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International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and Classification of Adenosine Receptors—An Update

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***Abstract*——In the 10 years since our previous International Union of Basic and Clinical Pharmacology report on the nomenclature and classification of adenosine receptors, no developments have led to major changes in the recommendations. However, there have been so many other developments that an update is needed. The fact that the structure of one of the adenosine receptors has recently been solved has already led to new ways of in silico screening of ligands. The evidence that adenosine receptors can form homo- and heteromultimers has accumulated, but the functional significance of such complexes remains unclear. The availability of mice with genetic modification of all the adenosine receptors has led to a clarification of the functional roles of adenosine, and to excellent means to study the specificity of drugs. There are also interesting associations between disease and structural variants in one or more of the adenosine receptors. Several new selective agonists and antagonists have become available. They provide improved possibilities for receptor classification. There are also developments hinting at the usefulness of allosteric modulators. Many drugs targeting adenosine receptors are in clinical trials, but the established therapeutic use is still very limited.**

I. Introduction

The Nomenclature Committee of the International Union of Basic and Clinical Pharmacology has had a subcommittee dealing with nomenclature and classification of adenosine receptors for more than 20 years, and two reports from the committee have previously been published in this journal—the first one dealing with receptors for both adenosine and nucleotides (Fredholm et al., 1994), the second with adenosine receptors only (Fredholm et al., 2001a). Although there have been rapid recent developments in the field of adenosine receptor signaling, most of what was stated in these early reviews remains valid, and the reader is referred to those texts for older data and a historical perspective. In

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the present update we focus on new aspects and recapitulate the older information only when absolutely needed for reference (for example as reference compounds for comparison with newer drugs).

There are four adenosine receptors among vertebrates, which have been denoted adenosine A_1, A_{2A}, A_{2B} and A_3 receptors (Fredholm et al., 2001a). Adenosine is a full agonist at all these receptors, and at A_1 and A_3 receptors, inosine can act as a partial agonist in functional assays (Jin et al., 1997; Fredholm et al., 2001b). There is no good evidence that adenine nucleotides can act on adenosine receptors without being degraded to nucleosides first. However, such breakdown is extremely rapid and efficient in most cells and tissues even when using so called "stable" ATP analogs, which rarely are stable in biological preparations. Thus, there is no reason to modify the recommended nomenclature.

To assess the roles of these receptors we must consider how the concentration of the endogenous agonist is regulated. There has been much progress in this field in recent years. Adenosine is known to take part in several different metabolic pathways, and intracellular concentrations of adenosine can never be zero. Furthermore, most, if not all, cells possess equilibrative nucleoside transporters (King et al., 2006). Therefore, there will be, by necessity, a finite level of adenosine in the extracellular space, even under the most basal conditions. This basal level has been estimated to be in the range of 30 to 200 nM (Ballarín et al., 1991). From this baseline level, adenosine can increase substantially via two mechanisms: formation intracellularly and export via transporters, and formation in the extracellular space from adenine nucleotides released from cells. The earlier literature on adenosine emphasized the former pathway (Newby, 1984), but more recently, interest has centered on the contribution of ATP as an important source of extracellular adenosine. Whereas the focus here was initially on the release of ATP as a neurotransmitter, stored together with other transmitters (Burnstock, 2006), several other mechanisms have now moved to the foreground. Among the other sources of extracellular adenosine to be considered are:

1. Cells with damage to the cell membrane [e.g., in necrotic or apoptotic (Elliott et al., 2009) cell death]. This can generate very high levels locally because intracellular ATP levels are 4 to 5 orders of magnitude higher than extracellular levels.
2. Storage vesicles containing hormones (and transmitter) also by so called kiss-and-run release, which generates ATP release nonsynchronously with hormone (neurotransmitter) release (MacDonald et al., 2006; Zhang et al., 2009).
3. Connexin/pannexin "hemichannels" (Spray et al., 2006).
4. Other channels, including maxi-anion channels, volume-regulated anion channels, or P2X7 receptor channels.
5. Transport vesicles delivering proteins to the cell membrane (Lazarowski et al., 2003).
6. A subset of lysosomes (Zhang et al., 2007).

It is well known that ATP is released from many cells in which cell membranes are subjected to stretch (Okada et al., 2006), perhaps via one of the abovementioned mechanisms. Once ATP (or ADP) is released, the phosphate groups of extracellular ATP/ADP are rapidly split off by ectoenzymes working in concert, first via nucleoside triphosphate diphosphohydrolases (NTDPases; e.g., CD39) (Robson et al., 2005), followed by hydrolysis via ecto-5′-nucleotidase, CD73 (Picher et al., 2003). Knockouts of these enzymes have revealed their importance in contributing to extracellular adenosine in different organs and situations (see Table 1).

The potency of adenosine at these receptors is obviously determined by the affinity of the endogenous ligand (adenosine) to the different receptors. Unfortunately, it is exceedingly difficult to determine this affinity, because adenosine is rapidly metabolized and rapidly formed in biological preparations, including membrane preparations. Therefore, if metabolism of adenosine is prevented, endogenous adenosine accumulates to confound the measurements. This endoge-

TABLE 1
Some genetically modified animals useful to delineate adenosine receptor functions

Gene Targeted	Comment	References
AdoRA1	Major portion of coding exon 2 + ~5 kb adjacent 3′ genomic seq; not lethal.	Johansson et al., 2001
	3′ portion of coding exon 1 + intron 5′ portion of coding exon 2, not lethal	Sun et al., 2001
AdoRA2A	Entire coding exon 2, not lethal	Ledent et al., 1997
	3′ portion of coding exon 2 ~1.0 kb immediate intron seq, not lethal	Chen et al., 1999, 2001a
AdoRA2B	Replacement of exon 1 of the A_{2B}AR with a reporter construct containing the *β-gal* gene; not lethal.	Yang et al., 2006
	Replacement of part of the intron and the entirety of exon 2 (amino acids 113–332) with a marker gene; not lethal.	Hua et al., 2007b
AdoRA3	Entire coding exon 1 + 7.5 kb immediate intron seq; not lethal	Salvatore et al., 2000
Adenosine kinase	In frame insertion at exon amino acid Gly169-Thr225; die at P4	Boison et al., 2002
Adenosine deaminase	In frame insertion at exon 5; perinatal death Die at 3 weeks after trophoblast rescue	Blackburn et al., 1998
CD39	Part of exon 1; not lethal.	Enjyoji et al., 1999
CD73	Entire exon 3 + intronic sequences; not lethal.	Thompson et al., 2004
	Entire exon 2; not lethal.	Koszalka et al., 2004

kb, kilobase(s).

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nous adenosine can be cryptically bound, especially to receptors coupling to G_i proteins, and also influences the apparent B_{max} values (Parkinson and Fredholm, 1992). Attempts have been made to assess the affinity of endogenous adenosine at A_1 receptors by examining the binding of another agonist in the presence or absence of adenosine deaminase (Cohen et al., 1996), but adenosine deaminase is a low-K_m enzyme, and A_1 receptors may also interact with it in other ways (e.g., Saura et al., 1996), and the estimate is uncertain. There have also been other attempts to indirectly estimate the binding affinity of adenosine, including an attempt to partly denature the receptor by urea and disrupting interactions with the G protein (May et al., 2005). Although binding of an antagonist was not markedly disrupted by treatment with urea, it is not certain that the same is true for an agonist. It is also not immediately obvious whether binding in the absence of GTP (which of course can never happen in vivo) or in its presence should be used to estimate the potency of agonists. Hence, we do not have truly reliable binding data on the comparative affinity of the endogenous agonist at the four adenosine receptors and must rely on determination of the potency of adenosine in functional assays (see an estimate based on functional studies in Table 3).

This introduces another important confounding factor: potency of the agonist is markedly influenced by the receptor number (Arslan et al., 1999; Johansson et al., 2001). The reason for this is that adenosine receptors are generally coupled via several amplification steps to the final response, and they therefore exhibit the behavior described by pharmacologists as "spare receptors." In such systems, alterations in receptor number are manifested by parallel shifts in the dose-response curve, not as alterations in the maximal response (except when receptor number or agonist efficacy are very low, when a further decrease causes a drop in maximum response). When one compares ligand potencies to modulate cAMP levels at comparative receptor densities, it is observed that adenosine is nearly equipotent at A_1, A_{2A}, and A_3 receptors but is some 50 times less potent at A_{2B} receptors (Fredholm et al., 2001b). If, by contrast, we instead examine the ability to activate mitogen-activated protein kinase (which all the adenosine receptors do), adenosine is equipotent at all of these (Schulte and Fredholm, 2000). Moreover, the expression levels of all the adenosine receptors is transcriptionally regulated and can change rapidly and dramatically in response to various stimuli (e.g., see Murphree et al., 2005). Thus, the potency of endogenous adenosine depends on receptor number and on the type of response measured. Hence, there is no rationale to divide the receptors into high- and low-affinity receptors as is frequently done.

II. Receptor Structure

The crystal structure of the adenosine A_{2A} receptor was recently determined (Jaakola et al., 2008). It is one of the very first "snapshots" of a GPCR[1] architec-

[1]Abbreviations: 2-AG, 2-arachidonylglycerol; AIA, aspirin-intolerant asthma; ATL146e, apadenoson [*trans*-4-{3-[6-amino-9-(*N*-ethyl-β-D-ribofuranosyluronamide)-9*H*-purin-2-yl]prop-2-ynyl}cyclohexanecarboxylic acid methyl ester]; ATL 802, *N*-(5-(1-cyclopropyl-2,6-dioxo-3-propyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)pyridin-2-yl)-*N*-methyl-6-(trifluoromethyl)nicotinamide; BAY 60-6583, 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide; BAY 68-4986, capadenoson [2-amino-6-({[2-(4-chlorophenyl)-1,3-thiazol-4-yl]methyl}sulfanyl)-4-[4-(2-hydroxyethoxy)phenyl]pyridine-3,5-dicarbonitrile]; BiFC, bimolecular fluorescence complementation; BG9228, 3-(4-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-bicyclo(2.20.2)oct-1-yl)propionic acid; BIIB014, vipadenant [3-(4-amino-3-methylbenzyl)-7-(2-furyl)-3*H*-[1,2,3]triazolo[4,5-*d*]pyrimidine-5-amine]; BRET, bioluminescence resonance energy transfer; CF102, Cl-IB-MECA; CGS 21680, 2-[*p*-2-(carbonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; CGS15943, 5-amino-9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazoline; CHO, Chinese hamster ovary; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; CPA, N^6-cyclopentyladenosine; CPFPX, 8-cyclopentyl-1-propyl-3-(3-fluoropropyl)xanthine; CV-3146, regadenoson [2-(4-((methylamino)carbonyl-1*H*-pyrazol-1-yl)adenosine]; CVT-6883, 3-ethyl-1-propyl-8-(1-(3-trifluoromethylbenzyl)-1*H*-pyrazolo-4-yl)xanthine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; DTI-0009, selodenoson [N^6-cyclopentyl-5′-(*N*-ethyl)carboxamidoadenosine]; DU124183, 2-cyclopentyl-4-phenylamino-1*H*-imidazo-[4,5-*c*]quinoline; FK-453, *R*-[(*E*)-3-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl)acryloyl]-2-piperidine ethanol; FRET, fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; GR79236, *N*-((1*S*,*trans*)-2-hydroxycyclopentyl)adenosine; GS9667 (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; GW493838, (2*R*,3*S*,4*R*,5*R*)-2-((5-(*tert*-butyl)-1,3,4-oxadiazol-2-yl)methyl)-5-(6-((4-chloro-2-fluorophenyl)amino)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol; HEK, human embryonic kidney; IB-MECA, N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine (CF101); KW-3902, rolofylline [1,3-dipropyl-8-(3-noradamantyl)xanthine]; KW6002, istradefylline [(*E*)-1,3-diethyl-8-(*m*,*p*-dimethoxystyryl)xanthine]; LAS38096, 2-amino-4-(2-furanyl)-5-(4-pyrimidinyl)pyrimidine; LPS, lipopolysaccharide; LUF6000, *N*-(3,4-dichlorophenyl)-2-cyclohexyl-1*H*-imidazo-[4,5-*c*]quinolin-4-amine; LUF6096, *N*-{2-[(3,4-dichlorophenyl)amino]quinoline-4-yl}cyclohexanecarboxamide; LUF6258, N^6-[2-amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-6-yl-9-nonyloxy-4-phenyl]adenosine; mGlu, metabotropic glutamate; MRE-0094, sonedenoson (2-[2-(4-chlorophenyl)ethoxy]adenosine); MRE-2029-F20, *N*-benzo[1,3]dioxol-5-yl-2-(5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydropurin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy)acetamide; MRE-3008-F20, 5-[[(4-methoxyphenyl)amino]carbonyl]amino-8-propyl-2-(2-furyl)pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine; MRS1191, 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl 5-(phenylmethyl) ester; MRS1220, *N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-yl]benzene acetamide; MRS1523, 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate; MRS1754, *N*-(4-cyanophenyl)-2-(4-(1,3-dipropylxanthin-8-yl)phenoxy)acetamide; MRS592, 2-chloro-N^6-(3-iodobenzyl)-adenosine; MSX-2, 3-(3-hydroxypropyl)-7-methyl-1-propargyl-8-(*m*-methoxystryryl)xanthine; MSX-3, phosphoric acid mono-(3-{8-[2-(3-methoxy-phenyl)-vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl}-propyl) ester; MSX-4, L-valine-3-{8-(*E*)-2-[3-methoxyphenyl)ethenyl]-7-methyl-1-propargylxanthine-3-yl}propyl ester hydrochloride; NAM, negative allosteric modulator; NECA, 5′-*N*-ethylcarboxamidoadenosine; nt, nucleotide(s); PAM, positive allosteric modulator; PD120,918, 4-methyl-2-oxo-2*H*-chromen-7-yl methylcarbamate; PD81,723, (2-amino-4,5-dimethyl-3-thienyl)(3-(trifluoromethyl)phenyl)-methanone; PSB-10, 8-ethyl-4-methyl-2-(2,3,5-trichlorophenyl)-(8*R*)-

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ture after structural determinations of rhodopsin and β_1- and β_2-adrenergic receptors (for review, see Rosenbaum et al., 2009).

A. Engineered Receptors

Except for rhodopsin, wild-type GPCRs tend to be too thermolabile to allow prolonged solubilization, purification, and X-ray studies. Magnani et al. (2008) elegantly addressed this issue by mutating the adenosine A_{2A} receptor sequentially to improve its thermostability. They managed to create relatively stable receptor variants with up to five amino acid substitutions, albeit with different patterns for either agonist or antagonist binding. A further obstacle is the receptors' inherent conformational flexibility and heterogeneity (especially upon agonist binding). Thus, Jaakola et al. (2008), in an approach different from that of Magnani et al. (2008), engineered the adenosine A_{2A} receptor to improve on these aspects by the insertion of the very stable lysozyme protein from T4 bacteriophage (T4L) for most of the third intracellular loop (Leu209–Ala221), and by deletion of the larger part of the very flexible C terminus (Ala317–Ser412). This construct (A_{2A}-T4L-ΔC) was further stabilized during purification with high concentrations of sodium chloride and cholesterol and a receptor-saturating concentration of the antagonist theophylline, which was replaced by the high-affinity 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) in the final purification step.

B. Receptor Crystallization

Crystallization was achieved through an in meso crystallization approach, in which the lipid phase consisted of a mixture of mono-olein and cholesterol. A total of approximately 100 crystals was obtained, of which the 13 best yielded diffraction data good enough for a 2.6-Å resolution. In Fig. 1A, a representation of the receptor structure (without T4L) is shown, with a closer look at the ligand binding site in Fig. 1B. The final refined structure thus refers to a fusion protein of the adenosine A_{2A} receptor with T4L (including amino acid residues Ile3 to Gln310 of the receptor and residues 2 to 161 of T4L), a number of lipids modeled as stearic acid molecules, sulfate ions, ordered water molecules, and the antagonist ZM241385. At some parts of the polypeptide chain, the weak electron density did not allow a precise structure determination. This was true for the first two amino acids of the receptor (Met1–Pro2), amino acids Glu311 to Ala316 in the carboxyl tail, and the tip of the second extracellular loop (Gln148–Ser156). Unlike the two β-adrenergic receptors, the A_{2A} receptor structure did not provide evidence for the presence of cholesterol molecules.

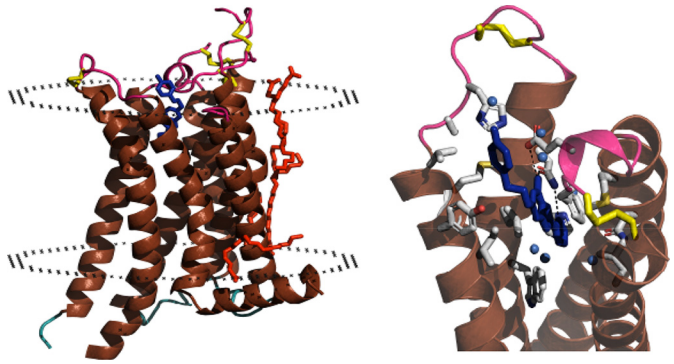

FIG. 1. Left, crystal structure of the adenosine A_{2A} receptor. Brown, α-helical elements; dark blue, ZM241385; yellow, disulfide bridges; pink, extracellular domain; red, stearic acid molecules; and light blue, intracellular domain. Membrane boundaries are represented as ellipsoids. Right, ligand binding site of the adenosine A_{2A} receptor. Brown, four transmembrane domains (helices 3, 5, 6, and 7); dark blue, ZM241385; blue spheres, explicit water molecules; pink, extracellular elements; yellow, disulfide bridges; and atom-coded colors, amino acid side chains involved in ligand binding.

C. Pharmacological Characterization

Such a highly engineered receptor construct necessitated a thorough pharmacological characterization with respect to signaling and ligand binding properties. Signaling, measured as modulation of cAMP production, was completely abrogated, most probably because of the insertion of T4L in the third intracellular loop of the receptor. Compared with the wild-type receptor, the construct displayed virtually identical affinity for the antagonist ZM241385 in radioligand binding studies, whereas agonist affinity was somewhat higher than observed for the wild-type construct. High sodium chloride concentrations, essential for the generation of crystals, did not affect antagonist affinity, whereas agonist affinity was reduced to a similar value for wild-type and engineered receptor, in line with earlier observations (Gao and IJzerman, 2000). This suggests that the antagonist-binding site in the crystal structure was not affected by the substantial modifications to the receptor protein.

