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N⁶-Substituted adenosine derivatives: selectivity, efficacy, and species differences at A₃ adenosine receptors

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Abstract

The activation of the human A₃ adenosine receptor (AR) by a wide range of N⁶-substituted adenosine derivatives was studied in intact CHO cells stably expressing this receptor. Selectivity of binding at rat and human ARs was also determined. Among N⁶-alkyl substitutions, small N⁶-alkyl groups were associated with selectivity for human A₃ARs vs. rat A₃ARs, and multiple points of branching were associated with decreased hA₃AR efficacy. N⁶-Cycloalkyl-substituted adenosines were full (≤5 carbons) or partial (≥6 carbons) hA₃AR agonists. N⁶-(endo-Norbornyl)adenosine **13** was the most selective for both rat and human A₁ARs. Numerous N⁶-arylmethyl analogues, including substituted benzyl, tended to be more potent in binding to A₁ and A₃ vs. A_{2A}ARs (with variable degrees of partial to full A₃AR agonisms). A chloro substituent decreased the efficacy depending on its position on the benzyl ring. The A₃AR affinity and efficacy of N⁶-arylethyl adenosines depended highly on stereochemistry, steric bulk, and ring constraints. Stereoselectivity of binding was demonstrated for N⁶-(R-1-phenylethyl)adenosine vs. N⁶-(S-1-phenylethyl)adenosine, as well as for the N⁶-(1-phenyl-2-pentyl)adenosine, at the rat, but not human A₃AR. Interestingly, DPMA, a potent agonist for the A_{2A}AR (*K_i* = 4 nM), was demonstrated to be a moderately potent antagonist for the human A₃AR (*K_i* = 106 nM). N⁶-[(1S, 2R)-2-Phenyl-1-cyclopropyl]adenosine **48** was 1100-fold more potent in binding to human (*K_i* = 0.63 nM) than rat A₃ARs. Dual acting A₁/A₃ agonists (N⁶-3-chlorobenzyl- **29**, N⁶-(S-1-phenylethyl)- **39**, and 2-chloro-N⁶-(R-phenylisopropyl)adenosine **53**) might be useful for cardioprotection.

Keywords

Purines; Nucleosides; GPCR; Cyclic AMP; Receptor binding; Structure–activity relationships

1. Introduction

The structure–activity relationships for adenosine derivatives as agonists at the ARs have been studied in both binding and functional assays [1,2]. Several of the four subtypes of adenosine receptors have cytoprotective effects when activated. The activation of the A₃AR by selective agonists has been demonstrated to be both cardioprotective and cerebroprotective [3,4]. In previous studies, a number of structural determinants for A₃AR activation have been identified [5–9], leading to the general conclusion that the ability of an adenosine derivative to activate the A₃AR is highly structure sensitive. In this study, we

further evaluated the binding affinity and functional properties of a wide range of N^6 -substituted adenosine derivatives at both human and rat A_3 ARs stably expressed in CHO cells.

The affinity and efficacy of many of these of N^6 -substituted adenosine derivatives have been evaluated at rat A_1 and/or A_{2A} ARs [10]. However, at A_3 ARs, the structure–activity relationships of a wide variety of N^6 -substituted adenosine derivatives has not been fully evaluated. Recent studies [5,6,8,9] suggested that various adenosine derivatives, previously assumed to be full/partial agonists at A_3 ARs, are indeed antagonists. Thus, the efficacy of adenosine derivatives at the A_3 AR appears to be more dependent on small structural changes than at other subtypes. In an effort to develop potent and selective A_3 receptor agonists and antagonists, the affinity and/or functional potency of these compounds for human and rat A_3 ARs were measured. In order to ascertain the selectivity within the same species, we also determined the binding affinity of selected adenosine derivatives at human and/or rat A_1 and A_{2A} ARs.

2. Materials and methods

2.1. Materials

[125 I] N^6 -(4-Amino-3-iodobenzyl)adenosine-5'- N -methyluronamide ([125 I]I-AB-MECA; 2000 Ci/mmol), [3 H]R-PIA (34 Ci/mmol), [3 H]DPCPX (120 Ci/mmol), and [3 H]cyclic AMP (40 Ci/mmol) were from Amersham Pharmacia Biotech. [3 H]CGS21680 (47 Ci/mmol) was from Perkin-Elmer Life Sciences. ENBA, NECA, CPA, CHA, DPMA (N^6 -[2-(3,5-dimethoxyphenyl)-2-(2-methylphenylethyl)] adenosine), ADAC, R - and S -PIA, N^6 -benzyl-NECA, and N^6 -benzyladenosine (Bn-Ado) were purchased from Sigma-RBI. Other N^6 -substituted adenosine derivatives were the kind gift of Dr. Ray A. Olsson (University of South Florida) and Dr. John W. Daly (NIDDK). All other chemicals were from standard commercial sources and of analytical grade.

2.2. Cell culture and membrane preparation

The CHO cells expressing recombinant human and rat A_3 ARs were cultured in DMEM and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μ g/mL streptomycin, 2 μ mol/mL glutamine, and 800 μ g/mL geneticin. After harvest and homogenization, cells were centrifuged at 500 g for 10 min, and the pellet was resuspended in 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. The suspension was homogenized with an electric homogenizer for 10 s, and was then re-centrifuged at 20,000 g for 20 min at 4°. The resultant pellets were resuspended in buffer in the presence of 3 U/mL adenosine deaminase, and the suspension was stored at –80° prior to the binding experiments. The membranes from rat forebrain and striatum were prepared as previously described [11]. Striatal and forebrain tissues from Wistar rats were homogenized in ice-cold 50 mM Tris–HCl buffer, pH 7.4, using an electric homogenizer. The homogenate was centrifuged at 20,000 g for 10 min at 4°, and the pellet was washed in fresh buffer. The final pellet was stored at –80° until the binding experiments. The protein concentration was measured using the Bradford assay [12].

