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Orally Active Adenosine A$_1$ Receptor Agonists with Antinociceptive Effects in Mice

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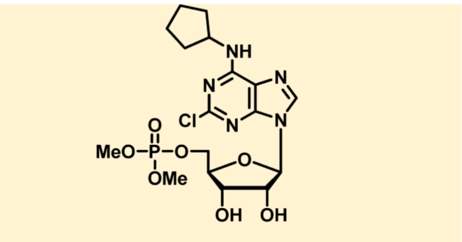
Abstract



A$_1$AR: K_i = 36 nM
> 100-fold selective for A$_1$AR over A$_{2A}$AR and A$_{2B}$AR
> 10-fold selective for A$_1$AR over A$_3$AR

Adenosine A$_1$ receptor (A$_1$AR) agonists have antinociceptive effects in multiple preclinical models of acute and chronic pain. Although numerous A$_1$AR agonists have been developed, clinical applications of these agents have been hampered by their cardiovascular side effects. Herein we report a series of novel A$_1$AR agonists, some of which are structurally related to adenosine 5′-monophosphate (5′-AMP), a naturally occurring nucleotide that itself activates A$_1$AR. These novel compounds potently activate A$_1$AR in several orthogonal in vitro assays and are subtype selective for A$_1$AR over A$_{2A}$AR, A$_{2B}$AR, and A$_3$AR. Among them, UNC32A (**3a**) is orally active and has dose-dependent antinociceptive effects in wild-type mice. The antinociceptive effects of **3a** were completely abolished in A$_1$AR knockout mice, revealing a strict dependence on A$_1$AR for activity. The apparent lack of cardiovascular side effects when

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ASSOCIATED CONTENT
Supporting Information
Cyclic AMP responses for 2′-, 3′-, and 5′-AMP in absence or presence of *αβ*-met-ADP. Effects of compound **3a** in the antinociceptive mouse model via IT and IV administrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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administered orally and high affinity (K_i of 36 nM for the human A_1AR) make this compound potentially suitable as a therapeutic.

INTRODUCTION

The adenosine A_1 receptor (A_1AR) belongs to a family of four G protein-coupled receptors that includes A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR.[1] A_1AR is expressed in the highest density in the brain, adipose tissue, kidney, and heart atria and to a lower extent in ventricles, lung, pancreas, liver, and GI tract.[2–4] A_1AR is also expressed in nociceptive dorsal root ganglia neurons.[5] A_1AR and A_3AR couple to inhibitory G_i/G_o proteins, which inhibit adenylate cyclase activity, thus reducing cellular levels of cyclic adenosine monophosphate (cAMP). On the other hand, A_{2A}AR and A_{2B}AR couple to stimulatory G_s proteins and stimulate adenylate cyclase activity.[6] Adenosine receptors also modulate phospholipase C, influencing inositol triphosphate and Ca^{2+} release from internal stores.[7] In addition, adenosine receptors regulate potassium and calcium channels.[8] Many pathophysiological states are associated with changes in adenosine levels, including asthma, neurodegenerative disorders, psychosis, anxiety, and chronic inflammatory disease.[9–12] A_1AR has been linked to a variety of human conditions. For example, adenosine is used clinically to treat supraventricular tachycardia and targets A_1AR in the atrioventicular node of the heart. This clinical application prompted development of selective A_1AR agonists as antiarrythmic agents.[13] Moreover, adenosine can reduce allodynia and hyperalgesia associated with chronic pain in rodents and humans.[14–19] Ectonucleotidases that dephosphorylate adenosine 5′-monophosphate (5′-AMP) to adenosine have potent, long-lasting, and entirely A_1AR-dependent thermal and mechanical antinociceptive effects when administered intrathecally.[20–22] Lastly, Goldman and co-workers found that A_1AR mediates the local antinociceptive effects of acupuncture, suggesting that peripheral activation of A_1ARs can contribute to analgesia.[23]

We recently found that 5′-AMP can directly activate A_1AR without being hydrolyzed to adenosine.[24] In view of this finding, we explored the structure–activity relationships (SAR) of 5′-AMP analogues with respect to their A_1AR agonist activity in our studies below. Furthermore, clinical applications of the available A_1AR agonists have been hampered by their cardiovascular side effects,[15,25] and few orally bioavailable compounds are known.[11,12,26] Herein we report a novel series of potent and selective A_1AR agonists, which are orally active in a mouse model of acute thermal nociception and lack apparent cardiovascular side effects.

RESULTS AND DISCUSSION

Chemistry

Isopropylidene-protected 2-chlorocyclopentyl adenosine **1a** was synthesized according to the literature procedure,[27] reacted with methyl, ethyl, and *tert*-butyl *N,N*-diisopropylphosphoramidite in the presence of tetrazole, and subsequently treated with hydrogen peroxide to produce phosphate diesters **2a–c** (Scheme 1). Isopropylidene protecting groups were then removed using *p*-TsOH in methanol to give **3a, 3b**, and **3c**. Compound **3c** was treated with TFA to cleave the *tert*-butyl protecting groups, and the resulting free phosphate was converted to its disodium salt **3d** by NaOH titration. Treatment of *tert*-butyl phosphate diester **2c** with TFA gave isopropylidene-protected phosphate **4**. The phosphate functionality in **4** was then converted to the dichloride via oxalyl chloride treatment, and the product reacted with glycine ethyl ester and phenethyl alcohol to produce compounds **3e** and **3f**, respectively, following *p*TsOH-assisted isopropylidene deprotection.

Pd/C and acetic acid-mediated dechlorination of **2a** followed by isopropylidene deprotection under standard conditions gave **5a** (Scheme 2). Isopropylidene-protected N^6-cyclopentyl adenosine **6** was synthesized according to published procedures[28] and reacted with *tert*-butyl *N,N*-diisopropylphosporamidite to give intermediate **7**, which was then taken through a sequence similar to above to arrive at the disodium salt **5c**.

We also developed a protocol for solvent-free coupling of 2,6-dichloropurine (**8**) and *β*-D-ribofuranose 1,2,3,5-tetraacetate (**9**) catalyzed by *p*-TsOH, carried out in a microwave reactor to furnish 2,6-dichloropurineriboside triacetate (**10**) (Scheme 3). Treatment of **10** with 2-amino-3-phenylpropanol in ethanol overnight gave intermediate **11**. Subsequently, acyl protecting groups in **11** were removed by methanolic ammonia treatment to give **12a** as a mixture of two diastereoisomers inseparable by column chromatography. Reaction of **11** with methyl and *tert*-butyl *N,N*-diisopropylphosporamidite followed by addition of hydrogen peroxide produced phosphate diesters, and the acyl protecting groups were similarly removed via methanolic ammonia treatment to furnish the desired products **12b** and **12c**. TFA treatment of **12b** followed by titrating sodium hydroxide into a methanolic solution of the resulting phosphate yielded the desired disodium salt **12d** as a mixture of two diastereoisomers.

Reaction of **10** with (1*R*,2*R*)-2-aminocyclopentanol produced intermediate **13**, which was phosphorylated using methyl and benzyl phosphoramidite to furnish **14a** and **14b**, respectively, after deprotection (Scheme 4). Benzyl protecting groups in **14b** were removed via Pd-mediated hydrogenation, and the resulting phosphate was treated with NaOH to give the desired phosphate disodium salt, compound **14c**.

Biological Evaluation

The newly synthesized or commercially available ligands were initially tested in a cAMP accumulation assay, which measures inhibition of isoproterenolor forskolin-stimulated cAMP accumulation in human embryonic kidney 293T (HEK293T) cells transiently transfected with human A_1AR. Efficacies of the tested compounds were compared to 2-chloro-N^6-cyclopentyladenosine (**3**), a potent full agonist of A_1AR.[29] All test compounds except **3b** and **14a** were full agonists at the highest concentration tested in this assay (Table 1). We previously reported that 5′-AMP is an A_1AR agonist,[24] consistent with other studies[30–32] showing that A_1AR can be activated by adenosine analogues with large and negatively charged groups at the 5′-position.

Interestingly, we found that adenosine 2′-monophosphate (2′-AMP) and adenosine 3′-monophosphate (3′-AMP) were also full agonists of A_1AR with similar potencies (EC_{50} values of 0.49 μM and 0.44 μM, respectively) (Table 1). This was not due to hydrolysis to adenosine for the following reasons: (1) Adenosine gives a characteristic “bimodal” response in the HEK293T cAMP assay due to its activation of endogenous G_s-coupled $A_{2A}AR$ in addition to the G_i-coupled ectopically expressed A_1AR.[24] If AMP hydrolysis to adenosine were occurring, we would expect to see a bimodal response for 2′-, 3′-, and 5′-AMP. However, we did not observe a bimodal response for 2′-, 3′-, or 5′-AMP (data not shown). (2) Inclusion of *α,β*-methylene adenosine 5′-diphosphate (*αβ*-met-ADP), a potent inhibitor of ecto-5′-nucleotidase (NT5E/CD73),[33] did not significantly alter the potencies of 2′-AMP or 3′-AMP (Table S1, Supporting Information).

Recent X-ray crystallography studies using $A_{2A}AR$ have identified positively charged histidine residues deep within the agonist binding pocket, which are conserved in A_1AR.[34,35] One of these residues, His278 in TM7, is proximal to the 2′ and 3′ hydroxyl groups in the ribose moiety of adenosine. Thus, a phosphate group at the 2′ or 3′ positions could form a stabilizing charge–charge interaction with His278, explaining this novel activity of 2′- and 3′-AMP.