4,5,7,8-tetrahydro-1*H*-imidazo[2.1-*i*]purin-5-one; PSB-11, 8-ethyl-4-methyl-2-phenyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2.1-*i*]purin-5-one; PSB-1115, 1-propyl-8-*p*-sulfophenylxanthine; PSB-36, 1-butyl-8-noradamant-3-yl-3-(3-hydroxypropyl)-3,7-dihydropurin-2,6-dione; PSB-603, 8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine; SAR, structure-activity relationship; SCH-420814, preladenant [2-(furan-2-yl)-7-(2-{4-[4-(2-methoxyethoxy)phenyl]piperazin-1-yl}ethyl)-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine]; SCH-442416, 2-(2-furyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine; SCH-58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]-pyrimidine; SLV320, 4-(4-hydroxycyclohexylamino)-6-phenylpyrrolo[2,3-*d*]pyrimidine; SNP, single nucleotide polymorphisms; ST-1535, 2-butyl-9-methyl-8-[1,2,3]triazol-2-yl-9*H*-purin-6-ylamine; SYN-115, 4-hydroxy-4-methylpiperidine-1-carboxylic acid-(4-methoxy-7-morpholin-4-ylbenzothiazol-2-yl)amide; T4L, lysozyme protein from T4 bacteriophage; T-62, 2-amino-3-(4-chlorobenzoyl)-5,6,7,8-tetrahydrobenzothiophene; VUF5455, 4-methoxy-*N*-(7-methyl-3-(2-pyridinyl)-1-isoquinolinyl)benzamide); VUF-5574, *N*-(2-methoxyphenyl)-*N*-[2-(3-pyridyl)quinazolin-4-yl]urea; WRC-04702, binodenoson [2-((cyclohexylmethylene) hydrazino)adenosine]; YFP, yellow fluorescent protein; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol.

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D. Structural Characteristics

1. The Ligand Binding Site. The A_{2A} receptor structure is quite different from the other GPCRs for which crystal structures are available. First, ZM241385 binds in an extended conformation protruding into the extracellular domain, whereas the ligand-binding site of β-receptor antagonists seems to reside within the transmembrane domain. Therefore, the amino acids interacting with the ligand are found mainly on helices 3, 5, 6, and 7 in the transmembrane domain as well as in extracellular loops 2 and 3 (see also Fig. 1B). Second, the architecture of the extracellular loops is markedly different, especially for the second one. It is best described as a spatially constrained random coil with three disulfide bridges to the first extracellular loop. Two of these are unique to the adenosine A_{2A} receptor, and the third one is conserved among virtually all class A GPCRs. An early mutagenesis study (Kim et al., 1996) predicted this. The second extracellular loop also holds a very short helical segment from which two amino acids (Phe168 and Glu169) have strong ligand interactions. The third extracellular loop has a fourth (intraloop) disulfide bridge. This extensive network of disulfide bridges yields a quite rigid but open architecture that might allow relatively unhindered access of ligand molecules.

2. "Toggle Switch" and "ionic Lock" The relative position of the seven transmembrane domains is somewhat different from the other receptor structures, most notably for helices 1 and 2, with a shift of ≤ 7 Å at the extracellular boundaries of the helices compared with the β-adrenergic receptors. Consequently, features that were generalized from, for example, the rhodopsin transmembrane structure, need to be reassessed for each new receptor structure. The conserved tryptophan residue in helix 6 (Trp246 or Trp6.48—the residue at the bottom of the cavity in Fig. 1B) has been proposed as a "toggle switch" between an active and inactive receptor state. This assumption is based largely on the position of retinal in rhodopsin, where it is near the tryptophan residue, keeping rhodopsin in an inactive form. However, in neither the β-adrenergic nor the adenosine A_{2A} receptors is this contact area between ligand and amino acid very prominent (see also Fig. 1B), hence casting some doubt on the unique role of Trp 6.48 in receptor activation. A similar generalization from the rhodopsin structure regards the so-called "ionic lock," the strong hydrogen bonding network between the conserved E/DRY motif at the cytoplasmic side of helix 3 and a glutamate residue in helix 6. This structural motif was proposed to restrain the receptor in its inactive form but takes alternative forms in the other receptor structures. In the adenosine A_{2A} receptor, Asp101 (D in DRY) forms a hydrogen bond with Tyr112 in a helical segment of the second intracellular loop and with Thr41 at the bottom of helix 2.

E. Receptor Structure and Receptor Homology Modeling

Overall, the findings described above suggest that the format of the ligand binding cavity may vary considerably between receptors. This caveat was firmly illustrated by a recent modeling assessment with the aim to evaluate GPCR structure prediction and ligand docking attempts (Michino et al., 2009). Before the release of the A_{2A} receptor crystal structure into the public domain, 29 research groups submitted more than 200 receptor models that were evaluated for overall protein architecture and their quality with respect to ligand docking. Whereas the transmembrane domains of the receptor were reasonably well aligned (usually within 4 Å root-mean-square deviation), the ligand-binding domain and the structure of the second extracellular loop were not well predicted at all. The majority of models had root-mean-square deviation values of more than 10 Å for the ligand binding site, and only one predicted the typical perpendicular orientation of ZM241385 halfway the extracellular and transmembrane domains. The reasons for this generally large divergence were implicitly examined by Ivanov et al. (2009). The authors noted that domain knowledge such as mutagenesis data yielded useful constraints when docking ZM241385 into a receptor homology model. The inclusion of the ordered water molecules from the crystal structure into the receptor model proved equally important to obtain acceptable ligand poses. From a different perspective, Mobarec et al. (2009) concluded that based on sequence identity the currently available structures may only provide reasonable homology models for a small minority of class A GPCRs. In two recent publications, new ligands interacting with the adenosine A_{2A} receptor were identified with the crystal structure as the template (Carlsson et al., 2010; Katritch et al., 2010). In a virtual, computer-based screening approach, millions of commercially available molecules were automatically docked into the ZM241385 binding site and ranked according to interaction energies. On the basis of these results, dozens of compounds were purchased and tested in radioligand binding studies on the adenosine receptors with a remarkably high "hit rate" of 30 to 40%. These included completely new chemotypes.

III. Receptor Oligomerization

Adenosine receptors, like other class A GPCRs, have long been thought to exclusively occur in a monomeric state. Monomeric receptors are sufficient to induce signaling (Chabre and le Maire 2005 Whorton et al., 2007, 2008, White et al., 2007). At least some studies suggest signaling via dimers occurs only at higher receptor densities (White et al., 2007). More recently, however, evi-

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dence is accumulating that adenosine receptors can form dimeric or, more generally speaking, multimeric or oligomeric structures. Through self-association, homo-oligomers ("homomers") can be formed. Hetero-oligomerization leading to "heteromers" may be the consequence of the association between adenosine receptors and preferred partners, mostly other GPCRs, including other adenosine receptor subtypes. This phenomenon has been demonstrated through a variety of experimental techniques, mostly in artificial cell lines. The use of overexpressed recombinant receptors may result in the creation of many more oligomers than naturally exist. In some cases, BRET may detect interactions between receptors that are in close proximity in a compact structure such as a coated pit, rather than forming true molecular dimers. Furthermore, GPCRs contain hydrophobic regions that can oligomerize, even after solubilization in SDS. Hence, receptor dimerization or oligomerization may occur after solubilization in detergent and not be representative of receptor structure and organization in the membrane.

Thus, it remains to be seen how relevant the findings are for (adenosine) receptors in their natural environment (i.e., primary cells and tissues in an intact animal or human body). This latter concern, which applies to all GPCRs, was eloquently brought forward by an ad hoc International Union of Basic and Clinical Pharmacology committee, which suggested two of the three following criteria to be fulfilled before accepting a given oligomer to be of physiologic significance (Pin et al., 2007):

- physical association of the receptor complex in native tissue or primary cells, indicated by colocalization in subcellular compartment (preferably with oligomer-specific receptor antibodies); energy transfer technologies [FRET, BRET, bimolecular fluorescence complementation (BiFC)] in native tissue (labeled ligands or antibodies);
- definition of a specific functional property of the receptor complex (e.g., allosteric modulation of one monomer by the other or activation of a particular signaling pathway); and
- occurrence in vivo (knockout animals, RNA interference technology, etc.).

Another initiative, although not linked to the International Union of Basic and Clinical Pharmacology, summarized similar criteria and recommendations along with further definitions for nomenclature, which we use here (Ferré et al., 2009). Before we discuss available evidence, it should be stressed that for the adenosine receptors, the criteria mentioned above have not yet been fully met. It should also be emphasized that interactions between different receptors on downstream signaling events do not constitute proof of interactions at the receptor level.

A. Adenosine Receptor Homomers

In theory, four homomeric pairs can be envisaged for adenosine receptors: A_1-A_1, A_{2A}-A_{2A}, A_{2B}-A_{2B}, and A_3-A_3. So far, only reports with experimental evidence for the occurrence of A_1-A_1 and A_{2A}-A_{2A} homomers have been published.

1. A_1-A_1. Two early reports hinted to A_1 receptor homodimerization. Both Ciruela et al. (1995) and Yoshioka et al. (2002) used (different) antibodies against the wild-type adenosine A_1 receptor to note that immunoprecipitation experiments analyzed with Western blotting revealed higher order bands in some instances (e.g., in HEK293 cells expressing the human adenosine A_1 receptor, but also in brain tissues). In a more recent publication (Suzuki et al., 2009), these findings were corroborated and extended with differently tagged receptors expressed in HEK293T cells. Both hemagglutinin- and myc-tagged adenosine A_1 receptors were used in coimmunoprecipitation experiments, providing evidence for both monomeric and dimeric structures. It is notable, however, that even after solubilization in SDS, adenosine receptors and many other GPCRs form aggregates upon heating. This indicates that homo- or heteroaggregates can form after detergent solubilization and may be artifacts. Green fluorescent protein- and *Renilla reniformis* luciferase-tagged receptors enabled BRET experiments to be performed in which bioluminescence signals were indicative for receptor-receptor association among overexpressed recombinant receptors. In another recent study, A_1-A_1 homomers, predominantly located at the cell surface, were identified with BiFC techniques in CHO cells expressing YFP-tagged receptors (Briddon et al., 2008).

2. A_{2A}-A_{2A}. The first evidence for A_{2A} receptor homodimerization was provided by Canals et al. (2004). The authors used both FRET and BRET techniques as well as immunoblotting to show that in transfected HEK293 cells, overexpressed recombinant adenosine A_{2A} receptors exist as both homodimers and monomers. They demonstrated, by means of cell surface biotinylation experiments, that after detergent solubilization, approximately 90% of the cell surface recombinant A_{2A}R species exists in the homodimeric form. The same pattern of dimer formation was observed for an engineered A_{2A} receptor lacking the C terminus, whereas this receptor mutant was no longer able to dimerize with the dopamine D_2 receptor (see section III.B.5). A_{2A} receptor homodimerization was also demonstrated with BiFC techniques by Vidi et al. (2008a), who also used a combination of FRET and BiFC techniques to demonstrate that recombinant adenosine A_{2A} receptors exist as higher order oligomers, consisting of at least three monomers, at the plasma membrane of differentiated neuronal cells (Vidi et al., 2008b). A similar conclusion was reached by Gandia et al. (2008), who combined BiFC with BRET techniques to detect the occurrence of aden-

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osine A_{2A} receptor oligomers with more than two monomers in HEK293 cells. In another recent study, recombinant A_{2A}-A_{2A} homodimers, predominantly located intracellularly, were identified with BiFC techniques in CHO cells expressing YFP-tagged receptors (Briddon et al., 2008).

B. Adenosine Receptor Heteromers

Available evidence points to the interaction of both adenosine A_1 and A_{2A} receptors with other GPCRs, whereas no direct data have been reported for adenosine A_{2B} and A_3 receptors.

1. A_1-A_{2A}. Ciruela et al. (2006) investigated the heteromerization of adenosine A_1 and A_{2A} receptors. The two receptors are colocalized in striatal glutamatergic nerve terminals, both pre- and postsynaptically. This was demonstrated in immunogold blotting and, after detergent solubilization, coimmunoprecipitation experiments. In HEK293 cells transfected with suitably tagged adenosine A_1 as well as A_{2A} receptors, evidence in BRET and TR-FRET experiments was found for a direct interaction between the two recombinant receptors. Radioligand binding studies in membranes of these HEK293 cells demonstrated that agonist binding to the adenosine A_{2A} receptor influenced the affinity of (*R*)-N^6-phenylisopropyladenosine for the adenosine A_1 receptor, but not vice versa. Ciruela et al. (2006) speculated that these findings constitute a mechanism for fine-tuning the release of glutamate by adenosine. BiFC techniques were used in a later study with CHO cells expressing both receptors fused with truncated YFP constructs, showing significant fluorescence at the plasma membrane and intracellularly for the recombinant A_1-A_{2A} heteromer (Briddon et al., 2008).

2. A_1-$P2Y_1$. The heteromerization of adenosine A_1 receptors with $P2Y_1$-purinergic receptors was studied by Yoshioka et al. (2001). The two detergent-solubilized recombinant receptors (hemagglutinin- and myc-tagged) were shown to coimmunoprecipitate in cotransfected HEK293T cells, unlike the negative control of A_1 and dopamine D_2 receptors. Colocalization was also demonstrated by double immunofluorescence experiments with confocal laser microscopy. This heteromerization had functional consequences. The affinities of the A_1 receptor agonist CPA and inverse agonist DPCPX were reduced in a [^{3}H]NECA radioligand binding assay, whereas the P2Y receptor agonist adenosine 5′-O-(2-thiodiphosphate) was capable of displacing the radioligand. Moreover, this agonist showed a concentration-dependent reduction of forskolin-stimulated cAMP accumulation in the cells that was sensitive to pertussis toxin and DPCPX. It is noteworthy that these findings were largely confirmed in/ex vivo, where the authors studied rat brain cortex, hippocampus, and cerebellum as well as primary cultures of cortical neurons (Yoshioka et al., 2002). In a later review, it was speculated that this heteromerization between adenosine A_1 and $P2Y_1$ receptor might be one of the mechanisms for the adenine nucleotide-mediated inhibition of neurotransmitter release (Nakata et al., 2005). However, the fact that the ATP-mediated inhibition of neurotransmitter release is eliminated in slices from A_1 knock-out mice suggests that a direct action at A_1 receptors after rapid breakdown is another likely explanation (Masino et al., 2002).

3. A_1-$P2Y_2$. A study similar to that described in section III.B.2 was done for the pair of adenosine A_1 and $P2Y_2$ receptors (Suzuki et al., 2006). Coimmunoprecipitation experiments of detergent-solubilized receptors provided evidence that these receptors directly associate in cotransfected HEK293T cells. Radioligand agonist but not antagonist binding at the A_1 receptor was significantly inhibited by high concentrations of the $P2Y_2$ agonist UTP in membranes from cotransfected cells. UTP also inhibited the reduction of forskolin-stimulated cAMP production by CPA in the cotransfected cells, again only at high concentrations. It is possible, however, that UTP can indirectly influence ATP and adenosine concentrations in membrane preparations (Lazarowski et al., 2000). The increase in intracellular Ca^{2+} levels induced by UTP via the $P2Y_2$ receptor was very much enhanced by the simultaneous addition of the adenosine receptor agonist NECA in the coexpressing cells. These results suggest either that heteromerization of these two receptors generates a unique protein architecture in which the simultaneous activation of the two receptors induces a differential effect on $A_1/G_{i/o}$ and $P2Y_2/G_{q/11}$ signaling or that monomeric receptors have additive effects of downstream signaling pathways.

4. A_1-D_1. Detergent-solubilized A_1 and D_1 receptors were shown to coimmunoprecipitate in cotransfected Ltk-fibroblast cells (Ginés et al., 2000). This interaction seemed specific, because no coimmunoprecipitation was detected between A_1 and D_2 receptors cotransfected in the same cells. The A_1–D_1 receptor coimmunoprecipitation was constitutive, in that it occurred in the absence of A_1 or D_1 receptor agonist, whereas D_1 receptor activation, but not that of A_1, led to disruption of the A_1–D_1 heteromer. In an earlier publication, Ferré et al., (1998), using the same cell system, found that the adenosine A_1 receptor agonist CPA induced a decrease in the proportion of radiolabeled dopamine D_1 receptors in the high affinity state, indicating possible interactions at the level of receptors or at the level of receptor-G protein coupling.

5. A_{2A}-D_2. The heteromeric pair of adenosine A_{2A} and dopamine D_2 receptor is probably the best studied combination. Hillion et al. (2002) performed double immunofluorescence experiments with confocal laser microscopy showing substantial colocalization of recombinant adenosine A_{2A} and dopamine D_2 receptors in cell membranes of SH-SY5Y human neuroblastoma cells stably transfected with human D_2 receptor as well as in cultured striatal cells. Heteromerization between the two detergent-solubilized receptors was demonstrated

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in coimmunoprecipitation experiments, for which membrane preparations were used from D_2 receptor-transfected SH-SY5Y cells and from mouse fibroblast Ltk^- cells stably transfected with the long form of the human D_2 receptor. In the latter case, the A_{2A} receptor (double-tagged with hemagglutinin) was transiently cotransfected. Similar studies were done by Kamiya et al. (2003) in HEK293 cells. Resonance energy transfer techniques (BRET and FRET) with suitably tagged receptors were used to demonstrate the same heteromerization in intact HEK293 cells (Canals et al., 2003; Kamiya et al., 2003). Heteromerization seemed to be constitutive and not ligand-induced, and involved the long C-terminal tail of the adenosine A_{2A} receptor (Canals et al., 2004), in contrast to A_{2A} receptor homomerization (see section III.A.2). BiFC technology to study A_{2A}-D_2 heteromers in a CAD neuronal cell background was introduced by Vidi et al. (2008a). Again, strong fluorescence signals were observed when the two tagged receptors were cotransfected. In contrast to the earlier studies, the level of heteromerization at the cell surface was influenced by (prolonged) incubation of ligands for the two receptors.

6. A_{2A}-CB_1 and A_{2A}-D_2-CB_1. There is one report on the heteromerization between A_{2A} and cannabinoid CB_1 receptors (Carriba et al., 2007). It was found that solubilized A_{2A} and CB_1 receptors coimmunoprecipitate from extracts of rat striatum, where they are colocalized in fibrillar structures. In cotransfected HEK293 cells, BRET measurements with tagged receptors provided evidence for a direct physical interaction in an A_{2A}-CB_1 receptor heteromer. In a later study, the combination of BiFC and BRET methods hinted at the oligomerization in HEK293 cells of this heteromer with the dopamine D_2 receptor (Navarro et al., 2008).

7. A_{2A}-D_2-$mGlu_5$. A combination of BiFC and BRET techniques and a newly developed sequential resonance energy transfer approach (Carriba et al., 2008) was vital in detecting higher order receptor heteromers in HEK293 cells, consisting of $mGlu_5$, D_2, and adenosine A_{2A} receptors (Cabello et al., 2009). By using a high-resolution immunogold technique, the authors showed the three receptors to codistribute within the plasma membrane of dendritic spines of striatal synapses. Glutamate receptors are constitutive dimers (Romano et al., 1996), and thus it may be that the $mGlu_5$ receptor homomer associates with the A_{2A}-D_2 receptor heteromer.

