Biophysical Fragment Screening of the \( \beta_1 \)-Adrenergic Receptor: Identification of High Affinity Arylpiperazine Leads Using Structure-Based Drug Design

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Supporting Information

ABSTRACT: Biophysical fragment screening of a thermostabilized \( \beta_1 \)-adrenergic receptor (\( \beta_1 \)AR) using surface plasmon resonance (SPR) enabled the identification of moderate affinity, high ligand efficiency (LE) arylpiperazine hits 7 and 8. Subsequent hit lead follow-up confirmed the activity of the chemotype, and a structure-based design approach using protein—ligand crystal structures of the \( \beta_1 \)AR resulted in the identification of several fragments that bound with higher affinity, including indole 19 and quinoline 20. In the first example of GPCR crystallography with ligands derived from fragment screening, structures of the stabilized \( \beta_1 \)AR complexed with 19 and 20 were determined at resolutions of 2.8 and 2.7 Å, respectively.

INTRODUCTION

G protein-coupled receptors (GPCRs) form a large and important protein family with 390 members (excluding olfactory receptors) in the human genome.5 GPCRs are critically involved in cell signaling in response to a wide range of endogenous ligands, including hormones, neurotransmitters, cytokines, odorants, and light. They fall into three major classes (families A, B, and C, of which family A, the rhodopsin family, is the largest). Historically, the GPCR arena has been a fruitful one for drug discovery, with a large number of approved new chemical entities (NCEs) in this period, being launched in the past 10 years.2 Despite this success, only one new GPCR target per year on average has been drugged in the past decade, indicating that GPCR research remains a difficult area and one in which there are many targets with clinical relevance and validation that are currently underserved by drug discovery efforts. Historically, the vast majority of GPCR drug discovery efforts have relied upon cell-based assays coupled with high-throughput screening of large compound libraries for hit identification. This approach has resulted in limited success for challenging, clinically valuable targets such as neuropeptide receptors, chemokine receptors, family B peptide-hormone receptors and family C metabotropic glutamate (mGlu) receptors.

Until recent years, in contrast to soluble protein classes such as enzymes, X-ray crystal structures of GPCRs had been lacking with only the structure of the visual pigment rhodopsin, first reported in 2000, being available to guide structure-based drug discovery efforts.3 After a gap of 7 years, structures of the \( \beta_2 \)-adrenergic receptor (\( \beta_2 \)AR) were published,4,5 rapidly followed by the revelation of medium to high resolution crystal structures of 14 additional receptors (\( \beta_1 \)-adrenergic (\( \beta_1 \)AR),6–8 adenosine A2A,9,10 sphingosine 1-phosphate 1 (SIP1),11 chemokine CXCR4,12 dopamine D3,13 histamine H1,14 muscarinic acetylcholine M1 and M3,15,16 neurotensin,17 opioid receptors \( \delta_8 \),18 \( \kappa_1 \),19 \( \mu_2 \),20 nociceptin,21 and protease-activated receptor 1 (PAR1).22 It has also recently been disclosed that the first example of a family B GPCR structure, that of the corticotropin releasing factor (CRF-1) receptor, has been solved.23 The significance of this recent upsurge in GPCR crystallographic information to drug discovery has been recently reviewed.24,25

The success in obtaining new X-ray crystal structures has been due to technical advances that have stabilized purified receptors in detergent solution and locked them in specific conformations, both of which are necessary for the formation of well-diffracting crystals.26,27 Two key approaches to enable the determination of GPCR structures have been devised in recent years. First, insertion of the enzyme T4 lysozyme (T4L) into receptors in detergent solution and locked them in specific conformations.26,27 Two key approaches to enable the determination of GPCR structures have been devised in recent years. First, insertion of the enzyme T4 lysozyme (T4L) into

