0022-3565/98/2851-0285$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS Vol. 285, No. 1

Printed in U.S.A.
JPET 285:285–292, 1998

Comparative Receptor Binding Analyses of Cannabinoid Agonists and Antagonists

BRIAN F. THOMAS, ANNE F. GILLIAM, DAVID F. BURCH, MICHAEL J. ROCHE and HERBERT H. SELTZMAN

Research Triangle Institute, Research Triangle Park, North Carolina

Accepted for publication December 23, 1997 This paper is available online at http://www.jpet.org

ABSTRACT

To further characterize neuronal cannabinoid receptors, we compared the ability of known and novel cannabinoid analogs to compete for receptor sites labeled with either [^{3}H]SR141716A or [^{3}H]CP-55,940. These efforts were also directed toward extending the structure-activity relationships for cannabinoid agonists and antagonists. A series of alternatively halogenated analogs of SR141716A were synthesized and tested in rat brain membrane binding assays along with the classical cannabinoids, Δ^9-tetrahydrocannabinol, cannabinol, cannabidiol, the nonclassical cannabinoid CP-55,940, the aminoalkylindole WIN55212–2 and the endogenous fatty acid ethanolamide, anandamide. Saturation binding isotherms were performed with both radioligands, as were displacement studies, allowing an accurate comparison to be made between the binding of these various compounds. Competition studies demonstrated that all of the compounds were able to displace the binding of [^{3}H]CP-55,940 with rank order potencies that agreed with previous studies. However, the rank order potencies of these compounds in competition studies with [^{3}H]SR141716A differed significantly from those determined with [^{3}H]CP-55,940. These results suggest that CP-55,940, WIN55212–2 and other agonists interact with cannabinoid binding sites within the brain which are distinguishable from the population of binding sites for SR141716A, its analogs and cannabidiol. Structural modification of SR141716A significantly altered the affinity of the compound and its relative ability to displace either [^{3}H]CP-55,940 or [^{3}H]SR141716A preferentially within the rat brain receptor membrane preparation.

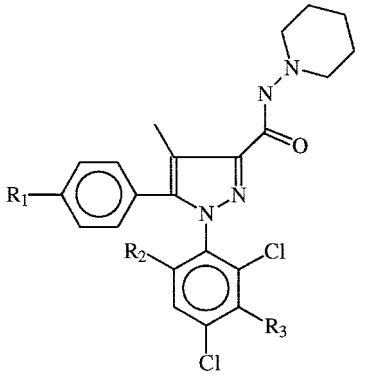
After many years of mechanistic studies involving marijuana and cannabinoids, unequivocal evidence for a cannabinoid receptor in brain was reported in the late 1980s (Devane *et al.*, 1988) with use of a receptor binding assay in membrane preparations and a synthetic cannabinoid ligand of high affinity ([^{3}H]CP-55940). Subsequent cloning and sequencing of this receptor, designated the CB1 receptor, indicated that it belonged to the superfamily of G-protein-coupled receptors (Matsuda *et al.*, 1990), which strengthened the hypothesis that the predominant signal transduction pathway for cannabinoids involved the G-protein-coupled inhibition of cyclic AMP (Howlett *et al.*, 1988). Comparison of the binding of other high-affinity ligands, including [^{3}H]11-OH-Δ^9-THC-DMH (Thomas *et al.*, 1992) and [^{3}H]WIN55212–2 (Ward *et al.*, 1991), further supported the widespread distribution of the CB1 site and its pharmacological relevance, yet failed to further discriminate receptor subtypes in the CNS. Similar binding studies with [^{3}H]CP-55940 in peripheral tissues (testes, spleen) indicated that these tissues and some blood cells (*e.g.*, lymphocytes) also possessed a cannabinoid receptor; however, these tissues expressed a receptor that differed in selectivity from the neuronal receptor (Munro *et al.*, 1993). This peripheral cannabinoid receptor, termed the CB2 receptor site, was cloned from HL60 cells and sequenced and found to have 44% sequence identity with the CB1 site. Although the CB2 site was thought to be localized exclusively in the periphery, Skaper *et al.* (1996) demonstrated that cerebellar granule cells express mRNA for both CB1 and CB2 receptors and provided data that suggested that WIN55212–2 bound to two receptor sites in cerebellar membrane preparations.

Received for publication February 27, 1997.

ABBREVIATIONS: CNS, central nervous system; K_i, affinity constant; K_d, dissociation constant; B_{max}, concentration of receptors; CB1, central cannabinoid receptor subtype; CB1A, splice variant of CB1 cannabinoid receptor subtype; CB2, peripheral cannabinoid receptor subtype; CBN, cannabinol; CBD, cannabidiol; THC, tetrahydrocannabinol; AMP, adenosine monophosphate; DMH, dimethylheptyl; BSA, bovine serum albumin; SR141716A, N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; [^{3}H]SR141716A, N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-([6-^{3}H]-2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; 6-I-SR141716A, N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichloro-6-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide; 3-I-SR141716A, N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichloro-3-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide; 4′-I-SR141716A, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; 4′,6-I_2-SR141716A, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichloro-6-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide; 4′,3-I_2-SR141716A, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichloro-3-iodophenyl)-4-methyl-1H-pyrazole-3-carboxamide; 4′-Br-SR141716A, N-(Piperidin-1-yl)-5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; 4′-H-SR141716A, N-(Piperidin-1-yl)-5-phenyl-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; PMSF, phenylmethanesulfonyl fluoride.

The discovery of additional cannabimimetic compounds whose structures differ from the classical and nonclassical cannabinoids has sustained the rapid expansion in the diversity of cannabimimetic compounds. These newly discovered compounds, such as the naturally occurring anandamides, have also been examined in receptor binding assays and autoradiographic analyses, but generally have failed to indicate cannabinoid receptor heterogeneity within the CNS (Adams *et al.*, 1995). Additionally, the affinity of cannabinoid compounds for the CB1 receptor typically is well correlated to the *in vivo* potencies of these compounds to produce a wide variety of cannabinoid effects, including analgesia, hypothermia, catalepsy and decreased locomotor activity (Compton *et al.*, 1993), which indicates that the CB1 receptor site is the primary transduction mechanism for the production of these central effects of cannabinoids.

