

The Third Transmembrane Helix of the Cannabinoid Receptor Plays a Role in the Selectivity of Aminoalkylindoles for CB2, Peripheral Cannabinoid Receptor¹

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ABSTRACT

Two subtypes of the human cannabinoid receptor have been identified. The CB1 receptor is primarily distributed in the central nervous system, whereas the CB2 receptor is associated with peripheral tissue, including the spleen. These two subtypes are also distinguished by their ligand-binding profiles. The goal of this study was to identify critical residues in transmembrane region III (TM3) of the receptors that contribute to subtype specificity in ligand binding. For this purpose, a chimeric cannabinoid receptor [CB1/2(TM3)] was generated in which the TM3 of CB1 was replaced with the corresponding region of CB2. These receptors were stably expressed in Chinese hamster ovary cells for evaluation. The binding affinities of CB1/2(TM3) and the wild-type CB1 receptor to several prototype ligands were similar with one notable exception: the chimeric receptor exhibited a 4-fold enhancement in binding affinity to WIN 55,212-2 ($K_d = 4.8$ nM) relative to that observed

with CB1 ($K_d = 21.7$ nM). Two additional aminoalkylindoles, JWH 015 and JWH 018, also bound the chimeric receptor ($K_i = 1.0$ μ M and 1.4 nM, respectively) with higher affinity compared with the wild-type CB1 ($K_i = 5.2$ μ M and 9.8 nM, respectively). Furthermore, the increase in binding affinities of the aminoalkylindoles were reflected in the EC_{50} values for the ligand-induced inhibition of intracellular cAMP levels mediated by the chimeric receptor. This pattern mirrors the selectivity of WIN 55,212-2 binding to CB2 compared with CB1. Site-specific mutagenesis of the most notable amino acid changes in the chimeric receptor, Gly195 to Ser and Ala198 to Met, revealed that the enhancement in WIN 55,212-2 binding is contributed to by the Ser but not by the Met residue. The data indicate that the amino acid differences in TM3 between CB1 and CB2 play a critical role in subtype selectivity for this class of compounds.

The psychoactive component of marijuana, Δ^9 -tetrahydrocannabinol (THC), exerts many of its effects through activation of specific G protein-coupled receptors. Two subtypes of cannabinoid receptors, CB1 and CB2, have been identified, and their cDNA has been cloned (Matsuda et al., 1990; Munro et al., 1993). Despite the high homology in their amino acid sequence [44% sequence identity overall and 68% in the transmembrane regions (TM)], they possess somewhat different pharmacological and biochemical properties. The CB1

receptor is mainly distributed in the central nervous system, and lesser amounts are found in peripheral tissues (Gerard et al., 1991). The CB2 receptor was found to be expressed predominantly in the immune system, including the marginal zone of the spleen, and in macrophages, which may be associated with the immunomodulation function of cannabinoids (Munro et al., 1993). The differential tissue distribution of the two subtypes suggests that CB1 and CB2 play different roles in mediating cannabinoid-induced effects.

Biochemical characterization of the signal transduction of CB1 and CB2 expressed in cultured cells has suggested that the activation of both receptors results in the inhibition of cAMP accumulation (Felder et al., 1995; Slipetz et al., 1995). This coincides with the notion that functional coupling to the inhibitory G protein (G_i) is one of the major signaling pathways for cannabinoid receptors (Howlett et al., 1986). CB1

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ABBREVIATIONS: THC, Δ^9 -tetrahydrocannabinol; TM, transmembrane region; AAI, aminoalkylindole; CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; CP 55,940, (–)-*cis*-3[2-hydroxy-4-(1',1'-dimethylheptyl)phenyl]*trans*-4-(3-hydroxypropyl)cyclohexanol; WIN 55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl][1-naphthalenyl]methanone monomethanesulfonate; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride; JWH 015, 1-propyl-2-methyl-3-(1-naphthoyl)indole; JWH 018, 1-pentyl-3-(1-naphthoyl)indole; (R)-methanandamide, (R)-(+)-arachidonyl-1'-hydroxy-2'-propylamide; MEM, minimal essential medium; CHO, Chinese hamster ovary.

has also been shown to mediate the inhibition of N-type calcium channels in neuroblastoma cells (Mackie and Hille, 1992), the inhibition of Q-type calcium channels, and the activation of inwardly rectifying potassium channels in AtT-20 cells (Mackie et al., 1995). These effects were not observed in AtT-20 cells transfected with CB2 (Felder et al., 1995). This suggests that CB1 and CB2 functionally couple to other effector systems that are independent of G_i and are not shared between the two subtypes.

To discriminate the physiological functions and cellular effects mediated by either CB1 or CB2, cannabinoid ligands that can selectively bind to one or the other of the subtypes have been developed. It was found that cannabinol has a 10-fold higher binding affinity to CB2 than to CB1 (Munro et al., 1993). $(-)-\Delta^9$ -THC, the active component of *Cannabis sativa*, has been shown to act as an agonist for CB1 but as a weak antagonist for CB2 (Bayewitch et al., 1996). Derivatives of Δ^8 -THC-dimethylheptyl- Δ^8 -THC lacking a phenolic hydroxyl were found to give rise to high selectivity for CB2 (Gareau et al., 1996; Huffman et al., 1996). It was also found that the C3 substituents of classical and nonclassical cannabinoids affected the selectivity for CB1 or CB2 (Lu et al., 1997). Selectivity for CB2 has also been observed for WIN 55,212-2 [(R) -(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)-methyl]pyrrolo[1,2,3-de]1,4-benzoxazin-6-yl](1-naphthalenyl)methanone monomethanesulfonate], the prototype of aminoalkylindoles (AAIs), as well as other analogs in this series of cannabimimetic ligands (Felder et al., 1995; Showalter et al., 1996). Anandamide, an endogenous ligand of the cannabinoid receptor isolated from porcine brain, has weak preference for CB1 over CB2. SR 141716A [N -(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride] and SR 144528 are potent antagonists specific for CB1 and CB2, respectively (Rinaldi-Carmona et al., 1994, 1998). Comparisons of pharmacological potency and binding affinities to CB1 and CB2 expressed in Chinese hamster ovary (CHO) cells have provided useful information on cannabinoid ligands that can discriminate between binding to CB1 or CB2 (Showalter et al., 1996). However, the molecular basis for the selectivity is not fully understood.