N^6 substitution in the purine moiety has been shown to improve binding affinities of ligands to A_1AR.[29] In our cAMP accumulation assay, *N*^{6}-cyclopentyl adenosine (**5**) was indeed more potent than adenosine (EC_{50}: 0.0063 μM versus 0.039 μM) (Table 1). The corresponding 5′-monophosphate derivative **5c** was less potent (EC_{50} = 0.10 μM) than compound **5**. This result is consistent with the potency changes from adenosine to 5′-AMP. Although the conversion of 5′-phosphate (**5c**) to 5′-phosphate dimethyl ester (**5a**) reduced A_1AR agonist potency, this dimethyl ester modification could potentially increase oral bioavailability of this series.

The 2-chloro-*N*^{6}-cyclopentyl adenosine subseries was also explored (Table 1). Compound **3** and its 5′-monophosphate analogue **3d** were equal to or slightly more potent than their corresponding des-chloro analogues (**5** and **5c**). The 2-chloro-*N*^{6}-cyclopentyl adenosine 5′-phosphate dimethyl ester **3a**, which was designed to improve oral bioavailability, was a full agonist in this assay with an EC_{50} value of 1.4 μM. The potency rank for 5′-alcohol, phosphate, and phosphate dimethyl ester is consistent for both the 2-chloro and des-chloro subseries. These SAR results further support our recent finding that 5′-AMP is a full agonist of A_1AR.[24] We next explored the SAR at the 5′ position by examining diethyl and diphenethyl esters **3b** and **3f** as well as phosphate diamide **3e**. Interestingly, phosphate diamide **3e** was slightly more potent than dimethyl ester **3a**. On the other hand, diethyl ester **3b** was significantly less potent than **3a** while diphenethyl ester **3f** was equal to or slightly less potent than **3a**. These SAR findings suggest that modifications to the ester or amide groups are tolerated in general, resulting in full agonists of A_1AR with moderate potencies with the exception of compound **3b**.

Two additional subseries which contain a phosphate or phosphate dimethyl ester-modified hydroxyl moiety in the N^6-substituent of the purine (compounds **12b, 12d, 14a**, and **14c**) were examined (Table 1). Consistent with the previously published results,[24,36–38] alcohols **12a** and GR-79236 (**14**) were potent A_1AR agonists in the cAMP accumulation assay (EC_{50} = 0.55 μM and 0.0059 μM). The corresponding phosphates **12d** and **14c** were in general less potent (EC_{50} = 1.5 μM and 0.12 μM) than the alcohols **12a** and **14**, but still showed significant agonist activity. The N^6 position projects toward the solvent-exposed extracellular face of A_1AR, so it is not surprising that a negatively charged phosphate group is tolerated at this position. The phosphate diesters **12b** and **14a** (EC_{50} = 5.4 μM and EC_{50} > 10 μM) were generally less potent than the phosphates **12d** and **14c**, a SAR trend similar to that observed in the two subseries described above.

We then selected compounds **3d** (a representative phosphate) and **3a** (a representative phosphate ester) and tested them in an orthogonal functional assay. This assay utilizes chimeric G proteins to visualize human A_1AR activation in real-time and at a single cell resolution by measuring Ca^{2+} mobilization (Figure 1).[24] The E_{max} of **3d** and **3a** were normalized to the E_{max} of adenosine in this assay. Both **3d** and **3a** were potent full agonists of A_1AR with EC_{50} values of 0.021 μM and 0.52 μM, respectively. Therefore, using two distinct and complementary assay platforms, we confirmed that compounds **3d** and **3a** directly activated A_1AR-mediated signaling.

Compounds **3a** and **3d** were next evaluated in a radioligand binding assay to determine their binding affinities to human A_1AR using tritiated compound 3 as the radioligand. Consistent with their functional activities in the cAMP accumulation and Ca^{2+} mobilization assays, compounds **3a** and **3d** had high affinities for the human A_1AR with K_i values of 36 nM and 25 nM, respectively (Figure 2).

To evaluate subtype selectivity (selectivity for A_1AR over A_{2A}AR, A_{2B}AR, and A_3AR) of compounds **3a** and **3d**, we tested **3a** and **3d** in human A_{2A}AR, A_{2B}AR, and A_3AR radioligand binding assays (Table 2). Both compounds had no binding affinity to A_{2B}AR (<10% inhibition at 50 μM and 10 μM, respectively). In addition, compounds **3a** and **3d** were >100-fold selective for A_1AR over A_{2A}AR and >10-fold selective for A_1AR over A_3AR. Compounds **3a** and **3d** are "compound **3**-like" agonists, containing both 2-chloro and N^6-cyclopentyl groups, which convey strong subtype selectivity for A_1AR. Compound **3** itself displays >100-fold selectivity for A_1AR over A_{2A}AR and A_{2B}AR.[29,39] As expected, compounds **3a** and **3d** retained this A_1AR selectivity after modification at the 5′ position. A_3AR is known to be a low-affinity adenosine receptor,[40] so the modestly decreased binding affinity of **3a** and **3d** at A_3AR is not surprising.

Having established that compounds **3a** and **3d** are potent and subtype selective A_1AR agonists, we next evaluated the antinociceptive effects of **3a** in mice. We focused on **3a** for in vivo studies because of its enhanced potential to demonstrate oral activity.[41] Notably, we found that oral administration of **3a** dose-dependently inhibited noxious thermal sensitivity (Figure 3A and 3B). Significant effects were observed even at a low dose (50 nmol/kg). The effects of **3a** were similar to **5** (Figure 3C and 3D), which was used as a positive control in this mouse model. In addition, intrathecal (IT) or intravenous (IV) injection of **3a** produced marked and dose-dependent inhibition of noxious thermal sensitivity (Figure S1, Supporting Information), similar to oral administration. Importantly, the thermal antinociceptive effect of **3a** was completely abolished in the A AR knockout ($A_1AR^{-/-}$) mice even at a high dose (5 nmol, IT injection) (Figure 4), strongly demonstrating that the biological effects of **3a** are A_1AR-mediated. Taken together, these results reveal that compound **3a** is an orally active compound with potent A_1AR-mediated antinociceptive activity in mice. It is worth noting that carboxylic acid esters are degraded rapidly in vivo by carboxylesterases; however, the corresponding alkyl esters of phosphates are typically metabolically stable.[41] Therefore, it is unlikely that the observed in vivo effects of the phosphate ester **3a** are due to hydrolysis of both alkyl esters to produce **3d** or the dephosphorylated product **3**.

Clinical applications of the currently available A_1AR agonists are hampered by their cardiovascular side effects.[32] To determine if compound **3a** affected cardiovascular function, we monitored heart rate and body temperature in wild-type and $A_1AR^{-/-}$ mice following oral administration (**5** was administered as a positive control). At the highest dose tested (5000 nmol/kg, oral administration), compound **3a** had negligible effects on heart rate and body temperature in wild-type and $A_1AR^{-/-}$ mice (Figure 5A and 5C). On the other hand, compound **5** elicited a statistically significant decrease in heart rate and body temperature which lasted for 4 to 6 h in wild-type mice (Figure 5B and 5D) but not in $A_1AR^{-/-}$ mice. Compound **5** caused a modest increase in heart rate in $A_1AR^{-/-}$ mice, possibly reflecting known off-target activation of stimulatory A_2AR.[42,43] Collectively, our results indicate that our novel A_1AR agonist **3a** has potent antinociceptive effects but minimal to no cardiovascular side effects when administered orally at a high 5000 nmol/kg dose. In contrast, the same high dose of **5** has antinociceptive effects and significant cardiovascular side effects. These data in turn suggest **3a** has a large therapeutic window and uniquely lacks cardiovascular side-effects that are associated with other A_1AR agonists such as compound **5**.

CONCLUSIONS

In summary, we designed and synthesized a novel series of A_1AR agonists which possess marked potency in several orthogonal in vitro assays and are subtype selective for A_1AR over A_{2A}AR, A_{2B}AR, and A_3AR. Surprisingly, our SAR studies revealed that the addition of a phosphate or, in some cases a phosphate ester group, to the 5′, 3′, 2′, or N^6 moiety of adenosine is tolerated. These findings reveal that A_1AR can be activated by diverse natural and non-natural nucleotide and nucleoside analogues. Among our novel A_1AR agonists, compound **3a** had potent, dose-dependent, and A_1AR-dependent antinociceptive activity in mice via oral administration. The apparent lack of cardiovascular side effects in vivo and nanomolar affinity at human A_1AR makes this novel compound potentially suitable for treating pain and other physiological processes that are modulated by A_1AR activation.

EXPERIMENTAL SECTION

General Procedure for Chemical Synthesis

HPLC data of all compounds were acquired using an Agilent 6110 Series system with the UV detector set to 220 nm. Samples were injected (<10 μL) onto an Agilent Eclipse Plus 4.6 × 50 mm, 1.8 μM, C^{18} column at room temperature. Mobile phases consisting of H_2O + 0.1% acetic acid (A) and MeOH + 0.1% acetic acid (B) were used. A linear gradient from 10% to 100% B during 5.0 min was used followed by 100% B for another 2 min with a flow rate of 1.0 mL/min. Mass spectra (MS) data were acquired in positive ion mode using an Agilent 6110 single quadrupole mass spectrometer with an electrospray ionization (ESI) source. High-resolution mass spectra (HRMS) were acquired using a Shimadzu LCMS-IT-TOF time-of-flight mass spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Mercury spectrometer at 400 MHz for proton (^{1}H NMR) and 100 MHz for carbon (^{13}C NMR); chemical shifts are reported in ppm (δ) relative to the solvent peaks.[44] Preparative HPLC was performed using an Agilent Prep 1200 series with the UV detector set to 220 nm. Samples were injected onto a Phenomenex Luna 75 × 30 mm, 5 μM, C-18 column at room temperature. Mobile phases consisting of H_2O + 0.1% TFA (A) and MeOH (B) were used at a flow rate of 30 mL/min. A linear gradient from 10% to 100% B in 17.0 min was followed by 100% B for another 3 min. Adenosine, 5′-AMP, 3′-AMP, 2′-AMP, **3, 5**, and **14** were purchased from Sigma-Aldrich. All synthesized compounds have >95% purity by the above analytical HPLC method unless specific purities are noted below.

