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Development and validation of opioid ligand–receptor interaction models: The structural basis of mu vs. delta selectivity

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Abstract: Opioid receptor binding conformations for two structurally related, conformationally constrained tetrapeptides, JOM-6 (μ receptor selective) and JOM-13 (δ receptor selective), were deduced using conformational analysis of these ligands and analogs with additional conformational restrictions. Docking of these ligands in their binding conformations to opioid receptor structural models, based upon the published rhodopsin X-ray structure, implicates specific structural features of the μ and δ receptor ligand binding sites as forming the basis for the μ selectivity of JOM-6 and the δ selectivity of JOM-13. In particular, the presence of E229 in the μ receptor (in place of the corresponding D210 of the δ receptor) causes an adverse electrostatic interaction with C-terminal carboxylate-containing ligands, resulting in the observed preference of ligands with an uncharged C-terminus for the μ receptor. In addition, the requirement that the Phe^3 side chain of JOM-13 assume a *gauche* orientation for optimal δ binding, whereas the Phe^3 side chain of JOM-6 must be in a *trans* orientation for high-affinity μ binding can be largely attributed to the steric effect of replacement of L300 of the δ receptor by W318 of the μ receptor. Testing this hypothesis by examining the binding of JOM-6 and several of its key analogs with specific μ receptor mutants is described. Our initial results are consistent with the proposed ligand–receptor interaction models.

In an effort to understand the details of opioid ligand–receptor interactions and the basis for observed differences in ligand structure activity (SAR) profiles at different opioid receptor subtypes, we have, over the past several years, followed two complementary paths. The first of these was directed toward elucidation of the bioactive conformation(s)

of conformationally restricted ligands selective for individual opioid receptor subtypes, while the second path was focused on the development of a reliable, accurate method for modeling the three-dimensional structure of the opioid receptors. Successful completion of these aims would allow the development of realistic models for the precise interactions of specific opioid ligands with individual opioid receptor subtypes, from which an understanding of the structural basis of receptor selectivity could follow. This, in turn, would provide a reasonable starting point for structure-based design of more potent and/or more selective opioid ligands.

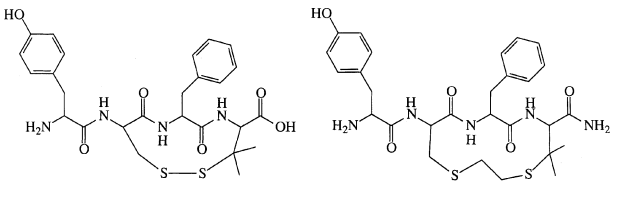
Our efforts have been focused primarily on μ and δ opioid receptor ligands and have been simplified by the development in our laboratory of two structurally related, conformationally constrained peptide series that differ markedly in their δ vs. μ receptor binding preferences. Results of conformational analyses, via NMR, X-ray crystallography, and molecular mechanics, as well as structure–activity differences in these series, which allow structural features underlying the relative receptor selectivity of the two series to be inferred, are summarized below. Also summarized below is the method we developed for structural modeling of the transmembrane 7-helical bundle of G-protein-coupled receptors (GPCRs) and its application to the μ and δ opioid receptors. The more recent publication of the X-ray structure of rhodopsin, the prototypical GPCR, confirmed the primary details of our GPCR models and allowed an alternative starting point for development of μ and δ receptor models.

Docking ligand bioactive conformation models to the independently developed receptor models allows hypotheses to be formulated regarding the basis for μ vs. δ receptor ligand selectivity. Receptor mutagenesis coupled with specifically modified ligands provides a means for testing these hypotheses. Initial studies probing these hypotheses are presented below.

