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Structural Insights from Recent CB1 X-Ray Crystal Structures

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Abstract

Over the past 2 years, X-ray crystal structures of the antagonist- and agonist-bound CB1 receptor have been reported. Such structures are expected to accelerate progress in the understanding of CB1 and should provide an exceptional starting point for structure-based drug discovery. This chapter examines the consistency of these X-ray structures with the CB1 experimental literature, including mutation, NMR and covalent labeling studies. These comparisons reveal discrepancies between this literature and the TMH1-2-3 region of each CB1 crystal structure. The chapter also examines crystal packing issues with each X-ray structure and shows that the discrepancies with the experimental literature can be attributed to crystal packing problems that force the N-terminus deep in the binding pocket of the two inactive state structures and force TMH2 to bend at G2.53/S2.54 and invade the binding pocket in the activated state structure. Revision is advisable before these structures are used for structure-based drug discovery.

Keywords: cannabinoid CB1 receptor, CB1 mutation, CB1 cross-linking, CB1 nuclear magnetic resonance, crystal packing

1. Introduction

The cannabinoid receptor type 1 (CB1) belongs to the G-protein coupled receptors (GPCRs) superfamily. GPCRs comprise the largest group of integral membrane proteins that mediate cellular responses to a wide spectrum of signaling molecules including peptides, lipids, neurotransmitters, glycoproteins, as well as light, taste and odor substances. They act via coupling and activating intracellular effector proteins including G-proteins and arrestins leading to an array of intracellular signaling cascades.
GPCRs have a common architecture of seven transmembrane helices (TMHs) joined by extracellular (EC) and intracellular (IC) loops of varied lengths, in addition to an extracellularly extending N terminus, and an intracellular C terminus that begins with an amphipathic alpha helical segment (Helix 8) oriented parallel to the cell membrane. In Class A GPCRs, the binding site for the endogenous ligand is generally formed by the EC core within the TMH bundle, and may extend to EC loops, referred to as the orthosteric binding site. Ligands may also bind to distinct (allosteric) binding sites in the receptor.

Due to the various physiological functions mediated by GPCRs, they are considered major targets for drug discovery and design of novel therapeutics. However, understanding the structure-function relationship of these proteins and the design of high affinity, selective ligands that target these receptors requires a detailed knowledge of the three-dimensional structure of the receptor in general and of the ligand binding site in specific. However, structural characterization for membrane proteins in general has been a challenge due to their low expression in recombinant hosts and their inherent instability in surfactants. It was not until the year 2000 that the first high resolution GPCR structure was resolved by X-ray crystallography, Rhodopsin in its inactive state [1]. The following 10 years witnessed the release of other inactive state crystal structures of class A GPCRs (e.g. the Adenosine A2A, and the β1 and β2 adrenergic receptors [2–4]), in addition to the release of the active state crystal structure of Rhodopsin in complex with a synthetic peptide resembling the C-terminus of the G-alpha subunit of transducing [5]. Available structures during that time served as templates for homology modeling for other GPCRs including the CB1 receptor. And parallel with biophysical studies, available crystal structures provided structural insights for their activation mechanism. A breakthrough in GPCR structural characterization has been achieved in the last 8 years with more than 200 structures for different GPCRs being deposited in the Protein Data Bank, including the CB1 inactive and active state crystal structures which have been resolved in 2016, and in 2017 respectively [6–8]. Before that, structural characterization of CB1 orthosteric as well as allosteric binding domains have been extensively studied via mutations, site-directed labeling, mass spectrometry, SAR studies, and in-silico methods, and will be discussed in detail throughout this chapter.

2. Structural divergence of the cannabinoid receptors from class A GPCRs

The CB1 receptor is a class A (Rhodopsin-like) GPCR (Figure 1). Different phylogenetic studies and multidimensional scaling analysis of Class A GPCRs classify cannabinoid receptors (CB1/CB2) into one cluster along with the endothelial differentiation G-protein coupled receptors (EDGRs) (including Sphingosine 1-phosphate receptors (S1P) and Lysophosphatidic acid receptors (LPA)) [9–12]. Receptors from those families, except for the LPA receptors, share common sequence divergence from other Class A GPCRs. Specifically, the absence of helix kinking proline residues in TMH2 and TMH5, and the absence of a disulfide bridge between the EC-2 loop and C3.25 at the EC end of TMH3. Instead, they share an internal disulfide bridge in the EC-2 loop, a conserved PxxGW motif at the EC end of TMH4, in addition to a Y5.39 that forms an aromatic pi-pi stack with W4.64 in that motif resulting in a similar shape of the EC2 loop as seen in the crystal structures for the CB1, S1P, and LPA receptors [6, 7, 13, 14]. At the
binding site, they share a common basic residue (K/R 3.28) on TMH3 and an aromatic residue (F/Y 2.57) on TMH2. In addition, the S1P receptors are like CB1/CB2 in the presence of E1.49 at TMH1. E1.49 has been reported to be a key interaction site for pregnenolone (an endogenous negative allosteric modulator that protects the brain from cannabis intoxication) with CB1 [15], while the LPA1–3 receptors share a W5.43 with CB1/CB2 that has been shown to affect antagonist binding to the cannabinoid receptors [16]. In addition, S1Pγ and the cannabinoid receptors recognize lipid-derived ligands that have been shown to bind to the receptor by diffusing from bulk lipid towards the binding site via a transmembrane portal [6, 7, 14, 17, 18].
3. CB1 receptor crystal structures

