Supporting Information:

Evaluation of Molecular Modeling of Agonist Binding in Light of the Crystallographic Structure of an Agonist-Bound A_{2A} Adenosine Receptor

Francesca Deflorian, T. Santhosh Kumar, Khai Phan, Zhan-Guo Gao, Fei Xu, Huixian Wu, Vsevolod Katritch, Raymond C. Stevens, and Kenneth A. Jacobson

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Table S1. Mutational Analysis of Residues Involved in Agonist Binding at the A_{2A} AR (Xu et al., 2011) and Comparison with the Predicted Agonist Binding Mode (Ivanov et al., 2009).

Position/ residue	A_{2A}AR (or other as noted) mutational results	Modeling prediction, with **4** or **14**	X-ray structure, with **1**
TM 1			
1.35	Y9		Forming the subpocket for the C2 substituent. The OH group is connected to the amide carbonyl oxygen of **1** C2-side chain through a water molecule (wat1204)
TM2			
2.61	A63		Forming the subpocket for the C2 substituent. The backbone CO group is H-bonded to the water molecule (wat1204) interacting with the amide carbonyl oxygen of **1**
2.64	I66A: reduced ZM241385 binding[12]		Forming the subpocket for C2 substituent, it is close to F168, A63, wat1204, and the C2-side chain (d=4.22Å)
2.65	S67		Located in the C2 substituent subpocket, in proximity from the C2-side chain of **1** (d=4.20Å)
TM3			
3.32	V84A/D: loss of ag and ant binding[4] V84L: reduction in ant affinity, no effect on ag [4]	Located in proximity of the ribose ring of **4** and **14**	Close hydrophobic contacts with ribose ring (d=4.10Å) of **1**
3.33	L85A: significantly reduced ZM241385[12]	Located in proximity of the ethylcarboxamide group of **14**	Located in proximity of the ribose ring (d=4.10Å) and the 5'-ethylcarboxamide group (d=3.93Å) of **1**
3.36	T88A/R/S: decrease of ag activity, no	The side chain OH-group located at	H-bond interaction between the OH

	effect on ant[5]	the distance of 4.5Å from the NH-group of the ethylcarboxamide moiety of NECA **14**.	group of T88 and the ethylcarboxamide NH group of **1**
3.37	Q89A: marginal decrease in ag and ant affinity[5] Q89L/N/S: marginal increase in ag affinity Q89H/R: affected ant affinity[5] Q89D: increase in ag but not ant activity[4]		Located in proximity of the ethylcarboxamide group of 1 (d=3.95 Å), interacts with N181 (d=3.47 Å)
3.40	I92		Forming the 5'-group subpocket, it is close to the ethylcarboxamide group of **1** (d=3.32 Å)
EL2			
	L167A: reduced ZM241385 binding[12]		Located in proximity of the C2-side chain of **1**
	F168A: loss of ag and ant binding[11] F168W/Y: moderate effects[11]	π-π stacking with the adenine ring of **4** and **14**	aromatic stacking with the adenine ring of **1**
	E169A: loss of ag and ant binding[6] E169Q: gain in N^6-substituted ag affinity[6]	H-bond interaction with the N^6 amino group of **4** and **14**	H-bond interaction with the urea group of **1**; H-bond interaction with the N^6 amino group of NECA and ADO in the crystal structures of NECA-A_{2A}AR and ADO-A_{2A}AR[10]
TM5			
5.35	M174		Located in the pocket for the N6 substituent of **1** (d=4.71 Å)
5.38	M177A: moderate reduced ant affinity; no significant effect on ag affinity[11]		Located close to the C^8 position of the adenine ring (d=3.32 Å) and the 5' substituent of **1** (d=4.13 Å)
5.42	N181A: significant reduced ant binding[12] N181S: modest reduction of ago affinity[7]		Located in the 5'-substituent subpocket close to the

	N181D: enhanced affinity of ag[8]		ethylcarboxamide group of **1** (d=3.32 Å)
5.46	C185		Located in the 5'-substituent subpocket
5.47	V186		Located in the 5'-substituent subpocket
TM6			
6.48	In A3, W243A/F mutant decrease ant affinity	Located close to the **4** and **14**, the indole ring moves outward from **14** after docking	"toggle-switch", close to the 5'-substituent of **1** (d=3.50 Å)
6.51	L249A: loss of ag and ant binding[11]		Non-polar contact with the adenine moiety (d=4.03 Å) and the ribose ring (d=3.65 Å) of **1**
6.52	H250A: no detectable binding[4,7] H250F/Y/N: modest changes in ag affinity; no effect on antagonist binding[7,4]	In proximity of the 5'-substituent of **4** and **14**	H-bond interaction with the amide carbonyl group of the 5'-substituent of **1**
6.54	I252		Located in the N^6-substituent hydrophobic subpocket (d=4.76 Å)
6.55	N253A/D: loss of ag and ant binding[7,8,12]	The oxygen atom of the side chain amido group is H-bonded to the ligand N^6-amino group; the NH_2-group of the amido group is H-bonded to the N^7-nitrogen atom of the adenine ring of **4** and **14**	The oxygen atom of the side chain amido group is H-bonded to the ligand N^6-amino group; the NH_2-group of the amido group is H-bonded to the N7-nitrogen atom of the adenine ring.
6.58	T256		Located in close proximity from the N^6 phenyl substituent of **1** (d=3.41 Å)
TM7			
7.32	L267		Non-polar interactions with the piperidine ring at the C2-substituent (d=3.74 Å) of **1**
7.35	M270		Non-polar interactions with the

			piperidine ring (d=3.66 Å) at the C2-substituent and the phenyl rings at N6-position (d=3.43 Å)
7.36	Y271A: reduced ant affinity[12]		Polar interaction with the urea moiety of **1**
7.39	I274A: loss of ag and ant affinity[7]		Non-polar interaction with the adenine ring and the C2-substituent of **1**
7.42	S277A: substantial reduction in only ag activity and potency[7] S277C/N/E/T: moderate changes in ligand affinity[5,6,7]	H-bond with 5'-OH of **4** and the 5'-ethylcarboxamide group of **14**; in close proximity to 3'-OH of **4** and **14**;	H-bond interaction with 3'-OH group
7.43	H278A: loss of ag and ant binding[7] H278D/E: marginal changes in binding[7] H278Y: modest reduction of ag affinity[9]	Imidazole of His278 can form H-bonds with 2′- and 3′-OH groups of the ligand ribose ring.	H-bond interaction with 2'-OH group of **1**

