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Structural determinants of diphenethylamines for interaction with the κ opioid receptor: Synthesis, pharmacology and molecular modeling studies

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ABSTRACT

The κ opioid (KOP) receptor crystal structure in an inactive state offers nowadays a valuable platform for inquiry into receptor function. We describe the synthesis, pharmacological evaluation and docking calculations of KOP receptor ligands from the class of diphenethylamines using an active-like structure of the KOP receptor attained by molecular dynamics simulations. The structure–activity relationships derived from computational studies was in accordance with pharmacological activities of targeted diphenethylamines at the KOP receptor established by competition binding and G protein activation in vitro assays. Our analysis identified that agonist binding results in breaking of the Arg156-Thr273 hydrogen bond, which stabilizes the inactive receptor conformation, and a crucial hydrogen bond with His291 is formed. Compounds with a phenolic 4-hydroxy group do not form the hydrogen bond with His291, an important residue for KOP affinity and agonist activity. The size of the N-substituent hosted by the hydrophobic pocket formed by Val108, Ile316 and Tyr320 considerably influences binding and selectivity, with the *n*-alkyl size limit being five carbon atoms, while bulky substituents turn KOP agonists in antagonists. Thus, combination of experimental and molecular modeling strategies provides an initial framework for understanding the structural features of diphenethylamines that are essential to promote binding affinity and selectivity for the KOP receptor, and may be involved in transduction of the ligand binding event into molecular changes, ultimately leading to receptor activation.

As a prominent member of the large family of seven transmembrane G protein-coupled receptors (7TM-GPCRs), the kappa opioid (KOP) receptor has been the subject of intense focus for drug discovery efforts over the past years. The KOP receptor is activated by the endogenous peptide-ligands, the dynorphins, and it signals through the heterotrimeric Gi/o proteins.[1] Given the importance of the KOP receptor/dynorphin system as a powerful regulator of a multitude of neurophysiological and behavioral responses, modulation of this system is considered as a promising strategy for the treatment of neuropsychiatric and other human disorders, including pain, drug addiction, mood disorders (e.g. depression and anxiety), neurological conditions (e.g. epilepsy) and itching skin diseases.[2–13] Numerous lines of evidence have been accumulated pointing to the KOP receptor as an important substrate in comorbidity between addictive and depressive disorders, or chronic pain and mood disorders.[9,10]

Differential strategies in modulating the downstream effects of KOP receptor signaling involve development of selective ligands that can either activate or block the receptor. KOP agonists attract considerable attention for their ability to produce analgesia without abuse potential. On the other hand, KOP agonists are limited by side effects (e.g. dysphoria, sedation, psychotomimetic effects),[1,5,14] whereas KOP antagonists and partial agonists have potential to emerge as antidepressants, anxiolytics and anti-addictive medications.[15–19]

Ligand–receptor interactions represent the basis of the mediation of many neuro-behavioral responses by both endogenous and exogenous ligands, and their tuning represents the goal of a large variety of pharmacotherapies. Molecular details of these interactions are nowadays emerging, with the recently

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solved crystal structure of the human KOP receptor,[20] therefore providing a novel platform for inquiry into receptor function and mechanism(s) of activation, and structural features promoting ligand binding affinity and selectivity.[21–26] Elucidation of ligand-receptor interactions and structure–function relationships of the KOP receptor represent important steps towards the development of improved pharmacotherapies for human disorders, where the KOP receptor plays a key role.

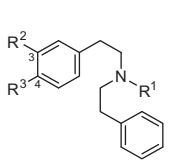
Hence, an important objective of our laboratories is the design and biological evaluation of structurally-distinct KOP receptor-targeted ligands in order to specifically assess the contribution of the KOP receptor in opioid system neurobiology, and furthermore, to provide novel opportunities for the discovery of potential therapeutic agents.[27–29] We recently disclosed a diphenethylamine series of KOP ligands, exemplified by **1** (HS665, Fig. 1), as one of the most interesting derivatives in terms of very high binding affinity and selectivity for the KOP receptor,[28,29] complemented by high potency and efficacy in vivo.[28] The *N*-cyclopropylmethyl (*N*-CPM) substituted analogue **2** (HS666, Fig. 1) also displayed favorable properties related to the activity at the KOP receptor as a potent partial agonist. We have described that cyclobutylmethyl (CBM) and CPM groups (**1** and **2**, respectively) at the nitrogen are more suitable for interaction with the KOP receptor than *n*-alkyl substituents (derivatives **3**–**6**, Fig. 1).[28]