2.3. Binding assay

For A_3 AR binding experiments, the procedures used were similar to those previously described [13]. Briefly, each tube contained 100 μ L of membrane suspension, 50 μ L of [125 I]I-AB-MECA (final concentration 0.5 nM), and 50 μ L of increasing concentrations of compounds in Tris–HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. Non-specific binding was determined using 10 μ M Cl-IB-MECA. The mixtures were incubated at 25° for 60 min. Binding reactions were terminated by filtration through

Whatman GF/B filters under reduced pressure using an MT-24 cell harvester (Brandell). Filters were washed three times with ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ -counter. The binding of [^3H]R-PIA to the rat forebrain and recombinant human A_1ARs and the binding of [^3H]CGS21680 to rat striatal and recombinant human $\text{A}_{2\text{A}}\text{ARs}$ were performed as previously described [11].

2.4. Cyclic AMP accumulation assay

Intracellular cyclic AMP levels were measured with a competitive protein binding method [14]. CHO cells expressing recombinant human and rat A_3ARs were harvested by trypsinization. After centrifugation and resuspension in medium, cells were planted in 24-well plates in 1.0 mL medium/well. After 24 hr, the medium was removed and cells were washed three times with 1 mL/well of DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with agonists and/or test compounds in the presence of rolipram (10 μM) and adenosine deaminase (3 U/mL). After 45 min forskolin (10 μM) was added to the medium, and incubation was continued for an additional 15 min. The reaction was terminated by removing the medium, and cells were lysed upon the addition of 200 μL /well of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at -20° . For determination of cyclic AMP production, protein kinase A (PKA) was incubated with [^3H]cyclic AMP (2 nM) in $\text{K}_2\text{HPO}_4/\text{EDTA}$ buffer (K_2HPO_4 , 150 mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL 0.1 M HCl, or 50 μL of cyclic AMP solution (0–16 pmol/200 μL for standard curve). Bound radioactivity was separated by rapid filtration through Whatman GF/C filters and washed once with cold buffer. Bound radioactivity was measured by liquid scintillation spectrometry.

2.5. Statistical analysis

Binding and functional parameters were estimated using GraphPAD Prism software (GraphPAD). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng–Prusoff equation [15]. Data were expressed as means $\pm$ standard error.

3. Results

3.1. Activation of A_3ARs by N^6 -substituted adenosine derivatives

The activation of A_3ARs by a wide range of the N^6 -substituted, halo-containing, and other adenosine derivatives (Tables 1 and 2) was examined by measuring their effects on forskolin-stimulated cyclic AMP accumulation in CHO cells stably expressing the human A_3AR . The efficacy of each of these adenosine derivatives was evaluated at a fixed concentration of 10 μM , and in some cases full concentration–response curves were measured. As shown in Fig. 1A, the non-selective agonist NECA **56** and the selective A_3AR agonist Cl-IB-MECA **59** both inhibited maximally the forskolin-stimulated cyclic AMP production by approximately 50%. Bn-Ado **21** was demonstrated to be less efficacious, while benzyl-NECA **57** [16] showed similar maximal efficacy as the full agonist Cl-IB-MECA. The A_1AR agonist, CHA **10**, was shown to be less efficacious than Cl-IB-MECA, while no significant activation by the $\text{A}_{2\text{A}}\text{AR}$ agonist DPMA **46** [17] was demonstrated. 2-Cl-R-PIA **53** [18] was also less than fully efficacious.

Various fluoro and chloro substitutions of the N^6 -benzyl group may increase the efficacy of Bn-Ado **21**. Chloro substitutions at the 2- or 4-position converted Bn-Ado **21** into full agonists **28** and **30**, while substitution at the 3-position increased efficacy only to a limited extent (Fig. 1B). The 2-fluorobenzyl substituent of **27** [19] also significantly increased the efficacy. In contrast, the chloro substituent at the 2-position of the adenine moiety might convert the full or partial agonists into antagonists, which have been demonstrated earlier [8,9].

Other N^6 -substituents that significantly decreased the efficacy (Tables 1 and 2) included: dicyclopropylmethyl **18**, 2-phenylethyl **22**, 2-phenylethoxy **23**, 2-methoxybenzyl **26**, 3-chlorobenzyl **29**, 2-pyridylmethyl **31**, 2-furanylmethyl **34**, 1-naphthylmethyl **37** *R*- and *S*-1-phenylethyl **38** and **39**, *R*-phenyl-isopentyl **45**, and (1*R*,2*S*)-2-phenyl-1-cyclopropyl **47**. The 3-pyridylmethyl derivative **32** was a potent, full agonist. 5'-Chloro-5'-deoxyadenosine **54** displayed low efficacy at the human A_3 AR. It should be noted that some compounds tested in this study could not be clearly identified as full or partial agonists due to low affinity for the human A_3 AR, although the efficacy observed at 10 μ M was reduced. These include the following N^6 substitutions: 2,2-dimethylpropyl **4**, 2,4-dimethyl-3-pentyl **7**, cyclooctyl **11**, (*S*)-*endo*-2-norbornyl **13**, 1-adamantyl **15**, 2-adamantyl **16**, cyclohexylmethyl **19**, *cis*- and *trans*-(1*R*,2*S*)-2-phenyl-cyclohexyl **49** and **50**.

3.2. Binding of the N^6 -substituted adenosine derivatives to A_1 , A_{2A} , and A_3 ARs

We initially measured the affinity and potency of the adenosine derivatives for the human A_3 AR. We also examined the selectivity and species differences by determining the binding affinity of these compounds for rat A_1 , A_{2A} , and A_3 ARs.

Fig. 2 shows that two compounds were found to be more potent at human than at rat A_3 ARs. N^6 -Methyladenosine **1** and N^6 -[(1*S*,2*R*)-2-phenyl-1-cyclopropyl]adenosine **48** were 687- and 1100-fold more potent, respectively, in binding to human A_3 ARs vs. rat A_3 ARs. A number of other compounds, including the simplest substituted adenosine derivatives CADO and FADO, were also found to be more potent for human than for the rat A_3 AR (Table 1). Small N^6 -substituents (**1–3**) were consistently associated with selectivity for human A_3 ARs vs. rat A_3 ARs.

