Supplemental Data:

"Structure activity relationships of the sustained effects of adenosine A2A receptor agonists driven by slow dissociation kinetics"

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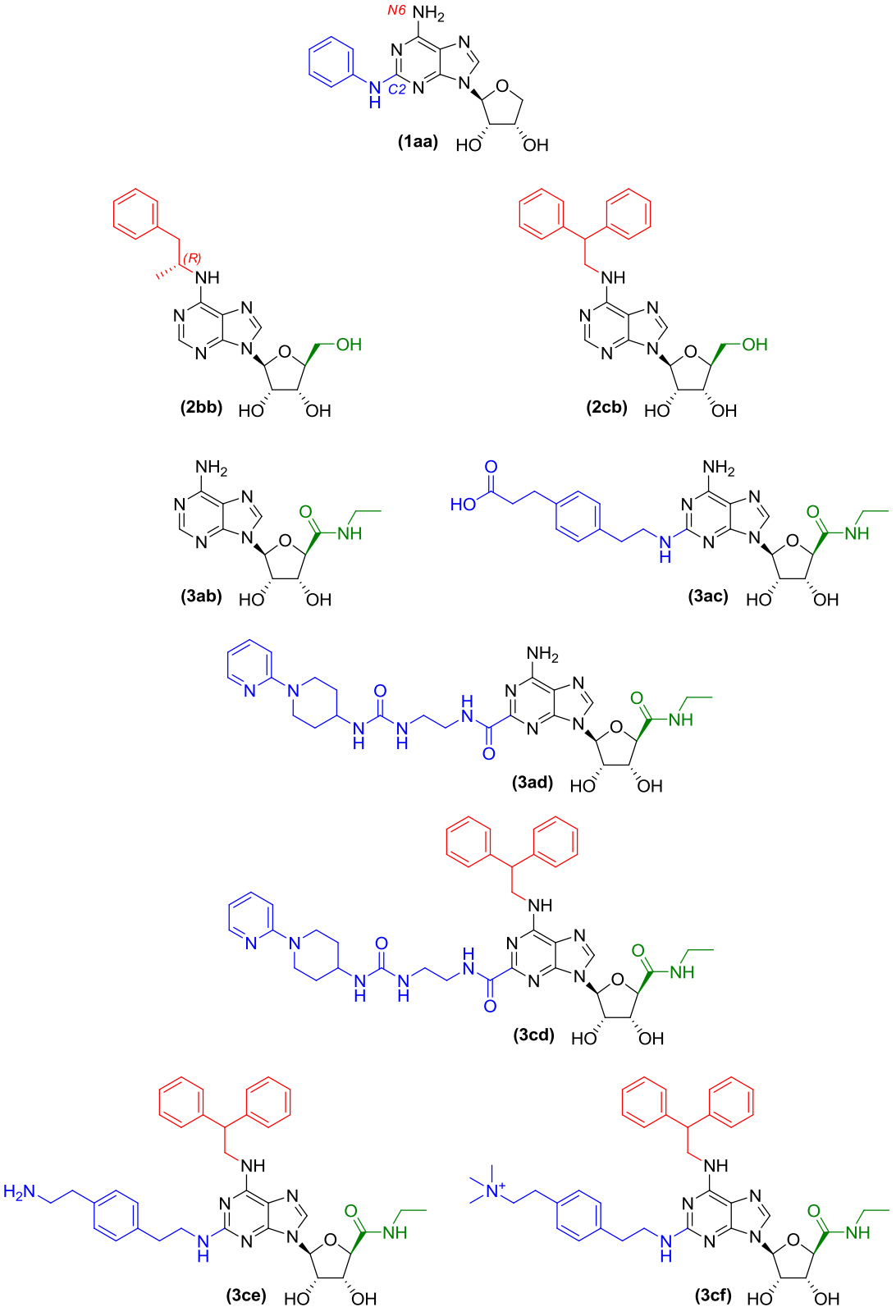
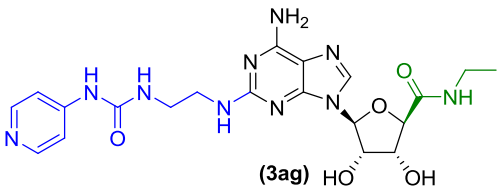
Supplemental Figure 1. The structures of the ligands investigated in this study. All the ligands have an adenosine scaffold (black), with substitution on the ribose 4 position (R1 green), N6 (R2; red), and/or C2 (R3; blue). Compound 4 is shown in magenta as it does not share this structural scaffold.





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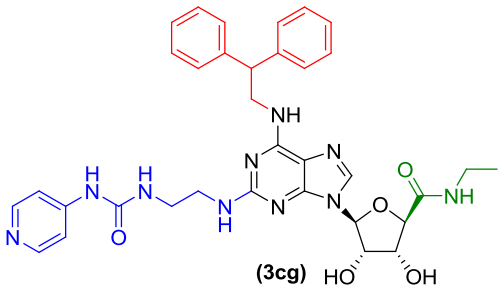

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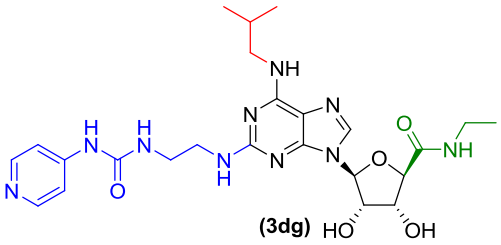

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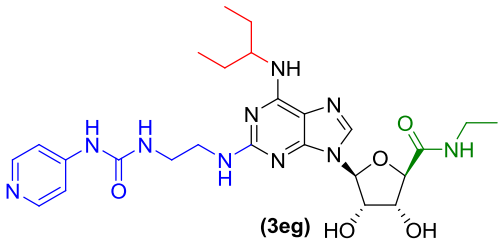

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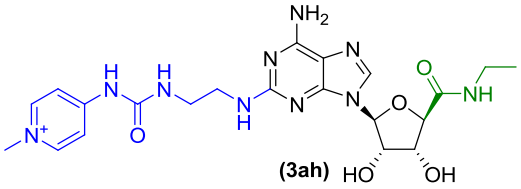

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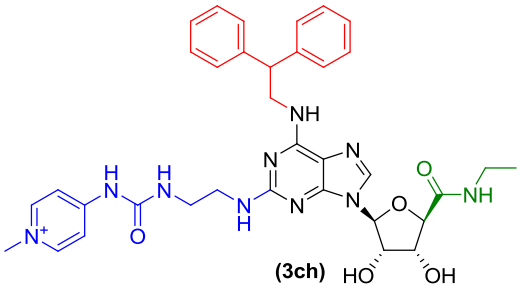

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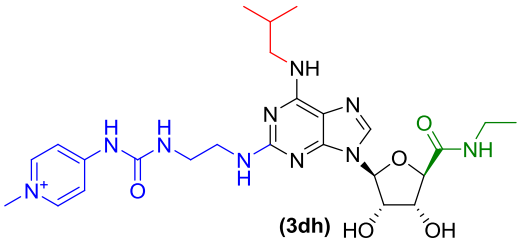

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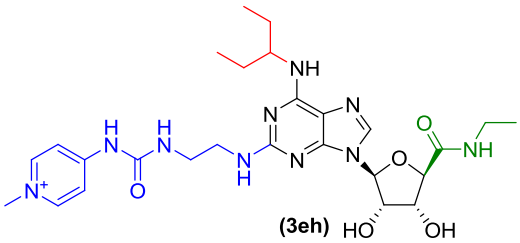

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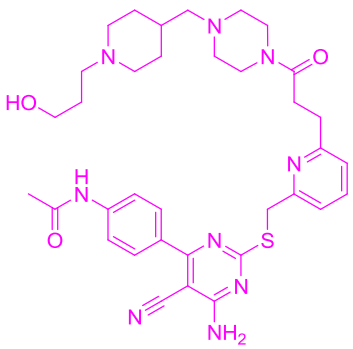
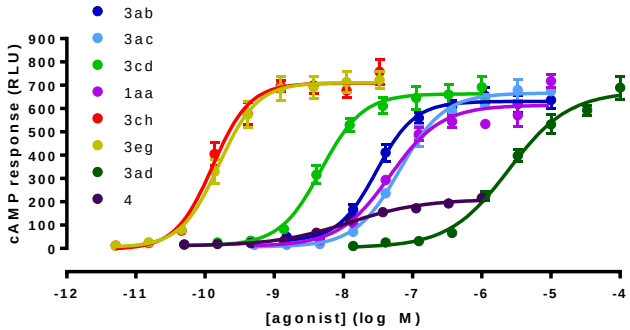


