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2-Substituted adenosine derivatives: affinity and efficacy at four subtypes of human adenosine receptors

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Abstract

The affinity and efficacy at four subtypes (A_1, A_{2A}, A_{2B} and A_3) of human adenosine receptors (ARs) of a wide range of 2-substituted adenosine derivatives were evaluated using radioligand binding assays and a cyclic AMP functional assay in intact CHO cells stably expressing these receptors. Similar to previous studies of the N^6-position, several 2-substituents were found to be critical structural determinants for the A_3AR activation. The following adenosine 2-ethers were moderately potent partial agonists (K_i, nM): benzyl (117), 3-chlorobenzyl (72), 2-(3-chlorophenyl)ethyl (41), and 2-(2-naphthyl)ethyl (130). The following adenosine 2-ethers were A_3AR antagonists: 2,2-diphenylethyl, 2-(2-norbornan)ethyl, *R*- and *S*-2-phenylbutyl, and 2-(2-chlorophenyl)ethyl. 2-(*S*-2-Phenylbutyloxy)adenosine as an A_3AR antagonist right-shifted the concentration–response curve for the inhibition by NECA of cyclic AMP accumulation with a K_B value of 212 nM, which is similar to its binding affinity (K_i = 175 nM). These 2-substituted adenosine derivatives were generally less potent at the A_1AR in comparison to the A_3AR, but fully efficacious, with binding K_i values over 100 nM. The 2-phenylethyl moiety resulted in higher A_3AR affinity (K_i in nM) when linked to the 2-position of adenosine through an ether group (54), than when linked through an amine (310) or thioether (1960). 2-[2-(l-Naphthyl)ethyloxy]adenosine (K_i = 3.8 nM) was found to be the most potent and selective (>50-fold) A_{2A} agonist in this series. Mixed A_{2A}/A_3AR agonists have been identified. Interestingly, although most of these compounds were extremely weak at the A_{2B}AR, 2-[2-(2-naphthyl)ethyloxy]adenosine (EC_{50} = 1.4 μM) and 2-[2-(2-thienyl)-ethyloxy]adenosine (EC_{50} = 1.8 (M) were found to be relatively potent A_{2B} agonists, although less potent than NECA (EC_{50} = 140 nM).

Keywords: Adenosine receptors; Purines; Nucleosides; GPCR; Efficacy; Structure–activity relationships

1. Introduction

Extracellular adenosine acts as a local modulator at four subtypes of receptors (A_1, A_{2A}, A_{2B}, and A_3), which are involved in numerous physiological and pathophysiological processes [1]. For example, adenosine attenuates the effects of ischemia in the heart and brain. Acting through the A_{2A} adenosine receptor (AR), it suppresses prolonged inflammation [2] and causes vasodilation and inhibits platelet aggregation, thus increasing the amount of oxygen available to an organ under stress. Adenosine agonists selective for the A_3AR are of interest as cerebroprotective [3], cardioprotective [4,5], and anticancer [6] agents.

Recently, we have characterized structure–efficacy relationships for adenosine derivatives as agonists at the A_3AR. The intrinsic efficacy of adenosine derivatives in activation of the A_3AR is more variable than at other subtypes [7–10]. Specific groups placed at the N^6-position and on the ribose moiety have reduced or completely abolished the ability to activate this receptor, while maintaining high binding affinity. Thus, it has been possible to design nucleoside-based antagonists [11], which in many cases are selective for both the human and rat A_3ARs. Such A_3AR antagonists include adenosine derivatives bearing: steric constraints of the ribose moiety or its 5′-amide modification [11], N^6-groups such as 2,2-diphenylethyl and cyclopropyl [12], and the combination of substituted N^6-benzyl groups and various small substituents at the adenine 2-position (such as chloro, cyano, and

Abbreviations: AR, adenosine receptor; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyladenosine; DMEM, Dulbeccos modified Eagles medium; I-AB-MECA, N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; PIA, N^6-phenylisopropyladenosine; PTLC, preparative thin layer chromatography

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methoxycarbonyl) [11,13]. A radically altered analogue in which the properly functionalized adenine moiety was shifted from the 1′-position to the 4′-carbon proved to be an A_3AR selective antagonist [9].

The aim of the present study is to expand knowledge of the structure activity relationships at the A_3AR and at other subtypes, both in relation to binding affinity and intrinsic efficacy, of adenosine derivatives modified in the 2-position. In this manner, it will be possible to design new, selective A_3AR agonists, partial agonists, and antagonists based on nucleoside structures. Derivatives of adenosine modified at the 2-position of the adenine ring have been studied in both binding and/or functional assays at the four AR subtypes. For this purpose, we have expressed the human ARs stably in Chinese hamster ovary (CHO) cells [14]. Most of these analogues are 2-ether substituted adenosine derivatives, which have been previously evaluated at the rat A_1 and A_{2A}ARs but not at the four human subtypes in a systematic manner [15–20]. From previous studies [7,13,21] and in greatly expanded form in the present study, it is clear that the intrinsic efficacy of adenosine derivatives at the A_3AR is dependent on structural changes at both the N^6-position and the 2-position. The intrinsic efficacy at the A_{2A}AR tended to be insensitive to the same structural changes. Additionally, here we have identified several substituents at the 2-position that contribute significantly to the A_{2B}AR activity.

