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Structural determinants of efficacy at A_3 adenosine receptors: modification of the ribose moiety

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Abstract

We have found previously that structural features of adenosine derivatives, particularly at the N^6- and 2-positions of adenine, determine the intrinsic efficacy as A_3 adenosine receptor (AR) agonists. Here, we have probed this phenomenon with respect to the ribose moiety using a series of ribose-modified adenosine derivatives, examining binding affinity and activation of the human A_3 AR expressed in CHO cells. Both 2′- and 3′-hydroxyl groups in the ribose moiety contribute to A_3 AR binding and activation, with 2′-OH being more essential. Thus, the 2′-fluoro substitution eliminated both binding and activation, while a 3′-fluoro substitution led to only a partial reduction of potency and efficacy at the A_3 AR. A 5′-uronamide group, known to restore full efficacy in other derivatives, failed to fully overcome the diminished efficacy of 3′-fluoro derivatives. The 4′-thio substitution, which generally enhanced A_3 AR potency and selectivity, resulted in 5′-CH_2OH analogues (**10** and **12**) which were partial agonists of the A_3 AR. Interestingly, the shifting of the N^6-(3-iodobenzyl)adenine moiety from the 1′- to 4′-position had a minor influence on A_3 AR selectivity, but transformed **15** into a potent antagonist (**16**) ($K_i = 4.3$ nM). Compound **16** antagonized human A_3 AR agonist-induced inhibition of cyclic AMP with a K_B value of 3.0 nM. A novel apio analogue (**20**) of neplanocin A, was a full A_3 AR agonist. The affinities of selected, novel analogues at rat ARs were examined, revealing species differences. In summary, critical structural determinants for human A_3 AR activation have been identified, which should prove useful for further understanding the mechanism of receptor activation and development of more potent and selective full agonists, partial agonists and antagonists for A_3 ARs.
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Keywords: Nucleosides; A_3 adenosine receptor agonist; A_3 adenosine receptor antagonist; Adenylyl cyclase; Phospholipase C; Partial agonist

1. Introduction

Pharmacological characterization of the four subtypes of adenosine receptors (ARs; A_1, A_{2A}, A_{2B} and A_3) has been facilitated by the availability of potent and selective agonists and antagonists [1]. The A_1 and A_3 subtypes are negatively coupled to adenylyl cyclase, while A_{2A} and A_{2B} subtypes are positively coupled to this enzyme. A_{2B} and A_3 ARs are also positively coupled to phospholipase C [1]. The A_3 AR is the most recently identified AR subtype [2] and is involved in a variety of physiological

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Abbreviations: Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine; CGS21680, 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; CCPA, 2-chloro-N^6-cyclopentyladenosine; DPMA, N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenylethyl)]adenosine; CADO, 2-chloroadenosine; DMSO, dimethylsulfoxide; GPCR, G protein-coupled receptor; Tris, tris(hydroxymethyl)aminomethane; I-AB-MECA, N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; MRS 542, 2-chloro-N^6-(3-iodobenzyl)adenosine; MRS 1743, N^6-(3-iodobenzyl)-(*N*)-methanocarbaadenosine; MRS 1760, 2-chloro-N^6-(3-iodobenzyl)-(*N*)-methanocarbaadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; *R*-PIA, *R*-N^6-[2-phenylisopropyl]adenosine.

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processes [1]. The selective activation of A_3 ARs has been demonstrated to be both cardioprotective [3,4] and cerebroprotective [5,6]. Both selective agonists and antagonists [7] for A_3 ARs are of potential clinical use.

In an effort to develop potent agonists and antagonists for A_3 ARs, a wide range of N^6- and 2′-substituted adenosine derivatives have recently been pharmacologically characterized [8–10]. We found that various adenosine derivatives, which were previously demonstrated to be agonists at A_1 and A_{2A} ARs and assumed to be full/partial agonists at A_3 ARs, are indeed antagonists at this subtype. Thus, the efficacy of adenosine derivatives appears to be more dependent on small structural changes at the A_3 AR than at other subtypes. Structural determinants critically involved in A_3 AR activation have been identified [8–13], leading to the conclusion that the ability of an adenosine derivative to activate the A_3 AR tends to be highly structure-sensitive. 3′-Fluoro has recently been found to interfere with A_3 AR binding [14], and certain 4′-thio nucleosides have been found to be potent and selective human A_3 AR agonists [15]. In this study, we further evaluated the binding affinity and functional properties of a series of ribose-modified adenosine derivatives, including some six previously reported compounds [14,15], at the human A_3 AR stably expressed in CHO cells.

2. Materials and methods

2.1. Cell culture and membrane preparation

Chinese Hamster Ovary (CHO) cells expressing recombinant human A_1 and A_3 ARs or HEK-293 cells expressing human A_{2A} ARs were cultured in DMEM and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 μmol mL^{-1} glutamine and 800 μg mL^{-1} geneticin. Cells were harvested and homogenized, and then centrifuged at 100 *g* for 5 min. The resulting pellet was re-suspended in 50 mM Tris–HCl buffer (pH 7.4) 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 units mL^{-1} 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 [10]. 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 [16].

