Modeling of κ-Opioid Receptor/Agonists Interactions Using Pharmacophore-Based and Docking Simulations

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The interaction of the κ-opioid receptor with arylacetamide and benzomorphan derivatives acting as agonists was modeled through pharmacophore-based and docking calculations. Potentially bioactive conformations of representative ligands (U-50,488 and its benzo-fused analogues **4** and **6** for arylacetamides and MPCB for benzomorphans) were identified by systematic conformational analysis and docked into a 3D model of the κ-receptor. The obtained complexes, refined by energy-minimization and molecular dynamics, were evaluated for their consistency with structure−activity relationships and site-directed mutagenesis data. The following interactions are hypothesized to govern the ligand−receptor recognition process: (i) a salt bridge between the Asp138 carboxylate and the protonated nitrogen of the bound agonist; (ii) a hydrogen bond donated by the Tyr312 hydroxyl to the carbonyl oxygen of arylacetamides and MPCB; (iii) hydrophobic interactions established by the dichlorophenyl moiety of arylacetamides and the pendant phenyl ring of MPCB with the surrounding side chains of Tyr312, Leu224, Leu295, and Ala298; (iv) a π-stacking contact between the Tyr312 side chain and the phenyl ring of arylacetamides; (v) a hydrogen bond linking the His291 imidazole ring to the phenolic hydroxy group featured by typical benzomorphans and the arylacetamides **4** and **6**.

Introduction

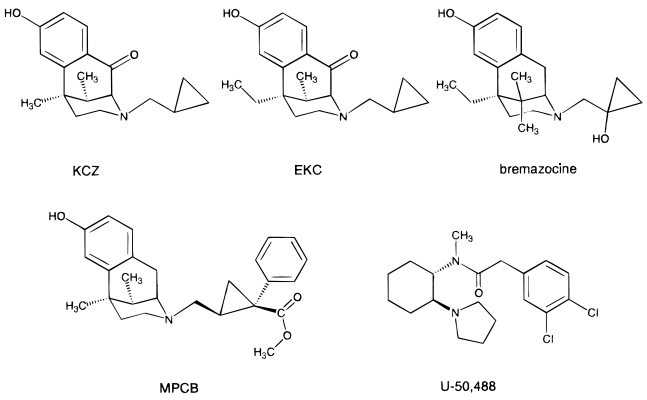
Today it is widely accepted that there are at least three opioid receptor subtypes, μ, κ and δ, which belong to the class of G-protein coupled receptors.[1,2] Increasing evidence has been accumulated during the past decade to support the hypothesis that a selective κ-opioid agonist would be a powerful analgesic agent without the clinically limiting side effects that characterize morphine (e.g. respiratory depression, constipation, and inhibition of gastrointestinal motility) and all other μ-opioid selective analgesic drugs. This rationale has provided the impetus for the discovery and preclinical development of selective non-peptide κ-opioid agonists.[3,4]

Most of the synthetic selective κ-agonists belong to the following chemical classes (Chart 1): (i) benzomorphans [ketocyclazocine (KCZ), ethylketocyclazocine (EKC), bremazocine, MPCB ((−)-*R*,*S*-6,11-dimethyl-1,2,3,4,5,6-hexahydro-3-[(2′-methoxycarbonyl-2′-phenylcyclopropyl)methyl]-2,6-methano-3-benzazocin-8-ol)][5] and (ii) arylacetamides, whose prototype is U-50,488.[6]

Previous attempts to deduce the bioactive conformations and the binding modes of κ-opioid agonists are reviewed in the Discussion section.[7−12] These binding models, based on different assumptions and methodological approaches, are neither convergent nor conclusive. Therefore, additional theoretical investigations still seem necessary in this field.

Herein, we describe the development of a model of interaction of the κ-opioid receptor with structurally different selective agonists (arylacetamides and benzomorphans) aimed at rationalizing structure−activity relationships (SARs) and site-directed mutagenesis data reported so far in the literature. Our work proceeded in two steps. First, candidate bioactive conformations of arylacetamides and benzomorphans were selected in the absence of the receptor through systematic conformational analysis. These structures were then submitted to docking calculations using an available model of κ-opioid receptor.[13]

Chart 1



Results

Derivation of a Pharmacophore Model for Arylacetamide κ-Agonists. A 3D pharmacophore model for arylacetamides was developed using compounds **1**−**8** listed in Figure 1 as representatives of this class of κ-agonists. Affinity data of the ligands and references are given in Table 1.

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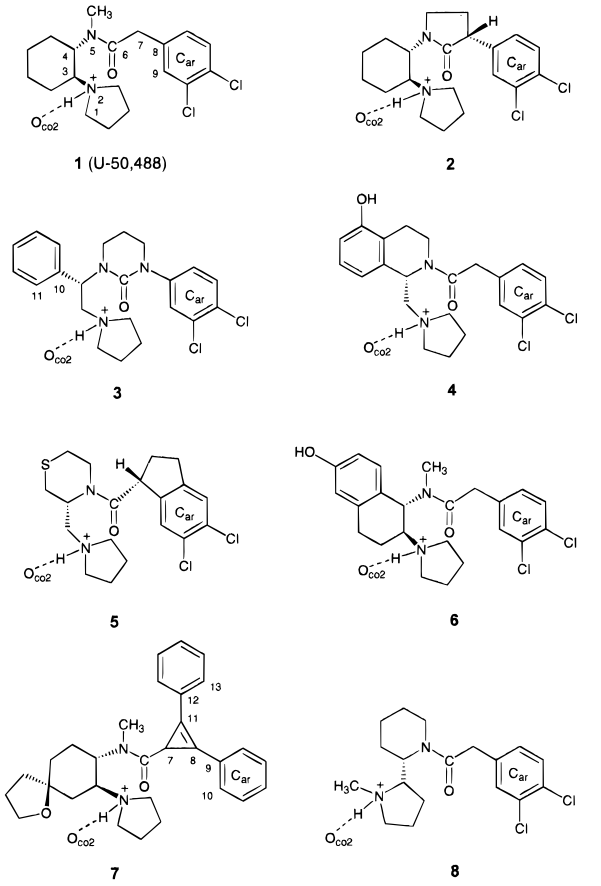

Figure 1. Chemical structures of κ-agonist arylacetamides analyzed to derive a 3D pharmacophore model. O_{co2}, C=O, and C_{ar} are the pharmacophoric points. The atom numbering scheme allows definition of torsion angles τ_1–τ_9 reported in Table 3.

