Supporting information

**Bitopic Fluorescent Antagonists of the A2A Adenosine Receptor Based on Pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine Functionalized Congeners**


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**Synthetic methods**

![Scheme S1](image)

Scheme S1. (a) BBr₃, CH₂Cl₂, rt, 4h; (b) sodium 4-(bromomethyl)benzenesulfonate, NaH, DMF, rt, 2h, 42%.

were obtained from the commercial sources specified in the parenthesis next to its name. All other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). NMR spectra were recorded on a Bruker 400 MHz spectrometer. Chemical shifts are given in ppm (δ), calibrated to the residual solvent signals or TMS. TLC analysis was carried out on glass sheets precoated with silica gel F 254 (0.2 mm) from Aldrich and spots were examined under ultraviolet light at 254 nm. Purification of final fluorescent compounds was performed by preparative HPLC with CH$_3$CN/H$_2$O as mobile phase (column A: Luna 5 μm C18(2) 100 Å, LC column 250 mm × 21.2 mm, flow rate of 5 mL/min; column B: Eclipse XDB-C18, 5 μm, 4.6 mm × 250 mm, flow rate of 5 mL/min). Column chromatography was performed on silica gel (40–63 μm, 60 Å). High resolution mass (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters). The purity of final derivatives was checked using a Hewlett-Packard 1100 HPLC equipped with a Zorbax SB-Aq 5 μm analytical column (50 × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). The mobile phase was as follows: linear gradient solvent system, 5 mM TBAP (tetrabutylammonium dihydrogen phosphate) – CH$_3$CN from 100:0 to 0:100 in 15 min; the flow rate was 0.5 mL/min. All derivatives tested for biological activity showed >95% purity by HPLC analysis with detection at 254 nm for molecules without fluorescent moieties and at 488 nm, 640 nm or 647 nm depending on the fluorescent ligands.

General procedure for synthesis of compounds 6a – 6e, by aminolysis of ester 8:

Compound 8 (1 eq., 0.047 mmol) was dissolved in a mixture of the corresponding dialkylamine and MeOH (7 mL, 1:9, v/v) and stirred overnight at room temperature before concentrated to dryness. The resulting residue was purified by silica gel column chromatography (20:78:2, MeOH:CH$_2$Cl$_2$:aq.NH$_3$, v/v/v) to afford the desired product.

2-(4-(3-(5-Amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)-N-(2-aminomethyl)acetamide 6a.

Compound 8 (1 eq., 12 mg, 0.0268 mmol) was dissolved in a mixture of ethylenediamine and MeOH (2 mL, 1:9, v/v). After stirring at room temperature overnight, the reaction mixture was concentrated to dryness, and the resulting residue was purified by silica gel column chromatography (20:78:2 MeOH:CH$_2$Cl$_2$:aq.NH$_3$, v/v/v) to afford a white solid (8 mg, 63 %) as a white solid. $^1$H NMR (MeOD-d$_4$, δ ppm) 8.10 (s, 1H), 7.77 (d, J = 0.7 Hz and J = 1.7 Hz, 1H), 7.26 (d, J = 0.7 Hz and J = 3.4 Hz, 1H), 7.10 (d, 2H, J = 8.7 Hz), 6.84 (d, 2H, J = 8.7 Hz), 6.68 (dd, 1H, J = 1.6 Hz and J = 3.2 Hz), 4.44 (s, 2H), 4.38 (t, 2H, J = 6.7 Hz), 3.33 (t, 2H, J = 6.2 Hz), 2.77 (t, 2H, J = 6.2 Hz), 2.62 (t, 2H, J = 6.2 Hz), 2.26-2.23 (m, 2H). ESI-HRMS calculated for C$_{23}$H$_{26}$N$_9$O$_3$ [M + H]$^+$, 476.2160; Calcd. 476.2159. HPLC purity 98% (R$_f$ = 6.9 min).

2-(4-(3-(5-Amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)-N-(3-aminopropyl)acetamide 6b.

Compound 8 (1 eq., 7 mg, 0.0157 mmol) was dissolved in a mixture of 1,3-diaminopropane and MeOH (2 mL, 1:9, v/v). After stirring at room temperature overnight, the reaction mixture was concentrated to dryness, and the resulting residue was purified by silica gel column chromatography (20:78:2 MeOH:CH$_2$Cl$_2$:aq.NH$_3$, v/v/v) to afford a white solid (4.5 mg, 59 %). $^1$H NMR (MeOD-d$_4$, δ ppm) 8.10 (s, 1H), 7.77 (d, J = 1.7 Hz, 1H), 7.26 (d, J = 3.2 Hz, 1H), 7.10 (d, 2H, J = 8.7 Hz), 6.68 (dd, 1H, J = 1.6 Hz and J = 3.2 Hz), 4.43 (s, 2H), 4.38 (t, 2H, J = 6.7 Hz), 3.33 (t, 2H, J = 6.2 Hz), 2.68 (t, 2H, J = 6.2 Hz), 2.62 (t, 2H, J = 6.2 Hz), 2.26-2.23 (m, 2H), 1.74-1.67 (m, 2H). ESI-HRMS calculated for C$_{24}$H$_{28}$N$_9$O$_3$ [M + H]$^+$, 490.2321; Calcd. 490.2315. HPLC purity 99% (R$_f$ = 6.9 min).
2-(4-(3-(5-Amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)-N-(4-aminobutyl)acetamide 6c.