Two inactive state crystal structures for the hCB1 receptor have been resolved. The first structure (PDB ID: 5TGZ) was resolved at 2.8 Å; the receptor was truncated at both the N-terminus (1–98) and the C-terminus (415–472), with a flavodoxin protein fused into the IC3 loop (V306, P332), the receptor was crystalized in complex with a biaryl-pyrazole derivative (AM6358, Figure 2) and using thermo-stabilizing mutations (T3.46A, E5.37K, T5.47V, and R6.32E) [6]. The second structure was resolved at 2.6 Å (PDB ID: 5U09) in complex with an acyclic high affinity inverse agonist of the CB1 receptor, taranabant (Figure 2) [7]. In this structure, fewer amino acid residues were truncated from the N-terminus (1–76) and the C-terminus (422–472), and *P. abysii* glycogen synthase protein was fused into the IC3 loop (A301, D333) of a single point mutant (T3.46A) hCB1 receptor [7]. In both structures, resolved residues were from E100 at the N-terminus to F412 at the C-terminus of the receptor.

![Figure 2. Compounds discussed in this chapter.](image-url)
Agonist bound hCB1 crystal structures (PDB IDs: 5XRA, 5XR8) were resolved at 2.80 and 2.95 Å resolution and in complex with the classical cannabinoids (AM11542, AM841) respectively (Figure 2). The receptor was constructed in a similar way to the AM6358-bound crystal structure. Resolved residues included D104-S414 and F102-S414 in the 2.80 and 2.95 Å resolution structures respectively [8].

Inactive state CB1 structures show a transmembrane portal for antagonist entry between TMH1 and TMH7 that is similar to the S1P1 structure. However, the membrane proximal region in the CB1 receptor forms a loop that extends towards the orthosteric binding site with two amino acid residues (F102, M103) invading unpredictably the binding site in the inactive state structures and forming Van der Waal (VDW) interactions with the antagonists (Figure 3) [6, 7].

Active state structures show characteristic conformational changes featuring class A GPCR activation including an outward movement and a counterclockwise rotation (EC view) of the IC end of TMH6, resulting in a break in the R3.50/D6.30 inactive state “ionic lock” [19, 20]. Unlike inactive state structures, a transmembrane portal is not present in active state structures due to the packing of the EC domain of TMH1 towards TMH7. In addition, the N-terminus resides at the top of the receptor with no invasion of the orthosteric binding site. On the other hand, the active state binding site displays a profound (53%) reduction in size that is resulting from an inward kink of the EC domain of TMH2 towards the orthosteric binding site, as well as, rotation of TMH3 towards TMH2 [8].

4. Mutation and labeling studies on CB1: consistency with CB1 crystal structures

Multiple mutation studies on either mCB1 or hCB1 were aimed to study the receptor’s binding site and to identify key residues for CB1 receptor activation (Figure 4). While different ligands where used in functional and binding affinity assessment, WIN55212, SR141716A and CP55940, were used primarily, due to the availability of tritiated versions of these compounds.
Figure 4. Mutations in or near the binding crevice of the CB1 receptor. The key represents changes in binding affinities of ligands to mutant CB1 receptor compared to WT. Residues are numbered using Ballesteros-Weinstein numbers. See Figure 1 for more details.
Other ligands characterized include HU210, Δ9-THC, tarianabant, and AM251. The discussion here will be focused on mutation and labeling studies near the orthosteric binding site and those affecting crystalized ligands or closely related structures such as SR141716A, HU201, or CP55940 (Figure 2).

4.1. K3.28 residue

One of the earliest mutational studies on the CB1 receptor targeted K3.28 [21, 22]. The lipophilic nature of CB1 ligands and the fact that the third TMH in CB1 has a V3.32 instead of an acidic residue at that position (as being conserved in aminergic receptors), directed the attention towards K3.28 to investigate its role in ligand binding.

