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Ligand Binding Sensitivity of the Extracellular Loop Two of the Cannabinoid Receptor 1

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Abstract

The cannabinoid receptor one (CB1) is a class A G-protein-coupled receptor thought to bind ligands primarily within its helical bundle. Evidence suggests, however, that the extracellular domain may also play a role. We have previously shown that the C-terminus of the extracellular loop 2 of CB1 is important in binding some compounds; receptors with mutations in this region (F268W, P269A, H270A, and I271A) bound some agonists with severely reduced affinity relative to the wild-type receptor. In the present work, we examine the impact of these mutations on binding a chemically diverse set of ligands. The receptors, F268W and I271A, exhibited a greater sensitivity to binding the inverse agonists/antagonists SLV319, AVE1625, NESS0327 relative to P269A and H270A, suggesting that the Pro and His are not involved in binding those compounds. In contrast, binding of the agonists, BAY593074 and WIN55212-2, was diminished in all four receptors, suggesting the conformational unit contributed by all four residues is important. A more marked loss in binding was observed for agonists of the nonclassical (CP55940) and classical (HU-210, JWH061, JWH179) cannabinoid classes and for a silent antagonist derivative (O-2050), pointing to the critical nature of this region for binding both the bicyclic/tricyclic core and the alkyl chain of these derivatives. However, moving the location of the alkyl chain on a series of pyrazole analogues shows it can be better accommodated in certain locations (O-1255) than others (O-1302, O-1690) and underscores the involvement of residues F268 and I271.

Keywords

G-protein-coupled receptor; cannabinoid receptor 1; extracellular loop 2; ligand binding

INTRODUCTION

G-protein-coupled receptors (GPCRs) such as the cannabinoid receptor 1 are of considerable importance, as they constitute a significant portion of the targets for FDA approved drugs [Overington et al., 2006]. Ever-increasing information implicates cannabinoid receptor modulation with appetite regulation, analgesia, intraocular pressure, and short-term memory [see Howlett et al., 2002]. Localized on presynaptic nerve terminals, cannabinoid receptors are thought to play a direct role in the inhibition of neurotransmitter release [Mackie and Hille, 1992]. Attempts to harness the therapeutic potential of the cannabinoid system have fallen short due to undesirable side effects [see Janero and Makriyannis, 2009; Pertwee, 2009]. However, recent advances suggest the possibility of selectively activating certain signaling pathways, a concept that may ultimately be used to assist in the development of

novel drugs that achieve the desirable medicinal benefits while minimizing the undesirable side effects.

Like all GPCRs, the cannabinoid receptors have seven hydrophobic transmembrane domains linked by three extracellular and three intracellular loops. Two cannabinoid receptor subtypes have been identified to date: the cannabinoid receptor 1 (CB1), which is found predominantly in the central nervous system [Devane et al., 1988; Matsuda et al., 1990], and the cannabinoid receptor 2 (CB2), which is localized mainly to immune tissue [Munro et al., 1993]. The two receptors have 44% sequence identity, and both couple predominantly to G_i proteins, but CB1 has been shown to signal through G_s and G_q subunits under certain conditions [Abadji et al., 1999; Bonhaus et al., 1998; Glass and Felder, 1997; Lauckner et al., 2005].

Capable of binding Δ^9 -THC, the main psychoactive constituent of the plant *Cannabis sativa*, also known as marijuana, CB1 is also activated by endogenous lipid-derived eicosanoids such as anandamide and 2-arachidonoylglycerol, aminoalkylindole-based compounds such as WIN55212-2, and synthetic nonclassical cannabinoids such as CP55940, as well as the tricyclic classical cannabinoids, including Δ^9 -THC, HU-210, and JWH061. Antagonists and inverse agonists for the cannabinoid receptors based on a pyrazole scaffold have also been developed. These include SR141716A (rimonabant), AM251, and AM281. Modifications of the alkyl chain of cannabinoid receptor agonists can affect both compound efficacy and their specificity for the CB receptor subtypes [Huffman et al., 1999, 2003].

