1995). Accordingly, A_1 AR KO mice showed increased anxiety-related behavior (Johansson et al. 2001), but this also holds true for A_{2A} AR KO mice (Ledent et al. 1997). A_1 and A_{2A} ARs are involved in benzodiazepine withdrawal signs. In mice, these signs of withdrawal are manifested by increased seizure susceptibility, and agonists of A_1 ARs (Listos et al. 2005) or A_{2A} ARs (Listos et al. 2008) attenuate them. The potential of A_1 AR agonists to reduce the anxiogenic effects during ethanol withdrawal has also been suggested (Prediger et al. 2006).

It is of interest that patients suffering from panic disorder, a serious form of anxiety disorder, appear to be particularly sensitive to small amounts of caffeine (Boulenger et al. 1984). Caffeine is well known to promote anxious behavior in humans and animal models, and can precipitate panic attacks (Klein et al. 1991). It is, however, worth noting that chronic and acute caffeine consumption may lead to quite different consequences with respect to the function of ARs (see above; Boulenger et al. 1983; Jacobson et al. 1996). The short-term anxiety-like effect of caffeine in mice may not be related solely to the blockade of A_1 and A_{2A} ARs, since it is not shared by selective antagonists of each receptor (El Yacoubi et al. 2000). In contrast, anxiolytic effects of a xanthine derivative have been reported, but this is most probably related to agonist activity at serotonin receptors (Daly 2007).

A significant association between self-reported anxiety after caffeine administration and two linked polymorphisms of the A_{2A} AR gene has been reported (Alsene et al. 2003). Furthermore, evidence for a susceptibility locus for panic disorder, either within the A_{2A} AR gene or in a nearby region of chromosome 22, was reported (Deckert et al. 1998, Hamilton et al. 2004). However, this positive association between A_{2A} AR gene polymorphism and panic disorder may not occur in the Asian population (Lam et al. 2005), suggesting an ethnicity-dependent association.

8.1.6 Depression

A_{2A} AR KO mice and wild-type mice injected with A_{2A} AR antagonists were found to be less sensitive to "depressant" challenges than controls (El Yacoubi et al. 2001),

suggesting that blockade of adenosine A_{2A} ARs might be an interesting target for the development of antidepressant agents. This antidepressant-like effect of selective A_{2A} AR antagonists is probably linked to an interaction with dopaminergic transmission, possibly in the frontal cortex, since administration of the dopamine D_2 receptor antagonist haloperidol prevented antidepressant-like effects elicited by selective A_{2A} AR antagonists in the forced swim test (putatively involving cortex), whereas it had no effect on stimulant motor effects of selective A_{2A} AR antagonists (putatively linked to ventral striatum) (El Yacoubi et al. 2003). Depression is frequently associated with loss of motivation and psychomotor slowing. In this context, it is interesting to note that A_{2A} ARs in the nucleus accumbens appear to regulate effort-related processes and action that could be related to modulation of the ventral striatopallidal pathway (Mingote et al. 2008).

Besides A_{2A} ARs, A_1 ARs are also probably involved in the antidepressant-like effect of adenosine (Kaster et al. 2004), which may be of consequence for interactions with the opioid system (Kaster et al. 2007).

It is worth noting that that deep brain stimulation, now widely used by neurosurgeons to treat tremor and other movement disorders, as well as in a number of psychiatric diseases, including obsessive–compulsive disorders and depression, produces its effects by inducing the release of ATP, which is subsequently converted extracellularly to adenosine (Bekar et al. 2008).

Results from clinical and basic studies have demonstrated that stress and depression decrease BDNF expression and neurogenesis, leading to the neurotrophic hypothesis of depression (Castrén et al. 2007; Kozisek et al. 2008). How adenosine A_{2A} AR-dependent facilitation of BDNF actions on hippocampal synapses (see Sect. 5.3), namely enhancement of synaptic transmission (Diógenes et al. 2004) and enhancement of synaptic plasticity (Fontinha et al. 2008), may contribute to these antidepressive actions of adenosine remains to be established.

8.1.7 Schizophrenia

No study, so far, has directly evaluated the influence of caffeine in schizophrenia, but there is growing evidence that adenosine dysfunction may contribute to the neurobiological and clinical features of schizophrenia (Lara et al. 2006). Indeed, adenosine, via activation of A_1 and A_{2A} ARs, is uniquely positioned to influence glutamatergic and dopaminergic neurotransmission, the two neurotransmitter systems that are most affected by the disease. It is possible that an adenosine inhibitory deficit may emerge, resulting in reduced control of dopamine activity and increased vulnerability to excitotoxic glutamate action in the mature brain. Interactions between A_{2A} ARs and D_2 receptors allow further opportunity for mutual modulation between the adenosine and dopamine systems (Fuxe et al. 2007). These mechanisms could provide a rationale for an antipsychotic-like profile for AR agonists, in particular A_{2A} AR agonists to promote a reduction in D_2 receptor signaling (Fuxe et al. 2007) and A_1 AR agonists to promote a reduction in dopamine release (Lara et al.

2006). Indeed, dypiridamole, a well-known inhibitor of adenosine transporters and therefore an enhancer of extracellular adenosine levels, may be of some therapeutic interest in schizophrenic patients (Akhondzadeh et al. 2000).

Reduced NMDA receptor function may contribute to the cognitive and negative symptoms of schizophrenia (Ross et al. 2006). The relationships between adenosine and NMDA receptor function are complex and may operate in opposite ways. Thus, NMDA receptor activation induces adenosine release (Hoehn and White 1989; Schotanus et al. 2006), and therefore NMDA receptor hypofunction may induce a decrease in adenosine-mediated actions. On the other hand, NMDA receptor activation suppresses neuronal sensitivity to adenosine (Nikbakht and Stone 2001). In addition, both A_1 and A_{2A} ARs can influence NMDA receptor functioning, with both receptors being able to inhibit NMDA currents in different brain areas (see Sect. 5.2.1 above).

8.1.8 Huntington's Disease

The role played by ARs in Huntington's disease was recently reviewed and discussed (Popoli et al. 2007) and is a topic in another chapter in this volume, Chap. 17, "Adenosine Receptors and Neurological Disease: Neuroprotection and Neurodegeneration" (by Stone et al.). Therefore, only a few considerations will be mentioned in this section. The complexity inherent to a genetically based, slowly progressing neurodegenerative disease; the different experimental models, which are very frequently nonchronic or subchronic models; as well as changes in receptor levels due to cell loss or to prolonged drug administration give an apparent contradictory picture of the AR involvement in this disease. The pre- versus postsynaptic localization of ARs, in particular A_{2A} ARs, which have highly distinct roles in striatal function according to their synaptic localization, may also contribute to conflicting neuroprotective/neurotoxic consequences of AR manipulation (Blum et al. 2003). Indeed, A_1 AR agonists (Blum et al. 2002), A_{2A} AR agonists (Popoli et al. 2007), as well as A_{2A} AR antagonists (Domenici et al. 2007) are all able to influence diverse symptoms in experimental models of Huntington's disease.

Another aspect that applies to all neurodegenerative diseases, and that may be particularly relevant in the case of Huntington's disease, is related to the loss of neurotrophic support. Huntington's disease is caused by a mutation in a protein named huntingtin that, in its mutated form, is neurotoxic. It happens that wild-type huntingtin upregulates transcription of BDNF (Zuccato et al. 2001), and decreased BDNF levels may be an initial cause of neuronal death in this disease. A_{2A} AR activation can facilitate or even trigger BDNF actions in the brain (Diógenes et al. 2004, 2007; Fontinha et al. 2008), pointing to the possibility that A_{2A} AR activation, at least in the early stages of the disease, may rescue striatal neurons from death due to diminished trophic support by BDNF. It is worth noting that A_{2A} ARs have a dual action in Huntington's disease (Popoli et al. 2007). The ability of A_{2A} ARs to facilitate the actions of BDNF, which is clearly deficient in this neurodegenerative disease (Zuccato and Cattaneo 2007), is most probably some of the positive influence that A_{2A} ARs have on the disease.

8.1.9 Parkinson's Disease

A significant association between higher caffeine intake and lower incidence of Parkinson's disease was reported some years ago (Ross et al. 2000). Moreover, the beneficial effects of caffeine in Parkinson's disease patients have also been reported (Kitagawa et al. 2007, see Table 1). Furthermore, caffeine administered before levodopa may improve its pharmacokinetics in some patients with Parkinson's disease (Deleu et al. 2006).

Caffeine has well known stimulatory actions upon locomotion due to the antagonism of A_{2A} and A_1 ARs in the striatum (Ferré 2008), and in most animal models of Parkinson's disease, antagonizing A_{2A} ARs attenuates some disease symptoms. Since a full chapter in this volume is devoted to ARs and Parkinson's disease, Chap. 18, "Adenosine A_{2A} Receptors and Parkinson's Disease" (by Morelli et al.), and since a recent sequence of reviews were published as proceedings of a meeting on the topic (Chen et al. 2007; Fredholm et al. 2007; Morelli et al. 2007; Schiffmann et al. 2007), we will only highlight a point that is focused upon less, which concerns interactions between adenosine and neurotrophic factors. The putative role of the neurotrophic factor GDNF in slowing or halting disease progression through the facilitation of neuronal survival (Peterson and Nutt 2008) and the facilitatory action of A_{2A} ARs upon the actions of GDNF in striatal dopaminergic nerve endings (Gomes et al. 2006) indicate the need for great caution when blocking A_{2A} ARs in the early phases of Parkinson's disease. Indeed, if the actions of GDNF in dopaminergic neurons depend upon coactivation of A_{2A} ARs (Gomes et al. 2006), it is highly probable that blockade of A_{2A} ARs will be deleterious during the time window when it is possible to rescue neurons with trophic support.

Another relevant consideration is related to the recent finding (Bekar et al. 2008) that deep brain stimulation, a procedure now used to reduce tremor in Parkinson's disease patients, involves the release of considerable amounts of ATP, with its subsequent extracellular metabolism to adenosine. Activation of A_1 ARs by adenosine during this procedure is an essential step in reducing tremor and controlling spread of excitability, thereby reducing the side effects of deep brain stimulation. However, since A_{2A} ARs are highly expressed in thalamic areas, it could be expected that A_{2A} ARs are also activated during deep brain stimulation. Thus, in the late stages of the disease, where it is desirable to prevent A_{2A} AR-mediated inhibition of dopamine D_2 receptor function, the use of an A_{2A} AR antagonist in combination with deep brain stimulation may be beneficial.

9 Drug Addiction and Substances of Abuse

It is currently believed that molecular adaptations of the corticoaccumbens glutamatergic synapses are involved in compulsive drug seeking and relapse. The high density of A_{2A} ARs that pre- and postsynaptically regulate glutamatergic transmission in this brain area lead to the proposal that A_{2A} AR-related compounds could

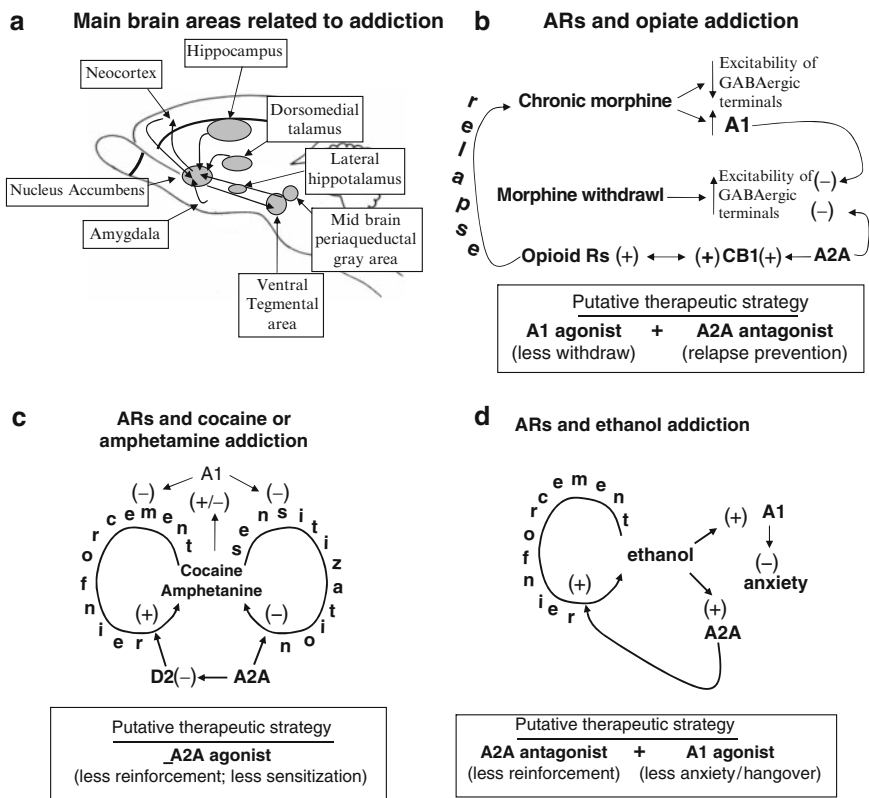


Fig. 5 a–d Brain areas mostly involved in addiction **a**, and the role of A₁ and A_{2A} adenosine receptors (ARs) in addictive behavior **b–d**. The putative therapeutic strategy based on the ARs is indicated *below each panel*. A *plus symbol* denotes facilitation and a *minus symbol* denotes inhibition. See text for references

become new therapeutic agents for drug addiction (Ferré et al. 2007b). Other brain areas involved in reinforcement, motivational and withdrawal consequences of drug use and abuse are the limbic areas, such as the hippocampus and amygdala (Fig. 5a). Accordingly, there is a growing body of evidence suggesting that adenosine is involved in drug addiction and withdrawal, that both A₁ and A₂ ARs may be involved (Hack and Christie 2003), and that a considerable degree of compensation may occur.

9.1 Opioids

Caffeine combined with the opioid antagonist naloxone produces a characteristic quasi-morphine withdrawal syndrome in opiate-naïve animals that is almost completely abolished in A_{2A}AR KO mice and has intermediate intensity in heterozygous

animals, suggesting an involvement of A_{2A} ARs in the withdrawal syndrome (Bilbao et al. 2006). These observations are in agreement with previous data that adenosine reduces morphine withdrawal in an acute model, while caffeine aggravates it (Capasso and Loizzo 2001).

Chronic treatment with opioids induces adaptations in neurons that lead to tolerance and dependence. Endogenous adenosine, through A_1 AR activation, reduces the hyperexcitability of GABAergic terminals of the midbrain periaqueductal gray area (Fig. 5b) that occurs during withdrawal from chronic morphine treatment (Hack et al. 2003). Chronic morphine treatment significantly increased the number of A_1 ARs (Kaplan et al. 1994) and adenosine transporters (Kaplan and Leite-Morris 1997) as well as the adenosine sensitivity in the nucleus accumbens (Brundege and Williams 2002). Surprisingly, chronic blockade of opioid receptors also causes up-regulation of A_1 ARs (Bailey et al. 2003), suggesting an adaptative mechanism in the purinergic system with chronic opioid receptor manipulation. Interestingly, A_{2A} AR levels in the striatum appear to be unaffected by chronic morphine (Kaplan et al. 1994) or chronic opioid antagonism (Bailey et al., 2003).

Both A_1 and A_{2A} AR agonists attenuate opiate withdrawal symptoms (Fig. 5b), but the specific symptoms affected by each AR are different, and the corresponding AR antagonists exacerbate those symptoms (Kaplan and Sears 1996), suggesting that AR agonists rather than AR antagonists may be useful as therapeutics for opioid withdrawal. In line with this idea is the observation that AK inhibitors attenuate opiate withdrawal symptoms (Kaplan and Coyle 1998). Adenosine also seems to act as a regulator of regional cerebral blood flow in both morphine-dependent rats and morphine withdrawal in rats (Khorasani et al., 2006).

Relapse is the most serious limitation of effective medical treatment of opiate addiction. In this respect, A_{2A} AR antagonists may prove useful since A_{2A} AR antagonists administered either directly into the nucleus accumbens or indirectly by intraperitoneal injection eliminate heroin-induced reinstatement in rats that are trained to self-administer heroin, a model of human craving and relapse (Yao et al. 2006). The mechanism wherein A_{2A} AR antagonists block heroin reinstatement most likely involves opiate receptors and their synergy with other GPCRs, namely crosstalk between CB_1 receptors and A_{2A} AR signaling, as well as β - γ dimers (see Sect. 5.1.4 and Fig. 5b).

9.2 Cocaine

Activation of A_{2A} ARs is required to develop the addictive effects to cocaine, since the lack of A_{2A} ARs diminishes the reinforcing efficacy of cocaine (Soria et al. 2006). On the other hand, A_{2A} AR activation protects against cocaine sensitization (Filip et al. 2006), which suggests a therapeutic potential of A_{2A} AR agonists in the treatment of cocaine dependence (Fig. 5c). This is not unexpected, since A_{2A} ARs inhibit D_2 receptor functioning, and these receptors are highly involved in brain-reinforcing circuits. In line with this idea are the observations that A_{2A} AR

agonists inhibit cocaine self-administration in rats (Knapp et al. 2001), and that a nonselective AR antagonist reinstates cocaine-seeking behavior and maintains self-administration in baboons (Weerts and Griffiths 2003). Interestingly, in high-risk situations, prophylactic activation of A_{2A}AR activation may prove beneficial, since A_{2A}AR agonists inhibit the initiation of cocaine self-administration in rats (Knapp et al. 2001). However, the ability of caffeine to prevent the extinction of cocaine-seeking behavior (Kuzmin et al. 1999) or even to reinstate extinguished cocaine self-administration (Green and Schenk 2002) may be related to its blocking effects on A₁ARs, rather than A_{2A}ARs. Moreover, in the nucleus accumbens, sorting and recycling of A₁ARs is dysregulated as a consequence of repeated cocaine administration, so that the amount of A₁AR protein and mRNA is upregulated but the number of membrane receptors, their coupling to G proteins, and their ability to form dimers with D₁ receptors is downregulated (Toda et al. 2003). Furthermore, adenosine uptake in the nucleus accumbens seems to be augmented after cocaine withdrawal (Manzoni et al. 1998).

9.3 Amphetamine

Daily treatment with amphetamine markedly enhances locomotor responses, and this enhancement remains after washout, a process known as sensitization. No sensitization to amphetamines occurs either in conditional A_{2A}AR KO mice or in the presence of A_{2A}AR activation (Bastia et al. 2005), indicating that A_{2A} receptors reduce sensitization (Fig. 5c). Also, selective A₁AR agonists may have some attenuating influence on the development of amphetamine dependence (Poleszak and Malec 2003).

9.4 Cannabinoids

Several studies have reported crosstalk between ARs and CB receptors, as mentioned above (see Sect. 5.1.4). In this section, only the studies specifically addressing the influence of ARs upon CB addiction or tolerance will be mentioned. Crosstolerance between A₁AR and CB₁ receptor agonists has been reported in motor incoordination induced by CBs (DeSanty and Dar 2001). A significant reduction in tetrahydrocannabinol-induced rewarding and aversive effects was found in mice lacking A_{2A}ARs, indicating a specific involvement of A_{2A}ARs in the addiction-related properties of CBs (Soria et al. 2004). Somatic manifestations of tetrahydrocannabinol withdrawal were also significantly attenuated in A_{2A}AR KO mice; however, antinociception, hypocomotion and hypothermia induced by acute tetrahydrocannabinol administration were not affected (Soria et al. 2004).

9.5 Ethanol

The anxiolytic properties of ethanol are generally accepted to be an important motivational factor in its consumption and the development of alcohol dependence. The anxiolytic-like effect induced by ethanol in mice involves the activation of A₁ARs but not A_{2A}ARs (Prediger et al. 2004). The anxiety-like behavior observed during acute ethanol withdrawal (hangover) in mice is attenuated by nonanxiolytic doses of A₁AR agonists (Prediger et al. 2006). Tolerance to ethanol-induced motor incoordination is prevented by A₁AR and dopamine D₁ receptor antagonists, but not by A_{2A}AR antagonists (Batista et al. 2005). However, the reinforcing properties of ethanol are partially mediated via an A_{2A}AR activation of cAMP/PKA signaling in the nucleus accumbens, indicating that administration of an A_{2A}AR antagonist may decrease ethanol reward and consumption (Fig. 5d). Indeed, A_{2A}AR antagonism produces a robust and behaviorally selective reduction of ethanol reinforcement (Thorsell et al. 2007).

10 Concluding Remarks

Several years ago, we (Sebastião and Ribeiro 2000) pointed out that “*In addition to its direct pre- and post-synaptic actions on neurones, adenosine is rich in nuances of priming, triggering and inhibiting the action of several neurotransmitters and neuromodulators (. . .) The harmonic way adenosine builds its influence at synapses to control neuronal communication is operated through fine-tuning, ‘synchronizing’ or ‘desynchronizing’ receptor activation. . .*”. In a recent review, Uhlhaas and Singer (2006) considered that abnormal neural synchronization is central to and the underlying basis for several neurological diseases such as epilepsy, schizophrenia, autism, Alzheimer’s disease, and Parkinson’s disease. These authors highlighted the role of GABAergic neurons and their pivotal role in the primary generation of high-frequency oscillations and local synchronization, the role of glutamatergic connections in controlling their strength, duration, and long-range synchronization, and the role of cholinergic modulation in the fast state-dependent facilitation of high-frequency oscillations and the associated response synchronization. As reviewed in the present work, adenosine is a molecule involved in brain homeostasis that has recently been proposed to be crucial to the effects of deep brain stimulation (Bekar et al. 2008), which mainly aims to affect neuronal synchronization and therefore influence several psychiatric and neurodegenerative diseases. This review suggests that adenosine is a sort of “universal modulator” or a “maestro;” the main molecule involved in coordinating and controlling the synchronization of the release and actions of many synaptic mediators. It also suggests that targeting approaches that increase adenosine levels to provide this synchronization, or targeting ARs with novel safe, selective, and effective therapeutics that are currently in (or are poised to enter) clinical trials, will enhance our understanding of the role of this important endogenous “universal modulator” signaling molecule and its receptors in cognition, neurodegenerative diseases, psychiatric diseases, and drug addiction.

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Adenosine Receptors and Neurological Disease: Neuroprotection and Neurodegeneration

Trevor W. Stone, Stefania Ceruti, and Mariapia P. Abbracchio

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Abstract Adenosine receptors modulate neuronal and synaptic function in a range of ways that may make them relevant to the occurrence, development and treatment of brain ischemic damage and degenerative disorders. A₁ adenosine receptors tend to suppress neural activity by a predominantly presynaptic action, while A_{2A} adenosine receptors are more likely to promote transmitter release and postsynaptic depolarization. A variety of interactions have also been described in which adenosine A₁ or A₂ adenosine receptors can modify cellular responses to conventional neurotransmitters or receptor agonists such as glutamate, NMDA, nitric oxide and P2 purine receptors. Part of the role of adenosine receptors seems to be in the regulation of inflammatory processes that often occur in the aftermath of a major insult or disease process. All of the adenosine receptors can modulate the release of cytokines such as interleukins and tumor necrosis factor- α from immune-competent leukocytes and glia. When examined directly as modifiers of brain damage, A₁ adenosine receptor (AR) agonists, A_{2A}AR agonists and antagonists, as well as A₃AR antagonists, can protect against a range of insults, both *in vitro* and *in vivo*. Intriguingly, acute and chronic treatments with these ligands can often produce diametrically opposite effects on damage outcome, probably resulting from adaptational changes in receptor number or properties. In some cases molecular approaches have identified the involvement of ERK and GSK-3 β pathways in the protection from damage. Much evidence argues for a role of adenosine receptors in neurological disease. Receptor densities are altered in patients with Alzheimer's disease, while many studies have demonstrated effects of adenosine and its antagonists on synaptic plasticity *in vitro*, or on learning adequacy *in vivo*. The combined effects of adenosine on neuronal viability and inflammatory processes have also led to considerations of their roles in Lesch–Nyhan syndrome, Creutzfeldt–Jakob disease, Huntington's disease and multiple sclerosis, as well as the brain damage associated with stroke. In addition to the potential pathological relevance of adenosine receptors, there are earnest attempts in progress to generate ligands that will target adenosine receptors as therapeutic agents to treat some of these disorders.

Keywords Neuroprotection · Neurodegeneration · Ischaemia · Alzheimer's disease · β -amyloid · Huntington's disease · Parkinson's disease · Neurotoxicity · Aging · Stroke · Lesch-Nyhan syndrome · Multiple sclerosis · Creutzfeldt-Jacob syndrome · Prion disease · Acute administration · Chronic administration · Receptor up-regulation · Receptor down-regulation

Abbreviations

ADAC	Adenosine amine congener
AMP	Adenosine monophosphate
AR	Adenosine receptor
BDNF	Brain-derived neurotrophic factor
BIIP20	<i>S</i> -(-)-8-(3-Oxocyclopentyl)-1,3-dipropyl-7 <i>H</i> -purine-2,6-dione
cAMP	Cyclic adenosine monophosphate
CCPA	2-Chloro- <i>N</i> ⁶ -cyclopentyladenosine
CGS15943	5-Amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5- <i>c</i>]quinazoline
CGS21680	2-[4-(2-Carboxyethyl)-phenylethylamino]-5' <i>N</i> -ethyl-carbox amido-adenosine
CHA	<i>N</i> ⁶ -Cyclohexyladenosine
CJD	Creutzfeldt–Jakob disease
Cl-IB-MECA	2-Chloro- <i>N</i> ⁶ -(3-iodobenzyl)adenosine-5' - <i>N</i> -methyluronamide
CNS	Central nervous system
CP66,713	4-Amino-1-phenyl[1,2,4]-triazolo[4,3- <i>a</i>]quinoxaline
CPA	Cyclopentyl adenosine
8-CPT	8-Cyclopentyltheophylline
CREB	Cyclic AMP responsive element binding protein
CSC	8-(3-Chloro styryl)caffeine
DMPX	3,7-Dimethyl-1-propargylxanthine
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
EAE	Allergic encephalomyelitis
ERK1/2	Extracellular signal-regulated kinases 1 and 2
GABA	Gamma-aminobutyric acid
HD	Huntington's disease
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
IB-MECA	<i>N</i> ⁶ -(3-Iodobenzyl)adenosine-5' - <i>N</i> -methyluronamide
IL	Interleukin
KFM19	<i>RS</i> -(-)-8-(3-oxocyclopentyl)-1,3-dipropyl-7 <i>H</i> -purine-2,6-dione
LNS	Lesch–Nyhan syndrome
MAP-2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinases
MCAo	Middle cerebral artery occlusion
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRS2179	<i>N</i> ⁶ -Methyl-2'-deoxyadenosine-3', 5'-bisphosphate
MRS1706	<i>N</i> -(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1 <i>H</i> -purin-8-yl)-phenoxy]acetamide
MS	Multiple sclerosis
NBTI	Nitrobenzylthioinosine
NECA	5' - <i>N</i> -Ethylcarboxamidoadenosine
NGF	Nerve growth factor
NMDA	<i>N</i> -Methyl-D-aspartate
3-NP	3-Nitro-propionic acid

PKC	Protein kinase C
PLC	Phospholipase C
R-PIA	<i>R</i> -Phenylisopropyladenosine
SAH	<i>S</i> -Adenosylhomocysteine
SCH58261	7-(2-Phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3- <i>e</i>]-1,2,4-triazolo[1,5- <i>c</i>]-pyrimidine
TNF- α	Tumor necrosis factor alpha
Trk	Tropomyosin-related kinase
ZM241385	4-(2-[7-Amino-2-(2-furyl)(1,2,4)-triazolo(2,3- <i>a</i>)-(1,3,5)triazin-5-yl-amino]ethyl)phenol

1 Introduction

As will be evident elsewhere in this volume, adenosine receptors are essentially ubiquitous, with almost all cell types expressing functional forms of at least one of the four known subtypes (A_1 , A_{2A} , A_{2B} , A_3). Each of these subtypes has been associated with a range of actions, some of which may become over- or underexpressed, over- or underactive. Such a change in activity could lead to abnormalities of tissue function, which may be severe enough to lead to overt disease. In this chapter, the evidence for a possible contribution of adenosine receptors to the processes of neurodegeneration and neurological disorders involving neurodegeneration will be addressed, together with the potential for developing adenosine receptor ligands as therapeutic agents to modify those disorders.

