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# RESEARCH PAPER

# MDA7: a novel selective agonist for $CB_2$ receptors that prevents allodynia in rat neuropathic pain models

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**Background and purpose:** There is growing interest in using cannabinoid type 2 ($CB_2$) receptor agonists for the treatment of neuropathic pain. In this report, we describe the pharmacological characteristics of MDA7 (1-[(3-benzyl-3-methyl-2,3-dihydro-1-benzofuran-6-yl)carbonyl]piperidine), a novel $CB_2$ receptor agonist.
**Experimental approach:** We characterized the pharmacological profile of MDA7 by using radioligand-binding assays and *in vitro* functional assays at human cannabinoid type 1 ($CB_1$) and $CB_2$ receptors. *In vitro* functional assays were performed at rat $CB_1$ and $CB_2$ receptors. The effects of MDA7 in reversing neuropathic pain were assessed in spinal nerve ligation and paclitaxel-induced neuropathy models in rats.
**Key results:** MDA7 exhibited selectivity and agonist affinity at human and rat $CB_2$ receptors. MDA7 treatment attenuated tactile allodynia produced by spinal nerve ligation or by paclitaxel in a dose-related manner. These effects were selectively antagonized by a $CB_2$ receptor antagonist but not by $CB_1$ or opioid receptor antagonists. MDA7 did not affect rat locomotor activity.
**Conclusion and implications:** MDA7, a novel selective $CB_2$ agonist, was effective in suppressing neuropathic nociception in two rat models without affecting locomotor behaviour. These results confirm the potential for $CB_2$ agonists in the treatment of neuropathic pain.
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**Keywords:** cannabinoid; $CB_2$; MDA7; neuropathic pain; allodynia; hyperalgesia; cancer; chemotherapy

**Abbreviations:** A-796260, [1-(2-morpholin-4-yl-ethyl)-*1H*-indol-3-yl]-(2,2,3,3-tetramethylcyclopropyl)-methanone; AM1241, (2-iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-*1H*-indol-3-yl-methanone; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methy l-*1H*-pyrazole-3-carboxamide; AM630, (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-y l](4-methoxyphenyl)methanone); CP55,940, 5-(1,1-dimethylheptyl)-2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxy-propyl)-cyclohexyl]-phenol; GW842166X, 2-[(2,4-dichlorophenyl)amino]-*N*-[(tetrahydro-*2H*-pyran-4-yl)methyl]-4-(trifluoromethyl)-5-pyrimidinecarboxamide; L-768,242 (GW405833), (2,3-dichloro-phenyl)-[5-methoxy-2-methyl-3-(2-morpholin-4-yl-ethyl)-indol-1-yl]-methanone; MDA7, 1-[(3-benzyl-3-methyl-2,3-dihydro-1-benzofuran-6-yl)carbonyl]piperidine; SR141716A, 5-(4-chloro-phenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*1H*-pyrazole-3-carboxylic acid piperidin-1-ylamide; WIN 55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de)-1,4-benzoxazin-6-yl]-1-napthalenylmethanone

## Introduction

Neuropathic pain can be a debilitating condition characterized by severe, persistent pain that is refractory to traditional analgesics. Neuropathic pain results from several different conditions affecting the peripheral or CNS and affects an estimated 8% of people worldwide (Torrance *et al.*, 2006). The annual U.S. health-care cost attributable to neuropathic pain is almost $40 billion (Turk, 2002). The currently available treatments for neuropathic pain—opioids, gabapentin and amitriptyline—are effective in fewer than 30% of patients (Warms *et al.*, 2002; Davis, 2007; Khaliq *et al.*, 2007; Tassone *et al.*, 2007).

Cannabis extracts, such as Marinol and Sativex, and synthetic derivatives, such as Cannador, have also been tried as treatments for neuropathic pain. However, these have limited efficacy in humans and they contain $\Delta^9$-THC, the natural constituent of hemp (*Cannabis sativa*), which produces adverse psychotropic effects (Karst *et al.*, 2003; Berman *et al.*, 2004; Woolridge *et al.*, 2005; Holdcroft *et al.*,

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2006; Abrams *et al*., 2007; Nurmikko *et al*., 2007). These cannabis extracts and synthetic derivatives have agonist activity for human cannabinoid type 1 ($CB_1$) receptors and inverse agonist activity at cannabinoid type 2 ($CB_2$) receptors (Govaerts *et al*., 2004; receptor nomenclature according to Alexander *et al*., 2008).

Both $CB_1$ and $CB_2$ receptors have been characterized and cloned (Devane *et al*., 1988; Matsuda *et al*., 1990; Munro *et al*., 1993). There is also evidence for the existence of additional pharmacologically identified, non-$CB_1$/$CB_2$ receptors (Overton *et al*., 2006; Ryberg *et al*., 2007). $CB_2$ receptors are expressed primarily in the peripheral immune system (Munro *et al*., 1993; Facci *et al*., 1995) and to a lesser degree in the CNS (Van Sickle *et al*., 2005; Onaivi *et al*., 2006). $CB_2$ receptor expression was found in the brainstem neurons of wild-type mice but was absent in $CB_2$-receptor knockout mice (Van Sickle *et al*., 2005). Onaivi *et al*. (2006) reported that $CB_2$ receptors were expressed in the brains of naive mice and that this expression was enhanced in the presence of chronic mild stress. Recently, a pharmacological magnetic resonance imaging study demonstrated that even if $CB_2$ receptors are expressed in the brain, they are not functional under normal physiological conditions (Chin *et al*., 2008). Human and rat $CB_1$ and $CB_2$ receptors show 97 and 81% amino-acid sequence identity, respectively (Matsuda *et al*., 1990; Gerard *et al*., 1991; Reggio, 2003). The $CB_2$ receptor shows 44% identity with the $CB_1$ receptor (Munro *et al*., 1993).

Several studies have demonstrated that $CB_2$-selective ligands have a significant function in the modulation of pain perception but do not produce the adverse psychoactive effects associated with $CB_1$ receptor activation (Huffman *et al*., 1996; Huffman, 2005; Salo *et al*., 2005; Valenzano *et al*., 2005; Manera *et al*., 2006; Murineddu *et al*., 2006; Giblin *et al*., 2007; Ohta *et al*., 2007a, b; Page *et al*., 2007; Yao *et al*., 2008). However, despite several publications concerning the therapeutic potential of $CB_2$-selective ligands as novel analgesic agents, conflicting results exist with respect to their *in vitro* and *in vivo* profiles. AM1241 [(2-iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-*1H*-indol-3-yl-methanone], which has been used as a reference compound to study the effects of $CB_2$ agonists (Ibrahim *et al*., 2003, 2006; Beltramo *et al*., 2006), appears to be a weak partial agonist for human $CB_2$ receptors and an inverse agonist for rat $CB_2$ receptors (Bingham *et al*., 2007). Furthermore, interactions of AM1241 with the opioid system may contribute to its *in vivo* properties (Ibrahim *et al*., 2005). Other $CB_2$-selective agonists that have been described include HU-308 (Hanus *et al*., 1999), GW405833 (L-768,242) [(2,3-dichloro-phenyl)-[5-methoxy-2-methyl-3-(2-morpholin-4-yl-ethyl)-indol-1-yl]-methanone] (Valenzano *et al*., 2005), Sch35966 (Gonsiorek *et al*., 2007), GW842166X (2-[(2,4-dichlorophenyl)amino]-*N*-[(tetrahydro-*2H*-pyran-4-yl)methyl]-4-(trifluoromethyl)-5-pyrimidinecarboxamide) (Giblin *et al*., 2007), A-796260 ([1-(2-morpholin-4-yl-ethyl)-*1H*-indol-3-yl]-(2,2,3,3-tetramethylcyclopropyl)-methanone) (Yao *et al*., 2008), AM1241 (Ibrahim *et al*., 2003) and PF-03550096 (Kikuchi *et al*., 2008). A dose of 50 mg kg$^{-1}$ of HU-308 given i.p. was reported to be effective in reversing the late but not the early phase of formalin-induced peripheral pain in mice and this effect was blocked by the $CB_2$ antagonist SR-144528 (Hanus *et al*., 1999). GW405833 (L-768,242) exhibited partial agonist activity at human $CB_2$ receptors and no functional data were reported on rat $CB_2$ receptors (Valenzano *et al*., 2005). The antiallodynic effect of GW405833 was found to be less than that of AM1241 (LaBuda *et al*., 2005). GW842166X was reported to be effective in the Freund's complete adjuvant model of inflammatory pain (with an oral $ED_{50}$ of 0.1 mg kg$^{-1}$) (Giblin *et al*., 2007). In a spinal nerve ligation neuropathic pain model, doses of up to 30 mg kg$^{-1}$ of GW842166X i.p. produced an effect comparable to that of 50 mg kg$^{-1}$ of gabapentin p.o. (Conti *et al*., 2007). PF-03550096 (Kikuchi *et al*., 2008) was effective in attenuating visceral hypersensitivity induced by 2,4,6-trinitrobenzene sulphonic acid but was not tested in a neuropathic pain model. Similarly, no *in vivo* activities were reported for Sch35966 (Gonsiorek *et al*., 2007). A-796260 (Yao *et al*., 2008) has been shown to be effective in a sciatic nerve chronic constriction neuropathic pain model. However, doses up to 35 mg kg$^{-1}$ of A-796260 i.p. were less effective than 6 mg kg$^{-1}$ of morphine i.p. in reversing allodynia (Yao *et al*., 2008).

