

Synthesis and Characterization of Potent and Selective Agonists of the Neuronal Cannabinoid Receptor (CB1)¹

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ABSTRACT

Two subtypes of the cannabinoid receptor (CB1 and CB2) are expressed in mammalian tissues. Although selective antagonists are available for each of the subtypes, most of the available cannabinoid agonists bind to both CB1 and CB2 with similar affinities. We have synthesized two analogs of *N*-arachidonyl ethanolamine (AEA), arachidonylcyclopropylamide (ACPA) and arachidonyl-2-chloroethylamide (ACEA), that bind to the CB1 receptor with very high affinity (K_i values of 2.2 ± 0.4 nM and 1.4 ± 0.3 nM, respectively) and to the CB2 receptor with low affinity (K_i values of 0.7 ± 0.01 μ M and 3.1 ± 1.0 μ M, respectively). Both ACPA and ACEA have the characteristics of agonists at the CB1 receptor; both inhibit forskolin-induced

accumulation of cAMP in Chinese hamster ovary cells expressing the human CB1 receptor, and both analogs increase the binding of [³⁵S]GTP γ S to cerebellar membranes and inhibit electrically evoked contractions of the mouse vas deferens. ACPA and ACEA produce hypothermia in mice, and this effect is inhibited by coadministration of the CB1 receptor antagonist SR141716A. Therefore, ACPA and ACEA are high-affinity agonists of the CB1 receptor but do not bind the CB2 receptor, suggesting that structural analogs of AEA can be designed with considerable selectivity for the CB1 receptor over the CB2 receptor.

Two cannabinoid receptors, CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993), have been identified. These two receptors form a distinct class within the family of G protein-coupled receptors (Matsuda 1997). They share 40 to 50% amino acid sequence homology and recognize many of the same ligands. In fact, most high-affinity agonists of the CB1 receptor bind to the CB2 receptor in the same concentration range (Munro et al., 1993; Felder et al., 1995). Selective antagonists of the two receptors have been identified: SR141716A, with at least 100-fold selectivity for CB1 over CB2 (Rinaldi-Carmona et al., 1994), and SR144528, a selective antagonist of the CB2 receptor (Rinaldi-Carmona et al., 1998). Several agonists have been identified with selectivity for the CB2 receptor over the CB1 receptor. These include the naturally occurring cannabinoid, cannabinol (Munro et al., 1993; Felder et al., 1995), and several deoxy derivatives of Δ^8 -tetrahydrocannabinol (Huffman et al., 1996, 1998). The

aminoalkylindole cannabimimetic Win 55212-2 binds to CB1 and CB2 receptors with similar affinity in membranes from tissues in which the receptors are expressed endogenously (Kuster et al., 1993; Slipetz et al., 1995) but has higher affinity for the cloned, expressed CB2 receptor than the CB1 receptor (Felder et al., 1995).

N-Arachidonyl ethanolamine (AEA or anandamide) is a brain-derived compound that binds and activates both the CB1 (Devane et al., 1992) and CB2 receptors (Felder et al., 1995; Slipetz et al., 1995). Several laboratories have synthesized and evaluated structural analogs of AEA for their ability to bind the CB1 receptor (see Hillard and Campbell, 1997, for review). To summarize, these studies have shown that analogs with modifications in the amide head group, particularly changes in which the head group becomes more hydrophobic, bind to the CB1 receptor with greater affinity than the parent compound. Less is known about the affinities of AEA analogs for the CB2 receptor; however, two anandamide analogs, arachidonyl-2-fluoroethylamide (Showalter et al., 1996) and (*R*)-methanandamide (Khanolkar et al., 1996) have been shown to bind with higher affinity to the CB1 than the CB2 receptor. These results suggest that modifications to the amide portion of AEA that enhance binding

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ABBREVIATIONS: AEA, *N*-arachidonyl ethanolamine; CB1, neuronal cannabinoid receptor; CB2, spleen cannabinoid receptor; CHO, Chinese hamster ovary; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; DMSO, dimethyl sulfoxide; ACPA, arachidonylcyclopropylamide; ACEA, arachidonyl-2-chloroethylamide; TME, Tris, magnesium, and EDTA.

to CB1 reduce binding to CB2. We have used this principle to design two analogs, arachidonylcyclopropylamide and arachidonyl-2-chloroethylamide, which would be predicted to be high-affinity CB1 ligands due to the hydrophobic character of the amide substitution. As predicted, these analogs bind to the CB1 receptor with very high affinity but have low affinity for the CB2 receptor, exhibiting selectivity ratios of 325 and 2200, respectively. We report in this article that both compounds are agonists of the CB1 receptor and, therefore, represent the first high-affinity cannabinoid agonists that exhibit significant selectivity for CB1 over CB2 receptors.

Experimental Procedures

Compound Syntheses. Arachidonylcyclopropylamide (ACPA; Fig. 1) was synthesized from arachidonic acid (0.033 mmol) in anhydrous tetrahydrofuran (200 μ l) and was stirred with 5 μ l of triethylamine (0.036 mmol) and isobutyl chloroformate (0.033 mmol) at 0°C for 30 min. Cyclopropylamine (5 μ l, 0.072 mmol) was added, and the reaction mixture was stirred at 0°C for 3 h. The reaction mixture was diluted with water and ether, and the ether extract was washed successively with water, 5% sodium bicarbonate solution, and water. After evaporation of the solvent, the product was purified by silica gel chromatography using 40% ethyl acetate in hexane as eluent; the yield was 88%. NMR was used to confirm the structural identity: ^1H NMR (300 MHz, CDCl_3) δ 0.49 (s, 2 H), 0.77 (d, J = 6.3 Hz, 2 H), 0.90 (t, J = 6.5 Hz, 3 H), 1.20 to 1.1.40 (m, 6 H), 1.65 to 1.80 (m, 2 H), 2.00 to 2.20 (m, 6 H), 2.71 (s, 1 H), 2.75 to 2.85 (m, 6 H), 5.30 to 5.45 (m, 8 H), 5.61 (broad s, 1 H); ^{13}C NMR (75 MHz, CDCl_3) δ 6.88 (2 C), 14.28, 22.78, 25.87, 25.82 (4 C), 26.84, 27.41, 29.51, 31.71, 36.11, 127.70, 128.04, 128.35 (2 C), 128.78, 128.93, 129.93, 130.70, 174.44.

