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A3 Adenosine Receptors as Modulators of Inflammation: From Medicinal Chemistry to Therapy

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Abstract

The A_3 adenosine receptor (A_3AR) subtype is a novel, promising therapeutic target for inflammatory diseases, such as rheumatoid arthritis (RA) and psoriasis, as well as liver cancer. A_3AR is coupled to inhibition of adenylyl cyclase and regulation of mitogen-activated protein kinase (MAPK) pathways, leading to modulation of transcription. Furthermore, A_3AR affects functions of almost all immune cells and the proliferation of cancer cells. Numerous A_3AR agonists, partial agonists, antagonists, and allosteric modulators have been reported, and their structure–activity relationships (SARs) have been studied culminating in the development of potent and selective molecules with drug-like characteristics. The efficacy of nucleoside agonists may be suppressed to produce antagonists, by structural modification of the ribose moiety. Diverse classes of heterocycles have been discovered as selective A_3AR blockers, although with large species differences. Thus, as a result of intense basic research efforts, the outlook for development of A_3AR modulators for human therapeutics is encouraging. Two prototypical selective agonists, N6-(3-Iodobenzyl)adenosine-5′-N-methyluronamide (IB-MECA; CF101) and 2-chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (Cl-IB-MECA; CF102), have progressed to advanced clinical trials. They were found safe and well tolerated in all preclinical and human clinical studies and showed promising results, particularly in psoriasis and RA, where the A_3AR is both a promising therapeutic target and a biologically predictive marker, suggesting a personalized medicine approach. Targeting the A_3AR may pave the way for safe and efficacious treatments for patient populations affected by inflammatory diseases, cancer, and other conditions.

Keywords

A_3 adenosine receptor; inflammation; cancer; drug development; therapy

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1 INTRODUCTION

The relevance of adenosine in the immune system has been established based on mounting scientific evidence showing that the nucleoside represents a paracrine inhibitor of inflammation, regulating the onset, extension, and termination of the inflammatory process and acting through four G protein coupled receptors (GPCRs), designated as A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (ARs).[1] Following inflammation, metabolic alterations occur leading to an increase of extracellular adenosine that is present in the low nanomolar range under physiological conditions, while in stressful conditions it can rise to micromolar levels.[2] Adenosine in the extracellular milieu is largely formed by hydrolysis/dephosphorylation of ATP, ADP, and AMP through specific ectonucleotidases termed ectonucleoside triphosphate diphosphohydrolase (CD39) and ecto-5′-nucleotidase (CD73).[3,4] Intracellular levels of adenosine are derived from hydrolysis of AMP and *S*-adenosylhomocysteine (SAH) through cytosolic 5′-nucleotidase, and SAH hydrolase, respectively. Adenosine activity is extinguished through its phosphorylation to AMP by adenosine kinase (AK) or deamination to inosine by adenosine deaminases (ADA1 and ADA2), with ADA present also extracellularly.[2] The existence of concentrative nucleoside transporters (CNTs) and equilibrative nucleoside transporters (ENTs) regulates the extra- and intracellular adenosine concentrations.[5]

The A_3AR, the last of the four subtypes to be discovered, was cloned sequentially in rat, sheep, and human,[6–8] but it was not shown to respond as an AR from the outset. One of the first activities of this receptor to be reported was the induction of histamine from rat basophilic cells.[9] The discovery and initial characterization of the A_3AR, and the exploration of its biological paradoxes, has led to the synthesis and biological characterization of a multitude of receptor probe molecules and clinically relevant candidate molecules, including orthosteric agonists and antagonists as well as allosteric enhancers. This discovery of the A_3AR as a fourth AR has spawned current and projected clinical trials of several A_3AR agonists and potentially of a selective A_3AR allosteric enhancer, as well.[10]

Using mechanisms triggered by adenosine to inhibit the immune system is a very exciting area of research, and increasing attention is focused on their elucidation in the context of developing new anti-inflammatory strategies. Thus, today the A_3ARsubtype is considered a novel, very promising therapeutic target and predictive biological marker, given its overexpression in inflammatory and cancer cells, compared to low levels found in healthy cells.[11]

The aim of this review is to summarize the state and the progress of the field of A_3AR modulators and their clinical development in the context of inflammation and cancer and other conditions, with an emphasis on rheumatoid arthritis (RA), psoriasis, and hepatocellular carcinoma.

2 MOLECULAR BIOLOGY OF A_3AR

The A_3AR, the only AR subtype cloned before its pharmacological identification, was initially isolated from rat testis and then from a variety of species. The A_3AR structure had a sequence homology of only 74% in rat versus sheep and human, versus 85% between sheep and human, suggesting significant interspecies differences in ligand recognition. This is manifested in different pharmacological profiles of the species homologs, especially with respect to antagonist binding, which have made the characterization of this AR subtype difficult.[12] There are also species differences in the biological roles of the A_3AR, for example, as the main mechanism for adenosine–induced release of inflammatory mediators in rat mast cells, but not in those of human.[13]

The $A_3AR$is located on human chromosome 1p21-p13 and consists of a single chain of 318 amino acids.[14] The A_3AR gene presents two exons separated by a single intron of about 2.2 kb.[15] Its promoter region has putative binding sites for multiple transcription factors: The upstream sequence has a CCAAT sequence, as well as consensus binding sites for SP1, NF-IL6, GATA1, and GATA3 transcription factors, the latter of which is important for the A_3AR-dependent role in immune function. Two species of mRNA code for the hA_3AR (sizes 2 and ~5 kb). Variants of the A_3AR have been shown to be associated with coronary heart disease, autism spectrum disorder, and aspirin-induced urticaria.[16–18] Recently, the A_3AR 3′-UTR (untranslated region) of the mRNA was found to be targeted by the proinflammatory microRNA (miR-206) in ulcerative colitis leading to downregulation of A_3AR mRNA/protein expression in colon cells.[19] The A_3AR is expressed in diverse tissues at relatively low levels, compared to A_1AR and $A_{2A}AR$. Genomic analysis of the expression of the A_3AR gene in various human tissues (Table 1A) shows highest levels in testes, the spinal cord, and various brain regions, bladder, lung, adipose tissue, and whole blood. The highest expression reached 12.4 RPKM (reads per kilobase of transcript per million mapped reads), while comparable data for A_1AR and $A_{2A}AR$ and exceeded 20 RPKM at maximal levels in specific tissues. This suggests that potential use of A_3AR ligands in pain and other nervous system disorders is supported by the presence of the receptor in these tissues, although the cell type is not determined in this RNA sequencing data. Various cancer tumors also show major alteration in A_3AR expression in comparison to normal tissue. As accessed from a public cancer database (Table 1B), in 393 unique genomic analyses of cancerous tumors, 25 showed a significant increase in A_3AR ($P < 10^{-4}$) and 28 showed a significant decrease compared to normal tissue of the same type. The most prominent increases were in brain cancer (particularly glioblastoma and astrocytoma) and kidney cancer (particularly renal clear cell carcinoma). Thus, the approach of using of A_3AR ligands in a wide range of cancers coincides with significant changes in the receptor expression level in tumors.

As is common to the GPCR superfamily, the A_3AR is characterized by seven transmembrane (TM) domains and an intracellular C-terminal region, with Ser and Thr residues serving as potential phosphorylation sites relevant for rapid receptor desensitization. Following agonist stimulation, the A_3AR undergoes phosphorylation at the C-terminus by GPCR kinases and subsequent internalization through clathrin-coated pits.[20–24] Interestingly, by mutational studies, it has been reported that the highly conserved Trp (W6.48) in TM6 is essential for the active conformation of A_3AR necessary to trigger a series of intracellular pathways for signal transmission, to interact with β-arrestin2, and to undergo receptor internalization.[25] Furthermore, use of a novel fluorescent A_3AR agonist has allowed for the observation of colocalization with internalized receptor–arrestin complexes.[26]

The energetics of A_3AR ligand interactions has been studied using a thermodynamic approach. The thermodynamic parameters of ligand binding at all ARs are similar within either agonist or antagonist classes, which reflects a common ligand receptor interaction mechanism with other ARs This commonality is proposed to explain the difficulty in designing selective adenosine ligands.[27–29]

3 DISTRIBUTION IN IMMUNE AND CANCER CELLS

The A_3AR is highly expressed in several immune cell types, as well as in cancer cells.[11] In particular, the native human A_3AR was first revealed in human eosinophils and subsequently in neutrophils, monocytes, macrophages, foam cells, dendritic cells, lymphocytes, splenocytes, bone marrow cells, lymphonodes, synoviocytes, chondrocytes, osteoblasts, and mast cells.[9,13,30–63] Overall, the presence of the A_3ARin almost all the cells involved in inflammatory processes suggests their potential involvement in a number of inflammatory pathologies, spanning from wound healing and remodeling to lung injury, inflammatory bone loss, autoimmune, and eye diseases.[2] In addition, high A_3AR expression has been observed using biochemical methods in many of types of cancer cells, including astrocytoma, melanoma, lymphoma, sarcoma, glioblastoma, colon, liver, pancreas, prostate, thyroid, lung, breast, and renal carcinomas.[64–89] This expression pattern reflects a demonstrated role for this subtype in tumor biology.

4 MEDICINAL CHEMISTRY OF THE A_3 ADENOSINE RECEPTOR

4.1 Adenosine derivatives as agonists of the A_3 adenosine receptor

The first efforts to develop A_3AR selective agonists (Table 2) were performed at the US National Institutes of Health (NIH),[90] and culminated in the report of *N*^{6}-(3-iodobenzyl)adenosine-5′-N-methyluronamide (IB-MECA **1,** Fig. 1), which is ~50-fold selective for the A_3AR in rat compared to the A_1AR and A_{2A}AR.[91,92] The first few years of medicinal chemical optimization of the affinity and selectivity of A_3AR agonists relied entirely on comparison of binding affinities at the rat ARs,[56] because the human homologues were not available initially.[7] A successful approach was to combine multiple A_3AR enhancing substitutions in adenosine analogues. Thus, IB-MECA contained a substituent that enhanced affinity at the ARs including A_3AR, for example, a 5′-*N*-alkyluronamide, with an *N*^{6}-benzyl substituent that maintained affinity at this subtype but reduced affinity at the A_1AR and A_{2A}AR. Initially, an unsubstituted *N*^{6}-benzyl group served this purpose, and later a halo atom at the 3-position of the benzyl ring was shown to increase A_3AR affinity and selectivity.[92] Optimization of the 5′-*N*-alkyluronamide demonstrated that methyl was more favorable for A_3AR binding than larger alkyl groups. A combination with 4-amino-3-iodo substitution of the *N*^{6}-benzyl group maintained high affinity, but not high selectivity at the A_3AR; thus, compound **3** became a widely used high-affinity radioligand in cells and membranes highly expressing this receptor.[56] A 3-isothiocyanatobenzyl group was also tolerated at the *N*^{6}-position, which provided the first selective chemically reactive affinity label of the rat A_3AR, termed ICBM(N6-(3-isothiocyanatobenzyl)-5.-N-methylcarboxamidoadenosine) **4**.[93] In a subsequent study of the structure–activity relationship (SAR), a third position of derivatization was explored: the C2-position.[94] It was noted that 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680 **16**[95]), originally introduced as an A_{2A}AR-selective agonist, surprisingly displayed affinities (nanomolar, all human homologues) in the order A_{2A}AR (27) > A_3AR (67) > A_1AR (289).[149] Based on this initial observation, it was apparent that the A_3AR binding site was flexible in the ability to accommodate a variety of C2 substituents, including sterically bulky groups. Thus, the order of potency of CGS21680 was A_{2A}AR > A_3AR > A_1AR ≫ A_{2B}AR. Nevertheless, the 2-chloro analogue **2** of IB-MECA was the focus at that time as an A_3AR agonist of increased selectivity, since other C2 modifications were not yet systematically explored. Later, an extended C2-alkynyl group, initially in the form of 6-hexynyl, was shown to be tolerated in adenosine derivatives in binding to the A_3AR.[96,97] This modification was also compatible with a 5′-*N*-ethyluronamide group, an observation that led to the identification of HE-NECA **7** as a potent, but nonselective A_3AR agonist.[98,99]

Unlike the C2-position, modification of the 2′ and 3′ hydroxyl groups was highly detrimental to A_3ARbinding affinity of simple adenosine analogues,[96] but a 3′-deoxy analogue of **2** (structure not shown) was later found to be a selective, full agonist at the rat A_3AR with a binding K_i value of 33 nM.[100] The ribose 5′-position was also amenable to modification beyond 4′-CH_2OH and 5′-*N*-alkyluronamides. For example, 5′-methyl ether analogue NNC53-0055 **6** was an agonist at the A_3AR,[101] and 5′-alkylthioethers were tolerated at the A_3AR.[102] Acylation of the N^6-NH reduced affinity at the A_3AR in comparison to the mono-alkylated analogues.[97] The flexibility of substitution at the N^6-position compatible with A_3AR affinity was higher than initially indicated in the report on **1**. For example, small alkyl and alkoxy groups, such as N^6-methyl in **10** and N^6-methyloxy in **6** and **11**, could be appended to the nitrogen.[99,101–104] However, a small alkyl group at the N^6-position often reduced affinity at the rat A_3AR compared to the human homologue. The human A_3AR tolerated larger N^6 substituents, such as the preferred 1*S*,2*R* stereoisomer of N^6-cyclopropylphenyl in **17**.[105] The adenine moiety could be replaced with other heterocyclic nucleobases, leading to the retention of A_3AR affinity and selectivity, but only in limited cases. For example, xanthine-7-ribosides such as DBXRM **5** were shown to fully activate the A_3AR by virtue of an intact 5′-*N*-methyluronamide.[106] Recently, in silico screening using an A_{2A}AR crystal structure identified alternative nucleobases that, when ribosylated, retained receptor affinity and efficacy at the A_3AR and other ARs. [107] Various pyridine-3,5-dicarbonitrile derivatives also bind to and activate ARs as atypical agonists, but they are not selective for the A_3AR.[108]