The reverse species dependence was also noted. Fig. 3 shows that 1-naphthylmethyladenosine **37** was approximately 10-fold more potent for rat than for human A_3 ARs. Some other compounds, including ENBA **13** and 2-fluorobenzyladenosine **27**, were also shown to be more potent for rat than for human A_3 ARs (Table 1).

Stereoselectivity of binding of adenosine derivatives has been established at A_1 and A_{2A} ARs [10], but not fully investigated at A_3 ARs. Fig. 4 shows compounds **38** and **39** N^6 -(*R*- and *S*-1-phenylethyl), as well as compounds **43** and **44** N^6 -(*R*- and *S*-1-phenyl-2-pentyl) showed stereoselectivity for rat but not for human A_3 ARs. In the case of compounds **38** and **39**, the potency of the *R*-isomer was similar for rat and human, while the *S*-isomer was 16-fold more potent at the human than at the rat A_3 AR. Similarly, in the case of compounds **43** and **44**, the potency of the *R*-isomer was similar for rat and human, while the *S*-isomer was 48-fold more potent for human than for rat A_3 ARs (Table 1).

Several compounds, including N^6 -cyclooctyl **11**, N^6 -(*exo*-norbornyl) **12**, ENBA **13**, and N^6 -(7-norbornyl) **14**, were found to be over 200-fold selective for rat A_1 vs. rat A_{2A} and A_3 ARs (Table 3). We further tested the possible selectivity of these compounds for the human A_1 AR. However, as shown in Table 3, most of these compounds only showed less than 100-fold selectivity for human A_1 vs. human A_{2A} and A_3 ARs. Surprisingly, ENBA was demonstrated to be the most potent and most selective compound for both human and rat A_1 ARs. Similar to CGS21680, DPMA, shown to be selective for the rat A_{2A} AR, was shown to be non-selective at human A_1 , A_{2A} , and A_3 ARs (Tables 1 and 3). Compound **29** (3-chlorobenzyl) was demonstrated to be over 100-fold selective for both human and rat A_1 and A_3 vs. A_{2A} ARs. Compounds **38** (*R*) and **39** (*S*), as well as **43** (*R*) and **44** (*S*), diastereoisomers which lost stereospecificity of binding at the human A_3 AR, were further evaluated at the human A_1 AR. In contrast to the human A_3 AR, stereospecific binding was preserved at the human A_1 AR (Table 3).

4. Discussion

4.1. Efficacy

It has been previously demonstrated that *N*⁶-(3-iodobenzyl) adenosine was a partial agonist for the human A₃AR [8]. However, it was not known whether the decrease in efficacy was due to the benzyl group or its iodo substitution. Here, we further demonstrated the *N*⁶-benzyl group itself decreased the maximal efficacy. Furthermore, it was demonstrated in this study that a number of other structural modifications of adenosine (Fig. 5) contributed variously to affinity and efficacy. The full agonist of the A₁AR, CHA, was shown to be a partial agonist of the A₃AR. A potent and full agonist for the A_{2A}AR, DPMA, was demonstrated to be a moderately potent antagonist of the A₃AR.

In previous studies, it has been demonstrated that 2-chloro substitution of the adenine ring may both increase the affinity and decrease the efficacy of the adenosine derivatives for the human A₃AR [6,8,9]. Here, we further demonstrated that the chloro substituent might alternately decrease, increase, or have no effect on the efficacy of the adenosine derivatives depending on the position of substitution. For example, R-PIA was a full agonist, while a chloro group substituted at the 2-position of the adenine moiety (2-Cl-R-PIA) significantly decreased the efficacy. In contrast, a chloro substituent at the 2- (**28**) or 4- (**30**) positions of the benzyl group of Bn-Ado (**21**, itself having 55% of maximal efficacy) significantly increased the efficacy, converting the partial agonist **21** into a full agonist. Chloro substitution at the 3-position of the benzyl group (**29**) also significantly increased the efficacy, although to a lesser extent. Similarly, a fluoro substituent at the 2-position of the benzyl ring (**27**) also induced a modest increase of the efficacy.

In contrast to the *N*⁶-benzyl group, which decreased the efficacy, the *N*⁶-phenyl group did not significantly influence the efficacy, thus *N*⁶-phenyladenosine **20** was still a full agonist. Also, similar to Cl-IB-MECA [8,21], the benzyl group did not influence the efficacy of NECA, and thus, *N*⁶-benzyl-NECA [16] was a full agonist.

The appending of an additional cyclopropyl group to compound **17**, obtaining compound **18**, which only caused a slight change of its affinity, dramatically diminished its maximal A₃AR efficacy. Similarly, bridging the methylene group in compound **25** to give **40**, thus introducing a ring constraint, also reduced A₃ efficacy. A comparison of compounds **17** and **19** suggested that the enlargement of a ring attached to the *N*⁶-methyl group appeared to lower both affinity and efficacy at the human A₃AR, while lengthening the chain between *N*⁶ and the phenyl group seemed to decrease efficacy, with minimum efficacy observed with compound **21**. Compared with compound **22**, the introduction of an oxygen (hydroxylamine linkage of compound **23**) seemed to induce a larger decrease in its affinity for all three AR subtypes, and a smaller decrease of its maximal A₃AR efficacy. This is in contrast to compound **2**, which showed enhanced potency at the rat A₃AR as a result of the oxygen inclusion [22]. Branching at the terminal alkyl position (compound **45** vs. compound **43**) did not change the A₃ affinity but diminished the A₃ efficacy. A 5'-Cl-5'-deoxy substitution (compound **54**), already reported for adenosine agonists [5,22], greatly reduced A₃AR efficacy.