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formation of crystal contacts has been used. The high resolution (2.4 Å) structure of the β2AR, previously solved at 3.4 Å in complex with an antibody fragment, was the first demonstration of this strategy. The T4L approach has been used in combination with the lipidic cubic phase (LCP) crystallization method. The second approach introduces a small number of point mutations into the receptor construct, significantly increasing thermostability of the protein and thereby enabling purification in the short chain detergents required for crystallization by vapor diffusion.38–40 Thermostabilized receptors form well-ordered crystals, as first demonstrated in the structure determination of the turkey β1AR at 2.7 Å resolution.7 The thermostabilization approach has the advantage of locking the receptor into a single homogeneous conformation, determined by the pharmacology of the ligand used in the stabilization process, greatly facilitating purification and structure determination. Stabilized receptors formed via this approach are also known as StaRs41 and typically contain a small number of thermostabilizing mutations, six in the case of the β1AR StaR (β1AR-m23), which is stabilized in an antagonist conformation.

Adrenergic receptors (adrenoceptors) are family A GPCRs that activate intracellular G proteins upon binding of endogenous catecholamine ligands such as adrenaline and noradrenaline.32 The adrenergic receptors are split into α and β classes, with the latter having β1, β2, and β3 subtypes. More than 4 decades of research and development in the area of β-adrenergic receptors have resulted in a legacy of clinically important agonist and antagonist molecules with varying degrees of selectivity.33 Antagonists of β-adrenergic receptors are frequently used in cardiovascular medicine, as well as in other areas such as migraine and anxiety, and β-agonists are used in the treatment of asthma and chronic obstructive pulmonary disease (COPD).36

Crystal structures of the human β2AR4,5 and turkey β1AR7 in complex with the inverse agonist carazolol 1 and antagonist cyanopindolol 2, respectively (Chart 1), were reported in 2007 and 2008. Subsequently, structures with the full agonists carmoterol 3 and isoprenaline 4 and the partial agonists salbutamol 5 and dobutamine 6 in the β1AR were published4 (Chart 1), as were structures with carazolol and the antagonist iodocyanopindolol in the same receptor.5 β2AR crystal structures with the inverse agonist ICI118,551 and agonist BI-167107, the latter in a nanobody-stabilized active state of the β2AR, have been published.37,38 More recently, structures of the stabilized β1AR bound to the β blockers bucindolol and carvedilol, which are classified as biased agonists because they stimulate G protein-independent signaling, have been reported.39 In comparison to other β1AR structures, both of these ligands make additional contacts to helix 7 and extracellular loop 2 (EL2) of the receptor, an observation that may provide an insight into the structural requirements of biased ligands. Taken together, the β1AR and β2AR structures provide a wealth of structural understanding within the β-adrenergic area, including insights into ligand-receptor interactions that impart antagonist, partial agonist, or full agonist pharmacologies.

The advent of GPCR crystal structures has initiated a new era of structure based drug design (SBDD) for this important target class. In concert with SBDD, fragment-based drug discovery (FBDD) as a strategy for identifying small (100–250 Da), efficient hit molecules is now a well established technique.40,41 Fragment hits, when coupled with a structural understanding of how they interact with their target protein, represent excellent starting points for medicinal chemistry, and many examples of how fragments have been successfully optimized to potent leads have been published.42–44 Indeed, multiple agents have been progressed into clinical trials in recent years and the first fragment-derived drug, vemurafenib, has reached the market.45,46 Biophysical methods such as SPR, NMR, and X-ray crystallography are among the mainstays of FBDD approaches used for soluble protein targets, but their application to the GPCR field has been highly challenging because of low expression and poor stability of the target receptor when isolated from the cell membrane. For these reasons, FBDD has been rarely utilized for GPCRs to date.47

### RESULTS AND DISCUSSION

Surface Plasmon Resonance (SPR) Fragment Screening. The engineering of StaRs with significantly increased thermostability has recently enabled fragment screening of GPCRs to be validated for the first time using a variety of biophysical and biochemical approaches. Screening of the β1AR and adenosine A2A receptors by target-immobilized NMR, SPR techniques, respectively, was reported in 2011, and fragment screening of the adenosine A2A receptor has been recently validated using the capillary electrophoresis (CE) approach.39

