Discovery of 1,2,4-Triazine Derivatives as Adenosine A\textsubscript{2A} Antagonists using Structure Based Drug Design

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Supporting Information

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### Table S1 - Crystallographic Table of Statistics.

| DATA COLLECTION | $A_{2\alpha}$ StaR2 – 4e | $A_{2\alpha}$ StaR2 – 4g |
|-----------------|--------------------------|--------------------------|
| Space Group     | I222                     | I222                     |
| Cell Dimensions (Å) | 110.70, 112.06, 126.90 | 111.81, 111.89, 125.84 |
| Cell Angles $\alpha$, $\beta$, $\gamma$ (°) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Resolution (Å) | 50.0 – 3.34 | 50.0 – 3.29 |
| $R_{\text{merge}}$ | 0.09 (0.85) | 0.11 (0.96) |
| $I / \sigma I$ * | 8.0 (1.6) | 7.3 (1.6) |
| Completeness (%) | 94.4 (96.1) | 93.6 (88.2) |
| Redundancy | 5.4 (5.5) | 6.1 (6.1) |
| **REFINEMENT** | | |
| Resolution (Å) | 19.80 – 3.34 | 19.93 – 3.29 |
| No. Reflections Working / Test | 10,797 / 511 | 11,568 / 548 |
| $R_{\text{work}} / R_{\text{free}}$ (%) | 28.2 / 29.0 | 29.3 / 33.4 |
| No. atoms | | |
| Protein | 2,250 | 2,250 |
| Ligand | 21 | 21 |
| $B$-factors Å$^2$ | | |
| Protein | 155.9 | 154.9 |
| Ligand | 116.7 | 118.3 |
| R.m.s deviations | | |
| Bond lengths (Å) | 0.002 | 0.002 |
| Bond Angles (°) | 0.392 | 0.466 |
**Chemical Synthesis**

General: 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. δ-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.

The chemical synthesis and analysis described in the experimental section of the main article were carried out by Oxygen Healthcare in India. The chemical syntheses described in this Supporting Information document were performed at Oxygen Healthcare (India), Syngnature Discovery (UK) and Heptares Therapeutics (UK). The stated HPLC purities and retention times were measured at the specified wavelengths under the following conditions: Instrument: Waters Alliance 2695. Column: Sunfire C-18, 250 x 4.6 mm, 5 µm, or equivalent; 1 mL/min flow rate. Gradient [time (min)/% solvent B in A]: 0.00/10, 9.00/90, 11.00/100, 20.00/100, 20.01/10, 25.00/10 (solvent A = 0.1% formic acid in water; solvent B = 0.1% formic acid in acetonitrile). HPLC purities were found to be ≥95% in all cases. Mass spectroscopy was carried out on a Shimadzu LCMS-2010 EV using electrospray ionisation conditions.

The LCMS analysis described in this Supporting Information document was carried out at Heptares Therapeutics 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 times and were determined to be ≥95% in all cases.
**General Procedure for the Bromination of 5-Aryl-1,2,4-Triazin-3-Amine Derivatives**

A solution of a 5-aryl-1,2,4-triazin-3-amine derivative (8.70 mmol) in DMF (15 mL) was cooled to -25 °C and treated with a solution of N-bromosuccinimide (26.6 mmol) in DMF (10 mL) by drop wise addition. The reaction was stirred overnight and monitored by TLC. After completion of the reaction, the mixture was poured into saturated bicarbonate solution (50 mL) and extracted with diethyl ether (25 × 3mL). The organic phases were combined, dried over Na$_2$SO$_4$ and concentrated in vacuo. The crude compound was purified by gradient flash chromatography, to afford the 6-bromo-5-aryl-1,2,4-triazin-3-amine derivative.

The following compounds were prepared according to this procedure:

| Product (yield)                                    | Prepared From                                         | LCMS       | $^1$H NMR                                                                 |
|----------------------------------------------------|-------------------------------------------------------|------------|--------------------------------------------------------------------------|
| 6-Bromo-5-(4-fluorophenyl)-1,2,4-triazin-3-amine (1.95 g, 49%) | 5-(4-fluorophenyl)-1,2,4-triazin-3-amine (2.8 g, 14.0 mmol) | 2.72 mins  | (400 MHz, DMSO) δ: 7.37 (m, 2H), 7.44 (bs, 2H), 7.84 (m, 2H). |
| 6-bromo-5-(3-fluorophenyl)-1,2,4-triazin-3-amine (1.27 g, 45%) | 5-(3-fluorophenyl)-1,2,4-triazin-3-amine (2.00 g, 10.2 mmol) | 2.74 mins  | (400 MHz, DMSO) δ: 7.41 (m, 1H), 7.48 (bs, 2H), 7.56-7.62 (m, 3H). |
| 6-bromo-5-(3,5-difluorophenyl)-1,2,4-triazin-3-amine (6.0 g, 24%) | 5-(3,5-difluorophenyl)-1,2,4-triazin-3-amine (18.0 g, 62.0 mmol) | 3.00 mins  | (400 MHz, DMSO) δ: 7.43-7.54 (m, 3H), 7.62 (bs, 2H). |

