Identification of Novel Adenosine A$_{2A}$ Receptor Antagonists
by Virtual Screening

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| Compound ID | MW   | MS data | HPLC Purity | NMR data                                                                 |
|------------|------|---------|-------------|--------------------------------------------------------------------------|
|            |      | m/z (ESI+) | m/z (ESI-) |                                                                 |
| 1          | 310.37 | 311.1   | 98%         | ¹H NMR δ (DMSO-d): 13.0 (1H, bs), 8.39 (1H, dd), 8.20 (1H, dd), 7.60 (1H, bs), 7.63 (1H, bs), 7.52 (1H, d), 7.41 (1H, dd), 6.99 (1H, dd), 6.85 (2H, m), 6.58 (1H, d), 2.49 (3H, s) |
| 2          | 222.26 | 223.2   | ≥95%         | H NMR δ (DMSO-d): 11.97 (1H, bs), 6.90 (1H, dd), 6.80-6.37 (3H, m), 5.02 (2H, bs), 4.76 (1H, dd), 3.21 (1H, dd), 2.86 (1H, dd) |
| 3          | 264.34 | 265.1   | 100%         | H NMR δ (DMSO-d): 10.29 (1H, s), 9.34 (1H, s), 7.03 (1H, s), 6.62 (1H, s), 4.54 (2H, s), 3.21 (1H, m), 2.19 (3H, s), 1.22 (6H, d) |
| 4          | 327.4  | 328.1   | 95%          | ¹H NMR δ (DMSO-d): 10.41 (1H, s), 7.31 (5H, m), 4.77 (2H, s), 4.37 (2H, s), 3.32 (m, 5H), 2.45 (m, 2H) |
| 5          | 331.34 | 330.2   | 100%         | ¹H NMR δ (DMSO-d): 9.00 (1H, s), 7.23 (1H, m), 6.78 (1H, d), 6.75 (1H, d), 4.26 (2H, s), 2.58 (3H, s), 2.42 (3H, s) |
| 6          | 367.86 | 368.1   | 98%          | consistent with structure* |
| 7          | 367.37 | 368.2   | 100%         | H NMR δ (DMSO-d): 13.37 (1H, bs), 10.30 (1H, bs), 7.207.06 (6H, m), 6.87 (1H, m), 6.67 (1H, m), 5.83 (1H, s), 3.82 (1H, m), 3.43 (1H, m), 3.26 (3H, s), 2.78 (1H, m) |
| 8          | 340.35 | 341.3   | 100%         | H NMR δ (DMSO-d): 12.21 (1H, bs), 8.32 (1H, m), 7.83 (2H, m), 7.62 (1H, m), 7.38 (1H, s), 7.32 (3H, m), 7.28 (1H, s), 6.86 (1H, s) 3.97 (2H, m), 3.17 (2H, m), 1.92 (2H, m) |
| 9          | 363.41 | 364.3   | 100%         | H NMR δ (DMSO-d): 7.33 (5H, m), 5.90 (2H, m), 5.25 (1H, m), 8.71 (1H, m), 3.22 (2H, m), 2.99 (3H, m), 2.47 (1H, m), 2.31 (3H, m), 1.89 (1H, m), 1.27 (1H, m) |
| 10         | 382.41 | 383.2   | ≥95%         | H NMR δ (DMSO-d): 12.88 (1H, bs), 8.48 (1H, m), 7.51 (2H, m), 7.41 (5H, m), 6.92 (2H, m), 4.49 (1H, m), 3.92 (1H, m), 3.81 (1H, m), 3.74 (3H, s), 3.28 (1H, m), 2.87 (1H, m), 2.62, (2H, m), 1.82 (1H, m) |
| 11         | 315.34 | 316.1   | ≥95%         | ¹H NMR δ (DMSO-d): 9.24 (1H, bs), 7.42 (1H, s), 7.31 (1H, s), 7.13 (1H, s), 2.62 (3H, s), 2.40 (3H, s), 2.27 (3H, s) |
| 12         | 343.4  | 344.2   | 99%          | ¹H NMR δ (DMSO-d): 9.23 (1H, s), 8.08 (1H, s), 7.61 (1H, s), 7.32 (1H, s), 2.63 (2H, m), 2.38 (3H, s), 2.35 (3H, s), 1.52 (2H, m), 0.87 (3H, t) |
| 13         | 387.45 | 388.2   | 100%         | ¹H NMR δ (DMSO-d): 9.23 (1H, s), 7.92 (1H, s), 7.35 (1H, s), 7.23 (1H, s), 4.98 (3H, m), 2.77 (2H, m), 2.40 (3H, s), 1.22 (9H, m) |
| 14         | 230.27 | 231.1   | 100%         | ¹H NMR δ (CHCl₃-d): 13.54 (1 H, s), 8.35 (1 H, dd), 7.41 (1 H, ddd), 7.00-6.87 (2 H, m), 5.48 (2H, s), 3.00-2.89 (1 H, m), 1.33 (6 H, t) |
Chemical Synthesis

