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SUPPORTING INFORMATION

New selective A_{2A} agonists and A_3 antagonists for human adenosine receptors. Synthesis, biological activity and molecular docking studies

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Experimental Section

General

NMR spectra were recorded at 300, 400, or 500 MHz for ^{1}H and 75 or 100 MHz for ^{13}C on a Varian Unity 300, Varian Mercury 400, or Varian Inova 500 spectrometer. The values are expressed in δ relative to the $CHCl_3$ present in the solvent (δ 7.24 ppm for ^{1}H and δ 77.0 ppm for ^{13}C). Alternatively, when CD_3OD was used, the values are expressed relative to CD_3OH signal at 3.31 ppm for ^{1}H and 49.0 ppm for ^{13}C. Coupling constants (J) are in Hz. High resolution mass spectra (HRMS) were run on a UPLC Acquity apparatus (Waters Corporation, Milford, MA, USA) coupled to a mass spectrometer LCT Premier XE (Waters) with a TOF analyzer using a 1.7 µm C18 (2.1 x 50 mm) column. Semipreparative HPLC was run on a Waters Alliance 2695 apparatus with a 996 UV photodiode array detector (Waters) using a Gemini 110 Å, 5 µm C-18 (250 x 10 mm) column (Phenomenex, Torrance, USA) connected to a Waters Fraction Collector III. For analytical HPLC, a Gemini 5520-65 110 Å, 5 µm C-18 (250 x 4.6 mm) (Phenomenex, Torrance, USA) column was used. All final compounds were checked for purity by analytical HPLC analysis and resulted to be >98% pure.

All reactions were carried out under inert atmosphere. All reagents were purchased from Sigma-Aldrich (Spain) and were used without further purification. THF was distilled from sodium-benzophenone ketyl, MeOH and DMF were distilled from CaH_2, *i*-Pr_2NEt was distilled from KOH, and DBU and DMSO were distilled from CaH_2. For flash column chromatography, silica gel (35-70 µm) (SDS) was used.

General procedure for preparation of compounds 1-7.

2-[1-(*R*)-Methyl-2-(4-methoxyphenyl)ethylamino]-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine ((*R*)-6). A mixture of (*R*)-1-(4-methoxyphenyl)propan-2-amine (**12f**) (14 mg, 0.089 mmol), compound **8** (15 mg, 0.041 mmol), anhydrous DMSO (38 µL) and *i*-Pr_2NEt (0.11 mL, 0.639 mmol) was heated under Ar at 145 ºC for 23 h. The mixture was cooled to room temperature, diluted with AcOEt (5 mL) and the volatile material was removed under vacuum. Then, AcOEt (5 mL) was added and the organic phase was washed with NaCl sat. soln. (4 x 10 mL). After drying ($MgSO_4$) and filtered, the solvent was evaporated. The crude was purified by flash column chromatography eluting with AcOEt:MeOH (95:5) followed by chromatography on a reverse phase C-18 Isolute column eluting with H_2O:MeCN:AcOH mixture (75:25:0.1). After evaporation of the solvent and lyophilization, the residue was finally purified by semipreparative HPLC using mixtures of MeOH:H_2O (50-95% MeOH) to afford compound (*R*)-**6** (6 mg, 30%). ^{1}H NMR (500 MHz, MeOD): δ 8.10 (s, 1H, NC*H*N), 7.17 (d, J= 8.7 Hz, 2H, C_6H_4), 6.82 (d, J = 9 Hz, 2H, C_6H_4), 6.13 (d, J = 4.5 Hz, 1H, C*H*O), 5.32 (d, J = 4.5 Hz, 1H, NCC*H*O), 4.74 (m, 1H, C*H*O), 4.71 (m, 1H, C*H*O), 4.69 (q, 2H, J = 7.5 Hz CH_2CH_3), 4.23 (m, 1H, C*H*), 3.65 (s, 3H, OCH_3), 2.89 (dd, J = 13.5 Hz, J´= 5.7 Hz, 1H, CH_2), 2.64 (dd, J = 13.2 Hz, J´= 7.2 Hz, 1H, CH_2), 1.60 (t, 3H, J = 7.5 Hz, CH_2CH_3), 1.13 (d, J = 6.6 Hz, CH_3) ppm. ^{13}C NMR (100 MHz, MeOD): δ 165.96, 160.65, 159.56, 157.32, 153.15, 137.63, 132.62, 131.50, 114.63, 89.92, 78.70, 75.67, 75.56, 55.61, 42.93, 20.31, 14.69 ppm. HRMS m/z calcd for $C_{22}H_{28}N_{10}O_4$ 497.2373 (M^+ +H), found 497.2378.