Table 1. Binding Affinity Data of Compounds **1**–**8** and MPCB

compd	K_i (nM)	ref
1 (U-50,488)	0.89[a]	6
2	10[b]	14
3	0.42[a]	15
4	0.09[a]	16
5	0.44[c]	17
6	1[a]	18
7	0.64[d]	19
8	0.33[a]	23
MPCB	240[e]	5

[a] Inhibition of [^{3}H]U-69593 binding in guinea pig brain homogenates. [b] Inhibition of [^{3}H]EKC binding in guinea pig forebrain. [c] IC_{50} related to inhibition of [^{3}H]U-69593 binding in guinea pig brain homogenates. [d] Inhibition of [^{3}H]U-69593 binding in cloned rat κ-opioid receptor expressed in CHO cell line. [e] Inhibition of [^{3}H]diprenorphine binding in guinea pig cerebella fraction. In this assay U-50,488 exhibited a K_i value of 20.5 nM.

SARs from various literature sources[14–31,34–36] allowed us to define the following pharmacophoric elements:

(i) The ammonium moiety, typical of all active opioid ligands, is believed to form a salt bridge with the Asp138 carboxylate group in TM3 domain of the κ-receptor.[32]

(ii) The amide carbonyl group is a crucial feature in the class of arylacetamides. In fact, replacement of the amide linkage with a reversed amide, reduced *N*-methyl amide, ester function, or *N*-methyl thioamide[33] significantly decreases or abolishes affinity.[21,34–36] These data

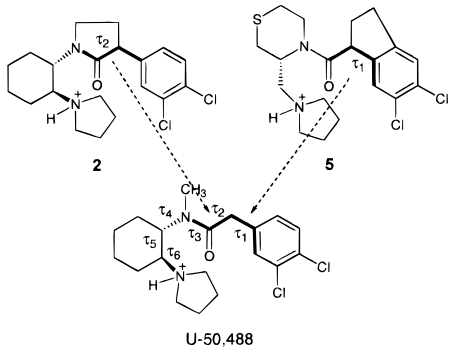

Figure 2. Pharmacophoric arrangement of the arylacetyl fragment results by combining the τ_1 and τ_2 torsion angles (defined by thick lines) of compounds **2** and **5**, respectively.

are consistent with the hypothesis that the carbonyl oxygen accepts a hydrogen bond from the receptor.

(iii) The benzene ring, generally substituted with two *o*-chlorine atoms, is required for a tight binding to the receptor.[23,26,30] It is likely that the aryl group is involved in a π-stacking interaction within a hydrophobic pocket made up of at least an aromatic amino acid side chain.

Pharmacophore identification is facilitated whenever rigid substructures of some molecules can be matched with corresponding flexible moieties of structurally related analogues. This procedure has been applied by Höltjie et al. on 5-HT_2 antagonists[37] as well as by Martin et al. on D_2 agonists.[38] Accordingly, the conformation of the arylacetyl fragment is of *synclinal*–*anticlinal* type by combining the τ_1 and τ_2 torsion angles of compounds **5** and **2**, wherein they are fixed to 39° and −139°, respectively (see Figure 2).

Since the putative ionic interaction between the ammonium moiety and the Asp138 carboxylate does not necessarily require coincidence of the nitrogens, we adopted the site point approach of Martin *et al.*[39] A site point O_{co2} was placed at 2 Å from the ammonium hydrogen along the N–H vector to simulate a carboxylate oxygen of Asp138. O_{co2} was given the van der Waals radius of the O.CO2 atom type defined within the TRIPOS force field.[40]

To identify the conformations of compounds **1**–**8** ensuring a common overlap of the O_{co2} pseudoatoms, we applied the active analogue approach[41] using the SYBYL/SEARCH module. The rotatable torsion angles τ_3, τ_4, τ_5, and τ_6, defined in Figure 2, were scanned. The aromatic ring position was defined by the centroid C_{ar}. The distances among the pharmacophoric points O_{co2}, C=O, and C_{ar} were recorded. This procedure did not yield manageable and interpretable output data because quite diverse conformations of each molecule were frequently associated with the same set of distances.

To solve this problem, we resorted to an alternative description of the 3D location of the O_{co2} point. All the molecules were first superimposed about the aryl ring and the carbonyl group and then placed into a box generated using the SYBYL/CoMFA module (the so-called region). The systematic scanning of the rotatable bonds was performed after anchoring the aryl ring and the carbonyl atoms at fixed positions within the box. For each conformation, we recorded the eight distances between O_{co2} and each corner of the box (see Figure 3).

This method produced 21 combinations of common distances among pharmacophoric elements, that is, 21

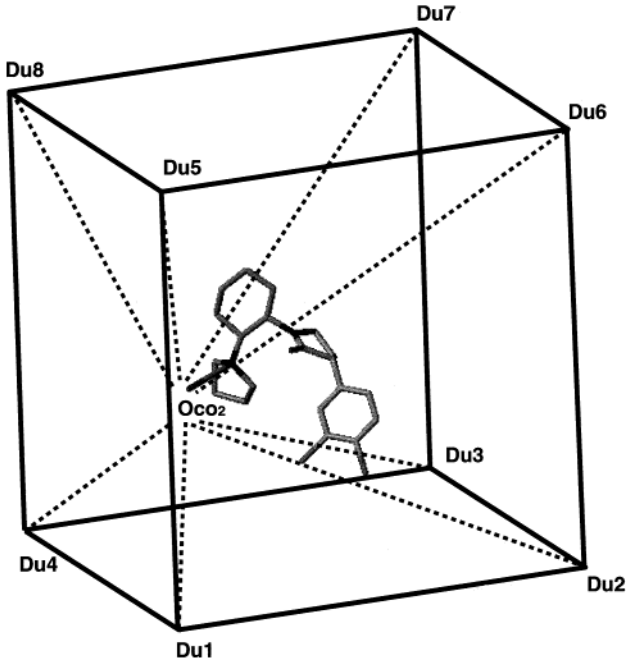

Figure 3. Strategy devised to describe the 3D location of the O_{co2} point in the active analogue approach applied to arylacetamides. Eight distances between O_{co2} and vertexes Du1−Du8 were recorded for each conformation.

potential pharmacophore patterns. For each solution, the lowest energy conformations were selected and submitted to the SYBYL/MULTIFIT routine to maximize the match of pharmacophoric elements. MULTIFIT maximizes the overlay of various molecules about user-specified atoms while simultaneously optimizing their geometries. The output structures were finally energy-minimized without any constraint to the nearest local minimum conformer.

Finding so many potential pharmacophore patterns was somehow unexpected, considering that such an issue had never been addressed or even mentioned in previous articles on the same topic.[7−12] To select unambiguously the best pharmacophore pattern among the 21 candidate models, we realized that alignments had to be examined by taking into account the general requirement of steric complementarity between ligand and receptor. In particular, it was assumed that bioactive conformations had to be oriented within the binding site to avoid steric clash with any of the protein atoms. This was checked by intersecting the volume occupied by the O_{co2} atom (conceptually part of the receptor) with the van der Waals envelop of the superimposed molecules.