Additionally, capture of the β1AR StaR and evaluation of the binding constants of small-molecule antagonists by SPR have been described, and by use of this approach, a subset of the Heptares fragment library (approximately 650 fragments) was screened in tandem against the β1AR StaR and adenosine A2A receptor StaR. Figure 1 shows the responses obtained from the β1AR surface plotted against responses from the A2A receptor surface. Most responses (some of those shown in gray squares) cluster around the origin. These are the compounds that showed no significant binding to either receptor. A number of responses (also shown in gray squares) showed binding to both A2A and β1AR. These nonselective binders track along the
A2A binders, those highlighted in yellow were selective binders to either receptor or the other; hits highlighted in red were selective to either. Molecules are typical by an ethanolamine side chain extending from an aromatic or heteroaromatic moiety (e.g., Chart 1, fi). SAR. Substituted phenylpiperazines have ample history within GPCR drug discovery and have been described as privileged structures for this target class. The analogous phenylpiperazines also have precedent within GPCR chemical space, for example, in the atypical antipsychotics aripiprazole and lurasidone (Chart 2). Despite this significance, phenylpiperazines have little precedent in the field of beta-adrenergic receptors, where the vast majority of clinically relevant molecules are typified by an ethanolamine side chain extending from an aromatic or heteroaromatic moiety (e.g., Chart 1, fi). Encouraged by the affinity and efficiency of SPR hits 7 and 8, we embarked upon a fragment hit-to-lead exercise to explore these phenylpiperazine fragments as novel hits for the beta AR.

Screening in an orthogonal assay format is a routine approach to confirm hits during FBDD, and in line with this strategy we assembled a set of commercially available analogues of similar size and complexity compared to 7 and 8 (see Modeling section). The set was screened in a radioligand binding assay with human wild-type beta AR and [3H]dihydroalprenolol, and affinity data (together with ligand efficiency (LE), ligand lipophilicity efficiency (LLE), and cLogP values) for selected analogues are shown in Table 1. All of the commercially available analogues tested have high ligand efficiencies and bind with moderate to high affinity. Unsubstituted phenylpiperazine 9 has good affinity, with the more polar 2-pyridyl and 2-pyrimidinyl analogues of 7 (10 and 11, respectively) being weaker and less ligand efficient than 9. Disubstituted phenyl groups yielded the highest affinities, with chloro (12–14) and methyl (15, 16) variants yielding higher affinities than the trifluoromethyl or methoxy derivatives 17 and 18. The chloro or methyl disubstituted analogues, where 2,3- or 3,5-regiochemistry is preferred over the 3,4-isomers, bound with moderate to high affinity and exceptional ligand efficiencies (>0.60). Indole 19 also has excellent affinity and ligand efficiency. Ligand lipophilicity efficiency (LLE = pKᵢ - cLogP) is another measure of drug likeness in general LLEs for the fragments are moderate to good (with the exception of the highly lipophilic 17). The LE and LLE values for several compounds in Table 1 compare favorably to those of the well characterized beta AR ligands carazolol 1 (LE = 0.62, LLE = 6.4), cyanopindolol 2 (LE = 0.56, LLE = 6.8), and carmoterol 3 (LE = 0.38, LLE = 5.6). Selected quinoline derivatives to explore initial SPR hit 8 were screened; 8 was included as a control and returned highly comparable affinity in the radioligand binding assay to that from the SPR screen (pKᵢ = 5.20 vs pKᵢ = 5.25, respectively). Installation of a methyl group at the 4-position (20) or substitution at the 8-position (21) yielded compounds with approximately 25-fold greater affinity than 8, whereas interchanging the methyl and piperazinyl groups of 20 to yield isomer 22 was only moderately effective. Compounds 23 and 24 were purchased to loosely mimic the carbazole core of carazolol; closer analogues were not readily available. Nevertheless, 23 and 24 returned submicromolar affinity, albeit with lower LLE than the parent hit 8. Selected compounds (12, 13, 19, and 20) were profiled in muscarinic M₁= M₄ acetylcholine receptor membrane binding and agonist functional assays to provide an initial indication of selectivity of the piperazine fragments against related family A GPCRs. All four compounds were inactive in the functional assay for each isoform and had only weak affinity at best in the binding assays (13, 19, and 20 M₁= M₄ pKᵢ < 4.7; 12 M₁= M₄ pKᵢ = 5.1–5.4, M₄ pKᵢ = 4.5). Further GPCR cross-screening is outside the scope of this.

