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# Antiemetic and motor-depressive actions of CP55,940: cannabinoid $CB_1$ receptor characterization, distribution, and G-protein activation

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**Abstract**

Dibenzopyran ($\Delta^9$-tetrahydrocannabinol) and aminoalkylindole [*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrolol[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl) methanone mesylate; (WIN55,212-2)] cannabinoids suppress vomiting produced by cisplatin via cannabinoid $CB_1$ receptors. This study investigates the antiemetic potential of the "nonclassical" cannabinoid CP55,940 [1α,2β-(*R*)-5α]-(−)-5-(1,1-dimethyl)-2-[5-hydroxy-2-(3-hydroxypropyl) cyclohexyl-phenol] against cisplatin-induced vomiting and assesses the presence and functionality of cannabinoid $CB_1$ receptors in the least shrew (*Cryptotis parva*) brain. CP55,940 (0.025–0.3 mg/kg) reduced both the frequency of cisplatin-induced emesis ($ID_{50}$ = 0.025 mg/kg) and the percentage of shrews vomiting ($ID_{50}$ = 0.09 mg/kg). CP55,940 also suppressed shrew motor behaviors ($ID_{50}$ = 0.06– 0.21 mg/kg) at such doses. The antiemetic and motor-suppressant actions of CP55,940 were countered by SR141716A [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide], indicating both effects are cannabinoid $CB_1$ receptor-mediated. Autoradiographic studies with [$^3$H]-SR141716A and [$^{35}$S]-GTPγS binding revealed that the distribution of the cannabinoid $CB_1$ receptor and its activation pattern are similar to rodent brain and significant levels are present in brain loci (e.g., nucleus tractus solitarius (NTS)) that control emesis. The affinity rank order of structurally diverse cannabinoid ligands for cannabinoid $CB_1$ receptor in shrew brain is similar to rodent brain: HU-210 = CP55,940 = SR141716A ≥ WIN55,212-2 ≥ delta-9-tetrahydrocannabinol>methanandamide = HU-211 = cannabidiol = 2-arachidonoylglycerol. This affinity order is also similar and is highly correlated to the cannabinoid $EC_{50}$ potency rank order for GTPγS stimulation except WIN55,212-2 and delta-9-tetrahydrocannabinol potency order were reversed. The affinity and the potency rank order of tested cannabinoids were significantly correlated with their antiemetic $ID_{50}$ potency order against cisplatin-induced vomiting (CP55,940>WIN55,212-2 = delta-9-tetrahydrocannabinol) as well as emesis produced by 2-arachidonoylglycerol or SR141716A (CP55,940>WIN55,212-2>delta-9-tetrahydrocannabinol).


*Keywords:* Vomiting; Locomotor activity; CP55,940; SR141716A; Cannabinoid $CB_1$ receptor; (Least shrew); G-protein; Potency; Efficacy

## 1. Introduction

Although the antiemetic potential of cannabinoids has been recognized for decades (Darmani, 2002b), the receptor mechanism by which cannabinoid receptor agonists (delta-9-tetrahydrocannabinol and WIN55,212-2 [(*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrolol[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl) methanone mesylate]) prevent vomiting was only recently ascertained (Darmani, 2001a,b,c; Simoneau et al., 2001; Van Sickle et al., 2001). At least two types of cannabinoid receptors, $CB_1$ and $CB_2$, have been cloned and the cannabinoid $CB_1$ receptor is the predominant cannabinoid receptor in the brain (Pertwee, 1999). Both cannabinoid receptors belong to the superfamily of G-protein-coupled membrane receptors. At least two endogenous ligands (endocannabinoids) for both cannabinoid $CB_1$ and $CB_2$ receptors, arachidonoylethanolamide (anandamide) and 2-arachidonoylglycerol, have been isolated and characterized (Giuffrida and Piomelli, 2000; Sugiura and Waku, 2000). The cannabinoid $CB_1$ receptor antagonist SR141761A

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[*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide] has been shown not only to reverse the antiemetic action of cannabinoids, but can also directly induce vomiting at high doses (Darmani, 2001a,b,c). These findings suggest an important role for endocannabinoids in emetic circuits. Indeed, anandamide and its more stable analog methanandamide appears to possess weak antiemetic effects (Darmani, 2002a; Van Sickle et al., 2001), whereas 2-arachidonoylglycerol is a potent emetogenic endocannabinoid (Darmani, 2002a).

Four chemically diverse groups of cannabinoid receptor agonists exist (Pertwee, 1999). Delta-9-tetrahydrocannabinol is a member of the "classical cannabinoids," whereas CP55,940 ([1α,2β-(*R*)-5α]-(−)-5-(1,1-dimethyl)-2-[5-hydroxy-2-(3-hydroxypropyl) cyclohexyl-phenol]) represents the "nonclassical cannabinoid" group. The aminoalkylindole cannabinoids comprise the pravadolline derivatives such as WIN55,212-2. The fourth group constitute arachidonic acid derivatives such as anandamide and 2-arachidonoylglycerol. Very little is known regarding the antiemetic structure activity relationship among different cannabinoids. Although WIN55,212-2 and delta-9-tetrahydrocannabinol have a similar antiemetic potency against cisplatin-induced emesis in the least shrew (*Cryptotis parva*) (Darmani, 2001b,c), WIN55,212-2 is four to nine times more effective than delta-9-tetrahydrocannabinol in preventing SR141716A- and 2-arachidonoylglycerol-induced vomiting in this insectivore species (Darmani, 2001a, 2002a). The antiemetic potency of the nonclassical cannabinoid CP55,940 against cisplatin-induced vomiting remains unknown.

The present work was undertaken to investigate the antiemetic potential of CP55,940 in the least shrew. The antiemetic action of cannabinoids in patients may be related to their sedative effects and/or production of a "psychological high" (Chang et al., 1979; Lucas and Laszlo, 1980; Sallan et al., 1980). Because the least shrew is very active and does not come to rest after acclimation to their environment, this species provides a good opportunity to evaluate whether the possible antiemetic activity of CP55,940 occurs at its motor-depressive doses. The cannabinoid $CB_1$ receptor appears to be the mediator of all the central nervous system (CNS) actions of cannabinoids because: (1) the structure activity relationship for the rodent tetrad of behaviors and receptor affinity is highly correlated (Compton et al., 1993); (2) the neurochemical localization of $CB_1$ receptors corresponds well with the central nervous system (CNS)-mediated effects of cannabinoids (Breivogel and Childers, 1998); and (3) most of the CNS-mediated actions of cannabinoids as well as their antiemetic effects are reversed by cannabinoid $CB_1$ receptor-selective antagonists (Compton et al., 1996; Darmani, 2001b,c, 2002a; Van Sickle et al., 2001). Furthermore, it has been suggested that delta-9-tetrahydrocannabinol and WIN55,212-2 inhibit emesis in the ferret via brain stem cannabinoid $CB_1$ receptors (Van Sickle et al., 2001).

The initial purpose of the current study was to determine whether the possible antiemetic effect of the "nonclassical cannabinoid" agonist CP55,940 is independent of its motor-suppressant actions, and whether these effects could be reversed by the cannabinoid $CB_1$ receptor-selective antagonist SR141716A. We further conducted a series of parallel biochemical studies designed to characterize both the distribution and pharmacology of cannabinoid $CB_1$ receptors as well as receptor-mediated G-protein activity of several representatives of the cited four classes of cannabinoid agonists using radioligand receptor binding and agonist-stimulated [$^{35}$S] guanosine-5′-*O*-(3-thio) triphosphate ([$^{35}$S] GTPγS) autoradiography in shrew brain sections and membrane homogenate binding assays (Sim et al., 1995; Breivogel et al., 1997). Thus, the antiemetic, motor-suppressant and cannabinoid $CB_1$ receptor affinity and functional activity of diverse cannabinoids could be compared and contrasted in this vomiting species.

