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# Catechins modulate the activity of mu opioid receptor (μOR): An in silico approach

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ABSTRACT

Treating addiction and withdrawal syndrome remains a research area of great interest since it is a persistent public health problem. Opiates account for the majority of addiction and withdrawal problems (at least 56% of reported people who consume drugs). Development of opiate addiction results mainly from activation of the mu-opioid receptor (MOR), and this entity is identified as a major target for the treatment of addiction and withdrawal syndrome. Based on our in silico analysis and calculations of binding energy, conformations adopted by catechins and contacts reached by ligand conformations could explain why opiates are capable of activating both β-arrestin and G-protein biased pathways, and therefore help to explain the phenomenon of tolerance, we can conclude that the family of catechins explored seem to be excellent modulators of the MOR.

## 1. Introduction

Substance use disorders (SUDs) are defined as a set of symptoms resulting from substance abuse. These symptoms include withdrawal, uncontrolled increasing intake, development of tolerance, and craving for the substance [1]. Treating addiction and withdrawal syndromes remains a research area of great interest because substance abuse is a persistent public health problem. Recently, the World Health Organization (WHO) estimated that at least 35 million people worldwide suffer from drug use disorders and require treatment services.

Opioid use disorder (OUD) is the maladaptive use of this class of agents resulting in impaired health or function over a 12 month period [2] with poor health outcomes such as liver disease, pancreatitis, cardiomyopathy, chronic obstructive pulmonary disease, cancer, cardiovascular disease and other chronic health conditions, depending on the agent, dose, and combination of drugs [3]. Opiate derivatives seem to be the most abused and generate most of the addiction and withdrawal problems (at least 56% of reported cases) [4] *(United Nations publication, Sales No. E.19. XI.8)*.

The mechanisms involved in the development of opiates addiction appear to include: 1) activation of the MOR, a G-protein coupled receptor (GPCR) linking to $G_i$, 2) inhibition of adenylyl cyclase and phosphorylation of cAMP-dependent proteins in the locus coeruleus (LC) (the brain region with the largest number of noradrenergic neurons) and, 3) activation of the reward circuit in the brain, involving the dopaminergic neurons of the ventral tegmental area and their extension towards the nucleus accumbens and the frontal regions of the cerebral cortex [5].

In response to the reduced synthesis of cyclic AMP under the influence of MOR stimulation, there appears to be a compensatory increase in the cellular capacity to produce cyclic AMP. As a consequence, during withdrawal of the agonist, there is an increase in adenylyl cyclase activity and the production of cyclic AMP beyond normal levels. There are also changes in brain areas to where the action of dopaminergic neurons extends, resulting in a compulsive search and "abuse" of the drug, as well as high probability of relapse, even after several years of abstinence [6].

*Abbreviations:* C, (+)-Catechin; EC, (-)-Epicatechin; ECG, (-)-Epicatechin gallate; EGC (-)-Epigallocatechin, EGCG; (-)-Epigallocatechin gallate, Fen: fentanyl; GPCR, G-protein coupled receptor; GR, Radius of gyration MD; Molecular dynamics, MOR; μ opioid receptor, Nal: Naloxone; RMSD, Root mean Square deviation; RMSF, Root Mean Square Fluctuation.

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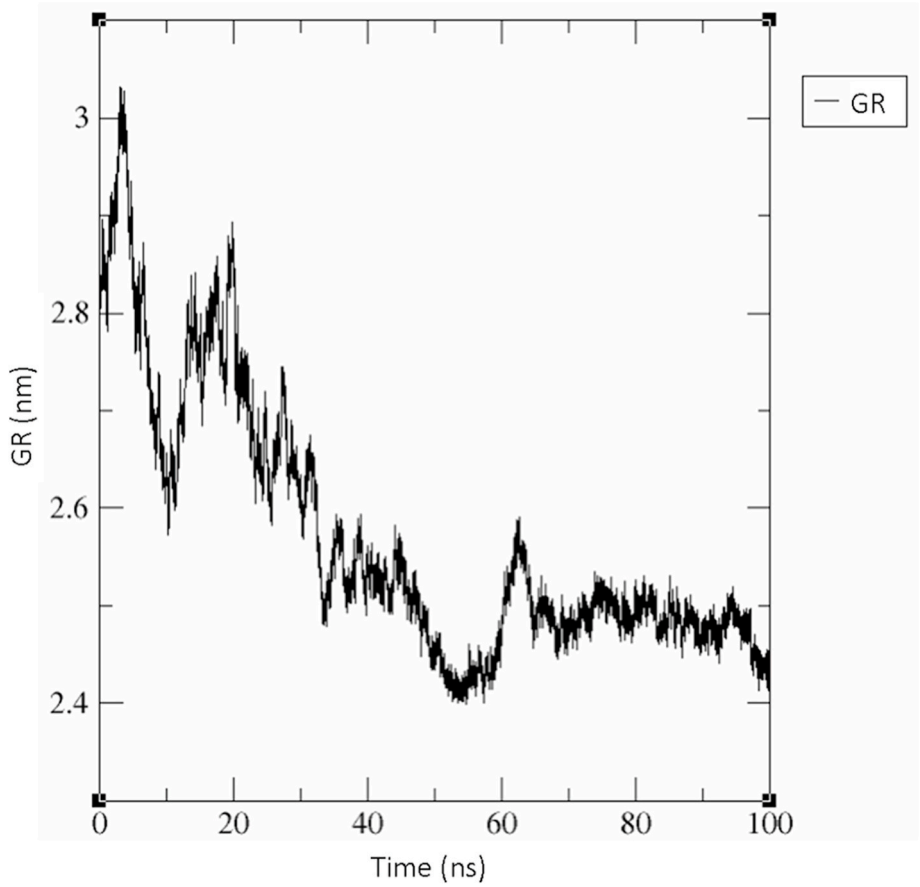


**Fig. 1.** Gyration radius (RG), a parameter which indicates a compact and organized structure, of 100 ns Molecular Dynamics of MOR.

