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Antinociceptive effects of the selective CB_2 agonist MT178 in inflammatory and chronic rodent pain models

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ABSTRACT

Cannabinoid CB_2 receptor activation by selective agonists has been shown to produce analgesic effects in preclinical models of inflammatory, neuropathic, and bone cancer pain. In this study the effect of a novel CB_2 agonist (MT178) was evaluated in different animal models of pain. First of all, in vitro competition binding experiments performed on rat, mouse, or human CB receptors revealed a high affinity, selectivity, and potency of MT178. The analgesic properties of the novel CB_2 agonist were evaluated in various in vivo experiments, such as writhing and formalin assays, showing a good efficacy comparable with that produced by the nonselective CB agonist WIN 55,212-2. A dose-dependent antiallodynic effect of the novel CB_2 compound in the streptozotocin-induced diabetic neuropathy was found. In a bone cancer pain model and in the acid-induced muscle pain model, MT178 was able to significantly reduce mechanical hyperalgesia in a dose-related manner. Notably, MT178 failed to provoke locomotor disturbance and catalepsy, which were observed following the administration of WIN 55,212-2. CB_2 receptor mechanism of action was investigated in dorsal root ganglia where MT178 mediated a reduction of $[^3H]$-D-aspartate release. MT178 was also able to inhibit capsaicin-induced substance P release and NF-κB activation. These results demonstrate that systemic administration of MT178 produced a robust analgesia in different pain models via CB_2 receptors, providing an interesting approach to analgesic therapy in inflammatory and chronic pain without CB_1-mediated central side effects.

1. Introduction

Neurotransmission and neuroinflammation are modulated by the endocannabinoid signaling system based on the CB_1 and CB_2 cannabinoid receptors [33,39]. CB_1 receptors are expressed at high levels in the central nervous system (CNS) and their activation results in hypothermia, sedation, catalepsy, and altered mental status [20,44]. CB_2 receptors were originally believed to be restricted to the periphery, primarily in the immune system, including mast cells, B and T cells, macrophages, and natural killer cells [28]. Low levels of CB_2 receptors were observed in different CNS sites, and an upregulation of these receptors in sites implicated in nociceptive process was found [4,18]. CB_2 receptors are also expressed in brain microglia, macrophage-like resident immune cells that are able to secrete proinflammatory cytokines, and several mediators such as nitric oxide, neurotrophins, and free radicals [12,56]. From the molecular point of view, the binding of agonist to CB receptors inhibits adenylyl cyclase activity by $G_{i/o}$ proteins and activates the mitogen-activated protein kinase pathway [10]. The stimulation of CB_2 receptors modified the cytokine milieu contributing to the accumulation of antiinflammatory mediators [8,25]. Therefore, CB_2 agonists could represent an attractive therapeutic target affecting a myriad of immune responses, from inflammation to neuroprotection [36]. The recent finding demonstrating the presence of CB_2 receptor immunoreactivity in dorsal root ganglion (DRG) neurons provides a platform to study functional effects of selective CB_2 agonists to better investigate their antinociceptive mechanism of action [2]. Several studies have demonstrated marked antinociceptive effects of CB agonists in different models of inflammatory or neuropathic pain [23]. These models could be very useful for the preclinical evaluation and validation of the therapeutic efficacy of novel putative analgesics [3]. Both CB_1 and CB_2 stimulation seem to be involved in the inhibition of inflammatory and/or neuropathic persistent pain [39,48]. However, most drugs interacting with CB_1 receptors showed marked central side effects

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that have prevented their widespread acceptance and therapeutic application [31]. The search for new compounds that activate the CB system and lack serious central side effects could be of relevance for chronic pain management [19]. As a consequence, the development and evaluation of selective CB_2 compounds provide direct support for the hypothesis that CB_2 activation produces antinociceptive effects in persistent pain states [42]. In addition, the use of CB_2 agonists could be positive without psychoactive or addictive effects and CNS side effects [4]. The aim of the present study was to determine the efficacy of the novel CB_2 selective agonist MT178 in 2 models of inflammatory pain such as writhing and formalin tests. The effect of MT178 was also investigated in chronic streptozotocin (STZ)-induced neuropathy, in bone cancer pain, and in acid-induced muscle pain (AIMP). Rotarod and catalepsy assays were performed to evaluate CNS adverse effects. The mechanisms underlying the analgesic properties of the novel CB_2 compound were studied in DRG neurons. In particular, the effects of CB_2 receptor stimulation on D-aspartate and substance P release, as well as nuclear factor (NF)-κB activation, were explored.

2. Methods

2.1. Drugs

MT178 (N-adamantyl-3-ethyl-3,7-dihydro-7-oxo-10-(pyrrolidin-1-yl)-2H-[1,4]oxazino[2,3,4-ij] quinoline-6-carboxamide) was recently synthesized [5], and the chemical structure is reported in Fig. 1. The nonselective CB agonist WIN 55,212-2, the selective CB_2 antagonist AM 630, and the selective CB_1 antagonist AM 251 were purchased from Tocris Biosciences (Bristol, UK). Drugs were dissolved in dimethyl sulfoxide and further diluted in water containing 5% Tween 20. The vehicle is composed of water containing 5% Tween 20 and 5% dimethyl sulfoxide. [^{3}H]-CP-55,940 (specific activity, 180 Ci/mmol) was obtained from Perkin Elmer (Boston, MA, USA). All other reagents were of analytical grade and obtained from commercial sources.

2.2. Animals

Male CD1 mice (22–24 g) and female or male Sprague-Dawley rats (170–200 g) were obtained from Charles River (Milan, Italy). The animals were kept under standard environmental temperature and humidity-controlled conditions (22 ± 2 °C) with 12-hour light/dark cycle (lights on at 6:00 am) with food and water ad libitum. The animals were acclimated to the laboratory settings for at least 1 hour before testing and were used only once throughout the experiments. All the procedures used in the present study were carried out in accordance with European Communities Council directives (86/609/EEC) and National Laws and Policies (D.L.116/92) with the authorization from the Italian Ministry for Health (181/2009-B and 122/2011-B). In addition, the experimental procedures were in agreement with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [60]. The number of animals and the intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatment.

2.3. In vitro assays

2.3.1. [^{3}H]-CP-55,940 competition binding assays

Competition binding experiments were performed by using [^{3}H]-CP-55,940 to CB_1 (1.0 nM) or CB_2 (0.5 nM) receptors in different tissues or cells. Briefly, rat or mouse brain and spleen were removed and suspended in 50 mM Tris HCl buffer, pH 7.4 at 4 °C. The suspension was homogenized, centrifuged for 10 minutes at 2000 × g and the supernatant was centrifuged again for 20 minutes at 40,000 × g. The pellet was resuspended in 50 mM Tris HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM $MgCl_2$, and 0.5% fatty acid free bovine serum albumin (BSA), pH 7.4 at 30°C containing 40 μg or 80 μg of protein/100 μL. MT178, WIN 55,212-2, AM 630, or AM 251 at different concentrations (1 nM–10 μM) were used in CB_1 or CB_2 competition binding experiments [45].