Arachidonyl-2-chloroethylamide (ACEA; Fig. 1) was synthesized

using essentially the same procedure. A mixture of 2-chloroethylamine monohydrochloride (0.043 mmol) and triethylamine (0.043 mmol) was added to the arachidonic acid reaction mixture described above and stirred at 0°C for 3 to 4 h. After dilution with ether and water, ether extract was washed successively with water, 1 N hydrochloric acid, water, 5% sodium bicarbonate, and water and dried over sodium sulfate. The product was purified by column chromatography over silica gel using 20% ethyl acetate in hexane as eluent; yield was 80%. The structural identity of the product was confirmed using NMR: ^1H NMR (300 MHz, CDCl_3) δ 0.9 (t, J = 6.5 Hz, 3 H), 1.20 to 1.40 (m, 6 H), 1.70 to 1.80 (m, 2 H), 2.0 to 2.20 (m, 4 H), 2.23 (t, J = 7.3 Hz, 2 H), 2.70 to 2.90 (m, 6 H), 3.50 to 3.70 (m, 4 H), 5.32 to 5.50 (m, 8 H), 5.82 (broad s, 1 H); ^{13}C NMR (75 MHz, CDCl_3) δ 14.29, 22.78, 25.59, 25.83 (3 C), 26.81, 27.42, 29.52, 31.72, 36.13, 41.32, 44.43, 127.72, 128.02, 128.32, 128.44, 128.80, 129.08, 129.18, 130.71, 173.23.

Radioligand Binding Methods. The affinities of the compounds for the CB1 receptor were determined using rat cerebellar membranes and [^3H]CP55940 as described previously (Hillard et al., 1995).

The affinities of the compounds for the CB2 receptor were determined in rat spleen membranes. Spleens were removed and homogenized in 10 ml of TME buffer (50 mM Tris-HCl, 1.0 mM EDTA, and 3.0 mM MgCl_2 , pH 7.4). The homogenate was centrifuged at 5000g for 5 min, and the resulting supernatant was recentrifuged at 17,500g for 20 min. The harvested membranes were resuspended in TME buffer and stored at -80°C for no more than 1 month. Spleen membranes (50 μ g of protein) were incubated with [^3H]CP55940 (0.5–1 nM) for 1 h at 30°C in a final volume of 0.2 ml of TME buffer containing 0.1% fatty acid free BSA. Nonspecific binding was defined as [^3H]CP55940 bound in the presence of 5 μM Win 55212-2. Bound and free radioligand were separated by filtration (Hillard et al., 1995).

For both the CB1- and CB2-binding assays, incubations were carried out in the presence of 150 μM phenylmethylsulfonyl fluoride to inhibit ligand catabolism by AEA amidohydrolase (Childers et al., 1994; Hillard et al., 1995). Competing ligands were added to the incubation in 1 μ l of dimethyl sulfoxide (DMSO). In each assay, binding was determined in triplicate or quadruplicate; IC_{50} values were calculated using nonlinear regression to fit the data to a one-site binding equation (Prism, GraphPad Software, San Diego). K_i values were calculated from the IC_{50} values using the formula of Cheng and Prusoff (1973).

The binding of [^{35}S]GTP γ S was carried out using a modification of previously published methods (Wieland and Jakobs 1994). Cerebellar membranes (5 μ g of protein) were incubated with 0.65 nM [^{35}S]GTP γ S in TME buffer containing 0.1% BSA, 10 μM GDP, 150 μM phenylmethylsulfonyl fluoride, and 150 mM NaCl. The incubation was carried out for 30 min at 37°C; bound and free [^{35}S]GTP γ S were separated by filtration. Nonspecific binding was defined using 10 μM Gpp[NH]p. Cannabinoid ligands were added to the incubations in 1 μ l of DMSO; control incubates contained DMSO alone. In each experiment, the percent increase in [^{35}S]GTP γ S binding in response to agonist was calculated using the DMSO-treated membranes as the control. The EC_{50} values and maximal agonist-induced increase in [^{35}S]GTP γ S binding were determined by fitting the data to a sigmoidal concentration-response curve using nonlinear regression (Prism, GraphPad Software, San Diego, CA).

Adenylyl Cyclase Assays. Cannabinoid receptor inhibition of adenylyl cyclase activity was determined in Chinese hamster ovary (CHO) cells stably transfected with cDNA encoding either the human CB1 or human CB2 receptors. The cells were kindly provided by Drs. G. Disney and A. Green (Glaxo Wellcome Research and Development, Medicines Research Center, Stevenage, England). Cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (Ham's F-12) supplemented with 2 mM glutamine, 600 $\mu\text{g}/\text{ml}$ geneticin, and 300 $\mu\text{g}/\text{ml}$ hygromycin.

Cells were preincubated for 30 min at 37°C with cannabinoid and

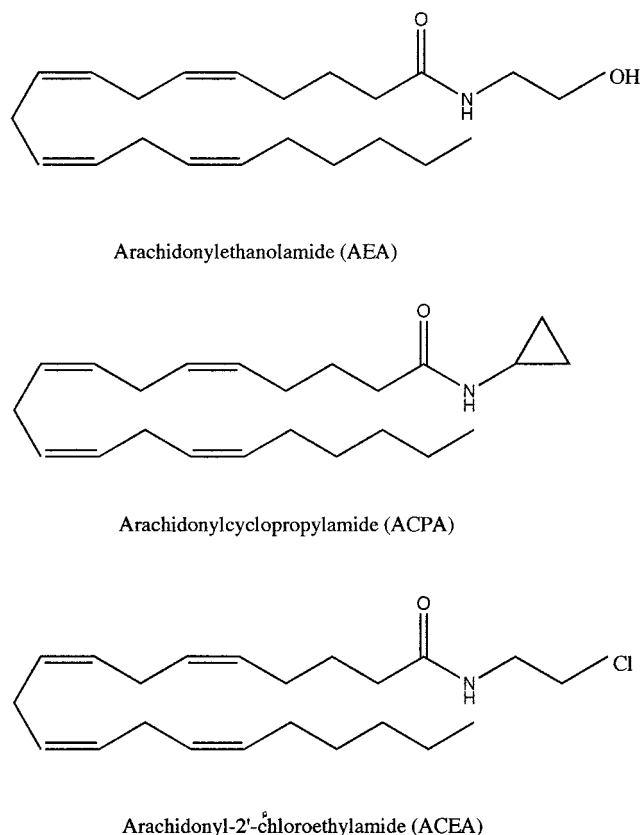


Fig. 1. Structures of the analogs of *N*-arachidonylethanolamine used in these studies.

isobutylmethylxanthine (50 μ M) in PBS containing 1 mg/ml BSA and 100 μ M phenylmethylsulfonyl fluoride. Forskolin was added (final concentration, 2 μ M), and the incubation was continued for 30 min. The reaction was terminated by addition of 0.1 M HCl followed by centrifugation to remove cell debris. The pH of the supernatant was adjusted to 8 to 9 using 1 M NaOH, and cyclic AMP (cAMP) content was measured by radioimmunoassay (Biotrak; Amersham, Arlington Heights, IL). Cannabinoids were dissolved in ethanol as 1-mg/ml stock solutions and diluted to final concentrations in assay buffer. Forskolin and isobutylmethylxanthine were dissolved in DMSO.