One goal of our study was to understand the molecular interactions between cannabinoid ligands and the receptor. A previous study on the CB1 receptor indicated the importance of Lys192 in TM3 for the binding of several cannabinoid agonists, including HU 210, CP 55,940 [$(-)$ -*cis*-3-[2-hydroxy-4-(1',1'-dimethylheptyl)phenyl]*trans*-4-(3-hydroxypropyl)cyclohexanol], and anandamide but not for the binding of WIN 55,212-2 (Song and Bonner, 1996). Furthermore, evidence from our laboratory suggests that a basic residue, in particular, at this position is essential to ensure the high-affinity binding of CP 55,940 to the CB1 receptor (Chin et al., 1998). To further explore the involvement of TM3 in ligand binding and to delineate its role in subtype specificity, we examined the selectivity displayed by several ligands for a chimeric mutant of CB1 in which the TM3 region was replaced by the corresponding region of CB2. Remarkably, several AAI compounds, including WIN 55,212-2, showed higher binding affinity for the chimeric receptor than for CB1. Our data indicate that amino acid differences in this segment, particularly Gly195 (CB1) versus Ser (CB2), contribute to the binding of AAIs and play a role in subtype selectivity.

Materials and Methods

Reagents. CP 55,940 was provided by Pfizer Inc. (Groton, CT). WIN 55,212-2, (R)-methanandamide [(R) -(+)-arachidonyl-1'-hydroxy-2'-propylamide], and CHO membrane expressing the CB2 receptor were purchased from Research Biochemical Inc. (Natick, MA). SR 141716A and [3 H]SR141716A (19.4 Ci/mmol) were provided by the National Institute on Drug Abuse (Bethesda, MD). JWH 015 and JWH 018 were provided by Dr. John Huffman of Clemson University (Bell et al., 1991; Huffman et al., 1994). Indomethacin morpholinamide was purchased from Cayman Chemical (Ann Arbor, MI). [3 H]CP 55,940 (165 Ci/mmol) and [3 H]WIN 55,212-2 (43 Ci/mmol) were purchased from DuPont-NEN (Boston, MA). G418 sulfate was obtained from Calbiochem (La Jolla, CA). Protease inhibitor cocktail was obtained from Sigma Chemical Co. (St. Louis, MO). The human CB1 cDNA was provided by Dr. Marc Parmentier (University Libre de Bruxelles, Bruxelles, Belgium). CHO-K1 cells were obtained from American Type Culture Collection (Rockville, MD). Minimal essential medium (MEM) and fetal bovine serum were purchased from Hyclone Laboratories, Inc. (Logan, UT).

Plasmid Construction and Stable Expression of Cannabinoid Receptor in CHO Cells. The human CB1 cDNA was subcloned into the pAlterI vector for site-directed mutagenesis using the Alter Sites in vitro mutagenesis system (Promega Corp., Madison, WI) as previously described (Chin et al., 1998). Eight new restriction enzyme sites were designed and introduced into the coding region of the CB1 receptor without changing the amino acid sequence. These restriction enzyme sites are unique in the coding region of CB1 to facilitate cassette mutagenesis. Two such sites, *Xba*I and *Age*I, were used in this study to generate the mutant CB1 receptor. Three pairs of oligonucleotides encoding the sequence of the TM3 of the CB2 receptor were inserted between these two sites, which resulted in the replacement of the TM3 of the CB1 receptor with that of the CB2 receptor. The mutated CB1 gene was then subcloned into the pcDNA3 vector (Invitrogen, San Diego, CA) for expression in mammalian cells. CHO cells were maintained in MEM supplemented with 0.1 mM MEM nonessential amino acid solution (Life Technologies, Inc., Gaithersburg, MD) and 5% fetal bovine serum under 5% CO_2 at 37°C. Stably transfected cell lines expressing CB1 or the mutant receptor were selected using reverse transcription-polymerase chain reaction as previously described (Chin et al., 1998) and maintained in the CHO cell growth medium with 0.5 mg/ml G418 sulfate. The single-site mutations, G195S and A198M, were introduced into the CB1 coding region using the QuikChange system (Stratagene, La Jolla, CA). These mutant receptors were evaluated after transient transfection of 293 cells with calcium phosphate.

Ligand Binding Assay and cAMP Determination. Membranes of CHO or 293 cells were prepared as previously described (Abadji et al., 1999). In brief, cells were grown to confluency and harvested by scraping in PBS with a cell scraper. The cell suspension was centrifuged at 1500g for 10 min at 4°C, and the pellet was resuspended in TME buffer (25 mM Tris · HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA) with 0.5% (v/v) protease inhibitor cocktail containing 100 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 4 mM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane, 2.2 mM leupeptin, 0.08 mM aprotinin, and 1.5 mM pepstatin A. The resuspended cells were then subjected to nitrogen cavitation at 750 psi for 5 min with a Parr Cell Disruption Bomb to rupture the cells. The fractionated cells were centrifuged at 1000g for 10 min to remove cell debris and unbroken nuclei. The supernatant containing cell membranes was stored at -70°C in aliquots of 1 to 2 mg protein/ml until use in the radioligand-binding assay.

The ligand-binding assays were carried out as previously described (Abadji et al., 1994). The reactions were conducted in a total volume of 200 μ l in silanized glass tubes. Approximately 40 μ g of membrane protein was added to the reaction and incubated at 30°C for 1 h with varying concentrations of radioligand and/or unlabeled ligand in TME buffer containing 0.1% fatty acid-free BSA. The con-

centrations of [3 H]CP 55,940, [3 H]WIN 55,212-2, or [3 H]SR 141716A in saturation binding studies ranged from 0.2 to 50 nM. Unlabeled ligands at a concentration of 2 μ M were used to determine the nonspecific binding. In competition binding studies, the concentrations of compounds tested ranged from 10^{-5} to 10^{-11} M, and 1.5 nM [3 H]CP 55,940 was used. The reaction was terminated by the addition of 250 μ l of cold TME containing 5% BSA immediately preceding the separation of bound and free radioligand via rapid filtration through GF/C filter paper (Whatman Inc.) on a 24-manifold Brandel cell harvester (Gaithersburg, MD). The filters were washed with 16 ml of cold TME buffer. Bound radioactivity was determined by scintillation counting. Specific binding accounted for >65% of the total binding at a [3 H]CP 55,940 concentration of 1.5 nM.

cAMP Determination. The accumulation of cAMP was measured as previously described (Chin et al., 1998). Forskolin was added at a concentration of 1 μ M to the reaction to stimulate adenylyl cyclase and elevate the level of cAMP. The amount of cAMP was determined using a [3 H]cAMP assay system (Amersham, Arlington Heights, IL).

Data Analysis. Data obtained from binding assays and cAMP assays were analyzed with the use of Prism (GraphPAD Software, San Diego, CA). The $B_{\max}$ and K_d values of saturation binding assays were determined using the template for fitting saturation binding curves to a single binding site. For competition binding assays, IC_{50} values were determined by the nonlinear regression analysis of the logarithm of ligand concentration versus percent displacement of radioligand binding. These values were then converted to K_i values according to the method of Cheng and Prusoff (1973). The statistical significance of differences between binding constants was determined with an unpaired t test. Differences with values of $P < .05$ were considered significant. Data obtained from cAMP assays were expressed as the percentage of forskolin-stimulated cAMP accumulation as a function of the logarithm of ligand concentration. The EC_{50} values were determined by nonlinear regression analysis.