8. A_{2A}-D_3. Torvinen et al. (2005) used confocal microscopy to show that green fluorescent protein-tagged A_{2A} and D_3 receptors colocalize in the plasma membrane of transfected HeLa cells. Furthermore, FRET analysis provided evidence for heteromeric A_{2A}/D_3 receptor complexes. In radioligand binding studies on CHO cell membranes stably expressing the two receptors, the A_{2A} receptor agonist 2-[*p*-2-(carbonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS-21680) reduced the affinity of the high-affinity agonist binding state of [^{3}H]dopamine.

C. Adenosine Receptor Heteromerization with Other Proteins

There is some evidence that heteromerization may also occur between adenosine receptors and proteins from other superfamilies, in particular adenosine deaminase and calmodulin.

1. A_1-D_1 with Adenosine Deaminase. Torvinen et al. (2002) used mouse fibroblast Ltk^- cells transfected with human D_1 receptor cDNA (D_1 cells) and with both human D_1 and human A_1 receptor cDNAs (A_1D_1 cells). Confocal laser microscopy analysis showed a high degree of adenosine deaminase immunoreactivity on the membrane of the A_1D_1 cells but not of the D_1 cells. In double immunolabeling experiments in A_1D_1 cells and rat embryonic cortical neurons, a marked overlap in the distribution of the A_1 receptor and adenosine deaminase immunoreactivities and of the D_1 receptor and adenosine deaminase immunoreactivities was found.

2. A_{2A}-D_2 with Calmodulin. Navarro et al. (2009) further explored the observation that calmodulin binds to cytoplasmic domains of some GPCRs, including the dopamine D_2 receptor. The same interaction was now demonstrated with BRET technology for the adenosine A_{2A} receptor. The sequential resonance energy transfer method developed by the same research group (Carriba et al., 2008) provided proof for a physical interaction between the three recombinant partners (i.e., calmodulin and the A_{2A}-D_2 heteromer). BRET competition experiments suggested that calmodulin preferentially binds to C-terminal tail of the A_{2A} receptor in the heteromer.

D. Functional Consequences

There is thus very good evidence that recombinant adenosine receptors can, and do, form homo- and heteromultimers. It is less clear, however, whether this has important pharmacological consequences in intact tissues. In addition to the concerns raised in the beginning of this section, it can be pointed out that many studies that demonstrate functional interactions between receptors were performed at low levels of GTP (significantly lower than 1 mM, normally present in intact cells). Under such circumstances, G proteins can become limiting, and unphysiological receptor-receptor interactions may occur (Chabre et al., 2009). In a dimeric complex, both receptors may not always (or even rarely) be simultaneously active and signaling (Rovira et al., 2010). This implies that the signaling consequences can be very complex. Multiple interactions can potentially occur in a given cell. It is unclear which rules determine how partnerships are formed. Thus, the fact that adenosine receptors can form multimeric complexes of different sorts will necessitate much more work on signaling in intact cells in vivo.

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IV. Receptor Polymorphisms and Disease Susceptibility

Among the many natural receptor variants possible, only single-nucleotide polymorphisms (SNPs) have been reported for adenosine receptors. SNPs in the A_1 and $A_{2A}R$ only have been associated with disease susceptibility. Significant differences were noted between healthy persons and patients with aspirin-intolerant asthma (AIA) in ADORA1 SNP genotype frequencies for 1405C>T ($P = 0.001$) and A102A ($P = 0.013$). In the haplotype analysis, ht[C-T-G] ($P = 0.003$) and ht[A-C-G] ($P = 0.032$) in ADORA1 and ht[A-T] in ADORA2A ($P = 0.013$) were significantly associated with AIA. These findings suggest that adenosine might play a role in the development of AIA through interactions with the A_1 and A_{2A} receptors (Kim et al., 2009a).

Other variants in the 3′-untranslated region of the *ADORA1* gene were associated with changes in infarct size in patients with ischemic cardiomyopathy (Tang et al., 2007). DNA samples from 273 subjects with ischemic cardiomyopathy and 203 healthy control subjects were screened, revealing that three variants (nt 1689 C/A, nt 2206 Tdel, nt 2683del36) were linked to changes in infarct size. The first two variants were associated with a decrease in infarct size, the latter with an increase. All three disease-associated variants were predicted to alter mRNA secondary structure, suggesting these variants have functional significance. The authors also addressed the limitation of such association studies. First, the occurrence of the three variants mentioned above was relatively rare. Second, the selection of patients introduced some bias. The group of patients in this study had survived a myocardial infarction, which may have enriched the population of those who had the wild-type phenotype, whereas the entry requirement of an ejection fraction ≤35% in this study might have biased the data by excluding those with small infarcts. In addition, some of the patients in the group with coronary artery disease might have had multiple infarctions, leading to larger infarct sizes.

Data from clinical and behavioral pharmacological studies have also implicated adenosine receptors in anxiety behaviors. Subjects from 70 panic disorder pedigrees and 83 child-parent "trios" were genotyped at five SNPs in and near the *ADORA2A* gene and were analyzed for genetic linkage and association. Linkage analysis revealed elevated logarithm of odds scores for a silent substitution (1083C/T = 1976C>T, SNP-4) in the second coding exon. Analyses carried out by broadening the panic disorder phenotype to include agoraphobia continued to support linkage to *ADORA2A*. These findings provide evidence for a susceptibility locus for panic disorder, possibly including agoraphobia, either within the *ADORA2A* gene or in a nearby region of chromosome 22. (Hamilton et al., 2004). Alsene et al. (2003) and Childs et al. (2008) found a significant association between self-reported anxiety after caffeine administration and a number of polymorphisms on the A_{2A} receptor gene, among others the known 1976C>T and the new 2592C>Tins polymorphisms. In both genotypes, greater increases in anxiety after caffeine administration were reported. These studies show that an adenosine receptor gene polymorphism that had been associated with panic disorder before (Deckert et al., 1998) is also linked to anxiogenic responses to caffeine. There is also an association between adenosine levels (Rétey et al., 2005) and adenosine A_{2A} receptors (Rétey et al., 2007) and sleep. Thus, there exist genetic associations between adenosine deaminase and deep sleep and between variants in the A_{2A} receptor and the effects of caffeine on sleep and anxiety (Rétey et al., 2005, 2007). Hohoff et al. (2010) studied the 1976C>T polymorphism and other potentially functionally relevant variants in subjects with Parkinson's disease or anxiety traits. The results support an important role of $A_{2A}R$ variants in the pathogenesis of anxiety disorders, suggesting their use as vulnerability factors for increased susceptibility to these conditions.

Five SNPs within *ADORA2A* were associated with stopping methotrexate therapy as a result of adverse events in patients with rheumatoid arthritis. Analysis by adverse event type showed that the association was specific for gastrointestinal toxicity. No association was observed between *ADORA2A* and efficacy outcomes. Knowledge of the *ADORA2A* genotype may help to improve identification of patients at high risk of gastrointestinal toxicity with methotrexate (Hider et al., 2008).

High adenosine plasma levels and high expression of adenosine A_{2A} receptors are observed in patients with unexplained syncope and a positive head-up tilt test. The most common SNP in the *$A_{2A}R$* gene is c.1364 T>C. Patients with a CC genotype had a higher incidence of spontaneous syncopal episodes (Saadjian et al., 2009).

V. Genetically Modified Animals

In the previous review on adenosine receptors in this journal, we presented initial information on mice lacking A_{2A}, A_1, and A_3 receptors (Ledent et al., 1997; Chen et al., 1999; Salvatore et al., 2000; Johansson et al., 2001; Sun et al., 2001). Since then, A_{2B} knockout mice (Yang et al., 2006; Hua et al., 2007b), several mice that allow tissue-specific targeting (Scammell et al., 2003; Bastia et al., 2005; Huang et al., 2006), and double knockouts have been described (Yang et al., 2009a). In addition, several mice that are deficient in the enzymes that form or degrade adenosine have been used to characterize the physiological significance of the receptors. There have also been reports on the use of small interfering RNA constructs (Popescu and Popescu, 2007). These animal models are briefly presented in Table 1 as

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well as in several reviews, including Fredholm et al., 2005; Yaar et al., 2005; Jacobson, 2009.

Here we provide a brief summary of key events in the history of adenosine receptor cloning, global deletion, and targeted deletion that led to important new insights about receptor function. The first adenosine receptors to be cloned were the canine A_1 and A_{2A} receptors, identified on the basis of homology to previously cloned GPCRs (Maenhaut et al., 1990; Libert et al., 1991). This was followed by identification of the *$A_{2B}R$* gene (Rivkees and Reppert, 1992) and the *A_3R* gene (Zhou et al., 1992).

A. A_1R

Targeted disruption of the *A_1AR* gene resulted in mice that gained weight normally and had normal heart rate, blood pressure, and body temperature but showed signs of increased anxiety (Johansson et al., 2001). Electrophysiological recordings from hippocampal slices revealed that adenosine-mediated inhibition of excitatory glutamatergic neurotransmission was abolished and the analgesic effect of intrathecal adenosine was lost. The decrease in neuronal activity upon hypoxia was reduced. These experiments established the importance of the A_1R under pathophysiological conditions including painful stimulation and hypoxia.

B. $A_{2A}R$

The $A_{2A}R$ was the first to be deleted. This was accomplished using the mouse CD1 strain (Ledent et al., 1997). In $A_{2A}R(-/-)$ mice, caffeine, which normally stimulates exploratory behavior, became a depressant. Knockout animals scored higher in anxiety tests, and male mice were much more aggressive toward intruders. Blood pressure, heart rate, and platelet aggregation were all increased. The agonist CGS-21680 lost its pharmacological activity. A second $A_{2A}R(-/-)$ C57BL/6 line was used to demonstrate deleterious effects of adenosine signaling in the brain. Although adenosine production generally protects tissues from injury, Chen et al. (1999) demonstrated that mice lacking the $A_{2A}R$ had reduced brain infarction in response to occlusion of the middle cerebral artery. This C57BL/6 $A_{2A}R(-/-)$ mouse was also used to address a controversial aspect of $A_{2A}R$ signaling. In the striatum, the $A_{2A}R$ is largely coexpressed with dopamine D2 receptors, where it modulates dopaminergic activity. These and other findings led to suggestions that heterodimers might be required for receptor function. However, mice deficient in $A_{2A}Rs$ or D_2Rs revealed that either receptor can elicit behavioral and biochemical activity in the absence of the other (Chen et al., 2001). The $A_{2A}R$ deletion has also been moved onto a Balb/C genetic background and deposited at the Jackson Laboratory (C;129S-*Adora2a^{tm1Jfc}*/J; Bar Harbor, ME).

A targeting vector to insert *loxP* sites flanking the first coding exon of the *$A_{2A}R$* gene was created. This floxed construct was electroporated into (129X1/SvJX129S1/Sv)F1-*Kitl$^+$* derived R1 embryonic stem cells. Correctly targeted embryonic stem cells were injected into recipient blastocysts. Resulting chimeric male animals were back-crossed to wild-type C57BL/6J mice using a marker-assisted selection. These mice have been used to establish a colony of floxed $A_{2A}R$ mice at The Jackson Laboratory (B6;129-*Adora2a^{tm1Dyj}*/J). A2a(flox/flox) × LysM-cre mice were used to demonstrate a role for granulocyte $A_{2A}R$ signaling in a model of lipopolysaccharide (LPS)-induced lung injury (Reutershan et al., 2007). Targeted $A_{2A}R$ deletion reversed the ability of an $A_{2A}R$ agonist to inhibit LPS-induced neutrophil recruitment, cytokine release, and vascular leak. To investigate central $A_{2A}R$ signaling, mice with a floxed $A_{2A}R$ were crossed with CaMKIIα-Cre mice to delete $A_{2A}Rs$ in both striatal neurons and other forebrain structures (Bastia et al., 2005), or Dlx5/6-Cre mice to selectively delete $A_{2A}Rs$ in the striatum (Shen et al., 2008). Cocaine- and phencyclidine-induced psychomotor activities were enhanced when striatal receptors were selectively deleted, but attenuated when receptors were more globally deleted. These results indicate that $A_{2A}Rs$ in striatal and extrastriatal neurons exert opposing modulation of psychostimulant effects.

C. $A_{2B}R$

$A_{2B}R$ (−/−) mice also containing a β-galactosidase knockin were produced by Yang et al. (2006). These mice displayed moderate inflammation as a result of elevated basal plasma tumor necrosis factor-α and an exaggerated response to LPS challenge. Based on β-galactosidase expression, these mice were also used to demonstrate high expression of $A_{2B}Rs$ on type II alveolar epithelial cells in the lung (Cagnina et al., 2009). NECA-stimulated interleukin-6 production detected in macrophages from wild-type mice was absent in another line of $A_{2B}R(-/-)$ mice prepared by Deltagen (San Mateo, CA). Thus, $A_{2B}R$ signaling has both pro- and anti-inflammatory elements. The $A_{2B}R(-/-)$ mice produced by Deltagen along with mice lacking the other adenosine receptor subtypes were used to determine which adenosine receptor subtype is most responsible for eliciting myocardial preconditioning (Eckle et al., 2007). The results suggest a major contribution by $A_{2B}R$ signaling.

D. A_3R

Mice with targeted disruption of the A_3R differed from wild-type mice in failing to enhance antigen-stimulated mast cell degranulation in response to Cl-IB-MECA (Salvatore et al., 2000). It is notable, however, that functional A_3Rs seem to be absent from human mast cells. A_3R deletion was initially reported to protect the heart from ischemic injury (Guo et al., 2001), possibly by reducing myocardial mast cell degranulation in the mouse (Rork et al., 2008). However, a recent study using con-

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genic (C57BL/6) A3R(−/−) mice revealed a cardioprotective effect of A3AR activation (Ge et al., 2006).

E. Adenosine Production

The generation of mice with deletions in CD39 and CD73, ecto-enzymes involved in adenine nucleotide metabolism, have altered the prevailing view of the predominant pathways of extracellular adenosine production. Although ADP enhances and adenosine inhibits platelet aggregation, deletion of CD39 caused prolonged bleeding times (Enjyoji et al., 1999). This was ascribed to desensitization of platelet P2Y12 receptors as a result of persistently high levels of ADP. Because several other P2 receptors undergo rapid desensitization, the effects of CD39 deletion on P2-receptor-mediated activities is generally unpredictable because of opposing effects of elevated ATP levels and desensitization of P2 receptor signaling.

In many instances, the results obtained with receptor-blocking drugs and those observed in the genetically modified animals to determine the roles of the adenosine receptors are entirely consistent. However, this is not always the case. One reason is that the drugs may not be as selective as was thought, and indeed the use of genetically modified animals to test the selectivity of drugs is becoming a more and more established practice. Another reason for discrepancy is that drugs rarely achieve a complete blockade of an adenosine receptor for any length of time and complete elimination of a response and partial blockade may have very different consequences. Therefore, it is of interest that there are some examples in which heterozygous mice can be used to mimic phenotypes of long-term drug treatment (Björklund et al., 2008; Yang et al., 2009b).

A third scenario is that the genetic modification results in major adaptive changes, and there is a common conception that genetic elimination of one of the adenosine receptors should lead to up-regulation of one or more of the other adenosine receptors. There are few, if any, examples of this type of adaptation. Instead, completely different processes may show adaptive changes. However, this also seems generally to occur only rarely and minimally (Johansson et al., 2001, 2007b; Lukashev et al., 2003), but examples of strong adaptive effects also exist (e.g., Teng et al., 2008). Perhaps one should expect only such processes that are physiologically regulated by an adenosine receptor to be compensated for in a targeted receptor deletion. There is little pressure to induce adaptations of processes that occur very rarely during a lifetime. Furthermore, not all physiological processes need to or can be compensated for. It is therefore of some interest that very few examples of adaptive compensations in receptor knockout mice have been noted, but there are numerous examples in which possible adaptations have been insufficiently studied (Table 2).

VI. Receptor Classification: New Drugs

Selective agonists and antagonists for all four adenosine receptor subtypes are available (Müller, 2000a,b; Yan et al., 2003; Jacobson and Gao, 2006; Müller and Ferré, 2007; Elzein and Zablocki, 2008; Müller and Jacobson, 2011). Affinities of the compounds are listed in Tables 3 and 4, and structures are shown in Figs. 2 to 9. Data from radioligand binding studies are presented; however, in a few cases, only functional data are available. The physiological agonist adenosine (**1**; bold numerals refer to chemical structures in Tables and Figures) and its close derivatives 2-chloroadenosine (**2**) and NECA (**3**) are nonselective; however, they are weaker at A_{2B} and—with the exception of NECA—also at rat A_3 receptors than at the other subtypes (see Table 3). In contrast to adenosine, **2** and **3** are metabolically quite stable (e.g., toward adenosine deaminase). Note, however, the difficulties of using binding assays to assess the potency of adenosine and the impact of receptor density on agonist potency discussed in section I. [^{3}H]NECA has been used as a radioligand for all four receptor subtypes. We foresee a shift from radioligands to fluorescent ligands for screening in the future (e.g., Kecskés et al., 2010).

A large number of studies over the past 20 years or so have used quite selective receptor agonists and antagonists in a variety of biological systems. It is beyond the scope of an update on receptor nomenclature and classification to review this vast literature. A recent volume in the *Handbook of Experimental Pharmacology* (Wilson and Mustafa, 2009) can provide a convenient inroad to it.

A_1-selective adenosine derivatives typically feature a cycloalkyl or an aromatic (phenyl or phenylisopropyl) residue at the exocyclic amino group (compounds **4–14**). Some compounds are additionally modified at the C2- and/or ribose structure. Selectivity versus A_{2A} and A_{2B} is often very high in such N^6 derivatives, but some compounds are less selective versus the A_3 receptor subtype. If possible, the selectivity should be at least 100-fold for a tool compound used for receptor characterization. [^{3}H](*R*)-N^6-Phenylisopropyladenosine, [^{3}H]N^6-cyclohexyladenosine, and [^{3}H]2-chloro-N^6-cyclopentyladenosine are used as standard radioligands for A_1 receptors. Bayer Schering Pharma (Wuppertal, Germany) identified 2-aminodicyanopyridines, such as capadenoson (**15**), as the first class of non–adenosine-derived, non-nucleoside adenosine receptor agonists (see also Beukers et al., 2004; Chang et al., 2005). Several A_1 agonists and partial agonists, including (2*R*,3*S*,4*R*,5*R*)-2-((5-(*tert*-butyl)-1,3,4-oxadiazol-2-yl)methyl)-5-(6-((4-chloro-2-fluorophenyl)amino)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol (GW493838; **5**), selodenoson (DTI-0009; **11**), *N*-((1*S*,*trans*)-2-hydroxycyclopentyl)adenosine (GR79236; **12**), tecadenoson (**13**), (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

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TABLE 2
Selected effects mediated by adenosine via the four adenosine receptors
For this table we have predominantly used data from genetically modified organisms.