Effects of the test compounds on forskolin-stimulated cAMP production have been expressed in percentage terms. This was calculated from the equation $[100 \times (f' - b)/(f - b)]$, where f' is cAMP production in the presence of forskolin and the test compound, f is cAMP production in the presence of forskolin alone, and b is basal cAMP production.

Mouse Vas Deferens Experiments. Vasa deferentia were obtained from albino MF1 mice weighing 32 to 42 g. Each tissue was mounted in a 4-ml organ bath at an initial tension of 0.5g as described previously (Pertwee et al., 1993). The baths contained Krebs' solution, which was kept at 37°C and bubbled with 95% O₂ and 5% CO₂. The composition of the Krebs' solution was 118.2 mM NaCl, 4.75 mM KCl, 1.19 mM KH₂PO₄, 25.0 mM NaHCO₃, 11.0 mM glucose, and 2.54 mM CaCl₂·6H₂O. Isometric contractions were evoked by stimulation with 0.5-s trains of three pulses of 110% maximal voltage (train frequency, 0.1 Hz; pulse duration, 0.5 ms) through platinum and stainless steel electrodes attached to the upper and lower ends of each bath, respectively. Stimuli were generated by a Grass S48 stimulator, then amplified (Med-Lab channel attenuator; Stag Instruments, Chalgrove, Oxford, UK) and divided to yield separate outputs to four organ baths (Med-Lab StimuSplitter). Contractions were monitored by computer (Apple Macintosh LC or Quadra 650, Cupertino, CA) using a data recording and analysis system (MacLab) that was linked via preamplifiers (Macbridge) to Dynamometer UF1 transducers (Harvard Apparatus, Edenbridge, Kent, UK).

Each tissue was subjected to several periods of stimulation. The first of these began after the tissue had equilibrated but before drug administration and was continued for 11 min. The stimulator was then switched off for 10 min, after which the tissues were subjected to further periods of stimulation, each lasting 5 min, and separated by a stimulation-free period. Drug additions were first made once the tissues were responding consistently to electrical stimulation, usually after the first 5-min stimulation period. Further additions were made after each subsequent 5-min stimulation period. The duration of the stimulation-free period that followed each addition of an agonist was 15 min. Because it is not possible to reverse the inhibitory effect of cannabinoids on the twitch response by washing them out of the organ bath, only one concentration-response curve was constructed per tissue. ACPA and ACEA were each mixed with 2 parts of Tween 80 by weight and dispersed in a 0.9% aqueous solution of NaCl as described previously for Δ^9 -tetrahydrocannabinol (Δ^9 -THC) (Pertwee et al., 1992). Drug additions were made in volumes of 10 or 31 μ l.

Inhibition of electrically evoked contractions has been calculated by comparing the amplitude of the twitch response after each addition of an agonist with its amplitude immediately before the first addition of the agonist. Values with their 95% CLs for the maximal degree of inhibition of the twitch response produced ($E_{\max}$) and for agonist concentrations producing 50% of the $E_{\max}$ (EC_{50}) were calculated using nonlinear regression analysis (Prism, GraphPad Software).

Determination of Mouse Rectal Temperature. Male ICR mice weighing between 25 and 35 g were used in these studies. Rectal temperatures were determined using a Yellow Springs thermister inserted to a depth of 2.5 cm. All drugs were dissolved in 100% ethanol and administered i.v. or i.p. to the mice in an emulsion of

ethanol/emulphor EL-60/saline (1:1:18). Vehicle solutions contained the same amounts of ethanol and emulphor.

Statistics. The binding and adenylyl cyclase data were analyzed for significant differences among mean values using one-way ANOVA followed by Tukey's post hoc test to compare individual means when warranted. The vas deferens data were compared using overlap of the 96% confidence intervals of the mean. The temperature data were analyzed by one-way ANOVA followed by Dunnett's modification of the t test to compare treatment means with a single control.

Materials. [³H]CP55940 (165 Ci/mmol) and [³⁵S]GTP γ S (1200 Ci/mmol) were purchased from NEN Life Sciences (Boston, MA). Emulphor EL-620 (now called Alkamuls EL-620) was kindly provided by Rhone-Poulenc (Cranbury, NJ). AEA was purchased from Cayman Chemical Company (Ann Arbor, MI). Win 55212-2 was purchased from RBI (Natick, MA). SR141716A and SR144528 were kindly provided by Sanofi Recherche (Montpellier, France); CP55940 was a gift from Pfizer Central Research (Groton, CT); and Δ^9 -THC was obtained from the National Institute on Drug Abuse (Rockville, MD). All other drugs and chemicals were of the highest grade possible and were purchased from standard commercial sources.

Results

ACPA and ACEA Have Higher Affinity for the CB1 Receptor Than the CB2 Receptor. The affinities of the arachidonyl amides for the CB1 receptor were determined in rat cerebellar membranes. Previous studies have demonstrated that rat cerebellar membranes are a rich source of CB1 receptors (Herkenham et al., 1990; Breivogel et al., 1997). CB2 receptor expression has been demonstrated in brain-derived microglial cells (Kearn and Hillard 1998); however, it is unlikely that these cells contribute significantly to the total cannabinoid receptor pool in cerebellar homogenates.

