Medicinal Chemistry of the A_3 Adenosine Receptor: Agonists, Antagonists, and Receptor Engineering

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Contents

Abstract A_3 adenosine receptor (A_3AR) ligands have been modified to optimize their interaction with the A_3AR. Most of these modifications have been made to the N^6 and C2 positions of adenine as well as the ribose moiety, and using a combination of these substitutions leads to the most efficacious, selective, and potent ligands. A_3AR agonists such as IB-MECA and Cl-IB-MECA are now advancing into Phase II clinical trials for treatments targeting diseases such as cancer, arthritis, and psoriasis. Also, a wide number of compounds exerting high potency and selectivity in antagonizing the human (h)A_3AR have been discovered. These molecules are generally characterized by a notable structural diversity, taking into account that aromatic nitrogen-containing monocyclic (thiazoles and thiadiazoles), bicyclic

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(isoquinoline, quinozalines, (aza)adenines), tricyclic systems (pyrazoloquinolines, triazoloquinoxalines, pyrazolotriazolopyrimidines, triazolopurines, tricyclic xanthines) and nucleoside derivatives have been identified as potent and selective A_3AR antagonists. Probably due to the "enigmatic" physiological role of A_3AR, whose activation may produce opposite effects (for example, concerning tissue protection in inflammatory and cancer cells) and may produce effects that are species dependent, only a few molecules have reached preclinical investigation. Indeed, the most advanced A_3AR antagonists remain in preclinical testing. Among the antagonists described above, compound OT-7999 is expected to enter clinical trials for the treatment of glaucoma, while several thiazole derivatives are in development as antiallergic, antiasthmatic and/or antiinflammatory drugs.

Keywords A_3 adenosine receptor · A_3 adenosine receptor agonist · A_3 adenosine receptor antagonist · Purines · Structure activity relationship · Nucleoside · G protein-coupled receptor · Neoceptor

Abbreviations

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

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

1 Introduction

The four subtypes of adenosine receptors (ARs), designated A_1, A_{2A}, A_{2B}, and A_3, are all seven-transmembrane spanning (7TM) receptors that couple to G proteins. The A_3AR inhibits adenylate cyclase through coupling to G_i. A_3AR activation may lead to an activation of the phospholipase C pathway through the β, γ subunit. The A_3AR is found at a high receptor density in the lungs, liver, and in immune cells such as neutrophils and macrophages, as well as at lower densities in the heart and brain (Fredholm et al. 2001) The A_3AR is expressed in neurons in the brain (Lopes et al. 2003; Yaar et al. 2002).

ARs in general, and the A_3AR in particular, are involved in many of the body's cytoprotective functions. Recently, agents that act at the A_3AR have been targeted for pharmaceutical development based on their anti-inflammatory, anticancer, and cardioprotective effects. For example, activation of the cardiac A_3AR preconditions cardiac myocytes against ischemic damage (Strickler et al. 1996; Tracey et al. 2003) and protects against apoptosis. Selective A_3AR agonists have been shown to protect cardiac muscle in various ischemic models and are protective against the cardiotoxic effects of the anticancer drug doxorubicin (Shneyvais et al. 2001). A_3AR antagonists are of interest as potential antiglaucoma agents (Yang et al. 2005) and as anticancer agents (Gessi et al. 2008).

Agonist ligands for the ARs, including the A_3AR, are almost exclusively nucleoside derivatives. The search for antagonists of the A_3AR in the early 1990s initially encountered an unanticipated difficulty: the lack of an obvious lead structure. Previously, efforts to develop antagonist ligands for the A_1 and A_{2A} ARs focused on xanthine derivatives. However, at the A_3AR, the prototypical AR antagonists (i.e., the xanthines) are typically much weaker in binding than at the other AR subtypes. This observation stimulated the screening of structurally diverse heterocyclic molecules as potential antagonists (Moro et al. 2006). Chemically diverse leads were discovered in this process that were subsequently optimized to achieve high antagonist selectivity for the A_3AR.

While the A_3AR may be activated by orthosteric agonists that are competitive with adenosine, the action of nucleosides at this receptor may also be enhanced by allosteric modulators. Several heterocyclic classes of positive allosteric modulators of the A_3AR, including 1*H*-imidazo-[4,5-*c*]quinolines such as LUF6000 (*N*-(3,4-dichloro-phenyl)-2-cyclohexyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine) and pyridinylisoquinolines, have been reported (Gao et al. 2005; Göblyös et al. 2006).

The structure–activity relationships (SARs) of nucleoside derivatives in binding to the A_3AR and other ARs have been extensively studied, leading to the development of both selective agonists and, more recently, antagonists. Most of the useful modifications of adenosine **1** (Fig. 1) to achieve high A_3AR affinity and selectivity have been made at the N^6 or C2 positions of adenine or on the ribose group of adenosine. The systematic probing of SAR of both adenosine derivatives and nonpurine antagonists is frequently guided by molecular modeling (Kim et al. 2003), in which the receptor protein is modeled based on structural homology to the light receptor, rhodopsin. The effects of substitution at various

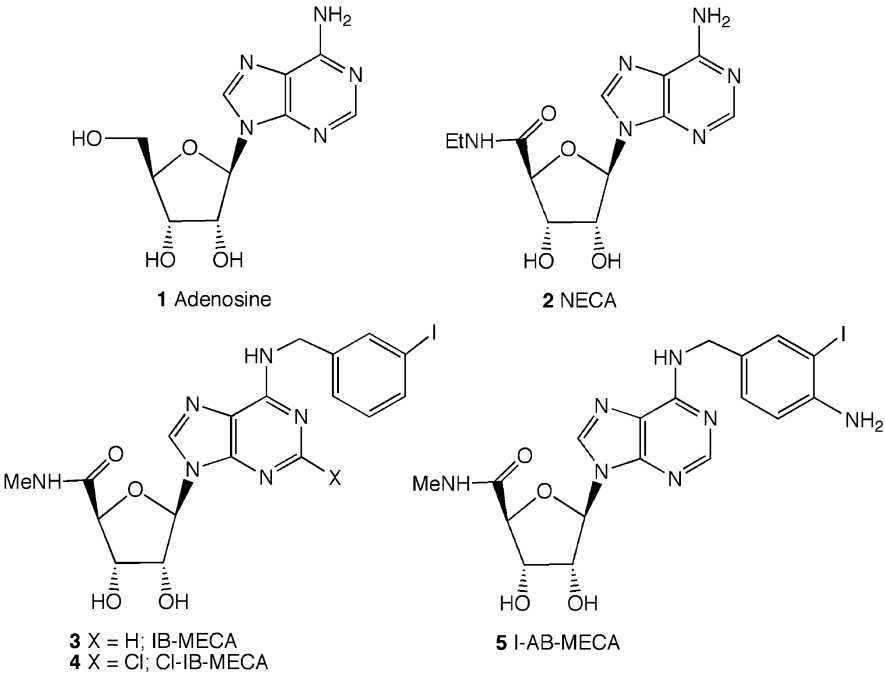


Fig. 1 Structures of adenosine and widely used agonist probes of the A_3AR

sites (i.e., on the nucleobase and ribose moiety) on both the affinity and relative efficacy of nucleoside derivatives at the A_3AR have been extensively probed (Gao et al. 2003, 2004). The approach initially taken to identify agonists for the newly cloned A_3AR was to screen known AR ligands in binding assays. The agonist NECA **2** (adenosine 5′-*N*-ethyluronamide) was found to be highly potent but nonselective for this receptor (Zhou et al. 1992). The structural features that promoted A_3AR potency were combined, leading to the first selective A_3 agonist, IB–MECA (N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine), developed in 1993 at the National Institutes of Health (Jacobson et al. 1993). This potent A_3AR agonist IB–MECA **3** and its more selective 2-chloro analog, Cl–IB–MECA **4** (2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine), are used widely as pharmacological tools. A related derivative **5** is widely used as an iodinated radioligand for the A_3AR. IB–MECA and Cl–IB–MECA have entered clinical trials for the treatment of rheumatoid arthritis and cancer (Baharav et al. 2005; Ohana et al. 2001).

One problem encountered in refining selective A_3AR ligands into pharmaceutically useful agents has been the species dependence of binding. This difference in affinity reflects the difference in sequence between the rodent and the human receptors, with only a 74% sequence identity between the rat (r) and human (h) A_3ARs (Fredholm et al. 2001). The species-dependence of A_3AR affinity is particularly

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

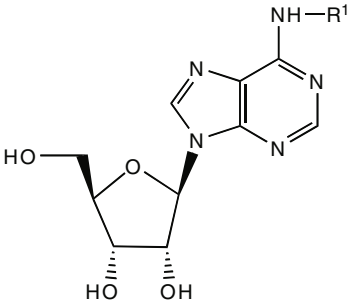
The ligand recognition within the putative binding site of the ARs has also been probed through extensive mutagenesis to confirm the predictions concerning ligand recognition made using molecular modeling (Kim et al. 2003). The hydrophobic environment surrounding the purine ring of AR agonists, as found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

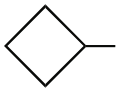
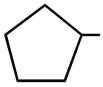
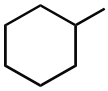
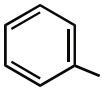
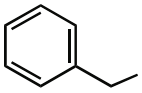
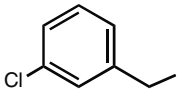
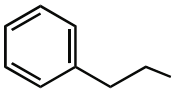
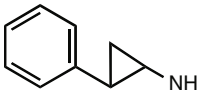
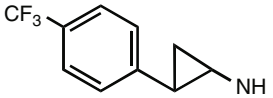
2 A_3AR Agonists

The subtype selectivity of adenosine derivatives as AR agonists has been probed extensively, principally through modification of the N^6-amine moiety (where large hydrophobic groups tend to produce A_1AR and A_3AR selectivity, Table 1) and the C2 position (where large hydrophobic groups tend to produce A_{2A}AR selectivity, but have also been shown to enhance A_3AR selectivity, Table 2). The ribose moiety is less amenable than the adenine moiety to the addition of steric bulk, although substitution of the 5′-CH_2OH moiety with certain amides, ethers, or other hydrophilic groups has resulted in enhancement of A_3AR selectivity.