Song and Bonner first reported that the binding of CP55940, HU210, and anandamide to a K3.28A mutant hCB1 expressed in HEK293 cells resulted in severe impairment, with more than 100-fold decrease in their potencies in inhibiting cAMP accumulation. On the other hand, the binding and the potency of WIN55212 at the K3.28A mutant receptor were comparable to WT, suggesting that the receptor is still functioning [21]. Shortly afterwards, Kendall’s Lab demonstrated retained binding affinity and potency for CP55940 in CHO cells expressing K3.28R hCB1 mutant, with no binding for up to 75 nM concentration in cells expressing K3.28Q or K3.28E mutants compared to cells expressing WT receptor ($K_d = 7.7 \pm 3.5$ nM). In the same study, WIN55212 displayed comparable affinity for the three mutants with more than one order of magnitude decrease in potency in the K3.28E mutant, while its potency in K3.28Q mutant was not determined due to low receptor density [22]. A significant loss of CP55940’s potency in stimulating [35S]GTPγS binding in HEK293 cells expressing the K3.28A hCB1 mutant were also reported where the $EC_{50}$ values for the WT and the mutant receptor were 1.3 and 225 nM respectively [23].

Results suggested that the loss of potencies of anandamide, and the classical and non-classical cannabinoids, but not WIN55212 at the K3.28A mutant is due to their low affinities to the receptor, and a basic residue at 3.28 is required for CP55940 binding. Based on mutation data, modeling studies suggested a hydrogen bond interaction between K3.28 and the amide oxygen of anandamide [16, 24], and with classical and non-classical cannabinoids [25–27]. While Shim argued later that K3.28 is important for stabilizing the binding site for the endocannabinoids and the classical and non-classical cannabinoids and not directly involved in their binding [28].

K3.28 mutations have also been demonstrated to affect affinities and deactivation profile of biaryl-pyrazole derivatives. The affinity of SR141716A to K3.28A hCB1 mutant has been reported to be 17-fold lower compared to the WT [29]. In addition, SR141716A was reported to act as neutral antagonist with loss of ability to turn off receptor’s basal activity in inhibiting $Ca^{2+}$ currents in SCG neurons microinjected with K3.28A hCB1 mutant cDNA [30]. This data prompted a mutant cycle study using an SR141716A analog (VCHSR) to test the hypothesis that an interaction between the carboxamide oxygen in SR141716A and K3.28 is essential for its inverse agonist activity. The results supported the hypothesis by demonstrating that VCHSR acts as neutral antagonist with comparable affinities to both K3.28A and WT receptor [29]. A set of SR141716A analogues were also designed later that support the hypothesis [31]. A K3.28L mutation at hCB1 has been also reported to lower the binding affinity of AM251 by 17-fold compared to the WT, while it had no effect on the affinity of the acyclic antagonist, tarianabant, to the receptor [32]. The discriminatory effect of K3.28 mutants on different classes
of antagonists may suggest different binding interactions within the receptor’s binding site, especially that taranabant acts also as an inverse agonist [33].

CB1 crystal structures, on the other hand, do not support proposed hydrogen bonding of ligands to K3.28. In both inactive state and the active state structures of CB1, K3.28 orients its side chain towards the TMH2/3 interface forming salt bridges with D184 in the EC1 loop and D2.63 at the top of TMH2. The K3.28/D2.63 interaction is only noticeable in the inactive state crystal structures (Figure 3).

4.2. D2.63 mutations

As described above, this residue forms a salt bridge with K3.28 in the inactive state crystal structures (Figure 4). This K3.28/D2.63 salt bridge has been previously proposed to be essential for CB1 basal activity [34]. However, an earlier study on K3.28A mutant reported a comparable basal activity to the WT receptor in inhibiting Ca\(^{2+}\) currents which does not support the role of K3.28/D2.63 salt bridge in controlling receptor’s basal activity [30]. Individual effects of D2.63 mutation on ligand binding and receptor activation have been also reported. In HEK293 cells expressing a D2.63N hCB1 mutant, binding affinities for the classical cannabinoid (HU210), non-classical cannabinoid (CP55940), and the amino alkyl indole (WIN55212) were not significantly different from WT, while the affinity for SR141716A was 5-fold decreased compared to the WT. In addition, the potencies of CP55940 and HU210 in stimulating \(^{[35]}\)S GTP\(_{\gamma}\)S binding were significantly different from WT with about 12-fold increase in their EC\(_{50}\) values, while the basal activity of the D2.63N mutant was not different from WT [35]. In a different study, a double hCB1 mutant (L3.43A/D2.63A) was shown to lower the affinity of CP55940 to the receptor by 7-fold, while increasing the affinity of SR141716A by 3-fold. The L3.43A single mutant had an opposite effect by increasing the affinity of CP55940 to the receptor by 6-fold and lowering the affinity of SR141716A by 7-fold. Knowing that L3.43A mutation has been shown to increase the basal signaling of CB1 receptor in stimulating \(^{[35]}\)S GTP\(_{\gamma}\)S binding, combining D2.63A with L3.43A mutation lowered the basal signaling below CB1 WT levels. Results suggest that D2.63 may be involved in receptor activation and that mutation into alanine stabilizes the inactive state of the receptor [34, 36]. A modeling and mutation study suggested that an ionic interaction between D2.63 and K373 in the EC-3 loop is important for receptor activation. In the study, a reciprocal mutant D2.63K/K373D resulted in similar potencies for CP55940 and WIN55212 in stimulation for \(^{[35]}\)S GTP\(_{\gamma}\)S compared to the WT receptor, while their potencies were more than 5-fold lower in the single and double alanine mutants [37]. Such an interaction is not present in the crystal structures.