2 Relevant General Features of Adenosine Receptor Actions

2.1 A_1 Adenosine Receptors

A_1 adenosine receptors occur throughout the central nervous system (CNS), with a high density in the hippocampus and neocortex. The widespread distribution of these receptors is seen in almost all mammalian species examined, including humans (Fastbom et al. 1986, 1987a, b). All cell types in the CNS possess these receptors, including both neurons and microglia (Goodman and Snyder 1982; Lee and Reddington 1986; Rivkees et al. 1995; Fiebich et al. 1996b; Svenningsson et al. 1997; Ochiishi et al. 1999a, b), with neuronal receptors existing on presynaptic terminals and postsynaptic membranes (Ochiishi et al. 1999a, b). Probably the most prominent consequence of activating the A_1 adenosine receptor (AR) is the inhibition of neurotransmitter release from synaptic terminals, an action that has been linked to the reduction of calcium influx in response to action potential invasion of the terminals (Wu and Saggau 1997). A_1 ARs are able to suppress the

release of a variety of neurotransmitters, including glutamate (Corradetti et al. 1984; Fastbom and Fredholm 1985; Andine et al. 1990; Butcher et al. 1990), acetylcholine (Spignoli et al. 1984; Brown et al. 1990) and dopamine (Michaelis et al. 1979; Chowdhury and Fillenz 1991). There is a significant degree of specificity in this action, however, since it seems to result primarily in a suppression of release of excitatory transmitters such as the major excitatory transmitter glutamate (Corradetti et al. 1984; Héron et al. 1993; Poli et al. 1991), rather than inhibitory transmitters such as gamma-aminobutyric acid (GABA). While a depression of GABA release can be demonstrated using A_1 AR agonists, the potency of these compounds and the amount of release inhibition that can be produced are far less than those that have been reported on glutamate release (Hollins and Stone 1980). This difference may be fundamentally important to understanding the relevance of adenosine receptors in neurodegeneration and neuroprotection, since the brain damage which follows strokes and traumatic (mechanical) injuries to the brain (Corsi et al. 1999a) has been attributed to a massive release of glutamate, and it is a suppression of this that may contribute to the neuroprotective efficacy of adenosine A_1 (and A_{2A}) AR. The much smaller effect on GABA release means that the risk of reinstating a degree of hyperexcitability, as a result of blocking inhibitory transmission, is greatly reduced.

Activation of A_1 AR reduces calcium influx, or inhibits calcium availability, as demonstrated in neuronal and cardiac tissues (Dolphin and Prestwich 1985; Fredholm and Dunwiddie 1988; Rudolphi et al. 1992; Scholz and Miller 1992). This may be related to the frequently observed ability of A_1 AR to modulate the potassium conductances of several types, including the ATP-sensitive potassium channels in heart and hippocampal neurons (Trussel and Jackson 1985; Regenold and Illes 1990; Hosseinzadeh and Stone 1998). There appear to be neuronal chloride conductances which are also sensitive to purines, resulting in an increased chloride influx which should contribute to neuronal inhibition in most areas of the brain (Mager et al. 1990; Schubert et al. 1991).

2.2 A_{2A} Adenosine Receptors

A population of A_{2A} ARs is usually distinguished from A_{2B} ARs on the basis of the higher affinity of A_{2A} ARs for the agonist ligand 2-[4-(2-carboxyethyl)-phenylethylamino]-5'-*N*-ethyl-carboxamido-adenosine (CGS21680). CGS21680 shows an approximately 140-fold selectivity for A_{2A} ARs relative to A_1 ARs, (Bridges et al. 1988; Hutchison et al. 1989; Merkel et al. 1992). The A_{2A} ARs occur predominantly on neurons in the striatum, especially the GABAergic striatopallidal projection neurons and on cholinergic interneurons (Jarvis and Williams 1989; Schiffmann et al. 1991; Cunha et al. 1994; Kurokawa et al. 1994; Latini et al. 1996; Ongini and Fredholm 1996; Moreau and Huber 1999). They are also found in the nucleus accumbens and olfactory tubercle, and the hippocampus and cerebral cortex (Cunha et al. 1994; Dixon et al. 1996), although in the last two areas there are significant pharmacological differences between the nominally A_{2A} sites and those

classically described in striatum (Cunha et al. 1996). A broadly similar distribution exists in human brain, since, although they were initially reported to exist primarily in striatal regions (Martinez-Mir et al. 1991), subsequent work has shown their presence more widely throughout the CNS (Svenningsson et al. 1997).

There is abundant evidence from a number of biochemical and electrophysiological investigations that the activation of $A_{2A}AR$ promotes the release of neurotransmitters, including glutamate (Sebastiao and Ribeiro 1992; Cunha et al. 1994), an effect probably produced by increasing presynaptic calcium influx (Goncalves et al. 1997). Administration of the $A_{2A}AR$ agonist CGS21680 *in vivo* does not itself alter the extracellular levels of glutamate in the CNS, but in the rat it can increase the efflux of glutamate triggered by ischemia (Fredholm and Dunwiddie 1988; O'Regan et al. 1992). Consistent with this, the AR antagonist 5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-*c*]quinazoline (CGS15943) can depress glutamate release, possibly by blocking the enhancing effect of endogenous adenosine at $A_{2A}AR$ (Fredholm and Dunwiddie 1988). The facilitation of release by $A_{2A}AR$ agonists has also been demonstrated for other transmitters such as GABA. Hence it is possible that neuroprotection by $A_{2A}AR$ agonists may result, at least in part, from increased extracellular levels of GABA causing generalized inhibition of cell activity, calcium influx and damage (Mayfield et al. 1993; Kurokawa et al. 1994).

2.3 A_{2B} Adenosine Receptors

The low-affinity $A_{2B}AR$ was cloned in the early 1990s, and has long remained the least known adenosine receptor subtype. The A_{2B} receptor positively couples to both adenylyl cyclase and phospholipase C (PLC), the latter occurring through G_q proteins and representing the most important pathway responsible for A_{2B} -mediated effects (Linden et al. 1999). The $A_{2B}AR$ is expressed at low levels in almost all tissues including brain and spinal cord, and its low affinity for the natural ligand suggests that it could be mainly recruited under pathological conditions.

In the CNS, $A_{2B}AR$ s have been suggested to mediate the outgrowth of dorsal spinal cord axons (Corset et al. 2000) and to interact with inflammatory cytokines in the induction of long-term brain responses to trauma and ischemia, such as reactive astrogliosis. A complex interaction between $A_{2B}AR$ and tumor necrosis factor alpha (TNF- α) has been reported, depending upon specific pathophysiological conditions. In particular, prolonged treatment of human astrocytes with the proinflammatory cytokine TNF- α increased the functional responsiveness of $A_{2B}AR$, which, in turn, synergized with the cytokine in inducing the morphological signs of chronic reactive gliosis (Trincavelli et al. 2004). Conversely, short-term exposure of astrocytes to TNF- α caused the phosphorylation of $A_{2B}AR$ and impairment in their coupling to G_s proteins, with consequent decreases of cyclic adenosine monophosphate (cAMP) production. TNF- α -mediated downregulation of $A_{2B}AR$ was demonstrated to occur via protein kinase C (PKC) intracellular kinase. This event likely represents a defense mechanism to counteract excessive A_{2B} receptor activation under acute

damage conditions characterized by massive release of both cytokines and adenosine, such as those occurring during trauma or ischemia (Trincavelli et al. 2008).

A_{2B}ARs have been also suggested to inhibit taurine release from pituicytes, the astroglial cells of the neurohypophyses. In whole rat neurohypophyses pre-loaded with [³H]taurine, taurine efflux elicited by hypotonic shocks was about 30–50% smaller in the presence of 10 mM adenosine or 1 mM NECA (5'-*N*-ethylcarboxamidoadenosine). The A_{2B}AR antagonists MRS1706 {*N*-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)-phenoxy]acetamide} or alloxazine partially reversed the inhibition of release by NECA, while neither agonists of the adenosine A₁, A_{2A} or A₃ ARs nor the A₁AR antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine) had any effect (Pierson et al. 2007). Based on evidence implicating taurine not only in cell osmoregulation but also in olfactory, auditory and visual development, as well as in long-term potentiation in the striatum (Warskulat et al. 2007), if confirmed by further studies, this observation may unveil entirely new pathophysiological roles for this as-yet neglected adenosine receptor subtype.

2.4 A₃ Adenosine Receptors

The A₃ARs (Zhou et al. 1992) have been less well studied than the A₁ and A_{2A} AR populations recognized earlier. A₃ sites exist primarily in peripheral tissues, but they are believed to occur on neuronal and glial cells membranes in most species examined, including human (Jacobson 1998), although at least one group has reported failing to find either the A₃ receptor protein or its mRNA in the CNS (Rivkees et al. 2000). This report was accompanied by claims that several of the purportedly selective ligands used in the functional study of A₃ receptor effects actually have significant activity at A₁AR that could complicate the interpretation of results, and could possibly account entirely for the supposed actions attributed to A₃AR.

2.5 Receptor Interactions

There is good evidence for interactions between receptors for adenosine and other neuroactive compounds. For instance, activation of *N*-methyl-D-aspartic acid (NMDA) receptors can inhibit the actions of A₁AR agonists on presynaptic terminals (Bartrup and Stone 1990; Bartrup et al. 1991; Nikbakht and Stone 2001). In a situation in which the levels of glutamate increase significantly, therefore, there is a real danger that the protective activity of endogenous adenosine could be compromised by NMDA receptors. The direction of receptor interactions is reversed at postsynaptic sites. On hippocampal and striatal neurons, for example, adenosine can depress the activation of NMDA receptors. This action can be produced by A₁ or A_{2A}AR (de Mendonça et al. 1995; Norenberg et al. 1997, 1998; Wirkner et al. 2000,

2004; Gerevich et al. 2002). The relevance of these interactions remains unclear, as do the circumstances under which one or the other would be dominant. Thus, if an increase in the ambient levels of glutamate occurs prior to any elevation of adenosine levels, then a loss of AR-mediated protection would be expected, leading to enhanced cell damage. If, however, any increase in adenosine levels precedes a change in glutamate, the purine could limit the release of the amino acid, and block the activation of NMDA receptors by the lower amounts of glutamate present.

There may be a significant contribution to A_{2A} AR antagonist neuroprotection by the modulation of responses to other neuroactive agents via influences directly on the receptors. Simpson et al. (1992) and later Cunha et al. (1994) reported the ability of A_{2A} AR to antagonize the activation of A_1 AR, a proposal subsequently confirmed and supported by other groups (Dixon et al. 1997; O'Kane and Stone 1998; Latini et al. 1999). The A_{2A} agonist CGS21680 inhibits neuronal responses to the A_1 ligand CPA (O'Kane and Stone 1998), an action that may be related to its ability to induce a low-affinity site for the highly selective A_1 receptor agonist 2-chloro- N^6 -cyclopentyladenosine (CCPA) (Dixon et al. 1997). This interaction may occur between the membrane receptors themselves, or via an intermediate, diffusible messenger. Both A_1 and A_{2A} ARs suppress the electrophysiological effects of glutamate or NMDA applied directly to neurons (de Mendonça et al. 1995; Norenberg et al. 1997, 1998; Gerevich et al. 2002; Wirkner et al. 2000, 2004), while CGS21680 reduces the increased postsynaptic influx of calcium induced by quinolinic acid, and the A_{2A} AR antagonist SCH58261 increases it (Popoli et al. 2002), although another antagonist 4-(2-[7-amino-2-(2-furyl)(1,2,4)-triazolo(2,3-*a*)-(1,3,5)triazin-5-yl-amino]-ethyl)phenol (ZM241385) appears not to do so (Tebano et al. 2004). One implication of this interaction is that, when the extracellular level of adenosine reaches levels sufficient to activate A_{2A} AR, as it can do after kainate administration or ischemia, it may inhibit A_1 receptor function. This phenomenon may explain the curious observation that neuroprotection by ZM241385 is lessened by DPCPX (Jones et al. 1998a,b). The protection by ZM241385 could be due to its blockade of A_{2A} AR, thus "releasing" A_1 AR from tonic suppression by A_{2A} AR. If the heightened activation of A_1 AR were then responsible for the neuroprotection, it would be prevented by DPCPX, as observed (Jones et al. 1998a, b).

As in the case of the A_{2A} agonists noted above, there is evidence that agonists at A_3 AR, administered acutely, may reduce responses to A_1 AR agonists, and thus decrease the protective activity of endogenous adenosine levels (von Lubitz et al. 1999a). On the contrary, a chronic activation of A_3 AR exerts protective effects, as detailed below (see Sect. 3.4). However, it is not known whether these effects are mediated by an opposite activity on the A_1 AR subtype.

Finally, there is evidence that some AR subtypes can physically interact with other neurotransmitter receptors, leading to the generation of receptor heteromers characterized by unique pharmacological properties. Yoshioka et al. (2001) co-expressed A_1 AR and P2Y₁ receptor for ADP in HEK293 cells. These receptors co-immunoprecipitated in western blots of whole cell membrane lysates. Coexpressing the P2Y₁ receptor did not alter surface expression of the A_1 receptor, but it did

inhibit the binding of radiolabeled A_1 AR agonists and antagonists in membrane preparations. This change was not seen in a mixture of membranes from cells expressing each receptor individually. Additionally, the binding of an A_1 AR agonist was displaced by the $P2Y_1$ agonist ADP β S and the $P2Y_1$ antagonist N^6 -methyl-2'-deoxyadenosine-3', 5'-bisphosphate (MRS2179) in cotransfected cells, but not in cells expressing the A_1 receptor only. Globally, these data indicate formation of a functional heteromeric complex where A_1 ARs physically interact with $P2Y_1$ receptors (Abbracchio et al. 2006).

A_1 ARs couple to G_i , mediating depression of intracellular cAMP levels, whereas $P2Y_1$ receptors interact with $G_{q/11}$ and have no effect on cAMP. ADP β S inhibited cAMP production in co-transfected cells only, an effect that was antagonized by the A_1 antagonist DPCPX, but not by MRS2179, and was abolished by pertussis toxin. Thus, ADP β S appears to have acted via the A_1 AR ligand-binding site; i.e., the $P2Y_1/A_1$ dimer has novel pharmacological properties compared with the parent receptors. Interestingly, although ADP β S induced inositol phosphate synthesis, the A_1 agonist cyclopentyl adenosine (CPA) did not. Thus, dimerization did not lead to a complete change in pharmacological properties in this case.

Using confocal laser microscopy to study the subcellular distribution of the $P2Y_1$ and A_1 AR, Yoshioka et al. (2001) showed that both were expressed mainly near the plasma membrane of HEK293 cells. Furthermore, there was a strong overlap in their distribution in individual cells. This was confirmed in a subsequent study using the biophysical technique of bioluminescence resonance energy transfer (Yoshioka et al. 2002b). In the absence of agonists, the receptors showed a homogeneous colocalization across the cells. Addition of ADP β S and CPA together, but not alone, induced an increase in the bioluminescence resonance energy transfer ratio over 10 min. Thus, although the receptors have a constitutive association, their coactivation increased the association. This association was also seen with native receptors in central neurons. Using confocal laser microscopy and double immunofluorescence, Yoshioka et al. (2002a) demonstrated that the $P2Y_1$ and A_1 AR colocalized in neurons of the rat cortex, hippocampus, and cerebellum. A direct association was then shown by their coimmunoprecipitation in membrane extracts from these regions.

The structural requirements for the receptor-receptor interaction are not known at present. The physiological roles of the $P2Y_1/A_1$ dimer also remain to be determined, although Nakata et al. (2003) have pointed out that its pharmacological properties resemble those of a presynaptic receptor that mediates inhibition of neurotransmitter release in some tissues. Finally, Yoshioka et al. (2001) reported that the rat $P2Y_2$ receptor also coimmunoprecipitated with the A_1 receptor when they were coexpressed in HEK293 cells. Thus, the formation of oligomers by A_1 AR receptors is likely to be widespread and to greatly increase the diversity of purinergic signaling.

In a similar way, A_{2A} ARs have been demonstrated to dimerize with D_2 receptors, an interaction which involved peculiar peptide residues (Canals et al. 2003). The formation of A_{2A}/D_2 receptor heteromers in the plasma membrane contributes

to explain the early observation of agonist affinity loss at the D₂ receptor after activation of the A_{2A}AR (Ferré et al. 1991) and provides a molecular explanation to the functional interaction between adenosine and dopamine in basal ganglia.

2.6 *Anti-inflammatory Effects*

One line of argument that tissue protection by purines is more dependent on modulation of the immune system than on neurotransmitter release or activity is that protection against damage is shown in a range of tissues besides the CNS. Adenosine antagonizes the release and actions of several proinflammatory cytokines such as TNF- α and complement (Lappin and Whaley 1984; Cronstein et al. 1992; LeVraux et al. 1993; Barnes et al. 1995; Ritchie et al. 1997). A_{2A}ARs specifically inhibit the production of IL-12 by human monocytes but increase the generation of IL-10 (Link et al. 2000). This ability to modulate the relative release of several cytokines could be a significant factor in determining the overall immune profile that occurs in response to different primary activating stimuli in different inflammatory situations. Adenosine suppresses phagocytosis, free radical generation and cell adherence by white blood cells activated by immune stimulation (Cronstein et al. 1985, 1987, 1990, 1992; Burkey and Webster 1993; Cronstein 1994). There is now clear evidence that A_{2A}AR play a major role in this form of cellular regulation (Dianzani et al. 1994; Hannon et al. 1998), probably acting via the activation of a serine/threonine protein phosphatase (Revan et al. 1996). Most strikingly, adenosine receptors protect the heart against damage occasioned by ischemia (Zhao et al. 1993; Matherne et al. 1997). Indeed, all anti-inflammatory actions of adenosine have been demonstrated in the myocardium, including suppression of TNF- α production (Meldrum et al. 1997; Wagner et al. 1998a, b; Cain et al. 1998) and regulation of neutrophil adherence to myocytes (Bullough et al. 1995). There is, however, some confusion as to the nature of the ARs involved. Human neutrophils possess A₁ and A_{2A}ARs (Varani et al. 1998) and Cronstein et al. (1992) have demonstrated that both receptors are able to modulate several aspects of the immune response, including chemotaxis. Lozza et al. (1997) have suggested that A₁ and A_{2A}AR agonists are both able to protect the heart against ischemia/reperfusion injury, but there are reports that A₁ agonists but not A_{2A}AR agonists provide cardiac protection (Casati et al. 1997), whereas other groups have claimed the opposite (Cargnoni et al. 1999). The former claim is more consistent with evidence that resistance to myocardial ischemia is correlated with the level of expression of A₁AR. In most cases, the two populations of receptor exhibit opposing actions, suggesting that their joint presence could be the basis of a control system in which low concentrations of adenosine, via A₁AR, are normally able to enhance the sensitivity of white blood cells to immune stimuli but, at the higher concentrations likely to occur at the time of an established immune response, A_{2A}AR can restrain the extent of cellular activity (Cronstein et al. 1992).

The regulation of cytokines by A₃AR is quite selective. Production of several cytokines, including some such as IL-1 β and IL-6, which are also proinflammatory, can be modified by A₃AR activation (Ramakers et al. 2006). A₃AR may also suppress the oxidative burst that accompanies the response of defensive leucocytes to immune activation. They can reduce superoxide generation in human eosinophils (Ezeamuzie and Philips 1999), for example, although there is apparently no similar suppression of oxidative activity in human neutrophils (Hannon et al. 1998). The former action could be secondary to an increase in the level of antioxidant enzymes, including superoxide dismutase, which has been shown to be produced by A₃AR agonists in endothelial cells (Maggirwar et al. 1994).

3 Role of Adenosine Receptors in Brain Cell Survival and in Neurodegenerative Diseases

3.1 A₁ Adenosine Receptors and Neuroprotection

Although much of the interest in the therapeutic value of purine receptor ligands has centered on protection following strokes, there remains the possibility that overactivation of glutamate receptors may contribute to chronic neurodegenerative disorders such as Alzheimer's disease and Huntington's disease. This possibility is the rationale for studying the protective effects of agents against excitotoxins, which are frequently used as a model of stroke and neurodegenerative disease. The most commonly used excitotoxins are kainic acid and quinolinic acid, a tryptophan metabolite for which the evidence for a role in some degenerative disorders is substantial (see Stone 1993, 2001; Stone and Darlington 2002 for reviews). Not only do they produce a controllable degree and extent of injury, but the mechanisms of damage have much in common with natural causes. Thus, even the damage produced by kainic acid probably involves a presynaptic action of kainate, which induces the release of endogenous compounds such as glutamate and aspartate (Kohler et al. 1978; Ferkany et al. 1982; Lehmann et al. 1983; Jacobson and Hamberger 1985; Connick and Stone 1986; Virgili et al. 1986; Okazaki and Nadler 1988). Whether this secondary release is the primary cause of cell death or only a contributory (perhaps permissive) component is irrelevant, since the essential issue is that the inhibition of their release by an agent such as an adenosine A₁AR agonist will have the same net protective activity.

Both A₁AR agonists and A_{2A}AR agonists and antagonists (see also below) can protect against kainic acid-induced damage (MacGregor and Stone 1993; Jones et al. 1998a, b). *R*-Phenylisopropyladenosine (*R*-PIA) protected against kainic acid neurotoxicity in several regions of the CNS in addition to the hippocampus (MacGregor and Stone 1993; MacGregor et al. 1993, 1996), the involvement of A₁AR being further confirmed by showing that protection could be prevented by the simultaneous administration of an A₁AR antagonist such as DPCPX. In addition to the

use of DPCPX to confirm the involvement of A₁AR, there have been several studies showing that DPCPX and other selective A₁AR blockers increase the amount of neuronal damage after ischemia or the administration of excitotoxins (Rudolphi et al. 1987; von Lubitz et al. 1994a; Phillis 1995). R-PIA prevented the kainate-induced damage in areas such as the basolateral amygdala, pyriform cortex and rhinal fissure. An observation that has not been pursued, but which may be of considerable pathological and therapeutic importance, was that some areas of the brain, especially those located in more caudal regions such as the entorhinal cortex, the posteromedial cortical amygdaloid nucleus and the amygdalopyriform transition, were not protected by A₁AR activation. It is still uncertain why R-PIA showed such regionally selective protection. There may be fewer A₁ARs in the resistant areas, or a greater susceptibility to damage which the adenosine agonist was unable to overcome at the doses used. The protection afforded by A₁ARs does not necessarily require the use of a selective exogenous agonist, since compounds which raise the concentrations of endogenous adenosine, either by inhibiting transporter function or adenosine metabolism, can also produce protection (Parkinson et al. 1994; Pazzagli et al. 1994).

There is an even greater contribution of presynaptic release in the neuronal damage caused by quinolinic acid, although its major action seems to be the activation of NMDA receptors and the generation of reactive oxygen species (Stone and Darlington 2002; Stone 2001).

A number of other studies have demonstrated protection by adenosine analogs against damage produced by toxins or excitotoxins (Arvin et al. 1989; Connick and Stone 1989; Finn et al. 1991). One especially interesting report showed that protection could be produced by the adenosine A₁ receptor agonist *N*⁶-cyclohexyladenosine (CHA) against the selective dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Lau and Mouradian 1993). This protection raises the real possibility that a selective A₁AR agonist could be useful in Parkinson's disease, where a proportion of cases may be caused by the exposure of patients to exogenous toxins with molecular structures and a propensity to generate oxidative stress similar to those of MPTP. However, the mechanism of protection against MPTP remains unclear, although antagonists at NMDA receptors can also block MPTP damage, raising the possibility that glutamate receptors may play a critical role comparable to that exhibited by them in stroke-induced damage, and against which A₁AR agonists are also effective.

3.2 A_{2A} Adenosine Receptors and Neuroprotection

At variance from the clearcut neuroprotective role exerted by A₁AR, contrasting data have been reported so far on the beneficial/detrimental roles mediated by A_{2A}AR on brain cells.

As with the A₁AR, very early studies indicated that agonists at A_{2A}AR can produce protection of the CNS against several insults, including ischemia (Phillis

1995; Sheardown and Knutsen 1996), and excitotoxins such as kainate (Sperk 1994; Jones et al. 1998a, b). However, protection by CGS21680 was largely prevented by 8-(*p*-sulfophenyl)-theophylline (8PST), a nonselective xanthine antagonist that blocks both A₁ and A₂ARs. Since this antagonist does not penetrate the blood–brain barrier, it was suggested that the protective activity of CGS21680 was generated via sites on the systemic rather than central side of the barrier. The effect was believed to be primarily exerted on the vascular system, modifying blood flow to the potentially damaged regions of brain, or on white blood cells of the immune system, reducing their penetration to and activation by the early neuronal damage. This conclusion was supported by findings that administering CGS21680 directly into the hippocampus did not induce protection.

On the other hand, neuroprotection by an antagonist at A_{2A}AR was first reported by Gao and Phillis (1994). They found that the nonselective A₂AR antagonist CGS15943 protected the gerbil brain against ischemic damage, an observation later supported using the more selective compounds 8-(3-chlorostyryl)caffeine (CSC) and 4-amino-1-phenyl[1,2,4]-triazolo[4,3-*a*]quinoxaline (CP66,713) (Phillis 1995). Many of the earlier studies examined protection against global cerebral ischemia, but protection has also been demonstrated against focal ischemic damage (Ongini et al. 1997). More recent work has involved a range of different receptor ligands and ischemic models (von Lubitz et al. 1995b; Sheardown and Knutsen 1996; Monopoli et al. 1998). In addition, protection by A_{2A}AR antagonists occurs against excitotoxins such as kainic acid, glutamate and quinolinic acid (Jones et al. 1998a, b). The ability of A_{2A}AR antagonists to protect the CNS has received strong support from the generation of transgenic mice lacking these receptors. These knockout animals exhibit a significantly lower level of brain injury following excitotoxins or ischemia (Bona et al. 1997; Chen et al. 1999).

Interestingly, a possible mechanism at the basis of the neuroprotective effects of A_{2A}AR antagonists may reside in blockade of A_{2A}AR-mediated glutamate release by astrocytes.

Adenosine causes a two- to threefold increase in glutamate release from cultured hippocampal astrocytes (Nishizaki et al. 2002; Nishizaki 2004). Such an effect is mimicked by the A_{2A}AR agonist CGS21680 and inhibited by the A_{2A}AR antagonist 3,7-dimethyl-1-propargylxanthine (DMPX), but not by the A₁AR antagonist 8-cyclopentyltheophylline (8-CPT) (Li et al. 2001; Nishizaki et al. 2002). These observations suggest that adenosine stimulates vesicular glutamate release from astrocytes via A_{2A}AR. This agrees with recent findings demonstrating that the A_{2A} receptor antagonist ZM241385 (5 nM via probe) completely prevents the increase in extracellular glutamate outflow induced by dihydrokainic acid, a blocker of glial glutamate uptake (Pintor et al. 2004).

More recently, however, the equation A_{2A} receptor blockade = neuroprotection has appeared too simplistic (in this respect, see Popoli et al. 2007). First, it is now definitely clear that, besides mediating “bad” responses (for example, stimulation of glutamate outflow and excessive glial activation), A_{2A}ARs also promote “good” responses (such as trophic and anti-inflammatory effects). This implies that blockade of A_{2A}AR can result in either protoxic or neuroprotective effects according to the mechanisms involved in a given experimental model.

Confirmation that A_{2A} AR activation could be neuroprotective came with the development of more selective compounds. Thus, ZM241385 is highly selective for A_{2A} AR, with an approximately 80-fold greater affinity at A_{2A} AR compared with A_{2B} AR. It has an affinity for A_{2A} AR that is around 1,000 times greater than for A_1 AR (Palmer et al. 1995). When examined for its ability to protect the CNS against kainic acid, ZM241385 was as effective as the agonist ligand CGS21680. Indeed, the agonist and antagonist together produced a synergistic protection leading to the complete protection of hippocampal neurones (Jones et al. 1998a, b).