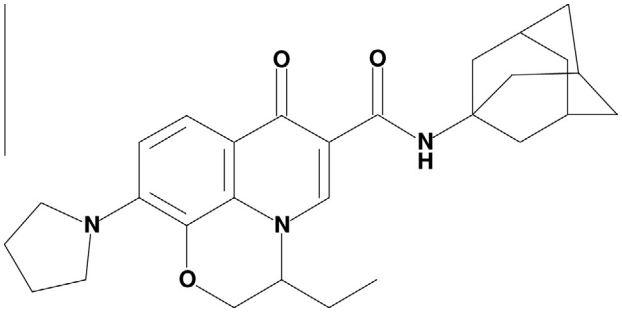

Fig. 1. Chemical structure of N-adamantyl-3-ethyl-3,7-dihydro-7-oxo-10-(pyrrolidin-1-yl)-2H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxamide (MT178).

Human CB_1 or CB_2 receptors were expressed in Chinese hamster ovary (CHO) cells (Perkin Elmer) that were grown adherently and maintained in Ham's F12 containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and geneticin (G418, 0.4 mg/mL) at 37°C in 5% CO_2/95% air [21,37]. For membrane preparation, the cells were washed with phosphate-buffered saline and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized and centrifuged for 10 minutes at 1000 × g, and the supernatant was centrifuged for 30 minutes at 100,000 × g. The membrane pellet was suspended in 50 mM Tris HCl buffer (pH 7.4) containing 0.5% BSA, 5 mM $MgCl_2$, 2.5 mM EDTA, or 1 mM EDTA for hCB_1 or hCB_2 receptors, respectively, and different concentrations of MT178, WIN 55,212-2, AM 630, or AM 251 were used in competition binding assays.

The incubation time was 90 or 60 minutes at 30°C for CB_1 or CB_2 receptors, respectively. Nonspecific binding was determined in the presence of WIN 55,212-2 (1 μM). Bound and free radioactivity was separated by filtering the assay mixture through Whatman GF/C glass fiber filters using a Brandel cell harvester (Brandel Instruments, Unterföhring, Germany). The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

2.3.2. Cyclic AMP assays

CHO cells transfected with human CB_1 or CB_2 receptors were washed with phosphate-buffered saline, detached with trypsin, and centrifuged for 10 minutes at 200 × g. The pellet containing CHO cells (1×10^6 cells/assay) was suspended in 0.5 mL of incubation mixture: NaCl 150 mM, KCl 2.7 mM, NaH_2PO_4 0.37 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, (4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid (HEPES) 5 mM, $MgCl_2$ 10 mM, and glucose 5 mM, pH 7.4 at 37°C. Then 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as a phosphodiesterase inhibitor was added and preincubated for 10 minutes in a shaking bath at 37°C. The potency of MT178 in comparison with the CB agonist (WIN 55,212-2) and antagonists (AM 630 and AM 251) was studied in the presence of forskolin 1 μM. The reaction was terminated by the addition of cold 6% trichloroacetic acid. The final aqueous solution was tested for cyclic adenosine monophosphate (AMP) levels by a competition protein binding assay [54].

2.4. Animal models of pain

2.4.1. Formalin assays

Mice received formalin injection (1.5% in saline, 20 μL) in the plantar surface of the hind paw. Each mouse was randomly assigned to one of the experimental groups (n = 6) and placed in a Plexiglas cage and allowed to move freely for 15–20 minutes. Lifting, favoring, licking, shaking, and flinching of the injected paw were recorded as nociceptive responses. The duration of those mentioned noxious behaviors were monitored by an observer blind to the experimental treatment for 45 minutes after formalin administration [1,23]. Fifteen minutes before formalin injection, mice received intraperitoneal (i.p.) administration of vehicle, MT178 (0.03, 0.1, 0.3, 1, 3 mg/kg, 100 μL/mouse) or WIN 55,212-2. The CB_2 antagonist AM 630 (3 mg/kg) or the CB_1 antagonist AM 251 (3 mg/kg) was administered alone or 10 minutes before the CB agonists.

2.4.2. Writhing test

The acetic acid-induced writhing response was performed after i.p. injection of 10 mL/kg of 0.6% acetic acid solution. A writhe is indicated by stretching of the abdomen followed by the extension of the hind limbs. The animals (6 mice per group) were placed singly in a glass cylinder and the number of writhing episodes in a 30-minute period was counted starting 5 minutes after the acetic acid administration. CB compounds were administered (i.p.) 15 minutes before injection of acetic acid solution. As expected, abdominal constrictions were not observed in saline-treated mice [34].

2.4.3. STZ-induced diabetes model

Diabetes was induced by using a 200-mg/kg i.p. injection of STZ, and blood glucose levels were assessed from 2 days to 2 weeks later [30]. A single administration of STZ induced insulin-dependent diabetes mellitus within 24–48 hours by destruction of pancreatic islet cells. Blood samples were used to monitor plasma glucose, and diabetes was confirmed by plasma glucose (over 300 mg/dL) using an Accu-Check blood glucose monitoring system (Roche Diagnostics, Indianapolis, IN, USA). All experiments were initiated 14 days after injection of STZ. To evaluate the effects of examined compounds, behavioral testing such as mechanical allodynia paw withdrawal test was used.

2.4.4. Bone cancer pain

An appropriate animal model based on MRMT-1 rat mammary gland carcinoma cells (Riken BRC, Tsukuba, Japan) injection in rat tibia that produced a progressive development of mechanical allodynia was used. Following complete induction of anesthesia, the left leg of the rat was shaved, the skin disinfected, a rostrocaudal incision was made, and the needle inserted at an angle to enable it to be pushed down the intramedullary canal of the bone. A volume of MRMT-1 cells (3 μL) or heat-killed cells was injected into the bone cavity with a 10-μL Hamilton syringe. The needle was lifted out of the bone to enable the cells to fill the space left in the bone cavity, taking care that no leakage of cells occurred outside the bone. Following injection, the site and the wound were closed and the animals placed on a heated pad until they regained consciousness, after which they were returned to their home cages [35].

2.4.5. Chronic musculoskeletal pain (acid-induced muscle pain)

Male rats were anesthetized and injected with 100 μL sterile saline (pH 4.0) into the gastrocnemius muscle on day 0 and on day 5. Responses of the paw to mechanical stimuli were measured 2 days after the second acid injection in the absence or in the presence of MT178, WIN 55,212-2, AM 630, or AM 251 [49].

2.5. Assessment of the mechanical allodynia

To assess mechanical allodynia, paw withdrawal thresholds were measured using the Dynamic Plantar Aesthesiometer (Ugo Basile, Milan, Italy). Mice or rats were placed individually in plastic cages with a wire mesh bottom and allowed to acclimatize for at least 2 hours. Increasing mechanical stimulation (0.25 g/s, cutoff force: 10 g) was applied to the plantar surface of a hind paw [17]. The nociceptive threshold is defined as the force, in grams, at which the animals withdraw their paws. When a withdrawal response occurred, the stimulus was terminated and the response threshold was electronically measured. Paw withdrawal threshold was recorded 45 minutes after i.p. injection of CB ligands. AM 630 or AM 251 (10 mg/kg) were also administered (i.p.) 10 minutes before the administration of CB agonists. Animals in the control group received an i.p. injection of vehicle.

2.6. Assessment of central side effects

2.6.1. Rotarod

Changes in motor performance were measured using a fixed-speed (12 rpm) rotarod (Ugo Basile). Mice received 2 training trials on 2 separate days prior to testing for acclimatization. Animals were trained to remain on the rotarod, eliminating those mice that did not remain on the bar for 2 consecutive periods of 300 seconds [13]. On the experimental day, the time that mice remained on the rotating bar (cutoff 300 seconds) was recorded 15 minutes after the i.p. injection of different concentrations of MT178 or WIN 55,212-2.