2. Materials and methods

2.1. Materials

[^{125}I]N^6-(4-Amino-3-iodobenzyl)adenosine-5′-N^6-methyluronamide ([^{125}I]I-AB-MECA; 2000 Ci/mmol), [^{3}H]*R*-PIA (*R*-N^6-[phenylisopropyl]adenosine, 34 Ci/mmol), [^{3}H]CGS21680 (2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-N^6-ethylcarboxamido-adenosine, 47 Ci/mmol) and [^{3}H]cyclic AMP (40 Ci/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, UK). NECA, CGS21680, CPA, and *R*-PIA were purchased from Sigma-RBI (St. Louis, MO). Most of the 2-substituted adenosine derivatives examined were the kind gift of Dr. Ray A. Olsson (University of South Florida) and Dr. John W. Daly (NIDDK). Compound **24** was prepared as described [37]. Other chemicals were from standard commercial sources and of analytical grade.

2.2. Chemical synthesis

General synthetic procedure for substitution at the 2-position of adenosine (for compounds **21** and **44**).

Adenosine derivatives were synthesized using the general method of Ueeda et al. [15]. A solution of appropriate alcohol or thiol (0.35 mmol) in 5 ml of dry 1,2-dimethoxyethane was cooled to 0 °C in an ice bath. To this solution was added 2.5 M *n*-BuLi (0.13 ml, 0.32 mmol), the reaction mixture was stirred for 1 h at 0 °C. The protected nucleoside 2-chloro-2′,3′-*O*-(ethoxymethylidene) adenosine (25 mg, 0.07 mmol) was added in one portion. The reaction mixture was refluxed for 4 days, at which time HPLC showed that starting material had almost completely disappeared.

The solvent was removed in vacuo, and a solution of the residue in 10 ml water was extracted with ethyl acetate (4 × 20 ml). The combined extracts were dried over Na_2SO_4 and evaporated in vacuo. The residue was purified with preparative thin layer chromatography (PTLC, silica gel) with the mobile phase consisting of mixtures of methanol (3% for **21** or 5% for **44**, by volume) and chloroform. Fractions containing products were concentrated and dissolved in methanol–water. Acetic acid or trifluoroacetic acid was added and the solution refluxed until HPLC showed that the nucleoside was completely deblocked. The solution was adjusted to pH 9 with NH_3–EtOH and was refluxed for 30 min. The solvent was removed, and the residue was purified with PTLC, with the mobile phase consisting of mixtures of methanol (20% for **21** or 10% for **44**, by volume) and chloroform, to afford the corresponding product (overall yield 25–34% in two steps). Proton NMR and mass spectra were consistent with the assigned structures.

2.3. Cell culture and membrane preparation

The CHO cells stably expressing recombinant ARs were cultured in DMEM and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μmol/ml glutamine and 800 μg/ml geneticin. After harvest and homogenization, cells were centrifuged at 500 × *g* for 10 min, and the 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 °C. The resultant pellets were resuspended in buffer in the presence of 3 units/ml adenosine deaminase, and the suspension was stored at −80 °C until the binding experiments. The protein concentration was measured using the Bradford assay [22].

2.4. Binding assays

For A_3AR binding assays [23], each tube contained 100 μl of membrane suspension, 50 μl of [^{125}I]I-AB-MECA (final concentration 0.5 nM), and 50 μl of increasing concentrations of compounds in Tris–HCl buffer (50 mM, pH 7.4) containing 10 mM MgC_2. Nonspecific binding was determined using 10 (M NECA. The mixtures were incubated at 25 °C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburg, MD). Filters were washed three

times with ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter. The binding of [^{3}H]R-PIA to A_1 receptors and the binding of [^{3}H]CGS21680 to A_{2A} receptors were as previously described [8].

2.5. Cyclic AMP accumulation assay

Intracellular cyclic AMP levels were measured with a competitive protein binding method [24]. CHO cells expressing four subtypes of recombinant ARs were harvested by trypsinization. After resupension in medium, cells were planted in 24-well plates in 0.5 ml medium. After 24 h, the medium was removed and cells were washed three times with 0.5 ml DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with agonists and/or test compounds in the presence of rolipram (10 (M) and adenosine deaminase (3 units/ml). In the case of A_1 and A_3ARs, after 45 min forskolin (10 (M) was added to the medium, and incubation was continued an additional 15 min. The reaction was terminated by removing the medium, and cells were lysed upon the addition of 200 (L of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at −20 °C. For determination of cyclic AMP production, protein kinase A (PKA) was incubated with [^{3}H]cyclic AMP (2 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 by liquid scintillation spectrometry.

2.6. Statistical analysis

Binding and functional parameters were estimated using Prism software (GraphPAD). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng–Prusoff equation [25]. For an antagonist, Schild analysis was carried out as reported [26]. Data were expressed as mean ± standard error.

3. Results

3.1. Nucleoside structures examined

The classes of compounds examined included 2-alkyloxy ethers **1**–**18**, 2-alkylaryl derivatives **19**–**46** including mainly ethers, however, an amino derivative **22** and a thioether **23** were included for comparison, two 5′,8-cyclo analogues **47** and **48**, and four standard reference AR agonists **49**–**52**. Although **47** and **48** were not substituted at the 2-position, they were included in this study, since conformational constraint in the ribose region has previously formed the basis of AR selectivity [27], and in these analogues the 2-position remains available for further derivatization.

Adenosine derivatives synthesized for this study were prepared by nucleophilic substitution by the appropriate alkoxy ion of the 2-chloro substituent in a ribose-protected derivative of 2-chloroadenosine, using methods similar to those reported previously [16].