To explain these puzzling results, several hypotheses have been invoked, including different degrees of presynaptic versus postsynaptic A_{2A} receptor blockade. The question of presynaptic versus postsynaptic sites of action of A_{2A} AR has been explored by Blum et al. (2003a), with the conclusion that the overall response will depend on the balance of involvement of the former, at which A_{2A} AR activation appears to be deleterious, whereas A_{2A} AR stimulation is protective at postsynaptic sites. In line with this hypothesis, the increase in intracellular calcium levels induced by quinolinic acid in striatal neurons (an effect mediated by postsynaptic NMDA receptors) is significantly potentiated by the A_{2A} AR antagonist 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]-pyrimidine (SCH58261) and prevented by the A_{2A} AR agonist CGS21680 (Popoli et al. 2002). In agreement, CGS-21680 was reported to reduce NMDA currents in striatal neurons (Norenberg et al. 1997; Wirkner et al. 2000). Moreover, ZM241385 potentiated NMDA-induced effects in rat corticostriatal slices (Tebano et al. 2004), and the A_{2A} AR antagonist CSC potentiated NMDA-induced toxicity in the hippocampus (Robledo et al. 1999). Thus, as far as NMDA-dependent toxicity is concerned, it seems that A_{2A} AR activation, rather than its blockade, can exert neuroprotective effects.

However, the activity of A_{2A} AR antagonists on the "postsynaptic side" of excitotoxicity appears to be far more problematic. At variance from protective receptors on postsynaptic neuronal cells, postsynaptic A_{2A} ARs localized on microglial inflammatory cells might play a detrimental role. In addition, A_{2A} ARs expressed by bone marrow-derived cells have been proposed as potential contributors to striatal damage induced by mitochondrial dysfunctions in Huntington's and Parkinson's disease (Huang et al. 2006), as was previously suggested in the ischemic context (Yu et al. 2004). In accordance with these findings, in an established in vitro model of reactive astrogliosis, blockade of A_{2A} ARs abolished growth-factor mediated astrocytic activation, an event that may potentially contribute to inflammation and neuronal damage in neurodegenerative diseases (Brambilla et al. 2003).

Finally, A_{2A} ARs can mediate neuroprotection by potentiating brain-derived neurotrophic factor (BDNF) survival signaling pathways. The first link between BDNF and adenosine was provided in 2001, with the demonstration that the activation of tropomyosin-related kinase (Trk)A receptors in PC12 cells and TrkB in hippocampal neurons could be obtained in the absence of neurotrophins by treatment with adenosine (Lee and Chao 2001). These effects were reproduced by using the adenosine agonist CGS21680 and were counteracted with the antagonist ZM241385, indicating that this transactivation by adenosine involves the A_{2A} AR subtype. At hippocampal synapses, presynaptic activity-dependent release

of adenosine, through the activation of $A_{2A}AR$, facilitates BDNF modulation of synaptic transmission (for a review, see: Popoli et al. 2007). A similar positive interaction has more recently been confirmed to occur at the neuromuscular junction, which possesses both adenosine $A_{2A}AR$ and BDNF TrkB receptors. The following sequence of events in what concerns cooperativity between $A_{2A}AR$ and TrkB receptors has been suggested: $A_{2A}AR$ s activate the PKA pathway, which promotes the action of BDNF through TrkB receptors coupled to PLC γ , leading to the enhancement of neuromuscular transmission (Pousinha et al. 2006; see also below). Preliminary data indicate that $A_{2A}AR$ s also regulate BDNF levels in the striatum. The importance of $A_{2A}AR$ in regulating BDNF has recently been strengthened by the demonstration that both BDNF levels and functions are significantly reduced in the brains of $A_{2A}AR$ knockout (KO) mice (Popoli et al. 2007).

The possible detrimental/beneficial effects elicited by $A_{2A}AR$ activation or blockade on different brain cell populations are summarized in Fig. 1.

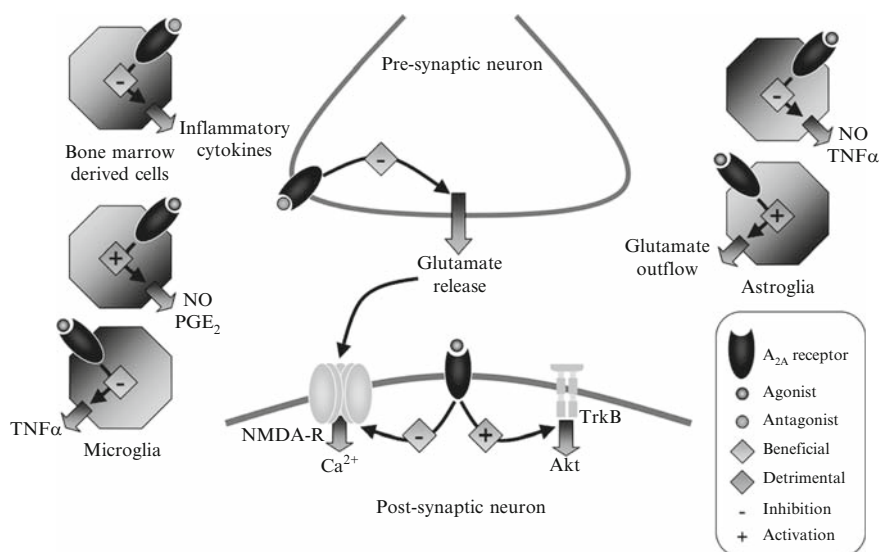


Fig. 1 Schematic representation of the possible effects elicited by A_{2A} adenosine receptor (AR) activation or blockade on different brain cell populations. In the presynaptic neurons, $A_{2A}AR$ blockade may exert beneficial effects through the inhibition of glutamate release. In the postsynaptic neurons, adenosine $A_{2A}AR$ s inhibit *N*-methyl-D-aspartate (NMDA) receptor currents and activate tropomyosin-related kinase (Trk)B receptors, both being potentially beneficial effects. The picture is further complicated by the different effects elicited by the stimulation or blockade of $A_{2A}AR$ s expressed on non-neuronal cells. In astrocytes, $A_{2A}AR$ stimulation can induce both deleterious effects by an increase in glutamate outflow (for a more detailed description of the effects elicited by $A_{2A}AR$ s on glial-mediated modulation of glutamate outflow, see the main text), and beneficial effects through an inhibition of nitric oxide (NO) and tumor necrosis factor alpha (TNF- α) release. This latter beneficial effect has been observed also in microglial cells, although the stimulation of $A_{2A}AR$ s can also induce potentially deleterious effects on this cell population (see also Saura et al. 2005). Finally, in bone marrow-derived cells, it seems to be the blockade of $A_{2A}AR$ s that, through the reduction of cytokine release, can induce beneficial effects. Reproduced and modified from Popoli et al. (2007) with permission from Elsevier

3.3 *A_{2B} Adenosine Receptors and Neuroprotection*

Far less is known about the role of A_{2B}ARs in neuroprotection compared to that of A₁ and of A_{2A}ARs. As already mentioned, expression of A_{2B}ARs on glial cells and their lower affinity for adenosine suggests a role under emergency conditions (when adenosine levels are massively increased) in mediating long-term inflammatory changes. In line with this hypothesis, A_{2B}ARs were found to synergize with the proinflammatory cytokine TNF- α in mediating the induction of reactive astrogliosis (see Trincavelli et al. 2004).

3.4 *A₃ Adenosine Receptors and Neuroprotection*

A dual, biphasic role of A₃AR in neuroprotection has been described in several experimental models, both in vivo and in vitro. In fact, von Lubitz et al. clearly demonstrated that an acute administration of the A₃AR selective agonist *N*⁶-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA) to gerbils dramatically worsened the outcome of a subsequent ischemic episode, whereas chronic stimulation of this receptor subtype protected the animals from stroke, probably through the induction of preconditioning (von Lubitz et al. 1999b; see also below). The protective action of A₃AR agonists against ischemic damage has been recently confirmed by Chen et al. (2006), who also showed that neuroprotection was completely lost in A₃ knockout mice, thus demonstrating the specific involvement of this receptor subtype. Similar results have been obtained in in vitro models. In fact, in non-neuronal cells, low concentrations of the A₃AR agonist 2-chloro-*N*⁶-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (Cl-IB-MECA; 10 nM or 1 μ M) protected against the cell death induced by selective antagonists at this receptor subtype (Yao et al. 1997). Thus, it is suggested that there is a tonic low level of A₃AR activation, possibly induced by the release of endogenous adenosine, which results in cell protection. Protection by Cl-IB-MECA against cell death has been also demonstrated in primary cortical cultures subjected to oxygen-glucose deprivation (Chen et al. 2006). Opposite toxic effects can be achieved when concentrations of agonists $\geq 10 \mu$ M are used. This has been proven true in several non-neuronal cell lines, with induction of apoptosis and Bak expression (Yao et al. 1997), but also in rat cerebellar granule cells (Sei et al. 1997) and in astrocytic cultures (Abbracchio et al. 1998; Di Iorio et al. 2002), where the reduction of the Bcl-2 expression and the activation of the proapoptotic enzyme caspase 3 by Cl-IB-MECA have been demonstrated (Appel et al. 2001).

3.5 *Adenosine Receptors and Therapeutic Possibilities*

These various findings have aroused great interest in the search for new drugs that could be used to slow or prevent the neuronal damage that characterizes

neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. That interest is attributable not only to the efficacy of the compounds available, but also to the fact that they should be relatively free of major side effects. Whereas the use of A₁AR agonists would lead to a suppression of transmitter release at many sites within the central and peripheral nervous system, whatever the physiopathological state of those sites, A_{2A}AR antagonists should only produce effects when the receptors are being activated by endogenous adenosine. In practice, this means that A_{2A}AR antagonists have little effect on heart rate, blood pressure, or other vital signs under normal conditions. During ischemia in the brain, however, the levels of adenosine may rise to levels at which A_{2A}AR are activated. Stimulation of A_{2A}AR increases the release of the excitotoxic amino acid glutamate (O'Regan et al. 1992; Popoli et al. 1995), which would tend to cause or facilitate the occurrence of damage. Under these circumstances, A_{2A}AR antagonists should reduce the enhanced release of glutamate and thus decrease the extent of neuronal damage. Their beneficial activity would therefore be restricted to those areas of the brain experiencing ischemia, with little or no effect on other areas of the brain or peripheral tissues.

A particularly exciting aspect of A_{2A}AR protection is that it may contribute to the long-term benefits of treating patients with Parkinson's disease with A_{2A} receptor antagonists. It is clear that A_{2A}ARs potently modulate cell sensitivity to dopamine receptors, accounting for the beneficial effects of adenosine antagonists in this disease (Mally and Stone 1994, 1996, 1998). This phenomenon has led to clinical trials with A_{2A}AR antagonists in Parkinson's disease with promising, though as-yet unpublished, results. In lower primates, A_{2A}AR antagonists are certainly effective against toxin-induced models of the disorder (Kanda et al. 1998; Grondin et al. 1999). The occurrence of protection has been supported strongly by the demonstration that MPTP was able to produce little damage in transgenic mice engineered to be deficient in A_{2A}AR (Ongini et al. 2001).

As noted earlier, the protective effect of A_{2A}AR antagonists may be the result of their removal of the A_{2A}AR suppression of A₁AR (Jones et al. 1998a, b; Pedata et al. 2001). Since there is clear evidence that A₁AR activation is protective, this interaction would explain both the protection by A_{2A}AR antagonists and the blockade of that protection by A₁AR antagonists (Jones et al. 1998a, b). A₁AR activation suppresses excitatory transmitter amino acid release, as does blockade of A_{2A}AR by CGS15943 (Simpson et al. 1992), whereas blockade of A₁AR or activation of A_{2A}AR enhances the release.

3.6 Molecular Basis of Neuroprotection

There has to date been little progress in identifying the molecular basis of the neuroprotective activity of adenosine receptors, quite apart from identifying the relative importance of neurons and glia in neuroprotection. Staurosporine is a well-recognized activator of apoptosis, and in many cells, including astrocytes, this

activity is accompanied by caspase 3, p38 mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 β (GSK3 β) activation (D'Alimonte et al. 2007). The induction of apoptosis can be prevented by CCPA at A₁ receptor-selective concentrations that are blocked by DPCPX. In addition, these authors noted that CCPA induced the phosphorylative activation of Akt and thus activation of phosphatidylinositol 3-kinase (PI3K), leading to the proposal that this action caused inhibition of the staurosporine effects. The same group has reported a similarly protective action of CCPA against astrocyte apoptosis induced by the quasi-ischemic procedure of oxygen/glucose deprivation (Ciccarelli et al. 2007). Abnormalities in both the p38 and GSK3 β pathways have been implicated in the neuronal damage following acute (stroke) and chronic (Alzheimer's disease) neurodegenerative conditions, so that the modulation of adenosine A₁ receptor function may have a more fundamental and direct relevance to cell protection in these cases than merely a global influence on cell excitability or transmitter release. Protection was again accompanied by activation of PI3K. Pharmacological modifiers of apoptosis led to the overall conclusion that A₁ receptor activation protects by activating the PI3K and extracellular signal-regulated kinase 1 and 2 (ERK1/2) MAPK pathways.

Some of the mechanisms at the basis of A_{2A}AR-mediated neuroprotection have already been described above (see Sect. 3.2). In addition, part of the neuroprotective effects of A_{2A}AR may stem from the reduction of nitric oxide production. Saura et al. (2005) reported that CGS21680 potentiated the lipopolysaccharide-induced increase of NOS expression and NO production in mixed neuron/glia cultures, whereas ZM241385 blocked the effect. Similarly, Fiebich et al. (1996b) have shown that the activation of A_{2A}AR can induce the expression of COX-2, a key proinflammatory molecule giving rise to eicosanoids and, indirectly, to increased oxidative stress. The A_{2A}AR antagonists could therefore suppress this expression as part of their neuroprotective mechanism (in this respect, see also Sect. 3.6).

The mechanisms underlying the protective effects exerted by low doses of A₃AR agonists have not been clearly understood. In an *in vivo* model of ischemia, protection by IB-MECA appears to be associated with preservation of cytoskeletal proteins (such as microtubule-associated protein) and increased deposition of glial fibrillary acidic protein in injured areas (von Lubitz et al. 1999b). This would accord with studies *in vitro*, using glial cultures, in which Cl-IB-MECA induced a number of cytoskeletal changes with the formation of actin filaments (the so-called "stress fibers") accompanied by alterations of cell morphology, such as the emission of long and thick processes in parallel with the alteration of cytoskeletal-associated RhoGTPases (Abbracchio et al. 1997). These changes resulted in a significant reduction of spontaneous apoptosis in culture (Abbracchio et al. 1998), suggesting that astrocytes exposed to nanomolar concentrations of A₃AR agonists are more resistant to cell death, probably due to increased adherence to the culture substrate. Therefore, it can be envisaged that neuroprotection observed *in vivo* could (at least in part) be due to the beneficial effects of A₃AR agonists on astrocytes, which might in turn help neurons to survive the ischemic episode.

3.7 Trophic Activity

It is possible that an important feature of adenosine receptor activation or blockade contributing to the regulation of neuronal and glial function and viability is the ability of these receptors to directly influence the growth and development of nerve and glial cells. Much of this work has been performed and reviewed by Rathbone et al. (1992, 1999). Both A_1 and A_{2A} ARs can promote neuritogenesis in neuroblastoma cells, the A_{2A} AR acting via PKA (Canals et al. 2005). Trophic effects are exerted by A_{2A} ARs via a positive synergistic interaction with BDNF prosurvival pathways (see above). This interaction occurred through activation of PI3K/Akt via a Trk-dependent mechanism, resulting in increased cell survival after nerve growth factor or brain-derived neurotrophic factor withdrawal.

The ability of adenosine to mediate trophic effects via activation of its receptors presents yet another factor to be considered in using adenosine ligands therapeutically, since antagonists might inhibit a valuable degree of structural reorganization and recovery following a brain insult or limit the degree of damage produced in a degenerative disorder.

4 Aging and Alzheimer's Disease

4.1 Changes of Adenosine Receptors with Aging

Since ARs can modify the neural release and actions of acetylcholine, one of the neurotransmitters most intimately associated with the loss of cortical afferent neurons arising from the nucleus basalis of Meynert, and therefore the transmitter most commonly linked to the development of Alzheimer's disease, they have attracted some attention in relation to dementias.

It is interesting to compare the range of studies that have examined adenosine receptors during the normal aging process with those that have concentrated selectively on changes found in the brains of patients with dementias. Animal studies to date have centered largely on A_1 AR presence and distribution in view of their ability to inhibit transmitter release. Reports of alterations with ageing have been confusing, no doubt (at least in part) due to the differing choices of species, brain region, methodology and ligands employed. Some groups have reported clear decreases in A_1 AR binding in limited regions of animal brain (Araki et al. 1993; Cunha et al. 1995), whereas others have found more generalized losses (Pagonopoulou and Angelatou 1992) or no change (Virus et al. 1984; Hara et al. 1992; Fredholm et al. 1998) with aging. In one of the earliest of these studies, the loss of a low-affinity subtype of A_1 AR was described (Corradetti et al. 1984), although binding was examined using an agonist ligand, and the pharmacological tools to explore the nature of the receptor in more detail were not yet available. A later study using gerbils classified as "middle aged" (16 months old), and which may not therefore have

direct relevance for neurodegeneration in the elderly, found significant reductions of A₁AR density in the hippocampus compared with young animals (1 month old), whereas increased binding was found in the neocortex (Araki et al. 1993). When changes in the presence of A₁AR were studied using quantitative autoradiography in the brains of young, old, and senescent rats (3, 24, or 30 months), the density of receptors diminished with age, although the dynamics of that reduction were very different in the various brain regions examined. Thus, while a gradual decline in receptor numbers was seen in hippocampus, cortical sites were lost only after 24 months of age (Meerlo et al. 2004). Fredholm et al. (1998) noted that, while they could find no change in receptor binding, mRNA for the A₁AR was decreased in aging rats, a finding which emphasizes the importance for interpretation of examining the receptor message as well as the protein and, ideally, a measure of receptor function.

Results with A_{2A}ARs have been more consistent, usually indicating a reduction in receptor binding in regions of high density such as striatum (Fredholm et al. 1998). Although these changes were statistically significant, the limited magnitude of the change (20% decrease between 6 and 99 weeks of age) leaves open the question of the functional meaning of that change in the light of the innate adaptive plasticity of the brain.

No data on the possible changes of A₃AR with age are available at the moment.

4.2 Alterations of Adenosine Receptors in Alzheimer's Patients

The examination of human brain tissue from patients who died with a confirmed diagnosis of Alzheimer's disease seems to consistently show a loss of A₁AR (Jansen et al. 1990; Kalaria et al. 1990; Ulas et al. 1993; Deckert et al. 1998), especially and most clearly in the hippocampus, a region of the brain most intimately involved in the processes of learning and memory.

Jansen et al. (1990) described a decrease in receptor densities for several neuroactive compounds in post-mortem tissue from Alzheimer's disease patients. Losses were found in receptors for most of these, including adenosine A₁ARs, which were reduced by 46% in the dentate gyrus. An autoradiographic study using DPCPX as a ligand also reported marked decreases in A₁AR binding in the outer layers of the dentate gyrus, probably reflecting the loss of perforant path input (Jaarsma et al. 1991). The surprising observation was made that the CA1 and CA3 regions showed no loss of A₁AR, despite clear cellular degeneration and reduced numbers of NMDA receptors. Although the difference was attributed to a dendritic location of the A₁AR, the recognized association of A₁AR with presynaptic terminals leaves open the question of whether the perforant path is far more profoundly affected by degeneration than intrinsic hippocampal fibers.

Ulas et al. (1993) also found a similar decrease in A₁ receptor binding in the hippocampus and parahippocampal gyrus of Alzheimer individuals and age-matched controls, with a loss of binding density, though not affinity, in the dentate gyrus

(molecular layer). However, decreases were also seen in the CA1 stratum oriens and outer layers of the para-hippocampal gyrus, with subnormal levels of antagonist binding in the CA3 region. Coupling to G proteins was similar in the control and patient populations, indicating a normal transduction pathway for the remaining receptors.

Striatal A₁ARs are also decreased in patients with Alzheimer's disease. Quantitative autoradiography in the post-mortem striatum indicated a reduction of A₁-binding sites in Alzheimer's disease patients compared with matched controls. No comparable change of another presynaptic site, that for kappa opiate receptors, was noted, but the loss of A₁AR showed a strong correlation with the decreased activity of choline acetyltransferase measured in the same tissue samples (Ikeda et al. 1993). In contrast, the levels of A₁AR and A₂ARs appear to be increased in the frontal cortex, in parallel with an increased functional activity of these receptors (Albasanz et al. 2008).

In a fascinating analysis of post-mortem neocortical and hippocampal tissue from patients with Alzheimer's disease, Angulo et al. (2003) reported a significant colocalization of A₁AR with β -amyloid in senile plaques. They also showed that, in human neuroblastoma cells, activation of A₁AR activated PKC, p21 Ras and ERK1/2, leading to increased formation of soluble β -amyloid fragments, raising the possibility that agonists at A₁AR might be valuable drugs in the treatment of established or late-stage Alzheimer's disease.

4.3 Adenosine Receptors and Cognition

In considering both the possible role of adenosine receptors in the symptomatology of Alzheimer's disease, and the potential value of adenosine ligands in treatment, it is clearly important to consider not only histologically or functionally defined neuronal damage but also the reflection of that damage at the behavioral level, especially for cognition.

There is increasing epidemiological evidence for a role of adenosine receptors in cognitive decline with aging. Much of this evidence relates to the use of coffee, which, in several recent studies, has been concluded to produce a protective effect against the cognitive decline in Alzheimer's disease (van Gelder et al. 2007; Quintana et al. 2007). It is clear, however, that the variations in methodology between studies rather confuse attempts to compare results. It is also clear that the relationship between coffee and cognition is not simple, with major questions remaining, such as the role of caffeine versus other constituents of the brew, and the existence of an optimal coffee intake, above and below which cognitive decline may be enhanced.

Despite this caveat, studies specifically focused on caffeine have reached similar conclusions. The risk of developing Alzheimer's disease, for example, is inversely related to caffeine consumption (Maia and de Mendonça 2002). Both caffeine and ZM241385 prevent the neuronal toxicity caused by β -amyloid peptide in vitro or in

vivo (Dall'igna et al. 2003, 2007). Caffeine was also effective in the Swedish mutation transgenic mouse model of Alzheimer's disease, in which cognitive deficits are associated with the induced overexpression of β -amyloid in the brain. Caffeine was able to reduce the β -amyloid load and behavioral indications of cognitive impairment in these mice (Arendash et al. 2006). Associated proteins such as presenilin 1 and β -secretase were also reduced. Confirmation that these effects were likely to be a direct result of actions on the neurons rather than glia or peripheral mechanisms was obtained by showing a similar reduction of β -amyloid formation in neuronal cultures with the same mutation.

Psychological studies have investigated the effects of caffeine on a range of behaviors in human subjects, including vigilance and aspects of learning, as well as in a variety of modified states, including subject age, frequency of caffeine use, level of tolerance or withdrawal, and state of sleep deprivation. However, the relevant doses and their molecular mechanism of action often remain unproven. In a representative study, Riedel et al. (1995) noted that, in healthy subjects, 250 mg of caffeine reduced the scopolamine-induced performance deficit in memory tasks. The provocative conclusion was drawn that any cognition-enhancing drug being considered for therapeutic use should be shown to be at least as active as this dose of caffeine: an amount equivalent to only three cups of coffee. Results of the many studies on caffeine are, however, often confusing. In one study of almost 1,000 people, it was reported that the consumption of (caffeinated) coffee was associated with improved cognitive performance in women, especially those aged over 80 years, but not men. A possible attribution of this finding to caffeine was made on the basis that decaffeinated coffee seemed to have no influence on cognitive function (Johnson-Kozlow et al. 2002).

It seems likely that the effects of adenosine antagonists, especially the nonselective ones such as caffeine, may have quite subtle effects on learning. Angelucci et al. (2002) suggested that this was due to an effect to improve memory retention, with less or no effect on memory acquisition, while Hauber and Bareiss (2001) showed an improvement by theophylline of spatial reference memory when acquisition was achieved under light conditions, but not in the dark.

Whereas most studies have found that agonists at A_1 ARs tend to impair learning and memory function (Normile and Barraco 1991; Zarrindast and Shafaghi 1994; Corodimas and Tomita 2001), there are occasional reports of learning facilitation or improvement after the acute (Hooper et al. 1996) or chronic (von Lubitz et al. 1993) administration of an agonist. Antagonists have clear ability to enhance cognition and to reverse induced cognitive deficits. One of the earliest studies on animal learning used the compound *RS*-(-)-8-(3-oxocyclopentyl)-1,3-dipropyl-7*H*-purine-2,6-dione (KFM19), an A_1 AR antagonist, which showed cognition-enhancing properties in a rat model (Schingnitz et al. 1991). In a more recent study of olfactory discrimination and social memory in rats, Prediger et al. (2005) demonstrated that deficits in the behaviors of both 12- and 18-month-old animals could be prevented by caffeine or ZM241385. Interestingly, A_1 AR blockade by DPCPX was ineffective. Similarly, DPCPX was reported not to affect the acquisition of a shock-induced avoidance task, even though caffeine, or a selective

A_{2A}AR antagonist, did so (Kopf et al. 1999). It is important to note, however, that knockout studies have not been consistent with many of the pharmacological studies using antagonists. Thus, mice lacking A₁AR exhibited normal learning of spatial tasks in the water maze (Gimenez-Llort et al. 2002).

Maemoto et al. (2004) have also shown recently that a new A₁AR-selective antagonist, FR194921 (2-(1-methyl-4-piperidinyl)-6-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl)-3(2*H*)-pyridazinone) was able to reverse scopolamine-induced deficits on a passive avoidance test, with little effect on behavioral paradigms related to anxiety and depression. Pitsikas and Borsini (1997) obtained similar results using the A₁AR antagonist *S*-(-)-8-(3-oxocyclopentyl)-1,3-dipropyl-7*H*-purine-2,6-dione (BIIP20). A number of detailed structure–activity studies have attempted to define the molecular requirements of A₁AR antagonism that are needed for cognition enhancement (Suzuki et al. 1993).

Few studies have been performed using A_{2A} or A₃ AR ligands in human subjects. Most recently, a specific relationship between A_{2A}AR and Alzheimer's disease was reported by Scatena et al. (2007). The administration of SCH58261 to mice in which β -amyloid (25–35) peptide was delivered into the cerebral ventricles was found able to prevent the subsequent neuronal loss, raising the possibility of reversing the neuronal loss in Alzheimer's disease that is attributable to β -amyloid accumulation. This is particularly interesting in relation to the report, described above, of the colocalization of A₁AR with β -amyloid in senile plaques and the ability of A₁AR to increase the formation of soluble β -amyloid fragments. Since there are several strands of evidence that A_{2A}AR can inhibit the activation of A₁AR (Cunha et al. 1994; Dixon et al. 1997; O'Kane and Stone 1998), it is possible that the effect of SCH58261 is the result of removing an A_{2A}AR-mediated suppression of A₁AR, unmasking the protective efficacy of A₁ARs.

Part of the difficulty in accounting for the detailed mechanism of A_{2A}AR antagonist protection lies in the fact that modulation of A_{2A}AR results in a plethora of actions, many of which are in functional opposition to each other. Thus, while A_{2A}AR agonists promote glutamate release (Sebastiao and Ribeiro 1996) and antagonists should therefore have a valuable action in suppressing excessive release (see also Sect. 3.2), the opposite applies to the inflammatory cytokines. Activation of A_{2A}AR inhibits both the initial calcium influx and the subsequent release of TNF- α induced by various stimuli, including the neurotoxic HIV protein Tat (Fotheringham et al. 2004). Blockade of A_{2A}AR should therefore increase the release of TNF- α and, presumably, related proinflammatory cytokines, thus potentially increasing cell damage. Perhaps the net neuroprotective effects of A_{2A}AR blockade are a complex result of pro- and anti-inflammatory activities, at least some of which may be time dependent, determined by whether the antagonists are present acutely or chronically.

The A₃AR agonist IB-MECA appeared to have little effect alone on measures of learning using simple tests such as spontaneous alternation and passive avoidance. However, this compound did prevent the deficits in these behaviors induced by scopolamine or dizocilpine (Rubaj et al. 2003).