Development of Opioid Ligand Pharmacophore Models

As an approach to reduce the flexibility of the backbone of the δ selective cyclic, disulfide-containing peptide, DPDPE (Tyr-c[D-Pen-Gly-Phe-D-Pen]OH) (1), attributable to its central glycine residue, a tetrapeptide series in which this residue was simply eliminated was explored (2). It was found that this glycine residue, thought to be essential from early enkephalin SAR studies, could in fact be removed as evidenced by the high δ selective binding affinity displayed by a key analog in the series, JOM-13 (Tyr-c[D-Cys-Phe-D-Pen]OH) (2) (Fig. 1). Conformational analysis, using both experimental and computational approaches, revealed that, although the 11-membered, tripeptide cycle within JOM-13 is indeed conformationally well defined, the exocyclic Tyr^1 residue and the side chain of Phe^3, which are key elements of the opioid pharmacophore, are quite flexible (3). To elucidate the bioactive conformations of these key elements of ligand–receptor recognition, new series analogs were prepared in which conformationally restricted replacements for Tyr or Phe were incorporated while maintaining the 11-membered, cyclic scaffold. In the first set of analogs (4), the Tyr^1 residue of JOM-13 was replaced by several conformationally constrained analogs of Tyr. Each of these Tyr replacements has reduced flexibility compared with Tyr and, importantly, each can sample a different subset of the conformational space available to Tyr. Consequently, if any of the analogs examined display similar binding affinity to JOM-13, then the bioactive conformation of the Tyr^1 residue of JOM-13 must lie within the more limited available conformational space of the Tyr replacement of this more constrained analog. If two or more of the analogs display similar binding affinity to JOM-13, then the search for the bioactive conformational features of Tyr^1 in JOM-13 is simplified to a search within the intersection of conformational space available to the Tyr^1 replacements in these active analogs. This approach proved to be quite successful, leading to a single proposed bioactive conformation of the Tyr^1 residue of JOM-13 (4). A similar approach was employed to deduce the side chain conformation of the Phe^3 residue when bound to the δ receptor (5,6). These studies indicated that a *gauche* ($\chi^1 = -60°$) conformation is preferred. Figure 2 shows the proposed bioactive conformation of JOM-13.

While the JOM-13 series SAR was evolving, we examined related analogs in which different cyclization approaches were employed to allow variation in ring size, while maintaining the D-Cys-Phe-D-Pen tripeptide cycle (2). This was achieved by cyclizing as a dithioether (rather than the



JOM-13 JOM-6

Figure 1. Structures of JOM-13 and JOM-6.

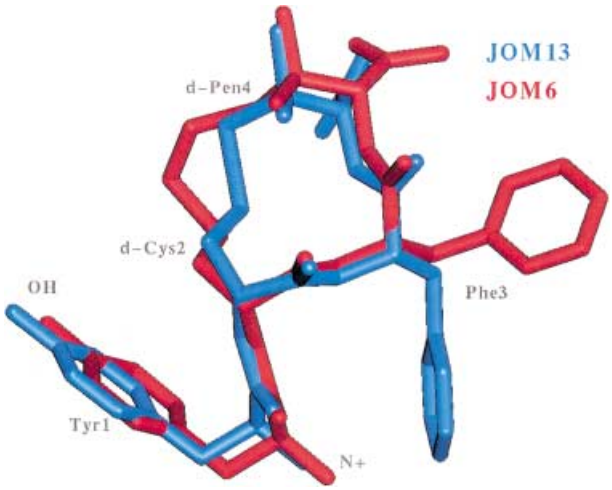


Figure 2. Superposition of bioactive conformations of JOM-13 in the δ receptor binding pocket (blue) and JOM-6 in the μ receptor pocket (red).

disulfide of JOM-13), which allows larger ring sizes to be readily explored. We discovered that simply replacing the 11-membered disulfide of JOM-13 by a 13-membered ethylene dithioether improved μ receptor binding affinity and decreased δ affinity (2). If this peptide is further modified by replacing the C-terminal carboxylate with a carboxamide (long known to favor μ vs. δ binding), the resulting compound, JOM-6 (Tyr-c(S-Et-S)[D-Cys-Phe-D-Pen]NH_2) (Fig. 1), displays high μ binding affinity and moderate μ selectivity (2). The combined result of two modifications (changes in ring size and C-terminus) is a 6000-fold selectivity shift (JOM-13: K_i (δ) = 0.74 nM; K_i (μ) = 52 nM. JOM-6: K_i (δ) = 24.8 nM; K_i (μ) = 0.29 nM).

