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Comparative modeling of human kappa opioid receptor and docking analysis with the peptide YFa

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ABSTRACT

The kappa opioid receptor belongs to the super family of G protein – coupled receptors that are of utmost significance in the development of potent analgesic drugs for the treatment of severe pain. An accurate evaluation of the ligand binding pathways into this receptor at molecular level may play a key role in the design of new molecules with more desirable properties and reduced side effects. In this study, homology model of the human kappa opioid receptor was developed by MODELLER using the X-ray crystal structure of bovine rhodopsin as template. Initial structure of the receptor was refined computationally with energy minimization and molecular dynamics simulation at 300 K in a pre-equilibrated phospholipid bilayer by GROMACS. The Met-enkaphalin-Arg-Phe based opioid peptide YFa (YGGFMKKKFMRF) designed and characterized by our laboratory was docked into the optimized model and the critical amino acids responsible for binding were identified. A number of low energy binding poses of YFa with the receptor were assessed after the molecular docking in which the peptide was observed to interact with the receptor's extracellular amino acids through hydrogen bonds. The human kappa opioid receptor model optimized in a phospholipid bilayer should provide a good starting point for further characterization of the binding modes of other opioid ligands. Furthermore, the biologically favorable molecular interactions between YFa and human kappa opioid receptor observed by our study might be able to justify the specificity of this peptide.

1. Introduction

Mu opioid receptors (MORs), delta opioid receptors (DORs) and kappa opioid receptors (KORs) are the three well defined opioid receptors (ORs) of the superfamily of G protein coupled receptors (GPCRs) [1,2]. They are widely distributed in the central nervous system with opioids as ligands [3] and are involved in a wide range of physiological symptoms like pain perception, mood regulation, physical dependence and development of analgesic tolerance [4]. ORs are negatively coupled to cAMP production mainly through $G_{i,o}$ [5] and play important role in the functioning of central and peripheral nervous systems. All of these receptors have the same general structure with a glycosylated extracellular N-terminus, palmitoylated intracellular C-terminus, seven transmembrane (TM) α helices connected by three extracellular loops i.e. extracellular loop1 (EL1), extracellular loop2 (EL2), extracellular loop3 (EL3) and three intracellular loops namely intracellular loop1 (IL1), intracellular loop2 (IL2) and intracellular loop3 (IL3).

The progress in resolving the three dimensional (3D) structures of the ORs is hindered by the challenges in isolating and solublizing these receptors [6,7]. In the absence of experimental data on 3D structure, indirect methods such as site directed mutagenesis and chimeric studies have been utilized for structure function analysis. Studies on chimeric receptors using either endogenous peptides or exogenous opiates show that the ORs utilize the negatively charged EL regions and TM domains to achieve differential binding affinities for peptide and non-peptide ligands [8–11]. The interaction of small ligands occurs in the core of the TM regions and is very different from that of the peptides which are dependent on the EL segments of the receptors [12]. The essential involvement of residues from EL2 and EL3 for the selectivity of the endogenous peptide dynorphin A (DynA), a HKOR specific agonist has been demonstrated using the human kappa opioid receptor (HKOR) chimera [13,14]. The combination of experimental studies and molecular modeling approaches should allow development of realistic 3D models of

Abbreviations: MOR, mu opioid receptor; KOR, kappa opioid receptor; DOR, delta opioid receptor; RCSB PDB, Research Collaboration of Structural Biology Protein Data Bank; IL, intracellular loop; EL, extracellular loop; DynA, dynorphin A; GPCR, G-protein coupled receptor; TM, transmembrane; 3D, three dimensional; BLAST, Basic Local Alignment Search Tool; ADIT, Auto Deep Input Tool; MD, molecular dynamics; RMSD, root mean square deviation.

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ligand–receptor complexes that will be helpful in elucidation of the molecular determinants for ligand affinity and selectivity.

We have preciously synthesized and characterized the interactions and the *in vivo* pharmacological effects of YFa, a chimeric opioid peptide YFa (Tyr^1-Gly^2-Gly^3-Phe^4-Met^5-Lys^6-Lys^7-Lys^8-Phe^9-Met^{10}-Arg^{11}-Phe^{12} or YGGFMKKKFMRF) based on the endogenous peptide, Met-enkephalin-Arg-Phe (Tyr-Gly-Gly-Phe-Met-Arg-Phe; MERF). This peptide contains the overlapping sequence of both opioid (Tyr-Gly-Gly-Phe or YGGF) and antiopioid (Phe-Met-Arg-Phe or FMRFamide) moiety. It is well reported that all endogenously occurring opioid peptides contain common tetra peptide sequence (YGGF) which works as allosteric sequence (message sequence) that activates the receptor for intracellular signaling pathways. The other half sequence FMRFamide is well reported for having the role in modulation of opioid effect and tolerance development as orthosteric site which also works as positive allosteric modulator by binding to antiopioid receptors [15–17]. To make this sequence more receptor selective three Lysine linker was attached between YGGF and FMRFamide. *In vivo* pharmacological studies of YFa showed that it induced naloxone-reversible antinociception and attenuated the development of tolerance to morphine analgesia indicating a possible role in pain modulation [18]. Further studies on YFa with specific MOR, DOR or KOR receptor antagonists and also effect of chronic administration of YFa (i.p.) revealed that YFa induced KOR specific antinociception, with no development of tolerance during 6 days of chronic treatment [19]. The tolerance and cross-tolerance effects of chimeric peptide, YFa on its own and morphine analgesic action was also extensively studied to demonstrate YFa as a kappa specific peptide and mu specific at higher concentration which could also be because of saturation of kappa receptor and have interaction with other receptors [20,21].

