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Identification of WIN55212-3 as a competitive neutral antagonist of the human cannabinoid CB_2 receptor

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1 Several G protein-coupled receptors (GPCRs), including cannabinoid CB_1 and CB_2 receptors, show constitutive activity under heterologous expression. Such a tonic response is generated in the absence of an activating ligand, and can be inhibited by inverse agonists. Neutral antagonists, however, are silent at such receptors, but can reverse both agonist and inverse agonist responses. To date, no neutral antagonist for the CB_2 receptor has been reported.

2 Here, by monitoring receptor-dependent G protein activation, we demonstrate that WIN55212-3 acts as a neutral antagonist at the human CB_2 (hCB_2) receptor. WIN55212-3 alone, at concentrations $\leqslant 10^{-4}$ M, behaved as a silent ligand exhibiting no agonist or inverse agonist activity. However, WIN55212-3 competitively antagonized cannabinoid agonist CP-55,940-stimulated responses (pA_2 6.1). Importantly, the inverse agonism evoked by SR144528 in hCB_2 was dose-dependently reversed by WIN55212-3 (pEC_{50} 5.3 ± 0.2), indicating true neutral antagonist behavior.

3 Furthermore, WIN55212-3 also antagonized CB_1 receptor signaling in a competitive manner (pA_2 5.6), but behaved as a partial inverse agonist (pIC_{50} 5.5 ± 0.1) at the constitutively active human CB_1.

4 Additionally, WIN55212-3 antagonized signaling of the human melatonin MT_1 receptor, with modest activity at the human muscarinic M4 receptor, but it was inactive towards several other GPCRs.

5 These data identify WIN55212-3 as a true neutral hCB_2 receptor antagonist. WIN55212-3 offers a valuable tool for further characterization of ligand activities at the CB_2 receptor and may serve as a lead compound in further efforts to develop more potent and selective neutral CB_2 receptor antagonists.

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Abbreviations: AEA, arachidonoylethanolamide; 2-AG, 2-arachidonoylglycerol; 2-AGE, 2-arachidonoylglycerylether, noladin ether; CB_1, neuronal cannabinoid receptor; CB_2, peripheral cannabinoid receptor; CHO, Chinese hamster ovary; CP-55,940, (−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-[3-hydroxypropyl] cyclohexan-1-ol; GTPγS, guanosine-5′-*O*-(3-thio)-triphosphate; [^{35}S]GTPγS, guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate; HU-210, (6a*R*)-*trans*-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol; MAFP, methyl arachidonoyl fluorophosphonate; PMSF, phenyl methyl sulfonylfluoride; SR141716, *N*-piperidin-*O*-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; SR144528, *N*-[1*S*)-endo-1,3,3-trimethyl-bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; WIN55212-2(*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone; WIN55212-3, (S)-(−)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone

Introduction

The occurrence of constitutively active G protein-coupled receptors (GPCR) has recently been described by different experiments and theories. The prevailing two-state-model theory suggests that GPCRs may fluctuate between active (*R**) and inactive (*R*) states. Affinity to *R** is higher with agonists and *R* with inverse agonists, whereas neutral antagonists (i.e., ligands lacking intrinsic activity) may bind to both receptor states equally. So far, constitutive receptor activity has been largely recognized in heterologous expression systems (Seifert & Wenzel-Seifert, 2002; Kenakin, 2004). In native tissues, however, the situation is more complex. In many experimental situations, it may be difficult to rule out the contribution of an endogenous agonist (Laitinen, 2004 and references therein).

Cannabinoid receptors (CB_1 and CB_2) are typical GPCRs, having seven-membrane spanning structures (Howlett *et al.*, 2002). The neuronal cannabinoid receptor (CB_1) is highly expressed in several areas of the mammalian brain (Matsuda *et al.*, 1990; Herkenham *et al.*, 1991). The low abundance cannabinoid receptor (CB_2) is mainly located in immune cells and lymphoid tissues, and may play a role in immune and inflammatory reactions (Munro *et al.*, 1993; Howlett *et al.*, 2002). So far, a few potent CB_2 agonists and inverse agonists

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have been characterized, but neutral CB_2 receptor antagonists have not been reported.

Herein are described cannabinoid ligand activities at the constitutively active human CB_2 (hCB2) receptor, stably expressed in Chinese hamster ovary (CHO) cells. By [^{35}S]GTPγS binding studies, the capacity of the major endocannabinoids (2-arachidonoylglycerol (2-AG), arachidonoylethanolamide (AEA) and noladin ether (2-arachidonoylglycerylether; 2-AGE)) (Devane *et al*., 1992; Mechoulam *et al*., 1995; Sugiura *et al*., 1995; Hanus *et al*., 2001) was determined, in addition to several synthetic cannabinoids, to influence CB_2-mediated G protein activity. WIN55212-3 (Figure 7b), which is known as the inactive isomer of the cannabinoid CB_1 and CB_2 receptor agonist WIN55212-2 (Figure 7a) (Pacheco *et al*., 1991; Compton *et al*., 1992; Felder *et al*., 1992; Slipetz *et al*., 1995; Sugiura *et al*., 2000), was unexpectedly found to behave as a competitive neutral antagonist of the hCB_2 receptor. In contrast, WIN55212-3 acts as a partial inverse agonist at the human CB_1 (hCB_1) receptor. We also show that WIN55212-3 blocked human melatonin MT_1 and human M4 muscarinic receptors, but was inactive towards other GPCRs. The present study is the first description of a true neutral antagonist for the cannabinoid CB_2 receptor.

Methods

Chemicals

2-AG, MAFP and anti-CB_2 polyclonal antibody were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). AEA and 2-AGE were synthesized in the Department of Pharmaceutical Chemistry, University of Kuopio. SR144528 and SR141716 were obtained from Sanofi Recherche (Montpellier, France). CP-55,940, HU-210 and luzindole were purchased from Tocris Cookson Ltd. (Bristol, U.K.). BSA (essentially fatty acid free), DTT, PMSF, GDP and GTPγS were purchased from Sigma (St Louis, MO, U.S.A.). Anti-Rabbit IgG-horseradish peroxidase conjugate was obtained from Santa-Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Adenosine deaminase (ADA) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). WIN55212-2 and WIN55212-3 were purchased from RBI/Sigma (Natick, MA, U.S.A.). [^{35}S]GTPγS (initial specific activity 1250 Ci mmol^{-1}) and [^{3}H]CP-55,940 (initial specific activity 158 Ci mmol^{-1}) were purchased from Perkin-Elmer Life Science Products, Inc. (Boston, MA, U.S.A.). Unless otherwise stated, all other chemicals were purchased from Sigma (St Louis, MO, U.S.A.) or Bio-Rad (Hercules, CA, U.S.A.), and were of highest purity available.