2.2. Radioligand binding assay

For A_3 AR binding experiments, the procedures used were similar to those previously described [17]. Briefly, each tube contained 100 μL of membrane suspension (20 μg protein), 50 μL of [^{125}I]I-AB-MECA (final concentration 1.0 nM), and 50 μL of increasing concentrations of compounds in Tris–HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. Nonspecific binding was determined using 10 μM Cl-IB-MECA. The mixtures were incubated at 25° for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell). Filters were washed three times with ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter. Binding assays were performed by methods previously described [10]. The binding to A_1 ARs utilized [^{3}H]*R*-PIA (1.0 nM, rat forebrain) or [^{3}H]CPX (0.5 nM, recombinant human A_1 AR), and the binding to A_{2A} ARs utilized [^{3}H]CGS21680 (15 nM, rat striatum) and [^{3}H]ZM241385 (2 nM, recombinant human A_1 AR).

2.3. Cyclic AMP accumulation assay

Intracellular cyclic AMP levels were measured with a competitive protein binding method [18]. CHO cell that expressed the recombinant human A_3 AR were harvested by trypsinization. After resuspension in medium, cells were planted in 24-well plates in 1 mL of medium. After 24 hr, the medium was removed and cells were washed with 1 mL DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with agonists and/or test compounds (for study of the antagonistic effect, antagonists were preincubated in the medium for 30 min before the addition of agonist) in the presence of rolipram (10 μM) and adenosine deaminase (3 units mL^{-1}). After 45 min forskolin (10 μM) was added to the medium, and the incubation was continued an additional 15 min. The reaction was terminated by removal of the supernatant, and cells were lysed upon addition of 200 μL of ice-cold 0.1 M HCl. The cell lysate was resuspended and stored at −20°. For determination of cyclic AMP production, protein kinase A (PKA) was incubated with [^{3}H]cyclic AMP (2.0 nM) in K_2HPO_4/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 using a liquid scintillation counter (LKB Wallace 1215 Rackbeta scintillation counter).

2.4. Inositol phosphate determination

The method used was similar to that previously described [13]. Briefly, cells were harvested by trypsinization and grown in six-well plates (∼10^6 cells per well; Costar) in

DMEM culture medium supplemented with 2 μCi mL^{-1} of *myo*-[^{3}H]inositol. The mixtures were swirled to ensure uniformity. After a labeling period of 24 hr, cells were preincubated in the presence of 3 units mL^{-1} adenosine deaminase for 30 min at 37° with 10 mM LiCl and for 20 min at room temperature. Following the addition of the test compounds, the cells were incubated for 30 min at 37°. The supernatants were removed by aspiration, and 500 μL of cold 20 mM formic acid was added to each well. Cell extracts were collected after a 30-min incubation at 4° and neutralized with 300 μL of 60 mM NH_4OH. The inositol monophosphate fraction was then isolated by anion exchange chromatography. The content of each well was applied to a small anion exchange column (AG-1-X8; BioRad) that had been pretreated with 15 mL of 0.1 M formic acid/3 M ammonium formate, followed by 15 mL of water. The columns were then washed with 15 mL of a solution containing 5 mM sodium borate and 60 mM sodium formate. [^{3}H]Inositol phosphates (IP) were eluted two times with 5 mL of 0.1 M formic acid/0.2 M ammonium formate and radioactivity was quantified by liquid scintillation counting (LKB Wallace 1215 Rackbeta scintillation counter).

2.5. Materials

[^{3}H]*R*-PIA (34 Ci $mmol^{-1}$), [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]I-AB-MECA; 2000 Ci $mmol^{-1}$), and [^{3}H]cyclic AMP (40 Ci $mmol^{-1}$) were from Amersham Pharmacia Biotech. [^{3}H]CGS21680 (47 Ci $mmol^{-1}$) was from Perkin-Elmer Life Sciences. Cl-IB-MECA, CPA, CCPA and NECA were purchased from Sigma-RBI. MRS 542, MRS 1743, and MRS 1760 were prepared as described [9]. All other reagents were from standard commercial sources and of analytical grade.

2.6. Statistical analysis

Binding and functional parameters were estimated using GraphPAD Prism software. IC_{50} values obtained from competition curves were converted to K_i values as described [19]. Data were expressed as mean ± standard error.

3. Results

3.1. Structures of ribose-modified nucleoside analogues

A series of ribose-modified analogues of adenosine agonists (Fig. 1) was synthesized [14,15,20,21] and compared in binding and functional assays (Table 1). The set of analogues included nucleosides having a 5′-uronamide modification (**1**, **2**, **8**, **9**, and **13–16**), modification of a hydroxy group either through chiral inversion (**3**) or through fluoro-substitution (**4–9**), or a 4′-thio modification