Figures

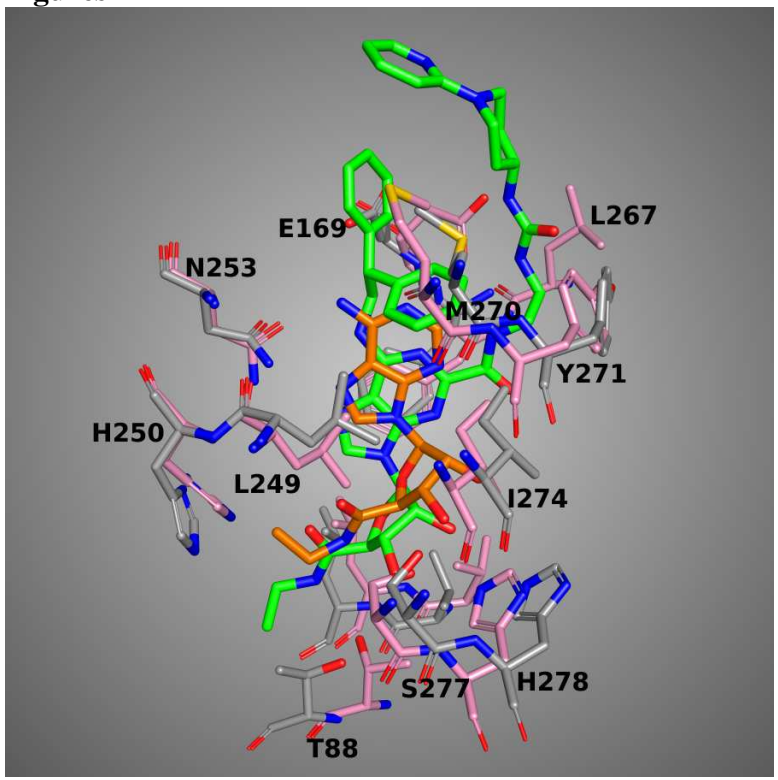


Figure S1. Details of UK-432097 binding site at the UK-A_{2A}AR structure and the previous NECA model in the antagonist-bound A_{2A}AR binding site after superimposition of the critical amino acid residues in the binding sites of the A_{2A}AR structures. The carbon atoms of residues of the UK-A_{2A}AR structure are shown in pink while UK-432097 carbons are colored in green. The C-atoms of residues of the NECA model in the antagonist-bound A_{2A}AR structure are shown in grey and the carbons of the docked pose of NECA are in orange.

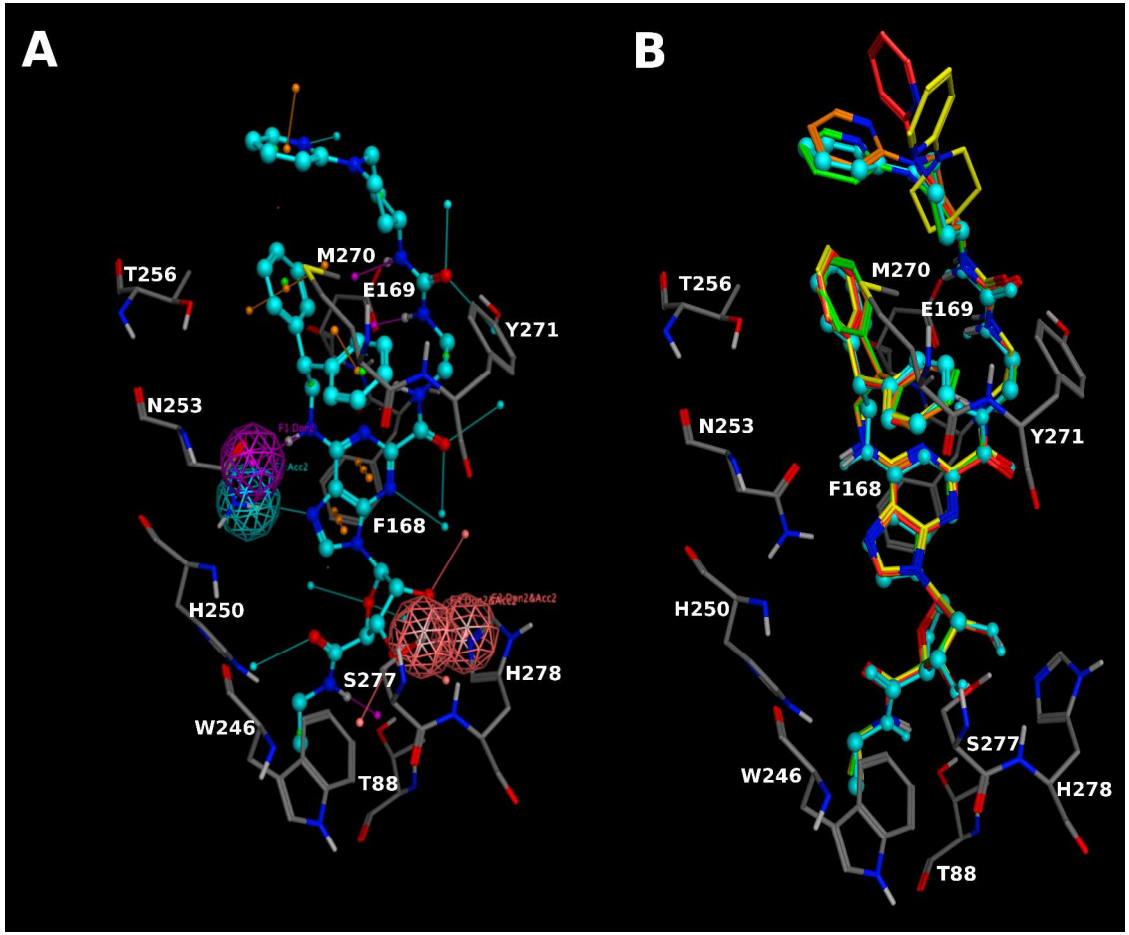


Fig. S2: Panel A: MOE pharmacophore query used for the MOE docking of UK-432097 in the binding site of the UK-A_{2A}AR structure. The pharmacophore features are represented as colored spheres. The amide group of Asn253 was defined as H-bond acceptor at the carbonyl group and H-bond donor at the amine group, while the Nε of His278 and the OH of Ser277 were considered

both H-bond donor and/or acceptor. The co-crystallized UK-432097 is shown with the ball-and-stick representation with Carbon atoms colored in cyan. Panel B: best MOE docking poses of UK-432097 to the UK-A_{2A}AR, represented as sticks with the carbons colored green, orange, red, and yellow. For comparison, the co-crystallized UK-432097 is shown as ball-and-stick with all the atoms colored in cyan. The residues in the binding cavity of UK-A_{2A}AR are shown colored with the C-atoms in grey.

