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SHORT COMMENTS

Exploring the structure of opioid receptors with homology modeling based on single and multiple templates and subsequent docking: A comparative study

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Abstract Opioid receptors are the principal targets for opioids, which have been used as analgesics for centuries. Opioid receptors belong to the rhodopsin family of G-protein coupled receptors (GPCRs). In the absence of crystal structures of opioid receptors, 3D homology models have been reported with bovine rhodopsin as a template, though the sequence homology is low. Recently, it has been reported that use of multiple templates results in a better model for a target having low sequence identity with a single template. With the objective of carrying out a comparative study on the structural quality of the 3D models based on single and multiple templates, the homology models for opioid receptors (mu, delta and kappa) were generated using bovine rhodopsin as single template and the recently deposited crystal structures of squid rhodopsin, turkey β-1 and human β-2 adrenoreceptors along with bovine rhodopsin as multiple templates. In this paper we report the results of comparison between the refined 3D models based on multiple sequence alignment (MSA) and models built with bovine rhodopsin as template, using validation programs PROCHECK, PROSA, Verify 3D, Molprobity and docking studies. The results indicate that homology models of mu and kappa with multiple templates are better than those built with only bovine rhodopsin as template, whereas, in many aspects, the homology model of delta opioid receptor with single template is better with respect to the model based on multiple templates. Three nonselective ligands were docked to both the models of mu, delta and kappa opioid receptors using GOLD 3.1. The results of docking complied well with the pharamacophore, reported for nonspecific opioid ligands. The comparison of docking results for models with multiple templates and those with single template have been discussed in detail. Three selective ligands for each receptor were also docked. As the crystallographic structures are not yet known, this comparison will help in choosing better homology models of opioid receptors for studying ligand receptor interactions to design new potent opioid antagonists.

Keywords Docking · Homology modeling · Multiple sequence alignment · Opioid antagonists · Opioid receptors

Introduction

Opioid receptors are expressed throughout the central nervous system (CNS) including the spinal cord. They are classified according to their ligand binding profile into three main types (mu, kappa and delta) [1]. These are characterized by seven transmembrane (TM) helices, with an extracellular (EC) amino-terminus and an intracellular carboxyl terminal end as present in all GPCRs. Opioid agonists are used as principle agents in treating pain. In addition to analgesia, the opioids generate a multitude of effects such as euphoria, sedation, depression, muscle rigidity and severe degrees of physical dependence or addiction [2]. Not all the ligands are equally efficacious in triggering opioid receptors. To design new anti-narcotics with better selectivity and reduced side effects, it is necessary to understand the receptor-ligand interactions more elaborately and for that it is necessary to have the 3D structure of receptors at atomic level. Since human opioid receptors are the principal targets of opiates and the crystal structures of

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opioid receptors are not available, we have to rely on homology modeled structures of opioid receptors for studying drug-receptor interactions and developing new ligands.

The basic first step in homology modeling is to align the sequence of the protein that has to be modeled (query protein) with sequences of homologous proteins of known structure (templates). The identity between these sequences is very important as it provides an indication of the reliability of the model. Earlier in the 1990s, the homology models of opioid receptors were constructed with bacteriorhodopsin as template [3]. Crystal structure of bovine rhodopsin was deposited in the late 1990s [4], since then bovine rhodopsin has been used as a template for modeling opioid receptors [5–7] and other GPCRs. Mu, delta and kappa possess 24%, 22%, 20% sequence identity, respectively, with bovine rhodopsin. Since accuracy of a homology model depends on sequence identity or strong similarity [8], rhodopsin based models are prone to errors. As the opioid receptors have less than 30 % identity with bovine rhodopsin, there always remains a scope for better model generation. With recent deposition of crystal structures of squid rhodopsin (2z73) [9], turkey beta-1 adrenoreceptor (2vt4) [10] and human beta-2 adrenoreceptor (2r4r) [11], more structures are available as templates for modeling opioid receptors. Recent studies by Mobarec et al. [12] states that when there is low sequence identity with the templates, using them in combination results in better homology model. So, an attempt was made to build homology models by combining both rhodopsins and adrenoreceptors as templates on the basis of multiple sequence alignment, so that number of gaps are reduced as much as possible and each residue gets aligned to the best possible similar residue. These new models were validated for accuracy, using a number of structure validation studies and compared with models, built with single template.

For further validation and to have deeper insight into the binding site of the opioid receptors, three non-selective ligands, butorphanol, naltrexone and naloxone were docked into the binding site of the modeled receptors, considering full ligand flexibility. It has been shown that nonspecific ligand recognition requires a protonated amine, two hydrophobic groups and a centroid of aromatic ring [13]. Three selective ligands naltrindole, cyprodime and guanidinonaltrindole were also docked. Docking was carried out to get clear insights of the key residues involved and their respective interactions.

Materials and methods

Computational tools

In this work the computational studies were performed by using the following software packages. Protein sequences of opioid receptors were obtained from UNIPROT. Homology modeling studies were carried out using Insight II/Homology [14] on SGI Fuel workstation, running on IRIX 6.5 operating system. Ligand preparation was done with Hyperchem 7.5 [15] and Cerius 2 v4.9 [16]. Docking studies were performed by using GOLD 3.1 (Genetic Optimization for Ligand Docking) [17], run on a Pentium 4 core2 Duo workstation using a Windows XP operating system. Accelrys DS Visualiser and Pymol [18] were used for generating figures.

Sequence alignment

The protein sequences for the opioid receptors (mu, delta and kappa) were obtained from the Swiss Prot database (accession numbers - P35372 (mu), P41143 (delta), and P41145 (kappa). For building homology models based on multiple templates, the template search was done with sequence search option of RCSB [19]. The recent deposition of crystallographic structures of human β-2 adrenergic receptors (PDB id: 2r4r and 2r4s), Turkey β-1 adrenergic receptor bound to cyanapindolol (PDB id: 2vt4) and squid rhodopsin (PDB id: 2z73) were proved to be better hits than the earlier used crystal structures of bovine rhodopsin (PDB id: 1f88) and bacteriorhodopsin for modeling of opioid receptors. The alignment of all the opioid receptors to a particular template (bovine or bacteriorhodopsin) resulted in some gaps. Additionally, a particular template did not give the best possible alignment with all the three opioid receptors individually, though all the three opioid receptors have high similarity. Hence, to retrieve the best possible alignment for each of the targets and reduce the gaps as much as possible, MSA was carried out with CLUSTALW using the templates found as top hits as a result of BLAST search. In CLUSTALW, the default alignment matrix GONNET 250 was used. The default parameter for gap open penalty was 10.2, gap extension penalty was 0.2 and gap distance was 4. For mu opioid receptor, MSA with 2r4r, 2vt4 and 1f88 proved to be the best alignment for the residues 72–358. For delta opioid receptor, MSA with 2vt4 and 1f88 proved to be the best alignment (Fig 1). For kappa opioid receptor, MSA with 2vt4 and 2z73 gave the best alignment. For building models based on single template, sequence of each of the target receptors was aligned to that of bovine rhodopsin and human β-2 adrenergic receptor using CLUSTALW.

