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Structure–activity relationships of truncated adenosine derivatives as highly potent and selective human A_3 adenosine receptor antagonists

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ABSTRACT

On the basis of potent and selective binding affinity of truncated 4′-thioadenosine derivatives at the human A_3 adenosine receptor (AR), their bioisosteric 4′-oxo derivatives were designed and synthesized from commercially available 2,3-*O*-isopropylidene-D-erythrono lactone. The derivatives tested in AR binding assays were substituted at the C2 and N^6 positions. All synthesized nucleosides exhibited potent and selective binding affinity at the human A_3 AR. They were less potent than the corresponding 4′-thio analogues, but showed still selective to other subtypes. The 2-Cl series generally were better than the 2-H series in view of binding affinity and selectivity. Among compounds tested, compound **5d** (X = Cl, R = 3-bromobenzyl) showed the highest binding affinity (K_i = 13.0 ± 6.9 nM) at the hA_3 AR with high selectivity (at least 88-fold) in comparison to other AR subtypes. Like the corresponding truncated 4′-thio series, compound **5d** antagonized the action of an agonist to inhibit forskolin-stimulated adenylate cyclase in hA_3 AR-expressing CHO cells. Although the 4′-oxo series were less potent than the 4′-thio series, this class of human A_3 AR antagonists is also regarded as another good template for the design of A_3 AR antagonists and for further drug development.

1. Introduction

Adenosine, an endogenous chemical messenger binds to four subtypes (A_1, A_{2A}, A_{2B}, and A_3) of adenosine receptors (ARs) and regulates many physiological functions in the cell.[1] A_3 AR activation stimulates phospholipase C (PLC), increasing the levels of inositol triphosphate (IP_3) and diacylglycerol (DAG) and inhibits adenylate cyclase, decreasing the level of cAMP. Since IP_3, DAG, and cAMP serve as second messengers important for cell signaling, A_3 AR has been promising targets for the treatment of several diseases associated with cell signaling.[2] Thus, A_3 AR agonists have the therapeutic potentials against cancer,[3] cerebral ischemia,[4] and myocardiac ischemia,[5] while A_3 AR antagonists can be developed as anti-inflammatory,[6] anti-asthma,[7] and anti-glaucoma agents.[8]

A number of purine and non-purine compounds have been developed as potent and selective A_3 AR ligands.[9,10] Most of adenosine derivatives were found to be A_3 AR agonists, among which compound **1** (Cl-IB-MECA)[11] showed high binding affinity (K_i for hA_3 = 1.4 nM) at the human (h) A_3 AR with high selectivities to other subtypes (Chart 1) and is under clinical trials as an anticancer agent. On the basis of a bioisosteric rationale, we designed and synthesized the 4′-thio analogue **2**[12] of Cl-IB-MECA, which exhibited better binding affinity (K_i for hA_3 = 0.38 nM) than compound **1**.

On the other hand, nonpurine heterocyclic compounds[13] have been reported to be better templates than purine derivatives in developing potent and selective hA_3 AR antagonists. However, these A_3 AR antagonists were weak or ineffective in binding to the rat A_3 AR, indicating that they were not suitable for efficacy evaluation in small animal models and thus as drug candidates.[14] Thus, searching for potent and selective species-independent A_3 AR antagonists has been an immediate goal. Because a few nucleoside derivatives have been reported to be species-independent A_3 AR antagonists[14], we began to search for novel potent and selective A_3 AR antagonists, derived from nucleoside templates.

As a result, the truncated 4′-thio analogue **3**[15] removing a hydrogen bonding donor ability of the NH in the 5′-uronamide required for the activation of the A_3 AR in compound **2** was found to be one of the best A_3 AR antagonists which bound selectively to the A_3 AR in a species-independent manner (K_i = 4.16 nM at the hA_3 AR and K_i = 3.9 nM at the rat A_3 AR) (Chart 1). Thus, it is of interest to design bioisosteric analogues of compound **3**, such as the truncated 4′-oxo derivatives **4** and **5**, and to compare their binding affinities at the hA_3 AR. In this article, we report the synthesis of truncated

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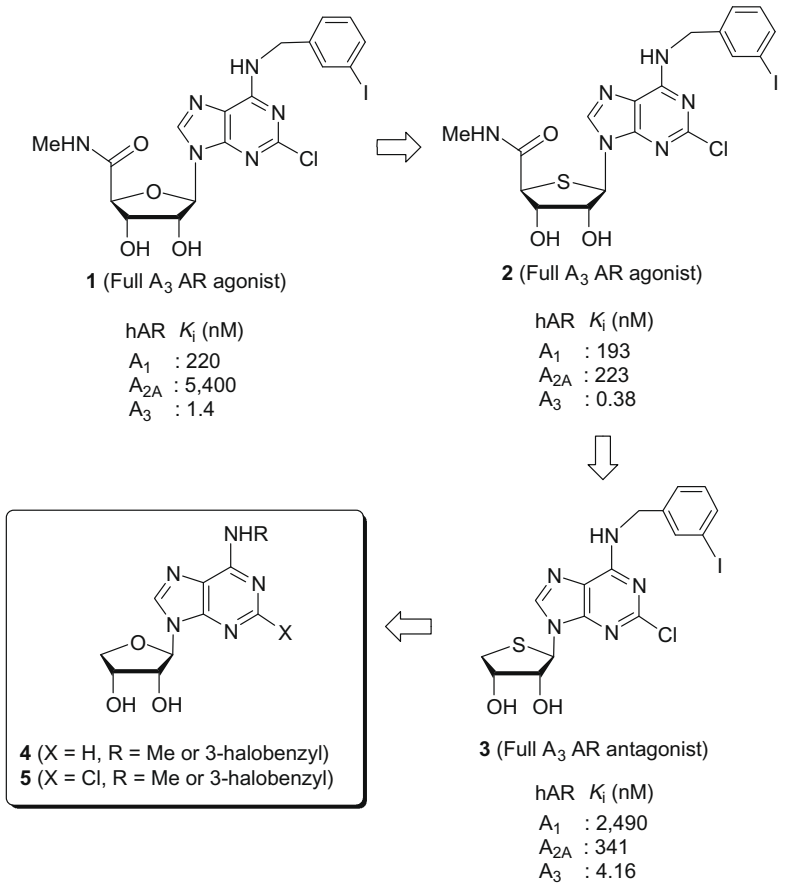


Chart 1. Binding affinity (A_1 AR, A_{2A} AR, A_3 AR) at human adenosine receptors.

adenosine derivatives **4** and **5** as potent and selective hA_3 AR antagonists.