**General Procedure for the Preparation of 5,6-Biaryl-3-amino-1,2,4-triazines**

A 6-bromo-5-aryl-1,2,4-triazin-3-amine derivative, (0.80 mmol) was dissolved in dioxane (2.0 mL) and treated with an arylboronic acid (0.92 mmol) and K$_2$CO$_3$ (0.23 g, 1.67 mmol). The resulting mixture was diluted with water (1.0 mL), degassed, treated with tetrakis triphenylphosphine palladium (0.05 g, 0.04 mmol) and stirred in a sealed vessel at 150 °C with monitoring by TLC. Upon completion of the reaction, the mixture was diluted with water (20 mL) and extracted with ethyl acetate (3 x 20 mL); the combined organic extracts were then dried over Na$_2$SO$_4$ and concentrated under reduced pressure. The crude residue was purified by gradient flash chromatography or preparative HPLC.
The following compounds were prepared according to this procedure:

| Table 1 ref | Product (yield) | Prepared From | LCMS | 'H NMR |
|-------------|-----------------|---------------|------|--------|
| 4a | 5,6-Diphenyl-1,2,4-triazin-3-amine (86.0 mg, 42%) | 6-bromo-5-phenyl-1,2,4-triazin-3-amine (0.21 g, 0.8 mmol) and phenyl boronic acid (0.11 g, 0.92 mmol) | 3.17 mins (100%), m/z 249 [M+H]^+ | (400 MHz, CDCl₃) δ: 5.49 (s, 2H), 7.30-7.37 (m, 5H), 7.40-7.43 (m, 3H), 7.45-7.46 (m, 1H), 7.47-7.51 (m, 1H). |
| 4b | 6-(3-Chlorophenyl)-5-phenyl-1,2,4-triazin-3-amine (110 mg, 49%) | 6-bromo-5-phenyl-1,2,4-triazin-3-amine (0.21 g, 0.80 mmol) and 3-chlorophenylboronic acid (0.12 g, 0.80 mmol) | 3.70 mins (100%), m/z 283/285 [M+H]^+ | (400 MHz, CDCl₃) δ: 5.50 (s, 2H), 7.18-7.26 (m, 2H), 7.30-7.36 (m, 3H), 7.42-7.47 (m, 3H), 7.53 (s, 1H). |
| 4c | 6-(3,5-Dichlorophenyl)-5-phenyl-1,2,4-triazin-3-amine (130 mg, 34%) | 6-bromo-5-phenyl-1,2,4-triazin-3-amine (0.30 g, 1.19 mmol) and 3,5-dichlorophenylboronic acid (0.42 g, 2.19 mmol) | 4.28 mins (100%), m/z 317/319/321 [M+H]^+ | (400 MHz, CDCl₃) δ: 5.55 (s, 2H), 7.33 (m, 3H), 7.38 (m, 2H), 7.42 (m, 1H), 7.48 (m, 2H). |
| 4d | 6-(3,5-Dimethylphenyl)-5-phenyl-1,2,4-triazin-3-amine (54 mg, 65%) | 6-bromo-5-phenyl-1,2,4-triazin-3-amine (75 mg, 0.299 mmol) and 3,5-dimethylphenylboronic acid (51.5 mg, 0.344 mmol) | 3.92 mins (100%), m/z 277 [M+H]^+ | (400 MHz, CDCl₃) δ: 2.06 (s, 6H), 5.64 (bs, 2H), 7.00 (m, 3H), 7.31 (m, 2H), 7.42 (m, 1H), 7.48 (m, 2H). |
| 4e | 4-(3-amino-5-phenyl-1,2,4-triazin-6-yl)-2-chlorophenol (26.8 mg, 0.085 mmol, 23%) | 6-bromo-5-phenyl-1,2,4-triazin-3-amine (90 mg, 0.358 mmol) and 3-chloro-4-hydroxyphenylboronic acid (64.9 mg, 0.376 mmol) | 1.07 mins (100%), m/z 299/301 [M+H]^+ | (400 MHz, DMSO) δ: 6.87 (d, J 8.4, 1H), 7.03 (dd, J 8.4, 2.2, 1H), 7.31 (d, J 2.2, 1H), 7.33-7.47 (m, 7H), 10.38 (s, 1H). |
| 4f | 4-(3-amino-5-phenyl-1,2,4-triazin-6-yl)-2,6-dimethylphenol (41.3 mg, 0.141 mmol, 39%) | 6-bromo-5-phenyl-1,2,4-triazin-3-amine (90 mg, 0.358 mmol) and 2,6-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenol (102 mg, 0.412 mmol) | 2.67 mins (98%), m/z 293 [M+H]^+ | (400 MHz, DMSO) δ: 2.06 (s, 6H), 6.87 (s, 2H), 7.31-7.64 (m, 7H), 8.46 (s, 1H). |
| 4h | 6-(2,6-dimethylpyridin-4-yl)-5-(4-fluorophenyl)-1,2,4-triazin-3-amine (23 mg, 23%) | 6-bromo-5-(4-fluorophenyl)-1,2,4-triazin-3-amine (90 mg, 0.334 mmol) and 2,6-dimethylpyridin-4-ylboronic acid (58.1 mg, 0.385 mmol) | 2.67 mins (100%), m/z 296 [M+H]^+ | (400 MHz, DMSO) δ: 2.40 (s, 6H), 7.10 (s, 2H), 7.20 – 7.31 (m, 2H), 7.40 – 7.53 (m, 2H), 7.68 (s, 2H). |
| 4i | 5-(3,5-difluorophenyl)-6-(2,6-dimethylpyridin-4-yl)-1,2,4-triazin-3-amine (50 mg, 49%) | 6-bromo-5-(3,5-difluorophenyl)-1,2,4-triazin-3-amine (90 mg, 0.314 mmol), 2,6-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine (84 mg, 0.361 mmol) | 2.82 mins (100%), m/z 314 [M+H]^+ | (400 MHz, DMSO) δ: 2.37 (s, 6H), 7.01 (s, 2H), 7.04 – 7.16 (m, 2H), 7.38 – 7.44 (m, 1H), 7.70 (s, 2H). |
| 4j | 6-(2,6-dimethylpyridin-4-yl)-5-(3-fluorophenyl)-1,2,4-triazin-3-amine (42 mg, 41%) | 6-bromo-5-(3-fluorophenyl)-1,2,4-triazin-3-amine (90 mg, 0.334 mmol) and 2,6-dimethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine (78 mg, 0.334 mmol) | 2.66 mins (100%), m/z 296 [M+H]^+ | (400 MHz, DMSO) δ: 2.36 (s, 6H), 7.02 (s, 2H), 7.17 (d, J 7.7 Hz, 1H), 7.25 – 7.36 (m, 2H), 7.38 – 7.45 (m, 1H), 7.66 (s, 2H). |
| 4l | 5-(4-fluorophenyl)-6-[2-methyl-6-(trifluoromethyl)pyridin-4-yl]-1,2,4-triazin-3-amine (78.9 mg, 56%) | 6-bromo-5-(4-fluorophenyl)-1,2,4-triazin-3-amine (108 mg, 0.40 mmol) and 2-methyl-6-trifluoromethyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine (121 mg, 0.42 mmol) | 3.58 mins (100%), m/z 350 [M+H]^+ | (400 MHz, CDCl3) δ: 2.61 (s, 3H), 5.58 (bs, 2H), 7.07 – 7.11 (m, 2H), 7.46 – 7.52 (m, 4H). |
Biological Experiments