In the studies described here, we tested the efficacy of a novel $CB_2$-selective agonist, MDA7 (1-[(3-benzyl-3-methyl-2,3-dihydro-1-benzofuran-6-yl)carbonyl]piperidine; Figure 1a), based on a 3,3-disubstituted-2,3-dihydro-1-benzofuran scaffold obtained by a colloidal palladium nanoparticle-catalysed tandem cyclization/cross-coupling reaction (Figure 1b). By using binding and functional assays and *in vivo* models of neuropathic pain, we demonstrated that MDA7 is a selective agonist for $CB_2$ receptors that suppressed neuropathic nociception.

## Methods

*Synthesis of MDA7*

MDA7 was synthesized as shown in Figure 1b. Briefly, 3-hydroxy-4-iodo-benzoic acid was obtained by iodination (NaI, NaOCl, NaOH, MeOH, 80% yield) of *meta*-hydroxybenzoic acid (Edgar and Falling, 1990). The corresponding methyl benzoate was obtained by esterification (MeOH, $H_2SO_4$). The phenol derivative was then coupled with 3-bromo-2-methyl-propene by using potassium carbonate in methylethylketone in 98% yield. The resulting compound was submitted to a palladium-catalysed tandem cyclization/Suzuki-coupling reaction (Szlosek-Pinaud *et al*., 2007) to afford the corresponding benzofuran in 95% yield. MDA7 was obtained after saponification (97% yield) and coupling with piperidine (71% yield).

*Analytical data for MDA7*

1-[(3-benzyl-3-methyl-2,3-dihydro-1-benzofuran-6-yl)carbonyl] piperidine, 1H NMR ($CDCl_3$): δ 1.37 (s, 3H), 1.58–1.69 (m, 6H), 2.8 (d, $J$ = 13.5 Hz, 1H), 2.90 (d, $J$ = 13.5 Hz, 1H), 3.34 (br s, 2H), 3.68 (br s, 2H), 4.09 (d, $J$ = 8.7 Hz, 1H), 4.53 (d, $J$ = 8.7 Hz, 1H), 6.75 (d, $J$ = 1.2 Hz, 1H), 6.88 (dd, $J$ = 1.2 Hz, $J$ = 7.5 Hz), 6.94 (d, $J$ = 7.5 Hz, 1H), 6.99–7.02 (m, 2H), 7.22–7.24 (m, 3H). $^{13}$C NMR ($CDCl_3$): δ 24.56 ($CH_3$), 24.64 ($CH_2$), 25.67 ($CH_2$), 26.56 ($CH_2$), 43.15 ($CH_2$), 46.22

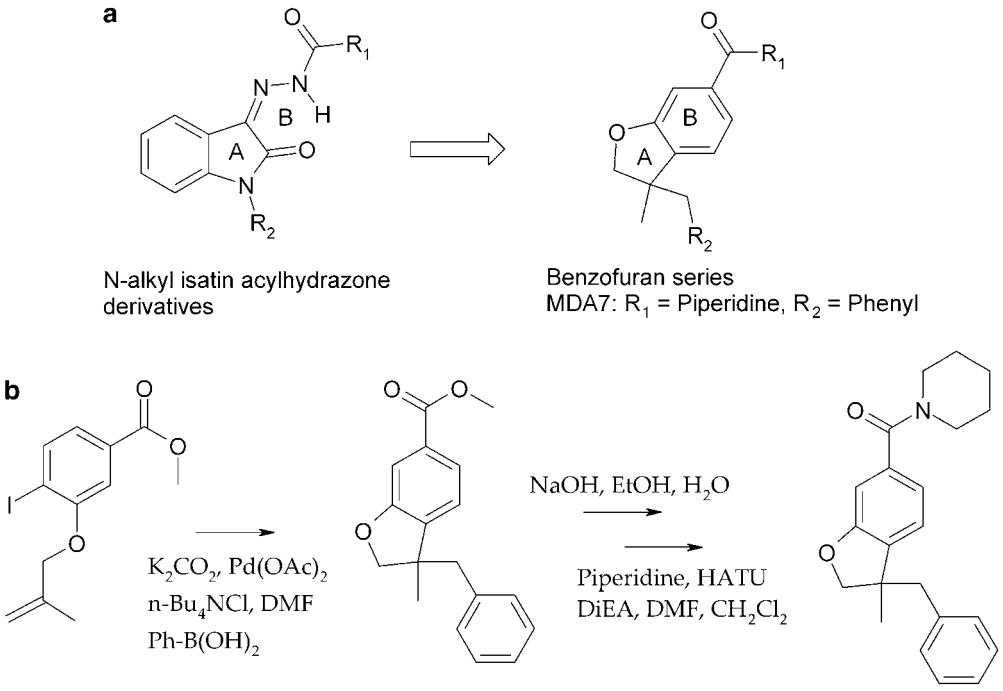


**Figure 1** (**a**) Comparison of isatin and benzofuran structures. Ring B from the benzofuran series is superimposable on the six-membered ring formed by the hydrazone and the indolone through an internal hydrogen bond. In this case, both five-membered rings (ring A) from the isatin and benzofuran structures also fit. (**b**) Summary of synthetic pathway for MDA7 (see Methods for details).

(C), 46.57 ($CH_2$), 48.75 ($CH_2$), 82.28 ($CH_2$), 108.20 (CH), 119.09 (CH), 123.43 (CH), 126.58 (CH), 127.99 (CH), 130.36 (CH), 136.21 (C), 136.75 (C), 137.26 (C), 159.47 (C), 170.19 (C=O). HRMS (ES+) calcd for $C_{22}H_{25}NO_2$ (M+ $H^+$), *m/e*, 336.1964; found, 336.1958.

In vitro *receptor radioligand-binding studies*

*Human $CB_1$ and $CB_2$ receptor-binding studies*. MDA7 was screened in a competitive binding experiment by using membranes of Chinese hamster ovarian cells (CHO-K1 cells) expressing human $CB_1$ receptors at different MDA7 concentrations in duplicate (Mukherjee *et al*., 2004). The competitive binding experiment was performed in 96-well plates (Masterblock, catalogue number 786201, Greiner Bio-One, San Diego, CA, USA) containing binding buffer (50 mM Tris, pH 7.4, 2.5 mM EDTA, 0.5% protease-free BSA, saponin 10 μg $mL^{-1}$), recombinant membrane extracts (2 μg protein per well) and 1 nM [$^3$H]SR141716A (5-(4-chloro-phenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1H-pyrazole-3-carboxylic acid piperidin-1-ylamide), a selective $CB_1$ receptor antagonist (GE Healthcare, TRK1028, 42 Ci $mmol^{-1}$, diluted in binding buffer). Nonspecific binding was determined in the presence of 10 μM CP55,940 (5-(1,1-dimethylheptyl)-2-[(1R, 2R,5R)-5-hydroxy-2-(3-hydroxy-propyl)-cyclohexyl]-phenol) (Tocris Bioscience, Ellisville, MO, USA). The sample was incubated in a final volume of 0.1 mL for 60 min at 25 °C and then filtered on a GF/C UniFilter microplate (Perkin Elmer, Waltham, MA, USA; catalogue number 6005177) presoaked in 0.05% Brij for 2 h at room temperature. Filters were washed six times with 4 mL of cold binding buffer, and bound [$^3$H]SR141716A was determined by liquid scintillation counting. The median $IC_{50}$ was determined by nonlinear regression by using the one-site competition equation. The inhibition constant ($K_i$) values were calculated by using the Cheng–Prusoff equation ($K_i = IC_{50}/(1+(L/K_D))$, where $L$ = concentration of radioligand in the assay and $K_D$ = affinity of the radioligand for the receptor.