[³H]CP55940 binds saturably to CB1 receptors in cerebellar membranes with a K_D of 0.5 ± 0.1 nM and $B_{\max}$ of 0.9 ± 0.1 pmol/mg protein. Both Win 55212-2 and AEA compete for binding with K_I values in close agreement with published values (Kuster et al., 1993; Childers et al., 1994) (Table 1 and Fig. 2). The CB2-selective antagonist SR144528 does not inhibit the binding of [³H]CP55940 to rat cerebellar membranes at a concentration of 100 nM (data not shown), supporting the argument that essentially all of the cannabinoid receptors in cerebellar membranes are the CB1 subtype. Both ACPA and ACEA bind to the CB1 receptor with very

TABLE 1
Affinities of ACPA and ACEA for CB1 and CB2 receptors

Ligand	$K_{D/I}$, CB1 Receptor ^a	$K_{D/I}$, CB2 Receptor ^b	Potency Ratio (CB2/CB1)
	nM		
CP55940	0.5 ± 0.1 (4)	2.8 ± 0.4 (4)	5.6
Win 55212-2	4.4 ± 1.3 (3) ^c	1.2 ± 0.25 (3)	0.27
AEA	71.7 ± 7.3 (4) ^d	279 ± 58 (3) ^d	3.9
ACPA	2.2 ± 0.4 (4)	715 ± 14 (2) ^d	325
ACEA	1.4 ± 0.3 (4)	>2000 (2)	>1400

^a Affinities for the CB1 receptor were determined either from saturation isotherms (CP55940) or from competition isotherms using [³H]CP55940 in rat cerebellar membranes. Membranes (10 μ g of protein/incubate) were incubated with 0.5 to 1 nM [³H]CP55940 for 60 min at room temperature.

^b Affinities for the CB2 receptor were determined either from saturation isotherms (CP55940) or from competition isotherms using [³H]CP55940 in rat spleen membranes. Membranes (10 μ g of protein/incubate) were incubated with 0.5 to 1 nM [³H]CP55940 for 60 min at 30°C.

^c Data are shown as mean $\pm$ S.E.M. Numbers in parentheses, number of replicate experiments.

^d Significantly different from K_D for CP55940 at $p < .05$ using Tukey's test.

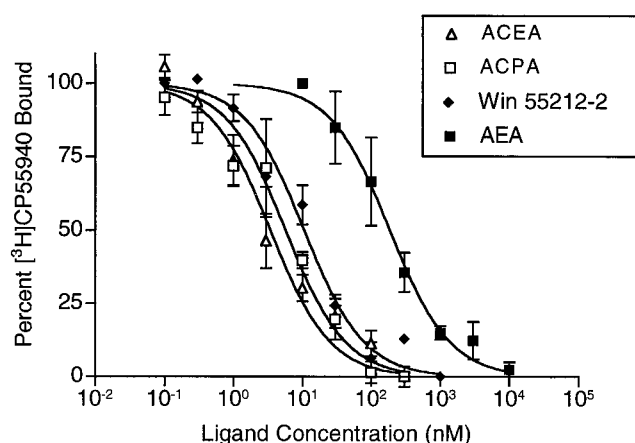


Fig. 2. Competition isotherms for arachidonyl amides and Win 55212-2 for binding to the cerebellar CB1 cannabinoid receptor. Cerebellar membranes were prepared from rat and incubated with [3 H]CP55940 (0.5–1 nM) for 60 min at room temperature. Bound and free ligand were separated by filtration; nonspecific binding was determined in the presence of 10 μ M THC and was subtracted. Each point is the mean of three to four experiments; vertical lines represent S.E.M.

high affinity, with K_i values of 2.2 and 1.4 nM, respectively (Table 1 and Fig. 2). The K_i values for ACPA and ACEA are not significantly different from each other or from the K_i for Win 55212-2, and both are significantly different from the K_i for AEA ($p < .05$).

The affinities of ACPA and ACEA for the CB2 receptor were determined in rat spleen, an abundant source of CB2 receptors (Munro et al., 1993). [3 H]CP55940 exhibits saturable binding to spleen membranes with a K_D of 2.8 ± 0.4 nM and B_{max} of 571 ± 21 fmol/mg protein. The CB2-selective antagonist SR144528 (Rinaldi-Carmona et al., 1998) competes for [3 H]CP55940 binding in spleen with a K_i of 4.23 ± 1.0 nM, whereas the CB1-selective antagonist/inverse agonist SR141716A (Rinaldi-Carmona et al., 1994; Bouaboula et al., 1997) has no effect on the binding of [3 H]CP55940 to spleen membranes at concentrations up to 1 μ M.

As expected, both Win 55212-2 and AEA compete for [3 H]CP55940 binding to CB2 receptors in spleen (Table 1 and Fig. 3). Win 55212-2 has significantly higher affinity for the

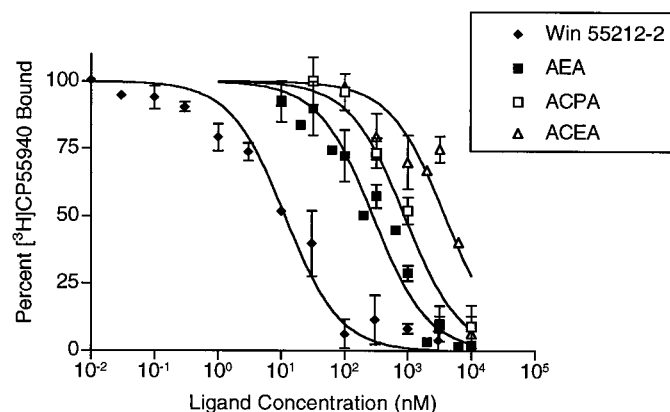


Fig. 3. Competition isotherms for arachidonyl amides and Win 55212-2 for binding to the spleen CB2 receptor. Spleen membranes were prepared from rat and incubated with [3 H]CP55940 (0.5–1 nM) for 60 min at 30°C. Bound and free ligand were separated by filtration; nonspecific binding was determined in the presence of 5 μ M Win 55212-2 and was subtracted. Each point is the mean of two to three experiments; vertical lines represent S.E.M.

CB2 receptor in spleen than the CB1 receptor in brain, whereas AEA has greater affinity for the brain CB1 receptor ($p < .05$). These results are in very close agreement with a previous report (Showalter et al., 1996). Both ACPA and ACEA compete for [3 H]CP55940 binding in spleen, however, with low affinity relative to both AEA and their affinity for the CB1 receptor (Table 1 and Fig. 3). The K_i value for ACPA is significantly greater than the K_i for AEA ($p < .05$).

ACPA and ACEA Are Agonists of the CB1 Receptor.

The CB1 receptor agonist activity of the arachidonyl amides was determined in several ways. In the first set of experiments, inhibition of forskolin-stimulated adenylyl cyclase activity was measured in CHO cells stably expressing either hCB1 or hCB2 receptor. The nonselective cannabinoid agonist CP55940 potently inhibits cAMP accumulation in both cell lines (Table 2). In agreement with the binding data, neither ACEA nor ACPA inhibit forskolin-stimulated adenylyl cyclase activity in cells expressing the CB2 receptor. However, both ACPA and ACEA are very potent inhibitors of cAMP accumulation in cells expressing the CB1 receptor. The IC_{50} values for ACPA and ACEA are not significantly different from each other and are both significantly less than the IC_{50} value for AEA ($p < .05$). All of the ligands investigated produced nearly complete inhibition of cAMP accumulation; the E_{max} values were not significantly different from each other.

