A peripherally restricted cannabinoid receptor agonist produces robust anti-nociceptive effects in rodent models of inflammatory and neuropathic pain

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ABSTRACT

Cannabinoids are analgesic in man, but their use is limited by their psychoactive properties. One way to avoid cannabinoid receptor subtype 1 (CB1R)-mediated central side-effects is to develop CB1R agonists with limited CNS penetration. Activation of peripheral CB1Rs has been proposed to be analgesic, but the relative contribution of peripheral CB1Rs to the analgesic effects of systemic cannabinoids remains unclear. Here we addressed this by exploring the analgesic properties and site of action of AZ11713908, a peripherally restricted CB1R agonist, in rodent pain models. Systemic administration of AZ11713908 produced robust efficacy in rat pain models, comparable to that produced by WIN 55, 212-2, a CNS-penetrant, mixed CB1R and CB2R agonist, but AZ11713908 generated fewer CNS side-effects than WIN 55, 212-in a rat Irwin test. Since AZ11713908 is also a CB2R inverse agonist in rat and a partial CB2R agonist in mouse, we tested the specificity of the effects in CB1R and CB2R knock-out (KO) mice. Analgesic effects produced by AZ11713908 in wild-type mice with Freund's complete adjuvant-induced inflammation of the tail were completely absent in CB1R KO mice, but fully preserved in CB2R KO mice. An in vivo electrophysiological assay showed that the major site of action of AZ11713908 was peripheral. Similarly, intraplantar AZ11713908 was also sufficient to induce robust analgesia. These results demonstrate that systemic administration of AZ11713908, produced robust analgesia in rodent pain models via peripheral CB1R. Peripherally restricted CB1R agonists provide an interesting novel approach to analgesic therapy for chronic pain.

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1. Introduction

Clinical trials and case reports have shown that cannabis extract (Sativex), Δ9-THC and synthetic derivatives (ajulemic acid, nabilone) are analgesic in chronic pain patients [3,16,24,28,30], and that the activation of cannabinoid receptors causes profound analgesic effects in animal pain models [4,29]. However, clinical use of cannabinoids is limited by their psychoactive properties, presumably mediated by cannabinoid receptors expressed in the CNS. Two types of cannabinoid receptors (CB), CB1R [9] and CB2R [21], have been identified. CB1R is highly expressed throughout the central nervous system (CNS), as well as in the peripheral nervous system (PNS), especially in sensory nerve fibers [14]. CB1R expression has also been reported in many peripheral tissues

including gastrointestinal tissues, reproductive tissues and some immune cells, but to a much lower extent when compared to neural tissues. CB2R receptors are located mainly in immune tissues and cells, with particularly high levels in B-cells and natural killer cells. It has been shown recently that CB2 is also expressed in CNS [6].

One approach proposed to dissociate cannabinoid analgesia from the cannabinoid-mediated psychoactive effects is to target CB1 receptors located in peripheral nerve fibers [17]. Activation of peripheral CB1Rs by topical application has been shown to be sufficient to reduce pain in a human experimental pain model [27]. Similarly, in preclinical rodent pain models, local administration of CB1R agonists has been reported to produce anti-nociceptive effects in both inflammatory and neuropathic conditions [12,22,25]. A recent study using a conditional gene targeting technique to selectively knock-out CB1 receptors in Nav1.8-expressing primary afferent fibers in mice has shown that the analgesic effects of systemically administered cannabinoids are reduced in the absence of this population of peripheral CB1 receptors [1]. Thus, although compensatory changes in the CB system in genetically

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manipulated mice cannot be excluded, peripheral CB1 receptors appear to be necessary for at least a proportion of the analgesic effects of systemic cannabinoids.

In the present study, we further explored this question using a novel peripherally restricted CB1R agonist, AZ11713908. We have extended the previous observations by demonstrating that systemic administration of a peripherally restricted CBR agonist has robust analgesic effects with reduced CNS side-effects predominately via a peripheral action on CB1 receptors in rodent pain models.

2. Materials and methods

2.1. Drugs

AZ11713908 (N-(1-(cyclohexylmethyl)-2-((5-ethoxypyridin-2-yl)methyl)-1H-benzo[d]imidazol-5-yl)-N-methylthiophene-2-sulfonamide) was synthesized at AstraZeneca R&D Montreal. Its chemical structure is shown in Fig. 1. AZ11713908 was mixed with 20% hydroxypropyl-beta cyclodextrin (HBC), administered by the sub-cutaneous (s.c.), or intraplantar (i.pl) route for pain behavioral measurements and was administered intravenously (i.v.) in the electrophysiological study. Peak drug effects were observed 30 min after s.c. or i.pl routes and 10 min following i.v. administration. WIN 55, 212-2 (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 1:1:8 emulphol: ethanol: water or in 20% hydroxypropyl-beta cyclodextrin (HBC) and administered by the s.c; animals were tested 1 h later.

