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Ligand Discovery for a Peptide-Binding GPCR by Structure-Based Screening of Fragment- and Lead-Like Chemical Libraries

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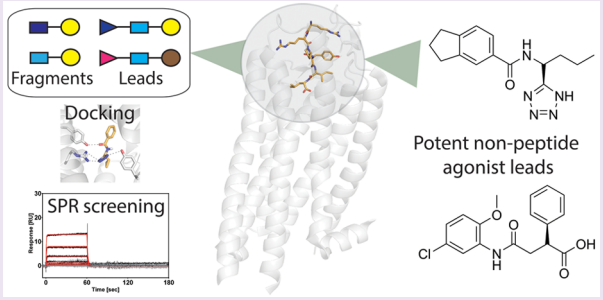
Supporting Information

ABSTRACT: Peptide-recognizing G protein-coupled receptors (GPCRs) are promising therapeutic targets but often resist drug discovery efforts. Determination of crystal structures for peptide-binding GPCRs has provided opportunities to explore structure-based methods in lead development. Molecular docking screens of two chemical libraries, containing either fragment- or lead-like compounds, against a neurotensin receptor 1 crystal structure allowed for a comparison between different drug development strategies for peptide-binding GPCRs. A total of 2.3 million molecules were screened computationally, and 25 fragments and 27 leads that were top-ranked in each library were selected for experimental evaluation. Of these, eight fragments and five leads were confirmed as ligands by surface plasmon resonance. The hit rate for the fragment screen (32%) was thus higher than for the lead-like library (19%), but the affinities of the fragments were ~100-fold lower. Both screens returned unique scaffolds and demonstrated that a crystal structure of a stabilized peptide-binding GPCR can guide the discovery of small-molecule agonists. The complementary advantages of exploring fragment- and lead-like chemical space suggest that these strategies should be applied synergistically in structure-based screens against challenging GPCR targets.



G protein-coupled receptors (GPCRs) constitute the largest superfamily of eukaryotic membrane proteins and play important roles in essential physiological processes.[1] Identification of small-molecule ligands that modulate GPCR signaling has been crucial for understanding receptor function and contributed to the development of numerous drugs. Ligand discovery has been particularly successful for GPCRs that have evolved to recognize small molecules, facilitating the generation of potent drug-like compounds.[2] Many of the GPCRs that remain unexplored as therapeutic targets are peptide- or protein-binding receptors. In these cases, identification of lead candidates has been more challenging due to the difficulties involved in developing small-molecule ligands based on the endogenous compound, which is a major hurdle for discovery of novel GPCR drugs.[3,4]

Recent advances in molecular and structural biology for membrane proteins have provided new avenues for drug development. Breakthroughs enabling stabilization of GPCRs for crystallography have led to the determination of high-resolution structures for pharmaceutically important receptors that recognize monoamine neurotransmitters.[5] Structure-based screens for ligands of these GPCRs have been remarkably successful, resulting in the discovery of potent leads to several therapeutic targets.[6−9] The recent crystallization of peptide-recognizing GPCRs provides opportunities for computer-aided ligand design guided by the binding site rather than by traditional techniques based on the natural agonist or mimetics thereof.[10,11] However, similar to protein−protein interfaces of soluble targets, identification of nonpeptide ligands to peptide-binding GPCRs is difficult, and it is particularly challenging to identify small-molecule agonists, as such compounds must mimic relevant interactions made by the significantly larger endogenous peptides to elicit the same conformational changes in the receptor.[12]

Lead discovery by library screening largely relies on two strategies based on fundamentally different philosophies. High-throughput screening (HTS) has been widely used to identify starting points for the development of pharmaceuticals.[13] However, screening campaigns using chemical libraries containing up to millions of drug-like compounds are costly

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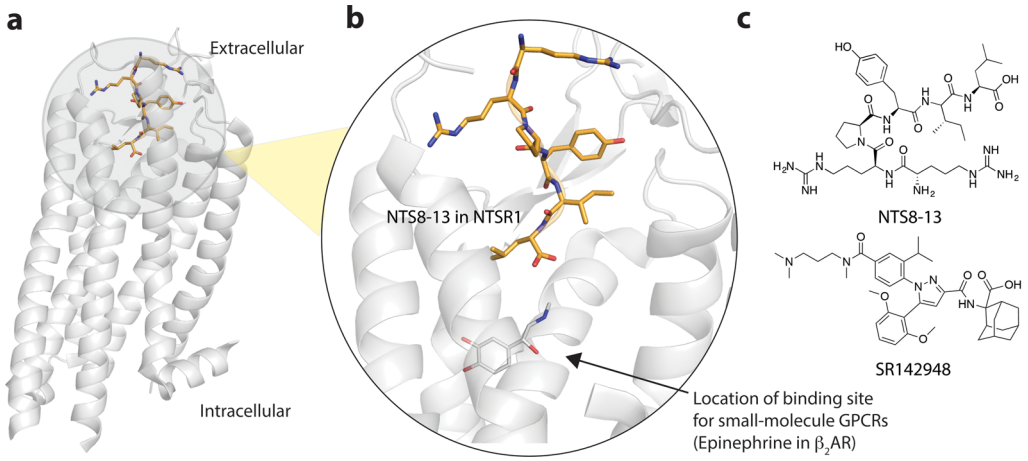

Figure 1. Crystal structure of NTSR1, comparison of binding sites for peptide and small-molecule binding GPCRs, and reference ligands of NTSR1. (a) The NTSR1 crystal structure (PDB accession code: 4BUO, white cartoon) in complex with agonist NTS8-13 (shown as sticks with carbon atoms in gold). (b) Binding mode of NTS8-13 in the NTSR1 binding site. The location of the binding site for a representative small-molecule binding GPCR, the β_2 adrenergic receptor (β_2AR), is shown to highlight the difference in the size of the endogenous ligands and the location of the orthosteric sites. The cocrystallized β_2AR ligand, epinephrine, is shown in lines with carbon atoms in white. (c) 2D representations of NTS8-13 and SR142948.

