International Union of Basic and Clinical Pharmacology. CXII: adenosine receptors: a further update

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1521-0081/74/2/340–372
PHARMACOLOGICAL REVIEWS

dx.doi.org/10.1124/pharmrev.121.000445
Pharmacol Rev 74:340–372, April 2022

ASSOCIATE EDITOR: ELIOT OHLSTEIN

International Union of Basic and Clinical Pharmacology. CXII: Adenosine Receptors: A Further Update[S]

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This research was supported in part by the Intramural Research Program of the National Institutes of Health [National Institute of Diabetes and Digestive and Kidney Diseases]. This work was supported by National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grant ZIADK31117]; the Deutsche Forschungs Gemeinschaft [Grants FOR2372, SFB1328, and GRK1873]; La Caixa Foundation [Grant LCF/PR/HP17/52190001]; Centro 2020 [Grants CENTRO-01-0145-FEDER-000008:BrainHealth 2020 and CENTRO-01-0246-FEDER-000010]; and Fundação para a Ciência e a Tecnologia [Grant UIDB/04539/2020].

Financial disclosure: Bruce N. Cronstein is founder and Chairman of the Scientific Advisory Board of Regenosine, LLC.

dx.doi.org/10.1124/pharmrev.121.000445.

***Abstract*——Our previous International Union of Basic and Clinical Pharmacology report on the nomenclature and classification of adenosine receptors (2011) contained a number of emerging developments with respect to this G protein-coupled receptor subfamily, including protein structure, protein oligomerization, protein diversity, and allosteric modulation by small molecules. Since then, a wealth of new data and results has been added, allowing us to explore novel concepts such as target binding kinetics and biased signaling of adenosine receptors, to examine a multitude of receptor structures and novel ligands, to gauge new pharmacology, and to evaluate clinical trials with adenosine receptor ligands. This review should therefore be considered a further update of our previous reports from 2001 and 2011.**

***Significance Statement*——Adenosine receptors (ARs) are of continuing interest for future treatment of chronic and acute disease conditions, including inflammatory diseases, neurodegenerative afflictions, and cancer. The design of AR agonists ("biased" or not) and antagonists is largely structure based now, thanks to the tremendous progress in AR structural biology. The A_{2A}- and A_{2B}AR appear to modulate the immune response in tumor biology. Many clinical trials for this indication are ongoing, whereas an A_{2A}AR antagonist (istradefylline) has been approved as an anti-Parkinson agent.**

I. Introduction

A decade has passed since our last International Union of Basic and Clinical Pharmacology report on the nomenclature and classification of adenosine receptors appeared (Fredholm et al., 2011), after the first one in 2001 (Fredholm et al., 2001). The field has matured to the extent that the recommendations on the nomenclature stand firmly and require neither changes nor refinements. Substantial developments, however, took place (Fredholm et al., 2021), and these alone warrant a further update already. The adenosine A_{2A} receptor (A_{2A}AR) has become a test case for G protein-coupled receptor (GPCR) structure elucidation, whereas structures of the adenosine A_1 receptor (A_1AR) have also become available. The structures have been obtained through either X-ray crystallography or a more recent development, cryo-electron microscopy (EM). These together constitute a huge variety, most of which were determined with different antagonist ligands, a few with agonistic ligands with or without (parts of the) G protein present, and one with a partial agonist. Secondly, the increasing awareness that the study of target binding kinetics reveals more details on the interaction between ligand and receptor has had its effect on the further and more detailed kinetic characterization of adenosine receptor ligands, both agonists and antagonists. Moreover, there is ample attention again for novel ligands interacting with adenosine receptors. Some of these newer and older ligands possess a preference for biased signaling (i.e., the preferred coupling to particular signaling pathways), most notably through different G proteins or β-arrestin. Furthermore, there is an in-depth analysis of the (patho)pharmacological aspects of adenosine receptors and their ligands, both in the periphery and the central nervous system (CNS), leading to an evaluation of the receptors' relevance in diverse disease states including COVID-19 infection and in aging. The report is concluded with a (nonexhaustive) overview of the clinical trials with adenosine receptor ligands in the last ten years. Disappointing were the outcomes for A_1AR partial agonists, with lack of efficacy in heart failure noted in advanced phase 2b clinical studies. On the other hand, an A_{2A}AR antagonist was approved in the United States as a new anti-Parkinsonian drug,

ABBREVIATIONS: ADA, adenosine deaminase; AdoK, adenosine kinase; AR, adenosine receptor; CCPA, 2-chloro-N^6-cyclopentyladenosine; CNS, central nervous system; EM, electron microscopy; GFR, glomerular filtration rate; GPCR, G protein-coupled receptor; h, human; K_D, equilibrium dissociation constant; MECA, methylcarboxamidoadenosine; NASH, nonalcoholic steatohepatitis; NECA, 5′-N-ethylcarboxamidoadenosine; PAM, positive allosteric modulator; PD, Parkinson's disease; PDB, Protein Data Bank; RT, residence time; TM, transmembrane domain; WT, wild-type.

and the role of adenosine receptors in immunology has led to a surge of ongoing studies in immuno-oncology, particularly with A_{2A}AR, A_{2B}AR, or dual A_{2A}AR/A_{2B}AR antagonists.

II. Receptor Ligands (for Structures, See Figs. 1, 2, 3, and 5)

Adenosine receptors (ARs) have become established drug targets. Adenosine (1, Fig. 1) itself is used as an injectable diagnostic for cardiac imaging to dilate the coronary arteries via A_{2A}AR activation of patients who cannot exercise on a treadmill (Singh and McKintosh, 2020). The short half-life of under 10 seconds prevents severe side effects of concomitant A_1AR activation, such as cardiac block. Moreover, adenosine is applied in supraventricular tachycardia due to its antiarrhythmic effects (Singh and McKintosh, 2020). The A_{2A} AR-selective agonist regadenoson (2, Table 1), used for the same purpose, displays a longer half-life of 2–3 minutes and is of benefit for patients who develop bronchospasms upon treatment with adenosine (Thomas et al., 2017; Patel and Alzahrani, 2020). The natural products caffeine (3) and theophylline (4), xanthine alkaloids present in plants (e.g., *Coffea arabica* and *Camellia sinensis*), are moderately potent, nonselective AR antagonists (see Table 2 for receptor affinities) that have been used for thousands of years (Daly, 2007; Müller and Jacobson, 2011b; van Dam et al., 2020). There is epidemiologic evidence linking coffee and tea consumption with different health benefits (Grosso et al., 2016; Poole et al., 2017; van Dam et al., 2020). Caffeine, probably the most widely applied psychoactive drug in the world and broadly used for recreational purposes, is therapeutically applied as a central nervous system (CNS) stimulant, for preterm infants to support breathing function, and in combination therapeutics with analgesics to treat pain and colds (Abo-Salem et al., 2004; Lipton et al., 2017; Alhersh et al., 2020; Evans et al., 2020; van Dam et al., 2020). Several ongoing clinical trials (see also Chapter VII) are evaluating caffeine for various indications including cognition, pain, obesity, cataract prevention, and others. Theophylline, which is less brain-permeant than caffeine, is used for the treatment of asthma, but due to its narrow therapeutic window and the availability of safer and more potent alternative therapeutics, it has lost its importance and nowadays serves as a third-line treatment of add-on therapy only (Barnes, 2003; Tilley, 2011; Journey and Bentley, 2020). Both caffeine and theophylline also interact with other targets (e.g., they inhibit phosphodiesterases), but many of these effects are only observed at high, nonphysiologic concentrations. Most of the desired effects of caffeine and theophylline can in fact be explained by a blockade of ARs. It has to be noted that both xanthine derivatives are about equally potent at all four human AR subtypes, but they are inactive at rodent A_3ARs (see Table 2). The A_{2A}AR-selective antagonist istradefylline (5), a xanthine derivative that is structurally derived from caffeine, has been approved for the treatment of Parkinson's disease (PD) in combination with levodopa, initially only in Japan (in 2013) but now also in the United States (in 2019), whereas the approval process in Europe is in progress (Takahashi et al., 2018; Chen and Cunha, 2020; Jenner et al., 2021). Due to intensive research for several decades aimed at developing AR ligands, a large number of subtype-selective agonists and antagonists has been developed (for reviews, see Müller and Jacobson, 2011a; Jacobson and Müller, 2016; Jacobson et al., 2019; Jacobson et al., 2021). The rather modest success in drug approvals despite a large number of clinical trials discouraged scientists and pharmaceutical companies. However, the recent approval of the A_{2A}AR antagonist istradefylline in the United States and, in particular, the 'gold rush fever' in immuno-oncology centered around adenosine as an immunosuppressant (Boison and Yegutkin, 2019; Borah et al., 2019; Allard et al., 2020; Willingham et al., 2020; Thompson and Powell, 2021) have newly energized and fueled the field.

This chapter will provide guidance in selecting tool compounds for research on ARs. Rather than presenting a comprehensive collection of AR ligands for which the reader be referred to previous review articles selected (Fredholm et al., 2011; Müller and Jacobson, 2011a; Jacobson and Müller, 2016; Jacobson et al., 2021), preferably well characterized ligands will be discussed that are recommended as tool compounds. Whenever possible, not only data for human ARs but also those for rat and mouse orthologs will be listed since considerable species differences have been observed in some cases, which are most pronounced for the A_3AR subtype (Alnouri et al., 2015; Du et al., 2018). For most receptor subtypes, at least two different agonists and antagonists will be included. In addition, useful physicochemical and pharmacokinetic data have been collected if available.

A. Adenosine Receptor Agonists

The physiologic agonist adenosine (1) is more potent at A_1-, A_{2A}- and A_3ARs than at A_{2B}ARs in most settings (see Table 1). However, reliable radioligand binding data cannot be obtained since adenosine is present in tissues, cells, and even cell membrane preparations and is constantly produced (e.g., from released ATP by ectonucleotidases) (Zimmermann, 2021). Therefore, it usually has to be removed, which is achieved by preincubation or addition of adenosine deaminase (ADA). Thus, ADA and its reaction product inosine are typically present during incubation with radioligand and test compound. ADA itself can allosterically modulate ARs (Gracia et al., 2013). In

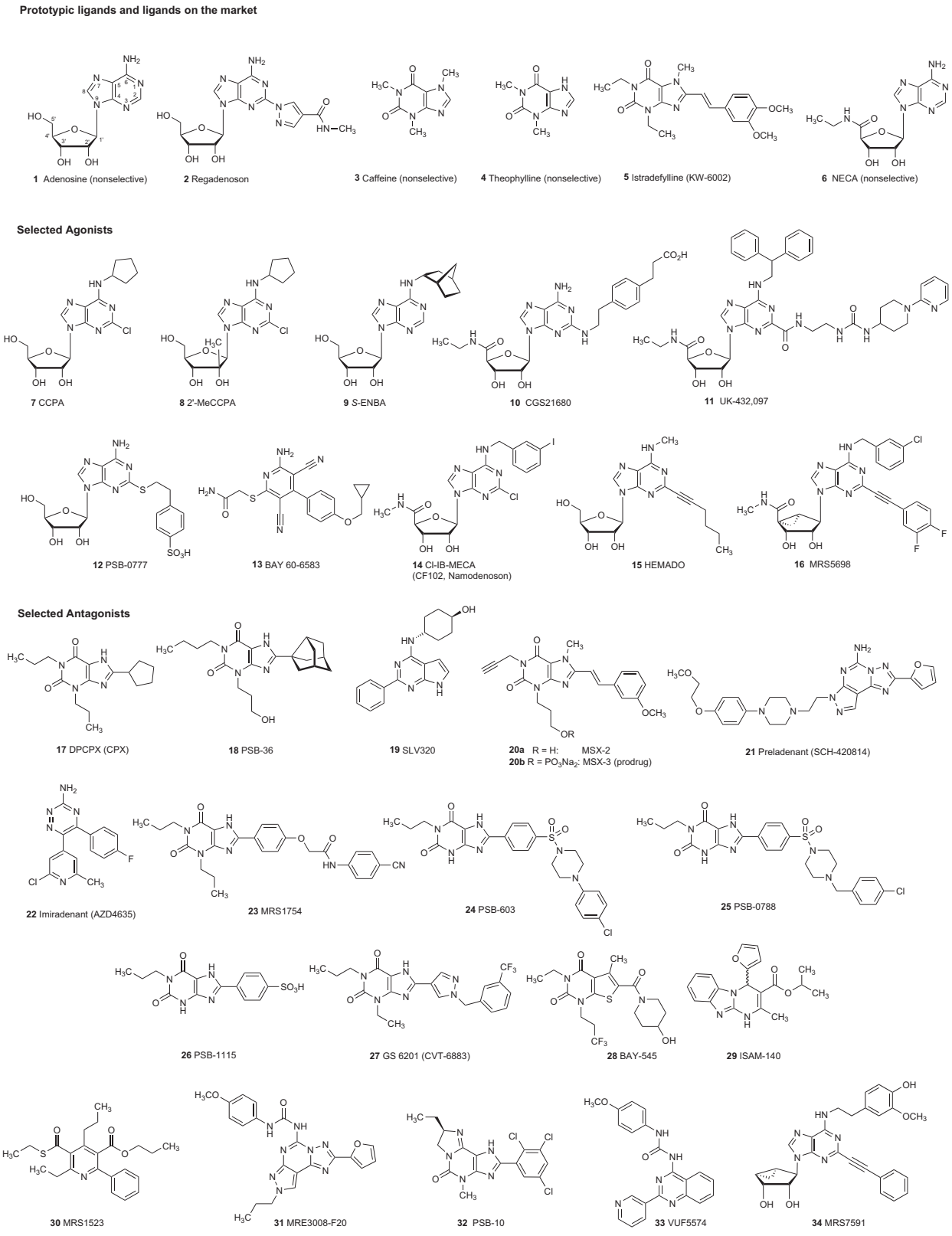

Fig. 1. Selected ligands for studying ARs.

contrast to radioligand binding data, potencies determined in functional, G protein-dependent assays such as cAMP accumulation studies depend on receptor expression levels and receptor reserve, and concentration-effect curves are shifted to the left with increased receptor expression levels (Fujioka and Omori, 2012).

