Virtual screening identifies novel high-affinity σ_1 receptor ligands

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Abstract:

The σ_1 receptor is a transmembrane protein implicated in several pathophysiological conditions, including neurodegenerative disease[1], drug addiction[2], cancer[3], and pain[4]. However, there are no high-throughput functional assays for σ_1 receptor drug discovery. Here, we assessed high-throughput structure-based computational docking for discovery of novel ligands of the σ_1 receptor. We screened a library of over 6 million compounds using the Schrödinger Glide package, followed by experimental characterization of top-scoring candidates. 77% of tested candidates bound σ_1 with high affinity (10-550 nM). These include compounds with high selectivity for the σ_1 receptor compared to the genetically unrelated but pharmacologically similar σ_2 receptor, as well as compounds with substantial cross-reactivity between the two receptors. These results establish structure-based virtual screening as a highly effective platform for σ_1 receptor ligand discovery.

Main text:

The σ_1 receptor is an enigmatic intracellular membrane protein that is an active drug target for the treatment of myriad conditions including neurodegenerative disease[1], pain[4], cancer[3], and drug abuse[2]. The receptor binds a chemically diverse set of ligands with high affinity, including many compounds that are designed to bind to other receptors, such as antidepressants, antipsychotics, and NMDA receptor ligands, among others[5]. Ligands targeting the σ_1 receptor are in clinical trials for treatment of neuropathic pain[6], ischemic stroke[7], and Alzheimer's disease[8]. The σ_1 receptor is expressed throughout the human body but is most highly expressed in the nervous system[5]. It is genetically unique, with no appreciable sequence similarity to any other gene in the human genome[5]. Even the pharmacologically similar σ_2 receptor is unrelated in sequence, despite its similar ligand affinity profile[9,10].

Despite decades of study, structural data for the σ_1 receptor have only recently become available[11]. The receptor crystallized as a homotrimer, though it is known to exist in multiple

32 oligomeric states[12-14]. Each monomer has an occluded ligand binding site containing Glu172 and
33 Asp126[11], which are essential for ligand binding[15]. The first crystal structure of the human σ_1
34 receptor was solved with the receptor in complex with PD144418 at 2.5 Å resolution (PDB:
35 5HK1). Four other receptor-ligand complexes with ligands 4-IBP, haloperidol, NE-100, and (+)-
36 pentazocine have been reported at crystallographic resolutions of 2.8-3.1 Å[11,16]. As is typical of
37 σ_1 receptor ligands, these co-crystallized ligands are structurally divergent from one another.
38 Nonetheless, all five σ_1 receptor-ligand complexes solved to date share some key features, the
39 most notable of which is an essential electrostatic interaction between a basic amine moiety in
40 the ligand and the side chain of receptor residue Glu172[11,16]. Given this constraint, we
41 hypothesized that the σ_1 receptor would be an ideal candidate for *in silico* ligand screening.
42 *In silico* screening uses software to estimate ligand binding energy, enabling efficient
43 prioritization of compounds while sampling a larger chemical space than is readily accessible to
44 experimental methods[17]. Schrödinger's Drug Discovery Platform Glide module[18,19] is a widely
45 used ligand docking software and is used as the preferred docking system by the structural
46 biology groups affiliated with SBGrid[20]. Glide converges upon optimally docked compounds by
47 sampling the ligand in various states and orientations within the user-defined binding site[18,19].
48 Candidate ligands that pass through initial space screens (*i.e.*, those that are sterically capable of
49 fitting into the target site), are scored at increasing precision levels until the final Glide Score is
50 calculated, with lower Glide Scores indicating a higher predicted binding affinity[19]. Glide
51 docking has three precision levels, High Throughput Virtual Screening (HTVS), Standard
52 Precision (SP), and Extra Precision (XP). HTVS is a less stringent method that eliminates ligands
53 from the sample pool if their volume exceeds the binding site volume. SP and XP are slower,
54 more intensive algorithms that predict binding chemistry. In the case of the σ_1 receptor, where no
55 high-throughput biological assays are available, computational docking is potentially useful for
56 identifying novel compounds that are chemically dissimilar to both one another and to known σ_1
57 receptor ligands.
58 Prior to docking experiments, a grid for Glide docking was established as a 10 Å cube
59 centered between the carboxylates of Glu172 and Asp126. In pilot docking test, this resulted in
60 known binders occupying a similar pose within the orthosteric site to that observed in crystal
61 structures. Enrichment plots were calculated to assess docking paradigms, following previously
62 reported approaches[17,21]. To compare enrichment with different approaches, we used 32

