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Pharmacological Characterization of a Novel Cannabinoid Ligand, MDA19, for Treatment of Neuropathic Pain

Jijun J. Xu, MD, PhD*, Philippe Diaz, PhD*, Fanny Astruc-Diaz, MSc*, Suzanne Craig, DVM†, Elizandro Munoz*, and Mohamed Naguib, MD*

*Department of Anesthesiology and Perioperative Medicine, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

†Department of Veterinary Medicine and Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Abstract

BACKGROUND—Cannabinoid receptor 2 (CB₂) agonists have recently gained attention as potential therapeutic targets in the management of neuropathic pain. In this study, we characterized the pharmacological profile of the novel compound *N'*-(3*Z*)-1-(1-hexyl)-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]benzohydrazide (MDA19), a CB₂ agonist.

METHODS—We used radioligand binding assays and multiple in vitro functional assays at human and rat CB₁ and CB₂ receptors. The effects of MDA19 in reversing neuropathic pain were assessed in various neuropathic pain models in rats and in CB₂^{+/+} and CB₂^{-/-} mice.

RESULTS—MDA19 displayed 4-fold-higher affinity at the human CB₂ than at the human CB₁ receptor ($K_i = 43.3 \pm 10.3$ vs 162.4 ± 7.6 nM) and nearly 70-fold-higher affinity at the rat CB₂ than at the rat CB₁ receptor ($K_i = 16.3 \pm 2.1$ vs 1130 ± 574 nM). In guanosine triphosphate (GTP) γ [³⁵S] functional assays, MDA19 behaved as an agonist at the human CB₁ and CB₂ receptors and at the rat CB₁ receptor but as an inverse agonist at the rat CB₂ receptor. In 3',5'-cyclic adenosine monophosphate (cAMP) assays, MDA19 behaved as an agonist at the rat CB₁ receptor and exhibited no functional activity at the rat CB₂ receptor. In extracellular signal-regulated kinases 1 and 2 activation assays, MDA19 behaved as an agonist at the rat CB₂ receptor. MDA19 attenuated tactile allodynia produced by spinal nerve ligation or paclitaxel in a dose-related manner in rats and CB₂^{+/+} mice but not in CB₂^{-/-} mice, indicating that CB₂ receptors mediated the effects of MDA19. MDA19 did not affect rat locomotor activity.

CONCLUSIONS—We found that MDA19 exhibited a distinctive in vitro functional profile at rat CB₂ receptors and behaved as a CB₁/CB₂ agonist in vivo, characteristics of a protean agonist. MDA19 has potential for alleviating neuropathic pain without producing adverse effects in the central nervous system.

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Address correspondence and reprint requests to Mohamed Naguib, MD, Department of General Anesthesiology, Institute of Anesthesiology, Cleveland Clinic, 9500 Euclid Ave. - E-31, Cleveland, OH 44195. naguibm@ccf.org. PD and FA-D are currently at the Department of Biomedical and Pharmaceutical Sciences, Core Laboratory for Neuromolecular Production, The University of Montana, Missoula, Montana. JJX is currently at the Anesthesiology Institute, Cleveland Clinic, Cleveland, Ohio.

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DISCLOSURE

Mohamed Naguib, MD, is the chief scientific officer and cofounder of CB2 Pharma, Inc., which has an option-to-license agreement with The University of Texas M. D. Anderson Cancer Center.

Cannabinoid (CB) receptor agonists have gained attention as potential therapeutic targets in the management of neuropathic pain.¹ Two CB receptors have been characterized and cloned: CB₁ (found predominantly in the brain)² and CB₂ (found primarily in the peripheral immune system³ and to a lesser degree in the central nervous system⁴). Both receptors belong to the G protein-coupled receptor (GPCR) superfamily.

CB receptor agonists have analgesic effects in various neuropathic pain models.^{5–9} However, CB₁ agonists are associated with altered psychological state and motor impairment, which has affected the pharmaceutical development and use of these agents.¹⁰ However, there is evidence that CB₂-selective ligands have a significant role in the modulation of pain perception but do not produce the psychoactive adverse effects associated with CB₁ receptor activation.^{7,8,11,12}

It is now recognized that a single receptor can mediate different signaling pathways and that a ligand (or a drug) can differentially target a receptor-directed signaling complex, behaving as an agonist, an inverse agonist, or an antagonist, depending on the receptor's constitutive activity (spontaneous activity in the absence of agonist). This selective behavior contradicts the classical concepts of pharmacological actions of drugs, in which in the absence of agonist, the system displays no or very little activity and in the presence of agonist, a single receptor-active state is produced.^{13,14}

CB₂ receptors have constitutively active conformations that are precoupled to G proteins. A ligand can produce positive agonism in quiescent systems and inverse agonism in constitutively active systems, and this differing activity is dependent on the ligand-induced conformation of the receptor.¹⁴ Such ligands have been coined *protean agonists*,¹⁵ a name based on the Greek mythological sea god *Proteus*, who could change shape and appearance at will. Protean agonists have been reported in vitro and in vivo at various receptor systems, including the histamine H₃ receptor,¹⁶ α 2A-adrenergic receptor,¹⁷ dopamine D₂ receptor,¹⁸ and CB₂ receptor.^{19,20} Depending on the cell context, a protean agonist ligand can be an agonist, neutral antagonist, or inverse agonist at the same receptor type. At the CB₂ receptor, this phenomenon has been described for the CB₂ agonist (2-iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-1*H*-indol-3-yl]methanone (AM1241).^{19,20} AM1241 has been effective in treating inflammatory and neuropathic pain in animal models.^{21–23} However, the antinociceptive effects of AM1241 have been shown to involve the μ -opioid receptor system and β -endorphin and to be blocked by administration of the opioid receptor antagonist naloxone or antiserum to β -endorphin.^{8,22}

We recently described a novel series of *N*-alkyl isatin acylhydrazone derivatives that act as CB₂-selective agonists for the treatment of neuropathic pain.²⁴ In this study, we characterized a novel compound, *N*'-[(3*Z*)-1-(1-hexyl)-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]benzohydrazide (MDA19; compound 33), from our isatin series²⁴ (Fig. 1). We showed that MDA19 exhibited the characteristics of a protean agonist at the rat CB₂ receptor. We tested the hypothesis that MDA19, which has functionally selective properties in vitro, is likely to have novel actions in vivo in treating neuropathic pain.

