Supporting information

Discovery of a High Affinity Adenosine A<sub>1</sub>/A<sub>3</sub> Receptor Antagonist with a Novel 7-Amino-Pyrazolo[3,4-d]pyridazine Scaffold

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Number of pages: 32
| Residues | 3.33 | 5.29 | 5.30 | 5.38 | 5.42 | 6.48 | 6.51 | 6.52 | 6.55 | 6.66 | 6.67 | 7.32 | 7.35 | 7.36 | 7.39 |
|----------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| A₁R      | L    | F    | E    | M    | N    | W    | L    | H    | N    | K    | K    | S    | T    | Y    | I    |
| A₃R      | L    | F    | V    | M    | S    | W    | L    | S    | N    | V    | V    | Q    | L    | Y    | I    |
| A₂B R    | L    | F    | V    | M    | N    | W    | V    | H    | N    | K    | N    | K    | M    | N    | I    |

**Scheme S1.** Sequence alignment of the residues surrounding the binding site of A₁R, A₃R,A₂B R (Ballesteros-Weinstein¹ numbering is applied). Colored in yellow columns show residues that differ inside chain polarity, volume, rigidity.
| Compound | A$_{2A}$R (10 μM) | A$_{2B}$R (10 μM) |
|----------|------------------|------------------|
|          | pEC$_{50}$ of NECA in presence of compound$^a$ | pEC$_{50}$ of NECA in presence of compound$^a$ | pK$_b$ |
| 10a      | 5.64 ± 0.38      | 7.01 ± 0.10      | 4.79 ± 0.15 |
| 10b      | 5.82 ± 0.33      | 6.39 ± 0.07*     | 5.76 ± 0.14 |
| 10c      | 5.60 ± 0.52      | 7.23 ± 0.10      | N.B.        |
| 15a      | 6.17 ± 0.47      | 6.87 ± 0.09      | 5.09 ± 0.15 |
| 15b      | 5.71 ± 0.46      | 7.22 ± 0.13      | N.B.        |


| Compound | pEC<sub>50</sub> (Vehicle) | pEC<sub>50</sub> (NECA) | Notes |
|----------|--------------------------|---------------------|-------|
| 15c      | 6.42 ± 0.17              | 7.22 ± 0.12         | -     |

*Mean ± SEM; Functional activities (pEC<sub>50</sub> values of NECA in the presence of either 1 µM ligands or vehicle) as mean ± standard error of the mean (SEM) of at least 3 independent repeats, conducted in duplicate. Statistical significance compared to NECA was determined, at p< 0.05, through One-Way ANOVA with Dunnett’s post-test (*, p< 0.05).

*Mean ± SEM; Equilibrium binding affinities of the ligands measured with NanoBRET against WT A<sub>2B</sub>R; NECA was used as positive control as described in ref. in the main manuscript.
Supplementary MD simulations and MM-GBSA calculations results

Representative MD simulations results for 10b and 15b against A1R, A3R and A2B R and for 10a, 10c against A1R are included (Table S2).

The MM-GBSA2-4 method with the OPLS20055,6 force field and using a hydrophobic slab7,8 as implicit membrane model and including waters in the orthosteric binding area, in a radius of 4 Å from the center of mass of the ligand provides a fair ranking for the binding of ligand 10b against the three receptors A1R, A3R or A2B R and for ligands binding 10a-10c inside A1R but did not differentiate 10b and 15b inside A1R (Table S2). This is due the lack of accuracy of the MM-GBSA3-4 method.

Table S2. MD simulations results of 7-amino-pyrazolo[3,4-d]pyridazines 10a-c, 15b in complex with A1R, A3R or A2B R and MM-GBSA calculations results for 10a-c in complex with A1R.

| Compound | A1R | A3R | A2B R |
|----------|-----|-----|-------|
|         | RMSD<sub>i</sub> g<sup>a</sup> | RMSD protein<sup>b</sup> | ΔG<sub>eff</sub><sup>c</sup> | RMSD<sub>i</sub> g<sup>a</sup> | RMSD protein<sup>b</sup> | ΔG<sub>eff</sub><sup>c</sup> | RMSD<sub>i</sub> g<sup>a</sup> | RMSD protein<sup>b</sup> | ΔG<sub>eff</sub><sup>c</sup> |
| 10a      | 2.21 ± 0.42 | 2.14 ± 0.14 | -94.50 ± 5.93 | - | - | - | - | - | - |
| 10b      | 1.29 ± 0.31 | 1.21 ± 0.17 | -96.42 ± 5.93 | 4.88 ± 0.66 | 2.01 ± 0.21 | -101.98 ± 5.02 | 1.53 ± 0.41 | 2.08 ± 0.56 | -94.19 ± 6.06 |
| 10c      | 1.53 ± 0.37 | 1.66 ± 0.24 | -85.35 ± 5.97 | - | - | - | - | - | - |
| 15b      | 2.38 ± 0.45 | 2.03 ± 0.19 | - | 3.19 ± 0.45 | 2.86 ± 0.15 | - | 6.77 ± 0.38 | 2.50 ± 0.14 | - |
Figure S1. $^1$H and $^{13}$C NMR spectra of compounds 10a-c in CDCl₃.
The mean frame from MD simulations was close to the starting docking pose in A<sub>2B</sub>R (RMSD<sub>lig</sub> = 1.53 Å). The interaction profile for 10b was very similar inside A<sub>2B</sub>R to that observed for the A<sub>1</sub>R (Figure S2).
B

A₃R - 10b

Representative frame from MD simulation
For 15b – A2BR complex a similar binding pose as 10b – A2BR was used. After 100ns molecular dynamic simulation, 15b leaves the binding pocket and enters the membrane area through an opening between TM6 and TM7. The RMSD of this ligand (6.77 ± 0.38 Å) is also indicative of a ligand translation from the starting position.
Figure S2. Docking poses and representative frames, receptor-ligand interaction frequency histograms and RMSD graphs of protein Ca (RMSD_{protein}; blue plots) and ligand heavy atoms (RMSD_{ligand}; red plots) from 100ns-MD simulations of 10b(A)-(B) inside the orthosteric binding area of WT A3R or A2B-R and (C),(D) 15b inside the orthosteric binding area of WT A2B-R,A3R, respectively. Bars are plotted only for residues with interaction.
frequencies $\geq 0.2$. Color scheme: Ligand=cyan sticks, receptor=white cartoon and sticks, hydrogen bonding interactions=yellow (dashes or bars), $\pi-\pi$ interactions=green (dashes or bars); hydrophobic interactions=grey; water bridges=blue. For the protein models of $A_3R$, $A_{2\beta}R$ were used the homology model derived from $A_{2\alpha}R$ (PDB ID 3EML) in complex with a highest-scored docking pose of tested compound.

A similar heterocyclic system with our compounds found in the literature is the 4-(2-phenethyl)amino 1-phenylethyl pyrazolo[3,4-b]pyridine which was found selective against $A_1R$. As has already been described in the literature the docking calculations showed that the $1H$-pyrazolo[3,4-b]pyridine central scaffold has a $\pi-\pi$ interaction with F171, a lipophilic interaction with V87 and L250, and 7-N forms a hydrogen bond with N254. The 4-(2-phenethyl)amino substituent is oriented towards the extracellular part of the receptor and interacted with I274 while 1-phenylethyl towards the bottom of the receptor.