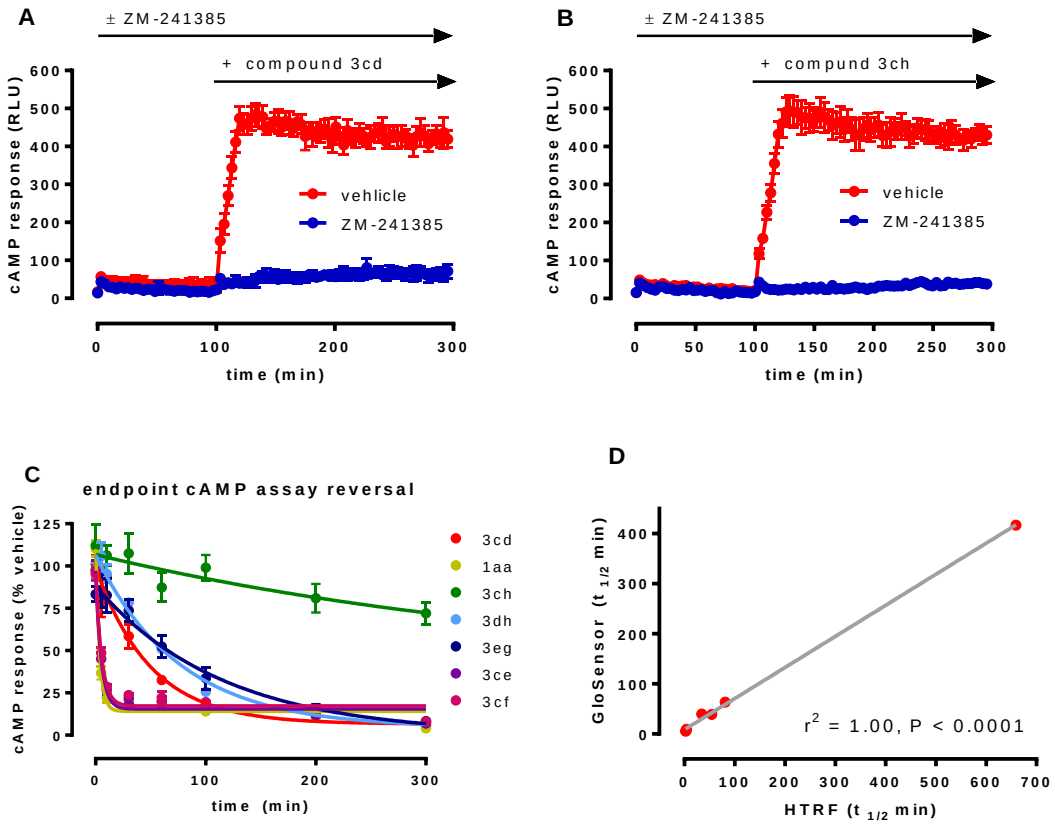
(4)

Supplemental Figure 2. Example concentration-response curves measured using the GloSensor cAMP assay in CHO-A2A cells (100 minutes, 20 °C) for the A2A agonists 3ab (dark blue), 3ac (light blue), 3cd (light green), 1aa (light purple), 3ch (red), 3eg (yellow), 3ad (dark red), and 4 (dark purple). Data represent the average of three experiments performed in triplicate, and derived pEC50 values are shown in Table 2 of main text.



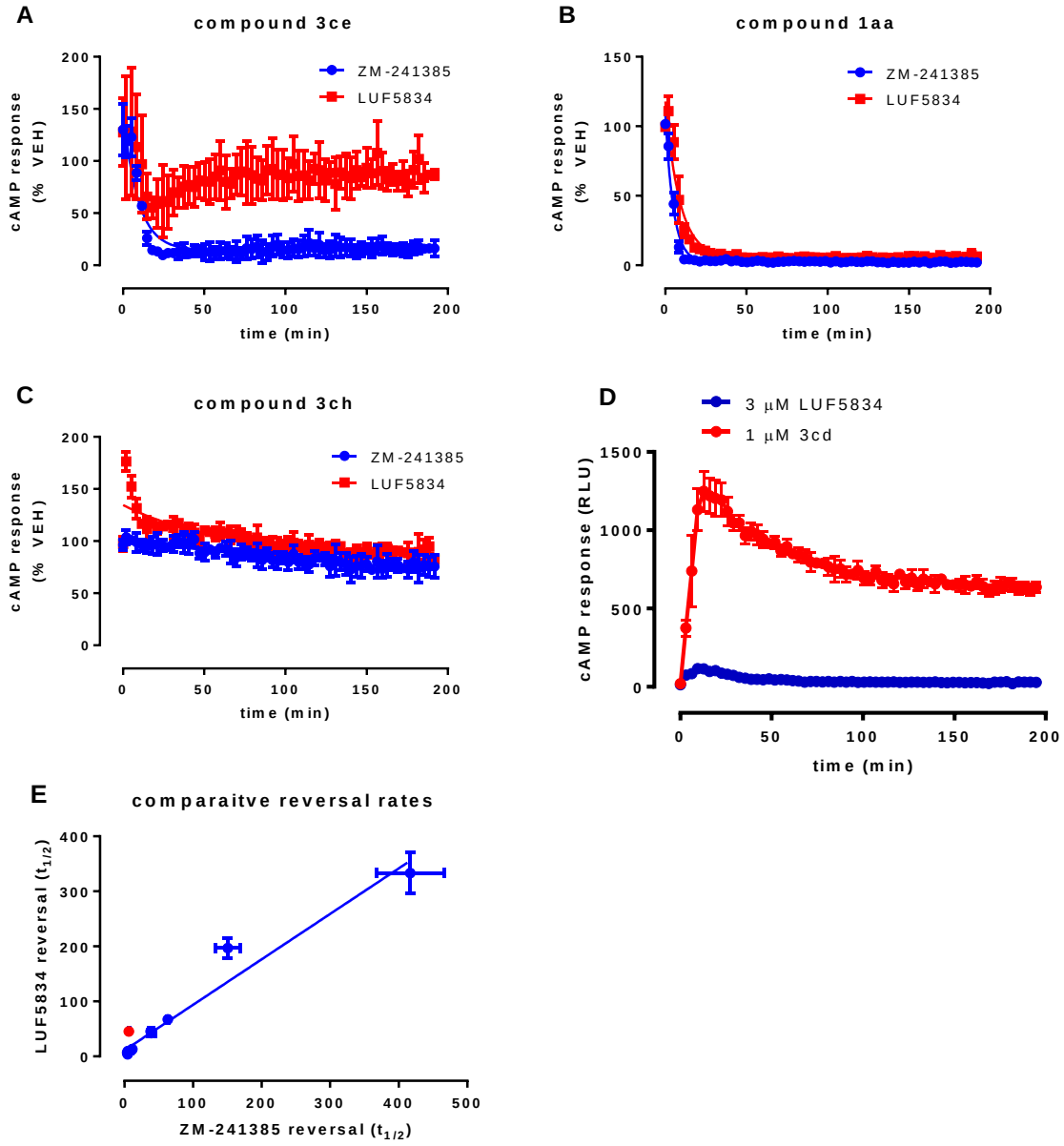
Supplemental Figure 2

Supplemental Figure 3. Control experiments verifying the GloSensor reversal assay to estimate agonist off-rate. **(A-B)** GloSensor traces (20 °C) demonstrating competitive antagonism of 3cd (A) or 3ch (B) agonist responses by ZM-241385. Cells were incubated with (red) or without (blue) 100 nM ZM-241385 for 100 minutes before direct addition of supramaximal concentrations (100 x EC_{50}) of each agonist and further incubation for 200 minutes. Pre-equilibration with antagonist prevents agonist cAMP responses, indicating competitive antagonism. Data represent an average of three experiments performed in triplicate. **(C-D)** For comparison in a separate assay format, reversal traces were also measured in the endpoint HTRF cAMP assay, with no PDE inclusion to measure dynamic changes in cellular cAMP. (C) Cells were incubated with EC_{90} concentrations of 3cd, 1aa, 3ch, 3dh, 3eg, 3ce, or 3cf for 2 hours at room temperature before direct addition of vehicle control of excess antagonist (100 nM ZM-241385) and incubation for a range of times up to 300 minutes at room temperature before cells were lysed and cAMP measured. Reversal responses with antagonist treatment were normalized to vehicle at each time-point and traces fitted to non-linear regression (one-phase exponential decay). Data represent an average of three experiments performed in triplicate. (D) Derived t½ values from the HTRF assay shown in (C) were plotted against those derived in the GloSensor assay (Table 2 in main text) and linear regression performed. There was a strong correlation between the two data sets ($r^2 = 1.00$, $P < 0.0001$, $n = 7$ ligands) indicating that the GloSensor assay accurately reflects cellular cAMP fluxes.



