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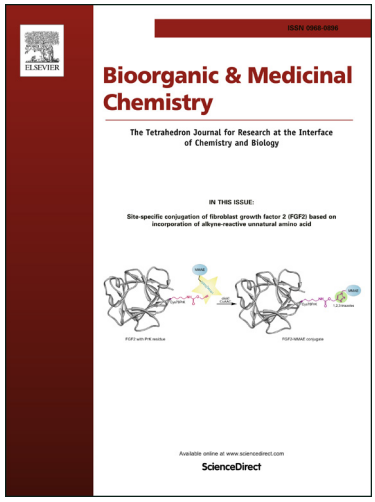

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Potent haloperidol derivatives covalently binding to the dopamine D2 receptor

*Tobias Schwalbe, Jonas Kaindl, Harald Hübner, Peter Gmeiner**

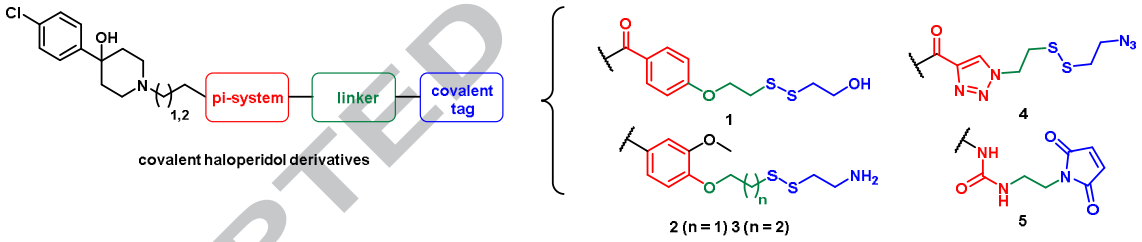
Department of Chemistry and Pharmacy, Emil Fischer Center, Friedrich-Alexander University Erlangen-Nürnberg, Schuhstraβe 19, D-91052 Erlangen, Germany

***Corresponding Author**. Phone: +49-(0)9131-85-24116. Email: peter.gmeiner@fau.de

Keywords

G protein-coupled receptor, dopamine D_2 receptor, haloperidol, covalent ligand, disulfide tethering, chemical probe

TOC Graphic



Abstract

The dopamine D_2 receptor (D_2R) is a common drug target for the treatment of a variety of neurological disorders including schizophrenia. Structure based design of subtype selective D_2R antagonists requires high resolution crystal structures of the receptor and pharmacological tools promoting a better understanding of the protein-ligand interactions. Recently, we reported the development of a chemically activated dopamine derivative (FAUC150) designed to covalently bind the L94C mutant of the dopamine D_2 receptor. Using FAUC150 as a template, we elaborated the design and synthesis of irreversible analogs of the potent antipsychotic drug haloperidol forming covalent D_2R-ligand complexes. The disulfide- and Michael acceptor-functionalized compounds showed significant receptor affinity and an irreversible binding profile in

radioligand depletion experiments.

1. Introduction

The selective covalent ligation of small molecules to pharmacological target proteins has gained of remarkable importance involving the application as irreversible drugs[1, 2] or as biochemical tools used for protein labelling (e.g. with fluorescent dyes)[3, 4] or protein crystallization.[5-7] Especially, the covalent tethering of drugs or neurotransmitters to specific receptor binding sites has gained in importance. The required covalent ligand is usually composed of a target-specific pharmacophore and a chemo- or photoreactive probe, which is designed to form a covalent bond with an adjacent amino acid residue of a wild-type receptor or a mutated variant.[8-10]

The structural elucidation of membrane proteins, in particular the class A of G-protein coupled receptors (GPCRs), has substantially benefited from covalent ligands. Irreversible binding allows the stabilization of particular functional states enabling the selection of state-specific nanobodies and homogenous crystallogenesis of GPCR complexes and, thus, facilitating X-ray crystallography. [6, 7, 11-13]

Very recently, we could synthesize and functionally investigate stable pairs of aminergic GPCRs bound to covalent neurotransmitter analogs. Hence, a covalent derivative of the endogenous ligand dopamine (FAUC150) could be tethered to a cysteine mutant of the dopamine D2 receptor ($D_2R^{L2.64C}$).[7] D_2R is involved in multiple neurological and psychiatric disorders including Parkinson's disease, schizophrenia and depression.[14] The potent D_2R antagonist haloperidol has been used as a typical antipsychotic drug for decades, although the compound exhibits severe neurological side effects.[14, 15] To design safer and more efficacious antipsychotics, a better understanding of the molecular origins of D_2R - haloperidol interactions is required. An X-ray crystal structure of the related D_3 dopamine receptor subtype in complex with the antagonist eticlopride was determined.[16] However, a high resolution structure of D_2R bound to an antagonist could not be resolved, yet. This illustrates that the structure-based design of novel drugs urgently requires powerful biochemical tools.

Taking advantage of our experience with the covalent attachment of the endogenous

agonist dopamine to the $D_2R^{L2.64C}$ receptor mutant, we were encouraged to apply this concept on the selective D_2R antagonist haloperidol (Fig. 1). Based on the chemical structure of haloperidol, we designed a small set of functionalized analogs **1**-**5** sharing the identical orthosteric head group of the parent compound. To facilitate covalent tethering, we intended to address the cysteine mutant in the position TM2.64 by functionalization with four different chemoreactive appendages. Here, we present the synthesis of the ligands **1-5**. Furthermore, we investigated the compounds for D_2R binding and tested their ability to irreversibly occupy the receptor binding site indicating covalent tethering.

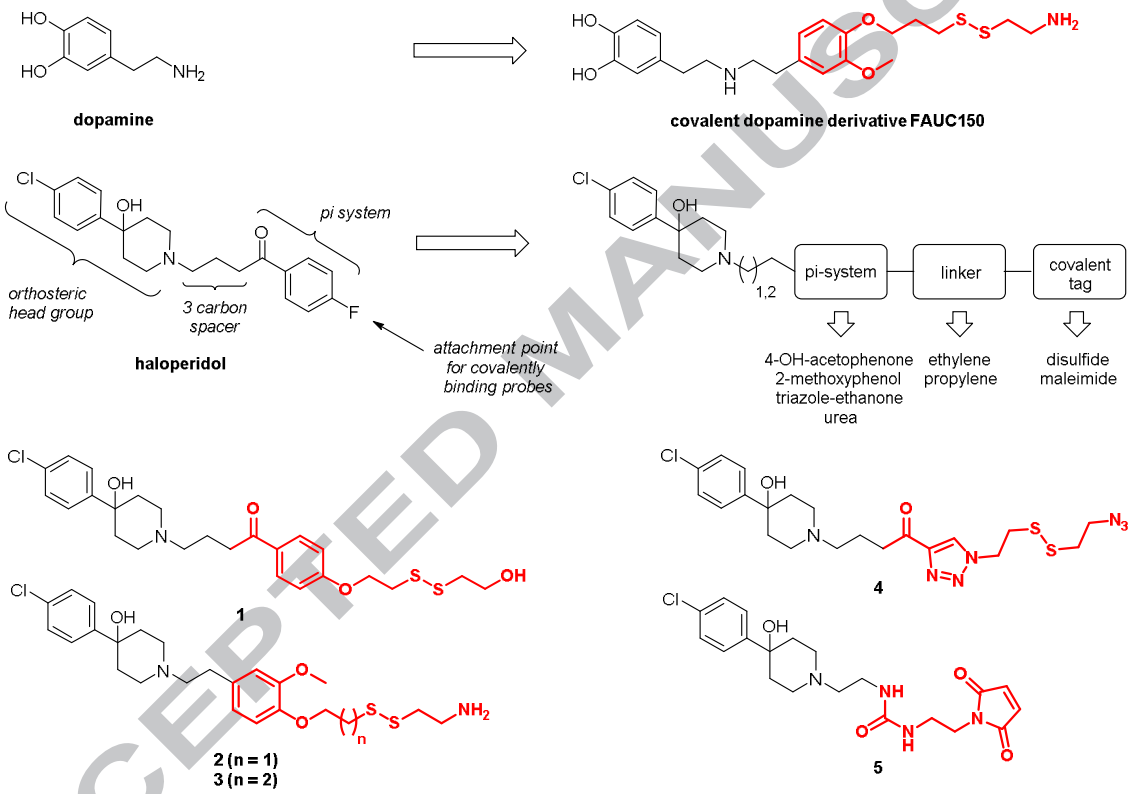


Figure 1: Ligand-based design of covalent D_2R antagonists derived from the lead compound haloperidol.

