Supporting Information:

Semi-Rational Design of Dual Acting A_1 and A_3 Adenosine Receptor Agonists for Cardioprotection

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Ligand design

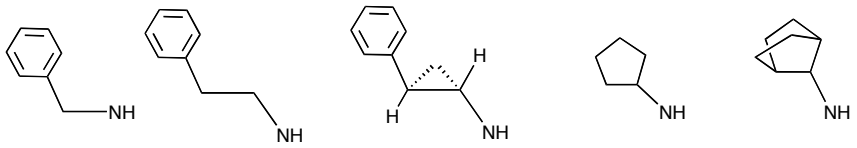
The selection of the N^6-substituents to achieve equipotency at A_1 and A_3 ARs was based on cumulative SAR data for adenosine derivatives binding to human and rat ARs.[21,23,24] Representative and illustrative examples of N^6-monosubstituted adenine-9-riboside derivatives are given in Table 3. The affinity at the A_3 AR is more variable depending on species than the affinity at the A_1 and A_{2A} ARs.

Synthetic approach

The synthetic scheme for the preparation of the nucleosides **2** and **3** from the key intermediate **7**, prepared in 8 steps from γ-erythronolactone,[21,25] is shown in Scheme 1A. The amine precursor **15** for the 7-norbornyl derivative **3** was synthesized as shown in Scheme 1B by a Curtius rearrangement of the acyl azide.

Table 3

Species Differences in Affinity at A_3AR Are More Pronounced than at A_1 or A_{2A}AR