MDA7 was also screened in a competitive binding experiment by using membranes of CHO-K1 cells selectively expressing the human $CB_2$ receptor at different MDA7 concentrations in duplicate (Mukherjee *et al*., 2004). 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 BSA), recombinant membrane extracts (0.25 μg protein per well) and 1 nM [$^3$H]CP55,940 (Perkin Elmer, NEX-1051, 161 Ci $mmol^{-1}$, diluted in binding buffer). Nonspecific binding was determined in the presence of 10 μM CP55,940 (Tocris Bioscience). The sample was incubated in a final volume of 0.1 mL for 60 min at 30 °C and then filtered on a GF/B UniFilter microplate (Perkin Elmer, catalogue number 6005177) presoaked in 0.5% polyethyleneimine for 2 h at room temperature. Filters were washed six times with 4 mL of cold binding buffer (50 mM Tris, pH 7.4, 2.5 mM EDTA, 0.5% protease-free BSA), and bound [$^3$H]CP55,940 was determined by liquid scintillation counting. The $IC_{50}$ and $K_i$ values were calculated as above.

*Rat $CB_1$ and $CB_2$ receptor-binding studies*. MDA7 was screened in a competitive binding experiment by using membranes of CHO-K1 cells selectively expressing the rat $CB_1$ receptor at different MDA7 concentrations in duplicate (Mukherjee

*et al.*, 2004). The competitive binding experiment was performed in 96-well plates (Masterblock) containing binding buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 0.5% protease-free BSA, 3 mM $MgCl_2$), recombinant membrane extracts (25 μg protein per well) and 1.25 nM [$^3$H]SR141716A (5-(4-chloro-phenyl)-1-(2,4-dichloro-phenyl)-4-methyl-1H-pyrazole-3-carboxylic acid piperidin-1-ylamide), a selective $CB_1$ receptor antagonist (GE Healthcare diluted in binding buffer). Non-specific binding was determined in the presence of 10 μM AM251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methy l-*1H*-pyrazole-3-carboxamide) (Tocris Bioscience). The sample was incubated in a final volume of 0.1 mL for 60 min at 25 °C and then filtered on a GF/C UniFilter microplate (Perkin Elmer, catalogue number 6005177) presoaked in 0.05% Brij for 2 h at room temperature. Filters were washed six times with 4 mL of cold binding buffer, and bound [$^3$H]SR141716A was determined by liquid scintillation counting. $IC_{50}$ was determined by nonlinear regression by using the one-site competition equation. The $K_i$ values were calculated using the Cheng–Prusoff equation.

MDA7 was also screened in a competitive binding experiment using membranes of CHO-K1 cells selectively expressing the rat $CB_2$ receptor at different MDA7 concentrations in duplicate (Mukherjee *et al.*, 2004). 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 BSA), recombinant membrane extracts (0.25 μg protein per well) and 1 nM [$^3$H]CP55,940 (Perkin Elmer, NEX-1051, 161 Ci mmol$^{-1}$, diluted in binding buffer). Nonspecific binding was determined in the presence of 10 μM CP55,940 (Tocris Bioscience). The sample was incubated in a final volume of 0.1 mL for 60 min at 30 °C and then filtered on a GF/B UniFilter microplate (Perkin Elmer, catalogue number 6005177) presoaked in 0.5% polyethyleneimine for 2 h at room temperature. Filters were washed six times with 4 mL of cold binding buffer (50 mM Tris, pH 7.4, 2.5 mM EDTA, 0.5% protease-free BSA), and bound [$^3$H]CP55,940 was determined by liquid scintillation counting. $IC_{50}$ and $K_i$ values were calculated as described previously.

### *GTPγ[$^{35}$S] functional assays*

Functional activity was evaluated by using a GTPγ[$^{35}$S] assay in CHO-K1 cell membrane extracts expressing recombinant human or rat $CB_1$ or $CB_2$ receptors. The assay relies on the binding of GTPγ[$^{35}$S], a radiolabeled nonhydrolyzable GTP analogue, to the G-protein upon binding of an agonist of the G-protein-coupled receptor. In this system, agonists stimulate GTPγ[$^{35}$S] binding, whereas neutral antagonists have no effect and inverse agonists decrease GTPγ[$^{35}$S] basal binding.

MDA7 was dissolved in 100% dimethylsulphoxide (DMSO) at a concentration of 10 mM within 4 h of the first testing session (master solution). A pre-dilution for the dose–response curve was performed in 100% DMSO, and this solution was diluted 100-fold in assay buffer to a concentration 2-fold higher than the concentration to be tested. MDA7 was tested for agonist and antagonist activities at eight concentrations in duplicate: 10, 3, 1, 0.3, 0.1, 0.03, 0.01 and 0.001 μM. CP55,940 (Tocris Bioscience) was the reference agonist. For GTPγS, 5 μg of membranes were mixed with guanosine-5′-diphosphate diluted in assay buffer to give 30 μM solution (volume/volume), and incubated for at least 15 min on ice. In parallel, GTPγ[$^{35}$S] (GE Healthcare, catalogue number SJ1308) was mixed with scintillation beads (PVT-WGA, GE Healthcare, catalogue number RPNQ001), diluted in assay buffer at 50 mg mL$^{-1}$ (0.5 mg per 10 μL$^{-1}$) (volume/volume) just before the reaction was started. The following reagents were successively added in the wells of an OptiPlate (Perkin Elmer): 50 μL of ligand or the reference antagonist (AM251, a $CB_1$ receptor-selective antagonist); 20 μL of the membrane/guanosine-5′-diphosphate mix; 10 μL of the reference agonist (CP55,940) at historical $EC_{80}$ (30 nM); and 20 μL of the GTPγ[$^{35}$S]:beads mix. The plates were covered with a topseal, shaken on an orbital shaker for 2 min and then incubated for 1 h at room temperature. Then the plates were centrifuged for 10 min at 800 *g* and counted for 1 min per well with a Perkin Elmer TopCount scintillation counter. Assay reproducibility was monitored by the use of the reference agonist, CP55,940. For replicate determinations, the maximum variability tolerated in the test was ±20% of the average of the replicates. Efficacies ($E_{max}$) for $CB_1$ or $CB_2$ receptors were expressed as a percentage relative to the efficacy of CP55,940.

### *Animals*

All animal procedures were approved by the Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. Animals were housed three per cage on a 12/12 h light/dark cycle with water and food pellets available *ad libitum*. Adult male Sprague–Dawley (Harlan Sprague Dawley, Indianapolis, IN, USA) rats weighing 120–150 g were used and all experiments were performed during the light cycle.

### *Spinal nerve (L5/6) ligation model of neuropathic pain*

All surgical procedures were performed under deep isoflurane anaesthesia in 100% $O_2$. The spinal nerve ligation was performed as described previously (Kim and Chung, 1992). 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). Behavioural experiments were conducted after neuropathy was established.

### *Paclitaxel-induced neuropathy model*

Groups of rats received either vehicle (10% Cremophor EL in saline) or 1.0 mg kg$^{-1}$ of paclitaxel daily i.p. for four consecutive days for a final cumulative dose of 4 mg kg$^{-1}$ (Polomano *et al.*, 2001); the injection volume was 1 mL kg$^{-1}$. Baseline responses to mechanical stimulation of the hind paw (see below) were established on day 0 and continued daily until the development of neuropathy was confirmed.

*Assessment of mechanical withdrawal thresholds*

Rats were placed in a compartment with a wire mesh bottom, and allowed to acclimatize for a minimum of 30 min before testing. Mechanical sensitivity was assessed by using a series of Von Frey filaments with logarithmic incremental stiffness (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50 and 15.1 g) (Stoelting Co., Wood Dale, IL, USA), as previously described (Chaplan *et al*., 1994), and 50% probability withdrawal thresholds were calculated with the up–down method (Dixon, 1965). In brief, beginning with the 2.0-g probe, filaments were applied to the plantar surface of a hind paw for 6–8 s, 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 withdrawal threshold (in grams). When response thresholds fell outside the range of detection, the withdrawal threshold was assigned as 15 g for continuous negative responses and 0.25 g for continuous positive responses. The percentage maximal possible effect (%MPE) was calculated as ((postdrug threshold−baseline threshold)/(cutoff threshold (15 g)−baseline threshold)) × 100. The baseline threshold was determined once. Different groups were tested in the same hour on consecutive days. The only exception is the 'vehicle + vehicle' group experiment, which was performed on a different day, separate from other groups. The drug groups were not randomized. Only one experimenter performed all behavioural tests and he, therefore, was not unaware of the different treatment groups.

