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RESEARCH PAPER

Sch35966 is a potent, selective agonist at the peripheral cannabinoid receptor (CB_2) in rodents and primates

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Background and purpose: The peripheral cannabinoid receptor (CB_2) is expressed on peripheral immune cells and is thought to have a role in the immunosuppressive effects of cannabinoids. Historically, there have been few potent, CB_2-selective agonists to assess the contribution of CB_2 to this phenomenon. The studies presented here describe the synthesis of 8,10-bis[(2,2-dimethyl-1-oxopropyl)oxy]-11-methyl-1234-tetrahydro-6H-benzo[β]quinolizin-6-one (Sch35966), which binds with low nanomolar potency to CB_2 in both primates and rodents.
Experimental approach: The affinity, potency and efficacy of Sch35966 and other cannabinoid ligands at CB_2 was assessed using competition binding assays vs [^{3}H]CP55,940, [^{35}S]GTPγS exchange, cAMP accumulation and cell chemotaxis assays.
Key results: We showed that Sch35966 has >450-fold selectivity for CB_2 binding vs the central cannabinoid receptor (CB_1) in primates (humans and cynomolgus monkeys) and rodents (rats and mice). Sch35966 is an agonist as it effectively inhibited forskolin-stimulated cAMP synthesis in CHO-hCB_2 cells, stimulated [^{35}S]GTPγS exchange and directed chemotaxis in cell membranes expressing CB_2. In all species examined, Sch35966 was more potent, more efficacious and more selective than JWH-015 (a commonly used CB_2-selective agonist).
Conclusions and implications: Taken together, the data show that Sch35966 is a potent and efficacious CB_2-selective agonist in rodents and primates.
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Keywords: peripheral cannabinoid receptor; CB_2-selective agonist; Sch35966

Abbreviations: 2-AG, 2-arachidonyl glycerol; CB_1, central cannabinoid receptor; CB_2, peripheral cannabinoid receptor; CHO, Chinese hamster ovary; FBS, fetal bovin serum; Δ^9-THC, Δ^9-tetrahydrocannabinol

Introduction

The actions of cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC), the endocannabinoids, anandamide and 2-arachidonyl glycerol (2-AG) and synthetic cannabinoids such as HU210 and (CP55,940) are mediated through activation of central cannabinoid receptor (CB_1) and peripheral cannabinoid receptor (CB_2) (Devane *et al*., 1992; Mechoulam *et al*., 1995; Sugiura *et al*., 1995; for review, see Howlett *et al*., 2002). Activation of either receptor subtype stimulates the inhibition of adenylyl cyclase (Bayewitch *et al*., 1995; Slipetz *et al*., 1995) and the activation of mitogen-activated protein kinase (Bouaboula *et al*., 1995, 1996). Originally cloned from rat brain (Matsuda *et al*., 1990; Gerard *et al*., 1991), CB_1 is expressed primarily in the central nervous system and mediates most of the psychotropic and analgesic effects associated with cannabinoid agonists. CB_2 was cloned from rat spleen and promyelocytic leukaemic HL60 cells (Munro *et al*., 1993) and is highly expressed in peripheral immune cells (Galiegue *et al*., 1995; Schatz *et al*., 1997). More recent work suggests that the receptor may also be expressed in neuronal cells involved in pain modulation and/or perception (Hanus *et al*., 1999; Malan *et al*., 2002; Hohmann *et al*., 2004; Ibrahim *et al*., 2005).

The recreational use of cannabinoids has been linked with diminished immune function (Kaminski, 1996, 1998; Klein *et al*., 1998) and there are studies suggesting that endocannabinoids may be immunomodulators (Cabral *et al*., 1995; Lee *et al*., 1995; Di Marzo *et al*., 1999). Owing to its expression pattern, the immunosuppressive effects of cannabinoids have been largely attributed to CB_2 activation.

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However, Smith *et al*. (2001b) recently showed that intracerebroventricular administration of the non-selective cannabinoid agonists HU210 or WIN55,212 to mice before an endotoxin challenge attenuated proinflammatory cytokine production and increased the levels of the anti-inflammatory cytokine interleukin (IL)-10. Co-administration of a CB_1-selective antagonist, SR141716A attenuated this reaction while the CB_2-selective antagonist SR144528 was ineffective in blocking the response.

Direct measurements of CB_1- and CB_2-specific effects have been hindered by the shortage of isotype-selective agonists. Indeed, until the recent isolation of 2-AG ether from porcine brain, there were no CB_1-selective agonists. 2-AG ether was characterized as an agonist with >140-fold selectivity for the CB_1 vs CB_2. Interestingly, there has been more success generating CB_2-selective agonists. For example, JWH-015 and JWH-133 are aminoalkylindole congeners of Δ^9-THC that are relatively selective (28 to 200-fold selectivity over CB_1) and potent ($K_i = 13.8$ and 3.4 nM, respectively) agonists (Showalter *et al*., 1996; Chin *et al*., 1999). L759633 and L759656 are aminoalkylindole congeners that have also been shown to be selective and potent CB_2 agonists (Ross *et al*., 1999). A bicyclic compound, HU-308, was recently described as a very selective CB_2 agonist (>400-fold selectivity over CB_1) although it has only moderate affinity for CB_2 (19–27 nM; Hanus *et al*., 1999). However, the CB_2-selective ligands described above are not widely accessible and until recently, only JWH-015 was available commercially. Data regarding the affinity, selectivity and efficacy of JWH-015 at CB_2 in human, nonhuman primates and rodents are lacking or incomplete.

In the studies described here, we assessed the utility of a benzoquinolizinone compound, 8,10-bis[(2,2-dimethyl-1-oxopropyl)oxy]-11-methyl-1234-tetrahydro-6*H*-benzo[*β*]quinolizin-6-one (Sch35966), as a CB_2-selective cannabinoid agonist in both rodents and primates. Using both functional and binding assays, we demonstrated that Sch35966 is both a potent and selective CB_2 agonist in rat, mouse, cynomolgus monkey and human.

