British Journal of Pharmacology (2007) 151, 1061–1070

www.brjpharmacol.org

RESEARCH PAPER

Species-specific *in vitro* pharmacological effects of the cannabinoid receptor 2 (CB_2) selective ligand AM1241 and its resolved enantiomers

B Bingham[1], PG Jones[1,4], AJ Uveges[1], S Kotnis[1], P Lu[1], VA Smith[1], S-C Sun[1], L Resnick[2], M Chlenov[3], Y He[2], BW Strassle[1], TA Cummons[1], MJ Piesla[1], JE Harrison[1], GT Whiteside[1] and JD Kennedy[1]

[1]*Neuroscience Discovery Research, Wyeth Research, Princeton, NJ, USA;* [2]*Chemical and Screening Sciences, Wyeth Research, Princeton, NJ, USA and* [3]*Discovery Analytical Chemistry, Wyeth Research, Princeton, NJ, USA*

Background and purpose: Racemic (R,S) AM1241 is a cannabinoid receptor 2 (CB_2)-selective aminoalkylindole with antinociceptive efficacy in animal pain models. The purpose of our studies was to provide a characterization of R,S-AM1241 and its resolved enantiomers *in vitro* and *in vivo*.
Experimental approach: Competition binding assays were performed using membranes from cell lines expressing recombinant human, rat, and mouse CB_2 receptors. Inhibition of cAMP was assayed using intact CB_2-expressing cells. A mouse model of visceral pain (para-phenylquinone, PPQ) and a rat model of acute inflammatory pain (carrageenan) were employed to characterize the compounds *in vivo*.
Key results: In cAMP inhibition assays, R,S-AM1241 was found to be an agonist at human CB_2, but an inverse agonist at rat and mouse CB_2 receptors. R-AM1241 bound with more than 40-fold higher affinity than S-AM1241, to all three CB_2 receptors and displayed a functional profile similar to that of the racemate. In contrast, S-AM1241 was an agonist at all three CB_2 receptors. In pain models, S-AM1241 was more efficacious than either R-AM1241 or the racemate. Antagonist blockade demonstrated that the *in vivo* effects of S-AM1241 were mediated by CB_2 receptors.
Conclusions and implications: These findings constitute the first *in vitro* functional assessment of R,S-AM1241 at rodent CB_2 receptors and the first characterization of the AM1241 enantiomers in recombinant cell systems and *in vivo*. The greater antinociceptive efficacy of S-AM1241, the functional CB_2 agonist enantiomer of AM1241, is consistent with previous observations that CB_2 agonists are effective in relief of pain.
British Journal of Pharmacology (2007) **151,** 1061–1070; doi:10.1038/sj.bjp.0707303; published online 4 June 2007

Keywords: AM1241; cannabinoid; CB_2; enantiomer; protean agonism

Abbreviations: PPQ, para-phenylquinone; SNL, spinal nerve ligation

Introduction

First cloned from a macrophage cell line from human spleen, the CB_2 cannabinoid receptor, a G-protein-coupled receptor (GPCR) that signals through G_i, is one of at least two cell surface receptors capable of transducing the signals of endocannabinoid ligands (Munro *et al.*, 1993). Another G_i-coupled GPCR, the CB_1 receptor is highly expressed in the central nervous system (CNS) (Howlett *et al.*, 2004), and preliminary evidence suggests that additional endocannabinoid receptors may exist (Fride *et al.*, 2003; Baker *et al.*, 2006). While CB_2 is expressed mainly in tissues of the immune system (Howlett *et al.*, 2004), recent reports provide evidence of expression in the CNS (Cabral and Marciano-Cabral, 2005; Van Sickle *et al.*, 2005; Beltramo *et al.*, 2006) and inducible expression in peripheral sensory neurons (Wotherspoon *et al.*, 2005). DNA sequence analysis of rodent orthologues of CB_2 (Shire *et al.*, 1996; Griffin *et al.*, 2000; Brown *et al.*, 2002) reveals mouse and rat CB_2 to be, respectively, 79 and 81% identical to human CB_2 in predicted primary amino-acid composition and 93% identical to each other.

Agonists of CB_2 are thought to possess therapeutic promise in a number of diseases, including cancer (Flygare *et al.*, 2005; Herrera *et al.*, 2005), osteoporosis (Ofek *et al.*, 2006), atherosclerosis (Steffens *et al.*, 2005) and amyotropic lateral

Correspondence: B Bingham, Neuroscience Discovery Research, Wyeth Research, CN 8000, Princeton, NJ 08543-8000, USA.
E-mail: binghab@wyeth.com
[4]Current address: Discovery Pharmacology, Sepracor Inc., 84 Waterford Dr, Marlborough, MA 01752, USA.
Received 11 January 2007; revised 2 April 2007; accepted 17 April 2007; published online 4 June 2007

sclerosis (Kim *et al.*, 2006; Shoemaker *et al.*, 2007). However, the therapeutic potential of agonists of the CB_2 receptor has been most strongly demonstrated in animal models of inflammatory and neuropathic pain. Much of this evidence has been generated using the racemic mixture of the synthetic ligand AM1241 (Makriyannis, 2002). The *in vitro* selectivity of R,S-AM1241 for CB_2 vs CB_1 receptors has been demonstrated to be approximately 80-fold in binding studies, employing natively expressing tissues (Ibrahim *et al.*, 2003) and recombinant cell systems (Yao *et al.*, 2006). In pain efficacy studies, the action of R,S-AM1241 at CB_2 receptors has been demonstrated either pharmacologically using CB_2-selective antagonists, such as AM630 or SR144528, or genetically, using animals lacking the CB_2 receptor ($CB_2^{-/-}$). Similarly, efficacy through CB_1 receptor activation has been ruled out through the use of either CB_1-selective antagonist compounds (AM251 or SR141716A) or $CB_1^{-/-}$ animals (Malan *et al.*, 2001; Ibrahim *et al.*, 2003, 2005, 2006; Nackley *et al.*, 2003, 2004; Quartilho *et al.*, 2003; Hohmann *et al.*, 2004; LaBuda *et al.*, 2005; Beltramo *et al.*, 2006).

In the present report, we provide an extensive *in vitro* pharmacological characterization of R,S-AM1241, measuring binding affinity and functional inhibition of forskolin stimulated cyclic adenosine monophosphate (cAMP) accumulation in CHO-K1 cell lines overexpressing human, rat or mouse CB_2. We reveal not only species-specific effects of R,S-AM1241, but in extending this analysis to the separated enantiomers of R,S-AM1241, we also demonstrate stereoisomer-specific pharmacology for this synthetic cannabinoid ligand both *in vitro* and *in vivo*.

Methods

Cloning and cell culture

CHO-K1 cells expressing hCB_1 and hCB_2 receptors (Eurosscreen, Gosselies, Belgium) were cultured in Hams F12 medium containing 10% foetal bovine serum (FBS), penicillin (10 IU ml^{-1})/streptomycin (10 μg ml^{-1}) and 400 μg ml^{-1} G418. Mouse and rat CB_2 receptor open reading frame sequences were PCR amplified from commercially prepared spleen cDNA (BD Biosciences, San Jose, CA, USA) using oligonucleotide primers spanning the start and stop sites designed from published sequences (GenBank accession numbers X86405 (mouse) and AF176350 (rat)). Restriction sites (5′ *Hin*dIII and 3′ *Eco*RI) were included in the sequence of the PCR primers to facilitate cloning into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA). Transfection of CHO-K1 cells was with Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Initial selection of transfectants was with 800 μg ml^{-1} G418. Cell lines stably expressing mCB_2 and rCB_2 receptors were cultured in Dulbecco's modified Eagle's medium containing 10% FBS, penicillin (10 IU ml^{-1})/streptomycin (10 μg ml^{-1}), non-essential amino acids and 500 μg ml^{-1} G418. All tissue culture reagents were from Invitrogen.

