Brief Article

Structure-Activity Relationships of Truncated d- and l-4#-Thioadenosine Derivatives as Species-Independent A3 Adenosine Receptor Antagonists(1)


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Structure–Activity Relationships of Truncated d- and L-4′-Thioadenosine Derivatives as Species-Independent A3 Adenosine Receptor Antagonists

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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-indepedent A3 adenosine receptor (AR) antagonists. Among the novel 4′-truncated 2-H nucleosides tested, a N6-(3-chlorobenzyl) derivative 7c was the most potent at the human A3 AR (Kᵢ = 1.5 nM), but a N6-(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 (A1, A2A, A2B, and A3) of adenosine receptors (ARs), a number of nucleoside analogues have been synthesized and evaluated as adenosine receptor ligands. Among these, IB-MECA1 and Cl-IB-MECA2 were discovered as potent and selective A3 AR full agonists (Kᵢ = 1.0 and 1.4 nM, respectively, at the human A3 AR) and are being developed as anti-inflammatory and anticancer agents. On the basis of the bioisosteric rationale, we reported the 4′-thionucleosides 3 and 4, derivatives of compounds 1 and 2, to also be highly potent and selective A3 AR full agonists. Compound 4 exhibited potent in vitro and in vivo antitumor activities, resulting from the inhibition of Wnt signaling pathway (Chart 1).

However, because of the structural similarity to adenosine, most of these nucleoside analogues were found to be A3 AR agonists. Only a few nucleoside derivatives have been reported to be A3 AR antagonists, but these generally exhibit weaker and less selective human A3 AR antagonism than nonpurine heterocyclic A3 AR antagonists. Although these nonpurine heterocyclic A3 AR antagonists bound with high affinity at the human A3 AR, they were weak or ineffective at the rat A3 AR, indicating that they were not ideal for evaluation in small animal models and thus as drug candidates. Therefore, it is highly desirable to develop A3 AR antagonists that are independent of species. The fact that nucleoside analogues show minimal species-dependence at the A3 AR prompted us to search for novel potent and selective A3 AR antagonists, derived from nucleoside templates.

A molecular modeling study of the A3 AR indicated that hydrogen of the 5′-uronamides of compounds 1–4 serves as a hydrogen-bonding donor in the binding site of the A3 AR, which is essential for the induced-fit required for the activation of the A3 AR. 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 A3 AR. As expected, these 5′-N,N-dialkyl amide derivatives displayed potent and selective A3 AR antagonism in which steric factors were crucial for affinity in binding to the A3 AR. Within this class, 5′-N,N-dimethylamide derivative 5 was discovered to be the most potent full A3 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–6c as highly potent and selective human A3 AR antagonists, which were more potent and selective than compound 5. Among these, compound 6c...
also showed species-independent binding affinity, as indicated by its high affinity at the rat A3 AR. On the basis of these results, it is of interest to systematically establish structure–activity relationships by modifying the C2 and N6 positions of the purine moiety of compounds 6a–6e in order to develop novel A3 AR antagonists. In this article, we extend previous observations that truncated d-4′-thioadenosine derivatives 6a–6e containing 2-Cl substitution are selective A3 AR antagonists. A series of 2-H analogues were prepared and characterized biologically. The binding affinities at the human A3 AR were compared with those at the rat A3 AR to develop species-independent A3 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 A3 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 the d-glycosyl donor 9 as a single diastereomer. The anomeric configuration of compound 9 was easily confirmed by 1H NOE experiment between 3′-H and 8-H. 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 N4-methyl derivative 7a and N4-3-halobenzyl derivatives 7b–7e by treating with methylamine and 3-halobenzylamines, respectively. This route parallels the synthesis of the 2-chloro-N4-substituted-4′-thiopurine analogues 6a–6e that we reported earlier.