Hit compounds 1-10 and follow-up compounds 11-14 were provided by Chembridge, Interchim, Asinex, BioFocus or Interbioscreen. The compounds were supplied with HPLC purities >95%, as determined by the vendors, or Heptares, as delineated in Table S1.

Chemical synthesis and analysis of compounds 15 and 16 were carried out at Oxygen Healthcare. Unless otherwise stated, all reagents were commercially available and were used as supplied, without further purification. \(^1\)H-NMR spectra were recorded at 400 MHz on a Bruker instrument. Chemical shift values are expressed in parts per million, i.e. $\delta$-values. The following abbreviations are used for the multiplicity for the NMR signals: $s$ = singlet, $b$ = broad, $d$ = doublet, $t$ = triplet, $q$ = quartet, $m$ = multiplet. Coupling constants are listed as $J$ values, measured in Hz. Chromatography refers to column chromatography performed using 60 - 120 mesh silica gel and executed under positive pressure (flash chromatography) conditions. Mass spectroscopy was carried out on a Shimadzu LCMS-2010 EV using electrospray ionisation conditions.

LCMS analysis of the final compounds was carried out at Heptares under the following conditions: Instruments: Waters Alliance 2795, Waters 2996 PDA detector, Micromass ZQ. Column: Waters X-Bridge C-18, 2.5micron, 2.1 x 20mm or Phenomenex Gemini-NX C-18, 3 micron, 2.0 x 30mm. Gradient [time (min)/solvent D in C (%)]: 0.00/2, 0.10/2, 8.40/95, 9.40/95, 9.50/2, 10.00/2 (solvent C = 1.58g ammonium formate in 2.5L water + 2.7mL ammonia solution; solvent D = 2.5L Acetonitrile + 132mL (5%) solvent C + 2.7mL ammonia solution). Injection volume 5 uL; UV detection 230 to 400nM; column temperature 45°C; 1.5 mL/min. LCMS purities were measured by diode array detection (AUC) at the specified retention time and were determined to be ≥95% in all cases.
**Typical Procedure for Suzuki Couplings**

A aryl halide derivative (1 eq) and an arylboronic acid derivative (1 eq) were mixed in 1,4-dioxane (10-20 mL per gram of chlorotriazine), and treated with an aqueous solution of Na$_2$CO$_3$ or K$_2$CO$_3$ (2 eq, 10% water v/v). The mixture was then de-gassed, treated with tetrakis triphenylphosphine palladium (5 mol%) at room temperature for 5 mintues, and then heated either conventionally at 70-100 °C, or in a microwave reactor, until the disappearance of starting materials was observed by TLC. The reaction was then cooled to room temperature and quenched with water (1 volume). The target compounds were isolated by extracting the mixture with an organic solvent (3 successive volumes), drying the combined organics over anhydrous sodium sulfate, then removing the solvent *in vacuo* and purifying the product by flash chromatography.

**Preparation of 2-{4-amino-6-[3-(4-methoxypiperidin-1-yl)phenyl]-1,3,5-triazin-2-yl}phenol**

A solution of cyanuric chloride (5.0 g, 27.11 mmol) in THF (20 mL) was cooled to -10 °C. DIPEA (3.5 g, 27.11 mmol) was added drop-wise and the resulting mixture was stirred at -10 °C for 5 min. A solution of ammonia in THF (2.6 M, 35 mL) was then added drop-wise and the reaction was followed by TLC for one hour. After completion, the reaction mixture was concentrated *in vacuo*, diluted with water (50 mL) and extracted with ethyl acetate (3 x 100 mL). The combined organic layers were dried over Na$_2$SO$_4$ and evaporated under reduced pressure. The crude compound was purified by gradient flash chromatography, eluting with up to 20% ethyl acetate in hexane, affording 4,6-dichloro-1,3,5-triazin-2-amine (4.35 g, 78%).