2.6.2. Catalepsy

Catalepsy was measured by placing the mice forelegs over a horizontal glass bar (0.5 cm diameter) elevated 4.5 cm from floor [48]. The time that mice remained with both forelegs on the bar was measured 15 minutes after the i.p. injection of various concentrations of MT178 or WIN 55,212-2.

2.7. Investigation of CB_2 receptor mechanisms

2.7.1. Culture of rat DRG cells

Rat DRG from the T1 to L6 levels were removed and placed in cold Dulbecco's modified eagle medium (DMEM) solution, containing (in mg/mL): 0.5 trypsin, 1 collagenase type IA, and 0.1 DNAse type IV (Sigma-Aldrich, Milan, Italy). DRG neurons were suspended in DMEM supplemented with 10% horse serum, 4 mM glutamine, 100 units/mL penicillin, 100 g/mL streptomycin, and 50 ng/mL 2.5S mouse salivary glands nerve growth factor. Cytosine-β-D-arabinofuranoside (Ara-C, 2.5 μM) was also added to the culture medium as a cytostatic agent to inhibit satellite cell proliferation. Cells were then plated on polylysine- (8.3 μM, Sigma-Aldrich)- and laminin- (5 μM, Sigma-Aldrich) coated 6-well plates and kept in an atmosphere of 5% CO_2/95% air at 37°C for 5 days before the experiments [6].

2.7.2. [^{3}H]-D-aspartate release

DRG neurons grown on coverslips were incubated for 2 hours in 2 mL of DMEM containing 4 μCi/mL [^{3}H]-D-aspartate (1 mCi/mL; specific activity 12 Ci/mmol, Perkin Elmer). Radiolabeled D-aspartate was used as a nonmetabolizable marker of intracellular glutamate and aspartate pools. The coverslips were inserted into a Lucite perfusion chamber and superfused with HEPES-buffered solution consisting of (in mM) 140 NaCl, 3.3 KCl, 0.4 $MgSO_4$, 1.3

$CaCl_2$, 1.2 KH_2PO_4, 10 glucose, and 25 HEPES (pH 7.4). KCl buffer (100 mM) was made by replacing Na^+ with equimolar K^+ [46]. Release of [^{3}H]-D-aspartate was evoked by K+ stimuli applied for 5 minutes at the 20th (S_1) and 50th (S_2) minute from the onset of perfusion. MT178 was present in the buffer solutions applied to the cells 5 minutes before and during S_2. The perfusate was collected in 1-minute intervals and the radioactivity content in each fraction was determined using a Perkin Elmer 2810TR liquid scintillation analyzer. At the end of the experiment, the cells were lysed in 1 N NaOH, which released any [^{3}H]-D-aspartate left in the cells. Percent fractional isotope release for each time point was determined by dividing radioactivity released at each 1-minute interval by the radioactivity remaining in the cells at this time point, as retroactively calculated using a custom computer program [27].

2.7.3. Substance P enzyme-linked immunosorbent assay

DRG neurons were treated with different concentrations of MT178 for 15 minutes (in the absence or in the presence of 10 μM AM 630) and subsequently exposed to 10 μM capsaicin for 10 minutes. Substance P levels were measured by using a highly sensitive enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions [58].

2.7.4. NF-κB assay

DRG neurons were treated with tumor necrosis factor (TNF)-α (10 ng/mL) for 1 hour in the absence or in the presence of MT178 (10 nM–1 μM) and AM 630 (10 μM). Nuclear extracts from DRG neuron cells were obtained by using a nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The NF-κB activation was evaluated by detecting phosphorylated p65 proteins in nuclear extracts by using the TransAM NF-κB kit (Active Motif). Phosphorylated NF-κB subunit specifically binds to the immobilized oligonucleotides containing the NF-κB consensus site (5′-GGGACTTTCC-3′). The primary antibody used to detect NF-κB recognized an epitope in the subunits that is accessible only after activation and bound to its DNA target. A horseradish peroxidase-conjugated secondary antibody provided a sensitive colorimetric readout that was quantified by spectrophotometry at 450 nm wavelength [53].

2.8. Statistics and data analysis

The protein concentration was determined according to a Bio-Rad method with bovine albumin as standard reference [53]. Inhibitory binding constant values, *K*i, were calculated from the IC_{50} according to the Cheng & Prusoff equation $K_i = IC_{50}/(1 + [C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant [54]. All data are expressed as the mean ± SEM of n = 4 independent experiments for in vitro assays and n = 6 for in vivo assays. Statistical analysis of the data was performed using unpaired 2-sided Student's *t*-test and, when appropriate, with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

3. Results

3.1. Affinity and potency of MT178 at cannabinoids receptors

Table 1 reports the affinity values of MT178 in comparison with the nonselective agonist WIN 55,212-2, the CB_2 selective antagonist AM 630, and the CB_1 selective antagonist AM 251. In [^{3}H]-CP55,940 competition binding experiments, the novel compound MT178 revealed a very good affinity for mouse, rat, and human CB_2 receptors with Ki values similar to those of the reference compound WIN 55,212-2. Notably, MT178 showed a remarkable selectivity to the CB_2 receptors with a CB_1/CB_2 Ki ratio > 1078. In contrast, the nonselective agonist WIN,55212-2 confirmed a similar affinity vs CB_1 or CB_2 receptors in all the substrates investigated (Table 1). Cyclic AMP assays revealed that MT178 behaves as a full agonist to the CB_2 receptors with an EC_{50} value of 25.31 nM and an efficacy comparable to WIN 55,212-2 (Emax 99 ± 5 and 100 ± 6, respectively). AM 630 displayed good affinity and selectivity (CB_1/CB_2 Ki ratio > 157) to mouse, rat, and human CB_2 receptors, making it suitable for in vivo studies. AM 251 confirmed the selectivity vs CB_1 receptor with a CB_2/CB_1 Ki ratio > 306 (Table 1).

3.2. MT178 elicited antinociceptive effects in inflammatory pain models

The formalin test was performed in mice after i.p. injection of vehicle, MT178, or WIN 55,212-2 in the absence or in the presence of AM 630 or AM 251. The time spent licking, shaking, flinching, and lifting the injected paw was recorded in 5-minute bins. MT178 resulted in a significant dose-dependent ($P < 0.001$, one-way ANOVA) suppression of pain behaviors relative to vehicle-treated mice in the second phase (15 to 60 minutes posttreatment) of the behavioral response to formalin (Fig. 2A). This effect was comparable to that obtained with the reference compound WIN 55,212-2. To investigate the involvement of CB_2 receptor activation in the antinociceptive effect elicited by MT178, mice were treated with the selective CB_2 antagonist AM 630 prior to the injection of the CB agonists. AM 630 was able to completely abrogate the effect of MT178 ($P < 0.01$), while it only partially blocked the effect of WIN 55,212-2, most likely due to residual CB_1 activation (Fig. 2B). The antinociceptive action of MT178 was not blocked by the CB_1 selective antagonist AM 251, confirming its selectivity vs CB_2 receptors. No effect was observed following the administration of AM 630 or AM 251 alone (Fig. 2B).