2.2. In vitro assays

Human and rat CB1Rs, and rat CB2R were expressed in HEK293 cells. Human CB2R was expressed in Sf9 cells.

2.2.1. ^{3}H-CP55,940 receptor binding assays

Compounds were tested in 10-point dose response curves. The assay was performed in 96-well plates, with each well containing 150 μl of buffer alone (50 mM tris, 2.5 mM EDTA, 5 mM MgCl2, and 0.05% BSA fatty acid free, pH 7.4) or the compound at varying concentrations, 80 μl of membranes from expressing human (2.8 pmol/mg) or rat (4.3 pmol/mg) CB1 receptors, human (22.7 pmol/mg), rat (28.6 pmol/mg) or mouse (2.2 pmol/mg) CB2 receptors, and from mouse brain (0.9 pmol/mg) preparation, and 70 μl of the tracer 3H-CP55,940 (~0.2 nM). Non-specific binding was determined in the presence of 0.2 μM HU 210, a potent non-selective cannabinoid agonist. Plates were then mixed on an orbital mixer, and incubated for 1 h at room temperature. Filtration was performed with a Packard cell harvester using 50 mM Tris, 5 mM MgCl2, 0.5 mg/ml BSA, pH 7.0. Filter plates (unifilter GF/B) were soaked in 0.1% PEI prior to filtration. Filter plates were then dried

Fig. 1. Chemical structure of AZ11713908 (N-(1-(cyclohexylmethyl)-2-((5-ethoxypyridin-2-yl)methyl)-1H-benzo[d]imidazol-5-yl)-N-methylthiophene-2-sulfonamide).

at 55 °C for 1 h before adding Microscint 20 (Perkin-Elmer) scintillation fluid. Filter plates were counted in a Packard Top Count. The inhibition of ^{3}H-CP55,940 binding observed for each concentration of compound was expressed as a percentage of maximal specific binding. ^{3}H-CP55,940 specific binding was calculated by subtracting the non-specific binding. Dose–response curves were fitted using a 3-parameter logistic fit to derive the IC$_{50}$, D_{max} and Hill slope (nH) parameters.

2.2.2. ^{35}S]GTPγS binding assays

The compounds were tested in 10-point dose response curves in a buffer consisting of 50 mM Hepes, 20 mM NaOH, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 0.1% BSA, and 30 μM GDP (hCB1) or 15 μM GDP (hCB2) or 5 μM GDP (rCB1 & rCB2) or 100 μM GDP (mouse brain) or 1 μM GDP (mCB2). The pH was set at 7.4 at room temperature. The assay, performed in 96-well plates, contained 150 μl of buffer alone or compound at varying concentrations, 80 μl of membranes (from cells expressing human or rat CB1 or CB2 receptors, mouse CB2 receptors and from mouse brain preparation) mixed with 3.75× of GDP final concentration. Finally, 70 μl of the tracer GTPγ^{35}S (~0.13 nM) was added to start the reaction. Maximal binding was determined using 10 μM WIN 55, 212-2. Plates were then mixed on an orbital mixer, and incubated for 1 h at room temperature. Filter plates (unifilter GF/B) were presoaked in deionized water. Filtration was performed with a Packard cell harvester using 50 mM Tris, 5 mM MgCl2, 50 mM NaCl, pH 7.4. Filter plates were then dried at 55 °C for 1 h before adding Microscint 20 (Packard Biosciences) scintillation fluid. Filter plates were counted in a Packard Top Count. The stimulation of GTPγ^{35}S binding observed for each concentration of compound was expressed as a percentage of maximal effect elicited by 10 μM WIN 55, 212-2 or 0.1 μM CP55,940 for rCB2 assay. GTPγ^{35}S specific binding is calculated by subtracting the basal activity. Dose–response curves were fitted using a 3-parameter logistic fit to derive the EC$_{50}$, E$_{max}$ and Hill slope (nH) parameters.

2.3. Animals

The AstraZeneca R&D Montreal Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines, approved all experiments dealing with animals. Rat subjects were male Sprague Dawley rats, weighing 175–350 g (Charles River, St Constant, QC, Canada and Harlan Inc., Indianapolis, IN, USA). Rats were housed in groups of six in a temperature- and humidity-controlled animal colony. Food and water were available ad libitum, and the room was illuminated with fluorescent lighting on a daily 12 h light/dark cycle.