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

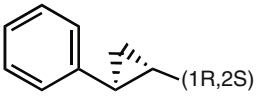
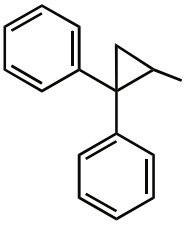
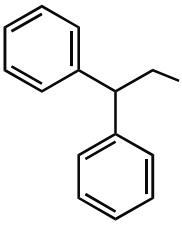
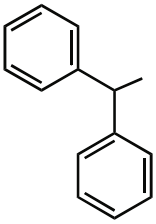
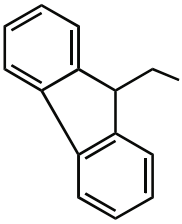
Table 1 Binding affinities of monosubstituted adenosine derivatives (N^6-substituted) at the human A_3AR expressed in CHO cells and at A_1 and A_{2A} ARs, and maximal A_3AR agonist effect



Compound	Substitution R^1 =	pK_i at A_1AR[a]	pK_i at A_{2A}AR[a]	pK_i at A_3AR[a]	%Activation, A_3AR[a]
6	CH_3	7.22[b]	<5[b]	8.03	96
7	CH_2CH_3	8.31[b]	5.05[b]	8.34	102
8	OCH_3	6.65[b]	<5[b]	7.55	107
9		9.15[b]	5.76[b]	8.19	100
10		9.35[b]	6.34[b]	7.14	97
11		9.05[b]	6.28[b]	7.14	76
12		8.48[b]	6.18[b]	7.83	102
13		6.76[b]	6.55[b]	7.38	55
14		7.35	<5[b]	8.36	80
15		7.89, 7.89[b]	7.17, 7.17[b]	8.68, 6.62[b]	84
16		6.91	5.60	9.06	101
17		6.98	5.60	8.72	101

(continued)

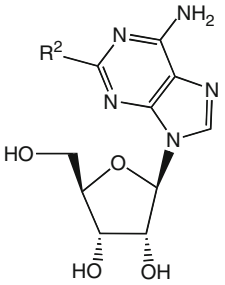
Table 1 (continued)

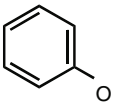
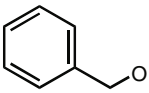
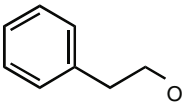
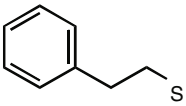
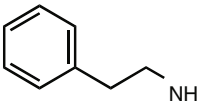
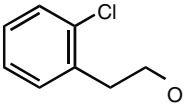
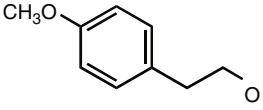
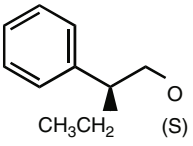
Compound	Substitution R^1 =	pK_i at A_1AR[a]	pK_i at $A_{2A}AR$[a]	pK_i at A_3AR[a]	%Activation, A_3AR[a]
18	 (1R,2S)	7.82[b]	5.52[b]	9.20	100
19	 (1S,2R)	7.93[b]	5.60[b]	7.62	87
20		8.15	6.24	7.04	100
21		7.30	6.29	8.41	0
22		6.31	<5	5.48	87
23		7.85	6.84	9.04	99

[a]A_3AR binding experiments were performed with membranes prepared from adherent CHO cells stably transfected with cDNA encoding the human A_3AR, using as radioligand [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]I–AB–MECA; 2000 Ci/mmol) at a final concentration of 0.5 nM, in Tris·HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. Nonspecific binding was determined using 10 μM Cl–IB–MECA. The mixtures were incubated at 25°C for 60 min. Maximal A_3AR agonist effect is the inhibition of forskolin-stimulated adenylate cyclase at 10 μM using a reference value for Cl–IB–MECA of 100%. (Gao et al. 2003; Tchilibon et al. 2004).

[b]In rat brain (Gao et al. 2003; Tchilibon et al. 2004).

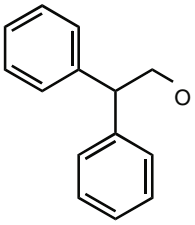
Table 2 Binding affinities of monosubstituted adenosine derivatives (2-ether-substituted) at the human A_3AR expressed in CHO cells and at A_1 and A_{2A} ARs, and maximal A_3AR agonist effect



Compound	Substitution R^1 =	pK_i at A_1AR[a]	pK_i at A_{2A}AR[a]	pK_i at A_3AR[a]	%Activation, A_3AR[a]
29	Cl	8.12	6.20	7.06	100
30		5.29	7.36	6.44	32
31		6.19	6.23	6.93	17
32		6.66	8.03	7.27	71
33		5.43	6.23	5.71	ND
34		6.28	7.21	6.51	72
35		6.66	7.75	6.85	1
36		6.54	7.19	6.98	91
37		5.32	7.57	6.76	0

(continued)

Table 2 (continued)

Compound	Substitution R^1 =	pK_i at A_1AR[a]	pK_i at A_{2A}AR[a]	pK_i at A_3AR[a]	%Activation, A_3AR[a]
38		<5	6.51	7.27	0

[a]A_3AR binding experiments were performed with membranes prepared from adherent CHO cells stably transfected with cDNA encoding the human A_3AR, using as radioligand [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]I–AB–MECA; 2000 Ci/mmol) at a final concentration of 0.5 nM, in Tris·HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. Nonspecific binding was determined using 10 μM Cl–IB–MECA. The mixtures were incubated at 25°C for 60 min. ND, not determined. Maximal A_3AR agonist effect is inhibition of forskolin-stimulated adenylate cyclase at 10 μM using a reference value for Cl–IB–MECA of 100% (Gao et al. 2004)

Modifications of the ribose moiety have also been explored for effects on both A_3AR binding affinity and efficacy (Gao et al. 2004; van Tilburg et al. 2002). SAR studies also indicate that flexibility in the ribose 5′ region is a prerequisite for A_3AR activation, in concert with a proposed required rotation of TM6 (Kim et al. 2006). Thus, with proper manipulation of groups at the N^6 and/or ribose moieties, a high-affinity agonist may be converted into a selective A_3AR antagonist. Conversely, agonist function may be maintained fully with proper derivatization of the ribose moiety. A flexible 5′-uronamide moiety is particularly well suited to maintaining efficacy, and even overcomes the reduction of efficacy induced by various adenine substituents at the N^6 and C2 positions.

2.1 Substitution of the Adenine Moiety of Adenine Nucleosides

2.1.1 N^6 Position

Multiple studies have been undertaken to optimize the N^6 position of adenosine in order to design selective A_3AR agonists (Table 1, **6–23**). Addition of small groups such as methyl (**6**) and oxymethyl (**8**) to the N^6 amine gave at least a tenfold increase in potency over adenosine and increased the selectivity of the ligand for the human A_3AR over other human ARs (Volpini et al. 2007). However, increasing the alkyl chain length to an ethyl group (**7**) increased the affinity of the ligand for both the A_3 and A_1 ARs, thus, decreasing the selectivity (Gao et al. 2003). Larger alkyl chains were not well tolerated at the N^6 position, and increased branching of the chain caused a decrease in A_3AR affinity and efficacy. Various cycloalkyl groups were

also appended to the N^6-amino group. Adding an N^6-cyclobutyl (**9**) or cyclopentyl (**10**) ring resulted in agonists that had greater affinity to the A_1AR than the A_3AR (Gao et al. 2003) but were full agonists at the A_3AR. Analogs bearing larger N^6-cycloalkyl rings such as **11** were only partially efficacious as A_3AR agonists. When a benzyl ring was attached to the N^6 amine (**13**), the compound was three- to four-fold selective in binding to the human A_3AR in comparison to the A_1 and A_{2A} ARs, but only displayed a 55% relative efficacy at the A_3AR. N^6-Phenyladenosine (**12**) was fully efficacious as an A_3AR agonist. N^6-(2-Phenylethyl)adenosine (**15**) was the most potent in binding to the A_3AR among a series of arylalkyl-substituted homologs. However, the N^6-benzyl and the N^6-phenyl substituents provided greater selectivity than 2-phenylethyl for the A_3AR. Generally, halogen substitution at the 3 position of the N^6-benzyl ring caused an increase in A_3AR affinity and selectivity. For example, N^6-(3-chlorobenzyl)adenosine (**14**) showed a tenfold selectivity for the A_3AR and a nanomolar affinity. Halogen substitution at other positions of the ring frequently decreased the A_3AR affinity.