and often result in low hit rates. Fragment-based drug discovery (FBDD) has emerged as a promising alternative to HTS.[14,15] FBDD relies on the idea that hit rates from libraries with low molecular weight (MW) compounds (<250 Da) will be higher than those from the drug-like compound sets used in HTS, due to better coverage of chemical space and reduced probability of steric mismatches with the binding site. Discovered (low affinity) fragment ligands are then elaborated to reach activities necessary for biological effects. In the first step of FBDD, biophysical methods are used to screen small libraries for ligands that occupy subpockets within a binding site. However, the use of fragment screening for GPCRs is still nascent due to the lack of sensitive cellular assays required to detect low affinity ligands. Stabilization of GPCRs for crystallization has also enabled development of fragment screening by biophysical methods applied to the purified receptor such as surface plasmon resonance (SPR)[16,17] and NMR.[18] SPR and NMR screening of fragment libraries, in some cases complemented by *in silico* approaches, have been successful for small-molecule binding GPCRs.[19,20] However, few studies have focused on peptide- or protein-binding targets, and to our knowledge, screens of fragment-[17] and lead-like[21] libraries have never been compared directly.

Access to a neurotensin receptor 1 (NTSR1) construct suitable for crystallization[22] and biophysical screening provided us with the opportunity to explore strategies for structure-based ligand discovery against peptide-binding GPCRs and address several questions relevant for drug development. First, can the success of molecular docking screening be extended beyond highly druggable small-molecule binding GPCRs? NTSR1 signals in response to a 13 amino-acid-long peptide and is a drug target for CNS diseases (e.g., Parkinson's disease and schizophrenia)[23,24] and analgesia.[25] However, only a few nonpeptide ligands have been identified.[26−30] Second, we wished to compare the results from structure-based computational screening of fragment- and lead-like libraries against a GPCR. What hit rates, ligand affinities, and chemotypes can be expected from docking screens of different libraries? If ligands emerge from the screens, can crystal structures guide optimization to potent leads, and what functional profiles will these show? To probe these questions, prospective docking screens of fragment- and lead-like libraries against a NTSR1 crystal structure were performed. Top-ranked compounds from each library were experimentally evaluated, followed by structure-guided ligand optimization. The implications of our results for drug development strategies against challenging GPCR targets will be discussed.

RESULTS AND DISCUSSION

Docking Screens of Fragment- and Lead-Like Libraries. Docking screens were carried out against a high-resolution crystal structure of NTSR1 that had been determined in complex with a truncated version of the natural peptide agonist, NTS8-13 (Figure 1a).[22] NTS8-13 spans multiple subpockets of the relatively shallow orthosteric site, violates most of Lipinski's rules of five,[31] and is 3-fold larger than a fragment (Figure 1b,c). In order to identify small-molecule starting points for lead development, two chemical libraries of commercially available compounds were screened computationally using DOCK3.6.[32] The fragment (MW < 250 Da) and lead-like (250 < MW < 350 Da) libraries from the ZINC database[33] with 0.5 and 1.8 million commercially available compounds, respectively, were docked to the most buried subpocket of the orthosteric site, which recognizes the C-terminal end of NTS8-13 (Figures 1 and 2). Several thousand orientations were sampled for each of the 2.3 million docked molecules using a flexible ligand algorithm[32] with the receptor held rigid, resulting in billions of predicted complexes. For each library, the 500 top-ranked compounds were inspected visually to identify candidates for experimental testing, taking into account terms neglected by the docking scoring function, such as receptor desolvation and internal energy.[6,19,21] Almost all of the top-ranked compounds had negatively charged moieties that anchored the molecules in the binding site *via* a salt bridge with Arg327$^{6.54}$. The lead-like compounds typically filled multiple subpockets of the orthosteric site, whereas the fragments tended to occupy these with smaller groups. The median number of heavy atoms (HAs) for the top-ranked compounds was 17 and 23 for the fragment- and lead-like compounds, respectively. Finally, a set

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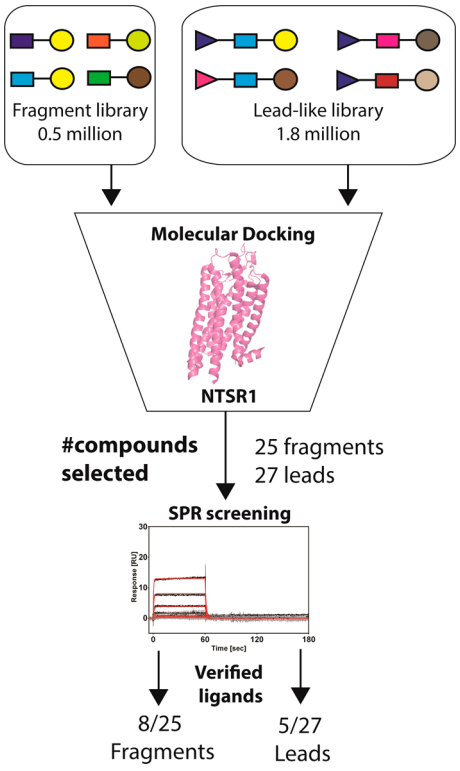

Figure 2. Docking screens of fragment- and lead-like libraries against NTSR1. Fragment- and lead-like chemical libraries were docked to a high-resolution crystal structure of NTSR1. Top-ranked compounds (25 fragments and 27 leads) were screened experimentally using SPR, yielding eight fragment- and five lead-like ligands.

of 52 compounds (**1**−**52**), consisting of 27 leads (**1**−**27**) and 25 fragments (**28**−**52**), was selected for experimental evaluation (Table S1, Figure 2).