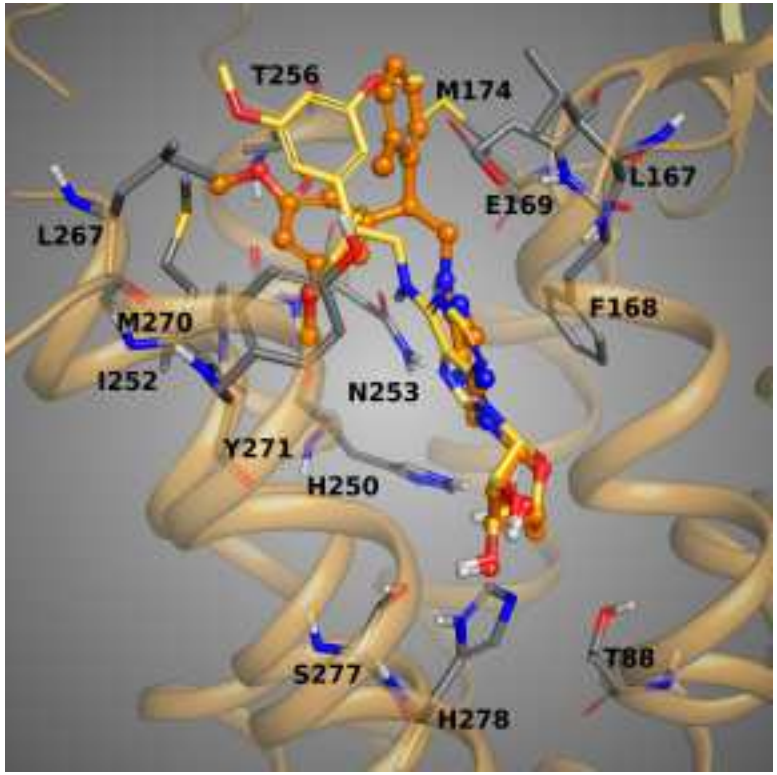


Fig. S3: Docking poses of **12** in the binding site of the UK-A_{2A}AR structure: the (S) enantiomer of **12** is represented as ball-and-stick with carbon atoms colored in orange. For comparison the docking pose of the (R) enantiomer of **12** is shown with the stick representation and carbons in yellow. The H-bond interactions between N253 and the adenine moiety of (S)-**12** are not as strong as for (R)-12 due to the suboptimal orientation of the bulky N^6 substituent in the (S) enantiomer docking pose.

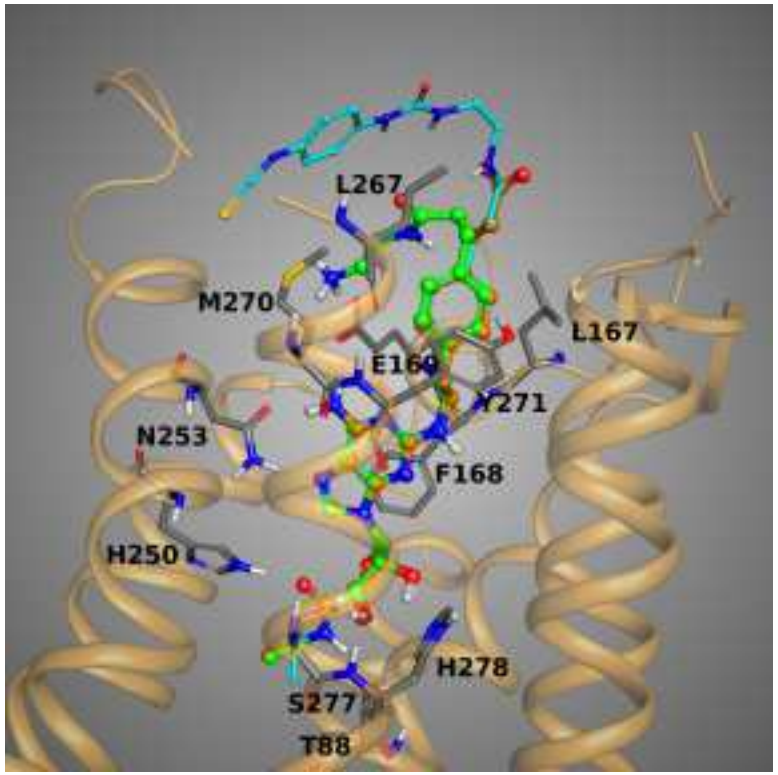
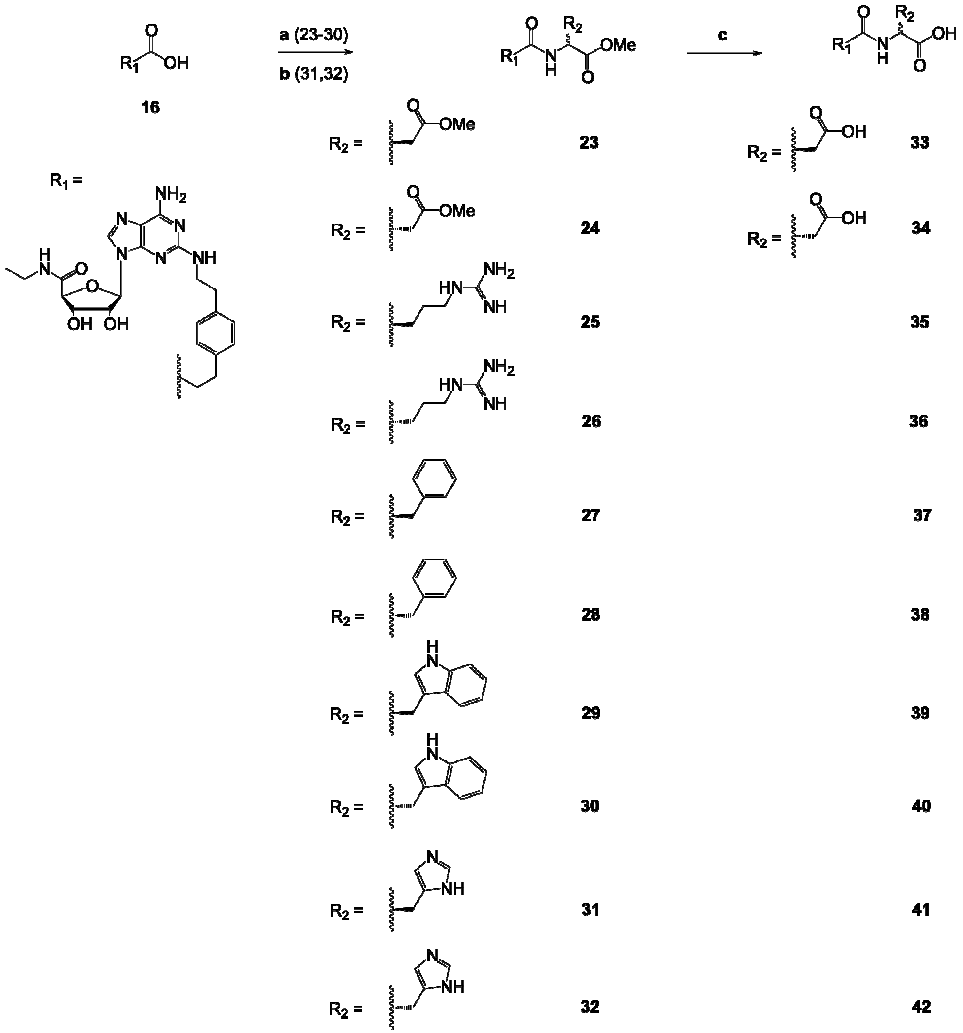


Fig. S4: Docking poses of **16** (with carbon atoms colored in orange), **17** (with carbons in green), and **18** (with carbons colored in cyan) in the binding site of the UK-A_{2A}AR structure. The lack of a portion of EL2 containing the positively charged residues K150 and K153 made the top opening of the binding cavity wide open leading to docking poses where the long and flexible C2 side chains of 16, 17, and 18 are freely oriented in the extracellular solvent environment.

