Surface plasmon resonance screening to identify active and selective adenosine receptor-binding fragments

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Supplementary Materials and Methods

Recombinant expression of wild-type adenosine receptors

Constructs of A_1, A_{2A}, A_{2B} and A_3 receptors were designed with a c-terminal 8-x-HIS or 10-x-HIS and Rho1D4 tag (TETSQVAPA). Codon optimisation for expression in HEK cells, gene synthesis and subcloning into a pcDNA3.1 and pcDNA3.4 vectors were performed by GeneArt® (Life Technologies). Receptors were expressed in Expi293F cells via transient transfection using an ExpiFectamine™293 transfection kit (Life Technologies). Cell count and viability were determined using a cellometer automated cell counter (Nexcelom Bioscience) 48 hours post-transfection. Cells were pelleted by centrifugation at 3,000 x g for 20 minutes at 4 °C and stored at -80 °C.

Purification of wild-type receptors

Frozen cell pellets were resuspended in solubilisation buffer as described previously[24] (50 mM Hepes pH 7.0, 300 mM NaCl, 10 % glycerol, 0.33% DDM, 0.33% CHAPS, 0.07% CHS, 0.33 mM DOPC:DOPS (7:3), plus one complete EDTA-free protease inhibitor tablet (Roche) per 50 mL buffer). Cell suspension was sonicated using a probe sonicator and incubated on a shaker at 4 °C for 2 hours followed by centrifugation at 3,500 x g for 20 minutes. Receptors were purified using standard Rho1D4 protocol from Cube Biotech. Rho1D4 agarose (Cube Biotech, Monheim, Germany) was equilibrated in purification buffer (50 mM Hepes pH 7.0, 300 mM NaCl, 10% glycerol, 0.1% DDM, 0.1% CHAPS, 0.02% CHS). Cell lysate was incubated with Rho1D4 resin on a shaker for 4 hours at 4 °C. The column was washed thoroughly with purification buffer followed by ATP/$MgCl_2$ (5/15 mM) mixture. Bound receptor was eluted by overnight incubation with purification buffer supplemented with 500 µM Rho1D4 peptide (Cube Biotech, Monheim, Germany). Eluted sample was concentrated using an Amicon 10K MWCO concentrator and stored at -80 °C.

SPR assay

Purified wild-type A_{2A}R was captured via the His tag on a Biacore Series S nickel-NTA sensor chip in running buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 50 µM EDTA, 0.1% DDM, 0.1% CHAPS, 0.02% CHS, 3% DMSO), to obtain capture levels 5,000 – 6,000 RU. Analysis temperature was set to 10 °C and the sample compartment to 15 °C to maintain stability of the receptor on the surface. To increase the stability of the receptor, hA_{2A}R was crosslinked to the surface by a 15 min injection of 1:1 ratio of 400 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 100 mM N-hydroxy succinimide (NHS) prior to the capture of A_{2A} onto activated Ni^{2+} surface. To confirm receptor activity,

binding of an agonist (adenosine) and antagonist (ZM241385) were measured by injecting ligands over the surface at 30 µL/min for 60 s and allowing to dissociate for 180 s. Adenosine was injected from 7.8 nM – 1 µM and ZM 241385 was injected in concentration series from 91.4 pM – 200 nM. To confirm assay sensitivity, tool fragments theophylline, caffeine and allopurinol were injected in concentration series from 0.823 – 200 µM. Theophylline and sulpiride were injected at 100 µM concentrations at 60-minute intervals for 35 hours to test stability of captured vs. capture-coupled receptor.

A library of 656 fragments was screened using Biacore S200 at 50 µM concentration in a running buffer composed of 50 mM Hepes pH 7.0, 150 mM NaCl, 50 µM EDTA, 0.1% DDM, 0.1% CHAPS, 0.02% CHS, 3% DMSO against the wild-type hA_{2A}R, blank surface and two additional reference receptors at a flow rate of 30 µL/min. Association was measured for 30 s and dissociation for 60 s. Theophylline was injected at 100 µM throughout the screen to determine receptor activity. To identify hits, sensorgrams were compared against all three surfaces (A_{2A}R, receptor 1 and receptor 2). Seventy-two compounds were then selected as potential hits and screened in concentration series from 0.586 – 75 µM.