Methods

Synthesis of Sch35966

Sch35966 was prepared from 3,5-bis(acetyloxy)benzoyl chloride and 2-(1,1-dimethoxyethyl)piperidine hydrochloride (Friary, 1990). The procedures described were used without further modification. The structure of Sch35966 is shown in Figure 1.

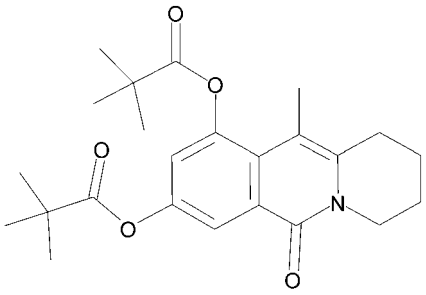

Figure 1 The structure of Sch35966.

Cloning CB_2 from rat, mouse and cynomolgus monkey

RNA was isolated from lymph node and spleen samples using RNA STAT-60 (Tel-Test, Friendsword, TX, USA) according to manufacturer's protocol. RNA was isolated from cynomolgus blood using the PAXgene blood RNA kit by PreAnalytiX (Hombrechtikon, CH). All samples were DNase treated using DNase 1 (Roche Diagnostics Corp., Indianapolis, IN, USA) before cDNA synthesis. cDNA was generated using SuperScript First Strand Synthesis System for reverse transcription-PCR (Invitrogen, Carlsbad, CA, USA). Both oligo-dT (Invitrogen) and random hexomers (Promega, Madison, WI, USA) were used to prime first strand synthesis. Mouse CB_2 cDNA was generated by amplifying mouse spleen cDNA with the forward primer ACGGCTAGCGAGGGATGCCGGGAGACA GAAGT (*Nhe*1 site) and the reverse primer GACACGCGGCC GCCTAGGTGGTTTTCACATCAGCCTCTG (*Not*1 site). The amplified gene was cloned into pme18neoCD8-FLAG. To obtain the 5′- and 3′-prime ends of the rat CB_2 gene, primers (forward primer GGCCGGAGCTGACTTTCCTGGCCAGCGT GATCTTT and reverse primer GCCAGCCCAGTAGGTAGTCG TTGGGGATCA) were designed for the rapid amplification of cDNA ends (RACE) based on the existing CB_2 sequences. Marathon RACE amplification (Gibco-BRL; Bethesda, MD, USA) was performed on RNA purified from rat spleen RNA. The forward primer ACGGCTAGCGCGGGATGCCGGGA GCTGGAG (with an *Nhe*1 site) and a reverse primer GTTG GCGGCCGCTCAGCAATTGGAGCAGCCCTGGTGTCT (with a *Not*1 site) were designed based on the RACE sequences. Rat CB_2 cDNA was then amplified by PCR of rat spleen cDNA and cloned into the pME18neoCD8FLAG vector. Cyno CB2 sequence was determined using SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) using the forward primer GCCTTCTGCTCCATGCTGTGCCTCGTCA and the reverse primer GCAGGCGGAGAAGAGTGCACAA CACGGC on cyno lymph node RNA. After sequencing of the RACE products, PCR primers (forward primer ACGGCTAGC GAGGAATGCTGGCTGACAGAGATA and reverse primer GT TGGCGGCCGCTCATCAGCAACTGGAGTGGTCTAG) were designed based on the sequence of the 5′ and 3′ end of cyno CB_2 to amplify the entire CB_2 cDNA from lymph node RNA. The cyno CB2 gene was then cloned into the pME18-neoCD8FLAG vector.

Cells and cell culture

The clonal Chinese hamster ovary (CHO)–hCB_2 cell line was generated by transfection of CHO-K1 cells with human cannabinoid type 2 receptor cDNA modified by placement of the haemagglutinin (HA) epitope on the N-terminus as described previously (Gonsiorek *et al*., 2000). Monolayer cultures were grown at 37°C in a humidified atmosphere (5% CO_2) in Dulbecco's modified Eagle F-12 medium containing L-glutamine and supplemented with 1% non-essential amino acids, 1% penicillin/streptomycin, 10% fetal bovine serum (FBS) (Gemini Bio-Products, Calabasas, CA, USA) and 0.2 mg ml^{-1} hygromycin B, pH 7.4. Ba/F3 cell lines transfected to express human CB_1 (Ba/F3-hCB_1) or CB_2

(Ba/F3-hCB_2) were generated as described previously (Lunn *et al*., 2006). Ba/F3 cell lines transfected to express rat, mouse or cyno CB_2 were generated and maintained similarly. Experimental cultures were used 1–2 days after seeding. Cell culture medium (RPMI-1640) was purchased from Gibco-BRL (Grand Island, NY, USA).

Membrane preparation

Cell membranes from brains of mice, rats and cynomolgus monkeys or transfected cell lines were prepared as described previously (Hipkin *et al*., 1997). Briefly, brains were minced on ice using a razor blade, rinsed with ice-cold phosphate-buffered saline (Invitrogen Corp., Carlsbad, CA, USA) to remove excess blood and kept on ice. CHO–hCB_2 cells were harvested using cell dissociation buffer according to the manufacturer's instructions (Invitrogen Corp.), collected by centrifugation and used immediately or stored at −80°C. Transfected Ba/F3 cells were pelleted by centrifugation and used immediately or stored at −80°C. Cell pellets were resuspended and incubated on ice for 30 min in cell homogenization buffer (10 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 3 mM ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*,-tetraacetic acid (EGTA), pH 7.6). Brain tissue was incubated with tissue homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, pH 7.6). Both cell and tissue homogenization buffer were supplemented with 1 mM phenylmethylsulphonyl fluoride. Cells and brain tissue were then homogenized with 15–20 strokes at 900 r.p.m. with a Dounce homogenizer using stirrer type RZR1 polytron homogenizer (Caframo, Wiarton, Ontario, Canada). Intact cells and nuclei were removed by low-speed centrifugation (500 *g* for 5 min at 4°C). Membranes in the supernatant were pelleted by centrifugation at 100 000 *g* for 30 min at 4°C and then resuspended in gly–gly buffer (20 mM glycylglycine, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.2) and stored at −80°C. Protein determinations were performed using the Bradford method (Bradford, 1976).