Only one solution was characterized by the point O_{co2} in a region of space not occupied by any of the superimposed ligands and therefore proposed as the most reliable pharmacophore pattern for κ-agonist arylacetamides (schematized in Figure 4). The hypothetical receptor-bound conformations of compounds **1**−**8** are superimposed in Figure 5. The distances among points O_{co2}, C═O, and C_{ar} are given in Table 2 for each molecule. Table 3 summarizes the most relevant torsional angles defining the bioactive conformations and the corresponding strain energies calculated by the TRIPOS force field. It is worth noting that the highest strain energy does not exceed 5 kcal/mol, a value generally considered as acceptable in most of pharmacophore-mapping studies.[42]

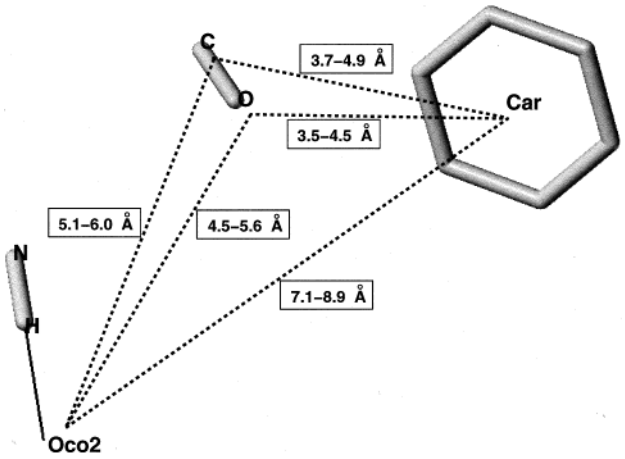

Figure 4. Distances among pharmacophoric points in the set of arylacetamides **1**−**8**.

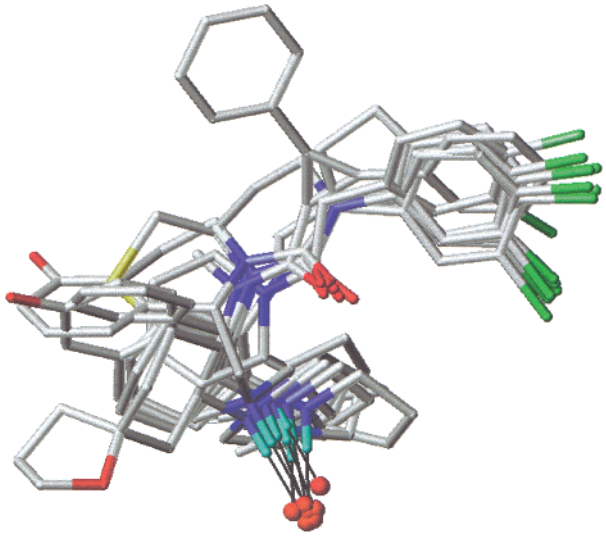

Figure 5. Structures of κ-arylacetamides **1**−**8** superimposed in their pharmacophore-consistent conformations.

Table 2. Distances (Å) among the Pharmacophoric Points O_{co2}, C═O, and C_{ar} in the Pharmacophore-Based Conformations of Compounds **1**−**8**

compd	$O_{co2}-C_{ar}$	$C_{ar}-O$	$O_{co2}-O$	$C_{ar}-C$	$O_{co2}-C$	rmsd[a]
1	7.8	3.6	5.3	3.8	5.7	0.0
2	8.4	4.0	5.6	3.8	5.9	0.3
3	8.0	3.6	4.9	3.7	5.5	0.4
4	8.1	3.5	5.5	3.8	6.0	0.1
5	7.1	3.7	4.5	3.9	5.1	0.3
6	8.1	3.6	5.2	3.8	5.7	0.2
7	8.9	4.5	5.4	4.9	5.8	0.5
8	8.5	3.6	5.1	3.8	5.5	0.6

[a] rmsd is the root-mean-square distance resulting from the fitting of the molecule on U-50,488 about the pharmacophoric points.

The putative pharmacophore pattern was evaluated for its ability to explain the inactivity of compound **9**,[20]

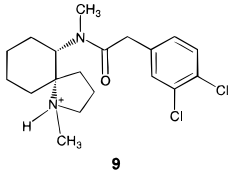

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Table 3. Main Torsion Angles[a] (deg) and Strain Energies of the Pharmacophore-Based Conformations of Compounds **1**−**8**

compd	τ_1	τ_2	τ_3	τ_4	τ_5	τ_6	τ_7	τ_8	τ_9	ΔE_{conf}[b]
1	63	−160	178	−129	68	−155				5.0
2	33	−139	−173	−132	67	−151				2.4
3	47	−179	−179	−119	74	−165			101	3.2
4	78	175	−178	−61	−54	−71				2.8
5	36	−160	170	−120	72	−175				1.2
6	57	−170	170	−117	82	−160				2.9
7	74	167	−178	−131	68	−152	9	28		5.0
8	46	−163	169	−95	176	−132				0.8

[a] The torsion angles are defined as follows on the basis of the atom numbering scheme reported in Figure 1: $\tau_1 = \tau(6,7,8,9)$; $\tau_2 = \tau(5,6,7,8)$; $\tau_3 = \tau(4,5,6,7)$; $\tau_4 = \tau(3,4,5,6)$; $\tau_5 = \tau(2,3,4,5)$; $\tau_6 = \tau(1,2,3,4)$; $\tau_7 = \tau(7,8,9,10)$; $\tau_8 = \tau(8,11,12,13)$; $\tau_9 = \tau(5,4,10,11)$. [b] ΔE_{conf} is the strain energy, calculated with the TRIPOS force field as the difference in energy between the conformation and the global minimum conformer.