2. Results and discussion

2.1. Design

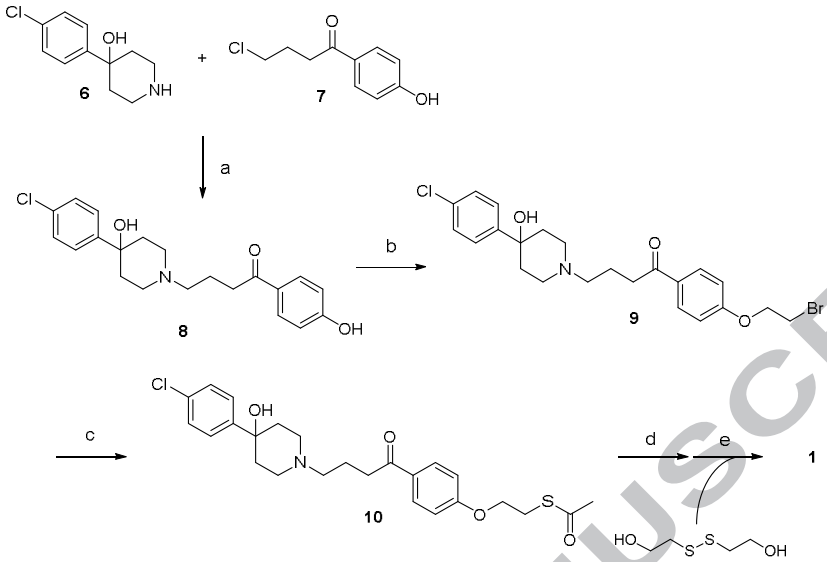
Due to its structural similarity to the endogenous ligand dopamine, we chose the *para*-chlorophenyl-substituted 4-hydroxypiperidine (**6**) substructure of haloperidol to be a recurring element throughout our ligand-based design (Fig. 1). We intended to

attach this orthosteric pharmacophore to a covalently binding appendage subdivided into three interconnected key elements mimicking the haloperidol tail: 1) a pi-system, 2) an appropriate linker to overcome the distance between the basic center and the nucleophilic residue of cysteine at position 2.64 of the receptor and 3) a chemoreactive probe.

In order to preserve the majority of the haloperidol pharmacophore, our initial investigations were directed to a formal replacement of the fluorine atom of the *4'*-fluoro-butyrophenone by an ethoxy linker attached to a disulfide based covalent function leading to compound **1**. To investigate a heterocyclic bioisostere, the benzene ring was replaced by a 1,2,3-triazole (compound **4**). Our previous investigations on the covalent dopamine ligand FAUC150, indicated that **1** might exceed the ideal spacer length between the basic amine center and the disulfide function, we considered to formally replace the dihydroxyphenethylamine scaffold of FAUC150 by the respective haloperidol substructure and to vary the linker length (C2 or C3 linker) towards the covalent tag (compounds **2** and **3**). All of our disulfide-based haloperidol derivatives are expected to bind the cysteine by a thiol-disulfide-interchange reaction, which results in a mainly affinity-driven ligation to the receptor.[17-19] To use an alternative cross-linking reaction, an irreversible analog of haloperidol replacing the disulfide group by a rather cysteine selective Michael acceptor was approached.[8, 20] Thus, we envisaged to link a maleimide chemical probe via a 1,3-diethyl urea group.

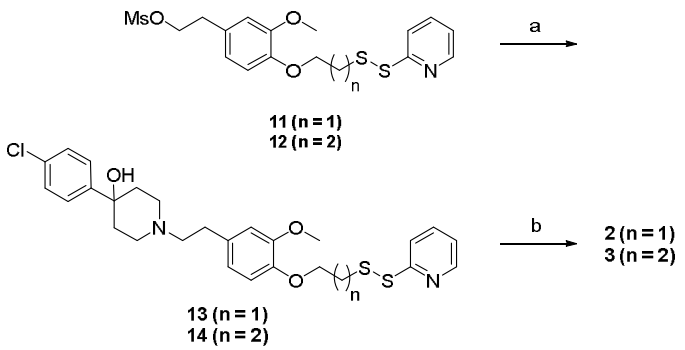
2.2. Chemistry

The synthesis of the haloperidol derivative **1** started from the 4'-chlorophenyl-4-hydroxypiperidine **6**, which was N-alkylated by the commercially available compound **7** to give the intermediate **8** (Scheme 1). Subsequent alkylation with different pre-functionalized 2-mercaptoethyl methane sulfonates gave undesired side products or failed. Therefore, we followed an alternative route by a stepwise integration of the ethylene linker and the disulfide tag. Alkylation of **8** with 1,2-dibromoethane afforded the intermediate **9**. Subsequent nucleophilic substitution by potassium thioacetate provided **10** in quantitative yield. Reaction of thioester **10** with sodium methoxide resulted in the deprotection of the aliphatic thiol, which was scavenged by the addition of bis(hydroxyethylene)disulfide to obtain the disulfide-functionalized ligand **1.**



Scheme 1. Synthesis of covalent haloperidol derivative **1**. Reagents and conditions: (a) KI, DIPEA, DMF, 70°C, 5h, 23% (b) 1,2-dibromoethane, K_2CO_3, CH_3CN, 70°C, 46h, 28% (c), potassium thioacetate, acetone, 60°C, 90min, 90% (d) NaOMe (0.5 M), MeOH, 2min, room temperature (e) bis(hydroxyethylene)disulfide, MeOH, 2h, room temperature, 20%

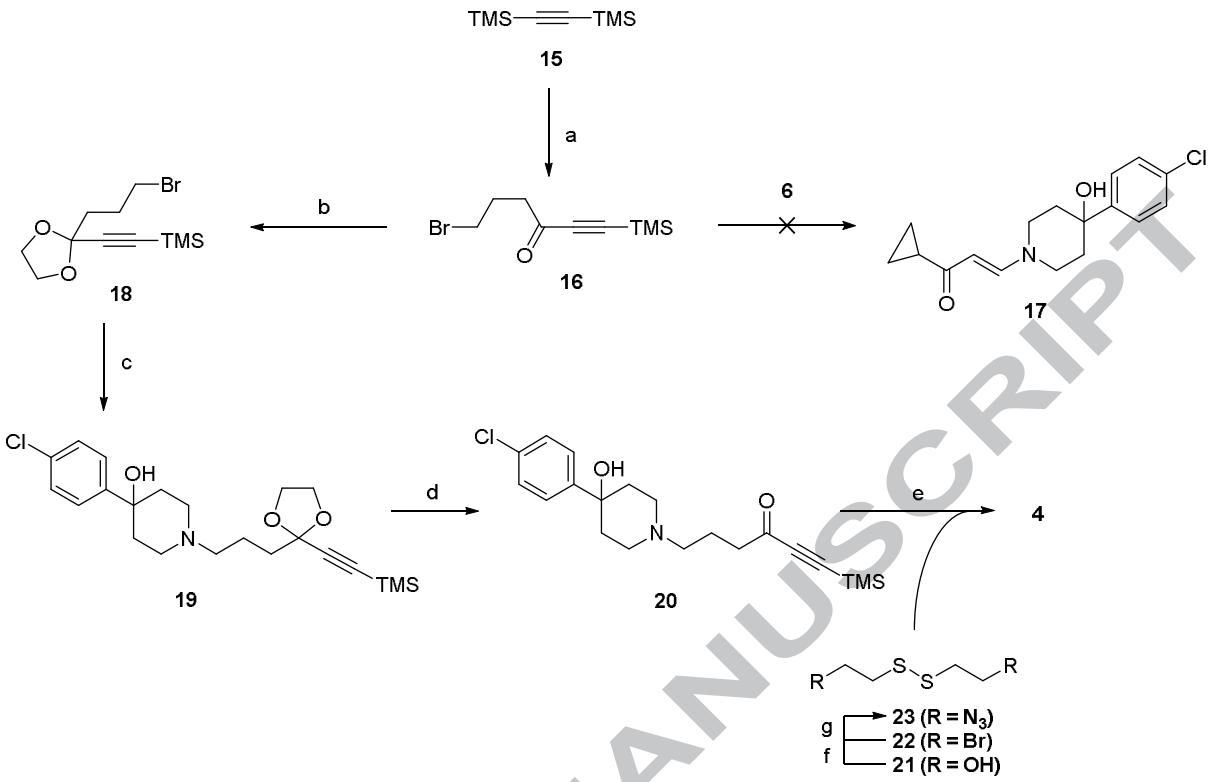
The haloperidol derivatives **2** and **3** (Scheme 2) were prepared in a similar manner as described for the covalent dopamine derivative DW150.[7] The methane sulfonates **11** and **12** consist of a thiopyridyl disulfide and an aromatic scaffold linked by an ethylene or propylene spacer, respectively, and were prepared as previously reported.[7] Compound **6** was alkylated with the electrophilic building blocks **11** and **12**. To initiate a disulfide interchange reaction, the resulting thiopyridyl disulfide precursor **13** and **14** were reacted with cysteamine hydrochloride leading to formation of the final products **2** and **3,** respectively.



Scheme 2. Synthesis of the covalent haloperidol derivatives **2** (n = 1) and **3** (n = 2).

