

Crystal Structure of the Human Cannabinoid Receptor CB₁

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<http://dx.doi.org/10.1016/j.cell.2016.10.004>

SUMMARY

Cannabinoid receptor 1 (CB₁) is the principal target of Δ^9 -tetrahydrocannabinol (THC), a psychoactive chemical from *Cannabis sativa* with a wide range of therapeutic applications and a long history of recreational use. CB₁ is activated by endocannabinoids and is a promising therapeutic target for pain management, inflammation, obesity, and substance abuse disorders. Here, we present the 2.8 Å crystal structure of human CB₁ in complex with AM6538, a stabilizing antagonist, synthesized and characterized for this structural study. The structure of the CB₁-AM6538 complex reveals key features of the receptor and critical interactions for antagonist binding. In combination with functional studies and molecular modeling, the structure provides insight into the binding mode of naturally occurring CB₁ ligands, such as THC, and synthetic cannabinoids. This enhances our understanding of the molecular basis for the physiological functions of CB₁ and provides new opportunities for the design of next-generation CB₁-targeting pharmaceuticals.

INTRODUCTION

Marijuana from *Cannabis sativa* L. has been used for both therapeutic and recreational purposes for many centuries (Lemberger, 1980; Li, 1973). In the 1940s, chemistry based on compounds isolated from the plant (Wollner et al., 1942) produced novel biologically active molecules (Adams et al., 1948; Ghosh et al., 1940); however, it was not until the 1960s that the active constituent of marijuana, Δ^9 -tetrahydrocannabinol (THC), a

terpenoid molecule, was isolated and characterized (Gaoni and Mechoulam, 1964). This provided an early molecular foundation for medicinal chemists to develop related structural analogs and new synthetic ligands (Makriyannis and Rapaka, 1990; Razdan, 1986). Initially, due to their lipophilic nature, it was assumed that cannabinoids exerted their effects by perturbing the physical properties of biological membranes (Makriyannis, 2014; Mavromoustakos et al., 1995). This assumption was challenged with the discovery, cloning, and expression of the first cannabinoid-specific membrane receptor, then designated as the cannabinoid receptor (CB) (Devane et al., 1988; Matsuda et al., 1990). With the subsequent identification of a second receptor, the designation evolved to CB₁ and CB₂ (Munro et al., 1993). The discovery of the endogenous agonists to the receptors, the endocannabinoids, anandamide (Devane et al., 1992), and 2-arachidonoyl glycerol (2-AG) (Mechoulam et al., 1995) soon followed.

Cannabinoid receptors belong to the class A G protein-coupled receptor (GPCR) family, signal through inhibitory G $\alpha_{i/o}$ heterotrimeric G proteins (Howlett, 1985), and interact with β -arrestins (Jin et al., 1999). CB₁ is the most highly expressed GPCR in the human brain and is expressed throughout the body, with the highest levels found in the central nervous system (Herkenham et al., 1990).

Cannabis has been used for centuries in many cultures to treat a wide range of medical conditions. More recently, therapeutic considerations have moved beyond the plant extract to explore and produce more pharmacologically refined compounds. CB₁-selective small-molecule agonists have shown therapeutic promise in a wide range of disorders, including pain and inflammation (Cravatt and Lichtman, 2004), multiple sclerosis (Pertwee, 2002; Pryce and Baker, 2015), and neurodegenerative disorders (Fernández-Ruiz et al., 2015). The first CB₁-selective antagonist/inverse agonist, rimonabant (SR141716, Acomplia [Sanofi-Aventis]) (Rinaldi-Carmona

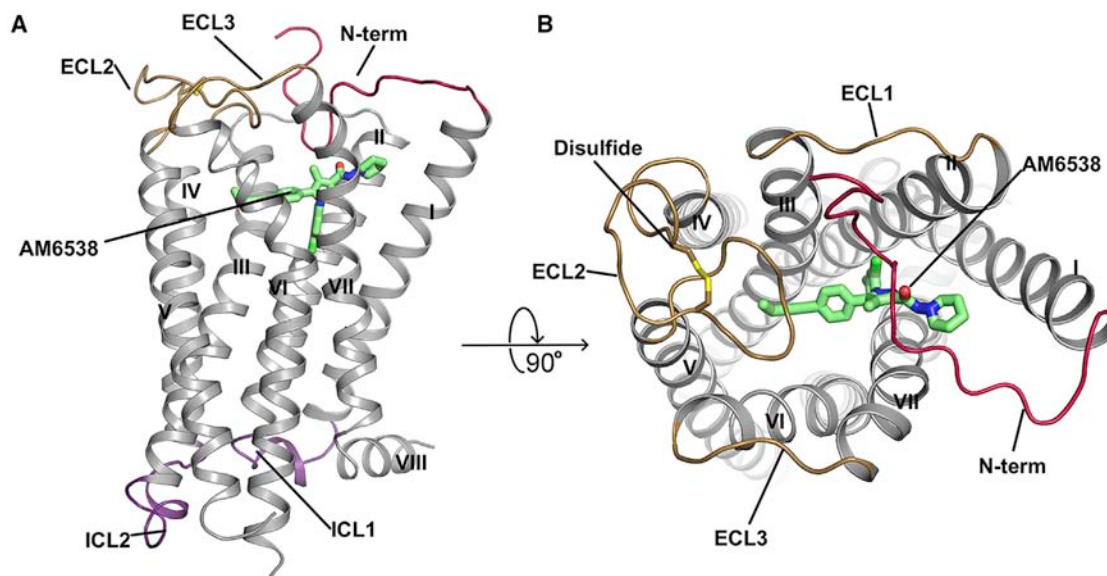


Figure 1. Overall Structure of CB₁-AM6538 Complex

(A) Side view of the CB₁-AM6538 complex. The receptor is shown in gray cartoon representation. The ligand AM6538, shown with green sticks, demarcates the binding pocket, which is partially occluded by the N-terminal loop (red). The nitrate group is not modeled in the experimental crystal structure, as the electron density was not observed. The extracellular loops (ECLs) are shown in brown and the intracellular loops (ICLs) are shown in purple.

(B) Top view of the extracellular side. The disulfide bond in ECL2 is shown as yellow sticks.

See also Figures S2 and S3.

et al., 1994), received approval from the European Medical Agency as an adjunct to diet and exercise for treating obesity (Janero and Makriyannis, 2009). Antagonists of CB₁ have been explored as potential therapeutics for obesity-related metabolic disorders (Mazier et al., 2015), mental illness (Black et al., 2011; Rubino et al., 2015), liver fibrosis (Mallat et al., 2013), and nicotine addiction (Schindler et al., 2016). However, rimonabant and other ligands in its class were not approved in the United States due to concerns about adverse events, such as increased anxiety, depression, and suicidal ideation.

Numerous studies have investigated how ligands binding CB₁ can mediate downstream signaling. While the variety of compounds exhibiting different pharmacological profiles have provided clues regarding CB₁ activation, the molecular details defining the binding modes of both endogenous and exogenous ligands are still largely unknown (Guo et al., 1994; Makriyannis, 2014; Picone et al., 2005). In order to address this deficit in understanding, we have determined the crystal structure of CB₁ in complex with a tight binding antagonist AM6538. In conjunction with molecular docking, the structure was used to elucidate the binding modes of a diverse set of antagonists/inverse agonists and agonists of CB₁. The structural details of the cannabinoid receptor reported herein improve our understanding of how ligands engage to modulate the cannabinoid system and provide a useful model to facilitate the design of next-generation pharmaceuticals to avoid unwanted side effects. The findings provide insight into mechanisms of slow dissociation of antagonists, which may potentially translate into long acting pharmacological effects.

RESULTS

Synthesis of CB₁ Stabilizing Antagonist AM6538 for Structural Studies

One of the key factors facilitating the structure determination of CB₁ (Figure 1) is utilization of the antagonist AM6538, the synthesis of which resulted from the strategic modification of rimonabant to enhance its ability to stabilize the ligand-receptor complex and promote CB₁ crystal formation. In contrast with rimonabant, the 5-phenyl ring substituent was modified so as to introduce motifs (ex. alkyne unit) that could favor increased affinity for the CB₁ receptor (Tam et al., 2010). The rimonabant analog, AM251, (1, Figure 2A) (Lan et al., 1999), a compound that has been used extensively as a pharmacological standard CB₁-selective antagonist, was used as the precursor in the AM6538 synthetic process. Synthesis of AM6538 involves the functionalization of the iodo substituent at the para position of the 5-phenyl ring in AM251 with an acetylenic chain system consisting of four carbons and substituted at the omega carbon. To this end, we initially focused on targeting cysteine residues within CB₁ by introducing suitable electrophilic groups (Janero et al., 2015; Li et al., 2005; Mercier et al., 2010; Picone et al., 2005; Szymanski et al., 2011) at the fourth carbon of the alkyne unit, capable of forming a covalent bond with the cysteine thiol group. For AM6538, we introduced at this position a nitrate group (ONO₂) whose role was to serve as a polar group, which may be displaced by a suitable nucleophile (e.g., thiol) (Pattison and Brown, 1956; Yeates et al., 1985) at or near the binding domain or alternatively bind as an intact group so as to obtain a non-covalent, near-irreversible attachment by interacting with hydrogen bonding amino acid residues, as well as

