

## Supplementary information

Molecular mechanism of allosteric modulation at G protein-coupled receptors: insight from a binding kinetics study at the human adenosine A<sub>1</sub> receptor

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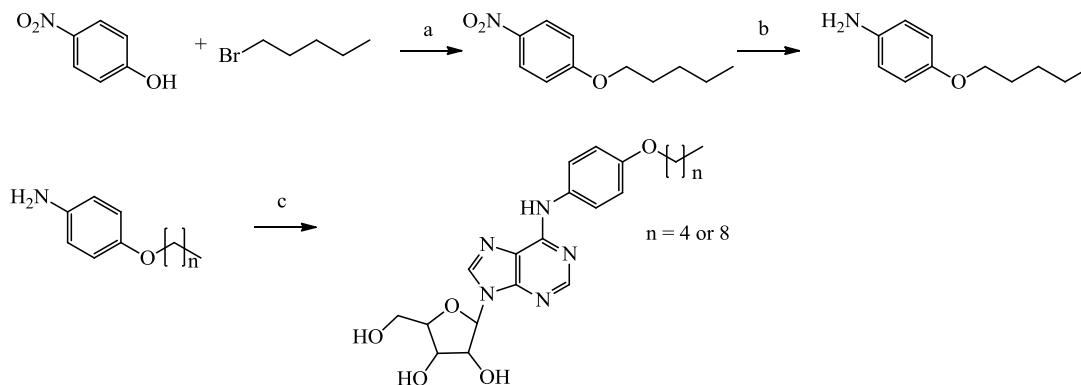
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## Synthesis

All solvents and reagents were purchased from commercial sources and were of analytical grade.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker AV 400 liquid spectrometer ( $^1\text{H}$  NMR, 400 MHz;  $^{13}\text{C}$  NMR, 100 MHz) at ambient temperature. Chemical shifts are reported in parts per million (ppm), are designated by  $\delta$  and are downfield to the internal standard tetramethylsilane (TMS). Coupling-constants are reported in Hz and are designated as J. High resolution mass spectrometry was performed by Leiden Institute of Chemistry and recorded by direct injection (2  $\mu\text{L}$  of a 2  $\mu\text{M}$  solution in water/MeCN; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275°C) with resolution  $R = 60000$  at  $m/z$  400 (mass range  $m/z = 150\text{-}2000$ ) and calibrated for dioctylphthalate ( $m/z = 391.28428$ ). Analytical purity of the final compounds was determined by high performance liquid chromatography (HPLC) with a Phenomenex Gemini 3u C18 110A column (50 x 4.6 mm, 3  $\mu\text{m}$ ), measuring UV absorbance at 254 nm. Sample preparation and HPLC method were as follows, unless stated otherwise: 0.3-0.8 mg of compound was dissolved in 1 mL of a 1:1:1 mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}/t\text{-BuOH}$  and eluted from the column within 15 minutes, with a three component system of  $\text{H}_2\text{O}/\text{CH}_3\text{CN}/1\%$  TFA in  $\text{H}_2\text{O}$ , decreasing polarity of the solvent mixture in time from 80/10/10 to 0/90/10. All compounds showed a single peak at the designated retention time and are at least 95% pure. Thin-layer chromatography (TLC) was routinely consulted to monitor the progress of reactions, using aluminum-coated Merck silica gel  $\text{F}_{254}$  plates. Purification by column chromatography was achieved by use of Grace

Davison Davisil silica column material (LC60A 30-200 micron). Microwave reactions (MW) were performed in the Biotage Initiator in a closed vessel at a set temperature.

### Scheme S1



a) K<sub>2</sub>CO<sub>3</sub>, acetone, 70 °C, overnight; b) H<sub>2</sub>, Pd/C(10%), MeOH, RT, overnight; c) 6-chloroadenosine, Et<sub>3</sub>N, EtOH, MW, 120 °C, 2.5h.

