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RESEARCH PAPER

Characterization of cannabinoid receptor ligands in tissues natively expressing cannabinoid CB2 receptors

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BACKGROUND AND PURPOSE

Although cannabinoid CB_2 receptor ligands have been widely characterized in recombinant systems *in vitro*, little pharmacological characterization has been performed in tissues natively expressing CB_2 receptors. The aim of this study was to compare the pharmacology of CB_2 receptor ligands in tissue natively expressing CB_2 receptors (human, rat and mouse spleen) and hCB_2-transfected CHO cells.

EXPERIMENTAL APPROACH

We tested the ability of well-known cannabinoid CB_2 receptor ligands to stimulate or inhibit [^{35}S]GTPγS binding to mouse, rat and human spleen membranes and to hCB_2-transfected CHO cell membranes. cAMP assays were also performed in hCB_2-CHO cells.

KEY RESULTS

The data presented demonstrate that: (i) CP 55,940, WIN 55,212-2 and JWH 133 behave as CB_2 receptor full agonists both in spleen and hCB_2-CHO cells, in both [^{35}S]GTPγS and cAMP assays; (ii) JWH 015 behaves as a low-efficacy agonist in spleen as well as in hCB_2-CHO cells when tested in the [^{35}S]GTPγS assay, while it displays full agonism when tested in the cAMP assay using hCB_2-CHO cells; (iii) (*R*)-AM 1241 and GW 405833 behave as agonists in the [^{35}S]GTPγS assay using spleen, instead it behaves as a low-efficacy inverse agonist in hCB_2-CHO cells; and (iv) SR 144528, AM 630 and JTE 907 behave as CB_2 receptor inverse agonists in all the tissues.

CONCLUSION AND IMPLICATIONS

Our results demonstrate that CB_2 receptor ligands can display differential pharmacology when assays are conducted in tissues that natively express CB_2 receptors and imply that conclusions from recombinant CB_2 receptors should be treated with caution.

Abbreviations

AM 630, 6-iodopravadoline; CP 55,940, (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol; DMSO, dimethyl sulphoxide; Forskolin (FSK), 7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one; G418, 3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl[oxy-2-hydroxycyclehexy]oxy-2-(1-hydroxyethyl0oxane-3,4-diol; GW 405833, 1-(2,3-dichlorobenzoyl)-5-methoxy-2-methyl-(3-(morpholin-4-yl)ethyl)-1H-indole hydrochloride; JTE 907, N-(1,3-benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2- oxo-8-(pentyloxy)-3-quinolinecarboxamide;

JWH 015, (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone; JWH 133, 3-(1,1-dimethylbutyl)-1-deoxy-Δ^8-tetrahydrocannabinol; PMSF, phenylmethylsulphonyl fluoride; (*R*)-AM 1241, (R,S)-3-(2-iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1H-indole; SR 144528, *N*-[(1S)-endo-1,3,3-trimethyl bicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; WIN 55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

Introduction

The endocannabinoid system has promising therapeutic targets. To date, two distinct cannabinoid receptors, designated CB_1 and CB_2, have been identified in mammalian tissues and have been cloned (Matsuda *et al.*, 1990; Munro *et al.*, 1993; Shire *et al.*, 1996; Brown *et al.*, 2002). The CB_1 and the CB_2 receptors belong to class A (rhodopsin-like) of the superfamily of GPCRs and share 44% overall homology and 68% homology in their transmembrane domain (Munro *et al.*, 1993; Shire *et al.*, 1996). The CB_1 receptor exhibits high amino-acid sequence identity across human, rat and mouse, while human CB_2 displays only 81 and 82% amino-acid identity with rat and mouse, respectively (Gérard *et al.*, 1991; Munro *et al.*, 1993; Shire *et al.*, 1996; Griffin *et al.*, 2000; Brown *et al.*, 2002; Liu *et al.*, 2009).

The CB_1 receptor is the most abundantly expressed GPCR in the brain with the highest density in hippocampus, cerebellum and striatum (Herkenham *et al.*, 1990). It is also found in various peripheral tissues including the gastrointestinal tract, pancreas, liver, kidney, prostate, testis, uterus, eye, lungs, adipose tissue and heart (Howlett, 2002). On the other hand, the CB_2 receptor is expressed mainly in the cells and tissues of the immune system including thymus, tonsils, B and T cells, macrophages, monocytes and NK cells and, to a far lesser extent, in brain (Van Sickle *et al.*, 2005; Cabral and Griffin-Thomas, 2009). In both, CNS and peripheral tissues, the CB_2 receptor is up-regulated during early inflammatory events (Guindon and Hohmann, 2008; Cabral and Griffin-Thomas, 2009).

The CB_2 receptor ligands can be classified as 'classical cannabinoids', 'non-classical cannabinoids', 'cannabimimetic indoles', 'pyrazoles' and finally '2-oxoquinolines' (Figure 1). The term 'classical cannabinoids' refers to Δ^9-THC-like tricyclic terpenoids; the selective CB_2 receptor agonist, JWH 133 [3-(1,1-dimethylbutyl)-1-deoxy-Δ^8-tetrahydrocannabinol] belonging to this class of compounds (Gareau *et al.*, 1996). Efforts directed towards simplification of THC's tricyclic structure while retaining or improving biological activity led to a second class of cannabinergic ligands possessing close similarity to classical cannabinoids. This group of compounds, generally designated as 'non-classical cannabi-

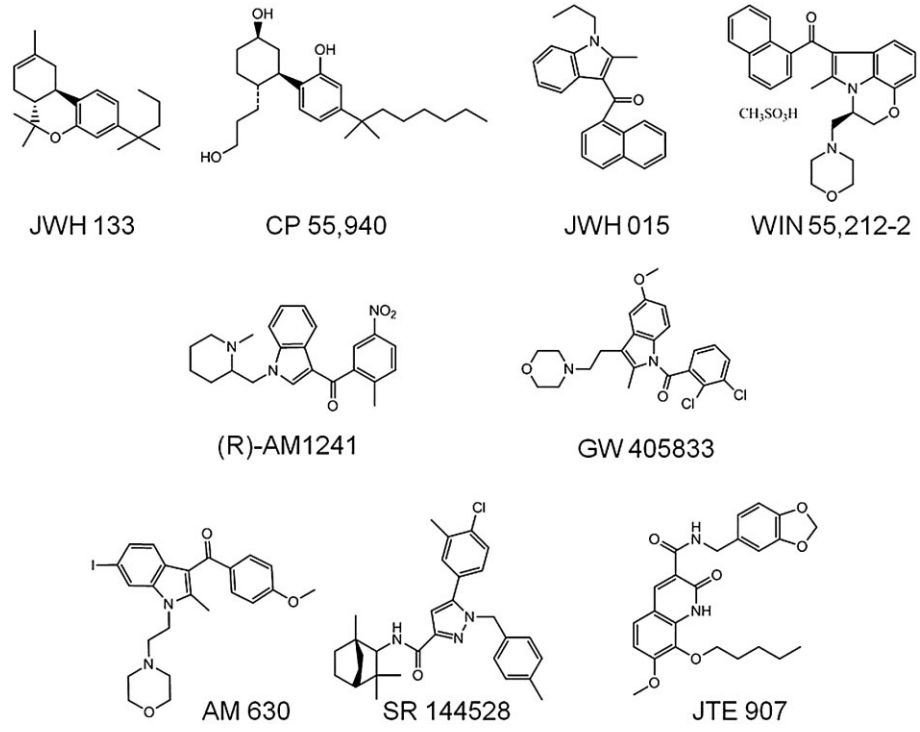

Figure 1

Cannabinoid CB_2 receptor ligands. The classical cannabinoid (Δ^9-THC-like terpenoid), JWH 133; non-classical cannabinoid (phenolic hydroxyl), CP 55,940; cannabimimetic indoles (aminoalkylindoles), WIN 55,212-2, (*R*)-AM 1241, JWH 015, GW 405833 and AM 630; pyrazole, SR 144528 and 2-oxoquinoline, JTE 907.