On the basis of the currently available crystal structure of the human KOP receptor in an inactive conformation,[20] the present study was undertaken to attain an active-like receptor state applying molecular dynamics (MD) simulations. The generated receptor model was next used to characterize the binding mode of KOP receptor-targeting diphenethylamine derivatives **1**–**6** (Fig. 1). Additionally, the importance of the substitution pattern at the nitrogen and the position of the phenolic hydroxyl group on the interaction with the KOP receptor were investigated after synthesis of the new derivatives **7**–**9** (Fig. 1). We herein report the in vitro binding and G protein activation of the KOP receptor by **7**–**9** in comparison to the previously reported analogues **1** and **2**, paralleled by binding mode investigations of these KOP ligands.

The new diphenethylamines **7**–**9** were prepared as depicted in Schemes 1 and 2. (3-Methoxyphenyl)-*N*-phenethaneamine (**10**) and (4-methoxyphenyl)-*N*-phenethaneamine (**11**) were prepared from 2-(3-methoxyphenyl)ethaneamine (**12**) and 2-(4-methoxyphenyl)ethaneamine (**13**), respectively by alkylation with phenethylbromide.[30] Next, **10** and **11** were alkylated with the respective alkyl bromide in DMF to afford **14** and **15**, and then converted into the respective phenols **7** and **9** by ether cleavage with sodium ethanethiolate in DMF (Scheme 1). Compound **8** was synthetized starting from 4-hydroxyphenylacetic acid (**16**), which was reacted with phenethylamine (**17**) in CH_2Cl_2 in the presence of EDCI and HOAt to afford amide **18**. BH_3 reduction in THF yielded amine **19**, which was *N*-alkylated with CBM bromide in CH_3CN in presence of $NaHCO_3$ to give the phenol **8** (Scheme 2).

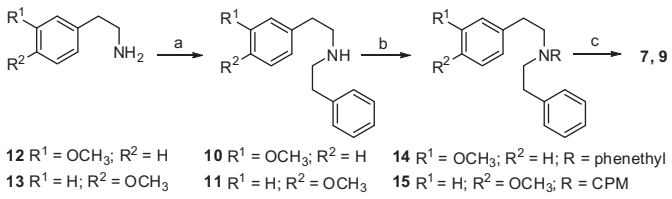
The new target diphenethylamines **7**–**9** were examined for binding affinity and selectivity for KOP, MOP and DOP receptors in competition binding assays using membranes from Chinese hamster ovary (CHO) cells stably expressing one of the human opioid receptors, according to the reported procedure.[28] Their functional activity was evaluated in G protein activation assays using guanosine 5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding and CHO cells expressing the human KOP receptors, as described earlier.[28] Their in vitro opioid activity profile is summarized in Table 1. To facilitate comparison to our previous SAR efforts, the diphenethylamines **1**–**6** were included. Receptor binding studies demonstrated that the introduction of a phenethyl group at the nitrogen led to a considerable reduction in KOP receptor affinity (**7**: K_i = 211 nM) when compared to the previously reported analogues (Table 1). It was found that the KOP receptor affinity of **7** was about 430- and 35-fold lower than that of the *N*-CBM and *N*-CPM substituted **1** and **2**, respectively. It was also evident that the presence of the *N*-phenethyl moiety in **7** has major consequences on the interaction with MOP and DOP receptors, by completely abolishing binding at these two receptors. In contrast to earlier developed diphenethylamines **1**–**6**,[28] the *N*-phenethyl derivative **7** exhibited no appreciable agonist activity at the KOP receptor, but it rather antagonized the stimulation of [^{35}S]GTPγS binding by the reference agonist U69,593 with relatively low potency (K_e = 1311 nM).