A persistent issue that confounds efforts to design "safe" opiates is that the MOR mediates both the therapeutic effects of opiates as well as their adverse effects [7]. One of the potential therapeutic strategies to treat addiction and withdrawal syndrome is the modulation of MOR's effects on adenylyl cyclase activity. Although there are drugs that are used to treat addiction and withdrawal (which characteristics are well described in diverse databases) [8,9], it is desirable to find alternative treatments that are possess minimal or lack adverse effects.

Natural compounds have been explored for their capacity to beneficially impact opiate addition and/or withdrawal syndromes. Epigallocatechin gallate (EGCG) has strong pharmacological activity against the development of morphine dependence, which can be partly explained by its inhibitory effects on the morphine-induced increase in the cAMP levels in the locus coeruleus and the signaling of the dopamine D2 receptor [10].

In this regard, the objective of the present work is to examine using in silico analysis, select flavonoids, particularly of the catechin family, as molecular modulators (i. e. agonists, antagonists or allosteric modulators) of MOR. This concept is based on the following:

**1) Molecular components**. It is known that most of the compounds that bind to MOR contain the following essential components: a) Nitrogen within their structure [11] which, is considered essential to the interaction with the receptor active site. b) Richness in hydrogen acceptors, i.e., oxygen atoms, such as the proposed catechins and c) Aromatic rings [12,13], which can bias a receptor-response toward a particular pathway. Biased agonists selectively activate one of several possible pathways, leading to a selective effect depending on the microsite reached. β-arrestin-biased signaling may be relevant in these processes [14]. Flavonoids are good candidates for this effect.

**2) Demonstrated effects**. Select non-central nervous system (CNS) catechin effects are mediated by their interaction with opioid receptors [15–18].

Considering the molecular moieties of catechins and these reports on their interaction with opioid receptors, we have modeled the potential interactions of catechins with MOR. The results may improve knowledge in the area of alternatives for opioids addiction, withdrawal syndrome, overdose and even some other actions involving the receptor and its effects on the CNS, such as modulating nociception [19].

## 2. Methods

### 2.1. MD simulations

The 3D model of the human MOR was obtained through the i-TASSER website, based on the protein sequence from Uniprot database [20–22]. The most viable model was chosen based on the Root Mean Square Deviation (RMSD) value of 0.84 Å, calculated from the template of the human delta opioid receptor (PDB ID: 4n6hA), and the parameters proper of the website. Non-polar hydrogen atoms were removed, and Gasteiger charges were assigned with AutoDock Tools v.4.2 (ADT). Prior to molecular dynamics (MD), the receptor was parameterized using the CHARMM-GUI online program, where the cell membrane was also parameterized and designed with 10% cholesterol, 45% phosphatidylcholine (PC) and 45% dipalmitoyl phosphatidylcholine (DPPC). The system was equilibrated under 100 ps in a constant volume ensemble (NVT) and 100 ps in a constant pressure ensemble (NPT) and simulated under the periodic boundary condition (PBC) in a cubic box and transferable intermolecular potential with a 3 points (TIP3P) water model. Then, a 100 ns MD run was performed using the GROMACS 5.1 package. The analysis of the produced trajectory from the simulation was rendered with Visual Molecular Dynamics software (VMD), along with some tools included in GROMACS software, Root Mean Square

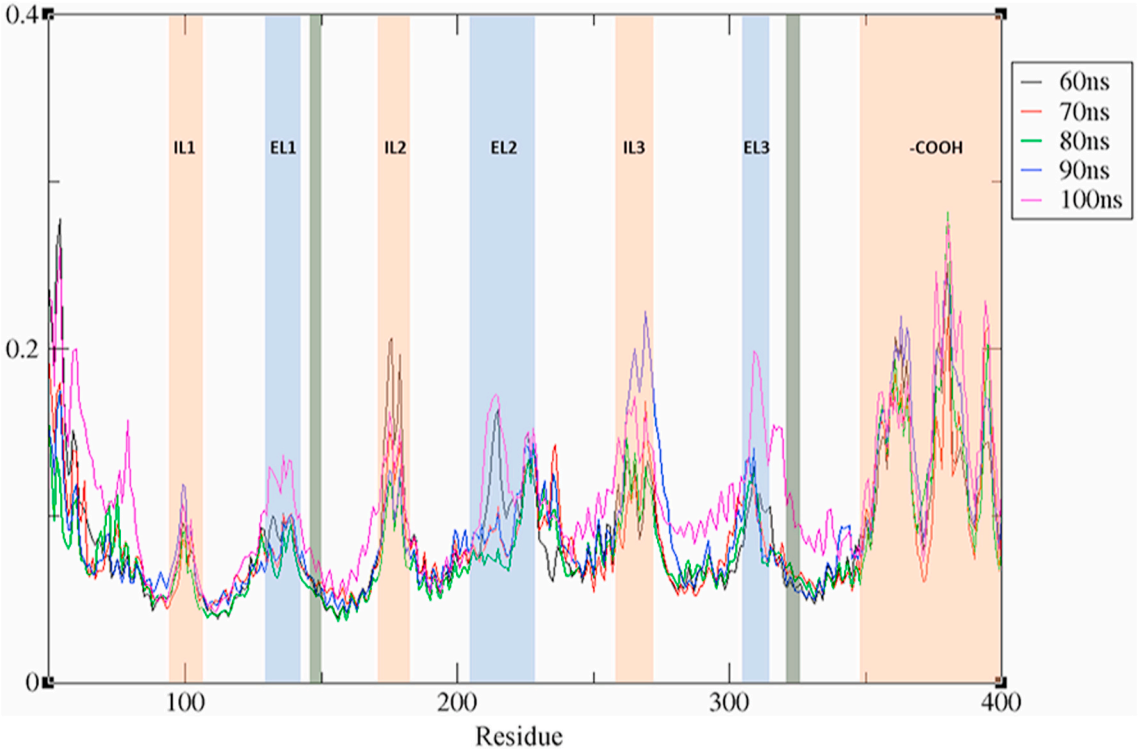


**Fig. 2.** Fluctuation of side chains for each amino acid (Root Mean Square Fluctuation). Green shaded rectangles mark D149 and W320, important amino acids into the binding site (ICL: Intracellular Loop. ECL: Extracellular Loop). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fluctuation (RMSF) and Radius of Gyration (GR) calculation. The frame from each 10 ns of the trajectory was extracted and used for the docking studies. To discern the type of conformation that each obtained frame of the receptor has (i.e., active, inactive or intermediate state), the volume cavity of the binding site was measured with UCSF Chimera v.1.14. and compared to those reported previously [23].