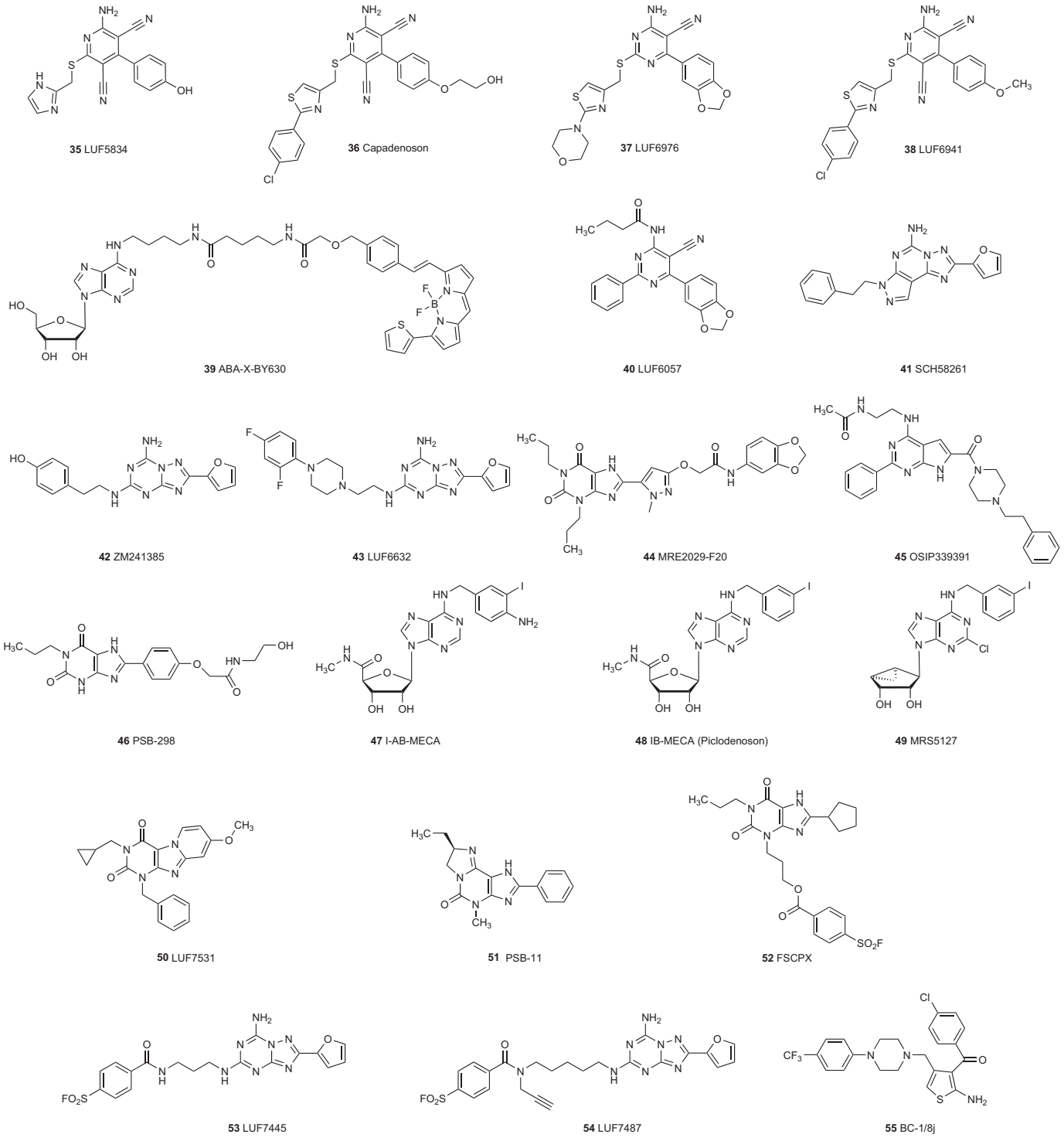

Fig. 2. Ligands investigated in kinetic studies.

Therefore, EC_{50} values of agonists obtained in different cellular systems are not comparable. As mentioned before, adenosine has a short half-life being metabolized by ADA or adenosine kinase (AdoK) after removal by cellular uptake through nucleoside transporters, which can additionally influence results. For that reason, metabolically (more) stable adenosine analogs have been developed. Nevertheless, it becomes increasingly clear that synthetic ligands do not necessarily induce the same effects at a certain receptor as the cognate agonist (e.g., regarding the activation of intracellular signaling pathways) (see also Chapter V). Therefore, if possible, adenosine should always be included in pharmacological studies besides more stable and selective synthetic

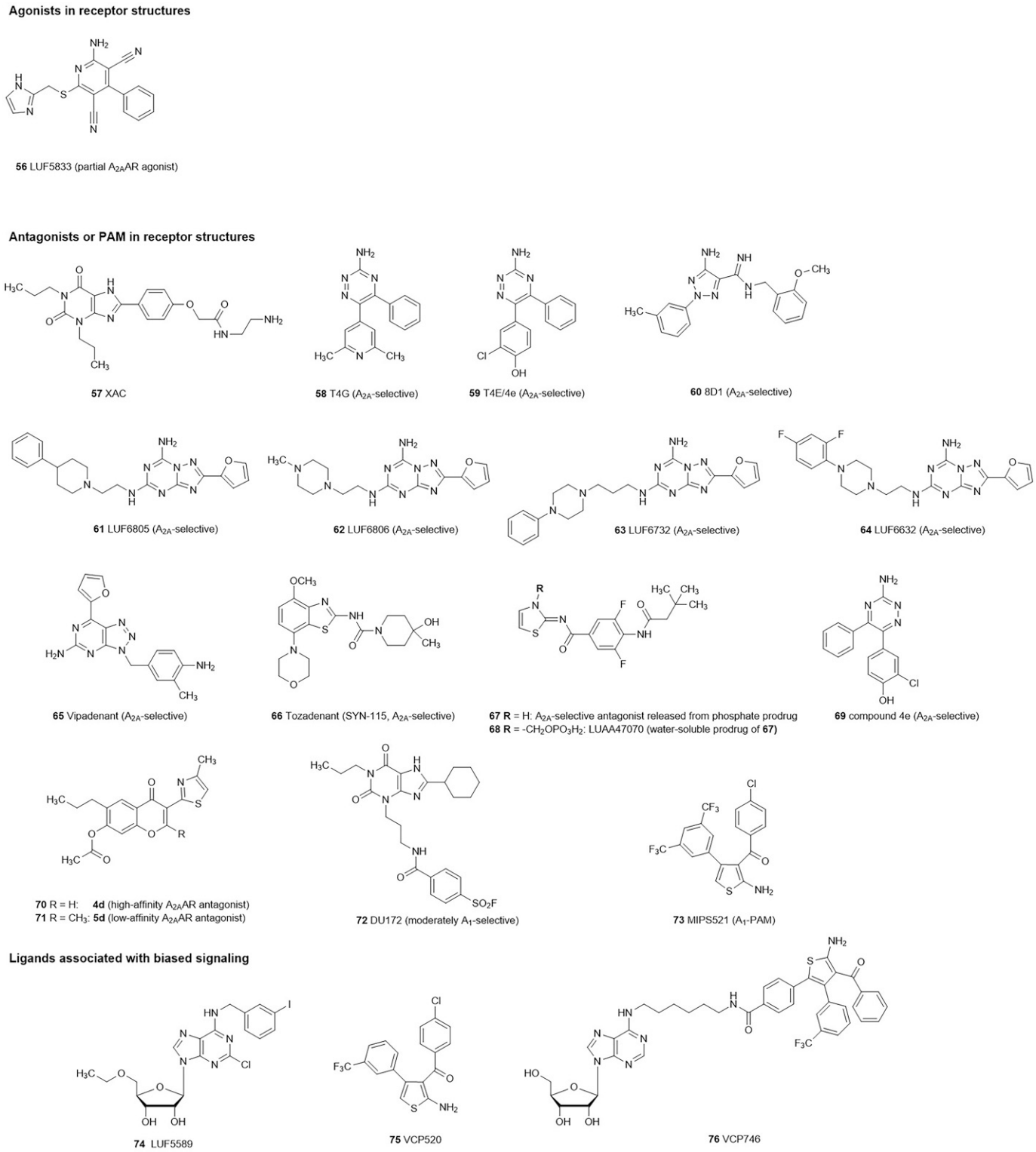

Fig. 3. Ligands in 3D receptor structures and ligands in biased signaling studies. (Note: In 67 and 68, the X-ray structure of "LUAA47070" was not obtained with the prodrug LUAA47070 but with the A_{2A}AR antagonist that is released from the prodrug upon hydrolysis.)

agonists. The closely related adenosine analog 5′-N-ethylcarboxamidoadenosine (NECA, 6) cannot be metabolized by ADA or AdoK. Similar to adenosine, NECA is significantly more potent at A_1-, A_{2A}-, and A_3ARs than at A_{2B}ARs. There is a lack of potent, selective, and fully efficacious A_{2B}AR agonists; NECA is still one of the more potent full agonists at the A_{2B}AR and represents a useful tool to study A_{2B}ARs in combination with selective antagonists for the other AR subtypes (Verzijl and IJzerman, 2011; Müller et al., 2018; Franco et al., 2021b).

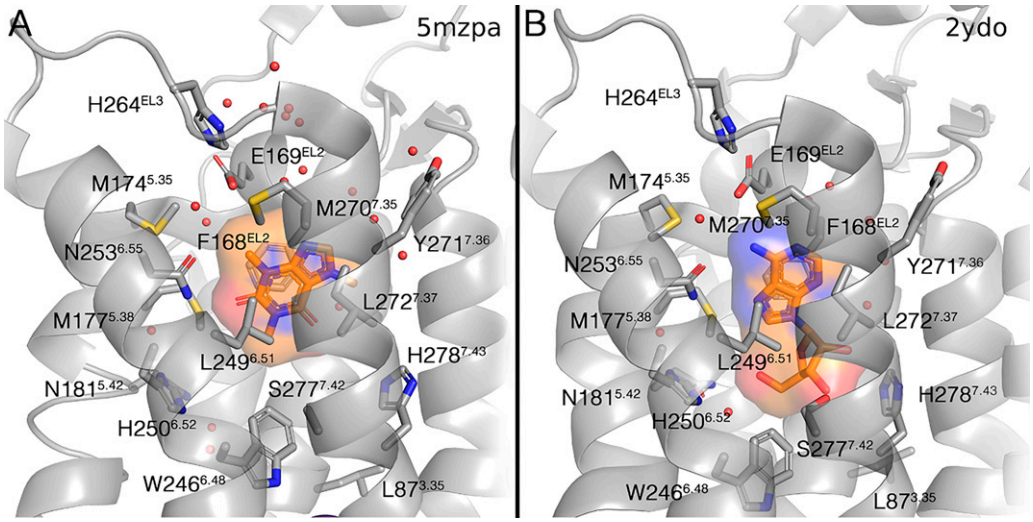

Fig. 4. Overview of the A_{2A}AR binding site, showing the first frame of two movies: (A) antagonist (Supplemental Video 1) and (B) agonist (Supplemental Video 2). All residues within 2Å of a given ligand in an A_{2A}AR structure were considered as the binding pocket, and this selection was maintained in all frames. The residues are labeled according to the wild-type (WT) sequence, and modifications made to the receptor (e.g., the thermostabilizing mutant $S277^{7.42}A$) are not taken into account; note that no labeling is used in the supplemental movie files. The Ballesteros-Weinstein numbering is given in superscript. Ligands are shown in orange, with a volumetric occupancy surface-colored on the atom type. Water atoms in the binding site are shown as red dots, and the sodium ion (when present) as a purple sphere. If alternate coordinates were given in the extracted PDB file, the 'A' coordinates were maintained, except in the case of caffeine, in which case we generated two separate frames (referred to as 5mzpa and 5mzpb) to show the two binding modes in the crystal structure. Only distinctly different binding modes of ZM241385 (as present in 4EIY and 3PWH) are included in the movie.

Potent, truly selective A_1AR agonists have been developed by N^6-substitution of adenosine (see Table 1 and Fig. 2). 2-chloro-N^6-cyclopentyladenosine (CCPA, 7) is suitable for rat and mouse studies, where it shows > 100-fold selectivity versus the other AR subtypes, whereas it is less selective in humans versus the A_3AR subtype (46-fold). For studies at the human A_1AR, its 2′-methyl-substituted derivative 2′-MeCCPA (8) can be used, which is more selective (>300-fold) in humans (Franchetti et al., 2009). Data at rat and mouse ARs are not available for this compound. Another potent and selective A_1AR agonist is (*S*)-ENBA (9), possessing a bulky bicyclo[2.2.1]hept-2-yl moiety at the N^6-position that confers A_1AR selectivity.

A_{2A}AR-selective agonists have been obtained by introducing large, bulky substituents into the 2-position of adenosine or NECA, in some cases in combination with an additional bulky N^6-substituent. Most of the developed compounds are only moderately selective in humans versus the A_1- or A_3AR subtypes. CGS21680 (10) is a potent and A_{2A}AR-selective agonist in rat and mouse but shows only moderate selectivity in humans (vs. A_1- and A_3ARs; see Table 1). However, in some studies on mouse brain, 10 has been observed to additionally bind to A_1ARs (Lopes et al., 2004). The reason for this observation is still unclear; one explanation could be the formation of heteromeric receptor complexes showing a different pharmacology. The 2,N^6-disubstituted NECA derivative 11 (UK-432,097; Table 1) is potent at the human A_{2A}AR and was reported to also be selective. Compound 11 is a relatively large and lipophilic molecule that is less water-soluble than other adenosine derivatives and analogs. It showed a long receptor residence time of 250 minutes at 5°C (see Table 3), which probably contributed to its successful cocrystallization with the human A_{2A}AR (Xu et al., 2011). PSB-0777 (12), bearing a phenylsulfonate group, is well soluble in water and has been useful for injection or for local application in the gut since it is not perorally absorbed due to its negative charge. It shows high selectivity in rats but not in humans and is thus useful for studies in rodents. Regadenoson (2) is only moderately potent but selective in humans and is clinically used as a diagnostic (see above). Importantly, in tissues with higher A_1AR versus A_{2A}AR density such as the brain, (moderately selective) A_{2A}AR agonists often bind to and activate A_1AR rather than A_{2A}AR (Zhang et al., 1994; Cunha et al., 1996; Halldner et al., 2004; Pliássova et al., 2020). Thus, potent and really selective A_{2A}AR agonists to target central A_{2A}ARs are still required.

So far, potent and selective full agonists for the A_{2B}AR are not available. BAY 60-6583 (13), a non-nucleoside aminopyridine derivative, behaves as a partial A_{2B}AR agonist (Hinz et al., 2014) but was shown to act as an antagonist at other AR subtypes (Alnouri et al., 2015). In the presence of high adenosine concentrations, it can even inhibit A_{2B}AR activation (Hinz et al., 2014; Alnouri et al., 2015). Data obtained with 13 are therefore difficult to interpret. BAY 60-6583 may induce a different A_{2B}AR conformation than adenosine or NECA; for example, it has been shown that BAY 60-6583 does not induce calcium mobilization via A_{2B}AR-mediated G_q protein activation in human embryonic kidney (HEK) cells with low endogenous A_{2B}AR expression in contrast to adenosine or NECA

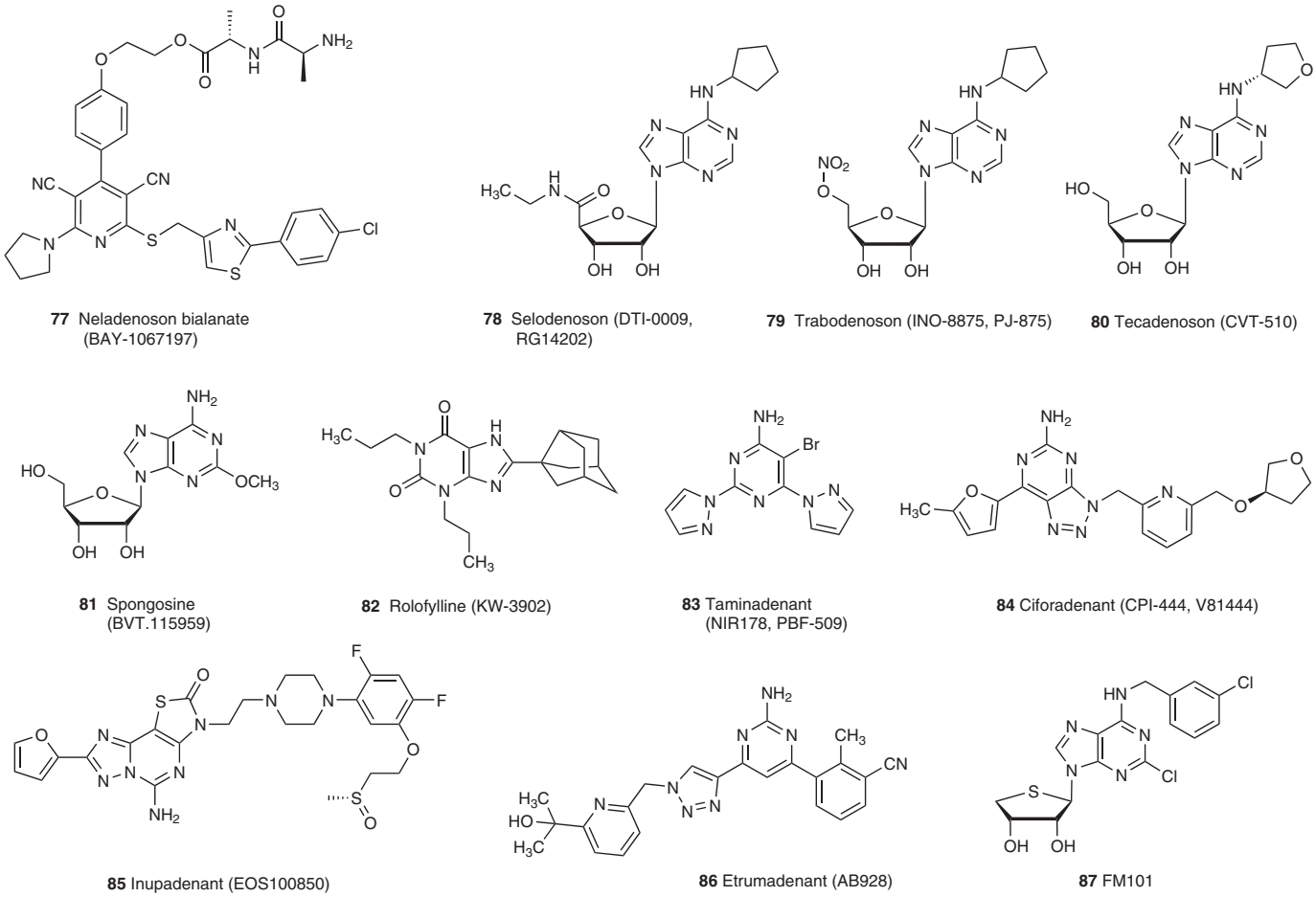

Fig. 5. Ligands in clinical studies.