Fragment hits confirmation and adenosine receptor selectivity assay

Purified wild-type A_1, A_{2A}, A_{2B} and A_3 were captured via His-tag on an NTA sensor chip surface in running buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 50 µM EDTA, 0.1% DDM, 0.1% CHAPS, 0.02% CHS (-CHS for A_1 receptor), 3% DMSO), to obtain capture levels 4,000 – 6,000 RU for A_{2A}, A_{2B} and A_3 and ~ 10,000 RU for A_1 receptors. Activity of captured receptors was assessed using control compounds SLV320 (A_1 receptor), CV1808 (A_{2A} receptor), LUF5834 (A_{2B} receptor) and adenosine (A_3 receptor) at 3-fold concentration series ranging from 4.57 nM - 10 µM for A_1, A_{2A} and A_{2B} and 0.0457 – 100 µM for A_3 compounds. Selected A_{2A} receptor fragment hits were screened in 3-fold concentration series with top concentrations ranging from 333 nM - 10 µM depending on compound affinity. Flow rate was set to 30 µL/min. Association and dissociation was measured for 60 s and 60 – 180 s, respectively. All experiments were run at 10 °C.

Kinase set screen and hit selectivity

Adenosine A_{2A} and a reference receptor were captured on an activated NTA surface of a Biacore T200 as described above. The GSK kinase set was screened at three concentrations for each compound (0.8, 4 and 20 µM) at flow rate 30 µL/min. Association and dissociation for each injection was measured

for 60 s and 90 s respectively. The selectivity assays of selected hits for A_1, A_{2A}, A_{2B} and A_3 receptors were run under the same buffer conditions as described above. Each compound was screened at 3-fold concentration series with top concentrations ranging from 500 nM to 10 μM depending on the compound affinity.

Live-cell pharmacology materials

Tissue culture and transfection techniques, live cell pharmacology buffers and reagents are described in full in supplemental methods.

Live-cell pharmacology tissue culture

HEK293T cells obtained from American Type Culture Collection were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% Pen-Strep at 37 °C, 5% CO_2. Cells were split every 2 – 3 days and were harvested at 60 – 70% confluency for transfections. Prior to transfection, 7 million cells were seeded into 10 cm cell culture dishes. Following a 5 – 7 h incubation, cells were transfected with either 0.25 μg of a plasmid encoding the hA_{2A} receptor and 4 μg of the GloSensor-22F plasmid or 1 μg of the A_{2A} receptor and 1 μg of the $G\alpha_S$ tricistronic TRUPATH plasmid. Transfections were performed with TransIT 2020 (Mirus Biosciences) as per manufacturer's protocol. The following day, cells were transferred to Poly-L-Lysine (PLK) coated 384- or 96-well white clear-bottom assay plates (Greiner) at densities of 20 – 25K cells and 60 – 65 K cells, respectively, in DMEM containing 1% dialysed FBS, 2 mM L-Glutamine and 1 % Pen-Strep, (40 μL or 100 μL, respectively). Live-cell assays were performed the following day. Live-cell pharmacology experiments utilised a plasmid encoding the human A_{2A} receptor with an N-terminal HA signal peptide under control of the CMV promoter, synthesised by Twist Biosciences (San Francisco, CA). cAMP accumulation experiments utilised the Promega Glosensor system. TRUPATH experiments utilised a tricistronic vector encoding human Gβ3-T2A-Gγ9-GFP2-IRES-GαsS-123-rLuc8 under control of the CMV promoter and was a generous gift from Bryan Roth's laboratory at University of North Carolina at Chapel Hill (UNC). Bovine Serum Albumin Fraction V fatty acid free (BSA), L-ascorbic acid and the Adenosine Deaminase (ADA) from bovine spleen were purchased from Sigma Aldrich. NECA, and ZM 241385 were purchased from Tocris Bioscience. The GloSensor™ cAMP assay reagent was purchased from Promega. BRET2 reagent Prolume Purple was purchased from Nanolight Technology (Arizona). For all live-cell experiments, compounds were solubilised in DMSO at 10 mM and were then diluted in 1x HBSS, 20 mM HEPES at pH 7.4 (assay buffer) to a final assay concentration

of 0.3% DMSO, which was supplemented with 1.0 mg/mL of BSA and 0.1 mg/mL L-ascorbic acid (drug buffer). All drug-plates were prepared using an Echo® Acoustic Liquid Handler. Drugs were resuspended by manual addition of drug buffer.

