British Journal of Pharmacology (2001) 132, 525–535

www.nature.com/bjp

Separation of cannabinoid receptor affinity and efficacy in delta-8-tetrahydrocannabinol side-chain analogues

*,[1]Graeme Griffin, [1]Stephanie Williams, [1]Mie Mie Aung, [2]Raj K. Razdan, [1]Billy R. Martin & [1,3]Mary E. Abood

[1]Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, VA 23298, U.S.A. and [2]Organix Inc., 240 Salem Street, Woburn, Massachusetts, MA 01801, U.S.A.

1 The activities of a number of side-chain analogues of delta-8-tetrahydrocannabinol (Δ^8-THC) in rat cerebellar membrane preparations were tested.

2 The affinities of each compound for the CB_1 receptor were compared by their respective abilities to displace [^{3}H]-SR141716A and their efficacies compared by stimulation of [^{35}S]-GTPγS binding.

3 It was found that the affinities varied from 0.19 ± 0.03 nM for 3-norpentyl-3-[6′-cyano,1′,1′-dimethyl]hexyl-Δ^8-THC to 395 ± 66.3 nM for 5′-[N-(4-chlorophenyl)]-1′,1′-dimethyl-carboxamido-Δ^8-THC.

4 The efficacies of these compounds varied greatly, ranging from the very low efficacy exhibited to acetylenic compounds such as 1′-heptyn-Δ^8-THC and 4′-octyn-Δ^8-THC to higher efficacy compounds such as 5′-(4-cyanophenoxy)-1′,1′-dimethyl-Δ^8-THC and 5′-[N-(4-aminosulphonylphenyl)]-1′,1′ dimethyl-carboxamido Δ^8-THC. All agonist activities were antagonized by the CB_1-selective antagonist SR141716A.

5 It was found that a ligand's CB_1 affinity and efficacy are differentially altered by modifications in the side-chain. Decreasing the flexibility of the side-chain reduced efficacy but largely did not alter affinity. Additionally, the positioning of electrostatic moieties, such as cyano groups, within the side-chain also has contrasting effects on these two properties.

6 In summary, this report details the characterization of a number of novel Δ^8-THC analogues in rat cerebellar membranes. It provides the first detailed pharmacological analysis of how the inclusion of electrostatic moieties in the side-chain and also how alteration of the side-chain's flexibility may differentially affect a CB_1 cannabinoid receptor ligand's affinity and efficacy.
British Journal of Pharmacology (2001) **132,** 525–535

Keywords: Cannabinoid receptors; [^{35}S]-GTPγS binding; radioligand binding; G-proteins; rat cerebellum; affinity; efficacy

Abbreviations: CP 55,940, (−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol; Δ^8-THC, delta-8-tetrahydrocannabinol; HU-210, (−)-11-OH-delta-8-tetrahydrocannabinol-dimethylheptyl; [^{35}S]GTPγS, guanosine-5′-O-(3-[^{35}S] thio)-triphosphate; O-581, 5′-cyano-1′,1′-dimethyl delta-8-tetrahydrocannabinol; O-606, 5′-[N-(2-(4-aminosulphonylphenyl)ethyl)]-1′,1′ dimethyl-carboxamido delta-8-tetrahydrocannabinol; O-607, 5′-carboxy-1′,1′ dimethyl-delta-8-tetrahydrocannabinol; O-704, 5′-(4-cyanophenoxy)-1′,1′-dimethyl delta-8-tetrahydrocannabinol; O-737, 3-norpentyl, 3-[6′cyano-1′,1′-dimethyl-6′-phenyl]hexyl delta-8-tetrahydrocannabinol; O-750, 3-norpentyl, 3[6′-cyano-1′,1′-dimethyl-6′-hydroxyl]hexyl delta-8-tetrahydrocannabinol; O-774, 3-norpentyl-3-[6′-cyano, 1′,1′-dimethyl]hexyl delta-8-tetrahydrocannabinol; O-775, 5′-(N,N-diethylcarboxamido)-1′,1′-dimethyl delta-8-tetrahydrocannabinol; O-856, 1′,1′-dimethyl-5′-[N-(piperidin-1-yl)carboxamido]-delta 8-tetrahydrocannabinol; O-964, 1′-heptyn-delta-8-tetrahydrocannabinol; O-1052, 4′-octyn-delta-8-tetrahydrocannabinol; O-1124, 5′[N-(4-aminosulphonylphenyl)methyl]-1′,1′ dimethyl-carboxamido delta-8-tetrahydrocannabinol; O-1125, 3-(1′,1′-dimethyl-6′-dimethylcarboxamido) delta-8-tetrahydrocannabinol; O-1126, 5′-[N-(4-aminosulphonylphenyl)]-1′,1′ dimethyl-carboxamido delta-8-tetrahydrocannabinol; O-1148, 5′-[N-(4-chlorophenyl)]-1′,1′-dimethyl-carboxamido delta-8-tetrahydrocannabinol; O-1149, 5′-[N-(2,4-dichlorphenyl)]-1′,1′-dimethyl carboxamido delta-8-tetrahydrocannabinol; O-1309, 3-[6′-(N-acetyl)-amino hex-2′-enyl]delta-8-tetrahydrocannabinol; O-1311, 3-(6′-hydroxyhex-2′-enyl) delta-8-tetrahydrocannabinol; O-1317, 3-(hept-1′-enyl) delta-8-tetrahydrocannabinol; O-1318, 3-(oct-2′-enyl) delta-8-tetrahydrocannabinol; O-1319, 3-(oct-3′-enyl) delta-8-tetrahydrocannabinol; SR 141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride; THC, Delta-9-tetrahydrocannabinol

Introduction

Delta-9-tetrahydrocannabinol (THC) is the prototypic cannabinoid compound. Initially identified as the major psychoactive constituent of marijuana (Gaoni & Mechoulam, 1964), it possesses a high affinity and a low efficacy for the cannabinoid receptor termed CB_1 (Martin *et al*., 1995; Selley *et al*., 1996). This receptor was first identified by Matsuda *et al*. (1990), and its localization throughout the central nervous system and the periphery was subsequently mapped (Herkenham *et al*., 1991). A member of the superfamily of G-protein

*Author for correspondence.
[3]Current address: Forbes Norris ALS Research Center, California Pacific Medical Center, 2351 Clay Street, Suite #416, San Francisco, CA 94115, U.S.A.

coupled receptors, it was recently joined by a second cannabinoid receptor, CB_2, which has, to date, only been shown to exist in the periphery (Munro *et al*., 1993). THC has also been shown to bind to CB_2 with approximately the same affinity as to CB_1 and also to possess low efficacy at this second cannabinoid receptor (Felder *et al*., 1995).