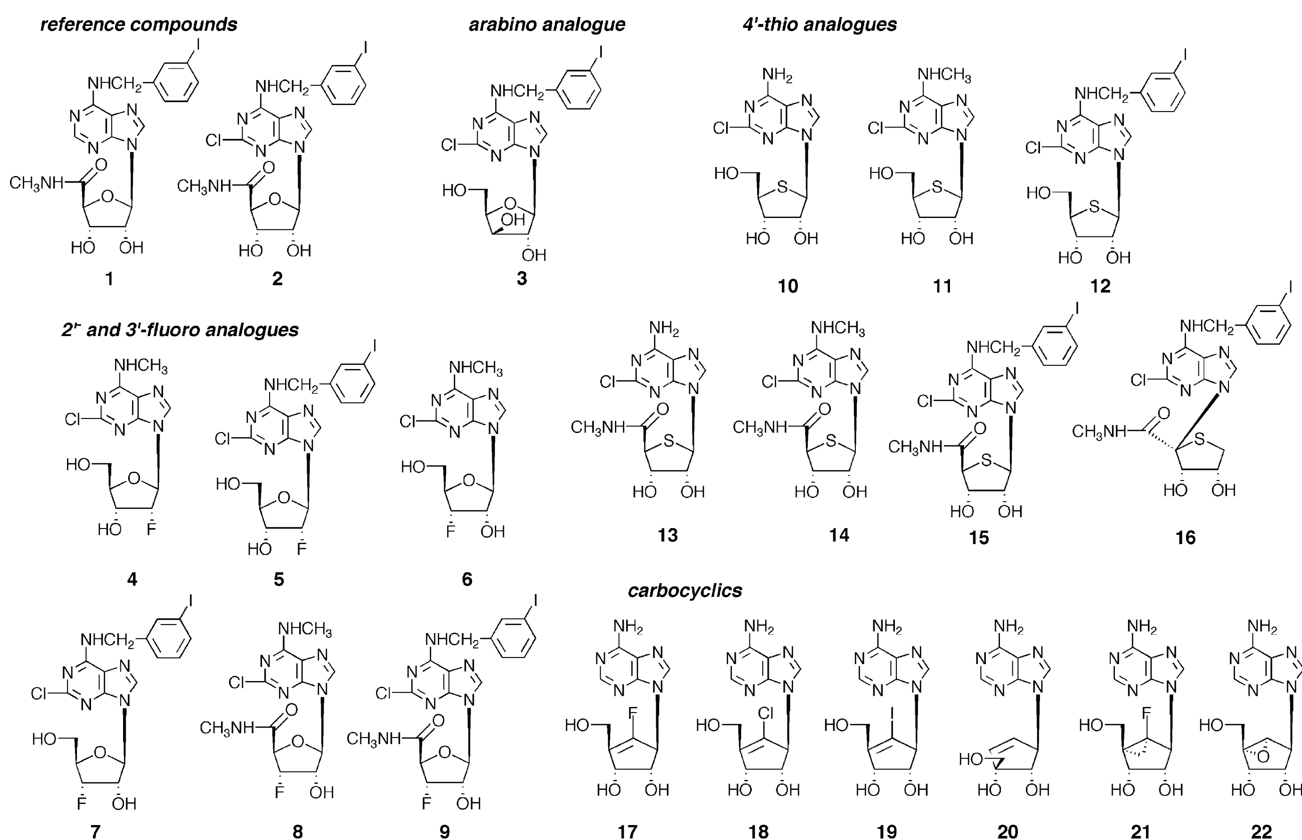


Fig. 1. Structures of the ribose-modified adenosine derivatives studied as agonists, partial agonists, and antagonists of the human A_3 AR.

Table 1
Binding affinity at human A_1, A_{2A} and A_3 ARs, and functional potency and efficacy (inhibition of forskolin-stimulated cyclic AMP accumulation at 10 μM) at human A_3 ARs[a]

Compound	K_i (nM) or percentage inhibition at 10 μM			EC_{50} (nM)	Efficacy
	hA_1	hA_{2A}	hA_3	hA_3	hA_3
1 IB-MECA	1620 ± 760	2910 ± 580	1.8 ± 0.7[b]	3.6 ± 1.3[b]	100%[b]
2 Cl-IB-MECA	1240 ± 320[c,d]	5360 ± 2470[c,d]	1.4 ± 0.3[b]	2.8 ± 1.4[b]	99 ± 6%[b]
3	23%	0%	3940 ± 1320	NA	0%
4	0%	0%	7%	NA	0%
5	0%	0%	44%	NA	6 ± 4%
6[d]	20%[c,e]	0%[c,e]	326 ± 112[e]	1380 ± 350	17 ± 8%
7[d]	4640 ± 340[c,e]	0%	75 ± 7	NA	0%
8	10%	7%[c,e]	127 ± 44[e]	1170 ± 350	54 ± 7%
9[d]	6%[c,e]	0%[c,e]	406 ± 60[e]	NA	0%
10	47%	2440 ± 316	4.9 ± 1.3	17.3 ± 3.4	64 ± 18%
11	629 ± 168	4%	0.8 ± 0.1	2.3 ± 0.8	96 ± 5%
12	554 ± 64	1190 ± 293	3.2 ± 0.9	3.0 ± 1.1	32 ± 7%
13	89.2 ± 11.7[c,d]	158 ± 29[c,d]	0.40 ± 0.06[d]	1.0 ± 0.3	100 ± 5%
14	1330 ± 242[c,d]	20%[c,d]	0.28 ± 0.09[d]	0.21 ± 0.04	119 ± 12%
15	193 ± 46[c,d]	223 ± 36[c,d]	0.38 ± 0.07[d]	0.38 ± 0.06	114 ± 9%
16	110 ± 12	47%	4.3 ± 1.2	NA	0%
17	7%	9%	49%	NA	28 ± 7%
18	7%	6%	3150 ± 680	1150 ± 380	42 ± 18%
19	5%	3%	6490 ± 1780	NA	8 ± 3%
20	8%	0%	628 ± 69	717 ± 221	101 ± 4%
21	1590 ± 121	5530 ± 693	26 ± 12	18.2 ± 7.1	33 ± 10%
22	1%	0%	1130 ± 350	NA	0%

