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Characterization of a Cannabinoid CB_2 Receptor-Selective Agonist, A-836339 [2,2,3,3-Tetramethyl-cyclopropanecarboxylic Acid [3-(2-Methoxy-ethyl)-4,5-dimethyl-3*H*-thiazol-(2*Z*)-ylidene]-amide], Using in Vitro Pharmacological Assays, in Vivo Pain Models, and Pharmacological Magnetic Resonance Imaging

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ABSTRACT

Studies demonstrating the antihyperalgesic and antiallodynic effects of cannabinoid CB_2 receptor activation have been largely derived from the use of receptor-selective ligands. Here, we report the identification of A-836339 [2,2,3,3-tetramethyl-cyclopropanecarboxylic acid [3-(2-methoxy-ethyl)-4,5-dimethyl-3*H*-thiazol-(2*Z*)-ylidene]-amide], a potent and selective CB_2 agonist as characterized in in vitro pharmacological assays and in in vivo models of pain and central nervous system (CNS) behavior models. In radioligand binding assays, A-836339 displays high affinities at CB_2 receptors and selectivity over CB_1 receptors in both human and rat. Likewise, A-836339 exhibits high potencies at CB_2 and selectivity over CB_1 receptors in recombinant fluorescence imaging plate reader and cyclase functional assays. In addition A-836339 exhibits a profile devoid of significant affinity at other G-protein-coupled receptors and ion channels. A-836339 was characterized extensively in various animal pain models. In the complete Freund's adjuvant model of inflammatory pain, A-836339 exhibits a potent CB_2 receptor-mediated antihyperalgesic effect that is independent of CB_1 or μ-opioid receptors. A-836339 has also demonstrated efficacies in the chronic constrain injury (CCI) model of neuropathic pain, skin incision, and capsaicin-induced secondary mechanical hyperalgesia models. Furthermore, no tolerance was developed in the CCI model after subchronic treatment with A-836339 for 5 days. In assessing CNS effects, A-836339 exhibited a CB_1 receptor-mediated decrease of spontaneous locomotor activities at a higher dose, a finding consistent with the CNS activation pattern observed by pharmacological magnetic resonance imaging. These data demonstrate that A-836339 is a useful tool for use of studying CB_2 receptor pharmacology and for investigation of the role of CB_2 receptor modulation for treatment of pain in preclinical animal models.

It is estimated that as high as 50% of the population will experience chronic pain during their lifetime, and the prevalence is likely to rise with the continued aging of the population (Markman and Philip, 2007). As a consequence, there exists an ever-growing demand for new therapies to provide safe and effective pain management. Despite intensive research to identify novel therapeutic approaches, there have been few major advances in pain therapy over the past sev-

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ABBREVIATIONS: CNS, central nervous system; CB, cannabinoid; GPCR, G-protein-coupled receptor; GW-842166X, 2-(2,4-dichlorophenylamino)-4-trifluoromethyl-pyrimidine-5-carboxylic acid (tetrahydro-pyran-4-ylmethyl)-amide; AM1241, (*R*,*S*-2-iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-1*H*-indol-3-yl]-methanone; JWH-015, (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenyl-methanone; L-768,242, (2,3-dichloro-phenyl)-[5-methoxy-2-methyl-3-(2-morpholin-4-yl-ethyl)-indol-1-yl]-methanone; A-796260, [1-(2-morpholin-4-yl-ethyl)-1*H*-indol-3-yl]-(2,2,3,3-tetramethyl-cyclopropyl)-methanone; A-836339, 2,2,3,3-tetramethyl-cyclopropanecarboxylic acid [3-(2-methoxy-ethyl)-4,5-dimethyl-3*H*-thiazol-(2*Z*)-ylidene]-amide; SR141716A, 5-(4-chloro-phenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1*H*-pyrazole-3-carboxylic acid piperidin-1-ylamide; SR144528, 5-(4-chloro-3-methyl-phenyl)-1-(4-methyl-benzyl)-1*H*-pyrazole-3-carboxylic acid ((1*S*,2*S*,4*R*)-1,3,3-trimethyl-bicyclo[2.2.1]hept-2-yl)-amide; CP 55,940, 5-(1,1-dimethyl-heptyl)-2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxy-propyl)-cyclohexyl]-phenol; AM630, [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)-methanone; HEK, human embryonic kidney; BSA, bovine serum albumin; FLIPR, fluorescence imaging plate reader; phMRI, pharmacological magnetic resonance imaging; CFA, complete Freund's adjuvant; PWL, paw withdrawal latency; CCI, chronic constrain injury; Cap-SMH, capsaicin-induced secondary mechanical hyperalgesia.

eral decades, and pain management continues to rely largely on nonsteroidal anti-inflammatory drugs, acetaminophen, opioids, and certain adjuvant analgesics.

The therapeutic potential of herbal and synthetic cannabinoids is being increasingly recognized (Fox and Bevan, 2005; Ibrahim et al., 2006), and herbal-based agents, such as Sativex, have shown analgesic effects in clinical trials (Russo et al., 2007). However, CNS side effects associated with these agents, such as sedation, euphoria, asthenia, and anxiety, have significantly limited their therapeutic utility. Both CB_1 and CB_2 receptors are $G_{i/o}$-coupled GPCRs, whose activation negatively couples to adenylyl cyclase (Felder et al., 1995; Slipetz et al., 1995). CB_1 and CB_2 receptors share only a moderate sequence identity, 32% for human and 34% for rat receptors, respectively (Matsuda et al., 1990; Munro et al., 1993). It is generally accepted (Shire et al., 1996) that the adverse CNS effects in humans and rodents are largely, if not exclusively, mediated through the activation of the CB_1 receptor, which is abundantly expressed in CNS tissues. In rodents, the CB_1-mediated effects of cannabinoids are manifested as decreased locomotor activity and coordination, catalepsy, and hypothermia. In contrast, the CB_2 receptor is expressed primarily in the normal peripheral immune tissues (Galiègue et al., 1995). Recent reports have indicated that the CB_2 receptor may also be expressed at limited levels in neuronal tissues (Duncan et al., 2004), and its expression in CNS may be up-regulated under pathological conditions (Zhang et al., 2003), CB_2 receptor activation is not expected to mediate the CNS side effects observed for nonselective cannabinoid ligands. Recent findings have demonstrated CB_2 receptor activation modulates pain perception in rodents (Malan et al., 2001; Hohmann et al., 2004), suggesting CB_2 receptor agonists may offer an attractive approach for the development of therapeutic agents devoid of psychotropic effects for treatment of pain. As a consequence, significant progress has been made toward the identification of CB_2-selective ligands, including a clinical candidate, GW-842166X, for treatment of chronic pain. GW-842166X exhibits modest functional potency for the CB_2 receptor subtype and efficacy in several rodent pain models (Giblin et al., 2007) but shows relatively weak (1000 nM) binding affinity for the human CB_2 receptor (Yao et al., 2008).

CB_2-selective agonists have been identified as pharmacological tools, falling into two general structural classes: the tetrahydrocannabinol mimetics (including JWH-133 and HU308) and indoles. JWH-133 has been characterized as a high-affinity CB_2 ligand (Huffman, 1999), exhibiting a CB_2-mediated efficacy in models of inflammatory (Elmes et al., 2005) and neuropathic pain (Yamamoto et al., 2008). HU308, described as a peripheral CB_2-selective ligand devoid of activity in the mouse tetrad behavioral test, demonstrated CB_2-mediated efficacy in the formalin-induced peripheral pain model (Hanus et al., 1999).

Indole-derived CB_2-selective agonists, including AM1241 (Malan et al., 2001), JWH-015 (Huffman, 1999), L-768,242 (Gallant et al., 1996; Valenzano et al., 2005; Whiteside et al., 2005), and A-796260 (Yao et al., 2008), are among the best characterized ligands. The pharmacological profile of AM1241 differentiates from other CB_2 ligands. It exhibits protean agonist activities (Yao et al., 2006; Bingham et al., 2007) in in vitro assays but apparent in vivo agonist activities in rodent pain models (Malan et al., 2001), that require a functional the μ-opioid receptor in addition to CB_2 (Ibrahim et al., 2005). Conversely, the analgesic activity of A-796260 and L-768,242 in various pain models has been shown to be independent of the μ-opioid receptor. Recently, Ohta et al. (2008) reported the synthesis of imine derivatives (represented by CBS0550) that exhibit >1000-fold CB_2 receptor selectivity and analgesic activity in a model of inflammatory pain.