3D model generation and validation

Based on the MSA derived with CLUSTALW, 3D models of the three opioid receptors were built using MODELLER of INSIGHT II package with default parameters. Ten models were generated initially. The model with best Kabs-Sanderasch score, also known as Verify 3D score, was chosen as the model for further studies. The Verify 3D score finds whether the given structure is compatible with the sequence. It is 3D-1D compatibility score. It calculates the

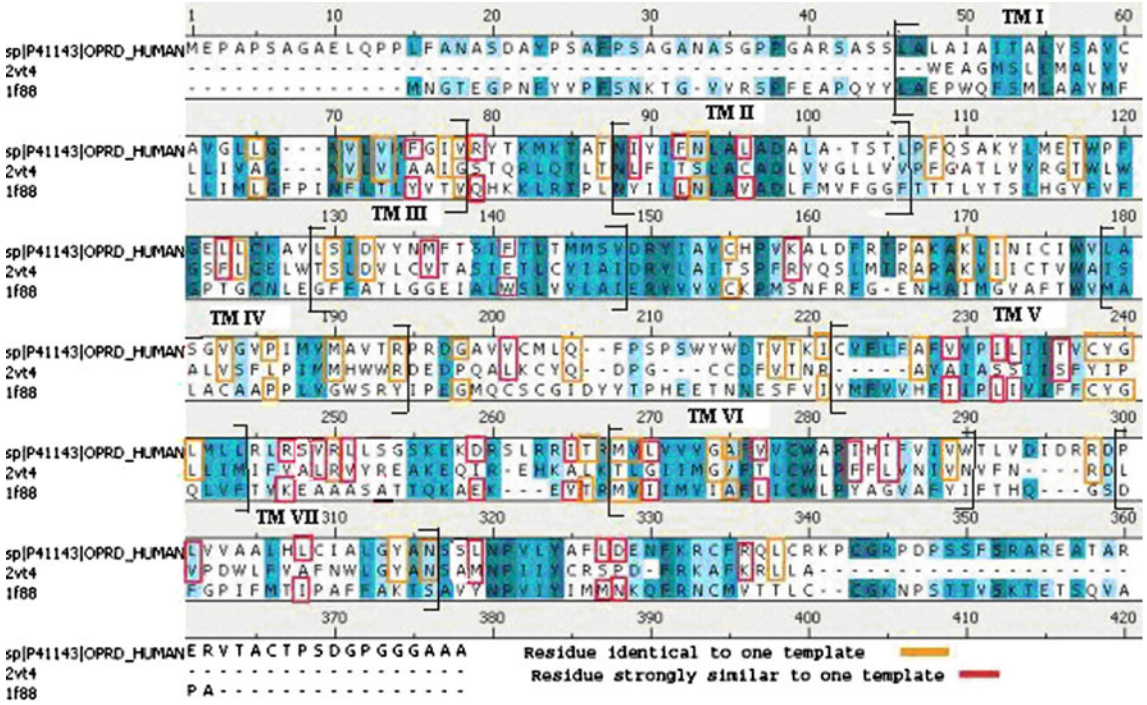


Fig. 1 MSA of delta opioid receptor with 2vt4 and 1f88 obtained from CLUSTALW. (Color code pattern: Dark blue - Identical with all templates, Medium blue - Conserved substituted residues all templates, Light blue - semi-conserved substituted all templates, Orange - Identical to one template, Red - Conserved substituted with one template). Alignment of only one receptor is shown for brevity

compatibility of each residue in a sequence with its predicted 3D environment. The 3D environment includes buried side chain area and side chain area that is exposed to polar atoms. The score is then normalized by the length of the sequence.

Models based on single template were built using alignment with single template. The refinement of models was done in subsequent steps. Hydrogens were added to the models at pH 7.4. Potentials of the modeled proteins were fixed with CVFF force field. Simple minimization was carried out with 100 steps of steepest descent and followed by 100 steps of conjugant gradient method with a gradient of 0.001 kcalmol^{-1} $Å^{-1}$.

Validation of the structural quality of the generated models was done using the programs: PROCHECK [20, 21], PROSA [22, 23], MOLPROBITY [24], secondary structure comparison [25, 26] and Verify 3D [27]. Stereochemical quality of backbone conformation was evaluated by PROCHECK analysis, PROSA uses knowledge based potentials of mean force to evaluate model accuracy and it shows local model quality by plotting energies as a function of amino acid sequence position. Secondary structure predictions for the opioid receptor sequences were done with consensus secondary structure prediction MLRC, while the secondary structures of the modeled proteins were determined by DSSP and a comparison was made. MolProbity score is a log-weighted combination of the clashscore, percentage Ramachandran not favored and percentage bad side-chain rotamers. The lower the score the better the structure. It also gives a percentile based on the score for each structure where 100th percentile is the best. Verify3D analyzes the compatibility of an atomic model (3D) with its own amino acid sequence (1D).

Docking of ligands

Three non-selective antagonists of opioid receptors, namely naltrexone, naloxone and butorphanol were selected for docking studies. Three selective ligands naltrindole for delta, cyprodime for mu and guanidinonaltrindole for kappa opioid receptors were docked to homology models based on both the templates. 3D structure of the ligands were modeled in Hyperchem7.5 and minimized initially with steepest descent followed by conjugant gradient. In order to get the lowest energy conformation, minimization was done including simulated annealing in Cerius 2 v4.9 at temperature 500 K at constant NVE (constant no. of atoms, volume and energy) until convergence was obtained. Ligands were protonated at physiological pH 7.4. The two dimensional structures of the ligands showing stereochemistry and binding affinity are shown in Table 1.

Docking was carried out with GOLD 3.1 software, which uses genetic algorithm and considers full ligand conformational flexibility and partial protein flexibility, i.e., flexibility of side chain residues only. For docking, the default settings of 1,00,000 genetic operations on a population size of 100 individuals, selection pressure 1.1 and mutation rate 95 were used. As evident from literature [28], the binding site for each

Table 1 Structure of ligands and binding affinity in Ki(nM)

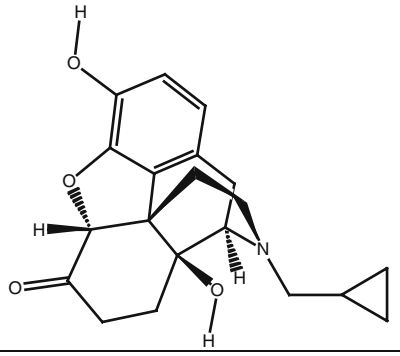
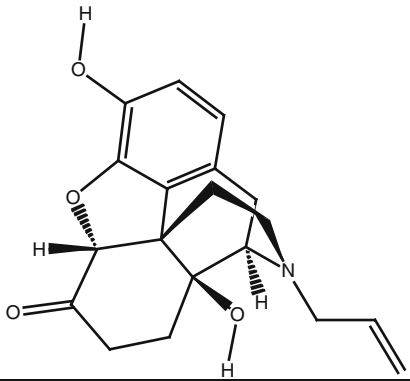
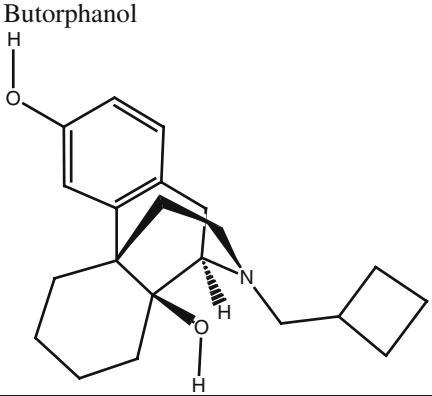
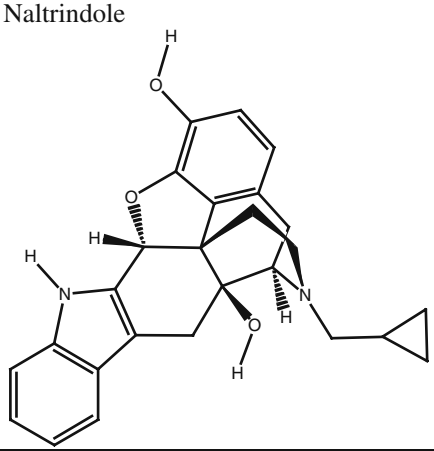
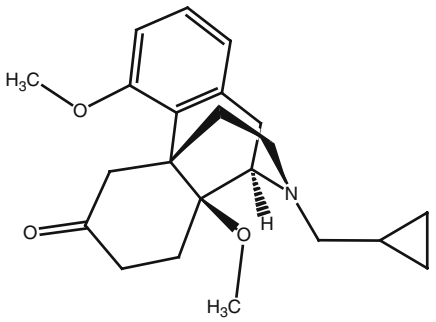
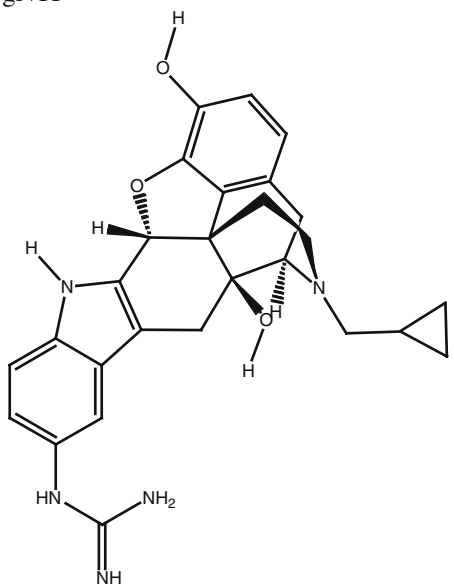
Ligand	Ki mu	Ki delta	Ki kappa	Ref.
1. Naltrexone 	2.5 ±0.21	39.5±3.0	7.0±0.18	[30]
2. Naloxone 	0.4	31.6	6.3	[31]
3. Butorphanol 	Not found	Not found	*15.7±0.9	[32]
4. Naltrindole 	99±4.6	0.41±0.09	35.8±4.0	[30]