2. Results and discussion

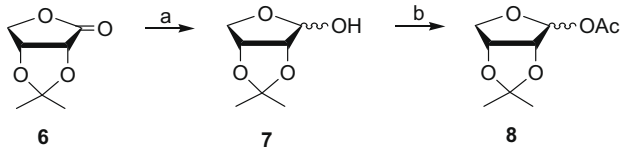
2.1. Chemistry

Our synthetic strategy to the final nucleosides was first to synthesize the glycosyl donor and then condense with purine bases. Synthesis of the glycosyl donor **7**[16] from commercially available 2,3-*O*-isopropylidene-D-erythronolactone (**6**) is shown in Scheme 1.

Reduction of **6** with DIBAL in toluene gave the lactol **7**[16], which was treated with acetic anhydride in pyridine to give the glycosyl donor **8**.

The glycosyl donor **8** was then condensed with silyalted 6-chloropurine and 2,6-dichloropurine to give 6-chloro derivative **9** and 2,6-dichloropurine derivative **10**, respectively (Scheme 2).

During condensation reaction, the initially formed N-3 isomer was smoothly converted to the N-9 isomer on refluxing. The anomeric assignment of **9** was easily achieved by ^{1}H NOE experiments. Strong NOE effect between H-8 and 2′-H in compound **9** was shown, indicating that it is β-anomer. Removals of the isopropylidene groups in **9** and **10** under acidic conditions afforded **11** and **12**, respectively.

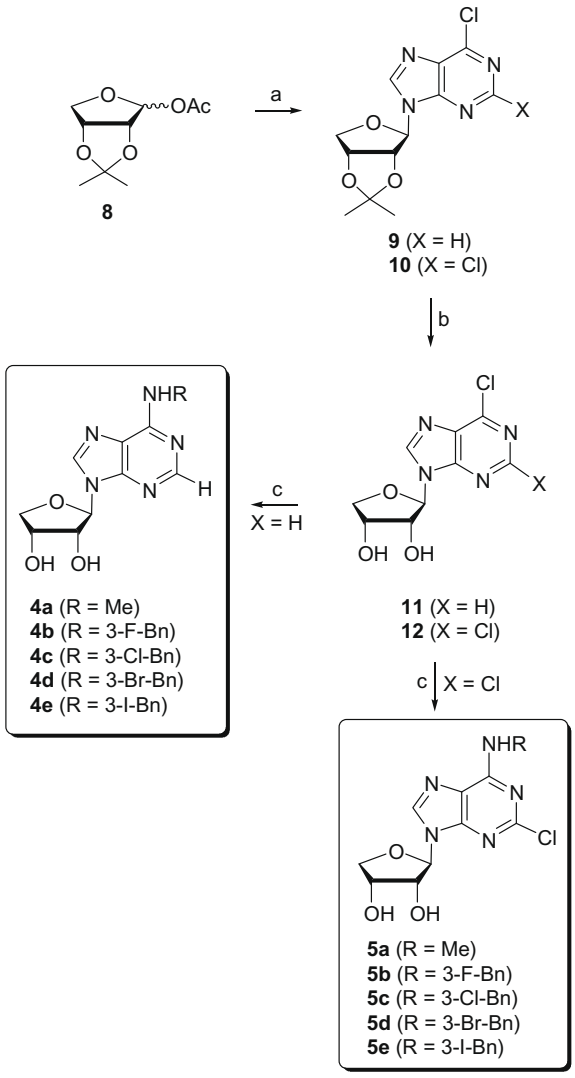


Scheme 1. Reagents and conditions: (a) DIBAL, toluene, −78 °C, 30 min; (b) Ac_2O, pyridine, rt, 3 h.

Treatment of **11** with methylamine and 3-halobenzyl amines yielded the N^6-methylamino derivative **4a** and the N^6-(3-halobenzyl)amino derivatives **4b**–**e**. Similarly, compound **12** was converted to the 2-chloro-N^6-methylamino derivative **5a** and the 2-chloro-N^6-(3-halobenzyl)amino derivatives **5b**–**e**.

2.2. Biological activity

Binding assays were carried out using adherent mammalian cells stably transfected with cDNA encoding the appropriate hARs (A_1 and A_3 in CHO cells and A_{2A} in HEK-293 cells) and standard radioligands (3.5 nM [^{3}H]NECA for A_1 AR, 10 nM [^{3}H]CGS21680 for A_{2A} AR, and 0.5 nM [^{125}I]I-AB-MECA for A_3 AR).[17,18] Table 1 shows that all synthesized compounds exhibited potent and selective binding affinity at the hA_3 AR, although they were less potent than the corresponding 4′-thio analogues. In general, the 2-Cl series showed higher binding affinity to the hA_3 AR and better selectivity to other subtypes (A_1 and A_{2A}) than the 2-H series. The similar trend was observed in the corresponding 4′-thio analogues. Within the 2-Cl series, compound **5d** (R = 3-bromobenzyl) showed the highest binding affinity (K_i = 13 nM) at the hA_3 AR with high selectivity to other subtypes, A_1 AR and A_{2A} AR. The magnitude of the binding affinity in the 3-halobenzyl series is decreased in the following order: Br-Bn > I-Bn > Cl-Bn > F-Bn, indicating that the



Scheme 2. Reagents and conditions: (a) silylated6-chloropurine or 2,6-dichloropurine, TMSOTf, 1,2-dichloroethane, rt to 80 °C; (b) 2 N HCl, THF, rt, overnight; (c) RNH_2, ET_3N, EtOH, rt, 1–3 days.

proper size of halogen is essential for optimal hydrophobic interaction at the hA_3 AR. The same trend was observed in the 2-H series.