The prototypical A_3AR selective agonists **1** (CF101, Piclidenoson) and 2-chloro-N6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (Cl-IB-MECA **2,** CF102, Namodenoson) are both in clinical trials for inflammation and cancer, respectively.[10] They have demonstrated safety and clinical efficacy in Phases I and II trials. IB-MECA is now about to enter larger Phase III trials for RA and psoriasis, while Cl-IB-MECA is now about to enter a Phase II trial for primary liver cancer. **1** and **2** have also become widely used pharmacological probes with **2** being more selective for the rat and human A_3AR. However, at the mouse A_3AR, an exceptionally high affinity with K_i value of 87 pM was noted for **1**, leading to 69-fold and >10,000-fold selectivity in comparison to mouse A_1AR and A_{2A}AR, respectively.[109] Two other agonists of the A_3AR, **12** and **13**, were considered for clinical application to anti-ischemic cardioprotection.[110] Compound **13** was unusually water soluble due to the presence of a 3′-amino group, which is largely protonated in physiological medium. Another highly selective A_3AR agonist **15b** containing a C2-pyrazolyl group was reported.[111]

In 2000, Jacobson et al. reported that conformationally constraining a ribose-like moiety in the form of bicyclic ring increased the A_3AR selectivity.[112] The methanocarba group, that is, a bicyclo[3.1.0]hexane in place of the tetrahydrofuryl group of native ribose, had been applied earlier to antiviral drugs,[113] and this was the first report of its incorporation in signaling ligands. Two isomeric forms of the methanocarba ring system, depending on the fusion point of the cyclopropane and cyclopentane rings, enforce either a North (N) (Fig. 2) or South (S) envelope conformation of the pseudoribose. A priori, the conformational preference of the ARs in binding ribose was not known, but the (N) isomer of simple adenosine was found to have >100-times the affinity of the corresponding (S) isomer at the A_3AR. Thus, this derivatization approach of using chemically constrained rings can be used to probe the conformational preference of a given receptor. Among the four ARs, the greatest affinity gain with the (N)-methanocarba ring occurred at the A_3AR. Numerous A_3AR selective agonists were subsequently reported that included this major modification that enhanced affinity and selectivity at the A_3AR compared to other ARs. The (N)-methanocarba modification is compatible with many other A_3AR affinity-enhancing groups, such as 5′-*N*-alkyluronamides and N^6-benzyl groups.[114] Incorporation of the (N)-methanocarba modification in IB-MECA **1** resulted in MRS1898 **18**, a potent and moderately selective A_3AR agonist that was later radiolabeled to provide a selective radioligand for the rodent A_3AR.[115] Other halogens could be placed at the 3-position, such as bromo **19** and chloro **20**; the 3-bromo equivalent **19** provided a selective A_3AR agonist as a ^{76}Br-labeled positron emitter for receptor imaging studies.[116]

The A_3AR-favoring (N)-methanocarba-5′-*N*-methyluronamide scaffold could also be combined with an A_1AR-favoring substituent (N^6-cyclopentyl) to provide a dual acting A_1/A_3AR agonist **22** that displayed cardioproprotective properties, as both receptors demonstrate anti-ischemic properties in heart.[117]

The C2-position could also be derivatized with (aryl)alkylthio groups, which resulted in moderate A_3AR affinity but not selectivity.[118] The C2-position substitution was expanded to include alkynyl and arylalkynyl groups that were compatible with both the riboside and (N)-methanocarba series.[97,103,104] C2-ethynyl and arylethynyl groups were shown to further increase A_3AR selectivity of adenosine analogues, that is, ribosides **7–11** and (N)-methanocarba derivatives such as **23–29**.[119–123] A carboxylic acid congener **21** is an A_3AR agonist that displayed greater water solubility and the option of conjugation without losing receptor affinity. A lower homologue of **21** was used as a carboxy-bearing pharmacophore to condense with an amine-functionalized Cy5 fluorophore in the fluorescent A_3AR-selective agonist MRS5218 **23** of high A_3AR affinity. **23** is selective for the A_3AR in human and mouse and is suitable for characterization of the receptor in live cells using flow cytometry.[124] Compound **24** contains a terminal alkyne group, as well as an alkyne at the C2 fusion position, and is suitable for click coupling to carriers such as gold nanoparticles with the retention of A_3AR affinity and selectivity. A 3,4-difluorophenyl ethynyl group in **25** was particularly conducive to attaining high A_3AR affinity in multiple species. Thus, the K_i value of **25** was approximately 3 nM at both the human and mouse A_3ARs. The tolerance of the receptor for extended C2 substituents was surprising—even a biphenyl substituent in **26** preserved high A_3AR affinity, which was explained based on conformational plasticity of TM2.[120,125] A_3AR-selective agonist **29** contains a sulfonate group that renders it unable to diffuse through biological barriers such as the blood–brain barrier. Thus, it is useful in vivo for distinguishing peripheral and central A_3AR effects.[122] The preferred placement of a sulfonate group on the scaffold of C2-arylethynyl-methanocarba-adenosine-5′-*N*-methyluronamides was predicted successfully using computational modeling of the receptor interactions at the human and mouse A_3ARs.

In 2003, the group of Lak Shin Jeong in South Korea synthesized thionucleoside analogues that were shown to be highly potent and selective as A_3AR agonists.[126] The SAR upon modification of thionucleosides at the C2, N^6 and 5′-positions was explored in detail. The 4′-thio modification of adenosine analogues was found to be compatible with many other A_3AR affinity-enhancing groups, such as 5′-*N*-alkyluronamides and a range of N^6 substitutions. Compounds **14a** and **14b** are analogues of IB-MECA **1** and Cl-IB-MECA **2**, respectively, which were found to be highly potent and selective in A_3AR binding. Compound **14b** has been shown to suppress angiogenesis, a property that might be beneficial in treating cancer, diabetic retinopathy, and inflammatory diseases.[127]

To summarize the SAR described, A_3AR affinity and selectivity of agonists are based on substitution at the C2, N^6, and 5′ positions of adenosine,[128] and only limited ribose functional group substitution of nucleosides is tolerated at this receptor. Some potent A_3AR agonists such as **13** contain a 3′-amino-3′-deoxy modification of adenosine,[110] but this modification does not apply universally. Although highly specific A_3AR agonists were obtained in SAR studies, their binding K_i values were not directly predictive of the magnitude of an in vivo protective response, for example, in reducing chronic neuropathic pain. Thus, it became necessary to measure parameters other than simple binding affinities (such as half-life, duration of response, and maximal efficacy in vivo) to select for molecules with translational potential. An in vivo phenotypic screen in real time of the action of A_3AR agonists to reduce or prevent chronic neuropathic pain was adopted for a comparison of diverse substitutions at these positions on the adenosine scaffold.[121] The data obtained in a mouse model of neuropathic pain, that is, chronic constriction injury (CCI) of Bennett and Xie,[129] allowed the chemists to steer the SAR in the direction of compounds that displayed high efficacy in reducing hyperalgesia and a long duration of action in vivo upon oral administration. The CCI model was ideally suited for the comparison of antinociceptive activity of A_3AR agonists because of their high potency (greater than the molar potency of other pain medications) and because they lacked activity in tests of acute pain, such as the hot plate test and tail flick assay.[130] The efficacy and duration of action of novel A_3AR agonists after p.o.per os administration indirectly indicated favorable oral bioavailability and pharmacokinetics, at least with respect to chronic neuropathic pain. Thus, this phenotypic screen proved to be an invaluable guide in the extension of the SAR in this compound series.

This in vivo phenotypic screen confirmed that C2-phenylalkynyl analogues were among the preferred A_3AR agonists. The terminal cyclic group in the C2-alkyne series was then varied to include diverse 6-membered rings, 5-membered heterocyclic rings, and cycloalkyl rings.[121] With respect to A_3AR binding and selectivity, many of these groups, including substituted phenyl rings, maintained high A_3AR affinity and selectivity. However, the in vivo phenotypic screen identified 5-membered heterocyclic rings, such as thienyl derivatives, as being particularly potent and efficacious in vivo in the chronic neuropathic pain model. A 5-chlorothienylethynyl group in MRS5980 **27** and MRS7154 **28** was found to prolong the protective action of A_3AR agonists in the CCI model. The substitution of the *N*1 group of the adenine moiety with CH in **31** was well tolerated in A_3AR binding and activation and in the CCI model.

Due to the possibility that the C2-arylethynyl group could serve as a Michael reaction acceptor in nucleophilic attacks, alternative bioisosteric extensions at the C2-position were compared. The aryltriazolyl group in MRS7138 **30** was found to mimic the geometry of the corresponding arylethynyl group when the (N)-methanocarba nucleoside was receptor bound,[131] and this substituent would not have liability as a potential Michael acceptor. This conformational relationship was predicted using molecular docking to a homology model of the A_3AR. C2-triazole substitution (two positional isomers) was previously found to be compatible with A_3AR binding in the riboside series, as in **15a** and **15b**.[132] As a postscript to that effort to replace the C2-arylethynyl group, this ethynyl group was found to be relatively unreactive toward thiols such as glutathione, and the risk of such compounds depleting liver glutathione was shown to be very small.[133]

The surprising finding that substitution of the exocylic NH of adenine with H or CH_3 in MRS5919 **32** allowed full activation of the A_3AR emphasized that the loss of otherwise important recognition elements in a ligand can be compensated by other affinity enhancing moieties on the nucleoside.[134] Moreover, the ribose moiety is the main effector of receptor activation, while adenine modifications tend to change the subtype selectivity but usually do not have a major effect on the agonist efficacy. However, there are exceptions to the above generalization, including various N^6 substituents and C2 substituents that produce partial agonism or antagonism at the A_3AR, as has been summarized,[135] and nucleoside derivatives with reduced efficacy are discussed below.

4.2 Nucleoside derivatives as partial agonists and antagonists of the A_3 adenosine receptor

Selected nucleoside derivatives that act as antagonists or low efficacy agonists at the A_3AR are shown in Figure 3. The truncation of hydroxyl groups of adenosine nucleosides, that were demonstrated to be A_3AR-selective agonists, was first explored in 1995.[96–99] The goal was to reduce the hydrophilicity of the nucleosides to increase bioavailability without loss of receptor affinity. A secondary goal was to probe the effect on intrinsic efficacy of the truncated nucleosides as A_3AR agonists, although it was not immediately achieved.[100] The conversion of adenosine agonists into antagonists by complete removal of the ribose ring, that is, in adenine derivatives, was previously demonstrated, but the pharmacological characteristics of intermediate structures, that is, those with partially truncated ribose moieties, were unknown. Among the four ARs, the A_3AR appears to be the easiest with respect to conversion of nucleoside agonists into antagonists, and numerous examples have been reported.[109,128,132,135–141] However, it should be noted that the degree of efficacy can vary, depending on the functional assay and the receptor expression level. Thus, a modified nucleoside that behaves as an A_3AR antagonist in one system, such as binding of a radiolabeled guanine nucleotide, might still activate the receptor under different circumstances, such as measurement of inhibition of adenylate cyclase.[115]

Cristalli and co-workers took another approach to achieve A_3AR antagonism. The presence of 8-alkynyl substituents on adenosine (4′-CH_2OH) analogues, such as **33**, reduced the ability of the A_3AR-selective nucleoside to activate the receptor, as is consistent with antagonism.[136,142] The theme of reduced efficacy in truncated nucleosides, rigid nucleosides, and otherwise modified ribosides was developed in pharmacological studies of Gao et al.[137] The requirement of an H-bond donating group at the 5′-position of nucleoside analogues for A_3AR activation was also demonstrated. A spirolactam **35** related structurally to IB-MECA **1** retained selectivity of binding to the rat and human A_3ARs, but completely lacked the ability to activate the receptors and was shown to be a functional antagonist. Thus, a degree of flexibility of the 5′-amide, which is capable of forming multiple H bonds with the receptor, was required for full A_3AR activation. Xanthine-7-riboside **34** was a partial agonist at the rat A_3AR but an antagonist at the A_1AR.[106] An N^6 substituent that converted A_3AR agonist activity into antagonism was the N^6-(2,2-diphenyl)ethyl group in **36**.[105] Curiously, the corresponding rigidified N^6-fluorenylmethyl analogue (structure not shown), upon addition to an aryl-aryl bind to **36**, became a full agonist. The combination of a N^6-benzyl-type substituent with 2-chloro in **38** reduced the efficacy at the A_3AR to nearly zero, although its residual efficacy could be expanded to full efficacy in the presence of A_3AR PAM LUF6000 **95** (where PAM is positive allosteric modulator).[108] Modification at the 5′-position as an ester **37** produced a low-efficacy partial agonist, while 5′-*N,N*-dialkyluronamide in **42** resulted in antagonism at the A_3AR.[109] Thus, conformational factors at various regions surrounding the adenosine core and H-bonding around the 5′-position are determinants of A_3AR efficacy.[128]

The 4′-truncation of the A_3AR nucleosides was explored in great detail for the 4′-thionucleosides [138,139] leading to compounds **39** and **40**, which were shown to be antagonists using a functional assay of guanine nucleotide binding. However, the 2-hexynyl group of compound **41** added a second activity to this series of A_3AR ligands, that is, **41** was a combined potent A_3AR antagonist and A_{2A}AR agonist.[143] Truncation of the nucleoside ribose-like moiety in the (N)-methanocarba series also led to A_3AR-selective antagonists and partial agonists,[109] including a radiolabeled 3-bromo analogue **44** for positron emission tomography (PET).[116] Compound **45** is a selective antagonist of both the human and mouse A_3ARs.[120,125] Compound **46** is a selective A_3AR antagonist with renal protective properties.[139] Compound **47** is a mixed A_1/A_3AR antagonist that also displays functional agonism at the A_{2A}AR, which displayed greater potency than predicted from its only moderate affinity in A_{2A}AR binding assays.[141]

4.3 Non-nucleoside derivatives as antagonists of the A_3 adenosine receptor

For more than 20 years, the advance of potent and selective A_3AR antagonists as promising therapeutic choices for a range of diseases has been a prime subject of medicinal chemistry research. The pharmaceutical industry and academic communities have focused on the synthesis and screening evaluation of numerous heterocyclic compounds to discover potent and highly selective A_3AR antagonists due to their potential therapeutic applications.[144]

A_3AR antagonists belong to different structural groups including monocyclic, bicyclic, and tricyclic aromatic compounds (Table 3). Several xanthine or purine analogues were examined first, but none showed significant affinity or selectivity at rat A_3AR.[96,100,144,145] Consequently, different classes of compounds, that could be classified as nonxanthine derivatives[135,144–149] and the lately nucleoside-derived antagonists, have been discovered as highly potent and selective A_3AR antagonists.