Supplemental Figure 3

Supplemental Figure 4. Compound 3ce is unique amongst the ligands tested in its ability to discriminate between antagonists in reversal experiments as it appears rapidly reversed when ZM-241385 is applied, but slowly/incompletely reversed when LUF5834 is applied. Thus, applying LUF5834 reversal data confirms that 3ce is a sustained wash-resistant agonist with a slow off-rate, thus explaining the anomaly highlighted in Figure 3H for this compound. **(A-C)** GloSensor antagonist reversal traces observed when ZM-241385 (blue; 100 nM) or LUF5834 (red; 6 µM) is added after generation of stable agonist responses. 3ce (A) is rapidly and completely reversed by ZM-241385 but only partially reversed by LUF5834. For comparison, 1aa (B) is rapidly reversed in a very similar manner by both antagonists, whilst 3ch (C) is poorly reversed by both. **(D)** GloSensor agonist stimulation responses of 3cd (red) and LUF5834 (blue) to demonstrate that LUF5834 has very weak efficacy in this system at a saturating concentration. **(E)** Comparison of reversal rates (t½) derived from GloSensor experiments using ZM-241385 (100 nM) and LUF5834 (6 µM) as reversal agents show strong correlation across 18 agonists tested, although 3ce (highlighted as a red circle) is a clear outlier. Linear regression analysis displayed an r^2 of 0.95 and $P < 0.001$. Therefore, with 3ce as an exception, consistent kinetic data was obtained regardless of the antagonist used, suggesting that derived reversal rates were not an artefact of the pharmacology of ZM-241385. All GloSensor traces represent an average of three experiments performed in triplicate.

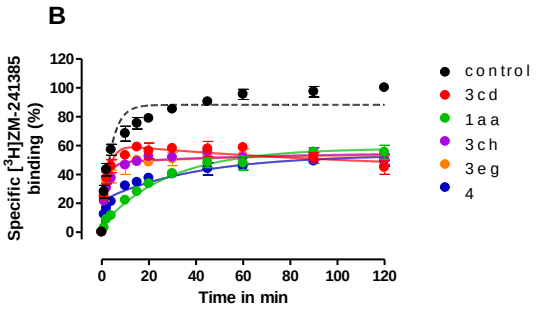


Supplemental Figure 4

Supplemental Figure 5. Control experiments supporting [^{3}H]ZM-241385 binding data. **(A)** [^{3}H]ZM-241385 association binding rate at 4 °C under control conditions (black) or in the presence of Ki concentrations of test compound. **(B)** Best-fit data derived from these curves is shown as a table. **(C-E)** Antagonist reversal of A2A agonist responses in membranes prepared from CHO-A2A cells. These experiments used the HTRF HiRange cAMP kit (CisBio). Methods to prepare membranes were based on those described previously (Palmer et al., 1994). Cells were grown to confluency in two 225 cm^2 flasks and washed twice with ice-cold PBS. Cells were then lysed and scraped in ice-cold lysis buffer (50 mM TRIS, 5 mM EDTA, pH 7.4), followed by incubation at 4 °C for 10 minutes. Cell lysates were homogenized using a glass-on-glass Dounce homogenizer, and then centrifuged at 42,000 x g for 15 minutes at 4 °C. The sample was then washed once with assay buffer (50 mM TRIS, pH 7.4) without disturbing the pellet, and then resuspended in 20 ml of assay buffer using the homogenizer and by passing through a fine needle. Membranes were prepared fresh immediately prior to each experiment. The membrane suspension was then supplemented with 5 mM $MgCl_2$ and 0.8 U/ml adenosine deaminase and 50 µl/well added to 96 well plates. 10 x concentrated agonist (final concentration EC_{90}) was then directly added and the plates incubated at room temperature for 2 hours. Antagonist (100 nM ZM-241385) was then directly added to these wells and incubated for a further 0, 1, 2, or 3 hours at room temperature. cAMP was then captured by direct addition of stimulation buffer in a 1:1 dilution to achieve final concentrations of 40 µM rolipram, 1 mM ATP, 5 mM $MgCl_2$, and 0.8 U/ml adenosine deaminase, and membranes incubated for a further 45 minutes at room temperature, before 10 µl of sample was added to a 384 well plate to measure cAMP levels by HTRF assay as per the manufacturer's instructions. In these experiments, A2A cAMP responses remained stable over time. The antagonist reversed 1aa (C) responses to near baseline within the first hour (this is likely a gross underestimation of reversal rate) with significantly reduced responses compared to vehicle ($P < 0.001$; ANOVA: $n = 3$). In marked contrast, whilst the antagonist reversed 3cd-mediated (D) cAMP responses to significantly decreased levels after 2 hours ($P < 0.05$; ANOVA: $n = 3$), this reversal was incomplete and reached only 79.56 ± 8.46 % of vehicle. 3ch (E) responses were not significantly affected by antagonist. Therefore, the slow off-rates of 3cd and 3ch are maintained in cAMP reversal responses in membranes, and the 3cd responses are more poorly reversed than in cells. Therefore, the

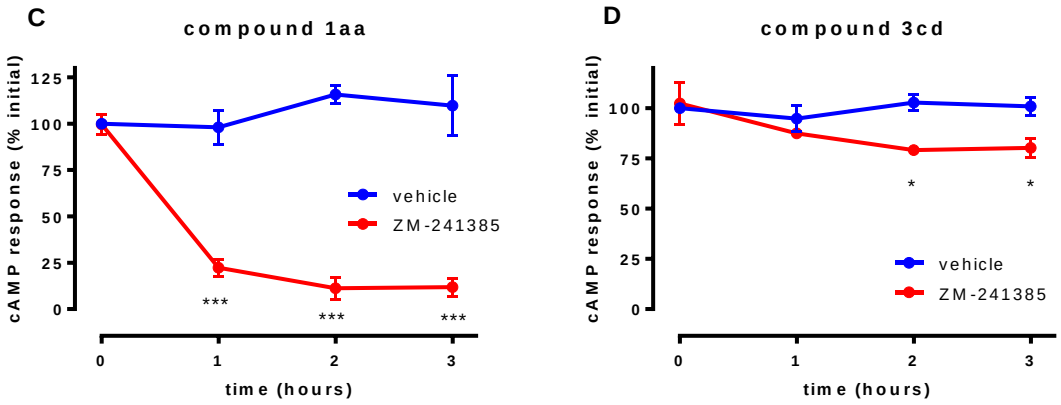
use of membranes, different cell background or non-equivalent buffer system compared to radioligand binding did not influence our findings.

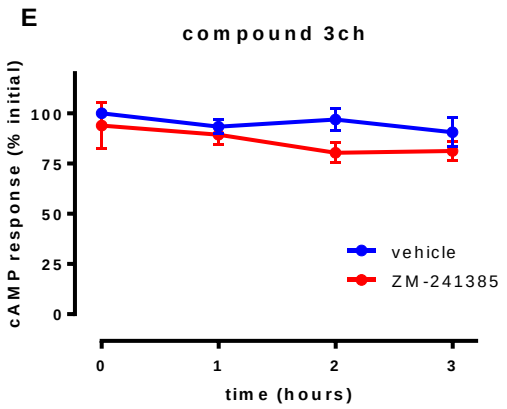
A



B

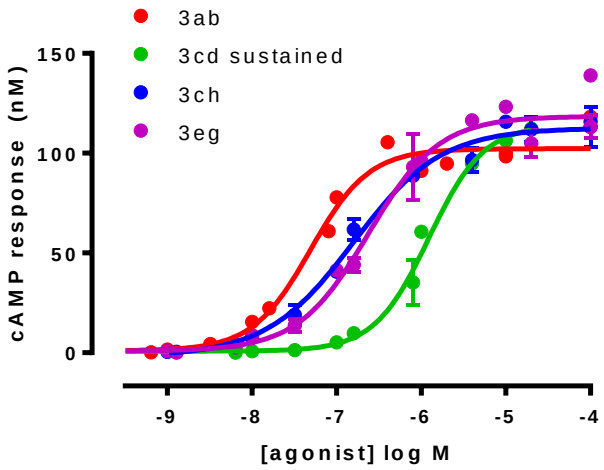
Compound	Affinity, K_i (nM)	k_{on} ($M^{-1}\cdot min^{-1}$)	k_{off} (min^{-1})	RT (min)
3cd	44 ± 5	$3.5 \pm 0.4 \times 10^5$	0.0057 ± 0.0024	175 ± 74
1aa	154 ± 31	$3.3 \pm 2.0 \times 10^6$	0.41 ± 0.26	2.4 ± 1.5
3ch	15 ± 2	$1.8 \pm 0.2 \times 10^6$	0.019 ± 0.005	53 ± 14
3eg	66 ± 11	$4.7 \pm 0.6 \times 10^5$	0.018 ± 0.005	56 ± 16
4	24 ± 1	$4.7 \pm 0.9 \times 10^6$	0.059 ± 0.014	17 ± 4