63 conformers of 15 known binders and 100,000 randomly selected eMolecules compounds of
64 similar molecular weight into a test subset (see Methods). The enrichment plots generated over
65 this subset confirmed that we were adequately predicting the binding mode of σ_1 ligands in the
66 docking protocol. The docking algorithm recovered (scored) over 90% of known binders, with a
67 high proportion of the known binders being in the top 10% of scored compounds. Additionally,
68 through visual confirmation of Glide successfully docking the co-crystallized ligand in a similar
69 configuration as it is bound (Figure 2A), the docking protocol demonstrated potential predictive
70 value.

71 Superimposition of the enrichment plots generated at the varying precision levels
72 demonstrated consistently higher recovery rates of known binders in flexible docking compared
73 to rigid docking. Extra precision flexible docking yielded the highest recovery rate (number of
74 known binders scored/total number of binders in subset tested) of known binders at 90.63%,
75 followed by standard precision flexible docking at just under 81.25%, extra precision standard
76 docking at 43.75%, and standard precision rigid docking at just below 33.33%. However,
77 flexible SP docking appeared better at recovering known binders early in the ranked database. It
78 is interesting that SP recovered more known binders quickly, as XP is considered to be the most
79 rigorous scoring paradigm, thus it would have been reasonable to see XP recover more known
80 binders overall and recover more in the top 10% of the tested ligands. The relationship between
81 SP and XP flexible and rigid docking is shown in Figure 2B.

82 In the docking experiment, compounds were selected from a starting library of over 6
83 million compounds through four sequential iterations of the docking protocol with increasing
84 precision levels. Approximately1.6 million compounds passed through high-throughput virtual
85 screening (HTVS) and went into SP, 71189 compounds from SP to XP, and 2625 compounds
86 from XP to Flexible XP docking (see Figure 2C). From HTVS to SP all ligands that scored better
87 than the re-docked co-crystallized ligand were selected. For the transition from SP to XP, the
88 mean of all output scores in SP was found and all scores beyond 2 standard deviations from that
89 mean were selected, and for all other precision levels chose ligands further than 1 standard
90 deviation from the mean.

91 K-means clustering was applied to ligands based on volume occupied in binding site in
92 an attempt to select a unique set of ligands to experimentally validate. After collapsing many
93 sparse clusters, we found 3 main clusters with a total of 250 chemically and structurally diverse