The existence of multiple receptor subtypes has necessitated the development of ligands selective for one or other of these receptors. Furthermore, it is also important to develop ligands of varying efficacies at each of these receptors in order to have a full range of pharmacological tools with which to enhance our understanding of the activity and roles of these receptors. To date, progress has been limited in the attainment of these goals. The most notable selective compounds so far developed are antagonists. SR141716A is an antagonist of the CB_1 receptor and possesses a negligible affinity for CB_2 (Rinaldi-Carmona *et al*., 1994). SR144528 is an antagonist at the CB_2 receptor, with a reported selectivity between 50 and 700 fold (Rinaldi-Carmona *et al*., 1998; Griffin *et al*., 1999b). AM 630 has also been reported to be a CB_2-selective antagonist (Pertwee *et al*., 1995). With respect to selectivity of agonists, fluoromethanandamide has been proposed to possess an approximately 30 fold selectivity for CB_1 and desoxy-HU-210 about a 50 fold selectivity for CB_2 (Showalter *et al*., 1996; Huffman *et al*., 1996). More recently, Hanus *et al*. (1999) presented data on a novel compound HU-308, which possesses >500 fold CB_2 selectivity. Present understanding of the structural influences on cannabinoid receptor efficacy is even less understood than the influences on receptor affinity. The concept of varying efficacies of cannabinoid receptor ligands was not conclusively defined until the studies of Mackie *et al*. (1993) and Shen *et al*. (1996) which described anandamide and CP 55,940 as partial agonists. Subsequently, the widely varying efficacies of traditional cannabinoid receptor agonists has been extensively characterized (Selley *et al*., 1996; Breivogel *et al*., 1998).

An early result in cannabinoid receptor ligand development was the synthesis of (−)-11-hydroxy-dimethylheptyl-delta-8-tetrahydrocannabinol (HU-210), whose principal structural alterations included an 11-OH and a dimethylheptyl (DMH) side-chain in the same structure (Mechoulam *et al*., 1987). The main factor behind the very high affinity and efficacy exhibited by this compound is most likely the DMH side-chain. Indeed this is a structural characteristic also possessed by other high affinity, high efficacy cannabinoid receptor ligands such as CP 55,940 and CP 55,244 (Martin *et al*., 1995). Additionally, the aforementioned compound desoxy-HU-210 also possesses this DMH side-chain and combined with the removal of a hydroxyl group, results in a highly CB_2 selective compound, suggesting that the CB_2 receptor is also sensitive to the composition of the classical cannabinoids side-chain. Other structure-activity relationship studies have further highlighted the influence of the tricyclic cannabinoids side-chain on cannabinoid activity (Busch-Peterson *et al*., 1996; Singer *et al*., 1998). Therefore, it is reasonable to assume that the side-chain of the classical tricyclic structure provides a unique basis from which to base the development of selective cannabinoid receptor ligands with emphasis on receptor affinity and efficacy.

In our previous study, Griffin *et al*., 1999a, we investigated the activities of nine novel analogues of Δ^8-THC, where the structural modifications were based around the side-chain. O-1125 was found to be a very high affinity and high efficacy compound at the CB_1 receptor. We also found that the inclusion of a cis-cis carbon double bond would reduce the efficacy of the molecule and, to an even greater extent, the inclusion of a carbon–carbon-triple bond would do likewise. As was particularly evident with the triple bond-containing compounds, this reduction in efficacy was not matched by a reduction in affinity, with these low efficacy compounds retaining a reasonably high affinity in the nano-molar range.

Molecular modelling analysis of a large number of Δ^8-THC side-chain analogues, including some detailed in this study supports these observations, with emphasis on the flexibility of the side-chain for both affinity and efficacy (Keimowitz *et al*., 2000). This was also observed in whole animal experiments with some of these same compounds (Martin *et al*., 1999). This observation that structural modifications centred around the aliphatic side-chain may discretely alter the two properties of ligand affinity and efficacy prompted further investigation.

The present study expands on the observations of the previous one, with 21 further side-chain analogues of Δ^8-THC synthesized and tested. The major structural alterations of the novel compounds included in this study were the addition of numerous electrostatic moieties within the side-chain (such as cyano- and sulphonylamine-groups) in order to determine their potential role in dictating the properties of affinity and efficacy. Furthermore, analogues were also synthesized with side-chain triple bonds at different positions from those previously tested in order to investigate how the orientation of planar rigidity may also influence these two drug properties.

Methods

Materials

Male Sprague-Dawley rats (200 g) were obtained from Harlan (Dublin, VA, U.S.A.). GDP and GTPγS were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). [^{35}S]-GTPγS (1000–1200 Ci/mmol) and [^{3}H]CP55,940 were purchased from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]-SR141716A (55 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). Other reagent grade chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Δ^8-THC and N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamidehydrochloride (SR141716A) were obtained from the National Institute on Drug Abuse (NIDA). (−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl]cyclohexan-1-ol (CP 55,940) was generously provided by Pfizer Inc., Groton, CT, U.S.A. (−)-11-OH-delta-8-tetrahydrocannabinol-dimethylheptyl (HU-210) was generously provided by Prof Raphael Mechoulam (Hebrew University, Jerusalem, Israel) and (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone (WIN 5512-2) was purchased from Research Biochemicals International (Natick, MA, U.S.A.). All delta-8-tetrahydrocannabinol analogues bearing O-prefixes were synthesized in our laboratory (RKR). All compounds were stored as 1 mg/ml solutions in ethanol at −20°C.

Membrane preparation

Membranes were prepared as previously described (Griffin *et al*., 1998). Briefly, cerebella were dissected on ice from male Sprague-Dawley rats. The tissue was homogenized in centrifugation buffer (in mM): Tris HCl 50, EGTA 1, $MgCl_2$ 3, pH 7.4 and the homogenate centrifuged at $42{,}000 \times g$ for 20 min at 4°C. The resulting pellet was then resuspended in GTPγS assay buffer (in mM): Tris HCl 50, $MgCl_2$ 9, EGTA 0.2, NaCl 150, pH 7.4, re-homogenized, and centrifuged at $42{,}000 \times g$ for 20 min at 4°C. The final pellet was then resuspended in GTPγS assay buffer, homogenized, and diluted to a concentration of approximately 2 μg/μl with assay buffer. Cerebellar membranes to be used for radioligand binding experiments were resuspended in binding buffer A (in mM): Tris-HCl 50, EDTA 1, $MgCl_2$ 3, 1 mg/ml fatty acid free bovine serum albumin (BSA), pH 7.4. Membranes from Chinese Hamster Ovary (CHO) cells transfected with the human CB_2 receptor were prepared as previously described (Griffin *et al*., 1999a). Aliquots were then stored at −80°C.

[^{35}S]–GTPγS binding

The methods for measuring agonist-stimulated [^{35}S]-GTPγS binding were adapted from those of Sim *et al*. (1995). Rat cerebellar membranes (10 μg) were incubated in assay buffer, or in sodium-free assay buffer, containing 0.1% BSA with GDP (100 μM), [^{35}S]-GTPγS (0.05 nM) and either cannabinoids or an ethanol control in siliconized glass tubes. If a compound produced no stimulation of GTPγS binding at 100 μM then the experiment was repeated with 10 μM GDP. This is stated where appropriate in the results. This was done as a reduction in the GDP concentration has been previously shown to increase the stimulation of GTPγS binding produced by lower efficacy agonists (Griffin *et al*., 1998). The total assay volume was 0.5 ml and this was incubated at 30°C for 30 min. The reaction was terminated by addition of 2 ml ice-cold wash buffer (50 mM Tris HCl, 5 mM $MgCl_2$; pH 7.4) followed by rapid filtration under vacuum through Whatman GF/C glass-fibre filters using a 12-well sampling manifold. The tubes were washed once with 2 ml of ice-cold wash buffer, and the filters were washed twice with 4 ml of ice-cold wash buffer. Filters were placed into 7 ml plastic scintillations vials and 5 ml BudgetSolve scintillation fluid added (RPI Corp., Mount Prospect, IL, U.S.A.). After shaking for 1 h, bound radioactivity was determined by liquid scintillation. Non-specific binding was determined using 10 μM GTPγS. Basal binding was assayed in the absence of agonist and in the presence of GDP. The stimulation by agonist was defined as a percentage increase above basal specific binding levels (i.e. [(d.p.m. (agonist) – d.p.m. (no agonist))/d.p.m. (no agonist)] × 100).