2-[1-(*R,S*)-Methyl-2-phenylethylamino]-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine (1). ^{1}H NMR (400 MHz, MeOD): δ 8.10 (s, 1H, NC*H*N), 7.26 (s, 5H, C_6H_5), 6.13 (d, J = 4.5 Hz, 1H, C*H*O), 5.32 (d, J = 4.5 Hz, 1H, NCC*H*O), 4.75 (m, 1H, C*H*O), 4.70 (m, 1H, C*H*O), 4.69 (q, 2H, J = 7.5 Hz CH_2CH_3), 4.29 (m, 1H, C*H*), 2.98 (dd, J = 13.5 Hz, J´= 5.7 Hz, 1H, CH_2), 2.71 (dd, J = 13.2 Hz, J´= 7.2 Hz, 1H, CH_2), 1.60 (t, 3H, J = 7.5 Hz, CH_2CH_3), 1.15 (d, J = 6.6 Hz, CH_3) ppm. HRMS m/z calcd for $C_{21}H_{26}N_{10}O_3$ 467.2268 (M^+ +H) found 467.2265.

2-[1-(*R,S*)-Methyl-2-(4-fluorophenyl)ethylamino]-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine (2). HRMS m/z calcd for $C_{21}H_{25}FN_{10}O_3$ 485.2173 (M^++H) found 485.2192 (Chart S1).

2-[1-(*R,S*)-Methyl-2-(4-chlorophenyl)ethylamino]-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine (3). ^{1}H NMR (400 MHz, MeOD): δ 8.10 (s, 1H, NC*H*N), 7.26 (d, J= 8.7 Hz, 2H, C_6H_4), 7.20 (d, J = 9 Hz, 2H, C_6H_4), 6.13 (d, J = 4.5 Hz, 1H, C*H*O), 5.32 (d, J = 4.5 Hz, 1H, NCC*H*O), 4.72 (m, 1H, C*H*O), 4.70 (m, 1H, C*H*O), 4.69 (q, 2H, J = 7.5 Hz CH_2CH_3), 4.23 (m, 1H, C*H*), 2.89 (dd, J = 13.5 Hz, J´= 5.7 Hz, 1H, CH_2), 2.72 (dd, J = 13.2 Hz, J´= 7.2 Hz, 1H, CH_2), 1.60 (t, 3H, J = 7.5 Hz, CH_2CH_3), 1.13 (d, J = 6.6 Hz, CH_3) ppm. HRMS m/z calcd for $C_{21}H_{25}ClN_{10}O_3$ 499.1721 (M^+ +H) found 499.1698.

