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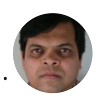

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Structure-Activity Relationships of Truncated D- and L-4′-Thioadenosine Derivatives as Species-Independent A$_3$ Adenosine Receptor Antagonists[1]

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Abstract

Novel D- and L-4′-thioadenosine derivatives lacking the 4′-hydroxymethyl moiety were synthesized, starting from D-mannose and D-gulonic γ-lactone, respectively, as potent and selective species-independent A$_3$ adenosine receptor (AR) antagonists. Among the novel 4′-truncated 2-H nucleosides tested, a N^6-(3-chlorobenzyl) derivative **7c** was the most potent at the human A$_3$ AR (K_i = 1.5 nM), but a N^6-(3-bromobenzyl) derivative **7d** showed the optimal species-independent binding affinity.

Introduction

On the basis of the structure of adenosine, an endogenous cell signaling molecule that binds to four specific subtypes (A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$) of adenosine receptors (ARs)[2], a number of nucleoside analogues have been synthesized and evaluated as adenosine receptor ligands.[3] Among these, IB-MECA[4] **1** and Cl-IB-MECA[5] **2** were discovered as potent and selective A$_3$ AR full agonists (K_i = 1.0 and 1.4 nM, respectively, at the human A$_3$ AR) and are being developed as antiinflammatory and anticancer agents. Based on the bioisosteric rationale, we reported the 4′-thionucleosides **3** and **4**, derivatives of compounds **1** and **2**, to also be highly potent and selective A$_3$ AR full agonists.[6] Compound **4** exhibited potent in vitro and in vivo antitumor activities,[7] resulting from the inhibition of Wnt signaling pathway (Chart 1).

However, because of the structural similarity to adenosine, most of these adenosine analogues were found to be A$_3$ AR agonists. Only a few nucleoside derivatives[8] have been reported to be A$_3$ AR antagonists, but these generally exhibit weaker and less selective human A$_3$ AR antagonism than nonpurine heterocyclic A$_3$ AR antagonists. Although these nonpurine heterocyclic A$_3$ AR antagonists[9] bound with high affinity at the human A$_3$ AR, they were weak or ineffective at the rat A$_3$ AR, indicating that they were not ideal for

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Supporting Information Available: Elemental analyses data for all unknown compounds and pharmacological methods. This material is available free of charge via the Internet at http://pubs.acs.org.

evaluation in small animal models and thus as drug candidates.[10] Therefore, it is highly desirable to develop A_3 AR antagonists that are independent of species. The fact[10] that nucleoside analogues show minimal species-dependence at the A_3 AR prompted us to search for novel potent and selective A_3 AR antagonists, derived from nucleoside templates.

A molecular modeling study of the A_3 AR indicated that hydrogen of the 5′-uronamides of compounds **1–4** serves as a hydrogen-bonding donor in the binding site of the A_3 AR, which is essential for the induced-fit required for the activation of the A_3 AR.[11] On the basis of these findings, we appended extra alkyl groups on the 5′-uronamides of compounds **1**–**4** to remove hydrogen-bonding ability at this site, thus precluding the conformational change required for activation of the A_3 AR. As expected, these 5′-*N,N*-dialkyl amide derivatives[12] displayed potent and selective A_3 AR antagonism, in which steric factors were crucial for affinity in binding to the A_3 AR. Within this class, 5′-*N,N*-dimethylamide derivative **5** was discovered to be the most potent full A_3 AR antagonist. Encouraged with these results, we designed and synthesized another new template to remove the 5′-uronamide group of compound **4** in order to minimize the steric repulsion at the binding region of the 5′-uronamide group and to abolish its hydrogen-bonding ability. This led to the discovery of compounds **6a – 6e** as highly potent and selective human A_3 AR antagonists, which was more potent and selective than compound **5**.[1] Among these, compound **6e** also showed species-independent binding affinity, as indicated by its high affinity at the rat A_3 AR.[1] On the basis of these results, it is of interest to systematically establish structure-activity relationships by modifying the C2 and N^6 positions of the purine moiety of compounds **6a – 6e** in order to develop novel A_3 AR antagonists. In this article, we extend previous observations that truncated D-4′-thioadenosine derivatives **6a – 6e** containing 2-Cl substitution are selective A_3 AR antagonists.[1] A series of 2-H analogues were prepared and characterized biologically. The binding affinities at the human A_3 AR were compared with those at the rat A_3 AR to develop species-independent A_3 AR antagonists. We also compared the binding affinities of D-4′-thionucleosides with those of the corresponding L-4′-thionucleosides to determine a stereochemical preference. Thus, here we report a full account of truncated D- and L-4′-thioadenosine derivatives **7** as highly potent and species-independent A_3 AR antagonists.

