REVIEW ARTICLE

Species dependence of A$_3$ adenosine receptor pharmacology and function

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Abstract

Efforts to fully understand pharmacological differences between G protein-coupled receptor (GPCR) species homologues are generally not pursued in detail during the drug development process. To date, many GPCRs that have been successfully targeted are relatively well-conserved across species in amino acid sequence and display minimal variability of biological effects. However, the A$_3$ adenosine receptor (AR), an exciting drug target for a multitude of diseases associated with tissue injury, ischemia, and inflammation, displays as little as 70% sequence identity among mammalian species (e.g., rodent vs. primate) commonly used in drug development. Consequently, the pharmacological properties of synthetic A$_3$AR ligands vary widely, not only in binding affinity, selectivity, and signaling efficacy, but to the extent that some function as agonists in some species and antagonists in others. Numerous heterocyclic antagonists that have nM affinity at the human A$_3$AR are inactive or weakly active at the rat and mouse A$_3$ARs. Positive allosteric modulators, including the imidazo [4,5-c]quinolin-4-amine derivative LUF6000, are only active at human and some larger animal species that have been evaluated (rabbit and dog), but not rodents. A$_3$AR agonists evoke systemic degranulation of rodent, but not human mast cells. The rat A$_3$AR undergoes desensitization faster than the human A$_3$AR, but the human homologue can be completely re-sensitized and recycled back to the cell surface. Thus, comprehensive pharmacological evaluation and awareness of potential A$_3$AR species differences are critical in studies to further understand the basic biological functions of this unique AR subtype. Recombinant A$_3$ARs from eight different species have been pharmacologically characterized thus far. In this review, we describe in detail current knowledge of species differences in genetic identity, G protein-coupling, receptor regulation, and both orthosteric and allosteric A$_3$AR pharmacology.

Keywords Nucleoside · Antagonist · G protein-coupled receptor · Adenosine receptor · Species differences

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Abbreviations

AR	Adenosine receptor
CHO	Chinese hamster ovary
Cl-IB-MECA	2-Chloro-N^6-(3-iodobenzyl) adenosine-5′-N-methylcarboxamide
GPCR	G protein-coupled receptor
[^{35}S]GTPγS	Guanosine 5′-[γ-[^{35}S]thio] triphosphate
HEK293	Human embryonic kidney 293

[^{125}I]I-AB-MECA	N^6-(4-amino-3-[^{125}I]iodobenzyl) adenosine-5′-N-methylcarboxamide
LUF6000	2-Cyclohexyl-N-(3,4-dichlorophenyl)-1H-imidazo [4,5-c] quinolin-4-amine
LUF6096	N-{2-[(3,4-Dichlorophenyl)amino]quinolin-4-yl} cyclohexanecarboxamide
NECA	Adenosine-5′-N-ethylcarboxamide
VUF5455	4-Methoxy-N-[7-methyl-3-(2-pyridyl)-1-isoquinoyl]-benzamide
PAM	Positive allosteric modulator

Introduction

G protein-coupled receptors (GPCRs) play essential physiological roles as signal transducers for regulation of organ function and sensory perception and are proven targets for therapeutic drugs [1]. In general, the amino acid sequence of a given GPCR is fairly well-conserved among mammalian species homologs, typically ~90%, particularly for most GPCRs that have been successfully targeted with therapeutic drugs. For example, the amino acid sequence identity for all five Class A muscarinic acetylcholine receptors (M_1–M_5) is 89–98% identical among mammalian species that are commonly used in drug development research (dog, rabbit, rat, mouse), and Class A human and rat β_1 adrenergic receptors are 88% identical. Nevertheless, awareness of significant species differences among various druggable GPCRs has emerged, including the Class A histamine receptor family [2] and the Class C metabotropic glutamate receptor family [3]. As an example, subtle differences in the functionally enhancing activity of the M_4 positive allosteric modulator (PAM) LY2033298 between human and mouse receptors has been described [4]. More striking species differences have been noted for D_1 dopamine receptor allosteric modulators [5, 6]. An evolutionary approach has been used to analyze conserved GPCR sequence-function relationships between distant species homologues [7].

Species differences among adenosine receptors

There are four subtypes of Class A adenosine receptors (ARs), termed A_1, A_{2A}, A_{2B}, and A_3. The A_1 and A_3ARs couple to $G_{i/o}$ inhibitory proteins, whereas A_{2A}ARs couple to G_s proteins. Uniquely, the A_{2B}AR dually couples to G_s and G_i in many cell types [8, 9] and couples to G_q proteins as well [10, 11]. For A_1, A_{2A}, and A_{2B}ARs, the sequence identity among human, dog, rat, and mouse is greater than 90%, so accordingly, there is relatively little pharmacological variability among these receptors, and only relatively

small differences in binding affinity of agonists and antagonists have been described. The A_1AR is a good example, where the binding affinity of N^6-adenine-substituted agonists such as R-PIA (Fig. 1) and C-8-substituted xanthine antagonist derivatives such as DPCPX (Fig. 2) remains potent across species, having only approximately 20-fold differences among human, dog, bovine, and rat. These species differences have been attributed to two amino acids in transmembrane helical domain (TM)7 using site-directed mutagenesis [12]. A well-studied A_1AR PAM, PD81,723, retains activity across all species that have been tested [13–15]. Other characteristics are also common to all A_1AR species, including activation and desensitization, which both occur slowly over several hours for this receptor [16, 17].

However, for the A_3AR, the amino acid sequence identity between human and rat homologues is only 72% (Table 1), and stark species differences in pharmacology have been observed for agonists, antagonists, and allosteric modulators [16–19]. Pharmacological differences between human and rodent (rat and mouse) A_3ARs are especially prominent (Table 2). For example, the binding affinities of N^6-methyladenosine at human and rat A_3ARs are 9.0 nM and 6.4 μM, respectively [16], a difference of ~700-fold. The binding affinity of the potent human A_3AR antagonist MRS1220 is 0.6 nM, i.e., 50,000-fold higher affinity than at the rat A_3AR, which is 30 μM [20]. Caffeine, theophylline, and other alkylxanthines are weak antagonists of all the human ARs but are inactive at rat or mouse A_3ARs at concentrations up to 100 μM [21–23].

The dramatic species-dependent variation in ligand potency between human and rodent A_3ARs is most likely because of the large genetic difference among species, as the amino acid identity between human and mouse A_3ARs is 73%. A higher degree of identity is found between human and canine A_3ARs (88%), between human and sheep A_3ARs (86%), and between rat and mouse A_3ARs (89%). The genetic differences may be reflected in functional variation among different A_3AR species homologues. Generally, A_3AR function is similar among more closely related mammalian species such as larger mammals, and among smaller mammals such as rodents, but differences are more pronounced between the two. This point should be earnestly considered in developing drugs that target the A_3AR. The application of various genetically modified small animal models, including global [28] and conditional mouse A_3AR gene knockout (KO) models [29], is necessary to implicate the A_3AR in specific human diseases and to validate effectiveness and specificity of potential clinical leads. The difference in A_3AR antagonist pharmacology between human and mouse led to the introduction of an "A_3AR functionally humanized" mouse model in which a human/mouse chimera that maintains faithful coupling to phosphoinositol 3-kinase
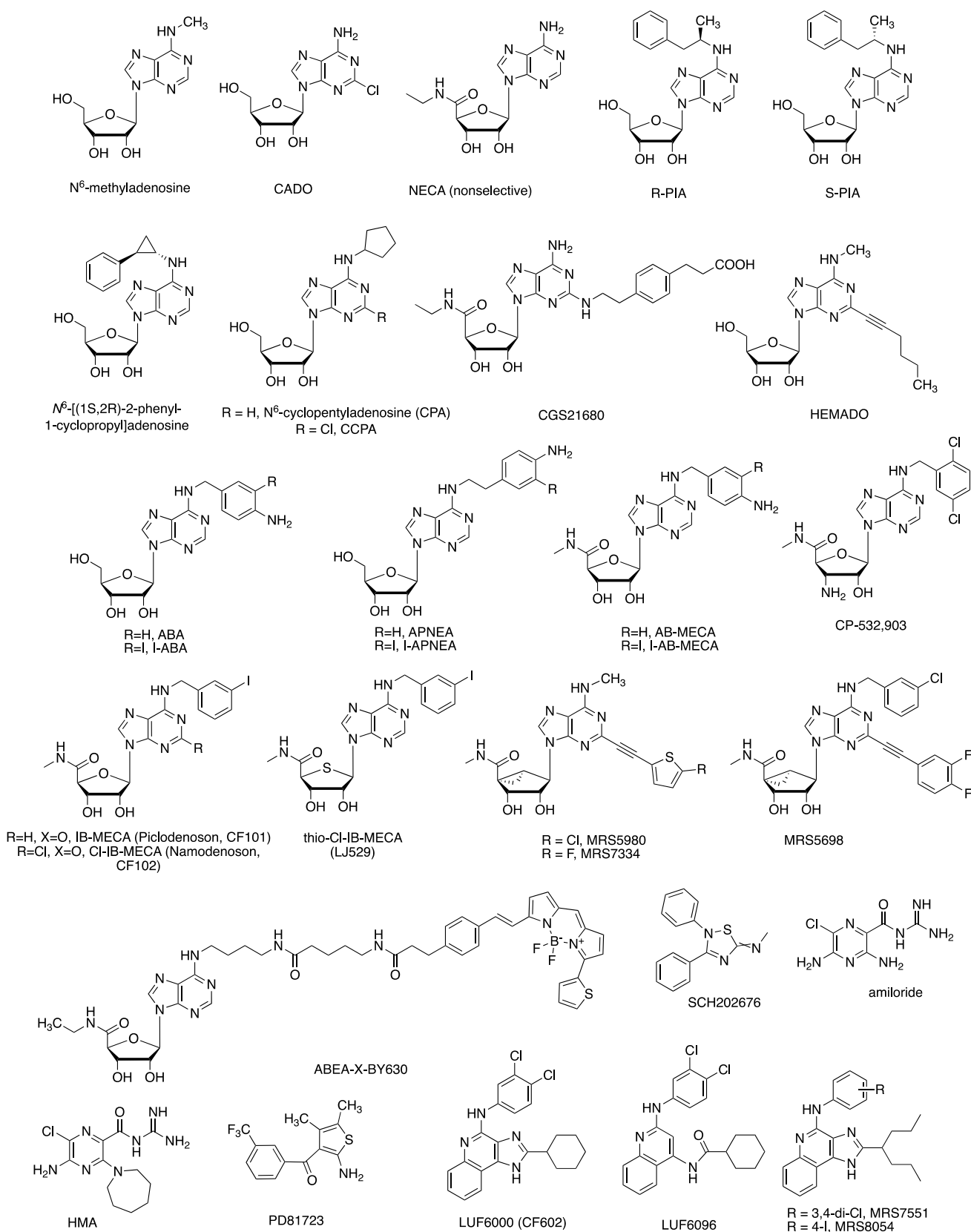
Fig. 1 Chemical structures of representative A_3AR agonists and allosteric modulators, including those that are commercially available (see text)

Fig. 2 Chemical structures of representative A₃AR antagonists, including those that are commercially available (see text). The four 4'-truncated nucleosides (bottom row, right) can display residual low efficacy agonism, depending on the model
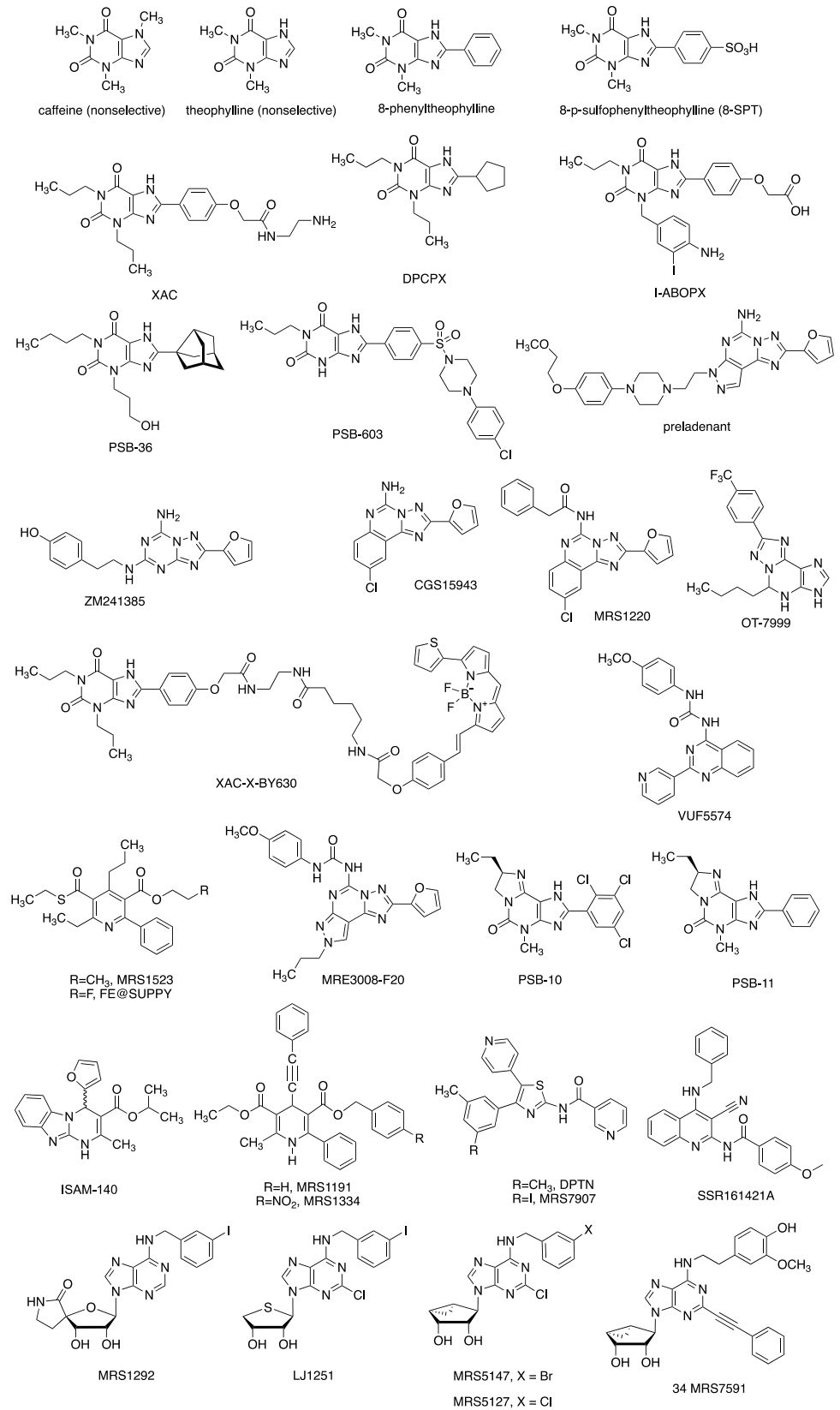
Table 1 Sequence identity between species of the A_3AR, as determined from protein sequences reported in the National Center for Biotechnology Information (NCBI, Bethesda, MD) website (https://www.ncbi.nlm.nih.gov/protein/, accessed Aug. 13, 2021), unless noted