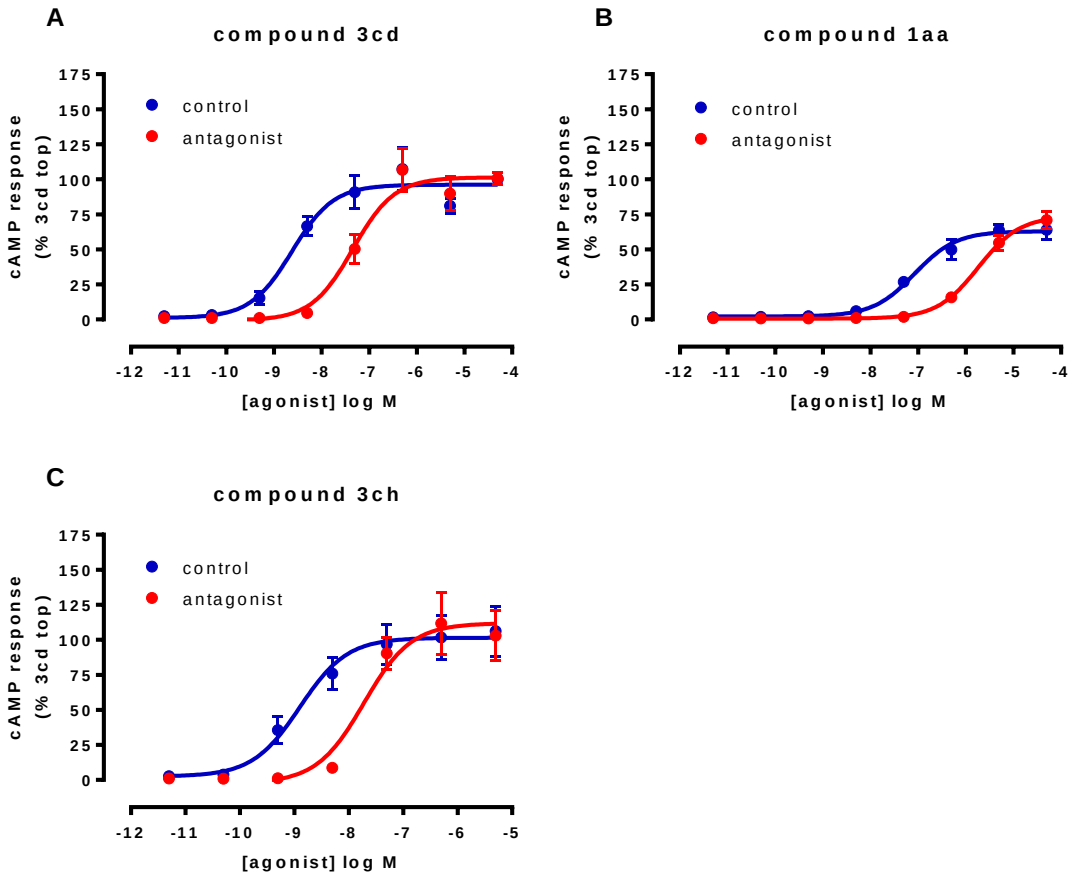
Supplemental Figure 5

Supplemental Figure 6. Concentration-response curves of agonists in CHO-A2B cells measured using the endpoint cAMP assay. 3ab (red), 3cd (green), 3ch (blue) or 3eg (purple) were incubated for 45 minutes at 37 °C with 40 µM rolipram before cAMP measured by HTRF. All agonists show robust and saturable A2B responses with response maxima comparable to 3ab.



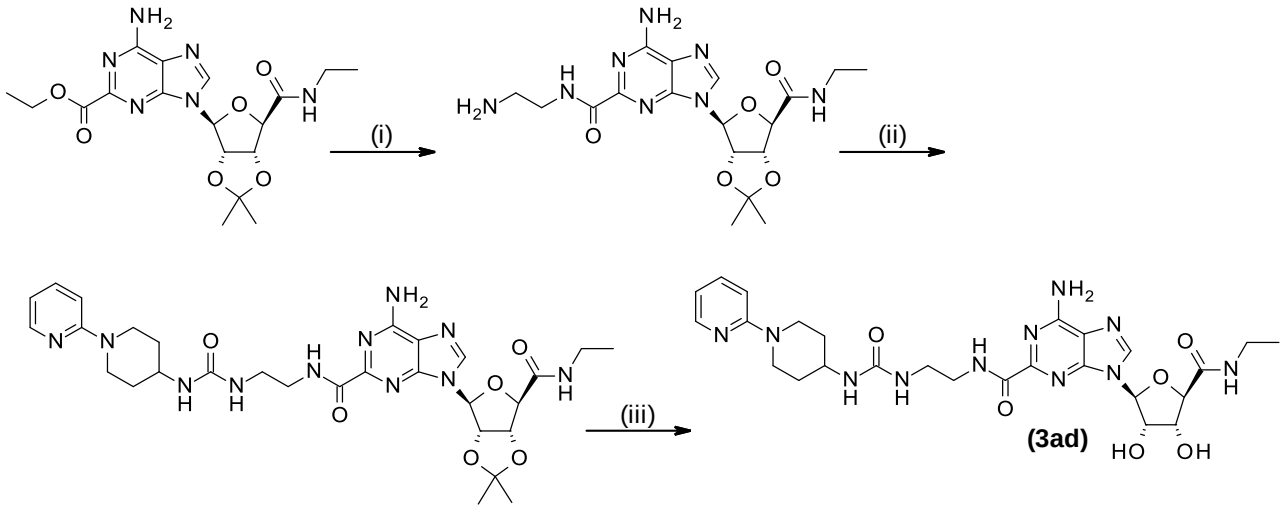
Supplemental Figure 6

Supplemental Figure 7. Concentration-response curves of A2A agonists in SH-SY5Y cells measured using the endpoint cAMP assay. To confirm receptor-specificity, cells were pre-treated with the A2A-selective antagonist SCH-442416 (100 nM) (red) or vehicle control (blue) for 30 minutes (37 °C) before addition of either 3cd (A), 1aa (B), or 3ch (C) for a further 45 minutes (37 °C) in the presence of 40 µM rolipram, and subsequent measurement of cAMP by HTRF. Derived pEC50 values are 8.59 ± 0.09, 7.00 ± 0.23, and 8.90 ± 0.05 for these agonists, respectively, consistent with their A2A-activity. Moreover, the antagonist caused a right-ward shift in the curves with dose ratios of > 15, supporting a role for A2A responses. Together, these findings suggest that the cAMP responses evoked by these ligands are at least predominantly A2A-mediated.



Supplemental Figure 7

Supplemental Schemes:



Supplemental Scheme 1: (i) ethane-1,2-diamine, acetone, 80°C, 3 h, 52%; (ii) *N*-(1-(pyridin-2-yl)piperidin-4-yl)-1*H*-imidazole-1-carboxamide, iPrOH, toluene, DCM, 24 h; (iii) HCl (1 M), 65°C, 1 h, 39% (over 2 steps).

6-amino-*N*-(2-aminoethyl)-9-((3a*S*,4*S*,6*R*,6a*R*)-6-(ethylcarbamoyl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)-9*H*-purine-2-carboxamide

A stirred mixture of ethyl 6-amino-9-((3a*S*,4*S*,6*R*,6a*R*)-6-(ethylcarbamoyl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)-9*H*-purine-2-carboxylate (0.500 g, 1.19 mmol) and etane-1,2-diamine (5 mL, 75 mmol) was heated to 80°C for 3 h. After cooling, the mixture was concentrated *in vacuo* and purified by preparative HPLC to give 6-amino-*N*-(2-aminoethyl)-9-((3a*S*,4*S*,6*R*,6a*R*)-6-(ethylcarbamoyl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)-9*H*-purine-2-carboxamide (0.268 g, 0.62 mmol, 52%).