GloSensor cAMP accumulation assay

Cell culture media was discarded from assay plates by a manual shake-out and cells were incubated for 1 h at room temperature with 20 µL/well of assay buffer containing 2 % GloSensor substrate (Promega) and 2 $U.mL^{-1}$ of ADA. Test compounds were then added to assay plates at 3x their final concentration at a final assay volume of 30 µL. Assay plates were read on a Pherastar FSX using the Lum Plus filterset. Measurement values for analysis were taken 10 minutes post-test compound addition.

Bioluminescence Resonance Energy Transfer (BRET) TRUPATH assay

Cell culture media was discarded from plates by a manual shake-out, and white backings (Perkin Elmer) were applied to the bottom of the plates before 60 µL of assay buffer containing 7.5 µM Prolume Purple was added to each well. Cells were then treated with 30 µL of 3x the final concentration of the test compounds, ZM 241385 and fragment N, in antagonist mode, whereby test compounds in concentration-response curves were interacted with 500 nM of the reference agonist, NECA, in drug buffer. Assay plates were read on a Pherastar FSX using the BRET2 Plus filterset. Measurement values for analysis were taken 38 minutes post-test compound addition. BRET2 ratios were computed as the ratio of GFP2 (515 – 530 nm) emission to RLuc8 (410 – 80 nm) emission.

Data analysis

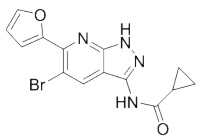
SPR data were referenced against blank surface and blank injections of running buffer. Scrubber 2 software (BioLogic Software) was used to process and analyse the data. To obtain kinetic parameters, data were fitted to a 1:1 binding model including mass transport coefficient. For steady state affinity values, data were fitted to a 1:1 equilibrium model. For slow off rate compounds, such as ZM 241385, the data were fitted using local R_{max} for each concentration.

GraphPad Prism version 9.20 (San Diego, CA) was used for statistical analysis and curve fitting for live-cell pharmacology assays. Agonist and antagonist concentration–response curves were analysed using a four-parameter fit logistic function.

Supplementary Figures

Figure S1. Chemical structures of kinase compounds

SB-739452



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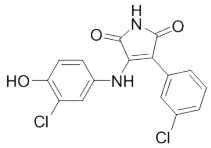

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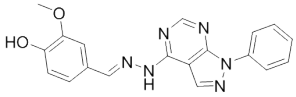

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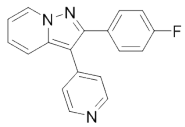


Figure S2. Affinity and kinetic fits for fragments against adenosine receptors. **A**. Affinity fit of fragments binding to the A_1 receptor. **B.** Affinity fit of fragments binding to the A_{2A} receptor. **C.** Affinity fit of fragments binding to the A_{2B} receptor. **D.** Affinity fit of fragments binding to the A_3 receptor. **E.** Kinetic fit for fragment J binding to the A_{2A} receptor. Black traces represent binding sensorgrams, orange traces represent 1:1 kinetic fit. Kinetic parameters were obtained at k_a = 1.03 (± 0.02) x 10^4 $M^{-1}s^{-1}$, k_d = 0.036 (± 0.05) s^{-1} and K_D = 3.45 (± 0.02) μM.