noids', lack the pyran ring of classical cannabinoids. The best known 'non-classical cannabinoid' is CP 55,940 [(–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol], a ligand exhibiting high affinity for both CB_1 and CB_2 receptors as well as a high degree of stereoselectivity (Devane *et al.*, 1988). Another major chemical class of cannabinoid ligands, the cannabimimetic indoles or amminoalkylindoles, are structurally distinct from 'classical cannabinoids' and were initially developed at Sterling Withrop (Eissenstat *et al.*, 1990; Bell *et al.*, 1991). WIN 55,212-2 [(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate; a potent agonist at both CB receptors with a preference for CB_2] (Eissenstat *et al.*, 1995), (*R*)-AM 1241[(R,S)-3-(2-Iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1H-indole; a highly CB_2 selective agonist] (Ibrahim *et al.*, 2003), JWH 015 [(2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone; with high affinity for the CB_2 receptor] (Marriott and Huffman, 2008) and GW 405833 [1-(2,3-dichlorobenzoyl)-5-methoxy-2-methyl-(3-(morpholin-4-yl)ethyl)-1H-indole hydrochloride; also known as L-768,242; Valenzano *et al.*, 2005)] belong to this class of compounds. AM 630, a CB_2 receptor inverse agonist also belongs to the cannabimimetic indole class (Ross *et al.*, 1999). One of the first discovered inverse agonists of the CB_2 receptor, SR 144528 [*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], is a pyrazole (Portier *et al.*, 1999) and is structurally related to the CB_1 receptor-specific inverse agonist SR 141716A. Since then, a number of additional chemotypes have been developed showing activity as inverse agonists including, JTE 907 [N-(1,3-Benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2- oxo-8-(pentyloxy)-3-quinolinecarboxamide], which is a 2-oxoquinoline (Iwamura *et al.*, 2001).

There is considerable evidence that CB_2 receptor agonists are effective in various animal models of pain (Guindon and Hohmann, 2008); however, the pharmacology of CB_2 ligands is complex (Yao *et al.*, 2006). Of particular interest is the atypical pharmacology of certain CB_2 receptor selective ligands, for example (*R*)-AM 1241. Consistent with the properties of a CB_2 receptor agonist, (*R*)-AM 1241 is efficacious in a variety of rat *in vivo* pain models (Ibrahim *et al.*, 2003; Malan *et al.*, 2003; Quartilho *et al.*, 2003; Hohmann *et al.*, 2004). Furthermore, the *in vivo* anti-nociceptive effects of (*R*)-AM 1241 appear to involve the CB_2 receptor, with no significant contribution from CB_1 activation (Malan *et al.*, 2001; Ibrahim *et al.*, 2003; 2005). However, *in vitro* characterization of (*R*)-AM 1241 in recombinant systems revealed that the compound is a protean agonist; thus, the efficacy of this compound varies depending on the level of constitutive activity in the assay system (Yao *et al.*, 2006). In line with this, Mancini *et al.* (2009) have found that both (*R*)-AM 1241 and GW 405833 (L-768,242) behave as agonists in recombinant rCB_2 and hCB_2, but only after constitutive activity is abolished. These data highlight the problem of making an accurate prediction of *in vivo* potency based on *in vitro* characterization in recombinant systems; the levels of constitutive activity in native systems being potentially different from those found in highly over-expressing recombinant systems. Furthermore, *in vivo* characterization in rat pain models may not directly correlate with the action in the human; the CB_2 receptor displays species differences in pharmacology (Bingham *et al.*, 2007). Added to this, there is the complexity of the possible differential levels of constitutive activity in each species. It is therefore crucial to determine the pharmacological profile of CB_2 receptor ligands in an *in vitro* system expressing native CB_2 receptors.

CB_2 receptors are highly expressed in spleen tissue (Galiègue *et al.*, 1995). While some studies have investigated the binding affinity of CB_2 ligands in the rodent spleen, analysis of the functional activity of CB_2 ligands in this tissue remains largely unexplored (Rayman *et al.*, 2004). In particular, there is no information on the pharmacology of CB_2 receptor agonists in human spleen. Here, we have investigated the pharmacological properties of some well-known CB_2 receptor ligands in mouse, rat and human spleen using [^{35}S]GTPγS assay. In addition, with the aim of obtaining a direct comparison of native and recombinant systems, the same compounds have been also tested in hCB_2-CHO cells, using both [^{35}S]GTPγS and cyclic AMP assays.

Materials and methods

Spleen tissue

All animal care and experimental procedures complied with EEC (O.J. of EC L358/1 18/12/1986) regulations on the protection of laboratory animals and with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines for the use of experimental animals. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Mouse spleen tissue was obtained from adult male C57BL/6J mice weighing 25–40 g and maintained on a 12/12 h light/dark cycle with free access to food and water. Spleen tissue was dissected and was stored at –80°C for 2–3 weeks prior to preparation of membranes.

Rat spleen tissue was obtained from adult male Wistar BRL rats weighing 175–200 g and maintained on a 12/12 h light/dark cycle with free access to food and water. Spleen tissue was dissected and was stored at –80°C for 2–3 weeks prior to preparation of membranes.

Human spleen tissue was obtained from Tissue Solutions Ltd, Bridge of Weir, Scotland, UK. Healthy spleen was obtained from surgical excess tissue from males. Surgical samples are typically frozen within 15–30 min of resection. Tissue was stored at –80°C for 4–6 weeks prior to preparation of membranes.

hCB_2-CHO cells

CHO cells transfected with cDNA encoding human cannabinoid CB_2 (Ross *et al.*, 1999) were maintained in Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM, supplemented with 1 mM L-glutamine, 10% FBS and 0.6% penicillin–streptomycin together with G418 [3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl[oxy-2-hydroxycyclehexy]oxy-2-(1-hydroxyethyl0oxane-3,4-diol; 400 mg·mL^{-1}]. Cells were maintained at 37°C and 5% CO_2

in the media, and were passage twice a week using non-enzymatic cell dissociation solution.

Membrane preparation

Spleen membranes. Frozen rat, human and mouse spleen tissue was cut in several pieces and placed in a Choi lysis buffer (Tris-HCl 20 mM, Sucrose 0.32 M, EDTA 0.2 mM, EGTA 0.5 mM, pH 7.5) containing Roche© protease inhibitor cocktail (1:40 v/v) and phenylmethylsulphonyl fluoride (PMSF; 150 μM) and then homogenized with a 1 mL handheld homogenizer. The homogenate was centrifuged at 500× *g* for 2 min and the resulting supernatant was re-centrifuged at 16 000× *g* for 20 min. The harvested membranes were re-suspended in TME buffer (50 mM Tris-HCl; EDTA 1.0 mM; $MgCl_2$ 3.0 mM; pH 7.4) and stored at –80°C for no more than 1 month.

hCB_2-CHO cell membranes. The hCB_2-CHO cells were removed from flasks by scraping and then frozen as a pellet at –20°C until required. Before use in a GTPγS assay, cells were defrosted in 50 mM Tris-buffer (pH 7.4) and homogenized with a 1 mL handheld homogenizer. Protein assays were performed using a Bio-Rad Dc Kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK).

