Supporting Information

Structure-Based Design of Reactive Nucleosides for Site-Specific Modification of the A_{2A} Adenosine Receptor

Steven M. Moss, P. Suresh Jayasekara, Silvia Paoletta, Zhan-Guo Gao, and Kenneth A. Jacobson

Contents:

Abbreviations: AR, adenosine receptor; cAMP, adenosine 3′,5′-cyclic phosphate; CHO, Chinese hamster ovary; DCC, *N,N′*-dicyclohexylcarbodiiimide; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMAP, 4-dimethylaminopyridine; DMEM, Dulbecco's modified Eagle's medium; EDC, *1*-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; EL, extracellular loop; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; I-AB-MECA, N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyl-uronamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRMS, high resolution mass spectroscopy; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PYBOP, benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate; *R*-PIA, N^6-R-phenylisopropyladenosine; TLC, thin layer chromatography; TM, transmembrane helical domain; WT, wild-type.

EXPERIMENTAL SECTION

Chemical Synthesis

^{1}H NMR spectra were obtained with a Bruker 400 MHz spectrometers using MeOD, $CDCl_3$ or DMSO-d_6 as a solvent. The chemical shifts are expressed as relative ppm from MeOD (4.78 ppm (s), 3.31 (m)).

High-resolution mass measurements were performed using a Micromass/Waters LCT Premier Electrospray Time of Flight (TOF) mass spectrometer coupled with a Waters HPLC system, unless noted. Purification of all the nucleotide analogues for biological testing was carried out on (diethylamino)ethyl (DEAE)-A25 Sephadex columns with a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase. The final reactive nucleosides (**3**) were additionally purified by HPLC with a Luna 5 μ RP-C18(2) semipreparative column (250 X 10.0 mm; Phenomenex, Torrance, CA) and using the following gradient conditions: flow rate of 2 mL/min; either 0.05% aqueous TFA:CH_3CN from 100:0 to 95:5 (System A) or up to 95:05 to 50:50 (System B) in 30 min. The final nucleosides isolated by HPLC were in the TFA salt form.

Purity of the compounds submitted for bioassay was checked using a Hewlett–Packard 1100 HPLC equipped with a Zorbax SB-Aq 5 μm analytical column (50 x 4.6 mm; Agilent Technologies, Inc., Palo Alto, CA). Mobile phase: linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH_3CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min. Peaks were detected by UV absorption with a diode array detector at 230, 254, and 280 nm. All derivatives tested for biological activity showed >98% purity by HPLC analysis (detection at 254 nm).

CGS 21680 hydrochloride (**1**) was purchased from Tocris Bioscience (Minneapolis, MN). 5-Azidopentanoic acid was purchased from Bachem (Torrance, CA). DIBO-Alexa and DIFO-TAMRA were purchased from Life Technologies (Grand Island, NY). All other reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

4-(3-(4-(2-((6-Amino-9-((2*R*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-2-nitrophenyl acetate (3a)
Compound **1** (5.45 mg, 0.01 mmol, 1 eq) and 4-amino-2-nitrophenyl acetate (**6a**, 3.92 mg, 0.02 mmol, 2 eq) were dissolved in anhydrous DMF (0.5 mL) in a 10 mL round bottom flask under N_2. DIPEA (4.5 μL, 0.025 mmol, 2.5 eq) was added at room temperature, and the mixture was stirred 10 min. PyBOP (5.5 mg, 0.01 mmol, 1 eq) was then added to the reaction mixture and stirring continued overnight. The solvent was evaporated, and product **3a** (MRS5854, 4.6 mg, 7 μmol, 70% yield) was isolated as a yellow/orange solid after HPLC separation (0.05% TFA:CH_3CN-system A). ^{1}H NMR (400 MHz, MeOD): 8.41(d, J_1 = 2.44 Hz, 1H), 8.14 (bs, 1H), 7.77 (dd, J_1 = 8.76 Hz, J_2 = 2.44 Hz, 1H), 7.19 (s, 4H), 7.13 (d, J_1 = 8.80 Hz, 1H), 5.95 (d, J_1 = 6.24 Hz, 1H), 4.44 (bs, 1H), 4.36 (bs, 1H), 3.72-3.56 (m, 2H), 3.23-3.15 (m, 3H), 2.97 (t, J_1 = 7.48 Hz, 2H), 2.95- 2.82 (m, 4H), 2.12 (s, 3H), 1.07 (t, J_1 = 7.24 Hz, 3H). m/z (M+ESI MS) found: 678.2599; calc for $C_{31}H_{36}N_9O_9$: 678.2636.

4-(3-(4-(2-((6-Amino-9-((2*R*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-2-nitrophenyl 5-azidopentanoate (3b)

Compound **3b** was prepared from **5** and 5-azidopentanoic acid using a procedure similar to the synthesis of **3c**. The product was isolated (2 µmol, 2.2 mg, 30% yield) after HPLC separation (0.05% TFA:CH_3CN-System B). ^{1}H NMR (400 MHz, MeOD): 8.37(d, J_1 = 2.48 Hz, 1H), 8.05 (bs, 1H), 7.60 (dd, J_1 = 9.00 Hz, J_2 = 2.56 Hz, 1H), 7.18 (s, 4H), 7.05 (d, J_1 = 9.00 Hz, 1H), 5.90 (d, J_1 = 5.64 Hz, 1H), 4.54 (d, J_1 = 3.64 Hz, 1H), 3.66-3.52 (m, 2H), 3.21-3.14 (m, 4H), 2.93 (t, J_1 = 7.64 Hz, 2H), 2.84 (t, J_1 = 7.16 Hz, 2H), 2.60 (t, J_1 = 7.60 Hz, 2H), 2.48 (t, J_1 = 7.84 Hz, 2H), 1.73-1.58 (m, 4H), 1.27 (t, J_1 = 7.20 Hz, 2H), 0.97 (t, J_1 = 6.00 Hz, 3H). m/z (M+ESI MS) found:761.3115; calc for $C_{34}H_{41}N_{12}O_9S$: 761.3114.