The discovery of SR141716A (Rinaldi-Carmona *et al.*, 1994; fig. 1) was unique because this study reported a cannabinoid antagonist possessing nanomolar affinity. This compound was shown to block the central effects of cannabinoids and to precipitate a withdrawal system in animals chronically exposed to cannabinoid agonists (Aceto *et al.*, 1995). Although some compounds such as cannabidiol, Δ^{9-11}-THC (Beardsley *et al.*, 1987), bromopravadoline (Casiano *et al.*, 1991) and AM630 (Pertwee *et al.*, 1995) previously have been reported to possess antagonistic activity, their potencies were low and they generally failed to act as antagonists in intact animals. Because SR141716A and its analogs constitute an additional family of compounds that interact with the cannabinoid receptor, it is of interest to determine whether these compounds interact with the same recognition site on the cannabinoid receptor and whether the population of neuronal receptor sites that is bound by SR141716A is the same as that with which classical and nonclassical cannabinoids interact.



R_1	R_2	R_3	NAME
Cl	H	H	SR141716A
Cl	^{3}H	H	[^{3}H]SR141716A
Cl	I	H	6-I-SR141716A
Cl	H	Cl	3-I-SR141716A
I	H	H	4'-I-SR141716A
I	I	H	4',6-I_2-SR141716A
I	H	I	4',3-I_2-SR141716A
Br	H	H	4'-Br-SR141716A
H	H	H	4'-H-SR141716A

Fig. 1. Structure and numbering system used for SR141716A and its halogenated analogs.

In the current study, we intended to compare the binding of the prototypical agonist, [^{3}H]CP-55,940, to the binding of a potent antagonist, [^{3}H]SR141716A (Seltzman *et al.*, 1995), as a means of further evaluating the cannabinoid receptor population with which they interact in the CNS. The compounds that were chosen for competition studies were selected because of their structural diversity and wide range of potencies. These compounds included the classical cannabinoids Δ^9-THC, CBN, CBD, the nonclassical cannabinoid CP-55,940, the aminoalkylindole WIN55212–2, the endogenous fatty acid ethanolamide anandamide, the antagonist SR141716A and halogenated analogs of SR141716A that were synthesized by a metalation/iodination procedure (Seltzman *et al.*, 1995). The binding analyses with these compounds enabled us to assess more closely for receptor binding heterogeneity and to further characterize the structure-activity relationships of both cannabinoid agonists and antagonists.

Materials and Methods

Chemicals. [^{3}H]CP-55,940 (101 Ci/mmol) was purchased from New England Nuclear (Boston, MA), and unlabeled CP-55,940 was the kind gift of Pfizer, Inc. (Groton, CT). SR141716A, both tritiated (22.4 Ci/mmol) and unlabeled, were synthesized at Research Triangle Institute (Research Triangle Park, NC). Anandamide, and the SR141716A analogs 4′-Br-SR141716A, 4′-I-SR141716A, 4′-H-SR141716A, 4′,6-I_2-SR141716A, 4′,3-I_2-SR141716A, 3-I-SR141716A and 6-I-SR141716A were also synthesized at Research Triangle Institute. Cannabidiol, cannabinol and Δ^9-THC were provided by the National Institute of Drug Abuse (NIDA), and WIN55212–2 was purchased from Research Biochemicals International (Natick, MA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). All drug dilutions for the assays were prepared in buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 3 mM $MgCl_2$, and 0.5% (w/v) BSA (buffer A).

Preparation of brain tissue. Male F344 rats (Charles River Laboratories, Raleigh, NC) weighing 200 to 225 g were sacrificed. The whole brains were quickly removed and placed into a 55 mL Potter-Elvehjem glass homogenizer tube maintained on ice. The tissue was subjected to homogenization and centrifugation procedure described previously (Devane *et al.*, 1988) to yield the final membrane preparation used in the binding assay. Total protein concentration of the resuspended membrane pellet was determined by a dye-binding assay commercially available from Bio-Rad Laboratories (Hercules, CA). Aliquots of the membrane preparation were stored at −70°C until use.

Saturation assays with [^{3}H]CP-55,940 and [^{3}H]SR141716A. Isothermal saturation binding assays were conducted with [^{3}H]CP-55,940 and [^{3}H]SR141716A by the following procedure. Dilutions of the tritiated compounds were prepared to yield final concentrations ranging from 5 pM to 10 nM. The unlabeled drugs for the determination of nonspecific binding were prepared to give a final concentration of 10 μM. To duplicate silated glass test tubes, a 100-μl aliquot each of the appropriate tritiated dilution was added, along with 100 μl of unlabeled drug (nonspecific binding) or 100 μl buffer A (total binding), and sufficient buffer A such that a total volume of 1 ml was achieved with the addition of brain extract. A 100-μl aliquot of each tritiated drug dilution was also removed for determination of total radioactivity (concentration). An aliquot of brain extract equivalent to 150 μg of protein was added to each tube to begin the reaction. After mixing by vortex, the reaction tubes were incubated at 30°C for 1 hr.

A 24-manifold Brandel Cell Harvester was prepared by priming approximately 1 l of cold 50 mM Tris-HCl, pH 7.4, containing 0.1% (w/v) BSA (buffer B) through the harvester. Filter paper (Whatman

GF/C) pretreated for 1 hr in 0.1% polyethylenimine was placed into the cell harvester. At the end of the incubation period, the reaction was terminated by vacuum filtration of the reaction mixture. The reaction tubes were then rinsed twice with approximately 4 ml of buffer B. After rinsing, the filter paper was removed and placed into liquid scintillation vials. To each vial was added 1 ml of H_2O and 10 ml of scintillation cocktail. The samples were placed on a shaker for 60 min and then counted in a liquid scintillation counter for a statistically appropriate amount of time.