[^{35}S]GTPγS binding assay

Spleen membranes. The assays were carried out with rat (20 μg protein per well), mouse (20 μg protein per well) and human (10 μg protein per well) spleen membranes, GTPγS binding buffer (50 mM Tris-HCl; 3 mM $MgCl_2$; 0.2 mM EGTA; 100 mM NaCl; 0.1% BSA), 0.1 nM [^{35}S]GTPγS and 30 μM GDP for rat and mouse spleen or 10 μM GDP for human spleen, in a final volume of 500 μL. Spleen membranes were preincubated for 30 min at 30°C with 0.5 U·mL^{-1} adenosine deaminase (200 U·mg^{-1}) to remove any endogenous adenosine. Binding was initiated by the addition of [^{35}S]GTPγS. Assays were performed at 30°C for 60 min (rat and mouse spleen) or 30 min (human spleen). The reaction was terminated by the addition of ice-cold Tris binding buffer and vacuum filtration using a 24-well sampling manifold (Brandel Cell Harvester, Alpha Biotech Ltd, London, UK) and Whatman GF/B glass-fibre filters that have been pre-soaked in wash buffer at 4°C for 24 h. Each reaction tube was washed three times with 4 mL aliquot of buffer. The filters were oven-dried for 60 min and then placed in 5 mL of scintillation fluid (Ultima Gold XR, Packard, PerkinElmer Ltd, Saxon Way Bar Hill, Cambridge, UK). Radioactivity was quantified by liquid scintillation spectometry. Nonspecific binding were measured in the presence of 30 or 10 μM GTPγS. Compounds under investigation were added to the incubations in 1 μL of dimethyl sulphoxide (DMSO); vehicle control contained DMSO alone. In each experiment, the percent increases in [^{35}S]GTPγS binding in response to ligands was calculated using the DMSO-treated membranes as the control. EC_{50} values were calculated using GraphPad Prism 5.0® (San Diego, CA, USA).

hCB_2-CHO cell membranes. The assays were carried out with hCB_2-CHO cell membranes (50 μg proteins per well), GTPγS binding buffer (50 mM Tris-HCl; 50 mM Tris base, 5 mM $MgCl_2$; 1 mM EDTA; 100 mM NaCl; 1 mM dithiothreitol [DTT], 0.1% BSA), 0.1 nM [^{35}S]GTPγS and 30 μM GDP in a final volume of 500 μL. Binding was initiated by the addition of [^{35}S]GTPγS. Assays were performed following the same steps used for the spleen membranes. Non-specific binding were measured in the presence of 30 μM GTPγS. Compounds under investigation were added to the incubations in 1 μL of DMSO; vehicle control contained DMSO alone. In each experiment, the percent increase in [^{35}S]GTPγS binding in response to ligands was calculated using the DMSO-treated membranes as the control. EC_{50} values were calculated using GraphPad Prism 5.0.

Cyclic AMP assay

The assays were performed using the HitHunter® cAMP assay kit according to the vendor's protocol. Briefly, CHO cells expressing the hCB_2 receptors were detached using cell dissociation buffer, counted and seeded at 2×10^4 cells per well in 100 μL of complete medium onto white 96-well plates and incubated at 37°C and 5% CO_2 for approximately 24 h before running the experiment. The assays and the drug dilutions were performed in a 1:1 mixture of DMEM and Ham's F12 medium without phenol red, containing 10 μM of rolipram and forskolin (FSK; 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one). Before running the assay, the medium was discarded and cells were washed once with D-MEM/F-12 medium. Then, cells were treated with the assigned drugs (30 μL per well) and incubated for 30 min at 37°C and 5% CO_2. Finally, cAMP standards and the appropriate mixture of kit components were added (as described by the manufacturer), DiscoveRx (DiscoveRx Corporation, Ltd, Aston, Birmingham, UK). Plates were incubated overnight at room temperature in the dark. Chemiluminescent signals were detected on a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). EC_{50} values were calculated using GraphPad Prism 5.0.

Statistical analysis

Values have been expressed as means and variability as SEM or as 95% confidence intervals (CIs). The EC_{50} values and maximal compound-induced increase in [^{35}S]GTPγS binding were determined by fitting the data to a sigmoidal concentration-response curve using nonlinear regression (Prism 5, Graph Pad Software). Analysis was by one-way ANOVA and Newman–Keuls multiple comparison tests, unless otherwise stated. A P-value of <0.05 was considered significant.

Materials

SR 144528 was kindly supplied by Sanofi-Aventis (Montpellier, France). CP 55,940, JWH 133, WIN 55,212-2, GW 405833, AM 630, JTE 907 were supplied by Tocris (Bristol, UK). JWH 015, G418 and FSK were supplied by Sigma-Aldrich (Poole, Dorset, UK). (*R*)-AM 1241 was supplied by Cayman (Ann Arbor, MI, USA).

Results

[^{35}S]GTPγS assay optimisation

The method used for measuring agonist-stimulated [^{35}S]GTPγS binding to mouse, rat and human spleen mem-