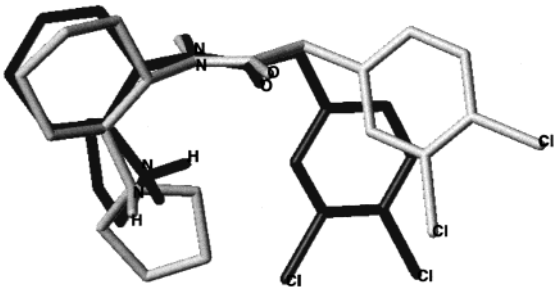
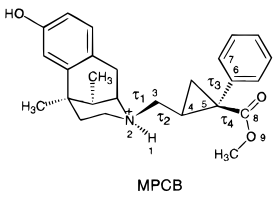


Figure 6. Putative receptor-bound conformation of U-50,488 (gray) superimposed on the crystal structure of the spiro compound **9** (black). Note that the ammonium N−H vectors of the two molecules point to quite different directions. The crystal structure of **9** was overlayed unmodified on U-50,488, using the nitrogen atom and the carbonyl group as fitting points. The aryl rings of the two molecules can be easily matched through a 30° increment of τ_2 in **9**.

whose crystal structure was retrieved from the CSD[43] (refcode VARBIZ). In this spiro compound the torsional angles τ_5 and τ_6 are fixed at values of 68° and −155°, respectively. Figure 6 shows an overlay of the crystal structure of **9** on the putative bioactive conformation of U-50,488. It can be noted that the ammonium N−H vectors of the two molecules point to different directions. The particular geometric arrangement of the ammonium moiety in **9** might prevent this ligand from making the supposedly vital hydrogen bond with the Asp138 carboxylate. An alternative reason for the inactivity of **9** could be related to the exceeding steric hindrance of the spiro system in the binding site. However, this latter hypothesis was discarded since the volume of **9** outside the union volume of the superimposed active ligands **1**−**8** was negligible.

Selection of Trial Conformations of the Benzomorphan MPCB for Docking Studies. Three independently published pharmacophore-based studies propose considerably divergent alignments of arylacetamides and benzomorphans.[7−9] This is perhaps not surprising since the two classes of ligands are not sufficiently similar to establish a straightforward and unbiased correspondence of pharmacophoric moieties.

The highly κ-selective benzomorphan MPCB (see Figure 7) is characteristic with respect to other members of its class in that it features a phenyl ring and an ester carbonyl proposed to mimic the dichlorophenyl and amide carbonyl of typical arylacetamides.[5] If such a hypothesis is correct, MPCB might represent an ideal



MPCB

Figure 7. Structure of the benzomorphan MPCB. The atom numbering scheme allows definition of torsion angles τ_1−τ_4 reported in Table 4.

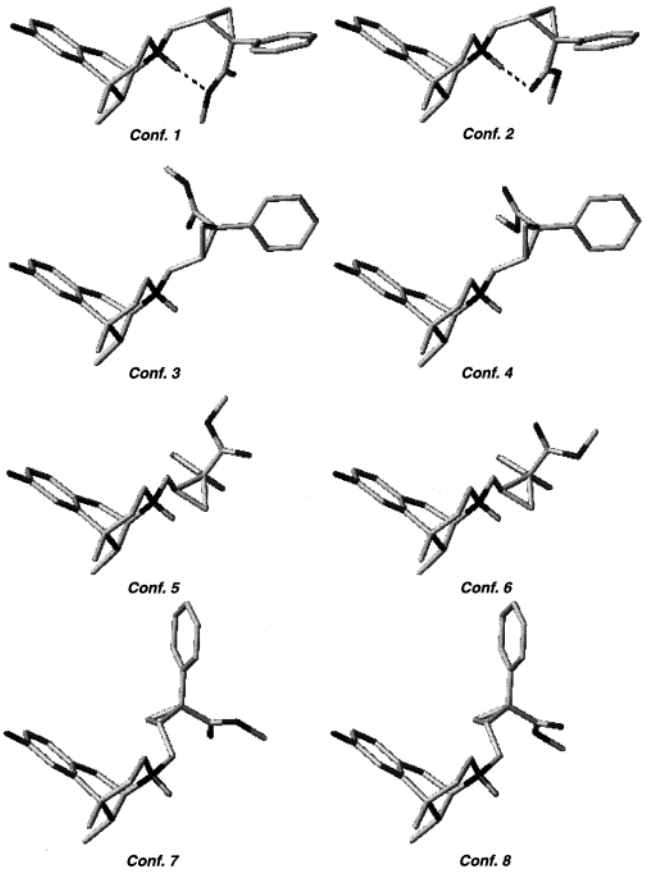


Figure 8. Conformers of MPCB identified by systematic conformational search.

link between benzomorphans and arylacetamides in the search of a mutual alignment of these two classes of ligands. It can be reasonably assumed that KCZ, EKC, and bremazocine (Chart 1) dock into the κ-receptor binding site similarly to MPCB as far as their common structural skeleton is concerned.

We had to select candidate bioactive conformations of MPCB according to criteria different from those relying on the pharmacophore concept since the active analogue approach cannot be applied to the benzomorphan derivatives shown in Chart 1 (their *N*-cyclopropylethyl chain is, conformationally speaking, an invariant feature). On the basis of these considerations, a set of energetically stable conformations, representative of the conformational chances of this molecule, was sought for docking calculations.

A systematic conformational analysis on MPCB indicated that eight possible orientations of the side chain on the nitrogen are energetically accessible for this compound (5 kcal/mol above the global minimum). Figure 8 shows these conformers, while Table 4 collects the values of their torsional angles and relative strain

Table 4. Main Torsion Angles[a] (deg) of Eight Representative Conformers of MPCB Derived by Systematic Conformational Search

conf	τ_1	τ_2	τ_3	τ_4	ΔE_{conf}[b]
1	−43	79	58	−138	0.0
2	−42	79	57	46	1.3
3	−48	−166	58	49	2.2
4	−48	−167	58	−141	1.8
5	71	−148	57	50	2.9
6	71	−149	59	−140	2.5
7	−168	−178	61	−81	4.0
8	−169	−177	60	106	4.6

[a] The torsion angles are defined as follows on the basis of the atom numbering scheme reported in Figure 7: $\tau_1 = \tau(1,2,3,4)$; $\tau_2 = \tau(2,3,4,6)$; $\tau_3 = \tau(5,6,7,8)$; $\tau_4 = \tau(4,6,9,10)$. [b] ΔE_{conf} is the strain energy, calculated with the TRIPOS force field as the difference in energy between the conformation and the global minimum conformer.

energies. The MPCB conformers can be grouped into two clusters: the first one (conformers 1 and 2) characterized by a folded arrangement of the flexible side chain wherein a hydrogen bond is formed between the ammonium and one of the ester oxygens; the second one (conformers 3−8) with the same side chain extending outward from the ammonium. The couples of conformers 1/2, 3/4, 5/6, and 7/8 differ only in the value of τ_4, the rotation of which implies an exchange of the positions of the ester oxygens. In all conformers the value of τ_3, controlling the orientation of the phenyl ring about the cyclopropane system, is locked in a gauche disposition. Differences in the value of τ_1 split the six extended conformations into couples 3/4 (*gauche*$^-$), 5/6 (*gauche*$^+$), and 7/8 (*trans*).

Indeed, each of the eight conformers so far discussed can give rise to a higher energy conformer by the swinging of τ_3 from ca. 40° to 60° to change the mutual disposition of the phenyl and cyclopropane rings from a "butterfly" to a skewed type (conformers not shown). However, these conformers were provisionally left out from the set of geometries submitted to docking, owing to the relatively high energetic cost (about 3 kcal/mol) associated with the skewed arrangement of the phenyl ring.