In a second set of experiments, efficacy of the compounds at the CB1 receptor was measured using ligand stimulation of [35 S]GTP γ S binding to cerebellar membranes. The first step in the activation of intracellular signaling by G protein-coupled receptors is the induction of an exchange of GDP for GTP on the guanine nucleotide binding site of the α subunit of a heterotrimeric G protein. The effects of various cannabinoid receptor agonists on GDP-GTP exchange can be determined from agonist-induced binding of the nonhydrolyzable GTP analog, [35 S]GTP γ S (Selley et al., 1996; Breivogel et al., 1997; Burkey et al., 1997). Both Win 55212-2 and AEA increase the binding of [35 S]GTP γ S; as has been reported previously (Selley et al., 1996; Burkey et al., 1997), Win 55212-2 is both more potent (i.e., has a lower EC_{50}) and more efficacious than AEA (Table 3). Both ACPA and ACEA increase the binding of [35 S]GTP γ S to cerebellar membranes (Table 3 and Fig. 4). The arachidonyl amides are significantly more potent than AEA ($p < .05$) but are not significantly more potent than Win 55212-2. ACPA and ACEA are equipotent, and the E_{max} values obtained in the presence of these analogs are not significantly different from the E_{max} value produced by either Win 55212-2 or CP55940. The increase in [35 S]GTP γ S binding to cerebellar membranes induced by both ACPA and ACEA is inhibited completely by coinubation of the membranes with the CB1-selective antagonist, SR141716A (Fig. 4).

ACPA and ACEA were also screened for cannabinoid agonist activity using the mouse vas deferens model. Cannabinoid agonists inhibit the electrically induced contractions of the mouse vas deferens via activation of inhibitory CB1 receptors present on the sympathetic nerve terminals (Pertwee, 1997). Both ACPA and ACEA potently inhibit electrically induced contractions (Table 4). There is no significant difference among the potencies of the three ligands. The E_{max} values for both ACPA and ACEA are significantly less than

TABLE 2

Inhibition of forskolin-stimulated cAMP production in CHO cells stably transfected with either CB1 or CB2 receptors^a

Ligand	IC ₅₀ , CB1 Receptor	IC ₅₀ , CB2 Receptor	Potency Ratio ^b	E _{max} , CB1 Receptor ^c
	<i>nM</i>			
CP55940	2.55 ± 0.96 (7)	2.85 ± 1.38 (9)	1.12	93.7 ± 1.3 (7)
AEA	444 ± 82 (3) ^d	>10 μM (3)	>20	83.3 ± 6 (3)
ACPA	2.06 ± 0.69 (3)	>10 μM (3)	>2000	95.2 ± 4.3 (3)
ACEA	4.52 ± 1.65 (3)	>10 μM (3)	>4000	97.1 ± 3.6 (3)

^a CHO cells stably transfected with either the human CB1 or human CB2 receptor were preincubated with cannabinoids for 30 min at 37°C in the presence of 50 μM isobutylmethylxanthine. Forskolin (2 μM) was used to activate adenylyl cyclase; activation period was 30 min. Six concentrations of ligand between 0.1 nM and 10 μM were used for each isotherm. Data shown are means ± S.E.M. Numbers in parentheses, number of replicate experiments.

^b Ratio of the IC₅₀ in CB2-expressing cells to IC₅₀ in CB1-expressing cells.

^c Maximal inhibition of adenylyl cyclase obtained in the same experiments in which the IC₅₀ values were determined.

^d Significantly different from IC₅₀ for CP55940 at *p* < .05 using Tukey's test.

TABLE 3

Potency and efficacy of ACPA and ACEA to increase [³⁵S]GTPγS binding in rat cerebellar membranes^a

Ligand	EC ₅₀	E _{max}
	<i>nM</i>	% control
CP55940 (4)	31 ± 5	200 ± 7
Win 55212-2 (10)	120 ± 28	217 ± 8
AEA (8)	276 ± 53 ^b	166 ± 6 ^c
ACPA (3)	37 ± 8	187 ± 17
ACEA (3)	51 ± 6	201 ± 14

^a Cerebellar membranes (5 μg/incubate) were incubated with 0.65 nM [³⁵S]GTPγS for 30 min at 37°C. Control [³⁵S]GTPγS binding was determined in the presence of DMSO vehicle (final concentration of DMSO, 0.4%). DMSO had no effect on [³⁵S]GTPγS binding at this concentration. Phenylmethylsulfonyl fluoride (0.15 mM) was included in the incubates when the arachidonyl amides were used. EC₅₀ and E_{max} values were determined in three separate experiments using nonlinear regression. Data shown are mean values ± S.E.M.

^b Significantly different from CP55940 at *p* < .05.

^c Significantly different from Win 55212-2 at *p* < .05.

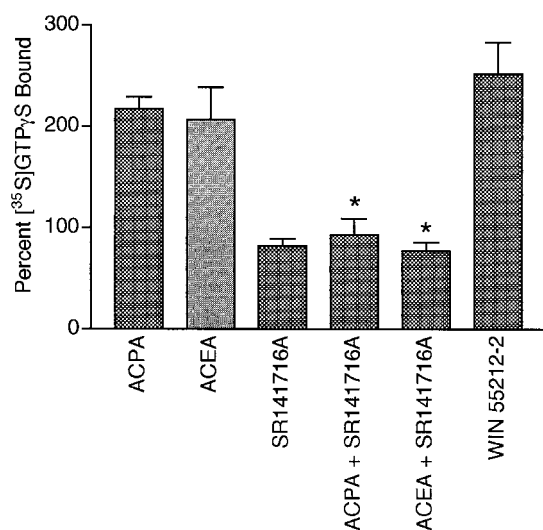


Fig. 4. The effects of ACPA and ACEA on [³⁵S]GTPγS binding are inhibited by the CB1 receptor antagonist SR141716A. Cerebellar membranes were incubated with cannabinoid and [³⁵S]GTPγS in buffer containing 100 mM NaCl and 10 μM GDP for 30 min at 37°C. The concentrations used were 200 nM ACPA and ACEA, 100 nM SR141716A, and 5 μM Win 55212-2. Control incubates contained DMSO and bound 117 ± 10 fmol/mg protein of [³⁵S]GTPγS. Data shown are percent of control; each bar is the mean of three experiments carried out in triplicate; vertical lines represent S.E.M. *Significantly different from the ACPA or ACEA treatment group with *p* < .05 using Tukey's test.