3.2. Assays of binding and activation of ARs

Binding at the A_1, A_{2A}, and A_3ARs was carried out using standard agonist radioligands [8,23] in membranes of transfected CHO cells [14]. The activation of A_1 and A_3ARs (G_i-coupled) by the 2-substituted adenosine derivatives (Table 1) was examined by measuring the inhibition of forskolin-stimulated cyclic AMP accumulation in intact CHO cells stably expressing these receptors. The activation of A_{2A} and A_{2B}ARs (G_s-coupled) in stably transfected CHO cells was measured in the absence of forskolin. The efficacy of each of these adenosine derivatives was evaluated at a fixed concentration of 10 (M and expressed as a percentage of the effect of a reference (full) agonist. In some cases, full concentration–response curves were measured. For compounds that bound weakly at a given AR, no indication of intrinsic efficacy was readily obtainable, however for the many examples of high affinity binding the degree of activation at 10 (M served as an approximate measure of intrinsic efficacy.

3.3. Affinity and potency at the A_3AR

Various 2-substituents were found to be critical structural determinants for activation of the A_3AR. Less than maximal efficacy or lack of efficacy was frequently observed for the 2-modified adenosine derivatives at the A_3AR. The following adenosine 2-ethers were moderately potent partial agonists (K_i): benzyl **20** (117 nM), 2-(3-chlorophenyl)ethyl **35** (41 nM), and 2-(2-naphthyl)ethyl **40** (130 nM). Fig. 1 shows the concentration–response curves for inhibition of cyclic AMP accumulation by a variety of 2-position ethers, indicating the variation in efficacy.

The following adenosine 2-ethers were A_3AR antagonists: 2,2-diphenylethyl **41**, *R*-2-phenylbutyl **37**, *S*-2-phenylbutyl **38**, 2-(2-chlorophenyl)ethyl **34** and 2-norzbornylethyl **9**. 2-(*S*-2-Phenylbutyloxy)adenosine **38** right-shifted the concentration–response curve for the inhibition by NECA of cyclic AMP accumulation with a K_B value of 212 nM (Fig. 2) calculated by Schild analysis [26], which is similar to its A_3AR affinity (K_i = 175 nM) determined in the binding assay.

Among small 2-alkyloxy groups, efficacy at the A_3AR remained nearly full with increasing size until the branched hexyl derivative **5**. Branching of the *O*-hexyl group more distally in **11** did not alter affinity, but increased efficacy. The straight-chain pentyl **14** and hexyl **16** ether derivatives

Table 1
Binding affinities of adenosine derivatives at human A_1, A_{2A}, and A_3ARs expressed in CHO cells (expressed as K_i value or percent displacement at 10 (M) and maximal agonist effects at 10 (M (% of full agonist) at the ARs