The amounts (nanomolar) of free, total bound and nonspecific bound drug were calculated from the counted radioactivity and plotted. For each concentration, the nonspecific bound was subtracted from the total bound to yield the specific bound amount. Saturation isotherms were generated by plotting the total, specific and nonspecific amounts bound as a function of the amount of free drug added. Scatchard analysis of the data was performed with EBDA Ligand software (Release 2.0, Biosoft). The K_d and B_{max} values were obtained and averaged ($n \geq 3$) and are provided with the standard error of the mean (S.E.M).

Competition assays. Six cannabinoid agonists, Δ^9-THC, CBN, CBD, anandamide, WIN55212–2, CP-55,940, the antagonist, SR141716A, and its halogenated analogs, 4′-I-SR141716A, 4′-Br-SR141716A, 4′-H-SR141716A, 3-I-SR141716A, 4′,3-I_2-SR141716A, 4′,6-I_2-SR141716A and 6-I-SR141716A, were evaluated for their ability to compete for the binding of [^{3}H]CP-55,940 or [^{3}H]SR141716A. Competing compounds were prepared in buffer A. In some instances, concentrations used for displacement were modified to better fit apparent inflection points in the displacement curves. Tritiated compounds were diluted in buffer A to yield a concentration of 7.2 nM for [^{3}H]CP-55,940 and 20 nM for [^{3}H]SR141716A, so that addition to the incubation mixture yielded a final concentration for assay of 0.72 nM and 2.0 nM, respectively. Unlabeled drug for determination of nonspecific binding in competition assays (unlabeled CP-55,940 in assays with [^{3}H]CP-55,940 and unlabeled SR141716A in assays with [^{3}H]SR141716A) was at a final concentration of 10 μM.

The competition assays were conducted in a total volume of 1 ml in silated glass test tubes. The reaction mixtures (in duplicate) consisted of 100 μl tritiated drug, 100 μl unlabeled drug dilution and sufficient buffer A such that a total volume of 1 ml was achieved with the addition of brain extract. Duplicate tubes for nonspecific binding and total binding were prepared by adding 100-μl aliquots of the unlabeled compound to be displaced and of buffer A, respectively. An aliquot of brain extract equivalent to 45 μg of protein was added to each tube. The final volume of the reaction mixture was brought to a total of 1 ml by the addition of buffer A. In the displacement curves conducted with anandamide, the incubation mixture also included 30 μM PMSF. After mixing by vortex, the reaction tubes were incubated at 30°C for 1 hr. After the incubation period was complete, the reaction tubes were processed and counted as described above.

The amount (nanomolar) of radiolabel specifically bound in the absence of competing compounds was calculated by subtracting nonspecific binding from total binding. The percentage of this specific binding was then calculated for the amount of radiolabel bound in the presence of various concentrations of each competing compound. The data were then analyzed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA) which fit the displacement data and calculated the K_i for the competing compounds. As for the K_d values, K_i values are presented as means ± S.E.M. ($n \geq 3$). Two-tailed t tests were performed to statistically compare the K_i values obtained between the two radioligands for all compounds.

Molecular models, energy minimization and structural comparisons. All molecular modeling was carried out on a Silicon Graphics Indigo ^{2}XZ or a Silicon Graphics Iris 4D/310 VGX workstation with SYBYL molecular modeling software (v 6.03, Tripos, Inc., St. Louis, MO). An initial structure of SR141716A was generated in SYBYL and energy minimized by use of the SYBYL force field and electrostatic charges based on the method of Gasteiger-Huckel (Gasteiger and Marsili, 1978). A similar process was used to generate molecular models for all other analogs of SR141716A as well as for Δ^9-THC. Energy minimization was allowed to proceed until the difference in energy between successive iterations was <0.01 kcal/mol. These energy-minimized conformations were imported into SPARTAN (WaveFunction, Inc., CA) where semiempirical calculations were performed to further compare the electrostatic properties of SR141716A and Δ^9-THC.

Results

Saturation assays with [^{3}H]CP-55,940 and [^{3}H]SR141716A. Scatchard transformation of the isothermal curves for [^{3}H]CP-55,940 and [^{3}H]SR141716A (figs. 2 and 3, respectively) produced data consistent with a single population of saturable binding sites. Both radioligands were found to possess a reasonably high degree of specific binding (≥ 80% at most concentrations). An average K_d value of 0.72 ± 0.02 nM (n = 3) was obtained for CP-55,940, and 1.20 ± 0.02 nM (n = 3) for SR141716A. B_{max} values of 40.1 ± 2.5 nM and 35.3 ± 2.0 nM were obtained with [^{3}H]CP-55,940 and [^{3}H]SR141716A, respectively. Finally, the Hill coefficients obtained for both compounds were close to unity (0.94 ± 0.04 for [^{3}H]CP-55,940 and 1.03 ± 0.04 for [^{3}H]SR141716A).