[^{35}S]GTPγS and [^{3}H]CP55,940 membrane binding

Binding reactions were carried out in 96-well microplates in a final volume of 100 μl. Cell membranes (1–25 μg, in triplicate) were incubated in the presence or absence of various compounds for 60 min at room temperature in binding buffer (50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, and 0.1% (1 mg l^{-1}) bovine serum albumin (BSA; factor V, lipid free), pH 7.4) containing 1–2 nM [^{3}H]CP55,940 (SA = 180 Ci $mmol^{-1}$). In some experiments, [^{3}H]CP55,940 binding was done in GTPγS-binding buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 100 mM NaCl, 5 mM $MgCl_2$, and 0.2% (2 mg l^{-1}) BSA, pH 7.4), supplemented with the indicated concentrations of GDP and 0.1 nM nonisotopic GTPγS. [^{3}H]CP55,940 binding was terminated by rapid filtration of the membranes through the microfiltration plates coated with 0.5% polyethylenimine (UniFilter GF/C filter plate; Packard, Meriden, CT, USA), using a Tomtek 96-well cell harvester (Hamden, CT, USA). The membranes were washed 10 times with ice-cold buffer (50 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA, pH 7.4). Membrane-bound radioligand was measured by liquid scintillation using a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard, Meriden, CT, USA).

For [^{35}S]GTPγS-binding experiments, membranes in GTPγS-binding buffer were incubated in the presence or absence of cannabinoids, 0.3 nM [^{35}S]GTPγS (SA = 1250 Ci $mmol^{-1}$ (NEN Boston, MA, USA) and the indicated concentrations of GDP. Membrane-bound [^{35}S]GTPγS was measured using WGA-SPA beads (300 μg) at 60 min by use of a 1450 Microbeta Trilux counter (Wallac, Gaithersburg, MD, USA) as described previously (Chou *et al*., 2002).

Cyclic AMP accumulation assay

Assays were performed as described previously (Gonsiorek *et al*., 2000). Briefly, cells, seeded in 96-well plates, were chilled on ice and washed two times with cold F-12 nutrient mixture (HAM) medium, containing 10 mM HEPES and 0.2% BSA, pH 7.4. Cells were then incubated for 15 min at 37°C in the above medium supplemented with 200 μM isobutylmethylxanthine (cAMP assay media), 5 μM forskolin and the indicated concentrations of cannabinoids. The media was removed and the cells lysed with 0.1 N HCl and rapid freezing. Intracellular cAMP in thawed lysates was measured by cAMP Enzyme Immunoassay (Biomol Research Laboratories, Plymouth Meeting, PA, USA) according to manufacturer's instructions. The results are expressed as a fraction of forskolin-stimulated cAMP accumulation measured in the absence of cannabinoids.

Cell chemotaxis assays

Cellular migration was measured as described previously (Lunn *et al*., 2006). Briefly, cannabinoids diluted in assay buffer (phenol red free-RPMI supplemented with 10% FBS) were dispensed (30 μl) into the bottom wells of disposable microchemotaxis plates (ChemoTx 101-5 sp; Neuroprobe Inc., Gaithersburg, MD, USA). Cell aliquots (25 μl; 50 000 Ba/F3-hCB_2 cells) in assay buffer were then applied to filters (5 μm pore size) in the top plate. After incubation for 90 min at 37°C, migrated cells were collected in the bottom well by centrifugation and transferred to wells of a flat-bottom Microlite (1+) luminometer plate (Thermo Electron Corporation, Waltham, MA, USA). Eighty microlitres of assay buffer and 100 μl of CellTiter Glo Reagent (Promega) were added per well, incubated for 10 min and the luminescence intensity was measured using a luminometer (Thermo Electron Corporation) at an excitation time of 100 ms. We found that a linear relationship exists between luminescence intensity and cell number (data not shown). Relative migration is expressed as a percentage of total input cell number. Data are presented as the mean of triplicate determinations.

Data analysis

Data are presented as mean values ± s.e.m. of at least three independent experiments, each of which was performed in triplicate. Nonlinear regression analysis of saturation data

and of concentration–response data was performed using Prism 2.0c software (GraphPad Software, San Diego, CA, USA) to calculate K_D, B_{max}, IC_{50} and EC_{50} values. IC_{50} values were converted to apparent K_i values by the method of Cheng and Prusoff (1973) using the K_D values for [^{3}H]CP55,940 determined from saturation experiments.

Materials

Sf9 membranes exogenously expressing $G\alpha_{i3}$, $\beta_1\gamma_2$ and hCB_2 (7–14 pmol mg^{-1}) or hCB_1 (0.7 pmol mg^{-1}) were purchased from NEN Life Sciences. HU210 ((6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol) was purchased from BIOMOL Research Laboratories. AM-630 (6-iodopravadoline) and JWH-015 (2-methyl-1-propyl-1H-indol-3-yl-1-naphthalenylmethanone) was purchased from Tocris Bioscience (Ellisville, Missouri, USA). CP55,940 ((1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol) was purchased from NEN. WIN55,212-2 ((*R*)-(+)-2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthaleneylmethanone mesylate) and SR144528 (N-[(1*S*)-endo-1,3,3-trimethyl bicycle[2,2,1] heptane-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3carboxamide) were synthesized in our Department of Chemistry at SPRI. All other reagents were of the best grade available and purchased from common suppliers.