## 2. Materials and methods

### 2.1. Animals and drugs

Shrews (*C. parva*) were bred and maintained in the animal facilities of the Kirksville College of Osteopathic Medicine. Both male and female shrews (4–6 g, 35–60 days old) were used throughout the study. The feeding and maintenance of shrews are fully described elsewhere (Darmani, 1998; Darmani et al., 1999). *R*(+)-WIN55,212-2 [*R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrolol[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl) methanone mesylate], *cis*-platinum (II) diamine dichloride ($Pt(NH_3)_2Cl_2$), delta-9-tetrahydrocannabinol, cannabinol, *R*(+) methanandamide, and GDP (for autoradiography) were purchased from Sigma/RBI (Natick, MA) or Biomol Research Laboratories (Plymouth Meeting, PA). SR 141716A [*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide], SR 144528 [*N*-[(1*S*)-endo-1,3,3-trimethylbicyclo [2.2.1] heptan-2-yl]5-(4-chloro-3-methylphenyl)-1-(4-methybenzyl) pyrazole-3-carboxamide] and [$^3$H] SR141716A (22.4 Ci/mmol) were generously donated by Sanofi Recherche (Montpellier, France) or NIDA. CP55,940 [1α,2β-(*R*)-5α]-(−)-5-(1,1-dimethyl)-2-[5-hydroxy-2-(3-hydroxypropyl) cyclohexyl-phenol] was provided by Pfizer (Groton, CT). HU-210 and HU-211 were a generous gift from Prof. Raphael Mechoulam (Hebrew University, Jerusalem, Israel). [$^{35}$S] Gaunosine-5′-*O*-(3-thio) triphosphate) ([$^{35}$S] GTPγS) (1250 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [$^3$H] CP55,940 (180 Ci/mmol) was obtained from Amersham Life Sciences (Arlington Heights, IL). GDP and GTPγS were purchased from Boehringer Mannheim (New York, NY). All other reagent grade chemicals and enzymes were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). For behavioral studies,

all drugs were initially dissolved to twice the stated drug concentrations in a 1:1:18 solution of ethanol:emulphor:0.9% saline. The drug concentrations were then diluted by the addition of an equal volume of saline. This procedure was necessary because the 1:1:18 vehicle mixture can cause emesis in up to 20% of animals by itself. The final vehicle mixture induced emesis in up to 10% of shrews. All drugs were administered at a volume of 0.1 ml/10 g of body weight. All animals received care according to the "Guide for the Care and Use of Laboratory Animals," DHSS Publication, revised, 1985.

### 2.2. Emesis studies

The present protocols were based upon our previous emesis studies (Darmani, 1998, 2001a,b,c; Darmani et al., 1999). On the test day, the shrews were transferred to the experimental room and were allowed to acclimate for at least 1 h prior to experimentation. To habituate the shrews to the test environment, each animal was randomly selected and transferred to a 20 × 18 × 21 cm clean clear plastic cage and offered four meal worms (*Tenebrio* sp.) 30 min prior to experimentation. To evaluate whether CP55,940 may induce emesis, different groups of shrews were injected intraperitoneally (i.p.) with vehicle ($n = 10$) or varying doses of CP55,940 (0, 0.025, 0.05, 0.1, and 0.3 mg/kg; 8–10 shrews/group). Immediately following injection, each shrew was placed in the observation cage and the frequency of vomiting (oral ejections of food or liquid; mean ± S.E.M.) was recorded for each individual shrew for the next 60 min.

To determine the antiemetic potential of CP55,940, a 20 mg/kg, i.p. dose of cisplatin was chosen to induce emesis (Darmani, 1998, 2001b,c). Shrews were offered four meal worms prior to drug administration. Different groups of shrews were injected i.p. with varying doses of CP55,940 (0, 0.025, 0.1, and 0.3; $n = 10–14$ per group) and a 20 mg/kg i.p. dose of cisplatin. Each shrew was then observed individually for the next 60 min immediately following the two simultaneous injections. The frequency of emesis was recorded as described above. A control group ($n = 11$) received two corresponding vehicle injections and were observed in an identical fashion. To demonstrate whether the antiemetic effects of CP55,940 is a cannabinoid $CB_1$ receptor-mediated event, nonemetic subcutaneous doses of SR141716A (1–10 mg/kg) were used to reverse the antiemetic effect of a fully effective dose of CP55,940 (0.3 mg/kg) against cisplatin (20 mg/kg, i.p.)-induced vomiting. Thus, at time 0, different groups of shrews were injected subcutaneously with either vehicle ($n = 10$) or varying doses of SR141716A (1, 5, and 10 mg/kg) and were then offered four meal worms. Ten minutes later, each shrew received i.p. CP55,940 (0.3 mg/kg) and cisplatin (20 mg/kg), and the emesis frequency was recorded for the next 60 min as described above. A 10 mg/kg subcutaneous dose of the cannabinoid $CB_2$ receptor antagonist SR144528 was also used to reverse the antiemetic effect of a 0.3 mg/kg dose of CP55,940 on cisplatin-induced emesis in the same manner as described for the cannabinoid $CB_1$ receptor antagonist.

### 2.3. Locomotor studies

On the test day, shrews were brought in their home cages from animal quarters and were allowed to acclimate for at least 1 h to a semidark environment. The reduced light condition was necessary for the computerized video tracking, motion analysis, and behavior recognition system [Ethovision (version 2.0), Noldus Information Technology, Costerweg, Netherlands] to work efficiently. The parameters of Ethovision were set to record the following triad of locomotion activities: (1) spontaneous locomotor activity in terms of the total distance moved in meters [moving was recorded when a shrew traveled a distance greater than 2 cm in the plane of the observation cage]; (2) total duration of movement in seconds [the total time recorded for any type of movement]; and (3) rearing frequency [a rearing event is recorded as a 20% decrease in surface area when shrews stand upright as seen by the overhead video camera (Darmani, 2001b)].

After acclimation to the dark laboratory environment, shrews were further acclimated in white plastic dummy observation cages (28 × 28 × 14 cm) for 1 h prior to testing. In the first experiment, different groups of shrews were injected intraperitoneally with either vehicle ($n = 12$) or varying doses of CP55,940 (0.025, 0.05, 0.1, 0.3, and 0.6 mg/kg, $n = 7–8$ per group). Then each shrew was individually placed in an observation cage of the same dimension and the discussed locomotor parameters were recorded for 50 min starting at the 10 min postinjection period. CP55,940 significantly reduced all 3 parameters in shrews at its 0.3 mg/kg dose. In the next experiment, the effect of different subcutaneous doses of SR141716A was investigated on the locomotor-reducing properties of the 0.3 mg/kg dose of CP55,940. Thus, at time 0, different shrews were injected with varying subcutaneous doses of SR141716A (0, 0.25, 1, and 5 mg/kg; $n = 8–15$ per group). At 30 min, each shrew received intraperitoneally a 0.3 mg/kg dose of CP55,940. Ten minutes later (i.e., at 40 min), the locomotor activity parameters were recorded for the next 50 min as described earlier.

### 2.4. [$^3$H]SR141716A autoradiography

Shrew brains were obtained by rapid decapitation and were immediately frozen and stored at −80° until use. Horizontal sections of brains from five shrews were cut on a cryostat and thaw mounted onto gelatin-coated slides. For assay, slides were brought to room temperature and rinsed in 50 mM Tris–HCl, pH 7.4, with 3 mM $MgCl_2$, 0.2 mM EGTA, and 100 mM NaCl (assay buffer) for 20 min at 30 °C. Slides were then incubated in assay buffer containing 0.8 nM [$^3$H] SR141716A at 30 °C for 90 min. Nonspecific binding was assessed with 5 μM unlabeled SR141716A.

Slides were rinsed four times for 10 min each in assay buffer at 25 °C, then for 30 s in deionized water on ice. Slides were dried and exposed to Kodak Biomax MS film in the presence of [$^3$H] microscales for 12 weeks. Binding values were converted to fmol/mg protein based on specific activity of the isotopes and the ratio of mg protein/mg tissue.

### 2.5. Agonist-stimulated [$^{35}$S]GTPγS autoradiography

Autoradiography for basal and CP55,940-stimulated [$^{35}$S]GTPγS binding was performed as previously described (Sim et al., 1995). Slides were equilibrated in assay buffer for 10 min at 25 °C. Slides were then incubated for 15 min in assay buffer with 0.5% bovine serum albumin, 2 mM GDP, and 9.5 mU/ml adenosine deaminase at 25 °C. This was followed by incubation in 0.04 nM [$^{35}$S]GTPγS, 2 mM GDP, and adenosine deaminase with CP55,940 (1 μM), or ethanol vehicle (basal) in assay buffer at 25 °C for 2 h. Slides were rinsed twice for 2 min each in 50 mM Tris–HCl buffer (pH 7.4) at 4 °C, then washed for 30 s in deionized $H_2O$ at 4 °C. Slides were dried completely and exposed to Kodak X-O-mat film in the presence of [$^{14}$C] microscales for 5 days. Films were digitized with a Sony XC-77 video camera and analyzed using the NIH Image program for Macintosh computers. Resulting densitometric values were corrected for [$^{35}$S] based upon incorporation of [$^{35}$S] into sections of frozen brain paste (Sim et al., 1996). Binding values were converted to fmol/mg protein based on specific activity of the isotope and the ratio of mg protein/mg tissue.