Results

Evaluation of Effects of TM3 Replacement on Ligand Binding Characteristics of CB1 Expressed in CHO Cells. A chimeric mutant of human CB1 was constructed in which the TM3 of CB1 was replaced with the corresponding region of CB2, as shown in Fig. 1. This replacement gave rise to a mutant CB1 receptor, CB1/2(TM3), containing six point mutations in the amino acid sequence of TM3 in CB1: F191L, L193I, G195S, A198M, S199T, and F207L (Fig. 1).

To examine the effects of the domain replacement on the ligand-binding properties of the CB1 receptor, binding to several different prototype ligands and related analogs was tested (Fig. 2). Binding assays were performed on membrane preparations of CHO cells stably expressing the CB1, CB1/2(TM3), or CB2 receptors. Using [3 H]CP 55,940 as a radioligand, the wild-type CB1 and CB2 receptors showed similar binding affinities with K_d values of 3.8 ± 0.8 and 4.2 ± 0.8 nM, respectively (Table 1). The affinities of [3 H]CP 55,940 for CB1 and CB2 are consistent with data previously reported from mammalian cell expression systems (2.6 nM for CB1 and 3.7 nM for CB2; Felder et al., 1995). The chimeric receptor CB1/2(TM3) exhibited binding of [3 H]CP 55,940 with a K_d value of 10.7 ± 1.5 nM (Table 1). The affinity of CP 55,940 for the chimeric mutant was similar to, although not quite as good as, the affinities for the wild-type CB1 and CB2. The CHO cell lines stably transfected with the wild-type or chimeric receptor tested in this study showed similar levels of expression with $B_{\max}$ values of 1124 ± 88 fmol/mg membrane protein for the wild-type CB1 and 1127 ± 60 fmol/mg mem-

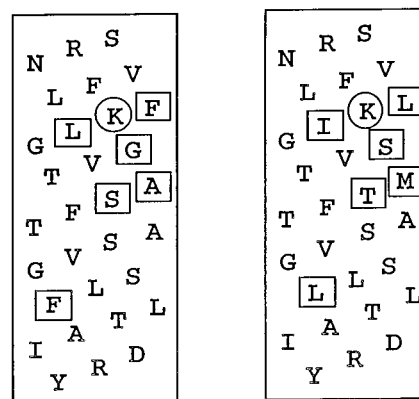
A

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XbaI                                     AgeI
tctaga.....acgggt
                                191 193 195 198 199      207
CB1  SRNVFLFKLGGVTASFTASVGSLFLTAIDRY
CB2  SRNVFLLKIGSVTMTFTASVGSLLLTAIDRY

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B



TM3 of CB1

TM3 of CB2

Fig. 1. A, comparison of the amino acid sequences in the TM3 of CB1 and CB2. Residues that are different between CB1 and CB2 are shown in bold and labeled with the number in the sequence of CB1. The introduced restriction enzyme sites, *Xba*I and *Age*I, used to create the domain replacement mutation of CB1 are shown in their corresponding positions in the DNA sequence, which are flanking the region encoding TM3. B, helical net representations of the TM3 of CB1 and CB2. Amino acid residues that are different in this region of CB1 and CB2 are indicated by squares, and Lys192, a key residue that is involving the binding of several cannabinoid ligands, is in a circle.

brane protein for the CB1/2(TM3) chimera. The membrane preparation from CHO cells expressing CB2 showed a higher expression level, with a $B_{\max}$ value of 2960 ± 210 fmol/mg membrane protein.

The results from the binding of CP 55,940 suggest that the mutations in CB1/2(TM3) did not disrupt the structural integrity of the receptor or cause the loss of critical amino acid residues for the binding of CP 55,940. To further investigate the affect of this chimeric mutation on the ligand-binding profile of CB1, we tested a variety of other cannabinoid ligands with different degrees of selectivity for CB1 or CB2 to compare the binding characteristics of CB1/2(TM3) with those of the wild-type CB1 and CB2 receptors. Ligands were assayed for affinity to each receptor type by a saturation binding assay or a competition binding assay with 1.5 nM [3 H]CP 55,940, and the binding constants of each compound are summarized in Table 1.

SR141716A, a biarylpyrazole, is a CB1-specific inverse agonist (Fig. 2; Bouaboula et al., 1997). Saturation binding analysis of [3 H]SR141716A to CB1 and CB1/2(TM3) yielded K_d values of 5.9 ± 0.7 and 11.8 ± 1.0 nM, respectively, showing that the mutation did not markedly affect the binding affinity of this ligand to the receptor (Table 1). (*R*)-Methanandamide is a chiral congener of anandamide that