Relatively little is known about the orientation of CB1 ligands at the active site of the receptor. Aromatic residues, such as F201, W280, and W357 among the transmembrane domains of mouse CB1, are critical for SR141716A and WIN55212-2 binding; however, evidence suggests that these do not directly interact with nonclassical agonists, such as CP55940 [McAllister et al., 2003]. We [Chin et al., 1998] and others [Song and Bonner, 1996] have previously demonstrated the importance of K192 in CP55940 but not WIN55212-2 binding. A hydrogen bonding interaction between the K192 and the phenolic hydroxyl group of classical and nonclassical agonists [Chin et al., 1998; Song and Bonner, 1996] or the carboxamide oxygen of SR141716A [Hurst et al., 2002, 2006] appears critical for ligand binding. Other residues of the receptor implicated in ligand binding include S383 which is thought to induce a bend in TM7 critical for nonclassical agonist binding [Kapur et al., 2007] as well as C386, also located in TM7, whose side chain precludes SR141716A binding when labeled with MTS reagents [Fay et al., 2005].

Recently, we have shown that the extracellular domains of CB1 can play an integral role in ligand binding [Ahn et al., 2009]. It had long been thought that class A GPCRs, including CB1, that bind small hydrophobic molecules, do so primarily in the transmembrane domain. However, we have shown that the C-terminal region of the extracellular loop 2 (EC2) differentiates agonist from inverse agonist binding. Point mutations at positions F268, P269, H270, and I271 of CB1 led to receptors that bound the agonists tested with substantially attenuated affinities but had no effect on inverse agonist binding affinity. Consistent with these findings, high concentrations of the nonclassical agonist CP55940 resulted in low EC₅₀ and E_{max} values for agonist-induced G-protein coupling activity. Outstanding questions include whether the decrease in agonist binding was caused by the loss of a functional group that made direct contact with the ligand or if each of the mutations led to a conformational change that indirectly hindered agonist binding. Moreover, it is unclear what aspects of the chemical structure of the agonists tested precluded binding. This is an important issue because molecular modeling of the receptor suggests that the F268 in this region of the receptor could project into the core of the receptor bundle and ligand binding cavity [Ahn et al., 2009].

Molecular modeling studies of CB1 ligands by Thomas et al. [1998] and Salo et al. [2004] suggest the aryl rings of the SR141716A molecule may overlap with the alkyl side chain of the classical and nonclassical cannabinoid receptor agonists in the ligand binding pocket. SAR studies to determine the role of the various substituents of SR141716A determined that the aryl ring at the C-5 substituent or the N-1 substituent of SR141716A is important for antagonism while the C-3 substituent modulates receptor activation [Wiley et al., 2001]. Interestingly, the presence of an alkyl chain in place of the piperidine ring at the C-3 substituent of SR141716A imparts partial agonism at the mouse CB1.

Understanding the interactions of CB1 ligands and specific amino acid residues of the receptor is critical for the development of high-affinity selective therapeutic agents. In the present study, we examine the binding profile of a structurally diverse set of ligands to a series of CB1 receptors with mutations in the C-terminus of EC2. Consistent with our previous findings [Ahn et al., 2009], the C-terminus of EC2 plays an important role in differentiating agonist binding from inverse agonist/antagonist binding. However, compounds with the bicyclic/tricyclic core, such as is found in the classical and nonclassical cannabinoids, exhibit more severely reduced binding than some other agonists. Moreover, we find that mutation of the two residues, F268 and I271, among the four assessed in this region display more sensitivity to binding ligands including some inverse agonists/antagonists.

MATERIALS AND METHODS

Plasmid Preparation

Site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA) was performed on a template of the human CB1 cDNA cloned into pcDNA3.1 according to the manufacturer's protocol. DNA sequencing verified the mutations.

CB1 Membrane Preparation

CB1 receptor-containing membrane preparation was obtained as previously described [Abadji et al., 1999]. In brief, HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 3.5 mg/ml glucose at 37°C in 5% CO₂. To transiently transfect HEK293T cells, 1 day before transfection, they were seeded at $\sim 0.9 \times 10^6$ cells/100-mm dish. The calcium phosphate precipitation method [Chen and Okayama, 1987] was used to transfect the cells. Approximately 24 h after transfection, the cells were harvested, then washed twice with phosphate-buffered saline (PBS). The cells were resuspended in a mammalian protease inhibitor cocktail (4-(2-aminoethyl)benzene-sulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin) (Sigma-Aldrich, St. Louis, MO) containing PBS solution and then lysed by nitrogen cavitation at 750 psi for 5 min, using a Parr cell disruption bomb. A 10-min spin at 4°C at 500 g was used to pellet cell debris and nuclei. The resulting supernatant was subsequently spun at 100,000 g for 45 min at 4°C. The membrane-containing pellet was then resuspended in TME buffer (25 mM Tris-HCl, 5 mM MgCl₂, and 1 mM EDTA, pH 7.4) containing 7% sucrose (w/v). The Bradford assay [Bradford, 1976] was used to determine protein concentration. The membrane preparation was stored at 0.6 mg/ml at -70°C.

