Synthesis and Anti-Renal Fibrosis Activity of Conformationally Locked Truncated 2-Hexynyl-\(N^6\)-Substituted-(\(N\))-Methanocarba-nucleosides as \(A_3\) Adenosine Receptor Antagonists and Partial Agonists

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ABSTRACT: Truncated \(N^6\)-substituted-(\(N\))-methanocarba-adenosine derivatives with 2-hexynyl substitution were synthesized to examine parallels with corresponding 4'-thioadenosines. Hydrophobic \(N^6\) and/or C2 substituents were tolerated in A2AR binding, but only an unsubstituted 6-amino group with a C2-hexynyl group promoted high hA2AR affinity. A small hydrophobic alkyl (4b and 4c) or \(N^6\)-cycloalkyl group (4d) showed excellent binding affinity at the hA2AR and was better than an unsubstituted free amino group (4a). A2AR affinities of 3-halobenzylamine derivatives 4f–4i did not differ significantly, with \(K_i\) values of 7.8–16.0 nM. \(N^6\)-Methyl derivative 4b (\(K_i = 4.9\) nM) was a highly selective, low efficacy partial A2AR agonist. All compounds were screened for renoprotective effects in human TGF-\(\beta\)-stimulated mProx tubular cells, a kidney fibrosis model. Most compounds strongly inhibited TGF-\(\beta\)-induced collagen I upregulation, and their A2AR binding affinities were proportional to antifibrotic effects; 4b was most potent (\(IC_{50} = 0.83\) \(\mu\)M), indicating its potential as a good therapeutic candidate for treating renal fibrosis.

INTRODUCTION

Extracellular adenosine acts as a signaling molecule with a generally cytoprotective function in the body. Adenosine mediates cell signaling through binding to four known subtypes (\(A_1\), \(A_2A\), \(A_2B\), and \(A_3\)) of adenosine receptors (ARs).\(^{1–4}\) \(A_1\), \(A_2A\), and \(A_2B\)Rs are activated by low levels of adenosine (\(EC_{50} = 0.01–1.0\) \(\mu\)M) similar to physiological levels of adenosine, whereas \(A_2B\)AR is activated by high levels of adenosine (\(EC_{50} = 24\) \(\mu\)M).\(^5\) \(A_1\) and \(A_2A\)Rs are \(G_i\)-coupled G protein-coupled receptors (GPCRs), and \(A_2A\) and \(A_2B\)Rs are \(G_s\)-coupled GPCRs. Binding of adenosine to the ARs modulates two messengers such as adenosine \(3',5'\)-cyclic phosphate (cAMP), inositol triphosphate (IP\(_3\)), and diacylglycerol (DAG).\(^{1–5}\) For example, the \(G_i\)-coupled \(A_2A\)AR inhibits adenylate cyclase (AC), resulting in cAMP down-regulation, while it stimuliates phospholipase C (PLC), which increases the levels of IP\(_3\) and DAG. Therefore, ARs have been attractive targets for the development of new therapeutic agents related to cell signaling.

Chronic kidney disease (CKD) is characterized by kidney fibrosis and is becoming a major health problem worldwide,\(^6\) and the use of renin–angiotensin–aldosterone system (RAAS) inhibitors\(^7,8\) is one of a few therapeutic options for the treatment of CKD. However, the efficacy of RAAS inhibitors is limited,\(^9\) it is, therefore, highly desirable to develop new therapeutic agents to improve the prognosis of CKD patients. Extracellular adenosine in the kidney dramatically increases in response to renal hypoxia and ischemia, and increased adenosine has been reported to be associated with CKD.\(^10\) ARs were upregulated in unilateral ureteral obstructed rat kidneys, which is a well-characterized model of CKD,\(^11\) and \(A_3\)AR knockout mice were protected against ischemia- and myoglobinuria-induced kidney failure.\(^10\) Therefore, \(A_2A\)AR antagonists may become effective renoprotective agents for the treatment of CKD.

Adenosine as a natural ligand has served as a good lead for the development of new AR ligands.\(^7\) Extensive modifications on the \(N^6\) and/or 4'-CH\(_2\)OH of adenosine have been explored, giving several potent and selective \(A_2A\)AR agonists,\(^12,13\) such as \(N^6\)-\(3\)-iodobenzyl)-\(5'\)-N-methylcarbamoyladenosine (IB-
MECA), 14 2-chloro-N^6-(3-iodobenzyl)-S''-N-methylcarbamoyl-adenosine (Cl-IB-MECA), 15 N^6-(3-iodobenzyl)-S''-N-methylcarbamoyl-4'-thioadenosine (thio-IB-MECA), 16 N^6-(3-iodobenzyl)-S''-N-methylcarbamoyl-4'-thioadenosine (thio-IB-MECA), 17 and 3'-amino-N^6-(5-chloro-2-(3-methylisoxazol-5-ylmethoxy)benzyl)-S''-N-methylcarbamoyl adenosine (CP-608039). 18 These compounds contain the potency- and efficacy-enhancing S'-methyluronamide moiety and the N^6-hydrophobic moiety. Also, AR agonists that combined N^6-alkyl and 2-alkynyl substitutions proved useful in the identification of A_3 or A_2B AR agonists with various selectivity profiles, depending on the type of 2-alkynyl substitution. 19 On the other hand, the truncated nucleosides where the S'-methyluronamide of the A_3AR agonists was deleted were converted into potent and selective A_3AR antagonists, because there was no S'-uronamide, which serves as the hydrogen bonding donor required for receptor activation. 20 Among these, compound 1 showed potent antiglaucoma 21 activity (Chart 1).