Competition studies with [^{3}H]CP-55,940. The results of the displacement assays are provided in table 1 and in figure 4. When competing for [^{3}H]CP-55,940, CP-55,940 had the highest affinity (K_i of 0.54 nM, as compared with its K_d of 0.72 nM determined in the Scatchard analysis). 4′-I-SR141716A, WIN55,212–2 and 4′-Br-SR141716A had similar apparent affinities, with K_i values of 2.42 nM, 2.48 nM and 2.96 nM, respectively. SR141716A did not compete as effectively for [^{3}H]CP-55,940 binding (K_i of 6.18 nM as compared with its K_d of 1.20 nM as determined by Scatchard analysis). Anandamide and Δ^9-THC were of intermediate affinity, with K_i values of 29.7 nM and 37.0 nM, respectively. When anandamide was assayed without the inclusion of PMSF to inhibit amidase activity, the K_i increased to 6984 ± 378 nM, which indicates the susceptibility of this compound to enzymatic hydrolysis in membrane preparations. The remaining 4′-analogs of SR141716A were of still lower apparent affinity, as were analogs of SR-141716A with iodinations at the 6 or 3 position, with only cannabidiol (with a K_i greater than 2000 nM) having a lower apparent affinity. Some of the

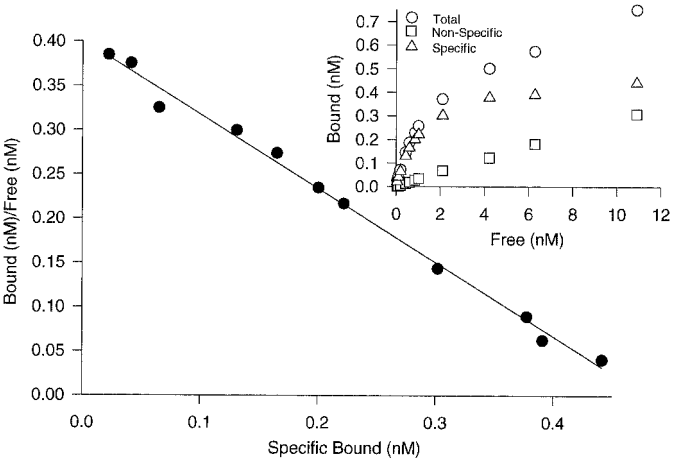


Fig. 2. Scatchard analysis and binding isotherm (inset) for CP-55940.

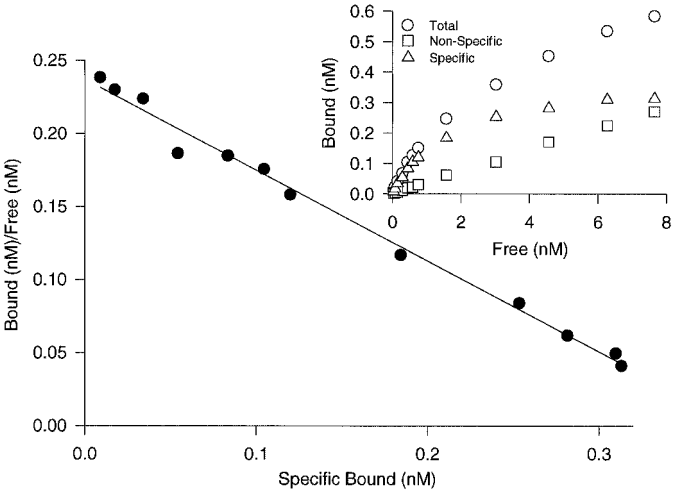


Fig. 3. Scatchard analysis and binding isotherm (inset) for SR141716A.

displacement curves shown in figure 4 for [^{3}H]CP-55,940 do not start at 100%. However, because the K_i values obtained with this experimental protocol were reproducible (as evidenced by the reasonably low standard error of the means for all compounds, as well as the similar K_i and K_d values obtained with CP-55,940), it seems that in these instances only the definition of 100% specific binding, and not the determination of the K_i value, was problematic.

Competition studies with [^{3}H]SR141716A. Despite the fact that SR141716A was of intermediate potency in competition studies with [^{3}H]CP-55,940, this analog, with a K_i of about 1.0 nM, had the greatest apparent affinity when tested in competition studies with [^{3}H]SR141716A. The apparent affinity of SR141716A was more than 17-fold higher than CP-55,940 (20.7 nM) and WIN55,212–2 (K_i = 21.8 nM). The 4′-I-SR141716A and 4′-Br-SR141716A analogs, which were some of the most potent displacers of [^{3}H]CP-55,940, were again characterized as high-affinity ligands with [^{3}H]SR141716A (K_i of 1.27 nM and 2.04 nM, respectively). 4′,6-I-SR141716A and 4′-H-SR141716A were found to possess apparent affinities of 62.6 nM and 71.5 nM, respectively, when competing against [^{3}H]SR141716A, and (as opposed to the data determined with [^{3}H]CP-55,940) are of higher apparent affinity than anandamide (103 nM) and Δ^9-THC (119 nM). Cannabidiol was again the least potent compound of the series tested with a K_i greater than 1000 nM. As seen with the displacement curves obtained with [^{3}H]CP-55,940, some of the displacement curves obtained with [^{3}H]SR141716A did not start at 100%. Although the reason for this was not apparent, the relatively low standard error of the means for all compounds, and the similar K_i and K_d values obtained with [^{3}H]SR141716A, indicates that despite this, the experimental procedures were accurate and reproducible in their determination of K_i values.

By comparing the displacement data, differences were noted in the ability of the various compounds to compete for the receptor site when labeled with [^{3}H]SR141716A as compared with when the site was labeled with [^{3}H]CP-55,940. For example, the affinity of CP-55,940 differed by more than 38-fold depending on what radioligand was used to determine its K_i. Similarly, WIN55212–2 had a greater ability to compete for the receptor when labeled with [^{3}H]CP-55,940. In contrast, SR141716A was more than 5-fold more effective when competing with [^{3}H]SR141716A than when competing with [^{3}H]CP-55,940. The ratio of each compound's affinity for the receptor binding sites when labeled with [^{3}H]CP-55,940 as opposed to the site when labeled with [^{3}H]SR141716A is provided in table 2. The marked differences in K_i values of these compounds suggests that their mode of binding, or the population of binding sites that are being occupied by these compounds and the radioligands, are significantly different. Also, the apparent selectivity of the SR141716A analogs for these sites varies from more "SR-selective" (K_i ratio of 6.18 with SR141716A) to less "SR-selective" (K_i ratio of 1.13 with 4′-H-SR141716A).