Addition of certain larger N^6 substituents also increased the potency and affinity of the ligands at the A_3AR. For instance, N^6-(*trans*-2-phenylcyclopropropyl)adenosine (**16**) was a full agonist with high selectivity and a subnanomolar potency (Gao et al. 2003). Further variations on this substituent were prepared, and the importance of conformational factors in the relative efficacy was demonstrated. The addition of one bond to bridge the phenyl rings could change an antagonist into an agonist. Thus, while N^6-(2,2-diphenylethyl)adenosine (**21**) was an antagonist at the A_3AR, adding a bond between the phenyl groups to create N^6-(9-fluorenylmethyl)adenosine (**23**) restored the efficacy. This compound also had a subnanomolar A_3AR affinity but was less selective than N^6-(*trans*-2-phenylcyclopropyl)adenosine (Tchilibon et al. 2004). The most selective compound of the series was N^6-(*trans*-2-(3-trifluoromethyl)phenyl)-1-cyclopropyl adenosine (**17**), which had a 100-fold selectivity at the A_3AR in comparison to the A_1AR.

Various labs have combined sterically bulky N^6 groups with a 5′-uronamide moiety on the ribose group to make potent, selective A_3AR agonists. The first A_3AR-selective compounds combined a 5′-*N*-alkyluronamide with an N^6-benzyl group (Gallo-Rodriguez et al. 1994; Jacobson et al. 1993; van Galen et al. 1994). One of the most common A_3 agonists, Cl–IB–MECA (Fig. 1), has an N^6-iodobenzyl group, a 2-chloro group, and a 5′-methylcarboxamido group. This compound has a K_i of 0.33 nM at the rat A_3AR, but K_i values of only 2,500 and 1,400 nM at rat A_1 and A_{2A} ARs, respectively (Kim et al. 1994a). At the human ARs, the binding affinities of Cl–IB–MECA are (nM): A_1AR 220, A_{2A}AR 5400, and A_3AR 1.4. Thus, Cl–IB–MECA is more selective for the rat A_3AR than the human A_3AR (Melman et al. 2008a). The ^{125}I form of I–AB–MECA (N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine, **5**, Fig. 1) is commonly used as a high-affinity radioligand for characterizing binding to the A_3AR of various species.

Baraldi et al. (1998) prepared a series of N^6-substituted-aminosulfonylphenyl derivatives of NECA (e.g., compound **24**). Among these compounds, the most favorable substituents of the sulfonamido group for increasing affinity at the A_3AR were small alkyl groups, such as ethyl or allyl moieties, and disubstitution of the

sulfonamido group. The A_3AR selectivity was increased by the addition of a saturated heterocyclic ring, such as piperidine or morpholine, to the sulfonamido moiety.

Finally, A_3 selective fluorescent probes have also been made by attaching 7-nitrobenzofurazan fluorophores to NECA derivatives using an alkyl spacer (e.g., compound **25**). These compounds displayed 500-fold selectivity at the A_3AR and bound in the low nanomolar range (Cordeaux et al. 2008).

2.1.2 Adenine 2 Position

Many modifications at the 2 position of adenosine (Table 2, **29–38**) tend to increase A_{2A}AR potency, but some additions have been found to contribute to A_3AR selectivity. Adding a simple 2-chloro group (**29**) increased the A_3AR affinity in comparison to adenosine, but it also significantly increased the potency at the A_1AR (Gao et al. 2004). Generally, 2-ether modifications decreased A_3AR affinity, with certain exceptions. For example, adding a 2-*i*-pentyloxy moiety increased A_3AR affinity threefold, and the compound was slightly selective. 2-Benzyloxy substitution (**31**) decreased the efficacy to 17% of the full agonist Cl–IB–MECA. 2-Phenylethyloxy substitution (**32**) often increased affinity at both the A_3 and A_{2A} ARs, but many such analogs displayed a decreased efficacy as A_3AR agonists. Other 2-ethers, such as 2-(2,2-diphenylethyloxy)adenosine (**38**), were A_3 AR antagonists, in curious parallel to the effect of the same group when placed at the N^6 position (Tchilibon et al. 2004).

Many other substitutions at the 2 position of adenosine were combined with previously introduced substitutions at the N^6 position of adenosine. For instance, adding a 2-cyano group to N^6-(3-iodobenzyl)adenosine created an A_3AR antagonist, but when the 2-cyano group was added to N^6-methyladenosine, the compound was a full agonist that was 30-fold selective for the human A_3AR in comparison to the A_1AR (Ohno et al. 2004). However, when other small modifications were made at the 2 position of N^6-methyladenosine, such as an amino or a trifluoromethyl group, there was a decrease in selectivity and affinity toward the A_3AR. Elzein et al. (2004) synthesized a series of 2-pyrazolyl-N^6-substituted adenosine derivatives that were very potent and selective for the A_3AR. Cosyn et al. (2006b) found that several 2-triazol-1-yl substitutions of N^6-methyladenosine increased affinity at the A_3AR. However, in order to maintain efficacy, a 5′-ethyluronamide was necessary. 9-(5-Ethylcarbamoyl-β-D-ribofuranosyl)-N^6-methyl-2-(4-pyridin-2-yl-1,2,3-triazol-1-yl)adenine **26** (LC257, Fig. 2) was a full agonist with a K_i of 1.8 nM at the A_3AR and a minimum of 900-fold selectivity over other ARs.

Additions at the 2 position of NECA often increased potency and/or selectivity. For instance, 2-(3-hydroxy-3-phenyl)propyn-1-yl-NECA **27** (PHPNECA) (Fig. 2) exhibited a subnanomolar affinity at the A_3 receptor (Volpini et al. 2002). Also, Zhu et al. (2006) made a series of N^6-ethyl-2-alkynyl-NECA derivatives which had sub- to low nanomolar affinities and were very selective in comparison to the A_{2A} and A_{2B} ARs, with some selectivity over the A_1AR. The most potent compound in that series (**28**) had a (*p*-(methoxy)phenyl)alkynyl substituent at the 2 position.

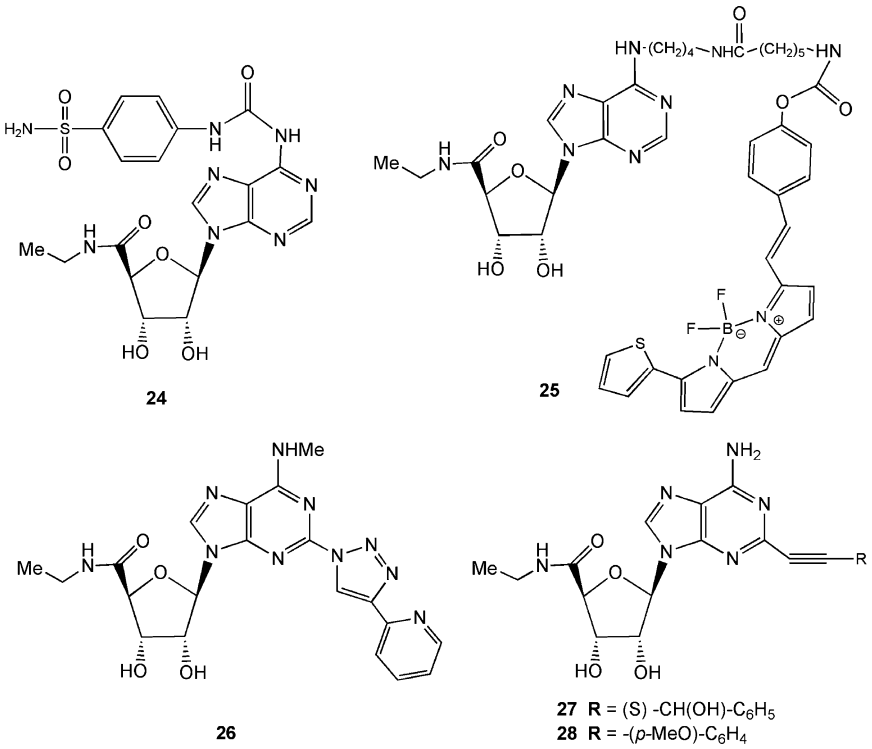


Fig. 2 Structures of a novel, multiply substituted A_3AR agonists

2.2 Ribose Modifications

2.2.1 Modification of Ribose Hydroxyl Groups

Many modifications have been made to the ribose ring. As mentioned above, the 5′-*N*-alkyluronamide modification has been particularly fruitful. Gallo-Rodriguez et al. (1994) initially found that adding a 5′-*N*-methyluronamide group to N^6-benzyl derivatives increased the binding affinity at all three ARs examined and resulted in several of the compounds gaining selectivity for the A_3AR. They also found that adding a 5′-*N*-ethyluronamide more than doubled the potency of several N^6-benzyl derivatives of adenosine. Other modifications at the ribose 5′ position, such as alkylthioethers (van Tilburg et al. 2002) have been found to modulate affinity and efficacy at the A_3AR.

