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# $N^6$-Substituted adenosine derivatives: selectivity, efficacy, and species differences at $A_3$ adenosine receptors

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**Abstract**

The activation of the human $A_3$ adenosine receptor (AR) by a wide range of $N^6$-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 $N^6$-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 ($\leq$5 carbons) or partial ($\geq$6 carbons) h$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 agonisms). 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. Stereoselectivity of binding was demonstrated for $N^6$-(*R*-1-phenylethyl)adenosine vs. $N^6$-(*S*-1-phenylethyl)adenosine, as well as for the $N^6$-(1-phenyl-2-pentyl)adenosine, at the rat, but not human $A_3$AR. 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 $A_3$AR ($K_i$ = 106 nM). $N^6$-[(1*S*,2*R*)-2-Phenyl-1-cyclopropyl]adenosine **48** was 1100-fold more potent in binding to human ($K_i$ = 0.63 nM) than rat $A_3$ARs. Dual acting $A_1/A_3$ agonists ($N^6$-3-chlorobenzyl- **29**, $N^6$-(*S*-1-phenylethyl)- **39**, and 2-chloro-$N^6$-(*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 $A_3$AR by selective agonists has been demonstrated to be both cardioprotective and cerebroprotective [3,4]. In previous studies, a number of structural determinants for $A_3$AR activation have been identified [5–9], leading to the general conclusion that the ability of an adenosine derivative to activate the $A_3$AR 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.

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*Abbreviations:* ADAC, $N^6$-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]-methyl]aniline]carbonyl]methyl]phenyl]adenosine; Bn-Ado, $N^6$-benzyladenosine; Cl-IB-MECA, 2-chloro-$N^6$-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine; CGS21680, 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine; CHA, $N^6$-cyclohexyladenosine; DBXRM, *N*-methyl-1,3-dibutylxanthine-7-β-D-ribofuronamide; CPA, $N^6$-cyclopentyladenosine; DPMA, $N^6$-[2-(3.5-dimethoxyphenyl)-2-(2-methylphenylethyl)]adenosine; CADO, 2-chloroadenosine; ENBA, (*S*)-*endo*-2-norbornyladenosine; FADO, 2-fluoroadenosine; 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 1220, *N*-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-yl]benzene-acetamide; NECA, 5′-*N*-ethylcarboxamidoadenosine; PIA, $N^6$-[phenylisopropyl]adenosine; RDT-39, $N^6$-[(1*S*,2*R*)-2-phenyl-1-cyclopropyl]adenosine.

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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 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 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 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 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. Non-specific 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 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 [$^3$H]R-PIA to the rat forebrain and recombinant human $A_1$ARs and the binding of [$^3$H]CGS21680 to rat striatal and recombinant human $A_{2A}$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 $A_3$ARs 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 [$^3$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.

### 2.5. 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 ± standard error.

## 3. Results

### 3.1. Activation of $A_3$ARs by $N^6$-substituted adenosine derivatives

The activation of $A_3$ARs 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 $A_3$AR. 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 $A_3$AR 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 $A_1$AR agonist, CHA **10**, was shown to be less efficacious than Cl-IB-MECA, while no significant activation by the

Table 1
Binding affinities of adenosine derivatives at rat $A_1$ and $A_{2A}$ARs and at human and rat $A_3$ARs and maximal agonist effects at human $A_3$ARs 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** | $CH_3O$ | 223 ± 32 | >10000 | 997 ± 343 | 28.6 ± 4.7 | 107 ± 13 |
| **3** | $CH_2CH_3$ | 4.9 ± 0.2 | 8900 ± 770 | 1050 ± 140 | 4.7 ± 1.9 | 102 ± 6 |
| **4** | $CH_2C(CH_3)_3$ | 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** | $CH(CH_2CH_3)_2$ | 0.8 ± 0.2 | 471 ± 200 | 147 ± 40 | 55.1 ± 9.5 | 99 ± 6 |
| **7** | $CH(CH((CH_3)_2)_2$ | 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** CPA | Cyclopentyl | 0.45 ± 0.04[b] | 462[c] | 240 ± 36[c] | 72 ± 12 | 97 ± 4 |
| **10** CHA | 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** | *exo*-2-Norbornyl | 0.7 ± 0.2 | 3400 ± 140 | 253 ± 30 | 85 ± 31 | 114 ± 6 |
| **13** ENBA | (*S*)-*endo*-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 |
| **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[e] | 210[c] | 185 ± 64 | 13.3 ± 3.0 | 103 ± 4 |
| **25** Metrifudil | 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 |