Preparation of ((2R,3S,4R,5R)-5-(2-Chloro-6-(cyclopentylamino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl) Phosphates (3a,b)—To a solution of 2-chloro-*N*^{6}-cyclopentyl-2′,3′-*O*-(1-methyl-ethylidene)-(9-Cl)adenosine (**1**) (0.73 mmol) in CH_2Cl_2 (15 mL) were added the appropriate phosphoramidite (1.5 mmol) and tetrazole (2.4 mmol). The reaction mixture was heated at 60 °C for 2 h and cooled to 0 °C, and hydrogen peroxide (0.3 mL, of 30% w/w solution) was added dropwise. The reaction mixture was stirred for 1 h at rt, diluted with CH_2Cl_2 (100 mL), and washed with 10% aqueous sodium metabisulfite (25 mL × 2), saturated aqueous sodium bicarbonate (25 mL × 2), water (25 mL × 2), and brine (25 mL × 2). The solvent was then removed under reduced pressure, and the residue was purified by preparative HPLC to afford the TFA salts of **2a,b**. To a solution of phosphates **2a,b** (0.32 mmol) in methanol (10 mL) was then added *p*-toluenesulfonic acid (0.03 mmol), the mixture was heated at 60 °C for 2 h, and the solvent was removed under reduced pressure. The residues were then purified by reverse phase preparative HPLC to afford the TFA salts of the title compounds.

((2R,3S,4R,5R)-5-(2-Chloro-6-(cyclopentylamino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl Dimethyl Phosphate (3a)—(22%) white solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.30 (s, 1H), 5.86 (d, J = 5.0 Hz, 1H), 5.75–5.50 (m, 1H), 4.58–4.48 (m, 1H), 4.50–4.35 (m, 1H), 4.30–4.40 (m, 4H), 3.72–3.53 (m, 6H), 2.11–1.89 (m, 2H), 1.89–1.51 (m, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 155.12, 153.78, 149.95, 139.89, 118.88, 87.92, 83.01, 82.94, 73.54, 70.35, 67.33, 54.64, 54.58, 54.52, 52.14, 33.45, 32.27, 23.93; HRMS calcd for $C_{17}H_{25}ClN_5O_7P$ + H: 478.1253; found: 478.1243 $[M + H]^+$.

((2R,3S,4R,5R)-5-(2-Chloro-6-(cyclopentylamino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl Diethyl Phosphate (3b)—(22%) white solid. ^{1}H NMR (400 MHz, CD_3OD): *δ* 8.21 (s, 1H), 5.97 (d, *J* = 4.3 Hz, 1H), 4.72–4.62 (m, 1H), 4.50 (brs, 1H), 4.44 (t, *J* = 5.2 Hz, 1H), 4.38–4.33 (m, 1H), 4.32–4.26 (m, 1H), 4.26–4.20 (m, 1H), 4.15–4.02 (m, 4H), 2.18–2.01 (m, 2H), 1.92–1.74 (m, 2H), 1.73–1.52 (m, 4H), 1.37–1.24 (m, 6H); HRMS calcd for $C_{19}H_{29}ClN_5O_7P$ + H: 506.1566; found: 506.1551 $[M + H]^+$.

Sodium ((2R,3S,4R,5R)-5-(2-Chloro-6-(cyclopentylamino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl Phosphate (3d)—To a solution of **1** (300 mg, 0.73 mmol) in CH_2Cl_2 (15 mL) were added *tert*-buytl *N,N*-diisopropylphosphoramidite (405 mg, 1.5 mmol) and tetrazole (5 mL of 0.45 M solution in ACN, 2.4 mmol), and the reaction mixture was heated at 60 °C for 2 h. The temperature was decreased to 0 °C, and hydrogen peroxide (0.3 mL, of 30% w/w solution) was added. The reaction mixture was stirred for 1 h at rt, CH_2Cl_2 (100 mL) was added, and the organic layer was washed with 10% sodium metabisulfite (25 mL × 2), saturated sodium bicarbonate (25 mL × 2), water (25 mL × 2), and brine (25 mL × 2). The solvent was removed under reduced pressure and purified by preparative HPLC to afford the TFA salt of compound **2c** (268 mg, 61%). To a solution of phosphate **2c** (44 mg, 0.09 mmol) in methanol (10 mL) was added *p*-toluenesulfonic acid (5 mg, 0.03 mmol), and the mixture was heated at 60 °C for 2 h. The solvent was removed under reduced pressure, trifluoroacetic acid (0.3 mL, 2.63 mmol) was added in CH_2Cl_2 (10 mL), and the reaction mixture was stirred for 1 h at rt. The solvent was removed under reduced pressure, and the residue was purified by preparative HPLC to afford the TFA salt of compound **3d**. The phosphate was then dissolved in methanol (5 mL), NaOH (0.90 mmol, 36 mg) in water (5 mL) was added, and the mixture was stirred for 30 min at rt. The solvent was removed under reduced pressure to afford the title compound **3d** (18 mg, 42% yield) as a white solid. ^{1}H NMR (300 MHz, CD_3OD): *δ* 8.47 (s, 1H), 6.02 (d, *J* = 5.7 Hz, 1H), 4.65–4.55 (m, 1H), 4.55–4.45 (m, 1H), 4.41–4.35 (m, 1H), 4.25–4.20 (m, 1H), 4.12–4.05(m, 3H), 2.23–2.07 (m, 2H), 1.86–1.69 (m, 7H); HRMS calcd for $C_{15}H_{21}ClN_5O_7P$ + H: 450.0945; found: 450.0911 $[M + H]^+$.

Ethyl 2-(((((2R,3S,4R,5R)-5-(2-Chloro-6-(cyclopentylamino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)((2-oxo-2-ethyloxyethyl)amino)phosphoryl) amino)acetate (3e)—To a solution of phosphate **4** (150 mg, 310 mmol) in CH_2Cl_2 (5 mL) were added oxalyl chloride (155 mg, 1.23 mmol) and DMF (1 drop), and the mixture was stirred at rt for 2 h. The solvent was removed under reduced pressure, and the residue was dissolved in DMF (10 mL) followed by addition of *N,N*-diisopropylethylamine (121 mg, 0.92 mmol) and ethyl 2-aminoacetate (119 mg, 0.78 mmol). The reaction mixture was then stirred for 4 h at rt, and the solvent was removed under reduced pressure. The residue was dissolved in water, extracted with ethyl acetate (3 × 50 mL), and dried over Na_2SO_4. The solvent was removed under reduced pressure, the residue was dissolved in methanol (10 mL), *p*-toluenesulfonic acid (1 mg, 0.009 mmol) was added, and the mixture was heated at 60 °C for 2 h. The solvent was removed under reduced pressure, and the residue was purified by preparative HPLC to afford the TFA salt of the title compound **3e** (15 mg, 7%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 8.54 (s, 1H), 7.39–7.26 (m, 1H), 6.06 (s, 1H), 5.62–4.82 (m, 5H), 4.66–4.45 (m, 3H), 4.40–4.20 (m, 2H), 4.16–4.05 (m, 3H), 3.75–3.60 (m, 4H), 2.16–2.05 (m, 2H), 1.84–1.44 (m, 5H), 1.23 (m, 6H); HRMS calcd for $C_{23}H_{35}ClN_7O_9P$ + H: 620.1995; found: 620.2008 $[M + H]^+$.

((2R,3S,4R,5R)-5-(2-Chloro-6-(cyclopentylamino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl Diphenethyl Phosphate (3f)—To a solution of phosphate **4** (150 mg, 310 mmol) in CH_2Cl_2 (5 mL) were added oxalyl chloride

(155 mg, 1.23 mmol) and DMF (1 drop), and the reaction mixture was stirred for 2 h at rt. The solvent was then removed under reduced pressure, and the residue was dissolved in DMF (10 mL) followed by addition of *N,N*-diisopropylethylamine (121 mg, 0.92 mmol) and phenylethyl alcohol (119 mg, 0.78 mmol). The reaction mixture was stirred at rt for 4 h, and the solvent was removed under reduced pressure. The residue was dissolved in water, extracted with ethyl acetate (3 × 50 mL), dried over Na_2SO_4, filtered, and concentrated in vacuo. To this were added methanol (10 mL) and *p*-toluenesulfonic acid (1 mg), and the mixture was heated at 60 °C for 2 h. The solvents were removed under reduced pressure, and the residue was purified by preparative HPLC to provide the TFA salt of the title compound **3f** (15 mg, 8%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 (s, 1H), 7.36–7.07 (m, 10H), 6.20 (brs, 1H), 5.89 (d, J = 4.5 Hz, 1H), 4.56 (brs, 1H), 4.49–4.43 (m, 1H), 4.39–4.35 (m, 1H), 4.30–4.25 (m, 1H), 4.17–4.03 (m, 4H), 4.03–3.95 (m, 1H), 2.98–2.76 (m, 5H), 2.22–2.04 (m, 2H), 1.87–1.61 (m, 4H), 1.60–1.45 (m, 2H), 1.39–1.13 (m, 2H); HRMS calcd for $C_{31}H_{37}ClN_5O_7P$ + H: 658.2192; found: 658.2173 $[M + H]^+$.