### 2.2. Docking assay

All ligand structures [fentanyl (Fen), naloxone (Nal), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG), catechin (C) and (-)-epicatechin (EC)] were obtained from the Chemspider online server. Ligands were energetically and geometrically optimized in Discovery Studio v.4 and ADT software. With a direct docking approach, the grid box employed was of 40 × 40 × 40 Å with 1 Å of spacing, centered at the αCarbon of D149, as this residue has been suggested to be crucial for the binding of drugs containing nitrogen in their structure [24]. 1000 independent docking assays were carried out with AutoDock Vina [25]. The results were analyzed with local UNIX scripts; clusters were built comparing the slight variation in the binding site with the equation used to calculate RMSD. The cluster with the highest number of repetitions was then analyzed to find potential patterns of interacting residues in the ligand-protein complex on 2D images built with Discovery Studio.

## 3. Results and discussion

### 3.1. Protein stability and analysis

GR and RMSF graphs (Figs. 1 and 2) show that, starting at 60 ns, the receptor begins to stabilize its movement adopting a compact and organized structure, reducing the fluctuation of each residue sidechain and maintaining the α-carbon backbone stability. Based on these arguments, the frames between 60 and 100 ns are the most reliable to analyze and to use for docking assays. In general, the MD approaches serve to determine whether the protein could reach relevant conformations to represent any possible active, inactive, or intermediate states [26]. A better exploration of these possibilities involves determination of the volume of the binding site cavity [23], which we calculated at 0, 70, 80, 90 and 100 ns in UCSF Chimera (Table 1). Comparing these volumes with those reported previously (which were determined in a 1μs MD of MOR embedded in a membrane) [23], our frames showed volumes between the values of 23,077.64 $A^3$ for an active conformation and 17,434.41 $A^3$ for an intermediate conformation [23]. Essentially, the cavities of the analyzed frames are composed by 39 amino acids. The main residues forming the interacting site are: D149, Y150, M153, W320, H321, I324 and Y328, some these residues are part of the orthosteric and/or allosteric site (each one described below). These amino acids coincide with those found in the docking analysis (Fig. 3 and Table 3). Interestingly, the conformations reached at 80 ns and 100 ns seem to have the bigger cavity, leading some residues of the extracellular loops to get in contact and stabilize the ligand at the binding site, as shown in Table 3.

**Table 1**
Calculated Volume of the main Cavity of MOR, showing volumes between an active and an intermediate state of the receptor (based on [23]].

| Nanosecond | Volume (Å3) |
|---|---|
| 70 | 19710 |
| 80 | 19870 |
| 90 | 19010 |
| 100 | 20510 |

Although, these results indicated that any of the conformations reached between 70 and 90 ns could represent a favorable state of the ligand-protein complex, we decided to look for an optimal conformation of the binding site. We analyzed docking results for each ns between 70