Species[a] (Adora3)	Compared to:	% Sequence identity
Rat	Human	72
Sheep	"	85
Rabbit	"	76
Canine	"	88
Mouse	"	73
Chick	"	52
Equine	"	83
Marmoset	"	92
Rhesus monkey[b]	"	97
Wild boar	"	90
Rat	Mouse	88
Canine	Sheep	86
Equine	Sheep	85
Sheep	Rat	72
Rat	Canine	72

[b]From nucleotide sequences coding for A_3AR of other species available from NCBI (https://www.ncbi.nlm.nih.gov/nuccore/, accessed Aug. 13, 2021)

(PI3K) ϒ-signaling in mast cells replaced the mouse A_3AR [30]. This is a particularly useful model to test the utility of antagonists for inflammatory and allergic diseases.

In this review, we first compare the protein sequence differences among the eight A_3AR species homologues that have been cloned and studied pharmacologically. We then assess the pharmacological profile of agonists, antagonists, and allosteric modulators at the A_3AR of various species where data are available. We also explore differences in receptor activation, desensitization, re-sensitization, internalization and downregulation, and the mechanistic differences behind these A_3AR-mediated events using human and rat A_3ARs as models. Finally, we discuss examples where lack of attention to fundamental A_3AR species differences may have led to data misinterpretation in A_3AR studies in experimental animals.

Discovery and genetic differences of A_3AR from various species

The original observation of an A_3AR pharmacological action was from Ali et al. [31], who reported that the relatively nonselective AR agonists NECA and R-PIA induced release of inositol phosphates (IP) and increased cytosolic Ca^{2+} levels in RBL-2H3 cells, a rat basophilic leukemia cell line. This activity was later ascribed to the cloned rat A_3AR [32]. Both NECA and R-PIA enhanced antigen (DNP-BSA)-induced IP and Ca^{2+} release in RBL-2H3 cells in a pertussis toxin-sensitive manner, whereas the effect of DNP-BSA was pertussis toxin-insensitive [31]. The prototypical AR antagonist theophylline did not substantially inhibit the stimulatory effects of NECA and R-PIA at concentrations up to 100 μM. 8-Phenyltheophylline, which binds to native rat A_1 and $A_{2A}ARs$ with affinities of 86 and 848 nM [33], respectively, was also ineffective (10 μM). Thus, the effects of NECA and R-PIA were sensitive to pertussis toxin but insensitive to alkyl xanthine antagonists. The authors proposed that NECA and R-PIA act via a novel AR subtype (termed "A_3 adenosine receptor") [31]. Since then, RBL-2H3 cells have been widely used as a model for studying the A_3AR, especially its role in mediating mast cell degranulation [34–36]. Even prior to the report by Ali et al. [31], Marquardt et al. [37] reported that adenosine (100 μM) enhanced cation ionophore (A23187)-induced histamine release in primary mast cells collected from rat thoracic and abdominal cavities. Adenosine-induced potentiation was partially inhibited by a theophylline concentration of 1 μM and completely blocked at 100 μM. The relatively xanthine-resistant effect of adenosine was suggestive of involvement of an atypical adenosine receptor.

Ribeiro and Sebastiao [38] studied the role of adenosine in the frog neuromuscular junction and proposed that an "A_3 adenosine receptor is a voltage-dependent calcium channel, which changes its conformation after binding adenosine." However, the term "A_3 adenosine receptor" described in this work does not seem to be related to the A_3AR identified in RBL-2H3 cells [31] or the cloned rat A_3AR, which will be described below. The term "A_3 adenosine receptor" has also been used in other instances in past years, but none are related to the one reported by Ali et al. [31] and Zhou et al. [32].

At the beginning of the molecular cloning era of GPCRs in the late 1980s and early 1990s, the rat A_3AR cDNA was first isolated from a rat testis cDNA library by Meyerhof et al. [39], but adenosine derivatives were not included among a panel of a dozen bioactive compounds that were initially tested as potential ligands, and thus the receptor was not deorphanized until later. Zhou et al. [32] re-cloned the rat A_3AR (318 amino acids) in 1992 from a rat brain cDNA library and then first discovered that it responded to adenosine and comprised a new AR subtype. Using in situ hybridization, the authors found highest expression of transcript in the testis, implicating for the first time a role of adenosine in reproduction. Zhou et al. [32] expressed the newly discovered cDNA in CHO cells and detected specific binding in isolated cell membranes using the agonist radioligand [^{125}I]I-APNEA. In subsequent competition binding assays with [^{125}I]I-APNEA, the rank order of potency of agonist AR ligands was as follows: R-PIA≈NECA > S-PIA > adenosine. Xanthine antagonists

Table 2 Agonist and antagonist affinity (K_i or K_d) or potency (EC_{50}, nM) at A_3ARs in 8 species from initial pharmacological characterization after cloning

Species	Rat	Sheep	Human	Rabbit	Canine	Mouse[b]	Chick	Equine
Reference	Zhou et al., 1992	Linden et al., 1993	Salvatore et al., 1993	Hill et al., 1997	Auchampach et al., 1997	Salvatore et al., 2000	Durand et al., 2001	Brandon et al., 2006
Cells	CHO	CHO or COS[a]	CHO	CHO	COS[a]	CHO	HEK	HEK
cAMP (ag)	R-PIA	I-ABA	I-ABA	R-PIA	ND	NECA	Cl-IB-MECA	IB-MECA
EC_{50} (nM)	18	50	35	20	ND	56	25	0.9
RL^2	APNEA	I-ABA	I-ABA	I-ABA	I-ABA	I-ABA	I-ABA	I-AB-MECA
RL-K_d	16	6	10	5	0.7	ND	1.2	ND
NECA	74	300	26	27	34	13	37	7.9
R-PIA	63	1210	34	49	3	10	1.1	ND
S-PIA	1140	13,720	324	ND	ND	ND	ND	ND
CPA	ND	>100,000	89	49	22	ND	2.6	ND
CGS21680	144	ND	114	ND	398	93	ND	110
XAC	>100,000	180	71	329	138	ND	ND	ND
DPCPX	>100,000	49,300	759	1120	115	>100,000	53.3	ND
I-ABOPX	1500[c]	3	18	18[c]	38	ND	ND	ND
BW-A1433	15,000[c]	21	55	746	1800	ND	37	ND
Theophylline	>100,000	424,000	22,300	>100,000	>100,000	ND	ND	ND
8-SPT	>100,000	ND	ND	37,800	25,300	ND	ND	30,000
MRS1220	>10,000[b]	ND	0.6	3.7[d]	ND	ND	ND	1200
MRS1191	>10,000[b,e]	ND	31	500[d]	ND	ND	1704	ND
IB-MECA	1.1[b]	ND	1.8[b]	2	0.5	0.2	3	5

Stably transfected cells were used if not mentioned otherwise. [a]Transient expression. [b][^{125}I]ABA used in Salvatore et al. [24]. [^{3}H]NECA was used in Alnouri et al. [18] for the measurement ligand affinity. [c]Auchampach et al. [25]. [d]Takano et al. [26]. The values are from the original A_3AR characterization reports, unless noted. [e]Ki 1.42 μM, Li et al. [27]. Ag, agonists. RL, radioligands. ND, not determined

including xanthine amine congener (XAC) and DPCPX displaced [^{125}I]I-APNEA binding with very low affinity. In functional assays with transfected cells, R-PIA and NECA potently inhibited forskolin-stimulated cAMP production in a pertussis toxin-dependent manner, demonstrating G_i protein coupling. Since the sequence and functional characterization of this receptor were unlike any previously cloned AR subtypes at the time, Zhou et al. [32] termed this receptor the A_3AR. In later studies, the A_3AR initially described in RBL-2H3 cells and the receptor encoded by the cDNA cloned by Meyerhof and colleagues from a rat testis cDNA library were confirmed to be the same A_3AR [32]. Both rat RBL-2H3 cells and mouse bone marrow-derived mast cells (BMMCs) express the A_3AR at high levels, as measured by both transcript (Northern blotting, RT-PCR) and protein (radioligand binding) expression [24], which will be described below.

As described previously, the A_3AR was first studied in the rat where transcripts were initially detected in testis [32, 39] by in situ hybridization. Later studies using the more sensitive technique of RT-PCR identified transcripts in the heart, lung, and a limited number of CNS regions [32] (Table 3). In a follow-up study, Dixon et al. [40] also found that rat A_3AR mRNA only highly expressed in testis using in situ hybridization. However, by using RT-PCR, A_3AR gene expression was also found to be widespread in the rat. In peripheral tissues, the A_3AR was found highly expressed in lung, spleen, uterus, and testis. Moderate levels occurred in liver and bladder and very low levels in the heart, aorta, stomach, jejunum, proximal colon, kidney, and eye [40]. RT-PCR also revealed the presence of low levels of A_3AR mRNA in all brain regions [40]. Efforts to detect A_3AR protein expression by radioligand binding have not been overly informative, because the available A_3AR radioligands lacked sufficient selectivity for this purpose. While Shearman and Weaver [41] detected specific binding using [^{125}I]I-AB-MECA using rat brain membranes, >95% of binding was displaced by XAC at a concentration (1 μM) that would not be expected to displace A_3AR binding. However, in similar studies, Ji et al. [42] detected residual [^{125}I]I-AB-MECA binding in the presence of 1 μM XAC in rabbit, gerbil, and rat brain. The AR expression profile in rat testis has been characterized in functional assays of receptor-induced regulation of cAMP generation, and the presence of A_3ARs has been confirmed in germ cells (spermatocytes and spermatids) but not spermatozoa [43]. A role of the A_3AR in spermatogenesis or sperm motility in rats has not been determined.

Table 3 Tissue distribution of A_3ARs in eight species. Tissue distribution was not reported for equine A_3AR [44]

Organ	Rat[a]	Sheep[b]	Human[c]	Rabbit[d]	Canine[e]	Mouse[f]	Chick[g]
Testes	+ + +	+	+	ND	+	− or +	ND
Spleen	+ +	+ +	−	ND	+ + or + + +	ND	ND
Lung	+ +	+ +	+ +	+ +	+	ND	ND
Heart	+	−	+	+ or + +	+	ND	ND
Liver	+ +	−	+ +	+ or + +	+	ND	ND
Kidneys	+	+	− (+)	ND	+	ND	ND
Brain	+	+	+	+	ND	+	+

Note that the expression detected weak or negative using in situ hybridization or Northern blot analysis was later shown to be positive or variable when using RT-PCR. − Very low or not detectable, + Low, + +, medium; + + +, high. [a]38, 41; [b]49–51; [d]18; [e]55; [f]detected in sperm or germ cells [45]; also detected expression in pancreas [46]; [g][47]

Haeusler et al. [48], using [^{125}I]I-AB-MECA, studied A_3AR distribution in rat and human tissues. Displacement of [^{125}I]I-AB-MECA specific binding by the A_3AR-selective 4-aryl-3,5-diacylpyridine derivatives MRS1523 and FE@SUPPY, first reported in Li et al. [27], was found in all rat peripheral tissues examined with the highest amounts in the spleen, lung, heart, and testes; lower amounts were found in rat brain and human brain. These observations are consistent with assays of mRNA expression. Kiesewetter et al. [49] showed that positron-emitting [^{76}Br]-labeled nucleoside A_3AR antagonist MRS5147 (designed based on the corresponding 3-chlorobenzyl analogue MRS5127) was taken up rapidly in the rat testes. Wadsak et al. [50] attempted to use [^{18}F]5-(2-fluoroethyl) 2,4-diethyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate (FE@SUPPY) to label rat brain A_3ARs. In this study, autoradiography was performed on rat brain slices in the presence or absence of Cl-IB-MECA. The authors suggested that [^{18}F]FE@SUPPY has the potential to serve as the first positron emission tomography (PET) tracer for the A_3AR, but the results were not definitive.

