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Ligand discovery from a dopamine D_3 receptor homology model and crystal structure

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G protein–coupled receptors (GPCRs) are intensely studied as drug targets and for their role in signaling. With the determination of the first crystal structures, interest in structure-based ligand discovery increased. Unfortunately, for most GPCRs no experimental structures are available. The determination of the D_3 receptor structure and the challenge to the community to predict it enabled a fully prospective comparison of ligand discovery from a modeled structure versus that of the subsequently released crystal structure. Over 3.3 million molecules were docked against a homology model, and 26 of the highest ranking were tested for binding. Six had affinities ranging from 0.2 to 3.1 μM. Subsequently, the crystal structure was released and the docking screen repeated. Of the 25 compounds selected, five had affinities ranging from 0.3 to 3.0 μM. One of the new ligands from the homology model screen was optimized for affinity to 81 nM. The feasibility of docking screens against modeled GPCRs more generally is considered.

GPCRs are a large family of membrane proteins that are critical for signal transduction. They have been a major focus of pharmaceutical research and are the primary targets of almost 30% of approved drugs[1]. All of these drugs were discovered without the aid of receptor structures by classical ligand-based medicinal chemistry. Accordingly, many of these drugs reflect their origins as mimics of natural signaling molecules. The determination of the first drug-relevant GPCR structures in the last 4 years[2–4] has opened up opportunities for structure-based discovery of more new scaffolds. Docking screens for these crystal structures have been unusually fruitful, with high hit rates returning unique and potent ligands[5–7]. Still, the structures of most GPCRs remain undetermined. There are thought to be just over 360 pharmaceutically relevant GPCRs in the human genome[8], and to date experimental structures have been determined for only six, all by dint of extraordinary effort and innovation. For structure-based efforts to have a meaningful impact on ligand discovery for most GPCRs, homology modeling of GPCR structures remains essential, especially in the near term.

In the past, the structure of rhodopsin (and before that, bacteriorhodopsin[9]) was used to explore GPCR function and ligand recognition[10–18]. Several efforts to use homology models for ligand discovery via docking have also been undertaken[19–25]. With rare exceptions[26,27], such docking screens use a hierarchy of pharmacophore filtering and ligand similarity to focus the molecules being docked. This typically reduces an 'unbiased' library by a factor of 10–100 to one that is dominated by precedented chemotypes. Although this approach can be effective, such a combination of filtering and docking perforce removes unexpected chemotypes that a stand-alone, structure-based approach might otherwise find. Interestingly, two of these early studies included work on dopamine receptors and used rhodopsin as a template[20,21]. Though both screens had high hit rates, pharmacophore filtering seems to bias the discovered ligands toward well-established chemotypes, a point to which we will return. More generally, the pharmacophore approach does not address those targets for which ligand information is weak and does not illuminate how these models compare to what might be achieved with an experimental structure.

The opportunity to prospectively investigate how homology models compare to experimental structures for ligand discovery, and by extension what fraction of GPCRs might be exploitable for ligand discovery, emerged recently by way of a community challenge[28]. After the determination of the structure of the dopamine D_3 (ref. 29) and CXCR4 GPCRs in complex with antagonists (for D_3, eticlopride, **1**, **Fig. 1**), the modeling community was asked to predict the structures of each complex before the coordinates were released. This provided an opportunity to not only predict the configuration of the single ligand bound to the complex but also use the homology model that emerged to discover new ligands, via structure-based docking screens, before the crystal structure was released. Once released, the same screen was prosecuted against the crystal structure. As the putative ligands would be tested for affinity in each screen, we could compare the two results to illuminate the success of the homology model compared to the crystal structure in a situation where the predictions were truly blind.

We thus undertook the following calculations and experiments. Once we had submitted models of the D_3–eticlopride complex, we turned to ligand-discovery calculations in which over 3 million commercially available molecules were screened by docking to identify putative ligands that complemented the structure of the homology model. Before the crystal structure was released, 26 high-scoring small molecules (compounds **2**–**27**) were purchased and tested for D_3 receptor affinity. When the crystal structure was released several months later, it was used to test a second docking campaign; 25 more high-scoring small molecules from this second screen (compounds **28**–**52**) were purchased and tested for D_3 receptor affinity.

These calculations and experiments enabled us to investigate whether a homology model—blinded to the (unknown) crystal

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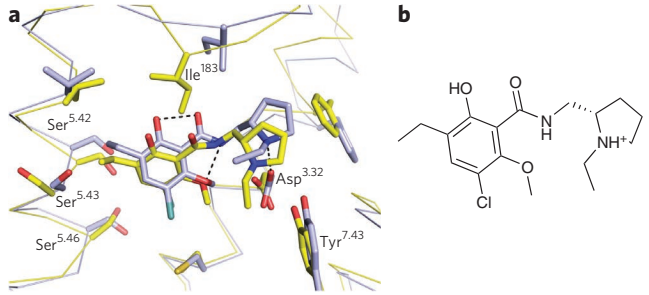


Figure 1 | Predicted structure of the dopamine D_3 receptor binding site. (**a**) Comparison of the homology model of the dopamine D_3 receptor in complex with eticlopride (light blue) to the crystal structure (yellow), visualized with PyMOL. The structures have been aligned using 15 binding-site residues. Polar interactions for the crystal structure are shown in black dashed lines. (**b**) Chemical structure of eticlopride (compound **1**).

structure—could template the discovery of new ligands and how well the homology model would compare to the subsequent crystal structure in terms of hit rate and affinity.

These questions directly addressed the possibility of using homology models for at least some of the vast majority of GPCRs whose structures are likely to remain undetermined in the near term. More subtly, because the homology model was refined for its ability to enrich known D_3 ligands, we wondered whether ligands discovered against it would be biased toward known D_3 chemotypes and therefore be less novel than those discovered using a crystal structure for docking-based discovery. From a chemical biology standpoint, we also wondered how ligand specificity would compare between the two screens. The template for the homology model was the β_2-adrenergic receptor, and one might predict that the screened molecules might retain an activity for this target or might be less specific than those screened against an experimental structure. From a chemical-probe standpoint, it is important to optimize for affinity, and we were unsure whether a homology model, selected for its ability to recognize general dopaminergic chemotypes, would be competent for such optimization. We explored these questions by undertaking prospective docking screens first against a homology-modeled structure of the D_3 receptor and subsequently against the crystal structure of the same receptor. As the crystal structure was released after the screen was completed against the model structure, both screens were fully prospective. To our surprise, we found that the hit rates against both the modeled and experimental structures were not only high but also essentially equivalent. Notwithstanding the opportunities for bias toward known chemotypes in optimizing the homology model, both screens returned new scaffolds at a similar rate.