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

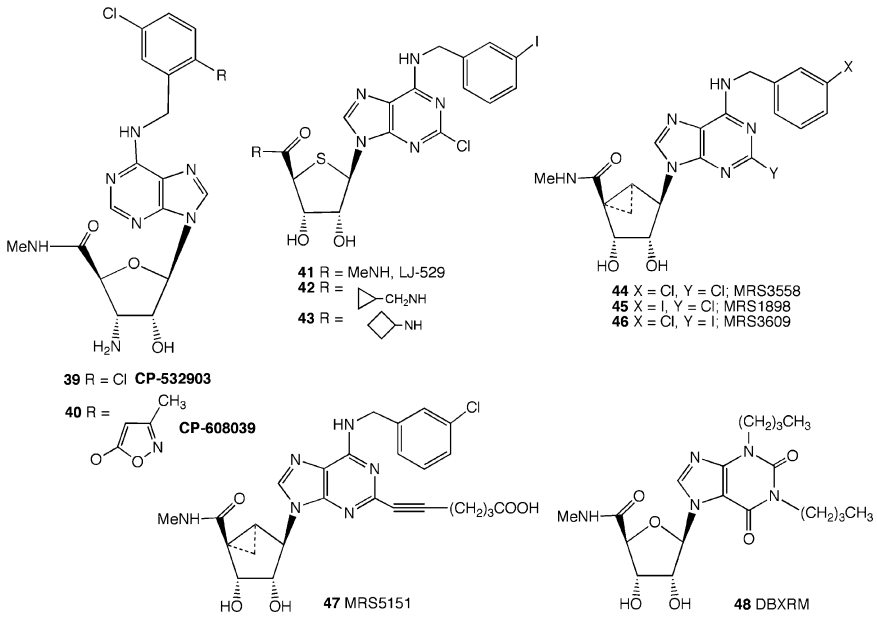


Fig. 3 Structures of ribose ring-modified selective A_3AR agonist probes

was added between the 3′-amino and ribose groups, there was a total loss of affinity (Van Rompaey et al. 2005). Also, 3′-deoxy-3′-acetylamino analogs were weak at the A_3AR. However, DeNinno et al. (2003, 2006) found that the 3′-amino substitution was tolerated and gave high selectivity when the 5′ and N^6 positions of adenosine were also appropriately modified in compounds **39** and **40** (Fig. 3). Replacement of the 3′-hydroxyl with an azido group generally abolished A_3AR activation. The 2′-hydroxyl group appeared to be more important than the 3′-hydroxyl group, because when it was replaced with the fluoro group there was no binding or activation of the A_3AR (Gao et al. 2004).

2.2.2 Modification of the Pentose Ring

4′-Thio derivatives were usually equipotent or slightly more potent at ARs than their oxygen equivalents (Jeong et al. 2006a). Many 4′-thio derivatives of adenosine have been found to be full agonists. For example, LJ-529 **41** (2-chloro-N^6-(3-iodobenzyl)-4′-thioadenosine-5′-methyluronamide) (Fig. 3) is a highly potent ligand (K_i = 0.38 nM against [^{125}I]–AB–MECA binding to the human A_3AR expressed in CHO cells). In the same 4′-thio-modified series, a wide variety of ribose 5′-alkyluronamides have shown that there is tolerance for groups larger than *N*-ethyl (Jeong et al. 2006a). For example, compounds **42** and **43** were full agonists with K_i values of 3.6 and 18 nM at the hA_3AR, respectively. The nature of the *N*-alkyl or *N*-arylalkyl group can modulate affinity and efficacy at the A_3AR (Jeong et al. 2008).

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

Ring-constrained nucleosides have been used to define conformational preferences at the A_3AR. Medicinal chemists frequently utilize the approach of conformationally constraining otherwise flexible molecules to probe the "active" conformation(s) and to increase ligand affinity by overcoming the energy barriers needed to attain this preferred conformation. Nucleoside analogs containing novel rigid ring systems in place of the ribose ring have been explored as ligands for the ARs. The focus on conformational factors of the ribose or ribose-like moiety allows the introduction of general modifications that lead to enhanced potency and selectivity at certain subtypes of these receptors. One ring system selected for this purpose is the methanocarba (bicyclo[3.1.0]hexane) ring system, which has been incorporated in either of two isomeric forms that adopt either a North (N) or South (S) envelope conformation (Jacobson et al. 2000; Marquez et al. 1996). These ribose modifications were combined with known enhancing modifications at other positions on the molecule to explore the resulting SARs. (N)-Methanocarba-adenosine was favored in binding at the A_3AR by 150-fold over the (S) conformation and by 2.5-fold over adenosine. Doubly modified nucleoside derivatives containing the (N)-methanocarba ring system have confirmed that this conformation of the pseudoribose ring is highly preferred over the (S) conformation for agonists at the A_3AR in general.

Introducing an (N)-methanocarba modification to adenosine 5′-ethyluronamide increased the human A_3AR binding affinity by sixfold. This modification also demonstrated that the ring oxygen is not required for binding or activation of the receptor (Lee et al. 2001).

Highly selective ring-constrained agonists of the A_3AR have been designed and synthesized based on the (N)-methanocarba ring system (Fig. 3). This led to the introduction of MRS3558 **44** ((1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4-{2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo-[3.1.0]-hexane-2,3-diol) as a full agonist with subnanomolar potency at the A_3AR and its congeners (e.g., **45** and **46**) as full agonists with nanomolar potency at the A_3AR (Tchilibon et al. 2005). The SAR of MRS3558 and related congeners as A_3AR agonists (Melman et al. 2008a) was recently explored in detail. The utility of MRS3558 in treating lung injury was shown in a model of ischemia reperfusion lung injury (Matot et al. 2006). In this series of (N)-methanocarba nucleosides, a 5′-uronamide moiety is needed in order to achieve full efficacy at the A_3AR. The corresponding 5′-alcohol is an antagonist of the A_3AR. The 5′-uronamide moiety overcomes the loss of efficacy associated with substitution of the N^6 and ribose ring moieties. Thus, in the (N)-methanocarba series, as in the ribose series, a freely rotating 5′-uronamide that is able to make and break multiple hydrogen bonds provides a necessary degree of flexibility during the receptor activation step.

2.3 Nonadenine Nucleosides and Nonnucleosides as A_3AR Agonists

Occasionally, nonadenine nucleotides are also found to activate the A_3AR. For instance, xanthines such as caffeine are generally found to act as antagonists, but *N*-methyl-1,3-dibutylxanthine 7-β-D-ribofuronamide **48** (DBXRM) acted as a moderately selective A_3AR agonist (Kim et al. 1994b).

A series of atypical, nonnucleoside agonist ligands that activated various ARs were reported (Chang et al. 2005). In addition to compounds in this family of pyridine-3,5-dicarbonitriles that were selective agonists of the A_1AR, various members of this series substantially activated the A_3AR.

2.4 Further Optimization of A_3AR Agonists Using Multiple Modifications

Interestingly, certain modifications (such as a 5′-alkylamide or an N^6-methyl group) can restore efficacy to previously modified compounds. For instance, adding a 2-chloro group to N^6-cyclopentyladenosine creates an A_3AR antagonist (Gao et al. 2002), but activation is restored by the 5′-methylcarboxamide and 4-thio substitutions. This is particularly interesting since 4′-thioadenosine is also an A_3AR antagonist, and 2-chloro-4-thioadenosine is only a partial agonist (Jeong et al. 2006b).

A series of (N)-methanocarba-2,N^6-disubstituted adenine nucleosides were made by Tchilibon et al. (2004), who found that adding the (N)-methanocarba, 2-chloro, and 5′-methyluronamido groups significantly improved the selectivity and efficacy of several compounds. For instance, N^6-(2,2-diphenylethyl)adenosine was an A_3AR antagonist with 12-fold and 130-fold selectivity over A_1 and A_{2A} ARs, respectively. However, by adding the above substitutions, the compound became a full agonist with a K_i of 0.69 nM and a selectivity of close to 2,000-fold over A_1 and A_{2A} AR (Tchilibon et al. 2004). Also, the 2-cyano derivative of N^6-methyl adenosine was a full agonist whereas the 2-cyano derivative of N^6-(2-phenylcyclopropyl) adenosine was an A_3AR antagonist (Ohno et al. 2004).

Adding several substitutions may also improve selectivity for the A_3AR. Adding an N^6-methyl group and 2-chloro group to 4′-thioadenosine-5′-methyluronamide created a compound with a K_i of 0.28 nM and a nearly 5,000-fold selectivity for the A_3AR (Jeong et al. 2006a). A series of these compounds was made by varying the N^6 and 5′ groups. While none of these derivatives could match the potency and selectivity of the original compound, it was found that 4′-thioadenosine derivatives were often more potent than their oxy counterparts. The most potent compound was 9-(3-amino-3-deoxy-5-methylcarbamoyl-β-D-ribofuranosyl)-2-amino-N^6-methylpurine. Another highly substituted yet extremely potent N^6-methyl derivative is 2-chloro-N^6-methyl-4-thioadenosine-5-methyluronamide,

which has a K_i of 0.28 nM (Jeong et al. 2006a). N^6-Methylation also seems to improve human A_3AR selectivity, as N^6-methyl-2-(2-phenylethyl)-adenosine is much more selective than 2-(2-phenylethyl)-NECA (Volpini et al. 2002). While large 2-position substitutions are not always tolerated, (2*R*,3*S*,4*R*)-tetra hydro-2-(hydroxymethyl)-5-(6-(methylamino)-2-(4-pyridin-2-yl)-1*H*-pyrazol-1-yl) -9*H*-purin-9-yl) furan-3,4-diol had a K_i of 2 nM and was extremely selective (Van Rompaey et al. 2005).