Compound 8 (1 eq., 21 mg, 0.047 mmol) was dissolved in a mixture of putrescine and MeOH (7 mL, 1:9, v/v) and stirred overnight at room temperature before concentrated to dryness. The resulting residue was purified by silica gel column chromatography (20:78:2 MeOH:CH₂Cl₂:aq.NH₃, v/v/v) to afford a white solid (22 mg, 94%). ¹H NMR (MeOD-d₄, δ ppm) 8.10 (s, 1H), 7.08 (d, J = 1.7 Hz, 1H), 7.26 (d, J = 0.7 Hz and J = 3.4 Hz, 1H), 1.01 (m, 4H). ESI-HRMS calculated for C₁₅H₁₇N₃O₂ [M + H]⁺, 254.1105; Calcd. 254.1105. HPLC purity 97% (Rₘ = 7.4 min).

2-(4-(3-(5-amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)-N-(5-aminopentyl)acetamide 6d.

Compound 8 (1 eq., 10 mg, 0.0224 mmol) was dissolved in a mixture of cadaverine and MeOH (5 mL, 1:9, v/v) and stirred overnight at room temperature before concentrated to dryness. The resulting residue was purified by silica gel column chromatography (20:78:2 MeOH:CH₂Cl₂:aq.NH₃, v/v/v) to afford a white solid (7.5 mg, 65%). ¹H NMR (MeOD-d₄, δ ppm) 8.10 (s, 1H), 7.78 (d, J = 0.7 Hz and J = 1.7 Hz, 1H), 7.26 (d, J = 0.7 Hz and J = 3.4 Hz, 1H), 7.10 (d, 2H, J = 8.7 Hz), 6.84 (d, 2H, J = 8.7 Hz), 6.68 (dd, 1H, J = 1.6 Hz and J = 3.2 Hz), 4.42 (s, 2H), 4.38 (t, 2H, J = 6.8 Hz), 3.27 (t, 2H, J = 7.2 Hz), 2.74-2.67 (m, 4H), 2.62 (t, 2H, J = 7.2 Hz), 2.26-2.23 (m, 2H), 2.16-1.68 (m, 4H). ESI-HRMS calculated for C₂₅H₃₀N₉O₃ [M + H]⁺, 504.2476; Calcd. 504.2472. HPLC purity 96% (Rₘ = 7.8 min).

2-(4-(3-(5-Amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)-N-(6-diaminohexyl)acetamide 6e.

Compound 8 (1 eq., 7 mg, 0.0157 mmol) was dissolved in a mixture of 1,6-diaminohexane and MeOH (2 mL, 1:9, v/v) and stirred overnight at room temperature before concentrated to dryness. The resulting residue was purified by silica gel column chromatography (20:78:2 MeOH:CH₂Cl₂:aq.NH₃, v/v/v) to afford a white solid (1.5 mg, 20%). ¹H NMR (MeOD-d₄, δ ppm) 8.10 (s, 1H), 7.78 (d, J = 0.7 Hz and J = 1.7 Hz, 1H), 7.26 (d, J = 0.7 Hz and J = 3.4 Hz, 1H), 7.10 (d, 2H, J = 8.7 Hz), 6.84 (d, 2H, J = 8.7 Hz), 6.68 (dd, 1H, J = 1.6 Hz and J = 3.2 Hz), 4.42 (s, 2H), 4.38 (t, 2H, J = 6.8 Hz), 3.27 (t, 2H, J = 7.2 Hz), 2.91 (t, 2H, J = 7.2 Hz), 2.62 (t, 2H, J = 7.2 Hz), 2.26-2.23 (m, 2H), 1.68-1.66 (m, 2H), 1.64-1.62 (m, 2H), 1.61-1.58 (m, 4H). ESI-HRMS calculated for C₂₇H₃₄N₉O₃ [M + H]⁺, 532.2776; Calcd. 532.2785. HPLC purity 96% (Rₘ = 7.5 min).

4-(3-(5-Amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenol 7.