Scheme 1. Synthesis of Truncated d-4′-Thioadenosine Derivatives 7a–7e

To determine whether a stereochemical preference exists in the binding to the A3 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. 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. 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 (A1 AR and A1 AR in CHO cells and A2AR AR in HEK-293 cells). Binding was carried out using 1 nM [3H]CCPA, 10 nM [3H]CGS-21680, or 0.5 nM [125I]I-AB-MECA as radioligands for A1, A2A, and A3 ARs, respectively. As shown in Table 1, most of the synthesized compounds exhibited high binding affinity at the human A3 AR with low binding affinities at the human A1 AR and human A2A AR. Among the novel 2-H truncated adenosine derivatives tested, compound 7c (R = 3-chlorobenzyl) showed the highest binding affinity (Ki = 1.5 ± 0.4 nM) at the human A3 AR with high selectivities versus the A1 AR (570-fold selective) and the A2A AR (290-fold selective). Compound 7e (R = 3-iodobenzyl) was also very potent (Ki = 2.5 ± 1.0 nM), with selectivities of 210- and 92-fold versus the A1 and A2A AR, respectively. N4-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 A3 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 A3 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. 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

Scheme 2. Synthesis of Truncated l-4′-Thioadenosine Derivatives 7f and 7g
Subtypes of ARs tested and was inactive as agonist or antagonist in a cyclic AMP response. In the 2-Cl-derivative, the full stimulation of cyclic AMP production, in comparison to 10 µM NECA. Among the 2-H nucleoside analogues tested, a compound exhibited the most potent binding affinity at the rat A3 AR, their binding affinity at the rat A3 AR was totally devoid of binding affinity in all subtypes of ARs, indicating that the d-series induced optimal interaction with all subtypes of ARs.

To determine if all final nucleosides show species-independent binding affinity at the A3 AR, their binding affinity at the rat A3 AR was expressed in CHO cells and A2AR in HEK-293 cells) or the rat A3 AR (CHO cells). Binding was carried out using 1 nM [ 3H]CCPA, 10 nM [3H]CGS-21680, or 0.5 nM [125I]I-AB-MECA as radioligands for A1, A2A, and A3 ARs, respectively. Values are expressed as mean ± SEM (or % inhibition at 10⁻⁵ M).

In a functional assay, percent inhibition at 10 µM forskolin-stimulated cyclic AMP production in CHO cells expressing the human A3 AR was measured as a mean percentage of the response of the full agonist (n = 1–3). None of the analogues 5–7 activated the hA3 AR (>10% of full agonist effect) by this criterion. Compound 6a 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 A3AR (ref 1).

### Table 1. Binding Affinities of Known A3 AR Agonists, 1–4 and Antagonist 5, and Truncated 4′-Thioadenosine Derivatives 6a–6e and 7a–7g at Three Subtypes of ARs

<table>
<thead>
<tr>
<th>compd</th>
<th>hA1</th>
<th>hA2A</th>
<th>rA3</th>
<th>hA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(IB-MECA)</td>
<td>51</td>
<td>2990</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>2 (Cl-IB-MECA)</td>
<td>222 ± 22</td>
<td>5360 ± 2470</td>
<td>0.33</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>3 (thio-IB-MECA)</td>
<td>17.3</td>
<td>ND</td>
<td>1.86 ± 0.36</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td>4 (thio-IB-MECA)</td>
<td>193 ± 46</td>
<td>223 ± 36</td>
<td>0.82 ± 0.27</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td>5</td>
<td>6220 ± 640</td>
<td>&gt;10,000</td>
<td>321 ± 74</td>
<td>15.5 ± 3.1</td>
</tr>
<tr>
<td>6a (R1 = Cl, R2 = methyl)</td>
<td>55.4 ± 1.8</td>
<td>45.0 ± 1.4</td>
<td>658 ± 160</td>
<td>3.69 ± 0.25</td>
</tr>
<tr>
<td>6b (R1 = Cl, R2 = 2-fluorobenzyl)</td>
<td>(20%) (48%)</td>
<td>36.2 ± 10.7</td>
<td>7.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>6c (R1 = Cl, R2 = 3-halobenzyl)</td>
<td>(38%) (18%)</td>
<td>6.2 ± 1.8</td>
<td>1.66 ± 0.90</td>
<td></td>
</tr>
<tr>
<td>6d (R1 = Cl, R2 = 3-bromobenzyl)</td>
<td>(34%)</td>
<td>6.1 ± 1.8</td>
<td>8.99 ± 5.17</td>
<td></td>
</tr>
<tr>
<td>6e (R1 = Cl, R2 = 3-iodobenzyl)</td>
<td>2490 ± 940</td>
<td>341 ± 75</td>
<td>3.89 ± 1.15</td>
<td>4.16 ± 0.50</td>
</tr>
<tr>
<td>7a (R1 = H, R2 = methyl)</td>
<td>1070 ± 180</td>
<td>(22 ± 5%)</td>
<td>(28 ± 10%)</td>
<td>4.8 ± 1.7</td>
</tr>
<tr>
<td>7b (R1 = H, R2 = 3-fluorobenzyl)</td>
<td>1430 ± 420</td>
<td>1260 ± 330</td>
<td>98 ± 28</td>
<td>7.3 ± 0.6</td>
</tr>
<tr>
<td>7c (R1 = H, R2 = 3-chlorobenzyl)</td>
<td>860 ± 210</td>
<td>440 ± 110</td>
<td>17 ± 5</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>7d (R1 = H, R2 = 3-bromobenzyl)</td>
<td>790 ± 190</td>
<td>420 ± 32</td>
<td>6.3 ± 1.3</td>
<td>6.8 ± 3.4</td>
</tr>
<tr>
<td>7e (R1 = H, R2 = 3-iodobenzyl)</td>
<td>530 ± 97</td>
<td>230 ± 65</td>
<td>48 ± 7</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>7f (R1 = Cl, R2 = 3-bromobenzyl)</td>
<td>(6.1%) (45.7%)</td>
<td>ND</td>
<td>(12.6%)</td>
<td>(18.4%)</td>
</tr>
<tr>
<td>7g (R1 = Cl, R2 = 3-iodobenzyl)</td>
<td>(−8.0%)</td>
<td>ND</td>
<td>(−0.95%)</td>
<td>ND</td>
</tr>
</tbody>
</table>