Generation of stably transfected cell lines

The coding sequence of the human cannabinoid CB_2 receptor (as a gift from Dr Sean Munro) was subcloned into pcDNA3 (Invitrogen) and sequenced. Recombinant plasmids were introduced into CHO cells by the Lipofectamine 2000 transfection reagent (Life Technologies/Gibco). Generation of human melatonin MT_1, $P2Y_{12}$ and muscarinic M4-receptors stably expressed in CHO cells is described elsewhere (Kokkola *et al*., 2005; Kokkola *et al*., manuscript in preparation). Transfected CHO cells were placed under G-418 selection (600 μg ml^{-1}) and several cell lines originating from single G-418 resistant cells were isolated. Cell clones with the highest levels of specific [^{3}H]CP-55,940 binding were maintained for subsequent experiments. No [^{3}H]CP-55,940 binding exceeding blank values (binding in the absence of cells) was detected in control CHO cells (data not shown). The G-418 resistant cell lines were cultured as monolayers with 100 μg ml^{-1} G-418 (Euroclone) in Ham's F-12 nutrient mixture (Euroclone), containing 10% fetal calf serum (Euroclone), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Euroclone) at 37°C in a humidified atmosphere of 5% CO_2/95% air.

[^{3}H]CP-55,940-binding assay

For saturation studies, [^{3}H]CP-55,940 binding to intact CHO-hCB_2 cell clones was performed as previously described (Gonsiorek *et al*., 2000), with some modifications. Briefly, the day before the assay, cells (20,000 cells per well) were seeded into 96-well plates. On the next day, after two washes with 200 μl of F-12 medium-based binding buffer containing 50 mM Tris-HCl, pH 7.4, 0.2% BSA and 150 mM NaCl, cells were incubated for 3 h at 20°C with gentle shaking in a binding buffer (200 μl) that contained different concentrations of [^{3}H]CP-55,940. Nonspecific binding was determined in the presence of 5 μM HU-210 and specific binding was obtained after subtraction of nonspecific from total binding. After three washes with binding buffer, cells were lysed with 0.5 M NaOH overnight at 20°C and then solubilized in a scintilliation liquid (HiSafe3, Wallac) before counting (1450 Microbeta liquid scintillation counter) at a 36% counting efficiency for [^{3}H]. For competitive binding experiments, CHO-hCB_2 cell membranes (10 μg) were incubated in the presence of 1.5 nM [^{3}H]CP-55,940 in the final volume of 400 μl, containing 55 mM Tris-HCl (pH 7.4), 1.1 mM EDTA, 5 mM $MgCl_2$ and 0.5% BSA with increasing concentrations of various cannabinoids dissolved in ethanol (final concentration 1%). Incubations were initiated by the addition of 40 μl membrane dilution and continued for 90 min at 25°C. Nonspecific binding was determined in the presence of 5 μM HU-210. The assay was terminated by three 4-ml washes with ice-cold washing buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 0.1% BSA), followed by rapid filtration through glass fiber filters (Whatman GF/B), pretreated overnight at +4°C with the washing buffer. The filters were transferred into scintillation vials together with 2 ml of scintillation cocktail (HiSafe, Perkin-Elmer/Wallac, Turku). After horizontal shaking for 15 min, the vials were centrifuged at 1000 × *g* for 10 min at room temperature to force the filters to the bottom. On the next day, vials were counted with a Wallac LKB 1214 Rackbeta counter. Specific binding represented 64 ± 2% (mean ± s.e.m., $n = 4$) of total.

Western blotting

Cell lysate samples from CHO clones (10,000 cells corresponding to ~1 μg protein), together with molecular weight markers, were fractionated by SDS–PAGE (10%) and transferred to a nitrocellulose membrane (Protran, Schleicher and Schell, Dassel, Germany). To block nonspecific binding, membranes were incubated with a 5% (w v^{-1}) fat-free milk solution for 1 h at 20°C. Next, membranes were treated overnight at +4°C with anti-CB_2-polyclonal antibody (1 : 250) and washed 4 × 10 min in Tris-buffered saline containing 0.1% Tween-20

(TBS-T). Membranes were then incubated with a secondary antibody (anti-rabbit IgG-HRP, 1 : 20,000) for 1 h at 20°C, followed by washing 4×10 min with TBS-T. Immunoblots were visualized by a Western blot chemiluminiscence reagent (Perkin Elmer, Boston, MA, U.S.A.) and quantified by image-analysis software (Scion image, Frederick, MA, U.S.A.).

Preparation of CHO cell membranes or lysates and rat cerebellar membranes

All animal experiments were approved by the local ethics committee. Rat cerebellar membranes were prepared as previously described (Savinainen *et al*., 2001). For membrane preparation, CHO cells stably expressing either hCB_2, melatonin MT_1, purinergic $P2Y_{12}$ or muscarinic M4 receptors were washed with and harvested in an ice-cold PBS solution containing 5 mM EDTA. Cells were then pelleted by centrifugation at $250 \times g$ for 10 min at $+4$°C and stored at -80°C until use. Membranes were prepared as previously described (Savinainen *et al*., 2001), except that the pellets were thawed in ice-cold 0.32 M sucrose and homogenized 3×20 s using Ultra-Turrax. Finally, membranes were resuspended in 50 mM Tris-HCl, pH 7.4 containing 1 mM EDTA and aliquoted for storage at -80°C. For the preparation of lysates, CHO cell monolayers were washed with PBS, trypsinized, and resuspended in the medium. Cell density was determined by hemocytometer. Cells were then centrifuged at $250 \times g$ for 10 min at 20°C. The resulting pellet was resuspended in, and washed with, PBS. Centrifugation was repeated and the pellet was resuspended in PBS. The suspension was then divided into aliquots (1.65×10^6 cells per aliquot), followed again by centrifugation. The supernatants were discarded and the dry pellets freeze–thawed twice and stored at -80°C until use. The protein concentrations of membranes and lysates were measured by the Bradford method (Bradford, 1976). Membranes prepared from CHO cells stably expressing the human CB_1 receptor (B_{max} 33 pmol mg^{-1} protein) were purchased from Euroscreen (Brussels, Belgium).