To a solution of 2-(furan-2-yl)-7-(3-(4-methoxyphenyl)propyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (3, 1 eq., 90 mg, 0.231 mmol) in DCM (12 mL) was added dropwise BBr₃ (5 eq., 1 M in CH₂Cl₂, 1.18 mL, 1.18 mmol) at 0 °C. The mixture was stirred for 4 h at room temperature, hydrolyzed carefully with MeOH at 0 °C and evaporated in vacuo to afford a brown solid, which was used without further purification in the next step (83 mg, 95%). ¹H NMR (MeOD-d₄, δ ppm) 8.12 (s, 1H), 7.78 (d, J = 1.1 Hz, 1H), 7.25 (d, J = 3.1 Hz, 1H), 6.92-7.10 (d, J = 8.5 Hz, 2H), 6.66 (d, J = 1.9 Hz, 1H), 6.61-6.65 (d, J = 8.5 Hz, 2H), 4.37 (t, J = 7.0 Hz, 2H), 2.52 (t, J = 7.0 Hz, 2H), 2.16-2.25 (m, 2H). ESI-HRMS calculated for C₁₀H₁₈N₇O₂ [M + H]⁺, 376.1532; Calcd. 376.1522.
Methyl 2-(4-(3-(5-amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)acetate 8.

To a suspension of 7 (1 eq., 18 mg, 0.048 mmol) in MeOH (3 mL) was added cesium carbonate (5 eq., 78.1 mg, 0.24 mmol). The mixture was stirred for 1 h at 40 °C and then methyl bromoacetate (12 eq., 0.055 mL, 0.58 mmol) was added. The mixture was stirred overnight at 40 °C and then concentrated in vacuo. The crude product was purified by silica gel column chromatography (DCM/MeOH : 99/1) to afford a white solid (12 mg, 90 %). 1H NMR (MeOD-d4, δ ppm): 8.21 (s, 1H), 7.65 (d, J = 1.8 Hz, 1H), 7.29-7.26 (m, 1H), 7.14-7.10 (d, 2H, J = 8.8 Hz), 6.85-6.80 (d, 2H, J = 8.8 Hz), 6.63 (dd, 1H, J = 1.6 Hz and J = 3.2 Hz), 5.97 (s, 2H), 4.62 (s, 3H), 4.37 (t, 2H, J = 7.0 Hz), 2.62 (t, 2H, J = 7.0 Hz), 2.30-2.19 (m, 2H). ESI-HRMS calculated for C23H22N7O4 [M + H]+, 448.1727; Calcd. 448.1733.

2-((1E,3E)-5-((E)-3-((2-(2-(4-(3-(5-amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)acetamido)ethyl)amino)-6-oxohexyl)-3-methyl-5-sulfonato-1-(3-sulfonatopropyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(3-sulfonatopropyl)-3H-indol-1-ium-5-sulfonate, triethylammonium salt 9.

To a solution of 6a (1 eq., 0.7 mg, 0.0015 mmol) in DMF (0.3 mL) was added Et3N (1.1 eq., 0.0002 mL, 0.0016 mmol) and Alexa Fluor® 647 NHS Ester (0.54 eq., 1.0 mg, 0.0008 mmol). The flask was protected from light, and the mixture was stirred overnight. The crude product was then directly purified by HPLC (column A, H2O/AN : from 100/0 to 70/30, 40 min, tr = 28.3 min) to afford a blue solid (1.2 mg, 62 %). 1H NMR (D2O-d4, δ ppm): 7.87-7.84 (m, 1H), 7.79-7.76 (m, 1H), 7.75-7.71 (m, 2H), 7.59-7.57 (m, 2H), 7.50-7.48 (m, 1H), 7.28 (d, 1H, J = 8.3 Hz), 7.06 (d, 1H, J = 3.3 Hz), 6.92 (d, 1H, J = 8.3 Hz), 6.64 (d, 2H, J = 8.7 Hz), 6.53 (dd, 1H, J = 1.6 Hz and J = 3.2 Hz), 6.29-6.19 (m, 4H), 5.87 (d, 1H, J = 13.7 Hz), 5.37 (s, 1H), 4.21-4.19 (m, 2H), 4.16-4.13 (m, 2H), 4.08-4.02 (q, NCH2), 3.96 (d, 1H, J = 14.6 Hz), 3.86-3.82 (m, 2H), 3.76 (d, 1H, J = 14.6 Hz), 3.66-3.52 (m, 1H), 3.57-3.54 (m, 1H), 3.11-3.00 (m, 2H), 2.94-2.91 (m, 2H), 2.84 (t, 2H, J = 7.25 Hz), 2.55 (m, 2H), 2.22-2.11 (m, 2H), 2.08 (t, 2H, J = 7.25 Hz), 1.99-1.94 (m, 3H), 1.82 (s, 1H), 1.54 (s, 3H), 1.49 (s, 3H), 1.41 (s, 3H), 1.34-1.26 (m, 2H), 1.23-1.18 (m, 5H), 1.16 (t, NCH3), 1.09-1.01 (m, 1H). ESI-HRMS calculated for C59H68N11O16S4 [M + H]+, 1314.3739; Calcd. 1314.3728. HPLC purity 99% (Rt = 14.0 min).