Another interesting outcome of our expanded SAR in the diphenethylamine series relates to the effect on the interaction with the KOP receptor upon shifting the position of the phenolic hydroxyl group. Switching the hydroxyl group from position 3 to 4 significantly decreased affinity and selectivity for the KOP receptor of **8** and **9**, in comparison to the 3-OH derivatives **1** and **2**, respectively (Table 1). Differential functional activity at the KOP receptor was also noted regarding G protein activation for the *N*-CBM, 3-hydroxy substituted **1** and its 4-hydroxy analogue **8**, as well as for the *N*-CPM, 3-hydroxy substituted **2** and its 4-hydroxy analogue **9**. While **1** is a highly potent and full KOP agonist,[28] the 4-hydroxy modification in **8** results in ca. 30-fold lower KOP potency, demonstrating properties of a low efficacy partial KOP agonist (Table 1). Furthermore, the presence of the phenolic 4-hydroxy group in **9** drastically altered the KOP-mediated G protein activation when compared to the corresponding analogue **2**, previously reported as a potent partial agonist at the KOP receptor.[28] Derivative **9** did not show any agonist activity at the KOP receptor,

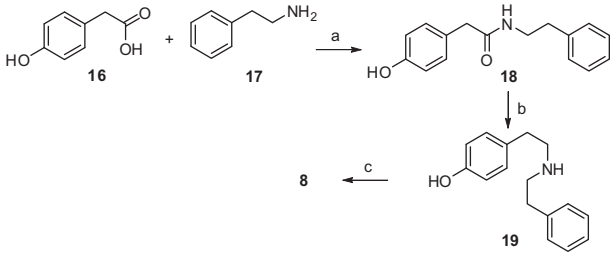


	R^1	R^2	R^3
1 (HS665)	CBM	OH	H
2 (HS666)	CPM	OH	H
3	*n*-C_3H_7	OH	H
4	*n*-C_4H_9	OH	H
5	*n*-C_5H_{11}	OH	H
6	*n*-C_6H_{13}	OH	H
7	phenylethyl	OH	H
8	CBM	H	OH
9	CPM	H	OH

Figure 1. Structures of investigated diphenethylamines **1**–**9**.



Scheme 1. Synthesis of diphenethylamine derivatives **7** and **9**. Reagents and conditions: (a) phenethyl bromide, K_2CO_3, DMF, 80 °C; (b) respective alkyl bromide, K_2CO_3, DMF, 80 °C; (c) sodium ethanethiolate, DMF, 130 °C.



Scheme 2. Synthesis of diphenethylamine derivative **8**. Reagents and conditions: (a) EDCI, HOAt, CH_2Cl_2, rt; (b) 1 M BH_3·THF, THF, reflux; (c) CBM bromide, $NaHCO_3$, CH_3CN, reflux.

Table 1
Binding affinities and functional activities of diphenethylamines **1**–**9** at the human opioid receptors

	Receptor binding (K_i, nM)[a]					Functional activity[b]		
	Affinity[b]			Selectivity		[^{35}S]GTPγS KOP		
	KOP	MOP	DOP	MOP/KOP	DOP/KOP	EC_{50} (nM)	% stim.[c]	K_e[d] (nM)
1 (HS665)[e]	0.49 ± 0.20	542 ± 239	>10,000	1106	>20000	3.62 ± 1.87	90.0 ± 3.7	
2 (HS666)[e]	5.90 ± 3.00	826 ± 98	>10,000	140	>1700	35.0 ± 5.3	53.4 ± 8.1	
3[e]	8.13 ± 0.32	594 ± 101	3713 ± 1266	73	457	49.1 ± 8.8	21.2 ± 0.1	
4[e]	10.9 ± 2.4	412 ± 19	2429 ± 837	38	223	46.2 ± 11.4	45.5 ± 5.9	
5[e]	12.6 ± 1.9	325 ± 26	1315 ± 364	26	104	86.4 ± 4.6	36.2 ± 2.7	
6[e]	141 ± 42	788 ± 175	3572 ± 222	5.6	25	647 ± 88	24.0 ± 1.3	
7	211 ± 106	>10,000	>10,000	>47	>47	>10,000	—[f]	1311 ± 593
8	36.3 ± 11.3	731 ± 235	2129 ± 266	20	59	109 ± 18	43.8 ± 6.2	
9	218 ± 27	1750 ± 893	2187 ± 1113	8	10	>10,000	—[f]	32.1 ± 8.4