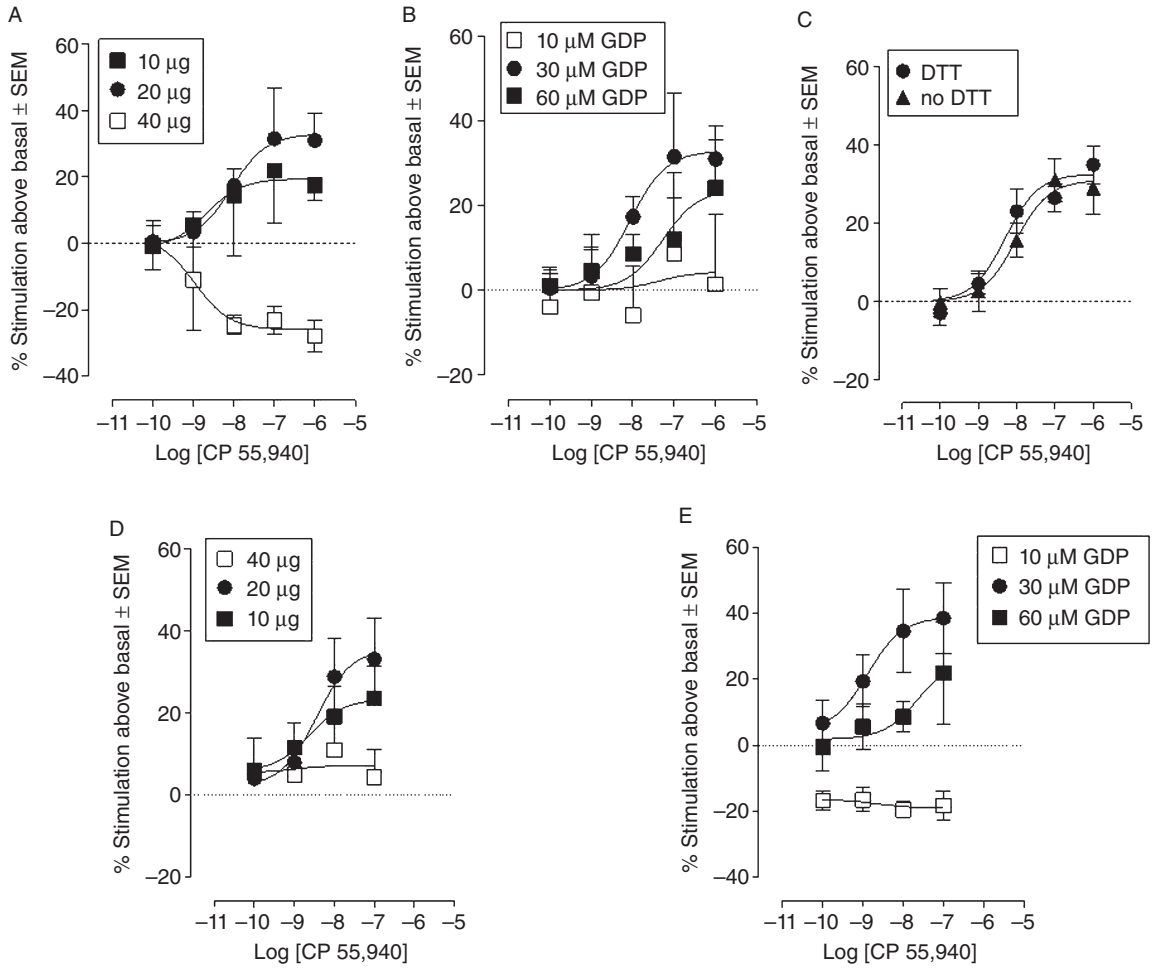

Figure 2

Assay optimization in mouse and rat spleen. Stimulation of [^{35}S]GTPγS binding by CP 55,940 (A) in mouse spleen membranes determined using 10, 20 and 40 µg of protein per well; (B) in mouse membranes using 10, 30 and 60 µM GDP. The membranes were incubates at 30°C for 60 min; (C) in mouse membranes in the presence of 20 µg of protein and 30 µM GDP in the presence and absence of DTT; (D) in rat spleen membranes determined using 10, 20 and 40 µg of protein per well; (E) in rat membranes determined using 10, 30 and 60 µM GDP. Each data point is the mean percentage value ± SEM.

branes was optimized following the experimental conditions described previously by Thomas *et al*. (2005). Specifically, the ability of the cannabinoid receptor agonist CP 55,940 to stimulate the [^{35}S]GTPγS binding to mouse, rat and human spleen membranes was determined using 10, 20 and 40 µg of proteins per well; 10, 30 and 60 µM GDP, 0.1 nM [^{35}S]GTPγS and 30 µM GTPγS in a final volume of 500 µL. The incubation was initially performed at 30°C for 60 min for spleen membranes from all species (Figures 2A–D and 3B).

The ability of CP 55,940 to stimulate the[^{35}S]GTPγS binding to both mouse (Figure 3A,B) and rat spleen membranes (Figure 3D,E) was maximum when 20 µg of proteins per well and 30 µM of GDP were used. E_{max} values with 95% CIs shown into brackets were 29.01% (15.06 & 42.97) and 34.96% (12.92 & 56.99), respectively. Maximum specific binding was 70–85% for both spleen membranes. DTT is routinely used in the [^{35}S]GTPγS binding assay; however, in this study, we aimed to maintain native conditions wherever possible. Optimization indicated that the E_{max} values in mouse spleen were similar in the presence and absence of DTT (Figure 2C).

Conversely, using 30 µM GDP and increasing amount of proteins (10, 20 and 40 µg per well), the ability of CP 55,940 to stimulate the [^{35}S]GTPγS binding to human spleen membranes was time dependent; it was maximum within the first 30 min (10 µg proteins per well) after the addition of the agonist (Figure 3A) and significantly decreased after 1 h incubation (Figure 3B). E_{max} value and 95% CIs into brackets was 21.14% (15.18 & 27.10%). Finally, maximum specific binding (70–85%) with the human spleen membranes was reached with 10 µM of GDP (Figure 3C).

For the hCB_2-CHO cells, condition were used as per previous optimization of these cells (50 µg proteins per well; 30 µM GDP). E_{max} value and 95% CIs into brackets was 65.50 (54.31–76.69). Basal levels of [^{35}S]GTPγS binding (pmol·mg^{-1}) were 3760 ± 760, 11 600 ± 2040, 7720 ± 970, 2820 ± 450 for mouse spleen, rat spleen, human spleen and hCB_2-CHO, respectively.

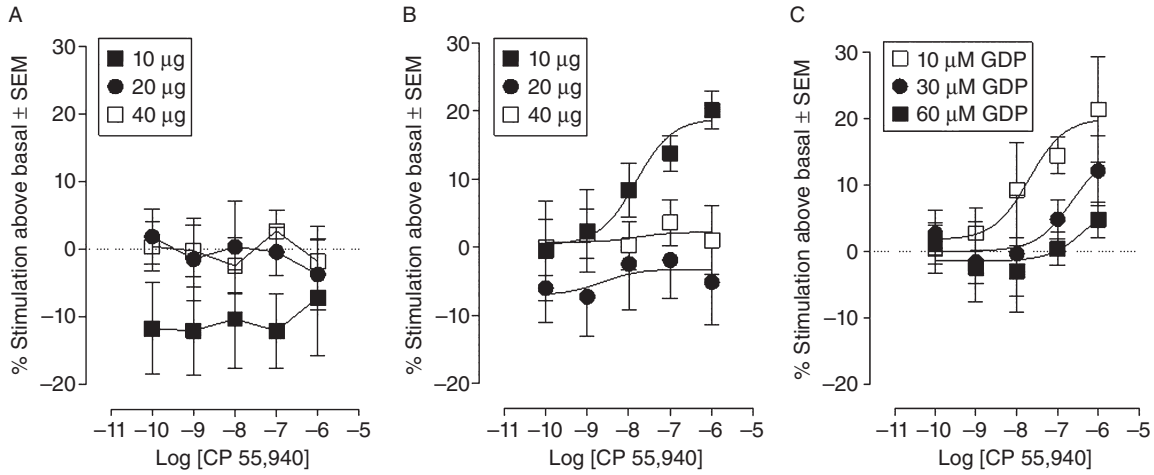

Figure 3

Assay optimization in human spleen. Stimulation of [^{35}S]GTPγS binding by CP 55,940 (A) in human spleen membranes determined using 10, 20 and 40 µg of protein per well with the membranes were incubates at 30°C for 60 min; (B) in human spleen membranes determined using 10, 20 and 40 µg of protein per well with the membranes were incubates at 30°C for 30 min (B) in human membranes determined using 10, 30 and 60 µM GDP. Each data point is the mean percentage value ± SEM.

Effects of CB_2 receptor agonists: CP 55,940, WIN 55,212-2, JWH 133, JWH 015

In the [^{35}S]GTPγS assay, CP 55,940 was a potent agonist in spleen membranes obtained from all species with EC_{50} values of 9.4 nM, 5.6 nM and 4.3 nM in mouse, rat and human spleen, respectively; pEC_{50} values were not significantly different (Table 1; Figure 4A–C). The efficacy (E_{max}) of CP 55,940 in native spleen tissues was also not significantly different from that obtained in the [^{35}S]GTPγS assay or the cAMP assay in cells over-expressing the hCB_2 receptor (Figures 4D and 5A; Table 2). Notably, in the rat spleen, CP 55,940 displayed a marked apparent desensitization at 1 µM such that the compound no longer stimulated [^{35}S]GTPγS binding (Table 3). In comparison, in mouse and human spleen, 1 µM CP 55,940 did not induce any detectable desensitization.