4.2. Selectivity

*N*⁶-(2-Methylbenzyl)adenosine (**25**) [20] was found to have comparable affinity at rat A₁, A_{2A}, and A₃ARs, while *N*⁶-(2-methoxybenzyl)adenosine **26** was found to be selective for both A₁ and A₃ but not A_{2A}ARs. *N*⁶-(2-Fluorobenzyl) adenosine (**27**) was >10-fold selective for A₁ over A_{2A} or A₃ARs, while *N*⁶-(2-chlorobenzyl)adenosine (**28**) was shown to have similar affinity for A₁ and A₃ARs. *N*⁶-(3-Chlorobenzyl)adenosine (**29**) was found to be extremely selective for A₁ and A₃ vs. A_{2A}ARs. *N*⁶-(Cyclooctyl)adenosine (**11**), the *N*⁶-

norbornyladenosines (**12–14**), and the N^6 -(1- and 2-adamantyl)adenosine derivatives (**15** and **16**) were demonstrated to be selective for the A_1 AR.

Some compounds shown to be selective agonists for a particular adenosine receptor subtype in the rat, were less selective among human ARs. For example, DPMA was selective for the A_{2A} AR only in the rat. R-PIA was selective for the rat A_1 AR, but had similar affinity for human A_1 and A_3 ARs [2]. Compared with R-PIA (**41**), the substitution of the propyl group by a pentyl group (**43**) induced a dramatic decrease in A_{2A} affinity, but only minor change in A_1 and A_3 AR affinity. CGS21680 **58**, originally shown to be a selective agonist for rat A_{2A} AR, was demonstrated to be equipotent at human A_{2A} and A_3 ARs [2].

4.3. Species difference: human and rat A_3 ARs

The identity in amino acid sequence between human and rat A_3 ARs is only 72% [23], which is much lower than the interspecies homology of A_1 , A_{2A} , and A_{2B} ARs. It is known that almost all non-nucleoside antagonists of the human A_3 AR showed extremely low affinity for rat A_3 receptors. For example, MRS 1220 [24], MRE 3008F20 [25], and (5-[[4-(pyridyl)amino]carbonyl]amino-8-methyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine hydrochloride) (K_i = 0.01 nM) [26] displayed exceptionally high affinity for the human A_3 AR, but extremely low affinity for the rat A_3 AR (K_i > 1000 nM). Adenosine derivatives were presumed to display less variability of affinity than non-nucleoside antagonists between human and rat A_3 ARs. However, as shown in the present study, species differences in binding were characterized for many of these compounds. For example, N^6 -[(1*S*,2*R*)-2-phenyl-1-cyclopropyl]adenosine **48** was demonstrated to be over 1000-fold more potent for human than rat A_3 AR. A small N^6 -substituent was demonstrated to be extremely important for the species difference between human and rat A_3 ARs. For example, N^6 -methyladenosine **1** was demonstrated to be approximately 700-fold more potent at human A_3 ARs vs. rat A_3 ARs. A number of other compounds, including **3** and **22**, were also demonstrated to be over 100-fold more potent at human than at rat A_3 ARs. Interestingly, several compounds were found to be more potent at the rat A_3 AR (compounds **7**, **13**, **27**, **37**, and **40**). Compounds **38** and **39** (N^6 -*R*- and *S*-1-phenylethyl) and compounds **43** and **44** (N^6 -*R*- and *S*-1-phenyl-2-pentyl) showed stereoselectivity for rat but not for human A_3 ARs. In each case the *R*-isomer was equipotent at rat and human A_3 ARs, while the *S*-isomer was more potent at human by 20- and 50-fold for **39** and **44**, respectively.

In conclusion, it was demonstrated in this study that a number of substitutions of adenosine contributed differently to affinity and efficacy. A number of full agonists for A_1 and A_{2A} ARs were demonstrated to be partial agonists or antagonists for the h A_3 AR. Among N^6 -alkyl substitutions, small N^6 -alkyl groups were associated with selectivity for human A_3 ARs vs. rat A_3 ARs, and multiple points of branching were associated with decreased h A_3 AR efficacy. N^6 -Cycloalkyl-substituted adenosines were full (≤ 5 carbons) or partial (≥ 6 carbons) h A_3 AR agonists. N^6 -(*endo*-Norbornyl)adenosine **13** was the most selective for both rat and human A_1 ARs. Numerous N^6 -arylmethyl analogues, including substituted benzyl, tended to be more potent in binding to A_1 and A_3 vs. A_{2A} ARs (with variable degrees of partial to full A_3 AR agonism). A chloro substituent decreased the efficacy depending on its position on the benzyl ring. The A_3 AR affinity and efficacy of N^6 -arylethyl adenosines depended highly on stereochemistry, steric bulk, and ring constraints. The identification of dual acting A_1/A_3 agonists, such as **29**, **39**, and **53**, might be useful for cardioprotection [3].

Abbreviations

ADAC

N^6 -[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]-methyl]aniline]carbonyl]methyl]phenyl]adenosine

Bn-Ado	<i>N</i> ⁶ -benzyladenosine
Cl-IB-MECA	2-chloro- <i>N</i> ⁶ -(3-iodobenzyl)-5'- <i>N</i> -methylcarbamoyladenosine
CGS21680	2-[<i>p</i> -(2-carboxyethyl)phenyl-ethylamino]-5'- <i>N</i> -ethylcarboxamidoadenosine
CHA	<i>N</i> ⁶ -cyclohexyladenosine
DBXRM	<i>N</i> -methyl-1,3-dibutylxanthine-7-β- <i>D</i> -ribofuronamide
CPA	<i>N</i> ⁶ -cyclopentyladenosine
DPMA	<i>N</i> ⁶ -[2-(3,5-dimethoxyphenyl)-2-(2-methylphenylethyl)]adenosine
CADO	2-chloroadenosine
ENBA	(<i>S</i>)-endo-2-norbornyladenosine
FADO	2-fluoroadenosine
GPCR	G protein-coupled receptor
Tris	tris(hydroxymethyl)aminomethane
I-AB-MECA	<i>N</i> ⁶ -(4-amino-3-iodobenzyl)-5'- <i>N</i> -methylcarboxamidoadenosine
MRS 542	2-chloro- <i>N</i> ⁶ -(3-iodobenzyl)adenosine
MRS 1220	<i>N</i> -[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5- <i>c</i>]quinazolin-5-yl]benzene-acetamide
NECA	5'- <i>N</i> -ethylcarboxamidoadenosine
PIA	<i>N</i> ⁶ -[phenylisopropyl]adenosine
RDT-39	<i>N</i> ⁶ -[(1 <i>S</i> ,2 <i>R</i>)-2-phenyl-1-cyclopropyl]adenosine