The representative ligand N9-methyl,N6-benzyl adenine binds $A_1R$. Our docking calculations show that the adenine ring has a $\pi-\pi$ interaction with F171, the imidazole nitrogen forms a hydrogen bond with N254 and that N6-benzyl is oriented towards the extracellular part of the receptor (Figure S3). We did not find in the literature any information for the activity of N7-methyl, N6-benzyl adenine against ARs; this compound had been developed against CDK kinases.

**N9-methyl,N6-benzyl adenine to $A_1R$**

*Figure S3.* N9-methyl,N6-benzyl adenine inside the orthosteric binding area of WT $A_1R$; from docking calculations. Color scheme: Ligand=light pink sticks, receptor=white cartoon and sticks, hydrogen bonding interactions=yellow (dashes), $\pi-\pi$ interactions=green (dashes). For the protein models of $A_1R$ the experimental structure of the inactive form for $A_1R$ in complex with an antagonist (PDB ID 5UEN) was used for homology modelling.
Figure S4. Representative frames from 100ns-MD simulations of (A) 10b inside the orthosteric binding area of WT A1R; (B) 10b inside mutant Y271A A1R. Receptor-ligand interaction frequency histogram and RMSD graphs of protein Ca (RMSDprotein; red plots) and ligand heavy atoms (RMSDligand; blue plots). Bars are plotted only for residues with interaction frequencies ≥ 0.2. Color scheme: Ligand=brown sticks, receptor=white cartoon and sticks, hydrogen bonding interactions=yellow (dashes or bars), π-π interactions=green (dashes or bars); hydrophobic interactions=grey; water bridges=blue. For the protein models of A1R the experimental structure of the inactive form for A1R in complex with an antagonist (PDB ID 5UEN 8) was used for homology modelling.

A small TM2 curvature already exists in the model of A3R generated using as template the crystallographic structure of A2AR (PDB ID 3EML)8.

Methods

Safety
No unexpected or unusually high safety hazards were encountered during the proceeding of this project.

Chemistry
Melting points were determined on a Büchi apparatus and are uncorrected. 1H NMR spectra and 2D spectra were recorded on a Bruker Avance III 600 or a Bruker Avance DRX 400 instrument, whereas 13C NMR spectra were recorded on a Bruker Avance III 600 or a Bruker AC 200 spectrometer in deuterated solvents and were referenced to TMS (δ scale). The signals of 1H and 13C spectra were unambiguously assigned by using the 2D NMR spectra COSY, NOESY, HMQC, and HMBC. Mass spectra were recorded with a LTQ Orbitrap Discovery instrument, possessing an Ionmax ionization source. The purity of all the target compounds was >95% as ascertained by 1H-NMR and elemental analysis. Elemental analyses were undertaken using a PerkinElmer PE 240C elemental analyzer and the measured values for C, H, and N were within ±0.4% of the theoretical values. Flash
chromatography was performed on Merck silica gel 60 (0.035 - 0.070 mm). Analytical thin layer chromatography (TLC) was carried out on precoated (0.25 mm) silica gel F-254 plates.

**General procedure for the synthesis of derivatives 4a-5a and 4b-5b**

Sodium hydride (1.30 g, 32.4 mmol, 60% dispersion in mineral oil) was added into a solution of pyrazole 3a or 3b (27.0 mmol) in anhydrous DMF (25 mL) at 0 °C, under argon, and this solution was stirred for 15 min, followed by dropwise addition of iodomethane (2.5 mL, 32.4 mmol). This reaction mixture was stirred at r.t for 1 h. Upon completion of the reaction, the solvent was evaporated under reduced pressure and the residue was extracted with dichloromethane (3 x 150 mL) and water (150 mL). The combined organic layers were dried over Na$_2$SO$_4$ and evaporated under reduced pressure. The crude product was then purified by column chromatography (silica gel) using a mixture of cyclohexane/ethyl acetate 10/1-10/2 as the eluent to provide pure compounds 4a–5a and 4b–5b.

**Ethyl 3-isopropyl-1-methyl-1H-pyrazole-5-carboxylate (4a):** Yield 28%. Colorless oil. $^1$H- and $^{13}$C-NMR spectra identical to those referred in bibliography.  

**Ethyl 5-isopropyl-1-methyl-1H-pyrazole-3-carboxylate (5a):** Yield 72%. Pale yellow oil.$^1$H-NMR (600 MHz, CDCl$_3$) δ (ppm) 6.63 (s, 1H, H-4), 4.31 (q, J = 7.1 Hz, 2H, CH$_2$CH$_3$), 4.10 (s, 3H, NCH$_3$), 2.95 (hept, J = 7.0 Hz, 1H, CH(CH$_3$)$_2$), 1.36 (t, J = 7.1 Hz, 3H, CH$_2$CH$_3$), 1.24 (d, J = 7.0 Hz, 6H, CH(CH$_3$)$_2$). $^{13}$C-NMR (151 MHz, CDCl$_3$) δ (ppm) 159.86 (C=O), 157.29 (C-3), 132.58 (C-5), 107.41 (C-4), 60.59 (CH$_2$CH$_3$), 38.93 (NCH$_3$), 27.52 (CH(CH$_3$)$_2$), 22.61 (CH(CH$_3$)$_2$), 14.12 (CH$_2$CH$_3$). HRMS (ESI) m/z: Calcd for C$_{11}$H$_{17}$N$_2$O$_2$: [M+H]$^+$ =197.1285, found 197.1281.

**Ethyl 1-methyl-3-phenyl-1H-pyrazole-5-carboxylate (4b):** Yield 63%. Pale yellow oil.$^1$H- and $^{13}$C-NMR spectra identical to those referred in bibliography. 

**Ethyl 1-methyl-5-phenyl-1H-pyrazole-3-carboxylate (5b):** Yield 32%. Pale yellow oil. $^1$H- and $^{13}$C-NMR spectra identical to those referred in bibliography.

**Ethyl 4-bromomethyl-3-isopropyl-1-methyl-1H-pyrazole-5-carboxylate (6a):** In a 33% HBr solution in acetic acid (3mL) were added the carboxylate 4a (0.2 g, 1.0 mmol) and paraformaldehyde (98 mg, 3.3 mmol) and the resulting mixture was heated at 90 °C for 3.5 h. The mixture was then poured into ice, made alkaline using NaHCO$_3$(pH~8) and extracted with ethyl acetate (3x30 mL). The organic solvent was dried (Na$_2$SO$_4$) and evaporated to dryness and the residue was purified by column chromatography (silica gel) using a mixture of dichloromethane/ethyl acetate 10/0.3-10/1 as the eluent to provide 0.1 g (33 %) of pure compound 6a as an oil. $^1$H-NMR (600 MHz, CDCl$_3$) δ (ppm) 4.67 (s, 2H, CH$_2$Br), 4.40 (q, J = 7.1 Hz, 2H, CH$_2$CH$_3$), 4.09 (s, 3H, NCH$_3$), 3.04 (hept, J = 7.0 Hz, 1H, CH(CH$_3$)$_2$), 1.43 (t, J = 7.1 Hz, 3H, CH$_2$CH$_3$), 1.30 (d, J = 7.0 Hz, 6H, CH(CH$_3$)$_2$). $^{13}$C-NMR (151 MHz, CDCl$_3$) δ (ppm) 159.87 (C=O), 156.17 (C-3), 130.48 (C-5), 119.21 (C-4), 61.41 (CH$_2$CH$_3$), 40.10 (NCH$_3$), 26.08 (CH(CH$_3$)$_2$), 23.14 (CH(CH$_3$)$_2$), 22.34 (CH$_2$Br), 14.26 (CH$_2$CH$_3$). HRMS (ESI) m/z: Calcd for C$_{11}$H$_{17}$BrN$_2$O$_2$: [M+H]$^+$ =289.0547, found 289.0542.
Ethyl 4-bromomethyl-1-methyl-3-phenyl-1H-pyrazole-5-carboxylate (6b): This compound was prepared following a method analogous to that of 6a, starting from 4b. Yield 72%. Mp: 47-49 °C (pentane). ^1H-NMR (600 MHz, CDCl₃) δ (ppm) 7.78 (d, J = 7.4 Hz, 2H, H-2', H-6'), 7.50 (t, J = 7.4 Hz, 2H, H-3', H-5'). 7.43 (t, J = 7.4 Hz, 1H, H-4'), 4.76 (s, 2H, CH₂Br), 4.49 (q, J = 7.1 Hz, 2H, CH₂CH₃), 4.24 (s, 3H, NCH₃), 1.51 (t, J = 7.1 Hz, 3H, CH₂CH₃). ^13C-NMR (151 MHz, CDCl₃) δ (ppm) 159.64 (C=O), 149.96 (C-3), 131.91 (C-1'), 131.55 (C-5), 128.84 (C-3', C-5'), 128.49 (C-4'), 128.21 (C-2', C-6'), 120.02 (C-5), 116.97 (C-3a), 37.73 (C-7a), 116.97 (C-3a), 37.73