[^{35}S]GTPγS-binding assays

When CHO lysates were used in [^{35}S]GTPγS-binding assay, dry pellets (1.65×10^6 cells) were thawed and resuspended in 5 mM Tris-HCl pH 7.4 containing 0.1 mM EDTA. Membranes and lysates were preincubated for 30 min at 25°C protected from light in the presence of 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 10 μM GDP and 0.5 U ml^{-1} ADA. Incubations were carried out as previously described (Savinainen *et al*., 2001) with the following modifications. The final incubation contained 5 μg membrane protein or lysate from 50,000 cells, 55 mM Tris-HCl pH 7.4, 1.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% (w v^{-1}) BSA, 11 μM GDP, 0.55 U ml^{-1} ADA, ~150 pM [^{35}S]GTPγS with the studied agonists in ethanol (final concentration 1%, v v^{-1}) or SR144528 and WIN55212-3 in DMSO (final concentration 0.5%, v v^{-1}). In experiments using MAFP or PMSF to inhibit 2-AG degradation, 0.5% (w v^{-1}) BSA was included in the preincubation step. Nontransfected CHO cells or CHO cells expressing noncannabinoid GPCRs did not respond to cannabinoid receptor agonists (data not shown). Incubations for measuring CB_1 receptor activities with rat cerebellar membranes were conducted under optimized conditions, essentially as previously described (Savinainen *et al*., 2003).

Molecular modeling

Structures of WIN55212-2 and WIN55212-3 were generated from a crystal template (refcode NADJAD in the Cambridge Structural Database) using the molecular modeling package SYBYL 6.9.2 (Tripos Associates, Inc.). The conformational analysis of the WIN55212 enantiomers was performed in vacuum utilizing the Tripos force field (Clark *et al*., 1989). For this purpose, the van der Waals radius of the hydrogen atoms was set to 1.1 Å, and the molecules were subjected to a simulated annealing protocol of 50 cycles (i.e., heating the molecule at 1000 K for 1000 fs, followed by an exponential cooling down of the molecule to 200 K for 1000 fs). Thereafter, the resulting 50 cooled-down conformers of both molecules were optimized in the MMFF94s force field (Halgren, 1999) using the BFGS minimizer (Broyden, 1970; Flethcer, 1970; Goldfarb, 1970; Shanno, 1970) for 500 iterations, or until the gradient change was less than 0.05 kcal (mol Å)$^{-1}$. The energy of the conformers was calculated in the Tripos force field. WIN55212-2 and WIN55212-3 conformers were superimposed on each other (50×50 superimpositions) with an in-house program that finds the best ways of superimposing the steric and electrostatic fields of the molecules. In addition, the lowest-energy conformers were superimposed at the indole ring system using the simple FIT ATOMS option in SYBYL.

Data analysis

For experimental data, results are presented as mean ± s.e.m. of at least three independent experiments performed in duplicate. Data analysis for dose–response curves were calculated as nonlinear regressions using sigmoidal dose-response fitting. Statistical differences between groups were tested using one-way ANOVA (unless otherwise stated), followed by Tukey's Multiple comparison test, with $P < 0.05$ considered as statistically significant. Data analysis was performed using GraphPad Prism 3.0 for Windows.

Results

Characterization of the hCB_2 receptor expressing cell lines

Dissociation constants (K_d) and maximal binding capacities (B_{max}) for the CHO-hCB_2 cell clones were determinated by the [^{3}H]CP-55,940 saturation binding studies, as described in Methods, and two clones with K_d values 4.8 ± 0.9 and 4.4 ± 0.9 nM (s.e.m., $n = 3$) and B_{max} values 20.4 ± 1.7 and 20.3 ± 1.7 pmol mg^{-1} (s.e.m., $n = 3$) were used in further experiments. Further identification of the hCB_2 receptor was performed by Western blotting, as described in Methods. The hCB_2 receptor was recognized as two bands (43 and 45 kDa) (Figure 1) that were not detected in control cells. Notable is that this antibody also recognized a relatively dense non-specific band (40 kDa) both in CB_2 receptor and non-CB_2 receptor cells (data not shown).

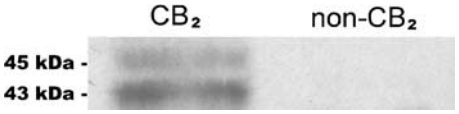

Figure 1 The identification of human CB_2 receptor from stable transfected CHO cells by Western blotting. CHO cells expressing muscarinic M4 receptor were used as a negative control (non-CB_2).

2-AG is the most potent and efficacious endocannabinoid activating the hCB$_2$ receptor