4-(3-(4-(2-((6-Amino-9-((2*R*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-2-nitrophenyl 5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate (3c)

Compound **5** (4 mg, 6 µmol, 1 eq), biotin (1.5 mg, 6 µmol, 1 eq), DCC (1.3 mg, 6 µmol, 1 eq) and DMAP (1 mg, 7 µmol, 1.2 eq) were dissolved in DMF (0.5 mL) in a 5 mL round bottom flask. The reaction mixture was stirred over night at room temperature. After the reaction was judged complete by TLC (silica, EtOAc:hexanes, 30:70), the mixture was added to 2 mL CH_2Cl_2 and washed with water and brine, and the organic layer was dried over Na_2SO_4. Solvent was removed, and the product **3c** (3.1 mg, 3 µmol, 50% yield) was isolated as a yellow/orange solid after HPLC separation (0.05% TFA:CH_3CN-System B). ^{1}H NMR (400 MHz, MeOD): 8.41(d, J_1 = 2.56 Hz, 1H), 8.08 (bs, 1H), 7.76 (dd, J_1 = 9.00 Hz, J_2 = 2.60 Hz, 1H), 7.16 (s, 4H), 7.06 (d, J_1 = 9.00 Hz, 1H), 5.93 (d, J_1 = 5.48 Hz, 1H), 4.56 (d, J_1 = 3.84 Hz, 1H), 4.43 (m, 1H), 4.24 (m, 1H), 4.17 (m, 1H), 3.72-3.56 (m, 2H), 3.16-3.09 (m, 4H), 2.91 (t, J_1 = 7.20 Hz, 2H), 2.88- 2.82 (m, 3H), 2.62 (m, 3H), 2.47 (t, J_1 = 7.24 Hz, 1H), 2.38 (t, J_1 = 7.04 Hz, 1H), 1.70 (m, 1H), 1.59 (m, 2H), 1.47 (m, 1H), 1.25 (m, 3H), 1.04 (t, J_1 = 7.20 Hz, 1H), 0.97 (t, J_1 = 7.24 Hz, 1H). m/z (M+ESI MS) found: 862.3328; calc for $C_{39}H_{48}N_{11}O_{10}S$: 862.3306.

(2*S*,5*R*)-5-(6-Amino-2-((4-(3-((4-hydroxy-3-nitrophenyl)amino)-3-oxopropyl)phenethyl)amino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide (5)

4-Amino-2-nitrophenol (**4**, 0.04 mmol, 6.16 mg, 2 eq) was added to a solution of **1** (10.9 mg, 0.02 mmol, 1 eq) in DMF (1 mL) under N_2. Then, EDC.HCl (0.1 mmol, 19.1 mg, 5 eq) was added slowly to the above reaction mixture at room temperature and stirring continued overnight. The reaction was complete as judged by analytical TLC (silica, EtOAc:hexanes, 30:70). The solvent was removed and product **5** (7.5 mg, 0.012 mmol, 60% yield) was isolated as a yellow/orange solid after column chromatography (silica gel, $CHCl_3$:MeOH gradient from 100:0 to 70:30). Purity (>95%) was demonstrated by analytical HPLC. Alternately, a final purification by HPLC (system B) was optionally used. ^{1}H NMR (400 MHz, MeOD): 8.36 (d, J_1 = 2.56 Hz, 1H), 8.19 (bs, 1H), 7.60 (dd, J_1 = 9.04 Hz, J_2 = 2.56 Hz, 1H), 7.16 (s, 4H), 7.05 (d, J_1 = 9.04 Hz, 1H), 5.95 (d, J_1 = 6.20 Hz, 1H), 4.44 (bs, 1H), 4.36 (bs, 1H), 3.71-3.56 (m, 2H), 3.23-3.15 (m, 3H), 2.92 (t, J_1 = 7.44 Hz, 2H), 2.87 (t, J_1 = 7.08 Hz, 2H), 2.59 (t, J_1 = 7.64 Hz, 2H), 1.07 (t, J_1 = 7.24 Hz, 3H). m/z (M+ESI MS) found: 636.2482; calc for $C_{29}H_{34}N_9O_8$: 636.2530.