Figure 1. Plot of SPR responses of Heptares fragment library against the adenosine A₂A StaR and beta AR StaR proteins: orange circles, beta AR selective hits; blue squares, beta AR control compound; red circles, A₂A selective hits; green squares, A₂A control compound.

Chart 2. SPR Hits 7, 8, and Arylpiperazine Atypical Antipsychotics Aripiprazole and Lurasidone.
initial communication. Given the precedent for related piperazines such as aripiprazole and lurasidone to have complex pharmacology with activities against α-adrenergic, dopaminergic, and serotonergic receptors, characterization of optimized compounds in the series against these GPCR subfamilies will be relevant.

**Modeling.** The advent of GPCR crystallographic information presents opportunities for structure-based design and discovery.\(^2\) We sought to use the available β₁AR crystal structure data to drive progress with the piperazine fragment hits. As it is not currently feasible to perform crystallography on the human β₁AR because of its instability, these data were obtained with the turkey β₁AR. However, the two receptors are 82% identical in the transmembrane and loop regions (with the exclusion of ICL3) and the residues that comprise the ligand binding pocket are 100% identical. The turkey receptor therefore provides an excellent model for studying ligand−receptor interactions.\(^3\) The ligand−receptor interactions observed in the β₁AR structures with cyanopindolol, carmoterol, and carazolol bound were of notable interest. During the hit-to-lead process, molecules were routinely docked into the liganded structure of the β₁AR\(^7\) and examined in relation to inverse agonist carazolol\(^1\) and agonist carmoterol\(^3\).\(^6\) Because of the small size of fragments and the multitude of ways they can fit into a receptor binding site, it is notoriously difficult to dock these ligands with any great confidence without examining the site in question in significant detail. A complete druggability analysis\(^5\) of the binding site was therefore carried out to identify the region most likely to be occupied by the fragment. Water and CH aromatic probes show the regions most favorably occupied by polar and hydrophobic portions of a ligand, respectively. Optimization of overlap of the docked molecule along with displacement of the least energetically favorable waters (shown in red in Figure 2) has been shown previously to be predictive for highly ligand efficient molecules.\(^5\) Thus, this strategy was also used in our analysis of the solutions obtained from dockings, as described below in further detail for representative hit 16. Additional compounds were selected from similarity and substructure searching approaches to complement the structure-based strategy and expand SAR in the series.

In the dockings of 16 with carazolol 1 and carmoterol 3 the carbon atom of the 3-methyl substituent of 16 overlays clearly

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**Table 1. β₁AR Binding Affinities, LE, LLE, and cLogP Values of 8−24**

| compd | R, R¹, R² | β₁AR pKᵢ | LE | cLogP' | LLE |
|-------|-----------|-----------|----|--------|-----|
| 8     | R¹, R² = H | 5.20      | 0.44| 1.59   | 3.61|
| 9     | R = H     | 5.87      | 0.67| 1.11   | 4.76|
| 10    |           | 5.63      | 0.48| 1.51   | 4.12|
| 11    |           | 5.60      | 0.48| 1.46   | 4.14|
| 12    | R = 3-Cl, 5-Cl | 7.07 | 0.69| 3.03   | 4.04|
| 13    | R = 2-Cl, 3-Cl | 6.97 | 0.68| 2.90   | 4.07|
| 14    | R = 3-Cl, 4-Cl | 6.67 | 0.66| 2.67   | 4.00|
| 15    | R = 3-Me, 4-Me | 6.13 | 0.60| 2.03   | 4.10|
| 16    | R = 2-Me, 3-Me | 6.27 | 0.61| 2.03   | 4.24|
| 17    | R = 3-CF₃, 5-CF₃ | 5.90 | 0.40| 4.03   | 1.87|
| 18    | R = 3-OMe, 5-OMe | 5.80 | 0.49| 0.79   | 5.01|
| 19    |           | 7.17      | 0.65| 1.03   | 6.14|
| 20    | R¹ = Me, R² = H | 6.65 | 0.53| 2.05   | 4.60|
| 21    | R¹ = H, R² = 2-thienyl | 6.60 | 0.43| 2.39   | 4.21|
| 22    |           | 5.80      | 0.47| 1.44   | 4.36|
| 23    |           | 6.70      | 0.43| 3.69   | 3.01|
| 24    |           | 6.17      | 0.40| 3.06   | 3.11|

