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Bioorganic & Medicinal Chemistry Letters 16 (2006) 596–601
Bioorganic & Medicinal Chemistry Letters

Conversion of A_3 adenosine receptor agonists into selective antagonists by modification of the 5′-ribofuran-uronamide moiety

Zhan-Guo Gao,[a] Bhalchandra V. Joshi,[a] Athena M. Klutz,[a] Soo-Kyung Kim,[a] Hyuk Woo Lee,[b] Hea Ok Kim,[b] Lak Shin Jeong[b] and Kenneth A. Jacobson[a,*]

[a]*Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA*
[b]*Laboratory of Medicinal Chemistry, College of Pharmacy, Ewha Womans University, Seoul 120-750, Republic of Korea*

Received 30 September 2005; revised 14 October 2005; accepted 14 October 2005
Available online 10 November 2005

Abstract—The highly selective agonists of the A_3 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 A_3AR, with K_i values of 29 nM (4′-O) and 15 nM (4′-S), showing >100-fold selectivity over A_1, A_{2A}, and A_{2B}ARs. Competitive antagonism was demonstrated by Schild analysis. The 2-(dimethylamino)-5′-(*N*,*N*-dimethyl)uronamido substitution also retained A_3AR selectivity but lowered affinity.

Antagonists of the A_3 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 A_3AR have recently been synthesized.[2–6] These human A_3AR antagonists were found to be weak or ineffective at the rat A_3AR[7,8] and were unsuitable for evaluation in small animal models or for further development as drugs. Thus, A_3AR 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 analogues, in contrast to nonpurine heterocyclic antagonists, could be species-independent, potent, and selective A_3AR antagonists.[9]

Among the four subtypes of receptors for adenosine **1**, the intrinsic efficacy of nucleosides acting at the A_3 subtype is known to be especially sensitive to structural changes. For example, substitution by a benzyl or a 3-iodobenzyl group at the N^6 position of adenosine was demonstrated to increase affinity but decrease efficacy at the human A_3AR, 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, N^6-(3-iodobenzyl)adenosine **2** was a partial agonist achieving only 46% of the maximal effect, while 2-chloro-N^6-(3-iodobenzyl)adenosine **3** was a potent antagonist for the A_3AR, albeit nonselective. A 5′-methyluronamide substitution of **3** restored its efficacy and rendered it selective for the A_3AR. Thus, Cl-IB-MECA **4** and its 4′-thio analogue LJ568 **5** are selective agonists for the A_3AR.[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 (see Chart 1). 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 amino-

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.

Keywords: Nucleoside; G protein-coupled receptor; Adenylyl cyclase; Molecular modeling; Radioligand binding.

* Corresponding author. Tel.: +301 496 9024; fax: +301 480 8422; e-mail: kajacobs@helix.nih.gov

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doi:10.1016/j.bmcl.2005.10.054