References:

1. Ballesteros, J.; Weinstein, H. Integrated methods for the construction of three-dimensional models of structure-function relations in G protein-coupled receptors. *Methods Neurosci.* **1995**, *25*, 366-428.
2. Kim, S.-K.; Gao, Z.-G.; Van Rompaey, P.; Gross, A. S.; Chen, A.; Van Calenbergh, S.; Jacobson, K. A. Modeling the adenosine receptors: comparison of the binding domains of A_{2A} agonists and antagonists. *J. Med. Chem.* **2003**, *46*, 4847-4859.
3. IJzerman, A. P.; Von Frijtag Drabbe Künzel, J. K.; Kim, J.; Jiang, Q.; Jacobson, K. A. Site-Directed Mutagenesis of the Human Adenosine A_{2A} Receptor. Critical Involvement of Glu13 in Agonist Recognition. *Eur. J. Pharmacol*. **1996**, *310*, 269-272.
4. Jiang, Q.; Lee, B. X.; Glashofer, M.; van Rhee, A. M.; Jacobson, K. A. Mutagenesis reveals structure-function parallels between human A_{2A}-adenosine receptors and the biogenic amine family. *J. Med. Chem*. **1997**, *40*, 2588-2595.
5. Jiang, Q.; van Rhee, M.; Kim. J.; Yehle, S.; Wess, J.; Jacobson, K. A. Hydrophilic side chains in the third and seventh transmembrane helical domains of human A_{2A} adenosine receptors are required for ligand recognition. *Mol. Pharmacol*. **1996**, *50*, 512-521.
6. Kim, J.; Jiang, Q.; Glashofer, M.; Yehle, S.; Wess, J.; Jacobson, K. A. Glutamate residues in the second extracellular loop of the human A_{2a} adenosine receptor are required for ligand recognition. *Mol. Pharmacol*. **1996**, *49*, 683-691.
7. Kim, J.; Wess, J.; van Rhee, M.; Schöneberg, T.; Jacobson, K. A. Site-directed mutagenesis identifies residues involved in ligand recognition in the human A_{2A} adenosine receptor. *J. Biol. Chem.* **1995**, *270*, 13987-13997.
8. Jacobson, K.A.; Ohno, M.; Duong, H.T.; Kim, S.K.; Tchilibon, S.; Cesnek, M.; Holy, A.; Gao, Z.G. A neoceptor approach to unraveling microscopic interactions between the human A_{2A} adenosine receptor and its agonists. *Chemistry and Biology* **2005**, 12, 237-247.
9. Gao, Z.-G.; Jiang, Q.; Jacobson, K. A.; IJzerman, A. P. Site-Directed Mutagenesis Studies of Human A_{2A} Adenosine Receptors. Involvement of Glu13 and His278 in Ligand Binding and Sodium Modulation. *Biochem. Pharmacol.* **2000**, *60*, 661-668.
10. Gao, Z.-G.; Chen, A.; Barak, D.; Kim, S.-K.; Müller, C. E.; Jacobson, K. A. Identification by Site-directed Mutagenesis of Residues Involved in Ligand Recognition and Activation of the Human A_3 Adenosine Receptor. *J. Biol. Chem*. **2002**, *277*, 19056-19063
11. Jaakola, V.P.; Lane, J.R.; Lin, J.Y.; Katrich, V.; IJzerman, A.P.; Stevens, R.C. Identification and characterization of amino acid residues essential for human A_{2A} adenosine receptor: ZM241385 binding and subtype selectivity. *J. Biol. Chem*. **2010**, *285*, 13032–13044.
12. Zhukov, A.; Andrews, S.P.; Errey, J.C.; Robertson, N.; Tehan, B.; Mason, J.S.; Marshall, F.H.; Weir, M.; Congreve, M. Biophysical mapping of the adenosine A_{2A} receptor. *J. Med. Chem.* **2011**, *54*, 4312-4323.

Scheme 1. Synthesis of amino acid conjugates of CGS21680 **16**.



Reagents and Conditions: a) *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl), corresponding amino acid, anhydrous DMF, rt; b) *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), Corresponding amino acid, *N*,*N*-diisopropylethylamine, anhydrous DMF, rt; c) 0.1 M aqueous NaOH, MeOH, rt.

Synthetic description:
Commercially available nucleoside carboxylic acid **16** (CGS21680, Tocris, Ellisville, MO) was coupled to various carboxylate-protected amino acids using either EDC.HCl (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) or HATU (2-(1H-7-azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium) as coupling agent in anhydrous DMF to obtain the corresponding amino acid coupled methyl ester derivatives **23**-**32**. Subsequently, methyl ester was hydrolyzed using 0.1M aqueous NaOH to give the target carboxylic acid derivatives **33**-**42**.

General Experimental section:
All reagents and solvents (regular and anhydrous) were of analytical grade and obtained from commercial suppliers and used without further purification. Reactions were conducted under an atmosphere of nitrogen whenever anhydrous solvents were used. All reactions were monitored by thin-layer chromatography (TLC) using silica gel coated plates with a fluorescence indicator which were visualized: (a) under UV light, (b) by dipping in 5% conc H_2SO_4 in absolute ethanol (v/v) followed by heating, or (c) by dipping in a solution of anisaldehyde: H_2SO_4 (1:2, v/v) in MeOH followed by heating. Silica gel column chromatography was performed with silica gel (SiO_2, 200-400 mesh, 60Å) using moderate air pressure. Evaporation of solvents was carried out under reduced pressure at a temperature below 50 ºC. After column chromatography, appropriate fractions were pooled, evaporated, and dried at high vacuum for at least 12 h to give the obtained products in high purity. ^{1}H NMR and analytical HPLC (>95%) ascertained sample purity. No corrections in yields were made for solvent of crystallization. ^{1}H NMR spectra were recorded at 300 MHz. Chemical shifts are reported in parts per million (ppm) relative to deuterated solvent as the internal standard. ESI-High resolution mass spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine. Observed mass accuracies are those expected on the basis of known performance of the instrument as well as the trends in masses of standard compounds observed at intervals during the series of measurements. Reported masses are observed masses uncorrected for this time-dependent drift in mass accuracy.

Purification of the compounds **33**-**36** and **41**, **42** for biological testing was performed by HPLC with a Luna 5 µ RP-C18(2) semipreparative column (250 mm × 10.0 mm; Phenomenex, Torrance, CA) under the following conditions: flow rate of 2 mL/min,

0.5% trifluoroacetic acid in H_2O-CH_3CN from 100:0 (v/v) to 50:50 (v/v) in 32 min. Analytical purity of compounds was checked using a Hewlett-Packard 1100 HPLC equipped with Zorbax SB-Aq 5 μm analytical column (50 mm × 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 254, 275, and 280 nm. All derivatives tested for biological activity showed >99% purity by HPLC analysis (detection at 254 nm).