In the light of these observations, we chose YFa for the docking studies with the HKOR to model the interactions in the receptor ligand complex. In the present study, we applied homology modeling and molecular dynamics simulation methods to develop an energetically favorable 3D molecular model of HKOR based on the high resolution crystal structure of bovine rhodopsin (BRHO) and analyzed the HKOR-YFa complex system through molecular docking to investigate the preferred binding orientation of YFa. We analyzed the interactions between YFa with charged as well as hydrophobic residues within the HKOR binding site. Furthermore, we analyzed the receptor–ligand interactions in terms of hydrogen-bond networks between HKOR and YFa. The intermolecular interactions between HKOR and YFa modeled at the individual residue level are discussed in the context of our previous *in vitro* studies.

2. Materials and methods

2.1. Homology modeling of HKOR

The 3D structure of HKOR was built by homology modeling with MODELLER 9v7 [22] using the X-ray crystal structure of BRHO at 2.2 Å resolution (PDB entry 1U19) as the structural template [23]. The pair-wise sequence alignment to predict the highly conserved functional residues in both target and template was generated by the program CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The resultant automatic alignment from CLUSTALW was manually corrected to bring into register as much overlapping residues as possible within the structurally conserved regions (TM regions) and the sequence gaps were shifted to the structurally non-conserved regions (loop regions). The IL1–IL3 and EL1–EL3 regions were modeled using the loop prediction approach implemented in MODLOOP [24] which built the loop regions satisfying the spatial restraints. For the specific case of EL2, the conserved disulfide bridge between Cys^{131} of TM3 and Cys^{210} of EL2 was manually introduced according to experimental data demonstrating the importance of this interaction in the ligand binding and structural stability of ORs [25]. Since the N-terminal and C-terminal regions of HKOR were longer than BRHO, these residues were partially truncated (specifically, 20 residues from the N-terminus and 10 residues from C terminus) to avoid any unnatural artifacts in the model, while the conserved helix 8 at the C terminal end was added to the HKOR model.

In the process of assigning coordinates for structurally non-conserved regions, the helical backbone structures of the receptors may be kinked, bent, or distorted. Such artifacts were removed by "Build/Check and Repair" tool of the WHATIF web server (http://swift.cmbi.ru.nl/servers/html/index.html). Model evaluation was done by AutoDep Input Tool (ADIT) validation server (http://deposit.pdb.org/validate/).

2.2. Molecular dynamics simulation of HKOR

The stability of structures was verified through the molecular dynamics (MD) simulation using GROMACS [26] in an explicit membrane aqueous system. The total system consisted of 121 molecules of DPPC lipids, 8920 water molecules, 350 amino acids and Na^+ ions. Energy minimization was carried out by steepest descent followed by conjugate gradient algorithms applying Berger lipids parameters for the lipid component in combination with the GROMOS representation of the protein [27].

In the initial round, the backbone atoms of the receptor model were held fixed during both steepest descent and conjugate gradient minimizations, and then the constraints were released. After 10,000 steps of steepest descent minimization, the models were further minimized for an additional 15,000 steps using the conjugate gradient procedure until the maximum force is less than 1000 kJ/mol/nm. The minimized models were imported into MD simulations which were carried out using leapfrog Verlet dynamics integrator and constant temperature dynamics using Berendsen weak coupling method (NVT) along with a step size of 0.002 picoseconds (ps). Non-bonded interactions were calculated by using a distance cutoff of 8.0 Å. Electrostatic energy was computed by Particle Mesh Edwald (PME) method and linear constraint solver (LINCS) algorithm was applied to fix all bonds involving hydrogen. The receptor model was gradually heated to 300 K over 500 ps, using strong constraints on the backbone Phi (φ) and Psi (ψ) angles. These constraints were gradually released over an additional 500 ps, producing relaxed structure for continued MD simulation till another 5 nanoseconds (ns). Two-dimensional (2D) root mean square deviation (RMSD) plots of the trajectory was used to monitor convergence and as a guide to extract average representative structure from the trajectory. The average structure which generally tends to be crude was minimized for 500 steps using conjugate gradient procedure by following the above mentioned parameters for energy minimization.

2.3. Molecular modeling of YFa

The starting conformation of the peptide YFa was generated by the BIOPOLYMER program of SYBYL 8.0 [28]. The structure of the peptide was then refined by energy minimization with appropriate charges and parameters. The different conformational spaces of the peptide were elucidated by performing *in vacuo* molecular dynamics simulation in SYBYL. The parameters for the simulation in the SYBYL program are as follows: non-bonded twin cutoff distance was 8.0 Å, time step was 0.001 ps, the dielectric constant was 8.0, Tripos force field and Kollman all-atom charges were applied. The MD simulations with the above parameters were performed

```
OPRK_HUMAN   CLPPNSSAWFPGWAEPDSNGSAGSEDAQLEPAHISPAIPVIITAVYSVVFVVGLVGNSLV 60
OPSD_BOVIN   MNGTEGPNFYVPFS--NKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLT 58
                .:.. ::  ::  :..* . *     :    .*    :::*   :::::*:  * *.

OPRK_HUMAN   MFVIIRYTKMKTATNIYIFNLALADALVTTTMPFQSTVYLMNSWPFGDV--LCKIVISID 118
OPSD_BOVIN   LYVTVQHKKLRTPLNYILLNLAVADLFMVFGGFTTTLYTSLHGY-FVFGPTGCNLEGFFA 117
             ::* :::.*::*. *  ::***:** ::.      :    ::.: *       *::   :

OPRK_HUMAN   YYNMFTSIFTLTMMSVDRYIAVCHPVKALDFRTPLKAKIINICIWLLSSSVGISAIVLGG 178
OPSD_BOVIN   TLGGEIALWSLVVLAIERYVVVCKPMS-NFRFGENHAIMGVAFTWVMALACAAPPLVGW- 175
               .   ::::*.::::::**:.**:*:.        :* :     *::: : . ..:*

OPRK_HUMAN   TKVREDVDVIECSLQFPDDDYSWWDLFMKICVFIFAFVIPVLIIIVCYTLMILRLKSVRL 238
OPSD_BOVIN   SRYIPEGMQCSCGIDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGQLVFTVKEAAA 235
             ::   :    .*.:::   . .  :  : * :*:. *:**:::*:.**  ::: :*..

OPRK_HUMAN   LSG-SREKDRNLRRITRLVLVVVAVFVVCWTPIHIFILVEALGSTSHSTAALSSYYFCIA 295
OPSD_BOVIN   QQQESATTQKAEKEVTRMVIIMVIAFLICWLPYAGVAFYIFTHQGS--DFGPIFMTIPAF 295
              .  *  .::  :.:**:*:::* .*::** *   . :     . *    .     :
OPRK_HUMAN   LGYTNSSLNPILYAFLDENFKRCFR---DFCFPLKMRMERQSTSRVRNTVQDPAYL- 350
OPSD_BOVIN   FAKTSAVYNPVIYIMMNKQFRNCMVTTLCCGKNPLG--DDEASTTVSKTETSQVAPA 348
             :. *.:  **::* :::::*:.*:              : :::: * :*  . .
```