*Assessment of paw withdrawal latencies in response to noxious heat*

To determine sensitivity to noxious heat, rats were placed in plexiglass enclosures on a transparent glass surface maintained at 30 °C, and allowed to acclimatize for 30 min. A thermal testing apparatus, consisting of a heat-emitting projector lamp and an electronic timer, was used. The device was activated after the lamp was placed directly beneath the plantar surface of the hind paw. The paw withdrawal latency in response to the radiant heat (50 W) was recorded using a digital timer. A cutoff of 30 s was used to prevent tissue damage. After the baseline paw withdrawal response to noxious heat was measured, three groups of naive rats ($n = 10$ per group) received 1, 3 or 10 mg kg$^{-1}$ of MDA7 i.p. Response latencies were determined for each rat before i.p. drug injection and at 5, 10, 15, 30, 45, 60, 90 and 120 min after i.p. drug injection. Each paw was only stimulated once per time point.

*Open-field chamber testing*

An automated open-field chamber (ENV-515 Test Environment, Med Associates, St Albans, VT, USA), 43.2 × 43.2 × 30.5 cm ($L \times W \times H$), equipped with three pairs of 16 infrared arrays that continually monitored each animal's movement, was used to determine potential CNS effects of MDA7, WIN 55,212-2 ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de)-1,4-benzoxazin-6-yl]-1-napthalenylmethanone), and haloperidol in naive rats. Rats were individually tested 15 min after i.p. drug administration. 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 four equal quadrants (zones), with data 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 moves 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 two sets of new zone photoelectric beams during an ambulatory movement.

*Data analysis*

Statistical analyses were carried out by using BMDP 2007 (Statistical Solutions, Saugus, MA, USA) and Graph Pad Prism (version 4.03; Graph Pad Software Inc., San Diego, CA, USA). Data were analysed by using one-way ANOVA. If findings on ANOVA were significant, Tukey–Kramer *post hoc* analysis was used for multiple group comparison. Area under the curve (AUC) was calculated using the trapezoidal rule. The results were presented as mean ± s.e.mean 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 Murray (1987), and included calculation of the $ED_{50}$ values and their 95% confidence intervals (CIs).

*Compounds and solutions*

AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-y l](4-methoxyphenyl)methanone) and AM251 were purchased from Tocris Bioscience. WIN 55,212-2, AM1241, naloxone, paclitaxel and all chemicals used for the synthesis of MDA7 were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA).

WIN 55,212-2, AM1241, AM630, AM251 and haloperidol were administered *in vivo* in 0.25 mL of 100% DMSO. MDA7 was administered *in vivo* in a vehicle consisting of *N*-methylpyrrolidone 25%, propylene glycol 25%, Cremophor ELP 10% and sterile water 40%. Briefly, MDA7 was dissolved in *N*-methylpyrrolidone. Propylene glycol and Cremophor were added dropwise at 40 °C. After the solution was stirred for 15 min, water was added dropwise. After an additional 15 min of stirring at 40 °C, the solution was cooled to room temperature. By this method, a solution of about 18 mM MDA7 could be prepared, enough for a 150 g rat.

## Results

In vitro *characterization of MDA7*

In the competitive binding assays performed in membranes of CHO-K1 cells selectively expressing the human $CB_2$ receptor, MDA7 potently displaced [$^3$H]CP55,940 from human receptors (Table 1). In the competitive binding assays performed in membranes of CHO-K1 cells selectively expressing human $CB_1$ receptors, MDA7 (up to 3 μM) did not demonstrate detectable radioligand displacement (Table 1). Corresponding $K_i$ values at CHO-K1 cells selectively

expressing the rat $CB_1$ and $CB_2$ receptors were about 10-fold higher for the $CB_1$ than for the $CB_2$ receptor (Table 1). In GTPγ[$^{35}$S] functional assays, MDA7 gave measurable $EC_{50}$ values (Table 2) at human and rat $CB_2$ receptors, but was without any activity at human or rat $CB_1$ receptors, up to 1 μM. Weak $CB_1$ partial agonist activity was detected at a concentration of more than 1 μM (Figure 2).

*Effects of MDA7 on thermal nociception in naive rats*

Administration of 1 or 3 mg kg$^{-1}$ of MDA7 i.p. did not block the nociceptive effect of a thermal stimulus applied to the hind paws of naive rats. Increasing the dose of MDA7 to 10 mg kg$^{-1}$ i.p. resulted in a short-lasting antinociceptive effect (Figure 3).

**Table 1** Radioligand competitive binding assays

| *Ligand* | *Mean $K_i$ (nM)* | | | |
|---|---|---|---|---|
| | *Human $CB_1$* | *Human $CB_2$* | *Rat $CB_1$* | *Rat $CB_2$* |
| MDA7 | >10,000 | 422 ± 123 | 2565 ± 695 | 238 ± 143 |
| CP55,940 | 3.4 | 1.8 ± 1.1 | ND | 1.1 ± 0.02 |
| AM251 | ND | ND | 0.58 ± 0.06 | ND |

Abbreviations: $CB_1$, cannabinoid receptor type 1; $CB_2$, cannabinoid receptor type 2; ND, not done.

**Table 2** GTPγ[$^{35}$S] functional assays

| *Ligands* | *Agonist $EC_{50}$ (nM) (mean ± s.e. mean) relative to CP55,940 (%)* | | | |
|---|---|---|---|---|
| | *GTPγ[$^{35}$S] functional assays* | | *GTPγ[$^{35}$S] functional assays* | |
| | *Human $CB_1$* | *Human $CB_2$* | *Rat $CB_1$* | *Rat $CB_2$* |
| MDA7 | NA | 128 ± 32 | NA | 67.4 ± 4.9 |
| CP55,940 | 9 ± 1.3 | 6.5 ± 2.1 | 8.2 ± 1.3 | 2.3 ± 1.8 |

Abbreviations: $CB_1$, cannabinoid receptor type 1; $CB_2$, cannabinoid receptor type 2; NA, not active at 1 μM.

*Effects of MDA7 on tactile allodynia in the spinal nerve ligation model of neuropathic pain*

In rats, spinal nerve ligation produced tactile allodynia 1 week after surgery, as demonstrated by a marked reduction in paw withdrawal threshold to mechanical stimulation with Von Frey filaments (Figure 4a). MDA7 (i.p.) attenuated tactile allodynia in a dose-related manner; the $ED_{50}$ was 7.5 mg kg$^{-1}$ i.p. (95% CI = 5.6–9.9 mg kg$^{-1}$). The higher doses of MDA7 (10 and 15 mg kg$^{-1}$) produced a significantly greater anti-allodynic effect than that noted with a dose of 5 mg kg$^{-1}$ (Figure 5).

The receptor specificity of MDA7 was investigated in this spinal nerve ligation model using receptor-selective antagonists (Figure 5c). Pretreatment with AM630 (5 mg kg$^{-1}$ i.p.), a selective $CB_2$ receptor antagonist (Hosohata *et al*., 1997; Ross *et al*., 1999), significantly reversed the antiallodynic effects induced by MDA7 (10 mg kg$^{-1}$ i.p.) ($P<0.001$). In contrast, pretreatment with AM251 (5 mg kg$^{-1}$ i.p.), a selective $CB_1$ receptor antagonist (Gatley *et al*., 1996), did not affect the antiallodynic effects induced by MDA7. The rats treated with $CB_1$ or $CB_2$ receptor antagonists alone at the doses used in these studies exhibited no significant change in paw withdrawal threshold compared with results from the vehicle-treated animals (Figure 5c).

