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Conversion of A₃ Adenosine Receptor Agonists into Selective Antagonists by Modification of the 5'-Ribofuran-uronamide Moiety

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Abstract

The highly selective agonists of the A₃ adenosine receptor (AR), Cl-IB-MECA (2-chloro-*N*⁶-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine) and its 4'-thio analogue, were successfully converted into selective antagonists simply by appending a second *N*-methyl group on the 5'-uronamide position. The 2-chloro-5'-(*N,N*-dimethyl)uronamido analogues bound to, but did not activate the human A₃AR, with K_i values of 29 nM (4'-O) and 15 (4'-S) nM, showing >100-fold selectivity over A₁, A_{2A}, and A_{2B}ARs. Competitive antagonism was demonstrated by Schild analysis. The 2-(dimethylamino)-5'-(*N,N*-dimethyl)uronamido substitution also retained A₃AR selectivity but lowered affinity.

Keywords

nucleoside; G protein-coupled receptor; adenylyl cyclase; molecular modeling; radioligand binding

ABBREVIATIONS

AR, adenosine receptor; CGS21680, 2-[p-(2-carboxyethyl)phenylethylamino]-5'-*N*-ethylcarboxamido-adenosine; CHO, Chinese hamster ovary; Cl-IB-MECA, 2-chloro-*N*⁶-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine; CPA, *N*⁶-cyclopentyladenosine; DMEM, Dulbecco's modified Eagle's medium; I-AB-MECA, *N*⁶-(4-amino-3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine; NECA, 5'-*N*-ethylcarboxamidoadenosine; PIA, *N*⁶-(phenylisopropyl)adenosine; PTLC, preparative thin layer chromatography

Antagonists of the A₃ adenosine receptor (AR) are of potential use for clinical targets, including the treatment of glaucoma, allergic conditions, and inflammation.¹ Potent and selective antagonists for the human A₃AR have recently been synthesized.^{2–6} These human A₃AR antagonists were found to be weak or ineffective at the rat A₃AR^{7,8} and were unsuitable for evaluation in small animal models or for further development as drugs. Thus, A₃AR antagonists of which the affinity and selectivity are independent of species are sought as drug candidates. In previous studies, it was found that antagonists derived from adenosine analogs, in contrast to nonpurine heterocyclic antagonists, could be species-independent, potent, and selective A₃AR antagonists.⁹

Among the four subtypes of receptors for adenosine **1**, the intrinsic efficacy of nucleosides acting at the A₃ subtype is known to be especially sensitive to structural changes. For example, substitution by a benzyl or a 3-iodobenzyl group at the N⁶ position of adenosine was demonstrated to increase affinity but decrease efficacy at the human A₃AR, in the inhibition of forskolin-stimulated adenylyl cyclase.^{9–11} Additional substitution by a 2-Cl substituent further increased affinity and decreased efficacy.^{9,10} As a result, N⁶-(3-iodobenzyl)adenosine **2** was a partial agonist achieving only 46% of the maximal effect, while 2-chloro-N⁶-(3-iodobenzyl)adenosine **3** was a potent antagonist for the A₃AR, albeit nonselective. A 5'-methyluronamide substitution of **3** restored its efficacy and rendered it selective for the A₃AR. Thus, Cl-IB-MECA **4** and its 4'-thio analogue LJ568 **5** are selective agonists for the A₃AR.^{9,12,13} Here, we report that **4** and **5** were successfully converted into selective antagonists by appending an additional methyl group on the 5'-uronamide nitrogen. The finding that removing H-binding ability in the region of the 5'-uronamide lowers efficacy is consistent with expectations derived from rhodopsin-based, dynamic molecular modeling and ligand docking.¹⁴

Synthetic routes to *N,N*-dimethylamide derivatives **6** – **8** are shown in Scheme 1. The 5'-ester group was aminolyzed with dimethylamine, which also led to a side product substituted at the 2-position.¹⁵ The 4'-S analogue was prepared from an acetoxy intermediate,¹⁶ similar to a reported series of derivatives.¹²

Binding assays were carried out using standard radioligands in Chinese hamster ovary (CHO) cells stably expressing a human AR subtype.¹⁷ The binding affinity at the human A₃AR of the 2-Cl antagonist derivatives **6** and **7** was shown to be 29 and 15 nM, respectively (Table 1). Thus, the compounds were demonstrated to be over 100-fold selective in binding to the human A₃AR in comparison to other AR subtypes. In addition, the 2-(dimethylamino) substitution in **8** resulted in A₃AR selectivity but with lower affinity.