Fig. 1. Domain wise sequence alignment between HKOR and BRHO. The highlighted regions are the TM helices (1–7) respectively and the red bold portions are the most conserved motifs. The underlined residues are the invariant residues observed in all proteins of GPCR superfamily. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

after the energy minimization by the conjugate gradient method until an RMS gradient tolerance of 0.05 kcal/mol*Å was reached.

2.4. Molecular docking of YFa

All the docking and scoring calculations were performed using the SYBYL 8.0 molecular modeling software suite. The receptor was prepared for docking using the "prepare protein" section of BIOPOLYMER tool. Based on the fact that the extracellular amino acids confer peptide selectivity in the GPCR superfamily, the active site was represented by picking the amino acids of EL1, EL2 and EL3 on the modeled receptor. Peptide docking to the HKOR homology model was performed using the Surflex-Dock module [29]. Post docking filtering of ligand poses with suitable binding orientations was performed using the consensus scoring approach with five different scoring functions provided within the SYBYL *C*-score module [30]. A total of 10 docked poses of the peptide were obtained and the best-fit pose was screened on the basis of the *C*-score. The best pose having highest score was taken for further analysis after complete energy minimization followed by 500 ps of MD simulation by following the parameters already described in Section 2.2.

3. Results and discussions

3.1. Homology modeling

We constructed the 3D structure of HKOR containing all the 7TM domains by deploying the homology modeling protocol based on the crystal structure of BRHO (1U19) that was completely stable during MD simulations. Target-template sequence alignment was performed by CLUSTALW web interface to identify the conserved motifs prior to homology modeling. Although, the overall homology of the opioid receptor was very low, the TM domains had sufficient identity (30–40%) to BRHO for carrying out homology modeling. In

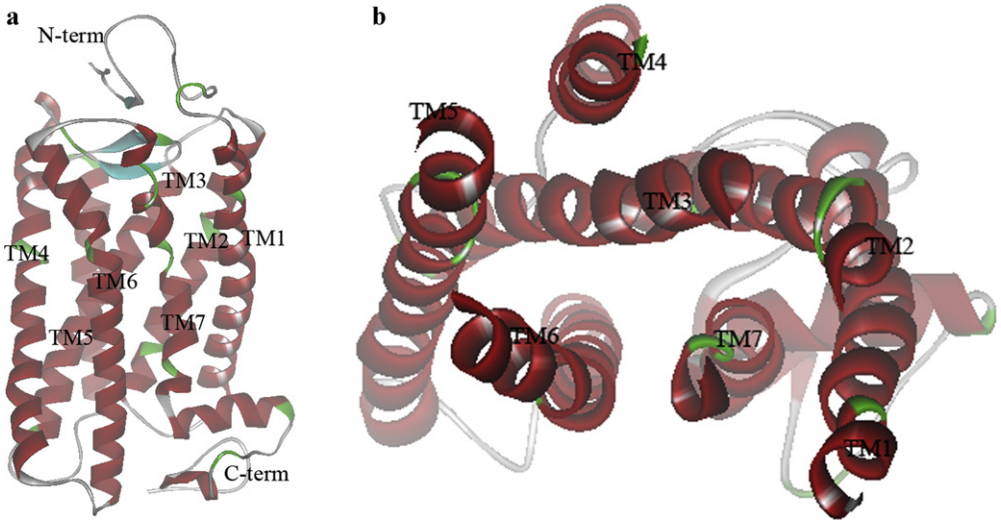

Fig. 2. Graphical representations of the HKOR homology model as seen from the side of the plane of the plasma membrane and the 7 helix orientations with the N-terminus above and the C-terminus below of the TM helix bundle. (The ribbon view of the model was prepared using Discovery studio visualizer 2.5.)

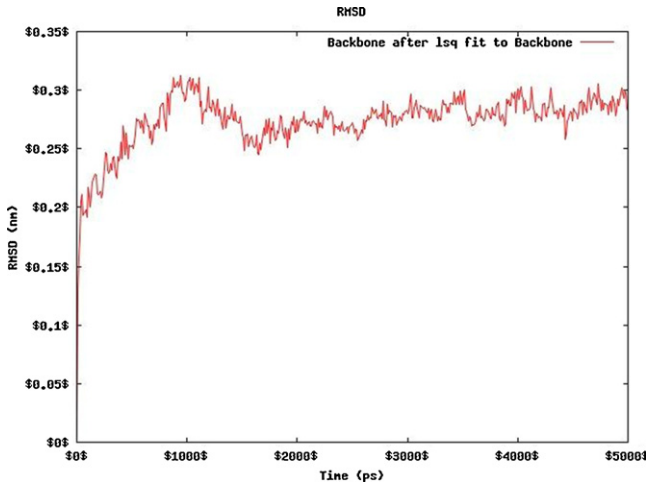

Fig. 3. 2D RMSD graph of HKOR simulations. The *X*-axis represents the time interval and the *Y*-axis shows the backbone deviation in terms of nanometer (nm). The graph indicates that the backbone of the HKOR model fluctuated till 2 ns and afterwards was stable since no change in the RMSD was observed indicating equilibrium.