Discussion

The results of the Scatchard analyses of [^{3}H]CP-55,940 and [^{3}H]SR141716A agreed with previous studies (Devane *et al.*, 1988; Rinaldi-Carmona, 1994; Showalter *et al.*, 1996). In this study, K_d values of 0.72 ± 0.02 nM and 1.20 ± 0.02 nM were obtained with [^{3}H]CP-55,940 and [^{3}H]SR141716A, respectively, which are similar to the values of 0.13 nM (Devane *et al.*, 1988) and 0.61 ± 0.06 nM (Rinaldi-Carmona *et al.*, 1995) reported for other synaptosomal preparations. In addition, the B_{max} values obtained with [^{3}H]CP-55,940 and [^{3}H]SR141716A reported herein were similar to the values obtained by Devane *et al.* (1988) and Rinaldi-Carmona *et al.* (1995), respectively. Finally, each radioligand appeared to bind with high affinity to a homogeneous population (Hill coefficients of 1.03 ± 0.04 and 0.94 ± 0.04 for SR141716A and CP-55,940, respectively) of saturable binding sites, as also reported previously. No evidence of two states of affinity could be detected, which indicates that the concentration of GTP in the membrane preparation was not sufficient to reveal GTP-dependent shifts in affinity. However, the fact that in our studies we obtain linear Scatchard plots with both radioligands does not exclude the possibility that a particular compound is binding to more than one site with approximately equal affinities. Further experimentation, such as competition assays with a wide variety of compounds, is necessary to fully evaluate a system for the presence or absence of receptor heterogeneity.

Competition studies with both [^{3}H]CP-55,940 and [^{3}H]SR141716A suggested that each radioligand could be displaced by a variety of cannabinoid analogs and defined rank order potencies of competing substances for each radioligand. It was found that the rank order potencies obtained for cannabinoid agonists in displacing [^{3}H]CP-55,940 were in accordance with their *in vivo* activity, which has been demonstrated previously with numerous cannabinoid compounds (Herkenham *et al.*, 1990; Howlett *et al.*, 1988; Compton *et al.*, 1993). Although there are relatively few compounds that can be compared, these rank order affinities are also in reasonable agreement with values obtained in CB1-transfected cell lines with [^{3}H]CP-55,940 (Showalter *et al.*, 1996; Felder *et al.*, 1995). However, the rank order of K_i values determined for these compounds when competing for sites labeled with [^{3}H]SR141716A was found to be significantly different. The compounds with the greatest disparity between the K_i values obtained with [^{3}H]CP-55940 and [^{3}H]SR141716A assays were found to be CP-55,940 (more than 38-fold selective for displacing [^{3}H]CP-55940), WIN55212–2 (more than 8-fold

TABLE 1
Displacement of [^{3}H]CP55,940 and [^{3}H]SR141716A

Compound	[^{3}H]CP55,940			Compound	[^{3}H]SR141716A		
	K_i	S.E.M.	n		K_i	S.E.M.	n
	nM				*nM*		
CP-55,940	0.54	0.03	9	SR141716A	1.18	0.10	9
4′-I-SR141716A	2.42	0.07	3	4′-I-SR141716A	1.27	0.09	3
WIN55212-2	2.48	0.29	3	4′-Br-SR141716A	2.04	0.51	6
4′-Br-SR141716A	2.96	1.12	6	CP-55,940	20.7	4.83	6
SR141716A	6.18	1.20	5	WIN55212-2	21.8	6.06	4
Anandamide	29.7	7.49	4	4′,6-I_2-SR141716A	62.6	9.79	4
Δ^9-THC	37.0	2.98	5	4′-H-SR141716A	71.5	10.0	3
4′-H-SR141716A	80.9	16.4	4	Anandamide	103	10.8	3
4′,6-I_2-SR141716A	126	19.4	5	6-I-SR141716A	108	12.0	3
6-I-SR141716A	166	28.2	4	4′,3-I_2-SR141716A	113	56.6	4
4′,3-I_2-SR141716A	198	36.0	4	Δ^9-THC	119	18.9	3
Cannabinol	247	18.1	4	3-I-SR141716A	197	32.9	3
3-I-SR141716A	282	32.9	3	Cannabinol	743	68.1	3
Cannabidiol	2283	453	4	Cannabidiol	1258	147	3

selective for displacing [^{3}H]CP-55940) and SR141716A (approximately 5-fold selective for displacing [^{3}H]SR141716A).

Felder *et al.* (1995) demonstrated that WIN55212–2 binds with approximately 20-fold higher affinity to the CB2 receptor than it does to the CB1 receptor expressed in transfected cell lines. Their research also indicated that SR141716A was approximately 82-fold selective for the CB1 receptor. The CB2 selectivity of WIN55212–2 has also been reported by Showalter *et al.* (1996). However, in these studies the selectivity of WIN55212–2 for the CB2 receptor was determined to be approximately 6-fold. In both studies, CP-55,940 was found to be relatively nonselective, binding to both CB1 and CB2 with similar affinity. Together, these findings could be interpreted to suggest that the radioligands used in our brain homogenate assay may be binding to different receptor populations in the brain which possess different selectivities. Indeed, there is evidence for the existence of neuronal CB2 receptor subtypes in the findings of Skaper *et al.* (1996), wherein they reported that cerebellar granule cells expressed both CB1 and CB2 mRNA and provided data that suggested that two cannabinoid binding sites could be detected in cerebellar membranes. In addition, preliminary studies with domain-specific antibodies and dot-immunoblot reductional analysis suggest the presence of a CB2-specific domain in rat brain (Cabral GA and Pettit DA, personal communication).

