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Steric interactions and the activity of fentanyl analogs at the μ-opioid receptor

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Abstract—Fentanyl is a highly potent and clinically widely used narcotic analgesic. The synthesis of its analogs remains a challenge in the attempt to develop highly selective μ-opioid receptor agonists with specific pharmacological properties. In this paper, the use of flexible molecular docking in a study of the formation of complexes between a series of active fentanyl analogs and the μ-opioid receptor is described. The optimal position and orientation of fourteen fentanyl analogs in the binding pocket of the μ-receptor were determined. The major receptor amino acids and the ligand functional groups participating in the complex formation were identified. Stereochemical effects on the potency and binding are explained. The proposed model of ligand–receptor binding is in agreement with point mutation experiments explaining the role of the amino acids: Asp147, Tyr148, Asn230, His297, Trp318, His319, Cys321, and Tyr326 in the complex formation. In addition, the following amino acids were identified as being important for ligand binding or receptor activation: Ile322, Gly325, Val300, Met203, Leu200, Val143, and Ile144.

1. Introduction

The μ-opioid receptor is the primary site of action in the brain for opioid neuropeptides as well as opiate drugs. It is a member of the seven trans-membrane (TM) domain, G protein-coupled (GPCR) receptor superfamily, Figure 1, which is believed to share a common topology and a common mechanism of action. One hypothesis[1b,k] proposes that an agonist binding to G protein-coupled receptors promotes a conformational change which leads to the formation of the activated receptor state. Another hypothesis[2] suggests that a rigid body movement of helices relative to one another is the key step in receptor activation. However, the character of these changes, which link agonist binding and G protein coupling and activation, is not known. It is the subject of intensive modeling[1] and experimental studies including receptor cloning, site-directed mutagenesis, and affinity labeling studies.[3]

Fentanyl is a highly selective μ-opioid agonist[4] with specific pharmacological properties.[5] Substitution on the piperidine ring of fentanyl was shown to dramatically affect the binding and activity of the ligand, Table 1. Substitution by an acetate,[6] or methoxymethyl[7] group at the piperidine 4-axial position, greatly increased the ligand binding and potency, while 4-methyl substitution[8] affected the potency to a lesser extent. Substitution of the *N*-phenethyl group generally prevented ligand binding and rendered the ligand impotent.[9] Fentanyl also displays great variation in enantiospecific binding and potency. The two enantiomers of *cis*-3-methylfentanyl exhibit a great variation in in vivo potency and binding,[1e,10] one being ca. twenty times more potent than fentanyl, and the other ca. five times less potent, Table 1. This is even more pronounced in the case of 2′-hydroxy-3-methylfentanyl,[11] where two of the eight stereoisomers belong to the 'super potent' class of μ-selective agonists (2′*R*,3*R*,4*S* and 2′*S*,3*R*,4*S*), while their antipodes are less potent than fentanyl and are only weakly bound to the receptor. On the other hand, both diastereoisomers of 3-carbomethoxy fentanyl exhibited in vivo potency weaker than fentanyl itself.[12]

On comparison of fentanyl with classical μ-opioids such as morphine, certain structural similarities may be noticed, that is, a protonated nitrogen and an aromatic

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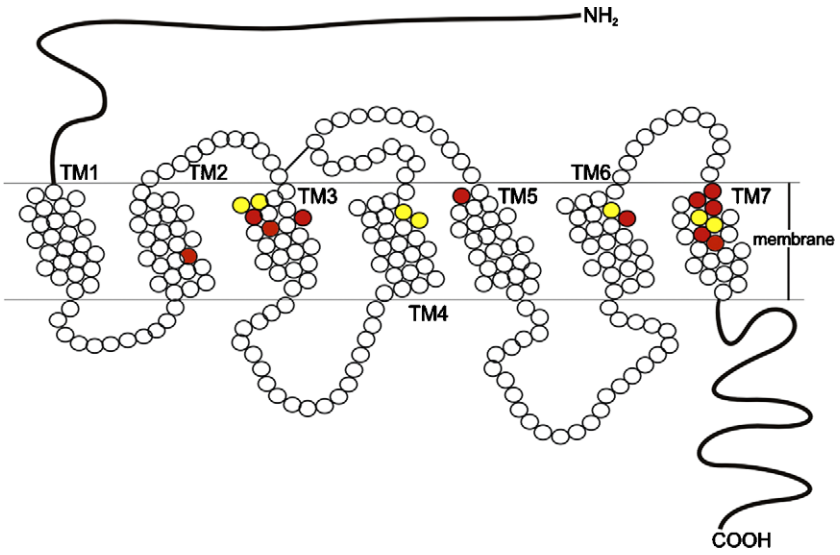


Figure 1. Model of the μ-opioid receptor. Important amino acids are in red (mutagenesis experiments) and in yellow (docking, this study).