((3aR,4R,6R,6aR)-6-(2-Chloro-6-(cyclopentylamino)-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl Dihydrogen Phosphate (4)—To a solution of phosphate **3c** (150 mg, 0.25 mmol) in CH_2Cl_2 (10 mL) was added TFA (0.3 mL, 2.63 mmol) in CH_2Cl_2 (10 mL), and the reaction mixture was stirred for 1 h at rt. The solvent was removed under reduced pressure to afford the title compound **4** (93 mg, 76% yield) as a white solid, carried on to the next step without further purification.

((2R,3S,4R,5R)-5-(6-(Cyclopentylamino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl Dimethyl Phosphate (5a)—To a solution of phosphate **2a** (600 mg, 1.16 mmol) in methanol (50 mL) were added 10% Pd/C (300 mg, 50% w/w) and acetic acid (50 mL), and the reaction flask was fitted with a hydrogen balloon. The reaction mixture was then stirred for 6 h at rt and filtered through Celite. The solvent was removed under reduced pressure, the residue was dissolved in methanol (10 mL), and *p*-toluenesulfonic acid (17 mg, 0.16 mmol) was added. The reaction mixture was heated at 60 °C for 2 h, the solvent was removed under reduced pressure, and the residue was purified via preparative HPLC to afford the TFA salt of the title compound **5a** (200 mg, 38% yield) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.41 (brs, 1H), 8.30 (s, 1H), 5.86 (d, J = 5.2 Hz, 1H), 4.61 (t, J = 5.4 Hz, 1H), 4.35–4.05 (m, 2H), 3.70–3.62 (m, 6H), 2.05–1.91 (m, 2H), 1.81–1.55 (m, 7 H); HRMS calcd for $C_{17}H_{26}N_5O_7P$ + H: 444.1643; found: 444.1630 $[M + H]^+$.

Sodium ((2R,3S,4R,5R)-5-(6-(Cyclopentylamino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl Phosphate (5c)—To a solution of **6** (281 mg 0.75 mmol) in CH_2Cl_2 (15 mL) were added *tert*-buytl-*N,N*-diisopropylphosphoramidite (405 mg, 1.5 mmol) and tetrazole (5 mL of 0.45 M solution in ACN, 2.4 mmol). The reaction mixture was heated at 60 °C for 2 h and then cooled to 0 °C, and hydrogen peroxide (0.3 mL, of 30% w/w solution) was added dropwise. The reaction mixture was then stirred for an additional 1 h, diluted with CH_2Cl_2 (100 mL), and washed with 10% sodium metabisulfite (25 mL × 2), saturated sodium bicarbonate (25 mL × 2), water (25 mL × 2), and brine (25 mL × 2). The solvent was removed under reduced pressure to afford compound **7** (217 mg, 51% yield), which was used in the following steps without further purificaiton. To a solution of phosphate **7** (145 mg, 0.26 mmol) in methanol (10 mL) was added *p*-toluenesulfonic acid (28 mg, 0.03 mmol), and the reaction mixture was heated at 60 °C for 2 h. The solvent was removed under reduced pressure, TFA (0.3 mL, 2.63 mmol) in CH_2Cl_2 (10 mL) was added, and the reaction mixture was stirred at rt for an additional 1 h. The solvent was again removed under reduced pressure, and the residue was purified by preparative HPLC to afford the TFA salt of the title compound. To a solution of the TFA salt (50 mg, 0.12 mmol) in methanol (5 mL) was added NaOH (9.6 mg, 0.24 mmol) in water (5 mL), and the reaction mixture was stirred for 30 min at rt. The solvent was removed under reduced pressure to afford the title compound **5c** (55 mg, 99% yield) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.52 (s, 1H), 8.30 (s, 1H), 7.47 (d, J = 6.1, 1H), 7.17 (d, J = 6.0, 1H), 5.95 (d, J = 6.0 Hz, 1H), 5.48 (brs, 2H), 4.55 (t, J = 6.0, 2.4 Hz, 1H), 4.40 (m, 1H), 4.29 (m, 1H), 2.19–1.92 (m, 2H), 1.80–1.54 (m, 7H); MS(ESI) *m/z* 460.4[M - H]$^-$

(2R,3R,4R,5R)-2-(Acetoxymethyl)-5-(2,6-dichloro-9H-purin-9-yl)-tetrahydrofuran-3,4-diyl Diacetate (10)—2,6-Dichloro-9*H*-purine (2.38 g, 12.6 mmol), (2*S*,3*R*,4*R*,5R)-5-(acetoxymethyl)-tetrahydrofuran-2,3,4-triyl triacetate (4.00 g, 12.6 mmol) and *p*-toluenesulfonic acid monohydrate (10 mg, 0.06 mmol) were ground to a smooth consistency using a mortar and pestle. The powder was transferred to a microwave vial and heated in the microwave reactor for 5 min at 100 °C at a 50 W power setting. The resulting brown oil was dissolved in methanol (25 mL) and stirred for 2 h. The precipitate was collected and washed with methanol to afford the title compound **10** (2.87 g, 51%) as a tan solid. ^{1}H NMR (300 MHz, DMSO): 8.92 (s, 1 H), 6.32 (d, J = 5.0 Hz, 1 H), 5.90 (t, J = 5.4 Hz, 1H), 5.62 (t, J = 5.5 Hz, 1H), 4.46–4.25 (m, 4H), 2.11(s, 1H), 2.05(s, 1H), 2.02(s, 1H); MS(ESI) *m/z* 447 [M + H]$^+$.

(2R,3R,4R,5R)-2-(Acetoxymethyl)-5-(2-chloro-6-((1-hydroxy-3-phenylpropan-2-yl)amino)-9H-purin-9-yl)tetrahydrofuran-3,4-diyl Diacetate (11)—To a solution of 2-amino-3-phenylpropanol (67.6 mg, 0.45 mmol) in ethanol (10 mL) were added 2,6-dichloropurine riboside (**10**) (100 mg, 0.22 mmol) and triethylamine (25 mg, 0.25 mmol). The reaction mixture was stirred at 70 °C for 12 h and partitioned between ethyl acetate (100 mL) and water (50 mL). The aqueous layer was extracted with ethyl acetate (100 mL × 2). The combined organics were washed with brine (10 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (50% EtOAc/hexanes) to afford the title compound **11** (clear oil) as an inseparable mixture of diastereomers (242 mg, 86%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.84–7.82 (m, 1H), 7.24–7.13 (m, 5H), 6.10–6.07 (m, 1H), 5.73–5.70 (m, 1H), 5.56–5.52 (m, 1H), 4.70–4.49 (m, 2H), 4.34–4.28 (m, 3H), 3.90–3.60 (m, 2H), 3.03–2.90 (m, 2H), 2.12–1.96 (m, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.4, 169.7, 169.6, 169.5, 169.4, 154.8, 138.1, 137.9, 129.4, 128.5, 126.5, 85.6, 85.7, 70.6, 67.1, 63.1, 62.4, 53.8, 37.2, 37.1, 20.8, 20.7, 20.6, 20.5, 20.5, 20.4; MS(ESI) *m/z* 562 [M + H]$^+$.

(2R,3R,4S,5R)-2-(2-Chloro-6-((1-hydroxy-3-phenylpropan-2-yl)-amino)-9H-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (12a)—To acetate **11** (65 mg, 0.116 mmol) was added methanolic ammonia (30 mL) at 0 °C, the reaction vessel was sealed, and the reaction mixture was stirred for 5 h at rt. The solvent was then removed under reduced pressure, and the residue was purified by preparative HPLC to afford the TFA salt of the title compound **12a** (30 mg, 60%) (white solid) as an inseparable mixture of diastereomers. ^{1}H NMR (400 MHz, DMSO): δ 8.42–8.34 (m, 1H), 8.08 (d, J = 8.3 Hz, 1H), 7.35–7.20 (m, 4H), 7.17–7.07 (m, 1H), 5.80 (d, J = 5.8 Hz, 1H), 4.55–4.34 (m, 2H), 4.15–4.06 (m, 1H), 3.98–3.89 (m, 1H), 3.65 (dd, J = 12.0, 4.0 Hz, 1H), 3.60–3.42 (m, 3H), 3.04–2,71 (m, 2H); ^{13}C NMR (126 MHz, DMSO) δ 158.91, 158.54, 155.91, 155.44, 153.49, 149.90, 140.22, 139.95, 139.65, 139.46, 129.49, 128.73, 128.53, 126.36, 118.89, 117.72, 87.86, 87.64, 86.16, 74.06, 70.81, 63.59, 62.82, 61.80, 56.27, 54.36, 40.58, 40.37, 40.16, 39.95, 39.74, 39.53, 39.32, 37.64, 36.71, 31.11.

Preparation of 2-((2-Chloro-9-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)-3-phenylpropyl Phosphates (12b,c) —To a solution of **11** (0.35 mmol) in CH_2Cl_2 (5 mL) were added the appropriate phosphoramidite (0.70 mmol) and tetrazole (0.45 M solution in THF, 2.22 mL, 1.02 mmol), and the mixture was heated for 2 h at 60 °C. The reaction was then cooled to 0 °C, H_2O_2 (0.2 mL of 30% solution, 0.225 mmol) was added, and the reaction mixture was stirred for an additional 1 h. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with 10% aq sodium metabisulfite (20 mL), sat. aq sodium bicarbonate (20 mL), water (20 mL), and brine (20 mL). The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (10% methanol/CH_2Cl_2) to afford the acetate. To the acetate (0.135 mmol) was added methanolic ammonia (20 mL) at 0 °C, the reaction vessel was sealed, and the reaction mixture was stirred for 5 h at rt. The solvent was then removed under reduced pressure, and the residue was purified by preparative HPLC to afford compounds **12b,c**.