Receptor	Effect	Physiology/ Pathophysiology	Evidence for Adaptation	Reference
A_1	Decreased renal blood flow, Tubuloglomerular feedback	Phys	No	Brown et al., 2001; Sun et al., 2001
	Inhibition of lipolysis	Phys	No	Johansson et al., 2008
	Vasoconstriction	?	?	Tawfik et al., 2005; Wang et al., 2010
	Inhibition of neurotransmitter release	Extreme phys	No?	Johansson et al., 2001
	Inhibition of insulin/glucagon release	Phys	No	Johansson et al., 2007a
	Reduced heart rate	Phys	No?	Yang et al., 2007
	Osteoclast activation, bone resorption	Phys?	No?	Kara et al., 2010a,b
	Sleep	Phys	Yes	Stenberg et al., 2003; Oishi et al., 2008 Goldman et al. 2010
	Acupuncture	Extreme phys?	?	
	Prevention of epilepsy	Path	?	Fedele et al. 2006; Li et al. 2007; Kochanek et al 2006 Hua et al., 2007a
	Bronchoconstriction	Path	?	
	Analgesia	Extreme phys	No	Johansson et al., 2001; Wu et al., 2005
	Preconditioning	Path	No	Schulte et al., 2004; Lankford et al., 2006 Wendler et al. 2007
	Tolerance to hypoxia	Path	No?	
A_{2A}	Wakefulness, locomotion	Phys	No	Ledent et al., 1997; El Yacoubi et al., 2000; Chen et al., 2000; Huang et al., 2005
	Neurodegeneration (including Parkinson's disease and Alzheimer's disease)	Path	No	Chen et al., 1999, 2001b, 2007
	Immunosuppression	Extreme/Path	No?	Ohta and Sitkovsky, 2001; Lappas et al., 2005; Ohta et al., 2007
	Vasodilatation/hypotension	Phys/Extreme	No?	Ledent et al., 1997
	Coronary vasodilatation	Phys/Extreme	Yes?	Morrison et al., 2002
	Inhibition of platelet aggregation	Extreme	No?	Ledent et al., 1997
	Angiogenesis	Extreme	No?	Montesinos et al., 2002
A_{2B}	Vascular integrity	Phys/Extreme	No	Yang et al., 2006, 2008
	Preconditioning	Extreme	No	Eckle et al., 2007
A_3	Increased mast cell activation	Extreme/Path	No?	Salvatore et al., 2000; Tilley et al., 2000
	Airway contraction	Path	No?	Hua et al., 2008
	Inflammatory pain	Extreme/Path	No?	Wu et al., 2002
	White cell chemotaxis	Extreme/Path	No?	Chen et al., 2006

(GS9667/CVT-3619; **14**), and capadenoson (BAY 68-4986; **15**) have been or are currently being evaluated in clinical trials for various indications (see section VIII).

A_{2A}-selective agonists have been developed by introducing bulky substituents in the 2-position of the adenosine scaffold (compounds **16–22**). CGS-21680 (**16**) has been widely used for studying A_{2A} receptor activation and is used as a standard A_{2A} radioligand in its tritium-labeled form. It is A_{2A}-selective in rat but shows relatively high affinity for the human A_3 receptor and thus low selectivity for the human A_{2A} subtype (Table 3). Regadenoson (**22**; Lexiscan, CVT 3146) is clinically used as a diagnostic agent for pharmacological stress testing because of its vasodilatory effects. Apadenoson (ATL-146e; **17**) is being developed for the same purpose. Several other A_{2A}-selective agonists are being or have been clinically evaluated, including UK-432097 (**19**), sonedenoson (MRE-0094; **20**), and binodenoson (WRC-0470; **21**).

Because adenosine analogs generally have a low affinity at A_{2B} receptors, it has been much harder to develop agonists with selectivity for the A_{2B} than for the other receptor subtypes. Only two A_{2B}-selective agonists have been developed so far, the adenosine derivative **23** and the 2-aminodicyanopyridine derivative 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy) phenyl]pyridin-2-ylsulfanyl]acetamide (BAY 60-6583; **24**). Both compounds have yet to be broadly characterized. A selective agonist radioligand for A_{2B} receptors is currently not available.

The development of A_3-selective agonists has been quite successful by optimizing the substitution pattern of the adenosine scaffold in the N^6, the C2, and the 5′ positions. In the most selective compounds, the ribose moiety is replaced by a bicycloalkyl ring structure (**30**, **31**). The standard A_3 agonists are IB-MECA (**25**) and Cl-IB-MECA (**26**), the 2-Cl analog **26** being more selective versus A_1 receptors than **25**. Several A_3 agonists are undergoing clinical trials (**25**, **26**, **27**, **30**). Iodinated agonist radioligands are frequently used for labeling A_3 receptors, but recently, the first ^{3}H-labeled compound, [^{3}H]2-(1-hexynyl)-*N*-methyladenosine (**28**), has been developed.

The classic nonselective adenosine receptor antagonists are the xanthine derivatives caffeine (**32**) and theophylline (**33**), both of which are used therapeutically. Compared with many of the synthetic alkylxanthines, they are only

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TABLE 3
Adenosine receptor affinities of agonists

Most data for A_{2B} are from functional studies.

		K_i			
		A_1	A_{2A}	A_{2B}	A_3
		nM			
Nonselective agonists					
1	Adenosine[a]	~100 (h)[b] 73 (r)[b]	310 (h)[b] 150 (r)[b]	15,000 (h)[b] 5100 (r)[b]	290 (h)[b] 6500 (r)[b]
2	2-Chloro-adenosine	6.7 (r)[c]	76 (r)[c]	24,000 (h)[d]	1890 (r)[e]
3	NECA	14 (h)[b] 5.1 (r)[f]	20 (h)[b] 9.7 (r)[f]	140 (h)[b] 1890 (h)[g] 1900 (m)[f]	25 (h)[b] 113 (r)[f]
A_1-selective agonists					
4	*R*-PIA	2.04 (h)[h] 1.2 (r)[c]	220 (r)[c]	150,000 (h)[d] 19,000 (m)[i]	33 (h)[i] 158 (r)[e]
5	GW493838	N.D.	N.D.	N.D.	N.D.
6	CHA	0.85 (r)[c]	460 (r)[c]	160,000 (h)[d]	1025 (h)[k] 176 (r)[e]
7	CPA	2.3 (h)[l]	794 (h)[l]	18,600 (h)[l]	72 (h)[l]
8	CCPA	0.83 (h)[l] 1.3 (r)[m] 0.1 (rb)[m]	2270 (h)[l] 950 (r)[m]	18,800 (h)[l]	38 (h)[l] 237 (r)[m] 37.7 (rb)[m]
9	TCPA	2.8 (h)[n]	210 (h)[n]	>10,000 (h)[n]	600 (h)[n]
10	2′-Me-CCPA	1.8 (c)[o]	3900 (c)[o]	N.D.	5000 (r)[o]
11	Selodenoson (DTI-0009)	N.D.	N.D.	N.D.	N.D.
12	GR79236	3.1 (r)[l]	1300 (h)[l]	N.D.	N.D.
13	Tecadenoson	6.5 (p)[l]	2315 (h)[l]	N.D.	N.D.
14	GS9667 (CVT-3619)	55 (h)[p]	>10,000 (h)[p]	>50,000 (h)[p]	>1000 (h)[p]
15	Capadenoson (BAY 68-4986)	N.D.	N.D.	N.D.	N.D.
A_{2A}-selective agonists					
16	CGS21680	289 (h)[l] 1800 (r)[m] 120 (rb)[m]	27 (h)[l] 19 (r)[m]	>10,000 (h)[l] >10,000 (r)[m]	67 (h)[l] 584 (r)[m] 673 (rb)[m]
17	Apadenoson (ATL-146e)	77 (h)[l]	0.5 (h)[l]	N.D.	45 (h)[l]
18	ATL-313	N.D.	N.D.	N.D.	N.D.
19	UK-432097	N.D.	4 (h)	N.D.	N.D.
20	Sonedenoson (MRE-0094)	N.D.	N.D.	N.D.	N.D.
21	Binodenoson (WRC-0470)	48,000 (h)[l]	270 (h)[l]	430,000 (h)[l]	903 (h)[l]
22	Regadenoson (CV-3146)	>10,000 (h)[l]	290 (h)[l]	>10,000 (h)[l]	>10,000 (h)[l]
A_{2B}-selective agonists					
23	A_{2B} agonist	1050 (h)[q]	1550 (h)[q]	82 (h)[q]	>5000 (h)[q]
24	BAY 60-6583	>10,000 (h)[a,r]	>10,000 (h)[a,r]	3–10 (h)[r] 330 (m)[s] 750 (d)[s] 340 (rb)[s]	>10,000 (h)[a,r]
A_3-selective agonist					
25	IB-MECA (CF101)	51 (h)[l]	2900 (h)[l]	11,000 (h)[l]	1.8 (h)[l]
26	Cl-IB-MECA CF102	220 (h)[l] 280 (r)[m] 35 (m)[m]	5360 (h)[l] 470 (r)[m] ~10,000 (m)[m]	>10,000 (h)[m] >10,000 (m)[m]	1.4 (h)[l] 0.33 (r)[m] 0.18 (m)[m]
27	CP608,039	7300 (h)[t] 1750 (rb)[t]	N.D.	N.D.	5.8 (h)[t] 83 (rb)[t]
28	HEMADO	330 (h)[u]	1200 (h)[u]	>30,000 (h)[u]	1.10 (h)[u]
29	2-Phenylethynyl-adenosine derivative	32,800 (h)[v]	41,700 (h)[v]	>30,000 (h)[v]	0.44 (h)[v]
30	MRS3558 (CF502)	260 (h)[l] 105 (r)[m] 15.8 (m)[m]	2330 (h)[l] 1080 (r)[m] 10,400 (m)[m]	>10,000 (h)[l]	0.29 (h)[l] 1.0 (r)[m] 1.49 (m)[m]
31	MRS5151	14,900 (h)[w] 10,500 (m)[w]	~10,000 (h)[w] >10,000 (m)[w]	N.D.	2.38 (h)[w] 24.4 (m)[w]

h, human; c, cow; d, dog; m, mouse; p, pig; r, rat; rb, rabbit; *R*-PIA, (*R*)-N^6-phenylisopropyladenosine; N.D., no data available; CHA, N^6-cyclohexyladenosine; CCPA, 2-chloro-N^6-cyclopentyladenosine; TCPA, N^6-cyclopentyl-2-(3-phenylaminocarbonyltriazene-1-yl)adenosine; ATL-313, 4-{3-[6-amino-9-(5-cyclopropylcarbamoyl-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl]prop-2-y nyl}piperidine-1-carboxylic acid methyl ester; CP608,039, (2*S*,3*S*,4*R*,5*R*)-3-amino-5-{6-[5-chloro-2-(3-methylisoxazol-5-ylmethoxy)benzylamino] purin-9-yl}-4-hydroxytetrahydrofuran-2-carboxylic acid methylamide; HEMADO, 2-(1-hexynyl)-*N*-methyladenosine; MRS3558, (1′S,2′R,3′S,4′R,5′S)-4′-{2-chloro-6-[(3-chlorophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.10.0]hexane-2,3-diol; 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[3.10.0]hexane-1′-carboxylic acid *N*-methylamide.

[a] Data are from functional studies.
[b] Yan et al., 2003.
[c] Daly et al., 1993.
[d] Bruns, 1980.
[e] van Galen et al., 1994.
[f] Müller and Stein, 1996.
[g] Data from radioligand binding studies versus the antagonist radioligand [^{3}H]PSB-603 (S. Hinz and C. E. Müller, unpublished data).
[h] Klotz et al., 1998.
[i] Salvatore et al., 1993.
[j] Brackett and Daly, 1994.
[k] Sajjadi et al., 1996.
[l] Jacobson and Gao, 2006.
[m] Liang et al., 2010.
[n] Beukers et al., 2003.
[o] Franchetti et al., 1998.
[p] Elzein and Zablocki, 2008.
[q] Baraldi et al., 2007.
[r] Kuno et al., 2007.
[s] Data from radioligand binding studies versus the antagonist radioligand [^{3}H]MRS1754 (Auchampach et al., 2009).
[t] Tracey et al., 2003.
[u] Volpini et al., 2002; Klotz et al., 2007.
[v] Volpini et al., 2009.
[w] Melman et al., 2008.

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TABLE 4
Adenosine receptor affinities of antagonists

A few A_{2B} data are from functional (cAMP) studies.

		K_i			
		A_1	A_{2A}	A_{2B}	A_3
		nM			
Nonselective antagonists					
32	Caffeine	10,700 (h)[a] 44,900 (h)[b] 41,000 (r)[f] 44,000 (r)[g] 47,000 (gp)[k] 44,000 (c)[k]	23,400 (h)[b] 9560 (h)[a] 45,000 (r)[g] 32,500 (r)[i] 48,000 (r)[a]	33,800 (h)[c] 10,400 (h)[d] 20,500 (h)[h] 30,000 (r)[j] 13,000 (m)[j]	13,300 (h)[a] >100,000 (r)[e]
33	Theophylline	6770 (h)[l] 14,000 (r)[m] 8740 (r)[a] 7060 (gp)[o] 4710 (rb)[o] 9050 (s)[o] 6330 (c)[o]	1710 (h)[l] 6700 (h)[a] 22,000 (r)[m] 25,300 (r)[a]	9070 (h)[d] 74,000 (h)[h] 15,100 (r)[d] 5630 (m)[n] 11,000 (gp)[p] 17,700 (rb)[n] 38,700 (d)[n]	22,300 (h)[a] 86,400 (h)[l] >100,000 (r)[e] 85,000 (r)[q] >100,000 (d)[r]
34	CGS15943	3.5 (h)[s] 6.4 (r)[s]	1.2 (h)[s]	32.4 (h)[n] 9.07 (m)[n]	35 (h)[s]
A_1-selective antagonists					
35	DPCPX (CPX)	3.0 (h)[t] 0.50 (r)[t] 1.0 (r)[d] 0.18 (r)[o] 1.06 (gp)[o] 3.9 (gp)[k] 0.21 (rb)[o] 0.10 (s)[o] 0.05 (c)[o] 0.29 (c)[k] 11.4 (d)[r]	129 (h)[l] 60 (h)[t] 157 (r)[i] 500 (r)[d]	51 (h)[t] 63.8 (h)[d] 186 (r)[d] 200 (r)[p] 86.2 (m)[n] 145 (gp)[p] 96.0 (rb)[n] 147 (d)[n] 132 (d)[p]	795 (h)[u] 243 (h)[t] 509 (h)[r] 3960 (h)[l] >10,000 (r)[t] 43,000 (r)[r] 708 (rb)[r] 115 (d)[c]
36	CPFPX	1.26 (h)[v] 0.63 (r)[v] 1.37(p)[v] 0.18 (c)[v]	940 (h)[v] 812 (r)[v]	nd[b]	N.D.
37	Rolofylline (KW-3902, NAX)	0.72 (h)[w] 8.0 (h)[x] 0.19 (r)[y] 12.6 (r)[w]	108 (h)[w] 673 (h)[x] 380 (r)[y] 510 (r)[w]	296 (h)[x]	4390 (h)[x]
38	PSB-36	0.7 (h)[t] 0.124 (r)[t]	980 (h)[t] 552 (r)[t]	187 (h)[t]	2300 (h)[t] 6500 (r)[t]
39	Toponafylline (BG-9928)	7.4 (h)[x] 3.9 (mk)[z] 1.3 (r)[x] 29 (d)[z]	6410 (h)[x] 943 (mk)[z] 2440 (r)[x] 4307 (d)[z]	90 (h)[x]	>10,000 (h)[x]
40	FK-453	18 (h)[aa]	1300 (h)[aa]	980 (h)[aa]	>10,000 (h)[aa]
41	SLV320	1.00 (h)[bb] 2.51 (r)[bb]	398 (h)[bb]	3981 (h)[bb] 501 (r)[bb]	200 (h)[bb]
42	LUF5981	0.90 (h)[cc]	194 (h)[cc]	>300 (h)[cc]	637 (h)[cc]
A_{2A}-selective antagonists					
43	Istradefylline (KW6002)	841 (h)[dd] 230 (r)[dd]	12 (h)[ee] 91.2 (h)[dd] 2.2 (r)[ff] 4.46 (r)[gg]	>10,000 (h)[dd]	4470 (h)[dd]
44	CSC (K_i MAO-B = 80.6 nM)[gg]	28,000 (r)[hh]	54 (r)[hh]	8200[ii]	>10,000 (r)[e]
45	MSX-2	900 (r)[ij] 2500 (h)[ij]	8.04 (r)[i,ij] 5.38 (h)[ij,kk] 14.5 (h)[ij,mm]	>10,000 (h)[ij] 2900 (h)[ll]	>10,000 (h)[ij]
46	SYN-115	N.D.	N.D.	N.D.	N.D.
47	BIIB014 (V2006)	68 (h)[nn]	1.3 (h)[nn]	63 (h)[nn]	1005 (h)[nn]
48	ZM-241385	774 (h)[aa]	1.6 (h)[aa]	75 (h)[aa]	743 (h)[aa]
49	ST-1535	71.8 (h)[oo]	6.6 (h)[oo]	352.3 (h)[oo]	>1000 (h)[oo]
50	SCH-58261	725 (h)[aa]	5.0 (h)[aa]	1110 (h)[aa]	1200 (h)[aa]
51	Preladenant (SCH-420814)	>1000 (h)[pp]	0.9 (h)[pp]	>1000 (h)[pp]	>1000 (h)[pp]
52	SCH-442416	1110 (h)[aa]	4.1 (h)[qq]	>10,000 (h)[aa]	>10,000 (h)[aa]
A_{2B}-selective antagonists					
53	MRS1754	403 (h)[rr] 16.8 (r)[rr]	503 (h)[rr] 612 (r)[rr]	1.97 (h)[rr] 12.8 (r)[rr] 16.6 (r)[p] 3.39 (m)[n] 9.12 (gp)[p] 1.79 (rb)[n] 12.8 (d)[n] 12.3 (d)[p]	570 (h)[rr]

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TABLE 4—*Continued.*

		K_i			
		A_1	A_{2A}	A_{2B}	A_3
			nM		
54	MRE-2029-F20	200 (h)[ss]	>1000 (h)[ss]	5.5 (h)[ss]	>1000 (h)[ss]
55	PSB-603	>10,000 (h)[tt] >10,000 (r)[tt]	>10,000 (h)[tt] >10,000 (r)[tt]	0.553 (h)[tt] K_D 0.403 (h)[tt] K_D 0.351 (m)[tt]	>10,000 (h)[tt]
56	CVT-6883	1940 (h)[uu]	3280 (h)[uu]	22 (h)[uu]	1070 (h)[uu]
57	PSB-1115	>10,000 (h)[u] 2200 (r)[m]	24,000 (r)[m]	53.4 (h)[u]	>10,000 (h)[u]
58	ATL 802	369 (h)[rr] 9583 (m)[rr]	654 (h)[rr] 8393 (m)[rr]	2.36 (h)[rr] 8.58 (m)[rr]	>1000 (h)[rr] >10,000 (m)[rr]
59	LAS38096	2821 (h)[vv]	>1000 (h)[vv]	17 (h)[vv]	1043 (h)[vv]
A_3-selective antagonists					
60	KF26777	1800 (h)[ww]	470 (h)[ww]	620 (h)[ww]	0.20 (h)[ww]
61	PSB-10	1700 (h)[xx] 805 (r)[yy]	2700 (h)[xx] 6040 (r)[yy]	N.D.	0.441 (h)[yy]
62	PSB-11	1640 (h)[xx] 440 (r)[xx]	1280 (h)[xx] 2100 (r)[xx]	2100 (m)[yy]	2.34 (h)[xx] K_D 4.9 (h)[zz]
63	MRE-3008-F20	1200 (h)[aa]	141 (h)[aa]	2100 (h)[aa]	0.82 (h)[aa]
64	MRS1523	>10,000 (h)[aaa] 15,600 (r)[aaa]	3660 (h)[aaa] 2050 (r)[aaa]	>10,000 (h)[aaa] >10,000 (m)[aaa]	18.9 (h)[aa] 113 (r)[aaa] 731 (m)[aaa]
65	MRS1191	>10,000 (h)[aaa] 40,100 (r)[aaa]	>10,000 (h)[aaa] >10,000 (r)[aaa]	>10,000 (h)[aaa]	31.4 (h)[aaa] 1850 (r)[aaa]
66	VUF-5574	≤10,000 (r)[bbb]	≤10,000 (r)[bbb]	N.D.	4.03 (h)[bbb]

h, human; c, cow; d, dog; gp, guinea pig; m, mouse; *r*, rat; rb, rabbit; N.D., no data available; LUF5981, 8-cyclohexyl-2,6-diphenyl-1-deazapurine; CSC, 8-(3-chlorostyryl)caffeine; KF26777, 2-(4-bromophenyl)-4-ethyl-4,5,7,8-tetrahydro-1*H*-imidazo[2.1-*i*]purin-5-one.