Table 1. Experimental potencies (relative to fentanyl) and binding constants (nM) of **1**–**14** for the μ-opioid receptor

Compound	Potency	K_i (exp)
1	1	3.97[d]
2	0.5[b], 7[i]	nd
3	30[b]	0.024[d]
4	3.8[a]	nd
5	19[c]	0.02[d,f]
6	0.16[c]	30.6[f]
7	3.3[c]	nd
8	0.8[c]	nd
9	0.47[e]	nd
10	0.13[e]	nd
11	30[g], 10[f]	0.66[h], 0.005[g]
12	130[g], 140–350[f]	0.55[h], 0.004[g]
13	Inactive[g], 0.2[f]	124[h], 5.85[g]
14	Inactive[g], inactive[f]	59.2[h], 2.89[g]

nd, not determined.
[a] Ref. 8.
[b] Ref. 14.
[c] Refs. 10 and 4a.
[d] Ref. 1e, and references therein.
[e] Ref. 12.
[f] Ref. 15.
[g] Ref. 11a.
[h] Ref. 11b.
[i] Ref. 20.

group. However, SAR studies[13] have suggested that fentanyl and morphine may exhibit qualitatively similar pharmacological profiles but through different mechanisms.

All this makes fentanyl analogs interesting probes for ascertaining the stereochemical requirements for ligand–receptor binding and for testing the mechanisms of receptor activation, in both experimental and modeling studies.

Experimental studies on fentanyl analogs and their interactions with μ-receptor include: site-directed mutagenesis,[16] studies of chimeric receptors,[17] and affinity labeling experiments.[18] Chimeric receptor studies[17] revealed that the major determinant for binding of μ-selective alkaloids exists in the region spanning the transmembrane segments TM5 to TM7. It was demonstrated[3c] using μ/κ chimeras that TM6 and TM7, as well as a third extracellular (E3) loop of μ-receptor, were important for the binding of agonists, such as morphine and fentanyl analogs, while the first extracellular loop (E1) is not important for μ-selective nonpeptide ligands. Site-directed mutagenesis studies of the μ-receptor indicated that Asp147 is the primary binding site, as the counterion for the protonated nitrogen of the opioid ligands. The importance of charged residues in TM2 (Asp 114), TM3 (Asp147), and TM6 (His297) has been demonstrated,[16] as has the modest involvement of N- and C-terminal domains in the ligand–receptor interactions. However, it was later[19] proven that the N-terminal domain of the μ-opioid receptor may be important for the binding of some alkaloid agents, such as fentanyl and methadone, and for some peptide agonists. Furthermore, site-directed mutagenesis studies[13c] established the importance of the TM7 residue Tyr326, especially for the fentanyl class of opiate ligands, while mutation of Asn150 to Ala produced only a slight increase in ligand binding. Additional site-directed mutagenesis studies[13c,21] stressed the importance of Trp318 and His319 in TM7 and Tyr148 in TM3 for the activation of μ-opioid receptor by fentanyl analogs, and the importance[22] of the boundary regions between the sixth and the seventh transmembrane domains, and the third extracellular loop, for the distinction between μ- and κ-opioid receptors. The Cys321 residue in TM7 of the μ-opioid receptor was found[23] to participate in ligand binding. In line with these studies, it was established[24] that Ser196 of TM4 is important not for ligand binding but, most likely, for maintaining the receptor conformation. It was also established by mutation experiments[25a] that Asn230 of TM5 is involved in the binding of morphine and, to a much lesser extent, of fentanyl. Binding of opioid peptides, on the other hand, was unaffected by mutation of Asn230 to Thr or Leu. Site-directed mutagenesis

experiments[25b] also suggested hydrophobic interactions of Ser329 (TM7) with the *N*-phenethyl group of the fentanyl molecule. The third intracellular loop was found[26] to be involved in activation of the μ-opioid receptor. Its interaction with G-proteins was found to be agonist-selective. In addition, as a result of some mutagenesis studies,[13b,27,28] it was suggested that the interaction of morphine with the μ-receptor during activation is different to that of fentanyl analogs.

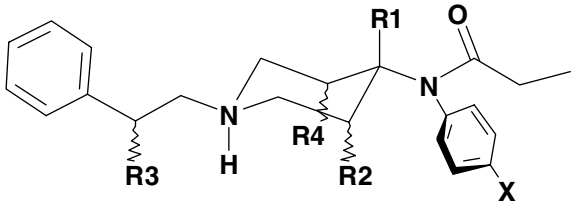
The GPCRs of pharmaceutical interest, including the μ-opioid receptor, are membrane-bound proteins but their 3D structures are unknown at present. Therefore, in order to gain a detailed insight into the key interactions between the ligand and the receptor, molecular models based on bacteriorhodopsin or rhodopsin templates of the various GPCRs have been developed.[1a,b,c] Despite inherent difficulties in the modeling of opioid receptors at the molecular level, several models of μ-opioid receptor are available.[1e,j,30,31] However, there are very few systematic studies of receptor–ligand interactions of fentanyl class of opiates. The existing two studies[1e,30] used 'manual docking' to a predefined binding cavity[30] or rigid ligand docking.[1e] The resulting bound conformation and orientation of *cis*-3-methylfentanyl were different in these two studies.