Results and discussion

The D-glycosyl donor **8** was subjected to the Lewis acid-catalyzed condensation for the synthesis of the final D-4′-thionucleosides lacking a 4′-hydroxymethyl group, as shown in Scheme 1. The D-glycosyl donor **8** was condensed with 6-chloropurine in the presence of TMSOTf as a Lewis acid to give β-6-chloropurine derivative **9** as a single diastereomer. The anomeric configuration of compound **9** was easily confirmed by ^{1}H NOE experiment between 3′-H and H-8. Removal of the isopropylidene group of **9** was achieved with 2 *N* HCl in THF to give **10**. The 2-H intermediate **10** was converted to the novel N^6-methyl derivative **7a** and N^6-3-halobenzyl derivatives **7b – 7e** by treating with methylamine and 3-halobenzylamines, respectively. This route parallels the synthesis of the 2-chloro-N^6-susbtituted-4′-thiopurine analogues **6a – 6e** that we reported earlier[1].

In order to determine whether a stereochemical preference exists in the binding to the A_3 AR, the L-enantiomers, **7f** and **7g** of D-4′-thionucleosides were synthesized as illustrated in Scheme 2. D-Gulonic γ-lactone was converted to the diol **11** according to our previously published procedure.[13] One-step conversion of the diol **11** into the L-glycosyl donor **12** was achieved using excess $Pb(OAc)_4$, indicating that oxidative diol cleavage, oxidation of the resulting aldehyde to the acid, and oxidative decarboxylation occurred simultaneously.[13] Using the same synthetic strategy shown in Scheme 1, L-4′-thioadenosine derivatives **7f** and **7g** were synthesized from L-glycosyl donor **12**.

Initial binding experiments were performed using adherent mammalian cells stably transfected with cDNA encoding the appropriate human ARs (A_1 AR and A_3 AR in CHO cells and A_{2A} AR in HEK-293 cells).[14,15] Binding was carried out using 1 nM [^{3}H]CCPA, 10 nM [^{3}H]CGS-21680, or 0.5 nM [^{125}I]I-AB-MECA as radioligands for A_1, A_{2A}, and A_3 ARs, respectively. As shown in Table 1, most of the synthesized compounds exhibited high binding affinity at the human A_3 AR with low binding affinities at the human A_1 AR and human A_{2A} AR. Among the novel 2-H truncated adenosine derivatives tested, compound **7c** (R = 3-chlorobenzyl) showed the highest binding affinity (K_i = 1.5 ± 0.4 nM) at the human A_3 AR with high selectivities versus the A_1 AR (570-fold selective) and the A_{2A} AR (290-fold selective). Compound **7e** (R = 3-iodobenzyl) was also very potent (K_i = 2.5 ± 1.0 nM), with selectivities of 210- and 92-fold versus the A_1 and A_{2A} AR, respectively. *N*^{6}-Substituted adenosine derivatives **7a** – **7e** without a 2-chloro substituent showed a very similar pattern to the corresponding 2-chloro derivatives **6a** – **6e** in the binding affinity at the human A_3 AR but showed less selectivity versus the other subtypes of ARs. In the 3-halobenzyl series, the order of binding affinity for 2-H analogues was as follows: Cl > I > Br > F, indicating that the size of halogen alone does not determine the binding affinity at the human A_3 AR. It is interesting to note that 2-H derivatives are less lipophilic than the corresponding 2-Cl derivatives, conferring more water solubility on the molecules for further biological evaluation. For example, the cLogP values of corresponding structures **6c** and **7c** are 1.84 and 1.12, respectively. In order to determine a stereochemical preference, the binding affinities of D-series were compared with those of L-series. As shown in Table 1, L-type nucleosides, **7f** and **7g** were totally devoid of binding affinities at all subtypes of ARs, indicating that the D-series induced optimal interaction with all subtypes of ARs.

In order to determine if all final nucleosides show species-independent binding affinity at the A_3 AR, their binding affinity at the rat A_3 AR expressed in CHO cells was also measured (Table 1). As expected, most of compounds exhibited species-independent binding affinity, indicating that they are suitable for evaluation in small animal models or for further drug development Among the 2-H nucleoside analogues tested, a *N*^{6}-(3-bromobenzyl) derivative **7d** exhibited the most potent binding affinity at the rat A_3 AR (K_i = 6.3 ± 1.3 nM) followed by *N*^{6}-(3-chlorobenzyl) derivative **7c**, *N*^{6}-(3-iodobenzyl) derivative **7e**, and *N*^{6}-(3-fluorobenzyl) derivative **7b**. *N*^{6}-Methyl derivative **7a** was totally devoid of A_3AR binding affinity in this species. In the 2-Cl-*N*^{6}-substituted adenosine series, the binding affinity was in the following order: I > Br ≈ Cl > F > Me. The 2-Cl derivatives generally showed more potent and species-indepndent binding affinity than the corresponding 2-H analogues. Compound **6e** exhibited the highest binding affinity at the rat A_3 AR (K_i = 3.89 ± 1.15 nM) among all compounds tested and was inactive as agonist or antagonist in a cyclic AMP functional assay[16,17] at the hA_{2B} AR. It is interesting to note that *N*^{6}-methyl derivatives **6a** and **7a** showing high binding affinities (K_i = 3.69 ± 0.25 nM and 4.8 ± 1.7 nM, respectively) at the human A_3 AR lost their binding affinities at the rat A_3 AR, indicating that there must be a larger *N*^{6} substituent for species-independent binding affinity at the A_3 AR.[18,19]

In a functional assay, percent inhibition at 10 μM forskolin-stimulated cyclic AMP production in CHO cells expressing the human A_3 AR was measured as a mean percentage of the response of the full agonist **3** (n = 1 – 3). None of the analogues **6** and **7** activated the human A_3 AR (> 10% of full agonist effect) by this criterion.