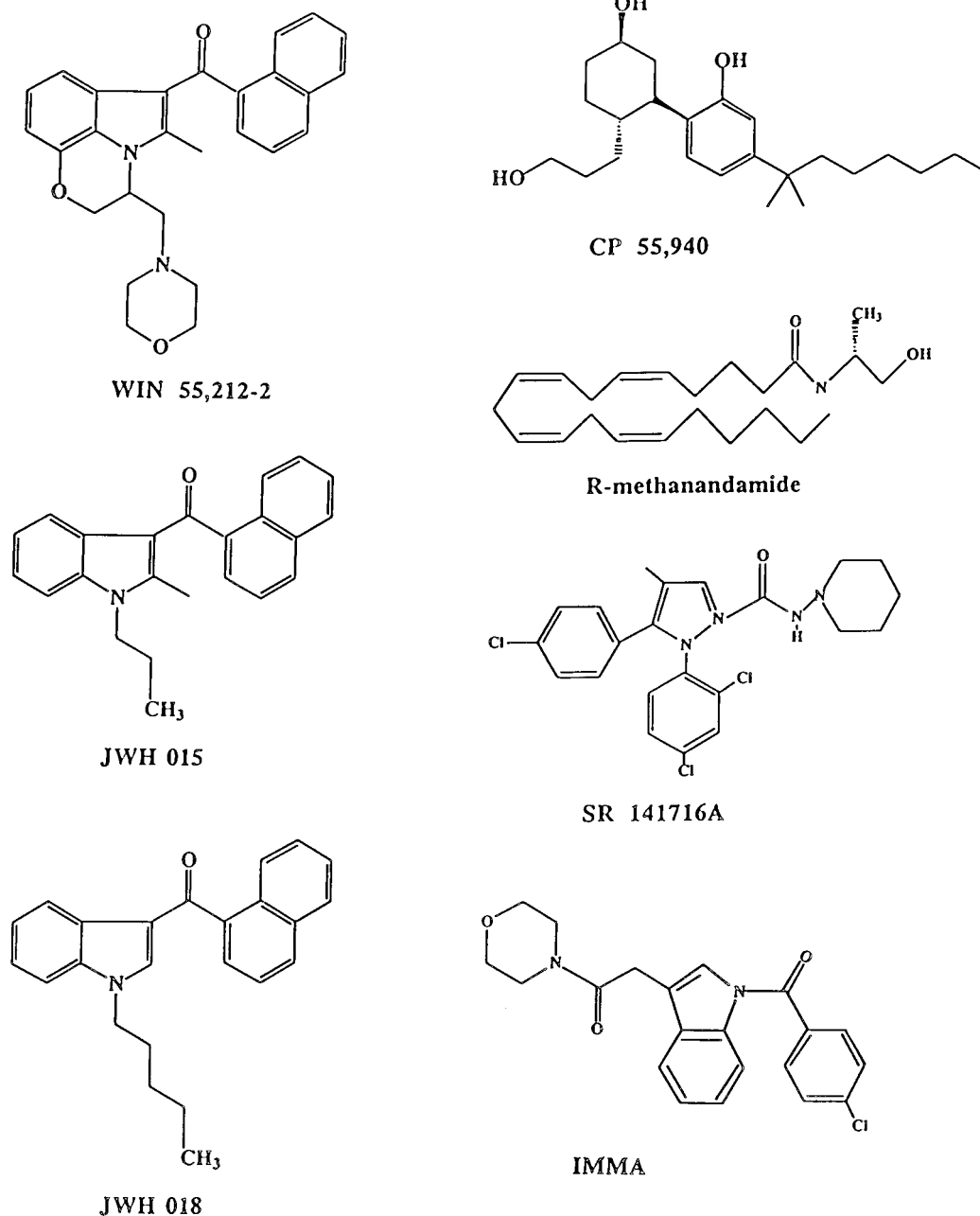


Fig. 2. The chemical structures of cannabinoid ligands used in this study.

displays higher affinity and better metabolic stability than anandamide (Abadji et al., 1994). Like anandamide, (*R*)-methanandamide (Fig. 2) possesses higher binding affinity to CB1 than to CB2 (Khanolkar et al., 1996). Our results showed that it binds to CB1/2(TM3) with affinity similar to that of the wild-type CB1 (Table 1). The K_i value for the wild-type CB1 is comparable to that previously reported (Abadji et al., 1994; Khanolkar et al., 1996). Indomethacin morpholinamide is a selective ligand for CB2 (Fig. 2), with a K_i value of 435 nM (Gallant, 1996). Our results showed that this compound possesses very low binding affinities to both the CB1 wild-type and mutant receptors ($K_i > 2 \mu\text{M}$; Table 1).

Overall, little or no changes in the binding affinities of the compounds tested above for CB1/2(TM3) were found relative to the wild-type CB1. In no case was a pattern of subtype

selectivity noted for these compounds; thus, it appears that residues in TM3 specific to CB2 are not critical to the selectivity of these compounds.

Selective Binding of AAI to CB2 Is Reflected in Binding to CB1/2(TM3) Chimera. WIN 55,212-2 has been reported as a selective ligand for CB2 with a 7- to 20-fold higher binding affinity than for CB1 (Fig. 2; Felder et al., 1995; Slipetz et al., 1995; Showalter et al., 1996). Selectivity of about 10-fold of WIN 55,212-2 for CB2 over CB1 was observed in our study ($P < .05$, unpaired *t* test). As shown in Fig. 3, saturation binding analyses for [^3H]WIN 55,212-2 yielded a K_d value of 21.7 ± 6.9 nM for CB1 and 2.3 ± 0.2 nM for CB2 (Fig. 3, top and middle; Table 1). The CB1/2(TM3) receptor bound [^3H]WIN 55,212-2 with a K_d value of 4.8 ± 0.5 nM (Fig. 3, bottom; Table 1). Interestingly, this indicates that the introduction of the TM3 of CB2 into CB1 resulted in a

TABLE 1

Comparison of binding affinities of cannabinoid ligands to CB1, CB2, and CB1/2(TMIII)

Binding constants are presented as mean $\pm$ S.E. values from at least two experiments performed in duplicate.

Cannabinoid Ligand	CB1 Wild Type K_i or K_d	CB2 Wild Type K_i or K_d	CB1/2(TMIII) Chimera K_i or K_d
	nM		
CP 55,940	3.8 $\pm$ 0.8	4.2 $\pm$ 0.8	10.7 $\pm$ 1.5
WIN 55,212-2	21.7 $\pm$ 6.9	2.3 $\pm$ 0.2	4.8 $\pm$ 0.5
JWH 015	5200 $\pm$ 1900 (383) ^a	13.8 ^c	1000 $\pm$ 300
JWH 018	9.8 $\pm$ 2.8	3.1 $\pm$ 0.3	1.4 $\pm$ 0.2
(R)-Methanandamide	35.1 $\pm$ 0.2	815 ^b	21.8 $\pm$ 5.4
Indomethacin	>2000	435 ^c	>2000
morpholinamide			
SR 141716A	5.9 $\pm$ 0.7	>1000 ^d	11.8 $\pm$ 1.0

^a K_i values from Showalter et al. (1996).

^b K_i value from Khanolkar et al. (1996).

^c K_i value from Gallant et al. (1996).

^d K_i value from Shire et al. (1996).

4-fold enhancement in the binding affinity of WIN 55,212-2 to the mutant receptor relative to CB1. Therefore, unlike other classes of cannabinoid compounds tested, WIN 55,212-2 is significantly more selective for the mutant receptor than for CB1 ($P < .05$, unpaired t test). The $B_{\max}$ values obtained from these data are 1563 $\pm$ 231 fmol/mg for CB1, 2389 $\pm$ 60 fmol/mg for CB2, and 1510 $\pm$ 50 fmol/mg for CB1/2(TM3). These values are consistent with the $B_{\max}$ values obtained for these CHO cell lines when assayed for [³H]CP 55,940 binding as described above.

Surprisingly, data from the study above indicate that one or more of the six residues unique to TM3 of CB2 plays a role, in particular, in the selectivity of WIN 55,212-2. To investigate whether this effect extended to AAIs as a whole, we tested two other AAI analogs: JWH 015 and JWH 018. As shown in Fig. 2, JWH 015 and JWH 018 are naphthoyl indole derivatives in which the morpholino ring of WIN 55,212-2 was replaced by the propyl or pentyl side chain, respectively. The alkyl chain substituents, such as 2-cyclohexylethyl, n -butyl, n -pentyl (JWH 018), or n -hexyl chain, for the morpholino moiety of WIN 55,212-2 do not affect its affinity or efficacy for CB1 (Huffman et al., 1994; Wiley et al., 1998). However, a propyl side chain at this position (JWH 015) caused a significant loss of binding affinity for CB1 but retained good binding affinity for CB2 (Showalter et al., 1996). Because JWH 015 and JWH 018 have been reported as selective ligands for CB2, although to different degrees, we examined the binding activities of these two AAI derivatives for CB1/2(TM3) relative to the wild-type CB1. As shown in Fig. 4A, although JWH 015 bound to CB1 and CB1/2(TM3) with relatively low affinities, lower than previously reported (Showalter et al., 1996), the K_i values suggest a trend toward selectivity for the mutant receptor (Table 1). JWH 018, on the other hand, displayed nearly a 7-fold selectivity for CB1/2(TM3) over the wild-type CB1 ($P < .05$, unpaired t test), with K_i values of 9.8 $\pm$ 2.8 nM for CB1, 3.1 $\pm$ 0.3 nM for CB2, and 1.4 $\pm$ 0.2 nM for CB1/2(TM3) (Fig. 4B; Table 1).