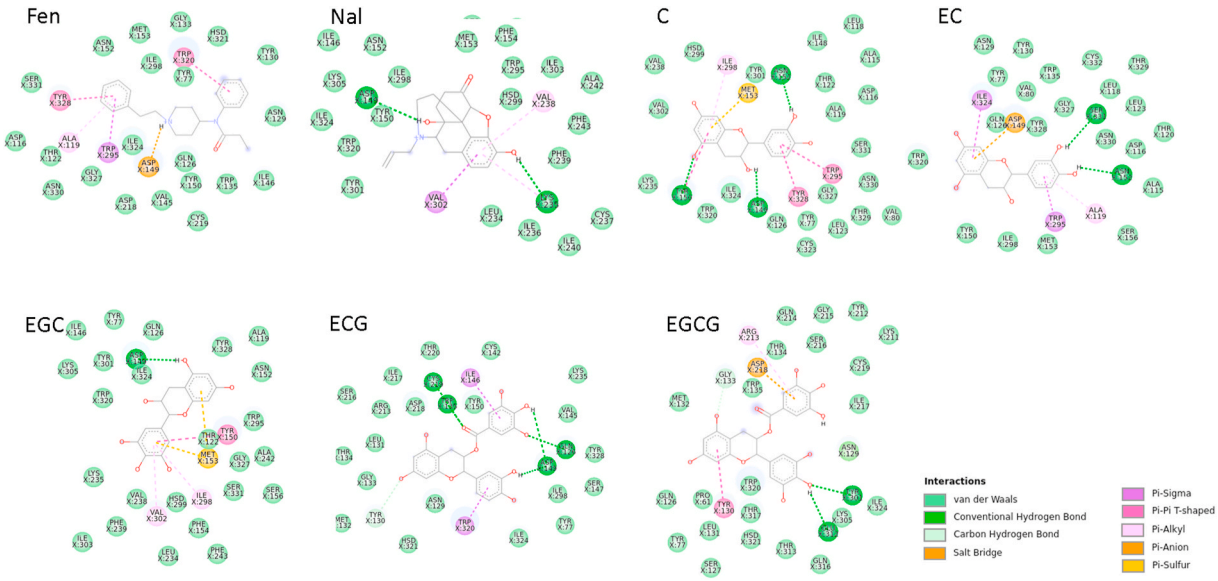


**Fig. 3.** Binding pockets and the predicted interaction of each ligand with the 80 ns conformation of MOR.

**Table 2**
Predicted interactions of evaluated ligands between 70 and 90 ns MOR conformations.

| Ligands | Conventional Hydrogen Bond | Non-conventional Hydrogen Bond | Pi-Anión | Salt-Bridge | Pi-Pi | Amide-Pi Stacked | Pi-Sigma | Pi-Alkyl | Pi-Sulfur |
|---|---|---|---|---|---|---|---|---|---|
| Nal | 28.57 | 14.29 | 14.29 | 7.14 | - | - | 7.14 | 28.57 | - |
| Fen | - | 20.00 | - | 10.00 | 40.00 | 10.00 | 10.00 | 10.00 | - |
| C | 23.53 | 11.76 | 5.88 | - | 29.41 | - | 5.88 | 17.65 | 5.88 |
| EC | 36.84 | 5.26 | 5.26 | - | 21.05 | 5.26 | 5.26 | 10.53 | 10.53 |
| ECG | 40.91 | 9.09 | 4.55 | - | 9.09 | - | 9.09 | 27.27 | - |
| EGCG | 36.36 | 9.09 | 13.64 | - | 18.18 | - | - | 22.73 | - |
| EGC | 33.33 | 5.56 | 5.56 | - | 16.67 | - | - | 27.78 | 11.11 |

and 90 ns, the reached amino acids and also the information of the GR graph (Fig. 1), which shows that movement of the protein becomes flatter from the 70 ns until reaching the 100 ns, where a disturbance in stability was found, and RMSF graph (Fig. 2) that shows a higher fluctuation of the side chains at 100 ns. Our results suggested that the 80 ns frame (Fig. 3) was the most accurate representation. This presumption was reinforced by the fact that fentanyl and naloxone are known to interact with the residues D149 and N152 in the binding site and also by results showing that all the evaluated ligands interact near this site (<1 A RMSD distance) [11].

### 3.2. *Defining possible activity of catechins based on structure and docking features*

One of the main objectives of this approach was to define a specific binding site for all evaluated ligands in order to define a specific interaction site for agonist, antagonist and probable allosteric modulators. We first found that all ligands interact at the same binding site cluster along the evaluated MOR conformations. Our results show that catechins interact with most of the conformations of the receptor that either expose residues that have been associated with the orthosteric site (D149) [24]. [27], the allosteric site (W320) [12] or with those that can activate the β-arrestin pathway (Q115 (2.60), W295 (6.48) and also W320 (7.43)) [14]. In this sense, it seems that there is little specificity amongst the compounds with respect to these sites.

The orthosteric ligand–binding site of the A family of GPCRs is a pocket in the extracellular-middle side of the receptor transmembrane (TM) region formed principally by TM3, TM5, TM6 and sometimes with TM7. The kinks and helical bulges at conserved proline and glycine residues at these TMs are essential for coupling ligand binding to global changes in conformation needed to achieve the active state, since they lower the energy barrier to main-chain conformational changes associated with the generation of different functional states (Weis and Kobilka, 2018). There are well-known agonists that binds only to the TM3, TM5, TM6, and TM7 helices (SNC-80). On the other hand, there are ligands, e. g. BMS-986121, with allosteric modulating activity that bind only to TM1, TM2 and TM7, defining an allosteric site (Burford et al., 2013; [12].

Rarely, some residues from extracellular loops (ECLs) contact the bound ligand and serve as an “anchor” that stabilizes the conformation adopted by the ligand, depending on the flexibility and the state of the receptor (i.e. active, intermediate or inactive). Some studies suggest that drugs targeting ECL2 and ECL3 could function as allosteric modulators with GPCR subtype-specific selectivity (LY2119620 to $M_2$ muscarinic receptor) (Fessler et al., 2008 [26]; O’connor, 2016; Weis and Kobilka, 2018).

We explored a possible interaction-activity pattern. The global percentage of the interactions at the binding pocket of the three snapshots (70 ns, 80 ns and 90 ns) of each ligand (Table 2), was calculated. This analysis shows an interesting result. Fentanyl (agonist) showed a high percentage of pi-pi binding interactions (40%) and naloxone (antagonist) showed an equal fraction of hydrogen bonds (28.57%) as Pi-Alkyl interactions. It is difficult to find a specific binding pattern for both ligands (fentanyl and naloxone); in general it is hypothesized that antagonists must make more hydrogen bonds [11]. This is supported by the relationship between structure and activity of the main agonist and antagonist: it seems that the not fused aromatic rings of fentanyl led the formation of exclusively Pi-Pi stacked and T-shaped interactions (doi: 10.3390/molecules24040740) (https://doi.org/10.1007/s00894-019-3999-2) and the fused rings of naloxone, also influenced by the

**Table 3**
Residues in the MOR cavity (pocket) and their location (relation of location and activity). Residues correspond to the reported in Fig. 5. (TM: trans membranal, ECL: extra cellular loop, N-term: amino terminal).