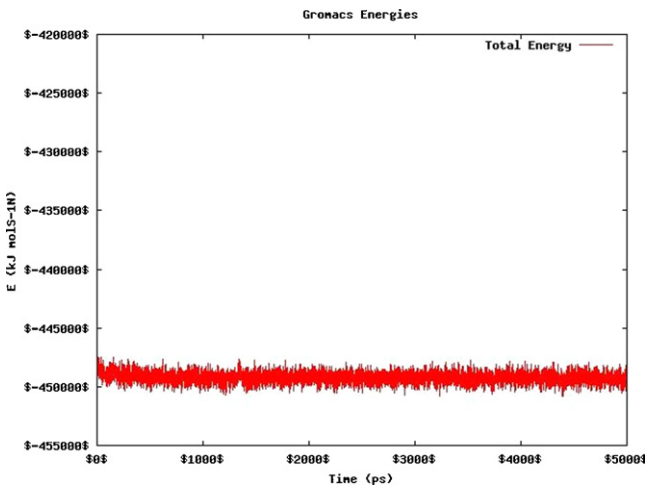

Fig. 4. 2D representation of the total energy variation during the HKOR molecular dynamics simulations. In the plot *X*-axis bears the simulation time whereas the *Y*-axis shows total energy in kJ mol^{-1}. The total energy of the HKOR molecular system was found to be constant after initial cycles of minimization.

the sequence alignment several highly conserved motifs were identified such as Asn^{77} of the GNXXV motif in TM1, Leu^{101} of the LXXXD motif in TM2, Arg^{157} of D/ERY motif in IL2, Asp^{183} in the middle of the TM4, Pro^{238} of FXXP motif in TM5, Pro^{289} of the CWXP motif in TM6, Pro^{327} of NPXXY motif in TM7 and the conserved disulfide bond linking Cys^{131} of TM3 and Cys^{210} of EL2. These highly conserved residues were used as reference points for aligning each helix during the alignment. The aligned sequences of TM 1–7 of HKOR with BRHO are shown in Fig. 1. For the refinement of loop regions MODLOOP web server was used which predicted the probable loop conformation satisfying spatial restraints. Fig. 2 shows side and top views of the HKOR homology model in which the heptahelical TM bundle lies roughly perpendicular to the plane of the membrane.

3.2. Molecular dynamics simulation

The HKOR model structure clearly evolved and diverged during the course of the MD simulations. By the end of the simulation, the backbone atoms of the simulated receptor model developed an average RMSD greater than 0.3 nm (3 Å) from the initial models. In general, the comparisons of the backbone atoms between average structures derived from the final 1.0 ns of the trajectories yield RMSD values less than 0.05 nm (0.5 Å). The structures generated by these trajectories have been stabilized, as indicated by analysis of 2D RMSD plots between snapshots from the trajectories and the backbone deviations during the final 1.0 ns portion of the simulations did not exceed 0.075 nm (0.75 Å) (Fig. 3). The stability of the model was further evaluated by calculating the total energy variation during simulation (Fig. 4). The conserved disulphide bond between the EL2 (Cys^{210}) and the TM3 (Cys^{131}) of the receptor remained stable throughout the simulation. Due to this disulphide bridge the EL2 acquires a unique turn which is a conserved tertiary fold observed in class A GPCRs [25]. This bond creates an additional geometric constraint in connecting TM3 with TM4 and TM5 and is important in the structural stability of ORs. The φ and ψ angles of the receptor backbone were also verified by Ramachandran plot [31] which indicated that more than 91.5% of the residues were in the most favorable regions (Fig. 5).

The average structures of the HKOR model obtained from the MD simulation maintained their similarity to the experimentally determined structures of BRHO in terms of the 7 TM helix orientations. The validation of the receptor model is based on the assumption that the GPCRs share significant structural commonalities and the high helical content of the initial model structure correlates with presence of 7 α helices in the TM regions which incorporate non-helical deviations induced by prolines (Pro^{113} and Pro^{238}) and other structural motifs [32]. During the simulations, α helical content of the HKOR model decreased only marginally, that reflects the balance between the stability of the helices and the structural dynamics during the simulation.

3.3. Interaction of YFa with HKOR

The initial conformations of both the peptides; YFa and DynA were selected randomly according to the results given by secondary structure prediction servers HNN, GOR and SOPMA at ExPASy proteomics server. The randomly predicted conformations were subjected to rounds of minimization and conformational analysis using molecular dynamics as described in Section 2. During MD

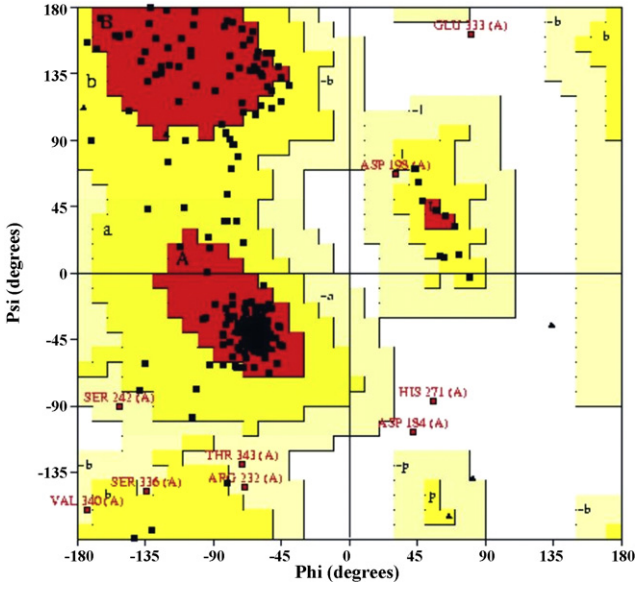

Fig. 5. 2D graphical representations of the Ramachandran plot showing φ and ψ angles of HKOR homology model. The plot statistics reported that more than 91.5% of the residues fall in the most favored regions of the plot.