Elucidation of the bioactive conformation of JOM-6 at the μ opioid receptor was pursued in the same manner as that of JOM-6 at the δ receptor (7). The resulting proposed bioactive conformation is shown in Fig. 2, overlaid with the corresponding δ receptor binding conformation of JOM-13. As is evident from the figure, the primary conformational difference between the two proposed binding conformations is in the Phe^3 side chain. For JOM-13 at the δ receptor, as noted above, the Phe^3 side chain assumes a $\chi^1 = -60°$ conformation, while for JOM-6 at the μ binding site $\chi^1 = 180°$. The significance of this difference in the context of structural differences in the μ and δ binding sites is discussed below.

Development of Opioid Receptor Structural Models

Several years ago we began exploring a new approach for the development of precise structural models of the seven transmembrane α-helical bundle of GPCRs by computational refinement of crude structures derived from low-resolution electron microscopy data and other experimental results (8). Our approach was based upon the observation that these transmembrane (TM) helices contain a significant fraction of polar residues that must form hydrogen bonds with other polar side chains or with the helix backbone. Analysis of GPCR multisequence alignments allowed us to assign putative hydrogen-bonding partners and these collected hydrogen bonds, in turn, served as distance constraints for distance geometry calculations, using the program DIANA, that allowed us to arrive at final models for the GPCR under consideration. The resulting models for rhodopsin (8), opioid receptors (9) and ≈20 additional GPCRs (10), agree well with experimental data. Indeed, our model of bovine rhodopsin deposited in the Protein Data Bank (1bok) superimposes well (11) with the recently determined rhodopsin crystal structure (12): the rmsd of all 186 common Cα atoms of the TM domain is 2.8 Å.

We recently developed alternative opioid receptor models using the rhodopsin X-ray structure as a template for the inactive state of these receptors. To retain the orientation of polar, conserved and functionally important residues inside the TM domain the distortions present in the rhodopsin structure in TM5 (α-aneurism at H211) and TM7 (2 turns of 3–10 helix near K296) were reproduced in the opioid receptor models, while the α-aneurism in TM2 (near G90) was omitted. Because the X-ray structure of rhodopsin reflects the inactive state of the receptor, structural alterations accompanying activation must be incorporated to obtain a realistic active-state receptor structure. For example, the active state must reproduce the observed changes in distance between residues V139 (TM3) and C247, C252 (TM6) during rhodopsin activation (13–15). After rotation of the χ^2 angle of W265 the binding pocket of the 'activated' receptor easily adopts the extended structure of the agonist, all-*trans* retinal, with the β-ionone ring oriented toward TM4. We have now employed this 'activated' rhodopsin model for modeling of 'active' opioid receptors, suitable for docking of opioid agonists, like JOM-6 and JOM-13. These 'homology' models agree well with our original distance geometry models of the μ and δ receptor and have similar binding pockets.

Docking of JOM-6 and JOM-13, in their proposed binding conformations, to the μ and δ receptors, respectively, was carried out manually using the QUANTA molecular modeling software. For both tetrapeptides, Tyr^1 was positioned in the bottom of the pocket to form an ionic interaction between the ligand N^+ and the conserved Asp residue from

TM3 (D147 in μ, D128 in δ), polar interactions with Tyr from TM7 (Y326 in μ, Y308 in δ), and H-bonds between the ligand Tyr[1] OH groups and a conserved Tyr from TM3 and His from TM6 (Y147 and H297 in μ, Y129 and H278 in δ). The importance of these four conserved residues of the binding pocket has been demonstrated by mutagenesis (16–20).

Figures 3 and 4 show the results of docking of JOM-13 to the δ and JOM-6 to the μ opioid receptor, respectively. Each figure also shows, in thin lines, the corresponding location of the 'other' ligand in its receptor binding site. The interactions displayed in these figures represent an abridged subset, meant to highlight the anchoring interactions noted above and interactions that, because of sequence differences between the μ and δ receptors, underlie the selectivity differences between JOM-13 and JOM-6. Notable in the latter category are differences in the regions of the μ and δ binding pockets that interact with the ligand C-terminus and the ligand Phe[3] side chain.