4.4 *The Enigma of Propentofylline*

The xanthine derivative propentofylline has been the subject of research for almost 20 years, yet in many respects it remains an enigma. It is also an enigma that requires decoding, since its activity may have significance for understanding the role of adenosine receptors in health and disease. Propentofylline is a weak antagonist at adenosine receptors. Its main actions seem to be an inhibition of adenosine uptake into cells, resulting in increased extracellular concentrations, and an inhibition of cyclic AMP phosphodiesterases. But at the level of the behaving animal, its overall effect is to promote cognitive function. Propentofylline has been shown to protect against cerebral ischemia in gerbils (Dux et al. 1990).

Even in humans, this compound is an effective cognition enhancer (Noble and Wagstaff 1997), and has been found to improve cognitive function in patients with vascular dementias (Mielke et al. 1996a, b). In animal models of Alzheimer's disease, it has been shown to prevent the cognitive impairment caused by intracerebral administration of β -amyloid (1–40) (Yamada et al. 1998). This effect was attributed to the promotion of nerve growth factor (NGF) production, which raises further questions about the relationship between this hypothesis and the activation of adenosine receptors. One possibility is that raised extracellular adenosine levels activate $A_{2A}AR$, and these in turn, as shown by Heese et al. (1997), then promote the generation of NGF and other neurotrophins. The balance of activation of adenosine receptors could be tipped from A_1AR to $A_{2A}AR$ activation by virtue of the inhibitory effect of propentofylline on phosphodiesterase (Schubert et al. 1997). This hypothesis would be consistent with the fact that propentofylline is able to suppress TNF- α production (Meiners et al. 2004), an action that could be mediated partly via the activation (direct or indirect) of $A_{2A}AR$.

There may also be more direct influences of propentofylline on microglial cells which regulate their degree of inflammatory activity (McRae et al. 1994; Schubert et al. 1996; Rudolphi and Schubert 1997), though the extent to which adenosine receptors might be involved in this also remains unclear. Some of these effects are almost certainly mediated via changes in calcium dynamics within neurons and glia (McLarnon et al. 2005).

4.5 *Adenosine, Homocysteinuria and Alzheimer's Disease*

Homocysteinuria has been widely linked to vascular abnormalities leading, directly or indirectly, to the compromise of neuronal function and cognitive dysfunction seen in vascular dementia and Alzheimer's disease, and there have been suggestions that a deficiency of adenosine may contribute to the neurological manifestations of increased homocysteine levels. One of the consequences of raised extracellular homocysteine is a parallel reduction of adenosine concentrations, possibly resulting from the formation of *S*-adenosylhomocysteine (SAH). A strong negative correlation between plasma levels of the two compounds has been recorded in Alzheimer's

disease patients (Selley 2004). It is possible, therefore, that a raised homocysteine level could induce a fall of adenosine concentrations to the extent that activation of protective receptors, including A_1 and A_{2A} ARs, is compromised.

4.6 Genetic Studies

In an attempt to assess the possible relevance to Alzheimer's disease of mutations in the A_{2A} receptor gene, Liu et al. (2005) have examined 174 patients and 141 controls for the presence of the 1976 T>C polymorphism. No significant differences were noted in the genotype distribution or allelic frequency of this molecule, implying that a change of A_{2A} AR function characterized by this mutation was not likely to be a major contributor to the Alzheimer's disease susceptibility. However, the numbers of patients are not high for this type of study, and there may be alternative polymorphisms that are more relevant.

5 Creutzfeldt–Jakob Disease

Creutzfeldt–Jakob disease (CJD) is one of the prion diseases, characterized by the presence of protease-resistant prion protein within the brain parenchyma, leading to neuronal degeneration, motor impairment and ultimately death. CJD is often considered to be the human equivalent of scrapie, a disease primarily of sheep and related animals, and bovine spongiform encephalopathy (BSE) in cattle. The involvement of transmitters and other endogenous neural molecules in the development of prion-induced brain damage has received rather little attention, other than a degree of focus on glutamate and its receptor subtypes. However, Rodriguez et al. (2006) have examined the levels of adenosine A_1 AR in the neocortex of 12 patients with CJD and six age-matched controls. Elevated numbers of A_1 AR were identified in the patient group, together with increased receptor activity in cyclic AMP assays but normal levels of mRNA, suggesting increased receptor efficacy together with a possible decrease in the rate of receptor turnover.

When similar measurements were made in mice expressing bovine BSE prion protein, a similar increase in A_1 AR number in the brain occurred in parallel with the appearance of prion protein and the development of motor symptoms (Rodriguez et al. 2006). A simplistic interpretation of these data would be consistent with an up-regulation of A_1 AR function as a protective adaptation to the potentially injurious prion protein. However, it will be important to assess how the changes in A_1 ARs compare with changes in other purine receptors, purine transporters and purine metabolic enzymes, in addition to other ARs and other neuroactive substances, before a significant role of A_1 ARs can be considered in isolation.

6 Lesch–Nyhan Syndrome

Lesch–Nyhan syndrome (LNS) is the result of an X-linked deficiency of hypoxanthine–guanine phosphoribosyltransferase (HGPRT). The lack of this major purine salvage enzyme results in high levels of hypoxanthine and uric acid, the latter producing a range of consequences in peripheral tissues, such as gouty arthritis and nephrolithiasis. In some cases, especially those with a complete absence of enzyme activity, there is also involvement of the CNS, with mental retardation and self-mutilation. Since the realization that the latter behavior could be induced by the administration of high doses of caffeine, the question has arisen of whether the various behavioral symptoms are due to a lack of adenosine or its receptors. To date, in spite of the increased *de novo* synthesis of purines, there is little evidence for any abnormality of adenosine levels or function, but it has been found that hypoxanthine can inhibit adenosine uptake. Levels of hypoxanthine comparable with those found in LNS patients suppress the equilibrative nucleoside transporters in human leucocytes, whether they are sensitive or not to nitrobenzylthioinosine (NBTI) (Torres et al. 2004; Prior et al. 2006). An examination of ARs in a mouse HGPRT knockout model of LNS has revealed an increase in the expression of A₁AR and a decrease of A_{2A}AR in the brain (Bertelli et al. 2006). What remains unclear is whether these receptor changes are induced by the alterations of adenosine uptake, and whether either of these phenomena can account for any of the behavioral symptoms in mouse models or human patients.

7 Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disorder that results in damage to areas of the CNS. It has been widely considered that the primary site of damage is the oligodendrocyte and myelin sheath surrounding central axons, but more recent work is beginning to indicate a significant involvement of neuronal damage, produced either directly by autoantibodies or occurring secondary to the loss of myelin.

The various adenosine receptors are effective modulators of cytokine release from immune-competent cells (Haskò and Cronstein 2004; Bours et al. 2006; Haskò et al. 2007). Adenosine levels in the blood of MS patients are lower than in controls (Mayne et al. 1999), raising the possibility that this could contribute to the induction of an autoimmune attack. The actions of adenosine on blood mononuclear cells also differ between patients and controls. Both groups of cells release similar amounts of the proinflammatory cytokines TNF- α and IL-6 in the resting state, but when activated, the increased production of TNF- α is reduced by A₁AR activation in controls but not patients with MS. Conversely, A₁ARs inhibit IL-6 but not TNF- α release in patients (Mayne et al. 1999). Both results are consistent with an apparently lower A₁AR density in cells from MS patients (Johnston et al. 2001). These data are also reflected in transgenic mice lacking A₁AR, which show a marked propensity to develop experimental allergic encephalomyelitis (EAE), a

condition widely recognized as the murine equivalent of MS (Tsutsui et al. 2004). The signs and symptoms of EAE develop in parallel with increased production of proinflammatory cytokines, consistent with the inhibitory activity of A₁AR activation in monocytes from control humans.

Some of this work may be translated into therapeutic application, since methylthioadenosine has now been shown to not only suppress proinflammatory cytokine production by human white blood cells but also prevent and reverse EAE in animals (Moreno et al. 2006). These effects were attributed to an interference with the activation of the nuclear transcription factor NF- κ B, and the involvement of AR activation of blockade was left open. Nevertheless, the potential implications of this activity of methylthioadenosine on MS treatment will no doubt encourage much further work on its molecular basis.

8 Huntington's Disease

Huntington's disease (HD) is an inherited neurodegenerative disease caused by loss of neurons in the striatum—especially medium spiny neurons containing GABA and enkephalin—and cortex. These changes result in motor abnormalities such as chorea, with the development of mental and psychological deterioration. The molecular origin of the degeneration has been ascribed to the production of an abnormal form of the protein huntingtin, in which an extended polyglutamine sequence (CAG triplets at the gene level) occurs.

Among the earliest proposals for the mechanism of neurodegeneration in HD was that excessive stimulation of glutamate receptors could be responsible for neuronal damage and death (Coyle and Schwarcz 1976; Lipton and Rosenberg 1994). Indeed, a large number of studies have demonstrated that overactivation of NMDA receptors in particular can produce many of the symptoms of HD in animals. The most effective agonist in this regard is quinolinic acid (Stone 2001), an endogenous metabolite of tryptophan that, unlike glutamate itself, is a selective agonist at the NMDA receptors (Stone and Perkins 1981; Stone and Darlington 2002). Administration of quinolinic acid into the striatum produces chronic neurodegeneration, which reproduces many of the electrophysiological, histological, motor and other behavioral symptoms of human HD (Beal et al. 1986, 1991; Ferrante et al. 1993; Popoli et al. 1994, 2002). Since ARs are important regulators of glutamate-mediated neurotransmission, there have been many suggestions that adenosine may be relevant to understanding HD, either as a key to the underlying cellular actions of huntingtin, and thus the molecular basis of the disorder, or as a means to treat the development or progress of the condition.

A second major hypothesis is that mutant huntingtin induces changes in mitochondrial function, and it is this that represents the primary cellular abnormality ("gain of function" hypothesis). A number of other potentially pathogenetic events have been attributed to mutant huntingtin. For example, proteolytic cleavage of mutant huntingtin generates fragments that aggregate into the nucleus and cytoplasm,

thus contributing to early neuropathology. Accumulation of proteolytic huntingtin fragments and their aggregation may also trigger a cascade of damaging processes, leading to increasing dysfunctions in neurons through oxidative injury, transcriptional dysregulation, glutamate receptor excitotoxicity and apoptotic signals (Popoli et al. 2007 and references therein). In addition to this toxicity, there may also be a “loss of function” effect due to the loss of some beneficial actions exerted by normal huntingtin, which has been shown to be antiapoptotic, essential for normal embryonic development, and stimulatory on the production of BDNF into the cortex and its delivery to the striatal targets (see Popoli et al. 2007 and references therein).

Animal models of HD have become widely used based on each of these defects, namely the intrastratial application of quinolinic acid or the administration (intrastratial or systemic) of the mitochondrial toxin 3-nitro-propionic acid (3-NP). In addition, there are several transgenic models involving the induced expression of mutant huntingtin, the R6/2 model being the most commonly used (Mangiarini et al. 1996).

8.1 Adenosine Receptors in HD

As noted above, A₁AR activation suppresses glutamate release from neurons. In line with the excitotoxic hypothesis, Blum et al. (2002) have reported that an A₁AR agonist, referred to as an adenosine amine congener (ADAC), was able to prevent the neuronal degeneration and motor sequelae of 3-NP administration to mice. Since no protection was apparent in cell cultures, the results were interpreted to indicate an action on presynaptic sites, presumably those at which the release of glutamate could be inhibited. Conversely, the A₁AR antagonist DPCPX exacerbates damage induced by a similar mitochondrial poison, malonate (Alfinito et al. 2003).

The ARs that have become of greatest interest in HD are the A_{2A}ARs. Activation of these promotes the release of glutamate, depending on the age of animals and the presence of a depolarizing stimulus (Corsi et al. 1999a, 2000), and increased numbers or functional activity of A_{2A}ARs could cause or contribute to an excitotoxic process (Domenici et al. 2007). The administration of CGS21680 itself increases extracellular glutamate levels (Popoli et al. 1995).

Consistent with this, A_{2A}AR antagonists have been shown to reduce the toxic consequences of quinolinic acid administration, an effect correlated with a reduction of glutamate release triggered by quinolinic acid (Reggio et al. 1999; Popoli et al. 2002; Scattoni et al. 2007). A similar phenomenon has been described in R6/2 mice, in which SCH58261 reduced the motor abnormalities and loss of brain tissue (Chou et al. 2005) and glutamate release in the striatum of R6/2 mice (Gianfriddo et al. 2004).

While this provides comforting support of the concept that quinolinic acid administration provides an acceptable model of HD, it is important to establish whether glutamate release is elevated in mutant mice or HD patients and, if so, the mechanism involved. The most obvious possibility, that of raised quinolinic acid levels,

has been supported directly by evidence from Guidetti et al. (2004), who measured increased amounts in patients at an early stage of HD. As to the mechanism, the presence of mutant huntingtin has been shown to reduce the uptake of glutamate by astrocytes (Behrens et al. 2002), a result that could cause increased activation of glutamate receptors, contributing to excitotoxicity.

Neuroprotection has also been demonstrated for the 3-NP model of HD. The blockade of $A_{2A}AR$ by CSC-protected mice treated with 3-NP against neuronal loss (Fink et al. 2004). Similarly, less cell death was seen when 3-NP was administered to $A_{2A}AR$ knockout mice compared with wild-type controls. Consistent with this, another inhibitor of mitochondrial complex II, malonate, produced a degeneration of striatal neurons that was also prevented by DMPX (Alfinito et al. 2003).

However, as already noted above (Fig. 1), the role of $A_{2A}AR$ s in HD is far more complex. Activation of $A_{2A}AR$ s has also been reported to mediate beneficial effects. The $A_{2A}AR$ agonist CGS21680 enhances the neurotrophic activity of growth factors such as BDNF, a key factor promoting the viability of striatal neurons, by facilitating TrkB receptor function (Lee and Chao 2001). Agonists at $A_{2A}AR$ are also associated with a normalization of cyclic AMP response element binding protein (CREB) in transgenic animals (Chiang et al. 2005).

Moreover, CGS21680 reduces the incidence of abnormal extracellular macromolecular deposits that are present in HD brains in a similar way to β -amyloid deposits in Alzheimer's disease and Lewy bodies in Parkinson's disease. In R6/2 mice, ubiquitinated deposits have indeed been demonstrated in striatal cells, both in vivo and in cell cultures, which appear to depend on the expression of mutant huntingtin protein (Chou et al. 2005). These deposits are reduced by CGS21680. In the same study, it was also noted that CGS21680 corrected the abnormally high levels of blood glucose and 5'-adenosine monophosphate (AMP)-activated protein kinase activity in the mutant mice, strongly suggesting a more fundamental role of $A_{2A}AR$ than had hitherto been suspected in the regulation of cellular biochemistry.

There is a significant depletion of $A_{2A}AR$ in the striatum of patients with HD and in transgenic mice (Blum et al. 2003b) or rats (Bauer et al. 2005) expressing mutant huntingtin. On the other hand, the density of $A_{2A}AR$ on blood platelets is increased in human HD patients (Varani et al. 2003), showing a significant correlation with both CAG repeat length (Maglione et al. 2006) and anticipation of symptoms between generations (Maglione et al. 2005). In a total of 126 HD gene-positive individuals, $A_{2A}AR$ B_{max} values were found to be robustly increased at all HD stages as well as in 32 presymptomatic subjects (Varani et al. 2007). The same abnormality is present also in other neurological diseases characterized by an extended polyglutamine sequence (polyQ), but not in non-polyQ inherited disorders (Varani et al. 2007). The same peripheral cells exhibited altered membrane fluidity, a finding that may explain the observed change in receptor density. Authors argue that the observed alteration in lymphocytes reflects the presence of the mutant protein and suggest that the measurement of the $A_{2A}AR$ binding activity might be of potential interest for a peripheral assessment of chemicals capable of interfering with the immediate toxic effects of the mutation.

There is clear evidence for increased activity of A_{2A} AR associated with HD. Striatal neurons expressing mutant huntingtin were found to show increased A_{2A} AR activation of adenylate cyclase (Varani et al. 2001), and a similar result was observed subsequently in blood cells of HD patients (Varani et al. 2003). The ability of A_{2A} AR stimulation to raise cyclic AMP levels is also increased in R6/2 mice (Chou et al. 2005; Tarditi et al. 2006), and in animals treated with 3-NP (Blum et al. 2003a). In the Tarditi et al. study, an increase in both the number of A_{2A} ARs as well as their activation of adenylate cyclase was reported, which was apparent within a few days of birth of R6/2 mice. Both of these parameters then fell to the values seen in wild-type animals. The mRNA for A_{2A} AR, in contrast, showed no change until 21 days postnatally, after which it decreased substantially. Two conclusions may be drawn from this work. Firstly, the mismatch between A_{2A} AR protein and mRNA could indicate changes in factors that affect translation or transcription of the A_{2A} AR, or which regulate receptor activity. Secondly, a loss rather than an increase of A_{2A} AR seems to be associated with older mice in which motor symptoms of HD are beginning to occur. A further intriguing observation in this study was that in the young mice, A_{2A} AR function was not prevented by ZM241385, whereas sensitivity to this antagonist was established in the older animals after 21 days of age. Whether this also implies differences in the regulation of receptor function, or different variant structures of the receptor protein at different ages, remains to be explored.

In conclusion, available data on the potential exploitation of A_{2A} AR ligands in HD are controversial and reflect the complexity of A_{2A} AR regulation in this disease (for further comments, see Popoli et al. 2007). The complex mutual relationship between AR activities mediating detrimental or beneficial effects (see also Sect. 3) makes it difficult to establish whether targeting A_{2A} AR would really be of interest to treat HD. Further basic research is needed to solve several specific questions, in particular: (1) neuronal versus non-neuronal receptor localization, and (2), for receptors expressed in neurons, pre- versus postsynaptic sites (see Fig. 1).

9 Cerebral Ischemia and Reperfusion: Stroke

9.1 Role of A_1 Adenosine Receptors

One of the earliest reports of neuroprotection against ischemia was that the nonselective agonist 2-chloroadenosine would prevent hippocampal damage in rats (Evans et al. 1987). Similar results were obtained subsequently using A_1 AR-selective agonists (von Lubitz et al. 1989; Phillis and O'Regan 1993; von Lubitz et al. 1995a), with suggestions that the protection could involve an inhibition of leukocyte adherence and extravasation (Grisham et al. 1989).

The finding that theophylline could increase the release of glutamate produced by ischemia certainly suggests that endogenous adenosine is exerting an inhibitory

action on glutamate release (Héron et al. 1993), although this could have been due to A₁ or A_{2A} AR blockade. The simultaneous measurement of purine and glutamate release into the extracellular space of brain, together with the neuronal damage and behavioral consequences of an ischemic episode, revealed a significant relationship between these parameters, with a lower extracellular glutamate being associated with less cell damage (Melani et al. 1999). It is interesting that several nonpurine compounds that can depress the release of excitatory amino acids are also protective against ischemic damage (Ochoa et al. 1992; Graham et al. 1993). Conversely, A₁AR blockade exacerbates ischemic damage (Phillis 1995).

On the other hand, it has been argued that the release of endogenous glutamate is not actually related to ischemic-induced brain damage. Systemic administration of R-PIA, CHA or an adenosine uptake inhibitor did not prevent the increase of glutamate levels in brain during ischemia (Héron et al. 1993, 1994; Cantor et al. 1992; Kano et al. 1994), although other groups have reported a decreased release using CPA (Simpson et al. 1992). The differences may depend on the pharmacokinetics of the agonists used or the model used for inducing damage.

Although little is known of the signaling pathways that underlie ischemic damage or adenosine-mediated protection in vivo, some clues may be gleaned from in vitro work. Di Capua et al. (2003), for example, found that A₁AR agonism protected primary rat neurons against “chemical ischemia” (produced by iodoacetate) via the activation of protein kinase C-epsilon. The activity of A₁ARs themselves may change under ischemic conditions. Adenosine A₁ARs are desensitized and internalized by a period of hypoxia in brain slices (Coelho et al. 2006). A period of ischemia in vivo followed by reperfusion has been said to result in no change in the number of A₁ARs or their inhibitory efficacy on presynaptic transmitter release (Shen et al. 2002), although Lai et al. (2005) have reported an increase in A₁AR expression in the cerebral cortex following ischemia in Wistar rats.

9.2 Role of A₂ Adenosine Receptors

The activation of A_{2A}AR can protect neurons against ischemia-induced damage. One of the best-tested A_{2A}AR agonists is ATL-146e, which prevents ischemic damage in the spinal cord (Cassada et al. 2001a; Reece et al. 2006) as well as damage induced by mechanical trauma (Reece et al. 2004; Okonkwo et al. 2006). This protection afforded by ATL-146e was accompanied by the normalization of several molecular markers, such as those for apoptosis (Cassada et al. 2001b), microtubule-associated protein 2 (MAP-2) and TNF- α levels (Reece et al. 2004). However, the protection is not completely prevented by ZM241385, implying that there are relevant sites of action other than A_{2A}AR. A period of ischemia of the spinal cord does, however, induce a highly significant increase in A_{2A}AR number, a finding that may contribute to the protective effect of A_{2A}AR agonists. There is also a greater inhibition of TNF- α levels in postischemic spinal cord, as well as reduced platelet adhesion to endothelial cells (Cassada et al. 2002), consistent with an important role of A_{2A}AR on blood cells

On the other hand, blockade of A_{2A}AR is also neuroprotective against ischemic damage caused by transient or permanent arterial occlusion (Gao and Phillis 1994; Phillis 1995; Monopoli et al. 1998; Pedata et al. 2005). Confirmation of the detrimental influence of A_{2A}AR has come from an examination of A_{2A}AR-deficient transgenic mice (Chen et al. 1999). These animals showed substantial resistance to ischemia-induced brain damage compared with their normal littermates.

An interesting observation reported by Corsi et al. (1999a, b) is that the agonist CGS-21680 only increased the spontaneous efflux of glutamate and GABA in young (not old) rats, although it enhanced potassium-evoked release similarly in both groups of animals. This may have implications for the utility of A_{2A}AR agonist and antagonist ligands in treating older patients after cerebral ischemia, since chronic treatment might show fewer side effects attributable to increased basal release of glutamate, while retaining neuroprotective activity against the depolarization-induced release occurring during and immediately after cerebral ischemia or trauma. The reason for the increased damage may depend, at least partly, on the increased release of glutamate and related amino acids that these compounds produced during cerebral ischemia (O'Regan et al. 1992)

It is interesting to note that, while most of the work in this area has employed adult rodents, there is some evidence that the reverse situation occurs for young animals. Thus, in neonatal rats, Aden et al. (2003) found that it was activation of A_{2A}AR that protected against a period of hypoxia and ischemia, with A_{2A}AR knockout mice showing greater brain damage than wild-type controls.

The release of proinflammatory cytokines such as TNF- α from macrophages is suppressed by activation of A_{2A}AR (Kreckler et al. 2006). Work by Chen and colleagues (Yu et al. 2004), however, has revealed a fascinating insight into the sites through which protection is mediated. By generating populations of rats lacking A_{2A}AR generally and replacing bone marrow tissue selectively with cells reconstituted to contain A_{2A}AR, they have been able to comment directly on the roles of receptors intrinsic to the CNS relative to those in the blood. The results showed that the presence of A_{2A}ARs on blood cells alone was sufficient to reverse the protective effect of generalized A_{2A}AR knockout, while wild-type mice given A_{2A}AR knockout bone marrow cells were protected against ischemic damage. This illuminating study strongly suggests that the A_{2A}ARs relevant to protection against ischemic damage are those on blood cells. This may also imply that the mechanism of A_{2A}AR antagonist protection is more strongly dependent on, for example, the release of inflammatory cytokines, than had previously been thought.

Although the A_{2B}AR has received relatively little attention with respect to neuroprotection, its activation has a number of consequences that could well contribute significantly to the phenomenon. For example, there is evidence that its activation of p38 MAPK leads to the increased expression of IL-6 in macrophages (Fiebich et al. 1996a, 2005). Since IL-6 is a cytokine that has been reported to protect neurons against a range of insults (Bensadoun et al. 2001; Carlson et al. 1999), its production, either in central glia or peripheral cells, may result in some protective efficacy.

Brain inflammation induced in rats by a chronic intraventricular infusion of LPS was associated with a loss of neuronal A_{2B}AR. This loss was prevented by a nitro

derivative of the anti-inflammatory drug flurbiprofen, while the parent compound was inactive (Rosi et al. 2003). The authors' conclusion was that an NO-releasing anti-inflammatory compound might be an effective inhibitor of brain inflammation in conditions such as Alzheimer's disease, and that changes in the density of A_{2B}AR might be involved. It is becoming increasingly clear that much more work is required to expand our knowledge of the effects of A_{2B}AR activation or loss on the overall profile of pro- and anti-inflammatory cytokines in the brain and elsewhere, especially in relation to the net effects on neurotransmission, β -amyloid production, and neuronal or glial cell viability.

9.3 Role of A₃ Adenosine Receptors

As already mentioned above (see Sect. 3.4), A₃AR activation can protect isolated cells from hypoxia-induced death (Chen et al. 2006), and it reduced infarct size in rats subjected to middle cerebral artery occlusion (MCAo). Conversely, animals lacking A₃AR exhibit substantially increased infarct volumes, suggesting that the activation of these receptors by endogenous adenosine normally acts as a physiological brake on those processes causing damage (Chen et al. 2006; Fedorova et al. 2003).

The chronic administration of an A₃AR agonist such as IB-MECA affords protection against a subsequent period of cerebral ischemia (von Lubitz et al. 1999b, 2001).

At least part of the protective activity of A₃AR agonists may involve modulation of immune-competent cells and the inflammatory reaction to cellular damage. Agonists have been shown to inhibit the generation of several proinflammatory cytokines from cells, including interleukin (IL) 10, IL-12, interferon- γ and TNF- α (Haskò et al. 1998; McWhinney et al. 1996). The latter action is sufficiently robust to have been developed as a screen for new agonist compounds (Knutsen et al. 1998). Indeed, it has been suggested that activation of A₃AR may be responsible for the reported inhibition by adenosine of TNF- α secretion in the human U937 macrophage cell line (Sajjadi et al. 1996).

The opposite effects obtained on the outcome of brain ischemia upon acute or chronic treatment with selective A₃AR agonists are discussed below (see Sect. 9.5).

9.4 Time Course of Protection Induced by Adenosine Receptor Ligands

One of the valuable features of neuroprotection by A₁AR activation is that it can be demonstrated for a period of several hours following the occurrence of a vascular or toxic insult. This is a major consideration for any drug intended for clinical use as a neuroprotectant following an acute incident such as a stroke, since the

expansion of damage from a limited central region into a more extensive penumbral area occurs over a period of hours or days, and it is essential to limit the degree of that expansion if patient recovery is to be optimized. Most authorities consider that there is a window of opportunity for neuroprotection of up to several hours after the occurrence of stroke. Several A_1 AR-selective agonists such as R-PIA certainly exhibit protection, even when administered up to 2 h after excitotoxic insults, indicating that the neuronal network and intracellular signaling processes that contribute to damage continue to operate over this time frame (Miller et al. 1994). Against ischemia-induced damage, cyclohexyladenosine (CHA) remains protective when administered up to at least 30 min following cerebral ischemia (von Lubitz et al. 1989), and ADAC similarly has a window of efficacy of several hours after cerebral ischemia in gerbils (von Lubitz et al. 1996). This latter compound is of special interest since it seems to possess fewer of the cardiovascular side effects associated with some other A_1 AR agonists (Bischofberger et al. 1997), and its efficacy is still apparent when administered chronically in very low doses (von Lubitz et al. 1999a). The importance of this finding is that many other purine receptor ligands produce opposite effects when used chronically rather than in a single acute dose paradigm. Since most patients needing neuroprotection may be taking the drugs for prolonged periods of time, this could be a highly significant advantage of ADAC and related compounds.

The timing of acute administration of A_3 AR agonists is also important. Treatment prior to ischemia increased infarct size, while postischemic administration reduced damage, probably as a result of altered dynamics of receptor activation, on neurons, glia and blood components (von Lubitz et al. 2001).