2-[1-(*R,S*)-Methyl-2-(4-bromophenyl)ethylamino]-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine (4). ^{1}H NMR (500 MHz, MeOD): δ 8.10 (s, 1H, NC*H*N), 7.40 (d, J= 7 Hz, 2H, C_6H_4), 7.20 (d, J = 8.5 Hz, 2H, C_6H_4), 6.13 (d, J = 4.5 Hz, 1H, C*H*O), 5.32 (d, J = 4.5 Hz, 1H, NCC*H*O), 4.74 (m, 1H, C*H*O), 4.72 (m, 1H, C*H*O), 4.70 (q, 2H, J = 7.5 Hz CH_2CH_3), 4.28 (m, 1H, C*H*), 2.94 (dd, J = 13.5 Hz, J´= 5.7 Hz, 1H, CH_2), 2.70 (dd, J = 13.2 Hz, J´= 7.2 Hz, 1H, CH_2), 1.60 (t, 3H, J = 7.5 Hz, CH_2CH_3), 1.15 (d, J = 6.6 Hz, CH_3) ppm. HRMS m/z calcd for $C_{21}H_{25}BrN_{10}O_3$ 543.1216 (M^+ -H) found 543.1236.

2-[1-(*R,S*)-Methyl-2-(4-trifluoromethylphenyl)ethylamino]-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine (5). HRMS m/z calcd for $C_{22}H_{25}F_3N_{10}O_3$ 535.2141 (M^+ +H) found 535.2154 (Chart S1).

2-[1-(*R,S*)-Methyl-2-(4-methoxyphenyl)ethylamino]-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine (*R*,*S*)-6). The NMR data were identical to the corresponding compound (*R*)-**6**. HRMS m/z calcd for $C_{22}H_{28}N_{10}O_4$ 497.2373 (M^+ +H) found 497. 2379.

2-[1-(*S*)-Methyl-2-(4-methoxyphenyl)ethylamino]-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine ((*S*)-6). The NMR data were identical to the corresponding compound (*R*)-**6**. HRMS m/z calcd for $C_{22}H_{28}N_{10}O_4$ 497.2373 (M^+ +H) found 497. 2357.

2-[1-(*R,S*)-Methyl-2-(3-methoxyphenyl)ethylamino]-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine (*R*,*S*)-7). ^{1}H NMR (400 MHz, MeOD): δ 8.10 (s, 1H, NC*H*N), 7.21 (t, J= 6.2 Hz, 1H, C_6H_4), 6.75 (m, 3H, C_6H_4), 6.15 (d, J = 4.5 Hz, 1H, C*H*O), 5.30 (d, J = 4.5 Hz, 1H, NCC*H*O), 4.72 (m, 1H, C*H*O), 4.71 (m, 1H, C*H*O), 4.69 (q, 2H, J = 7.5 Hz CH_2CH_3), 4.25 (m, 1H, C*H*), 3.75 (s, 3H, OCH_3), 2.89 (dd, J = 13.5 Hz, J´= 5.7 Hz, 1H, CH_2), 2.64 (dd, J = 13.2 Hz, J´= 7.2 Hz, 1H, CH_2), 1.61 (t, 3H, J = 7.5 Hz, CH_2CH_3), 1.15 (d, J = 6.6 Hz, CH_3) ppm. HRMS m/z calcd for $C_{22}H_{28}N_{10}O_4$ 497.2373 (M^+ +H) found 497. 2364.

2-Chloro-5'-(2-ethyl-2H-tetrazol-5-yl)adenosine (8). This compound was prepared as previously described.[1] ^{1}H-NMR (300 MHz, DMSO), δ: 8.40 (s, 1H, NC*H*N,), 7.81 (bs, 2H, NH_2), 6.04 (d, J=5.4 Hz, 1H, C*H*O), 5.78 (dd, J=6.9 Hz, J´=6 Hz, 1H, C*H*OH), 5.21 (d, J=4.2 Hz, 1H, C*H*CN), 4.79 (q, J=5.1 Hz, 1H, OH), 4.72 (q, J=7.5 Hz, 2H, CH_2CH_3), 4.57 (q, J=4.2 Hz, 1H, OH), 1.29 (t, J=7.5 Hz, 3H, CH_2CH_3) ppm. ^{13}C-NMR (50 MHz, d_6-DMSO), δ: 164.31, 156.99, 153.44, 150.75, 139.65, 118.17, 87.79, 77.40, 73.97, 73.69, 48.41, 14.25 ppm.