Conformers 1 and 2 were rejected from the set of the candidate bioactive conformations according to the same criteria adopted when analyzing the pharmacophore models of arylacetamides (i.e. the ammonium forms an intramolecular hydrogen bond which disables its interaction with the Asp138 carboxylate of the receptor). Docking calculations were therefore restricted to conformers 3−8.

Docking Calculations. Docking simulations were performed using the model of κ-receptor recently reported by Metzger and co-workers.[13] This model is unique in that secondary structures are taken from nonsequential alignments to the helical domains of bacteriorhodopsin,[44] thus allowing the conformational effects of the conserved prolines to be retained.

Calculations were performed using the automated DOCK 3.5 suite of programs,[45] following the same procedure described by Subramanian et al.[10] DOCK describes the receptor binding site as a cluster of spheres filling the binding cavity to conform to its shape. Docking is achieved by treating ligand and receptor as rigid entities and looking for matches between interatomic and intersphere distances. Several binding modes are generated, with each receiving a score dictated by the steric and electrostatic energies calculated by a molecular mechanics force field.[45] Although the force field scoring value is a useful descriptor of ligand receptor complementarity, the choice of the "best" docking model was ultimately dictated by its agreement with SARs and site-directed mutagenesis data.

Compound **6** was selected prioritarily as representative of arylacetamides for docking calculations because its phenolic hydroxyl is responsible for a 9-fold increase of affinity,[18] thus suggesting that it might form a hydrogen bond with the receptor. Compared with the other phenole derivative **4** included in the examined series of arylacetamides, **6** offered the advantage of being less flexible and therefore more suited for docking. Moreover, although **4** is more potent than **6**, the phenolic hydroxyl of the former has been reported to improve potency by only 2-fold,[16] which makes debatable its role as a hydrogen-bonding partner at the κ-receptor.

The putative pharmacophore-based conformation of compound **6** was directly docked. Out of the 1931 orientations obtained, 108 were within 10 kcal/mol of the best orientation based on the scoring function. Only 24 out of these 108 orientations were further considered, since they showed a salt bridge between the protonated nitrogen of the ligand and the carboxylate group of Asp138, in agreement with site-directed mutagenesis data.[32] From this cluster of structures, we selected one (the top scoring with a force field score of −35 kcal/mol) in which the phenolic moiety was engaged in a hydrogen bond with the His291 imidazole ring.

U-50,488 (**1**) and **4** (the archetypal and the most potent arylacetamide of the data set, respectively) were superimposed on the docked conformation of **6** by keeping the pharmacophore alignment shown in Figure 5.

Conformers 3−8 of MPCB (two with τ_1 in *gauche*$^-$, two with τ_1 in *gauche*$^+$, and two with τ_1 in *trans*), as obtained by the systematic conformational search, were considered for docking.[46] Inspection of the docked *gauche*$^+$ and *gauche*$^-$ conformers revealed that the best and many of the top scoring orientations placed the protonated nitrogen more than 7.5 Å away from the Asp138 carboxylate oxygens.

Docking of the trans conformer 7 generated 497 orientations. Out of these, 84 were within 10 kcal/mol of the best orientation. Among the top scoring orientations featuring a salt bridge with the Asp138 carboxylate, we extracted one (force field score −23 kcal/mol, 9 kcal/mol above the top scoring orientation) showing the phenolic hydroxyl of MPCB in vicinity of the His291 side chain. The trans conformer 8 led to significantly worse force field scores.

The trial complexes of **4**, **6**, and MPCB were refined by extensive energy minimization and molecular dynamics (MD) simulation cycles. Table 5 lists the amino acids in each TM helix within 5 Å from any atom of the ligand. Torsional angles and corresponding strain energies of the docked ligands are given in Table 6. Apart from the about 40° increment of τ_1 in U-50,488 and **6** as well as of τ_2 in **4**, the overall refinement procedure did not alter the starting geometries of the three ligands considerably.

Inspection of the theoretical models of compound **6** and MPCB bound to the κ-receptor, shown in Figure 9,

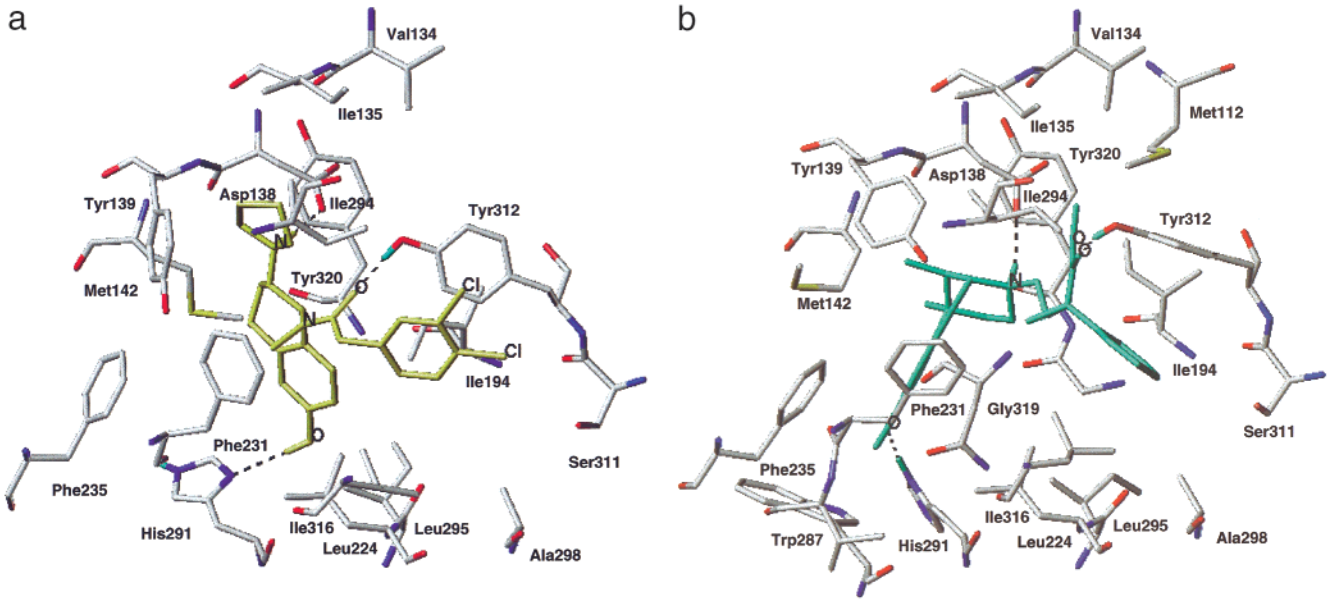


Figure 9. κ-Opioid receptor model with each of two docked ligands: arylacetamide **6** (a) and the benzomorphan MPCB (b). Only amino acids located within 5 Å from any atom of the bound ligand are displayed.