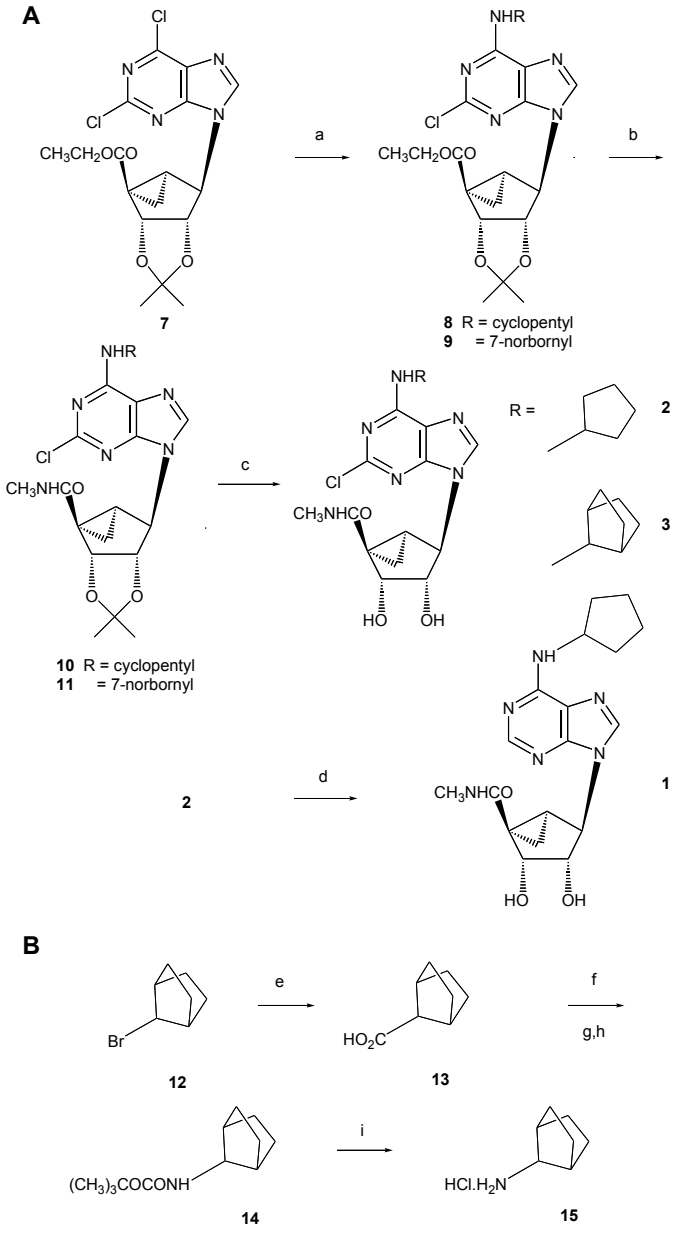


N^6-Substituted Adenosine:	Benzyl	2-Phenylethyl	(1S,2R)2-Phenyl-1-cyclopropyl	Cyclopentyl	7-Norbornyl
K_i (nM):					
Rat A_1	175	24	12	0.45	0.48
Human A_1	78	13	30	1.8	
Rat A_3	120	240	694	240	229
Human A_3	41	2.1	0.63	70	112
Rat A_{2A}	285	161	560	462	>10,000
Human A_{2A}	2180	676	2250	820	

Values are from references 23, 24, 38.

Scheme 2 (below). Synthetic route to three adenosine agonists **1** - **3** that were designed for high affinity at the A_1 and A_3 adenosine receptors and low affinity at the A_2 receptors. Both **2** and **3** contain the 2-chloro and 5'-uronamido-(N)-methanocarba substituents. In (A) the 2,6-dichloro 5'-ester **7** was treated first with a cycloalkylamine, which displaced selectively at the 6-position and subsequently with methylamine in excess, which displaced at the ester group to yield **10** and **11**. The final step was deprotection of the isopropylidene protecting group at the 2',3'-hydroxyl groups. The 2-Cl group of **2** was also hydrogenolyzed to give **1**. (B) outlines the preparation of the precursor 7-norbornylamine **15** in three steps from 7-norbornyl bromide using a Curtius

rearrangement. Reagents: a) RNH_2, MeOH, triethylamine; b) CH_3NH_2, MeOH; c) TFA, MeOH, 70˚C; d) 10% Pd/C/ H_2, MeOH; e) LDBB, CO_2; f) NaN_3, ethyl chloroformate; g) 90˚C; h) *t*-BuOH; i) HCl, dioxane.



Chemical synthesis

Materials and instrumentation. Reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). ^{1}H NMR spectra were obtained with a Varian Gemini

300 spectrometer using $CDCl_3$, D_2O, CD_3OD or DMSO-d_6 as solvents. Chemical shifts were expressed as ppm downfield from TMS. All melting points were determined with a Thomas-Hoover apparatus (A. H. Thomas Co.) and are uncorrected.

Purity of compounds was determined using a Hewlett–Packard 1100 HPLC equipped with a Luna 5 μ RP-C18(2) analytical column (250 X 4.6 mm; Phenomenex, Torrance, CA). System A parameters consisted of the following: linear gradient solvent system of H_2O/CH_3CN from 95/5 to 20/80 in 20 min; flow rate of 1 mL/min. System B parameters consisted of the following: linear gradient solvent system of 5 mM TBAP/CH_3CN from 80/20 to 20/80 in 20 min, then isocratic for 2 min; flow rate of 1 mL/min. Peaks were detected by UV absorption with a diode array detector. All derivatives tested for biological activity showed 96% or greater purity in the HPLC systems.

TLC analysis was carried out on aluminum sheets precoated with silica gel F_{254} (0.2 mm) from Aldrich. Low-resolution mass spectrometry was performed with a JEOL SX102 spectrometer with 6-kV Xe atoms following desorption from a glycerol matrix or on an Agilent LC/MS 1100, 1100 MSD, with a Waters Atlantis C18 column.

