CB_1 and CB_2 Cannabinoid Receptor Binding Studies Based on Modeling and Mutagenesis Approaches

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Abstract: In absence of X ray crystal structures of G-protein coupled receptors (GPCRs)-ligand complexes, computer-aided molecular modeling together with site-directed mutagenesis studies become of great importance in order to provide in-silico predictions that facilitate the development of new ligands. In this context, the present review addresses the application of these strategies to the CB_1 and CB_2 cannabinoid receptors. The combination of these complementary approaches represents a tool of considerable value which has allowed to understand the specific ligand-receptor interactions.

Keywords: Cannabinoid receptors, receptor-ligand interactions, site-directed mutagenesis, modeling studies.

1. INTRODUCTION

Cannabinoid receptor types are denoted by the abbreviation CB and numbered in the order of their discovery by a subscript. At present, two cannabinoid receptor types have been unequivocally identified, named CB_1 [1] and CB_2 [2]. Although they share a certain degree of structural homology, they differ in their signalling mechanisms and their tissue distribution. Additionally, during the last years it has been postulated the existence of additional types of cannabinoid receptors, mainly based on pharmacological results. However, other kinds of evidence are still lacking [3].

The CB_1 and CB_2 cannabinoid receptors belong to the rhodopsin (Rho) subfamily of G protein coupled receptors (GPCRs), targets for the approximate 30% of the clinically marketed drugs, which are active at this receptor familiy [4]. The elucidation of the 3D structure of the different receptors that belong to the GPCR superfamily is of great importance to allow the development of new potent and selective ligands. This objective requires the previous crystallization of the protein in order to carry out X ray studies. However, solving the 3D structure of GPCRs has been a significant obstacle in structural biology. A number of reasons have hindered efforts aimed at their purification, such as their large molecular weight, intricate interhelical packing or their membrane-associated topology. In the absence of pure protein, available in the native conformation, classical methods of structural analysis such as X-ray crystallography and nuclear magnetic resonance spectroscopy cannot be successfully used. Therefore, alternative methods must be explored to elucidate the structural features involved in ligand-receptor interactions. Among these, molecular modeling studies based on the alignment of the primary sequence of the receptor with the known Rho sequence [5] together with biological and site-directed mutagenesis data stand out as the most widely used approaches. These studies allow to propose hypothesis of binding modes and activation processes between the receptor and their ligands that subsequently can be validated experimentally. The main focus of this review is to overview the receptor-ligand interaction models and mutagenesis studies developed to date in relation with the CB_1 and CB_2 cannabinoid receptors.

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2. CANNABINOID CB_1 RECEPTOR

2.1. Aminoacid Residues Involved in Ligand Binding

The cannabinoid type 1 receptor (CB_1R) is one of the most abundant GPCRs in the central nervous system, with a high level of expression in cortex, hyppocampus, basal ganglia and cerebellum [6]. CB_1Rs are coupled to $G_{i/o}$ proteins, involved in inhibition of adenylyl cyclase, regulation of ion currents (inhibition of voltage-gated L, N, and P/Q Ca^{2+} currents, activation of K^+ currents), activation of focal adhesion kinase (FAK), mitogen activated protein kinase (MAPK) and induction of immediate early genes [3].

The CB_1R gene, designated Cnr1, has been localized on human chromosome 6 at 6q14-q15 (Fig. **1**) and the mouse CB_1 receptor gene on proximal chromosome 4. It is constituted by 472 (human) or 473 (mouse) residues and shares a certain degree of homology between species [7].

As all the members of the GPCRs superfamily, CB_1R is characterized by a membrane topology with seven hydrophobic alpha helix transmembrane segments, a long N terminus located extracellularly (comprised of 116 residues) and a C terminus situated intracellularly (Fig. **2**). Activation of the receptor is achieved by binding of the agonist ligand, which provokes a conformational change, leading to the active state of the receptor that is responsible for the signal transduction. However, there is an additional mechanism that can lead to the active state of the receptor in the absence of ligand. As numerous other GPCRs, CB_1R displays a high level of constitutive activity [8, 9], thus is, it can spontaneously adopt an active conformational state in the absence of agonist binding, keeping elevated basal levels of intracellular signaling. This pharmacological behaviour can be rationalized by the two state-model of receptor activation

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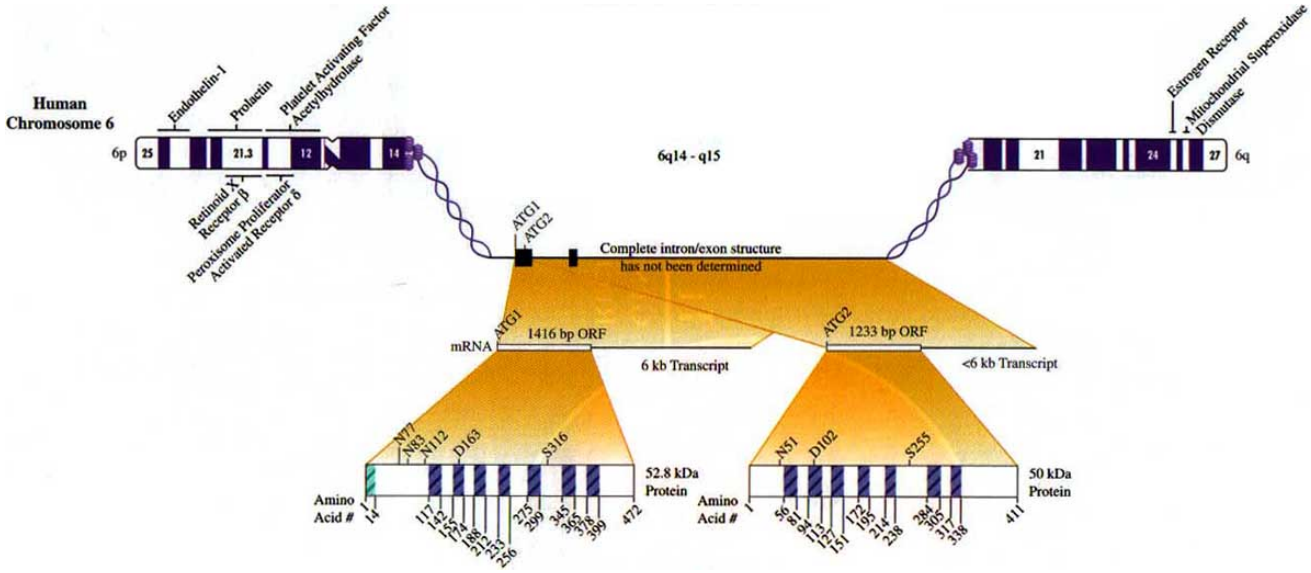


