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Use of the X-ray structure of the β_2-adrenergic receptor for drug discovery. Part 2: Identification of active compounds

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ABSTRACT

The recently published X-ray structures of the β_2-adrenergic receptor are the first examples of ligand-mediated GPCR crystal structures. We have previously performed computational studies that examine the potential viability of these structures for use in drug design, exploiting known ligand activities. Our previous study and a newly reported β_2/Timolol X-ray complex provide validation of the computational approaches. In the present work, we use the X-ray structures to extract, via in silico high-throughput docking, compounds from proprietary and commercial databases and demonstrate the successful identification of active compounds by radioligand binding.

In rational drug design, the most powerful approach and the method of choice when X-ray structures are available is the use of a target structure (typically a protein) for 'structure-based design.' X-ray structure-based design is now widely employed in the pharmaceutical field for soluble proteins. Unfortunately, the largest single class of drug targets, G-protein coupled receptors (GPCRs), has only recently yielded any X-ray structures, with the exception of rhodopsin. While the publication of the X-ray structure of rhodopsin[1,2] provided the first example of a high-resolution mammalian GPCR structure for use as a template in homology modeling, there remained concerns regarding the limitations of using a template from a structure whose activation mode differs significantly from that of receptors with diffusible ligands. For GPCRs, the utility of an X-ray structure of ligand-gated receptors remained to be seen. Potential limitations in the accuracy of homology models based on rhodopsin, which may have important structural differences for specific ligand-mediated GPCR target proteins, were also of concern.

The recently published X-ray structures of the β_2-adrenergic receptor[3–5] provided the first examples of a ligand-gated GPCR for use in structure-based design. This first high-resolution X-ray structure involved insertion of T4-Lysozyme into the third intracellular loop, to aid in protein stabilization. Since then, the X-ray structure of the β_1-adrenergic receptor has also been published.[6] Multiple point mutations were used to increase the stability of the β_1-adrenergic receptor. Despite these significant differences in the approach to protein stabilization, the β_1- and β_2-adrenergic X-ray structures are nevertheless very similar, thereby mitigating concerns about potential artifacts introduced by the stabilization approaches.

In a previous study,[7] using the above β_2-adrenergic X-ray structure, we had shown that commonly used docking procedures are able to accurately reproduce the X-ray binding pose of the bound ligand, Corazolol, to provide compelling models for known inhibitors, to efficiently extract known inhibitors through high-throughput docking of proprietary databases, and to identify possible alternative binding modes for new ligands. High-throughput docking provides a relatively quick (i.e., not requiring chemical syntheses) method to examine the usefulness of a protein structure in drug discovery. In this work, we present in vitro test results for compounds extracted via high-throughput docking of proprietary and commercial databases. These studies, using the β_2-adrenergic receptor as a prototypical system, provide an early view of the potential utility of X-ray structures of GPCR proteins. The availability of considerable ligand information from the literature, coupled with the experimental work presented, helps formulate this assessment.

The present docking studies were conducted with the Glide module from Schrödinger.[8] An X-ray structure of the β_2-adrenergic receptor[9] was used to generate a working model.[10] The X-ray structure contained the bound ligand Corazolol, a high-affinity inverse agonist. As part of our validation,[7] we docked Corazolol into the model. The binding of Corazolol in the X-ray structure is