Table 1 (continued)

5. Cyprodime 	10.6±0.7	414±27	109±4	[33]
6. gNTI 	1.20±0.2	5.8±0.65	0.4±0.06	[34]

* For butorphanol, IC50 in nM for kappa opioid receptor given

of the opioid receptors was defined as residues within the 10Å radius of Aspartic acid of third TM domain, which is involved in the most crucial interaction. Thus the binding site for mu opioid receptor consists of 10Å radius of Asp149, that of delta within 10Å of Asp 128 and for kappa opioid receptor within 10Å of Asp 138. As the reported pharmacophore [29] indicates that ligand binding in opioid receptors is favored by hydrophobic interactions, the Chemscore scoring function of GOLD was used. The Chemscore scoring function consists of four additive linear terms: hydrophobic, H-bonding, metal binding and entropic penalty. The conformation of ligand with highest Chemscore and best interaction was selected and interactions leading to binding were evaluated.

Results and discussion

Sequence alignment

The final MSA for all the receptors indicated that those are reasonably good to be used for homology modeling. The percentages of homologous residues in TM regions proved that these alignments are better (Table 2) than the alignment with only bovine rhodopsin as a template, used previously [6, 7] for building homology models of opioid receptors. The MSA alignment was also better than the alignment with human adrenoreceptor (Fig. 1 and Table 1 Supp. Inform.). Among the alignments based on single template only, alignment with bovine rhodopsin was better than that with human adrenoreceptor. In MSA it was found that in almost all the TM regions had sequence similarity greater than 50% (Table 2). These represent much better sequence homology for opioid receptors, which belong to GPCRs. Thus it could be expected that homology models built with these alignments would be more accurate.

Homology modeling

The 3D models, generated with both single (bovine rhodopsin) and multiple alignments for kappa, mu and delta opioid receptors from the respective templates, contain seven TM regions (Fig. 2), as evident in all

Table 2 Percentage of residues identical or strongly similar with single template (bovine rhodopsin) (**a**) and at least one of the templates (**b**) calculated from multiple sequence alignment for each domain

Domain	Mu		Delta		Kappa	
	A	B	A	B	A	B
TM1	60	80	37	63	59	67
TM2	61	72	56	68	59	64
TM3	35	60	37	59	36	73
TM4	38	50	25	56	30	65
TM5	74	70	74	87	68	68
TM6	57	70	61	87	58	79
TM7	35	53	24	47	32	73

GPCRs. Homology models were generated with the focus to get suitable 3D models for docking and other in silico experiments in the absence of crystallographic structures.

Superimposition of models, generated by both the methods, reveals the differences between the models (Fig. 3). For kappa receptor model with single template (bovine rhodopsin), the EC2 loop connecting TM IV and V is protruding inside whereas the same region in model with MSA is more flat. The EC region between TM VI and VII in model with single template is protruding outside. The TM VI of model with single template is closer to TM VII than in the model with multiple templates where it is bent inside and is closer to TM III. In the model with MSA, TM II isa bit farther from TM III than in the model with single template. For delta opioid receptor, in the model with single template (bovine rhodopsin), the EC region between TM VI and TM VII is flat whereas the same in model with MSA is more stretched and protruding outside. The EC region between TM IV and V of the model with single template is bent much toward the inside whereas in the model with MSA it is protruding outside. In the model with MSA, TM VI is farther from TM VII and also from TM V than in model with single template. In the model with MSA, EC I is bent toward the inside whereas it is not so in the case of the model with single template. For mu opioid receptor, in the model with single template (bovine rhodopsin), the EC