To probe species differences, the affinity of **6** and **7** was also measured at the rat A₃AR. Although the affinity decreased with respect to the affinity at the human A₃AR, these two compounds showed moderate affinity at the rat A₃AR. The K_i values were 286 ± 31 and 321 ± 74 nM for **6** and **7**, respectively.

In functional assays consisting of measuring inhibition of forskolin-stimulated production of 3',5'-cyclic-adenosine monophosphate (cAMP) in intact CHO cells heterologously expressing ARs,¹⁸ single concentration determinations (Table 1) indicated that A₃AR agonism was absent in compounds **6** – **8**. In contrast, the concentration-response curve for compound **4** indicated full agonism, as previously reported, with an EC₅₀ of 1.2 nM at the human A₃AR (Figure 1A). A Schild analysis¹⁹ indicated that **6** concentration-dependently antagonized the A₃ agonist **4** to inhibit forskolin-stimulated cAMP accumulation in CHO cells stably expressing the human A₃AR with a K_B value of 48 nM (Figure 1B,C). In contrast, **6**, at a concentration of 1 μM, had no significant effect on cAMP accumulation inhibited by the A₁ agonist CPA (N⁶-cyclopentyladenosine) in CHO cells expressing the human A₁AR under the similar conditions (data not shown).

Similar results were obtained in an assay of A₃AR-induced changes in intracellular [Ca²⁺].²⁰ The nonselective AR agonist NECA (5'-*N*-ethyl-carboxamidoadenosine) activated the human A₃AR expressed in CHO cells to induce a concentration-dependent rise in intracellular [Ca²⁺] with a potency corresponding to an EC₅₀ of 58 ± 16 nM (n=4), while **6** and **7** alone did not induce calcium transients (Figure 1D). Compound **8** also did not induce a Ca²⁺ response (data not shown). Increasing concentrations of **6** right-shifted the activation curves for NECA (not shown), and a K_B value for **6** of 20 nM was determined. The A₃AR antagonist **7** similarly shifted the NECA-induced Ca²⁺ response to the right (not shown).

In this study, two selective A₃AR agonists, Cl-IB-MECA and its 4'-thio analogue, have been successfully transformed into antagonists selective for the A₃AR by appending an additional *N*-methyl group on the 5'-uronamide position. Other related *N,N*-dialkyl substitution in the 4'-thio nucleoside series of adenosine agonists had more complex changes in affinity and efficacy.¹² Thus, it appears that the 5'-(*N,N*-dimethyl)uronamido group especially tends to preserve affinity and selectivity in *N*⁶-3-iodobenzyladenosine derivatives, while entirely abolishing activation of the human A₃AR. There was also an interdependence of the effects of an *N,N*-dimethyl moiety and the *N*⁶-substituent, since the *N*⁶-methyl analogue corresponding to **7** was a partial agonist.¹² A further advantage of the *N*⁶-benzyl substitution of **6** and **7** over many other smaller or larger groups is that the high A₃AR affinity tends to be retained in murine species,²¹ which was confirmed in the present study.

Molecular modeling of the A₃AR indicated that flexibility of the adenosine derivative in the 5'-region correlated with putative conformational changes of the receptor associated with activation. Although there is no global conformational model of the activated state of the receptor, local conformational changes have been proposed. One such change is the rotation, anticlockwise from the extracellular perspective of the receptor, of the conserved Trp243 in TM6.^{9,21} We have proposed that both flexibility of the 5'-uronamide and its ability to make and break multiple H-bonds as this conformational change occurs are needed for receptor activation. The low efficacy of 5'-thioether derivatives is also consistent with the need for H-bonding in this region in order to activate the A₃AR.²²

The present findings are consistent, in that we have removed the H-bond-donating ability of the 5'-uronamide with a relatively subtle structural alteration, resulting in the loss of ability to activate the A₃AR. From a comparison of the A_{2A} and A₃AR models,²³ the main difference in the docking complex was the preference of the χ angle; the A₃AR preferred nucleosides bound in an *anti*-form, while the A_{2A}AR preferred a high-*anti* conformation (approximately -70°) about the glycosidic bond. Another difference was the binding at the 5'-position. In the A_{2A}AR, the 5'-NH formed a H-bond with an important T88 residue in TM3, but in the model of the A₃AR complex, this was replaced by a stronger interaction proposed between the 5'-carbonyl group and S7.42. Accordingly, the 5'-dimethylamides displayed dramatically diminished binding affinity at the A_{2A}AR.