2-((2-Chloro-9-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)-3-phenylpropyl Dimethyl Phosphate (12b)—(38%) (clear oil) ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.28 (d, J = 51.4 Hz, 1H), 7.98 (dd, J = 23.7, 7.3 Hz, 1H), 7.35–7.15 (m, 5H), 5.83 (m, 1H), 4.79 (d, J = 29.4 Hz, 2H), 4.40 (s, 1H), 4.33–4.20 (m, 2H), 4.18–4.05 (m, 1H), 4.01–3.88 (m, 1H), 3.83–3.64 (m, 6H), 3.14–2.94 (m, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 160.9, 160.6, 155.7, 154.0, 148.5, 139.9, 139.6, 136.8, 129.4, 128.8, 127.1, 116.8, 116.5, 116.0, 113.9, 91.6, 87.9, 74.2, 72.1, 71.8, 67.9, 62.9, 62.6, 55.0, 52.5, 36.7; MS(ESI) m/z 544 $[M + H]^+$.

Di-tert-butyl (2-((2-Chloro-9-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)-3-phenylpropyl) Phosphate (12c)— (61%) (white solid). ^{1}H NMR (400 MHz, CD_3OD): δ 8.18 (s, 1H), 7.27–7.10 (m, 5H), 5.90 (m, 1H), 4.78 (br s, 1H), 4.64–4.56 (m, 1H), 4.26–4.20 (m, 1H), 4.15–4.05 (m, 2H), 4.03–3.90 (m, 1H), 3.85–3.80 (m, 1H), 3.74–3.65 (m, 1H), 3.30–3.22 (m, 2H), 3.02–2.90 (m, 2H) 1.37 (s, 9H), 1.33 (s, 9H); ^{13}C NMR (101 MHz, CD_3OD) δ 156.39, 155.20, 150.68, 141.78, 138.93, 130.40, 129.50, 129.39, 127.68, 127.51, 91.03, 90.97, 87.85, 84.64, 84.56, 75.55, 75.47, 72.35, 72.33, 68.23, 68.17, 63.31, 63.30, 53.35, 49.64, 49.43, 49.28, 49.21, 49.07, 49.00, 48.79, 48.57, 48.36, 37.93, 30.13, 30.09, 30.09, 30.05; HRMS calcd for $C_{27}H_{39}ClN_5O_8P$ + H: 628.2298; found: 628.2289 $[M + H]^+$.

Sodium 2-((2-Chloro-9-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)-3-phenylpropyl Phosphate (12d)—To a solution of phosphate **12c** (100 mg, 0.159 mmol) in CH_2Cl_2 (16 mL) was added trifluoroacetic acid (0.2 mL, 1.75 mmol), and the reaction mixture was stirred at rt for 1 h. The solvent was then removed under reduced pressure, and the residue was purified by preparative HPLC to afford the TFA salt of the title compound. The phosphate salt was then dissolved in methanol (5 mL), NaOH (9.6 mg, 0.24 mmol) in water (5 mL) was added, and the reaction mixture was stirred for 30 min at rt. The solvent was then removed under reduced pressure to afford the title compound **12d** (55 mg, 99%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 8.16 (s, 1H), 7.22–7.14 (m, 2H), 7.14–7.06 (m, 2H), 7.06–6.98 (m, 1H), 5.80–5.76 (m, 1H), 4.64 (br s, 1H), 4.52 (t, J = 5.5 Hz, 1H), 4.22–4.16 (m, 1H), 4.08–3.91 (m, 3H), 3.80–3.72 (m, 1H), 3.64–3.60 (m, 1H), 3.22–3.15 (m, 2H), 3.00–2.82 (m, 2H); ^{13}C NMR (126 MHz, D_2O) δ 155.7, 153.7, 148.6, 140.3, 138.4, 129.9, 128.1, 126.5, 118.8, 88.6, 85.9, 73.7, 70.7, 65.5, 61.7, 53.4, 37.6; HRMS calcd for $C_{19}H_{23}ClN_5O_8P$ + H: 516.1051; found: 516.1019 $[M + H]^+$.

((2R,3R,4R,5R)-2-(Acetoxymethyl)-5-(2-chloro-6-(((1R,2R)-2-hydroxycyclopentyl)amino)-9H-purin-9-yl)tetrahydrofuran-3,4-diyl Diacetate (13)—To a solution of (1*R*,2*R*)-2-aminocyclopentanol (346 mg, 3.42 mmol) in ethanol (3 mL) were added 2,6-dichloropurine riboside (**10**) (305 mg, 0.68 mmol) and triethylamine

(686 mg, 6.80 mmol). The reaction mixture was heated at 70 °C for 12 h and partitioned between ethyl acetate (100 mL) and water (50 mL). The aqueous layer was extracted with ethyl acetate (100 mL × 2). The combined organics were washed with brine (10 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (50% EtOAc/hexanes) to afford the title compound **13** (clear oil) (323 mg, 92%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.30 (s, 1H), 6.90 (s, 1H), 5.80–5.77 (m, 1H), 5.57–5.54 (m, 1H), 4.48–4.46 (m, 1H), 4.44–4.38 (m, 2H), 4.15–4.01 (m, 1H), 3.47–3.40 (m, 2H), 3.32–2.89 (m, 2H), 2.19 (s, 3H), 2.15–2.12 (m, 5H), 2.09–2.07 (m, 4H), 2.13–2.08 (m, 1H), 2.05–1.65 (m, 2H), MS(ESI) *m/z* 5.12 $[M + H]^+$.

(1R,2R)-2-((2-Chloro-9-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)-cyclopentyl Dimethyl Phosphate (14a)—To a solution of acetate **13** (60 mg, 0.12 mmol) in CH_2Cl_2 (5 mL) were added dimethyl diisopropylphosphoramidite (45 mg, 0.23 mmol) and tetrazole (0.45 M solution in THF, 0.83 mL, 0.37 mmol), and the mixture was heated at 60 °C for 2 h. The reaction was then cooled to 0 °C, H_2O_2 (0.1 mL of 30% solution, 0.12 mmol) was added, and the reaction mixture was stirred for an additional 1 h. The reaction was then diluted with CH_2Cl_2 (100 mL) and washed with 10% sodium metabisulfite, saturated sodium bicarbonate, water, and brine. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (10% methanol/CH_2Cl_2) to afford the phosphate as a clear oil. To the phosphate (60 mg, 0.097 mmol) was then added methanolic ammonia (30 mL) at 0 °C, the reaction vessel was sealed, and the reaction mixture was stirred for 5 h at rt. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (10% EtOAc/hexanes) to afford the title compound **14a** (white solid) (18 mg, 40%). ^1H NMR (400 MHz, CD_3OD): δ 8.30 (s, 1H), 5.92 (d, *J* = 6.0 Hz, 1H), 4.88–4.87 (m, 1H), 4.85–4.75 (m, 1H), 4.74–4.60 (m, 2H), 4.33–4.31 (m, 1H), 4.15 (q, *J* = 2.8 Hz, 1H), 3.89 (dd, *J* = 12.5, 2.7 Hz, 1H), 3.80–3.69 (m, 6H), 2.31–2.23 (m, 1H), 2.20–2.03 (m, 1H), 2.00–1.83 (m, 3H), 1.80–1.62 (m, 1H); HRMS calcd for $C_{17}H_{25}ClN_5O_8P + H$: 494.1208; found: 494.1219 $[M + H]^+$.

(2R,3R,4R,5R)-2-(Acetoxymethyl)-5-(2-chloro-6-((2-((di-tert-butoxyphosphoryl)oxy)cyclopentyl)amino)-9H-purin-9-yl)-tetrahydrofuran-3,4-diyl Diacetate (14b)—To a solution of **13** (429 mg, 0.838 mmol) in CH_2Cl_2 (5 mL) were added dibenzyl diisopropylphosphoramidite (579 mg, 1.68 mmol) and tetrazole (0.45 M solution in THF, 6.0 mL, 2.68 mmol) and the reaction mixture was heated for 2 h at 60 °C. The temperature was lowered to 0 °C, H_2O_2 (0.5 mL of 30% solution, 0.19 mmol) was added, and the reaction mixture was stirred for an additional 1 h. The reaction was then diluted with CH_2Cl_2 (100 mL) and washed with 10% aq sodium metabisulfite (30 mL), saturated aq sodium bicarbonate (30 mL), water (30 mL), and brine (30 mL). The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (10% methanol/CH_2Cl_2) to afford the title compound **14b** (clear oil) (100 mg, 15%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.01 (s, 1H), 7.35–7.11 (m, 10H), 6.09 (d, *J* = 5.5 Hz, 1H), 5.65 (t, *J* = 5.5 Hz, 2H), 5.49–5.46 (m, 1H), 5.01–4.89 (m, 4H), 4.80–4.70 (m, 1H), 4.65–4.50 (m, 1H), 4.43–4.36 (m, 1H), 4.35–4.36 (m, 2H), 2.25–2.15 (m, 1H), 2.13–2.04 (m, 6H), 2.01 (s, 3H), 1.99–1.90 (m, 1H), 1.86–1.67 (m, 3H), 1.63–1.44 (m, 1H); MS(ESI) *m/z* 772 $[M + H]^+$.