Recently, new potent and A_3-selective N^6,2-disubstituted adenosine derivatives have been reported. Volpini et al. (2007) made a series of N^6-methoxy-2-alkyladenosine derivatives, of which N^6-methoxy-2-*p*-acetylphenylethylMECA was the most potent and selective. This compound had a K_i of 2.5 nM at the human A_3AR and selectivities of 21,000 and 4,200 against A_1 and A_{2A} ARs, respectively. Recently, a series of water-soluble A_3AR agonists were synthesized (DeNinno et al. 2006). Of these compounds, (2*S*,3*S*,4*R*,5*R*)-3-amino-5-{6-[5-chloro-2-(2-oxo-2-piperazin-1-yl-ethoxy)-benzylamino]-purin-9-yl}-4-hydroxy-tetrahydro-furan-2-carboxylic acid methylamide was the most potent/selective derivative, with a K_i of 10 nM. Van Rompaey et al. (2005) found that adding additional substitutions to 3-amino-3-deoxyadenosine increased the potency, but these compounds were only partial agonists. 9-[3-Amino-3-deoxy-5-(methylcarbamoyl)-β-D-ribofuranosyl]-N^6-(5-chloro2-methoxybenzyl)adenine had a K_i of 27 nM and was extremely selective for the A_3AR, but had an efficacy of only 51%. Cosyn et al. (2006a) made a series of 3′-amino-3′-deoxy congeners that were highly selective for the A_3AR.

The selectivity at the mouse A_3AR of analogs containing the (N)-methanocarba ring system was reduced due to an increased tolerance of this ring system at the mouse A_1AR (Melman et al. 2008a). Substitution of the 2-chloro atom with iodo or hydrophobic alkynyl groups tended to increase the A_3AR selectivity (up to 430-fold) in mouse and preserve it in human. Extended and chemically functionalized alkynyl chains attached at the C2 position of the purine moiety preserved A_3AR selectivity more effectively than similar chains attached at the 3 position of the N^6-benzyl group. For example, the carboxylic acid congener MRS5151 **47** (Fig. 3) is a highly potent agonist (K_i 2.38 nM at hA_3AR) and is selective in binding at human (6,260-fold) and mouse (431-fold) A_3ARs in comparison to A_1ARs in the same species.

3 A_3AR Antagonists

Initial attempts at obtaining potent and highly selective A_3AR antagonists focused on wide pharmacological screening of different heterocyclic compounds (Jacobson et al. 1995; Ji et al. 1996; Siddiqi et al. 1995). One of the first nonxanthine heterocyclic derivatives (Fig. 4) found to be selective for the human A_3AR (K_i 0.65 nM) was MRS1220 (*N*-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-yl]benzeneacetamide) **49**, which was based on appropriate acylation of the exocyclic amino group of this class of known AR antagonists (Kim et al.

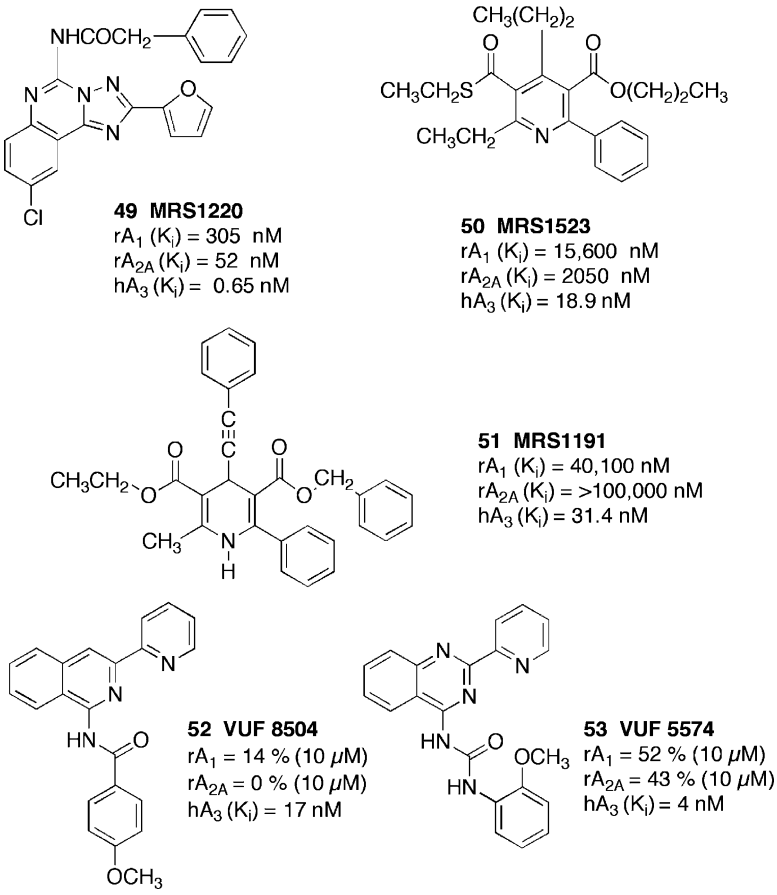


Fig. 4 Structures of heterocyclic derivatives that are widely used as selective human A_3AR antagonists

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

3.1 Recent Developments in Nonpurine Heterocycles

3.1.1 Thiazole and Thiadiazole

IJzerman and coworkers investigated a series of 3-(2-pyridinyl)-isoquinoline derivatives for their affinity at the A_3AR (Van Muijlwijk-Koezen et al. 2000). The effect of an additional nitrogen atom was valued by synthesizing bioisosteric quinazoline derivatives. The compounds VUF 8504 (4-methoxy-*N*-(3-(2-pyridinyl)-1-isoquinolinyl)benzamide, **52**) and VUF 5574, (*N*-(2-methoxyphenyl)-*N′*-(2-(3-pyridyl)quinazolin-4-yl)urea, **53**) (Fig. 4) display considerable A_3AR affinity and appreciable selectivity versus A_1 and A_{2A} AR subtypes.

The bicyclic system of isoquinoline and quinazoline has been replaced by several monocyclic rings (Van Muijlwijk-Koezen et al. 2001). Some thiazole and thiadiazole derivatives were shown to be most promising candidates for the identification of new A_3AR ligands.

The derivative *N*-[3-(4-methoxy-phenyl)-[1,2,4]thiadiazol-5-yl]-acetamide (**54**, Fig. 5) has been claimed to be the most potent A_3AR antagonist of the series, exhibiting a K_i value of 0.79 nM at hA_3AR and antagonistic properties in a cAMP functional assay (Jung et al. 2004). A series of potent and selective A_3AR antagonists have been obtained via an optimization study of compound **55** that revealed that a 5-(pyridine-4-yl) moiety on the 2-aminothiazole ring was optimal for enhanced receptor potency and selectivity (Press et al. 2004). Of particular note, *N*-[4-(3,4,5-trimethoxyphenyl)-5-pyridin-4-ylthiazol-2-yl]-acetamide **56** showed subnanomolar affinity at the human A_3AR as a competitive antagonist of [^{125}I]I–AB–MECA, binding with 1,000-fold selectivity versus the other ARs.

Binding affinity data on thiazole and thiadiazole derivatives at the hA_3AR have been subjected to QSAR analysis (Bhattacharya et al. 2005). This study disclosed the importance of the molecular electrostatic potential surface (Wang–Ford charges)

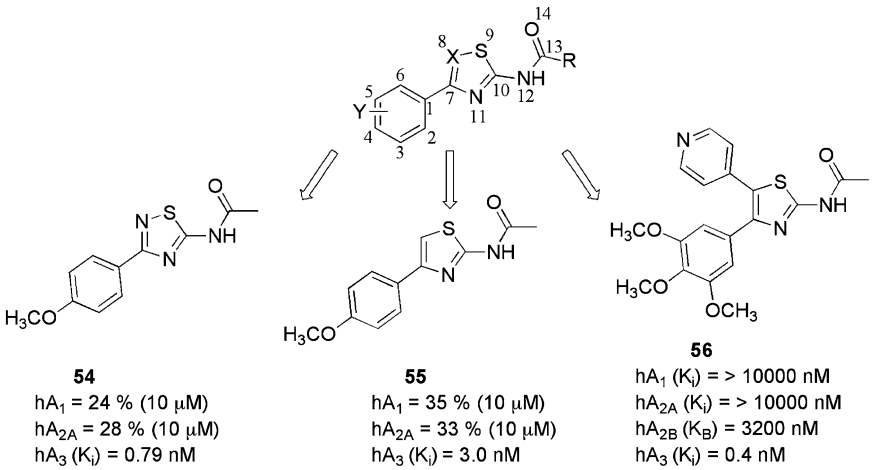


Fig. 5 Thiazole and thiadiazole derivatives as human A_3AR antagonists

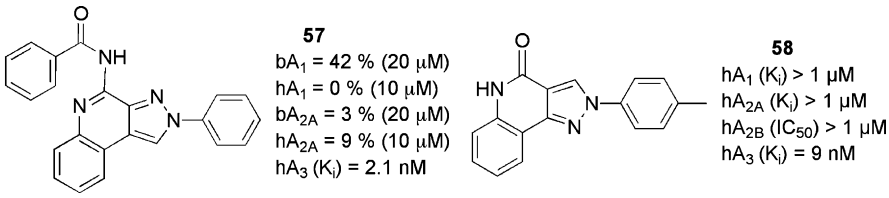


Fig. 6 Pyrazoloquinoline derivatives as human A_3AR antagonists

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

3.1.2 Pyrazoloquinolines

The binding affinities at bovine A_1 and A_{2A} ARs and at human cloned A_3ARs of some 2-arylpyrazolo[3,4-*c*]quinolin-4-ones along with their corresponding 4-amines and 4-substituted-amino derivatives were reported by Colotta et al. (2000). The 4-benzoylamido derivative **57** (Fig. 6) displayed one of the best binding profiles of the series of A_3AR antagonists. The same group recently reported an extension of the SAR study of this class of compounds (Colotta et al. 2007) which highlighted that bulky and lipophilic acyl–amino groups at the 4 position seemed able to promote hA_3AR potency and selectivity. Selected compounds of these series were tested in an in vitro rat model of cerebral ischemia and prevented the irreversible failure of synaptic activity induced by oxygen and glucose deficiency in the hippocampus, thus confirming that potent and selective A_3AR antagonists may substantially increase the tissue resistance to ischemic damage.