In the writhing test, the effect of increasing doses of MT178 or WIN 55,212-2 in mice relative to the vehicle group was reported (Fig. 3A). The i.p. administration of acetic acid induced 61 ± 2 abdominal constrictions in vehicle-treated mice. The antinociceptive effect of MT178 was demonstrated by a dose-dependent ($P < 0.001$, one-way ANOVA) reduction in the amount of writhing that reached a significant difference compared to vehicle starting at the dose of 0.03 mg/kg ($P < 0.01$). Similar results were obtained with the nonselective CB agonist WIN 55,212-2 (Fig. 3A). As expected, the effect of WIN 55,212-2 was only partially abrogated by the CB_2 selective antagonist AM 630, which was able, in contrast, to totally abrogate the effect of MT178 ($P < 0.01$). Moreover, AM 251 was not able to block the effect of MT178, but inhibited the effect of WIN 55,212-2 ($P < 0.01$, Fig. 3B).

3.3. MT178 reduced mechanical allodynia in chronic pain models

The effect of MT178 was evaluated in 3 different chronic pain models such as STZ-induced neuropathic pain, bone cancer pain, and AIMP. The injection of STZ produced a long-lasting mechanical allodynia and decreased the latency to paw withdrawal in comparison to naive mice. The i.p. administration of MT178 attenuated mechanical allodynia, demonstrating full efficacy at a dose of 0.3 mg/kg ($P < 0.001$ vs vehicle). Interestingly, statistically significant increases in paw withdrawal threshold were observed at doses from 0.1 to 3 mg/kg. A similar effect was also found following WIN 55,212-2 administration (Fig. 4A). Fig. 5A reports the effect of increasing doses of MT178 or WIN 55,212-2 in mechanical allodynia induced by bone cancer in rats. Our results reveal that MT178 was more effective than WIN 55,212-2, as suggested by

Table 1
Affinity and potency of examined compounds.

Compounds	hCB_1[a] Ki (nM)	rCB_1[b] Ki (nM)	mCB_1[c] Ki (nM)	hCB_2[d] Ki (nM)	rCB_2[e] Ki (nM)	mCB_2[f] Ki (nM)	hCB_1[a] IC_{50}(nM)	hCB_2[d] IC_{50} (nM)
MT178	>10,000	>10,000	>10,000	7.37 ± 0.65	9.28 ± 0.85	8.15 ± 0.78	>10,000	25.31 ± 2.23
WIN 55,212-2	12.73 ± 1.14	14.62 ± 1.43	13.81 ± 1.12	5.51 ± 0.52	7.67 ± 0.64	6.23 ± 0.57	42.36 ± 4.52	18.75 ± 1.68
AM 630	5652 ± 523	6235 ± 627	5967 ± 554	31.56 ± 3.12	39.71 ± 3.72	35.64 ± 3.26	>10000	122 ± 13
AM 251	11.32 ± 1.03	12.24 ± 1.14	10.53 ± 1.25	3642 ± 321	3748 ± 367	3769 ± 348	96 ± 8	>10,000

[^{3}H]-CP-55,940 competition binding experiments (Ki, nM) or cyclic AMP assays (IC_{50}, nM) were performed in different substrates. Data were presented as means ± SEM.
[a] hCB_1 expressed in CHO cells.
[b] Rat brain.
[c] Mouse brain.
[d] hCB_2 expressed in CHO cells.
[e] Rat spleen.
[f] Mouse spleen.

the efficacy of the 2 compounds (95% and 51%, respectively). In AIMP assays, MT178 was slightly more effective than WIN 55,212-2 ($P < 0.001$ and $P < 0.01$, respectively) in mechanical allodynia evoked by the injection of an acid solution in rat gastrocnemius muscle (Fig. 6A). In all these in vivo pain models, the receptor specificity was investigated using a CB selective antagonist. The pretreatment with AM 630 (3 mg/kg) significantly reversed ($P < 0.01$) the analgesic efficacy mediated by MT178 (0.3 mg/kg). In contrast, the CB_1 selective antagonist AM 251 was not able to abrogate the antinociceptive effect of MT178, as indicated in Figs. 4B, 5B, and 6B.

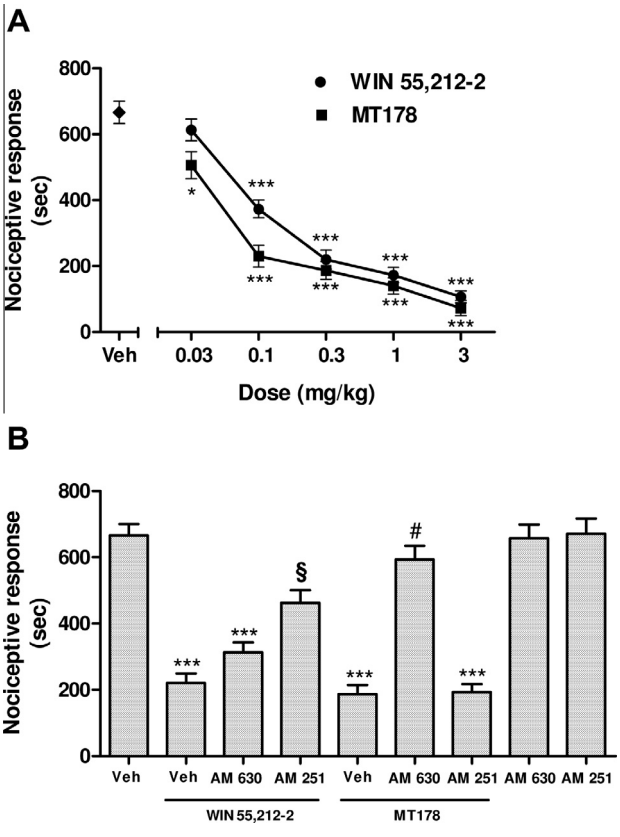

Fig. 2. Antinociceptive effect of MT178 in comparison to WIN 55,212-2 in formalin test. (A) MT178 and WIN 55,212-2 dose-dependently reverse formalin-induced nociceptive responses in mice. (B) Effect of AM 630 (3 mg/kg) or AM 251 (3 mg/kg) on MT178 (0.3 mg/kg) or WIN 55,212-2 (0.3 mg/kg) reversal of nociceptive behavior. Data are presented as mean ± SEM (n = 6 mice/group). Veh, vehicle. $^*P < 0.05$; $^{***}P < 0.001$ vs vehicle; $^{\S}P < 0.01$ vs WIN 55,212-2 (0.3 mg/kg); $^{\#}P < 0.01$ vs MT178 (0.3 mg/kg).

