A Structural Feature of the Non-Peptide Ligand Interactions with Mice Mu-Opioid Receptors

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Abstract: By binding to and activating the G-protein coupled µ-, κ- and δ-opioid receptors in the central nervous system, opiates are known to induce analgesic and sedative effects. In particular, non-peptide opioid ligands are often used in clinical applications to induce these therapeutically beneficial effects, due to their superior pharmacokinetics and bioavailability in comparison to endogenous neuropeptides. However, since opioid alkaloids are highly addictive substances, it is necessary to understand the exact mechanisms of their actions, specifically the ligand-binding properties of the target receptors, in order to safely apply opiates for therapeutic purposes. Using an in silico molecular docking approach (AutoDock Vina) combined with two-step cluster analysis, we have computationally obtained the docking scores and the ligand-binding pockets of twelve representative non-peptide non-endogenous agonists and antagonists at the crystallographically identified µ-opioid receptor. Our study predicts the existence of two main binding sites that are congruently present in all opioid receptor types. Interestingly, in terms of the agonist or antagonist properties of the substances on the receptors, the clustering analysis suggests a relationship with the position of the ligand-binding pockets, particularly its depth within the receptor structure. Furthermore, the binding affinity of the substances is directly correlated to the proximity of the binding pockets to the extracellular space. In conclusion, the results provide further insights into the structural features of the functional pharmacology of opioid receptors, suggesting the importance of the binding position of non-peptide agonists and antagonists- specifically the distance and the level of exposure to the extracellular space- to their dissociation kinetics and subsequent potency.

Keywords: Cluster analysis, GPCRs, molecular docking, morphine, mu-opioid receptor, opiates.

INTRODUCTION

For thousands of years, opium and its derivatives have been used as potent therapeutic and recreational agents, particularly to treat acute and chronic pain or to induce intense states of relaxation and euphoria. Previous gene disruption studies [1] suggest opiate interaction with G-protein coupled opioid receptors, particularly the µ-opioid receptor (MOR), as the primary cause of these effects. The µ-, κ-, and δ-opioid receptors are closely related members of the γ subfamily of class A G-protein coupled receptors, which have recently been characterized by x-ray diffraction method [2-4]. These receptors are comprised by seven transmembrane α-helices (TM) connected by three extracellular and three intracellular loops. Based on the strong structural congruence of the transmembrane domains within this receptor family (TM1: 62-69%; TM2: 82-95%; TM3: 90-100%; TM4: 33-57%; TM5: 77-85%; TM6: 64-73% and TM: 78-86% identity), we focused our method [2-4]. These receptors are comprised by seven α-helices (TM) connected by three extracellular and three intracellular loops. Based on the strong structural congruence of the transmembrane domains within this receptor family (TM1: 62-69%; TM2: 82-95%; TM3: 90-100%; TM4: 33-57%; TM5: 77-85%; TM6: 64-73% and TM: 78-86% identity), we focused our investigation without the loss of generality on the ligand-binding properties of Mus musculus MOR protein (PDB: 4DKL). This opioid receptor not only constitutes a key component in preclinical research on pain management, but has further been associated with the addictive side effects of opiates and even alcoholism [5]. Hence, the main aim of the present study was to identify significant structural features of the interactions of this receptor as a representative target of non-peptide opioid ligands. Despite the therapeutic potential of neuropeptides, these substances are relatively unimportant pharmacological agents (with exception of oxytocin and vasopressin and their derivatives) [6], as they scarcely survive the gastrointestinal tract and are readily degraded by enzymes. Therefore, a focus on the binding properties of non-peptides ligands is more appropriate from the clinical perspective, and may provide insights of interest for drug design and discovery procedures.

It is noteworthy that numerous computational studies have already investigated the binding properties of different ligands at the µ-, κ- and δ-opioid receptors (for review see [7-9]); however, a global analysis of the multi-dimensional factors of the opiate docking process at these receptors is lacking.

Therefore, state-of-the-art in silico docking software AutoDock Vina [10] was utilized in combination with two-step clustering analysis to 1) obtain likely binding modes and docking scores of twelve ligands representative of preclinical investigations and 2) computationally identify any significant correlations of the calculated binding affinities.
with the spatial orientation of the binding modes and functional pharmacology of the substances.