RESULTS

Prediction of the dopamine D_3–eticlopride structure

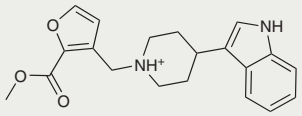
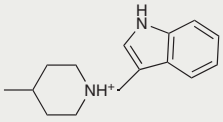
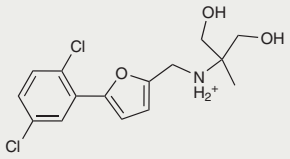
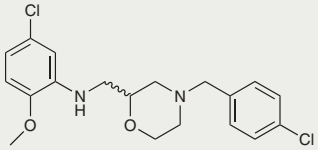
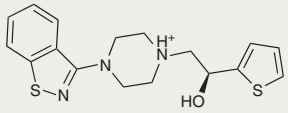
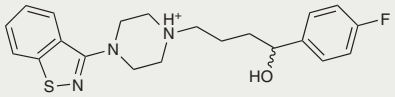
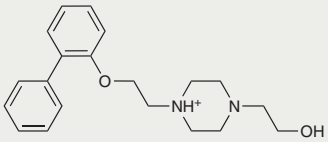
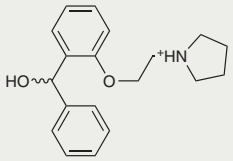
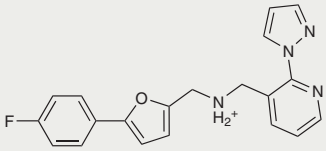
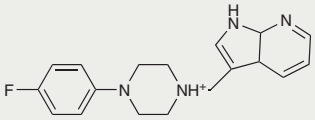
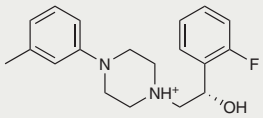
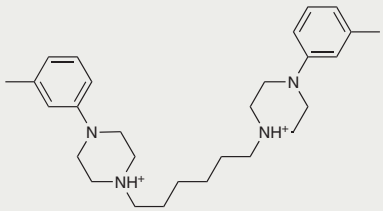
The results of the D_3–eticlopride structure prediction and docking challenge have been reported elsewhere[28] but will be briefly summarized here as they influence what follows. We were tasked with predicting the structure of the D_3–eticlopride complex without knowing the structure of either component. We used the docking enrichment of known ligands among the top-scoring molecules from a pool including a large number of decoys as a criterion of model accuracy[26,30,31]. The β_1- and β_2-adrenergic receptor crystal structures[2,3] and elastic network models calculated by the program 3K-ENM[32] were used as templates to build almost 200,000 homology models using the program Modeller-9v8 (ref. 33). The top-ranked 2,964 of these 200,000 models, judged by Modeller's internal DOPE score[34], were advanced for docking. Up to 1,300 known dopaminergic ligands, along with up to 110,000 property-matched decoys, were docked[35]. Modeled receptor structures were prioritized for their ability to highly rank the known ligands compared to the decoys in the screens. Although this demanded a substantial amount of docking—98,700,000 complexes calculated overall—the docking process was largely automated. Compared to the decoys, our top model enriched the known ligands by 32-fold over what is expected at random among the top-ranking 1% of molecules. This enrichment was substantially higher than that found for docking of dopaminergic ligands against the β_1- and β_2-adrenergic templates used for the modeling, where the enrichments had values of 2 and 1, respectively.

Eticlopride was docked into each of the top models, and five of them were selected for submission to the D_3–eticlopride structure prediction and docking[28]. As was true of the predictions from several other groups, our predicted structures showed overall fidelity to the crystal complex subsequently released. Our highest-ranked model had an overall Cα r.m.s. deviation of 3.4 Å, with r.m.s. deviations for eticlopride and the orthosteric-site residues both being 1.65 Å (**Fig. 1**). Most of the key ligand interactions[14] observed in the crystal structure[29] were also observed in this model, including the salt-bridge between the aminergic nitrogen of eticlopride and the recognition residue Asp110$^{3.32}$ (Ballesteros-Weinstein numbering[36]). Similarly, two internal hydrogen bonds in eticlopride were captured by both the model and the crystal structure[37]. Notably, all of the models that both enriched known ligands and docked eticlopride correctly were based on the templates from elastic network backbones; the higher range of motion explored by such models presumably contributed to the ultimate fidelity of the model to the experimental result. The 3K-ENM model captured backbone movements in several helices (III, IV, V and VI) that influence the shape of the binding site—for example, a 1-Å movement of transmembrane helix III in the region of Asp110$^{3.32}$.

Docking for new D_3 ligands

With this receptor model in hand, we next turned to ligand discovery. Over 3 million commercially available compounds were screened for complementarity to the receptor model, using DOCK3.6 (refs. 38,39). Each molecule was fit into the site in an average of 1,170 orientations relative to the receptor, and for each orientation an average of 789 conformations were tested (thus, over 900,000 configurations in total). Each configuration was scored for van der Waals and electrostatic complementarity and corrected for ligand desolvation[39]; thus, for the 3.1 million compounds screened, about 2 trillion complexes were evaluated. Prior to the release of the crystal coordinates, 26 top-ranking compounds (**2**–**27**), all among the top 0.02% of docking-ranked molecules, were selected for experimental testing. The top-scoring docking hits were dominated by monocationic molecules that all seemed to ion pair with the key aminergic recognition residue Asp110$^{3.32}$, and most had overall good van der Waals complementarity to the orthosteric site. In selecting the particular molecules for experimental testing, we corrected for energetic terms not included in the scoring function, such as high ligand internal energy or receptor desolvation (detailed in **Supplementary Methods**). As far as we know, none had been previously tested for activity against dopaminergic receptors. Six of these molecules, a hit rate of 23%, bound to D_3 measurably and had affinities ranging between 200 nM and 3.1 μM (**Table 1**, **Figs. 2** and **3**, **Supplementary Results** and **Supplementary Fig. 3**). The similarity of the new compounds versus dopamine receptor ligands was assessed by calculating the Tanimoto coefficient (T_c) to the 10,400 D_3 annotations in the ChEMBL database (http://www.ebi.ac.uk/chembl). Four of the active compounds (**2**, **4**, **5** and **7**) resembled known ligands, with extended connectivity fingerprint, maximum distance 4 (ECFP4)–based T_c values greater than 0.4.

