

## RESEARCH PAPER

# *In vitro* and *in vivo* characterization of A-796260: a selective cannabinoid CB<sub>2</sub> receptor agonist exhibiting analgesic activity in rodent pain models

BB Yao<sup>1</sup>, GC Hsieh<sup>1</sup>, JM Frost, Y Fan, TR Garrison, AV Daza, GK Grayson, CZ Zhu, M Pai, P Chandran, AK Salyers, EJ Wensink, P Honore, JP Sullivan, MJ Dart<sup>1</sup> and MD Meyer

Neurological Diseases Research, Global Pharmaceutical Research & Development, Abbott Laboratories, Abbott Park, IL, USA

**Background and purpose:** Selective cannabinoid CB<sub>2</sub> receptor agonists have demonstrated analgesic activity across multiple preclinical pain models. AM1241 is an indole derivative that exhibits high affinity and selectivity for the CB<sub>2</sub> binding site and broad spectrum analgesic activity in rodent models, but is not an antagonist of CB<sub>2</sub> *in vitro* functional assays. Additionally, its analgesic effects are  $\mu$ -opioid receptor-dependent. Herein, we describe the *in vitro* and *in vivo* pharmacological properties of A-796260, a novel CB<sub>2</sub> agonist.

**Experimental approach:** A-796260 was characterized in radioligand binding and *in vitro* functional assays at rat and human CB<sub>1</sub> and CB<sub>2</sub> receptors. The behavioural profile of A-796260 was assessed in models of inflammatory, post-operative, neuropathic, and osteoarthritic (OA) pain, as well as its effects on motor activity. The receptor specificity was confirmed using selective CB<sub>1</sub>, CB<sub>2</sub> and  $\mu$ -opioid receptor antagonists.

**Key results:** A-796260 exhibited high affinity and agonist efficacy at human and rat CB<sub>2</sub> receptors, and was selective for the CB<sub>2</sub> vs CB<sub>1</sub> subtype. Efficacy in models of inflammatory, post-operative, neuropathic and OA pain was demonstrated, and these activities were selectively blocked by CB<sub>2</sub>, but not CB<sub>1</sub> or  $\mu$ -opioid receptor-selective antagonists. Efficacy was achieved at doses that had no significant effects on motor activity.

**Conclusions and implications:** These results further confirm the therapeutic potential of CB<sub>2</sub> receptor-selective agonists for the treatment of pain. In addition, they demonstrate that A-796260 may be a useful new pharmacological compound for further studying CB<sub>2</sub> receptor pharmacology and for evaluating its role in the modulation of pain.

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**Abbreviations:** A-796260, [1-(2-morpholin-4-yl-ethyl)-1*H*-indol-3-yl]-(2,2,3,3-tetramethylcyclopropyl)-methanone; AM1241, (2-iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-1*H*-indol-3-yl]-methanone; CP 55,940, 5-(1,1-dimethylheptyl)-2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxy-propyl)-cyclohexyl]-phenol; GW-842166X, 2-(2,4-dichloro-phenyl-amino)-4-trifluoromethyl-pyrimidine-5-carboxylic acid (tetrahydro-pyran-4-ylmethyl)-amide; L-768,242 (GW405833), (2,3-dichloro-phenyl)-[5-methoxy-2-methyl-3-(2-morpholin-4-yl-ethyl)-indol-1-yl]-methanone; SR141716A (SR1), 5-(4-chloro-phenyl)-1-(2,4-dichloro-phenyl)-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; 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

## Introduction

Although the analgesic properties of cannabinoid ligands have been recognized for many years, the therapeutic potential of cannabis-based medicines is severely restricted

by the undesired psychotropic side-effects associated with this class of drugs (Pertwee, 2001; Fox and Bevan, 2005; Ibrahim *et al.*, 2006; Mackie, 2006; Whiteside *et al.*, 2007). The pharmacology of natural and synthetic cannabinoid ligands is derived from their interaction with two cannabinoid receptor subtypes, CB<sub>1</sub> and CB<sub>2</sub>. It is well established that the psychotropic effects of cannabis are derived largely, if not exclusively, by activity at the CB<sub>1</sub> subtype; and based on rodent studies, it is becoming apparent that the CB<sub>2</sub> receptor plays an important role in the mediation of pain

Correspondence: Dr MD Meyer, Neurological Diseases Research, Global Pharmaceutical Research & Development, Abbott Laboratories, R47W, AP9A-3, 100 Abbott Park Road, Abbott Park, IL 60064, USA.  
E-mail: mike.d.meyer@abbott.com

<sup>1</sup>These authors contributed equally to this work.

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processing (Malan *et al.*, 2002, 2003; Ibrahim *et al.*, 2005; Fox and Bevan, 2005). There is an emerging body of evidence to suggest that expression of the CB<sub>2</sub> receptor is upregulated as a consequence of tissue or nerve injury, supporting a potential role for CB<sub>2</sub>-selective ligands in the treatment of inflammatory, postoperative and neuropathic pain (Zhang *et al.*, 2003; Wotherspoon *et al.*, 2005; Romero-Sandoval and Eisenach, 2007).

Over the years, a number of CB<sub>2</sub>-selective ligands have emerged and have been shown to exhibit analgesic, antihyperalgesic and antiallodynic activity in various rodent pain models. JWH-133 and HU-308 are structurally related to  $\Delta^9$ -tetrahydrocannabinol, and display high radioligand-binding affinity and selectivity towards the human CB<sub>2</sub> receptor (Hanus *et al.*, 1999; Huffman *et al.*, 1999). JWH-133 has been characterized as a full agonist in a GTP $\gamma$ S-binding assay at the human CB<sub>2</sub> receptor (Huffman *et al.*, 2004), and HU-308 has been characterized as a selective full agonist in human CB<sub>2</sub> cyclic GMP assays (Hanus *et al.*, 1999). However, their activities at rodent CB<sub>1</sub> and CB<sub>2</sub> receptors have not been studied. In preclinical studies, JWH-133 has demonstrated efficacy in models of both inflammatory and neuropathic pain, and antagonist blockade studies have indicated that these activities are mediated through activation of the CB<sub>2</sub> subtype (Elmes *et al.*, 2004, 2005; Sagar *et al.*, 2005). HU-308 exhibited activity in a formalin-induced peripheral pain model (Hanus *et al.*, 1999) and a skin-incision model of postoperative pain (LaBuda *et al.*, 2005). In both these models, the effects of HU-308 were blocked by the CB<sub>2</sub> antagonist SR144528.

Several structurally related indole derivatives, including AM1241 and L-768,242 (GW405833) have been identified as CB<sub>2</sub>-selective ligands exhibiting activity in several rodent pain models. AM1241 has been found to exhibit high affinity at human, rat and mouse CB<sub>2</sub> receptors (Makriyannis and Deng, 2002; Ibrahim *et al.*, 2003a; Nackley *et al.*, 2003a; Yao *et al.*, 2006; Bingham *et al.*, 2007). However, the agonist efficacy profile of AM1241 (as the racemate) is complex and condition-dependent, ranging from inverse agonist to partial agonist at the human CB<sub>2</sub> receptor (Yao *et al.*, 2006; Bingham *et al.*, 2007) and is an apparent inverse agonist at the rat CB<sub>2</sub> receptor (Bingham *et al.*, 2007). Nevertheless, the behavioural profile of AM1241 across multiple pain models is consistent with CB<sub>2</sub> agonist activity. The efficacy of AM1241 in models of inflammatory (Malan *et al.*, 2001), neuropathic (Ibrahim *et al.*, 2003a) and postoperative pain (LaBuda *et al.*, 2005) has been shown to be mediated by the CB<sub>2</sub> receptor, as the effects are blocked by CB<sub>2</sub>- but not CB<sub>1</sub>-selective antagonists. Despite an apparent lack of affinity for the  $\mu$ -opioid receptor, the *in vivo* analgesic activity of AM1241 in a model of acute thermal pain is antagonized by both naloxone and  $\beta$ -endorphin antiserum, and AM1241 is inactive in the  $\mu$ -opioid receptor knockout mouse (Ibrahim *et al.*, 2005). L-768,242 is characterized by high affinity for human and rat CB<sub>2</sub> receptors and high selectivity vs the CB<sub>1</sub>-binding sites (Gallant *et al.*, 1996). Functionally, it displays partial agonist activity in human CB<sub>2</sub> receptor-mediated inhibition of forskolin-stimulated cAMP release assays (Valenzano *et al.*, 2005). Like AM1241, L-768,242 is effective in models of inflammatory, postoperative and neuropathic pain

(Valenzano *et al.*, 2005), but unlike AM1241, the effects of L-768,242 are not antagonized by a  $\mu$ -opioid receptor antagonist (Whiteside *et al.*, 2005).