(1'S, 2'R, 3'S, 4'S, 5'S)-4'-[6-(cyclopentylamino)-2-chloro-purin-9-yl]-2',3'-O-isopropylidene-bicyclo[3.1.0]hexane-1'-carboxylic acid ethyl ester (8). Cyclopentylamine hydrochloride (**15**) (45.5 mg, 0.53 mmol) was added to a solution of **7** (45 mg, 0.11 mmol) and triethylamine (1 mL) in methanol (3 mL). The mixture was stirred at room temperature for 3 h. Then it was concentrated *in vacuo* to dryness and the residue was purified by PTLC (chloroform/methanol 20:1) to give **8** (43 mg, 85%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.64 (s, 1H), 5.95 (br s, 1H), 5.87 (d, J= 7.2 Hz, 1H), 5.37 (d, J= 6.6 Hz, 1H), 4.85 (s, 1H), 4.72 (d, J= 6.9 Hz, 1H), 4.62-4.51 (m, 2H), 2.60-2.43 (m, 2H), 2.23-2.10 (m, 4H), 1.79-1.70 (m, 5H), 1.56 (s, 3H), 1.35 (t, J = 7.8, 3H), 1.32-1.24 (m, 6H). MS (*m*/*e*) (positive API-ES) 462.1 $(M+H)^+$.

(1'S, 2'R, 3'S, 4'S, 5'S)-4'-[6-(bicyclo[2.2.1]hept-7-yl-amino)-2-chloro-purin-9-yl]-2',3'-O-isopropylidene-bicyclo[3.1.0]hexane-1'-carboxylic acid ethyl ester (9).
^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.64 (s, 1H), 5.87 (m, 2H), 4.85 (s, 1H), 4.72 (d, J= 6.9 Hz, 1H), 4.32-4.12 (m, 2H), 3.74-3.68 (m, 1H), 3.48 (d, J= 4.5 Hz, 1H), 2.32-2.25 (br s, 2H), 2.23-2.15 (m, 1H), 1.18-1.67 (m, 4H), 1.63 (s, 3H), 1.55 (s, 3H), 1.41-1.22 (m, 7H). MS (*m*/*e*) (positive API-ES) 488.2 $(M+H)^+$.

(1'S, 2'R, 3'S, 4'S, 5'S)-4'-[6-(cyclopentylamino)-2-chloro-purin-9-yl]-2',3'-O-isopropylidene-bicyclo[3.1.0]hexane-1'-carboxylic acid methyl amide (10). The ester **3a** (35 mg, 0.07 mmol) was dissolved in methanol (3 mL) and treated with an aqueous solution of methylamine (1 mL, 40%). This mixture was stirred at room temperature overnight, then the solvent was evaporated to dryness, and the white residue was purified by PTLC (chloroform/methanol 9:1) to give the uronamide **10** (15.6 mg, 50%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.68 (s, 1H), 6.97 (br s, 1H), 6.02 (br s, 1H), 5.67 (d, J= 7.8 Hz, 1H), 4.79-4.76 (m, 2H), 4.61-4.54 (m, 1H), 2.91 (d, J= 5.1 Hz, 3H), 2.17-2.11 (m, 2H), 2.04-1.81 (m, 2H), 1.78-1.65 (m, 5H), 1.58-1.48 (m, 5H), 1.27 (s, 3H). MS (*m*/*e*) (positive API-ES) 447.2 $(M+1)^+$.

(1'S, 2'R, 3'S, 4'S, 5'S)-4'-[6-(bicyclo[2.2.1]hept-7-yl-amino)-2-chloro-purin-9-yl]-2',3'-O-isopropylidene-bicyclo[3.1.0]hexane-1'-carboxylic acid methyl amide (11).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.68 (s, 1H), 6.97 (br s, 1H), 6.01 (br s, 1H), 5.66 (d, J= 7.8 Hz, 1H), 4.14 (br s, 1H), 4.79-4.76 (m, 2H), 2.91 (d, J= 5.1 Hz, 3H), 2.31 (br s, 2H), 2.04-1.99 (m, 1H), 1.81-1.64 (m, 7H), 1.55 (s, 3H), 1.40 (d, J= 8.4 Hz, 3H), 1.27 (m, 4H). MS (*m*/*e*) (positive API-ES) 473.2 $(M+1)^+$.

(1'S, 2'R, 3'S, 4'S, 5'S)-4'-[6-(cyclopentylamino)-2-chloro-purin-9-yl]-2',3'-dihydroxy-bicyclo[3.1.0]hexane-1'-carboxylic acid methyl amide (2).