Table 1 | Ligands discovered from the docking screen against the dopamine D_3 receptor homology model

Cmpd.	Structure	T_c[a]	Rank[b] (model)	Rank[c] (crystal)	K_i[d] (μM)	Closest known ligand[f]
2		0.48	63	11,381	3.1	
3		0.23	118	9,519	1.6	
4		0.55	141	23,379	0.2	
5		0.47	236	19,806	1.8	
6		0.27	289	1,410	1.3	
7		0.51	484	100,067[e]	0.5	

Cmpd., compound.
[a]The ECFP4 Tanimoto similarity (*Tc*) to the most similar dopamine receptor ligand in the ChEMBL database. [b]Rank of the compound in the screen against the homology model. [c]Rank of the compound in the screen against the crystal structure (PDB accession code: 3PBL). [d]Measured affinity for the dopamine D_3 receptor. The uncertainty in each K_i is ± 30%. [e]This compound was not in the ZINC lead-like library at the time of the screen against the crystal structure. The rank has been calculated from a re-docking of the molecule. [f]The closest known dopamine receptor ligand from ChEMBL.

The two other active ligands, compounds **3** and **6**, were topologically dissimilar to dopamine receptor ligands in the ChEMBL database (best T_c <0.35 by ECFP4 fingerprints; their novelty was also observed using Daylight fingerprints, as shown in **Supplementary Table 2**). These therefore seemed to be new chemotypes for the D_3 receptor.

With access to the crystal structure (PDB code 3PBL)[29], we then carried out a second docking screen of the 3.6 million lead-like molecules from ZINC[40]. Unlike for the homology model, where side chain positions were optimized to enrich known ligands, the crystal structure heavy-atom positions were unmodified. We selected 25 molecules (compounds **28**–**52**) from among the top 0.02% results of the crystal structure–based screen for experimental testing. Five of these, molecules **28**–**32**, were active, with K_i values between 300 nM and 3 μM, a hit rate of 20% (**Table 2**, **Figs. 3** and **4** and **Supplementary Fig. 3**). Whereas two of these, **28** and **30**, resembled previously known scaffolds, and compound **32** was of intermediate similarity, two others, **29** and **31**, represented new scaffolds. Notably, though **29** explored new substituents distal to the aminergic group, its aryl-amide core resembled that of eticlopride, a chemotype that was not observed among the active molecules from the homology-model screen. We note that compound **6**, chosen from the homology model screen, also scored among the top 0.04% of the docking-prioritized molecules from the crystal structure screen.

Ligand bias in the docking screens

The homology model had been selected based on its ability to enrich known dopaminergic ligands, and in retrospective calculations it enriched known ligands substantially better than the crystal structure. Among the chemotypes most strongly enriched were the phenylpiperazines, which are characteristic for this target. The difference in enrichments between the screens was reflected in the compounds that were highly ranked in the prospective calculations. Overall, the overlap of the top 1,000 docking hits from each of the two screens consisted of only 90 molecules, two of which were selected for experimental evaluation and were found to be inactive. Eight of the compounds purchased for experimental testing from the homology model screen closely resembled known dopaminergic ligands, that is, their ECFP4 T_c values were greater than 0.45 with respect to annotated ligands in ChEMBL, but only four of the molecules purchased from the crystal structure screen had a comparable level of similarity. The one instance in which we observed a higher bias toward dopaminergic ligands from the crystal structure

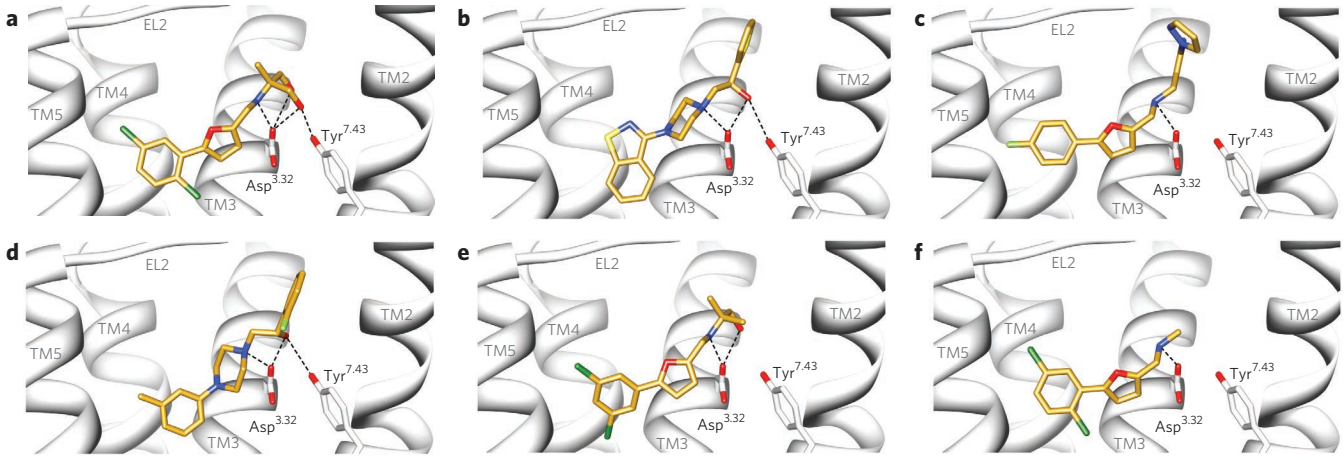


Figure 2 | Predicted binding modes of ligands found from the homology model screen. (**a**–**d**) Predicted binding poses for four ligands discovered in the docking screen against the dopamine D_3 receptor homology model, visualized with University of California San Francisco (UCSF) Chimera: **3** (**a**), **4** (**b**), **6** (**c**) and **7** (**d**). (**e**,**f**) Predicted binding modes for the two analogs of **3** based on docking to the homology model: **56** (**e**) and **57** (**f**). TM, transmembrane helices; EL2, extracellular loop 2.