#### 4-pentyloxy-nitrobenzene.

Procedure followed according to Narlawar *et al.* and Wada *et al.* (Narlawar *et al.*, 2010; Wada *et al.*, 2002). Yellow oil, quantitative yield.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 8.21 (d, *J* = 9.2 Hz, 2H), 6.94 (d, *J* = 9.2 Hz, 2H), 4.05 (t, *J* = 6.4 Hz, 2H), 1.86-1.58 (m, 2H), 1.50-1.35 (m, 4H), 0.94 (t, *J* = 7.2 Hz, 3H)

#### 4-pentyloxyaniline

Procedure based on Carrigan *et al.* (Carrigan *et al.*, 2002). 4-Pentyloxy-nitrobenzene (3.76 g, 18.0 mmol) was dissolved in MeOH, palladium (10% w/w on carbon) (38 mg,

1% w/w of starting material) was added and stirred overnight at room temperature in a Parr apparatus under a hydrogen (3 bar) atmosphere. By TLC (1 EtOAc: 4 Pet. ether) it was shown the reaction went to completion and subsequently the Pd/C was removed by filtration over celite. After evaporation of the filtrate *in vacuo* 3.16 g ( yield 98%) of a brown oil was obtained. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ 6.77 (d, *J* = 8.8 Hz, 2H), 6.67 (d, *J* = 8.8 Hz, 2H), 3.91 (t, *J* = 6.8 Hz, 2H), 3.31 (s br, 2H), 1.82-1.73 (m, 2H), 1.49-1.35 (m, 4H), 0.95 (t, *J* = 7.2 Hz, 3H)

#### **4-nonyloxyaniline**

Compound is commercially available from Alfa Aesar (Karlsruhe, Germany)

#### **N<sup>6</sup>-(4-pentyloxyphenyl)adenosine (LUF7161)**

Experimental procedure followed according to Narlawar *et al.* (Narlawar *et al.*, 2010).

White solid 356 mg, yield 82%. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 9.81 (s, 1H), 8.50 (s, 1H), 8.33 (s, 1H), 7.76 (d, *J* = 9.2 Hz, 2H), 6.90 (d, *J* = 9.2 Hz, 2H), 5.94 (d, *J* = 6.0 Hz, 1H), 5.49 (d, *J* = 6.4 Hz, 1H), 5.33 (dd, *J* = 6.8, 4.8 Hz, 1H), 5.22 (d, *J* = 4.4 Hz, 1H), 4.63 (dd, *J* = 11.2, 6.0 Hz, 1H), 4.18-4.14 (m, 1H), 3.99-3.96 (m, 1H), 3.94 (t, *J* = 6.4 Hz, 2H), 3.72-3.66 (m, 1H), 3.60-3.54 (m, 1H), 1.71 (pentet, *J* = 6.8 Hz, 2H), 1.44-1.30 (m, 4H), 0.90 (t, *J* = 6.8 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 154.8, 152.5, 152.2, 149.2, 140.6, 132.5, 122.9, 120.3, 114.3, 88.1, 86.1, 73.8, 70.8, 67.7, 61.8, 28.6, 27.9, 22.1, 14.1. HPLC: purity 99%, retention time 7.74 min. HRMS (ESI) *m/z* calcd for C<sub>21</sub>H<sub>27</sub>N<sub>5</sub>O<sub>5</sub><sup>+</sup>: 430.20850, found: 430.20807

***N*<sup>6</sup>-(4-nonyloxyphenyl) adenosine (LUF7160)**

Experimental procedure followed according to Narlawar *et al.* (Narlawar *et al.*, 2010).