Herein, we report the identification and characterization of a novel CB_2 receptor selectivity ligand, A-836339, in in vitro radioligand binding and functional assays and in in vivo animal pain and behavior models. We demonstrate that A-836339 is a potent agonist at the CB_2 receptor exhibiting improved in vitro selectivity versus the CB_1 receptor over currently available ligands and displays broad spectrum antihyperalgesic and antiallodynic efficacy across a range of rodent pain models.

Materials and Methods

Compounds

AM1241, A-796260, JWH-015, SR141716A, and SR144528 were prepared at Abbott Laboratories as described previously (Yao et al., 2006). CP 55,940 and AM630 were purchased from Tocris Bioscience (Ellisville, MO). L-768,242 and GW-842166X were synthesized as described by Valenzano et al. (2005) and by Giblin et al. (2007), respectively. A-836339 (Fig. 1) was synthesized as described by Dart et al. (2007).

Cell Culture

Reagents for cell culture needs were purchased from Invitrogen (Carlsbad, CA) unless indicated otherwise. Human embryonic kidney (HEK; American Type Culture Collection, Manassas, VA) cells stably expressing human CB_2, rat CB_2, or rat CB_1 receptors and HEK cells coexpressing the chimeric $G_{\alpha q/o5}$ protein with either the human or rat CB_2 receptors were generated and maintained as described previously (Yao et al., 2006). The Chinese hamster ovary cell lines stably expressing the human CB_1 receptor were purchased from Euroscreen SA (Brussels, Belgium), and the cells were grown under the conditions recommended by the vendor.

Radioligand Binding Assays

Membrane preparation from HEK cells stably expressing the human CB_2, rat CB_2, or rat CB_1 receptor and Chinese hamster ovary cells stably expressing the human CB_1 receptor and competition binding assays were performed as described previously (Yao et al., 2006, 2008) using 0.5 nM [^{3}H]CP 55,940 in the presence of test compounds and an assay buffer containing 50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 5 mM $MgCl_2$, and 0.05% fatty acid-free BSA. After incubation at 30°C for 90 min, the reaction was terminated by rapid vacuum filtration through UniFilter-96 GF/C

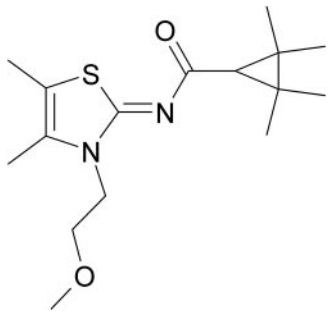

Fig. 1. Structure of A-836339.

filter plates (PerkinElmer Life and Analytical Sciences, Waltham, MA) and four washes with cold assay buffer. Nonspecific binding was defined by 10 μM unlabeled CP 55,940. K_i values and 95% confidence intervals were calculated from competition binding assays with one-site competition curve fitting using the Prism software (GraphPad Software Inc., San Diego, CA).

Cyclase Functional Assays

The cyclase functional assays were performed as described previously (Yao et al., 2006) using the HitHunter assay kit from DiscoveRx (Fremont, CA). In brief, cell suspensions were incubated at 37°C for 20 min with variable concentrations of test ligands or 10 μM CP 55,940-positive control in the presence of a fixed concentration of forskolin (18 μM for rat CB_2 and 37 μM for human CB_1 and CB_2 and rat CB_1) in Dulbecco's phosphate-buffered saline buffer (Invitrogen) supplemented with BSA (0.01% final concentration). The reactions were terminated by the addition of lysis buffer, and the luminescence was detected after the procedure according to the vendor's instruction. Receptor activation by ligands is expressed as percentage response compared with that of 10 μM CP 55,940. EC_{50} values and 95% confidence intervals were calculated using sigmoidal dose-response curve fitting using Prism (GraphPad Software Inc.) software.

Fluorescence Imaging Plate Reader Functional Assays

Fluorescence imaging plate reader (FLIPR) assays were performed using HEK cells stably coexpressing the chimeric $G\alpha_{q/o5}$ protein with either human or rat CB_2 receptors (Yao et al., 2008). In brief, cells were seeded at 75,000 cells/well 1 day before the assay, and assays were performed with no-wash dye (FLIPR Calcium Assay Kit; Molecular Devices, Sunnyvale, CA) following the vendor's instruction. Fluorescence responses of cells were measured with a FLIPR machine upon the addition of variable concentrations of test compounds, CP 55,940 (10 μM, positive control), or vehicle in the presence of an assay buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 2 mM $CaCl_2$, and 0.05% BSA). 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_{50} values and 95% confidence intervals were calculated with sigmoidal dose-response curve fitting using Prism.

Animals

Adult Sprague-Dawley rats (male, 250–300 g b.wt.) obtained from Charles River Breeding Laboratories (Portage, MI) were used for all experiments. Sprague-Dawley rats (male, 300–350 g b.wt.) from Charles River were used for all phMRI experiments. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities at Abbott Laboratories in a temperature-regulated environment under a controlled 12-h light/dark cycle, with lights on at 6:00 AM. Food and water were available ad libitum at all times except during testing. All testing was done after procedures outlined in protocols approved by Abbott's Institutional Animal Care and Use Committee.

In Vivo Pain Models

Complete Freund's Adjuvant-Induced Inflammatory Pain Model. Inflammatory thermal hyperalgesia was induced by injection of complete Freund's adjuvant (CFA; 150 μl, 50% solution in phosphate-buffered saline) into the plantar surface of the right hind paw of rats. Thermal hyperalgesia was assessed 48 h post-CFA injection using a commercially available thermal paw stimulator (UARDG; University of California, San Diego, CA) described by Hargreaves et al. (1988). In brief, rats were habituated for 20 min in individual plastic cubicles mounted on a glass surface maintained at 30°C. A thermal stimulus generated from a focused projection bulb (4.50 ± 0.05 amps) was applied to the plantar surface of each hind paw, with maximum exposure limited to 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 paw of each rat was 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 after A-836339 administration in both the CFA-treated and uninjured paw.

Skin Incision 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 toward the toes. The plantaris muscle was elevated and incised longitudinally, leaving the muscle origin and insertion points intact. After homeostasis 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 or 24 h after surgery, at which time mechanical allodynia was assessed. To test drug effects, rats were first acclimated for 20 min in inverted individual plastic containers (20 × 12.5 × 20 cm) on top of a suspended wire mesh grid, and A-836339 was injected intraperitoneally 30 min before testing for mechanical allodynia using calibrated von Frey filaments (Stoelting Co., Wood Dale, IL). 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 of the filament. Positive responses included an abrupt withdrawal of the hind paw from the stimulus or flinching behavior immediately after removal of the stimulus. A 50% withdrawal threshold was determined using an up-down procedure.

Capsaicin-Induced Secondary Mechanical Hyperalgesia Model. Capsaicin (10 μg/10 μl) was administered in vehicle (10% ethanol in 2-hydroxypropyl cyclodextrin) by intraplantar injection into the center of the right hind paw. Secondary mechanical hyperalgesia in a large region surrounding the injection site was measured at 3 h after capsaicin injection. A-836339 was administered intraperitoneally at 30 min before behavioral testing. Only rats with a baseline threshold score of less than 4.5 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.

Chronic Constriction Injury Model of Neuropathic Pain. A chronic constrain injury (CCI) model of neuropathic pain was produced by following the method of Bennett and Xie (1988). The right common sciatic nerve of rat was isolated at mid-thigh level and loosely ligated by four chromic gut (5-0) ties separated by an interval of 1 mm, and animals were allowed to recover for at least 2 to 4 weeks before testing for mechanical allodynia.