Receptor Binding

Inhibition binding assays were performed using 2.5 μg of membranes prepared from HEK293 cells transiently transfected with human adenosine A\textsubscript{2A} receptor or 10 μg of membranes prepared from CHO cells stably transfected with human adenosine A\textsubscript{1} receptor. Membranes were incubated in 50 mM Tris-HCl (HEK293-hA\textsubscript{2A}; pH 7.4) or 20 mM HEPES, 100 mM NaCl, 10 mM MgCl\textsubscript{2} (CHO-hA\textsubscript{1}; pH 7.4) in the presence of varying concentrations of test compound and 1 nM \textsuperscript{3}H]ZM241385 (HEK293-hA\textsubscript{2A}) or \textsuperscript{3}H]DPCPX (CHO-hA\textsubscript{1}) at 25°C for 1 h. The assay was then terminated by rapid filtration onto GF/B grade Unifilter plates using a TomTec cell harvester, followed by 5 x 0.5 ml washes with ddH\textsubscript{2}O. Nonspecific binding was defined in the presence of 1 μM CGS15943 (HEK293-hA\textsubscript{2A}) or 1 μM DPCPX (CHO-hA\textsubscript{1}). Bound radioactivity was determined by liquid scintillation counting and inhibition curves were analysed using a four-parameter logistic equation. IC\textsubscript{50} values were converted to K\textsubscript{i} values with the Cheng-Prusoff equation using a K\textsubscript{D} value derived from saturation binding studies.

