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Molecular modeling study of the opioid receptor interactions with series of cyclic deltorphin analogues

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In this study, ten tetra- and heptapeptide analogues of deltorphin containing the urea bridges between residues 2 and 4 have been docked into the δ- and μ-opioid receptors to explain their different biological activities. The important factors explaining particular ligand activity such as free energy of binding, conformation of the ligand, its location inside the binding pocket as well as the number and strength of the receptor–ligand interactions have been discussed. Several different binding modes for investigated ligands have been proposed. It appears that the binding site is not identical even for very similar ligands. Results of this study help to explain the differences in biological activity of the deltorphin analogues, their interaction with the opioid receptors at the molecular level and support designing a new generation of potent opioid drugs with improved selectivity.

Keywords: deltorphin; dermorphin; opioid receptors; GPCR; receptor–ligand interaction; molecular modeling

Introduction

Opioid receptors are members of the large superfamily of G protein-coupled receptors (GPCRs). Four opioid receptors, μ, δ, κ and nociceptin receptor (NOP) have been cloned [1]. Opioid receptors, being typical members of family A GPCR, are membrane-spanning proteins consisting of seven transmembrane helices (TM1–TM7), connected by alternating extracellular and intracellular loops (ELs and ILs respectively) [2]. They are about 60% identical to each other with greatest homology in the TM domain (73–76%). The greatest diversity is found in the *N*- and *C*-termini and ELs [3]. All four opioid receptors contain highly conserved motifs such as disulfide bridge linking TM3 and EL2, D(E)RY in TM3 or NPXXY in TM7 and numerous fingerprint residues [4]. They are coupled to the pertussis toxin-sensitive Gi and Go proteins and/or the pertussis toxin-insensitive Gz and G16 proteins.

Opioid receptors are responsible for pain perception being activated both by endogenous opioid peptides and by exogenous opiates, such as morphine. Opiates are among the most effective analgesics known, however, their clinical use is limited because they also produce many side effects such as nausea, vomiting, constipation, respiratory depression and, foremost, tolerance and addiction. The μ-opioid receptor activation causes analgesia accompanied by typical side effects of opiates, while the stimulation of δ-opioid receptor produces antinociceptive actions with reduced respiratory depression, low constipation and minimal potential for physical dependence, making this receptor subtype a promising target for the development of better tolerated analgesics [5–7].

On the basis of both experimental and theoretical studies, it has been suggested that opioid receptors share a common binding cavity located between TM2 and TM7 partially covered by the ELs [1,8]. It has been proposed that the common tyramine moiety of the ligand which constitute a 'message part' interacts with the conserved residues from TM domain being involved in the receptor activation. The chemically different 'address' parts of the ligands interact with the non-conserved residues being responsible for selective recognition of the ligands [8,9]. In particular, the opioid peptides are bound by the set of conserved aromatic residues that interact with the 'message' part of opioid molecules [10].

The deltorphins (Tyr-D-Ala-Phe-Asp/Glu-Val-Val-Gly-NH_2), isolated from the skin of South American frogs belonging to the subfamily Phyllomedusinae [11], are the most selective naturally occurring opioid agonists for δ-opioid receptors with potent analgesic activity.

In this study, ten cyclic analogues of deltorphin containing the urea bridge, substituted in positions 2 and 4 have been investigated (Figure 1). These analogues were previously synthesized and tested in the guinea-pig ileum (GPI) and mouse vas deferens (MVD) assays (Table 1) and their structures were determined by NMR [12,13]. In this study, their interaction with δ- and μ-opioid receptors has been analyzed in details to determine the probable receptor–ligand binding mode and to explain how the modifications of ligand molecules influence on receptor–ligand interaction.

Methods

Models Building

The δ- and μ-opioid receptor models were built on the β2-adrenergic receptor crystal structure template (PDB entry: 2RH1) [14]. The opioid receptors were aligned with the sequence of β2-adrenergic receptor using Multalin [15]. The computer

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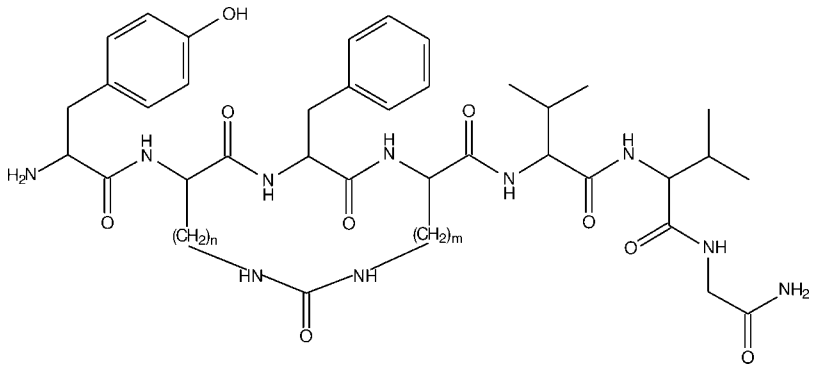

Figure 1. Chemical structures of the investigated ligands. **A**: Lys^2, Dab^4 ($n = 4$, $m = 2$); **B**: Lys^2, Dap^4 ($n = 4$, $m = 1$); **C**: Orn^2, Dap^4 ($n = 3$, $m = 1$); **D**: Lys^2, Orn^4 ($n = 4$, $m = 3$); **E**: Orn^2, Orn^4 ($n = 3$, $m = 3$); **a**: Lys^2, Dab^4 [1–4] ($n = 4$, $m = 2$); **b**: Lys^2, Dap^4 [1–4] ($n = 4$, $m = 1$); **c**: Orn^2, Dap^4 [1–4] ($n = 3$, $m = 1$); **d**: Lys^2, Orn^4 [1–4] ($n = 4$, $m = 3$); **e**: Orn^2, Orn^4 [1–4] ($n = 3$, $m = 3$).