Ethyl 4-formyl-3-isopropyl-1-methyl-1H-pyrazole-5-carboxylate (7a): To a solution of the bromide 6a (150 mg, 0.5 mmol) in dry acetonitrile (2 mL) was added under argon N-methylmorpholine-N-oxide (120 mg, 1.0 mmol) and the mixture was stirred for 24 h. The solvent was then vacuum-evaporated, water was added to the residue, and it was extracted with ethyl acetate. The organic phase was dried (Na₂SO₄) and concentrated to dryness to provide in high purity compound 7a (100 mg, 86 %) as an oil. ^1H-NMR (400 MHz, CDCl₃) δ (ppm) 10.32 (s, 1H, CHO), 4.40 (q, J = 7.1 Hz, 2H, CH₂CH₃), 4.07 (s, 3H, NCH₃), 3.47 (hept, J = 7.0 Hz, 1H, CH(CH₃)₂), 1.37 (t, J = 7.1 Hz, 3H, CH₂CH₃), 1.20 (d, J = 7.0 Hz, 6H, CH(CH₃)₂). ^13C-NMR (50 MHz, CDCl₃) δ (ppm) 187.34 (CHO), 159.19 (C=O), 158.82 (C-3), 135.57 (C-5), 120.36 (C-4), 62.18 (CH₂CH₃), 39.89 (NCH₃), 27.06 (CH(CH₃)₂), 14.13 (CH₂CH₃). HRMS (ESI) m/z: Calcd for C₁₄H₁₆BrN₂O₂: [M+H]^+ =325.0494, found 325.0494.

Ethyl 4-formyl-1-methyl-3-phenyl-1H-pyrazole-5-carboxylate (7b): This compound was prepared following a method analogous to that of 7a, starting from 6b. Yield 85%. Mp: 73-75 °C (CH₂Cl₂-pentane). ^1H-NMR (400 MHz, CDCl₃) δ (ppm) 10.37 (s, 1H, CHO), 7.73 (d, J = 7.4 Hz, 2H, H-2', H-6'), 7.47 – 7.36 (m, 3H, H-3', H-5', H-4'), 4.47 (q, J = 7.1 Hz, 2H, CH₂CH₃), 4.19 (s, 3H, NCH₃), 1.43 (t, J = 7.1 Hz, 3H, CH₂CH₃). ^13C-NMR (50 MHz, CDCl₃) δ (ppm) 186.09 (CHO), 159.14 (C=O), 151.67 (C-3), 136.31 (C-5), 131.36 (C-1'), 129.16 (C-2', C-6'), 129.05 (C-4'), 128.12 (C-3', C-5'), 120.83 (C-4), 62.49 (CH₂CH₃), 40.14 (NCH₃), 14.13 (CH₂CH₃). HRMS (ESI) m/z: Calcd for C₁₄H₁₅N₂O₃: [M+H]^+ =259.1077, found 259.1077.

3-Isopropyl-1-methyl-1,6-dihydro-7H-pyrazolo[3,4-d]pyridazin-7-one (8a): To a solution of the carboxylate 7a (0.11 g, 0.49 mmol) in ethanol (2.5 mL) was added dropwise hydrazine (20 µL, 0.49 mmol, 80 % in water) and a few drops of HCl 36 % and the mixture was heated at 90 °C for 1 h. The solvent was vacuum-evaporated, water was added to the residue and it was extracted with ethyl acetate (3x20 mL). The organic extracts were dried (Na₂SO₄) and evaporated to dryness and the residue was purified by column chromatography (silica gel) using a mixture of cyclohexane/ethyl acetate 8/2-6/4 as the eluent, to result in 60 mg (67 %) of pure compound 8a. M.p.: 178-180 °C (EtOAc). ^1H-NMR (400 MHz, DMSO-d₆) δ (ppm) 12.63 (s, D₂O exchang., 1H, NH), 8.38 (s, 1H, H-4), 4.18 (s, 3H, NCH₃), 3.26 (hept, J = 7.0 Hz, 1H, CH(CH₃)₂), 1.29 (d, J = 7.0 Hz, 6H, CH(CH₃)₂). ^13C-NMR (151 MHz, DMSO-d₆) δ (ppm) 154.40 (C=O), 151.68 (C-3), 133.08 (C-4), 132.83 (C-7a), 116.97 (C-3a), 37.73
1-Methyl-3-phenyl-1,6-dihydro-7H-pyrazolo[3,4-d]pyridazin-7-one (8b): This compound was prepared following a method analogous to that of 8a, starting from 7b. Yield 82%. Mp: 253-255 °C (EtOH). 1H-NMR (400 MHz, DMSO-d$_6$) δ (ppm) 12.83 (s, D$_2$O exchang., 1H, NH), 8.62 (s, 1H, H-4), 7.93 (d, J = 7.3 Hz, 2H, H-2', H-6'), 7.52 (t, J = 7.3 Hz, 2H, H-3', H-5'), 7.46 (t, J = 7.3 Hz, 1H, H-4'), 4.33 (s, 3H, NCH$_3$). 13C-NMR (151 MHz, DMSO-d$_6$) δ (ppm) 154.16 (C=O), 144.06 (C-3), 133.65 (C-7a), 133.03 (C-4), 131.22 (C-1'), 129.15 (C-3', C-5'), 128.88 (C-4'), 126.97 (C-2', C-6'), 116.87 (C-3a), 38.31 (NCH$_3$). HRMS (ESI) m/z: Calcd for C$_{12}$H$_{11}$N$_4$O: [M+H]$^+$ =227.0928, found 227.0926.

7-Chloro-3-isopropyl-1-methyl-1H-pyrazolo[3,4-d]pyridazine (9a): A mixture of compound 8a (40 mg, 0.2 mmol) in phosphorus oxychloride (2 mL) was heated at 110 °C for 8h. Upon completion of the reaction (determined by TLC), the excess of phosphorus oxychloride was vacuum-evaporated and the synthesized chloride 9a was used for the next step without further purification due to the relative ease of hydrolysis.