A₃AR antagonists are potentially useful therapeutically for a number of disorders.^{1,24,25} However, the A₃ antagonists have not been widely used in animal models due to their extremely weak potency in murine species.⁸ Thus, A₃AR antagonists independent of species are of high priority to be developed. For this reason, a nucleoside analog IB-MECA (2-H analogue of **4**) was converted to an antagonist by cyclization of the 5'-uronamide moiety into a spirolactam, resulting in a selective A₃AR antagonist of moderate affinity for both human and rat A₃ARs.⁹ Another advantage of nucleoside A₃AR antagonists over other heterocycles⁷ is increased aqueous compatibility. For example, the clog *p* values of **6** and **7** are 1.69 and 1.73, respectively, in comparison to 6.86 for MRS1191, a dihydropyridine antagonist of the A₃AR.⁷ Here, two additional selective antagonists with reasonable affinity for both human and rat A₃ARs were introduced, which increased the diversity of rat A₃AR antagonists.

It is clear that more potent and selective antagonists are needed for eventual therapeutic purposes. The newly synthesized A₃AR antagonists could be evaluated in models of a number of disorders related to the A₃AR, such as glaucoma and inflammation. Currently, existing nucleoside analogs should be good templates for further modification and development of potent and selective antagonists for the A₃ARs in diverse species.

Acknowledgements

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15. (2S,3S,4R,5R)-5-[2-Chloro-6-(3-iodo-benzylamino)-purin-9-yl]-3,4-dihydroxy-tetrahydrofuran-2-carboxylic acid dimethylamide (6). The methyl ester 10 (0.031 g, 0.05 mmol) was dissolved in MeOH (5 mL) and potassium carbonate (0.014 g, 0.1 mmol) was added and the mixture stirred at room temperature for 10 min. Acetic acid (0.2 mL) was added to neutralize the base and the resulting diol was treated in situ with aqueous dimethylamine (0.5 mL, 40%) and further stirred for 1 hr. The reaction mixture was concentrated under reduced pressure and subjected to preparative thin layer chromatography by using chloroform/methanol (9:1) as solvent to afford the dimethylamide 6 as a colorless solid (0.0072 g, 26%). ¹H NMR (CDCl₃) δ2.97 (s, 3 H, N-CH₃), 3.14 (s, 3 H, N-CH₃), 3.52-3.80 (m, 4 H), 4.45-4.85 (m, 3 H), 5.05 (d, 1 H, J = 5.5 Hz), 6.06 (d, 1 H, J = 5.1 Hz), 7.17 (t, 1 H, J = 7.8 Hz, 5'-H), 7.65-7.82 (m, 3 H), 8.21 (s, 1 H); TOFMS m/z 559.0353 (M + H⁺) (calculated for C₁₉H₂₁N₆O₄ClI⁺) 559.0358. (2S,3S,4R,5R)-5-[2-dimethylamino-6-(3-iodo-benzylamino)-purin-9-yl]-3,4-dihydroxy-tetrahydrofuran-2-carboxylic acid dimethylamide (8). Aqueous dimethylamine (0.5 mL, 40%) was added to methyl ester 10 (0.016 g, 0.025 mmol) and the resulting reaction mixture was stirred at room temperature for 6 h. The mixture was concentrated under reduced pressure and purified by preparative thin layer chromatography by using chloroform/methanol (9:1) as solvent to afford the dimethylamide 8 as a colorless solid (0.0058 g, 42%). ¹H NMR (CDCl₃) δ2.90-3.23 (m, 12 H), 3.79 (bs, 3 H), 4.42-4.95 (m, 4 H), 6.11 (d, 1 H, J = 5.1 Hz), 6.22 (bs, 1 H), 7.14 (t, 1 H, J = 7.5 Hz, 5'-H), 7.40 (d, 1 H, J = 7.6 Hz, 6'-H), 7.65 (d, 1 H, J = 7.8 Hz, 4'-H), 7.78 (s, 1 H, 2'-H), 8.22 (s, 1 H, H-8); TOFMS m/z 568.1162 (M + H⁺) (calculated for C₂₁H₂₇N₇O₄I⁺) 568.1169.