screen was in similarity to eticlopride itself. Indeed, nine of the molecules selected for testing had the aryl-amide-aminergic chemotype characteristic of eticlopride and its congeners, as did many high-ranking molecules from the crystal structure screen. Conversely, only one compound from the homology model screen had this aryl-amide-aminergic chemotype. Notwithstanding these apparent biases going into experimental testing, new chemotypes were ultimately confirmed for both screens.

The different ligands selected by the two screens reflect differences between the structures of the orthosteric sites in the homology model and the crystal structure. Though these two sites only differed by an r.m.s. deviation of 1.65 Å when superimposed, when they were interrogated at a docking level this difference was enough to change the identity, if not the nature, of the high-scoring docked molecules. The main difference between the two orthosteric sites is that in the homology model the site is slightly more open and thus larger. For instance, the distance between the Cα atoms of Asp189$^{3.32}$ and Ser192$^{5.42}$ was 11.9 Å in the crystal structure but 12.9 Å in the model. More locally, Ile183 differs by 3.6 Å between the two structures, whereas Val189$^{5.39}$, Phe345$^{6.51}$ and Phe346$^{6.52}$ differ by 0.8–1.5 Å. This opening of the overall structure reflects the way the model was optimized: we docked ligands of all sizes to the model and looked for enrichments. The model structure that was chosen can accommodate known ligands across a relatively wide size range, whereas the same site in the crystal structure more tightly encloses eticlopride, a relatively small ligand. Thus, many known phenylpiperazine ligands that were enriched well by the model would clash with residues such as Val189$^{5.39}$, Phe345$^{6.51}$ and Phe346$^{6.52}$ in the crystal structure.

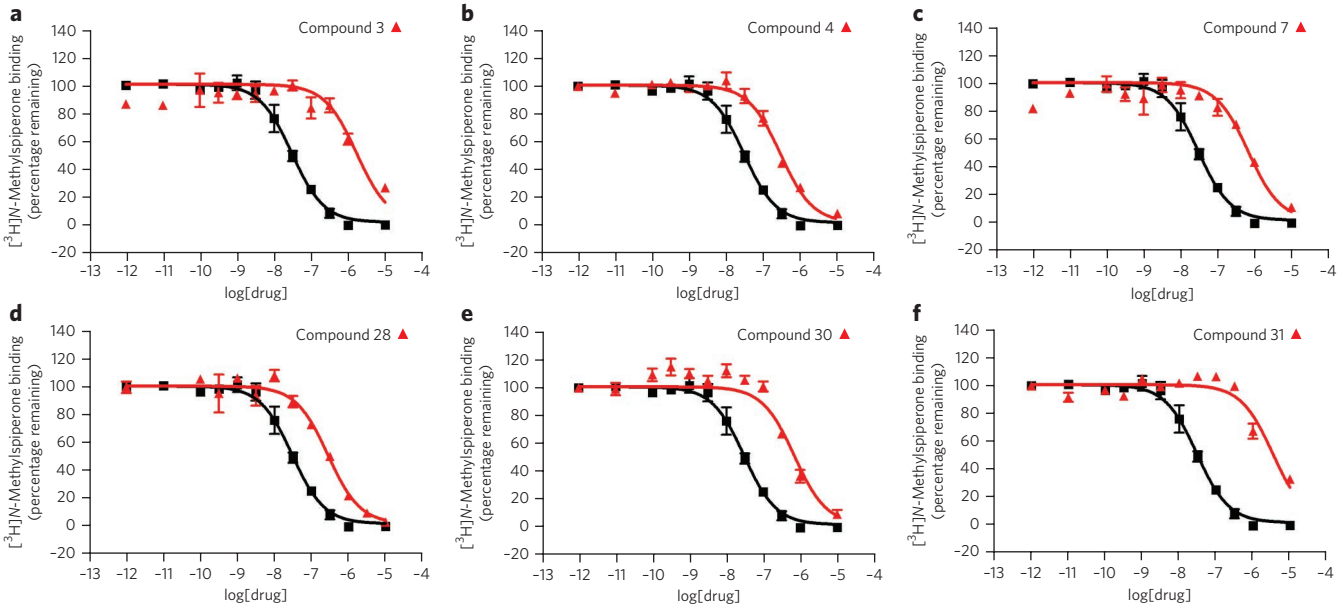
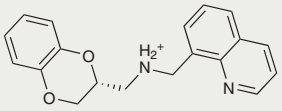
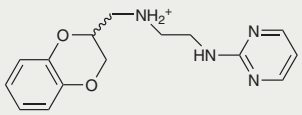
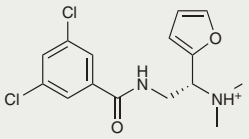
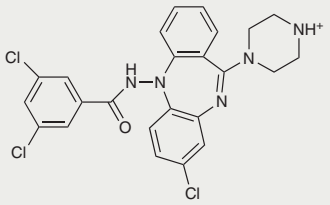
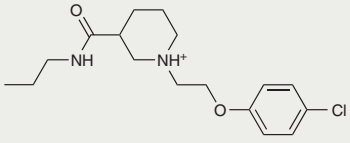
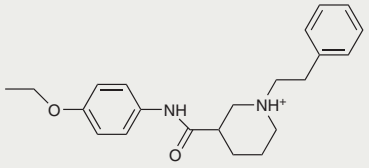
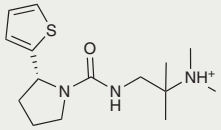
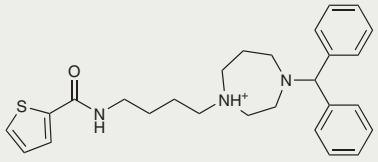
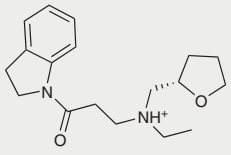
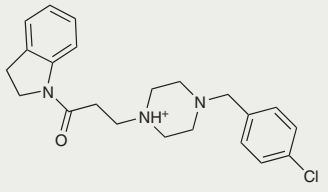