SPR Screening of Predicted Ligands. The 52 selected compounds were evaluated experimentally using SPR. After immobilization of NTSR1 onto the sensor chip, integrity and binding activity of the receptor were verified by injections of a mutated NTS8-13 peptide (NTS8-13−A11A12) and SR142948 (Figure S1). The 27 leads were evaluated at a single concentration of 50 μM, whereas the 25 fragments were assayed at a 10-fold higher concentration, which corresponded to conditions typical of HTS and fragment screening. Nonspecific interaction with the surface used for immobilization of NTSR1 was analyzed by including a blank reference without any receptor immobilized. The resulting sensorgrams were inspected, and 18 out of the 52 compounds (**1**−**5**, **28**−**40**) were selected for further evaluation in dose−response experiments. In order to assess specificity for the orthosteric binding site, these compounds were also tested on immobilized NTSR1 where the orthosteric binding site had essentially been blocked by pretreating with NTS8-13. Five leads (**1**−**5**) and eight fragments (**29**−**36**) showed dose-dependent responses and a clearly observable impairment of binding at the highest tested concentrations when analyzed on NTSR1 blocked with NTS8-13. These compounds thus behaved as orthosteric ligands, as predicted by docking. The remaining five compounds (**28**, **37**, **38**, **39**, and **40**) were all fragments and did not show any significant reduction of binding to the blocked receptor, which suggested that these were allosteric ligands. This screening result corresponded to hit rates (defined as the percentage of orthosteric ligands among those experimentally evaluated) of 19% and 32% from the lead- and fragment-like libraries, respectively. It should be noted that a few of the tested compounds were at the border between our MW-based definition of fragment- and lead-like compounds. However, if the results were analyzed using a HA-based definition of the two sets and without borderline cases (fragment-like compounds ≤17 HAs and lead-like compounds ≥23 HAs), the difference in hit rate was only marginally affected. Binding affinities for the five lead-like hits were estimated based on SPR measurements using up to 30 μM of the ligand (Figure S2), as at higher concentrations increasing levels of nonspecific binding were observed. The K_D values for the leads ranged from 1.2 to 42 μM, based on kinetic fits, and these affinities were essentially identical to those obtained using equilibrium analysis (Table 1). The eight fragment-sized ligands showed ∼100-fold weaker binding, but reliable K_D values could not be determined because saturation was not reached. Approximate K_D values for the fragments were estimated to range between 0.2 and 0.3 mM based on kinetic fits. The ligand efficiencies[34] (LE, defined as $-RT\ln(K_D)/N$, where K_D is the dissociation constant and N is the number of HAs), a metric for assessing ligands of different size, ranged from 0.28 to 0.34 kcal mol^{-1} atom^{-1} for the fragments (based on the approximate kinetic K_D values) and from 0.24 to 0.42 kcal mol^{-1} atom^{-1} for the leads. Predicted binding modes for discovered ligands are shown in Figure 3, and the experimental data for the lead- and fragment-like ligands are summarized in Tables 1 and 2.

The novelty of the discovered ligands was quantified based on Tanimoto similarity using ECFP4 fingerprints.[35] The similarity between each ligand identified in this work and all compounds in the ChEMBL21 database[36] that had been experimentally evaluated at NTSR1 was calculated. The Tanimoto similarity coefficient (T_c) is equal to 1 for two identical compounds, whereas values close to zero are obtained for unrelated molecules. The fragment ligands had T_c values ranging from 0.23 to 0.32, whereas the leads had somewhat higher similarity coefficients to previously evaluated compounds (T_c = 0.30−0.45). The compounds with the maximal T_c values are shown in Tables S2 and S3. Judged by visual inspection of the closest ligands, the least novel fragments contained a C-terminal amino acid connected to either aliphatic or aromatic rings and docked in an orientation similar to the cocrystallized peptide (Figure 3). Similarly, compound **1** from the lead-like library contained the substructure of a C-terminal leucine and was most similar to partial agonists (T_c = 0.45) discovered *via* ligand-based virtual screening.[27] However, there were also examples of novel chemotypes from each library. The lead-like compounds **2** and **3** did contain a carboxylate connected to an amide moiety but were topologically dissimilar to previously identified NTSR1 ligands. The fragment-sized ligands **34** and **36** were also considered to be novel, as these were both dissimilar to a C-terminal peptide and to the closest compound from the ChEMBL21 database. A subset of the experimentally evaluated fragment- and lead-like compounds was devoid of a carboxylate group and predicted by docking to be anchored in the site by other negatively charged groups, *e.g.*, phospinate and tetrazole moieties. Only one of these compounds, a tetrazole-containing fragment (**34**) with weak and specific binding to NTSR1 at high concentration, was identified as a ligand.