Sodium (1R,2R)-2-((2-Chloro-9-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-6-yl)amino)-cyclopentyl Phosphate (14c)—To a solution of phosphate **14b** (32 mg, 0.038 mmol) in methanol (10 mL) was added Pd–C (10%, 30 mg), and the reaction mixture was stirred for 12 h at rt in a pressure vessel under 60 psi of hydrogen gas. After 12 h, the mixture was filtered through

Celite to remove the catalyst. The solvent was removed under reduced pressure, and 6 N methanolic ammonia (25 mL) was added at 0 °C. The reaction was again sealed in a pressure vessel and stirred for 12 h at rt. The volatiles were removed under reduced pressure, and the residue was purified via preparative HPLC chromatography to give the TFA salt of the title compound. NaOH (13.5 mg, 0.34 mmol) in water (5 mL) was added, and the reaction mixture was stirred for 30 min at rt. The solvent was then removed under reduced pressure to afford the disodium salt of the title compound **14c** (80 mg, 99%) as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 8.10 (s, 1H), 5.83 (d, $J = 5.5$ Hz, 1H), 4.72–4.69 (m, 1H), 4.46–4.30 (m, 1H), 4.30–4.24 (m, 1H), 4.24–4.06 (m, 2H), 3.82–3.62 (m, 2H), 2.17–2.07 (m, 1H), 2.05–1.90 (m, 1H), 1.77–1.53 (m, 3H), 1.46–1.29 (m, 1H); HRMS calcd for $C_{15}H_{21}ClN_5O_8P$ + H: 466.0895; found: 466.0862 $[M + H]^+$.

cAMP Accumulation Assay

cAMP determinations were made using a modified GloSensor Luciferase detection system (Promega). Low passage, subconfluent HEK293T/17 cells (ATCC CRL-11268) were grown in Dulbecco's Modified Eagle's Medium without phenol red (Gibco #31053) and supplemented with 10% fetal bovine serum (Hyclone ‘Characterized’ #SH30071.03). Cells were reverse transfected by spotting a calcium phosphate DNA complex mixture containing 12.5 ng each of GloSensor 22F plasmid (Promega #E2301) and human adenosine A_1 receptor plasmid (Adora1, GenBank accession #AY136746, Missouri S&T Clone Collection (www.cdna.org) in 25 mM HEPES at pH 7.1, 140 mM sodium chloride, 0.75 mM disodium monophosphate, and 250 mM calcium chloride. Cells were immediately added at a density of 20 000 cells per well using a Multidrop 384 (Titertek) to 384-well white, clear-bottom tissue culture plates (Corning #3707). Cell plates were incubated for 24 h at 37 °C and 5% CO_2. Sixteen-point, 1:3 dilution curves of test compounds in the presence or absence of 10 μM final $\alpha\beta$-met-ADP (Sigma #M3763) were brought up to 8× final concentration in Hanks’ Balanced Salt Solution (Gibco #14175) supplemented with 2 mM HEPES, pH 7.4, and immediately added to the cell plates with a Multimek automated liquid handling device (Nanoscreen, Charleston, SC). Following a 10 min incubation at room temperature, 100 μM final 3-isobutyl-1-methylxanthine (Sigma) and 175 nM (–)-isoproterenol bitartrate (Sigma) or 1.5 μM forskolin (Tocris) were added by Multimek. Seven minutes later, GloSensor cAMP reagent (Promega #E1291) containing 0.1% final luciferin and supplemented with 0.3% final NP40 (Tergitol, Sigma #NP40S) to permeabilize the cells was added by Multimek along with a final 5 μL addition of 100% ethanol (Decon Laboratories) to eliminate bubbles. Luminescence was read on an Envision platereader (Perkin-Elmer) for 15 min. Data from approximately 95% of the maximal response for isoproterenol or forskolin (10–14 min post GloSensor reagent addition) were normalized for scale to 100% response equivalent to the response of 1 μM **3** and 0% response equal to the response from isoproterenol or forskolin alone. Normalized data were fit in GraphPad Prism with four parameter curves for EC_{50} determinations. Because HEK293T cells endogenously express A_2AR,[45,46] the cAMP response for adenosine and **5**, known agonists of both A_1AR (G_i coupled) and A_2AR (G_s coupled), was bimodal.[24] Concentrations below 1 μM were used to calculate potencies for adenosine, **5, 14**, and **14c**, against the A_1 receptor.

Ca^+ Mobilization

Assays of Ca^+ mobilization followed a published procedure.[24]

Radioligand Binding

Radioligand binding assays were performed by Cerep France http://www.cerep.fr/

Behavior Assay

All procedures and behavioral experiments involving vertebrate animals were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. C57BL/6 mice were purchased from Jackson Laboratories. $A_1AR^{-/-}$[47,48] mice were backcrossed to C57BL/6 mice (Jackson) for 12 generations. Male mice, 2–4 months old, were acclimated to the testing room, equipment, and experimenter for 1–3 days before behavioral testing. Noxious thermal sensitivity was measured by heating one hindpaw with a Plantar Test apparatus (IITC) following the Hargreaves method.[49] The radiant heat source intensity was calibrated so that a paw-withdrawal reflex was evoked in ~10 s, on average, in wild-type C57BL/6 mice. Cutoff time was 20 s. One measurement was taken from each paw at the indicated time points to determine paw withdrawal latency. Compounds (10 mM, dissolved in DMSO) were diluted in 0.9% saline then administered to unanesthetized mice via acute intrathecal injection (5 μL, direct lumbar puncture method[50]), intravenous tail vein injection, or oral gavage. The final DMSO concentration was 5% or less.

Telemetry

Data Sciences International ETA-F20 transmitters were implanted as follows: A 2 cm midline abdominal incision was made in anesthetized mice. The transmitter was placed intraabdominally on top of the intestines, parallel with the long axis of the body and the two leads pointing caudally. A large (14 gauge) needle was used to pass through the abdominal muscles on either side of the incision. The leads were passed through the lumen of the needle, one on each side, and the needle was withdrawn. The leads were placed (positive by the xiphoid and negative on the right pectoral) and anchored in place. The abdomen was closed with absorbable sutures and the skin with nonabsorbable sutures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS USED

A_1AR	Adenosine A_1 receptor
$A_{2A}AR$	adenosine A_{2A} receptor
$A_{2B}AR$	adenosine A_{2B} receptor
A_3AR	adenosine A_3 receptor
cAMP	cyclic adenosine monophosphate
5′-AMP	adenosine 5′-monophosphate
SAR	structure–activity relationships
2′-AMP	adenosine 2′-monophosphate
3′-AMP	adenosine 3′-monophosphate
$\alpha\beta$-met-ADP	α,β-methylene adenosine 5′-diphosphate

NT5E	ecto-5′-nucleotidase/CD73
IT	intrathecal
IV	intravenous

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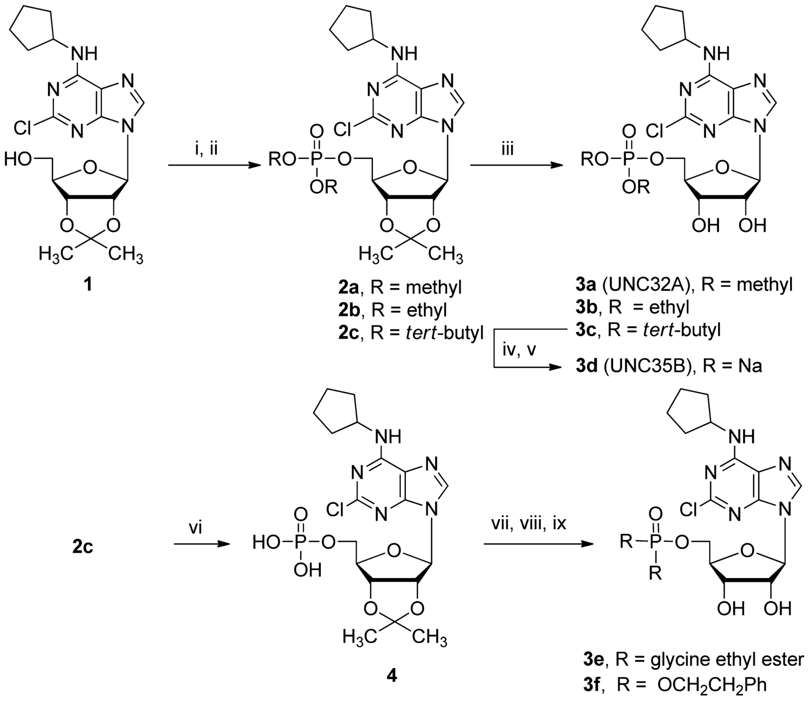
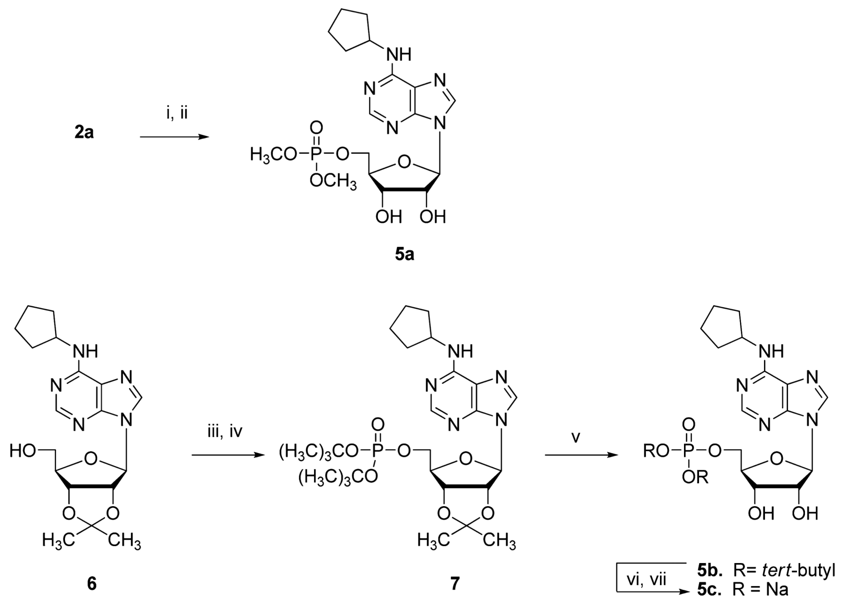


[a]Reagents and conditions: (i) R *N*,*N*-diisopropylphosphoramidite, tetrazole, CH_2Cl_2, 60 °C, 2 h; (ii) H_2O_2, 0 °C 1 h; (iii) *p*-TsOH, MeOH, 60 °C, 2 h; (iv) TFA, rt, 1 h; (v) NaOH, MeOH, rt, 30 min; (vi) TFA, CH_2Cl_2, rt, 1 h; (vii) oxalyl chloride, DMF, CH_2Cl_2, rt, 2 h; (viii) DIEA, glycine ethyl ester HCl or phenethyl alcohol, rt, 4 h; (ix) *p*-TsOH, MeOH, 60 °C, 2 h.