Finally, GW-842166X, a pyrimidine derivative structurally distinct from other known CB<sub>2</sub> agonists, has been shown to exhibit agonist activity at human and rat CB<sub>2</sub> receptors in a yeast reporter gene assay, and is effective in a complete Freund's adjuvant (CFA) model of chronic inflammatory pain (Giblin *et al.*, 2007). This compound has been selected as a clinical candidate for the treatment of pain associated with osteoarthritis and rheumatoid arthritis.

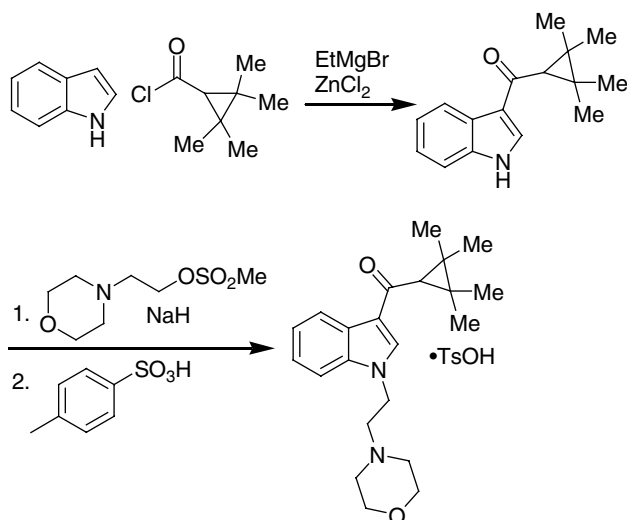
Despite supporting evidence provided by multiple CB<sub>2</sub>-selective ligands for the therapeutic potential of this pharmacological class as novel analgesic agents, inconsistencies exist with respect to both their *in vitro* and *in vivo* profiles. The compounds described above either fail to exhibit potent or robust agonist efficacy or have not been fully characterized in functional assays at rodent CB<sub>2</sub> receptors. Differences exist with respect to potential interactions with the opioid system, suggesting that different mechanisms may be contributing to the *in vivo* properties of these compounds. These shortcomings and inconsistencies highlight the need for additional compounds to help assess the potential that this pharmacological class may hold for the development of new drugs for the treatment of pain.

## Methods

### Compounds

Racemic AM1241, SR141716A and SR144528 were prepared at Abbott Laboratories as previously described (Yao *et al.*, 2006). CP 55,940, WIN 55,212-2 and AM630 were purchased from Tocris (Ellisville, MO, USA). L-768,242 was synthesized as described by Valenzano *et al.* (2005). GW-842166X was synthesized as described by Giblin *et al.* (2007). The paratoluenesulphonate salt of A-796260 was synthesized as depicted in the scheme and described herein. Briefly, acylation of indole with 2,2,3,3-tetramethylcyclopropane-carbonyl chloride afforded a 1.6:1 mixture of the desired C(3)- and undesired N-acylated regioisomers, which were separated by silica gel chromatography. Alkylation of the indole with the methanesulphonate ester of 2-morpholinoethanol gave the free base of A-796260 that was subsequently transformed into its toluenesulphonate salt, which was utilized in all biological experiments in the current study. Doses of A-796260 are presented as mg kg<sup>-1</sup> of the free base (mol wt = 354.50). Characterization of the free base and toluenesulphonate salt of A-796260 are as described below: Free base of A-796260, <sup>1</sup>H NMR (CD<sub>3</sub>OD, 300 MHz)  $\delta$  p.p.m. 1.33 (s, 12H), 2.13 (s, 1H), 2.46–2.54 (m, 4H), 2.79 (t, *J* = 6.4 Hz, 2H), 3.61–3.71 (m, 4H), 4.37 (t, *J* = 6.4 Hz, 2H), 7.16–7.30 (m, 2H), 7.45–7.53 (m, 1H), 8.11 (s, 1H), 8.20–8.30 (m, 1H), MS (DCI/NH<sub>3</sub>) *m/z* 355 (M + H)<sup>+</sup>; Tosylate salt of A-796260: [1-(2-morpholin-4-yl-ethyl)-1H-indol-3-yl]-(2,2,3,3-tetramethylcyclopropyl)-methanone *p*-toluenesulphonic acid, <sup>1</sup>H NMR (MeOH-d<sub>4</sub>, 300 MHz)  $\delta$  1.33 (s, 6H), 1.34 (s, 6H), 2.15 (s, 1H), 2.36 (s, 3H), 3.40 (m, 4H), 3.68 (dd, *J* = 7.1, 7.1 Hz, 2H), 3.90 (m, 4H), 4.73 (dd, *J* = 7.1, 7.1 Hz, 2H), 7.23 (br d, *J* = 7.8 Hz, 2H), 7.26 (ddd, *J* = 8.1, 8.1,

1.4 Hz, 1H), 7.33 (ddd,  $J=7.1, 7.1, 1.0$  Hz, 1H), 7.56 (br d,  $J=8.1$  Hz, 1H), 7.72 (br d,  $J=8.5$  Hz, 2H), 8.15 (s, 1H), 8.29 (dt,  $J=7.8, 1.0$  Hz, 1H); MS (DCI/NH<sub>3</sub>)  $m/z$  355 (M+H)<sup>+</sup>; anal (C<sub>22</sub>H<sub>30</sub>N<sub>2</sub>O<sub>2</sub>·C<sub>7</sub>H<sub>8</sub>O<sub>3</sub>S) C, H, N.



#### Cell culture

Reagents for cell culture were purchased from Invitrogen (Carlsbad, CA, USA) unless indicated otherwise. Human embryonic kidney (HEK; American Type Culture Collection, Rockville, MD, USA) cells stably expressing the human CB<sub>2</sub>, rat CB<sub>2</sub> or rat CB<sub>1</sub> receptor were grown in Dulbecco's modified Eagle's medium containing high glucose supplemented with 10% fetal bovine serum and 25 μg ml<sup>-1</sup> zeocin in a 37 °C incubator in the presence of 5% CO<sub>2</sub>. HEK cells stably co-expressing the chimeric G<sub>αq/o5</sub> protein with either the human or rat CB<sub>2</sub> receptor were grown under similar conditions, except that in addition to the components described above, the growth medium was further supplemented with 200 μg ml<sup>-1</sup> hygromycin. The Chinese hamster ovary cell lines stably expressing the human CB<sub>1</sub> receptor were purchased from Euroscreen (Brussels, Belgium), and the cells were grown under the conditions recommended by the vendor.

#### Radioligand-binding assays

Membrane samples prepared from HEK cells stably expressing the human CB<sub>2</sub>, rat CB<sub>2</sub> or rat CB<sub>1</sub> receptor, and Chinese hamster ovary cells stably expressing the human CB<sub>1</sub> receptor were used to perform radioligand-binding assays using [<sup>3</sup>H]-CP 55,940 ligand as previously described (Mukherjee *et al.*, 2004). Briefly, competition experiments were conducted using 0.5 nM [<sup>3</sup>H]-CP 55,940 in the presence of variable concentrations of test compounds in an assay buffer containing 50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 5 mM MgCl<sub>2</sub> and 0.05% fatty acid free BSA. After 90 min incubation at 30 °C, 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. Nonspecific binding was defined by 10 μM unlabelled CP 55,940. K<sub>i</sub> values from competition binding assays were determined with one-site competition curve fitting using the Prism software (GraphPad, San Diego, CA, USA).

#### Cyclase functional assays

The cyclase functional assays were performed using the HitHunter cAMP assay kit from DiscoverRx (Fremont, CA, USA) in suspension forms according to the manufacturer's protocol and as described previously (Yao *et al.*, 2006). Briefly, cell suspensions were incubated at 37 °C for 20 min with variable concentrations of test ligands or 10 μM CP 55,940 as a positive control in the presence of a fixed concentration of forskolin (18 μM for the rat CB<sub>2</sub> line; and 37 μM for human CB<sub>1</sub> and CB<sub>2</sub>, and rat CB<sub>1</sub> lines) in D-phosphate-buffered saline (PBS) buffer (Invitrogen) supplemented with BSA (0.01% final concentration). The reactions were terminated by the addition of lysis buffer and the luminescence was detected following the procedure according to the manufacturer's instructions. The positive control, CP 55,940 10 μM, produced significant inhibition of cAMP levels induced by forskolin in the cell lines expressing the human CB<sub>1</sub> (84% inhibition;  $n=10$ ), human CB<sub>2</sub> (71% inhibition;  $n=10$ ), rat CB<sub>1</sub> (90% inhibition;  $n=10$ ) and rat CB<sub>2</sub> receptors (63% inhibition;  $n=10$ ). Receptor activation by ligands is expressed as % response compared to that of 10 μM CP 55,940. EC<sub>50</sub> values were calculated by sigmoidal dose-response curve fitting using Prism (GraphPad) software.

