Towards high throughput GPCR crystallography: In Meso soaking of Adenosine A$_{2A}$ Receptor crystals

Prakash Rucktooa$^1$, Robert K. Y. Cheng$^{1,2}$, Elena Segala$^1$, Tian Geng$^1$, James C. Errey$^1$, Giles A. Brown$^1$, Robert M. Cooke$^1$, Fiona H. Marshall$^3$ & Andrew S. Doré$^1$

Here we report an efficient method to generate multiple co-structures of the A$_{2A}$ G protein-coupled receptor (GPCR) with small-molecules from a single preparation of a thermostabilised receptor crystallised in Lipidic Cubic Phase (LCP). Receptor crystallisation is achieved following purification using a low affinity “carrier” ligand (theophylline) and crystals are then soaked in solutions containing the desired (higher affinity) compounds. Complete datasets to high resolution can then be collected from single crystals and seven structures are reported here of which three are novel. The method significantly improves structural throughput for ligand screening using stabilised GPCRs, thereby actively driving Structure-Based Drug Discovery (SBDD).

Many of the world’s top selling drugs target G protein-coupled receptors (GPCRs) for indications including inflammatory, neurological, gastrointestinal, cardiovascular and respiratory diseases. Structural data on this clinically relevant membrane protein superfamily has increased dramatically over the last decade, resulting from pioneering research from a number of groups. High resolution crystal structures are now available for almost all major GPCR classes and are transformative from a pharmaceutical perspective, with several drug candidates generated by structure-based drug design (SBDD) techniques. Nevertheless, GPCR crystallography throughput lags behind that of soluble targets (e.g. kinases), in part due to the inherent conformational flexibility and instability of GPCRs when removed from the native cell membrane environment. To overcome this, receptors have been thermostabilised by introducing a small number of targeted point mutations using the StaR®, SABRE® or CHESS® technologies, or other mutagenesis approaches. These mutations enhance apparent thermostability and stabilise receptors in a specific pre-defined conformation, and detergent-resistant form. Such approaches were instrumental in solving structures of members of class B and C GPCRs. Receptors stabilised using the StaR® technology rely less on stability conferred by high affinity ligands to increase the chance of crystallogenesis. Co-crystal structures are thus obtainable even with low affinity compounds and fragments identified in early stages of discovery projects. This provides a unique opportunity to apply soaking techniques, successfully utilised for soluble targets (e.g. kinases), to GPCR crystals grown in meso by lipidic cubic phase crystallisation (LCP). The reliable production of multiple co-structures on a regular basis, in step with the medicinal chemistry cycle time, is fully enabling for SBDD.

Here, we report an in meso crystal soaking method developed to improve the crystallographic throughput for our work with the adenosine A$_{2A}$ receptor (A$_{2A}$R), including drug discovery activities. Previously, each ligand complex structure required a separate, bespoke A$_{2A}$R-ligand protein preparation. Now a single protein preparation can yield high resolution structural data for A$_{2A}$R in complex with up to a dozen different ligands.

Theophylline binds to the thermostabilised receptor used for crystallisation (A$_{2A}$-StaR2-$b_{562}$), with relatively low affinity ($pK_D = 5.71$), and with fast kinetics, whereas potent A$_{2A}$R-selective antagonists such as 1,2,4-triazine derivatives typically bind with higher affinity ($pK_D > 8$) and exhibit slow off-rates. Despite its low affinity for A$_{2A}$-StaR2-$b_{562}$, theophylline provides some thermostabilisation to the receptor in comparison to apo protein (Fig. 1A). This in meso soaking method uses theophylline as a low affinity carrier ligand, present throughout purification, to provide crystallisation-grade A$_{2A}$-StaR2-$b_{562}$ (Fig. 1B). The A$_{2A}$-StaR2-$b_{562}$-Theophylline complex

---

1Heptares Therapeutics Ltd, BioPark, Broadwater Road, Welwyn Garden City, Hertfordshire, AL7 3AX, UK. 2Present address: LeadXpro, Park InnovAARE, 5232, Villigen, Switzerland. Prakash Rucktooa, Robert K. Y. Cheng and Elena Segala contributed equally to this work. Correspondence and requests for materials should be addressed to F.H.M. (email: fiona.marshall@heptares.com)
readily crystallises in meso yielding thick ~60 µm long plates (Fig. 1C), typically diffracting to 2.0 Å and containing a theophylline molecule in the A2AR orthosteric binding site20 (Fig. 2A). Crystals with theophylline have also been used previously to generate a structure with another xanthine, PSB3620.