"See Experimental Section for assay conditions; pKᵢ = −log₁₀ Kᵢ.\(^b\) Reference 56.

Figure 2. Druggability analysis of the β₁AR ligand binding site,\(^5\) showing water molecules and their energies relative to bulk solvent (red, high; blue, low) combined with surfaces depicting water probe (green) and CH aromatic probe (yellow) hotspots, with the docking pose of 16 selected for comparison to the crystal structures.
with the carbazole nitrogen of 1 and is in the region of the quinolinone nitrogen of 3, suggesting an opportunity to introduce similar polar interactions with the receptor in this area of the molecule (Figure 3). This possibility is more clearly visualized in the structure of cyanopindolol 2 in the β1AR stabilized receptor7 (Figure 4), where hydrogen bond donation of the indole nitrogen to Ser211 5.42 (superscripts indicate Ballesteros−Weinstein nomenclature58), a nonpolar interaction of the indole phenyl ring with Phe3076.52, and interaction of the charged ethanolamine portion with Asp1213.32 and Asn3297.39 are observed. Inspired by these observations, we extended our studies to include a number of molecules with larger and more polar substituents in order to promote further interactions with residues in the β1AR ligand binding site and in particular polar interactions with Ser211 on helix 5. The strategy most notably resulted in identification of the simple indole derivative 19 (Table 1), which has both high affinity and ligand efficiency and reduced lipophilicity compared to parent molecule 16, a finding that validated our structure-driven approach and represents a very promising lead compound for further optimization.

Crystal Structures of β1AR Bound to Fragments 19 and 20. The identification of a series of piperazine-based fragments with high affinity and high solubility59 provided an invaluable opportunity to obtain co-structures of the molecules with the β1AR, as detailed structural information would confirm the modes of binding of the fragments, facilitate comparisons with existing structures, and indicate opportunities for further development. On the basis of a consideration of affinity and ligand efficiency, indole 19 and the structurally dissimilar quinoline 20 were selected for crystallography trials. Cocrystallization of thermostabilized β1AR with fragments 19 and 20 was performed as previously described6,39 and resulted in structures determined to resolutions of 2.8 and 2.7 Å, respectively (Supporting Information Table 2). The structures show that the piperazine rings in the two molecules are located between Asp1213.32 on helix 3 (H3) and Asn3297.39 on H7, and their phenyl substituents are positioned adjacent to H5 (Figure 5a and Figure 5b). The piperazine ring therefore substitutes for the ethanolamine core of conventional adrenergic receptor ligands, and the structural roles of the phenyl substituents are similar to those of the indolecarbonitrile and carbazole headgroups of cyanopindolol and carazolol (Figure 5c and Figure 5d). However, the total number and nature of ligand−receptor interactions observed in the β1AR-19 and β1AR-20 structures are reduced when compared to those observed in the crystal structures with cyanopindolol7 and carazolol8 (Supporting Information Table 3). With cyanopindolol and carazolol, the ethanolamine secondary amine and β-hydroxyl groups both
form hydrogen bonds to the Asp1213.32 and Asn3297.39 side chains. In contrast, with 19 and 20, only one secondary amine nitrogen in the piperazine ring is suitably positioned to interact with Asp1213.32 and the interactions are mostly weaker, polar interactions rather than hydrogen bonds (Supporting Information Table 3). Possibly as a consequence of the weaker interactions of fragments 19 and 20, there is greater variation in the orientation of the piperazine rings than is shown by the ethanolamine groups in carazolol and cyanopindolol. This can be observed in the superpositions of the different ligands and fragments complexed with the receptor depicted in Figure 5e. The variation in the positions of the piperazine rings in the two structures results in some differences in how 19 and 20 interact with Asp1213.32, Asn3297.39, Trp3036.48, and Tyr3337.43 (Supporting Information Table 3). The structures of β1AR bound to 19 and 20 do not show either the change in rotamer conformation of Ser2115.46 observed when full agonists are bound6 or a contraction of the ligand binding pocket (Supporting Information Table 3) characteristic of the binding of both full and partial agonists.6 These observations suggest that both of the fragments are antagonists, although data to support this hypothesis have not yet been generated. However, the interactions of the phenyl substituent headgroup with Ser2115.42 on H5 differ between the two fragment molecules, as only 19 can form a hydrogen bond to Ser2115.42. In the β1AR-20 structure, the conformation of Ser2115.42 is similar to that observed in the β1AR-carazolol structure (Figure 5b and Figure 5d), whereas in the β1AR-19 structure the conformation of Ser2115.42 corresponds to that observed in the β1AR-cyanopindolol structure (Figure 5a and Figure 5c). The differences in pharmacological activity between cyanopindolol (a partial agonist with sympathomimetic activity) and carazolol (an inverse agonist) may in part result from the differences in the conformation of Ser2115.42 observed in the structures with these ligands60 (Figure 5c and Figure 5d), and there may be similar differences in pharmacological activity between 19 and 20. Because of their relatively small size, fragments 19 and 20 interact with a subset of the residues that comprise the ligand binding pocket. This suggests that by use of the detailed structural information that has been made available, the targeted addition of further substituents to these fragments could serve to further increase their affinities, as well as to modulate their pharmacological activities.