### 2.6. [$^3$H]cannabinoid binding assays

Each shrew brain was homogenized with a Tissumizer (Tekmar, Cincinnati, OH) in cold membrane buffer (50 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA) and centrifuged at 48,000 × $g$ for 10 min at 4 °C. Pellets were resuspended in binding buffer, then centrifuged again at 48,000 × $g$ for 10 min at 4 °C. Pellets from second centrifugation were homogenized in binding buffer, then assayed for protein content before addition to assay tubes. Saturation analysis assays were conducted at 30 °C for 1 h in membrane buffer including 12 μg ([$^3$H]SR141716A) or 25 μg ([$^3$H]CP55,940) membrane protein with 0.1% (w/v) bovine serum albumin and 0.03–7.5 nM [$^3$H]SR141716A or 0.02–4.0 nM [$^3$H]CP55,940 in a final volume of 1 ml. Nonspecific binding was determined using 500 nM unlabeled SR141716A or CP55,940, respectively. $K_i$ values for other ligands were determined using the same assay conditions as for [$^3$H]CP55,940 saturation analysis except that 0.5 nM [$^3$H]CP55,940 and various concentrations of each other ligand were used. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by five washes with cold Tris–HCl buffer, pH 7.4, containing 0.05% (w/v) bovine serum albumin. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency for $^3$H after 1 h shaking of the filters in 4 ml BudgetSolve scintillation fluid.

### 2.7. Agonist-stimulated [$^{35}$S]GTPγS binding assays

Each shrew brain was homogenized with a Tissumizer (Tekmar, Cincinnati, OH) in cold membrane buffer (50 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.7) and centrifuged at 48,000 × $g$ for 10 min at 4 °C. Pellets were resuspended in membrane buffer, then centrifuged again at 48,000 × $g$ for 10 min at 4 °C. Pellets from second centrifugation were homogenized in membrane buffer and preincubated for 10 min at 30 °C in 0.004 units/ml adenosine deaminase (240 units/mg protein, Sigma) to remove endogenous adenosine, then assayed for protein content before addition to assay tubes. Assays were conducted at 30 °C for 1 h in membrane buffer including 10 μg membrane protein with 0.1% (w/v) bovine serum albumin, 30 μM GDP, 0.10 nM [$^{35}$S]GTPγS, and various concentrations of cannabinoid ligands in a final volume of 1.0 ml. Concentration–effect assays for WIN55,212-2 in the presence of 1 μM SR141716A also included a triplicate of 100 μM WIN55,212-2 in the absence of SR141716A. Nonspecific binding was determined in the absence of agonist and the presence of 30 μM unlabeled GTPγS. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with cold Tris–HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for $^{35}$S after 1 h shaking of the filters in 4 ml BudgetSolve scintillation fluid (RPI, Mount Prospect, IL).

### 2.8. Radioligand-binding analysis

Net agonist-stimulated [$^{35}$S]GTPγS-binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values. Specific binding of [$^3$H]cannabinoid was determined by subtracting binding values obtained in the presence from those obtained in the absence of unlabeled ligand. Data analyses of saturation-binding curves and agonist concentration–effect curves were conducted by iterative nonlinear regression using Prism for Windows (GraphPad Software, San Diego, CA) to obtain $K_D$ and $B_{max}$ values (saturation analysis) or $IC_{50}$, minima and maxima values (displacement assays). $K_i$ values for each ligand were calculated using the following equation: $K_i = IC50/[([L]/K_D) + 1]$, where $[L]$ is the concentration of [$^3$H]CP55,940, and $K_D$ is that determined by saturation analysis of [$^3$H]CP55,940. In the WIN55,212-2 concentration–effect assays that were analyzed for multiple components, data from each assay rack were normalized to the amount of stimulation obtained by a triplicate of 100 μM WIN55,212-2 in the absence of SR141716A. This was accomplished by dividing the net amount of stimulation at each concentration of WIN55,212-2 (in the presence or

absence of SR141716A) by that obtained with 100 μM WIN55,212-2 (in the absence of SR141716A) and multiplying by 100%. These data were then fitted to fixed (Hill slope equal to 1) and variable Hill slope models, and the goodness of fits compared with an *F* test using Prism. Data reported are fits to a one-site model when the data fit better to the model where the Hill slope was set equal to one, and to a two-site model when the data fit better to the variable Hill slope model (in all cases, Hill slopes were less than or equal to one). Data are presented as mean ± S.E.M. obtained from three to five experiments performed in duplicate ([$^{35}$S]GTPγS) or triplicate ([$^{3}$H]cannabinoid assays).

### 2.9. Statistical analysis

The frequency of emesis data were analyzed by the Kruskal–Wallis (KW) nonparametric one-way analysis of variance (ANOVA) and posthoc analysis by Dunn's multiple comparisons test. A $P$ value of $<0.05$ was necessary to achieve statistical significance. The incidence of emesis (number of animals vomiting) was analyzed by the Fisher's exact test to determine whether there were differences between groups. When appropriate, pairwise comparisons were also made by this method. For some emesis data, the two-tailed Mann–Whitney test was used. The $ID_{50}$ values (the inhibitory dose that prevented emesis in 50% of shrews, or the dose which reduced emesis frequency by 50%) were calculated by the use of a computerized program (GraphPad InPlot, San Diego, CA). A one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test were used to analyze the locomotor data.

## 3. Results

### 3.1. Emesis studies

Similar to our previous studies (Darmani, 2001a,b,c), intraperitoneal administration of the final concentration of the special solvent (ethanol: emulphor: saline) produced emesis in up to 10% of shrews. Intraperitoneal administration of 0.025, 0.05, 0.1, and 0.3 mg/kg doses of CP55,940 in the above solvent did not cause significant emesis.

The 20 mg/kg intraperitoneal dose of cisplatin induced vomiting in all of the tested shrews with a mean vomiting frequency of 7.7 ± 1.2 (Fig. 1). The Fisher's exact test showed that CP55,940 reduced the percentage of shrews vomiting in response to cisplatin in a dose-dependent manner with an $ID_{50}$ of 0.09 ± 3.8 mg/kg (Fig. 1A) ($\chi^2(4,52)=33.7$, $P<0.00000006$). Furthermore, relative to the vehicle-injected cisplatin-treated control group, significant reductions [79% ($P<0.0002$) and 90% ($P<0.0001$)] in the number of animals vomiting occurred in the 0.1 and 0.3 mg/kg exposed groups. CP55,940 pretreatment also significantly reduced the frequency of cisplatin-induced vomiting with an $ID_{50}$ value of 0.025 ± 1.3 mg/kg (KW(4,52) = 38.99, $P<0.0001$) (Fig. 1B). The pattern of reduction in the number of vomitings was also dose-dependent (51.9%, 91.7%, and 98.7%, respectively). Although reduction caused by the 0.025 mg/kg dose of CP55,940 failed to attain significance, larger doses of this cannabinoid significantly [0.1 ($P<0.001$), and 0.3 ($P<0.001$) mg/kg] attenuated the vomiting frequency. In addition, both the vehicle plus cisplatin-treated and 0.025 mg/kg CP55,940 plus cisplatin-treated groups were significantly different from vehicle plus vehicle injected group ($P<0.02$) (Fig. 1A,B).

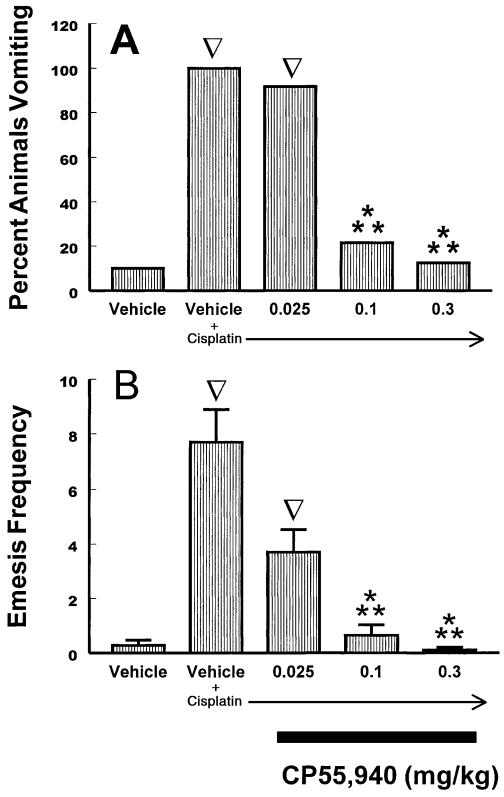


Fig. 1. The antiemetic dose–response effects of CP55,940 on cisplatin-induced emesis in the least shrew. Graph A depicts reduction in the percentage of shrews vomiting, whereas graph B shows attenuation in the frequency (mean ± S.E.M.) of vomiting. Vehicle = a control group which received two vehicle (i.p.) injections. Vehicle + cisplatin = a group receiving an i.p. injection of cisplatin (20 mg/kg) plus an i.p. injection of vehicle. The remaining groups received cisplatin and an i.p. injection of either 0.025, 0.1, or 0.3 mg/kg doses of CP55,940. Emesis parameters were recorded for 60 min post-injection. ***, Significantly different ($P<0.001$) from vehicle + cisplatin group. ∇, Significantly different from vehicle + vehicle group ($P<0.002$).