2-((1E,3E)-5-((E)-3-((4-(2-(4-(3-(5-amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)acetamido)butyl)amino)-6-oxohexyl)-3-methyl-5-sulfonato-1-(3-sulfonatopropyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-1-(3-sulfonatopropyl)-3H-indol-1-ium-5-sulfonate, triethylammonium salt 10.

To a solution of 6c (1 eq., 0.741 mg, 0.0015 mmol) in DMF (0.3 mL) was added Alexa Fluor® 647 NHS Ester (0.543 eq., 1 mg, 0.0008 mmol) and Et3N (1.1 eq., 0.0002 mL, 0.00162 mmol). The flask was protected from light, and the mixture was stirred overnight. The crude product was then directly purified by HPLC (column A, H2O/AN : from 100/0 to 70/30, 40 min, tr = 29.06 min) to afford a blue solid (1.5 mg, 76 %). 1H NMR (MeOD-d4, δ ppm): 7.83-7.65 (m, 5H), 7.56 (m, 2H), 7.48 (d, 1H, J = 8.4 Hz), 7.24 (d, 1H, J = 7.6 Hz), 6.98 (m, 1H), 6.91, (d, 1H, J = 7.6 Hz), 6.62 (d, 2H, J = 6.3 Hz), 6.51 (m, 1H), 6.23 (d, 2H, J = 8.8 Hz), 6.18-6.13 (m, 1H), 5.79 (d, 1H, J = 13.2 Hz), 4.10 (m, 4H), 3.94 (d, 1H, J = 14.4 Hz), 3.83-3.82 (m, 2H), 3.71 (d, 1H, J = 10.8 Hz), 3.10 (q, NCH2), 3.01 (m, 2H), 2.95-2.80 (m, 8H), 2.49 (m, 2H), 2.14-2.11 (m, 6H), 1.99-1.92 (m, 4H), 1.49 (s, 3H), 1.41 (s, 3H), 1.37 (s, 3H), 1.23-1.20 (m, 6H), 1.17 (t, NCH3), 1.06 (m, 2H). ESI-HRMS calculated for C61H72N11O16S4 [M + H]+, 1342.4052; Calcd. 1342.4041. HPLC purity 99% (Rt = 10.1 min).
(E)-N-(4-(2-(4-(3-(5-Amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)acetamido)butyl)-6-(2-(4-(2,5-difluoro-7-(thiophen-2-yl)-5H-4,5,6,7-tetrahydropyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-ylvinyl)phenoxy)acetamido)hexanamide 11.

To a solution of 6c (1 eq., 1.5 mg, 0.0029 mmol) in DMF (0.3 mL) was added Et3N (3.0 eq., 1.2 µL, 0.0087 mmol) and BODIPY® 630/650-X NHS Ester (0.8 eq., 1.57 mg, 0.0024 mmol). The flask was protected from light, and the mixture was stirred overnight. The crude product was then directly purified by HPLC (column A, H2O/AN : 50/50 to 0/100, 40 min, tR = 29.9 min) to afford after lyophilization a blue solid (1.9 mg; 61%). 1H NMR (MeOD-d4, δ ppm): 8.05 (m, 2H), 7.74 (m, 1H), 7.60 (d, 1H, J = 7.6 Hz), 7.56 (d, 2H, J = 7.6 Hz), 7.46 (d, 1H, J = 3.9 Hz), 7.31 (s, 1H), 7.23 (d, 1H, J = 3.2 Hz), 7.18 (m, 2H), 7.00 (d, 1H, J = 4.4 Hz), 7.07-7.03 (m, 4H), 6.99 (d, 2H, J = 8.8 Hz), 6.65 (m, 1H), 4.36 (m, 2H), 4.29 (t, 2H, J = 6.8 Hz), 3.22 (t, 2H, J = 6.4 Hz), 3.12-3.10 (m, 2H), 2.56 (t, 2H, J = 6.4 Hz), 2.19-2.13 (m, 4H), 2.15-1.43 (m, 8H), 1.28-1.26 (m, 2H), 1.21-1.19 (m, 2H). ESI-HRMS calculated for C54H56BF2N12O8S [M + H]+, 1049.4224; Calcd. 1049.4228. HPLC purity 96% (Rt = 13.6 min).

5-((4-(2-(4-(3-(5-Amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)acetamido)butyl)carbamoyl)-2-(6-amino-4-3-imino-4,5-disulfo-3H-xanthen-9-yl)benzoic acid, triethylammonium salt 12.