CB1R or CB2R KO mice were generated as previously described by conventional homologous recombination [32]. Briefly, targeted 129 ES-cells were injected into C57BL/6 blastocysts to give Chimera males. These males were crossed with C57BL/6 females. Heterozygous (HE, +/–) male offsprings were crossed with C57BL/6 females. HE couples were then bred to generate WT and KO mice for testing. The growth and general appearance of CB1R and CB2R null mouse line was normal. Mice were implanted with microchips for identification purpose at the time of blood sampling for genotyping. They were housed in same littermate groups of 2–6 in transparent plastic cages on CORNCOB 1/8 bedding, in a temperature- and humidity-controlled room on a 12 h light–dark cycle. Food and water were always ad libitum. Only male WT and KO mice were used for the behavioral testing.

2.4. Animal models of pain

2.4.1. Inflammatory

Rat carrageenan model: Carrageenan-lambda (Sigma) was dissolved in sterile saline 0.9% at a concentration of 1%. Rats were
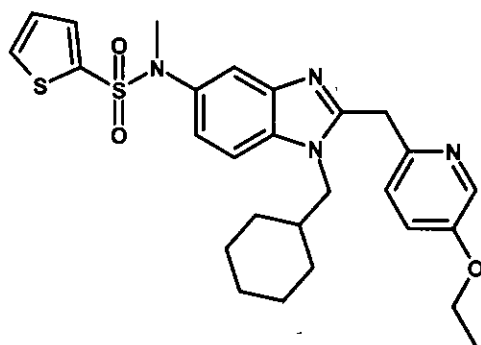
placed in a plexiglass chamber with 2% isoflurane at a flow rate of 0.8–1 l/h with oxygen, for approximately 60–90 s, until a light-medium depth of anesthesia is attained. One-hundred microliters of carrageenan solution was injected into the sub-cutaneous space of the dorsal aspect of the left hind paw, in the center of the four pads. This created inflammation, with accompanying edema and redness, as well as hyperalgesia, which was fully developed within 3 h, and remained stable for a total of 6 h following injection.

Rat and mouse FCA model: Freund's complete adjuvant (FCA, Sigma) was prepared from 1 mg of heat-killed Mycobacterium tuberculosis bacilli dissolved in 0.85 ml of paraffin oil and 0.15 ml of mannide oleate. An inflammation was induced by a single sub-cutaneous administration of 20 µl of FCA into the mice' tail or 100 µl into the rat's hindpaw, respectively. All experiments were conducted 24 h after FCA administration. Animals were sacrificed immediately after data acquisition.

2.4.2. Neuropathy

Rat spinal nerve ligation (SNL) model: the model was generated as described by Kim and Chung [19]. Briefly, animals were anesthetized with isoflurane (5% in O_2), and the left lumbar spinal nerves at the level of L5 and L6 were exposed by blunt dissection. Both nerves were tightly ligated (4–0 silk suture) distal to the dorsal root ganglion and prior to entrance into the sciatic. The incision was closed in layers, and the skin was sealed with tissue adhesive (Vetbond™). Compound testing occurred between post-operative days 7–25 and in order to assess behavioral pathology, baseline hyperalgesia or allodynia was determined 1–2 days before compound testing.

2.5. Nociceptive and Irwin tests

Thermal sensitivity of the plantar surface of the hind paw was assessed using a paw thermal stimulator system (University of California at San Diego, La Jolla) to measure paw withdrawal latency [13]. Two trials were conducted with 5 min intervals between each trial. A cut-off time of 20 s was used to prevent tissue injury. Baseline latencies were obtained for each rat before drug administration.

Thermal sensitivity of the mice tails (heat hyperalgesia) was measured by tail-immersion. Mice were restrained in a paper towel with the tail exteriorized and a thermal stimulus was applied by immersion of approximately 5 cm of the tail in a thermostatically controlled water-bath kept at a temperature of 48 ± 0.1 °C. Latencies for tail withdrawal were recorded for each animal before and after drug administration using a hand-held stopwatch. A 40 s cut-off time was imposed to avoid tissue damage. Prior to the induction of inflammatory model, baseline (control) latency was determined in both WT and KO mice. Twenty-four hours subsequent to FCA injection, tail withdrawal latencies at 48 °C were determined in both WT and KO FCA-injected mice.

The threshold for responses to punctate mechanical stimuli (mechanical allodynia) was tested according to the up-and-down method [8] by touching the plantar surface of the animal hind paw with a series of eight von Frey filaments (bending force ranging from 0.4 to 15 g).