Table 1 (*Continued*)

| Compound | $N^6$ substitution | $K_i$ ($rA_1AR$) (nM)[a] | $K_i$ ($rA_{2A}AR$) (nM)[a] | $K_i$ ($rA_3AR$) (nM)[a] | $K_i$ ($hA_3AR$) (nM)[a] | Percent activation ($hA_3AR$) |
|---|---|---|---|---|---|---|
| **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 ± 740412[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-methylphenylethyl | 142[c] | 4.4[c] | 3570 ± 1700[c] | 106 ± 22 | 0 |
| **47** | (1*R*,2*S*)-2-Phenyl-1-cyclopropyl | 15.2 ± 3.2[d] | 3040 ± 490 | 358 ± 33 | 24.1 ± 10.9 | 87 ± 4 |
| **48** | (1*S*,2*R*)-2-Phenyl-1-cyclopropyl | 11.8 ± 2.4[d] | 560 ± 232 | 694 ± 157 | 0.63 ± 0.17 | 117 ± 9 |
| **49** | *cis*-(1*R*,2*R*)-2-Phenylcyclohexyl | 15 ± 6 | >10000 | 1170 ± 30 | 1450 ± 241 | 73 ± 12 |
| **50** | *trans*-(1*R*,2*S*)-2-Phenylcyclohexyl | 6 ± 3 | 672 ± 51 | 279 ± 41 | 559 ± 96 | 72 ± 9 |

| Compound | Substitution | $K_i$ ($rA_1AR$) (nM) | $K_i$ ($rA_{2A}AR$) (nM) | $K_i$ ($rA_3AR$) (nM) | $K_i$ ($hA_3AR$) (nM) | Percent activation ($hA_3AR$) |
|---|---|---|---|---|---|---|
| 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 $A_3AR$ experiments were performed using adherent CHO cells stably transfected with cDNA encoding the human or rat $A_3$ receptor. Percent activation of the human $A_3AR$ was determined at 10 μM. Unless otherwise noted, $K_i$ values at $A_1AR$ are from Daly *et al*. [10]. Binding at $A_1$ 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 $A_1AR$ 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].

$A_{2A}AR$ 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

Table 2
Binding affinities of reference adenosine agonists at rat $A_1$ and $A_{2A}$ receptors and at human and rat $A_3$ receptors and maximal agonist effects at human $A_3$ receptors expressed in CHO cells

| Compound | $K_i$ ($rA_1AR$) (nM)[a] | $K_i$ ($rA_{2A}AR$) (nM)[a] | $K_i$ ($rA_3AR$) (nM)[a] | $K_i$ ($hA_3AR$) (nM)[a] | Percent activation ($hA_3AR$) |
|---|---|---|---|---|---|
| **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 $A_3AR$ experiments were performed using adherent CHO cells stably transfected with cDNA encoding the human or rat $A_3$ receptor. Binding at $A_1$ and $A_{2A}ARs$ was carried out as described in Section 2. Percent activation of the human $A_3AR$ 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].

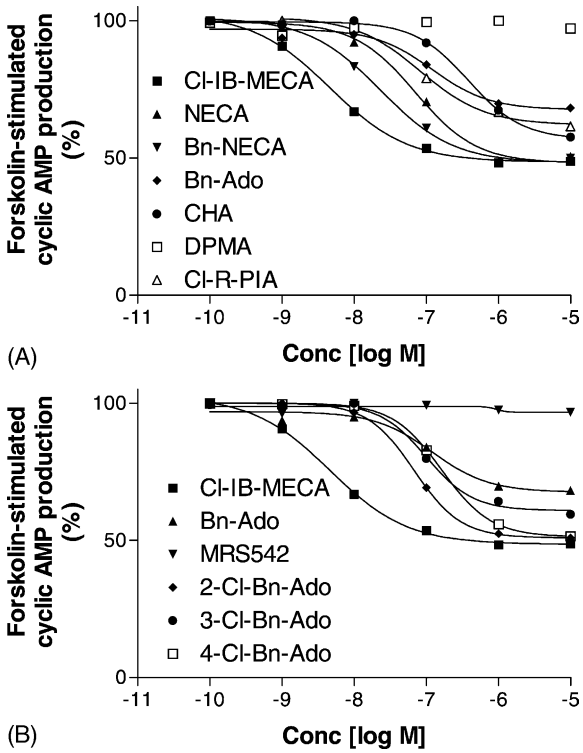


Fig. 1. Inhibition of forskolin-stimulated cyclic AMP production in CHO cells stably transfected with the human $A_3$AR, induced by various agonists (A) and by $N^6$-benzyl derivatives (B). All experiments were performed in the presence of 10 μM rolipram and 3 U/mL adenosine deaminase. Forskolin (10 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. $IC_{50}$ 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**, Cl-R-PIA, 76 ± 21; **56**, NECA, 62 ± 17; **57**, Bn-NECA, 18.2 ± 3.4; **59**, Cl-IB-MECA, 3.2 ± 1.4.