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depicted in Figure 1. As indicated schematically in Figure 1a, there is an extensive hydrophobic region, but there are only a few polar/hydrogen-bonding interactions. Looking at the hydrogen-bonding interactions (Fig. 1b), we see that both the hydroxyl and amino groups of Corazolol's alkylamine form hydrogen bonds with both Asp113 and Asn312. The nitrogen atom of Corazolol's carbazole moiety is 3.3 Å from the oxygen atom of the Ser203 hydroxyl group. This indicates a favorable polar interaction, albeit having a possibly longer than ideal hydrogen bond. Interestingly, the ability of a Ser residue to serve as either a proton donor or acceptor suggests that the carbazole nitrogen atom could be replaced by an oxygen atom. Figure 1c shows the enclosed nature of Corazolol in the binding site. Two nearby hydrophobic pockets are indicated in yellow ovals. The overlap of the docked Corazolol structure with its X-ray binding mode (Fig. 2) gave early, valuable validation support for the accuracy of the docking approach. Further support has now emerged. Among the known ligands whose binding poses were predicted via docking in the previous study[7] was the β_2-antagonist Timolol. An X-ray structure of the β_2 receptor complexed with Timolol has now been published.[11] Figure 3 shows the overlap of our previously reported[7] docked model of the β_2/Timolol complex with the newer X-ray structure. The clear overlap gives further support for the predictive accuracy of the approaches employed herein. The largest difference between the two structures is in the rotational conformation of the morpholine group.

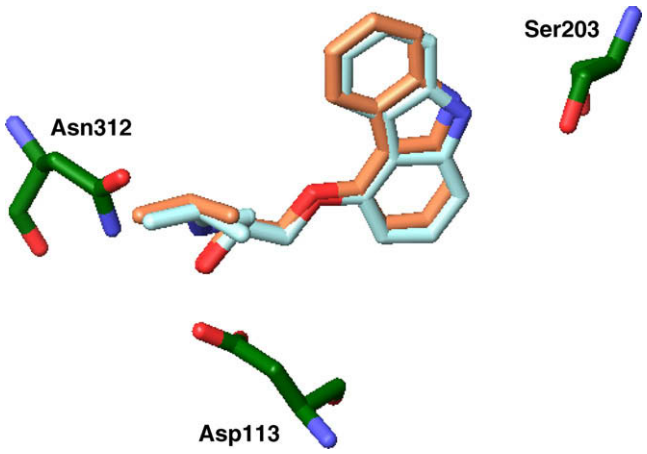


Figure 2. Comparison of Corazolol's Glide-docked binding pose (aqua) with the X-ray structure (brown).

We note that the ca. 180° rotation still places the morpholine oxygen atom at the same location. It is not uncommon for two rotational states of such a group to be within the resolution of the X-ray structure. While the estimated coordinate errors were not available for the 2.8 Å resolution X-ray structure,[11] our visual