[a] Jacobson et al., 1999.
[b] Abo-Salem et al., 2004.
[c] Borrmann et al., 2009.
[d] Kim et al., 2002.
[e] van Galen et al., 1994.
[f] Grahner et al., 1994.
[g] Daly et al., 1991.
[h] Bertarelli et al., 2006.
[i] Müller et al., 2000.
[j] Brackett and Daly, 1994.
[k] Ukena et al., 1986b.
[l] Klotz et al., 1998.
[m] Müller et al., 1993b.
[n] Auchampach et al., 2009.
[o] Klotz et al., 1991.
[p] Fozard et al., 2003.
[q] Jacobson et al., 1995.
[r] Auchampach et al., 1997.
[s] Müller, 2000a.
[t] Weyler et al., 2006.
[u] Hayallah et al., 2002.
[v] Holschbach et al., 2002.
[w] Pfister et al., 1997.
[x] Kiesman et al., 2006b.
[y] Kiesman et al., 2006a.
[z] Doggrell, 2005.
[aa] Jacobson and Gao, 2006.
[bb] Kalk et al., 2007.
[cc] Chang et al., 2007.
[dd] Müller et al., unpublished data.
[ee] Kase et al., 2003.
[ff] Shimada et al., 1997.
[gg] Pretorius et al., 2008.
[hh] Jacobson et al., 1993a.
[ii] Daly and Jacobson, 1995.
[jj] Sauer et al., 2000.
[kk] Recombinant receptors (expressed in CHO cells).
[ll] Solinas et al., 2005.
[mm] Native receptors (post mortem brain).
[nn] Gillespie et al., 2009.
[oo] Minetti et al., 2005.
[pp] Müller and Ferré, 2007.
[qq] Kecskés et al., 2010.
[rr] Kim et al., 2000.
[ss] Baraldi et al., 2004.
[tt] Borrmann et al., 2009.
[uu] Elzein et al., 2008.
[vv] Vidal et al., 2007; Eastwood et al., 2010.
[ww] Saki et al., 2002.
[xx] Ozola et al., 2003.
[yy] Müller et al., 2002a.
[zz] Müller et al., 2002b.
[aaa] Liang et al., 2010.
[bbb] van Muijlwijk-Koezen et al., 2000.

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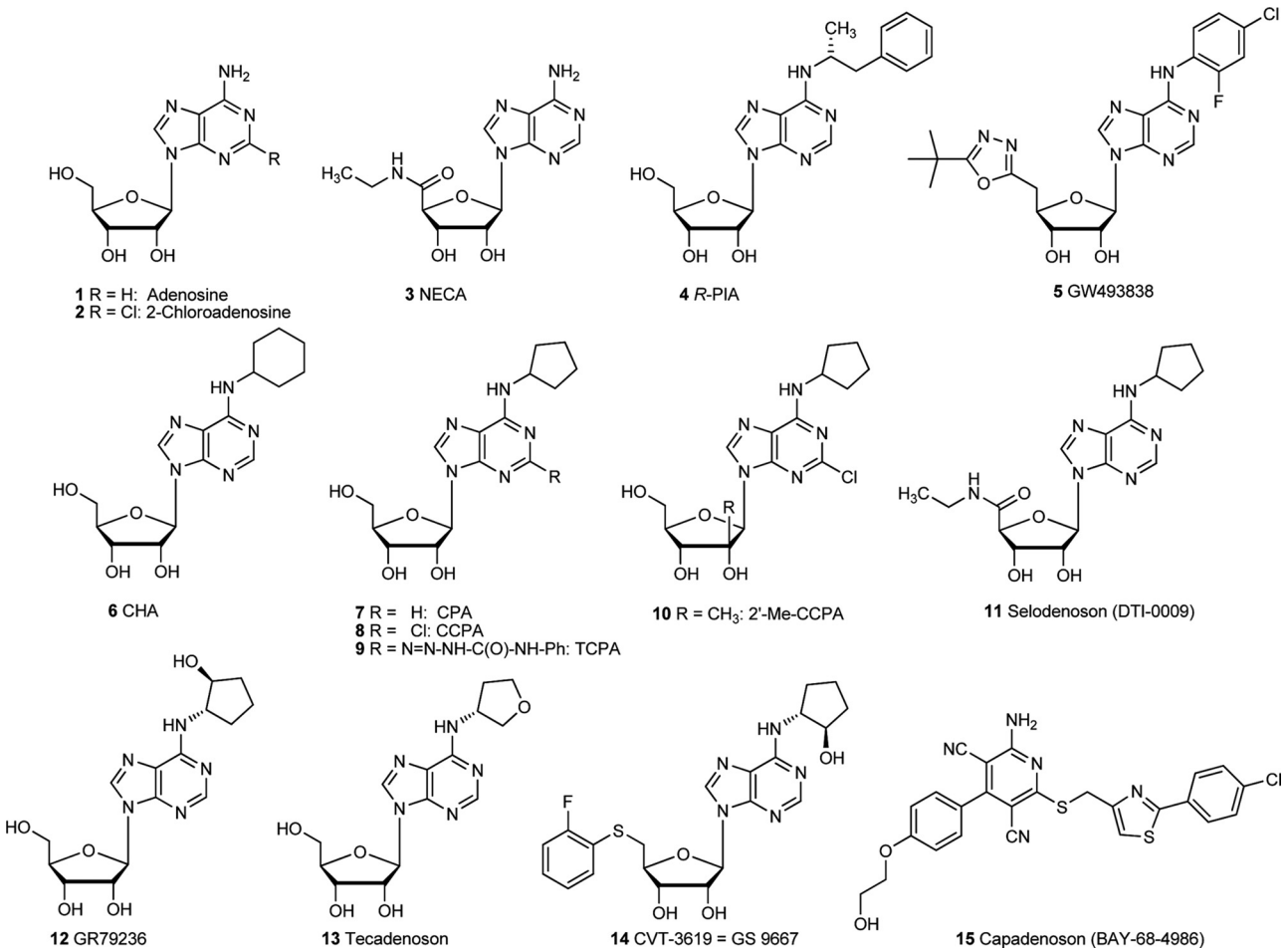

FIG. 2. Nonselective and A_1-selective agonists.

weakly potent at adenosine receptors. A more potent nonselective antagonist is the triazoloquinazoline derivative 5-amino-9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazoline (CGS15943; **34**). A very large number of A_1-selective antagonists has been developed derived from the xanthines (e.g., **35–39**) or with nonxanthine structure (e.g., **40–42**). A_1-selective xanthines typically feature a bulky cycloalkyl residue in the 8-position of the xanthine core. DPCPX (**35**) is the most widely used A_1 antagonist, although its selectivity is moderate in some species, [e.g., humans (see Table 4)]. It is also available in tritiated form as an A_1 antagonist radioligand. A fluorinated derivative—8-cyclopentyl-1-propyl-3-(3-[^{18}F]fluoropropyl)xanthine ([^{18}F]CPFPX; **36**)—has been developed as a tracer for positron emission tomography. The 8-(3-noradamantyl) derivative 1-butyl-8-noradamant-3-yl-3-(3-hydroxypropyl)-3,7-dihydropurin-2,6-dione (PSB-36; **38**) is more potent than **35** with a K_i value for the rat A_1 receptor in the picomolar range. A_1 antagonists including rolofylline (**37**), toponafylline (**39**), *R*-[(*E*)-3-(2-phenylpyrazolo[1,5-*a*]pyridin-3-yl) acryloyl]-2-piperidine ethanol (FK-453; **40**), and 4-(4-hydroxycyclohexylamino)-6-phenylpyrrolo[2,3-*d*]pyrimidine (SLV320; **41**) have been evaluated in clinical trials, but so far no A_1-selective antagonist has been approved as a drug.

Many A_{2A}-selective antagonists from different structural classes have been developed (**43–52**). One of the first A_{2A} antagonists was 8-(3-chlorostyryl)caffeine (**44**). However, **44** is also a potent inhibitor of monoamine oxidase B. Frequently used antagonists of the A_{2A} receptor are 3-(3-hydroxypropyl)-7-methyl-1-propargyl-8-(*m*-methoxystyryl)xanthine (MSX-2; **45**) and its water-soluble prodrugs phosphoric acid mono-(3-{8-[2-(3-methoxy-phenyl)-vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl}-propyl) ester (MSX-3; **45a**) and L-valine-3-{8-(*E*)-2-[3-methoxyphenyl)ethenyl]-7-methyl-1-propargylxanthine-3-yl}propyl ester hydrochloride (MSX-4; **45b**), ZM-241385 (**48**), and 5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]-pyrimidine (SCH-58261; **50**). Compounds **45**, **48**, and **50** have also been prepared in tritiated form and are used as A_{2A} antagonist radioligands. ZM-241385 (**48**) exhibits the lowest degree of selectivity among these three standard A_{2A} antagonists due to its affinity at the A_{2B} receptor. Several A_{2A} antagonists are in clinical trials for Parkinson's disease, including istradefylline (**43**), 4-hydroxy-4-methylpiperidine-1-carboxylic acid-(4-methoxy-7-morpholin-4-ylbenzothiazol-2-yl)amide (SYN-115; **46**), 2-butyl-9-methyl-8-[1,2,3]triazol-2-yl-9*H*-purin-6-ylamine (ST-1535; **49**), preladenant (**51**), and 2-(2-

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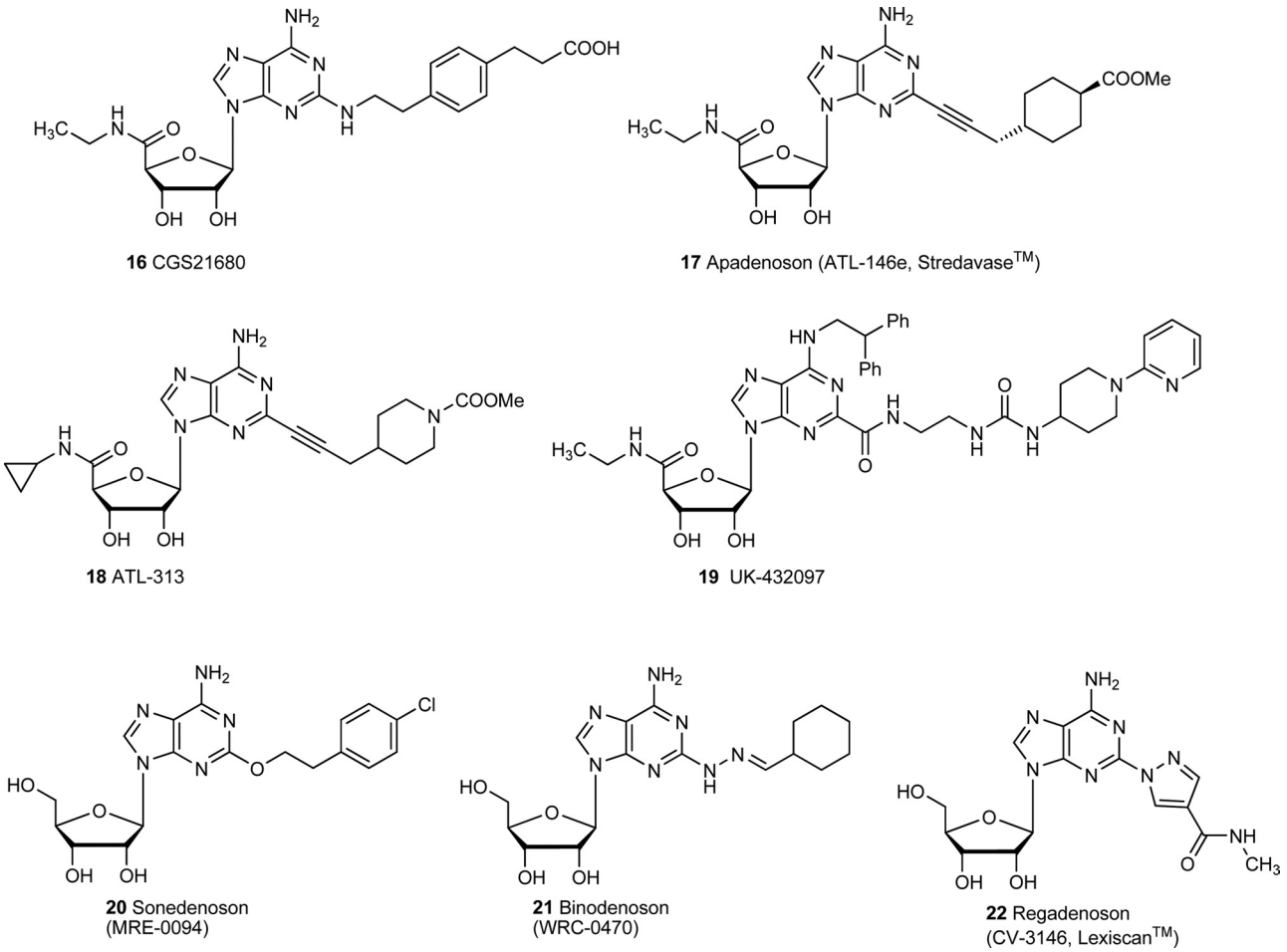

FIG. 3. A_{2A}-selective agonists.

furyl)-7-[3-(4-methoxyphenyl)propyl]-7*H*-pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine (SCH-442416; **52**).

During the past decade, potent and selective A_{2B} antagonists have been developed (**53–59**; Table 4). One of the first compounds was the xanthine derivative *N*-(4-cyanophenyl)-2-(4-(1,3-dipropylxanthin-8-yl)phenoxy-)acetamide (MRS1754; **53**), which is potent and selective in humans but not in other species (e.g., rat). Further potent and A_{2B}-selective xanthine derivatives include *N*-benzo[1,3]dioxol-5-yl-2-(5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydropurin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy)acetamide (MRE-2029-F20; **54**), 8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine (PSB-603; **55**), 3-ethyl-1-propyl-8-(1-(3-trifluoromethylbenzyl)-1*H*-pyrazolo-4-yl)xanthine (CVT-6883; **56**), 1-propyl-8-*p*-sulfophenylxanthine (PSB-1115; **57**), and *N*-(5-(1-cyclopropyl-2,6-dioxo-3-propyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)pyridin-2-yl)-*N*-methyl-6-(trifluoromethyl)nicotinamide (ATL 802; **58**). The most potent and selective A_{2B} antagonist yet developed is PSB-603 (**55**), which shows high affinity and selectivity not only in humans but also in rodents. Compounds **53** to **55** have also been prepared in

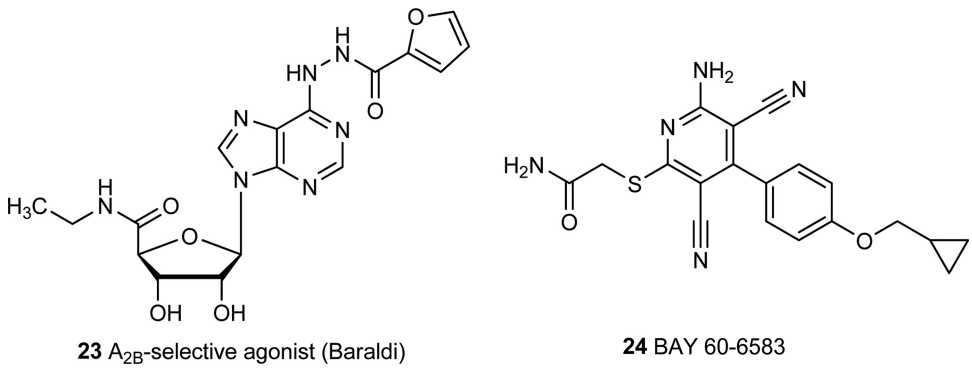

FIG. 4. A_{2B}-selective agonists.