Introduction of the 2-hexynyl group on the C2-position of 1 but no substitution on the N^6-position converted 1 into dually acting A_2AAR agonist and A_3AR antagonist 2. 22 Molecular modeling and empirical structure activity studies in both the ribose and the 4'-thioribose series indicated that the C2 binding sites of A_2AAR and A_3AR were spacious enough to accommodate the bulky substituent.

Truncated (N)-methanocarba-nucleosides 3 were also reported to be selective and potent A_3AR antagonists, indicating that compound 3 can also serve as a good template for the development of A_3AR ligands. Thus, we designed and synthesized the truncated C2-hexynyl-(N)-methanocarba-nucleosides 4, which hybridize the structure of C2-hexynyl derivative 2 with that of (N)-methanocarba-nucleoside 3 to determine if similar biological trends between 2 and 4 were observed. For the synthesis of the target nucleoside 4, copper-catalyzed 23 and palladium-catalyzed 24 cross-coupling reactions were employed as key steps for functionalization of the C2-position of 6-chloropurine nucleosides. Herein, we report the synthesis of truncated C2-hexynyl-N^6-substituted-(N)-methanocarba-nucleosides 4 as potent and selective A_3AR antagonists and their renoprotective effects using TGF-β1-stimulated mProx cells, a cell culture model for kidney fibrosis. 25

**RESULTS AND DISCUSSION**

**Chemistry.** The desired C2-hexynyl-methanocarba-adenosine derivatives 4a–4i were synthesized from our known cyclopentenone intermediate 5 using a palladium-catalyzed cross-coupling reaction as a key step (Scheme 1).

Scheme 1. Synthesis of Truncated (N)-Methanocarba-Nucleosides

![Scheme 1](image)

Reagents and conditions: (a) NaBH_4, CeCl_3−7H_2O, methanol, 0 °C, 2 h; (b) Et₂Zn, CH_2I_2, CH_2Cl_2, rt, 5 h; (c) 2-iodo-6-chloropurine, Ph_3P, DIAD, THF, rt, 18 h; (d) 1-hexyne, (Ph_3P)_4Pd, Cs_2CO_3, CuI, DMF, 50 °C, 6 h; (e) 2 N HCl/THF (1/1), 40 °C, 18 h; (f) R=NH_2, Et_3N, ethanol, 90 °C, 18 h.

The cyclopentenone derivative 5 was converted to the glycosyl donor 7 according to the reported procedure 27 developed by our laboratory. Direct condensation of 7 with 6-chloro-2-iodopurine 28 under the standard Mitsunobu conditions in THF afforded the β-anomer 8 in 67% yield, similar to a literature report. 29 The anomeric β-configuration of 8 was readily assigned by the diagnostic coupling constants typical of the boat conformation of the bicyclo[3.1.0]hexane system, which has been extensively confirmed by X-ray crystallography.
and NMR analysis. The coupling constants of the $J_{H1;H15}$ and $J_{H1;H145}$ should be zero, because both $H_1$-$C$-$C$-$H_2$ and $H_1$-$C$-$C$-$H_5$ dihedral angles with trans relationships are close to 90°, indicating that 1’-H of 8 should appear as a singlet. Indeed, $^1$H NMR of 8 showed that 1’-H appeared as a singlet at 5.03 ppm, confirming the structure of 8. Sonogashira coupling of 8 with 1-hexyne in the presence of palladium catalyst yielded the C2-hexynyl derivative 9 (56%). Treatment of 9 with 2 N HCl gave the 6-chloro derivative 10. Indeed, [125I] and a copper-rA3AR in this study was carried out using $[125I]$-

Indeed, 1H NMR of a catalyst yielded the C2-hexynyl derivative of the 6-position of human (h) A1 or A3AR, RBL-2H3 basophilic leukemia cells Chinese hamster ovary (CHO) cells stably expressing the 3H] (-)-2-phenylisopropyl adenosine (R-PIA, 14) or hA3AR using [3H]CGS21680 (2-[p-(2-carboxyethyl)phenyl-ethylenamino]-S'-N-ethylcarboxamidoadenosine, 15) was carried out. In cases of weak binding, the percent inhibition of radioligand binding to the hA1AR and hA2AR was determined at 10 µM. Nonspecific binding was defined using 10 µM of 5’-N-ethylcarboxamidoadenosine (NECA, 16).