One representative truncated 4′-oxo analogue **5d** was chosen for testing in a functional assay consisting of 10 μM forskolin-stimulated cyclic AMP production in CHO cells stably expressing the hA_3 AR. Compound **5d** was shown to be an antagonist of the hA_3 AR in this assay and did not activate the hA_3 AR. Figure 1 shows that the concentration–response curve for inhibition of adenylate cyclase by the full agonist Cl-IB-MECA **1** was right shifted by 300 nM **5d**. The EC_{50} value of **1** shifted from 1.14 to 11.9 nM in the presence of the antagonist.

The binding affinity of compound **5d** at the rat A_3 AR was also measured to determine if it shows species-independent binding affinity at the A_3 AR. This compound still showed high and species-independent binding affinity (K_i = 82.8 ± 22.9 nM) at the rat A_3 AR, although it was six times less potent than at the human A_3 AR. This result indicates that truncated 4′-oxo analogues are also suitable for evaluation in small animal models or for further drug development as truncated 4′-thio analogues.

3. Conclusions

On the basis of a bioisosteric rationale, novel truncated adenosine derivatives were designed and synthesized as potent and selective hA_3 AR antagonists. All the synthesized nucleosides showed high binding affinity at the hA_3 AR with high selectivity to the hA_1 and hA_{2A} ARs. Among compounds tested, compound **5d** (X = Cl, R = 3-bromobenzyl) showed the highest binding affinity (K_i = 13.0 ± 6.9 nM) at the hA_3 AR with high selectivity (at least 88-fold) in comparison to other AR subtypes. Like the corresponding truncated 4′-thio analogue series, compound **5d** was shown to be an antagonist of the hA_3 AR in an assay of adenylate cyclase. Although the truncated 4′-oxo series showed lower binding affinity at the hA_3 AR than the corresponding 4′-thio series, this class of potent hA_3 AR antagonists is also regarded as another good template for the design of A_3 AR antagonists and for further drug development.

4. Experimental

4.1. General methods

Melting points are uncorrected. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured in $CDCl_3$, CD_3OD or DMSO-d_6, and chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane as internal standard. Column chromatography was performed using Silica Gel 60 (230–400 mesh). Anhydrous solvents were purified by the standard procedures.

4.2. Synthesis

4.2.1. (3a*R*,4*R*,6a*R*)-2,2-Dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-ol (7)

To a stirred suspension of **6** (8.0 g, 50.58 mmol) in toluene (150 mL) was added DIBAL (61.1 mL, 61.1 mmol, 1 M solution in toluene) at −78 °C and the reaction mixture was stirred at the same temperature for 30 min and then quenched by the slow addition of methanol. The colloidal suspension was filtered on a Celite pad and washed with EtOAc. The combined organic layers were dried over $MgSO_4$, filtered, and evaporated. The residue was purified by flash silica gel column chromatography (hexane/EtOAc = 3:1) to give **7** (7.20 g, 89%) as a syrup, whose spectral data were identical with those of authentic sample.[16]

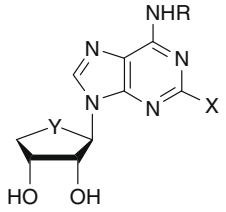
4.2.2. (3a*R*,4*R*,6a*R*)-2,2-Dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl-acetate (8)

To a stirred solution of **7**[16] (6.2 g, 38.7 mmol) in pyridine (30 mL) was added acetic anhydride (4.39 mL, 46.44 mmol) at 0 °C and the reaction mixture was stirred for 3 h at rt and evaporated. The residue was dissolved in EtOAc and washed with water. The organic layer was dried over $MgSO_4$ and evaporated. The residue was purified by flash silica gel column chromatography (hexane/EtOAc = 8:1) to give **8** (6.4 g, 82%) as a syrup: ^1H NMR ($CDCl_3$) δ 6.16 (s, 1H), 4.86 (dd, 1H, J = 3.6, 6.0 Hz), 4.66 (d, 1H, J = 6.0 Hz), 4.12 (d, 1H, J = 6.4 Hz), 3.99 (dd, 1H, J = 3.6, 10.8 Hz), 2.05 (s, 3H), 1.48 (s, 3H), 1.33 (s, 3H).