Table 5. Residues of the κ-Opioid Receptor Binding Site Located within 5 Å from Any Atom of the Docked Ligands (U-50,488, **4**, **6**, and MPCB)[a]

TM II	Met112
TM III	Val134, *Ile135*, Asp138, Tyr139, Asn141, Met142, Phe143
TM IV	*Ile191*, *Ile194*, Gly197, Gly198
TM V	*Leu224*, Lys227, Phe231, Phe235, *Val236*, Phe239
TM VI	Trp287, Hys291, *Ile294*, *Leu295*, *Glu297*, *Ala298*
TM VII	*Ser311*, *Tyr312*, Cys315, Ile316, Ala317, Gly319, Tyr320, Asn322

[a] κ-Receptor specific residues are italic.

Table 6. Main Torsion Angles[a] (deg) and Strain Energies of the Docked Conformations of Compounds U-50,488, **4**, **6**, and MPCB

compd	τ_1	τ_2	τ_3	τ_4	τ_5	τ_6	ΔE_{conf}[b]
U-50,488	101	154	−177	−129	68	−164	3.8
4	96	135	−175	−64	−58	−69	3.7
6	97	152	−171	−125	83	−167	3.4
MPCB	−171	−177	58	−81			5.5

[a] τ_1−τ_5 (U-50,488, **4**, and **6**) are defined in the legend of Table 3; τ_1−τ_4 (MPCB) are defined in the legend of Table 4. [b] ΔE_{conf} is the strain energy, calculated with the TRIPOS force field as the difference in energy between the conformation and the global minimum conformer.

enabled us to compare the following intermolecular interactions with experimental data reported in the literature.

(i) The protonated nitrogen of either arylacetamide and benzomorphan ligands forms a salt bridge with Asp138 carboxylate in TMIII, as suggested from site-directed mutagenesis data.[32,47,48]

(ii) A hydrogen bond occurs between the hydroxyl of Tyr312 (TMVII) and the carbonyl oxygen of arylacetamides and MPCB. This finding is in accordance with the pharmacophoric role that we assigned to the carbonyl group prompted by SAR data.[21,34−36] Moreover, the role of Tyr312 side chain as hydrogen-bond donor seems to be confirmed by the reduced affinity of arylacetamides toward the Tyr312/Ala mutant.[49]

(iii) The dichlorophenyl ring of **6** and the pendant phenyl ring of MPCB, although not exactly coincident in space, are both hosted in a hydrophobic pocket formed by Tyr312, Leu224 (TMV), Leu295 (TMVI), Ala298 (TMVI), Cys315 (TMVII), and Ile316 (TMVII). Ile316 and Cys315 are conserved among all the three opioid receptor subtypes, while the remaining residues are unique to the κ-receptor. It is likely that the hydrophobic character of this portion of the receptor and the specific interactions with Tyr312 at helix VII are important in conferring high κ-selectivity to arylacetamides and MPCB.

The phenyl ring of Tyr312 appears to be optimally oriented for a π-stacking interaction with the dichlorophenyl moiety of arylacetamides. The planes of the two aromatic rings are fairly parallel and separated by a distance of 4 Å. Such an interaction would be consistent with SARs of these compounds showing that lipophilic and electron-whithdrawing substituents at the *para* and/or *meta* positions of the benzene ring increase the affinity.[23,26,30] In fact, the electron-deficient halogenated rings would more favorably realize a π-stacking charge-transfer interaction with the electron rich ring of Tyr312. The electron-donating propensity of the Tyr312 might be further enhanced by the hydrogen bond that its phenolic hydroxyl engages with the carbonyl oxygen of arylacetamides.

(iv) The pyrrolidine ring of **6** is accommodated into a hydrophobic pocket of limited dimensions made up of side chains Ile135 (TMIII), Tyr139 (TMIII), and Ile194 (TMIV). An increased steric bulk on the pyrrolidine system has been reported to impact unfavorably on the binding affinity of arylacetamide derivatives.[18,23,24,26,30,31]

(v) The phenolic moiety of **6** lies just above a network of the aromatic side chains, including Trp287 (TMIV) and Phe235 (TMV), which are part of the conserved aromatic binding site "floor" as proposed by Metzger et al.[13] Similarly, the phenolic moiety of MPCB occupies the same locus in the κ-receptor, interposing between Phe235 and His291 (TMVI). The His291 imidazole ring contacts the hydroxy group of **6** and MPCB as hydrogen-bond acceptor (ϵ-tautomer) or donor (δ-tautomer), respectively. The importance of this histidine in EKC

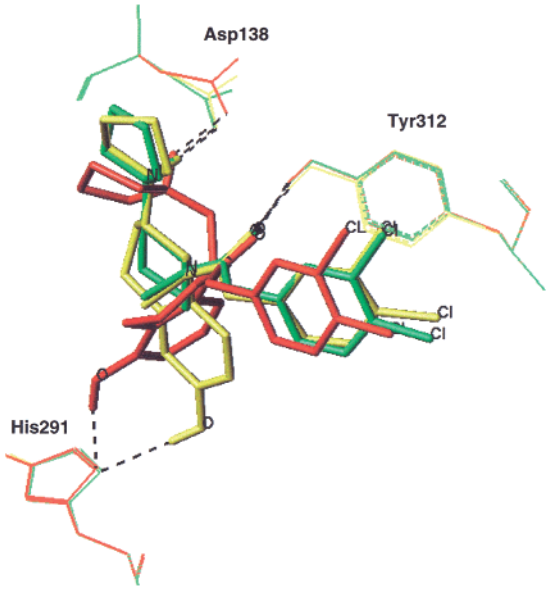


Figure 10. Overlay of the docked arylacetamides U-50,488 (green), **4** (red), and **6** (yellow). Only Asp138, Tyr312, and His291 residues are displayed (same color of the bound ligand).

binding has been underscored by site-directed mutagenesis experiments.[47,48]

Figure 10 shows an overlay of the docked conformations of U-50,488, **4**, and **6** together with a subset of κ-receptor amino acids more directly involved in the binding. It is interesting to note that the phenolic moieties of **4** and **6** are not coincident in space, although they occupy the same pocket and both donate a hydrogen bond to the His291 ϵ-nitrogen. Moreover, it can be appreciated that the phenolic hydroxyls of the two ligands lie out of the plane (compound **4**) or within the plane (compound **6**) of the His291 imidazole ring, thus suggesting that the different contribution of the phenolic oxygens to the binding affinity (9-[18] and 2-fold,[16] respectively) might be related to different geometries of these hypothetical hydrogen bonds.