Because binding affinity of similar (N)-methanocarba compounds was reported to be very weak or absent at the hA2BAR subtype, we did not include this receptor in the radioligand binding assays. To confirm that activity of the present chemical series is weak at the A2BAR, we performed a functional assay in CHO cells expressing the hA2AR. Compound 4b at 10 µM produced only 15.7 ± 12.6% of the activation of cAMP production seen with full agonist 16.

As shown in Table 1, a variety of N'-alkyl, cycloalkyl, and arylalkyl substituents in truncated (N)-methanocarba-nucleoside derivatives have produced nanomolar binding affinity at the hA3AR subtype, indicating that bulky C2 and N6 substituents could be tolerable in the binding site of A3AR. However, a hydrophobic substituent at the N6-position reduced the binding affinity greatly at the hA2A AR subtype in the presence of a hydrophobic C2-hexynyl group, and only an unsubstituted 6-amino group showed good binding affinity ($K_i = 100$ nM) at the hA2AR, indicating that the N6 binding site of hA2AR is small. This trend is similar to that of truncated 2- hexynyl-4'-thioadenosine (2), but truncated carbocarba derivative 4a was 14-fold less potent than truncated 4'-thioadenosine derivative 2. This result may be due to the fixed conformation of (N)-methanocarba-nucleosides unlike the flexible conformation of 4'-thioadenosine derivatives, hindering them from forming a favorable hydrophobic interaction in the binding site of A2AR. However, all compounds showed very weak binding affinity at the hA1AR, suggesting that the binding sites may not be large enough to accommodate the bulky C2 and/or N6 substituent. Among compounds tested, 4b (R = CH3) exhibited the highest binding affinity ($K_i = 4.9$ nM) at the hA2AR subtype with high selectivity for the hA1 and hA2ARs. The primary amine-substituted N'-alkyl- and N6-cycloalkyl-derivatives (4b–4e) generally exhibited better binding affinity at the hA3AR than the free amino derivative 4a, except cyclopentyl derivative 4e. The order of compounds showing high binding affinity at the hA3AR is as follows: 4b (R = CH3, $K_i = 4.6$ nM) > 4c (R = ethyl, $K_i = 6.7$ nM) > 4d (R = cyclopropyl, $K_i = 9.2$ nM) > 4a (R = H, $K_i = 16.2$ nM). The binding affinities of 3-halobenzylamine derivatives 4f–4i at the hA3AR did not differ significantly, with $K_i$ values of 7.8–16.0 nM. The binding affinity at the hA3AR in this series decreased in the following order: 3-CI derivative 4b, 3-Br derivative 4g > 3-I derivative 4f > 3-F derivative 4i. All synthesized compounds 4a–4i have also produced nanomolar binding affinity at the rA3AR, but they showed weaker binding affinity than that at the hA3AR. The N6-alkyl derivatives 4b and 4c exhibited lower binding affinity at the rA3AR than the free amino derivative 4a, the N6-cycloalkyl derivatives 4d and 4e, and the 3-halobenzylamine derivatives 4f–4i, which showed similar binding affinities at the rA3AR, with $K_i$ values in the range of 10.7–65 nM. The 3-chlorobenzyl derivative 4h exhibited the highest binding affinity ($K_i = 10.7$ nM) at the rA3AR, unlike the N6-methyl derivative 4b showing the highest affinity ($K_i = 4.9$ nM) at the hA3AR.

In a cAMP functional assay34 at the hA3AR expressed in CHO cells, the most potent compound 4b behaved as a partial agonist, in contrast to full agonists 2 and 3 (Figure 1). Compound 4b at 10 µM displayed an EC50 of 45.8 nM and a maximal stimulation of cAMP formation of 29.1 ± 5.0% relative to the full agonist 16 (= 100%). Similarly, other compounds
Table 1. Binding Affinities and Anti-Renal Fibrosis Activity of Truncated 2-Hexynyl-N\(^6\)-Substituted Derivatives 4a–4i and Reference Nucleosides 2 and 3 at hARs and rA3AR