9.5 Acute Versus Chronic Administration

Despite the evidence for a neuroprotective action of adenosine and A_1 AR agonists, caffeine—a nonselective antagonist at both A_1 and A_2 ARs—was also found to protect against ischemic damage in the CNS after its chronic administration (Rudolphi et al. 1989; Sutherland et al. 1991). Single, acute injections of more selective A_1 AR antagonists, including DPCPX, were also found to exacerbate ischemic damage (Phillis 1995; von Lubitz et al. 1994a), while their chronic administration reduced damage and produced neuroprotection (von Lubitz et al. 1994a). This dichotomy of response probably indicates compensatory changes of receptor density that follow the prolonged presence of any receptor ligand. However, such changes may be limited in extent, or restricted to certain cell subtypes, since no significant changes in A_1 AR binding were detected after chronic administration of antagonists (Traversa et al. 1994). However, others have reported that chronic administration of the AR antagonists caffeine and theophylline increase A_1 ARs in cerebral cortex (Murray 1982; Szot et al. 1987) and the hippocampal CA1 region (Rudolphi et al. 1989).

Chronic administration of low doses of ADAC generated the opposite result, with marked protection of the brain. The reasons for this difference from other A_1 AR

agonists is not entirely clear, although the authors point out the substantial difference in molecular structure between ADAC and other compounds, with the implication that it may yield a different spectrum or time course of action on a range of cellular targets whose balance determines the overall production of neuronal damage or protection (von Lubitz et al. 1999a).

The effects of acute and chronic treatment with A_{2A} AR ligands show less disparity than in the case of the A_1 AR ligands described above. Overall, the qualitative effects of agonists and antagonists are similar whether they are administered acutely or chronically. This assertion would be consistent with evidence that receptor numbers and affinities change little *in vivo* (von Lubitz et al. 1995b) or *in vitro* (Abbracchio et al. 1992) in the continued presence of A_{2A} AR ligands.

As mentioned above (see Sects. 3.4 and 3.5), the acute administration of agonists at A_3 ARs, and their application to neurons in cell culture, does appear to induce neuronal death (Sei et al. 1997). In addition, an A_3 AR agonist can potentiate the degree of CNS damage following cerebral ischemia (von Lubitz et al. 1994b). On the other hand, the maintained presence or chronic intermittent administration of A_3 AR agonists produces protection, probably as a result of compensatory adaptations in the number or sensitivity of receptors. Thus, acute administration of the selective agonist ligand IB-MECA significantly enhanced the extent of brain damage following ischemia in gerbils. Chronic administration of the same compound, however, resulted in a highly significant reduction in the ischemic damage (von Lubitz et al. 1994b; 1999b; Chen et al. 2006).

9.6 Therapeutic Implications of Preconditioning

Relatively short periods of hypoxia, hypoglycemia or ischemia can result in protection of tissues against a subsequent and more severe insult. This is the phenomenon of preconditioning. Neuronal preconditioning has been demonstrated using both *in vivo* and *in vitro* preparations (Schurr et al. 1986; Khaspekov et al. 1998). One factor contributing to this is a change in the number of A_1 ARs, which increases after the preconditioning period, probably as an adaptive protective development against further ischemia (Zhou et al. 2004). Adenosine is known to be involved in preconditioning, mainly through its opening of K_{ATP} channels (Yao and Gross 1994). In many models, even those in which it is induced by an anesthetic agent such as isoflurane (Liu et al. 2006), preconditioning can be prevented almost completely by A_1 AR blockers (Hiraide et al. 2001; Nakamura et al. 2002; Yoshida et al. 2004; Pugliese et al. 2003), although A_{2A} ARs seem to contribute little to the phenomenon. The lack of involvement of A_{2A} ARs is also surprising given the foregoing discussion on the clear neuroprotective activity of A_{2A} AR antagonists (Phillis 1995; Jones et al. 1998a, b), although it is likely that the differences between *in vitro* slice preparations and *in vivo* studies are largely responsible for this difference. Interestingly, A_3 AR antagonists can enhance neuronal recovery after simulated ischemia *in vitro*, consistent with the work quoted above that acute activation of A_3 AR worsens ischemic damage *in vivo* (Pugliese et al. 2007).

10 Prospects for Adenosine Receptor-Based Therapeutics

In summary, there is an increasingly acceptable rationale, at the cellular, biochemical and behavioral levels, for believing that changes in AR function might contribute to the symptoms and possibly progression of neurodegenerative disorders (Ribeiro et al. 2002), and that ligands acting at the various ARs may have a potential role in the therapeutic treatment of some of those disorders (Muller 1997, 2000; Mally and Stone 1998; Broadley 2000; Press et al. 2007; Baraldi et al. 2008). Of course, no receptor population is likely to function in isolation in the CNS. The activation or blockade of other neurotransmitter receptors may have significant effects on the number or efficacy of ARs.

For example, von Lubitz et al. (1995a) tested combinations of ligands acting at NMDA and A₁AR, using either acute or chronic treatments. The results revealed changes of animal responses with combined treatments that suggested important interactions between NMDA and A₁AR contributing to the changes of seizure generation and motor impairment. There were parallel changes of A₁AR density which indicated that the interactions were occurring at a deeper level of cellular function than merely a degree of nonspecificity in the ligand efficacy at the different receptors.

If ARs are indeed significant contributors to neuroprotection by responding to altered endogenous levels of the purine, or if they are used as targets for therapeutic agents that act directly upon them, it will be necessary to obtain information on the manner in which those receptors behave throughout the period of insult and subsequently. A range of factors, such as acidity, oxygen levels, cytokines, peptides, growth factors, and undoubtedly many more, could act to modify receptor responsiveness in a fashion that reduces or enhances the expected efficacy of agonists or antagonists. Examples of this include the report that tissue oxidation reduces the affinity of A₁AR antagonists, but not agonists, although the density of binding sites was decreased for both (Oliveira et al. 1995). Changes in the balance of agonist to antagonist activity could be produced in this way, which could significantly alter the anticipated response to ligands.

The ability of AR antagonists to reverse cognitive dysfunction has been taken to indicate that they may have a standalone place in the treatment of dementias. However, given the undoubted existence of this and many other receptor interaction phenomena, the generalized loss of neurons that can occur with aging or disease, and the complexity of neuronal interactions that underlie cognitive performance, it is likely that future attention will shift to compounds that retain specificity of action but act at a defined number of different sites (Van der Schyf et al. 2006). In this context, however, it seems likely that blockade of ARs would be one of the more valuable sites to include in the profile of optimum targets.

For the treatment of ischemic damage, however, there is clearly a potential use for A₁AR agonists and A_{2A}AR antagonists. An alternative approach to using conventional agonists for stroke-induced brain damage could be to inhibit adenosine kinase. This enzyme is a major route for the removal of adenosine, converting it to AMP. Consequently, the overexpression of the kinase results in an exacerbation of

ischemic damage (Pignataro et al. 2007), whereas inhibition has been found to raise extracellular adenosine levels and produce protection against damage (Jiang et al. 1997).

Whichever strategic approach is used, and whichever receptor subtype is selected, it seems likely that ARs will in the future represent a valuable series of targets for protection of the brain against a range of insults. However, we will have to solve some pending issues concerning the opposite (beneficial versus detrimental) effects exerted by some AR subtypes depending on their cellular and/or pre-versus postsynaptic localization. This especially applies to the A_{2A} AR subtype (see Fig. 1). Blockade of A_{2A} AR can result either in protoxic or neuroprotective effects according to the mechanisms involved in a given experimental model and, in some cases, to the disease stage. In this respect, it is envisaged that notable advances will be achieved by the availability of transgenic mice bearing selective defects of A_{2A} AR on specific cell populations (Yu et al. 2004). The use of these mice will help in addressing the therapeutic use of A_{2A} AR ligands in not only HD but also all other neurodegenerative diseases characterized by a dysfunction of the adenosinergic system.

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Adenosine A_{2A} Receptors and Parkinson's Disease

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Abstract The drug treatment of Parkinson's disease (PD) is accompanied by a loss of drug efficacy, the onset of motor complications, lack of effect on non-motor symptoms, and a failure to modify disease progression. As a consequence, novel approaches to therapy are sought, and adenosine A_{2A} receptors (A_{2A}ARs) provide

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a viable target. A_{2A}ARs are highly localized to the basal ganglia and specifically to the indirect output pathway, which is highly important in the control of voluntary movement. A_{2A}AR antagonists can modulate γ -aminobutyric acid (GABA) and glutamate release in basal ganglia and other key neurotransmitters that modulate motor activity. In both rodent and primate models of PD, A_{2A}AR antagonists produce alterations in motor behavior, either alone or in combination with dopaminergic drugs, which suggest that they will be effective in the symptomatic treatment of PD. In clinical trials, the A_{2A}AR antagonist istradefylline reduces “off” time in patients with PD receiving optimal dopaminergic therapy. However, these effects have proven difficult to demonstrate on a consistent basis, and further clinical trials are required to establish the clinical utility of this drug class. Based on preclinical studies, A_{2A}AR antagonists may also be neuroprotective and have utility in the treatment of neuropsychiatric disorders. We are only now starting to explore the range of potential uses of A_{2A}AR antagonists in central nervous system disorders, and their full utility is still to be uncovered.

Keywords A_{2A} antagonist · Clinical trial · Dyskinesia · Motor dysfunction · Basal ganglia · MPTP · 6-OHDA · Neuroprotection

Abbreviations

AIMs	Abnormal involuntary movements
A _{2A} AR	Adenosine A _{2A} receptor
AUC	Area under the curve
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BG	Basal ganglia
COMT	Catechol- <i>O</i> -methyl transferase
CPu	Caudate-putamen
CGI	Clinical global impression
DA	Dopamine
DYN	Dynorphin
ENK	Enkephalin
GABA	γ -Aminobutyric acid
GAD67	Glutamic acid decarboxylase
GP	Globus pallidus
GPe	Globus pallidus, external segment
GPi	Globus pallidus, internal segment
5-HT	5-Hydroxytryptamine
LOCF	Last observation carried forward
KO	Knockout
L-DOPA	3,4-Dihydroxy-L-phenylalanine
LTP/LDP	Long-term potentiation/long-term depression
mGlu5	Metabotropic glutamate subtype 5

MAO B	Monoamine oxidase B
6-OHDA	6-Hydroxydopamine
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD	Parkinson's disease
STN	Subthalamus
SNr	Substantia nigra pars reticulata
TJM	Tremulous jaw movement
UPDRS	Unified Parkinson's disease rating scale

1 Introduction

Increasing life expectancy will inevitably lead to an increase in the incidence of neurodegenerative illnesses, such as Parkinson's disease (PD), constituting an increasing social and economic burden (Dorsey et al. 2007). At the same time, the dopaminergic therapies currently used to treat the motor symptoms of PD, while effective in the initial stages of the illness, become inadequate as the disease progresses, do not reverse non-motor symptomatology, and become associated with adverse effects that prove difficult to manage (Fahn and Janlovic 2007; Jankovic 2006). In this situation, drug treatments that act beyond the damaged dopaminergic system, for example adenosine A_{2A} receptor (A_{2A}AR) antagonists, are becoming important targets for the treatment of PD since they may be effective in both the early and late stages of PD and avoid the unwanted side effects currently associated with chronic dopaminergic treatment.

2 Parkinson's Disease

PD affects 1 in 500 of the general population and 1 in 100 of those individuals aged 60 or over. The incidence of the illness is age related and this remains the only clearly established predisposing factor (Weintraub et al. 2008a). It is characterized by akinesia, rigidity, tremor and postural abnormalities, but increasingly there is awareness that it is a much broader illness that induces a range of non-motor symptoms such as sweating, falling, speech and swallowing difficulties, and neuropsychiatric components such as depression, anxiety and cognitive decline (Chaudhuri et al. 2005). Many of these features can precede the onset of motor symptoms and they, and others, are being actively investigated as early diagnostic features of those individuals that are likely to go on to develop clinical PD (Berg 2006; Siderowf and Stern 2006). The motor symptoms of PD are due primarily to the degeneration of the dopaminergic nigrostriatal pathway, with the mesolimbic/mesocortical dopaminergic pathways remaining relatively intact. However, pathology is widespread, with cell loss also occurring in many other brain areas, such as the locus coeruleus, raphe nuclei, dorsal motor nucleus of the vagus

and the ventral forebrain, leading to changes in a range of neurotransmitters, including noradrenaline, 5-hydroxytryptamine (5-HT) and acetylcholine (Agid 1991; Jellinger 2002). Precisely how these contribute to the symptomatology of PD is not known, but they may be the origin of the non-motor features of the illness. Recently, the suggestion was made that PD is a progressive pathological disorder that starts in the periphery and then affects the brain, sweeping from the brainstem through to the cortex and only leading to a diagnosis of PD when the pathological process starts to affect the basal ganglia (BG) (Braak et al. 2006a, b; Braak and Del 2008). Although this is controversial, it implies that treatment strategies should be more broadly based and that pathological change in the BG may be a later feature of PD than previously thought.

PD can be induced by gene defects in rare familial cases, but the bulk of the PD population is considered to have idiopathic disease (Gasser 2007; Hardy et al. 2006). In all probability, it is not a single disorder but a syndrome with multiple causes and with clear differences between, for example, young-onset PD and late-onset illness, and between tremor-dominant and akinetic manifestations. The usual description of PD is that it is due to a combination of genetic and environmental factors that can interact to varying degrees and at different levels (McCulloch et al. 2008). The pathogenic process responsible for neuronal loss in PD remains unknown, but contributing factors are oxidative and nitrate stress, mitochondrial dysfunction, excitotoxicity and altered proteolysis (Jenner and Olanow 2006; Litvan et al. 2007a, b). Cells are presumed to die by apoptosis, but this has not been conclusively demonstrated. There are, however, two key features of PD that probably provide the major clues to the underlying mechanisms. First, pathological change is always accompanied by the appearance of cytoplasmic inclusions, termed Lewy bodies, in surviving neurons (Wakabayashi et al. 2007), and second, there is a reactive microgliosis and to some extent astrocytosis that leads to inflammatory change and that may contribute to the progression of pathology in PD (McGeer and McGeer 2008).

The primary effect of dopaminergic loss in the striatum in PD leads to a disruption of the parallel processing loops between the motor cortex, basal ganglia, thalamus and back to pre-motor and motor cortex that are responsible for the integration of motor, sensory and cognitive information that controls voluntary movement (Obeso et al. 2000, 2004). Dopamine plays three important roles in the striatum that are lost in PD. It controls the activity of the corticostriatal glutamatergic input, it determines the activity of the GABAergic medium spiny neurons that make up the major striatal output pathways—the direct and indirect pathways (see below), and it plays a key role in motor programming through the maintenance of long-term potentiation or long-term depression (LTP/LDP)-type processes (Calabresi et al. 2006, 2007). All of these are key to how dopaminergic therapy reverses the motor symptoms of PD and to how non-dopaminergic drugs, such as adenosine antagonists, can also alter basal ganglia function in PD.

3 Treatment of PD and Limitations of Therapy

The current therapy for PD is based on dopaminergic replacement therapy using 3,4-dihydroxy-L-phenylalanine (L-DOPA) and dopamine agonists, notably ropinirole and pramipexole (Horstink et al. 2006a, b; Weintraub et al. 2008b). These lead to almost complete reversal of motor symptoms in the early stages of the disease, but the dopamine agonists do not possess as great an efficacy as L-DOPA. This may be related to their more selective effects on dopamine receptor subtypes, largely D₂/D₃ receptors, and to the fact that L-DOPA stimulates all dopamine receptor populations and also enhances noradrenergic and serotonergic transmission and can alter glutamate release among a range of actions. Adjuncts to dopaminergic therapy are the other major drug types used in PD. These are the catechol-*O*-methyl transferase (COMT) inhibitors entacapone and tolcapone, which prevent the metabolism of L-DOPA to 3-*O*-methyl-DOPA, as well as the monoamine oxidase B (MAO B) inhibitors selegiline and rasagiline, which prevent the breakdown of endogenous dopamine and dopamine derived from L-DOPA. Otherwise, the only other drugs routinely used to treat PD are anticholinergics, which are particularly effective against tremor, or the weak NMDA antagonist amantadine, which has some mild symptomatic actions but is usually employed to suppress dyskinesia (see below).

However, the symptomatic treatment of PD becomes more complex with disease progression and with chronic drug treatment (Fabbrini et al. 2007; Jankovic 2005; Jankovic and Stacy 2007; Stacy and Galbreath 2008; Stocchi 2003). Dopaminergic drugs show a shortening of duration of effect (wearing-off), and the clinical response becomes unpredictable and subject to rapid oscillations, with patients switching rapidly between mobility and immobility (on-off). This can be treated by using a longer-acting dopamine agonist drug or by adding a COMT inhibitor or MAO B inhibitor to therapy, but this is only a short-term measure. A significant proportion of PD patients develop involuntary movements or dyskinesia (chorea, dystonia, athetosis), particularly when treated with L-DOPA. Once established, these are evoked by every dose of dopaminergic medication that is administered. Treatment is usually by dose reduction, but this worsens PD; or by the addition of amantadine, but this is poorly tolerated by many patients; or by the use of continuous drug infusions (subcutaneous apomorphine or intraduodenal L-DOPA); or by referral for deep brain stimulation, employing electrode placement in the subthalamic nucleus (Guridi et al. 2008).

Dopaminergic medications induce a range of acute side effects that further complicate current treatment. These include acute effects such as nausea and vomiting and more prolonged changes in cardiovascular function and in hormonal status. Probably most worrying, however, are the neuropsychiatric complications of dopaminergic treatment usually seen after longer periods of treatment in more advanced patients with PD. Psychosis induced by dopaminergic medication, particularly in elderly patients showing cognitive decline, can become treatment limiting. More recently, dopaminergic dysregulation syndromes, such as compulsive gambling and hypersexuality, have been identified as affecting significant numbers of individuals (Stamey and Jankovic 2008; Stocchi 2005) and leading to legal action

that may limit the use of this drug class. All of this leads to the conclusion that new approaches to treatment are required. While dopaminergic medication is highly effective against the motor symptoms of PD, it has little effect on the non-motor components of PD, which are largely non-dopaminergic in origin. Cognitive decline in PD and the high incidence of anxiety and depression require particular attention (Weintraub et al. 2008c). These have become a major problem in treating PD, and novel therapeutic approaches are required.

All current treatment of PD is orientated towards symptomatic therapy. There are no proven treatments that alter the rate of progression of PD. A key objective is to find disease-modifying treatments that stop or slow disease progression. However, neuroprotection is proving a difficult issue, with drugs that look highly effective in preclinical models of PD turning out to be ineffective in clinical trials (Ahlskog 2007; Hung and Schwarzschild 2007; Kieburtz and Ravina 2007; LeWitt 2006; Schapira 2008; Stocchi and Olanow 2003). This has occurred with MAO B inhibitors, glutamate antagonists, inhibitors of apoptotic mechanisms, enhancers of mitochondrial function, trophic factors, and dopamine agonists, amongst others. The reasons for this are not entirely clear, but it may relate to the inappropriateness of the animal models or to the multiple causes of PD and the use of patient populations with different pathogenic mechanisms underlying the origin of their disease.

New approaches to neuroprotection are needed, and clues may be gained by looking at factors that are thought to reduce the risk of developing PD in the human population. Some of the more robust, although still controversial, include cigarette smoking, the use of nonsteroidal anti-inflammatory drugs, antihypertensive agents (notably calcium channel blockers), and caffeine (Becker et al. 2008; Bornebroek et al. 2007; Esposito et al. 2007; Hu et al. 2007; Powers et al. 2008; Ritz et al. 2007). The ability of caffeine to reduce risk may be highly relevant to the potential therapeutic effects of A_{2A} AR antagonists in the treatment of PD.

4 Basal Ganglia Organization

4.1 Localization of A_{2A} ARs in Basal Ganglia

The BG comprise a group of tightly interconnected forebrain nuclei, intercalated among the cerebral cortex, thalamus and brainstem, and mainly involved in motor control and sensorimotor integration. Within the last decade, a number of dedicated studies have extensively shown how dopamine and adenosine interact to modulate motor function at this level (Fuxe et al. 2007; Schwarzschild et al. 2006; Schiffmann et al. 2007).

Adenosine binds at least four different G-protein-coupled receptors, namely A_1 , A_{2A} , A_{2B} , A_3 (Fredholm et al. 1994). In contrast to the widespread distribution of A_1 , A_{2B} and A_3 adenosine receptors in the brain, A_{2A} ARs are more selectively distributed, being abundantly expressed in the BG, and reaching the

highest levels of expression in the caudate-putamen (CPu) (Rosin et al. 1998; Schiffmann et al. 1991). This selective distribution of A_{2A}ARs, involving a potentially low incidence of side effects, first led to the consideration of A_{2A}AR antagonists among the most promising non-dopaminergic agents for the treatment of PD motor symptoms.

The CPu is mainly composed of medium spiny GABAergic neurons, which are equally divided into two neuronal populations: striatonigral neurons, which connect the CPu with the substantia nigra pars reticulata (SNr) or globus pallidus (GP) internal segment (GPi), otherwise called the entopeduncular nucleus in rodents, and striatopallidal neurons, which connect the CPu with the GP or GPe (globus pallidus external segment) in primates (Fig. 1). Within this system, A_{2A}ARs are restricted to GABAergic neurons projecting to the GP which also selectively express the D₂ dopamine receptor and the peptide enkephalin (ENK) (Fink et al. 1992; Schiffmann et al. 1991). Conversely, striatonigral neurons, which selectively express the D₁ dopamine receptor and the peptide dynorphin (DYN), do not contain appreciable levels of A_{2A}AR. At the molecular level, G_s-coupled A_{2A}ARs activate adenylate cyclase, resulting in stimulation of neuronal activity, and opposing the dopamine-mediated inactivation of adenylate cyclase through the G_i-coupled D₂ receptor (Fredholm 1995). Recent studies have demonstrated that in striatopallidal neurons the A_{2A}AR can form heteromers with the D₂ receptor to attenuate coupling to the signaling pathway of the latter, offering a molecular mechanism of interaction which has compelling implications for PD treatment (Fuxe et al. 2005; Hillion et al. 2002).

The second most abundant neuronal population within the CPu are the large cholinergic aspiny interneurons, which represent about 5% of the entire population (Gerfen 1992). Striatal cholinergic nerve terminals express A_{2A}ARs, which, by modulating the release of acetylcholine in the rat CPu (Fig. 1), represent a novel interesting target for tremor control in PD models (see later).

4.2 Function of A_{2A}ARs in Basal Ganglia

In an intact CPu, adenosine via A_{2A}ARs excites striatopallidal neurons, opposing the inhibitory effect exerted by dopamine (Fig. 1). In PD, lack of dopamine generates an imbalance in the activity of striatal output pathways. Striatonigral neurons become hypoactive, whereas striatopallidal neurons, losing the inhibitory effect of dopamine while undergoing the stimulatory influence of adenosine, become hyperactive, boosting their inhibitory influence on GP neurons. Such imbalanced activity leads to a markedly increased inhibitory output from SNr/GPi to thalamocortical neurons, which produces hypokinetic symptoms in PD. Many authors have suggested that the positive effects of A_{2A}AR antagonists in PD rely on the blockade of A_{2A}ARs on striatopallidal neurons, which should dampen their excessive activity and restore some balance between striatonigral and striatopallidal neurons, consequently relieving thalamocortical activity. This mechanism offers a rationale

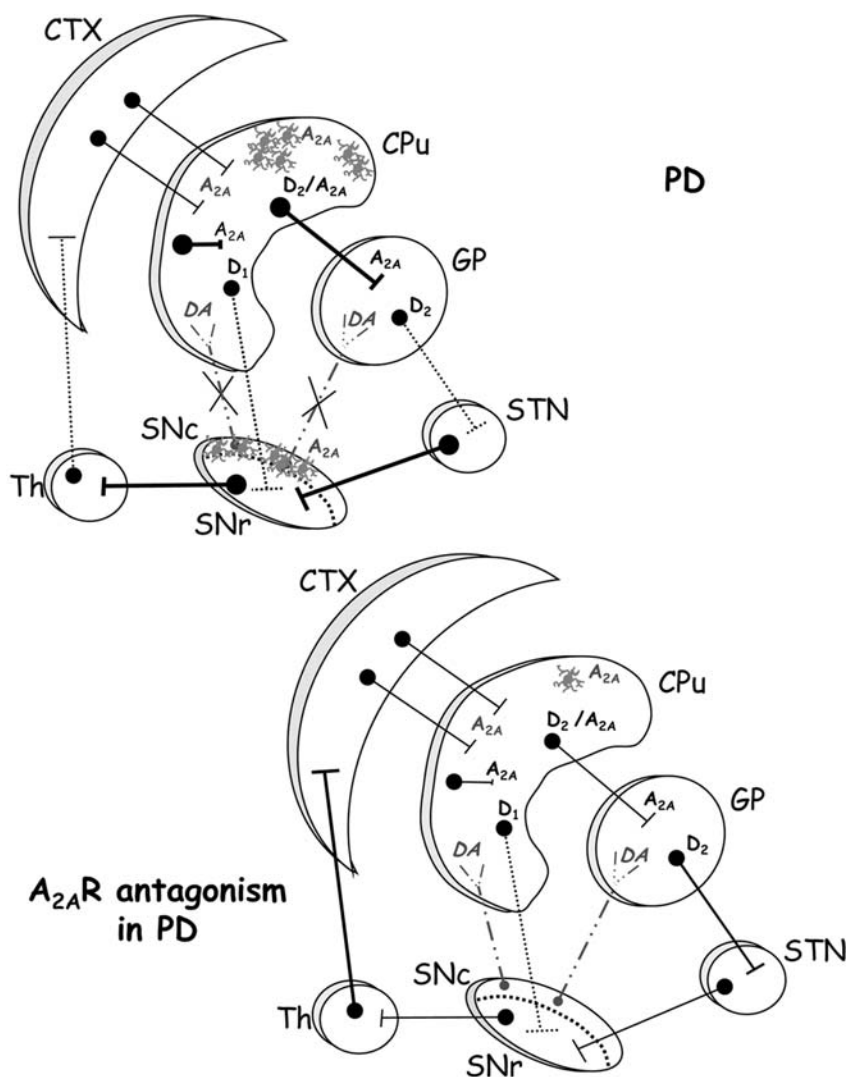


Fig. 1 Proposed mechanisms of adenosine A_{2A} receptor (A_{2A}AR) antagonist activity in Parkinson's disease (PD). Mechanisms of symptomatic effects are drawn in *black*, whereas mechanisms of neuroprotection are drawn in *gray*. In PD, lack of dopamine (DA) induces hypoactivity of striatonigral D₁-containing neurons and hyperactivity of striatopallidal D₂-containing neurons, resulting in subthalamus (STN) and substantia nigra pars reticulata (SNr) hyperactivity. Acetylcholine (ACh) interneurons in the caudate-putamen (CPu) are also hyperactive. The final outcome is depressed activity of thalamocortical (Th) projections, which produces characteristic symptoms of akinesia. A_{2A}AR blockade in striatopallidal neurons, and likely in the globus pallidus (GP), relieves their hyperactivity, restoring balance between the output pathways. As a consequence, SNr and Th-cortical neurons become normoactive, relieving the akinesia. Moreover, A_{2A}AR blockade in Ach interneurons restores Ach tone, which may contribute to counteracting tremor. In the parkinsonian state, glial proliferation is present in both the CPu and the substantia nigra pars compacta (SNc). As neuroprotective agents, A_{2A}AR antagonists attenuate dopaminergic cell degeneration through a mechanism that may involve A_{2A}ARs located presynaptically or alternatively A_{2A}ARs in glial cells

for the use of A_{2A}R antagonists as a monotherapy in PD, as well as for the synergistic effect observed upon the concurrent administration of A_{2A}AR antagonists with L-DOPA or dopaminergic agonists, which restore dopamine receptor stimulation (Jenner 2003; Morelli 2003).

Of great interest is the neuronal colocalization and synergistic interaction observed between striatal A_{2A} receptor and metabotropic glutamate subtype 5 (mGlu5), glutamate receptor, which itself represents one of the most promising targets for treatment of PD symptoms (Ferré et al. 2002; Rodrigues et al. 2005). A potentiation of motor activity has been reported upon combined administration of A_{2A} and mGlu5 receptor antagonists, together with a synergistic interaction at the level of signal transduction pathways (Coccurello et al. 2004; Ferré et al. 2002; Kachroo et al. 2005; Nishi et al. 2003). The recent discovery of A_{2A}-mGlu5 heteromers in CPu has further strengthened the rationale for studying antiparkinsonian strategies that simultaneously block A_{2A}ARs and mGlu5 receptors (Ferré et al. 2002).