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

3.1.3 Triazoloquinoxalines

Triazolo[4,3-*a*]quinoxalines

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

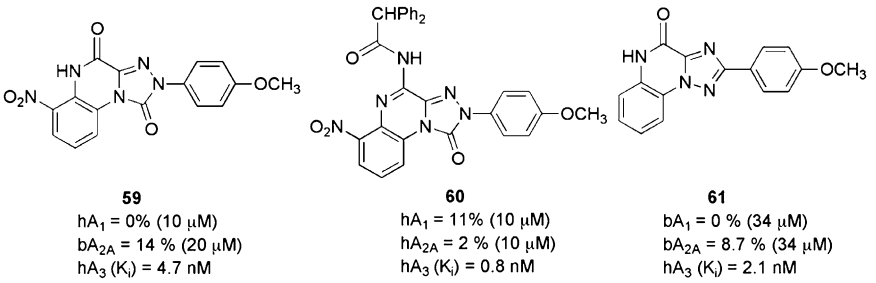


Fig. 7 Triazoloquinoxaline derivatives as A_3AR antagonists

scaffold for obtaining potent and selective hA_3AR antagonists (Colotta et al. 2004; Lenzi et al. 2006). Intensive efforts in the chemical synthesis of compounds based on the systematic substitution of the 2, 4 and 6 positions of the tricyclic template, along with molecular modeling investigations performed to rationalize the experimental SAR findings, led to the identification of optimal structural requirements for A_3AR affinity and selectivity. In particular, the introduction into the triazoloquinoxaline moiety of a 4-oxo (**59**) or a 4-*N*-amido (**60**, Fig. 7) function affords selective and/or potent A_3AR antagonists, indicating that a C = O group (either extranuclear or nuclear) is necessary for A_3AR affinity. This suggested that the probable engagement of this site of the molecule is a hydrogen bond with the A_3AR binding site. Hindering and lipophilic acyl–amino moieties at the 4 position showed enhanced A_3AR affinity (**60**). Substitution of the 4 position of the 2-phenyl ring with a methoxy or a nitro group and 6-nitro substitution, as well as the combination of these substituents, afforded nanomolar A_3AR affinity and better A_3AR selectivity. 1-Oxo, 6-nitro, and 4-amino groups have been proposed to be involved in hydrogen bonds that anchor the antagonists to the binding site.

Triazolo[1,5-*a*]quinoxalines

Some 2-aryl-8-chloro-1,2,4-triazolo[1,5-*a*]quinoxaline derivatives have been synthesized and tested in radioligand binding assays at bovine (b) A_1 and bA_{2A}ARs and at hA_1 and hA_3ARs (Catarzi et al. 2005a, b). The SAR of these compounds are in agreement with those of previously reported for 2-aryl-1,2,4-triazolo[4,3-*a*]quinoxalines and 2-arylpyrazolo[3,4/4,3-c]quinolines, thus suggesting a similar AR-binding mode. These studies provided some interesting compounds; among them, 2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one (**61**, Fig. 7) is the most potent and selective hA_3AR antagonist of this series.

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

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

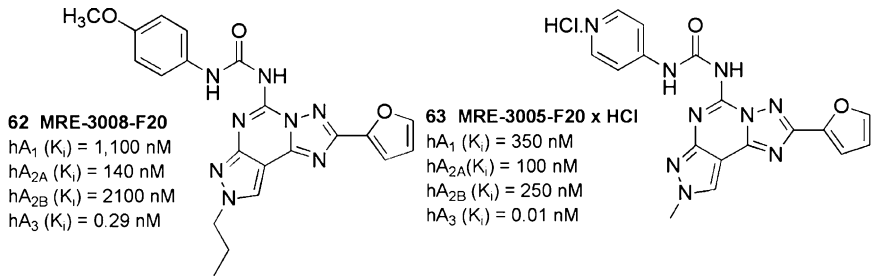


Fig. 8 A_3AR antagonists based on a pyrazolo-triazolo-pyrimidine scaffold

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

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

In order to rationally design and synthesize hA_3AR antagonists with improved binding and/or absorption, distribution, metabolism, and excretion (ADME) profiles, and as suitable clinical candidates, different molecular modeling investigations have been carried out in the last years. Particular attention has been paid to the pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine family, the most potent class of A_3AR antagonists ever reported (Tafi et al. 2006). A combined target-based (high-throughput molecular docking) and ligand-based (CoMFA) (comparative molecular field analysis) drug design approach has recently been performed by Moro and coworkers (Moro et al. 2005), which defined a novel "Y-shaped" binding motif for pyrazolo-triazolo-pyrimidines and rationally delineated some key ligand–receptor interactions for this class of molecules as follows: (1) steric control around the 3 and 4 positions of the N^5-phenyl ring justifies the decrease in affinity of 3- or 4-substituted-phenyl derivatives; (2) an important $\pi-\pi$ interaction takes place between the 2-furyl ring and two phenylalanine residues of the binding site; (3) a hydrophobic pocket, bordered by two hydrophilic amino acids, surrounds the N8 interaction area; and (4) strong hydrogen bonding is possible between a residue of Asn and the N4 of the triazolo ring.

3.1.5 Various Heterocycles

In the last few years, other classes of heterocyclic compounds have been identified as A_3AR antagonists, but large structural dissimilarities meant that none of these could be classified into particular family groups. The quinoxaline derivative **64** (Fig. 9) deserves to be mentioned here, not only because of its good binding profile as an A_3AR antagonist, but also (especially) due to the novelty of the strategy applied to its design, which was based on a 3D database-searching approach (Novellino et al. 2005). There is increasing evidence of the importance of 2D/3D database searching as a valuable tool to discover novel lead compounds for the A_3AR and for other G-protein-coupled receptors (GPCRs) (Costanzi et al. 2008).

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

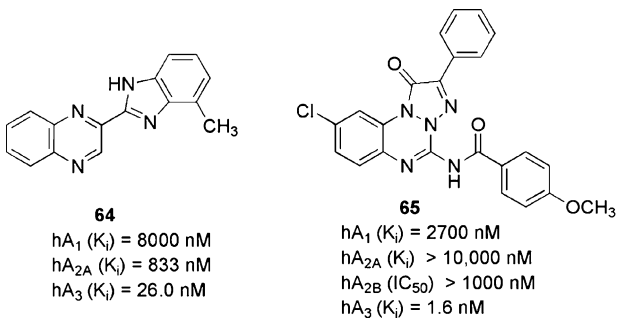


Fig. 9 A_3AR antagonists based on quinoxaline and triazolobenzotriazinone scaffolds

and coworkers to the identification of a series of aminophenyltriazolobenzotriazinones. Among these, compound **65**, a result of a systematic lead optimization, stands out for its remarkable potency and selectivity at the A_3AR (K_i values at the A_1, A_{2A}, A_3ARs of 2,700 > 10,000, 1.6 nM, respectively, and IC_{50} value from cAMP assay at the A_{2B} > 1,000 nM) (Da Settimo et al. 2007). Interestingly, the triazolobenzotriazinone nucleus is isomeric with that of the triazoloquinoxalinone series described above (compounds **59**–**61**, Fig. 7).

3.2 Purine Derivatives

3.2.1 Adenines

The first class of A_3AR-selective antagonists with a bicyclic structure strictly correlated to the adenine nucleus was claimed in 2005 by Biagi and coworkers (Biagi et al. 2005). The authors described the synthesis of a series of N^6-ureidosubstituted-2-phenyl-9-benzyl-8-azaadenines whose adenine-like structure was responsible for the antagonist activity and whose phenylcarbamoyl group ensures selectivity at the A_3AR. The structure–activity relationship studies were performed based on the systematic optimization of substituents at the 2, 6 and 9 positions of the bicyclic scaffold, and led to the desired enhancement of A_1/A_3 selectivity (compound **66**, Fig. 10).

Basing on the finding that the known differentiation agent "reversine" (2-(4-morpholinoanilino)-N^6-cyclohexyladenine) exerted a moderate antagonist activity at the hA_3AR (K_i value of 0.66 μM), Jacobson and coworkers developed a series of reversine analogs, focusing their attention on the substitution pattern at the 2 and N^6 positions of the adenine scaffold (Perreira et al. 2005). One of most interesting compounds in terms of hA_3AR affinity and selectivity, MRS3777, (2-(phenyloxy)-N^6-cyclohexyladenine, **67**), combines the N^6-cyclohexyl moiety of reversine with a 2-phenyloxy group. A few derivatives tested in binding assays to the rat A_3AR seemed to reflect the species dependence of the affinity typical of most known nonnucleoside A_3AR antagonists, and were shown to be inactive at 10 μM in this species.