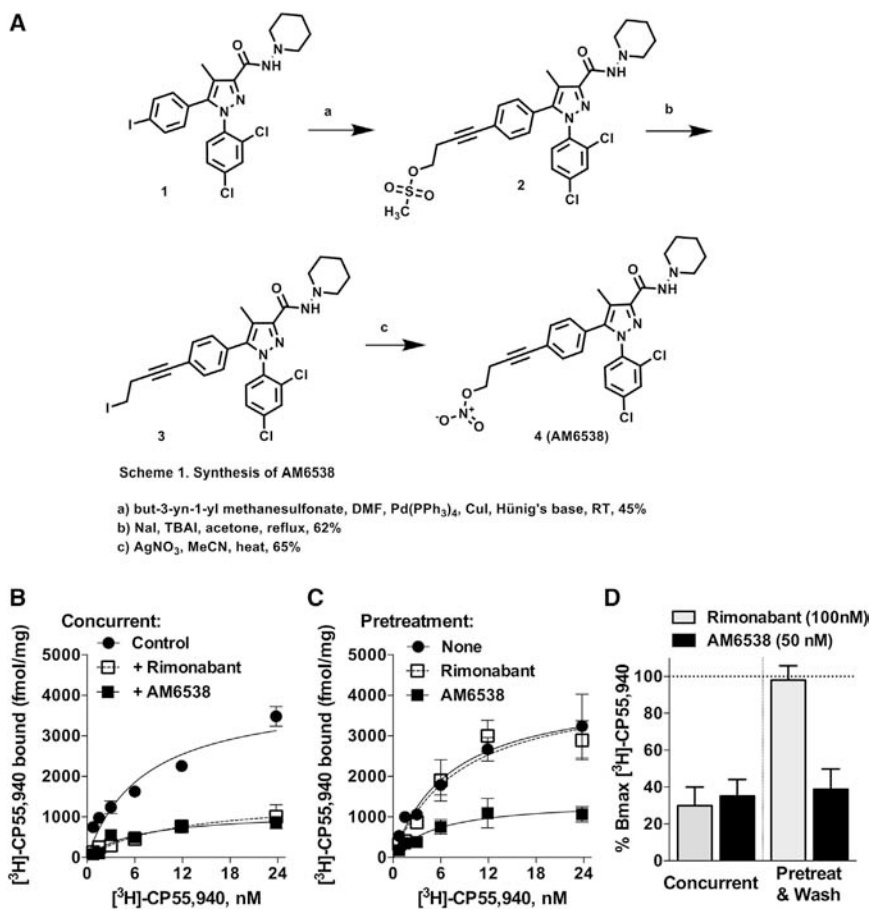


Figure 2. Synthesis and Characterization of AM6538

(A) Synthetic procedures for compound AM6538. (B) Saturation [³H]-CP55,940 binding assays in the absence (control) or presence of rimonabant (100 nM) or AM6538 (50 nM) demonstrates that both antagonists cause displacement of specific binding of the radioligand when present concurrently in the 1 hr binding assay.

(C) Following pretreatment of membranes (37°C, 6 hr) with buffer only (none), rimonabant (100 nM), or AM6538 (50 nM); membranes were washed with buffer 3× prior to [³H]-CP55,940 binding as described for (B).

(D) Percentage of remaining binding (B_{max}) detected following the conditions described in (B) (concurrent) and (C) (pretreat and wash). Both antagonists decrease the binding of [³H]-CP55,940 to ~30% when incubated concurrently during the 1 hr binding assay. Under pretreatment and washout conditions, rimonabant does not affect subsequent radioligand binding, while AM6538 continues to compete despite washing of the membranes.

See also Figure S1 and Table S1.

residues capable of π - π interactions. In the present study, affinity mass spectrometry analysis suggests that AM6538 reacts with CB₁ as an intact molecule with no evidence of covalent modification of relevant cysteine residues.

AM6538 was a strong candidate for crystallographic studies of the receptor based on its high affinity and wash-resistant binding to CB₁ as determined by radioligand competition assays against the tritiated agonist, [³H]-CP55,940 ($K_i = 3.4 \pm 1.0$ nM) (Figures 2B–2D). This is in contrast to rimonabant, which can be readily washed out of membranes, permitting subsequent radioligand binding (Figures 2B–2D). Importantly, the crystallized CB₁ construct (described below) has comparable affinity for AM6538 as the wild-type receptor ($K_i = 5.1 \pm 0.9$ nM).

In functional assays, AM6538 is a competitive antagonist of the effects of CP55,940 and THC on CB₁-mediated inhibition of adenylyl cyclase activity and β -arrestin2 recruitment in overexpression systems (Figures S1A and S1B and Table S1). Competitive antagonism was confirmed by the [³⁵S]-GTP γ S binding assays performed in mouse cerebellum (Figure S1C). For comparison purposes, competitive antagonism was demonstrated for rimonabant in the same systems (Figure S1 and Table S1).

Structure Determination of CB₁-AM6538 Complex

To facilitate crystallization, it was necessary to modify the wild-type (WT) CB₁ sequence. Construct optimization proced-

ures (Lv et al., 2016) identified Flavodoxin (Chun et al., 2012) as a stabilizing fusion partner when inserted within the receptor's third intracellular loop (ICL3) at Val306 and Pro332. Additionally, the WT receptor was truncated on both the N and C termini by 98 and 58 residues, respectively. Finally, in order to

improve the expression and thermostability of the receptor, four computationally predicted mutations (Thr210^{3.46}Ala [D'Antona et al., 2006], Glu273^{5.37}Lys, Thr283^{5.47}Val, and Arg340^{6.32}Glu) were introduced to the CB₁ sequence (Figures S2A–S2C) (superscripts denote amino acid position as described by Ballesteros and Weinstein [1995]). The modified CB₁ construct was inserted into a pTT5 vector for expression in HEK293F cells to generate protein (Figure S2D) that formed crystals in lipidic cubic phase supplemented with cholesterol (Figure S3E); the crystals diffracted to 2.8 Å (Table 1).

Based on affinity mass spectrometry analysis, intact AM6538 is associated with the CB₁ protein (Figure S3F). Electron density with three branches was observed near the orthosteric binding site and modeled as core AM6538 atoms with the terminal nitrate group omitted (Figures 3D and S3). A molecular dynamics (MD) simulation was performed on the CB₁-AM6538 complex with the nitrate group intact and modeled through docking analysis, and the results revealed that the root mean square fluctuation (RMSF) values for the nitrate group and the hinge carbon atom are higher, suggesting that the nitrate group is more mobile than other atoms in AM6538 (Figure S4B). As a modeled ligand with other possibilities to fit the electron density, further studies on ligand binding are under investigation.

Table 1. Crystallographic Data Collection and Refinement Statistics

Data Collection and Refinement Statistics	
Ligand	AM6538
Number of crystals	29
Data Collection	
Space group	C2
Cell dimensions	
a,b,c (Å)	116.56, 52.63, 143.63
β (°)	111.14
Number of reflections measured	160,794
Number of unique reflections	19,837
Resolution (Å)	47.30 - 2.80 (2.90-2.80)
R_{merge} ^a	0.126 (0.520)
Mean I/ σ (I)	10.1 (2.1)
Completeness (%)	97.4 (94.1)
Redundancy	8.1 (4.6)
CC _{1/2}	0.999 (0.44)
Refinement	
Resolution (Å)	47.05 - 2.80
Number of reflections (test set)	19,827 (985)
$R_{\text{work}}/R_{\text{free}}$	0.207/0.238
Number of Atoms	
CB ₁	2,312
Flavodoxin	1,103
AM6538 ^b	33
Lipid and other	102
Average B Factor (Å ²)	
Wilson	73.6
Overall	87.4
CB ₁	110.6
Flavodoxin	66.6
AM6538	119.5
Lipid and other	79.7
rmsds	
Bond lengths (Å)	0.004
Bond angles (°)	0.638
Ramachandran Plot Statistics (%) ^c	
Favored regions	97.7
Allowed regions	2.3
Disallowed regions	0

^aData for high-resolution shells are shown in parenthesis.^bNitrate group is excluded due to the absence of electron density.^cAs defined in MolProbity.**Structural Features of CB₁ in Complex with AM6538**

The overall CB₁ structural fold shares a similar architecture with previously solved class A GPCR structures, containing seven transmembrane (7TM) α -helices (I to VII) connected by three extracellular loops (ECL1–3), three intracellular loops (ICL1–3), and an amphipathic helix VIII (Figures 1A and S3A). The non-truncated

part of the N terminus of CB₁, residues 99–112, forms a V-shaped loop, which inserts into the ligand-binding pocket and functions as a plug, restricting access to the pocket from the extracellular side (Figures 1A and 1B). While the influence of crystal packing interactions on the conformation of the N terminus (Figure S3B) cannot be ruled out, it is interesting to note that the N terminus has been consistently observed in an ordered form in the structures of the related lipid receptors LPA₁ (lysophosphatidic acid receptor 1) (Chrencik et al., 2015) and S1P₁ (sphingosine 1-phosphate receptor 1) (Hanson et al., 2012) and that the function of CB₁ is very sensitive to the presence of the ordered portion of the N terminus (Andersson et al., 2003; Fay and Farrens, 2013). ECL2 in CB₁ consists of 21 residues folding into an intricate structure that projects four residues (268–271) into the binding pocket. Previous work has shown that the four residues are important for mediating interactions with certain classes of ligands (Ahn et al., 2009; Bertalovitz et al., 2010) and that the two cysteines (Cys257 and Cys264) in ECL2 are critical to the function of CB₁ (Fay et al., 2005). In the structure, the conformation of ECL2 is constrained by the presence of an intraloop disulfide bond (Cys257–Cys264) (Figure 1B) previously found in the structures of the closely related LPA₁ and S1P₁ receptors. The highly conserved disulfide bond between ECL2 and helix III (Cys^{3,25}) in most class A GPCRs is lacking in all three lipid receptor structures.

AM6538 Interactions in CB₁ Ligand-Binding Pocket

The position of the ligand-binding pocket of CB₁ is different from the previously described orthosteric binding sites of other class A GPCRs. AM6538 lies quite low in the binding pocket of CB₁, immediately above the conserved Trp356^{6,48} (Figures 3A and 3B). The ligand adopts an extended conformation with the ligand strain close to its local minimum as determined by quantum mechanical calculations (Figure S3D).