Fig. (1). Localization of CB_1 gene (Cnr1) on human chromosome 6.

(Fig. **3**), in which receptors are in equilibrium between two G-protein-coupled states, one of them inactive (RG_{GDP}) and the other one constitutively active (R^*G_{GTP}). An agonist stabilizes the active state, shifting the receptor population towards activation, a neutral antagonist binds with equal affinity to both active and inactive conformation, whereas an inverse agonist will preferentially stabilize the inactive state [10].

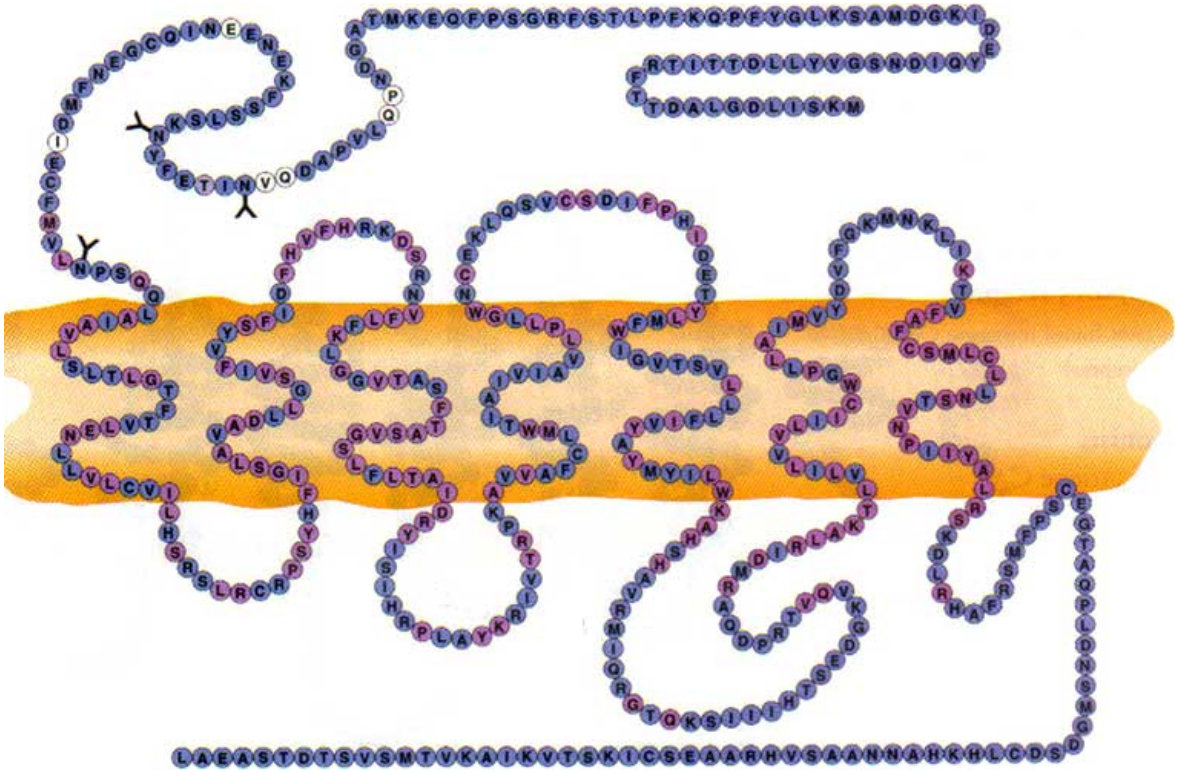


Fig. (2). Structure of human CB_1 cannabinoid receptor.