JWH 133 and WIN 55,212-2 displayed similar potency to CP 55,940 in native tissues from all species and in the assays in the recombinant systems; the efficacy (E_{max}) of these compounds was also not significantly different from that of CP 55,940 (Figure 4A–D, Table 1). At 1 µM, JWH 133 induced an apparent desensitization in spleen tissue form all species, such that there was no significant [^{35}S]GTPγS stimulation at this concentration; this is in comparison with a full agonist effect at 100 nM in mouse, rat and human spleen (Table 3). In the [^{35}S]GTPγS performed in the hCB_2-CHO cells, no desensitization was observed with JWH 133 at 1 µM. Desensitization was not observed with WIN 55,212-2 in any tissues (Table 3).

JWH 015 behaved as a partial agonist both in all the native systems we investigated and in hCB_2-CHO cell membranes, stimulating [^{35}S]GTPγS binding with significantly lower efficacy (E_{max} values) than CP 55,940; JWH 015 displayed least efficacy in the rat spleen (Figure 4A–C). In contrast, JWH 015 behaved as a full agonist in the cAMP assay performed with hCB_2-CHO cells, with an E_{max} that was not significantly different from that of CP 55,940 (Figure 5A; Tables 1 and 2). It is notable that the level of signal amplification will be significantly higher in the cAMP assay than in the [^{35}S]GTPγS binding; hence, the observation of higher efficacy of certain ligands in this assay.

Effects of CB_2 receptor 'protean' agonists (R)-AM 1241 and GW 405833

As mentioned in the introduction, both (*R*)-AM 1241 and GW 405833 (L-768,242) have been shown previously to be 'protean agonists' in recombinant systems, thus, behaving as agonists or inverse agonists depending on the levels of constitutive activity. In spleen membranes from mouse, rat and human, (*R*)-AM 1241 behaved as an agonist (Figure 4A–C). Its potency appeared to be somewhat lower in rat spleen (EC_{50} = 59.7 nM) than in mouse or human spleen (EC_{50} = 2.2 nM and 15.7 nM, respectively); however, this apparent difference is not statistically significant (Table 2). Notably, in mouse and human spleen, 1 µM (*R*)-AM 1241 seemingly induced a marked apparent desensitization as indicated by its ability to stimulate [^{35}S]GTPγS binding significantly at 100 nM, but not at 1 µM (Table 3). No such apparent desensitization was detected in rat spleen, a possible consequence of the lower potency that it displays in rat spleen than in mouse or human spleen. In marked contrast to all these findings, in the recombinant system over-expressing the hCB_2, (*R*)-AM 1241 behaved as an inverse agonist in both [^{35}S]GTPγS and cAMP assays (Figures 4D and 5A).

Similarly, GW 405833 behaved as a low-efficacy agonist in spleen membranes from all three species; indeed, because of the particularly low level of stimulation it induced in mouse and rat spleen, an accurate EC_{50} for this compound could only be determined in human spleen (734 nM) in which its efficacy was somewhat higher (Figure 4A–C, Table 1). GW 405833 was significantly less potent in human spleen than CP 55,940 ($P < 0.05$; one-way ANOVA). As found with (*R*)-AM 1241, GW 405833 behaved as an inverse agonist in hCB_2-CHO cell homogenates (Figures 4D and 5A). However, its

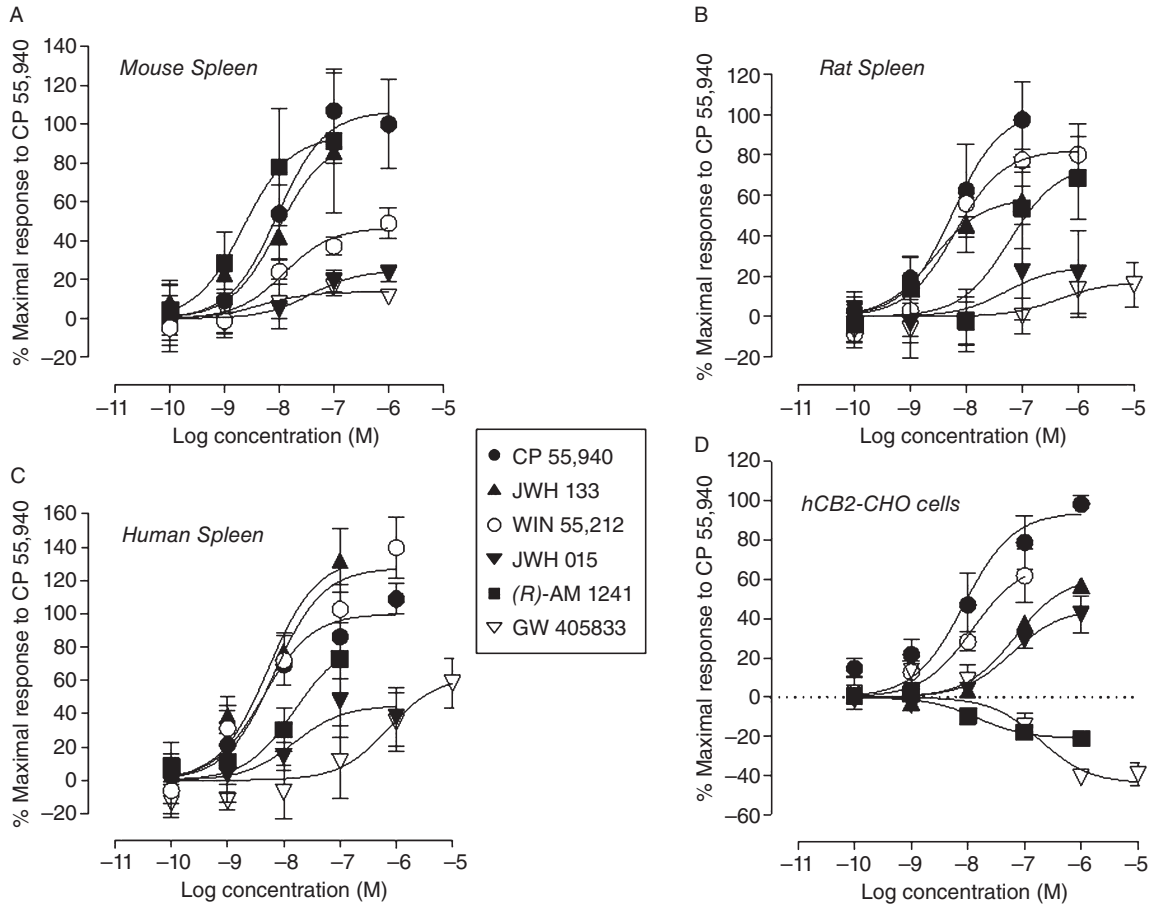

Figure 4

Effects of CP 55,940, JWH 133, WIN 55,212-2, (*R*)-AM 1241, JWH 015 and GW 405833 on [^{35}S]GTPγS binding using (A) mouse (B) rat (C) human spleen membrane homogenates and (D) human CB_2 cannabinoid receptor transfected CHO cell homogenates. Each data point is the mean percentage value ± SEM.

inverse efficacy in both the [^{35}S]GTPγS and the cAMP assay was significantly greater than that of (*R*)-AM 1241 (Figures 4A–D and 5A, Tables 1 and 2).

Effects of CB_2 receptor inverse agonists SR 144528, AM 630 and JTE 907

These compounds behaved as inverse agonists in all preparations (Figures 5B and 6). In the spleen, the potency of each of these compounds was not significantly affected by species (Figure 6, Table 1). However, in the rat and human spleens, the inverse efficacy of AM 630 was significantly lower than that of SR 144528. In contrast, in the [^{35}S]GTPγS assay performed with hCB_2-CHO cell homogenates, the EC_{50} of SR 144528 was significantly lower ($P < 0.001$, one-way ANOVA) than that of AM 630 and JTE 907; the efficacies of these three compounds did not differ from each other in the recombinant systems (Tables 1 and 2).