Table 1. Biological activities of the investigated analogues

Ligand	Ring size	Daa^2	Daa^4	GPI IC_{50} (nM)	MVD IC_{50} (nM)	GPI/MVD ratio	References
A	16	Lys	Dab	65.4 ± 9.6	0.640 ± 0.043	102	13
B	15	Lys	Dap	25.4 ± 2.0	0.483 ± 0.065	52.6	13
C	14	Orn	Dap	>10,000	27.1 ± 3.1	>369	13
D	17	Lys	Orn	>10,000	67.0 ± 6.9	>149	13
E	16	Orn	Orn	159 ± 23	0.814 ± 0.054	88	13
A	16	Lys	Dab	1.17 ± 0.25	5.02 ± 1.39	4.29	12
B	15	Lys	Dap	4.15 ± 0.36	19.5 ± 2.3	4.70	12
C	14	Orn	Dap	3.37 ± 0.09	7.76 ± 1.27	2.30	12
D	17	Lys	Orn	15.6 ± 1.6	47.1 ± 3.5	3.01	12
E	16	Orn	Orn	4.77 ± 0.71	11.4 ± 1.5	2.39	12
	[Leu^5]enkephalin			246 ± 39	11.4 ± 1.1	21.4	12

mutations, insertions and/or deletions necessary to obtain opioid receptor amino acid sequences were done using standard AMBER 10 tools [16] and PyMOL [17]. Subsequently, the raw receptor models were inserted into the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) membrane model consisting of 120 POPC lipids and about 3500 TIP3P water molecules (3467 and 3475 for δ- and μ-opioid receptor, respectively). To neutralize the charge of the receptor the counterions (Cl-) were used. The periodic boxes ready to be modeled had 118 Å × 67 Å × 122 Å initial size for δ-opioid receptor and 118 Å × 69 Å × 122 Å for μ-opioid receptor. For preliminary relaxation of the systems the energy minimization was done. Subsequently, the minimized systems were sent to MD simulation. Both systems were simulated for 10 ns using particle mesh Ewald (PME) summation method [18,19]. The simulations were performed using sander and pmemd programs from AMBER 10 package [16]. For POPC, the optimized potentials for liquid simulations (OPLS) [20] united atom parameters were applied. To prevent unfolding of the TM helices, flat-bottom soft harmonic-wall restraints were imposed onto the φ, ψ and ω peptide angles of the TM amino acid residues for whole simulation. They were centered around the initial φ, ψ and ω torsion and defined as 400 kcal (mol deg)$^{-1}$ with the angle limits: -20°, -10°, $+10^\circ$, $+20^\circ$ for φ, ψ, and -15°, -5°, $+5^\circ$, $+15^\circ$ for ω. The restraints resulted in about 130 kcal (mol deg)$^{-1}$ effective penalty per each of these torsions. The final snapshots were energy minimized.

The non-standard fragments of non-standard residues in positions 2 and 4 of the ligands were parameterized as proposed in AMBER 10 manual [16]. The point charges were optimized by fitting them to the *ab initio* molecular electrostatic potential (6-31G* basis set, GAMESS molecular orbital program package [21]) for two different conformations, followed by consecutively averaging the charges over all conformations, as recommended in RESP [22]. Full-atom ligand models were relaxed in Amber force field [16] by MD simulation (0.5 ns) and finally energy minimized. The experimental NMR constraints were used [13]. Structures of the ligands are presented in Figure 2.

Molecular Docking

In the next step, the ligands were docked to the δ- and μ-opioid receptors using AutoDock 4.2 [23,24]. Two thousand receptor–ligand complexes were generated. Subsequently, for each particular receptor–ligand pair the lowest-energy complex was selected. The relaxation of the selected receptor–ligand complexes using a constrained simulated annealing (CSA) protocol/energy minimization was performed in the AMBER 10 [16].

Nomenclature

The receptor residues are identified using one letter codes with the absolute numbers and/or universal Class A indices (Ballesteros-Weinstein numbering scheme; B-W) [25], e.g. in δ-opioid receptor V4.56, or V179 or V179(4.56). In the B-W scheme, the most conserved residue in the TM helix 'N' has been given the number 'N'50, and each residue is numbered according to its position relative to this conserved residue. Residues placed in loops are