4.2.3. 6-Chloro-9-((3a*R*,4*R*,6a*S*)-2,2-dimethyltetrahydrothieno[3,4-*d*][1,3]dioxol-4-yl)-9*H*-purine (9)

6-Chloropurine (2.29 g, 14.84 mmol), ammonium sulfate (98 mg, 0.742 mmol), and HMDS (25 mL) were refluxed overnight under inert and dry condition. The solution was evaporated under high vacuum. The resulting solid was re-dissolved in 1,2-dichloroethane (30 mL) and cooled in ice. To this mixture, a solution of **8**

Table 1
Binding affinities of known A_3 AR agonists, **1** and **2** and antagonist **3**, and truncated 4′-adenosine derivatives **4a–e** and **5a–e** at three subtypes of hARs



Compound	Affinity, K_i, nM ± SEM (or % inhibition)[a]		
	hA_1	hA_{2A}	hA_3
1 (Cl-IB-MECA)	220	5400	1.4
2 (thio-Cl-IB-MECA)	193	223	0.38
3 (X = Cl, Y = S, R = 3-iodobenzyl)	2490	341	4.16
4a (X = H, Y = O, R = methyl)	44 ± 2%	25 ± 5%	70.1 ± 18.3
4b (X = H, Y = O, R = 3-fluorobenzyl)	36 ± 1%	6560 ± 1560	537 ± 58
4c (X = H, Y = O, R = 3-chlorobenzyl)	3100 ± 890%	3410 ± 400	76.1 ± 3.9
4d (X = H, Y = O, R = 3-bromobenzyl)	1060 ± 180	1400 ± 120	59.0 ± 0.2
4e (X = H, Y = O, R = 3-iodobenzyl)	795 ± 101	997 ± 125	90.2 ± 39.8
5a (X = Cl, Y = O, R = methyl)	49 ± 1%	15 ± 5%	37.5 ± 5.0
5b (X = Cl, Y = O, R = 3-fluorobenzyl)	43 ± 1%	42 ± 4%	284 ± 37
5c (X = Cl, Y = O, R = 3-chlorobenzyl)	2480 ± 590	3630 ± 1080	75.0 ± 11.7
5d (X = Cl, Y = O, R = 3-bromobenzyl)	1150 ± 62	30%	13.0 ± 6.9
5e (X = Cl, Y = = O, R = 3-iodobenzyl)	2230 ± 700	43%	42.9 ± 8.9

[a] All binding experiments were performed using adherent mammalian cells stably transfected with cDNA encoding the appropriate hAR (A_1 AR and A_3 AR in CHO cells and A_{2A} AR in HEK-293 cells). Binding was carried out using 3.5 nM [^{3}H]NECA, 10 nM [^{3}H]CGS21680, or 0.5 nM [^{125}I]I-AB-MECA as radioligands for A_1, A_{2A}, and A_3 ARs, respectively. Values are expressed as mean ± sem, n = 3–4 (outliers eliminated), and normalized against a non-specific binder, 5′-*N*-ethylcarboxamidoadenosine (NECA, 10 μM). If a percentage is given, it represents the percent inhibition at a fixed concentration of 10 μM.

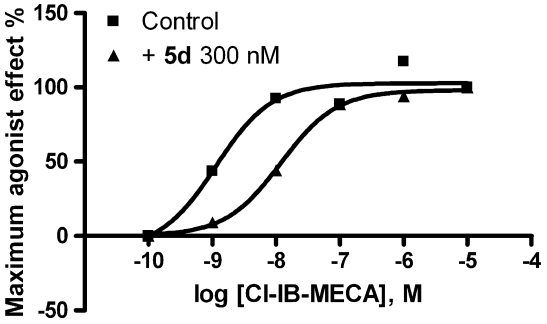


Figure 1. Right-shift of the concentration–response curves of the full agonist Cl-IB-MECA **1** by 300 nM of antagonist **5d**.

(1.5 g, 7.42 mmol) in 1,2-dichloroethane (10 mL) and TMSOTf (2.69 mL, 14.84 mmol) were successively added and the mixture was stirred at 0 °C for 30 min, at rt for 1 h, and then heated at 80 °C for 2 h. The mixture was cooled, diluted with CH_2Cl_2 and washed with saturated $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$ and evaporated. The yellowish syrup was purified by a flash silica gel column chromatography (CH_2Cl_2/MeOH = 50:1) to give **9** (1.5 g, 68%) as a white solid: mp 118.5–119.2 °C; UV (MeOH) λ_{max} 264.0 nm; ^{1}H NMR (CD_3OD) δ 8.73 (s, 1H), 8.62 (s, 1H), 6.27 (s, 1H), 5.52 (d, 1H, J = 5.6 Hz), 5.31 (dd, 1H, J = 3.6, 6.0 Hz), 4.27 (dd, 1H, J = 3.6, 10.4 Hz), 4.19 (d, 1H, J = 10.4 Hz), 1.54 (s, 3H), 1.38 (s, 3H); ^{13}C NMR (CD_3OD) δ 153.2, 152.8, 151.7, 147.9, 132.9, 114.3, 93.1, 85.9, 82.8, 77.0, 26.8, 25.1; $[\alpha]_D^{25.8}$ −45.04 (*c* 0.333, DMSO); FAB-MS *m/z* 297 [M+H]$^+$. Anal. Calcd for $C_{12}H_{13}ClN_4O_3$: C, 48.58; H, 4.42; N, 18.88. Found: C, 49.26; H, 4.61; N, 18.70.

4.2.4. 2,6-Dichloro-9-((3a*R*,6*R*,6aR)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-6-yl)-9*H*-purine (10)

Compound **8** (702 mg, 3.47 mmol) was condensed with 2,6-dichloropurine to give compound **10** (793 mg, 69%) as a foam according to the similar procedure used for the preparation of **9**: UV (MeOH) λ_{max} 276.5 nm; ^{1}H NMR ($CDCl_3$) δ 8.15 (s, 1H), 6.07 (s, 1H), 5.41 (d, 1H, J = 6.0 Hz), 5.29–5.26 (m, 1H), 4.31–4.25 (m, 2 H), 1.57 (s, 3 H), 1.41 (s, 3 H); $[\alpha]_D^{25}$ −21.00 (*c* 0.10, DMSO); FAB-MS *m/z* 331 [M+H]$^+$. Anal. Calcd for $C_{12}H_{12}Cl_2N_4O_3$: C, 43.52; H, 3.65; N, 16.92. Found: C, 43.08; H, 3.61; N, 16.70.