Values are means ± SEM. NA, not applicable.
[a]All A_3 AR experiments were performed using adherent CHO cells stably transfected with cDNA encoding the human A_3 AR using [^{125}I]I-AB-MECA as radioligand. Binding at A_1 and A_{2A} ARs was carried out as described in Section 2 using [^{3}H]CPX or [^{3}H]ZM241385 as radioligand, unless noted. Values from the present study are means ± SEM, N = 3.
[b]Data from Gao *et al.* [10].
[c]Binding using [^{3}H] PIA (A_1 AR) or [^{3}H]CGS21680 (A_{2A} AR).
[d]Data from Jeong *et al.* [15].
[e]Data from Lim *et al.* [14].

(**10–15**). 3′-Fluoro (**7–9**) and 5′-uronamide-4′-thionucleoside (**13–15**) analogues were reported previously and partially characterized pharmacologically [15]. Other species included were nucleoside-like analogues, i.e. a rearranged 4′-thionucleoside analogue (**16**) and those having carbocyclic rings (**17–22**). Substitution of the adenine moiety, where present, followed previously determined substitution patterns to obtain high affinity at the human A_3 AR, such as N^6-methyl and N^6-iodobenzyl groups, either alone or in combination with the 2-chloro substituent. Use of the N^6-methyl substituent for A_3 AR agonism by Cristalli and coworkers was usually accompanied by additional substitution at the 2-position [22]. The synthesis of the novel analogues in the present study (**3–6**, **10–12**, **16–19**, **21**) will be described in detail elsewhere,[1] however high purity and identity were ascertained through spectral means.

3.2. Binding affinity of ribose-modified nucleoside analogues

In binding (Table 1), high affinity for the human A_3 AR (K_i < 10 nM) was found for compounds **1** and **2** (the reference agonists IB-MECA and Cl-IB-MECA) and for 4′-thionucleosides **10–12** and **16**, extending previous observations obtained for the 5′-uronamides **13–15** [15]. Compound **16** is a novel structure having a drastic alteration in the connectivity of the structure, i.e. the adenine moiety has been shifted from the 1′- to 4′-position, but with the striking finding that the AR binding profiles and selectivity are similar in both cases. Several compounds were of intermediate affinity (K_i of 10 nM–1 μM) at the human A_3 AR, in the order of decreasing affinity: the novel 4′-fluoro-(*N*)-methanocarba derivative **21** > the 3′-fluoro derivatives **7**, **8** > **6**, **9**. Fluoro analogues **4** and **5**, with fluorine at the ribose 2′-position, and **17**, the 4′-fluoro analogue of neplanocin A, did not bind appreciably to the human A_3 AR. For comparison with affinities at other subtypes, the compounds were examined in binding experiments at the human A_1 and A_{2A} ARs. A high degree of selectivity (>100-fold) for the human A_3 AR vs. the human A_1 and A_{2A} ARs was found for 4′-thionucleosides **10–15**.

3.3. Functional potency and efficacy of ribose-modified nucleoside analogues

The activation of the human A_3 AR by this series of ribose-modified adenosine derivatives (Table 1) was examined by measuring their effects on forskolin-stimulated

[1] Jeong and coworkers, unpublished.

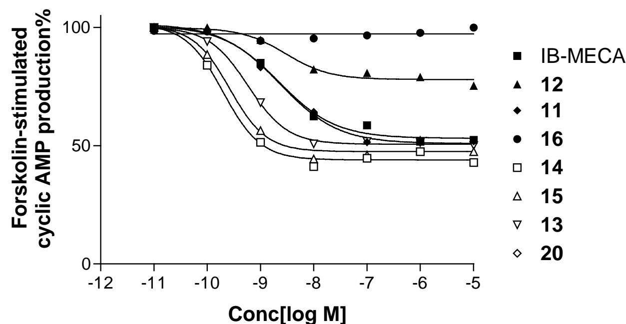


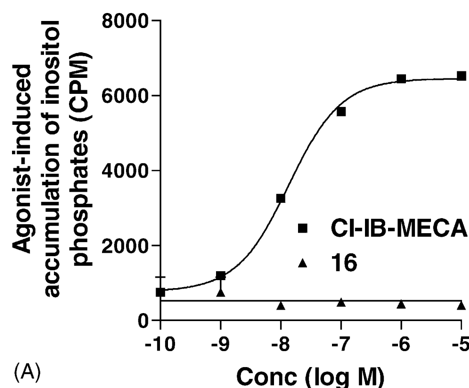
Fig. 2. Inhibition of forskolin-stimulated cyclic AMP production in CHO cells stably transfected with the human A_3 AR, induced by various adenosine derivatives. All experiments were performed in the presence of 10 μM rolipram and 3 units mL^{-1} adenosine deaminase. Forskolin (10 μM) was used to stimulate cyclic AMP levels. The level of cAMP corresponding to 100% was 220 ± 30 pmol mL^{-1}. The data shown were from one experiment performed in duplicate and are typical of three independent experiments giving similar results. EC_{50} values from three independent experiments were listed in Table 1.

cyclic AMP accumulation in CHO cells stably expressing the human A_3 AR. As shown in Fig. 2, the potent and selective A_3 AR agonist IB-MECA maximally inhibited the forskolin-stimulated cyclic AMP production by approximately 50%, and this level of inhibition of adenylate cyclase was taken as the 100% reference point in Table 1. Cl-IB-MECA was demonstrated to be equiefficacious as IB-MECA.