Administration of AM1241 (15 mg kg$^{-1}$ i.p.), a $CB_2$ ligand (Ibrahim *et al*., 2003), produced antiallodynic effects that were significantly different ($P<0.001$) from those of the vehicle (Figure 6). However, the antiallodynic effects of 10 mg kg$^{-1}$ of MDA7 i.p. were significantly greater ($P<0.001$) than those that were observed with AM1241. The antinociceptive effects of AM1241 have been shown to involve the

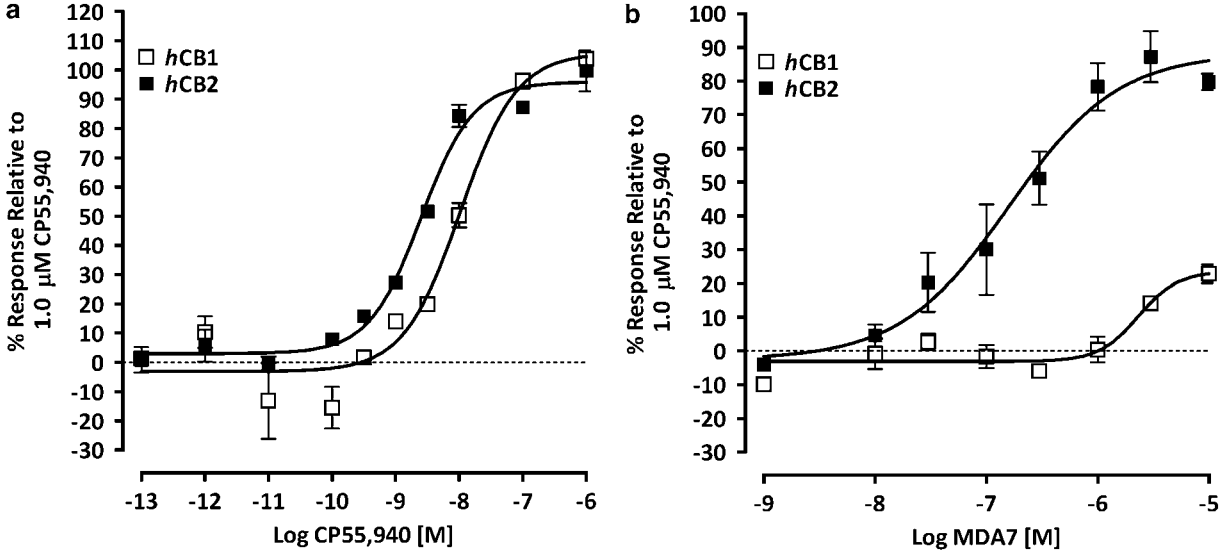


**Figure 2** Characterization of CP55,940 (**a**) and MDA7 (**b**) in recombinant human cannabinoid types 1and 2 ($CB_1$ and $CB_2$) ($hCB_1$ and $hCB_2$) GTPγ[$^{35}$S] assay systems. The levels of receptor activation were calculated and were expressed as a percentage relative to the response to 1.0 μM CP55,940.

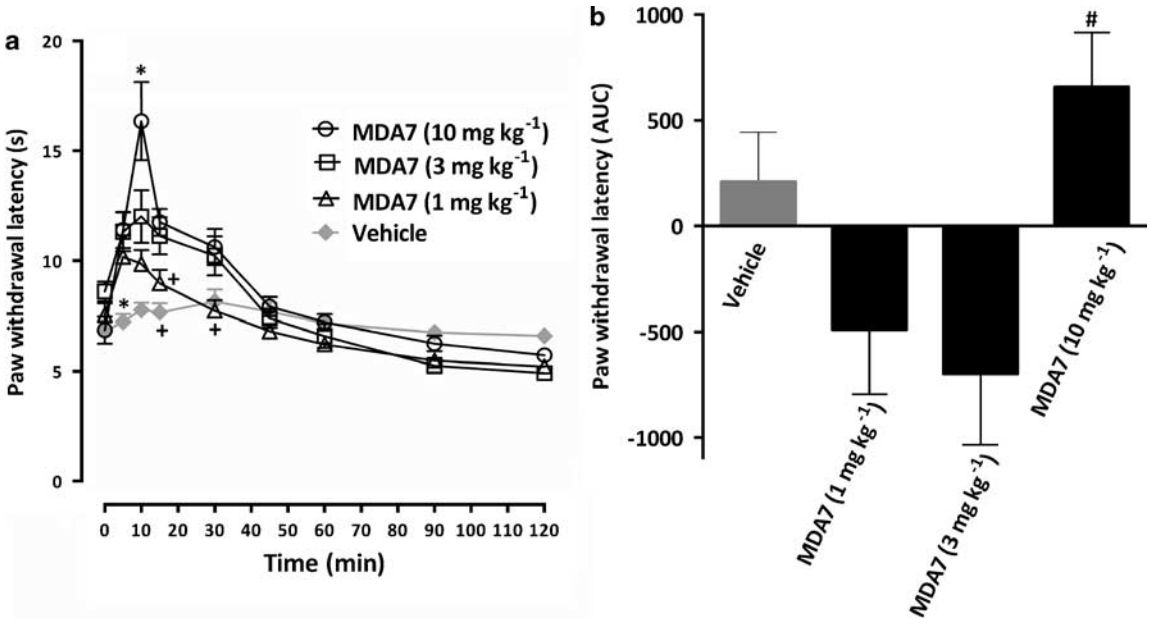


**Figure 3** Effects of i.p. administration of MDA7 on the hind paw withdrawal latency in response to noxious heat in naive rats ($n=10$ per group). (**a**) The time course of percent maximal possible effect (%MPE) and (**b**) the area under the curve (AUC) of 1.0, 3.0 and 10 mg kg$^{-1}$ of MDA7 and the vehicle. *$P<0.05$ compared with other groups. $^{+}P<0.05$ compared with 3.0 or 10 mg kg$^{-1}$ of MDA7 and the vehicle (ANOVA followed by Tukey–Kramer test for multiple group comparison). $^{\#}P<0.05$ compared with 1.0 and 3.0 mg kg$^{-1}$ of MDA7 (ANOVA followed by Tukey–Kramer test for multiple group comparison). Each point represents the mean ± s.e.mean.

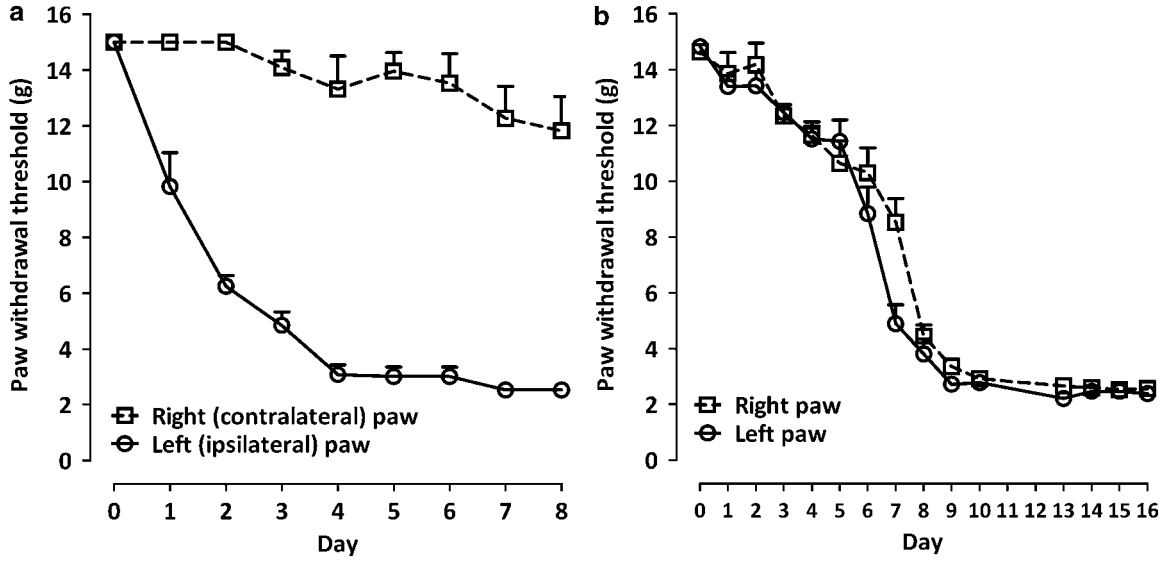


**Figure 4** Development of tactile allodynia (**a**) after spinal nerve ligation and (**b**) after i.p. administration of paclitaxel for 4 days ($n=10$ per group). Each point represents the mean ± s.e.mean.

μ-opioid receptor system and β-endorphin, and to be blocked by administration of the opioid receptor antagonist naloxone or antiserum to β-endorphin (Ibrahim *et al*., 2005). In this study, rats subjected to spinal nerve ligation were treated with naloxone (10 mg kg$^{-1}$ i.p.) 15 min before the administration of MDA7. Naloxone pretreatment had no effect on the antiallodynic activity of MDA7 (Figure 6). However, pretreatment with naloxone significantly reversed the antiallodynic activity of AM1241 (Figure 6, $P<0.01$), confirming that the antiallodynic effects of MDA7 were not mediated by μ-opioid receptor-dependent activity.