Scheme 1.

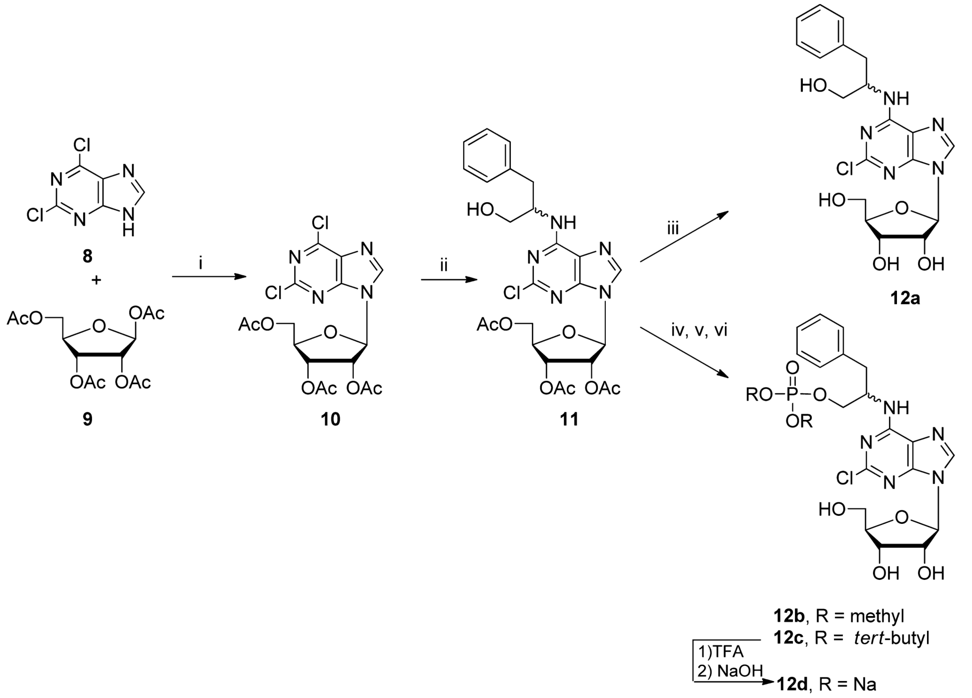
Synthesis of Compounds 3a–f[a]



[a]Reagents and conditions: (i) 10% Pd/C, H_2, AcOH, MeOH, rt, 18 h; (ii) *p*-TsOH, MeOH, 60 °C, 2 h; (iii) R *N,N*-diisopropylphosphoramidite tetrazole, CH_2Cl_2, 60 °C, 2 h; (iv) H_2O_2, 0 °C, 1 h; (v) *p*-TsOH, MeOH, 60 °C, 2 h; (vi) TFA, rt, 12 h; (vii) NaOH, MeOH, rt, 30 min.

Scheme 2.

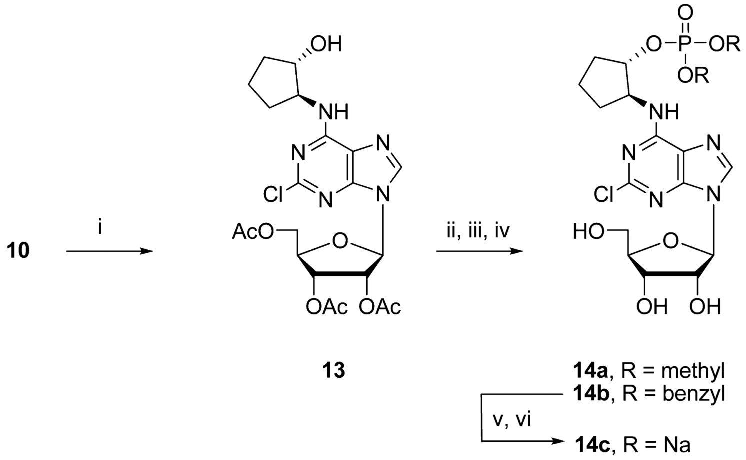
Synthesis of Compounds 5a–c[a]



[a]Reagents and conditions: (i) *p*-TsOH, mw; 10 min; (ii) 2-amino-3-phenylpropanol, EtOH, rt, 12 h; (iii) NH_3/MeOH, rt, 12 h; (iv) R *N,N*-diisopropylphosphoramidite, tetrazole, CH_2Cl_2, 60 °C, 2 h; (v) H_2O_2, 0 °C, 1 h; (vi) NH_3/MeOH, rt, 12 h.

Scheme 3.

Synthesis of Compounds 12a–d[a]



[a]Reagents and conditions: (i) (1*R*,2*R*)-2-aminocyclopentanol, EtOH, rt, 12 h; (ii) R *N*,*N*-diisopropylphosphoramidite, tetrazole, CH_2Cl_2, 60 °C, 2 h; (iii) H_2O_2, 0 °C, 1 h; (iv) NH_3/MeOH, rt, 12 h; (v) H_2, Pd/C, MeOH, rt, 12 h; (vi) NaOH, MeOH, rt, 30 min.

Scheme 4.

Synthesis of Compounds 14a–c[a]

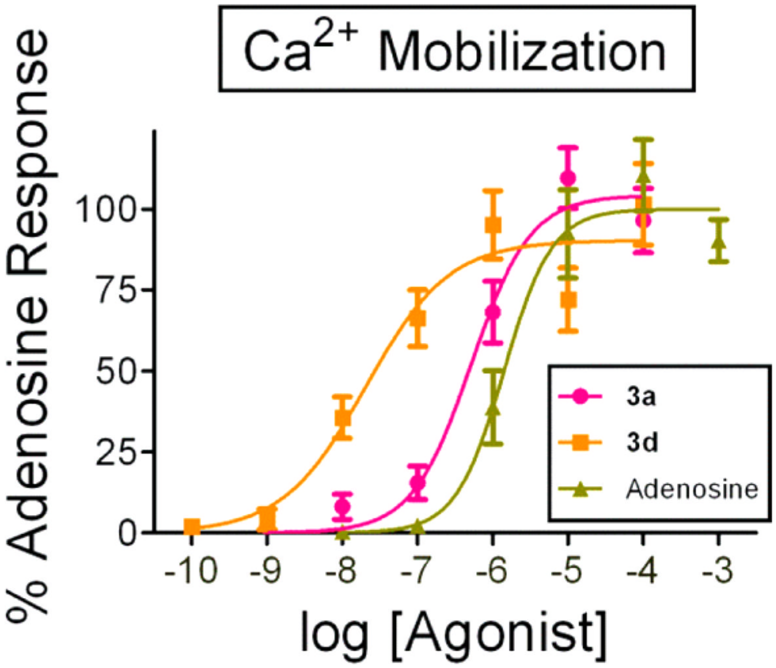


Figure 1.

Compounds **3d** and **3a** are full agonists of A_1AR in a Ca^{2+} mobilization assay. **3d**: EC_{50} = 0.021 μM; and **3a**: EC_{50} = 0.52 μM. Adenosine (EC_{50} = 1.41 μM) was used as a positive control. 27–50 cells per condition. All data are presented as mean ± standard error.

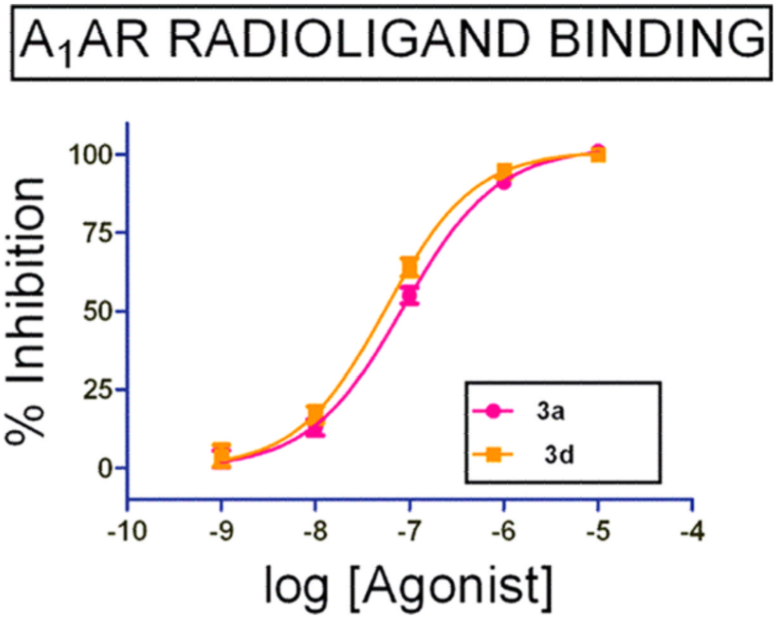


Figure 2.