Motor Coordination and CNS Functional Models. Rotorod performance was measured using an accelerating Rotorod apparatus (Omnitech Electronics Inc., Dartmouth, NS, Canada). Rats were placed on a 9-cm-diameter rod with an increasing rotating speed from 0 to 20 rpm over a 6-s period. Each rat was given three training sessions. The Rotorod performance was determined by the total amount of time within 60 s that rats stayed on the rod without falling off (the maximum score is 60 s). The effect of A-836339 was tested 30 min postcompound administration. Spontaneous exploratory behavior was examined in naive rats (30 min postcompound injection). Rats were individually placed into test chambers, and horizontal (locomotion) activity was recorded by a photobeam detector system for 30 min (AccuScan Instruments, Inc., Columbus, OH).

Data Analysis for in Vivo Tests. Statistical analyses were carried out using GraphPad Prism (version 4.03; GraphPad Software Inc.). The values were represented as mean ± S.E.M. Statistical significance on group means was derived by one-way analysis of variance, followed by Bonferroni post hoc analysis. In all cases, $p < 0.05$ (*; **, $p < 0.01$) was assumed as the level for statistical signif-

icance. ED_{50} values were also calculated by linear regression analysis (GraphPad Prism).

phMRI. Rats were divided into seven treatment groups ($n = 5$ per treatment group), where each group received either vehicle (5% dimethyl sulfoxide/polyethylene glycol 400 i.v.), A-836339 (at 3 or 10 μmol/kg i.v.), SR141716A (13 μmol/kg i.p.), AM630 (6 μmol/kg i.p.), A-836339 (10 μmol/kg i.v.) plus SR141716A (13 μmol/kg i.p.), or A-836339 (10 μmol/kg i.v.) plus AM630 (6 μmol/kg i.p.). In the groups where animals received A-836339 along with SR141716A or AM630, animals were pretreated with SR141716A or AM630 approximately 40 min before the infusion of A-836339. Cerebral blood volume-based spin-echo pharmacological phMRI experiments were carried out on a 7T Bruker Biospec MRI system (Karlsruhe, Germany) using an imaging protocol described previously (Chin et al., 2008). Data analysis was performed using the AFNI software package, and group brain activation maps were obtained by averaging the coregistered z score maps retrieved from individual animals with a threshold of $z > 1.96$ ($p < 0.05$). In addition, a satellite group of 15 rats were used for the bench-top PK study, in which rats were prepared and treated using the same procedures as the imaging experiment, and blood and brain samples were collected at 10 min postdrug, which corresponds to the time point that typically maximal change of MRI signal was observed.

Results

Characterization of A-836339 in Radioligand Binding and Functional Assays Employing in Vitro Recombinant Systems

The affinities of A-836339 at cannabinoid receptors were determined by the displacement of radioligand [^{3}H]CP 55,940 in competition binding assays using membranes prepared from recombinant HEK cells stably expressing the CB_1 or CB_2 receptor from human and rat. A-836339 exhibited high potencies at both human and rat CB_2 receptors, with K_i values of 0.64 and 0.76 nM, respectively (Table 1; Fig. 2), and 425- and 189-fold selectivity for the CB_2 receptor over the CB_1 receptor in human and rat, respectively. In addition, A-836339 did not exhibit significant species selectivity for either CB_1 or CB_2 receptors in human and rat. The rank order of affinities at the human CB_2 receptor was A-836339 > A-796260 ~ L-768,242 > AM1241 > JWH-015 > GW-842166X, and the rank order of selectivity at CB_2 over CB_1 was A-836339 > A-796260 ~ L-768,242 > AM1241 ~ JWH-015. GW-842166X showed weak binding affinities at both human and rat CB_2 and no detectable affinity at CB_1 receptors in human and rat.

In a previous publication (Yao et al., 2006), we have demonstrated that A-796260 and AM1241 lack binding affinity at the μ-opioid receptor as determined in [^{3}H]DAMGO binding assays. A-836339 was characterized in a similar assay, and like A-796260 and AM1241, A-836339 also failed to displace [^{3}H]DAMGO at the μ-opioid receptor at concentrations up to 10 μM (data not shown), whereas the μ-opioid receptor ligand naloxone exhibited a K_i value of 17 nM in the same experiment.

The functional potency and efficacy of A-836339 at human and rat cannabinoid receptors were assessed in in vitro FLIPR and cyclase functional assays (Table 2; Fig. 3). In human CB_2 receptor cyclase assays, A-836339 exhibited full agonist efficacy (E_{max} = 102%) with comparable potency (EC_{50} = 1.6 nM) as A-796260 (EC_{50} = 0.71 nM), greater potency than other CB_2-selective ligands including JWH-015 (EC_{50} = 46 nM) and GW-842166X (EC_{50} = 460 nM). A similar rank order of potencies (A-836339 ~ A-796260 > JWH-015 > GW-842166X) in human CB_2 cyclase assays was confirmed in human CB_2 receptor FLIPR functional assays. No significant species selectivity was observed between the human and rat CB_2 receptors for the ligands tested. Like A-796260, A-836339 exhibited partial agonist efficacy at the rat CB_2 receptor in cyclase assays (E_{max} = 73%) and in FLIPR assays (E_{max} = 60%). L-768,242 displayed inverse agonist efficacies in CB_2 cyclase assays for both human (E_{max} = −21%) and rat (E_{max} = −62%), consistent with the finding of lack of agonist efficacy in the human CB_2 FLIPR assay. In addition, A-836339 displayed weak but full agonist activities at human CB_1 (EC_{50} = 740 nM; E_{max} = 100%) and rat CB_1 (EC_{50} = 1200 nM; E_{max} = 130%) receptors in cyclase assays and full agonist activity in the rat CB_1 FLIPR assay (EC_{50} = 440 nM, E_{max} = 82%).

To investigate receptor specificity of A-836339, pharmacological profilings of A-836339, A-796260, and AM1241 were compared using Cerep binding assay platform containing a panel of 74 GPCRs and ion channels (Table 3). Both A-836339 and A-796260 (tested at 10 μM concentration) exhibited minimal off-target activities, with <50% inhibition for all targets except A_3 and 5-HT_{2C} for A-836339 (55 and 53% radioligand displacement, respectively) and the σ receptor for A-796260 (67% displacement). In contrast, AM1241 exhibited significant binding affinities at nine targets (≥81% radioligand displacement)

TABLE 1
Characterization of compounds in radioligand competition binding assays using [^{3}H]CP 55,940

Ligands	Mean K_i (nM)*, 95% Confidence Interval of K_i Values (nM)					
	hCB_2	hCB_1	hCB_2/hCB_1 Selectivity	rCB_2	rCB_1	rCB_2/rCB_1 Selectivity
A-836339	0.64 0.46–0.90	270 130–590	421	0.76 0.42–1.3	143 66–310	189
A-796260	4.6 2.2–9.6	950 580–1500	206	16 9.2–26	400 170–900	26
JWH-015	35 27–46	1200 760–1900	34	63 40–97	770 490–120	12
AM1241	10 6.8–16	1300 350–4500	125	3.4 1.6–7.3	120 7.7–1700	36
GW-842166X	2000 560–7100	>10000	N.A.	2600 990–6700	>10,000	N.A.
L-768,242 (GW405833)	7.6 2.2–26	1600 1100–2500	210	11 4.7–26	N.D.	N.A.

N.D., not determined; N.A., not applicable.
* All K_i values are derived from at least three studies performed in duplicate.