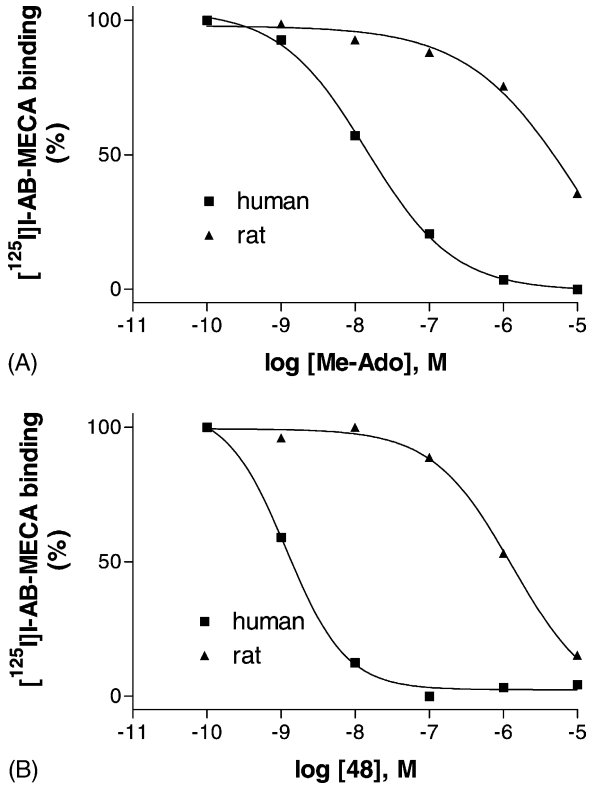


Fig. 2. Competition for radioligand binding ([$^{125}$I]I-AB-MECA) at $A_3$ARs in two species, by two representative adenosine analogues **1** and **48**, which were more potent at human than at rat $A_3$ARs. The procedures were described in "Section 2". The data points are from a representative experiment performed in duplicate. The mean $K_i$ values calculated from three independent experiments are listed in the text.

**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 μM 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-phenylcyclohexyl **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

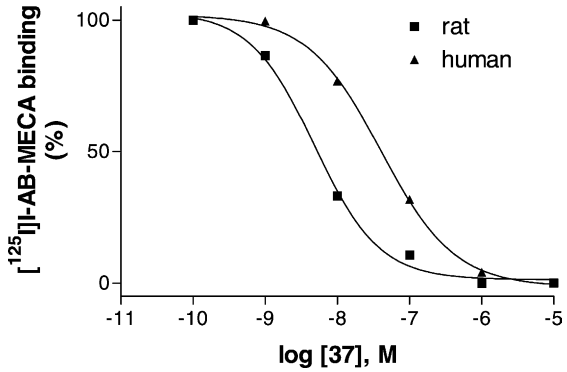


Fig. 3. Competition for radioligand binding ([$^{125}$I]I-AB-MECA) at $A_3$ARs in two species, by an adenosine analogue which was more potent at rat than at human $A_3$ARs. The procedures were described in "Section 2". The data points are from a representative experiment performed in duplicate. The mean $K_i$ values calculated from three independent experiments are listed in the text.

Table 3
Comparison of binding affinity of selected adenosine derivatives at human $A_1$, $A_{2A}$, and $A_3$ARs ($N^6$-substituent or compound abbreviation in parentheses)

| | $K_i$ (nM) | | |
|---|---|---|---|
| | $hA_1$ | $hA_{2A}$ | $hA_3$ |
| **11** (cyclooctyl) | 6.4 ± 1.4 | >10000 | 411 ± 55 |
| **12** (*exo*-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** (*R*-1-phenylethyl) | 28.6 ± 9.8 | 1750 ± 540 | 113 ± 22 |
| **39** (*S*-1-phenylethyl) | 94.5 ± 29.5 | >10000 | 68.7 ± 17.3 |
| **43** (*R*-1-phenyl-2-pentyl) | 16.9 ± 5.2 | 1120 ± 320 | 70.9 ± 26.2 |
| **44** (*S*-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.