4.3. Mutation studies on the CB1 N-terminus

The CB1 receptor is unique in having a relatively long (114 amino-acid residues) N-terminus compared to other class A GPCRs. Analysis of the amino acid sequence of the membrane proximal region (MPR) of the amino terminus reveals a remarkably high degree of conservation in that region (Figure 5).

Early studies on the N-terminus reported no effect on prolylglycine insertion in the N-terminus (at A73, L86, and E100) of hCB1 receptor expressed in HEK 293T cells on agonist (CP55940) and antagonist (SR141716A) binding. In addition, S1.30A and Q1.31A mutants at the N-terminal
Figure 5. Sequence alignment of the CB1 N-terminus of 11 different species downloaded from the UniProt online database (www.uniprot.org).
end of TMH1 did not affect the binding affinity for SR141716A, while they reduced the binding affinity of CP55940 by 5- and 10-fold respectively [38]. In addition, CP55940 binding to truncated receptor at the N-terminal region (Δ64, Δ80, Δ89, Δ103 shCB1) was comparable to the WT receptor [39, 40]. On the other hand, the binding affinity of SR141716A to the Δ103 synthetic hCB1 (shCB1) truncation mutant was higher compared to the WT with retained ability to inhibit basal signaling of the truncated mutant [39]. As described earlier, in the (inactive state CB1 X-ray crystal structures, two amino acid residues from the N-terminus occupy the receptor’s orthosteric binding site, forming strong VDW interactions with the antagonists, those are F102, M103. Affinity data of SR141716A to the Δ103 shCB1 truncation mutant was higher compared to the inactive state crystal structures.

Reduction of the proposed disulfide bridge at the N-terminus C98/C107 reduces CP55940 potency in [35S]GTPγS binding assay [39]. However, a previous study reported that a double mutant of the two cysteine residues into serine subtly affected CP55940 binding, but did not affect SR141716A binding [41]. It is worth-mentioning that the C98/C107 residues are conserved among all 11-CB1 species available from UniProt. This sulfide bridge is not apparent in crystal structures.

Interestingly, a recent mutational, and modeling study from the Kunos lab identified an N-terminal residue (M106 in rodent CB1 compared to I105 in hCB1) as the determinant of the species differential affinity of {5-(4-chlorophenyl)-N-{(1R,2R)-2-hydroxycyclohexyl}-6-(2-methoxyethoxy)-3-pyridinecarboxamide} (14 h) at the CB1 receptor [42]. The compound, has been described previously as a peripherally selective, high affinity CB1 receptor antagonist [43]. However, this compound has been shown to have higher affinity for the hCB1 receptor compared to mouse and rat CB1 receptor [42]. This residue faces the ligand binding site in crystal structures, but with a changed position in the different structures.

4.4. EC1 loop

Mutations of the EC1 loop negatively impacted CP55940 but not SR141716A binding, the Kᵢ value of CP55940 was 26-fold higher in D184A hCB1 mutant compared to the WT receptor expressed in HEK293 cells. Here, the Kᵢ was determined by competition binding against [3H]SR141716A [38]. This aspartate residue forms an ionic interaction with K3.28 in both the active and inactive state CB1 crystal structures (Figure 3) [6–8]. H181A, R182A, and K183A have also lowered CP55940 affinity by 3–4-fold compared with the WT [38]. None of the EC1 loop residues forms direct contact with crystallized ligands.

4.5. Aromatic residues lining the orthosteric binding site

The orthosteric binding site of CB1 is lined with multiple aromatic residues located on TMH2/3/5/6/7, as well as, F286 in the EC2 loop.