(Hinz et al., 2014; Gao et al., 2018b). Thus, a potent, selective, efficacious, and unbiased A_{2B}AR agonist is urgently needed. Instead of the partial agonist BAY 60-6583, the full, nonselective agonist NECA (6) may be used in the presence of antagonists for the other AR subtypes.

For the A_3AR, potent and selective agonists are available. Cl-IB-MECA (14, CF102, namodenoson), a 2-chloro-N^6-iodobenzyl-substituted methylcarboxamidoadenosine (MECA) derivative, is being evaluated in clinical trials for the treatment of hepatocellular carcinoma and nonalcoholic steatohepatitis (NASH). For pharmacological studies, especially in mice, the doses of 14 have to be carefully chosen in order not to activate the A_1AR as well (see Table 1). HEMADO (15) is similarly potent and selective in humans. A potent and at the same time selective A_3AR agonist, in human as well as in mouse, is MRS5698 (16).

B. Adenosine Receptor Antagonists

Many potent A_1AR-selective antagonists have been developed based on caffeine and theophylline as lead structures, such as DPCPX (17, CPX) and PSB-36 (18) (Müller and Jacobson, 2011b). Whereas DPCPX shows only moderate selectivity in humans, PSB-36 is highly selective in all three species: human, rat, and mouse (Alnouri et al., 2015). SLV320 (19) is an A_1AR antagonist with a 7-deaza-adenine core structure bearing a cyclohexyl moiety at the exocyclic amino function (Kalk et al., 2007). The compound is potent and selective in humans and displays similar potency in rat, but complete data in rat and mouse are not available.

The xanthine derivative istradefylline (5) was the first A_{2A}AR antagonist to be approved as a drug (Shimada et al., 1992; Takahashi et al., 2018). Its potency and selectivity for the A_{2A}AR is similar in human, rat, and mouse. Although it is highly selective versus the A_{2B}- and A_3AR subtypes, selectivity versus the A_1AR is somewhat lower (50- to 70-fold) (see Table 2). Like many other A_{2A}AR antagonists, it is moderately water-soluble. In addition, the double bond of its styryl residue can undergo light-induced *E*/*Z*-isomerization in dilute solution and is prone to light-induced dimerization in the solid state; therefore, it needs to be protected from light (Nonaka et al.,1993; Hockemeyer et al., 2004). The same is true for MSX-3 (20), a phosphate prodrug of MSX-2, which is, however, well soluble in water (Sauer et al., 2000; Faivre et al., 2018). The A_{2A}AR selectivity of MSX-2 is higher than that of istradefylline (see Table 2). The nonxanthine A_{2A}AR

TABLE 1
Affinities of selected adenosine receptor agonists

		K_i or EC_{50} (nM)[a]			
		A_1	A_{2A}	A_{2B}[b]	A_3
Nonselective Agonists					
1	Adenosine[c]	ca. 100 (h)	310 (h)	15,000 (h)	290 (h)
		73 (r)	150 (r)	5,100 (r)	6,500 (r)
6	NECA	14 (h)	20 (h)	1,890 (h)	25 (h)
		5.1 (r)	9.7 (r)	1,110 (r)	113 (r)
		2.49 (m)	43.4 (m)	656 (m)	13.2 (m)
A_1AR-Selective Agonists					
7	CCPA	0.83 (h)	2270 (h)	18,800 (h)	38 (h)
		1.3 (r)	950 (r)	6,160 (r)	237 (r)
		0.269 (m)	988 (m)	25.300 (m)	15.6 (m)
8	2′-MeCCPA	3.3 (h)	9,580 (h)	37.600 (h)	1.150 (h)
9	(*S*)-ENBA	n.d. (h)	n.d. (h)	n.d.	282 (h)
		0.34 (r)	477 (r)		915 (r)
A_{2A}AR-Selective Agonists					
10	CGS21680	289 (h)	27 (h)	>10,000 (h)	67 (h)
		1800 (r)	19 (r)	>10,000 (r)	584 (r)
		961 (m)	13.7 (m)	>10,000 (m)	93.0 (m)
11	UK-432,097	n.d.	4	n.d.	n.d.
12	PSB-0777	541 (h)	360 (h)	>10,000 (h)	>10,000 (h)
		≥10,000 (r)	44.4 (r)		
2	Regadenoson	>10,000 (h)	290 (h)	>10,000 (h)	>10,000 (h)
A_{2B}AR-Selective (Partial) Agonist					
13	BAY 60-6583	387 (h)	>10,000 (h)	3–10 (h, EC_{50})	223 (h)
		514 (r)	>10,000 (r)	114 (h)	2,750 (r)
		351 (m)	>10,000 (m)	100 (r)	3,920 (m)
				136 (m)	
A_3AR-Selective Agonists					
14	Cl-IB-MECA	220 (h)	5360 (h)	>10,000 (h)	1.4 (h)
	(CF102, Namodenoson)	280 (r)	470 (r)	1,210 (r)	0.33 (r)
		35 (m)	290 (m)	44,300 (m)	0.18 (m)
15	HEMADO	330 (h)	1200 (h)	>30,000 (h)	1.10 (h)
16	MRS5698	>10,000 (h)	>10,000 (h)	assumed to be inactive	3.49 (h)
		>10,000 (m)	>10,000 (m)		3.08 (m)

h, human; K_i, inhibition constant; m, mouse; n.d., no data; r, rat.
[a]data (if available from K_i values from radioligand binding assays) are taken from the literature cited in the text.
[b]most A_{2B}AR data are from functional studies (cAMP accumulation).
[c]adenosine data are from functional studies (cAMP accumulation).

antagonist preladenant (21, SCH-420814) is one of the most potent and selective A_{2A}AR antagonists. It has been evaluated in clinical trials for PD and was found to be well tolerated but did not show significant beneficial effects (Stocchi et al., 2017; LeWitt et al., 2020). As observed with istradefylline, the study design is most critical for these types of clinical PD studies and may have contributed to the negative outcome in the case of preladenant (Hauser et al., 2015). AZD4635 (22, imaradenant) is a potent A_{2A}AR antagonist with moderate selectivity versus the A_{2B}- and A_3ARs subtypes. Despite its relatively low molecular weight (315.7 g/mol) the compound is not readily soluble in water.

In recent years many A_{2B}AR-selective antagonists have been developed (Müller et al., 2018). The xanthine derivative MRS1754 (23) is a potent and selective A_{2B}AR antagonist in humans but not in rats and mice, where it additionally blocks the A_1AR (Kim et al., 2000; Alnouri et al., 2015). One of the most potent and selective A_{2B}AR antagonists in all three species is the 8-sulfophenylxanthine derivative PSB-603 (24) (Borrmann et al., 2009; Alnouri et al., 2015). The compound is metabolically highly stable in human, rat, and mouse. Its main drawback, however, is its low water solubility. The related A_{2B}AR antagonist PSB-0788 (25) (Borrmann et al., 2009; Alnouri et al., 2015) is better soluble, especially under weakly acidic conditions since it bears a basic nitrogen atom that can be protonated. However, it is less metabolically stable and therefore less suitable for in vivo studies. PSB-0788 is moderately selective for A_{2B}- versus A_1ARs in mouse (only about 60-fold) but highly A_{2B}AR-selective in human and rat. PSB-1115 (26) was developed as an A_{2B}AR antagonist with high water solubility due to its sulfonate group (Hayallah et al., 2002). Although the compound is potent and selective in human, it is not selective in rat and mouse and additionally blocks rodent A_1ARs (see Table 2) (Alnouri et al., 2015). The xanthine derivative GS6201 (27, CVT6883) which shows good potency and selectivity for human A_{2B}ARs (Elzein et al., 2008), was evaluated in a phase 1 clinical trial for pulmonary diseases, but further development has not been reported (Kalla and Zablocki, 2009). The compound displayed a half-life of 4 hours and a peroral

TABLE 2
Affinities of selected, useful adenosine receptor antagonists

		K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
Nonselective Antagonists					
3	Caffeine	44,900 (h) 41,000 (r) 50,700 (m)	23,400 (h) 43,000 (r) 11,100 (m)	33,800 (h) 30,000 (r) 23,000 (m)	13,300 (h) >100,000 (r) >100,000 (m)
4	Theophylline	6,770 (h) 14,000 (r)	6,700 (h) 22,000 (r)	9,070 (h) 15,100 (r) 5,630 (m)	22,300 (h) >100,000 (r)
A_1AR-Selective Antagonists					
17	DPCPX (CPX)	3.0 (h) 0.50 (r) 0.413 (m)	129 (h) 157 (r) 263 (m)	51 (h) 186 (r) 86.2 (m)	243 (h) >10,000 (r) >10,000 (m)
18	PSB-36	0.7 (h) 0.124 (r) 1.58 (m)	980 (h) 552 (r) 697 (m)	187 (h) 350 (r) 704 (m)	2,300 (h) 6,500 (r) >10,000 (m)
19	SLV320	1.00 (h) 2.51 (r)	398 (h)	3,981 (h) 501 (r)	200 (h)
A_{2A}AR-Selective Antagonists					
5	Istradefylline (KW6002)	841 (h) 230 (r) 438 (m)	12 (h) 4.46 (r) 6.83 (m)	>10,000 (h) 5,940 (r) 3,590 (m)	4,470 (h) >10,000 (r) >10,000 (m)
20	MSX-3 / MSX-2 (Data are for MSX-2)	2,500 (h) 900 (r)	5.38 (h) 8.04 (r)	>10,000 (h)	>10,000 (h)
21	Preladenant (SCH-420814)	>1,000 (h) >1,000 (h) 462 (m)	0.9 (h) 0.986 (r) 0.241 (m)	>1,000 (h) >1,000 (m) >1,000 (r)	>1,000 (h) >1,000 (m) >1,000 (r)
22	Imaradenant (AZD4635)	160 (h)	1.7 (h)	64 (h)	>10,000 (h)
A_{2B}AR-Selective Antagonists					
23	MRS1754	403 (h) 16.8 (r) 1.45 (m)	503 (h) 612 (r) >10,000 (m)	1.97 (h) 12.8 (r) 3.12 (m)	570 (h) >1,000 (m) >1,000 (r)
24	PSB-603	>10,000 (h) >10,000 (r) 42.4 (m)	>10,000 (h) >10,000 (r) >10,000 (m)	0.553 (h) 0.355 (r) 0.265 (m)	>10,000 (h) >10,000 (r) >10,000 (m)
25	PSB-0788	2,240 (h) 386 (r) 118 (m)	333 (h) 1,730 (r) 235 (m)	0.393 (h) 2.12 (r) 1.90 (m)	>1,000 (h) >10,000 (r) >10,000 (m)
26	PSB-1115	>10,000 (h) 2,200 (r) 591 (m)	3790 (h) 24,000 (r) >10,000 (m)	53.4 (h) 3,140 (r) 1,940 (m)	>10,000 (h) >10,000 (r) >10,000 (m)
27	GS 6201 (CVT-6883)	1,940 (h)	3,280 (h)	22 (h)	1,070 (h)
28	BAY-545	>1,000; 1,300 (h) n.d. (r) >6,700 (m)	>1,000; 820 (h) 750 (r) 470 (m)	59–97 (h) 280 (r) 400 (m)	>10,000 (h) n.d. (r) >6,700 (m)
29	ISAM-140	>1,000 (h)	>1,000 (h)	3.49 (h)	>1,000 (h)
A_3AR-Selective Antagonists					
30	MRS1523	>10,000 (h) 15,600 (r) >10,000 (m)	3660 (h) 2050 (r) >10,000 (m)	>10,000 (h) >10,000 (r) >10,000 (m)	18.9 (h) 113 (r) 731 (m)
31	MRE3008-F20	1200 (h)	141 (h)	2100 (h)	0.82 (h)
32	PSB-10	1,700 (h) 805 (r)	2,700 (h) 6,040 (r)	30,000 (h)	0.441 (h) 17,000 (r)
33	VUF5574	≥10,000 (r)	≥10,000 (r)	n.d.	4.03 (h)
34	MRS7591[b]	>10,000 (h) 590 (m)	>10,000 (h) n.d.	n.d. n.d.	10.9 (h) 17.8 (m)

h, human; K_i, inhibition constant; m, mouse; n.d., no data; r, rat.
[a]data are taken from the literature cited in the text.
[b]partial agonistic activity if receptor is highly expressed.

bioavailability of 35% in rat (Elzein et al., 2008); potency and selectivity in rodents have not been reported. BAY-545 (28) is a recently published A_{2B}AR antagonist with a new scaffold identified by high-throughput screening, although its thienopyrimidine-dione structure resembles the xanthine scaffold (Härter et al., 2019). The compound shows moderate affinity compared with other developed A_{2B}AR antagonists and is more potent at human than at rat and mouse A_{2B}ARs. It is more than 10-fold selective in human but is nonselective in mouse and rat (Härter et al., 2019). Another novel scaffold, a pyrimido[1,2-*a*]benzimidazole,

is represented by ISAM-140 (29), an A_{2B}AR antagonist that shows high potency and selectivity in human. Unfortunately, data from other species are not available (El Maatougui et al., 2016). Subsequently, related dihydropyrimidine derivatives have been developed that are similarly potent and selective (Majellaro et al., 2021).

The A_3AR typically shows large species differences for antagonists (Müller, 2003; Jacobson and Müller, 2016). Most published antagonists that are very potent at human A_3ARs are inactive at the rodent (rat and mouse) orthologs. One of the best A_3AR antagonists for rodent studies is MRS1523 (30). The compound is only moderately potent but very selective in human (>100-fold) and at least somewhat selective in rat (18-fold vs. A_{2A}, >100-fold vs. the other AR subtypes) and mouse (at least 14-fold vs. the other subtypes) (van Rhee et al., 1996; Li et al., 1998; Müller and Jacobson, 2011a; Alnouri et al., 2015; Jacobson and Müller, 2016). Further potent A_3AR antagonists, including MRE3008-F20 (31) (Baraldi et al., 2012; Borea et al., 2015), PSB-10 (32) (Ozola et al., 2003; Alnouri et al., 2015), and VUF5574 (33) (van Muijlwijk-Koezen et al., 2000) are highly potent and selective in human but virtually inactive at rodent A_3ARs (see Table 2). As species differences are more pronounced for A_3AR antagonists than for agonists, most of which are derivatives or analogs of adenosine, compounds with a truncated, furanyl, or carbocyclic moiety in place of the ribose ring of adenosine were investigated and optimized (Jeong et al., 2007; Lee et al., 2010; Nayak et al., 2014; An et al., 2020). Such adenosine analogs show reduced intrinsic activity or even block the receptors. Appropriate substitution on the adenine ring led to MRS7591 (34) showing high affinity for both human and mouse A_3ARs and good selectivity in human (>1000-fold) (Tosh et al., 2020). Selectivity in mouse has only been assessed against the A_1AR (33-fold). It has to be kept in mind that compound 50 behaved as a (weakly efficacious) partial agonist (Tosh et al., 2020).