10 (14 mg, 0.03 mmol) was treated with a solution of trifluoroacetic acid in MeOH (5 mL, 10%) and H_2O (0.5 mL), and the mixture was heated at 70^0C for 3 h. The solution was cooled and the solvent removed to dryness by coevaporation with toluene *in vacuo*. The white residue was purified by PTLC (chloroform/methanol 9:1) to give the final product **2** (10.1 mg, 80%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.81 (s, 1H), 6.96 (br s, 1H), 6.19 (br s,1H), 5.06 (br s, 1H), 4.88 (br s, 1H), 4.80 (s, 1H), 4.61-4.48 (m, 1H), 4.07 (d, J= 6.6 Hz, 1H), 3.62 (m, 1H), 2.91 (d, J= 4.8 Hz, 3H), 2.58-2.10 (m, 7H), 1.77-1.53 (m, 4H). MS (*m*/*e*) (positive API-ES) 407.1 $(M+1)^+$. HRMS $(M + Na)^+$: calculated 429.1418; found 429.1397. HPLC: (System A) 12.9 min (97%); (System B), 11.6 min (99%).

(1'S, 2'R, 3'S, 4'S, 5'S)-4'-[6-(bicyclo[2.2.1]hept-7-yl-amino)-2-chloro-purin-9-yl]-2',3'-dihydroxy-bicyclo[3.1.0]hexane-1'-carboxylic acid methyl amide (3).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.81 (s, 1H), 6.96 (br s, 1H), 6.15 (br s,1H), 5.05 (br s, 1H), 4.88 (br s, 1H), 4.79 (s, 1H), 4.13-4.06 (m, 2H), 3.60 (br s, 1H), 2.90 (d, J= 4.8 Hz, 3H), 2.31-2.15 (m, 6H), 1.41-1.22 (m, 5H). MS (*m*/*e*) (positive API-ES) 433.2 $(M+1)^+$. HRMS $(M + Na)^+$: calculated 455.1474; found 455.1554. HPLC: (System A) 14.2 min (97%); (System B), 14.2 min (98%).

(1'S, 2'R, 3'S, 4'S, 5'S)-4'-[6-(cyclopentylamino)-purin-9-yl]-2',3'-dihydroxy-bicyclo[3.1.0]hexane-1'-carboxylic acid methyl amide (1).

Compound **5** (5 mg, 0.007 mmol) in MeOH (5 mL) and 10% Pd/C (0.5 mg) were placed in a 250 mL pressure bottle, that was evacuated with a water-aspirator and then filled with 20 psi of hydrogen. The mixture was shaken for 15 h, the catalyst was filtered, and the solution was concentrated *in vacuo* to dryness. The residue was purified by PTLC (chloroform/methanol 10:1) to give the pure product **1** (2.1 mg, 80%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.30 (s, 1H), 7.86 (s, 1H), 6.82 (br s, 1H), 5.87 (br s,1H), 4.90 (s, 1H), 4.79 (d, J= 6.6 Hz, 1H), 4.07 (d, J= 6.6 Hz, 1H), 3.65 (m, 3H), 2.92 (d, J= 4.8 Hz, 3H), 2.38-2.33 (m, 1H), 2.18-2.11 (m, 2H), 1.77-1.59 (m, 4H), 1.36-1.26 (m, 2H), 0.95-0.86 (m, 2H). HRMS $(M+1)^+$: calculated 373.1988, found 373.1994. HPLC: (System A) 9.8 min (96%); (System B), 6.6 min (97%).