[a] Determined in competition binding assays against [^{3}H]U69,593 (KOP receptor), [^{3}H][D-Ala^2,Me-Phe^4,Gly-ol^5]enkephalin ([^{3}H]DAMGO, MOP receptor) and [^{3}H][D-Pen^2,pCl-Phe^4,D-Pen^5]enkephalin ([^{3}H]pCl-DPDPE, DOP receptor) with CHO cell membranes expressing human opioid receptors.
[b] Determined in [^{35}S]GTPγS binding experiments using CHO-hKOP cell membranes.
[c] Percentage stimulation (% stim.) relative to the reference KOP agonist U69,593.
[d] Determined by inhibition of U69,593-stimulated [^{35}S]GTPγS binding to CHO-hKOP cell membranes.
[e] Data taken from Ref. 28.
[f] No simulation up to 10 μM. Experimental data were analyzed using the GraphPad Prism Software. Values are the mean ± SEM of at least three independent experiments.

therefore it was tested for antagonism against U69,593-stimulated [^{35}S]GTPγS binding. In this study, **9** exhibited moderate KOP antagonist activity with a K_e value of 32.1 nM, which was about 40-fold higher than the antagonist potency of the *N*-phenethyl substituted **7** (Table 1).

The crystal structure of the human KOP receptor in complex with the selective antagonist JDTic (PDB code: 4DJH)[20] (see Supporting information, Fig. S3A) provides a valuable platform for in silico investigating binding characteristics of diphenethylamines **1**–**9** by means of molecular docking, and 3D-pharmacophores.[31]

It is well-recognized that GPCRs share a common molecular activation mechanism, although their ligands are chemically diverse and vary in their binding site locations. In particular, ligand binding results in molecular switches disrupting stabilized intramolecular interactions.[32] Prominent examples are (i) the Trp287 toggle-switch,[33] (ii) the ionic lock mimicking hydrogen bond between Arg156 and Thr273[20], and (iii) the so-called 3–7 lock, a hydrogen bond between residues Asp138 and Tyr320.[34] Thus, GPCRs could exhibit significant structural changes between inactive and active states.[35–37] With respect to opioid receptors, so far, the elucidated crystal structures of KOP,[20] MOP[38] and DOP[39] receptors represent the inactive receptor states, exception being the recently resolved crystal structure of the murine MOP receptor bound to a morphinan agonist.[40]

To begin our computational approach, the binding mode of the most active diphenethylamine of the series, **1** (Fig. 1 and Table 1), to the human KOP receptor was docked in the crystal structure to find a plausible starting point for a subsequent MD-based sampling to obtain a receptor model that resembles active-like properties.[41]

During the MD simulation process, the hydrogen bond between residues Arg156 and Thr273 was broken (Fig. 2A), which represents a molecular switch linked to the activation of the KOP receptor. For comparison, this hydrogen bond is present in the crystal structure of the KOP receptor in complex with the antagonist JDTic (distance of 3.5 Å), and it is proposed to stabilize the inactive state of the receptor.[20] Due to our focus on the ligand binding site, deviations of heavy atoms were calculated for key residues in ligand binding (Val108, Asp138, Tyr139, Trp287, Ile290, His291, Ile316 and Tyr320). After an initial structural rearrangement, we only noted smaller conformational changes (Fig. 2B). Since previous studies suggested a contribution of His291 to the interaction pattern of KOP ligands,[20,22,34,42–44] we interestingly observed a hydrogen bond between His291 and the hydroxyl group of **1** in nearly all conformations that present breaking of the Arg156-Thr273 hydrogen bond (Fig. 2B).