C. Allosteric Modulators of Adenosine Receptors

The development of allosteric modulators for GPCRs in general is an emerging field of research (Müller et al., 2012; Gao and Jacobson, 2013; Wootten et al., 2013). Positive allosteric modulators (PAMs) increasing the potency or efficacy of agonists, and negative allosteric modulators (NAMs) acting as noncompetitive antagonists, have been reported for various AR subtypes, especially for the A_1AR. The AR-PAMs that have been developed so far display only moderate potency or selectivity, and their usefulness is still unclear (Fredholm et al., 2011; Göblyös and IJzerman, 2011; Jacobson et al., 2011; Müller et al., 2012; Nguyen et al., 2016; Barresi et al., 2021). Interestingly, in a recent cryo-EM structure of the A_1AR, a PAM (MIPS521) was found to be localized in an extrahelical domain (Draper-Joyce et al., 2021). MIPS521's analgesic properties were evaluated in the same paper, reminiscent of earlier attempts to profile another PAM as a potential painkiller (Kiesman et al., 2009).

D. Inosine and Guanosine

Adenosine is metabolized to inosine by adenosine deaminases (ADA-1 and -2). Inosine has been reported by several groups to interact with ARs (e.g., with A_{2A}AR and A_3AR) but only at very high, nonphysiologic concentrations (>100 μM) (Welihinda et al., 2016). On the other hand, inosine (Lovász et al., 2021) as well as the nucleoside guanosine (Di Liberto et al., 2016) clearly show pharmacological effects, at least some of which seemed to be exerted by interaction with GPCRs. However, it is unlikely that these effects are mediated by direct activation of ARs. As an example, the hypothermic effects of inosine disappear completely in mice lacking either all four ARs or the A_3AR (Xiao et al., 2019). Alternatively, they may be due to inhibition of adenosine uptake through the equilibrative nucleoside transporter 1 (ENT1). Indirect effects are also conceivable (e.g., through allosteric modulation). Further research on inosine and guanosine as extracellular signaling molecules in their own right is warranted.

III. Receptor Binding Kinetics

It has been recognized in recent years that the study of target binding kinetics is crucial to reduce attrition rates in drug discovery (Copeland, 2016). Over the decades medicinal chemists have successfully synthesized lead compounds displaying high, often (sub)nanomolar affinity for a given target, including ARs. However, kinetic aspects of the ligand-receptor interaction have been studied in lesser detail. Although these can be very informative, the extra effort to obtain values for association (k_{on}) and dissociation (k_{off}) rate constants was and is substantial. This is because kinetic assays tend to be laborious although more efficient approaches (Guo et al., 2013) and methods are being developed, including scintillation proximity assays (Xia et al., 2016) and bioluminescence resonance energy transfer (BRET)-based ligand binding studies (Bouzo-Lorenzo et al., 2019; White et al., 2019). On the other hand, systematically evaluating the binding kinetics of a series of lead compounds that are otherwise chemically or biologically similar provides additional parameters for triage and advancement of molecules in the drug discovery process (Guo et al., 2016a; 2017). For instance, assessment of the lifetime of a ligand-receptor complex, coined residence time ($RT = 1/k_{off}$) (Copeland et al., 2006), has been shown predictive for drug efficacy and selectivity, including on ARs (Swinney,

2006a,b; Guo et al., 2014a; Zhang, 2015; Tonge, 2018). Drugs with long target RT are likely to produce a longer duration of action by more gradually reducing the decline of target occupancy than those with short RT (Dahl and Akerud, 2013; de Witte et al., 2016). Furthermore, a direct correlation between receptor RT and functional efficacy has been observed in some cases (Sykes et al., 2009; Guo et al., 2012). A thorough review of the kinetic characteristics of AR ligands, both orthosteric ligands and allosteric modulators, has recently appeared (Guo et al., 2017); hence, we will only provide a concise summary and update here.

A. Orthosteric Ligands and Adenosine Receptor Binding Kinetics

In Table 3, kinetic data [association and dissociation rate constants, kinetic equilibrium dissociation constants (K_D), and residence times] for (orthosteric) agonists and antagonists of the human adenosine receptors (hARs) are summarized. Their chemical structures, if not listed in Fig. 1, are assembled in Fig. 2. Most experiments were radioligand binding assays performed on membrane preparations, whereas lower than physiologic temperatures were employed in most cases due to practical limitations of the (radio)labeled probe used, such as a (too) fast dissociation at higher temperatures. There have been a few attempts to use surface plasmon resonance instrumentation for kinetic assays on $hA_{2A}R$, but these have not become routinely available since solubilized and purified receptor material is needed (Bocquet et al., 2015; Errey et al., 2015).

It is often thought that association rate constants for bimolecular encounters readily reach high values that are diffusion-limited only ($10^{10} \sim 10^{11}$ $M^{-1}{\cdot}min^{-1}$) (Smoluchowski, 1918; Alberty and Hammes, 1958). However, this is only true for reactant molecules that have isotropic reactivity, whereas the interaction between ligand and receptor, including ARs, is of a more constrained nature (e.g., due to the stereospecificity of recognition). This is obvious from Table 3, in which association rate constants vary from 5.0×10^5 (11, UK432,097) to 6.4×10^8 (41, SCH58261) $M^{-1}{\cdot}min^{-1}$, an over 1000-fold difference but still far from diffusion control. The latter compound is another selective $A_{2A}AR$ antagonist that has been extensively characterized in rodents. Association rate constants appear correlated with the onset of clinical action, in vivo target occupancy, and target rebinding (Vauquelin, 2018). However, this has not been demonstrated for AR ligands yet.

The dissociation rate constants or, for convenience, residence times also vary significantly, up to >5000-fold. There are ligands with ultra-short residence

TABLE 3
Association and dissociation rate constants of selected AR ligands

	Compound	Target (h, human; r, rat)	Temp (°C)	k_{on} ($M^{-1}{\cdot}min^{-1}$)	k_{off} (min^{-1})	RT (min)	Kinetic K_D (nM)[a]	Reference
7	CCPA	hA_1AR	25	9.6×10^6	1.2	0.9	131	(Guo et al., 2014b)
6	NECA	hA_1AR	25	9.0×10^5	0.47	2.1	522	(Guo et al., 2014b)
35	LUF5834	hA_1AR	25	2.0×10^8	0.92	1.1	4.6	(Guo et al., 2014b)
36	Capadenoson	hA_1AR	25	2.4×10^7	0.036	28	1.5	(Louvel et al., 2015)
37	LUF6976	hA_1AR	25	3.9×10^8	0.87	1.1	2.2	(Louvel et al., 2014)
38	LUF6941	hA_1AR	25	2.6×10^6	0.0076	132	2.9	(Louvel et al., 2015)
39	ABA-X-BY630	hA_1AR[b]	37	2.6×10^7	2.0	0.5	77	(May et al., 2010)
17	DPCPX	hA_1AR	25	1.4×10^8	0.21	4.8	1.5	(Guo et al., 2013)
17	DPCPX	rA_1R	25	9.6×10^7	0.045	22.2	0.50	(Guo et al., 2017)
10	CGS21680	$hA_{2A}AR$	25	5.0×10^4	0.02	50.0	380	(Guo et al., 2017)
10	CGS21680	$rA_{2A}AR$	23	2.1×10^7	0.033	30.3	1.6	(Guo et al., 2017)
40	LUF6057	hA_1AR	25	4.8×10^8	3.0	0.3	6.3	(Guo et al., 2013)
6	NECA	$hA_{2A}AR$	4	1.9×10^6	0.053	19	28	(Guo et al., 2015)
11	UK432,097	$hA_{2A}AR$	5	5.0×10^5	0.004	250	8.0	(Guo et al., 2012)
41	SCH58261	$hA_{2A}AR$	25	6.4×10^8	1.5	0.67	2.3	(Dionisotti et al., 1997)
42	ZM241385	$hA_{2A}AR$	4	1.3×10^8	0.014	71	0.11	(Guo et al., 2014c)
43	LUF6632	$hA_{2A}AR$	4	3.4×10^7	0.0031	323	0.091	(Guo et al., 2014c)
20a	MSX-2	$rA_{2A}AR$ (brain striatal membranes)	23	14.5×10^7	0.2839	3.52	1.95	(Müller et al., 2000)
6	NECA	$hA_{2B}AR$	4	n.d.	2.201	0.45	n.d.	(Hinz et al., 2018)
23	MRS1754	$hA_{2B}AR$	25	2.2×10^7	0.027	37	1.2	(Ji et al., 2001)
44	MRE2029-F20	$hA_{2B}AR$	4	1.7×10^7	0.031	32	1.8	(Baraldi et al., 2004)
45	OSIP339391	$hA_{2B}AR$	22	9.5×10^7	0.039	26	0.41	(Stewart et al., 2004)
24	PSB-603	$hA_{2B}AR$	21	11.4×10^7	0.02279	44	0.652	(Borrmann et al., 2009)
46	PSB-298	$hA_{2B}AR$	25	3.76×10^7	0.9533	1.05	25	(Bertarelli et al., 2006)
47	I-AB-MECA	hA_3AR	37	6.1×10^7	0.042	24	0.69	(Gao et al., 2001)
48	IB-MECA	hA_3AR	10	3.5×10^7	0.011	95	0.30	(Xia et al., 2018)
14	2-Cl-IB-MECA	hA_3AR	10	2.4×10^7	0.0043	231	0.18	(Xia et al., 2018)
49	MRS5127	hA_3AR	25	2.4×10^8	0.51	2.0	2.1	(Auchampach et al., 2010)
16	MRS5698	hA_3AR	10	7.8×10^6	5.1×10^{-4}	1961	0.068	(Xia et al., 2018)
31	MRE3008-F20	hA_3AR	4	7.6×10^7	0.042	24	0.55	(Varani et al., 2000)
50	LUF7531 (cmpd 2)	hA_3AR	10	1.7×10^8	0.0036	315	0.021	(Xia et al., 2017)
51	PSB-11	hA_3AR	25	2.35×10^8	0.2082	4.80	0.46	(Müller et al., 2002)

n.d., no data.
[a](kinetic) $K_D = k_{off}/k_{on}$.
[b]whole cells.

times [e.g., only seconds for antagonists LUF6057 (40, A_1AR) and SCH58261 (41, A_{2A}AR)], whereas agonists UK432,097 (11, A_{2A}AR), Cl-IB-MECA (14, A_3AR), and MRS5698 (16, A_3AR) as well as antagonists LUF6632 (43, A_{2A}AR) and LUF7531 (50, A_3AR) engage with the receptor for hours. In a recent study, Hothersall and coworkers (2017) identified UK432,097 analogs that displayed even longer target occupancy on hA_{2A}AR. Differences in RT for a number of A_{2A}AR antagonists have been linked to their differential modulation of the salt bridge strength between amino acids Glu^{169} and His^{264} in the egress pathway at the extracellular side of the receptor (Guo et al., 2016b; Segala et al., 2016).

B. Orthosteric Ligands Binding Covalently to Adenosine Receptors

Ligands that react covalently with ARs can be regarded as having infinite RT as long as the chemical bond between ligand and receptor "survives." Over the decades, such ligands have been developed as probes mostly (e.g., to identify the molecular weight of AR molecules, block the physiologic function of ARs or, more recently, help in AR structure elucidation). It remains to be investigated whether such ligands might have relevant therapeutic value.

Thus, both chemoreactive and photoaffinity agonists and antagonists were synthesized in early A_1AR studies and evaluated for their binding irreversibility using various assays and degrees of sophistication (Choca et al., 1985; Klotz et al., 1985; Earl et al., 1988; Patel et al., 1988; Stiles and Jacobson, 1988; Jacobson et al., 1989a; Boring et al., 1991; Scammells et al., 1994; Srinivas et al., 1996; Beauglehole et al., 2000; van Muijlwijk-Koezen et al., 2001; Jorg et al., 2016). Of these, FSCPX (52) has been most widely used, and a close derivative of it, DU172 (72) (Beauglehole et al., 2000), appeared crucial for the crystallographic structure elucidation of hA_1AR (Glukhova et al., 2017) (Chapter IV). DU172, through its fluorosulfonyl moiety, forms a covalent bond with amino acid $Y271^{7.36}$ at the extracellular end of the seventh transmembrane domain (TM7) of the receptor.

Likewise, similar efforts have been performed on A_{2A}AR for agonists (Jacobson et al., 1989b; Barrington et al., 1990; Jacobson et al., 1992; Niiya et al., 1993; Luthin et al., 1995; Moss et al., 2014) and antagonists (Ji et al., 1993; Muranaka et al., 2017; Yang et al., 2017). One of the covalent antagonists, LUF7445 (53), was equipped with a click handle to act as a chemical probe (54, LUF7487) for A_{2A}AR (Yang et al., 2018). This chemical biology approach allowed, among others, receptor visualization in hA_{2A}AR-expressing cell membranes.

The A_{2B}AR has not been subjected to covalent labeling yet, whereas the A_3AR has been the target for such studies, sampling both irreversibly binding agonist (Ji et al., 1994) and antagonist ligands (Li et al., 1999; Baraldi et al., 2001; Yang et al., 2019).

C. Allosteric Ligands and Adenosine Receptor Binding Kinetics

Ligands binding to an allosteric site distinct from the AR orthosteric binding pocket (see also Chapter II) may influence the binding kinetics of orthosteric ligands. Indeed, on many occasions it has been shown that positive allosteric modulators (PAMs) for the A_1AR retard the dissociation rate of orthosteric A_1AR agonists, as summarized in a number of reviews (Göblyös and IJzerman, 2011; Kimatrai-Salvador et al., 2013; Guo et al., 2017). For instance, one of the more potent A_1AR PAMs, BC-1/compound 8j (55) (Romagnoli et al., 2008), increased the residence time of CCPA up to 200-fold from 0.9 minutes (Table 2) to 172 minutes (Guo et al., 2014b). Unfortunately, the often modest, micromolar potency of PAMs and other allosteric ligands for ARs has so far precluded the assessment of the binding kinetics of these ligands per se.

D. Adenosine Receptor Target Binding Kinetics – Conclusions

Kinetic parameters are an additional factor in assessing the quality and nature of new chemical entities. Nearly all compounds in Table 3 have high affinity, but their kinetics can be very different. A striking example is the pair of LUF6976 (37, K_D = 2.2 nM for A_1AR, RT = 1.1 minutes) and LUF6941 (38, K_D = 2.9 nM for A_1AR, RT = 132 minutes), showing identical affinity but a more than 100-fold difference in residence time. Thus, many compounds are considered equivalent on the basis of affinity alone, whereas a further differentiation or even triage may be possible depending on their kinetic characteristics. For instance, A_{2A}AR antagonists are currently in clinical trials as potential adjuvants in cancer immunotherapy (see Chapter VII) to block adenosine's unwanted anti-inflammatory and immunosuppressive effects (Hatfield and Sitkovsky, 2016). The local adenosine concentration in the tumor may be so high that short-RT antagonists cannot productively compete, whereas a long-RT antagonist may lead to sufficient target engagement even in the presence of elevated adenosine concentrations. Likewise, A_{2A}AR antagonists have been developed for the treatment of PD in combination with levodopa/dopaminergic agonists, although clinical success has been limited so far (Morelli et al., 2009; Hickey and Stacy, 2012). In that setting, a compound with a long receptor RT could have some advantages, as it might yield a reduction in the "wear-off" effect (e.g., of levodopa in between doses) (Hickey and Stacy, 2012). Thus, information obtained from a kinetic perspective may provide additional rationales for the design of new AR ligands. At

the same time, one needs to realize that pharmacokinetic aspects are also governing in vivo effects and that an integration of aspects of target binding kinetics and of pharmacokinetics is required (Daryaee and Tonge, 2019).

