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Multiple fragment docking and linking in primary and secondary pockets of dopamine receptors

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ABSTRACT: A sequential docking methodology was applied to computationally predict starting points for fragment linking using the human dopamine D_3 receptor crystal structure and a human dopamine D_2 receptor homology model. Two focused fragment libraries were docked in the primary and secondary binding sites and best fragment combinations were enumerated. Similar top scoring fragments were found for the primary site, while secondary site fragments were predicted to convey selectivity. Three linked compounds were synthesized that had 9, 39 and 55-fold selectivity in favor of D_3 and the subtype selectivity of the compounds was assessed on a structural basis.

Fragment-based drug discovery (FBDD) has recently proved to have significant utility in early phase drug research.[1] Fragments are polar compounds of low molecular weight and low complexity enabling more efficient sampling of chemical space and exploring enthalpy dominated targeting of protein hot spots resulting in better physico-chemical and ADMET profiles of fragment derived leads and clinical candidates.[2] The two main strategies of fragment hit elaboration are growing and linking.[3] In the first one a single fragment is decorated with additional functionalities while in the second two (or more) fragments are identified that bind to the target simultaneously and in close proximity and are subsequently incorporated in a single molecule using a suitable linker moiety.

Although FBDD in the last decade has shown remarkable efficiency on enzyme targets, its applicability for membrane proteins has been limited by difficulties in obtaining structural information on membrane proteins and application of sensitive biophysical screening methods frequently used for fragment screening such as high-throughput X-ray screening, surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR). Recent advances in G protein-coupled receptor stabilization and structural investigation made it possible to develop biophysical assays for GPCRs and to utilize structural information in structure-based drug design. Several recent reports described experimental[4] and virtual fragment screening[5,6] as well as structure-guided optimization efforts[7] on GPCRs. It is expected that FBDD applied to GPCRs can provide novel and high quality compounds for this target family.

Recent clinical evidence supports the effectiveness of dual dopamine D_2 and D_3 antagonists or partial agonists in schizophrenia, depression and bipolar mania.[8] D D_2 antagonism is required for the antipsychotic effect and D_3 antagonism contributes to cognitive enhancement and reduced catalepsy. Finding the balance between D_3 and D_2 affinities is essential for a beneficial therapeutic effect and safety profile. Dual acting compounds should show higher affinity to the D_3 than to the D_2 receptors due to different expression levels of the two receptors in specific brain areas. Since the elucidation of the dopamine D_3 crystal structure in complex with eticlopride in 2010,[9] much attention has been directed towards the structure-based screening and design of D_3 ligands. We have recently evaluated the performance of a sequential docking methodology to computationally predict starting points for fragment linking.[10] In the present study we apply this methodology for fragment docking and linking to the D_3 crystal structure and a D_2 homology model and assess the subtype selectivity of the compounds on a structural basis. A similar methodology was also used by Abagyan et al. with dopamine as the fixed primary site ligand and no subsequent linking of the identified fragments.[11]

Homology modeling and protein structure preparation. The human dopamine D_2 receptor amino acid sequence from the UniProt server[12] was aligned to the sequence of the template, chain A of the 2.89 Å resolution X-ray structure of the human dopamine D_3 receptor crystallized with the D_2-D_3 dual antagonist eticlopride (PDB code: 3PBL) using Prime 3.2[13] (see alignment in Supporting Information). The third intracellular loop was not modeled and the eticlopride ligand was included in homology model building to prevent collapse of the binding site. Finally the whole structure was subjected to Impref restrained minimization in the Protein Preparation Wizard in the Schrödinger Suite 2013.[14] Chain A of the dopamine D_3 crystal structure was subjected to the full Protein Preparation Wizard workflow with default settings.

Ligand preparation and docking. An in-house focused library of 196 fragments was collected containing a basic amine moiety in an aliphatic ring connected directly or through a short linker to a substituted aryl or hetaryl moiety (see general formula in the Supporting Infor-

mation). Such compounds were believed to function as primary binding site ligands of the D_2 and D_3 receptors. It has been shown that the primary binding sites of the two receptors are nearly identical and selectivity can be achieved by modulation in the secondary binding pocket.[15] Another in-house focused library of 266 fragments were collected containing a cyclohexyl or piperidine ring (see general formula in the Supporting Information) as these fragments were believed to function as secondary binding site ligands based on known D_3 antagonists such as SB-277011[16] suitable for modulating selectivity. The two libraries were prepared for docking using LigPrep 2.6.[17] Protonation and tautomeric states at pH 7±2 were enumerated using Epik 2.4.[18] The Glide 5.9[19] software was used for sequential docking the two libraries to the two receptor structures according to the protocol described in ref. 10, briefly: the first library was docked to the apo receptor structures, then the docking poses were merged with the receptor, new grids were constructed including the merged ligands and the second fragment library was docked to the partially occupied binding sites (see Supporting Information for additional computational details). Top scoring fragment combinations were visually inspected; linked compounds were synthesized and tested in radioligand binding assays.