Catalepsy Studies

Studies were performed by Renasci, Nottingham, UK. Catalepsy was monitored individually in Sprague-Dawley rats (Charles River, UK; 200-250 g; n=8 / group) by gently placing each paw in turn on a large rubber bung. A score of 1 is given for each paw which remains in position for 15 seconds, giving each rat a maximum score of 4. Haloperidol (0.82 mg/kg, i.p.) induces significant cataleptic behaviour in rats. 30 mins later after dosing with haloperidol, animals were treated with 4k (0.3 – 3 mg/kg, p.o.) and after a further 60 mins the catalepsy score was measured above. Rats were re tested 60 minutes later in the same fashion.

In Vitro ADME

Kinetic solubility, rat microsomal stability and plasma protein binding assays were performed by BioFocus (Cambridge, U.K.).
Kinetic solubility.

Using a 10 mM stock solution of each compound in 100% DMSO, dilutions were prepared to a theoretical concentration of 50 µM in both Phosphate Buffered Saline (PBS), pH 7.4 (2% DMSO final) and in 100% DMSO and allowed to equilibrate at room temperature on an orbital shaker for two hours. The PBS dilutions were filtered and filtrate was analysed by LC-UV and LC-MS and compared to the DMSO dilutions to determine solubility.

Microsomal stability.

Test compounds (1 µM) were incubated with pooled microsomes (0.25 mg protein/mL) at 37 ºC for various time points. The reaction was terminated by addition of 100 µL of acetonitrile containing carbamazepine as analytical internal standard. Samples were centrifuged and the supernatant fractions analysed by LC-MS/MS to determine the percentage of compound remaining. Natural log plots of the % remaining were used to determine the half-life of the parent compounds.

Plasma protein binding.

Test compounds (10 µM) were added to rat plasma and dialysed against PBS (pH 7.4) for 6 hours at 37 ºC. After incubation the contents of each plasma and buffer compartment were removed and mixed with equal volumes of control buffer or plasma as appropriate to maintain matrix equivalence for analysis. Plasma proteins were then precipitated by the addition of acetonitrile containing carbamazepine as analytical internal standard, centrifuged and the supernatant removed for analysis by mass spectrometry (LC-MS/MS) to determine the percentage of drug bound.
Computational chemistry.

Homology models were constructed from the avian β₁ adrenergic GPCR crystal structure bound to cyanopindolol (PDB: 2VT4).¹² Owing to the relatively low percentage identity between the two proteins (25% overall, less than 20% around the ligand binding site), two initial homology models of the adenosine A₂A receptor were generated, using different methods (Modeller³ and MOE⁴) as described in main text and proceeding paper, providing a means to assess consistency in the alignments, the variability within the built structures and which regions of the models had higher and lower confidence associated with them. Further validation and improvement of the homology models was done within the Schrodinger Maestro package,⁵ using the induced-fit docking protocol, with an auto generated box size around the residues highlighted by in-house and external SDM as having a large effect on antagonist binding, namely I66².⁶⁴, V84³.³², L85³.³³, E151ECL², L167ECL², E169ECL², N181⁵.⁴², F182⁵.⁴³, H250⁶.⁵², N253⁶.⁵⁵, F257⁶.⁵⁹, Y27¹⁷.³⁶, I27⁴.³⁹ and H27⁸.⁴³. The resultant docking solutions in combination with the binding data from the thermostabilisation experiments were then used to select the in-house round of mutants for our BPM experiments; these mutations were chosen so as to best confirm or rule out differing possible binding orientations within the A₂A receptor.

The final docking experiments were guided by ligand SAR and our iterative process of assessing SDM and then designing our own biophysical mapping (BPM) mutants to confirm or rule out possible binding modes. The protein preparation and docking experiments were done within Maestro and the grid generation necessary for docking within Glide.⁶ The residues highlighted in SDM experiments (in-house and external) were used to 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. Glide XP docking was carried out on all of the ligands in question with 10 poses per ligand being stored. The poses were then assessed against the biophysical mapping data and the best solution identified; The BPM studies have been reported elsewhere and analogues 4e and 4g here equate to examples 3b and 3d in the earlier publication.⁷
Surface Plasmon Resonance Analysis

The assay was run on a Biacore T200 instrument at 25 °C using PBS, 0.05 mM EDTA, 0.1% DDM, 5% DMSO, pH 7.5 as the running buffer. The purified receptor (20 µg/ml in 10 mM MES, 0.1% DDM, pH 6.0) was amine-coupled to sensor chip CM5. Twofold dilution series of each compound (five concentrations) were injected in multicycle or single cycle format. The actual concentrations as well as contact and dissociation times varied between the ligands depending on the kinetics and affinity of interaction. The blank-subtracted data were fitted to 1:1 interaction model to obtain kinetic and affinity constants. With compound 4a, only steady-state affinity data could be obtained due to $k_a$ and $k_d$ falling beyond the instrument detection range. The sensorgrams and fits are shown below.
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