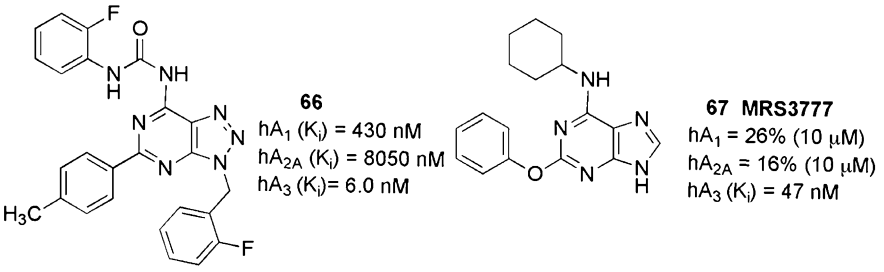
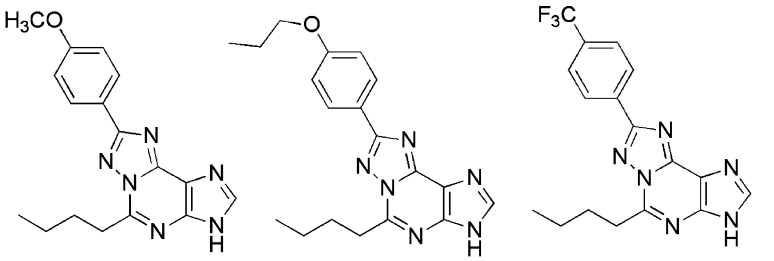


Fig. 10 A_3AR antagonists based on nonnucleoside adenine scaffolds



68
hA_1 (K_i) = 398 nM
hA_{2A} (K_i) = 892 nM
hA_{2B} (K_i) = 1030 nM
hA_3 (K_i) = 0.18 nM
hA_3 (IC_{50}) < 0.1 nM

69
hA_1 = 32 % (10 μM)
hA_{2A} = 49 % (10 μM)
hA_{2B} = 21 % (10 μM)
hA_3 (K_i) = 0.51 nM
hA_3 (IC_{50}) = 0.3 nM

70 OT-7999
hA_1 = 4 % (10 μM)
hA_{2A} = 31 % (10 μM)
hA_{2B} = 6 % (10 μM)
hA_3 (K_i) = 0.95 nM
hA_3 (IC_{50}) = 0.61 nM

Fig. 11 A_3AR antagonists based on triazolopurine scaffolds

3.2.2 Triazolopurines

Okamura et al. (Okamura et al. 2002, 2004a) recently reported the study of a new series of 1,2,4-triazolo[5,1-*i*]purines. This research group highlighted the structural similarity between the new class of compounds and the triazoloquinazoline derivatives and consequently evaluated the corresponding A_3AR affinities. These investigations led to potent and selective hA_3AR ligands, the most potent of which are reported in Fig. 11 (**68,69**). In particular, 5-*n*-butyl-8-(4-*n*-propoxyphenyl)-3*H*-[1,2,4]triazolo[5,1-*i*]purine (**69**) exhibited the best selectivity profile of this series (affinity ratios vs. other AR subtypes > 19,600). Compound (**70**), 5-*n*-butyl-8-(4-trifluoromethylphenyl)-3*H*-[1,2,4]triazolo-[5,1-*i*]purine (OT-7999), significantly reduced intraocular pressure in cynomolgus monkeys at 2–4 h following topical application (500 mcg) (Okamura et al. 2004b).

3.2.3 Tricyclic Xanthines

Natural antagonists for ARs such as caffeine and theophylline show, in general, low affinity for the A_3AR subtype (Baraldi et al. 2003a; van Galen et al. 1994). In a recent study, the approach based on the ring annelation of xanthine derivatives for the development of AR antagonists was considered in depth (Drabczyńska et al. 2003).

Some pyrido[2,1-*f*]purine-2,4-dione derivatives, which could be considered tricyclic xanthine derivatives, have been reported to exert subnanomolar affinity at the hA_3AR (Priego et al. 2002). The most potent compound of this recent series is the 1-benzyl-3-propyl-1*H*, 3*H*-pyrido[2,1-*f*]purine-2,4-dione derivative (**71**, Fig. 12), which presents a K_i value of 4.0 ± 0.3 nM at hA_3AR. The replacement of the benzyl nucleus at the 1 position with a methyl moiety caused dramatic losses of

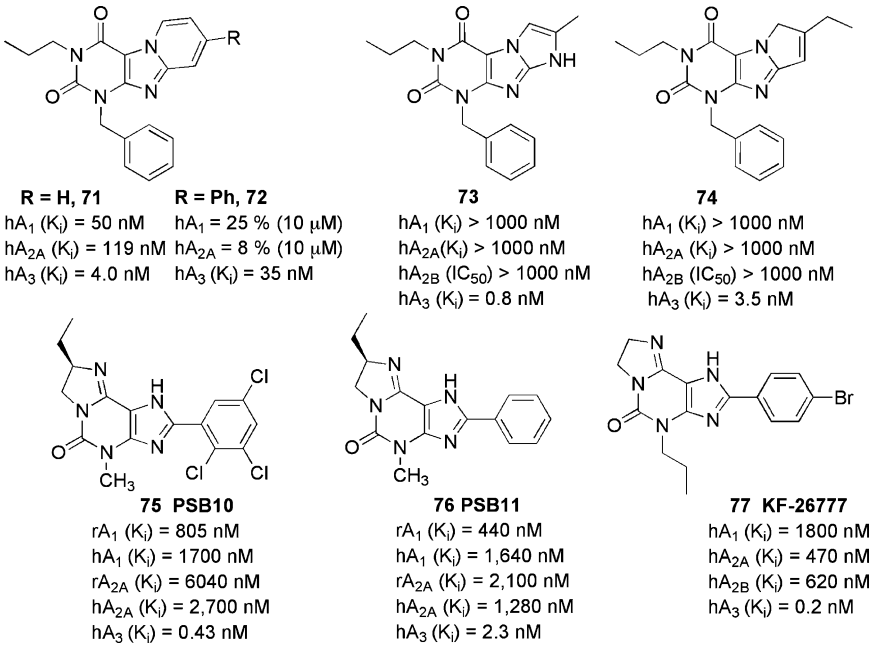


Fig. 12 A_3AR antagonists based on tricyclic xanthine scaffolds

both affinity and selectivity. The effect of the replacement of the pyridine ring of the pyrido[2,1-*f*]purine-2,4-dione core with different five-membered heterocycles was examined. In particular, the synthesis and the SAR profile at the ARs of a series of 1-benzyl-3-propyl-7-aryl/alkyl-1*H*,6*H*-pyrrolo[2,1-*f*]purine-2,4-dione and 1-benzyl-3-propyl-7-aryl/alkyl-1*H*,8*H*-imidazo[2,1-*f*]purine-2,4-dione derivatives were recently reported (Baraldi et al. 2005b). Among the examined tricycles, the imidazo[2,1-*f*]purine-2,4-dione derivatives were two- to tenfold more potent than the corresponding pyrrolo[2,1-*f*]purine-2,4-dione derivatives. The best results were obtained with the introduction of small alkyl chains at the 7 position (1-benzyl-7-methyl-3-propyl-1*H*,8*H*-imidazo[2,1-*f*]purine-2,4-dione **73**, 1-benzyl-7-ethyl-3-propyl-1*H*,6*H*-pyrrolo[2,1-*f*]purine-2,4-dione **74**, Fig. 12). Compound **73** shows a subnanomolar affinity towards the target A_3AR, with noteworthy selectivity with respect to the other AR subtypes (K_i (hA$_3$) = 0.8 nM, K_i (hA$_1$/hA$_3$) = 3, 163, K_i (hA$_{2A}$/hA$_3$) > 6,250, IC_{50} (hA$_{2B}$)/K_i (hA$_3$) = 2,570).

The synthesis and biological evaluation of a series of fused xanthine derivatives was investigated by Müller and coworkers (Müller et al. 2002a). In particular, the (*R*)-4-methyl-8-ethyl-2-phenyl-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-one (PSB-11 (**76**), Fig. 12) exhibited a K_i value of 2.3 nM for the A_3AR and good selectivity vs. all other AR subtypes. The radiolabeled derivative of this compound ([^{3}H]PSB-11) exhibited a K_d value of 4.9 nM and a B_{max} value of 3,500 fmol mg^{-1} of protein in human A_3AR binding in transfected CHO cells (Müller et al. 2002b). An important innovation of such a series, in comparison with

xanthines, is a significant increase in water solubility due to the introduction of a basic nitrogen atom, which can be protonated in physiological conditions. Compound PSB-10, bearing a 2,3,5-trichlorophenyl moiety at the 2 position, showed inverse agonist activity in binding studies in CHO cells expressing recombinant hA_3ARs ($IC_{50} = 4$ nM) (Ozola et al. 2003). The 2-(4-bromophenyl)-derivative named KF-26777 (**77**) with subnanomolar affinity at the hA_3AR ($K_i = 0.2$ nM) and high selectivity over A_1, A_{2A} and A_{2B} ARs (9,000-, 23,500-, 31,000-fold, respectively) was considered a potential lead molecule for development for the treatment of brain ischemia and inflammatory diseases such as asthma (Saki et al. 2002).

3.3 Nucleoside-Derived A_3AR Antagonists

Based on the observation that the relative efficacy of purine nucleosides depends on structural features (see Sect. 2), new subtype-selective nucleoside antagonists of the A_3AR have been designed. One of the first such antagonists was the rigid spirolactam MRS1292 (**78**) (Fig. 13, (2*R*,3*R*,4*S*,5*S*)-2-[N^6-3-iodobenzyl)adenos-9′-yl]-7-aza-1-oxa-6-oxospiro[4.4]-nonan-4,5-diol) (Gao et al. 2002), which binds potently and selectively to the rat and human A_3ARs but does not activate these receptors, and thus acts as an antagonist.