100%; only ACPA is significantly less efficacious than CP55940.

ACPA and ACEA Mimic the Effects of AEA on Body Temperature In Vivo. The cannabinoids produce robust

TABLE 4

Potency and efficacy of ACPA and ACEA to inhibit electrically induced contractions of the mouse vas deferens^a

Ligand	EC ₅₀	Maximal % Inhibition
	<i>pM</i>	
CP55940	46.2 (26, 84) ^b	94 (86, 102) ^b
ACPA	55.1 (26, 117)	74 (66, 82) ^c
ACEA	31.7 (20.4, 49.4)	84 (78, 89)

^a Vasa deferentia from MF1 mice were mounted in an organ bath and stimulated with 0.5-s trains of three pulses of 110% maximal voltage (0.1-Hz and 0.5-ms duration) through electrodes attached to the upper and lower ends of each bath. Cumulative concentration-response curves were constructed using concentrations between 3.16 pM and 100 nM. Cannabinoids were administered using Tween 80 vehicle; the vehicle had no effect on the response within the concentration range used. Inhibition was calculated as the ratio of the amplitude of the twitch response after the administration of drug to the amplitude immediately before the addition of any agonist.

^b Values are shown with 95% confidence intervals. Each value is the mean of six separate determinations.

^c Significantly different from CP55940; *p* < .05.

hypothermia in mice (Dewey, 1986). Like the other cannabinoid receptor agonists, AEA produces a significant hypothermia, although the effect is of shorter duration and lower maximum than the effect produced by Δ⁹-THC (Smith et al., 1994). ACPA, ACEA, and AEA all reduce rectal body temperature in mice in a dose-related manner (Fig. 5). All three compounds produced significant decreases in rectal temperature at a dose of 1 mg/kg. At 1 mg/kg, ACEA significantly reduced rectal temperature for at least 30 min following injection, whereas AEA was only significantly effective for the first 5 min. Two-way ANOVA of the 1 mg/kg data indicates no significant differences among the drugs.

To determine whether the in vivo effect was a result of activation of the CB1 receptor, mice were pretreated with the CB1 receptor antagonist, SR141716A (1 mg/kg i.p.) or an equivalent amount of vehicle (Fig. 6). Neither vehicle nor SR141716A had an effect on the rectal temperature. In the vehicle-pretreated mice, THC, AEA, ACPA, and ACEA (1 mg/kg i.v.) all produced significant decreases in rectal temperature at 7.5 min after injection (*p* < .05). In the SR141716A-pretreated mice, none of the four CB1 ligands produced a significant decrease in rectal temperature compared with the SR141716A/vehicle group.

Discussion

Previous studies have demonstrated that AEA binds to both the CB1 and CB2 receptors with moderate affinity (Devane et al., 1992; Felder et al., 1995). Furthermore, recent studies have suggested that modifications in the AEA structure that enhance ligand affinity for the CB1 receptor reduce

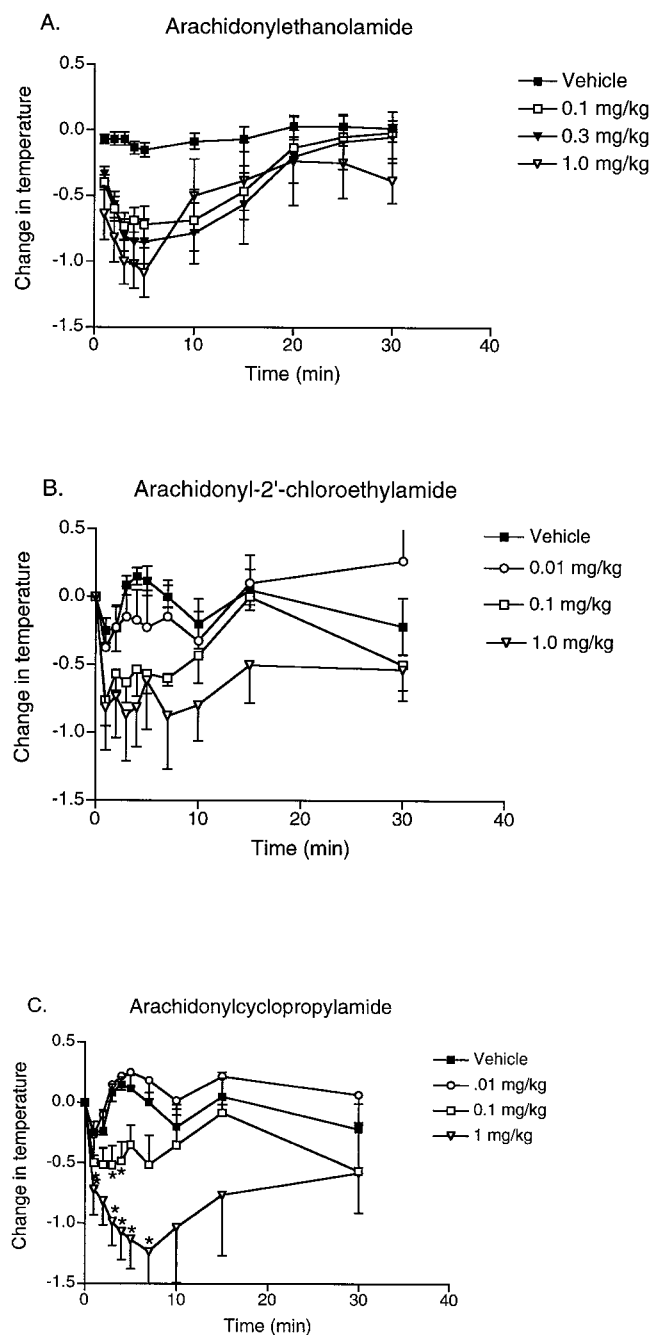


Fig. 5. AEA, ACEA, and ACPA produce hypothermia in mice. Male ICR mice were acclimatized to a 23°C chamber for 1 h before use. Rectal temperatures were recorded, and then mice were injected with cannabinoid i.v. in the tail vein. Cannabinoid were administered in an emulphor-ethanol vehicle. Data shown are the change in temperature at each time after injection from the initial (pretreatment) temperature. Data shown are the mean of six animals; vertical lines represent S.E.M. A, AEA produces significant decreases in rectal temperature at all doses at 2, 3, 4, and 5 min after injection and a significant decrease at 1 min in the 1-mg/kg dose group ($p < .05$ using Dunnett's test). B, ACEA produces significant decreases in body temperature at 1 mg/kg 3, 4, 5, 7, 10, 15, and 30 min after injection and 30 min after injection of a 0.1-mg/kg dose ($p < .05$ using Dunnett's test). C, significant changes are indicated by an asterisk ($p < .05$ using Dunnett's test).