In this study, a representative series of fentanyl analogs, Table 2, was flexibly docked to a model of the human μ-opioid receptor. The goal was to evaluate the binding orientations, and conformations of fentanyl and its analogs, and to study the effects of ligand stereochemistry on the formation of the receptor–ligand complex. The results are compared with the available experimental data in order to identify the key ligand–receptor interactions involved in the molecular recognition process. A possible mechanism of μ-opioid receptor activation is discussed.

2. Computational methods

All computations were performed using a Pentium IV computer. The μ-receptor model used in this study was the one suggested by Ferguson and co-workers[1e] and kindly made available through www.opiod.unm.edu. The rigid receptor model was used. The automated flexible ligand docking experiments were made with the AutoDock 3.0.5. program.[32] The starting geometries of the neutral ligands were taken from previous studies.[33a,b] These geometries satisfy the suggested fentanyl pharmacophore[33a] by having the piperidine ring in the chair conformation, the *N*-phenethyl and *N*-phenylpropanamide substituents both equatorial, with the anilido phenyl α-oriented. The amide bond had the *trans* configuration and the *N*-phenethyl substituent adopted an extended conformation, Table 2. Based on the pK_a values of several fentanyl derivatives,[34] these starting

Table 2. The fentanyl analogs studied



	Compound	R1	R2	R3	R4	X
1	Fentanyl	H	H	H	H	H
2	4-Fluorofentanyl	H	H	H	H	F
3	Carfentanil	$COOCH_3$	H	H	H	H
4	4-Methylfentanyl	CH_3	H	H	H	H
	cis-3-Methylfentanyl					
5	3*R*,4*S*	H	ax-CH_3	H	H	H
6	3*S*,4*R*	H	H	H	ax-CH_3	H
	trans-3-Methylfentanyl					
7	3*S*,4*S*	H	eq-CH_3	H	H	H
8	3*R*,4*R*	H	H	H	eq-CH_3	H
9	*cis*-3-Carbomethoxyfentanyl	H	ax-$COOCH_3$	H	H	H
		H	H	H	ax-$COOCH_3$	H
10	*trans*-3-Carbomethoxyfentanyl	H	eq-$COOCH_3$	H	H	H
		H	H	H	eq-$COOCH_3$	H
	2′-Hydroxy-3-methylfentanyl					
11	2′*R*,3*R*,4*S*	H	ax-CH_3	OH	H	H
12	2′*S*,3*R*,4*S*	H	ax-CH_3	OH	H	H
13	2′*R*,3*S*,4*R*	H	H	OH	ax-CH_3	H
14	2′*S*,3*S*,4*R*	H	H	OH	ax-CH_3	H

geometries were protonated and the protonated geometries of **1**–**14** were optimized using the semiempirical AM1 method of the HyperChem program.[35] The ab initio like partial charges, calculated by the Vcharge program,[36] were assigned to the ligand atoms. The $60 \times 60 \times 60$ grid was centered on one of the Asp147 oxygen atoms and the Lamarckian genetic algorithm (LGA) was used in all docking calculations. The docking process was performed in two steps. In the first short step, consisting of 200 LGA runs, the initial position of the ligand was random. The population was 50, the maximum number of generations was 27,000, and the maximum number of energy evaluations was limited to 250,000. The best ligand orientation in the first step, based on the score criteria, was used as the input position for the second docking step, where the number of energy evaluations was 2.5×10^6. The second step provided the most probable ligand geometries and orientations in the binding pocket. The resultant ligand orientations and conformations were scored based on the docking and binding energies (the cutoff value for the energies was 2 kcal/mol) and on the distance of Asp147 to the protonated nitrogen of a ligand (the cutoff value for the distance was 4.0 Å). Site-directed mutagenesis studies have shown[37] that Asp147 to Ala/Asn or Glu point mutations lead to diminished binding affinities, presumably due to the loss of a salt bridge or an electrostatic interaction between the negatively charged Asp147 and the protonated nitrogen of the ligand.

3. Results and discussion

Automated docking of compounds **1**–**14** to the TM domain of the μ-opioid receptor resulted in several plausible docking orientations and conformations for each ligand. The resulting ligand orientations and conformations were scored based on the docking and binding energies and the distance between Asp147 and the protonated nitrogen of the ligand. Only a few met these criteria and they were further evaluated based on experimental results indicating the important amino acids constituting the ligand binding site within the receptor. All the selected ('best') ligand orientations have low energies (within 2 kcal/mol above the minimum energy orientation). Hence, stable complexes are formed between the receptor and a ligand in the selected orientation. However, the correlation between the calculated binding energies and the experimentally determined binding affinities was poor. Also, the calculated binding energies were consistently higher than the experimentally determined binding affinities. This problem may be due to uncertainties in the side-chain conformations of the receptor model,[1e] as well as to the fact that possible conformational rearrangements of the receptor upon ligand binding were neglected.[32]