7-Chloro-1-methyl-3-phenyl-1H-pyrazolo[3,4-d]pyridazine (9b): This compound was prepared following a method analogous to that of 9a, starting from 8b. In this case the reaction was completed in 2.5 h and the resulting chloride was again not purified.

N-Benzyl-3-isopropyl-1-methyl-1H-pyrazolo[3,4-d]pyridazin-7-amine (10a): To a solution of the chloropyrazolopyridazine 9a (42 mg, 0.2 mmol) in absolute ethanol (3 mL), was added benzylamine (66 µL, 0.6 mmol) and the mixture was heated at 80 °C for 2h. The solvent was then vacuum-evaporated, water was added to the residue, and it was extracted with dichloromethane (3x20 mL). The organic extracts were dried (Na$_2$SO$_4$) and evaporated to dryness and the residue was purified by column chromatography (silica gel) using a mixture of cyclohexane/ethyl acetate 4/6 as the eluent, to result in 23 mg (41 %) of pure compound 10a. M.p.: 137-140 °C (Et$_2$O). 1H-NMR (600 MHz, CDCl$_3$) δ (ppm) 8.94 (s, 1H, H-4), 7.44 (d, J = 7.4 Hz, 2H, H-2, benzyl H-6), 7.36 (t, J = 7.4 Hz, 2H, benzyl H-3, H-5), 7.30 (t, J = 7.4 Hz, 1H, benzyl H-4), 5.00 (s, 1H, NH), 4.86 (s, 2H, benzyl CH$_2$), 4.24 (s, 3H, NCH$_3$), 3.32 (hept, J = 7.0 Hz, 1H, CH(CH$_3$_2)), 1.42 (d, J = 7.0 Hz, 6H, CH(CH$_3$_2)). 13C-NMR (151 MHz, CDCl$_3$) δ (ppm) 151.51 (C-3), 147.15 (C-7), 139.75 (C-4), 138.80 (benzyl C-1), 128.83 (benzyl C-3, C-5), 128.72 (C-7a), 128.17 (benzyl C-2, C-6), 127.66 (benzyl C-4), 118.57 (C-3a), 46.11 (benzyl CH$_2$), 38.84 (NCH$_3$), 27.75 (CH(CH$_3$_2)), 22.33 (CH(CH$_3$_2)). Anal. Calcd for C$_{16}$H$_{19}$N$_5$: C, 68.30; H, 6.81; N, 24.89. Found: C, 68.54; H, 6.88; N, 24.55.
N-Benzyl-1-methyl-3-phenyl-1H-pyrazolo[3,4-d]pyridazin-7-amine (10b): This compound was prepared following a method analogous to that of 10a, starting from 9b. Yield 33%. Mp: 208-210 °C (CH2Cl2-Et2O). 1H-NMR (600 MHz, CDCl3) δ (ppm) 9.06 (s, 1H, H-4), 7.80 (d, J = 7.6 Hz, 2H, H-2', H-6’), 7.43 (t, J = 7.6 Hz, 2H, H-3', H-5'), 7.36 (m, 3H, benzyl H-2, H-6, H-4), 7.28 (t, J = 7.8 Hz, 2H, benzyl H-3, H-5), 7.22 (t, J = 7.6 Hz, 1H, H-4'), 5.19 (s, 1H, NH), 4.80 (s, 2H, benzyl CH2), 4.29 (s, 3H, NCH3), 3.99 (t, 4H, morpholine H), 3.48 (t, 4H, morpholine H). Anal. Calcd for C21H19BrN3O: C, 65.07; H, 5.80; N, 19.72. Found: C, 65.07; H, 5.80; N, 19.72.

Ethyl 4-bromomethyl-5-isopropyl-1-methyl-1H-pyrazole-3-carboxylate (11a): This compound was prepared following a method analogous to that of 6a, starting from 5a. Yield 60%. Oil. 1H-NMR (600 MHz, CDCl3) δ (ppm) 4.79 (s, 2H, CH2Br), 4.38 (q, J = 7.1 Hz, 2H, CH2CH3), 3.88 (s, 3H, NCH3), 3.19 (hept, J = 7.0 Hz, 1H, CH(CH3)2), 1.36-1.39 (m, 9H, CH2CH3, CH(CH3)2). 13C-NMR (151 MHz, CDCl3) δ (ppm) 162.22 (C=O), 148.01 (C-5), 139.48 (C-3), 118.08 (C-4), 60.95 (CH2CH3), 38.46 (NCH3), 25.78 (CH(CH3)2), 23.01 (CH2Br), 20.47 (CH(CH3)2), 14.39 (CH2CH3). HRMS (ESI) m/z: Calcd for C11H18BrN2O2: [M+H]+ = 289.0547, found 289.0545.

Ethyl 4-bromomethyl-1-methyl-5-phenyl-1H-pyrazole-3-carboxylate (11b): This compound was prepared following a method analogous to that of 6a, starting from 5b. Yield 48%. Oil. 1H-NMR (400 MHz, CDCl3) δ (ppm) 7.55 – 7.45 (m, 3H, H-3’, H-5’, H-4’), 7.42 (d, J = 7.4 Hz, 2H, H-2’, H-6’), 4.56 (s, 2H, CH2Br), 4.44 (q, J = 7.1 Hz, 2H, CH2CH3), 3.80 (s, 3H, NCH3), 1.41 (t, J = 7.1 Hz, 3H, CH2CH3). 13C-NMR (50 MHz, CDCl3) δ (ppm) 162.01 (C=O), 144.20 (C-5), 139.90 (C-3), 129.74 (C-4’), 129.48 (C-3’, C-5’), 129.07(C-2’, C-6’), 127.82 (C-1’), 119.59 (C-4), 61.07 (CH2CH3), 38.09 (NCH3), 23.26 (CH2Br), 14.36 (CH2CH3). HRMS (ESI) m/z: Calcd for C14H16BrN2O2: [M+H]+ = 323.0390, found 323.0384.
Ethyl 4-formyl-5-isopropyl-1-methyl-1H-pyrazole-3-carboxylate (12a): This compound was prepared following a method analogous to that of 7a, starting from 11a. Yield 61%. Oil. 1H-NMR (600 MHz, CDCl₃) δ (ppm) 10.47 (s, 1H, CHO), 4.44 (q, J = 7.1 Hz, 2H, CH₂CH₃), 3.96 (s, 3H, NCH₃), 3.65 (hept, J = 7.2 Hz, 1H, CH(CH₃)₂), 1.40 (t, J = 7.1 Hz, 3H, CH₂CH₃), 1.35 (d, J = 7.2 Hz, 6H, CH₂CH₃). 13C NMR (151 MHz, CDCl₃) δ (ppm) 187.61 (CHO), 161.72 (C=O), 157.57 (C=O), 141.41 (C=O), 130.08 (C≡N). HRMS (ESI) m/z: Calcd for C₁₁H₁₇N₂O₃: [M+H]+ =225.1234, found 225.1229.