2-Dimethylamino 5'-(2-ethyl-2H-tetrazol-5-yl)adenosine (9). A mixture of compound **8** (50 mg, 0.136 mmol), 1-phenylpropan-2-amine (**12a**) (40 mg, 0.299 mmol), *i*-Pr_2NEt (240 µL, 1.36 mmol), DMF (1.3 mL) and NaI as catalyst was heated under Ar at 145ºC for 70 h. The mixture was treated as above and the residue was purified through an Isolute C-18 cartridge followed by semipreparative HPLC using mixtures of MeOH:H_2O (50-95% MeOH) to give compound **9** (21 mg, 41% yield). ^{1}H NMR (500 MHz, MeOD): δ 8.10 (s, 1H, NC*H*N), 6.13 (d, J = 4.5 Hz, 1H, C*H*O), 5.29 (d, J = 5 Hz, 1H, C*H*O), 4.93 (t, J = 5 Hz, 1H, CH_3), 4.79(t, J = 4.5 Hz, 1H, C*H*O), 4.72 (dd, J = 7 Hz, J´= 14.5 Hz, 2H, CH_2CH_3), 3.14(S, 6H, CH_3), 1.62 (t, J = 7.5 Hz, 3H, CH_3CH_2) ppm.^{13}C NMR (100 MHz, MeOD): δ 164.2, 159.5, 155.6, 151.8, 136.1, 112.6, 87.5, 76.5, 73.7, 73.1, 48.2, 37.1, 14.2 ppm. HRMS m/z calcd for $C_{14}H_{19}N_{10}O_3$ 375.1642 (M^+ -H) found 375.1635.

2-(1-Methyl-2-phenylethylamino)adenosine (10). A mixture of compound **13** (25 mg, 0.083 mmol), 1-phenylpropan-2-amine (**12a**) (25 mg, 0.183 mmol), Et_3N (190 µL, 1.33 mmol), DMSO (50 µL) and NaI as catalyst was heated under Ar at 145ºC for 48 h. The mixture was cooled to room temperature, diluted with AcOEt (5 mL), and the volatile materials were removed under vacuum. The residue was diluted with CH_2Cl_2 (5 mL), washed with brine (3 x 5 mL) and the organic phase was dried ($MgSO_4$). After filtering, the solvent was stripped off. The residue was purified through an Isolute column C18 cartridge, followed by semipreparative HPLC using an isocratic mixture of H_2O:MeOH (40:60) to afford compound **10** (11.9 mg, 36%). ^{1}H NMR (400 MHz, d_6-DMSO): δ 7.90 (s, 1H, NC*H*N), 7.17 (s, 1H, N*H*), 6.72 (s, 2H, NH_2), 6.04 (s, 1H, C*H*O), 5.73 (d, J = 5.6 Hz, 1H, C*H*O), 5.37 (m, 1H, C*H*O), 5.12 (s, 2H, OH), 4.57 (m, 1H, C*H*O), 4.12 (s, 2H, CH_2), 3.88 (s, 1H, O*H*), 3.65 (m, 2H, CH_2), 2.90 (m, 1H, C*H*), 106 (d, J = 5.2 Hz, CH_3) ppm. HRMS m/z calcd for $C_{19}H_{24}N_6O_4$ 399.1781 (M^+ -H) found 399.1766.