A re-docking of **1** (Fig. 2C) and the first four amino acids of the endogenous KOP ligand dynorphin (Tyr-Gly-Gly-Phe) was carried out for the validation of the active-like properties. The essential polar interaction with Asp138, that was reported by O'Conner et al.,[45] played also an essential role for diphenethylamine **1**. We observed that the phenolic group of **1** forms a hydrogen bond with His291 and it was projected into a pocket formed by residues Trp287, Ile294, and Tyr139. The positively ionizable nitrogen forms a salt bridge to Asp138, and additionally showed a π–cation interaction with Tyr139. The *N*-CBM group is projected into a pocket formed by the residues Val108, Ile316 and Tyr320, while the other phenyl moiety deeply interacts with the residues Trp287, Val108 and Ile290 (Fig. 2C). The binding orientation of **1** and dynorphin turned out to be comparable (Fig. 2D) regarding the essential interactions. The subsequent binding mode investigations explain (i) the agonist activity of the diphenethylamine derivatives (Table 1), where the hydrogen bond to His291 is linked to the active-like conformation of the receptor, and (ii) the subtype selectivity for the KOP receptor, as the residues Val108 and Ile294 differ in the other closely related opioid receptors (MOP[38] and DOP[39], and are thought to contribute to the subtype selectivity of several) KOP selective ligands.[20,21]

The position of the phenolic hydroxyl group strongly influences the KOP receptor affinity, as it was experimentally observed for **1** and **8**, and **2** and **9** (Fig. 1 and Table 1). The 3-OH substituted **1** is the most active compound of the series, and the only one that shows full agonist behavior,[28] indicating that a *N*-CBM substitution is favorable for KOP receptor binding and activation. Its analogue **8**, in which the phenolic hydroxyl group is located at position 4, shows reduced KOP affinity and selectivity, and acts as a partial agonist (Table 1). A similar phenomenon was observed for compounds **2** and **9** having an *N*-CPM moiety. In this case shifting the position of the phenolic hydroxyl group from 3 to 4, a partial KOP agonist (**2**) was converted into an antagonist (**9**) that has reduced affinity and selectivity for the KOP receptor. Our 3D-pharmacophores indicate that the 4-OH substituted compounds (**8** and **9**) are not likely to form a hydrogen bond with residue His291, which is important for KOP affinity and agonist activity (Fig. 3).

The hydrophobic pocket formed by the residues Val108, Ile316 and Tyr320 is important for hosting the N-substituent. The *N*-CBM (in **1**) and *N*-CPM (in **2**) groups appear to have the optimal size.