94 compounds. After visual inspection of the docked structures, the top 100 compounds were
95 ranked based on glide score and the chemical plausibility of the docked pose. Of these, 17
96 representative compounds that were chemically divergent from one another and significantly
97 different from existing σ_1 receptor ligands were chosen for experimental assessment.
98 Among compounds purchased, 9 passed LC/MS inspection for purity and expected
99 molecular weight and were characterized further (Figures S1 and S2). Figure S3 depicts the
100 results from our initial screen of the compounds. Of the 9 compounds experimentally tested, 7
101 bound the σ_1 receptor with <1 μM affinity (Table 1 and Figure S3B). Compound **2** bound with
102 10 nM affinity (Table 1). Compounds **3**, **4**, **5** and **9** also bound with < 100 nM affinity, while
103 compounds **6** and **8** bound with 132 and 550 nM affinity respectively.
104 Though the σ_1 and σ_2 receptors are genetically unrelated, they have very similar
105 pharmacological profiles and development of selective compounds can be challenging[22]. We
106 reasoned that our structure-guided approach and the chemical diversity of our ligands might
107 allow the discovery of subtype-selective probes. The 9 compounds tested for σ_1 receptor binding
108 were also tested for cross-reactivity with the σ_2 receptor (Figures S3C and S3D, Table 1). Of the
109 compounds that bound well to σ_1 receptor, only compound **2** bound σ_2 with <100 nM affinity
110 (Table 1). The other compounds did not bind as well to σ_2 in the initial screen as they did to σ_1,
111 with most exhibiting >1 μM affinity for the σ_2 receptor.
112 To our knowledge, this work represents the first purely structure-based virtual screen on
113 the σ_1 receptor without the use of additional pharmacophore modeling. We found several ligands
114 that bound with high affinity and had good selectivity for the σ_1 receptor over the
115 pharmacologically similar σ_2 receptor. This work demonstrates that the σ_1 receptor is highly
116 amenable to *in silico* structure-based drug discovery, and that such approaches represent a viable
117 way to identify novel σ_1 receptor ligands at relatively low cost.
118 The σ_1 receptor is an especially interesting target for virtual screening because of its
119 potential therapeutic relevance and the lack of functional assays suited to experimental high-
120 throughput screening. Previous work has identified the minimal chemical features of σ_1 ligands
121 to be a basic amine flanked by two hydrophobic regions, one that is 6-10 Å and another that is
122 2.5-3.9 Å[23]. Later work has refined this model, but the basic elements have remained
123 unchanged[24-27].

124 There have been previous attempts to design and discover σ_1 receptor ligands through
125 computational pharmacophore modeling[24,25]. These studies found high-affinity binders but were
126 limited to the discovery of compounds with very similar chemical features to reference ligands.
127 In another effort, a computationally predicted structural model of the σ_1 receptor was developed
128 and used for ligand screening[28]. However, this model showed little similarity to the
129 experimentally determined σ_1 receptor structure, and it only led to the identification of
130 compounds with poorer affinity than that of a reference ligand, indicating that it offered little if
131 any value in guiding ligand discovery.
132 More recent efforts have combined structural information from the σ_1 receptor crystal
133 structure with pharmacophore modelling or quantitative structure-affinity relationship (QSAR)
134 approaches[26,27]. One recent report used the crystal structure of the σ_1 receptor bound to
135 PD144418 (PDB ID: 5HK1) to align 180 σ_1 receptor antagonists and develop a 3D-QSAR model
136 for σ_1 antagonists[26]. This model was able to accurately predict ligands that would bind the σ_1
137 receptor with high affinity from the Drugbank Database of FDA approved drugs, with the top
138 hits binding the receptor with 58 nM and 160 nM affinity[26]. Similarly, another recent study used
139 the same crystal structure to develop a predictive pharmacophore model, which was able to
140 accurately predict binders from a set of 3,707,672 conformers of 25,676 compounds previously
141 screened for σ_1 receptor affinity[27]. Interestingly, this study found that the pharmacophore model
142 derived from the σ_1 crystal structure was more accurate for this set of compounds than docking
143 into the structure directly using LibDock[27,29,30]. Neither of these models were used to identify
144 uncharacterized compounds, though some of the FDA approved drugs were not known to bind
145 σ_1 receptor previously[26,27].
146 Here, we show that Schrödinger Glide docking allows for the identification of novel σ_1
147 receptor ligands. Starting with a computational library of over 6 million compounds, we were
148 able to select only 9 for experimental characterization and still found 7 binders with sub-
149 micromolar affinity, corresponding to a success rate of approximately 77%. This compares
150 favorably to other docking studies. Of 54 recent docking studies, only 8 had higher hit rates and
151 only 3 found compounds with better affinity than the best hit of 10 nM reported here, despite the
152 use of significantly larger screening libraries in some cases[31]. This may be because the σ_1
153 receptor is particularly well suited to computational docking. Most high-affinity ligands found to
154 date bind with only a single electrostatic interaction between a primary amine and Glu172, and