Linden et al. [51] cloned the sheep A_3AR (317 amino acids) in 1993 and found that the sequence is only 72% identical to the rat A_3AR. The largest differences between sheep and rat were found in the C-terminal segment and the second and third extracellular loops (EL2 and EL3). These interspecies differences suggested that desensitization, signaling, and ligand binding might differ between these two species homologues. In contrast to the distribution observed in rat, where A_3AR mRNA transcript was found primarily in testis, the A_3AR transcripts in sheep were found to be most abundant in lung, spleen, and pineal gland. Moderate levels were detected in the brain, kidney, and testis [51]. Agonists were found to inhibit forskolin-stimulated cAMP accumulation in CHO cells stably expressing the sheep A_3AR, confirming coupling to G_i proteins. The potency order of agonists in displacing [^{125}I]I-ABA binding was NECA > R-PIA > S-PIA > CPA.

However, several xanthine derivatives, which do not bind to the rat A_3AR, were potent sheep A_3AR antagonists with a potency order of I-ABOPX > XAC > DPCPX > theophylline. Thus, the sheep A_3AR showed clear differences from the rat A_3AR in ligand binding, amino acid sequence, tissue distribution, and possibly receptor regulation.

Salvatore et al. [52] cloned the human A_3AR in 1993 from a striatal cDNA library (318 amino acids). The human A_3AR exhibits 72% and 85% overall identity with the rat and sheep A_3AR sequences, respectively. In CHO cells expressing the human A_3AR, agonists inhibited forskolin-stimulated cAMP accumulation with the order of potencies of NECA > R-PIA > CPA > S-PIA. In human A_3AR radioligand binding assays, several xanthine antagonists bound with high affinity with a rank order of potency of I-ABOPX > XAC > DPCPX. The human A_3AR transcript is widespread with the most abundant expression in the lung and liver, which was similar to the profile found in sheep. Abundant A_3AR expression in lung tissue raised the possibility for the first time that the A_3AR may play a prominent role in pulmonary pathophysiology in some species including humans.

Sajjadi and Firestein [53] cloned a human A_3AR homolog from a human heart cDNA library. The receptor shared a low degree of sequence homology to the rat A_3AR (71%). Northern analysis of various human tissues showed the human A_3AR gene to be expressed primarily in the lung, liver, kidney and heart, with very low expression in the brain and skeletal muscle. Comparable variants have not been identified in other species. It is generally viewed that this variant likely plays no physiological role and likely is not functionally expressed. Murrison et al. [54] isolated the human A_3AR gene from a HepG2 cell genomic DNA library, which encodes a protein of 319 amino acids identical in sequence to that of the A_3AR described by Salvatore et al. [52] but differing by a single nucleotide (G rather than A at position 310) from that described by Sajjadi and Firestein [53]. Atkinson et al. [55] also cloned the human A_3AR gene using fluorescence

in situ hybridization to map the ADORA3 gene to chromosomal locus 1p13.3. Northern blot studies showed that the A_3AR is widely expressed and is most abundant in brain and some endocrine tissues. The authors found no evidence of alternate splicing in the 5′ untranslated region of the ADORA3 gene. Thus, the observed A_3AR distribution patterns in humans from different reports were not entirely consistent. The finding of high A_3AR expression in human brain deserves further exploration.

Hill et al. [56] cloned the rabbit A_3AR (320 amino acids). The amino acid identities with human, sheep and rat A_3ARs were 76%, 75%, and 68%, respectively. Rabbit A_3AR mRNA was identified in lung, liver, brain, and heart. In CHO cells expressing the rabbit A_3AR, R-PIA inhibited forskolin-stimulated cAMP accumulation with an EC_{50} of 25 nM. The binding affinities (K_i, nM) of selected ligands based on the inhibition of [^{125}I]I-ABA binding were IB-MECA (2), NECA (27), R-PIA (49), CPA (49), XAC (329), and DPCPX (1120). The rabbit A_3AR pharmacological profile is closer to the human A_3AR in comparison to the rat A_3AR.

Auchampach et al. [25] cloned and characterized the canine A_3AR (314 amino acids), which was 88%, 86%, 72%, and 77% identical to the human, sheep, rat, and rabbit A_3ARs, respectively. Of the six A_3AR species homologs cloned before 1997, the human A_3AR amino acid sequence was most similar to the canine and least similar to the rat A_3AR. Canine A_3AR transcripts were found predominantly in the lungs, spleen, liver, and testes. In COS-7 cells transiently expressing the canine A_3AR, it is interesting that [^{125}I]I-ABA bound to two affinity states of the receptor with K_d values of 0.7 and 16 nM, respectively. Since [^{125}I]I-ABA bound to a single low-affinity state with a K_d of 17.4 nM when GTPγS was included to effect receptor-G protein-uncoupling, the two affinity states likely reflect binding of [^{125}I]I-ABA to high affinity G protein-coupled receptors and low-affinity G protein-uncoupled receptors. The potency order of agonists for the canine A_3AR was IB-MECA > I-ABA > R-PIA. The canine A_3AR bound xanthine antagonists with a potency order of I-ABOPX > XAC≈DPCPX > 8-SPT.

Salvatore et al. [24] cloned the mouse A_3AR gene encoding a 319-amino acid protein, which exhibits 89% overall identity to the rat, 76% to the sheep, and 73% to the human A_3AR. Although A_3AR transcripts are abundant in rat testis and a role in reproduction has been suggested [32], the A_3AR KO mice bred and matured normally, indicating that the mouse A_3AR probably does not play a critical role in reproduction. Also, specific A_3AR binding of a newly developed species-general A_3AR antagonist radioligand [^3H]MRS7799 (DPTN) to membranes from mouse testis or brain was below the detection limit (Gao and Jacobson, unpublished data). However, Burnett et al. [45] reported that both IB-MECA and Cl-IB-MECA accelerate mouse sperm motility. We are not aware of any substantial progress toward the development of A_3AR ligands applied to reproduction or contraception. It is important to consider that disparities in results of mouse A_3AR expression studies (and perhaps other species) may be explained by the discovery in 2010 that the mouse A_3AR gene is embedded within the gene encoding transmembrane and immunoglobulin domain containing 3 (TMIGD3) [57]. Both genes share coding exons that create at least one TMIGD3/A_3AR hybrid variant (Adora3i3). Interestingly, TMIGD3 is abundantly expressed in mouse testes, and the Adora3i3 variant, which is encoded by exon 1 of the TMIGD3 gene and exon 2 of the A_3AR gene, is possibly controlled by the TMIGD3 promoter. Functionality of the hybrid TMIGD3 splice variant remains highly questionable since it is unlikely to bind adenosine or to transduce signals to G proteins. Expression studies based on probes or PCR primer pairs targeting areas that are shared between TMIGD3 and A_3AR splice variants may produce misleading information on A_3AR distribution. Notably, TMIGD3 and A_3AR splice variants are also found in other species including humans.

Alnouri et al. [18] reported the binding affinity of commonly used agonists and antagonists at the mouse A_3AR expressed in CHO cells. The agonist affinities (nM) were as follows: NECA (13.2), R-PIA (9.98), IB-MECA (0.21), and Cl-IB-MECA (0.18). The antagonist affinities (nM) at mouse A_3AR were as follows: CGS15943 (2970), MRS1523 (1980), and xanthine antagonists, i.e., caffeine, theophylline, and DPCPX (all > 100,000 nM). Thus, the ligand potency profile at the mouse A_3AR is similar to that of the rat A_3AR, a conclusion confirmed by Gao et al. [17].

To further investigate mouse A_3AR distribution, Yamano et al. [30, 46] generated a transgenic mouse with whole-body expression of jellyfish apoaequorin under control of the A_3AR gene promoter. The authors succeeded in detecting adenosine ligand-induced light emission by aequorin in response to increases in cytoplasmic Ca^{2+} in the pancreas, brain, and testis. Yamano et al. [46] suggested that the pancreas is one of the main tissues that expresses the A_3AR.

Durand and Green [47] cloned the chick A_3AR cDNA showing 52.1%, 57.0%, and 52.2% homology to the rat, sheep, and human A_3ARs, respectively. As a comparison, the chick A_3AR was 43.8% homologous to the chick A_1AR. In HEK293 cells expressing the chick A_3AR, CHA and Cl-IB-MECA inhibited isoproterenol-stimulated cAMP accumulation with $EC_{50}s$ of 16.2 and 25.1 nM, respectively, confirming G_i coupling. In radioligand binding assays using [^{125}I]I-ABA, the following K_i values (nM) were determined: IB-MECA, 2.8; Cl-IB-MECA, 14.9; NECA, 37; CPA, 2.6; R-PIA, 1.1; DPCPX, 53; and MRS1191, 1704. Thus, the binding profile of agonists and antagonists at the chick A_3AR differed from other cloned A_3ARs. Most notably, the chick A_3AR bound both A_3 (IB-MECA, Cl-IB-MECA)

and A$_1$AR (CPA, R-PIA) agonist ligands with high affinity. We are not aware of any further reports regarding the chick A$_3$AR ligand binding profile.

Other A$_3$AR species homologues have been reported, but with less extensive pharmacological characterization. Brandon et al. [44] cloned and pharmacologically characterized the equine A$_3$AR, although no data related to the tissue distribution of this receptor has been provided. The equine A$_3$AR amino acid sequence is 82.5% and 84.7% identical to human and sheep A$_3$ARs, respectively. In radioligand binding studies with transfected HEK293 cells, the rank order of potency of agonists to displace [^{125}I]I-AB-MECA binding was as follows: IB-MECA (5.2 nM) > NECA (7.9 nM) > CGS21680 (110 nM), which roughly agreed with published findings with all other mammalian nonrodent A$_3$ARs. The rank order of antagonist potency was as follows: MRS1220 (1.2 μM) > ZM241385 > 8-p-sulfophenyltheophylline, which was inconsistent with other nonrodent A$_3$ARs.

Thus, among the eight species that have been characterized, it is of note that the A$_3$AR tissue distribution varies substantially. For example, A$_3$AR expression is high in rat testes but low in mouse and sheep testes. Expression of A$_3$AR transcripts in human liver is abundant, whereas it is low in sheep [32, 51, 52, 56, 58]. In general, A$_3$AR abundance in the lungs is consistent across many species, including rabbit, dog, sheep, and human. However, even within the same species, reports of the A$_3$AR distribution profile vary (especially in the rat and human). It must be kept in mind that many earlier studies utilized techniques (in situ hybridization, Northern blotting) that are less sensitive compared to modern molecular techniques that are now used widely (PCR, qPCR, direct sequencing). Regarding new technology, exon expression by RNA sequencing for the A$_3$AR gene in postmortem human tissues is now available [58]; interestingly, high expression was detected in the spinal cord. Moreover, several public single-cell RNA sequencing databases have become available in recent years. Interestingly, the Tabula Muris database (https://tabula-muris.ds.czbiohub.org/), a compendium of single-cell transcriptome data from the mouse, reports that A$_3$AR mRNA expression is most abundant in microglial cells in the brain and is also found in a number of different immune cell populations including granulocytic cells (basophils, neutrophils) and other myeloid cell populations (monocytes and tissue-resident macrophages). The Human Protein Atlas (www.proteinatlas.org) reports predominant A$_3$AR expression in myeloid cells (granulocytes, monocytes, eosinophils, basophils) and tissue resident macrophages as well as in Kuppfer cells and Hofbauer cells. These findings raise the possibility that prior expression analyses using whole-tissue samples may reflect A$_3$AR presence in immune cell populations within the tissues. With earlier published studies, expression levels

in different species were not compared directly using the same methodology under the same experimental conditions. As suggested previously, variability may be related to detection of TMIGD3/A$_3$AR splice variants that are likely non-functional.

To summarize thus far, the initial A$_3$AR molecular cloning studies raised awareness of large differences in A$_3$AR protein sequence, tissue distribution, and pharmacology. These early studies in particular identified stark differences in A$_3$AR properties between humans and rodents, species commonly used during the course of drug development. Thus, it was soon appreciated that observations in rodent models, must be confirmed in additional larger mammalian species, such as dogs, sheep, or rabbits [20, 25, 59–61].

Species dependence of A$_3$AR agonists

In most initial studies with recombinant A$_3$ARs from various species, the 5′-substituted adenosine derivative NECA and the N^6-substituted adenosine derivative R-PIA were found to be full, relatively potent agonists. The non-selective radioligands [^{125}I]I-ABA [25, 56] and [^{125}I]I-APNEA, used in the initial A$_3$AR cloning [32], are bound with high affinity at rat, mouse, canine, rabbit, and human homologues. The 5′- and N^6-substituted radioligand [^{125}I]I-AB-MECA was found to bind with nearly nM affinity at the A$_3$ARs from various species [42, 44, 62, 63]. In subsequent studies after 1997, IB-MECA and/or Cl-IB-MECA, which exhibit moderate A$_3$AR selectivity, were also used to initially characterize cloned A$_3$ARs from a number of different species. It is notable that IB-MECA and Cl-IB-MECA have been demonstrated to act as human A$_3$AR partial agonists in several independent assay systems [64–66]. However, they are likely full agonists for rat and mouse A$_3$ARs [58, 67], although this has not been systematically studied or strictly compared.