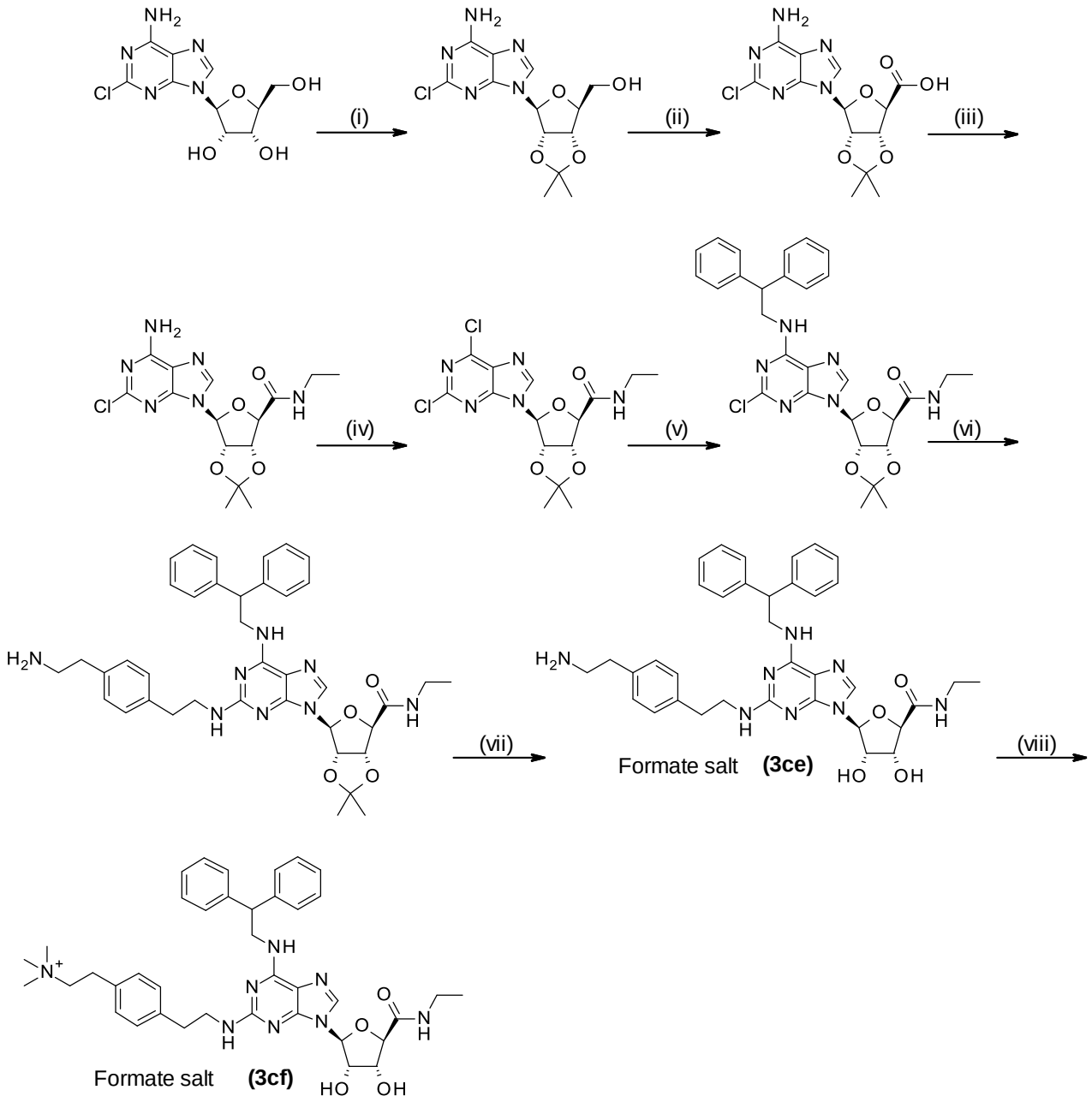
6-amino-9-((3a*S*,4*S*,6*R*,6a*R*)-6-(ethylcarbamoyl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)-*N*-(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9*H*-purine-2-carboxamide

A stirred solution of 6-amino-*N*-(2-aminoethyl)-9-((3a*S*,4*S*,6*R*,6a*R*)-6-(ethylcarbamoyl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)-9*H*-purine-2-carboxamide (0.079 g, 0.18 mmol) in toluene (4 mL) and iPrOH (1 mL) was treated with a solution of *N*-(1-(pyridin-2-yl)piperidin-4-yl)-1*H*-imidazole-1-carboxamide (0.049 g, 0.18 mmol) in DCM (2.5 mL). The mixture was stirred at

room tempertautre for 24 h then concentrated *in vacuo* to give a residue containing 6-amino-9-((3a*S*,4*S*,6*R*,6a*R*)-6-(ethylcarbamoyl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)-*N*-(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9*H*-purine-2-carboxamide, which was taken on to the next step without purification.

6-amino-9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-*N*-(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9*H*-purine-2-carboxamide

A residue containing 6-amino-9-((3a*S*,4*S*,6*R*,6a*R*)-6-(ethylcarbamoyl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)-*N*-(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9*H*-purine-2-carboxamide (0.18 mmol) was treated with HCl (1 M) (1.5 mL) and heated at 65^{o}C. After stirring for 1 h the mixture was concentrated *in vacuo* and purified by preparative HPLC to give 6-amino-9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-*N*-(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9*H*-purine-2-carboxamide (0.042 g, 0.070 mmol, 39% over two steps).



Supplemental Scheme 2: (i) 2,2-dimethoxypropane, TsOH.H_2O, acetone, r.t., 20 h, 88%; (ii) $KMnO_4$, KOH, MeCN, H_2O, r.t., 20 h, then H_2O_2 (30%), followed by HCl (2 M), 73%; (iii) Thionyl chloride, DCM, DMF (cat.) 50°C, 4 h, then ethylamine, DCM, 0-5°C, 2 h, 74%; (iv) *t*-Butyl nitrite, CCl_4, MeCN, 80°C, 4 h, 30%; (v) 2,2-diphenylethanamine, EtOH, r.t., 20 h, 94%; (vi) 2,2'-(1,4-phenylene)diethanamine, 130°C, 2 h, 67%; (vii) HCl (1 M), 60°C, 1 h, 50%; (viii) Iodomethane, K_2CO_3, MeOH, 30°C, 7 h, 47%.

((3a*S*,4*S*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methanol

A stirred mixture of (2*S*,3*S*,4*R*,5*S*)-2-(6-amino-2-chloro-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (50 g, 166 mmol), *p*-toluenesulfonic acid monohydrate (31.45 g, 166 mmol), 2,2-dimethoxypropane (357 mL, 2903 mmol), and acetone (690 mL) was stirred at room temperature. After 20 h water (400 mL) was added and the mixture adjusted to pH =7-8 by the addition of ammonia solution. The solvent was removed *in vacuo*, and the residue partitioned between EtOAc and water. The organic extracts were concentrated *in vacuo* to give ((3a*S*,4*S*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methanol (50 g, 146 mmol, 88%) as a white solid.

(3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxylic acid

A stirred mixture of ((3a*S*,4*S*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl)methanol (50 g, 146 mmol) and potassium hydroxide (24.6 g, 439 mmol) in water/MeCN (400 mL/1600 ml) was treated with a solution of $KMnO_4$ (69.3 g, 439 mmol) in water (720 mL). The resulting mixture was stirred at room temperature for 20 h, then filtered through Celite. Hydrogen peroxide (30%) (100 mL) was added to the filtrate. The organic solvent was removed *in vacuo*, and the remaining aqueous adjusted to pH = 4 by the addition of HCl (1 M). The resulting precipitate was removed by filtration and dried *in vacuo* to afford (3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxylic acid (38 g, 107 mmol, 73%) as a white solid.

(3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred mixture of (3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxylic acid (15 g, 42 mmol), DMF (0.6 mL) and thionyl chloride (75 mL, 1034 mmol) was heated to 50^{o}C for 4 h, then the solvent was removed *in vacuo*. The residue was suspended in DCM (240 mL), and then added in portions to a solution of

ethylamine (65-70% solution in H_2O) (48 mL) dissolved in DCM (200 mL) at 0-5°C. After stirring for 2 h the mixture was poured onto water. The organic layer was dried (Na_2SO_4), filtered, and concentrated *in vacuo* to give (3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (12 g, 31 mmol, 74%) as a white solid.

(3a*R*,4*R*,6*S*,6a*S*)-6-(2,6-dichloro-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred mixture of (3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (10 g, 26 mmol) and carbon tetrachloride (270 mL) in acetonitrile (67 mL) was treated with *tert*-butyl nitrite (9.31 mL, 78 mmol). The mixture was stirred for 4 h at 80°C, then concentrated *in vacuo*. The residue was purified by flash chromatography (silica), eluting with methanol in DCM (2%), to give (3a*R*,4*R*,6*S*,6a*S*)-6-(2,6-dichloro-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxole-4-carboxamide (3.2 g, 7.96 mmol, 30%) as a yellow solid.

(3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

To a stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-6-(2,6-dichloro-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (1.6 g, 4.0 mmol) in ethanol (40 mL) was added 2,2-diphenylethylamine (7.8 g, 39 mmol). The mixture was stirred at room temperature for 20 h then concentrated *in vacuo*. The resulting residue was dissolved in EtOAc and washed with HCl (2 M) (50 mL). The organic layer was dried (Na_2SO_4), filtered, and concentrated *in vacuo* to give a residue which was purified by flash chromatography (silica), eluting with methanol in DCM (2%), to afford (3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (2.1 g, 3.7 mmol, 94%) as a yellow solid.