In this section, we summarize the medicinal chemistry of A_3AR antagonists updating our previous reviews on this field.[150–153]

4.3.1 Monocycles

1,4-Dihydropyridines and pyridines: After the first evidence that 1,4-dihydropyridines (DHPs) exerted antagonistic activity at the A_3AR in addition to L-type calcium channel inhibition, Jacobson et al. designed a series of substituted DHPs in the attempt to separate the two different activities.[154] In this study, the replacement of the methyl ester at the 5-position of nifedipine with a bulkier 4-nitrobenzyl ester, along with the introduction of phenylethynyl and phenyl moieties at positions 4 and 6, respectively, led to **48** (MRS1334, Fig. 4). This compound showed high affinity and selectivity as an A_3AR antagonist without inhibiting L-type calcium channels.[155] In addition, a 3,5-diacyl-2,4-dialkylpyridine series was delineated by the oxidation of the corresponding DHP derivatives, and the best profile against A_3AR was achieved with **49** (MRS1505, Fig. 4) in which the position 4 of the pyridine ring was substituted with small alkyl groups such as ethyl chain.[156,157] General SAR of pyridine derivatives revealed that structural requirements responsible for enhancement of A_3AR affinity and selectivity did not completely reflect that of the DHP parent compounds.[158] Among this series, were also reported fluorinated and hydroxylated pyridine derivatives[159] and an extension of this study performed by Jacobson and co-workers described a series of *N*-alkylpyridinium salts as water soluble A_3AR antagonists although with lower potency than the pyridine analogues.[160] A pyridine-based A_3AR antagonist PET ligand [^{18}F]FE@SUPPY was introduced.[161]

Pyrimidines: Within the classes of bi- and tricyclic ARs antagonists, the pyrimidine nucleus present in the endogenous modulator adenosine, is a frequent substructural scaffold. 4-Amino-6-hydroxy-2 mercaptopyrimidines derived from chain opening of a series of triazolopyrimidinones have been synthesized by Cosimelli et al.[162] Introduction of the propylsulfanyl and *p*-chlorobenzyloxy moieties at 2 and 6 positions, respectively, combined with an acetamide group at 4-position of the pyrimidine ring led to compound **50** (Fig. 4), a potent and selective human A_3R antagonist (K_i = 3.5 nM). Similar structures characterized by two regioisomeric series of diaryl 2- or 4-amidopyrimidines such as *N*-[2,6-bis(4-

methoxyphenyl)pyrimidin-4-yl]acetamide **51** (ISVY130, Fig. 4) were reported by Sotelo and co-workers. Some of the ligands in this series exhibited good selectivity and affinity with K_i values of < 10 nM at the A_3AR.[163,164]

Pyrazin-2(1H)-ones: Very recently, Sotelo and co-workers published a novel series of compounds, including a simplified pyrazin-2(1*H*)one scaffold, as A_3AR antagonists and with better pharmacokinetic properties.[165] These new derivatives obtained by the Ugi-based multicomponent reaction were less potent than many other A_3AR antagonists reported in the literature. The entire library of compounds, including the most potent compound of this series with a K_i value of 386 nM (**52**, SYJA385, Fig. 4), was subjected to a computational study to determine a rational hypothesis for their binding model.[165]

Thiadiazole and thiazoles: IJzerman co-workers identified thiadiazole and thiazole analogues as A_3AR antagonists by chemical structure simplification of corresponding bicyclic quinazoline and isoquinoline nuclei, respectively.[166] Among them, compound *N*-[3-(4-methoxyphenyl)-[1,2,4]thiadiazol-5-yl]acetamide **53** (Fig. 4) was claimed as the best compound of the series exhibiting a K_i value of 0.79 nM and acting as antagonist in a cyclic AMP (cAMP) functional assay.[167] SAR optimization by introduction of a 5-(pyridine)-4-yl moiety on the 2-aminothiazole ring revealed a series of potent and selective compounds such as *N*-[4-(3,4,5-trimethoxyphenyl)-5-(pyridin-4-yl)thiazol-2-yl]-acetamide **54**, with subnanomolar affinity for human A_3AR (K_i = 0.4 nM) and 1000-fold selectivity against the other AR subtypes.[168]

The QSAR analysis of thiazole and thiadiazole A_3AR antagonists indicated that their binding affinity increased with decreasing lipophilicity and in the presence of small alkyl moieties such as amide functions (acetamide or propionamide).[168] In addition, the introduction of substituents, such as benzoyl, nicotinoyl (e.g., compound **55**, Fig. 4) and isonicotinoyl moieties in position 2 of the thiazole ring, led to potent and selective antagonists at both human and rat A_3ARs.

4.3.2 Bicycles

Quinazolines, phthalazines, and quinoxalines: A structure–affinity study reported by IJzerman co-workers indicated that introduction of a phenyl or heteroaryl substituent on the 2-position of the quinazoline scaffold or the equivalent 3-position of the isoquinoline improved the A_3AR affinity in comparison to the unsubstituted derivatives. Combinations of the best substituents in the two series led to the potent and selective human A_3AR antagonist *N*-(2-methoxyphenyl)-*N*-(2-(pyridin-3-yl)quinazolin-4-yl)urea **56** (VUF5574; Fig. 5) with a K_i value of 4 nM. [169]

Subsequently, the 2-amino/2-oxoquinazoline-4-carboxamide compounds, resulting from an in silico molecular simplification approach of the 2-aryl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one skeleton, were published by Morizzo et al. as

A_3AR antagonists. One example of this series is compound **57 (**Fig. 5) that showed good affinity and selectivity at the A_3AR.[170] With a similar approach on the triazoloquinazolinone nucleus, a new series of 2-phenylphthalazin-ones was identified as promising A_3AR

antagonists. Molecular manipulations by introduction of amide and ureide functional groups at the 4-position of the phthalazinone ring led to compound **58** (Fig. 5) with the best activity profile.[171] Additionally, the 2-(4-methyl-1*H*-benzo[*d*]imidazol-2-yl)-quinoxaline **59** (Fig. 5) is noteworthy for the novelty of its design strategy utilizing a 3D database searching approach.[172]

Imidazo[1,2-a]pyrazines: Very recently, the imidazo[1,2-*a*]pyrazine nucleus was reported as a suitable core for the design of new AR antagonists. Within this series of compounds, a *N*-(2,6-diphenylimidazo[1,2-*a*]pyrazin-8-yl)-4-methoxybenzamide **60** (Fig. 5) showed good A_3AR affinity with a K_i value of 25 nM. The molecular docking study of these compounds was also carried out to describe the potential binding mode of the new derivatives to their refined target receptor model.[173]

Adenines and adenine-like derivatives: The first class of selective A_3ARantagonists characterized by a bicyclic structure strictly correlated to the adenine core was identified by Biagi et al.[174] The adenine-like structure of these new N^6-ureidosubstituted-2-phenyl-9-benzyl-8-azaadenines was responsible for their activity as antagonists, while the phenylcarbamoyl group ensured selectivity at the A_3AR. The SAR studies based on the systematic substitutions of 2, 6, and 9 positions of the bicyclic system led to improved A_1/A_3 selectivity with compound **61** (Fig. 5).

Starting from reversine, 2-(4-morpholinoanilino)-N^6-cyclohexyladenine, with a moderate activity as A_3AR antagonists (K_i = 0.66 μM), Perreira et al. explored the SAR of related derivatives in order to improve A_3AR potency and selectivity.[175] A series of reversine analogues was synthesized by substitution of 2- and N^6-positions of the adenine core. One of the most remarkable compounds in terms of hA_3AR affinity and selectivity resulted when the N^6-cyclohexyl moiety of reversine was combined with a 2-phenyloxy group (compound **62**, MRS3777, Fig. 5).[175] Some analogues from this study were shown to be inactive at 10 μM in the rat, reflecting the typical species dependence of binding of most known nonnucleoside A_3AR antagonists.

The pyrazolo[3,4-*d*]pyrimidine scaffold, a bicyclic system structurally connected to the adenine nucleus that resulted from simplification of tricycles pirazolo-triazolo-pyrimidine, was recently described by Taliani et al.[176] The SAR profile of this series indicated that the presence of an amide or ureide functionality at the 4-position (compounds **63** and **64**, respectively) along with a phenyl ring at the 6-position was essential for promoting A_3AR affinity and selectivity. The N^2-position was characterized by substantial steric tolerance; in fact, both small methyl group (**63**, Fig. 5) and bulkier benzyl moiety (**64**, Fig. 5) were well tolerated. Compound **63**, which showed a subnanomolar A_3AR affinity and high selectivity versus the other AR subtypes, has been suggested as a promising lead compound for the development of adjuvant agents in glioma chemotherapy. In a related effort, a series of junction isomers of pyrazolo[3,4-*d*]pyrimidine derivatives were synthesized by Lenzi et al. applying a molecular simplification approach to the tricyclic pyrazolo[3,4-*c*]quinolin-4-one skeleton.[177] The binding results of the junction isomers were successful and the new derivatives maintained high affinity for the hA_3AR increasing also the selectivity versus the other AR subtypes. Aryl/arylalkyl substitution at the 5-position of such derivatives was poorly tolerated for A_3AR binding affinity, while small groups at the same position were shown to enhance the ligand–receptor interaction. In addition, the substitution of the 2-phenyl ring with a 4-methoxy group led to 2-(4-methoxyphenyl)-5-methyl-2*H*-pyrazolo[4,3-*d*]pyrimidin-7(6*H*)-one **65** (Fig. 5), the most potent compound of this series. Very recently, a large number of 2-arylpyrazolo[4,3-*d*]pyrimidin-7-amine or 7-acylamine derivatives were synthesized as potent A_3AR antagonists.[178,179] The pyrazolopyrimidines bearing a 4-methoxyphenyl or a 2-thienyl group at the 5-position showed high hA_3AR affinity and selectivity. 4-Methoxy-*N*-(2-phenyl-5-(thiophen-2-yl)-2*H*-pyrazolo[4,3-*d*]pyrimidin-7-yl)benzamide **66** (K_i = 0.027 nM, Fig. 5) is one of the most potent and selective A_3AR antagonists in this structural class.[179]

4.3.3 Tricycles

Triazoloquinazoline: Jacobson and co-workers first described the N^5-acylation of the free amino group of well-known 9-chloro-2-(furan-2-yl)-[1,2,4]triazolo[1,5-*c*]quinazoline-5-amine **67** (CGS15943, Fig. 6).[180,181] This structural modification yielded **68** (MRS1220, Fig. 6), which was enhanced in both affinity and A_3AR selectivity.[181,182] The removal of the chlorine atom at 9-position of the triazoloquinazoline **67** along with the replacement of the 5-phenylacetamido and the 2-furyl moieties with a linear alkyl chain and a 4-Br-phenyl group, respectively, led to **69** (Fig. 6). This compound was found to be a potent and selective A_3AR antagonist.[183]

Very recently, a new series of triazoloquinazolines was reported as A_3AR antagonists. Two examples are the 3,5-diphenyl[1,2,4]triazolo[4,3-*c*]quinazoline **70** (K_i =1.16 nM, Fig. 6) and the 5′-phenyl-1,2-dihydro-3′*H*-spiro[indole-3,2′-[1,2,4]triazolo[1,5-*c*]quinazolin]-2-one **71** (K_i = 6.94 nM, Fig. 6).[184]

Pyrazolo[3,4-c]/[4,3-c]quinolines: 2-Arylpyrazolo[3,4-*c*]quinolin-4-ones, 4-amines, and 4-amino-substituted derivatives are reported as potent and selective hA_3AR antagonists.[185] Most of them showed a nanomolar hA_3AR affinity and different degrees of selectivity that were strictly dependent on the presence and nature of the substituent on the 4-amino group. The benzoamide derivative **72** (Fig. 6) was the most potent and selective among the three reported series of compounds.

The pyrazole[4,3-*c*]quinoline-4-one scaffold was adapted to A_3AR antagonists as structural isomers of the previous nucleus by Baraldi et al. Among the pyrazole[4,3-*c*]quinoline-4-ones, compounds that contained a 2-*p*-substituted phenyl (CH_3, OCH_3, and Cl) group showed good hA_3AR affinity and excellent selectivity in comparison to the other AR subtypes (e.g., compound **73**, Fig. 6).[186]

Triazolo[4,3-a]/[1,5-a]quinoxaline: Colotta et al. identified 1,2,4-triazolo[4,3-*a*]quinoxalin-1-one derivatives as promising hA_3AR antagonists.[187] The SAR study recognized the appropriate substitutions at 2, 4, and 6 positions of the tricyclic template. In particular, the introduction of the 4-oxo or 4-*N*-amide functions afforded selective and potent A_3AR antagonists such as compounds **74**[188] and **75**[189], respectively, confirming the importance of both nuclear or extranuclear carbonyl functionality for A_3AR affinity (Fig. 6).