4.5.1. F2.57, F2.61, and F2.64

F2.57 is two turns extracellular to the conserved D2.50, facing the orthosteric binding site. In the inactive state CB1 crystal structures, this residue has been shown to form an aromatic
π-π stack with the 2,4-dichlorophenyl ring in AM6538 [6], and with the cyanophenyl ring in taranabant [7]. Mutation data show a reduced affinity for taranabant and AM251 (a diarylpyrazole antagonist) by 30- and 97-fold respectively in F2.57 A hCB1 mutant [32]. In addition, both SR141716A and AM6538 failed to antagonize 100 nM CP55940-induced inhibition of cAMP in F2.57 A hCB1 mutant while preserving their abilities in F2.57 W hCB1 mutant [6]. Results indicate a major role for this residue in antagonist binding via aromatic interactions and in shaping the antagonist binding site. On the other hand, while this residue shows a major contact with the agonists (A-ring, Figure 2) in the AM11542 and the AM841 bound crystal structures, [8] CP55940 displayed similar potency for inhibition of cAMP in both F2.57 A, and F2.57 W mutants compared to WT [8].

Mutations on F2.61 revealed effects on antagonist and agonist binding and potencies. In the inactive state CB1 structures, this residue is rotated towards TMH1 and its side chain is at the TMH1/TMH2 interface, yet it forms moderate VDW interactions with the piperedine and with the trifluoro-methyl pyridine in AM6538 and taranabant respectively [6, 7]. While in the active state structures, this residue faces the binding site and forms strong VDW interactions with the agonists (AM11542 and AM841) B-ring (Figure 2) [8]. SR141716A displayed only 5-fold higher $K_d$ value in F2.61 A hCB1 mutant transiently expressed in HEK293 cells [44], but both SR141716A and AM6538 failed to inhibit 100 nM CP55940-induced inhibition of cAMP in F2.61 A mutant while preserving their potencies in F2.61 W mutants (mutations were on hCB1, and functional assays were done in stably transfected CHO cells) [6]. Also, CP55940 displayed similar potency in both F2.61 A and F2.61 W in inhibition of cAMP compared to the hCB1 WT stably transfected in CHO cells, [8] while the binding affinities for CP55940, HU210, and Δ9-THC determined against $[^3H]$SR141716A were severely affected by F2.61A mutation transiently transfected in HEK293 cells [45]. In the same study, the potency of HU210 in inducing $[^35S]$GTP$\gamma$S binding has been reported to be 30-fold less in F2.61 A hCB1 mutant compared to the WT [45].

The F2.64 A mutation has also been shown to be detrimental for agonists (HU210, CP55940, and Δ9-THC) binding [45]. CP55940, AM841, and AM11542 displayed about an order of magnitude lower potency in inhibition of forskolin-stimulated cAMP in CHO cells expressing the mutant receptor [8]. In crystal structures, this residue forms major contacts with the agonists’ (C-ring, Figure 2) [8], and does not display any contact with the antagonists due to the presence of the N-terminus [6, 7], and no mutation data are available to characterize antagonists binding or potency in this mutant.

4.5.2. F3.25

Different studies determined binding affinity of CP55940 to F3.25 A mutant; in one study, the binding affinity of CP55940 determined by saturation binding against $[^3H]$SR141716A was 60-fold lower in F3.25 A hCB1 stably transfected in CHO-K1 cells compared to WT [38]. In other studies, CP55940 affinity was not affected in F3.25 A mCB1 receptor stably transfected into HEK293 cells, affinity was determined using $[^3H]$CP55940 [16, 46]. The discrepancy in binding affinities here could be due to species differences. F3.25 A did not affect SR141616A binding in those studies [16, 38, 46]. Basal $[^35S]$GTP$\gamma$S binding was also determined for the F3.25 A mCB1 mutant stably transfected in HEK293 cells and was not significant from WT, while the WIN55212-2 induced $[^35S]$GTP$\gamma$S binding was lower in the mutant with $EC_{50}$ value
being 6-fold higher compared to the WT. In crystal structures, this residue shows moderate 
VDW interactions with the crystallized agonists (C-ring, Figure 2) [8], and no direct interac-
tions with the antagonists [6, 7].