As expected, most of the 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 N²-(3-bromobenzyl) derivative 7d exhibited the most potent binding affinity at the rat A3 AR (Kᵢ = 6.3 ± 1.3 nM) followed by N²-(3-chlorobenzyl) derivative 7c, N²-(3-iodobenzyl) derivative 7e, and N²-(3-fluorobenzyl) derivative 7b. Methyl derivative 7a was totally devoid of A3 AR binding affinity in this species. In the 2-Cl-N²-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-independent binding affinity than the corresponding 2-H analogues. Compound 6e exhibited the highest binding affinity at the rat A3 AR (Kᵢ = 3.89 ± 1.15 nM) among all compounds tested and was inactive as agonist or antagonist in a cyclic AMP functional assay at the hA3AR. It is interesting to note that N⁶-methyl derivatives 6a and 7a showing high binding affinities (Kᵢ = 3.69 ± 0.25 nM and 4.8 ± 1.7 nM, respectively) at the human A3 AR lost their binding affinities at the rat A3 AR, indicating that there must be a larger N⁶ substituent for species-independent binding affinity at the A3 AR. In a functional assay, percent inhibition at 10 µM forskolin-stimulated cyclic AMP production in CHO cells expressing the human A3 AR was measured as a mean percentage of the response of the full agonist (n = 1–3). None of the analogues 6 and 7 activated the human A3 AR (>10% of full agonist effect) by this criterion.

### Conclusion

We have established structure–activity relationships of novel truncated d- and l-thionucleoside analogues as potent species-independent A3 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-N⁶-(3-halobenzyl) derivatives 7b–7e exhibited high binding affinities at the human A3 AR as well as at the rat A3 AR with very low binding affinities at the human A1 and A2A ARs and a N⁶-(3-chlorobenzyl) derivative 7c was the most potent at the human A3 AR, but at the rat A3 AR 3-bromobenzyl derivative, 7d was the most potent. Among both 2-H and 2-Cl analogues tested, 2-chloro-N⁶-(3-iodobenzyl) derivative 6e was found to exhibit the most potent binding affinity at the rat A3 AR. Because this class of potent nucleoside human A3 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.

1H NMR (400 MHz) and 13C NMR (100 MHz) spectra were measured in CDCl3, CD3OD or DMSO-d6, 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 ChemDraw Ultra, version 11.0 (CambridgeSoft).