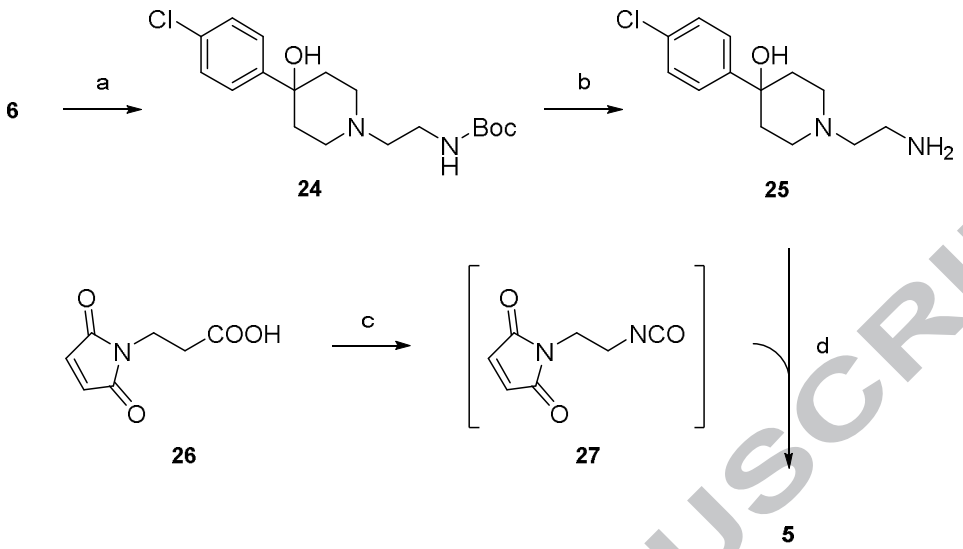
Reagents and conditions: (a) DMSO, 70°C, 16h (b) MeOH, cysteamine hydrochloride, rt, 1h, 8-27%

The bioisosteric replacement of the butyrophenone scaffold of haloperidol by a butyryl substituted 1,2,3-triazole required the preparation of the disulfide-functionalized ligand **4**, which is outlined in Scheme 3. The synthesis began with the acylation of double TMS-protected acetylene **15** with 4-bromobutyryl chloride giving compound **16**, according to a protocol described by Gronnier et al.[21] Unfortunately, the subsequent reaction of **16** with the 4-hydroxypiperidine **6** led to the precipitation of the side product **17**. ^{1}H-NMR and LCMS analysis suggested hetero-Michael addition and cyclopropane formation. Modification of solvent (DMF, DMSO, toluene), base (K_2CO_3, $NaHCO_3$, w/ or w/o KI additive), reaction time (6h to overnight) and temperature (room temperature to 70°C) did not lead to formation of the desired product **20**. Masking the ketone of **16** with 1,2-ethanediol to form the intermediate **18** induced deactivation of the Michael system and reduction of the CH-acidity of ketone **16**. The following alkylation of **6** with **18** afforded the tertiary amine **19** almost quantitatively. Acidic cleavage of the acetal group provided the ketone **20**. Finally, the chemoreactive disulfide probe was attached by copper assisted click chemistry (CuAAC) of the TMS-protected alkyne[22] with the symmetric bis(2-azidoethylene)disulfide **23**, which was prepared according to literature.[23] The TMS-protective group of **20** was cleaved under aqueous conditions and the resulting intermediate underwent a triazole cycloaddition affording the trans-isomer of **4**, which was structurally confirmed by 2D-NMR (Supplementary Data).



Scheme 3. Synthesis of covalent haloperidol derivative **4**. Reagents and conditions: (a) 4-Bromobutyryl chloride, $AlCl_3$, CH_2Cl_2, 0°C to room temperature, 57% (b) 1,2-Dihydroxyethan, pTsOH, toluene, 100°C, 62% (c) 4-(4-Chlorophenyl)piperidin-4-ol, DIPEA, KI, DMF, room temperature, 97% (d) HCl (2M) in MeOH, room temperature, 19h, 60% (e) **23**, sodium L-ascorbate, $CuCl_2$ x H_4O_2, tBuOH/H_2O, N_2, 16h, room temperature, 19% (f) H_2SO_4 (conc), HBr (wt 48%), 100°C, 3h, 69% (g) NaN_3, DMF, 16h, 80°C, 82%.

In order to obtain a maleimide-based covalent derivative of haloperidol, the synthesis started from the building block **6**, which was treated with the commercially available 2-(Boc-amino)ethyl bromide providing compound **24** (Scheme 4). Subsequently, **24** converted to **25** by acidic Boc-deprotection following a described protocol.[24, 25] The maleimide building block **26** was transformed to the corresponding isocyanate **27** via Curtius rearrangement using sodium azide in combination with ethyl chloroformiate.[26] Reaction of the crude isocyanate **27** with the amine **25** yielded the urea derivative **5**.



Scheme 4. Synthesis of covalent haloperidol derivative **5**. Reagents and conditions: (a) 2-(Boc-amino)ethyl bromide, NaI, DIPEA, acetonitrile, N_2, 14h, reflux, 50% (b) HCl in dioxane (4M), 15h, room temperature, 98% (c) NaN_3, ethyl chloroformate, TEA, acetone 60min, 0°C (d) **27**, TEA, DMF, 50h, room temperature, 7%

2.3. Receptor binding studies

2.3.1. Reversible binding affinity to D_2-like receptor subtypes

To assess binding affinity of our designed haloperidol derivatives **1**-**5**, we conducted radioligand binding assays using CHO cells stably expressing the subtypes of the human dopamine D_2-like receptor family including the isoforms $D_{2Short}R$ and $D_{2Long}R$ as well as the closely related D_3R and $D_{4.4}R$ subtypes. Additionally, we tested these compounds on the dopamine receptor mutant $D_{2short}R^{L.2.64C}$ transiently expressed in HEK 293T cells. Binding assays were performed with the radioligand [^{3}H]spiperone and the resulting binding affinities of the covalent ligands were compared to that of the reference antagonist haloperidol (Table 1).

The D_2-type receptor affinities of the test compounds **1**–**5** were significantly lower than for the parent compound haloperidol. Nevertheless, the K_i values in the nanomolar or low micromolar range clearly indicated significant specific binding. The disulfide derivatives **1** and **4** showed the highest affinities (**1**: K_i ($D_{2long}R$) = 240 nM, K_i ($D_{2short}R$) = 73 nM; **4**: K_i ($D_{2long}R$) = 260 nM, K_i ($D_{2short}R$) = 140 nM). This indicates

that subtle structural modifications such as the substitution of the fluorine atom or the bioisosteric replacement of the fluoro-phenyl ring were well tolerated by the receptors. Additionally, the subtype selectivity for D_2R over D_3R showed a 4-6-fold reduction when compared to haloperidol ($D_3R/D_{2short}R$ = 1.9 for **1** and 1.5 for **4**, respectively versus $D_3R/D_{2short}R$ = 9.2 for haloperidol), whereas the distinct D_2R/D_4R-selectivity of haloperidol ($D_4R/D_{2short}R$ = 23) was retained for both ligands ($D_4R/D_{2short}R$ = 13 for **1** and 23 for **4**, respectively). Binding experiments with the $D_{2short}R^{L2.64C}$ mutant with the compounds **1** and **4** led only to a minor loss in affinity relative to the wildtype receptor $D_{2short}R$ (0.22- to 0.44-fold), though, compounds **2**, **3**, and **5** slightly gained in affinity (1.1- to 2.7-fold).

Table 1. Receptor binding affinities of the covalent ligands **1-5** in comparison to the reference haloperidol for the human receptor subtypes of the D_2 family ($D_{2long}R$, $D_{2short}R$, D_3R, and D_4R) and for the mutant $D_{2short}R^{L2.64C}$.

	K_i values (nM±SD)[a]					selectivity pattern		
compd.	$hD_{2long}R$	$hD_{2short}R$	$hD_{2short}R^{L2.64C}$	hD_3R	$hD_{4.4}R$	D_3R / $D_{2short}R$[b]	D_4R / $D_{2short}R$[c]	$D_{2short}R$ / $D_{2short}R^{L2.64C}$[d]
1	240±250	73±52	330±21	140±100	970±190	1.9	13	0.22
2	1500±140	1500±0	550±91	390±50	1100±140	0.26	0.73	2.7
3	1000±120	940±230	850±220	600±430	1500±280	0.63	1.6	1.1
4	260±84	140±79	320±130	210±49	3200±2400	1.5	23	0.44
5	2200±0	1500±280	860±84	690±330	>50000	0.46	>33	1.7
haloperidol[e]	0.42±0.087	0.25±0.027	*n.d.*	2.3±0.11	7.2±2.2	9.2	29	*n.d.*

[a] K_i values as means of two independent experiments each done in triplicate and displayed in nM±SD. [b] Selectivity for $D_{2short}R$ over D_3R calculated by the ratio of $K_i(D_3R)/K_i(D_{2short}R)$. [c] Selectivity for $D_{2short}R$ over D_4R calculated by the ratio of $K_i(D_4R)/K_i(D_{2short}R)$. [d] Selectivity for $D_{2short}R^{L2.64C}$ mutant over the $D_{2short}R$ calculated by the ratio of $K_i(D_{2short}R)/K_i(D_{2short}R^{L2.64C})$. [e] K_i values as means of 4-6 independent experiments each done in triplicate and displayed in nM±S.E.M.. *n.d.* = not determined