4.3 Role of Globus Pallidus A_{2A} Adenosine Receptors

An important function of A_{2A}ARs located outside the CPu, particularly in the GP, has been evidenced by the positive effects displayed by A_{2A}AR antagonists when administered in association with dopaminergic therapies. In recent years, several works have led to a reconsideration of the role played by the GP in BG circuits, with this nucleus now placed at a critical functional position to modulate the excitability of afferent (CPu and STN) and efferent (SNr) nuclei (Obeso et al. 2006). The infusion of GABA agonists directly into the GP has been found to severely hamper motor function, whereas the antagonism of pallidal GABAergic transmission results in beneficial motor effects (Hauber 1998). The GP receives a direct dopaminergic innervation, being enriched in D₂ dopamine receptors. In the parkinsonian state, in which the GP discharge rate and oscillatory activity are altered, intrapallidal dopaminergic antagonists produce akinesia, whereas dopamine stops this symptom (Galvan et al. 2001; Hauber and Lutz 1999), suggesting that dopamine depletion either directly or indirectly disrupts the modulatory function of GP within the BG. A_{2A}ARs are highly expressed in the GP, mainly in the neuropil, where they can regulate pallidal extracellular GABA concentration and, thereafter, GP activity (Rosin et al. 1998; 2003). While stimulation of pallidal A_{2A}ARs enhances striatopallidal GABA outflow, their blockade reduces it (Ochi et al. 2004; Shindou et al. 2003). Recently, it was reported that while intrapallidal infusion of A_{2A}AR antagonists in 6-hydroxydopamine (6-OHDA)-lesioned rats does not elicit any motor response per se, it does potentiate motor activity induced by L-DOPA or dopaminergic agonists, suggesting that the beneficial effect exerted by these compounds in PD might also rely on the blockade of pallidal A_{2A}ARs (Simola et al. 2006; 2008). It might be hypothesized that in PD, the blockade of pallidal A_{2A}ARs, by reducing extracellular GABA, may contribute to restoring GP activity and in turn subthalamic

nucleus activity, leading to a more balanced activation of direct and indirect pathways and, when associated with dopaminergic agonists, an enhancement of their motor-stimulating effects.

5 Motor-Behavioral Effects of A_{2A}AR Antagonists in Animal Models of Parkinson's Disease

5.1 Effects of Acute A_{2A}AR Antagonism on Motor Deficits

The highly enriched distribution of adenosine A_{2A}ARs in striatopallidal neurons, and their ability to form functional heteromeric complexes with dopamine D₂ and metabotropic glutamate mGlu5 receptors, mean that A_{2A}AR antagonists are of particular interest for the modulation of motor behavior, whilst at the same time they display a low predisposition to induce non-motor side effects.

Research performed to evaluate the effects produced by AR ligands on motor behavior in experimental rodents has provided the first evidence that adenosine is implicated in the modulation of movement. The critical role of A_{2A}AR in the regulation of motor behavior was first highlighted by data showing inhibition of motor behavior by the A_{2A}AR agonist 2-*p*-[(2-carboxyethyl)-phenethylamino]-5'-*N*-ethylcarboxamidoadenosine (CGS-21680), while the A_{2A}AR antagonist 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH-58261) was found to stimulate motor activity (Morelli et al. 1994; Pollack and Fink 1996; Pinna et al. 1996).

A large number of A_{2A}AR antagonists have been demonstrated to affect motor behavior by reversing catalepsy in rodents (reducing its duration and severity), hence accounting for an improvement in parkinsonian motor deficit by these drugs. Moreover, combined administration of the A_{2A}AR antagonists with L-DOPA has been shown to potentiate the L-DOPA-induced anticataleptic effect, indicating the existence of a synergistic interaction between L-DOPA and A_{2A}AR antagonists (Kanda et al. 1994; Shiozaki et al. 1999; Wardas et al. 2001).

In line with results obtained in the catalepsy protocol, A_{2A}AR antagonists showed motor-facilitatory activity in animals rendered parkinsonian by the administration of dopaminergic neurotoxins, such as 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which induce the degeneration of nigrostriatal dopaminergic neurons, resulting in models of parkinsonian-like disabilities (akinesia, bradykinesia, etc.) in the animals treated.

Acute administration of the A_{2A}AR agonist CGS 21680 to unilaterally 6-OHDA-lesioned rats has been shown to significantly reduce the turning behavior induced by L-DOPA and either D₁ or D₂ dopamine receptor agonists (Morelli et al. 1994). Conversely, the A_{2A} receptor antagonist SCH 58261, when administered acutely to 6-OHDA-lesioned rats, has been demonstrated to significantly potentiate turning behavior induced by L-DOPA and either D₁ or D₂ dopamine

receptor agonists (Pinna et al. 1996). An increase in the turning behavior stimulated by L-DOPA or apomorphine was observed following acute A_{2A}AR blockade by 1,3-dipropyl-7-methyl-8-(3,4-dimethoxystyryl)xanthine (KF-17837), 2-butyl-9-methyl-8-(2*H*-1,2,3-triazol-2-yl)-9*H*-purin-6-ylamine (ST-1535) or (*E*)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1*H*-purine-2,6-dione (KW-6002) (Koga et al. 2000; Rose et al. 2007; Tronci et al. 2007).

Besides turning behavior, subtle aspects of PD symptomatology develop in rats as a consequence of dopamine neuron degeneration, such as forelimb akinesia, gait impairment and sensory-motor integration deficits that are considered analogous to the PD symptoms seen in humans. Acute administration of the A_{2A}AR antagonists SCH-58261 and ST-1535, in a similar manner to L-DOPA although with a lower intensity, counteracted the lesion-induced impairments to the initiation time of the stepping test, to adjusting steps, and to vibrissae-evoked forelimb placing (Pinna et al. 2007). These results suggest that A_{2A}AR antagonists might ameliorate parkinsonian symptoms in PD patients, even when used as a monotherapy.

Most importantly, the efficacy of A_{2A}AR antagonists in MPTP-treated nonhuman primates, provided the impetus for experimenting with these compounds in clinical trials. Acute administration of the A_{2A}AR antagonist KW-6002 counteracted motor impairments and increased locomotor activity in primates previously treated with MPTP (Kanda et al. 1998a, b). Furthermore, a synergistic interaction between A_{2A}AR antagonists and L-DOPA, as well as dopaminergic agonists, in decreasing motor impairment has been observed in MPTP-treated common marmosets (Kanda et al. 2000; Rose et al. 2007).

The crucial role of CPu in the effects of A_{2A}AR antagonists has been confirmed by data indicating that the intrastriatal infusion of the A_{2A}AR antagonist MSX-3 significantly counteracted catalepsy produced by D₁ or D₂ receptor antagonists (Hauber et al. 2001). However, further to the well-documented role of CPu in mediating motor facilitation produced by A_{2A}AR antagonists, extrastriatal circuits may also be involved in this effect (see Sects. 4.3 and 5.5).

5.2 Efficacy of A_{2A}AR Antagonists in Relieving Parkinsonian Tremor and Muscular Rigidity

To date, tremor and rigidity are devoid of adequate pharmacological treatments, and so preclinical evidence showing that A_{2A}AR antagonists may be effective in relieving rigidity as well as resting tremor, one of the first symptoms manifested in individuals affected by PD, has greatly increased the attention directed towards A_{2A}AR antagonist compounds.

Promising effects of A_{2A}AR antagonists have been observed in rat models of parkinsonian-like muscular rigidity. Haloperidol and reserpine induce a muscular stiffness that displays electromyographic and mechanographic features that partly overlap with those of parkinsonian muscular rigidity. Both effects are attenuated by

the administration of the A_{2A} AR antagonist SCH-58261, suggesting the existence of a potential beneficial effect of A_{2A} AR blockade on parkinsonian-like muscular rigidity (Wardas et al. 2001).

Blockade of A_{2A} ARs effectively counteracts tremulous jaw movements (TJM), a valuable model for the screening of new antitremorigenic agents in rats. Administration of either the A_{2A} AR antagonist SCH-58261 or ST-1535 has been demonstrated to significantly suppress tacrine-induced TJM and, in line with this finding, antagonism of A_{2A} AR by KF-17837 has been reported to relieve TJM elicited by haloperidol, suggesting a beneficial use of these drugs as specific agents against this parkinsonian symptom (Correa et al. 2004; Mally and Stone 1996; Simola et al. 2004). In addition, intracranial infusion of A_{2A} AR antagonists revealed a critical role of the ventrolateral portion of the CPu in counteracting TJM (Simola et al. 2004). Interestingly, a specific increase in A_{2A} AR mRNA expression in this striatal portion was detected following dopamine denervation in the 6-OHDA model of PD (Pinna et al. 2002).

In order to explain the antitremorigenic effect, it should be noted that striatal cholinergic nerve terminals express A_{2A} ARs, and A_{2A} AR antagonists can reduce the evoked release of acetylcholine in rat CPu (Kurokawa et al. 1996), whereas increased acetylcholine transmission, particularly in the ventrolateral portion of CPu, is believed to play an important role in the genesis of TJM in rats (Salamone et al. 1998).

5.3 Effects of Chronic A_{2A} AR Antagonism on Motor Complications and Dyskinesia

In line with data obtained following acute administration, long-term treatment with A_{2A} AR antagonists has been shown to significantly counteract motor disabilities in rodent and nonhuman primate PD models (Kanda et al. 1998b; Pinna et al. 2001). Moreover, chronic A_{2A} AR antagonism has been shown not to induce tolerance to motor-stimulant effects in both rats and primates (Halldner et al. 2000; Jenner 2003; Pinna et al. 2001). Lack of tolerance to motor-stimulant effects of A_{2A} AR antagonists is of particular significance in PD, in which the motor-improving properties of therapeutic agents are required to persist during the chronic regimen.

A major finding emerging from studies on chronic A_{2A} AR antagonists is represented by the results reported on motor fluctuations (“wearing off”) and dyskinesia in experimental animals treated with A_{2A} AR antagonists and L-DOPA (Koga et al. 2000). The wearing off of L-DOPA that is observed in humans is mimicked in 6-OHDA-lesioned rats, where the duration of rotational behavior elicited by L-DOPA is progressively reduced during chronic administration. Combined administration of the A_{2A} AR antagonist KW-6002 prevented the shortening of rotational behavior, reflecting a potential beneficial influence of A_{2A} AR blockade on L-DOPA wearing off (Koga et al. 2000). At the same time, sensitization of rotational behavior and development of abnormal involuntary movements (AIMs) is thought to mimic

dyskinetic effects elicited by L-DOPA. In this paradigm, interesting results concerning the modulation of dyskinesia by A_{2A}AR blockade have been obtained by comparing the rotational behavior elicited by long-term administration of a higher dose of L-DOPA to an equipotent combination of a lower dose of L-DOPA plus the A_{2A}AR antagonist ST-1535 (Rose et al. 2007; Tronci et al. 2007). Although both L-DOPA (high dose) and L-DOPA (lower dose) plus ST-1535 produced a comparable degree of rotations on the first administration, sensitization of rotational behavior and AIMs were observed only in response to chronic L-DOPA alone, not to chronic L-DOPA plus ST-1535, suggesting that the association between the two drugs represents a treatment with low dyskinetic potential (Tronci et al. 2007). These results have been strengthened by studies showing that genetic deletion of the A_{2A}AR prevents the sensitization of rotational behavior stimulated by L-DOPA in 6-OHDA-lesioned A_{2A}AR knockout (KO) mice (Fredduzzi et al. 2002).

Results obtained in MPTP-treated primates confirm and further extend those deriving from 6-OHDA-lesioned rats. First, A_{2A}AR antagonists do not induce dyskinesia per se, since administration of KW-6002 to parkinsonian primates relieved motor disability without stimulating abnormal movements (Grondin et al. 1999; Kanda et al. 1998, 2000). Second, in MPTP-treated marmosets previously exposed to chronic L-DOPA in order to develop dyskinesia, motor stimulation induced by KW-6002 was not associated with an exacerbation of dyskinetic movements (Kanda et al. 1998). Furthermore, no sign of apomorphine-induced dyskinesia was observed in parkinsonian cynomolgus monkeys chronically treated with a combination of apomorphine and KW-6002 (Bibbiani et al. 2003). Interestingly, when KW-6002 (but not apomorphine) administration was interrupted, primates previously treated with KW-6002 displayed apomorphine-induced dyskinesia only 10–12 days after KW-6002 discontinuation, thus accounting for a potential preventive effect of A_{2A}AR blockade on the development of dyskinesia (Bibbiani et al. 2003; Morelli 2003). It should be noted, however, that while A_{2A}AR antagonists associated with a low nondyskinetic dosage of L-DOPA may achieve satisfactory results in motor stimulation, whilst at the same time limiting the severity of L-DOPA-induced dyskinesia, no study has yet demonstrated the ability of A_{2A}AR antagonists to revert an already established dyskinesia in animal models.

In this regard, in MPTP-treated common marmosets previously rendered dyskinetic by chronic L-DOPA, it has been shown that the relief of motor impairment produced by an optimal dose of L-DOPA presenting a high dyskinetic potential was adequately mimicked by a combination of KW-6002 plus a suboptimal dose of L-DOPA, which, in contrast, was associated with weak induction of dyskinesia (Bibbiani et al. 2003).

Taken together, data obtained from several preclinical studies indicate the existence of beneficial effects of chronic A_{2A}AR antagonists on PD motor disability and on motor complications produced by long-term L-DOPA. These effects are of considerable interest in light of the fact that motor complications are one of the intrinsic limitations of L-DOPA therapy, and are often insensitive to pharmacological manipulation.

5.4 *Effects of Acute and Chronic A_{2A}AR Antagonism on Biochemical Parameters*

The study of the effects of A_{2A}AR antagonists on behavioral parameters in both rat and primate models has been paralleled by the analysis of the influence of A_{2A}AR blockade on the biochemical modifications induced by chronic L-DOPA in 6-OHDA lesioned rats in the basal ganglia. Prolonged administration of L-DOPA, according to a regimen capable of inducing a sensitized (dyskinetic-like) rotational response and AIMs, has been shown to modify the expression of the neuropeptides ENK and DYN as well as of the enzyme glutamic acid decarboxylase (GAD67) in the basal ganglia of 6-OHDA-lesioned rats (Carta et al. 2002; Cenci et al. 1998). Although a direct relationship between these biochemical changes and L-DOPA-induced dyskinesia onset has not been unequivocally demonstrated, they have nevertheless been postulated to reflect a more general aberrant functionality of BG produced by long-term L-DOPA, which is thought to underlie the dyskinesia elicited by this drug.

Interestingly, combined administration of low doses of L-DOPA with the A_{2A}AR antagonists SCH-58261 or ST-1535, which (as reported above) induce the same degree of contralateral rotation upon the first administration, did not induce the modifications in the striatal levels of ENK, DYN and GAD67 mRNAs produced by chronic higher doses of L-DOPA in 6-OHDA-lesioned rats (Carta et al. 2002; Tronci et al. 2007).

Moreover, beneficial effects of A_{2A}AR blockade on the regulation of the phosphorylation state of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type of glutamate receptor by L-DOPA have been described. Hyperphosphorylation of the striatal AMPA receptor consequent to chronic administration of L-DOPA to 6-OHDA-lesioned rats is in fact prevented by combined administration with KW-6002 (Chase et al. 2003).

In addition to the postulated A_{2A}AR regulatory effects on neuronal responsiveness following prolonged dopaminergic stimuli, it should be considered that A_{2A}AR antagonists, by potentiating the motor effects of L-DOPA or dopamine agonist drugs, allow the use of dopaminomimetic compounds at low nondyskinetic doses. Therefore, the sparing of these agents produced by combined administration with A_{2A}AR antagonists might contribute towards reducing, or at least delaying, the onset of neuroplastic modifications in BG.

5.5 *Biochemical Changes in Extrastriatal Basal Ganglia Areas*

In the context described above, the increase in GAD67 mRNA in the GP of 6-OHDA-lesioned rats treated subchronically with L-DOPA (full effective dose) but not with SCH-58261 plus L-DOPA (threshold dose) is particularly important, indicating that chronic L-DOPA—but not an equally effective combination of SCH-58261 plus L-DOPA—elicits abnormal modifications of GP neuronal activity (Carta et al. 2003).

Moreover, subchronic studies have shown that, while a fully effective dose of L-DOPA reduces the 6-OHDA lesion-induced increase in GAD67 mRNA in SNr, it simultaneously reduces GAD67 mRNA values to below the levels present on the intact side, producing an excessive inhibition of SNr efferent neurons (Carta et al. 2003). In contrast, the combined subchronic administration of SCH-58261 plus L-DOPA reduces GAD67 mRNA to a lesser extent, decreasing GAD67 mRNA to levels similar to those present on the intact nonlesioned side (Carta et al. 2003). Excessive inhibition of SNr in rodents and GP internal segment in primates, together with an altered firing pattern, is correlated with the onset of dyskinetic movements after L-DOPA (Boraud et al. 2001; Papa et al. 1999). Thus, the ability of subchronic SCH-58261 plus L-DOPA to produce a decrease in GAD67 mRNA values to levels similar to those present in nonlesioned SNr may correlate with the presence of contralateral turning (index of therapeutic response) and to the failure to produce sensitization in contralateral turning (index of dyskinetic movements).

The results of those studies underline the importance of the role played by the indirect CPu-GP-STN-SNr pathway in eliciting the therapeutic response of A_{2A}AR receptor antagonists, and its involvement in abnormal motor responses produced by subchronic L-DOPA.

6 Clinical Actions of Adenosine A_{2A}AR Antagonists

The anatomic localization of A_{2A}ARs and their biochemical and pharmacological properties suggest that modulation of striatal GABAergic output will modify motor function in PD, and that this should occur with no risk of the development or expression of dyskinesia (Kase et al. 2003). The activity of A_{2A}ARs in functional models of PD also points to actions of the A_{2A}AR antagonists as monotherapy and as adjuncts to L-DOPA and dopamine agonists. Only one A_{2A}AR antagonist has undergone detailed clinical evaluation so far: istradefylline (KW-6002).

In healthy subjects, istradefylline (40, 60, 80 and 160 mg per day for 14 days) showed dose-proportional increases in the area under the curve (AUC) and a C_{max} with a half-life (*t*_{1/2}) of 67–95 h, suggesting that once-daily dosing should be effective (Rao et al. 2005a). Similar studies in patients with PD showed that istradefylline (60 and 80 mg per day for 14 days) also exhibits a dose-proportional pharmacokinetic profile (Rao et al. 2005b). The occupation of striatal A_{2A}ARs by istradefylline was shown using ¹¹C-istradefylline as a ligand for PET investigations in healthy subjects (Brooks et al. 2008). These studies showed >90% occupation of A_{2A}ARs at doses of istradefylline exceeding 5 mg, while this decreased proportionally at lower doses. From these studies, it was concluded that 20 or 40 mg per day istradefylline would provide consistent A_{2A}AR occupation, and that this would be an appropriate dosage for subsequent clinical investigations.

Some early clinical efficacy studies to establish proof of concept in patients with PD took place prior to the completion of the PET A_{2A}AR imaging investigations, and so these studies utilized higher doses. These involved studies of the effects of

istradefylline (40 or 80 mg per day over four weeks) alone or in combination with subsequent steady-state intravenous infusions of L-DOPA using an optimal or low infusion rate (Bara-Jimenez et al. 2003). Perhaps surprisingly, istradefylline alone had no effect on motor disability. This finding contrasts with the mild symptomatic effects of istradefylline seen in MPTP-treated primates, but is more consistent with the absence of significant rotation in 6-OHDA-lesioned rats. The data suggest that the drug would not be effective as monotherapy in the treatment of PD, but there is only one recent report on the efficacy of istradefylline as sustained monotherapy, which was inconclusive (Fernandez et al. 2008).

The results of the effects of istradefylline in conjunction with L-DOPA infusions gave the first indication of the clinical actions of the effect of A_{2A}AR receptor occupation. Istradefylline in conjunction with an optimal L-DOPA infusion had no effect on the severity of motor deficits (Bara-Jimenez et al. 2003). However, when combined with a low dose of L-DOPA, istradefylline (80 mg per day) potentiated the improvement in motor function by 36% while dyskinesia was unchanged. All primary motor symptoms of PD were improved by the addition of istradefylline. Istradefylline also increased the duration of efficacy of L-DOPA by 76%, as judged by the length of time patients remained mobile ("on" time) following cessation of L-DOPA infusion.

These findings are interesting, as they strongly support the results of the preclinical investigations in 6-OHDA-lesioned rats and in MPTP-treated primates, which showed that istradefylline potentiated the effects of low-threshold doses of L-DOPA but that little effect was seen when combined with high effective doses of the drug. The implication is that the optimal clinical effects would therefore be observed under similar conditions, but, as will be seen, the major clinical trials were undertaken in patients receiving optimal administration of dopaminergic therapy for regulatory reasons related to the need to demonstrate efficacy as a decrease in the length of time patients were immobile during the waking day ("off" time) in a group not adequately controlled by currently available medication.

In a 12-week exploratory study of safety and efficacy in advanced PD patients receiving L-DOPA therapy and other dopaminergic agents with both motor fluctuations and peak dose dyskinesia, istradefylline (up to 20 or 40 mg per day) reduced off time by 1.2 h during the waking day in the later stages of the study, as assessed using a home diary, although no change in the unified Parkinson's disease rating scale (UPDRS) scores for motor function or clinical global impression (CGI) of improvement in parkinsonian symptoms was found (Hauser et al. 2003; Hauser and Schwarzschild 2005). This is similar to the reductions produced by the COMT inhibitor entacapone when added to L-DOPA therapy. No overall increase in dyskinesia was observed, but perhaps surprisingly based on the preclinical findings, there was an increase in the amount of on time during which dyskinesia occurred. The overall success of this study then paved the way for a series of longer-term clinical investigations in larger patient populations.

These studies have largely confirmed the effects seen in the initial investigations with istradefylline. In a double-blind multicenter study, in PD patients with prominent end-of-dose wearing off, istradefylline (40 mg per day) reduced off time

during the waking day by 1.2 h compared to placebo (LeWitt et al. 2004, 2008; Stacy et al. 2004). There was no increase in dyskinesia that was disabling to the patient, but on time with dyskinesia was increased as a result of an increase in mild dyskinesia that was not troublesome to the patient and did not impair mobility. This was not unexpected on the basis of the earlier clinical studies, but it does conflict with the preclinical data on dyskinesia in MPTP-treated primates, although these studies were largely carried out using low doses of L-DOPA. In another study of istradefylline in PD patients with motor complications using 20 or 60 mg per day istradefylline versus placebo, almost identical findings were obtained except that the decreases in off time were 0.64 and 0.72 h, respectively, for the 20 and 60 mg per day doses, respectively (LeWitt et al. 2004; Stacy et al. 2004, 2008). A long-term open-label efficacy study lasting 52 weeks in advanced-stage PD patients who had previously completed a double-blind placebo-controlled investigation showed that the efficacy of the drug in reducing off time in doses of between 20 and 60 mg per day was maintained in patients who were already taking the drug at the start of the study (Mark et al. 2005). In those patients from the placebo arm of the previous double-blind study who started istradefylline, or those who had been off the drug for more than two weeks and were restarted on the drug, off time was reduced after two weeks and then maintained. The findings of these studies have more or less set the scene for the clinical effects of this A_{2A}AR antagonist in advanced PD patient populations.

However, problems have recently been encountered relating to the efficacy of istradefylline in other Phase III clinical studies, which are probably due to the problem of large and maintained placebo effects in PD and the modest duration of the decrease in off time seen throughout the clinical development. In patients with advanced PD exhibiting motor fluctuations, as defined by an average of at least 3 h off time, 20 mg per day istradefylline reduced the off times at two and four weeks but not at eight or twelve weeks (Hauser et al. 2006, 2008; Shulman et al. 2006; Trugman et al. 2006), although the effect was significant at the end-point (determined by the last observation carried forward, LOCF), with a 0.73 h reduction in off time. An analysis of secondary end-points showed a reduction in UPDRS Part 3 for motor symptoms at four weeks, a trend at two and eight weeks, and no effect at twelve weeks. Similarly, in patients with PD showing motor complications that were not adequately controlled by L-DOPA, istradefylline (10, 20 or 40 mg per day) did not decrease off time compared to a larger than expected placebo effect, although a trend for the improvement in response to increase with increasing istradefylline dosage (a dose-ordered response) was observed between the istradefylline-treated groups (Guttman et al. 2006; Pourcher et al. 2006). The results from these studies have led the FDA to issue a nonapprovable letter for the use of istradefylline in late-stage PD.

Since istradefylline is the only A_{2A}AR antagonist with results from clinical trials for PD reported to date, it is difficult to know whether the profile seen with this drug is typical of this class of drugs, or whether the design of the clinical trials in line with regulatory end-points will provide further insights into the efficacy of this class of drugs for PD. A number of other A_{2A}AR antagonists are in clinical trials at this

time, such as V2006 and SCH-58261, and the results of these studies are eagerly awaited. Based on its preclinical profile, istradefylline would have been expected to have some modest symptomatic effects as a monotherapy, but this needs further investigation. Moreover, based on preclinical investigations, istradefylline should produce an additive effect with L-DOPA, but perhaps the necessity of undertaking the clinical studies in patients on optimal dopaminergic medication has masked its ability to potentiate the effects of low-threshold doses of L-DOPA, an effect that was clearly demonstrated in preclinical studies. Thus, the design of clinical trials for istradefylline with this in mind may have provided a different outcome.

7 Future Directions

7.1 *Effects on Cognition*

Clinical evidence demonstrates the occurrence of cognitive impairments irrespective of motor disability in parkinsonian patients, including both overt dementia during later stages of the disease and less marked deficits displayed by the majority of subjects during the early stages. PD-associated cognitive symptoms involve abnormalities in visuospatial performance and memory deficits, with both short- and long-term memory being affected. Alterations in organization, planning, regulation of goal-directed behaviors and information retrieval and attention are widely observed in PD patients and are key events triggering the manifestations of PD-associated cognitive decline (Appollonio et al. 1994).

L-DOPA has been found to exert contradictory effects, if any, on cognitive deficits in PD, improving several symptoms whilst worsening others. Thus, the development of new therapeutic options currently constitutes an important requirement in the treatment of cognitive decline observed in PD, and $A_{2A}AR$ antagonists may represent a valid option. Several data obtained in experimental animals have evidenced how counteracting $A_{2A}AR$ -mediated signaling by drugs or genetic deletion of the gene encoding for the $A_{2A}AR$ may significantly improve cognitive functions, whereas working memory deficits have been demonstrated in rats overexpressing the $A_{2A}AR$ (Giménez-Llort et al. 2007; Wang et al. 2006). Moreover, studies employing the $A_{2A}AR$ antagonists KW-6002 and SCH-412348 have revealed how $A_{2A}AR$ blockade exerts beneficial effects on cognition-related functions other than memory, enhancing both motivation and attention, facilitating reward-related behaviors, increasing motor readiness, and speeding up motor-preparatory responses (O'Neill and Brown 2006; Takahashi et al. 2008).

Several authors have hypothesized how a defective functionality of the frontostriatal dopaminergic circuit connecting the CPu to the frontal cortex contributes towards cognitive deficits associated with PD (Gao and Goldman-Rakic 2003; Kulisevsky et al. 2000). $A_{2A}AR$ s are particularly abundant in the CPu, and are also (although to a lesser extent) expressed in the frontal cortex (Rosin et al. 2003).