155 affinity is likely driven largely by hydrophobic interactions and steric constraints. This relatively
156 simple binding mode may be easier to accurately predict by docking than more complex ones.
157 Indeed, constraining ligands to be docked near Glu172 and Asp126 was sufficient to successfully
158 find binders, without requiring a more complex pharmacophore model.
159 In addition to having high affinity for the σ_1 receptor, most compounds tested here
160 exhibited high selectivity for the σ_1 receptor over the pharmacologically similar σ_2 receptor. Few
161 compounds bound to σ_2 with sub-micromolar affinity, and almost all tested compounds had
162 higher affinity for the σ_1 receptor than the σ_2 receptor. For example, Compound **5** exhibited 81
163 nM affinity for the σ_1 receptor yet bound with >10 μM affinity to the σ_2 receptor. In contrast,
164 compounds 2 and 6 showed high affinity for both σ_1 and the σ_2 receptor, indicating that non-
165 selective ligands can also be identified by virtual screening even against just one receptor type.
166 While we focused here on the σ_1 receptor, relatively few highly σ_2 selective compounds are
167 currently available. The σ_2 structure may allow identification of highly σ_2-selective ligands using
168 similar approaches to those described here, further expanding the suite of molecular tools
169 available for σ receptor research.
170
171 **Materials and Methods**
172 ***Computational Methods***
173 Prior to docking, the σ_1 receptor structure (PDB ID: 5HK1) was put through the Protein
174 Preparation Protocol[32]. This added hydrogens, removed co-crystallized water molecules,
175 enumerated bond orders, optimized the receptor's hydrogen bond network, and ran a restrained
176 minimization to alleviate backbone clashes within the receptor backbone. Additionally, Asp126
177 was protonated in our docking screens on the basis of its apparent hydrogen bond with Glu172 in
178 the crystal structure of the receptor[11].
179 The eMolecules ligand library was prepared using the Ligand Preparation Protocol[32] to
180 generate three-dimensional geometries, assign proper bond orders, and generate accessible
181 tautomer and ionization states for the ligands prior to virtual screening[33].
182 The grid generation protocol in Glide Docking defines the target area (binding site) for
183 each ligand to be tested within during the docking program. This was centered between the
184 carboxylates of Glu172 and Asp126 and was kept constant as the docking precision was
185 increased. Docking began with high throughput virtual screening (HTVS), in which all

186 compounds in the eMolecules library were tested. All ligands with Glide scores more favorable
187 than the score of the re-docked co-crystallized ligand PD144418 were put through standard
188 precision screening (SP). All compounds scoring better than 1 standard deviation from the mean
189 were tested in extra precision (XP), and the top 10% of XP ligands were put through flexible XP
190 docking, where the receptor backbone was given freedom to shift in order to fit ligands to the
191 orthosteric site.
192 To preform enrichment analysis similar to previous approaches[34], a set of 15 known σ_1
193 receptor binders were collected from the PDSP Ki Database[35] and put through the ligand
194 preparation protocol. These ligands were then tested in varying precision levels after being
195 merged with a set of 100,000 eMolecules ligands of similar weight to the known binders. The
196 average molecular weight of the known binders taken from the PDSP Ki Database is 351.07
197 g/mol, and the sample of ligands that were selected for enrichment with known binders had
198 molecular weights between 300 and 400 g/mol in an attempt to force Glide to focus on
199 differences in chemical composition rather than physicochemical descriptors.
200 Schrodinger Drug Discovery Platform installation was supported by SBGrid Consortium and all
201 data was generated and processed on SBGrid servers.[20]
202
203 **Recombinant receptor expression and preparation of *Sf*9 cell membranes**

204 Membranes were prepared from *Sf*9 insect cells infected with baculovirus encoding either
205 recombinant σ_1 or σ_2 receptor, using a protocol adapted from that of Vilner et al., as previously
206 described[10,11,22]. Briefly, either the σ_1 receptor or σ_2 receptor, which was recently identified as
207 TMEM97[10], were expressed in Sf9 cells using the pFastBac baculovirus expression system. Cells
208 were lysed by osmotic shock and membrane fractions were separated by repeated centrifugation
209 and washing steps, followed by dounce homogenization. Total protein content was quantified
210 using the BioRad DC protein assay, and membranes were stored in 100 µL aliquots at a
211 concentration of 3-10 mg/mL at -80 °C until use.