The utility of the in meso soaking system for diverse ligands from chemical series other than xanthines was then investigated. A 2A- StaR2- bRIL 562-Theophylline crystals were soaked in mother liquor supplemented with A 2A antagonists Tozadenant21 (pK\textsubscript{D} = 8.4), LUAA4707022 (pK\textsubscript{D} = 6.5) or Vipadenant23 (pK\textsubscript{D} = 9.0), and their diffraction characterised. Crystals from these experiments diffracted in spacegroup C2\textsubscript{2}2\textsubscript{1} to 2.0–3.1 Å resolution (Table 1).

Tozadenant, LUAA47070 and Vipadenant are all well defined in the electron density maps (Fig. 2B–D). For these ligands the basal region of the orthosteric site is delimited by Trp246 6.48, which engages in Van der Waals contacts to the Tozadenant benzothiazole ring, the LUAA47070 thiazole ring or the Vipadenant furan ring (Fig. 2B–D). These ligands explore different regions at the apical end of the orthosteric site. The 4-hydroxy,4-methylpiperidine moiety of Tozadenant sits upright on the benzothiazole ring, and hydrogen bonds to Thr256 6.58. The N,2,2-trimethylpropanamide group of LUAA47070 extends obliquely towards transmembrane helix 1 (TM1), and engages in water-mediated contacts with ECL2 Glu169 and Tyr9 1.35. Further this structure shows how the experimentally defined water mediated interactions of the amide group of LUAA47070 to both Asn253 6.55 and His278 7.42 contribute to this ligand binding pose. Finally, the Vipadenant 2-methylaniline moiety points laterally towards TM1, and is hydrogen-bonded to Tyr9 1.35. We find that, despite adopting a range of orientations in the orthosteric binding site, ligands from different chemical series can be effectively soaked into A2A-StaR2- bRIL 562-Theophylline crystals, and used in crystallographic structural studies to identify their binding modes. Contrary to poorly diffracting, bespoke A2A-StaR2- bRIL 562-Tozadenant crystals, likely resulting from the disruption of the salt bridge between extracellular loop 2 (ECL2) Glu169 and ECL3 His264, interfering with crystal packing, co-crystals from soaking experiments yielded good quality structural data, highlighting the versatility of the in meso soaking system.

The validity of structural results obtained by the in meso soaking method was checked using ZM24138524 (pK\textsubscript{D} = 8.6), a well-characterised A2AR antagonist that increases A2A-StaR2- bRIL 562 stability by ~12 °C (Fig. 1A). The crystal structure of the receptor in complex with ZM241385 resulting from in meso soaking, was compared with similar complexes obtained from bespoke crystallisation setups using either A2A-StaR2- bRIL 56225 or A2A- bRIL 56226 (Fig. 2E). Overlaying these structures shows a remarkably similar structural conformation of residues in the orthosteric located within 5 Å of the ligand with an all atom r.m.s.d. of only 0.074 Å (soaked vs
bespoke \(A_2\alpha\)-StaR2-b\(\text{EIL}\)562 (PDB: 5IU4)) or 0.118 Å (soaked v/s bespoke \(A_2\alpha\)-StaR2-b\(\text{EIL}\)562 (PDB: 4EIY)) (Fig. 2E).

Such a high degree of structural conservation across different crystallisation methods (and \(A_2\alpha\)R constructs) benchmarks and underlines the robustness of the in meso soaking system described here.