The fact that 2-AG and AEA are endocannabinoids at CB_1 and CB_2 receptors is already well established by several functional studies (Sugiura *et al*., 1999; 2000; Gonsiorek *et al*., 2000; Savinainen *et al*., 2001). However, the ability of 2-AGE (the third putative endocannabinoid) to stimulate G proteins *via* human CB_2 receptor is not yet clear. In the present study, the CB_2 receptor activities of 2-AG, AEA and 2-AGE, together with structurally different synthetic cannabinoid agonists (HU-210, WIN55212-2 and CP-55,940), were determined in [^{35}S]GTPγS-binding experiments. In addition, the enzymatic stabilities of endocannabinoids in CHO cell preparations were tested by HPLC analysis, as previously described (Savinainen *et al*., 2001; 2003). These studies revealed that 2-AG is more stable in lysates than in membranes, whereas AEA and noladin ether (2-AGE) were stable in both preparations (data not shown). The partial enzymatic degradation of 2-AG was fully blocked by pretreatment with monoacylglycerol lipase (MGL) and fatty acid amide hydrolase (FAAH) inhibitors MAFP (1 μM) and PMSF (1 mM). However, both enzyme inhibitors also increased basal [^{35}S]GTPγS binding CB_2 receptor-dependently (data not shown) and, therefore, were not used in further experiments. As shown in Figure 2 and Table 1, HU-210 was the most potent ($EC_{50} = 0.2$ nM) cannabinoid with full agonist properties to stimulate [^{35}S]GTPγS binding in CHO-hCB_2 cell lysates. Another synthetic cannabinoid, CP-55,940 was the most efficacious agonist exhibiting super agonist behavior. 2-AG was the most potent ($EC_{50} = 40$ nM) and efficacious endocannabinoid studied. 2-AGE was the second most potent ($EC_{50} = 270$ nM) endocannabinoid, with an efficacy similar to that of 2-AG, whereas AEA was the least potent endocannabinoid ($EC_{50} = 345$ nM) and behaved as a partial agonist. Also, noteworthy was the observation that the full CB_1 receptor agonist WIN55212-2 (Glass & Northup, 1999) behaved as a partial agonist at the CB_2 receptor, but concomitantly, its potency was similar to that of CP-55,940 ($EC_{50} = 0.6$ nM).

WIN55212-3 is a neutral CB$_2$ antagonist and a partial inverse CB$_1$ agonist

Unlike the WIN55212-2 enantiomer, which is a potent and high-affinity cannabinoid agonist, WIN55212-3 has been reported to have both a low receptor affinity and low capacity to modulate second messenger responses *via* cannabinoid CB_1 and CB_2 receptors (Pacheco *et al*., 1991; Compton *et al*., 1992; Felder *et al*., 1992; Slipetz *et al*., 1995; Sugiura *et al*., 2000). Consistent with these results, but in contrast to a very recent study with exceptionally high hCB_2 receptor density

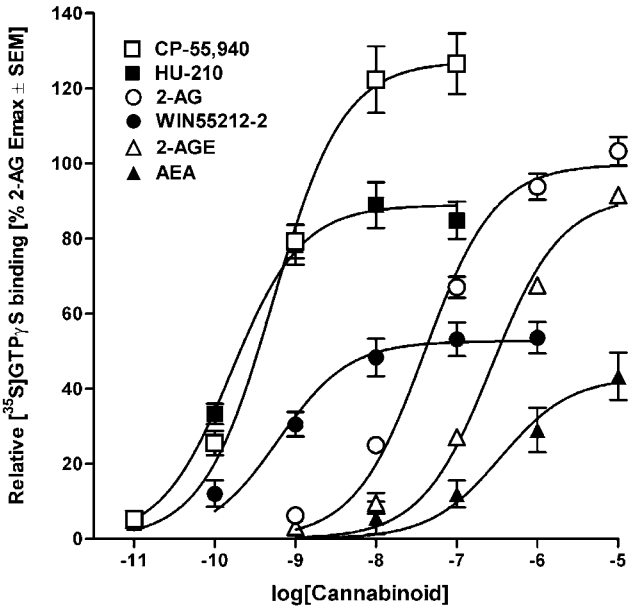

Figure 2 Dose–response curves for the endogenous and synthetic cannabinoids tested in CHO-hCB_2 cell lysates. The [^{35}S]GTPγS-binding experiments were performed as described in Methods. The data represent the relative [^{35}S]GTPγS binding (% from maximal 2-AG response ± s.e.m.) from at least three independent experiments performed in duplicate.

Table 1 Potency and relative efficacy of endogenous and synthetic cannabinoids, tested in lysates prepared from CHO-hCB_2 cells

Agonist	*Potency* ($-Log\ EC_{50}$ ± s.e.m.)	*Relative* E_{max} (*%2-AG-*E_{max} ± s.e.m.)	*Agonism*
2-AG	7.4 ± 0.1	100 ± 2[a]	Full
2-AGE	6.6 ± 0.1	91 ± 3[a]	Full
AEA	6.5 ± 0.2	43 ± 5[b]	Partial
CP-55,940	9.3 ± 0.1	127 ± 5	Super
WIN55212-2	9.2 ± 0.1	53 ± 2[b]	Partial
HU-210	9.8 ± 0.1	89 ± 3[a]	Full

[a,b]No significant ($P > 0.05$) difference in efficacy.
The data represent the mean (± s.e.m.) of [^{35}S]GTPγS binding from the maximal response evoked by 2-AG and are from at least three independent experiments performed in duplicate.

($B_{max} \sim 110$ pmol mg^{-1} protein) (Govaerts *et al*., 2004), WIN55212-3 exhibited no agonist or inverse agonist activities at the hCB_2 receptor (Figures 3 and 4b). However, to test whether WIN55212-3 shows neutral antagonism at the CB_2 receptor, we challenged both agonist CP-55,940 and inverse agonist SR144528 (Portier *et al*., 1999) responses to increasing concentrations of WIN55212-3. As depicted in Figure 4a, dose–response curves to CP-55,940 were right-shifted with increasing concentrations of WIN55212-3, indicating a competitive mode of antagonism at the hCB_2 receptor with pA_2 value 6.1. Consistently, half-maximal responses evoked by other types of cannabinoid agonists, 2-AG (10^{-7} M), HU-210 (3×10^{-9} M) and WIN55212-2 (10^{-9} M), were abolished by WIN55212-3 (10^{-4} M) as well (data not shown). To further test whether antagonism at CB_2 is neutral-type, response to inverse

agonist SR144528 (which alone inhibited basal [^{35}S]GTPγS binding by $\sim$40% with an IC_{50} value of 3 nM) was challenged with WIN55212-3. As shown in Figure 4b, the SR144528 response (evoked at 3 nM) was reversed dose-dependently to basal (pEC_{50} 5.3$\pm$0.2), indicating true neutral antagonism at the hCB_2 receptor. The presently used approach did not allow the examination of WIN55212-3 behavior in native cellular environment (rat spleen or RAW-264 macrophages) as CB_2-dependent G protein activity was undetectable in membranes of these native tissues (data not shown).