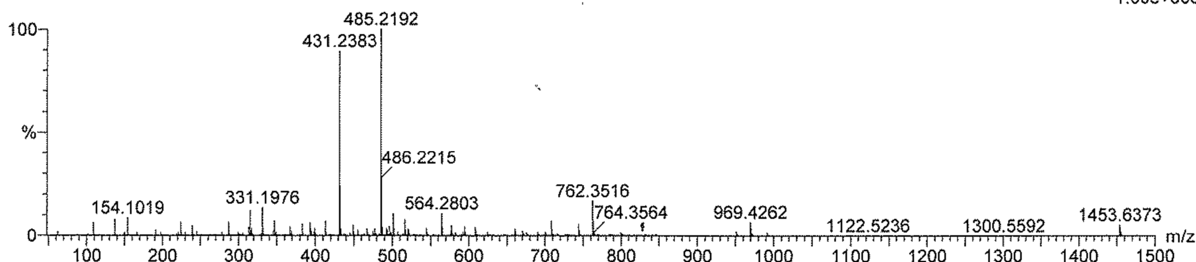
Radioligand binding assays

Radioligand binding competition assays were performed *in vitro* using A_1, A_{2A}, A_{2B} and A_3 human receptors expressed in transfected CHO (A_1), HeLa (A_{2A} and A_3) and HEK-293 (A_{2B}) cells. The experimental conditions used are summarized in Table 1S. In each instance aliquots of membranes (15 µg for A_1, 8 µg for A_{2A}, 40 µg for A_{2B} and 100 µg for A_3) in buffer A (see Table 1S) were incubated for the specified period at 25 ºC with the radioligand (2-35 nM) and 6 different concentrations (ranging from 0.1 nM to 1 µM) of the test molecule or standard in a final volume of 200 µl. The binding reaction was stopped by rapid filtration in a multiscreen manifold system (Millipore Ibérica, Madrid, Spain). Unbound radioligand was removed by washing 4x with 250 µl of ice-cold buffer B for A_1, A_{2A} and A_{2B} receptors, and 6x 250 µl of ice-cold buffer B for A_3 receptor (see Table 1S). Non-specific binding was determined using a 50 µM NECA solution for A_{2A} receptors and 10-1000 µM R-PIA solution for A_1, A_{2A} and A_3 receptors. Radioactivity retained on filters was determined by liquid scintillation counting using Universol (ICN

Elemental Composition Report

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
43 formula(e) evaluated with 2 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 15-25 H: 20-30 N: 8-11 O: 0-4 F: 0-2
AR136c
AR136c 29 (0.297) Cm (27:33)

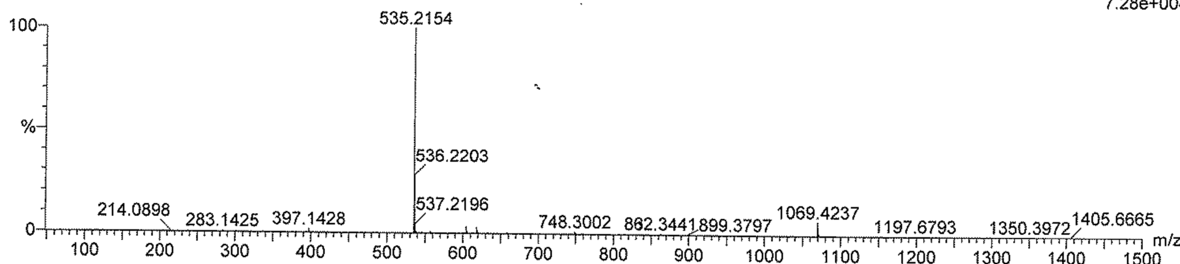


Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
485.2192	485.2173	1.9	3.9	13.5	449.7	0.0	C21 H26 N10 O3 F
	485.2185	0.7	1.4	9.5	455.6	5.9	C18 H27 N10 O4 F2

Elemental Composition Report

Single Mass Analysis
Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
53 formula(e) evaluated with 2 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 20-24 H: 20-30 N: 8-12 O: 0-3 F: 0-4
AR138
AR138 12 (0.187) Cm (12:13)



Minimum:				-1.5			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
535.2154	535.2141	1.3	2.4	13.5	244.1	0.0	C22 H26 N10 O3 F3
	535.2193	-3.9	-7.3	13.5	248.4	4.3	C24 H27 N8 O2 F4