Ethyl 4-formyl-1-methyl-5-phenyl-1H-pyrazole-3-carboxylate (12b): This compound was prepared following a method analogous to that of 7a, starting from 11b. Yield 89%. M.p.: 79-81 °C (CH₂Cl₂-Et₂O). 1H-NMR (400 MHz, CDCl₃) δ (ppm) 10.37 (s, 1H, CHO), 7.50 – 7.42 (m,3H, H-3', H-5', H-4'), 7.34 (d, J = 7.4 Hz,2H, H-2', H-6'), 4.46 (q, J = 7.1 Hz, 2H, CH₂CH₃), 3.77 (s, 3H, NCH₃), 1.40 (t, J = 7.1 Hz, 3H, CH₂CH₃). 13C-NMR (50 MHz, CDCl₃) δ (ppm) 186.60 (CHO), 161.61 (C=O), 146.73 (C-5), 143.39 (C-3), 130.08 (C-4'), 129.77 (C-3', C-5'), 128.64 (C-2', C-6'), 127.54 (C-1'), 120.96 (C-4), 61.73 (CH₂CH₃), 37.75 (NCH₃), 14.30 (CH₂CH₃). HRMS (ESI) m/z: Calcd for C₁₄H₁₅N₂O₃: [M+H]+ =259.1078, found 259.1070.

3-Isopropyl-2-methyl-2,6-dihydro-7H-pyrazolo[3,4-d]pyridazin-7-one (13a): This compound was prepared following a method analogous to that of 8a, starting from 12a. Yield 69%. M.p.: 214-216 °C (EtOAc). 1H-NMR (600 MHz, CDCl₃) δ (ppm) 8.27 (s, 1H, H-4), 4.11 (s, 3H, NCH₃), 3.34 (hept, J = 7.0 Hz, 1H, CH(CH₃)₂), 1.48 (d, J = 7.0 Hz, 6H, CH(CH₃)₂). 13C NMR (151 MHz, CDCl₃) δ (ppm) 157.57 (C=O), 145.37 (C-3), 141.41 (C-7a), 133.64 (C-4), 116.12 (C-3a), 38.36 (NCH₃), 26.98 (CH(CH₃)₂), 22.10 (CH(CH₃)₂). HRMS (ESI) m/z: Calcd for C₁₀H₁₃N₃O: [M+H]+ =193.1084, found 193.1075.

2-Methyl-3-phenyl-2,6-dihydro-7H-pyrazolo[3,4-d]pyridazin-7-one (13b): This compound was prepared following a method analogous to that of 8a, starting from 12b. Yield 68%. M.p.: 239-241 °C (EtOH). 1H-NMR (600 MHz, DMSO-d₆) δ (ppm) 12.34 (s, D₂O exchang., 1H, NH), 8.09 (s, 1H, H-4), 7.70 (d, J = 7.6 Hz, 2H, H-2', H-6'), 7.62 (m, 3H, H-3', H-4', H-5'), 4.11 (s, 1H, NCH₃). 13C-NMR (151 MHz, DMSO-d₆) δ (ppm) 156.43 (C=O), 140.88 (C-7a), 138.90 (C-3), 132.59 (C-4), 129.85 (C-4'), 129.53 (C-2', C-6'), 129.30 (C-3', C-5'), 127.12 (C-1'), 117.23 (C-3a), 39.10 (NCH₃). HRMS (ESI) m/z: Calcd for C₁₂H₁₃N₄O: [M+H]+ =227.0928, found 227.0921.

7-Chloro-3-isopropyl-2-methyl-2H-pyrazolo[3,4-d]pyridazine (14a): This compound was prepared following a method analogous to that of 9a, starting from 13a. Reaction was completed in 2 h and the compound was used to the next step with no further purification.

7-Chloro-2-methyl-3-phenyl-2H-pyrazolo[3,4-d]pyridazine (14b): This compound was prepared following a method analogous to that of 9a, starting from 13b. Reaction was completed in 2 h and the compound was used to the next step with no further purification.
**N-Benzyl-3-isopropyl-2-methyl-2H-pyrazolo[3,4-d]pyridazin-7-amine (15a):** This compound was prepared following a method analogous to that of 10a, starting from 14a. Yield 37%. Oil. $^1$H-NMR (600 MHz, CDCl$_3$) $\delta$ (ppm) 8.90 (s, 1H, H-4), 7.44 (d, $J$ = 7.4 Hz, 2H, benzyl H-2, H-6), 7.33 (t, $J$ = 7.4 Hz, 2H, benzyl H-3, H-5), 7.27 (t, $J$ = 7.4 Hz, 1H, benzyl H-4), 4.95 (s, 2H, benzyl CH$_2$), 4.09 (s, 3H, NCH$_3$), 3.36 (hept, $J$ = 7.2 Hz, 1H, CH(CH$_3$)$_2$), 1.50 (d, $J$ = 7.2 Hz, 6H, CH(CH$_3$)$_2$). $^{13}$C-NMR (151 MHz, CDCl$_3$) $\delta$ (ppm) 150.53 (C-7), 144.34 (C-3), 138.85 (benzyl C-1), 138.70 (C-4), 135.42 (C-7a), 128.74 (benzyl C-3, C-5), 128.29 (benzyl C-2, C-6), 127.58 (benzyl C-4), 114.94 (C-3a), 45.69 (benzyl CH$_2$), 38.34 (NCH$_3$), 27.22 (CH(CH$_3$)$_2$), 22.12 (CH(CH$_3$)$_2$). Anal. Calcd for C$_{16}$H$_{19}$N$_5$: C, 68.30; H, 6.81; N, 24.89. Found: C, 68.58; H, 6.90; N, 24.50.

**N-Benzyl-2-methyl-3-phenyl-2H-pyrazolo[3,4-d]pyridazin-7-amine (15b):** This compound was prepared following a method analogous to that of 10a, starting from 14b. Yield 28%. M.p.: 211-214 °C (CH$_2$Cl$_2$-Et$_2$O). $^1$H-NMR (600 MHz, CDCl$_3$) $\delta$ (ppm) 10.61 (s, 1H, H-4), 7.78 (d, $J$ = 7.6 Hz, 2H, H-2', H-6'), 7.65 (t, $J$ = 7.6 Hz, 2H, H-3', H-5'), 7.55 (t, $J$ = 7.6 Hz, 1H, H-4'), 7.43 (d, $J$ = 7.4 Hz, 2H, benzyl H-2, H-6), 7.36 (t, $J$ = 7.4 Hz, 2H, benzyl H-3, H-5), 7.31 (t, $J$ = 7.4 Hz, 1H, benzyl H-4), 4.78 (s, 2H, benzyl CH$_2$), 4.26 (s, 3H, NCH$_3$). $^{13}$C-NMR (151 MHz, CDCl$_3$) $\delta$ (ppm) 150.88 (C-7), 146.18 (C-3), 140.49 (C-4), 136.62 (benzyl C-1), 135.03 (C-7a), 131.83 (C-4'), 130.28 (C-3', C-5'), 130.05 (C-2', C-6'), 129.10 (benzyl C-3, C-5), 128.31 (benzyl C-4), 128.27 (benzyl C-2, C-6), 124.70 (C-1'), 115.79 (C-3a), 45.69 (benzyl CH$_2$), 40.28 (NCH$_3$). Anal. Calcd for C$_{19}$H$_{17}$N$_5$: C, 72.36; H, 5.43; N, 22.21. Found: C, 72.61; H, 5.47; N, 21.90.

