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RESEARCH PAPER

In vitro pharmacological characterization of AM1241: a protean agonist at the cannabinoid CB_2 receptor?

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Background and purpose: The CB_2 receptor has been proposed as a novel target for the treatment of pain, and CB_2 receptor agonists defined in *in vitro* assays have demonstrated analgesic activity in animal models. Based on its *in vivo* analgesic efficacy, AM1241 has been classified as a CB_2-selective agonist. However, *in vitro* characterization of AM1241 in functional assays has not been reported.
Experimental approach: In this study, AM1241 was characterized across multiple *in vitro* assays employing heterologous recombinant receptor expression systems to assess its binding potencies at the human CB_2 and CB_1 receptors and its functional efficacies at the human CB_2 receptor.
Key results: AM1241 exhibited distinct functional properties depending on the assay conditions employed, a unique profile in contrast to those of the agonist CP 55,940 and the inverse agonist SR144528. AM1241 displayed neutral antagonist activities in FLIPR and cyclase assays. However, when cyclase assays were performed using lower forskolin concentrations for stimulation, AM1241 exhibited partial agonist efficacy. In addition, it behaved as a partial agonist in ERK (or MAP) kinase assays.
Conclusions and implications: The unusual phenomenon of inconsistent functional efficacies suggests that AM1241 is a protean agonist at the CB_2 receptor. We postulate that functional efficacies displayed by protean agonists in various assay systems may depend on the levels of receptor constitutive activities exhibited in the assay systems, and therefore, efficacies observed in *in vitro* assays may not predict *in vivo* activities.
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Keywords: AM1241; CB_2 receptor; cannabinoid; protean agonist; receptor constitutive activity; GPCR

Abbreviations: AM1241, (2-iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-1*H*-indol-3-yl]-methanone; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; CHO cells, Chinese hamster ovary cells; DMSO, dimethyl sulfoxide; CP 55,940, 5-(1,1-dimethyl-heptyl)-2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxy-propyl)-cyclohexyl]-phenol; DMEM, Dulbecco's modified Eagle's medium; D-PBS, Dulbecco's phosphate-buffered saline; ERK (or MAP) kinase, extracellular signal-regulated protein kinase; FLIPR, fluorometric image plate reader; FSK, forskolin; G protein, guanine nucleotide binding protein; GPCR, G-protein coupled receptors; HEK cells, human embryonic kidney cells; HuT 78 line, human T lymphoma line; PBS, phosphate-buffered saline; PTX, pertussis toxin; SR141716A (SR1), 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxylic acid piperidin-1-ylamide; SR144528 (SR2), 5-(4-chloro-3-methyl-phenyl)-1-(4-methyl-benzyl)-1*H*-pyrazole-3-carboxylic acid ((1*S*,2*S*,4*R*)-1,3,3-trimethyl-bicyclo[2.2.1]hept-2-yl)amide

Introduction

The analgesic properties of herbal and synthetic cannabinoid agonists have been well established (Pertwee, 2001; Rice, 2001; Baker *et al*., 2003; Croxford, 2003; Howlett *et al*., 2004). However, undesirable psychotropic effects produced by these ligands have limited their therapeutic potential (Baker *et al*., 2003). Cloning and functional characterization of cannabinoid receptors have led to a better understanding of the underlining physiological function of this receptor class.

Two types of cannabinoid receptors, CB_1 and CB_2, have been characterized to date (Wess, 1998; Karnik *et al*., 2003). Both are G-protein coupled receptors (GPCRs) coupling to $G_{i/o}$, whose activation leads to the inhibition of the adenylyl cyclase activity. Activation of CB_1 and CB_2 receptors has been demonstrated to activate extracellular signal-regulated

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protein (ERK or MAP) kinase (Bouaboula *et al*., 1999a, b; Powles *et al*., 2005). The CB_1 receptor is predominantly located in the CNS and the peripheral CB_2 receptor is abundant in the immune system (Matsuda *et al*., 1990; Munro *et al*., 1993; Shire *et al*., 1996; Griffin *et al*., 2000; Brown *et al*., 2002). The activation of the CB_1 receptor subtype has been clearly linked to the sedation, anxiety, paranoia, increased appetite, mild euphoria and impaired short-term memory associated with cannabis use. However, activation of both the CB_1 and CB_2 receptor subtypes has been shown to be involved in the analgesic activity of nonselective cannabinoids (Fox *et al*., 2001; Malan *et al*., 2001; Scott *et al*., 2004). An increasing body of evidence supports the premise that the CB_2 receptor may be the better target for the treatment of inflammatory and neuropathic pain, since CB_2 receptor activation with receptor-selective agonists has been shown in animals to be devoid of the centrally-mediated side effects of hypothermia, catalepsy and loss of motor coordination associated with the nonselective cannabinoids (Jaggar *et al*., 1998; Malan *et al*., 2003; Walter *et al*., 2003; Fox and Bevan, 2005). A possible third cannabinoid receptor has recently been recognized in the orphan receptor, GPR55, and is expressed in the smooth muscle cells surrounding the blood vessels, as well as in the CNS (Sawzdargo *et al*., 1999; Brown *et al*., 2005; Lauckner *et al*., 2005; Reyes *et al*., 2005; Sjogren *et al*., 2005). Although some herbal and synthetic cannabinoid ligands exhibit affinity for GPR55, this receptor remains largely uncharacterized.