AM6538 forms mainly hydrophobic interactions with ECL2 and the N terminus, as well as with all CB₁ helices except helix IV (Figures 3A and 3B). As described above, the ligand has a pyrazole ring core with three functional groups. For clarity, we have termed the 2,4-dichlorophenyl ring “arm 1,” the 4-aliphatic chain substituted phenyl ring “arm 2,” and the piperidin-1-ylcarbonyl “arm 3” (Figure 3C). The pyrazole ring core (including the 4-methyl group) is situated between helices II and VII, forming hydrophobic interactions with the side chains of Phe170^{2,57}, Phe379^{7,35}, and Ser383^{7,39} (Figure 3C) and is capped by the N-terminal loop interactions (Met103^{N-term}). Arm 1 is located in a narrow side pocket (Figure 3B) formed by helices II, III, VI, and VII and forms edge-face π - π interactions with the side chain of Phe170^{2,57} and with the backbone amide bond between Gly166^{2,53} and Ser167^{2,54}. This substituted ring moiety forms hydrophobic interactions with Val196^{3,32}, Trp356^{6,48}, Cys386^{7,42}, Leu387^{7,43}, and Met103^{N-term} (Figure 3C). The 2,4-dichlorophenyl ring in arm 1 fits well into the shape of the narrow side pocket (Figure S3C), which explains why 2,4-dichloro or 2-chloro substitutions result in optimal binding (Lange and Kruse, 2005).

Arm 2 of the ligand extends toward a long, narrow channel (Figure 3B) formed by helices III, V, VI, and ECL2. The phenyl group in arm 2 establishes π - π interactions with Phe102^{N-term}, Phe268^{ECL2}, and Trp356^{6,48}; hydrophobic interactions with

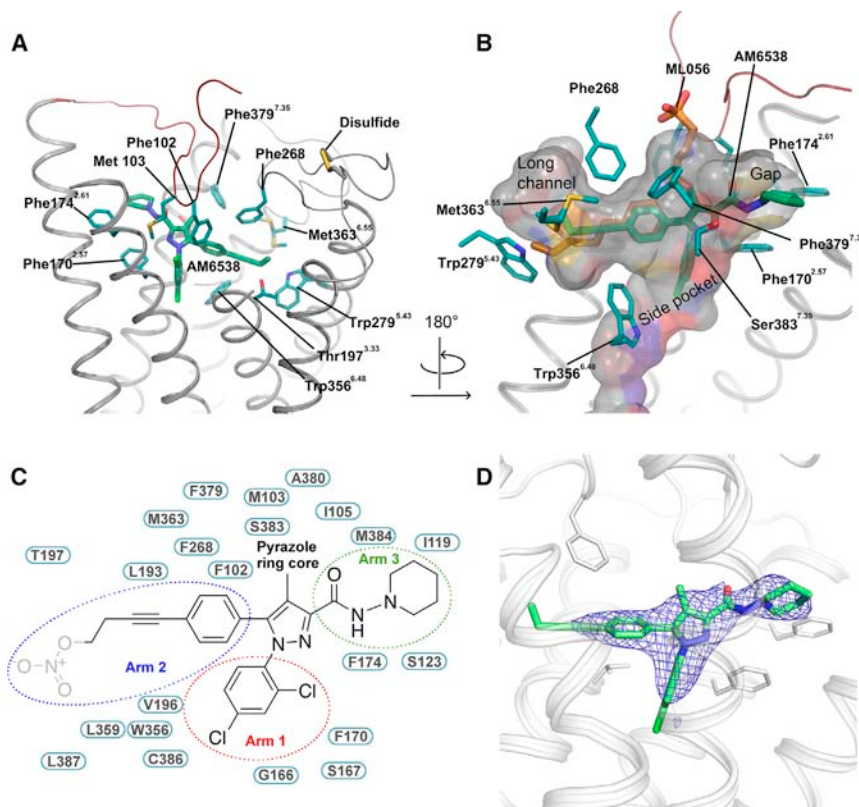


Figure 3. Analysis of the Ligand Binding Pocket of CB₁

(A) Key residues in CB₁ for AM6538 binding. AM6538 (green carbons) and CB₁ residues (teal carbons) involved in ligand binding are shown in stick representation. The receptor is shown in gray cartoon representation.

(B) The shape of the ligand binding pocket. AM6538 (green carbons) and ML056 (brown carbons) are shown in stick representation.

(C) Schematic representation of interactions between CB₁ and AM6538. The 2,4-dichlorophenyl ring in the red circle is termed as arm 1; the 4-aliphatic chain substituted phenyl ring in the blue circle is termed as arm 2; the piperidin-1-ylcarbamoyl in the green circle is termed as arm 3. The nitrate group, which was not observed in the electron density, is shown in gray.

(D) Electron density maps calculated from the refined structure of the CB₁-AM6538 complex. [Fo]-[Fc] omit map (blue mesh) of the ligand AM6538 is shown (contoured at 3 σ).

See also Figure S3.

Leu193^{3.29}, Val196^{3.32}, and Leu359^{6.51} are observed. A triple bond within the long aliphatic chain in arm 2 forms π - π interactions with Phe268^{ECL2} and Trp356^{6.48} and forms hydrophobic interactions with several residues, including Leu193^{3.29}, Val196^{3.32}, Thr197^{3.33}, Leu359^{6.51}, and Met363^{6.55} (Figure 3C). Interestingly, the binding mode of the long hydrophobic chain is similar to that of ML056 with the S1P₁ receptor (Figure 3B), implying that this could be a conserved binding pocket for long aliphatic chains in lipid-binding receptors. In regards to the nitrate group that has not been observed in the crystallographic structure, our docking experiments define a domain in which the nitrate group is interacting with residues Thr197^{3.33}, Tyr275^{5.39}, and Trp279^{5.43} through hydrogen bonding and π - π interactions (Figure S4A).

Finally, arm 3 extends toward a gap constituted by helices I, II, VII capped by the N-terminal loop (Figure 3B). It forms interactions with hydrophobic residues, Met103^{N-term}, Ile105^{N-term}, Ile119^{1.35}, Ser123^{1.39}, Phe170^{2.57}, Phe174^{2.61}, Ala380^{7.36}, Ser383^{7.39}, and Met384^{7.40} (Figure 3C). Unlike the π - π interactions formed by the other two arms, the interactions between arm 3 and the receptor are non-specific.

Among the interactions between AM6538 and CB₁, Phe170^{2.57} plays an important role by interacting with the pyrazole ring core, as well as rings in arm 1 and arm 3. Moreover, Phe170^{2.57} is pushed by the ligand to move toward helix I, resulting in a tilt of the last two turns (residues 170–177) of helix II toward helix I, compared to S1P₁ and LPA₁, the two closest homologs (Figures 4A and 4B). This tilted helix II, in turn, pushes

helix I by about 7 Å, mainly due to interactions with the two bulky residues Phe170^{2.57} and Phe174^{2.61}.

The role of Lys192^{3.28} in CB₁ has been intensively researched. It was reported that Lys192Ala/Lys192Gln/Lys192Glu mutants decreased the affinities of several agonists such as CP55,940, HU-210, and anandamide (Chin et al., 1998; Hurst et al., 2002; Pan et al., 1998; Song and Bonner, 1996). Previously, it was suggested that Lys192^{3.28} has direct interactions with CB₁ ligands. However, in our CB₁ structure, Lys192^{3.28} does not interact directly with AM6538. Instead, it forms a salt bridge/hydrogen bond network that stabilizes the conformation of ECL1, the N terminus, and the extracellular parts of helices II and III. The side chain of Lys192^{3.28} points away from the binding pocket and forms salt bridges with Asp176^{2.63} and Asp184^{ECL1}, while Asp184^{ECL1} further stabilizes the N terminus by forming a hydrogen bond with the backbone of Phe102^{N-term} (Figure 4C).

Structural Comparison of the CB₁, LPA₁, and S1P₁ Receptors

CB₁, LPA₁, and S1P₁ receptors all bind lipid-derived endogenous ligands (anandamide/sn-2-arachidonoylglycerol, sphingosine-1-phosphate, lysophosphatidic acid) (Shimizu, 2009). Early sequence analysis revealed that CB₁ has a moderate sequence identity with LPA₁ (13% overall, 28% in TM regions) and S1P₁ (14% overall, 27% in TM regions) (Bramblett et al., 1995; Isberg et al., 2016) (Figure S5). Crystal structures of LPA₁ (Chrencik et al., 2015) and S1P₁ (Hanson et al., 2012) have been recently determined. The main structural difference between the three lipid receptors occurs in the extracellular portion, with the most striking being the unique conformation of the N-terminal loop of CB₁ (Figures 4A and 4B). For all three receptors, the N terminus has a role in ligand recognition. Comparing the ligand binding