#### Fluorescence imaging plate reader functional assays

Fluorescence imaging plate reader (FLIPR) assays were performed using HEK cells stably co-expressing the chimeric G<sub>αq/o5</sub> protein with either human or rat CB<sub>2</sub> receptors (Mukherjee *et al.*, 2004). Briefly, cells were seeded at 75 000 cells per well 1 day prior to the assay and the assays were performed with no-wash dye (FLIPR Calcium Assay Kit; Molecular Device, Sunnyvale, CA, USA) following the manufacturer's instructions. Variable concentrations of test compounds (0.3 nM to 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<sub>2</sub>, 5 mM KCl, 2 mM CaCl<sub>2</sub> and 0.05% BSA), and fluorescence responses were measured immediately with a FLIPR machine. Net peak responses were compared with that of 10 μM CP 55,940 and expressed as percentages of the CP 55,940-evoked response. EC<sub>50</sub> values were analysed with sigmoidal dose-response curve fitting using Prism.

#### Animals

Adult Sprague-Dawley rats (male, 250–300 g body weight; obtained from Charles River, Portage, MI, USA) were used for all experiments. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) approved facilities at Abbott Laboratories in a temperature-regulated environment under a controlled

12 h:12 h light–dark cycle, with lights on at 0600 h. Food and water were available *ad libitum* at all times except during testing. All testing was carried out following procedures outlined in protocols approved by Abbott's Institutional Animal Care and Use Committee.

#### *In vivo pain models*

**Chronic inflammatory pain model.** Chronic inflammatory thermal hyperalgesia was induced by injection of 150 µl of CFA solution (50%) in PBS into the plantar surface of the right hind paw of the rats. Thermal hyperalgesia was assessed 48 h post-CFA injection.

Thermal hyperalgesia was determined using a commercially available thermal paw stimulator (UARDG; University of California, San Diego, CA, USA) described by Hargreaves *et al.* (1988). Rats were placed in individual plastic cubicles mounted on a glass surface maintained at 30 °C, and allowed a 20 min habituation period. A thermal stimulus, in the form of radiant heat emitted from a focused projection bulb, was then applied to the plantar surface of each hind paw. The stimulus current was maintained at  $4.50 \pm 0.05$  A, and the maximum time of exposure was set at 20.48 s to limit possible tissue damage. The elapsed time until a brisk withdrawal of the hind paw from the thermal stimulus was recorded automatically using photodiode motion sensors. The right and left hind paws of each rat were tested in three sequential trials at approximately 5-min intervals. Paw withdrawal latency (PWL) was calculated as the mean of the two shortest latencies. PWL was measured 30 min post-A-796260 administration in both the CFA-treated and uninjected paws.

**Neuropathic pain model.** A model of chronic constriction injury (CCI)-induced neuropathic pain was produced by following the method of Bennett and Xie (1988). The right common sciatic nerve was isolated at mid-thigh level, and loosely ligated by four chromic gut (5-0) ties separated by an interval of 1 mm. All animals were left to recover for at least 2 weeks and no more than 5 weeks prior to testing tactile allodynia.

A-796260 was injected *i.p.* 30 min before the test for tactile allodynia. Tactile allodynia was measured using calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA) as previously described (Chaplan *et al.*, 1994). Rats were placed into inverted individual plastic containers (20 cm × 12.5 cm × 20 cm) on top of a suspended wire mesh grid, and acclimatized to the test chambers for 20 min. von Frey filaments were presented perpendicularly to the plantar surface of the selected hind paw, and then held in this position for approximately 8 s with enough force to cause a slight bend in the filament. Positive responses included an abrupt withdrawal of the hind paw from the stimulus, or flinching behaviour immediately following removal of the stimulus. A 50% withdrawal threshold was determined using an up–down procedure (Dixon, 1980). Only rats with a baseline threshold score of less than 5 g were used in this study, and animals demonstrating motor deficit were excluded. A 15 g threshold was used as the maximal possible

effect in this assay. Drug effects in this model were conducted in a randomized, blind manner.

**Postoperative pain model.** A model of postoperative pain was performed as described by Brennan *et al.* (1996). The plantar aspect of the rat left hind paw was exposed through a hole in a sterile plastic drape, and a 1 cm longitudinal incision was made through the skin and fascia, starting 0.5 cm from the proximal edge of the heel and extending towards the toes. The plantaris muscle was elevated and incised longitudinally, leaving the muscle origin and insertion points intact. After homoeostasis by application of gentle pressure, the skin was apposed with two mattress sutures using 5-0 nylon. Animals were then allowed to recover for 2 h after surgery, at which time tactile allodynia was assessed as described above. Drug effects in this model were conducted in a randomized, blind manner.

**Knee joint osteoarthritic pain model.** Unilateral knee joint osteoarthritis was induced in the rats by a single intra-articular (*i.a.*) injection of sodium monoiodoacetate (MIA) (Sigma-Aldrich, St Louis, MO, USA) (3 mg in 0.05 ml sterile isotonic saline) into the joint cavity under light (1–3%) isoflurane anaesthesia using a 26 G needle (Guzman *et al.*, 2003; Pomonis *et al.*, 2005). Following the injection, the animals were allowed to recover from anaesthesia (usually 5–10 min) before being returned to their home cages. To maintain uniformity across the study, the right knee joint of each animal was injected with MIA. Hindlimb grip force assessment was carried out 20 days following MIA injection as discussed below. The dose of the MIA (3 mg per *i.a.* injection) was selected based on results obtained from preliminary studies wherein optimal pain behaviour was observed at this dose.

Pain behavioural assessment of hindlimb grip force following unilateral injection of MIA was used as a behavioural measure of activity-induced pain in adult osteoarthritic (OA) rats (body weight 20 days following MIA injection: 325–350 g). Measurements of peak hindlimb grip force were conducted by recording the maximum compressive force exerted on the hindlimb strain gauge set up, in a commercially available grip force measurement system (Columbus Instruments, Columbus, OH, USA). During testing, each rat was gently restrained by grasping it around its rib cage and then allowed to grasp the wire mesh frame attached to the strain gauge. The experimenter then moved the animal in a rostral-to-caudal direction until the grip was broken. Each rat was sequentially tested twice at approximately 2–3 min interval to obtain a raw mean grip force (CF<sub>max</sub>). This raw mean grip force data were converted to a maximum hindlimb cumulative compressive force (CF<sub>max</sub>) (g force) per kg body weight for each animal. For assessing the effects of A-796260, the evaluation of hindlimb grip force was conducted 20 days following the *i.a.* injection of MIA. A group of age-matched naive animals were added to each experiment and the data obtained from the different dose groups for the compound being tested were compared to the naive group. Experiments were conducted in a

randomized, blind manner, and drug effects are expressed as raw mean grip force (CF<sub>max</sub>)/body weight (kg).

**Locomotor activity.** To determine potential CNS side-effects of A-796260, spontaneous exploratory behaviour was examined in naive rats (30 min post-compound injection). Rats were individually placed into test chambers, and horizontal (locomotion) activity was recorded by a photo beam detector system for 30 min (AccuScan Instruments, Columbus, OH, USA).

**Data analysis.** Statistical analyses were carried out using Graph Pad Prism (version 4.03; Graph Pad Software Inc., San Diego, CA, USA). The results are presented as mean ± s.e.mean. Statistical significance between group means was measured by one-way ANOVA, followed by Bonferroni *post hoc* analysis. In all cases,  $P < 0.05$  was assumed as the level for statistical significance. ED<sub>50</sub> values were also calculated by linear regression analysis and presented with CI 95% (Graph Pad).

## Results

### *Ligand affinities and selectivity at CB<sub>2</sub> receptors in radioligand-binding assays*

The receptor-binding potencies of A-796260, WIN 55,212-2, CP 55,940, AM1241, SR144528, SR141716A, GW-842166X and L-768,242 (GW405833) were determined in [<sup>3</sup>H]-CP 55,940 competition binding assays performed with membrane preparations generated from recombinant cell lines expressing the human CB<sub>1</sub>, human CB<sub>2</sub>, rat CB<sub>1</sub> or rat CB<sub>2</sub> receptor, respectively. The results are summarized in Table 1. A-796260 exhibited high binding potencies at both human and rat CB<sub>2</sub> receptors with  $K_i$  values of 4.37 and 13.0 nM, respectively. Selectivity for the CB<sub>2</sub>- over the CB<sub>1</sub>-binding site was 193-fold in human and 30-fold in rat samples. This is comparable to the potencies and selectivity exhibited by AM1241. GW-842166X did not demonstrate detectable [<sup>3</sup>H]-CP 55,940 displacement at either human or rat CB<sub>1</sub> (up to 10 μM) and had weak affinities at the human and rat CB<sub>2</sub> receptors (2000 and 2580 nM, respectively) in the current assays, whereas L-768,242 showed good binding affinity at the human and rat CB<sub>2</sub> receptors. The binding potencies of the non-selective agonists WIN 55,212-2 and CP 55,940, CB<sub>1</sub>-selective antagonist SR141716A and CB<sub>2</sub>-selective antagonist SR144528 displayed binding properties similar to those published previously (Howlett *et al.*, 2002).