Figure 3 | Dose-response curves of discovered ligands. (**a**–**f**) Representative radioligand ([^{3}H]*N*-Methylspiperone) competition binding isotherms for compounds **3** (**a**), **4** (**b**), **7** (**c**), **28** (**d**), **30** (**e**) and **31** (**f**). Data for a reference compound (chlorpromazine, black curve) are shown along with data for the test compound (red curve). Assays are performed using a final radioligand concentration between 0.5 × K_D and 1 × K_D, where K_D equals the radioligand dissociation constant, which is determined for each crude membrane preparation by radioligand saturation binding analysis. Data represent mean values ± s.e.m, performed on triplicate experiments.

Table 2 | Discovered ligands from the docking screen against the dopamine D_3 receptor crystal structure

Cmpd.	Structure	T_c[a]	Rank[c] (model)	Rank[b] (crystal)	K_i[c] (μM)	Closest known ligand[f]
28		0.57	7,661	772	0.3	
29		0.33	44,768[e]	829	2.2	
30		0.48	1,490[e]	483	0.3	
31		0.29	51,761[e]	707	1.6	
32		0.39	5,169[e]	110	3.0	

Cmpd., compound.
[a]The ECFP4 Tanimoto similarity (T_c) to the most similar dopamine receptor ligand in the ChEMBL database. [b]Rank of the compound in the screen against the homology model. [c]Rank of the compound in the screen against the crystal structure (PDB accession code: 3PBL.) [d]Measured affinity for the dopamine D_3 receptor. The uncertainty in each K_i is ± 30%. [e]This compound was not in the ZINC lead-like library at the time of the screen against the homology model. The rank has been calculated from a redocking of the molecule. [f]The closest known dopamine receptor ligand from ChEMBL.

Ligand selectivity

An important challenge in dopaminergic receptor pharmacology is finding ligands that are specific for the D_3 rather than the D_2 receptor. With few exceptions[41], most D_3 receptor ligands are also active in D_2, making their use as chemical probes problematic. Methodologically, we were interested to learn whether the new ligands derived from homology-model docking retained activity for the β_2-adrenergic receptor, from whose template the D_3 model was derived. Active ligands were therefore counterscreened against the D_2 and β_2 receptors (**Table 3**). None had measurable

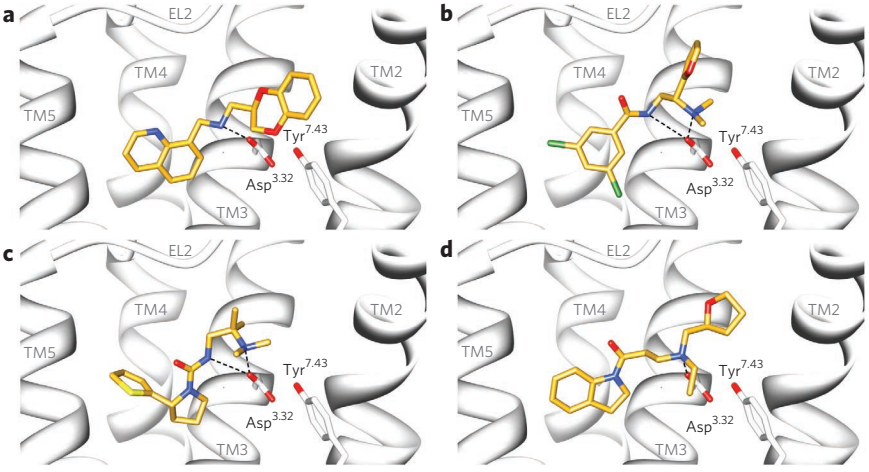


Figure 4 | Predicted binding modes of ligands found from the crystal structure screen. Predicted binding poses for the ligands discovered in the docking screen against the dopamine D_3 receptor crystal structure, visualized with UCSF Chimera: **28** (**a**), **29** (**b**), **31** (**c**) and **32** (**d**).

Table 3 | Ligand selectivity for the dopamine D_3, D_2 and the β_2-adrenergic receptor

	Receptor affinity (K_i, μM)[a]		
	Dopamine		Adrenergic
Cmpd.	D_3	D_2	β_2
2	3.1	1.3	>10
3	1.6	>10	>10
4	0.2	0.4	>10
5	1.8	0.4	>10
6	1.3	4.5	>10
7	0.5	>10	>10
28	0.3	0.9	n.d
29	2.2	3.9	n.d
30	0.3	>10	n.d
31	1.6	>10	n.d
32	3.0	3.8	n.d

n.d., not determined; Cmpd., compound.
[a]The uncertainty in each measured K_i is ± 30%.

activity against β_2-adrenergic receptor at 10μM, suggesting that no substantial template bias remained and also that ligands specific for dopaminergic receptors had emerged. No effort was made to find D_3-selective ligands, so achieving D_3 selectivity among the dopamine-receptor subtypes would be fortuitous. Whereas most compounds showed little selectivity between D_3 and D_2, a few of the more unique scaffolds did; compounds **3** and **7** had affinities 6- and 20-fold stronger, respectively, for the D_3 over the D_2 receptor (**Table 3**).