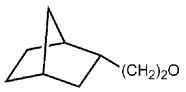
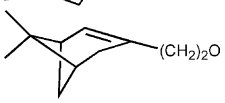
Compound	2-Substitution	K_i at A_1AR (nM[a]) (% activation)	K_i at A_{2A}AR (nM[a]) (% activation)	% activation at A_{2B}AR[a] (and % inhibition), unless noted	K_i at A_3AR (nM[a]) (% activation)
2-Alkoxy analogues					
1	CH_3O	155 ± 32 (119)	970 ± 310 (78.6)	−2.7 ± 5.3 (−0.2)	156 ± 37 (75.2 ± 5.1)
2	CH_3CH_2O	2640 ± 540 (81.3)	360 ± 139 (92.4)	−0.8 ± 1.9 (−6.7)	568 ± 205 (99.1 ± 4.2)
3	$(CH_3)_2CHO$	16% (15.9)	927 ± 204 (87.5)	0.4 ± 2.9 (−3.2)	457 ± 154 (101 ± 5)
4	$(CH_3)_2CHCH_2O$	4410 ± 1150 (44.6)	222 ± 89 (93.2)	−1.2 ± 8.8 (−3.4)	84.2 ± 8.4 (108 ± 10)
5	$(CH_3CH_2)_2CHCH_2O$	42% (1.0)	45.8 ± 22.1 (90.0)	−4.4 ± 4.2 (2.0)	336 ± 116 (4.9 ± 5.2)
6	Cyclohexyl-CH_2O	3350 ± 390 (27.5)	342 ± 22 (92.8)	−0.9 ± 2.3 (−4.7)	143 ± 32 (27.1 ± 4.9)
7	$(CH_3)_2CH(CH_2)_2O$	3560 ± 1120 (29)	37.7 ± 4.9 (92.4)	−0.6 ± 1.8 (−8.4)	81.1 ± 9.0 (96.3 ± 3.0)
8	Cyclohexyl-$(CH_2)_2O$	36% (27.8)	579 ± 250 (102)	−4.6 ± 8.8 (0.4)	578 ± 182 (51.6 ± 3.4)
9		3590 ± 670 (0)	137 ± 31 (98.6)	−0.1 ± 2.6 (2.8)	149 ± 45 (−3.5 ± 9.0)
10		3350 ± 720 (25.9)	21.2 ± 12.7 (97.7)	−0.5 ± 4.4 (2.8)	341 ± 132 (−0.2 ± 5.9)
11	$(CH_3)_2CH(CH_2)_3O$	3700 ± 790 (18.3)	77.8 ± 20.9 (96.4)	−1.7 ± 2.8 (0.0)	105 ± 31 (49.6 ± 8.1)
12	Cyclohexyl-$(CH_2)_3O$	1730 ± 330 (33.5)	92.0 ± 52.5 (105)	−2.5 ± 4.6 (2.2)	83.3 ± 8.4 (21.2 ± 11.8)
13	Cyclohexyl-$(CH_2)_4O$	883 ± 99 (109)	291 ± 73 (98.5)	−0.27 ± 2.8 (−0.5)	105 ± 13 (12.7 ± 1.5)
14	$CH_3(CH_2)_4O$	2430 ± 620 (48.7)	6.9 ± 1.3 (94.1)	−2.2 ± 2.6 (9.9)	222 ± 68 (92.8 ± 13.4)
15	$CH_3C{\equiv}C–(CH_2)_2O$	583 ± 78 (49.8)	63.2 ± 50.3 (95.9)	1.4 ± 3.7 (−7.9)	90.2 ± 36.2 (73.4 ± 4.4)
16	$CH_3(CH_2)_5O$	1830 ± 340 (11.8)	156 ± 89 (100)	12.6 ± 0.5 (−6.5)	124 ± 28 (43.7 ± 6.3)
17	$(CH_3)_2C{=}CH(CH_2)_2CH(R\text{-}CH_3)(CH_2)_2O$	34% (23.6)	382 ± 100 (91.3)	0.3 ± 3.4 (−5.0)	431 ± 130 (−1.5 ± 1.5)
18	$(CH_3)_2C{=}CH(CH_2)_2CH(S\text{-}CH_3)(CH_2)_2O$	4550 ± 950 (21.3)	78.3 ± 10.3 (90.2)	−0.4 ± 1.7 (−1.7)	74.4 ± 22.5 (31.5 ± 6.4)
2-Aryl- and arylalkyloxo (or amino)					
19	Phenyl-O	5140 ± 1110 (57.6)	44 ± 12 (100)	0.7 ± 1.8 (−0.9)	364 ± 96 (32.2 ± 3.5)
20	Benzyl-O	642 ± 79 (11.5)	585 ± 155 (85.1)	−3.3 ± 0.6 (9.5)	117 ± 8 (16.9 ± 3.9)
21	3-Chlorobenzyl-O	27.4 ± 3.9 (46)	228 ± 66	7.4 ± 2.1	71.6 ± 24.6 (16.2 ± 3.8)
22	Phenyl-$(CH_2)_2O$	221 ± 57 (112)	9.3 ± 2.9 (99.6)	3490 ± 1490[c] (0.0)	54.2 ± 14.3 (70.7 ± 2.7)
23	Phenyl-$(CH_2)_2NH$	530 ± 88 (70.5)	62.0 ± 17.6 (105)	−1.7 ± 3.1 (−6.9)	310 ± 163 (72.0 ± 3.2)
24	Phenyl-$(CH_2)_2S$	3700 ± 770	590 ± 260	ND	1960 ± 310
25	2-Methylphenyl-$(CH_2)_2O$	396 ± 83 (74.6)	17.4 ± 7.4 (110)	−1.0 ± 2.7 (3.8)	214 ± 47 (7.3 ± 6.0)
26	3-Methylphenyl-$(CH_2)_2O$	295 ± 8 (106)	41.6 ± 22.0 (98.4)	15.3 ± 2.5 (0.6)	242 ± 55 (70.9 ± 3.9)
27	4-Methylphenyl-$(CH_2)_2O$	1250 ± 250 (61.8)	118 ± 95 (98.3)	12.1 ± 1.9 (−12.2)	470 ± 81 (95.5 ± 15.1)
28	2-Methyloxyphenyl-$(CH_2)_2O$	490 ± 114 (111)	274 ± 142 (94.2)	7.4 ± 1.3 (0.6)	940 ± 354 (3.6 ± 6.7)
29	3-Methyloxyphenyl-$(CH_2)_2O$	246 ± 34 (114)	32.1 ± 1.6 (103)	0.4 ± 4.1 (0.0)	231 ± 53 (85.1 ± 5.8)
30	4-Methyloxyphenyl-$(CH_2)_2O$	288 ± 22 (109)	64.3 ± 7.8 (97.6)	11.3 ± 1.8 (−4.4)	105 ± 20 (91.1 ± 9.2)
31	3,4-Dimethyloxyphenyl-$(CH_2)_2O$	469 ± 118 (101)	30.3 ± 2.8 (99.8)	−1.4 ± 2.1 (6.2)	863 ± 313 (66.5 ± 3.7)
32	2-Fluorophenyl-$(CH_2)_2O$	331 ± 22 (18.9)	58.1 ± 24.9 (99.1)	16.6 ± 2.5 (0.3)	77.8 ± 13.5 (44.5 ± 5.1)
33	4-Fluorophenyl-$(CH_2)_2O$	467 ± 100 (115)	56.8 ± 16.3 (97.9)	17.3 ± 3.1 (−7.2)	112 ± 16 (73.4 ± 5.2)
34	2-Chlorophenyl-$(CH_2)_2O$	366 ± 33 (31.8)	17.9 ± 6.1 (94.9)	1.0 ± 3.4 (6.9)	144 ± 22 (1.4 ± 2.7)
35	3-Chlorophenyl-$(CH_2)_2O$	372 ± 116 (85.3)	11.5 ± 5.3 (96.7)	28.0 ± 4.9 (2.9)	41.0 ± 7.8 (31.0 ± 7.0)
36	4-Chlorophenyl-$(CH_2)_2O$	331 ± 51 (113)	58.5 ± 8.0 (97.4)	17.5 ± 2.8 (5.7)	116 ± 23 (69.0 ± 6.3)
37	*R*-2-Phenylbutyl-O	28% (24.5)	503 ± 98 (97.7)	−0.2 ± 6.0 (4.6)	201 ± 61 (−1.6 ± 3.8)
38	*S*-2-Phenylbutyl-O	4780 ± 990 (16.0)	26.9 ± 6.9 (96.0)	0.4 ± 5.6 (−5.5)	175 ± 31 (−3.9 ± 3.9)
39	2-(l-Naphthyl)ethyl-O	220 ± 18 (101)	3.8 ± 1.4 (102)	−2.1 ± 5.1 (−3.7)	205 ± 19 (12.8 ± 5.9)
40	2-(2-Naphthyl)ethyl-O	141 ± 51 (102)	16.1 ± 7.0 (105)	1440 ± 70[c]	130 ± 8 (45.1 ± 8.5)
41	2,2-Diphenylethyl-O	39% (10.4)	310 ± 119 (97.5)	−1.8 ± 5.6 (0.8)	53.6 ± 10.4 (−0.0 ± 0.6)
42	2-(2-Thienyl)ethyl-O	174 ± 20 (112)	10.9 ± 4.8 (105)	1780 ± 260[c]	93.3 ± 16.8 (79.7 ± 4.5)
43	2-(3-Thienyl)ethyl-O	280 ± 72 (117)	13.3 ± 4.1 (106)	8.9 ± 5.5 (−2.0)	101 ± 34 (61.6 ± 15.1)
44	*trans*-2-Phenylcyclopropyl-O	367 ± 27 (19.1)	2050 ± 700	1.2 ± 2.3	292 ± 46 (0.4 ± 1.6)
45	4-Phenylbutyl-O	1100 ± 230 (24.6)	243 ± 166 (103)	−0.4 ± 2.3 (−7.8)	251 ± 80 (19.3 ± 6.2)
46	5-Phenylpentyl-O	700 ± 126 (104)	249 ± 54 (101)	6.2 ± 3.6 (5.4)	429 ± 159 (9.8 ± 8.8)
5′,8-Cyclo analogues	Name				
47	Cyclo-CPA[b]	418 ± 62 (117)	12% (11.7)	−0.6 (−2.9)	25% (11.7 ± 0.3)
48	Cyclo-*R*-PIA[b]	49% (26.3)	18% (27.6)	4.5 (−9.6)	27% (1.7 ± 1.7)