The 4′-thionucleosides **11** and **13–15**, and compound **20** were found to be full agonists at the human A_3 AR. Compound **20** is a novel apio analogue of neplanocin A [21] in which the 4′-CH_2OH group has been displaced to the 3′-position. Nevertheless, this highly modified carbocyclic nucleoside analogue fully activated the human A_3 AR.

Partial agonists were also identified among the novel analogues. For example, the 4′-thionucleoside **10**, which is similar to the reference agonist 2-chloroadenosine (CADO), was shown to be less efficacious than the full agonist CADO [9]. The 4′-thionucleoside **12**, which is similar to the reference nucleoside MRS 542 [9], was shown to be more efficacious than its oxygen analogue. A 3′-fluoro group was less detrimental for recognition by the human A_3 AR than a 2′-fluoro group [14], however the reduction of efficacy by the 3′-fluoro group was substantial. Thus, compounds **7** and **9** were antagonists, while compounds **6** and **8** were partial agonists at the human A_3 AR. Other partial agonists found were: a 4′-chloro neplanocin A derivative **18** and the 4′-fluoro-(*N*)-methanocarba derivative (**21**). The 4′-iodoneplanocin A derivative (**19**) was weaker in binding to the A_3 AR than its 4′-chloro analogue, however there was an indication of partial agonism for that derivative as well.

Strikingly, it was evident that structural changes in the ribose moiety, in addition to the 3′-fluoro substitution, were able to completely eliminate activation of the human A_3 AR without elimination of binding affinity. Thus, compounds **16** and **22** were antagonists at this receptor. At the human A_1 AR, compound **16** displayed a K_i value of 110 ± 12 nM, while at the human A_{2A} AR, the displacement reached only less than 50% at 10 μM. Thus, a significant degree of A_3 AR binding selectivity (26-fold vs. A_1 AR, >100-fold vs. A_{2A} AR) was seen among the human subtypes. Compound **22**, neplanocin C [20], although weaker in binding to the A_3 AR than the antagonist **16**, was noteworthy in that it was otherwise unsubstituted on the adenine moiety.



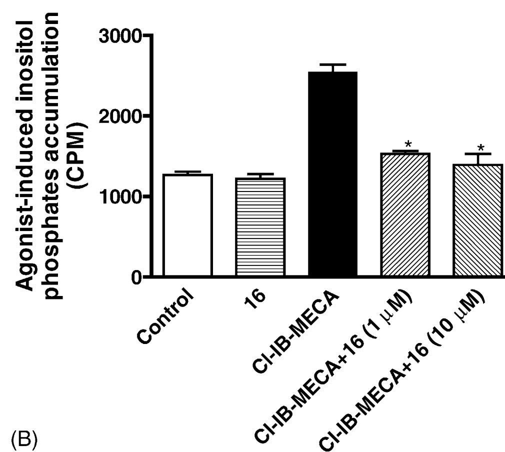


Fig. 3. (A) Stimulation of phospholipase C activity in CHO cells stably transfected with the human A_3 AR by Cl-IB-MECA **2** ($EC_{50} = 16.6 \pm 2.6$ nM, N = 4) and lack of stimulation by **16**. (B) Antagonism of the effects of Cl-IB-MECA (100 nM) by **16** at two concentrations. The procedures were described in Section 2. The data shown are from one representative experiment performed in duplicate. The EC_{50} value was from four independent experiments performed in duplicate.

The structurally unique compound **16** was examined in another functional assay. The activation of phospholipase C (PLC) was studied in CHO cells expressing the human A_3 AR (Fig. 3A). While Cl-IB-MECA displayed an EC_{50} of 16.6 ± 2.6 nM (N = 4), compound **16** (0.01–10 μM) failed to stimulate, suggesting that it may be an antagonist. This was confirmed (Fig. 3B) in an experiment in which compound **16** effectively antagonized the effects of Cl-IB-MECA to activate PLC. Furthermore, compound **16** antagonized the effects of Cl-IB-MECA on forskolin-stimulated cyclic AMP production (Fig. 4). Schild analysis [23] indicated a K_B value of 3.0 nM for inhibition of this functional effect of the human A_3 AR. In contrast to