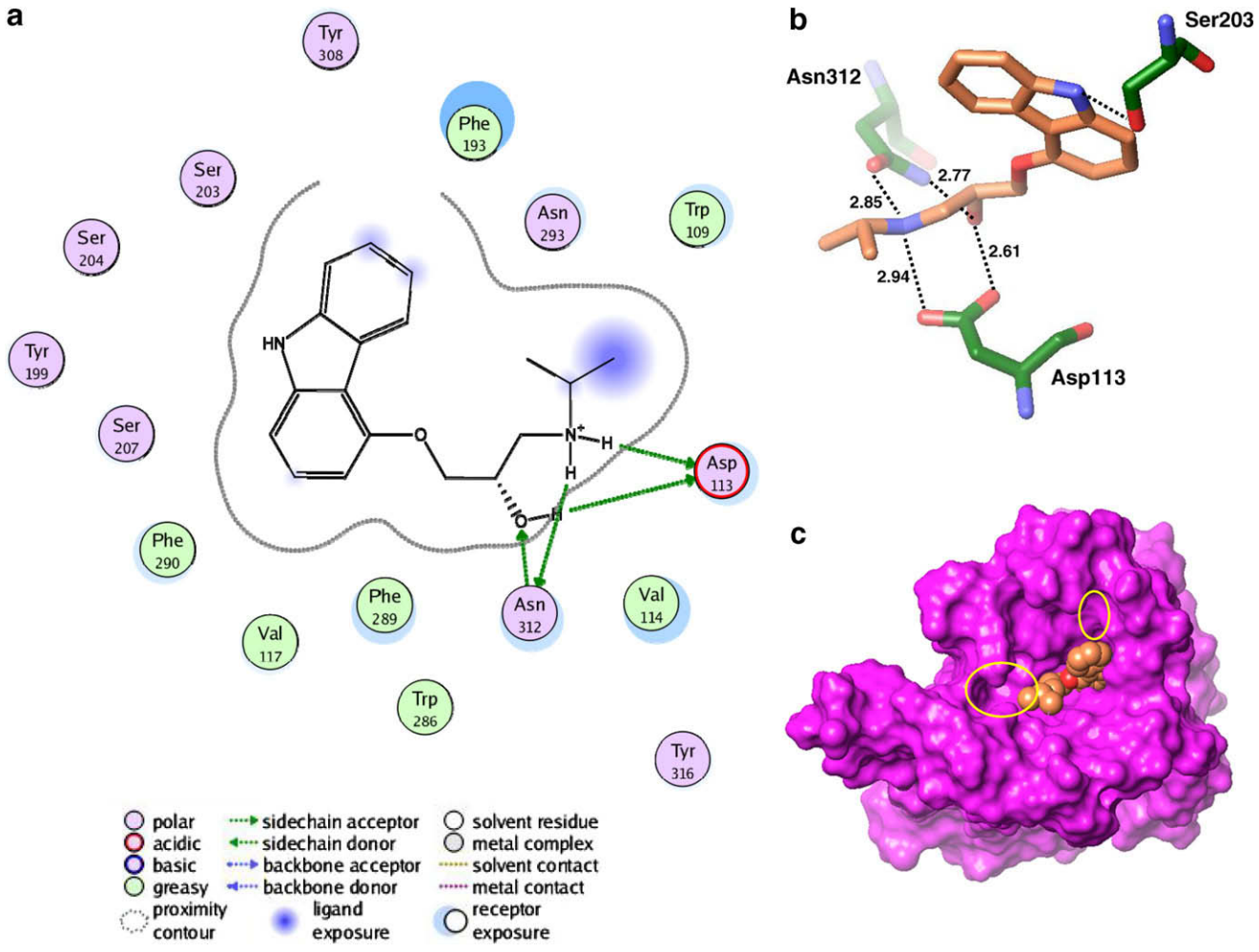


Figure 1. X-ray structure of Corazolol. (a) Schematic representation of key interactions. (b) Hydrogen-bond interactions between Corazolol and the β_2 receptor. Heavy-atom distances are shown in Å. (c) Depiction of the buried nature of Corazolol. The extracellular loop 2 has been removed for easier visualization. Neighboring pockets are indicated with yellow ovals.

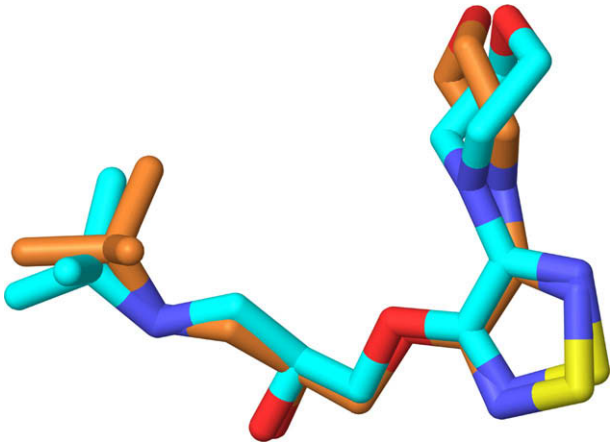


Figure 3. Superposition of the ligands in the previously predicted[7] binding model of the Timolol/β_2 complex (aqua) and the corresponding, subsequently published X-ray structure (brown).[11] The coordinates of the α-carbon atoms in the active site were selected for a 3D alignment of the two complexes. For clarity, only the ligands are shown.