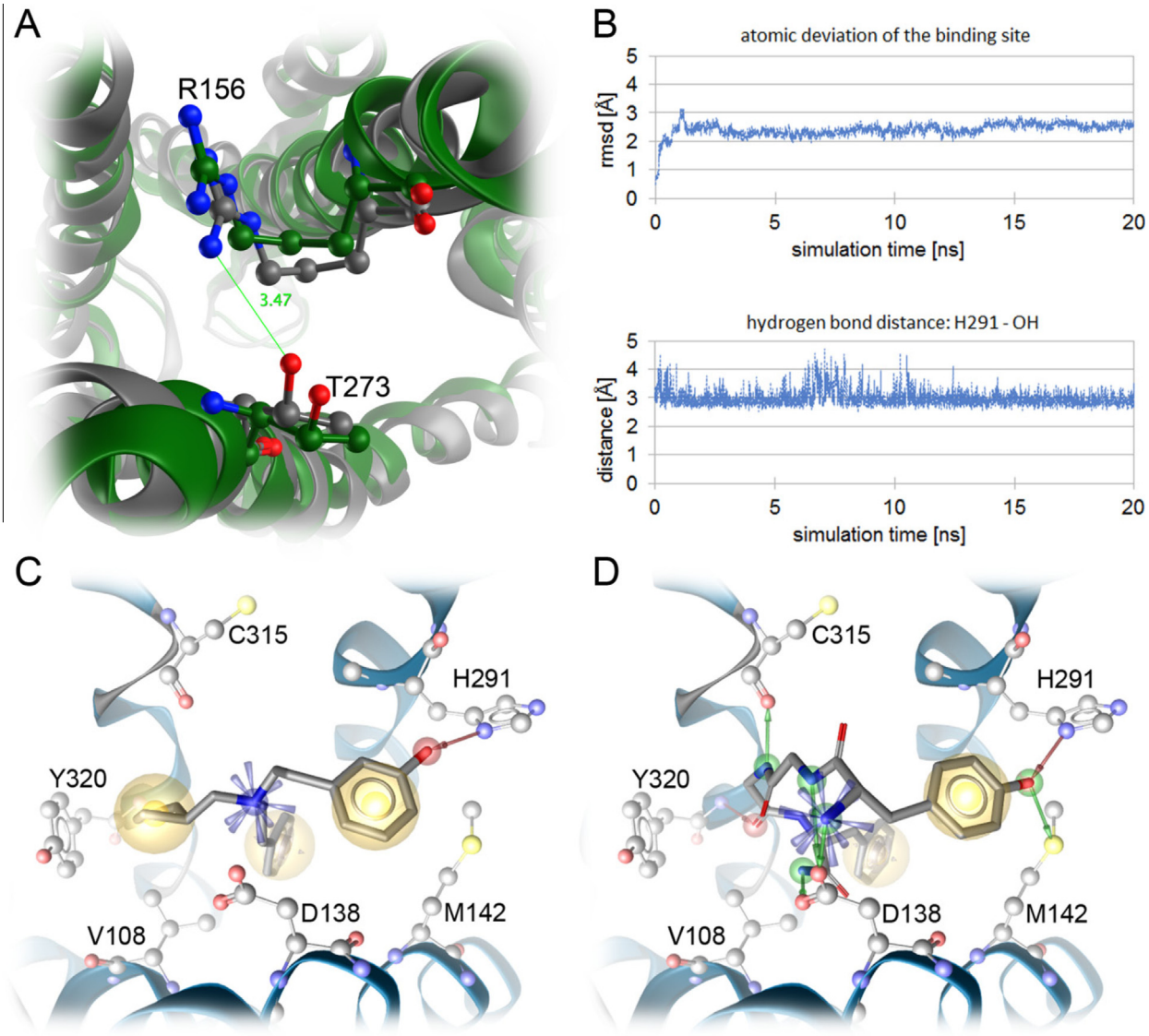


Figure 2. MD-guided generation of an active-like KOP receptor model. (A) A hydrogen bond between Arg156 with Thr273 is proposed to stabilize the inactive state of the human KOP receptor (grey). The breakage of this 'ionic lock' could be found in a representative conformation of the active-like KOP receptor model (green). (B) The upper plot shows the atomic deviation of key residues of the binding site. The plot below shows the distance between the nitrogen of His291 and the phenolic hydroxyl group of the diphenethylamine **1**. (C and D) 3D-pharmacophore analysis of **1** (C) and dynorphin (D) in the active-like KOP receptor model. Yellow spheres indicate lipophilic contacts, green arrows hydrogen bond donors, red arrows hydrogen bond acceptors and positive ionizable centres are shown as blue spheres.

Concerning the linear substituents (from *n*-C_3H_7 to *n*-C_6H_{13}), increasing the chain length results in a decrease of the KOP affinity (K_i = 8.3 nM (**3**) < 10.9 nM (**4**) < 12.6 nM (**5**) < 141 nM (**6**), Table 1). While the KOP affinities of the *n*-propyl, *n*-butyl, and *n*-pentyl *N*-substituted derivatives are in the same range, the presence of the *n*-hexyl chain at the nitrogen in **6** produces a markedly decreased KOP affinity (Table 1), indicating that five carbon atoms in linear alkyl substituents is the critical size for agonist activation of the KOP receptor (Fig. 3A). The *N*-phenethyl group in analogue **7** is rather bulky to be hosted by the hydrophobic pocket, which results in a different orientation of the phenolic moiety (Fig. 3B), and making this compound a weak KOP antagonist (Table 1).