The change in the rank order potencies of the competing compounds, therefore, could reflect an effect caused by varying proportions of receptor subtype populations being occupied at a given concentration of radioligand in combination with the selectivity of the individual unlabeled compounds for each receptor subtype. When a compound such as WIN55212–2, which has higher affinity for the CB2 receptor than the CB1 receptor, is used to displace each radioligand, a marked difference in affinities is observed because of the proportion of receptor subtypes bound with [^{3}H]CP-55,940 as opposed to the relatively selective binding of [^{3}H]SR141716A. Because [^{3}H]CP-55,940 binds with similar affinity to CB1 and CB2, WIN55212–2 would be able to more readily displace this binding compared with the CB1 selective [^{3}H]SR141716A, which is what was observed here. (The K_i values of WIN55212–2 for displacing [^{3}H]CP-55940 and [^{3}H]SR141716A was 2.48 nM and 21.8 nM, respectively.) When SR141716A is used as the displacing compound, it would be anticipated to compete more readily with [^{3}H]SR141716A (which is selectively labeling CB1) than it would against [^{3}H]CP-55,940, which would be in equilibrium with both CB1 and CB2 receptor populations.

Because CB2 transcripts have been detected only after polymerase chain reaction (Skaper *et al.*, 1996), the existence of neuronal receptor subtypes is still equivocal, and there are alternative explanations for these differences in binding characteristics that cannot be disregarded at this time. For example, CB2 receptors have been reported to be present on rat microglial cells (Kearn and Hilliard, 1997). These cells may be contributing to the appearance of neuronal receptor subtypes in our rat brain preparation. Given the relatively high density of CB1 receptors in rat brain, the extent to which this cellular population would affect our results would be anticipated to be relatively low, yet remains to be determined. Furthermore, WIN55212–2 previously has been reported to more readily displace [^{3}H]WIN55212–2 than [^{3}H]SR141716A (Petitet *et al.*, 1996), findings which were interpreted as indicating that the two radioligands are not identical with respect to their recognition pocket in the CB1 receptor or that their binding mechanism is different. Finally, because antagonists (or inverse agonists) and agonists recognize different forms of a particular receptor, the differences in affinities also could be interpreted as indicating that different affinity states of the same receptor are being occupied by the different radioligands and the displacers. However, the observation that the K_i ratios (table 2) among SR141716A analogs varied quite dramatically with relatively subtle structural changes suggests that differences in the recognition pocket between [^{3}H]SR141716A and its analogs and [^{3}H]CP-55,940 would not be sufficient to explain the differences in binding affinity. Furthermore, CBD possesses a classical cannabinoid structure, yet produced a K_i ratio greater than 1, which again suggests that differences in recognition sites or binding mechanisms may not be sufficient to explain the observed differences in K_i.

With regard to the structure-activity relationships investigated within the SR141716A analogs, it was determined that the 4′-Br-SR141716A analog had the highest affinity for the binding sites, as determined by its rank order potency in the displacement studies with [^{3}H]CP-55,940. After this compound, 4′-I-SR141716A was also of high affinity, followed by the chloro-analog (*i.e.*, SR141716A), and finally, 4′-H-SR141716A. The analogs with C3 and C6 substituents were