Adenosine derivatives that act as agonists generally display much less human/rat species variability as compared to non-nucleoside antagonists, but more subtle differences have been noted [16, 68]. The affinity of adenosine itself cannot easily be measured precisely, because it is rapidly degraded by adenosine deaminase found in all cells and tissues. Alternatively, 2-chloroadenosine (CADO), which is resistant to the actions of adenosine deaminase, has been used as a surrogate. The K$_i$ values of CADO are 1890 nM at the rat A$_3$AR and 87 nM at the human A$_3$AR; thus, under the assumption that CADO faithfully replicates the actions of adenosine, these results predict A$_3$AR species variability for its endogenous agonist, which may have evolved as a survival advantage to attenuate A$_3$AR-mediated mast cell degranulation that occurs in rodents. Various N^6-substituted agonists have been demonstrated to be > 100-fold more potent at human than at rat A$_3$ARs (Table 4). For

Table 4 Affinity of selected commercially available and commonly used A_3AR agonists and antagonists and the allosteric enhancer LUF6000 at human, mouse, and rat A_1, A_{2A}, A_{2B}, and A_3ARs, as a reference guide for selection of appropriate concentrations

Ligand	A_1			A_{2A}			A_{2B}			A_3		
Species	h	m	r	h	m	r	h	m	r	h	m	r
Me-ADO[a]	ND	ND	60	ND	ND	>10,000	ND	ND	ND	9.3	ND	6390
HEMADO[b]	330	ND	ND	1200	ND	ND	>10,000	ND	ND	1.1	ND	6440
IB-MECA[c]	51	5.9	54	2900	326	948	11,000	3340	12,400	1.8	0.087	1.1
Cl-IB-MECA[c]	220	35	820	2100	290	470	>10,000	1210	43,000	1.4	0.180	0.3
MRS5698[c]	>10,000	>10,000	ND	>10,000	>10,000	ND	ND	ND	ND	3.5	3.1	ND
MRS1191[c,g]	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	49.1	245[i]	856[i]
MRS1220[e,g]	135	168	161	22.8	106	29.2	1800	774	1280	0.96	>10,000	>10,000
MRS1523[c,g,h]	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	>10,000	43.9	349	216
DPTN[g]	162	411	333	121	830	1150	230	189	163	1.65	9.61	8.53
MRS7907[i]	8770	2640	ND	>10,000	>10,000	ND	ND	ND	ND	6.12	12.4	ND
MRE3008F20[d,g]	1830	930	1010	1197	618	926	527	542	480	1.13	>10,000	>10,000
PSB10[e,g]	1530	4660	7890	2120	2180	6850	1120	411	1530	1.56	>10,000	>10,000
VUF5574[f,g]	7390	>10,000	4660	6500	>10,000	7850	941	976	1150	3.84	>10,000	>10,000
LUF6000[i]	>10,000	ND	ND	>10,000	ND	ND	>10,000	ND	ND	>10,000	ND	ND

[a] N^6-Methyladenosine, Gao et al. [16]; [b] Volpini et al. [73]; [c] Ge et al. [23]; [d] Baraldi et al. [74]; [e] Ozola et al. [75]; [f] van Muijlwijk-Koezen et al. [76]. [g] Gao et al. [17]. [h] Other different K_i (nM) values for MRS1523 were reported: for mouse A_1 (5330) and A_3 (702), using [^{125}I]I-AB-MECA [69]); for mouse A_3AR (1980 nM, using [^{3}H]NECA [18]); and for rat $A_{2A}AR$ (2050 nM, using [^{3}H]CGS21680 [27]). [i] Values listed are effects on the human A_3AR orthosteric site (using [^{125}I]I-AB-MECA) [77]. For allosteric effect of LUF6000, see Goblyos et al. [77]. [j] Suresh et al. [78]. Note: K_i values determined using different radioligands and different cell types to express the receptors may produce variability [17, 18, 79]

example, N^6-[(1S,2R)-2-phenyl-1-cyclopropyl]adenosine ($K_i = 0.63$ nM for hA$_3$AR) is > 1000-fold more potent for human than rat A$_3$ARs. A small N^6-alkyl substituent, as with N^6-methyladenosine, has been demonstrated to be extremely important for the species difference between human and rat A$_3$ARs ($K_i = 9.3$ and 6390 nM, for human and rat A$_3$AR, respectively) [16]. Some N^6-benzyl substituted agonists, such as 5′-methylamides IB-MECA and Cl-IB-MECA and the corresponding 5′-CH$_2$OH nucleosides, are potent at both human and rat A$_3$ARs. Furthermore, in the (North, N)-methanocarba series containing a sterically constrained bicyclic ribose substitution, an N^6-benzyl group was incorporated in adenosine 5′-uronamides to achieve species-independent A$_3$AR receptor-selectivity [68]. Thus, it seems that the N^6-benzyl group promotes potency at rodent A$_3$ARs [16–25, 27–68]. More recently, Alnouri et al. [18] compared some commonly used agonists in radioligand binding studies using membranes from CHO cells expressing four AR subtypes of three species: human, rat, and mouse. The authors confirmed that IB-MECA and Cl-IB-MECA are indeed potent agonists for human, rat or mouse A$_3$ARs, although the A$_3$AR selectivity is variable at different species. Ge et al. [69] and Carlin et al. [23], using HEK293 cells expressing mouse A$_3$AR, also showed that both IB-MECA and Cl-IB-MECA are very highly potent A$_3$AR agonists in the mouse (K_i 87 and 180 pM, respectively). Therefore, it should be kept in mind that it is best to use a concentration of ~ 1 nM or lower to activate the mouse or rat A$_3$AR, since IB-MECA and Cl-IB-MECA are also potent mouse A$_1$AR agonists with K_i values of 5.9 and 35 nM, respectively [69]. At a higher concentration, such as 1 μM, IB-MECA can also act as an A$_{2A}$AR agonist, which will be described later in this review. The commercially available and moderately selective A$_3$AR agonist HEMADO is potent at human A$_3$AR (1.1 nM). Since the N^6-substitution of HEMADO is a methyl group, it is very weak at rat or mouse A$_3$ARs, and the tritiated radioligand was unsuitable for these species [70]. Its potency at mouse A$_3$AR has not been examined, but HEMADO binds to rat A$_3$AR with a K_i of 6.44 ± 1.18 μM (unpublished data). The introduction of H-bonding groups (4-hydroxy-3-methoxy) in a truncated N^6-(2-phenylethyl)adenosine analogue leading to MRS7591 was found to dramatically increase the mouse A$_3$AR affinity, as predicted using molecular modeling based on the multiple polar H-bonding side chains in mouse A$_3$AR EL residues. There has been no report of selective A$_3$AR agonists from the non-ribose class compounds [71, 72].

The (N)-methanocarba ring system (consisting of a fused cyclopropyl and cyclopentyl ring) in place of the furanose ring of adenosine is a general method for enhancing both A$_3$AR affinity selectivity, as in a recently available commercially N^6-(3-chlorobenzyl) derivative, MRS5698. MRS5698, containing a 5′-methylamide, was shown to be potent and selective at human, mouse and rat A$_3$ARs with K_i values of ~ 3 nM,

and > 3000-fold selectivity versus A$_1$, A$_{2A}$, and A$_{2B}$ARs [17, 23]. Recently reported analogues based on that observation include the highly potent MRS5980 (> 10,000-fold selective for human A$_3$AR compared to A$_1$/A$_{2A}$, $K_i = 0.7$ nM) [80]. However, as an N^6-methyl analogue, the mouse A$_3$AR affinity of MRS5980 ($K_i = 36$ nM) is 51-fold lower than at human A$_3$AR [23], but it remains highly selective. The human A$_3$AR affinity is further enhanced by substitution of Cl with F in MRS7334 ($K_i = 0.28$ nM) [80]. Thionucleoside LJ529, an analogue of Cl-IB-MECA, is a highly potent A$_3$AR agonist [81].

The residues of the human and mouse A$_3$AR sequences that are predicted to be in proximity (distance ≤ 4 Å) to the partial agonist nucleoside ligand MRS7591 [82, Fig. 2] are shown in bold in Fig. 3 for both the human and mouse sequences. Residues of the human A$_3$AR sequence that are shown in red were found to diminish agonist binding or activation [83]. There is a close commonality across adenosine receptor subtypes in the residues that are involved in orthosteric ligand recognition [78, 82]. For example, the following residues that were identified as important for agonist action at the human A$_3$AR [83] are conserved in all 13 species in Fig. 3, including chick: T3.36, F168 (EL2), M5.38, W6.48, L6.51, N6.55, I7.39, S7.42, and H7.43 (using Ballesteros-Weinstein numbering, shown in red in the aligned human sequence). Recently, the binding site for the A$_3$AR-selective PAM LUF6000 was localized to the intracellular side of the receptor [84], although specific residues involved were not identified. LUF6000 is inactive at the mouse A$_3$AR, which allowed use of chimeric mouse-human A$_3$ARs to identify the allosteric binding region on the half of the receptor facing the cytosol. Thus, there is need for additional mutagenesis experiments to better characterize the basis for species differences in both orthosteric ligand recognition and allosteric modulation.