In order to investigate structural features for KOP receptor selectivity of diphenethylamines, we built mutant receptor models using MOE (Molecular Operating Environment 2014.09; Chemical Computing Group Inc.). The amino acid residue Val108 was virtually mutated into Ala, which represents the corresponding residue in MOP and DOP receptors.[38,39] Docking of **1** into the mutant receptor indicated that the crucial hydrogen bond of the phenolic group with His291 was lost, and **1** showed a reduction of the hydrophobic contact with the receptor (see Supporting information, Fig. S3B). Thus, a reduction of Val108 by two carbon atoms (Ala) leads to an extension of the hydrophobic pocket, in which volume and shape are important for hosting the N-substituent. Interestingly, we observed decreasing MOP/KOP and DOP/KOP selectivity ratios for diphenethylamines **1**–**6** (Table 1) with increasing the chain length from C3 to C6, which is in accordance with our computational outcomes for the mutation of Val108 to Ala, showing more space in the hydrophobic pocket (binding site volume: $KO_{wildtype}$: 521.3 Å^3, $KOP_{Val108/Ala}$: 549.8 Å^3).

Based on our computational studies applying molecular docking and MD simulations, we can draw a SAR pattern on the investigated diphenethylamines **1**–**9** as KOP receptor ligands summarized in Table 2. The main ligand–receptor interactions are represented by the hydrogen bond between the phenolic hydroxyl group and His291, the salt bridge formed by the protonable nitrogen with Asp138, and the projection of the N-substituent into the hydrophobic pocket formed by Val108, Ile316, and Tyr320.

