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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-3H-thiazol-(2Z)-ylidene]-amide], a potent and selective CB$_2$ agonist as characterized in in vitro pharmacological assays and 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 constriction 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 several years.

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-(4-chloro-5-nitrophenyl)-1-[1-(2,3-dichloro-phenyl)-5-methoxy-2-methyl-3-(2-morpholin-4-yl-ethyl)-1-naphthalenyl]-methanone; JWH-015, [2-(methyl-1-propyl-1H-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)-1H-indol-3-yl]-5-hydroxy-2-(3-hydroxy-propyl)-cyclohexyl]-phenol; AM630, [6-iodo-2-methyl-1-[2-(4-morpholinyl)-ethyl]-1H-indol-3-yl]-4-(4-methylphenyl)-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 constriction 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 CB1 and CB2 receptors are G protein-coupled GPCRs, whose activation negatively couples to adenylyl cyclase (Felder et al., 1995; Slivetz et al., 1995). CB1 and CB2 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 CB1 receptor, which is abundantly expressed in CNS tissues. In rodents, the CB1-mediated effects of cannabinoids are manifested as decreased locomotor activity and coordination, catalepsy, and hypothermia. In contrast, the CB2 receptor is expressed primarily in the normal peripheral immune tissues (Galiègue et al., 1995). Recent reports have indicated that the CB2 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). CB2 receptor activation is not expected to mediate the CNS side effects observed for nonselective cannabinoid ligands. Recent findings have demonstrated CB2 receptor activation modulates pain perception in rodents (Malan et al., 2001; Hofmann et al., 2004), suggesting CB2 receptor agonists may offer an attractive approach for the development of therapeutic agents devoid of psychotrop effects for treatment of pain. As a consequence, significant progress has been made toward the identification of CB2-selective ligands, including a clinical candidate, GW-842166X, as a novel CB2 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 CB2 receptor exhibiting improved in vitro selectivity versus the CB1 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 CB2, rat CB2, or rat CB1 receptors and HEK cells coexpressing the chimeric Gαsop5 protein with either the human or rat CB2 receptors were generated and maintained as described previously (Yao et al., 2006). The Chinese hamster ovary cell lines stably expressing the human CB2 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 CB2, rat CB1, or rat CB2 receptor and Chinese hamster ovary cells stably expressing the human CB2 receptor and competition binding assays were performed as described previously (Yao et al., 2006, 2008) using 0.5 nM 3H/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 MgCl2, 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 filters and counted in a scintillation counter.

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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 CB2 and 37 μM for human CB2 and CB3) 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 a percentage response compared with that of 10 μM CP 55,940. EC50 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 Gsalpha protein with either human or rat CB2 receptors (Yao et al., 2006). 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 MgCl2, 5 mM KCl, 2 mM CaCl2, and 0.05% BSA). Peak responses were compared with that of 10 μM CP 55,940 and expressed as percentages of the CP 55,940-evoked response. EC50 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 stimulus (UARDG; University of California, San Diego, CA) described by Hargreaves et al. (1988). In brief, rats were habituated for 20 min in individual plastic cubes 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 1-g threshold was used as the maximal possible effect in this assay.