Hence, by facilitating dopamine receptor-mediated effects, A_{2A}AR antagonists may boost neurotransmission at the level of the frontostriatal circuit, eventually exerting a positive influence on parkinsonian cognitive deficits. Moreover, in addition to the modulation of dopaminergic transmission by A_{2A}ARs, cholinergic system functioning may also be affected. Interestingly, the A_{2A}AR antagonist SCH-58261 has been found to increase acetylcholine release in rat frontal cortex (Acquas et al. 2002). The latter finding may be potentially relevant to the treatment of cognitive deficits in PD, suggesting the potential ability of A_{2A}AR antagonism to modify hypofunctionality of the frontal cortex cholinergic system, implicated to some extent in cognitive decline in PD, an effect which may contribute towards improving this specific symptom of PD. These results do not exclude a potential role of adenosine A₁ receptor in contrasting cognitive decline in PD (Mihara et al. 2007).

7.2 *Neuroprotective Potential*

One of the major limitations of the current pharmacological treatment of PD is represented by its substantial ineffectiveness in counteracting the degeneration of dopaminergic neurons, which underlies this condition. In this regard, it has recently been emphasized that the blockade of adenosine A_{2A}ARs may potentially represent a valuable approach in counteracting neuronal death in PD (Chen et al. 2007).

Neuroprotective effects have been obtained in different PD animal models by drug administration or in A_{2A}AR KO mice. In the MPTP mouse model, blockade of A_{2A}ARs by either SCH-58261 or KW-6002 or deletion of the gene encoding for the A_{2A}AR has been shown to substantially reduce both the demise of dopaminergic nigral neurons and the fall in striatal dopamine concentration elicited by MPTP administration (Chen et al. 2001; Ikeda et al. 2002; Pierri et al. 2005).

Despite the fact that neuroprotection elicited by A_{2A}AR antagonists in PD animal models is clearly manifested, the neuronal mechanisms underlying this effect have not yet been ascertained, although they would seem to differ from those mediating the motor-stimulating effects of these agents.

An abnormal increase in glutamate outflow may be implicated in triggering the demise of dopaminergic neurons observed in PD, and so an involvement of glutamate in A_{2A}AR blockade-mediated neuroprotection has been suggested, since A_{2A}ARs located presynaptically on glutamatergic terminals control glutamate release in a negative way (Cunha 2001; Popoli et al. 2002). It should nevertheless be taken into account that mechanisms other than that regulating glutamate release may be involved in the neuroprotection mediated by A_{2A}AR blockade, in view of the modulation by A_{2A}ARs of a large number of brain functions. The ability of A_{2A}ARs to modulate the activity of non-neuronal cell types (e.g., microglia or astroglia) is of particular interest to this regard, in view of the crucial role played by glia-mediated neuroinflammation in PD. Therefore, interference with glial-released neurotoxic factors might confer protective properties on these agents

as well, leading to the compelling possibility that a unique broad mechanism might subserve A_{2A} AR-mediated neuroprotection in diverse neurodegenerative pathologies (Kust et al. 1999; Nishizaki et al. 2002).

To date no clinical studies have been carried out to investigate potential neuroprotective effects on the dopaminergic system following the administration of A_{2A} AR antagonists. However, epidemiological studies have demonstrated how the incidence of idiopathic PD negatively correlates with caffeine intake, being significantly lower in individuals that regularly consume caffeine throughout their lifetime (Ascherio et al. 2001).

Therefore, direct evidence of neuroprotection mediated by A_{2A} AR antagonists in experimental animals, as well as data from epidemiological studies, provide new insights into the study of the antiparkinsonian potential of these drugs. It can therefore be postulated that A_{2A} AR antagonists may not only relieve motor deficits in established PD but may also potentially prevent the pathology from progressing by arresting the degeneration of dopaminergic mesencephalic neurons.

8 Conclusions

Although the neuroprotective and symptomatic effects of A_{2A} AR antagonists on parkinsonian neuronal demise appear to be most promising, it should be noted that (i) by acting on A_{2A} ARs to produce vasodilation, adenosine affects oxygen supply:demand, (ii) by acting on A_{2A} ARs on inflammatory cells, adenosine produces anti-inflammatory responses, and (iii) by acting on A_{2A} ARs on endothelial cells, adenosine decreases endothelial permeability. Therefore, blockade of A_{2A} ARs may produce adverse effects in regions other than the brain, such as the heart, kidney, lung and inflammatory responses in general. For more information on A_{2A} ARs in other organs, please refer to other chapters in this volume, such as those focusing on adenosine receptors and the kidney (Chap. 15), heart (Chaps. 6 and 7), asthma (Chap. 11), and inflammation (Chap. 8). As a consequence, more detailed studies should be undertaken in the future in both experimental animals and humans to clarify whether (and under which specific conditions) A_{2A} AR antagonists may be used as safe and effective agents in the treatment of PD.

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Adenosine Receptor Ligands and PET Imaging of the CNS

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Abstract Advances in radiotracer chemistry have resulted in the development of novel molecular imaging probes for adenosine receptors (ARs). With the availability of these molecules, the function of ARs in human pathophysiology as well as the safety and efficacy of approaches to the different AR targets can now be determined. Molecular imaging is a rapidly growing field of research that allows the identification of molecular targets and functional processes in vivo. It is therefore gaining increasing interest as a tool in drug development because it permits the process of evaluating promising therapeutic targets to be stratified. Further, molecular imaging has the potential to evolve into a useful diagnostic tool, particularly for neurological and psychiatric disorders. This chapter focuses on currently available AR

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ligands that are suitable for molecular neuroimaging and describes first applications in healthy subjects and patients using positron emission tomography (PET).

Keywords Adenosine receptors · Brain disorders · Drug development · Molecular imaging · Positron emission tomography · Radioligands · Radiosynthesis

Abbreviations

AMP	Adenosine monophosphate
AR	Adenosine receptor
A ₁ AR	A ₁ adenosine receptor
A _{2A} AR	A _{2A} adenosine receptor
A _{2B} AR	A _{2B} adenosine receptor
A ₃ AR	A ₃ adenosine receptor
AD	Alzheimer's disease
BS–DMPX	(<i>E</i>)-8-(3-Bromostyryl)-3,7-dimethyl-1-propargylxanthine
Bq	Becquerel
CNS	Central nervous system
CPFPX	8-Cyclopentyl-3-(3-fluoropropyl)-1-propylxanthine
CSC	(<i>E</i>)-8-Chlorostyryl-1,3,7-trimethylxanthine (8-chlorostyrylcaffeine)
D ₂ R	Dopamine D ₂ receptor
DMPX	3,7-Dimethyl-1-propylxanthine
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
ED ₅₀	50% Efficient dose
EPDX	2-Ethyl-8-dicyclopropylmethyl-3-propylxanthine
FDG	2-Deoxy-2-fluoro-D-glucose
[¹⁸ F]FE@SUPPY	5-(2-[¹⁸ F]fluoroethyl)-2,4-diethyl-3-(ethylsulfonylcarbonyl)-6-phenylpyridine-5-carboxylate
FR194921	2-(1-Methyl-4-piperidinyl)-6-(2-phenylpyrazolo [1,5- <i>a</i>]pyridin-3-yl)-3(2 <i>H</i>)-pyridazinone
IS–DMPX	(<i>E</i>)-3,7-Dimethyl-8-(3-iodostyryl)-1-propargylxanthine
keV	Kiloelectron volt
KF15372	8-Dicyclopropylmethyl-1,3-dipropylxanthine
MPDX	8-Dicyclopropylmethyl-1-methyl-3-propylxanthine
KF17837	(<i>E</i>)-8-(3,4-Dimethoxystyryl)-1,3-dipropyl-7-methylxanthine
KF18446 (TMSX)	(<i>E</i>)-8-(3,4,5-Trimethoxystyryl)-1,3,7-trimethylxanthine
KF19631	(<i>E</i>)-1,3-Diallyl-7-methyl-8-(3,4,5-trimethoxystyryl)xanthine

KF21213	(<i>E</i>)-8-(2,3-Dimethyl-4-methoxystyryl)-1,3,7-trimethylxanthine
KF21652	3-[1-(6,7-Dimethoxyquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4(<i>IH</i> , <i>3H</i>)-quinazolinedione
KW-6002 (istradefylline)	(<i>E</i>)-1,3-Diethyl-8-(3,4-dimethoxystyryl)-7-methylxanthine
PET	Positron emission tomography
PD	Parkinson's disease
SCH442416	5-Amino-7-(3-(4-methoxyphenyl)propyl)-2-(2-furyl)-pyrazolo[4,3- <i>e</i>]-1,2,4-triazolo[1,5- <i>c</i>]pyrimidine
SCH 58261	7-(2-Phenylethyl)-5-amino-2-(2-furyl)-pyrazolo[4,3- <i>e</i>]-1,2,4-triazolo[1,5- <i>c</i>]pyrimidine
SPECT	Single-photon emission computed tomography
SUV	Standard uptake value
Sv	Sievert

1 Introduction

Adenosine contributes to many physiological processes, particularly in excitable tissues such as the heart and brain. In the brain, adenosine acts as a neuromodulator and seems to have an inhibitory net effect on neuronal tissue (Dunwiddie and Masino 2001). It participates in the autoregulation of cerebral blood flow (Berne et al. 1981; Dirnagl et al. 1994), functions as a retrograde synaptic messenger (Brundege and Dunwiddie 1997), and is involved in the induction and maintenance of sleep and the regulation of arousal (Elmenhorst et al. 2007b; Porkka-Heiskanen 1999; Portas et al. 1997). Given the broad range of adenosine involvement in physiological and pathophysiological processes, numerous agonists and antagonists of adenosine receptors (ARs) are presently under evaluation in order to explore their therapeutic and diagnostic potential.

Molecular imaging is a means to get access to these processes in vivo in the human body. It will, therefore, aid in stratifying the process of evaluating promising therapeutic compounds from bench to market, and it has also the potential to evolve into a useful diagnostic tool of adenosine-related diseases, particularly, neurodegenerative disorders [e.g., Parkinson's disease (PD) and Alzheimer's disease (AD)], and brain pathologies including epilepsy, ischemia, and sleep disorders (Jacobson and Gao 2006). This chapter will primarily focus on AR-related ligands suitable for molecular neuroimaging, and their research and clinical applications using positron emission tomography (PET).

Adenosine exerts its physiological actions through four subtypes of G-protein-coupled receptor ARs (A_1 , A_{2A} , A_{2B} , and A_3) (Fredholm et al. 1997, 2001; Olah and Stiles 2000). The A_1 adenosine receptor (A_1 AR) is densely and heterogeneously expressed in the brain. High densities occur in thalamus and basal ganglia, as well as in neocortical and allocortical regions. A_1 AR density is low in cerebellum,

midbrain, and brain stem (Chaudhuri et al. 1998; Deckert et al. 1998; Fastbom et al. 1986; Glass et al. 1996; Schindler et al. 2001; Svenningsson et al. 1997). Pre- and postsynaptic A₁ARs mediate the depressant, sedative, and anticonvulsant effects of cerebral adenosine. A₁ARs are involved in the pathology of seizure disorders (Franklin et al. 1989; Moraidis and Bingmann 1994) and are reduced in cerebral inflammatory diseases (Johnston et al. 2001). In AD there are reports of regional losses of A₁AR binding sites (Deckert et al. 1998; Jaarsma et al. 1991; Schubert et al. 2001; Ulas et al. 1993) and local increases of A₁AR immunoreactivity (Albasanz et al. 2008; Angulo et al. 2003), which could reflect a specific regional and stage-related pattern of cerebral A₁AR involvement in AD. Therefore, evidence is accumulating that cerebral A₁ARs are potential targets for diagnostic imaging and therapeutic interventions in these diseases (Abbracchio and Cattabeni 1999; Fukumitsu et al. 2008; Ribeiro et al. 2003; Schubert et al. 1997).

The interaction and coexpression of A_{2A} adenosine receptors (A_{2A}ARs) and D₂ dopamine receptors (D₂Rs) in medium-sized cells of the striatum have drawn attention to the therapeutic potentials of A_{2A}AR antagonists. Treatment with these compounds alleviates symptoms in PD and seems to decelerate the neurodegenerative process (Xu et al. 2005). Given the importance of A₁ARs and A_{2A}ARs in brain physiology and pathology, they were the first AR subtypes to be successfully visualized in the human brain *in vivo* (Bauer et al. 2003; Fukumitsu et al. 2003, 2005; Ishiwata et al. 2005a; Mishina et al. 2007).

Adenosine A_{2B} receptors (A_{2B}ARs) and A₃ receptors (A₃ARs) seem to be primarily activated under pathological conditions, such as ischemia and various types of cancer. For both AR subtypes, there is currently no radiotracer that has successfully been applied in the human brain.

Molecular imaging methods, such as PET and single-photon emission computed tomography (SPECT), are characterized by a high sensitivity that allows the visualization of receptors of neurotransmitters and neuromodulators (e.g., adenosine; i.e., ARs) *in vivo* with excellent temporal and reasonable spatial resolution, respectively. PET is based on the imaging of radiopharmaceuticals labeled with positron-emitting radionuclides such as ¹¹C, ¹⁵O, and ¹⁸F, and on measuring the annihilation radiation using a coincidence technique. Two 511 keV γ -rays are emitted at $\sim 180^\circ$ as a result of the collision between a positron emitted from a radionuclide and a nearby electron. The two 511 keV γ -rays are detected by external coincidence circuits. Importantly, the nanomolar amount of mass for the radionuclide that is injected intravenously is too small to affect the steady state of the biochemical process under investigation. Therefore, the advantage of PET is its ability to measure low-density binding sites without perturbing the biochemistry of the system. Besides, PET can determine the pharmacokinetics of labeled drugs and assess the effects of drugs on metabolism *in vivo* in a quantitative manner. Because only very low amounts of the radiolabeled drug have to be administered (far below toxicity levels) human studies can be carried out even before the drug is entered in Phase I clinical trials. Such studies can provide cost-effective predictive toxicology data and information on the metabolism and mode of action of drugs. Especially valuable is the contribution of PET to bridge the gap between molecular biology/pathophysiology and the design

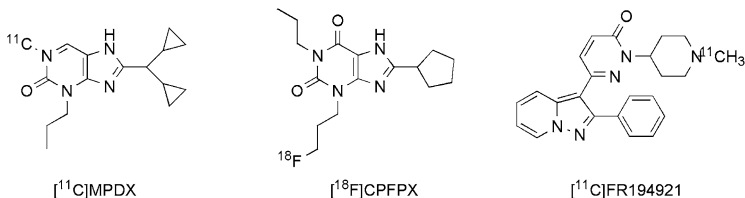
of new drugs. Regarding ARs, there are several reports of successful visualizations of A₁ARs, A_{2A}ARs (in humans and different animal species), and recently A₃ARs (in the rat) using PET, which clearly demonstrate the feasibility of these powerful modalities to further enhance the role of radiotracer studies in drug-effect monitoring. However, so far, all of these applications are of an experimental nature and have not yet reached the arena of clinical diagnostic use.

This chapter provides an overview of the current status regarding the development of both PET radioligands for mapping ARs and new lead compounds for potential PET radioligands. It also summarizes preclinical and clinical results that have so far been obtained by molecular imaging of ARs.

2 Development of PET Radioligands

For the last two decades, ARs have been extensively studied biologically and pharmacologically, and advancements in the synthesis and screening of a large number of compounds have resulted in the identification of selective ligands with high affinity and high specific binding for each receptor subtype. Since 1995, several PET ligands with xanthine-type structures, which are expected to penetrate the blood–brain barrier, have been proposed for mapping A₁ARs (Furuta et al. 1996; Holschbach et al. 1998; Ishiwata et al. 1995; Noguchi et al. 1997) and A_{2A}ARs (Hirani et al. 2001; Ishiwata et al. 1996, 2000a, b, d, 2003a; Marian et al. 1999; Noguchi et al. 1998; Stone-Elander et al. 1997; Wang et al. 2000) in the central nervous system (CNS). Later, nonxanthine-type ligands were also developed (Matsuya et al. 2005; Todde et al. 2000). Among them, at least five PET ligands for A₁AR and A_{2A}AR subtypes have been applied to clinical studies (Fig. 1) (Bauer

Adenosine A₁ receptor PET ligands



Adenosine A_{2A} receptor PET ligands

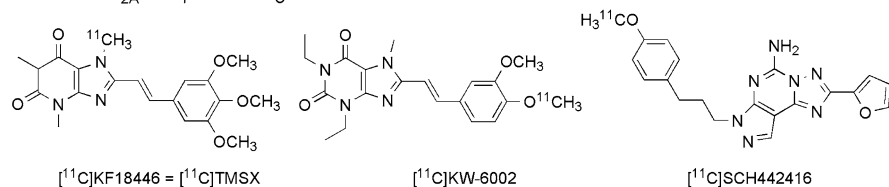


Fig. 1 Representative PET ligands for mapping adenosine A₁ receptors (A₁ARs) and adenosine A_{2A} receptors (A_{2A}ARs). All ligands except for [¹¹C]JFR194921 have been used clinically, but only preliminary results have been published for [¹¹C]SCH442416

et al. 2003; Fukumitsu et al. 2003; Hunter 2006; Ishiwata et al. 2005a). On the other hand, PET ligands for the A₃AR subtype (Wadsak et al. 2008) and the adenosine uptake site (Ishiwata et al. 2001; Mathews et al. 2005) are limited, and no PET ligand for the A_{2B}AR subtype has been reported until now. Early works on the development of PET ligands have been described (Holschbach and Olsson 2002; Ishiwata et al. 2002c; Suzuki and Ishiwata 1998), and recent advances in the development of PET ligands and medicinal chemistry, including candidates for this purpose, have been reviewed (Ishiwata et al. 2008).

2.1 Adenosine A₁ Receptor Ligands

In Table 1, in vitro and in vivo properties of A₁AR PET ligands are summarized. Xanthine derivatives such as 8-dicyclopropylmethyl-1,3-dipropylxanthine (KF15372) (Shimada et al. 1991; Suzuki et al. 1992) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Bruns et al. 1987; Lohse et al. 1987) are selected as leading compounds for PET ligands. [³H]DPCPX has been used in vitro as a radioligand with high affinity and selectivity for the A₁AR (Deckert et al. 1998; Jaarsma et al. 1991; Svenningsson et al. 1997; Ulas et al. 1993). Both compounds have two propyl groups, each of which can potentially be labeled with ¹¹C (half-life of 20.4 min). Ishiwata et al. prepared [¹¹C]KF15372 and its [¹¹C]ethyl and [¹¹C]methyl derivatives (2-[¹¹C]ethyl-8-dicyclopropylmethyl-3-propylxanthine ([¹¹C]EPDX) and 8-dicyclopropylmethyl-1-[¹¹C]methyl-3-propylxanthine ([¹¹C]MPDX), respectively) (Furuta et al. 1996; Ishiwata et al. 1995; Noguchi et al. 1997). [¹¹C]MPDX (Fig. 1) showed a slightly lower affinity for A₁ARs than [¹¹C]KF15372; however, [¹¹C]MPDX was selected for further investigations among the three ligands because of a high radiochemical yield and easy penetration through the blood–brain barrier. Later, Holschbach et al. examined a series of DPCPX analogs and found several candidates containing fluorine or iodine (Holschbach et al. 1998). The selected ligand was [¹⁸F]8-cyclopentyl-3-(3-fluoropropyl)-1-propylxanthine ([¹⁸F]CPFPX) (Fig. 1) (¹⁸F, half-life of 110 min), in which a [¹⁸F]fluoropropyl group was incorporated into DPCPX instead of ¹¹C labeling a propyl group (Holschbach et al. 2002). This substitution greatly enhanced the affinity and selectivity for A₁ARs. Radioiodine-labeled ligands may be used for PET (¹²⁴I, half-life of 4.18 days) and SPECT (¹²³I, half-life of 13.3 h). Recently, nonxanthine-type pyrazolpyridine compounds were proposed for A₁AR ligands (Kuroda et al. 2001; Maemoto et al. 2004), and Matsuya et al. prepared [¹¹C]2-(1-methyl-4-piperidinyl)-6-(2-phenylpyrazolo [1,5-*a*]pyridin-3-yl)-3(2*H*)-pyridazinone ([¹¹C]FR194921) (Fig. 1) (Matsuya et al. 2005).

Among five ligands, [¹⁸F]CPFPX shows the highest affinity and selectivity in vitro as well as high uptake and specific binding in vivo (Table 1). In mice, the brain uptake was rapid and remained constant for 40 min after injection, followed by a gradual decrease because of high affinity, suggesting that a long PET scan covering

Table 1 PET ligands for the A₁ adenosine receptor (A₁AR)

	In vitro studies				In vivo studies		
	Affinity (K _i nM)		Selectivity A _{2A} /A ₁	Reference	Uptake ^a (cerebral cortex) (SUV)	Specific binding ^b (%)	Reference
	A ₁	A _{2A}					
DPCPX	6.4	590	92	Shimada et al. (1991); Suzuki et al. (1992)			
[¹¹ C]KF15372	3.0	430	143	Shimada et al. (1991); Suzuki et al. (1992)	0.43 (m, 15 min)	57 (m, 15 min) ^d	Noguchi et al. (1997)
[¹¹ C]EPDX	1.7	> 100	> 59	Noguchi et al. (1997)	0.66 (m, 15 min)	47 (m, 15 min) ^d	Noguchi et al. (1997)
[¹¹ C]MPDX	4.2	> 100	> 24	Noguchi et al. (1997)	0.54 (m, 15 min)	43 (m, 15 min) ^d	Noguchi et al. (1997)
[¹⁸ F]CPPFX	0.183			Holschbach et al. (1998)		61–64 (r, 15 min) ^{e, c}	Wang et al. (2003)
	0.63–1.37 (K _d)	812–940 (K _d)	> 700	Holschbach et al. (2002)	0.88 (m, 40 min)	70–80 (m, 10–40 min) ^{e, f}	Holschbach et al. (2002)
[¹¹ C]FR194921	2.91	> 100	> 34	Matsuya et al. (2005); Maemoto et al. (2004)	0.3 (r, 30 min)	50 (r, 30 min) ^e	Matsuya et al. (2005)

^aUptake was normalized as the standardized uptake value [SUV, (tissue activity/total injected activity) × (gram body weight/gram tissue weight)], assuming the body weights of rats and mice were 300 g and 35 g, respectively. In the parentheses, “r” and “m” express the uptakes in the brain of rats and mice, respectively, which were killed at the indicated time after injection of the tracer. The tissue uptake was measured by the tissue dissection method, except in one case (marked by ^c), where it was measured by ex vivo autoradiography

^bThe reduced percentages of the uptake by blockade with injection of selective appropriate adenosine A₁ receptor ligand together with the tracer^d or before^e or after^f injection of the tracer

the pseudoequilibrium state of the ligand–receptor binding may be preferable. The other ligands showed reasonable brain uptake and specific binding due to the affinity *in vitro* and the lipophilicity.

Xanthine derivatives are unstable in relation to peripheral metabolism. Percentages of the unchanged form in rodent plasma were <30% for both [^{11}C]MPDX and [^{18}F]CPFPX 30 min postinjection, whereas [^{11}C]FR194921 was much more stable (87% at 60 min) (Bier et al. 2006; Matsuya et al. 2005; Noguchi et al. 1997). The metabolic pathway of [^{18}F]CPFPX was extensively investigated (Bier et al. 2006), and Matusch et al. (2006) identified that cytochrome P-450 1A2 catalyzed the metabolism of it. Later [^{11}C]MPDX was confirmed to be much more stable in human plasma (75% was unchanged at 60 min) (Fukumitsu et al. 2005), while [^{18}F]CPFPX was metabolized faster in humans (Bauer et al. 2003).

However, the evaluation of PET ligands at a single or a limited number of time points after injection, as shown in Table 1, was not adequate when comparing several ligands. Dynamic PET studies in monkeys or cats were carried out for [^{11}C]KF15372 (Wakabayashi et al. 2000), [^{11}C]MPDX (Ishiwata et al. 2002a; Shimada et al. 2002) and [^{11}C]FR194921 (Matsuya et al. 2005). Although [^{11}C]KF15372 and [^{11}C]FR194921 have similar affinities *in vitro*, the brain kinetics were considerably different in monkeys. [^{11}C]KF15372 accumulated and reached a maximum at 10 min followed by a gradual decrease, while [^{11}C]FR194921 accumulated over 60 min. In the time frame of a PET scan using a ^{11}C -labeled tracer (60–90 min), [^{11}C]KF15372 showed preferable brain kinetics for quantitative evaluation of the ligand–receptor binding, while the affinity of [^{11}C]FR194921 may be too high. Compared with [^{11}C]KF15372, [^{11}C]MPDX showed a faster brain clearance in monkeys and cats, but quantitative evaluation of A_1 ARs in the cat brain was nevertheless successfully performed by PET.

The other radioligands labeled with positron emitters are 5'-*N*-(2-[^{18}F]fluoroethyl)-carboxamidoadenosine and 5'-(methyl[^{75}Se]seleno)-*N*⁶-cyclopentyladenosine (^{75}Se , half-life of 7.1 h) (Lehel et al. 2000; Blum et al. 2004). Although the biological evaluation of these tracers has not been reported, they may be suitable ligands for peripheral organs but not for the CNS, if available for PET studies; however, 5'-*N*-(2-[^{18}F]fluoroethyl)-carboxamidoadenosine may not be a selective ligand for A_1 ARs (Lehel et al. 2000).

2.2 Adenosine $\text{A}_{2\text{A}}$ Receptor Ligands

Considering 3,7-dimethyl-1-propylxanthine (DMPX) as a lead for $\text{A}_{2\text{A}}$ AR-selective antagonists (Seale et al. 1988), Shimada et al. have discovered that xanthines with the styryl group in the 8 position have selective $\text{A}_{2\text{A}}$ AR antagonistic properties (Nonaka et al. 1994; Shimada et al. 1992). Later, Müller et al. also introduced brominated and chlorinated styryl groups in the 8 position of DMPX to produce $\text{A}_{2\text{A}}$ AR-selectivity (Müller et al. 1997, 1998). The representative compound (*E*)-8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxanthine (KF17837) has been used for pharmacological and neurochemical studies as a selective antagonist for

A_{2A}ARs (Correa et al. 2004; Hayaishi 1999; Koga et al. 2000). So far KF17837 and seven other derivatives have been labeled with ¹¹C, and these radiotracers were investigated as potential PET ligands (Ishiwata et al. 1996, 2000a, b; Noguchi et al. 1998; Stone-Elander et al. 1997; Wang et al. 2000) (Table 2). [¹¹C](*E*)-8-(3-Bromostyryl)-3,7-dimethyl-1-propargylxanthine ([¹¹C]BS-DMPX) and [¹¹C](*E*)-3,7-dimethyl-8-(3-iodostyryl)-1-propargylxanthine ([¹¹C]IS-DMPX) (Ishiwata et al. 2000d) can potentially be labeled with radiolabeled bromines (⁷⁵Br, *t*_{1/2} = 1.7 h or ⁷⁶Br, *t*_{1/2} = 16.1 h) and iodines (¹²⁴I, half-life of 4.18 days, and ¹²³I, half-life of 13.3 h), respectively, for PET or SPECT. Most of these studies were done by Ishiwata et al. in collaboration with Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Later, Kyowa Hakko Kogyo chose the selective A_{2A}AR antagonist (*E*)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KW-6002), known as istradefylline, for clinical evaluation as an antiPD agent (Bara-Jimenez et al. 2003; Hauser et al. 2003) after an experimental study of [¹¹C]KW-6002 (Fig. 1) (Hirani et al. 2001). It was noted that photoisomerization occurred in the styryl group at the 8 positions of xanthine-type A_{2A}AR-selective ligands such as (*E*)-8-(3,4,5-trimethoxystyryl)-1,3,7-trimethylxanthine ([¹¹C]KF18446), later designated [¹¹C]TMSX (Fig. 1) (Ishiwata et al. 2003b; Nonaka et al. 1993). Consequently, all procedures in PET studies were carried out under dim light until injection and also during plasma metabolite analysis.

Besides xanthine derivatives, a number of nonxanthine heterocycles have also been synthesized as A_{2A}AR antagonists. 7-(2-Phenylethyl)-5-amino-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH 58261) is a representative ligand with a high and selective affinity for the A_{2A}AR (Zocchi et al. 1996a, b); however, it does not have an appropriate synthon for labeling with positron emitters. Todde et al. used 5-amino-7-(3-(4-methoxyphenyl)propyl)-2-(2-furyl)pyrazolo [4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH442416) with its 4-methoxyphenylpropyl group, and prepared [¹¹C]SCH442416 (Fig. 1) by *O*-[¹¹C]methylation (Todde et al. 2000).