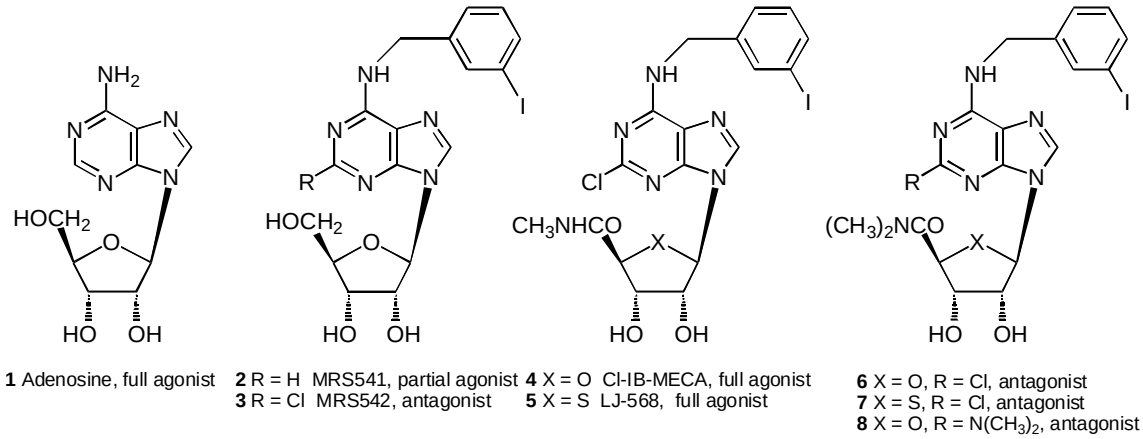
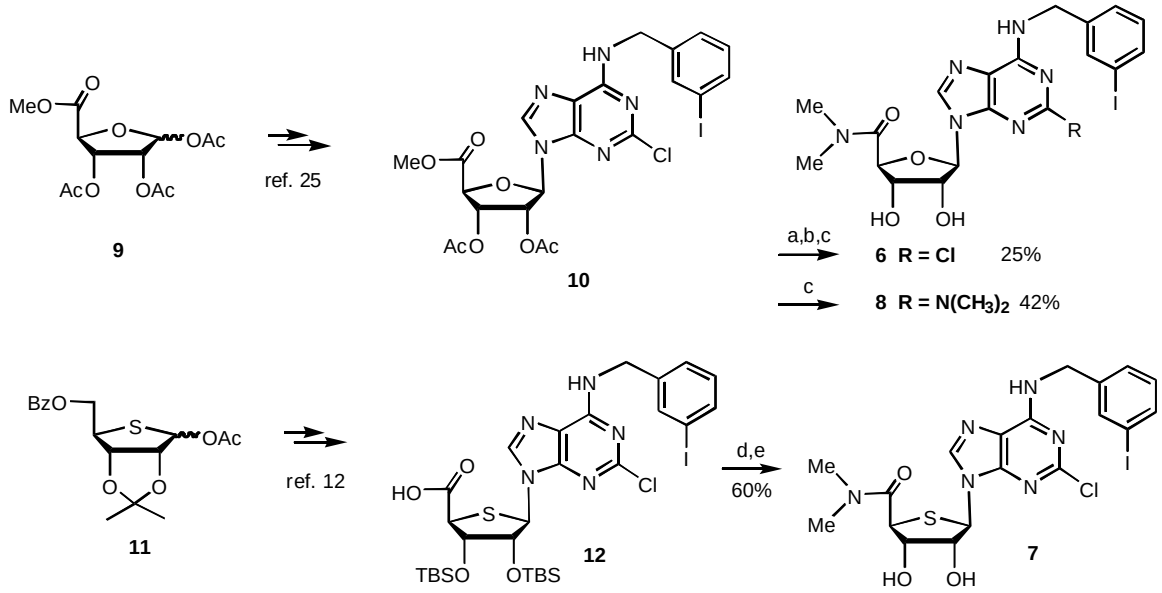


Chart 1.



Scheme 1. Reagents and conditions: (a) K_2CO_3, MeOH, rt; (b) HOAc; (c) 40% aq. Me_2NH, rt; (d) Me_2NH, EDC, HOBT, DIPEA; (e) TBAF, THF.

lyzed 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 A_3AR 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 A_3AR in comparison to other AR subtypes. In addition, the 2-(dimethylamino) substitution in **8** resulted in A_3AR selectivity but with lower affinity.

To probe species differences, the affinity of **6** and **7** was also measured at the rat A_3AR. Although the affinity decreased with respect to the affinity at the human A_3AR, these two compounds showed moderate affinity at the rat A_3AR. 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 A_3AR agonism was absent in compounds **6**–**8**. In contrast, the concentration–response curve for compound **4** indicates full agonism, as previously reported, with an EC_{50} of 1.2 nM at the human A_3AR (Fig. 1A). A Schild analysis[19] indicated that **6** concentration-dependently antagonized the A_3 agonist **4** to inhibit forskolin-stimulated cAMP accumulation in CHO cells stably expressing the human A_3AR with a K_B value of 48 nM (Figs. 1B and C). In contrast, **6**, at a concentration of 1 μM, had no significant effect on cAMP accumulation inhibited by the A_1 agonist CPA (N^6-cyclopentyladenosine) in CHO cells expressing the human A_1AR under the similar conditions (data not shown).

Table 1. Potency of a series of adenosine derivatives for four subtypes of human ARs and the efficacy at the A_3AR

Compound	Potency (K_i or EC_{50}, nM)				Efficacy[c]
	A_1[d]	A_{2A}[d]	A_{2B}[e]	A_3[d]	A_3 (%)
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	>110,000	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 A_1, A_{2A}, and A_3ARs was expressed as K_i values and was determined by using agonist radioligands ([^{3}H]R-PIA), ([^{3}H]CGS21680), and [^{125}I]I-AB-MECA, respectively. The potency at the A_{2B}AR was expressed as EC_{50} values and was determined by stimulation of cAMP production in AR-transfected CHO cells. The efficacy at A_3ARs was determined by inhibition of forskolin-stimulated cAMP production in AR-transfected CHO cells, as described in the text. Data are expressed as means ± standard error.