4.2.5. (2*R*,3R,4*S*)-2-(6-Chloro-9*H*-purin-9-yl)-tetrahydrothiophene-3,4-diol (11)

To a solution of **9** (300 mg, 1.01 mmol) in THF (5 mL) was added 2 N hydrochloric acid (2.5 mL) and the mixture was stirred at rt overnight. The mixture was neutralized with 1 N NaOH solution, and then carefully evaporated. The mixture was purified by a flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **11** (165 mg, 64%) as a white solid: mp 165.5–166.7 °C; UV (MeOH) λ_{max} 264.0 nm; ^{1}H NMR (DMSO-d_6) δ 8.95 (s, 1H), 8.82 (s, 1H), 6.01 (d, 1H, J = 6.4 Hz), 5.54 (d, 1H, J = 6.4 Hz), 5.29 (d, 1H, J = 4 Hz), 4.83 (ddd, 1H, J = 4.8, 6.4, 10.8 Hz), 4.41 (dd, 1H, J = 3.2, 9.2 Hz), 4.33–4.29 (m, 1H), 3.86 (dd, 1H, J = 1.6, 9.2 Hz); ^{13}C NMR (DMSO-d_6) δ 151.8, 151.7, 149.4, 146.5, 131.5, 88.4, 74.6, 74.1, 70.3; $[\alpha]_D^{25.6}$ −95.71 (*c* 0.14, DMSO); FAB-MS *m/z* 257 [M+H]$^+$. Anal. Calcd for $C_9H_9ClN_4O_3$: C, 42.12; H, 3.53; N, 21.83. Found: C, 42.17; H, 3.83; N, 20.43.

4.2.6. (2*R*,3*R*,4*R*)-2-(2,6-Dichloro-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol (12)

Compound **10** (683 mg, 2.06 mmol) was converted to **12** (344 mg, 57%) as a white solid according to the similar procedure used for the preparation of **11**: mp 122.7–123.4 °C; UV (MeOH) λ_{max} 276.5 nm; ^{1}H NMR (DMSO-d_6) δ 8.98 (s, 1H), 5.96 (d, 1H, J = 6.4 Hz), 5.57 (d, 1H, J = 6.0 Hz), 5.32 (d, 1H, J = 4.0 Hz), 4.74–4.69 (m, 1H), 4.41 (dd, 1H, J = 3.6, 9.2 Hz), 4.32–4.29 (m, 1H), 3.87 (dd, 1H, J = 2.0, 9.6 Hz); ^{13}C NMR (DMSO-d_6) δ 153.2, 151.2, 150.04, 147.1, 131.2, 88.4, 74.8, 74.1, 70.1; $[\alpha]_D^{25}$ −68.09 (*c* 0.14, DMSO); FAB-MS *m/z* 291 [M+H]$^+$. Anal. Calcd for $C_9H_8Cl_2N_4O_3$: C, 37.13; H, 2.77; N, 19.25. Found: C, 37.23; H, 3.11; N, 19.45.

4.3. General procedure for the synthesis of 4a–e and 5a–e

To a solution of **11** and **12** in EtOH (5 mL) was added appropriate amines (1.5 equiv) at room temperature and the mixture was stirred at room temperature for 1 d to 3 d and evaporated. The residue was purified by a flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give **4a–e** and **5a–e**, respectively.

4.3.1. (2*R*,3*R*,4*R*)-Tetrahydro-2-(6-(methylamino)-9*H*-purin-9-yl)-furan-3,4-diol (4a)

73% Yield; white solid; mp 208.5–209.0 °C; UV (MeOH) λ_{max} 265.0 nm; ^{1}H NMR (DMSO-d_6) δ 8.33 (s, 1H), 8.23 (s, 1H), 7.73 (br s, 1H), 5.85 (d, 1H, J = 6.4 Hz), 5.43 (d, 1H, J = 6.4 Hz), 5.19 (d, 1H, J = 4.0 Hz), 4.87–4.82 (m, 1H), 3.34 (dd, 1H, J = 4.0, 9.6 Hz), 4.27–4.25 (m, 1H), 3.79 (dd, 1H, J = 1.2, 9.2 Hz), 2.95 (br s, 3H); ^{13}C NMR (DMSO-d_6) δ 155.0, 152.6, 148.5, 139.9, 119.9, 87.7, 74.2, 73.6, 70.3, 26.9; $[\alpha]_D^{25.8}$ −115.45 (*c* 0.11, DMSO); FAB-MS *m/z* 252 [M+H]$^+$. Anal. Calcd for $C_{10}H_{13}N_5O_3$: C, 47.81; H, 5.22; N, 27.87. Found: C, 47.50; H, 5.55; N, 27.66.

4.3.2. (2*R*,3*R*,4R)-2-(6-(3-Fluorobenzylamino)-9*H*-purin-9-yl)tetrahydrofuran-3,4-diol (4b)

80% yield; white solid; mp 196.3–197.0 °C; UV (MeOH) λ_{max} 268.0 nm; ^{1}H NMR (DMSO-d_6) δ 8.42 (br s, 1H), 8.39 (s, 1H), 8.21 (s, 1H), 7.36–7.30 (m, 1H), 7.18–7.11 (m, 2 H), 7.03 (dt, 1H, J = 2.0,

8.4 Hz), 5.86 (d, 1H, *J* = 6.4 Hz), 5.43 (d, 1H, *J* = 6.4 Hz), 5.18 (d, 1H, *J* = 4.0 Hz), 4.88–4.83 (m, 1H), 4.71 (br s, 2 H), 4.34 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.28–4.25 (m, 1H), 3.79 (dd, 1H, *J* = 1.6, 9.2 Hz); ^{13}C NMR (DMSO-d_6) δ 163.4, 160.9, 154.3, 152.5, 148.9, 143.2, 140.3, 130.13 (d, *J* = 31.6 Hz), 123.1 (d, *J* = 8.0 Hz), 119.8, 113.5 (dd, *J* = 82.4, 143.6 Hz), 87.8, 74.2, 73.7, 70.3, 42.5; $[\alpha]_D^{25.7}$ −96.52 (*c* 0.115, DMSO); FAB-MS *m/z* 346 $[M+H]^+$. Anal. Calcd for $C_{16}H_{16}FN_5O_3$: C, 55.65; H, 4.67; N, 20.28. Found: C, 55.29; H, 4.75; N, 19.93.