Discussion

Theoretical models of κ-agonists binding have already been reported wherein receptor-bound conformations are deduced on the basis of energetic criteria (stability of conformations in vacuo and/or solution).[7−12] Some authors have superimposed ligands by matching the pharmacophoric elements without taking into account their interactions with the κ-receptor.[7−9] Others have performed docking studies using trial conformations of ligands as they were solved by X-ray crystallography or calculated as the most stable in vacuo or in solution.[9−12]

Higginbottom et al.[7] have speculated on the active conformation of chemically diverse κ-agonists in absence of the structure of the receptor, not yet cloned at that time. While details of the molecular conformations were not given in their paper, a visual inspection of the proposed alignment suggests that the bioactive conformation of the arylacetamide compounds is somewhat similar to ours. However, the relative orientation of benzomorphans and arylacetamides differs from that presented by us. Specifically, these authors matched the phenolic ring of KCZ with the dichlorophenyl ring of arylacetamides. Indeed, published SARs[23,26,30] clearly indicate that these two rings occupy well-distinct sites within the receptor. In the benzomorphan series, a hydroxy group on this ring is beneficial to affinity, whereas in the class of arylacetamides electron-withdrawing and lipophilic substituents on the pharmacophoric aromatic moiety improve potency.

Froimowitz et al.[8] proposed the receptor-bound conformation of U-50,488 and related analogues. A drawback with this model is that the pharmacophore alignment emphasizes the overlap of the ammonium nitrogens rather than the direction of the corresponding N−H vectors. Directionality of the ligand N−H should actually be a crucial parameter controlling the putative charge-reinforced hydrogen bond between the ligand ammonium and the receptor Asp138 carboxylate. Froimowitz and co-workers modeled several arylacetamides in a conformation wherein the N−H fragment points to the interior of the ligand itself, a type of arrangement which we have supposed incompatible with the intermolecular ligand N−H/receptor Asp138 carboxylate salt bridge. Additionally, these authors aligned U-50,488 and ketazocine by fitting their aromatic rings similarly to what was done by Higginbottom et al.[7] The shortcoming of such a superimposition has been discussed above.

Brandt et al.[9] proposed, 4 years later, bioactive conformations of arylacetamides similar to those published by Froimowitz's group.[8]

Recently, Subramanian et al.,[10] Pogozheva et al.,[11] and Cappelli et al.[12] developed, independently, theoretical models for ligand−receptor interactions of arylacetamides. Docking calculations of these ligands performed by Subramanian[10] and Pogozheva[11] led to geometries of the ammonium/Asp138 carboxylate salt bridge significantly divergent from well-documented experimental patterns,[50] as detailed below.

The strength of a salt bridge depends on at least two geometric parameters: (i) the distance between the donor (D) and the hydrogen (H) of the acceptor (A); (ii) the angle A···H−D. Various studies[50] have shown that the optimum value for the A···H distance is 1.8−2.0 Å, the optimum value for the A···H−D angle is around 180°.

In our model of U-50,488, **4**, and **6** docked into the κ-receptor, the A···H distance and A···H−D angle are within 1.8−2.0 Å and 145−177°. In the other two models (Mosberg and Subramanian), the same salt bridge is characterized by a A···H distance of 5.9 and 4.2 Å, respectively (the value of the A···H−D angle is not reported in either of the two papers).

Unfortunately, no details of the molecular conformation and docking model were given in the paper by Cappelli et al.[12]

Different hypotheses have been formulated to rationalize SARs pointing out the contribution of the carbonyl group of arylacetamides to the binding affinity. Cappelli et al.[12] suggested that this carbonyl oxygen accepts a hydrogen bond from the imidazole ring of His291, whereas Pogozheva et al.[11] proposed, in this regard, Tyr139 (TMIII) as a hydrogen-bond donor. In contrast, Subramanian et al.[10] excluded any involvement of His291 in the binding of arylacetamides. In this latter model, it appears that, except for some of the

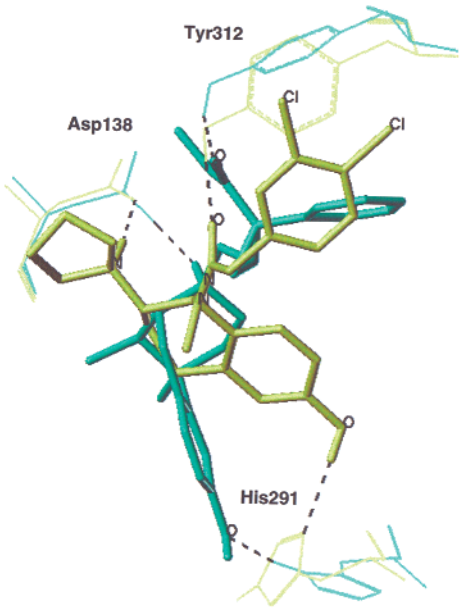


Figure 11. Overlay of the docked ligands **6** (yellow) and MPCB (cyan). Only Asp138, Tyr312, and His291 residues are displayed (same color of the bound ligand).

examined arylacetamide ligands, the carbonyl group does not make well-defined hydrogen bonds. Overall, these three ligand−receptor models[10−12] yield bound conformations of arylacetamide ligands different from that proposed by us.

Our model revealed that the Tyr312 hydroxyl is able to donate a hydrogen bond to the carbonyl group of both arylacetamides and MPCB, as well as to give rise to a π-stacking interaction with the dichlorophenyl ring of arylacetamides. None of the above-mentioned studies[10−12] reported the involvement of the Tyr312 side chain in the binding of κ-agonists.

Any model developed by superimposing structures of isolated ligands (pharmacophore mapping) or by docking a ligand conformation selected merely on energetic criteria (stability in vacuo, in solution or at the solid state) relies on working hypotheses typical of each of these approaches. In particular, the former methods generally assume that all the considered ligands can be overlapped about common pharmacophoric elements. Owing to the flexibility of target proteins,[51] the same receptor subsite (e.g. a side chain) may interact with nonoverlapping "equivalent" pharmacophoric groups of different ligands. Moreover, the possibility of alternative binding modes for similar molecules is hardly taken into account when trying to build up a pharmacophore model.[52,53]

It is worth noting that in our docking model the arylacetamide **6** and the benzomorphan MPCB do not overlap precisely about the protonated nitrogens, the carbonyl oxygens, and the phenolic hydroxyls. Nevertheless, each of these pharmacophoric functions engages the same receptor amino acid side chain, conformationally tailored to the docked ligand. Figure 11 shows what described above as an overlay of the receptor-bound ligands and selected residues of the receptor binding site (Asp138, His291, and Tyr312).