4.5.3. Y5.39, W5.43

Y5.39 is a conserved residue in many class A GPCRs. In the active state crystal structures, Y5.39 
interacts with the agonists and forms a hydrogen bond interaction with the isothiocyanate 
moiety in AM841. Mutation data published along with the crystal structures show that mutation 
of this residue in hCB1 into phenylalanine or alanine results in significant reduction in the 
potencies of CP55940, AM841, and AM11542 in the inhibition of forskolin-induced cAMP, 
with pEC\textsubscript{50} values for CP55940 being 8.3 ± 0.15 for the WT and 6.7 ± 0.13 and 5.4 ± 0.95 for the 
Y5.39F and Y5.39A mutants respectively [8]. Efficacy data for CP55940 are consistent with 
previous report from Abood’s Lab [47]. In this report, WIN55212-2 has been shown to retain its 
WT potency in the Y5.39F mutant. In addition, the Y5.39F hCB1 mutant generally retained WT 
binding affinities for CP55940, Δ9-THC, WIN55212-2, and SR141716A and resulted in 17-fold 
lower K\textsubscript{i} value for anandamide. On the other hand, Y5.39I hCB1 mutant resulted in loss of 
ligand binding. Authors concluded that aromaticity is required at this position [47]. Results 
from Abood’s lab suggest that aromaticity is required for ligand binding generally, while the 
phenolic ring is required for signal transduction for classical and non-classical cannabinoids.

The W5.43A mutation in mCB1 was detrimental for the binding of SR141716A [16, 46], this 
mutation also negatively affected the binding affinity of AM251 to the mutant hCB1 with 54-fold 
lower affinity, while it resulted in only 7-fold lower affinity for taranabant [32]. This mutant 
resulted in 16-fold reduction in affinity of WIN55212-2, but did not affect either CP55940 or 
anandamide binding [16, 46]. The potency of CP55940 in stimulation of [\textsuperscript{35}S]GTP\gammaS, however, 
was 66-fold lower in the mutant receptor compared to the WT, while the basal [\textsuperscript{35}S]GTP\gammaS 
binding for the W5.43A mutant being comparable to WT [46]. In active state crystal structures, 
this residue forms strong VDW interaction with AM841 and AM11542 aliphatic tails. In inactive 
state structures, the residue forms moderate VDW interactions with the 4-chlorophenyl 
ring in taranabant and the aliphatic chain-substituted phenyl ring in AM6538, an interaction 
that is inconsistent with the mutation data which suggests that W5.43 stabilizes the binding 
site of the antagonists, rather than being a strong interaction site with the antagonists.

4.5.4. W6.48, F3.36: the rotamer toggle switch

W6.48 belongs to the conserved CWXP hinge motif in TMH6. A W6.48 χ1 rotameric state 
change from g+ to trans has been proposed to be the binding pocket trigger for the hinge 
motion of TMH6 that occurs during receptor activation. Here the IC end of TMH6 moves away 
from the TMH bundle, providing an opening into which the alpha-5 helix of the G-protein can 
insert [48–52]. This rotameric change is manifest for class A GPCRs in Molecular Dynamics 
(MD) simulations [18, 53–55], even though available active state crystal structures of class A 
GPCRs do not show evidence for this rotameric change. The W6.48A mCB1 mutation resulted 
in a 7-fold increase in binding affinity (K\textsubscript{i}) of SR141716A compared to the WT receptor, while 
it had no effect on the dissociation constant of CP55940 [16, 46]. In the CB1 crystal structures, 
only antagonists show mild VDW interaction with W6.48.
Computational modeling and mutation studies targeting F3.36 in CB1 receptor suggested that the F3.36/W6.48 interaction represents a toggle switch that stabilizes the inactive state of the receptor [46, 56]. Consistent with the inactive and active state CB1 crystal structures, the modeling study suggested that F3.36/W6.48 contact is broken during activation with a rotameric change of the $\chi_1$ dihedral of F3.36 from trans in the inactive state to g+ upon activation. The F3.36A CB1 mutation resulted in increased basal signaling of the receptor and did not affect the CP55940 dissociation constant, but reduced the binding affinity of SR141716A [49, 57, 58]. An F3.36L mutation generally restored the binding affinity of SR141716A to the receptor [57]. In a different study, the F3.36L mutation resulted in a 7- and 9-fold lower binding affinities for taranabant and AM251 respectively [32]. In the CB1 crystal structures, only agonists show mild VDW interaction with F3.36 via their dimethyl substituent. Thus, the reduction in the binding affinity of SR141716A to the F3.36A mutant could be a result of shifting the equilibrium towards active state.

While the rotameric change of F3.36 only and not W6.48 is evident in the CB1 crystal structures, it is essential to notice that this change requires a synchronized rotameric change in the $\chi_1$, as well as, the $\chi_2$ dihedrals of W6.48. Thus, it could be proposed that a transient rotameric change in $\chi_1$ dihedral of W6.48 from g+ to trans or vice-versa is required to permit conformational changes in F3.36. In addition, the major rotameric change in F3.36 is associated with a rotational movement of TMH3 towards TMH2. Agonists appear to stabilize this conformational change in TMH3 by blocking F3.36 in g+, thus stabilizing the active state of the receptor. While in the inactive state structures, it could be noticed that the antagonists seem to prohibit the rotameric change of W6.48 into trans, thus acting as inverse agonists at the CB1 receptor.