Conclusion

We have established structure-activity relationships of novel truncated D- and L-4′-thionucleoside analogues as potent species-independent A_3 AR antagonists. The glycosyl donors **8** and **12** were efficiently synthesized from D-mannose and D-gulonic γ-lactone, respectively, using ring closure of dimesylate with sodium sulfide and one step conversion of the diol into the acetate with lead tetraacetate as key steps. Among the novel 4′-truncated 2-H nucleosides tested, D-*N*^{6}-(3-halobenzyl) derivatives **7b** – **7e** exhibited high binding affinities at the human A_3 AR as well as at the rat A_3 AR with very low binding affinities at the human A_1 and A_{2A} ARs and a *N*^{6}-(3-chlorobenzyl) derivative **7c** was the most potent at the human A_3 AR, but at the rat A_3 AR 3-bromobenzyl derivative **7d** was the most potent. Among both 2-H and 2-Cl analogues tested, 2-chloro-*N*^{6}-(3-iodobenzyl) derivative **6e** was found to exhibit the most potent binding affinity at the rat A_3 AR. Since this class of potent nucleoside human A_3 AR antagonists showed species-independence in interaction at this AR subtype, they are regarded as good candidates for efficacy evaluation in small animal models and for further drug development.

Experimental Section

General methods

Melting points are uncorrected. ^{1}H 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. cLogP values were calculated using ChemDrawUltra, version 11.0 (CambridgeSoft).

Synthesis

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 (3.91 g, 25.3 mmol), ammonium sulfate (84 mg, 0.63 mmol) and HMDS (50 mL) were refluxed under inert and dry conditions overnight. The solution was evaporated under high vacuum. The resulting solid was re-dissolved in 1,2-dichloroethane (20 mL) cooled in ice. The solution of **8**[1] (2.76 g, 12.6 mmol) in 1,2-dichloroethane (20 mL) was added to this mixture dropwise. TMSOTf (4.6 mL, 25.3 mmol) was added dropwise to the mixture. 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 with anhydrous $MgSO_4$ and evaporated under reduced pressure. The yellowish syrup was subjected to a flash silica gel column chromatography (CH_2Cl_2:MeOH = 50:1) to give **9** (3.59 g, 90%) as a foam: $[\alpha]^{23.6}{}_D$-157.63 (*c* 0.144, DMSO); FAB-MS *m/z* 313 $[M+H]^+$; UV (MeOH) λ_{max} 265.0 nm; ^{1}H NMR ($CDCl_3$) *δ* 8.67 (s, 1 H), 8.23 (s, 1 H), 5.88 (s, 1 H), 5.25-5.19 (m, 1 H), 3.69 (dd, 1 H, *J* = 4.0, 13.2 Hz), 3.18 (d, 1 H, *J* = 12.8 Hz), 1.51 (s, 3 H), 1.28 (s, 3 H). ^{13}C NMR ($CDCl_3$) δ 152.0, 151.4, 151.1, 144.3, 132.6, 111.9, 89.6, 84.3, 70.3, 40.8, 26.4, 24.6. Anal. ($C_{12}H_{13}ClN_4O_2S$) C, H, N, S.

(2*R*,3R,4*S*)-2-(6-Chloro-9*H*-purin-9-yl)-tetrahydrothiophene-3,4-diol (10): 2 *N* Hydrochloric acid (12 mL) was added to a solution of **9** (2.59 g, 8.28 mmol) in THF (20 mL), and the mixture was stirred at room temperature overnight. The mixture was neutralized with 1 *N* NaOH solution, and then the volatiles were carefully evaporated under reduced pressure. The mixture was subjected to a flash silica gel column chromatography (CH_2Cl_2:MeOH = 20:1) to give **10** (1.79 g, 79%) as a white solid: $[\alpha]^{23.5}{}_D$-109.14 (*c* 0.164, DMSO); FAB-MS *m/z* 273 $[M+H]^+$; mp 192.3–192.8 °C; UV (MeOH) λ_{max} 264.5 nm; ^{1}H NMR (DMSO-d_6) *δ* 9.02 (s, 1 H), 8.81 (s, 1 H), 6.02 (d, 1 H, *J* = 7.2 Hz), 5.62 (d, 1 H, *J* = 6.0 Hz, D_2O exchangeable), 5.43 (d, 1 H, *J* = 4.1 Hz, D_2O exchangeable), 4.74-4.70 (m, 1 H), 4.40-4.36 (m, 1 H), 3.47 (dd, 1 H, *J* = 4.0, 11.2 Hz), 2.83 (dd, 1 H, *J* = 2.8, 11.2 Hz). ^{13}C NMR (DMSO-d_6) 152.1, 151.6, 149.2, 146.6, 131.3, 78.6, 72.1, 62.4, 34.7. Anal. ($C_9H_9ClN_4O_2S$) C, H, N, S.