Table 1 (*Continued*)

Compound	2-Substitution	K_i at A_1AR (nM[a]) (% activation)	K_i at A_{2A}AR (nM[a]) (% activation)	% activation at A_{2B}AR[a] (and % inhibition), unless noted	K_i at A_3AR (nM[a]) (% activation)
Reference compounds	Name				
49	CPA	1.8 ± 0.5 (112)	820 ± 216 (98)	8.6 ± 2.9 (0.0)	72 ± 12 (99 ± 6)
50	*R*-PIA	2.0 ± 0.3 (101)	884 ± 188 (101)	1680 ± 498[c]	8.7 ± 0.9 (102 ± 6)
51	CGS21680	1570 ± 460 (99)	8.8 ± 1.6 (100)	4.7 ± 1.8 (0.0)	114 ± 16 (98 ± 5)
52	NECA	6.8 ± 2.4 (100)	2.2 ± 0.6 (100)	140 ± 19[c] (0.0)	16.0 ± 5.4 (100)

At the A_{2B}AR, EC_{50} values or the percent stimulation at 1 (M (and in parentheses percent inhibition at 1 (M of the effects of 100 nM NECA) are shown.

[a] All experiments were performed using adherent CHO cells stably transfected with cDNA encoding a human adenosine receptor. Percent activation of the human A_1 A_{2A} and A_3AR was determined at 10 μM. Binding at A_1, A_{2A} and A_3ARs was carried out as described in Section 2. The A_1 and A_{2A}AR activation results were expressed as the mean values from two separate experiments, while the A_3AR activation results were from three separate experiments. The K_i and EC_{50} values from the present study are means ± S.E.M., N = 3–5.

[b] Structures shown in Fig. 4; ND: not determined.

[c] EC_{50} (nM) for activation of the A_{2B}AR.

were full and partial agonists, respectively. In the series of *O*-alkylcyclohexyl derivatives of varying chain length **6**, **8**, **12**, and **13**, all were partial agonists at the A_3AR with similar affinity (100–600 nM), but with efficacy diminishing as chain length increased beyond two methylenes. A diastereomeric pair of branched decenyl ethers **17** and **18** differed greatly in both affinity (see below) and efficacy at the A_3AR.

The series of phenyl **19** and phenylalkyl (**20**, **22**, **45**, and **46**) ethers showed high A_3AR efficacy only at intermediate length, i.e. the 2-phenylethyl ether **22**, which also demonstrated the highest A_3AR affinity among the five analogues with a K_i value of 54 nM. Nevertheless, **22** was not selective for the A_3AR. The benzyl ether **20** was modestly potent as a partial agonist at the A_3AR and slightly selective (5–6-fold) in comparison to A_1 and A_{2A}ARs. The 3-chlorobenzyl ether **21** was approximately equipotent to **20** at the A_3AR with a K_i value of 71.6 nM, but 23-fold more potent in A_1AR binding. The 2-phenylethyl amino derivative **23** was less potent than corresponding *O*-ether **22** at all AR subtypes, with K_i values at the A_3AR of 310 and 54 nM, respectively. The corresponding 2-phenylethylthio derivative **24** was less potent at three AR subtypes. Thus, the 2-*O*-ether linkage was selected for further modification.