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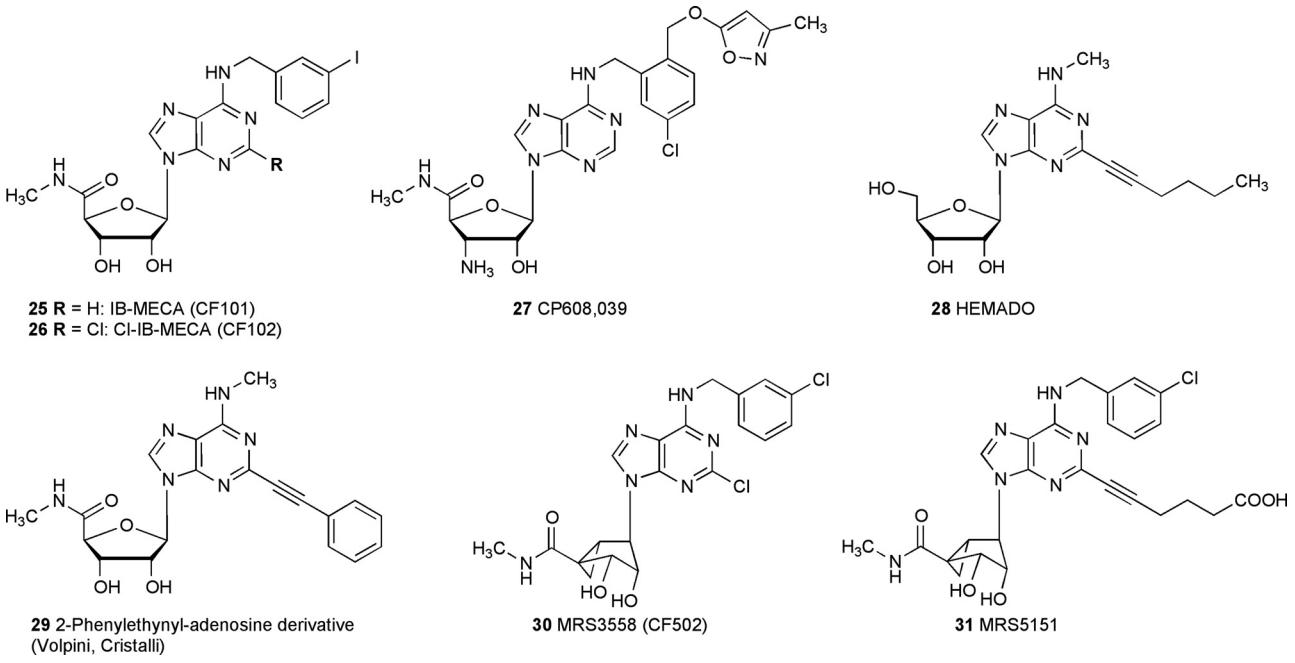

FIG. 5. A_3-selective agonists.

tritium-labeled form as antagonist A_{2B} radioligands. PSB-1115 (**57**) is particularly useful for in vivo studies because it shows high water solubility; however, its A_{2B} affinity and selectivity are lower than those of the more lipophilic compounds. The xanthine CVT-6883 (**56**) is in clinical trials for the treatment of chronic obstructive pulmonary disorder. Besides xanthines derivatives, nonxanthine A_{2B} antagonists have recently been developed, such as 2-amino-4-(2-furanyl)-5-(4-pyrimidinyl)pyrimidine (LAS38096; **59**).

The A_3 receptor is characterized by particularly large species differences between human and rodent orthologs, especially for antagonists. Known heterocyclic A_3 antagonists are typically much more potent at the human compared with the rat receptor. Therefore, their use in mouse or rat studies is questionable. An exception is the pyridine derivative 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate (MRS1523; **64**), which is quite potent at rat A_3 receptors as well. Frequently used potent and A_3-selective tool compounds include 8-ethyl-4-methyl-2-(2,3,5-trichlorophenyl)-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2.1-*i*]purin-5-one (PSB-10; **61**), 8-ethyl-4-methyl-2-phenyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2.1-*i*]purin-5-one (PSB-11; **62**), 5-[[(4-methoxyphenyl)amino]carbonyl]amino-8-propyl-2-(2-furyl)pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine (MRE3008-F20; **63**), 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl 5-(phenylmethyl) ester (MRS1191; **65**) and *N*-(2-methoxyphenyl)-*N*-[2-(3-pyridyl)quinazolin-4-yl]urea (VUF-5574; **66**). PSB-11 (**62**) and MRE3008-F20 (**63**) have been prepared in tritiated form as A_3 antagonist radioligands. The imidazopurine derivatives **60** to **62** have the advantage of being less lipophilic and more water-soluble than most of the other A_3 antagonists because of a basic nitrogen atom that can be protonated.

VII. Allosterism

A. Allosteric versus Orthosteric Ligands

Most of the efforts in the medicinal chemistry and pharmacology of adenosine receptors have concentrated on competitive agonist or antagonist ligands that occupy the principle (orthosteric) binding sites of the receptors. More recently, the area of allosteric modulation of the action of a native agonist has become the focus of widespread efforts in ligand design and pharmacology of these receptors. Each of the four subtypes of the ARs now has selective agonist and antagonist ligands. Allosteric modulators, on the other hand, are well explored only for A_1 and A_3 receptors, with isolated reports of examples for allosteric or "noncompetitive" (i.e., potentially allosteric) ligands for other subtypes of purine receptors (Gao et al., 2005). Most of the examples of allosteric modulators of adenosine receptors are positive allosteric modulators (PAMs) (i.e., they increase the affinity, potency, and/or efficacy of the agonist).

The justification for studying allosteric modulation of ARs is clear:

1. First, adenosine receptors are widely distributed throughout the body, yielding a risk for side effects when direct orthosteric agonist is administered. At least for the ARs, the problem of a lack of selective ligands, as has plagued the muscarinic acetylcholine receptor field, is not a limitation for the AR field. However, the ubiquity of the ARs does present a problem of lack of selectivity even for highly selective agonists.
2. Native adenosine is rapidly degraded and does not migrate beyond the target site. In stress situations, the extracellular concentration of adenosine is ele-

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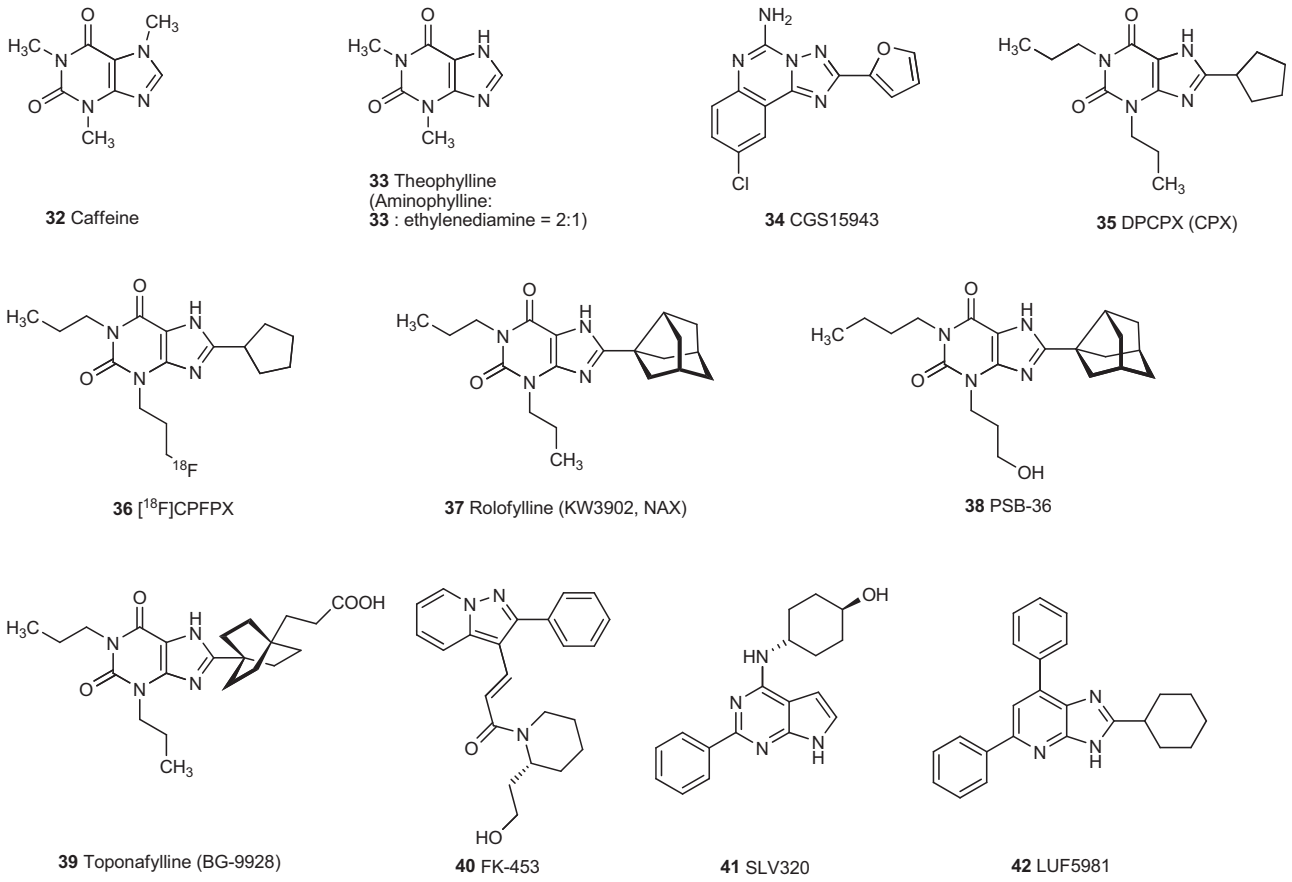

FIG. 6. Nonselective and A_1-selective antagonists.

vated locally. Thus, the action of a PAM is expected to be more tissue-specific than the action of a stable, exogenously administered orthosteric agonist, which would circulate throughout the body (Conn et al., 2009).

3. Adenosine agonists do not readily penetrate the blood-brain barrier. The brain entry of nucleoside derivatives is typically only 1 to 2% of free passage across the blood-brain barrier. Thus, for induction of adenosine receptor activation in the brain, where adenosine levels can be greatly elevated in response to stress or hypoxia, a freely penetrating PAM would be more effective.

These factors make allosteric enhancement of receptor activation by endogenous adenosine a particularly attractive option—leading to site-specific and event-specific action, with a particular advantage in the central nervous system.

Assay methods used to identify allosteric modulators of the adenosine receptors have included both radioligand binding and functional assays. Initially, screening typically has consisted of looking for an increase in the level of binding of radioligand to membranes expressing a given receptor subtype. A more labor-intensive binding method has been to look for alteration of the dissociation rate of a radioligand. Thus, slowing the off rate of an agonist is one indication of a PAM, although it is not conclusive until at least one functional assay is carried out. Such functional assays might consist of the enhancement (for a PAM) or reduction [for a negative allosteric modulator (NAM)] of binding of a radiolabeled guanine nucleotide in response to a known receptor agonist or effects on agonist-induced changes in adenylate cyclase or other second-messenger systems.

B. A_1 Receptor Allosteric Modulators

The benzoylthiophenes were the first AR allosteric modulators to be identified (Bruns et al., 1990). They have been extensively modified in subsequent studies, and their structure-activity relationship (SAR) as PAMs has been documented. The prototypical benzoylthiophene to act as an A_1 receptor allosteric modulator is (2-amino-4,5-dimethyl-3-thienyl)(3-(trifluoromethyl)phenyl)-methanone (PD81,723; Fig. 10), and many analogs have been prepared (Kourounakis et al., 2000; Baraldi et al., 2007a,b). The 2-amino-3-carbonylthiophene moiety is required as a minimal pharmacophore. The aroyl group can be substituted with other phenyl and heteroaromatic groups. The 4,5-alkyl substituents of the thiophene ring may be cyclized, particularly with cyclohexyl group (van der Klein et al., 1999), although larger rings are also tolerated (Nikolakopoulos et al., 2006). In certain cases, the aroyl group

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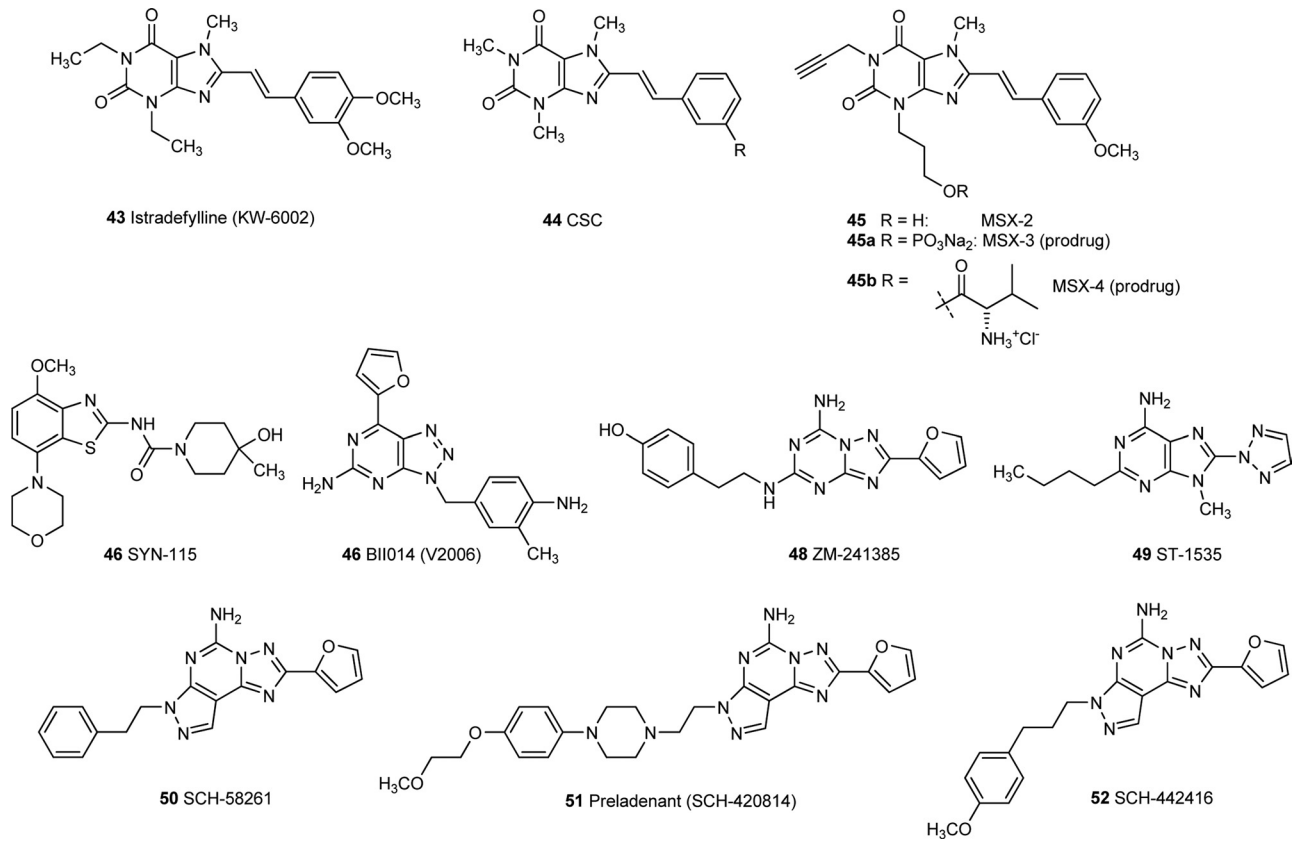

FIG. 7. A_{2A}-selective antagonists.

can be simplified in the form of a carboxylic acid. An atypical structural class, 2-aminothiazoles, were reported as AR allosteric enhancers, but their activity as PAMs is not always evident and seems to be limited to specific salt forms (Chordia et al., 2005; Göblyös et al., 2005).

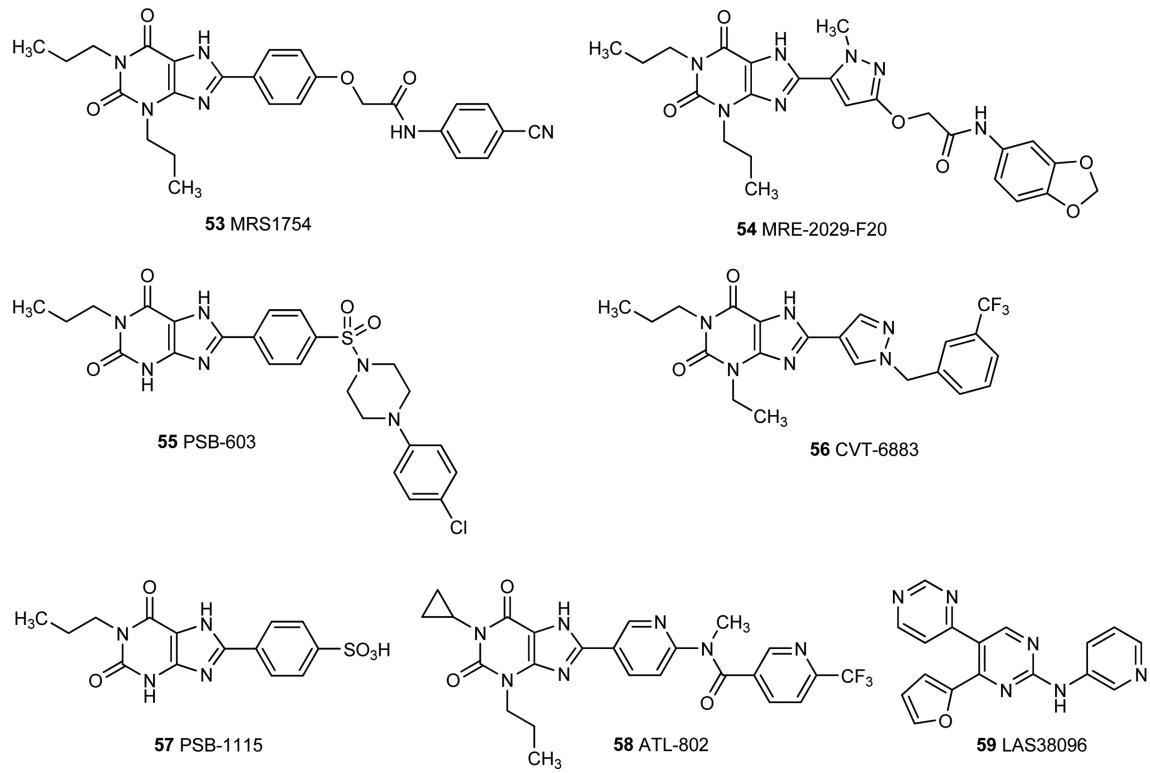

FIG. 8. A_{2B}- selective antagonists.

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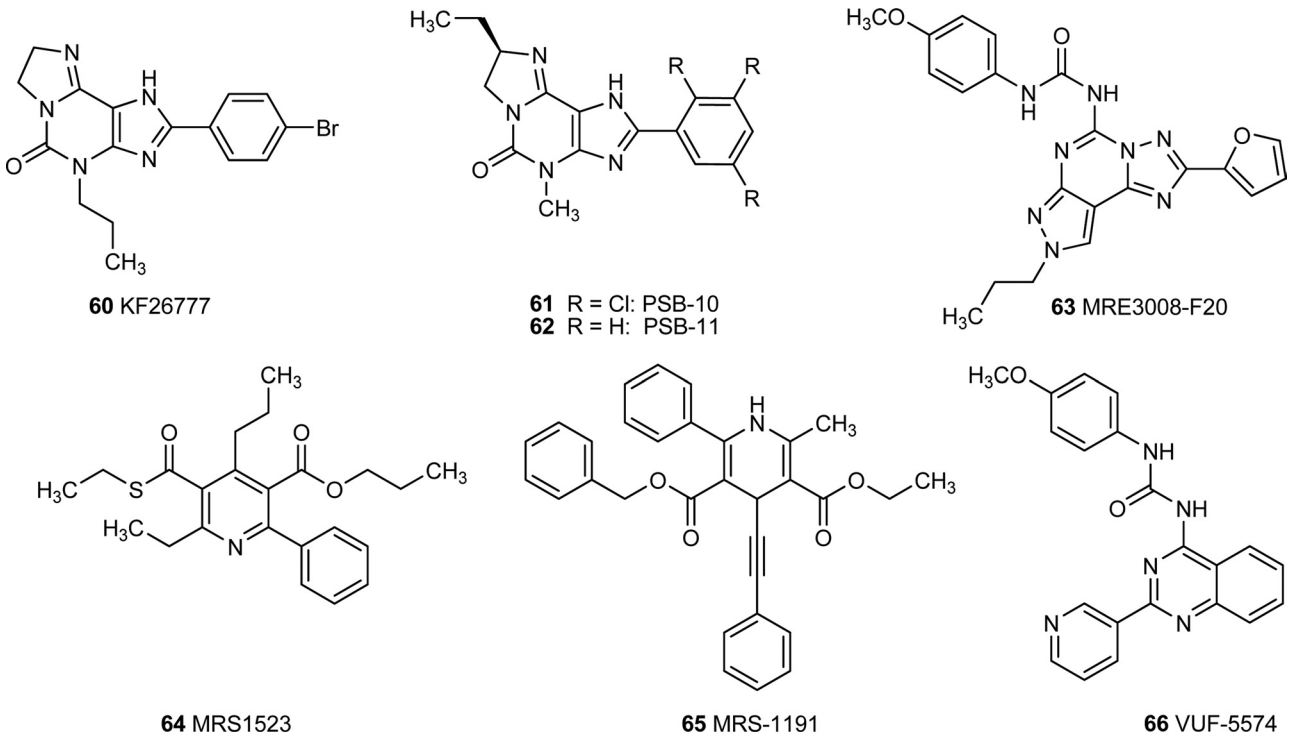

FIG. 9. A_3-selective antagonists.