Modeling/mutagenesis of ARs has focused on distinct residues related to ligand binding and the relative efficacy of adenosine derivatives, and on a conserved Trp residue (6.48) which is involved in the activation process (termed a "rotamer switch," Shi et al. 2002). Docking studies of agonists suggest that the activation pathway of the A_3AR involves a characteristic anticlockwise rotation of this residue, as viewed from the exofacial side (Kim et al. 2006). The docking of MRS1292 (**78**) to the A_3AR model is not accompanied by rotation of this residue, as occurs with nucleoside agonists, consistent with its action as an antagonist (Kim et al. 2006). Moreover, the affinity and selectivity of MRS1292 occurs across species, unlike most other heterocyclic antagonists for the A_3AR reported. This allows its use in nonprimate (e.g., murine) experimental animals used as clinical models. For example, MRS1292 applied directly to the eye in mouse has been shown to be effective in reducing intraocular pressure, which may be predictive of its utility as an antiglaucoma agent (Yang et al. 2005).

The removal of the ability of the 5′-*N*-alkyluronamide to donate a hydrogen bond was found to convert agonists into selective antagonists (Gao et al. 2006a). In both the 4′-oxo and the 4′-thio series, *N*-methylation of an *N*-methylamide (i.e., to form a dimethylamide) resulted in potent and selective A_3AR antagonists. Recently, nucleosides that are truncated at the 4′ position were found to act as A_3AR antagonists. For example, (2*R*, 3*R*, 4*S*)-2-(2-chloro-6-(3-chlorobenzylamino)-9*H*-purin-9-yl)tetrahydrothiophene-3,4-diol (LJ-1416, **80**) and (2*R*, 3*R*, 4*S*)-2- (2-chloro-6-(3-iodobenzylamino)-9*H*-purin-9-yl)tetrahydrothiophene-3,4-diol (LJ-1251, **81**) (Fig. 13) (Jeong et al. 2007) displayed K_i values of 1.66 and 4.16 nM, respectively, at the human A_3AR,

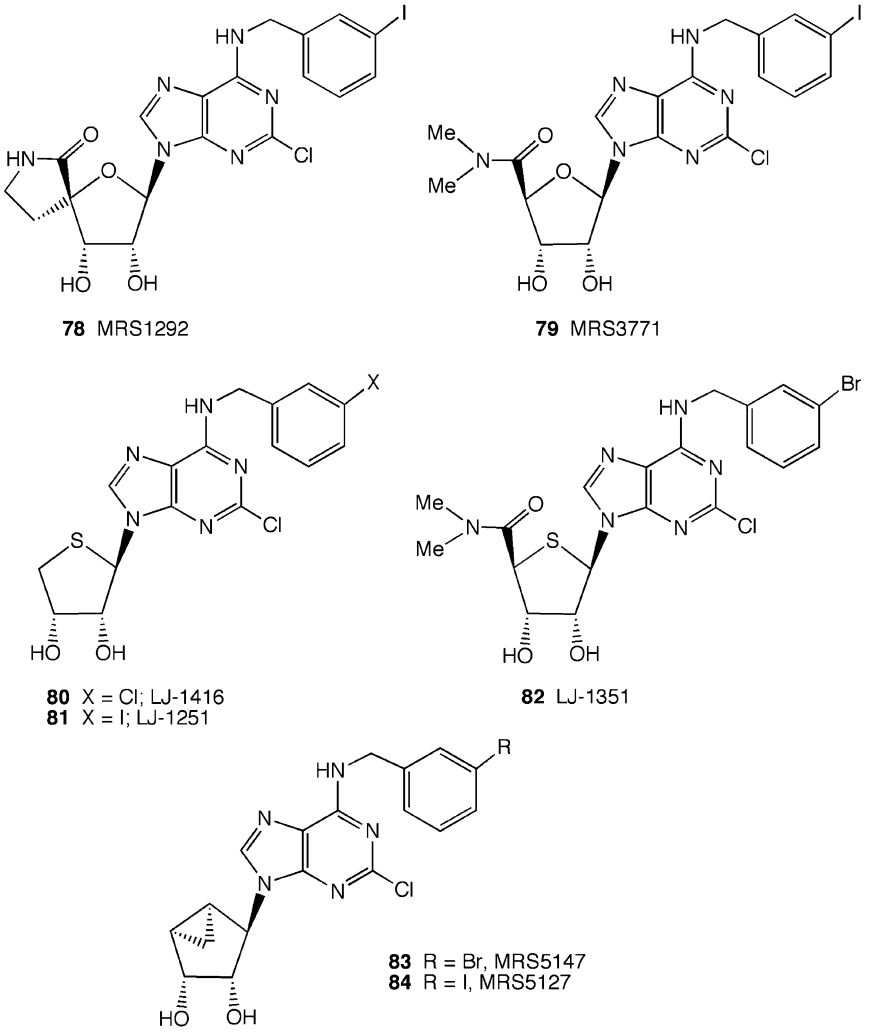


Fig. 13 A_3AR antagonists based on nucleoside scaffolds

with >600-fold selectivity in comparison to the A_1AR. LJ-1251 was shown to have neuroprotective properties in an ischemia model in the rat hippocampus (Pugliese et al. 2007). Truncation at the 4′ position of A_3AR agonist in the (N)-methanocarba series produces potent and selective A_3AR antagonists (Melman et al. 2008b), such as the 3-bromo derivative 1′*R*, 2′*R*, 3′*S*, 4′*R*, 5′*S*)-4′-[2-chloro-6-(3-bromobenzylamino)-purine]-2′, 3′-*O*-dihydroxybicyclo-[3.1.0]hexane (MRS5147) (**83**, Fig. 13) (2,900-fold selective for hA_3 vs. hA_1AR) or its 3-iodo analog, MRS5127 (**84**) (2,400-fold selective for hA_3 vs. hA_1AR). MRS5127 (**84**) displayed a K_B (Schild constant) value of 8.9 nM as an antagonist of the human A_3AR in a functional assay.

4 Engineering of the A_3AR to Avoid Side Effects of Conventional Synthetic Agonists

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

Since ARs are widespread in the body, in order to overcome inherent nonselectivity of activating the native ARs using synthetic agonists, we have introduced the concept of neoceptors, by which the putative ligand binding site of a 7TM receptor is re-engineered for activation by synthetic agonists (neoligands) that are built to have a structural complementarity. This is a molecular modeling approach to receptor engineering by which a mutant receptor (neoceptor) is designed for selective activation by a novel synthetic ligand (neoligand) at concentrations that do not activate the native receptor. An amino acid residue of the receptor and a functional group of the ligand moiety thought to be in close proximity can be modified in a complementary fashion so that the two groups exhibit a novel mode of interaction (e.g., reversing the polarity in a salt bridge or introducing unique hydrogen-bonding sites). If a stabilizing interaction exists between these two groups, an increase in affinity is expected at the mutant receptor relative to the native receptor. This strategy is intended for eventual use in gene therapy and may also be useful in mechanistic elucidation, using neoceptor–neoligand pairs that are pharmacologically orthogonal with respect to the native species. Neoceptors have so far been applied successfully to A_{2A} and A_3 ARs (Gao et al. 2006b; Jacobson et al. 2001, 2005). Compounds **85–87** (Fig. 14) were found to interact selectively with the H272E mutant hA_3AR. All three compounds activated this neoceptor.

5 Conclusions

A_3AR ligands have been modified to optimize their interaction with the A_3AR. Most of these modifications have been made to the N^6 and 2 positions of adenine as well as the ribose moiety, and using a combination of these substitutions leads to the most efficacious, selective, and potent ligands. A_3AR agonists such as IB–MECA and Cl–IB–MECA are now advancing into Phase II clinical trials for treatments targeting diseases such as cancer, arthritis, and psoriasis.

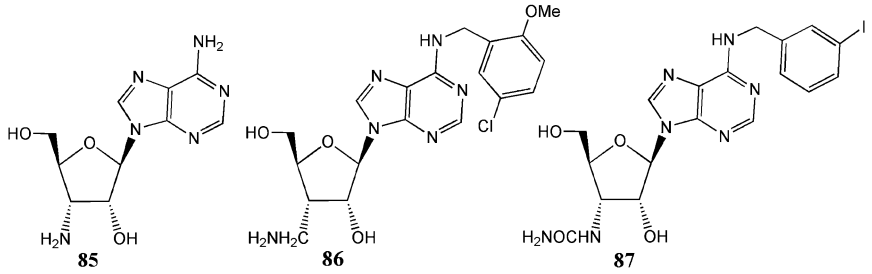


Fig. 14 Compounds that interact selectively with the H272E mutant hA_3AR neoceptor

Also, a wide number of compounds exerting high potency and selectivity in antagonizing the hA_3AR have been discovered. These molecules are generally characterized by a notable structural diversity, taking into account that aromatic nitrogen-containing monocyclic (thiazoles and thiadiazoles), bicyclic (isoquinoline, quinozalines, (aza)adenines), tricyclic systems (pyrazoloquinolines, triazoloquinoxalines, pyrazolotriazolopyrimidines, triazolopurines, tricyclic xanthines) and nucleoside derivatives have been identified as potent and selective A_3AR antagonists. Probably due to the "enigmatic" physiological role of A_3AR, whose activation may produce opposite effects (for example, concerning tissue protection in inflammatory and cancer cells) and may produce effects that are species dependent, only a few molecules have reached preclinical investigation. Indeed, the most advanced A_3AR antagonists remain in preclinical biological testing. Among the antagonists described above, compound OT-7999 is expected to enter clinical trials for the treatment of glaucoma, while several thiazole derivatives are in development as antiallergic, antiasthmatic and/or anti-inflammatory drugs.

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