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

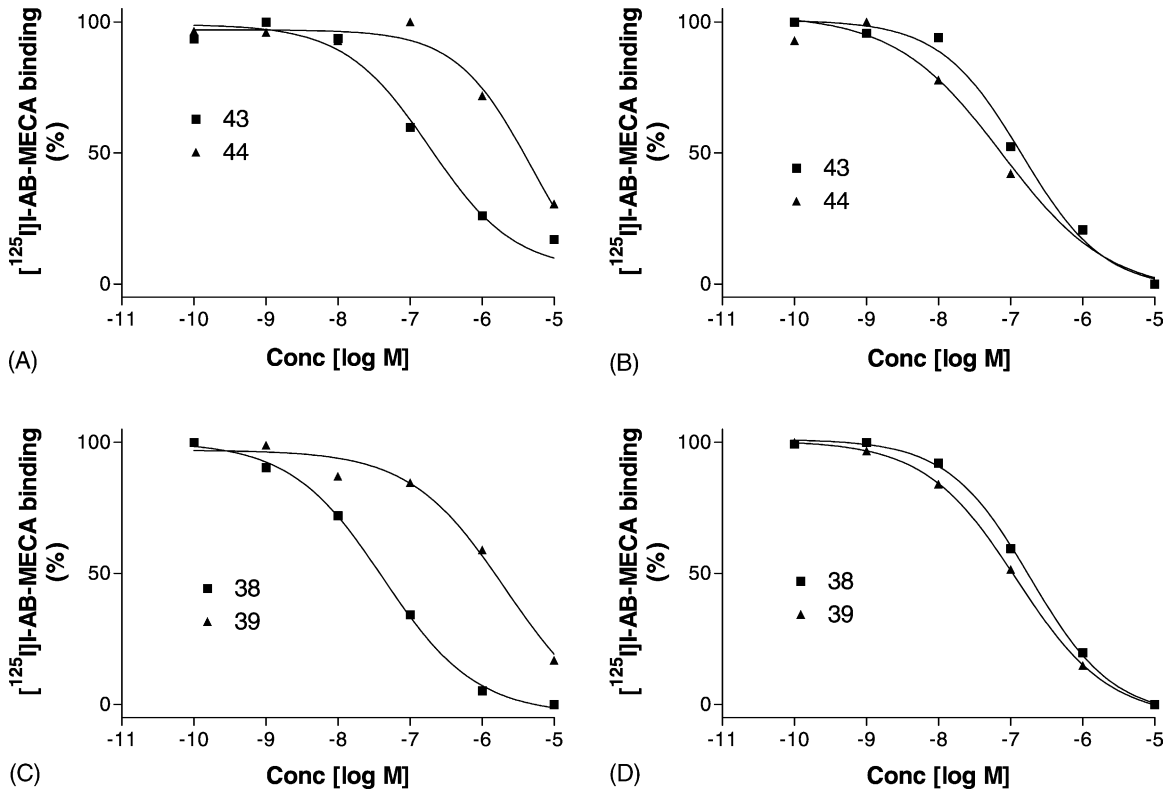


Fig. 4. Competition for radioligand binding ([$^{125}$I]I-AB-MECA) at $A_3$ARs by pairs of *R*- and *S*-diastereoisomers ($N^6$-(1-phenyl-2-pentyl)adenosines (A) and (B); $N^6$-(1-phenylethyl)adenosines (C) and (D) at rat (A) and (C) and at human (B) and (D) $A_3$ARs, demonstrating that stereoselectivity 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.

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 $A_3$AR [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 $A_1$AR, CHA, was shown to be a partial agonist of the $A_3$AR. A potent and full agonist for the $A_{2A}$AR, DPMA, was demonstrated to be a moderately potent antagonist of the $A_3$AR.

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 $A_3$AR [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 $A_3$AR efficacy. Similarly, bridging the methylene group in compound **25** to give **40**, thus introducing a ring constraint, also reduced $A_3$ 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 $A_3$AR, 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 $A_3$AR efficacy. This is in contrast to compound **2**, which showed enhanced potency at the rat $A_3$AR as a result of the oxygen inclusion [22]. Branching at the terminal alkyl position (compound **45** vs. compound **43**) did not change the $A_3$ affinity but diminished the $A_3$ efficacy. A 5′-Cl-5′-deoxy substitution (compound **54**), already reported for adenosine agonists [5,22], greatly reduced $A_3$AR efficacy.