| Ligand | 70ns | Location | 80ns | Location | 90ns | Location | 100ns | Location |
|---|---|---|---|---|---|---|---|---|
| C | Y77 | TM1 | I298 | TM6 | D149 | TM3 | D218 | ECL2 |
| | Y130 | TM2 | M153 | TM3 | A119 | TM2 | W135 | ECL1 |
| | H321 | TM7 | Y150 | TM3 | M153 | TM3 | Q126 | TM2 |
| | I324 | TM7 | D149 | TM3 | W295 | TM6 | A208 | ECL2 |
| | | | Y328 | TM7 | | | I146 | TM3 |
| | | | W295 | TM6 | | | T220 | ECL2 |
| | | | N152 | TM3 | | | | |
| EC | N129 | TM2 | I324 | TM7 | Y328 | TM7 | D149 | TM3 |
| | T328 | TM7 | D149 | TM3 | A119 | TM2 | L221 | ECL2 |
| | Y130 | TM2 | S331 | TM7 | T122 | TM2 | A208 | ECL2 |
| | I324 | TM7 | G327 | TM7 | D149 | TM3 | C219 | ECL2 |
| | G133 | ECL1 | A119 | TM2 | W295 | TM6 | I146 | TM3 |
| | | | W295 | TM6 | N152 | TM3 | | |
| ECG | N129 | TM2 | C219 | ECL2 | W295 | TM6 | W135 | ECL1 |
| | D218 | ECL2 | W135 | ECL1 | A119 | TM2 | N129 | TM2 |
| | A70 | TM1 | I146 | TM3 | T122 | TM2 | T317 | TM7 |
| | | | Q126 | TM2 | D149 | TM3 | E312 | ECL3 |
| | | | D149 | TM3 | Y328 | TM7 | | |
| | | | W320 | TM7 | I298 | TM6 | | |
| | | | Y130 | TM2 | D116 | TM2 | | |
| | | | | | H299 | TM6 | | |
| EGC | Y328 | TM7 | D149 | TM3 | T122 | TM2 | D149 | TM3 |
| | Y77 | TM1 | Y150 | TM3 | A119 | TM2 | W135 | ECL1 |
| | Y130 | TM2 | M153 | TM3 | D149 | TM3 | I146 | TM3 |
| | I324 | TM7 | I298 | TM6 | M153 | TM3 | L221 | ECL2 |
| | G133 | ECL1 | V302 | TM6 | W295 | TM6 | | |
| | D218 | ECL2 | | | | | | |
| | N129 | TM2 | | | | | | |
| EGCG | N129 | TM2 | R213 | ECL2 | H299 | TM6 | D218 | ECL2 |
| | Y301 | TM6 | D218 | ECL2 | M153 | TM3 | W135 | ECL1 |
| | T317 | TM7 | G133 | ECL1 | A119 | TM2 | I217 | ECL2 |
| | E312 | ECL3 | Y130 | TM2 | Y328 | TM7 | G133 | ECL1 |
| | P61 | N-term | E312 | ECL3 | D149 | TM3 | N129 | TM2 |
| | S66 | N-term | Y301 | TM6 | I324 | TM7 | W320 | TM7 |
| | W320 | TM7 | N129 | TM2 | W295 | TM6 | T317 | TM7 |
| Fen | M132 | TM2 | W320 | TM7 | P61 | N-term | L234 | TM5 |
| | Y130 | TM2 | D149 | TM3 | Y130 | TM2 | Y150 | TM3 |
| | N129 | TM2 | W295 | TM6 | | | W320 | TM7 |
| | S66 | N-term | A119 | TM2 | | | | |
| | | | Y328 | TM7 | | | | |
| Nal | V302 | TM6 | N152 | TM3 | A119 | TM2 | W135 | ECL1 |
| | V238 | TM5 | A119 | TM2 | N152 | TM3 | N129 | TM2 |
| | K235 | TM5 | D149 | TM3 | D149 | TM3 | | |
| | D149 | TM3 | | | | | | |

**Table 4**
Percentage of the interaction associated with ECL2, orthosteric or allosteric site.

| Site | Nal | Fen | C | EC | ECG | EGC | EGCG |
|---|---|---|---|---|---|---|---|
| Allosteric | 27 | 73 | 37 | 50 | 58 | 37 | 52 |
| Orthosteric | 73 | 27 | 53 | 36 | 47 | 53 | 29 |
| ECL2 | 0 | 0 | 11 | 14 | 11 | 11 | 19 |

electron donors near the aromatic milieu, favor the formation of Pi-alkyl or Pi-Anion interactions, conserving mainly, hydrophobic interactions. However, with this approach it is complicated to stablish if the evaluated compounds can interact with MOR as agonists or antagonists, since this flavonoid family has a fused ring system and also a not fused ring, with electrodonors attached. As shown in Table 4, we observe that fentanyl forms a greater probabilistic binding percentage to an allosteric site; however, fentanyl is not an allosteric modulator, leading us to reinforce the idea of pseudo sites, and thereby, decreasing the idea of defined sites. Looking at the interactions of fentanyl in Fig. 3, one notices that it binds to D149, associated with an orthosteric site, but also binds to W320, which is associated with an allosteric site and with activation of β-arrestin pathway. Perhaps the interaction with a site classified as allosteric is not as relevant a binding property as fentanyl finding stability amongst the pseudo sites. Could these contacts could explain why opiates are capable of activating both β-arrestin and G protein pathways? Could this duality explain the phenomenon of tolerance? Of course, as this is a theoretical approach it is necessary to consider the complex network of events that happen in vivo, such as the concentration of the opioid (bioavailability) or the its multiple conformers.

In contrast, catechins do not to behave as fentanyl or naloxone. At first, the formation of hydrogen bonds by the catechins at the binding site (C 23.53%, EC 36.84%, ECG 40.91%, EGCG 36.36%, EGC 33.33%), suggest that they could exert an antagonist activity, but the fentanyl-like participation of the pi orbitals of aromatic rings of catechins in the formation of interactions complicates the interpretation. Although the formation of hydrogen bonds suggests antagonist activity, some reports indicate that this type of interaction occurs with all ligands that interact with MOR [24]. In addition, Table 4 shows the percentage of binding of all the ligands to a TM associated with allosteric (TM1 and TM2), orthosteric (TM3, TM5 and TM6), and extracellular loops (ECL2) within the time interval 70–90 ns. As mentioned before, ECL2 could function as a binding site for allosteric modulators [26]. All catechins (C, EC, ECG, EGC and EGCG) assayed interact with ECL2, and if we add the percentage of this interaction to the percentage of allosteric interaction, we obtain a high probability that they may be acting as allosteric modulators.