Chronic Constriction Injury Model of Neuropathic Pain. A chronic constriction 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 at ASPET Journals on July 10, 2017 jpet.aspetjournals.org Downloaded from
characterization. ED<sub>50</sub> 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 [3H]CP 55,940 in competition binding assays using membranes prepared from recombinant HEK cells stably expressing the CB<sub>1</sub> or CB<sub>2</sub> receptor from human and rat. A-836339 exhibited high potencies at both human and rat CB<sub>2</sub> receptors, with K<sub>i</sub> values of 0.64 and 0.76 nM, respectively (Table 1; Fig. 2), and 425- and 189-fold selectivity for the CB<sub>2</sub> receptor over the CB<sub>1</sub> receptor in human and rat, respectively. In addition, A-836339 did not exhibit significant species selectivity for either CB<sub>1</sub> or CB<sub>2</sub> receptors in human and rat. The rank order of affinities at the human CB<sub>2</sub> receptor was A-836339 > JWH-015 > GW-842166X, and the rank order of selectivity at CB<sub>2</sub> over CB<sub>1</sub> was A-836339 > A-796260 > L-768,242 > AM1241 > JWH-015 > GW-842166X, and the rank order of selectivity at CB<sub>2</sub> over CB<sub>1</sub> was A-836339 > A-796260 > L-768,242 > AM1241 > JWH-015. GW-842166X showed weak binding affinities at both human and rat CB<sub>2</sub> and no detectable affinity at CB<sub>1</sub> 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 [3H]DAMGO binding assays. A-836339 was characterized in a similar assay, and like A-796260 and AM1241, A-836339 also failed to displace [3H]DAMGO at the μ-opioid receptor at concentrations up to 10 μM (data not shown), whereas the μ-opioid receptor ligand naltrexone exhibited a K<sub>i</sub> 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<sub>2</sub> receptor cyclase assays, A-836339 exhibited full agonist efficacy (E<sub>max</sub> = 102%) with comparable potency (EC<sub>50</sub> = 1.6 nM) as A-796260 (EC<sub>50</sub> = 0.71 nM), greater potency than other CB<sub>2</sub>-selective ligands including JWH-015 (EC<sub>50</sub> = 46 nM) and GW-842166X (EC<sub>50</sub> = 460 nM). A similar rank order of potencies (A-836339 > A-796260 > JWH-015 > GW-842166X) in human CB<sub>2</sub> cyclase assays was confirmed in human CB<sub>2</sub> receptor FLIPR functional assays. No significant species selectivity was observed between the human and rat CB<sub>2</sub> receptors for the ligands tested. Like A-796260, A-836339 exhibited partial agonist efficacy at the rat CB<sub>2</sub> receptor in cyclase assays (E<sub>max</sub> = 73%) and in FLIPR assays (E<sub>max</sub> = 60%). L-768,242 displayed inverse agonist efficacies in CB<sub>2</sub> cyclase assays for both human (E<sub>max</sub> = −21%) and rat (E<sub>max</sub> = −62%), consistent with the finding of lack of agonist efficacy in the human CB<sub>2</sub> FLIPR assay. In addition, A-836339 displayed weak but full agonist activities at human CB<sub>1</sub> (EC<sub>50</sub> = 740 nM; E<sub>max</sub> = 100%) and rat CB<sub>1</sub> (EC<sub>50</sub> = 1200 nM; E<sub>max</sub> = 130%) receptors in cyclase assays and full agonist activity in the rat CB<sub>1</sub> FLIPR assay (EC<sub>50</sub> = 440 nM, E<sub>max</sub> = 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<sub>2a</sub> and 5-HT<sub>2C</sub> 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

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Mean K&lt;sub&gt;i&lt;/sub&gt; (nM)*, 95% Confidence Interval of K&lt;sub&gt;i&lt;/sub&gt; Values (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-836339</td>
<td>0.64, 0.46–0.90, 130–590, 421, 0.42–1.3, 66–310, 143, 189</td>
</tr>
<tr>
<td>A-796260</td>
<td>4.6, 2.2–9.6, 580–1500, 206, 16, 400, 26</td>
</tr>
<tr>
<td>JWH-015</td>
<td>35, 27–46, 760–1900, 34, 63, 770, 12</td>
</tr>
<tr>
<td>AM1241</td>
<td>10, 6.8–16, 350–450, 125, 3.4, 120, 36</td>
</tr>
<tr>
<td>GW-842166X</td>
<td>2000, &gt;10000, 560–7100, N.A., 2600, &gt;10000, N.A.</td>
</tr>
<tr>
<td>L-768,242 (GW405833)</td>
<td>7.6, 2.2–26, 1100–2500, 210, 11, 4.7–26</td>
</tr>
</tbody>
</table>

N.D., not determined; N.A., not applicable.

* All K<sub>i</sub> values are derived from at least three studies performed in duplicate.
including muscarinic receptors M1, M3, and M4, the NK2 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 ED50 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, CB2 and CB1 receptor-selective antagonists were employed. Systemic administration of SR144528 (10 μmol/kg i.p.), a CB2 receptor-selective antagonist, fully reversed the A-836339-evoked antihyperalgesic effect in the CFA model, whereas SR141716A (30 μmol/kg i.p.), a CB1 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-
The antihyperalgesic effects of AM1241 in the CFA model are antagonized by naloxone (Yao et al., 2008). 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 ED50 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 ED50 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 (Tao et al., 2006) and has been characterized as a model of central sensitization. Capsaicin injection (10 μg in 10 μl of 10% ethanol/hydroxypropyl-β-cyclodextrin) in the 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 ED50 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 ED50 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

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**TABLE 3**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Total Targets Assayed</th>
<th>Percentage Inhibition of Control at 10 μM</th>
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<tbody>
<tr>
<td></td>
<td>≤50%</td>
<td>51–60%</td>
</tr>
<tr>
<td></td>
<td>61–80%</td>
<td>81–100%</td>
</tr>
<tr>
<td>A-836339</td>
<td>74</td>
<td>72</td>
</tr>
<tr>
<td>A-796260</td>
<td>74</td>
<td>73</td>
</tr>
<tr>
<td>AM1241</td>
<td>48</td>
<td>α1 (60%); D3 (51%); D5 (52%); ML1 (53%); M1 (58%); M2 (57%); 5-HT3 (53%); 5-HT2A (53%); Ca2+ channel (57%); NE transporter (55%)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>σ (67%)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>M1 (92%); M2 (87%); M3 (92%); M4 (92%); NK1 (93%); x (83%); 5-HT1A (92%); 5-HT1B (81%); α (97%); Na+ channel site 2 (92%)</td>
</tr>
</tbody>
</table>