Taken together, the selectivity of these compounds for the CB1/2(TM3) mutant receptor suggests that the TM3 of the cannabinoid receptor plays a fundamental role in the selectivity of AAIs to CB2. This selectivity is further reflected in the efficacy of these compounds to mediate changes in cAMP levels.

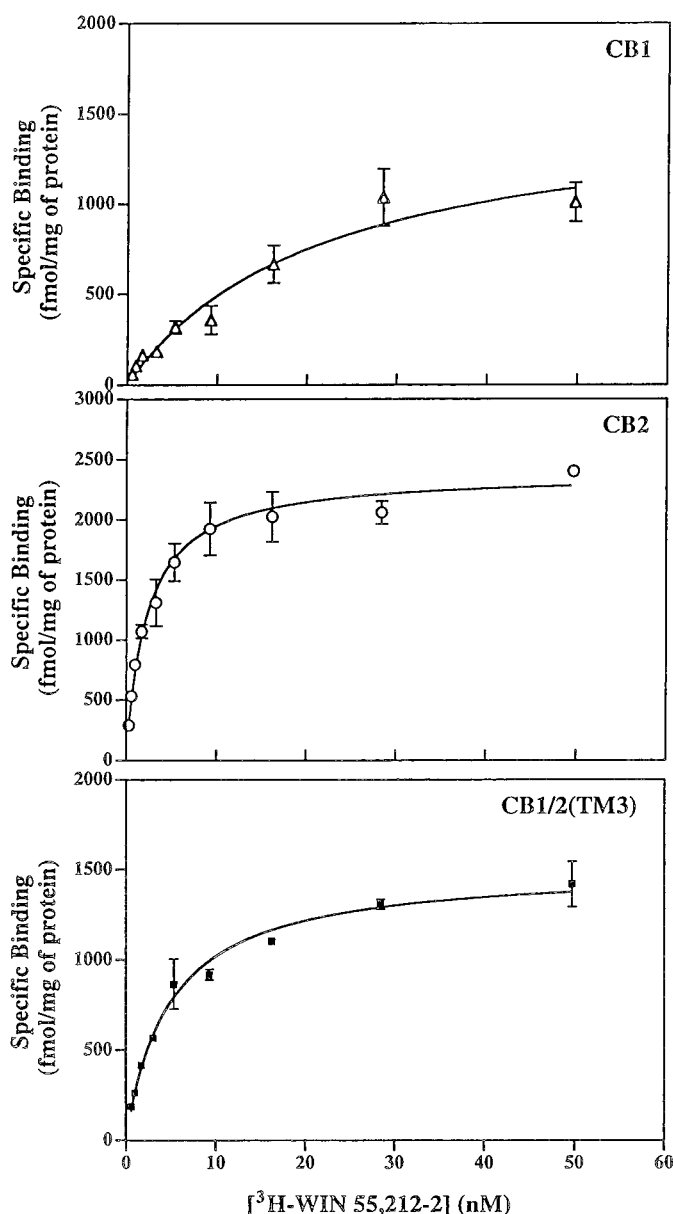


Fig. 3. Saturation analysis of [³H]WIN 55,212-2 binding to membranes of CHO cells expressing the CB1 receptor (Δ), the CB2 receptor ($\circ$), and the CB1/2(TM3) receptor ($\blacksquare$). Binding assays were performed over a concentration range of 0.2 to 50 nM [³H]WIN 55,212-2. Specific binding is the difference between the binding in the absence and presence of 2 μ M unlabeled WIN 55,212-2. Saturation binding isotherms were analyzed with nonlinear regression analysis as described in *Materials and Methods*. Data presented are the mean $\pm$ S.E. of three independent experiments, with each performed in duplicate.

Efficacy of Inhibition of Forskolin-Stimulated cAMP Levels Mediated by CB1/2(TM3) Chimera Reflect CB2 Selectivity for AAIs. To examine whether this mutant also changes the efficacy of CB1 to activate G_i in response to the binding of ligands, cAMP assays were performed as described in *Materials and Methods*. Concentration-dependent inhibition of the level of forskolin-stimulated cAMP by CP 55,940, WIN 55,212-2, and JWH 018 in CHO cells expressing either the wild-type CB1 or the CB1/2(TM3) receptor are shown in Fig. 5.

Using CP 55,940 as a point of comparison, nonlinear regression analysis of the data yields EC_{50} values of 5.5 and

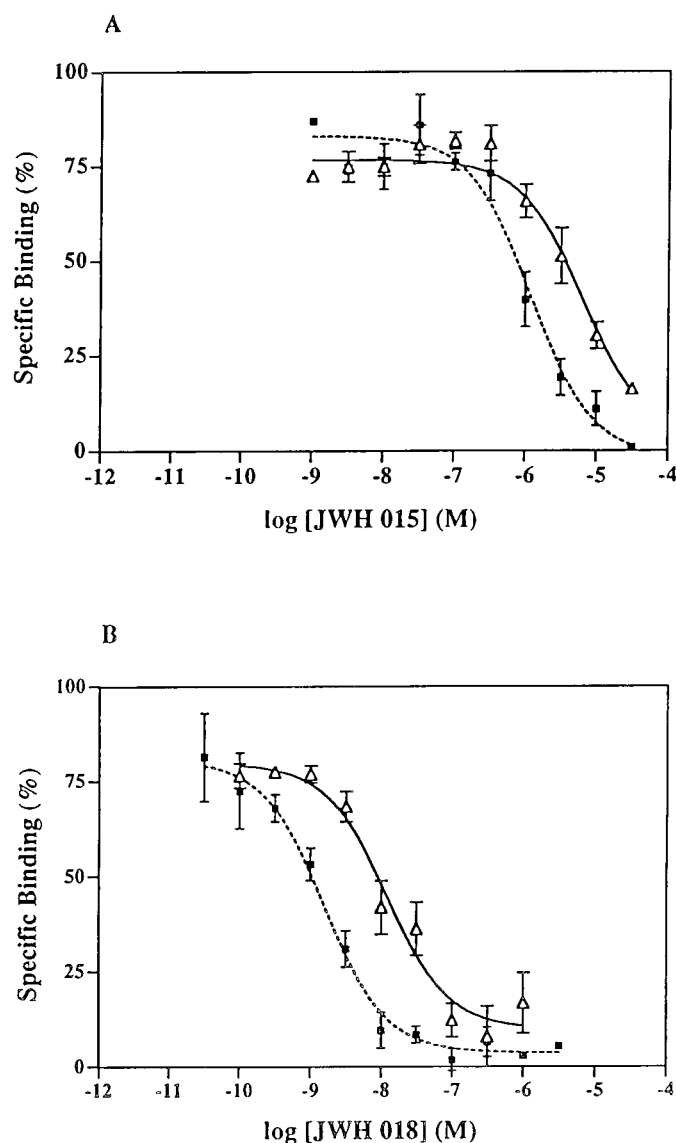


Fig. 4. Competition binding experiments performed on membranes of CHO cells expressing the wild-type CB1 (Δ) or the CB1/CB2(TM3) ($\blacksquare$) receptor. Data shown are the displacement of JWH 015 (A) and of JWH 018 (B) with 1.5 nM [3 H]CP 55,940. Data points represent the mean $\pm$ S.E. of three independent experiments, with each performed in duplicate. The K_i values are presented in Table 1.