To investigate if the scaffolds represented by the discovered fragments or lead-like ligands could have been identified from

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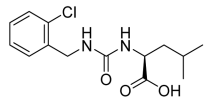
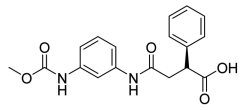
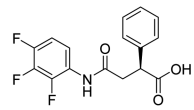
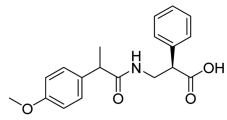
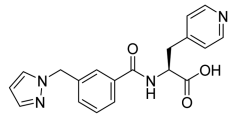
Table 1. Ligands Identified from the Docking Screen of a Lead-Like Library

ID	Docking rank[a]	Ligand structure	$K_{D,kinetic}$ (μM)[b]	$K_{D,equilibrium}$ (μM)[c]	LE[d]	2D similarity (T_c)[e]
NTS8-13-A11A12	-	-[f]	0.043	-	0.20	-
SR142948	-	-[f]	0.0004	-	0.27	-
1	143		1.2 ± 0.09	1.0 ± 0.1	0.42	0.45
2	291		1.6 ± 0.03	2.8 ± 0.4	0.33	0.38
3	165		2.1 ± 0.04	1.9 ± 0.4	0.35	0.30
4	148		18 ± 0.11	17.6 ± 1.5	0.28	0.36
5	157		42 ± 3.44	41.5 ± 6.9	0.24	0.34

[a]Rank from docking screen of commercially available leads from the ZINC database. [b]K_D from kinetic analysis. Calculated errors represent the SD from duplicates. [c]K_D from equilibrium analysis. Calculated errors represent the SD from duplicates. [d]LE (kcal mol^{-1} atom^{-1}) calculated as $-RT \ln(K_{D,kinetic})/N$, where N is the number of ligand HAs. [e]The maximal Tanimoto coefficient (ECFP4) when compared with all compounds tested at NTSR1 in the ChEMBL21 database. [f]2D structures for NTS8-13 and SR142948 are shown in Figure 1.

both libraries, the docking screens were analyzed in more detail. For the eight fragment ligands (**29**−**36**), molecules that represented superstructures of these in the lead-like set from the ZINC database were identified.[33] Similarly, we identified fragment-like compounds that were substructures of the five discovered lead-like ligands (**1**−**5**). There were 1872 compounds in the lead-like library that represented superstructures of the eight fragment ligands. However, none of these were among the 500 top-ranked compounds considered for experimental evaluation. In fact, even if an order of magnitude more compounds had been considered, only 18 compounds (representing five of the eight discovered ligands) would have been top-ranked. Conversely, there were 529 molecules from the fragment library that were substructures of the five lead-like ligands, and one of these was ranked high enough to be considered for experimental evaluation. The 16 fragments (representing all five discovered lead-like ligands) that reached the top 5000 by docking rank possessed a carboxylate moiety that anchored the compound in the site, whereas the compounds with the worst ranks lacked this characteristic. To summarize, the discovered ligand chemotypes were typically available in both the fragment- and lead-like libraries but were often only top-ranked in one of these.

Fragment and Lead Optimization. To probe the tractability of structure-guided optimization for fragments and leads, one nonpeptide chemotype from each library was further elaborated. The tetrazole-containing compound **34** was selected from the fragment screen as it represented an unusual ligand chemotype. From the lead-like ligands, compounds **2** and **3** were selected, as these represented the most novel scaffold from this set.

The fragment selected for optimization (**34**) was a weakly binding compound (approximate K_D = 0.2 mM) but showed a considerable loss of binding for the blocked receptor at high concentration, supporting that it was an orthosteric ligand. The tetrazole group of compound **34** was predicted to form a salt bridge with Arg327$^{6.54}$ and a hydrogen bond to Tyr146$^{3.29}$, while the amide group formed a hydrogen bond to the side

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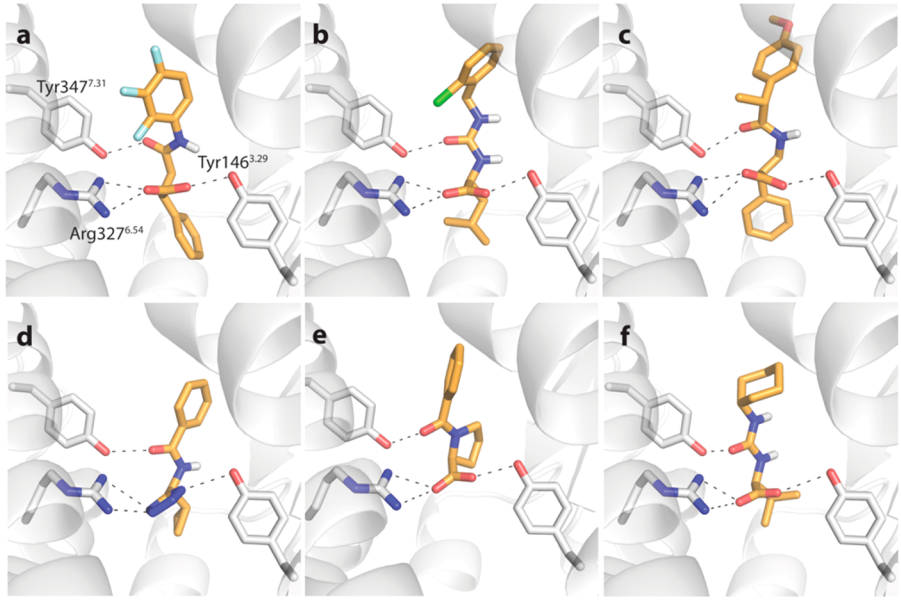

Figure 3. Predicted binding modes for six discovered ligands. Top panel: Docking poses for the lead-like ligands (a) **3**, (b) **1**, and (c) **4**. Bottom panel: Docking poses for fragment ligands (d) **34**, (e) **30**, and (f) **29**. The ligands are shown as sticks with carbon atoms in gold. NTSR1 is shown as a white cartoon, with key residues in sticks and carbon atoms in white. Hydrogen bonds are indicated with black dashed lines.

chain of Tyr347$^{7.31}$ (Figure 3d). The cyclopropyl group was positioned in a hydrophobic cleft created by Met208$^{4.64}$, Phe331$^{6.58}$, and Leu234$^{5.35}$, whereas the phenyl ring was predicted to form an edge-to-face interaction with Phe128$^{2.65}$. On the basis of molecular docking, 14 analogs (**53**–**66**, Table S4 and Figure S3) were selected for experimental evaluation. A majority of the analogs turned out to be NTSR1 ligands, and as these had up to ∼45-fold higher affinities compared to the original fragment hit (**34**), K_D values could be determined more accurately (Figure S3). In addition, binding was almost completely abolished in the experiments carried out for NTSR1 blocked with NTS8-13, demonstrating that the compounds were orthosteric ligands. Compound **65** had the highest affinity (K_D = 4.4 μM), corresponding to an LE value of 0.36 kcal mol^{-1} atom^{-1} (Figure 4a).