In a recent publication (Ibrahim *et al.*, 2005), AM1241 has been shown to stimulate β-endorphin release via a μ-opioid receptor-dependent pathway. To investigate if CB<sub>2</sub>-selective ligands directly interact with the μ-opioid receptor, [<sup>3</sup>H]-DAMGO-binding assays were performed according to the method described by Spetea *et al.* (1998). Neither A-796260, AM1241 nor SR144528 exhibited affinity at the μ-opioid receptor, up to ligand concentrations of 10 μM, in this assay, whereas μ-opioid receptor ligand naloxone had a  $K_i$  value of 17 nM in these experiments.

### *In vitro ligand characterization in cyclase and FLIPR functional assays*

To assess agonist properties of these ligands, receptor-coupled adenylyl cyclase activity and calcium influx response were measured, respectively, in cyclase and FLIPR functional assays, employing cell lines that express the recombinant cannabinoid receptors. In cyclase assays, A-796260 behaved as a potent and efficacious agonist at human and rat CB<sub>2</sub> receptors with EC<sub>50</sub> values of 0.710 nM ( $E_{max}$  of 78%) and 1.63 nM ( $E_{max}$  of 69%), respectively (Table 2). A-796260 is highly selective at CB<sub>2</sub> receptors, exhibiting 1380- and 175-fold selectivity at human and rat CB<sub>2</sub> receptors compared to CB<sub>1</sub> receptors of the respective species. In contrast, AM1241 did not exhibit either agonist or inverse agonist activity at human and rat CB<sub>2</sub> receptors in these assays, but it was shown to produce a full agonist efficacy at both human and rat CB<sub>1</sub> receptors at high concentrations (human CB<sub>1</sub> EC<sub>50</sub> = 2650 nM,  $E_{max}$  = 101%; and rat CB<sub>1</sub> EC<sub>50</sub> = 1970 nM,  $E_{max}$  = 81%). L-768,242 was a potent inverse agonist at both human (EC<sub>50</sub> = 44.4 nM) and rat (EC<sub>50</sub> = 3.26 nM) CB<sub>2</sub> receptors, producing maximum (-30% (human) and -39% (rat)) negative efficacy relative to that of CP 55,940. In contrast, it was a weak, but efficacious, agonist at the rat CB<sub>1</sub> receptor. GW-842166X was inactive at human and weakly efficacious at rat CB<sub>1</sub> receptors; however, it exhibited full agonist activity at human and rat CB<sub>2</sub> receptors. Reference standards, WIN 55,212-2, CP 55,940, SR144528 and SR141716A, exhibited properties similar to previously published results (Felder *et al.*, 1995; Bonhaus *et al.*, 1998; Tao and Abood, 1998; Lunn *et al.*, 2006).

In FLIPR assays, where a chimeric G<sub>αq/o</sub> protein was employed to facilitate redirection of the G<sub>αi/o</sub> signalling to intracellular calcium release responses (Yao *et al.*, 2006), receptor activation was readily measured by the use of a FLIPR machine. To characterize A-796260 and other cannabinoid receptor ligands further, FLIPR functional assays were performed. A-796260 displayed high potency and full efficacy at the human CB<sub>2</sub> receptor. In contrast, AM1241 and L-768,242 failed to exhibit agonist efficacy, and GW-842166X exhibited weak agonist potency with an EC<sub>50</sub> of 7780 nM and  $E_{max}$  of 84%. Non-selective agonists, WIN 55,212-2 and CP 55,940, exhibited high potencies and full efficacy, whereas SR144528 and SR141716A were inactive. WIN 55,212-2, a partial agonist at the rat CB<sub>2</sub> receptor in cyclase assays, was inactive in this assay. Similar to the result with the human CB<sub>2</sub> receptor, AM1241 was also inactive at the rat CB<sub>2</sub> receptor in FLIPR assays (Figure 1).

### *Effects of A-796260 on CFA-induced chronic inflammatory thermal hyperalgesia*

Two days following CFA injection into the hind paw, a significant decrease in PWL to thermal stimulation was observed (PWL uninflamed: 10.7 ± 0.3 s vs PWL inflamed: 5.3 ± 0.1 s,  $P < 0.01$ ), demonstrating the development of thermal hyperalgesia (Figure 2a). A-796260 dose-dependently attenuated CFA-induced thermal hyperalgesia (Figure 2a;  $F_{7,88} = 68.2$ ,  $P < 0.0001$ , ED<sub>50</sub> = 2.8 mg kg<sup>-1</sup> i.p., CI 95% = 2.1–3.4 mg kg<sup>-1</sup>), demonstrating full efficacy

**Table 1** Radioligand competition binding assays using [<sup>3</sup>H]CP 55,940

Ligands	Mean K <sub>i</sub> , nM (n)					
	95% CI of K <sub>i</sub> values, nM					
	hCB <sub>1</sub>	hCB <sub>2</sub>	hCB <sub>2</sub> /hCB <sub>1</sub> selectivity	rCB <sub>1</sub>	rCB <sub>2</sub>	rCB <sub>2</sub> /rCB <sub>1</sub> selectivity
A-796260	845 (13) 410–1740	4.37 (14) 1.87–10.2	193	395 (5) 173–899	13.0 (5) 6.35–26.6	30
WIN 55,212-2	13.3 (27) 7.5–23.6	1.30 (44) 0.971–1.74	10	4.47 (11) 3.02–6.65	1.64 (19) 0.871–3.11	2.7
CP 55,940	2.11 (71) 1.62–2.77	1.08 (99) 0.925–1.27	2.0	1.54 (29) 1.15–2.07	0.987 (56) 0.764–1.28	1.6
AM1241	1270 (10) 354–4545	11.5 (21) 6.99–18.8	110	115 (4) 7.75–1710	3.37 (15) 1.24–9.15	34
SR144528	264 (12) 175–398	5.66 (17) 3.86–8.31	47	428 (6) 159–1150	1.80 (7) 0.477–6.81	238
SR141716A	2.36 (29) 1.41–3.95	429 (13) 270–682	182 × CB <sub>1</sub> selective	2.80 (23) 1.94–4.03	127 (4) 35.4–452	45 × CB <sub>1</sub> selective
GW-842166X	>10 000 (2)	2000 (7) 561–7140	NA	>10 000 (2)	2580 (8) 993–6700	NA
L-768,242 (GW405833)	282 (2) 1370–5830	7.62 (4) 2.23–26.1	37	ND	11.1 (4) 4.74–25.9	NA

Abbreviation: NA, not available.

**Table 2** Cyclase and FLIPR functional assays

Ligands	Mean agonist EC <sub>50</sub> , nM (n), efficacy % relative to CP 55,940 (%)					
	95% CI of EC <sub>50</sub> values, nM					
	Cyclase assays				FLIPR assays	
	hCB <sub>1</sub>	hCB <sub>2</sub>	rCB <sub>1</sub>	rCB <sub>2</sub>	hCB <sub>2</sub>	rCB <sub>2</sub>
A-796260	983 (7), 118 618–1560	0.710 (6), 78 0.315–1.60	286 (8), 97 206–397	1.63 (7), 69 0.505–5.28	17.3 (8), 71 10.64–28.2	31.0 (4), 39 7.87–125
WIN 55,212-2	47.2 (5), 113 23.5–94.9	0.650 (5), 98 0.156–2.72	16.5 (5), 114 3.74–72.5	1.16 (6), 46 0.367–3.65	119 (10), 74 57.94–245	>10 000 (13)
CP 55,940	1.81 (12), 103 0.777–4.21	0.864 (41), 106 0.626–1.19	4.12 (17), 103 2.51–6.76	0.682 (42), 99 0.444–1.05	31.3 (212), 99 28.3–34.5	13.0 (29), 106 9.44–17.8
AM1241	2650 (5), 101 1730–4050	>1000 (9)	1970 (6), 81 842–4610	>1000 (8)	>10 000 (18)	>10 000 (9)
SR144528	9420 (4), 52 4340–20 400	73.4 (20), –106 48.4–111	5960 (3), 39 1780–20 000	34.3 (17), –75 24.9–47.3	>10 000 (12)	>10 000 (6)
SR141716A	21.3 (4), –67 5.73–79.1	>3000 (4)	31.6 (7), –61 16.2–61.8	>3000 (2)	>10 000 (3)	>10 000 (6)
GW-842166X	>25 000 (2)	133 (4), 101 94–187	26 000 (4), 33 24 200–27 900	96.2 (6), 95 65.5–141	7780 (5), 84 2700–22 400	ND
L-768,242 (GW405833)	ND	44.4 (2), –30 22.4–88.1	916 (2), 70 839–1000	3.26 (4), –39 1.09–9.78	>10 000 (4)	ND