2.3.2. Irreversible radioligand depletion

We designed the haloperidol derivatives **1**-**5** to form a covalent bond with the thiol residue of a cysteine amino acid introduced by a single L94C mutation in the transmembrane helix 2 of the dopamine D_2 receptor. To investigate the reactivity of our covalently binding probes, we performed a radioligand depletion assay. In a screening approach HEK293T cells transiently expressing the $D_{2short}R^{L2.64C}$ mutant were incubated for 120 min with compounds **1**-**5** at two different concentrations both representing the 5-fold and 20-fold concentration according to the corresponding K_i values for binding at $D_{2short}R$. Once a ligand is covalently bound to the receptor mutant, the binding crevice of the receptor remains blocked even after several steps of careful washing, which causes a complete removal of reversibly bound ligand. Subsequent radioligand binding experiments reveal a reduced binding of the radioligand [^{3}H]spiperone relative to the amount of blocked binding site. Figure 2 shows the screening results for the covalent ligands **1-5** relative to the reversible effect of the reference haloperidol. All test compounds display a dose-dependent blocking of $D_{2short}R^{L2.64C}$ within a range of 20 to 95%. For the butyrophenone derivative **1**, only minor blocking (20%) was determined at low concentration revealing a weaker tendency to form a stable covalent bond, while at high concentration blocking was improved up to 70%. Compounds **2-5** exhibited substantial irreversible binding at low concentration in the range of 60% to 85%, while increasing the concentration of active reagent led to an inhibition of radioligand binding in the range of 70% to 95%. Among the tested ligands, the disulfide **3** and the maleimide **5** showed superior covalent binding, leading to a remaining radioligand binding of only 10% and 5%, respectively. Despite their weak affinity for all D_2-subtypes, compound **3** and **5** exhibit strong covalent binding of >90% conferring an optimized orientation of the reactive linker to the mutated amino acid and an effective reaction with the cysteine in position 2.64. To learn more about the cross-linking process, we chose to examine the covalent binding kinetics of the most promising disulfide-based ligands **2-4** as well as those of the maleimide-based derivative **5** (Figure 3). Therefore, we performed time-dependent radioligand depletion experiments incubating the receptor together with the covalent ligand for four different time periods. Interestingly, all haloperidol analogs **2-5** showed a very fast

onset of covalent ligation with an amount of blocking in the range of 70% to 85% after 30 minutes with the oxypropyldisulfide **3** exhibiting best blocking yield (87% at 30 min). Extending the time of incubation for up to 240 min did not facilitate any distinct increase of blocking for the disulfide derivatives **2-4** (for **2**: 72% at 30 min to 82% at 240 min, for **3**: 87% at 30 min to 85% at 240 min, for **4**: 80% at 30 min to 86% at 120 min) (Figure 3A-C). By contrast a continuous increase of blocking could be observed for the maleimide **5** revealing an inhibition of the receptor of more than 90% after 120 min (Figure 3D). This may be due the fact that the disulfides **2-4** show some reactivity with constituents within the assay resulting in a partial removal of reactive compound from the receptor during extended incubation. This may not be the case for the maleimide **5** leading to nearly complete blocking of the receptor binding site.

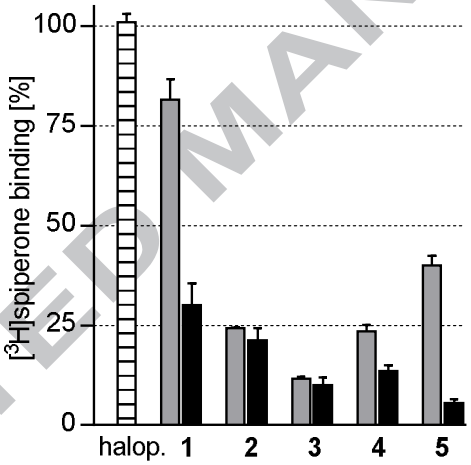


Figure 2. Screening of the blocking behavior of **1-5** in comparison to haloperidol at the mutant receptor $D_{2short}R^{L2.64C}$. Membranes were preincubated with two different concentrations of compounds for 120 min. After washing specific binding of [^{3}H]spiperone was measured indicating the amount of blocking of the receptor binding site. Incubation was run at 20 nM for haloperidol (striped bar), 1 µM (light grey) and 10 µM (black) for **1**, 5 µM (light grey) and 20 µM (black) for **2, 3** and **5**, and 1 µM (light grey) and 5 µM (black) for **4**, respectively. Bars represent the average values (±SD) of two to six individual experiments each done in quadruplicate.

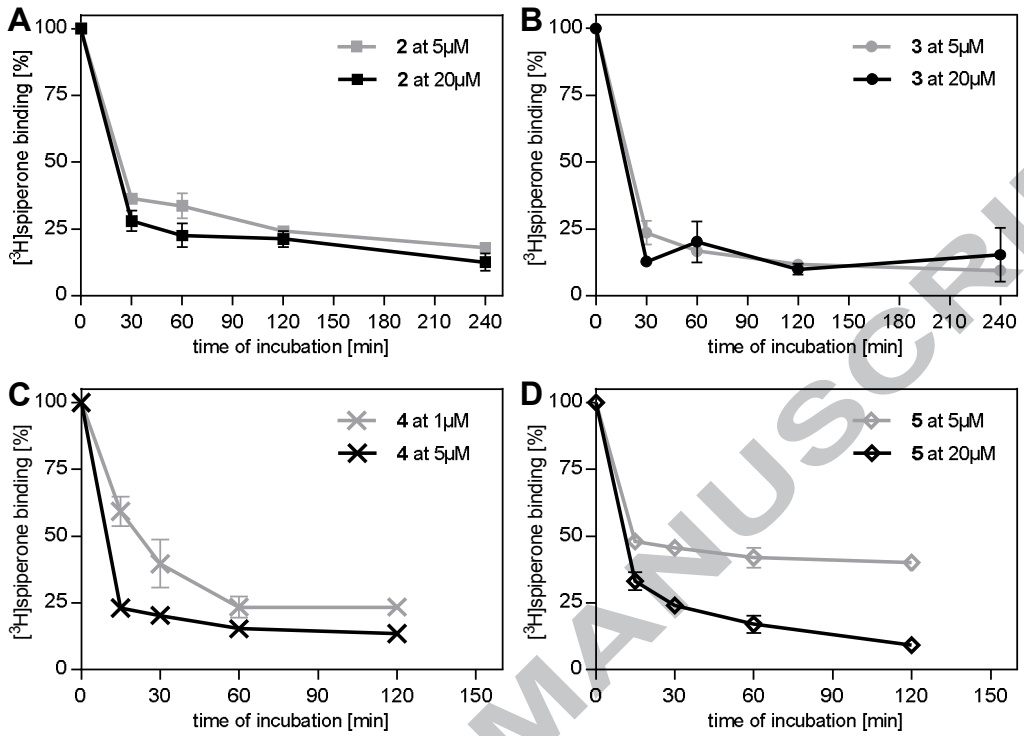


Figure 3. Kinetics of blocking behavior of the test compounds **2-5** at $D_{2short}R^{L2.64C}$. Radioligand depletion was measured time dependently at two different concentrations for compound **2** (**A**), **3** (**B**), **4** (**C**), and **5** (**D**) indicating a fast reaction of all test ligands to covalently block the receptor binding site. Graphs show the average data of pooled curves derived from two to four independent experiments each done in quadruplicate.

2.1. Ligand-Receptor Interactions

To investigate the binding mode of compound **3** and **4**, docking studies were performed using a recently established homology model of D_2R[27] in which we introduced the L2.64C mutation. The consistent para-chlorophenyl-substituted 4-hydroxypiperidine moiety occupies the orthosteric binding pocket formed by residues of transmembrane helices (TMs) 3, 5, 6, 7 and extracellular loop 2 (EL2) showing hydrophobic interactions to Phe6.51, Phe6.52, His6.55 and Phe7.38 as well as Ile183 and Ile184 of EL2 (Figure 4). The canonical salt bridge between their positively charged nitrogen and the carboxylate of Asp3.32 is present for the two ligands. Additionally, a hydrogen bond is formed between their hydroxyl group and

the backbone oxygen of Asp3.32. As expected, further interactions of compounds **3** and **4** to residues located in TM2 and TM7 (Glu2.65 and Tyr7.35, respectively) position the disulfide-based cysteine binding probe in close proximity to Cys2.64, allowing the covalent binding to the mutant receptor.

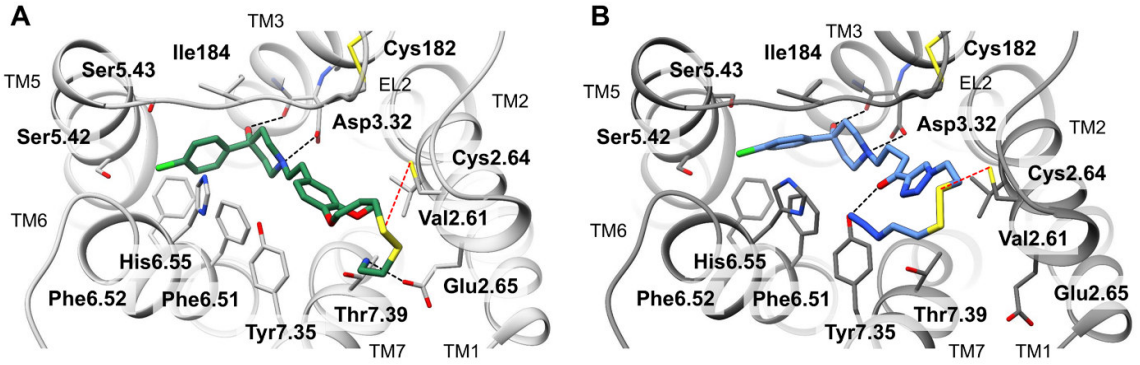


Figure 4. Ligand-receptor interactions of compounds **3** and **4** at D_2R-L2.64C (light gray and dark gray ribbons, respectively). Amino acids expected to interact with the two compounds are displayed as light gray and dark gray sticks. Compound **3** within D_2R is shown by green sticks (A), while compound **4** within D_2R is shown by blue sticks (B). The Ballesteros-Weinstein numbering has been used for transmembrane (TM) residues, whereas sequence numbers are given for residues of extracellular loops (EL).