In summary, two prototypical AR agonists R-PIA and NECA, although nonselective, were used in the initial pharmacological characterization of A$_3$ARs cloned from various species. N^6-methyl substituted adenosine derivatives in the ribose series, such as N^6-methyladenosine and HEMADO, are potent at human but weak at rat A$_3$ARs. N^6-Methyladenosine is a 690-fold weaker in binding to the rat A$_3$AR compared to the human homologue [16]. N^6-Benzyl substituted derivatives such as IB-MECA and Cl-IB-MECA are potent across species, although somewhat more potent at rat and mouse A$_3$AR than at human A$_3$AR. The A$_3$AR selectivity of IB-MECA and Cl-IB-MECA is limited; this limitation should be kept in mind when used in studies with mice or rats. For example, the affinity of IB-MECA for the mouse A$_1$AR is 5.9 nM [69]. Accordingly, IB-MECA should be used at a concentration of ≤ 1 nM in studies with mouse cells/tissues to achieve an A$_3$AR-selective concentration. MRS5698 is a potent and selective agonist across all species tested thus far and is the best agonist that is readily available to study the A$_3$AR in mice or rats. Potent and selective A$_3$AR agonists from the class of non-nucleoside agonists [72] have not been developed, which should be a future effort.

```
human (1-63)          M-----------PN-------NST-ALSLA-NVTYITMEIFIGLCAIVGNVLVICVVKLNPSLQTTTFYFIVSLALADIAVGV
                                               TM1                          TM2
chimpanzee (1-63)     M-----------PN-------NST-ALSLA-NVTYITMEIFIGLCAIVGNVLVICVVKLNPSLQTTTFYFIVSLALADIAVGV
rabbit (1-64)         M-----------PD-------NST-TLFLAIRASYIVFEIVIGVCAVVGNVLVIWVIKLNPSLKTTTFYFIFSLALADIAVGF
dog (1-63)            M-----------AV-------NGT-ALLLA-NVTYITVEILIGLCAIVGNVLVIWVVKLNPSLQTTTFYFIVSLALADIAVGV
camel (1-63)          M-----------PV-------NST-TMSLA-NITYITVEILIGLCAIVGNVLVIWVVKLNPSLQTTTFYFIVSLALADIAVGV
marmoset (1-63)       M-----------SV-------NST-APSLA-NITYITMEIFIGLCAIVGNVLVIWVIKLNPSLQSTTFYFIGSLALADIAVGM
cat (1-63)            M-----------AV-------NST-ALLLA-NVTYITVEILIGLCAIVGNVLVIWVVKLNPSLQTTTFYFIVSLALADIAVGV
guinea pig (1-70)     M-----------LFQDSVVFNNTS-TPRWD-DITYIIMEVLIGLSAVVGNVLVIWVVKLNPSLRTTTFYFIVSLALADIAVGI
hamster (1-64)        M-----------KP------DNTT-TALWL-KITYISMEVVIGLCAVVGNMLVIWVVKLNPSLRTTTFYFIVSLALADIAVGV
gerbil (1-64)         M-----------KP------DNTT-TALWL-NITYISMEVVIGLCAVVGNMLVIWVVKLNPSLRTTTFYFIVSLALADIAVGV
mouse (1-65)          M-----------EA------DNTTETDW-L-NITYITMEAAIGLCAVVGNMLVWVVKLNPTLRTTTFYFIVSLALADIAVGV
rat (1-65)            M-----------KA------NNTTTSALWL-QITYITMEAAIGLCAVVGNMLVIWVVKLNRTLRTTTFYFIVSLALADIAVGV
chicken (1-80)        MCPQGSPCPASSSPINVTF---NTS-QVMGSIDVIYISAECLVALLAALGNIPVVWAVKLNAAFHNTTMYFIASLALADIAVGV

human (64-147)        LVMPLAIVVSLGITIHFYSCLFMTCLLLIFTHASIMSLLAIAVDRYLRVKLTVRYKRVTTHRRIWLALGLCWLVSFLVGLTPMF
                                      TM3                                                TM4
chimpanzee (64-147)   LVMPLAIVVSLGITIHFYNCLFMTCLLLIFTHASIMSLLAIAVDRYLRVKLTVRYKRVTTHRRIWLALGLCWLVSFLVGLTPMF
rabbit (65-148)       LVMPLAIVISLGITIGFYSCLVMSCLLLVFTHASIMSLLAIAVDRYLRVKLTVRYRRVTTQRRIWLALGLCWVVSLLVGFTPMF
dog (64-147)          LVMPLAIVISLGITIQFYNCLFMTCLLLIFTHASIMSLLAIAVDRYLRVKLTVRYRRVTTQRRIWLALGLCWLVSFLVGLTPMF
camel (64-147)        LVMPLAVVISLGITVHFYSCLLMTCLLLIFTHASIMSLLAIAVDRYLRVKLTVRYRRVTTQRRIWLALGLCWLVSFLVGLTPMF
marmoset (64-147)     LVMPLAIVISLGITIHFYSCLFMSCLLLIFTHASIMSLLAIAVDRYLRVKLTVRYRRVTTHRRIWLGLGLCWLVSFLVGLTPMF
cat (64-147)          LVMPLAIVISLGITIHFYNCLFMTCLLLIFTHASIMSLLAIAVDRYLRVKLTVRYRRITTQRRIWLALGLCWLVSFLVGLTPMF
guinea pig (71-154)   LVIPFAIVINLHFPMHFYSCLFMCCVLLIFTHASIMSLLAIAVDRYLRVKLTVRYKTVTTQKRIWLALGLCWLVSLLVGLTPMF
hamster (65-148)      LVMPLAIAVSLEVEMHFYVCLFMSCVLLIFTHASIMSLLAIAIDRYLRVKLTVRYRTVTTQRRIWLSLGLCWLLSFLVGLTPMF
gerbil (65-148)       LVIPLAIAVSLEVKMHFYTCLFMSCVLLIFTHASIMSLLAIAVDRYLRVKLTVRYKTVTTQRRIWLSLGLCWLVSFLVGLTPMF
mouse (66-149)        LVTPLAIAVSLQVKMHFYACLFMSCVLLIFTHASIMSLLAIAVDRYLRVKLTVRYRTVTTQRRIWLFLGLCWLVSFLVGLTPMF
rat (66-149)          LVIPLAIAVSLEVQMHFYACLFMSCVLLVFTHASIMSLLAIAVDRYLRVKLTVRYRTVTTQRRIWLFLGLCWLVSFLVGLTPMF
chicken (81-164)      FVVPLAVLVSLQVSIPFHFCLFLCCLMVVFTQASILSLLAIAIDRYLRVKLPIRYKIISTERRIWGALGLCWSLSLLVGLTPMF

human (148-227)       GWNMKLTSEYHRNVTFLSCQFVSVMRMDYMVYFSFLTWIFIPLVVMCAIYLDIFYIIRNKLSLNLSNSKETGAFYGREFK
                                                         TM5
chimpanzee (148-227)  GWNMKLTSEYHRNVTFLSCQFVSVMRMDYMVYFSFLTWIFIPLVVMCAIYLDIFYIIRNKLSLNLSNSKETGAFYGREFK
rabbit (149-228)      GWNMKPTLESARNYSDFQCKFDSVIPMEYMVFFSFFTWILIPLLLMCALYVYIFYIIRNKLVQSFSSFKETGAFYRREFK
dog (148-227)         GWNMKLTSEHQRNVTFLSCQFSSVMRMDYMVYFSFFTWILIPLVVMCAIYLDIFYVIRNKLNQNFSSSKETGAFYGREFK
camel (148-227)       GWNMKLTSEYHRNITFLPCQFSRVMRMDYMVYFSFFTWIFIPLVVMCAIYLDIFYIIRNKLSQNFSSSKETGAFYGREFK
marmoset (148-227)    GWNMKLTSEYYRNVTFLSCRFPSVMSMDYMVYFSFFTWIFVIMCAIYLDIFYIIRSKLRQNFSNSKETGAFYGREFK
cat (148-227)         GWNMKLTSEHYRNVTSLSCQFRSVMTMDYMVYFSFFTWILIPLVVIMCAIYLDIFYIIRNKLNQSFSSSKETGTFYGREFK
guinea pig (155-232)  GWNGKLTSKEFQNVTV--CSFSRIISMEYMVFFSFFTWIFIPLVVMCAIYLNIFYVIRNKLSQNLSASKETGAFYGREFK
hamster (149-245)     GWNKKMTSGLSPNSTTLSCHFRSVVPLDYMVFFSFITWILIPLIVMCIIYLDIFYILRNKLSQNLSGFRETRAFYGREFK

gerbil (149-228)      GWNKKVTSGLPQNATTLLCHFRSVVCLDYMVFFSFITWILIPLIVMCVIYLDIFYIIRHKLSQNLSGFRETRAFYGREFK
mouse (150-229)       GWNRKATLASSQNSSTLLCHFRSVVSLDYMVFFSFVTWILVPLVVMCVIYLDIFYIIRNKLSQNLSGFRETRAFYGREFK
rat (150-246)         GWNRKVTLELSQNSSTLSCHFRSVVGLDYMVFFSFITWILIPLVVMCIIYLDIFYIIRNKLSQNLTGFRETRAFYGREFK
chicken (165-238)     GWN------KQRSAPYHTCGFTSVIRMDYMVYFSFFAWTLIPLSIMCALYVAVFYIIRTKLSQGATGARGAGTFYGKEFR

human (228-305)       TAKSLFLVLFLFALSWLPLSIINCIIYFNGE--VPQLVLYMGILLSHANSMMNPIVYAYKIKKFKETYLLILKACVVCHP
                                      TM6                    TM7
chimpanzee (228-305)  TAKSLFLVLFLFALSWLPLSIINCIIYFNGE--VPQLVLYMGILLSHANSMMNPIVYAYKIKKFKETYLLILKACVVCHP
rabbit (229-306)      TAKSLFLVLALFAGCWLPLSIINCVTYFKCK--VPDVVLHVGILLSHANSMMNPIVYACKIQKFKETYLLIFKARVTCQP
dog (228-305)         TAKSLFLVLFLFAFSWLPLSIINCITYFHGE--VPQIILYLGILLSHANSMMNPIVYAYKIKKFKETYLLIFKTYMICQS
camel (228-305)       TAKSLFLVLFLFALSWLPLSIINCITYFNGE--VPQVVLNLGILLSHANSMMNPIVYAYKIRKFKETYLLILKACVVCQP
marmoset (228-305)    TAKSLFLVLFLFALSWLPLSIVNCVTYFHGE--VPQLVLHLGILLSHANSMMNPIVVYVYKIKKFKETYLLILKACVVCHP
cat (228-305)         TAKSLFLVLFLFALSWLPLSIINCITYFHGE--VPQIILYLGILLSHANSMMNPIVYAYKIKKFKETYVLIFKTCMICQS
guinea pig (233-310)  TAKSLFLVLFLFALCWLPLSIINCALYFKVK--ITKDVLYLSILLSHANSMMNPIIYACKIKRFKETCFLILKDCRVCQL
hamster (246-323)     TAKSLFLVLFLFALCWLPLSINNLVSYFNVD--IPEPAMCLGILLSHANSMMNPIVYACKIKKFKETYFVILKACRVCQT
gerbil (229-306)      TAKSLFLVLFLFASCWLPLSIINFVSYFQVE--IPEVAMCLGILLSHANSMMNPIVYACKIKKFKETYSLILRACRVCQT
mouse (230-307)       TAKSLFLVLFLFALCWLPLSIINFVSYFDVK--IPDVAMCLGILLSHANSMMNPIVYACKIKKFKETYFLILRALRLCQT
rat (247-324)         TAKSLFLVLFLFALCWLPLSIINFVSYFNVK--IPEIAMCLGILLSHANSMMNPIVYACKIKKFKETYFVILRACRLCQT
chicken (239-318)     KAKSLALVLFLFAVSWLPLCIMNCVLYFHPEYKIPKPWIFLGILLSHANSAMNPVVYACKIKKFKTTYLLILRTYILCRK

human (306-318)       SDSLDTSIEK-------NSE
                           H8
chimpanzee (306-318)  SDSLDTSIEK-------NSE
rabbit (307-319)      SDSLDPSSEQ-------NSE
dog (306-314)         SDSLDSSTE-----------
camel (306-318)       SDSLDPSIEQ-------ASE
marmoset (306-318)    SDSLDTSIEK-------NSE
cat (306-318)         SDSLDPSTDQ-------TSE
guinea pig (311-323)  SNSLDPNIEQ-------ISE
hamster (324-336)     SVSLDSNIEQ-------TTD
gerbil (307-319)      SDSLDSNIEQ-------TTE
mouse (308-319)       SDSLDSNMEQ-------TTE
rat (325-337)         SDSLDSNLEQ-------TTE
chicken (319-338)     KPQAMPSSYRLNTPAVVDTE
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◄**Fig. 3** Sequence alignment of thirteen A_3AR species homologues (Species identifiers (https://www.ncbi.nlm.nih.gov/): NP_000668.1, Homo sapiens (human); NP_001289704.2,Pan troglodytes (chimpanzee); NP_001075527.1, Oryctolagus cuniculus (rabbit); NP_001003178.1,Canis lupus familiaris (dog); XP_006182940.1, Camelus ferus (camel); NP_001289720.1,Callithrix jacchus (marmoset); XP_006935064.2, Felis catus (cat); XP_012998623.1,Cavia porcellus (guinea pig); XP_035293911.1, Cricetulus griseus (hamster); XP_021514447.1,Meriones unguiculatus (Mongolian gerbil); mouse (source: Fisher et al. [84]); NP_001289680.1,Rattus novegicus (Norwegian rat); NP_989482.2, Gallus gallus (chicken). TMs are indicated in cyan, and extracellular loops in yellow highlight. The definition of helical regions and residues (bold, human and mouse sequences) corresponding to those in contact (distance ≤ 4Å) with partial agonist ligand MRS7591 (Figure2) are according to Tosh et al. [82]. Ala mutation of red residues in the humanA$_3$AR sequence diminished agonist binding or activation [85]: L3.32,T3.36, F168 (EL2), M5.38, W6.48, L6.51, N6.55, L7.35, I7.39, S7.42, H7.43. Note: The green highlighted L in the mouse sequence is the correct residue [82]. On GPCRdb (https://gpcrdb.org/) this residue is incorrectly shown as a valine (V301$^{8.61}$). Note that residue numbers shown in this alignment may differ from other reports

Species dependence of A_3AR antagonists

Methylxanthines, such as caffeine and theophylline, are generally weak antagonists for all human and rodent AR subtypes. However, caffeine and theophylline function as weak human A_3AR antagonists but are essentially inactive at the rat A_3AR [18, 32, 52]. At human, rat, and mouse A_1, A_{2A}, and $A_{2B}ARs$, the selective antagonists PSB-36 (A_1), preladenant (A_{2A}), and PSB-603 (A_{2B}) displayed high selectivity across species [18]. Thus, there remains a need to discover pan-species, highly potent, and selective non-nucleoside A_3AR antagonists.

Pyridine derivative MRS1523 is one of the few A_3AR antagonists suitable for rat ($K_i = 0.5$ μM; mean value of all published data) or mouse ($K_i = 0.35$ [17], 0.70 [86], 1.98 μM [18]) studies, with limited selectivity versus A_1, A_{2A}, and $A_{2B}ARs$ [17, 18]. Another known AR antagonist CGS15943 was also demonstrated to be a weak antagonist at rat (1.3 μM) and mouse (3.0 μM), but it is more potent at A_1, A_{2A}, and $A_{2B}ARs$ [18]. Other commonly used A_3AR antagonists, such as MRS1220 ($K_i = 0.65$ nM for human A_3AR [87], a derivative of the triazoloquinazoline antagonist CGS15943, and 1,4-dihydropyridine MRS1191 [88, 89]), exhibit low affinity or are inactive (< 30% inhibition of radioligand binding at 10 μM) at the mouse or rat A_3AR. Carlin et al. [23] compared MRS1191 and MRS1523 at the mouse and human A_1, A_{2A}, and A_3ARs and found that MRS1523 and MRS1191 are indeed selective for human A_3AR, but MRS1523 is a weak antagonist for mouse A_1AR (5 μM) and mouse A_3AR (1.1 μM). MRS1191 is essentially inactive at mouse A_3AR (32% inhibition of radioligand binding at the concentration of 10 μM) and weak at the rat A_3AR (K_i 1.42 μM) [90]. A congener MRS1334 that was 12-fold more potent than MRS1191 in human A_3AR binding was not enhanced at rat A_3AR (K_i 3.85 μM) [90].

Miwatashi et al. [91] synthesized a novel series of 4-phenyl-5-pyridyl-1,3-thiazole derivatives with human A_3AR affinity and found that the acyl moiety at the 2-position of thiazole is important for affinity of rA_3AR. Several thiazol-2-amines, including DPTN (MRS7799) and MRS7907, were shown to be potent at rat A_3AR (around 1 nM) and with selectivity against A_1 and $A_{2A}ARs$ [91]. Recently, the K_i values (nM) of DPTN at A_1, A_{2A}, A_{2B}, and A_3 receptors were reported: 162, 121, 230, and 1.65 (human); 411, 830, 189, and 9.61 (mouse); and 333, 1147, 163, and 8.53 (rat) [17].

The signaling efficacy of nucleosides for the A_3AR can be modulated by various structural changes to the ribose ring system, for example, 4′-truncated adenosine derivatives tend to have low or no efficacy [82, 92]. The class of adenosine-based antagonists, such as spiro-nucleoside MRS1292 and 4′-truncated LJ1251, were initially discovered as human A_3AR antagonists [92, 93]. However, later studies demonstrated that some 4′-truncated nucleoside antagonists studied earlier still possess some residual agonist activity [93, 94]. For the A_3AR and unlike other AR subtypes, there are multiple sites on the nucleoside scaffold for modification to reduce efficacy without removing affinity. Furthermore, although some nucleoside antagonists are shown to be potent across A_3AR species, the E_{max} at various species has not been carefully examined. Individual follow-up studies demonstrated that at some of them could be partial agonists at species other than human [82, 94]. Several selective nonnucleoside A_3AR antagonists that are commercially available include MRS1220, MRE 3008F20, MRS1334, VUF5574, PSB10, PSB11, and MRS1523. To our knowledge, among these, only DPTN and MRS1523 have been confirmed as A_3AR antagonists for rat or mouse albeit with limited selectivity, and the remaining A_3AR antagonists listed are either inactive or not yet examined at the mouse or rat A_3AR [18].

In summary, only MRS1523 ($K_i = 0.5$ μM for rat A_3AR and 0.7 μM for mouse A_3AR) and DPTN ($K_i = 9.61$ nM for rat A_3AR and 8.53 nM for mouse A_3AR) and its 3-iodo analogue MRS7907 exhibit selective A_3AR antagonism at both rat and mouse A_3ARs. It is also notable that at a high concentration of 10 μM, MRS1523 also antagonizes mouse and rat A_1 and $A_{2A}ARs$. At a concentration higher than 100 nM, DPTN may also antagonize other AR subtypes (Table 4). Thus, MRS1523 and DPTN must be used judiciously to discriminate A_3AR-mediated responses in rodent model systems. Other commercially available A_3AR antagonists have K_i values at mouse or rat A_3ARs in the μM range at best. MRS1220 should not be used for mouse or rat A_3ARs, since it is largely inactive. In general, antagonist pharmacology is highly species dependent. Affinity and selectivity within the species of interest must be carefully considered before an antagonist be applied to investigate A_3AR function.