4.3.3. (2*R*,3*R*,4*R*)-2-(6-(3-Chlorobenzylamino)-9*H*-purin-9-yl)-tetrahydrofuran-3,4-diol (4c)

83% Yield; white solid; mp 187.0–187.7 °C; UV (MeOH) λ_{max} 267.0 nm; ^{1}H NMR (DMSO-d_6) δ 8.43 (br s, 1H), 8.39 (s, 1H), 8.22 (s, 1H), 7.38 (s, 1H), 7.34–7.24 (m, 3 H), 5.85 (d, 1H, *J* = 6.4 Hz), 5.44 (d, 1H, *J* = 6.8 Hz), 5.20 (d, 1H, *J* = 3.6 Hz), 4.85 (dd, 1H, *J* = 6.4, 10.8 Hz), 4.70 (s, 2H), 4.35 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.28–4.25 (m, 1H), 3.80 (dd, 1H, *J* = 1.6, 9.2 Hz); ^{13}C NMR (DMSO-d_6) δ 154.3, 152.5, 148.9, 142.8, 140.4, 132.9, 130.2, 126.9, 126.6, 125.8, 119.8, 87.8, 74.3, 73.7, 70.4, 42.4; $[\alpha]_D^{25.8}$ −92.92 (*c* 0.099, DMSO); FAB-MS *m/z* 362 $[M+H]^+$. Anal. Calcd for $C_{16}H_{16}ClN_5O_3$: C, 53.12; H, 4.46; N, 19.36. Found: C, 53.12; H, 4.57; N, 18.96.

4.3.4. (2*R*,3*R*,4R)-2-(6-(3-Bromobenzylamino)-9*H*-purin-9-yl)-tetrahydrofuran-3,4-diol (4d)

86% Yield; white solid; mp 174.0–174.9 °C; UV (MeOH) λ_{max} 267.0 nm; ^{1}H NMR (DMSO-d_6) δ 8.44 (br s, 1H), 8.39 (s, 1H), 8.21 (s, 1 H), 7.52 (s, 1H), 7.42 (dt, 1H, *J* = 2.0, 8.8 Hz), 7.36 (d, 1H, *J* = 8.0 Hz), 7.25 (d, 1 H, *J* = 8.0 Hz), 5.86 (d, 1H, *J* = 6.4 Hz), 5.43 (d, 1 H, *J* = 6.4 Hz), 5.19 (d, 1H, *J* = 3.6 Hz), 4.88–4.82 (m, 1H), 4.69 (br s, 2 H), 4.35 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.28–4.25 (m, 1H), 3.80 (dd, 1H, *J* = 1.6, 9.6 Hz); ^{13}C NMR (DMSO-d_6) δ 154.3, 152.5, 148.9, 143.0, 140.4, 130.4, 129.8, 129.5, 126.2, 121.5, 119.8, 87.8, 74.2, 73.7, 70.3, 42.3; $[\alpha]^{23.3}{}_D$ -88.00 (*c* 0.15, DMSO); FAB-MS *m/z* 406 $[M]^+$. Anal. Calcd for $C_{16}H_{16}BrN_5O_3$: C, 47.31; H, 3.97; N, 17.24. Found: C, 47.30; H, 3.94; N, 17.47.

4.3.5. (2*R*,3*R*,4*R*)-2-(6-(3-Iodobenzylamino)-9*H*-purin-9-yl)-tetrahydrofuran-3,4-diol (4e)

77% Yield; white solid; mp 202.8–203.5 °C; UV (MeOH) λ_{max} 267.0 nm; ^{1}H NMR (DMSO-d_6) δ 8.42 (br s, 1H), 8.39 (s, 1H), 8.21 (s, 1H), 7.71 (s, 1H), 7.57 (dt, 1H, *J* = 2.8, 8.0 Hz), 7.35 (d, 1H, *J* = 7.6 Hz), 7.10 (d, 1H, *J* = 7.6 Hz), 5.86 (d, 1H, *J* = 6.4 Hz), 5.44 (d, 1H, *J* = 6.4 Hz), 5.19 (d, 1H, *J* = 3.6 Hz), 4.88–4.83 (m, 1H), 4.66 (br s, 2 H), 3.35 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.27–4.25 (m, 1H), 3.80 (dd, 1H, *J* = 1.6, 9.6 Hz); ^{13}C NMR (DMSO-d_6) δ 154.3, 152.5, 148.9, 142.9, 140.37, 135.7, 135.3, 130.5, 126.6, 119.8, 94.7, 87.8, 74.2, 73.7, 70.3, 42.2. $[\alpha]_D^{25.6}$ −67.74 (*c* 0.124, DMSO); FAB-MS *m/z* 454 $[M+H]^+$. Anal. Calcd for $C_{16}H_{16}IN_5O_3$: C, 42.40; H, 3.56; N, 15.45. Found: C, 42.44; H, 3.59; N, 15.22.