Fig. 2 describes the ability of subcutaneously administered SR141716A to reverse the antiemetic action of CP55,940 (0.3 mg/kg, i.p.) against cisplatin (20 mg/kg, i.p.)-induced vomiting. In the absence of SR141716A, the 0.3 mg/kg dose of CP55,940 prevented emesis in all shrews tested (Fig. 2A). The percentage of shrews vomiting increased [44.4% ($P>0.05$), 70% ($P<0.003$), and 78% ($P<0.0007$), respectively) in response to preadministration

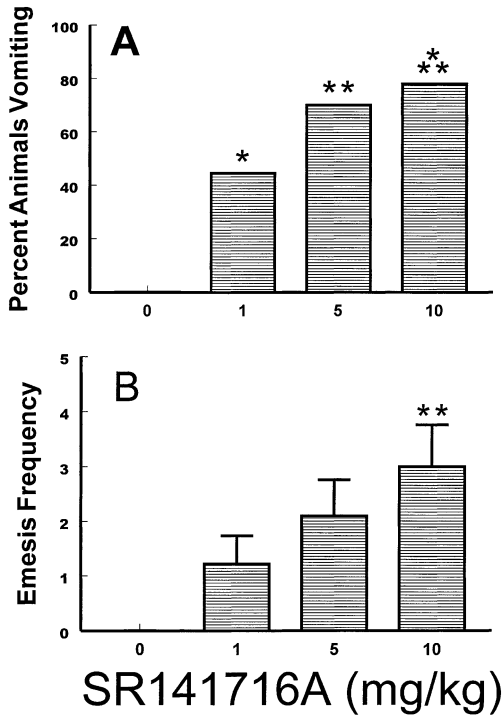


Fig. 2. The ability of different subcutaneous doses of SR141716A (0, 1, 5, and 10 mg/kg) to reverse the antiemetic effects of a 0.3 mg/kg (i.p.) dose of CP55,940 against cisplatin (20 mg/kg, i.p.)-induced emesis. SR141716A blocked the ability of CP55,940 to protect shrews from vomiting (graph A) as well as reversing the CP55,940-induced reduction in emesis frequency (mean ± S.E.M.) (graph B). A time 0, shrews received either vehicle or the cited doses of SR141716A and, 10 min later, CP55,940 plus cisplatin. Emesis parameters were recorded for the next 60 min. Significantly different from the 0.3 mg/kg CP55,940 plus 20 mg/kg cisplatin-treated control group which had received no SR141716A (i.e., column 0) at $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***).

of different (1, 5, and 10 mg/kg) doses of SR141716A with an $ID_{50}$ of $0.9 \pm 1.1$ mg/kg ($\chi^2(3,34)=14.42$, $P<0.012$). The frequency of vomiting also increased (122% ($P>0.05$), 210% ($P<0.05$), and 300% ($P<0.01$) relative to control, respectively) with increasing doses of SR141716A with an $ID_{50}$ of $2.5 \pm 2.3$ mg/kg (KW(3,34) = 13.62, $P<0.0035$) (Fig. 2B). A 10 mg/kg dose of the $CB_2$ antagonist SR144528 failed to alter the ability of a 0.3 mg/kg dose of CP55,940 to prevent cisplatin (20 mg/kg)-induced vomiting. Indeed, in the control group ($n=7$), none of the shrews vomited, whereas only one of the SR144528-injected animals ($n=7$) vomited.

### 3.2. Locomotion

One-way analysis of variance (ANOVA) indicated that intraperitoneal administration of 0.025 to 0.6 mg/kg doses of CP55,940 significantly attenuated spontaneous locomotor activity (i.e., total distance moved) with an $ID_{50}$ of $0.13 \pm 1.18$ mg/kg in the least shrew in the 50-min observation period (Fig. 3A) ($F(5,43)=18.3$, $P<0.0001$). However, the reduction in locomotor activity appears to be biphasic because the 0.1 mg/kg dose failed to induce a significant effect, whereas the 0.05, 0.3, and 0.6 mg/kg doses of CP55,940 produced dose-dependent decreases [32% ($P<0.01$), 96% ($P<0.01$), and 97% ($P<0.01$), respectively) in locomotion. CP55,940 also significantly reduced the total duration of movement in shrews ($ID_{50}=0.21 \pm 1.18$ mg/kg) (Fig. 3B) ($F(5,43)=17.9$, $P<0.0001$). However, significant effects (62% and 76% reduction, $P<0.001$) were only seen at 0.3 and 0.6 mg/kg doses. In a similar manner, CP55,940 administration dose-dependently reduced the frequency of rearings with an $ID_{50}$ of $0.06 \pm 1.2$ mg/kg in the

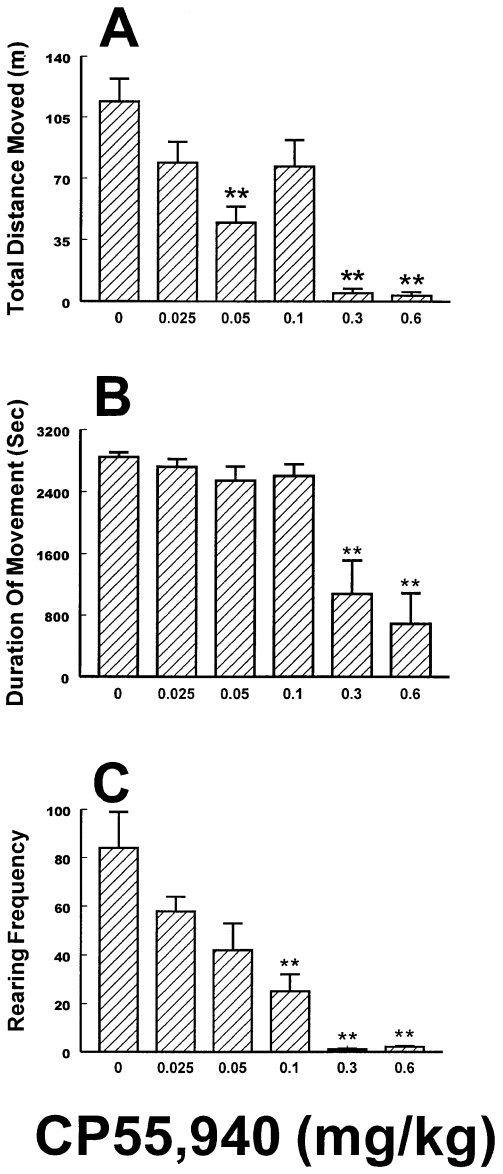


Fig. 3. The dose–response effects of CP55,940 on the triad of motor behaviors (mean ± S.E.M) in the least shrew. The motor behaviors were recorded for 50 min by a computerized video tracking, motion analysis, and behavior recognition system (Ethovision) 10 min after CP55,940 administration. **, Significantly different from vehicle-injected control group at $P<0.01$.

least shrew (Fig. 3C) ($F(5,43)=12.52$, $P<0.0001$). Dunnett's $t$ test revealed that the 0.1 mg/kg (70%, $P<0.001$), 0.3 mg/kg (99%, $P<0.001$), and 0.6 (98%, $P<0.001$) mg/kg doses of CP55,940 caused significant inhibition. Vomiting by itself did not appear to reduce any of the cited motor parameters because the duration of vomiting (2–4 s/vomit) is negligible relative to the total duration of motor observation (i.e., 3000 s).

Fig. 4 represents the ability of the cited subcutaneous doses of SR141716A to reverse the motor inhibitory effects of a 0.3 mg/kg intraperitoneal dose of CP55,940. Also shown in this figure is an additional control representing the possible effects of the vehicle injections (i.e., one i.p. and one s.c.). The cited doses of SR141716A reversed the inhibitory action of CP55,940 on the cited motor parameters in a dose-dependent fashion. Indeed, relative to the control group (i.e., 0 mg/kg SR141716A + 0.3 mg/kg CP55,940), SR141716A significantly reversed the CP55,940-induced suppression of spontaneous locomotor activity, and significant reversals [626% ($P<0.01$) and 797% ($P<0.05$), respectively] occurred at the 0.25 and 1 mg/kg doses ($F(3,30)=5.3$, $P<0.004$) (Fig. 4A). Although the 5 mg/kg dose of SR141716A also tended to reverse the induced motor suppression, the reversal did not attain significance. Varying doses of SR141716A (0.25, 1, and 5 mg/kg) also reversed [99% ($P<0.05$), 156% ($P<0.01$), and 115% ($P<0.05$), respectively] the ability of CP55,940 to reduce the duration of movement (Fig. 4B) ($F(3,30)=6.49$, $P<0.0016$). Likewise, SR141716A (0.25, 1 and 5 mg/kg) pretreatment reversed the inhibition of the rearing behavior produced by CP55,940 (Fig. 4C) ($F(3,30)=7.5$, $P<0.0007$). However, partial reversals were seen at the 1 mg/kg (1400%, $P<0.01$) and 5 mg/kg (1395%, $P<0.01$) doses.