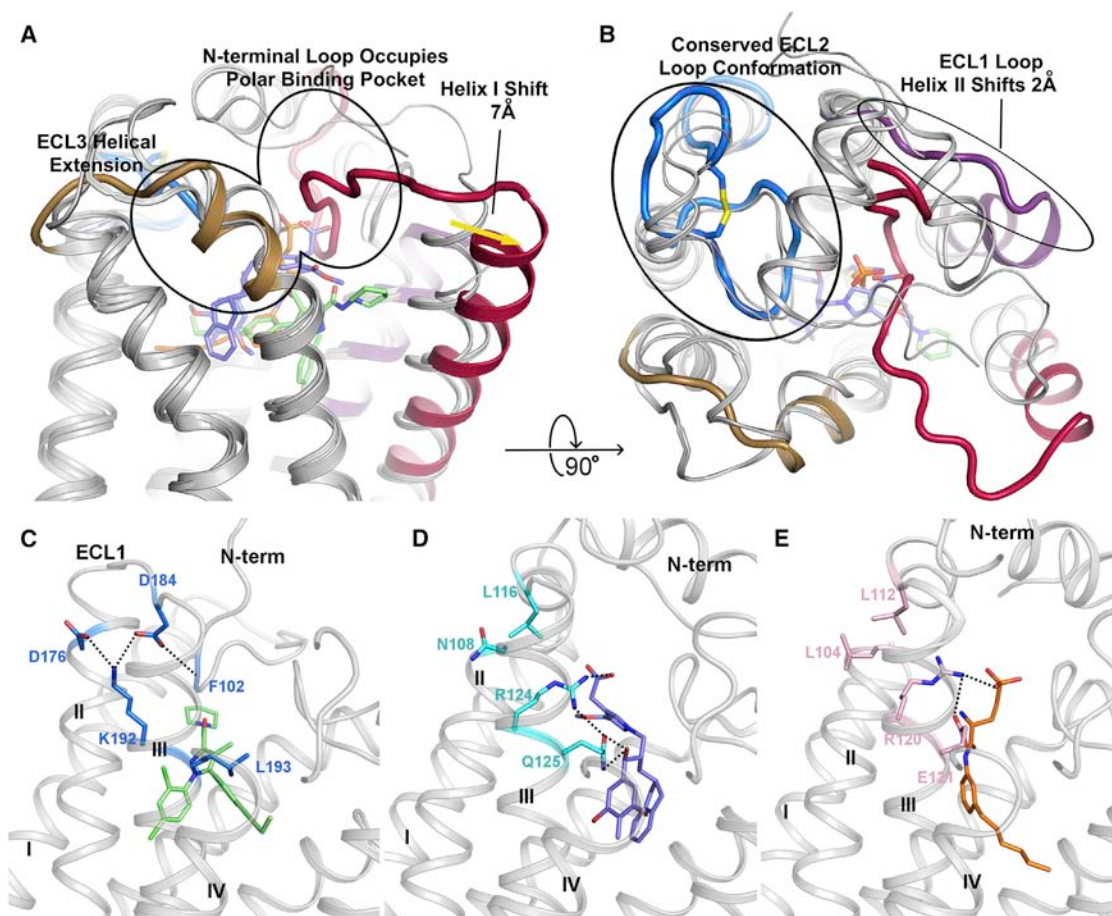


Figure 4. Comparison of CB₁, LPA₁, and S1P₁ Structural Features

(A) Side view of CB₁ with structurally divergent regions of LPA₁ (PDB: 4Z34) and S1P₁ (PDB: 3V2Y) overlaid. LPA₁ and S1P₁ receptors are shown in gray cartoons. The CB₁ N-terminal loop (red) occupies the polar binding pocket, helix I (red helix) is shifted out 7 Å compared with the other two receptors. ECL3 of CB₁ shows a three helical turn extension of helix VII (brown helix).

(B) 90° rotation of (A) for a top view of CB₁ with structurally divergent regions of LPA₁ (4Z34) and S1P₁ (3V2Y) overlaid. CB₁ shows a conserved conformation of ECL2 (blue) with the other two receptors, helix II is shifted out 2 Å (purple helix).

(C–E) The interaction network of position 3.28 of CB₁ (K192), LPA₁ (R124), and S1P₁ (R120). Polar interactions are represented by black dashed lines. (C) CB₁ is shown in gray cartoon, AM6538 is shown in green sticks and the key residues are shown in blue sticks; (D) LPA₁ is shown in gray cartoon, ONO-9780307 is shown in purple-blue sticks and the key residues are shown in cyan sticks; (E) S1P₁ is shown in gray cartoon, ML056 is shown in orange sticks and the key residues are shown in pink sticks.

See also Figure S5.

positions of the three receptors, AM6538 lies more horizontally than the ligands in LPA₁ and S1P₁, with arm 1 inserted deeper into the side pocket (Figure 4A). Consistently, the N-terminal loop in CB₁ is positioned deeper into the binding pocket compared to the N-terminal helices of LPA₁ and S1P₁, which are both positioned as a cap on their respective ligand binding pockets. As a consequence, helix I of CB₁ is pushed outward ~7 Å relative to LPA₁ and S1P₁ by arm 2, opening a wider gap between helices I and VII than what was observed in LPA₁ and S1P₁ (Figure 4A). Moreover, helix II and ECL1 change their conformation in CB₁, with helix II shifting 2 Å further from the binding pocket compared with LPA₁ and S1P₁, and ECL1 changing from a short helical region in LPA₁ and S1P₁ to a loop in CB₁ (Figure 4B). These conformational changes effectively enlarge the binding pocket of

CB₁, allowing access to the re-entrant N-terminal loop, and contribute to the extensive surface area and multiple sub-pockets associated with CB₁. Finally, the ECL3 region of CB₁ differs from that of its related receptors by a three helical turn extension of helix VII, which increases the rigidity and presumably decreases the flexibility of this loop region in CB₁ (Figure 4A).

The arrangement of Lys192^{3.28} in CB₁ is unique when compared with its equivalent residue Arg^{3.28} in LPA₁ and S1P₁. In LPA₁ and S1P₁, Arg^{3.28} points into the binding pocket forming a strong interaction with the phosphate head group of the ligands (Figures 4D and 4E); it is stabilized by the negatively charged or polar residue 3.29 (Gln125 in LPA₁ and Glu121 in S1P₁). However, in CB₁ the environment near Leu193^{3.29} and the ligand is hydrophobic, thus, it is energetically favorable for the positively

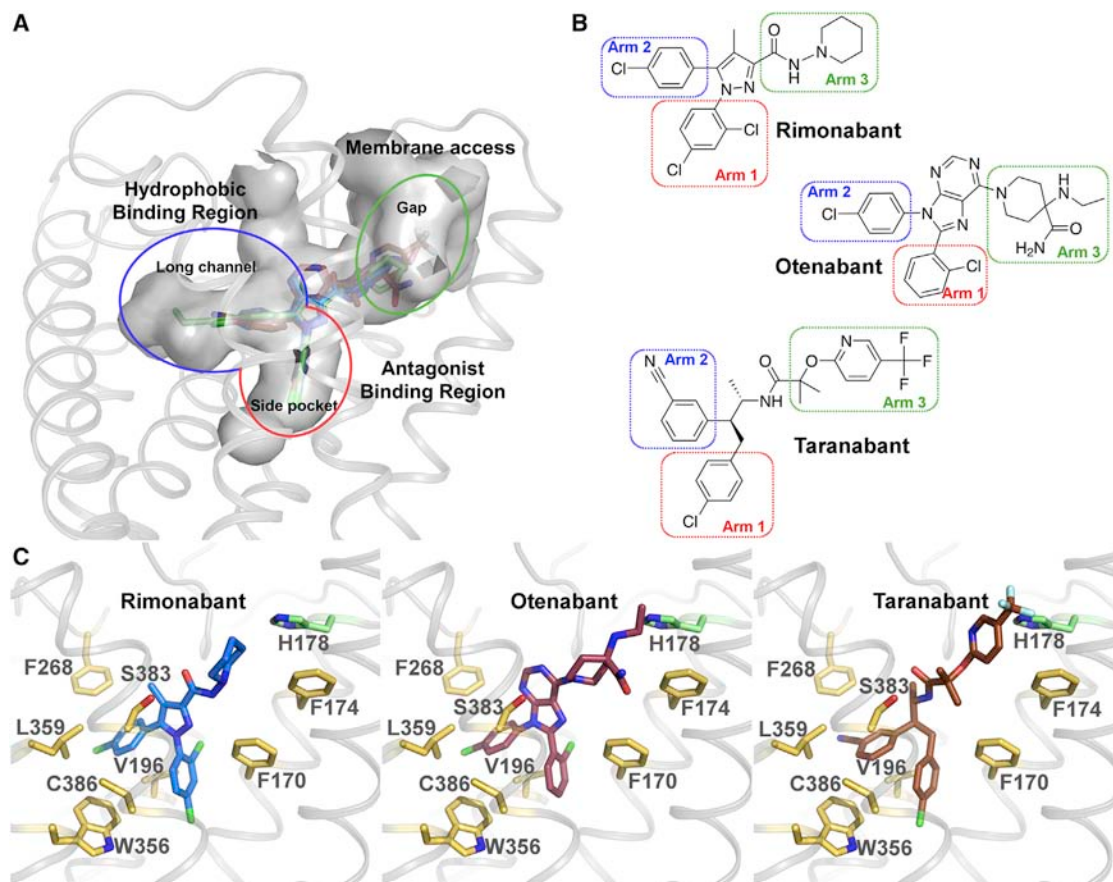


Figure 5. Docking of Different Antagonists in the CB₁ Crystal Structure

(A) CB₁ binding pocket with rimonabant (blue sticks), otenabant (raspberry sticks), and taranabant (brown sticks) are shown in gray surface representation. (B) Chemical structures of rimonabant, otenabant, and taranabant. The red/blue/green rectangles highlight previously described “arms” of the molecule termed arm 1/arm 2/arm 3 (see Figure 3C). (C) Predicted binding modes of rimonabant (blue sticks), otenabant (raspberry sticks), and taranabant (brown sticks) with CB₁. The interacting residues are shown in yellow sticks, and H178 is shown in green sticks. See also Figure S4 and Table S2.

charged Lys192^{3,28} to point away from the binding pocket (Figure 4C). In fact, Lys192^{3,28} functions as a stabilization anchor by forming a salt bridge/hydrogen bond network instead of directly interacting with the ligand as Arg^{3,28} in LPA₁ and S1P₁. Another major difference of the endogenous ligands of CB₁ (anandamide, 2-AG), LPA₁ (LPA) and S1P₁ (S1P) is the head group. The heads of LPA₁ and S1P₁ ligands are negatively charged phosphate groups, while the heads of CB₁ ligands are neutral. In fact, phosphorylation of the head group of CB₁ ligands anandamide and 2-AG would transform them into ligands of LPA₁ (Chrencik et al., 2015).