Numerous modifications were included on the 2-phenylethyl moiety of ether **22**, including phenyl substitution with methyl **25**–**27**, methyloxy **28**–**31**, and halo **32**–**36**. In a number of these cases (**25**, **28**, and **34**), the 2-substitution of the phenyl ring resulted in the greatest reduction of A_3AR

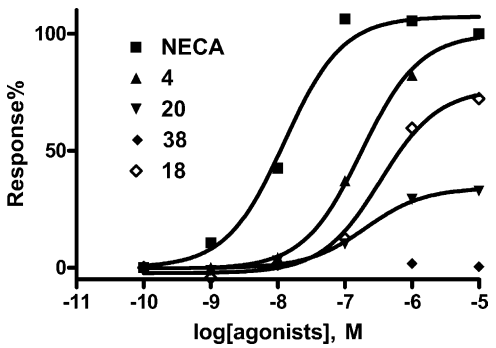


Fig. 1. Inhibition of forskolin-stimulated cyclic AMP production in CHO cells stably transfected with the human A_3AR, induced by various agonists. All experiments were performed in the presence of 10 μM rolipram and 3 units/ml adenosine deaminase. Forskolin (10 (M) was used to stimulate cyclic AMP levels. 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): NECA, 20.0 ± 4.5; 2-isobutyloxy derivative 4, 150 ± 32; 2-*S*-(3,7-dimethyl)oct-6-enyloxy derivative 18, 187 ± 66; 2-benzyloxy derivative 20, 160 ± 40; 2-*S*-(2-phenyl)butyloxy derivative 38, not applicable.

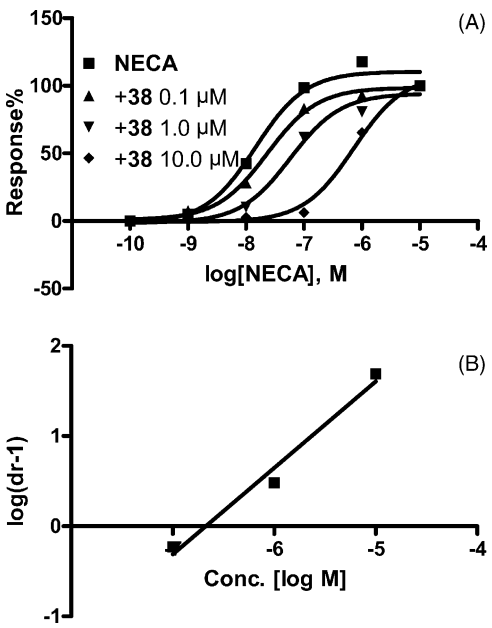


Fig. 2. Antagonism by compound 38 of the inhibition of cyclic AMP production elicited by NEC in CHO cells stably transfected with the human A_3AR (A) and Schild analysis of the data (B). The experiment was performed in the presence of 10 μM rolipram and 3 units/ml adenosine deaminase. Forskolin (10 (M) was used to stimulate cyclic AMP levels. The level of cAMP corresponding to 100% was 220 ± 30 pmol ml^{-1}. The K_B value for compound 38 was calculated to be 212 nM.

efficacy. Methoxy substitution at both 3- and 4-positions of the phenyl ring in **31** reduced A_3AR affinity in comparison of substitution of either position alone (i.e. **29**, **30**). Substitution of the phenylethyl ring with groups having electron withdrawing character (i.e. F) versus donating (i.e. methoxy or methyl) had little effect on A_3AR affinity, however the efficacy of 3- and 4-substituted halo derivatives tended to be less than the corresponding methoxy or methyl derivatives. Also, the phenyl ring was substituted with other ring systems, as in **39**, **40**, and **42–44**, all of which had reduced efficacy at the A_3AR. Substitution at the 2-position of the alkyl (ethyl) group of **22** with ethyl (the diastereomers **38** and **38**) or with phenyl 41 completely abolished efficacy at the A_3AR.

3.4. Affinity and potency at the A_1AR

These 2-substituted adenosine derivatives were generally less potent at the A_1AR than at the A_3AR with binding K_i values over 100 nM. Among the most potent derivatives at the ($K_i < 200$ nM) were **1**, **40**, **42** and **44**. Among the two 5′,8-cyclo derivatives **47** and **48**, only moderate affinity was observed for the N^6-cyclopentyl analogue **47** at the A_1AR. Compound **47** was a somewhat selective A_1AR agonist.

Those 2-modified compounds with high to moderate affinity at the A_1AR tended to be fully efficacious at that subtype. However, the alkynyl ether **15**, 2-phenylethyl amine **23**, 2-methoxyphenylethyl ether **25**, 2-fluorophenylethyl ether **32**, and 2-chlorophenylethyl ether **34** were not fully efficacious at the A_1AR.

3.5. Affinity and potency at the A_{2A}AR

Consistent with previous studies [15,16], the 2-substituted ether derivatives of adenosine were generally fully efficacious at the A_{2A}AR. A number of substitutions at the 2-position, which were previously found to contribute to the affinity for the rat A_{2A}AR [20,28,29], were also demonstrated to be important for the affinity and selectivity at the human A_{2A}AR homologue. A single substitution at the 2-position might contribute significantly to both A_{2A}AR affinity and selectivity. For example, 2-[2-(1-naphthyl)ethyloxy]adenosine **39** was found to be the most potent (K_i = 3.8 nM) and selective (>50-fold) A_{2A} agonist in this series. Thus, **39** was roughly as potent as an A_{2A}AR agonist as the nonselective agonist NECA **52**. The 2-naphthyl isomer **40** was also potent at the A_{2A}AR.