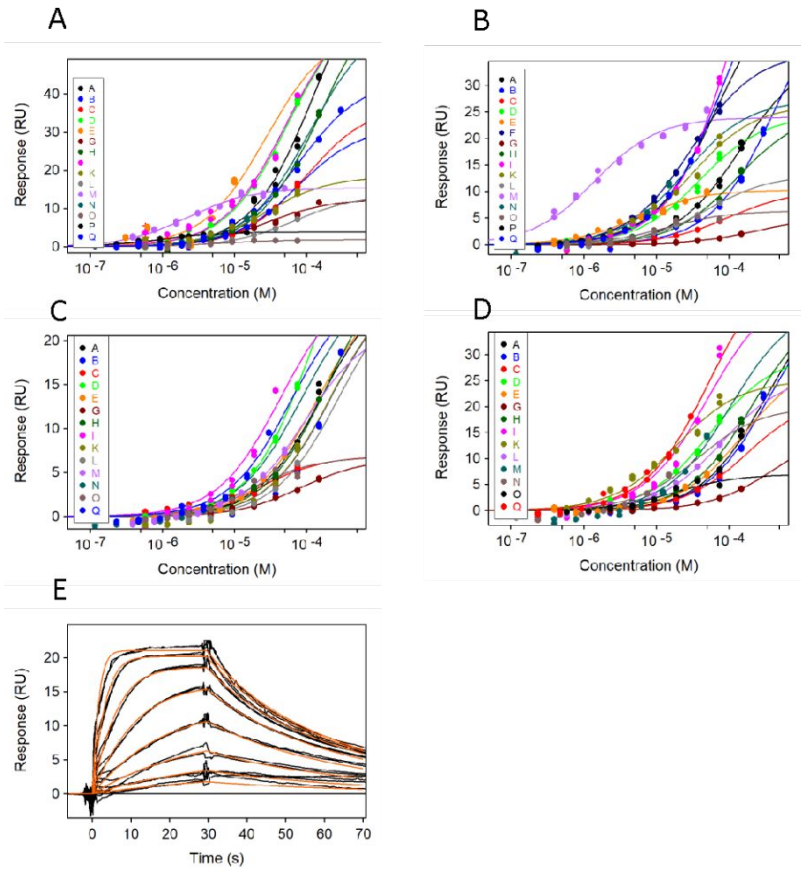
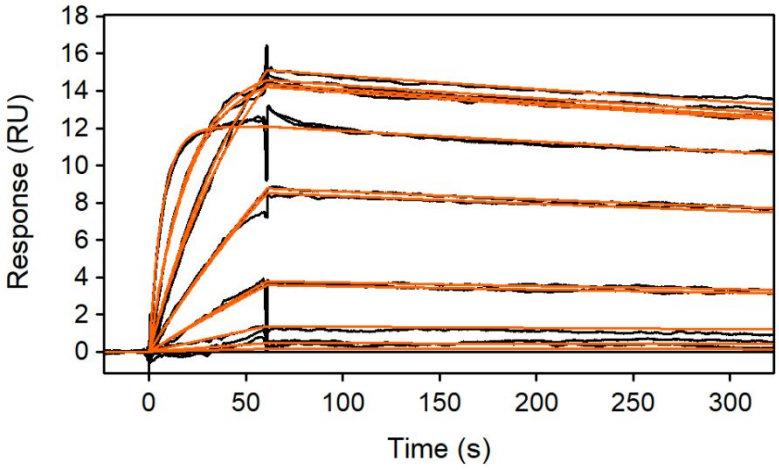


Figure S3. NECA binding to the A_{2A} receptor. NECA was injected in a 3-fold concentration series from 4.6 nM to 10 µM. Black lines represent sensorgrams and orange lines 1:1 kinetic fit.



k_a=1.47(±0.04)x10^4 M^{-1} s^{-1}, k_d=4.96(±0.01) x10^{-4} s^{-1}, K_D=33.81(±0.09) nM

Figure S4. A-B A_{2A} receptor–mediated cAMP accumulation as measured by a GloSensor assay. Ability of the A_{2A} receptor agonist, NECA, **A.** fragment library hits or **B.** kinase library hits to accumulate cAMP as measured by a GloSensor assay in cells transiently expressing $hA_{2A}R$. **C.** Activity of A_{2A} receptor test compounds in cells transiently transfected with pcDNA in a cAMP accumulation GloSensor assay. **D.** Lack of activity of ZM 241385 and fragment D in a control (pcDNA-transfected) Gs TRUPATH assay showing no non-specific activity/interference. Data are expressed as either a percentage of the maximum NECA response as determined at the A_{2A} receptor A-C or as raw BRET values D and presented as mean ± S.E.M. of at least three independent experiments performed in technical duplicate.