Species dependence of A_3AR allosteric modulators

There are dramatic species differences among a number of different A_3AR allosteric modulators that have been studied to date. One class of A_3AR PAMs, i.e., 3-(2-pyridinyl)isoquinolines including VUF5455, has been shown to decrease the rate of dissociation of [^{125}I]I-AB-MECA from the human A_3AR, and to a lesser extent from the rat A_3AR [95]. Amiloride and several of its analogs have been shown to decrease the dissociation rate of the agonist radioligand [^{125}I]I-AB-MECA and to increase the dissociation rat of the antagonist radioligand [^{3}H]PSB-11 from the human A_3AR [96]. The potency of amiloride is weak at both human and rat A_3ARs (> 100 μM), but the amiloride analog hexamethylene amiloride (HMA) binds with higher affinity to both human (7.0 μM) and rat A_3AR (5.7 μM) [96]. Also, HMA slowed the dissociation of [^{125}I]I-AB-MECA from both human and rat A_3ARs. Unlike amilorides, thiadiazole derivative SCH202676 accelerated the dissociation of [^{125}I]I-AB-MECA from the human A_3AR [97], but its effect at the rat A_3AR has yet to be tested. Unlike amiloride analogs, SCH202676, a modulator of diverse GPCRs, did not show any effect on antagonist radioligand [^{3}H]PSB-11 dissociation. May et al. [98] identified a diazo polyanionic food dye, Brilliant Black BN (structure not shown), as a novel A_3AR negative allosteric modulator (NAM). Interestingly, at concentrations ranging from 5 to 500 μM, Brilliant Black BN had no substantial effect on NECA-induced calcium mobilization in CHO cells expressing the human A_3AR. However, it decreased the affinity of several structurally unrelated antagonists, i.e., MRS1220, XAC, and XAC-X-BY630. Brilliant Black BN accelerated the dissociation rate of the fluorescent XAC derivative, XAC-X-BY630 (useful probe at both human A_1AR and A_3AR), suggesting that the decrease in antagonist affinity is probably due to its ability to increase the antagonist dissociation rate. Again, the effects of Brilliant Black BN at rat A_3AR have not yet been tested.

The human A_3AR PAMs LUF6000 (a 1*H*-imidazo[4,5-c]quinolin-4-amine) and LUF6096 (an *N*-(2-(arylamino)quinolin-4-yl)cyclohexanecarboxamide), at concentrations up to 10 μM, are inactive or only weakly active at mouse or rat A_3ARs, while they are fully active at human, dog and rabbit A_3ARs [19, 64, 77, 99, 100]. The enhancer LUF6096 was shown to reduce infarct size in a barbital-anesthetized dog model of myocardial ischemia/reperfusion injury [100]. In [^{35}S]GTPγS binding assays with cell membranes isolated from HEK293 cells stably expressing recombinant A_3ARs, Du et al. [19] showed that both LUF6000 and LUF6096 greatly enhanced agonist efficacy at human, dog, and rabbit A_3ARs but only had a minor effect at mouse A_3ARs (in enriched membranes). The EL1 region, which displays sequence variability, is not responsible for this species difference based on studies with mutant human/mouse A_3ARs. Nevertheless, homology modeling and molecular dynamics simulation suggested interaction of LUF6000 with species-variable EL1 and agonist-contacting EL2 [101], although current work more accurately addresses the mechanism of action of these compounds. In recent studies using human/mouse chimeric receptors [84, 102], a distal intramembrane site of LUF6000 binding was indicated. The same study introduced novel A_3AR PAMs, including MRS7551 and MRS8054. A_3AR allosteric enhancers that exhibit activity versus rat or mouse A_3ARs have yet to be developed.

In brief, amiloride analogs are allosteric modulators at both human and rat A_3ARs. However, LUF6000 and LUF6096 exhibit species variability and PAM activity with human, dog, and rabbit A_3ARs, but lack activity versus rat and mouse A_3ARs. Other classes of allosteric modulators have not been well characterized in different species. There is a need to develop pan-species A_3AR PAMs and negative allosteric modulators (NAMs).

Species differences in G protein coupling and A_3AR activation

Both A_1 and A_3ARs couple to activation of G_i proteins. Regarding the A_1AR, coupling to the various G_i-like protein isoforms has been relatively well-documented, with evidence supporting that it is able to activate $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{OA}$, $G\alpha_{OB}$, and $G\alpha_Z$ [103, 104]. Specific α G protein isoforms are known to mediate A_1AR actions within tissues. For example, A_1AR-mediated cat detrusor muscle contraction depends on $G\alpha_{i3}$, PLC-β_3 and the release of intracellular Ca^{2+} [105]. A_1AR-mediated PLC-β_3 activation occurs via both $G\alpha_{i3}$ as well as βγ subunits in rabbit intestinal muscle [106]. Tateyama and Kubo [107] proposed that the agonist-bound A_1AR active conformation is unstable in the absence of G protein binding and that the stabilizing effects differ among the G_i isoproteins.

In all species examined thus far, the A_3AR has been reported to couple to activation of G α $_i$ proteins, and limited studies suggest that it predominantly couples to the $G\alpha_{i2}$ and $G\alpha_{i3}$ isoforms [108, 109]. In CHO cells stably expressing rat A_3ARs, Palmer et al. [108] demonstrated that treatment with NECA stimulated GTP binding to both $G\alpha_{i2}$ and $G\alpha_{i3}$ isoproteins, very little to G α $_{q/11}$, and essentially none to G α $_s$. Interestingly, incubation with NECA reduced expression of the $G\alpha_{i3}$ and Gβγ subunits without changing levels of $G\alpha_{i2}$ (or $G\alpha_s$ or G α $_{q/11}$), suggesting that even though the rat A_3AR is coupled to activation of both $G_{\alpha i2}$ and $G_{\alpha i3}$ isoproteins, sustained agonist exposure selectively downregulates

$G\alpha_{i3}$. In rat peritoneal mast cells and the HMC-1 human mast cell line, Baram and colleagues demonstrated that A_3AR-induced activation of ERK1/2 and other signaling pathways linked to mRNA expression of cytokines, chemokines, and growth factors is inhibited by a cell-permeable C-terminal inhibitory peptide directed toward $G\alpha_{i3}$ [109]. Thus, this study identified functionally relevant A_3AR-$G\alpha_{i3}$ isoprotein coupling within native human and rat cell systems. In a recent work by our groups [84], we utilized a novel BRET-based reporter system and demonstrated that, in addition to $G\alpha_{i1-3}$ isoproteins, the human A_3AR also weakly couples to $G\alpha_{OA}$ and $G\alpha_{OB}$ isoproteins. The A_3AR PAMs LUF6000 and LUF6096 preferentially enhance signaling through $G\alpha_{i3}$ and $G\alpha_{OA}$ isoproteins [84].

While we are not aware of any specific reports describing species differences in A_3AR-G isoprotein coupling in vivo, it is important to discuss the work by Yamato and colleagues [110] who ventured to develop a humanized A_3AR mouse line to aid discovery of potent human A_3AR antagonists for treating asthma and other inflammatory disorders. The strategy involved "knocking in" the human A_3AR coding sequence into the mouse genome in place of the mouse sequence. Unfortunately, these investigators discovered that A_3AR activation no longer induced degranulation of bone marrow-derived mast cells as is it does in wild-type mice, likely explained by an inability of the human A_3AR to activate ERK1/2 and PI3Kγ signaling events (yet it stimulated calcium mobilization). It was also discovered that the rate of A_3AR internalization was much slower in mast cells from the humanized A_3AR mouse line. Subsequently, a second mouse line was generated in which only the receptor extracellular regions that comprise the orthosteric ligand binding site were replaced with the human sequence leaving the intracellular regions that interact with G proteins unchanged. Mast cells from these mice responded to adenosine agonist exposure similarly to those from wild-type mice (i.e., ERK1/2 and PI3K γ activation, calcium mobilization, granule release). In addition, the chimeric receptor maintained an antagonist binding profile similar to the wild-type human A_3AR. On one hand, these studies suggest that, in addition to ligand binding, there may be differences in G protein coupling between human and mouse A_3ARs. On the other hand, they may illustrate the consequences of utilizing heterologous expression systems to investigate A_3AR signaling.

Species differences in desensitization, internalization, and resensitization

Phosphorylation of activated receptors by GPCR kinases (GRKs) followed by β-arrestin binding is a common mechanism to desensitize/sequester and, via clathrin-coated pits, internalize GPCRs, thereby terminating signaling mediated by G proteins. The internalized receptors can be redirected to lysosomes where they are degraded resulting in a net loss of cell-surface expression (down-regulation), or they can be dephosphorylated and recycled back to the cell surface. In the meantime, β-arrestin binding via scaffolding can also initiate G protein-independent signaling events such as ERK1/2 activation. Prior work has shown that the rates and extents of desensitization of the four AR subtypes are markedly different [111, 112]. A_1AR desensitization occurs very slowly, typically with a half-life of ~ 8 h, whereas the half-life of A_{2A} and $A_{2B}AR$ desensitization is ~ 1 h. The A_3AR is unique in comparison and has been shown to desensitize ($t_{1/2}$ of 5–30 min) and re-sensitize (~ 2 h) very rapidly [113]. Because the A_3AR desensitization process is so rapid, it has been speculated that desensitization in response to agonist exposure can therapeutically resemble antagonism, which makes the A_3AR a very complex and intriguing therapeutic target. A_1AR regulation has been investigated most extensively, and no species differences have been identified. For example, 30-min agonist treatment did not alter the potency or efficacy of agonist-stimulated inhibition of forskolin-stimulated adenylyl cyclase activity in CHO cells expressing the human A_1AR [114]. A similar rate of agonist-induced desensitization ($t_{1/2}$ of 7–9 h) was observed in DDT1-MF2 cells (hamster smooth muscle cell line [115]) and isolated chick atrial myocytes [116] that natively express the A_1AR. β-Arrestin-1, but not β-arrestin-2, appears responsible for agonist-induced A_1AR desensitization [117–119].

In contrast, there are subtle species differs in the rates of A_3AR desensitization. In terms of speed, current evidence indicates that the rat receptor desensitizes more rapidly (~ 1–10 min) compared to the human A_3AR (~ 10–30 min), which might be explained by the involvement of different molecular mechanisms that control surface expression. In CHO cells exogenously expressing the rat A_3AR, Palmer et al. [113, 114] showed that exposure to NECA induced rapid ($t_{1/2} = ~ 1$ min) phosphorylation on serine and threonine residues within the receptor C-terminal region by GRK2. This was associated with a 30–40% reduction in the number of high-affinity agonist binding sites as determined in radioligand binding assays and an eightfold decrease in the potency of NECA to inhibit forskolin-induced cAMP production [113, 114]. In similar studies with transfected CHO cells, Ferguson et al. [120] reported that the rat A_3AR was rapidly ($t_{1/2} = 10$ min) phosphorylated by GRK2 and internalized after agonist exposure. These responses were blocked by the A_3AR antagonist MRS1523 and were not observed in CHO cells expressing a non-phosphorylatable A_3AR mutant, indicating that the internalization process is phosphorylation-dependent. While all of the studies described thus far were conducted with heterologous expression systems, Ali et al. [31] similarly observed rapid agonist-induced functional desensitization in studies with

RBL-2H3 rat mast cell-like cells that endogenously expressing the A_3AR. In this study, agonist-induced mobilization of intracellular calcium was abolished after only 3 min of NECA exposure [31]. Curiously, current evidence suggests that desensitization responses of the rat A_3AR expressed endogenously in RBL-2H3 cells or heterologously in HEK293 cells are not dependent on arrestin binding [119, 121], although this issue needs to be addressed more thoroughly using newer tools that are available to study arrestin translocation and arrestin-receptor binding in cells.

With the human A_3AR, it is well-established that receptor desensitization and internalization are dependent on phosphorylation by GRKs as well as by β-arrestin-2 binding [94, 113, 122–125]. Using a fluorescent agonist and confocal imaging, Stoddart et al. [124] directly visualized internalization of the human A_3AR and its colocalization with β-arrestin-2. In this study, the fluorescent agonist BY630-X-(D)-A-(D)-A-G-ABEA induced A_3AR internalization, which was blocked by the A_3AR antagonist MRS1220. In a separate study [123], Stoddart and colleagues also showed that both NECA and HEMADO induced rapid internalization of the human A_3AR into punctuate intracellular granules located near the nucleus and that HEMADO-induced internalization was not apparent in studies with a W243F (residue 6.48) mutant receptor. Other mutagenesis studies have shown that agonist-induced β-arrestin-2 recruitment to the human A_3AR is dependent on the C-terminus as well as the "DRY" motif located at the distal portion of TM3 [125]. The ability of structurally diverse agonists to stimulate β-arrestin-2 translocation in engineered CHO cells expressing the human A_3AR has been investigated, which revealed that the agonist potency order and efficacy to induce β-arrestin-2 translocation and G protein activation were similar [94]. Collectively, current evidence suggests that mechanisms that mediate human A_3AR internalization involve β-arrestin-2, but that β-arrestin-2 may not be critical for rat A_3AR internalization.