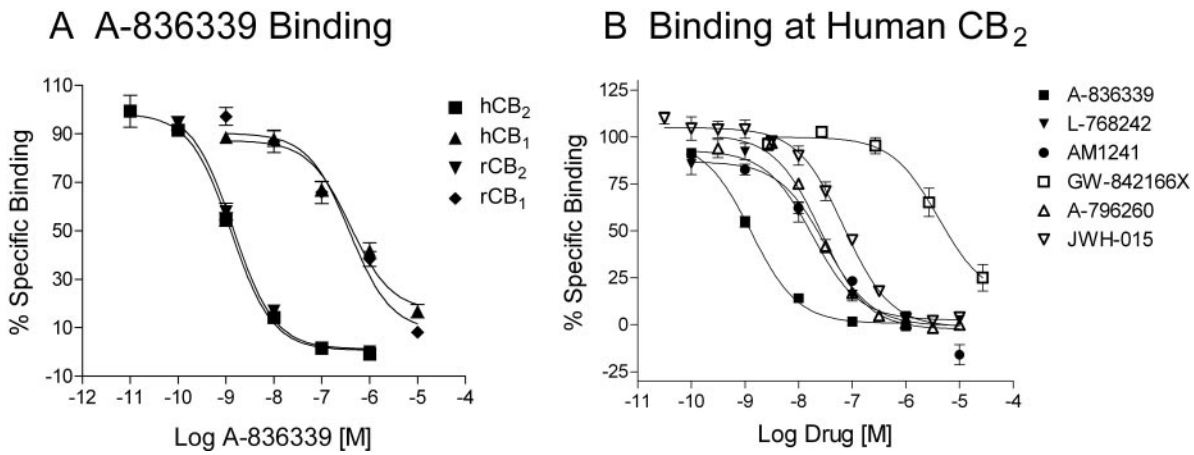

Fig. 2. [^{3}H]CP 55,940 radioligand competition binding assays. A, displacement of radioligand [^{3}H]CP 55,940 binding at human (h) and rat (r) CB_2 and CB_1 receptors by A-836339. B, comparison of ligand affinities at the human CB_2 receptor in [^{3}H]CP 55,940 competition binding assays. Nonspecific binding was defined by 10 μM cold CP 55,940. All data represent at least three independent measurements in duplicate.

TABLE 2
Characterization of compounds in cyclase and FLIPR functional assays

Ligands	Mean EC_{50} (nM)*, E_{max} Relative to CP 55,940; 95% Confidence Interval of EC_{50} (nM)					
	Cyclase Assays				FLIPR Assays	
	hCB_2	hCB_1	rCB_2	rCB_1	rCB_2	rCB_1
A-836339	0.42, 100% 0.14–1.3	740, 100% 430–1300	0.42, 73% 0.090–2.0	1200, 130% 850–1600	5.3, 60% 3.1–9.2	440, 82% 83–2400
A-796260	0.71, 78% 0.32–1.6	980, 120% 620–1600	2.5, 70% 0.86–7.2	290, 97% 210–400	31, 39% 7.9–120	1800, 78% 880–3900
JWH-015	46, 96% 30–72	1100, 94% 450–2800	8.9, 63% 4.2–19	500, 100% 260–960	1100, 58% 550–2000	51% at 10,000
AM1241	>1800	2700, 101% 1700–4100	>1000	2000, 81% 840–4600	>10,000	>22,000
GW-842166X	460, 96% 120–1800	4300, 89% 1600–12,000	100, 89% 48–217	>25000	N.D.	>10,000
L-768,242 (GW405833)	29, −21% 9.5–86	950, 80% 580–1600	2.9, −62% 1.6–5.4	560, 76% 200–1600	N.D.	>10,000

N.D., not determined; N.A., not applicable.
* All EC_{50} values are derived from at least three studies performed in duplicate.

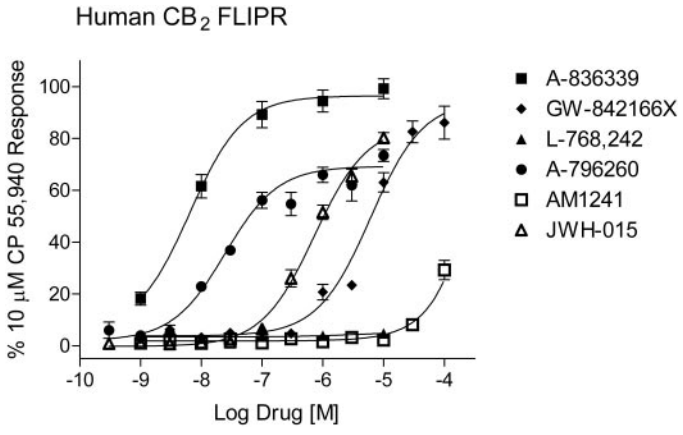

Fig. 3. Comparison of agonist activities at the human CB_2 receptor in FLIPR assays. Receptor activation is expressed as percentage of net peak florescence responses evoked by test compounds relative to that of 10 μM CP 55,940. All data represent at least three independent measurements in duplicate.

including muscarinic receptors M_1, M_3, and M_4, the NK_2 receptor, κ and σ receptors, 5-HT_{1A} and 5-HT_7, and Na^+ channel site 2, moderate affinities at seven targets (61–80%), and lower affinities for 10 targets (51–60%).

Characterization of A-836339 in Preclinical Animal Models of Nociceptive and Neuropathic Pain

CFA Model. In the CFA model, the induction of inflammation and thermal hyperalgesia was achieved by the administration of CFA (150 μl, 50% solution) in the rat hind paw 2 days before the hot-box test. Increased sensitivity to thermal stimulation, expressed as the reduction of PWL on the hot plate, was observed with the ipsilateral paw (PWL = 5.4 s) but not contralateral paw (PWL = 10.4 s) relative to the CFA injection. Systemic administration of A-836339 (1, 3, and 10 μmol/kg i.p.) dose-dependently reversed thermal hyperalgesia by 31, 68, and 80% with an ED_{50} of 1.96 μmol/kg (Fig. 4A) without affecting response time in the uninjured paw. To determine the receptor specificity for the A-836339-evoked antihyperalgesic effect, CB_2 and CB_1 receptor-selective antagonists were employed. Systemic administration of SR144528 (10 μmol/kg i.p.), a CB_2 receptor-selective antagonist, fully reversed the A-836339-evoked antihyperalgesic effect in the CFA model, whereas SR141716A (30 μmol/kg i.p.), a CB_1 receptor-selective antagonist, did not show a significant effect (Fig. 4B). Previous studies from Ibrahim et al. (2005) have demonstrated that the μ-opioid receptor plays an important role in the antinociceptive effect mediated through AM1241. In addition, we have demonstrated previ-

TABLE 3
Cerep profiles

Ligand	Total Targets* Assayed	Percentage Inhibition of Control at 10 μM			
		≤50%	51–60%	61–80%	81–100%
A-836339	74	72	A_3 (55%); 5-HT_{2C} (53%)	0	0
A-796260	74	73	0	σ (67%)	0
AM1241	74	48	α_1 (60%); D3 (51%); D5 (52%); ML_1 (53%); M_2 (58%); δ_2 (57%); 5-HT_3 (53%); 5-HT_{5A} (53%); Ca^{2+} channel (57%); NE transporter (55%)	α_2 (66%); BZD central (62%); CB_1 (65%); D1 (71%); H_1 (66%); H_2 (68%); 5-HT_{2A} (65%)	M_1 (82%); M_3 (87%); M_4 (92%); NK_2 (93%); κ (83%); 5-HT_{1A} (92%); 5-HT_7 (81%); σ (97%); Na^+ channel site 2 (92%)

* A_1 (h); A_{2A} (h); A_3 (h); α_1 (nonselective); α_2 (nonselective); β_1 (h); β_2 (h); AT_1 (h); AT_2 (h); BZD (central); BZD (peripheral); BB (nonselective); B_2 (h); CGRP (h); CB_1 (h); CCK_A (h) (CCK1); CCK_B (h) (CCK2); D1 (h); D2S (h); D3 (h); D4.4 (h); D5 (h); ET_A (h); ET_B (h); GABA (nonselective); GAL1 (h); GAL2 (h); PDGF; IL-8B (h) (CXCR2); TNF-α (h); CCR1 (h); H_1 (h); H_2 (h); MC_4 (h); ML_1; M_1 (h); M_2 (h); M_3 (h); M_4 (h); M_5 (h); NK_1 (h); NK_2 (h); NK_3 (h); Y_1 (h); Y_2 (h); NT_1 (h) (NTS1); δ_2 (h) (DOP); κ (KOP); μ (h) (MOP); ORL1 (h) (NOP); PACAP (PAC1); PCP; TXA_2/PGH_2 (h) (TP); P2X; P2Y; 5-HT_{1A} (h); 5-HT_{1B}; 5-HT_{2A} (h); 5-HT_{2C} (h); 5-HT_3 (h); 5-HT_{5A} (h) (5-ht_{5A}); 5-HT_6 (h); 5-HT_7 (h); σ (nonselective); sst (nonselective); VIP_1 (h) (VPAC1); V_{1a} (h); Ca^{2+} channel (L, verapamil site) (phenylalkylamines); K_V^+ channel; SK_{Ca}^+ channel; Na^+ channel (site 2); Cl^- channel; NE transporter (h); DA transporter (h).