(3a*R*,4*R*,6*S*,6a*S*)-6-(2-(4-(2-aminoethyl)phenethylamino)-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

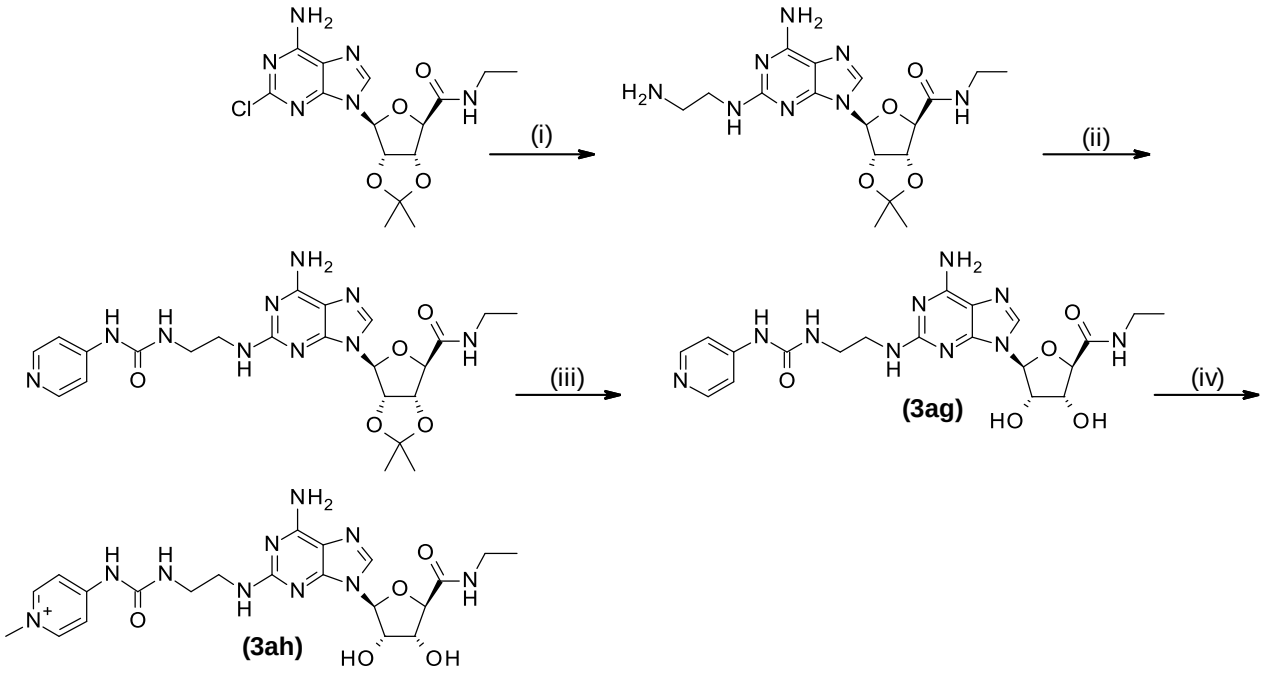
A stirred mixture of (3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.489 g, 0.87 mmol) and 2,2'-(1,4-phenylene)diethanamine (1.0 g, 6.1 mmol) was heated to 130°C. After 2 h the mixture was allowed to cool, then purified by flash chromatography (silica), eluting with methanol in DCM (16%), to afford (3a*R*,4*R*,6*S*,6a*S*)-6-(2-(4-(2-aminoethyl)phenethylamino)-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.400 g, 0.579 mmol, 67%) as a yellow solid.

(2*R*,3*R*,4*S*,5*S*)-5-(2-(4-(2-aminoethyl)phenethylamino)-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide formate salt

A stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-6-(2-(4-(2-aminoethyl)phenethylamino)-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.400 g, 0.579 mmol) in HCl (1 M) (20 mL) was heated to 60°C. After 1 h the mixture was concentrated *in vacuo* and purified by preparative HPLC to give (2*R*,3*R*,4*S*,5*S*)-5-(2-(4-(2-aminoethyl)phenethylamino)-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide formate salt (0.200 g, 0.287 mmol, 50%) as a white solid.

2-(4-(2-(6-(2,2-diphenylethylamino)-9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-ylamino)ethyl)phenyl)-*N*,*N*,*N*-trimethylethanaminium formate

A stirred solution of (2*R*,3*R*,4*S*,5*S*)-5-(2-(4-(2-aminoethyl)phenethylamino)-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide formate salt (0.100 g, 0.144 mmol, in methanol (15 mL) was treated with potassium carbonate (0.191 g, 1.38 mmol) and iodomethane (1 mL, 16.1 mmol), then heated to 30°C. After 7 h the mixture was concentrated *in vacuo* and purified by preparative HPLC to afford 2-(4-(2-(6-(2,2-diphenylethylamino)-9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-ylamino)ethyl)phenyl)-*N*,*N*,*N*-trimethylethanaminium formate (0.050 g, 0.068 mmol, 47%) as a white solid.



Supplemental Scheme 3: (i) ethane-1,2-diamine, 80°C, 3 h, 84%; (ii) 4-isocyanatopyridine, DCM, 0°C – r.t., 1 h; (iii) HCl (1 M), 65°C, 1 h, 49%; (iv) Iodomethane, MeCN, 38°C, 20 h, 80%.

(3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-(2-aminoethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred mixture of (3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-chloro-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.500 g, 1.31 mmol) and ethane-1,2-diamine (5 mL, 75 mmol) was heated to 80°C for 3 h. The mixture was concentrated *in vacuo* then purified by preparative HPLC to give (3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-(2-aminoethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.445 g, 1.09 mmol, 84%) as a solid.

(3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-(2-aminoethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide in DCM at 0°C was treated with 4-isocyanatopyridine. The mixture was allowed to wam to room temperature then concentrated *in vacuo* to give a residue which was purified to afford (3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-(2-(3-pyridin-4-

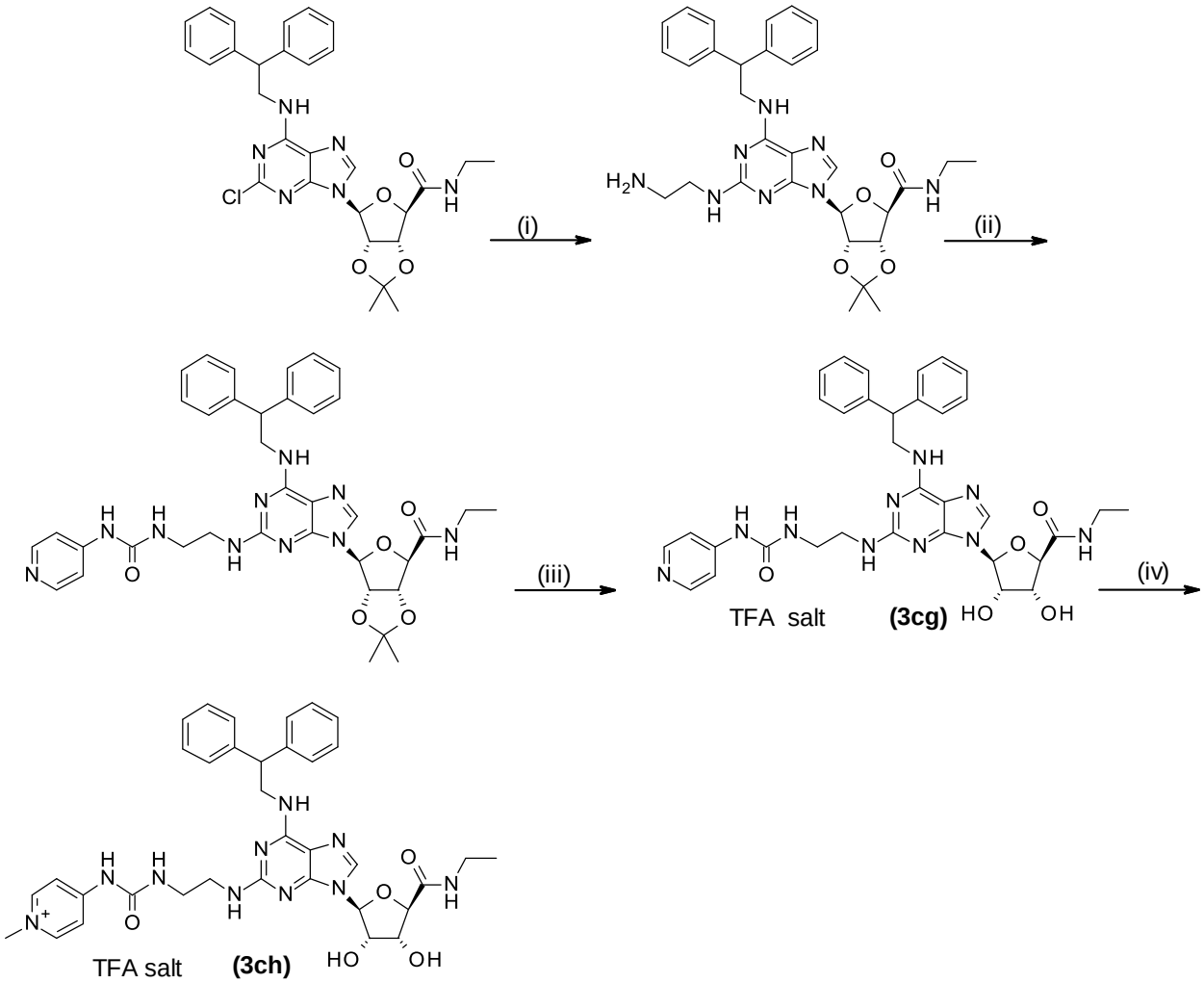
ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide as a solid.

(2*R*,3*R*,4*S*,5*S*)-5-(6-amino-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide

A stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-6-(6-amino-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.452 g, 0.858 mmol) in HCl (1 M) (2.3 mL) was heated at 65°C for 1 h. The mixture was allowed to cool to room temperature then purified by preparative HPLC to afford (2*R*,3*R*,4*S*,5*S*)-5-(6-amino-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide (0.203 g, 0.417 mmol, 49%) as a white solid.