Coupling of the amino acid:

Method A. Nucleoside **14** (1.0 molar equivalents) was coevaporated with anhydrous toulene (2 × 5 mL) and dissolved in anhydrous DMF (1 mL). *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl, 2.0 molar equivalents) and corresponding amino acid (2.0 molar equivalents) were added. After stirring at rt for 6-18 h, the reaction mixture was diluted with distilled H_2O (20 mL) and extracted with EtOAc (4 × 25 mL). The combined organic phase was evaporated to dryness and the resulting crude residue purified by silica gel column chromatography (MeOH in CH_2Cl_2, v/v) to afford amino acid coupled nucleosides **23-30**.

Method B: Nucleoside **7** (1.0 molar equivalents), *O*-(7-Azabenzotriazole-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HATU, 2.0 molar equivalents) and *N*,*N'*-diisopropylethylamine (2.0 molar equivalents) were dissolved in anhydrous DMF (1 mL). Corresponding amino acid (2.0 molar equivalents) was added to this reaction mixure. After stirring at rt for 6 h, the reaction mixture was evaporated to dryness and the resulting crude residue was purified by silica gel column chromatography (MeOH in CH_2Cl_2, v/v) to afford amino acid coupled nucleosides **31,32**.

(*S*)-Dimethyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)succinate (23)

Compound purified by silica gel column chromatography (0-15% MeOH in CH_2Cl_2, v/v), R_f = 0.5 (15% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 643.2850 ($[M + H]^+$, $C_{29}H_{38}N_8O_9 \cdot H^+$ Calcd 670.2840; ^{1}H NMR (MeOD) δ 7.99 (s, 1H), 7.12-7.22 (s, 4H), 5.94 (d, *J* = 6.5 Hz, 1H), 5.01 (t, *J* = 5.3 Hz, 1H), 4.76 (t, *J* = 6.7 Hz, 1H), 4.48-4.52 (m, 1H), 4.40 (d, *J* = 2.8 Hz, 1H), 3.71 (s, 3H), 3.67 (s, 3H), 3.59-3.66 (m, 1H), 3.48-3.55 (m, 1H), 3.25-3.29 (m, 1H), 3.09-3.16 (m, 1H), 2.68-2.89 (m, 6H), 2.51 (t, *J* = 8.2 Hz, 2H), 1.04 (t, *J* = 7.3 Hz, 3H).

(*R*)-Dimethyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)succinate (24)

Compound purified by silica gel column chromatography (0-15% MeOH in CH_2Cl_2, v/v), R_f = 0.5 (15% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 643.2866 ($[M + H]^+$, $C_{29}H_{38}N_8O_9 \cdot H^+$ Calcd 670.2840; ^{1}H NMR (MeOD) δ 7.99 (s, 1H), 7.13-7.20 (s, 4H), 5.94 (d, *J* = 6.5 Hz, 1H), 5.00 (t, *J* = 5.5 Hz, 1H), 4.76 (t, *J* = 5.5 Hz, 1H), 4.48-4.52 (m, 1H), 4.40 (d, *J* = 2.8 Hz, 1H), 3.74 (s, 3H), 3.70 (s, 3H),

3.59-3.64 (m, 1H), 3.48-3.55 (m, 1H), 3.25-3.30 (m, 1H), 3.09-3.17 (m, 1H), 2.69-2.91 (m, 6H), 2.51 (t, *J* = 8.2 Hz, 2H), 1.04 (t, *J* = 7.3 Hz, 3H).

(*S*)-Methyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-5-guanidinopentanoate (25)
Compound purified by silica gel column chromatography (0-25% MeOH in CH_2Cl_2, v/v), R_f = 0.3 (20% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 670.3408 ($[M + H]^+$, $C_{30}H_{43}N_{11}O_7 \cdot H^+$ Calcd 670.3425; ^{1}H NMR (MeOD) δ 8.01 (s, 1H), 7.17 (s, 4H), 5.96 (d, *J* = 5.5 Hz, 1H), 4.97 (t, *J* = 5.5 Hz, 1H), 4.52-4.55 (m, 1H), 4.37-4.44 (m, 2H), 3.71 (s, 3H), 3.48-3.65 (m, 2H), 3.15-3.30 (m, 4H), 2.85-2.92 (m, 2H), 2.56 (t, *J* = 8.2 Hz, 2H), 1.85-1.92 (m, 1H), 1.64-1.69 (m, 1H), 1.54-1.61 (m, 2H), 1.11 (t, *J* = 7.3 Hz, 3H).

(*R*)-Methyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-5-guanidinopentanoate (26)
Compound purified by silica gel column chromatography (0-25% MeOH in CH_2Cl_2, v/v), R_f = 0.3 (20% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 670.3405 ($[M + H]^+$, $C_{30}H_{43}N_{11}O_7 \cdot H^+$ Calcd 670.3425; ^{1}H NMR (MeOD) δ 8.01 (s, 1H), 7.16-7.19 (m, 4H), 5.94 (d, *J* = 6.1 Hz, 1H), 4.97 (t, *J* = 5.8 Hz, 1H), 4.52-4.55 (m, 1H), 4.39-4.45 (m, 2H), 3.72 (s, 3H), 3.58-3.67 (m, 1H), 3.51-3.56 (m, 1H), 3.25-3.31 (m, 1H), 3.12-3.19 (m, 3H), 2.84-2.93 (m, 4H), 2.56 (t, *J* = 7.9 Hz, 2H), 1.83-1.89 (m, 1H), 1.62-1.75 (m, 1H), 1.52-1.58 (m, 2H), 1.04 (t, *J* = 7.3 Hz, 3H).

(*S*)-Methyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-phenylpropanoate (27)
Compound purified by silica gel column chromatography (0-15% MeOH in CH_2Cl_2, v/v), R_f = 0.4 (15% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 661.3086 ($[M + H]^+$, $C_{33}H_{40}N_8O_7 \cdot H^+$ Calcd 661.3098; ^{1}H NMR (MeOD) δ 7.99 (s, 1H), 7.07-7.29 (m, 9H), 5.94 (d, *J* = 6.5 Hz, 1H), 5.01 (t, *J* = 6.5 Hz, 1H), 4.66 (dd, *J* = 3.0 Hz, 5.5 Hz, 1H), 4.47-4.51 (m, 1H), 4.40 (d, *J* = 3.0 Hz, 1H), 3.58-3.66 (m, 1H), 3.45-3.54 (m, 1H), 3.24-3.31 (m, 1H), 3.07-3.16 (m, 2H), 2.88-2.96 (m, 1H), 2.87 (t, *J* = 8.3 Hz, 2H), 2.79 (t, *J* = 7.1 Hz, 2H) 2.45 (t, *J* = 8.1 Hz, 2H), 1.03 (t, *J* = 7.3 Hz, 3H). HPLC purity analysis retention time 6.46 min (>95% purity).