3.6. Potency at the A_{2B}AR

All of nucleosides were tested at a fixed concentration for the ability to stimulate or inhibit adenylate cyclase mediated by the A_{2B}AR, and activation curves were determined for only a few compounds (Fig. 3). Since an agonist radioligand for the A_{2B}AR is not yet available, the binding affinities of these nucleosides at this subtype have not been determined. Interestingly, although most of these compounds were extremely weak at the A_{2B}AR, 2-[2-(2-naphthyl)ethyloxy]adenosine **40** (EC_{50} = 1.44 (M) and 2-[2-(2-thienyl)ethyloxy]adenosine **42** (EC_{50} = 1.78 (M) were found to be moderately potent A_{2B}AR agonists, although less potent than NECA (EC_{50} = 140 nM). Another 2-ether having substantial potency at the A_{2B}AR was the 2-phenylethyl ether **22**, which displayed an EC_{50} value of 3.49 (M. At the A_{2A}AR **39** was a more potent agonist than **40**, while the opposite order was seen at the A_{2B}AR.

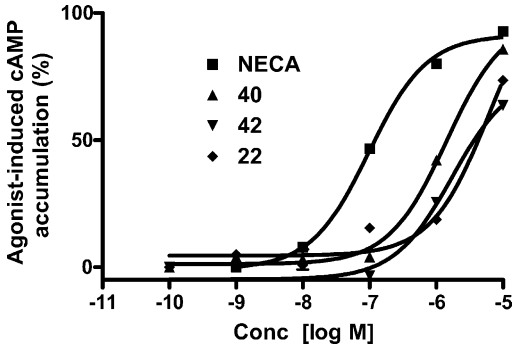


Fig. 3. Stimulation of cyclic AMP production elicited by NECA and compounds 39 and 40 in CHO cells stably transfected with the human A_{2B}AR. The experiment was performed in the presence of 10 (M rolipram and 3 units/ml adenosine deaminase. The data shown were from one experiment performed in duplicate and are typical of three independent experiments giving similar results. The EC_{50} values were listed in Table 1.

4. Discussion

Although in the present study we have not identified highly selective ligands for a given AR, we have found that substitution at the 2-position greatly modulates the pharmacological characteristics at the A_3AR. Thus, the aim of the study has been partially achieved, and in future studies, specific 2-ether groups identified here may be studied in combination with other modifications of adenosine known to provide subtype selectivity. Until present, the modifications of the 2-position to be included in A_3AR selective agonists have been somewhat limited (e.g. chloro, methylthio, and alkynyl groups). The SAR of alkynyl groups at the 2-position based on affinity at several subtypes of ARs has been explored [21,29].

Several of the nucleosides studied here were found to have K_i values at the A_3AR of approximately 50 nM and compared favorably in selectivity to other analogues of adenosine in which only single sites have been modified [11–13,21]. For example, compound **41** was >100-fold selective for the A_3AR in comparison to both the A_1 and A_{2B}ARs. Alkyl ethers **4** and **7** were moderately selective full agonists of the A_3AR in comparison to the A_1AR. Compounds **13** and **20** were slightly selective for the A_3AR in comparison to the A_1AR and other subtypes.

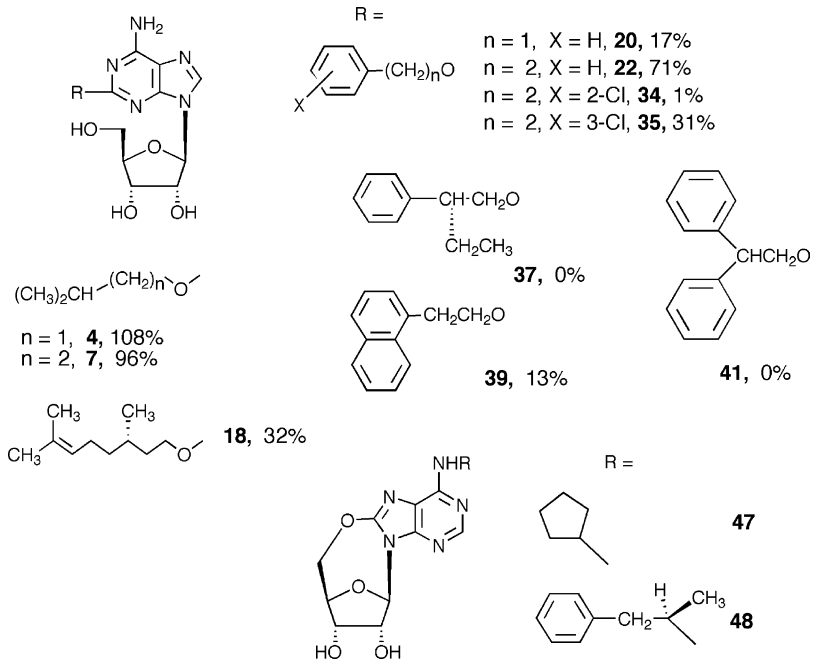


Fig. 4. Representative adenosine derivatives examined in this study. Compounds having A_3AR selectivity in comparison to at least two other AR subtypes (4, 7, 35, 37, and 41) and/or reduced efficacy (2-*O*-benzyl ether 20 and others) at the A_3AR are included. A striking dependence of the efficacy on the position of substitution of 2-*O*-phenylethyl ethers (22 in comparison to 34, 35, 37, 37, and 41) is illustrated. The full agonism of alkyl ethers (4 and 7) is in contrast to the branched decenyl ether 18, which is a partial agonist. 5′,8-Cyclo analogues (47 and 48) of known adenosine agonists CPA and R-PIA are also shown. The percent of maximal activation of the human A_3AR at 10 (M is indicated, where applicable.