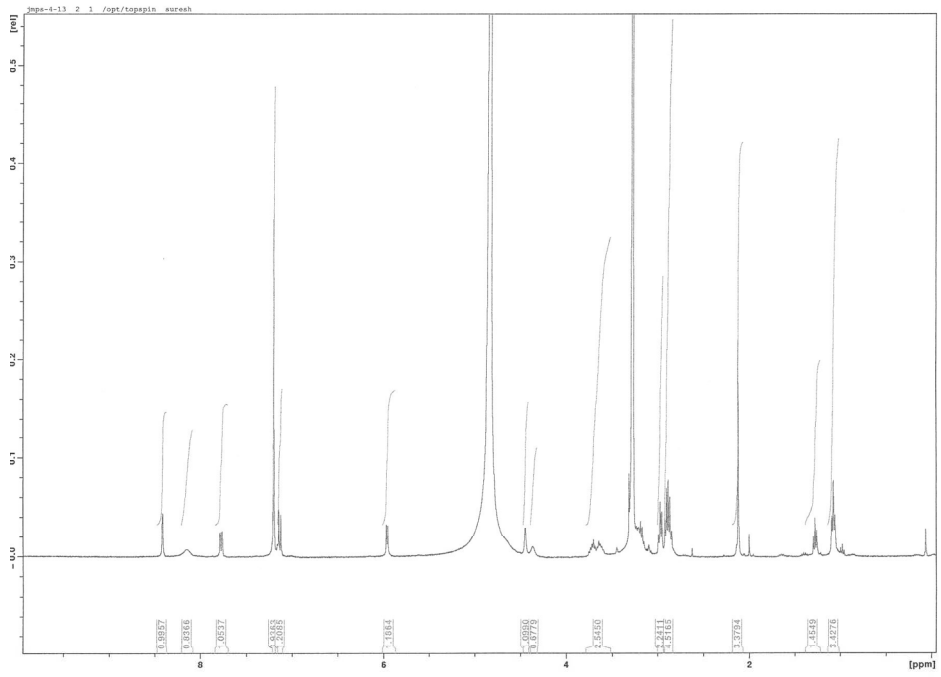
4-Amino-2-nitrophenyl acetate (6a)

4-Amino-2-nitrophenol (**4**, 100 mg, 0.65 mmol, 1 eq) and anhydrous pyridine (4 mL) were added to a round bottom flask under N_2. Acetic anhydride (61 μL, 0.65 mmol, 1 eq) was added to the reaction mixture at room temperature and stirring continued overnight. The solvent was removed by evaporation to dryness, and **6a** was isolated as a yellow/orange solid (38 mg, 30% yield) after column chromatography (silica gel, EtOAc:hexanes: 40:60). ^{1}H NMR (400 MHz, MeOD): 8.22 (d, J_1 = 2.56 Hz, 1H), 7.72 (dd, J_1 = 9.04 Hz, J_2 = 2.56 Hz, 1H), 7.09 (d, J_1 = 9.04 Hz, 1H), 2.16 (s, 3H). m/z (M+ESI MS) found: 197.0558; calc for $C_8H_9N_2O_4$: 197.0557.

NMR and Mass Spectra

4-(3-(4-(2-(((6-Amino-9-((2R,5S)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)- 9H-purin-2-yl)amino)ethyl)phenyl)propanamido)-2-nitrophenyl acetate (3a)

^{1}H NMR



Elemental Composition Report Page 1

Single Mass Analysis
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Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
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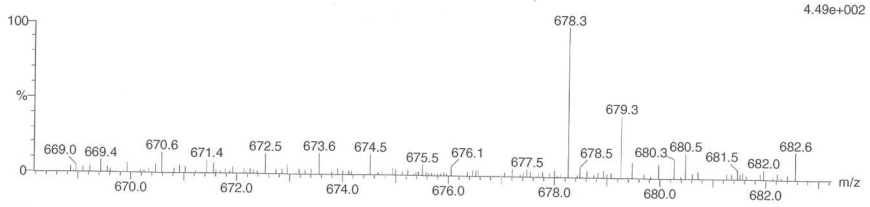
TOF MS ES+



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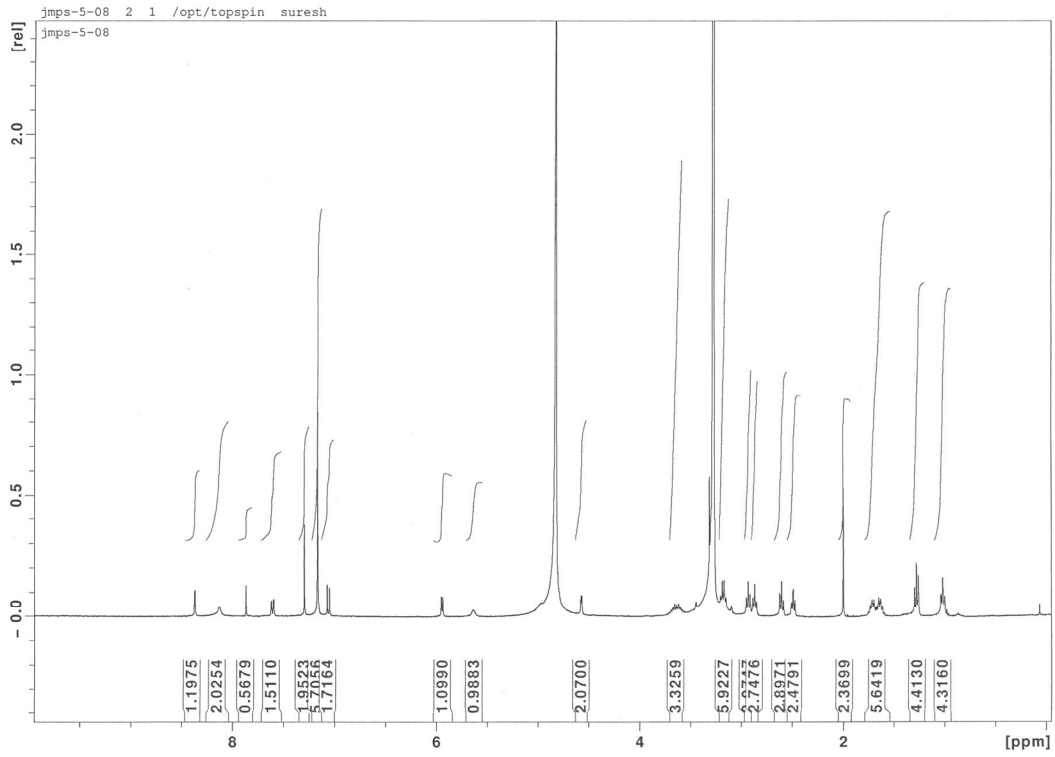
	678.2564	3.5	5.2	22.5	0.7	C37 H36 N5 O8
	678.2636	-3.7	-5.5	18.5	2.1	C31 H36 N9 O9
	678.2537	6.2	9.1	23.5	1.5	C33 H32 N11 O6
	678.2524	7.5	11.1	18.5	1.8	C32 H36 N7 O10
	678.2676	-7.7	-11.4	22.5	1.1	C36 H36 N7 O7
	678.2716	-11.7	-17.3	26.5	2.3	C41 H36 N5 O5