Table 1
Surflex docking scores of the 10 best poses of YFa in the binding site of HKOR. First 6 poses have equal and highest *C*-scores but differ in terms of interaction energies. 2nd pose having highest *C*-score of 4 and best interaction energy was considered for further study.

Poses	Total_score	Crash	Polar	*D*_score	PMF-score	*G*_score	Chemscore	*C*-score	Interaction energy (kcal/mol)
2	34.26	−28.32	0.61	−513.09	63.62	−752.02	−45.23	4	−34,848.91
3	34.26	−28.32	0.61	−513.09	63.62	−752.02	−45.23	4	−34,591.39
1	34.26	−28.32	0.61	−513.09	63.62	−752.02	−45.23	4	−34,580.02
4	34.26	−28.32	0.61	−706.07	293.17	−926.78	−51.5	4	−34,579.55
7	34.6	−42.01	2.48	−706.07	293.17	−926.78	−51.5	4	−34,344.87
8	34.6	−42.01	2.48	−513.09	63.62	−752.02	−45.23	4	−34,235.06
5	34.6	−42.01	2.48	−322.1	120.95	−555.03	−13.32	2	−34,170.03
6	34.6	−42.01	2.48	−322.1	120.95	−555.03	−13.32	2	−34,142.05
9	36.34	−51.74	1.57	−322.1	120.95	−555.03	−13.32	1	−33,816.77
10	36.34	−51.74	1.57	−322.1	120.95	−555.03	−13.32	1	−33,728.74

simulations the peptides evolved into realistic structural organizations and developed RMSD of 0.3 nm from initial conformation. The peptides were then docked into the homology modeled structure of HKOR using Surflex-Dock and among the 10 best receptor–ligand poses 6 poses have the highest *C*-scores i.e. 4 with varying interaction energies (Table 1). All these poses were visually inspected for close intermolecular interactions of the binding residues. The 2nd pose having greater interaction energy was found to be most plausible ligand–receptor complex. This initial docking mode was then used as the starting structure for subsequent energy minimization and molecular dynamics simulations as described in Section 2. It is known that the opioid receptors employ their highly negative extracellular loops for recognition of larger peptides, whereas small molecules interact at the hydrophobic core of the TM regions [12]. Studies of OR chimeras and site-directed mutants revealed that the ligand binding pocket is located between TMs 2–7 and is covered by EL1, EL2, and EL3. Published mutagenesis data on ORs claim that peptide selectivity occurs at the EL1–EL3 regions [33–35]. For the specific case of DynA it has been reported that residues from EL2 and EL3 confer the site selectivity to HKOR [13,33,36,37]. The HKOR ELs are negatively charged, while YFa has four positively charged side chains; Lys^6, Lys^7, Lys^8 and Arg^{11}. Therefore it is reasonable to expect complementarities between the positively charged residues of YFa and negatively charged residues of kappa EL regions. Recognition of YFa by the HKOR occurs between residues 4–10 of YFa and various residues of kappa EL. After molecular docking, the positively charged residues of YFa were found docked into the negatively charged regions of HKOR ELs which was surrounded by the amino acids namely Asn^{25}, Ser^{26}, Arg^{202}, Asp^{204}, Pro^{215}, Asp^{216}, Glu^{297}, Ser^{311}, Ser^{311}, Ala^{308}, Ser^{311}, Ser^{310}, Ala^{307}, Thr^{306}, His^{304}, Ser^{303}, Ala^{307} and the orientation of the peptide was observed to favor its stability required for interaction with the receptor. A list of inter atomic contacts between YFa and HKOR is shown in Table 2. Table 3 lists the amino acids of HKOR within 5 Å of each residue of YFa that might contribute to the stability of the peptide in the binding site. The hydrophobic residues of YFa (Tyr^1, Phe^4, Phe^9, Phe^{12} Met^5, and Met^{10}) make van der Waals contacts with residues of kappa ELs as well as several other hydrophobic and aromatic residues at the extracellular ends of TM6 and TM7 of HKOR. The side chain hydroxyl group of Tyr^1 forms a hydrogen bond (H bond) with the side chain amino group of Arg^{202} of EL2. The two glysine residues (Gly^1, Gly^2) having no side chains do not involve in the interaction with HKOR, however, the backbone oxygen and nitrogen atoms of both these amino acids contribute to the stability of the peptide by forming hydrogen bonds with Asn^{25} and Ser^{26} of N terminus of HKOR. The side chain aromatic ring of the fourth phenylalanine of YFa was found to be entangled within a hydrophobic cleft formed by Pro^{23}, Pro^{24}, Pro^{31} of N terminal domain and Pro^{215}

Table 2
The inter-molecular hydrogen bonds between the residues of HKOR and the residues of YFa along with the bond distance. SCG, side chain guanidine group of arginine; NT, N terminal; MC, main chain; SC, side chain.