**2-Methyl-7-(morpholin-4-yl)-3-phenyl-2H-pyrazolo[3,4-d]pyridazine (15c):** This compound was prepared following a method analogous to that of 10a, starting from 14b. Yield 46%. M.p.: 184-186 °C (CH$_2$Cl$_2$-Et$_2$O). $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ (ppm) 8.82 (s, 1H, H-4), 7.59 (m, 3H, H-3', H-5', H-4'), 7.51 (d, $J$ = 7.2 Hz, 2H, H-2', H-6'), 4.23 (t, 4H, morpholine H), 4.19 (s, 3H, NCH$_3$), 3.91 (t, 4H, morpholine H). $^{13}$C-NMR (151 MHz, CDCl$_3$) $\delta$ (ppm) 151.57 (C-7), 139.53 (C-4), 137.81 (C-3), 136.67 (C-7a), 130.07 (C-4'), 129.54 (C-2', C-6', C-3', C-5'), 127.75 (C-1'), 118.21 (C-3a), 67.20 (morpholine C), 47.00 (morpholine C), 38.93 (NCH$_3$). Anal. Calcd for C$_{16}$H$_{17}$N$_5$: C, 65.07; H, 5.80; N, 23.71. Found: C, 65.22; H, 5.84; N, 23.55.

**Pharmacological methods**

**Cell lines**

Stable Flp-In-CHO cell lines expressing the WT A$_3$R were generated and maintained as previously described$^{13,14}$. CHO-K1 cells stably expressing WT A$_1$R, A$_2a$R or A$_2b$R were routinely cultured in Ham's F-12, supplemented with 10% Foetal bovine serum (FBS). All were annually checked for mycoplasma infection using an EZ-PCR mycoplasma test kit (Biological Industries, Kibbutz Beit-Haemek, Israel). Production and analysis of the mutant versions of the A$_1$R were as described in ref 15.

**Compounds**

NECA was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO). All compounds used for the in vitro testing were >95% pure by elemental analysis.
cAMP accumulation assay

cAMP inhibition experiments were performed using a LANCE® cAMP kit as described previously\textsuperscript{13,14}. Briefly, Flp-In-CHO cells expressing WT A\textsubscript{3}R and CHO-K1 cells expressing WT A\textsubscript{1}R, A\textsubscript{2A}R or A\textsubscript{2B}R were seeded in a white 384-well optiplate at a density of 2,000 cells per well and stimulated for 30 min with a range of NECA concentrations, with or without potential antagonists, in the presence of 0.1% BSA and 25 μM rolipram, and 10 μM forskolin (to enable detection of A\textsubscript{1}R- or A\textsubscript{3}R-mediated inhibition of cAMP). Since the A\textsubscript{2A}R and A\textsubscript{2B}R promote cAMP accumulation,\textsuperscript{16} the addition of forskolin was not included when assaying these receptors.

Schild analysis

NECA concentration-dependent response curves were constructed in the presence of either DMSO alone or multiple concentrations of 10b at the A\textsubscript{1}R and A\textsubscript{3}R. NECA concentrations ranged from 1 pM to 1 μM. Estimates of the pEC\textsubscript{50} values in the presence and absence of the antagonist were determined using the three-parameter logistic equation built into Prism.

NanoBRET assays for binding

NanoBRET competition binding assays were conducted to determine the affinity (pK\textsubscript{i}) of various potential antagonists at the A\textsubscript{1}R and A\textsubscript{3}R as described previously\textsuperscript{9}. For both the A\textsubscript{1}R and A\textsubscript{3}R, the CellAura fluorescent A\textsubscript{3}R antagonist (CA200645) with a xanthine amine congener (XAC) structure was used at 20 nM and 5 nM concentration for A\textsubscript{1}R and A\textsubscript{3}R, respectively, since it has a slow off rate. Kinetic data was fitted with the ‘kinetic of competitive binding’ model (see ref.\textsuperscript{17}; built into Prism) to determine affinity (pK\textsubscript{i}) values and the association rate constant (K\textsubscript{on}) and dissociation rates (K\textsubscript{off}) for unlabelled A\textsubscript{3}R antagonists. In agreement with our previous studies\textsuperscript{15,18} we determined the pK\textsubscript{d} of CA200645 at the A\textsubscript{1}R to be 18.29 ± 2.4 nM and at the A\textsubscript{3}R 26.95 ± 3.2 nM.\textsuperscript{18} The BRET ratio at 10 min poststimulation was fitted with the “one-site–K\textsubscript{i} model” derived from the Cheng and Prusoff correction, built into Prism to determine the affinity (pK\textsubscript{i}) constant at equilibrium values for all unlabelled antagonists at the A\textsubscript{1}R and A\textsubscript{3}Rs.

Data and Statistical analysis

All in vitro assay data were analyzed using Prism 9.0 (GraphPad software, San Diego, CA), with all dose-inhibition curves being fitted using a three-parameter logistic equation to calculate response range and pEC\textsubscript{50}. Experimental design ensured random distribution of treatments across 96/384-well plates to avoid systematic bias. Agonist stimulation alone was used as an intrinsic control across all experiments. Dose-inhibition/dose-response curves were normalized to forskolin stimulation (A\textsubscript{2A}R and A\textsubscript{2B}R) or forskolin inhibition (A\textsubscript{1}R and A\textsubscript{3}R) relative to NECA (agonist allowing comparison across AR subtypes), expressed as percentage forskolin inhibition for G\textsubscript{i}-coupled A\textsubscript{1}R and A\textsubscript{3}R (1 μM or 10 μM, respectively) or stimulation for A\textsubscript{2A}R and A\textsubscript{2B}R (100 μM, representing the maximum cAMP accumulation of the system), relative to NECA. For cAMP experiments on A\textsubscript{1}R mutants, data was normalized to 100 μM forskolin, representing the maximum cAMP accumulation possible for each cell line.
Schild analysis, when using a single concentration of antagonist was performed to obtain, the dissociation constant ($pK_d$) using eq. (1) \(^{19}\)

$$\frac{D'}{D} = 1 + [A]K_2,$$  \hspace{1cm} (1)

where $D'$ and $D = EC_{50}$ values of NECA with and without antagonist present, respectively, $[A] = the concentration of antagonist present$, and $K_2 = the affinity constant (K_A)$ of the antagonist used\(^{19}\). Receptor binding kinetics was determined as described previously \(^{18}\) using the Motulsky and Mahan method \(^{17}\) (built into Prism 9.0) to determine the test compound association rate constant and dissociation rate constant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology\(^{20}\). Statistical significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ****, $p < 0.0001$) was calculated using a one-way ANOVA with a Dunnett’s post-test for multiple comparisons. All statistical analysis was performed using Prism 9.0 on data which were acquired from experiments performed a minimum of five times, conducted in duplicate.

### Computational Methods

#### 3D Similarity Calculations

All of the 3D similarity calculations were performed with Canvas program (Schrödinger Release 2021-1: Canvas, Schrödinger, LLC, New York, NY, 2021)\(^{21}\), searching in CHEMBL\(^{21,22}\) database. The similar compounds structure was ranked according to the TanimotoCombo \(^{10}\) coefficient as metric\(^{24,25}\).

#### Models of the complexes between $A_1$R or $A_3$R or $A_{2B}$R with an antagonist

We used for $A_{2A}$R in inactive state the experimental structure of $A_{2A}$R in complex with ZM241385 which was resolved by X-ray crystallography (PDB ID 3EML\(^8\)). We used for $A_1$R in inactive state the experimental structure of $A_1$R in complex with DU172 which as resolved by X-ray crystallography (PDB ID 5UEN\(^{26}\)). Their models were retrieved from Adenosineland web service\(^{26}\).