IV. Receptor Structures

Over the last decade, the elucidation of receptor architecture has been one of the hallmarks in GPCR research (Venkatakrishnan et al., 2013). The A_{2A}AR was one of the first structures solved, through X-ray crystallography (Jaakola et al., 2008), and since then many adenosine receptor structures have been reported (see Table 4 and references therein). Typical characteristics of GPCRs such as their thermolability and fragility have dictated the use of highly engineered proteins and protein constructs for structure elucidation as well as of highly sophisticated technologies (Grisshammer, 2017). At least three approaches have been used widely. First, thermostabilization of GPCRs (Magnani et al., 2016), including the A_{2A}AR, has yielded material sufficient for crystallization by combining amino acid mutations to raise the protein melting temperature. Secondly, fusion of the A_{2A}AR with proteins that crystallize "easily," such as T4 lysozyme (T4L) (Jaakola et al., 2008) or apocytochrome b_{562}RIL (bRIL) (Liu et al., 2012), has been instrumental to generate crystalline material. Thirdly, complexation of the A_{2A}AR with antibodies raised against epitopes of the receptor provided sufficient stability to render X-ray crystallography feasible (Hino et al., 2012). In recent years, cryogenic electron microscopy (EM), particularly single-particle cryo-EM (Cheng, 2018; Ceska et al., 2019), has been employed to study membrane protein structures as well, including agonist-bound structures of the A_1AR (Draper-Joyce et al., 2018; 2021) and A_{2A}AR (Garcia-Nafria et al., 2018) in complex with G protein variants.

A. Resolution

The overall resolution of the AR crystal structures varies between 1.7 Å and 3.6 Å (see Table 4). A high resolution (lower Å values) provides more structural details, particularly the presence or absence of explicit water molecules. It has been shown that a minimum resolution of ~3.0 Å is required to see any water molecules in a protein crystal structure, whereas on average one water molecule per amino acid residue can be detected at 2.0 Å (Carugo and Bordo, 1999). This means that most adenosine receptor crystal structures lack information on the role that water molecules play in ligand binding. However, >60 explicit water molecules are observed in the 1.8 Å resolution A_{2A}AR-ZM241385 (42) complex (4EIY, Table 4), showing a wide distribution throughout the protein, including the ligand binding site, in which water molecules hydrogen-bond to both ligand and amino acid residues (Liu et al., 2012). In fact, the receptor structure is suggestive of a water-filled pore or channel. The channel has two bottlenecks around $Trp246^{6.48}$ and $Tyr288^{7.53}$, slightly less in size than the diameter of one water molecule. These amino acids are part of two general motifs related to GPCR activation, the "rotamer/toggle switch" and the NPxxY sequence, respectively. Interestingly, recent developments show that molecular dynamics and other calculations can make up for the absence of water molecules in a low-resolution protein structure (Matter and Gussregen, 2018). Due to its high resolution, the same 4EIY structure was the first to show the presence of an allosterically binding sodium ion, interacting in a cavity containing a strongly conserved aspartic acid ($Asp52^{2.50}$). As this domain is generic among most class A GPCRs, it is expected that other GPCRs bind sodium ions at this site as well (e.g., as was demonstrated for the human δ-opioid receptor) (Fenalti et al., 2014).

B. Ligand Binding Site

The ARs' orthosteric binding site (i.e., the binding site for endogenous agonist adenosine) accommodates a range of ligands with diverse scaffolds and different sizes (see Table 4; Fig. 4). In fact, the A_{2A}AR is the GPCR with the most structures available in the Protein Data Bank (PDB) (Vass et al., 2018), allowing an unprecedented view on the conformational flexibility of the ligand binding site.

In total >15 different antagonists have been cocrystallized with hA_{2A}AR so far (Table 4), compared with just one structure with ZM241385 (PDB: 3EML) in the previous update. The receptor binding site appears flexible as these antagonists take slightly different positions therein (see Supplemental Video 1). The four agonists cocrystallized with hA_{2A}AR until now (Table 4) all have a ribose moiety, pointing deeper into the ligand binding pocket and displacing explicit water molecules present in the antagonist-occupied receptor structures (see Supplemental Video 2). The agonist-bound structures crystallized in the absence of G protein are now regarded as representing intermediate states in the process of receptor activation. The presence of an engineered G protein makes the cytoplasmic end of TM6 move away considerably from the receptor core by ~14 Å compared with the other agonist-bound structures, with little impact on the extracellular side of the receptor and the ligand binding pocket (Carpenter et al., 2016; Garcia-Nafria et al., 2018). This is most pronounced for the NECA (6)-bound cryo-EM structure with engineered G protein and nanobody Nb35 (Garcia-Nafria et al., 2018). The thermodynamic contributions of a single, conserved water molecule bridging the 2′-hydroxyl and 3-aza groups of adenosine were analyzed, which led to the design of a modified, potent agonist containing a mimic of this water (Matricon et al., 2020). Recently, the first X-ray structure of A_{2A}AR with

TABLE 4
Reported structures of adenosine receptor subtypes

PDB	Engineering	Ligand	Resolution (Å)	Technique	Remarks	Reference
A_{2A}AR Antagonist Structures						
3PWH	TS	ZM241385 (42)	3.3	X-ray		(Dore et al., 2011)
3REY	TS	XAC (57)	3.3	X-ray		(Dore et al., 2011)
3RFM	TS	Caffeine (3)	3.6	X-ray		(Dore et al., 2011)
3UZA	TS	T4G (58)	3.3	X-ray	T4G: 6-(2,6-dimethylpyridin-4-yl)-5-phenyl-1,2,4-triazin-3-amine	(Congreve et al., 2012)
3UZC	TS	T4E (59)	3.3	X-ray	T4E: 4-(3-amino-5-phenyl-1,2,4-triazin-6-yl)-2-chlorophenol	(Congreve et al., 2012)
3EML	FP (T4L)	ZM241385	2.6	X-ray		(Jaakola et al., 2008)
4EIY	FP (bRIL)	ZM241385	1.8	X-ray		(Liu et al., 2012)
5UIG	FP (bRIL)	8D1 (60)	3.5	X-ray	8D1: 5-amino-N-[(2-methoxyphenyl)methyl]-2-(3-methylphenyl)-2H-1,2,3-triazole-4-carboximidamide	(Sun et al., 2017)
5K2A	FP (bRIL)	ZM241385	2.5	X-ray/SFX/XFEL, sulfur SAD phasing	SFX: serial femtosecond crystallography; XFEL: X-ray free-electron laser; SAD: single-wavelength anomalous diffraction	(Batyuk et al., 2016)
5K2B	FP (bRIL)	ZM241385	2.5	X-ray/SFX/XFEL, MR phasing	MR: molecular replacement	(Batyuk et al., 2016)
5K2C	FP (bRIL)	ZM241385	1.9	X-ray/SFX/XFEL, sulfur SAD phasing and phase extension		(Batyuk et al., 2016)
5K2D	FP (bRIL)	ZM241385	1.9	X-ray/SFX/XFEL, MR phasing		(Batyuk et al., 2016)
5VRA	FP (bRIL)	ZM241385	2.4	X-ray in situ	in situ: film sandwich plates at room temperature	(Broecker et al., 2018)
5JTB	FP (bRIL)	ZM241385	2.8	X-ray/I-SAD	I-SAD: iodide-single-wavelength anomalous diffraction	(Melnikov et al., 2017)
5UVI	FP (bRIL)	ZM241385	3.2	X-ray millisec	millisec: serial millisecond crystallography using synchrotron radiation	(Martin-Garcia et al., 2017)
6AQF	FP (bRIL)	ZM241385	2.5	X-ray		(Eddy et al., 2018b)
7RM5	FP (bRIL)	ZM241385	2.8	Microcrystal electron diffraction		(Martynowycz et al., 2021)
5NM2	TS-FP (bRIL)	ZM241385	2.0	X-ray millisec (cryo)		(Weinert et al., 2017)
5NLX	TS-FP (bRIL)	ZM241385	2.1	X-ray millisec (room temp)		(Weinert et al., 2017)
5NM4	TS-FP (bRIL)	ZM241385	1.7	X-ray femtosec (room temp)	Serial femtosecond crystallography using XFEL	(Weinert et al., 2017)
5MZJ	TS-FP (bRIL)	Theophylline (4)	2.0	X-ray		(Cheng et al., 2017)
5MZP	TS-FP (bRIL)	Caffeine (3)	2.1	X-ray		(Cheng et al., 2017)
5N2R	TS-FP (bRIL)	PSB-36 (18)	2.8	X-ray		(Cheng et al., 2017)
5IU4	TS-FP (bRIL)	ZM241385	1.7	X-ray		(Segala et al., 2016)
5IU7	TS-FP (bRIL)	12c (61, LUF6805)	1.9	X-ray	12c: 2-(furan-2-yl)-N^5-(2-(4-phenylpiperidin-1-yl)ethyl)[1,2,4]triazolo[1,5-a][1,3,5]triazine-5,7-diamine	(Segala et al., 2016)
5IU8	TS-FP (bRIL)	12f (62, LUF6806)	2.0	X-ray	12f: 2-(furan-2-yl)-N^5-(2-(4-methylpiperazin-1-yl)ethyl)[1,2,4]triazolo[1,5-a][1,3,5]triazine-5,7-diamine	(Segala et al., 2016)
5IUA	TS-FP (bRIL)	12b (63, LUF6732)	2.2	X-ray	12b: 2-(furan-2-yl)-N^5-(3-(4-phenylpiperazin-1-yl)propyl)[1,2,4]triazolo[1,5-a][1,3,5]triazine-5,7-diamine	(Segala et al., 2016)
5IUB	TS-FP (bRIL)	12x (64, LUF6632)	2.1	X-ray	12x: N^5-(2-(4-(2,4-difluorophenyl)piperazin-1-yl)ethyl)-2-(furan2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazine-5,7-diamine	(Segala et al., 2016)
5OLG	TS-FP (bRIL)	ZM241385	1.9	X-ray, soaking	soaking of ligand to displace theophylline in the crystals	(Rucktooa et al., 2018)
5OLH	TS-FP (bRIL)	Vipadenant (65)	2.6	X-ray, soaking for 24 hr		(Rucktooa et al., 2018)
5OLO	TS-FP (bRIL)	Tozadenant (66)	3.1	X-ray, soaking for 24 hr		(Rucktooa et al., 2018)
5OLV	TS-FP (bRIL)	LUAA47070 (analog) (67/68)	2.0	X-ray, soaking for 24 hr		(Rucktooa et al., 2018)

(*continued*)

TABLE 4—*Continued*

PDB	Engineering	Ligand	Resolution (Å)	Technique	Remarks	Reference
5OLZ	TS-FP (bRIL)	4e (69)	1.9	X-ray	4e: 4-(3-amino-5-phenyl-1,2,4-triazin-6-yl)-2-chlorophenol	(Rucktooa et al., 2018)
5OM1	TS-FP (bRIL)	4e	2.1	X-ray, soaking for 1 hr		(Rucktooa et al., 2018)
5OM4	TS-FP (bRIL)	4e	2.0	X-ray, soaking for 24 hr		(Rucktooa et al., 2018)
6LPJ/K/L	FP (bRIL)	ZM241385	1.8–2.0	Serial femtosecond crystallography using XFEL	$EROCOC_{17+4}$ as crystallization matrix	(Ihara et al., 2020)
6ZDR	TS-FP (bRIL)	Chromone 4d (70)	1.9	X-ray		(Jespers et al., 2020)
6ZDV	TS-FP (bRIL)	Chromone 5d (71)	2.1	X-ray		(Jespers et al., 2020)
6GT3	TS-FP (bRIL)	AZD4635 (22)	2.0	X-ray		(Borodovsky et al., 2020)
6S0Q	TS-FP (bRIL)	ZM241385	2.7	Native SAD	SAD: single-wavelength anomalous diffraction	(Nass et al., 2020)
3VG9	antibody-stab	ZM241385	2.7	X-ray		(Hino et al., 2012)
3VGA	antibody-stab	ZM241385	3.1	X-ray		(Hino et al., 2012)
A_{2A}AR Agonist Structures						
2YDO	TS	Adenosine (1)	3.0	X-ray		(Lebon et al., 2011)
2YDV	TS	NECA (6)	2.6	X-ray		(Lebon et al., 2011)
4UG2	TS	CGS21680 (10)	2.6	X-ray		(Lebon et al., 2015)
4UHR	TS	CGS21680	2.6	X-ray		(Lebon et al., 2015)
3QAK	FP (T4L)	UK432097 (11)	2.7	X-ray	WT receptor	(Xu et al., 2011)
5WF5	FP (bRIL)	UK432097	2.6	X-ray	D52N mutant	(White et al., 2018)
5WF6	FP (bRIL)	UK432097	2.9	X-ray	S91A mutant	(White et al., 2018)
5G53	truncated and tagged WT	NECA (6)	3.4	X-ray	with engineered G protein (mini-Gs)	(Carpenter et al., 2016)
6GDG	FP (thioredoxin)	NECA	4.1	cryo-EM	with engineered G protein (mini-Gs-$\beta_1\gamma_2$); also includes nanobody Nb35	(Garcia-Nafria et al., 2018)
7ARO	TS-FP (bRIL)	LUF5833 (56)	3.1	X-ray	LUF5833 is a partial agonist	(Amelia et al., 2021)
A_1AR Antagonist Structures						
5N2S	TS	PSB-36 (18)	3.3	X-ray		(Cheng et al., 2017)
5UEN	FP (bRIL)	DU172 (72)	3.2	X-ray		(Glukhova et al., 2017)
A_1AR Agonist Structures						
6D9H	tagged WT receptor	Adenosine (1)	3.6	cryo-EM	with engineered G_{i2} protein	(Draper-Joyce et al., 2018)
7LD3/4	tagged WT receptor	Adenosine (1) +/− MIPS521 (73)	3.3–3.4	cryo-EM	with engineered G_{i2} protein	(Draper-Joyce et al., 2021)

FP, fusion protein; PDB, Protein Data Bank four-digit entry number; TS, thermostabilization.
Chemical structures of ligands are depicted in Fig. 3, if not already in Figs. 1 and 2.

a nonriboside, 3,5-dicyanopyridine partial agonist (56, LUF5833) was reported (Amelia et al., 2021).

The structure elucidation of the hA_1AR is another achievement. Two crystal structures of antagonist-bound receptor are available (Cheng et al., 2017; Glukhova et al., 2017), whereas one cryo-EM structure with an agonist (adenosine, 1) bound has been reported, the latter in the presence of an engineered G_i protein (Draper-Joyce et al., 2018). The latter structure was later complemented with a positive allosteric modulator, MIPS521 (73), as well, the binding site of which appeared to be extrahelically located involving TM domains 1, 6, and 7 (Draper-Joyce et al., 2021). In the antagonist structures, it was noted that there are differences in the extracellular loop regions, particularly the second one, relative to the $hA_{2A}AR$ structure. The ligand binding cavity is relatively wide, again in comparison with $hA_{2A}AR$. Differences in pocket shape between the two receptors may determine selectivity more than the (very similar) amino acids lining the pockets. There is a tightening of the orthosteric binding site induced by an ~4 Å inward movement of the extracellular ends of TMs 1 and 2 in the adenosine-bound, active structure compared with the antagonist-bound, inactive hA_1AR. At the intracellular surface, the engineered G protein present causes a 10.5 Å outward movement of TM6 in the hA_1AR, quite comparable to the similar large shift in active $hA_{2A}AR$.

All agonists and antagonists are anchored by two amino acids in particular in both receptors [i.e., $Asn253^{6.55}$ (numbering as in $hA_{2A}AR$) and Phe168 in EL2]. A further summary of relevant amino acids for ligand binding, also focusing on selectivity issues between ARs, has been provided recently (Jespers et al., 2018).

C. NMR Studies

Although X-ray crystallography and cryo-EM methods provide important information on AR architecture, NMR

spectroscopy has the potential to reveal additional structural dynamics data. Two main approaches have been used so far: 1) indole ^{15}N-^{1}H chemical shifts are monitored after introducing extrinsic (^{15}N-labeled) tryptophan residues at relevant sites, or 2) by incorporating ^{19}F reporter tags onto cysteine residues in the protein, ^{19}F-^{1}H resonances are assessed. In both cases, distinct conformational $A_{2A}AR$ states were observed upon interaction with G protein (Prosser et al., 2017; Huang et al., 2021), cations (Ye et al., 2018), full and partial agonists (Eddy et al., 2018a; 2021; Sušac et al., 2018), and allosteric modulators/sites (Eddy et al., 2018b). The next challenge will be to begin and address other aspects of AR dynamics and functioning, such as the impact of the lipid membrane environment (Guixà-González et al., 2017).