To a solution of 6c (1 eq., 0.71 mg, 0.0014 mmol) in DMF (0.14 mL) was added Et3N (1.1 eq., 0.0002 mL, 0.0016 mmol) and Alexa Fluor® 488 Carboxylic Acid, 2,3,5,6-Tetrafluorophenyl Ester, 5-isomer (0.8 eq., 1 mg, 0.0011 mmol). The flask was protected from light, and the mixture was stirred overnight. The crude product was then directly purified by HPLC (column B, H2O/AN : 100/0 to 70/30, 20 min, tR = 10.7 min) to afford after lyophilization an orange solid (0.47 mg; 33%). 1H NMR (D2O, δ ppm): 8.07 (s, 1H), 7.82 (s, 1H), 7.70 (m, 1H), 7.58 (s, 1H), 7.02 (d, 1H, J = 3.6 Hz), 6.85 (d, 2H, J = 9.6 Hz), 6.69-6.66 (m, 5H), 6.53 (m, 1H), 6.39 (d, 2H, J = 8.4 Hz), 4.09 (m, 4H), 3.24 (s, 2H), 3.10 (q, NCH2), 3.07-3.05 (m, 2H), 2.27-2.25 (m, 2H), 2.03 (m, 2H), 1.39 (m, 4H), 1.17 (t, NCH3). ESI-HRMS calculated for C46H40N11O13S2 [M + H]+, 1020.2411; Calcd. 1020.2405. HPLC purity 99% (Rt = 10.1 min).

4-((4-(3-(5-Amino-2-(furan-2-yl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-7-yl)propyl)phenoxy)methyl)benzenesulfonate ammonium salt 13.

To a solution of 7 (1 eq., 10 mg, 0.0266 mmol) in DMF (4.29 mL) under N2 was added NaH (1 eq., 0.64 mg, 0.027 mmol) and the mixture was stirred for 15 min at room temperature before adding sodium 4-(bromomethyl)benzenesulfonate (1.1 eq., 8 mg, 0.0293 mmol). After 1 h stirring at room temperature, sodium 4-(bromomethyl)benzenesulfonate (1.1 eq., 8 mg, 0.0293 mmol) was added again, and the mixture was stirred for 45 min. The mixture was then treated with MeOH, concentrated in vacuo and purified by silica gel column chromatography (DCM/MeOH/ NH3: 85/15/1) to afford a white solid (6.3 mg, 42%). 1H NMR (MeOD-d4, δ ppm): 8.12 (s, 1H), 7.77-7.76 (m, 3H), 7.76 (m, 1H), 7.49 (d, 1H, J = 8.0 Hz), 7.45 (d, 2H, J = 8.4 Hz), 7.24 (dd, 1H, J = 0.4 Hz and J = 3.2 Hz), 7.04 (d, 2H, J = 8.8 Hz), 6.80 (d, 2H, J = 8.8 Hz), 6.67 (dd, 1H, J = 1.6 Hz and J = 3.2 Hz), 4.99 (s, 2H), 4.69 (br s, 1H), 4.38 (t, 2H, J = 7.0 Hz), 2.62 (t, 2H, J = 7.0 Hz), 2.27-2.24 (m, 2H). ESI HRMS calculated for C26H22N4O2S·[M-H]−, 544.1401; Calcd. 544.1403. HPLC purity 96% (Rt = 10.8 min).
Pharmacological assays:

Cell culture for membrane binding assays and flow cytometry: HEK-293 cells stably expressing the A2AAR were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 0.500 mg/mL G418 Sulfate (Geneticin). Cells were maintained in a humidified atmosphere and sterile incubation conditions held at 37 ℃ and 5% CO2 (g). A day prior to the experiment, cells were plated on a 96-well clear and flat bottom plate at 80-90% confluency in 100 µL of medium.

Radioligand binding assays: Cell membranes were prepared as reported.1

Binding assays were carried out using standard radioligands and membrane preparations from HEK-293 cells stably expressing the human (h) A1, A2A or A3ARs or mouse (m) A1, A2A or A3ARs. The radioligands used were: A1AR, [3H]8-cyclopentyl-1,3-dipropylxanthine 14; A2AAR, [3H]2; A3AR, [125I]N6-(4-amino-3-iodobenzyl)adenosine-5’-N-methyluronamide 16. The radioligand for archival A2AAR affinity data presented in Table 1 was [3H]2-[p-(2-carboxyethyl)phenyl-ethylamino]-5’-N-ethylcarboxamidoadenosine 15. Nonspecific binding was determined using 10 µM 8-[4-[[[(2-aminoethyl)amino]carbonyl][methyl]oxy]phenyl]-1,3-dipropylxanthine 17 (A1AR and A2AAR) or 10 µM adenosine-5’-N-ethyluronamide 18 (A3AR). HEK-293 cells expressing recombinant mA1, A2A, or A3AR were used.

Protein was determined as reported.2 In all the binding experiments, IC50 values and Kd values were calculated using GraphPad Prism software (San Diego, CA). Values are expressed as mean±SEM.