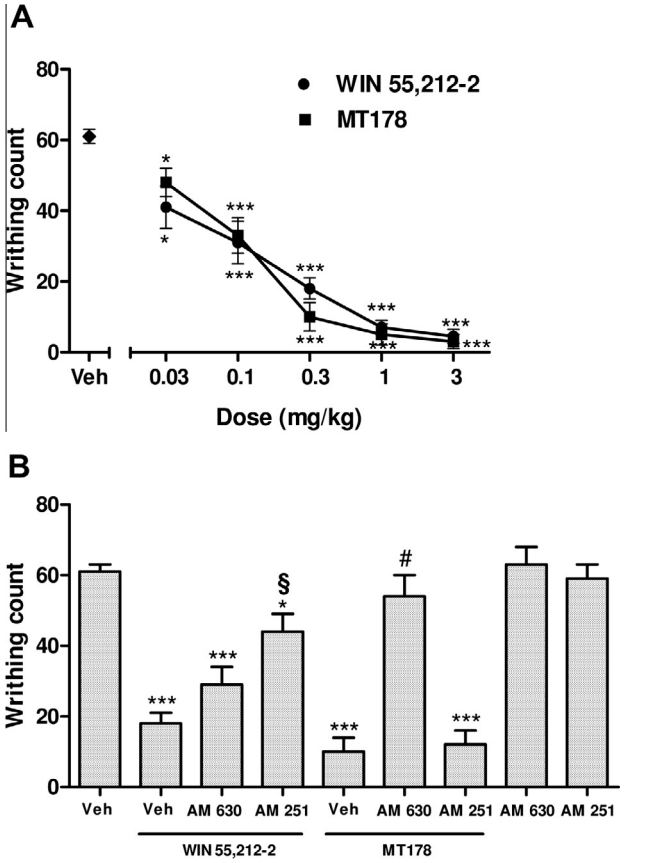

Fig. 3. Analgesic effect of MT178 and WIN 55,212-2 in writhing test. (A) MT178 and WIN 55,212-2 (0.03–3 mg/kg) reduce acid acetic-induced abdominal constriction in mice. (B) Effect of AM 630 (3 mg/kg) or AM 251 (3 mg/kg) on MT178 (0.3 mg/kg) or WIN 55,212-2 (0.3 mg/kg) in writing test. Data are presented as mean ± SEM (n = 6 mice/group). Veh, vehicle; $^*P < 0.05$; $^{***}P < 0.001$ vs vehicle; $^{\S}P < 0.01$ vs WIN 55,212-2 (0.3 mg/kg); $^{\#}P < 0.01$ vs MT178 (0.3 mg/kg).

3.4. MT178 did not display central side effects

The effect of CB agonists MT178 and WIN 55,212-2 on motor performance was measured using the rotarod assay. MT178 failed to provoke any locomotor disturbance following administration of doses up to 30 mg/kg. On the contrary, the nonselective CB agonist WIN 55,212-2 exhibited a marked reduction in the latency to fall off the rotarod in a dose-dependent manner ($P < 0.001$, one-way ANOVA, Fig. 7A). To determine the potential activation of central CB_1 receptors, the activity of MT178 or WIN 55,212-2 was evaluated in a mouse catalepsy assay. MT178 (0.3 to 30 mg/kg) failed to cause catalepsy-like behavior, whilst WIN 55,212-2 produced

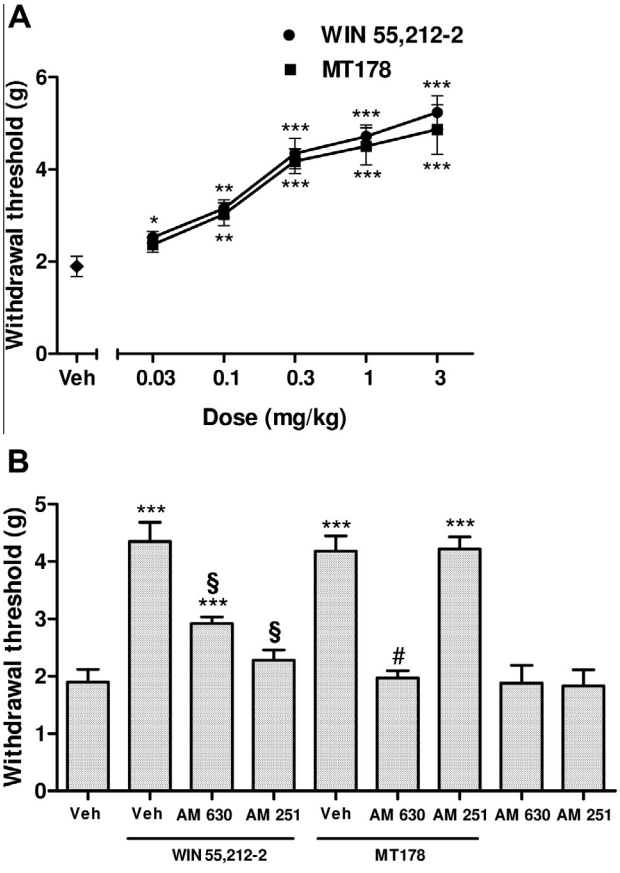

Fig. 4. Effect of MT178 in streptozotocin-induced neuropathic pain in comparison to WIN 55,212-2. (A) MT178 and WIN 55,212-2 (0.03–3 mg/kg) decrease mechanical allodynia as indicated by the increase of paw withdrawal threshold relative to vehicle. (B) AM 630 (3 mg/kg) partially blocked the antiallodynic effect of WIN 55,212-2 (0.3 mg/kg) and completed abrogated MT178 effect (0.3 mg/kg). AM 251 (3 mg/kg) was not able to block the effect of MT178. Data are presented as mean ± SEM (n = 6 mice/group). Veh, vehicle; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs vehicle; $^{\S}P < 0.01$ vs WIN 55,212-2 (0.3 mg/kg); $^{\#}P < 0.01$ vs MT178 (0.3 mg/kg).

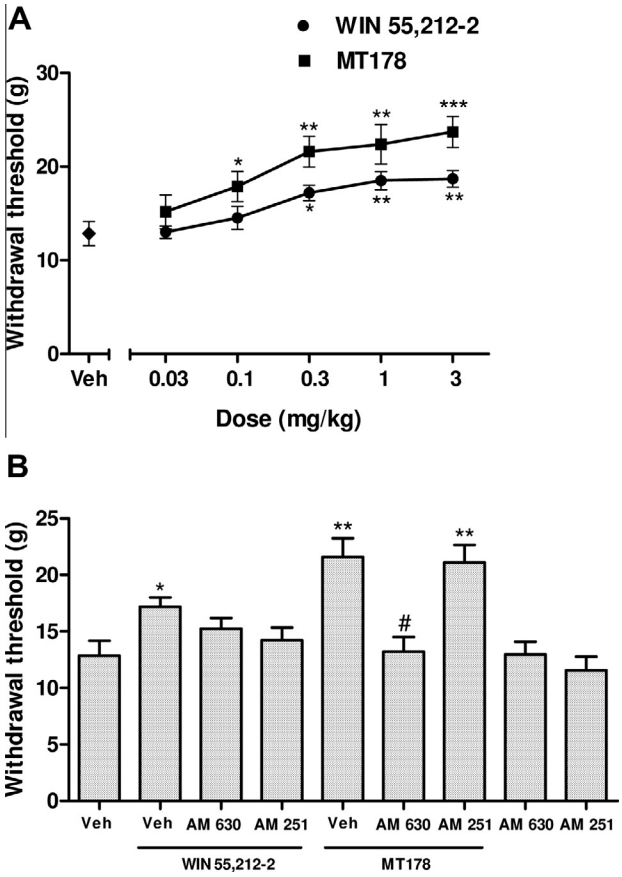

Fig. 5. Effect of MT178 and WIN 55,212-2 in bone cancer pain evaluated 14 days after MRMT-1 cell injection. (A) MT178 and WIN 55,212-2 (0.03–3 mg/kg) increase paw withdrawal threshold relative to vehicle reducing mechanical allodynia. (B) AM 630 (3 mg/kg), but not AM 251 (3 mg/kg), completely blocked the antiallodynic effect of MT178 (0.3 mg/kg). The same antagonists partially reduced WIN 55,212-2 effect (0.3 mg/kg). Data are presented as mean ± SEM (n = 6 mice/group). Veh, vehicle; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs vehicle; $^{\#}P < 0.01$ vs MT178 (0.3 mg/kg).

a significant cataleptic effect at 3 ($P < 0.05$), 10 ($P < 0.001$), or 30 mg/kg ($P < 0.001$), suggesting an involvement of central CB_1 receptors (Fig. 7B).