4-(3-(4-(2-((6-Amino-9-((2R,5S)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran 2-yl)-9H-purin-2-yl)amino)ethyl)phenyl)propanamido)-2-nitrophenyl 5-azidopentanoate (3b)

^{1}H NMR



Elemental Composition Report Page 1

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20-Mar-2013 15:31:55
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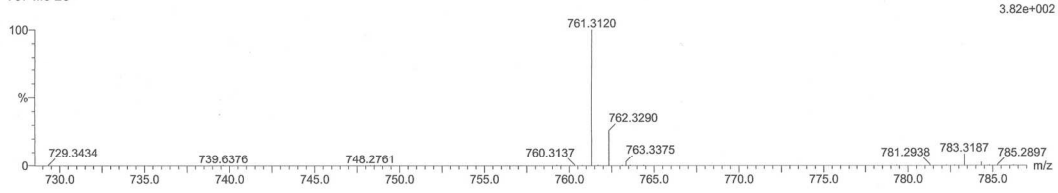
3.82e+002



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Maximum:		8.0	10.0	50.0		
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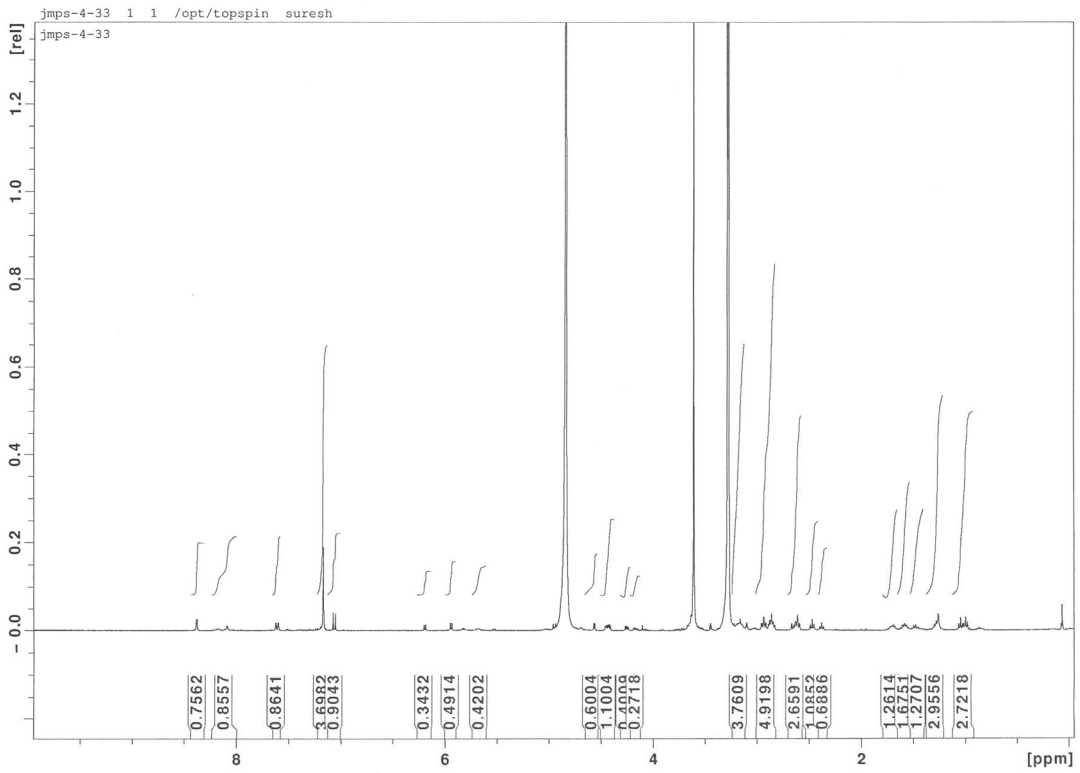
761.3120	761.3119	0.1	0.1	20.5	37.5	C34 H41 N12 O9
	761.3061	5.9	7.7	29.5	54.2	C41 H37 N12 O4