Structural Basis of μ vs. δ Selectivity

Receptor environment of ligand C-terminus

It can be seen in Figs 3 and 4 that the environment of the ligand C-terminus of JOM-13 in the δ binding site and JOM-6 in the μ binding site differ slightly in our two ligand–receptor interaction models. As seen in Fig. 3, the amine-containing side chain of K214 (TM5) of the δ receptor forms an ionic interaction with the C-terminal carboxylate of JOM-13, consistent with the observation that a negatively

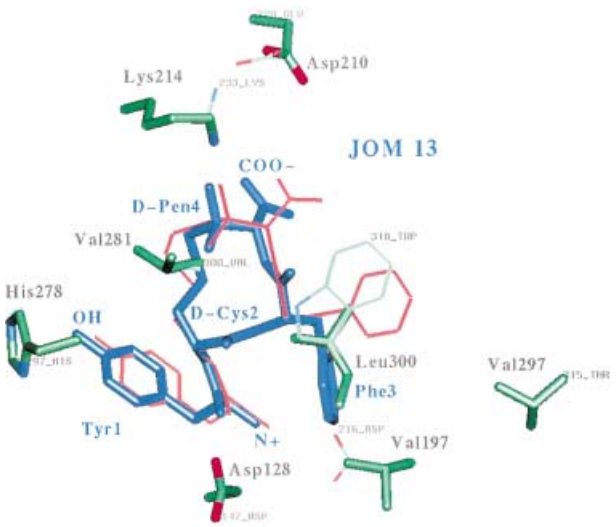


Figure 3. JOM-13 (blue) in δ receptor binding site (thick lines). JOM-6 (thin red lines) in μ binding site (thin lines) is also depicted.

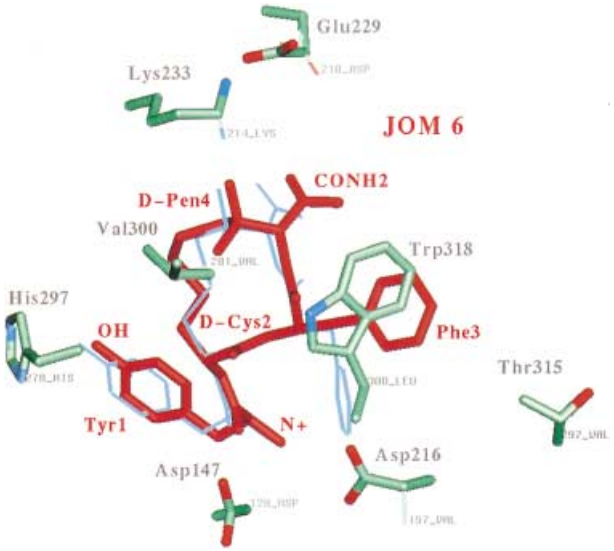


Figure 4. JOM-6 (red) in μ receptor binding site (thick lines). JOM-13 (thin blue lines) in δ binding site (thin lines) is also depicted.

charged C-terminus enhances ligand affinity for the δ receptor. Figure 4 demonstrates that the longer cycle of JOM-6 leads to a slightly different positioning of the ligand C-terminus, slightly farther from the corresponding μ receptor residue, K233. In this case, the K233 interacts, instead, with the receptor E229 residue. In the δ receptor, the residue corresponding to the μ receptor's E229 is D210. In our ligand–receptor interaction models, the longer side chain of the E229 (compared with the δ receptor's D210) and the shifted location of the ligand C-terminus in the two receptors results in a closer proximity between these groups in the μ binding site. Because of this proximity, C-terminal carboxylic acid-containing ligands encounter ionic repulsion at the μ binding site, resulting in lower affinity. This is entirely consistent with a large body of structure activity data for μ ligands.