General procedure for the synthesis of 7a – 7e—To a solution of **10** in EtOH (5 mL) was added appropriate amine (1.5 equiv) at room temperature and the mixture was stirred at rt for a time period ranging from 2 h to 3 d and evaporated. The residue was purified by a flash silica gel column chromatography (CH_2Cl_2:MeOH = 20:1) to give **7a – 7e**.

(2*R*,3*R*,4*S*)-Tetrahydro-2-(6-(methylamino)-9*H*-purin-9-yl)thiophene-3,4-diol (7a): 83% yield; $[\alpha]^{22.8}_D$-175.60 (*c* 0.123, DMSO); FAB-MS *m/z* 268 $[M+H]^+$; mp 223.9–224.8 °C; UV (MeOH) λ_{max} 266.0 nm; ^1H NMR (DMSO-d_6) δ 8.40 (s, 1 H), 8.23 (s, 1 H), 7.72 (br s, 1 H, D_2O exchangeable), 5.89 (d, 1 H, *J* = 7.2 Hz), 5.51 (d, 1 H, *J* = 6.4 Hz, D_2O exchangeable), 5. 32 (d, 1 H, *J* = 4.4 Hz, D_2O exchangeable), 4.70-4.64 (m, 1 H), 4.37-4.33 (m, 1 H), 3.40 (dd, 1 H, *J* = 4.0, 10.8 Hz), 2.95 (s, 3 H), 2.79 (dd, 1 H, *J* = 3.2, 10.8 Hz). ^{13}C NMR (DMSO-d_6) δ 154.9, 152.5, 148.8, 139.5, 119.5, 78.3, 72.2, 61.5, 34.6, 27.0. Anal. ($C_{10}H_{13}N_5O_2S$) C, H, N, S.

(2*R*,3*R*,4*S*)-2-(6-(3-Fluorobenzylamino)-9*H*-purin-9-yl)tetrahydrothiophene-3,4-diol (7b): 82% yield; $[\alpha]^{23.7}_D$-141.22 (*c* 0.114, DMSO); FAB-MS *m/z* 362 $[M+H]^+$; mp 180.5–180.7 °C; UV (MeOH) λ_{max} 273.5 nm; ^1H NMR (DMSO-d_6) δ 8.45 (s, 1 H), 8.43 (br s, 1 H, D_2O exchangeable), 8.21 (s, 1 H), 7.36-7.30 (m, 1 H), 7.18-7.11 (m, 2 H), 7.03 (dt, 1 H, *J* = 2.4, 8.4 Hz), 5.90 (d, 1 H, *J* = 7.2 Hz), 5.53 (d, 1 H, *J* = 6.4 Hz, D_2O exchangeable), 5.35 (d, 1 H, *J* = 4.0 Hz, D_2O exchangeable), 4.70-4.66 (m, 2 H), 4.36-4.33 (m, 1 H), 3.41 (dd, 1 H, *J* = 4.0, 10.8 Hz), 3.17 (d, 1 H, *J* = 5.2 Hz), 2.79 (dd, 1 H, *J* = 2.8, 10.8 Hz). ^{13}C NMR (DMSO-d_6) δ 163.4, 160.9, 152.4, 143.2, 140.0, 130.2, 130.1, 123.1, 123.1, 113.8, 113.6, 113.4, 113.2, 78.3, 72.2, 61.6, 48.6, 34.4. Anal. ($C_{16}H_{16}FN_5O_2S$) C, H, N, S.

(2*R*,3*R*,4*S*)-2-(6-(3-Chlorobenzylamino)-9*H*-purin-9-yl)-tetrahydrothiophene-3,4-diol (7c): 85% yield; $[\alpha]^{23.9}_D$-162.5 (*c* 0.096, DMSO); FAB-MS *m/z* 378 $[M+H]^+$; mp 165.0–165.3 °C; UV (MeOH) λ_{max} 274.5 nm; ^1H NMR (DMSO-d_6) δ 8.46 (s, 1 H), 8.44 (br s, 1 H, D_2O exchangeable), 8.22 (s, 1 H), 7.39-7.24 (m, 4 H), 5.90 (d, 1 H, *J* = 10.4 Hz), 5.53 (d, 1 H, *J* = 6.4 Hz, D_2O exchangeable), 5.35 (d, 1 H, *J* = 4.0 Hz, D_2O exchangeable), 4.71-4.67 (m, 2 H), 4.38-4.33 (m, 1 H), 3.47-3.31 (m, 2 H), 2.80 (dd, 1 H, *J* = 3.2, 10.8 Hz). ^{13}C NMR (DMSO-d_6) δ 154.3, 152.4, 142.8, 140.0, 132.8, 130.1, 126.9, 126.6, 125.8, 78.3, 72.2, 61.6, 56.0, 34.4. Anal. ($C_{16}H_{16}ClN_5O_2S$) C, H, N, S.