*Effects of MDA7 on tactile allodynia in a paclitaxel-induced neuropathic pain model*

Tactile allodynia developed in 100% of rats 10 days after the start of paclitaxel administration, as demonstrated by a reduction in paw withdrawal threshold to mechanical

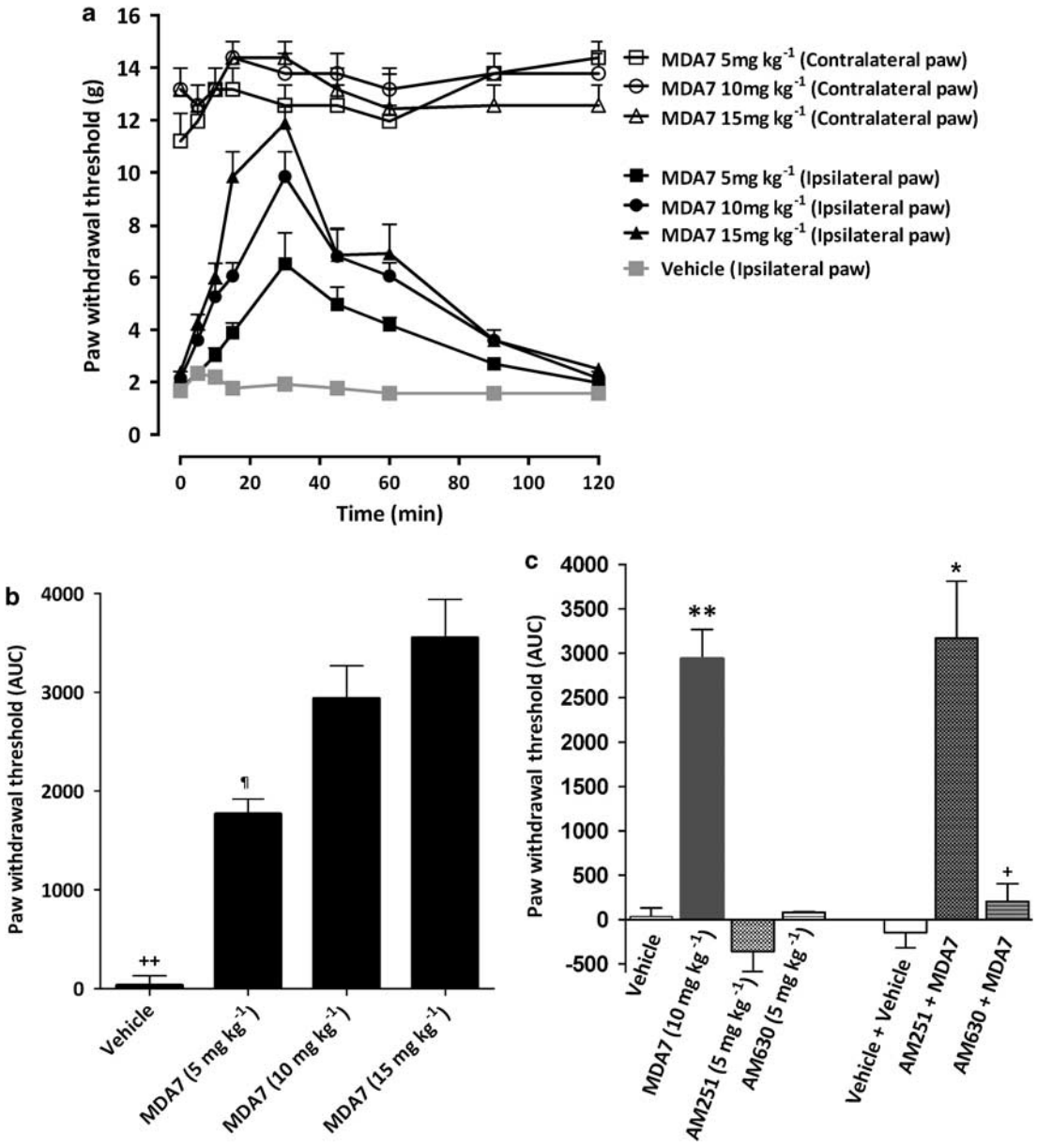


**Figure 5** Effects of MDA7 and selective antagonists for cannabinoid types 1 and 2 ($CB_1$ and $CB_2$) receptors, administered by i.p., on tactile allodynia in a spinal nerve ligation model of neuropathic pain in rats ($n=6$ per group). MDA7 induced increases in the withdrawal threshold of the nerve-injured paw in a dose-dependent manner. (**a**) The time course of 5, 10 and 15 mg kg$^{-1}$ of MDA7. (**b**) Area under the curve (AUC). (**c**) Effects of selective antagonists for $CB_1$ and $CB_2$ receptors on the antiallodynic effects of 10 mg kg$^{-1}$ of MDA7, shown as AUC ($n=6$ per group). All drugs were administered by i.p. injection. Administration of 5 mg kg$^{-1}$ of AM251, a selective $CB_1$ receptor antagonist, or 5 mg kg$^{-1}$ of AM630, a selective $CB_2$ receptor antagonist, had no effect. Administration of 5 mg kg$^{-1}$ of AM630 15 min before administration of MDA7 (AM630 + MDA7) reversed the antiallodynic effects of MDA7. Administration of 5 mg kg$^{-1}$ of AM251 15 min before administration of MDA7 (AM251 + MDA7) did not affect the antiallodynic effects of MDA7. Data expressed as mean ± s.e.mean. $^{++}P<0.001$ versus all other groups (ANOVA followed by Tukey–Kramer test for multiple group comparison). $^{¶}P<0.05$ versus MDA7 10 mg kg$^{-1}$ and MDA7 15 mg kg$^{-1}$ (ANOVA followed by Tukey–Kramer test for multiple group comparison). $^{**}P<0.001$ versus vehicle, AM251, AM630 (ANOVA followed by Tukey–Kramer test for multiple group comparison). $^{*}P<0.001$ versus vehicle + vehicle and AM251. $^{+}P<0.001$ versus MDA7 10 mg kg$^{-1}$ (ANOVA followed by Tukey–Kramer test for multiple group comparison).

stimulation with Von Frey filaments to 2.9 ± 0.19 and 2.8 ± 0.15 g for the right and left paws, respectively (Figure 4b). In paclitaxel-treated rats, i.p. administration of MDA7 suppressed mechanical allodynia (Figure 7) in a dose-dependent manner, as indicated by an increase in the %MPE withdrawal threshold AUC (Figure 7) and an $ED_{50}$ of 24 mg kg$^{-1}$ i.p. The %MPE for reversing mechanical allodynia, observed at 30 min with 15 mg kg$^{-1}$ of MDA7 i.p., was 42 ± 8.8%.

*Open-field chamber testing*

In contrast to MDA7 (15 mg kg$^{-1}$ i.p.), both WIN 55,212-2 (7 mg kg$^{-1}$ i.p.) (Herzberg *et al.*, 1997) and haloperidol

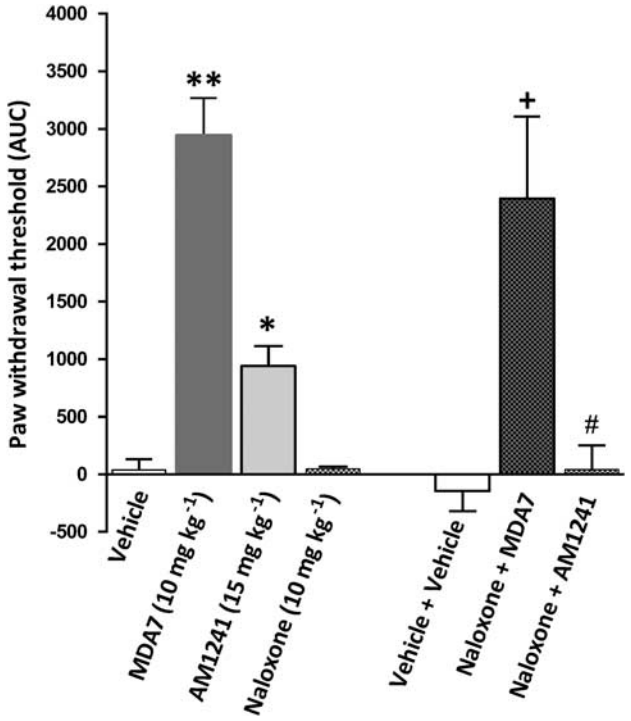


**Figure 6** Effects of the opioid receptor antagonist naloxone on MDA7- and AM1241-induced antiallodynic effects in a spinal nerve ligation model of neuropathic pain in rats ($n=6$ per group). All drugs were administered by i.p. injection. The antiallodynic effects of MDA7 (10 mg kg$^{-1}$) were significantly greater than were those of a cannabinoid type 2 ($CB_2$) receptor-selective agonist, AM1241 (15 mg kg$^{-1}$). Administration of the opioid antagonist naloxone (10 mg kg$^{-1}$) did not affect the paw withdrawal threshold. Pretreatment with naloxone (10 mg kg$^{-1}$) followed 15 min later by MDA7 (10 mg kg$^{-1}$) (naloxone + MDA7) did not affect the antiallodynic effects of MDA7. In contrast, pretreatment with naloxone (10 mg kg$^{-1}$) followed 15 min later by AM1241 (15 mg kg$^{-1}$) (naloxone + AM1241) reversed the antiallodynic effects of AM1241. $^{**}P<0.001$ compared with vehicle, AM1241 and naloxone (ANOVA followed by Tukey–Kramer test for multiple group comparison). $^{*}P<0.001$ compared with vehicle and naloxone (ANOVA followed by Tukey–Kramer test for multiple group comparison). $^{+}P<0.01$ compared with vehicle + vehicle and naloxone. $^{\#}P<0.01$ compared with AM1241 (ANOVA followed by Tukey–Kramer test for multiple group comparison).