ND, not determined.

[a] Values from Ref. 24.

[b] Values from Refs. 9,21.

[c] At a concentration of 10 μM, in comparison to the maximal effect of a full agonist NECA at 10 μM.

[d] K_i in binding, unless noted.

[e] cAMP assay.

[f] K_i values from Ref. 13.

[g] **6**, MRS3771; **7**, LJ-1256.

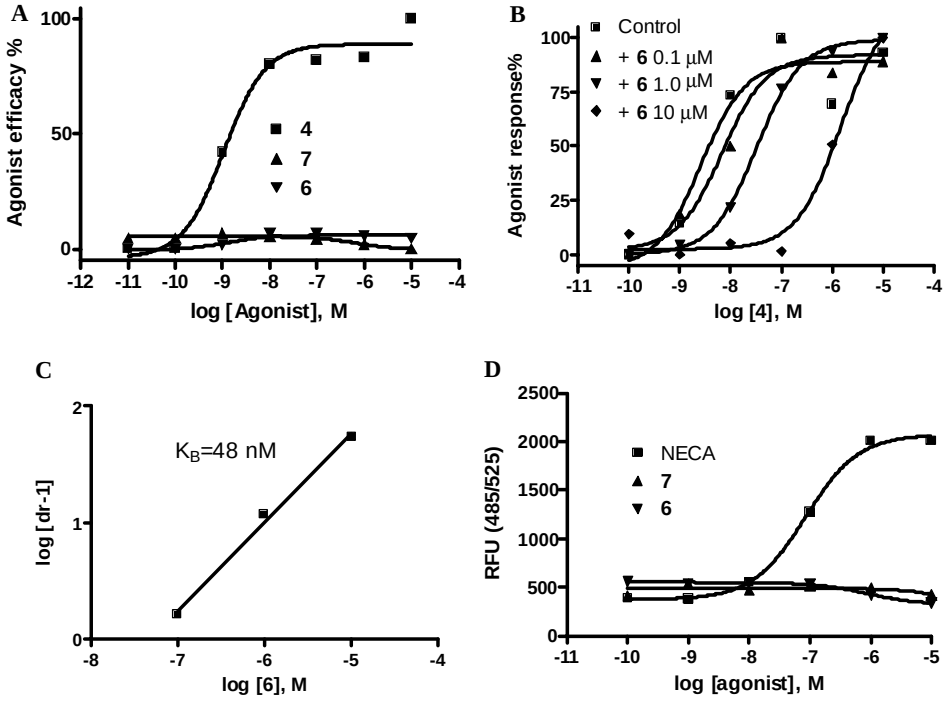


Figure 1. (A) Inhibition by nucleoside derivatives of forskolin-stimulated cAMP accumulation in CHO cells stably expressing the human A_3AR. Cl-IB-MECA **4** concentration-dependently inhibited with an EC_{50} of 1.2 ± 0.3 nM (n = 3), while **6** and **7** alone had no effect. (B) Right-shift of the concentration–response curves and (C) Schild analysis for the concentration-dependent inhibition by **6** of the effect of Cl-IB-MECA **4** on forskolin-stimulated cAMP accumulation in CHO cells stably expressing the human A_3AR. (D) A_3AR-induced changes in intracellular [Ca^{2+}] in A_3AR-expressing CHO cells (proportional to relative fluorescence units, RFU) upon activation by nucleosides. In control CHO cells, there was no response to NECA.

Similar results were obtained in an assay of A_3AR-induced changes in intracellular [Ca^{2+}].[20] The nonselective AR agonist NECA (5′-*N*-ethyl-carboxamidoadenosine) activated the human A_3AR expressed in CHO cells to induce a concentration-dependent rise in intracellular [Ca^{2+}] with a potency corresponding to an EC_{50} of 58 ± 16 nM (n = 4), while **6** and **7** alone did not induce calcium transients (Fig. 1D). Compound **8** also did not induce a Ca^{2+} 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 A_3AR antagonist **7** similar-

ly shifted the NECA-induced Ca^{2+} response to the right (not shown).

In this study, two selective A_3AR agonists, Cl-IB-MECA and its 4′-thio analogue, have been successfully transformed into antagonists selective for the A_3AR 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 A_3AR. 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 A_3AR affinity tends to be retained in murine species,[21] which was confirmed in the present study.