Results

Functional characterization of Sch35966

To assess if Sch35966 is an agonist, inverse agonist or neutral antagonist, we tested the effect of known cannabinoid agonists (HU210, WIN55,212-2), Sch35966 and an inverse agonist (SR144528) on forskolin-stimulated cAMP accumulation in CHO–hCB_2 cells, (Figure 2) which we had previously used to characterize endocannabinoid pharmacology (Gonsiorek *et al*., 2000). As shown in Figure 2a, Sch35966 inhibited forskolin-stimulated cAMP accumulation with an efficacy similar to HU210 and WIN55,212-2, although the latter ligands were more potent. It is difficult to conclude if Sch35966 is a full or partial agonist due to the high constitutive activity of CB_2, which suppressed the endogenous adenylyl cyclase activity in these cells. The constitutive activity of CB_2 was apparent upon co-incubation of these cells with SR144528 which relieved this tonic suppression as witnessed by a dramatic increase in forskolin-stimulated cAMP levels. In parallel, we assessed the effect of these ligands on forskolin-stimulated cAMP accumulation in CHO–hCB_2 cells preincubated with pertussis toxin (PTX) to inactivate the Gi transducers (Figure 2b). Interestingly, incubation of PTX-pretreated cells with cannabinoid agonists resulted in stimulation of cAMP levels. The stimulatory effects of the inverse agonist were largely abolished. These data suggest that hCB_2 expressed in our cell line interacts with both inhibitory (G_i) and stimulatory G proteins (G_s). A similar observation was made for CB1 in the brain (Glass and Felder, 1997).

Lastly, we tested the chemotactic response of Ba/F3-hCB_2 in response to Sch35966 and HU210 as cannabinoids have been shown to stimulate cell chemotaxis via CB_2 (Sacerdote *et al*., 2000; Kishimoto *et al*., 2003; Lunn *et al*., 2006). Both Sch35966 and HU210 stimulated Ba/F3-hCB_2 cell chemotaxis with the classic bell-shaped concentration–response curve (data not shown).

We next assessed the pharmacology of Sch35966 using [^{35}S]GTPγS-exchange assays (as described in the Methods section). Breivogel *et al*. (1998) previously established that relative to full agonists, the efficacy of partial agonists at the cannabinoid CB_1 to stimulate [^{35}S]GTPγS exchange was more susceptible to inhibition with elevated GDP concentrations. Therefore, we assessed the effect of increasing concentrations of GDP on the [^{35}S]GTPγS exchange in CHO–hCB_2 membranes in response to 100 nM HU210,

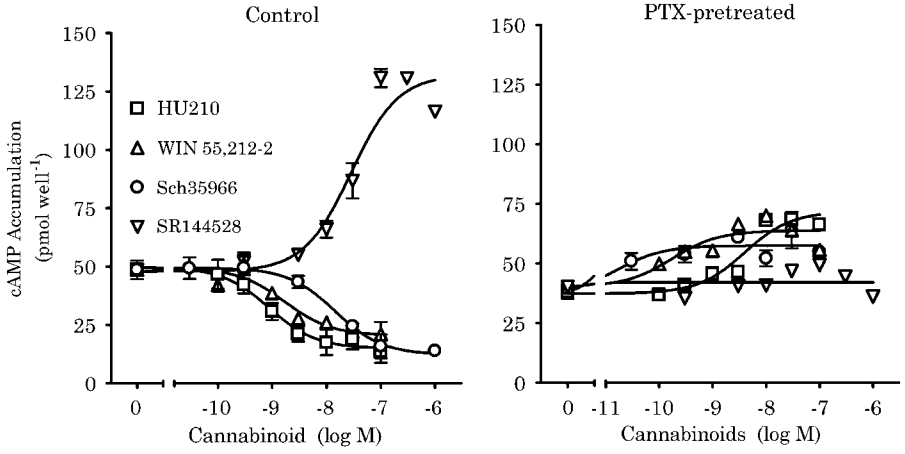

Figure 2 The effect of cannabinoids on cAMP accumulation in CHO–hCB_2 cells. Cells in 96-well plates were pretreated in the absence (left) or presence (right) of 100 ng ml^{-1} PTX for 24 h (as described in the Methods section). Cells were washed and incubated for 15 min at 37°C in cAMP assay buffer containing 5 μM forskolin and the indicated concentrations of HU210, Sch35966, WIN 55,212-2 or SR144528. Following incubation, intracellular cAMP was measured by enzyme immunoassay. Data represent the mean fraction of forskolin-stimulated cAMP (pmol/well)±range of triplicate determinations from a representative experiment.

CP55,940, WIN55,212-2, SR144528, AM-630, Sch35966 or 1 μM JWH-015 (Figure 3a) and expressed the effect of the ligands relative to basal binding (Figure 3b). CP55,940 was the most efficacious ligand tested in stimulating GTPγS exchange. While HU210 appeared to be (at least) as effective as CP55,940 with $\leqslant 1\,\mu$M GDP, its relative efficacy declined in the face of higher GDP levels. Sch35966, WIN55,212-2 and JWH-015 were less efficacious. As would be expected, the inverse agonists SR144528 and AM-630 decreased constitutive GTPγS exchange. From these experiments, we selected 5 μM GDP and 0.3 nM GTPγS for the more extensive assessment of the potency, efficacy and affinity of HU210, CP55,940, Sch35966 and JWH-015 at hCB_2. Representative data are shown in Figure 4. Consistent with the data from the GDP titration experiments (Figure 3), CP55,940 was a slightly more efficacious agonist than HU210 and Sch35966 although the latter compound was less potent. In this particular set of data, Sch35966 stimulated GTPγS exchange to levels which are equivalent to that seen with HU210, although taken together the results from our experiments suggest that HU210 is slightly more efficacious than Sch35966 (Figure 3b; data not shown). HU210, CP55,940 and Sch35966 were all more potent than JWH-015 in stimulating GTPγS exchange. The binding affinities in buffer containing 5 μM GDP and 0.3 nM nonisotopic GTPγS were in general agreement with the functional potencies.