Effects in spleen membranes from $CB_2^{-/-}$ mice

At concentration, which produced a significant effect in spleen membranes prepared from wild-type mice, none of the ligands tested had a significant effect on [^{35}S]GTPγS binding in spleen membranes prepared from mice lacking the CB_2 receptor (Figure 7).

Discussion

To our knowledge, this is the first full *in vitro* characterization of a panel of CB_2 receptor ligands in spleen tissues, which natively express this receptor and, in particular, in the human spleen. The results confirm previous observations in recombinant systems demonstrating that CB_2 receptor ligands that are known to be anti-nociceptive in animal models are indeed CB_2 receptor agonists in native systems. This is in marked contrast to the 'protean' nature of a subgroup of these compounds that has been observed in over-expressing recombinant systems (Yao *et al.*, 2006; Mancini *et al.*, 2009).

It is notable in the current study that, even after extensive assay optimization in each tissue, there is a low signal in the assays conducted in spleen tissue, which expresses native CB_2 receptors. Thus, the percentage stimulation of [^{35}S]GTPγS binding with the high efficacy CB_2 receptor agonist, CP 55,940 is only 21, 29 and 35% in human, mouse and rat

Table 1

Effect of CB_2 receptor ligands in the [^{35}S]GTPγS binding assay using spleen membranes from mouse, rat and human. pEC_{50} with SEM and E_{max} (maximum response, %) values with 95% CI were determined from GraphPad Prism

	Mouse		Rat		Human	
Compound	**pEC_{50} ± SEM (*n*)**	**E_{max} (%) (95% CI)**	**pEC_{50} ± SEM (*n*)**	**E_{max} (95% CI)**	**pEC_{50} ± SEM (*n*)**	**E_{max} (95% CI)**
CP 55,940	8.03 ± 0.31 (48)	99.99 (70.54 & 129.40)	8.25 ± 0.33 (24)	101.90 (65.68 & 138.10)	8.37 ± 0.24 (16)	99.97 (81.06 & 118.90)
JWH 133	8.02 ± 0.21 (16)	89.07 (64.33 & 113.80)	8.59 ± 0.37 (20)	58.30 (37.54 & 79.07)	8.27 ± 0.21 (14)	133.60 (101.70 & 165.50)
WIN 55,212-2	7.90 ± 0.31 (16)	43.84 (32.01 & 55.68)	8.21 ± 0.23 (16)	82.25 (66.59 & 97.91)	8.17 ± 0.24 (12)	127.50 (102.90 & 152.20)
JWH 015	7.43 ± 0.43 (16)	24.67 (13.12 & 36.22)	–	24.71 (–15.46 & 64.88)	7.82 ± 0.60 (12)	44.81 (19.08 & 70.53)
(*R*)-AM 1241	8.65 ± 0.57 (32)	94.03 (44.62 & 143.4)	7.22 ± 0.50 (24)	75.26 (33.95 & 116.6)	7.80 ± 0.51 (12)	84.01 (28.24 & 139.80)
GW 405833	–	13.69 (–7.07 & 34.45)	–	17.05 (–4.57 & 38.68)	6.13 ± 0.50 (12)	62.21 (26.55 & 97.87)
SR 144528	8.39 ± 0.23 (12)	–63.83 (–76.82 & –50.83)	7.85 ± 0.19 (12)	–93.01 (–109.80 & –76.19)	8.87 ± 0.17 (12)	–102.90 (–116.7 & –89.16)
AM 630	8.78 ± 0.27 (12)	–51.00 (–61.43 & –40.56)	8.29 ± 0.23 (12)	–38.95 (–46.39 & –31.51)	8.18 ± 0.75 (12)	–39.04 (–64.74 & –13.33)
JTE 907	8.54 ± 0.25 (12)	–55.74 (–67.51 & –43.96)	8.66 ± 0.55 (12)	–54.79 (–77.58 & –31.99)	8.09 ± 0.28 (12)	–86.14 (–107.90 & 64.36)

Figure 5

Effects of cannabinoid receptor ligands on forskolin-stimulated cAMP production in hCB_2-CHO cells. The concentration of forskolin used in these experiments was 10 μM. Each point represents the mean ± SEM percentage of forskolin-stimulated cAMP production.

spleens, respectively. This is in line with a recent report demonstrating a low signal detected with CB_2 receptor ligands in rat spleen cells using the [^{35}S]GTPγS binding assay (Geiger *et al.*, 2010). However, motivated by the imperative for assessment of CB_2 ligand pharmacology in native tissues, we progressed with compound characterization. The data obtained are sufficiently reproducible to draw conclusions related to the pharmacological profile of the various ligands tested. Furthermore, the lack of effect of any of the ligands in spleen taken from $CB_2^{-/-}$ mice indicates that the results are meaningful despite the low signal.

Our observation that (*R*)-AM 1241 behaves as a CB_2 receptor agonist in systems that natively express this receptor is in line with the hypothesis that CB_2 receptor agonists are anti-nociceptive (Guindon and Hohmann, 2008; Beltramo, 2009). (*R*)-AM 1241 is anti-nociceptive in a variety of *in vivo* rat pain models; an effect that is blocked by CB_2 receptor antagonists and is absent from $CB_2^{-/-}$ mice, with no significant component of CB_1 receptor activation (Ibrahim *et al.*, 2003; 2005; Malan *et al.*, 2003; Quartilho *et al.*, 2003; Hohmann *et al.*, 2004). In line with this finding, the CB_2 receptor ligands JWH 133 (Elmes *et al.*, 2004) and GW 405833 (Valenzano *et al.*, 2005), characterized as agonists in native tissues in this study,

Table 2

Effect of CB_2 receptor ligands in the [^{35}S]GTPγS binding assay and cAMP assay using hCB_2-CHO cells. pEC_{50} with SEM and E_{max} (maximum response, %) values with 95% CI were determined from GraphPad Prism

	[^{35}S]GTPγS binding assay		cAMP assay	
Compound	**pEC_{50} ± SEM (*n*)**	**E_{max} (95% CI)**	**pEC_{50} ± SEM (*n*)**	**E_{max} (95% CI)**
CP 55,940	8.07 ± 0.20 (12)	94.28 (75.70 & 112.90)	8.36 ± 0.31 (12)	100.40 (79.29 & 121.50)
JWH 133	7.16 ± 0.09 (12)	61.51 (55.12 & 67.90)	7.89 ± 0.26 (12)	105.80 (85.36 & 126.20)
WIN 55,212-2	7.92 ± 0.31 (12)	68.62 (42.34 & 94.90)	7.63 ± 0.11 (12)	103.10 (94.30 & 111.80)
JWH 015	7.22 ± 0.28 (12)	45.19 (31.61 & 58.77)	8.59 ± 0.36 (12)	104.10 (79.46 & 128.60)
(*R*)-AM 1241	7.83 ± 0.18 (16)	–21.08 (–24.70 & –17.45)	7.74 ± 1.64 (12)	–10.59 (–23.58 & 2.40)
GW 405833	6.72 ± 0.31 (16)	–43.98 (–57.93 & –30.03)	6.97 ± 0.61 (12)	–54.53 (–86.53 & –22.53)
SR 144528	8.19 ± 0.12 (12)	–66.23 (–73.40 & –59.05)	7.14 ± 0.17 (12)	–273.90 (–313.80 & –233.90)
AM 630	6.97 ± 0.21 (12)	–53.06 (–67.33 & –38.80)	6.37 ± 0.31 (12)	–190.20 (–269.80 & –110.70)
JTE 907	6.59 ± 0.16 (12)	–67.17 (–83.61 & –50.73)	6.63 ± 0.22 (12)	–254.10 (–319.90 & –189.30)