3. Conclusions

Despite the apparent structural similarity of the endogenous neurotransmitter dopamine and the 4-(4-chlorophenyl)-piperidin-4-ol pharmacophore of haloperidol, the exact binding mode of haloperidol and structurally similar antipsychotic drugs is an open question.[28, 29] Due to the current absence of crystal structures of an antagonist-bound dopamine D_2 receptor, substantial efforts have to be invested to learn more about the receptor-ligand interactions responsible for the stabilization of the receptor in its inactive state. To our knowledge, only a few covalent ligands have been reported to irreversibly antagonize the D_2-receptors.[30-32] In this work, we synthesized a set of covalent ligands derived from the classical antipsychotic drug haloperidol and the covalent dopamine analog FAUC150 irreversibly binding to a L94C cysteine mutant of the dopamine D_2 receptor.[7] We envisaged to apply different

disulfide-based cysteine binding probes as well as a maleimide-based Michael acceptor together with structural modifications including a partial shortening of the haloperidol lead structure. The formal replacement of the single fluorine atom of haloperidol by an ethoxy linker and a disulfide appendage led to moderate binding affinity of the ligand (**1**), however, the rate of covalent binding was comparatively low. Bioisosteric replacement of the butyrophenone moiety to an acylated 1,2,3-triazole or transferring the homovanillyl appendage of FAUC150 increased the efficiency of covalent binding significantly (**2 - 4**). The shortest haloperidol derivative modified with a maleimide covalent probe (**5**) clearly lost in binding affinity, however, it exhibited high covalent binding efficiency. In summary, we were able to show that our haloperidol derivatives covalently bind the dopamine receptor mutant $D_2^{L2.64C}$ and that these ligands compete with the radioligand [^{3}H]-spiperone for the same binding site since the binding of most of our derivatives could not be reversed by adding an excess of radioligand. Future studies will aim for the structural optimization of the reported covalent analogs. Moreover, we plan to implement these ligands in structural and functional investigations of dopamine receptors.

4. Experimental section

4.1. General

Unless otherwise noted, reactions were performed under nitrogen atmosphere and reagents as well as dry solvents were of commercial quality as purchased. MS was run on a BRUKER ESQUIRE 2000 using electrospray ionization (ESI). HRMS-ESI was run on an AB Sciex Triple TOF660 SCiex, source type ESI or at the Chair of Organic Chemistry, Friedrich Alexander University Erlangen-Nürnberg on a Bruker Daltonik micrOTOF II focus or Bruker Daltonik maXis 4G, source type ESI or APPI. NMR spectra were obtained on a Bruker Avance 400 (400 MHz for ^{1}H and 101 MHz for ^{13}C) or a Bruker Avance 600 (600 MHz for ^{1}H and 151 MHz for ^{13}C) spectrometer at 300 K in the solvents indicated. The chemical shifts are reported in ppm (δ) relative to TMS. Purification via column chromatography was performed using silica gel 60. TLC analyses were performed on Merck 60 F254 aluminum plates in combination with UV detection (254 nm) and/or TLC staining (Ninhydrin, $KMnO_4$, Vanillin, $FeCl_3$). Analytical HPLC was conducted on an Agilent 1200 HPLC system employing a DAD detector and with a ZORBAX ECLIPSE XDB-C8 (4.6 x 150 mm, 5 µm) column and

using one of the following binary solvent systems. System 1: eluent, methanol/0.1% aq formic acid, 10% methanol for 3 min, to 100% in 15 min, 100% for 6 min, to 10% in 3min, then 10% for 3min, flow rate 0.5 mL/min; System 2: CH_3CN/0.1% aq TFA, 10% CH_3CN for 3 min, to 100% in 15 min, 100% for 6 min, to 10% in 3min, then 10% for 3min, flow rate 0.5 mL/min; System 3: methanol/0.1% aq TFA, 10% methanol for 3 min, to 100% in 15 min, 100% for 6 min, to 10% in 3min, then 10% for 3min, flow rate 0.5 mL/min; Preparative HPLC was performed on an Agilent 1100 Preparative Series, using a *ZORBAX ECLIPSE XDB-C8 PrepHT* column (21.5 x 150 mm, 5 µm, flow rate 10 mL/min) , a *VP 250/32 NUCLEODUR C18 HTec* column (5µm, flow rate 32 mL/min) or a *VP 250/10 NUCLEODUR C18 HTec* column (5µm, flow rate 2-4 mL/min) with the solvent systems indicated and a detection wavelength of 220 nm or 254 nm.

4.2. Synthesis

4.2.1. 4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-1-(4-hydroxyphenyl)butan-1-one (8)

4(4-chlorophenyl)-piperidin-4-ol (**6**) and 4-Chloro-4'-hydroxybutyrophenone (**7**) were commercially available. The amine **6** (2.0g, 9.5 mmol), compound **7** (2.1 g, 10 mmol), DIPEA (4.1 mL, 24 mmol) and potassium iodide (94 mg, 0.57 mmol) were placed in a Schlenk flask and dissolved in 60 mL anhydrous DMF. After stirring for 16 hours at 70°C the solvent was evaporated under reduced pressure and the residue was taken up in ethyl acetate and acidified with 2N HCl (pH = 3). The resulting precipitate was filtered and washed with CH_2Cl_2 /methanol (2/1) and dried in vacuum to give **8** as a pale yellow solid. (0.80 g, 23%). ^{1}H NMR (600 MHz, DMSO-d6) δ 7.82 (d, J = 8.7 Hz, 2H), 7.40 (d, J = 8.6 Hz, 2H), 7.33 (d, J = 8.6 Hz, 2H), 6.85 (d, J = 8.4 Hz, 2H), 2.87 (t, J = 6.9 Hz, 2H), 2.64 – 2.55 (m, 2H), 2.36 – 2.28 (m, 4H), 1.84 – 1.70 (m, 3H), 1.53 – 1.42 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d6) δ 197.91, 163.05, 149.26, 130.66, 130.35, 127.90, 127.65, 126.81, 115.35, 69.59, 57.43, 48.94, 37.87, 35.10, 22.08; ESI-MS *m/z* 374.15 $[M+H]^+$. HPLC (system 1, 220 nm): t_R = 15.3 min, purity: 96%, (system 2, 220 nm): t_R = 14.9 min, purity: 98%. HR-ESI-MS *m/z* $[M+H]^+$ calcd for $C_{21}H_{24}ClNO_3$, 374.1517; found, 374.1515

4.2.2. 1-(4-(2-bromoethoxy)phenyl)-4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)butan-1-one (9)

Compound **8** (0.20 g, 0.54 mmol), 1,2-dibromoethane (0.23 mL, 2.7 mmol) and K_2CO_3 (0.15 g, 1.1 mmol) were dissolved in acetonitrile (20mL) and stirred at 70°C for 41 hours. An additional portion of 1,2-dibromoethane (0.09 mL, 1.1 mmol) was added to the mixture and stirring/heating was continued for 5 hours. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The residue was taken up in H_2O and extracted multiple times with CH_2Cl_2. After drying (Na_2SO_4) the solvent was evaporated and the crude product was purified via flash column chromatography (eluents: CH_2Cl_2/methanol 9/1) to afford compound **9** (74 mg, 28%). ^{1}H NMR (600 MHz, DMSO-d6) δ 7.97 (d, J = 8.8 Hz, 2H), 7.39 (d, J = 8.3 Hz, 2H), 7.35 (d, J = 8.3 Hz, 2H), 7.09 (d, J = 8.8 Hz, 2H), 4.91 (s, 1H), 4.42 (t, J = 5.4 Hz, 2H), 3.83 (t, J = 5.3 Hz, 2H), 2.96 (t, J = 6.9 Hz, 2H), 2.81 – 2.58 (m, 2H), 2.58 – 2.24 (m, 4H), 1.89 – 1.81 (m, 2H), 1.77 (s, 2H), 1.58 – 1.47 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d6) δ 198.05, 161.54, 149.43, 130.81, 130.40, 130.24, 127.72, 126.74, 114.41, 69.27, 68.00, 62.79, 48.82, 37.32, 35.24, 31.21, 21.55; ESI-MS *m/z* 480.1/482.1 $[M+H]^+$, HR-ESI-MS *m/z* $[M+H]^+$ calcd for $C_{23}H_{27}BrNO_3$, 480.0936; found, 480.0936

4.2.3. **S-(2-(4-(4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)butanoyl)phenoxy)-ethyl)ethanethioate (10)**

Compound **8** (67 mg, 0.14 mmol) and potassium thioacetate (48 mg, 0.42 mmol) were dissolved in 4.0 mL dry acetone and heated at 60°C for 90 minutes. The reaction was allowed to cool to room temperature and the solvent was removed under reduced pressure. The residue was taken up in H_2O and extracted with CH_2Cl_2. The combined organic fractions were dried (Na_2SO_4) and the solvent was evaporated to give compound **10** without further purification step (60 mg, 90%). ^{1}H NMR (600 MHz, DMSO-d6) δ 8.00 – 7.92 (m, 2H), 7.45 – 7.37 (m, 2H), 7.35 – 7.28 (m, 2H), 7.00 – 6.91 (m, 2H), 4.16 (t, J = 6.5 Hz, 2H), 3.29 (t, J = 6.5 Hz, 2H), 3.00 (t, J = 6.9 Hz, 2H), 2.99 – 2.94 (m, 3H), 2.68 – 2.56 (m, 4H), 2.38 (s, 3H), 2.22 – 2.13 (m, 2H), 2.09 – 2.01 (m, 2H), 1.79 – 1.68 (m, 2H) ^{13}C NMR (100 MHz, $CDCl_3$) 198.58, 195.45, 162.25, 146.85, 132.94, 130.73, 130.51, 128.54, 126.24, 114.37, 71.16, 66.79, 57.97, 49.41, 38.31, 36.10, 30.73, 28.37, 21.86; HPLC (system 1, 254 nm): t_R = 17.6 min, purity: 93%; (system 2, 254 nm) t_R = 16.3 min, purity: 92%; ESI-MS *m/z* 476.2 $[M+H]^+$; HR-ESI-MS *m/z* $[M+H]^+$ calcd for $C_{25}H_{30}ClNO_4S$, 476.1657; found, 476.1657