4-(3-(4-(2-((6-Amino-9-((2R,5S)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9H-purin-2-yl)amino)ethyl)phenyl)propanamido)-2-nitrophenyl 5-(2-oxohexahydro-1Hthieno[3,4-d]imidazol-4-yl)pentanoate (3c)



Elemental Composition Report Page

Single Mass Analysis
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Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
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15-Aug-2012 16:05:00
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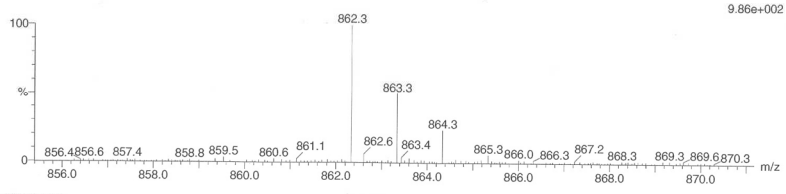

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Maximum:		12.0	10.0	150.0		
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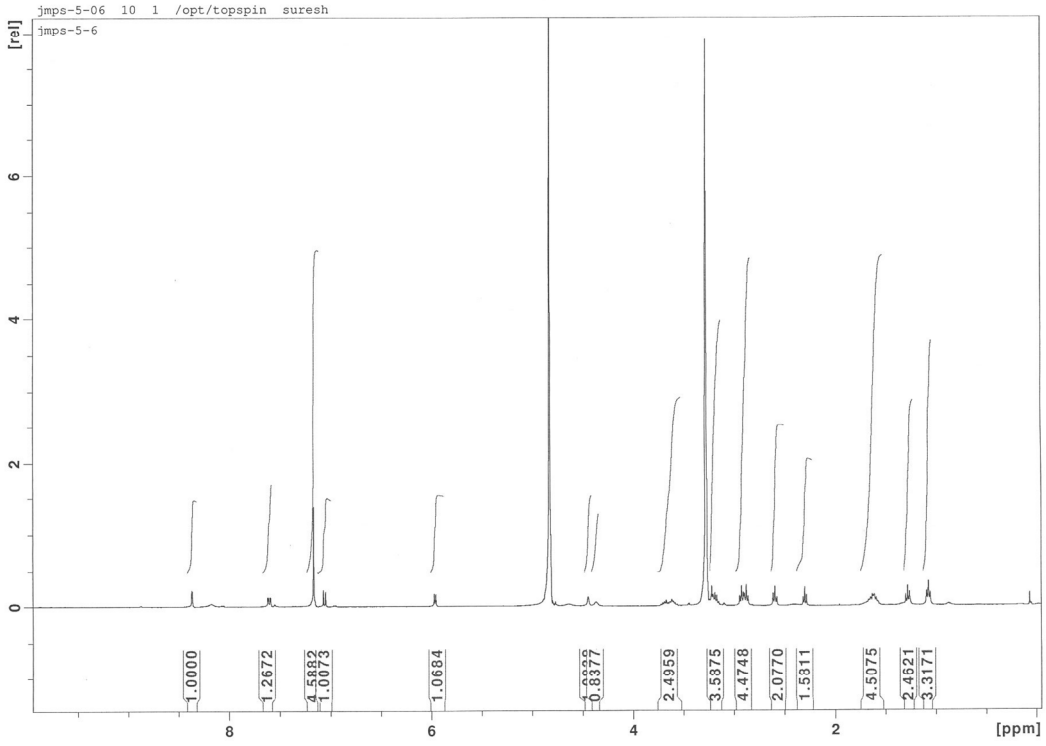
	862.3282	5.4	6.3	18.5	7.3	C37 H49 N11 O10 Na S

(2S,5R)-5-(6-Amino-2-((4-(3-((4-hydroxy-3-nitrophenyl)amino)-3-oxopropyl)phenethyl)amino)-9H-purin-9-yl)-N-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide (5)



lemental Composition Report

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Monoisotopic Mass, Even Electron Ions
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09-May-2012 12:54:44
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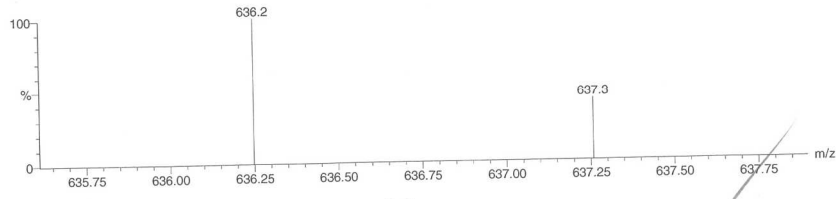

Minimum:				-1.5		
Maximum:		22.0	10.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
636.2482	636.2530	-4.8	-7.5	17.5	n/a	C29 H34 N9 O8
	636.2431	5.1	8.0	22.5	n/a	C31 H30 N11 O5
	636.2418	6.4	10.1	17.5	n/a	C30 H34 N7 O9
	636.2571	-8.9	-14.0	21.5	n/a	C34 H34 N7 O6
	636.2643	-16.1	-25.3	17.5	n/a	C28 H34 N11 O7
	636.2319	16.3	25.6	22.5	n/a	C32 H30 N9 O6
	636.2683	-20.1	-31.6	21.5	n/a	C33 H34 N9 O5
	636.2279	20.3	31.9	18.5	n/a	C27 H30 N11 O8

Molecular modeling: To perform docking studies, we used a $hA_{2A}AR$ crystal structure in complex with the agonist **10** (PDB ID: 2YDV).[12] Among all the available $hA_{2A}AR$ crystallographic structures, this particular structure was selected for several reasons: it is agonist-bound as the ligands explored in this study; it has highest resolution (2.6 Å); it has a completely solved EL2, that is the region we are interested in because is the possible site of interaction for the active ester of our compounds.