(2*R*,3*R*,4*S*)-2-(6-(3-Bromobenzylamino)-9*H*-purin-9-yl)-tetrahydrothiophene-3,4-diol (7d): 71% yield; $[\alpha]^{23.7}_D$-100.71 (*c* 0.139, DMSO); FAB-MS *m/z* 422 $[M]^+$; mp 183.0–184.0 °C; UV (MeOH) λ_{max} 270.0 nm; ^1H NMR (DMSO-d_6) δ 8.46 (s, 1 H), 8.43 (br s, 1 H, D_2O exchangeable), 8.21 (s, 1 H), 7.53 (s, 1H) 7.42-7.24 (m, 3 H), 5.90 (d, 1 H, *J* = 7.2 Hz), 5.53 (d, 1 H, *J* = 6.4 Hz, D_2O exchangeable), 5.35 (d, 1 H, *J* = 4.0 Hz, D_2O exchangeable), 4.71-4.66 (m, 2 H), 4.37-4.34 (m, 1 H), 3.41 (dd, 1 H, *J* = 4.0, 10.8 Hz), 3.06 (q, 1 H, *J* = 7.2 Hz). 2.79 (dd, 1 H, *J* = 2.8, 10.8 Hz). ^{13}C NMR (DMSO-d_6) δ 154.2, 152.4, 143.0, 140.0, 130.4, 129.8, 129.4, 126.2, 121.5, 78.3, 72.2, 61.6, 45.5, 34.5. Anal. ($C_{16}H_{16}BrN_5O_2S$) C, H, N, S.

(2*R*,3*R*,4*S*)-2-(6-(3-Iodobenzylamino)-9*H*-purin-9-yl)-tetrahydrothiophene-3,4-diol (7e): 88% yield; $[\alpha]^{23.8}_D$-97.08 (*c* 0.137, DMSO); FAB-MS *m/z* 370 $[M+H]^+$; mp 198.8–199.8 °C; UV (MeOH) λ_{max} 271.5 nm; ^1H NMR (DMSO-d_6) δ 8.45 (s, 1 H), 8.43 (br s, 1 H, D_2O exchangeable), 8.21 (s, 1 H), 7.72 (s, 1 H), 7.56 (d, 1 H, *J* = 7.2 Hz), 7.35 (d, 1 H, *J* = 7.6 Hz), 7.10 (merged dd, 1 H, *J* = 7.6 Hz), 5.90 (d, 1 H, *J* = 7.2 Hz), 5.53 (d, 1 H, *J* = 6.4 Hz, D_2O exchangeable), 5. 35 (d, 1 H, *J* = 4.4 Hz, D_2O exchangeable), 4.71-4.66 (m, 2 H), 4.37-4.34 (m, 1 H), 3.41 (dd, 1 H, *J* = 2.8, 10.8 Hz), 3.15 (d, 1 H, *J* = 5.2 Hz), 2.79 (dd, 1 H, *J* = 2.8, 10.8 Hz). ^{13}C NMR (DMSO-d_6) δ 154.2, 152.4, 149.2, 142.9, 140.0, 137.0, 135.7, 135.3, 130.4, 126.6, 94.7, 78.3, 72.2, 61.6, 42.2, 34.4. Anal. ($C_{16}H_{16}IN_5O_2S$) C, H, N, S.

L-4-Thiosugar acetate **12** was synthesized from D-gulonic acid γ-lactone according to a similar procedure[1,13] used for the preparation of **8** (Scheme 1). Then L-4-thiosugar acetate **12** was converted to **13** according to a similar procedure used for the preparation of **9**. The final L-4′-thio nucleosides **7f** and **7g** were synthesized from **12** according to the described general procedure for the synthesis of **7a – 7e**.

The ^{1}H, ^{13}C NMR, UV, and mp data of L-series compounds were the same as for the D-series of compounds as described above, except that the specific optical rotations were in the opposite direction. Yields of the L-series compounds were comparable with those of the D-series of compounds.

Binding assays[1,6]

Human A_1 and A_{2A} ARs: For binding to human A_1 AR, [^{3}H]CCPA (1 nM) was incubated with membranes (40 μg/tube) from CHO cells stably expressing human A_1 ARs at 25 °C for 60 min in 50 mM Tris·HCl buffer (pH 7.4; $MgCl_2$, 10 mM) in a total assay volume of 200 μL. Nonspecific binding was determined using 10 μM of NECA. For human A_{2A} AR binding, membranes (20 μg/tube) from HEK-293 cells stably expressing human A_{2A} ARs were incubated with 15 nM [^{3}H]CGS21680 at 25 °C for 60 min in 200 μL 50 mM Tris·HCl, pH 7.4, containing 10 mM $MgCl_2$. NECA (10 μM) was used to define nonspecific binding. Reaction was terminated by filtration with GF/B filters.

Human and Rat A_3 ARs: For competitive binding assay, each tube contained 100 μL of membrane suspension (from CHO cells stably expressing the human or rat A_3 AR, 20 μg protein), 50 μL of [^{125}I]I-AB-MECA (0.5 nM), and 50 μL of increasing concentrations of the nucleoside derivative in Tris·HCl buffer (50 mM, pH 7.4) containing 10 mM $MgCl_2$. Nonspecific binding was determined using 10 μM of NECA in the buffer. 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, Gaithersburgh, MD, USA). Filters were washed three times with 9 mL ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter.