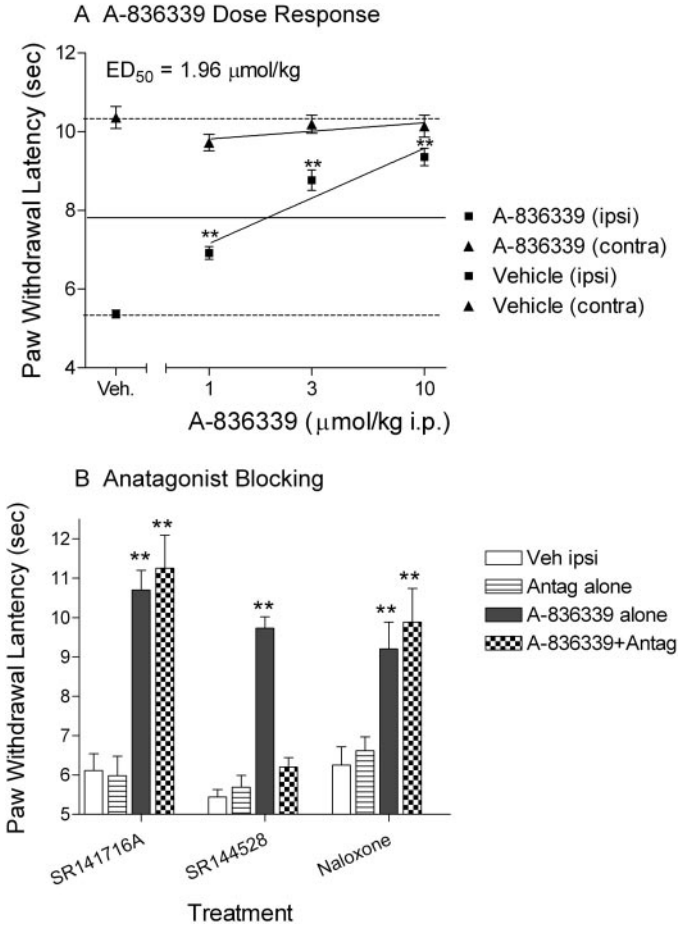

Fig. 4. Efficacy of A-836339 in the CFA-induced inflammatory pain model. Hyperalgesia induced by the injection of CFA in hind paws of animals was demonstrated in the hot-box test shown by the reduction of PWL (seconds) in the paws ipsilateral (■), but not contralateral (▲), to the CFA injection side. A, dose-dependent reversal of the decreased PWL of ipsilateral paws upon A-836339 treatment (1, 3, and 10 μmol/kg i.p.) versus vehicle treatment (Veh). B, antagonism of the effect of A-836339 (10 μmol/kg i.p.) by SR144528 (10 μmol/kg i.p.), SR141716A (30 μmol/kg i.p.), and naloxone (10 μmol/kg i.p.). Responses of only the ipsilateral paws of the treated animals were shown. Responses of the respective contralateral paws of all treatment groups are similar to those of the vehicle-treated contralateral paws (data not shown). Data are expressed as means ± S.E.M., with $n \geq 6$. **, $p < 0.01$ versus vehicle control.

ously that the antihyperalgesic effects of AM1241 in the CFA model are antagonized by naloxone (Yao et al., 2008). The involvement of the μ-opioid receptor in A-836339-evoked antihyperalgesic effect was investigated in the current studies. Naloxone (10 μmol/kg i.p.) did not antagonize the A-836339-evoked antihyperalgesic effect in the CFA model (Fig. 4B).

Skin Incision Model. To assess the efficacy of A-836339 in modulating postoperative pain, the rat skin incision model was employed (Brennan et al., 1996). Mechanical allodynia was induced within 2 h after the incision surgery for the ipsilateral paw relative to the uninjured contralateral side as evidenced by a significant reduction of paw withdrawal threshold (PWT = 1.9 g) upon von Frey stimulation, compared with the contralateral paw (PWT = 15 g) (Fig. 5). The efficacy of A-836339 was evaluated at 2- or 24-h time points after the incision surgery. Systemic administration of A-836339 (3, 10, and 30 μmol/kg i.p.) administered 30 min before efficacy testing reversed mechanical allodynia of the ipsilateral paw by 23, 53, and 60% at 2 h after the incision surgery with an ED_{50} of 12.6 μmol/kg. Likewise, at 24 h after the incision surgery, A-836339 reversed mechanical allodynia of the ipsilateral paw by 21, 41, and 73% with an ED_{50} of 12.0 μmol/kg.

Capsaicin-Induced Secondary Mechanical Hyperalgesia Model. The capsaicin-induced secondary mechanical hyperalgesia (Cap-SMH) model has been proposed as a surrogate model predictive of the efficacy of known drugs that have exhibited utility in the treatment of neuropathic pain (Joshi et al., 2006) and has been characterized as a model of central sensitization. Capsaicin injection (10 μg in 10 μl of 10% ethanol/hydroxyl-β-cyclodextrin) in the rat hind paw induced mechanical allodynia, as shown by the significant reduction of PWT (5.5 g) in the ipsilateral paw upon von Frey stimulation. Systemic administration of A-836339 (3, 10, and 30 μmol/kg i.p.) significantly reversed the capsaicin-induced reduction of PWT by 26, 55, and 73%, respectively, with an ED_{50} of 10.4 μmol/kg (Fig. 6).

CCI Model of Neuropathic Pain. A-836339 was assessed in the rat CCI model of neuropathic pain. In the CCI model, 2 weeks after nerve injury, significant mechanical allodynia developed at the ipsilateral paw (PWT = 2.8 g), whereas no significant change to pain stimulation was observed on the contralateral paw (data not shown). Acute systemic administration of A-836339 (3, 10, and 30 μmol/kg i.p.) 30 min before von Frey testing dose-dependently reversed mechanical allodynia by 15, 38, and 75%, with an ED_{50} value of 12.9 μmol/kg (Fig. 7). Gabapentin (500 μmol/kg i.p., positive control) exhibited a statistically significant 54% reversal of allodynia. The A-836339 (30

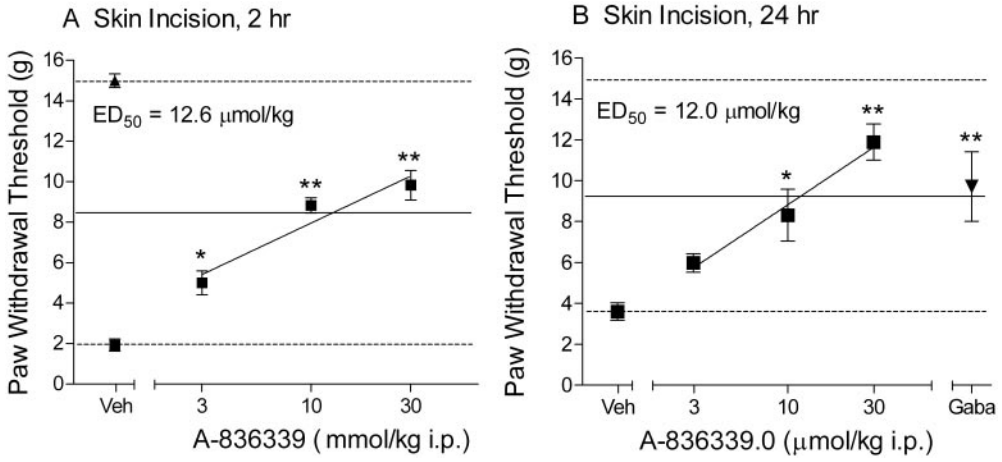

Fig. 5. Efficacy of A-836339 in skin incision postoperative pain model. Mechanical allodynia was induced by an incision surgery. Dose-dependent response of mechanical allodynia expressed as PWT (grams) for ipsilateral paws (■) and contralateral paws (▲) of A-836339-treated (1, 3, and 10 μmol/kg i.p.) versus vehicle-treated (Veh) animals in skin incision models at 2 (A) or 24 h (B) after the incision surgery. Gabapentin (▼, Gaba, 500 μmol/kg i.p.) was used as a positive control. Responses of ipsilateral paws for drug-treated animals were shown. Responses of the respective contralateral paws of each treatment group are similar to those of the vehicle-treated contralateral paws (data not shown). Data are expressed as means ± S.E.M., with $n \geq 6$. **, $p < 0.01$ versus vehicle control; *, $p < 0.05$ versus vehicle control.