Radioligand binding

The methods used for radioligand binding were those described by Griffin *et al*. (1999a). Binding was initiated by the addition of 20 μg membrane protein to siliconized tubes containing 0.35 nM [^{3}H]-SR141716A, the competing ligand and a sufficient volume of buffer A (in mM): Tris HCl 50, $MgCl_2$ 3, EDTA 1, 0.1% BSA, pH 7.4) to bring the total volume to 0.5 ml. The addition of 1 μM SR141716A was used to assess non-specific binding. Radioligand binding using hCB_2 CHO cell membranes were conducted as previously described (Griffin *et al*., 1999a).

Data analysis

Data are reported as means ± standard error of the means of four to 10 experiments, performed in triplicate. Non-linear regression analysis of concentration-response data was performed using Prism 2.0 software for the Macintosh (GraphPad Software, San Diego, CA, U.S.A.) in order to calculate and compare E_{max} and EC_{50} values. The equilibrium dissociation constant (K_B) for the interaction of the antagonist and the receptor has been calculated from the equation $K_B = [B]/(\text{dose ratio} - 1)$, where [B] is the concentration of the antagonist used in the experiment (Schild, 1949). EC_{50} values are presented with 95% confidence limits indicated by parentheses. Displacement IC_{50} values were determined originally by unweighted least-squares non-linear regression of log concentration-percentage of displacement data and then converted to K_i values using the method of Cheng and Prusoff (1973). Student's *t*-test, two-tailed (unpaired) was used for comparison of K_i values ($P < 0.05$).

Results

Methodological rationale

The efficacy of each compound at CB_1 receptors was assessed using the GTPγS binding assay in rat cerebellar membranes. This assay was chosen as it has previously been demonstrated to be able to detect discrete differences in agonist efficacies (Griffin *et al*., 1998; Breivogel *et al*., 1998). Figures 1–4 show the ability of each of these compounds to stimulate GTPγS binding. Unless otherwise specified, 100 μM GDP was used in the assay. It has previously been shown that compounds with lower efficacies may not stimulate GTPγS binding at this GDP concentration, but when 10 μM GDP is used instead, then a significant concentration-related stimulation of GTPγS binding may be seen. One hundred μM GDP was used for the original assessment of efficacy as this has been shown to maximize efficacy differences between ligands (Griffin *et al*., 1998). Displacement of [^{3}H]-SR141716A from rat cerebellar membranes was used to determine CB_1 receptor binding affinity (Table 1) and Figure 1. This tissue was chosen as it is known to contain a large population of CB_1 receptors and thought to be absent of CB_2 receptors (Herkenham *et al*., 1991; Griffin *et al*., 1999b). Specific binding to cerebellar membranes averaged 83% at a [^{3}H]-SR141716A concentration of 0.5 nM and 78% at a [^{3}H]-CP55940 concentration of 1 nM in hCB_2 CHO membranes.

Carboxamide-containing side-chain analogues of delta-8-tetrahydrocannabinol

Our previous study described a carboxamide-containing side-chain Δ8-THC compound, O-1125, which demonstrated both a high affinity and also acted as a full agonist in the GTPγS binding assay (Griffin *et al*., 1999a). Following this observation, several further analogues were also synthesized

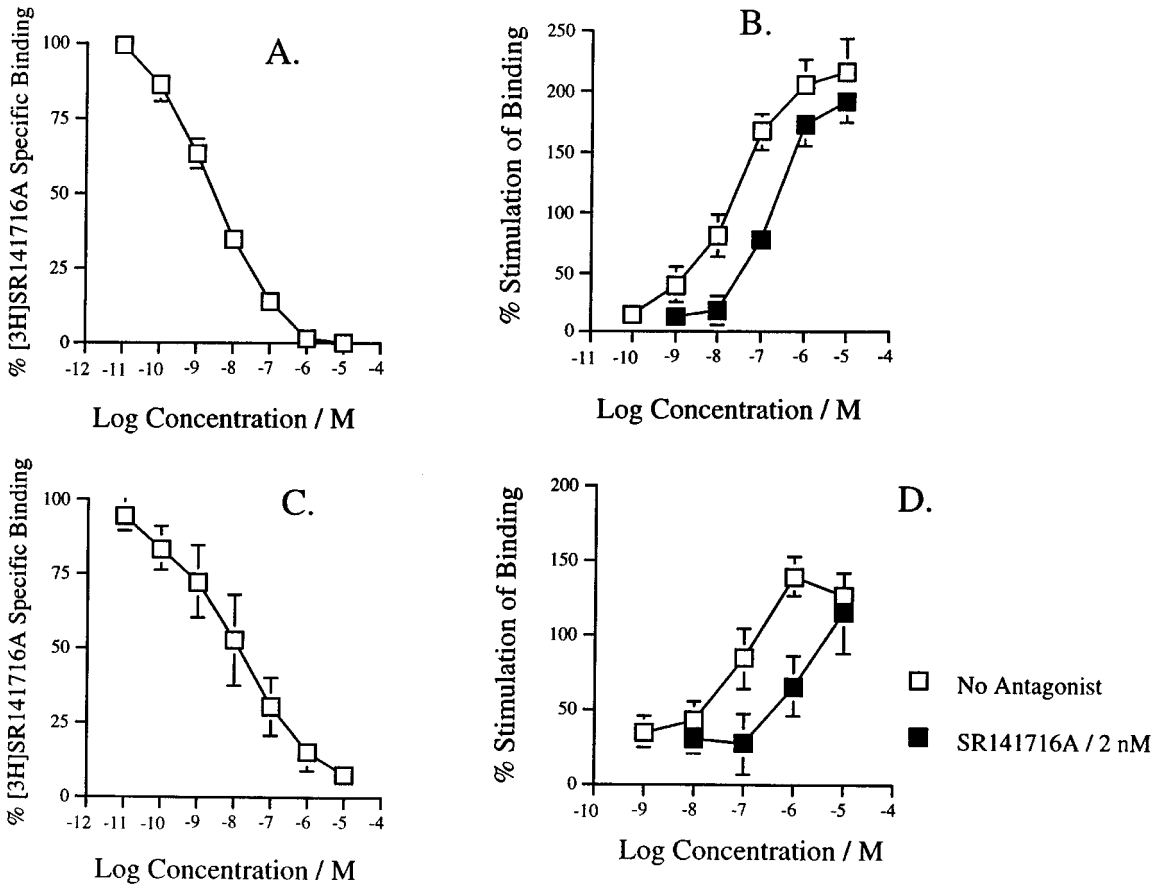


Figure 1 Affinity and efficacy of O-1125 and O-775 in rat cerebellar membrane homogenates. Displacement of bound [^{3}H]-SR141716A by O-1125 (A), O-775 (C). The data are expressed as percentage displacement of specific binding; 0.35 nM [^{3}H]-SR141716A was the concentration of radioligand used. Non-specific binding was measured in the presence of 1 μM SR141716A. Effect of SR141716A, at a concentration of 2 nM on the ability of O-1125 (B) and O-775 (D) to stimulate [^{35}S]-GTPγS binding in rat cerebellar membrane homogenates. Data represent percentage stimulation over basal levels. Data points are the means$\pm$s.e.mean of 4–6 experiments performed in triplicate.