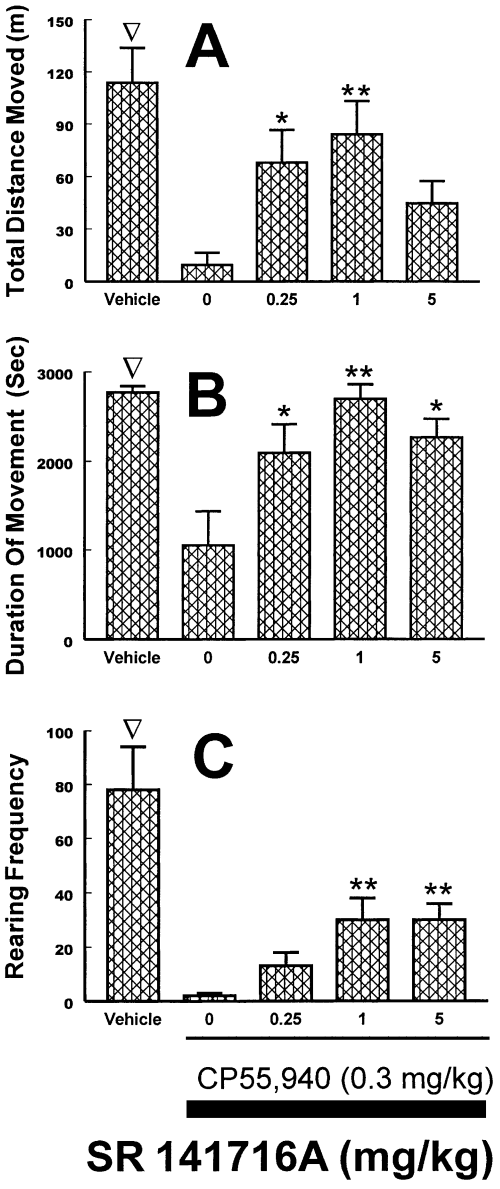


Fig. 4. The ability of s.c. doses of SR141716A (0, 0.25, 1, and 5 mg/kg) to reverse the motor depressant effects of a 0.3 mg/kg (i.p.) dose of CP55,940 on the cited triad of motor parameters. Vehicle = a control group which had received both an i.p. and s.c. injections of vehicle; 0 = a control group which received an 0.3 mg/kg (i.p.) dose of CP55,940 plus a subcutaneous injection of vehicle. Significantly different from control (0) at $P<0.05$ (*) and $P<0.01$ (**). Data shown are mean ± S.E.M.

### 3.3. Receptor ligand-binding and [$^{35}$S] GTPγS studies

This study characterized the distribution and pharmacology of cannabinoid receptors and receptor-mediated G-protein activity in shrew brain using receptor- and agonist-stimulated [$^{35}$S]GTPγS binding in brain sections and membrane homogenates. Autoradiographic results showed that [$^{3}$H]SR141716A- and CP55,940-stimulated [$^{35}$S]GTPγS bindings exhibited similar distributions to those previously seen in rat and mouse (Fig. 5). [$^{3}$H]SR141716A- and CP55,940-stimulated [$^{35}$S]GTPγS bindings in brain sections were analyzed using computer-assisted densitometry to determine the levels of binding in several brain regions (Table 1). The highest levels of cannabinoid receptors and receptor-activated G-proteins were found in the cerebellum (molecular layer) and hippocampus. Moderate levels of binding were found in the cortex and striatum. [$^{3}$H]SR141716A- and CP55,940-stimulated [$^{35}$S]GTPγS bindings were low in the pons and medulla, with the exception of the nucleus tractus solitarius (NTS) (Fig. 5). Basal [$^{35}$S]GTPγS binding was also high in the NTS, with levels approximately twice as high as other regions measured (data not shown). [$^{3}$H]SR141716A binding was not evaluated in the NTS due to the limited number of sections available. In general, the distribution and relative levels of [$^{3}$H]SR141716A binding were similar to that seen for CP55,940-stimulated [$^{35}$S]GTPγS binding (Table 1). The one exception was the cortex, where CP55,940-stimulated [$^{35}$S]GTPγS binding appeared higher relative to [$^{3}$H]SR141716A binding than in other regions. This difference was most apparent in the deep layers of the cortex (Fig. 5).

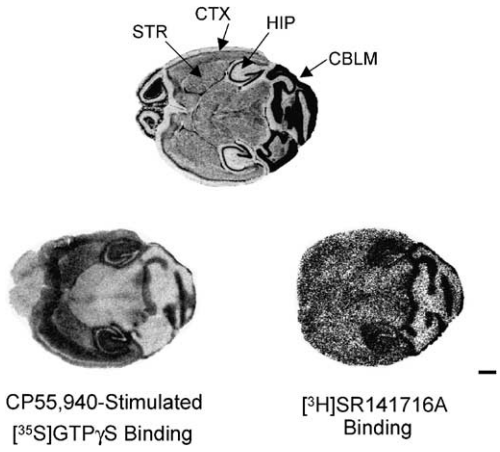


Fig. 5. Cannabinoid-stimulated [$^{35}$S]GTPγS and receptor binding in horizontal sections of shrew brain. Autoradiography was performed using CP55,940 to stimulate [$^{35}$S] GTPγS binding (bottom left) and [$^{3}$H] SR141716A (bottom right) as described in Methods. The highest levels of binding are visible in the cerebellum (CBLM) and hippocampus (HIP), with moderate levels in cortex (CTX), striatum (STR), and nucleus tractus solitarius. These sections are identified on a near-adjacent Nissel-stained section (top row). Bar = 0.7 mm.

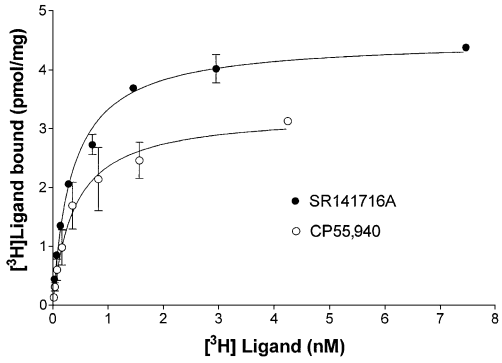


Fig. 6. Saturation analysis of the binding of [$^{3}$H]SR141716A and [$^{3}$H]CP55,940 to whole shrew brain membrane homogenates. Various concentrations of each ligand were incubated with membranes in the presence and absence of 500 nM unlabelled ligand. Values were obtained by subtracting nonspecific binding of each ligand (determined in the presence of unlabelled ligand) from total binding (obtained in the absence of unlabelled ligand). Curves were fit by nonlinear regression analysis of the data as described in Methods. Data shown are mean ± S.E.M. from binding experiments done in three different shrew brain homogenates.

Receptor- and agonist-stimulated [$^{35}$S]GTPγS binding were also measured in membrane homogenates prepared from whole shrew brain. Receptor density was determined by saturation analysis of both [$^{3}$H]SR141716A and [$^{3}$H]CP55,940 binding (Fig. 6). Values obtained for $B_{max}$ were $4.5 \pm 0.2$ and $3.2 \pm 0.2$ pmol/mg, respectively. $K_D$ values for each were $0.46 \pm 0.1$ and $0.43 \pm 0.1$ nM, respectively. Both absolute and relative receptor-binding values between [$^{3}$H]antagonist (SR141716A) and [$^{3}$H]agonist (CP55,940) were similar to those previously reported in rat brain membranes. Receptor affinity was also determined for a number of other cannabinoid receptor ligands and related compounds including cannabinoids obtained from *Cannabis sativa*, delta-9-tetrahydrocannabinol, and cannabidiol; an analog of the putative endogenous cannabinoid ligand, methanandamide; synthetic cannabinoids WIN 55,212-2 and HU-210; and the inactive isomer of HU-210, HU-211 (Fig. 7). Affinity ($K_i$) values were determined by displacement of [$^{3}$H]CP55,940 binding by various concentrations of each ligand (Table 2). Results indicated that the order of ligand affinity was HU-210 = CP55,940 = SR141716A ≥ WIN55,212-2 = delta-9-tetrahydrocannabinol> methanandamide = HU-211 = cannabidiol = 2-arachidonoylglycerol ($F = 57.04$, $P < 0.0001$).