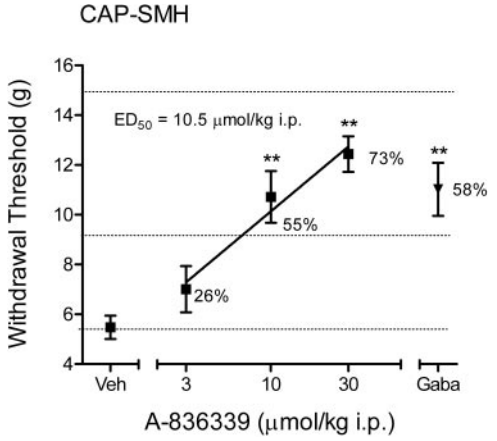

Fig. 6. Efficacy of A-836339 in the Cap-SMH model. Dose-dependent response of mechanical allodynia expressed as PWT (grams) for ipsilateral paws (■) of A-836339-treated (3, 10, and 30 μmol/kg i.p.) versus vehicle-treated (Veh) animals in the Cap-SMH model. Gabapentin (▼, Gaba, 500 μmol/kg i.p.) was used as a positive control. Responses of ipsilateral paws for drug-treated animals were shown. Responses of the respective contralateral paws of each treatment group are similar to those of the vehicle-treated contralateral paws (data not shown). Data are expressed as means ± S.E.M., with $n = 12$. **, $p < 0.01$ versus vehicle control.

μmol/kg i.p.)-evoked antiallodynic effect in the CCI model is fully reversed by the CB_2 receptor-selective antagonist SR144528 (10 μmol/kg i.p.).

Opioids have been well characterized with respect to the rapid development of tolerance (within 5 days) after repeat dosing in rodent pain models (Buntin-Mushock et al., 2005). To assess the potential for the development of tolerance, A-836339 was evaluated in a repeat dosing paradigm in the CCI neuropathic pain model (5 days, b.i.d. treatment at 10 μmol/kg i.p.). Mechanical allodynia was assessed at days 1, 3, and 5 for animals that either received vehicle or A-836339 throughout the 5-day treatment. Vehicle-treated animals exhibited allodynia at days 1, 3, and 5, with a PWT of 2.9, 2.3, and 2.3 g, respectively (Fig. 8). A-836339-treated animals demonstrated reversal of allodynia in the ipsilateral paw at days 1, 3, and 5, with a PWT of 8.5, 6.7, and 9.3 g, respectively, exhibiting no loss of efficacy over time. Animals that received vehicle for the first 4 days and A-836339 on day 5 exhibited a PWT of 6.4 s, which was not significantly different from the PWT of the acute A-836339 treatment (day 1, PWT = 8.5 s). Animals that received A-836339 for the first 4 days and vehicle on day 5 exhibited allodynia (PWT = 3.2 g), similar to the vehicle-treated group (PWT = 2.3 g). Gabapentin (500 μmol/kg i.p.) was administered on days 1, 3, and 5 as a positive control. Under identical testing conditions, the antiallodynic effects of morphine have been shown previously to fully tolerate (Contet et al., 2008).

Assessment of the Effect of A-836339 in the Rotorod Test of Motor Coordination. Nonselective cannabinoid agonists are well characterized to produce deficits in motor coordination within an analgesic dose range, an effect attributed to activation of the CB_1 receptor subtype (Fox et al., 2001). A-836339 was evaluated for effects on motor coordination in the Rotorod test across the behaviorally effective doses used in the pain assays. In the Rotorod test, animals treated with A-836339 up to 45 μmol/kg i.p., a supratherapeutic dose in the pain models, displayed normal motor coordination function compared with vehicle-treated group, as measured by the mean time animals stayed on the bar (Fig. 9).

Assessment of the Effect of A-836339 on Horizontal Locomotor Activity. Because activation of the central CB_1 receptor also has been demonstrated to be associated with sedation and immobility, A-836339 was evaluated for effects on horizontal locomotor activity for an initial 30-min period after being placed in a novel environment. Compared with vehicle-treated animals, A-836339-treated animals (15 and 45 μmol/kg i.p.) produced dose-related reduction in horizontal motor activity by 30 and 70%, respectively (Fig. 10A). The reduction of locomotor activity was blocked by the treatment of the CB_1 receptor antagonist, SR141716A (30 μmol/kg i.p.) (Fig. 10B).

phMRI Imaging. To assess the activation of central CB_1 receptors evoked by treatment with A-836339 at a higher dose, phMRI was employed to visualize regional changes in cerebral blood volume. A high dose of A-836339 (10 μmol/kg i.v.) produced a CNS activation pattern consistent with that

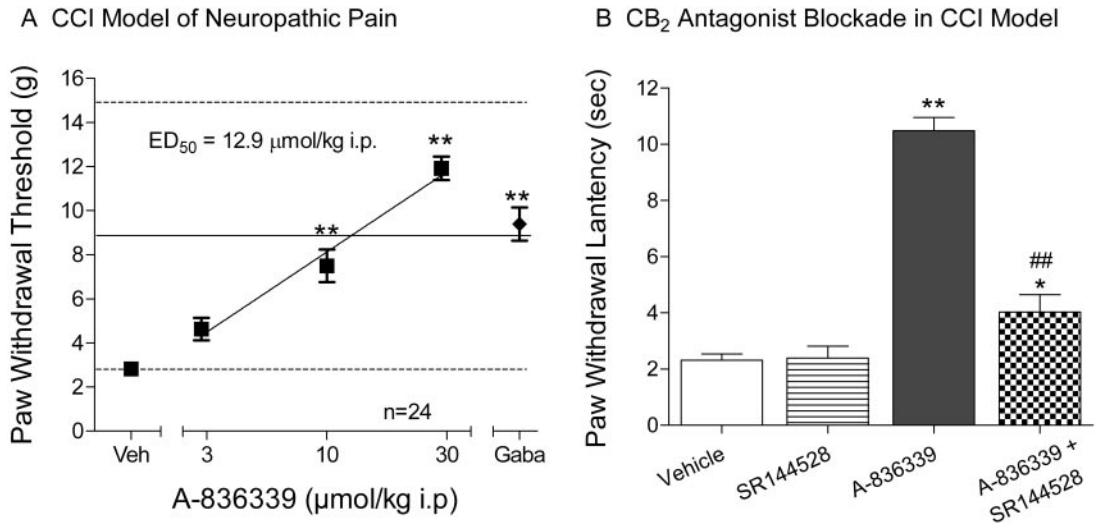

Fig. 7. Efficacy of A-836339 in CCI model of neuropathic pain. A, dose-dependent response of mechanical allodynia expressed as PWT (g) for ipsilateral paws (■) of A-836339-treated (3, 10, and 30 μmol/kg i.p.) versus vehicle-treated (Veh) animal. Gabapentin (▼, Gaba, 500 μmol/kg i.p.) was used as a positive control. Responses of ipsilateral paws for drug-treated animals were shown. Responses of the respective contralateral paws of each treatment group are similar to those of the vehicle-treated contralateral paws (data not shown). Data are expressed as means ± S.E.M. (n = 24). B, reversal of A-836339 (30 μmol/kg i.p.) antiallodynic effect by CB_2 antagonist SR144528 (10 μmol/kg i.p.). **, $p < 0.01$ versus vehicle control; *, $p < 0.05$ versus vehicle control; ##, $p < 0.01$ versus A-836339 treatment group.