<table>
<thead>
<tr>
<th>compd no.</th>
<th>R</th>
<th>hA1AR (nM)</th>
<th>hA2AAR (nM)</th>
<th>hA3AR (nM)</th>
<th>rA3AR (nM)</th>
<th>IC(_{50}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2(^b)</td>
<td></td>
<td>39 ± 10%</td>
<td>7.19 ± 0.6</td>
<td>11.8 ± 1.3</td>
<td>ND(^e)</td>
<td>ND(^e)</td>
</tr>
<tr>
<td>3(^c)</td>
<td>H</td>
<td>3040 ± 610</td>
<td>1080 ± 310</td>
<td>1.44 ± 0.6</td>
<td>ND(^e)</td>
<td>18.6</td>
</tr>
<tr>
<td>4a</td>
<td>methyl</td>
<td>29% ± 6%</td>
<td>100 ± 10</td>
<td>16.2 ± 6.7</td>
<td>65 ± 18</td>
<td>6.12</td>
</tr>
<tr>
<td>4b</td>
<td>ethyl</td>
<td>14% ± 4%</td>
<td>7490 ± 590</td>
<td>4.90 ± 1.30</td>
<td>231 ± 81</td>
<td>0.83</td>
</tr>
<tr>
<td>4c</td>
<td>cyclopentyl</td>
<td>31% ± 7%</td>
<td>2860 ± 1060</td>
<td>6.70 ± 1.80</td>
<td>176 ± 47</td>
<td>0.84</td>
</tr>
<tr>
<td>4d</td>
<td>cyclopropyl</td>
<td>2170 ± 510</td>
<td>2200 ± 660</td>
<td>9.20 ± 0.40</td>
<td>39 ± 19</td>
<td>11.8</td>
</tr>
<tr>
<td>4e</td>
<td>cyclopentyl</td>
<td>1580 ± 240</td>
<td>1760 ± 410</td>
<td>160 ± 50</td>
<td>58 ± 39</td>
<td>&gt;50</td>
</tr>
<tr>
<td>4f</td>
<td>3-iodobenzyl</td>
<td>48% ± 5%</td>
<td>2530 ± 170</td>
<td>12.0 ± 6.0</td>
<td>26 ± 22</td>
<td>7.88</td>
</tr>
<tr>
<td>4g</td>
<td>3-bromobenzyl</td>
<td>38% ± 6%</td>
<td>3150 ± 170</td>
<td>8.60 ± 4.80</td>
<td>59 ± 37</td>
<td>10.4</td>
</tr>
<tr>
<td>4h</td>
<td>3-chlorobenzyl</td>
<td>19% ± 8%</td>
<td>3310 ± 1220</td>
<td>7.80 ± 1.70</td>
<td>10.7 ± 1.6</td>
<td>2.87</td>
</tr>
<tr>
<td>4i</td>
<td>3-fluorobenzyl</td>
<td>21% ± 4%</td>
<td>2770 ± 5%</td>
<td>16.0 ± 10.0</td>
<td>43 ± 30</td>
<td>3.17</td>
</tr>
</tbody>
</table>

\(^{a}\)All binding experiments were performed using adherent mammalian cells stably transfected with cDNA encoding the appropriate hA1AR and hA2AAR in CHO cells and \(A_3\)AR in HEK-293 cells) or \(A_3\)AR expressed endogenously in RBL-2H3 cells. Binding was carried out using 1 nM [\(^{3}\)H]14, 10 nM [\(^{3}\)H]15, or 0.5 nM [\(^{3}\)H]13 as radioligands for hA1AR, hA2AAR and hA3ARs, respectively. Values expressed as a percentage in italics refer to percent inhibition of specific radioligand binding at 10 \(\mu\)M for 3 = 5 duplicate determinations, with nonspecific binding defined using 10 \(\mu\)M 16. \(^{b}\)Ref 22. \(^{c}\)Ref 20a. \(^{d}\)Concentration to inhibit the TGF-\(\beta\)-induced collagen I mRNA expression by 50%. \(^{e}\)Not determined.

Molecular Docking Study. The truncated C2-substituted thio-ribose compound 2 (\(A_3\)AR \(K_i = 7.19 \text{nM}\)) exhibited excellent binding affinity, and the methanocarba analogue 4a (\(A_3\)AR \(K_i = 100 \text{nM}\)) showed \(\approx 14\)-fold less binding affinity at the hA3AR. In addition, the presence of the 3-iodobenzyl group at the \(N^6\) position in 4f led to a substantial decrease in its binding affinity at the hA2AAR with a \(K_i\) of 2530 nM. In view of the observed variations in the hA2AAR binding affinities among these compounds, molecular docking and binding free energy calculations were carried out considering the X-ray structure of the hA2AAR complexed with an agonist, 16 (PDB code 2YDV\(^{23}\)). The common interactions among \(N^6\)-unsubstituted compounds 2 and 4a at the hA2AAR includes: (i) the adenine ring stabilized through \(\pi\)-\(\pi\) stacking interaction with Phe168 (extracellular loop 2) and a H-bonding interaction with Asn253\(^{35}\), (ii) the exocyclic 6-amino group H-bonded with Asn253\(^{35}\) and Glu169, and (iii) the projection of C2-hexynyl group toward the extracellular side exhibiting hydrophobic interaction with Phe168, Ile66\(^{25}\), Leu267\(^{2.55}\), Leu267\(^{7.32}\), Met270\(^{7.36}\), and Tyr271\(^{7.38}\) residues (Figure 3).