Each of the above ligands was also assayed for the stimulation of [$^{35}$S]GTPγS binding in whole shrew brain

Table 1
[$^{3}$H]SR141716A- and CP55,940-stimulated [$^{35}$S]GTPγS autoradiography in shrew brain

| Brain region | [$^{3}$H]SR141716A binding (pmol/mg) | CP55,940-stimulated [$^{35}$S]GTPγS (fmol/mg) |
|---|---|---|
| Striatum | 3.42 ± 0.32 | 3.66 ± 0.41 |
| Cortex | 2.67 ± 0.48 | 5.01 ± 0.30 |
| Hippocampus | 4.95 ± 0.43 | 5.97 ± 0.53 |
| Brainstem | 1.87 ± 0.16 | 0.77 ± 0.20 |
| Nucleus tractus solitarius | ND | 2.71 ± 0.58 |
| Cerebellum | 6.80 ± 0.28 | 6.26 ± 0.19 |

Receptor- and agonist-stimulated [$^{35}$S]GTPγS binding were performed as described in Methods. Data represent mean values ± S.E.M. of binding in brains from three to five animals. [$^{3}$H]SR141716A binding is expressed as pmol/mg and agonist-stimulated [$^{35}$S]GTPγS binding is expressed as fmol/mg. ND = not determined.

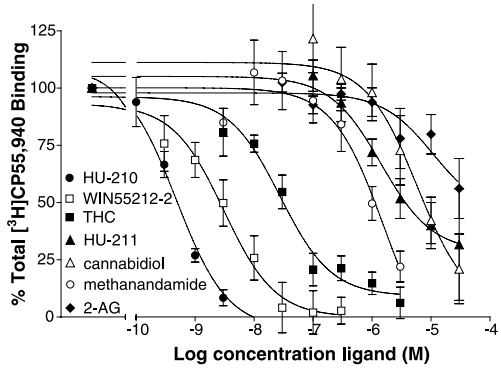


Fig. 7. Determination of the affinity of various cannabinoid- and cannabinoid-related ligands in whole shrew brain membrane homogenates. Various concentrations of each ligand were incubated with 0.5 nM [$^{3}$H]CP55,940. Percent total [$^{3}$H]CP55,940 binding values were obtained by dividing binding obtained at each concentration of ligand by total specific binding values for [$^{3}$H]CP55,940. Curves were obtained by nonlinear regression analysis of the data as described in Methods. Data shown are mean ± S.E.M. from binding experiments done in three different shrew brain homogenates.

Table 2
Cannabinoid receptor- and cannabinoid ligand-stimulated [$^{35}$S]GTPγS binding values in shrew brain membrane homogenates

| | $CB_1$ binding | Ligand-stimulated [$^{35}$S]GTPγS binding | | |
|---|---|---|---|---|
| | log$K_i$ or log$K_D$ | log$EC_{50}$ | $E_{max}$(%) | %HU-210$E_{max}$ |
| HU-210 | − 9.47 ± 0.06 a | − 9.72 ± 0.43 a | 120 ± 5 | 100 ± 0 a |
| CP55,940 | − 9.40 ± 0.12 ab | − 8.28 ± 0.11 b | 105 ± 1 | 110 ± 9 a |
| SR141716A | − 9.38 ± 0.13 ab | ND | ND | ND |
| WIN55,212-2 | − 8.33 ± 0.11 bc | − 6.97 ± 0.40 bc | 104 ± 8 | 90 ± 5 a |
| delta-9-Tetrahydrocannabinol | − 7.43 ± 0.15 c | − 7.44 ± 0.08 b | 53 ± 14 | 45 ± 10 b |
| Methanandamide | − 5.82 ± 0.18 d | − 5.91 ± 0.23 cd | 120 ± 25 | 100 ± 3 a |
| Cannabidiol | 5.74 ± 0.46 d | None | None | None |
| HU-211 | − 5.49 ± 0.16 de | None | None | None |
| 2-Arachidonoylglycerol | − 4.43 ± 0.25 e | − 5.12 ± 0.09 d | 68 ± 4 | 58 ± 2 b |

Receptor binding affinity values were determined either by saturation analysis (SR141716A and CP55,940, yielding log$K_D$ values, see Fig. 6) or displacement of [$^3$H]CP55,940 binding by various concentrations of each (log$K_i$ values, see Fig. 7). Ligand stimulation of [$^{35}$S]GTPγS binding was determined using various concentrations of each ligand in the presence of 0.1 nM [$^{35}$S]GTPγS and 30 μM GDP as described in Methods (see Fig. 9), except for the values for WIN55,212-2, which were determined as described in Methods and in Table 2 and in Fig. 8. log$K_D$, log$K_i$, log$EC_{50}$ and $E_{max}$ values (as percent stimulation of binding over basal values) were determined by nonlinear regression analysis of the data. Relative $E_{max}$ values (%HU-210) for each ligand were calculated as the percent of the $E_{max}$ value obtained with HU-210 in each experiment and pooled. ND = not determined. Values in a column marked with the same letter are not significantly different.

membrane homogenates (Fig. 8 and Table 2). From the concentration–effect curves, $EC_{50}$ and $E_{max}$ values were obtained for each compound except SR141716A, which was not assayed because it is a known $CB_1$ antagonist, and HU-211 and cannabidiol, which were assayed but exhibited no activity. The results of agonist stimulation of [$^{35}$S]GTPγS binding were somewhat confounded by the recent discovery that WIN55,212-2 activated a second (non-$CB_1$) G-protein-coupled receptor in mouse (Breivogel et al., 2001), rat and shrew brain (C.S. Breivogel, B. Parekh, and N.A. Darmani, unpublished observations). Therefore, more complete concentration–effect curves were generated for WIN55,212-2 in the presence and absence of 1 μM SR141716A, a concentration of antagonist sufficient to completely block activation of $CB_1$ by WIN55,212-2 (Fig. 9). Comparison of the fits of the data with Hill slope constrained to 1 and the Hill slope unconstrained indicated that WIN55,212-2 alone stimulated [$^{35}$S]GTPγS binding at more than one site (Hill slope = 0.77 ± 0.02, $F$ test $P < 0.05$), but that in the presence of SR141716A, WIN55,212-2 acted at only one site (Hill slope = 1.1 ± 0.4, $F$ test $P > 0.05$). As stimulation by WIN55,212-2 represents $CB_1$ plus non-$CB_1$-mediated activity, and stimulation by WIN55,212-2 in the presence of SR141716A represents only non-$CB_1$ activity, $CB_1$-only-mediated activity was estimated by subtracting net-stimulated binding values obtained in the presence of SR141716A from those obtained in the absence of SR141716A (Fig. 9). Analysis of the resulting concentration–effect curve indicated a better fit to a one-site than a two-site model, and the Hill slope was 1.2 ± 0.1. All three curves were analyzed by

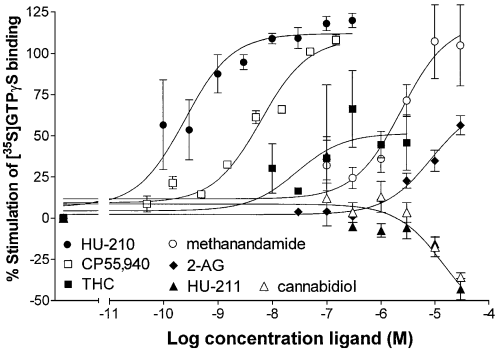


Fig. 8. Determination of the efficacy and potency of various cannabinoid- and cannabinoid-related ligands for the stimulation of [$^{35}$S]GTPγS binding to whole shrew brain membrane homogenates. Various concentrations of each ligand were incubated with 0.1 nM [$^{35}$S]GTPγS. Percent stimulation values were obtained by dividing specific binding values obtained at each concentration of ligand by specific basal binding values obtained in the absence of ligand. Curves were obtained by nonlinear regression analysis of the data as described in Methods. Data shown are mean ± S.E.M. from binding experiments done in three different shrew brain homogenates.

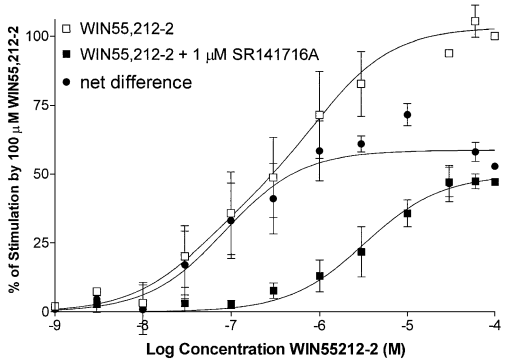


Fig. 9. Determination of the efficacy and potency of WIN55,212-2 for the stimulation of [$^{35}$S]GTPγS binding to whole shrew brain membrane homogenates. Various concentrations of WIN552,212-2 were incubated in the absence and presence of SR141716A with 0.1 nM [$^{35}$S]GTPγS and 30 μM GDP. Values shown are percent of net stimulation obtained with 100 μM WIN55,212-2 in each assay rack. Curves were obtained by nonlinear regression analysis of the data as fit to one- or two- site models as described in Methods.