Mass spectroscopy: (ESI +ve) 165 [M]$^+$, (ESI -ve) 163 [M-H]$^-$

4-chloro-6-[3-(4-methoxypiperidin-1-yl)phenyl]-1,3,5-triazin-2-amine (1.60 g, 40%) was prepared from 4,6-dichloro-1,3,5-triazin-2-amine (2.1 g, 12.80 mmol) and 3-(4-methoxypiperidine-1-yl) phenyl boronic acid (33.00 g, 144.81 mmol) according to the typical procedure for Suzuki reactions. Mass spectroscopy: (ESI +ve) 320.1 [M]$^+$
2-{4-amino-6-[3-(4-methoxypiperidin-1-yl)phenyl]-1,3,5-triazin-2-yl}phenol (0.078 g, 4.5%) was prepared from 4-chloro-6-[3-(4-methoxypiperidin-1-yl)phenyl]-1,3,5-triazin-2-amine (1.50 g, 4.69 mmol) and 2-hydroxyphenyl boronic acid (1.10 g, 8.00 mmol) according to the typical procedure for Suzuki reactions. LCMS: 4.56 minutes (100%), m/z 378 [M+H]⁺; ¹H NMR: (400 MHz, d₆-DMSO) δ: 1.50-1.58 (m, 2H), 1.94-1.96 (m, 2H), 2.93-2.99 (m, 2H), 3.35-3.37 (m, 4H), 3.51-3.56 (m, 2H), 6.96-6.98 (m, 2H), 7.19-7.22 (m, 1H), 7.36-7.47 (m, 2H), 7.76-7.78 (d, 1H), 7.96-8.08 (m, 3H), 8.41 (m, 1H) 13.5 (s, 1H).

Preparation of {3-[4-amino-6-(2-phenol)-1,3,5-triazin-2-yl]phenyl}(4-methylpiperazin-1-yl)methanone

A three-necked, round-bottomed flask was charged with toluene (30 mL), 1,3-dibromo benzene (8.2 g, 34.70 mmol) and 4-methylpiperidine (4.9 g, 34.70 mmol). The reaction mixture was stirred for 10 min at RT. BINAP (217 mg, 0.35 mmol) and Pd₂(dba) (106 mg, 0.115 mmol) were then added, and the reaction mixture was stirred for a further 10 min at RT. Following the addition of DBU (4.33 g, 28.5 mmol), the mixture was heated to 60 °C, treated with NaO'Bu (5.0 g, 52.5 mmol) and refluxed overnight. After completion of the reaction (TLC: ethyl acetate/hexane mixtures), the reaction was cooled to RT, quenched by drop-wise addition of water (100 mL) and extracted with ethyl acetate (2 x 250 mL). The combined organics were dried over Na₂SO₄ and concentrated in vacuo, affording crude 1-(3-bromophenyl)-4-methoxypiperidine (5.0 g, 53%) which was used without further purification. Mass spectroscopy: (ESI +ve) 270.9 [M+H]⁺.

A three-necked, round-bottomed flask was charged with 1-(3-bromophenyl)-4-methoxypiperidine (5.0 g, 18.52 mmol), and THF (100 mL) under N₂ atmosphere. The mixture was cooled to -78 °C, treated with n-BuLi (18 mL, 26% solution in hexane, 48 mmol), and stirred for 2 hrs. at -78 °C. Triisopropyl borate (2.93 g, 55 mmol) was then added and the mixture was stirred overnight at RT. After completion (TLC: ethyl acetate/hexane mixtures), the reaction was quenched by drop-wise addition of saturated NH₄Cl solution (40
mL), extracted with ethyl acetate (2 x 100 mL) and the combined organics were dried over Na₂SO₄, concentrated *in vacuo* and triturated with hexane (4 x 50 mL) to afford 4-methoxypiperidin-1-yl)phenylboronic acid (4.0 g, 90%) which was used in the next step without any further purification. Mass spectroscopy: (ESI +ve) 235.9 [M+H]⁺.

4-[2-(benzyloxy)phenyl]-6-chloro-1,3,5-triazin-2-amine (10.85 g, 23%) was prepared from 4,6-dichloro-1,3,5-triazin-2-amine (25.0 g, 152.43 mmol), 2-benzyloxy phenyl boronic acid (33.00 g, 144.81 mmol), Na₂CO₃ solution (40.0 g, 378.78 mmol in 40 mL H₂O) and tetrakis triphenylphosphine palladium (3.50 g, 3.03 mmol) at 70 °C for 3 hrs, according to the typical procedure for performing a Suzuki reaction. Mass spectroscopy: (ESI +ve) 312.9 [M]⁺

{3-[4-amino-6-(2-(benzyloxy)phenyl)-1,3,5-triazin-2-yl]phenyl}(4-methylpiperazin-1-yl)methanone was prepared from 4-[2-(benzyloxy)phenyl]-6-chloro-1,3,5-triazin-2-amine (0.50 g, 1.60 mmol) and 3-[4-(methylpiperazine-1-carbonyl)phenyl]boronic acid hydrochloride (0.67 g, 2.40 mmol) at 90 °C for 16 hours, according to the typical procedure for Suzuki reactions. Crude yield: 1.00 g, 78%; Mass spectroscopy: (ESI +ve) 480.9 [M]⁺.