In contrast, they exhibited different binding modes at the ribose binding site formed by Val843\(^{32}\), Leu853\(^{33}\), Trp246\(^{6.48}\), Leu249\(^{5.51}\) and Ile274\(^{7.39}\), Ser277\(^{7.42}\), and His278\(^{7.43}\) residues (Figure 3A), whereas 4a lost one of the key H-bond interactions with Ser277\(^{7.42}\) (Figure 3B). This residue Ser277 is a key residue and was reported to be important for hA2AAR agonistic activity and potency using site-directed mutagenesis. \(^{36-38}\) It appears that a decrease in the binding affinity of 4a at the hA2AAR could be due to the loss of H-bonding with Ser277\(^{7.42}\) at the ribose binding site. The loss of H-bonding with Ser277\(^{7.42}\) may particularly be attributed to the methanocarba ring (4a), being

![Schematic diagram](image-url)
less flexible than the thio-ribose ring (2). Furthermore, the calculated prime MM-GBSA binding free energies (ΔG_{bind}) for 2 and 4a were -104.16 and -90.37 kcal/mol, respectively, which are in good agreement with their observed binding affinities at the hA2AR. However, 4f with a bulky group at the N6-position did not fit well at the binding site of the hA2AR. These results show that the H-bond interactions with both Ser277.42 and His278.43 at the ribose binding site are important for high affinity and potency, and the bulky group at the N6-position is unfavorable toward high binding affinity at the hA2AR.

In addition, we also performed the molecular docking studies of the analogues 2 and 4a to hA3AR (see Figure 1S in Supporting Information). Because the X-ray crystal structure of hA3AR is not available yet, the homology model available in the Protein Data Bank (PDB code 1OEA) was used. The docking results showed that the binding modes of the analogues in hA3AR are flipped compared to those in hA2AAR. In hA3AR, the bulky C2-hexynyl group positions toward the middle of the trans-membrane region exhibited hydrophobic interactions. However, in hA2AAR, there is limited space at the bottom of the pocket, making the bulky hexynyl group face toward the extracellular side. The NH2 group at N6-position forms the hydrogen bonding with Asn6.55 in both hA2AAR (Asn253) and hA3AR (Asn250). Interestingly, there is a relatively bigger space near this region in hA3AR, whereas the NH2 group binds tightly in the pocket of hA2AAR. It appears according to this docking mode that this is why the N6-substituted derivatives (4b−4i) maintained their binding affinity at the hA3AR, but not at the hA2AAR.

**CONCLUSIONS**

The series of truncated N6-substituted-(N)-methanocarba-adenosine derivatives, 4a−4i with 2-hexynyl group were synthesized in order to examine if this class of nucleosides behaves as the corresponding 4′-thioadenosine derivatives. The functionalization at the C2-position of 6-chloropurine derivatives was achieved using lithiation-mediated stannyl transfer and copper- or palladium-catalyzed cross-coupling reactions. It was revealed that all synthesized nucleosides showed very high binding affinity at the hA3AR as well as at the rA3AR, as in the case of the corresponding 4′-thioadenosine derivatives, indicating that the hydrophobic N6 and/or C2 substituent could be tolerable in the binding site of the A3AR. However,
only an unsubstituted 6-amino group in the presence of a bulky C2-hexyl group was associated with high binding affinity at the hA3AR (compound 4a). This trend is similar to that of the corresponding 4′-thioadenosine derivatives, serving as dual acting A2A and A3AR ligands. However, the binding affinity at the hA3AR of the truncated (N)-methanocarba-nucleoside 4a is 14-fold less potent than the truncated 4′-thioadenosine derivative 2. It is attributed to the loss of key hydrogen bonding due to the rigid structure, which was confirmed by a hA3AR molecular docking study.

The specific structure—activity relationship for this series of conformationally constrained nucleosides might arise from the molecule of lacking in the flexibility required for optimal interaction in the binding site because of the rigidity of (N)-methanocarba-nucleosides. From this study, N6-methyl derivative 4b was discovered as a preferred hA3AR ligand (low efficacy partial agonist) with high selectivity, whereas 3-chlorobenzyl derivative 4h was discovered as the most potent/selective rA3AR ligand in this series.

The nature of the N6 substituent in this chemical series modulates the level of hA3AR agonist efficacy (ranging from nearly 0% to 29% of full agonist). For these assays, we used a CHO cell with a high level of stable expression of the hA3AR, which would tend to amplify partial agonist action. Because even these partial agonists have a relatively low efficacy, they can be expected to behave similarly to full antagonists in some pharmacological models, especially in cases of low receptor expression.39

A3AR antagonist 1 was recently shown to inhibit unilateral ureteral obstruction-induced renal fibrosis and collagen I upregulation.40 This suggests that A3AR antagonists might be useful therapeutically to block the development and attenuate the progression of renal fibrosis. All of the compounds synthesized here were screened for renoprotective activity. Among compounds tested, 4b exhibited the most potent inhibitory activity (IC50 = 0.83 μM) against TGF-β1-induced collagen I upregulation. These findings indicate that this series of truncated (N)-methanocarba-nucleoside derivatives acting as partial agonists of low efficacy or as antagonists, which show high binding affinity at the human A3AR, can serve as a good lead for the development of antifibrosis agents.