Results of docking methods are heavily sensitive to the quality of the scoring functions (knowledge-based rules or force field calculations), the allowed degrees of torsional freedom (one or both partners of the complex can be treated as rigid or flexible entities), and the algorithm employed to find candidate orientations (systematic, deterministic, or stochastic).[54]

A combination of pharmacophore modeling and docking procedures, such as that described in this paper, should reduce the undeterminateness of the system and allow the limitations of one approach to be compensated by the strengths of the other, thus leading to a more reliable solution than those obtained by each method separately. However, the agreement between the model and all the available experimental data is the ultimate criterion through which the reliability of the model itself can be judged.

Conclusions

Using molecular modeling methods, we have deduced the receptor-bound conformations and the mutual alignment of various arylacetamides analogues of U-50,488 and the benzomorphan MPCB binding selectively to the κ-opioid receptor.

Docking calculations into a 3D model of κ-receptor[13] revealed that the Asp138 carboxylate forms a salt bridge with the protonated nitrogen of both classes of ligands. The Tyr312 side chain is involved in a π-stacking interaction with the dichlorophenyl ring of arylacetamides and in a hydrogen bond with the carbonyl oxygen of both ligands. In agreement with SARs data,[23,26,30] our model shows that the dichlorophenyl ring of arylacetamides and the phenolic moiety of the benzomorphan MPCB occupy distinct sites within the receptor. A hydrophobic pocket, consisting of Tyr312, Leu224, Leu295, and Ala298 side chains, hosts the diclorophenyl ring of arylacetamides and the pendant phenyl ring of MPCB. Finally, the His291 imidazole ring makes a hydrogen bond with the phenolic hydroxyl of benzomorphans and, if present, of arylacetamides.

Experimental Section

Derivation of a Pharmacophore Model for Arylacetamide κ-Agonists. Construction of molecular models, geometry optimization, conformational search, and molecular superimposition were performed using the molecular modeling software package SYBYL[55] running on a Silicon Graphics R10000 workstation. Ligands were modeled in their nitrogen-protonated form.

The crystal structure of U-50,488 was retrieved from the April 1997 release (3D graphics 5.13 version for UNIX platforms) of the Cambridge Structural Database (CSD)[43] (refcode JEDXIZ). Analogues of U-50,488 were built by modifying this basic structure.

Geometries of the ligands were optimized with the standard TRIPOS force field[40] in which the electrostatic contribution to the total energy was completely disregarded.[56] The BFGS (Broyden, Fletcher, Goldfarb, and Shanno) algorithm[57] was used for geometry optimizations, setting a root-mean-square gradient of the forces acting on each atom of 0.05 kcal/mol Å as the convergence criterion.

Pharmacophore-consistent conformations of the structures reported in Figure 1 were identified with the SYBYL/SEARCH routine based on Marshall's active analogue approach.[41]

With reference to Figure 2, the rotatable bonds defined by τ_4 and τ_6 were generally scanned with 20° increments (10° increments for the aliphatic ring bond τ_5) within a 0−340° interval. The amidic rotatable bond defined by τ_3 was scanned with 180° increments. A 0.75 van der Waals scaling factor was used to "soften" steric contacts in the rigid rotamers. A 10 kcal/

mol energy window (energy difference between the generated conformation and the current minimum) was set to reduce the number of conformations to be examined.

Input conformations, all overlapping about the aryl ring and the carbonyl group, were placed in a box defined by eight corners (dummy atoms Du1-Du8). The box was built following an automatic procedure, featured by the SYBYL/CoMFA routine, which ensures that the box walls extend beyond the dimensions of each structure by 4 Å in all directions. Distances O_{co2}−Du*X* (where *X* varies from one to eight) were recorded in each search run at 0.7 Å resolution (see Figure 3). The distance map of the more rigid compound was utilized to constrain the conformational search performed on the more flexible molecule.

The output conformations associated with a specific pharmacophoric pattern were clustered in the grid space of O_{co2}−Du*X* distances into a smaller number of families using the FAMILY option of the SYBYL/SPREADSHEET routine. The lowest energy conformation from each family was selected for further calculations. The match of pharmacophoric points was improved by using the SYBYL/MULTIFIT command, setting spring force constants of 20 kcal/mol Å^2. After each MULTIFIT run, the molecules were fully relaxed to their nearest local minimum conformer and rigidly fitted on the structure of U-50,488 about the pharmacophoric points. Molecular volume manipulations were performed using the SYBYL/MVOLUME command.

Conformational Analysis of MPCB. A systematic conformational search was carried out on the structure of MPCB shown in Figure 7 aimed at identifying conformers to be docked into the available model of the κ-receptor.

A starting model of MPCB was obtained by modifying the crystal structure of cyclazocine, retrieved from the CSD[43] (refcode CYLAZE). This initial structure was minimized by the BFGS method[57] within the MAXIMIN2 option using the TRIPOS force field[40] with neglect of electrostatics.[56]

With reference to Figure 7, torsional angles τ_1, τ_2, and τ_4 were scanned with 20° increments in the range of 0−340°; the absolute interval of variation of τ_3, associated with the rotation of a phenyl ring, was restricted to 160°; the torsional angle about the ester bond was kept in a standard trans geometry. The van der Waals scaling factor was set to 0.75. A 10 kcal/mol energy window was applied to reduce the number of output conformations.

The resulting conformations were grouped into eight families according to the values of their torsional angles. The lowest energy conformation from each family was geometry optimized.

Docking Calculations. Partial atomic charges of the ligands were generated by restrained electrostatic potential (RESP) fitting[58] using the HF/6-31G* basis set in the GAUSSIAN94 program.[59]

The κ-opioid receptor model used for docking was that developed by Metzger et al.[13] A detailed description of the model, including the coordinates and site-directed mutagenesis data used in model building, is available on the Internet site at http://www.opioid.umn.edu.

The DOCK 3.5 program[44] was employed to dock candidate bioactive conformations of the analyzed ligands into the κ-receptor model.

Ligand−receptor complexes yielded by DOCK were refined by in vacuo energy minimization using a distance-dependent dielectric function of $\epsilon = 4r$ and tethering the backbone atoms through a force constant of 5 kcal/mol.

MD simulations on the complexes were performed using the AMBER 4.1 suite of programs[60] based on the Cornell force field.[61] Each simulation was run for 2 ns under the following settings: 1 fs time step; nonbonded pairlist updated every 25 fs; temperature kept at 300 K using the Beredensen algorithm;[62] 0.2 ps as the coupling constant; the protein backbone atoms constrained as done in the energy minimization step. Four snapshots, extracted each 250 ps from the last 1 ns MD simulation, were very similar one another in terms of root-mean-square deviation. An average structure was calculated from the last 1 ns trajectory and energy-minimized using the steepest descent and conjugate gradient methods available within the SANDER module of AMBER 4.1 as specified above.

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