The $A_{2A}AR$ structure was prepared using the Protein Preparation Wizard[30] tool implemented in the Schrödinger suite.[31] The orientation of polar hydrogens was optimized, the protein protonation states were adjusted and the overall structure was minimized with harmonic restraints on the heavy atoms, to remove strain. Hetero groups and water molecules were deleted.

Ligand structures were prepared for docking using the build panel and the LigPrep panel implemented in the Schrödinger suite. Molecular docking of ligands at the $hA_{2A}AR$ crystal structure was performed by means of the Glide[32] package from the Schrödinger suite. In particular, a Glide Grid was centered on the centroid of the co-crystallized ligand. The Glide Grid was built using an inner box (ligand diameter midpoint box) of 10 Å x 16 Å x 10 Å and an outer box that extended 30 Å in each direction from the inner one. Docking of ligands was performed in the rigid binding site using the XP (extra precision) procedure. The top scoring docking conformations for each ligand were subjected to visual inspection and analysis of protein-ligand interactions to select the proposed binding conformations in agreement with the experimental data.

Additional references:

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Pharmacological procedures

Transfections: Transient transfections of the wild-type (WT) and mutant A_{2A}AR were performed in COS-7 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Following the transfection according to the manufacturer's instructions, the membranes were harvested[21] and stored at -80°C.

Membrane binding assays: Radioligand binding was performed using mammalian membranes expressing the hA_1, hA_{2A} or hA_3AR in a competitive model following published procedures.[21] Effects of preincubation of membranes with various agonist ligands on A_{2A}AR binding was examined in detail. 10 μg of membrane from HEK-293 cell lines overexpressing the A_{2A}AR were used per test tube. The membrane was incubated separately with a 1 μM concentration of the various *o*-nitrophenyl ester derivatives or various control compounds diluted with Tris-HCl buffer (50 mM, pH 7.5) containing 10 mM $MgCl_2$. This was done for 30 min at 25°C in an ultracentrifuge tube. The mixture was then centrifuged at 65,000 x g and 4°C for 25 min. The supernatant was then removed and the pellet was resuspended in 10 mL of the Tris-HCl buffer, and the suspension was incubated for 30 min and 25°C. The centrifugation and resuspension was repeated twice or until there were only trace amounts of nucleoside derivative remaining in the supernatant. The final supernatant was subjected to an A_{2A}AR radioligand binding assay to confirm that that there was no significant binding activity remaining. After removing excess nucleoside derivative, the remaining membranes were resuspended and tested at 25°C against either a single concentration of the antagonist radioligand [^{3}H]4-[2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-a][1,3,5]triazin-5-yl-amino]ethylphenol **7** ([^{3}H]ZM241385, American Radiolabeled Chemicals, St. Louis, MO, 50 Ci/mmol)[26] or agonist radioligand [^{3}H]CGS21680 **1** (39 Ci/mmol, American Radiolabeled Chemicals).[27] Alternately, the membranes were subjected to a saturation experiment with concentrations ranging from 0.1 to 10 nM of antagonist radioligand. To control for loss of protein during the centrifugation steps, the protein concentration was measured again at the end of the procedure. Any variations on this procedure are noted in the figure legends.

Whole cell preincubation with nucleoside derivatives followed by radioligand binding assay of membranes: HEK-293 cell lines overexpressing the A_{2A}AR were grown in 20 mm tissue culture dishes until they reached 80-90% confluency. The media was replaced with fresh media containing a 1 μM concentration of various nucleoside derivatives or controls xand incubated for 1 h at 37°C. Following the incubation, the membranes were harvested following previously published procedures[21] and the membranes were tested tested in saturation binding assays in the same manner as above.

Cyclic AMP accumulation assay: Intracellular cAMP levels were measured with a competitive protein binding method.[28] CHO cells expressing the recombinant hA_{2A}AR were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 μmol/ml glutamine. Cells were plated in 96-well plates in 100 μl medium. After 24 h, the medium was removed and cells were washed three times with 100 μl DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated for 30 min with agonists at 37ºC in the presence of rolipram (10 μM) and adenosine deaminase (3 units/ml). The reaction was terminated by removal of the supernatant, and cells were lysed upon the addition of 100 μl of lysis buffer (0.3% Tween-20). For determination of cAMP production, an AlphaScreen cAMP kit (PerkinElmer, Waltham, MA) was used according manufacturer's instructions.

Statistical analysis: Binding and functional parameters were calculated using Prism 5.0 software (GraphPAD, San Diego, CA, USA). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng-Prusoff equation.[29] Data were expressed as mean ± standard error of the mean.

Fig. S1. Stimulation of cAMP formation by carboxylic acid **1** and acetyl ester **3a** in CHO cells stably expressing the $hA_{2A}AR$. EC_{50} values (nM) are: **1**, 5.86±1.29; **3a**, 2.12±0.81.