4.3.6. (2*R*,3*R*,4*R*)-2-(2-Chloro-6-(methylamino)-9*H*-purin-9-yl)-furan-3,4-diol (5a)

85% Yield; white solid; mp 247 °C (dec); UV (MeOH) λ_{max} 269.0 nm; ^{1}H NMR (DMSO-d_6) δ 8.38 (s, 1H), 8.25 (q, 1H, *J* = 4.4 Hz), 5.79 (d, 1H, *J* = 6.8 Hz), 5.48 (d, 1H, *J* = 6.4 Hz), 5.22 (d, 1H, *J* = 4.0 Hz), 4.73–4.69 (m, 1H), 4.30 (dd, 1 H, *J* = 3.6, 9.2 Hz), 4.25–4.23 (m, 1H), 3.79 (dd, 1H, *J* = 1.6, 9.2 Hz), 2.92 (d, 3H, *J* = 4.4 Hz); ^{13}C NMR (DMSO-d_6) δ 155.5, 153.3, 149.4, 140.2, 118.8, 87.4, 74.4, 73.6, 70.2, 27.1; $[\alpha]_D^{23.5}$ −40.77 (*c* 0.13, DMSO); FAB-MS *m/z* 286 $[M+H]^+$. Anal. Calcd for $C_{10}H_{12}ClN_5O_3$: C, 42.04; H, 4.23; N, 24.51. Found: C, 42.38; H, 4.14; N, 24.27.

4.3.7. (2*R*,3*R*,4*R*)-2-(2-Chloro-6-(3-fluorobenzylamino)-9*H*-purin-9-yl)-tetrahydrofuran-3,4-diol (5b)

83% Yield; white solid; mp 187.0–187.9 °C; UV (MeOH) λ_{max} 271.0 nm; ^{1}H NMR (DMSO-d_6) δ 8.92 (t, 1H, *J* = 6.0 Hz), 8.43 (s, 1H), 7.39–7.33 (m, 1H), 7.18–7.13 (m, 2 H), 7.06 (dt, 1H, *J* = 2.0, 8.4 Hz), 5.81 (d, 1H, *J* = 6.8 Hz), 5.47 (d, 1H, *J* = 6.0 Hz), 5.22 (d, 1H, *J* = 4.0 Hz), 4.74–4.64 (m, 3 H), 4.41 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.25 (br s, 1H), 3.80 (dd, 1H, *J* = 1.2, 9.6 Hz); ^{13}C NMR (DMSO-d_6) δ 163.4, 163.9, 154.9, 153.1, 149.9, 142.2 (d, *J* = 26.8 Hz), 140.7, 130.3 (d, *J* = 34.8 Hz), 123.2, 118.8, 113.8 (dd, *J* = 82.4, 122.0 Hz), 87.5, 74.4, 73.7, 70.2, 42.7; $[\alpha]^{23.2}{}_D$ -73.40 (*c* 0.094, DMSO); FAB-MS *m/z* 380 $[M+H]^+$. Anal. Calcd for $C_{16}H_{15}ClFN_5O_3$: C, 50.60; H, 3.98; N, 18.44. Found: C, 50.30; H, 4.01; N, 18.04.

4.3.8. (2*R*,3*R*,4*R*)-2-(2-Chloro-6-(3-chlorobenzylamino)-9*H*-purin-9-yl)-tetrahydrofuran-3,4-diol (5c)

76% Yield; white solid; mp 196.4–197.0 °C; UV (MeOH) λ_{max} 271.5 nm; ^{1}H NMR (DMSO-d_6) δ 8.92 (t, 1H, *J* = 6.0 Hz), 8.43 (s, 1H), 7.39 (s, 1H), 7.37–7.28 (m, 3H), 5.80 (d, 1 H, *J* = 6.8 Hz), 5.47 (d, 1H, *J* = 6.4 Hz), 5.22 (d, 1H, *J* = 4.0 Hz), 4.73–4.64 (m, 3H), 4.32 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.24 (br s, 1H), 3.79 (dd, 1H, *J* = 1.6, 9.2 Hz); ^{13}C NMR (DMSO-d_6) δ 154.8, 153.1, 149.9, 141.8, 140.7, 132.9, 130.2, 127.1, 126.8, 125.9, 118.7, 87.5, 74.4, 73.7, 70.2, 42.7; $[\alpha]_D^{22.9}$ −78.19 (*c* 0.133, DMSO); FAB-MS *m/z* 396 $[M]^+$. Anal. Calcd for $C_{16}H_{15}Cl_2N_5O_3$: C, 48.50; H, 3.82; N, 17.68. Found: C, 48.56; H, 3.89; N, 17.12.

4.3.9. (2*R*,3*R*,4*R*)-2-(2-Chloro-6-(3-bromobenzylamino)-9*H*-purin-9-yl)-tetrahydrofuran-3,4-diol (5d)

81% Yield; white solid; mp 181.5–181.7 °C; UV (MeOH) λ_{max} 274.5 nm; ^{1}H NMR (DMSO-d_6) δ 8.92 (t, 1H, *J* = 6.0 Hz), 8.43 (s, 1H), 7.55 (s, 1H), 7.44 (d, 1H, *J* = 8.0 Hz), 7.35–7.33 (m, 1H), 7.30–7.26 (m, 1H), 5.81 (d, 1H, *J* = 6.4 Hz), 5.47 (d, 1H, *J* = 6.4 Hz), 5.22 (d, 1H, *J* = 4.0 Hz), 4.69–4.66 (m, 1H), 4.62 (s, 2H), 4.32 (dd, 1H, *J* = 3.6, 9.2 Hz), 4.25 (br s, 1H), 3.80 (dd, 1H, *J* = 1.6, 9.2 Hz); ^{13}C NMR (DMSO-d_6) δ 154.9, 153.2, 149.9, 142.1, 140.7, 130.6, 130.1, 129.8, 126.4, 121.6, 118.8, 87.5, 74.4, 73.7, 70.2, 42.6; $[\alpha]_D^{25}$ −62.75 (*c* 0.10, DMSO); FAB-MS *m/z* 440 $[M+H]^+$. Anal. Calcd for $C_{16}H_{15}ClBrN_5O_3$: C, 43.61; H, 3.43; N, 15.89. Found: C, 43.92; H, 3.44; N, 16.05.