examination suggests that both rotamers would fit the density map equally well. In this X-ray structure, a hydrogen bond between a nearby Asn amide nitrogen atom and the morpholine oxygen atom would favor the X-ray conformation. This Asn side chain has moved from the corresponding location in the β_2-adrenergic/Corazolol X-ray structure used in our docking studies.[7]

Previously, we conducted[7] high-throughput docking calculations using in-house proprietary and commercial databases of approximately 400 thousand and 4 million compounds, respectively. For the proprietary database, we initially selected 10 of the top-scoring 100 compounds based on the Glide docking scores. One of these was in fact Corazolol. Also found in the top-scoring 100 compounds was Carvedilol, a potent β_2 antagonist. We now have sought to test the top-scoring 150 compounds each from both in-house and commercial databases for binding affinity at the human β_2 receptor.

Of the 150 compounds selected from the in-house database, 56 were physically available for testing in the radioligand binding assay. Of these, 19 produced at least 35% inhibition at 10 μM.[12] K_i determinations[13] with these compounds yielded a range of activities from 0.11 nM to 21 μM (see Table 1). High-throughput docking identified 20 β_2 antagonists in total, including Carvedilol (unavailable for this study), representing a hit rate of 36%. Of the 150 compounds selected from the commercial database, 94 were available for testing. Of these, 17 produced greater than 35% inhibition at 10 μM. Eleven of these yielded measurable binding affinities that ranged from 13.7 nM to 4.3 μM, representing a hit rate of 12%. For comparison, we screened a randomly selected, diverse set of 320 compounds from the in-house collection. One of these compounds produced greater than 35% inhibition at 10 μM, which is equivalent to a 0.3% hit rate. The K_i value of this compound was determined to be 257 nM.

It is interesting to examine some of the binding characteristics predicted from the docked structures. Examples of the hits which bind in the same region as Corazolol are shown in Figure 4. The known β-blocker Carvedilol was ranked 20th among the top hits in the in-house database. Figure 5 shows the docked structure of Carvedilol. Not surprisingly, the features that Carvedilol and Corazolol have in common (i.e., the carbazole and alkylamine moieties) overlap. The methoxy phenyl group of Carvedilol nestles into one the hydrophobic pockets described above, adjacent to the location of Corazolol in the X-ray structure. The docked structure of compound **2**, ranking 30th in our in-house database docking, is shown in Figure 6 (superimposed on Corazolol.) In this structure, the atoms that are in common with Corazolol overlap very closely. In compound **2**, the proton–donor NH of Corazolol's carbazole group, which is close to Ser203, has been replaced by an oxygen atom. The efficacy of this NH-to-O switch from the Corazolol structure to compound **2** is substantiated by the potency of this compound, i.e., 0.15 nM. These possibilities, which were readily identifiable from the use of the X-ray structure model, could not have been predicted in the absence of a protein-structure model.

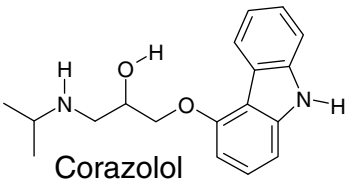
Other active compounds were identified in our in-house database docking effort and are predicted to bind similarly to Corazolol but do not have an acyclic amine (see, e.g., compounds **3** and **11** in Fig. 4). Similar compounds have been reported in the literature.[14] The predicted binding mode for compound **3** is shown in Figure 7. In this model, one of the piperazine nitrogen atoms plays the role of the primary amine of Corazolol. The diphenyl-methyl moiety is predicted to occupy one of the hydrophobic regions identified above. Of the 20 hits from our in-house database, 9 are devoid of the hydroxyl-alkylamine (cyclic or acyclic) binding feature. For the commercial database, this is true for 1 of the 11 active compounds.