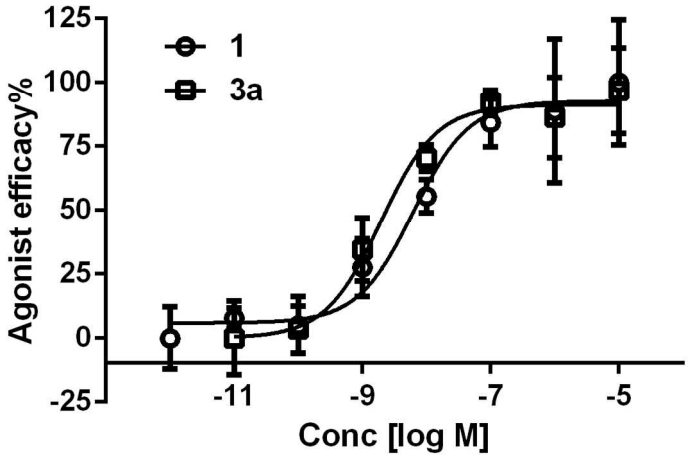


Fig. S2. Results of antagonist ([^{3}H]**7**) radioligand saturation binding experiments with HEK-293 cells overexpressing the WT A_{2A}AR (A) following cell membrane preincubation with nucleoside derivatives or control. Pharmacological parameters (K_d and B_{max} values) from these curves are shown in Table 2A.

A

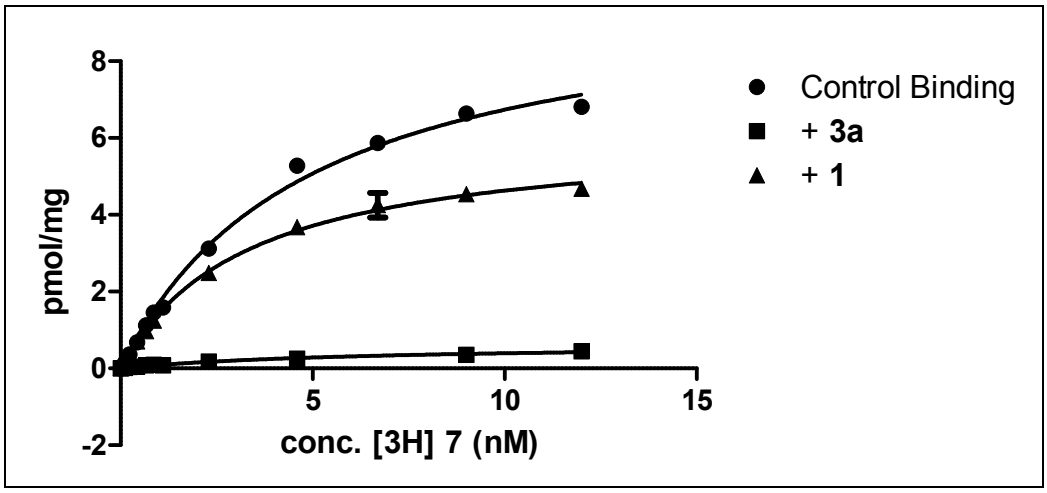
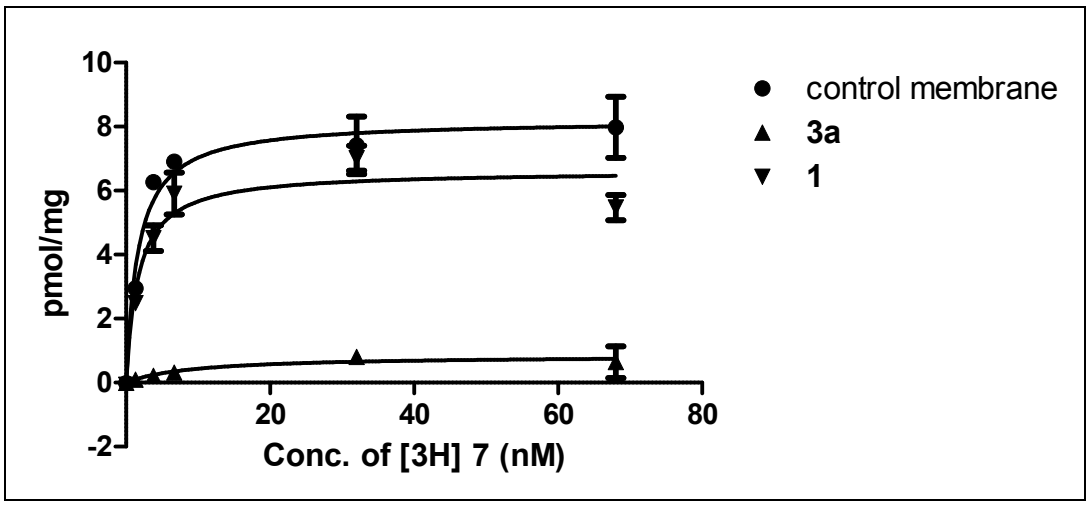


Fig. S3. Radioligand ([^{3}H]**7**) saturation binding experiments in membranes of HEK-293 cells overexpressing the WT A_{2A}AR following whole cell preincubation with nucleoside derivatives or control (A). The accompanying table (B) shows the comparative K_d and B_{max} values post-treatment for the various compounds.