Fluorescent binding studies: All binding studies were done in triplicate. For saturation binding studies, cells were treated with 50 µL of 11 (MRS 7396) or 12 (MRS 7416), to achieve a final concentration from 0.19 to 400 nM, and 50 µL of Tris-HCl buffer containing 10 mM MgCl2. Non-specific binding was determined with SCH442416 3 (final concentration of 10 µM, in Tris-HCl buffer). For displacement experiments, cells were incubated simultaneously with 50 µL of 40 nM 11 or 12 (final concentration 10 nM) and 50 µL of the non-labeled displacing ligand at increasing concentrations. The total binding was measured in the absence of a displacing ligand, and non-specific binding was determined with 10 µM 3. After 1 h at 37 ℃ (for both the saturation and displacement experiments), the medium was removed and the cells were carefully washed two times with 150 µL of ice-cold PBS (not containing Mg2+ or Ca2+). The cells were treated with 40 µL of Corning Cellstripper (Mediatech, Manassas, VA) per well and then incubated at 37 ℃ for 10 min. To each well was subsequently added 160 µL of PBS (not containing Mg2+ or Ca2+), and the cell fluorescence was analyzed with a BD FACScalibur flow cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ) with excitation at 635 nm (red diode laser, for 11) or 488 nm (blue laser, for 12) in conjunction with the software from BD Bioscience PlateManager and CellQuest. Data analysis was performed with the Prism 5 (GraphPad, San Diego, CA) software.


Molecular Modeling Methods

Protein preparation. The high-resolution hA2AAR X-ray structure in complex with the triazolo-triazine antagonist ZM241385, structurally related to the reference compound 3, was retrieved from the Protein Data Bank (PDB) (ID: 4EIY). Hydrogen atoms were added using the Protein Preparation Wizard tool implemented in the Schrödinger suite. During the protein preparation, co-crystallized hetero groups and the fusion partner (BRIL) were removed. The protonation states of titrable residues were determined according to H-bond patterns with surrounding residues. To this aim, all water molecules present in the X-ray construct were retained during the protein preparation procedure. However, for the subsequent docking analysis only water molecules in the first solvation sphere of the ligand were kept. According to H-bond pattern analysis His75/278/306 and His155/230/250 were protonated on the N and Ne, respectively, whereas His264 (establishing a salt bridge with Glu169) was considered doubly protonated. The native sequence of the hA2AAR as well as missing side chains of residues whose backbone coordinates were observed in the X-ray structure were restored by building a homology model with Prime.

Docking. Structures of selected ligands were built and prepared for docking using the Builder and the LigPrep tools implemented in the Schrödinger suite. The structures were minimized using the OPLS 2005 force field. Molecular docking was performed with the Glide package from the Schrödinger suite, with the barycenter of the co-crystallized ligand representing the center of the Glide Grid (inner box: 14 x 14 x 14 Å; outer box extended by 20 Å in each direction from the inner box). Docking was performed considering the protein binding sites residues rigid by using the standard precision (SP) scoring function. Ligands were docked at the hA2AAR by retaining a variable number (depending upon the specific ligand considered) of non-overlapping water molecules according to the following protocol: ligands were first docked at the hA2AAR structure without water molecules; the best docking poses so obtained were superimposed with the hA2AAR structure containing water molecules in the first solvation sphere of the co-crystallized ligand; after the superimposition, non-overlapping water molecules were identified; ligands were therefore redocked at the hA2AAR containing those water molecules. In a few cases, iterative cycles of removal of non-overlapping water molecules and ligand docking were performed until the SP score did not further improve.

Molecular Dynamics. MD system setup, equilibration, and production were performed with the HTMD module (Acellera, Barcelona Spain, version 1.5.4). The ligand-protein complexes were embedded into an 80 x 80 x 80 Å 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) membrane leaflet generated through the VMD Membrane Plugin tool. Overlapping lipids (within 0.6 Å) were removed upon protein insertion and the systems were solvated with TIP3P water and neutralized by Na+/Cl- counter-ions (final concentration 0.154 M). MD simulations with periodic boundaries conditions were carried out with the ACEMD engine (Acellera, version 2016.10.27) using the CHARMM36/C2FF(3.0.1) force fields for lipid and protein, and ligand atoms, respectively. Ligand parameters were retrieved from the ParamChem service (https://cgenff.paramchem.org, accessed 04/2017, version 1.0.0) with no further optimization. After initial validation, the atom types for compounds were manually assigned to enforce the equivalency of the atoms on the two terminal aryl rings of the fluorophore moiety, consistently with previous MD studies performed on AlexaFluor488. As for the specific purpose of this study atomic charges on the so-defined atom types were not optimized, the electrostatic contribution to the total ligand-protein interaction energy for this ligand was evaluated only qualitatively and will not be described in detail. The systems were equilibrated through a 5000-step minimization followed by 40 ns of MD simulation in the NPT ensemble by applying initial constrains (0.8 for the ligand atoms, 0.85 for alpha carbon atoms, and 0.4 for the other protein atoms) that were linearly reduced after 20 ns. During the equilibration procedure, the temperature was maintained at 310 K using a Langevin thermostat with a low damping constant of 1 ps⁻¹, and the pressure was maintained at 1 atm using a Berendensen barostat. Bond
lengths involving hydrogen atoms were constrained using the M-SHAKE\textsuperscript{16} algorithm. The equilibrated systems were subjected to 30 ns of unrestrained MD simulations run in triplicate for each ligand-protein complex (NVT ensemble, timestep = 2 fs, damping constant = 0.1 ps\textsuperscript{-1}). Long-range Coulomb interactions were handled using the particle mesh Ewald summation method (PME)\textsuperscript{17} with grid size rounded to the approximate integer value of cell wall dimensions. A non-bonded cutoff distance of 9 Å with a switching distance of 7.5 Å was used. All simulations were run on three NVIDIA GeForce GTX (970, 980Ti, and 1080).