(*R*)-Methyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-phenylpropanoate (28)
Compound purified by silica gel column chromatography (0-15% MeOH in CH_2Cl_2, v/v), R_f = 0.4 (15% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 661.3099 ($[M + H]^+$, $C_{33}H_{40}N_8O_7 \cdot H^+$ Calcd 661.3098; ^{1}H NMR (MeOD) δ 7.99 (s, 1H), 7.02-7.30 (m, 9H), 5.94 (d, *J* = 6.5 Hz, 1H), 5.01 (t, *J* = 5.5 Hz, 1H), 4.67 (dd, *J* = 3.0 Hz, 5.5 Hz, 1H), 4.46-4.51 (m, 1H), 4.40 (d, *J* = 3.0 Hz, 1H), 3.64 (s, 3H),

3.59-3.62 (m, 1H), 3.46-3.55 (m, 1H), 3.24-3.30 (m, 1H), 3.09-3.17 (m, 1H), 2.84-3.01 (m, 1H), 2.79 (t, *J* = 8.5 Hz, 2H), 2.44 (t, *J* = 8.1 Hz, 2H), 1.03 (t, *J* = 7.3 Hz, 3H). HPLC purity analysis retention time 6.43 min (>95% purity).

(*S*)-Methyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-(1*H*-indol-3-yl)propanoate (29)

Compound purified by silica gel column chromatography (0-15% MeOH in CH_2Cl_2, v/v), R_f = 0.5 (15% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 700.3206 ($[M + H]^+$, $C_{35}H_{41}N_9O_7 \cdot H^+$ Calcd 700.3207; ^{1}H NMR (MeOD) δ 7.98 (s, 1H), 7.50 (d, *J* = 8.0 Hz, 1H), 7.33 (d, *J* = 8.0 Hz, 1H), 6.98-7.14 (m, 7H), 5.94 (d, *J* = 6.4 Hz, 1H), 5.00 (t, *J* = 5.5 Hz, 1H), 4.74 (dd, *J* = 2.0 Hz, 5.5 Hz, 1H), 4.48-4.52 (m, 1H), 4.40 (d, *J* = 2.8 Hz, 1H), 3.67 (s, 3H), 3.57-3.66 (m, 1H), 3.46-3.52 (m, 1H), 3.22-3.30 (m, 2H), 3.08-3.17 (m, 2H), 2.84 (t, *J* = 7.2 Hz, 2H), 2.78 (t, *J* = 8.5 Hz, 2H), 2.45 (t, *J* = 8.1 Hz, 2H), 1.02 (t, *J* = 8.1 Hz, 3H). HPLC purity analysis retention time 6.57 min (>95% purity).

(*R*)-Methyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-(1*H*-indol-3-yl)propanoate (30)

Compound purified by silica gel column chromatography (0-15% MeOH in CH_2Cl_2, v/v), R_f = 0.5 (15% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 700.3183 ($[M + H]^+$, $C_{35}H_{41}N_9O_7 \cdot H^+$ Calcd 700.3207; ^{1}H NMR (MeOD) δ 7.99 (s, 1H), 7.48 (d, *J* = 7.8 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 6.98-7.14 (m, 7H), 5.94 (d, *J* = 6.5 Hz, 1H), 5.01 (t, *J* = 5.5 Hz, 1H), 4.74 (dd, *J* = 2.0 Hz, 5.5 Hz, 1H), 4.48-4.52 (m, 1H), 4.40 (d, *J* = 2.8 Hz, 1H), 3.62 (s, 3H), 3.56-3.61 (m, 1H), 3.46-3.52 (m, 1H), 3.22-3.30 (m, 2H), 3.08-3.13 (m, 2H), 2.84 (t, *J* = 6.9 Hz, 2H), 2.78 (t, *J* = 6.9 Hz, 2H), 2.45 (t, *J* = 7.9 Hz, 2H), 1.03 (t, *J* = 7.5 Hz, 3H). HPLC purity analysis retention time 6.55 min (>95% purity).

(*S*)-Methyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-(1*H*-imidazol-4-yl)propanoate (31)

Compound purified by silica gel column chromatography (0-30% MeOH in CH_2Cl_2, v/v), R_f = 0.1 (25% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 651.2988 ($[M + H]^+$, $C_{30}H_{38}N_{10}O_7 \cdot H^+$ Calcd 651.3003; ^{1}H NMR (MeOD) δ 8.76 (s, 1H), 8.19 (s, 1H), 7.11-7.21 (s, 5H), 5.99 (d, *J* = 6.1 Hz, 1H), 4.74-4.81 (m, 2H), 4.45-4.49 (m, 2H), 3.74 (s, 3H), 3.57-3.70 (m, 2H), 3.20-3.28 (m, 3H), 3.03-3.10 (m, 1H), 2.91 (t, *J* = 7.5 Hz, 2H), 2.84 (t, *J* = 7.5 Hz, 2H), 2.49 (t, *J* = 7.5 Hz, 2H), 1.10 (t, *J* = 7.5 Hz, 3H).

(*R*)-Methyl 2-(3-(4-(2-((6-amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-(1*H*-imidazol-4-yl)propanoate (32)

Compound purified by silica gel column chromatography (0-30% MeOH in CH_2Cl_2, v/v), R_f = 0.1 (25% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 651.2990 ($[M + H]^+$, $C_{30}H_{38}N_{10}O_7 \cdot H^+$ Calcd 651.3003; ^{1}H NMR (MeOD) δ 8.01 (s, 1H), 7.69 (s, 1H), 7.09-7.18 (s, 4H), 6.84 (s, 1H), 5.96 (d, *J* = 6.2 Hz, 1H), 4.99-5.02 (m, 1H), 4.68 (dd, *J* = 3.1, 5.2 Hz, 1H), 4.51-4.55 (m, 1H), 4.41 (d, *J* = 3.1 Hz, 1H), 3.71 (s, 3H), 3.57-3.67 (m, 1H), 3.48-3.55 (m, 1H), 2.98-3.28 (m, 4H), 2.80-2.88 (m, 4H), 2.49 (t, *J* = 8.2 Hz, 2H), 1.04 (t, *J* = 7.3 Hz, 3H).

Hydrolysis of methyl ester:
Each methyl ester nucleoside (**23-32**) was dissolved in MeOH (1 mL), and 0.1 M aqueous NaOH (1 mL) solution was added to this solution. After stirring the reaction mixture for 2 to 8 h, reaction mixture neutralized with 0.1M HCl and purified either by silica gel column chromatography (**37**-**40**, 0-30% MeOH in CH_2Cl_2, v/v) or by semi preparative HPLC (**33**-**36** and **41**, **42**) to afford desired compounds.

(*S*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)succinic acid (33)
Compound purified by semi preparative HPLC (retention time of the compound is 23.10 min), ESI-HRMS *m/z* 615.2523 ($[M + H]^+$, $C_{27}H_{34}N_8O_9 \cdot H^+$ Calcd 615.2527; ^{1}H NMR (MeOD) δ 8.52 (s, 1H), 7.14-7.23 (s, 4H), 6.05 (d, *J* = 5.7 Hz, 1H), 4.74 (t, *J* = 5.5 Hz, 1H), 4.69 (s, 1H), 4.51-4.54 (m, 1H), 4.44 (s, 1H), 3.64-3.79 (m, 2H), 3.21-3.30 (m, 2H), 2.85-2.95 (m, 4H), 2.75-2.80 (m, 2H), 2.51 (t, *J* = 8.2 Hz, 2H), 1.14 (t, *J* = 8.3 Hz, 3H). HPLC purity analysis retention time 4.96 min (>95% purity).