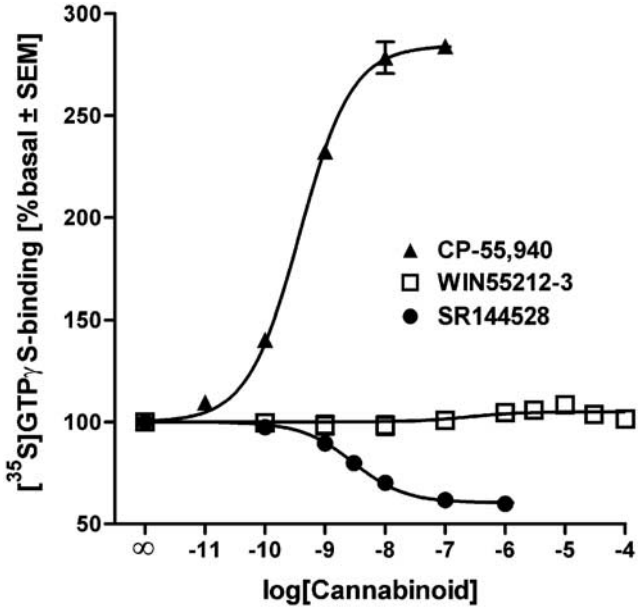

Figure 3 Unlike the cannabinoid agonist CP-55,940 or the inverse CB_2 receptor agonist SR144528, WIN55212-3 is silent with respect to G protein activity in CHO-hCB_2 cell membranes. The data represent the [^{35}S]GTPγS binding (% basal$\pm$s.e.m.) from at least three independent experiments performed in duplicate.

The displacement binding experiments using CHO hCB_2 membranes (Figure 5) revealed that WIN55212-3 inhibited [^{3}H]CP-55,940 binding with relatively low, but expected potency ($IC_{50}=13.2\,\mu$M). In contrast, the active enantiomer WIN55212-2 ($IC_{50}=4.1$ nM) was approximately 3200-fold more potent. The inhibition of [^{3}H]CP-55,940 binding by WIN55212-3 was not complete at the used concentrations ($\leqslant 10^{-4}$ M) (Figure 5). Higher concentrations of WIN55212-3 could not be tested due to solubility problems. From the endocannabinoids, 2-AGE showed the highest affinity ($IC_{50}=1.7\,\mu$M) at the hCB2 receptor while 2-AG and AEA exhibited equal affinity ($IC_{50}\sim 5\,\mu$M). From the synthetic cannabinoids, CP-55,940 and HU-210 were the most potent (IC_{50} values 1.8 and 2.6 nM, respectively), whereas SR144528 was the least potent ($IC_{50}=21.3$ nM) synthetic compound. The potencies obtained from the [^{35}S]GTPγS-binding studies (Table 1) were somewhat higher than those obtained from the affinity study (Figure 5). However, when the values from these separate assays were plotted against each other, they showed a significant positive correlation ($r^2=0.91$, $n=7$; $P<0.001$ as compared to a slope with zero-value).

The activity of WIN55212-3 was also tested against rat cerebellar CB_1 and human CB_1 receptors. Under the assay conditions employed, the native rat CB_1 receptor shows no constitutive activity (Savinainen *et al*., 2001; 2003). As with the CB_2 receptor, dose–responses to CP-55,940 in rat cerebellar membranes were competitively antagonized by WIN55212-3 (pA_2 value 5.6) (Figure 6a). WIN55212-3 itself was inactive in cerebellar membranes (data not shown). However, in CHO cell membranes over-expressing the hCB_1 receptor, WIN55212-3 dose-dependently inhibited basal G protein activity (pIC_{50} 5.5$\pm$0.1), indicating that it is an inverse CB_1 agonist (Figure 6b). This inverse agonism was confirmed by the finding that the inhibitory response evoked by 1 nM SR141716 ($\sim IC_{50}$ value) was further enhanced by WIN55212-3 in a dose-dependent manner, and to the same maximum as that evoked by WIN55212-3 alone (Figure 6b). When comparing maximal inhibitory effects produced by the inverse CB_1 agonist SR141716 (MacLennan *et al*., 1998) and WIN55212-3 (44$\pm$1

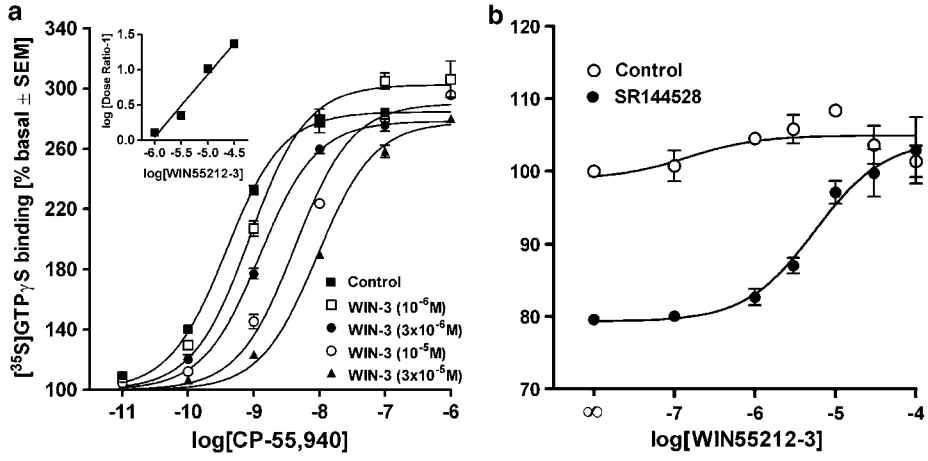

Figure 4 (a) WIN55212-3 competitively antagonizes CP-55,940 responses at the hCB_2 receptor. pA_2 value (6.1) for WIN55212-3 was calculated from the Schild plot. The slope (0.88$\pm$0.11) does not differ significantly from unity ($P>0.05$, unpaired *t*-test). (b) WIN55212-3 is a neutral hCB_2 receptor antagonist, reversing the inverse agonism evoked by SR144528 (3 nM). The data represent the [^{35}S]GTPγS binding (% basal$\pm$s.e.m.) in hCB_2 membranes from at least three independent experiments performed in duplicate.