Molecular docking is a computational method to predict non-covalent binding of macromolecules. In other words, it is a computational framework to efficiently investigate the binding properties of small ligands to receptors, reflected by an energy scoring function that accurately describes the ligand-protein interaction, starting with their unbound structures, structures obtained from MD simulations, or homology modeling [11]. Via the determination of the scoring functions, the different ligand orientations and conformations are ranked based on evaluation of the binding tightness of any putative complex [12].

The overall findings of this study not only improve our current understanding of the structural features of the functional pharmacology of opiates, but further highlight the power and appropriateness of computer-aided pharmacology for preclinical and clinical research.

MATERIALS AND METHODS

Non-Peptide Ligands and Receptor

Within this study, we investigate the docking properties of twelve non-peptide non-endogenous opioid agonists and antagonists (Fig. 1; Beta-Funaltrexamine [13]; Bremazocine [14]; Buprenorphine [15]; Diprenorphine, Etorphine, Fentanyl [16]; Morphine [17]; Naloxone [18]; Naltrexone [19]; Nor-binaltorphimine [20]; Oxymorphone [21]; U-69,593 [22]) on the backbone crystal structure (2.8 Å) of the mouse MOR in the monomer configuration [2].

Although MOR crystallizes as a two-fold symmetric dimer, molecular docking on the dimer structure did not show any binding modes specific to the dimer configuration (for instance, in the interface area formed by the transmembrane domains 5 and 6), which justifies our focus on the monomer structure of the μ-opioid receptors. All required coordinate (PDB) files for the ligands were obtained from ZINC database [23].

Molecular Docking Study

In this study, the docking software AutoDock Vina 1.1.2 [10] was utilized to investigate the binding modes of non-peptide opioids at the mice μ-receptor. AutoDock Vina treats receptors as rigid bodies and ligands as flexible molecules and provides an accurate algorithm (78% accuracy for binding mode predictions) to efficiently calculate the “empirical and knowledge-based” scoring function

\[ c = \sum_{i \in C} f_{i,t_i}(r_{ij}) \]

as an approximate for the standard chemical potentials of the system (i.e. an optimal sum of inter- and intramolecular contributions). As Trott and Olson [10] introduced, the summation is over all atom pairings that can move relative to each other, normally excluding 1–4 interactions, i.e. atoms separated by three consecutive covalent bonds. Hereby, each atom \( i \) is assigned to a type \( t_i \), and a symmetric set of interaction functions \( f_{i,t_i}(r_{ij}) \) (including van der Waals interactions, hydrogen bonding, deformation penalty and hydrophobic effects) of the interatomic distance \( r_{ij} \). Of thousands of putative ligand binding orientations/conformations at the active site of the receptor, the algorithm utilizes an efficient quasi-Newtonian optimization method (Broyden-Fletcher-Goldfarb-Shanno method) using the scoring function and its derivatives with respect to the position and orientation of the ligand to identify the binding mode with the highest likelihood. If scoring function is the energy, its derivatives with respect to the position, orientation and torsions denote the negative total force acting on the ligand, the negative total torque and negative torque projections [10].

To use AutoDock Vina to calculate ligand-receptor binding modes, hydrogen atoms were added to a randomized conformation of MOR (PDB:4DKL) and the structure file was converted to PDBQT format using AutoDockTools 1.5.6 [24]. Furthermore, the software was configured with variable search space size (a 3-dimensional volume containing the protein in total or partially, which effectively restricts where the movable atoms, including those in the flexible side chains, should lie) between 27000 and 64000 Å³ was utilized to ensure complete coverage of all possible docking modes for non-peptide ligands and an exhaustiveness value of 1000 (default value=8; the 125-fold enhancement lead to a linear increase of calculation time, while it decreases the probability of not finding the minimum exponentially within the enlarged search space) to identify the most likely binding sites. The calculations were performed on a Dell Precision T7500 Workstation with 6 quadcore Xeon CPUs (each 3.33 GHz).

Post Docking Analysis

The spatial orientations of the docking modes of opiates were considered as a function of continuous numerical and discrete categorical variables. In order to discover patterns in mixed datasets derived from the literature, we applied a two-step clustering algorithm [25], a part of IBM SPSS statistics software. This clustering technique allows clustering data with both continuous and categorical attributes and uses a distance measure derived from a probabilistic model. The distance between two clusters is equivalent to the decrease in log-likelihood function. In a first step, a k-means procedure was applied to pre-cluster the data. Subsequently, we conducted a modified hierarchical agglomerative clustering procedure combining the objects sequentially to form homogenous clusters. Furthermore, using Bayesian Information Criterion (BIC), the procedure indicated the importance of each variable (predictor) for the formation of a specific cluster. To guarantee the robustness of this analysis, we investigated the datasets statistically with respect to the clustering parameters by one-way analysis of variance (ANOVA) using the Holm-Bonferroni method with a global level of significance of \( \alpha=0.05 \) and identified significant heterogeneity factors.