METHODS

Compounds

6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl] (4-methoxyphenyl)methanone (AM630) and *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) were purchased from Tocris Bioscience (Ellisville, MO). (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone] (WIN 55,212-2) and all chemicals used for the

synthesis of MDA19 were purchased from Sigma-Aldrich (St. Louis, MO). 1-[(3-Benzyl-3-methyl-2,3-dihydro-1-benzofuran-6-yl)carbonyl]piperidine (MDA7) was prepared as described before.^{8,12} AM630, AM251, WIN 55,212-2, and haloperidol were administered in 0.25 mL dimethyl sulfoxide (DMSO). MDA19 was dissolved in *N*-methylpyrrolidone (30%). Propylene glycol (30%), ethyl alcohol (10%), and Cremophor (10%) were added dropwise at 40°C. After the solution was stirred for 15 minutes, water was added dropwise. After an additional 15 minutes of stirring at 40°C, the solution was cooled to room temperature.

In Vitro Characterization of MDA19

MDA19 Binding Affinity Studies at Rat CB₁ and CB₂ Receptor—MDA19 was screened in a competitive binding experiment by using membranes of Chinese hamster ovary (CHO)-K1 cells selectively expressing the rat CB₁ receptor at different MDA19 concentrations in duplicate.²⁵ The competitive binding experiment was performed in 96-well plates (Masterblock, catalog number 786,201; Greiner Bio-One, San Diego, CA) containing binding buffer (50 mM Tris [pH 7.4], 1 mM EDTA, 0.5% protease-free bovine serum albumin, 3 mM MgCl₂), recombinant membrane extracts (25 µg protein/well), and 1.25 nM 5-(4-chloro-phenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1*H*-pyrazole-3-carboxylic acid piperidin-1-ylamide ([³H]SR141716A), a CB₁ receptor-selective antagonist (TRK1028, 42 Ci/mmol, diluted in binding buffer; GE Healthcare). Nonspecific binding was determined in the presence of 10 µM AM251, a CB₁-selective antagonist (Tocris Bioscience). The sample was incubated in a final volume of 0.1 mL for 60 minutes at 25°C and then filtered on a GF/C UniFilter microplate (catalog number 6005177; PerkinElmer) presoaked in 0.05% Brij for 2 hours at room temperature. Filters were washed 6 times with 4 mL cold binding buffer, and the amount of bound [³H]SR141716A was determined by liquid scintillation counting. The half-maximal inhibitory concentration was determined by nonlinear regression by using the 1-site competition equation. *K_i* values were calculated by using the Cheng-Prusoff equation. The same protocol was repeated using 0.5 nM of the agonist [³H]CP 55,940 (NEX-1051, 161 Ci/mmol, diluted in binding buffer; PerkinElmer). Nonspecific binding was determined in the presence of WIN 55,212-2 (10 µM). The sample was incubated for 120 minutes at 37°C.

MDA19 was also screened in a competitive binding experiment by using membranes of CHO-K1 cells selectively expressing rat CB₂ at different MDA19 concentrations in duplicate.²⁵ The competitive binding experiment was performed in 96-well plates (Masterblock) containing binding buffer (50 mM Tris [pH 7.4], 2.5 mM EDTA, 0.5% protease-free bovine serum albumin), recombinant membrane extracts (0.25 µg protein/well), and 1 nM [³H]CP 55,940. Nonspecific binding was determined in the presence of 10 µM CP 55,940 (Tocris Bioscience). The sample was incubated in a final volume of 0.1 mL for 60 minutes at 30°C and then filtered on a GF/B UniFilter microplate (catalog number 6005177; PerkinElmer) presoaked in 0.5% polyethylenimine for 2 hours at room temperature. Filters were washed 6 times with 4 mL cold binding buffer (50 mM Tris [pH 7.4], 2.5 mM EDTA, 0.5% protease-free bovine serum albumin), and the amount of bound [³H]CP 55,940 was determined by liquid scintillation counting. The half-maximal inhibitory concentration and *K_i* values were calculated as described in the preceding paragraph.

GTPγ[³⁵S] Functional Assays—Functional activity was evaluated by using a guanosine triphosphate (GTP)γ[³⁵S] assay in CHO-K1 cell membrane extracts expressing recombinant human or rat CB₁ or CB₂ receptors. The assay relies on the binding of GTPγ[³⁵S], a radiolabeled nonhydrolyzable GTP analog, to the G protein upon binding of an agonist of the GPCR. In this system, agonists stimulate GTPγ[³⁵S] binding, whereas neutral antagonists have no effect and inverse agonists decrease GTPγ[³⁵S] basal binding.