The effect of Sch35966 on [^{3}H]CP55,940 binding and [^{35}S]GTPγS exchange in membranes expressing primate or rodent CB_2

We measured the binding affinities and intrinsic efficacies of Sch35966, HU210, JWH-015 using membranes from a cell line transfected to express CB_2 from human (Ba/F3-hCB_2), cynomolgus monkey (Ba/F3-cynoCB_2), rat (Ba/F3-rCB_2) or mouse (Ba/F3-mCB_2). Saturation analysis (Figure 5) showed that [^{3}H]CP55,940 bound with high affinity to CB_2 from mouse (mCB_2 $K_D = 0.5$ nM), rat (rCB_2 $K_D = 0.6$ nM) and cynomolgus monkey (cynoCB_2 $K_D = 0.7$ nM). In GTPγS exchange assays (Figure 6, Table 1), Sch35966 was less potent than HU210 and demonstrably more potent than JWH-015. In competition binding assays (Figure 7 and Table 2), HU210 bound with highest affinity in all species (0.40–0.60 nM) followed by Sch35966 (2–7 nM). JWH-015 bound CB_2 with considerably lower affinity ($\sim$30–400 nM).

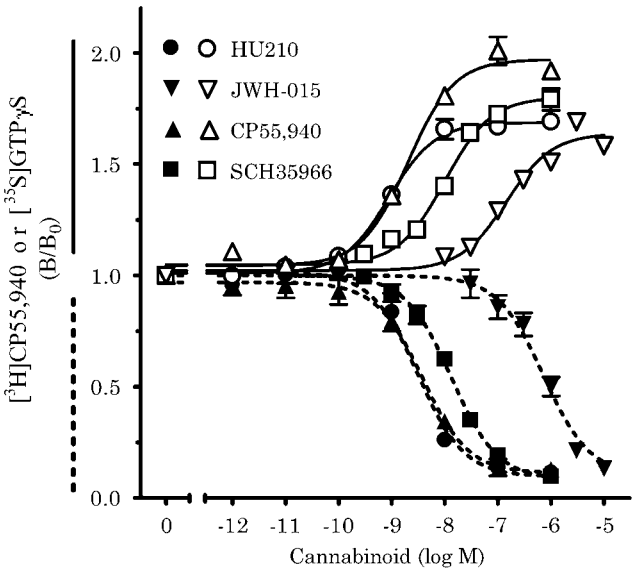

Figure 4 The effect of cannabinoids on [^{35}S]GTPγS exchange and [^{3}H]CP55,940 binding in CHO–hCB_2 membranes. Membranes (4 μg) were incubated at 30°C for 60 min in binding buffer containing 5 μM GDP, the indicated concentrations of HU210, CP55,940, WIN 55,212 or Sch35966. In GTPγS exchange assays, the incubation contained 0.1 nM [^{35}S]GTPγS (open symbols, solid lines). In competition binding assays, the incubation contained 0.1 nM nonisotopic GTPγS and 1–2 nM [^{3}H]CP55,940 (closed symbols, broken lines), Following filtration, the membrane-associated radioactivity was measured by liquid scintillation. Data (expressed as B/B_0) represent the mean specific binding $\pm$ range of triplicate determinations from a representative experiment ($n = 2$–4).

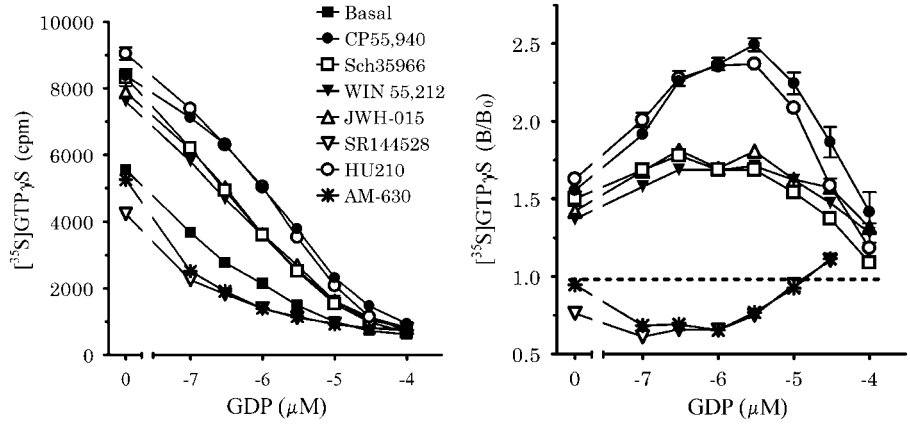

Figure 3 The effect of GDP concentration on [^{35}S]GTPγS exchange in response to cannabinoids in CHO–hCB_2 membranes. Membranes expressing hCB_2 (4 μg) prebound to WGA-SPA beads were incubated in GTPγS binding buffer (as described in the Methods section) containing 0.1 nM [^{35}S]GTPγS and the indicated concentration of GDP in the absence or presence of cannabinoids at either 100 nM (HU210, CP55,940, WIN 55,212, SR144528) or 1 μM (Sch35966, JWH-015) for 60 min at room temperature. The mean binding $\pm$ range of triplicate determinations from a representative experiment is shown on the left panel. The same data are presented in the right panel expressed as total/basal binding at each GDP concentration (B/B_0). Membrane-associated radioactivity was measured by WGA-SPA scintillation.