affinity for the CB2 receptor (Showalter et al., 1996; Edmond et al., 1998). In the present study, we report the synthesis and characterization of two analogs of AEA, ACPA and ACEA. Both of these arachidonyl amides are high-affinity

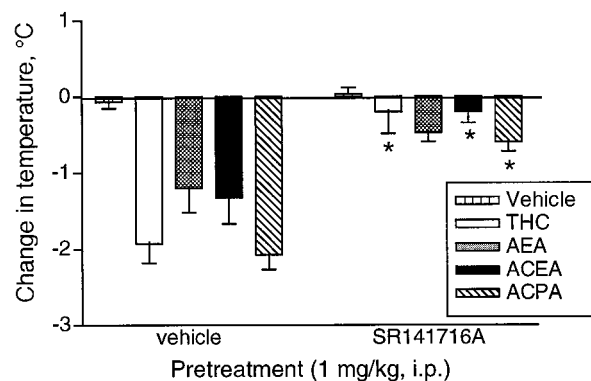


Fig. 6. Pretreatment of mice with SR141716A (1 mg/kg i.p.) inhibits the hypothermic response to AEA, THC, ACEA, and ACPA. AEA, THC, ACEA, and ACPA were administered i.v. in the tail vein at a dose of 1 mg/kg in emulphor-ethanol vehicle 30 min after the pretreatment injection. Rectal temperatures were recorded before the i.v. injection and 7.5 min following the injection; data shown are the mean of three animals; vertical lines represent S.E.M. *Significantly different from the vehicle pretreatment group at $p < .05$ using unpaired t test.

agonists of the CB1 receptor: 1) These compounds compete for binding with [3 H]CP55940 to cerebellar membranes with K_i values in the low nanomolar range. 2) They inhibit forskolin-stimulated adenylyl cyclase activity in CHO cells transfected with hCB1 at low nanomolar concentrations. 3) They inhibit the electrically induced contractions of mouse vasa deferentia at picomolar concentrations. In addition, both analogs induce the exchange of GDP for GTP at nanomolar concentrations, and this effect is blocked by the CB1-selective antagonist SR141716A (Rinaldi-Carmona et al., 1994). Taken together, these results strongly support the contention that both ACPA and ACEA are high-affinity agonists of the CB1 receptor.

In contrast, the analogs bind with low affinity to the CB2 receptor and do not affect adenylyl cyclase activity in CHO cells transfected with the CB2 receptor. The calculated potency ratios, based upon the dissociation constants of the ligands for the two receptors, indicate that ACPA has greater than 300-fold selectivity for CB1, and ACEA has greater than 2000-fold selectivity. The only other cannabinoid ligands with reported selectivity of greater than 25-fold are SR141716A (Rinaldi-Carmona et al., 1994; Felder et al., 1995; Showalter et al., 1996), (*R*)-methanandamide (Khanolkar et al., 1996), and arachidonyl-(2'-fluoroethyl)amide (Showalter et al., 1996).

As has been shown previously by other investigators (Selley et al., 1996; Burkey et al., 1997), AEA is significantly less efficacious than both Win 55212-2 and CP55940 when [35 S]GTP γ S binding is used to assess efficacy. AEA has also been shown to be a partial agonist in studies of calcium channel inhibition by the CB1 receptor (Mackie et al., 1993). Neither ACPA nor ACEA behaves as a partial agonist in the [35 S]GTP γ S assay (i.e., produce a lower E_{max} than the full agonist Win 55212-2). Similarly, ACEA and ACPA produce E_{max} values that are not different from CP55940 in the adenylyl cyclase assay carried out using transfected CHO cells. However, in the vas deferens assay, ACPA has a significantly lower E_{max} value than CP55940, which indicates that it is not a fully efficacious agonist in this assay. The E_{max} values of a partial agonist vary from assay to assay due

to differences in the density of receptors; partial agonists are more apparent in preparations that are "receptor limited" or have a low number of spare receptors. In sum, our data suggest an efficacy order of Win 55212-2 $\approx$ CP55940 $\approx$ ACEA > ACPA > AEA.

As has been shown previously, THC and AEA produce significant decreases in rectal temperature following i.v. administration in mice (Adams et al., 1998). This effect is mimicked by ACEA and ACPA. The hypothermia produced by ACEA and ACPA is blocked by the CB1 receptor antagonist SR141716A, supporting the involvement of the CB1 receptor in this effect. Interestingly, the hypothermia produced by AEA was attenuated by SR141716A but did not reach statistical significance. Similarly, Adams et al. (1998) reported that SR141716A reversed the hypothermic effects of Δ^9 -THC and CP55940 but not AEA. It is possible, as the authors of that study point out, that the conversion of AEA to arachidonic acid and its metabolites at high doses may account for some noncannabinoid temperature effects.

ACEA and ACPA have 35- to 50-fold higher affinity for the CB1 receptor than AEA, yet produce the same degree of hypothermia in vivo at a dose of 1 mg/kg. These data suggest that bioavailability plays a significant role in the in vivo potency of ACEA and ACPA. Indeed, Willoughby et al. (1997) have demonstrated that only a small fraction of [3 H]AEA administered to mice is found in the brain. They found that AEA was very rapidly degraded to arachidonic acid and other more polar metabolites. It is very likely that the same is true of ACPA and ACEA; both analogs inhibit the hydrolysis of AEA to arachidonic acid and ethanolamine (data not shown), which suggests they may be substrates of the amidohydrolase that catabolizes AEA (Deutsch and Chin 1993; Desarnaud et al., 1995; Hillard et al., 1995).

In summary, both ACPA and ACEA are high-affinity agonists of the CB1 receptor. In contrast, they are very poor ligands for the CB2 receptor and, therefore, represent the most selective CB1 agonist currently available. Both analogs produce hypothermia in vivo that is reversed by the CB1-selective antagonist SR141716A. These analogs represent the most selective ligands for the CB1 receptor over the CB2 receptor yet reported and provide interesting lead compounds for further structure activity studies.