A body of evidence supporting the role of CB_2 receptor activation in pain is demonstrated by the efficacies of CB_2 receptor-selective agonist ligands such as HU-308 (Hanus *et al*., 1999), JWH-133 (Elmes *et al*., 2004), GW405833 (Valenzano *et al*., 2005) and AM1241 (2-iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-1*H*-indol-3-yl]-methanone) (Ibrahim *et al*., 2003) in preclinical models of inflammatory and neuropathic pain. Although HU-308, JWH-133, GW405833 have been functionally characterized as agonists at the CB_2 receptor in *in vitro* assay systems, *in vitro* characterization of AM1241 has not been reported. Nevertheless, a large body of evidence in the literature has established that AM1241 is efficacious in a variety of *in vivo* animal pain models, a profile that is consistent with the properties of an agonist at the CB_2 receptor. For example, AM1241 has been shown to produce antinociceptive effects in an acute thermal pain model (Malan *et al*., 2003) and antihyperalgesic effects in a spinal nerve ligation model of neuropathic pain (Ibrahim *et al*., 2003). AM1241 is also efficacious in the carrageenan-induced thermal hypersensitivity model (Quartilho *et al*., 2003), as well as capsaicin-induced tactile thermal hyperalgesia and tactile allodynia (Hohmann *et al*., 2004). Further, using receptor-selective antagonists, the *in vivo* analgesic effects of AM1241 have been demonstrated to involve the CB_2 receptor, with no significant contribution from CB_1 activation (Malan *et al*., 2001; Ibrahim *et al*., 2003; Ibrahim *et al*., 2005).

This study characterizes AM1241 in various *in vitro* assay systems including radioligand binding assays and receptor functional assays using a cell line expressing the recombinant human CB_2 receptor. Unlike the reference ligands, CP 55,940 (5-(1,1-dimethyl-heptyl)-2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-phenol) and SR144528 (5-(4-chloro-3-methyl-phenyl)-1-(4-methyl-benzyl)-1*H*-pyrazole-3-carboxylic acid ((1*S*,2*S*,4*R*)-1,3,3-trimethyl-bicyclo[2.2.1]hept-2-yl)amide), which exhibit consistent pharmacology across various functional assays, AM1241 behaves as an apparent agonist, an inverse agonist, or a neutral antagonist depending on the specific assay systems employed. This unique property of inconsistent efficacies across functional assays suggests that AM1241 behaves as a protean agonist (Kenakin, 2001) at the CB_2 receptor.

Methods

Cell culture

Human embryonic kidney (HEK, American Type Culture Collection, Rockville, MD, USA) cells stably expressing the human CB_2 receptor were grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose supplemented with 10% fetal bovine serum and 25 μg ml^{-1} zeocin in a 37°C incubator in the presence of 5% CO_2. HEK cells stably co-expressing the human CB_2 receptor and the chimeric $G_{\alpha q/o5}$ protein were grown under similar conditions except that in addition to the components described above, the growth medium was further supplemented with 200 μg ml^{-1} hygromycin. The Chinese hamster ovary (CHO) cell line stably expressing the human CB_1 receptor was purchased from Euroscreen (Brussels, Belgium), and the cells were grown under the conditions recommended by the vendor.

Radioligand binding assay

Membrane samples were prepared from HEK cells stably expressing the human CB_2 receptors previously generated (Mukherjee *et al*., 2004), or the CHO cell line that stably expresses the human CB_1 receptor. Radioligand binding assays were performed as described previously (Mukherjee *et al*., 2004). Briefly, the cells were harvested and homogenized using a Polytron for 2×10 s bursts in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, and 1 mM EDTA in the presence of protease inhibitors followed by centrifugation at 45 000 *g* for 20 min. The membrane pellets were washed and frozen at −80°C in aliquots until use. Saturation binding reactions were performed at 30°C for 90 min using [^{3}H]CP 55,940 (0.01–8 nM) in an assay buffer containing 50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 5 mM $MgCl_2$, and 0.05% fatty acid free bovine serum albumin (BSA) and the reactions were terminated by rapid vacuum filtration through UniFilter-96 GF/C filter plates (Perkin-Elmer Boston, MA, USA) and four washes with cold assay buffer. Competition experiments were conducted using 0.5 nM [^{3}H]CP 55,940 in the presence of test compounds (0.1 nM–10 μM). Nonspecific binding was defined by 10 μM unlabeled CP 55,940. K_D values from saturation binding assays and K_i values from competition binding assays were determined with one site binding or one site competition curve fitting using the Prism software (GraphPad, San Diego, CA, USA).

Fluorometric image plate reader (FLIPR) functional assay

FLIPR assays were performed using HEK cells stably expressing the human CB_2 receptor and chimeric $G_{\alpha q/o5}$ protein

(Mukherjee *et al*., 2004) with modification. Briefly, cells were seeded at 7.5×10^4 cells per well in Biocoat 96-well poly-L-lysine coated clear-bottomed black wall plates (BD Biosciences, San Jose, CA, USA) 1 day prior to the assay. The cells were incubated with No-Wash Dye following vendor's instruction. For agonist assays, variable concentrations of test compounds (0.3 nM–10 μM), CP 55,940 (at 10 μM final concentration) positive control or vehicle negative control were added to cells in the presence of assay buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 2 mM $CaCl_2$, 0.05% BSA), and maximum fluorescence responses were measured with a FLIPR machine immediately following addition of compounds. Agonist responses were adjusted for the fluorescence background with vehicle controls and the activities were expressed as percentages of the CP 55,940 response. For antagonist assays, vehicle or variable concentration of test compounds (0.3 nM–10 μM) were added to the cells at the first addition and CP 55,940 (at 1 μM final concentration) was added to all cells at the second addition. The interval between the first and the second additions was 5 min. For antagonist assays, the maximum fluorescence response was measured immediately after the addition of CP 55,940 at the second addition and the response was compared with the control where vehicle instead of test compounds was added at the first addition. EC_{50} and K_b values were analyzed with sigmoidal dose response curve fitting using Prism.