Bicyclo[2.2.1]heptane-7-carboxylic acid (13). Several pieces of lithium metal (49 mg, 7 mmol), previously scraped under THF to remove any oxide and nitride from its surface, were added under nitrogen to a solution of DBB (1.67 g, 6.28 mmol) in THF (10 mL). The mixture was then cooled to 0^0C, and the blue-green color of the radical

anion solution appeared within 30 min. After 3 h all the lithium had reacted. This dark green solution of lithium 4,4'-di-*t*-butylbiphenylide (LDBB) was added under nitrogen, through a cannula, to a solution of 7-bromonorbornane (**12**) (600 mg, 3.42 mmol) in THF (3 mL), cooled to -78^{0}C. During the addition of the LDBB solution, the reaction was first colorless and then turned red. This red solution was stirred for additional 30 min at -78^{0}C and then quickly poured on freshly crushed dry CO_2. The resulting light yellow solution was allowed to warm to room temperature and the THF was removed under reduced pressure. Et_2O (20 mL) was added and the organic layer was extracted with 1M aqueous NaOH (15 mL x 2). The combined aqueous extracts were acidified by concentrated HCl and extracted with Et_2O (10 mL x 3). The combined organic solutions were washed with water and brine, dried over Na_2SO_4, filtered and concentrated *in vacuo*, to give **13** as a white solid (320 mg, 67% yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.48 (s, 3H), 1.82 (d, J = 8.4 Hz, 2H), 1.63 (d, J = 8.4 Hz, 2H), 1.29- 1.25 (m, 4H).

Bicyclo[2.2.1]hept-7-yl-carbamic acid *tert*-butyl ester (14). A mixture of **13** (290 mg, 2.1 mmol), triethylamine (0.4 mL, 2.8 mmol), and ethyl chloroformate (0.3 mL, 3.1 mmol) in dry acetone (3.6 mL) was stirred at -10^{0}C for 3 h. Then a solution of NaN_3 (224 mg, 3.4 mmol) in H_2O (1.5 mL) was added and the mixture was stirred for additional 1.5 h. H_2O (10 mL) was added and the solution was concentrated and extracted with toluene (10 mL x 3). The combined organic layers were dried over Na_2SO_4, filtered and concentrated to about 50% to remove remaining traces of water. The resulting solution was heated at 90^{0}C until the evolution of nitrogen ceased (2 h)

and the toluene evaporated. The resulting isocyanate was dissolved in dry *t*-BuOH (10 mL) and the solution was refluxed for 20 h. The reaction mixture was concentrated and the crude *t*-butyl carbamate was purified by a flash chromatography on a column using ethyl acetate/ petroleum ether (1/8) to give **14** as a pure compound (217 mg, 50% yield)). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.48 (br s, 1H), 3.58 (br s, 1H), 2.09 (s, 2H), 1.72-1.61 (m, 4H), 1.45 (s, 9H), 1.30 (d, J = 8.4 Hz, 2H), 1.19 (d, J = 8.4 Hz, 2H). MS (m/e) positive API-ES) 212 $(M+1)^+$.

Bicyclo[2.2.1]hept-7-yl-ammonium chloride (15). Dry HCl in dioxane (2 mL, 4N) was added to **14** (150 mg, 0.7 mmol) and the mixture stirred for 0.5 h. The dioxane was evaporated, Et_2O was added, and the suspension was filtered. The filtrate washed with Et_2O to give **15** as a white solid (82 mg, 78% yield). ^{1}H NMR (D_20, 300 MHz) δ 3.29 (s, 1H), 2.29 (br s, 2H), 1.74-1.66 (m, 4H), 1.50 (d, J = 7.5 Hz, 2H), 1.19 (d, J = 7.5 Hz, 2H).

Receptor binding and functional assays

[^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5'-*N*-methyluronamide (I-AB-MECA; 2000 Ci/mmol), [^{3}H]cyclic AMP (40 Ci/mmol), and other radioligands were from GE Healthcare (Piscataway, NJ). [^{3}H]CCPA (2-chloro-N^6-cyclopentyladenosine) was a custom synthesis product.