Off white solid 381 mg, yield 78%. <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 9.82 (s, 1H), 8.50 (s, 1H), 8.33 (s, 1H), 7.76 (d, *J* = 8.8 Hz, 2H), 6.89 (d, *J* = 9.2 Hz, 2H), 5.94 (d, *J* = 6.4 Hz, 1H), 5.49 (d, *J* = 6.4 Hz, 1H), 5.33 (dd, *J* = 6.8, 4.8 Hz, 1H), 5.22 (d, *J* = 4.8 Hz, 1H), 4.63 (dd, *J* = 11.2, 6.0 Hz, 1H), 4.18-4.14 (m, 1H), 3.99-3.96 (m, 1H), 3.93 (t, *J* = 6.4 Hz, 2H), 3.72-3.66 (m, 1H), 3.60-3.54 (m, 1H), 1.70 (pentet, *J* = 7.2 Hz, 2H), 1.43-1.36 (m, 2H), 1.35-1.20 (m, 10H), 0.86 (t, *J* = 6.8 Hz, 3H). <sup>13</sup>C NMR (101 MHz, DMSO-d<sub>6</sub>): δ 154.7, 152.4, 152.2, 149.2, 140.6, 132.5, 122.8, 120.3, 114.3, 88.1, 86.1, 73.8, 70.8, 67.7, 61.8, 31.5, 29.2, 29.0, 28.9, 28.8, 25.7, 22.3, 14.1. HPLC: purity 100%, retention time 10.63 min. HRMS (ESI) *m/z* calcd for C<sub>25</sub>H<sub>35</sub>N<sub>5</sub>O<sub>5</sub><sup>+</sup>: 486.27110, found: 486.270840

## Data simulation

**Table S1.** Data simulation of the binding of radioligand, 'c', during a period of 50 min, either alone or in the presence of increasing concentrations of the bitopic ligand, 'ab'. The microscopic binding kinetics of the bitopic ligand 'ab' was defined as  $k_{+a} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-a} = 1 \text{ min}^{-1}$ ,  $k_{+b} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-b} = 1 \text{ min}^{-1}$  and  $[L] = 0.29 \text{ mM}$ . The binding kinetics of the monovalent ligand 'c' was defined as  $k_{+c} = 1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-c} = 0.3 \text{ min}^{-1}$  and  $[c] = 100 \text{ nM}$ . The cooperativity factors of 'ab' were set as  $\alpha_{ab} = 10$ .

time	Concentration of 'ab'(nM)						
	0	100	300	1000	3000	10000	30000
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	36.77	36.60	36.26	35.11	32.10	24.04	12.74
1.0	55.96	55.47	54.51	51.31	43.54	26.85	11.60
1.5	65.98	65.15	63.54	58.34	46.53	25.24	10.23
2.0	71.21	70.07	67.87	60.93	46.09	22.86	9.02
2.5	73.94	72.52	69.79	61.35	44.30	20.52	7.98
3.0	75.37	73.68	70.47	60.74	42.02	18.39	7.08
3.5	76.11	74.18	70.53	59.64	39.64	16.50	6.30
4.0	76.50	74.33	70.26	58.31	37.30	14.82	5.63
4.5	76.70	74.30	69.82	56.91	35.07	13.33	5.04
5.0	76.8	74.8	71.0	60.0	41.3	21.6	13.0
10	76.92	72.18	63.78	42.99	18.37	4.97	2.08
15	76.92	70.21	58.88	33.94	11.26	2.94	1.51
20	76.92	68.40	54.60	27.42	7.81	2.36	1.38
25	76.92	66.72	50.88	22.73	6.13	2.19	1.35
30	76.92	65.16	47.64	19.35	5.32	2.14	1.34
35	76.92	63.72	44.81	16.91	4.92	2.13	1.34
40	76.92	62.39	42.35	15.16	4.73	2.12	1.34
45	76.92	61.16	40.21	13.89	4.64	2.12	1.34
50	76.92	60.02	38.34	12.98	4.59	2.12	1.34

Data are shown as the percentage of  $B_{\text{max}}$ .