Cyclase functional assay

The cyclase functional assays were performed using the HitHunter cAMP assay kit according to vendor's protocols. Briefly, HEK cells expressing the human CB_2 receptor were detached using cell dissociation buffer, dispersed and placed in suspension at 10^4 cells per well in 96-well plates prior to the assay. For agonist and inverse agonist assays, cells were treated for 20 min with variable concentrations of test ligands (6.4 pM–25 μM) and forskolin (8 or 37 μM) in Dulbecco's phosphate-buffered saline (D-PBS) supplemented with BSA (0.01% final concentration). In experiments assessing the antagonist properties of AM1241, variable concentrations of AM1241 (6.4 pM–25 μM) and forskolin (37 μM) were added to the cells together with a fixed concentration of either CP 55,940 (final concentration at 4 nM) or SR144528 (final concentration at 100 nM). The concentration of forskolin used to stimulate the cAMP level in cyclase assays was 37 μM unless indicated otherwise. Reactions were incubated for 20 min at 37°C and terminated by the addition of lysis buffer and the luminescence was detected following the procedure according to vendor's instructions. The cyclase activities were expressed as percent responses over the forskolin-stimulated control levels, where cells received vehicle instead of test compounds. EC_{50} values were calculated using sigmoidal dose response curve fitting from Prism (GraphPad).

ERK activation assay

HEK cells stably expressing the human CB_2 receptor were seeded at 2×10^5 cells per well in six-well plates, serum-starved in DMEM plus 0.1% BSA and 1% penicillin–streptomycin overnight with 0.1 ng μl^{-1} pertussis toxin (PTX) included where indicated. Following pretreatment for 10 min with 10 μM SR141716A (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxylic acid piperidin-1-ylamide), SR144528 or vehicle where indicated, the cells were treated for 5 min with 100 nM CP 55,940 or 1 μM AM1241. Cells were washed with cold PBS, solublized in a lysis buffer (5 mM HEPES, pH 7.4, 0.5% NP-40, 250 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol 1 × complete protease inhibitor Cocktail) and the lysate clarified by centrifugation at 4°C for 15 min at 14 000 r.p.m. Equal amounts of proteins were separated on 4–12% Novex Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and transferred onto nitrocellulose membranes for immunoblotting. Phosphorylated p42/44 ERK and total ERK proteins were detected by immunoblotting with polyclonal anti-phospho-p44/42 ERK antibodies at 1:2000 dilution, or anti-ERK antibodies at 1:10 000 dilution. Chemiluminescent detection was performed using the SuperSignal West Pico reagent.

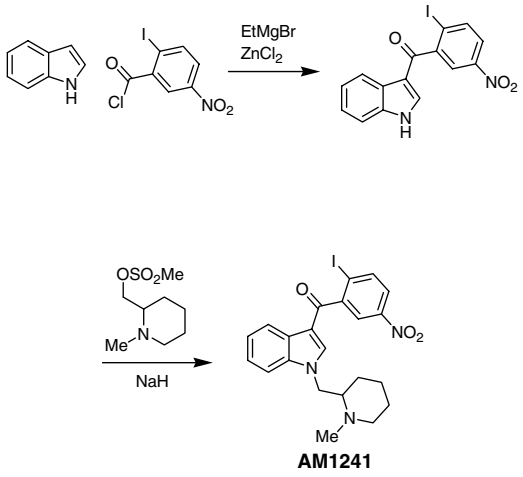
Statistical analysis

Results are shown as means ± s.e.m. or means with 95% confidence interval (CI).

Compounds synthesized

Racemic AM1241 (Makriyannis and Deng, 2001), SR144528 (Barth *et al*., 1997) and SR141716A (Barth *et al*., 1995) were synthesized at Abbott Laboratories according the methods described, and their structures characterized by ^{1}H NMR spectroscopy, mass spectrometry and elemental analysis.

The synthesis of racemic AM1241 used in the current study is shown in the scheme below, and its characterization described here.



Briefly, acylation of indole with 2-iodo-5-nitrobenzoyl chloride followed by *N*-alkylation with the mesylate derived from (1-methylpiperidin-2-yl)methanol afforded racemic AM1241 as a yellow powder: ^{1}H NMR (500 MHz, CD_3OD) δ p.p.m. 1.10–1.29 (m, 3H), 1.45–1.71 (m, 3H), 2.20 (dt,

$J=11.2$, 3.7 Hz, 1H), 2.43 (s, 3H), 2.51 (m, 1H), 2.89 (m, 1H), 4.00 (dd, $J=13.9$, 9.2 Hz, 1H), 4.67 (dd, $J=13.9$, 4.7 Hz, 1H), 7.37 (m, 2H), 7.57 (m, 1H), 7.69 (s, 1H), 8.07 (dd, $J=8.8$, 2.7 Hz, 1H), 8.22 (d, $J=2.5$ Hz, 1H), 8.26 (d, $J=8.8$ Hz, 1H), 8.28 (m, 1H); MS (DCI) m/z 504 $(M+H)^+$.

Analysis: Calculated for $C_{22}H_{22}IN_3O_3$: C, 52.50; H, 4.41; N, 8.35. Found: C, 52.23; H, 4.59; N, 8.09.