### EXPERIMENTAL SECTION

**Chemical Synthesis. General Methods.** 1H NMR spectra (CD3CN, CD3OD, or DMSO-d6) were recorded on a Varian Unity Inova 400 MHz instrument. The 1H NMR data are reported as peak multiplicities: s for singlet, d for doublet, dd for doublet of doublets, t for triplet, q for quartet, brs for broad singlet, and m for multiplet. Coupling constants are reported in hertz. 13C NMR spectra (CD3CN, CD3OD, or DMSO-d6) were recorded on a Varian Unity Inova 100 MHz instrument. 13C NMR spectra (CD3CN, CD3OD) were recorded on a Varian Unity Inova 376 MHz instrument. The chemical shifts were reported as parts per million (δ) relative to the solvent peak. Optical rotations were determined on Jasco III in appropriate solvent. UV spectra were recorded on U-3000 made by Hitachi in methanol or water. Infrared spectra were recorded on FT-IR (FTS-135) made by Bio-Rad. Melting points were determined on a Büchi B-540 instrument and are uncorrected. Elemental analyses (C, H, and N) were used to determine the purity of all synthesized compounds, and the results were within ±0.4% of the calculated values, confirming ≥95% purity. Reactions were checked with TLC (Merck precoated 60F254 plates). Flash column chromatography was performed on silica gel 60 (230–400 mesh, Merck). Reagents were purchased from Aldrich Chemical Co. Solvents were obtained from local suppliers. All the anhydrous solvents used were redistilled over CaH2, P2O5 or sodium/benzophenone prior to the reaction.

6-Chloro-2-(hex-1-ynyl)-9-[(3aR,3bR,4aS,5R,5aS)-hexahydro-2,2-dimethyldibicyclo[3.1.0] hex-1(5)-eno[3,2-d][1,3]dioxol-5-yl]-9H-purine 9 was prepared by a hA2AAR-mediated phosphorylation of the anhydrous solvents used were redistilled over CaH2, P2O5 or sodium/benzophenone prior to the reaction.
16 h. The reaction mixture was evaporated, and the residue was purified by flash silica gel column chromatography (CH₂Cl₂:MeOH = 9:1) to give 4a (0.103 g, 87%) as a white solid; mp 122–124 °C; UV (MeOH) \( \lambda_{max} \) 278 nm. MS (ESI⁺): [M + H⁺]⁺ calcd for C₃₅H₄₀N₆O₄, 568.2974; found, 568.2975; \( [\delta]_{D}^{25} \) = +2.5 (c 0.2, MeOH). 1H NMR (CDCl₃, 500 MHz) \( \delta \): 0.73–0.79 (m, 1 H), 0.96–0.91 (t, 3 H, J = 7.2 Hz), 1.33–1.55 (m, 1 H), 1.49–1.50 (m, 1 H), 1.50–1.51 (m, 1 H), 1.71–1.73 (m, 1 H), 1.73–1.75 (m, 1 H), 1.95–2.12 (m, 2 H), 2.12–2.14 (m, 2 H), 2.24–2.26 (m, 1 H), 2.26–2.28 (m, 1 H), 4.64–4.65 (t, 1 H, J = 6.8 Hz), 4.65–4.67 (t, 1 H, J = 6.8 Hz), 4.81 (s, 1 H), 8.18 (s, 1 H). 13C NMR (CDCl₃, 125 MHz) \( \delta \): 7.7, 7.9, 14.0, 19.6, 19.7, 23.2, 24.6, 24.8, 31.6, 63.8, 73.0, 77.2, 81.6, 88.2, 120.1, 140.7, 148.0, 149.8, 157.1. Anal. (C₃₅H₄₀N₆O₄) C, H, N.

**General Procedure for the Synthesis of 4b–4i.** To a solution of 1 (1 equiv) in EtOH (10 mL) were added Et₃N (3 equiv) and the mixture was stirred at 90 °C for 18 h in a steel bomb. The reaction mixture was evaporated, and the residue was purified by flash silica gel column chromatography (CH₂Cl₂:MeOH = 12:1) to give 4b–4i.

\( \text{(1R,2R,3S,4R,5S)-4-(2-(Hex-1-ynyl)-6-(methylamino)-9H-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol (4d)} \)