MDA19 was solubilized in 100% DMSO at a concentration of 10 mM within 4 hours of the first testing session (master solution). A predilution for the dose-response curve was performed in 100% DMSO, and the solution was then diluted by a factor of 100 in assay buffer at a concentration 2-fold higher than the concentration to be tested. MDA19 was tested in duplicate for agonist activity at 8 concentrations: 10, 3, 1, 0.3, 0.1, 0.03, 0.01, and 0.001 μ M. CP 55,940 was the reference agonist. For GTP γ S, 5- μ g membrane extracts were mixed with guanosine diphosphate (GDP) diluted in assay buffer to give a 30- μ M solution (volume/volume) that was incubated for at least 15 minutes on ice. In parallel, GTP γ [35 S] (catalog number SJ1308; GE Healthcare) was mixed with scintillation beads (PVT-WGA, catalog number RPNQ001; GE Healthcare) diluted in assay buffer at 50 mg/mL (0.5 mg/10 μ L) (volume/volume) just before the reaction was started.

The following reagents were successively added to the wells of an OptiPlate (PerkinElmer): 50 μ L of ligand or the reference antagonist (AM251, a CB₁ receptor-selective antagonist); 20 μ L of the membrane/GDP mix; 10 μ L of the reference agonist (CP 55,940) at historical 80% effective concentration (EC₈₀) (30 nM); and 20 μ L of the GTP γ [35 S]/beads mix. The plates were covered with a TopSeal tape (PerkinElmer), shaken on an orbital shaker for 2 minutes, and then incubated for 1 hour at room temperature. The plates were then centrifuged for 10 minutes at 2000 rpm and counted for 1 minute/well with a PerkinElmer TopCount scintillation counter. Assay reproducibility was monitored by use of CP 55,940. For replicate determinations, the maximum variability tolerated in the test was $\pm 20\%$ of the average of the replicates. Efficacies (E_{max}) for CB₁ and CB₂ were expressed as percentages relative to the efficacy of CP 55,940.

cAMP Activation Assays—MDA19 was tested for agonist activity at rat CB₁ and CB₂ receptors at 8 MDA19 concentrations: 10, 3, 1, 0.3, 0.1, 0.03, 0.01, and 0.001 μ M. Recombinant cells grown to mid-log phase in culture medium without antibiotics were detached with phosphate-buffered saline containing 5 mM EDTA, centrifuged, and resuspended in assay buffer at a concentration of 4.16×10^5 cells/mL. The test was performed in 96-well plates. For testing, 12 μ L of cells (5×10^3 cells/well) were mixed with 6 μ L of the reference agonist (CP 55,940) at increasing concentrations and 6 μ L forskolin (10- μ M final concentration). The plates were then incubated for 30 minutes at room temperature. After addition of lysis buffer, 3',5'-cyclic adenosine monophosphate (cAMP) assay concentrations were estimated by using a Homogeneous Time-Resolved Fluorescence kit from Cisbio Bioassays (catalog number 62AM2PEB; Bedford, MA) according to the manufacturer's specifications.

Extracellular Signal-Regulated Kinases 1 and 2 (or Mitogen-Activated Protein Kinase) Activation Assays—CB₂ receptors inhibit the activity of the intracellular effector adenylyl cyclase via pertussis toxin (PTX)-sensitive G_i/G_o subunits²⁶ and stimulate the activity of the extracellular signal-regulated kinase (Erk) subgroup of the mitogen-activated protein kinase (MAPK). We were interested in examining the effect of MDA19 on this pathway.

CHO cells stably expressing rat CB₂ receptors were cultured at 2×10^5 cells per well in HAM-F12 medium (Mediatech, Manassas, VA) containing 10% fetal bovine serum, 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 400 μ g/mL G418. Cells were maintained in a humidified atmosphere of 90% air–10% CO₂. Cells were treated with 10 μ M MDA7 (a CB₂ receptor-selective agonist⁸) for 30 minutes; 10 μ M MDA19 for 30 minutes; or 20 μ M AM251 (a CB₁ receptor-selective antagonist²⁷) for 30 minutes, 20 μ M AM630 (a CB₂ receptor-selective antagonist²⁸) for 30 minutes, or 200 ng/mL PTX (List Biological Laboratories, CA) for 16 hours, followed by MDA19 for 30 minutes. PTX inactivates the G

proteins of the G_i and G_o families and hence blocks downstream effects mediated through these proteins.

After treatment, cells were collected and lysed in buffer (1.0% Nonidet P-40, 20 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerol, 60 mM octyl β -glucoside, 10 mM NaF, 1.0 mM Na_3VO_4 , 2.5 mM nitrophenyl phosphate, 1 mM phenylmethyl sulfonyl fluoride, 0.7 $\mu\text{g}/\text{mL}$ pepstatin, and a protease inhibitor cocktail tablet [Roche, Mannheim, Germany]) and centrifuged at 12,000 rpm at 4°C for 15 minutes. The supernatants (lysate) were collected. From the lysate, total protein was taken and dissolved in $2 \times$ Laemmli buffer. The samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes (Whatman, Florham Park, NJ). Antibodies to total and phosphorylated Erk1/2 (1:1500; Cell Signaling Technology, Boston, MA) were used as the primary antibodies, and horseradish peroxidase–conjugated antibodies were used as the secondary antibodies. Band detection was conducted by using an enhanced chemiluminescence detection system (Amersham Biosciences, Buckinghamshire, UK).