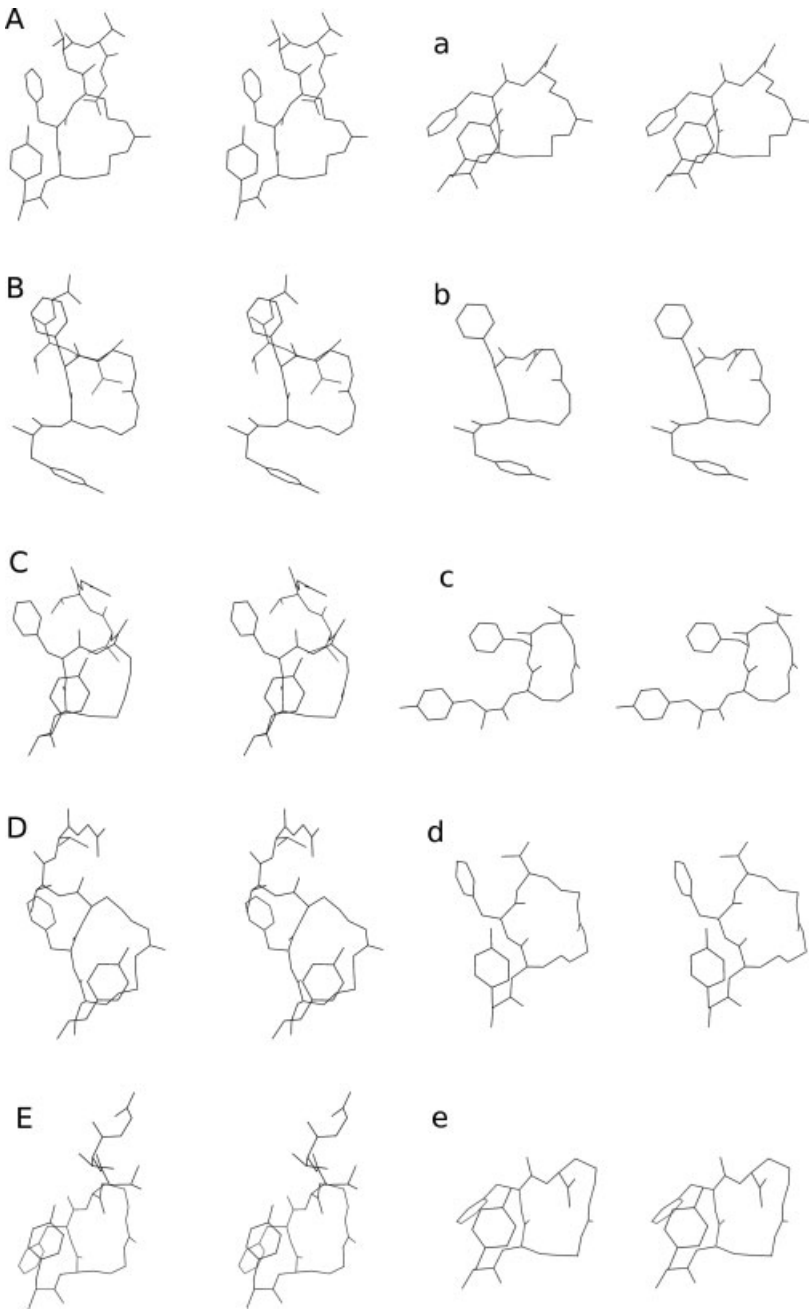

Figure 2. Stereodiagrams of the ligand conformations before the docking procedure.

indicated using one letter codes, followed only by the residue absolute number. Ligand residues are identified using three letter codes with residue number in superscript.

Results and Discussion

Energy of Binding

After the docking procedure 2000 receptor–ligand complexes have been obtained. An important factor validating the docking correctness is free energy of binding estimated by AutoDock. The best-scored (lowest energy) docking has been considered to be the most likely position of ligand in the binding pocket. In Table 2, free energy of binding for the lowest-energy complex for each particular receptor–ligand complex is given. As one may see, there is observable difference in energy of binding between the heptapeptides *versus* tetrapeptides. In all complexes formed with tetrapeptides regardless of the receptor type, there are similar low energy values in range from −6 kcal/mol to −10 kcal/mol approximately (Table 2). This is not significant difference, especially that the standard deviation of energy estimated by AutoDock is 2.5 kcal/mol. All tetrapeptide ligands appear to interact with receptors with comparable strength. These results are basically in accordance with biological activity of the tetrapeptides determined in GPI and MVD assays (Table 1). Only small difference is observed for ligand **d** which is somewhat less active in both biological assays, but its energy of binding does not differ from other tetrapeptides. In complexes of heptapeptides, the energy

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Table 2. The estimated binding energy (ΔG_{AD}) for the lowest-energy complexes computed in AutoDock with the standard deviation 2.5 kcal/mol

Ligand	ΔG_{AD} (kcal/mol)	
	μ-opioid receptor	δ-opioid receptor
A	6.29	−1.24
B	3.71	−6.06
C	4.42	3.38
D	20.78	4.04
E	19.06	−7.28
A	−8.56	−10.64
B	−7.34	−6.55
C	−6.47	−7.37
D	−6.16	−9.19
E	−6.82	−8.72

values are not as evidently correlated with biological activity as for tetrapeptides (Table 2). All complexes of heptapeptides with μ-opioid receptor show higher energy values than tetrapeptides which is in accordance with biological data. Also for both low active ligands **C** and **D** (IC_{50} > 10 000 nM, Table 1) obtained binding energy is comparable to energy of biologically active ligands. The energies for the heptapeptides docked in δ-opioid receptor are different. Three ligands: **A, B** and **E** show the lowest-energy values in accordance with their highest biological activity (Table 1). As one may observe, the majority of complexes with heptapeptides have been scored with positive energy values. It is probably a result of the high flexibility of the ligands (more rotatable bonds than in smaller tetrapeptides) and also their high molecular weight. These both factors may cause positive energies of binding. This problem has been already reported [26,27]. Therefore, binding energy estimated by AutoDock should be treated very carefully, especially for the heptapeptides and energy values should not be the only interpretation to assess ligand activity.

Location of the Ligands Inside the Binding Cavity

The other factor describing the quality of complex and determining proper receptor–ligand interactions is location of the ligand inside the receptor cavity. In both receptors, the ligands are located inside the binding pocket formed by the extracellular parts of TM2–TM7 helices and EL (EL2 and/or EL3) (Figures 3 and 4). In complexes of tetrapeptide analogues with δ-opioid receptor, the locations of ligands **a, b, c** and **e** inside the receptor are similar, with *N*-terminal tyramine part docked in the depth of the binding cavity (Figure 3). Only ligand **d** is located inversely with the tyramine moiety directed towards the EL part of the receptor. Similarly, for the heptapeptide analogues there are observable differences in ligand locations inside the δ-opioid receptor binding pocket. Ligands **A, B** and **E** show that higher biological activity are docked inside the binding cavity with the aromatic rings of Tyr^1 and Phe^3 located at the bottom of the cavity and *C*-terminus protruding towards the extracellular domain (Figure 3). All ligands adopt rather extended conformation being situated parallel to the longer axis of the receptor. Location of the ligand **C** and **D** is exactly opposite to the *C*-terminal part of molecule docked in the depth of the binding pocket. This reverse location of the analogues **d, C** and **D** may explain their lower activity at the δ-opioid receptor (Table 1). In this position they can not interact with the crucial receptor residues (which in turn interact with **A, B** and **E**; see below) resulting in their low activity. Therefore, efficient interaction of the investigated analogues with δ-opioid receptor appears to be dependent on proper ligand location. In μ-opioid receptor, among the tetrapeptides, the location of ligand **d** is again slightly different from other tetrapeptides according to its slightly lower biological activity (Figure 4). Interestingly, for the heptapeptide ligands in μ-opioid receptor quite the contrary situation exists. As one may see in Figure 4, there is diverse location of the all heptapeptides inside the binding cavity. Any ligand does not adopt an extended conformation and it is situated rather perpendicular to the longer axis of the receptor. In complexes with **C** and **D**, the *N*-terminal amino group of the ligand is docked at the bottom of the binding cavity. In the remaining (interestingly, more active complexes) it is docked shallower (Figure 4). Obviously, the location of the biologically non-active ligands **C** and **D** (Table 1) should not be closer as discussed here. During the docking in AutoDock, where the ligand is instantly generated in the optimal location inside the binding cavity still there is a possibility to fit any ligand inside the binding pocket one way or another. However, both **C** and **D** are presented in Figure 4 to point out that their conformations also make possible to fit them within the receptor pocket and lack of biological activity is probably a result of their too weak interaction with μ-opioid receptor and fast diffusion from receptor cavity rather than impossibility of docking. For ligands **A, B** and **E**, despite of their location, there still may be a possibility to interact strong enough with the μ-opioid receptor (as described below).