3.5. CB_2 receptors activation by MT178 inhibited the release of [^{3}H]-D-aspartate, substance P and NF-κB activation

The mechanism underlying the antinociceptive effect of MT178 was studied in DRG neurons, which have a key role in pain signal processing. First of all, the effect of MT178 was evaluated on the release of [^{3}H]-D-aspartate, a nonmetabolizable marker of glutamate, which is the main neurotransmitter involved in *pain* sensation and transmission. Interestingly, MT178 at the 1-μM concentration was able to significantly inhibit the K^+-evoked [^{3}H]-D-aspartate release from cultured DRG neurons ($P < 0.001$ vs vehicle). In particular, the ratio between the fractional [^{3}H]-aspartate release in the absence and in the presence of MT178 (S2/S1 ratio) showed a decrease of 52% relative to the vehicle-treated DRG neurons. The effect of MT178 was blocked by the CB_2 antagonist AM 630 ($P < 0.01$), suggesting an involvement of CB_2 receptors, as indicated in Fig. 8. Next, we investigated the effect of MT178 on the release of substance P, a small peptide that transmits pain signals from the sensory nerves to the CNS. Notably, the capsaicin-induced release of substance P from DRG neurons was significantly inhibited by the treatment with MT178 at the 1-μM concentration, reaching a reduction of 70% ($P < 0.01$). The specificity of this effect was demonstrated using the CB_2 selective antagonist AM 630 (10 μM), that completely abrogated the effect of MT178 ($P < 0.01$, Fig. 9A). Considering that NF-κB pathway has been suggested to be involved in inflammatory and/or chronic pain, we have evaluated the effect of MT178 in DRG neurons. MT178 (1 μM) was able to significantly inhibit ($P < 0.01$) NF-κB p65 subunit activation induced by TNF-α (10 ng/mL) and this effect was blocked by AM 630 ($P < 0.01$, Fig. 9B).

4. Discussion

The present study demonstrates that MT178 is a high affinity agonist for human, rat, or mouse CB_2 receptors and has antihyperalgesic activity in various rodent models of inflammatory and chronic pain. Although the analgesic properties of CB ligands have been well documented, their therapeutic potential is restricted by the unwanted psychotropic side effects associated with the CB_1 activation [39]. Interestingly, MT178 showed a high CB_2 selectivity, making it suitable as an antinociceptive drug without CNS side effects. Moreover, the novel CB_2 compound behaves as a potent full agonist, as indicated by cyclic AMP experiments performed in human CB_2 receptors expressed in CHO cells. As to CB_2 selective agonists, those most frequently used as pharmacological tools are JWH-133, a classical CB with a higher CB_2 (Ki = 3.4 nM) than CB_1 (Ki = 677 nM) affinity and HU-308, a nonclassical CB with a good affinity vs CB_2 (Ki = 22.7 nM) receptors without binding CB_1

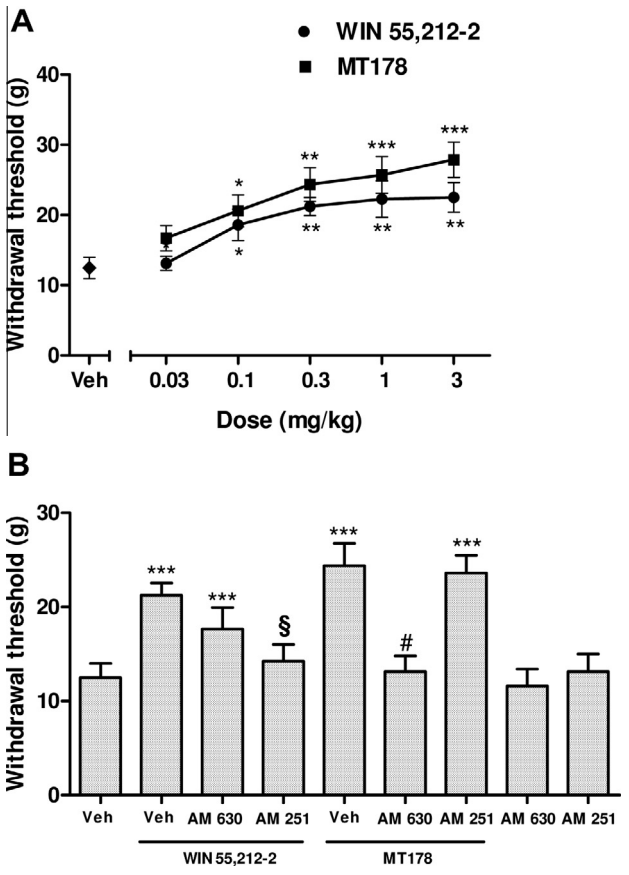

Fig. 6. Antinociceptive effect of MT178 and WIN 55,212-2 in AIMP model in rats. (A) MT178 and WIN 55,212-2 (0.03–3 mg/kg) increase paw withdrawal threshold relative to vehicle reducing mechanical allodynia. (B) AM 630 (3 mg/kg) completely blocked the antiallodynic effect of MT178 (0.3 mg/kg) while it partially reduced WIN 55,212-2 effect (0.3 mg/kg). AM 251 (3 mg/kg) did not abrogate the effect of MT178. Data are presented as mean ± SEM (n = 6 mice/group). Veh, vehicle; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs vehicle; $^{\S}P < 0.01$ vs WIN 55,212-2 (0.3 mg/kg); $^{\#}P < 0.01$ vs MT178 (0.3 mg/kg).