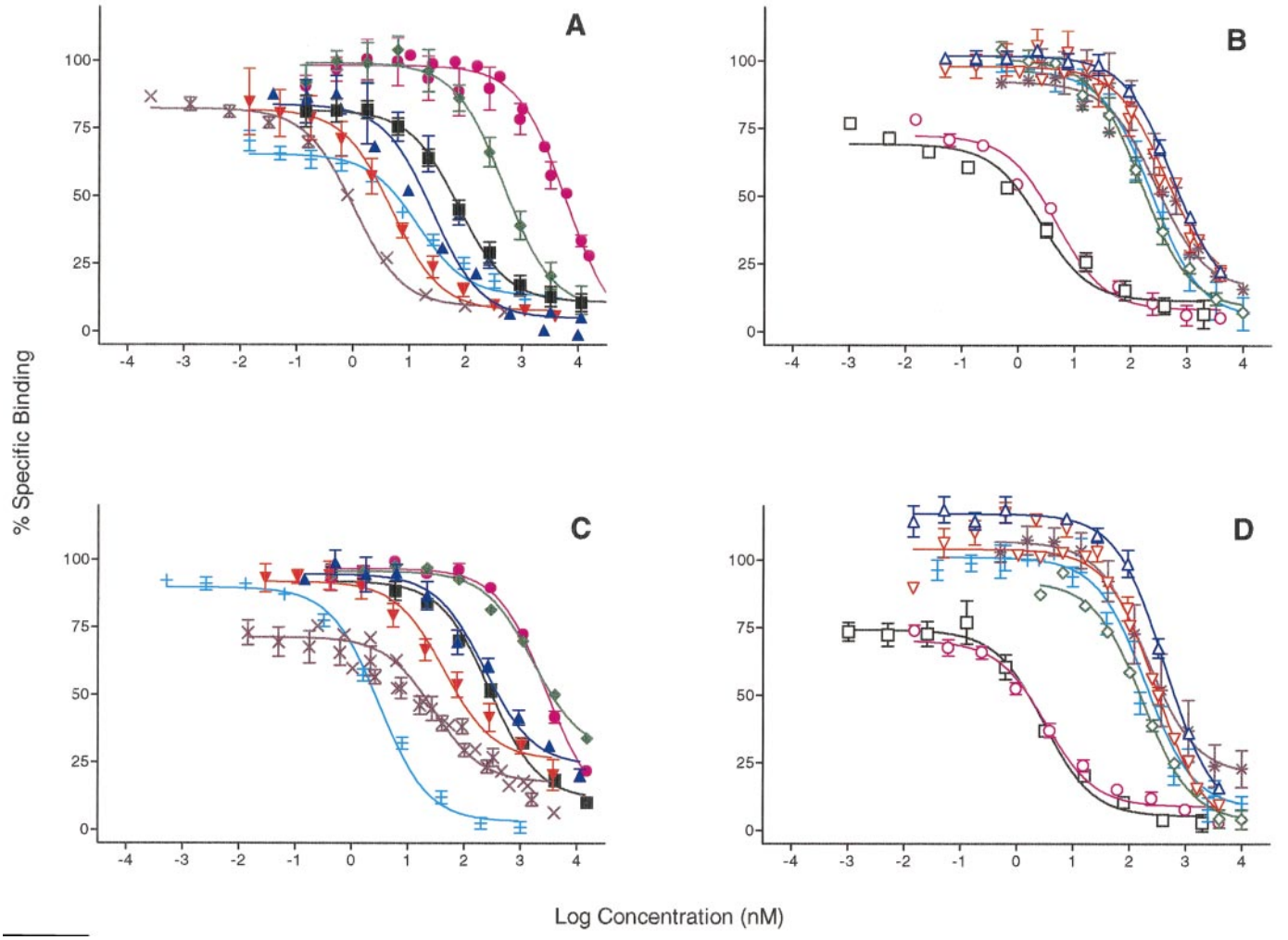


Fig. 4. Displacement curves for cannabinoid analogs. The mean values ($n \geq 3$) are presented in each plot with y-error bars indicating the variability (S.E.M.) of the assay. Panel A provides displacement curves obtained with [^{3}H]CP-55,940 for ▲, anandamide; υ, cannabinol; λ, cannabidiol; ν, Δ^9-THC; ▼, WIN55212–2; +, SR141716A; ×, CP-55,940. Panel B provides displacement curves obtained with [^{3}H]CP-55,940 for the SR141716A analogs o, 4′-Br-SR141716A; ◇, 4′-H-SR141716A; μ, 4′-I-SR141716A; *, 4′,3-I-SR141716A; +, 4′,6-I-SR141716A; △, 3-I-SR141716A; ▽, 6-I-SR141716A. In panels C and D, displacement curves obtained with [^{3}H]SR141716A are provided with the symbols and the color-coding of the symbols as provided for panels A and B, respectively. For some compounds, the concentrations used for displacement were modified to better fit apparent inflection points in the displacement curves, resulting in two sets of experimental data points for a specific compound.

of the lowest affinity. The structure-activity relationships determined with further ring iodination indicate an additivity of effects. That is, substitution with an iodine at either C6 or C3 (replacement of a proton by iodine) results in a marked decrease in activity, which is somewhat offset by the presence of the 4′-halogen, as indicated by the intermediate potency of the bisubstituted analogs. It also appears that the presence of increased steric bulk and/or decreased electronegativity caused by halogen substitution or addition causes a marked change in the selectivity of this compound for the binding sites labeled with either [^{3}H]CP-55,940 or [^{3}H]SR141716A. Thus, these data indicate that modification of SR141716A can significantly alter the apparent selectivity of the antagonist. Because the structure-activity relationships with these SR analogs are relatively limited in scope, further experimentation is needed to continue to define the structural requirements for binding affinity and selectivity.

It is also of interest to discern whether the structure-activity relationships determined for SR141716A can be placed in the context of the pre-existing structure-activity relationships for cannabinoid agonists. Efforts such as these may facilitate the elucidation and characterization of the unique and/or common binding domains on the cannabinoid

TABLE 2

K_i ratio for receptor when labeled with either [^{3}H]CP-55,940 or [^{3}H]SR141716A

Compound	K_i ratio (CP/SR)
CP-55,940	0.03[a]
WIN55212-2	0.11[a]
Anandamide	0.29[a]
Δ^9-THC	0.31[a]
Cannabinol	0.33[a]
4′-H-SR141716A	1.1
4′-Br-SR141716A	1.2[a]
3-I-SR141716A	1.4[a]
6-I-SR141716A	1.5
4′,3-I_2-SR141716A	1.8[a]
Cannabidiol	1.8[a]
4′-I-SR141716A	1.9[a]
4′,6-I_2-SR141716A	2.0
SR141716A	5.2[a]

[a] Difference in K_i values found to be statistically different ($P < .05$).

receptor for these two classes of compounds and enable us to predict more accurately the effect of structural modification on behavioral potency and binding affinity. The structure-activity relationships of cannabinoid agonists have been investigated extensively and reviewed (Razdan *et al.*, 1986; Melvin *et al.*, 1993), and quantitative structure-activity analyses have resulted in the development of pharmacophores and three-dimensional models which can accommodate (*i.e.*, fit, or predict the activity of) a large variety of cannabimimetic compounds (Howlett *et al.*, 1988; Melvin *et al.*, 1993; Reggio, 1993, 1987; Thomas *et al.*, 1991, 1996). The relatively recent discovery of SR141716A presents the opportunity to further examine the cannabinoid pharmacophore in its relation to the binding domain of cannabinoid antagonists. The structure-activity relationships described here for the SR141716A analogs are consistent with a pharmacophoric alignment of SR141716A analogs as shown in figure 5, wherein the 4′ position of SR141716A is overlaid with the pentyl side-chain of Δ^9-THC. Specifically, the relevance of the pharmacophore alignment is supported by previously reported cannabinoid pharmacophore models (Thomas *et al.*, 1991; Howlett *et al.*, 1988) which would correctly predict increased cannabinoid binding affinity with extension at the 4′ position (*i.e.*, substitution of larger atoms H < Cl < Br < I). Furthermore, this superposition is achieved with low-energy conformations of both molecules and allows a relatively high degree of molecular volume overlap. Finally, this superposition results in the similar positioning in space of the lone pair electrons associated with the carbonyl oxygen in SR141716A and the pyran oxygen in Δ^9-THC, as well as by the superpositioning of the lone pair electrons of the pyrazole pyridine nitrogen in SR141716A and the phenolic hydroxyl in Δ^9-THC.