Conformation of the ligand is critical to recognition and binding to the receptor. The analysis of the ligand structures may help explain low activity of the ligands **C** and **D**. In both molecules (and exclusively in these two) the aromatic ring of Tyr^2 is situated over the plane of the macrocyclic ring (Figure 2). Thus, it is possible that low biological activity of **C** and **D** is a result of unfavorable location of the Tyr^2 making impossible its interaction with crucial receptor residues.

Receptor–Ligand Interactions

The receptor–ligand interactions have been analyzed in details. The receptors residues forming the putative ligand binding pockets have been selected using the distance criterion: the receptor residues whose nearest atom was not farther than 3.0 Å from any atom of ligand molecule have been considered.

Complexes of the heptatpeptides with δ-opioid receptor

In the complexes of heptapeptides with δ-opioid receptor it appears initially that there are only two general binding modes. One mode is observed for **A, B** and **E** and a second for less active ligands **C** and **D.** This is closely correlated with ligand locations inside the binding cavity (see above). However, after detailed analysis as many as four different binding modes have been observed for five ligands. Only **A** and **E** (both with macrocyclic ring consisted of 16 atoms) are bound in δ-opioid receptor in very similar manner. *N*-terminal amino group of the ligand is immersed into the depth of the cavity being involved in salt bridge with the D128 (Figure 5, MODE 1). Six hydrogen bonds between the ligand and receptor have been observed. The phenol group of Tyr^1 is involved in hydrogen bonding with the side chain of H278, carbonyl oxygen of Phe^3 interacts via hydrogen bond with amide nitrogen of the W207 and urea oxygen is hydrogen bonded to side chain of H301. The remaining three hydrogen bonds are observed

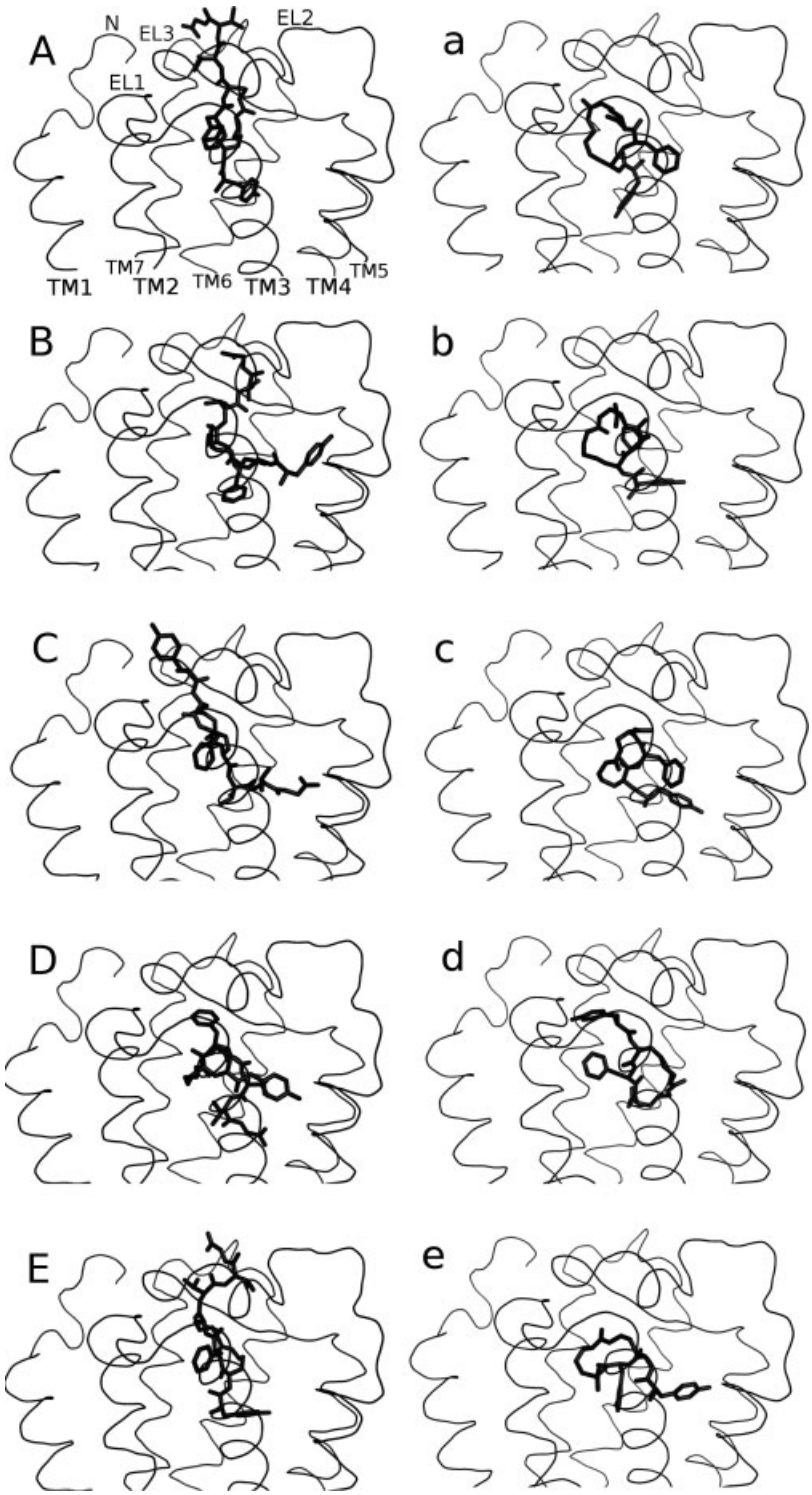

Figure 3. Location of the ligands docked in δ-opioid receptor. Only the extracellular halves of the receptor are shown. The helices, *N*-terminus and ELs are marked.