Ligand Binding

Ligand binding assays were performed as previously described [Abadji et al., 1999]. Briefly, for saturation binding assays, ~39 mg of membrane was incubated with increasing concentrations of [³H]SR141716A (43 Ci/mmol; GE Healthcare, Piscataway, NJ) for 90 min at 30°C in TME+0.1% fatty acid-free bovine serum albumin (BSA) in a final volume of 200 µl. Nonspecific binding was typically determined with a 5 µM concentration of unlabeled

ligand. For competition binding assays, ~25–40 µg of membranes was incubated with 4 nM [³H]SR141716A and increasing concentrations of displacing ligand for 90 min at 30°C in TME+0.1% fatty acid-free BSA in a final volume of 200 µl. Nonspecific binding was typically determined with 5 µM of unlabeled ligand. The addition of 250 µl of TME+5% BSA was used to terminate the reaction before separating bound from unbound ligand by filtering with a 24-manifold cell harvester (Brandel, Gaithersburg, MD). Filters were washed with cold TME buffer. Scintillation counting was then used to determine the bound radioactivity.

Data Analysis

Ligand binding assays were performed in duplicate. The data are presented as the median with the corresponding 95% confidence limits from at least three independent experiments. IC₅₀ values were calculated by nonlinear regression (fitted to a one-site competition model) using GraphPad Prism (GraphPad Software, San Diego, CA). The Cheng–Prusoff equation [Cheng and Prusoff, 1973] was used to calculate the K_i values using K_d values for the tracer obtained from saturation binding analyses. Analysis of variance (ANOVA) followed by Dunn's post hoc test was used to compare the wild-type K_i values with those of the mutant receptors. *P*-values of <0.05 were considered statistically significant.

RESULTS

The sequence of CB1 EC2 is shown in Figure 1. Using alanine scanning mutagenesis, we previously found mutations in the C-terminus of EC2 of CB1 that were sensitive to agonist but not to inverse agonist binding [Ahn et al., 2009]; P269A, H270A, and I271A receptors exhibited diminished binding for the agonists CP55940, methanandamide, and HU-210 but bound inverse agonists, SR141716A, AM251, and LY320135 with wild-type receptor affinity. The neighboring mutation, yielding the F268A receptor, displayed both impaired localization and ligand binding, while characterization of other amino acid substitutions at 268 identified a F268W receptor that was trafficked to the cell surface yet displayed the differential binding preference for inverse agonists comparable to the P269A, H270A, and I271A receptors. Therefore, we used this set of CB1 receptor mutants, F268W, P269A, H270A, and I271A, to reveal the molecular features for ligand binding within this region.

While our previous work suggested a clear demarcation in the binding affinity of inverse agonists and agonists to the EC2 mutant receptors, relative to wild-type, this may reflect commonalities in the structures tested as opposed to preferences for ligands with particular pharmacological effects. Therefore, we evaluated the binding affinity of a spectrum of agonists, antagonists, and inverse agonists with a range of structures to the CB1 mutant receptors. Since we had shown that the diarylpyrazole ligands, SR141716A and AM251, retained high-affinity binding to the C-terminal EC2 mutants, we tested the diarylpyrazoline SLV319, a CB1 inverse agonist that is more hydrophilic than SR141716A, the benzhydrylazetidine, AVE1625, and NESS0327 which has a 3-carbon linker between the monochlorinated aryl ring of SR141716A and the pyrazole core, and thus an antagonist with limited flexibility relative to the diarylpyrazole ligands. The K_i values from competition binding studies using [³H]SR141716A as tracer are shown in Table 1. For each of these compounds, the P269A and H270A receptors bound the ligand with an affinity comparable to the wild-type receptor (SLV319, K_i = 1.8 nM; AVE1625, K_i = 0.64 nM; NESS0327, K_i 5 4.6 nM) or at most 2-fold more weakly. The F268W and I271A receptors displayed greater differences; the F268W receptor showed a 3- and 5-fold loss in affinity for AVE1625 and NESS0327, respectively, and the I271A receptor a 5- and 3-fold loss for SLV319 and AVE1625, respectively.