(*R*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)succinic acid (34)
Compound purified by semi preparative HPLC (retention time of the compound is 23.16 min), ESI-HRMS *m/z* 615.2509 ($[M + H]^+$, $C_{27}H_{34}N_8O_9 \cdot H^+$ Calcd 615.2527; ^{1}H NMR (MeOD) δ 8.25 (s, 1H), 7.16-7.25 (s, 4H), 6.02 (d, *J* = 6.1 Hz, 1H), 4.72 (t, *J* = 6.1 Hz, 1H), 4.64-4.69 (m, 1H), 4.49 (s, 1H), 4.43 (s, 1H), 3.70-3.80 (m, 1H), 3.61-3.70 (m, 1H) 3.20-3.27 (m, 2H), 2.85-2.95 (m, 4H), 2.75-2.80 (m, 2H), 2.50 (t, *J* = 7.9 Hz, 2H), 1.14 (t, *J* = 8.3 Hz, 3H). HPLC purity analysis retention time 4.16 min (>95% purity).

(*S*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-5-guanidinopentanoic acid (35)
Compound purified by semi preparative HPLC (retention time of the compound is 21.85 min), ESI-HRMS *m/z* 656.3274 ($[M + H]^+$, $C_{29}H_{41}N_{11}O_7 \cdot H^+$ Calcd 656.3269; ^{1}H NMR (MeOD) δ 8.24 (s, 1H), 7.17 (s, 4H), 6.01 (d, *J* = 5.9 Hz, 1H), 4.76 (t, *J* = 4.1 Hz, 1H), 4.47 (s, 2H), 4.38 (dd, *J* = 4.8 Hz, 1H), 3.70-3.77 (m, 1H), 3.58-3.65 (m, 1H), 3.10-3.29 (m, 4H), 2.86-2.92 (m, 4H), 2.50-2.57 (m,

2H), 1.85-1.92 (m, 1H), 1.64-1.69 (m, 1H), 1.54-1.61 (m, 2H), 1.11 (t, *J* = 7.3 Hz, 3H). HPLC purity analysis retention time 1.40 min (>95% purity).

(*R*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-5-guanidinopentanoic acid (36)

Compound purified by semi preparative HPLC (retention time of the compound is 21.6 min), ESI-HRMS *m/z* 656.3264 ($[M + H]^+$, $C_{29}H_{41}N_{11}O_7 \cdot H^+$ Calcd 656.3269; ^{1}H NMR (MeOD) δ 8.26 (s, 1H), 7.15-7.22 (m, 4H), 6.01 (d, *J* = 6.1 Hz, 1H), 4.71 (brs, 1H), 4.49 (s, 1H), 4.46 (s, 1H), 4.42 (dd, *J* = 5.0 Hz, 1H), 3.70-3.75 (m, 1H), 3.60-3.68 (m, 1H), 3.15-3.24 (m, 4H), 2.86-2.95 (m, 4H), 2.51-2.56 (m, 2H), 1.88-1.93 (m, 1H), 1.64-1.70 (m, 1H), 1.54-1.60 (m, 2H), 1.12 (t, *J* = 7.3 Hz, 3H). HPLC purity analysis retention time 1.34 min (>95% purity).

(*S*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-phenylpropanoic acid (37)

Compound purified by silica gel column chromatography (0-20% MeOH in CH_2Cl_2, v/v), R_f = 0.3 (15% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 647.2945 ($[M + H]^+$, $C_{32}H_{38}N_8O_7 \cdot H^+$ Calcd 647.2942; ^{1}H NMR (MeOD) δ 8.08 (s, 1H), 7.06-7.24 (m, 9H), 5.96 (d, *J* = 6.1 Hz, 1H), 4.93 (t, *J* = 5.5 Hz, 1H), 4.52-4.57 (m, 2H), 4.51 (d, *J* = 3.0 Hz, 1H), 3.66-3.71 (m, 1H), 3.50-3.57 (m, 1H), 3.24-3.30 (m, 1H), 3.12-3.19 (m, 2H), 2.88-2.96 (m, 1H), 2.76-2.96 (m, 4H), 2.43 (t, *J* = 7.2 Hz, 2H), 1.06 (t, *J* = 7.3 Hz, 3H). HPLC purity analysis retention time 6.03 min (>95% purity).

(*R*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-phenylpropanoic acid (38)

Compound purified by silica gel column chromatography (0-20% MeOH in CH_2Cl_2, v/v), R_f = 0.3 (15% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 647.2936 ($[M + H]^+$, $C_{32}H_{38}N_8O_7 \cdot H^+$ Calcd 647.2942; ^{1}H NMR (MeOD) δ 8.08 (s, 1H), 7.05-7.25 (m, 9H), 5.96 (d, *J* = 6.3 Hz, 1H), 4.96 (t, *J* = 5.5 Hz, 1H), 4.50-4.55 (m, 2H), 4.43 (d, *J* = 3.0 Hz, 1H), 3.66-3.71 (m, 1H), 3.40-3.44 (m, 1H), 3.24-3.30 (m, 1H), 3.12-3.19 (m, 2H), 2.90-2.96 (m, 1H), 2.76-2.89 (m, 4H), 2.43 (t, *J* = 7.2 Hz, 2H), 1.03 (t, *J* = 7.3 Hz, 3H). HPLC purity analysis retention time 5.96 min (>95% purity).

(*S*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-(1*H*-indol-3-yl)propanoic acid (39)

Compound purified by silica gel column chromatography (0-25% MeOH in CH_2Cl_2, v/v), R_f = 0.3 (20% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 686.3042 ($[M + H]^+$, $C_{34}H_{39}N_9O_7 \cdot H^+$ Calcd 686.3051; ^{1}H NMR (MeOD) δ 8.02 (s, 1H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 6.95-7.09 (m, 7H), 5.96 (d, *J* = 6.3 Hz, 1H), 4.97 (t, *J* = 5.6 Hz, 1H), 4.55-4.60 (m, 1H), 4.50-4.53 (m, 1H), 4.40 (d, *J*

= 3.1 Hz, 1H), 3.53-3.58 (m, 2H), 3.20-3.29 (m, 2H), 3.09-3.16 (m, 2H), 2.73-2.85 (m,4H), 2.40-2.47 (m, 2H), 1.03 (t, *J* = 7.3 Hz, 3H). HPLC purity analysis retention time 5.88 min (>95% purity).

(*R*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-(1*H*-indol-3-yl)propanoic acid (40)

Compound purified by silica gel column chromatography (0-25% MeOH in CH_2Cl_2, v/v), R_f = 0.3 (20% MeOH in CH_2Cl_2, v/v), ESI-HRMS *m/z* 686.3046 ($[M + H]^+$, $C_{34}H_{39}N_9O_7 \cdot H^+$ Calcd 686.3051; ^{1}H NMR (MeOD) δ 7.99 (s, 1H), 7.57 (d, *J* = 7.8 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 6.99-7.11 (m, 7H), 5.93 (d, *J* = 5.9 Hz, 1H), 5.01 (t, *J* = 5.9 Hz, 1H), 4.55-4.59 (m, 1H), 4.52-4.54 (m, 1H), 4.40 (d, *J* = 2.9 Hz, 1H), 3.59-3.64 (m, 1H), 3.45-3.50 (m, 1H), 3.20-3.29 (m, 1H), 3.08-3.16 (m, 2H), 2.76-2.90 (m, 5H), 2.45 (t, *J* = 7.3 Hz, 2H), 1.01 (t, *J* = 7.3 Hz, 3H). HPLC purity analysis retention time 5.96 min (>95% purity).