between the side chain of D290 and amide nitrogens from Val^6 and Gly^7 and *C*-terminal carboxamide, simultaneously. The aromatic and hydrophobic residues of the receptor are strongly involved in ligand binding. Aromatic side chain of Tyr^1 strongly interacts with Y129 and I304 and aromatic ring of Phe^3 with L125 and hydrophobic part of K108 side chain. The hydrocarbon chain of ligand Lys^2 interacts with V281 and L300. V297 and A298 strongly interact with Val^5 of the ligand and I289 with Val^6. Second binding mode is observed in the δ-opioid receptor–**B** complex (Figure 5, MODE 2). In this complex *N*-terminal amino group is also immersed into the binding cavity but interacts via hydrogen bond with the carbonyl oxygen of L125, besides four other hydrogen bonds exist. The urea oxygen interacts with amino group of K108, carbonyl oxygen of Gly^7 is hydrogen bonded to hydroxyl group

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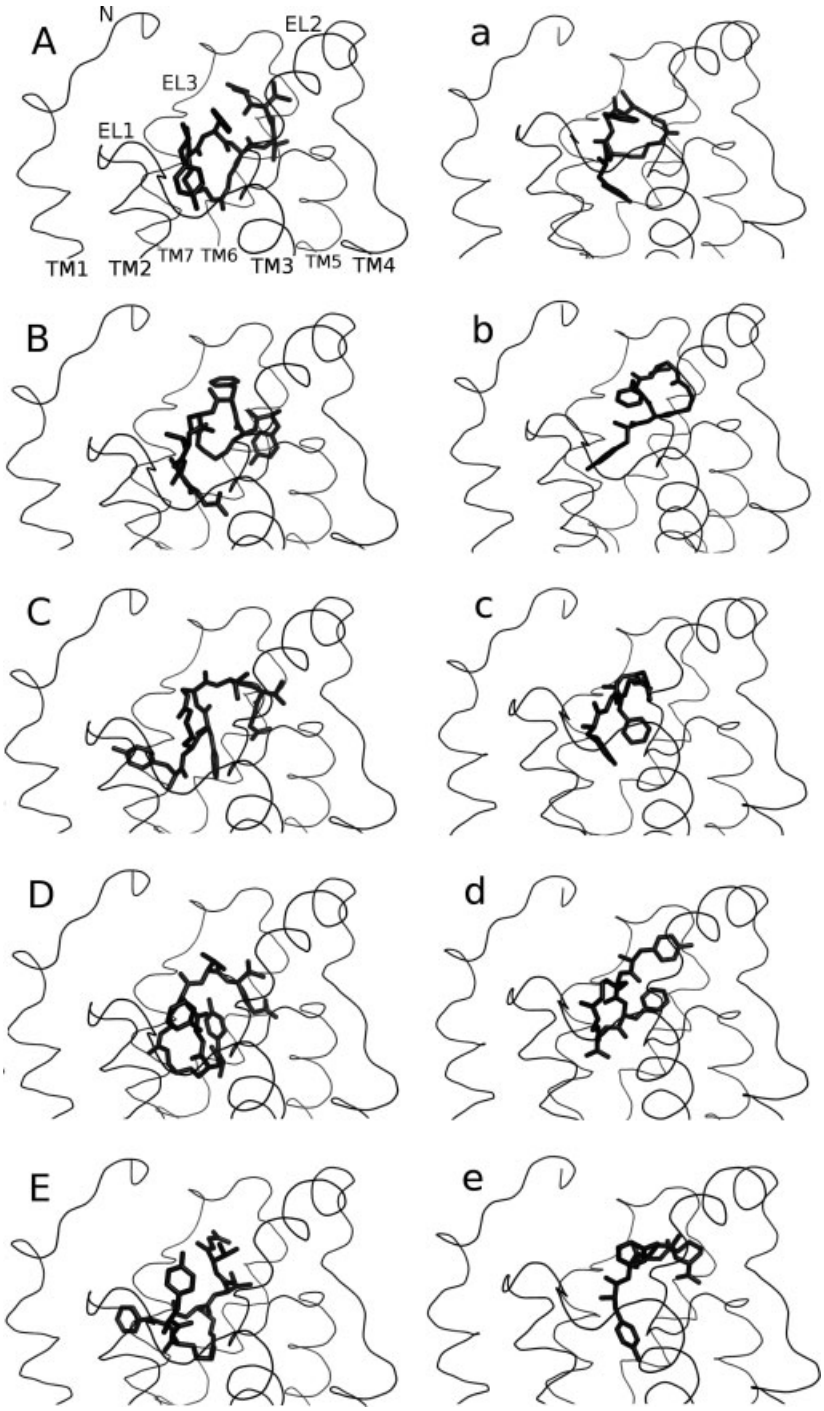

Figure 4. Location of the ligands docked in μ-opioid receptor. Only the extracellular halves of the receptor are shown. The helices, *N*-terminus and ELs are marked.