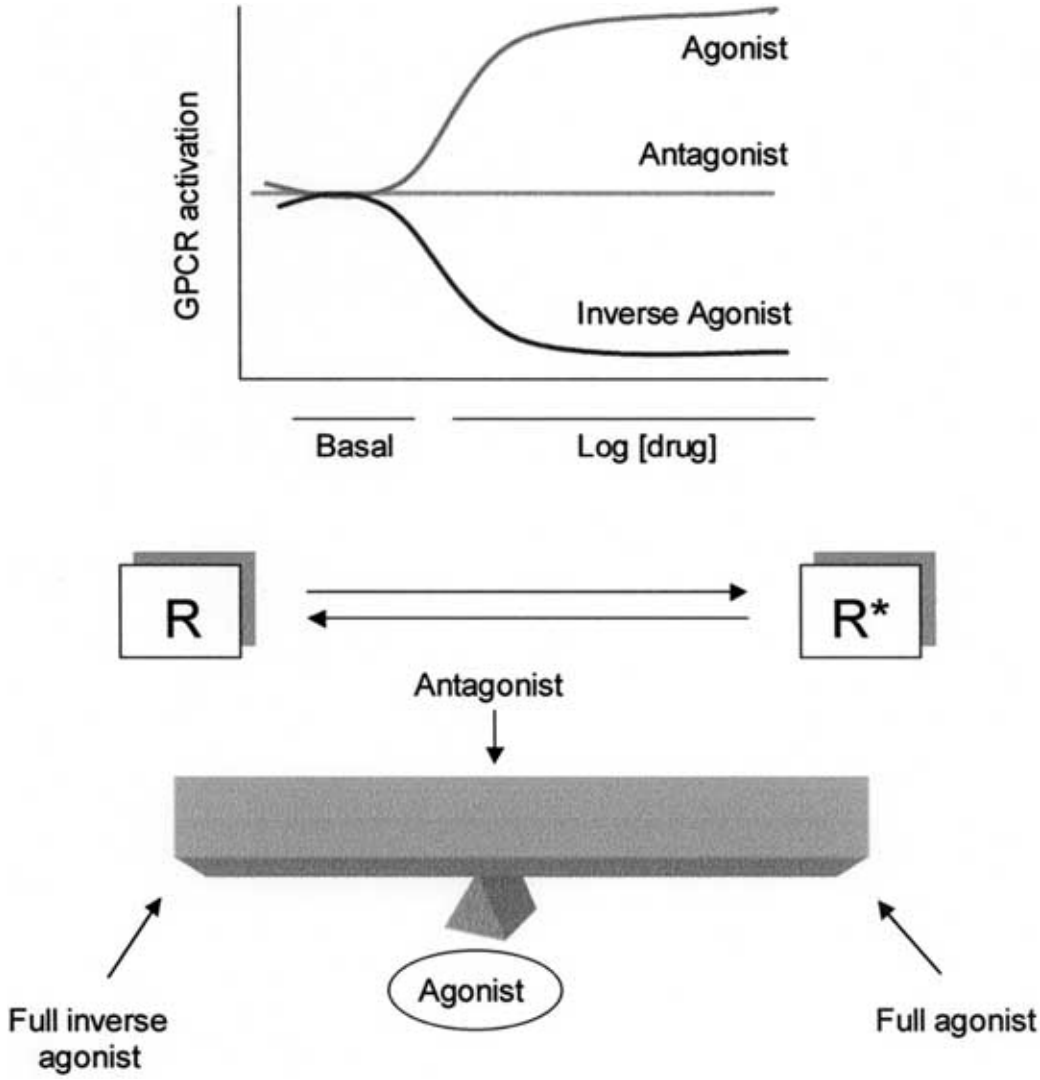


Fig. (3). Schematic representation of the two state-model of receptor activation.

An interesting feature of CB_1 is its ability to be activated by a number of structurally different classes of compounds, thus, raising the possibility of multiple activated forms of the receptor. The currently known classes of cannabinoid agonists include i) the tricyclic classical cannabinoids, based on the structure of Δ^9-tetrahydrocannabinol (Δ^9-THC), ii) the nonclassical group of bicyclic terpenoids such as CP55940, iii) the aminoalkylindoles (AAIs) as, for instance, WIN552122 and iv) the endogenous cannabinoid ligands derived from arachidonic acid, being anandamide the most representative one. Regarding antagonism/inverse agonism, the family of biarylpyrazoles has been deeply studied, leading to the characterization of one of the most widely used pharmacological tools, SR141716A (Fig. **4**).

In spite of the high degree of structural disimilarity among these ligands, the fact that all of them can be specifically recognized by the same receptor, supports the existence of similar interactions between chemical groups present in the ligands and specific residues located in determined regions of the receptor. Therefore, the understanding of the residues involved in the recognition between the protein and their ligands is relevant to rationalize the design and synthesis of new compounds with predetermined affinities, activities and selectivities. The availability of X ray structures of ligand-receptor complexes would be extremely helpful in achieving this objective. Unfortunately, attemps to obtain such GPCRs crystal complexes have often failed, which has made necessary the development of alternative methods. Among these, one of the most reliable approach involves in silico prediction based on 3D homology models which are subsequently experimentally confirmed by mutagenesis studies. The integration of data obtained from these two complementary approaches will allow to establish the main regions responsible for ligand binding and receptor activation. In 2000 was described the unique GPCR crystal structure that has been obtained to date, the bovine rhodopsine, solved at 2.8Å resolution [5]. This crystal structure can be used as a template to model the transmembrane domains (TM) of other GPCRs, which, along with mutagenesis studies constitutes a tool to investigate the receptor-ligand interactions. Using this methodology, it has been elaborated several rhodopsine-based CB_1 models depending on the different ways of constructing the receptor [11-13] that have allowed to identify some of the most important residues involved in the binding of the different structural types of ligands.