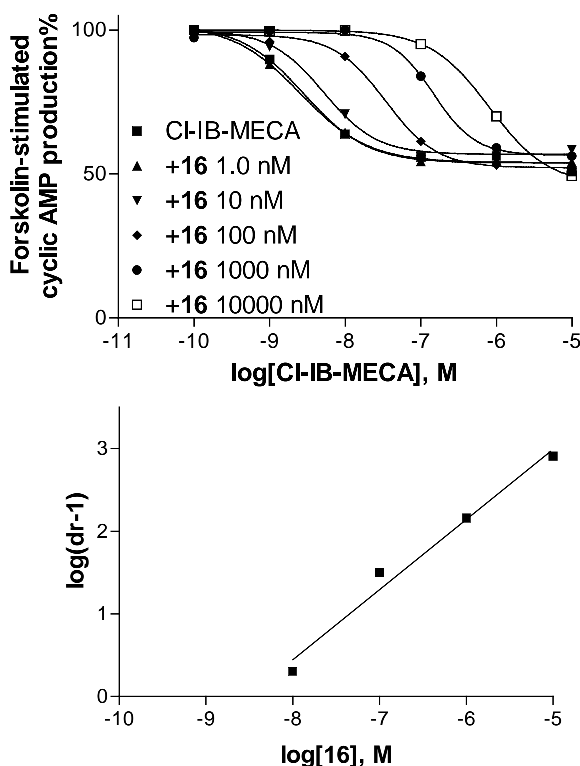


Fig. 4. Antagonism by **16** of the inhibition of cyclic AMP production elicited by Cl-IB-MECA in CHO cells stably transfected with the human A_3 AR. All experiments were performed in the presence of 10 μM rolipram and 3 units mL^{-1} adenosine deaminase. Forskolin (10 μM) was used to stimulate cyclic AMP levels. The level of cAMP corresponding to 100% was 220 ± 30 pmol mL^{-1}. The data shown were from one experiment performed in duplicate and are typical of three independent experiments giving similar results. The K_B value for **16** was calculated to be 3.0 nM.

compound **16**, the 4′-thionucleoside (**15**) was a potent full agonist (Table 1).

3.4. Activity of selected ribose-modified nucleoside analogues at other ARs

Compound **16** has moderate affinity for the human A_1 AR, however, it was not known whether it was an agonist or antagonist at this subtype. Hence, we further examined its effect on forskolin-stimulated cyclic AMP production in CHO cells expressing human A_1 ARs. As shown in Fig. 5, both CPA and CCPA are potent agonists for human A_1 ARs, while no significant activation by compound **16** was demonstrated. In comparison, MRS 1743 and MRS 1760, which are potent, yet low efficacy partial agonist/antagonists at the human A_3 AR [9], were shown to fully activate the human A_1 AR.

3.5. Species differences: comparison with rat ARs

As demonstrated earlier, pronounced species differences of some adenosine derivatives have been found among human and rat A_3 ARs [10]. Selected compounds were tested in binding to three subtypes of rat ARs (Table 2).

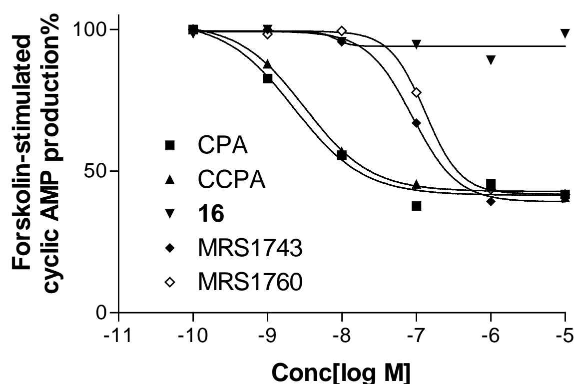


Fig. 5. Inhibition of forskolin-stimulated cyclic AMP production in CHO cells stably transfected with the human A_1 AR, induced by various adenosine derivatives. All experiments were performed in the presence of 10 μM rolipram and 3 units mL^{-1} adenosine deaminase. Forskolin (10 μM) was used to stimulate cyclic AMP levels. The level of cAMP corresponding to 100% was 247 ± 52 pmol mL^{-1}. The data shown were from one experiment performed in duplicate and are typical of three independent experiments giving similar results. EC_{50} values were (nM): CPA, 2.2 ± 0.5; CCPA, 2.8 ± 0.8; MRS 1743, 78 ± 23; MRS 1760, 97 ± 36.

Here we show that species differences were also pronounced for some compounds of this series, especially at A_3 ARs (Fig. 6). Compounds **7**, **13**, **14**, **16**, and **21** showed much higher affinity for human than for rat A_3 ARs (12-, 13-, 85-, 22-, and 22-fold, respectively). The A_1 and A_{2A} AR affinities of **13** were also diminished in rat compared to human. Compound **15** showed a similar affinity for human and rat A_3 ARs (approximately 2-fold difference), and thus retained A_3 AR selectivity in the rat.