V. Cellular Pharmacology – Biased Signaling of Adenosine Receptors

Each GPCR potentially couples to multiple G proteins, as was demonstrated for the endogenous $A_{2A}AR$ (Cunha et al., 1999) and $A_{2B}AR$ (Gao et al., 2018b), and to non-G protein dependent pathways, such as β-arrestins (Michel and Charlton, 2018; Vecchio et al., 2018). In some cases, the net effect of each of these signaling cascades induced by the same endogenously expressed AR in different cells may be opposite, such as with the $A_{2B}AR$ (Gao et al., 2018b). In theory, the ability of a GPCR agonist to consistently distinguish among multiple intracellular signaling pathways provides advantages when used in a therapeutic mode if the preferred pathway is associated with the beneficial action at the receptor. Such an agonist is termed biased, which implies a nonequivalence in the potency or efficacy across the signaling pathways. In principle, side effects that are associated with the nonpreferred pathways would be avoided. Signaling bias might also affect the kinetics of GPCR trafficking, as internalized receptors can also signal, or gene transcription.

Biased signaling depends on multiple active GPCR conformations, each of which would couple to its own spectrum of second messenger pathways. Thus, biased agonists, also at ARs, achieve signaling selectivity by interacting with or stabilizing a subset of the possible active receptor conformations, and this subset has characteristic pharmacology distinct from other conformations of the same receptor (Verzijl and IJzerman, 2011). Biased agonism has been reported at adenosine A_1-, A_{2B}-, and A_3ARs for both nucleoside agonists and two classes of non-nucleoside AR agonists, the 3,5-dicyanopyridines and 5-cyanopyrimidines (Langemeijer et al., 2013). Tissue-dependent $A_{2A}AR$ signaling was observed in neurons of different brain areas through engineered optogenetic signaling (Li et al., 2015). Inhibitors of GPCR signaling could be biased as well, for example, as shown for the A_1AR using a suramin derivative (Kudlacek et al., 2002). Allosteric GPCR modulators, such as A_1AR enhancers (PAMs) in the 2-amino-3-benzoylthiophene family or A_3AR PAMs in the imidazoquinolinamine family, can show biased effects on agonist-induced signaling (Gao et al., 2011).

A_1AR: In a broad screen of AR agonists, nucleoside agonist LUF5589 (74, 2-chloro-5′-O-ethyl-N^6-(3-iodobenzyl)adenosine) tended toward a signaling bias for the G_i protein-dependent pathway in comparison with the β-arrestin pathway (Langemeijer et al., 2013). Biased agonism at the A_1AR was also explored by Baltos and coworkers (2016). PAM VCP520 (75) potentiated A_1AR agonist-induced Ca^{2+} mobilization more effectively than extracellular signal-regulated kinase 1/2 activation (Valant et al., 2010). Identification of biased agonism (i.e., cardioprotective efficacy without hemodynamic side effect) associated with an A_1AR PAM conjugated to an agonist, VCP746 (76), suggested that this bitopic ligand might be bridging orthosteric and allosteric sites on the receptor (Valant et al., 2014). A recent structure determined for A_1AR (Glukhova et al., 2017) shows that it possesses at least one allosteric site, potentially the site that has been exploited to promote biased agonism (Valant et al., 2014).

$A_{2A}AR$: The biased signaling of $A_{2A}AR$ is rather peculiar among ARs since it seems to be a property of the environment of the $A_{2A}AR$, probably related to the numerous G protein-interacting proteins that are associated with $A_{2A}AR$. In fact, at least six G protein-interacting proteins (actinin, calmodulin, NECAB2, translin-associated protein X, ARNO/cytohesin-2, and ubiquitin-specific protease-4) have been reported to interact with the long $A_{2A}AR$ C terminus (Keuerleber et al., 2011). The exploitation of constructs with an altered C-terminal tail revealed a biased $A_{2A}AR$-mediated signaling with PSB-0777 (12) and LUF5834 (35) (Navarro et al., 2020). Also, inosine has been proposed to activate $A_{2A}AR$ in a biased manner in CHO-K1 cells heterologously expressing $hA_{2A}AR$ (Welihinda et al., 2016). Still, $A_{2A}AR$ agonists with biased properties have been scarcely explored, although they would be of clear interest to potentially optimize immunomodulatory functions without cytotoxic or vascular effects.

$A_{2B}AR$: Nucleoside agonists distinguish among different G protein-dependent signaling pathways of the $A_{2B}AR$ (Gao et al., 2014). Extracellular signal-regulated kinase 1/2 activation may result from β-arrestin mobilization or from G_q- or G_s-protein coupling. In fact, entirely different signaling pathways are activated depending on whether the receptor is endogenously occurring or introduced by transfection (Gao et al., 2018b). $A_{2B}AR$ activation in muscle and brown fat had a beneficial effect on energy expenditure and

increased muscle mass, suggesting the application of selective A_{2B}AR agonists that principally activate cAMP for treating obesity (Gnad et al., 2020). A_{2B}AR activation also reduces cardiac fibrosis via the PKCδ to p38-MAPK pathway and protects the ischemic heart by stabilizing HIF-1α (Campos-Martins et al., 2021). Thus, translational opportunities are conceivable if selective and biased A_{2B}AR agonists could be developed for these signaling pathways.

A_3AR: Storme and coworkers (2018), using an engineered arrestin-reporter cell line, compared the G_i-dependent and β-arrestin2-dependent signaling of 19 nucleoside agonists at the A_3AR to show a tendency toward weak bias for the G protein pathway in a few analogs. Similar conclusions were reported in an earlier study comparing known A_3AR agonists (Gao and Jacobson, 2008), which noted differences in the kinetics of receptor activation.

VI. Pharmacology – Novel Developments

This chapter addresses select aspects of AR pharmacology. In this update, we focus on the therapeutic targeting of ARs and elaborate on their relevance in disease states. In the previous update, dimerization/oligomerization of ARs was particularly emphasized (Fredholm et al., 2011). This potentially critical variable to selectively modulate AR activity has been detailed in a number of recent reviews (Vecchio et al., 2018; Ferré and Ciruela, 2019; Franco et al., 2021a).

A. Therapeutic Targeting of Adenosine Receptors

Adenosine receptors have been targeted in the treatment of a number of (peripheral and CNS) diseases including PD, cardiac arrhythmias, asthma, and infant apnea (Kreutzer and Bassler, 2014). Adenosine receptors are also targeted for diagnostic studies of coronary circulation in individuals unable to manage a treadmill. Over the years, targeting adenosine receptors has been tested in animal models of diabetes, inflammatory diseases, wound healing, sickle cell disease, congestive heart failure, Alzheimer's disease, depression, and grand mal epilepsies, as well as in human trials. Other potential disease targets for agents targeted to adenosine receptors have recently been identified. Below we identify some of the most promising applications for adenosine receptor agents described over the past 10 years (Borea et al., 2018). It should be kept in mind, however, that a knowledge gap exists between advanced animal studies, which are many, and the limited number of reports on native human cells and tissues. From a translational perspective toward successful clinical studies, it seems essential to close this gap.

B. Therapeutic Targeting of Peripheral Adenosine Receptors

1. Adenosine A_1 Receptors and Congestive Heart Failure. Adenosine, generated within the kidney and acting at A_1AR, induces vasoconstriction of afferent arterioles reducing renal blood flow and glomerular filtration rate (GFR), further stimulating renin release. Moreover, activation of A_1AR increases proximal tubular reabsorption of sodium ions (Vallon et al., 2006). In congestive heart failure A_1AR activation was postulated to play a role in the reduced GFR and sodium retention that characterize congestive heart failure, and it was suggested that blockade of A_1AR could alleviate the symptoms of congestive heart failure by increasing GFR and promoting sodium elimination (Vallon et al., 2008). When tested in the clinic, however, short courses of rolofylline, a selective A_1AR antagonist, provided no benefit in the treatment of congestive heart failure, and a number of patients suffered seizures, a known potential adverse effect of A_1AR antagonists (Massie et al., 2010). Subsequently, it was noted that A_1AR stimulation could enhance cardiac myocyte function by improving mitochondrial function and the function of the Ca^{2+}-ATPase (SERCA2a), and the use of a partial agonist could potentially avoid the cardiac dysrhythmias induced by A_1AR (full) agonists (Voors et al., 2018). Unfortunately, the partial A_1AR agonist neladenoson (BAY1067197) did not improve exercise tolerance (see also Chapter VII) in patients with heart failure (Shah et al., 2019).

2. Adenosine A_{2A} Receptors and Cancer. Severe impairment of the cellular immune system was first associated with deficiency of adenosine deaminase in 1972 (Giblett et al., 1972). Whereas adenosine deaminase deficiency is toxic to T cells, in many subsequent studies the immunosuppressive effects of adenosine at concentrations that are not toxic to T cells have been further confirmed. Moreover, the A_{2A}AR has been implicated as the mediator by which adaptive immunity is suppressed (Huang et al., 1997), the T cell subtypes affected have been identified, and the intracellular signaling mechanisms have been investigated (Cronstein and Sitkovsky, 2017). The impact of A_{2A}AR in cancer development is best heralded by the pioneering report in which melanoma and lymphoma cell lines were completely rejected in A_{2A}AR knockout mice (Ohta et al., 2006) through a mechanism involving the control of the antitumor effects of CD8 T cells. Although it had previously been established that high concentrations of adenosine were present in the extracellular fluid of solid tumors (Blay et al., 1997), the significance of that finding was not fully appreciated until the report by Ohta and colleagues. Moreover, a number of more recent studies suggest that A_{2A}AR antagonists interact with anti-PD1 and anti-CTLA4 therapy to further

enhance tumor immunity and promote tumor regression (Iannone et al., 2014; Beavis et al., 2015; Gessi et al., 2017). Indeed, A_{2A}AR antagonists bolster cytokine release by CAR-T cells increasing their antitumor efficiency (Beavis et al., 2017). Currently, a number of A_{2A}AR, A_{2B}AR, and dual antagonists are at various stages of clinical development (see Chapter VII) (Yu et al., 2020). In addition, other therapeutic approaches targeting adenosine production from adenine nucleotides by ecto-5′nucleotidase (CD73) are making their way to the clinic as well (Congreve et al., 2018).

3. Adenosine Receptors and Autoimmune and Inflammatory Diseases. The potential anti-inflammatory effects of adenosine, acting at A_{2A}AR, have been known since 1983 (Cronstein et al., 1983). Subsequently adenosine, acting at both A_{2A}AR and A_3AR, was shown to mediate many of the anti-inflammatory and immunosuppressive effects of low-dose methotrexate therapy, the gold standard in the therapy of rheumatoid arthritis and psoriasis (Cronstein and Sitkovsky, 2017). Administration of A_{2A}AR agonists, although potentially useful for treatment of inflammatory diseases, would likely have too many side effects to be tolerated, mainly due to their strong hypotensive action, so other approaches have been taken. Thus, one approach has been to develop a prodrug of an A_{2A}AR agonist that is liberated by the action of ecto-5′-nucleotidase (CD73). Such an agent was shown to suppress inflammatory arthritis in animal models (Flögel et al., 2012) and suggests a promising approach to development of new anti-inflammatory agents.

In contrast, A_3AR agonists do not appear to have the same potential for systemic toxicity, as receptor expression is not as widespread as for the A_{2A}AR. Thus, relatively selective A_3AR agonists have been tested in both animal models and the clinic for their anti-inflammatory effects. Potential clinical utility with minimal toxicity has been reported for A_3AR agonists in the treatment of rheumatoid arthritis, psoriasis, and liver conditions, and thus agents remain in development for the treatment of these autoimmune disorders (reviewed in Jacobson et al., 2018).

4. Adenosine Receptors and Infectious Diseases. The anti-inflammatory and immunosuppressive effects of adenosine, acting at A_{2A}AR, have not gone unnoticed by microorganisms. Thus, adenosine has been identified as a virulence factor in *Candida albicans* (Smail et al., 1992; Rodrigues et al., 2016), *Staphylococcus aureus*, (Thammavongsa et al., 2009), and *Streptococcus suis* (Liu et al., 2014) that mitigates the effects of the host immune and inflammatory response on these microorganisms. *Leishmania amazonensis* also exploits the adenosine system to elude detection by dendritic cells, in this case through A_{2B}AR (Figueiredo et al., 2021). To date, A_{2A}AR or A_{2B}AR have not been targeted as a means to enhance host responses to microorganisms for the treatment of infectious diseases for resistant organisms.

In contrast, it is increasingly clear that much of the injury associated with infections comes as a result of the active host response to the infection with tissue damage in affected tissues, much like the tissue injury triggered by inflammatory and autoimmune diseases. First postulated as a potential therapy for COVID-19 pneumonia (Abouelkhair, 2020; Falcone et al., 2020), Correale and colleagues (2020) reported on the beneficial effects of administration of aerosolized adenosine in patients with COVID-19 pneumonia. They treated 14 patients with COVID-19 interstitial pneumonitis with aerosolized adenosine and observed improved oxygenation in 13 of 14 patients (compared with 7 of 52 control patients) and improved imaging studies, although the RNA load of SARS-CoV-2 increased in 13 of 14 patients. There was one death in the adenosine-treated patients compared with 11 of 52 patients in the historic control group. Bronchospasm was observed in one of the treated patients. The authors concluded that aerosolized adenosine might be a useful adjunct to other therapies for the treatment of SARS-CoV-2 pneumonia and might be similarly effective in other types of viral pneumonia. Although it is likely that the actions of adenosine in viral pneumonitis are mediated by the actions of an adenosine receptor, it is unclear which receptor(s) that might be, although the actions of A_{2A}AR, A_{2B}AR, and A_3AR could account for the anti-inflammatory effects observed, as noted above.

5. Adenosine A_{2A} Receptors and Retinal Disease. The retinopathy of prematurity is the most common cause of childhood blindness. A_{2A}AR stimulation in the retina promotes retinal vascular overgrowth, and results of recent studies indicate that A_{2A}ARs play a significant role in the development of oxygen toxicity-induced retinal angiogenesis (Taomoto et al., 2000; Liu et al., 2010; 2017). Caffeine, which is commonly used to treat apnea in neonates, was recently shown to prevent oxygen toxicity-induced retinal angiogenesis in animal models and has been suggested as a therapeutic approach to prevent retinopathy of prematurity (Zhang et al., 2017), an effect mimicked by the selective antagonism of A_{2A}ARs (Zhou et al., 2018). The antagonism of A_{2A}ARs also emerges as a novel promising strategy to dampen the local inflammatory processes involved in the degeneration of ganglion neurons in ischemic eye diseases and glaucoma that are a prevalent cause of blindness in the elderly (Liu et al., 2016; Madeira et al., 2016; Boia et al., 2017). A_1AR agonists, which prevent neuronal damage from pressure and ischemia in animal models, have been tested in the treatment of glaucoma but failed in phase 3 trials to reduce intraocular pressure

better than placebo (ClinicalTrials.gov Identifier: NCT02565173).

6. Adenosine Receptors and Bone. Adenosine A_1-, A_{2A}-, and A_{2B}-ARs play a role in regulating bone biology by modulating osteoclast differentiation and bone remodeling as well as osteoblast differentiation and production of new bone (Strazzulla and Cronstein, 2016). A_1AR stimulation is required for osteoclast differentiation, and A_1AR knockout mice have mild osteopetrosis (Kara et al., 2010a,b). In contrast, A_{2A}AR and A_{2B}AR stimulation diminish osteoclast differentiation and stimulate new bone formation by osteoblasts (Mediero et al., 2012b; 2013; 2015b; Corciulo et al., 2016). More importantly, an A_1AR antagonist, an A_{2A}AR agonist, or dipyridamole, which blocks adenosine uptake via the equilibrative nucleoside transport protein ENT1 (SLC29A1) and thereby increases extracellular adenosine levels, stimulate bone regeneration in critical bone defects, whether applied topically or as a coating for 3D-printed β-tricalcium phosphate scaffolds (Mediero et al., 2015b; Ishack et al., 2017). Currently, dipyridamole-coated scaffolds are undergoing preclinical testing for restoration of bone.