212 **Screening docking hits for σ_1 and σ_2 receptor binding**

213 Membrane radioligand binding assays were performed as described with slight
214 modifications[10,11,36]. All compounds were diluted to 10 µM in 50 mM Tris pH 8.0. Membrane

215 samples were also diluted and homogenized in 50 mM Tris pH 8.0 using a needle and syringe. A
216 total of 2.5 μg of protein was added to each well of a 96-well block in a final volume of 100 μL,
217 which also contained 1 μM of the compound and 10 nM of the appropriate radioligand (^{3}H (+)-
218 pentazocine for the σ_1 receptor, and ^{3}H DTG for the σ_2 receptor). All reactions were performed
219 in triplicate. Haloperidol was used as a positive control, and buffer alone served as a negative
220 control. Samples were incubated at room temperature with shaking for 1.5 h. The reaction was
221 then terminated and samples were then applied to glass fiber filters (Merck Millipore) using a
222 Brandel cell harvester. Filters were soaked with 0.3% (w/v) polyethylimine prior to use. After
223 harvesting, filters were soaked overnight in scintillation vials containing 5 mL of Cytoscint
224 scintillation fluid and measured on a Beckman Coulter LS 6500 scintillation counter.

225 **Saturation binding in *Sf*9 cell membranes.**

226 ^{3}H (+)-pentazocine and ^{3}H DTG saturation binding to *Sf*9 membranes respectively
227 expressing σ_1 or σ_2 receptor were determined using a saturation binding assay similar to that
228 described by Chu and Ruoho[36],which we have described previously[10,11]. The membranes (2.5 μg
229 and 0.5 μg total protein per reaction for σ_1 and σ_2 expressing membranes, respectively) were
230 incubated in a 100 μL reaction buffered with 50 mM Tris pH 8.0, containing 0-300 nM ^{3}H (+)-
231 pentazocine for σ_1 binding, or 0-300 nM ^{3}H DTG for σ_2 receptor binding. In the σ_2 receptor
232 binding assay, concentrations of 100 and 300 nM DTG were achieved by isotopic dilution to
233 minimize ^{3}H DTG expenditure. For both receptors, nonspecific binding was assayed by parallel
234 reactions containing 2 μM haloperidol. Reactions were incubated at 37 °C for 1.5 h and then
235 terminated, filtered, and measured as for the single-point binding described previously. All
236 reactions were performed in triplicate in a 96-well block. K_D values were calculated using non-
237 linear regression tools in Graphpad Prism.

238 **Competition binding assays in *Sf*9 membranes.**

239 ^{3}H (+)-pentazocine or ^{3}H DTG competition curves testing the binding the docked
240 compounds to the σ_1 and σ_2 receptors respectively, were performed similarly to the protocol
241 described by Chu and Ruoho[36],with slight modifications we have used previously[10,11]. Briefly,
242 *Sf9* insect membranes overexpressing either σ_1 receptor (2.5 μg of total protein per reaction) or
243 σ_2 receptor (0.5 -- 2.5 μg total protein per reaction) were incubated in a 100 μL reaction buffered

with 50 mM Tris pH 8.0, with the appropriate radioligand at a 10 nM concentration and eight competing cold compound concentrations ranging from 10 pM – 100 μM. Reactions were incubated at 37 °C for 1.5 h to reach equilibrium, and then terminated by filtration as described previously. K_i values were computed by directly fitting the data and using the experimentally determined probe K_D to calculate a K_i value, using the Graphpad Prism software. This process implicitly uses a Cheng-Prusoff correction, so no secondary correction was applied.

LC/MS analysis of compound purity

Purchased compounds were analyzed on an Agilent QTOF 6530. Samples were separated by reverse-phase HPLC (Agilent Extend C18 column 1.8 μM, 50 x 2.1 mm) at a flow rate of 0.3 mL min^{-1} in H_2O with 0.1% formic acid for 15 min followed by a 5 min elution in 10-100% acetonitrile. Data were analyzed using Agilent MassHunter Quantitative Analysis Software.