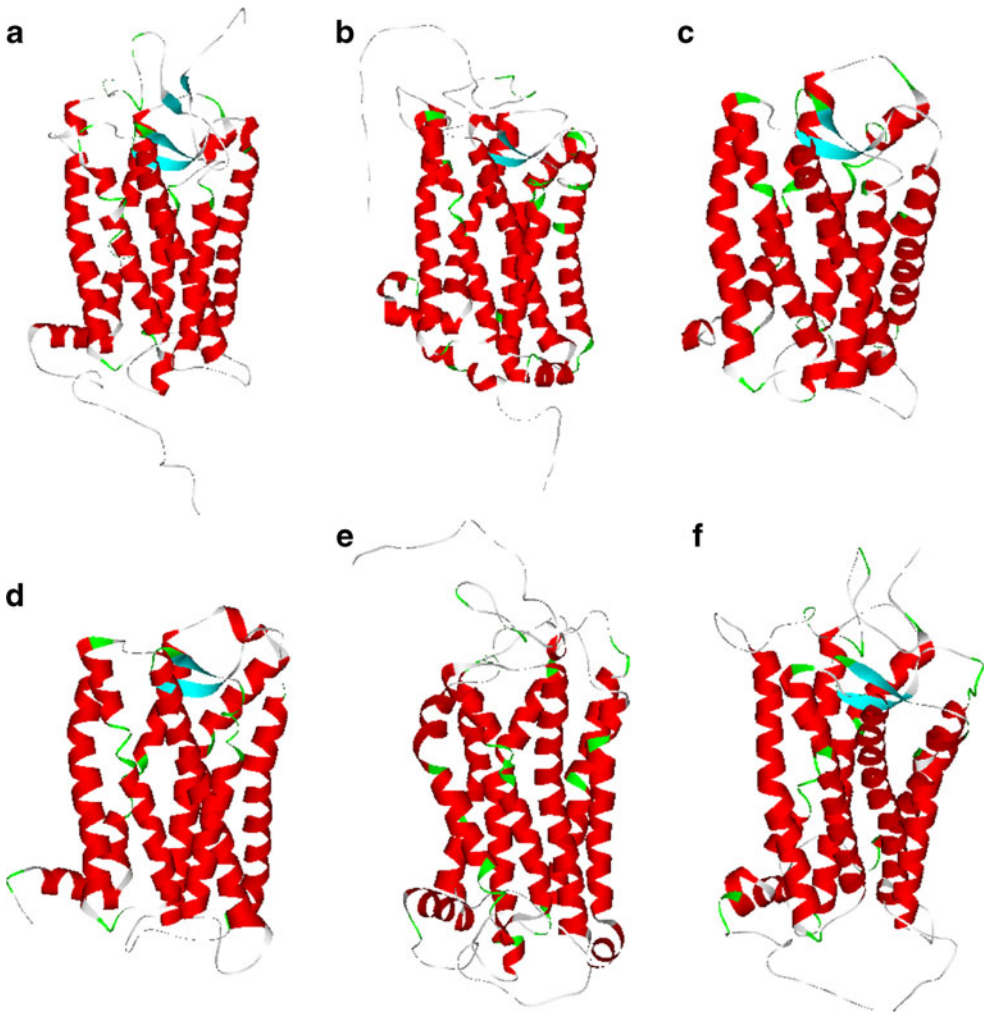


Fig. 2 The 3D-structure of homology modeled kappa (**a**, **b**), mu (**c**, **d**) and delta (**e** ,**f**) opioid receptors **a**) Kappa model with single template **b**) Kappa model with multiple templates **c**) Mu model with single template **d**) Mu model with multiple templates **e**) Delta model with single template **f**) Delta model with multiple templates

between TM IV and TM V, is protruding a little inside, which is not the case in the model with MSA. The TM VI and TM , in model with single template, are far from each other than in model with MSA. The TM I and TM II are closer to each other in the model with single template.

Evaluations of the 3-D models were done for various levels of structural organization. The results of stereochemical check of backbone with PROCHECK show that models built with MSA for mu and kappa opioid receptors are better than models based on single template. For mu opioid receptor, in the model with multiple templates, no residue was in the disallowed region (Fig. 4), whereas the model with single template (bovine rhodopsin) has two residues in the disallowed region. The residues in the disallowed region are Thr 209 and Met 207 belonging to TM IV. For kappa opioid receptor, model with single template (bovine rhodopsin) has eight residues in disallowed region, whereas model with multiple templates has only 2 residues in the disallowed region. Eight residues in the disallowed region of the model with single template are Ile 57 and 58 belonging to EC region, Val 60 belonging to TM I, Phe 114 of TM II, Ser 19, Cys 21, Thr 199 and Val 205 of EC region. The residues Asp 37 of EC and Ala 368 are part of the disallowed region in model with MSA. However, for delta opioid receptor, model with single template (bovine rhodopsin) has only one residue, Asp 193, belonging to EC, in the disallowed region whereas model with MSA has three residues in the disallowed region. The residues in the disallowed region are Val 188 of TM IV, Ala 318 and Asp 341 of cytoplasmic region. PROCHECK results are shown in Table 3.

For all the modeled structures, the residue interaction energy of each residue with respect to the rest of the protein was calculated using PROSA program. The results show that the models generated with multiple templates for mu and kappa opioid receptors have better PROSA Z-score than the models generated with single template (bovine rhodopsin) (Table 4). However, in the case of delta opioid receptor PROSA Z score indicates that model based on only bovine rhodopsin is better than that generated with multiple templates. The residue interaction energy profile for mu receptor with multiple templates is shown in Fig. 5. Verify 3D score was calculated for the proteins modeled with both the alignments. The results show that homology models with single template for mu and kappa opioid receptor did not cross the threshold score (Table 5).

However for delta opioid receptor, models based on both the alignments have Verify 3D score above the cut off score. The detailed analysis of Verify 3D results reveals the regions having negative score. The proper score for each residue should be above zero. In kappa model with single template (bovine rhodopsin), the residues 65–69 of TM I, 169–186 of cytoplasmic and TM IV, 198–205 of EC, 218–237 of EC and TM V, 241–250 of TM V and cytoplasmic and 370–380 of cytoplasmic regions have a negative score. In kappa model with MSA, residues 1–11 of EC, 172–175 of TM IV, 258–263, 352–359 and 370–380 of cytoplasmic regions have a negative score. In mu receptor model with single template (bovine rhodopsin), residues 72–91 from TM I, 121–124 from TM II, 134–136 from EC and 236–243 from TM V have a score below zero. Whereas in mu model with MSA, residues 174–176 of cytoplasmic, 179–189 of cytoplasmic, 196–199 of TM 4 and 308–314 of EC region have a negative score. In the case of delta opioid model with single template (bovine rhodopsin), residues 153–154 of cytoplasmic region have a negative value and in the model with MSA, residues 153–154 of cytoplasmic and 288 of EC have a negative score. Thus the results clearly indicate that in kappa and mu opioid receptor models with single template (bovine rhodopsin), more residues of TM regions have a negative score than in models with multiple templates. None of the models with only human adrenoreceptor as template cross the threshold score (Table 2 Supple Inform.).

The results of Molprobity score calculation shows that in the case of mu opioid receptor, homology model with single template (bovine rhodopsin) got only 16th percentile whereas the model with multiple templates got 76th

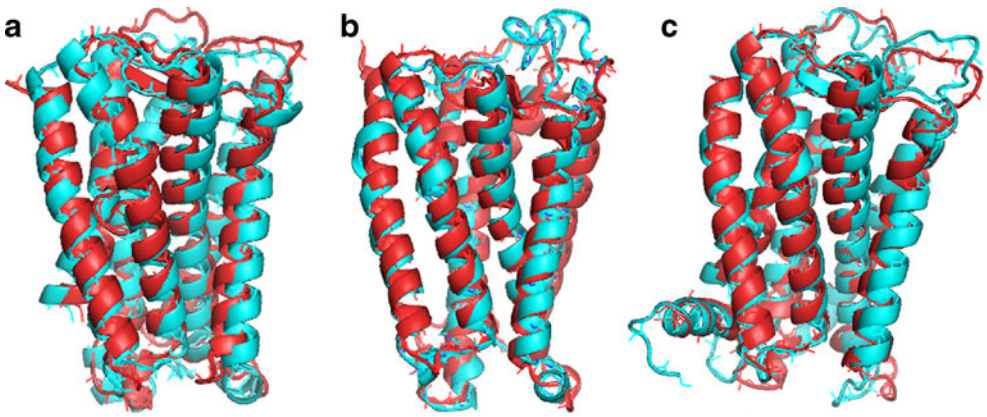


Fig. 3 Superimposition of homology models based on single with bovine rhodopsin (red) and multiple (cyan) templates from TM1 to TM7 **a**) kappa **b**) delta **c**) mu

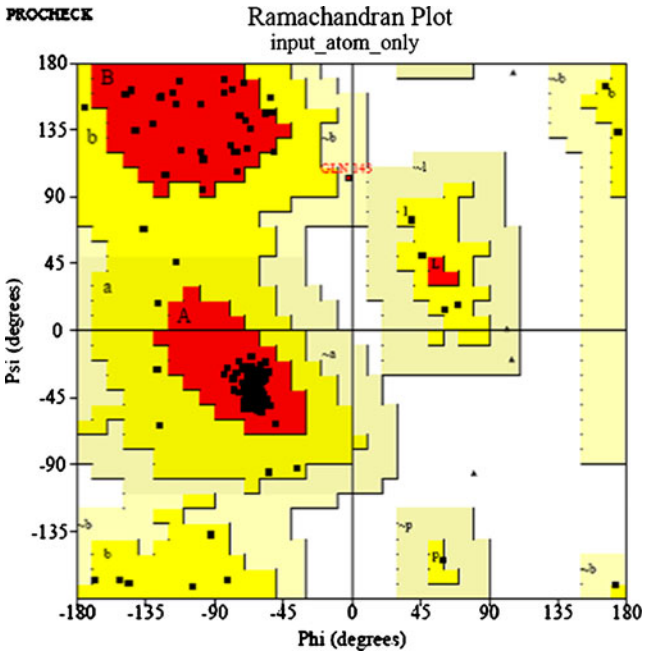


Fig. 4 Ramachandran plot of mu opioid receptor, based on multiple templates, obtained by PROCHECK. Plot for only one shown for brevity

percentile. For kappa opioid receptor, homology model with single template (bovine rhodopsin) and MSA got 62nd and 79th percentile, respectively. However, for delta opioid receptor, the model with single (bovine rhodopsin) got 89th percentile whereas model with MSA got 66th percentile (Table 6).