All compounds used were dissolved in dimethyl sulfoxide (DMSO) at a 10 mM concentration and stored at −20°C until use. The initial dilutions from 10 mM stock solution were made in D-PBS supplemented with fatty acid free BSA at 0.01% final concentration at the assay for cyclase and ERK activation assays, or in assay buffers for radioligand binding and FLIPR assays at a greater than 1:100 fold dilution to avoid compound precipitation. Subsequent serial dilutions were performed in assay buffers according to the concentration range tested.

Other materials

CP 55,940 (5-(1,1-dimethyl-heptyl)-2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxy-propyl)-cyclohexyl]-phenol) was purchased from Tocris Inc. (Ellisville, MI, USA) and [^{3}H]CP 55,940 (specific activity, 6.67 TBq/mmol) from Perkin Elmer Boston, MA, USA.

D-PBS, cell dissociation buffer, penicillin–streptomycin and other reagents for cell culture were purchased from Invitrogen (Carlsbad, CA, USA). Fatty acid free BSA was from Sigma (St Louis, MO, USA) and the No-Wash Dye for the FLIPR Calcium Assay Kit was from Molecular Device, (Sunnyvale, CA, USA). The HitHunter cAMP assay kit was purchased from DiscoveRx (Fremont, CA, USA), PTX from List Biological Laboratories, Inc. (Campbell, CA, USA) and the complete protease Inhibitor Cocktail from Roche Applied Sciences (Indianapolis, IN, USA). Polyclonal anti-phospho-p44/42 ERK antibody was from Cell Signaling Technology, Beverly, MA, USA and anti-ERK antibodies from Upstate Group Inc., Lake Placid, NY, USA). The SuperSignal West Pico reagent for chemiluminescent detection was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA).

Results

AM1241 is selective at the human CB_2 receptor

The binding selectivity of AM1241 at the CB_2 cannabinoid receptor has been reported previously (Ibrahim *et al*., 2003) and was confirmed in the current [^{3}H]CP 55,940 radioligand binding studies using membrane preparations from stable HEK and CHO cell lines expressing the recombinant human CB_2 and CB_1 receptors, respectively. In saturation binding assays, [^{3}H]CP 55,940 exhibited high potencies at these cannabinoid receptors (Table 1). The host HEK and CHO cells do not exhibit significant specific binding to the [^{3}H]CP 55,940 ligand (data not shown).

In [^{3}H]CP 55,940 competition binding assays, AM1241 displayed high affinity at the human CB_2 receptor with a K_i value of about 7 nM, whereas its affinity at the human CB_1 receptor was more than 80-fold weaker (Table 2). For comparison, SR144528 and CP 55,940 were also tested in competition binding assays and these results are also summarized in Table 2. As reported previously by Howlett *et al*. (2002), SR144528 exhibited high selectivity at the human CB_2 receptor over the human CB_1 receptor. In contrast, CP 55,940 was essentially nonselective with high potencies at both human CB_1 and CB_2 receptors.

Table 1 Summary of compiled K_D in [^{3}H]CP 55,940 saturation binding assays

Receptor	n	K_D *(nM) (mean with 95% CI)*	B_{max} *(pmol (mg protein)$^{-1}$) (mean±s.e.m.)*
CB_2	11	0.75 (0.57–0.98)	9.4±4.9
CB_1	7	2.4 (2.0–2.9)	4.9±2.2

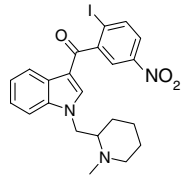
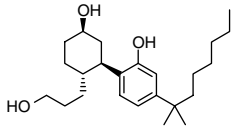
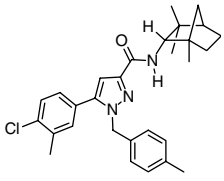
AM1241 is an apparent antagonist at the human CB_2 receptor in FLIPR assays

In order to assess the functional efficacy of AM1241 at the human CB_2 receptor, a FLIPR functional assay was performed using an HEK cell line as previously described (Mukherjee *et al*., 2004), which co-expresses the human CB_2 receptor and the chimeric $G_{\alpha q/o5}$ protein. The chimeric $G_{\alpha q/o5}$ protein facilitates the increase of intracellular Ca^{2+} level upon activation of $G_{\alpha i/o}$-coupled GPCRs, which can be readily measured by a FLIPR machine (Conklin *et al*., 1993). The stable HEK cell line used in FLIPR assays was developed by introducing chimeric $G_{\alpha q/o5}$ into the HEK cell line that expresses the human CB_2 receptor alone. Saturation binding assays indicated that the resulting cell line co-expressing the human CB_2 receptor and chimeric $G_{\alpha q/o5}$ exhibited [^{3}H]CP 55,940 radioligand binding profiles comparable to that of the parent cell line expressing the human CB_2 receptor alone with a similar K_D value (0.60 nM) and slightly lower B_{max} value (3.5 pmol mg^{-1} protein). In FLIPR assays, AM1241 exhibited antagonist activity, blocking the agonist CP 55,940-evoked Ca^{2+} response (Figure 1a) in a concentration dependent manner with a K_b value of 63 nM (Figure 1b). Similarly, SR144528 exhibited antagonist activity at the human CB_2 receptor with a K_b value of 22 nM, whereas CP 55,940 was an agonist at the human CB_2 receptor with an EC_{50} of 55 nM (Figures 1a and b).