HRMS (ESI+) *m/z* calcd for $C_{20}H_{31}N_3O$ [M + H] 342.2540, found 342.2545 (error 1.5 ppm);
HRMS (ESI+) *m/z* calcd for $C_{20}H_{26}N_2O$ [M + H] 311.2118, found 311.2117 (error 0.3 ppm);
HRMS (ESI+) *m/z* calcd for $C_{18}H_{22}N_4OS$ [M + H] 343.1587, found 343.1587 (error <0.3 ppm);
HRMS (ESI+) *m/z* calcd for $C_{18}H_{15}N_2OF_3$ [M + H] 333.1209, found 333.1231 (error 6.6 ppm);
HRMS (ESI+) *m/z* calcd for $C_{19}H_{21}N_3O$ [M + H] 308.1757, found 308.1764 (error 2.3 ppm);
HRMS (ESI+) *m/z* calcd for $C_{18}H_{19}N_3OClF$ [M + H] 348.1273, found 348.1279 (error 1.7 ppm);
HRMS (ESI+) *m/z* calcd for $C_{22}H_{23}N_3O$ [M + H] 346.1914, found 346.1912 (error 0.6 ppm);
HRMS (ESI+) *m/z* calcd for $C_{19}H_{19}N_2OF_3$ [M + H] 349.1522, found 349.1538 (error 4.6 ppm);
HRMS (ESI+) *m/z* calcd for $C_{20}H_{19}N_2OF_3$ [M + H] 361.1522, found 361.1545 (error 6.4 ppm).

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Supporting Information: Binding data, Compounds tested in binding assays, eMolecules ligand library information, Compound similarity

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Author Contributions
D.A.G. designed and carried out computational docking and analysis of results, and together with H.R.S. performed radioligand binding assays and associated data analysis. H.R.S. conducted LC-MS for assessment of compound purity. P.S. Co-designed the computational pipeline and co-supervised the docking experiments. A.C.K. supervised the work and together with D.A.G. and H.R.S. wrote the manuscript.

Acknowledgements:
We thank Dr. Matthew Henke and the Harvard Medical School Analytical Chemistry core, and Dr. Meredith Skiba for assistance with LC/MS. This work was supported by NIH grant R01GM119185 (A.C.K.), and grant number FG-2017-9226 from the Alfred P. Sloan Foundation (A.C.K.). H.R.S is supported by an NSF Graduate Research Fellowship DGE1745303. D.A.G is

supported by BCMP Scholars Internship Program and Molecular Biophysics Training Grant NIGMS T32 GM008313.

Abbreviations Used: NMDA, N-methyl-D-aspartate; ECFP, Extended Connectivity Fingerprints; HTVS, High Throughput Virtual Screening; SP, Standard Precision Virtual Screening; XP, Extra Precision Virtual Screening; 4-IBP, N-(benzylpiperidin-4yl)-4-iodobenzamide; PD144418, 1,2,3,6-tetrahydro-5-[3-(4-methylphenyl)-5-isoxazolyl]-1-propylpyridine; NE-100, *N*, *N*-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ehthylamine monohydrochloride; DTG, 1,3-Di-(2-tolyl)guanidine