Primary site docking results. Docking of the first focused library of basic fragments produced results similar binding modes as in ref. 15. All of the 196 fragments could be docked into the inner binding site of the D_3 receptor, of which 145 produced an ionic hydrogen bond to the characteristic Asp110$^{3.32}$ in the D_3 crystal structure and the aromatic moiety encased between hydrophobic residues Phe345$^{6.51}$, Phe346$^{6.52}$, Val111$^{3.33}$ and Ile183$^{45.52}$. The top 15 fragments in D_3 docking also achieved high ranks when the same library was docked to the D_2 crystal structure, particularly the highest scoring 1-(3-cyano-5-trifluoromethylphenyl)piperazine was identical in both receptors providing good docking scores (-8.576 for D_3 and -8.745 for D_2) and identical binding modes. This is in line with the highly conserved nature of the primary binding site. Docking seemed to favor a *meta*-trifluoromethyl substituent in further high ranking fragments as well. Binding modes of the four top ranked compounds are depicted in Figure 1.

Secondary site docking results. The 145 well-docked fragments were merged with the apo D_3 structure allowing for 145 new grids to be constructed and the second focused fragment library was docked to all of these new grids of partially occupied binding site. Docking scores of

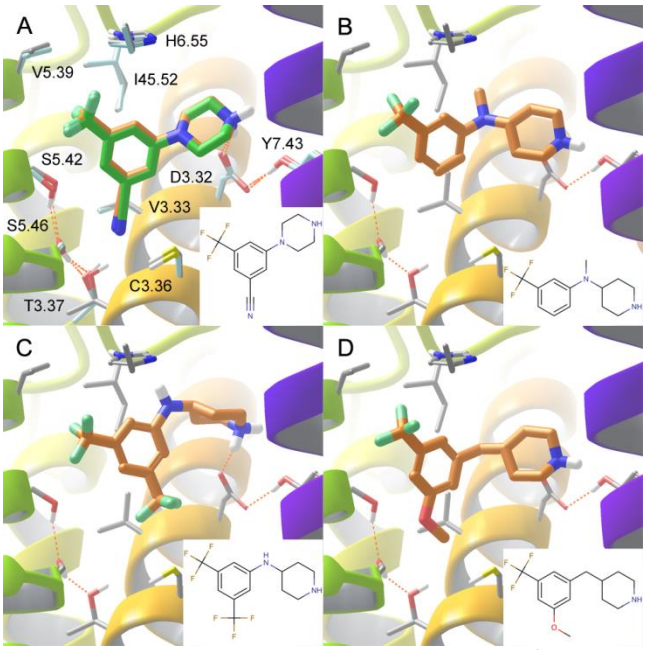


Figure 1. Binding modes of the top four fragments (from A to D) in D_3 primary site docking. In A) the D_3 and D_2 binding sites are overlaid in grey and light blue carbons respectively, as well as docked poses of the ligand in orange and green carbons respectively. From B) to D) only D_3 results are shown. Helix 6 is omitted for clarity. Compound structures are shown as insets.

the 266 fragments in all 145 D_3 grids were averaged and ranked by this mean docking score. Since docking might be sensitive to small differences of the grid used this procedure was used identifying secondary site fragments that bind next to different primary site fragments and furthermore for the more robust estimation of the GlideScore. The single best primary site ligand was also merged with the apo D_2 homology model and the second library was docked into this partially occupied structure to assess structural determinants of selectivity. Top ranking secondary site binders in the D_3 receptor and their binding modes in the D_2 receptor were visually inspected. The binding modes of the top three fragments by mean D_3 docking score in ten D_3 grids and the single D_2 grid are shown in Figure 2. It can be seen that these fragments produce extensive H-bonding patterns in the secondary binding site of the D_3 crystal structure. Carbonyl groups of the cyclohexylurea (mean docking score: -6.574) and the cyclohexylglycinamide (mean docking score: -6.230) and one of the S=O groups of the cyclohexylaminosulfonamide (mean docking score: -6.087) act as acceptors for Thr369$^{7.39}$ in D_3 and the homologous Thr412$^{7.39}$ in D_2. Two NH groups of all three ligands interact as donors to Glu90$^{2.65}$ and Ser366$^{7.36}$ in D_3 as well as the same Glu95$^{2.65}$ and Ser409$^{7.36}$ amino acids in D_2. The only interaction different between the two receptor subtypes is the second S=O group of the cyclohexylaminosulfonamide fragment, which acts as an acceptor for Tyr36$^{1.39}$ in D_3, while in the homologous position of D_2 Leu41$^{1.39}$ can be found incapable of forming a hydrogen bond with the ligand.