In Vivo Characterization of MDA19

Animals—Adult male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 120 to 150 g and male $\text{CB}_2^{-/-}$ and $\text{CB}_2^{+/+}$ mice weighing 20 to 25 g were used in experimental procedures approved by the Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center. Animals were housed 3 per cage for rats and 5 per cage for mice on a 12/12-hour light/dark cycle with water and food pellets available ad libitum. We obtained CB_2 knockout ($\text{CB}_2^{-/-}$), wild-type ($\text{CB}_2^{+/+}$), and heterozygote mice from Nancy E. Buckley, PhD (Biological Sciences Department, California State Polytechnic University, Pomona, CA). Mice breeding and genotyping were performed as described by Buckley et al.²⁹

Lumbar 5–6 Spinal Nerve Ligation Neuropathic Pain Model in Rats—All surgical procedures were performed under deep isoflurane anesthesia in 100% O_2 . Spinal nerve ligation was performed as described previously.³⁰ Briefly, a midline incision was made above the lumbar spine to expose the left L6 transverse process. The process was then removed, the left L5 and L6 spinal nerves were isolated, and both nerves were tightly ligated with 6-0 silk thread. The development of neuropathy was confirmed by daily measurement of the paw-withdrawal threshold by using Von Frey filaments (see below). Behavioral experiments were conducted after neuropathy was established.

Paclitaxel-Induced Neuropathy Model (Rats and Mice)—Groups of rats received either vehicle (10% Cremophor EL in saline) or 1.0 mg/kg paclitaxel daily by intraperitoneal (IP) injection for 4 consecutive days for a final cumulative dose of 4 mg/kg³¹; the injection volume was 1 mL/kg. Baseline responses to mechanical stimulation of the hind-paw (see below) were established on day 0, and responses were measured daily until the development of neuropathy was confirmed.

Assessment of Tactile Paw-Withdrawal Thresholds—Rats or mice were placed in a compartment with a wire mesh bottom and allowed to acclimate for a minimum of 30 minutes before testing. Sensory thresholds for the development of allodynia to mechanical stimuli were assessed. Mechanical sensitivity was assessed by using a series of Von Frey filaments with logarithmic incremental stiffness (Stoelting Co., Wood Dale, IL), as previously described,³² and 50% probability paw-withdrawal thresholds were calculated with the up-down method.³³ In brief, filaments were applied to the plantar surface of a hindpaw for approximately 6 seconds in an ascending or descending order after a negative or positive withdrawal response, respectively. Six consecutive responses after the first

change in response were used to calculate the paw-withdrawal threshold (in grams). In rats, when response thresholds occurred outside the range of detection, the paw-withdrawal threshold was assigned at 15 g for continuous negative responses and at 0.25 g for continuous positive responses. Corresponding values in mice were 2.5 g and 0.02 g, respectively.³⁴ The percentage maximal possible effect was calculated as $[(\text{postdrug threshold} - \text{baseline threshold}) / (\text{cutoff threshold (15 g)} - \text{baseline threshold})] \times 100$. The drug groups were not randomized.

Open-Field Chamber Testing (Rats)—An automated open-field chamber (ENV-515 Test Environment; Med Associates, St. Albans, VT), $43.2 \times 43.2 \times 30.5$ cm (length $\times$ width $\times$ height), equipped with 3 pairs of 16 infrared arrays that continually monitored each animal's movement, was used to determine potential central nervous system effects of WIN 55,212-2 (a CB₁ and CB₂ agonist), haloperidol, and DMSO in naïve rats. These data were compared with our historical data for MDA19 and the vehicle.²⁴ Rats were individually tested 15 minutes after drugs were administered IP. The infrared beams were set 2.5 cm apart horizontally and at a height of 3 cm above the floor, with the rearing array set at 12 cm from the floor. The area in the box was divided into 4 equal quadrants (zones), and data were collected within each quadrant and across quadrants (zone entries). An ambulatory movement was defined as a motion of at least 5 cm and was coded by quadrant. Vertical movements were counted when the rat moved vertically at least 12 cm from the floor. Zone entries were defined as an entry into a zone from another zone. Entry into a zone was counted when the rat was far enough into the new zone to break 2 sets of new zone photoelectric beams during an ambulatory movement.

Data Analyses

Statistical analyses were performed by using BMDP 2007 (Statistical Solutions, Saugus, MA). The concentration-response curves were fitted by using GraphPad Prism (version 4.03; GraphPad Software, San Diego, CA). Data were analyzed by using unpaired 2-tailed Student *t* test or 1-way analysis of variance for repeated measures where appropriate. If findings on analysis of variance were significant, Tukey-Kramer post hoc analysis was used for multiple group comparison. The area under the curve was calculated by using the trapezoidal rule. The results were presented as mean $\pm$ SEM and were considered significant at $P < 0.05$. Analyses of the dose-response curves and statistics were obtained by using the pharmacological software programs of Tallarida and Murray³⁵ and included calculation of the 50% effective dose (ED₅₀) values and their 95% confidence intervals (CIs).

RESULTS

In Vitro Characterization of MDA19

Binding Affinity Studies—To determine whether MDA19 exhibited affinities for both CB₁ and CB₂ receptors, a binding study was performed. In the competitive binding assays performed in membranes of CHO-K1 cells selectively expressing the human CB₂ receptor, MDA19 displaced [³H]CP 55,940 from human receptors with a mean *K_i* value of 43.3 ± 10.3 nM. However, its affinity at the human CB₁ receptor was approximately 4 times weaker, with a *K_i* value of 162.4 ± 7.6 nM (Table 1). It should be noted that *K_i* values were in the same range using [³H]CP 55,940 or [³H]SR141716A. Rat CB₁ and CB₂ binding affinities were determined to correlate in vivo results and the in vitro profile. Corresponding *K_i* values for CHO-K1 cells selectively expressing the rat CB₁ and CB₂ receptors were 1130 ± 574 and 16.3 ± 2.1 nM, respectively (Table 1). Thus, MDA19's affinity for the rat CB₁ receptor was nearly 70 times weaker than its affinity for the rat CB₂ receptor.