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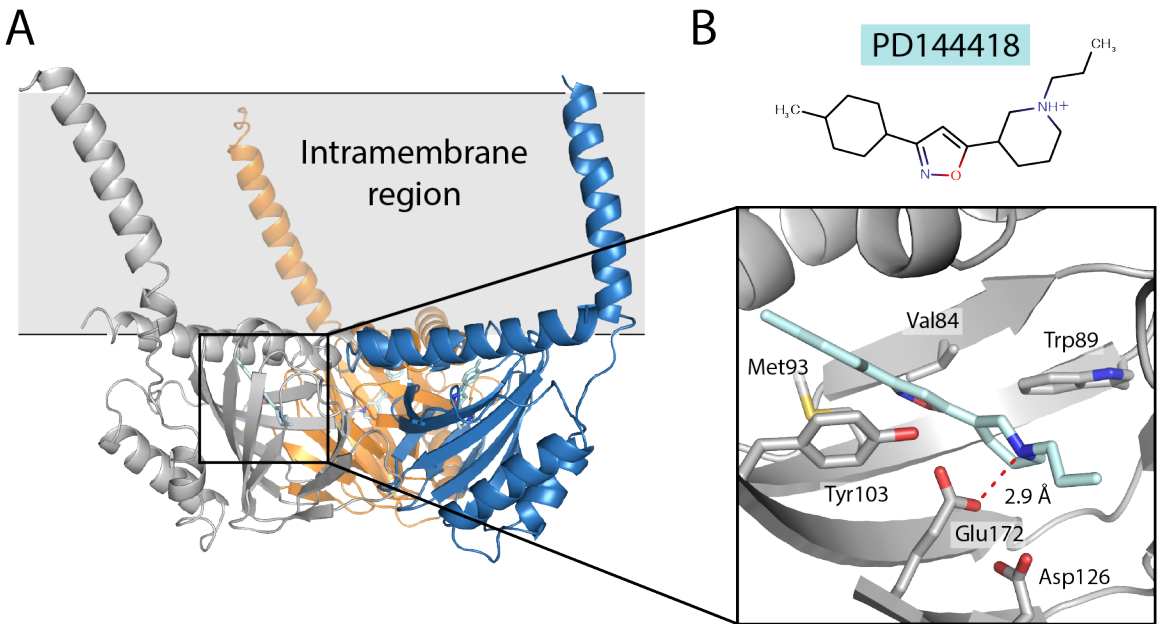


Figure 1. Structure of the human σ_1 receptor bound to PD144418 (PDB: 5HK1). (A) Overall architecture of the human σ_1 receptor. The receptor crystallized as a homotrimer, with each monomer binding one ligand molecule in an occluded binding pocket. (B) Close-up of the σ_1 receptor's ligand binding pocket with several amino acid side chains depicted as sticks and PD144418 shown in cyan.