Debenzylation of {3-[4-amino-6-(2-(benzyloxy)phenyl)-1,3,5-triazin-2-yl]phenyl}(4-methylpiperazin-1-yl)methanone was achieved by dissolving the crude compound (0.10 g, 0.21 mmol) in ethyl acetate (0.5 mL) and treating the resulting solution with palladium hydroxide on carbon (0.10 g, 0.70 mmol) and 1,4-cyclohexadiene (0.17 g, 2.10 mmol) in microwave reactor at 140 °C for 1 hr. The crude mixture was filtered through a hyflow bed and washed with ethyl acetate (25 mL). The filtrate was evaporated under reduced pressure and the product was purified by preparative TLC, affording {3-[4-amino-6-(2-phenol)-1,3,5-triazin-2-yl]phenyl}(4-methylpiperazin-1-yl)methanone (50 mg, 51 %).

LCMS: 3.32 minutes (100%), m/z 391 [M+H]⁺; ¹H NMR: (400 MHz, DMSO) δ: 2.19 (m, 3H), 2.30-2.60 (m, 4H), 3.25 (m, 2H), 3.64 (m, 2H), 6.96 (m, 2H), 7.45 (m, 1H), 7.65 (m, 2H), 8.06 (s, 1H), 8.17 (s, 1H), 8.37 (s, 1H), 8.45 (m, 2H), 13.29 (s, 1H).
Computational Methods

As described in the main text, ligand datasets were drawn from CAP (Chemicals Available for Purchase), a collection of vendor catalogues giving details of screening samples for purchase (http://accelrys.com/products/datasheets/chemicals-available-for-purchase.pdf). A subset of the BioFocus SoftFocus™ library collections were also screened, after excluding compounds designed to target GPCRs. The compounds from CAP were pre-filtered to remove those molecules containing unwanted chemical functionality. Physicochemical profiles for the dataset were biased towards a CNS-like profile and the properties of a set of literature A_{2a} antagonists as indicated in the main text. Up to 545K compounds were prepared for screening and docked into each of the models using the SP algorithm within the Schrodinger Glide software, running on a 28 cpu linux cluster. The total number of compounds screened were: Model 1 (MODELLER) was used to screen 545,410 compounds from CAP and the BioFocus SoftFocus compounds, which met the CNS filters, with Model 2 (MOE) being used to screen 59,245 compounds from CAP and the BioFocus SoftFocus compounds. In the latter case stricter criteria for calculated properties for predicting oral penetration were applied using the StarDrop Oral CNS Profile (http://www.optibrium.com/stardrop.php) and the remaining dataset was then clustered, resulting in the smaller set of molecules. The results were combined and top ranking poses were selected as follows:

Model1 (MODELLER) – 50,000 poses progressed (top ranking ~3%)

Model2 (MOE) – 18,000 poses progressed (top ranking ~10%)

The protein preparation and docking experiments were done within the Schrodinger Maestro package. The grid generation necessary for docking was done within Glide. The residues highlighted in SDM experiments (in-house and external) were used to further define the cavity of the grid. However, no constraints were added in the grid generation to ensure subsequent dockings were not biased in any way. As standard, up to 3 poses per molecular structure were stored for analysis as detailed above. For some compound subsets, Glide XP docking was carried out on the ligands with 10 poses per ligand being stored. A selection of a total of 632 commercial vendor compounds (CAP) and 52 SoftFocus compounds, combined from all screens after further filtering by molecular properties and visual inspection as described in the main text, were initially selected for sourcing and a set of 372 virtual hits was finally prioritised for purchasing, following manual inspection and subsequent triaging by medicinal chemistry of the most promising docking solutions. The overall virtual process is shown schematically in figure S2, with details of the workflows.
used for the docking and analysis shown in figures S3 and S4. 232 compounds were finally screened after logistical criteria (cost and availability etc).