4-(3-(2-(6-amino-9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-ylamino)ethyl)ureido)-1-methylpyridinium

A stirred solution of (2*R*,3*R*,4*S*,5*S*)-5-(6-amino-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide (0.020 g, 0.041 mmol) in acetonitrile (0.5 mL) was treated with iodomethane (0.1 mL, 1.60 mmol), then heated to 38°C. After 20 h the mixture was concentrated *in vacuo* then purified by preparative HPLC to give 4-(3-(2-(6-amino-9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-ylamino)ethyl)ureido)-1-methylpyridinium (0.016 g, 0.033 mmol, 80%) as a solid.



Supplemental Scheme 4: (i) ethane-1,2-diamine, 70°C, 20 h, 61%; (ii) 4-isocyanatopyridine, MeCN, 80°C, 20 h, 96%; (iii) HCl (1 M), 65°C, 1 h, 77%; (iv) Iodomethane, MeCN, MeOH, 35°C, 20 h, 54%.

(3a*R*,4*R*,6*S*,6a*S*)-6-(2-(2-aminoethylamino)-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred mixture of (3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (2.99 g, 5.31 mmol) and ethane-1,2-diamine (30 mL, 449 mmol) was heated at 70°C for 20 h. The mixture was concentrated *in vacuo* then purified by flash chromatography (silica), eluting with methanolic aqueous ammonia solution (2%) in DCM, to give (3a*R*,4*R*,6*S*,6a*S*)-6-(2-(2-aminoethylamino)-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (1.89 g, 3.22 mmol, 61%) as a yellow solid.

(3a*R*,4*R*,6*S*,6a*S*)-6-(6-(2,2-diphenylethylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-6-(2-(2-aminoethylamino)-6-(2,2-diphenylethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (1.00 g, 1.70 mmol) in acetonitrile (40 mL) was treated with 4-isocyanatopyridine (0.205 g, 1.71 mmol) then heated to 80°C. After heating for 20 h the mixture was concentrated *in vacuo* to give a residue which was purified by flash chromatography (silica), eluting with methanolic aqueous ammonia solution (2%) in DCM, to afford (3a*R*,4*R*,6*S*,6a*S*)-6-(6-(2,2-diphenylethylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (1.16 g, 1.64 mmol, 96%) as a white solid.

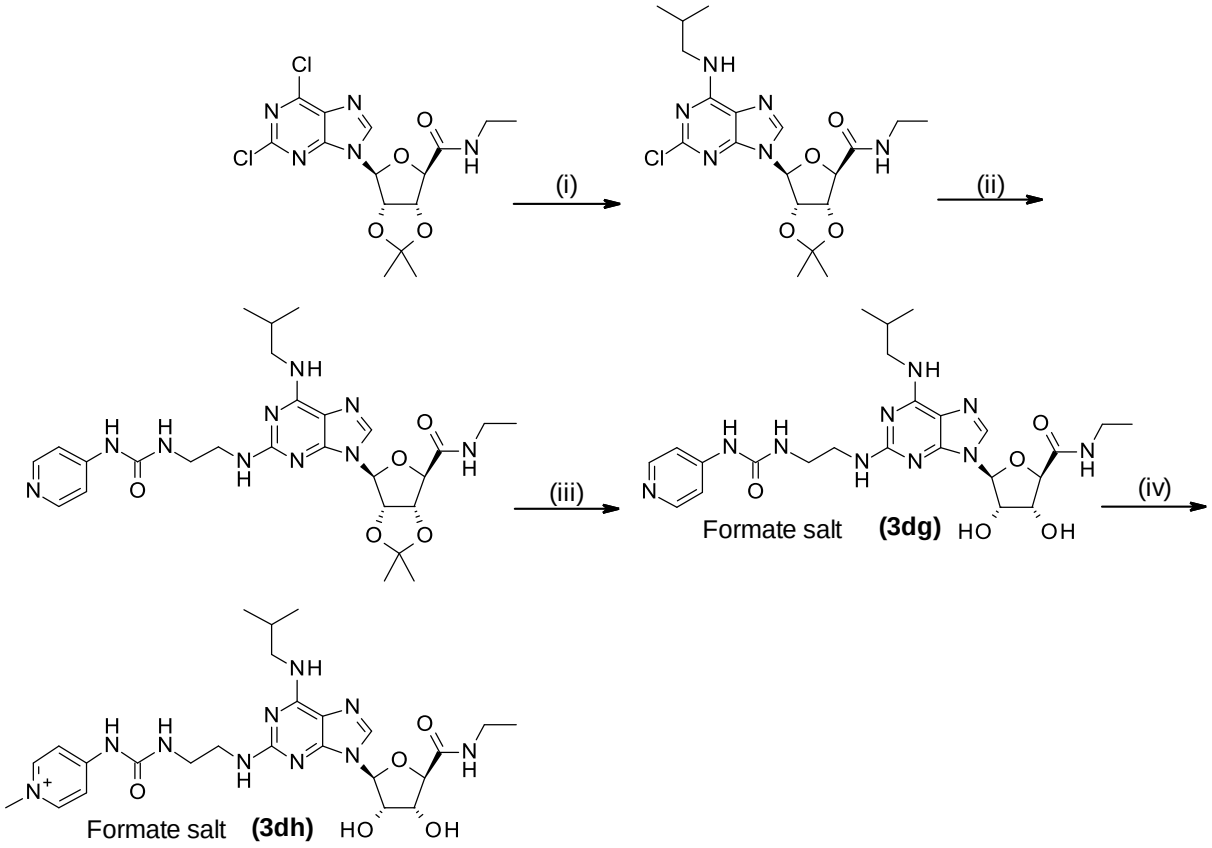
(2*R*,3*R*,4*S*,5*S*)-5-(6-(2,2-diphenylethylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide trifluoroacetate salt

A stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-6-(6-(2,2-diphenylethylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.61 g, 0.86 mmol) in HCl (1 M) (12 mL) was heated at 65°C for 1 h. The mixture was allowed to cool to room temperature then treated with saturated Na_2CO_3 solution until the pH = 7. The mixture was then concentrated *in vacuo* to give a residue which was purified by preparative HPLC to afford (2*R*,3*R*,4*S*,5*S*)-5-(6-(2,2-diphenylethylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide trifluoroacetate salt (0.51 g, 0.65 mmol, 77%) as a white solid.

4-(3-(2-(6-(2,2-diphenylethylamino)-9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-ylamino)ethyl)ureido)-1-methylpyridinium trifluoroacetate salt

A stirred solution of (2*R*,3*R*,4*S*,5*S*)-5-(6-(2,2-diphenylethylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-*N*-ethyl-3,4-dihydroxytetrahydrofuran-2-carboxamide trifluoroacetate salt (0.51 g, 0.65 mmol) in acetonitrile/methanol (20 mL/5 mL) was treated with iodomethane (3 mL, 48.2 mmol), then heated to 35°C. After 20 h the mixture was concentrated *in*

vacuo then purified by preparative HPLC to give 4-(3-(2-(6-(2,2-diphenylethylamino)-9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-9*H*-purin-2-ylamino)ethyl)ureido)-1-methylpyridinium trifluoroacetate salt (0.279 g, 0.551 mmol, 54%) as a white solid.



Supplemental Scheme 5: (i) 2-methylpropan-1-amine, EtOH, r.t., 1 h, 55%; (ii) 1-(2-aminoethyl)-3-(pyridin-4-yl)urea, 130°C, 1 h, 30%; (iii) HCl (1 M), 60°C, 1 h, 43%; (iv) Iodomethane, K_2CO_3, MeOH, 35°C, 20 h, 41% (over 2 steps).

(3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(isobutylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred mixture of (3a*R*,4*R*,6*S*,6a*S*)-6-(2,6-dichloro-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (2.0 g, 4.97 mmol) in ethanol (80 mL) was treated with 2-methylpropan-1-amine (2.47 mL, 24.9 mmol). After 1 h at room temperature the mixture was concentrated *in vacuo* then purified by flash chromatography (silica), eluting with methanol in DCM (3%), to afford (3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(isobutylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (1.20 g, 2.73 mmol, 55%) as a solid.

(3a*R*,4*R*,6*S*,6a*S*)-*N*-ethyl-6-(6-(isobutylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred mixture of (3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(isobutylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.50 g, 1.14 mmol) and 1-(2-aminoethyl)-3-(pyridin-4-yl)urea (1.50 g, 8.32 mmol) was heated at 130°C. After 1 h the mixture was purified by flash chromatography (silica), eluting with methanol in DCM (1%), to give (3a*R*,4*R*,6*S*,6a*S*)-*N*-ethyl-6-(6-(isobutylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.20 g, 0.34 mmol, 30%) as a solid.