4.5.5. F7.35

This residue has been shown to mildly affect SR141716A binding with ~4-fold increase in $K_d$ in F7.35A hCB1 mutant [44]. Potencies of SR141716A and AM6538 in inhibiting 100 nM CP55940 activity were also retained in F7.35A and F7.35W hCB1 mutations [6]. However, the potency of CP55940, AM841, and AM11542 in inhibition of forskolin-induced cAMP has been shown to be around one order of magnitude affected by F7.35W mutation which might be due to steric hindrance, while their potencies were majorly affected by a 7.35A mutation [6, 8]. This residue shows moderate VDW interactions with the gem dimethyl group at C1’ of agonists and very mild VDW interactions with the antagonists in the active and inactive state crystal structures respectively.

4.6. EC2 loop residues

The CB1 EC2 loop lines the binding site with five amino acid residues residing on top of the ligand binding site; 267-IFPHI-271. Mutations at the EC2 loop have been shown to affect CP55940 binding generally and have no effect on SR141716A binding. Replacement of the entire hCB1 EC2 loop (254-GWNCEKLQSVCSDFPHIDETYL-276) by the hCB2 EC2 loop (GWTCPRP - - CSELFPLIPNDYL) did not affect SR141716A binding but resulted in a complete loss of CP55940 binding, while replacing EKLQSV in CB1 by CPRP (CB2/EC2) resulted in receptor sequestration [41]. In addition, the C257/C264 internal disulfide bridge has been determined to be required for membrane expression [41, 59]. Single point alanine mutations
were investigated for the majority of the EC2 loop. Among the residues that face the binding site, F268A/N hCB1 mutation impaired receptor membrane expression. F268Y hCB1 mutation had no effect on ligand binding, while F268W mutation drastically affected CP55940 binding with no effect on SR141716A binding. In addition, P267A, H270A, and I271A mutants showed no effect on SR141716A binding while drastically affecting CP55940 binding [60]. In crystal structures, F268 forms strong VDW interaction with both agonists and antagonists in addition to an aromatic stacking with the agonists. P267 and I271 form week VDW interaction with agonists while the H270 side chain points towards TMH3 and is packed against F3.25. In addition, due to closer packing of the EC end of TMH5/6 towards the ligand binding site in the active state compared to the inactive state, and the fact that agonists are cupping F268 compared to the antagonists, it could be interpreted that F268W mutation data regarding the binding of agonists versus the antagonists could be consistent with crystal structures.

4.7. Cysteine residues in the EC domain of CB1; labeling and mutation studies

Among the 13 cysteine residues in the CB1 receptor, C6.47, C7.38, and C7.42 reside in the EC transmembrane domain and are not engaged in a disulfide bond. C6.47 is only available in the binding pocket in the activated state of Class A GPCRs. Consistent with this, the earliest CB1 cysteine reactivity study using the isothiocyanate derivatized agonist, AM841, showed that AM841 labels C6.47 [61]. A subsequent study showed that AM841 also labels C6.47 in CB2 [62]. The isothiocyanate derivatized anandamide analog, AM3677, was also found to label C6.47 [63]. This has led to the hypothesis that cannabinoid agonists enter CB1 via a portal between TMH6 and TMH7 at the level of C6.47. The active state crystal structure, is not consistent with cysteine crosslinking studies of AM841, since the AM841 alkyl tail points towards Y5.39 in the crystal structure.

In another cysteine reactivity study, C7.42, was found reactive, suggesting that it faces the binding pocket. Mutation of C7.42 to a larger amino acid resulted in loss of SR141716A binding, but not CP55940 binding. In all reported crystal structures, C7.42 faces into the binding pocket. Further, if C7.42 is mutated to M in the active state structure, it does not affect the agonist binding pocket. However, a methionine residue at that position in the inactive state structures clashes severely with the antagonists and surrounding residues, such clashes are not relieved by rotameric changes for nearby residues.