The Table (S1) below indicates the closest published Adenosine A<sub>2A</sub> receptor antagonist in terms of Tanimoto similarity, and calculated logBB values (blood-brain barrier penetration). The similarity analysis was performed by generating a database of published A<sub>2A</sub> antagonists from the publically available ChEMBL data source (https://www.ebi.ac.uk/chembl/) and for each hit molecule querying to identify the most similar molecules reported. The searches were carried out using Pipeline Pilot 8.5 (http://accelrys.com/products/pipeline-pilot/) using SciTegic extended-connectivity functional class fingerprints with a maximum bond distance of 4 from the central atom (FCFP_4). In each case the nearest similar molecule has very low similarity (~0.3 or less) indicating that the virtual screen had been successful in identifying distinct chemotypes as antagonists of the protein target. The logBB prediction was performed using two methods, the first value using the method of Clark<sup>11</sup> (polar surface area and clogP) and the second using StarDrop. Predicted values were not used during the filtering process, the variability between the methods being evident, but taking for example the first method, all values are > -1 and an analysis of 64 CNS drugs by this method showed 41% to be in the -1 to 0 range, with 15% actually between -1.5 to -1.
Table S2. Nearest known Adenosine A\textsubscript{2A} antagonist to each hit molecule by Tanimoto similarity and predicted logBB (blood-brain barrier penetration) by the methods of Clark\textsuperscript{11} and StarDrop.

| Hit | Structure | Similarity to nearest reported adenosine A\textsubscript{2A} inhibitor | Structure | Reference | Predicted logBB (two methods) |
|-----|-----------|-------------------------------------------------|----------|----------|-------------------------------|
| 1   | ![Structure 1](image1.png) | 0.31 | ![Structure 2](image2.png) | 1 | -0.65 \(-0.22\) |
| 2   | ![Structure 3](image3.png) | 0.28 | ![Structure 4](image4.png) | 2 | -0.82 \(-0.49\) |
| 3   | ![Structure 5](image5.png) | 0.19 | ![Structure 6](image6.png) | 3 | -0.48 \(-0.05\) |
| 4   | ![Structure 7](image7.png) | 0.26 | ![Structure 8](image8.png) | 4 | -0.39 \(-0.94\) |
| 5   | ![Structure 9](image9.png) | 0.22 | ![Structure 10](image10.png) | 5 | -0.84 \(-1.20\) |
Figure S1. Concentration-dependent inhibition of specific [\(^3\)H]-ZM241385 binding in HEK293 membranes transiently expressing the human A$_{2A}$ receptor by hits 1-10. Data are from the mean of two independent experiments; open squares represent total binding (0.1% DMSO vehicle control).
Figure S2. Overall workflow used for the A2A virtual screening using the two different homology models and compound sets created from commercially available vendor compounds (CAP) and Biofocus DPI SoftFocus™ compounds. Prior to docking, the tautomers and ionisation states were enumerated using either Pipeline Pilot (model 1 work) or Ligprep (model 2 work). The numbers refer to the datasets after compounds with general unwanted chemical functionality were removed but before this enumeration, so represent the numbers of different compounds screened; the final medicinal chemist check was however more stringent on unwanted chemical functionality. More compounds were chosen based on the Model 2 work than Model 1 (of the 632 CAP compounds selected, 414 were selected from the Model 2 work) as more experimental SDM data was used in the screening and analysis process; an improved ligand preparation protocol was also used. Clustering was done using Pipeline Pilot extended connectivity fingerprints.
Figure S3. Workflow of Docking & Analysis Process 1 used for the A_{2A} virtual screening

Model preparation → Docking validation literature compounds → Screening using Glide SP algorithm: 20,000 poses selected, up to 3 per unique molecule

Selection progressed on score components and consensus scoring: E-model and Glide_score and at least 1 hydrogen bond. Datasets selected on: Asp170 interaction + Volume overlap with buried region of site

7,500 compounds re-scored with Glide XP and filtered as before

Further filtering: Score/molecular weight plotted by molecular weight bins

Manual review of ~2,500 docked poses

Further chemistry triage

Figure S4. Workflow of Docking & Analysis Process 2 used for the A_{2A} virtual screening

Model preparation → Docking validation literature compounds → Screening using Glide SP algorithm: 10,000 poses progressed to analysis, up to 3 per unique molecule

Incorporation of literature and Heptares SDM data and biasing against E2 loop as poorly defined

Compounds selected based on: Glide_score and balanced score components (hydrophilic, lipophilic and strain), distance filters (<6Å) to Leu85, Met270 and His278 and binned score/molecular weight plots

Manual inspection of ~2000 docked poses and chemical inspection of the ligands
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