(1 mg kg$^{-1}$ i.p.) significantly ($P<0.05$) decreased exploratory behaviour in rats, as shown by a reduction in the total distance travelled (Figure 8a), time spent ambulating (Figure 8b), vertical movements (Figure 8c) and zone entries (Figure 8d).

## Discussion

The results of this study indicate that MDA7 is a selective $CB_2$ agonist that suppresses allodynia in rats with spinal nerve ligation or paclitaxel-induced neuropathic pain without affecting their locomotor activity. MDA7 showed good affinity and excellent selectivity for $CB_2$ receptors and low affinity and efficacy at $CB_1$ receptors. The effects of MDA7 were inhibited by the $CB_2$ receptor-selective antagonist AM630 but not by the $CB_1$ receptor-selective antagonist

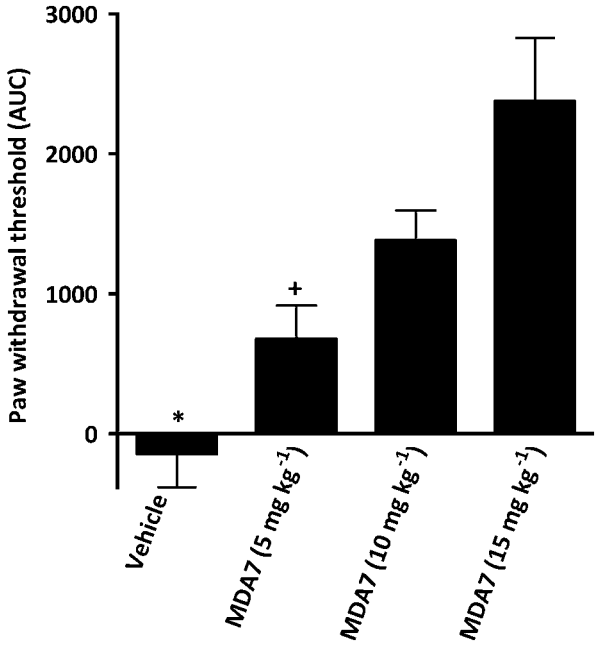


**Figure 7** Effects of MDA7 administered by i.p. injection on tactile allodynia in a paclitaxel-induced neuropathic pain model in rats ($n=8$ per group). MDA7 dose-dependently attenuated tactile allodynia in this model, as shown by an increase in the percentage maximal possible effect (%MPE) withdrawal threshold area under the curve (AUC). $^{*}P\leqslant0.05$ compared with 10 and 15 mg kg$^{-1}$ of MDA7 (ANOVA followed by Tukey–Kramer test for multiple group comparison). $^{+}P\leqslant0.05$, $P<0.05$ compared with 15 mg kg$^{-1}$ of MDA7 (ANOVA followed by Tukey–Kramer test for multiple group comparison).

AM251 or by the opioid receptor antagonist naloxone, indicating that the effects of MDA7 are mediated by $CB_2$ receptors.

The $CB_2$ receptor has emerged as a new target for treating neuropathic pain, and $CB_2$ receptor agonists have the advantage of lacking the adverse psychotropic effects normally seen with the $CB_1$ receptor agonists (Beltramo *et al*., 2006; Cox *et al*., 2007; Whiteside *et al*., 2007; Guindon and Hohmann, 2008). The mechanism by which $CB_2$ receptors modulate neuropathic pain is still poorly understood. Peripheral nerve injury induces $CB_2$ protein expression in rat sensory neurons (Wotherspoon *et al*., 2005). The mRNA for $CB_2$ receptors is expressed in the dorsal root ganglia of neuropathic rats and is upregulated in their spinal cords. Expression of $CB_2$ receptor mRNA was also found in cultured spinal cord microglia (Walczak *et al*., 2005; Beltramo *et al*., 2006) and was upregulated in reactive microglia (Ashton and Class, 2007; Romero-Sandoval and Eisenach, 2007).

The efficacy of selective $CB_2$ receptor agonists in attenuating neuropathic pain has been reported with only a few compounds, including A-796260 (Yao *et al*., 2008), AM1241 (Malan *et al*., 2001; Ibrahim *et al*., 2003) and GW405833 (Valenzano *et al*., 2005). In this study, MDA7 exhibited specificity for human $CB_2$ receptors; indeed $CB_1$ receptor-mediated functional activity for MDA7 was so low that we were not able to calculate the corresponding $EC_{50}$ for MDA7 at human $CB_1$ receptors. The $CB_2$ agonist GW842166X

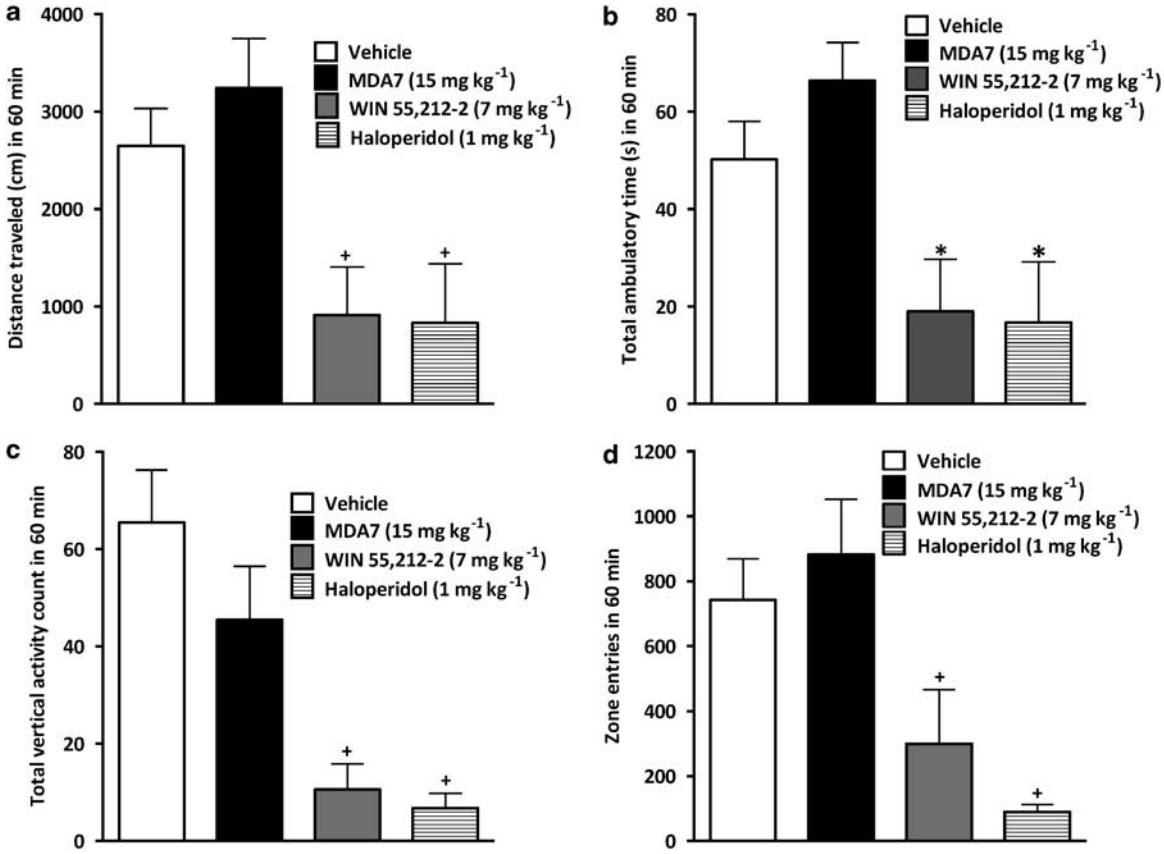


**Figure 8** Effect of MDA7 on locomotor activity in rats. Exploratory behaviour was tested in an open-field chamber following i.p. administration of vehicle, MDA7, WIN 55,212-2 or haloperidol ($n = 6$ per group). The following parameters were scored for 60 min: distance travelled (**a**), ambulatory time (**b**), vertical activity (**c**) and number of zone entries (**d**). $^{+}P < 0.05$ versus vehicle and MDA7 (ANOVA followed by Tukey–Kramer test for multiple group comparison). $^{*}P < 0.05$ versus MDA7 (ANOVA followed by Tukey–Kramer test for multiple group comparison).