Interacting residues (hydrogen bonds)							
HKOR				YFa			Distance (Å)
No#	Residue	Group	Atom	Atom	Group	Residues	
1	Arg^{202}	N (SCG)	N	H	OH (SC)	Tyr^1	2.8
2	Asp^{216}	OH (SC)	OD1	H	NH (NT)	Tyr^1	2.4
3	Asn^{25}	NH (SC)	H	O	CO (MC)	Gly^2	1.8
4	Ser^{26}	NH (MC)	H	O	CO (MC)	Gly^2	2.5
2	Asn^{25}	NH (SC)	H	O	CO (MC)	Gly^3	1.9
3	Asn^{25}	CO (SC)	OD1	H	NH (MC)	Phe^4	2.5
4	Asp^{216}	NH (MC)	H	O	CO (MC)	Phe^4	1.5
5	Pro^{215}	CO (MC)	O	H	NH (MC)	Met^5	1.8
6	Pro^{215}	CO (MC)	O	H	NH (MC)	Lys^6	2.0
7	Glu^{297}	CO (SC)	OE2	H	NH (MC)	Lys^8	2.8
8	Glu^{297}	OH (SC)	OE1	H	NH (MC)	Lys^8	2.1
9	Asp^{204}	CO (MC)	H	H	NH (SC)	Lys^8	2.4
10	Glu^{297}	OH (SC)	OE1	H	NH (MC)	Phe^9	2.2
11	Ser^{311}	OH (SC)	H	O	CO (MC)	Met^{10}	2.2
12	Ser^{311}	NH (MC)	H	O	CO (MC)	Met^{10}	2.3
13	Ala^{308}	CO (MC)	O	H	NH (MC)	Arg^{11}	2.9
14	Ser^{311}	NH (MC)	H	O	CO (MC)	Arg^{11}	2.4
15	Ser^{310}	NH (MC)	H	O	CO (MC)	Arg^{11}	2.6
16	Ala^{307}	NH (MC)	H	N	N (SCG)	Arg^{11}	1.8
17	Thr^{306}	NH (MC)	H	N	N (SCG)	Arg^{11}	1.7
18	His^{304}	CO (MC)	O	H	NH_2 (SCG)	Arg^{11}	2.7
19	Ser^{303}	CO (MC)	O	H	NH_2 (SCG)	Arg^{11}	2.3
20	Ala^{307}	CO (MC)	O	H	NH (MC)	Phe^{12}	2.3

Table 3
The amino acids of HKOR present within 5 Å of each residues of YFa. There are several polar and negatively charged amino acids of HKOR such as serine, aspartic acid and glutamic acid that frequently occur within the 5 Å range of YFa imposing strong electrostatic interactions with the positively charged amino acids of YFa (lysine and arginine). Also an ensemble of hydrophobic residues (bold marked) present in the 5 Å radius of each YFa may exert hydrophobic attraction on the amino acid residues tyrosine, phenylalanine and methionine present in YFa.

Residues of YFa	Residues of HKOR	Domain of HKOR
Tyr[1]	Asn[25], Ser[26], Ser[27], **Trp[29]**, **Phe[30]**	N-terminal
	Arg[202], Gln[213], **Phe[214]**, **Pro[215]**, Asp[216], Asp[217], Asp[218]	EL2
Gly[2]	Asn[25], Ser[26], **Trp[29]**	N-terminal
	Arg[202], Asp[216]	EL2
Gly[3]	**Pro[23]**, **Pro[24]**, Asn[25], Ser[26], **Trp[29]**, **Phe[30]**, **Pro[31]**	N-terminal
	Arg[202]	EL2
Phe[4]	**Pro[23]**, Asn[25], **Trp[29]**, **Phe[30]**, **Pro[31]**	N-terminal
	Arg[202], Glu[203], Asp[204], **Pro[215]**, Asp[216]	EL2
Met[5]	**Cys[21]**, **Pro[23]**, Asn[25], **Phe[30]**	N-terminal
	Pro[215], Asp[216]	EL2
	Glu[297]	TM6
	Gly[300], Ser[301]	EL3
Lys[6]	**Phe[30]**	N-terminal
	Gln[213], **Phe[214]**, **Pro[215]**, Asp[216]	EL2
	Asp[223], Lys[227]	TM4
	Glu[297], **Ala[298]**	TM6
Lys[7]	**Phe[214]**, **Pro[215]**, Gly[300], Ser[301], Thr[302]	EL2
	Phe[293], Ile[294], Val[296], Glu[297], Ala[298]	TM6
	Tyr[312]	TM7
Lys[8]	**Phe[30]**, Gly[32],	N terminal
	Asp[204], **Val[205]**, Asp[206], **Pro[215]**	EL2
	Glu[297]	TM6
	Thr[302], Ser[305], **Ala[308]**	EL3
	Tyr[312]	TM7
Phe[9]	**Phe[293]**, Glu[297]	TM6
	Thr[302], Ser[303], His[304], Thr[306], **Ala[307]**, **Ala[308]**	EL3
	Tyr[312]	TM7
Met[10]	**Val[205]**, Ser[211]	EL2
	Ile[290], **Phe[293]**, **Ile[294]**	TM6
	Ala[308], **Leu[309]**	EL3
	Ser[311], **Tyr[312]**	TM7
Arg[11]	Gly[32]	N-terminal
	Val[205]	EL2
	Phe[293]	TM6
	Ser[303], His[304], Ser[305], Thr[306], **Ala[307]**, **Ala[308]**, **Leu[309]**, Ser[310]	EL3
	Ser[311]	TM7
Phe[12]	**Phe[293]**	TM6
	Thr[306], **Ala[307]**, **Ala[308]**, **Leu[309]**	EL3