The best fentanyl (**1**) docking orientation positions the piperidine ring vertically in the region between the transmembrane helices TM3 and TM7. The protonated nitrogen is close to Asp147 of TM3 ($^{+}NH\cdots O^{-}$ distance is 3.38 Å). The *N*-phenylpropanamide group is oriented toward the extracellular side of the cavity, while the *N*-phenethyl group is not extended but adopts a *gauche* conformation placing the phenyl group between TM6 and TM7, Figure 2a. The only other fentanyl orientation which meets the energy and distance criteria ($HN^{+}\cdots O^{-}$ distance 3.80 Å) is the one in which the fentanyl molecule is positioned horizontally across the region between TM3,TM4,TM5, and TM7, at the extracellular edge of the transmembrane domain, Figure 2b. However, the vertically aligned structure is not only slightly lower in energy but also complies better with the experimental data. In addition, the salt bridge formed between Asp147 and the *N*-phenethyl group is oriented so that its phenyl group is close to His297 in TM6 and may have a strong donor–acceptor interaction with the imidazole ring. These two ligand–receptor interactions may be very important for μ-receptor activation by fentanyl and its analogs. Electron paramagnetic resonance spectroscopy on rhodopsin[2] suggested, for instance, rigid body movement of helices TM3 and

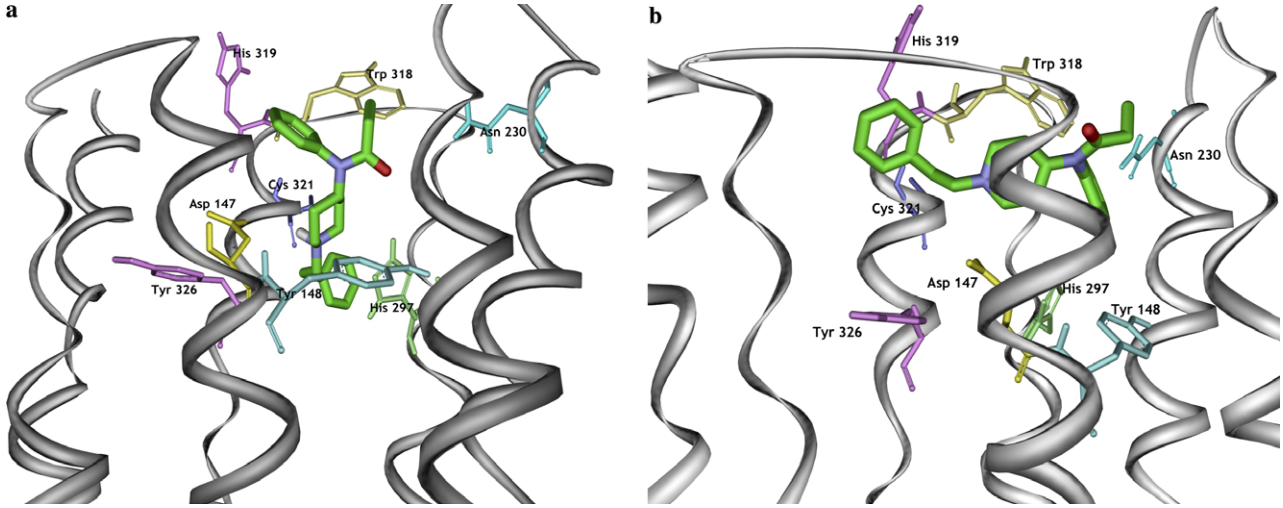


Figure 2. (a) The best orientation of (1) in the binding pocket. C-atom green, N-blue, O-red, and H-white. (b) An alternative, less stable, orientation of (1) in the binding pocket.

TM6 relative to each other, upon light activation. On the other hand, the *N*-phenylpropanamide group is close to Trp318 and His319 of TM7, and oriented in such a way that the ethyl group may have non-polar (van der Waals) interactions with the aromatic part of Trp318, while the phenyl group is oriented toward His319. The Tyr148 of TM3 is close to the hydrocarbon part of the piperidine ring, and the Tyr326 of TM7 is close to the $–CH_2–CH_2–$ bridge of the *N*-phenethyl group. Moreover, the strong effect of the mutation of Tyr326 to Phe on the binding of fentanyl[13c] suggests that, in addition to this nonpolar interaction with the *N*-phenethyl group of the ligand, the Tyr326 may play a role in the stabilization of receptor conformation. According to the receptor model used, Tyr326 (TM7) forms a hydrogen bond with Ala117 (TM2). Its mutation to Phe would prevent the formation of this hydrogen bond and, possibly, affect the conformation of the receptor in this region. The carbonyl group of Cys321 (TM7) is close to the *N*-phenethyl phenyl group of the ligand, suggesting that any change in the electron density of the phenyl may affect this interaction. Finally, Asn230 (TM5) is close to the alkyl group of the *N*-phenylpropanamide group of the ligand, which is in agreement with the experimental finding that mutation of Asn230 (TM5) to the more hydrophobic Thr or Leu increased the potency of the fentanyl, presumably by strengthening hydrophobic ligand–receptor interactions. Hence, this orientation of fentanyl was taken as the most probable, the 'best,' fentanyl alignment in the binding pocket of the μ-receptor, Figure 2a. The other amino acid residues, not selected for point mutation experiments, but important according to our docking studies are: hydrophobic Val300 (TM6) and Ile322 (TM7), as well as Gly325 (TM7) surrounding the *N*-phenethyl phenyl group of the ligand. The Ile322 (TM7) and Gly (TM7) together with Tyr326 (TM7) are also close to the $–CH_2CH_2–$ bridge of the *N*-phenethyl group. The protonated nitrogen, NH^+ group, in addition to being close to Asp147 (TM3), is also close to Ile322 (TM7). This amino acid, Ile322 (TM7), is also close to the other piperidine ring atoms and seems to be the major group responsible for the specific potency of *cis*-3-methylfentanyl. The ethyl group of the *N*-phenylpropanamide substituent of the ligand is in the pocket surrounded by Met203 (TM4), Leu200(TM4), Asn230 (TM5) and the aromatic ring of Trp318 (TM7). The phenyl ring of the *N*-phenylpropanamide substituent is surrounded by Trp318 (TM7) and His319 (TM7) on the one side, and Val143 (TM3) and Ile144 (TM3) on the other side.