CONCLUSIONS

Fragment screening is a well-established and powerful approach to the discovery of a new lead series that is now starting to be utilized for membrane-bound receptor targets. Recent virtual screening efforts have demonstrated that, at least for aminergic family A GPCRs, fragment hits can be identified, now that the
details of how small molecules bind to these receptors are better understood. Furthermore, studies on, for example, the histamine H1 receptor (H1R) have shown, in a manner analogous to the work exemplified here, that fragment-sized ligands can be bound to the receptor, crystallized and their binding modes established. In the research presented here, biophysical fragment screening of β1AR using SPR was enabled by receptor stabilization and led to the identification of arylpiperazine hits 7 and 8. Selection of analogues for screening in an orthogonal wild-type radioligand membrane binding assay through similarity and substructural searching approaches was complemented by the use of β-adrenergic receptor crystallographic information, allowing a parallel structure-based design strategy. High affinity and ligand efficient fragments were identified, including indole 19 and quinoline 20, which were identified and subsequently co-crystallized with the stabilized β1AR, yielding structures at 2.8 and 2.7 Å, respectively. Overall, these results demonstrate, for the first time, that a true fragment based drug discovery paradigm, encompassing biophysical screening with a direct-binding platform of a diverse fragment library, structure-guided fragment optimization, and co-crystallization of fragment hits, can now be applied to GPCR targets.

# EXPERIMENTAL SECTION

Expression of β1AR and Membrane Preparation. HEK293 cells were transiently transfected with cDNA encoding human β1AR using Genejuice (Novagen) according to the manufacturer’s instructions to achieve approximately 2 × 10^6 post-transfected cells. After 48 h of incubation the cells were dissociated using TrypLE Express cell dissociation fluid and washed with PBS, pelleted down, and stored at −80 °C until required. The pellet was resuspended in chilled buffer consisting of 20 mM HEPES, 10 mM EDTA containing 1X Complete protease inhibitor cocktail tablet per 50 mL of buffer (pH 7.4). All subsequent procedures were carried out at 4 °C. The suspension was homogenized for 15 s and centrifuged at 4000g for 15 min. The pellet was suspended in buffer containing 20 mM HEPES and 0.1 mM EDTA (pH 7.4) and homogenized for 30 s. After centrifugation at 4000g for 45 min, the pellet was resuspended in the same buffer and homogenized for a further 30 s. Protein concentration was estimated against the standard bovine serum albumin using the BCA method (Pierce, Socochim, Lausanne, Switzerland) and then frozen to −80 °C at 2 mg/mL prior to use.