4.3.10. (2*R*,3*R*,4*R*)-2-(2-Chloro-6-(3-iodobenzylamino)-9*H*-purin-9-yl)-tetrahydrofuran-3,4-diol (5e)

78% Yield; white solid; mp 195.5–195.8 °C; UV (MeOH) λ_{max} 274.0 nm; ^{1}H NMR (DMSO-d_6) δ 8.91 (t, 1H, *J* = 6.4 Hz), 8.44 (s, 1H), 7.75 (s, 1H), 7.61 (d, 1H, *J* = 8.0 Hz), 7.36 (d, 1H, *J* = 7.6 Hz), 7.13 (t, 1H, *J* = 4.0 Hz), 5.81 (d, 1H, *J* = 6.8 Hz), 5.47 (d, 1H, *J* = 6.8 Hz), 5.23 (d, 1H, *J* = 4.0 Hz), 4.72 (dd, 1H, *J* = 6.4, 10.8 Hz), 4.61 (d, 1H, *J* = 6.0 Hz), 4.34 (dd, 1H, *J* = 3.6, 9.2 Hz), 3.81 (dd, 1H, *J* = 1.2, 9.2 Hz); ^{13}C NMR (DMSO-d_6) δ 154.8, 153.2, 149.9, 141.9, 140.7, 136.0, 135.6, 130.6, 126.8, 118.8, 94.8, 87.5, 74.4, 73.7, 70.2, 42.5; $[\alpha]_D^{25}$ −68.07 (*c* 0.12, DMSO); FAB-MS *m/z* 488 $[M+H]^+$. Anal. Calcd for $C_{16}H_{15}ClIN_5O_3$: C, 39.41; H, 3.10; N, 14.36. Found: C, 39.66; H, 3.08; N, 14.53.

4.4. Pharmacological methods

[^{125}I]N^6-(4-Amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide (I-AB-MECA; 2200 Ci/mmol), and [^{3}H]CGS21680 (2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamido-adenosine) (40.5 Ci/mmol) were purchased from Perkin–Elmer Life and Analytical Science (Boston, MA). [^{3}H]NECA (18.64 Ci/mmol) was a custom synthesis product (Perkin Elmer). Test compounds were prepared as 5 mM stock solutions in DMSO and stored frozen.

Cell Culture and Membrane Preparation: CHO (Chinese hamster ovary) cells stably expressing the recombinant human A_1 and A_3, and HEK-293 (Human Embryonic Kidney) cells stably expressing human A_{2A} were cultured in Dulbecco's modified Eagle medium (DMEM) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 μmol/mL

glutamine. In addition, 800 μg/mL geneticin was added to the A_{2A} media, while 800 μg/mL hygromycin was added to the A_1 and A_3 media. After harvesting, cells were homogenized and suspended in PBS. Cells were then centrifuged at 240*g* for 5 min, and the pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The suspension was homogenized and was then ultra-centrifuged at 14,330*g* for 30 min at 4 °C. The resultant pellets were resuspended in Tris buffer, incubated with adenosine deaminase (3 units/mL) for 30 min at 37 °C. The suspension was homogenized with an electric homogenizer for 10 s, pipet into 1 mL vials and then stored at −80 °C until the binding experiments. The protein concentration was measured using the BCA Protein Assay Kit from Pierce Biotechnology, Inc. (Rockford, 1000 mL).[19]

Binding assays: Into each tube in the binding assay was added 50 μL of increasing concentrations of the test ligand in Tris–HCl buffer (50 mM, pH 7.5) containing 10 mM $MgCl_2$, 50 μL of the appropriate agonist radioligand, and finally 100 μL of membrane suspension. For A_1 receptors (22 μg of protein/tube) the radioligand used was [^{3}H]NECA (3.5 nM). For A_{2A} receptors (20 μg/tube) the radioligand used was [^{3}H]CGS21680 (10 nM). For A_3 receptors (21 μg/tube) the radioligand used was ^{125}I-AB-MECA (0.34 nM). Nonspecific binding was determined using a final concentration of 10 μM 5′-*N*-ethylcarboxamidoadenosine (NECA) diluted with the buffer. The mixtures were incubated at 25 °C for 60 min in a shaking water bath. Binding reactions were terminated by filtration through Brandel GF/B filters under a reduced pressure using a M-24 cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with 3 mL of 50 mM ice-cold Tris–HCl buffer (pH 7.5). Filters for A_1 and A_{2A} AR binding were placed in scintillation vials containing 5 mL of Hydrofluor scintillation buffer and counted using a Perkin Elmer Liquid Scintillation Analyzer (Tri-Carb 2810TR). Filters for A_3 AR binding were counted using a Packard Cobra II γ-counter. The K_i values were determined using GraphPad Prism for all assays.

Cyclic AMP accumulation assay: Intracellular cyclic AMP levels were measured with a competitive protein binding method.[20] CHO cells that expressed the recombinant hA_3AR were harvested by trypsinization. After centrifugation and resuspended in medium, cells were planted in 24-well plates in 1.0 mL medium. After 24 h, the medium was removed and cells were washed three times with 1 mL DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with the agonist NECA and/or test compound (e.g., **5d**) in the presence of rolipram (10 μM) and adenosine deaminase (3 units/mL). After 30 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 supernatant, 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 cyclic AMP production, a cyclic AMP kit (Sigma) was used.

4.4.1. Statistical analysis

Binding and functional parameters were calculated using Prism 4.0 software (GraphPAD, San Diego, CA, USA). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng–Prusoff equation.[21] Data were expressed as mean ± standard error.

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