Chiral separation of R,S-AM1241

The enantiomers of R,S-AM1241 were separated by chiral HPLC on a 2 × 25 cm Chiralcel OD column (elution solvent: 20% isopropanol/0.1% diethylamine in hexane, 22 ml min^{-1}). S-AM1241 eluted at 12.2 min, and R-AM1241 eluted at 17.26 min. Optical rotations were obtained with a Jasco P-1020 polarimeter with a 5 cm cell. S-AM1241: $[\alpha]_D^{25} = -46^\circ$(*c 1.0*, dimethyl sulphoxide (DMSO)); R-AM1241 $[\alpha]_D^{25} = +40^\circ$ (*c 1.0*, DMSO).

The absolute stereochemistry of the enantiomers was determined by vibrational circular dichroism (VCD). The VCD spectra were measured with the VCD instrument, ChiralIR (BioTools Inc., Wauconda, IL, USA). Each sample was dissolved in $CDCl_3$ (10 mM) and placed in a BaF_2 cell with a 0.1 mm pathlength. The VCD spectrum of each sample and solvent was measured for 4 h with a 4 cm^{-1} resolution and the photo elastic modulators optimized at 1400 cm^{-1}. The VCD baseline was obtained by subtracting the VCD of one enantiomer from that of the other, then dividing by two. The infrared (IR) baseline was obtained by subtracting the IR spectrum of $CDCl_3$ from that of the sample.

The (S)-conformers of the molecule and a truncated molecule were built with Hyperchem 7 (Hypercube Inc., Gainesville, FL, USA). (Truncation accelerates the geometry optimizations and the VCD calculations). The conformational search was performed with the semi-empirical PM3 method and resulted in 15 conformers for the whole molecule and 18 conformers for the truncated molecule. Six conformers of the truncated molecule have matches among the conformers of the whole molecule. The geometry optimization and VCD spectra of the six (S)-conformers were calculated with Gaussian 03 (Frisch *et al.*, 2003) at density functional theory (DFT) level with the b3lyp/6–31G(d) basis set. The average and the Boltzmann sum of the VCD and IR spectra of the six conformers were calculated and compared with the measured spectra. S-AM1241 was confirmed as the S-enantiomer, and R-AM1241 was confirmed as the R-enantiomer.

Membrane preparation

Confluent 245 cm^2 dishes of cells were washed twice with cold phosphate-buffered saline (PBS). Cells were scraped in 10 ml cold buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA)), homogenized in a Dounce homogenizer and pelleted at 32 000 *g*. Cell pellets were resuspended in storage buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM MgCl), homogenized again, aliquoted and frozen at −80°C. Protein concentrations were determined using Bio-Rad Protein Assay reagents as per the manufacturer's instructions (Biorad, Hercules, CA, USA).

Radioligand binding

Binding assays were conducted using 30 μg (hCB_2), 50 μg (rodent CB_2) or 12 μg (hCB_1) membrane protein per tube and 1–3 nM [^{3}H]-CP55,940 (Perkin Elmer, Boston, MA, USA) as the radioligand; compounds were diluted to 10 × concentrations in 4% DMSO/H_2O, and all reagents were combined in the assay buffer (50 mM Tris, pH 7.5, 2.5% bovine serum albumin, 2.5 mM EDTA). The assay was incubated at 30°C for 60 min and filtered on Whatman GFB filter mats treated with 0.15% polyethyleneimine using a Brandel 96-channel harvester (Brandel, Gaithersburg, MD, USA). Radioactivity was determined by liquid scintillation counting.

cAMP inhibition assays
Cells cultured in T-175 flasks were harvested by washing twice with PBS, followed by addition of 5 ml cell dissociation solution (Mediatech, Herndon, VA, USA). After 3–5 min incubation at room temperature, the dissociated cells were removed, mixed with 10 ml Krebs assay buffer (118 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM glucose, 1.2 mM $MgSO_4$, 2.4 mM $CaCl_2$) and pelleted. Cell pellets were resuspended in Krebs and counted. Cannabinoid ligands were serially diluted in Krebs containing 1 μM forskolin. Per well of a 96-well plate (Corning 3912), the ligand/forskolin mixture was combined with 1.5×10^4 cells and incubated at 37°C for 30 min. cAMP determinations were performed using the HitHunter cAMP XS Assay according to the manufacturer's protocol (DiscoveRx, Fremont, CA, USA). Chemiluminescence was counted using a Wallac Victor V after a 3 h incubation. For the *Pertussis* toxin study, cells were incubated in the presence of 100 ng ml^{-1} *Pertussis* toxin for 4 h before forskolin stimulation.

In vivo *studies*
All animal procedures were approved by an institutional animal care and use committee and were conducted in accordance with the International Association for the Study of Pain guidelines on the use of animals in experimental research (Zimmermann, 1983).

Acute analgesia (tail flick and hot plate)
Acute analgesia was investigated using the tail-flick (D'Amour and Smith, 1941) and hot-plate assays (Woolfe and MacDonald, 1944). For the tail-flick assay, male Sprague–Dawley rats ($n = 10$ per group) were placed on the apparatus (Ugo Basile, Varese, Italy), and an infrared beam was focused 5 cm from the tip of the tail. The latency to tail flick was measured to the nearest 0.1 s with a cutoff of 20 s. For the hot-plate assay, male Sprague–Dawley rats ($n = 10$ per group) were placed on a metal plate (Ugo Basile, Varese, Italy) maintained at 52°C. The latency to nocifensive response, defined as hindpaw lift, flutter, licking or escape behaviour, was measured to the nearest 0.1 s with a cutoff of 30 s. Approximately, 1 h after determination of baseline latency, animals received a single intraperitoneal (i.p.) dose of vehicle (0.5% methylcellulose and 2% Tween, 2 ml kg^{-1}) or 1, 3 or 10 mg kg^{-1} R,S-AM1241, R-AM1241 or S-AM1241. Dosing of the positive control (10 mg kg^{-1} morphine) was by subcutaneous (s.c.) injection. Tail-flick and hot-plate latencies were determined 30 and 90 min after drug administration.

Acute visceral pain (PPQ)
The ability of compounds to attenuate painful abdominal stretching (also referred to as writhing) was assessed in male CD-1 mice following i.p. injection of 2 mg kg^{-1} paraphenylquinone (PPQ) (dissolved in 4% ethanol). Delivery of R,S-AM1241, R-AM1241 or S-AM1241 (s.c.) was as a suspension in vehicle (1% DMSO, 0.5% methylcellulose and 2% Tween) 30 min before PPQ injection. Following PPQ administration, mice were placed individually in a Plexiglas observation cage, and stretching movements were recorded for two periods of 1 min each, at 5 and 10 min post-injection. Percent blockade was calculated according to the following equation:

$$\%\ \text{Blockade} = [(\text{meanvehicle}) - (\text{meandrug})] \times (\text{meanvehicle})^{-1} \times 100\%$$