β1AR Radioligand Binding Assay. Cell membranes were incubated with [3H]dihydroalprenolol in assay buffer (50 mM HEPES, 15 mM MgCl2, 150 mM NaCl, pH 7.4) in a total assay volume of 0.25 mL with a final DMSO concentration of 1%. After 90 min of incubation at room temperature the reaction was terminated by rapid filtration through GF/B 96-well glass fiber plates with 5 × 0.25 mL washes with doubly distilled H2O using a Tomtec cell harvester. Bound radioactivity was determined through liquid scintillation using Lablogic SafeScint and detected on a MicroBeta liquid scintillation counter. Nonspecific binding was determined in insect cells and purification were all performed as described previously. For crystallization, the detergent was exchanged to hydrogen bond acceptor hotspots, and amide NH probe (N1) for hydrophobic hotspots, water probe (OH2) for water hotspots, carbonyl group probe (O) for hydrogen bond acceptor hotspots, and amide NH probe (N1) for hydrogen-bond donor hotspots. Similarity and substructure searches were conducted within Canvas from Schrodinger. The MolPrint2D algorithm as implemented in Canvas was used for the similarity searching.

Expression, Purification, and Crystallization of Thermolabeled β1AR. The turkey (Meleagris gallopavo) β1AR (StAR) construct that was used in crystallization experiments, β44-m23, contains six thermostabilizing point mutations and truncations at the N terminus, inner loop 3, and C terminus. Receptor expression in insect cells and purification were all performed as described previously. For crystallization, the detergent was exchanged to hydrogen bond acceptor hotspots, and amide NH probe (N1) for hydrophobic hotspots, water probe (OH2) for water hotspots, carbonyl group probe (O) for hydrogen bond acceptor hotspots, and amide NH probe (N1) for hydrogen-bond donor hotspots. For crystallization, Hega-10 and cholesteryl hemisuccinate were added to 0.5% and 1 mg/mL respectively. Crystals were grown at 4 °C by vapor diffusion in sitting drops with 150 nL of receptor + 150 nL of precipitant (0.1 M bicine, pH 9.0, 24% PEG 600 in both cases) and cryoprotected by addition of 60% PEG 600 for 1 min before mounting on Hampton CrystalCap HT loops and cryocooling in liquid nitrogen.

Data Collection, Structure Solution, and Refinement. For both β1AR-19 and β1AR-20 structures, diffraction data were collected from a single cryocooled crystal (100 K) using a 10 μm focused beam at I24, Diamond Light Source, Oxford, U.K. Images were processed with MOSFLM and SCALA. Both structures were solved by molecular replacement with Phaser- using the structure of β1AR with carbodiilol bound (PDB code 4AMJ) as a starting model. Refinement, rebuilding, and validation were carried out with REFMAC5, Coot, and MolProbity. In both the β1AR-19 and β1AR-20 structures there is a distortion of the ligand binding pocket in monomer A due to lattice contacts, and monomer B represents the more physiologically relevant conformation. The β1AR-19 and β1AR-20 crystal structures were determined at a resolution of 2.8 and 2.7 Å, respectively. This resolution was more than adequate to provide clear electron densities that enabled unequivocal placement of the ligands in the structures (Supporting Information Figure 1), as well as further details such as specifically bound detergent and lipid molecules and ordered internal water molecules and sodium ions (Supporting Information Table 2).
**ASSOCIATED CONTENT**

Supporting Information
Supplier information and LCMS QC data for all compounds, NMR data for selected compounds, muscarinic M1–M4 acetylcholine receptor membrane binding and agonist functional assays, and Andrew the Medical Research Council (MRC U105197215) and a 2007 Nature ■ REFERENCES

ABBREVIATIONS USED

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The authors declare no competing financial interest.

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Notes
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