Yield: 90%; white solid; mp 124–126 °C; UV (MeOH) \( \lambda_{max} \) 278 nm. MS (ESI⁺): [M + H⁺]⁺ calcd for C₃₅H₄₀F₇N₆O₄, 620.2888; found, 620.2885; \( [\delta]_{D}^{25} \) = +20.2 (c 0.2, MeOH). 1H NMR (CDCl₃, 500 MHz) \( \delta \): 0.74–0.76 (m, 1 H), 0.96–1.00 (t, 3 H, J = 7.2 Hz), 1.34–1.37 (m, 1 H), 1.50–1.56 (m, 2 H), 1.98–2.01 (m, 1 H), 2.45–2.48 (t, 2 H, J = 7.2 Hz), 3.05–3.08 (brs, 1 H), 3.84–3.88 (brs, 1 H, J = 6.8 Hz), 4.65–4.66 (t, 1 H, J = 6.8 Hz), 4.83 (s, 1 H), 8.24 (s, 1 H). 13C NMR (CDCl₃) \( \delta \): 8.2, 14.1, 19.6, 19.7, 23.2, 24.7, 31.6, 64.0, 73.0, 77.4, 81.3, 88.6, 120.3, 141.4, 147.9, 157.2, 167.2. Anal. (C₂₃H₂₆ClN₅O₄) C, H, N.
reaction mixture, and the mixture was stirred at the same temperature for another 2 h. The resulting dark solution was quenched by dropwise addition of a saturated aqueous NH4Cl solution (15 mL). After the mixture was stirred at room temperature for 15 h, the mixture was diluted with CH2Cl2 (15 mL). The organic layer was washed with saturated NaHCO3 solution, dried over anhydrous MgSO4, and filtered. The DMF was evaporated under reduced pressure. The crude syrup was purified by flash silica gel column chromatography (hexane/EtOAc = 3:1) to give 12 (0.68 g, 70%) as a colorless syrup: UV (MeOH) λmax 269 nm. MS (ESI)-: [M + H]+ calculated for C23H33ClIN5O4Sn, 597.2011; found, 595.2020; [α]D25: −36.5 ([c 0.2, MeOH).1H NMR (CDCl3): δ 6.45-6.49 (m, 2 H), 2.75 (m, 4 H), 2.19 (m, 10 H), 0.89 (t, 3 H).13C NMR (CDCl3): δ 93.4, 109.9, 132.3, 141.8, 150.5, 181.0, 190.2.

The solvent was evaporated under reduced pressure, and the residue was purified by flash silica gel column chromatography (hexane/EtOAc = 3:1) to give 9 (0.155 g, 60%) as a white foam, whose spectral data were identical to those of authentic sample.

**Biological Assays.** Cell Culture and Membrane Preparation. CHO cells expressing the recombinant hA1 or A3R and HEK-293 cells expressing the hA2AR were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 μg/mL glutamine. RBL-2H3 cells endogenously expressing rA3AR were cultured as described.41 Cells were harvested by trypsinization. After homogenization and suspension, cells were centrifuged at 500g for 10 min, and the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl2. The suspension was homogenized with an electric homogenizer for 10 s and was then recentrifuged at 20 000 g for 20 min at 4 °C. The resultant pellets were resuspended in buffer containing 3% Triton X-100 and the suspension was stored at −80 °C until the binding experiments. The protein concentration was measured using the Bradford assay.42

**Binding Assays at the hA1 and hA2ARs.** For binding to the hA1AR, 50 μL of increasing concentrations of a test ligand and 50 μL of [3H]D4 (2 nM, PerkinElmer, Boston, MA) were incubated with membranes (40 μg/tube) from CHO cells stably expressing the hA1AR were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 μM penicillin, 100 μg/mL streptomycin, and 2 μg/mL glutamine. RBL-2H3 cells endogenously expressing rA3AR were cultured as described.41 Cells were harvested by trypsinization. After homogenization and suspension, cells were centrifuged at 500g for 10 min, and the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl2. The suspension was homogenized with an electric homogenizer for 10 s and was then recentrifuged at 20 000 g for 20 min at 4 °C. The resultant pellets were resuspended in buffer containing 3% Triton X-100 and the suspension was stored at −80 °C until the binding experiments. The protein concentration was measured using the Bradford assay.42

**Binding Assays at the hA1 and hA3ARs.** Each tube in the competitive binding assay contained 100 μL membrane suspension (20 μg protein), 50 μL [3H]D4 (1.0 nM, PerkinElmer, Boston, MA), and 50 μL of increasing concentrations of the test ligands in Tris-HCl buffer (50 mM, pH 8.0) containing 10 mM MgCl2, 1 mM EDTA.52 Nonspecific binding was determined using 10 μM of 16 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, USA). Filters were washed three times with 9 mL ice-cold buffer. Filters for A1AR binding were counted using a PerkinElmer Cobra γ-counter.

**Cyclic AMP Accumulation Assay.** Intracellular cAMP levels were measured with a competitive protein binding method.43 CHO cells that expressed the recombinant hA2AR or hA3AR were harvested by trypsinization. After centrifugation and resuspension in medium, cells were plated in 24-well plates in 0.5 mL medium. After 24 h, the medium was removed, and cells were washed three times with 1 mL DMEM, containing 50 mM N-(2-hydroxyethyl)piperazin-N’-2-ethanesulfonic acid (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 units/mL). For assay of the hA2AR but not the hA3AR, forskolin (10 μM) was added to the medium after 45 min. After the addition of forskolin, the incubation was continued an additional 15 min. The reaction was terminated upon removal of 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 cAMP production, protein kinase A (PKA) was incubated with [3H]cAMP (2 nM) in K2HPO4/EDTA buffer (K2HPO4, 150 mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL of 0.1 M HCl or 50 μL of 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.