Acute inflammatory pain (carrageenan)
Latency of paw withdrawal from a thermal stimulus (Hargreaves *et al*., 1988) was assessed in male Sprague–Dawley rats in response to focusing a radiant heat source (ITTC, Woodland Hills, CA, USA) on the plantar surface of the left hindpaw. Intraplantar injection of 2% carrageenan (50 μl volume) into the left hindpaw took place under anaesthesia (3% isoflurane/O_2), 24 h after baseline withdrawal latency was measured. Following a 30 min habituation period on a heated glass surface (32°C), withdrawal latency was measured to the nearest 0.1 s, with a cutoff of 20 s to avoid tissue damage. Delivery of R,S-AM1241, R-AM1241 or S-AM1241 (i.p., 2.5 h after carrageenan administration) was as a solution in a vehicle of 0.5% methylcellulose and 2% Tween. Three withdrawal latency measurements were taken for each rat 30 min post-drug administration. Paw volume was measured with a plethysmometer (Ugo Basile, Varese, Italy) before and 3.5 h after carrageenan injection. Percent reversal was calculated according to the following equation:

$$\%\ \text{Reversal} = [(\text{meandrug, post}) - (\text{meanvehicle, post})] \; [(\text{meanvehicle, baseline}) - (\text{meanvehicle, post})]^{-1} \times 100\%$$

For the antagonist experiments, two consecutive i.p. injections were administered 2.5 h post-carrageenan. The first injection was either vehicle (0.5% methylcellulose and 2% Tween) or 10 mg kg^{-1} S-AM1241 in vehicle; the second injection was either vehicle (1% DMSO, 0.5% methylcellulose and 2% Tween) or 1 mg kg^{-1} AM630 (Tocris Bioscience, Ellisville, MO, USA) in vehicle. A positive control group (indomethacin, Sigma, St Louis, MO, 10 mg kg^{-1}, per os) was included.

Statistical analysis of data
From the radioligand binding experiments, K_i values were determined using GraphPad Prism (GraphPad Software, San Diego, CA, USA). From the cAMP inhibition experiments, EC_{50} values were determined using GraphPad Prism (GraphPad Software, San Diego, CA, USA). For all *in vivo* pain studies, raw data were analysed by one-way ANOVA using a customized SAS-Excel application (SAS Institute, Cary, NC, USA). Significant ($P < 0.05$) main effects were analysed further *post hoc*, using least significant difference analysis.

Results

R,S-AM1241 binds to CB_2 receptors
The human, rat and mouse CB_2 receptors were expressed stably in CHO-K1 cells. Radioligand saturation binding

analysis using [^{3}H]-CP55,940 indicated that the levels of expression were comparable (7.8, 9.5 and 23.1 pmol mg^{-1}, respectively). In binding studies, the control compound WIN55,212-2 displaced [^{3}H]-CP55,940 from human, rat and mouse receptors with K_i values of 2.8±0.6, 129±36 and 209±34 nM, respectively (data not shown). This increased affinity for the human receptor was not reflected by the functional studies, in which WIN55,212-2 was nearly equipotent at all three receptors (see below). R,S-AM1241 displaced [^{3}H]-CP55,940 from all three CB_2 receptors with near-equal affinity (K_i values 20–30 nM) (Table 1, Figure 1).

Table 1 Affinity of R,S-AM1241 and its enantiomers, R-AM1241 and S-AM1241, for the human, rat and mouse CB_2 receptor and the human CB_1 receptor

Species of receptor	*R,S-AM1241* K_i *(nM)*	*R-AM1241* K_i *(nM)*	*S-AM1241* K_i *(nM)*
hCB_2	28.7±2.01	15.1±4.18	658±44.2
rCB_2	26.7±0.44	12.0±1.27	893±58.5
mCB_2	23.8±4.36	13.2±0.76	577±58.4
hCB_1	$>10\times10^3$	$5.0\times10^3\pm300$	$>10\times10^3$

Membranes prepared from CHO-K1 cells stably expressing human, rat or mouse CB_2 or human CB_1 receptors were incubated in the presence of drug and 1–3 nM [^{3}H]-CP55,940 for 1 h. The membranes were filtered onto Whatman GFB paper and radioactivity determined. K_i values were calculated using the Cheng–Prusoff equation. Data are expressed as mean±s.e.m. for three independent experiments.

To investigate the pharmacology of R,S-AM1241 further, we resolved its enantiomers. R-AM1241 had similar affinities at all three species of CB_2 receptors, although these affinities were approximately twofold greater for R-AM1241 than the racemate, as reflected by K_i values. S-AM1241 had a much lower affinity, with K_i values ranging from 600 to 900 nM. The K_i value of R-AM1241 for the hCB_1 receptor was approximately 5 μM, while the corresponding values for racemic AM1241 and S-AM1241 exceeded 10 μM (Table 1).

CB_2 receptor agonists decrease cAMP levels

For all CB_2 functional assays, 1 μM forskolin was used to stimulate cAMP production. The effects of the non-selective cannabinoid agonist WIN55,212-2 on forskolin stimulated cAMP accumulation are shown in Figure 2a. A robust response was seen in cells with the human receptors, with a maximal inhibition of approximately 80%. However, stimulation of the rat and mouse CB_2 receptor resulted in a smaller inhibition of cAMP formation (approximately 40%), despite the high level of expression in the murine cell line. The inverse agonist SR144528 (Rinaldi-Carmona *et al*., 1998), which increased forskolin-stimulated cAMP by 50–100% in cells expressing any of the three CB_2 receptors (Figure 2b, Table 2), provided evidence for constitutive activity of the CB_2 receptors, with the mouse CB_2 receptor displaying the greatest amount.