Optimization for affinity

We were also interested in progressing a novel series for affinity, both as an end in itself and to explore whether model-based approaches could effectively guide this effort. Twenty analogs of compound **3**, which is among the most dissimilar to known dopaminergic ligands, were found that had good complementarity to the D_3-modeled structure (**Table 4** and **Supplementary Table 4**, compounds **53** through **72**). In its docked position, the two hydroxyls and the aliphatic amine of compound **3** interacted with Asp110$^{3.32}$ and Tyr373$^{7.43}$. As it was difficult for these two residues to optimally interact with all three ligand donors, we wanted to explore the possibility that the hydroxyls were not crucial for affinity. Commercially available analogs of compound **3** with varying numbers of hydroxyl groups were extracted from the ZINC database[40] and docked to the orthosteric site of the homology model. We inspected the docked poses and selected a set of analogs representing the diversity found in the database for testing. All of these compounds retained the key Asp110$^{3.32}$ cationic interaction, but we explored variations in the hydroxyl groups and the substituents of the phenyl ring. In particular, we focused on compounds that preserved the *meta* substituent on the phenyl ring, which fills a hydrophobic pocket formed by the side chains of residues Phe164$^{3.28}$, Val165$^{3.29}$ and Ser168$^{4.57}$. Eleven analogs had substantially improved affinities, ranging from 4- to 20-fold better than the lead compound **3**, with the most active reaching 81 nM (**Table 4**, **Figs. 2** and **3** and **Supplementary Fig. 3**).

Functional activity of the docking hits

In previous docking screens against the GPCR crystal structures[5–7], there has been a close correspondence between the function of the ligand cocrystallized with the receptor, either inverse agonist or neutral antagonist, and the functional efficacy of docking hits. To explore whether this (presumably structural) bias was present in the D_3 screens undertaken here, we investigated the docking hits (compounds **2**–**7** and **28**–**32**) and several analogs of compound **3** (**55**–**57** and **63**) for agonism of the D_3 and D_2 receptors. With the possible exception of compound **28**, which showed very weak partial agonism (**Supplementary Fig. 4** and **Supplementary Table 3**), none of these 15 compounds were agonists against either receptor, and all seemed to function as antagonists. This finding corresponds with the known function of eticlopride, which was cocrystallized with the receptor and the ligands that were used to predicate the model's binding site.

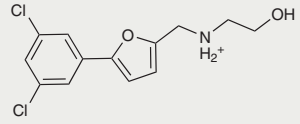
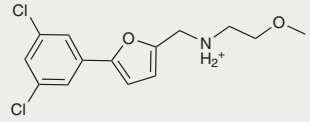
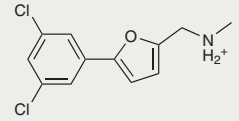
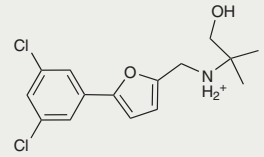
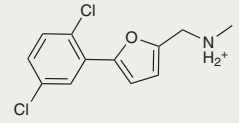
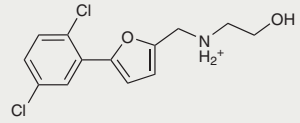
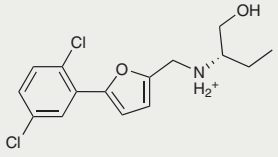
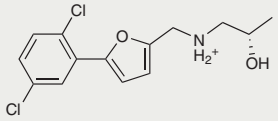
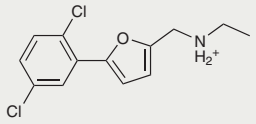
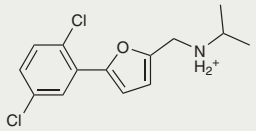
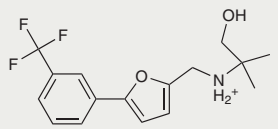
DISCUSSION

The determination of the structures of pharmacologically relevant GPCRs[2–4] has sparked intense interest[42]. It is crucial to not only identify how these structures may themselves be exploited for ligand discovery but also determine what range of homologous targets they illuminate. An astonishing result of this study was that the docking screen against the homology model was no less effective than that against the crystal structure, which was certainly not a result we expected or hoped for. The hit rates for the screens on both the homology model and the crystal structure were high, at 23% and 20%, respectively, and their affinity ranges fully overlapped, with several molecules from each screen having 200–300 nM affinity. The geometries occupied by the new ligands likely reflect their predicted docking poses, at least grossly: their poses overlap with those adopted by eticlopride in its D_3 complex, and this complex was itself well-predicted in the original blinded challenge. Homology models of proteins have been previously used for discovery of ligands, including those of GPCRs[19–27]. What was unusual and perhaps unique to this study was that a docking screen was prosecuted prospectively against a homology model and then, subsequently, the crystal structure. The results were thus doubly unbiased—the crystal structure was unknown at the time of the docking, and what we ultimately compared were new, experimentally tested ligands.

A concern that we had was whether the active molecules from the model-based screen—assuming any would be found—would be highly biased toward known dopaminergic ligands. A criterion for selecting effective models was their ability to enrich known ligands, and it seemed possible, even probable, that any active molecules that emerged from such a screen against it would simply recapitulate known D_3 ligands. Indeed, the high-ranking molecules from the homology-model screen more closely resembled known dopaminergic ligands than did those from the crystal structure screen. Some of this bias can be seen among the experimentally tested molecules: with the model, three aryl-piperazines were confirmed as active, whereas with the crystal structure screen one eticlopride-like ligand was confirmed, as is consistent with the structure's own conformational bias. In this sense, the concern regarding bias was justified and may affect future studies. In the end, however, the experimentally *active* molecules were no more biased toward known ligands in one screen than in the other. Meanwhile, each screen yielded two novel scaffolds, resulting in four overall, and these differed not only from known ligands but also among themselves. It was equally noteworthy that the active molecules from the homology model screen showed no measurable affinity for the β_2-adrenergic receptor, which was the template for the model (**Table 3**). The model thus seemed to have not only captured the broad similarity that exists among aminergic GPCR targets but also represented features specific to the D_3 receptor. The lack of overlap among the hits from the two screens and the observation that many ligands that dock well into the modeled structure did not fit into the corresponding crystallographic site may reflect the many low-energy conformations that GPCRs sample, both active and inactive[43]. The modeled and the experimental structures may thus represent different but viable low energy D_3 receptor conformations, both likely inactive ones. This was also consistent with the satisfactory fidelity of the original D_3–eticlopride structure prediction that was the point of departure for this study (**Fig. 1**).