Despite the remarkable success of joint replacement therapy, approximately 25% of implanted hip and knee prostheses will require revision due to erosion of the bone surrounding the prosthesis (Bozic et al., 2010). Application of A_{2A}AR agonists markedly 1) diminishes the inflammation due to prosthesis wear particles, the most common cause of bone destruction leading to prosthetic joint replacement, and 2) by inhibiting osteoclast differentiation, diminishes wear particle-induced bone destruction in a murine model (Mediero et al., 2012a). Moreover, weekly low doses of methotrexate, a commonly used anti-inflammatory drug that inhibits inflammation by increasing local adenosine concentrations, similarly alleviates wear particle-induced bone destruction in mice (Mediero et al., 2015a) by an A_{2A}AR-dependent mechanism.

7. Adenosine Receptors and Cartilage. In recent studies in both mice (Corciulo et al., 2017) and humans (St Hilaire et al., 2011), premature development of osteoarthritis has been described, and in mice, loss of A_{2A}ARs leads to spontaneous development of osteoarthritis (Corciulo et al., 2017), indicating that endogenous adenosine production acts in an autocrine fashion to maintain chondrocyte homeostasis. Moreover, treatment of rats with post-traumatic osteoarthritis with intra-articular injections of liposomal adenosine preparations prevents progression of osteoarthritis (Corciulo et al., 2017). Similarly, loss of A_3ARs leads to the development of osteoarthritis in mice (Shkhyan et al., 2018), and treatment of chemically induced osteoarthritis with an A_3AR agonist inhibits development of osteoarthritis (Bar-Yehuda et al., 2009). These events suggest that targeting A_{2A}Rs or A_3ARs in the joint may be useful approaches to the treatment of osteoarthritis, a disabling condition affecting as many as 150 million people worldwide.

8. Adenosine Receptors and Fibrosis. Fibrosis is a common condition in a number of organs, and recent studies indicate that blockade of A_{2A}ARs can diminish excessive fibrosis in the skin, liver, and other organs in response to injury, ionizing radiation, or exposure to toxins (Shaikh and Cronstein, 2016). Indeed, in recent studies, topical application of an A_{2A}AR antagonist prevents both scarring and radiation fibrosis in the skin (Perez-Aso et al., 2012; 2016). In some organs, A_{2B}AR blockade can also diminish fibrosis (Shaikh and Cronstein, 2016), but recent studies suggest that in Peyronie's disease, which involves fibrosis of the shaft of the penis, A_{2B}AR stimulation prevents myofibroblast production of collagen (Mateus et al., 2018), suggesting that an A_{2B}AR agonist could prevent the development of Peyronie's disease.

9. Adenosine A_{2A} Receptors and Sickle Cell Disease. Patients with sickle cell disease suffer from focal areas of vascular obstruction leading to localized regions of poor perfusion and resulting ischemia. In these hypoxic foci, invariant natural killer T cells can induce further tissue injury, and A_{2A}AR stimulation inhibits invariant natural killer T cell function and tissue injury. Studies in humanized mice with sickle cell disease demonstrated that infusion of an A_{2A}AR agonist, regadenoson, reduced the tissue injury associated with sickle cell disease (Nathan et al., 2012). Although preclinical studies showed promise in these patients, the results of a clinical trial of regadenoson infusions for sickle cell disease did not show any evidence of shortened hospital stay or reduction in respiratory symptoms or opioid use (ClinicalTrials.gov Identifier: NCT01788631).

10. Summary. ARs are expressed ubiquitously in the periphery and play a variety of roles. ARs remain targets for clinical development despite recent failures in treatment of congestive heart failure and sickle cell disease. Immunostimulatory blockade of A_{2A}AR for the treatment of cancer shows real promise in early clinical trials, and development of other adenosine receptor targets is moving out of the laboratory into the clinic.

C. Therapeutic Targeting of Central Nervous System Adenosine Receptors

Although ARs are present throughout the human body, their density is far greater in the brain. Accordingly, manipulating ARs upon moderate intake of caffeine (Fredholm et al., 1999) mainly results in brain-associated effects, typified by increased arousal and attention with faster reaction time, decreased fatigue, more efficient working memory and memory recall, and better mood (Smith et al., 2005; McLellan et al.,

2016). These effects of caffeine are mostly mediated by brain ARs, namely A_1ARs and A_{2A}ARs (Fredholm et al., 2005), as heralded by the elimination of the effects of caffeine on synaptic transmission and plasticity upon blockade of A_1AR and A_{2A}AR (Lopes et al., 2019). Although also present in glia cells, A_1AR and A_{2A}AR are mostly colocated in excitatory synapses where they cooperate to encode information salience in neuronal circuits through a combined A_1AR-mediated inhibition of synaptic transmission (decreasing noise) and an A_{2A}AR-mediated facilitation of synaptic plasticity (increasing encoding) (Cunha, 2016).

1. Acute Brain Dysfunction – Ischemia and Epilepsy. Apart from their physiologic role, ARs also have an impact on brain dysfunction and damage, in accordance with the universal utilization of ATP (Rodrigues et al., 2015) and adenosine (Cunha, 2001) to signal stress or increased cellular workload in the brain. Thus, in conditions of metabolic stress such as upon ischemic stroke, both the acute A_1AR activation and A_{2A}AR blockade afford a robust neuroprotection but through different mechanisms. A_1AR activation increases the hurdle for onset of brain dysfunction by hyperpolarizing neurons. In contrast, A_{2A}AR blockade restrains neurodegeneration, probably as a result of the combined inhibition of glutamate release and decreased activation of N-methyl-D-aspartate (NMDA) receptors (Cunha, 2016), together with an attenuation of neuroinflammation (Rebola et al., 2011) and decreased neuronal apoptosis (Silva et al., 2007). A similar dual and opposite control by A_1ARs and A_{2A}ARs occurs upon abnormal increased workload typified by epileptic conditions (Tescarollo et al., 2020). Acute A_1AR activation attenuates the onset of seizures and, conversely, acute A_1AR inhibition decreases seizures threshold, whereas A_{2A}ARs control seizure-induced neurodegeneration (Canas et al., 2018). This dual control of the onset and evolution of brain damage by A_1ARs and A_{2A}ARs prompts the suggestion that a combined activation of A_1ARs and blockade of A_{2A}ARs might have a superior efficacy to limit acute brain damage (Cunha, 2005). However, timing of intervention might be of key importance since A_1ARs desensitize and their function decreases in the injured brain, which may result in paradoxical effects (Jacobson et al., 1996). In contrast, central A_{2A}ARs are upregulated in noxious brain condition (Cunha, 2016), justifying the interest in A_{2A}AR antagonists to control brain damage.

2. Neurodegenerative Diseases – Parkinson's and Motor Diseases. The particularly high density of A_{2A}ARs in the basal ganglia and their tight antagonistic interaction with dopamine D_2 receptors typified by the formation of A_{2A}AR-D_2 receptor heteromers (Ferré and Ciruela, 2019) prompted targeting A_{2A}ARs to alleviate dopaminergic depletion characteristic of PD. Indeed, A_{2A}AR antagonists dampen PD features in animal models, and the regular consumption of moderate doses of caffeine attenuates PD features in humans (Schwarzschild et al., 2006). As mentioned above, this preclinical evidence, together with the safety profile of A_{2A}AR antagonists, supported the US Food and Drug Administration's recent approval of istradefylline as an add-on therapy to manage PD patients (Chen and Cunha, 2020). This offers novel possibilities of carrying out phase 4 trials to directly test the role of A_{2A}ARs in the control of nonmotor PD symptoms, such as cognitive deficits and mood dysfunction. It should be mentioned that the clinical trajectory of istradefylline has been long and windy. After its introduction in Japan in 2013, market access to the United States has only recently (in 2019) been granted after an earlier rejection and is limited to treating "off" episodes with levodopa only. Other A_{2A}AR antagonists such as preladenant have failed to obtain market authorization from the Food and Drug Administration, as clinical efficacy was not convincingly demonstrated. This might be due to our insufficient knowledge of the role that different A_{2A}R populations have in the control of altered motor function and to lack of patient stratification in the clinical studies.

Selective A_{2A}AR antagonists also attenuate other motor conditions, such as catalepsy and tremor (Salamone et al., 2008), akathisia (Varty et al., 2008), dystonia (Maltese et al., 2017), cocaine or MK801-induced psychomotor activity (Shen et al., 2008; Yu et al., 2008), cerebrospinal type 3 ataxia or Machado-Joseph's disease (Gonçalves et al., 2013; Gonçalves et al., 2017), or amyotrophic lateral sclerosis (Ng et al., 2015). Their impact on Huntington's disease is less clear and might depend on the phase of the disease (Popoli et al., 2008). This broader ability of A_{2A}AR antagonists to control different motor disorders that might not directly result from dopaminergic depletion prompts the involvement of a control of glutamate excitotoxicity rather than only the control of dopamine D_2 receptors (Schiffmann et al., 2007; Cunha, 2016).

3. Neurodegenerative Diseases – Alzheimer's Disease and Cognitive Dysfunction. The pharmacological or the genetic blockade of A_{2A}ARs prevents memory deficits in different animal models of Alzheimer's disease (Canas et al., 2009; Laurent et al., 2016; Viana da Silva et al., 2016). A_{2A}AR antagonism also prevents memory dysfunction associated with other conditions, such as convulsions (Cognato et al., 2010), diabetes (Duarte et al., 2012), hypoxia (Chen et al., 2018), traumatic brain injury (Zhao et al., 2017), demyelination conditions (Akbari et al., 2018), repeated stress or depression (Batalha et al., 2013; Kaster et al., 2015; Machado et al., 2017), PD (Hu et al., 2016; Carmo et al., 2019), or cannabis exposure (Mouro et al., 2019). A_{2A}ARs are not only necessary but

actually sufficient to impair memory since their increased activity driven by genetic (Temido-Ferreira et al., 2020), optogenetic (Li et al., 2015), or pharmacological strategies (Pagnussat et al., 2015) impairs memory in normal animals. This converges with several findings in humans, namely: 1) Caffeine intake prevents cognitive deterioration upon aging (Ritchie et al., 2007; Dong et al., 2020) and is inversely associated with the onset (Eskelinen et al., 2009; Sugiyama et al., 2016) or neuropathological hallmarks of dementia (Gelber et al., 2011); 2) A_{2A}ARs are upregulated in the brains of demented patients (Temido-Ferreira et al., 2020); and 3) A_{2A}AR polymorphisms are associated with memory phenotypes (Beste et al., 2012; Horgusluoglu-Moloch et al., 2017). However, it is still unknown if A_{2A}AR antagonists ameliorate memory deficits in dementia patients.

4. Neuropsychiatric Diseases – Major Depression and Suicide. Caffeine, A_{2A}AR antagonists, and the genetic deletion of A_{2A}ARs selectively in forebrain neurons abrogate the onset of depressive-like symptoms and can also reverse these symptoms in mice subject to chronic unpredictable stress (Kaster et al., 2015). Accordingly, coffee intake is inversely correlated with the incidence of depression (Grosso et al., 2016; Lucas et al., 2011) and its major consequence suicide (Lucas et al., 2014), and the incidence of major depression is associated with A_{2A}AR haplotypes (Oliveira et al., 2019). In parallel, the upregulation of A_1ARs bolsters the resilience toward depressive-like behavior and, conversely, knocking out A_1ARs increased depressive-like behavior and eliminated the antidepressant effects of sleep deprivation (Serchov et al., 2015). Furthermore, A_1ARs in the amygdala are also involved in neuroimmune-driven depression (Fan et al., 2019).

5. Other Neuropsychiatric Diseases. The elegant work of Chen and colleagues revealed a temporal ability of A_{2A}ARs to modulate instrumental behavior, formatting the sensitivity to goal-directed valuation (Li et al., 2016; 2018). Thus, A_{2A}AR-mediated overactivation of striatopallidal neurons disrupts the homeostatic control of goal-directed behavior, with impaired decision-making and behavioral disinhibition with loss of flexibility, which are at the core of psychiatric symptoms (Li et al., 2016; 2020; He et al., 2020). Indeed, A_{2A}ARs control addiction (Ferré, 2016; Borroto-Escuela et al., 2018) and preservative and obsessive-compulsive behaviors (Bleickardt et al., 2014; Asaoka et al., 2019) that are transversal to most neuropsychiatric diseases. Accordingly, A_{2A}AR polymorphisms are associated with anxiety (Alsene et al., 2003; Fraporti et al., 2019), depression (Oliveira et al., 2019), phobia (Deckert et al., 1998; Hamilton et al., 2004), preservative/obsessive disorders (Freitag et al., 2010; Janik et al., 2015), or addictive profiles (Kobayashi et al., 2010). This A_{2A}AR-mediated control of behavioral inhibition may be associated with their ability to modulate arousal (Lazarus et al., 2012) and enhanced motivation (He et al., 2020), which are founding behaviors of decision-making and cognitive performance.

6. Brain Aging. Aging is by far the major risk factor for most prevalent chronic brain diseases, namely depressive, cerebrovascular, and neurodegenerative diseases. The adenosine modulation system in the forebrain is modified upon aging with a decreased density and functional efficiency of A_1ARs (Sperlagh et al., 1997; Sebastião et al., 2000; Costenla et al., 2011) and an increased density and efficiency of A_{2A}ARs (Rebola et al., 2003; Canas et al., 2009; Costenla et al., 2011). These alterations posit a contribution of the adenosine modulation system to the deterioration of brain function since hyperactivity of A_{2A}ARs is sufficient to trigger brain dysfunction (Li et al., 2015; Carvalho et al., 2019; Temido-Ferreira et al., 2020) and a hypofunction of A_1ARs increases excitability (noise) of brain networks and bolsters the spreading of excitotoxicity (Tescarollo et al., 2020). Indeed, the intake of caffeinated coffee is inversely associated with memory deterioration upon aging (Hameleers et al., 2000; Ritchie et al., 2007; van Gelder et al., 2007; Arab et al, 2011; Dong et al., 2020), which was shown in animal models to be reverted by caffeine and by selective A_{2A}AR antagonists (Prediger et al., 2005). This stresses the particular association between increased A_{2A}AR activity with the deterioration of brain function upon aging, which might underlie the increased susceptibility for the emergence of age-associated brain diseases.

Hyperactivity of A_{2A}ARs in the aged brain is further reinforced by the parallel increase of the pathway responsible for the formation of the pool of adenosine selectively associated with the activation of A_{2A}ARs, namely ecto-5′-nucleotidase (CD73)-mediated formation of ATP-derived extracellular adenosine (Augusto et al., 2013; Carmo et al., 2019; Gonçalves et al., 2019). In fact, different studies reported a robust increase of the activity of CD73 in the aged brain (Fuchs, 1991; Cunha et al., 2001; Mackiewicz et al., 2006), as best heralded by the inverse association of CD73 activity with the probability of reaching centenarian ages (Crooke et al., 2017). In parallel, the activity of AdoK is decreased in the aged brain (Mackiewicz et al., 2006). This bolsters the availability of the extracellular adenosine (Cunha et al., 2001; Murillo-Rodriguez et al., 2004), possibly to compensate the decreased density of A_1ARs. Indeed, the alteration of purinergic metabolism seems to be a prominent characteristic—a metabolic fingerprint of the aging process in different organisms (Furman et al., 2017; Gao et al., 2018a). Precocious modifications of adenosine metabolism occur in the brain of aged rodents (Ivanisevic et al., 2016) and in an

animal model of accelerated aging (Sanchez-Melgar et al., 2020).