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References

1. Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev.* 2001; 53:527–552. [PubMed: 11734617]
2. Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, Lohse MJ. Comparative pharmacology of human adenosine receptor subtypes—characterization of stably transfected receptors in CHO cells. *Naunyn Schmiedeberg Arch Pharmacol.* 1998; 357:1–9. [PubMed: 9459566]
3. Liang BT, Jacobson KA. A physiological role of the adenosine A₃ receptor: sustained cardioprotection. *Proc Natl Acad Sci USA.* 1998; 95:6995–6999. [PubMed: 9618527]
4. Von Lubitz DK, Lin RC, Popik P, Carter MF, Jacobson KA. Adenosine A₃ receptor stimulation and cerebral ischemia. *Eur J Pharmacol.* 1994; 263:59–67. [PubMed: 7821362]
5. van Tilburg EW, von Frijtag Drabbe Künzel J, de Groote M, Vollinga RC, Lorenzen A, IJzerman AP. *N*⁶,5'-Disubstituted adenosine derivatives as partial agonists for the human adenosine A₃ receptor. *J Med Chem.* 1999; 42:1393–1400. [PubMed: 10212125]
6. van Tilburg EW, van der Klein PA, von Frijtag Drabbe Künzel J, de Groote M, Stannek C, Lorenzen A, IJzerman AP. 5'-*O*-Alkyl ethers of *N*,2-substituted adenosine derivatives: partial

- agonists for the adenosine A₁ and A₃ receptors. *J Med Chem.* 2001; 44:2966–2975. [PubMed: 11520205]
7. Gao ZG, Chen A, Barak D, Kim SK, Müller CE, Jacobson KA. Identification by site-directed mutagenesis of residues involved in ligand recognition and activation of the human A₃ adenosine receptor. *J Biol Chem.* 2002; 277:19056–19063. [PubMed: 11891221]
8. Gao ZG, Kim SK, Biadatti T, Chen W, Lee K, Barak D, Kim SG, Johnson CR, Jacobson KA. Structural determinants of A₃ adenosine receptor activation: nucleoside ligands at the agonist/antagonist boundary. *J Med Chem.* 2002; 45:4471–4484. [PubMed: 12238926]
9. Gao ZG, Jacobson KA. 2-Chloro-*N*⁶-cyclopentyladenosine, adenosine A₁ receptor agonist, antagonizes the adenosine A₃ receptor. *Eur J Pharmacol.* 2002; 443:39–42. [PubMed: 12044789]
10. Daly JW, Padgett W, Thompson RD, Kusachi S, Bungi WJ, Olsson RA. Structure–activity relationships for *N*⁶-substituted adenosines at a brain A₁-adenosine receptor with a comparison to an A₂-adenosine receptor regulating coronary blood flow. *Biochem Pharmacol.* 1986; 35:2467–2481. [PubMed: 3017353]
11. Gao ZG, Van Muijlwijk-Koezen JE, Chen A, Muller CE, IJzerman AP, Jacobson KA. Allosteric modulation of A₃ adenosine receptors by a series of 3-(2-pyridinyl)isoquinoline derivatives. *Mol Pharmacol.* 2001; 60:1057–1063. [PubMed: 11641434]
12. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
13. Olah ME, Gallo-Rodriguez C, Jacobson KA, Stiles GL. ¹²⁵I-4-Aminobenzyl-5'-*N*-methylcarboxamidoadenosine, a high affinity radioligand for the rat A₃ adenosine receptor. *Mol Pharmacol.* 1994; 45:978–982. [PubMed: 8190112]
14. Nordstedt C, Fredholm BB. A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal Biochem.* 1990; 189:231–234. [PubMed: 2177960]
15. Cheng Y-C, Prusoff WH. Relationship between the inhibition constant (*K_i*) and the concentration of inhibitor which causes 50 percent inhibition (*I*₅₀) of an enzymatic reaction. *Biochem Pharmacol.* 1973; 22:3099–3108. [PubMed: 4202581]
16. Van Galen PJM, van Bergen AH, Gallo-Rodriguez C, Melman N, Olah ME, IJzerman AP, Stiles GL, Jacobson KA. A binding site model and structure–activity relationships for the rat A₃ adenosine receptor. *Mol Pharmacol.* 1994; 45:1101–1111. [PubMed: 8022403]
17. Trivedi BK, Blankley CJ, Bristol JA, Hamilton HW, Patt WC, Kramer WJ, Johnson SA, Bruns RF, Cohen DM, Ryan MJ. *N*⁶-Substituted adenosine receptor agonists: potential antihypertensive agents. *J Med Chem.* 1991; 34:1043–1049. [PubMed: 2002448]
18. Thompson RD, Secunda S, Daly JW, Olsson RA. Activity of *N*⁶-substituted 2-chloroadenosines at A₁ and A₂ adenosine receptors. *J Med Chem.* 1991; 34:3388–3390. [PubMed: 1766003]
19. Daly JW, Padgett W. Agonist activity of 2- and 5'-substituted adenosine analogs and their *N*⁶-cycloalkyl derivatives at A₁- and A₂-adenosine receptors coupled to adenylate cyclase. *Biochem Pharmacol.* 1992; 43:1089–1093. [PubMed: 1554381]
20. Siddiqi SM, Jacobson KA, Esker JL, Olah ME, Ji X, Melman N, Tiwari KN, Secrist JA III, Schneller SW, Cristalli G, Stiles GL, Johnson CR, IJzerman AP. Search for newpurine- and ribose-modified adenosine analogues as selective agonists and antagonists at adenosine receptors. *J Med Chem.* 1995; 38:1174–1188. [PubMed: 7707320]
21. Kim HO, Ji X-d, Siddiqi SM, Olah ME, Stiles GL, Jacobson KA. 2-Substitution of *N*⁶-benzyladenosine-5'-uronamides enhances selectivity for A₃-adenosine receptors. *J Med Chem.* 1994; 37:3614–3621. [PubMed: 7932588]
22. Mogensen JP, Roberts SM, Bowler AN, Thomsen C, Knutsen LJ. The synthesis of new adenosine A₃ selective ligands containing bioisosteric isoxazoles. *Bioorg Med Chem Lett.* 1998; 8:1767–1770. [PubMed: 9873431]
23. Salvatore CA, Jacobson MA, Taylor HE, Linden J, Johnson RG. Molecular cloning and characterization of the human A₃ adenosine receptor. *Proc Natl Acad Sci USA.* 1993; 90:10365–10369. [PubMed: 8234299]