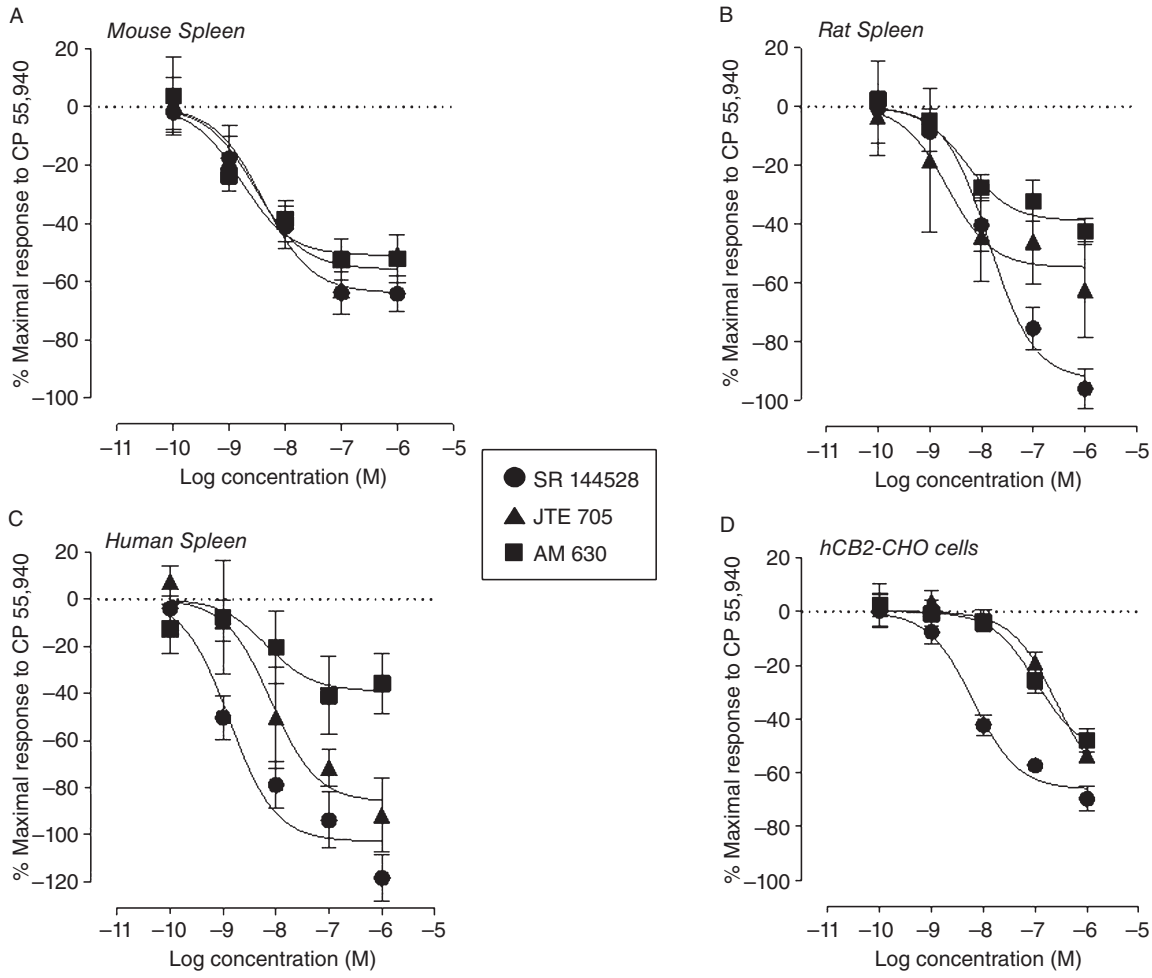

Figure 6

Effects of SR 144528, AM 630 and JTE 907 on [^{35}S]GTPγS binding using (A) mouse (B) rat (C) human spleen membranes homogenates and (D) human CB_2 cannabinoid receptor transfected CHO cell homogenates. Each data point is the mean percentage value ± SEM.

Table 3

A comparison of the % response at 100 nM and 1 μM of CB_2 receptor ligands in the [^{35}S]GTPγS binding assay using spleen membranes from mouse, rat and human

	Mouse (% response, 95% CI)		Rat (% response, 95% CI)		Human (% response, 95% CI)	
Compound	**100 nM**	**1 μM**	**100 nM**	**1 μM**	**100 nM**	**1 μM**
CP 55,940	100.21 ± 19	93.71 ± 23	97.42 ± 18.4	–9.98 ± 10.5 *** Signal loss	86.04 ± 8.6	108.9 ± 9.0
JWH 133	85.7 ± 5.9	11.35 ± 7.1*** Signal loss	57.31 ± 6.9	0.55 ± 10.4 *** Signal loss	138 ± 21.5	–16.2 ± 18*** Signal loss
WIN 55,212-2	37.3 ± 4.4	49.1 ± 7.8	77.33 ± 5.7	80.09 ± 15.2	102.7 ± 13.5	131.7 ± 18.2
JWH 015	14.2 ± 2.8	19.4 ± 2.9	21.76 ± 23.7	21.10 ± 21.6	47.56 ± 22	38.02 ± 17.4
(*R*)-AM 1241	91.41 ± 27	–12.76 ± 10.2* Signal loss	53.73 ± 20.4	68.41 ± 19.8	72.93 ± 30.4	–21.9 ± 12.2* Signal loss
GW 405833	11.07 ± 4.5	15.99 ± 3.2	13.30 ± 11.9	15.58 ± 10.9	34.89 ± 20.4	58.29 ± 18.0

*$P < 0.05$, ***$P < 0.001$, unpaired Student's *t*-test comparing response at 100 nM with that at 1 μM.

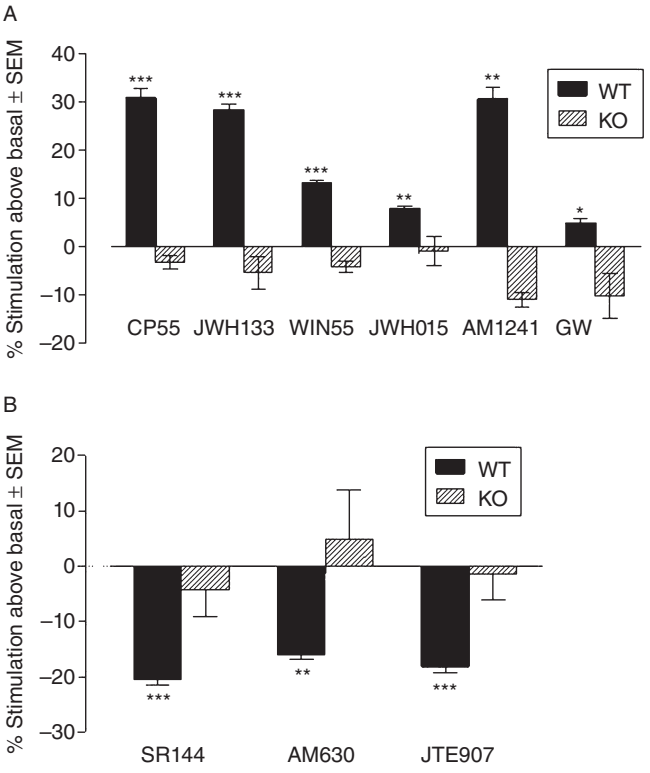

Figure 7

Stimualtion of [^{35}S]GTPγS binding by CP 55,940 in mouse spleen membranes from wild-type mice (WT) and $CB_2^{-/-}$ mice (KO) with CP 55,950 (1 μM), JWH133 (100 nM), WIN55212 (1 μM), JWH015 (1 μM), AM1241 (100 nM) and GW 405833 (10 μM). Each data point is the mean percentage value ± SEM. ***$P < 0.001$, **$P < 0.01$ significantly different from basal, one-sample *t*-test.

are also effective in preclinical models of inflammatory and neuropathic pain.