Binding Modes of Representative Antagonists to CB₁

We performed docking of AM6538 and three CB₁ antagonists: rimonabant, otenabant, and taranabant (Figure 5, Table S2), which represent diverse scaffolds of CB₁ antagonists used in clinical trials. For each compound, the top one ranked pose was used for analysis. The docking pose of AM6538 reproduces the crystallographic pose (Figure S4A), with a root mean

square deviation (RMSD) of 0.55 Å. For the three antagonists, their first-ranked docking poses resemble that of AM6538 in the crystal structure, with three arms that fit into the three branches of the binding pocket, as described in the AM6538 binding mode (Figure 5A). As denoted for AM6538, the arms in the side pocket, long channel, and gap are termed arm 1, arm 2, and arm 3 (Figure 5B). The scaffolds of arm 1 and arm 2 of the three antagonists are very similar to each other, and so are the docking poses. The biggest difference is from arm 3, yet they are all quite bulky, which we speculate is the signature for CB₁ antagonists. Taranabant has the highest affinity with CB₁ among the three ligands (Table S2). It does not have a rigid aromatic ring at the core, allowing its arm 2 and arm 3 to have more freedom to form stronger interactions with surrounding residues (Figure 5C). Mutagenesis of Phe170^{2,57}Ala or Phe174^{2,61}Ala results in dramatically reduced functional affinity for rimonabant and AM6538, while neither mutation alters the potency of the agonist, CP55,940. More conservative mutations to tryptophan (Phe170^{2,57}Trp or Phe174^{2,61}Trp) at either site have no

appreciable effect on antagonist binding, further supporting the importance of the hydrophobic interactions at this site (Figures S4G and S4H). In addition, we performed 50 ns MD simulations to visually assess the predicted ligand-receptor interactions, starting from the docking poses. The RMSD values of AM6538, rimonabant, and otenabant are about 1.4 Å. For taranabant, the value is larger (about 3 Å), in accordance with its lack of the core aromatic ring (Figures S4C–S4F). The predicted interactions of the ligands are conserved during the short MD simulations. The central structure of AM6538 in MD simulation is closest to the docking pose.

Docking Poses of Representative Agonists of CB₁

Although the crystal structure we present is in the inactive state, we are able to investigate how representative agonists likely bind to the orthosteric pocket of CB₁ by integrating molecular docking, mutagenesis, and SAR data (Ahn et al., 2009; Aung et al., 2000; Bertalovitz et al., 2010). Six CB₁ agonists (Table S2)—the classical cannabinoids, THC and CP55,940; the endogenous agonists, anandamide and 2-AG; and indole derivatives, JWH-018 and WIN 55,212-2—were selected for docking studies. These agonists mainly interact with ECL2, N-terminal loop, helices III, VI, and VII, and they do not interact with helices I and II. Their predicted binding modes are shown in Figure 6. For THC and CP55,940, the rings reside between the N-terminal loop and ECL2, forming π - π interactions with Phe268^{ECL2} (Figures 6A and 6B), and the carbon chains extend into the long channel and interact with residues from helices III, VI, and VII. CP55,940 does not interact with helices I and II, which is supported by our mutagenesis studies: mutations on Phe170 and Phe174 (Phe170^{2,57}Ala/Trp or Phe174^{2,61}Ala/Trp) do not alter the potency of CP55,940 (Figure S4G). Anandamide and 2-AG were predicted to adopt a C-shaped conformation and occupy a similar space as THC (Figures 6C and 6D). Their hydrophilic heads are sandwiched between the N-terminal loop and ECL2, and their long aliphatic tails extend deeper into the long channel. JWH-018 and WIN 55,212-2, however, are predicted to bind deeper in the pocket than THC (Figures 6E and 6F). Both the indole rings and naphthalene rings form π - π interactions with Phe268^{ECL2}. The N-substituents reach the end of the long channel and interact with helix V. The binding mode of JWH-018 and WIN 55,212-2 is supported by mutations on helix V (McAllister et al., 2003; Song et al., 1999) and SAR study of N-alkyl chain length (Aung et al., 2000). Notably, all of the agonists interact with Phe268^{ECL2} and Phe379^{7,35} in our docking poses, which is consistent with mutagenesis studies on ECL2 (Ahn et al., 2009) and Phe379^{7,35} (Figure S4I). Any of the following individual mutations—Phe268^{ECL2}Trp, Pro269^{ECL2}Ala, His270^{ECL2}Ala, Ile271^{ECL2}Ala, or breaking of the disulfide bond Cys257-Cys264 on ECL2—dramatically decreased the binding of all three different types of agonists, yet had little impact on antagonist binding (Ahn et al., 2009). The prominent role of Phe379^{7,35} to facilitate CP55,940 functional affinity is supported by the loss of CP55,940 potency with the Phe379^{7,35} Trp mutation and an even greater loss of agonist activity with the Phe379^{7,35}Ala mutation (Figure S4I). While these mutations dramatically affected CP55,940 agonism, they had no impact on antagonist/inverse agonist (AM6538 or rimonabant) displacement of agonist (Fig-

ure S4J), further supporting the predicted binding pose of CP55,940 (Figure 6G).

DISCUSSION

The ligand used in this study, AM6538, was designed with the aim of stabilizing the ligand-CB₁ receptor complex and promoting CB₁ crystal formation. For this purpose, our approach focused on the use of the substituted biarylpyrazole chemotype based on the structure of rimonabant for obtaining highly utilized proprietary probes. Within this class of compounds, a slight modification of the chemotype led to AM251, a commonly used CB₁ inverse agonist/antagonist (Lan et al., 1999), and AM281, a CB₁ antagonist whose radiolabeling produced the first in vivo imaging agent for labeling CB₁ in nonhuman primates and humans (Berding et al., 2004; Gatley et al., 1998). AM6538 acts as a CB₁ stabilizing antagonist, and the ligand was effective in allowing structural determination of CB₁. AM6538 reacts as an intact molecule with no crystallographic evidence of covalent binding while at the same time not revealing the location of the terminal nitrate group in the X-ray structure. While radioligand binding studies demonstrate that AM6538 binds tightly to the receptor, the precise mode of action for stabilizing the receptor remains to be determined.

To date, there remains considerable controversy with regards to CB₁ ligands and their diverse medical applications. This is likely due in part to the wide availability and illicit nature of the most famous CB₁ pharmaceutical, marijuana. Marijuana has been widely used across many cultures to treat multiple conditions, with most of the results relayed via oral tradition, anecdote, political position, or with economic interest preventing an objective interpretation of therapeutic efficacy in any particular disease state (Whiting et al., 2015). The medicinal marijuana movement continues to gain support, and clinical trials with well-defined endpoints will continue to educate the medical and pharmaceutical communities regarding the relative benefits and drawbacks of targeting this physiological system. The crystal structure of CB₁ in complex with AM6538 reveals an expansive and complicated binding pocket network consisting of multiple sub-pockets and channels to various regions of the receptor. The three-arm ligand structure is common to CB₁ antagonists and inverse agonists and may be critical for stabilizing the inherent flexibility of the native receptor in a non-signaling conformation. Combining the 3D structure of CB₁ and molecular docking of the three representative antagonists, which act as inverse agonists, rimonabant, otenabant, and taranabant, the role of each arm is clearly illustrated. Arm 1 is crucial for high affinity binding, while arm 2 extends into the long channel. An aliphatic or aromatic ring on arm 3 pushes on helices I and II, causing them to bend outward, and potentially modulating the pharmacological signaling state of the receptor. Together with structure and modeling data, we speculate that a bulky ring on arm 3 is essential for CB₁ antagonism. This observation provides direction for designing more diverse compounds as we have learned that variable chemical groups are tolerated at the core of arm 3, a long carbon chain can be added at the *para*-position of the phenyl ring in arm 2. For example, introduction of a 4-cyanobut-1-ynyl at arm 2