AM1241 behaves as a neutral antagonist at the human CB_2 receptor in cyclase assays

In order to further assess the efficacy of AM1241 at the human CB_2 receptor, cyclase functional assays were performed and activation of the human CB_2 receptor was measured using the stable HEK cell line expressing the human CB_2 receptor. Forskolin induced a concentration-dependent increase in cAMP levels in HEK cells expressing the human CB_2 receptor with an EC_{50} value of 15 μM (data not shown). Forskolin, at ~EC_{70} concentration (37 μM), was used to stimulate cAMP production in cyclase assays, and the abilities of test ligands to modulate cyclase activity were measured and expressed as percent responses over the forskolin-stimulated cAMP levels. AM1241 exhibited no agonist or inverse agonist activities in the concentration

Table 2 Radioligand competition binding assays using [^{3}H]CP 55,940

Ligand	*Structure*	*Human CB_2*			*Human CB_1*		
		K_i *(nM)*	*95% CI*	n	K_i *(nM)*	*95% CI*	n
AM1241		7.1	4.0–13	13	580	61–5600	5
CP 55,940		0.79	0.63–0.99	49	1.9	1.3–2.7	41
SR144528		2.7	1.3–5.3	4	320	260–380	4

range tested at the human CB_2 receptor in the cyclase assays (Figure 2a and Table 3). In contrast, under similar assay conditions, CP 55,940 displayed potent agonist activity with an EC_{50} value of 0.36 nM reducing cyclase activity by 70% of the forskolin-induced level, whereas SR144528 exhibited an inverse agonist activity with EC_{50} value of 92 nM increasing cyclase activity by 74% of the forskolin-induced level. The lack of robust functional efficacy of AM1241 at the human CB_2 receptor may indicate that AM1241 is a neutral antagonist in this assay.

Subsequently, AM1241 was evaluated for its ability to antagonize the effects of agonist CP 55,940 and the inverse agonist SR144528. AM1241 dose-dependently blocked the agonist activity of CP 55,940 (4 nM) and the inverse agonist activity of SR144528 (100 nM) at the human CB_2 receptor with K_b values at 27 and 11 nM, respectively (Figure 2b). Schild analysis was performed to ascertain the competitive nature of AM1241 inhibition. Rightward shifts of the CP 55,940 concentration–response curve were observed in the presence of increasing concentrations of AM1241 ranging from 0.1 to 10 μM (Figure 2c). The Schild plot of these results (Figure 2d) gave a pA2 value of 7.9, similar to the pK_b value of 7.6 ($K_b = 27$ nM) with the Hill slope approaching unity, indicating that under the conditions tested, AM1241 functions as a competitive antagonist of CP 55,940.

At lower forskolin concentrations, AM1241 behaves as an agonist at the human CB_2 receptor in cyclase assays

To determine if the assay conditions affected the functional properties of AM1241 at the CB_2 receptor, cyclase assays were performed at a lower forskolin concentration (8 μM, $\sim EC_{35}$). In contrast to the neutral antagonist behavior observed with a higher (37 μM) forskolin concentration, AM1241 now exhibited partial agonist activity at the human CB_2 receptor, in the presence of 8 μM forskolin (Figure 3). Conversely, CP 55,940 and SR144528 still exhibited agonist and inverse agonist properties, respectively, independent of the forskolin concentration used (Figure 3).

AM1241 is an apparent agonist at the human CB_2 receptor in ERK activation assays

It has been reported previously that activation of the human CB_2 receptor enhances phosphorylation and activation of p42/p44 ERK (Bouaboula *et al*., 1999b). Unlike the cyclase and FLIPR assays described above, AM1241 demonstrated partial agonist activity, inducing the phosphorylation of p42/p44 ERK but to a lesser extent than that induced by CP 55,940 (Figure 4a). ERK phosphorylation induced by AM1241 and CP 55,940 was blocked by SR144528 but not by SR141716A, demonstrating that the ERK activation is mediated through the CB_2 receptor (Figure 4a). Further, PTX abolished the ERK phosphorylation induced by AM1241 and CP 55,940, indicating that the activation of ERK by AM1241 and CP 55,940 was mediated by the $G_{i/o}$ protein (Figure 4b).

Discussion

An emerging model of a protean agonist describes a ligand that changes its apparent behavior, and depending on the assay systems, can operate as an agonist, antagonist or