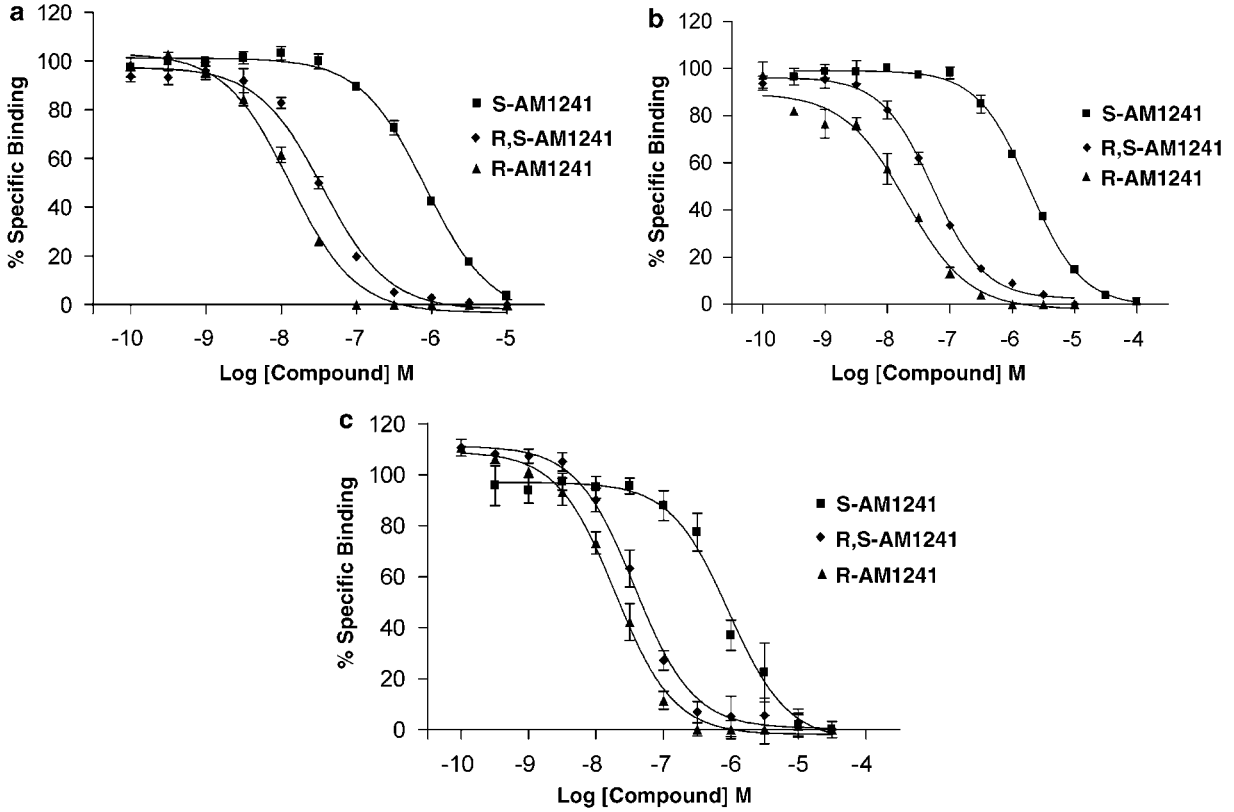

Figure 1 Radioligand displacement curves for R,S-AM1241, R-AM1241 and S-AM1241 at the human (**a**), rat (**b**) and mouse (**c**) CB_2 receptor. Membranes prepared from CHO-K1 cells expressing the human, rat or mouse CB_2 receptor were incubated in the presence of drug and 1–3 nM [^{3}H]-CP55,940 for 1 h. Membranes were filtered onto Whatman GFB paper and radioactivity determined. K_i values were calculated using the Cheng–Prusoff equation. Data are expressed as mean±s.e.m. for three independent experiments.

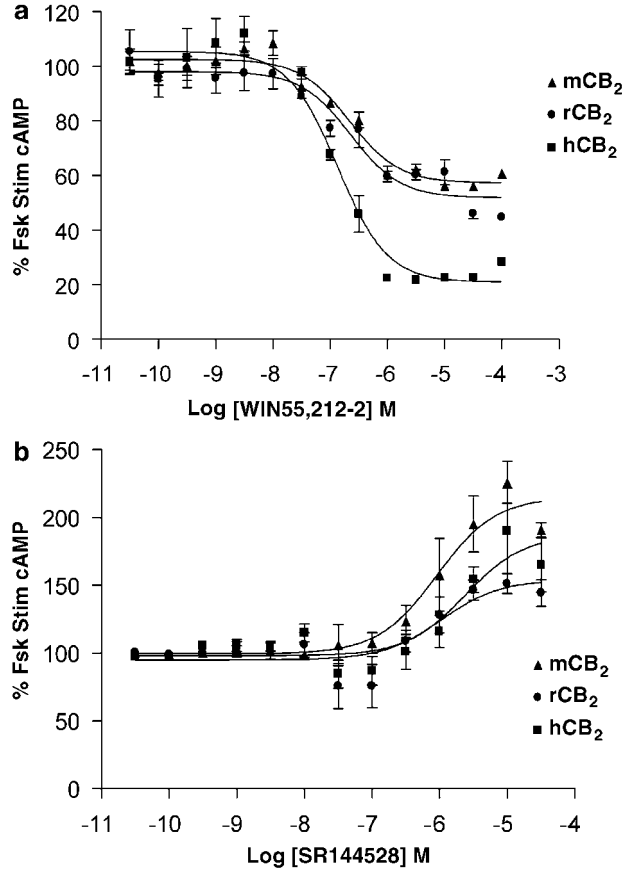

Figure 2 Effects of the non-selective cannabinoid agonist WIN55,212-2 (**a**) and the CB_2-selective antagonist/inverse agonist SR144528 (**b**) on cAMP levels in cells expressing the human, rat or mouse CB_2 receptor. Cells were stimulated in the presence of 1 μM forskolin for 30 min. Data are expressed as mean$\pm$s.e.m. from a representative dose–response curve.

R,S-AM1241 and its enantiomers display species-dependent in vitro *pharmacology*

At the human CB_2 receptor, R,S-AM1241 demonstrated partial agonist activity with a decrease of forskolin-stimulated cAMP by a maximum of 60% with an EC_{50} of 28 nM; in comparison, WIN55,212-2 produced a maximal inhibition of approximately 80%. Surprisingly, an opposite effect was observed when either rodent CB_2 receptor was stimulated. At these receptors, R,S-AM1241 acted as an inverse agonist, increasing forskolin-stimulated cAMP levels by 30–70% (Figure 3a). Interestingly, stereoisomer-specific pharmacology was observed at the rodent receptors. As seen with the racemate, R-AM1241 was an agonist at the human receptor and an inverse agonist at each of the rodent receptors. Similar to SR144528, R-AM1241 increased the levels of cAMP to a greater extent in the mouse cell line than the rat (Figure 3b). S-AM1241 was a potent (131 nM) agonist at the human receptor, but in contrast to the R-enantiomer, was also an agonist at the rodent receptors, albeit with lower potency than at the human receptor (Figure 3c, Table 3). The CB_2-specificity of the effects of R,S-AM1241 and its enantiomers was demonstrated by the absence of effects on forskolin-stimulated cAMP in parental CHO-K1 cells (data not shown). The effects of all three ligands in all three CB_2-expressing cells were sensitive to *Pertussis* toxin (data not shown), indicating that the observed inverse agonist effects of R,S-AM1241 and R-AM1241 were the result of G_i-coupled signalling and not the result of rodent CB_2 receptors signalling through an alternative G-protein in response to these ligands.

Table 2 Effects of a non-selective cannabinoid agonist (WIN55,212-2) and a CB_2-selective antagonist/inverse agonist on cAMP levels in cells expressing the human, rat or mouse CB_2 receptor

Species of receptor	*WIN 55,212-2 EC_{50} (nM)*	*SR144528 EC_{50} (nM)*
hCB_2	103$\pm$22.0	1720$\pm$879
rCB_2	234$\pm$29.4	1050$\pm$467
mCB_2	134$\pm$25.0	1240$\pm$758

Abbreviation: cAMP, cyclic adenosine monophosphate.
Cells were stimulated in the presence of 1 μM forskolin for 30 min. cAMP levels were determined using HitHunter. Data are expressed as mean$\pm$s.e.m. for three independent experiments.

R,S-AM1241 and its enantiomers are not analgesic

R,S-AM1241 and its separated enantiomers were tested for acute nociception in rats using the tail-flick and hot-plate assays (data not shown). I.p. administration of each of R,S-AM1241, R-AM1241 and S-AM1241 did not affect hot-plate or tail-flick latency at 30 or 90 min following administration of doses up to 10 mg kg^{-1}. In contrast, morphine (10 mg kg^{-1}, s.c.), a positive control in these assays, produced a significant increase in both the tail-flick and hot-plate latencies at both 30 and 90 min post-administration (data not shown).

S-AM1241 blocks visceral pain and thermal hyperalgesia associated with chemical irritants

R,S-AM1241 and its enantiomers, R-AM1241 and S-AM1241, were evaluated in a dose–response study in the PPQ model of acute visceral pain. R,S-AM1241 did not produce a statistically significant blockade of PPQ induced stretching at the doses tested. At the 10 mg kg^{-1} dose, R-AM1241 produced a small (17%) reversal, 30 min post-PPQ injection, while S-AM1241 produced a relatively greater (35%) reversal of stretching (Figure 4a).

In the rat carrageenan model of inflammatory pain, R,S-AM1241 produced a reversal of carrageenan-induced thermal hyperalgesia, but only at the two highest doses tested. R-AM1241 did not reverse thermal hyperalgesia at any dose tested. In contrast, S-AM1241 was more efficacious than the racemate, producing a reversal of thermal hyperalgesia at all doses (Figure 4b). Neither the racemate nor either of the enantiomers produced a significant change in carrageenan-induced paw oedema at any of the doses tested (data not shown).