containing this moiety in order to try and better understand the role of this group in the conference of high affinity and high efficacy on O-1125. O-775 differed from O-1125 in the replacement of the two terminal methyl groups with ethyl groups. This lengthening of the side-chain resulted in a reduction in both affinity (10 fold) and efficacy (E_{max} 163$\pm$44% compared to O-1125 (225$\pm$9%)). This tends to suggest that the lengthening of the side-chain seen with O-1125 appears to be optimal (at least with the compounds presented in this study) for maximal efficacy and further lengthening appears to begin to reduce efficacy. O-1148 and O-1149 vary from O-1125 in the replacement of the dimethyl group with a 4-chloro- and 2,4-dichloro-phenyl group respectively. Both of these compounds showed reduced efficacy and affinity compared to O-1125, although the presence of the additional chloride ion in O-1149 increased the affinity compared to O-1148 by 10 fold and also increased the efficacy. It appears that this additional chloride group, and likely its associated negative charge, may play a significant role in the interaction of the ligand with the receptor. O-1126 replaces the 4-chloro ion of O-1148 with a sulphonylamide group. This increases both the affinity (10 fold) and quadruples the E_{max} of the compound. Progressive addition of one and two carbon atoms between the carboxamide group and the phenyl ring causes a concurrent reduction in the affinity and efficacy (O-1124 and O-606). However, O-1124 and O-606 do not have a significantly different affinity or efficacy suggesting that once the optimal chain length is surpassed, further additions, within the small range investigated with these two compounds does not appear to have further detrimental effects on these two properties. The final compound in this series differs from O-1148 by the replacement of the chlorophenyl group with a piperidine group. This effect is 2 fold. Firstly, the affinity for the CB_1 receptor is markedly increased (approximately 100 fold) and secondly, the efficacy is very much higher (E_{max} 157$\pm$38%).

Cyano- and carboxylic acid-containing side-chain analogues of delta-8-tetrahydrocannabinol

The inclusion of moieties such as the strongly electronegative cyano-group and the less electronegative carboxylic acid residue into the side-chain was an attempt to investigate whether or not the inclusion of a charged moiety influenced either the affinity or the efficacy of the resulting compound. To this extent, six compounds were synthesized, five with a cyano-group and one with a carboxylic acid. All of these

substitutions occurred at the terminal carbon of a straight chain with the exception of O-750 and O-737 where a double substitution occurred on the terminal carbon with a cyano and a hydroxyl group (O-737) and a cyano and a phenyl group (O-750). In general, the inclusion of the cyano group was associated with high affinity compounds irrespective of the placement of the cyano, although when it was included in the double substitution, this caused about a 10 fold decrease in the affinity of the compound. The inclusion of the carboxylic acid group (O-607) caused an approximately 150 fold reduction in affinity over the corresponding cyano-compound (O-581). Efficacy, however, seemed to be affected to a greater extent by the placement of the cyano group within the side-chain, and in particular, the length of the side-chain prior to the cyano group. Specifically, the extension of the side-chain by a single carbon atom (O-774 vs O-581) caused a marked reduction in efficacy although the affinities were affected in the reverse direction with the affinity of O-774 actually slightly increased. Contrastingly, the inclusion of a phenyl group in the side-chain prior to the cyano group and thus extending the side-chain by the inclusion of the spacially bulkier phenyl group actually increased the efficacy slightly. This further suggests that it is the placement of the cyano group and its spacial interaction with the receptor that may significantly dictate its effect on efficacy. O-750 and O-737 represent compounds that have a double terminal substitution, with the inclusion of two electronegative components, a cyano and a hydroxyl group (O-750) and the single electronegative cyano/phenyl combination (O-737). Both compounds had almost identical affinities for the receptor but the double negative charge of O-750 caused a dramatic decrease in efficacy compared to the single negative-charge substitution of O-737. The final compound in this series, O-607, which contained the carboxylic acid group maintained a reasonably high efficacy despite the large reduction in affinity compared to the similar compounds O-774 and O-581.

Unsaturated side-chain analogues of delta-8-tetrahydrocannabinol

We have previously described that analogues with side-chains containing either cisplatin carbon double bonds or acetylenic bonds had a decreased efficacy but maintained affinity compared to other similar saturated side-chains. We hypothesized that this may be as a result of decreasing the flexibility of the side-chain and, in particular, its rotational angle in relationship to the body of the molecule (Griffin *et al*., 1999a). To further investigate how a carbon–carbon double bond within the side chain affects the activity of cannabinoid ligands, five compounds were tested. These consisted of four compounds with the double bond situated between carbons 2 and 3 with varying chain lengths and terminal substitutions. The other compound possessed a double bond that connected carbons 3 and 4. None of the compounds had the dimethyl addition that was seen in all of the previous molecules. O-1309 and O-1311 have side-chain lengths of seven carbon atoms with terminal substitutions of a hydroxyl (O-1311) or a methylcarboxamide (O-1309). O-1317 has a chain length of seven carbon atoms with no terminal substituent. Of these three compounds, the unsubstituted O-1317 had the highest affinity but the lowest efficacy. The methylcarboxamide group caused a reduction in affinity and an increase in efficacy, which occurred to an even greater extent with the hydroxyl moeity that possessed the lowest affinity but highest efficacy. O-1318 differs from O-1317 by the extension of the side-chain from seven to eight carbon atoms. The affinity, as a result of this change, is not significantly affected but the efficacy is increased. Similarly, O-1319 also has an eight-carbon side chain except that the double bond was situated between carbons 3 and 4. Neither the affinity nor the efficacy was affected compared to O-1318.

We previously described the activities of five triple-bond containing compounds as high affinity but very low efficacy agonists in the GTPγS assay, unable to stimulate binding under various assay conditions and suggested only to possess some efficacy based on observations from other experimental models. Each of those compounds contained a triple bond situated on carbon 2 of the side-chain.

In order to test whether the induced rigidity of the side-chain and/or the particular spatial orientation of the side-chain caused by the triple bond was responsible for this observation, two further triple bond compounds were tested in this study with the triple bond placed on either carbon 1 (O-964) or carbon 3 (O-1052). Both of these compounds possess a reduced affinity over O-584 (eight carbon chain length, triple bond on C2) and O-823 (seven carbon chain length with cyano terminal substitution, triple bond on C2) (Griffin *et al*., 1999a). However, both showed a low efficacy albeit, increased over both O-584 and O-823.