* A1 (h); A2A (h); A3 (h); α1 (nonselective); α2 (nonselective); β1 (h); β2 (h); AT1 (h); AT2 (h); BDZ (central); BDZ (peripheral); BB (nonselective); B2 (h); CGRP (h); CB1 (h); CCK1 (h); CCK2 (h); D1 (h); D2 (h); D3 (h); D5 (h); D2S (h); D3 (h); D4 (h); D5 (h); ET1 (h); ET2 (h); GABA (nonselective); GALL1 (h); GAL2 (h); PDGF; IL-8 (h); CCK2R; TNF-α (h); CR1 (h); H2 (h); H3 (h); MC1 (h); MC4 (h); ML1; M1 (h); M2 (h); M3 (h); M4 (h); M5 (h); NK1 (h); NK2 (h); NK3 (h); Y1 (h); Y2 (h); NT (h); NT1 (h); Y1 (h); Y2 (h); NT (h); NT1 (h); NT2 (h); NT3 (h); 5-HT1A (h); 5-HT1B; 5-HT2A (h); 5-HT2C (h); 5-HT3 (h); 5-HT5A (h); 5-HT6 (h); 5-HT7 (h); 5-HT3 (h) (500 μmol/kg); Naloxone (10 μmol/kg i.p.) did not antagonize the A-836339-evoked antihyperalgesic effect in the CFA model (Fig. 4B).
expressed as means ± S.E.M., with n = 6. **, p < 0.01 versus vehicle control; *, p < 0.05 versus vehicle control.

μmol/kg i.p.)-evoked antiallodynic effect in the CCI model is fully reversed by the CB2 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 CB1 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 CB2 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 CB2 receptor antagonist, SR141716A (30 μmol/kg i.p.) (Fig. 10B).

phMRI Imaging. To assess the activation of central CB2 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
of A-836339, a previously characterized, nonselective agonist that activates the CB₁ 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 CB₁-selective antagonist, SR141716A, and the CB₂ 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 CB₂ 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 CB₂-selective agonists has been well established (Guindon and Hohmann, 2008). However, both the site and mechanism of action of CB₂-mediated efficacy in pain modulation remains poorly understood. CB₂-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,
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 CB2-selective ligands, A-836339 displayed a pharmacological profile of high potency and full agonist efficacy at human and rat CB2 receptors and improved selectivity versus human and rat CB1 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 CB2-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 CB2 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 $\mu M$), a radioligand used in Cerep CB1 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 CB2, but not a CB1, receptor-selective antagonist, indicating that the antihyperalgesic effects of A-836339 are mediated through the CB2, but not the CB1, 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$_{50} > 10$ $\mu M$), 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 CB2 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 CB1 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 demonstrated 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 CB1 and CB2 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 CB2 receptor.

Although A-836339 has been shown in the current study to be a CB2 receptor-selective agonist, it does display weak agonist properties at the rat CB1 receptor in in vitro cyclase and FLIPR functional assays. The activation of CB1 receptors by A-836339 in vivo was demonstrated by the CB1 receptor-mediated reduction of locomotor activity. Although Rotorod performance is commonly used to assess CB1-mediated CNS effects, locomotor deficit appears to be more sensitive for use in detecting the effect of activation of the central CB1 receptor. In addition, A-836339, after intravenous administration (10 µmol/kg i.v.), produced a CNS activation pattern shown by phMRI consistent with the CB1 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 CB1 receptor in the CNS. In addition, the CNS activation patterns observed in the phMRI studies with A-836339 have been demonstrated to be CB1 receptor-mediated because only the CB1 receptor-selective antagonist, SR141716A, but not the CB2 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 CB2 receptors, but not CB1 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 CB1 receptor, not CB2. Therefore, the lack of CNS activation with the lower dose of A-836339 demonstrates that there is unlikely a functional CB2 receptor in the CNS, consistent with the findings using AM1241 (Chin et al., 2008) and other CB2 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 CB1 receptor activation. Additional evidence supporting that the antiallodynic effects are CB2 mediated was provided by McGarvaugthy 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 CB2 antagonist SR144528, but not by CB1 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 CB2 receptor over the CB1 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 CB1 receptor in the CNS tissues that leads to a significantly enhanced ligand sensitivity of the CB1 receptor in these tissues and, therefore, CB1 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 CB2 receptors and an excellent selectivity against the CB1 receptors and other targets in the Cerep panel. It does not show significant human and rat species differences for both the CB1 and CB2 receptors. A-836339 demonstrates a CB2 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 CB2 receptor pharmacology in vitro and for use of interrogating the biological consequence of CB2 receptor activation in animals.

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References


Characterization of CB2 Receptor-Selective Agonist, A-836339

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