To probe whether the cannabinoid agonists CP55940 and HU-210 previously found to bind receptors F268W, P269A, H270A, and I271A, with attenuated affinity [Ahn et al., 2009] did so as a result of a shift in the receptor toward the inactive state, we determined the affinity of the “silent” antagonist O-2050 for these EC2 mutant receptors (Table 1). The structure of O-2050 is based on a tricyclic ring structure more similar to a classical CB receptor agonist than a diapyrazole inverse agonist such as SR141716A. However, it is considered devoid of any agonism at the CB1 receptor, likely attributable to the introduction of a triple bond in its alkyl side chain reducing the chain flexibility potentially needed for CB1 activation by classical and nonclassical agonists [Griffin et al., 1999; Reggio, 2003]. O-2050, which should bind the inactive and active forms of the CB1 receptor with comparable affinity, was found to bind to F268W, P269A, H270A, and I271A receptors with substantially reduced affinity (44-, 20-, 12-, and 49-fold, respectively) relative to the wild-type receptor ($K_i = 3.4$ nM). This suggests that binding the tricyclic ring structure and/or the bulky alkyl chain require the conformation and/or contacts of the wild-type EC2 C-terminus and that the mutants in this region are not simply shifted to the inactive state.

To examine the affinity of the EC2 mutant receptors for other structurally diverse agonists, BAY593074 (wild-type receptor $K_i = 249$ nM), and WIN55212-2, an aminoalkylindole (wild-type receptor $K_i = 510$ nM), were tested. The binding affinity was diminished relative to that of the wild-type receptor (up to 12-fold for BAY593074 and up to 6-fold for WIN55212-2) but not as substantially as with O-2050. Thus, binding the classical and nonclassical agonists (and their analogues), relative to these agonists, seems more sensitive to alterations in the composition of CB1 EC2. Interestingly, both WIN55212-2 and SR141716A are thought to form aromatic stacking interactions critical for high-affinity ligand binding [McAllister et al., 2003] and mutations that inhibit the affinity of one ligand typically also decrease the other's affinity for the receptor [McAllister et al., 2003]. Here, the C-terminal EC2 mutations do not affect SR141716A binding affinity and although they decrease the affinity of WIN55212-2 to the receptor, the attenuation is not as severe as to the classical and nonclassical agonists.

To gain insight on whether the length of the alkyl side chain of CB receptor agonists was a factor in the attenuation of binding affinity, we tested the effect of shortening the alkyl side chain of the classical cannabinoid receptor agonist structure. The 5-carbon-long dimethylpentyl Δ^8 -THC (JWH061) chain is shorter than the 7-carbon-long dimethylheptyl side chain common to the nonclassical compound CP55940 and on the classical compound HU-210. This is reduced by 3 carbons to yield dimethyl Δ^8 -THC (JWH179) containing a 2-carbon-long chain [Huffman et al., 2003]. Shortening the alkyl side chain did not attenuate the loss of binding affinity for any of the EC2 mutants and the fold-loss was found to be comparable to the reduction in binding observed for CP55940 and HU-210 to the F268W, P269A, H270A, and I271A receptors relative to the wild-type CB1 receptor (Table 2). These data suggest the length of the alkyl chain as configured on the Δ^8 -THC series was not the sole determinant of the diminished agonist binding affinity and underscores the need for the C-terminus EC2 residues in accommodating the bicyclic/tricyclic core.