24. Jacobson KA, Park KS, Jiang J-l, Kim Y-C, Olah ME, Stiles GL, Ji X-d. Pharmacological characterization of novel A₃ adenosine receptor-selective antagonists. *Neuropharmacology*. 1997; 36:1157–1165. [PubMed: 9364471]
25. Varani K, Merighi S, Gessi S, Klotz KN, Leung E, Baraldi PG, Cacciari B, Romagnoli R, Spalluto G, Borea PA. [³H]MRE 3008F20: a novel antagonist radioligand for the pharmacological and biochemical characterization of human A₃ adenosine receptors. *Mol Pharmacol*. 2000; 57:968–975. [PubMed: 10779381]
26. Maconi A, Pastorin G, Da Ros T, Spalluto G, Gao ZG, Jacobson KA, Baraldi PG, Cacciari B, Varani K, Moro S, Borea PA. Synthesis, biological properties, and molecular modeling investigation of the first potent, selective, and water-soluble human A₃ adenosine receptor antagonist. *J Med Chem*. 2002; 45:3579–3582. [PubMed: 12166930]

Fig. 1.

Inhibition of forskolin-stimulated cyclic AMP production in CHO cells stably transfected with the human A₃AR, induced by various agonists (A) and by *N*⁶-benzyl derivatives (B). All experiments were performed in the presence of 10 μM rolipram and 3 U/mL adenosine deaminase. Forskolin (10 μM) was used to stimulate cyclic AMP levels. The level of cyclic AMP corresponding to 100% was 220 ± 30 pmol/mL. The data shown were from one experiment performed in duplicate and are typical of three independent experiments giving similar results. IC₅₀ values were (in nM): **10**, CHA, 354 ± 116; **21**, Bn-Ado, 120 ± 34; **28**, 2-Cl-Bn-Ado, 81 ± 19; **29**, 3-Cl-Bn-Ado, 103 ± 22; **30**, 4-Cl-Bn-Ado, 146 ± 31; **53**, CI-R-PIA, 76 ± 21; **56**, NECA, 62 ± 17; **57**, Bn-NECA, 18.2 ± 3.4; **59**, CI-IB-MECA, 3.2 ± 1.4.

(A)

(B)

Fig. 2. Competition for radioligand binding ($[^{125}\text{I}]\text{-AB-MECA}$) at A_3ARs in two species, by two representative adenosine analogues **1** and **48**, which were more potent at human than at rat A_3ARs . The procedures were described in "Section 2". The data points are from a representative experiment performed in duplicate. The mean K_i values calculated from three independent experiments are listed in the text.

Fig. 3. Competition for radioligand binding ($[^{125}\text{I}]\text{-AB-MECA}$) at A_3ARs in two species, by an adenosine analogue which was more potent at rat than at human A_3ARs . The procedures were described in "Section 2". The data points are from a representative experiment performed in duplicate. The mean K_i values calculated from three independent experiments are listed in the text.

Fig. 4. Competition for radioligand binding ($[^{125}\text{I}]\text{I-AB-MECA}$) at A_3ARs by pairs of *R*- and *S*-diastereoisomers (N^6 -(1-phenyl-2-pentyl)adenosines (A) and (B); N^6 -(1-phenylethyl)adenosines (C) and (D) at rat (A) and (C) and at human (B) and (D) A_3ARs , demonstrating that stereoselectivity of binding was species dependent. The procedures were described in "Section 2". The data points are from a representative experiment performed in duplicate. The mean K_i values calculated from three independent experiments are listed in the text.

Fig. 5.
Structures of representative adenosine derivatives and their efficacy (% of inhibition of adenylate cyclase at 10 μM compared to the effect of 10 μM Cl-IB-MECA) as agonists of the human A₃AR.

Table 1

Binding affinities of adenosine derivatives at rat A₁ and A_{2A}ARs and at human and rat A₃ARs and maximal agonist effects at human A₃ARs expressed in CHO cells