To determine the feasibility of using the in meso soaking method system to support optimisation of novel \(A_2\alpha\)R antagonists for drug discovery, Compound 4e, a 1,2,4-triazine derivative, was investigated. Compound 4e is a low nanomolar affinity ligand (\(pK_d = 9.6\)) for \(A_2\alpha\)R and increases \(A_2\alpha\)-StaR2-b\(\text{EIL}\)562 stability by \(\sim 19^\circ \text{C}\) when compared to apo protein (Fig. 1A) and co-crystals were generated using either a bespoke protein preparation or by soaking \(A_2\alpha\)-StaR2-b\(\text{EIL}\)562-Theophylline crystals in mother liquor supplemented with Compound 4e for 1 or 24 hours (Fig. 1D,E). Crystal morphology remained unchanged regardless of soaking times (Fig. 1D,E) and crystals from these three experiments diffracted to 1.9–2.1 Å in spacegroup \(C222_1\). Structures generated from
| Data collection | Compound 4e (1 hour soak) 5OM1 | Compound 4e (24 hour soak) 5OM4 | Compound 4e (Bespoke) SOZ | Tozadenant (24 hour soak) 5OL | Vipadenant (24 hour soak) 5OLH | LUAA47070 (24 hour soak) 5OLV | ZM241385 (24 hour soak) 5OLG |
|----------------|--------------------------------|-------------------------------|-------------------------|-----------------|---------------------|-------------------|-------------------|
| Space group    | C222,                          | C222,                         | C222,                   | C222,           | C222,               | C222,             | C222,             |
| Cell dimensions|                               |                               |                         |                 |                     |                   |                   |
| a, b, c (Å)    | 39.54, 179.85, 140.32          | 39.47, 179.11, 140.03         | 39.37, 179.25, 140.07  | 39.38, 181.10, 141.14 | 39.40, 179.33, 141.14 | 39.43, 180.77, 140.90 | 39.45, 179.39, 139.60 |
| a, b, c (°)    | 90, 90, 90                     | 90, 90, 90                    | 90, 90, 90              | 90, 90, 90      | 90, 90, 90          | 90, 90, 90        | 90, 90, 90        |
| Resolution (Å) | 33.83–2.10 (2.16–2.10)°        | 32.92–2.00 (2.05–2.00)°       | 33.71–1.90 (1.94–1.90)° | 38.48–3.10 (3.21–3.10)° | 29.82–2.60 (2.72–2.60)° | 76.08–2.00 (2.05–2.00)° | 46.53–1.85 (1.89–1.85)° |
| R_{free} (%)   | 0.061 (0.564)                  | 0.059 (0.635)                 | 0.040 (0.583)           | 0.078 (0.559)   | 0.097 (0.547)       | 0.059 (0.624)     | 0.068 (0.935)     |
| R<sub>f</sub>  | 10.0 (1.5)°                    | 10.3 (1.3)°                   | 11.3 (1.4)°             | 8.3 (1.5)°      | 8.3 (1.6)°          | 8.0 (1.3)°        | 7.7 (1.0)°        |
| CC<sub>i,j</sub> | 0.997 (0.524)°                | 0.998 (0.439)°                | 0.999 (0.421)°          | 0.977 (0.480)°  | 0.981 (0.515)°      | 0.957 (0.420)°    | 0.994 (0.372)°    |
| Completeness (%) | 100.0 (100.0)°               | 98.4 (98.8)°                  | 99.3 (99.6)°            | 99.9 (100.0)°   | 99.5 (98.7)°        | 98.5 (94.1)°      | 99.7 (100.0)°     |
| Redundancy     | 6.6 (6.7)°                    | 6.5 (6.5)°                    | 4.7 (4.8)°              | 5.7 (5.9)°      | 5.9 (5.1)°          | 3.5 (3.7)°        | 6.2 (6.4)°        |
| Reflections    | 35.83–2.10                     | 32.92–2.00                    | 33.71–1.90              | 38.48–3.10      | 29.82–2.60          | 41.68–2.00        | 41.31–1.86        |
| No. reflections | 56388                         | 63810                        | 74939                   | 9645            | 15827               | 34169             | 41376             |
| R<sub>merge</sub>/R<sub>free</sub> | 0.1882/0.2097°               | 0.1831/0.2049°               | 0.1727/0.1963          | 0.1987/0.2448   | 0.2000/0.2487       | 0.1801/0.2081     | 0.1921/0.2332     |
| No. atoms      | 3082                          | 3083                         | 3104                    | 2983            | 3047                | 3083              | 3097              |
| Protein        | 21                            | 21                           | 21                      | 21              | 24                  | 24                | 25                |
| Ligand         | 687                           | 727                          | 684                     | 397             | 488                 | 607               | 597               |
| Solvent        |                               |                               |                         |                 |                     |                   |                   |
| R.m.s.d. deviations |                                    |                               |                         |                 |                     |                   |                   |
| Bond lengths (Å) | 0.002                        | 0.003                        | 0.004                   | 0.002           | 0.003               | 0.009             | 0.014             |
| Bond angles (°) | 0.92                         | 0.93                         | 0.99                    | 0.74            | 0.75                | 1.19              | 1.34              |