To test this aspect of our binding models, the binding affinities of JOM-6 and JOM-18 (Tyr-c[S-Et-S][D-Cys-Phe-D-Pen]OH), in which the C-terminal carboxamide of JOM-6 is replaced by a carboxylate, were determined. Binding to both the wild-type μ opioid receptor and the E229D μ receptor mutant was examined. Our ligand–μ receptor interaction model would predict that JOM-18 should bind poorly to the wild-type μ receptor because of ionic repulsion, but that this effect should be less dramatic in the E229D mutant, as the shorter Asp side chain attenuates this repulsion. Table 1 summarizes the results of these binding assays. As expected, binding of JOM-18 to the wild-type μ receptor is poor; compared with JOM-6, the affinity of JOM-18 is reduced ≈4400-fold. By contrast, JOM-18 binding to the E229D μ receptor mutant is an order of magnitude

Table 1. **Binding affinities of JOM-*6* analogs to wild-type and mutant μ opioid receptors**

Analog	MOR K_i (nM)	E229D K_i (nM)	W318L K_i (nM)
JOM-6	0.32	1.8	
JOM-18	1400	160	
[Δ^EPhe^3]JOM-6	2.8		319
[Δ^ZPhe^3]JOM-6	107		96

For each K_i value, SEM was < 10%. K_D values for [^{3}H]DAMGO: 1.1 nM (MOR); 1.4 nM (E229D); 0.94 (W319L).

stronger than to wild-type, and is only 90-fold weaker than that of JOM-6. Thus *compared with JOM-6* (whose affinity decreases > 6-fold owing to the E229D mutation), the *relative* binding affinity of JOM-18 improves 50-fold when E229 is mutated to Asp. This strongly supports our binding interaction model and implies that the change from D210 in the δ receptor to the corresponding E229 in μ is chiefly responsible for the well documented difference in receptor preference of opioid peptides with anionic vs. neutral C-terminal groups.

Receptor environment of Phe^3 side chain

Examination of Figs 3 and 4 suggests the basis for the observed difference in Phe^3 side chain orientation preference at the δ vs. μ opioid receptors. As depicted in Fig. 3 our models predict that a *gauche* ($\chi^1 = -60°$) orientation of Phe^3 of JOM-13 is favored because it places the Phe^3 side chain in a nonpolar region of the receptor, near L300. JOM-6 in the μ receptor binding site interacts with the corresponding receptor residue, W318. However, because of the slightly shifted orientation of JOM-6 in its binding site, this interaction requires that the JOM-6 Phe^3 side chain be in a *trans* ($\chi^1 = 180°$) orientation. The binding models are consistent with the observation that [Δ^ZPhe^3]JOM-13, in which the residue 3 side chain is constrained in a *gauche*-like orientation, binds much better to the δ receptor than does [Δ^EPhe^3]JOM-13, in which the residue 3 side chain is *trans*, and with the observation that the opposite preferences are observed for the corresponding JOM-6 analogs binding to the μ receptor.

Our models suggest that the replacement of the δ receptor L300 by W318 in μ is critical for the favorable μ binding of JOM-6, because the bulkier Leu residue would result in adverse steric interactions with the *trans* Phe^3 rotamer. This prediction was evaluated by examining the binding of [Δ^EPhe^3]JOM-6 and [Δ^ZPhe^3]JOM-6 to the wild-type μ opioid receptor and to the W318L mutant. If our model is correct, the W318L mutation should block optimal interaction of the Δ^EPhe^3 side chain, resulting in diminished binding affinity of [Δ^EPhe^3]JOM-6, while the mutation should have little effect on the binding of [Δ^ZPhe^3]JOM-6, as the *gauche*-like orientation of the Δ^ZPhe^3 interacts poorly with either W318 or its Leu replacement, due to the shift of the ligand in the μ binding site. Results shown in Table 1 are consistent with these predictions. As predicted, the binding of [Δ^ZPhe^3]JOM-6 is weak to both wild-type and mutant μ receptor, with similar affinities observed for the two receptors. In contrast, [Δ^EPhe^3]JOM-6, which binds well to the wild-type μ receptor, displays a > 100-fold reduction in affinity to the W318L mutant.

While falling short of unequivocally proving our ligand–receptor interaction models, the results described above clearly provide support for these models and provide valuable insights into the basis of ligand selectivity at μ and δ opioid receptors. Further validation of the models is in progress as is structure-based ligand design using the models as the foundation.