15.8 nM for the wild-type CB1 and the CB1/2(TM3) receptors, respectively (Fig. 5A; Table 2). Remarkably, WIN 55,212-2 displays a nearly 5-fold enhancement in the EC_{50} values for the inhibition of the accumulation of cAMP for cells expressing CB1/2(TM3) relative to CB1: 7.0 and 38.9 nM, respectively (Fig. 5B; Table 2). These data again suggest that the mutations introduced into the TM3 of CB1 enhance the ability of the receptor to bind and respond to WIN 55,212-2. In addition, the JWH 018-induced inhibition of the levels of cAMP followed a similar trend. The wild-type CB1 and the CB1/2(TM3) receptors yield EC_{50} values of 14.7 and 4.7 nM, respectively, with this compound (Fig. 5C; Table 2). A comparison of the data in Tables 1 and 2 reveals that the differences in the EC_{50} values of WIN 55,940 and JWH 018 mirrored the differences in their binding affinities, suggesting that the enhancement in the efficacy of CB1/2(TM3) to these two AAs, relative to the wild-type CB1, is directly due to the

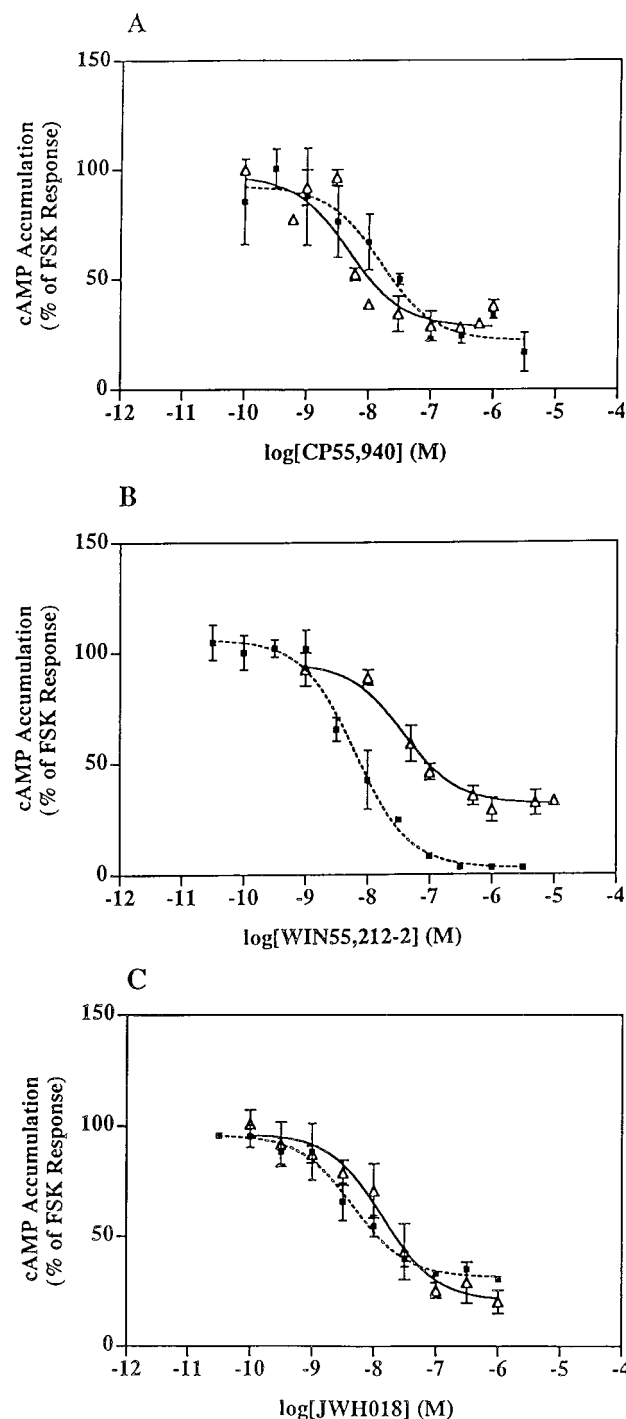


Fig. 5. cAMP accumulation in CHO cells expressing the wild-type CB1 (Δ) receptor or the CB1/CB2(TM3) receptor ($\blacksquare$). The levels of cAMP were determined in the presence of 1 μ M forskolin and varying concentrations of CP 55,940 (A), WIN 55,212-2 (B), or JWH 018 (C). The data are presented as the percent change from forskolin-stimulated activity and shown as the mean $\pm$ S.E. of at least two independent experiments, with each performed in duplicate. The EC_{50} values are presented in Table 2.

higher binding affinity rather than to mutation-induced changes in the coupling to G_i protein.

Evaluation of Individual Amino Acid Changes Gly195 to Ser and Ala198 to Met within TM3 of CB1 for WIN 55,212-2 Binding. Assessment of the chemical nature of the six amino acids in TM3 that differ in CB1 and CB2

TABLE 2

Cannabinoid ligand-induced inhibition of forskolin-stimulated cAMP accumulation in CHO cell lines expressing CB1 or CB1/2(TMIII)

Results are presented as mean $\pm$ S.E. of EC₅₀ values from at least two experiments performed in duplicate.