The CB_2-selective antagonist AM630 was used to confirm the CB_2 specificity of the S-AM1241 anti-hyperalgesic effects in the carrageenan model (Figure 4c). S-AM1241 at a 10 mg kg^{-1} dose produced a complete reversal of carrageenan-induced thermal hyperalgesia, similar to that produced

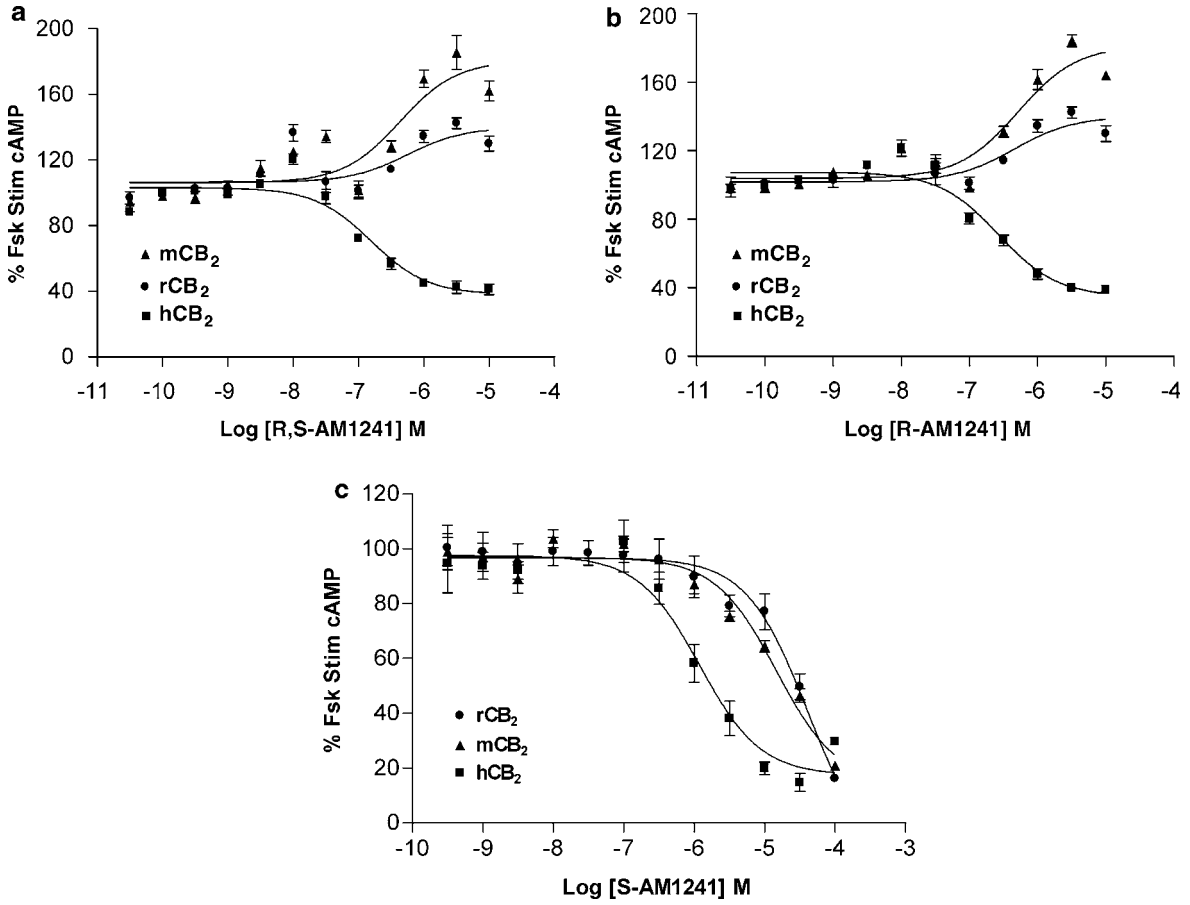

Figure 3 Effects of R,S-AM1241 (**a**) and its enantiomers, R-AM1241 (**b**) and S-AM1241 (**c**) on cAMP accumulation in CHO-K1 cells expressing the human, rat or mouse CB_2 receptor. Cells were stimulated in the presence of 1 μM forskolin for 30 min. Data are expressed as mean$\pm$s.e.m. for three independent experiments. cAMP, cyclic adenosine monophosphate.

Table 3 Effects of R,S-AM1241 and its enantiomers, R-AM1241 and S-AM1241, on cAMP levels in cells expressing the human, rat or mouse CB_2 receptor

Species of receptor	*R,S-AM1241 EC_{50} (nM)*	*R-AM1241 EC_{50} (nM)*	*S-AM1241 EC_{50} (nM)*
hCB_2	190$\pm$184 (agonist)	118$\pm$112 (agonist)	131$\pm$46.9 (agonist)
rCB_2	216$\pm$71.9 (inverse agonist)	315$\pm$180 (inverse agonist)	785$\pm$564 (agonist)
mCB_2	463$\pm$199 (inverse agonist)	341$\pm$94.4 (inverse agonist)	2000$\pm$475 (agonist)

Abbreviation: cAMP, cyclic adenosine monophosphate.
Cells were stimulated in the presence of 1 μM forskolin for 30 min, and cAMP levels were determined using the HitHunter assay. In parentheses, the agonist or inverse agonist designation is given. Data are expressed as mean$\pm$s.e.m. for three independent experiments.

by the positive control, treatment with indomethacin. This anti-hyperalgesic effect of S-AM1241 was blocked by the antagonist, AM630 at 1 mg kg^{-1}. The paw withdrawal latency resulting from co-administration of S-AM1241 and AM630 was not different from that resulting from administration of AM630 alone.

Discussion and conclusions

In this paper, we describe the *in vitro* and *in vivo* pharmacology of R,S-AM1241 and its resolved enantiomers, as summarized in Table 4. The affinity of R,S-AM1241 for the murine CB_2 receptor (28 nM) was lower than a previous report of 2 nM in mouse spleen membranes (Nackley *et al.*, 2003). This discrepancy may reflect differences in the G-protein coupling of the CB_2 receptors between native and heterologous expression systems, wherein any differences in stoichiometry of the receptor, G-proteins and other signalling molecules may be expected to affect agonist affinity. We were unable to distinguish between high- and low-affinity states, consistent with the report of a single K_i in mouse spleen (Nackley *et al.*, 2003).

Consistent with the coupling of CB_2 receptors to the inhibitory G-protein α-subunit G_i, stimulation of the receptor resulted in decreased cAMP levels following activa-