MD Trajectory Analysis. MD trajectory analysis was performed with an in-house script exploiting the NAMD 2.10\textsuperscript{18} mdenergy function and the RMSD trajectory tool (RSMDTT) implemented in VMD\textsuperscript{7}. All simulations were run in triplicate and selection of representative trajectories and of lowest interaction energy (IE) ligand-protein complexes were based upon the total ligand–protein interaction energy (IE\textsubscript{tot}) expressed as the sum of van der Waals (IE\textsubscript{vdW}) and electrostatic (IE\textsubscript{ele}) contribution as previously described\textsuperscript{19}. IE vs simulation time graph was generated with an in-house script exploiting Gnuplot\textsuperscript{20}.

Modeling References


**Molecular Modeling Results: Tables and Figures**

Table S1. Parameters considered for the selection of a representative trajectory among three replicas: protein alpha carbon atoms (Cα) average RMSD, ligand average RMSD, and slope of the dynamic scoring function (DSFslope). RMSD values are in Å and DSF is adimensional. Selected runs are marked in bold.

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Figure S1. Most energetically favored ligand-protein structure (Interaction Energy = -89.544 kcal/mol) obtained for 2-hA2AAR complex in the selected MD run starting from the docking pose. In this snapshot the ligand features the same interaction pattern observed for the initial docking pose, thus validating the quality of the ligand-protein interaction predicted by docking. Side view facing TM6, TM7, and TM1 (from the left).
Figure S2. Two alternative binding modes obtained for compound 6c, the synthetic precursor of 11, at the hA2aAR. In the most energetically favored docking complex (orange carbon sticks, docking score = -12.077 kcal/mol) the points toward TM4 and TM5, the amide moiety establishes a H-bond with the sidechain of E169, and the terminal amine group engages in H-bond interactions with the backbone of E169 (EL2) and the sidechain of K150 (EL2). In the alternative binding mode (green carbon sticks, docking score = -10.994 kcal/mol), the tail points toward TM1 and TM2 and does not establish additional interactions. Residues establishing polar (dashed orange lines) and π-π interactions with the docked ligands are represented as thin sticks. Non-polar hydrogen atoms are omitted.
Figure S3. Three-dimensional representation (A) and schematic depiction (B) of the distance between the terminal ammine group and the centroids of the aromatic moieties in the fluorophore group of 11. (C) Most energetically favored ligand-protein complexes obtained after MD simulation, starting from 6c-hA_{2}A_{2}AR docked complexes. Aromatic (solid surface) and hydrophobic (wireframe surface) regions in the proximity of the terminal amine moiety are colored according to the distance from the nitrogen atom as follows: 5 Å = magenta, 13 Å = green, and 14 Å = yellow. As depicted, only in one orientation the proximity of aromatic/hydrophobic regions in the protein (colored arrow) are compatible with the placement of the aromatic moieties of the fluorophore group of 11. Side view, facing TM6, TM7, and TM1 (from the left).
Figure S4. Superimposition of the most energetically favorable 12-hA$_{2A}$AR complexes obtained after three MD simulation starting from BM2: the three replicas converged in a unique binding mode. The structures are colored according to the IE value, the lower (more favorable) the value the darker the color.
Figure S5. (A) Superimposition of the most energetically favorable 13-hA2AR complexes obtained after MD simulation starting from three different binding modes (BM1 = cyan, BM2 = magenta, BM3 = orange): the three different initial poses converged in a unique binding mode. (B) Ligand-protein complex with the lowest interaction energy (IE = -201.59 kcal/mol) obtained after MD simulation starting from BM3: with respect to its initial conformation, the 7-phenylpropyl ring of the ligand moves toward TM7 and establishes a π-π stacking interaction with Y271 (7.36). Both A and B are a side view facing TM6, TM7, and TM1 (from the left).
Pharmacological Results

Inhibition of whole cell binding of fluorescent probe 12 by agonists:

Although the inhibition of binding of AlexaFluor488 conjugate 12 provided the expected affinities when employing antagonists, the inhibition by agonists was complex, possibly due to multiple affinity states of this GPCR for agonists. Further study is required.

Agonists 2-[(p-(2-carboxyethyl)phenyl-ethylamino)-5′-N-ethylcarboxamidoadenosine 15 and 6-(2,2-diphenylethylamino)-9-((2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-N-(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9H-purine-2-carboxamide 19 bound with Ki values, respectively, of 4.0 and 99 nM.