The relatively high affinities of the docking hits undoubtedly reflected the bias, even among large commercial libraries, toward molecules resembling known aminergic GPCR ligands[5,7]. It was encouraging that new chemotypes could nevertheless be discovered. This is, after all, the promise of the structure-based enterprise: that on the basis of complementarity to a protein structure, new ligands can be discovered from which unique biologies might emerge. In this sense, it was instructive to compare the results of this study, which leveraged complementarity to a modeled structure alone in ligand selection, with a study that used a pipeline of pharmacophore

Table 4 | Binding affinities for 11 analogs of compound 3 against the dopamine D_2 and D_3 receptors

Cmpd.	Structure	T_c[a]	Receptor affinity (K_i, μM)[b]	
			D_3	D_2
53		0.24	0.20	0.50
54		0.29	0.20	0.20
55		0.28	0.20	0.20
56		0.25	0.08	0.30
57		0.24	0.30	2.60
58		0.27	0.30	0.80
59		0.24	0.30	1.20
60		0.24	0.10	0.60
61		0.23	0.10	0.20
62		0.26	0.10	0.60
63		0.33	0.50	1.70

[a]The Tanimoto similarity (T_c) to the most similar dopamine receptor ligand in the ChEMBL database. [b]The uncertainty in each measured K_i is ± 30%.

filtering for dopaminergic chemotypes followed by docking[20]. Whereas this earlier study was in many ways path-breaking, had a high hit rate and high affinities, the molecules it discovered were typically much more similar to known dopaminergic ligands than those found here. This similarity can be seen by inspection of the structures and comparison to the previously known ligands (**Supplementary Table 5**), or more quantitatively by considering the ECFP4 Tanimoto coefficient values (T_c values). The T_c values averaged 0.55 to the most similar known dopaminergic molecules, whereas for the molecules discovered here the average T_c value to the nearest known dopaminergic molecule was 0.42—a large difference for this fingerprint that is borne out by visual inspection (**Table 1** and **Supplementary Table 5**).

Admittedly, the K_i of compound **3**, which was among the most unique, was only 1.6 μM, a value probably too high (poor) to be useful as a probe or lead. As a new chemotype for this target, we wondered whether its affinity could be improved. Structure-guided analog exploration led to the discovery of derivatives of **3** with up to 20-fold improved affinity (**Table 4**), which may be due to elimination of one of the ethanolic groups and exploration of small groups on the meta position of the aryl ring. The most potent of these analogs, compound **56**, had a K_i of 81 nM, which is much more in the probe range and as potent as many approved dopaminergic drugs. With only 20 non-hydrogen atoms, **56** is only slightly larger than a fragment, and thus is far from optimized; its ligand efficiency of 0.49 is promising for further elaboration[44]. This series may merit further consideration as D_3 receptor probes.

Apart from bias in the ligand libraries, docking hits against GPCRs have previously recapitulated the functional properties of the ligand with which the receptor was cocrystallized, presumably reflecting a bias in the receptor structure used. Thus, earlier structure-based screens against inactive structures of GPCRs found only antagonists and inverse agonists[5–7]. This was true here too: both the ligands discovered against the homology model and those discovered against the crystal structure were essentially antagonists. This likely reflected the inactive D_3 conformation selected by eticlopride, from among those sampled in solution, in the experimental structure and the bias toward such a structure from the modeling of a conformation competent to recognize the drug in the homology model. Additionally, most of the known ligands chosen for docking against the homology model were also antagonists.

GPCRs are central to cell signaling and are key targets for medicinal chemistry. The determination of the structures of pharmacologically relevant GPCRs illuminates why they are so fruitful for drug discovery—their orthosteric sites are particularly well-suited to accommodate small organic molecules. This and the substantial bias of chemical libraries toward the ligands of these targets explains the high potency of hits emerging from structure-based, and indeed high-throughput, screens against them[5–7]. In this sense, the docking screens against the D_3 receptor reprise what we learned from those against the β_2-adrenergic and A_{2A} adenosine receptors: hit rates are high, as are the affinities of the hits.

What was new to this study, in addition to the particular ligands discovered, was the direct, prospective comparison of the ability of homology models of GPCRs to template ligand discovery. Though the model used here was fully blinded from the crystal structure, it was ultimately as effective in prioritizing active D_3 ligands, as judged by the hit rate, potency and novelty of the new ligands. Although we did not expect this result, it was encouraging for the structure-based enterprise against GPCRs. These receptors have advantages for homology modeling: the conservation of the seven transmembrane helices and the strong conservation of several residues, such as the DRY and NPXXY motifs, allow registry in sequence alignment to be determined with greater confidence than is typically possible. At a conservative cut-off of 35% transmembrane sequence identity, the five structures determined to date resemble 59 other GPCRs[45] (**Supplementary Table 6**). Whereas each new GPCR crystal structure will provide a rich vein for ligand discovery, together their luster may reflect on a much larger number of exploitable targets.

METHODS

Homology models. The initial alignment was generated using PROMALS3D[46] using a sequence profile that included all dopamine receptor sequences as well as the β_1- and β_2-adrenergic receptor sequences (PDB: 2VT4 (chain B)[3] and 2RH1 (chain A)[2]). The initial alignment was manually refined to correctly align the residues forming the conserved disulfide bonds (Cys103–Cys181 and Cys355–Cys358). Alternative alignments of the extracellular loop 2 (EL2), which contacts the binding site[14–16], were evaluated, resulting in the final alignment (**Supplementary Fig. 2**). All homology models were built with Modeller-9v8 (ref. 33). The models were based on two types of templates: the crystal structures of the β_1[3]- and β_2[2]-adrenergic receptors and 710 elastic network models produced by 3K-ENM[32], based on each of these two crystal structures. This led to almost 200,000 homology models. These were scored using DOPE[34] resulting in 2,964 models that scored well by modeling criteria, 4 from each 3K-ENM backbone and 64 from each crystal structure backbone.