**Statistical Analysis.** Binding and functional parameters were calculated using Prism 5.0 software (GraphPad, San Diego, CA, USA). IC50 values obtained from competition curves were converted to Ks values using the Cheng–Prusoff equation.54 Data were expressed as mean ± standard error of the mean.

**Antibiosis Assay.** Immortalized murine proximal tubular cells (mProx24) derived from microdissected proximal tubular segments of C57BL/6j adult mouse kidneys were supplied from Dr. Sugaya at St. Marianna University School of Medicine, Kanagawa, Japan. mProx24 were maintained in DMEM supplemented with 10% fetal calf serum (FCS; Gibco), 100 μU/mL penicillin, 100 μg/mL streptomycin, and 44 mM NaHCO3 under 5% CO2 environment at 37 °C. Cells were cultured in 6-well plate for mRNA analysis. At next day after seeding cell on 6-well plate, the cultured cells were growth-arrested with a DMEM medium containing 0.15% FCS for 24 h. Each synthesized compound was dissolved in DMSO to 50 mM and it was diluted to 20 mM, 10 mM, and 1 mM. After cells were pretreated with the synthesized compound dissolved in DMEM containing 0.15% FCS for 1 h, treated with recombinant human transforming growth factor-β1 (hTGF β1, R&D Systems) 5 ng/mL for 6 h. Total RNA was extracted from mProx24 using Trizol (Invitrogen) according to the standard protocol. mRNA expressions were measured by real-time PCR using StepOnePlus (Applied Biosystems) with 20 μL reaction volume consisting of cDNA transcripts, primer pairs, and SYBR Green PCR Master Mix (Applied Biosystems). Quantifications were normalized to 18S. The sequences of mouse collagen Iα1 primer pairs are 5'-GAAGATCCACTACCA CTGCA-3' and 5'-GTTCGGATGCGG- GAGTTTA-3'.

**Molecular Modeling.** The X-ray crystal structure of the human A2A AR complex with an agonist, 16 (PDB ID: 2DYD)55 was retrieved from the protein data bank (PDB) and prepared using the Protein Preparation Wizard in Maestro v9.2 (Schrodinger, LLC, NY, U.S.A.), where water and ions were removed, hydrogen atoms were added and optimized, and then the protein was minimized using the Optimized Potentials for Liquid Simulations-all atom (OPLS-AA) 2005 force field. The structures of the molecules were sketched in the Maestro and energy minimized using Impact v5.3 (Schrodinger, LLC, NY, U.S.A.) considering conjuant gradient algorithm with the maximum minimization cycles of 1000 and convergence gradient of 0.001 kJ/mol·Å. The four docking programs Glide-SP (standard precision), Glide-XP (extra precision), GOLD, and Sulfex-dock showed...
consistent results, and the Glide-XP docking results are presented. The receptor grid box with 10 Å around the centroid of the cocrystallized NECA was generated. The best binding poses of 2, 4a, and 4f were selected for the calculation of the receptor–ligand binding free energy (ΔG BIND) using Prime molecular mechanics-generalized Born surface area (MM-GBSA) module (Schrödinger, LLC, NY, U.S.A.).

The Ballesteros–Weinstein double-numbering system44 is used to describe the transmembrane (TM) location of the amino acids. Along with numbering their positions in the primary amino acid sequence, the residues have numbers in parentheses (X.YZ) that indicate their position in each transmembrane (TM) helix (X), relative to a conserved reference residue in that TM helix (YZ).

## ASSOCIATED CONTENT

### Supporting Information
Elemental analyses, molecular docking studies in the hA3AR homology model, and 1H and 13C NMR copies of tensin 4a at http://pubs.acs.org.

### Notes
The authors declare no competing financial interest.

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### ABBREVIATIONS

**AR**, adenosine receptor; **TG**, transforming growth factor; mProx, murine proximal; cAMP, cyclic adenosine-5′-monophosphate; IP3, inositol triphosphate; DAG, diacylglycerol; AC, adenylyl cyclase; PLC, phospholipase C; CKD, renin–angiotensin–aldosterone system; RAAS, Chronic kidney disease; CL-IB-MECA, 2-chloro-N6-(3-iodobenzyl)-5′-N-methylcarbamoyladenosine; thio-CL-IB-MECA, 2-chloro-N6-(3-iodobenzyl)-5′-N-methylcarbamoyl-4′-thioadenosine; LiTMP, lithium tetramethylpyrophosphoramide; CHO, Chinese hamster ovary; HEK, human embryonic kidney; I-AB-MECA, N6-(3-iodo-4-aminobenzyl)-5′-N-methylcarboxamidoadenosine; R-PIA, (−)-N6-2-phenylisopropyl adenosine; CGS21680, 2′-[p-(2-carboxyethyl)-phenylethylaminol]-3′-N-ethylcarboxamidoadenosine; NECA, 5′-N-ethylcarboxamidoadenosine; DMEM, Dulbecco’s modified Eagle’s medium; HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; OPLS-AA, optimized potentials for liquid simulations-all atom; MM-GBSA, molecular mechanics-generalized Born surface area; TM, transmembrane

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