The results of secondary structure prediction for the homology models, using DSSP, and the putative secondary structures for the sequences of opioid receptors, predicted with MLRC, were compared. In the 3D model of delta opioid receptor with multiple templates, 18 of the residues, supposed to get helical structure, were not predicted as helix, whereas, in case of the model from single template (bovine rhodopsin), 17 of the residues did not get helical structure. The residues not forming helix in delta model with MSA template are 41–48 from EC and TM I, 208–209 from cytoplasmic, 238–242 from cytoplasmic, 256–257 and 322 from cytoplasmic regions. Whereas in model with single template residues not forming helix are 41–46 from

Table 4 PROSA Z-score for homology models with single (bovine rhodopsin) and multiple templates

	Delta	Mu	Kappa
With single	−2.91	−1.34	−1.28
With MSA	−2.05	−2.05	−2.8

EC and TM I, 208–211, 238–242, 322, 252 from cytoplasmic regions. On the other hand, for kappa opioid receptor model, based on MSA, only nine residues were not predicted as helix whereas in the model based on one template (bovine rhodopsin), 29 residues, supposed to be helix, were not part of the helix. The residues not part of helix in kappa model with MSA are 127 from EC, 225–227 from TM V, 334, 352–355 from cytoplasmic regions. In model with single template, residues 55–61 from TM I and EC , 127 from EC, 251–255 from cytoplasmic, 221–222, 268–270 from cytoplasmic, 291–292 from TM VI and 352–360 from cytoplasmic regions are not part of helix. In mu opioid receptor model based on MSA, only 15 of the residues, supposed to be helix, were not part of the helix, whereas, in the model based on single template, 38 residues were not part of the helix. In mu model with MSA, residues 137–138 from EC region, 228–232 from EC, 273–275, 263 from cytoplasmic, 315–316 from TM VII, and 341–342 from cytoplasmic do not form helix. In the model with single template, 137–138, 228–233 and 300–303 from EC, 255–263 from TM V and cytoplasmic, 315–316 from TM VII and 341–352 from cytoplasmic region do not form helix. Results show that more residues from TM regions are not part of helix in the case of models with single template for mu and kappa opioid receptors.

Thus based on all these structural verifications of 3D models, it was inferred that for mu and kappa opioid receptors, the homology models with multiple templates are better than those with single template of bovine rhodopsin. However, for delta opioid receptor, model with single template with bovine rhodopsin is better than that with multiple templates in many aspects. However, the models with only human adrenoreceptor as template were worse than those based on bovine rhodopsin as template. So these models were not used for further studies.

Table 3 PROCHECK results for modeled opioid receptors **a**) With MSA **b**) with single template (bovine rhodopsin)

Position of the region	% In Mu A B		% In Delta A B		% In Kappa A B	
Most favored regions	91.3	87.5	87.1	87.7	90.0	85.5
Additional allowed regions	8.3	10.2	10.1	9.5	7.3	10
Generously allowed regions	0.4	1.8	1.2	2.5	2.1	1.8
Disallowed regions	0.0	2.3	0.9	0.3	0.6	2.3

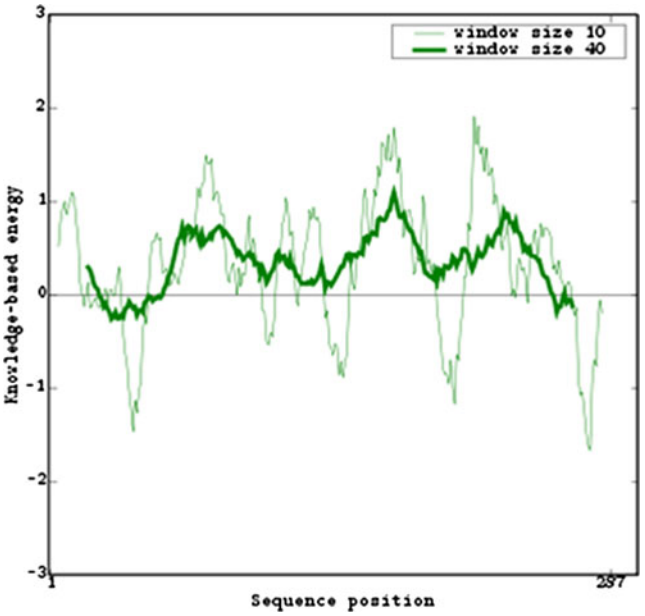


Fig. 5 Residue interaction energy profile for the mu opioid receptor drawn with PROSA Profile for only one shown for brevity

Docking results

The results of docking studies with three nonselective ligands for antagonistic activities, using GOLD, were compared with the results of mutational studies [28, 29]. An earlier study has shown that aspartic acid of TM III is an important residue for ligand recognition [28]. Studies by Befort et al. [29] have shown that aromatic residues spanning from TM III to VII are important for ligand binding. Pharmacophoric studies by Filizola et al. [13] has shown that four components, a protonated amine, centroid of the aromatic ring, and two hydrophobic pockets are necessary for recognition of nonspecific ligands. In silico studies by Habibi -Nezhad et al. [3] have shown that hydrophobic residues of TM III–VII form hydrophobic pockets of ligand binding site. Docking results of individual ligands with both the models for each of the receptors are described below. The results of docking with selective ligands cyprodime, naltrindole and guanidinonaltrindole are also discussed.

Table 5 Verify 3D score

Opioid receptor	Cut off	With MSA	With single template (bovine)
Mu	58.74	66.5	46.43
Delta	76.30	93.94	86.49
Kappa	77.95	87.85	73.88

Table 6 Molprobity score for homology models with single (bovine rhodopsin) and multiple templates

	Delta	Mu	Kappa
With single	1.70(89th percentile)	3.21(16th percentile)	2.24(62nd percentile)
With MSA	2.18(66th percentile)	1.99(76th percentile)	1.93(79th percentile)

Binding of naltrexone

With model based on MSA

The aspartic acid (Asp 149 in mu, Asp128 in delta and Asp138 in kappa) was involved in forming the ionic interaction with the protonated nitrogen of naltrexone. The residues, Tyr 328 in the case of mu, Trp 287 in kappa and Trp274 in delta opioid receptors, were responsible for pi-pi interaction with aromatic ring of naltrexone. In addition to the ionic and pi-pi interactions, there are two other hydrophobic pockets involved in ligand binding. In mu opioid receptor, residues Ile146, Tyr150, Met153 form one of the hydrophobic pockets. The other hydrophobic pocket consists of Ser156, Trp 295, Trp320 and Ile 324. In delta opioid receptor, first hydrophobic pocket consists of Leu125, Ile127, Tyr129, while the key residues of another hydrophobic pocket are Leu295, Val296, Leu300 and Tyr 308. The first hydrophobic pocket in kappa opioid receptor consists of Ile 137, Ile 135 and Phe 214 and the key residues of the second hydrophobic pocket are His291, Ile292, Ile 294 and Leu 295 (Fig. 6). Figure with backbone trace shown in supplementary information Fig. 2.