All the other studied active analogs of fentanyl adopted very similar conformations and alignments in the binding pocket.

3.1. *p*-Fluorofentanyl (2)

The lowest energy *p*-fluorofentanyl-receptor complex has the *p*-fluorofentanyl molecule aligned so that it overlaps the 'best' fentanyl structure. The fluorine atom in the *para* position is oriented toward the region between Val143 (TM3) and His319 (TM7). The electrostatic interactions between the polar C–F bond and the positively charged His319 (TM7) may facilitate ligand binding and therefore explain the enhanced potency of *p*-fluorofentanyl relative to fentanyl and its sensitivity to His319 (TM7) mutation.[20]

3.2. Carfentanil (3)

Carfentanil is one of the most potent fentanyl analogs. Earlier pharmacophore modeling studies[33] suggested that the activity of fentanyl analogs is highly sensitive to the negative charge density in the region between the anilido oxygen and the C_4 substituent of the piperidine ring. The docking results support this suggestion. The 'best' carfentanil conformation overlaps the 'best' fentanyl conformation and positions the partially negative methoxy oxygen close to the protonated nitrogen of His297 ($O^-\cdots HN^+$ distance 3.9 Å). This interaction underlines the importance of the relative position of TM3 and TM6 for μ-receptor activation by fentanyl analogs.

3.3. 4-Methylfentanyl (4)

As with the other studied fentanyl analogs, 4-methylfentanyl has a number of possible orientations between the seven transmembrane helices. In the lowest energy ligand–receptor complex, the 4-methylfentanyl molecule is positioned in such a way that the protonated nitrogen of the piperidine ring was far from Asp147 (distance > 7.0 Å). The alternative ligand orientation, having a slightly higher energy, overlaps the 'best' fentanyl conformation, except that the piperidine ring is rotated relative to fentanyl by about 60 degrees, positioning the 4-methyl group in the hydrophobic pocket between Tyr148 (TM3), Ile144 (TM3), and Leu200 (TM4). In this orientation, the protonated nitrogen and Asp147 (TM3) have an acceptable distance ($O^-\cdots{}^+NH$ distance 4.2 Å) and additional hydrophobic interactions of the 4-methyl group may be related to the threefold increase in the potency of 4-methylfentanyl relative to fentanyl.

3.4. 3-Methylfentanyls (5–8)

Substitution at position 3 of the piperidine ring produced some of the most potent fentanyl analogs. The (3*R*,4*S*)-*cis*-3-methylfentanyl (**5**) (R2=ax-CH_3) is about twenty times more potent than fentanyl. However, the potencies of 3-alkylfentanyls are highly stereosensitive, hence, the (3*S*,4*S*)-*trans* isomer (**7**) (R2=eq-CH_3) is only three times more active than fentanyl, while the (3*S*,4*R*)-*cis*-3-methylfentanyl (**6**) (R4=ax-CH_3) and (3*R*,4*R*)-*trans*-3-methylfentanyl (**8**) (R4=eq-CH_3) are both less active than fentanyl. The potency is known to depend on the size of the alkyl group: 3-propyl- and 3-allyl-substitution leads to diminished activity,[14] suggesting the existence of a small hydrophobic pocket in the receptor. According to the present docking studies, the geometries of the three isomers of low potency; (**6**), (**7**), and (**8**) in the binding pocket are very similar and overlap the 'best' fentanyl orientation, while that of the most potent of the 3-methylfentanyls, (**5**), is different.

The molecule of (3*S*,4*S*)-*trans*-3-methylfentanyl (**7**) overlaps that of fentanyl and places the equatorial 3-methyl group (R2=eq-CH_3) toward the hydrophobic pocket surrounded by Trp318 (TM7), Ile322 (TM7), Ile301 (TM6), and Phe237 (TM5), that is, between the transmembrane helices TM5, TM6, and TM7. Ile 322 in TM7 seems to be the key residue for the discrimination between the stereoisomers of 3-methylfentanyl. Its location near the 3-H_{ax} (R2=H_{ax}) in the 'best' fentanyl orientation makes this orientation inaccessible to any analog with a voluminous substituent at the 3-ax (R2=ax-substituent) position, forcing the molecule to adopt a different orientation. Steric interactions have also been proposed[38] as being responsible for the selectivity of naltrexone-derived ligands against the three opioid receptors. The increased potency of the (3*S*,4*S*)-*trans* isomer (**7**), Figure 3, relative to fentanyl, is probably due to favorable hydrophobic interactions of the methyl group in the pocket.