A series of 2-aryl-8-chloro-1,2,4-triazolo[1,5-*a*]quinoxalines has also been synthesized and evaluated in radioligand binding assay at both bovine and human ARs[190,191] showing a similar SAR profile to that of the 2-arylpyrazolo[3,4/4,3-*c*]quinolines[185, 186] and triazolo[4,3-*a*]quinoxaline.[189] One of the representative derivatives was a 2-(4-methoxyphenyl)-[1,2,4]triazolo[1,5-*a*]quinoxalin-4(5*H*)-one **76** (Fig. 6).

1,2,3-Triazolo[1,2-a][1,2,4]benzotriazolones: A_3AR antagonists based on aminophenyl-triazolobenzotriazinone have been reported by Da Settimo et al. A lead optimization strategy focused on the structural modifications provided that the suitable groups were attached to the 5-amino group and in the 4′-and/or 9-positions. The best result was obtained with compound **77** (Fig. 6), which showed a K_i value of 1.6 nM at the A_3AR and no significant affinity at the other ARs.[192]

Pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidines: The pyrazolo-triazolo-pyrimidine (PTP) scaffold, due to its strong structural correlation with the nonselective antagonists CGS15943 (**67**, Fig. 6) [180] and to the adenine nucleus present in the endogenous modulator adenosine, has been investigated in depth as a prototypical template for adenosine antagonists.

Rigorous research efforts were made on this scaffold in order to obtain potent A_{2A}- and A_3AR antagonists.[153] A series of PTP derivatives (MRE series) reported by Baraldi's group were obtained by the structure–activity optimization based on the introduction of different substituents at the 5, 7, 8, and 9 positions.[145,149,193,194,196–200] The N^7-substituted derivatives proved to be predominantly hA_{2A}AR antagonists, while the combination of a small alkyl chain at the N^8-pyrazole position with a (substituted)phenylcarbamoyl chain at the N^5-position led to potent and selective hA_3AR antagonists. [198] One of the most active and selective compounds in this series was **78** (MRE3008-F20, Fig. 7) with a K_i value of 0.29 nM.[193] The corresponding tritium-labeled analogue as the first potent and selective radiolabeled antagonist for the A_3AR was prepared. It bound to the hA_3AR expressed in Chinese hamster ovary (CHO) cells with a K_D value of 0.82 nM (B_{max} = 297 fmol/mg protein).[201,202] The isosteric replacement of the phenyl ring with a 4-pyridylmoiety yielded **79** (MRE3005-F20, Fig. 7) that maintained the high affinity at the A_3AR with enhanced water solubility.[203] Subsequently, replacement of pyridin-4-ylmoiety of MRE3005-F20 with substituted piperidine rings led to the preparation of the hydrochloride salt of 1-(cyclohexylmethyl)piperidin-4-yl (**80**, Fig. 7).[149]

Synthesis of fluorosulfonyl- and bis(β-chloroethyl)amino-phenylamino-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidines as irreversible A_3AR antagonists was performed by Baraldi's group to provide useful tools for structure–activity studies. Electrophilic groups, specifically sulfonyl fluoride and nitrogen mustard (bis-(β-chloroethyl)amino) moieties, have been incorporated at the 4-position of the aryl urea group (compounds **81** and **82,** respectively, Fig. 7).[204] While compounds containing a fluorosulfonyl moiety proved to be irreversible antagonists at the hA_3AR (at 100 nM, 79% of inhibition), the corresponding nitrogen mustard derivatives were unable to covalently bind the target receptor subtype. This difference in the receptor interaction between the **81** and **82** series has been explained on the basis of chemical reactivity of the two different groups.

Very recently a new series of *N,N′*-disubstituted guanidines of the PTP scaffold, prepared in a one-pot reaction, was described as A_3AR antagonists. The best compound of this series was **83** (Fig. 7) bearing a *N,N*-(4-nitrophenyl)guanidine moiety at 5-position of the tricyclic nuleus.[196]

Triazolopurines: [1,2,4]Triazolo[5,1-*i*]purines structurally related to the triazoloquinazolines have been identified as A_3AR antagonists.[183,205] Within this family, the 5-*n*-butyl-8-(4-*n*-propoxyphenyl)-3*H*-[1,2,4]triazolo[5,1-*i*]purine **84** (Fig. 7) showed a good potency and selectivity at A_3AR. Among the reported structures, compound **85** (OT-7999**,** Fig. 7) demonstrated a significant reduction of intraocular pressure in cynomolgus monkeys at 2–4 hr following topical application.[205]

Tricyclic xanthines: Caffeine and theophylline are the classical nonselective xanthine antagonists of ARs that display micromolar affinity at human AR subtypes. At the rat A_3AR, caffeine and theophylline are weaker in affinity.

Initial SAR studies at the A_3AR were carried out using multiple substituted xanthines, many of which retained selectivity for the A_3AR.[90] An interesting approach was based on the ring annelation of xanthine derivatives, which permitted several research groups to discover different tricyclic systems that showed dissimilar affinity to AR subtypes.[206] A series of imidazo[2,1-*i*]purinones as tricyclic xanthines (PSB series) was developed by Müller et al. as human A_3AR antagonists with improved water solubility.[207] For example, **86** (PSB-10, Fig. 7) showed a subnanomolar A_3AR affinity and a good selectivity compared to the other AR subtypes, and its tritiated form ([^{3}H]PSB-11) exhibited a K_D value of 4.9 nM (B_{max} = 3500 fmol/mg of protein).[208] Another similar compound is 2-(4-bromophenyl)-4-propyl-7,8-dihydro-1*H*-imidazo[2,1-*i*]purin-5(4*H*)-one **87**, also designated KF-26777 (Fig. 7), was endowed with high affinity and selectivity.[209]

The pyrido[2,1-*f*]purine-2,4-diones, another series of tricyclic xanthines, have been reported to exert affinity at A_3AR in the low nanomolar range. Different substituents at the 1 and 8 positions of the new scaffold were evaluated, and the SAR studies led to 3-(cyclopropylmethyl)-1-(4-methylbenzyl)pyrido[2,1-*f*]purine-2,4(1*H*,3*H*)-dione **88** (Fig. 7) showing the best A_3AR binding profile of the series with total selectivity.[210,211]

Replacement of the pyridine nucleus of the pyrido[2,1-*f*]purine-2,4-dione scaffold with different 5-membered heterocycles has been extensively examined by Baraldi's group. The SAR studies led to both series of pyrrolo[2,1-*f*]purine-2,4-dione and imidazo[2,1-*f*]purine-2,4-diones as A_3AR antagonists.[199] Among the examined molecules, the imidazo[2,1-*f*]purine-ones were 2- to 10-fold more potent than the corresponding pyrrolo[2,1-*f*]purine-ones (e.g., **89** vs. **90**, respectively, Fig. 7) and the most favorable affinity and selectivity at A_3AR was obtained by introduction of small alkyl chain at the 7-position of the main scaffold (compound **89**).[200]

More recently, replacement of the trichlorophenyl ring at 2-position of PSB-10 **86** and congeners with differently substituted five-membered heterocycles, like 1,3- and 1,5-disubstituted pyrazoles or 3-substituted isoxazoles (*R-enantiomer* **91** and **92**, respectively,

Fig. 7) was investigated by Baraldi's group.[195] The 2-heterocyclic substitution induced excellent affinity and selectivity for the hA_3AR subtype. Docking of the most potent compound (**91**) in complex with a hA_3ARhomology model furnished a general survey of the hypothetical binding mode of the newly described derivatives.[195]

4.4 Allosteric modulators of the A_3 adenosine receptor

Allosteric modulators of GPCRs bind at a location that is distinct from the binding site for a native ligand, that is, the orthosteric site, and this phenomenon has been reported for the A_3AR.[212] In theory, the modulation may be positive, that is, with a PAM enhancing the activity of a directly acting (orthosteric) agonist, or negative, in the case of a NAM. A_3AR PAMs have been shown to enhance the potency and/or efficacy of agonists. When administered in vivo, they would be expected to be silent, with respect to A_3AR activity, unless either an endogenous or exogenous agonist would be present. Therefore, treatment with an A_3AR-selective PAM might display greater event- or site-specific action than an exogenous agonist, because it would magnify the effect of locally released adenosine, that is, in response to stress of an organ or tissue.

The SAR of three classes of A_3AR PAMs have been explored in detail: 1*H*-imidazo-[4,5-c]quinolin-4-amines (**93** – **97**),[213–215] 2,4-disubstituted quinolines (**98**), [216] and 3-(2-pyridinyl)isoquinolines (**99**, **100**).[217] Representative key members of these structural classes are shown in Figure 8. In addition to these heterocycles, allosteric modulation of this receptor by compounds and ions that are not specific to the A_3AR, such as amiloride analogues and sodium, have been studied.[212,218]

The principal assay used to screen for allosteric modulators of the A_3AR has been to examine effects on the dissociation rate of an agonist radioligand, $[^{125}I]$I-AB-MECA **3**.[212] Many of the PAMs that have been reported also compete with the radioligand for specific binding. Thus, the objective in early studies was to identify lead molecules that impeded the dissociation rate, with minimal competitive binding potency.[213,217] At a concentration of 10 μM, the imidazoquinolinamines and DU124182 **93** and DU124183 **94** reduced the dissociation rate of the radioligand from the receptor by roughly half, and the phenylamino derivative **94** was more potent as an allosteric enhancer.[213] Dichloro substitution and expanding the cycloalkyl group in LUF6000 **95**, MRS5049 **96**, and MRS5190 **97** were later shown to produce potent allosteric enhancement with less prominent competitive inhibition.[214,215] An amide LUF6096 **98** was particularly selective for allosteric enhancement of the A_3AR compared to **95**, but it displayed a short half-life in vivo. The residues of the A_3AR that are associated with the allosteric enhancement compared to orthosteric ligand binding were probed using stite-directed mutagenesis.[218]

When multiple effector mechanisms induced by agonist Cl-IB-MECA were compared, **95** was found to modulate each activity in a different manner, that is, this imidazoquinolinamine appeared to be a functionally biased PAM.[108,219] For example, **95** had no effect on phosphorylation of ERK1/2, a small effect on β-arrestin2 translocation, and intense effects on cAMP inhibition and cell hyperpolarization. The agonist-enhancing effect of **95** was probe-dependent, that is, it had different degrees of modulation of different AR agonists, considered receptor "probes." Notably, **95** greatly increased the efficacy of the naturally occurring nucleoside inosine, which is normally a weak and only partially efficacious agonist at the A_3AR. Inosine is a metabolite of adenosine and, like adenosine, is elevated in concentration when stress to an organ is present. Thus, **95** could produce therapeutic benefit in vivo partially from its enhancing action on inosine, as well as endogenous adenosine. **95** also enhanced the efficacy of nucleoside antagonists of the A_3AR, such as MRS542 **38**, to produce full agonism at the A_3AR. This indicates that nucleoside antagonists might behave as antagonists in a given functional model, but there is a latent agonism that can be amplified in the presence of a PAM. In contrast, nonnucleoside A_3AR antagonists did not display any latent agonism in the presence of an A_3AR PAM.

LUF6000 **95** has anti-inflammatory effects in rat models of arthritis and a model of liver inflammation in mice,[220] suggesting its potential use in the treatment of autoimmune inflammatory diseases. LUF6096 **98** was shown to protect the heart in a model of canine cardiac ischemia,[218] suggesting the potential use of A_3AR PAMs in the treatment of ischemic conditions.

4.5 Structural characterization of the A_3 adenosine receptor

Although there is currently no X-ray crystallographic structure of the A_3AR available, effective use of modeling techniques has provided a window into its orthosteric binding site (Fig. 9). In particular, docking of agonists in conjunction with data from site-directed mutagenesis has demonstrated the close similarity of the A_3AR to the X-ray crystallographic structure of the $hA_{2A}AR$.[221] Complexes of the $A_{2A}AR$ with four different agonists, namely 6-(2,2-diphenylethylamino)-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-*N*-(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9*H*-purine-2-carboxamide (UK432,097); adenosine; 5′-*N*ethylcarboxamidoadenosine (NECA); and 2-[*p*-(2-carboxyethyl)phenylethyl-amino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680) (PDB IDs: 3QAK, 2YDO, 2YDV and 4UG2, respectively), have been crystallized and their structures determined.[222–224] The complexes revealed a common interaction pattern anchoring the adenosine moiety in the orthosteric binding site of the $A_{2A}AR$ involving several conserved amino acid residues that are predicted to serve the same function in the A_3AR binding site. For example, the side chain Asn6.55 (using standard notation for numbering of TMs[225]) coordinates by H-bonding in a bidentate fashion with the adenine N^6H and *N*7 in both ARs. His7.43 is in contact with the 2′-hydroxyl group of ribose. Also, Phe168 (EL2) is predicted to form π–π stacking with the adenine ring, as in the $A_{2A}AR$. However, there are some differences in the interaction of nucleoside ligands with A_3AR compared to $A_{2A}AR$ that account for pharmacological differences of such ligands, with respect to their affinity, selectivity, and efficacy. For example, His6.52, which forms an H-bond to the 5′-carbonyl group of potent $A_{2A}AR$ agonists occurs as Ser6.52 in the A_3AR and is not predicted to closely associate with typical nucleoside ligands. This might explain why binding of 4′-truncated nucleosides is maintained at the A_3AR relative to other ARs.