4.2.4. 4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-1-(4-(2-((2-hydroxyethyl) disulfanyl)ethoxy)phenyl)butan-1-one x HCOOH (1)

Compound **10** (51 mg, 0.11 mmol) was dissolved in 2.0 mL anhydrous MeOH and a 0.5M solution of NaOMe (0.26 mL, 0.13 mmol) was added while stirring. After 2 minutes Bis(hydroxyethylene)disulfide (30µL, 0.21 mmol) was added with a microsyringe and the reaction was stirred for additional 2 hours at room temperature. Purification by preparative HPLC (C8 eluents: MeOH/0.1% aq. HCOOH 25-100%) yielded the formic acid salt of product **1 (**12 mg, 20%**)** as a colorless solid. ^{1}H NMR (400 MHz, DMSO-d6) δ 8.18 (s, 1H), 8.03 – 7.88 (m, 2H), 7.49 – 7.38 (m, 2H), 7.38 – 7.30 (m, 2H), 7.16 – 7.01 (m, 2H), 4.32 (t, J = 6.2 Hz, 2H), 3.68 – 3.57 (m, 2H), 3.20 – 3.08 (m, 2H), 3.04 – 2.92 (m, 2H), 2.86 – 2.72 (m, 4H), 2.62 – 2.52 (m, 3H), 1.94 – 1.73 (m, 4H), 1.63 – 1.47 (m, 2H) ^{13}C NMR (101 MHz, DMSO-d6) δ 197.91, 161.84, 148.63, 130.90, 130.25, 130.15, 127.77, 126.75, 114.35, 69.13, 66.02, 59.55, 59.36, 56.78, 48.70, 41.11, 37.04, 35.17, 21.15; HPLC (system 1, 220 nm): t_R = 14.0 min, purity: >99%, (system 2, 220 nm): t_R = 16.0 min, purity: 96%; ESI-MS *m/z* 510.2 $[M+H]^+$; HR-ESI-MS *m/z* $[M+H]^+$ calcd for $C_{25}H_{32}ClNO_4S_2$, 510.1534; found, 510.1534

4.2.5. 1-(4-(2-((2-aminoethyl)disulfanyl)ethoxy)-3-methoxyphenethyl)-4-(4-chlorophenyl)piperidin-4-ol x 2·TFA (2)

Compounds **6** (153 mg, 722 µmol) and **11** (100 mg, 241 µmol) were placed in a sealed tube and dissolved in 2 mL of anhydrous DMSO. The solution was heated at 70°C for 16 hours. The reaction mixture was allowed to cool to room temperature followed by the addition of H_2O and subsequent lyophilization. After a first purification step via flash column chromatography on silica gel (eluents: CH_2Cl_2/MeOH 98/2 to 90/10) the resulting oil was redissolved in dry MeOH (1mL) and cysteamine hydrochloride (10 mg, 88 µmol) was added. The mixture was stirred for 1 hour at room temperature and the product **2** was isolated by preparative HPLC (C8 eluents: CH3CN /aq. TFA 0.1%; 5-45%) to give the corresponding TFA salt (12 mg, 8% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.53 – 7.47 (m, 2H), 7.41 – 7.35 (m, 2H), 6.97 (d, J = 2.1 Hz, 1H), 6.96 (d, J = 8.2 Hz, 1H), 6.86 (dd, J = 8.2, 2.0 Hz, 1H), 4.26 (t, J = 6.1 Hz, 2H), 3.86 (s, 3H), 3.64 – 3.57 (m, 2H), 3.52 – 3.44 (m, 2H), 3.44 – 3.38 (m, 2H), 3.32 – 3.28 (m, 2H), 3.14 (t, J = 6.1 Hz, 2H), 3.10 – 3.04 (m, 2H), 3.00 (t, J = 6.6 Hz, 2H), 2.34 (td, J = 14.1, 4.3 Hz, 2H), 2.02 – 1.95 (m, 2H) ^{13}C NMR (151 MHz, CD_3OD) δ 151.42, 148.48, 146.99, 134.30, 131.35, 129.53, 127.41, 122.28, 115.93,

114.22, 69.21, 68.68, 59.28, 56.57, 50.33, 39.09, 38.96, 36.67, 35.41, 31.09. HPLC (system 2, 220 nm): t_R = 14.0 min, purity: >99%; (system 3, 220 nm): t_R = 16.2 min, purity: >99%; ESI-MS *m/z* 497.2 $[M+H]^+$; HR-ESI-MS *m/z* $[M+1]^+$ calcd for $C_{24}H_{34}ClN_2O_3S_2$, 497.169389; found, 497.168036.

4.2.6. 1-(4-(3-((2-aminoethyl)disulfanyl)propoxy)-3-methoxyphenethyl)-4-(4-chlorophenyl)piperidin-4-ol x 2·TFA (3)

Compounds **6** (0.15g, 0.70 mmol) and **12** (0.10 g, 0.23 mmol) were placed in a sealed tube and dissolved in 2 mL of anhydrous DMSO. The solution was heated at 70°C for 20 hours. The reaction mixture was allowed to cool to room temperature followed by the addition of H_2O and subsequent lyophilization. After a first purification step via flash chromatography on silica gel (eluents: CH_2Cl_2/MeOH; 98/2 to 90/10) the resulting oil was redissolved in dry MeOH (1mL) and cysteamine hydrochloride (10 mg, 88 µmol) was added. The mixture was stirred for 1 hour at room temperature and the product was isolated by preparative HPLC (C8 eluents: CH_3CN /aq. TFA 0.1%; 5-45%) as the corresponding TFA salt (39 mg, 27% yield). ^{1}H NMR (600 MHz, CD_3OD) δ 7.50 (d, J = 8.6 Hz, 2H), 7.38 (d, J = 8.6 Hz, 2H), 6.94 (d, J = 1.9 Hz, 1H), 6.93 (d, J = 8.2 Hz, 1H), 6.85 (dd, J = 8.2, 2.0 Hz, 1H), 4.09 (t, J = 6.0 Hz, 2H), 3.85 (s, 3H), 3.64 – 3.57 (m, 2H), 3.52 – 3.43 (m, 2H), 3.44 – 3.38 (m, 2H), 3.32 – 3.26 (m, 2H), 3.10 – 3.03 (m, 2H), 2.99 – 2.90 (m, 4H), 2.38 – 2.28 (m, 2H), 2.23 – 2.13 (m, 2H), 2.05 – 1.92 (m, 2H). ^{13}C NMR (151 MHz, CD_3OD) δ 151.37, 148.93, 146.98, 134.31, 130.85, 129.53, 127.41, 122.25, 115.54, 114.04, 69.20, 68.48, 59.32, 56.57, 50.33, 39.28, 36.67, 35.49, 35.15, 31.10, 29.93; HPLC (system 2, 220 nm): t_R = 14.2 min, purity: >99%, (system 3, 220 nm): t_R = 16.5 min, purity: >99%; ESI-MS *m/z* 511.2 $[M+H]^+$ HR-ESI-MS *m/z* $[M+H]^+$ calcd for $C_{25}H_{36}ClN_2O_3S_2$, 511.185039; found, 511.185663.

4.2.7. 6-bromo-1-(trimethylsilyl)hex-1-yn-3-one (16)

Compound **16** was synthesized following the literature.[33] Anhydrous $AlCl_3$ (1.88 g, 14.1 mmol) was placed in a Schlenk flask and stirred in 16 mL methylene chloride at 0°C under argon atmosphere. 4-Bromobutyryl chloride (1.49 mL, 12.9 mmol) was added to the cooled solution followed by the addition of bis(trimethylsilyl)acetylene (2.00 g, 11.7 mmol) in small portions. After stirring for 1 hour at 0°C the reaction mixture was quenched with cold H_2O and extracted with methylene chloride. The

combined organic layers were washed with H_2O and Brine, dried over Na_2SO_4 and the solvent was removed under reduced pressure. Flash column chromatography (eluent: n-hexane/EtOAc 100/1, $KMnO_4$ staining) yielded the product **16** as a colorless oil (1.65 g, 57%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.45 (t, J = 6.4 Hz, 2H), 2.78 (t, J = 7.0 Hz, 2H), 2.25 – 2.16 (m, 2H), 0.25 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 186.14, 101.87, 98.66, 43.53, 32.64, 26.70, -0.64. HPLC (system 2, 254 nm): t_R = 21.4 min, purity: 96%; ESI-MS *m/z* 246.9/248.9 $[M+H]^+$

4.2.8. ((2-(3-bromopropyl)-1,3-dioxolan-2-yl)ethynyl)trimethylsilane (18)