GTP γ [³⁵S] Functional Assays with Human and Rat Receptors—Having shown that MDA19 exhibited affinities for both rat and human CB₁ and CB₂ receptors, we next determined whether MDA19 was behaving as an agonist or an antagonist for these receptors. For this purpose, we used a GTP γ [³⁵S] functional assay. The assay relies on the binding of GTP γ [³⁵S], a radiolabeled nonhydrolyzable GTP analog, to the G protein upon binding of an agonist of the GPCR. In this system, agonists stimulate GTP γ [³⁵S] binding, whereas antagonists have no effect and inverse agonists decrease GTP γ [³⁵S] basal binding. This assay, therefore, measures the functional consequence of receptor occupancy at one of the earliest receptor-mediated events. In our GTP γ [³⁵S] functional assays, MDA19 behaved as an agonist at the human CB₁ and CB₂ receptors, with 50% effective concentration (EC₅₀) values of 922 ± 56 and 83 ± 19 nM at the human CB₁ and CB₂ receptors, respectively (Table 2 and Fig. 2). MDA19 also behaved as an agonist at the rat CB₁ receptor, with an EC₅₀ value of 427 ± 35 nM; however, it behaved as an inverse agonist at the rat CB₂ receptor, with an EC₅₀ value of 19.7 ± 14 nM (Table 2 and Fig. 3). CP 55,940 exhibited agonist activity at the rat CB₁ and CB₂ receptors (Table 2 and Fig. 3).

cAMP Activation Assays—Both CB₁ and CB₂ receptors are negatively coupled to adenylate cyclase. Activation of the G protein G_i inhibits the cAMP-dependent pathway by inhibiting the enzyme adenylate cyclase. cAMP has an important role in the transmission of signals by functioning as a second messenger. In cAMP activation assays, MDA19 behaved as an agonist at the rat CB₁ receptor, with an EC₅₀ of 814 ± 83 nM, but exhibited no activity at the rat CB₂ receptor (Table 2 and Fig. 4). In contrast, CP 55,940 exhibited strong agonist activity at both the rat CB₁ receptor and the rat CB₂ receptor (Table 2 and Fig. 3).

Erk1/2 Activation Assay in Rat CHO Cells Stably Expressing Rat CB₂ Receptors—Both the CB₁ and CB₂ receptors are coupled with G_i or G_o protein, positively to MAPK.^{36,37} In Erk1/2 activation studies, MDA19 behaved as an agonist and induced phosphorylation of Erk1/2 in rat CHO cells stably expressing rat CB₂ receptors (CHO-rCB₂ cells). Activation of Erk1/2 by MDA19 in CHO-rCB₂ cells was blocked by AM630 and by PTX, but not by AM251 (Fig. 5).

In Vivo Characterization of MDA19

Effects of MDA19 on Tactile Allodynia in a Lumbar 5–6 Spinal Nerve Ligation Neuropathic Pain Model—Next, we tested our hypothesis that MDA19, a compound with a wide spectrum of activity ranging from agonism to inverse agonism in vitro, is likely to have novel actions in vivo in treating neuropathic pain. In the spinal nerve ligation neuropathic pain model, MDA19 (IP) attenuated tactile allodynia in a dose-related manner; the ED₅₀ was 9.1 mg/kg IP (95% CI = 6.9–11.8 mg/kg) at 30 minutes (Fig. 6, A and B). Area under the curve analyses revealed that the highest doses of MDA19 (15 mg/kg) produced a significantly ($P < 0.01$) greater antiallodynic effect than that noted with a dose of 5 mg/kg or with the vehicle (data not shown). Pretreatment with AM630 (5 mg/kg IP), a CB₂ receptor-selective antagonist,²⁸ significantly reversed the antiallodynic effects induced by MDA19 (10 mg/kg IP) ($P < 0.05$) (Fig. 6C). The rats treated with CB₂ receptor antagonists alone exhibited no significant change in paw withdrawal threshold compared with results in the vehicle-treated animals (Fig. 6C).

WIN 55,212 is a potent CB₁ and CB₂ agonist with high stereoselectivity and a slight preference for CB₂.^{7,38} Administration of WIN 55,212-2 attenuated tactile allodynia in a dose-related manner; the ED₅₀ was 0.7 (95% CI = 0.5–0.9 mg/kg IP) at 15 minutes (data not shown). The antiallodynic effects of 15 mg/kg MDA19 IP were not significantly different from those observed with 1 mg/kg WIN 55,212-2 IP (Fig. 7). Administration of a higher dose (3 mg/kg) of WIN 55,212-2 IP resulted in adverse effects such as immobility and

increased sensitivity to noise and handling. Because it was difficult to discriminate the analgesic effects from adverse effects (immobility), we decided not to include these data in the analysis.

Effects of MDA19 on Tactile Allodynia in a Rat Model of Paclitaxel-Induced Neuropathic Pain

—To confirm the results obtained in the spinal nerve ligation neuropathic pain model, MDA19 was tested in a second model of neuropathic pain, namely, the paclitaxel-induced neuropathic pain model. In this model, tactile allodynia developed in 100% of rats within 10 days after the start of paclitaxel administration.⁸ In paclitaxel-treated rats, IP administration of MDA19 suppressed mechanical allodynia compared with findings in the vehicle-treated rats (Fig. 8).