Only one allosteric modulator of adenosine receptors, the benzoylthiophene analog 2-amino-3-(4-chlorobenzoyl)-5,6,7,8-tetrahydrobenzothiophene (T-62; Baraldi et al., 2000), has until now been in clinical trials. A main envisioned application of T-62 is in the central nervous system, and it seems to alleviate chronic pain. In brain slices, this PAM selectively enhances the coupling of the A_1 receptor to its G_i protein. T-62 itself has agonist properties, because it also raises the basal levels of guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding. An antinociceptive effect has been studied after T-62 administered intrathecally in carrageenan-inflamed rats. This allosteric adenosine receptor modulation reduced hypersensitivity after peripheral inflammation by a central mechanism (Li et al., 2003). T-62 has been radiolabeled, and its binding properties are indicative of allosteric binding (Baraldi et al., 2006).

Another possible advantage of PAMs over orthosteric agonists is the possibility to alter the spectrum of second-messenger effects, or produce a bias toward a particular pathway based on conformational variation of the receptor in its activated state. In this context, a new 3,5-di(trifluoromethyl)benzoylthiophene derivative was shown to act as an alloagonist of the A1AR (Aurelio et al., 2009). Activation of the extracellular signal-regulated kinase phosphorylation pathway required higher concentrations of the derivative than for G protein modulation (based on guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding), suggesting the possibility of signaling bias. Such functional selectivity was corroborated in a recent study with a number of allosteric modulators, in which it was also shown that some modulators are direct inhibitors of G protein function (Valant et al., 2010).

Bivalent ligands linking both orthosteric (adenosine-like) and allosteric (PD81,723-like) pharmacophores were recently synthesized (Narlawar et al., 2010). N^6-[2-Amino-3-(3,4-dichlorobenzoyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-6-yl-9-nonyloxy-4-phenyl]adenosine (LUF6258; Fig. 11) with a nine-carbon atom linker between the two pharmacophores showed no significant changes in affinity or potency in the presence of PD81,723, indicating that LUF6258 bridged both orthosteric and allosteric binding sites on the receptor.

C. A_{2A} Receptor Allosteric Modulators

Amiloride analogs have been characterized as allosteric modulators of the A_{2A} adenosine receptor (Gao and IJzerman, 2000). 5-(*N*,*N*-Dialkyl)amiloride derivatives, such as 5-(*N*,*N*-hexamethylene)amiloride (Fig. 10), increase the dissociation rate of antagonist radioligand at this subtype. However, these compounds are not selective for this subtype or for adenosine receptors in general. They also allosterically modulate action at both A_1 and A_3 receptors (Gao et al., 2003b). At the A_1 adenosine receptor, their behavior is similar to that at the A_{2A} receptor. At the A_3 receptor, they additionally decrease the dissociation rate of agonist radioligand. They also compete for orthosteric binding at these three subtypes. Thus, amiloride analogs are of limited use as allosteric pharmacological probes.

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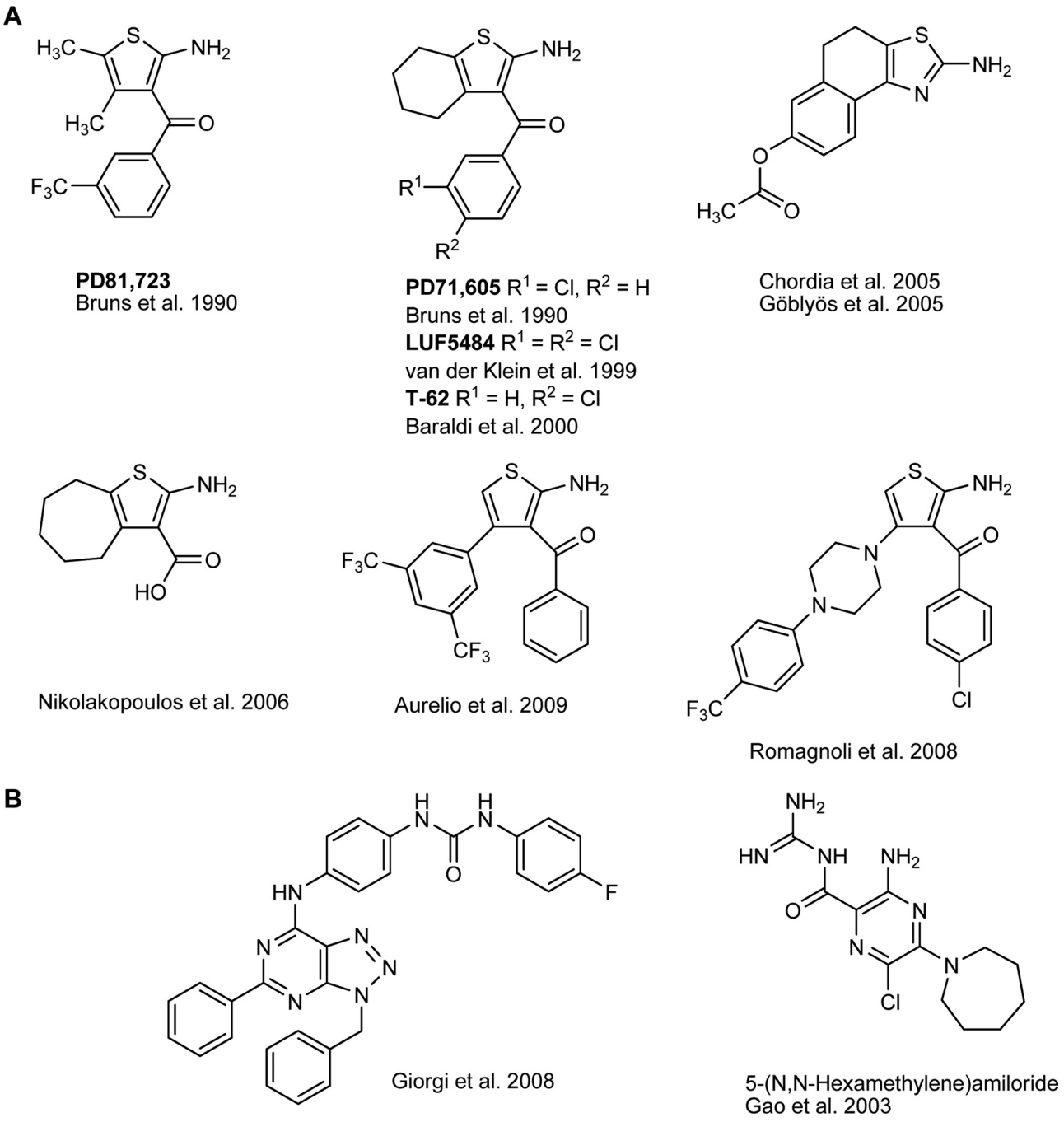

FIG. 10. Selected allosteric enhancers of action of agonist at the A_1 adenosine receptor (A) and at the A_{2A} receptor (B).

A 2-phenyl-9-benzyl-8-azaadenine derivative (Fig. 10) was reported to be an allosteric enhancer of radioligand binding at the A_{2A} adenosine receptor and agonist-induced relaxation of rat aortic rings (Giorgi et al., 2008). The benzopyran-2-one derivative 4-methyl-2-oxo-2*H*-chromen-7-yl methylcarbamate (PD120,918) was also reported to be an enhancer of binding at the A_{2A} receptor (Gao et al., 2005).

D. A_3 Receptor Allosteric Modulators

Structurally diverse classes of allosteric modulators for the A_3 adenosine receptor were discovered during screening of known ligands of this subtype (Gao et al., 2001, 2002). Prototypical PAMs of the A_3 receptor include 3-(2-pyridinyl)isoquinolines [e.g., 4-methoxy-*N*-(7-methyl-3-(2-pyridinyl)-1-isoquinolinyl)benzamide) (VUF5455)] and 1*H*-imidazo-[4,5-*c*]quinolin-4-amines [e.g., 2-cyclopentyl-4-phenylamino-1*H*-imidazo-[4,5-*c*]quinoline (DU124183) and *N*-(3,4-dichlorophenyl)-2-cyclohexyl-1*H*-imidazo-[4,5-*c*]quinolin-4-amine (LUF6000) (Göblyös et al., 2006)].

The A_3AR selective antagonist VUF-5574 (K_i, 4 nM) belongs to the class of pyridinyl isoquinolines, a chemical series that was originally introduced as antago-

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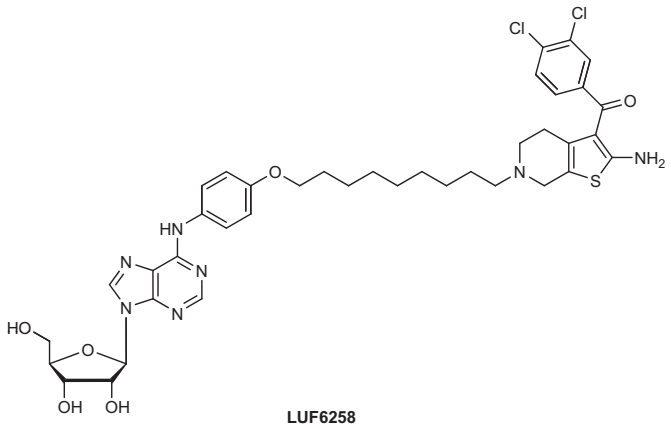

FIG. 11. Bivalent ligand bridging orthosteric and allosteric site on the human A_1 adenosine receptor.

nists of this subtype (van Muijlwijk-Koezen et al., 1998). In binding assay screens of diverse ligands designed to detect enhancement, as well as inhibition, members of the pyridinyl isoquinoline class were found to exhibit allosteric as well as orthosteric properties in interaction with the receptor (Fig. 12). The SAR of pyridinyl isoquinolines in orthosteric binding to the A_3 adenosine receptor (presumed from the competitive displacement of orthosteric radioligands) is distinct from SAR in allosteric enhancement. Because many structural homologues were already available, it was feasible to characterize their profile as allosteric enhancers of A_3 adenosine receptor.

Another structural class of A_3 adenosine receptor PAMs are the imidazoquinolinamines (Gao et al., 2002). van Galen et al. (1991) originally introduced the imidazoquinolinamines as A_1 receptor antagonists. In addition to orthosteric binding competitive with the native ligand adenosine, the compound DU124183 was an allosteric enhancer of radioligand binding at the A_3 adenosine receptor.

The SAR of a series of imidazoquinolinamines as allosteric enhancers of the A_3 adenosine receptor has been explored in detail. Modification of the 4 position shows a divergence of the structural requirements for orthosteric and allosteric actions. Combination of the most favorable groups for allosteric enhancement is shown in Fig. 3, resulting in the prototypical A_3 receptor PAM of the imidazoquinolinamine class, LUF6000. Allosteric enhancement was demonstrated by reducing the dissociation rate of agonist radioligand and by enhancement of maximal guanine nucleotide binding in

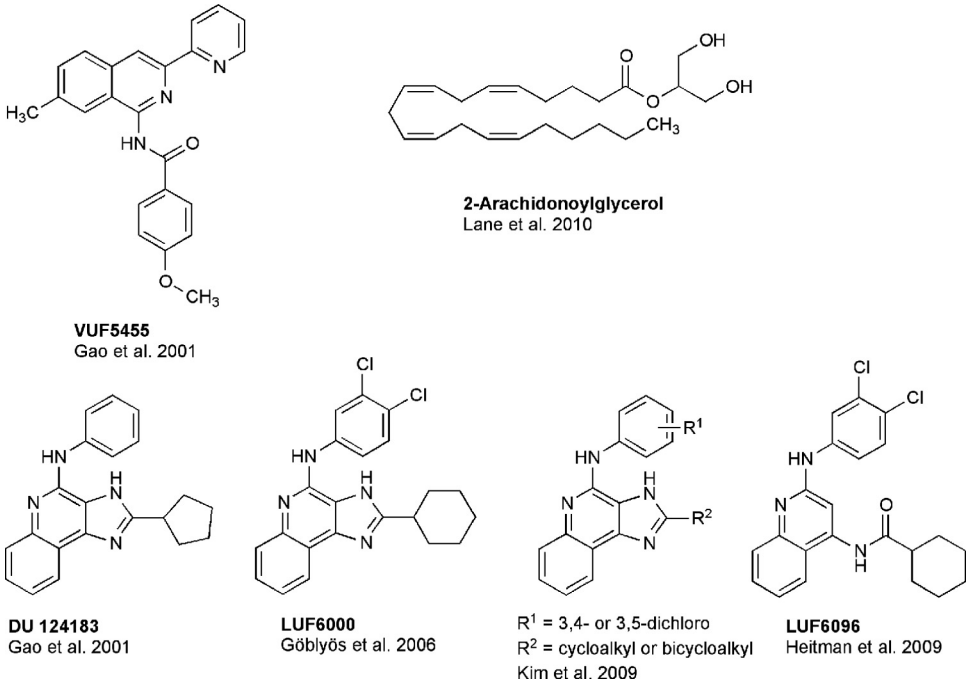

FIG. 12. Structures of pyridinylisoquinoline and imidizoquinolinamine derivatives that act as PAMs of of the human A_3 adenosine receptor and structure of 2-AG, which acts as a NAM of the A_3 receptor.

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the presence of a known agonist. No major effect on potency of prototypical agonist Cl-IB-MECA at the human A_3 adenosine receptor by LUF6000 was observed. The allosteric effects were increased without increasing the orthosteric inhibition by altering the size of the 2-cycloalkyl ring and by substitution of the 4-phenylamino group. Further exploration of steric and electronic effects of substitution at the 2 and 4 positions of a series of imidazoquinolinamines as allosteric enhancers of the A_3AR was reported (Kim et al., 2009b). Enhancement was observed by two bridged bicycloalkyl derivatives, indicating that rigid steric bulk is tolerated at the 2 position. Hydrophobicity is also a requirement at that position. Scission of the imidazole ring in the structure of LUF6000 (Fig. 12) led to a series of 2,4-disubstituted quinolines as allosteric enhancers of the adenosine A_3 receptor. The same substitution pattern as in LUF6000 led to the most potent allosteric modulator [*N*-{2-[(3,4-dichlorophenyl)amino]quinoline-4-yl}cyclohexanecarboxamide (LUF6096)] in the series with negligible orthosteric activity on A_1 and A_3 receptors, even less than observed for LUF6000 (Heitman et al., 2009).

The ability of nucleoside derivatives to activate as well as bind to the A_3 adenosine receptor is highly structure-dependent. It is even possible to modify nucleoside agonists to become selective A_3 adenosine receptor antagonists. The allosteric enhancer LUF6000 was found to "revive" a potently binding nucleoside antagonist at the human A_3 receptor, 2-chloro-N^6-(3-iodobenzyl)-adenosine (MRS592) (Gao et al., 2008). This nucleoside derivative, although having an intact ribose moiety, contains two modifications that diminish the efficacy of the compound at the A_3 receptor but do not prevent its high-affinity receptor binding. Coadministration of LUF6000, up to 10 μM, converted this full antagonist into a full agonist in a concentration-dependent manner, which is an example of a complete reversal of the nature of the action of an antagonist by a PAM of a GPCR. This phenomenon was observed only with nucleoside-based antagonists, and not with heterocyclic antagonists of the A_3 receptor such as *N*-[9-chloro-2-(2-furanyl)[1,2,4]-triazolo[1,5-*c*]quinazolin-5-yl]benzene acetamide (MRS1220).

Some endogenous cannabinoid ligands also modulate the A_3 receptor (Lane et al., 2010). 2-Arachidonylglycerol (2-AG) was able to inhibit agonist ^{125}I-4-aminobenzyl-5′-*N*-methyl-carboxamidoadenosine binding at the human A_3 adenosine receptor. In the presence of 2-AG, the rate of ^{125}I-4-aminobenzyl-5′-*N*-methyl-carboxamidoadenosine dissociation was increased, suggesting that 2-AG acts as a NAM. Because the human A_3 adenosine receptor is expressed in astrocytes and microglia, these findings may be relevant in cerebral ischemia, a pathological condition in which levels of 2-AG are raised.

E. Evidence for Mode of Binding of Adenosine Receptor Allosteric Modulators

Site-directed mutagenesis of the human A_1 adenosine receptor (Barbhaiya et al., 1996; Kourounakis et al., 2001; de Ligt et al., 2005; Heitman et al., 2006) and chimera with the A_{2A} receptor (Bhattacharya et al., 2006) have provided some insight into the structural basis of allosteric modulation. On this receptor subtype, Asp55 in TM2 is probably responsible for allosteric regulation of ligand binding by sodium ions and amilorides, whereas upon G14T (TM1) and T277A (TM7) mutagenesis, PD81,723 loses its enhancing activity with respect to CPA. However, the potency of CPA alone is also drastically diminished by these mutations; hence, it remains unclear whether these two amino acids are also part of the PD81,723 binding site.

Site-directed mutagenesis of the human A_3 adenosine receptor has shown that His in TM3 and Phe in TM5 are important for orthosteric binding of the imidazoquinolinamine DU124183 (Gao et al., 2003a). His in TM7 is required for radioligand binding. Other residues, such as Trp243 and Asn30, were modulatory. It was determined that Asn274 in TM7 was required for allosteric binding of the imidazoquinolinamine but not for maintaining the orthosteric binding site. A conserved His residue in TM7 is required for A_3 radioligand binding; therefore, it was not possible to establish the effect of its mutation to Ala on allosteric enhancement.