Compound	N ⁶ substitution	K _i (rA ₁ AR) (nM) ^a	K _i (rA _{2A} AR) (nM) ^a	K _i (rA ₃ AR) (nM) ^a	K _i (hA ₃ AR) (nM) ^a	Percent activation (hA ₃ AR)
N ⁶ -Substituted analogues						
Alkyl						
1	CH ₃	60 ± 11	>10000	6390 ± 1630	9.3 ± 0.4	96 ± 3
2	CH ₃ O	223 ± 32	>10000	997 ± 343	28.6 ± 4.7	107 ± 13
3	CH ₂ CH ₃	4.9 ± 0.2	8900 ± 770	1050 ± 140	4.7 ± 1.9	102 ± 6
4	CH ₂ C(CH ₃) ₃	17 ± 6	>10000	1870 ± 320	306 ± 80	76 ± 4
5	CH(CH ₃) ₂	1.9 ± 0.1	2030 ± 510	201 ± 38	18.3 ± 5.5	111 ± 4
6	CH(CH ₂ CH ₃) ₂	0.8 ± 0.2	471 ± 200	147 ± 40	55.1 ± 9.5	99 ± 6
7	CH(CH ₂ CH(CH ₃) ₂) ₂	3.8 ± 0.8	2170 ± 490	753 ± 384	3760 ± 840	21 ± 2
Cycloalkyl						
8	Cyclobutyl	0.7 ± 0.1	1740 ± 170	144 ± 64	6.4 ± 1.0	100 ± 7
9	Cyclopentyl	0.45 ± 0.04 ^b	462 ^c	240 ± 36 ^c	72 ± 12	97 ± 4
10	Cyclohexyl	0.9 ± 0.2	514 ^c	167 ± 26 ^c	73 ± 23	76 ± 2
11	Cyclooctyl	1.7 ± 0.1	6200 ± 1120	498 ± 197	411 ± 55	49 ± 5
12	<i>exo</i> -2-Norbornyl	0.7 ± 0.2	3400 ± 140	253 ± 30	85 ± 31	114 ± 6
13	ENBA (<i>S</i>)- <i>endo</i> -2-Norbornyl	0.34 ± 0.06	477 ± 72	282 ± 101	915 ± 299	23 ± 10
14	7-Norbornyl	0.48 ± 0.01	>10000	229 ± 76	112 ± 25	103 ± 1
15	1-Adamantyl	73 ± 3	14800 ± 3500	>10000	>10000	0
16	2-Adamantyl	46 ± 5	>10000	>10000	>10000	0
17	Cyclopropylmethyl	0.8 ± 0.3	1370 ± 410	608 ± 242	10.2 ± 4.1	108 ± 4
18	Dicyclopropylmethyl	0.8 ± 0.2	590 ± 30	772 ± 200	41.3 ± 5.3	31 ± 6
19	Cyclohexylmethyl	19 ± 7	>10000	2550 ± 1610	263 ± 73	38 ± 3
Aryl-containing						
20	Phenyl	3.3 ± 0.3	663 ^c	802 ± 279 ^c	14.9 ± 3.1	102 ± 9
21	Benzyl	175 ± 20	285 ^c	120 ± 20 ^c	41.3 ± 5.3	55 ± 3

Compound	N ⁶ substitution	K _i (nA ₁ AR) (nM) ^a	K _i (αA _{2A} AR) (nM) ^a	K _i (αA ₃ AR) (nM) ^a	K _i (hA ₃ AR) (nM) ^a	Percent activation (hA ₃ AR)
22	2-Phenylethyl	24.0 ± 8.8 ^d	161 ^c	240 ± 58 ^c	2.1 ± 0.4	84 ± 5
23	2-Phenylethoxy	225 ± 45	>10000	8660 ± 2900	88.7 ± 7.4	73 ± 6
24 ADAC	4-[[[4-[[[(2-Aminoethyl)amino]-carbonyl]-methyl]aniline]-carbonyl]methyl]phenyl	0.85 ^c	210 ^c	185 ± 64	13.3 ± 3.0	103 ± 4
25 Meritfudil	2-Methylbenzyl	59.6 ± 14.3 ^e	24.1 ± 1.8 ^e	35 ± 15	47.2 ± 10.8	100 ± 3
26	2-Methoxybenzyl	36 ± 2	761 ± 460	29 ± 11	32.5 ± 4.6	81 ± 8
27	2-Fluorobenzyl	6 ± 1	551 ± 282	60 ± 8	339 ± 5	67 ± 7
28	2-Chlorobenzyl	17 ± 3	93 ± 16	13 ± 1	17.3 ± 3.2	95 ± 1
29	3-Chlorobenzyl	45 ± 10	>10000	35 ± 20	4.4 ± 1.7	80 ± 3
30	4-Chlorobenzyl	61 ± 3	5120 ± 1230	96 ± 38	47.5 ± 4.1	96 ± 2
31	2-Pyridylmethyl	225 ± 5	>10000	111 ± 32	115 ± 33	73 ± 15
32	3-Pyridylmethyl	115 ± 4	3220 ± 1210	288 ± 67	4.5 ± 1.1	100 ± 6
33	4-Pyridylmethyl	70 ± 5	3860 ± 1520	67 ± 9	80.1 ± 20.0	99 ± 12
34	2-Furanylmethyl	95 ± 9	>10000	301 ± 72	21.8 ± 3.9	54 ± 10
35	2-Thienylmethyl	36 ± 7	734 ± 60	112 ± 33	59.8 ± 25.7	92 ± 2
36	3-Thienylmethyl	45 ± 9	3300 ± 450	297 ± 53	26.3 ± 8.2	97 ± 8
37	1-Naphthylmethyl	13 ± 3	1120 ± 580	2.7 ± 0.5	25.2 ± 9.7	67 ± 8
38	R-1-Phenylethyl	3.4 ± 0.4	1300 ± 620	60 ± 25	113 ± 22	76 ± 10
39	S-1-Phenylethyl	195 ± 20	>10000	1110 ± 460	68.7 ± 17.3	83 ± 5
40	R-1-Indanyl	65 ± 10	2480 ± 740413 ^f	79 ± 12	233 ± 27	82 ± 5
41 R-PIA	R-1-Phenyl-2-propyl	1.2 ± 0.1	124 ^c	158 ± 52 ^c	8.7 ± 0.9	102 ± 6
42 S-PIA	S-1-Phenyl-2-propyl	49.3 ^c	1820 ^c	920 ± 311 ^c	68 ± 12	97 ± 3
43	R-1-Phenyl-2-pentyl	3.34 ± 0.66 ^d	>10000	76 ± 18	70.9 ± 26.2	92 ± 9
44	S-1-Phenyl-2-pentyl	282 ± 17 ^d	>10000	1810 ± 530	37 ± 13	101 ± 10
45	R-1-Phenyl-isopentyl	8.89 ± 1.97 ^d	3250 ± 580	103 ± 43	96 ± 17	77 ± 4
46 DPMA	2-(3,5-Dimethoxy-phenyl)-2-(2-methylphenyl)ethyl	142 ^c	4.4 ^c	3570 ± 1700 ^c	106 ± 22	0
47	(1R,2S)-2-Phenyl-1-cyclopropyl	15.2 ± 3.2 ^d	3040 ± 490	358 ± 33	24.1 ± 10.9	87 ± 4
48	(1S,2R)-2-Phenyl-1-cyclopropyl	11.8 ± 2.4 ^d	560 ± 232	694 ± 157	0.63 ± 0.17	117 ± 9
49	cis-(1R,2R)-2-Phenylcyclohexyl	15 ± 6	>10000	1170 ± 30	1450 ± 241	73 ± 12