Chart S1

Biochemicals, Inc.). The binding affinities were determined using [^{3}H]-DPCPX (120 Ci/mmol; NEN-Perkin Elmer Life Sciences, Madrid, Spain) as the radioligand for A_1 and A_{2B}, [^{3}H]-ZM241385 (50 Ci/mmol; ARC-ITISA, Madrid, Spain) for A_{2A} and [^{3}H]-NECA (18.6 Ci/mmol; NEN-Perkin Elmer Life Sciences, Madrid, Spain) for A_3. The inhibition constant (K_i) of each compound was calculated by the expression $K_i = IC_{50} / (1+(C/K_D))$, where IC_{50} is the concentration of compound that displaces the binding of radioligand by 50%, C is the free concentration of radioligand and K_D is the apparent dissociation constant of each radioligand.

cAMP assays

cAMP assays were performed on transfected adenosine receptors using a cAMP enzyme immunoassay kit (GE-Healthcare). Because A_{2A} are Gs-coupled receptors, competition curves were performed based on the NECA-induced cAMP accumulation. In turn, A_3 are Gi/o-coupled receptors and, therefore, competition curves were obtained over the NECA-induced inhibition of the cAMP accumulation produced by forskolin.

Human A_{2A} receptors

CHO-A_{2A} cells were seeded (10000 cells/well) in 96-well culture plates and incubated at 37ºC in an atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM F-12), containing 10% fetal calf serum (FCS) and 1% L-glutamine. Cells were washed 3x with 200 µl of assay medium (DMEM-F12 and 25 mM HEPES pH=7.4), and pre-incubated with the assay medium containing 30 µM Rolipram and the test compounds at 37ºC for 15 min. NECA was incubated for 15 min at 37ºC (total incubation time 30 min). The reaction was stopped with the lysis buffer supplied with the kit and detection of intracellular cAMP was performed by the enzyme immunoassay at 450 nm in an M1000 Pro Reader (Tecan). Data were fitted by non-linear regression using GraphPad Prism v2.01 (GraphPad Software).

Human A_3 receptors

CHO-A_3 cells were seeded (20000 cells/well) in 96-well culture plates and incubated at 37ºC in an atmosphere with 5% CO_2 Dulbecco's Medium Nutrient F-12 (DMEM F-12), containing 10% FCS and 1% L-glutamine, as above. Cells were washed and pre-incubated with the test compounds as cited for A_{2A} receptors. NECA was incubated also at 37ºC for 15 min and forskolin (10 µM) was also incubated for 3 min (total incubation time 33 min). The reaction was stopped with the lysis buffer supplied with the kit and the enzyme immunoassay performed as cited above. Data were also fitted by non-linear regression using GraphPad Prism v2.01.

Table 1S Experimental conditions used for radioligand binding assays using A_1, A_{2A}, A_{2B}, and A_3 human receptors.

	A_1	A_{2A}	A_{2B}	A_3
Cell line	CHO-A_1	HeLa-A_{2A}	HEK-A_{2B}	HeLa-A_3
Buffer A	20 mM Hepes, 100 mM NaCl, 10 mM $MgCl_2$, 2 units/ml adenosine deaminase (pH=7.4)	50 mM Tris-HCl, 1mM EDTA, 10 mM $MgCl_2$, 2 units/ml adenosine deaminase (pH=7.4)	50 mM Tris-HCl, 1mM EDTA, 5 mM $MgCl_2$, 100µg/ml Bacitracin, 2 units/ml adenosine deaminase (pH=6.5)	50 mM Tris-HCl, 1mM EDTA, 5 mM $MgCl_2$, 2 units/ml adenosine deaminase (pH=7.4)
Buffer B	20 mM Hepes, 100 mM NaCl, 10 mM $MgCl_2$, (pH=7.4)	50 mM Tris-HCl, 1mM EDTA, 10 mM $MgCl_2$ (pH=7.4)	50 mM Tris-HCl 1mM EDTA, 5mM $MgCl_2$ (pH=6.5)	50 mM Tris-HCl (pH=7.4)
Plate	GF/C	GF/C	GF/C	GF/B
Radioligand	[^{3}H]DPCPX 2 nM	[^{3}H]ZM241385 3 nM	[^{3}H]DPCPX 25 nM	[^{3}H]NECA 30 nM
Non-specific binding	10 µM (R)-PIA	50 µM NECA	1000 µM (R)-PIA	100 µM (R)-PIA
Incubation	25°C/60 min	25°C/30 min	25°C/30 min	25°C/180 min