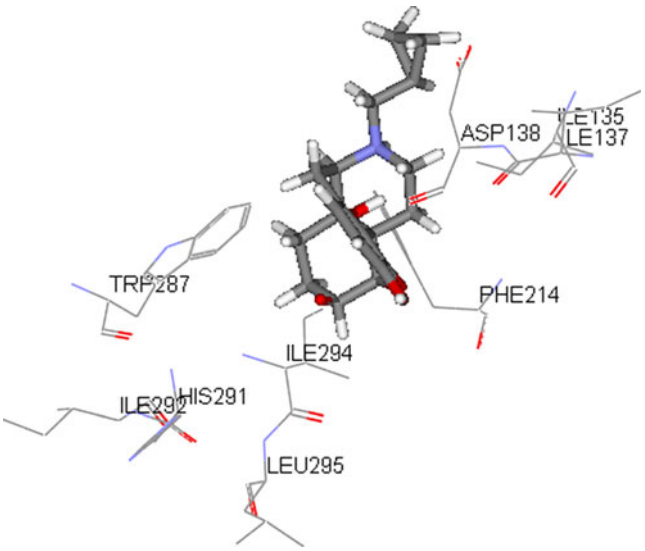


Fig. 6 Key residues involved in binding of naltrexone to kappa opioid receptor model based on MSA. Interaction with only one shown for brevity

Table 7 Important residues involved in binding of Naltrexone

Interaction	Mu single	Mu MSA	Delta single	Delta MSA	Kappa single	Kappa MSA
Ionic	Asp 149	Asp 149	Asp 128	Asp 128	Asp 138	Asp 138
Aromatic-aromatic	Tyr 77	Tyr 328	Phe 202	Trp 284	Tyr 312	Trp 287
Hydrophobic	Leu 123,Cys 332, Val 80	Ile 146,Tyr 150, Met 153	Leu 102,Val 197,Tyr 56	Leu 125,Ile 127, Tyr 129	Ser 211,Leu 212,Tyr 139	Ile 137, Ile 135, Phe 214
Hydrophobic	Ile 144,Val 145, Leu 326, Tyr 328	Val 260,Ser 156, Trp 295,Trp 320	Tyr 129, Lys 214,Val 217	Leu 295,Val 296, Leu 300,Tyr 308	Met 142, Phe 143, Trp 287	His 291, Ile 292, Ile 294, Leu 295

With model based on single template

The aspartic acid of TM III (Asp 149 in mu, Asp128 in delta and Asp138 in kappa) was involved in putative ionic interaction with the protonated nitrogen of naltrexone. The pi-pi interactions to aromatic ring of naltrexone were formed by Tyr 77 in the case of mu, Phe 202 of delta and Tyr 312 of kappa opioid receptors. The first hydrophobic pocket of mu opioid receptor is formed by Leu 123, Pro 124, Val 80 and Cys 332 and the other is formed by Ile 144, Val 145, Leu 326 and Tyr 328. For delta opioid receptor, the residues Tyr 129, Val 217, Lys 214 form the first hydrophobic pocket, whereas the other pocket consists of Tyr 56, Leu 102, and Val 197. The first hydrophobic pocket of kappa consists of Cys 315, Met 142, Phe 143 and Trp 287 whereas the residues Ser 211, Leu 212 and Tyr 139 form the second hydrophobic pocket. The interactions are mentioned in Table 7.

Binding of butorphanol

With model based on MSA

The aspartic acid of TM III (for mu 149, delta 128 and kappa 138) served the purpose of ionic interaction with butorphanol. Tyr 328 of mu opioid receptor, Trp 287 of kappa opioid receptor and Tyr 308 of delta are involved in aromatic interaction with butorphanol. The first hydrophobic domain of mu consists of Ala119, Ser121, Leu150, and Ser156. Leu123, Ser127, Val290 and Ile298 mediate the second hydrophobic interaction. The first hydrophobic pocket in delta opioid receptor consists of Leu125, Ile127, Phe133 and Met 132 and the second one consists of Ile277, Trp284, Leu300 and His301. The key residues of first hydrophobic pocket of kappa are Ile137, Ile135, Met226 and Phe214 while the other hydrophobic domain consists of Ile294, Leu295 and Val 296 (Fig. 7). Figure with backbone trace shown in supplementary information Fig. 2. The interactions are mentioned in Table 8.

With model based on single template

The residues Asp 149, Asp 138 and Asp 128 of mu, kappa and delta opioid receptors respectively form the ionic interaction with butorphanol. The pi-pi interactions are mediated by Tyr 77, Trp 114, Tyr 312 for mu, delta and kappa receptors respectively. The first hydrophobic pocket in mu opioid receptor consists of Ile 79, Val 80 Leu 221 and Tyr 328 and the second one consists of Val 83, Ala 119, Ser 147 and Ile 148. The residues mediating first hydrophobic interaction in delta are Leu 102, Phe 104, Val 124 and Ser 135 whereas the second pocket consists of Val 196, Ile 304 and Ala 309. The important residues in first hydrophobic pocket of kappa are Ile 135, Tyr 139, Phe 235 and Val 236 and those in second are Ser 211, Ile 290 and His 291.

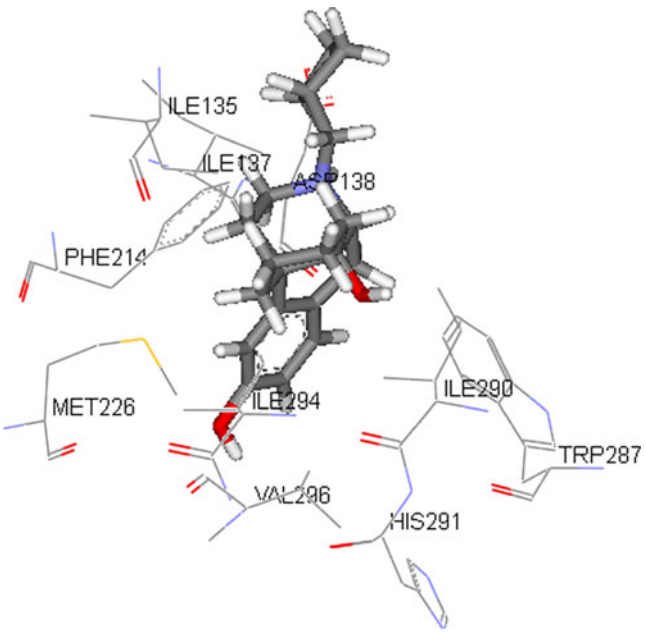


Fig. 7 Binding of butorphanol to kappa opioid receptor model with MSA. Interaction with only one receptor shown for brevity

Table 8 Important residues involved in binding of Butorphanol

Interaction	Mu single	Mu MSA	Delta single	Delta MSA	Kappa single	Kappa MSA
Ionic	Asp 149	Asp 149	Asp 95	Asp 128	Asp 138	Asp 138
Aromatic-aromatic	Tyr 77	Tyr 328	Trp 114	Tyr 308	Tyr 312	Trp 287
Hydrophobic	Ile 79, Val 80, Leu 221,Tyr 328	Ala 119, Ser 121, Leu 150, Ser 156	Leu 102, Phe 104, Val 124	Leu 125,Ile 127, Phe 133	Ser 211, Ile 290, His 291	Ile 137, Ile 135, Phe 214
Hydrophobic	Val 83, Ala 119, Ser 147, Ile 148	Leu 123, Ser 127, Val 290, Ile 298	Val 196, Ala 309, Ile 304	Trp 284, Leu 300, His 301	Ile 135, Phe 235, Val 236	Ile 291, Leu 295, Val 296

Binding of naloxone

With model based on MSA

The Aspartic acid 116 of mu, 128 of delta and 138 of kappa opioid receptor form the ionic interactions with protonated nitrogen of naloxone. The aromatic interaction is mediated by Tyr328 of mu, Trp287 of kappa and Trp274 of delta opioid receptor with phenyl ring of naloxone. The analysis of hydrobhobic pockets in mu opioid receptor shows that Ser156, Ile157, Val290 and Ser331 form the first pocket whereas Met153, Phe154, Ser197, and Trp295 form the second pocket. The first hydrophobic domain in kappa consists of Ile137, Ile135 and Phe 214 and the key residues of the second one are Ile290, Ile 292, Leu295, Met226 and Phe 293. The first hydrophobic pocket of delta consists of His301, Ile304 and Tyr308, while the second hydrophobic pocket is made up of Leu125, Leu102 and Ile127 (Fig. 8). Figure with backbone trace shown in supplementary information Fig. 2. The interactions are mentioned in Table 9.