Trincavelli et al. [126] showed that the human A_3AR is rapidly internalized ($t_{1/2}$ of 16.9 min) after treatment with NECA in transfected CHO cells, resulting in a > 80% reduction in the efficacy of IB-MECA to inhibit forskolin-stimulated cAMP production. After the removal of NECA, the A_3AR recycled back to the cell surface within 60 min fully restoring functional coupling to adenylyl cyclase inhibition, thus providing evidence that the human A_3AR can rapidly re-sensitize and recycle back to the cell surface after it has undergone agonist-induced internalization. This was subsequently attributed to a signaling mechanism involving the MAPK cascade and GRK2-mediated receptor phosphorylation. Short-term exposure to Cl-IB-MECA (100 nM) of human astrocytoma cells endogenously expressing the human A_3AR caused rapid desensitization (15 min), internalization (30 min), and the receptor recycled back to the

cell surface within 120 min after agonist removal [113]. While the rat A_3AR also appears to rapidly return to the cell surface after agonist-induced internalization, Ferguson and colleagues showed that the proportion of receptors that are recycled is much less after agonist removal as compared to the human A_3AR. This study found that the rat A_3AR accumulates in transferrin receptor-positive endosomal compartments, which is dependent upon GRK-mediated phosphorylation of the receptor's carboxyl terminus but is independent of β-arrestin-2 translocation [119, 121].

In summary, current evidence indicates that the A_3AR is highly regulated, particularly when compared with the other AR subtypes. It undergoes rapid desensitization and internalization, and intricate mechanisms control its fate once it is internalized. Published studies suggest subtle differences in the processes that regulate G protein coupling and surface expression of the A_3AR, based on published studies of the human and rat A_3ARs. These subtle variations are related to differences in the rates of receptor desensitization, the extent of receptor recycling, and the molecular mechanisms that control A_3AR function. Overall, the rat A_3AR undergoes desensitization faster than the human A_3AR, but the human A_3AR can be completely re-sensitized and recycled back to the cell surface, whereas the rat A_3AR is only partially re-recycled back to the cell surface within a short period of time after agonist removal. Current evidence suggests that β-arrestin-2 is critical for human A_3AR but not rat A_3AR internalization.

A_3AR functions in cells, tissues, or animals from various species

This discussion of the biological functions of the A_3AR does not cover all topics, which have been reviewed elsewhere [58, 67], but rather emphasizes those where species differences have been identified. The involvement of the A_3AR in organ systems and during disease in nearly all cases is related to its role in regulation of circulating or tissue-resident immune cells.

A_3AR in mast cells, allergy, and asthma

Adenosine has been implicated in a variety of inflammatory processes, exerting both pro- and anti-inflammatory actions, especially in allergy and asthma. Holgate and colleagues first demonstrated that adenosine potentiated IgE-induced mediator release from rodent mast cells in 1980 [127]. Relevance of adenosine/mast cell interactions to human asthma was further suggested in studies that documented the ability of inhaled adenosine to induce bronchoconstriction in asthmatic, but not normal patients [128]. It is notable that adenosine-induced bronchospasm in humans is partially

attenuated by several classes of anti-asthmatic drugs [128] and the mechanism of adenosine-induced bronchospasm has been linked to mediators released from mast cells [129]. While there is a strong association between asthma, adenosine, and mast cells, understanding of the identity of specific AR subtypes that mediate adenosine-mediated bronchospasm remains to be fully understood and is clearly variable among species [129].

A central role of the A_3AR in controlling mast cell responses and adenosine-induced bronchoconstriction has essentially been confirmed in rodent species by employing adenosine receptor KO mice and c-Kit mutant mice that are deficient in mast cells. Specifically, bronchoconstriction elicited by aerosolized adenosine or NECA is significantly attenuated in both A_3AR KO mice and in mast cell-deficient mice [130]. However, full responsiveness to NECA is restored in mast cell-deficient mice that are reconstituted with mast cells from wild-type mice but not from A_3AR KO mice. Thus, it is suggested that adenosine-induced airway hyperresponsiveness in rodents primarily occurs indirectly by activating A_3ARs on mast cells. In further support of this idea, studies with isolated rodent mast cells (bone marrow-derived, alveolar, rodent mast cell lines including RBL-2H3 cells) have shown that subtype selective A_3AR agonists induce degranulation and chemotaxis of via the A_3AR [31, 131]. Moreover, intravenous administration of A_3AR agonists induces systemic mast cell degranulation resulting in increased plasma histamine levels [29, 69, 132] or, if applied intradermally, induces localized vascular leakage [133]. Notably, certain actions of A_3AR agonists have been attributed to actions of mediators released from mast cells such as histamine. As two examples, intravenous administration of A_3AR agonists including IB-MECA and Cl-IB-MECA reduce blood pressure and produce reflex tachycardia, responses that are mast cell dependent and likely mediated by histamine and other vasoactive components stored in mast cells [69, 132]. Similarly, systemic administration of A_3AR agonists reduces body temperature in mice by mast cell-derived histamine acting centrally via histamine receptors [23]. Past work by Duling's group reported that topical application of adenosine or its deaminated metabolite inosine [134], which is a relatively potent agonist of rodent A_3ARs, to the hamster cheek pouch preparation induces localized constriction of arterioles by stimulating degranulation of perivascular mast cells via the A_3AR. Notably, it is assumed that rodent mast cells also express the $A_{2A}AR$, which via the production of cAMP attenuates mast cells responses.

In contrast to rodent species, the mechanism of action of adenosine-induced bronchoconstriction and mast cell responses in larger mammals is less clear. Interestingly, in humans, the A_1AR and $A_{2B}AR$ [129] rather than the A_3AR have been implicated in adenosine-induced bronchoconstriction and mast cell activation. This is based on studies with isolated human mast cell lines, where the $A_{2B}AR$ induces production of various pro-inflammatory cytokines including IL-4, IL-8, and IL-13, and on the fact that enprofylline and theophylline, two effective anti-asthmatic drugs with low activity at the human A_3AR, relatively potently block the $A_{2B}AR$. In allergic rabbit and guinea pig models, the mechanism of adenosine-induced bronchoconstriction has been proposed to include direct constriction of bronchiolar smooth muscle via the A_1AR [135]. Unlike rodent species, systemic administration of A_3AR agonists (nor $A_{2B}AR$ agonists) does not evoke mast cell degranulation in larger mammals that have been investigated (rabbit, dog) and therefore do not alter systemic hemodynamics [136, 137]. A_3AR agonists including IB-MECA and Cl-IB-MECA have been evaluated in several clinical trials of inflammatory diseases and have been found at the doses tested to be well-tolerated, safe, and devoid of hemodynamic effects [138].

A_3AR in cardiac and brain ischemia

An anti-ischemic role of the A_3AR in heart studies has been suggested in several animal species. However, because this conclusion is largely based on findings with first-generation A_3AR agonists (IB-MECA, Cl-IB-MECA) that are only moderately selective, contributions of additional or other ARs in many instances cannot be excluded. As examples, IB-MECA has been shown to protect against contractile dysfunction induced by brief episodes of ischemia (i.e., myocardial "stunning") in conscious rabbits, to reduced infarct size induced by sustained ischemia and reperfusion in conscious rabbits and anesthetized dogs, and to induce "late" preconditioning in conscious rabbits [85, 136, 137, 139–141]. In the anesthetized dog model, IB-MECA effectively reduced infarct size to an equivalent extent if it was administered prior to induction of ischemia as compared to if it was administered when reperfusion was initiated, indicating protection against both ischemic and reperfusion-mediated injury [137]. In these studies, IB-MECA was effective at doses that did not cause hemodynamic changes [85, 136, 137, 139–141], which prevents clinical development of A_1 and $A_{2A}AR$ agonists for ischemic heart disease as well other diseases where they have shown efficacy in animal models.

Efforts to utilize AR gene KO mouse models to define the A_3AR as the site of action of A_3AR agonists in models of infarction are complicated by the effect of A_3AR agonists to evoke systemic degranulation of mast cells in mice. Nevertheless, the A_3AR agonist Cl-IB-MECA was shown to lose the ability to reduce infarct size in A_3AR KO mice but to retain efficacy in mice depleted of mast cell contents by chronic pretreatment with compound 48/80, suggesting an A_3AR-dependent but mast cell-independent cardioprotective mechanism [69]. Studies with bone marrow-chimeric

mice and mice with deletion of the gene encoding the A_3AR specifically in cardiomyocytes utilizing the LoxP/Cre recombinase strategy implicate that A_3AR-mediated mechanisms are partially explained by direct protection of ischemic muscle and suppression of reperfusion-mediated inflammatory injury [29, 142].

With respect to the brain in the context of stroke, several A_3AR actions have been reported, some of which appear to be species-dependent and may be related to A_3AR-mediated mast cell degranulation. It has been suggested that the A_3AR is involved in the regulation of cerebral blood flow in gerbils [143]. Mice lacking the A_3AR were shown to be sensitive to hypoxic neurodegeneration, and A_3AR KO mice were more vulnerable to hippocampal pyramidal neuron damage following episodes of carbon monoxide (CO)-induced hypoxia [144]. Using primary cultured rat retinal ganglion cells, Nakashima et al. [145] demonstrated that stimulation of the A_3AR, not the A_1, A_{2A}, or $A_{2B}AR$, promotes neurite outgrowth of retinal ganglion cells. A_3AR activation may have great potential in the management of retinal neurodegenerative diseases characterized by retinal ganglion cell (RGC) death, as occurs in glaucoma, diabetic retinopathy, and ischemic diseases. Luo et al. [146] showed that A_3AR agonist Cl-IB-MECA reduces early brain injury in rat subarachnoid hemorrhage. It is to be noted that Cl-IB-MECA (≥ 10 μM) increased cell death in cultured rat cerebellar granular neurons, and the acute pre-administration of IB-MECA increased damage in a global ischemia model in gerbils [147]. This might suggest that both a downregulation or desensitization and an activation-related mechanism may be involved in the neuroprotective effect by A_3AR agonists. Nevertheless, the cerebroprotective effects in rat of postischemic administration of selective A_3AR agonist LJ529, a thio analogue of Cl-IB-MECA, were abolished with coadministration of A_3AR antagonist MRS1523, supporting receptor activation as the protective signal [81]. Furthermore, in the mouse transient middle cerebral artery occlusion (MCAO) ligation model, increased cerebral damage was noted in A_3AR KO compared to WT mice [131]. Although the cerebroprotective effects of A_3AR agonists were initially only demonstrated in mice, gerbils and rats [148, 149], recently the efficacy of a mixed A_1/A_3 agonist AST-004 (MRS4322) was observed in the MCAO model in macaques [150]. Thus, the role of the A_3AR in cardiac and brain ischemia has been confirmed in several species.

A_3AR in immune cells and cancer cells

As discussed previously, the A_3AR is highly expressed in several types of immune cells, which suggests its participation in immune responses and inflammation. A_3AR expression has also been observed in many cancer cells, such as leukemia, melanoma, and colon cancer cells, which

may indicate a vital role in cancer biology. A_3AR agonists entered into clinical trials for psoriasis, rheumatoid arthritis, hepatocellular carcinoma (HCC), nonalcoholic steatohepatitis (NASH), and COVID-19 [151]. Anticancer mechanisms have been suggested to involve, at least in part, immune-enhancing activity following A_3AR activation [152] and the Wnt signaling pathway [152]. It has been shown that adenosine and IFN-α synergistically increase IFN-γ production of human NK cells [153]. However, both IFN-γ production and up-regulation of IL-10 were found in cultured splenocytes derived from CF101 (IB-MECA)-treated animals [154], thus suggesting that IB-MECA may produce both immune-enhancing and suppressive effects, although it is not clear if this due to effects on the A_3AR or to non-specific activation of the immunosuppressive $A_{2A}AR$ [155, 156].