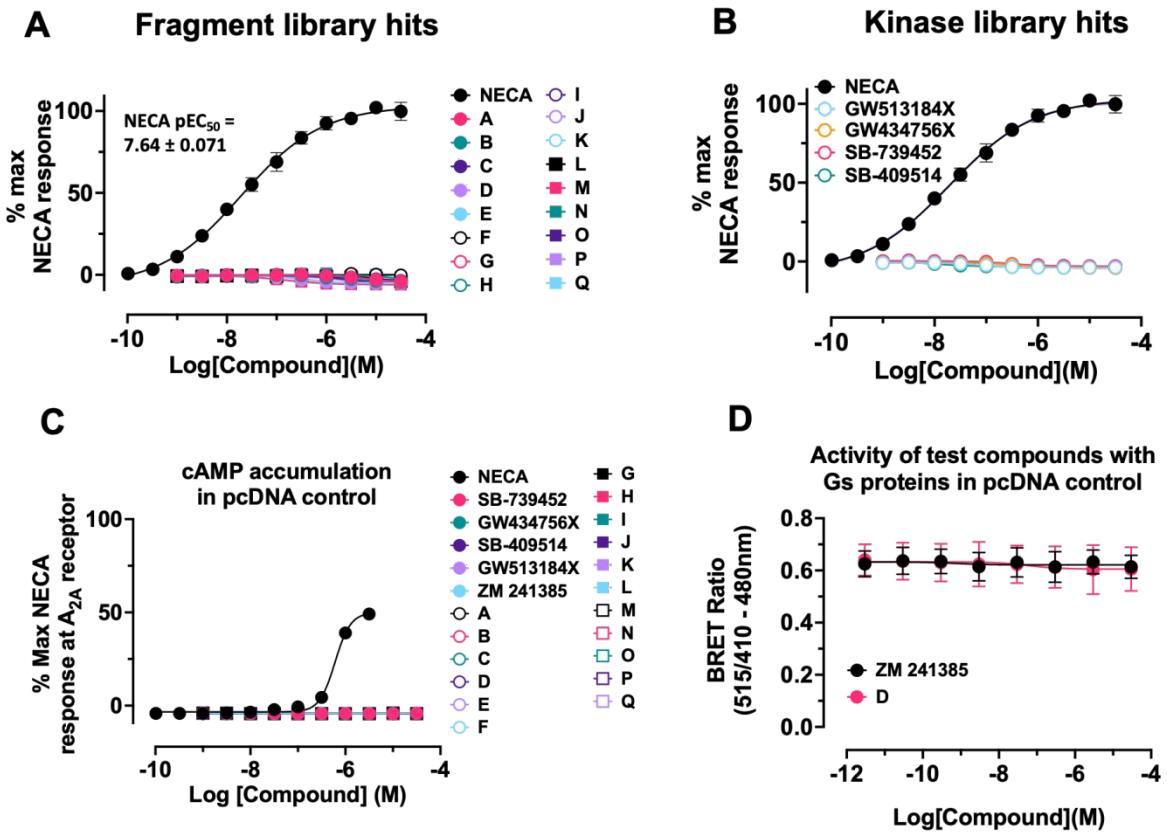
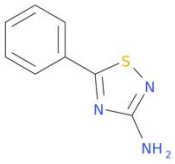
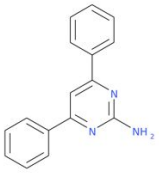
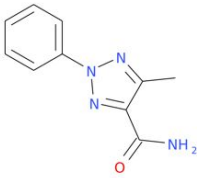
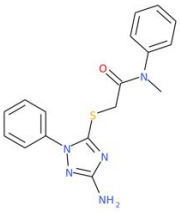
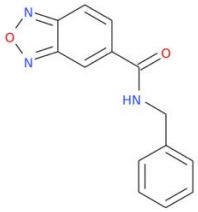
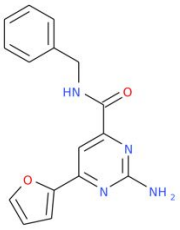
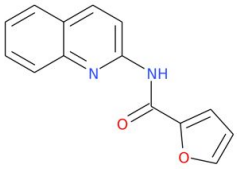
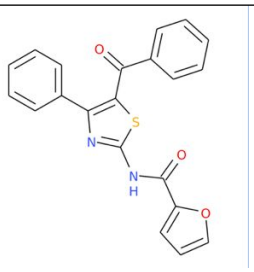
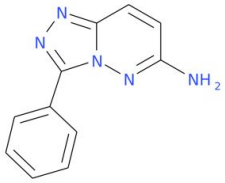
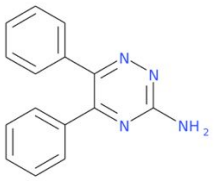
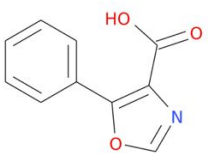
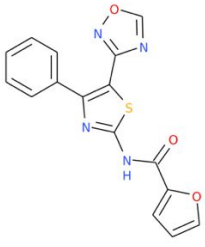
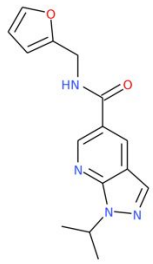
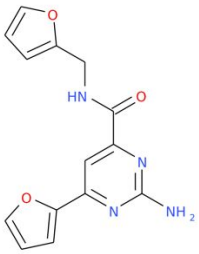
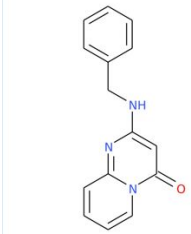
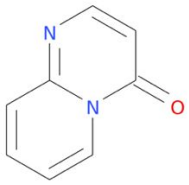
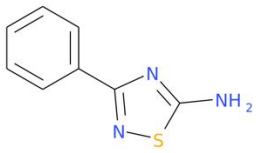
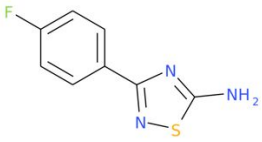
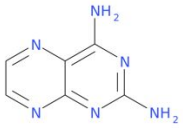
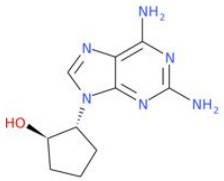
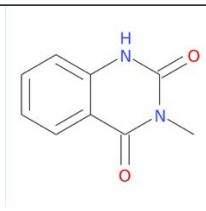
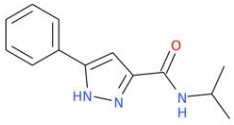
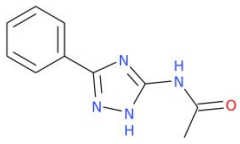