We superimposed the experimental crystal structure ZM241385 - $A_{2A}$R complex (PDB ID 3EML\(^8\)) to the WT $A_3$R or WT $A_{2B}$R models from Adenosineland web-service\(^{27}\). (Residue numbers in parenthesis refer to the Ballesteros–Weinstein numbering)\(^{28}\). Then, the $A_{2A}$R (PDB ID 3EML\(^8\)) protein was removed resulting in the WT $A_3$R - ZM241385 or WT $A_{2B}$R - ZM241385 models used in the study.

In the next step, the WT $A_1$R - PSB36, $A_3$R - ZM241385, $A_{2B}$R - ZM241385 structures were optimized using the Protein Preparation Wizard implementation in Schrodinger suite\(^{29}\). In this process, the bond orders and disulfide bonds were assigned, and missing hydrogen atoms were added. Additionally, N- and C-termini of the protein model were capped by acetyl and N-methyl-amino groups, respectively. All His were treated with imidazoles protonated at $N\_e$ except $H278^{7.43}$ at $N\_\delta$. Each protein was subjected in an all atom minimization using the OPLS 2005 force field\(^{30}\) with heavy atom RMSD value constrained to 0.30 Å until the r.m.s of conjugate-gradient value reached $< 0.05$ kcal mol\(^{-1}\) Å\(^{-1}\). The side chain of V169\(^{5.30}\) in the WT $A_3$R complex was rotated to fit the conformer as was suggested\(^{31}\) in order to increase the free space for the accommodation of ligands with bulky substitutions.
Docking calculations

The molecular docking calculations of the 6 tested 7-amino-pyrazolo[3,4-d]pyridazines (Table 1) (the protocol used for the preparation of models of the tested ligands is reported in the Supporting Information) with A1R, A3R, A2B R were performed using GOLD software\(^{32}\) (GOLD Suite, Version 5.2; Cambridge Crystallographic Data Centre: Cambridge, U.K., 2015. GOLD Suite, version 5.2; Cambridge Crystallogr. Data Cent. Cambridge, U.K., 2015) and ChemScore\(^{33}\) as the scoring function. The models of WT A1R - DU172, WT A3R - ZM241385, WT A2B R - ZM241385 were used as templates for the molecular docking calculations of the antagonists to the binding area of each of the receptors. Each compound was docked in the binding site of ZM241385 in the A3R-ZM241385 model or DU172 in A1R - DU172, model or ZM241385 in the A2B R-ZM241385 model in an area of 15 Å around the ligand using the experimental coordinates of ZM241385 or DU172 and 20 genetic algorithm runs were applied for each docking calculation. The top-scoring docking poses were used for MD simulations to investigate the binding profile of the 6 tested 7-amino-pyrazolo[3,4-d]pyridazines (Table 1) at A1R or A3R or A2B R.

MD Simulations

Each protein-ligand complex was inserted in a pre-equilibrated hydrated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) membrane bilayer according to OPM ( Orientations of Proteins in Membranes) database\(^{34}\). The orthorhombic periodic box boundaries were set 12 Å away from the protein using the System Builder utility of Desmond v4.9 (Schrödinger Release 2021-1: Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2021. Maestro-Desmond Interoperability Tools, Schrödinger, New York, NY, 2021). The membrane bilayer consisted by ca. 180 lipids and 16,000 TIP3P\(^{35}\) water molecules. Sodium and chloride ions were added randomly in the water phase to neutralize the system and reach the experimental salt concentration of 0.150 M NaCl. The total number of atoms of the complex was approximately 75,000 and the simulation box dimensions was (88 x 75 x 110Å\(^3\)). We used the Desmond Viparr tool to assign the amber99sb\(^{36,37}\) force field parameters for the calculation of the protein, lipids and intermolecular interactions, and the Generalized Amber Force Field (GAFF)\(^{38}\) parameters for the ligands. Ligand atomic charges were computed using the RESP\(^{39}\) fitting for the electrostatic potentials calculated with Gaussian03\(^{40}\) at the HF/6-31G*\(^{41}\) level of theory and the antechamber of AmberTools18, 42

100 ns MD simulations at constant pressure (NPT) were performed for the 6 ligands (Table 1) in complex with A1R or A3R or A2B R embedded in POPE bilayers using Desmond v4.9 software, the Desmond MD algorithm\(^{43}\) with amber99sb\(^{44}\) force field to investigate their binding interactions. The protocol used to calculate interactions and run the MD simulations and the visualization of the trajectories is described in the Supporting Information. Within the 100ns-MD simulation time, the total energy and RMSD of the protein backbone C\(_\alpha\) atoms reached a plateau, and the systems were considered equilibrated and suitable for statistical analysis (Figure S4).
Two MD simulations were performed for each complex using the same starting structure and applying randomized velocities. All the MD simulations with Desmond software were run on GTX 1060 GPUs in lab workstations or the ARIS Supercomputer.

Computational Biochemistry Methods

Ligand preparation
The 2D structures of the 6 compounds were sketched with Marvin Program (Marvin version 21.17.0, ChemAxon (https://www.chemaxon.com)) and model-built with Schrödinger 2021-1 platform (Schrödinger Release 2021-1: Protein Preparation Wizard; Epik, Schrödinger, LLC, New York, NY, 2021; Impact, Schrödinger, LLC, New York, NY; Prime, Schrödinger, LLC, New York, NY, 2021) and minimized using the CG method, the MMFF94 force field and a distance-dependent dielectric constant of 4.0 until a convergence threshold of $2.4 \times 10^{-5}$ kcal mol$^{-1}$ Å$^{-1}$ was reached. Ionization states of the compounds at pH 7.5 were tested using Epik program implemented in Schrödinger suite (Prime, Schrödinger, LLC, New York, NY, 2021). Energy minimization of the compounds' 3D structures was performed using the OPLS2005 force field.

MD simulations protocol
The MD simulation of each protein-ligand complex inside the lipid bilayer was performed using the default protocol provided with Desmond v4.9 program. The MD simulation protocol consists of a series of MD simulations designed to relax the system, while not deviating substantially from the initial coordinates. During the first stage, a simulation was run for 200 ps at 10 K in the NVT ensemble (constant volume, temperature and number of atoms), with solute-heavy atoms restrained by a force constant of 50 kcal mol Å$^{-2}$. The temperature was raised to 310 K during a 200 ps MD simulation in the NPT ensemble (constant pressure, temperature and number of atoms), with the same force constant applied to the solute atoms. The temperature of 310 K was used in MD simulations in order to ensure that the membrane state is above the main phase transition temperature of 298 K for POPE bilayers. The heating was then followed by equilibration simulations. First, two 1 ns stages of NPT equilibration were performed. In the first 1 ns stage, the heavy atoms of the system were restrained by applying a force constant of 10 kcal mol$^{-1}$ Å$^{-2}$, and in the second 1 ns stage, the heavy atoms of the protein-ligand complex were restrained by applying a force constant of 2 kcal mol$^{-1}$ Å$^{-2}$ to equilibrate water and lipid molecules. In the production phase, the relaxed systems were simulated without restraints in the NPT ensemble for 100 ns. Replicas of the system were saved every 10 ps.