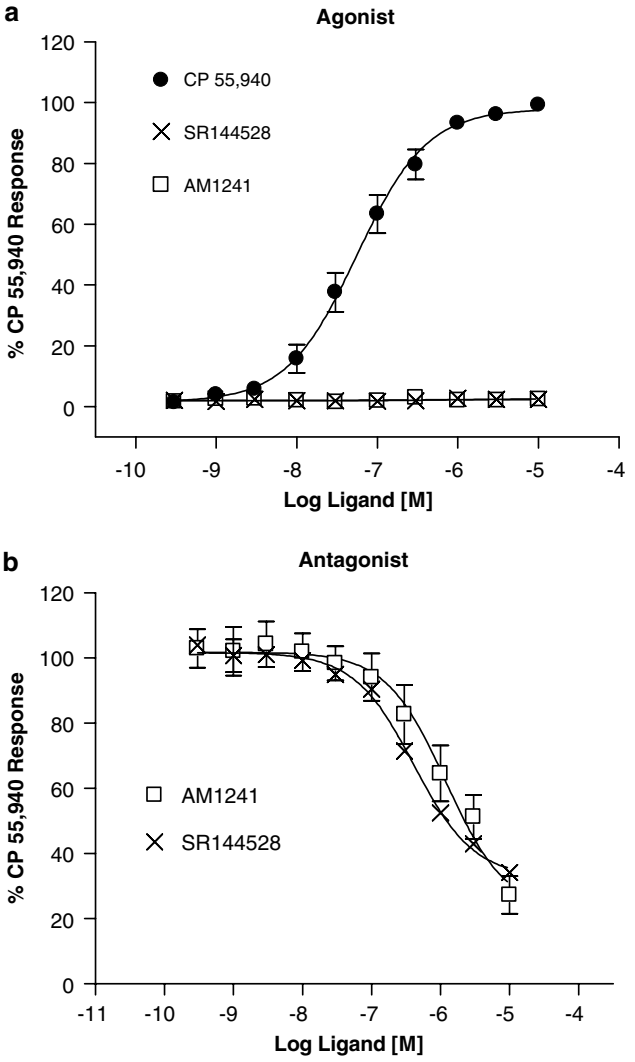


Figure 1 Characterization of AM1241 in FLIPR functional assays at the human CB_2 receptor. The FLIPR assays were performed using stable HEK cell line co-expressing the human CB_2 receptor and chimeric $G_{\propto q/o5}$ protein. (**a**) In agonist assays, the maximum fluorescence responses were expressed as the percent of positive control, 10 μM CP 55,940. The results are the average of three experiments performed in duplicates. (**b**) In antagonist assays, compounds or vehicle were added at the first addition and 1 μM CP 55,940 was added at the second addition. The maximum fluorescence responses following the CP 55,940 addition were measured and expressed as the percentage of the CP 55,940 response when vehicle instead of compounds was added in the first addition. The results are the average of at least three experiments, performed in duplicates.

inverse agonist at the same receptors (Kenakin, 2001, 2004; Prather, 2004). Protean agonists have been reported for the histamine H_3 receptor (Gbahou *et al*., 2003; Baldi *et al*., 2005) and α_{2A}-adrenergic receptor (Jansson *et al*., 1998; Pauwels *et al*., 2002). The present studies provide evidence that AM1241 behaves as a protean agonist at the human CB_2 receptor.

AM1241, a CB_2-selective ligand, consistently demonstrates broad-spectrum analgesic activities in various preclinical animal pain models (Malan *et al*., 2001), and is a reference standard for CB_2 receptor activation in these assays. Multiple structures have been published for AM1241 (Goutopoula and Makriyannis, 2003; Ibrahim *et al*., 2003). The structure of AM1241 used in the current studies is consistent with that published by Makriyannis and Deng (2001) and the compound is now available from Alexis Corporation (Lausen, Switzerland).

Our present studies employing recombinant CB_2 receptor systems showed that AM1241 exhibited inconsistent functional efficacies. In ERK activation assays, AM1241 exhibited $G_{i/o}$-dependent partial agonist activity at the CB_2 receptor, stimulating ERK activation at a level lower than that of CP 55,940. In contrast, AM1241 was an apparent antagonist in FLIPR assays, blocking the CP 55,940-evoked calcium influx through the CB_2 receptor, similar to the effect observed with SR144528. In cyclase assays, AM1241 produced inconsistent efficacies that were dependent upon the assay conditions used. When a higher forskolin concentration (37 μM, $\sim EC_{75}$) was used to stimulate the adenylyl cyclase, AM1241 failed to produce a change in efficacy. However, AM1241 reversed the effects of agonist CP 55,940 and inverse agonist SR144528 in a concentration-dependent manner, demonstrating that AM1241 behaved as a neutral antagonist. When assays were performed using lower forskolin concentrations (8 μM, $\sim EC_{35}$), AM1241 consistently exhibited agonist efficacy, reducing the cAMP level, as did CP 55,940. The divergence among functional properties of AM1241 in various *in vitro* assays and the lack of robust CB_2 agonist efficacies may suggest that AM1241 is a protean agonist at the CB_2 receptor. In contrast, the agonist CP 55,940 and inverse agonist SR144528 exhibited consistent functional efficacies across different assay systems.

In order to provide a direct comparison to preclinical animal studies (Malan *et al*., 2001; Ibrahim *et al*., 2003; Ibrahim *et al*., 2005), a racemic mixture of AM1241 has been used in the current studies. In addition, it has been shown by Uveges *et al*. (2005) that the individual enantiomers exhibit similar potencies and efficacies at the human CB_2 receptor in cyclase assays compared with those of the racemic mixture, indicating that neither enantiomer is likely to produce confounding functional properties.

CP 55,940 failed to show agonist activity in cyclase assays in native cell lines such as HuT 78 (human T lymphoma) that expresses the human CB2 receptor gene (data not shown). In contrast, the recombinant HEK cell line used in the current studies expresses the human CB2 receptor at a high level, allowing readily detection of both agonists and antagonists. According to the current receptor activation theory (Chen *et al*., 2000; Seifert and Wenzel-Seifert, 2002; Milligan, 2003; Prather, 2004), the increased receptor availability in recombinant systems increases the absolute number of receptors activated by agonist ligands, leading to significant augmentation of the signalling pathway as well as the detection capability of the assay system, resulting in significant amplification in maximal agonist efficacies (Figure 5). Concomitantly, the higher level of receptor expression in recombinant systems increases the total number of spontaneously activated receptors regardless of the presence of agonist ligands, and thus the assay system displays higher levels of constitutive activity. The level of