Cell culture and membrane preparation

CHO (Chinese hamster ovary) cells expressing recombinant the human A_3AR were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal

bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 μmol/mL glutamine and 800 μg/mL geneticin. The CHO cells expressing rat A_3ARs were cultured in DMEM and F12 (1:1). Cells were harvested by trypsinization. After homogenization and suspension, cells were centrifuged at 500 *g* for 10 min, and the pellet was re-suspended in 50 mM Tris·HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA and 0.1 mg/mL CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid). The suspension was homogenized with an electric homogenizer for 10 sec, and was then re-centrifuged at 20,000 *g* for 20 min at 4°C. The resultant pellets were resuspended in buffer in the presence of 3 Units/mL adenosine deaminase, and the suspension was stored at -80°C until the binding experiments. The protein concentration was measured using the Bradford assay.[37]

Binding assays at the A_1 and A_{2A} receptors

For binding to rat A_1 receptors,[24] the radioligand [^{3}H]CCPA (0.5 nM) was incubated with rat brain membranes as described. For binding to human A_1 receptors,[23] [^{3}H]R-PIA (N^6-[(R)-phenylisopropyl]adenosine, 2 nM) was incubated with membranes (40 μg/tube) from CHO cells stably expressing human A_1 receptors at 25°C for 60 min in 50 mM Tris·HCl buffer (pH 7.4; $MgCl_2$, 10 mM) in a total assay volume of 200 μl. Nonspecific binding was determined using 10 μM of CPA (N^6-cyclopentyladenosine). For human A_{2A} receptor binding,[22] membranes (20 μg/tube) from HEK-293 cells stably expressing human A_{2A} receptors were incubated with [^{3}H]CGS21680 (2-[p-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamido-adenosine, 15 nM) at 25°C for 60 min in 200 μl 50 mM Tris·HCl, pH 7.4, containing 10 mM $MgCl_2$. NECA (10 μM) was used to define nonspecific binding. Reaction was terminated by filtration with GF/B filters.

Binding assay at the human A_3 receptor

For competitive binding assay, each tube contained 50 μL membrane suspension (20 μg protein), 25 μL of [^{125}I]I-AB-MECA (1.0 nM), and 25 μL of increasing concentrations of the test ligands in Tris·HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. Nonspecific binding was determined using 10 μM of Cl-IB-MECA in the buffer. The mixtures were incubated at 37°C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburgh, MD, USA). Filters were washed three times with 9 mL ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter. IC_{50} values were converted to K_i values as described.[17]

Cyclic AMP accumulation assay

Intracellular cyclic AMP levels were measured with a competitive protein binding method.[18,19] CHO cell that expressed recombinant the human or rat A_3AR or the human A_1 or A_{2B}AR were harvested by trypsinization. After centrifugation and resuspended in medium, cells were planted in 24-well plates in 1.0 mL medium. After 24 h, the medium was removed and cells were washed three times with 1 mL DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with agonists and/or test compounds in the presence of rolipram (10 μM) and adenosine deaminase (3 units/mL). After 45 min forskolin (10 μM) was added to the medium, and incubation was continued an additional 15 min. The reaction was terminated by removing the supernatant, and cells were lysed upon the addition of 200 μL of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at -20°C. For determination of cyclic AMP production, protein kinase A (PKA) was incubated with [^{3}H]cyclic AMP (2 nM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150

mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL 0.1 M HCl or 50 μL of cyclic AMP 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. Concentration response curves by for inhibition of cyclic AMP formation compound **2** are given in Figure 3.

Anti-ischemic Cardioprotection

Langendorff perfusion of the mouse hearts was carried out as previously described.[27,30,34-36] Mice were anesthetized with 60 mg/kg sodium pentobarbital (i.p.), and heart excised in ice-cold perfusion buffer.[8,13,27] The aorta was cannulated and retrograde perfused in the Langendorff mode at a pressure of 55 mmHg. To assess isovolumic function, fluid-filled polyvinyl plastic film balloons were inserted into the left ventricle via the left atrium. Balloons were connected to a fluid-filled pressure transducer to measure the left ventricular developed pressure (LVDP), +dP/dt, -dP/dt, and heart rate. To induce normothermic global ischemia and reperfusion, retrograde perfusion of the aorta was stopped, and hearts were immersed in the same perfusion buffer in a water-jacketed bath maintained at 37˚C. After 35-min ischemia and 30-min reperfusion, the recovery of left ventricular function was determined by quantifying the LVDP, +dP/dt, and –dP/dt. To quantify the infarct, the hearts were retrograde perfused with 1% triphenyltetrazolium chloride (TTC) in perfusion buffer for 10 minutes after 120-min reperfusion, which allowed washout of pyridine nucleotides from necrotic cells as required for TTC staining.[30] The heart was then immersed in the same TTC buffer for