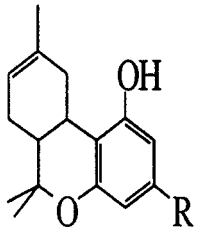
Other observations with delta-8-TCH analogues

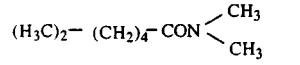
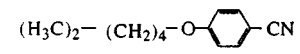
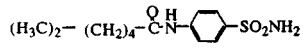
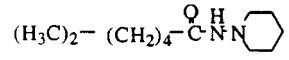
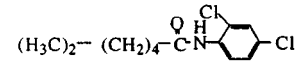
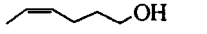
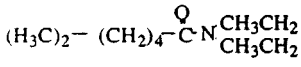
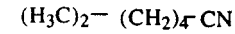
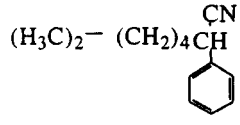
All compounds that produced a significant stimulation of GTPγS binding were tested for sensitivity to antagonism by the CB_1-selective antagonist SR141716A. The two acetylenic compounds O-964 and O-1052 were not tested due to their lower potencies and efficacies and the resulting difficulty in constructing a complete concentration response curve in the presence of antagonist. With each of the other analogues, however, it was found that despite some small individual variations in the calculated K_b of SR141716A, all were antagonized by the antagonist at a K_b (0.07–0.39 nM) that would be expected for an activity *via* the CB_1 receptor (data not shown). This would suggest that all these analogues were likely acting at the same receptor site, CB_1, in these experiments.

Ability of novel delta-8-THC analogues to displace [^{3}H]-CP 55,940 from hCB$_2$-CHO cell membranes

A number of these compounds were also tested for the ability to displace [^{3}H]-CP 55,940 from membranes prepared from CHO cells transfected with human CB_2 receptors in order to determine whether or not these compounds demonstrated any level of CB_1 and/or CB_2 selectivity. Although only the saturated side-chain analogues were tested for their ability to displace [^{3}H]-CP 55,940 at CB_2 receptors, several trends were observed. Every compound tested had a high affinity for the CB_2 receptor in the range 1.14 ± 0.56 nM (5′-(4-cyanophenoxy)-1′,1′-dimethyl delta-8-tetrahydrocannabinol (O-704)) through 37.2 ± 4.41 nM (5′-[N-(2,4-dichlorophenyl)]-1′,1′-dimethyl-carboxamido delta-8-tetrahydrocannabinol (O-1149)).

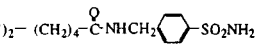
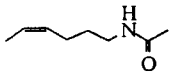
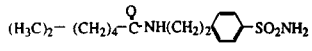
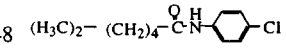
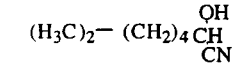
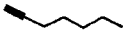
Table 1 Effects of side-chain modifications on receptor affinity, efficacy and potency



Compound	*R*	*Emax*	*EC50 / nM*	*CB1 Affinity / nM*	*CB2 Affinity / nM*
O-1125		225 ± 9.00 %	20.1 (8.86-45.9)	2.47 ± 0.53	1.98 ± 0.26
O-704		167 ± 7.56 %	55.2 (35.9-85.9)	1.50 ± 0.31	1.14 ± 0.54
O-1126		163 ± 27.5 %	84.8 (26.9-266)	41.5 ± 15.9	10.3 ± 0.80
O-856		157 ± 38.5 %	34.8 (10.1-119)	4.51 ± 1.73	3.23 ± 0.29
O-1149		155 ± 30.2 %	322 (123-844)	41.2 ± 11.2	37.2 ± 4.41
O-1311		143 % @10μM	ND	125 ± 42.8	ND
O-1318		138 % @10μM	ND	4.55 ± 1.08	ND
O-1319		135 % @10μM	ND	6.88 ± 1.06	ND
O-775		146 ± 44.3 %	29.9 (3.84-233)	23.9 ± 11.0	2.47 ± 1.85
O-581		139 ± 8.08 %	3.46 (1.58-7.60)	1.75 ± 0.58	1.10 ± 0.57
O-737		135 ± 77.7 %	239 (44.7-1290)	18.4 ± 4.50	5.75 ± 2.22

The majority of the compounds did not demonstrate a marked selectivity for either subtype of cannabinoid receptor, although interestingly, the phenylsulphonylamine compounds (O-1126, O-1124 and O-606) all demonstrated a higher degree

Table 1 continued

Compound	R	Emax	EC50 / nM	CB1 Affinity / nM	CB2 Affinity / nM
O-1124		96.2 ± 12.0 %	83.3 (20.5-339)	180 ± 101	2.27 ± 0.75
O-1309		99.0 ± 17.5 %	336 (56.9-1990)	43.4 ± 6.07	ND
O-606		96.1 ± 18.8 %	171 (43.9-664)	246 ± 70.6	ND
O-607	$(H_3C)_2-(CH_2)_4-COOH$	89.6 ± 8.69 %	89.6 (36.0-223)	222 ± 63.6	4.00 ± 1.35
O-774	$(H_3C)_2-(CH_2)_5-CN$	88.7 ± 8.40 %	1.25 (0.64-2.45)	0.19 ± 0.03	2.94 ± 1.40
O-1317		69.6 ± 9.54 %	3.38 (0.5-24.1)	1.53 ± 0.28	ND
O-1148		42.6 ± 12.1 %	19.9 (3.05-123)	395 ± 66.3	11.3 ± 4.18
O-750		62.3 ± 2.88% (10μMGDP)	6.05 (3.18-11.5)	21.3 ± 4.2	3.22 ± 1.38
O-1052		48.7 % @10μM (10μMGDP)	ND	20.9 ± 1.13	ND
O-964		41.9 %@10μM (10μMGDP)	ND	172 ± 62.1	ND

of selectivity for the CB_2 receptor (4.03, 79.3 and 55.5 fold respectively).

Discussion

The present study focuses upon the activities of a number of analogues of delta-8-tetrahydrocannabinol. Our previous study (Griffin *et al*., 1999a) demonstrated how structural modification of the side-chain, with the inclusion of double and triple bonds into the side-chain as well as the addition of numerous terminal carbon substitutions gives rise to reasonably predictable changes in ligand affinity and efficacy, in particular. The novelty of this study lies not only in the expansion of our previous findings with the additional double-bond and acetylenic cannabinoids but also in the more detailed investigation of the effects of introducing substitutions into the side-chain, specifically those of both physically bulkier and electrostatic groups.

To summarize the effects of the compounds containing ring-structures, it is clear that the addition of a bulky ring group into the side-chain does not itself appear to particularly effect the pharmacological properties of the compound, as these structures all demonstrate a wide range of efficacies and affinities. More likely it would appear that a combination of the length, orientation and presence of electrostatic moieties within the side-chain have a greater effect on the compound's activities. The localization of such moieties within the side-chain appears to be the integral factor in determining the behaviour of the compound. An interesting observation with these compounds is the fact that the three phenylsulphonylamine compounds (O-1124, O-1126