Although the relevance of this superposition is strengthened by the atom alignment and structure-activity relationships, it is important to note that studies with receptor mutations and chimeric cannabinoid receptors (CB1/CB2 constructs) demonstrated that the e2 domain (second extracellular loop region) can affect CP-55,940 binding without modifying SR141716A binding (Shire *et al.*, 1996). This observation, and the results of their chimeric receptor studies, contributed to the authors' conclusion that because SR141716A is structurally dissimilar to CP-55,940, the antagonist probably binds to quite different amino acids. However, it is also possible that perturbations of the three-dimensional structure of the cannabinoid receptors (CB1 and CB2) caused by the amino acid alterations within these receptor constructs could result in the selective elimination of radioligand binding, despite the fact that the substituted amino acids are not involved directly in interaction with the ligands. Indeed, as Shire *et al.* (1996) pointed out, relatively minor local conformational perturbations are responsible for species selectivity in substance P antagonists. Therefore, the hypothesis that Δ^9-THC and SR141716A interact within overlapping binding sites can not be rejected with the data available at this time. Thus, it remains possible that despite the similarities between the structures of SR141716A and the prototype cannabinoid agonist Δ^9-THC when aligned as shown in figure 5, there are sufficient differences in their molecular volume and electrostatic potential which enable these compounds (including CP-55,940) to interact with unique, yet overlapping binding sites involving some of the same amino acids within the cannabinoid receptor. Indeed, in our pharmacophore alignment, the dichlorinated ring system could be inferred to be the "antagonist-conferring" region of SR141716A, because it is the most unique region when compared to Δ^9-THC as shown in figure 5. Replacement of this aromatic functionality with nonaromatic, alkyl chains has been shown to produce compounds that are no longer antagonists, but appear to be agonists in GTPγS studies (Houston *et al.*, 1997), thereby supporting the idea that this region may confer antagonist activity. Clearly, extensive mutagenesis, continued structure-activity relationship analyses and receptor modeling such as that of Bramblett *et al.* (1995) will be required if we are to identify the specific amino acids with which a particular compound interacts.

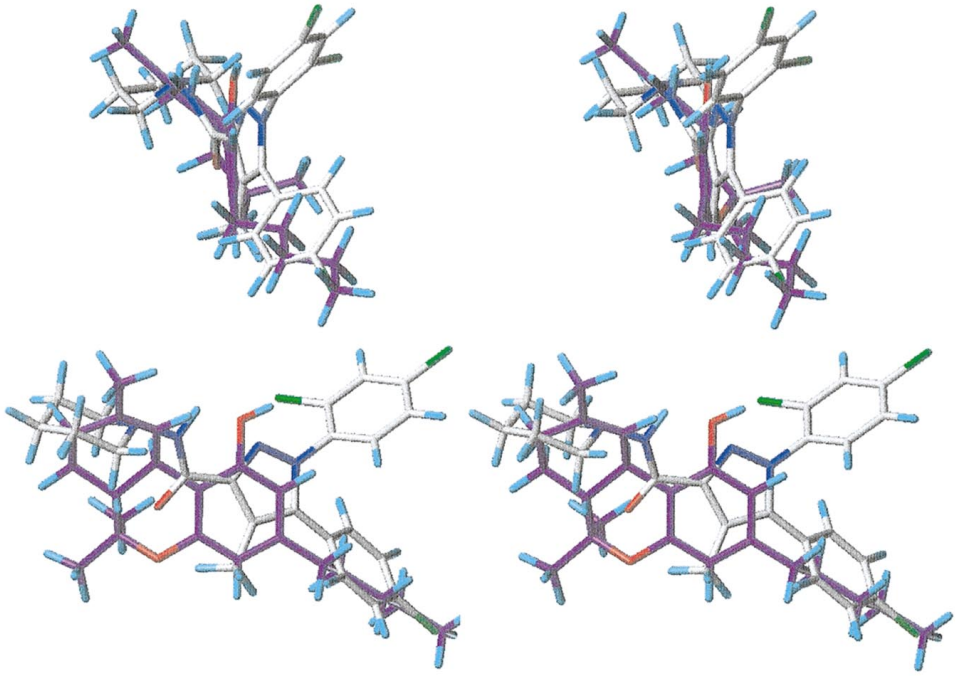


Fig. 5. Stereoviews of the structures of SR141716A and Δ^9-THC overlaid to maximize the overlap in their molecular volumes. The image at the top has been rotated 90° to produce the image at the bottom.

In conclusion, our studies suggest the existence of distinguishable populations of binding sites or thermodynamic interactions with which these various cannabinoid compounds interact. It remains possible that the apparent receptor heterogeneity is caused by the presence of CB2 in the CNS, or a receptor subtype with some sequence homology with the CB2 receptor, or an alternative, yet uncharacterized, receptor protein, because neither the protein nor the mRNA which was reported by Skaper *et al.* (1996) or Cabral and Pettit (personal communication) has been fully characterized (*e.g.,* mRNA sequencing, or definitive resolution of the CB2-specific domain identified with domain-specific antibody). It is also plausible that these differences are revealing differences in receptor binding sites on CB1, or arise from thermodynamic differences in G-protein interactions which might be anticipated between agonists and antagonists. Studies in CB1 and CB2 transfected cell lines using both [^{3}H]CP-55,940 and [^{3}H]SR141716A may assist in determining whether the differences in the binding characteristics of cannabinoids are caused by the presence of CB2 in neuronal preparations. Finally, if receptor heterogeneity is what is being detected in our displacement assays, the proportion of a receptor subtype, and/or the selectivity of the compounds for these receptors, is sufficiently high to be detected, which suggests that this heterogeneity could be pharmacologically significant. Therefore, we plan to evaluate the analogs of SR141716A as both agonists and antagonists in cannabinoid-specific tissue and behavioral assays. Because it appears that structural modification of SR-141716A from a 4′-chlorine to a 4′-iodine results in a compound of increased affinity and altered selectivity of binding, these compounds could be unique in their ability to produce or antagonize particular pharmacological effects.

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Send reprint requests to: Brian F. Thomas, Ph.D., Research Pharmacologist, Research Triangle Institute, P.O. Box 12194, Research Triangle Park, NC 27709.