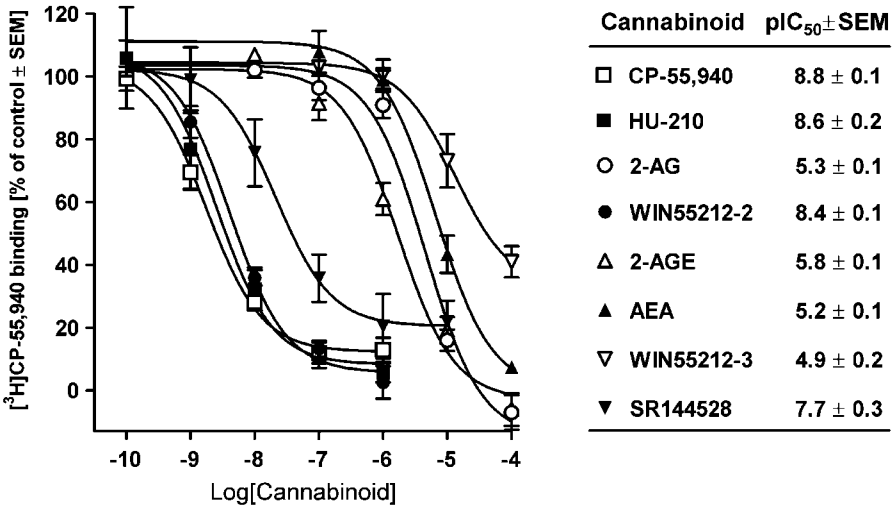

Figure 5 Affinities (pIC_{50}) of various cannabinoids in displacing [^{3}H]CP-55,940 (1.5 nM) binding to CHO hCB_2 cell membranes. Experiments were performed as described in Methods, and the data represent the specific binding (% from control ± s.e.m.) from three independent experiments performed in duplicate.

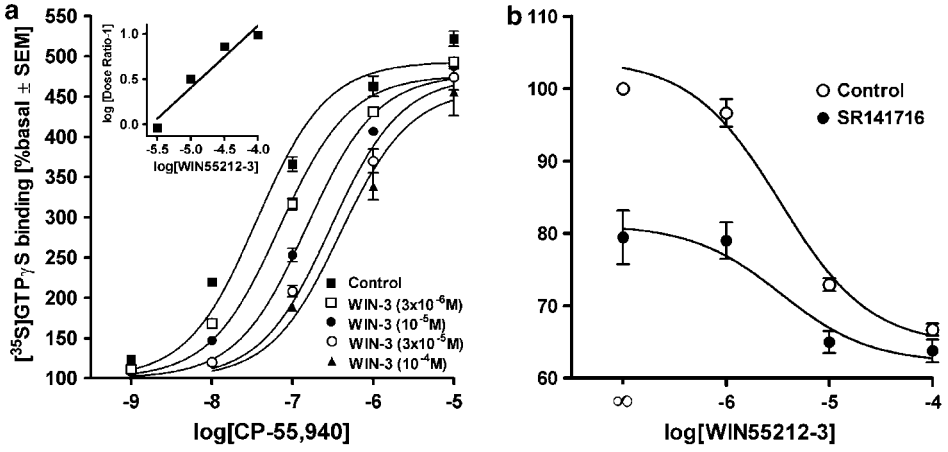

Figure 6 (a) WIN55212-3 competitively antagonizes responses to CP-55,940 at the rat cerebellar CB_1 receptor. pA_2 value (5.6) for WIN55212-3 was calculated from Schild plot. The slope (0.69 ± 0.13) does not differ significantly from unity ($P > 0.05$, unpaired *t*-test). (b) WIN55212-3 is a partial inverse agonist of the human CB_1 receptor and enhances the inhibitory effect evoked by the CB_1 inverse agonist SR141716 (1 nM). The data represent the [^{35}S]GTPγS binding (% basal ± s.e.m.) in rat cerebellar membranes (a) or human CB_1 membranes (b) from at least three independent experiments performed in duplicate.

and 67 ± 1% basal, respectively), WIN55212-3 can be classified as a partial inverse CB_1 agonist.

Molecular modeling of WIN55212-2 and WIN55212-3

In order to get insight into the dramatic difference in activity produced by the two enantiomers (i.e., high-potency agonism vs low-potency antagonism), the 3D structures of WIN55212-2 and WIN55212-3 were modeled. Both enantiomers can adopt comparable conformations, even though the greatest difference comes from the preferred location of the morpholine ring (Figure 7a and b). The lowest-energy conformers of WIN55212-2 and WIN55212-3 are exact mirror images of each other (Figure 7c). With the in-house program (see Methods), it was possible to find such conformers for WIN55212-2 and WIN55212-3 where the structural moieties fit fairly well to each other. However, the energies of such conformers were significantly (over 10 kcal mol^{-1}) higher than those of the lowest-energy conformers.

Effects of WIN55212-3 on other GPCRs

The selectivity of WIN55212-3 as a cannabinoid receptor antagonist was further examined against several other GPCRs. These studies revealed that WIN55212-3 (at 10^{-4} M) antagonized signaling of the human recombinant MT_1 receptor, and that of the human recombinant muscarinic M4 receptor to a lesser degree (Figure 8). In contrast, WIN55212-3 did not affect rat brain adenosine A_1, sphingosine-1-phosphate, $GABA_B$ and opioid receptors, or human recombinant $P2Y_{12}$ or endogenous lysophosphatidic acid receptors expressed in CHO cells (Figure 8). WIN55212-3 alone (10^{-4} M) had no