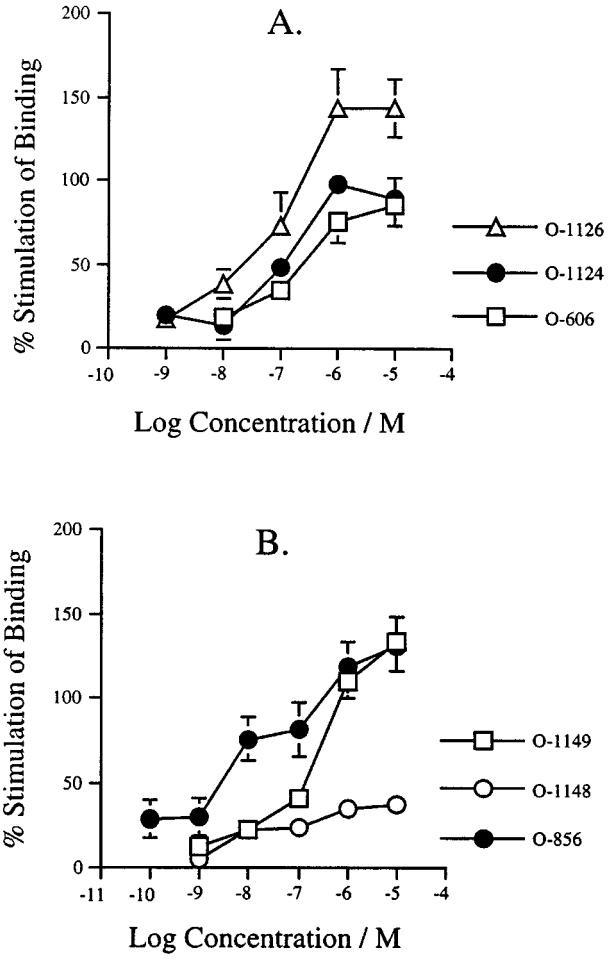


Figure 2 Ability of O-606, O-1124 and O-1126 (A) and O-856, O-1148 and O-1149 (B) to stimulate [^{35}S]-GTPγS binding in rat cerebellar membrane homogenates. Data represent percentage stimulation over basal levels. Results are presented as means $\pm$s.e.mean for 4–7 experiments, performed in triplicate.

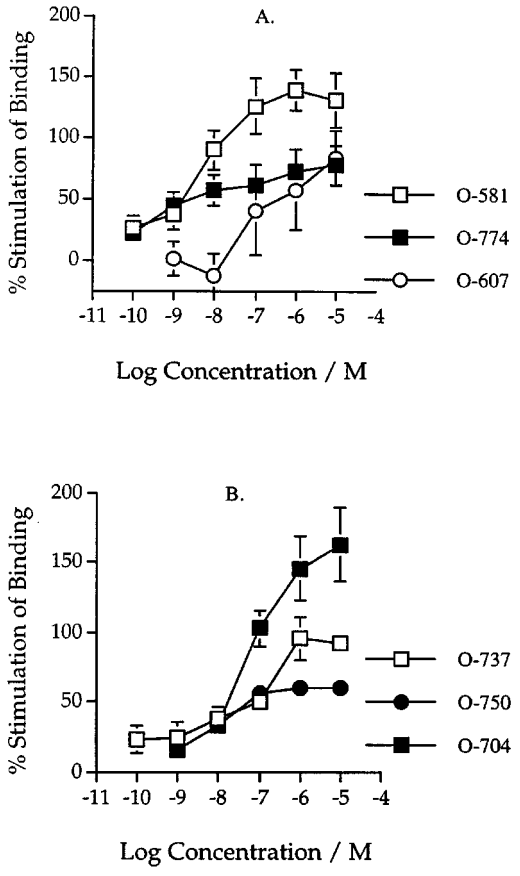


Figure 3 Ability of O-581, O-774 and O-607 (A) and O-704, O-737 and O-750 (B) to stimulate [^{35}S]-GTPγS binding in rat cerebellar membrane homogenates. The concentration-response curve of O-750 was constructed in the presence of 10 μM GDP. Data represent percentage stimulation over basal levels. Results are presented as means $\pm$s.e.mean for 4–7 experiments, performed in triplicate.

and O-606 all possess a high selectivity for the CB_2 receptor, in particular the two longer side-chain compounds) O-1126 and O-606. Although there have been recent developments in the development of CB_2-selective compounds based round the DMH side-chain (desoxy-HU-210) and around the resorcinol structure (Huffman *et al*., 1996; Hanus *et al*., 1999), there is still the requirement for developing an accurate CB_2 pharmacophore. These two compounds may aid in the elucidation of this and the subsequent development of increasingly selective ligands for the CB_2 receptor.

The theory of an optimal side-chain length for the properties of affinity and efficacy is further supported by the activities of O-1125 and O-775. These two compounds share a similar structure as the ring-containing structures as far as the carboxyamide grouping where the ring is replaced by either two methyl groups or two ethyl groups (O-1125 and O-775 respectively). O-1125 was the most efficacious compound with one of the highest affinities of the series and both affinity and efficacy are reduced in O-775. It may be that the carboxamide group interacts with a positively charged amino acid in the binding pocket and that the longer ethyl groups of O-775, as well as the bulkier phenyl groups (and additional electronegative groups of the previous compounds), may hamper this interaction, possibly by spatially 'pushing' the carboxamide grouping away from this site. However, it must be noted that compounds such as HU-210 have the basic DMH side-chain but contain no electrical charges, and this compound retains high affinity and efficacy.

The two compounds O-750 and O-737, which have very similar structures with the difference being that O-750 possesses a terminal hydroxyl and cyano group, whereas O-737 has only the cyano. The differences in activities between these two compounds are very significant. Both share an almost identical affinity for the receptor but the inclusion of the extra electronegative hydroxyl group of O-750 has the effect of markedly lowering efficacy, clearly showing a disparity between the two properties of affinity and efficacy. If there is an amino acid that is closely involved in the binding of these two compounds, it would only appear to be involved in receptor activation rather than receptor binding. Whether the two negative charges on O-750 result in a competition for this site or whether the bulkier phenyl group of O-737 orients the cyano group, into a more favourable position for receptor activation cannot be determined from this data.

In support of the involvement of a key positively charged amino acid being involved for both affinity and efficacy are the observations with the compounds with straight side-

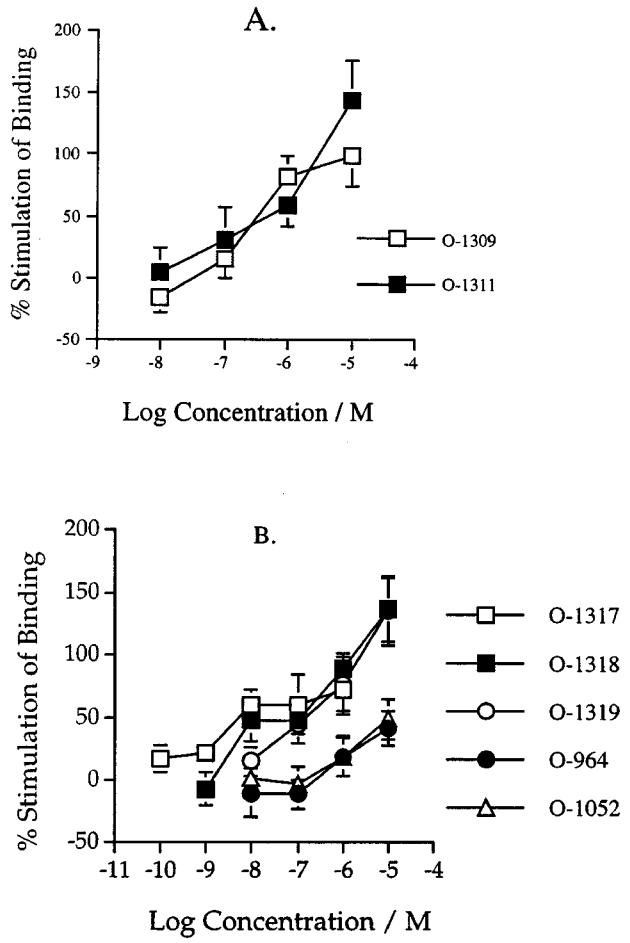


Figure 4 Ability of O-1309 and O-1311 (A) and O-1317, O-1318, O-1319, O-964 and O-1052 (B) to stimulate [^{35}S]-GTPγS binding in rat cerebellar membrane homogenates. The concentration-response curves of O-964 and O-1052 were constructed in the presence of 10 μM GDP. Data represent percentage stimulation over basal levels. Results are presented as means$\pm$s.e.mean for 4–7 experiments, performed in triplicate.