Table 1. Data collection and refinement statistics. *Values in parentheses are for highest-resolution shell. All data presented above were collected from single crystals except for the A<sub>2A</sub>-StaR2-b<sub>HiL</sub>562-Vipadenant complex, where data was merged from three different crystals.

bespoke crystallisation or from the soaking experiments were essentially equivalent (r.m.s.d. ~0.1 Å over 297 residues). Compound 4e was well defined in electron density maps from the resultant three co-structures and binds in the same orientation in the orthosteric site (Fig. 2F–H), displaying similar β factors (17.8–19.8 Å<sup>2</sup>) (Table 1). Compound 4e sits lower in the orthosteric site than theophylline, with the triazine ring π stacking against Phe168 from ECL2, while also engaging in polar contacts with an extensive water network. The amine moiety on the triazine ring is further hydrogen-bonded to ECL2 Glu169 and Asn253<sup>35,55</sup>, whereas the hydroxyl group on the chlorophenol ring makes a hydrogen bond with His278<sup>7,41</sup>. In the basal region of the orthosteric site, the ligand benzyl ring makes Van der Waals interactions with Trp246<sup>6,48</sup>.

A pairwise comparison of residues located within 5 Å of all the different liganded structures presented here demonstrates all atom r.m.s.d. values ranging from 0.48 Å (between the A<sub>2A</sub>-StaR2-b<sub>HiL</sub>562-Compound 4e and -LUAA47070 structures) to 1.05 Å (between the A<sub>2A</sub>-StaR2-b<sub>HiL</sub>562-ZM241385 and -Tozadenant structures). Altogether, most of the mobility stems from Tyr271<sup>17,38</sup>, involved in water-mediated interactions with ZM241385, and from Glu169 in ECL2 and His264 which adopt different rotamer orientations in the A<sub>2A</sub>-StaR2-b<sub>HiL</sub>562-Tozadenant structure compared to the other ligand complexes.

In drug development, high-throughput X-ray crystallography expedites the elaboration of novel hits into lead compounds and drug candidates by providing multiple high resolution views of ligand-receptor complexes, which are key for understanding critical intermolecular interactions alongside interpretation of ligand-induced receptor conformational changes<sup>37</sup>. The accelerated availability of multiple receptor-ligand complexes provides a data-rich starting point for SBDD and medicinal chemistry<sup>38</sup> which, when correlated with in vitro biological activity, allows rapid incorporation of molecular modifications towards increasing ligand affinity for the binding site or improvement of their absorption, distribution, metabolism, excretion and toxicity (ADMET) properties.

We have demonstrated that an in meso ligand soaking methodology can rapidly and efficiently yield multiple high-resolution co-crystal structures from a diverse set of ligands in complex with a given GPCR. Such soaking techniques have also been employed in-house for other discovery projects. The method described here has general applicability to further discovery campaigns with stabilised membrane proteins using LCP crystallisation setups, provided high quality crystals exist for the target in complex with low affinity stabilising carrier ligands with fast off-rates.