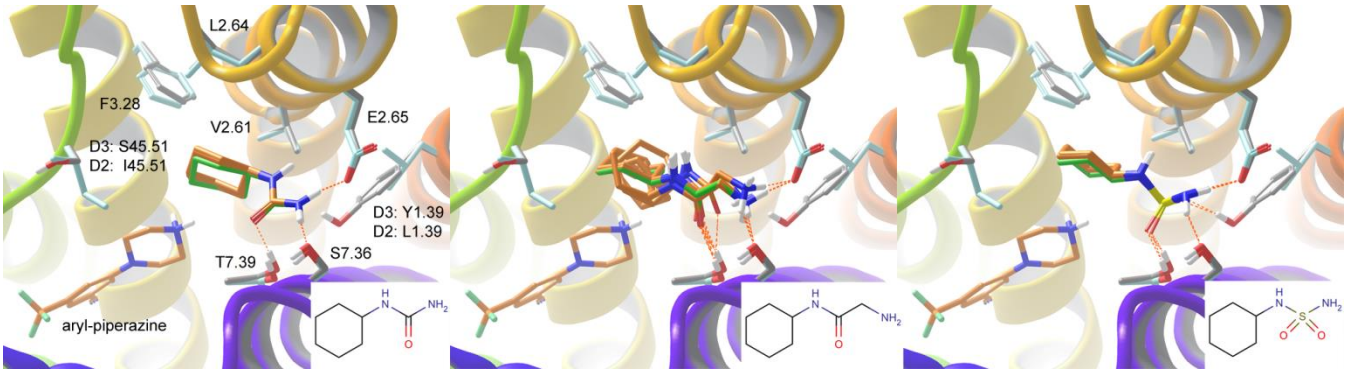
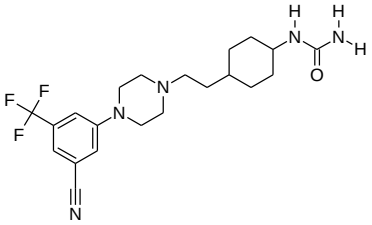
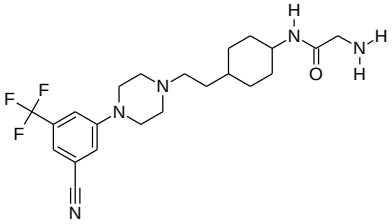
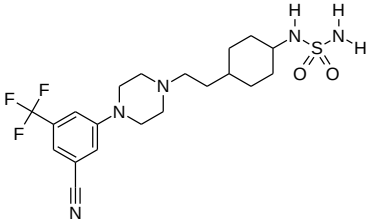


Figure 2. Binding modes of the top three fragments in D_3 and D_2 secondary site docking. The D_3 and D_2 binding sites are overlaid in grey and light blue carbons respectively, as well as an ensemble of 10 docked poses of the ligand in D_3 in orange carbons and a single docked pose of the ligand in D_2 in green carbons. Only the top ranked primary site ligand is included for clarity. Compound structures are shown as insets.

Table 1. Experimental and docking data of linked compounds.

Entry	Compound structure	hD_3R K_i (nM)[a]	hD_3R docking score	hD_2R K_i (nM)[a]	hD_2R docking score	Selectivity
1		0.75 ± 0.10	-10.393	30 ± 11	-10.664	39
2		0.62 ± 0.23	-10.488	5.4 ± 0.2	-11.210	9
3		0.67 ± 0.15	-10.514	37 ± 9	-10.833	55

[a]Inhibition constants from binding experiments on recombinant human D_2 and D_3 receptors. For details on the assays, see the Supporting Information. The data are derived from at least three independent experiments; the standard error of the mean is indicated.