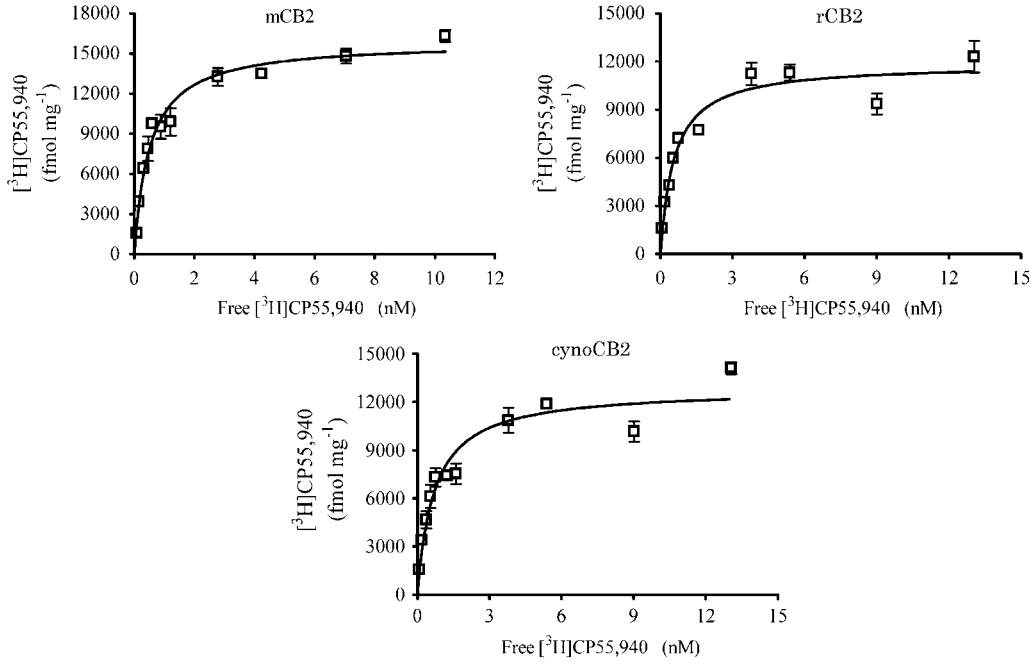

Figure 5 Saturation binding isotherms for mouse, rat and monkey CB_2. Membranes from Ba/F3 cells transfected to express CB_2 from the mouse (mCB_2), rat (rCB_2) or monkey ($cynoCB_2$) were incubated for 30 min at 30°C in binding buffer (as described in the Methods section) with the indicated concentrations of [^{3}H]CP55,940 in the presence or absence of excess unlabelled ligand. Following filtration, the membrane-associated radioactivity was measured by liquid scintillation. Data represent the mean total binding ± range of triplicate determinations from two independent experiments.

To assess CB_2 selectivity, we measured the binding affinity of these compounds to hCB_1 (Ba/F3-hCB_1 membranes), rCB_1 (rat brain membranes) and $cynoCB_1$ (cynomolgus monkey brain membranes) by competition with [^{3}H]CP55,940 (Figure 7). Again, HU210 bound with high affinity in all of the membrane preparations (0.7–1.1 nM; Table 3). Both Sch35966 and JWH-015 displaced [^{3}H]CP55,940 from CB_1 with low affinity. On the basis of its higher affinity for CB_2, Sch35966 is more CB_2 selective than is JWH-015 in all species tested.

Discussion

The studies presented here describe the characterization of a novel benzoquinolizinone, Sch35966, which binds with low nM potency to CB_2 in both primates (human and cynomolgus monkey) and rodents (rat and mouse). Further, we demonstrated that Sch35966 has >450-fold selectivity for CB_2 binding vs the CB_1. Sch35966 is an agonist as it effectively inhibited forskolin-stimulated cAMP synthesis in CHO–hCB_2 cells and stimulated [^{35}S]GTPγS exchange in cell membranes expressing human, monkey, rat and mouse CB_2. In all species examined, Sch35966 was more potent and selective than JWH-015 (a commonly used CB_2-selective agonist).

Benzoquinolizinones have been used to treat a number of medical conditions. The compounds were identified early on as novel partial agonists for the benzodiazepine receptors (Jenck *et al*., 1992) and developed for the treatment of anxiety disorders. More recent examples of this class of compound can be seen in the development of a class of non-steroidal inhibitors of human steroid 5α-reductases 1 and 2 (Ferrali *et al*., 2005), progressed for the treatment of prostatic hyperplasia, acne and alopecia. We identified the benzoquinolizinone Sch35966 by screening a group of compounds using a recombinant CB_2 membrane preparation in a ligand-binding assay, while seeking to identify a novel class of cannabinoid CB_2 ligands. The interest in developing cannabinoid CB_2-specific agonists was based on the hypothesis that the relatively high expression of the receptor mRNA in immune cells vs its expression in the CNS could allow the generation of an immunomodulatory cannabinoid compound that lacked the psychoactive effects mediated by the central CB_1. Data supporting this approach continue to be demonstrated – in a recent publication, Correa *et al*. (2005) showed that a cannabinoid CB_2-specific agonist inhibited lipopolysaccharide/interferon-γ (IFN-γ) induced IL-12p40 and enhanced IL-10 release.