To further probe the impact of the alkyl side chain on binding affinity to the EC2 receptor mutants, a series of SR141716A derivatives with an alkyl chain linked to different constituents was examined (Table 3). Thus, the bulk of these compounds resembles SR141716A that bound the mutant receptors with wild-type like affinity ($K_i = 5.2$ nM). All three compounds (O-1302, O-1690, and O-1255) were bound by the mutant receptors (P269A and H270A) like the wild-type (O-1302, $K_i = 1.7$ nM; O-1690, $K_i = 1.3$ nM; O-1255, $K_i = 83.4$ nM). In contrast, the F268W and I271A receptors displayed significantly diminished affinity for O-1302 and O-1690, though by only 4- and 6-fold, respectively, for O-1255. This suggests that the position of the alkyl chain relative to the cyclic core of the

molecule impacts binding affinity and thus contributes to the loss of binding observed for CP55940 and HU-210 especially by the F268W and I271A receptors.

DISCUSSION

Taken together, these data suggest the C-terminus of CB1 EC2 participates in ligand binding and is particularly important for accommodating both the bicyclic/tricyclic core and alkyl side chain of the classical and nonclassical cannabinoids. Data from binding isotherms to the O-1255 series further suggest that the composition and location of both components are problematic as the alkyl chain in a different orientation on O-1255 was bound by the receptors without the substantial loss in affinity observed for CP55940 and HU-210. Moreover, the JWH061 and JWH179 series, with shorter alkyl side chains, displayed binding loss comparable to that for CP55940 and HU-210.

We find that mutation of F268 and I271 yield receptors that are especially sensitive to ligand binding, while mutation of the two residues internal to these, P269 and H270, yielded receptors that retained wild-type receptor like affinity for many compounds tested. This is consistent with the notion that the F268 and I271 residues project more deeply into the ligand binding pocket and/or form a hydrophobic lid on the ligand [Ahn et al., 2009].

In addition to the classical and nonclassical cannabinoids, we have previously shown that mutations in the C-terminus of EC2 display substantially reduced binding affinity for methanandamide, the C-2 methylated anandamide analogue. Interestingly, McAllister et al. [2003] showed the alanine substitution at F190 (corresponding to F189 in human CB1) in mouse CB1 extracellular loop 1 (EC1) resulted in a 6-fold loss in anandamide binding. Together with computational modeling, the results suggest that F189 is a key residue for anandamide binding as a CH- π interacts with the C5-C6 double bond of anandamide. Based on our previous molecular modeling, we have proposed an intramolecular interaction between F268 and two residues from EC1, F189, and H178. It is possible that the mutations in the C-terminus of EC2 influence the interaction with EC1, resulting in impaired methanandamide binding. However, we cannot rule out the possibility that the C-terminus of EC2 plays a role in agonist-specific receptor activation. In this study, we also demonstrate that the C-terminal EC2 mutant receptors exhibited reduction in binding affinity for the aminoalkylindole agonist, WIN55212-2 though not as markedly as for other classes of agonists. It is believed that the binding pocket for WIN55212-2 overlaps that of SR141716A. High affinity binding for both ligands appears to require a network of aromatic residues for receptor stabilization and/or aromatic stacking interaction with ligands.

The Cys-X-X-X-Ar (where Ar is aromatic) motif of EC2 that is conserved among GPCRs that bind biogenic amines and peptides is also found in CB1 with F268 serving as the aromatic residue. In other receptors, the aromatic residue is thought to contribute indirectly [Cherezov et al., 2007] or directly [Lim et al., 2008; Palczewski et al., 2000] to ligand binding. For example, in the β_2 -adrenergic receptor, the analogous residue phenylalanine 194 participates in hydrophobic interactions thought critical for stabilizing phenylalanine 193 for its interaction with ligand [Cherezov et al., 2007; Rosenbaum et al., 2007]. A more direct role for this residue is observed in bovine rhodopsin in which the corresponding residue, tyrosine 191, is part of the β_4 strand that contributes to the chromophore binding pocket [Palczewski et al., 2000]. In addition, F209, the corresponding residue in the V1a vasopressin receptor is thought to project into the binding crevice and mutation of this residue impairs ligand binding and intracellular signaling [Conner et al., 2007]. A recent reporter cysteine accessibility mapping (RCAM) study suggests the EC2 residues, especially among the C-X-X-X-Ar motif, are responsible for a conformational change upon the agonist binding [Unal et al., 2010].