The two least active 3-methylfentanyls, (**8**) and (**6**), overlap the 'best' fentanyl orientation but the 3-methyl-group (R4=ax-CH_3 or eq-CH_3) is oriented away from the hydrophobic pocket, and toward Asp147, where it has unfavorable steric interactions. This steric crowding is particularly unfavorable in (**6**), increasing its docking energy and may be responsible for its reduced potency, relative to other isomers. The most potent of the four stereoisomers is (**5**). Although it occupies the same binding pocket as the other three isomers, the position and orientation of the molecule are different, Figure 4. It is rotated and shifted relative to the other three isomers in order to relieve the steric interactions of the *axial* 3-methyl group (R2=ax-CH_3) and Ile322 (TM7) in the hydrophobic pocket between helices TM5, TM6, and TM7. While maintaining a good salt bridge ($HN^+\cdots O^-$ distance 3.73 Å), a molecule in this orientation connects TM3 and TM6 through the *N*-phenethyl group, which has favorable edge-to-face interactions with the imidazole ring in His297 (TM6). Simultaneously, the *N*-phenylpropanamide phenyl group is much closer to Trp318 (TM7) and His319 (TM7) than in the case of fentanyl itself. Considering the importance of the boundary region between TM6, TM7, and the third extracellular loop for both the activity and selectivity of opioid ligands, this proximity may explain the exceptional potency of this isomer of 3-methylfentanyl.

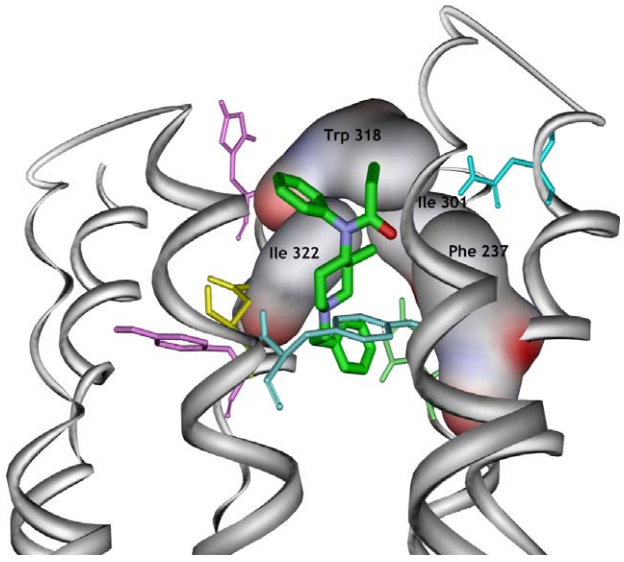


Figure 3. (3*S*,4*S*)-3-Methylfentanyl (**7**), with the 3-equatorial methyl group in the hydrophobic pocket.

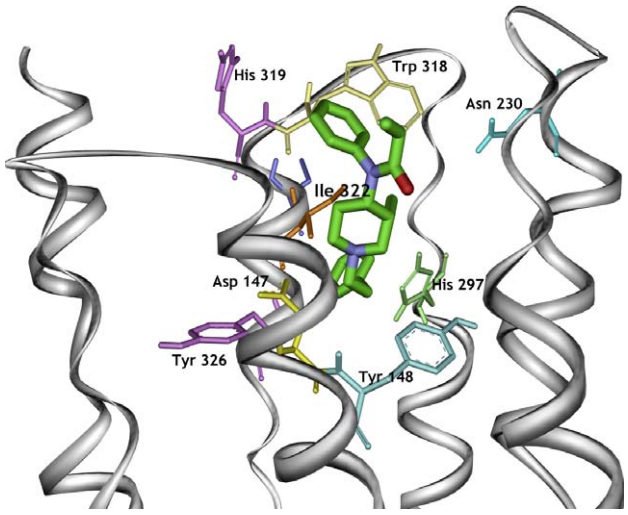


Figure 4. (3*R*,4*S*)-3-Methylfentanyl (**5**) in the binding pocket.

3.5. 3-Carbomethoxy fentanyl (9–10)

In order to test the hypothesis of a hydrophobic pocket accommodating the 3-alkyl group of 3-alkylfentanyls, the synthesis and pharmacological evaluation of 3-carbomethoxy fentanyl isomers (**9**) and (**10**) were performed.[12] Both stereoisomers, (**9**) and (**10**), were considerably less potent, Table 1, than their 3-methyl counterparts. In the present docking studies on (3*R*,4*S*)-**9** (R2=ax-$COOCH_3$) and (3*S*,4*S*)-**10** (R2=eq-$COOCH_3$) 3-carbomethoxy fentanyls, it was found that the best orientation of the (3*R*,4*S*)-**9** overlaps the 'best' orientation of (**5**). The polar carbomethoxy group occupies the hydrophobic pocket of the receptor, which may explain its reduced activity. It was found earlier[12] that the *trans*-3-carbomethoxy fentanyl is more conformationally flexible than the *cis* isomer, and its global minimum conformation has the anilido phenyl β-oriented, which is known to be an inactive conformation.[29] Its orientation in the binding pocket is equivalent to that of the less potent 3-methylfentanyl (**7**).