Molecular modelling

The three available agonist-bound crystal structures of the A_{2A}AR were initially considered, i.e. receptors co-crystallized with adenosine (PDB code 2YDO), 5'-*N*-ethyl-carboxamidoadenosine (NECA, PDB code 2YDV) and UK432097 (a derivative of NECA with bulky N6 and C2 substitutions, PDB code 3QAK). A first docking exploration with the agonist series reported[1] (Fig. S1), revealed a more consistent binding mode when using the NECA-bound complex, probably due to the more enclosed conformation of the extracellular loops compared to the UK432097-bound structure.

The A_{2A}AR-NECA complex was equilibrated using the molecular dynamics (MD) protocol implemented in GPCR-ModSim (http://gpcr-modsim.org),[2] which is here explained in brief. First, missing protein atoms and residue protonation states were modelled for the A_{2A}AR structure with tools from the Schrödinger software suite.[3] The coordinates of the crystallographic receptor, ligand and water molecules observed in the binding cavity were then prepared to be simulated in GROMACS v4.0.5.[4] The complex was embedded in a pre-equilibrated hydrated POPC (1-palmitoyl-2-oleoylphosphatidylcholine) lipid bilayer, following the appropriate orientation adopted by GPCRs in the membrane. The system was then neutralized with counterions, and arranged in a simulation box with a hexagonal prism-shaped geometry comprising ~50,000 atoms (74% solvent molecules, 15% lipids, and 11% protein and ligand atoms). The OPLS all-atom (OPLS-AA) force field[5] was used for the protein, the corresponding parameters were generated for the ligand with Macromodel,[3] and the

double-pairlist half-epsilon method[6] was followed to make the Berger united-atom parameters used for the POPC lipids[7] compatible with the rest of the system. After a steepest-descent energy minimization, 5 ns of partially-restrained MD simulation were performed with GROMACS4.0.5.[4] This equilibration protocol consists of a first stage of 2.5 ns where positional restraints on the heavy atoms of the protein, ligand, and crystallographic waters are gradually reduced, followed by 2.5 ns with only weak positional restraints on the protein Cα atoms. Very small rearrangements were observed for NECA and binding site residues, while water molecules further optimized an internal-ligand and receptor-ligand interaction networks (Fig. S2).

Compounds described in the text were docked to the MD-equilibrated A_{2A}AR receptor, including two binding site water molecules, with the protein-ligand docking program GOLD version 4.1.[8] The 3D conformers of each ligand, including their possible *R* and *S* enantiomers, were built and optimized using the Maestro graphical interface and the LigPrep utility from the Schrödinger suite.[3] Twenty genetic algorithm (GA) runs were performed with GOLD. High accuracy search parameters were used, allowing full flexibility of the ligand, e.g. flipping of amide bonds. A search sphere with a radius of 15 Å was centered on the side chain (CD1) of Ile274$^{7.39}$, which comprises the agonist binding site observed in active-like structures of the A_{2A}AR. Ligand conformations from docking experiments were selected according to a combination of their predicted binding energies with the ChemScore scoring function, and the population (convergence) of the solutions according to an RMSD clustering criteria of 1 Å. Finally, the ligands were energy-minimized in the binding site with Macromodel,[3] with the crystallographic water and receptor coordinates held rigid.