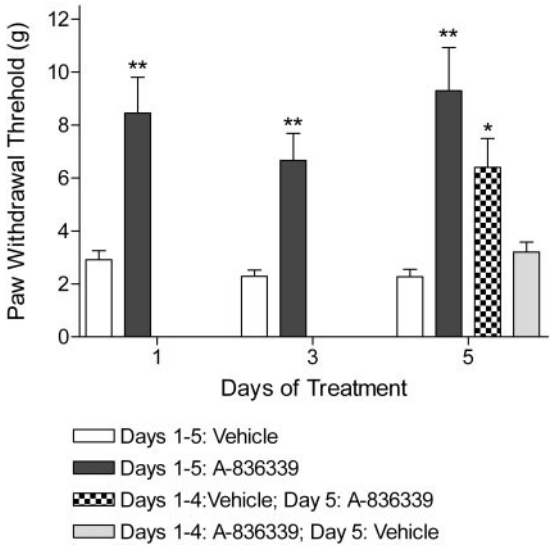

Fig. 8. Lack of tolerance during 5-day treatment with A-836339 in the CCI model of neuropathic pain. Dose-dependent response of mechanical allodynia expressed as PWT (g) for ipsilateral paws of animals treated with vehicle for 5 days, A-836339 (15 μmol/kg i.p. b.i.d.) for 5 days, vehicle on days 1 to 4 and A-836339 (15 μmol/kg i.p.) on day 5, and A-836339 (15 μmol/kg i.p. b.i.d.) on days 1 to 4 and vehicle on day 5. Responses of ipsilateral paws of all treatment groups were shown. Responses of the respective contralateral paws of each treatment group are similar to those of the vehicle-treated contralateral paws (data not shown). Data are expressed as means ± S.E.M., with n = 6 for each treatment group. **, $p < 0.01$ versus vehicle control; *, $p < 0.05$ versus vehicle control.

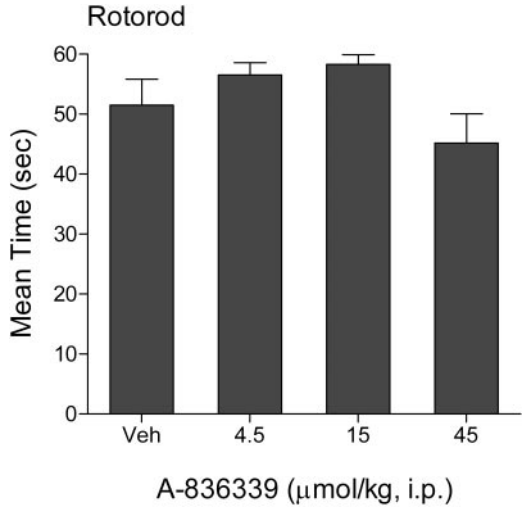

Fig. 9. Lack of performance deficit of A-836339 in the Rotorod test. Animals were treated with vehicle (Veh) or A-836339 (4.5, 15, and 45 μmol/kg i.p.) 30 min before testing. The mean time (seconds) that animals stayed on the rod within 60 s was recorded. Data are expressed as means ± S.E.M., with n = 6.

of A-834735, a previously characterized, nonselective agonist that activates the CB_1 receptor (Chin et al., 2008) (Fig. 10C). A lower dose of A-836339 (3 μmol/kg i.v.) did not produce a significant CNS activation pattern. To study the receptor specificity in CNS activation, the CB_1-selective antagonist, SR141716A, and the CB_2 receptor-selective antagonist, AM630, were employed. Although SR141716A (13 μmol/kg i.p.) or AM630 (6 μmol/kg i.p.) infusion did not produce an effect alone, SR141716A, but not AM630, completely reversed the CNS activation pattern induced by A-836339.

Discussion

Recent advances in pain research have led to the identification of novel molecular targets for treatment of nociceptive and neuropathic pain states (Gillen and Maul, 2002). Over the past several years, a number of CB_2 subtype-selective ligands have been identified and characterized in preclinical models of inflammatory and neuropathic pain, and from a preclinical perspective, the potential utility of CB_2-selective agonists has been well established (Guindon and Hohmann, 2008). However, both the site and mechanism of action of CB_2-mediated efficacy in pain modulation remains pooly understood. CB_2-selective ligands, such as AM1241, L-768,242, and A-796260, have demonstrated efficacy in a variety of preclinical pain models, yet appear to be mechanistically distinct with respect to in vitro agonist efficacy profile, site,

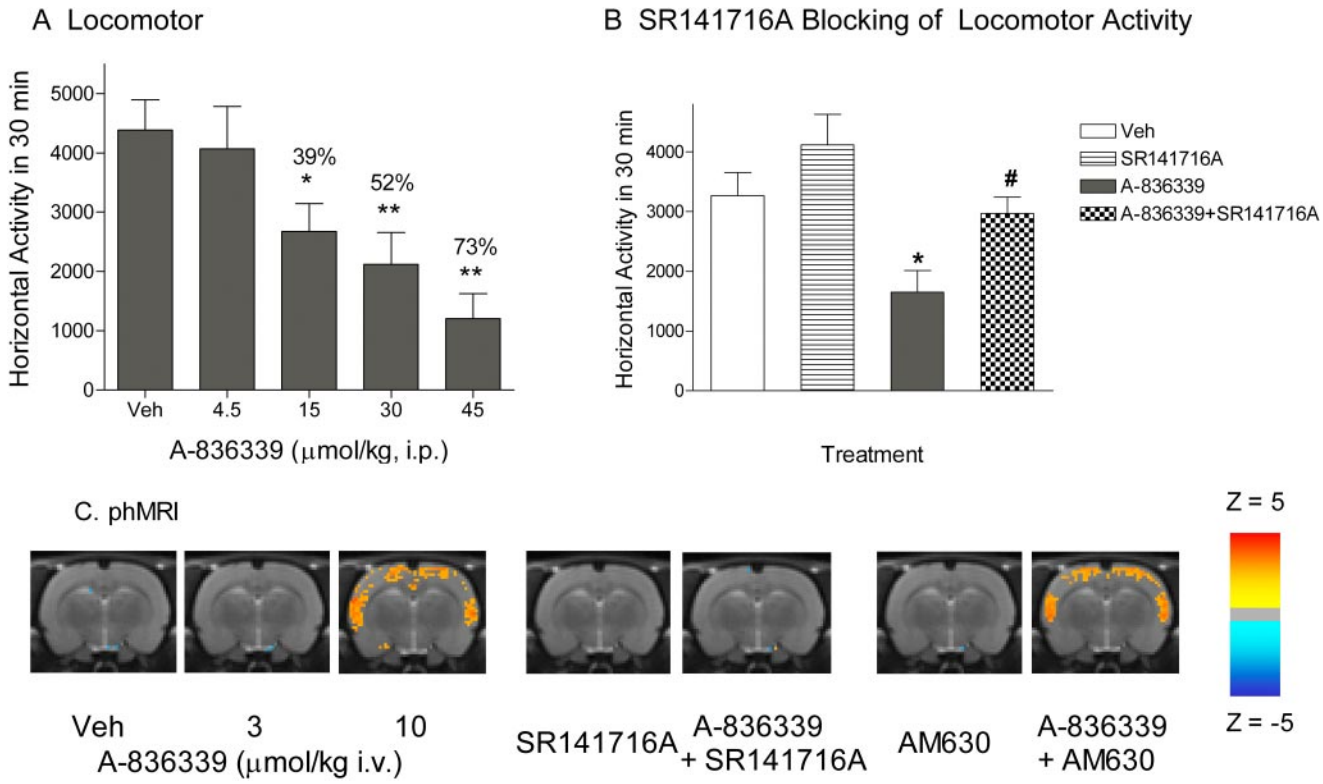

Fig. 10. Locomotor deficit and CNS activation by A-836339 is mediated by the CB_1 receptor. A, dose-dependent responses of locomotor deficit induced by treatment with A-836339. B, reversal of A-836339-mediated effect (45 μmol/kg i.p.) by SR141716A. C, group average phMRI maps (z score; threshold, $z > 1.96$; *, $p < 0.05$; $n = 5$ per group; one of 13 brain slices) obtained from vehicle, A-836339 treatments (3, 10 μmol/kg i.v.), or A-836339 treatment (10 μmol/kg i.v.) in the presence of CB_1 receptor-selective antagonist (SR141716A, 13 μmol/kg i.p.) or CB_2 (AM630, 6 μmol/kg i.p.). **, $p < 0.01$ versus vehicle control; *, $p < 0.05$ versus vehicle control.

and mechanism of action. As a consequence, new and structurally diverse ligands will continue to be important tools to further the understanding of this potentially important new class. Here, we present the characterization of A-836339 in in vitro radioligand binding and functional assays and in in vivo animal models of nociceptive and neuropathic pain.