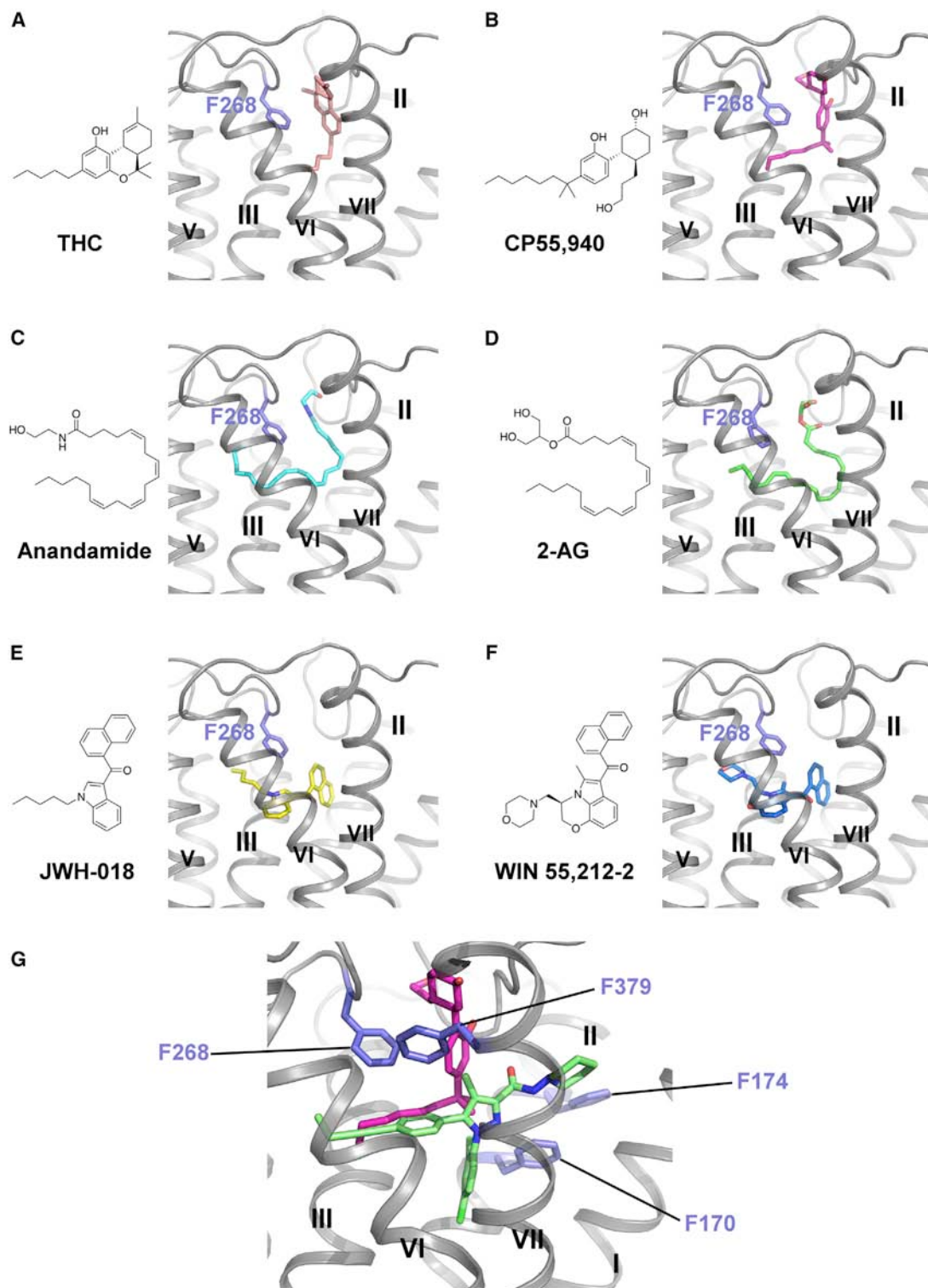


Figure 6. Docking Poses of Different Cannabinoid Receptor Agonists

(A–F) Chemical structures and predicted binding poses of THC (pink sticks) (A), CP55,940 (magenta sticks) (B), Anandamide (cyan sticks) (C), 2-AG (green sticks) (D), JWH-018 (yellow sticks) (E), and WIN 55,212-2 (blue sticks) (F).

(G) Zoom-in view of predicted CP55,940 binding pose. CP55,940 is shown in magenta sticks, AM6538 is shown in green sticks, and the key residues are shown in slate sticks.

See also [Figure S4](#) and [Table S2](#).

produces AM6545 (Table S2), a high-affinity CB₁ neutral antagonist (Tam et al., 2010).

Understanding the nuances of CB₁ binding and activation is important, as human use has noted differences between the phytocannabinoid agonist THC and the synthetic cannabinoid constituents of “Spice” or “K-2” such as JWH-018. In general, cannabinoid agonists are routinely abused substances; yet, while overdose of THC/marijuana has not been documented, there have been cases of severe and even deadly responses to the ingestion of such synthetic mixtures resulting in federal restrictions by many countries, including the US. It remains unclear as to why THC can have such a high safety margin, while the synthetic cannabinoid constituents can prove toxic with varying severities of serious side effects (Hermanns-Clausen et al., 2013). Going forward, the study of cannabinoids present in *Cannabis sativa* will provide clues to its high efficacy and safety margins and may continue to inspire a rich source of pharmacologically refined compounds and novel therapeutics; the utility of the crystal structure may provide inspiration for drug design toward refining efficacy and avoiding adverse events.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2016.10.004>.

AUTHOR CONTRIBUTIONS

Conceptualization, L.M.B., A.M., R.C.S., Z.-J.L.; Methodology, K.V., A.M. J.-H.H., R.B.L., E.L.S., B.W., Q.Z.; Validation, G.W.H.; Formal Analysis, T.H., M.P., L.Q., G.W.H., Y.W., S.Z., W.S., S.L., R.B.L., E.L.S., N.Z., B.W., Q.Z., M.A.H., L.M.B., A.M., Z.-J.L.; Investigation, T.H., K.V., L.Q., G.W.H., W.S., S.L., A.K., R.B.L., E.L.S., J.-H.H., N.Z., H.Z., I.K.; Writing – Original Draft, T.H.; Writing, Reviewing & Editing, T.H., K.V., Y.W., S.Z., W.S., I.K., M.A.H., L.M.B., A.M., R.C.S., Z.-J.L.; Visualization, T.H., M.P., M.A.H.; Supervision, L.M.B., A.M., R.C.S., Z.-J.L.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Science and Technology of China grants 2014CB910400 and 2015CB910104 and The National Nature Science Foundation of China grant 31330019 to (Z.-J.L.); National Institutes of Health grants P01DA009158 (A.M. and L.M.B.), R37DA023142 (A.M.), and R01AI118985 (I.K.); and NSF grants. We thank the Shanghai Municipal Government, ShanghaiTech University, and GPCR Consortium for financial support. The synchrotron radiation experiments were performed at the BL41XU of Spring-8 with approval of the Japan Synchrotron Radiation Research Institute (JASRI) (proposal numbers 2015B1031 and 2016A2731), GM/CA@APS of Argonne National Lab, which is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under contract number DE-AC02-06CH11357, and beamline BL17U1 (Shanghai Synchrotron Radiation Facility [SSRF]). We thank the Cloning, Cell Expression, and Protein Purification Core Facilities of iHuman Institute for their support. We thank A. Walker for assistance with the manuscript; we also thank F. Xu, V. Cherezov, V. Katritch, A. Ishchenko, H. Tao, J. Cheng, D. Liu, W. Zhong, and W. Liu for helpful discussions. The NIDA Drug supply program provided standards used in this study. A.M. is a founder of MAKScientific, LLC, a research company engaged in discovering medications for metabolic disorders with an interest in CB₁. R.C.S. is a board member and shareholder with Birdrock Bio, an antibody therapeutic company with an interest in CB₁.

Received: July 15, 2016

Revised: September 6, 2016

Accepted: October 3, 2016

Published: October 20, 2016

SUPPORTING CITATIONS

The following references appear in the Supplemental Information: Ben-Shabat et al., 1998; Felder et al., 1995; Fong et al., 2007; Griffith et al., 2009; Kuster et al., 1993; Showalter et al., 1996; Wiley et al., 1998.

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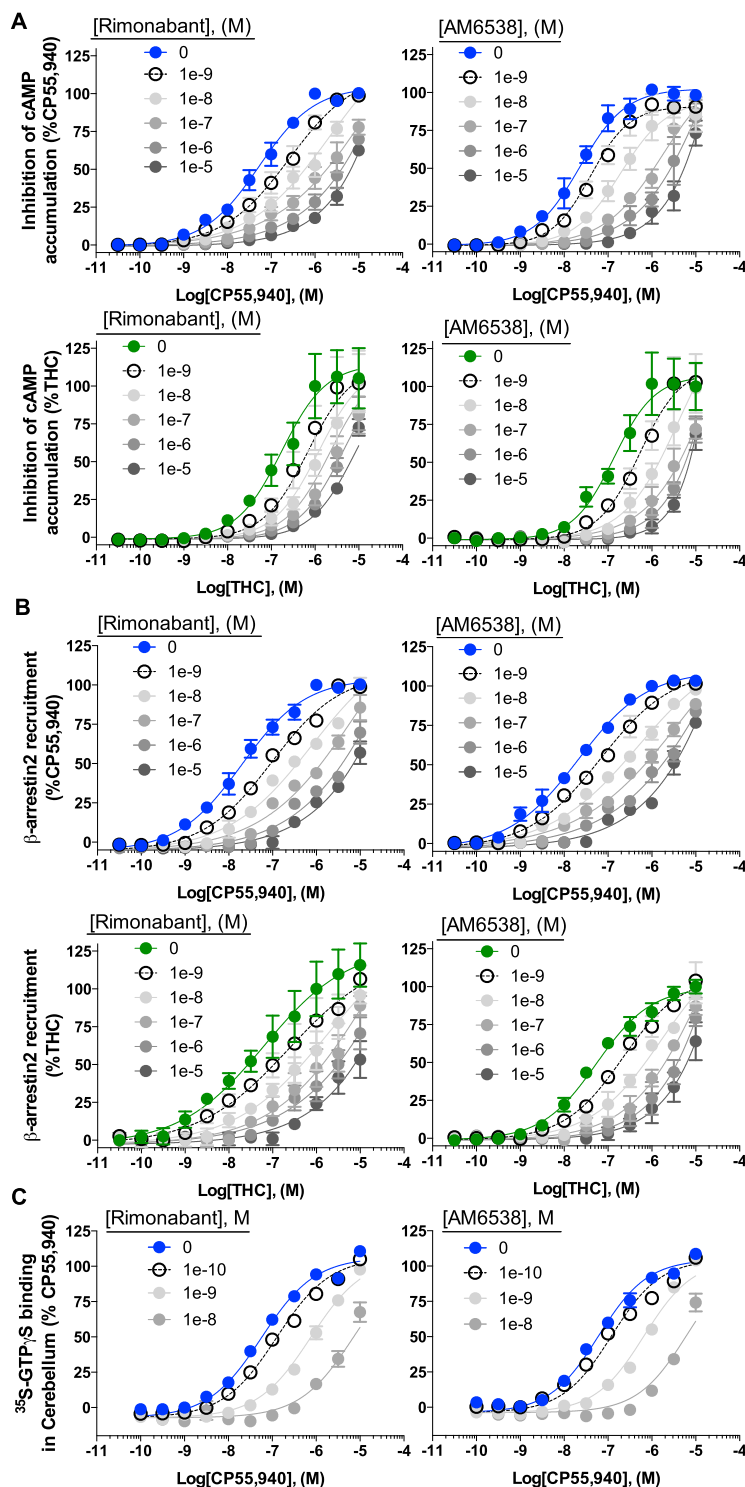


Figure S1. Rimonabant and AM6538 Are Competitive Antagonists of CP55,940 and THC, Related to Figure 2

(A and B) Rimonabant (left) and AM6538 (right) competitively antagonize CP55,940 (top) and THC (bottom)-induced (A) Inhibition of forskolin-stimulated cAMP accumulation in hCB₁-CHO cells and (B) β -arrestin 2-recruitment in hCB₁-CHO DiscoverX PathHunter cells.