Although the CB1 receptor belongs to the class A GPCR family, the CB1 EC2 loop is somewhat unusual relative to other receptors in this class. It is short (18 residues), whereas the EC2 loops of the adrenergic receptors (about 25 residues) and rhodopsin (about 26 residues) are significantly longer. CB1 has an EC2 intraloop disulfide bond and no disulfide linkage between EC2 and TM3. Moreover, receptor modeling [Ahn et al., 2009] suggests that the CB1 EC2 adopts a small helical segment, while other receptors such as rhodopsin exhibit an antiparallel β -hairpin structure. Whereas the binding pocket for most ligands is thought to reside within the TM domain, it appears that CB1 EC2, especially F268 and I271, is highly organized to assist in forming the ligand binding site. Any therapeutic strategy targeting CB1 will require consideration of the conformation of this region.

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Abbreviations used

AVE1625	<i>N</i> -{1-[bis(4-chloro-phenyl)-methyl]azetidin-3-yl}- <i>N</i> -(3,5-difluorophenyl)methanesulfonamide
BAY593074	3-[2-cyano-3-(trifluoromethyl)phenoxy]phenyl-4,4,4-trifluoro-1-butanefulfonate
CB1	cannabinoid receptor 1
CP55940	(1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol
EC2	extracellular loop 2
GPCR	G-protein-coupled receptor
HEK293T	human embryonic kidney cell
HU-210	(-)-11-hydroxydimethylheptyl- Δ 8-tetrahydrocannabinol
JWH061	3-(1',1'-dimethylpentyl)- Δ 8-tetrahydrocannabinol
JWH179	3-(1',1'-dimethylethyl)- Δ 8-tetrahydrocannabinol
NESS0327	<i>N</i> -piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-xtetrahydrobenzo [6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide
O - 1255	[I] N [I] - pentylphenyl - 5 - (4-chloro-phenyl)-3- ([I]N[I]-piperidin-1-yl)carboxamide-4-methyl-pyrazole
O-1302	[I]N[I]-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-4-methyl-5-(4-pentylphenyl)-1H-pyrazole-3-carboxamide
O-1690	<i>N</i> -(<i>N</i> -piperdy)-3-(2,4-dichlorophenyl)-4-(4'-(1'-methylpentyl)phenyl)-5-methylpyrazole O-2050, (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran
PBS	phosphate-buffered saline
SLV319	4S-(α)-3-(4-chloroenyl) - <i>N</i> - methyl - <i>N</i> ' - [(4 - chlorophenyl) sulfonyl] -4 - phenyl - 4,5-dihydro-1H-pyrazole-1-carboxamidine

SR141716A	<i>N</i> -(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
THC	tetrahydrocannabinol
TME	Tris/Mg ²⁺ /EDTA
WIN 55212-2	(<i>R</i>)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone

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Fig. 1. The EC2 loop of CB1. The dotted line represents the disulfide bridge between residues C257 and C264. Residues comprising the C-X-X-X-Ar motif (where Ar is any aromatic residue) are noted by the hatched marked circles. Mutating the residues in grey results in variants with attenuated ligand binding properties [Ahn et al., 2009] and were examined for SAFIR (structure-affinity relationship) here.

TABLE 1
Binding Affinity of Various Ligands to CB1 Receptors With Mutations in Positions 268–271 in EC2

Receptor	SLV319	AVE1625	NESS0327	O-2050	BAY593074	WIN55212-2
WT	1.8 (1.2–2.6)	0.64 (0.37–1.1)	4.6 (3.2–6.5)	3.4 (2.6–4.6)	249 (140–445)	510 (395–658)
F268W	2.0 (1.5–2.5)	2.2 (1.7–2.7)*	21.0 (18.3–24.2)*	148 (87.4–251)*	1857 (841–4101)*	1829 (1296–2579)*
[WT:F268W] ^a	[1:1]	[1:3]	[1:5]	[1:44]	[1:7]	[1:4]
P269A	2.1 (1.7–2.7)	0.88 (0.70–1.1)	3.0 (2.7–3.4)	68.6 (48.5–97.1)*	341 (275–422)	2030 (1193–3455)*
[WT:P269A] ^a	[1:1]	[1:1]	[1:1]	[1:20]	[1:1]	[1:4]
H270A	4.0 (2.8–5.8)*	1.2 (0.89–1.7)*	6.1 (5.2–7.3)	40.2 (28.7–56.1)*	412 (348–487)	1999 (1609–2484)*
[WT:H270A] ^a	[1:2]	[1:2]	[1:1]	[1:12]	[1:2]	[1:4]
I271A	8.6 (7.4–10.1)*	1.8 (1.4–2.3)*	11.2 (9.8–12.9)*	166 (119–229)*	2872 (1543–5348)*	2825 (2516–3173)*
[WT:I271A] ^a	[1:5]	[1:3]	[1:2]	[1:49]	[1:12]	[1:6]

Data are the median and corresponding 95% confidence limits of three independent experiments performed in duplicate. *K_i* values for each ligand were determined using 4 nM [³H]SR141716A.