RESULTS AND DISCUSSION

The docking study was performed on the 2.8 Å crystal structure of the mouse μ-opioid receptor in complex with the
Fig. (1). Chemical structure of the investigated opioid agonists and antagonists.
irreversible morphinan antagonist Beta-funaltrexamine. In order to validate the results of our study, we investigated the docking properties of Beta-funaltrexamine (betaFNA) and compared the calculated optimal docking mode with the experimentally obtained binding position [2]. Both the experimental and in silico study of docking of betaFNA agree (RMSD=0.089 Å) in their determination of its binding site facing toward transmembrane domains 3, 5, and 6 (Fig. 2).

Therefore, we used the docking position of this antagonist as the reference value (center of the coordinate system) for statistical comparison of the center of masses of the agents under investigation.

The calculated binding affinities as well as the centers of masses of the ligands at the binding sites are presented in Table 1. We further applied a two-step clustering analysis to identify significant chemical and/or structural properties shared by the functional groups of the ligands. In general, the docking study suggests the presence of two binding pockets (Fig. 3) that are preferred by the majority of ligands under investigation.

Thereby, the orientation of the pocket 1 as a volume spanned between transmembrane domains TM3, TM4, TM5 and TM7 and pocket 2 as a volume spanned between TM2 and TM3 are confirmed (see Fig. 4 for the amino acids that are involved in the docking processes).

In particular, the study suggests 11 residues lying in close proximity to the ligands that are most likely involved in the ligand-receptor interactions. These are: Tyr 128, Asp 147, Tyr 148, Ile 155, Trp 293, Ile 296, Val 300, Trp 318, His 319, and Gly 325. For five ligands (betaFNA, fentanyl, etorphine, naltrexone and U69593), this prediction is in agreement with previous studies [26-42].

Furthermore, the clustering analysis showed a statistical difference in the spatial orientation of the agonists and antagonists. While both substance groups are buried deeply within solvent-exposed pockets of the transmembrane domain (Fig. 3), the analysis suggests that the binding sites (and most dominantly within pocket 1) of agonists lie on average 3.81±1.1 Å deeper (with respect to y-axis) than the antagonists. In particular, the antagonists in pocket 1 and pocket 2 were on average 4.92±0.8 Å and 1.59 ±0.2 Å, respectively, closer to the extracellular space.

Table 1. Calculated docking parameters for the opioid agonists and antagonists. COM refers to the position of the center of mass of the ligands in their respective binding pockets, which are clustered into two main groups (1 and 2), with respect to the experimentally confirmed position of Beta-Funaltrexamine. Y-axis represents the depth of binding within the receptor, whereas x- and z-axis span y-tangential planes. The investigation shows that agonists are buried much deeper inside the receptor than antagonists.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Pocket Cluster</th>
<th>Docking Score (kcal/mol)</th>
<th>Function</th>
<th>COM (x)</th>
<th>COM (y)</th>
<th>COM (z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprenorphine</td>
<td>1</td>
<td>-5.9</td>
<td>Agonist</td>
<td>0.52</td>
<td>-5.96</td>
<td>1.09</td>
</tr>
<tr>
<td>Etorphine</td>
<td>1</td>
<td>-5.8</td>
<td>Agonist</td>
<td>0.21</td>
<td>-7.14</td>
<td>0.52</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>1</td>
<td>-5.3</td>
<td>Agonist</td>
<td>0.16</td>
<td>-8.0</td>
<td>2.55</td>
</tr>
<tr>
<td>Oxymorphone</td>
<td>1</td>
<td>-6.2</td>
<td>Agonist</td>
<td>-0.84</td>
<td>-5.04</td>
<td>2.28</td>
</tr>
<tr>
<td>U69593</td>
<td>1</td>
<td>-5.4</td>
<td>Agonist</td>
<td>-0.08</td>
<td>-5.5</td>
<td>1.58</td>
</tr>
<tr>
<td>Beta-Funaltrexamine</td>
<td>1</td>
<td>-6.2</td>
<td>Antagonist</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diprenorphine</td>
<td>1</td>
<td>-6.0</td>
<td>Antagonist</td>
<td>-0.84</td>
<td>-4.09</td>
<td>0.42</td>
</tr>
<tr>
<td>Nor-binaltorphimine</td>
<td>1</td>
<td>-7.4</td>
<td>Antagonist</td>
<td>-0.4</td>
<td>0.15</td>
<td>0.36</td>
</tr>
<tr>
<td>Bremazocine</td>
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<td>-5.4</td>
<td>Agonist</td>
<td>4.67</td>
<td>-3.92</td>
<td>-9.66</td>
</tr>
<tr>
<td>Morphine</td>
<td>2</td>
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<td>Agonist</td>
<td>4.95</td>
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<tr>
<td>Naloxone</td>
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<td>Antagonist</td>
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<td>-8.45</td>
</tr>
<tr>
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<td>-6.1</td>
<td>Antagonist</td>
<td>6.2</td>
<td>-2.53</td>
<td>-8.03</td>
</tr>
</tbody>
</table>