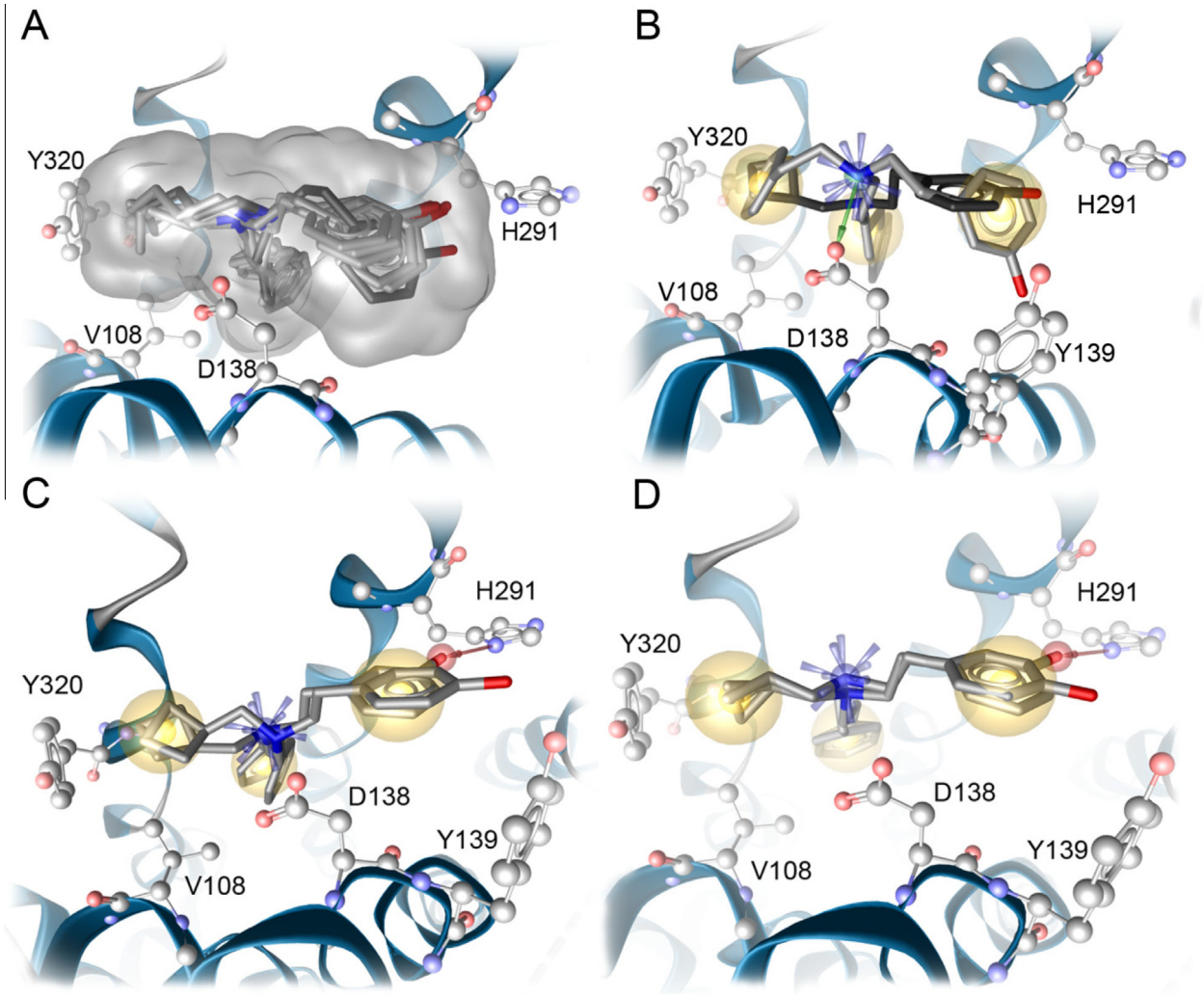


Figure 3. Binding mode analysis of diphenethylamines. (A) Superimposition of diphenethylamines **1**–**5** (light grey) and **6** (dark grey). (B–D) 3D-pharmacophore analysis of diphenethylamines. Yellow spheres indicate lipophilic contacts, green arrows hydrogen bond donors, red arrows hydrogen bond acceptors and positive ionizable centres are shown as blue spheres. (B) Diphenethylamine derivative **7** (light grey) in superimposition with **1** (dark grey) indicating the different orientation of the phenolic hydroxyl group of **7** compared to **1** due to the bulky N-substituent. (C and D) Binding mode comparison of diphenylethylamines **1** and **8** (C), and **2** and **9**. (D) Compounds without the hydroxyl group in position 3 cannot form the crucial hydrogen bond with residue His291, which results in lower KOP receptor affinity of **8** and **9**.

Table 2
Overview of the ligand-receptor interactions observed for diphenethylamines **1**–**9** with the human KOP receptor

Moiety	Interactions with the KOP receptor	Relevance
Phenolic hydroxyl group at position 3	Hydrogen bond with His291	KOP receptor affinity
Protonable nitrogen	Salt bridge with Asp138	Essential for KOP receptor binding
N-substituents	Hydrophobic interactions with Val108, Ile316, Tyr320	KOP receptor selectivity (linear substituents and small rings)
Lipophilic moiety	Phenyl ring: Hydrophobic interactions with Trp287, Val108 and Ile 290	KOP receptor selectivity

Concerning the N-substituent, the *n*-alkyl size limit is five carbon atoms, and bulky substituents turn KOP receptor agonists in antagonists, since they prevent the interaction between the protonable nitrogen and Asp138, essential in anchoring positively ionizable KOP receptor ligands.

In summary, our current findings expanded the SAR exploration in the series of diphenethylamines as KOP receptor ligands. The SAR pattern derived from molecular modeling is supported by the in vitro pharmacological profile of the target ligands. The size of the N-substituent hosted by the hydrophobic pocket formed by the residues Val108, Ile316 and Tyr320 influences ligand binding and selectivity. The hydrogen bond formed by the phenolic 3-OH group of **1** with His291 is essential for binding affinity and agonist activity at the KOP receptor. Shifting the phenolic hydroxyl group from position 3 to 4 resulted in reduced KOP binding affinity due to the absence of a hydrogen bond with His291. Potency and efficacy to activate the KOP receptor was strongly dependent on the 3-OH→4-OH switch, with remarkable consequences as this modification turned a highly potent full agonist (**1**) into a much less potent partial agonist (**8**) or even eradicated essentially all KOP activity by converting a potent partial agonist (**2**) into a moderate antagonist (**9**).

Combination of experimental and molecular modeling strategies offers fundamental insights into the structural features of diphenethylamines that are necessary to promote binding affinity, agonist efficacy and selectivity for the KOP receptor. These structural characteristics may be also involved in transduction of the ligand binding event into molecular changes, ultimately leading to receptor activation. The present results are useful for guiding drug design, and the structural simplicity and readily accessible synthesis of these scaffolds compared to those of currently utilized KOP agents make them attractive for further exploration.

Supplementary data

Supplementary data (experimental section with synthetic procedures, spectroscopic characterization data, biological assays and molecular modeling) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.08.031.

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