Compounds	CB1		CB1/2(TMIII)	
	EC ₅₀	Maximum Inhibition	EC ₅₀	Maximum Inhibition
	nM	%	nM	%
CP 55,940	5.5 $\pm$ 2.9	72	15.8 $\pm$ 6.8	78
WIN 55,212-2	38.9 $\pm$ 8.2	68	7.0 $\pm$ 0.6	97
JWH 018	14.7 $\pm$ 3.9	79	4.7 $\pm$ 0.8	70

points to only a few candidates that are likely to contribute to the enhanced binding of the chimeric receptor with the AAIs. For example, L193I and S199T are conservative changes and retain the general characteristics of the amino acid side chain that was used. More notable are the changes of G195S and A195M; these changes provide an extra hydroxyl group (G195S) and a longer and more hydrophobic side chain (A195M). To examine the role of these two amino acid changes in WIN 55,212-2 binding, mutant CB1 receptors were generated in which only the Gly-to-Ser or the Ala-to-Met substitution was made. The resulting G195S and A195M receptors were separately evaluated after transient transfection of 293 cells. Membrane preparations of the wild-type CB1 receptor expressed in 293 cells versus CHO cells exhibit no differences in CP 55,940 binding (data not shown) or WIN 55,212-2 (Table 3). The ability of [³H]WIN 55,212-2 to bind to the A195M and G195S receptors was examined using saturation binding assays. Substitution of A195 in CB1 with Met produced no significant change in the binding isotherm relative to the wild-type receptor with a K_d value of 20.4 ± 5.1 nM and a B_{max} value of 1949 ± 185 fmol/mg of protein (Table 3). In contrast, the G195S receptor bound [³H]WIN 55,212-2 with enhanced affinity relative to CB1 ($P < .05$, unpaired t test; Table 3). The K_d value of 5.6 ± 0.6 nM ($B_{max} = 1534 \pm 56$ fmol/mg protein) suggests that the Gly195-to-Ser exchange contributes substantially to the observed enhancement in WIN 55,212-2 binding by the CB1/2(TM3) chimera relative to the wild-type CB1 receptor.

Discussion

In this study, we demonstrate that amino acid differences in the TM3 of CB1 and CB2 affect the binding profiles of some cannabinoid ligands. For most of the compounds, we found either no difference or only a small loss in binding affinity. Surprisingly, however, the exchange of TM3 resulted in a distinct enhancement in the binding affinity and efficacy of AAI analogs; this includes a 5-fold increase for WIN 55,212-2,

TABLE 3

Binding parameters of CB1 wild type, CB1 A195M, and CB1 G195S to [³H]WIN 55,212-2

Results are obtained from membrane preparations of 293 cells expressing the wild-type and mutant CB1 receptors, and data are presented as mean $\pm$ S.E. values from at least four saturation binding experiments performed in duplicate.

Receptor	K_d	B_{max}
	nM	fmol/mg protein
CB1 wild type	20.4 $\pm$ 3.6	1926 $\pm$ 149
CB1 A195M	20.4 $\pm$ 5.1	1949 $\pm$ 185
CB1 G195S	5.6 $\pm$ 0.6	1534 $\pm$ 56

a 5-fold increase for JWH 015, and a 7-fold increase for JWH 018 relative to binding to the wild-type CB1. This increase in binding affinity was also reflected in the EC₅₀ values for the inhibition of intracellular levels of cAMP and further validates that the interactions of these ligands with the receptor were augmented in this mutant. This observation suggests that at least a part of the elements in CB2 responsible for the subtype selectivity of AAIs has been introduced into CB1 via the mutations that we generated.

A priori, sequence inspection of TM3 in CB1 and CB2 points to Lys192 in CB1 (Lys109 in CB2) as the most likely potential contact point for cannabinoid ligand/receptor interactions. The Lys residue stands out as being the only charged residue in the TM regions that is not conserved in the G protein-coupled receptor family yet is shared by both subtypes of the cannabinoid receptor. However, previous work involving single-site mutations of this residue has demonstrated that at least for CB1, Lys192 is not critical for WIN 55,212-2 binding (Song and Bonner, 1996; Chin et al., 1998). Furthermore, we have been able to change this residue to Arg, Gln, and Glu and still detect WIN 55,212-2 binding activity to CB1 (Chin et al., 1998). Consequently, many investigators have emphasized the involvement of amino acid residues in other TMs for the interaction with AAIs (Abood et al., 1998; Song and Reggio, 1998). For example, molecular modeling studies have pointed to the potential role of residues such as Phe and Tyr in TM5 of CB2 that could stabilize an interaction with WIN 55,212-2 through aromatic stacking. However, the data presented here have shown that the residues of TM3 also contribute substantially to the binding of AAIs, and residues other than Lys192 in this TM appear critical.

Although our conceptual strategy was to switch the entire TM3 domain from CB1 to CB2, in effect, this amounts to only six amino acid changes. However, modeling indicates that these changes are largely clustered in one area of TM3, as seen in the helical net representation (Fig. 1B). This cluster is positioned proximal to and on the same helical face as Lys192, which has been implicated as a key residue for the binding of other classes of cannabinoid ligands. This intriguing observation therefore suggests that this cluster is very likely to constitute a part of the ligand-binding pocket for the cannabinoid receptor. To assess the contribution of the individual residue differences in this region in TM3, two single-site mutations were made in which a residue in CB1 was exchanged for the corresponding residue from CB2: G195S and A195M. The A195M receptor was indistinguishable from wild-type CB1 in WIN 55,212-2 binding. In contrast, the G195S receptor exhibited enhanced WIN 55,212-2 binding, suggesting that this residue plays a critical role in the CB2-like binding characteristics of the CB1/2(TM3) chimeric receptor with AAIs.

The change from Gly to Ser could alter the interaction with AAIs by introducing a new hydrogen-bonding group. Previous SAR studies demonstrated that the removal of the carbonyl oxygen of AAIs did not affect their binding to CB1 (Kumar et al., 1995; Reggio et al., 1998), suggesting that this moiety is not required for the binding of AAIs to CB1. However, these AAI analogs lacking the carbonyl oxygen displayed a 7- to 10-fold loss of binding affinity for CB2 compared with the binding of WIN 55,212-2 and, thus, loss of selectivity for CB2 (Reggio et al., 1998). This suggested the

possibility that hydrogen bonding of WIN 55,212-2 occurs with the amino acid residues of CB2, which may be absent in CB1. Indeed, this may explain the higher binding affinity to CB2, and the change from Gly to Ser in our receptor may provide an additional contact point in the receptor for such interactions.

The parallelism of the results from the cAMP assays and the data from the binding assays suggest that the effects caused by the TM3 switch are mainly on the ligand-binding sites. For CP 55,940, the EC_{50} value for inhibition of cAMP accumulation is similar for the chimera and wild-type receptors, and an enhancement with the chimera is observed only with the AAI. Unlike the K192E mutant of CB1 in which the mutational effect propagates from the TM region to the G protein-coupling region (Chin et al., 1998), the G protein-coupling efficiency does not appear to be affected by the mutations in TM3 generated here.

Coupled with previous studies identifying the involvement of Lys192 in binding other cannabinoid agonists (Song and Bonner, 1996; Chin et al., 1998), we demonstrate that this region of TM3 of the cannabinoid receptor is also critical for binding AAIs. This indicates that different classes of ligands for the cannabinoid receptor have at least partially overlapping binding pockets, although the functional groups within these domains that are involved in making key contacts may be ligand specific. This information is important for the development of future generations of ligands with enhanced binding affinity and receptor subtype selectivity.