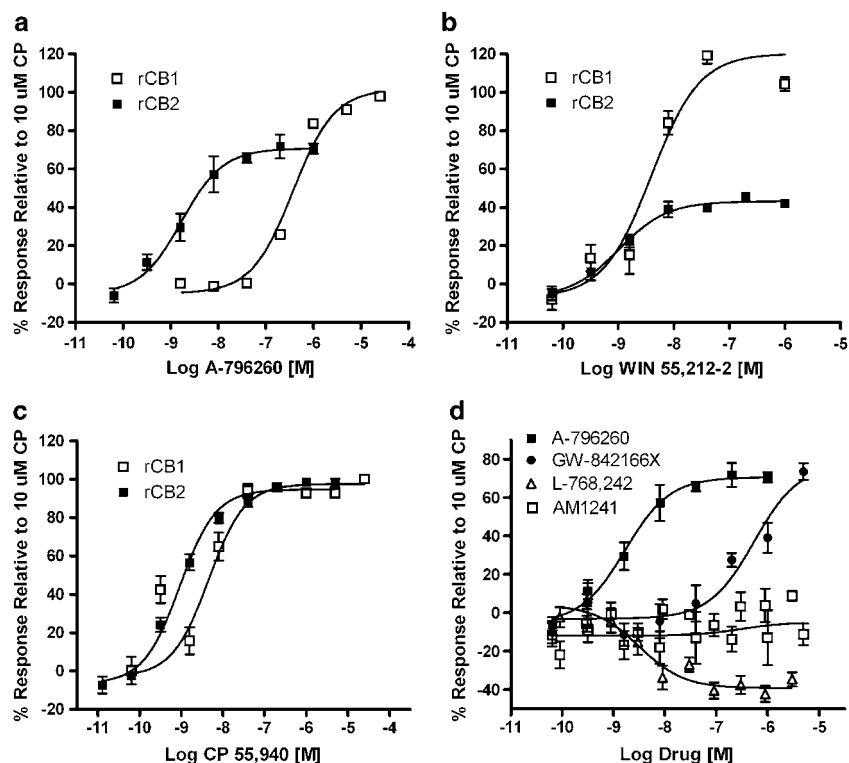
Abbreviation: ND, not determined.

(94 ± 7%) at 11 mg kg<sup>-1</sup>. No effect was observed in the uninflamed hind paw.

Using this *in vivo* inflammatory pain model, the receptor specificity was investigated using receptor-selective antagonists (Figure 2b). Pretreatment with SR144528 (4.8 mg kg<sup>-1</sup> i.p.), a CB<sub>2</sub> receptor-selective antagonist (Rinaldi-Carmona *et al.*, 1998), significantly reversed the analgesic efficacy induced by treatment with A-796260 at 11 mg kg<sup>-1</sup> i.p. (F<sub>7,40</sub> = 112.2, P < 0.0001). AM630, another CB<sub>2</sub> receptor-selective antagonist (Hosohata *et al.*, 1997; Ross *et al.*, 1999), when pre-administered at 3 mg kg<sup>-1</sup> i.p., also significantly blocked the antihyperalgesic activity induced by A-796260 (F<sub>7,38</sub> = 77.7, P < 0.0001). In contrast, pretreatment with SR141716A (14 mg kg<sup>-1</sup> i.p.), a selective CB<sub>1</sub> receptor

antagonist (Rinaldi-Carmona *et al.*, 1994), had no effect on the analgesia induced by A-796260 (F<sub>7,40</sub> = 13.2, P < 0.0001). The rats treated with CB<sub>2</sub> receptor antagonists alone at the doses used in the present studies did not exhibit any change in PWL as compared with the vehicle-treated animals.

Release of endogenous opioids has been proposed as a mechanism of action for the analgesic activities of CB<sub>2</sub> agonists, and the effects of the CB<sub>2</sub> ligand AM1241 have been shown to be dependent on β-endorphin release and require a functional μ-opioid receptor (Ibrahim *et al.*, 2005). To investigate whether the analgesic effects of A-796260 are mediated via μ-opioid receptor-dependent activity, CFA-treated rats were injected with the opioid receptor antagonist naloxone (10 mg kg<sup>-1</sup> i.p.) prior to the administration of



**Figure 1** Characterization of cannabinoid ligands in recombinant rat CB<sub>1</sub> and CB<sub>2</sub> cyclase assay systems. A-796260 (a), WIN 55,212-2 (b) and CP 55,940 (c) were tested in rat CB<sub>1</sub> and CB<sub>2</sub> cyclase assays. Levels of receptor activation are calculated as the reduction of cAMP levels and are expressed as a percentage relative to the response of 10  $\mu$ M CP 55,940. Typically, 10  $\mu$ M CP 55,940 inhibits forskolin-induced cAMP levels in rat CB<sub>1</sub> and CB<sub>2</sub> cyclase assays by 90 and 63%, respectively; these responses were normalized to 100% activation of receptor activity. (d) The effects of A-796260, AM1241, GW-842166X and L-768,242 (GW405833) at the rat CB<sub>2</sub> receptor.

A-796260 (11 mg kg<sup>-1</sup> i.p.). Naloxone pretreatment had no effect on the antihyperalgesic activity of A-796260 (Figure 2c;  $F_{7,52} = 14.1$ ,  $P < 0.0001$ ). However, under similar conditions, pretreatment with naloxone significantly reversed the analgesic effects induced by AM1241 at 15 mg kg<sup>-1</sup> i.p. (Figure 2c;  $F_{7,52} = 51.5$ ,  $P < 0.0001$ ).

#### Effects of A-796260 in the skin incision model of acute postoperative pain

Two hours following skin incision surgery, tactile allodynia was observed on the injured paw ( $PWT_{\text{vonfrey}} = 1.91 \pm 0.17$  g). Skin incision did not affect tactile PWT in the uninjured paw. A-796260, administered i.p. 1.5 h post-surgery, dose-dependently attenuated tactile allodynia (Figure 3a;  $F_{4,48} = 39.8$ ,  $P < 0.0001$ ,  $ED_{50} = 18$  mg kg<sup>-1</sup>, CI 95% = 13–25 mg kg<sup>-1</sup>), with a maximal efficacy of  $68 \pm 7\%$  ( $P < 0.01$  vs vehicle) at the highest dose tested (Figure 3a). Intraperitoneal administration of morphine (6 mg kg<sup>-1</sup>) also produced a statistically significant reversal of allodynia 30 min post-dosing.

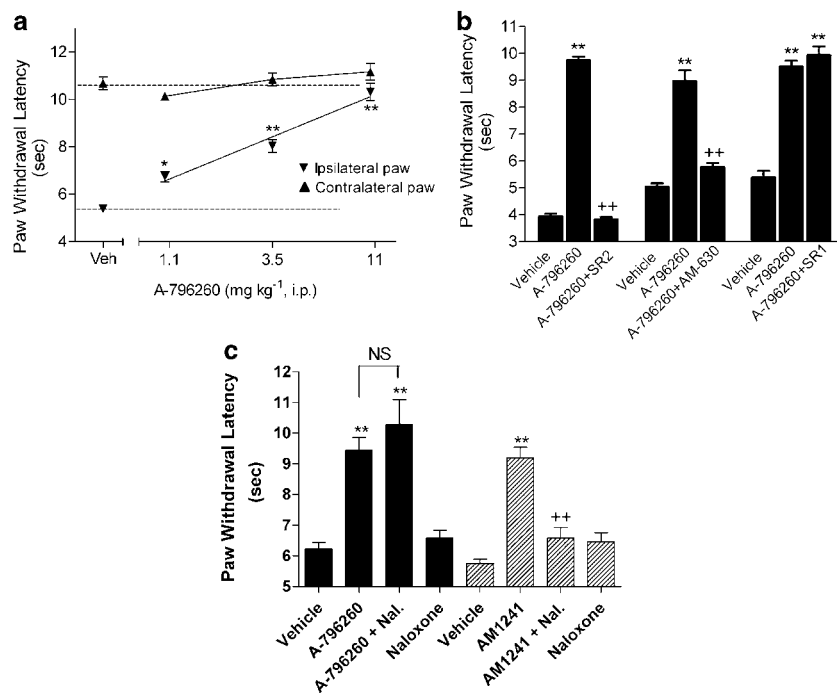
A separate study was conducted to determine whether the analgesic effect of A-796260 was maintained after repeat administration (twice daily for 5 days). The results, shown in Figure 3b, indicate that tolerance to the analgesic effect of A-796260, 35 mg kg<sup>-1</sup> i.p., did not develop following repeated administration for 5 days ( $64 \pm 4\%$ ,  $P < 0.01$  vs vehicle for acute dosing vs  $60 \pm 10\%$ ,  $P < 0.01$  vs vehicle for repeated dosing). The plasma levels of A-796260 were measured after

the behavioural testing, and no difference was seen between the two treatment groups (data not shown). In contrast to the results with A-796260, the analgesic effects of morphine were almost completely abolished after the repeated administration with the same dosing regime (Figure 3b;  $F_{5,41} = 29.4$ ,  $P < 0.0001$ ).