Furthermore, these ligands were found to produce robust binding modes in most of the 145 D_3 grids. In fact, we identified 115, 109 and 94 out of 145 binding poses for urea, glycinamide and sulfonamide fragments, respectively in the different grids within 1.5 Å RMSD of the pose docked to the grid with the best primary site ligand included. As can be seen from Figure 2 the predicted binding modes of the second-site ligands in the grids containing the top ten primary site ligands are almost identical in the case of the urea and the sulfonamide fragment and show little variability for the glycinamide derivative. Robust ensembles of docking poses have been associated with higher reliability of the predicted binding mode[20] and a higher entropy change upon binding.[21] It is our experience and also shown in the literature that docking in some cases can be sensitive to grid centering, grid spacing, small differences in input geometries and even atom numbering.[22] Therefore a binding mode of a fragment that is seen in many similar grids of the protein is considered being more reliable than a binding mode that is only produced with specific grids. In the present case it also suggests that such secondary site fragments might bind proximally to different primary site fragments. Other fragments produced less robust binding modes. Therefore these top

three fragments predicted to bind the secondary site were selected for linking with the top primary aryl-piperazine fragment. Docking suggested possible linking of the basic aryl-piperazine nitrogen with either the para or the meta position of the cyclohexyl rings of the secondary fragments. The distance of the para positions in the various docking poses ranged from 3.8 to 4.5 Å while the distance of the meta positions ranged from 3.4 to 3.6 Å, thus both seemed to be suitable linking points. Because of synthetic accessibility and fewer possible stereoisomers, linking was carried out at the symmetric para position.

Biological activities. Linked compounds **1-3** were synthesized and tested in *in vitro* [^{3}H]raclopride binding experiments against recombinant human D_2 and D_3 receptors. See Supporting Information for synthetic routes and experimental details. The ligand displacement experiments were repeated at least three times. K_i values and derived selectivities of the compounds are shown in Table 1. The linked compounds possessed subnanomolar activities against the D_3 receptor and low- to mid-nanomolar activity against the D_2 receptor. The selectivity of compound **2** was lowest, only 9 times higher K_i was measured for D_2 than for D_3, which is in line with the higher flexibility and less robust predicted binding mode of the secondary site fragment. On the other hand, compound **3** showed a 55 times higher K_i for D_2 than to D_3, which is also supported by the docking results. This was the only compound featuring an extra D_3 specific interaction, namely the H-bond with Tyr36$^{1.39}$, which is not present in D_2. Docking of the linked compounds to the apo structures provided similar binding modes and the same H-bonding pattern as the original unlinked fragments (data not shown). Only a small upward shift of the aryl-piperazine fragment was evident in the primary binding site (RMSD: 1.7 Å) and very small deviations were seen in the secondary fragment binding modes (RMSD: 0.57 Å for the urea, 1.06 Å for the glycinamide and 0.84 Å for the sulfonamide fragment). The docking scores of the linked compounds were very high, and in this particular case the relative values of compounds at both receptors were in accordance with the experimental data (see Table 1). However, their selectivity could not be predicted, probably due to the different grids used. Relative scales of docking scores might be slightly different even for two grids of the same receptor. The prediction of selectivity using docking scores only is usually more reliable if selectivity ratios are larger. Finally, we note that the linked compounds have favorable physico-chemical properties. The water-octanol partition coefficient (clogP) calculated by ChemAxon *cxcalc*[23] of **2** and **3** is 2.5 and 3.1 for compound **1** lying in the optimal range for orally active drugs. Accordingly, they exhibit favorable ligand lipophilicity efficiency values (LLE = pK_i-clogP > 5 is favorable) and ligand-efficiency-dependent lipophilicity values (LELP = 0.73·clogP·HAC/pK_i < 10 is favorable). LLE is 6.0 for **1** and 6.7 for **2** and **3**. LELP is 7.6 for **1** and 6.2 for **2** and **3** anticipating a favorable safety profile.[24]

In conclusion we have applied our sequential fragment docking methodology to identify fragments to link in a GPCR target, namely the dopamine D_3 receptor binding site. A homology model was also built for the D_2 receptor subtype and docking of the fragments as well as the full linked compounds was carried out to both receptors in order to assess the structural basis of subtype selectivity of the predicted binders. Three linked compounds were synthesized and docking predictions were validated by the experimental results. Thus it has been shown that multiple fragment docking can provide starting points for linking for GPCR targets with elucidated 3D structures, and subtype selectivity has been achieved by virtual secondary site fragment screening and fragment linking.

ASSOCIATED CONTENT

Supporting Information. General formulae of the focused fragment libraries, synthetic and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

FBDD, Fragment-based drug discovery; ADMET, absorption – distribution – metabolism – excretion – toxicity; GPCR, G protein-coupled receptor.

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