VII. Current and Recent Clinical Trials

Both AR agonists and antagonists have been in clinical trials dating back to the late 1960s for a wide range of conditions (Borah et al., 2019; Jacobson et al., 2019). Although most of these clinical trials were unsuccessful, the therapeutic focus of AR-based therapeutics has shifted since the early days, and new trials are underway for more recently identified indications (Table 5; Fig. 5). There is reason for optimism that this situation can be remedied in future clinical trials based on current pharmaceutical technology. Firstly, the availability of high-resolution experimental structures of two of the adenosine receptors allows the discovery and optimization of compounds of extremely high selectivity for each of the four receptors. Furthermore, the lack of efficacy in clinical trials often results from inadequate pharmacokinetics, which has been greatly improved in the recent generation of adenosine receptor ligands, which are also much more structurally diverse than in the past. Additionally, the expanded range of allosteric modulators of the adenosine receptors and indirect adenosine modulators (e.g., enzyme and transport inhibitors) promises to provide clinical candidate molecules that are more temporally and spatially selective than orthosteric agonists. More specifically, further research may lead to new therapeutics, including: A_{2A}- and A_{2B}AR antagonists for treating cancer and neurodegenerative conditions such as Parkinson's disease and Alzheimer's disease; A_{2B}- and A_3AR agonists for treating chronic conditions such as obesity and NASH, respectively; A_1- and A_3AR agonists for treating chronic pain; A_3AR antagonists for treating glaucoma; and A_{2A}- and A_{2B}AR agonists for treating musculoskeletal conditions such as osteoporosis. These are potential therapeutic approaches that have matured scientifically in recent years.

A. Clinical Trials of Adenosine Receptor Agonists

AR agonists and partial agonists have been considered for pharmaceutical development in the treatment of: pain, seizures, arrhythmias, atrial fibrillation, diabetes, chronic heart failure, glaucoma (A_1AR); hypertension (and diagnosis), inflammation, atrial fibrillation, ischemic conditions, sickle cell disease (A_{2A}AR); neurodegeneration, inflammation, hepatocellular carcinoma, ischemic conditions, NASH, and chronic neuropathic pain (A_3AR). There are no clinical trials of A_{2B}AR agonists, but their use as antidiabetic agents or in cardioprotection, lung injury, diabetes, pulmonary hypertension, and other vascular conditions has been suggested (Eckle et al., 2007; Koscso et al., 2013; Merighi et al., 2015; Bessa-Gonçalves et al., 2018).

The antiarrhythmic effects of adenosine acting at the A_1AR led to its approval for treating supraventricular tachycardia (Jacobson et al., 2019). More selective nucleoside-based A_1AR agonists were also considered for this application, but their clinical trials failed. A partial A_1AR agonist, CVT-3619, was predicted to display fewer side effects as an antiarrhythmic agent, but the clinical trial was discontinued (Jacobson et al., 2019). A clinical trial of A_1AR agonist selodenoson (78, DTI-0009) for atrial fibrillation was discontinued. Non-nucleosides have also been developed as A_1AR agonists. 3,5-Dicyanopyridine derivative capadenoson was in a clinical trial for heart failure, but it was later supplanted by an ongoing trial of neladenoson (77, BAY1067197), a newer, more selective prodrug derivative of the same structural class (Shah et al., 2019). However, the compound failed to meet the clinical endpoint. A phase 3 trial of A_1AR agonist trabodenoson (79) also failed to demonstrate efficacy (Jacobson and Civan, 2016). A_1AR agonists, including selective agonist GW493838 and intrathecally administered adenosine, and a PAM (T-62) were also studied for their application in pain, but three clinical trials failed to demonstrate efficacy (Miao et al., 2018; Jacobson et al., 2019).

The vasodilatory effects of A_{2A}AR agonists have resulted in the approval of adenosine (1) itself and regadenoson (2, CVT-3146) for coronary stress imaging in patients not suitable for exercise-induced vasodilation. However, clinical trials for chronic obstructive pulmonary disease, asthma, and sickle cell disease, based on the anti-inflammatory effects of A_{2A}AR agonists, were unsuccessful (Jacobson et al., 2019).

A_3AR activation has been the subject of many clinical trials, and several are ongoing (Jacobson et al., 2019). Two prototypical A_3AR agonists, IB-MECA (48, CF101, piclodenoson) and Cl-IB-MECA (14, CF102, namodenoson) have progressed to clinical phases 3 and 2 for autoimmune inflammatory diseases and liver diseases, respectively. A_3AR agonists also have protective effects in models of chronic pain. Piclodenoson is in phase 3 trials for rheumatoid arthritis/psoriasis. Namodenoson is in phase 2 trials for hepatocellular carcinoma and nonalcoholic steatohepatitis (NASH).

B. Clinical Trials of Adenosine Receptor Antagonists

AR antagonists have been considered for pharmaceutical development in the treatment of: asthma, renal dysfunction (A_1AR); neurodegeneration, cancer (A_{2A}AR); cancer, asthma, diabetes (A_{2B}AR); glaucoma, psoriasis, NASH, and ulcerative colitis (A_3AR). There are not yet therapeutic antibodies proposed for use in blocking ARs.

Various clinical trials of A_1AR antagonists for treatment of heart failure were eventually discontinued. These A_1AR antagonists include rolofylline (82,

TABLE 5
Representative adenosine receptor modulators in clinical trials, currently and previously, according to clinicaltrials.gov

Compound	Action	Activity	Phase, National Clinical Trial Number
Agonists			
Adenosine (1)	Nonselective agonist	headache/migraine	-, 04577443
		ADCY5-related dyskinesia	-, 04469283
Neladenoson bialanate (77, BAY1067197)	A_1AR agonist	heart failure	2, 02040233, PARSiFAL 2, 02992288, PANTHEON 2, 03098979, PANACHE
Selodenoson (78, DTI-0009, RG14202)	A_1AR agonist	atrial fibrillation	2, 00040001
Trabodenoson (79, INO-8875; PJ-875)	A_1AR agonist	glaucoma	3, 02565173
Regadenoson (2, CVT 3146)	A_{2A}AR agonist	sickle cell anemia	2, 01788631
		COVID-19	1/2, 0460609
		pulmonary hypertension	-, 02220634
		glioma (disrupting BBB)	1, 03971734
Tecadenoson (80, CVT-510)	A_{2A}AR agonist	atrial fibrillation	2, 00713401
Spongosine (81, BVT.115959)	A_{2A}AR agonist	diabetic nerve pain	2, 00452777
UK-432,097 (11)	A_{2A}AR agonist	chronic obstructive pulmonary disease	2, 00430300
Piclodenoson (48, IB-MECA, CF-101)	A_3AR agonist	rheumatoid arthritis	3, 02647762
		psoriasis	3, 03168256
		COVID-19	2, 04333472
Namodenoson (14, Cl-IB-MECA, CF-102)	A_3AR agonist	hepatocellular carcinoma	2, 02128958
		NASH (non-alcoholic steatohepatitis)	2, 02927314
Antagonists			
Caffeine (3)	Nonselective antagonist	hypoxic-ischemic encephalopathy	1, 03913221
		ADCY5-related dyskinesia	-, 04469283
		Alzheimer's disease	3, 04570085
		radiation-induced fibrosis	2, 03768492
		glaucoma	-, 03675412
Theophylline (4)	Nonselective antagonist	acute kidney injury	3, 03897335
		smell in COVID-19	2, 04789499
		depression	1, 04309877
		anaesthesia recovery	1, 04151381
Rolofylline (82, KW-3902)	A_1AR antagonist	congestive heart failure	3, 00328692 (PROTECT-1) 3, 00354458 (PROTECT-2)
SLV320 (19)	A_1AR antagonist	heart failure and	2, 00744341
		renal dysfunction	2, 00160134
		combined with furosemide	-, 00568009[a]
PBF-680	A_1AR antagonist	asthma	2, 03774290, ADENOASMA
Istradefylline (5, KW-6002)	A_{2A}AR antagonist	Parkinson's disease (alone)	2, 00250393
		Parkinson's disease (with L-dopa)	3, 00955526, 6002-009 3, 01968031
Preladenant (21, MK-3814, SCH 420814)	A_{2A}AR antagonist	Parkinson's disease	3, 01155479, PARADYSE
		antipsychotic drug side effects	2, 00686699, P04628
		advanced solid tumors (alone and in combination with pembrolizumab)	1, 0309916
BIIB014 (65)	A_{2A}AR antagonist	Parkinson's disease	2, NCT00438607
Tozadenant (66)	A_{2A}AR antagonist	Parkinson's disease	3, NCT03051607
Taminadenant (83, NIR178, PBF-509)	A_{2A}AR antagonist	Parkinson's disease, non-small cell lung cancer (with PDR001*b*)	1, 02111330 1/2, 02403193, AdenONCO
		various cancers*c* (with PDR001)	3, 03207867
Ciforadenant (84, CPI-444, V81444)	A_{2A}AR antagonist	advanced cancers (in combination with CD73 blocker, CPI-0006)	1, 03454451
		(in combination with PD-L1/PD-[1]*b*)	1/2, 03337698
Imaradenant (22, AZD4635, HTL1071)	A_{2A}AR antagonist	cancer, alone	1, 03980821
		(in combination with CD73 blocker, MEDI9447*d*)	1/2, 03381274
		(in combination with anticancer drugs)	1, 02740985 2, 04089553
Inupadenant (85, EOS100850)	A_{2A}AR antagonist	solid tumors	1, 03873883
Etrumadenant (86, AB928)	A_{2A}/A_{2B} antagonist	various cancers (with AB122[b])	1, 03629756
		(in combination with anticancer drugs)	1/2, 04381832
PBF-1129	A_{2B}AR antagonist	non-small cell lung cancer	1, 03274479
PBF-677	A_3AR antagonist	glaucoma	1, 02639975
		ulcerative colitis	2, 03773952, ADENOIBD
PBF-1650	A_3AR antagonist	psoriasis, NASH	1, 03798236, ADENOIMMUNE
FM101 (87)	A_3AR antag/part agonist	glaucoma	1/2, 04585100
		NASH	2, 04710524

Note that this list is not all-inclusive (e.g., dipyridamole has been omitted). Other compounds are reviewed elsewhere (Borah et al., 2019; Jacobson et al., 2019). Structures, when disclosed, are shown in Figs. 1, 2, 3, and 5.

[a]terminated additional enrollment criteria made patient recruitment unfeasible.

[b]checkpoint inhibitor.

[c]triple negative breast cancer, pancreatic ductal adenocarcinoma, non-small cell lung cancer, renal cell cancer, urothelial cancer, head and neck cancer, diffused large B cell lymphoma, microsatellite stable colon cancer, non-Hodgkin lymphoma.

[d]oleclumab.

KW-3902) for congestive heart failure and SLV320 (19) for renal dysfunction/heart failure.

Initially, when A_{2A}AR antagonists were first reported in the early 1990s, the principal target was PD. Several selective antagonists were in clinical trials, some of which indicated a relatively modest effect, whereas others did not reach statistical significance. Antagonists in this group were tozadenant (66), preladenant (21), and istradefylline (5). A phase 3 trial of tozadenant was discontinued after five fatalities from agranulocytosis had occurred. The caffeine-like antagonist istradefylline was first approved in Japan for use as a cotherapy in treating PD to reduce off-time. Additional clinical evidence of a beneficial effect of istradefylline has accrued, leading to its recent approval in the United States, as mentioned before (Chen and Cunha, 2020).

Currently, the most excitement surrounds the use of A_{2A}AR antagonists as adjuvants in cancer immunotherapy. Adenosine forms an immunosuppressive "cloud" in the tumor microenvironment for both solid malignancies and hematologic cancer (Sek et al., 2018). The adenosine acts through both A_{2A}AR and A_{2B}AR to induce an anti-inflammatory phenotype in T cells, macrophages, and other cells; and antagonists have a beneficial effect when combined with immunotherapy (Congreve et al., 2018). Ongoing clinical trials include: NIR178 (83, PBF-509, now taminadenant) for PD, NSCLC, and various other cancers in combination with a checkpoint inhibitor; CPI-444 (84, formerly V81444, now ciforadenant) for advanced cancers in combination with a checkpoint inhibitor; phase 1/1b study of inupadenant (85, EOS100850) for solid tumors (NCT03873883) (Houthuys et al., 2018); PBF-1129 for non-small cell lung cancer (NSCLC, structure not disclosed); and mixed A_{2A}AR/A_{2B}AR antagonist AB928 (86, now etrumadenant) for various cancers in combination with checkpoint inhibitor AB122. A_{2A}AR antagonist preladenant (21) has been repurposed from a failed phase 3 trial in PD to treating advanced solid tumors (also in combination with pembrolizumab) but the data did not support study endpoints. A_{2A}AR antagonist AZD4635 (22, formerly HTL1071, now imaradenant) was developed by rational drug design based on A_{2A}AR X-ray structures. Its envisioned application was attention deficit hyperactivity disorder (ADHD), but it is currently being applied to cancer immunotherapy (Borodovsky et al., 2020).

VIII. Concluding Remarks

The number of scientific publications with "adenosine receptor" as a topic in the Web of Science database has remained relatively stable over the years. With close to 4500 articles in each of the two decades covered by this and the previous report, one captures the field as relatively mature and significant. This number is quite comparable to other GPCRs (e.g., ~4000 in the last decade for serotonin/5-HT and ~6000 for dopamine), more than for histamine receptors (~1000) but fewer than for chemokine receptors (close to 13,000 publications in the last decade). Even a relatively comprehensive report like this one can only pinpoint to some of these many references, however, with a focus on particular topics that have gained traction over the years. We invite readers to draw our attention to other focal points for future inclusion. Such focal points in this report not or hardly covered before are target binding kinetics, receptor structure, and biased signaling. It turns out that high-affinity ligands, be it agonists or antagonists, may have very divergent kinetic profiles. Some have relatively short residence times (defined as $1/k_{off}$) at one or more of the four AR subtypes, whereas others show long residence times, up to several hours. It should be mentioned that many of these studies have been performed at lower than physiologic temperature, which impedes a reliable estimation of in vivo residence times and target engagement. The ultimate in this respect is covalent binding, a feature that is now well established in clinically approved kinase inhibitors but that has not found much application yet in clinical studies of GPCRs. With only one receptor structure discussed in the previous report, remarkable progress has been made, particularly with respect to the A_{2A}AR. By combinations of fusion proteins and thermostabilizing receptor mutations, this receptor has become one of the more easily accessed GPCRs for structure elucidation. Today it has become possible, as with cytosolic proteins, to soak/exchange receptor crystals to enable multiple crystal structures at the same time. On the other hand, the A_{2B}AR and A_3AR have not been successfully subjected to structure elucidation. Biased signaling has been a hot topic and heavily studied aspect of GPCR signaling in the last decade, but less so for ARs. Compared with the opioid receptor field, for example, most AR agonists appear to display less outspoken preferences, also with a less obvious separation between desired and side effects. Still, the potential of developing adenosine receptor agonists that are biased for particular signaling pathways associated with treatment modalities promises to alleviate the problem of side effects of adenosine agonists, as noted in multiple clinical trials. However, in most cases, the precise G protein-dependent or independent pathways involved in the salutary effects of adenosine agonists are unexplored. Recently, an A_1AR agonist that was reported to selectively activate G_{ob} versus the other five $G\alpha_{i/o}$ subtypes and without β-arrestin recruitment was discovered (Wall et al., 2020). It appears to act as a potent analgesic without sedation or cardiorespiratory depression. This can serve as a model for applying biased signaling to other adenosine receptor subtypes. Finally, clinical development takes a long time, and this is also true for AR ligands. Istradefylline

was only recently allowed access to the United States (Food and Drug Administration) and is in the approval process for the European market (European Medicines Agency) for the treatment of motor effects in PD long after its introduction in Japan. It had been reviewed as an early clinical candidate in our 2001 report, whereas in the 2011 update it was again mentioned as a clinical candidate, then in large phase 3 trials. This suggests that there will be room for a fourth update a decade from now.

Acknowledgments

Figure 4 and Supplemental Videos 1 and 2 were assembled and configured by Willem Jespers (Uppsala University, Sweden/Leiden University, The Netherlands), which is gratefully acknowledged.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: IJzerman, Jacobson, Müller, Cronstein, Cunha.

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