Effects of MDA19 on Exploratory Behavior in Open-Field Chamber Testing

—To exclude any possible central effect of MDA19 that could be attributed to the antiallodynic effect of MDA19 in both rat models of neuropathic pain, we decided to test the effect of MDA19 on exploratory behavior. MDA19 (15 mg/kg IP) had no effect on exploratory behavior in open-field chamber testing, whereas WIN 55,212-2 (1 mg/kg IP)³⁹ and haloperidol (1 mg/kg IP) significantly decreased exploratory behavior in naïve rats, as evidenced by a reduction in the total distance traveled (Fig. 9A), time spent ambulating (Fig. 9B), vertical movements (Fig. 9C), and zone entries (Fig. 9D).

Effects of MDA19 on Tactile Allodynia in a Mouse Model of Paclitaxel-Induced Neuropathic Pain Model

—In the aforementioned studies, MDA19 was effective in alleviating allodynia in 2 models of neuropathic pain in rats. To exclude any off-target effect and involvement of other receptors, we tested MDA19 in CB₂^{-/-} mice and compared the effect with CB₂^{+/+} mice. Tactile allodynia developed in 100% of CB₂^{+/+} and CB₂^{-/-} mice within 10 days after the start of paclitaxel administration. In paclitaxel-treated CB₂^{+/+} mice, IP administration of 10 mg/kg MDA19 suppressed mechanical allodynia (Fig. 10). The peak effect for reversing mechanical allodynia was observed at 30 minutes with 10 mg/kg MDA19 IP. In contrast, IP administration of MDA19 had no effect in CB₂^{-/-} mice.

DISCUSSION

Our study results support the hypothesis that MDA19, which has functionally selective properties *in vitro*, is likely to have novel actions *in vivo* in treating neuropathic pain. We found that MDA19 displayed a spectrum of activity ranging from agonism to inverse agonism at the rat CB₂ receptor. In GTPγ[³⁵S] functional assays, we found that MDA19 behaved as an agonist at the human CB₁ and CB₂ receptors and at the rat CB₁ receptor, but as an inverse agonist at the rat CB₂ receptor. In cAMP assays, MDA19 behaved as an agonist at the rat CB₁ receptor and exhibited no activity at the rat CB₂ receptor. In addition, in Erk1/2 activation assays using CHO cells expressing rat CB₂ receptors, MDA19 behaved as an agonist at the rat CB₂ receptor. In the *in vivo* studies, MDA19 was effective in attenuating tactile allodynia in 2 models of neuropathic pain. This effect was dependent on the presence of the CB₂ receptor because MDA19 showed no effect in CB₂^{-/-} mice.

Our results suggest that MDA19 is a ligand that has a protean effect at the CB₂ receptor. The phenomenon of protean agonism refers to a ligand that can display a wide array of activities at the same GPCR: acting as a partial agonist, full agonist, neutral antagonist, partial inverse agonist, or full inverse agonist depending on the level of intrinsic activity of the ligand and constitutive activity of the receptor.^{19,20,40} A protean agonist induces a receptor conformation of lower efficacy than the constitutively active conformation. In the absence of constitutively active receptors, it acts as an agonist and as an antagonist, but in the presence of constitutively active receptors, it acts as an inverse agonist.

AM1241, a CB₂-selective agonist, has been shown to have the characteristics of a protean agonist. Yao et al.¹⁹ found AM1241 to be (1) an apparent antagonist at the human CB₂ receptor in fluorometric image plate reader functional assays; (2) a neutral antagonist at the human CB₂ receptor in cyclase assays; (3) an agonist at the human CB₂ receptor at lower forskolin concentrations in cyclase assays; and (4) an apparent agonist at the human CB₂ receptor in Erk activation assays. Similarly, Bingham et al.²⁰ reported that AM1241 behaved as an agonist at the human CB₂ receptor but as an inverse agonist at rat and mouse CB₂ receptors in cAMP inhibition assays. Nevertheless, the efficacy of AM1241 in rodent pain models and the fact that this activity was reversed by CB₂ antagonists indicated that AM1241 acted as an agonist at the CB₂ receptor. Recently, Mancini et al.⁴¹ demonstrated that (+)AM1241 and L768242 are protean agonists at both the human and rat CB₂ receptors. After abolishment of CB₂ receptor constitutive activity, both (+)AM1241 and L768242 behaved as agonists.

We noted that MDA19 behaved similarly to AM1241, as an agonist at the human CB₂ receptor but as an inverse agonist at the rat CB₂ receptor in GTPγ[³⁵S] functional assays. This change in activity was seen despite similar affinities at the rat and human CB₂ receptors and use of the same GTPγ[³⁵S] concentration in both rat and human assays. Without the use of GTPγ[³⁵S], which may affect receptor conformation stabilization, MDA19 showed no agonist activity in the cAMP assay. The Erk1/2 activation assay results seemed to be more correlated to the in vivo results as MDA19 behaved as a CB₂ agonist.

Despite being characterized as an inverse agonist at rodent CB₂ receptors, AM1241 attenuated tactile allodynia in a dose-related manner in rodents with spinal nerve ligation.^{22,42} Similarly, we noted that MDA19 was effective in attenuating tactile allodynia in neuropathic rats. In the spinal nerve ligation rat model, we noted that MDA19 attenuated tactile allodynia in a dose-related manner, with an ED₅₀ of 9.1 mg/kg IP (95% CI = 6.9 – 11.8 mg/kg) and an efficacy of 70% ± 10% after a 15 mg/kg dose of MDA19 IP (Fig. 5). This in vivo activity of MDA19 at the CB₂ receptor seems to contradict the in vitro characterization of MDA19 as an inverse agonist at the rat CB₂ receptor in GTPγ[³⁵S] functional assays or as a ligand with no activity at the rat CB₂ receptor in cAMP functional assays. AM1241 and its resolved enantiomers had been shown to exhibit similar CB₂-mediated behavior in mice and rats in in vitro functional assays and in in vivo models.²⁰ In cAMP inhibition assays, (*R,S*)-AM1241 was found to be an agonist at human CB₂ receptors but was an inverse agonist at rat and mouse CB₂ receptors.