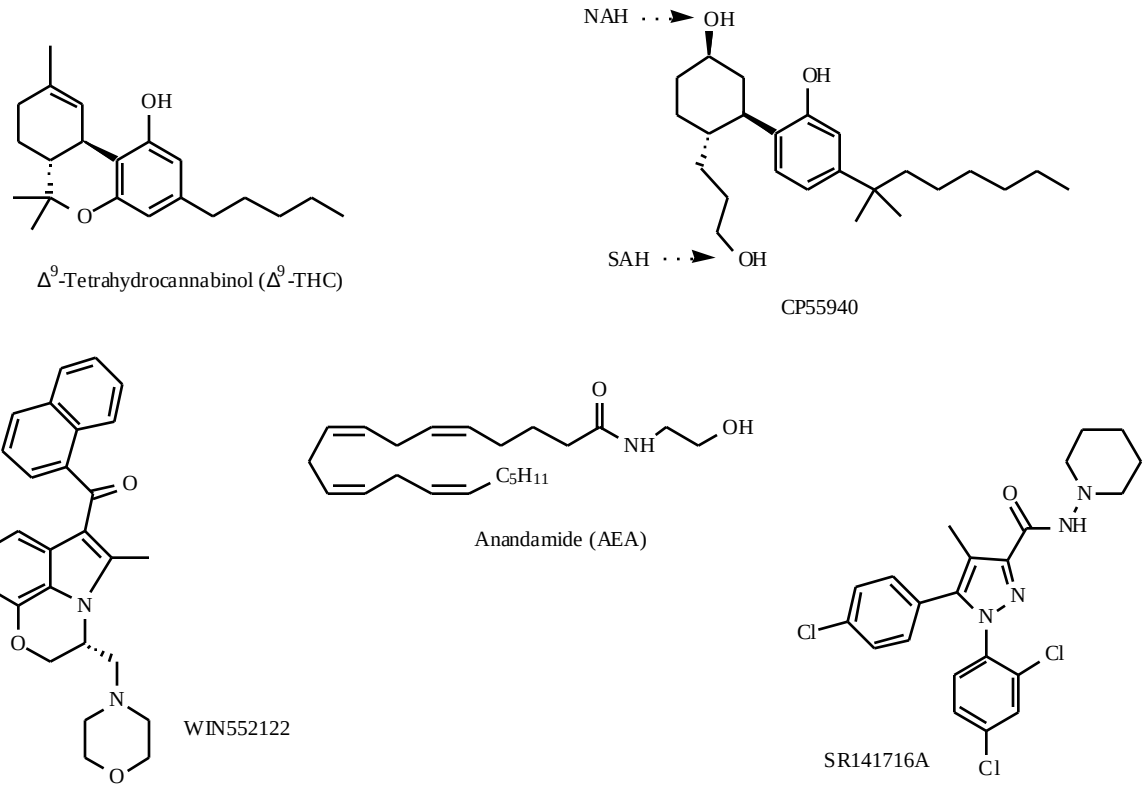
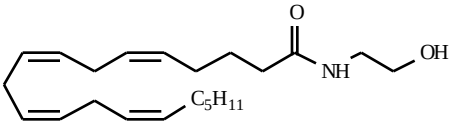


Fig. (4). Structures of the most representative cannabinoid lignads.

i) Endogenous Cannabinoids

Considering anandamide as the prototypical arachidonic acid-derived endocannabinoid, an important number of studies aimed at elucidating its binding mode have been carried out (Table **1**). In particular, it has been confirmed by site directed mutagenesis data [14] that Lys 192 (K3.28, following the nomenclature by Weinstein and Ballesteros [15]), plays a crucial role in the binding of anandamide. K3.28 has been proposed to form a hydrogen bond with the oxygen of either the carbonyl [16] or the hydroxy [13] groups of anandamide. In this interaction, K3.28 forms a hydrogen bond with the amide oxygen of anandamide (N to amide O distance 2.6 Å; N-H-O angle 158º). Simultaneously, the hydroxyl headgroup of anandamide is engaged in an intramolecular hydrogen bond with the amide oxygen (O to O distance 2.7 Å; O-H-O angle 130º) [16]. However, it is also possible that the hydroxy group of anandamide forms a hydrogen bond with K3.28 and in this model, S7.39 is located opposite to the critical lysine and may also be involved in forming H-bonds with the carbonyl oxygen of anandamide [13]. Regarding the lipophilic side chain of anandamide, it has been described that this moiety of the molecule is buried into a highly hydrophobic binding pocket constituted by residues from the helices 2, 3, 6 and 7 (F2.57, F3.25, L3.29, V3.32, Y6.57, F6.60, F7.35, A7.36 and S7.39) [16]. Among these residues, only F3.25 has been confirmed to be part of the anandamide binding pocket. It has been suggested the existence of a C-H···π interaction between the F3.25 and the C5-C6 double bond of the chain [16] since mutation of F3.25 for alanine seems to decrease about 7-fold the binding affinity of anandamide [11, 16]. However, in the model proposed by Salo *et al*. [13] anandamide does not present any interactions with F3.25.

Table 1. Binding interactions of anandamide docked in CB_1R models[a]



	Donor	Acceptor
H bonds	K3.28	Carbonyl oxygen
		Hydroxyl oxygen
	S7.39	Carbonyl oxygen
C-H···π interactions	F3.25	C5-C6 double bond

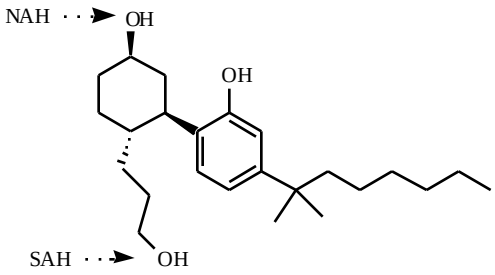
[a]Models from ref. 13 and 16

ii) Non Classical Bicyclic Agonists

This class of ligands is represented by CP55940. Three different models (Table **2**) have been proposed to explain the binding mode of this agonist [12, 13, 16]. Docking studies of CP55940 into CB_1R* carried out by McAllister *et al*. [16] involve hydrogen bonding interactions of the southern aliphatic hydroxyl (SAH) with K3.28, the northern aliphatic hydroxyl (NAH) with K260 of the second extracellular (EC-2) loop and the phenolic hydroxyl with D6.58. However, in the conformation proposed by Shim *et al*. [12], CP55940 would hydrogen bond with K3.28 and E258, as well as with Q261, by involving its phenolic hydroxyl and the SAH groups. The importance of K3.28 has been also remarked by Salo *et al*., who suggest that this residue could interact with the phenolic, SAH or even NAH hydroxyl groups [13].