In addition to its role in immune responses [156–158], adenosine acting via the A_3AR has been shown to involve several other aspects of cancer biology including cell proliferation, apoptosis, angiogenesis, and metastasis. A_3AR activation has been shown to trigger phosphorylation of protein kinase B and protect rat basophilic leukemia 2H3 mast cells from apoptosis [159]. In A172 human glioma cells, Kim et al. [160] showed that treatment with A_3AR agonist CI-IB-MECA at μM concentrations induced cell death as a result of increased intracellular Ca^{2+} followed by enhanced reactive oxygen species (ROS) generation. Using A_3AR agonist IB-MECA and antagonist VUF5574 in an in vitro model of malignant undifferentiated and all-trans retinoic acid (RA)-differentiated SH-SY5Y cells, Maugeri et al. [161] showed that A_3AR activation induced HIF and VEGF expression via ERK1/2 signaling. Both adenosine and IB-MECA have been demonstrated to arrest motility of MDA-MB-231 breast cancer cells [162]. Adenosine was shown to promote migration of human neutrophils [131] via the A_3AR activation. CF101 has been shown to inhibit primary colon cancer cell growth and liver metastasis [163], which may suggest a possible role of A_3AR in tumor metastasis.

In addition to functions clearly mediated via the A_3AR, the ability of A_3AR agonists, such as Cl-IB-MECA at high μM concentrations to induce apoptosis in many cell types, is complexed mechanistically. Both A_3AR-dependent and A_3AR-independent apoptosis can be induced by Cl-IB-MECA [164, 165], and at lower doses A_3AR agonists can protect from apoptosis [166]. IB-MECA completely inhibited the growth of the human breast cancer cell lines MCF-7 and ZR-75 while inducing apoptosis in T47D and Hs578T cells, which do not express A_3AR mRNA [167]. In MCF-7 cells, A_3AR overexpression did not increase the sensitivity to drug treatment, and an A_3AR antagonist did not abolish the IB-MECA effect. The authors concluded that IB-MECA acts via estrogen receptor α. Kim et al. [165] showed that apoptosis in HL-60 cells induced by high concentrations of Cl-IB-MECA is A_3AR independent,

as neither MRS1220 nor MRS1523 blocked that effect. Thus, the role of A_3AR agonists in cancer progression has been examined in cells and tissues from both humans and rodents, but the mechanisms related to role of A_3AR are still somewhat controversial, and more work is still needed to clarify the controversy.

A_3AR in pain

All four AR subtypes have been reported to participate in the nociceptive process in rodents. With regard to the A_3AR, Sawynok and colleagues first proposed that A_3AR activation produces nociceptive behavior in rats [168]. Wu et al. [169] showed that mice lacking the A_3AR exhibit deficits in generating the localized inflammatory response to carrageenan, suggesting a pro-inflammatory role of A_3AR in peripheral tissues. In another study, A_3AR KO mice showed evidence of decreased nociception in the hot plate test, but not in the tail-flick test [144]. Yoon et al. [170] demonstrated that systemically administered IB-MECA exerts a significant antinociceptive effect during the second phase of the formalin test without altering protective nociceptive responses. Since MRS1220 prevented the antinociceptive actions of adenosine in the second phase of the formalin test, a role of spinal A_3ARs was suggested [170]. However, it is likely that these results implicate the $A_{2A}AR$ since in rodents MRS1220 is a fairly potent at the $A_{2A}AR$ (K_i values are 106 and 29 nM, respectively, for mouse and rat $A_{2A}ARs$ (Table 4)), but inactive at mouse or rat A_3ARs (Table 4).

More direct involvement of the A_3AR in pain biology has been promoted by Salvemini's group based on findings using Cl-IB-MECA as well as the more selective A_3AR agonist MRS5698. These investigators discovered that administration of A_3AR agonists reduces neuropathic pain induced by mechanical nerve injury and by chemotherapeutic agents [171]. Mechanistic studies suggest involvement of both central and peripheral A_3ARs, with central effects due to activation of inhibitory signals in the spinal cord and anti-inflammation possibly related to increased IL-10 production and peripheral effects potentially due to suppressed lymphocyte activation [172–176]. This work has implicated the possibility that A_3AR agonists may be a safe and successful non-opioid-based analgesic treatment strategy for patients suffering from chronic pain. Other studies by Terayama et al. [177] have proposed that A_3AR agonists attenuate neuropathic pain induced by nerve injury by suppressing microglial activation and nociceptive inputs in the spinal dorsal horn. Thus, it seems that probably both A_3ARs play a role in antinociception in mice, but it is not remains undetermined whether this is also the case in other species including humans. While there is evidence to support the possibility that the A_3AR plays a role in chronic pain, as discussed by Alles and Smith [178], many parallel, interdependent, and time-dependent processes (neuroimmune interactions at the peripheral, supraspinal, and spinal levels) contribute to the etiology of this complex condition. It may be necessary for drugs to target multiple processes rather than a single specific target to provide the greatest therapeutic benefit. Regarding the A_3AR, it seems necessary to examine the effect of selective agonists in pain models from multiple species, since to date all previous studies on this topic have been conducted in mice or rats. Considering that both G protein and arrestin signaling contribute to nociception [179], and β-arrestin2 appears to be necessary to mediate desensitization of the human A_3AR but not rat A_3AR, and different G_i proteins are involved in human and rat A_3AR signaling, it is possible that agonists found to be effective in rodents may or may not be active in humans.

A_3AR in glaucoma

Kvanta et al. [180], using in situ hybridization, have demonstrated the expression of mRNA for A_1, A_{2A}, and A_{2B} ARs, but not A_3AR mRNA in the rat eye. Mitchell et al. [181] detected A_3AR mRNA in both immortalized human non-pigmented cells and rabbit ciliary processes using RT-PCR. A_3AR was shown to regulate Cl- channels of nonpigmented ciliary epithelial cells. It is likely that the discrepancy was due to the sensitivity of detecting methods used, as A_3AR can still be detected with RT-PCR, although expression is possibly lower than other AR subtypes. Avila [182] demonstrated that A_3AR KO reduces mouse intraocular pressure (IOP) to further confirm its role. Okamura et al. [183] identified series of potent and selective A_3AR antagonists, and one compound, a 4,5-dihydro-3H-[1,2,4]triazolo[5,1-i]purine OT-7999, was evaluated in a monkey model of intraocular pressure (IOP) by topical administration. OT-7999 was found to significantly decrease IOP. Schlötzer-Schrehardt et al. [184] showed selective A_3AR upregulation in human eyes with pseudoexfoliation syndrome and glaucoma. Yang et al. [93] found that topical application of the A_3AR agonist IB-MECA increased mouse IOP, while the nucleoside antagonist MRS1292 reduced IOP. MRS1292 also inhibited adenosine-triggered human nonpigmented ciliary epithelial cell fluid release. Zhang et al. [185] found adenosine acting at A_3AR can attenuate rat retinal ganglion cell death. Wang et al. [22] showed that A_3AR antagonist LJ1251, a 4′-truncated adenosine derivative, lowers mouse IOP and display efficacy in both mouse and bovine cells. Thus, plenty of evidence support that A_3AR antagonists reduce IOP in several animal models and in humans. However, Fishman et al. [186] reported that CF101, which was in a Phase II clinical trial for dry eye syndrome that later failed to show efficacy, was found to decrease IOP in patients. In a subsequent Phase II clinical trial for glaucoma, CF101 had no effect on IOP. Park et al. [187] reported that FM101 proposed for the treatment of glaucoma is an agonist in cAMP pathway,

but an antagonist in arrestin translocation. The mechanism behind the agonist-decreased IOP could be a result of desensitization, which is similar in a way to A_3AR antagonism, but this remains to be further confirmed [188]. Thus, it seems convincing that A_3AR antagonists rather than agonists may reduce IOP, since it has been demonstrated in four species: mouse, rat, monkey and human.

A_3AR in pancreatic islets

The A_3AR has been found highly expressed in mouse pancreas [46]. Amisten et al. [189] systematically compared the GPCR transcriptome of human and mouse pancreatic islets to determine to what extent mouse islets can be used as surrogates to investigate the roles of specific GPCRs in human islet cell biology. Interestingly, from these analyses, the A_3AR was identified as one of several GPCRs with species-specific expression, as it was found to only be expressed in mouse islets and not human islets. In accord with these findings, MRS5698 was found to only inhibit glucose-induced insulin secretion from mouse islets but not from human islets [189].

Examples of data interpretation based on ambiguous use of A_3AR agonists and antagonists

As mentioned earlier, the commonly used A_3AR agonist IB-MECA is potent at the mouse A_3AR ($K_i = 0.2$ nM) (Table 4), but it is also potent for mouse A_1 ($K_i = 5.9$ nM) and mouse $A_{2A}ARs$ ($K_i = 300$ nM). Thus, if a concentration of 10 nM is used, it can activate both A_1 and A_3ARs. If a concentration of 1 μM is used in any experiments, mouse A_1, A_{2A}, and A_3ARs all can be fully activated, and mouse $A_{2B}ARs$ can be partially activated [69]. Thus, many controversies from earlier publications may have stemmed from agonist selectivity and affinity at different species. On the other hand, MRS1220 is sometimes used as an A_3AR antagonist in mouse or rat, assuming incorrectly that it is an A_3AR antagonist across multiple species ($K_i = 0.65$ nM for human A_3AR). For example, MRS1220 was used to antagonize the antinociceptive action of adenosine in rat, Cl-IB-MECA-facilitated epileptiform discharges in rat hippocampal slices, cordycepin (3′-deoxyadenosine)-inhibited B16-BL6 mouse melanoma cell growth, and IB-MECA's effect on bone destruction in rats [170, 190–192]. However, it should be kept in mind that MRS1220 is an antagonist of mouse and rat A_1ARs with respective binding K_i values of 81 and 305 nM, respectively, and mouse and rat $A_{2A}ARs$ with K_i values of 9.1 and 52 nM, respectively. Its affinity at the mouse or rat A_3AR is > 10 μM [17, 193]. In addition to MRS1220, there is also some controversy about the

selectivity of another commonly used A_3AR antagonist MRS1523. Unlike MRS1220, MRS1523 is a confirmed antagonist of both mouse and rat A_3ARs [17, 18], although with variable selectivity versus other AR subtypes (Table 4), which could be due to the use of different expression systems or radioligands (especially agonist vs. antagonist radioligands), etc. in the various studies (Table 4).

It is notable that that not all studies in the past using IB-MECA or Cl-IB-MECA drew a conclusion related to a role of the A_3AR, reinforcing the need for applying appropriately selective AR antagonists. For example, Lasley et al. [194] observed that IB-MECA-induced rat coronary dilation was completely blocked by the $A_{2A}AR$ antagonist SCH58261 (50 nM) and thus the authors concluded that coronary dilatory effect of IB-MECA in the rat heart appear to be caused by $A_{2A}AR$ activation. IB-MECA was found to inhibit LPS-induced TNF-α production via $A_{2A}ARs$ but not A_3ARs in equine monocytes [195]. In mouse macrophages, LPS-induced TNF-α production was potently inhibited by IB-MECA (1 μM) via activation of A_{2A} but not A_3ARs [86].

Conclusions

There are large genetic differences of the A_3AR from various species, especially between human and mouse or rat, achieving only ~70% amino acid similarity. A_3AR tissue distribution varies substantially among species. Different mechanisms are involved in human and rat A_3AR activation, desensitization and re-sensitization. Some agonist structures, such as N^6-methyladenosine, are ~700-fold more potent at human compared to rat A_3ARs, while some agonists, such as N^6-(3-halobenzyl)-substituted adenosines (IB-MECA, Cl-IB-MECA and MRS5698), maintain high affinity at human, mouse, and rat A_3ARs. However, most A_3AR antagonists are inactive or weakly active at mouse and rat A_3ARs; DPTN and MRS1523 are the only two confirmed A_3AR antagonists albeit with moderate selectivity against A_1, A_{2A}, or $A_{2B}ARs$. Most allosteric modulators, including LUF6000 and LUF6096, are only active at human and large animal species, but not rodents, but amiloride analogs retain allosteric activity across species. Considering that many species differences were identified between human and mouse or rat, some A_3AR functions identified using mouse or rat models need to be confirmed in larger animals, such as dogs or sheep. These species differences in the pharmacological properties of synthetic A_3AR ligands, e.g., their binding affinity, selectivity, and signaling efficacy, as well as the species-dependent role and regulation of the receptor, must be taken into consideration during drug development.

Declarations

Competing interests The authors declare no competing interests.

Conflicts of interest Zhan-Guo Gao declares that there is no conflict of interest. John A. Auchampach declares that there is no conflict of interest. Kenneth A. Jacobson declares that there is no conflict of interest.

Ethical approval Not applicable.

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