Table S1. Similarity analysis of the structures of fragments A to Q to known adenosine receptor binders reported in the ChEMBL database (www.ebi.ac.uk/chembl).

The search was conducted using Tanimoto similarity of each fragment to the nearest neighbours in the ChEMBL database with reported adenosine receptor binding activity.

Fragment	Structure	Nearest ChEMBL active neighbour Tanimoto Similarity	Structure of Nearest ChEMBL active neighbour	Target	Log Activity (pKi)
A		0.43333		Human Adenosine A1 receptor	7.75
B		0.32075		Human Adenosine A2a receptor	4
C		0.43396		Human Adenosine A2a receptor	8.12
D		0.4717		Human Adenosine A1 receptor	8.24
E		0.33333		Human Adenosine A2a receptor	6.93
F		0.32727		Human Adenosine A1 receptor	6.64

G		0.44262		Human Adenosine A2a receptor	7.85
H		0.37778		Human Adenosine A3 receptor	4.32
I		0.65517		Human Adenosine A2a receptor	7.15
J		0.30233		Rat Adenosine A2a receptor	4.68
K		0.5		Rat Adenosine A1 receptor	5.54
L		0.3913		Human Adenosine A3 receptor	6.52

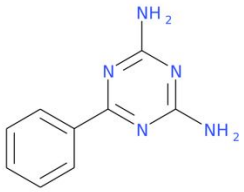
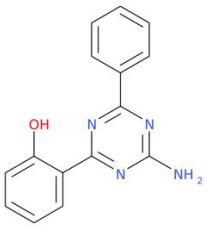
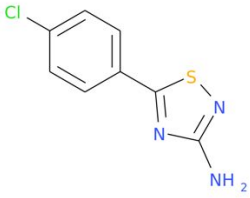
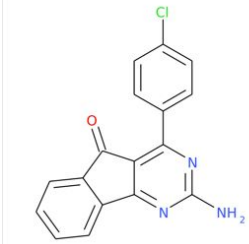
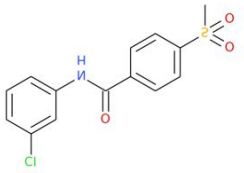
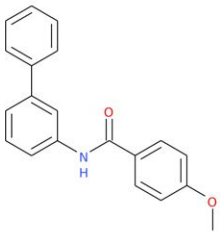
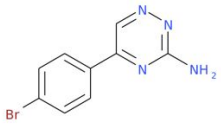
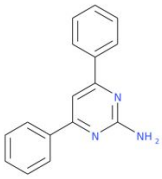
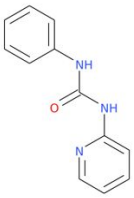
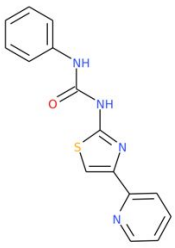
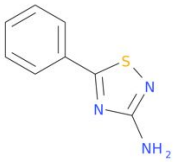