Table 2 summarizes the in vitro and in vivo properties of A_{2A}AR PET ligands. The highest affinity for A_{2A}ARs was found in SCH442416, followed by KF17837, KW-6002, and (*E*)-8-(2,3-dimethyl-4-methoxystyryl)-1,3,7-trimethylxanthine (KF21213). SCH442416, KF21213 and IS-DMPX showed superior A_{2A}AR selectivity. (*E*)-1,3-Diallyl-7-methyl-8-(3,4,5-trimethoxystyryl)xanthine (KF19631), TMSX, (*E*)-8-chlorostyryl)-1,3,7-trimethylxanthine (8-chlorostyrylcaffeine, CSC), and BS-DMPX showed moderate selectivity, but their affinities for the A₁ARs were too low to bind in vivo. In evaluation studies in rodents, all radioligands showed A_{2A}AR-selective uptake in the striatum where the expression of A_{2A}ARs is high; however, specific binding was also observed in the cerebral cortex as well as cerebellum to a certain extent for most radioligands except for [¹¹C]KF21213. Thus, the highest A_{2A}AR selectivity in vivo was observed in [¹¹C]KF21213, followed by [¹¹C]SCH442416 and [¹¹C]TMSX, when evaluated based on the uptake ratio of receptor-rich striatum to receptor-poor cerebellum.

Compared with A₁AR receptor ligands, a slow peripheral degradation of two xanthine compounds was confirmed in the metabolite analysis in plasma;

Table 2 PET ligands for the A_{2A} adenosine receptor (A_{2A}AR)

	In vitro studies				In vivo studies			
	Affinity (K _i)		Selectivity A ₁ /A _{2A}	References	Striatal uptake ^a (SUV)	Selectivity ^b (Str/Cer)	Specific binding ^c (%)	References
	A ₁	A _{2A}						
DMPX	12,000	8,600	1.4	Shimada et al. (1991); Suzuki et al. (1992)				
[¹¹ C]KF17837	62	1.0	62	Nonaka et al. (1994)	0.82(m, 15 min)	2.0 (m, 60 min) 1.2 (r, 15 min) ^d	43 (m, 15) ^e	Noguchi et al. (1998)
[¹¹ C]KF19631	860	3.5	250	Ishiwata et al. (2002c)	0.33 (m, 15 min)	1.2 (m, 60 min) 1.2 (r, 15 min) ^d	31 (m, 15) ^e	Ishiwata et al. (2000a)
[¹¹ C]KF18446 =	1,600	5.9	270	Ishiwata et al. (2000a)	1.54 (m, 15 min) 1.68 (r, 15 min)	2.8 (m, 15 min)	72 (m, 15) ^e	Ishiwata et al. (2000a)
[¹¹ C]TMSX					ND	ND	ND	
[¹¹ C]CSC	28,000	54	520	Nonaka et al. (1994)				
[¹¹ C]BS-DMPX	2,300	7.7	300	Ishiwata et al. (2000d)	0.90 (m, 15 min)	1.2 (m, 60 min)	51 (m, 15) ^e	Ishiwata et al. (2000d)
[¹¹ C]JS-DMPX	>10,000	8.9	>1,100	Ishiwata et al. (2000d)	0.70 (m, 15 min)	1.2 (m, 60 min)	17 ^{ns} (m, 15) ^e	Ishiwata et al. (2000d)
[¹¹ C]KF21213	>10,000	3.0	>3,300	Wang et al. (2000)	1.40 (m, 15 min)	10.5 (m, 60 min)	69 (m, 15) ^e	Wang et al. (2000)

[¹¹ C]KW-6002	150	2.2	68	Nonaka et al. (1994)	2.9 (r, 15 min)	1.1 (r, 75 min)	88 (r, 75) ^f	Hirani et al. (2001)
SCH-58261	121	2.3	53	Zocchi et al. (1996a)				
[¹¹ C]SCH442416	1,800	0.50	3,630	Todde et al. (2000)	1.15 (r, 15 min)	4.6 (r, 15 min)	ND	Todde et al. (2000)
					ca. 1 (r, 15 min)	4.97 (r, 15 min)	31–86 (r, 15) ^f	Moresco et al. (2005)

^aUptake was normalized as the standardized uptake value [SUV, (tissue activity/total injected activity) × (gram body weight/gram tissue weight)], assuming the body weights of rats and mice were 300 g and 35 g, respectively. In the parentheses, “r” and “m” express the uptakes in rat and mouse brain, respectively, which were killed at the indicated time after injection of the tracer. The tissue uptake was measured by the tissue dissection method, except in some cases (marked by ^d), where it was measured by *ex vivo* autoradiography

^bSelectivity was determined as the uptake ratio of striatum to cerebellum (Str/Cer). This concept is based on the finding that the striatum is rich in A_{2A}ARs, while the expression of A_{2A}ARs is low or negligible in the cerebellum

^cReduced percentages of uptake by the blockade with injection of selective appropriate A_{2A}AR ligand together with the tracer^e or before^f injection of the tracer
^dND, not determined; *ns*, no significance (control versus blocked animals)

percentages of the unchanged form were 81% for [^{11}C]TMSX at 30 min in mice (Ishiwata et al. 2000a) and 66% for [^{11}C]KW-6002 at 45 min in rats (Hirani et al. 2001). [^{11}C]SCH442416 was slightly unstable: 40% was unchanged at 30 min in rats (Todde et al. 2000). Later, [^{11}C]TMSX was confirmed to be much more stable in human plasma (>90% of the unchanged form at 60 min) (Mishina et al. 2007).

Dynamic PET studies in monkeys were carried out for [^{11}C]KF17837, [^{11}C]TMSX and [^{11}C]SCH442416. The striatal uptake of [^{11}C]TMSX was approximately tenfold higher at 5–10 min compared with [^{11}C]KF17837, and the uptake ratios of striatum to cortex and striatum to cerebellum for [^{11}C]TMSX were slightly higher than those for [^{11}C]KF17837 (Ishiwata et al. 2000a). A slightly lower affinity of [^{11}C]TMSX resulted in a faster clearance of the radioligand from the striatum compared to [^{11}C]KF17837. Because it exhibited the highest affinity among the three ligands, [^{11}C]SCH442416 showed more preferable brain kinetics for quantitative evaluating the ligand–receptor binding (Moresco et al. 2005). Although [^{11}C]KF21213 showed the most preferable properties in rodents, in a preliminary PET study using monkeys [^{11}C]TMSX showed better brain kinetics than [^{11}C]KF21213 (Ishiwata et al. 2005b).

Most studies of radioligands have focused on ARs in the CNS. On the other hand, Ishiwata et al. demonstrated that xanthine-type ligands can be applicable to studies on peripheral A_{2A} ARs (Ishiwata et al. 1997, 2003a, 2004). In rodents, specific binding of [^{11}C]TMSX was observed in the muscle and heart. Swimming exercise caused fluctuations in [^{11}C]TMSX-receptor binding in these tissues, and the specific binding of [^{11}C]TMSX to these tissues was also preliminarily demonstrated clinically (Ishiwata et al. 2004). Furthermore, the [^{11}C]TMSX-receptor binding in the cardiac and skeletal muscles was greater in endurance-trained men than in untrained men (Mizuno et al. 2005).

2.3 Adenosine A_3 Receptor Ligands

Recently, Wadsak et al. (2008) reported on the synthesis of 5-(2-[^{18}F]fluoroethyl)-2,4-diethyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate ([^{18}F]FE@SUPPY) for the A_3 AR and a preliminary evaluation. The tracer was taken up in the rat brain at intermediate levels and bound to rat brain slices *in vitro*; however, further *in vivo* studies are essential for the evaluation of its specificity and selectivity.

2.4 Ligands for the Adenosine Uptake Site

[1-Methyl- ^{11}C]-3-[1-(6,7-dimethoxyquinazolin-4-yl)piperidin-4-yl]-1,6-dimethyl-2,4-(1*H*, 3*H*)-quinazolin-2(1*H*)-one ([^{11}C]KF21652), with a K_i value of 13 nM, was prepared by *N*-[^{11}C]methylation (Ishiwata et al. 2001). The brain uptake of [^{11}C]KF21652 was very low *in vivo*, probably because of its relatively high

lipophilicity ($\log P$ 3.6), although in vitro autoradiography showed specific binding to adenosine uptake sites to a certain extent (less than 25% of total binding). Peripherally, only the liver showed carrier-saturable uptake. The compound is not a suitable PET ligand.

Another potential labeled tracer for adenosine uptake sites is [^{11}C]adenosine monophosphate ([^{11}C]AMP) (Mathews et al. 2005). In mice, this tracer was not incorporated in the brain, and the highest uptake was observed in the lung, blood, and heart. The lung uptake was significantly reduced to about 40% by blocking with dipyridamole, a ligand for adenosine uptake sites. The putative value of this ligand needs to be investigated further.

2.5 Radiosynthesis

All ^{11}C -labeled ligands except for [^{11}C]AMP have been synthesized by *N*- or *O*-alkylation with [^{11}C]methyl iodide or [^{11}C]alkyl iodide. Practically speaking, the production of [^{11}C]methyl iodide is much easier than those of [^{11}C]ethyl iodide and [^{11}C]propyl iodide, which were used for the syntheses of [^{11}C]EPDX and [^{11}C]KF15372, respectively, and usually achieved high radiochemical yields of the ligands (Noguchi et al. 1997). [^{11}C]Methyl triflate is a highly reactive alternative to [^{11}C]methyl iodide (Kawamura and Ishiwata 2004). [^{11}C]AMP was produced by reacting [^{11}C]formaldehyde with the corresponding amino-imidazolyl-carboxamide, giving a low radiochemical yield (Mathews et al. 2005).

On the other hand, ^{18}F -labeled ligands were prepared by nucleophilic, cryptate-mediated substitution using ^{18}F anion. In general, ^{18}F -labeled ligands have practical advantages: the specific activity is usually higher than that of ^{11}C -labeled ligands, fluorine-18 provides slightly better resolution of the images, and its longer half-life is more suitable for clinical purposes than that of ^{11}C -labeled tracers. On the other hand, ^{11}C -labeled ligands provide reduced radiation doses for human subjects compared to ^{18}F -labeled ligands. Also, the shorter half-life of ^{11}C can allow successive PET measurements experimentally (Nariai et al. 2003) and clinically (Ishiwata et al. 2005a) on the same day.

3 Experimental Studies

Several studies using experimental animals have demonstrated the usefulness of AR ligands and PET. In the rat model, in which monocular enucleation was performed in order to destroy the anterior visual input, a loss of A_1ARs was detected by ex vivo autoradiography using [^{11}C]MPDX (Kiyosawa et al. 2001). The decrease in presynaptic A_1ARs in the superior colliculus following enucleation was coupled with an upregulation of postsynaptic benzodiazepine receptors (Wang et al. 2003). In an occlusion and reperfusion model of the cat, [^{11}C]MPDX PET was more sensitive

to the detection of severe cerebral ischemic insult than [^{11}C]flumazenil PET when measuring central benzodiazepine receptors (Nariai et al. 2003).

In a glioma-bearing rat model, Bauer et al. found that the binding of [^{18}F]CPFPX was increased in the zone surrounding tumors (136–146% as compared to control brain tissue) due to the upregulation of A_1ARs in activated astrocytes (Bauer et al. 2005; Dehnhardt et al. 2007). Furthermore, in a preliminary study, the same group demonstrated A_1AR occupancy by caffeine in the rat brain by [^{18}F]CPFPX PET (Meyer et al. 2003).

In a Huntington's disease model, induced by intrastriatal injection of quinolinic acid and consecutive degeneration of striatopallidal γ -aminobutyric acid/enkephalin neurons, degeneration of $\text{A}_{2\text{A}}\text{ARs}$ in the lesioned striatum was detected to a similar extent as degeneration of D_2Rs using PET and ex vivo and in vitro autoradiography with [^{11}C]TMSX (Ishiwata et al. 2002b). Another $\text{A}_{2\text{A}}\text{AR}$ ligand, [^{11}C]SCH442416, was applied to the same rat model of Huntington's disease (Moresco et al. 2005), demonstrating that the striatal uptake of [^{11}C]SCH442416 was reduced on the quinolinic acid-lesioned side. Furthermore, an ex vivo autoradiography study showed that [^{11}C]TMSX, but not [^{11}C]raclopride for D_2Rs , was incorporated into the globus pallidus to a lesser extent (the striatum-to-globus pallidus uptake ratio was approximately 0.6), and showed a remarkably reduced uptake in both the striatum and globus pallidus for the lesioned side in the rat model of Huntington's disease (Ishiwata et al. 2000c). These findings suggest that [^{11}C]TMSX is a candidate tracer for imaging the pallidal terminals of striatal projection neurons.

4 Clinical Studies

A large number of selective AR agonists and antagonists have been discovered, and some of them have been taken to the next level and evaluated in Phase I, II, and III clinical trials. So far, no compound has received regulatory approval. The same is true of adenosine and AR-based ligands used as PET tracers, which are under evaluation for diagnostic purposes or as markers to evaluate the efficacy of therapeutics.

4.1 Adenosine A_1 Receptor Imaging

To date, two PET ligands have successfully been applied for the visualization of A_1ARs in the human brain, [^{18}F]CPFPX (Bauer et al. 2003) and [^{11}C]MPDX (Fukumitsu et al. 2003, 2005). A direct comparison of postmortem brain material using autoradiography demonstrated a close correlation between the regional [^{18}F]CPFPX binding potential and the cerebral [^3H]CPFPX distribution (Bauer et al. 2003). Consistent with results from [^3H]CPFPX autoradiography, high A_1AR

densities were found in the putamen and mediodorsal thalamus using [^{18}F]CPFPX PET. Neocortical areas showed regional differences in [^{18}F]CPFPX binding, with high accumulation in temporal > occipital > parietal > frontal lobes and a lower level of binding in the sensorimotor cortex. Ligand accumulation was low in the cerebellum, midbrain, and brain stem (Bauer et al. 2003; Meyer et al. 2004). The specificity of [^{18}F]CPFPX binding was established in a displacement study using cold CPFPX (Meyer et al. 2006).

The clinical applicability of [^{18}F]CPFPX was assured by test–retest (Elmenhorst et al. 2007a) and dosimetrical studies (Herzog et al. 2008), respectively. The dosimetrical studies showed that an injection of 3×10^8 Bq [^{18}F]CPFPX resulted in an effective dose of 5.3×10^{-3} Sv, which is comparable to other ^{18}F -labeled ligands and thus suitable for clinical applications. Test–retest evaluations were performed in order to study the physiological intrasubject variability of [^{18}F]CPFPX binding. This factor is extremely important for the definition of the normal range of cerebral receptor binding and thus highly accountable for the discriminative power of the method as a diagnostic tool. Elmenhorst et al. (2007a) demonstrated that test–retest variability was low (5.9–13.2% on average) and therefore highly suitable for diagnostic purposes. They also showed that noninvasive quantification (i.e., without the need to take blood samples during the PET scan) is even superior to invasive measurements, which greatly improves the clinical applicability of [^{18}F]CPFPX PET. A series of horizontal planes of the cerebral [^{18}F]CPFPX distribution as well as a three-dimensional reconstruction of the neocortical surface of the brain of a healthy subject are depicted in Fig. 2.

The spatial distribution of [^{11}C]MPDX differed significantly from the regional cerebral blood flow measured by PET using [^{15}O]H $_2$ O and the regional cerebral metabolism of glucose evaluated using 2-deoxy-2- [^{18}F]fluoro-D-glucose ([^{18}F]FDG), and was in good agreement with autoradiographic data from other highly specific A $_1$ AR ligands (Fukumitsu et al. 2003). Moreover, this A $_1$ AR radiotracer showed a better metabolic stability than [^{18}F]CPFPX but had a lower affinity to A $_1$ ARs (4.2 nM in comparison to 0.183 nM).

For both tracers, quantitative methods have been developed to measure the A $_1$ AR binding potential *in vivo* in the human brain (Kimura et al. 2004; Meyer et al. 2005a, b). For clinical applications, noninvasive but fully quantitative methods with significantly shortened scan durations and without blood sampling have been developed (Naganawa et al. 2008; Meyer et al. 2005b).

With respect to the use of AR-based PET tracers in humans to define the role of ARs in neuropathology, only a limited number of clinical studies have been published so far. Boy et al. (2008) reported lower cortical and subcortical A $_1$ AR binding in patients suffering from liver cirrhosis and hepatic encephalopathy in comparison to controls. They concluded that regional cerebral adenosinergic neuromodulation is heterogeneously altered in cirrhotic patients, and that the decrease in cerebral A $_1$ AR binding may further aggravate neurotransmitter imbalance at the synaptic cleft in hepatic encephalopathy.

In a recent study utilizing an A $_1$ AR-based PET tracer, Fukumitsu et al. (2008) reported on changes in A $_1$ ARs in the brains of patients with AD. They applied two

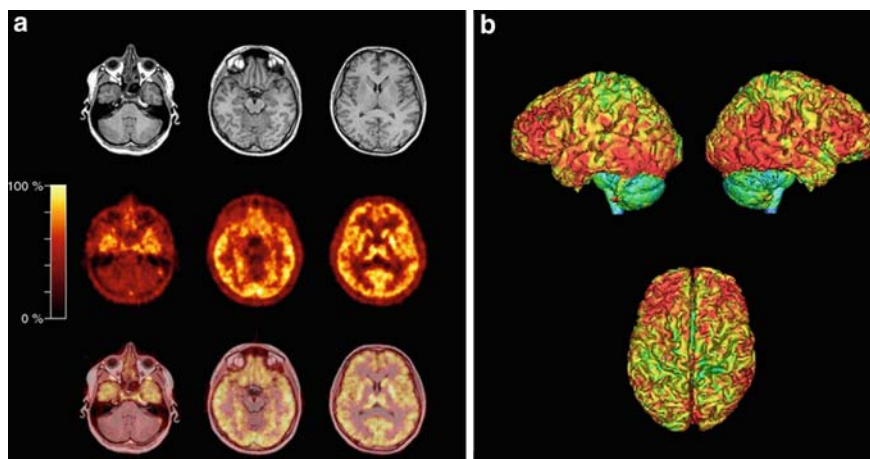


Fig. 2 a–b Distribution of adenosine A₁ receptors (A₁ARs) in the human brain. **a** Serial horizontal MRI (*upper line*) and coregistered PET images (*middle line*) from a healthy subject. Summed data from 5 to 60 min after intravenous injection of [¹⁸F]CPFPX are depicted. The fusion images (*bottom line*) show high ligand binding in neocortical areas as well as thalamus and basal ganglia (as indicated by *bright yellow colors*); low binding is found in the cerebellum (depicted by *dark orange colors*). **b** Three-dimensional reconstruction of the brain surface generated from serial planes from the same PET scan as in **a**. Note that A₁ARs are ubiquitously but not homogeneously distributed in the neocortex. There are clusters with high A₁AR binding in prefrontal and temporoparietal cortices (high binding is indicated by *red and orange colors*, low binding is indicated by *green and cyan colors*)

PET scans with [¹¹C]MPDX and [¹⁸F]FDG to the same patients to directly compare A₁ARs and glucose metabolism reflecting neural activity in the brain. There was significantly reduced binding of [¹¹C]MPDX in patients with AD in the temporal and medial temporal cortices and in the thalamus. Thus, the regional pattern of A₁AR changes in AD was different from the well known and previously reported hypometabolic brain regions (temporoparietal cortex and posterior cingulate gyrus), where [¹⁸F]FDG uptake was typically decreased in AD. This pilot study was the first study to show with the use of a PET tracer for A₁ARs that A₁ARs are reduced in AD. It clearly demonstrates that A₁AR PET ligands could become valuable tools for the investigation of neurodegenerative disorders like AD.

An interesting example of the scientific potential of A₁AR imaging in neuroscience has been published in a study on the effect of sleep deprivation for 24 h on healthy subjects, which shows promise for clinical applications in sleep disorders (Elmenhorst et al. 2007b). It is currently hypothesized that adenosine is involved in the induction of sleep after prolonged wakefulness. This effect is partially reversed by the application of caffeine, which is a nonselective blocker of ARs. Elmenhorst et al. (2007b) report that the A₁AR is upregulated after 24 h of sleep deprivation in a region-specific pattern in a broad spectrum of brain regions, with a maximum increase in the orbitofrontal cortex. There were no changes in the control group, who had regular sleep. Thus, the study provides *in vivo* evidence for an A₁AR

contribution to the homeostatic regulation of sleep in humans. Molecular imaging using A_1 AR ligands therefore shows significant potential for sleep research and, in the long run, sleep medicine.

These findings are also of importance regarding the role of caffeine as a neurostimulant and nonselective antagonist of adenosine effects at A_1 ARs and A_{2A} ARs. Throughout the world, caffeine is the most widely used pharmacological agent; it is present in beverages such as coffee, tea, and soft drinks. As a stimulant, caffeine promotes wakefulness and reduces sleep and sleep propensity (Fredholm et al. 1999; Landolt 2008; Schwierin et al. 1996; Virus et al. 1990; Yanik and Radulovacki 1987). Molecular imaging using adenosine tracers has great potential to provide insights into the regional and temporal modes of caffeine action in the human brain. In vivo A_1 AR occupancy by caffeine has so far only been demonstrated in the rat brain by [18 F]CPFPX PET (Meyer et al. 2003).

4.2 Adenosine A_{2A} Receptor Imaging

With regard to adenosine A_{2A} AR imaging, the most promising clinical application is currently PD. Striatopallidal A_{2A} ARs have been implicated in the modulation of motor functions because they partially antagonize the functions of striatal D_2 Rs. Since A_{2A} ARs show a highly enriched distribution in basal ganglia cells and are able to form functional heteromeric complexes with D_2 Rs and metabotropic glutamate mGluR5 receptors, A_{2A} ARs are of particular interest with regard to the nondopaminergic modulation of motor behavior (Ferré and Fuxe 1992; Fuxe et al. 1993; Marino et al. 2003). Additional evidence for an adenosinergic contribution to PD comes from epidemiological studies showing that chronic consumption of caffeine, a nonselective AR antagonist, is able to reduce the risk of developing PD (Ascherio et al. 2001; Ross et al. 2000). Given the relevance of A_{2A} ARs in PD, an important advance was made by Ishiwata et al. (2005a), who were able to introduce [11 C]TMSX, allowing A_{2A} ARs to be imaged in the living human brain for the first time. The specificity of [11 C]TMSX PET was confirmed by theophylline challenge (Ishiwata et al. 2005a), and the cerebral distribution pattern was consistent with previous autoradiographic findings in human postmortem brain. The binding potential was largest in the anterior (1.25) and posterior putamen (1.20), followed by the head of caudate nucleus (1.05) and thalamus (1.03). Low ligand binding was found in the cerebral cortex, particularly in the frontal lobe (0.46). Interestingly, the binding of [11 C]TMSX was relatively large in the thalamus in comparison with previous reports for other mammals (Mishina et al. 2007). For clinical purposes, the authors developed a modeling method (Naganawa et al. 2007) and proposed recently a noninvasive method for in vivo receptor quantification (Naganawa et al. 2008). A preliminary application of [11 C]TMSX to patients suffering from PD was presented at an international meeting (Mishina et al. 2006). Figure 3 depicts [11 C]TMSX PET images of a unilaterally affected patient with early-stage PD and a healthy control subject. [11 C]TMSX binding was reduced in the left putamen, which

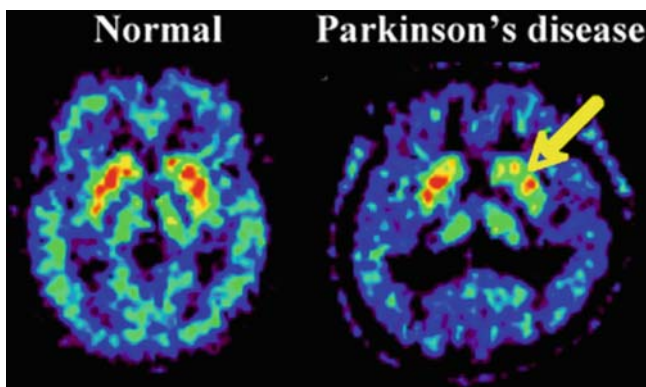


Fig. 3 Distribution of adenosine A_{2A} receptors ($A_{2A}ARs$) in the human brain: a normal subject (*left*) and a patient with Parkinson's disease (PD) (*right*). The binding potential of [^{11}C]TMSX (Naganawa et al. 2007) in a patient with early-stage PD (*right*) was lower in the putamen of the left hemisphere (*arrow*), which was consistent with more severe clinical symptoms on the right body side. In contrast, the binding of [^{11}C]raclopride to dopamine D_2 receptors (D_2Rs) was slightly increased in the left putamen (Mishina et al. 2006). See text for comments on the findings of this PET study in humans

is contralateral to the primarily affected body side, while binding of [^{11}C]raclopride to D_2Rs was slightly increased. Upregulation of D_2Rs most likely reflects a post-synaptic compensation to impaired presynaptic dopamine release. Simultaneous downregulation of $A_{2A}ARs$ and upregulation of D_2Rs is therefore likely to reflect an imbalance of adenosinergic and dopaminergic transmission at the postsynaptic site as a consequence of PD pathophysiology. This study suggests that PET imaging with $A_{2A}AR$ -selective radiotracer PET ligands may be used to monitor the natural history and progression of PD in both animal models of PD and humans with PD, and may serve as guide for therapy with $A_{2A}AR$ antagonists in patients with PD. Moreover, PET imaging with $A_{2A}AR$ -selective radiotracer PET ligands may be used to stratify patients for recruitment into clinical trials (i.e., patients with early versus later stages of PD), in order to determine the safety and efficacy of $A_{2A}AR$ antagonists in this patient population.

The above mentioned development of the selective $A_{2A}AR$ antagonist istradefylline (KW-6002) as a nondopaminergic drug for PD (Kase et al. 2003) is another good example of the usefulness of PET imaging in the process of drug development. In a study of healthy subjects, seven groups received doses of cold istradefylline ranging from 0 to 40 mg per day for 14 days (Brooks et al. 2008). Thereafter, ^{11}C -labeled istradefylline ([^{11}C]KW-6002) and PET were applied in order to determine the binding potential of [^{11}C]KW-6002. Estimates of the striatal binding potential were used to derive saturation kinetics in the presence of cold KW-6002, assuming that nonspecific binding was constant across subjects and the binding potential was proportional to the concentration of available $A_{2A}AR$ binding sites.

Brain [^{11}C]KW-6002 uptake was well characterized by a two-tissue compartmental model with a blood volume term, and the 50% efficient dose (ED_{50}) of cold KW-6002 was 0.5 mg in the striatum. The study revealed that over 90% receptor occupancy was achieved with daily oral doses of greater than 5 mg.

5 Conclusion

Both basic neuroscience and clinical research have established substantial evidence for an important role of adenosine and its receptors in the pathophysiology of the brain. Molecular *in vivo* imaging of ARs in the human brain is therefore an attractive means to study the role of adenosine, its receptor subtypes and their alterations under disease conditions in patients suffering from neurologic and psychiatric disorders, sleep disorders, and perhaps drug addiction. The first two high-affinity and subtype-selective AR ligands dedicated for use in PET, [^{18}F]CPFPX and [^{11}C]MPDX permit quantitative measurements of A_1ARs in the living human brain. The clinically important $\text{A}_{2\text{A}}\text{AR}$ has been made accessible through the use of [^{11}C]TMSX and [^{11}C]KW-6002, a radiolabeled drug. Reports on human applications are currently focused on A_1ARs and $\text{A}_{2\text{A}}\text{ARs}$, reflecting current understanding of their specific implications in cerebral neuropathology and their potential as neuroprotective targets. Regarding $\text{A}_{2\text{B}}\text{ARs}$ and A_3ARs , their relatively low densities and their disease-specific appearance make it more challenging to assess them *in vivo*. However, given that it is now clear that adenosine plays a greater role in the pathophysiology of neurological and psychiatric disorders than previously thought, and the systematic and intensive search that is now underway for ligands with high affinity and selectivity, the molecular imaging of ARs will become an increasingly important tool in clinically oriented research.

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