Additionally, these studies indicate the existence of a hydrophobic pocket located at the helices 3, 5, 6 and 7 where the C3 alkyl chain could interact, although two different conformational possibilities for this chain have been suggested depending on its relative orientation towards the inner or the outer part of receptor [12, 13, 16].

Table 2. Hydrogen bonding interactions of CP55940 docked in CB_1R models



	Donor	Acceptor
McAllister *et al.* model (Ref. 16)	K3.28	SAH
	K260	NAH
	D6.58	Phenolic hydroxyl
Shim *et al.* model (Ref. 12)	K3.28 E258 Q261	SAH Phenolic hydroxyl
Salo *et al.* model (Ref. 13)	K3.28	SAH[a],NAH or phenolic hydroxyl

[a]If C3 alkyl chain is positioned into the hydrophobic part of the binding pocket

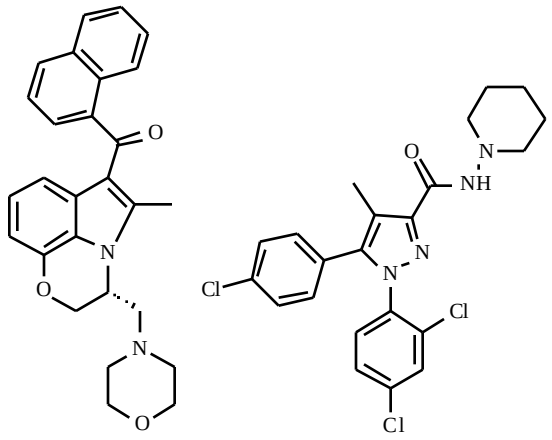
iii) Aminoalkylindoles

The most representative ligand of this class is WIN552122 (*R* enantiomer). Due to the fact that the AAIs are highly aromatic ligands and because K3.28 is not an interaction site for WIN552122 [14], it has been hypothesized that aromatic stacking, rather than hydrogen bonding interactions, is the primary interaction for the AAIs at CB_1.The main interactions responsible for WIN552122 binding are the aromatic stacking interactions (Table **3**) between the naphtalene and indole rings with F3.36, W5.43, and W6.48 residues [13, 16], since the replacement of these aromatic residues for alanine directly affects binding affinity [16]. This binding site is consistent with the experimental fact that the enantiomer of WIN552122, the so-called WIN552123, does not show cannabinoid effects. The lack of activity of WIN552123 is justified because the morpholino alkyl tail would be sterically blocked by the TM6 backbone and residues V6.59 and M6.55 [16].

iv) Diarylpyrazoles Class of CB_1R Antagonists

SR141716A stands out as the most widely studied potent and selective CB_1R antagonist. In the inactive state of CB_1, the binding site of the inverse agonist/antagonist SR141716A is within the TM3-4-5-6 aromatic microdomain and involves direct aromatic stacking interactions with F3.36, Y5.39 and W5.43 as well as hydrogen bonding with K3.28 [13, 16]. Remarkably, these modelling studies suggest that in the active state of CB_1, the agonist WIN552122 binds in the same aromatic microdomain with direct aromatic stacking interactions with F3.36, W5.43 and W6.48 (Table **3**). The difference between WIN552122 and SR141716A is the interactions with K3.28 and W6.48. SR141716A stabilizes transmembrane helix 6 in its inactive conformation via aromatic stacking with F3.36/W6.48. In this binding site, SR141716A would exhibit higher affinity for CB_1 receptor due to a hydrogen bond between the SR141716A C3 substituent and K3.28, a residue available to SR141716A only in the inactive state [17].

Table 3. Ligand-aromatic stacking interactions of WIN552122 and SR141716A docked in CB_1R models[a]



WIN552122 SR141716A

	WIN552122 in R*		SR141716A in R	
	d^b_{NAP} (Å)	d_{IND} (Å)	d_{MC} (Å)	d_{DC} (Å)
F3.36	**6.4**[c]	**6.3**	7.9	**5.0**
Y5.39	10.7	9.7	**6.5**	10.0
W5.43	**4.5**	6.1	**4.8**	**4.8**
W6.48	**4.2**	8.1	11.7	7.6

[a]Models from ref. 16. NAP = naphtyl ring, IND = indolyl ring, MC = monochlorophenyl ring, DC = dichlorophenyl ring. [b]d = distance between aromatic ring centroids. [c]Distances (d) for aromatic systems that meet the criteria for aromatic stacking interactions are highlighted in bold.

Taken together, these results support modeling studies that identify the TMH3-4-5-6 aromatic microdomain as the binding region of SR141716A and WIN552122 but not of anandamide. Anandamide binds in the TM2-3-6-7 region in which hydrogen bonding and C-H···π interactions appear to be important. Only one TM3 aromatic residue, F3.25, was found to be part of the anandamide binding pocket.