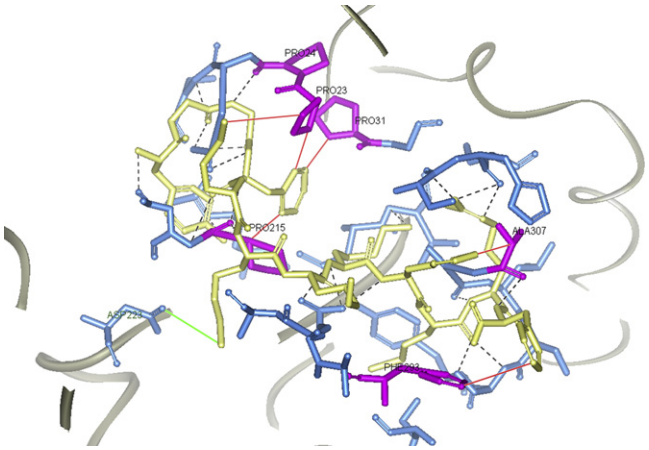

Fig. 6. YFa interactions with HKOR. The image illustrates inter-molecular hydrogen bonds between the peptide YFa and HKOR binding site residues. From the figure it can be seen that the peptide interacts with the charged residues of the receptor through H bonds, hydrophobic interactions and salt bridges. H-bonds are represented as dashed lines, salt bridge as solid green line, solid red lines for hydrophobic interactions, hydrophobic amino acids are tinted as magenta, the peptide is colored in light yellow and the binding site residues of HKOR are in light blue shade for visual assistance.

of EL2 of HKOR (Fig. 6). By observing the orientation and distance of the side chain of Met[5] of YFa in relation to Pro[23] a hydrophobic attraction with a atomic distance of 4.8 Å between them can be reliably predicted. The side chain amino group (NH_2) of Lys[6] of YFa was situated at a distance of 4.2 Å from the side chain carboxyl group (COOH) of Asp[223] of HKOR and was assumed to form a salt bridge which contributes to the additional stability of the peptide in the binding pocket of HKOR. The side chain NH_2 group of Lys[7] of YFa interacts through an H bond with the backbone CO group of Asp[204]. Glu[297] present in the HKOR TM6 stabilizes the peptide by forming three H bonds with Lys and Phe of YFa in which, the two oxygen atoms (OE1 and OE2) form H bonds with the main chain NH group of Lys[8] and the OE1 atom forms H bond with backbone NH of Phe[9]. Met[10] of YFa was found at a distance of 3 Å from Tyr[312] and the side chain of Met[10] was observed to be placed such that a hydrophobic interaction could be predicted between the two residues. Arg[11] of YFa forms a strong H bond network with the backbone NH and CO groups of several amino acids of the EL3 of HKOR. The amino acids of HKOR EL3 involved in the binding of Arg[11] of YFa were Ser[303], His[304], Thr[306], Ala[307], Ala[308], Ser[310], and Ser[311] of which the side chain guanidine group of Arg interacts with His, Thr and Ala. A 2D plot of the inter-atomic hydrophobic and electrostatic or polar interactions is shown using ligplot (Supporting data, ligplot.tif).

For the additional validation of the docking mode of YFa, molecular modeling of DynA (1–10), an endogenously occurring kappa specific peptide was carried out. Though in the initial design we have shown that the YFa peptide was derived from Met-enkaphlin but for the structural comparison of docked peptides we chose DynA for comparison as [Met] – and [Leu]enkephalin have high affinities for delta-receptors, ten-fold lower affinities for mu receptors and negligible affinity for kappa receptors. Other products of processing of pro-enkephalin, which are N-terminal extensions of [Met]enkephalin, have a decreased preference for the delta receptor with some products, e.g. metorphamide displaying highest affinity for the mu receptor. The opioid fragments of prodynorphin, particularly dynorphin A and dynorphin B, have high affinity for kappa receptors but also have significant affinity for mu and delta receptors [38]. The protocols of modeling and docking of DynA (1–10) were similar to that of YFa described in Section 2.3. Tables 1 and 3 show that both the peptide agonists bind in the same general region of the receptor. In addition, the binding site on the receptor can accommodate each of these structurally similar ligands in such a way that the common recognition interactions are preserved (Asn[25], Ser[26], Arg[202], Pro[215], Glu[297] and Ser[311]). The specific residues of the receptor that interact with the DynA (1–10), further validate that both DynA and YFa are recognized by the receptor through the interaction of similar residues (Table 4). Several negatively charged side chains from EL2 (Asp[204], Glu[209], Asp[216]) and EL3 (Glu[297]) have been suggested to form the lining in the binding cavity [35] and one of the specific interaction of Glu[297] with arginine of Dyn was also observed in our case is specific for KOR interaction. We observed H bond formation involving Try1 of both the peptides with Arg[202] in contrast to another known interaction [35] with Asp[138] which was missing. Although both the peptides did not bind to a specific residue Asp[138] in orthosteric binding site but in our case this could be attributed to conformation of the proposed EL2 which in turn prevents the ligand

Table 4
The inter molecular residue contacts between DynA (1–10) and HKOR homology model. SCG, side chain guanidine group of arginine; NT, N terminal; MC, main chain; SC, side chain.