The experimental determination of binding affinities of the docking study's top-ranked compounds confirmed the predictive value of the X-ray structure for drug discovery. Typically, we find hit rates, as defined in this study, from random high-throughput screens to be 0.1% or less (unpublished results). We obtained a

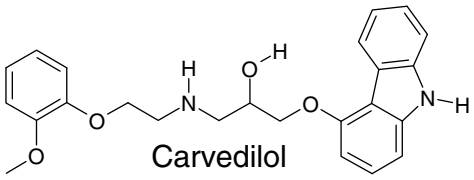
Table 1
Disposition of hits, defined by K_i values, for three libraries: Internal, External, and Random

Compound	Rank	K_i (nM)
Internal Database Docking: 20/56 (36%)		
1	18	0.114
2	30	0.145
3	4	0.311
4	20	0.398[18]
5	2	1.04
6	40	10.4
7	3	19.7
8	76	23.5
9	34	24.4
10	79	35.3
11	5	57.3
12	61	123
13	68	131
14	73	350
15	54	559
16	81	1600
17	31	2300
18	32	2510
19	69	2700
20	70	21,000
External Database Docking: 11/94 (12%)		
21	105	13.7
22	134	21.2
23	61	208
24	2	232
25	67	290
26	4	498
27	46	935
28	30	970
29	11	1000
30	42	1280
31	15	4300
Random Selection from Internal Database: 1/320 (0.3%)		
32		257

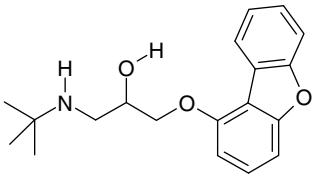
All values are means from two independent determinations. A number in parentheses indicates the hit rate for that compound set. Compounds **17–20** were retested at concentrations up to 100 μM. The K_i value for compound **4** (Carvedilol) was obtained from Ref. 18.



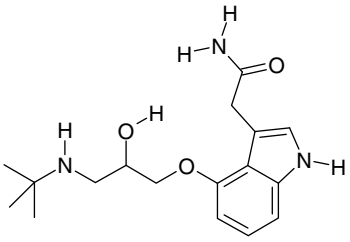
Compound **1**, ranked 18
0.114 ± 0.05 nM (n=2)



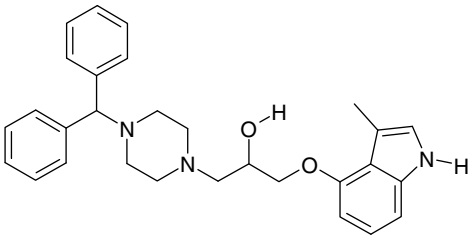
Compound **4**, ranked 20
(Not tested)



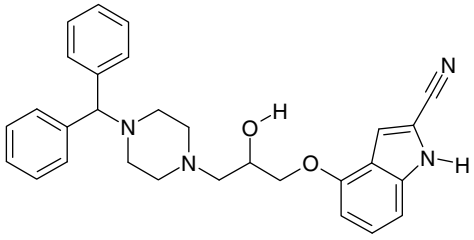
Compound **2**, ranked 30
0.145 ± 0.06 nM (n=2)



Compound **8**, ranked 76
23.5 ± 13.8 nM (n=2)



Compound **3**, ranked 4
0.311 ± 0.09 nM (n=2)



Compound **11**, ranked 5
57.3 ± 1.6 nM (n=2)

Figure 4. Sample, high-affinity ligands predicted by the docking studies to bind like Corazolol.