Compound **16** (0.50 g, 2.0 mmol) 1,2-ethanediol (1.3 g, 20 mmol) and para-toluenesulfonic acid (77 mg, 0.40 mmol) were dissolved in 50 mL toluene and stirred at 100°C for 16 hours. The reaction mixture was allowed to cool to room temperature and the solvent was evaporated under reduced pressure. The crude product was purified via flash column chromatography on silica gel (eluent: n-hexane/ethyl acetate 20/1, $KMnO_4$ staining) to yield compound **18** (0.37 g, 62%) as colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.14 – 4.05 (m, 2H), 4.01 – 3.94 (m, 2H), 3.48 (t, J = 6.7 Hz, 2H), 2.16 – 2.09 (m, 2H), 2.06 – 2.02 (m, 2H), 0.18 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 102.56, 102.34, 89.21, 64.83, 37.75, 33.59, 27.57, -0.08 ESI-MS *m/z* 290.6/292.6 $[M+H]^+$

4.2.9. 4-(4-chlorophenyl)-1-(3-(2-((trimethylsilyl)ethynyl)-1,3-dioxolan-2-yl)propyl)piperidin-4-ol (19)

Compound **18** (0.20 g, 0.69 mmol) and amine **6** (0.73 g, 3.4 mmol) were dissolved in 2.0 mL anhydrous DMF. Potassium iodide (23 mg, 0.14 mmol) and DIPEA (0.60 mL, 3.4 mmol) were added to the reaction mixture and stirred for 13 hours at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by flash column chromatography on silica gel (eluents: CH_2Cl_2 / MeOH 95/5) to give product **19** (0.28 g, 97%) as a pale yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.51 – 7.44 (m, 2H), 7.35 – 7.30 (m, 2H), 4.12 – 4.03 (m, 2H), 4.06 – 3.97 (m, 2H), 3.38 – 3.29 (m, 2H), 3.18 (t, J = 12.4 Hz, 2H), 3.04 – 2.97 (m, 2H), 2.77 (t, J = 14.4 Hz, 2H), 2.16 – 2.06 (m, 2H), 1.99 (t, J = 7.1 Hz, 2H), 1.95 – 1.87 (m, 2H), 0.19 (s, 9H) ^{13}C NMR (151 MHz, $CDCl_3$) δ 144.77, 133.73, 128.90, 126.16, 102.32, 101.94, 89.65, 69.86, 64.88, 57.33, 48.95, 36.07, 35.83, 19.12, -0.04. HPLC (system 1, 210 nm): t_R = 18.2 min, purity: 86%, (system 2, 210 nm): t_R = 17.4 min, purity:

86%; ESI-MS *m/z* 422.17 $[M+H]^+$; HR-ESI-MS *m/z* $[M+H]^+$ calcd for $C_{22}H_{33}ClNO_3Si$, 422.191275; found, 422.190363.

4.2.10. 6-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-1-(trimethylsilyl)-hex-1-yn-3-one trifluoroacetate (20)

19 (0.10 g, 0.24 mmol) was dissolved in 0.5 mL MeOH and treated with 2N aq. HCl (1.0mL, 2.0 mmol) for 2 hours at room temperature. The mixture was diluted with 5mL H_2O and lyophilized to dryness. The crude product was purified via preparative HPLC (C18, eluents: CH_3CN / 0.1% aq. TFA, 10-45%) to give **20** as a white solid (70 mg, 60%). ^1H NMR (600 MHz, $CDCl_3$) δ 12.40 (s, 1H), 7.44 – 7.38 (m, 2H), 7.37 – 7.31 (m, 2H), 3.54 – 3.46 (m, 2H), 3.30 – 3.21 (m, 3H), 3.04 (t, J = 8.0 Hz, 2H), 2.77 (t, J = 6.5 Hz, 2H), 2.57 (td, J = 14.0, 4.1 Hz, 2H), 2.36 (s, 3H), 2.15 – 2.06 (m, 2H), 1.97 – 1.84 (m, 2H), 0.25 (s, 9H) ^{13}C NMR (91 MHz, $CDCl_3$) δ 185.36, 144.29, 134.09, 129.06, 125.96, 101.46, 99.95, 69.39, 56.25, 48.81, 41.93, 35.52, 17.98, -0.75. ESI-MS *m/z* 378.18 $[M+H]^+$; HPLC (system 1, 230nm) : t_R = 17.9 min, purity: 98%, (system 2, 230 nm): t_R = 17.1 min, purity: 73%; HR-ESI-MS *m/z* $[M+H]^+$ calcd for $C_{20}H_{28}ClNO_2Si$, 377.1578; found, 377.1578.

4.2.11. Bis(2-bromoethyl)disulfide (22)

Bis(2-bromoethyl)disulfide (**22**) was synthesized following the literature[23]. To an ice-cooled solution of conc. H_2SO_4 (20 mL) was slowly added HBr (48% wt, 28 mL) under stirring. Subsequently, Bis(2-hydroxyethyl)disulfide **21** (0.52 mL, 4.3 mmol) was added dropwise and the solution was stirred for 16 hours at room temperature followed by stirring at 100°C for 4 hours. The mixture was allowed to cool to room temperature and extracted with methylene chloride. The combined organic solvents were washed with H_2O, 10% Na_2CO_3 solution and dried (Na_2SO_4). Evaporation of the solvent under reduced pressure gave compound **21** (0.83 g, 69%) which was used in the next step without further purification. ^1H NMR (600 MHz, $CDCl_3$) δ 3.67 – 3.58 (m, 4H), 3.15 – 3.07 (m, 4H). Analytical data were in agreement with the literature.

4.2.12. Bis(2-azidoethyl)disulfide (23)

Bis(2-azidoethyl)disulfide (**23**) was synthesized following the literature[23]. Compound **22** (0.15 g, 0.54 mmol) and sodium azide (0.17 g, 2.7 mmol) were dissolved in 8.0 mL DMF and heated at 80°C for 16 hours. After cooling to room temperature the

reaction mixture was extracted with diethyl ether, washed with brine and dried over Na_2SO_4. Careful evaporation of the solvent (no heating, slow rotation, potentially explosive!) gave the crude product **23** which was used in the next step without further purification (90 mg, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.6 (t, J = 6.7 Hz, 4H), 2.88 (t, J = 6.7 Hz, 4H). Analytical data were in agreement with the literature.

4.2.13. 1-(1-(2-((2-azidoethyl)disulfanyl)ethyl)-1H-1,2,3-triazol-4-yl)-4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)butan-1-one x TFA (4)

Compound **20** (62 mg, 0.13 mmol), sodium L-ascorbate (5.0 mg, 25 µmol), $CuCl_2$ x H_4O_2 (2.5 mg, 13 µmol) and bis(azidoethylene)disulfide (31 mg, 0.15 mmol) were placed in a Schlenk flask and dissolved In 1.0 mL CH_3CN/H_2O (4/1) under argon atmosphere. After stirring for 2 hours at 70°C the reaction mixture was allowed to cool to room temperature and the desired product **4** was purified via preparative HPLC (C8, eluents: CH_3CN / 0.1% aq. TFA; 16 mg, 19%). ^{1}H NMR (600 MHz, DMSO-d6) δ 9.37 (s, 1H), 8.85 (s, 1H), 7.51 – 7.45 (m, 2H), 7.48 – 7.42 (m, 2H), 5.62 (s, 1H), 4.77 (t, J = 6.5 Hz, 2H), 3.60 (t, J = 6.4 Hz, 2H), 3.51 – 3.49 (m, 4H), 3.32 (t, J = 6.5 Hz, 2H), 3.29 – 3.21 (m, 2H), 3.18 (t, J = 7.2 Hz, 2H), 2.97 (t, J = 6.4 Hz, 2H), 2.18 (m, J = 14.1, 4.4 Hz, 2H), 2.11 – 2.02 (m, 2H), 1.84 (d, J = 14.2 Hz, 2H). ^{13}C NMR (91 MHz, DMSO-d6) δ 192.65, 146.70, 146.54, 131.61, 128.18, 127.54, 126.61, 67.68, 55.36, 49.13, 48.42, 48.38, 36.76, 36.66, 35.87, 35.03, 17.77; HPLC (system 1, 254 nm): t_R = 16.6 min, purity: 97%, (system 2, 254 nm): t_R = 16.4 min, purity: 98%; ESI-MS *m/z* 510.20 $[M+H]^+$; HR-ESI-MS *m/z* $[M+H]^+$ calcd for $C_{21}H_{28}ClN_7O_2S_2$, 510.150; found, 510.1507

4.2.14. tert-butyl-(2-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-ethyl)-carbamate (24)

Compound **24** was prepared as described in the literature.[24] Amine **6** (0.40 mg, 1.9 mmol), sodium iodide (0.28 g, 1.9 mmol) and DIPEA (0.31 mL, 1.9 mmol) were dissolved in 40mL acetonitrile. A solution of 2-(Boc-amino)ethylbromide (0.55 g, 2.5 mmol) in 2.5 mL acetonitrile was added dropwise and the mixture was heated to reflux for 14 hours. After the reaction was allowed to cool to room temperature the

solvent was removed under reduced pressure. The residue was taken up in K_2CO_3 solution (1M, 50 mL) and ethyl acetate. The combined organic fractions were washed with H_2O, Brine and dried (Na_2SO_4). Evaporation of the solvent under reduced pressure gave the crude product which was purified via flash column chromatography on silica gel (eluents: n-hexane/ethyl acetate 2/3 to 1/4) to yield compound **24** (338 mg, 50%). ^{1}H NMR (600 MHz, DMSO-d6) δ 7.51 – 7.45 (m, 2H), 7.38 – 7.32 (m, 2H), 6.62 (t, J = 5.8 Hz, 1H), 4.87 (s, 1H), 3.08 – 3.01 (m, 2H), 2.67 – 2.60 (m, 2H), 2.42 – 2.35 (m, 2H), 2.35 (t, J = 7.1 Hz, 2H), 1.88 (td, J = 12.8, 4.3 Hz, 2H), 1.59 – 1.51 (m, 2H), 1.38 (s, 9H) ^{13}C NMR (101 MHz, DMSO-d6) δ 155.53, 149.19, 130.73, 127.71, 126.82, 77.49, 69.45, 57.61, 49.10, 37.90, 37.67, 28.26; HPLC (system 1, 210 nm): t_R = 16.9 min, purity: 88%; ESI-MS *m/z* 355.0 $[M+H]^+$ Analytical data were in agreement with the literature.