(C) Rimonabant (left) and AM6538 (right) competitively antagonize CP55,940-stimulated [^{35}S]-GTP γ S binding in mouse cerebellum membrane preparations. Data are presented as the mean $\pm$ S. E. M. of 3 or more assays run in duplicate or triplicate. Competitive nonlinear analysis was performed in GraphPad Prism 6.0 and parameters are included in Table S1. Characterization of agonist effects in the cells and cerebellum are provided in the supplemental methods.

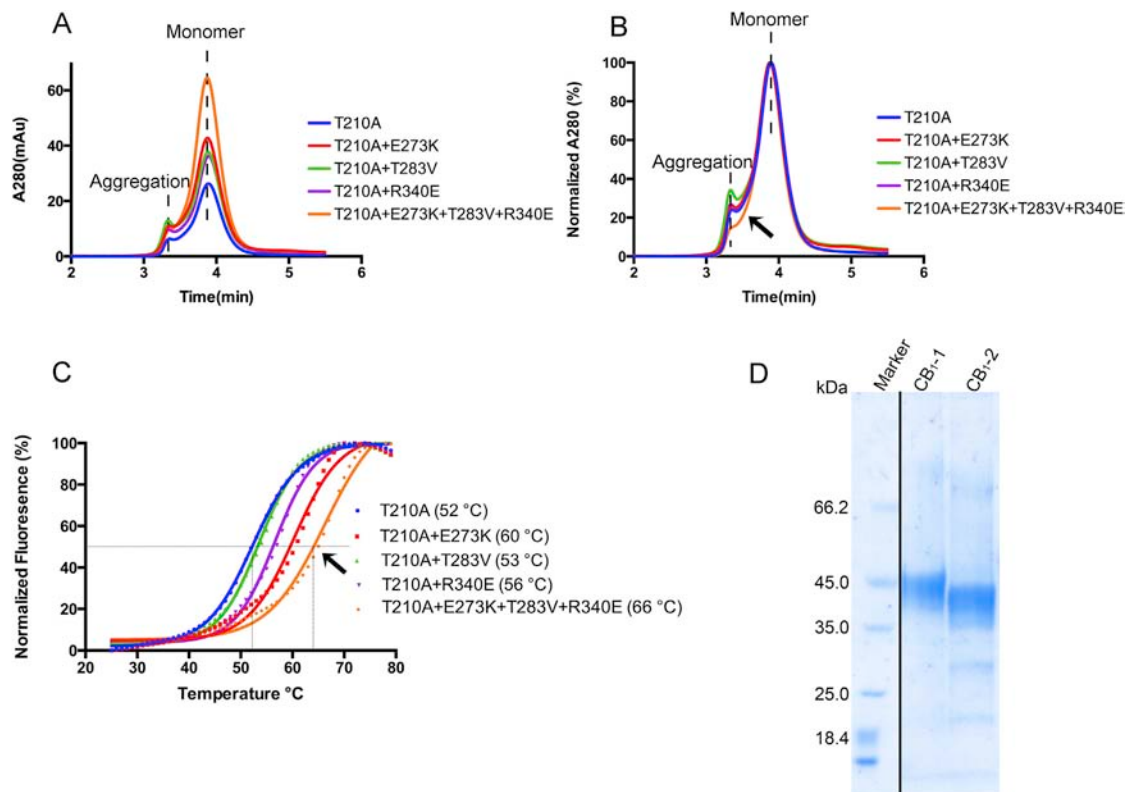


Figure S2. Stability Assay of CB₁ Mutants, Related to Figure 1

(A and B) Analytical size exclusion chromatography (aSEC) of CB₁ mutants testing the effects of mutations and mutation combination on protein homogeneity. (A) The raw data show that the CB₁ mutant containing all four mutations (orange trace) has the highest protein yield. (B) The normalized data show that the combination (orange trace) improves protein homogeneity.

(C) CPM ramping assay of CB₁ mutants testing the effects of mutations and mutation combinations on protein thermostability. The T_m value of the CB₁ mutant containing all four mutations (orange trace) is higher than other mutants, indicating that this mutation combination improves protein thermostability.

(D) Coomassie-stained polyacrylamide gel electrophoresis (PAGE) of the purified crystallization-grade CB₁. CB₁-1: Coomassie-stained PAGE of sample purified by cobalt immobilized-metal affinity chromatography (TALON-IMAC); CB₁-2: Coomassie-stained PAGE of sample after TEV protease digestion. The black line indicates where three intervening lanes from the original gel were removed during figure preparation for clarity of presentation. Three additional lanes following CB₁ were omitted as well.

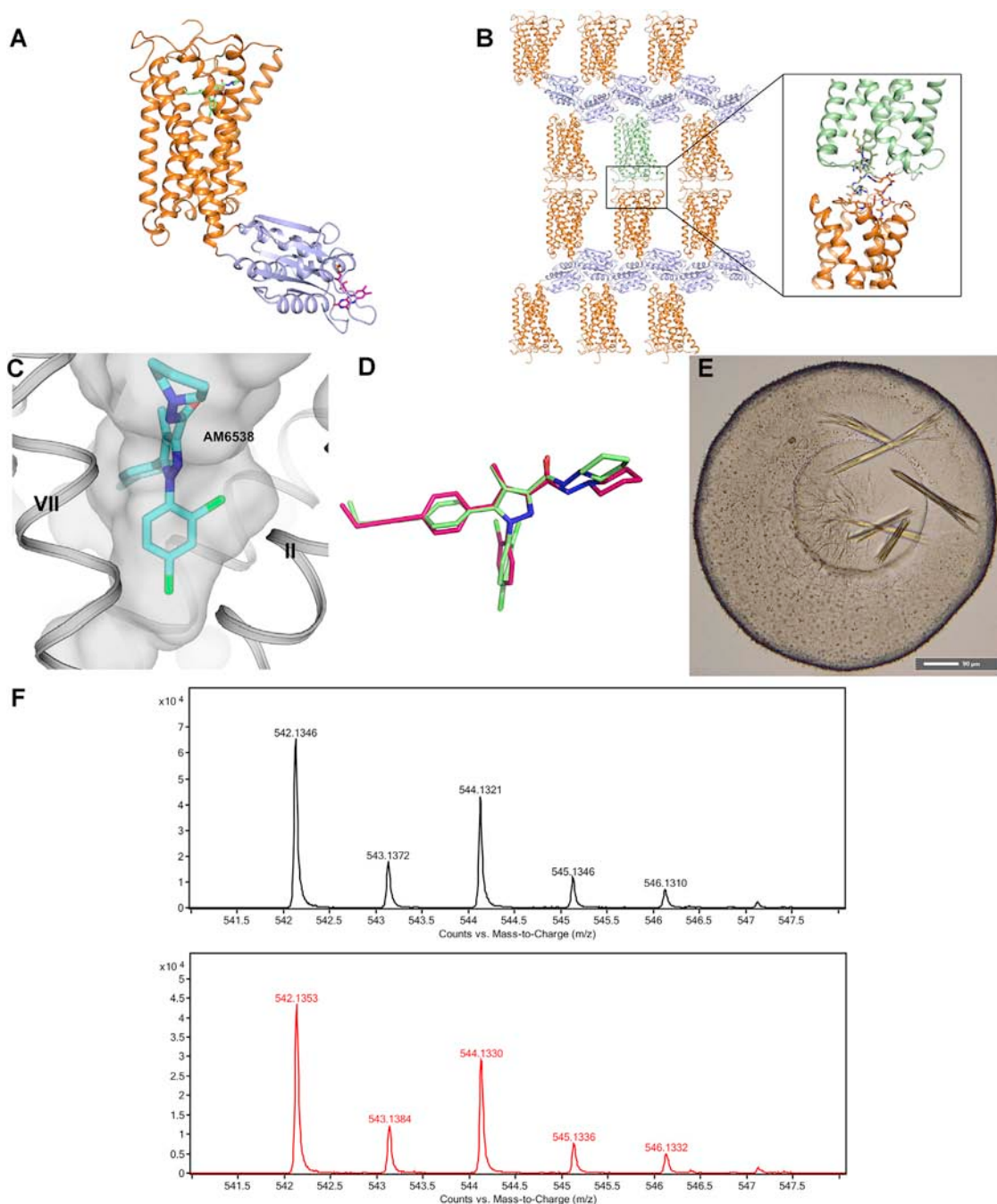


Figure S3. Fusion Protein Engineering and Crystal Packing of CB₁-AM6538 Complex, Related to Figures 1 and 3

(A) Overall structure of CB₁-AM6538 complex. CB₁ and Flavodoxin are colored in orange and slate, respectively. AM6538 is shown in green stick representation and FMN (the substrate of Flavodoxin) is shown in magenta stick representation.