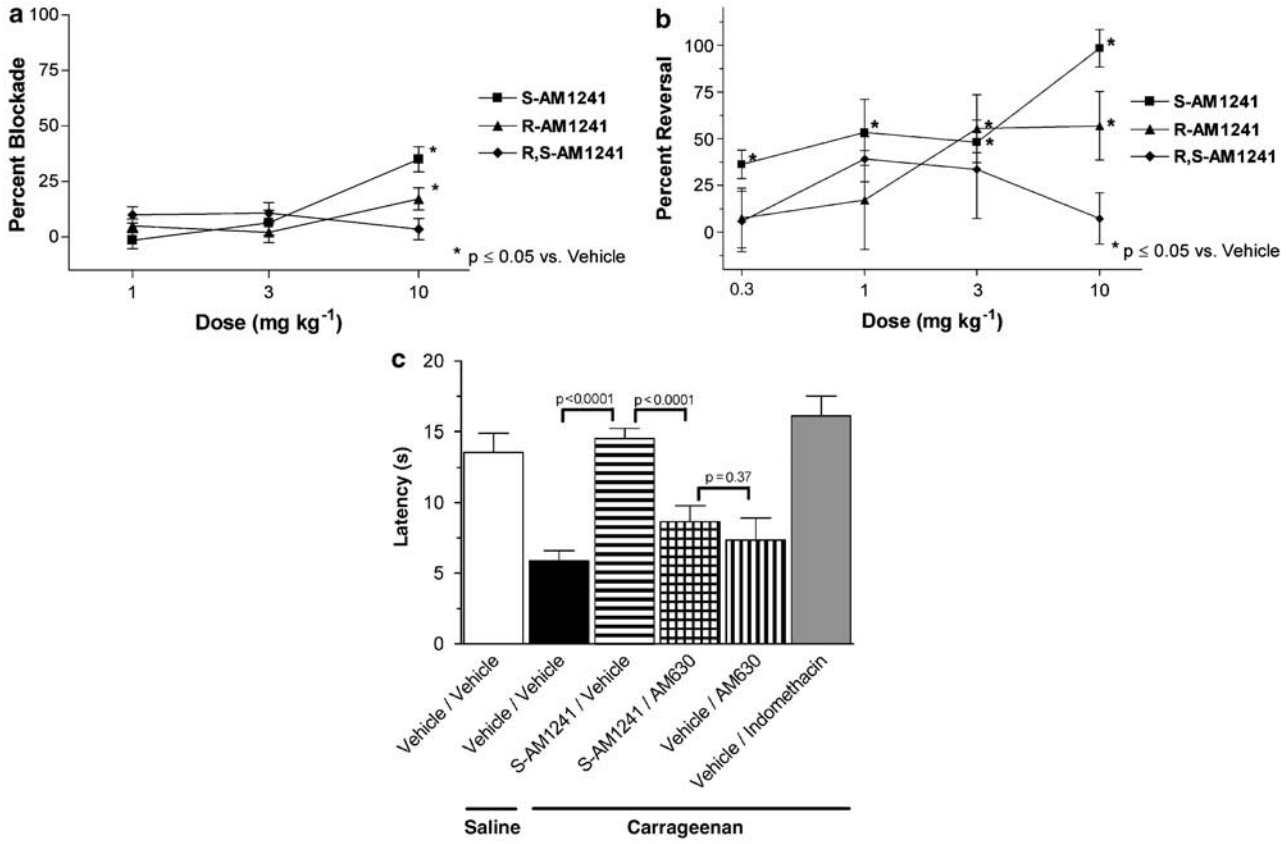

Figure 4 Effects of R,S-AM1241 and its enantiomers, R-AM1241 and S-AM1241, on visceral pain and thermal hyperalgesia associated with chemical irritants. (**a**) Male CD-1 mice (25–30 g; $n=10$ per group) were pretreated with vehicle or compound (s.c.) 30 min before PPQ administration and tested 10 min post-administration. Data (mean ± s.e.m.) are expressed as percent blockade relative to vehicle-treated mice. (**b**) Male SD rats (220–250 g; $n=8$ per group) received an intraplantar injection of 50 μl of saline or 2% carrageenan into the hindpaw, followed 2.5 h later by i.p. administration of vehicle, R,S-AM1241, R-AM1241 or S-AM1241. Data (mean ± s.e.m.) are expressed as percent reversal relative to vehicle-treated rats. (**c**) Male SD rats (220–250 g; $n=10$ per group) received an intraplantar injection of 50 μl of saline or 2% carrageenan into the hindpaw, followed 2.5 h later by i.p. administration of either vehicle or 10 mg kg^{-1} S-AM1241 and either vehicle or 1 mg kg^{-1} AM630. Data (mean ± s.e.m.) are expressed as paw withdrawal latencies; pre-carrageenan baseline data not shown. I.p., intraperitoneal; SD rat, Sprague–Dawley rat.

Table 4 Qualitative summary of *in vitro* and *in vivo* results for R,S-AM2141 and its enantiomers, R-AM2141 and S-AM1241

	In vitro *activity*			In vivo *efficacy*	
	hCB$_2$	*rCB$_2$*	*mCB$_2$*	*Carrageenan*	*PPQ*
R,S-AM1241	Agonist	Inverse agonist	Inverse agonist	+ +	−
R-AM1241	Agonist	Inverse agonist	Inverse agonist	−	+
S-AM1241	Agonist	Agonist	Agonist	+ + +	+ +

Abbreviation: PPQ, para-phenylquinone.
In vitro pharmacological characterizations are as measured in Table 3. Categories of *in vivo* efficacy: −, no statistically significant reversal of the chemically induced pain state; +, statistically significant reversal of the pain state; + +, statistically significant >30% reversal of the pain state; + + +, statistically significant >60% reversal of the pain state.

tion of adenylyl cyclases by forskolin. In agreement with previous data (Pertwee, 1997), the agonist WIN55,212-2 decreased cAMP formation by 80% in hCB$_2$-expressing cells. The reason for the more modest 40–50% decrease seen in both rodent CB$_2$ cell lines is not clear, but may be due to differences in coupling of the receptor to the G-protein complex. An increase in cAMP levels above those stimulated by forskolin was observed in response to the CB$_2$ antagonist SR144528, as would be expected based on this compound's characterization as an inverse agonist (Rhee and Kim, 2002). Inverse agonism is an operative term used to describe inhibition of basal coupling or constitutive activity of the ligand-unbound receptor. As shown by its higher maximal response to either SR144528 or R-AM1241, the cells with the mCB$_2$ receptors would appear to have a higher level of constitutive activity than those with the human or rat receptors, perhaps corresponding to a more effective coupling of this receptor to the cellular signal transduction machinery.

R,S-AM1241 inhibited cAMP production stimulated by treatment of the h CB$_2$-expressing cell line with 1 μM forskolin, consistent with this racemate acting as an agonist of hCB$_2$ receptors. The forskolin concentration used in our studies was lower than those used in a similar study (Yao *et al*., 2006), wherein it was reported that the function of R,S-AM1241 (partial agonist or neutral antagonist) in cyclase assays was sensitive to the concentration of forskolin (8 or 37 μM, respectively) used to stimulate hCB$_2$-expressing cells. In our characterization of the rodent receptors, R,S-AM1241

demonstrated inverse agonist properties at the same concentration of forskolin (1 μM) that was associated with agonist activity at the hCB_2 receptors. S-AM1241 was seen to be an agonist at human, mouse and rat CB_2 receptors, whereas R-AM1241 was observed to be an agonist at the human receptor and an inverse agonist in the cells with the rodent receptors. The functional properties of the racemate are dominated by those of the R-enantiomer, reflecting its more than 40-fold higher CB_2 affinity compared with the S-enantiomer.

In an analysis of racemic AM1241 in hCB_2 receptor assays (Yao *et al*., 2006), functional activity varied depending on the end point that was measured (antagonist of calcium mobilization; agonist of extracellular signal-regulated kinase activation; and antagonist or partial agonist of cAMP inhibition, depending on the level of stimulation). The authors proposed the diverse functional effects of R,S-AM1241 as a case of protean agonism (Yao *et al*., 2006), a phenomenon wherein the state of constitutive receptor activity can determine the functional effect of a ligand-receptor interaction (Kenakin, 2001). Under the protean agonist hypothesis, two receptor states, a ligand-bound and a constitutively active, ligand-unbound form, compete for G-proteins. If the efficacy of the constitutively active receptor is higher than that of the ligand-bound receptor, then the protean agonist, by inducing a less active receptor conformation, will appear as an inverse agonist. In the absence of constitutive activity, the same ligand will act as a partial agonist. Differing levels of receptor activation in different cell-based assay systems can thus suffice to produce varying functional outcomes. It is tempting, therefore, to speculate that the inverse agonist activity of R-AM1241 at the rodent CB_2 receptors, in contrast to its agonist activity at the human receptor, results from different levels of CB_2 constitutive activity between our rodent and human receptor expression systems, giving rise to a case of protean agonism. However, the observation that the human receptor displays higher basal activity than the rat receptor is at odds with this hypothesis and suggests that other, as yet undefined, mechanisms may be involved.