4.8. Serine residues in CB1

Mutation of S7.39 in hCB1 to alanine in was generally detrimental for CP55940, HU201 and AM4056 binding to the CB1 receptor, while it had no effect on the binding affinities for SR141716A, AM251, as well as, WIN55212 [32, 57, 64]. On the other hand, it resulted in a profound reduction in the binding affinity of tataanabant to the receptor [32]. In the inactive state crystal structure in complex with tanaanabant, as well as, the active state crystal structure, there is a hydrogen bond interaction between S7.39 and the ligand. The residue adopts a g− χ1 dihedral that allows this interaction. In the AM6358/inactive state crystal structure, this residue adopts a g+ χ1 dihedral. In this structure, the ligand is incapable of forming a hydrogen bond interaction with S7.39, since such an interaction requires a high energy conformation of the antagonist.
Mutation data show that the S2.60A mutation in hCB1 has no effect on the binding affinities of both CP55940 and SR141716A [64]. S2.60 does not seem to be involved in any interactions with ligands in the crystal structures. This is due to the rotation of TMH2 towards TMH3 caused by the G2.53/S2.54 motif in TMH2 allowing a wider turn in that region.


L3.29 faces the ligand binding site and has been shown to interact with both agonists and antagonists. Such interactions are stronger in the active state due to the rotation of TMH3 towards TMH2, allowing L3.29 to be more oriented towards the binding site. The L3.29A mutation in hCB1 has been shown to mildly affect the binding affinity of SR141716A to the receptor, while having a profound effect on the binding of CP55940, HU210 and ∆9-THC. The L3.29A mutations resulted in reduced efficacy of both HU210 in stimulation of [35S]GTPγS binding and in the efficacy of CP55940 in the inhibition of forskolin-induced cAMP accumulation [8, 44, 45].

Both T3.33A and M6.55A mutations did not have any effect on the binding affinity of SR141716A which is consistent with the inactive state crystal structures [44]. M6.55A mutation in hCB1 resulted in a 15- and 4-fold reduction in the affinity of HU210 and CP55940 respectively while it did not affect the affinity of ∆9-THC [45]. This residue shows moderate VDW interactions with the agonists in the crystal structures.

5. NMR and circular dichroism (CD) studies on the C-terminus

Both NMR and CD studies have been performed on the C-terminus of CB1 employing peptide segments that correspond to that receptor region. Results show a helical segment resembling helix 8 that is parallel to the plane of the membrane [65–67]. Ahn et al., reported two amphipathic α-helical domains; S410-F412 that corresponds to helix 8, and a second helical segment (A440-M461) that is also parallel to the membrane, (Figure 1) [65].

6. Crystal contacts

In the inactive and active state CB1 crystal structures, crystal packing impinges on the ligand binding site (Figure 6). In the first published CB1 inactive state structure [6], receptor bundles are crystallized top-to-top, forcing the N-terminus to invade the binding pocket and flattening the EC loops. In the second inactive state CB1 structure [7], adjacent bundles impinge on receptor EC loops and N-terminus around the “rim” of the receptor’s EC domain (Figure 6).

The effect on CB1 structure is similar to that discussed above for the first inactive crystal structure. Crystal packing in the active state structure [8] also causes an impact on the CB1 binding pocket. Packing causes TMH2 to hinge at G2.53/S2.54 (S2.54 has a χ1 = g−) and invade the binding pocket. Packing also impacts the N-terminus, TMH1 above N1.50, the EC top of TMH3, the EC-2 loop and the EC end of TMH4.
Such packing issues can promote non-genuine conformations in the structure that is promoted by the crystalline low energy state. A recently published crystal structure of the μ-opioid receptor (MOR) has revealed a histidine H54 residue in the N-terminus of the receptor that...
is positioned 2.6 Å from the secondary amine of the bound agonist. Mutation of this residue into alanine did not affect the affinity of the ligand to the receptor, suggesting that the resulting conformation of the N-terminus in MOR structure is a result of crystallization and not relevant in the real state [68].

7. Conclusions

Because X-ray crystal structures are used frequently for drug design projects, it is critical to identify any issues with these structures, such as crystal packing effects and to evaluate how consistent these structures are with the body of structural information in the literature for a given receptor, such as mutation, cross-linking and NMR studies. Results presented in this chapter show that crystal packing issues impact both of the CB1 inactive state crystal structures and the activated state CB1 crystal structures. Impacts include N-terminus insertions deep into the binding pocket seen in the CB1 inactive state structures, as well as, TMH1 and TMH2 bending into the binding pocket seen in the activated state structures. Not surprisingly, we find here that the CB1 structures have important inconsistencies with mutation data, particularly in their TMH1-2-3 regions. In addition, the CB1 crystal structures do not capture the movement of W6.48 during receptor activation, or the existence of a ligand portal in the activated state; however, X-ray structures by their very nature will not capture all transient changes. In conclusion, then, the CB1 crystal structures are an important contribution to the drug design field, but revisions are advisable before these structures are used for structure-based drug discovery.

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