The results reported here can be compared with those reported by

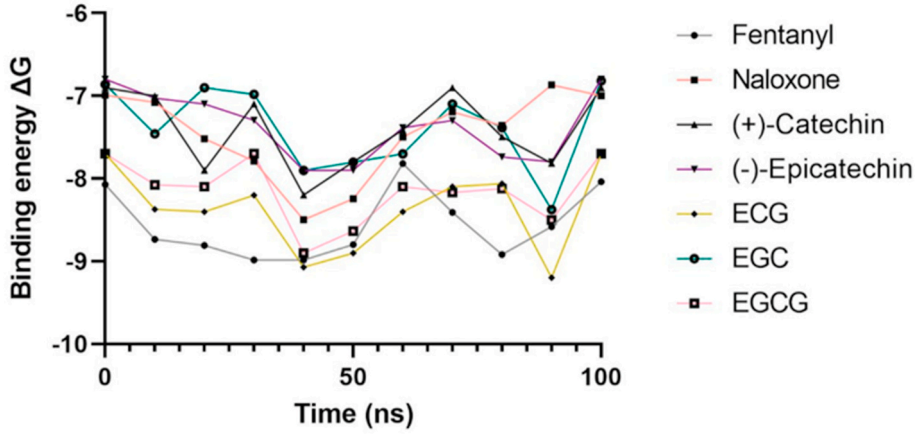


**Fig. 4.** Calculated binding energy of all ligands.

**Table 5**
Average delta G values for each ligand at most feasible selected times. The more negative the value, the more effective the binding is.

| Ligand | Average Delta G (70–100ns) |
|---|---|
| Fentanyl | −8.49 |
| Naloxone | −7.11 |
| EC | −7.41 |
| C | −7.28 |
| EGCG | −8.12 |
| ECG | −8.26 |
| EGC | −7.42 |

Katavic [15] evaluating catechins activity. They showed that no compound acts as an agonist, they showed activity as antagonists.

ECG and EGC have a Kd value of 1500 and 300 nM respectively our results show (using ΔG values obtained in the docking analysis) a theoretical Kd of 887 nM and 3660 nM for ECG and EGC respectively. Even when the values are not equal they are in the same range, however, for a more realistic affinity constant calculation it is necessary to consider occupation of receptor and agonist concentration.

Both studies suggested that EGC is more potent that ECG, and that both molecules seem to be behave as antagonists.

Another important point is the analysis of binding energy (ΔG). Looking at Fig. 4, the binding energy of all the ligands is like that of naloxone and fentanyl, especially the ECG with the closest values throughout the DM to fentanyl values. The more negative the value, the greater the affinity of the binding; when observing the average values of the binding energy for each ligand (Table 5), one can notice that all the values of the catechins are between the(ΔG of naloxone and fentanyl. Giving the guideline to establish that their unions are highly effective.

### 3.3. *Finding "pseudo sites" as a possibility of interaction-activity pattern*

With the described results and the global analysis of the binding site, we found a sequence of residues with similar chemical characteristics

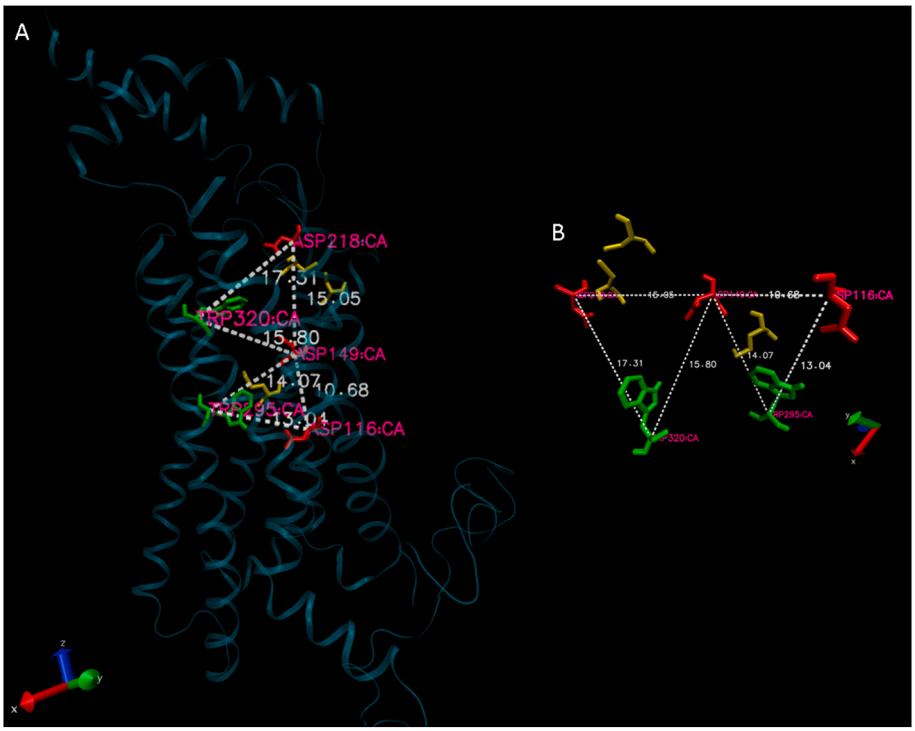


**Fig. 5.** (A) MOR in a lateral view highlighting the "pseudo sites". With a white dotted line, the distances between residues. In green, tryptophans; in red, aspartates; and in yellow the sulfur containing residues (methionine and cysteine). (B) Zoom of the pseudo sites. White dotted line, the distances between residues. In green, tryptophans; in red, aspartates; and in yellow, the sulfur containing residues (methionine and cysteine). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

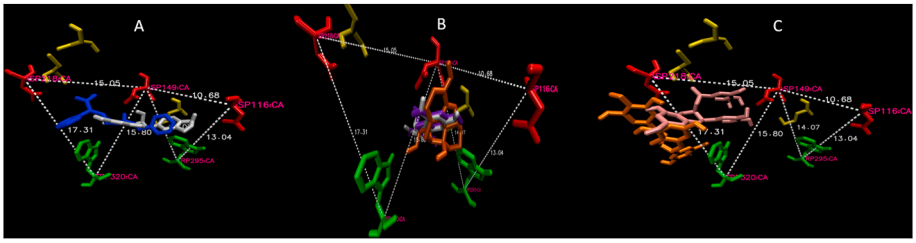


**Fig. 6.** Conformations of the evaluated compounds at the "pseudo sites". (A) Fentanyl (blue) and EC (white). (B) Naloxone (orange), C (white) and EGC (purple). (C) ECG (orange) and EGCG (pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