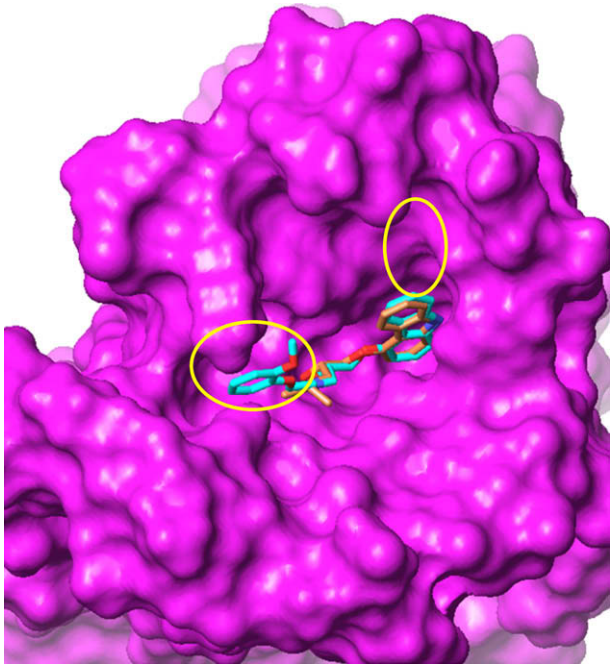


Figure 5. Docked structure of Carvedilol (aqua) overlapped with the X-ray structure of Corazolol (brown). The protein surface has been cropped; the extracellular loop 2 has been removed for easier visualization. Neighboring pockets are indicated with yellow ovals.

similar hit rate with the random set of compounds in this study. In contrast, selection of compounds using high-throughput docking with the X-ray structure yielded a vastly improved hit rate of 36%. Because the Lundbeck compound collection may be biased toward GPCR ligands and pharmacological tool compounds, it is reasonable to speculate that a high hit rate reflects the disproportionate number of these β_2 ligands. High-throughput docking of a much larger set of external compounds, however, also

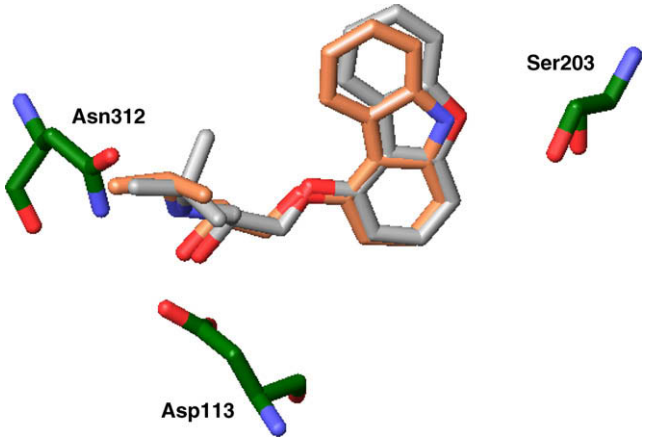


Figure 6. Comparison of the predicted binding of compound **2** (gray) with Corazolol's X-ray structure (brown).

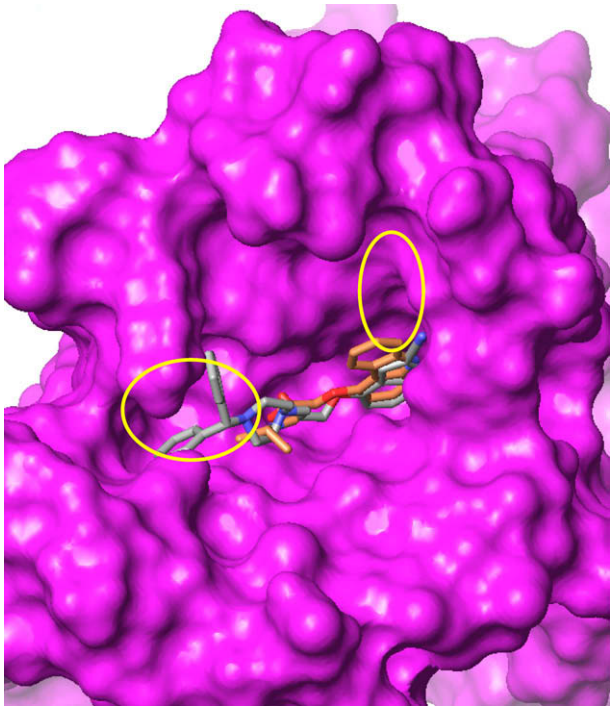


Figure 7. Docked structure of compound **11** (gray) overlapped with the X-ray structure of Corazolol (brown). The protein surface has been cropped; the extracellular loop 2 has been removed for easier visualization. Neighboring pockets are indicated with yellow ovals.