A

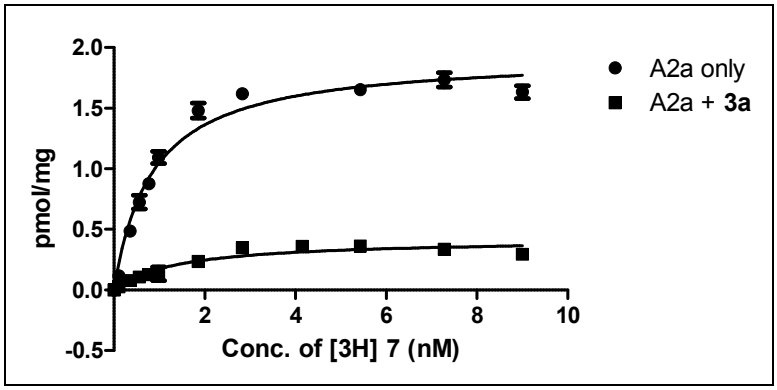


B

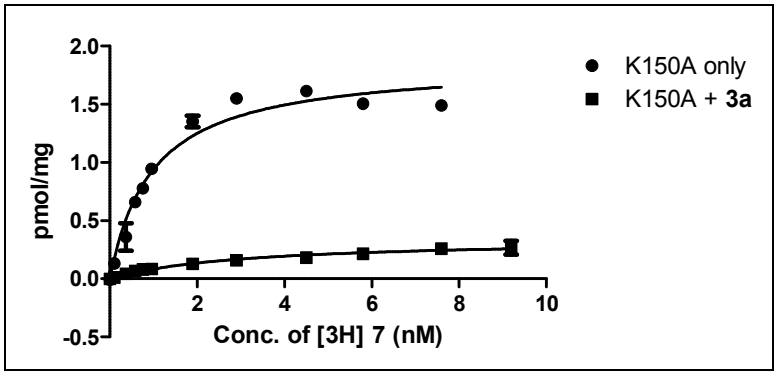
Compound	K_d (nM)	B_{max} (pmol/mg)
WT	1.67	8.20
WT + 3a	9.43	0.85
WT + 1	1.69	6.62

Fig. S4. Radioligand saturation curves with WT (A), K150A (B) and K153A (C) $A_{2A}AR$ mutant receptors, using an antagonist radioligand, [^{3}H]**7** following cell membrane preincubation with nucleoside derivative **3a** or control. Transfections were performed on COS-7 cells as described in the methods section. Pharmacological parameters (K_d and B_{max} values) from these curves are shown in Table 2B.

A



B



C

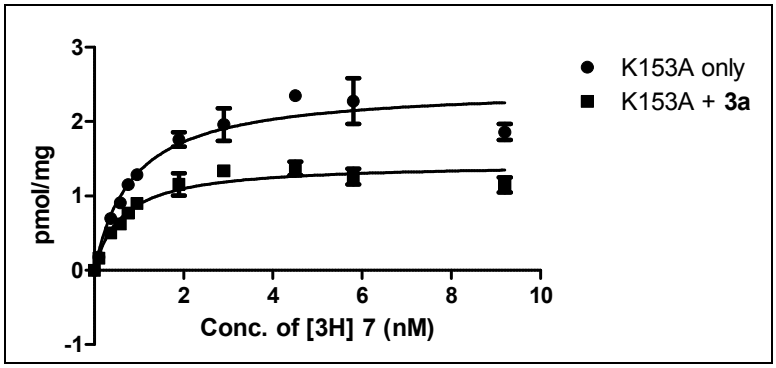
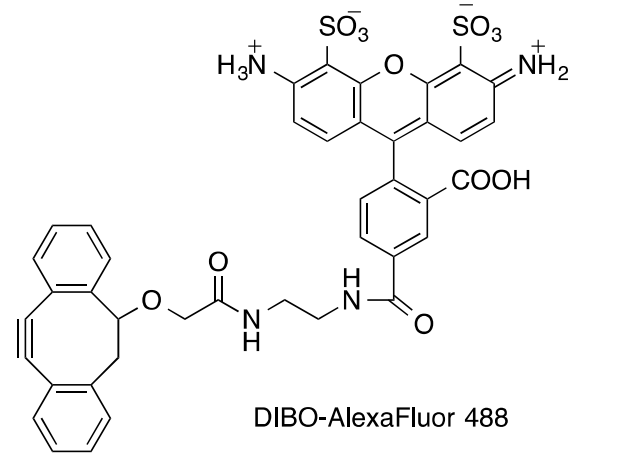
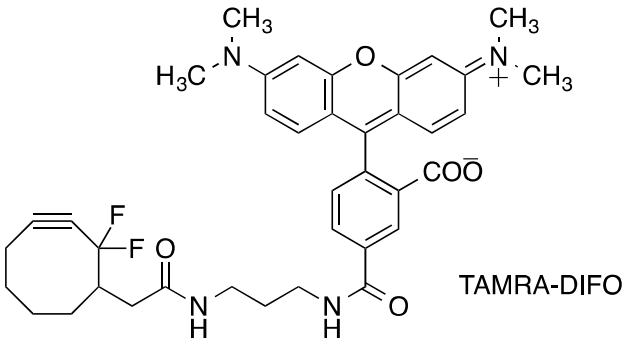


Fig. S5. Structures of the cyclooctane reagents for Cu-free click reactions.



DIBO-AlexaFluor 488



TAMRA-DIFO