In spite of the extensive *in vitro* data supporting an immunomodulatory role for cannabinoid agonists, in recent clinical trials no immunological deficits were observed following short-term dosing with plant cannabinoid agonists. Abrams *et al*. (2003) showed that Δ^9-THC had no significant effect on the number of peripheral $CD4^+$ and $CD8^+$ cells, and no effect on viral load in patients using cannabis to control AIDS-related wasting syndrome. Likewise, Katona *et al*. (2005) found no effect on serum IFN-γ, IL-10, IL-12 or C-reactive protein in patients participating in CAMS, the multicentre randomized placebo-controlled trial on the effect of Δ^9-THC on the symptoms of multiple sclerosis. In addition, trials for the only cannabinoid

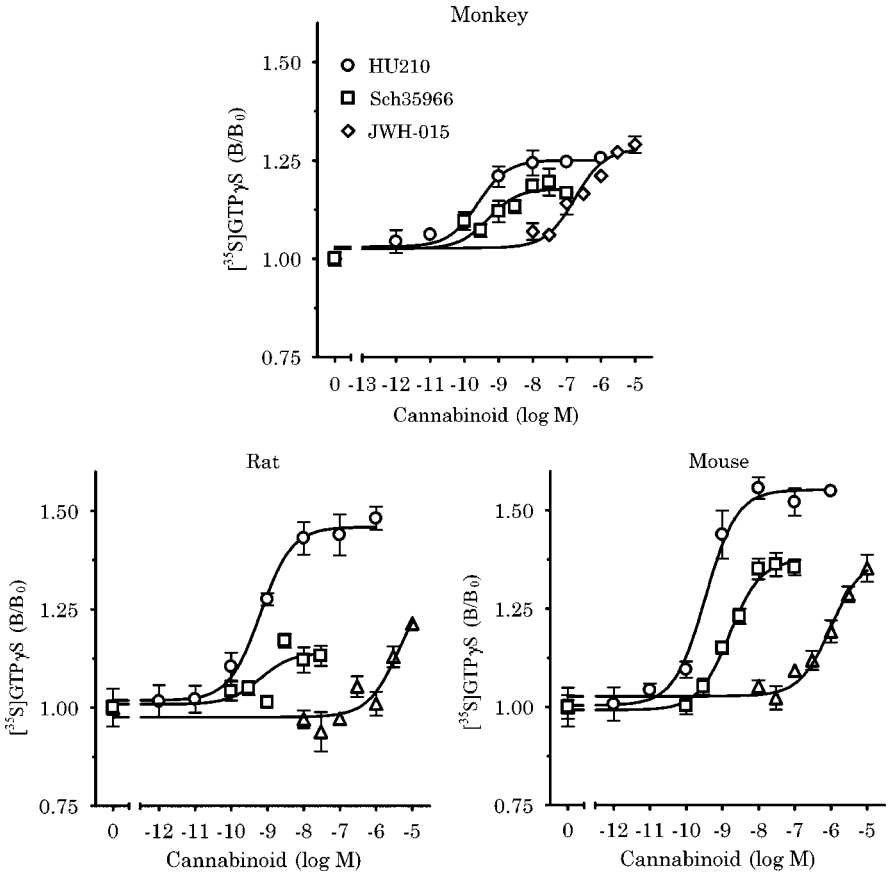

Figure 6 The effect of cannabinoids on [^{35}S]GTPγS exchange in membranes-expressing rodent or monkey CB_2. Membranes from Ba/F3 cells transfected to express CB_2 from the mouse (mCB_2), rat (rCB_2) or monkey ($cynoCB_2$) were incubated at 30°C for 60 min in binding buffer containing 5 μM GDP and the indicated concentrations of HU210, Sch35966 or JWH-015. The incubation contained 0.1 nM [^{35}S]GTPγS. Following filtration, the membrane-associated radioactivity was measured by liquid scintillation. Data represent the mean specific binding $\pm$ range of triplicate determinations from a representative experiment ($n = 2$–4).

Table 1 Comparison of functional potencies (EC_{50}) of cannabinoid ligands in stimulating [^{35}S]GTPγS exchange in membranes expressing monkey, rat or mouse CB_2

Ligand	*Monkey*	*Rat*	*Mouse*
HU-210	0.32 $\pm$ 0.17	0.28 $\pm$ 0.22	0.24 $\pm$ 0.09
Sch35966	1.5 $\pm$ 0.98	6203 $\pm$ 7597	2.4 $\pm$ 1.4
JWH-015	106 $\pm$ 41	4407 $\pm$ 3090	537 $\pm$ 253

Abbreviation: CB_2, peripheral cannabinoid receptor.
Potency data (nM) are presented as mean $\pm$ s.e./(range) values from two or three independent experiments performed in triplicate.

compounds approved for clinical use, marinol and sativex, have reported no drug-related immunological deficits in an immunocompromised patient population (Guy and Stout, 2005). It is important to note that the lack of profound immunological effects upon administration of Δ^9-THC may well reflect its lack of activity as an agonist at hCB_2 (Bayewitch *et al*., 1995, 1996; Govaerts *et al*., 2004; for review, see Howlett *et al*., 2002). Interestingly, Smith *et al*. (2000, 2001a) demonstrated that *in vivo* cytokine regulation in mice challenged with endotoxin involved the central CB_1. Studies in models of thioglycolate-induced or staphylococcus enterotoxin A-induced peritoneal inflammation demonstrated roles for both CB_1 and CB_2 ligands in mediating inflammation (Smith *et al*., 2001b). Our experiments have also suggested that cannabinoid CB_2 inverse agonists may mediate immune cell motility following an immune insult (Lavey *et al*., 2005; Lunn *et al*., 2006), supporting the idea that CB_2 agonists may not be the only productive strategy for cannabinoid-based immunoregulation.