yielded a similarly substantial hit rate of 12%, which is roughly 100-fold higher than the results of a random screen. Therefore, the high hit rates observed using docking as a filter validate the utility of this method for rapidly identifying high-affinity ligands. Similar results have been obtained with crystal structures of soluble proteins such as kinases.[15,16]

The availability of X-ray structures for the β_2-adrenergic receptor now affords the opportunity to evaluate whether ligand-mediated GPCR X-ray structures will be as useful as X-ray structures of, e.g., soluble proteins. With high-throughput docking studies, we have found that known, high-affinity ligands do indeed distill to the top-scoring ranks when the ligands are represented within large databases. The binding modes of known inhibitors are compelling. As direct evidence of the predictive power of these approaches when used with X-ray structures, the docking-predicted binding mode of Timolol overlaps very well with that of the subsequently published X-ray structure. Related ligands, such as Carvedilol, are predicted to bind similarly. This confirms that structure-based in silico screening can indeed be a powerful tool for high-affinity compound identification, when X-ray structures are available, and offers great promise for instances where ligands are not known.

Other utilizations of the X-ray structure of the β_2-adrenergic receptor have begun to emerge. Audet and Bouvier propose correlations between the characteristics of the docked structures of ligands to the β_2-adrenergic X-ray structure with their varying roles in modulating adenylyl cyclase activity versus the mitogen-activated protein kinase (MAPK) cascade.[19] An example of the use of the β_2-adrenergic X-ray structure in homology modeling has been published by Selent et al.[20] In their work, Clozapine and Olanzapine were examined in homology models they developed for 14 endogenous amine GPCRs to explore the structural basis of the selective behavior among these targets. de Graaf and Rognan have examined the use of the β_2-adrenergic X-ray structure to develop models for full and partial agonists.[21] It is becoming clear that high-resolution X-ray structures of GPCRs will significantly enhance our understanding of the modes of action of agonists, inverse agonists, and antagonists, and thereby accelerate drug discovery efforts aimed toward this important class of proteins.

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10. Hydrogen atoms were added to the X-ray structure's coordinates of the β_2-adrenergic receptor, and those hydrogen atoms that appeared to be pointed in unfavorable directions were adjusted manually. Then all the coordinates were slightly relaxed by using the Protein Preparation module in the Schrödinger software suite.[8] CORINA[17] was used to prepare the proprietary and commercial databases for docking by converting 2D coordinates to 3D structures, adding hydrogen atoms, generating stereoisomers, and sampling ring conformations. The LigPrep module in the Schrödinger software suite[8] was used to adjust protonation states to pH 7. Some of the docked binding complexes using the commercial database contained obvious computational artifacts (e.g., strained geometries or clashes between the ligand and the protein) and were removed in the selection. Thus, the 150 selected compounds were taken from the 177 top-ranked structures, i.e., 22 were removed.
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12. Active compounds were defined as producing $\geqslant$35% inhibition at 10 μM and yielding an IC_{50} value, when re-tested at multiple concentrations, $\leqslant$50% of the highest test concentration. Moreover, all compounds tested in-house gave IC_{50} values.
13. Competition binding assays were performed by using 0.5 nM [3H]DHA (GE Healthcare, TRK649), various concentrations of test compounds, and membranes prepared from cells expressing the human β_2 adrenoceptor (Perkin-Elmer, cat no. RBHBE2M400UA). The binding reactions were carried out for 2 h at room temperature. Binding was terminated by filtering under vacuum on GF/A filters (Millipore, Bedford, MA). Radioactivity bound to the filters was measured by using a liquid scintillation counter. Radioligand binding data obtained from serially diluted compounds were analyzed by nonlinear regression analysis to determine the IC_{50} and K_i values using PRISM software (GraphPad Software, San Diego, CA).
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