**Methods**

**StaR generation.** The thermostabilisation of the human A<sub>2A</sub> receptor (resulting in A<sub>2A</sub>-StaR2) using a mutagenesis approach<sup>8</sup>, has been previously described<sup>29</sup>.
Expression, membrane preparation and protein purification. The A2A-StaR2-b_{86}562 construct has been described previously\(^2\) and harbours eight thermostabilising mutations (A54L\(^{3,22}\), T88A\(^{3,28}\), R107A\(^{3,25}\), K122A\(^{4,27}\), L202A\(^{4,36}\), L235A\(^{4,27}\), V239A\(^{4,41}\) and S277A\(^{7,42}\)) as well as a mutation to remove a glycosylation site (N154A). The construct further comprises an Apo CONTRIBUTORS: b_{562} fusion between transmembrane helices 5 and 6 and a C-terminal decahistidine tag. The receptor was expressed using the Bac to Bac Expression System (Invitrogen) in Trichoplusia ni Tni PRO cells using ESF 921 medium (Expression Systems) supplemented with 5% (v/v) fetal bovine serum (Sigma-Aldrich) and 1% (v/v) Penicillin/Streptomycin (PAA Laboratories). Cells were infected at a density of 2.6 × 10^6 cells/ml with virus at an approximate multiplicity of infection of 1. Cultures were grown at 27°C with constant shaking and harvested by centrifugation 48 hours post infection. All subsequent protein purification steps were carried out at 4°C unless otherwise stated.

For each protein preparation, cells from 2 L cultures were resuspended in 40 mM TRIS buffer at pH 7.6 supplemented with 1 mM EDTA and Complete EDTA-free protease inhibitor cocktail tablets (Roche). Cells were disrupted at ~15 000 psi using a microfluidizer (Processor M-110L Pneumatic, Microfluidics). Membranes pelleted by ultra-centrifugation at 200 000 g for 50 minutes, were subjected to a high salt wash in a buffer containing 40 mM Tris pH 7.6, 1 M NaCl and Complete EDTA-free protease inhibitor cocktail tablets, before they were centrifuged at 200,000 g for 50 minutes. Washed membranes were resuspended in 50 mM 40 Tris pH 7.6 supplemented with Complete EDTA-free protease inhibitor cocktail tablets and stored at −80°C until further use.

Protein preparations intended for soaking experiments were carried out in the presence of theophylline whereas the bespoke preparation of A2A-StaR2-b_{86}562 in complex with Compound 4e was done in the presence of 5 µM ligand.

Membranes were thawed, resuspended in a total volume of 150 ml with 40 mM Tris–HCl pH 7.6, Complete EDTA-free protease inhibitor cocktail tablets (Roche), 3 mM theophylline (Sigma-Aldrich) (or 5 µM Compound 4e), and incubated for 2 hours at room temperature. Membranes were then solubilized by addition of 1.5% n-Decyl-β-D-maltopyranoside (DM, Anatrace), and incubation for 2 hours at 4°C, followed by centrifugation at 145 000 g for 60 min to harvest solubilised material.

The solubilised material was applied to a 5 ml Ni-NTA (nickel-nitrilotriacetic acid) Superflow cartridge (Qiagen) pre-equilibrated in 40 mM TRIS buffer pH 7.4, 200 mM NaCl, 0.15% DM, 1 mM theophylline (or 5 µM Compound 4e), and the column was washed with 25 column volumes of buffer 40 mM Tris pH 7.4, 200 mM NaCl, 0.15% DM, 70 mM imidazole, 1 mM theophylline (or 5 µM Compound 4e), and then the protein was eluted with 40 mM Tris pH 7.4, 200 mM NaCl, 0.15% DM, 280 mM imidazole, 1 mM theophylline (or 5 µM Compound 4e).

Collected fractions were analyzed by SDS PAGE and fractions containing A2A-StaR2-b_{86}562 were pooled and concentrated using an Amicon Ultra Ultracell 50 K ultrafiltration membrane to a final volume of ~800 µl. The protein sample was ultra-centrifuged at 436 000 g for 10 minutes before being applied to a Superdex200 size exclusion column (GE Healthcare) pre-equilibrated with 40 mM Tris pH 7.4, 200 mM NaCl, 0.15% DM, 1 mM theophylline (or 5 µM Compound 4e). Eluted fractions containing the protein were analyzed by SDS PAGE, pooled and concentrated to ~35 mg/ml using an Amicon Ultra Ultracell 50 K ultrafiltration membrane and subjected to a ultra-centrifugation at 436 000 g prior to crystallisation. Protein concentrations were measured using the DC assay (Bio-Rad), and confirmed using quantitative amino acid analysis.