The 2,964 models were then evaluated for their ability to enrich known ligands among a large number of decoys. The models were ranked on the basis of their adjusted logAUC and the enrichment factor at 1% (EF1) of the database[39]. Models had to score in the top quartile for logAUC and EF1, and more than 60% of the best-scoring ligands had to form the conserved salt-bridge interaction with Asp110$^{3.32}$ to be considered as a final model. The conformational sampling of eticlopride was restricted to conserve the internal hydrogen bonds observed in the Cambridge Structural Database[47]. Before the release of the crystallographic structure, five modeled eticlopride–D_3 structures were submitted to the GPCRDOCK2010 competition[28]. Of these, models no. 1 and no. 4 had ligand poses and orthosteric residue positions resembling that of the crystal structure (to 1.65 Å or better).

Molecular docking screens. A version of DOCK3.5.54 with improved treatment of ligand solvation and speed, DOCK3.6 (ref. 38,39) (http://dock.compbio.ucsf.edu/), modified with scripting drawn from DOCK Blaster (http://blaster.docking.org/), was used in docking calculations against the homology model and the crystallographic structure of the dopamine D_3 receptor (PDB 3PBL[29]). The flexible-ligand sampling algorithm in DOCK3.6 superimposes atoms of the docked molecule onto binding site matching spheres, which represent favorable positions for individual ligand atoms. Forty-five matching spheres were used; for the crystal structure these were derived from the position of eticlopride, whereas the spheres for the homology models were derived from overlaid docking poses of known ligands. The degree of ligand sampling is determined by the bin size, bin size overlap and distance tolerance, set to 0.4 Å, 0.1 Å and 1.5 Å, respectively, for both the matching spheres and the docked molecules. Complementarity of each ligand pose is scored as the sum of the receptor-ligand electrostatic and van der Waals interaction energy and corrected for ligand desolvation[39]. The best-scoring conformation of each docked molecule is then subjected to 100 steps of rigid-body minimization. Partial charges from the united-atom AMBER force field were used for all receptor atoms except for Ser192$^{5.42}$, Ser193$^{5.43}$ and Ser196$^{5.46}$, for which the dipole moment was increased as previously described[5]. From the ZINC lead-like set of commercially available molecules, over 3 million compounds were docked. Prior to the selection of compounds for experimental testing, the hit list was filtered to remove a previously known high-internal-energy motif that results in unreasonably favorable docking scores[48], using automated scripts. The rankings reported here reflect this filtering (details are in **Supplementary Methods**).

Binding affinity and functional activity of the docking-predicted compounds. Affinities for D_3-dopaminergic, D_2-dopaminergic and β_2-adrenergic receptors were determined by radioligand competition binding at the National Institute of Mental Health **P**sychoactive Drug Screening Program[49]. Briefly, crude P2 (21,000*g*) membrane preparations were prepared from cell lines transiently expressing recombinant human GPCRs at about 50 μg protein per μl of 50 mM Tris, 1% BSA, pH 7.4 (assayed by Bradford method using a BSA standard). Fifty microliters of membrane suspension were added to the wells of a 96-well plate containing 100 μl of binding assay buffer, 50 μl of radioligand present at five times its K_d, and 50 μl of candidate ligand at a concentration five times that desired in the assay (**Supplementary Table 1**). Reactions were incubated for 60–90 min at room temperature (~22 °C) in the dark and then harvested onto 0.3% PEI–treated GF/A filter mats (Wallac). After three washes with ice-cold wash buffer (50 mM Tris, pH 7.4), filter mats were dried in a microwave oven and impregnated with Meltilex scintillant (Wallac). Residual radioligand binding, measured by scintillation using a TriLux microbeta counter (Wallac), was plotted as a function of competitor and regressed using 'one-site competition' in Prism 4.0 (GraphPad) to obtain IC_{50} values. K_i values were calculated from the IC_{50} values using the Cheng-Prusoff approximation.

To investigate the functional activity of the new ligands (that is, agonism or antagonism) at D_2 and D_3 receptors, we measured recruitment of β-arrestin 2 to agonist-occupied receptors using the Tango assay[50] (summarized in

Supplementary Methods). HTLA cells were transfected with plasmid encoding either the hD_2V_2 or the hD_3V_2 Tango receptor. As a negative control, cells were transfected with pEYFP-N1 (Clontech). Subsequently, the cells were trypsinized, resuspended to 1×10^4 cells per 50 µl growth medium and seeded in poly-D-lysine–coated glass-bottomed 384-well plates (Costar). The next day, the medium was replaced with serum-free DMEM (Cellgro), and the cells were stimulated with reference agonist (quinpirole), reference antagonist (chlorpromazine) or test compounds. Assay concentrations of all compounds ranged from 3 pM to 30 µM. After an overnight incubation with reference or test compounds, the medium was removed and replaced with 1× Brite-Glo (Promega). Luminescence was counted using a TriLux (PerkinElmer) plate reader. Quinpirole (Sigma-Aldrich), chlorpromazine (Sigma-Aldrich) and the test compounds were all inactive on HTLA cells not expressing a Tango receptor. In additional control experiments, HTLA cells were transfected with a plasmid encoding the human V_2 Tango receptor[50]; quinpirole and chlorpromazine had no effect on HTLA cells expressing this receptor.

Supplementary information. Additional methods, both computational and experimental, are detailed in **Supplementary Methods**. Compound sourcing and purity are also described in **Supplementary Methods. Supplementary Results** include the sequence alignment used during modeling, the results of the functional assays, a table describing GPCRs with high sequence identity and known ligands, and all compounds tested for both screens and analogs are presented. The top lists produced by each docking screen are also presented in their entirety as **Supplementary Data Set 1**. Coordinates for the discovered ligands docked to the homology model and crystal structure are also supplied as **Supplementary Data Set 2**.

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Author contributions

Docking and homology modeling was conducted by J.C. and R.G.C., the latter with assistance from H.F., A. Schlessinger and A. Sali, J.J.I. and B.K.S. assisted with compound selection and strategy, J.J.I. lent expertise with the DOCK Blaster toolchain and fixed problems with ZINC. B.L.R. and V.S. were responsible for the pharmacological guidance as to the appropriate specificity tests, while V.S. conducted all the experiments. All authors contributed to the writing of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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