16. (2S,3S,4R,5R)-5-[2-Chloro-6-(3-iodo-benzylamino)-purin-9-yl]-3,4-dihydroxy-tetrahydrothiophene-2-carboxylic acid dimethylamide (7). To a solution of 12 (483.0 mg, 0.622 mmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 179 mg, 0.933 mmol), 1-hydroxybenzotriazole (HOBt, 126 mg, 0.933 mmol), and dimethylamine.HCl (76 mg, 0.933 mmol) in CH₂Cl₂ (20 mL) was added N,N-diisopropylethylamine (DIPEA, 0.325 mL, 1.87 mmol) and the mixture was stirred at room temperature for 12 h. The reaction mixture was evaporated and the residue was purified by a silica gel column chromatography (hexane/EtOAc = 10:1-5:1) to give the silyl-protected amide intermediate as a white foam. To a stirred solution of the silyl amide (414 mg, 0.516 mmol) in THF (10 mL) was added tetrabutylammonium fluoride (1.29 mL, 1.29 mmol, 1 M THF solution) and the reaction mixture was stirred at room temperature for 1 h. The solvent was evaporated and the resulting residue was purified by silica gel column chromatography (CH₂Cl₂/MeOH = 10:1) to give 7 (214 mg, 64%): white solid; mp 186.1-186.3 °C; [α]_D²⁰ -12.4 (c 0.10, MeOH); UV (MeOH) $\lambda_{\max}$ 274 nm (pH 7); ¹H NMR (DMSO-d₆) δ 2.95 (s, 3 H, N-CH₃), 3.04 (s, 3H, N-CH₃), 4.30 (d, 1 H, J = 4.6 Hz, 2-H), 4.52 (br dd, 1 H, J = 4.6, 8.4 Hz, 3-H), 4.58 (m, 1 H, 4-H), 4.65 (d, 2 H, J = 5.7 Hz, N-CH₂), 5.59 (d, 1 H, J = 5.5 Hz, exchangeable with D₂O, OH), 5.86 (d, 1 H, J = 5.1 Hz, exchangeable with D₂O, OH), 5.91 (d, 1 H, J = 5.4 Hz, 5-H), 7.17 (t, 1 H, J = 7.8 Hz, 5'-H), 7.40 (d, 1 H, J = 7.6 Hz, 6'-H), 7.65 (d, 1 H, J = 7.8 Hz, 4'-H), 7.78 (s, 1 H, 2'-H), 8.52 (s, 1 H, H-8), 9.00 (br t, 1 H, J = 6.1 Hz, exchangeable with D₂O, NH); ¹³C NMR (CD₃OD) δ 36.6, 38.2, 44.5, 64.6, 77.2, 80.7, 95.0, 119.7, 128.3, 131.5, 137.5, 138.0, 141.5, 142.8, 145.2, 151.7, 155.8, 156.5, 172.7; FAB-MS m/z 575 (M⁺+1); Anal. (C₁₉H₂₀ClIN₆O₃S) C, H, N, S.
17. 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 μ g/mL streptomycin, 2 μ mol/ml glutamine and 800 μ g/ml geneticin. After harvest and homogenization, cells were centrifuged at 500 g for 10 min, and the pellet was re-suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂, 1 mM EDTA. The suspension was homogenized with an electric homogenizer for 10 sec, 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 as described [Bradford, M.M. Anal. Biochem. 1976, 72, 248]. For A₃AR binding assays, each tube contained 100 μ l of membrane suspension, 50 μ l of [¹²⁵I]I-AB-MECA (final concentration 0.5 nM), and 50 μ l of increasing concentrations of compounds in Tris-HCl buffer (50 mM, pH 7.4) containing 10 mM MgCl₂. Nonspecific binding was determined using 10 μ 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 [³H]R-PIA to A₁ARs and the binding of [³H]CGS21680 to A_{2A}ARs were as previously described.¹⁰ IC₅₀ values were converted to K_i values as described [Cheng Y-C, Prusoff WH. Biochem. Pharmacol. 1973, 22, 3099.]. [¹²⁵I]N⁶-(4-amino-3-iodo-benzyl)adenosine-5'-N-methyluronamide ([¹²⁵I]I-AB-MECA; 2000 Ci/mmol), [³H]R-PIA (R-N⁶-[phenylisopropyl]adenosine, 34 Ci/mmol), [³H]CGS21680 (2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamido-adenosine, 47 Ci/mmol) and [³H]cAMP (40 Ci/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, UK). NECA, CGS21680, CPA, and R-PIA were purchased from Sigma-RBI (St. Louis, MO). Other chemicals were from standard commercial sources and of analytical grade.
18. Intracellular cAMP levels were measured with a competitive protein binding method [Nordstedt, C.; Fredholm, B.B., Anal. Biochem. 1990, 189, 231]. CHO cells expressing one of four subtypes of recombinant ARs were harvested by trypsinization. After resuspension in medium, cells were planted in 24-well plates in 0.5 ml medium. After 24 hr, 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 μ M) and adenosine deaminase (3 units/mL). After 45 min forskolin (10 μ 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 μ L of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at -20°C. For determination of cAMP production, protein kinase A was incubated with [³H]cAMP (2 nM) in K₂HPO₄/EDTA buffer (K₂HPO₄, 150 mM; EDTA, 10 mM), 20 μ L of the cell lysate, and 30 μ L 0.1 M HCl or 50 μ L of cAMP solution (0-16 pmol/200 μ L 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.