Conformational plasticity of the A_3AR has been proposed to account for the high-affinity binding of rigid C2-arylalkynyl agonists such as MRS5980 **27**.[120] When docking this class of compounds in a homology model of the A_3AR derived exclusively from the agonist-bound "active-like" A_{2A}AR structure, there was a steric clash with the extracellular tip of TM2. An outward movement of TM2, as observed in several other active GPCR structures such as the β_2-adrenergic receptor and opsin, was therefore hypothesized to occur also for the A_3AR. Thus, a hybrid homology model in which TM2 assumed its highly displaced position in opsin was required to dock biphenyl derivative MRS5679 **26**, and the other TMs followed their orientation in the agonist-bound A_{2A}AR.[226] This proposed outward movement of TM2 was also associated with the degree of activational bias of C2-extended A_3AR agonists for cell survival, in a comparison of five different functional readouts.[131]

Molecular dynamics (MD) simulation of the A_3AR in complex with agonists have been reported.[227] The analysis of the conformational changes of conserved Trp243 (6.48) as a result of agonist (Cl-IB-MECA) binding suggested that the ligand was able to promote and stabilize an expected conformational switch involved in receptor activation.

A supervised MD (SuMD) study simulated the approach of PAM LUF6000 **95** toward a putative allosteric binding site on the A_3AR that contained an adenosine molecule in the orthosteric site.[228] In the modeled ternary complex, **95** occupied the upper region of the orthosteric binding site by directly interacting with the agonist. The study suggested that **95** might exert its PAM activity by acting as "pocket cap."

GPCRs may form characteristic dimers, either homo- or heterodimers, and the pharmacological implications of such dimerization remain to be fully characterized. Homodimers of the A_3AR have been detected using fluorescent methods.[229] Indications are that in the future, the discovery of A_3AR ligands will rely heavily on computational methods to define the dynamic interaction of the receptor with its ligands and with other proteins, including other GPCR protomers.

5 INTRACELLULAR PATHWAYS IN IMMUNE AND CANCER CELLS

A schematic diagram showing the main signaling pathways triggered by adenosine through A_3AR activation in different cellular types is reported in Figure 10. The A_3AR preferentially couples to Gi protein to inhibit cAMP accumulation, and it may also couple to Gq protein to mediate stimulation of phospholipase C (PLC), which then increases calcium concentrations.[230] The activation of PLC, and even the Ca^{2+} effects observed at high concentrations of A_3AR agonists, could conceivably be triggered by mechanisms other than Gq, such as $G\beta\gamma$ subunits. In addition, a Gq protein kinase C (PKC) dependent mechanism has been related to the apoptosis-inducing factor upregulation mediated by high doses of adenosine and of an A_3AR agonist in a human bladder cancer cell line.[78] However, PKC was recruited by A_3AR stimulation in order to increase tumor necrosis factor alpha (TNF-α) release in activated macrophages.[231]

A huge amount of data supports a link between A_3AR and MAPKs in several cellular models.[232] A_3AR-mediated activation of extracellular signal-regulated kinases (ERK1/2) and mitogenesis modulation was found in human fetal astrocytes, CHO cells expressing the human A_3AR (CHO-hA_3), microglia, colon carcinoma, glioblastoma, melanoma and in foam cells.[43,74,233–240] However, an inhibition of ERK activation has been revealed in A375 melanoma, prostate cancer, and glioma cells, leading to a reduction of cell proliferation as well as a decrease of TNF-α release in RAW 264.7 cells.[22,73,241,242] The A_3AR also activates p38 MAPKs in different cellular models including CHO-hA_3, hypoxic melanoma, glioblastoma, and colon carcinoma cells,[235,237,243] while reducing p38 MAPKs in human synoviocytes.[53] Concerning c-Jun N-terminal kinase (JNK), its activation by A_3AR has been retrieved in microglia and glioblastoma cells, leading to cell migration and matrix metalloproteinase 9 (MMP-9) stimulation, respectively.[74,244] MEK/ERK1/2 and PI3K/Akt signaling pathways downstream of the A_3AR control multiple resistance-associated protein 1 (MRP1) transporter substrate in glioblastoma cells, demonstrating a chemosensitizing effect by pharmacological blockade of A_3AR and consequent reduction in tumor size.[245]

A pathway involving Akt phosphorylation protects RBL-2H3 and glioblastoma cells from apoptosis, while the same pathway produced an antiproliferative effect and increase in MMP-9 in A375 and glioblastoma cells, respectively.[74,236,246,247] On the other hand, A_3AR-mediated activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt signaling pathway enhances pigmentation in B16melanoma cells and in human skin explants.[85]

The PI3K/Akt and nuclear factor (NF) κB signaling pathways are the mediators of the anti-inflammatory A_3AR-mediated effects, as observed in activated BV2 microglial cells, monocytes, adjuvant-induced arthritis, and in mesothelioma.[75,248–253] Akt inhibition is involved in the reduction of A_3AR-mediated of hypoxia inducible factor 1 (HIF-1α) accumulation in murine astrocytes.[254] In addition, the block of PI3K/Akt/mammalian target of rapamycin (mTOR) signaling through A_3AR suppresses angiogenesis in endothelial cells.[127]

Furthermore, an A_3AR-mediated decrease in the protein kinase A (PKA) level was responsible for: (i) an increase in glycogen synthase kinase 3β (GSK-3β), leading to a downregulation of beta-catenin and its transcriptional gene products cyclin D1 and c-Myc, and (ii) a decrease in the level of NF-κB DNA-binding capacity. This effect through A_3AR activation has been reported in melanoma, hepatocellular carcinoma, synoviocytes from RA patients, and in adjuvant-induced arthritis rats.[89,255–257] Interestingly, a downregulation of A_3AR mRNA/protein expression in colon cells after ulcerative colitis by miR-206 led to an increase of NF-κB and the downstream cytokine (TNF-α/IL-8/IL-1β) expression in the mouse colon, producing a proinflammatory effect.[19]

6. BIOLOGICAL ROLE AND THERAPEUTIC APPLICATIONS IN MODELS OF IMMUNE DISORDERS AND CANCER

6.1 Preclinical studies in immune cells

The A_3AR is expressed in almost all cells of the immune system acting as essential mediators of adenosine's role in inflammation.[258–260]

A growing body of evidence suggests a key role of the A_3AR in *neutrophil* behavior. In recent years, it has been reported that the A_3AR is distributed in a highly polarized fashion on the neutrophils cell membrane and contribute to their chemotaxis and migration. Specifically, A_3AR is translocated to the neutrophil leading edge, and adenosine generated by ecto-ATPases/nucleotidases results in autocrine stimulation of A_3AR-enhanced polarized migration, following its initiation by ATP activating the $P2Y_2$ receptor.[34,261–263] This was confirmed also in a sepsis model of A_3ARknockout (KO) mice where a reduction in the recruitment of neutrophils to the lung and peritoneum was observed.[264] In contrast, in human breast cancer cells, the A_3AR is expressed in multiple leading edges where it promotes cell migration with numerous directional changes. Indeed, exogenous adenosine may simultaneously stimulate A_3AR on all leading edges of the cell, inducing it to spread out in opposing directions resulting in arrest of cell motility.[263] Furthermore, it has been found that the endogenous A_3AR aggregates into plaque-like microdomains that affect cytoskeletal remodeling. In addition, they promote the formation of membrane protrusions, also named cytonemes, which enable neutrophils to capture pathogens.[36] In contrast, data in early literature reported that chemotaxis and, in addition, oxidative burst were inhibited by A_3AR activation with anti-inflammatory effects.[32,33,35]

Adenosine modulates *monocyte-macrophage* functions, being responsible for both inflammatory mediator production and resolution induction. For example, A_3AR stimulation inhibits the respiratory burst, interleukin (IL) 1β, TNF-α, chemokine macrophage inflammatory protein (MIP) 1α, interferon regulatory factor 1, iNOS (inducible nitric oxide synthase), and CD36 gene expression.[38,40,41,249,265–267] However, adenosine reduced the expression of adhesion molecules on monocytes and decreased cytokine production, effects that were potentiated by an A_3AR antagonist.[268] In addition, A_3AR stimulation increased TNF-α production in activated macrophages.[231]

A functional A_3AR is expressed in *dendritic cells*, antigen-presenting entities activating naive T lymphocytes and starting primary immune responses.[269,270] In particular, the A_3AR in the human immature elements has been found to induce elevated Ca^{2+} levels, actin polymerization, and chemotaxis, while in mature dendritic cells, the A_3AR is down-regulated and decreases TNF-α release.[44,46]

T cells represent the major actors in adaptive immunity and play a crucial role in the battle against infections and tumors. Both cytotoxic ($CD8^+$) and helper ($CD4^+$) T cells express A_3AR.[48,271] Initial studies attributed to the A_3AR an immunosuppressive role toward T cell mediated immune responses, but the mechanisms involved have not been investigated.[272,273] Interestingly, oral administration of an A_3AR agonist increased the cytotoxic activity of mouse NK cells and serum IL-12, thus reducing in vivo growth of melanoma cells.[274] Indeed, ex vivo activation of $CD8^+$ T cells with an A_3AR agonist improves adoptive immunotherapy for melanoma.[275] A_3AR has been found to be upregulated in peripheral blood mononuclear cells (PBMCs) obtained from patients affected by different autoimmune disorders such as RA, Crohn's disease, and psoriasis. This effect has been ascribed to the increase of TNF-α resulting in an upregulation of NF-κB and the cAMP response element binding protein (CREB), acting as A_3AR transcription factors.[276] Therefore, A_3AR could be indicated as a biological predictive marker in autoimmune inflammatory pathologies. Furthermore, an immunosuppressive effect has been confirmed in lymphocytes derived from patients affected by RA, where the A_3AR, upregulated with respect to healthy subjects, inhibited the NF-κB signaling, inflammatory cytokines, and MMPs production. Their density inversely correlated with indexes used to assess RA disease activity, supporting the importance of A_3AR in the control of RA joint inflammation.[277] Furthermore, A_3AR activation in rat models hampered damage of cartilage, formation of osteoclast/osteophyte, bone destruction, and generation of pannus and of lymphocyte formation.[278,279] Similarly, an A_3AR anti-inflammatory effect, that is, NF-κB-TNF-α-dependent has also been found in synoviocytes of RA patients.[257] Interestingly, this mechanism has been suggested also in A_3AR-induced hepatoprotection against ischemia/reperfusion (IR) injury.[280]

The A_3ARhas long been recognized as a major contributor of rodent *mast cell* activation by increasing their degranulation and, more recently, this factor has been observed also in both primary human and LAD2 mast cells.[9,13,63,82–84,281] A_3AR activation with an agonist significantly increased IL6, IL8, VEGF, amphiregulin, and osteopontin genes in human mast cells, affecting severe asthma.[285,286] Indeed, prolonged treatment with an agonist produced A_3AR downregulation responsible for the suppression of its basal inhibitory effect on cytokine production. This response was obtained only at a transcriptional level, suggesting that, at variance with rodents, in humans the primary role of the A_3AR is to act as a modulator rather than a stimulator of mast cell responses.[287]

Activation of the A_3AR induced hypothermia through the induction of mast cell degranulation, consequent histamine release, and activation of central histamine H_1 receptors.[288]

6.2 Preclinical studies in cancer cells

The role of A_3AR in cancer has been extensively studied using selective agonist and antagonist ligands, often with contrasting results. The efficacy of antagonists as anticancer drugs has been supported starting by the concept that hypoxia, characteristic of solid tumors, increases adenosine levels and stabilizes HIF-1α, the most important factor regulating cellular responses to the lack of oxygen.[289] In this context, it has been reported that A_3AR stimulation induced HIF-1α accumulation in different cancer cell lines.[235,243] This has been seen to lead to an increase in angiopoietin-2 and/or VEGF, depending on the cell model investigated.[237] Accordingly, it has been found that A_3AR stimulation increased microvessel density, expression of proangiogenic factors, macrophage tumor infiltration, and cytokine production in in vivo melanoma tumor models.[290] Furthermore, a basal stimulation through A_3AR was responsible for an increased MRP1 expression in glioblastoma cells. As a consequence, A_3AR antagonist administration provoked a potentiating effect on the reduction in tumor size induced by the chemotherapic vincristine.[245] In addition, an increase of glioblastoma cell invasion, through A_3AR and MMP-9 stimulation, was found, as already shown in macrophages.[74,291] However, a potential therapeutic effect of agonists in cancer has been also reported moving from initial studies on the observation that tumor metastases were infrequent in striated muscles. Interestingly, it has been found that in addition to adenosine, natural agonists of A_3AR were secreted from muscle cells, contributing to the systemic anticancer and chemoprotective activity exerted by muscle-conditioned medium. This evidence explained the rarity of tumor metastases in muscle and represented the rationale for the utility of A_3AR agonists in cancer.[292,293] Further studies reported that

A_3AR activation inhibited telomerase activity and exerted cytostatic effects in tumor cells.[10,255,294,295] Interestingly, cancer tissues and the interstitial fluid of several tumors contained high levels of adenosine able to activate ARs, among which the A_3AR subtype was the most expressed.[230,296,297] For example, an A_3AR upregulation has been found in human colorectal and hepatocellular carcinomas that was reflected in peripheral blood cells, thus making this AR subtype a possible marker for cancer, reflecting receptor status in remote tumor tissue.[87–89] As for the role of A_3AR in tumors, pro- and antiproliferative effects due to their activation have been documented in several cancer cell types.[69,74,75,298–307] A_3AR activation decreased prostate cancer cell migration in vitro and in vivo and inhibited cell proliferation, inducing G1 cell cycle arrest and apoptosis.[70,73,308]