of S206 and finally *C*-terminal carboxamide and amide nitrogen of Gly^7 both interact with side chain of D290. Aromatic ring of Tyr^1 interacts with I183 and W209. The side chain of Phe^3 is involved in strong aromatic–hydrophobic interaction with I304, I277 and V281. Another aromatic hydrophobic interaction involves the aromatic ring of Y208 and Val^6. The hydrocarbon part of the macrocyclic ring interacts with the side chain of L125. The binding mode of the less active **C** analogue is completely different (Figure 5, MODE 3), several polar contacts are observed. The *N*-terminal part of the molecule protrudes towards the extracellular domain and amino group forms a salt bridge with E112. The latter is involved in two hydrogen bonds: with carbonyl oxygen and amide nitrogen of Orn^2. Carbonyl oxygens of Tyr^1 and Phe^3 are hydrogen bonded to S206 hydroxyl group and K108 ε-amino group, respectively. Amide nitrogen of Val^5 forms hydrogen bond with D128 and K214 ε-amino group interacts via hydrogen bonds with three carbonyl oxygens of Val^5, Val^6 and Gly^7. The hydrophobic interactions involve Val^5 and I304 and V281, whereas Val^6 interacts with L125, W207 and W209. Aromatic ring of Phe^3 interacts with side chains of L125, V124 and K108 and in fact there is no any significant interaction involving the

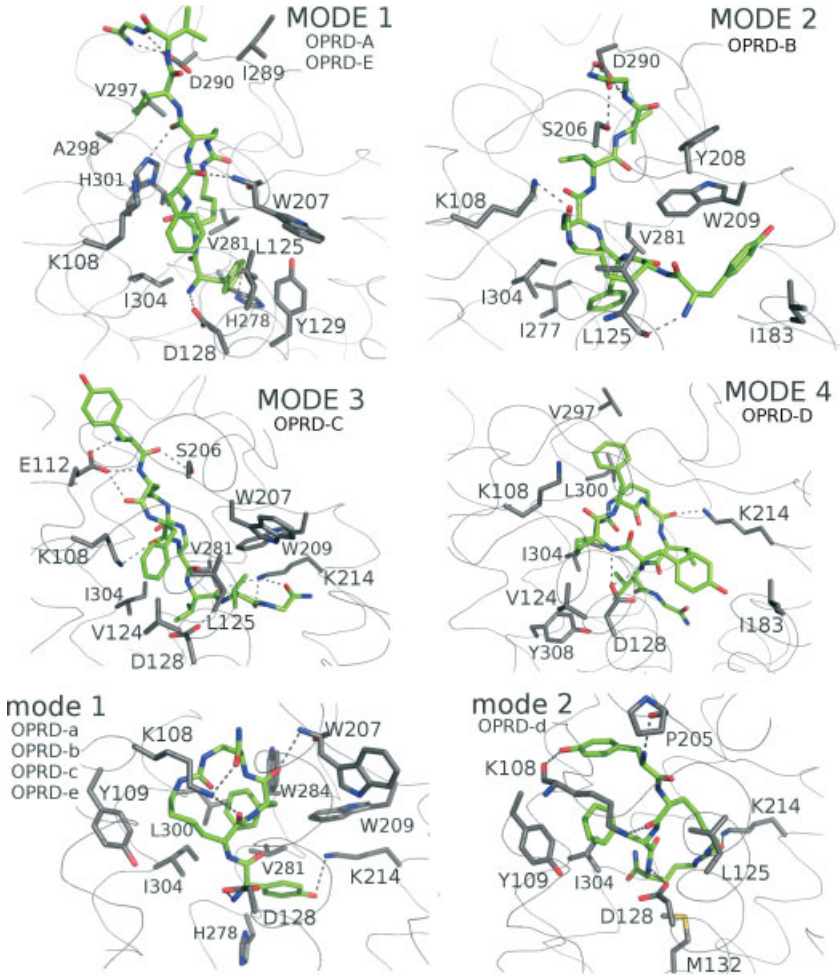

Figure 5. Various modes of interaction in the complexes with δ-opioid receptor. The ligand is coloured light green. Binding receptor residues are exposed and coloured grey. The hydrogen bonds are shown.

Tyr[1] aromatic ring. In the δ-opioid receptor–**D** complex (Figure 5, MODE 4), both the *N*- and *C*-terminus are immersed into the depth of the binding cavity. The amino group is involved in salt bridge with D108, which interacts also with amide nitrogen of Lys[2]. In this complex only one more hydrogen bond is formed, between ε-amino group of K214 and carbonyl oxygen of Orn[4]. There are no strong interactions involving aromatic rings of Tyr[1] or Phe[3], only very weak contacts with I183 and V297, respectively. The hydrocarbon macrocyclic ring interacts with L300. Val[6] is involved in interaction with V124, I304 and Y308, and Val[5] interacts with the hydrophobic part of the K108 side chain.

It seems that the most probable model of receptor–ligand interaction for heptapeptides in δ-opioid receptor is MODE 1. Several hydrogen bonds as well as hydrophobic/aromatic interactions between the ligand and receptor crucial residues are observed. Moreover, the receptor–ligand interactions in MODE 1 appear to be the most efficient and both ligands (**A** and **E**) binding to the δ-opioid receptor in this way show the highest biological activity among all investigated ligands.

Complexes of the tetrapeptides with δ-opioid receptor

In the complexes of tetrapeptides with δ-opioid receptor there are two binding modes observed. First binding mode is observed for ligands **a, b, c** and **e** and second for ligand **d** (Figure 5, mode 1). In the first mode, the *N*-tyramine moiety is docked in the bottom of the binding pocket being involved in salt bridge with D128 in complexes with **b** and **e**. Alternatively, D128 may be hydrogen bonded to macrocyclic ring (in complexes with **a** and **c**). Phenol group of Tyr[1] is hydrogen bonded with the ε-amino group of K214, carbonyl oxygen of Tyr[1] interacts with amide nitrogen of W207 and the charged ε-amino of K108 is involved in hydrogen bonds with carbonyl oxygens of Lys[2] and Dap[4]. The hydrocarbon chain of macrocyclic ring interacts with I304 and Y109. Aromatic ring of Tyr[1] is involved in interaction with H278 and V281. The aromatic ring of Phe[3] is in close contact with aromatic (W284, W209) and hydrophobic (V281, L300) residues. In slightly less active δ-opioid receptor–**d** complex (Figure 5, mode 2), the *N*-terminal amino group is directed towards the extracellular domain and interacts via hydrogen bond with carbonyl oxygen of P205. Phenol group of Tyr[1] is hydrogen bonded with carbonyl oxygen of K108, whose ε-amino group in turn interacts with carbonyl oxygen of Lys[2]. The side chain of D128 is hydrogen bonded to Orn[4] amide nitrogen. One more hydrogen bond is observed between urea oxygen and ε-amino group of K214. Hydrocarbon parts of the macrocyclic ring interact with M132 and L125. The aromatic ring of Phe[3] interacts with Y109, I304 and hydrocarbon part of the side chain of K108. Aromatic ring of Tyr[1] is not involved in any important interaction. The highly probable model of tetrapeptides interaction with δ-