Molecular modeling of the A_3AR 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 W243 in TM6(6.48).[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 A_3AR.[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 A_3AR. From a comparison of the A_{2A} and A_3AR models,[23] the main difference in the docking complex was the preference of the χ angle; the A_3AR preferred nucleosides bound in an *anti*-form (185°), 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(3.36), but the model of the A_3AR complex featured a stronger interaction proposed between the 5′-carbonyl group and S271 in TM7(7.42). Accordingly, the 5′-dimethylamides displayed a dramatically diminished binding affinity at the A_{2A}AR.

A_3AR antagonists are potentially useful therapeutically for a number of disorders.[1,8,24] However, the A_3 antagonists have not been widely used in animal models due to their extremely weak potency in murine species.[8] Thus, A_3AR antagonists independent of species are of high priority to be developed. For this reason, a nucleoside analogue 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 A_3AR antagonist of moderate affinity for both human and rat A_3ARs.[9] Another advantage of nucleoside A_3AR antagonists over other heterocycles[7] is increased aqueous compatibility. For example, the C log *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 A_3AR.[7] Here, two additional selective antagonists with reasonable affinity for both human and rat A_3ARs were introduced, which increased the diversity of rat A_3AR antagonists.

It is clear that more potent and selective antagonists are needed for eventual therapeutic purposes. The newly synthesized A_3AR antagonists could be evaluated in models of a number of disorders related to the A_3AR, such as glaucoma and inflammation. Currently, existing nucleoside analogues should be good templates for further modification and development of potent and selective antagonists for the A_3ARs in diverse species.

Acknowledgments

B.V. Joshi thanks Gilead Sciences (Foster City, CA) for financial support. This research was supported in part by the Intramural Research Program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases, and by the Korea Health R&D Project, Ministry of Health & Welfare, Korea (03-PJ2-PG4-BD02-0001). We thank Prof. Mortimer Civan (University of Pennsylvania) for helpful discussion.

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14. Jacobson, K. A.; Kim, S. K.; Costanzi, S.; Gao, Z. G. *Mol. Interventions* **2004**, *4*, 337.
15. (2*S*,3*S*,4*R*,5*R*)-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), potassium carbonate (0.014 g, 0.1 mmol) was added, and the mixture was 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 h. 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%). ^{1}H NMR ($CDCl_3$) δ 2.97 (s, 3 H, N-CH_3), 3.14 (s, 3 H, N-CH_3), 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 $C_{19}H_{21}N_6O_4ClI^+$) 559.0358. (2*S*,3*S*,4*R*,5*R*)-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%). ^{1}H NMR ($CDCl_3$) δ 2.90–3.23 (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 $C_{21}H_{27}N_7O_4I^+$) 568.1169.
16. (2*S*,3*S*,4*R*,5*R*)-5-[2-Chloro-6-(3-iodo-benzylamino)-purin-9-yl]-3,4-dihydroxy-tetrahydro-thiophene-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 CH_2Cl_2 (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 (CH_2Cl_2/MeOH = 10:1) to give **7** (214 mg, 64%): white solid; mp 186.1–186.3 °C; $[\alpha]_D^{20}$ −12.4 (*c* 0.10, MeOH); UV (MeOH) λ_{max} 274 nm (pH 7); ^{1}H NMR (DMSO-d_6) δ 2.95 (s, 3 H, N-CH_3), 3.04 (s, 3H, N-CH_3), 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-CH_2), 5.59 (d, 1 H, *J* = 5.5 Hz, exchangeable with D_2O, OH), 5.86 (d, 1 H, *J* = 5.1 Hz, exchangeable with D_2O, 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 D_2O, NH); ^{13}C NMR (CD_3OD) 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. ($C_{19}H_{20}ClIN_6O_3S$) 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 U/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 U/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 A_3AR binding assays, each tube contained 100 μl of membrane suspension, 50 μl [^{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 $MgCl_2$. Non-specific binding was determined using 10 μM 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_1ARs and the binding of [^{3}H]CGS21680 to A_{2A}ARs were as previously described.[10] IC_{50} values were converted to K_i values as described [Cheng Y.-C., Prusoff W. H. *Biochem. Pharmacol.*; **1973**, *22*, 3099]. [^{125}I]*N*^{6}-(4-amino-3-iodo-benzyl)adenosine-5′-*N*-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*-ethylcarboxamido-adenosine, 47 Ci/mmol), and [^{3}H]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 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 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 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 [^{3}H]cAMP (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 of 0.1 M HCl or 50 μL cAMP 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.

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20. CHO cells stably expressing human A_3ARs were grown overnight in 100 μl media in 96-well flat-bottomed plates at 37 °C at 5% CO_2 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 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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