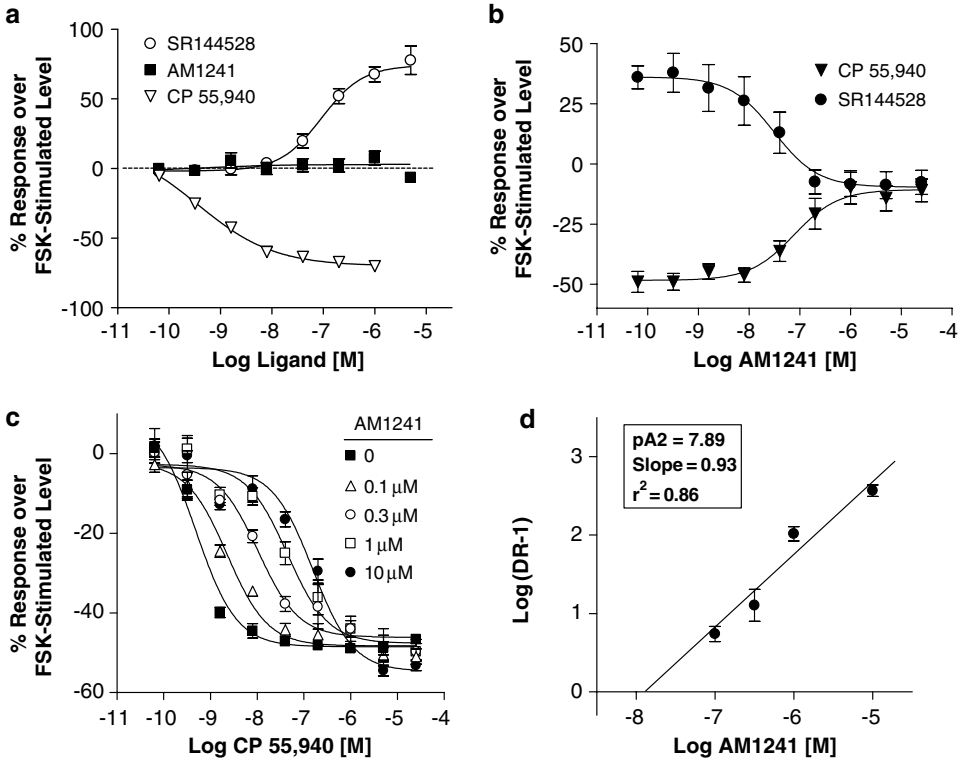


Figure 2 Characterization of AM1241 in cyclase functional assays at the human CB_2 receptor. The cyclase functional assays were performed using stable HEK cell lines expressing the human CB_2 receptor. (**a**) In agonist/inverse agonist assays, compounds were added together with 37 μM forskolin. The luminescence responses in the presence of test compounds were compared with those when compounds were replaced with the vehicle and expressed as percent responses over the forskolin-stimulated level. (**b**) AM1241 was tested as an antagonist to block the agonist activity of CP 55,940 and the inverse agonist activity of SR144528. In antagonist assays, a range of AM1241 concentration was used in conjunction with 4 nM CP 55,940 or 100 nM SR144528 in the presence of 37 μM forskolin. The luminescence responses in the presence of test compounds were compared with those when compounds were replaced with the vehicle and expressed as percent responses over the forskolin-stimulated level. (**c**) Rightward shift of concentration dependent curves of CP 55,940 with increased concentration of AM1241. (**d**) Schild analysis of results shown in (**c**). Results from all above experiments are the average of at least four experiments performed in duplicate.

Table 3 Agonist and inverse agonist activities in human CB_2 cyclase assays

Ligand	*EC_{50} (nM)*	*95% CI of K_i (nM)*	n	*Efficacy over FSK-stimulated level (%)*
AM1241	>1600	NA[a]–NA	7	NA
CP 55,940	9	5.4–15	16	−70
SR144528	88	61–127	16	74

[a]NA: not available.

spontaneous activation of receptors is dependent upon multiple factors – the thermodynamic nature of receptor conformational changes from quiescent state to active state, the cellular background, such as repertoires of G proteins, GPCR binding and effector proteins, as well as the conditions under which the cells were grown and the assays performed (Chen *et al*., 2000; Kenakin, 2001; Seifert and Wenzel-Seifert, 2002; Milligan, 2003).

In contrast to native systems where the receptors are quiescent in general and do not display significant constitutive activity (*R0* state, Figure 5), recombinant systems often show variable levels of constitutive activity (*R1* and *R2* states, Figure 5). Such constitutive systems increase the detection capabilities not only for agonists (Figure 5, B and C), but also permit the characterization of inverse agonists. It has been hypothesized that the ligand-induced receptor conformation state is dependent upon the intrinsic characteristics of the ligand, regardless of the assay system employed (Kenakin, 2001, 2004; Perez and Karnik, 2005). Therefore, the pharmacological definition of a ligand may vary depending on the constitutive activity present in the assay system. Due to its high intrinsic activity, the hypothetical ligand C behaves as an agonist in native and recombinant systems. Without any intrinsic activity, the hypothetical ligand A is an antagonist in the native system, and behaves as an inverse agonist in systems containing constitutively active receptors. In contrast, the hypothetical ligand B, possessing a low level of intrinsic activity, can behave as a partial agonist in a system with a lower relative level of constitutive activity (*R1* state), but as an inverse agonist when the receptor constitutive activity is higher (*R2* state). By definition, ligand B is a protean agonist, as its observed functional efficacy is dependent upon the relative level of constitutive activity exhibited by the system. Therefore, it is possible that inverse agonists defined in recombinant systems *in vitro* may actually behave as antagonists or even partial agonists in native systems that exhibit low levels