For binding at all three subtypes, K_i values are expressed as mean ± sem, n = 3–4 (outliers eliminated), and normalized against a non-specific binder, 5′-*N*-ethylcarboxamidoadenosine (NECA, 10 μM). Alternately, for weak binding a percent inhibition of specific radioligand binding at 10 μM, relative to inhibition by 10 μM NECA assigned as 100%, is given.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

AR	adenosine receptor
CCPA	2-chloro-N^6-cyclopentyladenosine

CHO	Chinese hamster ovary
IB-MECA	*N*^{6}-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine
Cl-IB-MECA	2-chloro-*N*^{6}-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine
I-AB-MECA	2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine
NECA	5′-*N*ethylcarboxamidoadenosine

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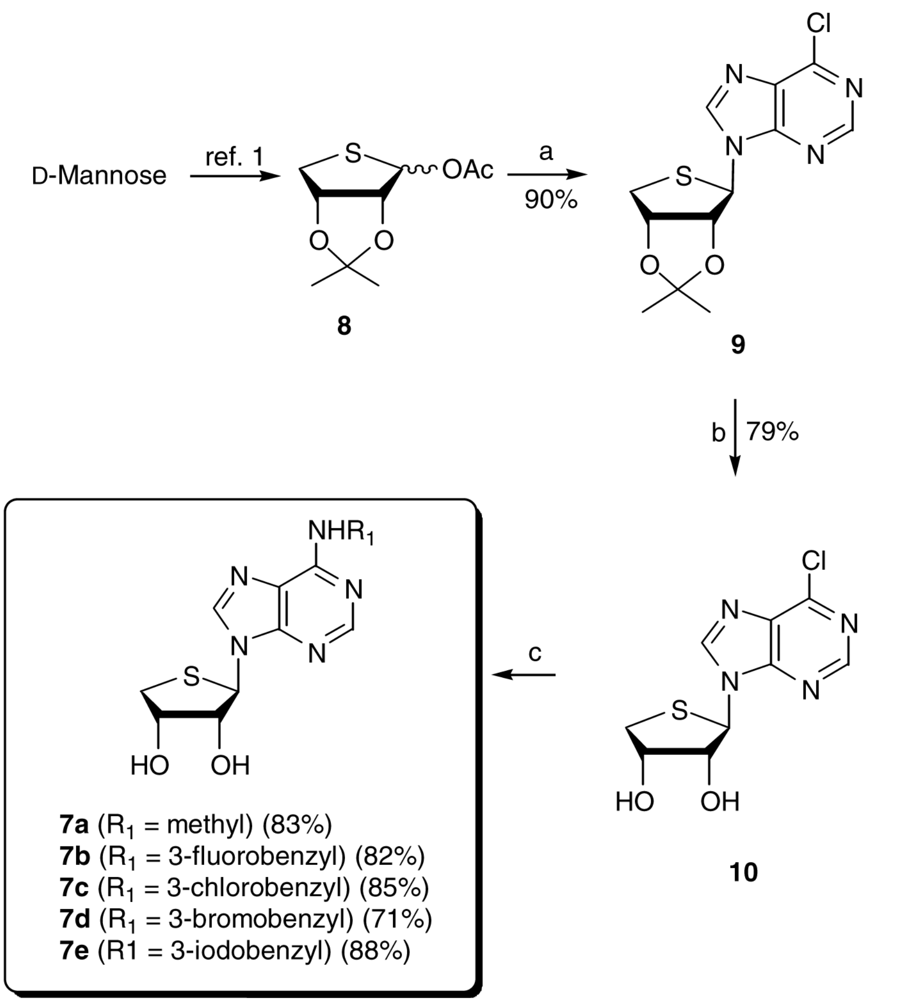
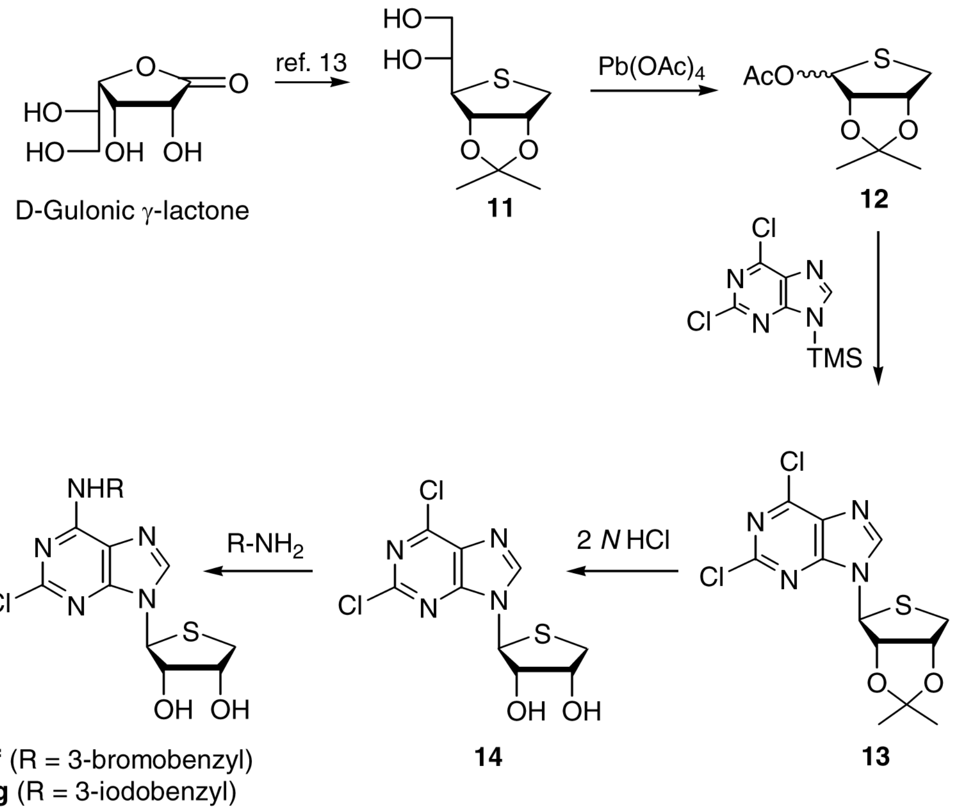