Several different groups of animals (and their corresponding naïve controls) were used throughout this study. After testing baselines, animals were allocated to counterbalanced treatment groups. In all cases the experimenter was blind to the treatment received. Data shown are pooled from >1 experimental occasion, performed by different experimenters to ensure that the results were reproducible. Each individual experiment was run under the same testing protocol and the data were pooled only under the condition that all individual results were consistent (no significant differences in baseline and vehicle-treated values). Data were analyzed using Excel (Microsoft Corp, WA, USA) and SigmaStat (SPSS, Chicago, IL, USA). Raw data underwent significant one-way or two-way RM ANOVA, followed by post hoc pair-wise multiple comparison using the Holms-Sidak test. Data were converted to maximal% effects using the following formula: ((value-vehicle)/(naïve-vehicle)) * 100 for plotting graphs.

Irwin test: for each animal, the observational battery of tests was performed at 15 min, 30 min, 60 min, 120 min, 240 min, and a 24 h post-drug administration. For each test, both the number of animals (at each dose) displaying a symptom deficit and the severity of the symptom were recorded (ratings of 1–3 with 1 being the least pronounced and 3 being the most affected). At each time point subsequent to drug injection, rats were observed for the following behaviors: sniffing, grooming, scratching, vocalization, stereotypy (excessive repetition or lack of variation in movements), rearing, and body posture. Animals were also examined for signs of ptosis (drooping of the upper eyelid), exophthalmos (abnormal protrusion of the eyeball), lacrimation, salivation, piloerection, and signs of diarrhea. Biting reaction was assessed by presenting a pencil horizontally in front of the animal while "response to touch" was assessed by poking the rat with the blunt side of a pencil. Catalepsy was evaluated by placing the animal in a vertical manner with forepaws on a plastic block and subsequently observing whether the animal remained in this position. For evaluating traction response, the forepaws of the animal were placed on an elevated mesh grid and then the ability to stay on the grid was assessed. While on the grid, gait, presence of Straub tail, tremor, twitches, convulsions and signs of sedation were assessed. Grip strength was evaluated by having the animal hold onto a mesh grid angled at 60 degrees. Body tonus and hind leg position were evaluated by holding the rat by hand vertically. At each time point, the total score represents the sum of all behaviors for all testing animals ($n = 6$/group) with their ratings; for example, if 6 animals had a rating of 1 at 15 min for vocalization and 3 animals had a rating of 2 for catalepsy, the total score would be 12. The sum of total scores represents the sum of scores for all time points.

2.6. Electrophysiological recording of motoneuron activity

The experiments were performed on FCA rats (24 h post injection). Under isoflurane anesthesia, one carotid artery, jugular vein and the trachea were cannulated. The rat was decerebrated by aspiration of all the cranial contents rostral to the mesencephalon and spinalized via a laminectomy at T3–T4. The anesthetic was then discontinued, and the animals were paralyzed with gallamine (Rhone-Poulenc Rorer, France) and artificially ventilated. Rectal temperature, heart rate, ECG and blood pressure were monitored throughout the entire period of the experiment.

The activity of flexor α-motoneurons was recorded extracellularly from the nerve to the posterior biceps femoris/semitendinosus muscles. The nerve was exposed in the popliteal fossa, a very fine terminal branch dissected free in the muscle and cut, and the central end placed on a silver recording electrode. The sural nerve was dissected free and placed in continuity on a pair of silver wire stimulating electrodes. The experiment was started 1 h after preparation, to allow for recovery from the anesthetic and stabilization of the preparation.

Single units were detected by using a conventional window discriminator. Spikes were counted with a pulse integrator, and the spike shape monitored continuously by an analog delay line. To measure the excitability of the flexor reflex, the following measurements were made of: (1) spontaneous activity (for 10 s); (2) the response to pinch stimulation applied to the middle 3 toes; (3) the response to noxious heat (water 52 °C) stimulation to the hind paw; (4) the total discharge evoked by electrical stimulation of the sural nerve (1 Hz for 5–10 s). The above test protocols were

Table 1
In vitro binding of compounds to CB1R and CB2R (pIC50).[a]

Compound	hCB1	rCB1	mCB1[b]	hCB2	rCB2	mCB2
AZ11713908	8.4 ± 0.1	8.4 ± 0.2	8.8 ± 0.2	9.0 ± 0.2	8.8 ± 0.2	8.8 ± 0.1
Win 55, 212-2	7.2 ± 0.2	8.0 ± 0.3	7.7 ± 0.3	8.5 ± 0.2	8.1 ± 0.2	8.0 ± 0.2

Data were presented as means ± SD, N = 4–459.
[a] Log M.
[b] From mouse brain.

applied at 5-min intervals during the entire course of the experiment.