With model based on single template

The Asp residues 149, 128 and 138 of mu, delta and kappa opioid receptors respectively form the ionic interactions. Tyr 77, Phe 104 and Tyr 312 mediate the pi-pi interactions in mu, delta and kappa receptors respectively. The residues Ser 78, Ile 79, Val 80 and Ser 121 form the first hydrophobic pocket of mu opioid receptor whereas the residues Ser 147, Ile 148, and Tyr 328 form the second hydrophobic pocket. The first hydrophobic pocket of delta opioid receptor consists of Leu 302, Ala 305 and Cys 303 while the residues Ala123, Val 124, Leu 300 and His 301 form the second hydrophobic pocket. In kappa opioid receptor, the residues Val 134, Ile 290 and His 291 form the first hydrophobic pocket whereas residues Ile 135, Phe 235, Val 236 and Leu 212 mediate the second hydrophobic interaction.

The results for docking with models generated from multiple templates complied well with experimental studies. The results show that the Asp of TM III forms the ionic interactions. Trp of TM VI or Tyr of TM VII mediates the pi-pi interactions. The hydrophobic residues of TM III, TM VI, and TM VII mainly form the hydrophobic pockets. Docking results for models with single template show that not all the interacting regions are complying with the experimental studies. Though the ionic interaction, here also, is mediated by Asp of TM 3, the residues involved in pi-pi interaction in mu and delta are located in TM I and EC regions, respectively. Mutation studies did not report EC and TM I regions as important parts of binding pocket. Though according to previous studies hydrophobic pockets are mainly supposed to be composed of residues from TM III to TM VII, some residues of hydrophobic pockets of delta opioid receptor are also part of TM I and EC regions. A few residues from EC and TM1 regions are part of hydrophobic pocket of mu receptor.

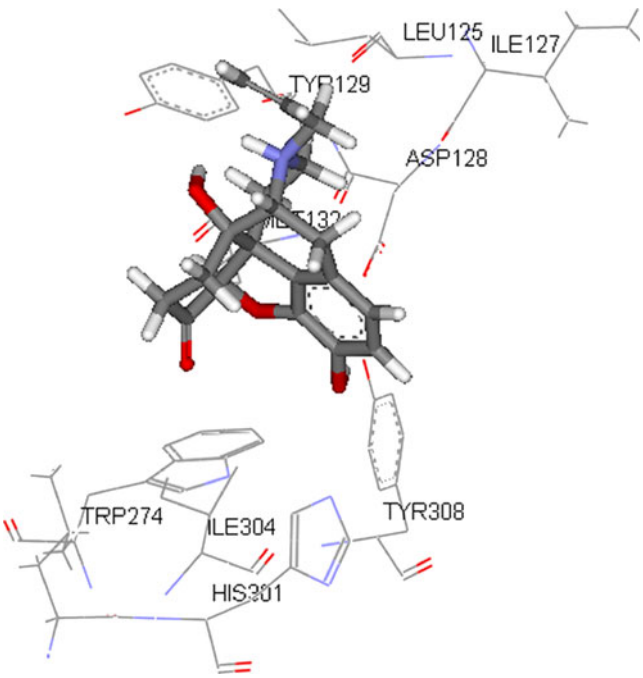


Fig. 8 Binding of naloxone to delta opioid receptor with MSA. Only one shown for brevity

Table 9 Important residues involved in binding of Naloxone

Interaction	Mu single	Mu MSA	Delta single	Delta MSA	Kappa single	Kappa MSA
Ionic	Asp 149	Asp 116	Asp 128	Asp 128	Asp 138	Asp 138
Aromatic-aromatic	Tyr 77	Tyr 328	Phe 104	Trp 274	Tyr 312	Trp 287
Hydrophobic	Ile 79, Ser 121, Leu 125	Ser 156,Ile 157, Val 290	Leu 302, Cys 303, Ala 305	Leu 102, Leu 125, Ile 127	Ile 135, Phe 235, Val 236	Ile 135, Ile 137, Phe 214
Hydrophobic	Ser 147, Ile 148, Tyr 328	Met 153,Phe 154, Ser 197	Ala 123, Val 124, Leu 300, His 301	His 301, Ile 304, Tyr 308	Val 134, Ile 290, His 291	Ile 290, Ile 292, Leu 295

In mu opioid receptor with single template, TM I is closer to TM II. So residues of TM I are also part of binding site. A hydrophobic pocket of kappa opioid receptor also has a few residues from EC region. The presence of EC region residues in case of models with single template can be explained with conformation of EC2 where they are protruding a little inside.

Binding of naltrindole

Binding results show that aspartic acids 128, 149 and 138 of delta, mu and kappa opioid receptors, respectively, form the ionic interaction with protonated nitrogen of naltrindole. The residues Tyr 308, Tyr 328 and Phe 231 of delta, mu and kappa opioid receptors, respectively, form pi-pi interaction. The first hydrophobic pocket of delta consists of Val 124, Leu 125 and Leu 200 and the second hydrophobic pocket consists of Met 132, Ala 195, and Val 196. The residues Leu 80, Val 81 and Tyr 150 form the first hydrophobic pocket of mu and Trp 320, Ile 324 and Phe 223 form the second one. For kappa opioid receptor, the residues Val 134, Ile 135 and Phe 231 forms first hydrophobic pocket. And the second hydrophobic pocket consists of Ile 290, His 291 and Tyr 320. In addition to these there is additional interaction for the indole ring of naltrindole. In delta opioid receptor, the residues Trp 274, Trp 284 and Val 281 are making hydrophobic interaction with the indole ring (Fig. 9). In mu receptor, Phe 291and Trp 295 are interacting with the indole ring. The residues Trp 124, Phe 126 are ineracting with the indole ring of naltrindole. It can be hypothesized that the indole ring of naltrindole is selectively interacting with delta opioid receptor. However, the insights regarding the contribution of specific residue/s could be obtained only through molecular dynamic studies of the complex.