To examine whether the analgesic effects of A-796260 in the skin incision model of postoperative pain are mediated via CB<sub>2</sub> receptors, studies were conducted in conscious rats treated with A-796260, 35 mg kg<sup>-1</sup> i.p., before and after pretreatment with the CB<sub>2</sub> antagonist SR144528. A-796260 produced  $75 \pm 11\%$  analgesia ( $P < 0.01$  vs vehicle) when given alone, whereas the same dose of A-796260 had no significant effect ( $26 \pm 6\%$ ) in rats pretreated with SR144528 4.8 mg kg<sup>-1</sup> i.p. (Figure 3c;  $F_{3,20} = 29.9$ ,  $P < 0.0001$ ).

#### Effects of A-796260 on tactile allodynia in a neuropathic pain model

In rats, CCI of the sciatic nerve (Bennett and Xie, 1988) produced a decrease in PWT to mechanical stimulation 2 weeks following surgery ( $PWT_{\text{vonfrey}} = 3.7 \pm 0.4$  g), demonstrating the development of tactile allodynia. A-796260 treatment attenuated CCI-induced tactile allodynia in a dose-related manner with an  $ED_{50}$  of 15 mg kg<sup>-1</sup> i.p. (CI 95% = 5.3–26 mg kg<sup>-1</sup>) and an efficacy of  $66 \pm 9\%$  ( $P < 0.01$  vs vehicle) at the highest dose tested (Figure 4;  $F_{4,54} = 18.0$ ,  $P < 0.0001$ ). Under the same conditions, morphine



**Figure 2** Effects of A-796260 (i.p.) on complete Freund's adjuvant (CFA)-induced chronic inflammatory thermal hyperalgesia. (a) At 48 h following CFA injection, A-796260 was injected 30 min before testing. Data represent mean  $\pm$  s.e.mean. \* $P < 0.05$ , \*\* $P < 0.01$  as compared to vehicle-treated rats ( $n = 12$  per group). (b) Reversal of A-796260-evoked analgesia by pretreatment with CB<sub>2</sub>-selective antagonists SR144528 or AM630 (SR2, 4.8 mg kg<sup>-1</sup> i.p.; AM630, 3.0 mg kg<sup>-1</sup> i.p.), but the CB<sub>1</sub>-selective antagonist SR141716A (SR1, 14 mg kg<sup>-1</sup> i.p.) had no effect. The antagonists were administered 15 min before A-796260 injection. (c) Effects of the opioid antagonist naloxone (Nal) on the analgesic activity induced by A-796260 or AM1241. Naloxone (10 mg kg<sup>-1</sup> i.p.) was administered 15 min before the injection of A-796260 (11 mg kg<sup>-1</sup> i.p.) or AM1241 (15 mg kg<sup>-1</sup> i.p.). In panels b and c data are expressed as mean  $\pm$  s.e.mean ( $n = 6$ ). \*\* $P < 0.01$  as compared to vehicle-treated rats, ++ $P < 0.01$  as compared to A-796260 control.

(6 mg kg<sup>-1</sup> i.p.) also produced a statistically significant reversal of allodynia 30 min post-administration.

#### Effects of A-796260 in an OA pain model

The analgesic effects of A-796260 on activity-induced pain behaviour were evaluated in a rat model of MIA-induced OA joint pain (Guzman *et al.*, 2003; Pomonis *et al.*, 2005), observed 20 days following the i.a. injection of MIA. Previously we showed that i.a. administration of MIA produces a long lasting decrease in hindlimb grip force when compared to saline-injected animals (Chandran *et al.*, 2006). A-796260 (i.p.) reversed, in a dose-related manner, MIA-induced decrease in the grip force with 56  $\pm$  7% effect at 35 mg kg<sup>-1</sup> ( $P < 0.01$  vs the vehicle CF<sub>max</sub> 370  $\pm$  87 g). The effects were comparable to the 62% efficacy elicited by celecoxib (38 mg kg<sup>-1</sup> i.p.), a clinically effective analgesic for OA pain (Figure 5;  $F_{5,30} = 31.6$ ,  $P < 0.0001$ ).

#### Effects of A-796260 on locomotion

Locomotor activity in rats was evaluated by automatically recording distance travelled in an open field. Systemic administration of A-796260 (1–35 mg kg<sup>-1</sup> i.p.) did not significantly affect locomotor activity (Figure 6). The highest dose tested in this study has been shown to produce full or nearly full efficacy across models of chronic inflammatory, postoperative, neuropathic and OA pain. In this study,

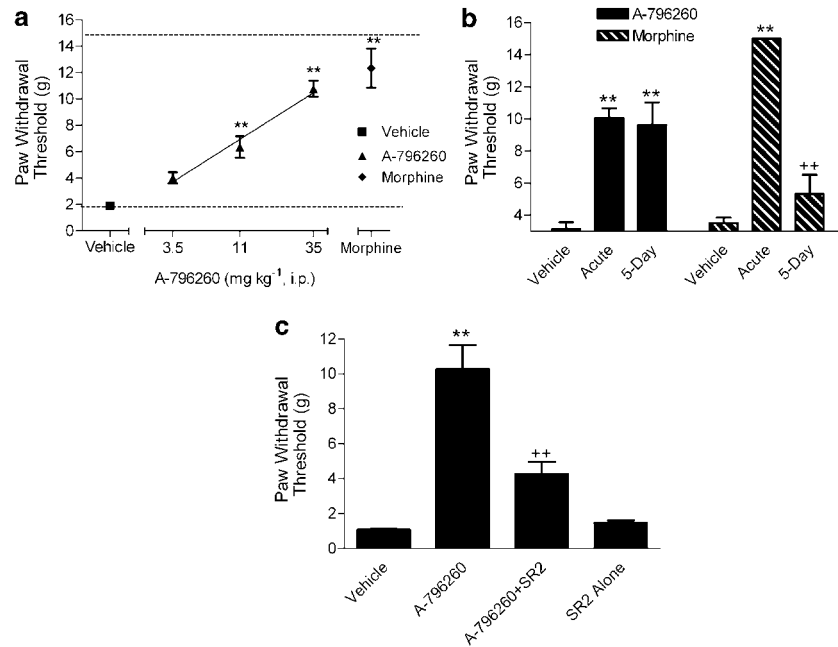
haloperidol (1 mg kg<sup>-1</sup> i.p.) significantly decreased locomotion in rats (Figure 6;  $F_{5,42} = 7.10$ ,  $P < 0.0001$ ).

## Discussion and conclusions

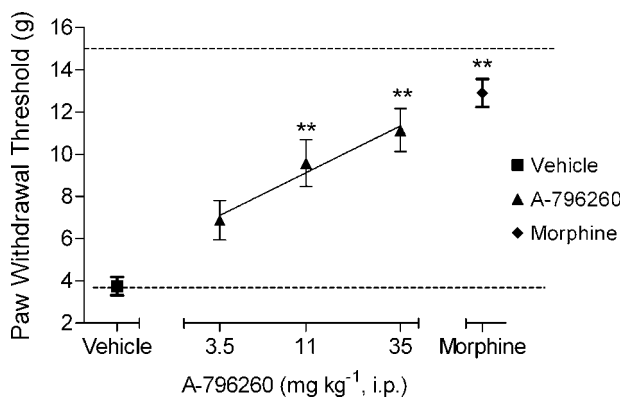
Selective activation of the cannabinoid CB<sub>2</sub> receptor has been proposed as a new mechanism for the treatment of pain, with potential for the development of broad-spectrum analgesics devoid of the CNS-mediated side-effects associated with non-selective cannabinoid ligands. The evidence supporting this hypothesis has been largely derived from the study of a handful of CB<sub>2</sub>-selective ligands that share efficacy across multiple rodent pain models, but have significantly different *in vitro* pharmacological profiles at the CB<sub>2</sub> receptor and also differ with respect to the potential involvement of the  $\mu$ -opioid receptor in their mechanism of action. In the present study, we describe the *in vitro* and *in vivo* properties of a new CB<sub>2</sub>-selective ligand, A-796260.