2.7. Pharmacokinetic analysis

Animals were treated with WIN 55, 212-2 or AZ11713908 (s.c.) and 60 min or 30 min after drug administration; blood was taken by decapitation and the brains were immediately harvested. The skull was opened with a bone trimmer, brain tissue was collected with a spatula and placed in a plastic bag into nitrogen liquid before storage at −80 °C pending analysis. Blood samples (500 µl) were collected in microcentrifuge tubes (Eppendorf) containing Heparin and immediately placed on ice. Following centrifugation for 10 min at 3000 rpm and 4 °C, plasma was recovered from the supernatant and transferred to 1.5 mL Eppendorf tubes and finally stored at −80 °C pending analysis.

The determination of the total plasma or brain concentration of WIN 55, 212-2 and AZ11713908 was performed by protein precipitation (after homogenization of brain samples), followed by reversed-phase liquid chromatography and electrospray mass spectrometry. The limit of quantification in plasma was 1.22 nmol/L with a 30 µl sample volume. AZ11713908 determinations were performed in a range of 1.22 to 10,000 nmol/L. The limit of quantification in brain was 3.66 pmol/g with a 30 µl homogenate volume. WIN 55, 212-2 and AZ11713908 determinations were performed in a range of 3.66 to 30,000 pmol/g.

3. Results

3.1. In vitro activity at cannabinoid receptors

AZ11713908 bound with high affinity to human, rat and mouse cannabinoid receptors (Table 1). AZ11713908 was active as full agonist at human, rat and mouse CB1R using the GTPγS assay to test functional activity. Compared to WIN 55, 212-2, AZ11713908 exhibited better potency at human, rat and mouse CB1R. AZ11713908 acted as a partial agonist against the human (E_{max} = 74%) and mouse (E_{max} = 38%) CB2R, but acted as an inverse agonist at the rat CB2R (Table 2).

3.2. Brain/plasma ratios of AZ11713908 and WIN 55, 212-2 following sub-cutaneous administration

Plasma and brain levels of AZ11713908 and WIN 55, 212-2, as well as brain/plasma ratios of both drugs following s.c. administration in rat and mouse are listed in the Table 3. Measurements were

made at the time after dosing corresponding to the time of testing in the behavioral experiments. Compared to WIN 55, 212-2, AZ11713908 showed minimal brain penetration. There was between 2- and 4-fold more WIN 55, 212-2 in brain than plasma, whereas in marked contrast, the brain concentration of AZ11713908 was less than 10% of the plasma concentration (Table 3).

3.3. AZ11713908 showed anti-nociceptive effects comparable to WIN 55, 212-2 in both rat inflammatory and neuropathic pain models

When tested in the rat carrageenan model, AZ11713908 (0.6–1.2 µmol/kg, s.c.) produced robust anti-heat hyperalgesia. The efficacy of AZ11713908 was comparable to that of the CNS-penetrant compound, WIN 55, 212-2 (Fig. 2A), and the potency was higher. Likewise, when tested in the rat spinal nerve ligation model, systemic administration of AZ11713908 (0.6–2.5 µmol/kg) reduced the mechanical allodynia with a maximum efficacy of 100% (Fig. 2B). This efficacy was comparable to the effect of the WIN 55, 212-2, and again was observed at somewhat lower doses. Thus AZ11713908 shows equivalent efficacy to WIN 55,212-2, despite its low brain penetration.

3.4. Anti-nociceptive effects of AZ11713908 are mediated by CB1R, but not CB2R

AZ11713908 binds human, rat and mouse CB1R and CB2R with high affinity and exhibits full CB1R agonism and partial CB2R agonism. In order to identify which subtype of CB receptor mediated the anti-nociceptive effects in animal pain models, the compound was tested in CB1R and CB2R KO mice. FCA-induced tail inflammation produced equivalent hyperalgesia in WT and both CB1R and CB2R KO mice (Fig. 3). Based on previous experiments with AZ11713908 in wild-type mice in this model (data not shown) we selected a 3 µmol/kg dose of AZ11713908 given s.c. which was expected to give ~80% reversal, or ~ED80.

As expected, we found that AZ11713908 showed robust antihyperalgesic effects in WT littermate mice (Fig. 3). This effect was completely absent in CB1R KO mice (Fig. 3A) whereas it was completely preserved in CB2R KO mice (Fig. 3B), suggesting that the effects of AZ11713908 are mediated by CR1R.

3.5. AZ11713908 showed a better margin to CNS side-effects than the CNS-penetrant compound, WIN 55, 212-2

At anti-hyperalgesic doses, WIN 55-212, 2 (2–8 µmol/kg s.c.) produced more pronounced side-effects as measured by scores in

Table 2
In vitro functional activities of compounds on CB1R and CB2R (GTPγS assay).