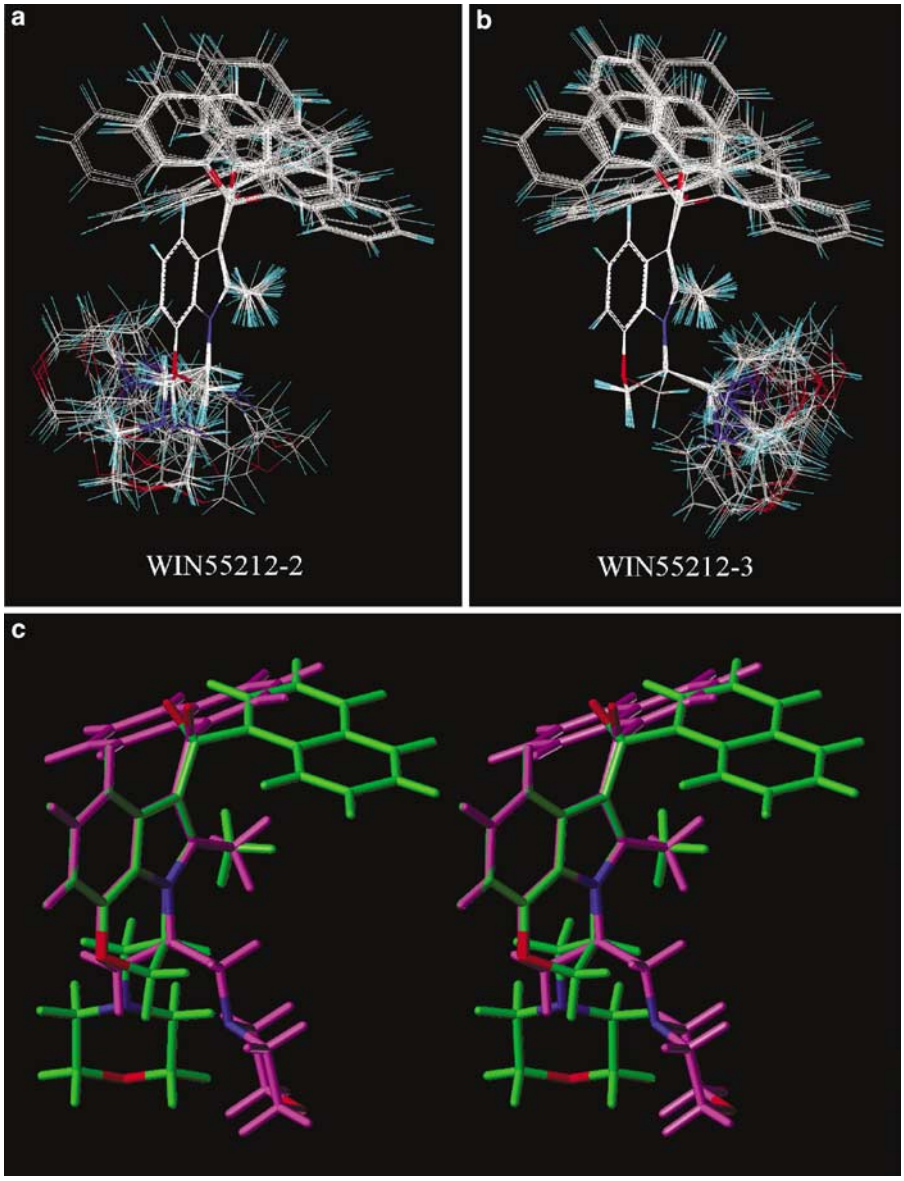

Figure 7 Different conformations of (a) WIN55212-2 and (b) WIN55212-3 produced in the simulated annealing procedure. Atom color code: carbon, grey; oxygen, red; nitrogen, blue; hydrogen, cyan. (c) Stereoview for the superimposed lowest-energy conformers of WIN55212-2 (green) and WIN55212-3 (magenta).

effects on basal G protein activity in any of the above-studied receptor models (data not shown). As WIN55212-3 clearly antagonized MT_1 receptor responses, its relative potency was tested against the MT receptor antagonist luzindole. As evident from Figure 9, luzindole was approximately 27 times more potent than WIN55212-3 in reversing hMT_1 receptor responses.

Discussion

The traditional concept of antagonism at GPCRs has changed considerably with the discovery of inverse agonism. By recent estimation, most of the antagonists that target different GPCRs appear to behave as inverse agonists, and only a small fraction ($\sim$15%) are recognized as neutral antagonists (Kenakin, 2004). To date, a few potential neutral antagonists for the CB_1 receptor have been suggested (Hurst *et al*., 2002; Thomas *et al*., 2004), but none has been reported for the CB_2 receptor. Among the neutral CB_1 antagonist candidates, VCHSR (an SR141716 derivative), has been reported to exhibit a high binding affinity for the CB_1 receptor ($K_i = 31.3$ nM) but the neutral antagonistic properties were, however, demonstrated only by using a single, and much higher concentration (1 μM) of the compound (Hurst *et al*., 2002). In our study, WIN55212-3 was clearly identified as a true, neutral CB_2 receptor antagonist. WIN55212-3 fulfilled all the basic requirements at the hCB_2 receptor that are needed for a neutral antagonist, including an inability to produce receptor activity itself, as well as an ability to dose-dependently inhibit both agonist and inverse agonist responses. Additionally, in agreement with the recent report (Govaerts *et al*., 2004),

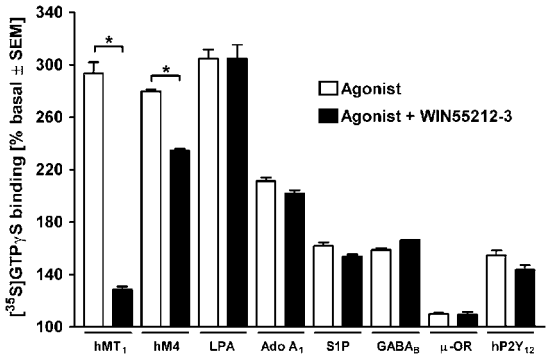

Figure 8 WIN55212-3 (10^{-4} M) significantly antagonizes agonist responses at the human recombinant melatonin MT_1 (melatonin at 10^{-9} M) and human recombinant muscarinic M4 ACh (carbachol at 10^{-5} M) receptors, but does not affect rat brain adenosine A_1 (2-chloroadenosine at 3×10^{-6} M), sphingosine-1-phosphate (S1P at 10^{-7} M), $GABA_B$ (R(+)-baclofen at 10^{-5} M) and opioid receptors (μ-OR; DAMGO at 10^{-7} M), or human recombinant $P2Y_{12}$ (2MeSADP at 10^{-9} M) or endogenous lysophosphatidic acid (LPA at 10^{-7} M) receptors in CHO cells. The data represent the [^{35}S]GTPγS binding (% basal ± s.e.m.) in membranes expressing appropriate receptors (hMT1, hM4, $hP2Y_{12}$ or LPA) or in rat cerebellar membranes from at least three independent experiments performed in duplicate. An asterisk denotes a statistically significant difference ($P<0.05$).