4. Discussion

In previous studies it has been demonstrated that the intrinsic efficacy of adenosine derivatives in human A_3 AR activation depended upon both N^6- and 2-substitutions at the adenine moiety [8–12]. For example, N^6-substituents

Table 2
Binding affinities of selected compounds at rat A_1, A_{2A} and A_3 ARs (K_i, nM)[a]

Compound	rA_1	rA_{2A}	rA_3
7	1350 ± 350[b]	>10,000[b]	910 ± 240
13	294 ± 115[c]	442 ± 148	13.4 ± 4.3
14	198 ± 14[c]	$6,340 \pm 90$[c]	23.8 ± 11.2
15	140 ± 43[c]	348 ± 110[c]	0.82 ± 0.27
16	188 ± 40	>10,000	95 ± 12
21	417 ± 119	>10,000	570 ± 240

[a]All A_3 AR experiments were performed using adherent CHO cells stably transfected with cDNA encoding the rat A_3 AR using [^{125}I]I-AB-MECA as radioligand. Binding at rat forebrain A_1AR and rat striatal A_{2A} AR in this study was carried out as described in Section 2 using [^{3}H] PIA or [^{3}H]CGS21680 as radioligand. Values from the present study are means ± SEM, N = 3.

[b]Data from Lim *et al.* [14].

[c]Data from Jeong *et al.* [15].

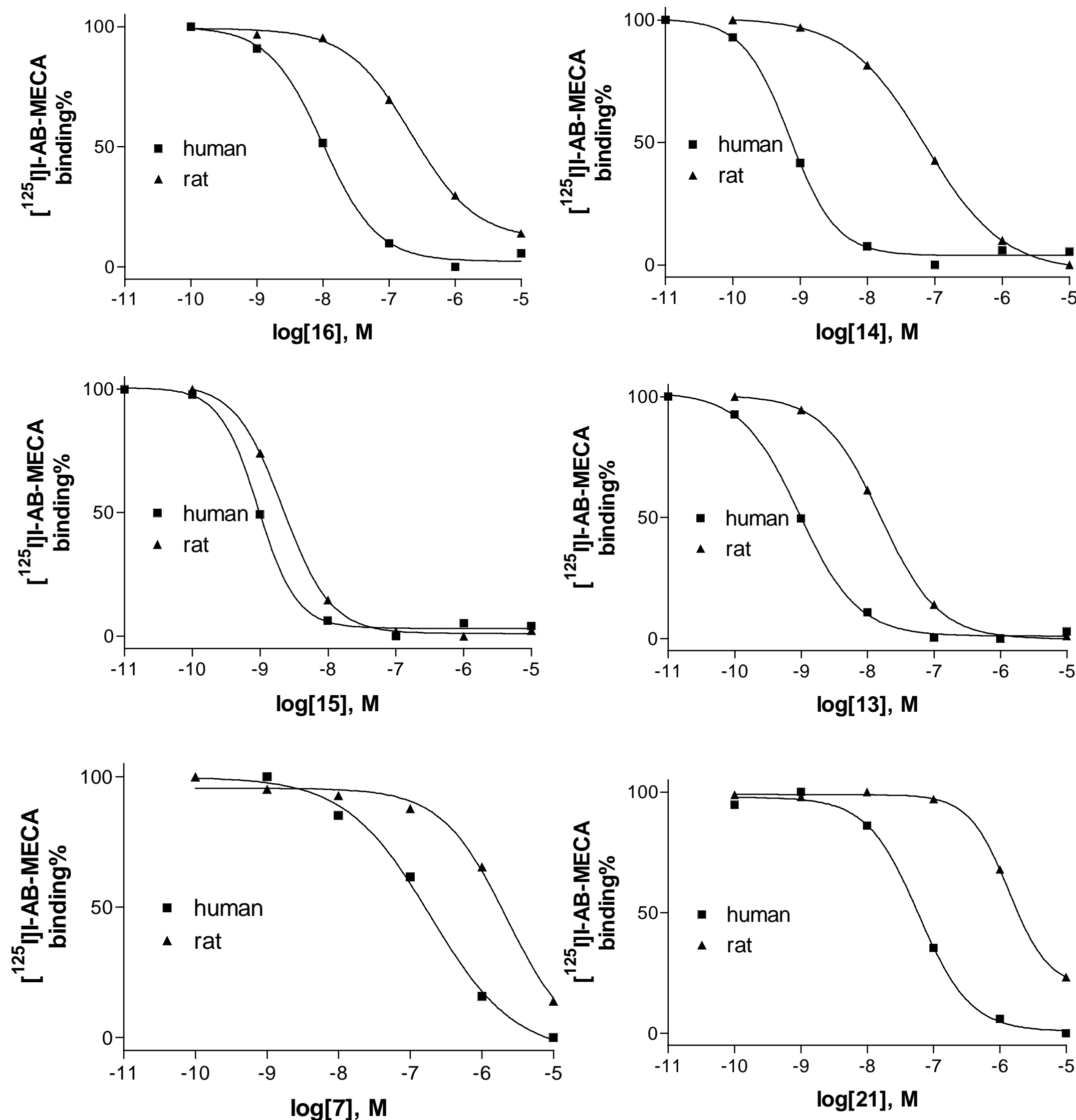


Fig. 6. Species differences in affinity as illustrated by competition for radioligand binding ([^{125}I]I-AB-MECA) at rat and human A_3 ARs, by six representative adenosine analogues **7**, **13–16** and **21**. All except **15** were significantly more potent at human than at rat A_3 ARs. The procedures were described in Section 2. The data points are from a representative experiment performed in duplicate. The mean K_i values for rat A_3 ARs calculated from three independent experiments are provided in Table 2.

consisting of a large cycloalkyl group (>5 carbons) reduced efficacy compared to smaller rings in the activation of the human A_3 AR. Here, we further demonstrated that the simultaneous exocyclic and cyclic substitution of the ribose moiety also contributed differentially to the affinity and efficacy at the human A_3 AR.