Thermal unfolding experiments. A2A-StaR2-b_{86}562 purified in DM in the presence of 500 µM theophylline was used for thermal unfolding experiments. The protein was diluted in 40 mM Tris pH 7.4, 200 mM NaCl, 0.15% DM to a final concentration of 0.2 mg/ml. Following heavy dilution (~70-fold) of the protein in a buffer without ligand, the sample was considered to be in an apo-like state. Samples were supplemented with the respective ligands to a final concentration of 50 µM, with a final DMSO concentration of 5% (v/v). The control sample was supplemented with DMSO to a final concentration of 5% (v/v). Samples were incubated 30 minutes on ice before being applied to a Superdex200 size exclusion column (GE Healthcare) pre-equilibrated with 40 mM Tris pH 7.4, 200 mM NaCl, 0.15% DM, 1 mM theophylline (or 5 µM Compound 4e). The solubilised protein sample was ultra-centrifuged at 436 000 g for 10 minutes before being applied to a Superdex200 size exclusion column (GE Healthcare) pre-equilibrated with 40 mM Tris pH 7.4, 200 mM NaCl, 0.15% DM, 1 mM theophylline (or 5 µM Compound 4e).

The A2A-StaR2-b_{86}562 in complex with either theophylline or Compound 4e was crystallized in lipidic cubic phase at 20°C. Concentrated protein was mixed with monoolein (Nu-Chek) supplemented with 10% (w/w) cholesterol (Sigma Aldrich) and 10 µM theophylline (or 5 µM Compound 4e) using the twin-syringe method\(^9\). The final protein:lipid ratio was 40:60 (w/w). 40 ml boli were dispensed onto 96-well Laminex Glass Bases (Molecular Dimensions Ltd) using a Mosquito LCP crystallization robot (TTP Labtech) and overlaid with 800 nl precipitant solution. Glass bases were sealed using Laminex Film covers (Molecular Dimensions Ltd). 60–80 µm long plate-shaped crystals grew within 2 weeks in 0.1 M tri-sodium citrate pH 5.3–5.4, 0.05 M sodium thiocyanate, 29–32% PEG400, 2% (v/v) 2,5-hexanediol and 0.5 mM theophylline (or 5 µM Compound 4e).

In meso soaking and crystal harvesting. For soaking experiments, incisions were made into the Laminex cover over base wells containing crystals identified for harvesting and these wells were flooded with 10 µl motherliquor supplemented by 1 mM ligand. The crystals are soaked in motherliquor with a final ligand concentration of 925 µM, and a final theophylline concentration of 74 µM. Flooded wells were then re-sealed using Crystal Clear Sealing Tape (Hampton Research), and plates were incubated for 1 hour or 24 hours at 20°C. Single crystals were mounted in LithoLoops (Molecular Dimensions Ltd) and flash-frozen in liquid nitrogen without the addition of further cryoprotectant.
Diffraction data collection and processing. X-ray diffraction data were measured on a Pilatus 6 M detector at beamline 124 (Diamond Light Source) using a 6 × 9 μm beam size of for crystals of A_{2A}-StaR2-b_{RIL}562 in complex with Compound 4e, Tozadenant or LUAA47070. Complete datasets were acquired from a single crystal for each of these complexes at wavelengths 0.96857 Å (Compound 4e and LUAA47070) or 0.96862 Å (Tozadenant), using an unattenuated beam and 0.2° oscillation per frame, with an exposure of 0.1 second per degree of oscillation. Diffraction data for the A_{2A}-StaR2-b_{RIL}562-Vipadenant complex were acquired from 3 different crystals on an Eiger 16 M detector at beamline X06SA (Swiss Light Source) at a wavelength of 1 Å, using 10% beam transmission and 0.1° oscillation per frame, with an exposure of 1 second per degree of oscillation. The A_{2A}-StaR2-b_{RIL}562-ZM241385 data was collected from a single crystal on an Eiger 16 M detector at beamline ×06SA at a wavelength of 1 Å, using 20% beam transmission and 0.25° oscillation per frame, with an exposure of 0.24 second per degree of oscillation. Data from individual crystals were integrated using XDS53, merged and scaled using AIMLESS55 from the CCP4 suite51. Data collection statistics are reported in Table 1.