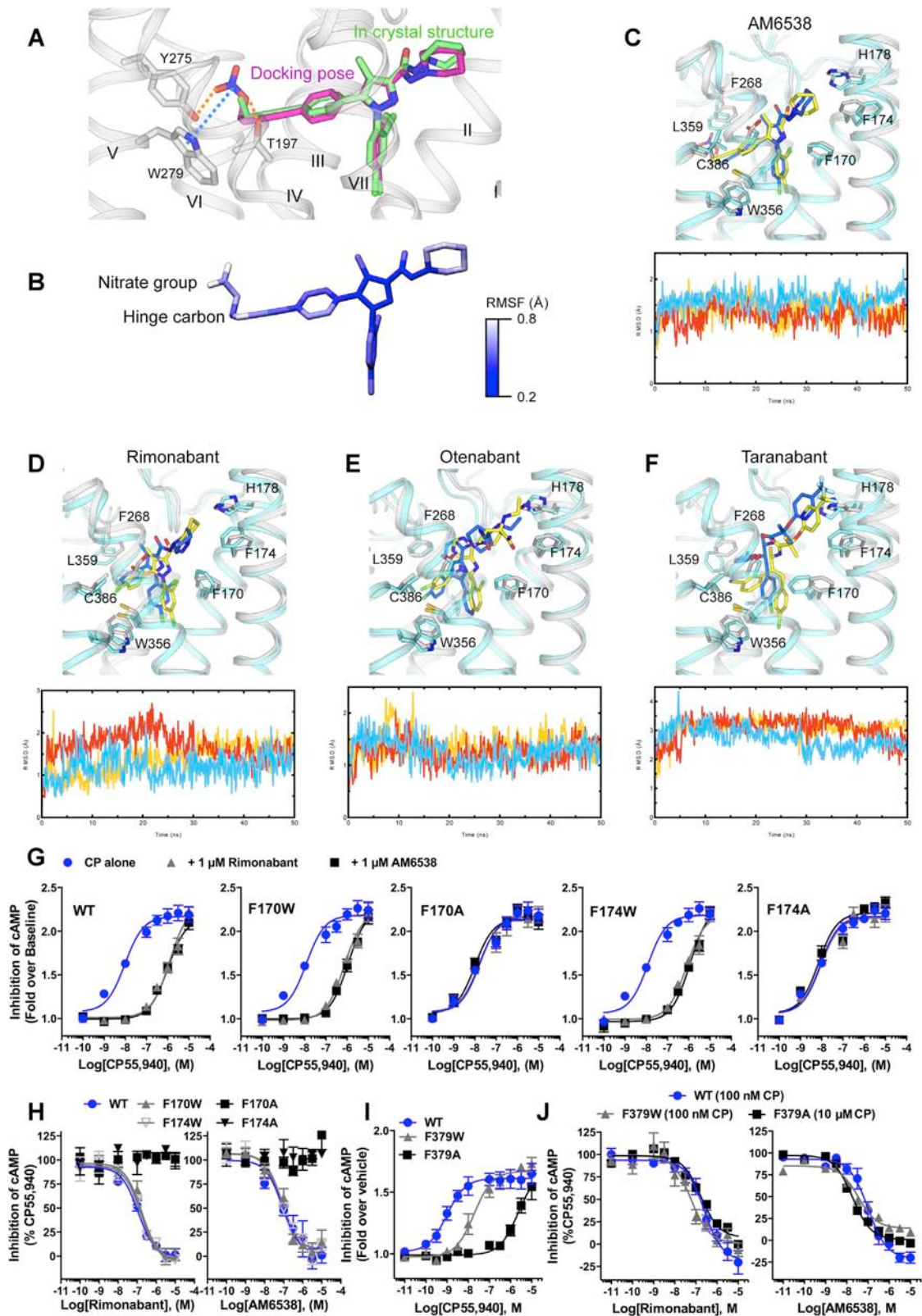
(B) Crystal packing of the CB₁-AM6538 complex, N terminus involved in the crystal packing.

(C) The 2,4-dichlorophenyl ring of AM6538 fits well into the shape of the side-pocket. The receptor is shown in gray cartoon and gray surface, AM6538 is shown in cyan carbons.

(D) Superposition of AM6538 in crystal structure (green) and local minimum calculated by quantum mechanics (magenta).

(E) Crystal images for CB₁ in complex with AM6538 (scale bar is 90 μm).

(F) The high-resolution mass spectrometry spectra of the pure standard AM6538 (top) and the ligand dissociated from CB₁ complex (bottom). The theoretical m/z for the monoisotopic peak of AM6538 is 542.1356.



(legend on next page)

Figure S4. MD Simulations of the Predicted Antagonists and Mutagenesis Validation of the Docking Predictions, Related to Figures 5 and 6

(A) Docking pose of AM6538 (magenta sticks) and predicted hydrogen bond (orange dashed lines) and π - π interaction (blue dashed lines) in comparison with AM6538 in crystal structure (green sticks).

(B) RMSF of AM6538 in MD simulation.

(C–F) AM6538 (C), rimonabant (D), otenabant (E) and taranabant (F) Central structures in MD (protein is shown in light cyan cartoon; ligands are shown in blue sticks) versus docking poses (protein is shown in white cartoon and ligands are shown in yellow sticks), and ligand RMSD (three independent runs).

(G) Mutations of hCB₁ at Phe170Ala or Phe174Ala disrupts the ability of AM6538 and rimonabant to displace CP55,940 while preserving its functional activity. The corresponding tryptophan mutations have no effect on agonist or antagonist actions.

(H) A loss of antagonist affinity is further demonstrated in competition response curves agonist 100 nM CP55,940.

(I) Mutation of Phe379Trp and Phe379Ala progressively worsen the functional affinity of CP55,940.

(J) When tested against concentrations of CP55,940 that maximally stimulate the receptor, AM6538 and rimonabant maintain their potency as antagonists (100 nM CP55,940 for WT and Phe379Trp and 10 μ M CP55,940 for Phe379Ala; normalization is made per mutant). Data are presented as mean $\pm$ S. E. M. of 3 or more assays performed in duplicate or triplicate.

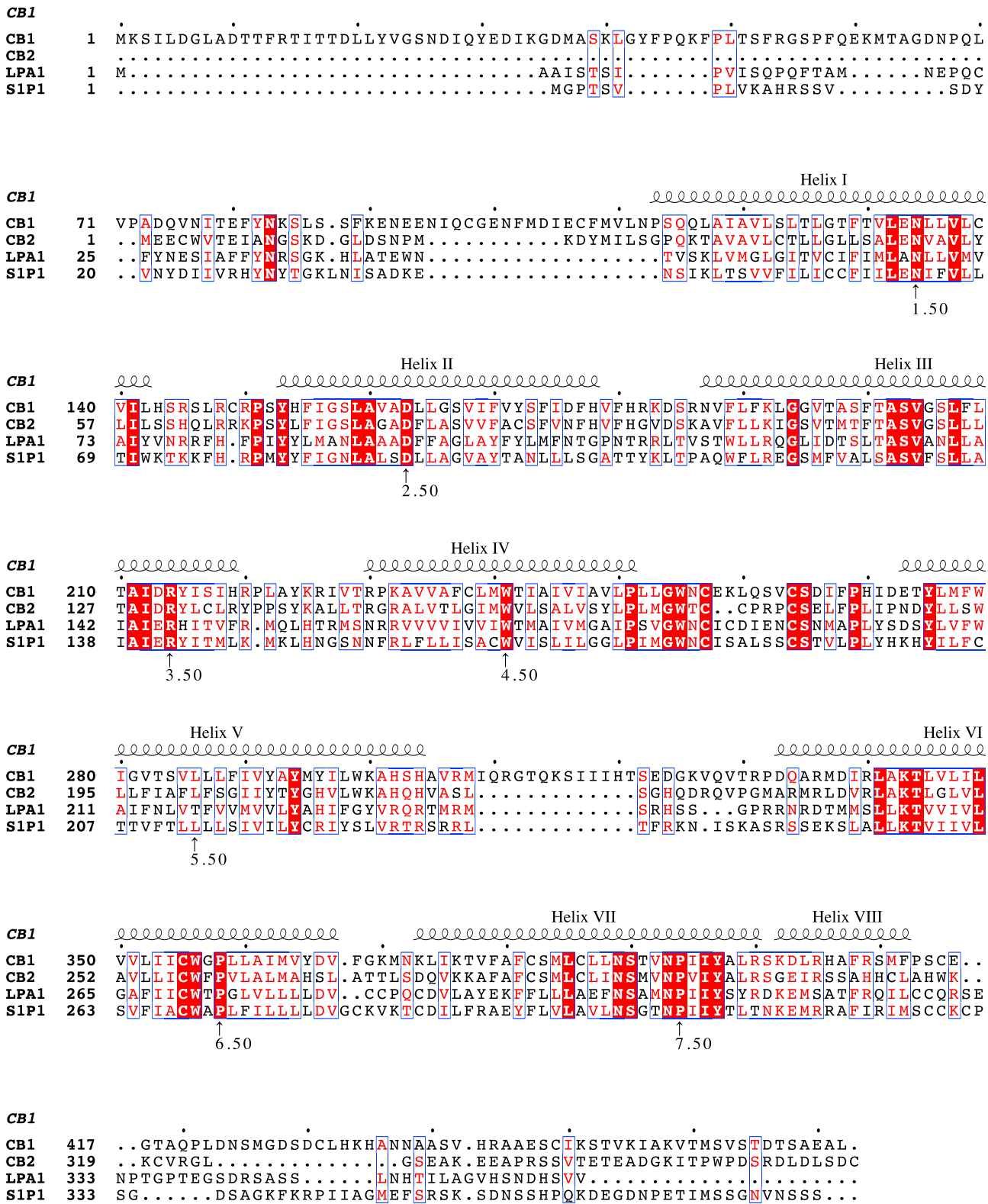


Figure S5. Multiple Sequence Alignment of Human CB₁ with CB₂, LPA₁, and S1P₁ Receptors, Related to Figure 4

Positions that are identical between the receptors are highlighted with a red background. Alignment was done by MAFFT v7.299b (Kato and Standley, 2013) and the graphics were prepared using ESPrnt 3.0 server (Robert and Gouet, 2014).