Our earlier studies [8–10] demonstrated that some full A_1 AR agonists, including 2-chloro analogues, have reduced intrinsic efficacy at the human A_3 AR. For example, 2-chloro-*R*-PIA was a partial A_3 AR agonist, and CCPA was an antagonist. The N^6-derivative DPMA, a potent A_{2A} AR agonist [24], was also demonstrated to be a moderately potent antagonist at the A_3 AR [10]. In the present study, the newly synthesized adenosine derivative **16** was demonstrated to be a potent antagonist for human A_3 AR. In contrast to CCPA and DPMA, **16** is an antagonist for both human A_1 and human A_3 ARs. It is not yet known whether **16** is an agonist or antagonist at the A_{2A} AR, due to its extremely low affinity at this receptor.

The stereochemistry and substitution of the ribose hydroxyl groups greatly affected the intrinsic efficacy. The arabino derivative (**3**) displayed greatly reduced A_3 AR binding affinity due to inversion of stereochemistry at the 3′-carbon. Like its stereoisomer MRS 542 [9], it did not display agonist action at the human A_3 AR. In compounds **4** and **5**, a 2′-fluoro group greatly attenuated human A_3 AR binding. The 3′-fluoro substitution, as in **6**, allowed a greater degree of agonism than when fluoro was present at the 2′-position. The human A_3 AR affinity of the 3′-fluoro analogue **7** was 42-fold weaker than the affinity of the corresponding riboside MRS 542, and, like MRS 542, it was an antagonist at this subtype. The loss of intrinsic

efficacy of 3′-fluoro analogues (**6**, **7**) could be partly overcome by the presence of a 5′-uronamide group for an N^6-methyl analogue (**8**), but not for a N^6-(3-iodobenzyl) analogue (**9**). Previously, it was found that the agonism of other low efficacy A_3 AR agonists was fully restored by a 5′-uronamide group [9], unlike the present case.

The 4′-thio modification [15] generally enhanced potency (as in **10**), yet decreased efficacy. Thus, 4′-thio substituted 5′-CH_2OH analogues **10** and **12** were partial agonists of the A_3 AR. The N^6-methyl group in **11** counteracted the potency-reducing effect of a 2-Cl group; thus **11** was a very potent agonist. The 4′-thio group of **12** increased efficacy in comparison to its oxygen analogue MRS 542. Compounds **13–15** were potent, full agonists, consistent with the ability of a flexible 5′-amide group to overcome various efficacy-reducing structural changes [9]. The rearranged analogue **16** was a potent, selective antagonist. The SAR comparison of **10–12** vs. compounds **13–15** suggests that an analogue of **16** where the *N*-methylcarboxamide is replaced by a hydroxymethyl group would be an interesting compound. Carbocyclic derivatives were found to be of variable affinity and intrinsic efficacy. Although compound **17** was inactive as either agonist or antagonist, compound **18** was a weak agonist. Compound **20** was the first example of this structural class shown to interact with adenosine receptors.

The present study together with our earlier studies [10] suggested that the N^6-methyl group is extremely favorable for human A_3 AR affinity and selectivity. Thus, both **11** and **14** are potent and selective agonists for the A_3 AR. This finding extended our previous observation that N^6-iodobenzyl group is optimal for A_3 AR affinity and selectivity [25]. However, it should be noted that the N^6-methyl group is somewhat detrimental to the binding affinity at the rat A_3 AR.

Recent findings indicated that some compounds might activate two different subtypes of a certain receptor family, both leading to beneficial effects. Some other compounds might activate a specific receptor subtype that is beneficial, while blocking another subtype that might lead to the harmful effects associated with adenosine. The dual-acting ligands of ARs may have considerable promise as novel approaches to treat ischemic conditions. It has been suggested that activation of both A_1 and A_3 ARs may have cardioprotective effects but via different mechanisms [26,27]. Hence, dual acting agonists for A_1 and A_3 ARs might be useful for cardioprotection. In contrast to the effect of AR activation on the heart, pretreatment with an A_1 agonist or an A_3 antagonist protects the kidney from a subsequent prolonged ischemic insult [28]. The role of the A_3 AR in kidney function was further confirmed by the fact that A_3 AR knockout mice are protected against ischemia- and myoglobinuria-induced renal failure [29]. Hence, dual-acting compounds (activating A_1 and blocking A_3 ARs), such as CCPA, might be useful for protection of ischemic condition of kidney. It may be speculated that agonists blocking both A_1 and A_3 ARs while activating A_{2A} ARs may also have potential clinical use. Compound **16** could serve as a starting point for this series. Novel and unique ligands may be identified by optimizing the selectivity, efficacy and binding affinity of the adenosine derivatives at different AR subtypes.

In conclusion, critical structural determinants for A_3 AR activation have been identified by modifying the ribose moiety of adenosine or adenosine derivatives, which should prove useful for further understanding of the mechanism of receptor activation and development of more potent and selective full agonists, partial agonists and antagonists for A_3 ARs.

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