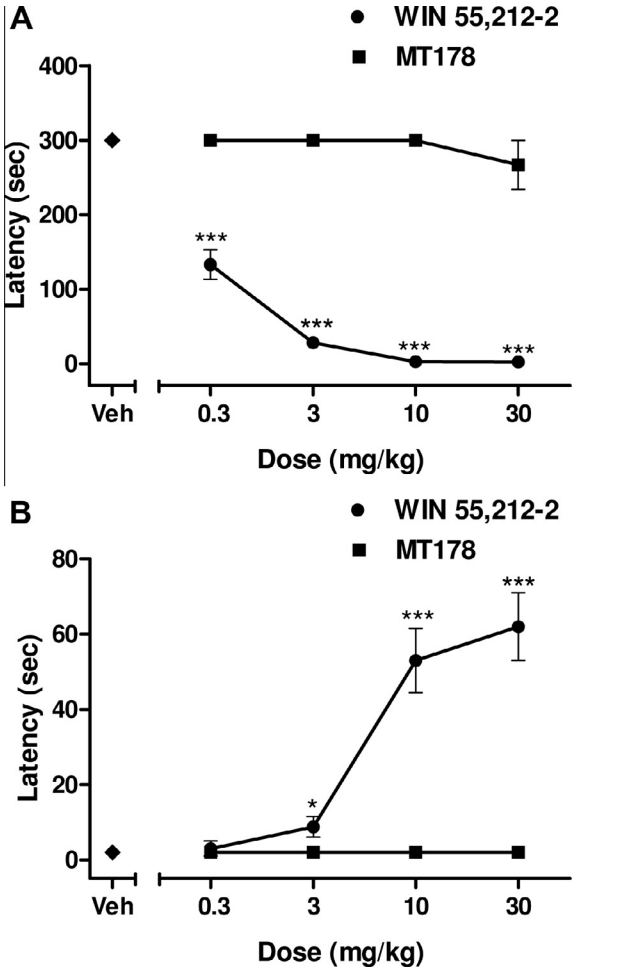

Fig. 7. Activity of MT178 and WIN 55,212-2 in specific tests for assessment central side effects. (A) MT178 (0.3–30 mg/kg) did not produce locomotor disturbance evaluated in rotarod assay whilst WIN 55,212-2 (0.3–30 mg/kg) mediated a marked reduction of the latency to fall off the rotarod. (B) MT178 (0.3–30 mg/kg) did not produce cataleptic effect while WIN 55,212-2 (0.3–30 mg/kg) evoked catalepsy starting from the dose of 3 mg/kg. Data are presented as mean ± SEM (n = 6 mice/group). Veh, vehicle; $^{*}P < 0.05$; $^{***}P < 0.001$ vs vehicle.

receptors. Moreover, JWH-015 and AM 1241 showed a low selectivity vs CB_2 receptors <100 [43]. Compared with the CB_2 ligands studied previously, MT178 revealed a high affinity (Ki = 7.37 nM, hCB_2) and selectivity (>1357) vs CB_2 receptors.

As a consequence, we have performed in vivo assays to better investigate the analgesic effect of MT178 in comparison with a well-known nonselective agonist, WIN 55,212-2. In 2 of the most used inflammatory pain models such as formalin and writhing tests, MT178 produced a dose-dependent antihyperalgesic effect in a similar way to the reference compound, WIN 55,212-2, as demonstrated by one-way ANOVA. Moreover, the effect of MT178 was reversed by the selective CB_2 antagonist AM 630, but not by the selective CB_1 antagonist AM 251 supporting a CB_2-mediated mechanism of action. Our data confirm previous results on the antinociceptive effect of CB_2 agonists such as HU-308 or JWH-133 in the formalin test [24,26].

It is well known that STZ-induced diabetes has been increasingly used as a model of painful *neuropathy* to assess the efficacy of novel potential compounds [57]. In this study, MT178 significantly reduced mechanical allodynia elicited by STZ injection in a dose-dependent manner with an efficacy comparable to that obtained with WIN 55,212-2. Previous papers have reported that single or chronic administration of nonselective CB agonist WIN 55,212-2, as well as the selective CB_2 agonist AM1241, dose-dependently alleviated STZ hyperalgesia [11,55].

Many epithelial-derived cancers, including sarcoma, breast, prostate, and lung, commonly metastasize to bone [14]. Once cancer metastasis occurs, bone pain can significantly impact the quality of life and functional status of the patient [50]. Here we utilized an animal model of bone cancer pain based on the injection of syngeneic MRMT-1 cells in rat tibia that results in behavioral signs of evoked pain [35]. We have found the effectiveness of CB_2 selective agonist MT178 in suppressing the bone cancer-induced mechanical allodynia in a manner that was blocked by CB_2 antagonist AM 630, but not by the CB_1 antagonist AM 251. The effect of WIN 55,212-2 was less evident than MT178 in the reversal of paw withdrawal threshold decrease. Recently, it has been reported that the withdrawal threshold was dose-dependently increased by the intrathecal CB agonist WIN 55,212-2. The selective CB_1 or CB_2 antagonists (AM 251 or AM 630) significantly blocked the antinociceptive effect of WIN 55,212-2 on bone tumor pain in the spinal cord [15]. On the other hand, various studies have demonstrated the major involvement of CB_2 in bone cancer pain. Systemic administration of the CB_2 receptor agonist AM1241 blocked both tumor-induced thermal hyperalgesia and mechanical allodynia through the selective stimulation of CB_2, and not CB_1 receptors, as only the systemic administration of the CB_2 antagonist abolished analgesic effects of AM 1241 [16,32].

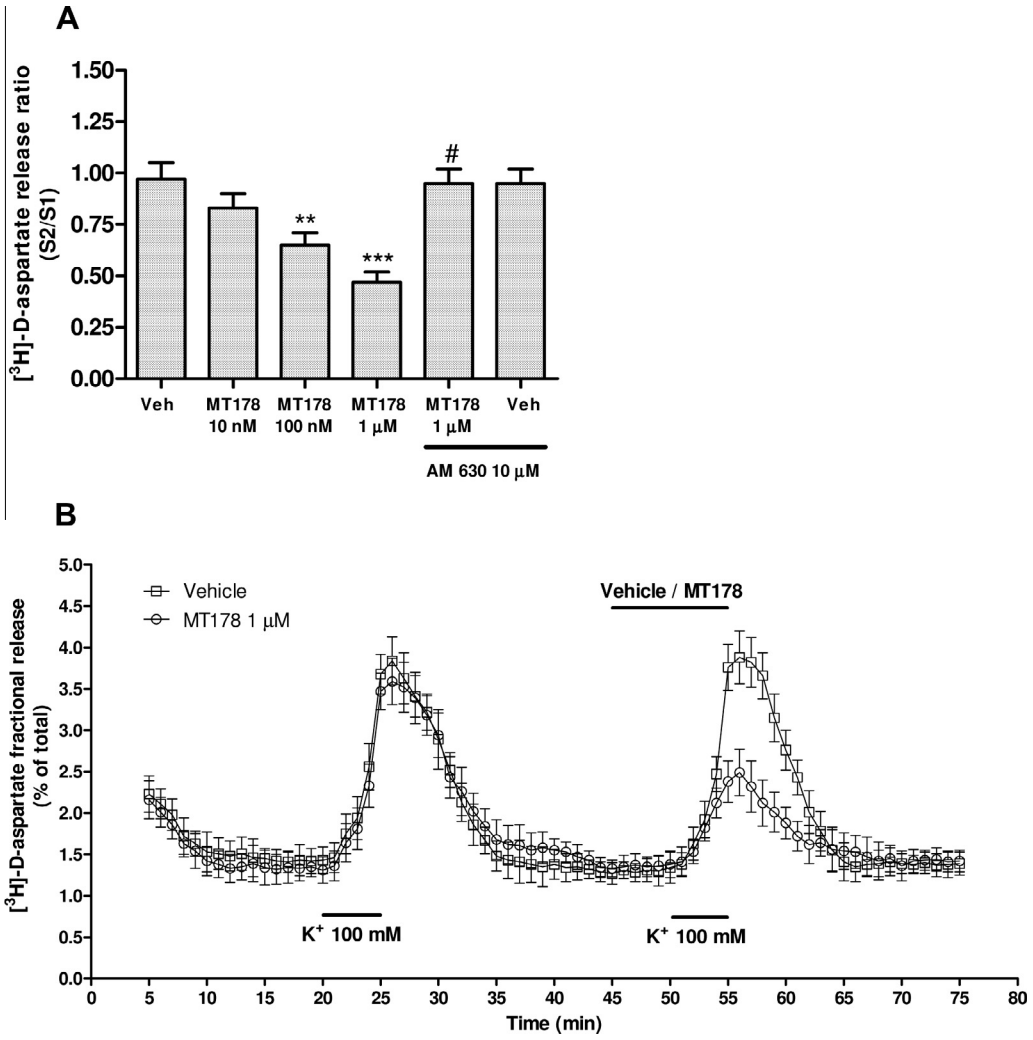

Fig. 8. MT 178 (10 nM–1 μM) inhibited [^{3}H]-D-aspartate release from dorsal root ganglion neurons. (A) The graph of S2/S1 ratio indicated that the effect of MT178 (1 μM) was completely abrogated by AM 630 (10 μM). (B) Release profile showing the effect of MT178 (1 μM) in the inhibition of K^+-induced [^{3}H]-D-aspartate release. Data are presented as mean ± SEM (n = 4). $^{**}P < 0.01$; $^{***}P < 0.001$ vs vehicle; $^{\#}P < 0.01$ vs MT178 (1 μM).