Similar to the structurally related indole derivatives AM1241 and L-768,242 (GW405833), A-796260 is characterized by high affinity for human and rat CB<sub>2</sub> receptors and moderate to excellent selectivity vs the CB<sub>1</sub> receptor subtypes. Previous studies on AM1241 have shown it has affinities for both the human and mouse CB<sub>2</sub> receptors in the range from 1.6 (Makriyannis and Deng, 2002) to 28.7 nM (Bingham *et al.*, 2007) for the human receptor, and from 2 (Nackley *et al.*, 2003b) to 28.8 nM (Bingham *et al.*, 2007) for



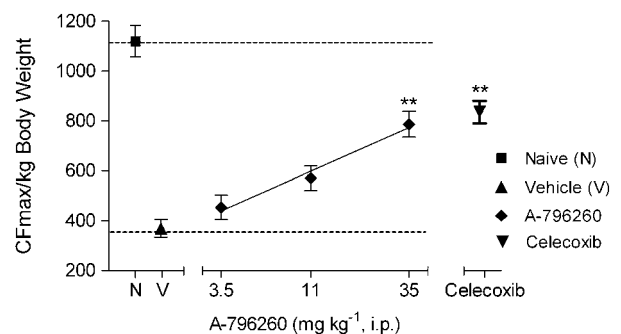


**Figure 3** Effects of A-796260 on skin incision-induced acute postoperative pain. (a) A-796260, administered 1.5 h post-surgery, dose-dependently attenuated tactile allodynia ( $ED_{50} = 18 \text{ mg kg}^{-1} \text{ i.p.}$ ). Morphine ( $6 \text{ mg kg}^{-1} \text{ i.p.}$ ) was included in the same study. Data represent mean  $\pm$  s.e.mean.  $**P < 0.01$  as compared to vehicle-treated animals ( $n = 12$  per group). (b) Analgesic effect of A-796260 ( $35 \text{ mg kg}^{-1} \text{ i.p.}$ ) and morphine ( $3 \text{ mg kg}^{-1} \text{ s.c.}$ ) in rats after repeated administration for 5 days. Data are expressed as mean  $\pm$  s.e.mean ( $n = 6$ ).  $**P < 0.01$  as compared to vehicle-treated animals;  $^{+}P < 0.01$  as compared to the acute single dosing group. (c) Reversal of A-796260 analgesic effects by CB<sub>2</sub>-selective antagonist, SR144528 (SR2,  $4.8 \text{ mg kg}^{-1} \text{ i.p.}$ ) was administered 15 min before A-796260 injection ( $35 \text{ mg kg}^{-1} \text{ i.p.}$ ). Data are expressed as mean  $\pm$  s.e.mean ( $n = 6$ ).  $**P < 0.01$  as compared to vehicle-treated animals;  $^{+}P < 0.01$  as compared to A-796260 control.



**Figure 4** Effects of A-796260 on tactile allodynia in a rat neuropathic pain model, sciatic nerve chronic constriction injury (CCI). A-796260 dose-dependently attenuated neuropathic pain in this model ( $ED_{50} = 12 \text{ mg kg}^{-1} \text{ i.p.}$ ), which is seen as an increase in the withdrawal threshold of the nerve-injured paw. Data are expressed as mean  $\pm$  s.e.mean.  $**P < 0.01$  vs vehicle-treated group,  $n = 12$  per group.

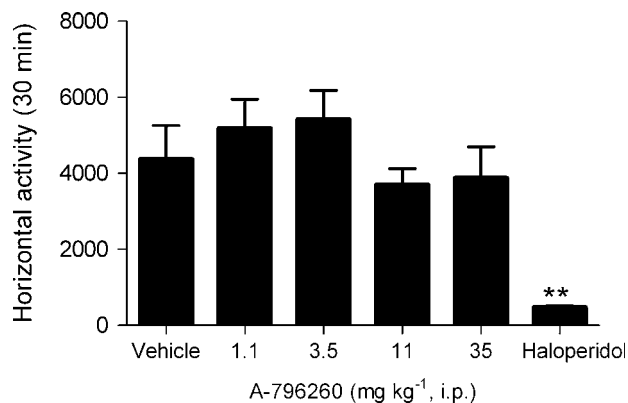
the mouse receptor, and 26.7 nM for the rat CB<sub>2</sub> receptor (Bingham *et al.*, 2007). In this study, we observed comparable affinities and selectivity ratios to these values for AM1241 (see Table 1). However, in previous studies discrepancies in binding affinity data for L-768,242 have been obtained; Valenzano *et al.* (2005) reported a  $K_i$  of 3.92 nM at the human CB<sub>2</sub>-binding site and  $>1200$ -fold selectivity vs the human CB<sub>1</sub> site, whereas Gallant *et al.* (1996) reported an affinity of 12 nM at the human CB<sub>2</sub> receptor and 160-fold selectivity vs



**Figure 5** Effects of A-796260 on hindlimb grip force in rats 20 days following injection of monoiodoacetate (MIA). A-796260 demonstrated dose-related reversal of activity-induced pain behaviour in osteoarthritic (OA) rats with effects comparable to celecoxib ( $38 \text{ mg kg}^{-1} \text{ i.p.}$ ), a clinically relevant analgesic for OA pain. Data are expressed as mean  $\pm$  s.e.mean.  $**P < 0.01$  vs vehicle-treated group,  $n = 6$  per group.

the human CB<sub>1</sub> site. The values obtained in this study are intermediate between these two results (Table 1). Based on radioligand-binding experiments, A-796260 exhibits a comparable level of affinity and selectivity for the human and rat CB<sub>2</sub> receptors relative to previously described CB<sub>2</sub>-selective ligands.

In *in vitro* functional assays, A-796260 exhibits a profile distinct from other available CB<sub>2</sub> ligands, characterized by high potencies and full or near full agonist efficacy at human and rat CB<sub>2</sub> receptors, and a high degree of selectivity vs CB<sub>1</sub> receptors (Table 2). This is in contrast to structurally related



**Figure 6** Effects of A-796260 treatment on spontaneous horizontal locomotor activity. Average ( $\pm$  s.e.mean;  $n=8$  rats per group) levels of horizontal locomotor activity exhibited by rats 30 min following compound injection (i.p.) are shown. Haloperidol ( $1 \text{ mg kg}^{-1}$  i.p.) was included as a positive control. \*\* $P < 0.01$ , vs vehicle control.

ligands that, in this study, either exhibit no agonist efficacy (AM1241) at either the human or rat CB<sub>2</sub> receptor or display inverse agonist activity (L-768,242) at the rat CB<sub>2</sub> receptor. Bingham *et al.* (2007) showed that AM1241 exhibits partial agonist efficacy when a low ( $1 \mu\text{M}$ ) concentration of forskolin was used to stimulate the cAMP release, a finding consistent with that of Yao *et al.* (2006), where it was demonstrated that at the human CB<sub>2</sub> receptor, AM1241 exhibits no antagonistic activity or very low efficacy as an inverse agonist at high forskolin concentrations ( $37 \mu\text{M}$ ), but is a partial agonist at lower ( $8 \mu\text{M}$ ) forskolin concentrations. Further, the effects of AM1241 are species-dependent; it is a partial agonist at human CB<sub>2</sub> receptor and an inverse agonist at the rat CB<sub>2</sub> receptor (Bingham *et al.*, 2007). L-768,242 has been shown to exhibit partial agonist efficacy at the recombinant human CB<sub>2</sub> receptor (Valenzano *et al.*, 2005), but its effects on rat CB<sub>1</sub> and CB<sub>2</sub> receptors had not been characterized previously. In this study, L-768,242 exhibited inverse agonist activities at both human and rat CB<sub>2</sub> receptors. The pharmacological properties of AM1241 and L-768,242 are consistent with those of protean agonists. In contrast, the fully efficacious agonist CP 55,940 and the inverse agonist SR144528 are unaffected by the assay conditions used and the level of receptor constitutive receptor activity (Yao *et al.*, 2006). GW-842166X, despite its low potency for displacement of [<sup>3</sup>H]-CP 55,940 from the human and rat CB<sub>2</sub> receptors (2000 and 2580 nM, respectively), did exhibit full agonist efficacy in cyclase assays in the present study (Table 2), exhibiting potencies similar to those found by Giblin *et al.* (2007) in a yeast reporter gene assay (human CB<sub>2</sub> EC<sub>50</sub> = 63 nM,  $E_{\text{max}}$  = 95%; rat CB<sub>2</sub> EC<sub>50</sub> = 91 nM,  $E_{\text{max}}$  = 100%). It is possible that GW-842166X may activate signalling through another site in addition to the CP 55,940-binding site on the CB<sub>2</sub> receptor. Compared with the CB<sub>2</sub> ligands studied previously, the consistent pharmacology, robust and fully efficacious agonist activities, as well as selectivity at CB<sub>2</sub> receptors displayed by A-796260 demonstrate that this compound may be a useful tool for studying CB<sub>2</sub> receptor pharmacology and for evaluating the therapeutic potential of CB<sub>2</sub> agonists in rodent models.