A docking hypothesis for a pyridinylisoquinoline PAM, VUF5455, in the agonist-occupied human A_3 adenosine receptor was reported based on an energetically favorable interaction of this heterocyclic derivative with the outer portions of the receptor—near the extracellular loops (Gao et al., 2003a). This would allow the simultaneous binding of both agonist and PAM to different regions of the receptor protein, as has been shown for allosteric modulation of muscarinic receptors (Conn et al., 2009). An alternate hypothesis for the binding of allosteric enhancers in family A GPCRs is that agonists and PAMs might bind on opposite protomers of homodimeric receptor pairs (Schwartz and Holst, 2006). The applicability of this hypothesis to PAMs of the adenosine receptors has yet to be demonstrated.

In conclusion, 2-amino-3-aroylthiophene derivatives (such as T-62 for chronic pain) are under development as allosteric enhancers of the A_1 adenosine receptor. These derivatives tend to act as allosteric agonists, as well as PAMs. Two classes of A_3 AR allosteric modulators have been explored: 3-(2-pyridinyl) isoquinolines (e.g., VUF5455) and 1*H*-imidazo-[4,5-c]quinolin-4-amines (e.g., DU124183 and LUF6000), which selectively decreased the agonist dissociation rate at human A_3ARs but not at A_1ARs and A_{2A}ARs. The antagonist properties have been minimized in SAR studies. Nucleoside derivatives that are A_3-selective antagonists and low-efficacy agonists can be

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converted into full agonists by coadministration of allosteric enhancer LUF6000. Site-directed mutagenesis of A_1 and A_3 receptors has identified residues associated with the allosteric effect. Distinct amino acid residues affect orthosteric versus allosteric binding. Thus, there are clear advantages to the design of allosteric modulators of ARs.

VIII. Drugs in the Clinic

What follows is a survey of some of the compounds targeting adenosine receptors that have been investigated in ongoing or recently completed clinical trials. The list is not complete because several early trials in Europe and Japan are not included. Where appropriate, we have included the National Institutes of Health clinical trial identifier.

A. Adenosine

Newly identified targets of adenosine signaling have triggered new ideas for human investigation of adenosine itself. It is well known that adenosine has long been registered for treatment of supraventricular tachyarrhythmia; therefore, a suitable preparation for use in humans is available. The following is a summary of some of these studies.

1. Inflammation. Investigators at Vanderbilt University are studying the *Role of Adenosine in the Release of VEGF and Cytokines* (www.clinicaltrials.gov identifier NCT00580905). This trial will determine whether intravenous or intradermal adenosine influences cytokine production in man. This is based on research demonstrating that in animals, adenosine enhances the release of cytokines by activating A_{2B} or A_3 receptors (Ryzhov et al., 2008). Related to this is a study at Radboud University entitled *A Possible Therapeutic Role for Adenosine During Inflammation* (www.clinicaltrials.gov identifier NCT00513110). This study is based on the observation that a C34T-polymorphism of the enzyme AMP-deaminase alters adenosine metabolism. The hypothesis to be tested is that individuals with polymorphisms that result in increased adenosine will display less inflammation in response to LPS and that caffeine consumption will enhance inflammation in response to LPS.

2. Cardioprotection. Investigators at the University of Ottawa Heart Institute are investigating *Prophylactic Intracoronary Adenosine to Prevent Postcoronary Artery Stenting Myonecrosis* (www.clinicaltrials.gov identifier NCT00612521). Adenosine acting at A_{2A} or A_3 receptors has been found to reduce reperfusion injury after coronary artery occlusion (Yang et al., 2006; Wan et al., 2008). The aim of this study is to assess whether the use of intracoronary adenosine given directly into the coronary artery before stenting can reduce the incidence of myocardial necrosis and achieve better outcomes at 30-day follow-up. A similar study is under way at the University Hospital, Gasthuisberg, entitled *Salvage: Postconditioning with Adenosine for STEMI* (www.clinicaltrials.gov identifier NCT00284323). The goal of this study is to investigate the effect of intracoronary administration of adenosine on myocardial salvage and microvascular integrity in the setting of acute myocardial infarction. Another cardioprotection study is underway at Hospital Universitari Vall d'Hebron Research Institute, entitled *Myocardial Protection with Adenosine during Primary Percutaneous Coronary Intervention in Pts with STEMI* (*PROMISE*) (www.clinicaltrials.gov identifier NCT00781404). This study will test the effect of a brief intracoronary infusion of adenosine applied at the time of reperfusion to limit infarct size and left ventricular remodelling in patients with acute coronary syndrome with ST segment elevation. Endpoints are infarct size measured by NMR and changes in left ventricular volume and ejection fraction.

3. Pain. Investigators at Wake Forest University are investigating *Clonidine versus Adenosine to Treat Neuropathic Pain* (www.clinicaltrials.gov identifier NCT00349921). Activation of adenosine receptors has been shown to inhibit pain via A_1 receptors in the spine (Borghi et al., 2002; Hussey et al., 2007) or secondary to inhibiting inflammation as a consequence of A_{2A}R activation. The purpose of this study is to compare the effects of intrathecal clonidine and adenosine on neuropathic thermal pain. Another pain study was conducted by Xsira Pharmaceuticals (Morrisville, NC) entitled *Dose Response of Adenosine for Perioperative Pain* (www.clinicaltrials.gov identifier NCT00298636). This was a dose-response trial of intravenous adenosine for perioperative analgesia in women undergoing abdominal hysterectomy or myomectomy.

B. Dipyridamole

The therapeutic use of adenosine is limited by its very short half-life in vivo. Dipyridamole continues to be investigated as an agent that can elevate adenosine levels for several hours as a result of its activity as an inhibitor of the equilibrative nucleoside transporter.

1. Coronary Vasodilation. Investigators at Hillel Yaffe Medical Center are exploring *"Normal Coronary Artery" with Slow Flow Improved by Adenosine Injection, Dipyridamole Treatment, and Clinical Follow-up* (www.clinicaltrials.gov identifier NCT00960817). Patients undergoing coronary angiography with normal coronary but slow blood flow that was normalized after adenosine injection into the coronary artery will be selected for this study. The investigators believe that these patients have small-vessel coronary disease that precedes anatomical narrowing of large vessels. To alleviate this phenomenon, the investigators will examine the long-term clinical response to treatment with dipyridamole.

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2. Schizophrenia. Investigators at the University of Maryland are conducting a *Clinical Trial of Dipyridamole in Schizophrenia* (www.clinicaltrials.gov identifier NCT00349973). In the central nervous system, adenosine signaling through A_{2A} receptors generally counteracts dopamine signaling. Published data suggest efficacy of dipyridamole in treating psychosis when added to haloperidol treatment. This trial will test oral dipyridamole in symptomatic patients with a *Diagnostic and Statistical Manual of Mental Disorders* (American Psychiatric Association, 1994) diagnosis of schizophrenia, schizoaffective disorder, or schizophreniform disorder. The study aims to provide preliminary estimates of whether the effect sizes of dipyridamole on positive symptoms, negative symptoms, and cognitive deficits differ between patients with schizophrenia treated with dipyridamole and those treated with olanzapine.

C. Synthetic Adenosine Receptor Agonists

A number of synthetic agonists for the adenosine receptors have been or are being evaluated in clinical studies.

1. A_1 Partial Agonists. A_1 agonists have the potential to produce heart block by effects on cardiomyocytes, reduce pain by effects on neurons in the spine, and inhibit lipolysis by effects on adipocytes. Partial agonists are being investigated in an attempt to influence pain and lipolysis without producing heart block.

*a. GW493838 (**5**).* Investigators at GlaxoSmithKline designed *The Study of GW493838, an Adenosine A_1 Agonist, in Peripheral Neuropathic Pain* (www.clinicaltrials.gov identifier NCT00376454). The purpose of this study was to determine the analgesic effect of GW493838 in patients with postherpetic neuralgia or peripheral nerve injury caused by trauma or surgery.

*b. GS9667 (**14**).* Investigators at Gilead Sciences are investigating this partial A_1 agonist as a possible treatment for hypertriglyceridemia associated with diabetes.

*c. Capadenoson (**15**).* Bayer Schering Pharma is in phase II for the treatment of atrial fibrillation (www.clinicaltrials.gov identifier NCT00568945).

2. A_{2A} Agonists. Clinical uses of A_{2A} agonists exploit the effects of A_{2A} receptor activation to produce vasodilation and to reduce inflammation by activating receptors on several cells of the immune system.

*a. Regadenoson (**22**).* In 2008, the Food and Drug Administration approved Regadenoson (CVT 3146) for stress testing in conjunction with myocardial perfusion imaging (Al Jaroudi and Iskandrian, 2009). The side effects of regadenoson are similar to adenosine, but unlike adenosine, which is infused in the coronary artery, regadenoson can be given as an intravenous bolus. Apadenoson is currently in phase III clinical trails for the same indication. In phase II clinical trials, apadenoson displayed a much improved side-effect profile compared with adenosine.

b. BVT.115959. Biovitrum has completed a clinical trial entitled *Efficacy and Tolerability of Novel A_{2A} Agonist in Treatment of Diabetic Neuropathic Pain* (www.clinicaltrials.gov identifier NCT00452777). The purpose of this study was to evaluate the efficacy and tolerability of BVT.115959 in patients with diabetes with neuropathic pain.

3. A_3 Agonists. A_3 receptors have been found to be highly expressed on some tumors and activated neutrophils (Ohaion et al., 2009; Varani et al., 2010, 2011). The physiology of A_3 signaling is especially complex because of major differences between rodent and primate species in receptor function and tissue distribution. Orally active agonists are being tested in inflammatory diseases such as psoriasis and rheumatoid arthritis, and in cancer, and efficacy has been demonstrated in phase II trial for dry eye disease (Avni et al., 2010).

*a. CF101 (**25**).* Investigators at Can-Fite BioPharma have designed *Oral CF101 Tablets Treatment in Patients with Rheumatoid Arthritis* (www.clinicaltrials.gov identifier NCT00556894). This trial tested the hypothesis that the administration of IB-MECA (CF101) reduces inflammation in patients with rheumatoid arthritis. In the *Safety and Efficacy of Daily CF101 Administered Orally in Subjects with Elevated Intraocular Pressure* (www.clinicaltrials.gov identifier NCT01033422) study, treatment will be with CF101 or placebo for 16 weeks in a phase II trial to test the hypothesis that CF101, administered orally, will reduce intraocular pressure in patients with ocular hypertension and/or glaucoma. The *Trial of CF101 to Treat Patients with Dry Eye Disease* (www.clinicaltrials.gov identifier NCT01235234) will involved treatment with CF101 or placebo for 24 weeks in a phase III trial. Disease activity will be assessed using evaluations of ocular surface integrity, tear production, and patient symptoms.

*b. CF102 (**26**). A Phase 1–2 Study of CF102, an A_3 Agonist, in Patients with Advanced Hepatocellular Carcinoma* (www.clinicaltrials.gov identifier NCT00790218) will test the safety and efficacy of CF102 (Cl-IB-MECA) in patients with advanced liver cancer. Successive groups of patients will be given higher doses of CF102 by mouth on a twice-daily basis. Treatment will be assessed for adverse effects and for effects on the tumor. *A Phase 1–2 Study of CF102 in Patients with Chronic Hepatitis C Genotype 1* (www.clinicaltrials.gov identifier NCT00790673) will test the hypothesis that CF102 can safely and effectively suppress viral load in patients with chronic hepatitis C and high circulating levels of virus. The trial will monitor the safety of twice-daily oral dosing with CF102 over a 16-week period, will measure changes in viral load during therapy, and will measure blood concentrations of CF102 at various time points during dosing.

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D. Adenosine Receptor Antagonists

The approved methylxanthines theophylline, aminophylline (theophylline ethylenediamine), and caffeine are all nonselective adenosine receptor antagonists that are being used to investigate the consequences of adenosine receptor signaling in man.

1. Caffeine. Investigators at Radboud University/ The Netherlands Organization for Health Research and Development evaluated *The Effect of Caffeine on Ischemic Preconditioning* (www.clinicaltrials.gov identifier NCT00184912). Brief periods of ischemia render the myocardium more resistant to a subsequent more prolonged period of ischemia and reperfusion. This phenomenon is referred to as ischemic preconditioning. Animal studies have provided evidence that adenosine receptor stimulation is an important mediator of ischemic preconditioning. Because caffeine is an effective adenosine receptor antagonist at concentrations reached after coffee consumption, this study showed that caffeine impairs preconditioning in humans in vivo as assessed using ^{99m}Tc-annexin A5 scintigraphy in forearm skeletal muscle (Riksen et al., 2006).

Investigators at McMaster University have designed the *Caffeine for Apnea of Prematurity-Sleep* (*CAP-S*) *Study* (www.clinicaltrials.gov identifier NCT01020357). Apnea of prematurity is a common condition that is usually treated with methylxanthines. Little is known about the long-term effects of methylxanthines on the developing brain. This is a placebo-controlled randomized trial of methylxanthine therapy for apnea of prematurity. This substudy is designed to take advantage of a cohort of ex-premature 5 -to 7-year-old children who were randomized at birth to receive either caffeine or placebo and are currently receiving detailed neurocognitive and behavioral assessments in the CAP trial.

2. Aminophylline. Investigators at King Faisal University investigated *Aminophylline for Entropy Recovery after Sevoflurane Anaesthesia* (www.clinicaltrials.gov identifier NCT01022151). The use of general anesthesia in fast-tracking outpatient setting represents a great challenge because the residual anesthetic effects may delay home discharge after surgery. Sevoflurane has been advocated for the routine anesthesia for ambulatory surgery patients. Sevoflurane activates adenosine A_1 receptors in primary rat hippocampal cultures through the liberation of adenosine secondary to the interaction with adenosine transporters or key enzymes in adenosine metabolism. Aminophylline in doses of 3 to 5 mg/kg hastens recovery from sevoflurane anesthesia and improves bispectral index scores with associated significant increases in heart rate. This study investigates the use of lower doses of aminophylline (2–3 mg/kg) to hasten the recovery from sevoflurane anesthesia with the possible avoidance of significant tachycardia.

3. Selective A_1 Antagonists. Preglomerular arterioles in the kidney are unusual in that they constrict in response to adenosine A_1 receptor activation. Several companies are evaluating A_1 antagonists as diuretics.

*a. Rolofylline (**37**).* Investigators at NovaCardia and Merck have designed *PROTECT-2: A Study of the Selective A_1 Adenosine Receptor Antagonist KW-3902 for Patients Hospitalized with Acute HF and Volume Overload to Assess Treatment Effect on Congestion and Renal Function.* This study demonstrated that rolofylline [1,3-dipropyl-8-(3-noradamantyl)xanthine (KW-3902)] administered intravenously resulted in improvement in signs and symptoms of heart failure, with less treatment failure than standard therapy (Cotter et al., 2008). However, PROTECT-2 did not demonstrate clinical efficacy.

Other A_1 antagonists being investigated for this indication include *trans*-4-[(2-phenyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino]cyclohexanol [SLV320 (**41**); Abbott Laboratories] and 3-(4-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-bicyclo(2.20.2)oct-1-yl)propionic acid [BG9928 (**39**); tonapofylline; Biogen ldec, Weston, MA], but the negative outcome in PROTECT-2 may influence their fate.

4. Selective A_{2A} Antagonists. Selective A_{2A} blockers are being evaluated for the treatment of Parkinson's disease and for drug addiction on the basis of these compounds' ability to simulate activation of dopamine D_2 receptors (Svenningsson et al., 1999; Jenner et al., 2009; Pinna, 2009). The selective A_{2A} antagonists (*E*)-1,3-diethyl-8-(*m*,*p*-dimethoxystyryl)xanthine [KW6002 (**43**); istradefylline; Kyowa-Hakko Kogyo, Tokyo, Japan] has been extensively studied in large phase III studies (Hauser et al., 2008; LeWitt et al., 2008; Stacy et al., 2008) Other antagonists including vipadenant [BIIB014 (**47**), V2006; Vernalis and Biogen-Idec], SCH442416 (**52**), preladenant [SCH-420814 (**51**); Schering Plough, Kenilworth, NJ], and ST-1535 (**49**) have been in phase I and II clinical trials for Parkinson's disease. Vipadenant and preladenant are effective in reducing the waking time during the Off state in patients with late-stage Parkinson's disease who are receiving L-DOPA. Vipadenant is also an effective monotherapy during the early stages of Parkinson's disease, but its development has been stopped because of preclinical toxicological issues.

Investigators at the National Institute on Drug Abuse are conducting *An fMRI Study of SYN115 in Cocaine Dependent Subjects* (www.clinicaltrials.gov identifier NCT00783276). The dopamine system is critical in modulation of reward and has been implicated in the initiation and maintenance of addiction. Medications that increase dopamine either directly or indirectly may have efficacy at reducing cocaine use in cocaine dependent subjects. This study examines the acute effects of A_{2A} receptor antagonist 4-hydroxy-4-methyl-piperidine-1-carboxylic acid-(4-methoxy-7-morpholin-4-yl-benzothiazol-2-yl)-amide (SYN115; **44**) (Synosia Therapeutics, Basel, Switzerland) on brain function and behavior in cocaine-dependent subjects with the use of functional magnetic resonance imaging.

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5. Selective A_{2B} Antagonists. A_{2B} receptor blockers inhibit the degranulation of activated human mast cells and the release of cytokines for several other cell types. In addition, A_{2B} blockade reverses insulin resistance in animal models of type II diabetes. Hence, A_{2B} blockers are in development for the treatment of asthma and diabetes. A selective A_{2B} antagonist is being developed in a partnership between Clinical Data, Inc. (Newton, MA) and Novartis (Basel, Switzerland). GS 6201 [CVT-6883 (**56**)] is being developed by Gilead Sciences (Foster City, CA).

Thus, there are several recent interesting developments in the efforts to bring adenosine-receptor directed drugs to the clinic.

IX. Concluding Remarks

Adenosine receptors constitute one of the best studied and characterized subfamilies of 7TM receptors. The physiological roles have been examined in vivo and in vitro using both genetic and pharmacological tools. There are rather good agonists and antagonists for all four receptors. There is evidence that adenosine receptors are potential drug targets in several areas, and several clinical studies are ongoing. The fact that adenosine receptors influence a large number of processes, both physiological and pathological, introduces a measure of caution regarding widespread long-term use. Conversely, the widespread consumption of caffeine in doses that do block adenosine receptors, with few apparent negative consequences, argues that adenosine receptors can be targeted.

The following are among the more important open pharmacological questions:

- How are the levels of adenosine regulated in different tissues and different conditions (physiological and pathological)?
- What is the functional significance of multimeric (homomeric or heteromeric) adenosine receptors? Do they offer pharmacological opportunities?
- Do all antagonists bind to the same states and sites? Are there major differences between receptors?
- Is it possible to tap into the potential for specificity offered by allosteric ligands or partial agonists?
- What will happen in the several clinical trials?
- Should drugs targeting adenosine receptors be used alone or as adjuvant to other therapies?

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