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References

- Adams IB, Compton DR and Martin BR (1998) Assessment of anandamide interaction with the cannabinoid brain receptor: SR 141716A antagonism studies in mice and autoradiographic analysis of receptor binding in rat brain. *J Pharmacol Exp Ther* **284**:1209–1217.
- Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecqueur F, Lupker J, Maffrand J-P, Fur GL and Casellas P (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. *J Biol Chem* **272**:22330–22339.
- Breivogel C, Sim L and Childers S (1997) Regional differences in cannabinoid receptor/G-protein coupling in rat brain. *J Pharmacol Exp Ther* **282**:1632–1642.
- Burkey TH, Quock RM, Consroe P, Ehler FJ, Hosohata Y, Roeske WR and Yamamura HI (1997) Relative efficacies of cannabinoid CB1 receptor agonists in the mouse brain. *Eur J Pharmacol* **336**:295–298.
- Cheng YC and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**:711–715.
- Childers S, Sexton T and Roy M (1994) Effects of anandamide on cannabinoid receptors in rat brain membranes. *Biochem Pharmacol* **47**:711–715.
- Desarnaud F, Cadas H and Piomelli D (1995) Anandamide amidohydrolase activity in rat brain microsomes. *J Biol Chem* **270**:6030–6035.
- Deutsch D and Chin S (1993) Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem Pharmacol* **46**:791–796.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (Wash DC)* **258**:1946–1949.
- Dewey WL (1986) Cannabinoid pharmacology. *Pharmacol Rev* **38**:151–178.
- Edgemond WS, Hillard CJ, Falck JR, Kearn CS and Campbell WB (1998) Human platelets and polymorphonuclear leukocytes synthesize oxygenated derivatives of arachidonyl ethanolamine (anandamide): Their affinities for cannabinoid receptors and pathways of inactivation. *Mol Pharmacol* **54**:180–188.
- Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma AL and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB1 and CB2 receptors. *Mol Pharmacol* **48**:443–450.
- Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, DeCosta BR and Rice KC (1990) Cannabinoid receptor localization in brain. *Proc Natl Acad Sci USA* **87**:1932–1936.
- Hillard C and Campbell W (1997) Biochemistry and pharmacology of arachidonyl ethanolamine, a putative endogenous cannabinoid. *J Lipid Res* **38**:2383–2398.
- Hillard CJ, Edgemond WS and Campbell WB (1995) Characterization of ligand binding to the cannabinoid receptor of rat brain membranes using a novel method: Application to anandamide. *J Neurochem* **64**:677–683.
- Hillard C, Wilkison D, Edgemond W and Campbell W (1995) Characterization of the kinetics and distribution of *N*-arachidonyl ethanolamine (anandamide) hydrolysis by rat brain. *Biochim Biophys Acta* **1257**:249–256.
- Huffman JW, Yu S, Liddle J, Wiley JL, Abood ME, Martin BR and Aung MM (1998) 1-Deoxy-1',1'-dimethylalkyl- Δ^8 -THC derivatives: Selective ligands for the CB2 receptor. 1998 Symposium on the Cannabinoids, La Grande Motte, France, International Cannabinoid Research Society, Burlington, VT.
- Huffman JW, Yu S, Showalter V, Abood ME, Wiley JL, Compton DR, Martin BR, Bramblett RD and Reggio PH (1996) Synthesis and pharmacology of a very potent cannabinoid lacking a phenolic hydroxyl with high affinity for the CB2 receptor. *J Med Chem* **39**:3875–3877.
- Kearn CS and Hillard CJ (1998) Evidence for peripheral-type cannabinoid receptors (CB2) on rat microglial cells. *NIDA Res Monogr Ser* **178**:93.
- Khanolkar AD, Abadi V, Lin S, Hill WAG, Taha G, Abouzid K, Meng Z, Fan P and Makryannis A (1996) Head group analogs of arachidonyl ethanolamine, the endogenous cannabinoid ligand. *J Med Chem* **39**:4514–4519.
- Kuster J, Stevenson J, Ward S, D'Ambra T and Haycock D (1993) Aminoalkylindole binding in rat cerebellum: Selective displacement by natural and synthetic cannabinoids. *J Pharmacol Exp Ther* **264**:1352–1363.
- Mackie K, Devane WA and Hille B (1993) Anandamide, an endogenous cannabinoid, inhibits calcium currents as a partial agonist in N18 neuroblastoma cells. *Mol Pharmacol* **44**:498–503.
- Matsuda LA (1997) Molecular aspects of cannabinoid receptors. *Crit Rev Neurobiol* **11**:143–166.
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature (Lond)* **346**:561–564.
- Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature (Lond)* **365**:61–65.
- Pertwee RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* **74**:129–180.
- Pertwee RG, Stevenson LA, Elrick DB, Mechoulam R and Corbett AD (1992) Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. *Br J Pharmacol* **105**:980–984.
- Pertwee R, Stevenson L and Griffin G (1993) Cross-tolerance between delta-9-tetrahydrocannabinol and the cannabinomimetic agents, CP 55,940, WIN 55,212-2 and anandamide. *Br J Pharmacol* **110**:1483–1490.
- 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 and LeFur G (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Letts* **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 and Le Fur G (1998) SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther* **284**:644–650.
- Selley D, Stark S, Sim L and Childers S (1996) Cannabinoid receptor stimulation of guanosine-5'-O-(γ -[35S]thio)triphosphate binding in rat brain membranes. *Life Sci* **59**:659–668.
- Showalter VM, Compton DR, Martin BR and Abood ME (1996) Evaluation of binding in a transfected cell line expressing a peripheral cannabinoid receptor (CB2): Identification of cannabinoid receptor subtype specific ligands. *J Pharmacol Exp Ther* **278**:989–999.
- Slipetz DM, O'Neill GP, Favreau L, Dufresne C, Gallant M, Gareau Y, Guay D, Labelle M and Metters KM (1995) Activation of the human peripheral cannabinoid receptor results in inhibition of adenylyl cyclase. *Mol Pharmacol* **48**:352–361.
- Smith PB, Compton DR, Welch SP, Razdan RK, Mechoulam R and Martin BR (1994) The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *J Pharmacol Exp Ther* **270**:219–227.
- Wieland T and Jakobs K (1994) Measurement of receptor-stimulated guanosine 5'-O-(γ -thio)triphosphate binding by G proteins. *Methods Enzymol* **237**:3–26.
- Willoughby KA, Moore SF, Martin BR and Ellis EF (1997) The biodisposition and metabolism of anandamide in mice. *J Pharmacol Exp Ther* **282**:243–247.

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