(*S*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-(1*H*-imidazol-4-yl)propanoic acid (41)

Compound purified by semi preparative HPLC (retention time of the compound is 21.36 min), ESI-HRMS *m/z* 637.2817($[M + H]^+$, $C_{29}H_{36}N_{10}O_7 \cdot H^+$ Calcd 637.2847. ^{1}H NMR (MeOD) δ 8.83 (s, 1H), 8.32 (s, 1H), 7.12-7.23 (s, 5H), 5.99 (d, *J* = 5.9 Hz, 1H), 4.77 (dd, *J* = 5.1 Hz, 1H), 4.74 (s, 1H), 4.51 (s, 1H), 4.47 (s, 1H), 3.62-3.75 (m, 2H), 3.19-3.28 (m, 3H), 3.07-3.16 (m, 1H), 2.93 (t, *J* = 7.2 Hz, 2H), 2.84 (t, *J* = 7.2 Hz, 2H), 2.50 (t, *J* = 7.2 Hz, 2H), 1.13 (t, *J* = 7.2 Hz, 3H). HPLC purity analysis retention time 1.39 min (>95% purity).

(*R*)-2-(3-(4-(2-((6-Amino-9-((2*R*,3*R*,4*S*,5*S*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl)amino)ethyl)phenyl)propanamido)-3-(1*H*-imidazol-4-yl)propanoic acid (42)

Compound purified by semi preparative HPLC (retention time of the compound is 21.36 min), ESI-HRMS *m/z* 637.2830 ($[M + H]^+$, $C_{29}H_{36}N_{10}O_7 \cdot H^+$ Calcd 637.2847. ^{1}H NMR (MeOD) δ 8.79 (s, 1H), 8.25 (s, 1H), 7.14-7.22 (s, 5H), 6.01 (d, *J* = 6.5 Hz, 1H), 4.70-4.75 (m, 2H), 4.47-4.50 (m, 2H), 3.75-3.84 (m, 1H), 3.54-3.60 (m, 1H), 3.19-3.30 (m, 3H), 3.01-3.09 (m, 1H), 2.85-2.91 (m, 2H), 2.85 (t, *J* = 7.2 Hz, 2H), 2.48 (t, *J* = 7.2 Hz, 2H), 1.12 (t, *J* = 7.2 Hz, 3H). HPLC purity analysis retention time 1.31 min (>95% purity).

Table S2. Retention times of the amino acid conjugates from HPLC purity analysis.[a]

Compound Number	Retention time (min)
27	6.46
28	6.43
29	6.57
30	6.55
33	4.96
34	4.16
35	1.28
36	1.34
37	6.03
38	5.96
39	5.88
40	5.96
41	1.39
42	1.31

a - Conditions: Hewlett-Packard 1100 HPLC equipped with Zorbax SB-Aq 5 μm analytical column (50mm×4.6mm). 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.

Pharmacological Methods

[^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide (I-AB-MECA; 2000 Ci/mmol), [^{3}H]R-PIA (N^6-phenylisopropyladenosine, 63 Ci/mmol), [^{3}H]CGS21680 (2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamido-adenosine, 47 Ci/mmol) and [^{3}H]cyclic AMP (40 Ci/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell culture and membrane preparation

Adherent mammalian cells stably transfected with cDNA encoding the appropriate human AR were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 µmol/mL glutamine and 800 µg/mL geneticin. The recombinant A_1, A_{2B}, and A_3 ARs were expressed in CHO (Chinese hamster ovary) cells[1] and the A_{2A} AR in HEK-293 (human embryonic kidney) cells.[2] 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 7.4) containing 10 mM $MgCl_2$, 1 mM EDTA and 0.1 mg/mL CHAPS. 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.[3]

Binding assay

Human A_1 and A_{2A} Receptors: For binding to human A_1 receptors, [^{3}H]R-PIA (1 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. For human A_{2A} receptor binding, membranes (20 µg/tube) from HEK-293 cells stably expressing human A_{2A} receptors were incubated with 15 nM [^{3}H]CGS21680 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.

Human A_3 Receptor: For competitive binding assay, each tube contained 100 µL membrane suspension (20 µg protein), 50 µL of [^{125}I]I-AB-MECA (0.5 nM), and 50 µL of increasing concentrations of the nucleoside derivative in Tris·HCl buffer (50 mM, pH 7.4) 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 25°C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburgh, MD, USA). Filters were washed three times with 9 mL ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter.

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

Cyclic AMP accumulation assay

Intracellular cyclic AMP levels were measured with a competitive protein binding method.[4,5] CHO cells that expressed recombinant human A_3ARs were harvested by trypsinization. After centrifugation and resuspension in medium, cells were plated in 24-well plates in 1.0 mL medium. After 24 hr, 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 upon removal of the supernatant, and cells were lysed upon the addition of 200 μL of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at -20°C. For determination of 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.

A functional assay carried out at the hA_{2A}AR subtype consisted of stimulation of cyclic AMP production in CHO cells expressing this receptor, as a mean percentage of the response of the full agonist CGS21680 (10 μM). The activity was determined at a single concentration of 10 μM.

Statistical analysis

Binding and functional parameters were calculated using Prism 4.0 software (GraphPAD, San Diego, CA, USA). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng-Prusoff equation.[6] Data were expressed as mean ± standard error.

References:

1. a) Klotz, K.-N.; Hessling, J.; Hegler, J.; Owman, C.; Kull, B.; Fredholm, B.B.; Lohse, M.J. Comparative pharmacology of human adenosine receptor subtypes – characterization of stably transfected receptors in CHO cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998**, *357*, 1-9. b) Gao, Z.G.; Mamedova, L.; Chen, P.; Jacobson, K.A. 2-Substituted adenosine derivatives: Affinity and efficacy at four subtypes of human adenosine receptors. *Biochem. Pharmacol.* **2004**, *68*, 1985-1993.
2. Kim, Y.C.; Ji, X.-d.; Melman, N.; Linden, J.; Jacobson, K.A. Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A_{2B} adenosine receptors. *J. Med. Chem.* **2000**, *43*, 1165-1172.
3. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248-254.
4. Nordstedt, C.; Fredholm, B.B. A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal. Biochem.* **1990**, *189,* 231-234.
5. Post, S.R.; Ostrom, RS.; Insel, P.A. Biochemical methods for detection and measurement of cyclic AMP and adenylyl cyclase activity. *Methods Mol. Biol.* **2000**, *126*, 363-374.
6. Cheng, Y.-C.; Prusoff, W.H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973***, 22*, 3099-3108.