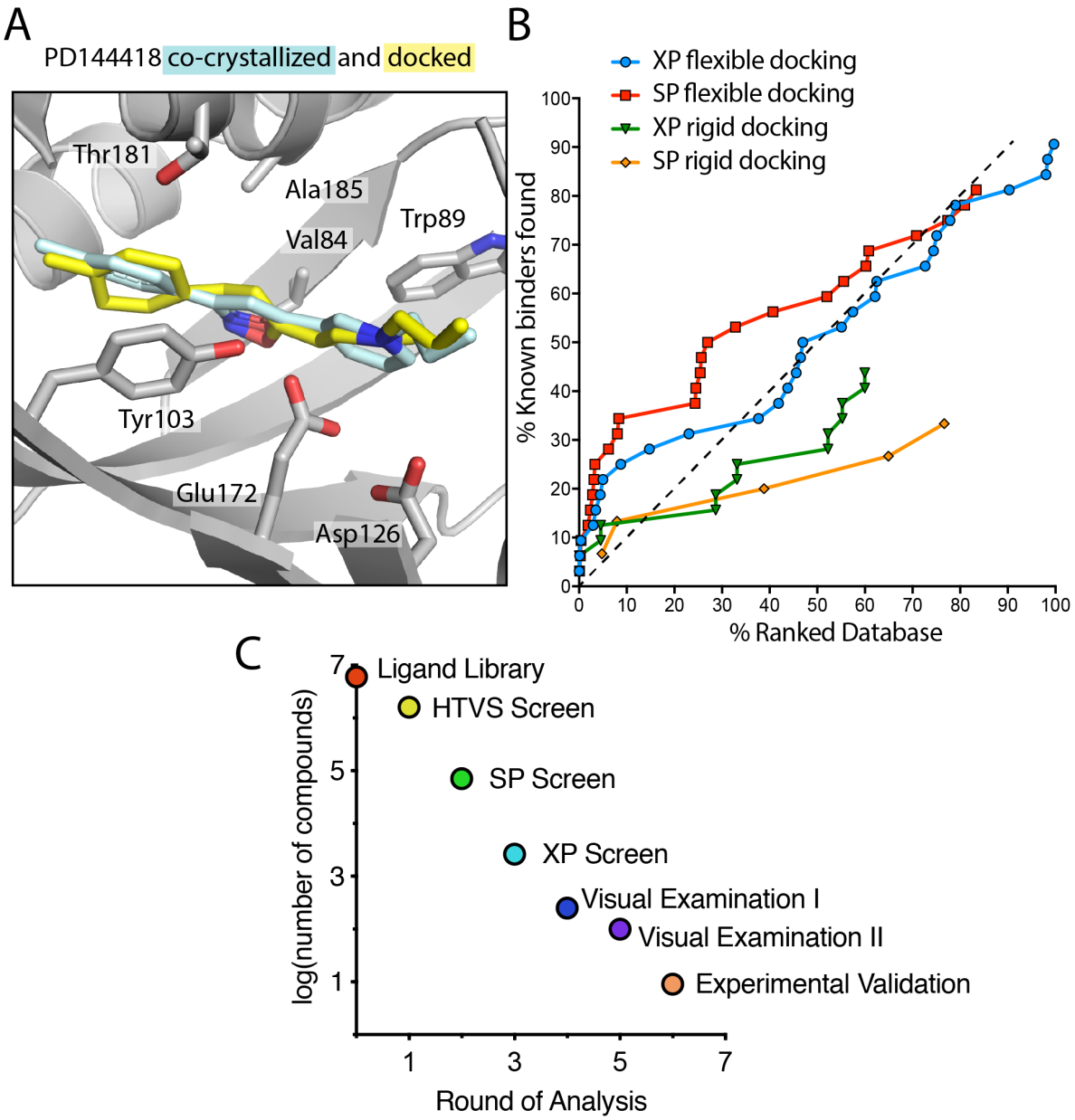
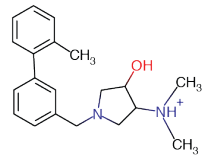
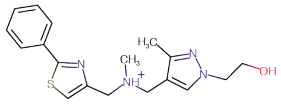
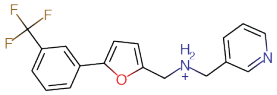
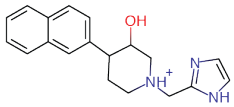
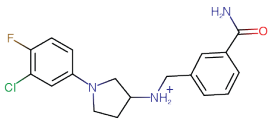
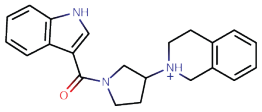
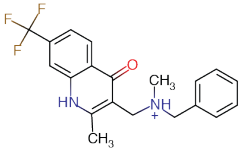


Figure 2. Ligands can be accurately docked into the σ_1 receptor structure. (A) Superposition of the structures of PD144418 from the σ1 receptor crystal structure (cyan, PDB: 5HK1) and the highest-ranked docked pose (yellow). (B) Enrichment Plot showing the number of ligands recovered by the docking algorithm in the ranked database as a function of number of ligands sampled with the grid centered between Glu172 and Asp126. Docking types shown are XP flexible docking (blue circles), SP flexible docking (red squares), XP rigid docking (green triangles), and SP rigid docking (orange diamonds). The black line of slope 1 indicates performance expected via random selection of compounds by the docking algorithm. (C) Log-scale plot of the number of compounds remaining in each round of the docking pipeline.

Table 1. Summary of experimental characterization of docked compounds. All affinity values are representative results from at least two independent experiments performed in triplicate. “N.D.” indicates that the affinity was not determined.

Compound	Glide Score	$\sigma_1$receptor K_i (nM)	$\sigma_2$receptor K_i (nM)	Selectivity (σ_1/σ_2)	Structure
2	-10.828	10	92	0.11	
3	-10.693	71	2373	0.03	
4	-10.830	59	2482	0.02	
5	-10.466	81	>10,000	<0.01	
6	-11.405	132	250	0.52	
7	-10.750	550	N.D.	n/a	
9	-10.950	53	1233	0.04