chains, O-581, O-607 and O-774. These structures have virtually identical straight side-chains with the only differences being terminal substitutions which may not be likely to have significant spatial effects on the molecule. The results also suggest that there may be differential effects of moieties on affinity and efficacy. The replacement of the cyano group of O-581 with a carboxylic acid group markedly reduces affinity and efficacy but the elongation of the chain by a single carbon atom and retaining the cyano group (O-774) causes an increase in affinity but decrease in efficacy compared to O-581.

Another aspect of these experiments was to further the previous data obtained when including double and triple bonds in the side-chain as in our previous study. We suggested that the introduction of a cis-double bond or an acetylenic triple bond would likely impart a degree of planar rigidity to the side-chain which, in turn, may introduce certain constraints or changes on the binding of the ligand such that it effectively reduces the ligand's ability to activate the receptor. The data in this study largely support the theory that side-chain flexibility is required for high efficacy but not affecting affinity. The compounds examined in the present study have differing side-chain lengths after the cisplatin bond and different terminal substitutions than those examined in the previous study (Griffin *et al*., 1999a). It was observed that by increasing the length of the tail, the efficacy might be increased, thus largely overcoming these suggested constraints. Additional groups on the terminus appear to reduce affinity but in the case of O-1311, do not affect efficacy. The acetylenic compounds used in this study differed from our previous study in that the triple bond was centred on carbon atoms 1 and 3 of the chain rather than on the second carbon. This would certainly cause a longer flexible trip to the molecule (after carbon 1 compared to being placed on carbon 2) or a shorter tip (triple bond on carbon 3) and may change the angle at which the side-chain lies. Both compounds tested in this study maintained the low efficacy described for the compounds we previously described but this low efficacy is significantly greater than when the triple bond is on carbon 2. The data for both the double- and triple-bond containing compounds further support the hypothesis that the angle and flexibility of the side-chain is important. If the effects of the electrostatic groups of O-1309 and O-1311 are also considered, this further agrees with the possibility of the involvement of charged amino acids and their divergent roles in conferring affinity and efficacy.

One important aspect of this study relates to the three-point model of receptor interaction, which has been used to predict the activity of cannabinoid compounds in the past. The side-chain has often been suggested to have the role of a hydrophobic interaction with the receptor, but these data demonstrate that this interaction may actually be more complicated than this. The most significant observation of this study is the fact that the inclusion of electronegative groups in the side-chain of the classical cannabinoid structure can markedly affect both affinity and efficacy of molecules. One possible explanation for this is that these negatively charged moieties might be interacting with positively charged amino acids within the ligand binding pocket. As described by Tao *et al.* (1999), CP 55,940, which also possesses a dimethylheptyl side-chain and a similar body to these compounds, is thought to dock within a largely hydrophobic cluster of residues in transmembrane regions 6 and 7. Assuming that these compounds bind to the receptor in a similar manner then there may be evidence of such positively charged amino acids in close proximity to this within this particular model. Similarly, previous studies by our group demonstrated that the D163N mutation in the human CB_1 receptor (mutation of a positively charged aspartate to a asparagine residue) causes uncoupling of cannabinoid ligands but does not affect their binding, although the analogous mutation in the rat only caused uncoupling of certain transduction mechanisms (Tao and Abood, 1998; Roche *et al*., 1999). Again this or a similar amino acid may prove to be a candidate for the modulatory effect of these charged side-chains on efficacy. Furthermore, from the data presented here, it would appear that these electronegative moieties additionally affect affinity also, and that should this be the result of an amino acid interaction then it may not be the same residue(s). Close analysis of the CB_1 receptor sequence and similar docking analyses may be able to isolate candidates for this amino acid and subsequent point mutation studies may answer this question definitively. However, an alternative explanation for these results lies in

the possibility that the additional groups contained in the side-chain may not themselves directly interact with the receptor binding pocket, or if they do so, they interact in such a way that the body of the molecule is forced into other parts of the binding pocket. This may cause these sections to interact in a different manner than with the parent compound and thus alter the pharmacological properties. Whichever the reason, molecular modelling and ligand docking studies utilizing this pharmacological data may prove to be valuable in further characterizing the CB_1 receptor pharmacophore.

As aspect of this study that must be carefully considered relates to the use of the GTPγS binding assay for the functional data. Although this assay is one of the most effective at measuring efficacy differences between ligands, it does not differentiated between G-proteins and will reflect coupling to whichever G-proteins are present in the tissue being studied. In cerebellar membranes it may be anticipated that Go will be the most predominant G-protein. However, it is thought that the majority of cannabinoid receptor effects are mediated by Gi. It is not inconceivable that alterations in the side chain may affect a receptor's ability to interact with Go but have little effect on coupling with Gi. Although this may not be predicted to alter the interpretation of the results within the scope of this particular study, it may be of consequence when considering the behavioural effects of these analogues.

In summary, this report details the pharmacological effects of a number of delta-8-tetrahydrocannabinol side-chain analogues. Furthermore, this report shows how structural modifications of the side-chain of these compounds may have profound effects on the ligand's affinities and efficacies. Importantly, it also demonstrates how the effects on each of these properties may be independent of one another.

This work was supported in part by National Institute of Drug Abuse grants DA-09978, DA-05274, DA-09789 and DA-05488.

References

BREIVOGEL, C.S., SELLEY, D.E. & CHILDERS, S.R. (1998). Cannabinoid receptor agonist efficacy for stimulating [35S]GTPgammaS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. *Biol. Chem.,* **273,** 16865 – 16873.

BUSCH-PETERSEN, J., HILL, W.A., FAN, P., KHANOLKAR, A., XIE, X.Q., TIUS, M.A. & MAKRIYANNIS, A. (1996). Unsaturated side chain beta-11-hydroxyhexahydrocannabinol analogs. *J. Med. Chem.,* **39,** 3790 – 3796.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) on an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099 – 3108.

FELDER, C.C., JOYCE, K.E., BRILEY, E.M., MANSOURI, J., MACKIE, K., BLOND, O., LAI, Y., MA, A.L. & MITCHELL, R.L. (1995). Comparison of the pharmacology and signal transduction of the human cannabinoid CB_1 and CB_2 receptors. *Mol. Pharmacol.,* **48,** 443 – 450.

GAONI, Y. & MECHOULAM, R. (1964). Isolation, structure and partial synthesis of an active constituent of hashish. *J. Amer. Chem. Soc.,* **86,** 1646 – 1647.