Mutation studies have shown that CB_1/CB_2 subtype differences in TM3 and TM5 contribute to the CB_2 selectivity of WIN552122. A recent mutant cycle study identified K3.28 as an important interaction site for SR141716A at CB_1, whereas CB_1 Y5.39F/Y5.39I mutation studies have underscored the structural importance of aromaticity at position 5.39 [16, 17].

2.2. Aminoacid Residues Involved in Activation and Life Cycle of CB_1

Additionally to the aminoacids identified as being important for CB_1 ligand binding, another area that deserves further exploration is to establish the residues that participate in the capacity of the receptor to acquire its active conformation, to recognize and interact with the corresponding G protein and that govern the receptor stability, its cell surface expression and its dynamic life cycle and recycling processes.

Modeling studies on the CB_1R suggest that aspartate D2.50 interacts with asparagine N7.49 only in the active receptor conformation. This aspartate residue plays a critical role in G-protein binding by allowing the receptor the intrinsic flexibility to switch from an inactive state uncoupled to G-proteins into two G-protein-coupled states, an inactive RG_{GDP} state responsible for $G_{i/o}$-protein sequestration and an active R^*G_{GTP} state responsible for constitutive activity. The aspartate-to-asparagine mutation in the second transmembrane domain shifts the CB_1 cannabinoid receptor into the G-protein-uncoupled state, whereas truncation of the distal C terminal promotes the constitutively active R^*G_{GTP} receptor conformation [18].

The N-terminal region of the CB_1 receptor is involved in regulating the synthesis, degradation, folding and trafficking (stability and surface expression) of CB_1 [19].

The terminal CB_1 region TM5-IL3-TM6 is in general an important determinant in the dynamic life cycle of GPCRs, including the activation, internalization, desensitization, and resensitization processes. In particular, the third intracellular loop (IL3) is mostly unstructured, in contrast with the well-defined α-helices forming the cytoplasmic ends of TM5 and TM6. This result suggests that in CB_1 intact receptor, the G protein is activated by the cytoplasmic ends of TM5 or TM6 and not by the unstructured central region of the IL3. Interestingly, most of the residues identified as being important for the activation of the G proteins are located at the beginning of the TM6, supporting a translation of the helix into the cytoplasmic space upon receptor activation [20]. Additional experimental evidence indicates that TM6 straightens during activation. It has been described that activation of the cannabinoid CB_1 receptor may involve a W6.48/F3.36 rotamer toggle switch [21].

A recent paper describes the recycling cycle of CB_1. Due to its natural constitutive activity, CB_1R permanently and constitutively cycles between plasma membrane and endosomes, leading to a predominantly intracellular localization at steady-state. This constitutive endocytosis is Rab5 dependent while constitutive recycling is mediated by Rab4 [22].

3. CANNABINOID CB_2 RECEPTOR

The homology degree between CB_1 and CB_2 is only a 44% in their amino acid sequence identity overall, percentage that rises to a 68% in the transmembrane regions [2]. However, most of prototypical cannabinoid ligands (Δ^9-THC, anandamide, CP55940, WIN552122) show affinity for both receptors, although several selective ligands have been developed during the last years [23].

The cannabinoid type 2 receptor (CB_2R) is specially abundant in the immune system. Its expression has been reported in multiple lymphoid organs such as thymus, tonsils, bone marrow, pancreas, splenic macrophage/monocyte preparations, mast cells, peripheral blood leukocytes and in a variety of cultured immune cell models [3].

CB_2, as well as CB_1Rs, is also coupled to $G_{i/o}$ proteins and involved in inhibition of adenylyl cyclase. Other important responses induced by CB_2 activation include the stimulation of the expression of immediate early genes such as Krox-24 and the inhibition of the inducible nitric oxide synthase (iNOS) as described in RAW 264.7 cells [3].

The CB_2R gene, designated Cnr2, has been localized on human chromosome 1p36 and the mouse CB_2 receptor gene on distal chromosome 4. It is constituted by 360 (human) or 347 (mouse) residues and shares a certain degree of homology between species [7].

As all the members of the GPCRs superfamily, CB_2R is characterized by a membrane topology with seven hydrophobic alpha helix transmembrane segments, a C terminus situated intracellularly and a N terminal domain (Fig. **5**), which is much shorter than the corresponding CB_1R N terminus and which does not show significant sequence conservation.

3.1. Residues Involved in Binding Affinity

From previous studies performed in CB_1 receptor, TM3 was identified as an important region in the binding of ligands. In particular, it was known that K3.28 played a key role in binding of HU210, CP55940 and anandamide, but not of WIN552122. Therefore, some studies were developed to find the residues in TM3 that accounted for the binding and partial selectivity of WIN552122 for CB_2. With this goal, Chin *et al*. [24] first identified the residues that were different between CB_1 and CB_2. Assessment of chemical nature of the six aminoacids in TM3 that differed in both cannabinoid receptors pointed to Ser 112 (S3.31) and Met 115 (M3.34) as the most probable candidates for selectivity, since the other four residues represent quite conservative changes. From these two residues, the most important resulted to be Ser 112, which corresponds to Gly 195 in CB_1, and it is responsible for the experimentally observed 7 to 20-fold selectivity of WIN552122 for CB_2. It has been hypothesized that the change from Gly to Ser could alter the interaction with AAIs by introducing a new hydrogen-bonding group [24].