The other two stereoisomers (3*S*,4*R*)-**9** (R4=ax-$COOCH_3$) and (3*R*,4*R*)-**10** (R4=eq-$COOCH_3$) cannot adopt the 'best' fentanyl-like orientation at all, due to the unfavorable steric and electrostatic interactions of the 3-carbomethoxy group with Asp147 (TM3). This further diminishes the pharmacological potency of 3-carbomethoxy fentanyl.

3.6. 2′-Hydroxy-3-methylfentanyl (11–14)

The four stereoisomers (**11**–**14**) of the *N*-[1-(2-hydroxy-2-phenylethyl)-3-methyl-4-piperidinyl]-*N*-phenylpro-

panamide (2′-hydroxy-3-methylfentanyl) derived from *cis*-3-methylfentanyls (**5**) and (**6**) were studied here. The introduction of the 2′-hydroxy group has a pronounced effect on the potency. The (2′*R*,3*R*,4*S*) isomer (**11**) (R2=ax-CH_3, R3=2′*R*-OH) has reduced activity relative to (**5**), while the (2′*S*,3*R*,4*S*) isomer (**12**) (R2=ax-CH_3, R3=2′*S*-OH) has an activity considerably higher than that of (**5**). The docking studies revealed that both molecules have their best orientation similar to that of (**5**). However, the 2′-hydroxy-group is oriented in the opposite direction in the two isomers, Figure 5a. In (**12**), it is oriented toward Asp147, allowing an additional hydrogen bond to be formed ($OH\cdots O^-$ distance is 3.91 Å). Hence, the protonated nitrogen ($^+NH\cdots O^-$ distance 3.89 Å) and the 2′-hydroxy-group in (**12**) work cooperatively to establish a better association between the ligand and the receptor. However, the action of these two groups is opposed in (**11**). The 2′-hydroxy-group forms a hydrogen bond with His297 ($OH\cdots HN^+$ distance 3.47 Å), but at the expense of increasing the distance between Asp147 and the protonated nitrogen ($^+NH\cdots O^-$ distance 4.37 Å) of the ligand, thus weakening their interaction.

The other two isomers, the (2′*R*,3*S*,4*R*) isomer (**13**) (R4=ax-CH_3, R3=2′*R*-OH) and the (2′*S*,3*S*,4*R*) isomer (**14**) (R4=ax-CH_3, R3=2′*S*-OH), were expected to have low potency since they were derived from (**6**), a relatively barely active 3-methylfentanyl, with only 0.16 of the activity of fentanyl. According to the docking experiment the best orientation of (**13**) is similar to the best orientation of (**6**). The 2′*R*-hydroxy group is not so close to Asp147 ($OH\cdots O^-$ distance is 4.31 Å) to make an important contribution. On the other hand (**14**), although inactive, binds better to the μ-receptor than (**13**), Table 1. Its binding to the receptor, in the fentanyl-like orientation, is assisted by the 2′*S*-hydroxy group, Figure 5b.

However, the reduced activities of (**11**), (**13**), and (**14**), relative to the parent isomers of 3-methylfentanyl, are probably due to the existence of other low energy molecular orientations hydrogen bonded to Asn147 through the 2′-hydroxy group. The binding of some of the 2′-hydroxy-3-methylfentanyl isomers to the region between TM1, TM2, and TM3 was suggested earlier,[11b] based on experimental data. Contrary to (**12**), where the molecular orientations fitting the TM1, TM2, and TM3 region have binding and docking energies higher than the energies of the fentanyl-like orientation, the situation is opposite in the case of (**11**), (**13**), and (**14**), where these orientations (horizontal) dominate, presumably leading to inactive receptor–ligand complexes.

It has been suggested[2] that rigid body movement of helices TM3 and TM6 relative to each other is essential for rhodopsin activation. Also, random mutations of the human δ-opioid receptor,[39] demonstrated that a number of different mutations may activate GPCRs to various extents. Four groups of activating mutations have been identified,[39] one very close to the region of the binding pocket of fentanyl analogs in the μ-receptor. The movement of helices TM3, TM6, and TM7 during the activation process was suggested.[39] By analogy, the mechanism of μ-opioid receptor activation by fentanyl analogs may be described. The molecule slips into the binding pocket with the *N*-phenethyl group leading. On entering the binding pocket, the ligand binds to TM3 and TM6 causing the two helices to move relative to each other, while, at the same time, the *N*-phenylpropanamide group interacts with TM2, TM6, and TM7 amino acids near the extracellular side of the receptor, destabilizing the mutual interaction between TM6 and TM7, as well as with amino acids of the E3 extracellular loop.