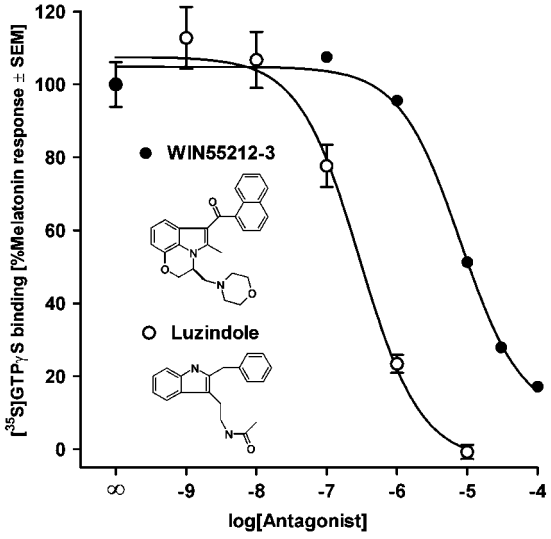

Figure 9 Albeit being less potent than the melatonin receptor antagonist luzindole (IC_{50} 0.3 μM), WIN55212-3 (IC_{50} 8 μM) dose-dependently antagonizes melatonin response (1 nM) at the human melatonin MT_1 receptor. The data represent the [^{35}S]GTPγS (% basal ± s.e.m.) in hMT_1 membranes from at least three independent experiments performed in duplicate.

WIN55212-3 behaved as a partial inverse agonist at the hCB_1 receptor. As an evidence of receptor-mediated effects, WIN55212-3 showed antagonistic properties with a potency that is matching with our affinity results at hCB_2 receptor and reported affinities (>1 μM) at native and recombinant cannabinoid CB_1 and CB_2 receptors (Felder *et al*., 1992; Slipetz *et al*., 1995; Govaerts *et al*., 2004).

Against the two-state receptor model, our results indicate that WIN55212-3 preferentially binds to the inactive state of hCB_1 (partial inverse agonist), but binds equally well to the inactive and active states of the hCB_2 receptor (a neutral CB_2 antagonist). In addition, our data from the molecular modeling suggest that the bioactive conformations of WIN55212 enantiomers are not superimposable, which may explain their significantly different activity and binding properties. The finding is supported with the previous observation that in the preferred orientation in solution, the morpholinyl substituent of WIN55212 has its plane off the indole ring (Xie *et al*., 1999), whereas in the closest superpositioning of the enantiomers, the planes of the morpholinyl substituents approached the plane of the indole ring.

Noteworthy, WIN55212-3 is often used as a negative control in *in vivo* experiments to ensure cannabimetic activity produced by WIN55212-2 (Song & Slowey, 2000; Pfitzer *et al*., 2004; and references therein). Our data do not contradict this: due to the huge potency difference between the two enantiomers, it is presumable that comparable (low) doses of WIN55212-3 used in these studies are indeed insufficient to evoke significant antagonistic effects *in vivo*. Nevertheless, as WIN55212-3 antagonized cannabinoid receptor activity with relatively low potency, its specificity was determined towards several other GPCRs. Among these, WIN55212-3 showed antagonist activity at the melatonin MT_1 receptor.

In full accordance with the previous reports measuring CB_2 receptor-mediated G protein activity and second-messenger responses in stably transfected CHO cells (Gonsiorek *et al*., 2000), or measuring intracellular Ca^{2+} levels in intact HL-60 cells endogenously expressing the human CB_2 receptor (Sugiura *et al*., 2000), our results showed that the efficacy and potency of 2-AG to stimulate CB_2 receptor-mediated G protein activity was superior as compared to AEA. Also, in agreement with the study determining endocannabinoid activity in intact HL-60 cells (Sugiura *et al*., 2000), noladin ether appeared as the second efficacious endocannabinoid at the human CB_2 receptor. Furthermore, observed differences in potency and efficacy between these endocannabinoids were similar to those in rat cerebellar CB_1 receptor, except that 2-AGE was only a partial agonist at CB_1 when tested under optimized conditions where the enzymatic degradation of endocannabinoids was eliminated (Savinainen *et al*., 2001; 2003). Against these findings, our result that 2-AGE exhibited a relatively high efficacy also at the hCB_2 receptor was expected, even though 2-AGE has been reported to show over 100-fold selectivity in affinity towards the CB_1 receptor in radioligand binding assays (Hanus *et al*., 2001). Notable is that the reported low CB_2 affinity ($K_i>3$ μM) (Hanus *et al*., 2001), is in full agreement with the affinity obtained in our study ($IC_{50}=1.7$ μM). Interesting observation was that CP-55,940 showed superior agonism over endogenous full agonist 2-AG. Similar super-agonism (over endogenous full agonist) has been recognized at least at α2-adrenoceptors (Ge *et al*., 2003) and somatostatin subtype 4 receptors (Engstrom *et al*., 2005). Collectively, the potency and efficacy of the tested ligands was consistent with results from a previous report measuring intracellular Ca^{2+} levels in intact HL-60 cells endogenously expressing human CB_2 receptor (Sugiura *et al*., 2000).

To conclude, in this study, WIN55212-3 was clearly identified as a true, neutral CB_2 receptor antagonist. Despite its relatively low potency and compromised specificity, WIN55212-3 may serve as an important lead molecule in attempts to design novel and more potent neutral CB_2 antagonists. Moreover, as our experimental data clearly show, WIN55212-3 can be used as a pharmacological tool in

functional *in vitro* studies exploring ligand-dependent and constitutive signaling at CB_2 receptors. Such studies should shed more light on the important question of constitutive vs ligand-dependent receptor activity also in native cellular environment. Of note: after the submission of this work, a review on inverse agonism and neutral antagonism of cannabinoid CB_1 receptors has appeared (Pertwee, 2005).

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