* Statistically significant differences from wild-type (*P* < 0.05) using analysis of variance followed by Dunn's post hoc test.

^a Values represent the *K_i* ratio for WT to EC2 mutant receptors for the specified compound.

TABLE 2

Effects of Truncating the Alkyl Side Chain of Dimethylpentyl Δ^8 -THC on Ligand Binding Affinity to Cannabinoid Receptors With Mutations in Positions 268–271 in EC2

	K_i (nM)			
Receptor	CP55940 ^a	HU-210 ^a	JWH061	JWH179
WT	24.8 (14.6–42.3)	3.7 (2.1–6.5)	29.9 (18.5–48.3)	7.3 (5.4–9.8)
F268W	8132 (5793–11421) *	1672 (757.9–3689) *	3459 (2619–4568) *	1112 (922–1342) *
[WT:F268W] ^b	[1:328]	[1:452]	[1:116]	[1:152]
P269A	2081 (1256–3446) *	22.6 (10.3–49.5) *	1728 (1414–2111) *	798 (620–1027) *
WT:P269A ^b	[1:84]	[1:6]	[1:58]	[1:109]
H270A	188 (152–232) *	13.8 (9.4–20.1) *	373 (226–618) *	90.0 (79.3–102) *
WT:H270A ^b	[1:8]	[1:4]	[1:12]	[1:12]
I271A	3306 (1422–7686) *	310 (117–822) *	773 (623–958) *	194 (158–240) *
WT:I271A ^b	[1:133]	[1:84]	[1:26]	[1:27]

Data are the median and corresponding 95% confidence limits of three independent experiments performed in duplicate. K_i values for each ligand were determined using 4 nM [³H]SR141716A.

* Statistically significant differences from wild-type ($P < 0.05$) using analysis of variance followed by Dunn's post hoc test.

^a K_i values from Ahn et al. [2009], except for H270A, which was obtained here.

^b Values represent the K_i ratio for WT to EC2 mutant receptors for the specified compound.

TABLE 3

K_i Values for Binding SR141716A Analogues to the CB1 Receptors With Mutations in Positions 268–271 in EC2

Receptor	<div> </div> K_i (nM)			
	SR141716A ^a	O-1302	O-1690	O-1255
WT	5.2 (3.4–8.1)	1.7 (1.2–2.3)	1.3 (0.8–2.0)	83.4 (49.7–139.9)
F268W	7.3 (6.4–8.3)	26.2 (20.2–34.1) *	23.3 (16.9–32.0) *	310.2 (242.8–396.2) *
[WT:F268W] ^b	[1:1]	[1:15]	[1:18]	[1:4]
P269A	5.3 (3.8–7.3)	1.1 (0.82–1.5)	0.96 (0.71–1.3)	74.7 (28.5–195.8)
[WT:P269A] ^b	[1:1]	[1:1]	[1:1]	[1:1]
H270A	3.5 (2.5–5.0)	2.6 (1.6–4.2)	2.0 (1.4–2.8)	113 (87.0–147)
[WT:H270A] ^b	[1:1]	[1:1]	[1:1]	[1:1]
I271A	2.4 (1.9–2.9)	38.4 (33.2–44.6) *	77.3 (72.0–82.9) *	460 (361–587) *
[WT:I271A] ^b	[2:1]	[1:23]	[1:59]	[1:6]

Data are the median and corresponding 95% confidence limits of three independent experiments performed in duplicate. K_i values for each ligand were determined using 4 nM [³H]SR141716A.

* Statistically significant differences from wild-type ($P < 0.05$) using analysis of variance followed by Dunn's post hoc test.

^a K_i values from Ahn et al. [2009], except for H270A, which was obtained here.

^b Values represent the K_i ratio for WT to EC2 mutant receptors for the specified compound.