(Giblin *et al*., 2007) is scheduled to undergo phase 2 studies in Europe for treatment of thermal pain. It is worth noting that Yao *et al*. (2008) found that GW842166X exhibits low potency for displacement of [$^3$H]CP55,940 from the human and rat $CB_2$ receptors (2000 and 2580 nM, respectively) yet exhibits full agonist efficacy in functional assays.

In a partial sciatic nerve ligation model of neuropathic pain, Yao *et al*. (2008) reported that the $ED_{50}$ for reversal of tactile allodynia for A-796260 was 15 mg kg$^{-1}$ i.p. (95% CI = 5.3–26 mg kg$^{-1}$), with an efficacy of 66 ± 9% for the highest dose used (35 mg kg$^{-1}$ i.p.). In the same model, Valenzano *et al*. (2005) assessed the effects of 0.1, 0.3, 1, 10 and 30 mg kg$^{-1}$ i.p. of GW405833 on the reversal of mechanical hyperalgesia. The maximum percentage reversal (63.6 ± 9.2%) was achieved 1 h after administration of the 10 mg kg$^{-1}$ dose. The effect of the 30 mg kg$^{-1}$ dose of GW405833 was less than that noted with the 10 mg kg$^{-1}$ dose.

We recently described a series of N-alkyl isatin acylhydrazone derivatives that act as selective $CB_2$ agonists (Diaz *et al*., 2008). This series was based on a parallel synthesis approach to generate structure-activity relationships. A second novel series based on a racemic mixture of 3,3-disubstituted-2, 3-dihydro-1-benzofuran compounds was then designed to increase bioavailability compared with that of the isatin series. Comparing benzofuran with isatin structures, it was assumed that the benzofuran scaffold might mimic the isatin scaffold. In Figure 1a, ring B from the benzofuran series is superimposable on the six-membered ring formed by the hydrazone, and the indolone through an internal hydrogen bond. In this case, both the five-membered rings (ring A) from the isatin and benzofuran structures fit. Structure-activity relationships based on the isatin series have been used to shorten the optimization time for the benzofuran series. The piperidine moiety was used to mimic the cyclohexyl group borne by the hydrazoic moiety in the isatin series ($R_1$ in Figure 1a). Because the isatin compound with a benzyl moiety on the nitrogen of the isatin ring ($R_2$ in Figure 1a) exhibited one of the best ratios in terms of selectivity and activity for $CB_2$ receptors, we retained this substituent in the benzofuran series, resulting in the synthesis of MDA7.

In our spinal nerve ligation model in rats, we noted that MDA7 treatment attenuated tactile allodynia in a dose-related manner, with an $ED_{50}$ of 7.5 mg kg$^{-1}$ i.p. (95% CI = 5.6–9.9 mg kg$^{-1}$) and an efficacy of 75.1 ± 5% after the 15 mg kg$^{-1}$ dose of MDA7 i.p. In the same model, AM1241 had a %MPE of 100% in suppressing allodynia at a dose of 3 mg kg$^{-1}$ i.p. (Ibrahim *et al*., 2005). Using the same model in this study, we noted that the %MPE of a larger dose

(15 mg kg$^{-1}$ i.p.) of AM1241 was only 25% at 10 min (data not shown). In fact, the effect of 15 mg kg$^{-1}$ of AM1241 i.p. was significantly less ($P<0.01$) than that of 10 mg kg$^{-1}$ of MDA7 i.p. in suppressing allodynia in the spinal nerve ligation model (Figure 6). In accordance with our results (Figure 6), Yao *et al*. (2008) noted that the effects of AM1241 were antagonized by naloxone. The antiallodynic effects of MDA7 were not affected by naloxone. It is not clear how AM1241 interacts with the opioid system, as it has no affinity for the μ-opioid receptor (Ibrahim *et al*., 2005).

In this study, paclitaxel did not affect the baseline paw withdrawal latency in response to thermal stimulation compared with naive rats. Similar observations were previously reported in a vincristine-induced neuropathy model (Rahn *et al*., 2007). In our study, MDA7 suppressed paclitaxel-induced mechanical allodynia relative to treatment with the vehicle in a dose-dependent manner (Figure 7). The effects of $CB_2$ receptor ligands on acute nociception have not yet been fully explored. Some reports have suggested that some $CB_2$ receptor ligands have antinociceptive effects in naive animals. AM1241 produced dose-dependent antinociception in response to a thermal stimulus in rats and mice (Malan *et al*., 2001; Ibrahim *et al*., 2005). These effects were absent in $CB_2$ knockout mice (Ibrahim *et al*., 2006). The antinociceptive effect was reversed with the $CB_2$ receptor-selective antagonist AM630 (Malan *et al*., 2001) or with naloxone or antiserum to β-endorphin (Ibrahim *et al*., 2005). In contrast, administration of the $CB_2$ receptor-selective agonist HU-308 did not affect acute nociception in mice, when thermal withdrawal latency was measured on a 55 °C hot plate (Hanus *et al*., 1999). Treatment with GW405833 i.p. did not affect hot plate or tail flick latency after doses of up to 30 mg kg$^{-1}$. However, 100 mg kg$^{-1}$ of GW405833 resulted in a significant increase in both tail flick and hot plate latencies 1 h after administration (Valenzano *et al*., 2005). The 100 mg kg$^{-1}$ dose of GW405833 that resulted in an analgesic effect on tail flick and hot plate test has also been shown to induce typical $CB_1$ receptor-mediated effects (Valenzano *et al*., 2005)—not supporting a $CB_2$-mediated effect in acute nociception. In this study, low doses of MDA7 (1 and 3 mg kg$^{-1}$ i.p.) failed to suppress thermal nociception in naive rats. Only higher doses (10 mg kg$^{-1}$ of MDA7 i.p.) resulted in modest antinociceptive effects (Figure 3).

We used 0.25 mL (0.25 g) of 100% DMSO as a vehicle in this study and observed no analgesic effects. The behavioural effects (motor activity, grip strength, paw pressure, mechanical non-nociceptive thresholds with the von Frey hair test) after repeated i.p. injections of 10 mL kg$^{-1}$ of various concentrations of DMSO (1.8, 3.6, 7.2%) for 10 days were studied in male Sprague–Dawley rats (Authier *et al*., 2002). The maximum dose of DMSO used in the latter studies was 0.144 g daily for 10 days (total dose, 1.44 g) for a rat weighing 200 g. Results from behavioural tests were not different between the treated and control groups except for the withdrawal thresholds to von Frey hairs, which demonstrated a slight but significant decrease only after the 10th cumulative dose (Authier *et al*., 2002). DMSO is known to be a cumulative drug (Hucker *et al*., 1967). It should be noted that Authier *et al*. (2002) used von Frey hairs in normal (not neuropathic) rats in their experiments. The reported median motor activity of mice (measured as revolutions per hour of activity wheel) after oral or i.p. administration of 5 g kg$^{-1}$ of DMSO were 38 and 71.1, respectively, and the corresponding control (saline) values were 271 and 51.1 respectively (Kocsis *et al*., 1975).

MDA7 did not inhibit ambulation or rearing in the open-field testing. Others studies have shown that other selective $CB_2$ receptor agonists also did not affect locomotor activity (Yao *et al*., 2008) or cause catalepsy (Hanus *et al*., 1999).

In summary, we have described the synthesis and pharmacological characterization of a novel selective $CB_2$ agonist, MDA7. We found that MDA7 was effective in treating various models of neuropathic pain in rats by activating $CB_2$ receptors without affecting the locomotor behaviour of the animals. Our study supports the role of selective $CB_2$ ligands in modulating neuropathic pain.

## Conflict of interest

The authors state no conflict of interest.

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