Similar to previous studies of the N^6-position [8] and the ribose moiety [9,30,31], structural determinants at the 2-position of adenosine have been found to be critical for A_3AR recognition and activation. Fig. 4 shows the structures of selected analogues having A_3AR selectivity in comparison to at least two other AR subtypes and/or reduced efficacy at the A_3AR and the 5′,8-cyclo analogues (**47** and **48**). A striking dependence of the A_3AR efficacy on relatively minor structural changes of the 2-ether group, for example, substitution of 2-*O*-phenylalkyl ethers, is illustrated. The benzyl ether **20** is a low efficacy partial agonist. Homologation to give **22** restored most of the ability to activate the A_3AR, however simple substitution of this ring in **34** and **35** or additional aryl substitution in **37**, **39**, and **41** greatly reduced the intrinsic efficacy. The alkyl ether **7** is structurally related to **18**, however, the efficacy at the A_3AR was greatly reduced upon chain elaboration.

In binding experiments, typically these 2-substituted nucleosides displaced AR radioligand binding with the following order of potency: $A_{2A} > A_3 > A_1$. The benzyl ether **20** displayed selectivity (5–6-fold) for the A_3AR. Mixed A_{2A}/A_3AR agonists have been identified. For example, **7** was a mixed agonist with full A_3AR intrinsic efficacy. Compounds **11**, **12**, and **18** were mixed agonists, but with partial intrinsic efficacy at the human A_3AR. Finally, the 2-norbornanethyl ether **9**, the branched decenyl ether **17**, and the 2-phenylbutyl ethers **37** and **38** were full agonists at the A_{2A}AR and antagonists at the human A_3AR, with affinities roughly matched at the two subtypes and having selectivity over the A_1 and A_{2B} ARs. Nucleosides with combined action in activating the A_{2A}AR and antagonizing the A_3AR may be of use in treating inflammation. Several arylethyloxy substituents at the 2-position were found to contribute significantly to the A_{2B}AR activity, for which there are not yet any selective agonists although the SAR has been explored [32,33].

In the absence of a physically-determined structure for the A_3AR, the use of molecular modeling [12,13,34] will be necessary to understand the structural basis for the loss of efficacy associated with certain 2-ether groups, such as benzyl, 2,2-diphenylethyl, and *S*-2-phenylbutyl. There were parallels between the effects on A_3AR binding and activation of the same substitution at either the 2-position (in the form of an ether) or at the N^6-position (in the form of a secondary arylamine). For example, 2-(2,2-diphenylethyloxy)-adenosine and N^6-(2,2-diphenylethyl)adenosine were both antagonists of the A_3AR [12].

Also, in the series of 2-phenylalkylethers, the benzyl ether had more favorable affinity particularly at the A_3AR in comparison to other subtypes and was a partial agonist. The same was observed for N^6-benzyladenosine derivatives [11]. This raises the possible explanation, already invoked by Olsson and coworkers [16] prior to any knowledge of the receptor binding site, that these two substitutions might be overlayed in their receptor-bound positions [13,34]. However, the *trans*-2-phenylcyclopropyl group at the N^6-position resulted in high A_3AR affinity as an agonist, while at the 2-ether position (**44**), only moderate affinity was observed with no activation of the A_3AR.

Thus, we have demonstrated that affinity at the A_3AR may be enhanced by modifying the nucleoside in a systematic fashion at a position that was not previously explored for ether groups. The 2-ether modification of adenosine, previously known as a means of enhancing potency at the A_{2A}AR, has now been shown to variably enhance potency at the A_3AR. Typically, adenosine 2-benzyl and 2-phenylethyl ethers showed favorable binding affinity at the A_3AR and, depending on substitution, ranged from agonists to partial agonists to antagonists at this receptor. The 2-substituted adenosine derivatives examined in this study were generally less potent at the A_1AR in comparison to the A_3AR, but fully efficacious. However, a single substitution at the 2-position could also lead to a potent and selective A_1AR agonist. For example, compound **21** is somewhat selective for the A_1AR. Mixed A_2A/A_3AR agonists have been identified. Additionally, we have identified several substituents at the 2-position that contribute significantly to the A_{2B}AR activity.

In conclusion, a number of novel structural determinants for the A_3AR activation have been identified. Given the interest in A_3AR agonists as antiischemic agents [35] and antagonists as antiglaucoma agents [36] and for other therapeutic applications, there is need for additional selective ligands to interact with the receptor. Presently, only one A_3AR agonist (IB-MECA) is in clinical trials [6], and the drug-like properties of most of the analogues in this study are unexplored. Selective agonists and antagonists, especially those whose selectivity extend across species, are needed both as receptor probes for research and as clinical candidates. In some cases, nucleoside AR ligands of mixed selectivity (e.g. mixed A_1/A_3AR agonists for cardioprotection) would be desirable for a particular clinical application. Structural insights gained in the present study may now be extended to multiple substitutions of adenosine.

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