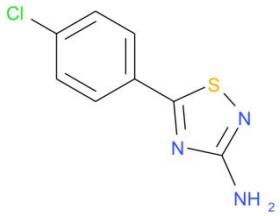
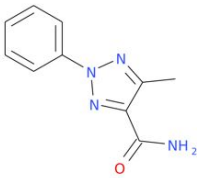
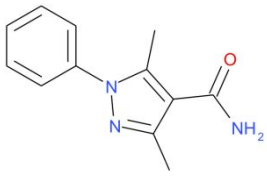
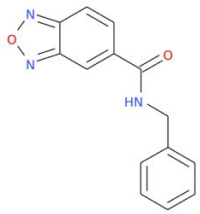
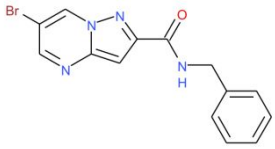
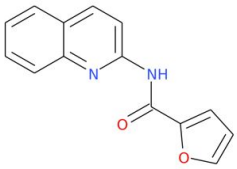
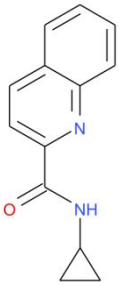
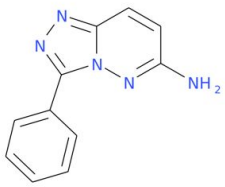
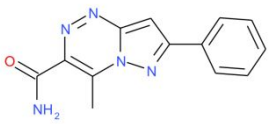
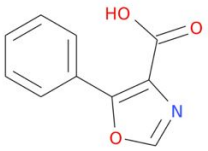
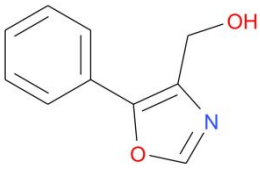
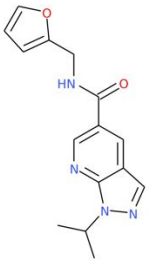
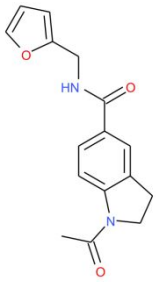
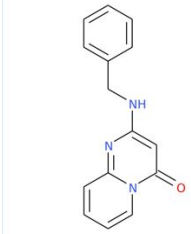
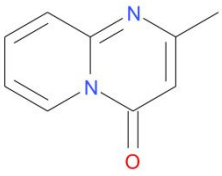
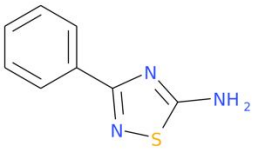
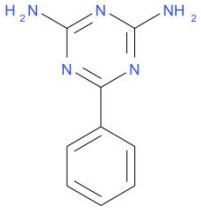
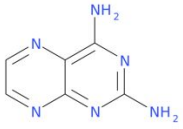
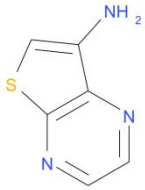
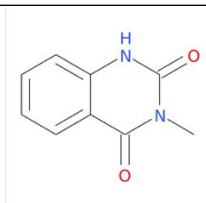
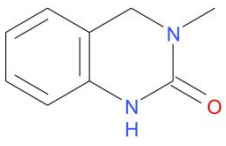
M		0.65385		Human Adenosine A1 receptor	8.14
N		0.35714		Human Adenosine A2a receptor	8.26
O		0.44681		Human Adenosine A3 receptor	5
P		0.35294		Human Adenosine A1 receptor	7.75
Q		0.52381		Rat Adenosine A2a receptor	6.04

Table S2. Similarity analysis of the structures of fragments A to Q to nearest inactive neighbour from the fragment library. The search was conducted using Tanimoto similarity of each fragment to the nearest neighbours.

Fragment	Structure	Nearest library inactive neighbour Tanimoto Similarity	Structure of nearest library inactive neighbour
A		0.65517	
B		0.5	
C		0.40741	
D		0.39583	
E		0.34043	