Even though contrasting results about the role of A_3AR agonists and antagonists in cancer still are present in literature data, only the therapeutic utility of A_3AR agonists has been supported by in vivo studies. These studies encompassed syngeneic xenograft, orthotopic and metastatic experimental animal models of colon, prostate, melanoma, and hepatocellular carcinomas, in which IB-MECA and Cl-IB-MECA were administered orally in view of their stability and bioavailability. These agonists inhibited cell growth in syngeneic and lung metastatic models of murine melanoma, and potentiated cyclophosphamide effect.[10] Also in xenograft models, IB-MECA inhibited the development of human colon and prostate tumors in nude mice and increased 5-fluorouracil or taxol antitumor effect. Furthermore, it blocked primary and liver metastases of colon carcinoma cells inoculated in the spleen. Finally, Cl-IB-MECA reduced hepatocellular tumor growth, liver inflammation, and cancer pain in rat bone residing breast cancer.[10,76,86,89]

6.3 Clinical trials

The scientific evidence obtained through in vitro and in vivo experiments led to the progression of A_3AR agonists in clinical studies for the therapy of inflammatory and cancer pathologies. Importantly, they were found safe and well tolerated in all preclinical, Phases I and II human clinical studies. According to clinicaltrials.gov, the agonist IB-MECA (Piclidenoson, CF101) entered in the following clinical trials (NCT numbers):

1. RA (Phase II, NCT00280917; Phase II, NCT01034306; Phase II, NCT00556894) in which it showed significant antirheumatic effect as a standalone drug. Interestingly, a direct significant correlation was found between receptor expression at baseline and patients' response to the drug. CF101 treatment resulted in an ACR20 (American College of Rheumatology Criteria for disease improvement) of 48.6%, statistically significantly higher than that of the placebo group (25%) at week 12. CF101 treatment also showed superiority in ACR50 and ACR70 values versus placebo. These data suggest that the A_3AR is a promising therapeutic target in RA and can be used also as a biologicalmarker to predict patients' response to CF101. This is a unique type of a personalized medicine approach, which may pave the way for a safe and efficacious treatment for this patient population.
2. Plaque psoriasis (Phase II, NCT00428974; Phase II/III, NCT01265667) in which even though the primary endpoint, which was a 75% reduction in the psoriasis

area and severity score (PASI75) at week 12, was not reached, however 24.7% of subjects achieved PASI90 at week 32.[309,310] In addition, CF101 was more efficacious than apremilast (Otezla), a PDE4 inhibitor, on week 32 while having an excellent safety profile, suggesting its promise as a chronic treatment.

3. Ocular hypertension (NCT01033422), keratoconjunctivitis sicca (dry eye disease; Phase II, NCT00349466; Phase III, NCT01235234), RA in cotreatment with methotrexate (NCT00280917) failed to reach their endpoints.

Furthermore, additional trials are expected in the near future for the treatment of: RA (Phase III, NCT02647762); osteoarthritis of the knee (Phase II, NCT00837291).

The agonist Cl-IB-MECA (Namodenoson, CF102) has been in the following clinical trials:

1. Advanced hepatocellular carcinoma (Phase I/II, NCT00790218), finding that it is able to induce a median overall survival (OS) of 7.8 months.[311] In patients with advanced HCC and Child Puh B, CF102 induced an OS of 9.3 months (literature data demonstrate OS of 3.5 months). A global Phase II study in this patient population is currently ongoing.
2. Chronic hepatitis C genotype 1 (Phase I/II, NCT00790673).

Additional trials of CF102 are expected soon for the treatment of: nonalcoholic steatohepatitis (Phase II, NCT02927314) and hepatocellular carcinoma (Phase II, NCT02128958).

An A_3AR allosteric enhancer (CF602, LUF6000 **95**) demonstrated an anti-inflammatory effect in vivo. It is currently under development for treating sexual dysfunction.

7 CONCLUSIONS

The impact of the A_3AR on the drug discovery process and development is rapidly expanding, and its significance for human health should not be underestimated. Purine scientists are well advised to remain optimistic for the three following reasons. First, A_3AR is overexpressed in cancer and inflammatory cells in comparison to healthy cells, findings that are mirrored in the PBMCs of patients affected by these pathologies. Second, highly selective A_3AR agonists are now available as tool compounds and potential clinical molecules. They induce both anti-inflammatory/anticancer effects in pathological cells, as well as protective functions in normal cells, have been synthesized. Third, clinical data that correlate a high expression of A_3AR at baseline prior to agonist treatment with a beneficial response in patients suggest that modulation of the A_3AR may lead to a personalized medicine approach. Thus, new findings with A_3AR ligands appear to open new opportunities to fight inflammatory diseases, cancer, and other conditions.

Abbreviations

ADA	adenosine deaminase
AK	adenosine kinase

AR	adenosine receptor
cAMP	cyclic AMP
CCI	chronic constriction injury
CD73	ecto-5′-nucleotidase
CHO-hA$_3$	Chinese hamster ovary cells expressing the human A$_3$AR
Cl-IB-MECA	2-Chloro-N6-(3-iodobenzyl)-adenosine-5′-Nmethyluronamide
CNT	concentrative nucleoside transporter
CREB	cAMP response elements binding protein
ENT	equilibrative nucleoside transporter
ERK1/2	extracellular signal-regulated kinases
GPCR	G protein coupled receptor
GSK-3β	glycogen synthase kinase-3β
HIF-1α	hypoxia inducible factor 1
IB-MECA	N6-(3-Iodobenzyl)adenosine-5′-N-methyluronamide
IL	interleukin
IR	ischemia/reperfusion
JNK	c-Jun N-terminal kinase
KO	knockout
MIP	macrophage inflammatory protein
MMP-9	matrix metalloproteinase 9
MRP1	multiple resistance-associated protein 1
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor κB
PET	positron emission tomography
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C

SAH	*S*-adenosylhomocysteine
TM	transmembrane domain
TNF-α	tumor necrosis factor α
PBMC	peripheral blood mononuclear cell
RA	rheumatoid arthritis.

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Biographies

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Pier Andrea Borea received his degree in Chemistry from the University of Ferrara in 1967. He is currently President of the Evaluation Board of the University of Ferrara. He contributed to some 400 publications in international journals and about 20 chapters in international books. His main field of interest is represented by the study, at the molecular level, of drug–receptor interactions.

Stefania Baraldi received her doctoral degree in Pharmaceutical Chemistry and Technology with 110 cum Laude (University of Ferrara, 2005), her Ph.D. in Pharmaceutical Science/ medicinal Chemistry (University of Ferrara, 2009) and her second-level master's degree in Cosmetic Science and Technology with Top Marks (University of Ferrara, 2012). Her scientific interests have focused on the design and synthesis of ligands in the adenosine field and Endocannabinoid System modulators for the treatment of pain and inflammation. Her research activity has been supported by the company King Pharmaceutical (North Carolina, USA) now part of Pfizer.

Mojgan Aghazadeh Tabrizi received her doctoral degree in Pharmacy (University of Ferrara, 1987), Ph.D. in Medicinal Chemistry (University of Ferrara, 1992). She has worked at the Department of Pharmaceutical Sciences of Ferrara University focusing her interests on the adenosine receptors ligands and antitumor compounds in collaboration with the company King Pharmaceuticals (North Carolina, USA), now part of Pfizer. Since 2008, she has been part of a research project on the treatment of pain and inflammation involving the endocannabinoid system. She is author/coauthor of several scientific publications in the field of medicinal chemistry.

Romeo Romagnoli graduated in Pharmaceutical Chemistry and Technology at the University of Ferrara in 1990, and in 1995 he received his Ph.D. in Organic Chemistry. From 1999 to 2014, he has held a position of Assistant Professor in Pharmaceutical Chemistry at the University of Ferrara. Actually he is Associate Professor in Pharmaceutical Chemistry at the same University. His scientific interests have focused on new synthetic methods for the preparation of natural substances or biologically active analogues, the medicinal chemistry of ligands for adenosine receptors subtypes and antitumor agents. He has published more than 230 research papers and 20 international patents.

Pier Giovanni Baraldi received his doctoral degree in Chemistry in 1974 from the University of Ferrara where he is currently Full Professor of Medicinal Chemistry. He has published more than 400 scientific papers including about 50 patents and he participated in more than 90 Medicinal Chemistry meetings as plenary speaker. His research interests have focused on the design and synthesis of minor groove alkylating agents, combretastatin analogs, ligands for ARs, cannabinoid receptors, and TRP channel modulators. He has been promoter of several scientific collaborations with national and international pharmaceutical companies.

Antonella Ciancetta received hermaster's degree in Pharmaceutical Chemistry and Technology in 2007 and her Ph.D. degree in Drug Sciences in 2010 from the University of Chieti, Italy. She is currently a Visiting Fellow at the National Institutes of Health in Bethesda, Maryland, USA in Dr. Jacobson's laboratory. Her research focuses on the application and development of structure-based molecular modeling techniques for the design of GPCR ligands and the elucidation of their binding mechanisms.

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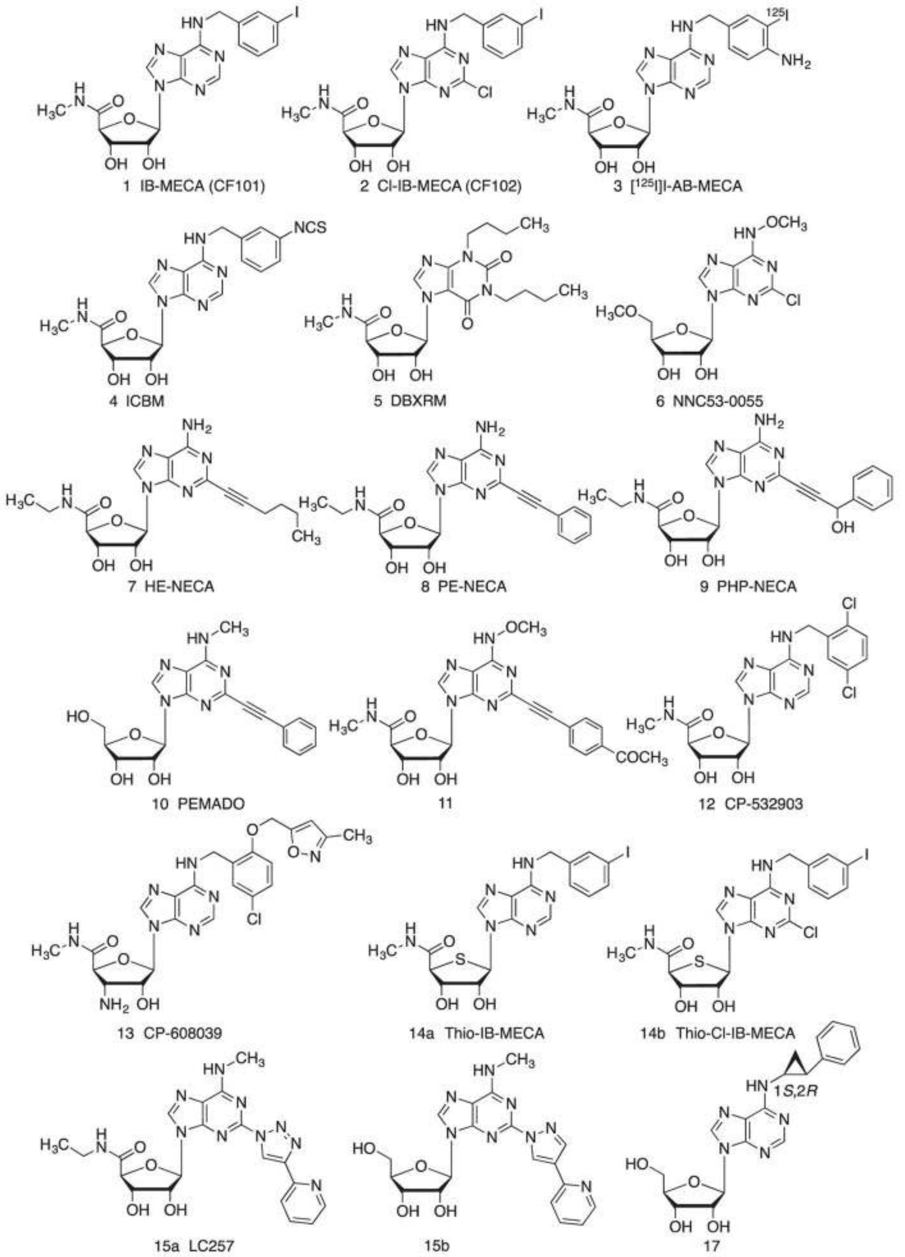


1 IB-MECA (CF101)
2 Cl-IB-MECA (CF102)
3 [^{125}I]I-AB-MECA
4 ICBM
5 DBXRM
6 NNC53-0055
7 HE-NECA
8 PE-NECA
9 PHP-NECA
10 PEMADO
11
12 CP-532903
13 CP-608039
14a Thio-IB-MECA
14b Thio-Cl-IB-MECA
15a LC257
15b
17

FIGURE 1.