### 4.2. Selectivity

$N^6$-(2-Methylbenzyl)adenosine (**25**) [20] was found to have comparable affinity at rat $A_1$, $A_{2A}$, and $A_3$ARs, while $N^6$-(2-methoxybenzyl)adenosine **26** was found to be selective for both $A_1$ and $A_3$ but not $A_{2A}$ARs. $N^6$-(2-Fluorobenzyl)adenosine (**27**) was >10-fold selective for $A_1$ over $A_{2A}$ or $A_3$ARs, while $N^6$-(2-chlorobenzyl)adenosine (**28**) was shown to have similar affinity for $A_1$ and $A_3$ARs. $N^6$-(3-Chlorobenzyl)adenosine (**29**) was found to be extremely selective for $A_1$ and $A_3$ 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 that 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 $A_3$

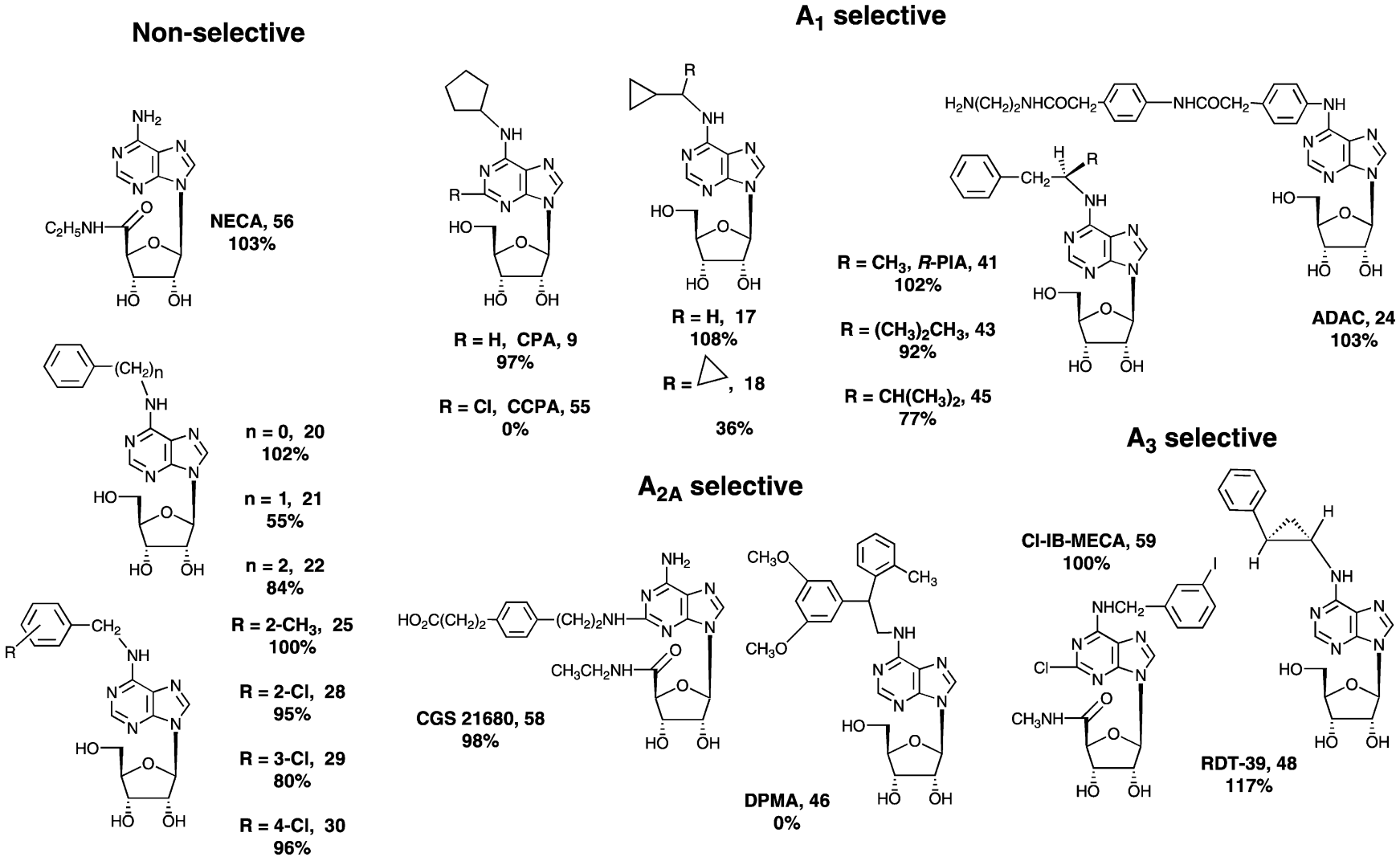


Fig. 5. Structures of representative adenosine derivatives and their efficacy (% of inhibition of adenylate cyclase at 10 μM compared to the effect of 10 μM Cl-IB-MECA) as agonists of the human $A_3$AR.

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 ($\leq$5 carbons) or partial ($\geq$6 carbons) h$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].

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