Compound	N ⁶ substitution	K _i (rA ₁ AR) (nM) ^a	K _i (rA _{2A} AR) (nM) ^a	K _i (rA ₃ AR) (nM) ^a	K _i (hA ₃ AR) (nM) ^a	Percent activation (hA ₃ AR)
50	<i>trans</i> -(1 <i>R</i> ,2 <i>S</i>)-2-Phenylcyclohexyl	6 ± 3	672 ± 51	279 ± 41	559 ± 96	72 ± 9
Compound	Substitution	K _i (rA ₁ AR) (nM)	K _i (rA _{2A} AR) (nM)	K _i (rA ₃ AR) (nM)	K _i (hA ₃ AR) (nM)	Percent activation (hA ₃ AR)
Other halo analogues						
51	CADO 2-Chloro	6.7 ± 1.0 ^b	63 ^e	1890 ± 900 ^e	87 ± 24	100 ± 7
52	FADO 2-Fluoro	68.3 ± 18.9 ^d	28 ^c	4590 ± 2410	99 ± 13	31 ± 3
53	2-Chloro-R-PIA	0.86 ± 0.14 ^d , 1.4 ± 0.1 ^g	1070 ± 250	34 ± 10	13.1 ± 0.9	76 ± 13
54	5'-Chloro-5'-deoxyadenosine	20 ± 1 ^b	62.7 ± 14.4	4590 ± 2410	107 ± 6	9 ± 4

^a All A₃AR experiments were performed using adherent CHO cells stably transfected with cDNA encoding the human or rat A₃ receptor. Percent activation of the human A₃AR was determined at 10 μM. Unless otherwise noted, K_i values at A₁AR are from Daly *et al.* [10]. Binding at A₁ and A_{2A}ARs was carried out as described in Section 2. Values from the present study are means ± SEM, N = 3–5.

^b Data from Daly and Padgett [19].

^c Data from Van Galen *et al.* [16].

^d K_i values at the A₁AR determined in the present study.

^e Data from Siddiqi *et al.* [20].

^f Data from Trivedi *et al.* [17].

^g Data from Thompson *et al.* [18].

Binding affinities of reference adenosine agonists at rat A₁ and A_{2A} receptors and at human and rat A₃ receptors and maximal agonist effects at human A₃ receptors expressed in CHO cells

Table 2

Compound	K _i (rA ₁ AR) (nM) ^a	K _i (rA _{2A} AR) (nM) ^a	K _i (rA ₃ AR) (nM) ^a	K _i (hA ₃ AR) (nM) ^a	Percent activation (hA ₃ AR)
55 CCPA	0.6 ^b	950 ^b	237 ± 71 ^b	38 ± 6 ^c	0 ^d
56 NECA	6.3 ^b	10.3 ^b	113 ± 34 ^b	35 ± 12	103 ± 6
57 N ⁶ -Bn-NECA	87 ± 14 ^b	95 ± 25 ^b	6.8 ± 2.5 ^b	10.8 ± 1.5	107 ± 8
58 CGS21680	2600 ^b	15 ^b	584 ± 32 ^b	114 ± 16 ^d	98 ± 5
59 Cl-IB-MECA	820 ^e	470 ^e	0.33 ± 0.08 ^e	1.4 ± 0.3 ^d	100
60 MRS 542	18.5 ^e	38.5 ^e	1.41 ± 0.17 ^e	1.8 ± 0.1 ^d	0 ^d

^a All A₃AR experiments were performed using adherent CHO cells stably transfected with cDNA encoding the human or rat A₃ receptor. Binding at A₁ and A_{2A} ARs was carried out as described in Section 2. Percent activation of the human A₃AR was determined at 10 μM. Values from the present study are means ± SEM, N = 3.

^b Data from Van Galen *et al.* [16].

^c Data from Gao and Jacobson [9].

^d Data from Gao *et al.* [8].

^e Data from Kim *et al.* [21].

Table 3

Comparison of binding affinity of selected adenosine derivatives at human A₁, A_{2A}, and A₃ARs (*N*⁶-substituent or compound abbreviation in parentheses)

	<i>K_i</i> (nM)		
	hA ₁	hA _{2A}	hA ₃
11 (cyclooctyl)	6.4 ± 1.4	>10000	411 ± 55
12 (<i>exo</i> -norbornyl)	2.4 ± 0.8	>10000	85 ± 31
13 ENBA	0.38 ± 0.19	>10000	915 ± 299
14 (7-norbornyl)	2.1 ± 0.5	>10000	112 ± 25
29 (3-chlorobenzyl)	34.9 ± 9.6	>10000	4.4 ± 1.7
38 (<i>R</i> -1-phenylethyl)	28.6 ± 9.8	1750 ± 540	113 ± 22
39 (<i>S</i> -1-phenylethyl)	94.5 ± 29.5	>10000	68.7 ± 17.3
43 (<i>R</i> -1-phenyl-2-pentyl)	16.9 ± 5.2	1120 ± 320	70.9 ± 26.2
44 (<i>S</i> -1-phenyl-2-pentyl)	113 ± 18	6840 ± 2350	37 ± 13
46 DPMA	168 ± 29	153 ± 26	106 ± 22
58 CGS21680	ND	43 ± 18	114 ± 16
59 Cl-IB-MECA	1240 ± 320	5360 ± 2470	1.4 ± 0.3

Values from the present study are means ± SEM, N = 3.