Reagents and conditions: a) 6-chloropurine, ammonium sulfate, HMDS, 170 ^{o}C, 15 h, then TMSOTf, DCE, rt to 80 ^{o}C, 3 h; b) 2 *N* HCl, THF, rt, 15 h; c) RNH_2, Et_3N, EtOH, rt, 1-3 d.

Scheme 1.

Synthesis of truncated D-4′-thioadenosine derivatives **7a**–**7e**.

Reagents and conditions: a) 6-chloropurine, ammonium sulfate, HMDS, 170 °C, 15 h, then TMSOTf, DCE, rt to 80 °C, 3 h; b) 2 *N* HCl, THF, rt, 15 h; c) RNH_2, Et_3N, EtOH, rt, 1–3 d.



Scheme 2.
Synthesis of truncated L-4′-thioadenosine derivatives **7f** and **7g**.

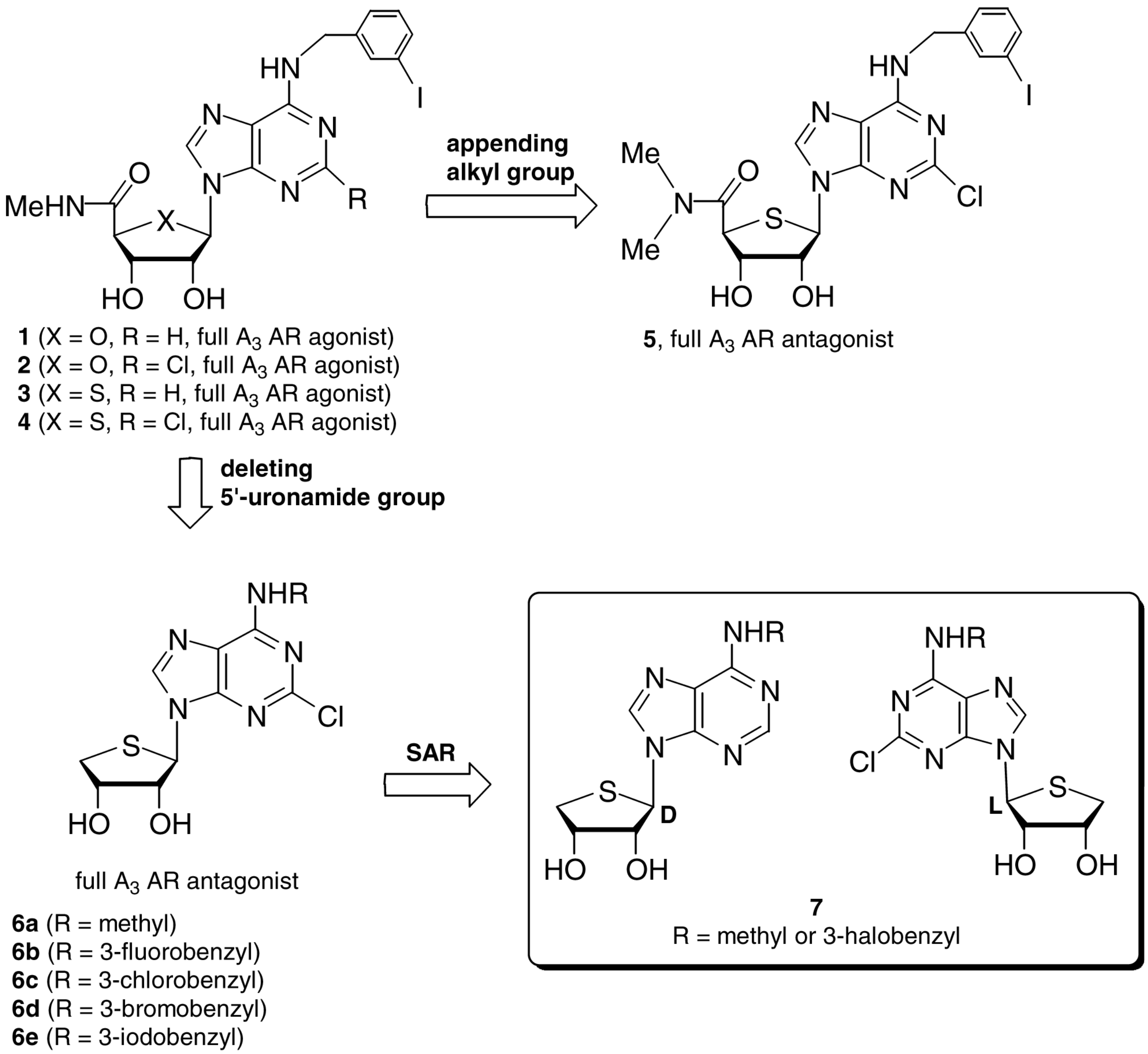
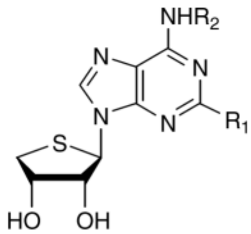