Structures of adenosine or 1,3-dialkylxanthine riboside derivatives that act as agonists of the A_3AR. Compound **16** (CGS21680, not shown) is an A_{2A}AR agonist

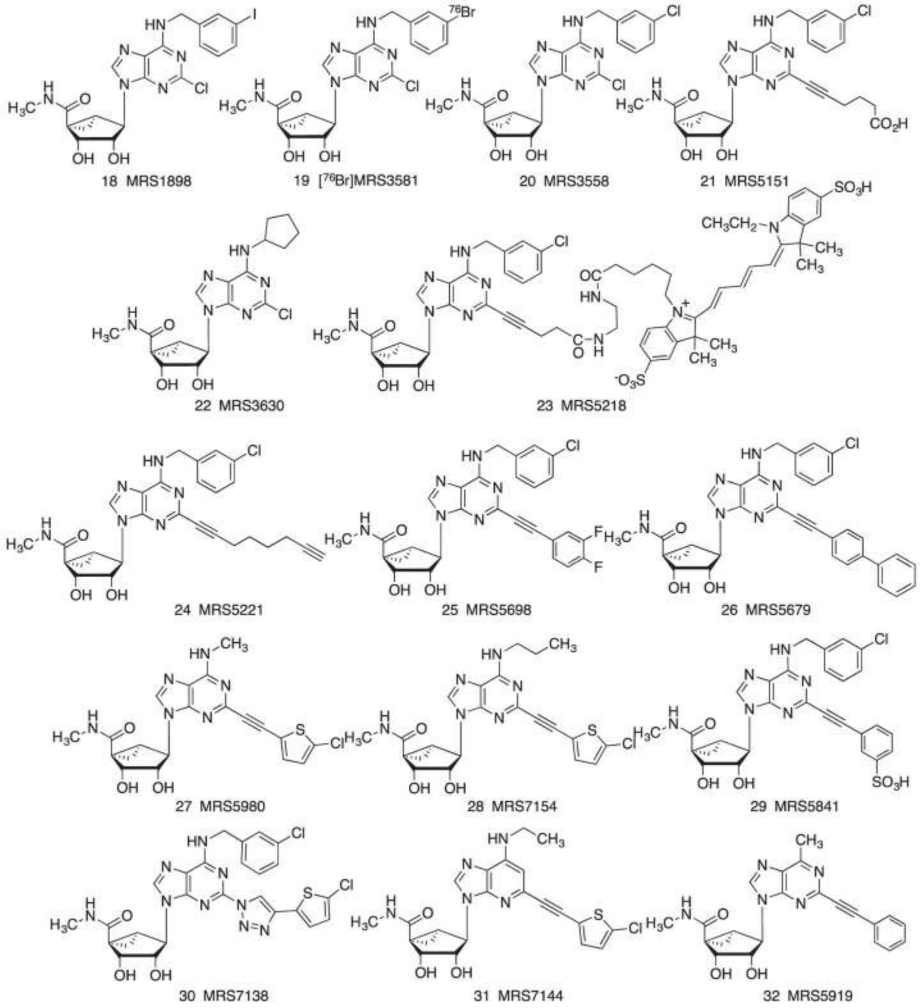


FIGURE 2.

Structures of (N)-methanocarba-adenosine derivatives that act as agonists of the A_3AR

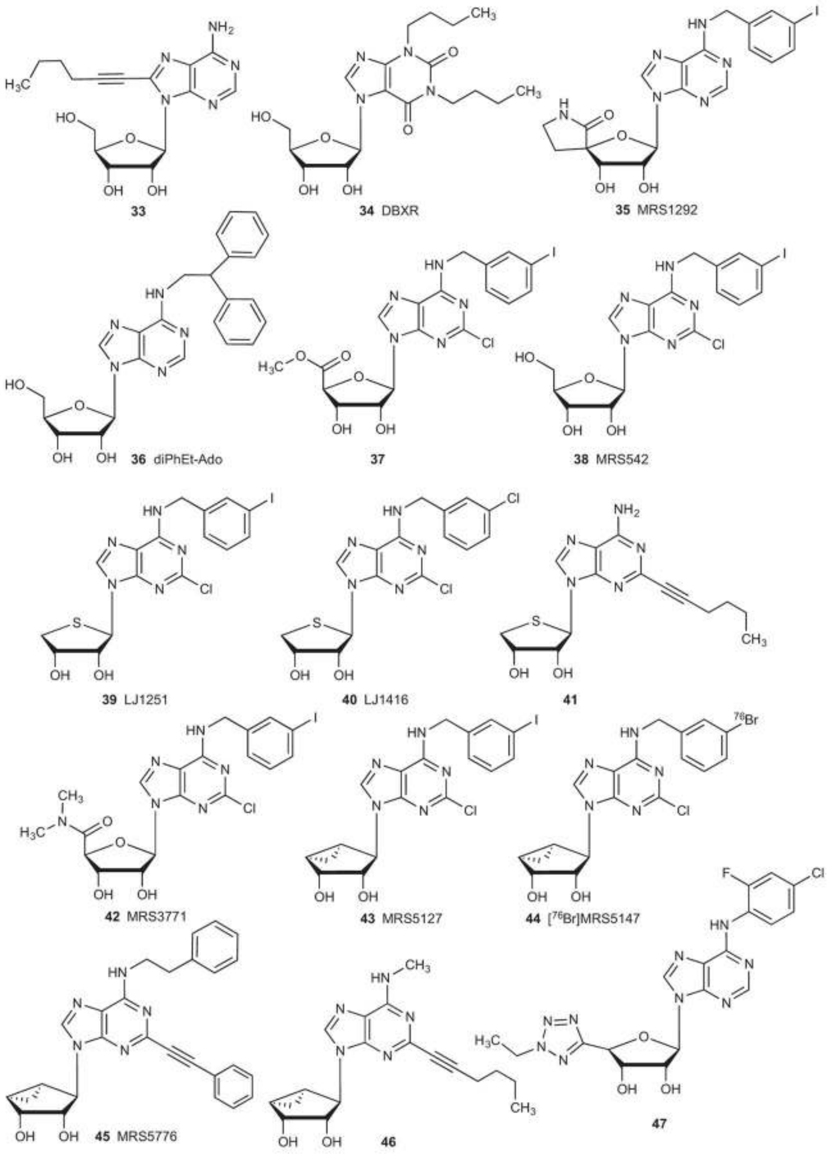


FIGURE 3.

Structures of (N)-methanocarba and riboside derivatives of adenosine or 1,3-dialkylxanthine that act as partial agonists or antagonists of the A_3AR

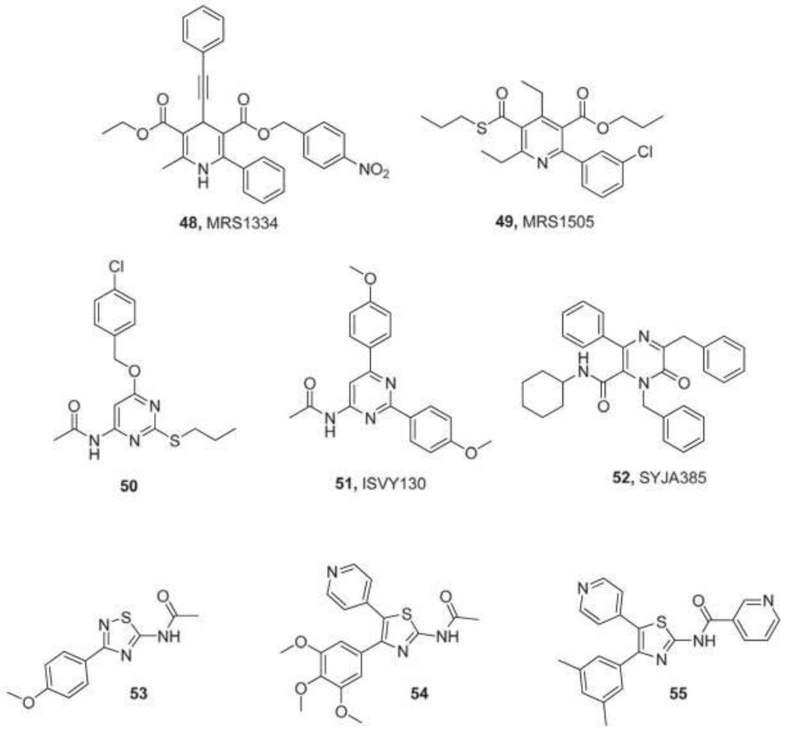


FIGURE 4.

Monocycle-based A_3AR antagonists

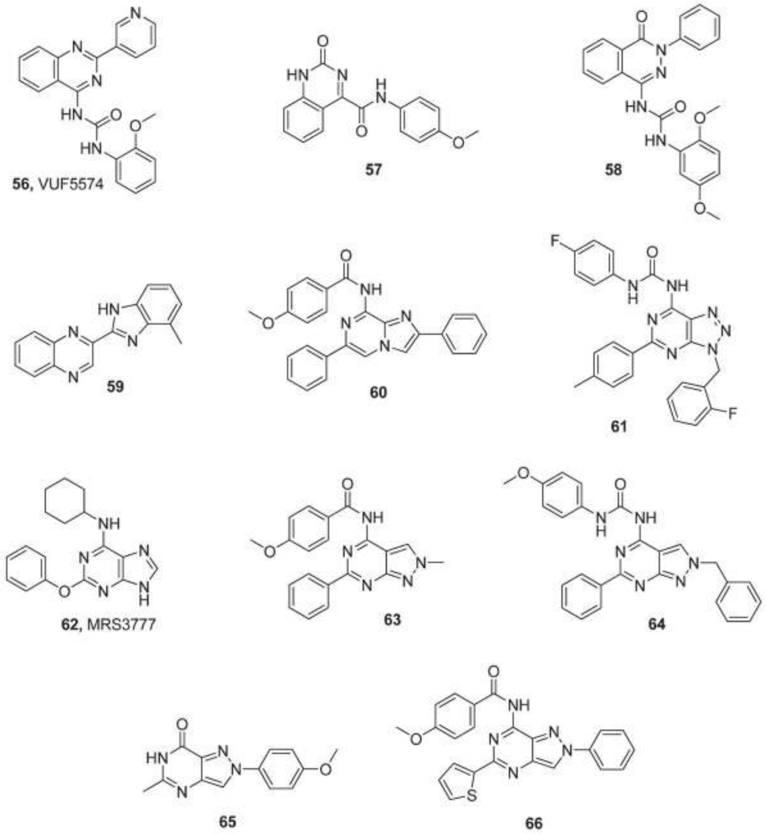


FIGURE 5.

Bicycle-based A_3AR antagonists

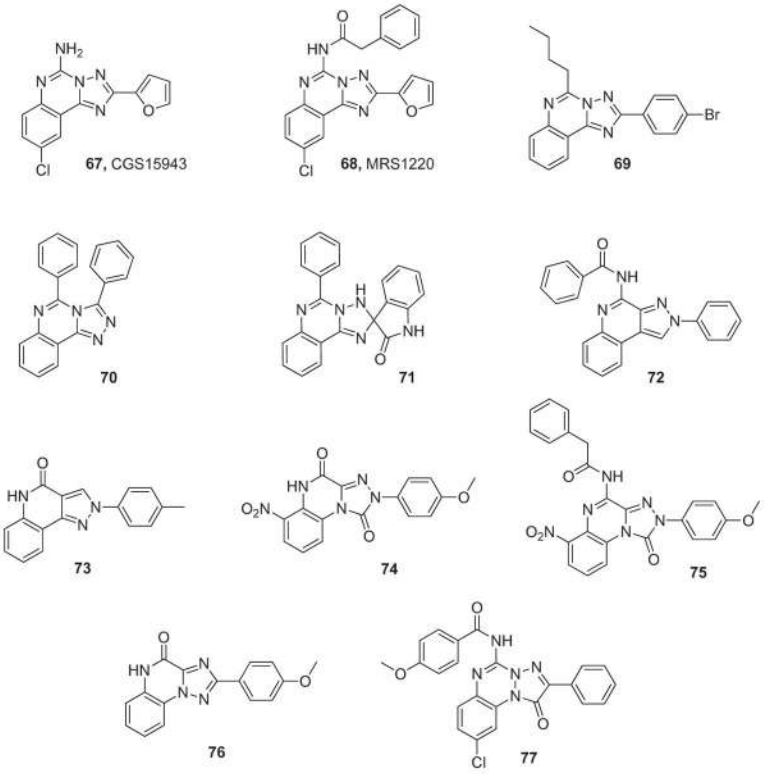
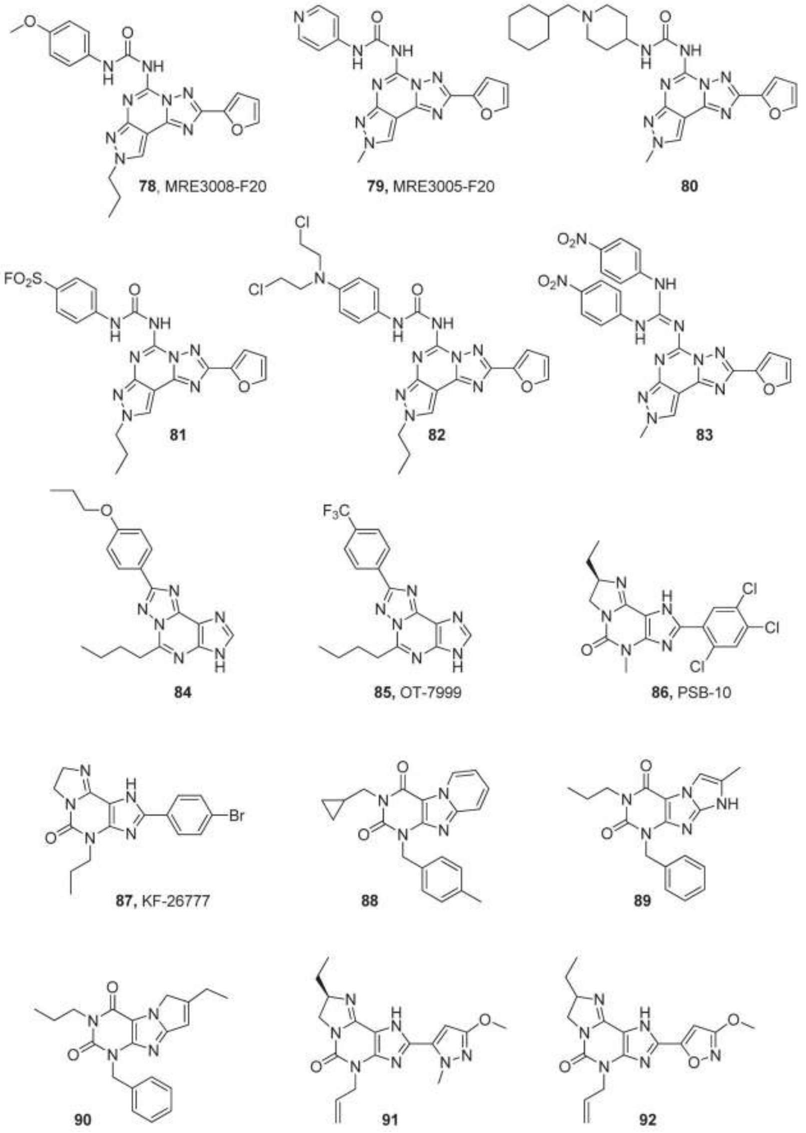


FIGURE 6.
Tricycle-based A_3AR antagonists



78, MRE3008-F20 **79,** MRE3005-F20 **80**

81 **82** **83**

84 **85,** OT-7999 **86,** PSB-10

87, KF-26777 **88** **89**

90 **91** **92**

FIGURE 7.