Structure solution and refinement. The structures of the different A_{2A}-StaR2-b_{RIL}562-ligand complexes were solved by molecular replacement (MR) with Phaser34 using the A_{2A}-StaR2-b_{RIL}562-theophylline complex structure39 as the search model (PDB code: 5MZJ). Iterative rounds of model refinement performed using phenix.refine36, were interspersed with manual model building in COOT56. Both xray and B-factor restraint weights were optimised in phenix.refine, and 2 TLS groups corresponding to the receptor and to the b_{RIL}562 respectively were defined during refinement. Refinement was with positional and individual isotropic B-factor refinement. The final models were validated using MolProbity37. The final refinement statistics are presented in Table 1. Structure figures were generated using PyMOL38. The three structures of the A_{2A}-StaR2-b_{RIL}562-tozadenant complex reported here generated from crystals grown using LCP, are comparable with the previously reported A_{2A}-StaR2-Compound 4e structure (PDB: 3UZC)39 solved from crystals grown using the vapour diffusion technique, with an all atom r.m.s.d. of 0.91 Å over 276 residues.

Data Availability Statement. The data that support the findings of this study are available from the corresponding author upon reasonable request. Co-ordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 5OM1, 5OM4, 5OLZ, 5OLV, 5OLO, 5OLH and 5OLG.