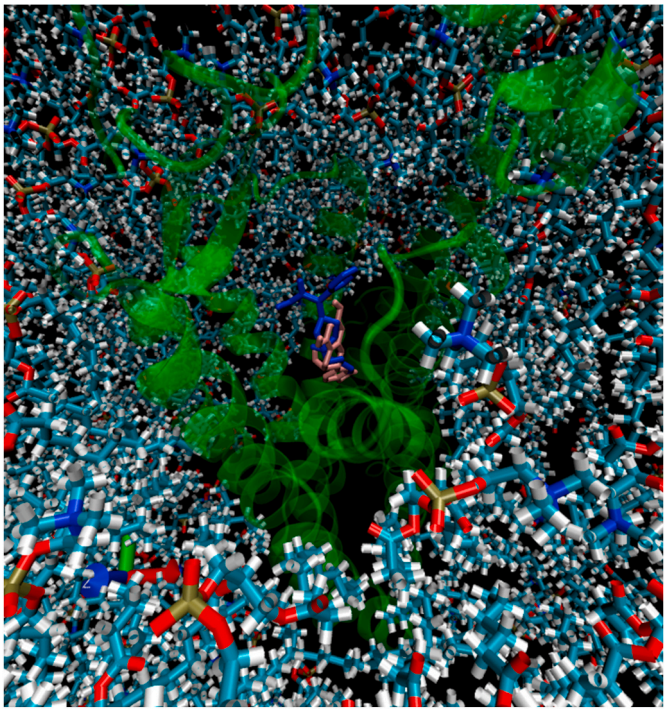

**Fig. 7.** EC (pink) and Fen (blue) inside the binding pocket of the receptor embedded in the membrane. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that seem to form "pseudo sites" inside the binding site. All evaluated compounds (except the antagonist naloxone), in at least one of the 70–90 ns protein conformations, interact with D149 (Table 3), with an aromatic residue (i.e. tryptophan), and also with a cysteine or a methionine (which contain a sulfur atom in their structures). These three amino acids repeat several times in different places and may interact with other residues. Interestingly, these pseudo sites conserve geometry; imaginary lines (representing the distance between the α-carbon of each amino acid) can be drawn and two inverted triangles with two tryptophan at the base (W295 and W320), three aspartates at the top (D116, D149, D218), and one methionine (M153) and two cysteines (C142 and C219) can be drawn (Fig. 5). This geometrical perspective led us to hypothesize that the adopted allocation of each ligand at these "pseudo sites" influences the activity of each compound, producing different modes or degrees of signaling (see Fig. 6).

One important approach in describing these "pseudo sites" is to establish the possibility that the binding site is governed by a group of residues with similar characteristics which are repeated in the each "pseudo site", setting aside that just one amino acid may be necessary for contact in order to observe an effect. In this scenario, it would be possible that D149 or even similar amino acids D218 and D116 could serve as main residue, in addition or in place of the previously described binding pocket or transmembrane-associated binding with an orthosteric or allosteric site.

Based in our "pseudo sites" proposal, we analyzed 80 ns conformation (Fig. 7) and found that agonists (fentanyl) accommodate through the two "pseudo sites" and the antagonists (naloxone) allocate between both "triangles", accommodating the rest of the structure out the binding pocket. Interestingly, the catechin family adopts different conformations through these sites: EC, accommodates similarly to the agonist site; EGC and C, similarly to the antagonist and ECG and EGCG accommodate to the left side of the "pseudo site" without losing contact with D149, which could be defined as the allosteric site, taking into account that these ligands also interact with W320. Describing the existence of these pseudo sites within the cavity may contribute to a better understanding of MOR binding site.

## 4. Conclusion

Based on the results of our modeling of catechin, naloxone, and fentanyl binding, we can establish three probable outcomes: 1) based on the proportion of established hydrogen bonds, which corroborate the interaction at binding site, we suggest that these flavonoids act probably as modulators of the orthosteric site, 2) according to the binding at the "pseudo sites", we propose that EGCG and EGC could act as allosteric modulators, with the rest of the catechin family acting as agonist or antagonist, depending of their adopted conformation and, 3) taking into account that some amino acids favor the activity, molecules that poses a gallate moiety could act as allosteric modulators. Nevertheless, considering the results reported by Ref. [15]; it seems that catechins that include a gallate moiety in its structure act as antagonist of MOR. However, more work in vitro and/or in vivo is necessary to reach complete conclusions.

On other hand, our data lead us to hypothesize that contacts reached by ligand conformations could explain why opiates are capable of activating both β-arrestin and G-protein biased pathways, and therefore help to explain the phenomenon of tolerance.

Finally, we can conclude that the family of catechins explored seem to be excellent modulators of the MOR, based on binding energy values, the interactions reached, and the conformations adopted. It is necessary to perform more studies in order to find which conformation could describe fully the effects observed. Further experiments could enable us to find specific molecular characteristics that could lead to finding new compounds that bind this receptor. Most importantly, our data also suggest the possibility that catechins can function as treatment for withdrawal symptoms and as compounds for substitution therapy in addiction treatment.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## References