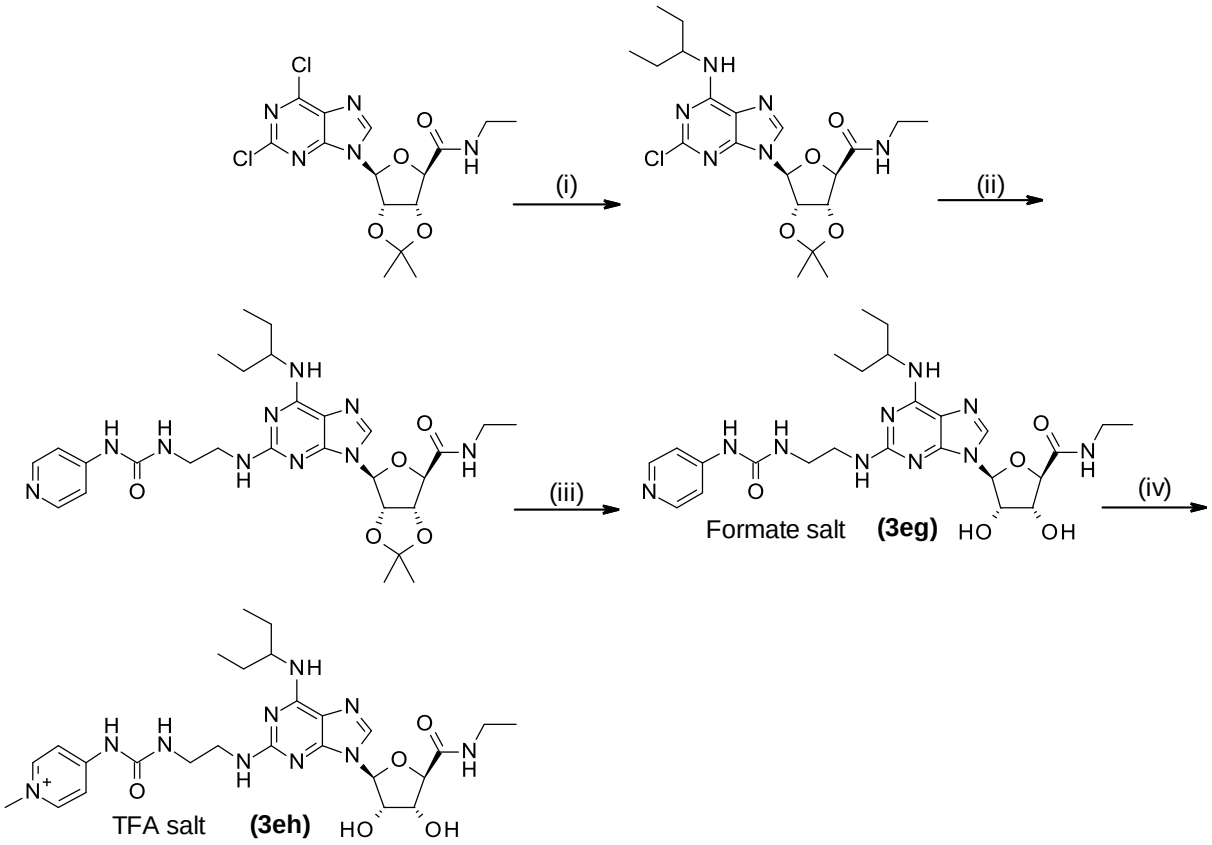
(2*R*,3*R*,4*S*,5*S*)-*N*-ethyl-3,4-dihydroxy-5-(6-(isobutylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)tetrahydrofuran-2-carboxamide formate salt

A stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-*N*-ethyl-6-(6-(isobutylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.065 g, 0.111 mmol) in HCl (1 M) (4 mL) was heated at 60°C. After 1 h the mixture was concentrated *in vacuo* then purified by preparative HPLC (acetonitrile/water/formic acid [0.05%]) to afford (2*R*,3*R*,4*S*,5*S*)-*N*-ethyl-3,4-dihydroxy-5-(6-(isobutylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)tetrahydrofuran-2-carboxamide formate salt (0.028 g, 0.048 mmol, 43%) as a solid.

4-(3-(2-(9-((2S,3S,4R,5R)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-6-(isobutylamino)-9H-purin-2-ylamino)ethyl)ureido)-1-methylpyridinium formate salt

A stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-*N*-ethyl-6-(6-(isobutylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.100 g, 0.171 mmol) in HCl (1 M) (6 mL) was heated at 60°C. After 1 h the mixture was concentrated *in vacuo* then the residue dissolved in methanol (10 mL) and treated with potassium carbonate (0.118 g, 0.855 mmol) and iodomethane (0.242 g, 1.70 mmol). The mixture was heated at 35°C for 20 h then concentrated *in vacuo* and purified by preparative HPLC (acetonitrile/water/formic acid [0.05%]) to afford 4-(3-(2-(9-((2S,3S,4R,5R)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-

2-yl)-6-(isobutylamino)-9H-purin-2-ylamino)ethyl)ureido)-1-methylpyridinium formate salt (0.042 g, 0.070 mmol, 41%) as a light yellow solid.



Supplemental Scheme 6: (i) pentane-3-amine, EtOH, r.t., 2 h, 41%; (ii) 1-(2-aminoethyl)-3-(pyridin-4-yl)urea, 130°C, 1 h, 27%; (iii) HCl (1 M), 60°C, 1 h, 23%; (iv) Iodomethane, K_2CO_3, MeOH, 35°C, 20 h, 26%.

(3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(pentan-3-ylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-6-(2,6-dichloro-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (3.0 g, 7.45 mmol) in ethanol (200 mL) was treated dropwise with pentan-3-amine (2.00 mL, 17.16 mmol). After 2 h at room temperature the mixture was concentrated *in vacuo* then purified by flash chromatography (silica), eluting with methanol in DCM (2%), to afford (3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(pentan-3-ylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (1.40 g, 3.09 mmol, 41%) as a solid.

(3a*R*,4*R*,6*S*,6a*S*)-*N*-ethyl-2,2-dimethyl-6-(6-(pentan-3-ylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)tetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide

A stirred mixture of (3a*R*,4*R*,6*S*,6a*S*)-6-(2-chloro-6-(pentan-3-ylamino)-9*H*-purin-9-yl)-*N*-ethyl-2,2-dimethyltetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.500 g, 1.10 mmol) and 1-(2-aminoethyl)-3-(pyridin-4-yl)urea (1.00 g, 5.55 mmol) was heated at 130°C. After 2.5 h the mixture was allowed to cool to room temperature then purified by flash chromatography (silica), eluting with methanol in DCM (5%), to give (3a*R*,4*R*,6*S*,6a*S*)-*N*-ethyl-2,2-dimethyl-6-(6-(pentan-3-ylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)tetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.27 g, 0.45 mmol, 41%) as a yellow solid.

(2*R*,3*R*,4*S*,5*S*)-*N*-ethyl-3,4-dihydroxy-5-(6-(pentan-3-ylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)tetrahydrofuran-2-carboxamide formate salt

A stirred solution of (3a*R*,4*R*,6*S*,6a*S*)-*N*-ethyl-2,2-dimethyl-6-(6-(pentan-3-ylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)tetrahydrofuro[3,4-*d*][1,3]dioxole-4-carboxamide (0.960 g, 1.61 mmol) in HCl (1 M) (25 mL) was heated at 60°C. After 1 h the mixture was concentrated *in vacuo* then purified by preparative HPLC to afford (2*R*,3*R*,4*S*,5*S*)-*N*-ethyl-3,4-dihydroxy-5-(6-(pentan-3-ylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)tetrahydrofuran-2-carboxamide formate salt (0.220 g, 0.365 mmol, 23%) as a solid.

4-(3-(2-(9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-6-(pentan-3-ylamino)-9*H*-purin-2-ylamino)ethyl)ureido)-1-methylpyridinium trifluoroacetate salt

A stirred mixture of (2*R*,3*R*,4*S*,5*S*)-*N*-ethyl-3,4-dihydroxy-5-(6-(pentan-3-ylamino)-2-(2-(3-pyridin-4-ylureido)ethylamino)-9*H*-purin-9-yl)tetrahydrofuran-2-carboxamide formate salt (0.200 g, 0.332 mmol), potassium carbonate (0.500 g, 3.62 mmol) and iodomethane (5.0 mL, 80.3 mmol) in methanol (20 mL) was heated at 35°C. After 20 h the mixture was concentrated *in vacuo* and purified by preparative HPLC to afford 4-(3-(2-(9-((2*S*,3*S*,4*R*,5*R*)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)-6-(pentan-3-ylamino)-9*H*-purin-2-ylamino)ethyl)ureido)-1-methylpyridinium trifluoroacetate salt (0.060 g, 0.088 mmol, 26%) as a white solid.

Reference List

Palmer TM, Gettys TW, Jacobson KA and Stiles GL (1994) Desensitization of the canine A2a adenosine receptor: delineation of multiple processes. *Molecular pharmacology* **45**(6): 1082-1094.