The two ligands from the lead-like library (**2** and **3**) shared a 4-oxo-2-phenyl-4-[(phenyl)amino]-butanoic-acid scaffold. These compounds were predicted to be anchored in the site by phenyl and carboxylate groups, and the amide moiety formed a hydrogen bond to the side chain of Tyr347$^{7.31}$ (Figure 3a). A series of 11 analogs (**67**–**77**, Table S5), with different substituents on the 4-phenyl ring and alternative hydrophobic anchors, was evaluated experimentally. Several analogs had submicromolar binding affinities (Table S5 and Figure S2), and the SPR data for compound **75** (0.6 μM, LE = 0.39 kcal mol^{-1} atom^{-1}) are shown in Figure 4b,d.

Functional Assays for Discovered Ligands. The fragment and lead compounds selected for optimization (**2**, **3**, and **34**) along with their analogs (**53**–**77**) were evaluated in functional assays for their ability to elicit an intracellular signaling response by measuring changes in levels of cAMP (G_s pathway) or inositol monophosphate (IP1, a metabolite of inositol trisphosphate, IP3, G_q pathway). Both the G_s and G_q pathways are known to be activated by the natural peptide agonist NTS, with preference for activation of the G_q mediated signaling.[37,38] The ability of the compounds to stimulate signaling was assessed in HEK293 cells stably expressing the rat NTSR1 wild-type receptor. Stimulation of G_s- and G_q-mediated signaling *via* NTSR1 was tested at a single concentration of 100 μM for each compound. None of the tested compounds resulted in a measurable increase of cAMP through activation of the G_s pathway at this concentration. In contrast, the series of compounds based on **2** and **3** (**67**–**75**) and one tetrazole analog (**65**) displayed agonistic activity *via* the G_q/IP3 pathway. The most potent ligands from each series, compounds **65** and **75**, had EC_{50} values of 228 and 68 μM, respectively (Figure 4e,f). Both these ligands were also evaluated in competition experiments, in which NTSR1 signaling *via* the G_q/IP3 pathway was measured in the presence and absence of the antagonist SR142948 (Figure 5a,b). The addition of SR142948 reduced the response to a background level for compounds **65**, **75**, and the control peptide NTS8-13, as expected for competitive antagonism.

Discussion. The main goal of this study was to assess structure-based screening strategies for peptide-binding GPCRs. Prospective molecular docking screens of chemical libraries containing either fragment- or lead-like compounds were performed to identify ligands of the neuropeptide receptor NTSR1, a challenging therapeutic target relevant for drug development against Parkinson's disease and schizophrenia.[23,24] Top-ranked compounds from the virtual screens were experimentally evaluated by SPR and in functional assays. Three key results emerged from these experiments. First, high hit rates were obtained for fragments and leads, respectively, with new chemotypes identified from both libraries. Second, structure-guided optimization was successful for fragments as well as lead-like ligands. A new scaffold from the fragment screen was optimized to an affinity of 4 μM, an approximately 45-fold improvement compared to the parent ligand. Similarly, a chemotype from the lead-like library was optimized to submicromolar affinity. Finally, the two optimized scaffolds were evaluated in functional assays, and small-molecule agonists were obtained from both series.

The rapid advancements in GPCR structural biology have ushered in a new era in rational drug design for these important therapeutic targets. A majority of available GPCR crystal

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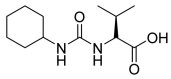
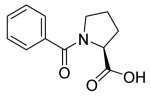
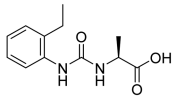
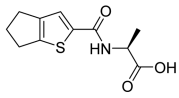
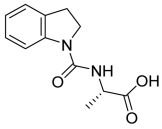
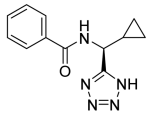
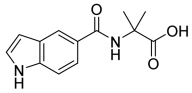
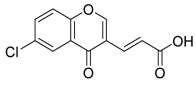
Table 2. Ligands Identified from the Docking Screen of a Fragment Library[a]

ID	Docking rank[b]	Ligand structure	2D similarity (T_c)[c]
29	337		0.32
30	388		0.30
31	81		0.32
32	308		0.27
33	80		0.29
34	317		0.28
35	491		0.23
36	78		0.29

[a] K_D values for the fragments could not be determined. Approximate K_D values from kinetic analysis range from 0.2 to 0.3 mM. [b] Rank from docking screen of commercially available fragments from the ZINC database. [c] The maximal Tanimoto coefficient (ECFP4) when compared with all the compounds tested at NTSR1 in the ChEMBL21 database.