We noted in this study that the administration of 15 mg/kg MDA19 IP (in contrast to 1 mg/kg WIN 55,212-2 IP) did not inhibit ambulation or rearing in the open-field testing. We also noted that a 3 mg/kg dose of WIN 55,212-2 IP induced adverse effects in rats' central nervous systems, such as immobility and increased sensitivity to noise and handling, as expected for a CB₁/CB₂ receptor agonist. In agreement with our results, other investigators have reported adverse effects in the central nervous system with WIN 55,212-2 at doses >1 mg/kg IP.^{43,44}

To confirm CB₂ involvement and discount any off-target effect for MDA19, we tested MDA19 in a paclitaxel-induced neuropathy mouse model using CB₂^{-/-} and CB₂^{+/+} mice. MDA19 clearly attenuated tactile allodynia at a 10 mg/kg dose in CB₂^{+/+} mice but showed no effect in CB₂^{-/-} mice, discounting off-target and CB₁ effects. As discussed earlier, other investigators have reported similar discrepancies between in vivo effects and in vitro characterization of various CBs, such as the protean agonist AM124120 and other CB ligands.⁴⁵ The endocannabinoid concentration is expected to be high in rats experiencing neuropathic pain, increasing CB₂ functional activity. This CB₂ functional activity may also, at least partially, explain the discrepancy between the in vitro and in vivo profiles. Despite

the fact that AM630 did not worsen pain in our in vivo model, it is difficult to anticipate the behavior of a compound based on in vitro results because of the putative basal CB₂ activity and endocannabinoid concentration.

Considering previous results for AM1241 that clearly demonstrate that AM1241 is a protean agonist and despite the inverse agonist behaviors we observed in functional assays, MDA19 should be considered a protean agonist. The ability of a ligand to selectively activate distinct signaling pathways (e.g., agonist-directed trafficking of response phenomena) could explain the behavior of MDA19. Evidence suggests that a ligand can differentially regulate intracellular effectors and have different intrinsic activity depending on a second messenger.⁴⁶ Our data provide evidence for agonist-directed trafficking of response by MDA19. We noted that MDA19 would activate the Erk pathway, behaving as an agonist, whereas exhibiting no agonist activity in the cAMP pathway. Because of the similarities in the profiles of MDA19 and AM1241, MDA19 appears to be a protean agonist.

The concept of protean agonism (i.e., functional selectivity) has led to important implications for drug discovery.⁴⁷ First, target validation for drug discovery should include more information on the downstream signaling pathway(s) and not only the involvement of a given receptor in the pathological and/or therapeutic process. Otherwise, compounds with the appropriate activity in a clinically relevant pathway could be missed. Furthermore, with functionally selective drugs, it would be possible to improve therapeutic profiles and to separate the desired from the undesired effects of a single molecule acting through a single receptor.

In summary, we have provided in vitro and in vivo pharmacological characterization of a novel CB₂ agonist, MDA19. Our study results support the hypothesis that MDA19, which has functionally selective properties in vitro, is likely to have novel actions in vivo in treating neuropathic pain. We found that MDA19 behaves as a CB₂ protean agonist and exhibits various functional activities, depending on the functional assay as well as the various responses at the human and rodent CB₂ receptors. Therefore, useful therapeutics could be overlooked if CB₂ ligands that behave as inverse agonists in vitro are not tested in vivo. MDA19 was effective in treating allodynia in rat and mice models of neuropathic pain by activating CB₂ receptors, but did not affect the locomotor behavior of the animals at the active dose. MDA19 thus has potential for alleviating neuropathic pain without producing adverse effects in the central nervous system.

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Figure 1. Structure of MDA19 (*N'*-[*(3Z)*-1-(1-hexyl)-2-oxo-1,2-dihydro-3*H*-indol-3-ylidene]benzohydrazide). For details on synthesis and structure-activity relationships, see the article by Diaz et al.²⁴

Figure 2. Characterization of CP 55,940 (A) and MDA19 (B) in recombinant human CB₁ and CB₂ (hCB₁ and hCB₂) GTPγ[³⁵S] assay systems. Levels of receptor activation were calculated and were expressed as percentages relative to the response to 1.0 μM CP 55,940. In this assay, MDA19 behaved as an agonist at the human CB₂ receptor.

Figure 3.

Characterization of CP 55,940 (A) and MDA19 (B) in recombinant rat CB₁ and CB₂ (rCB₁ and rCB₂) GTPγ[³⁵S] assay systems. Levels of receptor activation were calculated and were expressed as percentages relative to the response to 1.0 μM CP 55,940. In this assay, MDA19 behaved as an inverse agonist at the CB₂ receptor. The corresponding curve does not look like a typical sigmoidal curve because of 3 outliers. If we had excluded these outliers, the curve would have looked sigmoidal.

Figure 4. Characterization of CP 55,940 (A) and MDA19 (B) in cAMP activation assays at the rat CB₁ and CB₂ receptors. In this assay, MDA19 exhibited no activity at the rat CB₂ receptor.