In the MD simulations the Particle Mesh Ewald (PME) method was employed to calculate long-range electrostatic interactions with a grid spacing of 0.8 Å. The SHAKE method was used to constrain heavy atom-hydrogen bonds at ideal lengths and angles. Van der Waals and short-range electrostatic interactions were smoothly truncated at 12 Å. The Nosé-Hoover thermostat was utilized to maintain a constant temperature in all MD simulations, and the Martyna-Tobias-Klein method was used to control the pressure. The equations of motion were integrated using the multistep reversible reference system propagator algorithms (RESPA) integrator with an inner time step of 2 fs for bonded
interactions and non-bonded interactions within the cutoff of 12 Å. An outer time step of 6.0 fs was used for non-bonded interactions beyond the cutoff.

The visualization of the MD simulation trajectories was performed using the graphical user interface (GUI) of Maestro and the protein-ligand interaction analysis was carried out with the Simulation Interaction Diagram (SID) tool, available with Desmond v4.9 program. For the calculation of hydrogen bond interactions were considered, a distance 2.5 Å between donor and acceptor heavy atoms, and angle ≥120° between donor-hydrogen-acceptor atoms and ≥ 90° between hydrogen-acceptor-bonded atom. Non-specific hydrophobic contacts were identified when the side chain of a hydrophobic residue fell within 3.6 Å from a ligand’s aromatic or aliphatic carbon, while π-π interactions were characterized by stacking of two aromatic groups face-to-face or face-to-edge. Water-mediated interactions were characterized when the distance between donor and acceptor atoms was 2.7 Å, as well as an angle ≥ 110° between donor-hydrogen-acceptor atoms and ≥ 80° between hydrogen-acceptor-bonded atom.

**MM-GBSA calculations**

The MD simulation trajectories were used for the calculation of approximate binding free energies of ligand – protein complexes using the 1-trajectory MM-GBSA method, the OPLS2005 force field and 20 waters in the vicinity of the ligand. The MD trajectories were processed with the Python library MDAnalysis in order to extract the 20 water molecules closest to any atom in the ligand for each of the 501 frames. We applied a dielectric constant \( \varepsilon_{\text{solute}} = 1 \) to the binding area and to account for the lipophilic environment of the protein an heterogeneous dielectric implicit membrane model was used along the bilayer z-axis.

For this, structural ensembles were extracted in intervals of 40 ps from the 20 ns MD simulation for each complex. Prior to the calculations all water molecules, ions, and lipids were removed, except 20 waters in the vicinity of the ligand, and the structures were positioned such that the geometric centre of each complex was located at the coordinate origin. The MD trajectories were processed with the Python library MDAnalysis in order to extract the 20 water molecules closest to any atom in the ligand for each of the 501 frames. During the MM-GBSA calculations, the explicit water molecules were considered as being part of the protein. Binding free energies of compounds in complex with A1R and A3R were estimated using the 1-trajectory MM-GBSA approach.

For the calculation of binding free energy for each complex eqs (1)-(4) were used

\[
\Delta G_{\text{bind}} = \langle G_{\text{complex}} - G_{\text{protein}} - G_{\text{ligand}} \rangle_{\text{complex}} \tag{1}
\]

\[
G_i = V_{\text{MM}} - T \langle S_{\text{MM}} \rangle + \Delta G_{\text{solv}} \tag{2}
\]

\[
V_{\text{MM}} = V_{\text{bonded}} + V_{\text{coul}} + V_{\text{LJ}} \tag{3}
\]

\[
\Delta G_{\text{solv}} = \Delta G_{\text{p}} + \Delta G_{\text{NP}} \tag{4}
\]

The binding free energy for each complex was thus calculated using eq. (5)

\[
\Delta G_{\text{bind}} = \langle \Delta E_{\text{coul}} + \Delta E_{\text{LJ}} \rangle - T \langle \Delta S_{\text{MM}} \rangle + \Delta \Delta G_{\text{solv}} \tag{5}
\]
In eqs (1)-(4) $G_i$ is the free energy of system $i$, that being the ligand, the protein, or the complex; $V_{\text{MM}}$ is the potential energy in vacuum as defined by the molecular mechanics (MM) model, which is composed of the bonded potential energy terms ($V_{\text{bonded}}$) and nonbonded Coulombic ($V_{\text{coul}}$) and Lennard-Jones ($V_{\text{LJ}}$) terms; $S_{\text{MM}}$ is the entropy; $\Delta G_{\text{solv}}$ is the free energy of solvation for transferring the ligand from water in the binding area calculated using the PBSA model, composed by a polar ($\Delta G_p$) and nonpolar ($\Delta G_{\text{NP}}$) term; $T$ is the temperature and angle brackets represent an ensemble average. Molecular mechanics energies for Lennard-Jones ($V_{\text{LJ}}$) and Coulombic electrostatic ($V_{\text{coul}}$) were calculated with OPLS2005 \cite{3,4} force field; in these calculations $\Delta V_{\text{bonded}} = 0$ as the single trajectory method was adopted and $\Delta V_{\text{MM}} = \Delta V_{\text{LJ}}$ and $\Delta V_{\text{coul}}$. The polar part of the solvation free energy was determined by calculations using the Generalized-Born model. \cite{5} The nonpolar term was considered proportional to the solvent accessible surface area (SASA), $\Delta G_{\text{NP}} = \gamma \cdot \text{SASA}$, where $\gamma = 0.0227$ kJ mol$^{-1}$ Å$^{-2}$. The entropy term was neglected since we were interested for binding free energies between ligands in the same series and in this case $\Delta G_{\text{bind}}$ is termed as effective binding energy, $\Delta G_{\text{eff}}$. The post-processing thermal_mmbgsa.py script of the Schrodinger Suite was used which takes snapshots from the MD simulations trajectory and calculates $\Delta G_{\text{eff}}$.

**Cell viability assays**

Human HCT116 colon cancer cell line and PC-3 prostate cancer cell linewere obtained from the American Type Cell Culture (ATCC, Bethesda, Md). Both cell lines were grown in 75-cm$^2$ culture flasks at 37°C in 5% CO$_2$ using Roswell Park Memorial Institute 1640 medium (RPMI 1640, Gibco) and Dulbecco’s modified Eagle’s medium F/12 (DMEM/F-12, Gibco) respectively, containing 10% fetal bovine serum (FBS, Gibco). To test the inhibitory activities of compounds using a cell-based assay, HCT116 cells were plated at a density of 1500 per well, while PC-3 cells were plated at a density of 750 per well in a 96-well plate. After 24 h, cells were treated with the indicated compounds in a dose-dependent manner for 72h and 96h (all tested compounds provided clear solutions). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma M-5655) was added at a final concentration of 0.5 mg/mL directly to each well for 4 hours at 37°C. The medium was aspirated and the blue MTT formazan precipitate was dissolved in dimethyl sulfoxide (DMSO). Absorbance was determined in a Powerwave microplate spectrophotometer (Biotek Instruments, Inc.) at 540 nm. Viable cell numbers were determined by tetrazolium conversion to its formazan dye. The IC$_{50}$ was calculated by Microsoft Excel equation and confirmed by GraphPad Prism (7.0). Each experiment was performed in triplicate and mean values ± SD are reported.

**Abbreviations**

AcOEt, ethyl acetate; CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; CPT, 8-cyclopentyl-1,3-dimethylxanthine; cryo-em, cryogenic electron microscopy; 2D NMR, two-dimensional NMR, EL2, Extracellular loop 2; MAPK, mitogen-activated protein kinase; OPM, Orientations of Proteins in Membranes; PD, Parkinson's disease; PME, particle mesh Ewald method; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; RESPA, Reversible multiple time scale molecular dynamics.
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