Tricycle-based A_3AR antagonists

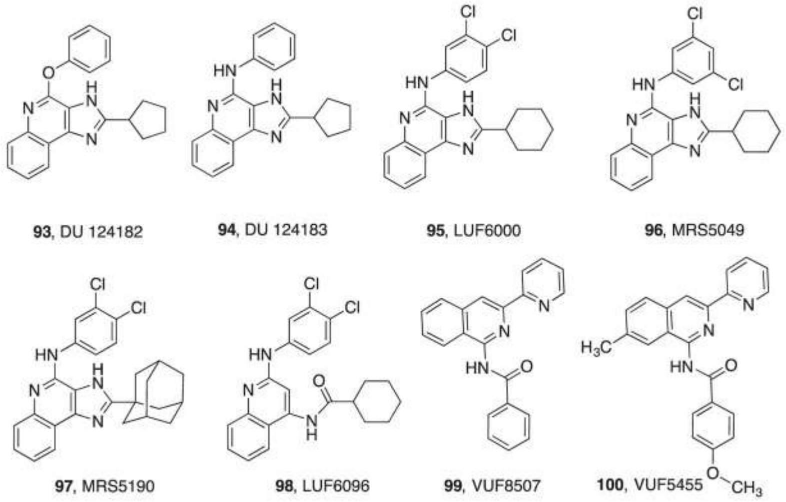


FIGURE 8.

Allosteric modulators of the A_3AR

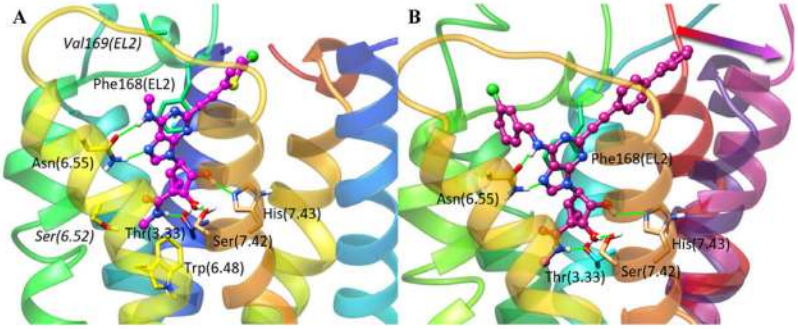


FIGURE 9.

(A) Docking pose of 3,4-difluorophenyl agonist analogue MRS5980 (**27**) at the hA_3AR homology model, in which TM2 is based on its position in the active β^2 adrenergic receptor. Residues interacting with the ligand (magenta carbon atoms) are labeled. H-bond and π–π interactions are represented as green solid and cyan dashed lines, respectively. Nonconserved ARs residues are in italics. (B) Docking pose of biphenyl agonist analogue MRS5679 (**26**) at the hA_3AR homology model. Residues interacting with the ligand (violet carbon atoms) are labeled and H-bond interactions are represented as green solid lines. The degree of displacement of TM2 with respect to the TM bundle in hA_3AR homology models based on the $hA_{2A}AR$ (red ribbon), hybrid $hA_{2A}AR$-β_2 adrenergic receptor (purple ribbon), and hybrid $hA_{2A}AR$-opsin (violet ribbon) templates is highlighted with an arrow. TM1 is omitted to aid visualization

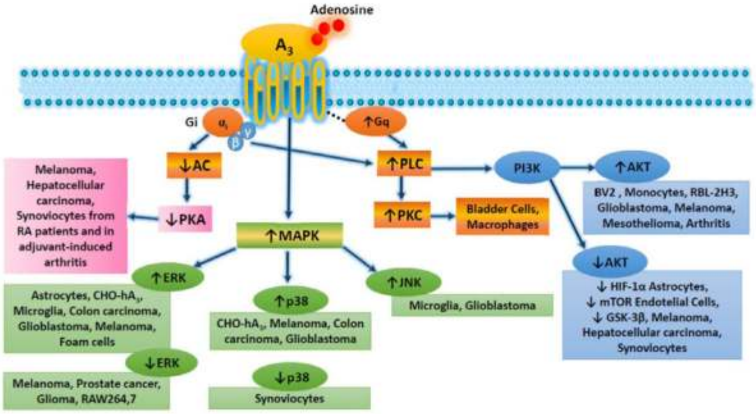


FIGURE 10.

Intracellular pathways of A_3AR in immune and cancer cells. Schematic diagram showing the main signaling pathways triggered by adenosine through A_3AR activation in different cellular types. A_3AR preferentially couples to Gi. PLC activation, and even the Ca^{2+} effects observed at high concentrations of A_3AR agonists, could conceivably be triggered by mechanisms other than Gq, such as $G\beta\gamma$ subunits

TABLE 1

Expression of the A_3AR RNA in Normal and Cancerous Tissues from Public Databases

(A) Exon expression for the A_3AR gene in various postmortem human tissues, from RNA sequencing data[a,b]	
Tissue	**RPKM**[b]
Testes	12.401
Brain (spinal cord, cervical C-1)	5.612
Brain (substantia nigra)	4.268
Adrenal gland	3.884
Spleen	3.495
Small intestine (terminal ileum)	2.778
Brain (amygdala)	2.405
Brain (hypothalamus)	2.201
Nerve (tibial)	2.102
Brain (hippocampus)	1.99
Bladder	1.764
Lung	1.747
Adipose (subcutaneous)	1.73
Whole blood	1.709
Colon (transverse)	1.604
Artery (coronary)	1.517
(B) Alteration in the level of A_3AR in cancerous tumors compared to normal tissue[c]	
Tumor	**Percentile (no. of analyses)**[c]
Upregulation	
Brain and CNS cancer[d]	1% (4/29)
Kidney cancer	1% (6/20)
Breast cancer	5% (11/43)
Esophageal cancer	10% (1/9)
Downregulation	
Bladder cancer	5% (3/10)
Colorectal cancer	5% (12/33)
Sarcoma	5% (1/31)
Brain and CNS cancer	10% (1/29)
Cervical cancer	10% (1/10)
Myeloma	10% (1/8)

[a] Data from The Genotype-Tissue Expression (GTEx) Project. The data were accessed from the GTEx portal (http://www.gtexportal.org/home/) on February 9, 2017, GTEx Analysis Release V6p (dbGaP Accession phs000424.v6.p1).

[b] RPKM stands for reads per kilobase of transcript per million mapped reads for the A_3AR gene (ADORA3, gencode ID ENSG00000121933.13). The highest 16 values are shown from a total of 53 tissues assayed.

[c] Percentile refers to the best gene rank percentile for the analyses within the cell. The data were accessed from http://oncomine.org on February 9, 2017, using gene summary visualization for ADORA3. Ratio refers to the number of analyses out of the total number that met the criterion of $p < 10^{-4}$ for the change in expression in cancer versus normal tissue.

[d] Highly significant upregulation of ADORA3 noted in numerous analyses of glioblastoma and astrocytoma.

TABLE 2

Affinity of Selected Nucleoside Derivatives in Binding at Human ARs

		pK_i value		
Compound		**A_1AR**	**A_{2A}AR**	**A_3AR**
Agonists				
1[120]	IB-MECA	7.29	5.50	8.74
2[120]	Cl-IB-MECA	6.66	5.27	8.85
7[99,103]	HE-NECA	7.22	8.19	8.62
8[99,103]	PE-NECA	6.25	6.21	8.21
9[99,103]	PHP-NECA (*R*,*S*)	8.57	8.51	9.38
10[99,103]	PEMADO	4.48	4.38	8.52
11[104]		4.27	4.98	8.60
13[110]	CP-608,039	5.14	<4.3	8.24
14a[126]		<5	<5	7.81
14b[126]		6.71	5.36	9.42
15a[132]	LC257	5.79	<4	8.74
15b[132]		5.42	<5.30	8.70
16[95]	CGS21680	6.54	7.57	7.17
20[105]	MRS3558	6.59	5.64	9.54
21[119]	MRS5151	4.83	~5	8.62
22[117]	MRS3630	7.74	5.49	8.43
25[120,121]	MRS5698	<5	<5	8.52
26[121]	MRS5679	<5	<5	8.51
27[121]	MRS5980	<5	<5	9.15
29[122]	MRS5841	<5	<5	8.72
32[134]	MRS5919	<5	<5	8.22
Antagonists and partial agonists				
33[136,142]		<4	<4	6.19
35[128]	MRS1292	ND	ND	7.53[a]
37[109]		5.80	5.32	7.91
39[139]		5.60	6.47	8.38
41[143]		<4	8.14	7.93
42[175]	MRS3771	5.23	<5	7.54
45[125]	MRS5776	<5	<5	7.70
46[140]		<5	5.13	8.31
47[141]		9.36	7.11	8.52

[a] pK_i at rat A_3AR = 7.31.

ND, not determined.

TABLE 3

Affinity of Selected A$_3$AR Antagonists

		pK$_i$ value or *% inhibition at 10 μM*		
	Compound	**A$_1$AR**	**A$_{2A}$AR**	**A$_3$AR**
Monocyclic systems				
48[155]	MRS1334	5.54 (r)	*<10%*(r)	5.41 (r)
				8.57 (h)
49[158]	MRS1505	4.38 (r)	4.62 (r)	6.09 (r)
				8.10 (h)
50[162]		*17%* (h)	*43%*(h)	8.46 (h)
51[164]	ISVY130	*1%* (h)	*10%* (h)	8.44 (h)
52[165]	SYJA385	*7%* (h)	*10%*(h)	6.41 (h)
53[167]		*24%* (h)	*28%* (h)	9.10 (h)
54[146]		<5 (h)	<5 (h)	9.39 (h)
55[146]		<6.18 (h)	<6.08 (h)	9.44 (h)
				8.80 (r)
Bicyclic systems				
56[169]	VUF5574	*52%* (r)	*43%*(r)	8.39 (h)
57[170]		*4%* (h)	*1%* (h)	7.71 (h)
58[171]		*0%* (h)	*19%*(h)	9.11 (h)
59[135]		5.10 (h)	6.08 (h)	7.59 (h)
60[173]		*6%* (h)	*8%*(h)	7.60 (h)
61[174]		6.37 (h)	5.09 (h)	8.22 (h)
62[175]	MRS3777	*26%* (h)	*16%*(h)	7.33 (h)
63[176]		5.98 (h)	5.50 (h)	9.74 (h)
64[176]		<5 (h)	<5 (h)	8.54 (h)
65[177]		*5%* (h)	*1%* (h)	8.92 (h)
66[179]		*1%* (h)	*1%*(h)	10.57 (h)
Tricyclic systems				
67[180]	CGS15943	8.46 (h)	9.40 (h)	7.02 (h)
68[182]	MRS1220	7.28 (r)	8.00 (r)	9.19 (h)
69[183]		<5 (h)	<5 (h)	8.09 (h)
70[184]		<6 (h)	6.98 (h)	8.94 (h)
71[184]		<6 (h)	<6 (h)	8.16 (h)
72[185]		*42%* (b)	*3%* (b)	8.68 (h)
73[186]		<6 (h)	<6 (h)	8.05 (h)
74[188]		*25%* (b)	*14%* (b)	8.33 (h)

		pK$_i$ value or *% inhibition at 10 μM*		
	Compound	**A$_1$AR**	**A$_{2A}$AR**	**A$_3$AR**
		0% (h)		
75[189]		6.59 (b)	*0%* (b)	9.10 (h)
		7.96 (h)	*2%* (h)	
76[190]		*0%* (b)	8.06 (b)	8.68 (h)
77[192]		5.57 (h)	<5 (h)	8.80 (h)
78[193]	MRE3008-F20	<5 (r)	5.70 (r)	9.54 (h)
79[203]	MRE3005-F20	6.60 (h)	7.22 (h)	10.40 (h)
80[149]		5.47 (h)	<5.3	8.01 (h)
81[204]		ND	ND	*79%* (h)
82[204]		ND	ND	*16%* (h)
83[196]		<6 (h)	<6 (h)	7.74 (h)
84[143]		*32%* (h)	*49%* (h)	9.29 (h)
85[143]	OT-7999	*4%* (h)	*31%*(h)	9.02 (h)
86[207]	PSB-10	5.77 (h)	5.56 (h)	9.36 (h)
87[209]	KF-26777	5.74 (h)	6.33 (h)	9.70 (h)
88[211]		*24%* (h)	*0%* (h)	8.66 (h)
89[200]		<6 (h)	<6 (h)	9.10 (h)
90[200]		<6 (h)	<6 (h)	8.46 (h)
91[195]		5.60 (h)	<5.3 (h)	8.84 (h)
92[195]		5.52 (h)	5.82 (h)	8.71 (h)

h, human; r, rat; and b, bovin.

ND = not determined.