**Table S2** Data simulation of the binding of radioligand, 'c', during a period of 50 min, either alone or in the presence of increasing concentrations of the bitopic ligand, 'ab'. The microscopic binding kinetics of the bitopic ligand 'ab' was defined as  $k_{+a} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-a} = 1 \text{ min}^{-1}$ ,  $k_{+b} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-b} = 1 \text{ min}^{-1}$  and  $[L] = 0.29 \text{ mM}$ . The binding kinetics of the monovalent ligand 'c' was defined as  $k_{+c} = 1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-c} = 0.3 \text{ min}^{-1}$  and  $[c] = 100 \text{ nM}$ . The cooperativity factors of 'ab' were set as  $\alpha_{ab} = 0.1$ .

time	Concentration of 'ab'(nM)						
	0	100	300	1000	3000	10000	30000
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	36.77	36.67	36.48	35.82	34.07	29.24	21.79
1.0	55.96	55.69	55.16	53.39	48.96	38.71	27.41
1.5	65.98	65.56	64.74	62.06	55.70	42.77	30.89
2.0	71.21	70.69	69.67	66.38	58.93	45.13	33.60
2.5	73.94	73.35	72.21	68.57	60.59	46.77	35.75
3.0	75.37	74.74	73.52	69.69	61.52	48.00	37.47
3.5	76.11	75.46	74.20	70.27	62.08	48.96	38.84
4.0	76.50	75.83	74.55	70.58	62.44	49.70	39.94
4.5	76.70	76.03	74.73	70.75	62.70	50.29	40.82
5.0	76.81	76.13	74.83	70.85	62.88	50.75	41.53
10	76.92	76.24	74.93	71.01	63.42	52.28	44.03
15	76.92	76.24	74.93	71.02	63.47	52.42	44.29
20	76.92	76.24	74.93	71.02	63.47	52.43	44.32
25	76.92	76.24	74.93	71.02	63.47	52.43	44.33
30	76.92	76.24	74.93	71.02	63.47	52.43	44.33
35	76.92	76.24	74.93	71.02	63.47	52.43	44.33
40	76.92	76.24	74.93	71.02	63.47	52.43	44.33
45	76.92	76.24	74.93	71.02	63.47	52.43	44.33
50	76.92	76.24	74.93	71.02	63.47	52.43	44.33

Data are shown as the percentage of  $B_{\text{max}}$ .

**Table S3** Data simulation of the binding of radioligand, 'c', during a period of 50 min, either alone or in the presence of increasing concentrations of the bitopic ligand, 'ab'. The microscopic binding kinetics of the bitopic ligand 'ab' was defined as  $k_{+a} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-a} = 1 \text{ min}^{-1}$ ,  $k_{+b} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-b} = 1 \text{ min}^{-1}$  and  $[L] = 0.29 \text{ mM}$ . The binding kinetics of the monovalent ligand 'c' was defined as  $k_{+c} = 1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-c} = 0.3 \text{ min}^{-1}$  and  $[c] = 100 \text{ nM}$ . The cooperativity factors of 'ab' were set as  $\alpha'_{ab} = 10$ .

time	Concentration of 'ab'(nM)						
	0	100	300	1000	3000	10000	30000
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	36.77	36.61	36.30	35.25	32.47	24.93	13.99
1.0	55.96	55.50	54.59	51.58	44.20	28.07	12.81
1.5	65.98	65.20	63.67	58.72	47.38	26.51	11.32
2.0	71.21	70.13	68.03	61.39	47.06	24.11	10.00
2.5	73.94	72.59	69.98	61.89	45.34	21.71	8.86
3.0	75.37	73.76	70.69	61.33	43.12	19.52	7.86
3.5	76.11	74.27	70.77	60.28	40.77	17.56	7.00
4.0	76.50	74.43	70.52	59.01	38.45	15.81	6.25
4.5	76.70	74.41	70.11	57.64	36.23	14.26	5.60
5.0	76.81	74.30	69.62	56.26	34.13	12.88	5.04
10	76.92	72.37	64.28	44.00	19.35	5.35	2.23
15	76.92	70.47	59.51	35.00	11.95	3.09	1.56
20	76.92	68.71	55.32	28.43	8.25	2.41	1.39
25	76.92	67.08	51.66	23.63	6.40	2.21	1.35
30	76.92	65.56	48.45	20.12	5.47	2.15	1.34
35	76.92	64.15	45.64	17.56	5.01	2.13	1.34
40	76.92	62.84	43.17	15.70	4.78	2.12	1.34
45	76.92	61.63	41.01	14.33	4.66	2.12	1.34
50	76.92	60.50	39.12	13.34	4.60	2.12	1.34