F		0.51429	
G		0.43077	
H		0.48837	
I		0.51852	
J		0.3125	
K		0.45714	

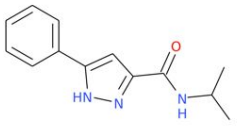
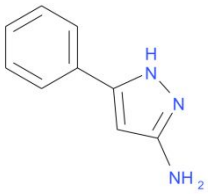
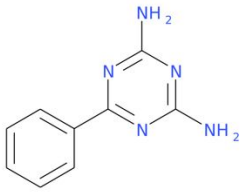
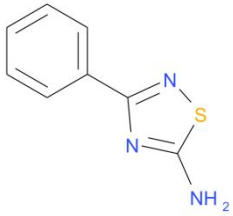
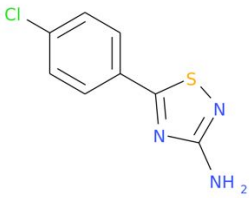
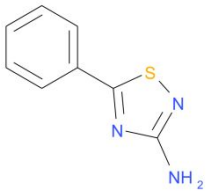
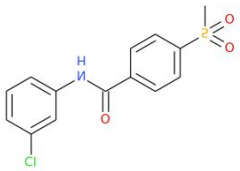
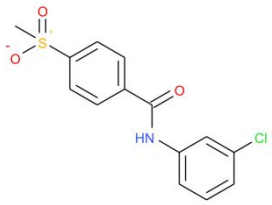
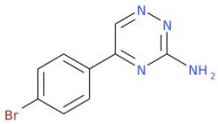
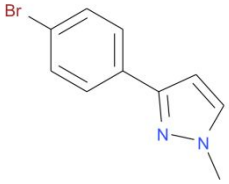
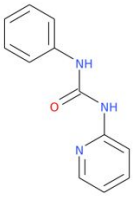
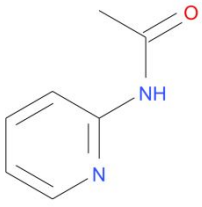
L		0.425	
M		0.51852	
N		0.65517	
O		0.62791	
P		0.38889	
Q		0.59375	

Table S3. Inhibitory potency and efficacy estimates of fragment and kinase hits in a cAMP GloSensor assay at the A2A receptor as determined by a standard four-parameter fit logistic function. Data represent the mean ± S.E.M. of at least three individual experiments performed in duplicate. *value estimates the putative PAM effect (activating potency) of Fragment N on the response of a fixed concentration of the A_{2A} receptor agonist, NECA.

	Inhibitory potency of an EC_{80} of NECA			**Inhibitory potency of an EC_{20} of NECA**		
Compound	**pIC_{50}**	**I_{max}**	**ACT_{max}**	**pIC_{50}**	**I_{max}**	**ACT_{max}**
ZM 241385	8.39 ± 0.80	104 ± 0.82	103 ± 0.21	9.63 ± 0.03	111 ± 0.18	111 + 0.15
A	n.d.	n.d.	n.d.	>31.7	n.d.	64.70 ± 2.36
B	5.83 ± 0.18	-25.3 ± 2.88	9.35 ± 2.14	5.06 + 0.03	105 ± 6.54	91.2 ± 0.65
C	n.d.	n.d.	n.d.	4.91 ± 0.04	74.4 ± 5.01	61.90 ± 1.64
E	>31.7	n.d.	49.2 ± 1.91	6.36 ± 0.02	107 ± 0.60	110 ± 0.12
F	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G	n.d.	n.d.	n.d.	>31.7	n.d.	47.5 ± 2.6**0**
H	n.d.	n.d.	n.d.	>31.7	n.d.	62.9 ± 2.61
I	n.d.	n.d.	n.d.	>31.7	n.d.	99.3 ± 1.19
J	n.d.	n.d.	n.d.	>31.7	n.d.	99.6 ± 1.83
K	n.d.	n.d.	n.d.	>31.7	n.d.	102 ± 1.15
L	>31.7	n.d.	55.5 ± 1.49	>31.7	n.d.	84.9 ± 0.98
M	>31.7	n.d.	40.9 ± 1.34	6.70 ± 0.02	111 ± 0.33	111 ± 0.23
N*	5.83 ± 0.21	-58.9 ± 7.71	12.1 ± 2.26	6.67 ± 0.15	-79.0 ± 11.2	-2.24 ± 2.24
O	>31.7	n.d.	31.1 ± 4.35	>31.7	n.d.	43.8 ± 3.51
P	n.d.	n.d.	n.d.	5.56 ± 0.02	85.5 ± 6.53	80.5 ± 4.44

Q	>31.7	n.d.	56.4 ± 2.83	>31.7	n.d.	53.0 ± 4.07
ZM 241385	8.45 ± 0.10	104 ± 0.90	104 ± 0.13	9.53 ± 0.03	111 ± 0.18	112 ± 1.31
GW513184X	n.d.	n.d.	n.d.	7.20 ± 0.05	109 ± 2.77	100 ± 0.71
SB-739452	n.d.	n.d.	n.d.	6.08 ± 0.01	111 ± 0.87	110 ± 0.20
GW434756X	>31.7	n.d.	57.2 ± 3.71	>0.63	n.d.	72.6 ± 2.41
SB-409514	5.70 ± 0.07	120 ± 6.69	97.7 ± 0.25	7.58 ± 0.01	110 ± 0.94	111 ± 0.31