The *in vivo* efficacy of R,S-AM1241 and its enantiomers was assessed in rodent models of acute, inflammatory and visceral pain. Neither R,S-AM1241 nor either of its enantiomers showed evidence of acute nociception in either the tail-flick or hot-plate assay. This is the first report of the effects of the AM1241 enantiomers in an assay of acute nociceptive pain. Our results, although in contrast with an earlier report demonstrating analgesic effects of racemic AM1241 (Malan *et al*., 2001), are consistent with studies demonstrating that other CB_2 agonists are not analgesic *in vivo* (Valenzano *et al*., 2005; Whiteside *et al*., 2005).

S-AM1241 was efficacious in the mouse PPQ model, as was R-AM1241. However, the latter compound had only a modest antinociceptive effect, and the racemate had no statistically significant effect in this model. The lone previous report of *in vivo* efficacy of a resolved stereoisomer of AM1241 was an investigation of (+)-AM1241 (the R-enantiomer) in a mouse pain model that used intraplantar formalin injection (Beltramo *et al*., 2006). In light of our characterization of the resolved enantiomers, particularly the antinociceptive effects of S-AM1241, it would be of interest to compare the efficacy of both enantiomers in the formalin-induced pain model.

In the rat carrageenan model of inflammatory pain, S-AM1241, an agonist at rCB_2 receptors, was more efficacious than the racemate against thermal hyperalgesia, whereas R-AM1241, an inverse agonist, lacked statistically significant efficacy. The antihyperalgesic effect of S-AM1241 was blocked by the CB_2 antagonist AM630, demonstrating that the activity of S-AM1241 was mediated by CB_2 receptors. Additional off-target effects of S-AM1241 cannot be ruled out, but the magnitude of the AM630-induced blockade should be interpreted as evidence that any non-CB_2 components of this effect would be minor in comparison to the CB_2 component. Our results in the carrageenan model are consistent not only with previous reports of antinociceptive efficacy following administration of racemic AM1241, but also with reports of efficacy achieved with other CB_2 agonists in models of inflammatory pain (Clayton *et al*., 2002; Elmes *et al*., 2005; Valenzano *et al*., 2005).

Whereas the *in vivo* efficacy of S-AM1241 in rodent pain models is consistent with the *in vitro* functional characterization of this enantiomer as a rodent CB_2 agonist, the *in vivo* efficacy of R,S-AM1241 and R-AM1241 in the same rodent pain models appears to be inconsistent with their *in vitro* characterization as inverse agonists. In the absence of constitutive CB_2 receptor activity *in vivo*, the prediction following from the protean agonist hypothesis is that R-AM1241 would behave as a partial agonist. In that case, the efficacy of R-AM1241 in the mouse formalin (Beltramo *et al*., 2006) and PPQ models and the efficacy of the racemate in multiple pain models would be consistent with the *in vitro* characterization of these compounds. However, constitutive activation of receptors is an elusive property to measure *in vivo*. In one case in which this property has been deduced for CB_2 receptors, the *in vivo* efficacy of CB_2-selective inverse agonists in the inhibition of leucocyte trafficking (Lunn *et al*., 2006) provides evidence of the existence of constitutive CB_2 receptor activity in rodents. This condition, if it holds in our rodent pain models, would argue against any expectation of partial agonist properties of R-AM1241 *in vivo*.

It is noteworthy that our study is not the first reported example of a discrepancy between the *in vitro* characterization of cannabinoid ligands and their *in vivo* effects. Formalin-induced hyperalgesia in mice was shown to be exacerbated by each of two fatty acid-derived compounds whose *in vitro* properties indicate them to be CB_1 partial agonists (Cascio *et al*., 2006), an observation that is not consistent with the expectation of CB_1 receptor agonism being antihyperalgesic. Expectations about the effects of cannabinoid receptor inverse agonist compounds are further confused by reports of anti-inflammatory effects of CB_2 inverse agonists (Iwamura *et al*., 2001; Ueda *et al*., 2005). Without direct *in vivo* measurements of the basal state of CB_2 receptor activation, in particular, in cell types known to mediate the responses to exogenous CB_2 ligands, the behavioural studies we report herein can at the best be viewed as a characterization of R,S-AM1241 and its enantiomers, and not as a direct test of the protean agonist hypothesis.

In summary, we have reported for the first time an *in vitro* functional characterization of R,S-AM1241 in rodent CB_2 heterologous expression systems. In addition, we have provided the first *in vitro* and *in vivo* pharmacological assessment of this compound's resolved enantiomers. Despite the observation that S-AM1241, the enantiomer that displayed rodent CB_2 receptor agonist properties, was more efficacious than either R-AM1241 or the racemate in rodent pain models, a full understanding of the relevance of the species-dependent and stereoisomer-dependent pharmacology we present herein will require further characterization.

Acknowledgements

We thank Drs Alexandros Makriyannis, Adam M Gilbert and Kathryn E Rogers for beneficial discussion of this work.

Conflict of interest

The authors state no conflict of interest.

References

Baker D, Pryce G, Davies WL, Hiley CR (2006). *In silico* patent searching reveals a new cannabinoid receptor. *Trends Pharmacol Sci* **27**: 1–4.

Beltramo M, Bernardini N, Bertorelli R, Campanella M, Nicolussi E, Fredduzzi S *et al.* (2006). CB_2 receptor-mediated antihyperalgesia: possible direct involvement of neural mechanisms. *Eur J Neurosci* **26**: 1530–1538.

Brown SM, Wager-Miller J, Mackie K (2002). Cloning and molecular characterization of the rat CB_2 cannabinoid receptor. *Biochim Biophys Acta* **1576**: 255–264.

Cabral GA, Marciano-Cabral F (2005). Cannabinoid receptors in microglia of the central nervous system: immune functional relevance. *J Leukoc Biol* **78**: 1192–1197.

Cascio MG, Bisogno T, Palazzo E, Thomas A, van der Stelt M, Brizzi A *et al.* (2006). *In vitro* and *in vivo* pharmacology of synthetic olivetol- or resorcinol-derived cannabinoid receptor ligands. *Br J Pharmacol* **149**: 431–440.

Clayton N, Marshall FH, Bountra C, O'Shaughnessy CT (2002). CB_1 and CB_2 cannabinoid receptors are implicated in inflammatory pain. *Pain* **96**: 253–260.

D'Amour FE, Smith D (1941). A method for determining loss of pain sensation. *J Pharmacol Exp Ther* **72**: 74–79.

Elmes SJ, Winyard LA, Medhurst SJ, Clayton NM, Wilson AW, Kendall DA *et al.* (2005). Activation of CB_1 and CB_2 receptors attenuates the induction and maintenance of inflammatory pain in the rat. *Pain* **118**: 327–335.

Flygare J, Gustafsson K, Kimby E, Christensson B, Sander B (2005). Cannabinoid receptor ligands mediate growth inhibition and cell death in mantle cell lymphoma. *FEBS Letters* **579**: 6885–6889.

Fride E, Foox A, Rosenberg E, Faigenboom M, Cohen V, Barda L *et al.* (2003). Milk intake and survival in newborn cannabinoid CB_1 receptor knockout mice: evidence for a ''CB3'' receptor. *Eur J Pharmacol* **461**: 27–34.

Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR *et al.* (2003). *Gaussian* Revision B.03. Pittsburgh, PA: Gaussian, Inc.

Griffin G, Tao Q, Abood ME (2000). Cloning and pharmacological characterization of the rat CB(2) cannabinoid receptor. *J Pharmacol Exp Ther* **292**: 886–894.

Hargreaves K, Dubner R, Brown F, Flores C, Joris J (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**: 77–88.

Herrera B, Carracedo A, Diez-Zaera M, Guzman M, Velasco G (2005). p38 MAPK is involved in CB_2 receptor-induced apoptosis of human leukaemia cells. *FEBS Lett* **579**: 5084–5088.

Hohmann AG, Farthing JN, Zvonok AM, Makriyannis A (2004). Selective activation of cannabinoid CB_2 receptors suppresses hyperalgesia evoked by intradermal capsaicin. *J Pharmacol Exp Ther* **308**: 446–453.

Howlett AC, Breivogel CS, Childers SR, Deadwyler SA, Hampson RE, Porrino LJ (2004). Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacology* **47**: 345–358.

Ibrahim M, Porreca F, Lai J, Albrecht P, Rice F, Khodorova A *et al.* (2005). CB_2 cannabinoid activation produces antinociception by stimulating peripheral release of endogenous opioids. *Proc Natl Acad Sci USA* **102**: 3093–3098.

Ibrahim M, Rude M, Stagg N, Mata H, Lai J, Vanderah T *et al.* (2006). CB_2 cannabinoid receptor mediation of antinociception. *Pain* **122**: 36–42.

Ibrahim MM, Deng H, Zvonok A, Cockayne DA, Kwan J, Mata HP *et al.* (2003). Activation of CB_2 cannabinoid receptors by AM1241 inhibits experimental neuropathic pain: pain inhibition by receptors not present in the CNS. *Proc Natl Acad Sci USA* **100**: 10529–10533.

Iwamura H, Suzuki H, Ueda Y, Kaya T, Inaba T (2001). *In vitro* and *in vivo* pharmacological characterization of JTE-907, a novel selective ligand for cannabinoid CB_2 receptor. *J Pharmacol Exp Ther* **296**: 420–425.

Kenakin T (2001). Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J* **15**: 598–611.

Kim K, Moore DH, Makriyannis A, Abood ME (2006). AM1241, a cannabinoid CB_2 receptor selective compound, delays disease progression in a mouse model of amyotrophic lateral sclerosis. *Eur J Pharmacol* **542**: 100–105.

LaBuda CJ, Koblish M, Little PJ (2005). Cannabinoid CB_2 receptor agonist activity in the hindpaw incision model of postoperative pain. *Eur J Pharmacol* **527**: 172–174.

Lunn CA, Fine JS, Rojas-Triana A, Jackson JV, Fan X, Kung TT *et al.* (2006). A novel cannabinoid peripheral cannabinoid receptor-selective inverse agonist blocks leukocyte recruitment *in vivo*. *J Pharmacol Exp Ther* **316**: 780–788.

Makriyannis A (2002). University of Connecticut, Receptor selective cannabimimetic aminoalkylindoles. WO 02060447 A1.

Malan TP, Ibrahim MM, Deng H, Liu Q, Mata HP, Vanderah T *et al.* (2001). CB_2 cannabinoid receptor-mediated peripheral antinociception. *Pain* **93**: 239–245.

Munro S, Thomas KL, Abu-Shaar M (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature* **365**: 61–65.

Nackley A, Zvonok A, Makriyannis A, Hohmann A (2004). Activation of cannabinoid CB_2 receptors suppresses C-fiber responses and windup in spinal wide dynamic range neurons in the absense and presence of inflammation. *J Neurophysiol* **92**: 3562–3574.

Nackley AG, Makriyannis A, Hohmann AG (2003). Selective activation of cannabinoid CB(2) receptors suppresses spinal fos protein expression and pain behavior in a rat model of inflammation. *Neuroscience* **119**: 747–757.

Ofek O, Karsak M, Leclerc N, Fogel M, Frenkel B, Wright K *et al.* (2006). Peripheral cannabinoid receptor, CB_2, regulates bone mass. *Proc Natl Acad Sci USA* **103**: 696–701.

Pertwee RG (1997). Pharmacology of cannabinoid CB_1 and CB_2 receptors. *Pharmacol Ther* **74**: 129–180.

Quartilho A, Mata HP, Ibrahim MM, Vanderah TW, Porreca F, Makriyannis A *et al.* (2003). Inhibition of inflammatory hyperalgesia by activation of peripheral CB_2 cannabinoid receptors. *Anesthesiology* **99**: 955–960.

Rhee MH, Kim SK (2002). SR144528 as inverse agonist of CB_2 cannabinoid receptor. *J Vet Sci* **3**: 179–184.

Rinaldi-Carmona M, Barth F, Millan J, Derocq JM, Casellas P, Congy C *et al.* (1998). SR 144528, the first potent and selective antagonist of the CB_2 cannabinoid receptor. *J Pharmacol Exp Ther* **284**: 644–650.

Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Pessegue B, Bonnin-Cabanne O *et al*. (1996). Molecular cloning, expression and function of the murine CB_2 peripheral cannabinoid receptor. *Biochim Biophys Acta* **1307**: 132–136.

Shoemaker JL, Seely KA, Reed RL, Crow JP, Prather PL (2007). The CB_2 cannabinoid agonist AM-1241 prolongs survival in a transgenic mouse model of amyotrophic lateral sclerosis when initiated at symptom onset. *J Neurochem* **101**: 87–98.

Steffens S, Veillard NR, Arnaud C, Pelli G, Burger F, Staub C *et al*. (2005). Low dose oral cannabinoid therapy reduces progression of atherosclerosis in mice. *Nature* **434**: 782–786.

Ueda Y, Miyagawa N, Matsui T, Kaya T, Iwamura H (2005). Involvement of cannabinoid CB(2) receptor-mediated response and efficacy of cannabinoid CB(2) receptor inverse agonist, JTE-907, in cutaneous inflammation in mice. *Eur J Pharmacol* **520**: 164–171.

Valenzano KJ, Tafesse L, Lee G, Harrison JE, Boulet JM, Gottshall SL *et al*. (2005). Pharmacological and pharmacokinetic characterization of the cannabinoid receptor 2 agonist, GW405833, utilizing rodent models of acute and chronic pain, anxiety, ataxia and catelepsy. *Neuropharmacology* **48**: 658–672.

Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K *et al*. (2005). Indentification and functional characterization of brainstem cannabinoid CB_2 receptors. *Science* **310**: 329–332.

Whiteside GT, Gottshall SL, Boulet JM, Chaffer SM, Harrison JE, Pearson MS *et al*. (2005). A role for cannabinoid receptors, but not endogenous opioids, in the antinociceptive activity of the CB_2-selective agonist, GW405833. *Eur J Pharmacol* **528**: 65–72.

Woolfe G, MacDonald AD (1944). The evaluation of the analgesic action of pethidine. *J Pharmacol Exp Ther* **80**: 300–307.

Wotherspoon G, Fox A, McIntyre P, Colley S, Bevan S, Winter J (2005). Peripheral nerve injury induces cannabinoid receptor 2 protein expression in rat sensory neurons. *Neuroscience* **135**: 235–245.

Yao BB, Mukherjee S, Fan Y, Garrison TR, Daza AV, Grayson GK *et al*. (2006). *In vitro* pharmacological characterization of AM1241: a protean agonist at the cannabinoid CB(2) receptor? *Br J Pharmacol* **149**: 145–154.

Zimmermann M (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **16**: 109–110.