Fig. (2). The comparison of the experimentally obtained and computationally calculated binding mode of beta-funaltrexamine at the µ-opioid receptor show a high level of conformity (RMSD (28)=0.089 Å) between both binding modes (in silico binding shown in yellow and co-crystallized binding in blue).
Structural Feature of the Non-Peptide Ligand


Fig. (3). The positions of ligand-binding pockets in the structure of opioid receptors. Pocket 1 constitutes a volume between the transmembrane domains (TM) 3 to 5 and 7, whereas pocket 2 takes a much smaller volume stretched between TM2 and TM3. In comparison to antagonists (blue), opioid agonists (green) are buried deeper in the GPCR helices, which is most likely associated with their slow dissociation kinetics. Moreover, the positioning of antagonists appears to hinder the access of agonists to their binding sites rather than altering the binding affinities by conformational changes.

In addition to the structural differences, the calculated docking scores of the different ligand types differed significantly ($F(1,10) = 5.433; p<0.01$; Agonists: -5.59 kcal/mol; Antagonists: -6.36 kcal/mol). Hereby, $F$ denotes the ratio between the variance between agonists and antagonists and the variance with the agonist and antagonist groups. The statistical analysis of the differences between agonist and antagonist binding suggests that on average, antagonists bind significantly stronger to the mu-receptor.

These results suggest that proximity and exposure to the extracellular space is in direct correlation with the calculated binding affinities and the subsequent potency of the drugs, a hypothesis that has also been proposed by Manglik and colleagues [2] based on the observation that extremely potent opioids such as buprenorphine (Ki 740pM), diprenorphine (Ki 72pM), and etorphine (Ki 230 pM) present rapid dissociation half-lives of 44 minutes to less than 1 minute. This proposed structural basis of the pharmacology of opioid receptors is further supported by the observation that opioid antagonists bind closely to the extracellular space and thereby hinder the access of agonists to their respective binding sites.

In conclusion, this *in silico* study presents a novel hypothesis of the possible mechanisms of action of non-peptide drugs at opioid receptors and thus provides a quantitative structural feature of the functional pharmacology of opioid receptors that may be beneficial for the development of novel analgesic and anti-addiction compounds. In particular, the study suggests the importance of the proximity of the opioid agents to the extracellular space as an indicator of their efficacy. This finding, together with the observation that the binding modes of antagonists (most prominently within pocket 1) are closer to the extracellular space than the agonists, suggests that in addition to the chemical interactions, the inhibition of opiate actions may be due to a physical barrier (formed by the binding of antagonists) above the binding sites of agonists.

Despite the advantages of computer-implemented docking investigations, this study was restricted to a specific static state of the receptor and does not provide any further information on the dynamics of the ligand-binding processes. Particularly, the time-course of the substance-induced effects

Fig. (4). 11 residues (Tyr 128, Asp 147, Tyr 148, Ile 155, Trp 293, Ile 296, Val 300, Trp 318, His 319, and Gly 325) lying in close proximity to the ligands, which are most likely involved in the ligand-receptor interactions (right pocket: cluster 1; left pocket: cluster 2).
on the receptor in a natural environment (e.g. embedded in a neuronal membrane) has not been considered in the present study, a major limitation that suggests the need for future in silico investigations of this subject, such as those using molecular dynamics simulations [9, 43]. Furthermore, due to the limited number of ligands under investigation, the robustness of the observed pattern and statistical conclusions should be interpreted with special care.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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