Compound	hCB1	rCB1	mCB1[a]	hCB2	rCB2	mCB2
pEC50 (Log M) [Emax (%)]						
AZ11713908	8.2 ± 0.0	8.2 ± 0.1	7.3 ± 0.1	9.1 ± 0.1 0.2	8.4 ± 0.3	8.8 ± 0.3
	[115 ± 4]	[104 ± 3]	[133 ± 24]	[74 ± 3]	[−21 ± 5]	[38 ± 12]
Win 55, 212-2	7.0 ± 0.2	7.4 ± 0.1	6.5 ± 0.2	9.0 ± 0.2	8.7 ± 0.2	8.5 ± 0.2
	[102 ± 8]	[101 ± 3]	[100 ± 2]	[101 ± 4]	[26 ± 7]	[50 ± 7]

Data were presented as means ± SD; N = 3–408.
[a] From mouse brain.

Table 3
In vivo PK profile (s.c.) of AZ3908 & WIN 55-212, 2.

Compound	Dose (µmol/kg)	Cpl (µM)	Cbr (µM)	Cbr/Cpl (means ± SD)
AZ11713908-Rat	3.0	0.28	0.02	0.07[a]
AZ11713908-Mouse	0.5–27	0.1–5.7	0.01–0.4[b]	0.05 ± 0.01
WIN 55-212, 2 Rat	1–8	0.03–0.3	0.1–0.8	2.2 ± 2.6[c]
WIN 55-212, 2 Mouse	1–16	0.01–1.0	0.08–1.9[d]	4.2 ± 2.6

[a] $N = 1$.
[b] $N = 8$.
[c] $N = 15$.
[d] $N = 16$.

the Irwin test than AZ11713908 (0.6–2.5 µmol/kg s.c.). Indeed, plotting of equipotent anti-nociceptive doses versus percentage anti-nociceptive effect and total Irwin score for each of these two compounds (Fig. 4) reveals that whereas the margin between in vivo efficacy and side-effects is minimal for WIN55,212-2 (Fig. 4A), a better margin exists between anti-nociceptive potency and the side-effects profile for AZ11713908 (Fig. 4B).

3.6. Local peripheral administration of AZ11713908 is sufficient to produce anti-nociceptive effects in rat inflammatory pain models

We investigated whether a peripheral action of AZ11713908 was sufficient to induce anti-nociceptive effects by measuring the effect after local administration of the compound in rat inflammatory pain models. Intraplantar injection of AZ11713908 (40 nmol/20 µl) in the inflamed paw significantly reversed the heat hyperalgesia induced by either FCA or Carrageenan injections. Subcutaneous administration of same amount of AZ11713908 at another body site produced no effect on heat hyperalgesia in the paw in either animal pain model (Fig. 5).

3.7. The anti-nociceptive effects of AZ11713908 is mediated largely via peripheral CB1R in rat FCA model

The peripheral site of action of AZ11713908 was also assessed in an in vivo electrophysiological study in rat FCA model. Extracellular recordings of flexor α-motoneuron activity were made from the nerve to the Posterior Biceps Femoris/SemiTendinous (PBF/ST) muscles, following various noxious stimuli to the periphery. AZ11713908 (10 µmol/kg, i.v.) inhibited the spinal reflex responses to noxious heat and mechanical stimulation by >75%, but did not significantly inhibit spinal reflex responses to electrical stimulation

of the peripheral nerve (Fig. 6A). As electrical stimulation of a peripheral nerve effectively 'by-passes' the peripheral terminal and generates action potentials directly in the axon, it would be expected that an agent acting at the nociceptor terminal would not affect the response to this stimulus. In contrast, heat and mechanical stimulation activate the peripheral terminals via transduction elements to generate action potentials, thus the inhibition of responses to these stimuli would be consistent with a site of action at these peripheral nociceptor terminals (Fig. 6A). In contrast to AZ11713908, WIN 55, 212-2 (0.5 µmol/kg, i.v.) exhibited a significant central effect as judged by the fact that it significantly inhibited spinal reflex responses to electrical stimulation of the peripheral nerve (Fig. 6B).