Binding of guanidinonaltrindole (gNTI)

In kappa selective ligand, gNTI, guanidine moiety is attached to naltrindole. This guanidine moiety confers selectivity to the kappa opioid receptor. One of the nitrogens of guanidine moiety gets protonated at physiological pH7.4. Thus, in gNTI there are two protonated

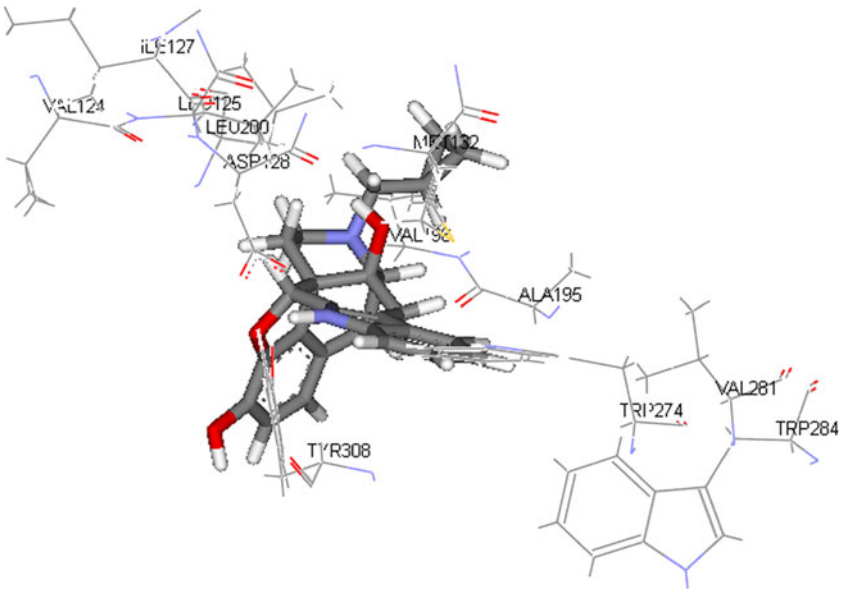


Fig. 9 Binding of naltrindole to delta opioid receptor with MSA

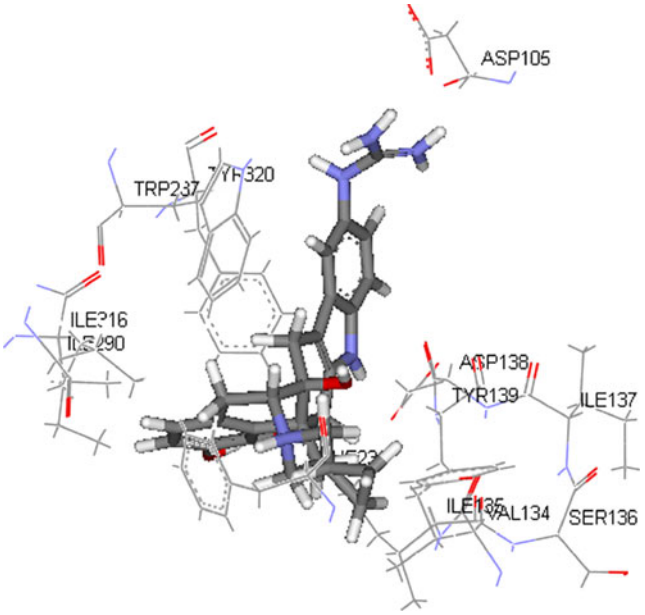


Fig. 10 Binding of gNTI to kappa opioid receptor with MSA

nitrogens which form ionic interactions. In case of kappa opioid receptor, Asp 105 forms the ionic interaction with protonated nitrogen of guanidine (Fig. 10). However, in the case of mu and delta opioid receptor, there were no ionic interactions with the protonated nitrogen of guanidine.

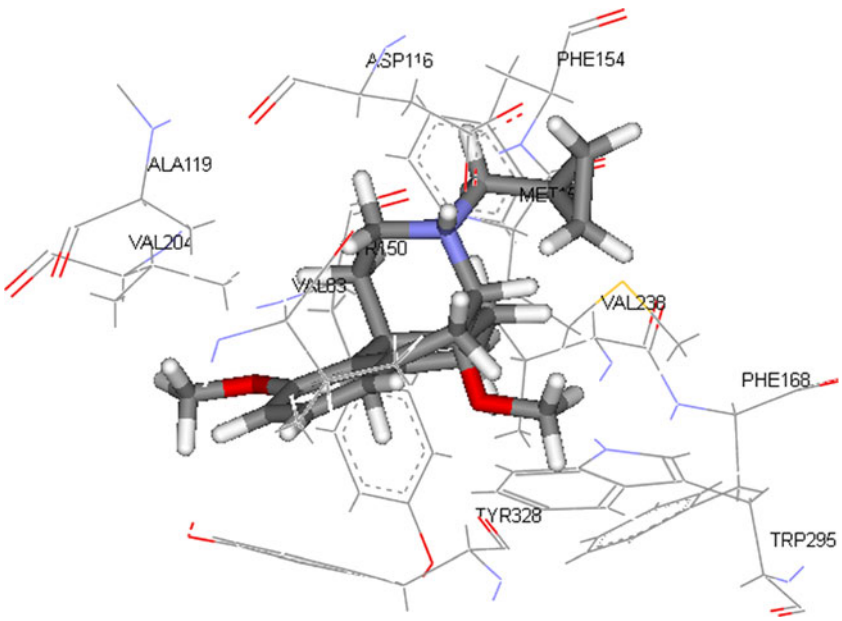
Binding of cyprodime

For mu selective antagonist, cyprodime, residues Asp 116, Asp 128 and Asp 138 of mu, delta and kappa opioid receptors, respectively, form the ionic interaction with protonated nitrogen. The residue Tyr 328 of mu and Trp 287 of kappa opioid receotor form the pi-pi interaction. However, in the case of delta receptor, no such well-defined pi-pi interaction was present. Besides these, two hydrophobic pockets were also present. In the case of mu opioid receptor, residues Trp 295, Phe 237, Tyr 150 and Val 238 form one hydrophobic pocket. Val 83, Val 133 and Phe 154 form the other hydrophobic pocket (Fig. 11). The two hydrophobic pockets of kappa opioid receptor are comprised of residues Ile 135, Ser 136, Ile 137, Ile 290, Phe 293 and Ile 294. Whereas in delta, residues Leu 125, Ile 127, Ser 126, His 301, Ile 304 and Leu 306 form the two hydrophobic pockets. It may be hypothesized that the difference of extent of hydrophobic interaction may be responsible for selectivity of cyprodime toward mu opioid receptor. Further detailed molecular dynamics studies are needed to reach a definite conclusion.

Conclusions

In this paper, we present a comparative study on the homology models of opioid receptors based on single and multiple templates. 3D models based on single template were built using bovine rhodopsin as a template. For models based on multiple templates, recently deposited crystal structures of GPCRs were taken into consideration, along with bovine rhodopsin. Using multiple templates in combination helped us to improve the sequence homology of TM regions. To check whether the structural quality of models has improved with an increase in sequence homology, both the models were assessed by a number of

Fig. 11 Binding of cyprodime to mu opioid receptor with MSA



validation programs like PROCHECK, PROSA, Verify 3D and Molprobity. The results of these validation studies prove that the models with multiple templates are better than those of single template for mu and kappa opioid receptors. However, for delta opioid receptor, though the sequence homology has improved with multiple templates, the model with single template showed structurally better results in most of the validation studies. Thus it is not always true that the model with multiple templates is structurally better as in the present case of the GPCRs.

For further validation of the quality of the models, three non-specific ligands were docked into the binding site of both the models for each receptor. The docking results for all three non-specific ligands, with both models, complied well with established pharmacophore. However, in models with single template some residues from TM 1 and EC region are the part of the binding pocket though these residues are supposed to be confined within TM III to TM VII. Thus for all three cases the docking results of models with multiple templates complied better with earlier studies. Additionally, three selective ligands, one for each of the mu, delta and kappa opioid receptors, were also docked to the models with multiple templates. The selectivity for the receptors was hypothesized, but further detailed molecular dynamics study will lead to better understanding of selectivity of opioid receptors. The better 3D models of opioid receptors can be used further for in silico studies to develop potent antagonists with minimal side effects.

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