Experimental Procedures

Materials

The pCMV expression vectors containing the coding sequence for the μ opioid receptor were obtained from Professor Huda Akil at the University of Michigan. Pfu turbo DNA polymerase, *Dpn*I restriction endonuclease and XL1-blue super-competent *E. coli* cells were purchased from Stratagene. Nucleotide primers, antibiotics, 1 M Tris–HCl buffer, Lipofectamine Plus reagent and cell culture media and reagents were purchased from Life Technologies. [^{3}H] DAMGO was purchased from NEN. Ninety-six-well glass-fiber filter plates were purchased from Millipore. Protected amino acids, reagents and resins for peptide synthesis were obtained from Advanced ChemTech, or Peptides International. All other reagents were from Sigma-Aldrich unless otherwise indicated.

Peptide synthesis

All peptides were prepared using standard solid-phase methods similar to those described previously for the synthesis of JOM-6 (2), using chloromethylated polystyrene (Merrifield) resin cross-linked with 1% divinylbenzene.

Trifluoroacetic acid (TFA) was used for deprotection, and dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) were employed to facilitate coupling. Alpha-amino functions were *t*-butyloxycarbonyl (Boc) protected, and *p*-methylbenzene protection was employed for the labile side chain sulfhydyl groups of Cys and Pen. Simultaneous deprotection and cleavage from the resin were accomplished by treatment with anhydrous hydrogen fluoride in the presence of 5% *p*-cresol and 5% *p*-thiocresol. Cyclization to dithioether-containing analogs was accomplished by treatment of a dilute solution of the linear free sulfhydryl-containing species in dimethyl formamide with potassium *tert*-butoxide followed by addition of dibromoethane. All peptides were then purified by RP-HPLC. Final product confirmation was obtained by fast atom bombardment mass spectrometry (FAB-MS).

Site-directed mutagenesis

Single, double and triple point mutations of the μ opioid receptor were generated from the μ/pCMV expression vector using the QuickChange Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Each mutation was verified by DNA sequencing.

Cell culture and transfection

Cos-1 cells were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% FBS and incubated at 37°C in 5% CO_2. At 80% confluency, the cells were transiently transfected with 8–10 μg per 75 cm^2 flask of the wild-type and μ opioid/pCMV mutants using Lipofectamine Plus reagent (20 and 30 μL of Plus reagent and Lipofectamine, respectively).

Cos-1 membranes

Forty-eight hours after transfection, the Cos-1 membranes were prepared for assay as described previously (21). Briefly, the Cos-1 cells were scraped into 50 mM Tris–HC1, pH 7.4 containing 0.1 μg/mL PMSF (ice-cold) and homogenized using a Polytron homogenizer. Following centrifugation at 15 000 ***g*** for 30 min at 4°C, the membranes were resuspended to a concentration of 0.2 μg/mL in the homogenization buffer.

Radioligand binding assays

We used 40–50 μg of the membrane preparations in 200 μL for all binding studies. For all binding assays, the membranes were incubated with 25 μL aliquots of [^{3}H] DAMGO in 50 mM Tris–HC1, pH 7.4 in 96-well polypropylene microtiter plates. 0.1–20 nM of the radioligands was used for saturation binding studies. Competition binding assays were carried out in the presence of 2 nM radioligand and 0.1 nM to 30 μM of peptide ligands. Nonspecific binding was determined in the presence of 2 μM unlabeled Naloxone. After incubating for 1.5 h at room temperature, the samples were transferred to 96-well glass-fiber filter plates, filtered, and washed with 2 × 200 μL ice-cold 50 mM Tris–HC1, pH 7.4. Filter plates were counted using a Wallac Trilux1450 Microbeta scintillation counter.

Data analysis

Saturation binding results were analyzed and K_d values for the wild-type and receptor mutants were determined using the LIGAND program. Competition binding curves were analyzed by nonlinear regression using SIGMAPLOT 7.0 (SPSS Science, Chicago, IL, USA), and IC_{50} values were converted to K_i values using the Cheng-Prusoff correction (22).

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