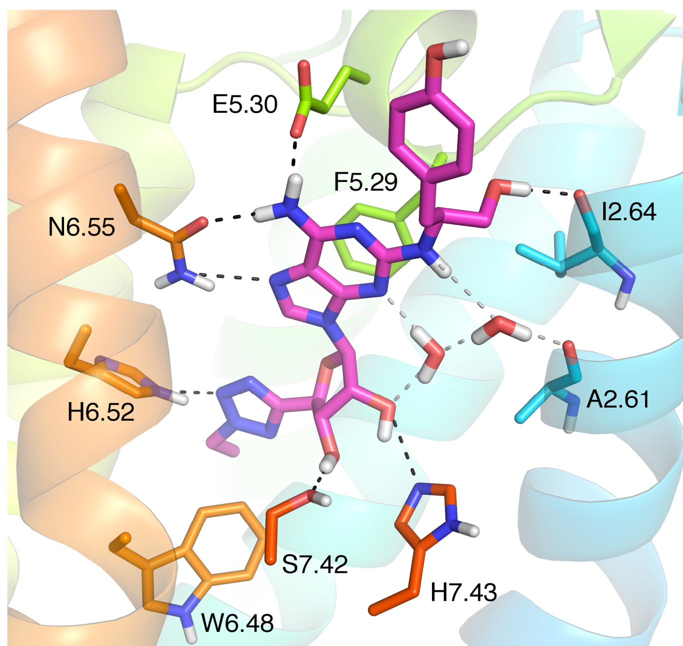


Fig. S1. Predicted binding mode for compound **5** in Bosch et al.[1]

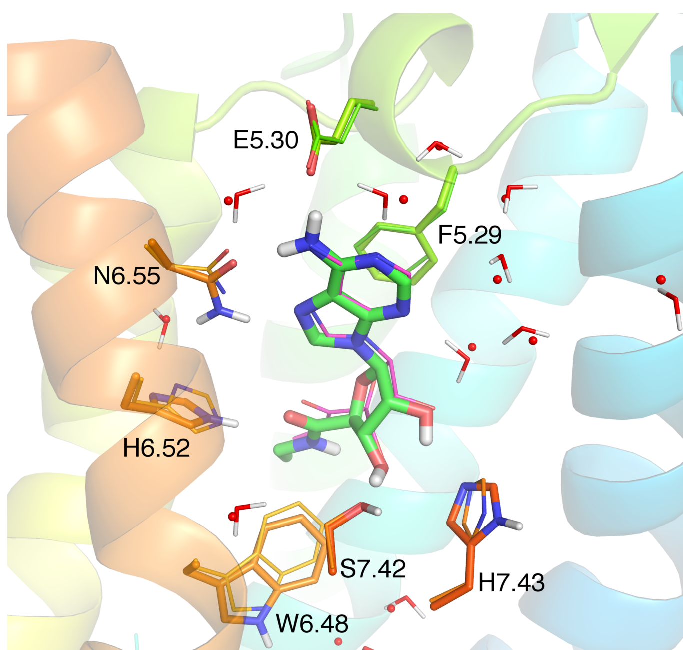


Fig. S2. MD equilibration of the A_{2A}AR-NECA crystallographic coordinates. Binding site residues and NECA are represented in lines and sticks for the pre- and post-MD coordinates, respectively. The crystallographic waters included in the simulations are shown in spheres, while their MD-refined coordinates are represented in sticks.

Abbreviations

UPLC = ultra-performance liquid chromatography; TOF = time of flight; *i*-Pr_2NEt = diisopropylethylamine; DBU = 1,8-diazabicyclo[5,4,0]undec-7-ene; DMSO = dimethyl sulfoxide; DMF = N,N-dimethyl formamide; cAMP = 3’-5’ cyclic adenosine monophosphate; NECA = 5’-(N-ethylcarboxamido)adenosine; DPCPX = 1,3-dipropyl-8-cyclopentylxanthine; ZM241385 = (7-amino-2-(2-furyl)-5-[2-(4-hydroxyphenyl)ethyl]amino-[1,2,4]-triazolo[1,5-a][1,3,5]triazine; GPCR = G-protein coupled receptor.

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