Table 3
Cannabinoid $CB_1$ receptor-mediated and non-$CB_1$-mediated stimulation of [$^{35}$S]GTPγS binding by WIN55,212-2

| | $E_{max}$ high | $logEC_{50}$ high | $E_{max}$ low | $logEC_{50}$ low |
|---|---|---|---|---|
| WIN55,212-2 | 28 ± 18% | −8.3 ± 0.4 | 104 ± 1.4% | −6.2 ± 0.2 |
| WIN + SR141716A | | | 51 ± 0.2% | −5.5 ± 0.3 |
| Net difference | 60 ± 1.3% | −7.0 ± 0.4 | | |

Ligand stimulation of [$^{35}$S]GTPγS binding was determined by incubating shrew brain membranes with various concentrations of WIN55,212-2 in the presence and the absence of 1 μM SR141716A with 0.1 nM [$^{35}$S]GTPγS and 30 μM GDP as described in Methods and as shown in Fig. 8. The contribution of cannabinoid $CB_1$ receptors to the activity of WIN55,212-2 (Net difference) was estimated by subtracting (prior to nonlinear fitting) values obtained in the presence of SR141716A (non-$CB_1$-mediated) from values obtained in the absence of SR141716A ($CB_1$ plus non-$CB_1$-mediated) at each concentration of WIN55,212-2. $logEC_{50}$ values and $E_{max}$ values (as percent of binding stimulated by 100 μM WIN55,212-2 determined in each assay rack) were determined by nonlinear fitting of the data to a two ("WIN55,212-2") or one ("WIN + SR141716A" and "Net difference") site model. The "high" and "low" following "$E_{max}$" and "$logEC_{50}$" refer to the high and/or the low potency exhibited by WIN55,212-2 in each case. WIN + SR141716A, WIN55,212-2 concentration–effect in the presence of 1 μM SR141716A.

nonlinear regression to appropriate models and results of these analyses are shown in Table 3.

Results indicated that the rank order of ligand potency was similar to that of ligand affinity for those compounds that displayed stimulation of [$^{35}$S]GTPγS binding: HU-210>CP55,940 = delta-9-tetrahydrocannabinol = WIN55, 212-2>methanandamide = 2-arachidonoylglycerol. WIN55, 212-2 and delta-9-tetrahydrocannabinol were reversed in rank order compared to the affinity values, these two compounds were not significantly different from each other in either affinity or potency in these assays. The $E_{max}$ and relative $E_{max}$ values, which were calculated as a percent of the $E_{max}$ value obtained for HU-210 in each experiment prior to averaging the data, are also shown (Table 2). These data indicate the relative $E_{max}$ values for these ligands was methanandamide = CP55,940 = HU-210 = WIN55,212-2>2-arachidonoylglycerol = delta-9-tetrahydrocannabinol. Neither HU-211 nor cannabidiol displayed any activity in shrew brain except to cause decrease in [$^{35}$S]GTPγS binding at very high concentrations (10–30 μM). These compounds also do not display the spectrum of cannabinoid activity in other in vitro and in vivo assays. The effect of decreasing [$^{35}$S]GTPγS binding at high concentrations appears to be a common nonspecific effect of such inactive compounds or cannabinoid antagonists (Christopher Breivogel, unpublished observations).

## 4. Discussion

This study demonstrates dose-dependent antiemetic efficacy of the "nonclassical" cannabinoid prototype CP55,940 against cisplatin-induced emesis in the least shrew model of emesis. CP55,940 appears to be a potent antiemetic cannabinoid because low doses of this agent (0.1–0.3 mg/kg) reduced the vomiting frequency by 92–99%. Indeed, CP55,940 is 13–20 times more potent against cisplatin-induced emesis than WIN55,212-2 or delta-9-tetrahydrocannabinol with the $ID_{50}$ potency rank order: CP55,940> WIN55,212-2 = delta-9-tetrahydrocannabinol (Darmani, 2001b,c). Likewise, CP55,940 is 4–25 times more potent than delta-9-tetrahydrocannabinol and WIN55,212-2 in producing the tetrad of behaviors in mice (Abood and Martin, 1992). As with delta-9-tetrahydrocannabinol and WIN55, 212-2 (Darmani, 2001b,c), the antivomiting effect of CP55,940 appears to be cannabinoid $CB_1$ receptor-mediated because the selective $CB_1$ (SR141716A)—and not the $CB_2$ (SR144528)-receptor antagonist dose-dependently counteracted the antimetic action of an effective dose of CP55,940 in cisplatin-treated shrews. CP55,940 suppressed both the triad of motor (spontaneous locomotor activity, duration of movement, and rearing) and antiemetic parameters at equivalent doses in shrews. However, locomotor suppression per se does not account for the antivomiting effects of other cannabinoids because both delta-9-tetrahydrocannabinol and WIN55,212-2 prevent cisplatin-induced vomiting at lower doses relative to their motor-suppressant actions (Darmani, 2001b,c). The motor-depressant effects of the discussed cannabinoid agonists are also $CB_1$ receptor-mediated because SR141716A dose-dependently reversed the induced effects.

The presence of cannabinoid $CB_1$ receptors in shrew whole brain homogenates was confirmed by saturation analysis of both [$^3$H]-SR141716A and [$^3$H]-CP55,940 binding. The respective absolute and relative cannabinoid $CB_1$ receptor densities (4.5 ± 0.2 and 3.2 ± 0.2 pmol/mg protein) and $K_D$ values (0.46 ± 0.1 and 0.43 ± 0.1 nM) are similar to those previously reported in rat brain homogenates (Breivogel et al., 1997). The regional distribution of cannabinoid $CB_1$ receptors in shrew brain was revealed by autoradiographic studies with [$^3$H]-SR141716A. Highest levels of $CB_1$ receptors were found in the cerebellum and hippocampus, with moderate levels in the cortex and striatum. The distribution of cannabinoid $CB_1$ receptor activation of G-proteins was demonstrated by autoradiography via CP55,940-stimulated binding of [$^{35}$S] GTPγS to G-proteins α-subunits in shrew brain sections. In general, the distribution of G-protein activation mirrored the distribution and levels of [$^3$H]-SR141716A binding. Although [$^3$H] SR1 41716A- and CP55,940-stimulated [$^{35}$S] GTPγS binding were low in pons and medulla (brain stem), the nucleus tractus solitarius exhibited moderate G-protein activation. The area postrema, the medullary formation, and the nucleus tractus solitarius are structures involved in the regulation of emesis (Mitchelson, 1992). While the brain stem appears to be relatively $CB_1$ receptor sparse, the nucleus tractus solitarius and area postrema contain significantly moderate amounts of cannabinoid receptors in several emetic species (Van Sickle et al., 2001; Glass et al., 1997; present study). These loci in the brain stem are probably

involved both in cannabinoid-induced reductions in gastric tone (Krowicki et al., 1999) and their antiemetic action (Van Sickle et al., 2001).