There is strong evidence from recombinant systems that (*R*)-AM 1241 and GW 405833 (L-768,242) behave as 'protean agonists' (Kenakin, 2001); thus, these compounds display different profiles of agonism, antagonism or inverse agonism depending on the assay conditions (Yao *et al.*, 2006; Mancini *et al.*, 2009). Mancini *et al.* (2009) demonstrated that, under basal conditions, GW 405833 (L-768,242) behaves as an inverse agonist in human and rat CB_2 receptor recombinant systems. When constitutive activity is abolished, the compound behaves as an agonist. The authors propose that differences in the levels of constitutive activity in native versus recombinant systems explain the fact that compounds, which are apparent CB_2 receptor inverse agonists, have antinociceptive actions *in vivo*; an effect that is clearly associated with CB_2 agonism (Guindon and Hohmann, 2008; Beltramo, 2009). In line with this hypothesis, we demonstrate that this compound is an agonist in the human spleen, but an inverse agonist in cells over-expressing hCB_2. These finding are also in line with the significantly lower basal levels of [^{35}S]GTPγS binding in the human spleen (GW 405833 is an agonist) as compared with the rat spleen (GW 405833 has little effect) and hCB_2-CHO cells (GW 405833 is an inverse agonist). Also, in line with the findings of Mancini *et al.* (2009), we show that GW 405833 (L-768,242) displays an apparently higher inverse efficacy in recombinant systems than that observed with (*R*)-AM 1241. It is also a lower potency agonist in spleen tissue expressing native CB_2 receptors.

Clearly, this study further highlights that in order to be predicative of *in vivo* efficacy, it is important to conduct pharmacological characterization of ligand function in native systems. However, it is also important to note that levels of receptor expression and constitutive activity are known to change in disease; thus, healthy native tissue *in vitro* may still provide a flawed representation of the behaviour of protean compounds in disease (Smit *et al.*, 2007).

It is well established from recombinant systems that SR 144528, AM 630 and JTE 907 are CB_2 receptor inverse agonists (Portier *et al.*, 1999; Ross *et al.*, 1999; Iwamura *et al.*, 2001). AM 630 has recently been demonstrated to behave as a protean ligand in recombinant systems, whereby the profile of the compound is altered depending on the level of constitutive activity (Bolognini *et al.*, 2012). Here we find that, in

spleen tissue from rat-, mouse- and human-expressing native CB_2 receptors, AM 630, SR 144528 and JTE 907 behave as inverse agonists. In the rat and human spleen, AM 630 has significantly lower inverse efficacy than SR 144528, a difference that is not mirrored in hCB_2 recombinant systems where the three compounds have similar inverse efficacy. As is the case with agonists, the results highlight the importance of compound characterization using natively expressing systems.

Bingham *et al*. (2007) suggest that (*R*)-AM 1241 displays species-specific effects based on a comparison of pharmacology in recombinant rat, mouse and human systems. However, here we show that in native systems, there is little difference in the pharmacology of the compound. However, GW 405833 has significantly higher efficacy in human spleen than in rodent spleen. This may reflect the fact that human CB_2 displays only 81 and 82% amino-acid identity with rat and mouse, respectively (Gérard *et al*., 1991; Munro *et al*., 1993; Shire *et al*., 1996; Griffin *et al*., 2000; Brown *et al*., 2002; Liu *et al*., 2009). Alternatively, as mentioned earlier, this may reflect the lower levels of constitutive activity in human spleen that allows an agonist effect of this protean compound to be revealed. Here we see that in the over-expressing hCB_2-CHO cells, all the compounds display a similar inverse efficacy. In contrast, in the human spleen, AM630 has significantly lower efficacy; this may indicate that lower levels of constitutive activity in this tissue allow discrimination between partial and full inverse agonists.

Of note in this study are the differential levels of signal loss observed with various agonists at increasing concentrations. Here we report that in the rat spleen, CP 55,940 and JWH 133 appear to induce a marked signal loss; this is in line with the findings of Atwood *et al*. (2012) in rCB_2-HEK internalization studies. Notably, we did not observe such signal loss or apparent desensitization, with CP 55,940 in the mouse or human spleen tissue or in hCB_2-CHO cell homogenates, suggesting a species-specific effect in natively expressed CB_2 receptors. Again, in line with the findings of Atwood *et al*. (2012), we find that the aminoalkylindoles WIN 55,212-2 and JWH 015 do not display signal loss in spleen from mouse, rat or human. Atwood *et al*. (2012) reported that (*R*)-AM 1241 causes little receptor internalization in rCB_2-HEK cells. Here we report that, while (*R*)-AM 1241 does not display signal loss in rat spleen tissue, it induces a marked signal loss at higher concentrations in mouse and human spleen.

These data highlight ligand- and species-specific differences in the observed signal loss, which may reflect differential desensitization. However, classic desensitization of GPCRs occurs in a time- and concentration-dependent manner and these characteristics were very clearly demonstrated for receptor internalization in the study by Atwood *et al*. (2012). In contrast, here we observe an apparent 'all-or-none' response in which the signal is lost at the highest agonist concentration tested rather than a graded, concentration-dependent effect. In order to establish the exact nature of the mechanism underlying the observed signal loss observed further, more detailed, characterization of the concentration- and time-dependant nature of the effect would be required.

In conclusion, here we present the first functional characterization of a panel of CB_2 receptor ligands in rodent and human spleen tissue, which express the CB_2 receptor. It is notable that the spleen may also express non-CB_2 receptors with which these compounds may interact. However, we have demonstrated here that all ligands tested are devoid of significant effects in spleen derived from $CB_2^{-/-}$ mice. Thus, the data appear to confirm the hypothesis that compounds that produce anti-nociceptive action in animal models of pain are also CB_2 receptor agonists in native tissues *in vitro*. This is in marked contrast to the complex, protean behaviour that is observed with certain ligands in over-expressing recombinant systems. It is notable that the potency and efficacy of CB_2 receptor ligands is known to be influenced by various factors including tethering of G-protein subunits, RGS proteins (G-protein signalling regulators), receptor phosphorylation (by GPCR kinase, GRK) and compartmentalization of signalling elements within the membrane (Sutor *et al*., 2011). Such factors may explain the differences in the observed pharmacological profile of ligands between species and, in particular, may account for the marked differences between the native and recombinant cell systems. Thus, despite the low signal obtained in the native tissues, it is important to attempt a full pharmacological characterization in native tissues, which reflect the *in vivo* physicochemical nature of the receptor state. Taken together, the data emphasize that, in order to gain an accurate reflection of CB_2 ligand pharmacology, it is necessary to characterize ligands in assay systems that use human native and diseased tissues. This is particularly important in light of the increasing portfolio of diseases that may be amenable to treatment with CB_2 receptor agonists, most recently including the treatment of cocaine addiction (Onaivi *et al*., 2008; Adamczyk *et al*., 2012; Xi *et al*. 2011).

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Conflict of interest

The authors have no conflict of interest to declare.

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