4.2.15. 1-(2-aminoethyl)-4-(4-chlorophenyl)piperidin-4-ol hydrochloride (25)

The Boc-protected amine **24** (0.31 g, 0.87 mmol) was stirred in a 4M solution of HCl in 1,4-dioxane (2.0 mL, 7.9 mmol) for 15 hours at room temperature. Evaporation of the solvent under reduced pressure gave the hydrochloric salt of **25** (0.28 g, 98%). ^{1}H NMR (600 MHz, DMSO-d6) δ 7.52 (d, J = 8.5 Hz, 2H), 7.38 (d, J = 8.5 Hz, 2H), 3.62 (d, J = 12.0 Hz, 2H), 3.51 (m, 6H), 2.53 (td, J = 14.1, 4.3 Hz, 2H), 2.02 – 1.94 (m, 2H) ^{13}C NMR (101 MHz, DMSO-d6) δ 146.87, 134.32, 129.52, 127.47, 69.15, 68.14, 54.75, 51.03, 36.58, 35.33; ESI-MS *m/z* 255.0 $[M+H]^+$ Analytical data were in agreement with the literature.[25]

4.2.16. 1-(2-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)ethyl)-3-(2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)urea (5)

3-maleimidepropionic acid **26** (0.10 g, 0.59 mmol) and TEA (90 µL, 0.65 mmol) were dissolved in 1.2 mL acetone and cooled to -5°C. After 2 minutes ethyl chloroformate (60µL, 0.65 mmol) was added dropwise and stirred for additional 10 minutes. Sodium azide (38 mg, 0.59 mmol) was added and the reaction was stirred for 45 minutes. Subsequently, the mixture was diluted with H2O, extracted with toluene (30 mL) and dried with Na_2SO_4. The organic phase was heated at 130°C for 100 minutes and allowed to cool to room temperature afterwards. The solvent was removed under reduced pressure and the crude oil was taken up in DMF (4 mL) and used in the next step without further purification. The hydrochloric salt of amine **25** (0.12 mg, 0.37

mmol) was added to the solution and stirred for 50 hours at room temperature. Evaporation of the solvent and purification by preparative HPLC (C8, eluents: CH_3CN/0.1% aq. TFA) gave the TFA salt of **5** as white solid (13 mg, 7%). ^{1}H NMR (600 MHz, DMSO-d6) δ 8.21 (br s, 1H), 7.53 – 7.45 (m, 2H), 7.44 – 7.35 (m, 2H), 7.00 (s, 2H), 6.25 (t, J = 6.1 Hz, 1H), 5.99 (d, J = 7.5 Hz, 1H), 5.18 (s, 1H), 3.43 (t, J = 5.9 Hz, 2H), 3.21 – 3.11 (m, 4H), 3.00 – 2.89 (m, 2H), 2.80 – 2.70 (m, 2H), 2.69 – 2.61 (m, 2H), 2.08 – 1.96 (m, 2H), 1.76 – 1.61 (m, 2H) ^{13}C NMR (101 MHz, DMSO-d6) δ 171.06, 158.14, 148.18, 134.50, 131.08, 127.89, 126.74, 68.75, 57.19, 48.81, 37.95, 37.58, 36.59, 36.09; HPLC (system 1, 210 nm): t_R = 15.4 min, purity: >99%; (system 2, 210 nm): t_R = 13.8 min, purity: >99%; ESI-MS *m/z* 421.2 $[M+H]^+$; HR-ESI-MS *m/z* $[M+H]^+$ calcd for $C_{20}H_{26}ClN_4O_4$, 421.1637; found, 421.1637.

4.3. Biological Investigation

4.3.1. Receptor Binding Experiments

Receptor binding studies were carried out as described previously.[34, 35] In brief, competition binding experiments with the human $D_{2long}R$, $D_{2short}R$,[36] D_3R[37] and $D_{4.4}R$[38] receptors were perform when using preparations of membranes from CHO cells stably expressing the corresponding receptor together with [^{3}H]spiperone (specific activity = 81 Ci/mmol, PerkinElmer, Rodgau, Germany) at a final concentration of 0.15-0.30 nM. The assays were carried out at protein concentrations of 5-6 µg/assay tube, B_{max} values of 800-1500 fmol/µg and K_D values of 0.060-0.10 nM for $D_{2long}R$, concentrations of 1-4 µg/assay tube, B_{max} values of 2600-8500 fmol/µg and K_D values of 0.050-0.090 nM for $D_{2short}R$, concentrations of 1-3 µg/assay tube, B_{max} values of 2300-8500 fmol/µg and K_D values of 0.080-0.15 nM for D_3R, and concentrations of 4-10 µg/assay tube, B_{max} values of 1000-1700 fmol/µg and K_D values of 0.14-0.25 nM for $D_{4.4}R$, respectively. Radioligand binding assays with the mutant receptor $D_{2short}R^{L2.64C}$ were performed using homogenates of membranes from HEK 293T cells, which were transiently transfected with the pcDNA3.1 vector containing the appropriate mutation using the Mirus TransIT-293 transfection reagent (MoBiTec, Goettingen, Germany).[7] Unspecific binding was determined in the presence of 10 µM haloperidol, protein concentration was established by the method of Lowry using bovine serum albumin as standard.[39] The resulting competition curves of the receptor binding experiments were analyzed by nonlinear regression using the algorithms in PRISM 6.0 (GraphPad Software, San Diego, CA). The data were

initially fit using a sigmoid model to provide an IC_{50} value, representing the concentration corresponding to 50% of maximal inhibition. IC_{50} values were transformed to K_i values according to the equation of Cheng and Prusoff.[40]

4.3.2. Radioliogand Depletion Assays.

Tests on covalent blocking of the receptor were carried out as described previously.[7] Membranes from HEK 293T cells transiently expressing the human $D_{2short}R^{L2.64C}$ were preincubated in binding buffer (50 mM Tris, 1 mM EDTA, 5 mM MgCl2, 100 µg/mL bacitracin, 5 µg/mL soybean trypsin inhibitor at pH 7.4) at 37°C at a protein concentration of 50 µg/mL and the test compounds applying two different concentrations roughly representing the 5-fold or 20-fold K_i value derived from the binding experiment with the wild-type receptor. In particular the compounds **2**, **3** and **5** were tested at concentrations of 5 µM and 20 µM, ligand **1** at 1 µM and 10 µM, and compound **4** at 1 µM and 5 µM, respectively. As a reference haloperidol was used at 20 nM (100-fold). For screening of the test compounds preincubation was run for 120 min, while kinetic studies were performed with incubation times from 15 min to 240 min. Generally incubation was stopped by centrifugation and the amount of reversibly bound ligand was washed out for four times (resuspension of the memebranes in buffer for 30 min followed by centrifugation). Washed membranes were used for radioligand binding experiments with [^{3}H]spiperone to determine the remaining specific binding at the mutant receptor according to the standard protocols for radioligand binding. Non-specific binding was determined in the presence of 10 µM haloperidol. Data analysis was performed by normalizing the receptor bound radioactivity derived from unspecific binding equal to 0% and total binding equal to 100%. Average values were calculated from two to four individual experiments each done in quadruplicate and displayed in % ± SD.

4.1. Molecular Docking

Docking studies were performed using a recently published homology model of D_2R.[27] We introduced the L2.64C mutation utilizing UCSF Chimera[41] at which we selected the most probable rotamer of the dunbrack rotamer library.[42] The receptor

structure was protonated by means of the H++ server (http://biophysics.cs.vt.edu/H++)[43] for pH 7 applying the default parameters. The investigated compounds **3** and **4** were geometry-optimized by means of Gaussian 09[44] at the B3LYP/6-31G(d) level (attributing a formal charge of +2 and +1, respectively) and subsequently docked into the modified D_2R model using AutoDock Vina.[45] We applied a search space of 26 Å × 24 Å × 30 Å to ensure a complete coverage of the binding pocket. The ligands were subjected to the docking procedure using an exhaustiveness value of 32 and a randomly selected starting position. Twenty conformations of each ligand were obtained and inspected manually. On the basis of the scoring function of AutoDock Vina and the presence of the canonical salt bridge to Asp3.32, we selected one final conformation for each ligand. The ligand-receptor complexes were submitted to an energy minimization procedure as described.[27] Figures were prepared using UCSF Chimera.

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Supplementary data

Supplementary data associated with this article can be found in the online version.

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Potent haloperidol derivatives covalently binding to the dopamine D2 receptor

*Tobias Schwalbe, Harald Hübner, Peter Gmeiner**

Department of Chemistry and Pharmacy, Emil Fischer Center, Friedrich-Alexander University Erlangen-Nürnberg, Schuhstraβe 19, D-91052 Erlangen, Germany

***Corresponding Author**. Phone: +49-(0)9131-85-24116. Email: peter.gmeiner@fau.de

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