structures belong to the group of small-molecule binding receptors that have already been successfully targeted in drug discovery.[39] These GPCRs have proved to be excellent targets for structure-based methods, and docking screens of fragment libraries have been fruitful.[19,40−42] Empirical screens of fragment libraries have also identified very potent ligands of small-molecule binding GPCRs,[18,20] but with significantly lower hit rates than molecular docking, demonstrating the potential of structure-based approaches for such targets. The excellent results obtained for small-molecule binding GPCRs can partly be attributed to their binding sites having evolved to recognize fragment-sized compounds and a bias toward ligand-like chemotypes in commercial chemical libraries.[7,43] Recently, crystal structures of GPCRs that signal in response to peptides and proteins have been determined, in some cases even with peptides bound,[22,44,45] providing opportunities to design novel ligands to targets such as NTSR1.[46,47] Several of the peptide-binding GPCRs have been recognized as attractive but difficult targets for drug development, which is likely due to their large and often solvent exposed binding sites.[11] In the case of NTSR1, the challenges involved in the discovery of small molecule ligands were clearly exemplified by a recent HTS campaign of 332 000 compounds, which resulted in a hit rate of <0.06%, and only one of the discovered ligands was considered suitable for further elaboration.[26] Similarly, a ligand-based virtual screen identified two drug-sized ligands with modest functional activity after experimental evaluation of 170 compounds.[27] These observations are consistent with our findings that the affinities and functional activity of ligands identified from the lead-like library were lower than those obtained for small-molecule binding receptors.[7,8,43] Similar results have been obtained from prospective docking screens against the protein/peptide-binding CXCR4 and κ-opioid receptors.[21,48] Fragment screening has been proposed as an alternative strategy for targets where HTS of lead-like libraries has fallen short, including protein- and peptide-binding interfaces of soluble proteins.[49] However, the accuracy of molecular docking for fragment-sized compounds has been questioned as scoring functions were developed for drug-like molecules.[15] To evaluate computational strategies for structure-based lead discovery for challenging targets, we carried out docking screens of two compound libraries against NTSR1, a peptide-binding GPCR with few known small-molecule ligands.[26−30] The unique aspect of our study was not only that it was the first structure-based screen of fragment libraries against a peptide-binding GPCR but also that the efficiency of FBDD was directly compared to results from a lead-like library. As ligands were discovered from both screens, the strengths and weaknesses of these widely used strategies in drug development could be probed.

An interesting result from the screens was the difference in hit rate for the fragment- and lead-like library. However, the somewhat higher hit rate obtained for fragments was achieved at the expense of binding affinity, which was approximately 100-fold lower. Judged by the less size-dependent LE metric, the discovered ligands represented promising starting points for further elaboration irrespective of the library from which they originated. A majority of the discovered lead-like ligands had LE values >0.3 kcal mol^{-1} $atom^{-1}$, which is considered a promising starting point for optimization[34] and is also higher compared to reference antagonists of NTSR1, *e.g.*, SR142948 (LE = 0.27 kcal mol^{-1} $atom^{-1}$, Figure 1).[30] On the basis of the approximate K_D values for the fragments, the ranges of LE values for the ligands from the two screens appeared to be overlapping. Considering that LE rarely improves during optimization[34,50] and the challenges involved in fragment optimization, the high-affinity leads may be preferred as starting points for further elaboration. Taken together, the results suggest that, whereas screens of lead-like libraries offer the possibility to identify attractive starting points for drug development, fragment screening may be the preferred strategy for the most challenging targets. In such cases, the hit rates for larger (lead- or drug-like) compounds could approach zero while fragment screens may still be successful, as previously observed for a soluble enzyme.[51] The encouraging result from this study was that discovery of small-molecule ligands of a peptide-recognizing GPCR was within reach for molecular docking from both fragment- and lead-like libraries.

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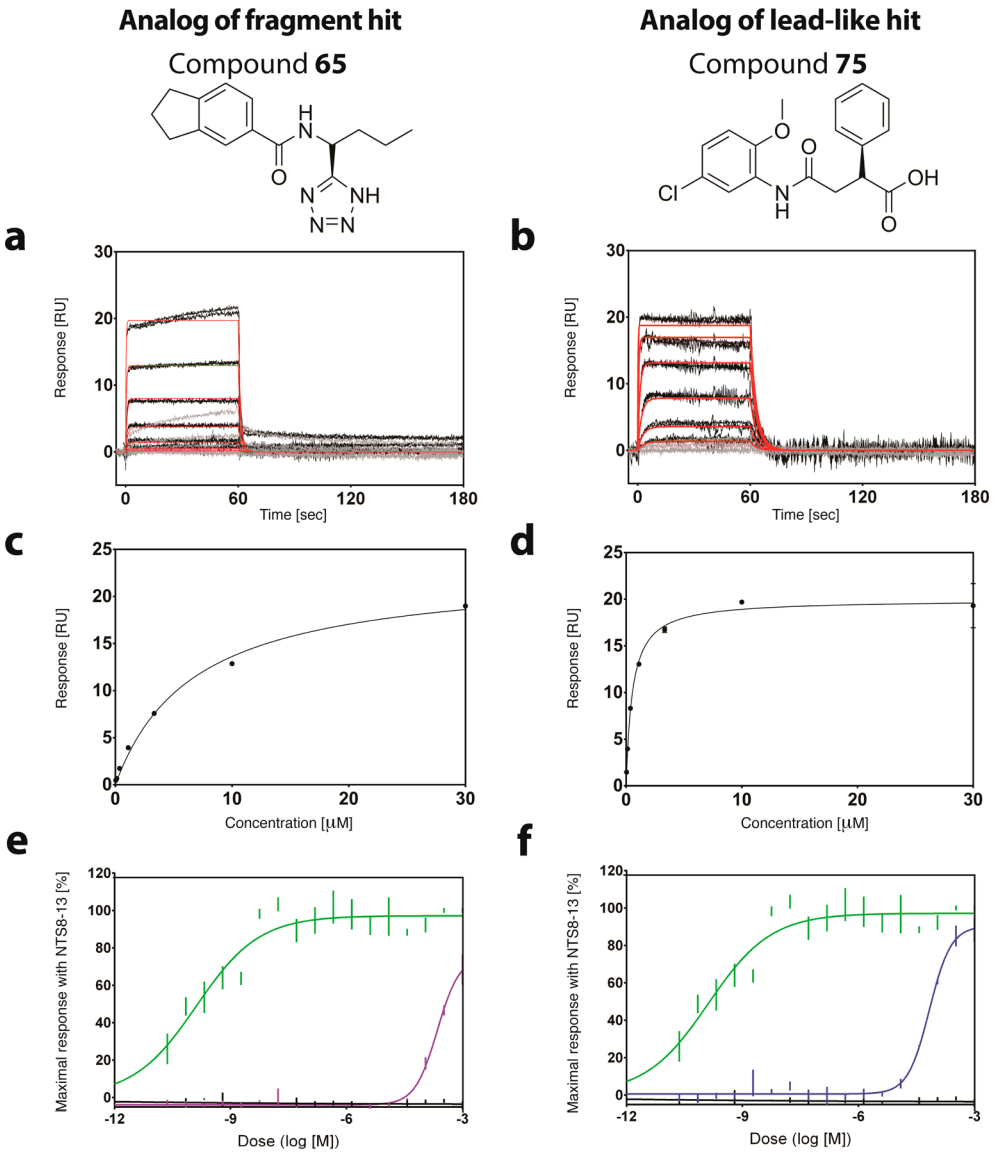