4. Discussion

The present study demonstrates that AZ11713908 binds with high affinity to rat, mouse and human CB1R and CB2R in vitro. AZ11713908 is a full agonist at CB1R in these three species but is a partial agonist at the human and mouse CB2R, and is inactive at the rat CB2R. AZ11713908 produces potent in vivo anti-hyperalgesic activity in inflammatory and chronic neuropathic pain models in rats. The specificity of the analgesic effect of AZ11713908 was addressed using CB1R and CB2R KO mice. The anti-hyperalgesic effect of AZ11713908 in a mouse FCA induced inflammatory pain model completely disappeared in CB1R KO mice but were fully preserved in CB2R KO mice. Therefore the analgesic activity of AZ11713908 appears to depend on actions at CB1R in this model, but since AZ11713908 is a weak partial agonist (38%) on mouse CB2R and inactive at rat CBR2, we are not able to draw any firm conclusions regarding the role of peripheral CB2R in rodent pain models from our results.
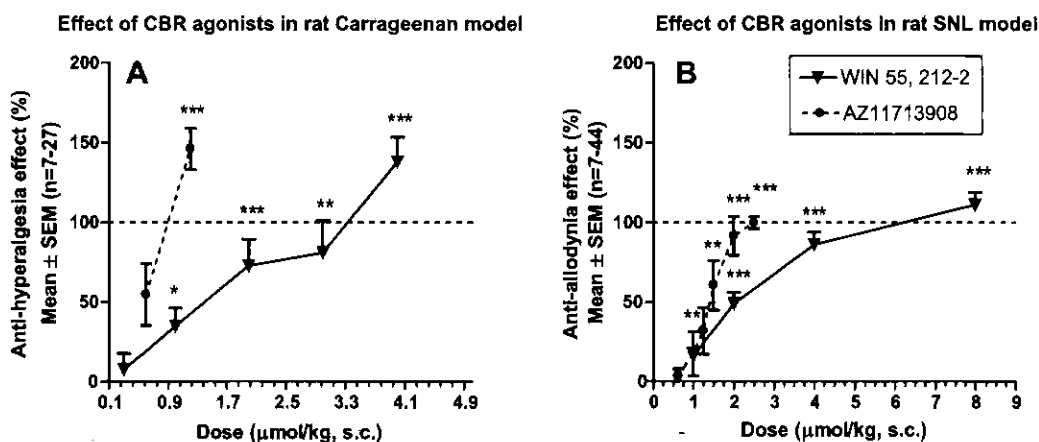
Effect of CBR agonists in rat Carrageenan model

Effect of CBR agonists in rat SNL model

Fig. 2. Anti-nociceptive effects of AZ11713908 (s.c.) in both inflammatory (A) and neuropathic pain models (B). Data are shown as means ± SEM (n = 7–27 for Fig. 2A and n = 7–44 for Fig. 2B). (A) $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. vehicle group by one-way ANOVA. (B) $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs. vehicle group by two-way RM ANOVA followed by Holm-Sidak test (baseline data not shown).
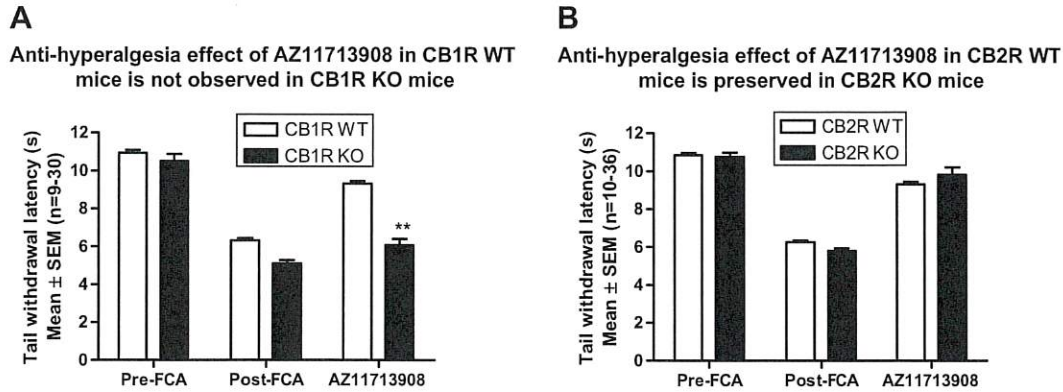
A

Anti-hyperalgesia effect of AZ11713908 in CB1R WT mice is not observed in CB1R KO mice

B

Anti-hyperalgesia effect of AZ11713908 in CB2R WT mice is preserved in CB2R KO mice

Fig. 3. Anti-nociceptive effect of AZ11713908 at 3 μmol/kg (s.c.) in CB1R and CBR2 mice. The effect was observed in CB1R WT mice, but not in CB1R KO mice (A) as well as in CBR2 WT and KO mice (B). Data are shown as means ± SEM (n = 9–30 for Fig. 3A and n = 10–36 for Fig. 3B). **$p < 0.01$ vs. CB1R KO group by unpaired t-test.