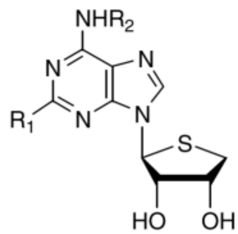
Chart 1.
The rationale for the design of the target nucleosides **7**.

Table 1

Binding affinities of known A_3 AR agonists, **1 – 4** and antagonist **5**, and truncated 4′-thioadenosine derivatives **6a** – **6e** and **7a** – **7g** at three subtypes of ARs.



6a-6e and **7a-7e**



7f and **7g**

Compound	Affinity, K_i, nM ± SEM (or % Inhibition at 10^{-5}M)[a,b]			
	hA_1	hA_{2A}	rA_3	hA_{3c}
1(IB-MECA)	51	2900	1.1	1.0
2 (Cl-IB-MECA)	222 ± 22	5360 ± 2470	0.33	1.4 ± 0.3
3 (thio-IB-MECA)	17.3	ND	1.86 ± 0.36	0.25 ± 0.06
4 (thio-Cl-IB-MECA)	193 ± 46	223 ± 36	0.82 ± 0.27	0.38 ± 0.07
5	6220 ± 640	> 10,000	321 ± 74	15.5 ± 3.1
6a (R_1 = Cl, R_2 = methyl)	55.4 ± 1.8	45.0 ± 1.4	658 ± 160	3.69 ± 0.25
6b (R_1 = Cl, R_2 = 3-fluorobenzyl)	(20%)	(48%)	36.2 ± 10.7	7.4 ± 1.3
6c (R_1 = Cl, R_2 = 3-chlorobenzyl)	(38%)	(18%)	6.2 ± 1.8	1.66 ± 0.90
6d (R_1 = Cl, R_2 = 3-bromobenzyl)	(34%)	(18%)	6.1 ± 1.8	8.99 ± 5.17
6e[d] (R_1 = Cl, R_2 = 3-iodobenzyl)	2490 ± 940	341 ± 75	3.89 ± 1.15	4.16 ± 0.50
7a (R_1 = H, R_2 = methyl)	1070 ± 180	(22 ± 5%)	(28 ± 10%)	4.8 ±1.7
7b (R_1 = H, R_2 = 3-fluorobenzyl)	1430 ± 420	1260 ± 330	98 ± 28	7.3 ± 0.6
7c (R_1 = H, R_2 = 3-chlorobenzyl)	860 ± 210	440 ± 110	17 ± 5	1.5 ± 0.4
7d (R_1 = H, R_2 = 3-bromobenzyl)	790 ± 190	420 ± 32	6.3 ± 1.3	6.8 ± 3.4
7e (R_1 = H, R_2 = 3-iodobenzyl)	530 ± 97	45.0 ± 1.4	658 ± 160	3.69 ± 0.25
7f (R_1 = Cl, R_2 = 3-bromobenzyl)	(6.1%)	(45.7%)	ND	(12.6%)
7g (R_1 = Cl, R_2 = 3-iodobenzyl)	(−8.0%)	(−0.95%)	ND	(18.4%)

ND: Not determined.

[a]All binding experiments were performed using adherent mammalian cells stably transfected with cDNA encoding the appropriate human AR (A_1 AR and A_3 AR in CHO cells and A_{2A} AR in HEK-293 cells) or the rat A_3 AR (CHO cells). Binding was carried out using 1 nM [^{3}H]CCPA, 10 nM [^{3}H]CGS-21680, 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). Data for compounds **6a** – **6e** at the human ARs and compound **6e** at the rat A_3 AR were reported in ref. 1.

[b]When a value expressed as a percentage refers to percent inhibition of specific radioligand binding at 10 μM, with nonspecific binding defined using 10 μM NECA.

[c]A functional assay was also carried out at this subtype: percent inhibition at 10 μM forskolin-stimulated cyclic AMP production in CHO cells expressing the human A_3 AR, as a mean percentage of the response of the full agonist **3** (n = 1 – 3). None of the analogues **5** – **7** activated the hA_3AR (>10% of full agonist effect) by this criterion.

[d]Compound **6e** at 10 μM displayed <10% of the full stimulation of cyclic AMP production, in comparison to 10 μM NECA; no inhibition of the stimulatory effect of 150 nM NECA in CHO cells expressing human A_{2B} AR (ref. 1).