From the present study and previous reports in mouse brain membranes (Breivogel et al., 2001), it appears that WIN55,212-2 activates not only cannabinoid $CB_1$ receptors, but also a non-$CB_1$ G-protein-coupled receptor. While the presence of an additional site for WIN55,212-2 for the stimulation of [$^{35}$S]GTPγS binding complicates the analysis of WIN55,212-2 activity, it is not an issue in the receptor-binding experiments as this was measured by the binding of [$^3$H]CP55,940 or [$^3$H]SR141716A, neither of which exhibits significant binding to non-cannabinoid $CB_1$ receptor sites at the concentrations used in mouse brain membranes (Breivogel et al., 2001). Further evidence that [$^3$H]CP55,940 is only binding to cannabinoid receptors in shrew brain membranes comes from the observation that most ligands (especially the more potent ones or which a saturating concentration could be achieved) completely displaced [$^3$H]CP55,940 binding. The rank order of cannabinoid $CB_1$ receptor affinity ($K_D$ or $K_i$) of structurally diverse cannabinoid ligands in shrew whole brain homogenates is similar to rat brain homogenates (Breivogel and Childers, 2000; Breivogel et al., 2001): HU-210 = CP55,940 = SR141716A ≥ WIN55,212-2 ≥ delta-9-tetrahydrocannabinol>methanandamide = cannabidiol = HU-211 = 2-arachidonoylglycerol. The overall pharmacological potency at producing the characteristic activity profile of diverse cannabinoid agonists attained from several in vivo functional assays (e.g., the tetrad of behaviors in mice) has been demonstrated to be highly correlated with their affinity binding at $CB_1$ receptors ($r^2 = 0.86$, $P < 0.05$) (Compton et al., 1993; Wiley et al., 1998). Likewise, the antiemetic $ID_{50}$ potency order of tested cannabinoids in reducing the incidence of vomiting significantly correlated with their corresponding affinity order for the cannabinoid $CB_1$ receptor [CP55,940>WIN55,212-2 ≥ delta-9-tetrahydrocannabinol; $r^2 = 0.99$, $P < 0.05$] (Darmani, 2001b,c). However, the potency of these cannabinoids in reducing the frequency of emesis failed to attain a significant correlation because the $ID_{50}$ dose of WIN55,212-2 was greater than that of delta-9-tetrahydrocannabinol. In contrast, when correlated with the ability to prevent 2-arachidonoylglycerol or SR141716A-induced emesis, the affinity of the tested cannabinoids is highly correlated with their antiemetic $ID_{50}$ potency orders for both parameters of emesis (Darmani, 2001a, 2002a) [($r^2 = 0.99–1.0$; $P < 0.03–0.001$) and ($r^2 = 0.97–0.98$; $P < 0.03–0.001$), respectively] with the following rank order: CP55,940>WIN55, 212-2>delta-9-tetrahydrocannabinol.

Addressing the issue of the potency and efficacy of WIN55,212-2 became the focus of an additional set of experiments in the present study. In this regard, some work had already been done in mouse and rat brain membranes. Thus, both anandamide and WIN55,212-2 have been shown to induce SR141716A-insensitive stimulation of [$^{35}$S]GTPγS binding to brain membranes from mice (including transgenic C57BL/6 mice that lacked cannabinoid $CB_1$ receptors) (Breivogel et al., 2001), indicating the presence of an additional G-protein-coupled target for these ligands. However, none of the other cannabinoid ligands tested in the present study (even methanandamide) were able to stimulate [$^{35}$S]GTPγS binding to membranes lacking cannabinoid $CB_1$ receptors. As a control, and similar to results obtained in the mouse, there was no SR141716A-insensitive stimulation of [$^{35}$S]GTPγS binding by CP55,940 to shrew or rat brain membranes (C. Breivogel, B. Parekh, and N.A. Darmani, unpublished observations).

To determine the activity of WIN55,212-2 at cannabinoid $CB_1$ receptors for comparison to other cannabinoid ligands, it was necessary to distinguish $CB_1$-mediated activity from that mediated by the non-$CB_1$ receptor. To this end, we determined both total and SR141716A-insensitive WIN55,212-2-stimulated [$^{35}$S]GTPγS binding, and calculated the difference to obtain the amount of [$^{35}$S]GTPγS binding stimulated by cannabinoid $CB_1$ receptors at each concentration of the agonist. As was measured previously in mouse brain, the potency of WIN55,212-2 at the non-$CB_1$ site was considerably lower than at cannabinoid $CB_1$, and accounted for most, if not all, of the low-potency stimulation of [$^{35}$S]GTPγS binding by WIN55,212-2 and for the low Hill slope values observed in these assays. Previous reports, which did not account for the activity of WIN55,212-2 at the non-$CB_1$ receptor, have indicated that WIN55,212-2 was usually more efficacious than other cannabinoid ligands for the stimulation of [$^{35}$S]GTPγS binding (Breivogel and Childers, 2000). Most other ligands appeared to exhibit intermediate efficacy, but $\Delta^9$-tetrahydrocannabinol has consistently been characterized as a low efficacy agonist (Breivogel et al., 1998; Burkey et al., 1997a,b; Sim et al., 1996). In the present study, the efficacy of WIN55,212-2 at cannabinoid $CB_1$ receptors appeared to be similar to most other agonists tested including HU-210, CP55940, and methanandamide; thus, each of these, including WIN55,212-2, appeared to be a full agonist. Two of the remaining ligands, delta-9-tetrahydrocannabinol and 2-arachidonoyl glycerol appeared to be partial agonists, and cannabinol and HU-211 exhibited no activity that could be conclusively attributed to cannabinoid $CB_1$ receptors.

Results for both receptor binding and ligand-stimulated [$^{35}$S]GTPγS binding were similar to values previously reported in rat (Breivogel and Childers, 2000) and mouse (Breivogel et al., 2001) brain, where cannabinoid $CB_1$ receptors have been cloned and fully characterized (Matsuda et al., 1990). This strongly implies that the binding and activity of cannabinoid ligands measured in the present study was attributable to shrew brain cannabinoid $CB_1$ receptors although these proteins have not yet been cloned in this species. The rank order of ligand potency ($EC_{50}$ for GTPγS stimulation) was similar ($r^2 = 0.88$, $P < 0.006$) to that of ligand affinity ($K_D$ or $K_i$ for $CB_1$

receptor) for those compounds that displayed stimulation of [$^{35}$S] GTPγS binding: HU-210>CP55,940 = delta-9-tetrahydrocannabinol = WIN55,212-2 ≥ methanandamide = 2-arachidonoylglycerol. However, the exception was that WIN55,212-2 and delta-9-tetrahydrocannabinol potency order were reversed because WIN55,212-2 displayed higher affinity but lower potency than delta-9-tetrahydrocannabinol. Such differences in the cited rank orders have also been observed in rodent brain (Burkey et al., 1997a; Griffin et al., 1998). Because of the latter reversal, linear regression analysis yielded a significant correlation ($r^2 = 0.99$, $P < 0.045$) between cannabinoid GTPγS stimulation and their antiemetic $ID_{50}$ potency in reducing the frequency of vomiting rather than the incidence of vomiting as was earlier discussed for the cannabinoid $CB_1$ receptor affinity order.

An important aspect of the current biochemical studies were to shed further light as to why the endocannabinoid 2-arachidonoylglycerol induces vomiting, whereas other cannabinoid $CB_1$ agonists tend to possess antiemetic properties. To this end, three hypothesis were recently proposed (Darmani, 2002a). First, 2-arachidonoylglycerol possesses full efficacy and its emetic activity can be blocked by cannabinoid $CB_1$ receptor partial agonists. Support for this notion comes from studies in which 2-arachidonoylglycerol maximally increases intracelular levels of $Ca^{2+}$, while other cannabinoid $CB_1$ receptor agonists block the induced effect via partial agonism (Sugiura and Waku, 2000). However, the current and other published findings (e.g., Breivogel et al., 2001) do not support this notion as 2-arachidonoylglycerol poorly stimulates GTPγS binding in both shrew and rodent brain. Second, 2-arachidonoylglycerol is a partial agonist of cannabinoid $CB_1$ receptors and suppresses the activity of an endogenous emetic tone by some yet-to-be discovered endocannabinoid, which is more efficacious than 2-arachidonoylglycerol. This notion helps to explain why more efficacious cannabinoid agonists are antiemetic and block 2-arachidonoylglycerol-induced vomiting. The current GTPγS findings seem to support the latter notion. However, although 2-arachidonoylglycerol exhibits low affinity for cannabinoid $CB_1$ receptors, most studies indicate 2-arachidonoylglycerol possesses full efficacy (Hillard, 2000). The final and most likely hypothesis is that one or more metabolite(s) of 2-arachidonoylglycerol is/are emetogenic. Indeed, the main metabolite of 2-arachidonoylglycerol is arachidonic acid which is also a potent emetic agent (Darmani, 2002a).

In summary, CP55,940 is a very potent antiemetic cannabinoid against cisplatin-induced emesis. However, unlike dela-9-tetrahydrocannabinol and WIN55,212-2, the antiemetic activity of CP55,940 occurs at motor-suppressant doses. The cannabinoid $CB_1$ receptor seems to mediate both the antiemetic and motor-depressant actions of CP55,940 because both effects are countered by the cannabinoid $CB_1$ receptor antagonist SR141716A. The cannabinoid $CB_1$ receptor is both present and functionally active throughout the shrew brain, and significant amounts are found in specific loci controlling emesis in the brain stem. Both cannabinoid $CB_1$ receptor affinity and ligand potency ($EC_{50}$ for GTPγS stimulation) rank orders of tested cannabinids appear to be related to their antiemetic $ID_{50}$ potency rank order for certain parameters of cisplatin-induced vomiting. The latter correlation are more highly correlated when vomiting was induced by 2-arachidonoylglycerol (Darmani, 2002a) or SR141716A (Darmani, 2001a). This is probably not surprising as cisplatin induces vomiting via a multitude of mechanisms (Veyrat-Follet et al., 1997), and each of the cited cannabinoids may have a differential inhibition pattern on the diversely activated emetic stimuli.

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