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20. CHO cells stably expressing human A₃ARs were grown overnight in 100 µl of media in 96 well flat bottom plates at 37°C at 5% CO₂ to reach approx. 90% confluency. The calcium assay kit (Molecular Devices) was used as directed with no washing of cells, and with probenecid added to the loading dye at a final concentration of 2.5 mM to increase dye retention. Cells were loaded with 50 µl of dye with probenecid to each well and incubated for 60 minutes at room temperature. The compound plate was prepared using dilutions of various compounds in Hanks Buffer. For antagonist studies, both agonist and antagonist were added to the sample plate. Samples were run in duplicate using a Molecular Devices Flexstation I at room temperature. Cell fluorescence (Excitation = 485 nm; Emission = 525 nm) was monitored following exposure to compound. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure.
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Figure 1.

A) Inhibition by nucleoside derivatives of forskolin-stimulated cAMP accumulation in CHO cells stably expressing the human A₃AR. CI-IB-MECA **4** concentration-dependently inhibited with an EC₅₀ of 1.2±0.3 nM (n=3), while **6** and **7** alone had no effect. B) Right-shift of the concentration-response curves and C) Schild analysis for the concentration-dependent inhibition by **6** of the effect of CI-IB-MECA **4** on forskolin-stimulated cAMP accumulation in CHO cells stably expressing the human A₃AR. D) A₃AR-induced changes in intracellular [Ca²⁺] in A₃AR-expressing CHO cells (proportional to relative fluorescence units, RFU) upon activation by nucleosides. In control CHO cells there was no response to NECA.

Scheme 1a.

^aReagents & conditions: a) K₂CO₃, MeOH, r.t.; b) HOAc; c) 40% aq. Me₂NH, r.t.; d) Me₂NH, EDC, HOBT, DIPEA; e) TBAF, THF.

Chart 1.

Table 1Potency of a series of adenosine derivatives for four subtypes of human ARs and the efficacy at the A₃AR.

Compound	A ₁ ^d	A _{2A} ^d	Potency (K _i or EC ₅₀ , nM)		Efficacy ^c A ₃
			A _{2B} ^e	A ₃ ^d	
1 ^a	310 ^e	700 ^e	24,000	290 ^e	100
2 ^b	7.4 ± 1.7	132 ± 22	~10,000	5.8 ± 0.4	46 ± 8
3 ^b	16.8 ± 2.2	197 ± 34	>10,000	1.8 ± 0.1	0
4 ^b	222 ± 22	5360 ± 2470	>10,000	1.4 ± 0.3	100
5 ^f	193 ± 46	223 ± 36	ND	0.38 ± 0.07	114 ± 9
6 ^g	5870 ± 930	>10,000	>10,000	29.0 ± 4.9	0
7 ^g	6220 ± 640	>10,000	>10,000	15.5 ± 3.1	0
8	>10,000	>10,000	>10,000	315 ± 19	0

All experiments were done on CHO cells stably expressing one of four subtypes of human ARs. The binding affinity for A₁, A_{2A} and A₃ARs was expressed as K_i values and was determined by using agonist radioligands ([³H]R-PIA), ([³H]CGS21680), [¹²⁵I]I-AB-MECA, respectively. The potency at the A_{2B}AR was expressed as EC₅₀ values and was determined by stimulation of cAMP production in AR-transfected CHO cells. The efficacy at A₃ARs was determined by inhibition of forskolin-stimulated cAMP production in AR-transfected CHO cells, as described in the text. Data are expressed as mean ± standard error.

^aValues from reference 21.

^bValues from references 9, 21.

^cAt a concentration of 10 μM, in comparison to the maximal effect of a full agonist NECA at 10 μM.

^dK_i in binding, unless noted.

^ecAMP assay.

^fK_i values from reference 13.

^g6, MRS3771; 7, LJ-1256.

ND not determined.