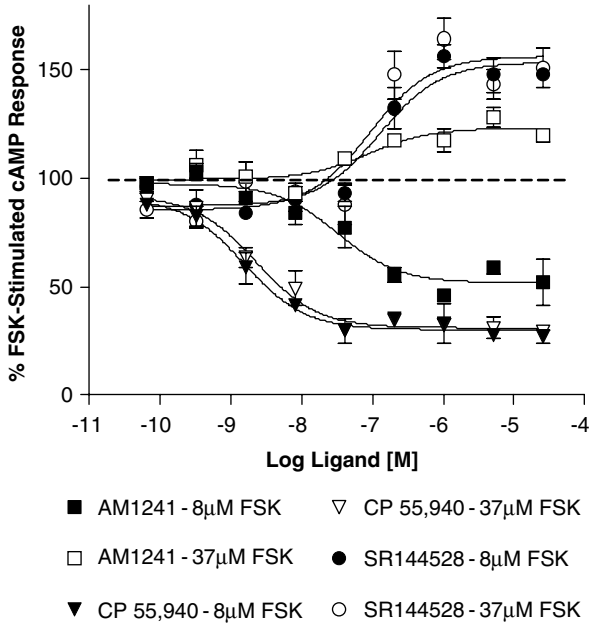


Figure 3 Efficacy of AM1241 in cyclase assays depends on level of stimulus. All three compounds, AM1241, CP55,940 and SR144528, were re-assessed in cyclase assays at a low (8 μM) concentration of forskolin and compared with their effects at the higher concentration of forskolin (37 μM; see Figure 2a).

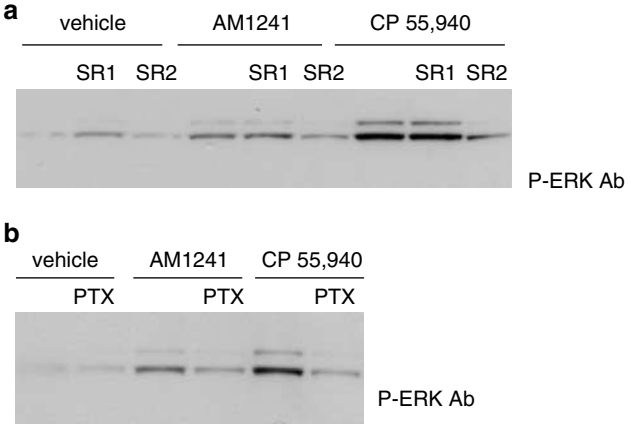


Figure 4 AM1241 behaves as an agonist at the human CB_2 receptor in ERK (MAPK) activation assays. (**a**) Stable HEK cell line expressing the human CB_2 receptor was treated with either 10 μM SR141716A (SR1), 10 μM SR144528 (SR2) or vehicle followed by 5 min treatment of 1 μM AM1241 or 100 nM CP 55,940. (**b**) AM1241 or CP 55,940-induced ERK activation is blocked by treatment with PTX (0.1 ng μl^{-1}).

of constitutive receptor activity. The behavior of AM1241 as an agonist *in vivo* and as a weakly efficacious agonist or antagonist/inverse agonist in recombinant systems is consistent with this classification as a protean agonist.

In vitro recombinant systems have been shown to be indispensable tools in drug discovery for high throughput screening and functional characterization of compounds at target receptors. While high levels of receptor expression in *in vitro* systems make functional assays feasible, the increased receptor tone in these systems compared with the native

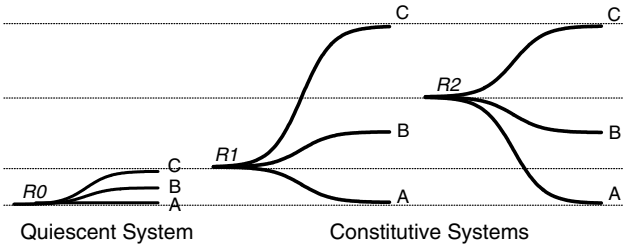


Figure 5 A model of receptor activation theory. *R0* represents the receptor resting state in a quiescent system lacking receptor constitutive activities, and *R1* and *R2* represent resting states in systems with receptor constitutive activities. A, B and C represent hypothetical ligands that possess different levels of intrinsic activities and produce different levels of functional efficacies at the receptor.

receptor systems can produce confounding results for compounds with low efficacies. In addition, the distinct repertoire of G proteins and other proteins that interact with target receptors in cell lines employed may also contribute to the inconsistent pharmacology between *in vitro* and *in vivo* systems. Therefore, physiologically relevant assay systems, ideally derived from target tissues, should be employed to evaluate the predictability of *in vitro* assay systems, and, ultimately, *in vivo* assays are necessary for compound selection for advancing through the drug discovery process.

In summary, although efficacious agonists and antagonists/inverse agonists can be identified using recombinant systems, characterizing protean agonists may be more complex and require multiple functional assay systems. Further, physiologically relevant *in vitro* assay systems with correlations to *in vivo* testing are essential for the accurate prediction of compound efficacies *in vivo*. While both agonists and inverse agonists have proven utility in regulating receptor activities, the therapeutic potential of protean agonists is not clear. Perhaps their unique properties of promoting a lower level of ligand-specific receptor activation states may be advantageous over fully efficacious agonists and inverse agonists, whose therapeutic utility may be limited by the development of tolerance (Kenakin, 2001).

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Conflict of interest

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