Moreover, other studies have obtained deeper insights into the global TM3-4-5 cluster, proving that important residues involved in binding of selective compounds for CB_2 receptor are located at these helices. Regarding the TM4, it has been pointed out the importance of several Trp residues: W158 (W4.50), completely conserved in all currently known GPCRs and W172 (W4.64), conserved in both cannabinoid receptors, play an important role in CB_2 binding and signalling. It has been indicated, using site directed mutagenesis studies, that W4.64 is involved in ligand binding whereas W4.50 in CB_2 is very important for receptor activity [25]. With respect to helix 5, residue F5.46 (Phe 197) has been proposed to enhance the aromatic

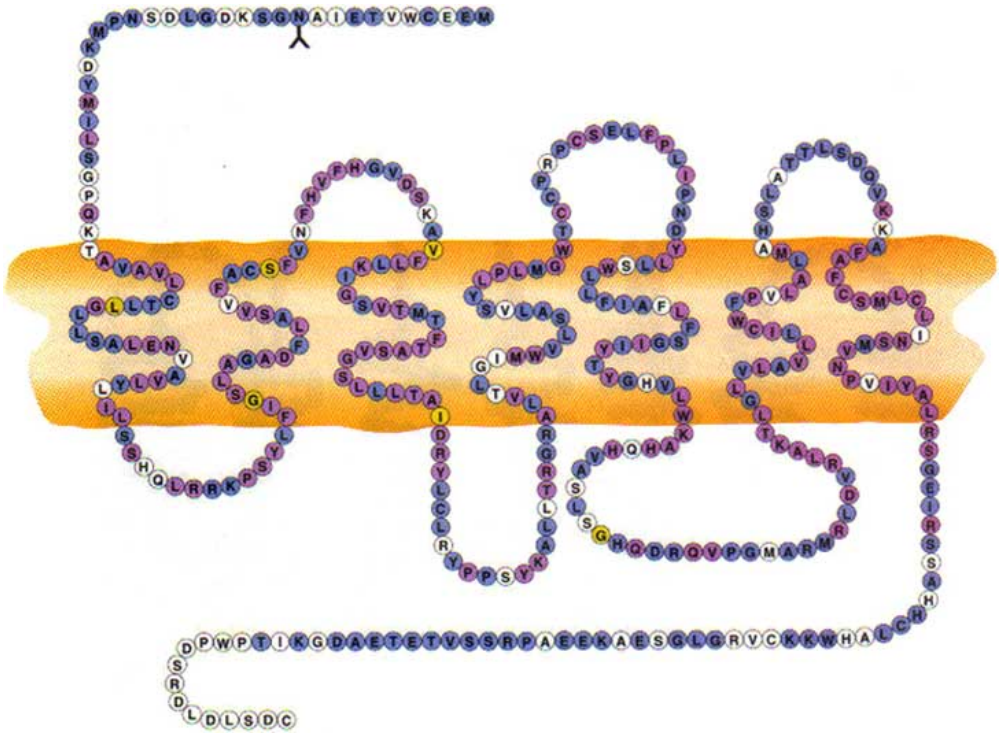


Fig. (5). Structure of human CB_2 cannabinoid receptor.

stacking of WIN552122 with CB_2, interaction whose importance has been confirmed by mutagenesis studies, since the replacement of Phe for Val in CB_2 sequence implies a 14-fold loss of affinity of WIN552122, and, conversely, the substitution of Val for Phe in CB_1 leads to a 12-fold increase in its affinity. However, none of these mutations affected to the binding affinity of HU210, CP55940 or anandamide [26].

3.2. Activation of CB_2 Receptor

Additionally to docking and mutagenesis studies focused on the understanding of the residues that are responsible for ligand binding, a different number of studies have also addressed the question of which aminoacids play key roles in the activation of cannabinoid receptors. In this context, some residues have been proposed and identified in helices 3-5-6-7.

Regarding TM3, site directed mutagenesis studies indicate that CB_2 R3.50 in the DRY motif, is crucial for signal transduction, whereas D3.49 and A6.34 do not seem to be important to keep the receptor in an inactive state, although they are involved in ligand binding and all three residues are responsible for the constitutive activity of the wild type CB_2 receptor [27]. However, other authors have described that R3.50A mutation only affected cannabinoid agonist-induced inhibition of cAMP accumulation in a weak manner [28].

With respect to TM5, L5.50 and Y5.58 are important for the function of CB_2. Mutating L5.50 to a proline abolished ligand binding, whereas mutating Y5.58 to an alanine resulted in a rightward shift of the competition binding curves. Both of these mutations led to a complete loss of the ability of cannabinoid agonists to inhibit cAMP accumulation [29].

In TM7, Song *et al*. have studied the role of the $NP(X)_nY$ motif of this helix together with cysteine residues in the C terminal juxtamembrane region, highly conserved in GPCRs. Their results indicate that Y299, residue that belongs to the $NP(X)_nY$ motif, is critical for binding and functional coupling to adenylyl cyclase. These authors also demonstrate that C313 and C320, from the C terminus, are critical for coupling to adenylyl cyclase but not for ligand binding and receptor desensitisation [30]. Another important residue in TM7 is serine 292, which has been suggested to be involved in G-protein signaling, in particular in the conformational change of receptor after agonist binding [31].

4. CONCLUDING REMARKS

During the last years the combined approaches of molecular modeling and site-directed mutagenesis have allowed to postulate and experimentally identify the main aminoacid residues that are responsible for binding and selectivity of ligands for cannabinoid receptors as well as their involvement in the receptor activation and dynamic cycle processes. Taken together, these results will provide the rational basis for the design and synthesis of new cannabinoid ligands with predetermined affinities, selectivities and activities.