Compounds **3a** and **3d** have high binding affinities to human A_1AR. Concentration–response curves of **3a** and **3d** in the radioligand binding assay using tritiated compound **3** as the radioligand, and 5′-*N*-ethylcarboxamidoadenosine (NECA) as a positive control (data not shown).

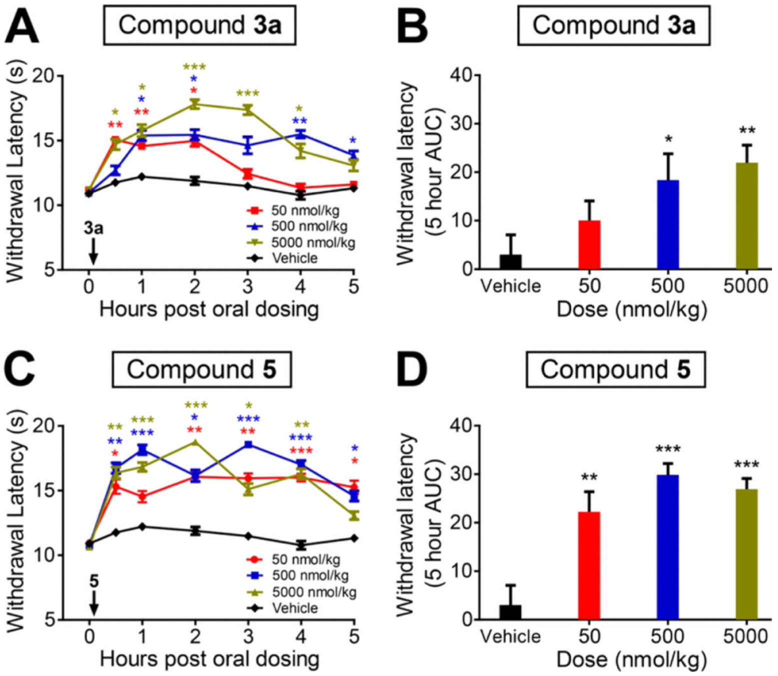


Figure 3.

Oral administration of compound **3a** dose-dependently decreases thermal sensitivity in wild-type mice. (A) Compound **3a** and (C) compound **5** were orally administrated at 50, 500, or 5000 nmol/kg immediately after determination of the baseline time point. Paw withdrawal latency was assessed at the indicated times, using Hargreaves apparatus to deliver noxious thermal stimulus. Ten C57BL/6 male mice per dose group. *t* tests were conducted relative to the corresponding vehicle time point. (B, D) Area under curve (AUC) analysis of data shown in (A, C). Five hour AUC measurements were calculated relative to baseline paw withdrawal latency for each mouse and averaged for each treatment condition. All data are presented as mean ± standard error. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

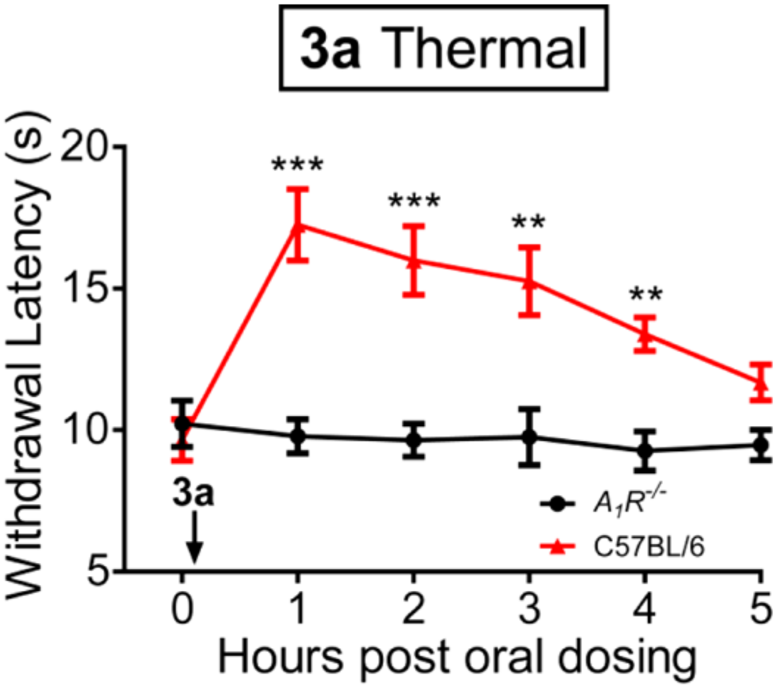


Figure 4.

Effects of **3a** on thermal sensitivity in wild-type mice are completely abolished in $A_1AR^{-/-}$ mice. Time course of effects of **3a** on on thermal paw withdrawal latency. **3a** (5 nmol) was administered via intrathecal injection immediately after determination of the baseline time point. Thermal paw withdrawal latency in wild type and $A_1AR^{-/-}$ mice were monitored using Hargreaves apparatus. Ten C57BL/6 male mice per group. All data are presented as mean ± standard error. *t* tests were conducted relative to the baseline time point. ** $p < 0.005$. *** $p < 0.0005$.

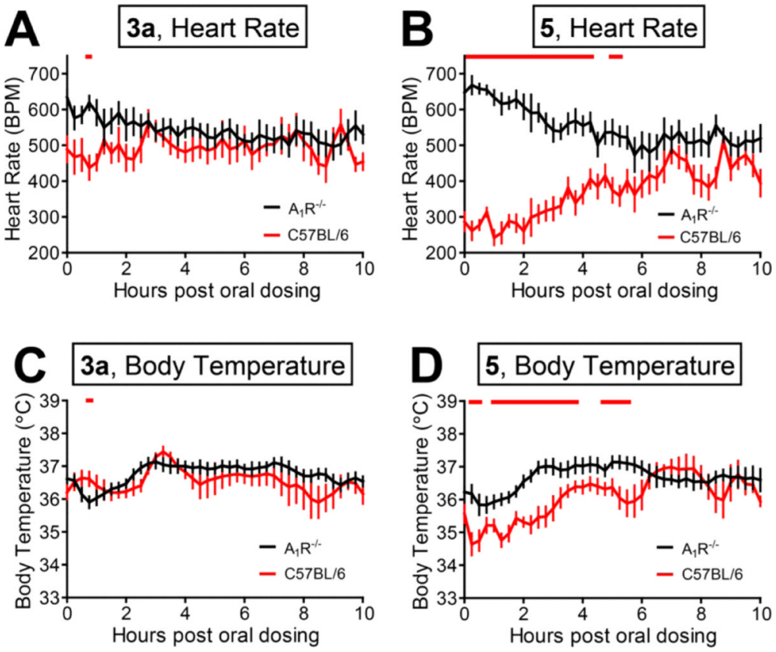
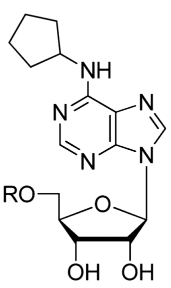


Figure 5.

Compound **3a** does not have long-lasting effects on heart rate or body temperature while compound **5** causes a significant decrease in heart rate and body temperature in wild-type mice. (A, C) Effects of **3a** on (A) heart rate and (C) body temperature in wild-type (red) and $A_1AR^{-/-}$ (black) mice. (B, D) Effects of **5** on (B) heart rate and (D) body temperature in wild-type (red) and $A_1AR^{-/-}$ (black) mice. Compounds were orally administered at 5000 nmol/kg immediately before telemetry recording began. Eight C57BL/6 male mice per group for $A_1AR^{-/-}$ body temperature measurements, and six male mice per group for all other conditions. Red bar: $p < 0.05$.

Table 1

cAMP Accumulation Assay Results



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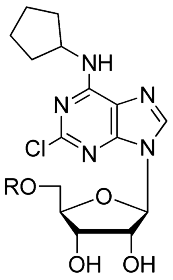

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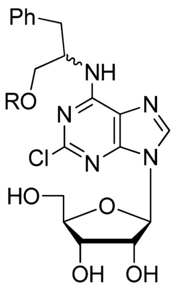

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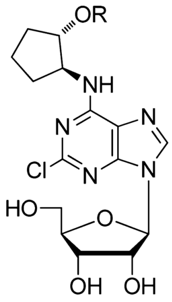

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compd	R	EC$_{50}$ (μM)[a]
adenosine	–	0.039
2′-AMP	–	0.49
3′-AMP	–	0.44
5′-AMP	–	0.50
5	H	0.0063[b]
5c	$PO(OH)_2$	0.10
5a	$PO(OMe)_2$	4.4
3	H	0.014/0.0046[b]
3d	$PO(OH)_2$	0.072
3a	$PO(OMe)_2$	1.4
3b	$PO(OEt)_2$	> 10
3f	$PO(OCH_2CH_2Ph)_2$	6.1
3e	$PO(NHCH_2CO_2Et)_2$	0.53
12a	H	0.55
12d	$PO(OH)_2$	1.5
12b	$PO(OMe)_2$	5.4
14	H	0.0059[b]
14c	$PO(OH)_2$	0.12[b]
14a	$PO(OMe)_2$	>10[b]

[a] EC$_{50}$ values are the average of at least two independent experiments with SD values that are 3-fold less than the average.

[b] Forskolin substituted for isoproterenol stimulation.

Table 2

Subtype Selectivity of Compounds 3a and 3d Assessed Using Radioligand Binding Assays

	binding affinity (K_i (nM))				binding affinity ratio	
compound	**A_1**	**A_{2A}**	**A_3**	**% inhibition/[concentration] at A_{2B}**	**A_{2A}/A_1**	**A_3/A_1**
3a	36	4700	450	7%/[50 μM]	130	12
3d	25	1600	1300	0%/[10 μM]	640	52