Interacting residues (hydrogen bonds)							
HKOR			DynA (1–10)				Distance (Å)
No#	Residue	Group	Atom	Atom	Group	Residues	
1	Ser^{26}	OH (SC)	O	H	NH_2 (NT)	Tyr^1	1.5
2	Trp^{29}	CO (MC)	O	O	CO (MC)	Tyr^1	2.6
3	Trp^{29}	CO (MC)	O	H	NH (MC)	Gly^2	2.3
4	Arg^{202}	NH_2 (SCG)	H	O	CO (MC)	Gly^2	1.6
5	Asn^{25}	NH (MC)	H	O	CO (MC)	Gly^2	1.5
6	Asn^{25}	CO (MC)	O	H	NH (MC)	Phe^4	2.7
7	Pro^{215}	CO (MC)	O	H	NH (MC)	Leu^5	2
8	Ser^{301}	NH (MC)	H	O	CO (MC)	Arg^6	2.1
9	Glu^{297}	OH (SC)	OE1	H	NH_2 (SCG)	Arg^7	2.6
10	Glu^{297}	CO (MC)	OE2	H	NH_2 (SCG)	Arg^7	2.6
11	Leu^{299}	CO (MC)	O	H	NH (MC)	Ile^8	1.5
12	Ser^{301}	OH (SC)	O	H	NH_2 (SCG)	Arg^9	1.6
13	Glu^{297}	OH (SC)	OE1	H	NH_2 (SCG)	Arg^9	2.4
14	Glu^{297}	CO (SC)	OE2	H	NH_2 (SCG)	Arg^9	2.5
15	Tyr^{312}	OH (SC)	H	N	N (SCG)	Arg^9	1.7
16	Leu^{212}	CO (MC)	O	H	NH_2 (SCG)	Arg^9	2.5
17	Ser^{311}	OH (SC)	H	O	CO (MC)	Pro^{10}	2
18	Tyr^{312}	NH (MC)	H	O	CO (MC)	Pro^{10}	2.2

entry into the TM core. These explicit ligand receptor complexes obtained by docking simulations provide a comparison between the mode of interaction of a synthetic and an endogenous ligand which shows that the two peptides bind to similar regions of the receptors largely having similar requirements for recognition with only minor variations (Figs. 7 and 8). There is some evidence of dynorphin binding to kappa opioid receptor in dualsteric based on computational modeling [39] but in the receptor model proposed by us the EL2 will restrict the entrance of the message part to the aromatic core between the TM helix. This is also supported by literature [35] as binding pocket in KOR is quite smaller in regions between EL2 and TM7 and between EL2 and TM5.

Hence the 3D model of the kappa opioid receptor and the candidate binding site identified for YFa appears to be consistent with its bioactivity observed in earlier experiments [20,21]. This model can also serve as a template for analyzing the effect of mutations on HKOR, which in turn can be used to assess and refine the model further. This iterative process involving structure based selection of candidates for mutation and refinement of model structures using

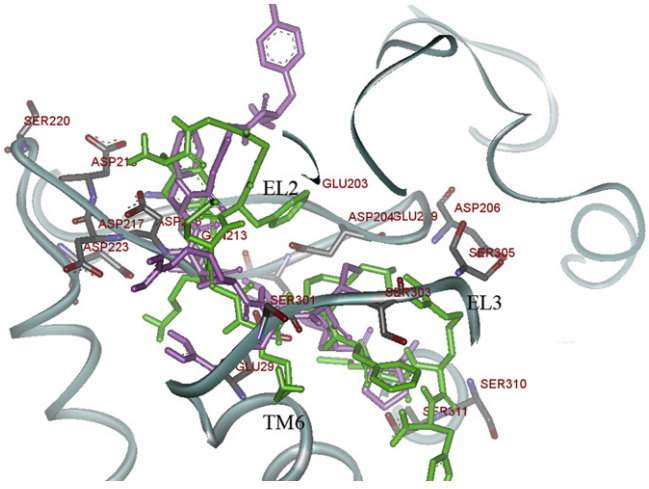

Fig. 7. Illustration of the DynA (1–10) superimposed with YFa at the binding site of HKOR homology model. The graphical interface of Discovery Studio molecular modeling software was used to capture these pictures. Yellow color represents YFa and purple color represents DynA (1–10). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

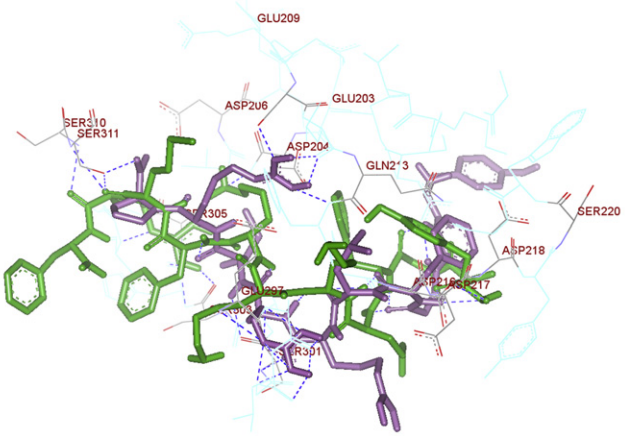

Fig. 8. Intermolecular H bonds between DynA (1–10) and YFa at the binding site of HKOR. Asn^{25}, Ser^{26}, Arg^{202}, Pro^{215}, Glu^{297} and Ser^{311} of HKOR are commonly found interacting with both the peptide showing some degree of similarity observed in the orientation of these two peptides. Yellow color represents YFa and purple color represents DynA (1–10). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

these results should ultimately lead to a 3D model sufficiently reliable to provide mechanistic insight into ligand recognition and activation of the opioid receptors and the design of novel kappa-selective ligands.

4. Conclusion

We have successfully modeled the 3D coordinates of HKOR and studied the molecular interaction with the peptide agonist YFa that was previously synthesized and characterized in our laboratory. This computational study revealed the importance of the positively charged amino acids incorporated in the peptide. The Lys^6 of YFa formed a salt bridge with Asp^{223} of HKOR, Lys7 increase the stability by forming H bond with Glu of HKOR. The residues conserved between the endogenous and the synthetic ligands i.e. YGGF, interact with the residues of EL2 whereas the anti-opioid segment chiefly acted on the EL3 portion of HKOR. Thus it can be concluded that the binding of the bioactive peptide YFa to the HKOR is defined by the EL regions, which is the biologically favorable site

for larger peptides as indicated by the chimeric receptor study. The correlation between results of the previous pharmacological and interaction studies and the computational modeling discussed here validate the kappa specific interaction of YFa, a potential analgesic which does not lead to tolerance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2011.10.007.

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