References
1. Cooke, R. M., Brown, A. J., Marshall, F. H. & Mason, J. S. Structures of G protein–coupled receptors reveal new opportunities for drug discovery. Drug discovery today 20, 1355–1364, https://doi.org/10.1016/j.drudis.2015.08.003 (2015).
2. Jazayeri, A. et al. Extra-helical binding site of a glucagon receptor antagonist. Nature 533, 274–277, https://doi.org/10.1038/nature17414 (2016).
3. Stevens, R. C. et al. The GPCR Network: a large-scale collaboration to determine human GPCR structure and function. Nature reviews. Drug discovery 12, 25–34, https://doi.org/10.1038/nrd3859 (2013).
4. Xiang, J. et al. Successful Strategies to Determine High-Resolution Structures of GPCRs. Trends in pharmacological sciences 37, 1055–1069, https://doi.org/10.1016/j.tips.2016.09.009 (2016).
5. Congreve, M., Dias, J. M. & Marshall, F. H. Structure-based drug design for G protein-coupled receptors. Progress in medicinal chemistry 53, 1–63, https://doi.org/10.1016/j.978-0-44-63380-4.00001-9 (2014).
6. Shoichet, B. K. & Kobilka, B. K. Structure-based drug screening for G-protein-coupled receptors. Trends in pharmacological sciences 33, 268–272, https://doi.org/10.1016/j.tips.2012.03.007 (2012).
7. van Montfort, R. L. & Workman, P. Structure-based design of molecular cancer therapeutics. Trends in biotechnology 27, 315–328, https://doi.org/10.1016/j.tibtech.2009.02.003 (2009).
8. Robertson, N. et al. The properties of thermostabilised G protein–coupled receptors (StaRs) and their use in drug discovery. Neuropharmacology 60, 36–44, https://doi.org/10.1016/j.neuropharm.2010.07.001 (2011).
9. Schutz, M. et al. Directed evolution of G protein-coupled receptors in yeast for higher functional production in eukaryotic expression hosts. Scientific reports 6, 21508, https://doi.org/10.1038/srep21508 (2016).
10. Scott, D. J., Kummer, L., Egloff, P., Bathgate, R. A. & Pluckthun, A. Improving the apo-state detergent stability of NTS1 with CHESS for pharmacological and structural studies. Biochimica et biophysica acta 1838, 2817–2824, https://doi.org/10.1016/j.bbamem.2014.07.015 (2014).
11. Müller, I. L. & Tate, C. G. Engineering an ultra-thermostable beta(1)-adrenoceptor. Journal of molecular biology 413, 628–638, https://doi.org/10.1016/j.jmb.2011.08.057 (2011).
12. Alexandrov, A. I., Mileni, M., Chien, E. Y., Hanson, M. A. & Stevens, R. C. Microscale fluorescent thermal stability assay for membrane proteins. Structure 16, 351–359, https://doi.org/10.1016/j.str.2008.02.004 (2008).
13. Yasuda, S. et al. Hot-Spot Residues to be Mutated Common in G Protein-Coupled Receptors of Class A: Identification of Thermostabilizing Mutations Followed by Determination of Three-Dimensional Structures for Two Example Receptors. The journal of physical chemistry. B, https://doi.org/10.1021/jpacs.7b02997 (2017).
14. Serrano-Vega, M. J., Magnani, F., Shibata, Y. & Tate, C. G. Conformational thermostabilization of the betal-adrenergic receptor in a detergent-resistant form. Proceedings of the National Academy of Sciences of the United States of America 105, 877–882, https://doi.org/10.1073/pnas.0711253105 (2008).
15. Hollenstein, K. A. et al. Structure of class B GPCR corticotropin-releasing factor receptor 1. Nature 499, 438–443, https://doi.org/10.1038/nature12357 (2013).
16. Dore, A. S. et al. Structure of class C GPCR metabotropic glutamate receptor 5 transmembrane domain. Nature 511, 557–562, https://doi.org/10.1038/nature13396 (2014).
17. Christopher, J. A. et al. Biophysical fragment screening of the betal-adrenergic receptor: identification of high affinity aryppiperazine leads using structure-based drug design. Journal of medicinal chemistry 56, 3446–3455, https://doi.org/10.1021/jm000140q (2013).
18. Segala, E., Errey, J. C., Fiez-Vandael, C., Zhubov, A. & Cooke, R. M. Biosensor-based affinities and binding kinetics of small molecule antagonists to the adenosine A2A receptor reconstituted in HDL like particles. FEBS letters 589, 1399–1405, https://doi.org/10.1016/j.febslet.2015.04.030 (2015).
19. Congreve, M. et al. Discovery of 1,2,4-triazine derivatives as adenosine A2A antagonists using structure based drug design. Journal of medicinal chemistry 55, 1898–1903, https://doi.org/10.1021/jm03767w (2012).
20. Cheng, K. Y. K. et al. Structures of Human A1 and A2A Adenosine Receptors with Xanthines Reveal Determinants of Selectivity. Structure. https://doi.org/10.1016/j.str.2017.06.012 (2017).
21. Fehse, T., Moreau, J.-L., Poli, S. M., Riemer, C. & Steward, L. 4-hydroxy-4-methyl-piperidine-1-carboxylic acid (4-methoxy-7-morpholin-4-yl-benzothiazol-2-yl)-amidine. US 20050261289 A1 (2008).
22. Cheng, R. K. Y. et al. Structures of Human A1 and A2A Adenosine Receptors with Xanthines Reveal Determinants of Selectivity. Structure. https://doi.org/10.1016/j.str.2017.06.012 (2017).
Acknowledgements

The research leading to these results has received support from the Innovative Medicines Initiative Joint Undertaking under K4DD (www.k4dd.eu), grant agreement no. 115366, resources of which are composed of non-statutory support from the European Union’s Seventh Framework Programme (FP7/2007–2013) and EFPIA companies’ in kind contribution. More info: www.imi.europa.eu. We thank D. Axford, R. Owen and D. Sherrell at I24, Diamond Light Source, Oxford, UK and M. Wang at beamline X06SA, Swiss Light Source, Villigen, Switzerland for technical support. We thank colleagues at Heptares Therapeutics Ltd. for suggestions and comments.

Author Contributions

R.K.Y.C. devised initial soaking experiments, performed LCP crystallization, designed crystal optimization, performed in meso soaking experiments, collected and processed X-ray diffraction data, solved and refined the structures. E.S. established the protein expression and purification protocols and performed LCP crystallization. T.G. performed and optimized protein purification. P.R. and T.G. optimized and performed in meso soaking experiments, collected and processed X-ray diffraction data and solved and refined structures. Project management was carried out by A.S.D., J.C.E., G.A.B., R.M.C., and F.H.M. The manuscript was prepared by P.R., A.S.D. and F.H.M.

Additional Information

Competing Interests: The authors are shareholders of Sosei Group Corporation and declare competing financial interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017