However, the employed receptor model had neither extracellular or intracellular loops nor N- and C-terminal domains, hence their effects on ligand binding could not be modeled. Furthermore, some amino acid residues, known from point mutation studies to affect ligand binding of fentanyls, such as Asn150 (TM3)

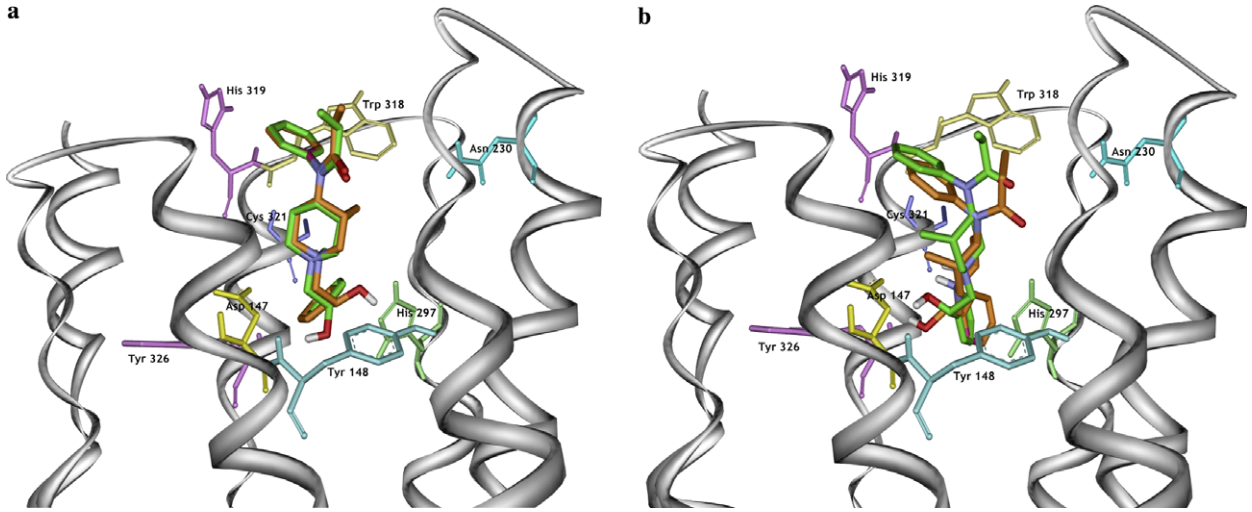


Figure 5. (a) The compounds (**12**) (C-atoms green) and (**11**) (C-atoms orange) in the binding pocket. (b) The compounds (**14**) (C-atoms green) and (**13**) (C-atoms orange) in the binding pocket.

and Ser329 (TM7), or binding of some other μ-selective alkaloids, such as Asp114 (TM2), are far from the binding pocket located in this study. These three amino acids are in close proximity to each other, suggesting the potential existence of the second binding pocket between helices TM2, TM3, and TM7, which is supported by chimeric receptor studies.[11b] It is also possible that mutation of Asn150 and Asp114, both forming hydrogen bonds connecting TM2 and TM3, makes the formation of these hydrogen bonds impossible, which affects the conformation of the receptor.

4. Conclusion

In the present study, an automated docking procedure was applied in order to determine the optimal position and orientation of the fourteen fentanyl analogs in the binding pocket of the μ-opioid receptor, and to identify the major amino acids and the major functional groups of the ligand which participate in the formation of a receptor–ligand complex. The quality of the models of the receptor–ligand complexes was estimated on the basis of their binding and docking energies, the distance between Asp147 (TM3) and the protonated amine nitrogen of the ligand, and the agreement with point-mutation experimental data.

It was shown that all the active compounds occupy the same binding pocket in the receptor, located near the extracellular region and between the transmembrane helices TM3 to TM7. The ligand molecule is parallel to the transmembrane helices, with the *N*-phenylpropanamide group pointing to the extracellular region, and the *N*-phenethyl group placed deep in the pocket. The *N*-phenethyl group is in the *gauche* conformation, positioning the phenyl group in the region between TM6 and TM7.

The model is in agreement with point mutation experiments and explains the role and importance of the following amino acids: Asp147 and Tyr148 in TM3, Asn230 in TM5, His297 in TM6, and Trp318, His319, Cys321, and Tyr326 in TM7 to the formation and stability of a complex. In addition, the following amino acids were predicted to be involved in ligand binding or activation, by the modeling studies described here: Ile322 and Gly325 in TM7, Val300 in TM6, Met203 and Leu200 inTM4, and Val143 and Ile144 in TM3. Steric interactions in the binding pocket have a major impact on the potency of fentanyl analogs.

We believe that the model proposed here satisfactorily explains the structure–activity relationships observed in the fentanyl class of opiates, as well as most of the available site-directed mutagenesis experiments. The major amino acids participating in the complex formation were identified and may be used to guide point mutations. The results related to the functional groups of the ligands and the type of their interactions with the receptor may suggest modifications which may lead to the discovery of new active analogs of fentanyl.

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