Relative to other known CB_2-selective ligands, A-836339 displayed a pharmacological profile of high potency and full agonist efficacy at human and rat CB_2 receptors and improved selectivity versus human and rat CB_1 receptors. In Cerep (http://www.cerep.fr) radioligand binding assays that were used to assess receptor selectivity against a panel of 74 GPCRs/ion channels, A-836339 was shown to exhibit relatively few off-target interactions, which is in contrast to the CB_2-selective ligand, AM1241, which exhibited significant radioligand binding affinity to a large number of additional GPCR and ion channel targets (Table 3). Interestingly, A-836339, although exhibiting significant binding affinity at the human CB_1 receptor (K_i = 270 nM) in competition binding assays using [^{3}H]CP 55,940 (Table 1) did not significantly displace [^{3}H]WIN 55,212-2 (38% at 10 μM), a radioligand used in Cerep CB_1 radioligand binding assays. It is possible that the WIN 55,212-2 binding site only partially overlaps with the CP 55,940 site (Monory et al., 2002) and, therefore, results in an inefficient displacement by A-836339.

In this study, A-836339 exhibited a broad spectrum of efficacy in in vivo animal models of nociceptive, postsurgical, and neuropathic pain and a capsaicin-induced model of central sensitization. A-836339 was highly efficacious in the CFA model of chronic inflammatory pain. Although A-836339 has not been tested in acute nociceptive pain models, it is fully efficacious in reversing the CFA-induced temperature sensitivity of the injured paw without affecting that of the uninjured paw, indicating that A-836339 is antihyperalgesic in this model, with little effect on normal nociceptive processing. Furthermore, the A-836339-evoked effects were blocked by a CB_2, but not a CB_1, receptor-selective antagonist, indicating that the antihyperalgesic effects of A-836339 are mediated through the CB_2, but not the CB_1, receptor. In addition, unlike AM1241 (Ibrahim et al., 2005; Yao et al., 2008), the effects evoked by A-836339 do not involve the μ-opioid receptor, a finding similar to those previously reported for A-796260 (Yao et al., 2008) and L-768,242 (Whiteside et al., 2005). Although none of these ligands exhibits appreciable binding affinity to the μ-opioid receptor (IC_{50} > 10 μM), AM1241-mediated effect is fully blocked by naloxone. Given the distinct Cerep binding profiles of AM1241 from those of A-836339 and A-796260, it is possible that AM1241 may interact with additional targets that may contribute to the antinociceptive efficacy through an indirect regulation of the opioid receptor pathway.

In the postsurgical incision pain model (Brennan et al., 1996), A-836339 was efficacious at either 2 or 24 h postsurgery, demonstrating the potential utility of CB_2 receptor agonists in postoperative pain. Capsaicin-induced secondary mechanical or thermal hyperalgesia models have been used to assess the efficacy of compounds in reducing central sensitization. It has been demonstrated that the pharmacological mechanism underlying capsaicin-induced secondary mechanical hyperalgesia also contributes to certain neuronal

mechanisms underlying neuropathic pain states (Joshi et al., 2006). In the CCI model of neuropathic pain, A-836339 demonstrated efficacies consistent with those of CFA, skin incision, and Cap-SMH. In a previous report, a lack of tolerance to A-796260 has been demonstrated in the skin incision model after chronic dosing for 5 days (Yao et al., 2008). In this study, we demonstrated that there was no development of tolerance in the CCI model after chronic dosing of A-836339 for 5 days. In addition, after 4 consecutive days of A-836339 administration, replacing A-836339 with vehicle on the 5th day of treatment, animals restored hyperalgesia to a similar level as untreated animals, indicating a lack of drug accumulation during the chronic treatment.

The Rotorod test was employed to determine whether motor deficits may be generating a false positive in the pain behavior studies and to assess whether CB_1 receptor activation was occurring within the analgesic dose range. The lack of deficit in Rotorod performance for A-836339 at a dose (45 μmol/kg i.p.) higher than that used in the pain efficacy studies demonstrates that the reversal of PWL and PWT reduction induced by pain states was not because of impaired motor function for these animals. These data, in combination with the CB_1 and CB_2 receptor antagonist blocking studies, further confirm that the restoration of PWL and PWT in rat pain models after the administration of A-836339 is mediated through the activation of the CB_2 receptor.

Although A-836339 has been shown in the current study to be a CB_2 receptor-selective agonist, it does display weak agonist properties at the rat CB_1 receptor in in vitro cyclase and FLIPR functional assays. The activation of CB_1 receptors by A-836339 in vivo was demonstrated by the CB_1 receptor-mediated reduction of locomotor activity. Although Rotorod performance is commonly used to assess CB_1-mediated CNS effects, locomotor deficit appears to be more sensitive for use in detecting the effect of activation of the central CB_1 receptor. In addition, A-836339, after intravenous administration (10 μmol/kg i.v.), produced a CNS activation pattern shown by phMRI consistent with the CB_1 receptor localization (Chin et al., 2008), with corresponding plasma and brain drug levels of 193 and 408 nM, respectively (data not shown). The exposure of A-836339 in the brain is consistent with a concentration that would be anticipated to activate the CB_1 receptor in the CNS. In addition, the CNS activation patterns observed in the phMRI studies with A-836339 have been demonstrated to be CB_1 receptor-mediated because only the CB_1 receptor-selective antagonist, SR141716A, but not the CB_2 receptor-selective antagonist, AM630, was able to block these effects. It is worth mentioning that A-836339, at a lower dose (3 μmol/kg i.v.), achieved a level that is sufficient to activate CB_2 receptors, but not CB_1 receptors, and at such a level, A-836339 failed to produce a positive CNS activation pattern, suggesting that the phMRI signal observed at a higher dose of A-836339 is mediated through the CB_1 receptor, not CB_2. Therefore, the lack of CNS activation with the lower dose of A-836339 demonstrates that there is unlikely a functional CB_2 receptor in the CNS, consistent with the findings using AM1241 (Chin et al., 2008) and other CB_2 receptor-selective compounds (not shown). In addition, plasma exposures of A-836339 observed in various pain models are significantly lower (e.g., plasma level of A-836339 at ED_{50} in the CFA model is 20 nM) than those achieved from all doses used in phMRI studies. As a consequence, these data provide additional evidence that the antiallodynic and antihyperalgesic effects evoked by A-836339 are not mediated through central CB_1 receptor activation. Additional evidence supporting that the antiallodynic effects are CB_2 mediated was provided by McGaraughty et al. (2008). Using electrophysiology techniques, these authors demonstrated that in the spinal nerve ligation model of neuropathic pain, both evoked and spontaneous firing of wide dynamic range neurons are reversed by A-836339, and these effects are selectively blocked by the CB_2 antagonist SR144528, but not by CB_1 antagonist SR141716A.

Despite the confirmation of receptor specificity for in vivo pain efficacy tests and in vivo CNS effect, it is intriguing that the high level of selectivity of A-836339 for the CB_2 receptor over the CB_1 receptor (189-fold) defined in in vitro assays is reduced significantly in animal models. This is particularly evident in the effects of A-836339 on horizontal motor activity, where significant effects are observed at relatively low multiples of the analgesic doses. This may be in part because of the abundant expression of the CB_1 receptor in the CNS tissues that leads to a significantly enhanced ligand sensitivity of the CB_1 receptor in these tissues and, therefore, CB_1 receptor-mediated biological effects in animals.

In summary, A-836339 has been characterized extensively in in vitro binding and functional assays and in vivo pain models and in CNS behavior tests and phMRI studies. A-836339 demonstrates high affinities and agonist potencies at CB_2 receptors and an excellent selectivity against the CB_1 receptors and other targets in the Cerep panel. It does not show significant human and rat species differences for both the CB_1 and CB_2 receptors. A-836339 demonstrates a CB_2 receptor-mediated broad spectrum of efficacy in in vivo animal models of nociceptive pain, neuropathic pain, and post-surgical pain, including CFA, Cap-SMH, and CCI models, and the skin incision model. Therefore, A-836339 provides a useful tool for studying the CB_2 receptor pharmacology in vitro and for use of interrogating the biological consequence of CB_2 receptor activation in animals.

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