Data are shown as the percentage of  $B_{\text{max}}$ .



**Table S4** Data simulation of the binding of radioligand, 'c', during a period of 50 min, either alone or in the presence of increasing concentrations of the bitopic ligand, 'ab'. The microscopic binding kinetics of the bitopic ligand 'ab' was defined as  $k_{+a} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-a} = 1 \text{ min}^{-1}$ ,  $k_{+b} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-b} = 1 \text{ min}^{-1}$  and  $[L] = 0.29 \text{ mM}$ . The binding kinetics of the monovalent ligand 'c' was defined as  $k_{+c} = 1 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-c} = 0.3 \text{ min}^{-1}$  and  $[c] = 100 \text{ nM}$ . The cooperativity factors of 'ab' were set as  $\alpha'_{ab} = 0.1$ .

time	Concentration of 'ab'(nM)						
	0	100	300	1000	3000	10000	30000
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	36.77	36.65	36.41	35.60	33.46	27.64	19.03
1.0	55.96	55.65	55.03	52.99	47.95	36.67	25.05
1.5	65.98	65.51	64.60	61.62	54.68	41.15	29.45
2.0	71.21	70.64	69.53	65.97	58.07	44.04	32.84
2.5	73.94	73.31	72.08	68.22	59.93	46.12	35.46
3.0	75.37	74.70	73.42	69.41	61.03	47.66	37.48
3.5	76.11	75.43	74.11	70.05	61.74	48.82	39.04
4.0	76.50	75.81	74.48	70.42	62.22	49.70	40.24
4.5	76.70	76.01	74.68	70.63	62.55	50.36	41.17
5.0	76.81	76.11	74.79	70.76	62.79	50.86	41.89
10	76.92	76.23	74.93	71.01	63.43	52.33	44.14
15	76.92	76.24	74.93	71.02	63.47	52.42	44.31
20	76.92	76.24	74.93	71.02	63.47	52.43	44.33
25	76.92	76.24	74.93	71.02	63.47	52.43	44.33
30	76.92	76.24	74.93	71.02	63.47	52.43	44.33
35	76.92	76.24	74.93	71.02	63.47	52.43	44.33
40	76.92	76.24	74.93	71.02	63.47	52.43	44.33
45	76.92	76.24	74.93	71.02	63.47	52.43	44.33
50	76.92	76.24	74.93	71.02	63.47	52.43	44.33

Data are shown as the percentage of  $B_{\text{max}}$ .

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## Figure S1

Competition association assay of a radioligand, 'c' (with  $k_{+c} = 1 \cdot 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-c} = 0.3 \text{ min}^{-1}$  and  $[c] = 100 \text{ nM}$ ), during a period of 50 min, either alone or in the presence of increasing concentrations of 'ab' ( $k_{+a} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-a} = 1 \text{ min}^{-1}$ ,  $k_{+b} = 1 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ ,  $k_{-b} = 1 \text{ min}^{-1}$  and  $[L] = 0.29 \text{ mM}$ ) with different cooperativity factors: 1)  $\alpha_{ab} = 10$ ; 2)  $\alpha_{ab} = 0.1$ ; 3)  $\alpha'_{ab} = 10$ ; 4)  $\alpha'_{ab} = 0.1$ . Data input into this analysis were from Table S1 to S4, respectively.