Figure 5. Characterization of MDA19 at the rat CB₂ receptor in Erk (MAPK) activation assays. Stable Chinese hamster ovary cells expressing rat CB₂ receptors were treated with MDA7 (a CB₂ receptor-selective agonist); MDA19; or AM251 (a CB₁ receptor-selective antagonist), AM630 (a CB₂ receptor-selective antagonist), or pertussis toxin (PTX), followed by MDA19.

Figure 6.

Effects of MDA19 administered by intraperitoneal (IP) injection on tactile allodynia in a spinal nerve ligation neuropathic pain model in rats. A, MDA19 increased the paw withdrawal threshold of the nerve-injured paw in a dose-dependent manner ($n = 12$ per group). B, MDA19 attenuated tactile allodynia in a dose-dependent manner (at 30 minutes, 50% effective dose = 9.1 mg/kg IP; 95% confidence interval = 6.9–11.8 mg/kg; $n = 12$ per group). C, Effects of CB₂-selective antagonists on the antiallodynic effects of 10 mg/kg MDA19 in a spinal nerve ligation neuropathic pain model in rats ($n = 6$ per group). All drugs were administered by IP injection. Administration of 5 mg/kg AM630, a selective CB₂ antagonist, had no effect. Administration of 5 mg/kg AM630 15 minutes before

administration of MDA19 (AM630 + MDA19) reversed the antiallodynic effects of MDA19 ($P < 0.05$). Data are expressed as mean $\pm$ SEM. Vehicle = vehicle used for MDA19. Dimethyl sulfoxide (DMSO) was the solvent used for other compounds. %MPE = maximum peak effect; AUC = area under the curve.

Figure 7.

Effects of WIN 55,212-2 administered by intraperitoneal (IP) injection on tactile allodynia in a spinal nerve ligation neuropathic pain model in rats. A, The time course of paw withdrawal threshold. B, WIN 55,212-2 attenuated tactile allodynia in a dose-dependent manner (at 15 minutes, 50% effective dose = 0.7mg/kg IP; 95% confidence interval = 0.5–0.9 mg/kg; $n = 6$ per group). Data are expressed as mean $\pm$ SEM. DMSO = dimethyl sulfoxide.

Figure 8.

Effects of MDA19 administered by intraperitoneal injection on tactile allodynia in a paclitaxel-induced neuropathic pain model in rats ($n = 5$ per group). MDA19 produced antiallodynic effects that were significantly different ($P < 0.05$) (analysis of variance followed by the Tukey-Kramer test for multiple group comparison) from those of the vehicle, as evidenced by an increase in the %MPE (maximum peak effect) paw withdrawal threshold area under the curve (AUC).

Figure 9.

Effect of MDA19 on locomotor activity. Exploratory behavior was tested in naïve animals in an open-field chamber after IP administration of the vehicle, MDA19, dimethyl sulfoxide (DMSO), WIN 55,212-2, or haloperidol ($n = 6$ per group). The following variables were scored for 60 minutes: distance traveled (A), ambulatory time (B), vertical activity (C), and number of zone entries (D). Statistical analyses were performed by analysis of variance followed by the Tukey-Kramer test for multiple group comparison. (Data presented for MDA19 and the vehicle were reproduced from Diaz et al.²⁴ with permission from the *Journal of Medicinal Chemistry*.)

Figure 10.

Effects of 10 mg/kg MDA19 administered by intraperitoneal injection on tactile allodynia in a paclitaxel-induced neuropathic pain model in CB₂^{+/+} and CB₂^{-/-} mice ($n = 5$ per group). MDA19 suppressed mechanical allodynia in paclitaxel-treated CB₂^{+/+} mice but did not show any effect in CB₂^{-/-} mice. * $P < 0.05$, *** $P < 0.001$ versus other groups, and ** $P < 0.01$ versus CB₂^{-/-} mice groups (repeated-measures analysis of variance followed by the Tukey-Kramer test for multiple group comparison).

Table 1**Radioligand Competitive Binding Assay Results**

Ligand	Mean K_i (nM)			
	Human CB₁	Human CB₂	Rat CB₁	Rat CB₂
MDA19 ^a	162.4 ± 7.6	43.3 ± 10.3	1130 ± 574	16.3 ± 2.1
CP 55,940 ^a	3.4	1.8 ± 1.1	ND	1.1 ± 0.02
AM251	ND	ND	0.58 ± 0.04	ND
MDA19 ^b	150	ND	ND	ND
CP 55,940 ^b	3.0 ± 0.55	ND	ND	ND

CB₁ = cannabinoid receptor type 1; CB₂ = cannabinoid receptor type 2; ND = not determined.

Data are presented as mean ± SEM (obtained from 2 or 3 independent experiments).

^a[³H]SR141716A was used.

^b[³H]CP 55,940 was used.

Table 2

GTPγ[³⁵S] Functional and cAMP Activation Assays

Ligands	GTPγ[³⁵ S] functional assays				cAMP activation assays			
	Human CB ₁		Human CB ₂		Rat CB ₁		Rat CB ₂	
	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)	EC ₅₀ (nM)	E _{max} (%)
MDA19	922 ± 56	64.5	83 ± 19	65.4	427 ± 35	54	19.7 ± 14 ^a	-42
CP 55,940	9.6 ± 1.2	107	3.1 ± 1.1	94	8.2 ± 1.3	116	2.3 ± 1.8	112

CB₁ = cannabinoid receptor type 1; CB₂ = cannabinoid receptor type 2; EC₅₀ = 50% effective concentration; NA = not active at 1 μM.

Data are presented as mean ± SEM (obtained from 2 or 3 independent experiments).

^aEC₅₀ for inverse agonism.