In this study, A-796260 exhibited robust analgesic activity in rat models of inflammatory, postoperative, neuropathic and OA pain. In the CFA model of chronic inflammatory pain (Figure 2a), A-796260 produced a dose-dependent antihyperalgesic effect, but did not increase the response time to thermal stimulus in the uninjured paw. The antihyperalgesic effects of A-796260 were reversed by the CB<sub>2</sub>-selective antagonists AM630 and SR144528 but not by the CB<sub>1</sub>-selective antagonist SR141716A (Figure 2b), supporting a CB<sub>2</sub>-mediated mechanism of action. However, unlike AM1241, whose analgesic activity requires a functional  $\mu$ -opioid receptor, A-796260-mediated analgesic effects were not dependent on  $\mu$ -opioid receptors. Extending the previous studies by Ibrahim *et al.* (2005), we demonstrated that the effects of AM1241 in the CFA model are blocked by the  $\mu$ -opioid antagonist naloxone (Figure 2c), whereas the antihyperalgesic effects of A-796260 were unaffected by naloxone under identical conditions, suggesting that the mechanisms of action of these two CB<sub>2</sub>-selective indole derivatives are clearly distinct. However, although the mechanism whereby  $\mu$ -opioid receptors are involved in AM1241-evoked analgesic activity is not clearly understood, it does not appear to involve a direct receptor interaction as AM1241 has no affinity for the  $\mu$ -opioid receptor.

Similarly, in a skin-incision model of postoperative pain (Brennan *et al.*, 1996), A-796260 was efficacious in reversing allodynia (2 h post-incision), with a maximal efficacy comparable to that observed with morphine (Figure 3a). This effect of A-796260 was completely inhibited by the CB<sub>2</sub>-selective antagonist SR144528, indicating that CB<sub>2</sub> receptors are involved in this model as well (Figure 3c). The efficacy of cannabinoid agonists has previously been demonstrated in a postoperative pain model (Romero-Sandoval and Eisenach, 2007); it was demonstrated that both CB<sub>1</sub> and CB<sub>2</sub> activation can contribute to reversal of incisional pain and the antiallodynic effects of the non-selective ligand CP 55,940 were partially reversed by both CB<sub>1</sub> and CB<sub>2</sub> antagonists, whereas the effects of the CB<sub>2</sub>-selective agonist JWH-015 were antagonized by the CB<sub>2</sub> antagonist and not the CB<sub>1</sub> antagonist. Consistent with this work, we also observed that systemic administration of the CB<sub>1</sub> full agonist and CB<sub>2</sub> partial agonist WIN 55,212 (see Table 2) produced a reversal of allodynia in the skin incision model, and that these effects were preferentially blocked by the CB<sub>1</sub> antagonist SR141716A (data not shown). In addition, L-768,242 has also been shown to be active in this model at a 24 h time point post-surgical incision (Valenzano *et al.*, 2005). These results provide further support for the potential of CB<sub>2</sub> agonists in the management of post-surgical pain.

Tolerance develops rapidly to the analgesic effects of morphine, particularly in animal models. In the present study, tolerance to the effects of A-796260 did not develop at a dosing protocol shown to induce tolerance to the analgesic effects of morphine. Although chronic dosing studies of a substantially longer duration are required before it can be concluded that tolerance to the analgesic effects of A-796260 does not occur, these preliminary data suggest that this compound has less propensity for the development of tolerance than opioid agonists.

In the CCI of the sciatic nerve model of neuropathic pain (Bennett and Xie, 1988), A-796260 produced a dose-dependent reversal of tactile allodynia, suggesting that CB<sub>2</sub> receptor agonists have potential as a treatment of neuropathic pain (Figure 4). These findings are consistent with the effects observed of other CB<sub>2</sub>-selective ligands, such as AM1241 (Ibrahim *et al.*, 2003a) and L-768,242 (Valenzano *et al.*, 2005) in models of neuropathic pain.

I.a. injection of sodium monoiodoacetate into the knee joint of rats has been used to induce morphological and behavioural changes that mimic OA in humans (Guingamp *et al.*, 1997; Guzman *et al.*, 2003; Combe *et al.*, 2004). A behavioural end point that measures grip force in rats has been characterized for several classes of drugs with demonstrated clinical efficacy in the management of pain associated with OA (Chandran *et al.*, 2006). In this model, A-796260 exhibited dose-dependent restoration of performance comparable to the clinically effective COX-2 inhibitor celecoxib. To our knowledge, this is the first time a CB<sub>2</sub> agonist has been demonstrated to be an effective analgesic in a model of chronic OA pain. Compounds, such as morphine and non-selective agonists, which produce sedation or decreased motor coordination, also have a negative effect on the grip force task that the animals are required to perform even in uninjured animals (data not shown). In contrast, the present findings indicate that, within the dose range used, A-796260 has minimal effects that could be attributed to activation of central CB<sub>1</sub> receptors.

To address the behavioural consequences of the modest selectivity exhibited by A-796260 in rat radioligand-binding studies (30-fold selective rat CB<sub>2</sub> vs rat CB<sub>1</sub>) directly, its effects on spontaneous horizontal motor activity were assessed across a behaviourally effective range. No significant effects on motor activity were observed, indicating a separation of the analgesic effect mediated by the activation of CB<sub>2</sub> receptor *in vivo* from the locomotor deficit attributable to CB<sub>1</sub> receptor activation.

The pain models described in this study are all models of tissue or nerve injury, and thus compound-mediated effects in these models represent reversal of hyperalgesia or allodynia, and not antinociceptive activity. Indeed, in the CFA model, the inability of A-796260 to increase PWL to a thermal stimulus in the contralateral paw within the behaviourally effective range for reversal of hyperalgesia (as measured in the ipsilateral paw; Figure 3a) suggests that A-796260 does not possess significant antinociceptive activity, at least within this dose range. In a separate experiment in a naive rat model of acute thermal nociception (data not shown), A-796260 exhibited no effect on PWL at 3.5 and 11 mg kg<sup>-1</sup>, and only a modest increase in PWL (45% of a maximum possible effect) at a dose of 35 mg kg<sup>-1</sup> i.p. These data contrast with the antinociceptive activity of AM1241 observed in a model of acute thermal pain (Malan *et al.*, 2001). Also, within the same dose range AM1241 has been shown to reverse capsaicin-induced hyperalgesia (Hohmann *et al.*, 2004) and tactile allodynia in a model of neuropathic pain (Ibrahim *et al.*, 2003b). These different effects of AM1241 compared to A-796260 may be due to the involvement of an opioid mechanism in the effects of AM1241 but not A-796260.

In this study, we describe the properties of a new ligand, A-796260, with attributes suggesting it could be useful compound for the further exploration of CB<sub>2</sub>-selective receptor agonists in the treatment of pain. A-796260 was found to be a potent and selective CB<sub>2</sub> agonist with broad-spectrum antihyperalgesic and antiallodynic activity across models of chronic inflammatory, postoperative, neuropathic and OA pain, and these effects were shown to be mediated through the CB<sub>2</sub> receptor and independent of an interaction with the  $\mu$ -opioid receptor. These properties differentiate A-796260 from many of the existing CB<sub>2</sub> agonists both with respect to its agonist efficacy profile *in vitro* and lack of opioid receptor dependence *in vivo*. The studies described here also add additional support for the hypothesis that selective CB<sub>2</sub> receptor agonists may be an attractive approach for the development of new drugs for the treatment of chronic pain; they are less likely to cause side-effects compared to the currently available treatments.

## Conflict of interest

All authors are employees of Abbott Laboratories, which funded the research.

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