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References

- Abadji V, Lin S, Taha G, Griffin G, Stevenson LA, Pertwee RG and Makriyannis A (1994) (*R*)-Methanandamide: A chiral novel anandamide possessing higher potency and metabolic stability. *J Med Chem* **37**:1889–1893.
- Abadji V, Lucas-Lenard JM, Chin CN and Kendall DA (1999) Involvement of the carboxyl terminus of the third intracellular loop of the cannabinoid CB1 receptor in constitutive activation of G_s . *J Neurochem* **72**:2032–2038.
- Abood ME, Tao Q, McAllister SD and Hurst DP (1998) A critical role for a tyrosine residue in the cannabinoid receptors for recognition of several compounds excluding the indoles. 1998 Symposium on the Cannabinoids International Cannabinoid Research Society, Burlington, VT.
- Bayewitch M, Rhee MH, Avidor-Reiss T, Breuer A, Mechoulam R and Vogel Z (1996) ($-$)- Δ^9 -Tetrahydrocannabinol antagonizes the peripheral cannabinoid receptor-mediated inhibition of adenylyl cyclase. *J Biol Chem* **271**:9902–9905.
- Bell MR, D'Ambra TE, Kumar V, Eissenstat MA, Herrmann JL Jr, Wtzel JR, Rosi D, Phillion RE, Daum SJ, Hlasta DJ, Kullnig RK, Ackerman JH, Haubrich DR, Luttinger DA, Baizman ER, Miller MS and Ward SJ (1991) Antinociceptive (aminoalkyl)indoles. *J Med Chem* **34**:1099–1110.
- Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Peceuf F, Lupker J, Maffrand JP, Le Fur G and Casellas P (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1: Evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272**:22330–22339.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzyme reaction. *Biochem Pharmacol* **22**:2099–3108.
- Chin CN, Lucas-Lenard JM, Abadji V and Kendall DA (1998) Ligand binding and modulation of cyclic AMP levels depend on the chemical nature of residue 192 of the human cannabinoid receptor 1. *J Neurochem* **70**:366–373.
- Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma AL and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol* **48**:443–450.
- Gallant M (1996) New class of potent ligands for the human peripheral cannabinoid receptor. *Biol Med Chem Lett* **6**:2263–2268.
- Gareau Y, Dufresne C, Gallant M, Rochette C, Sawyer N, Slipetz DM, Tremblay N, Weech PK, Metters KM and Labelle M (1996) Structure activity relationships of tetrahydrocannabinol analogues on human cannabinoid receptors. *Biol Med Chem Lett* **6**:189–194.
- Gerard CM, Mollereau C, Vassart G and Parmentier M (1991) Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem J* **279**:129–134.
- Howlett AC, Quail JM and Khachatrian LL (1986) Involvement of G_i in the inhibition of adenylyl cyclase by cannabinimetic drugs. *Mol Pharmacol* **29**:307–313.
- Huffman JW, Dai D, Martin BR and Compton DR (1994) Design, synthesis and pharmacology of cannabinimetic indoles. *Biol Med Chem Lett* **4**:563–566.
- Huffman JW, Yu S, Showalter V, Abood ME, Bramblett RD and Reggio PH (1996) Synthesis and pharmacology of a very potent cannabinoid lacking a phenolic hydroxyl with high affinity for the CB2 receptor. *J Med Chem* **39**:3875–3877.
- Khanolkar AD, Abadji V, Lin S, Hill WA, Taha G, Abouzid K, Meng Z, Fan P and Makriyannis A (1996) Head group analogs of arachidonylethanolamide, the endogenous cannabinoid ligand. *J Med Chem* **39**:4515–4519.
- Kumar V, Alexander MD, Bell MR, Eissenstat MA, Casiano FM, Chippari SM, Haycock DA, Luttinger DA, Kuster JE, Miller MS, Stevenson JI and Ward SJ (1995) Morpholinoalkylindenes as antinociceptive agents: Novel cannabinoid receptor agonists. *Bioorg Med Chem Lett* **5**:381–386.
- Lu D, Khanolkar A, Meng Z, Fan P, Reggio PH and Makriyannis A (1997) CB1/CB2 selective cannabinoids by conformational constraint of the side chain. 1997 Symposium on the Cannabinoids, International Cannabinoid Research Society, Burlington, VT.
- Mackie K and Hille B (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc Natl Acad Sci USA* **89**:3825–3829.
- Mackie K, Lai Y, Westenbroek R and Mitchell R (1995) Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *J Neurosci* **15**:6552–6561.
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature (Lond)* **346**:561–564.
- Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature (Lond)* **365**:61–65.
- Reggio PH, Basu-Dutt S, Barnett-Norris J, Castro MT, Hurst DP, Seltzman HH, Roche MJ, Gilliam AF, Thomas BF, Stevenson LA, Pertwee RG and Abood ME (1998) The bioactive conformation of aminoalkylindoles at the cannabinoid CB1 and CB2 receptors: Insights gained from (E)- and (Z)-naphthyliden indenes. *J Med Chem* **41**:5177–5187.
- Rinaldi-Carmona M, Barth F, Heaulme M, Shire D, Calandra B, Congy C, Martinez S, Maruani J and Neliat G (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett* **350**:240–244.
- Rinaldi-Carmona M, Barth F, Millan J, Derocq JM, Casellas P, Congy C, Oustric D, Sarrazin M, Bouaboula M, Calandra B, Portie RM, Shire D, Breliere JC and Le Fur G (1998) SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther* **284**:644–650.
- Shire D, Calandra B, Delpech M, Dumont X, Kaghad M, Le Fur G, Caput D and Ferrara P (1996) Structure features of the central cannabinoid CB1 receptor involved in the binding of the specific CB1 antagonist SR141716A. *J Biol Chem* **271**:6941–6946.
- Showalter VM, Compton DR, Martin BR and Abood ME (1996) Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): Identification of cannabinoid receptor subtype selective ligands. *J Pharmacol Exp Ther* **278**:989–999.
- Slipetz DM, O'Neill GP, Favreau L, Dufresne C, Gallant M, Gareau Y, Guay D, Labelle M and Metters KM (1995) Activation of the human peripheral cannabinoid receptor results in inhibition of adenylyl cyclase. *Mol Pharmacol* **48**:352–361.
- Song ZH and Bonner TI (1996) A lysine residue of the cannabinoid receptor is critical to receptor recognition by several agonists but not WIN 55,212-2. *Mol Pharmacol* **49**:891–896.
- Song ZH and Reggio PH (1998) The difference between the CB1 and CB2 cannabinoid receptors at position 5.46 is crucial for the selectivity of WIN 55,212-2 for CB2. 1998 Symposium on the Cannabinoids. International Cannabinoid Research Society, Burlington, VT.
- Wiley JL, Compton DR, Dai D, Lainton JA, Phillips M, Huffman JW and Martin BR (1998) Structure-activity relationships of indole- and pyrrole-derived cannabinoids. *J Pharmacol Exp Ther* **285**:995–1004.

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