To complete the analysis on chronic pain, we have investigated the antinociceptive effect of MT178 and WIN 55,212-2 in the AIMP model, revealing a good efficacy in the reduction of the mechanical allodynia. To our knowledge, this is the first study reporting the analgesic effect of the systemic administration of a CB_2 agonist in the AIMP model. However, the effectiveness of local administration of WIN 55,212-2 and JWH-015 in the masseter and gastrocnemius muscle pain models induced by hypertonic saline injection was recently shown [47].

It is well reported that CB_1 agonists may exhibit psychotropic side effects that limit their use in pain treatment. This is evident in animal studies where there is little separation between the potency of central CB agonists in tests as rotarod and catalepsy assays and in chronic pain models [17,22]. As expected, WIN 55,212-2 showed marked central side effects in rotarod or catalepsy assays at a very low dose of 0.3 or 3 mg/kg, respectively. Of high relevance, the treatment with MT178 did not cause any locomotor disturbance or catalepsy even at doses up to 100-fold the doses that caused antinociception. This evidence suggests that MT178 could have a wide therapeutic window due to the lack of CB_1-mediated central side effects. These results confirm the potential beneficial use of CB_2 selective agonists that promise to have clear medical implications. In particular, CB_2 agonists could be considered a useful tool for the medical practice because they are effective in treating pain and inflammation without CNS side effects.

To elucidate the mechanism underlying the antinociceptive effect of MT178 and to further support the role of CB_2 activation in pain processing, we have used DRG neurons as a cellular model. CB_2 receptors are well expressed in DRG neurons, as confirmed by previous works that reported CB_2 mRNA expression [7,29]. Intense noxious stimuli or inflammatory conditions cause primary afferent terminals to release glutamate, thereby initiating a nociceptive response [38]. To verify the hypothesis that CB_2 activation could inhibit glutamate release, we have demonstrated that MT178 was able to significantly reduce K^+-evoked [^{3}H]-D-aspartate from DRG neurons. D-aspartate was chosen as a transportable but nonmetabolizable analogue of glutamate [9]. In addition to excitatory amino acid neurotransmitters, pain-related neuropeptides such as substance P are known to be released from DRG cells, potentiating excitatory input to nociceptive neurons in response to noxious stimuli [52]. In DRG neurons, we have found that MT178 markedly decreased capsaicin-induced substance P release. Our data are in agreement with those previously found where the CB_2 agonist JWH 133 blocked the capsaicin-induced release of substance P-like immunoreactivity from guinea pig airway tissues [59]. Another study indicated that CB_1 agonist HU210 inhibited

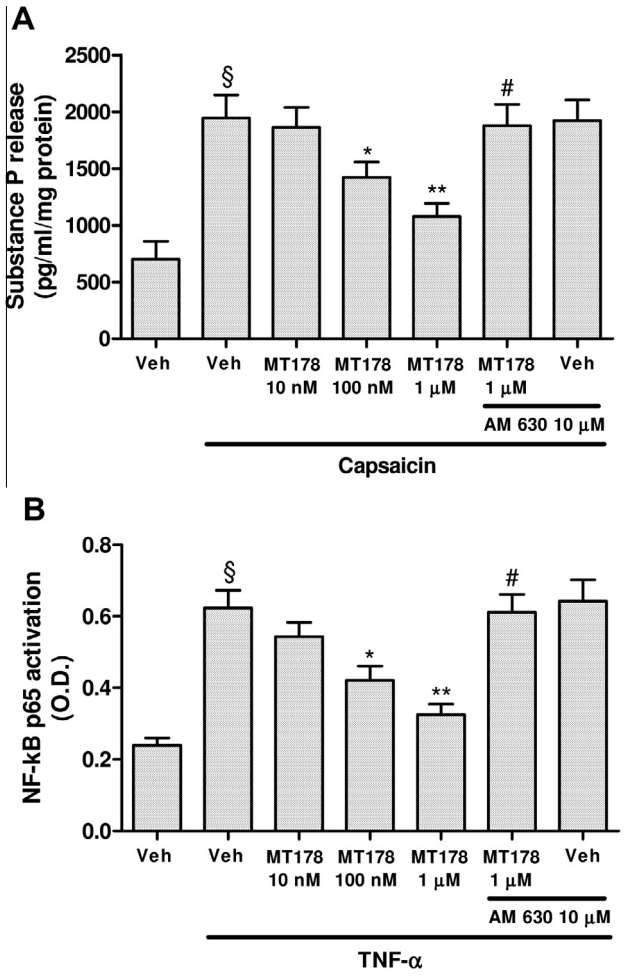

Fig. 9. Effect of MT 178 (10 nM–1 μM) on substance P release and NF-κB activation. (A) MT178 (1 μM) significantly reduced the capsaicin-mediated release of substance P from dorsal root ganglion neurons, an effect that was abolished by AM 630 (10 μM). (B) MT178 (100 nM and 1 μM) significantly inhibited NF-κB p65 subunit activation induced by tumor necrosis factor (TNF)-α (10 ng/mL). Data are presented as mean ± SEM (n = 4). $^{§}P < 0.01$ vs vehicle; (B,C,D); $^{*}P < 0.05$; $^{**}P < 0.01$ vs capsaicin or TNF-α; $^{\#}P < 0.01$ vs MT178 (1 μM).

the capsaicin-induced Ca^{2+} influx and substance P-like immunoreactivity release in cultured rat DRG cells [41]. Finally, we tested the effect of MT178 on NF-κB p65 activation, revealing an inhibitory response elicited by the novel CB_2 agonist in DRG neurons following TNF-α stimulation. Increasing evidence suggested an important role of *NF-κB* in *pain regulation* and substances that inhibit the NF-κB-activating cascade are capable of reducing the nociceptive response in different animal models [40]. Increased NF-κB activation has been observed in rat DRG neurons after partial sciatic nerve injury and intrathecal injection of antisense oligonucleotides to p65 subunit of NF-κB-alleviated neuropathic pain [51].

In summary, MT178 is a potent and selective CB_2 full agonist that is highly effective in inflammatory and chronic rodent pain models. The novel CB_2 agonist failed to provoke any locomotor disturbance or cataleptic effects confirming the high selectivity vs CB_2 receptors and suggesting that MT178 may represent an ideal tool in the control pathological pain status.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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