another 10 minutes. The hearts were frozen, cut into six or seven 1 mm slices from apex to base, and scanned for measurement of infarct size by computer morphometry (Image-Pro Plus, version 5.0, Media Cybernetics, Inc, Silver Spring, MD).[26] The infarct size was quantified as the area of necrosis normalized to the total ventricular area. To assess the anti-ischemic cardioprotective effect of adenosine receptor agonists, vehicle (0.1% DMSO in perfusion buffer), or compound **2** (30 nM) or compound **5** (100 nM) dissolved in DMSO and diluted in buffer were administered in Langendorff mode for five minutes prior to the normothermic global ischemia/reperfusion.

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Functional effects of Compound **2**:

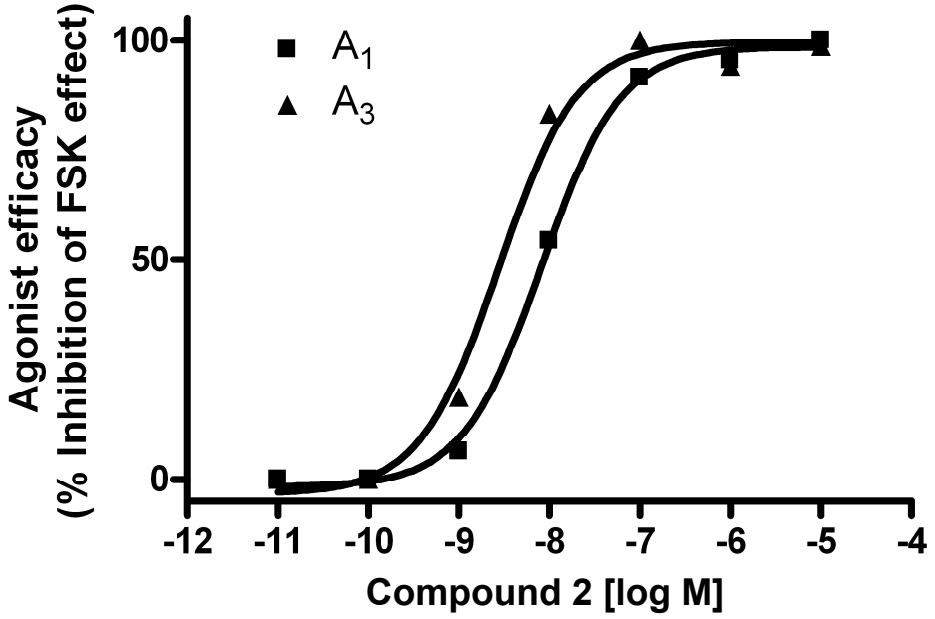

Figure 3. Inhibition of forskolin-stimulated cyclic AMP production induced by compound **2** in CHO cells stably transfected with the human A_1 (■) or A_3 (▲) AR. All experiments were performed in the presence of 10 μM rolipram and 3 Units/ml adenosine deaminase. Forskolin (10 μM) was used to stimulate cyclic AMP levels. The level of cAMP corresponding to 100% was 220 ± 30 pmol/mL. The data shown were from one experiment performed in duplicate and are typical of two independent experiments giving similar results. EC_{50} values were 8.2 and 2.8 nM at the A_1 or A_3 AR, respectively. 100% values are normalized to the maximal effect of NECA (10 μM) as a full agonist at either the A_1 or A_3 AR.