[1] Wang S. Historical review: opiate addiction and opioid receptors. Cell Transplant 2019;28(3):233–8. https://doi.org/10.1177/0963689718811060.
[2] Connery HS. Medication-assisted treatment of opioid use disorder. Harv Rev Psychiatr 2015;23(2):63–75. https://doi.org/10.1097/HRP.0000000000000075.
[3] Sarvet AL, Hasin D. The natural history of substance use disorders. Current opinion in psychiatry, 29. Lippincott Williams and Wilkins; 2016. p. 250–7. https://doi.org/10.1097/YCO.0000000000000257. Issue 4.
[4] World Drug Report. (United Nations publication, *Sales No. E.19.XI.8)*. (n.d.). Retrieved april 27, 2020. from, https://wdr.unodc.org/wdr2019/; 2019.
[5] Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, Schulz S, Koch T, Evans CJ, Christie MJ. Regulation of μ-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. Pharmacol Rev 2013;65(1):223–54. https://doi.org/10.1124/pr.112.005942. American Society for Pharmacology and Experimental Therapy.
[6] Nestler EJ. Historical review: molecular and cellular mechanisms of opiate and cocaine addiction. Trends in pharmacological sciences, 25. Elsevier Current Trends; 2004. p. 210–8. https://doi.org/10.1016/j.tips.2004.02.005. Issue 4.
[7] Contet C, Kieffer BL, Befort K. Mu opioid receptor: a gateway to drug addiction. Curr Opin Neurobiol 2004;14(3):370–8. https://doi.org/10.1016/j.conb.2004.05.005.
[8] Alexander SPH, Christopoulos A, Davenport AP, Kelly E, Mathie A, Peters JA, Veale EL, Armstrong JF, Faccenda E, Harding SD, Pawson AJ, Sharman JL, Southan C, Davies JA, Abbracchio MP, Alexander W, Al-hosaini K, Bäck M, Beaulieu JM, Yao C. The concise guide to pharmacology 2019/20: G protein-coupled receptors. Br J Pharmacol 2019;176(S1):S21–141. https://doi.org/10.1111/bph.14748.
[9] Wishart DS. DrugBank: a comprehensive resource for in silico drug discovery and exploration. Nucleic Acids Res 2006;34:D668–72. https://doi.org/10.1093/nar/gkj067 (90001).
[10] Oh K-W, et al. Effects of (-)-epigallocatechin gallate on the development of morphine-induced physical dependence. Arch Pharm Res (Seoul) 2007;30:1111–5. https://doi.org/10.1007/BF02980245.
[11] Kaserer T, Lantero A, Schmidhammer H, Spetea M, Schuster D. μ Opioid receptor: novel antagonists and structural modeling. Sci Rep 2016;6(1):1–15. https://doi.org/10.1038/srep21548.
[12] Shang Y, Yeatman HR, Provasi D, Alt A, Christopoulos A, Canals M, Filizola M. Proposed mode of binding and action of positive allosteric modulators at opioid receptors. ACS Chem Biol 2016;11(5):1220–9. https://doi.org/10.1021/acschembio.5b00712.
[13] Remesic M, Hruby VJ, Porreca F, Lee YS. Recent advances in the realm of allosteric modulators for opioid receptors for future therapeutics. ACS Chem Neurosci 2017;8(6):1147–58. https://doi.org/10.1021/acschemneuro.7b00090. . American Chemical Society.
[14] Bartuzi D, Kaczor AA, Matosiuk D. Activation and allosteric modulation of human μ opioid receptor in molecular dynamics. J Chem Inf Model 2015;55(11):2421–34. https://doi.org/10.1021/acs.jcim.5b00280.
[15] Katavic PL, Lamb K, Navarro H, Prisinzano TE. Flavonoids as opioid receptor ligands: identification and preliminary structure-activity relationships. J Nat Prod 2007;70(8):1278–82. https://doi.org/10.1021/np070194x.
[16] Panneerselvam M, Ali SS, Finley JC, Kellerhals SE, Migita MY, Head BP, Patel PM, Roth DM, Patel HH. Epicatechin regulation of mitochondrial structure and function is opioid receptor dependent. Mol Nutr Food Res 2013;57(6):1007–14. https://doi.org/10.1002/mnfr.201300026.
[17] Panneerselvam M, Tsutsumi YM, Bonds JA, Horikawa YT, Saldana M, Dalton ND, Head BP, Patel PM, Roth DM, Patel HH. Dark chocolate receptors: epicatechin-induced cardiac protection is dependent on δ-opioid receptor stimulation. Am J Physiol Heart Circ Physiol 2010;299(5):H1604–9. https://doi.org/10.1152/ajpheart.00073.2010.
[18] MacRae K, Connolly K, Vella R, Fenning A. Epicatechin's cardiovascular protective effects are mediated via opioid receptors and nitric oxide. Eur J Nutr 2019;58(2):515–27. https://doi.org/10.1007/s00394-018-1650-0.
[19] Quinõnez-Bastidas GN, Pineda-Farias JB, Flores-Murrieta FJ, Rodriguez-Silverio J, Reyes-Garcia JG, Godínez-Chaparro B, Granados-Soto V, Rocha-Gonzalez HI. Antinociceptive effect of (-)-epicatechin in inflammatory and neuropathic pain in rats. Behav Pharmacol 2018;29:270–9. https://doi.org/10.1097/FBP.0000000000000320.
[20] Zhang Y. I-TASSER: fully automated protein structure prediction in CASP8. Proteins: Structure, Function and Bioinformatics 2009;77(SUPPL. 9):100–13. https://doi.org/10.1002/prot.22588.
[21] Yang J, Zhang Y. I-TASSER server: new development for protein structure and function predictions. Nucleic Acids Res 2015;43(W1):W174–81. https://doi.org/10.1093/nar/gkv342.
[22] Roy A, Yang J, Zhang Y. COFACTOR: an accurate comparative algorithm for structure-based protein function annotation. Nucleic Acids Res 2012;40(W1):W471–7. https://doi.org/10.1093/nar/gks372.
[23] Liao J-M, Wang Y-T. In silico studies of conformational dynamics of Mu opioid receptor performed using Gaussian accelerated molecular dynamics. J Biomol Struct Dyn 2019;37(1):166–77. https://doi.org/10.1080/07391102.2017.1422025.
[24] Cui X, Yeliseev A, Liu R. Ligand interaction, binding site and G protein activation of the mu opioid receptor. Eur J Pharmacol 2013;702(1–3):309–15. https://doi.org/10.1016/j.ejphar.2013.01.060.
[25] Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, NA-NA 2009. https://doi.org/10.1002/jcc.21334.
[26] Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, Madan Babu M. Molecular signatures of G-protein-coupled receptors. Nature 2013;494(7436):185–94. https://doi.org/10.1038/nature11896. Nature Publishing Group.
[27] Berríos-Cárcamo P, et al. Molecular modeling of salsolinol, a full Gi protein agonist of the μ-opioid receptor, within the receptor binding site. Chemical biology & drug design, 94. John Wiley & Sons, Ltd; 2019. p. 1467–77. https://doi.org/10.1111/cbdd.13523 (2).