Figure 4. Binding and functional data for compounds **65** and **75**. (a,b) SPR binding curves on a free (black) and agonist-blocked (gray) receptor surface. Red lines represent kinetic fits using the 1:1 Langmuir binding model. (c,d) Corresponding equilibrium fits. (e,f) Dose-dependent stimulation of IP1 production (a metabolite of IP3) in HEK293 cells for compounds **65** (purple) and **75** (blue), the peptide agonist (NTS8-13, green), and an antagonist (SR142948, black). All data points represent mean ± SEM from measured duplicates. Curves were fitted using a nonlinear regression curve fit with a variable slope (four parameters) in GraphPad Prism.

Another important metric of success for library screening is the novelty of the discovered ligands, and encouragingly new chemotypes emerged from both fragment- and lead-like chemical space. In addition, unique ligands were identified from each screen, reflecting that the top-ranked compounds only partially overlapped in terms of chemotypes. A more detailed analysis of both screening libraries revealed that lead-like compounds representing superstructures of the fragment ligands were typically available in commercial chemical space. However, in agreement with the notion of FBDD,[52] the lead-like superstructures frequently had substituents that prevented optimal receptor−ligand complementarity, resulting in unfavorable docking energies. This observation is consistent with the idea that the smaller size of fragments will result in higher hit rates, leading to the discovery of scaffolds unlikely to emerge from lead-like libraries. An illustrative example was the discovered tetrazole scaffold. None of the seven tetrazole-containing molecules selected from the lead-like library were identified as ligands by SPR, whereas a tetrazole fragment (**34**) was discovered and optimized to a high-affinity lead (**65**). Although compound **65** was ranked 2806 (top 0.1%) in the lead-like library, it could not have been discovered from this screen as it was outside the group of 500 top-ranked molecules considered for experimental evaluation. In light of the higher hit rates from the fragment screen, another relevant question is, could the scaffolds represented by the lead-like ligands have been identified from the fragment library? The fact that only one out of 529 commercially available compounds representing substructures of the five discovered lead-like ligands was ranked high enough to be considered for experimental evaluation suggests that this is not the case. We attribute this to the fact that most substructures lack key moieties for ligand binding and are thus likely to be inactive, as previously observed in experimental fragment screening studies.[53]

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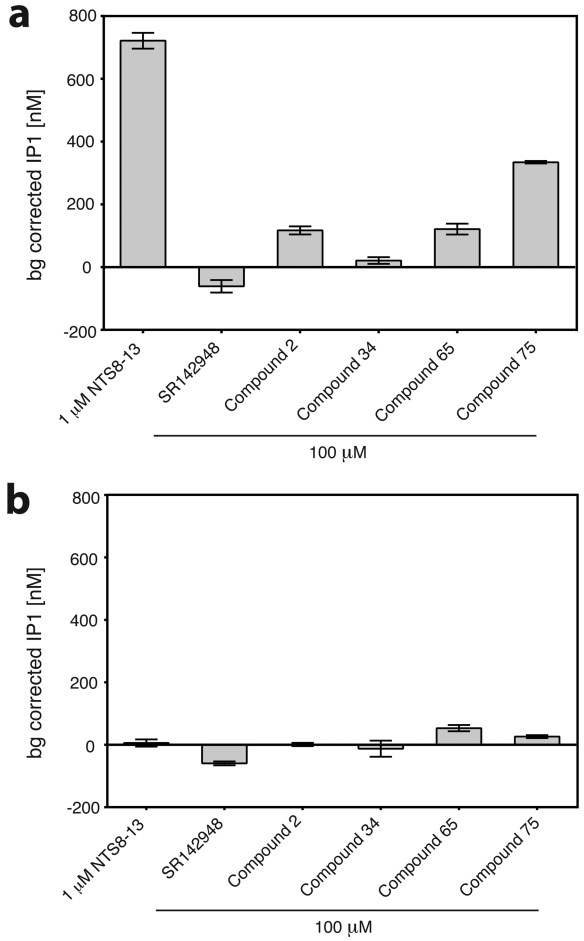

Figure 5. Functional competition experiments for reference and discovered ligands. Signaling assays measuring IP1 production in HEK293 cells for NTS8-13 (1 μM); SR142948 (100 μM); and compounds **2**, **34**, **65**, and **75** (100 μM) in the (a) absence and (b) presence of the antagonist SR142948 (100 μM, cells preincubated), corrected for background (bg) signal.