Demonstration of antagonist action at the hA2AAR:

Figure S6. Right shifts of the hA2AAR curve for activation, i.e. cyclic AMP accumulation, by CGS21680 15, induced by antagonists 11 (A) and 13 (B). Results are expressed and mean±SEM from 2-3 experiments performed in duplicate. The EC50 of CGS21680 15 alone was 0.89±0.17 nM, in the presence of 11 (1000 nM), EC50 = 128±35 nM; in the presence of 13 (100 nM), EC50 = 10.2±2.3 nM.
Chinese hamster ovary (CHO) cells stably expressing the human A2AAR were cultured in Dulbecco's Modified Eagle Medium (Mediatech, Manassas, VA) 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 treated with antagonists (or medium for control) in the presence of rolipram (10 µM) and adenosine deaminase (3 units/ml) and 5 min later with agonist for 20 min. 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). cAMP was measured using ALPHAScreen cAMP kits (PerkinElmer, Boston, MA) as instructed by the manufacturer.

**Off-target interactions for selected compounds (Kᵢ in radioligand binding inhibition < 10 µM)**
Refer to: [http://pdspdb.unc.edu](http://pdspdb.unc.edu) for full list of comprehensive screen at 45 targets.

PDSP 46674, MRS7354 (6c)
5HT₂A serotonin receptor:

PDSP 48400, MRS7352 (13)
None detected.
6b, MRS7353

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9-Dec-2016
18:19Date:18-01-17 127 (2.349) Cl:29 (2.349) C: 0.50, Ar: 5.90, Sm (6E, 1-e0.00): 6b (19.500)

TOF MS E5+
2777308

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Maximum
Calc. Mass eDa
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450.2237 5.3 -10.8 6.5 80.9
430.2221 10.0 20.4 2.5

Formulas
C24 H26 N3 O3
C17 H12 N9 O8
C14 H12 N9 O11

RDX217-meod (12-29-2016) - meod

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Signal 1: DAD1 A, Sig=254.8 Ref=360.100

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6c, MRS7354

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504.2530  504.2532  0.4  10.7  6.5  18.8  C18 E34 N9 O8
504.2378  504.2378  5.8  19.4  2.5  59.2  C14 E34 N9 O11

RD018-2-meod (12-23-2016)-meod

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6e, MRS7356

Monoisotopic Mass, Even Electron Ions
103 formula(s) evaluated with 3 results within limits (up to 10 closest results for each mass)
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03-Jan-2017
n=03j=17-020 131 (2.423) Cn (Cen,5, 50.00, Ar), Sm(SG, ,x3.00); Sb (12.5.00)

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9, MRS7322

Figure 3: "Chemical shift and integration spectrum of MRS7322."
### 10, MRS7395

Monoisotopic Mass, Even Electron Ions
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20-Jan-2017

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**Diagram:**
- Spectrum of the Mass Spectral Data
- Peaks at various ppm values
- Interpretation of the spectral data for molecular structure

**Additional Information:**
- Mass values ranging from 8.5 to 15 ppm
- Specific peaks at 3.43, 3.45, 7.54, and 2.57 ppm
- Additional peaks at 1.39, 1.44, and 1.78 ppm
11, MRS7396

Monoisotopic Mass, Even Electron Ions
320 formula(s) evaluated with 5 results within limits (up to 10 closest results for each mass)

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31-Jan-2017
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1049.4174  -5.2 -5.0  23.5  118.0  C48 H60 N10 012 F2 328 15B
1049.4057  6.5  6.2  41.5  352.8  C12 H52 N10 012 F2 328 15B

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12, MRS7416

Monoisotopic Mass, Even Electron Ions
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03-Mar-2017
ref: 03mar17-086  111 (2.182) Cr (Cen.5, 56.00, Ar); Sm (150, 3x5.00); Sb (125.00)

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[Graphical representation of NMR spectra with peaks at various ppm values]
Lambda Maximum at 630 nm
Absorption: 3.4
Extinction Coefficient of Alexa 647: 270,000 cm$^{-1}$M$^{-1}$
Path Length: 1 cm
MW: 1382.45
Volume: 500 μL

\[ A = \varepsilon bc \]
\[ 3.4 = 270000 \times 1 \times c \]
\[ c = 3.4/270000 = 1.26 \times 10^{-5} \text{ M} \]
\[ c = 12.6 \text{ μM} \]

\[ c = m/v \]
\[ 1.26 \times 10^{-5} = m/5 \times 10^{-4} \]
\[ m = 6.3 \times 10^{-9} \text{ mol} \]

\[ m = \text{amount}/\text{M.W} \]
\[ 6.3 \times 10^{-9} \text{ M} = \text{amount}/1382.45 \]
\[ \text{amount} = 8.71 \text{ μg} \]