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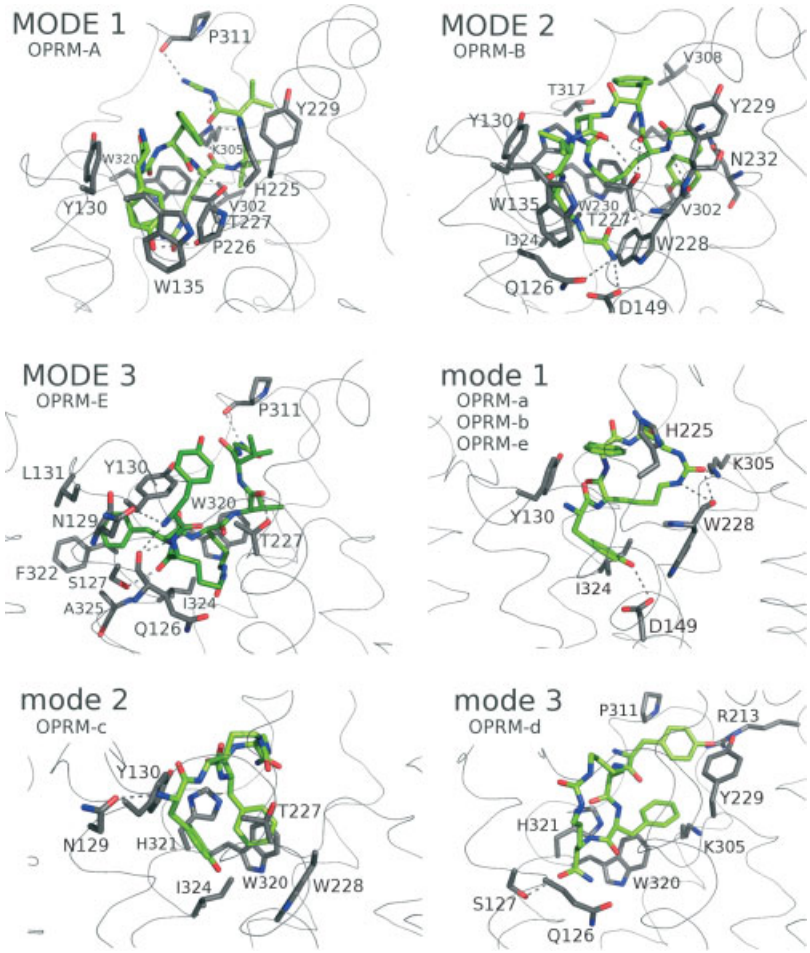

Figure 6. Various modes of interaction in the complexes with μ-opioid receptor. The ligand is coloured light green. Binding receptor residues are exposed and coloured grey. The hydrogen bonds are shown.

opioid receptor is mode 1. As many as four tetrapeptides interact with receptor in this way and several points of interaction between the ligand and receptor exist.

Complexes of the heptapeptides with μ-opioid receptor

The ligands **C** and **D** (non-active in μ-opioid receptor; Table 1) have been omitted. Complex with ligand **A** represents first binding mode of heptapeptides in μ-opioid receptor (Figure 6, MODE 1). The *N*-terminal amino group is directed towards the extracellular domain and does not interact with the receptor. Phenol group of Tyr^1 and *C*-terminal carboxamide form hydrogen bonds with carbonyl oxygens of P226 and P311, respectively. Side chain of T227 is involved in hydrogen bond with carbonyl oxygen of Phe^3. Moreover, ε-amino group of K305 interacts with three oxygen atoms of the ligand, those from urea bridge and both Val^5 and Val^6 residues. One part of the hydrocarbon macrocyclic ring interacts with Y130, whereas second is in close contact with W320. Aromatic–aromatic interaction is observed between rings of Tyr^1 and W135, whereas Phe^3 interacts with H225. Hydrophobic side chain of Val^5 is involved in interaction with V302 and hydrocarbon chain of K305, whereas Val^6 interacts with Y229. The second mode of heptapeptide binding in μ-opioid receptor is observed in μ-opioid receptor–**B** complex (Figure 6, MODE 2). *N*-terminal amino group is directed towards the extracellular side and interacts via hydrogen bond with carbonyl oxygen of Y229. Amide nitrogen of Lys^2 is involved in hydrogen bond with carbonyl oxygen of W228, whose amide nitrogen in turn interacts with carbonyl oxygen of Gly^7. Hydroxyl group of T227 is hydrogen bonded to carbonyl oxygens of Lys^2 and Dap^4, simultaneously. Another hydrogen bond is formed between side chain of H321 and urea oxygen. *C*-terminal carboxamide is involved in two hydrogen bonds with acidic side chain of D149 and carboxamide of Q126, simultaneously. The aromatic side chain of Tyr^2 interacts with V302, hydrocarbon chains of K305 and N232 and is in close contact with W320. The aromatic ring of Phe^3 is involved in strong cation–π interaction with K305, moreover, it weakly interacts with V308 and T317. The hydrocarbon chain of macrocyclic ring interacts with I324. Val^5 interacts with aromatic side chains of W135 and Y130, the latter is also in close contact with Val^6 which in turn is involved in interaction with I324. Third binding mode in μ-opioid receptor is observed for ligand E (Figure 6, MODE 3). *N*-terminal amine is hydrogen bonded to carboxamide of N129 and carbonyl oxygen of Q126. The latter interacts also with amide nitrogen of Orn^2. Hydroxyl groups of S127 and T227 are involved in hydrogen bonds with carbonyl oxygen of Orn^2 and Val^5, respectively. Last hydrogen bond is formed between *C*-terminal carboxamide of the ligand and carbonyl oxygen of P311. Aromatic ring of Phe^3 weakly interacts with side chains of F322, L131 and A325, similarly, there is no strong interaction with participation aromatic side