Successful application of FBDD relies on efficient optimization of weak ligands to potent leads. The discovery of both fragment- and lead-like ligands provided an opportunity to probe if optimization could be guided by predicted binding modes. This is particularly important for GPCR targets as structure determination for screening hits is still very difficult, considering their modest affinities and lower contribution to receptor stability, making full receptor occupancy challenging under crystallization conditions. One chemotype from each library was taken forward, and initially, the leads (**2** and **3**) were several orders of magnitude more potent than the fragment (**34**). Optimization of the fragment containing a tetrazole led to ligands with affinities similar to those of the leads, and subsequent to our docking screens, more potent NTSR1 agonists utilizing this bioisostere have also been described in the literature.[29] A series of analogs of a ligand from our screen of the lead-like library also improved the affinity of this series, yielding submicromolar leads. Compounds **65** and **75** have excellent LE values (0.36 and 0.39 kcal mol^{-1} $atom^{-1}$, respectively), suggesting great potential to further progress both these leads.

One of the challenges in lead discovery for GPCRs is that ligands with particular functional effects are sought. In the case of peptide-binding receptors, identification of agonists has proven particularly difficult and, as leads identified from HTS tend to be antagonists, drug discovery efforts have primarily focused on modification of peptides.[12] Access to crystal structures for peptide-binding GPCRs could open up new avenues for ligand discovery, and two recent prospective docking screens have identified small-molecule agonists of μ- and κ-opioid receptors.[48,54] For these reasons, we were interested in what functional properties the fragment- and lead-like compounds would show. The lead-like scaffold selected for optimization and many of its derivatives activated G_q protein signaling, demonstrating that the conformation captured by the crystal structure of a stabilized peptide-binding GPCR can enable discovery of nonpeptide agonists. However, the tetrazole identified from the fragment library did not show any effect in functional assays. This result demonstrates that access to a sensitive biophysical assay was critical for discovery of this scaffold. Furthermore, as fragments will typically have too low affinity to show functional activity, such data cannot be used to prioritize which compounds to elaborate. Instead, resources have to be invested to optimize fragments in the absence of this information. In the case of the tetrazole scaffold, optimization efforts proved fruitful, as the ligand with the highest affinity in this series was an agonist. Remarkably, two scaffolds with agonistic activity were thus discovered despite the fact that these were less than half the size of the cocrystallized peptide ligand.

Molecular docking offers the opportunity to screen millions of compounds at low expense. A total of 2.3 million molecules were screened *in silico* against NTSR1, a number that is currently out of reach for most experimental campaigns but can be evaluated by molecular docking in less than 24 h on a supercomputer cluster. Virtual screening of fragment- and lead-like tranches of chemical space have distinct advantages over their experimental counterparts. Compared to costly HTS campaigns of drug-like libraries, prefiltering with docking can reduce the number of compounds that need to be evaluated and drastically increases hit rates. Whereas empirical fragment screening, at least for soluble targets, is becoming widely available at reasonable cost, the docked library of >500 000 fragments is several orders of magnitude larger than those considered experimentally, offering improved coverage of chemical space.[55] The complementary advantages observed for docking screens of fragment- and lead-like libraries suggest that these techniques should be used in parallel. Fragment screens will provide higher hit rates, but it still remains very challenging to detect weakly binding ligands experimentally, and optimization will be required to uncover the true potential of each discovered scaffold. Screens of lead-like libraries will return fewer ligands, but each hit may represent a stronger starting point for drug development. The fact that both screening strategies were successful, even for a peptide-binding site, suggests that the greatest impact of the revolution in structural biology is to facilitate lead discovery for difficult targets and thereby expand the druggable GPCRome.

METHODS

Molecular Docking Screening. All docking calculations were carried out with the program DOCK3.6[32] against a high-resolution crystal structure of NTSR1.[22] Sets of commercially available compounds from the ZINC database[33] were used in the prospective screens. The fragment- (molecular weight $\leq$ 250, log P $\leq$ 3.5, and rotatable bonds $\leq$ 5) and lead-like (250 < molecular weight $\leq$ 350, log P $\leq$ 3.5, and rotatable bonds $\leq$ 7) libraries contained 0.5 and 1.8

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million unique compounds, respectively. For detailed computational methods, see the Supporting Information Methods.

SPR Screening and Functional Assays. The rat NTSR1 variant NTSR1-H4 was first expressed in *E. coli* using a derivative of the vector pRG/III-hsMBP. This construct had a C-terminally fused Avi-tag, which, due to its cytoplasmic location, is biotinylated *in vivo* in *E. coli*.[22] The incorporation of biotin allowed a direct coupling of the purified receptor on a streptavidin-coated surface. All surface plasmon resonance (SPR) measurements were performed on a Biacore T100 instrument. Screening was performed against immobilized free receptor and blocked receptor (bound to NTS8-13), as well as a blank reference surface. For functional assays, HEK293 cells stably expressing rat NTSR1 wild-type were used. For detailed experimental methods, see the Supporting Information Methods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00646.

Detailed materials and methods concerning molecular docking, similarity calculations, SPR, and functional assays, Supporting Tables S1−S5 and Figures S1−S3 (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GPCR, G protein-coupled receptor; HTS, high throughput screening; FBDD, fragment-based drug discovery; NTSR1, neurotensin receptor 1; SPR, surface plasmon resonance; MW, molecular weight; HA, heavy atom; LE, ligand efficiency; ECFP4, extended chemical fingerprints up to four atoms; T_c, Tanimoto coefficient; IP, inositol phosphate; cAMP, cyclic adenosine monophosphate

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