In recent years, another CB_2-mediated physiological effect has been identified, further intensifying the search for novel CB_2-specific agonists. CB_2 agonists have been found to be effective in a number of animal models of pain, including formalin-induced and capsaicin-induced pain (Hanus *et al*., 1999; Hohmann *et al*., 2004), inflammatory pain (Clayton *et al*., 2002; Quartilho *et al*., 2003), neuropathic pain (Malan *et al*., 2002; Ibrahim *et al*., 2005) and a hindpaw incision model of postoperative pain (Labuda *et al*., 2005). The mechanism by which CB_2 mediates pain probably varies between the different classes of pain and remains an active area of investigation. Because of the highly selective expression of CB_2 in peripheral immune cells, most models target these cells. Malan *et al*. (2003) suggested that the CB_2 agonists could act on immune cells proximal to the site of insult, blocking mediator release. Ibrahim *et al*. (2005) showed that the pain response was blocked by μ-receptor

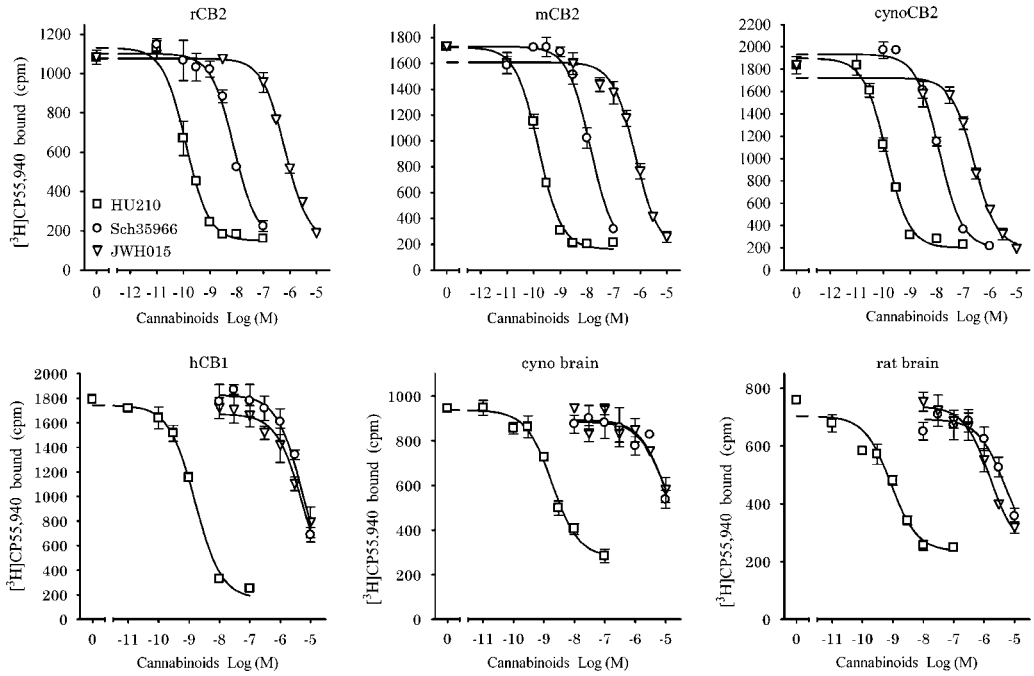

Figure 7 The effect of cannabinoids on [^{3}H]CP55,940 binding to human, rat and monkey CB_1 and CB_2. Membranes from Ba/F3 cells transfected to express rCB_2, mCB2, cynoCB_2 or hCB_1 or from rat or monkey brain were incubated at 30°C for 60 min in binding buffer containing 2 nM [^{3}H]CP55,940 and the indicated concentrations of HU210, Sch35966 or JWH-015. Following filtration, the membrane-associated radioactivity was measured by liquid scintillation. Data represent the mean specific binding ± range of triplicate determinations from a representative experiment ($n = 2$–4).

Table 2 Comparison of binding affinities (K_i) of cannabinoid ligands to CB_2 in human, monkey, rat and mouse

Ligand	*Human*	*Monkey*	*Rat*	*Mouse*
HU-210	0.45 ± 0.01	0.53 ± 0.1	0.41 ± 0.12	0.50 ± 0.09
Sch35966	6.8 ± 2.3	5.4 ± 0.4	2.4 ± 0.5	4.8 ± 1.6
JWH-015	180 ± 70	34 ± 6	340 ± 111	373 ± 176

Abbreviation: CB_2, peripheral cannabinoid receptor.
Binding constants (nM) are presented as mean ± s.d. values from at least three independent experiments performed in triplicate.

Table 3 Comparison of binding affinities (K_i) of cannabinoid ligands to human, monkey and rat CB_1

Ligand	*Human*	*Monkey*	*Rat*
HU-210	0.7 ± 0.11	0.94 ± 0.03	1.00 ± 0.18
Sch35966	2633 ± 829	5100 ± 718	3000 ± 982
JWH-015	2300 ± 442	4400 ± 1227	1257 ± 400

Binding constants (nM) are presented as mean ± s.e. values from at least three independent experiments performed in triplicate.

antagonists or anti-β-endorphin, suggesting the modulation of β-endorphin production by neighbouring tissue was involved. With increasing evidence for CB_2 expression in neural tissue (Van Sickle *et al*., 2005), other non-peripheral models for the role of CB_2 in pain have been proposed. Beltramo *et al*. (2006) showed that CB_2-specific agonists reduce capsaicin-induced calcitonin gene-related peptide (CGRP) release from cultures of spinal cord microglia cells. This effect appeared to be mediated by the CB_2, as SR144528 induced a rightward shift of the agonist dose–response curve. In addition, studies in CB_1-depleted mice ruled out a role for the central CB_1. These studies support a potential role for CB_2-specific agonists as a novel new class of drug for the induction of pain relief without psychoactive effects (Malan *et al*., 2003).

In conclusion, the benzoquinolizinone compound Sch35966 is a novel agonist at the CB_2. Sch35966 potently activates CB_2 from both primates (human and cynomolgus monkey) and rodents (rat and mouse) with >450-fold selectivity vs the CB_1. In all species examined, Sch35966 was more potent and selective than JWH-015 (a commonly used CB_2-selective agonist).

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Conflict of interest

The authors are or were employees of the Schering-Plough Research Institute.

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