chain of Tyr[2], which only weakly interact with Y130. Hydrocarbon part of macrocyclic ring and Val[6] are in close contact with W320, whereas Val[5] is not involved in any significant interaction with receptor. In summary, there is no one and only the most probable binding mode among the complexes of the μ-opioid receptor with heptapeptides.

Complexes of the tetrapeptides with μ-opioid receptor

In the complexes of tetrapeptides with μ-opioid receptor there are three binding modes. First binding mode is observed for ligands **a, b** and **e**, second for ligand **e** and third for **d.** In the first mode, the *N*-terminal amino group is docked at the bottom of the binding cavity, but interestingly it is neither involved in salt bridge nor even in hydrogen bond with any receptor residue (Figure 6, mode 1). Phenol group of Tyr[1] is strongly hydrogen bonded to D149. Urea oxygen interacts via hydrogen bonds with ε-amino group of K305 and urea nitrogen of Lys[2] with carbonyl oxygen of W228. Hydrocarbon macrocyclic side chain interacts with I324 which also interacts with aromatic ring of Tyr[1]. Side chain of Phe[3] is involved in aromatic–aromatic interaction with H225 and Y130. Second mode of binding of tetrapeptides in μ-opioid receptor is observed in complex with **c** (Figure 6, mode 2). This mode is predominantly hydrophobic. There is only one hydrogen bond between receptor and ligand involving *N*-terminal amino group of the ligand and carboxamide of N129. The remaining interactions are hydrophobic/aromatic in character. Aromatic ring of Phe[3] strongly interacts with set of aromatic residues: W320, W228 and H321. There is also interaction of both Phe[3] and Tyr[1] aromatic rings with I324. Moreover, Tyr[1] is involved in interaction with Y130 and methyl group of T227. In μ-opioid receptor–**d** complex (Figure 6, mode 3), the *N*-terminal amino group of the ligand is directed towards the EL domain and does not interact with the receptor. Phenol group of Tyr[1] is hydrogen bonded with guanidinium group of R213. Carbonyl oxygen of Orn[4] is involved in hydrogen bond with hydroxyl group of S127. Aromatic ring of Phe[3] interacts with aromatic side chains of W320 and H321, moreover, it is involved in cation–π interaction with ε-amino group of K305. Tyr[1] aromatic side chain interacts with Y229 and P311. Hydrocarbon part of macrocyclic ring interacts with hydrocarbon side chain of Q126.

For tetrapeptides docked in μ-opioid receptor, it appears that the most likely model of receptor–ligand interaction is presented by mode 1.

Validation of the Results

The majority of the receptor residues proposed in this paper as interacting with the ligands were previously identified as important for binding in site-directed mutagenesis studies: K108(2.63) [28,29], D128(3.32) [30–32], Y129(3.33) [10,36], K214(5.39) [51], F218(5.43) [10], W274(6.48) [10,34], I277(6.51) [51], H278(6.52) [51], W284(6.58) [33,35,36], I289(EL3) [37], V296(EL3) [35], V297(7.32) [35], L300(7.35) [38], I304(7.39) [51], Y308(7.43) [10,36] in δ-opioid receptor and Q126(2.60) [39], N129(2.63) [29], D149(3.32) [40,41], H225(EL2) [42], E231(EL2) [39], K235(5.39) [43], V302(6.55) [39], K305(6.58) [44–46], E312(EL3) [39], T317(7.32) [39], W320(7.35) [39,44,47,48], H321(7.36) [48], I324(7.39) [39], Y328(7.45) [49,50] in μ-opioid receptor. These experimental data provide useful information to validate obtained models. Some residues identified previously in site-directed mutagenesis studies as important for some ligands binding in δ-opioid receptor such as R291 and V297 [36] play a minor role in receptor–ligand interactions proposed in this study. In obtained models these residues are located within the extracellular domain of the receptor, thus, they may interact with ligands only on the first stage of recognition, before ligand entry into the receptor pocket, as the authors suggest themselves [36]. Moreover, the other reason why some residues do not occur in some complexes is that the binding site may be different for various ligands in the same receptor [10,28]. The remaining receptor residues proposed in this paper as interacting with ligands may provide guidelines for future experimental site-directed mutagenesis.

Conclusions

The most important factors explaining particular ligand activity such as free energy of binding, conformation of the ligand, its location inside the binding pocket as well as the number and strength of the receptor–ligand interactions have been discussed above. Several different binding modes for investigated ligands have been found and the most probable binding modes have been proposed. Obviously, the remaining binding modes may also occur. It has been previously demonstrated that there is no unique binding site for opioid ligands and this site is not identical even for the same receptor type [10,51]. Moreover, the binding site is not identical even for very similar ligands as investigated in this study. In general, it appears that the shorter tetrapeptide ligands are better accommodated inside the receptor cavities, thus interact stronger with receptor. Moreover, the binding of the tetrapeptides is less specific and no important differences in ligand binding to δ- *versus* μ-opioid receptor are observed. The heptapeptides show higher selectivity towards the δ-opioid receptor, probably because of the ligand *C*-terminus interaction with receptor residues from the extracellular parts of the helices and ELs. Results of this study help to explain the differences in biological activity of the deltorphin analogues, their interaction with the opioid receptors at the molecular level and support designing a new generation of potent opioid drugs with improved selectivity.

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