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REFERENCES

[1] Matsuda, L. A.; Lolait, S. J.; Brownstein, M. J.; Young, A. C.; Bonner, T. I. *Nature* **1990**, *346*, 561-564.

[2] Munro, S.; Thomas, K. L.; Abu-Shaar, M. *Nature* **1993**, *365*, 61-65.

[3] Howlett, A. C.; Barth, F.; Bonner, T. I.; Cabral, G.; Casellas, P.; Devane, W. A.; Felder, C. C.; Herkenham, M.; Mackie, K.; Martin, B. R.; Mechoulam, R.; Pertwee, R. G. *Pharmacol. Rev.* **2002**, *54*, 161-202.

[4] Wise, A.; Gearing, K.; Rees, S. *Drug Discovery Today* **2002**, *7*, 235-246.

[5] Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Le Trong, I.; Teller, D. C.; Okada, T.; Stenkamp, R. E.; Yamamoto, M.; Miyano, M. *Science* **2000**, *289*, 739-745.

[6] Herkenham, M.; Lynn, A. B.; Little, M. D.; Johnson, M. R.; Melvin, L. S.; De Costa, B. R.; Rice, K. C. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 1932-1936.

[7] Onaivi, E. S.; Leonard, C. M.; Ishiguro, H.; Zhang, P. W.; Lin, Z.; Akinshola, B. E.; Uhl, G. R. *Prog. Neurobiol.* **2002**, *66*, 307-344.

[8] Pan, X.; Ikeda, S. R.; Lewis, D. L. *Mol. Pharmacol.* **1998**, *54*, 1064-1072.

[9] Hillard, C. J.; Munthian, S.; Kearn, C. S. *FEBS Lett.* **1999**, *459*, 277-281.

[10] Milligan, G.; Bond, R. A. *Trends Pharmacol. Sci.* **1997**, *18*, 468-474.

[11] Barnett-Norris, J; Hurst, D. P.; Lynch, D. L.; Guarnieri, F.; Makriyannis, A.; Reggio, P. H. *J. Med. Chem.* **2002**, *45*, 3649-3659.

[12] Shim, J. Y.; Welsh, W. J.; Howlett, A. C. *Biopolymers* **2003**, *71*, 169-189.

[13] Salo, O. M. H.; Lahtela-Kakkonen, M.; Gynther, J.; Järvinen, T.; Poso, A. *J. Med. Chem.* **2004**, *47*, 3048-3057.

[14] Song, Z. H.; Bonner, T. I. *Mol. Pharmacol.* **1996**, *49*, 891-896.

[15] Ballesteros, J. A.; Weinstein, H. In *Methods in Neuroscience*, Conn and Sealfon Eds. Academic Press. San Diego, **1995**; Vol. *25*, pp. 366-428.

[16] McAllister, S. D.; Rizvi, G.; Anavi-Goffer, S.; Hurst, D. P.; Barnett-Norris, J.; Lynch, D. L.; Reggio, P. H.; Abood, M. E. *J. Med. Chem.* **2003**, *46*, 5139-5152.

[17] Hurst, D. P.; Lynch, D. L.; Barnett-Norris, J.; Hyatt, S. M.; Seltzman, H. H.; Zhong, M.; Song, Z.-H.; Nie, J.; Lewis, D.; Reggio, P. H. *Mol. Pharmacol.* **2002**, *62*, 1274-1287.

[18] Nie, J.; Lewis, D. L. *J. Neurosci.* **2001**, *21*, 8758-8764.

[19] Andersson, H.; D'Antona, A. M.; Kendall, D. A.; Von Heijne, G.; Chin, C. N. *Mol. Pharmacol.* **2003**, *64*, 570-577.

[20] Ulfers, A. L.; McMurry, J. L.; Kendall, D. A.; Mierke, D. F. *Biochemistry* **2002**, *41*, 11344-11350.

[21] Singh, R.; Hurst, D. P.; Barnett-Norris, J.; Lynch, D. L.; Reggio, P. H.; Guarnieri, F. *J. Peptide Res.* **2002**, *60*, 357-370.

[22] Leterrier, C.; Bonnard, D.; Carrel, D.; Rossier, J.; Lenkei, Z. *J. Biol. Chem.* **2004**, DOI 10.1074/jbc.M403990200.

[23] Goutopoulos, A.; Makriyannis, A. *Pharmacol. Therapeut.* **2002**, *95*, 103-117.

[24] Chin, C.-N.; Murphy, J. W.; Huffman, J. W.; Kendall, D. A. *J. Pharmacol. Exp. Ther.* **1999**, *291*, 837-844.

[25] Rhee, M.-H.; Nevo, I.; Bayewitch, M. L.; Zagoory, O.; Vogel, Z. *J. Neurochem.* **2000**, *75*, 2485-2491.

[26] Song, Z. H.; Slowey, C.-A.; Hurst, D. P.; Reggio, P. H. *Mol. Pharmacol.* **1999**, *56*, 834-840.

[27] Feng, W.; Song, Z. H. *Biochem. Pharmacol.* **2003**, *65*, 1077-1085.

[28] Rhee, M. H.; Nevo, I.; Levy, R.; Vogel, Z. *FEBS Lett.* **2000**, *466*, 300-304.

[29] Song, Z. H.; Feng, W. *FEBS Lett.* **2002**, *531*, 290-294.

[30] Feng, W.; Song, Z. H. *FEBS Lett.* **2001**, *501*, 166-170.

[31] Rhee, M.-H. *J. Vet. Sci.* **2002**, *3*, 185-191.