**Synthesis.** 6-Chloro-9-(3aR,4R,6aS)-2,2-dimethyltetrahydrodieno[3,4-d][1,3]dioxol-4-y1)-9H-furin-9-y1-tetrahydrophosphine-3,4-diol (7e). Yield 85%; [α]23.5° c 100.71 (c 0.139, DMSO); FAB-MS m/z 378 [M + H]+; mp 165.0–165.3 °C. UV (MeOH) 7max 274.5 nm. 1H NMR (DMSO-d6) δ, 8.46 (s, 1 H), 8.44 (br s, 1 H, D2O 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, D2O exchangeable), 5.35 (d, 1 H, J = 4.0 Hz, D2O exchangeable), 4.71–4.67 (m, 2 H), 4.38–4.33 (m, 1 H), 3.47–3.34 (m, 1 H), 3.48 (d, 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).

13C NMR (DMSO-d6) δ 154.3, 152.4, 142.8, 140.0, 130.1, 129.4, 128.9, 126.8, 126.5, 78.5, 72.3, 61.6, 56.4, 34.3. Anal. (C16H16BrN5O2S) C, H, N, S.

**(2R,3R,4S)-2-(6-(3-Chlorobenzylamino)-9H-furin-9-y1-tetrahydrophosphine-3,4-diol (7d).** Yield 71%; [α]23.5° c -100.71 (c 0.139, DMSO); FAB-MS m/z 422 [M]+; mp 183.0–184.0 °C. UV (MeOH) 7max 270.0 nm. 1H NMR (DMSO-d6) δ 8.46 (s, 1 H), 8.43 (br s, 1 H, D2O exchangeable), 8.21 (s, 1 H), 7.53 (s, 1 H), 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, D2O exchangeable), 5.35 (d, 1 H, J = 4.0 Hz, D2O exchangeable), 4.71–4.66 (m, 2 H), 4.37–4.34 (m, 1 H), 3.34 (dd, 1 H, J = 4.0, 10.8 Hz). Anal. (C16H16BrN5O2S) 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 flash silica gel column chromatography (CH2Cl2:MeOH = 20:1) to give 7a–7e.

**(2R,3R,4S)-Tetrahydro-2-(6-(methylamino)-9H-furin-9-y1-tetrahydrophosphine-3,4-diol (7a).** Yield 83%; [α]23.5° c -175.60 (c 0.123, DMSO); FAB-MS m/z 268 [M + H]+; mp 223.9–224.8 °C. UV (MeOH) 7max 266.0 nm. 1H NMR (DMSO-d6) δ 8.40 (s, 1 H), 8.23 (s, 1 H), 7.72 (br s, 1 H, D2O exchangeable), 5.89 (d, 1 H, J = 7.2 Hz), 5.51 (d, 1 H, J = 6.4 Hz, D2O exchangeable), 5.32 (d, 1 H, J = 4.4 Hz, D2O 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). 13C NMR (DMSO-d6) δ 152.1, 151.6, 149.2, 146.6, 131.3, 78.6, 72.1, 62.4, 34.7. Anal. (C16H17N3O2S) C, H, N, S.

**Binding Assays.** 1. Human A1 and A2A ARs. For binding to human A1 AR, [3H]CCPA (1 nM) was incubated with membranes (40 µg/tube) from CHO cells stably expressing human A1 AR at 25 °C for 60 min in 50 mM Tris-HCl buffer (pH 7.4; MgCl2, 10 mM) in a total assay volume of 200 µL. Nonspecific binding was determined using 10 µM of NECA. For human A2A AR binding, membranes (20 µg/tube) from HEK-293 cells stably expressing human A2A ARs were incubated with 15 nM [3H]CGS21680 at 25 °C for 60 min in 200 µL 50 mM Tris-HCl, pH 7.4, containing 10 mM MgCl2. NECA (10 µM) was used to define nonspecific binding. Reaction was terminated by filtration with GF/B filters.

Human and Rat A1 ARs. For competitive binding assay, each tube contained 100 µL of membrane suspension (from CHO cells
stably expressing the human or rat \( \alpha_1 \) AR, 20 \( \mu \)g protein), 50 \( \mu \)L of [\(^{125}\)I]-AB-MECA (0.5 nM), and 50 \( \mu \)L 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 \( \mu \)M 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, Gaithersburg, MD). Filters were washed three times with 9 mL of ice-cold buffer. Radioactivity was determined in a Beckman 5500B \( \gamma \)-counter.

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

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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.

References