GRIFFIN, G., ATKINSON, P.J., SHOWALTER, V.M., MARTIN, B.R. & ABOOD, M.E. (1998). Evaluation of cannabinoid receptor agonists and antagonists using the [^{35}S]GTPγS binding assay in rat cerebellar membranes. *J. Pharmacol. Exp. Ther.,* **285,** 553 – 560.

GRIFFIN, G., WRAY, E.J., RORRER, W.K., CROCKER, P.J., RYAN, W.R., SAHA, B., RAZDAN, R.K., MARTIN, B.M. & ABOOD, M.E. (1999a). An investigation into the structural determinants of cannabinoid receptor efficacy. *Br. J. Pharmacol.,* **126,** 1575 – 1584.

GRIFFIN, G., WRAY, E.J., TAO, Q., MCALLISTER, S.D., RORRER, W.K., AUNG, M., MARTIN, B.R. & ABOOD, M.E. (1999b). Evaluation of the cannabinoid CB_2 receptor-selective antagonist, SR144528: further evidence for cannabinoid CB_2 receptor absence in the rat central nervous system. *Eur. J. Pharmacol.,* **377,** 117 – 125.

HANUS, L., BREUER, A., TCHILIBON, S., SHILOAH, S., GOLDENBERG, D., HOROWITZ, M., PERTWEE, R.G., ROSS, R.A., MECHOULAM, R. & FRIDE, E. (1999). HU-308: A specific agonist for CB(2), a peripheral cannabinoid receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 14228 – 14233.

HERKENHAM, M., LYNN, A.B., JOHNSON, M.R., MELVIN, L.S., DE COSTA, B.R. & RICE, K.C. (1991). Characterization and localization of cannabinoid receptors in rat brain: A quantitative in vitro autographic study. *J. Neurosci.,* **11,** 563 – 583.

HUFFMAN, J.W., YU, S., SHOWALTER, V., ABOOD, M.E., WILEY, J.L., COMPTON, D.R., MARTIN, B.R., BRAMBLETT, R.D. & REGGIO, P.H. (1996). Synthesis and pharmacology of a very potent cannabinoid lacking a phenolic hydroxyl with a high affinity for the CB_2 receptor. *J. Med. Chem.,* **39,** 3875 – 3877.

KEIMOWITZ, A.R., MARTIN, B.R., RAZDAN, R.K., CROCKER, P.J., MASCARELLA, S.W. & THOMAS, B.F. (2000). QSAR analysis of Delta(8)-THC analogues: relationship of side-chain conformation to cannabinoid receptor affinity and pharmacological potency. *J. Med. Chem.,* **43,** 59 – 70.

MACKIE, K., DEVANE, W.A. & HILLE, B. (1993). Anandamine, an endogenous cannabinoid, inhibits calcium currents as a partial agonist in N18 neuroblastoma cells. *Mol. Pharmacol.,* **44,** 498 – 503.

MARTIN, B.R., JEFFERSON, R., WINCKLER, R., WILEY, J.L., HUFFMAN, J.W., CROCKER, P.J., SAHA, B. & RAZDAN, R.K. (1999). Manipulation of the tetrahydrocannabinol side chain delineates agonists, partial agonists, and antagonists. *J. Pharmacol. Exp. Ther.,* **290,** 1065 – 1079.

MARTIN, B.R., THOMAS, B.F. & RAZDAN, R.K. (1995). Structural requirements for cannabinoid receptor probes. In *Cannabinoid Receptors*. ed. Pertwee R.G., pp. 35 – 86. London: Academic Press.

MATSUDA, L.A., LOLAIT, S.J., BROWNSTEIN, M.J., YOUNG, A.C. & BONNER, T.I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature,* **346,** 561 – 564.

MECHOULAM, R., LANDER, N., SREBNIK, M., BREUER, A., SEGAL, M., FEIGENBAUM, J.J., JARBE, T.U. & CONSROE, P. (1987). Stereochemical requirements for cannabimimetic activity. *NIDA Res. Monogr.,* **79,** 15 – 30.

MUNRO, S., THOMAS, K.L. & ABU-SHAAR, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature,* **365,** 61 – 64.

PERTWEE, R., GRIFFIN, G., FERNANDO, S., LI, X., HILL, A. & MAKRIYANNIS, A. (1995). AM630, a competitive cannabinoid receptor antagonist. *Life. Sci.,* **56,** 1949 – 1955.

RINALDI-CARMONA, M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D., FERRARA, P., SOUBRIE, P., BRELIERE, J.C. & LE FUR, 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, J.M., CASELLAS, P., CONGY, C., OUSTRIC, D., SARRAN, M., BOUABOULA, M., CALANDRA, B., PORTIER, M., SHIRE, D., BRELIERE, J.C. & LE FUR, G.L. (1998). SR 144528, the first potent and selective antagonist of the CB_2 cannabinoid receptor. *J. Pharmacol. Exp. Ther.,* **284,** 644–650.

ROCHE, J.P., BOUNDS, S., BROWN, S. & MACKIE, K. (1999). A mutation in the second transmembrane region of the CB_1 receptor selectively disrupts G protein signaling and prevents receptor internalization. *Mol. Pharmacol.,* **56,** 611–618.

SCHILD, H.O. (1949). pAx and competitive drug antagonism. *Br. J. Pharmacol.,* **4,** 277–280.

SELLEY, D.E., STARK, S., SIM, L.J. & CHILDERS, S.R. (1996). Cannabinoid receptor stimulation of guanosine-5′-O-(3-[^{35}S]thio)triphosphate binding in rat brain membranes. *Life Sci.,* **59,** 659–668.

SHEN, M., PISER, T.M., SEYBOLD, V.S. & THAYER, S.A. (1996). Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. *J. Neurosci.,* **16,** 4322–4334.

SHOWALTER, V.M., COMPTON, D.R., MARTIN, B.R. & ABOOD, M.E. (1996). Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB_2): Identification of cannabinoid receptor subtype selective ligands. *J. Pharmacol. Exp. Ther.,* **278,** 989–999.

SIM, L.J., SELLEY, D.E. & CHILDERS, S.R. (1995). *In vitro* autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[g-[^{35}S]thio]-triphosphate binding. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7242–7246.

SINGER, M., RYAN, W.J., SAHA, B., MARTIN, B.R. & RAZDAN, R.K. (1998). Potent cyano and carboxamido side-chain analogues of 1′,1′-dimethyl-D8-tetrahydrocannabinol. *J. Med. Chem.,* **41,** 4400–4407.

TAO, Q. & ABOOD, M.E. (1998). Mutation of a highly conserved aspartate residue in the second transmembrane domain of the cannabinoid receptors, CB_1 and CB_2, disrupts G-protein coupling. *J. Pharmacol. Exp. Ther.,* **285,** 651–658.

TAO, Q., MCALLISTER, S.D., ANDREASSI, J., NOWELL, K.W., CABRAL, G.A., HURST, D.P., BACHTEL, K., EKMAN, M.C., REGGIO, P.H. & ABOOD, M.E. (1999). Role of a conserved lysing residue in the peripheral cannabinoid receptor (CB_2): evidence for subtype specificity. *Mol. Pharmacol.,* **55,** 605–613.

(Received August 7, 2000
Revised October 16, 2000
Accepted November 6, 2000)