Considerable evidence in clinical studies indicate that THC or cannabis extracts produce analgesic activity in neuropathic pain patients, and in other pain conditions, for example a recent randomized double-blind placebo controlled trial also showed that the cannabinoid, nabilone, significantly relieved pain and improved quality of life in patients with fibromyalgia [28]. Despite these good effects, the use of these CNS-penetrant drugs is limited by cannabis-like adverse events, produced by the activity of CB1R in the CNS [3,16,30]. This lack of therapeutic window is also observable in animal studies – here we demonstrated that there was no separation between the potency of a centrally penetrant CB agonist (WIN 55, 212-2) in the Irwin tests and anti-hyperalgesic effects in chronic pain models. As described in the introduction, one approach to improve the tolerability profile of cannabinoids while retaining the analgesic effects is to target peripheral CB1 receptors.

There is considerable evidence in rodent pain models indicating that activation of CB1 receptors on peripheral sensory nerves by local application of CB agonists inhibits neuronal activity with a consequent reduction in pain behaviors [7,12,15,18,20,25]. It has also been reported that topical administration of HU210 reduced thermal and mechanical hyperalgesia and allodynia, induced by capsaicin, in an experimental human pain model, and no psychoactive effects were observed [27]. Consistent with these studies, our results clearly showed that local administration of AZ11713908

was sufficient to produce full reversal of heat hyperalgesia in a rat inflammatory pain model. Therefore, previous studies and our present data show that activation of peripheral CB1 receptors is sufficient to induce analgesic effects; however, local concentrations of CB agonists in these studies are likely higher than those achieved after systemic administration.

Data from mice with a selective knock-out of CB1 receptors in Nav1.8-expressing afferents suggests the analgesia produced by systemically administered brain penetrant cannabinoids is partly mediated by peripheral CB1 receptors [1]. However, can a peripherally acting CB1R agonist produce sufficient analgesia after systemic administration? CT-3 (ajulemic acid), a THC derivative, has been reported active in a phase II clinical trial for neuropathic pain patients [5,16] and has reduced CNS penetration compared to THC in animal pain models (Cbrain/Cplasma ratio ~30–40%) [10]. Recently, a novel CB agonist with limited CNS penetration has been reported to show robust analgesic effects in animal pain models without obvious side-effects [11]. These results support a strategy aimed at developing a new class of analgesic drugs with reduced side-effects by limiting CNS penetration.

In the present study, we used a CB1R agonist, AZ11713908, with a very low CNS penetration of <10%, compared to WIN 55, 212-2, which, like THC, concentrates in the brain with 2–4-fold higher levels in brain than in plasma. Despite the marked difference in brain
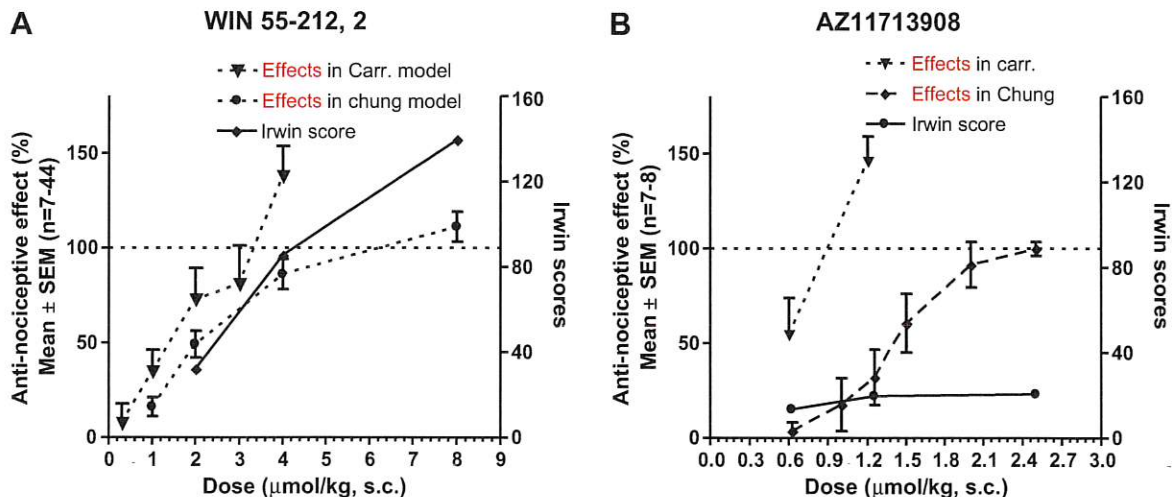
A   **WIN 55-212, 2**

B   **AZ11713908**

Fig. 4. Comparison of anti-nociceptive effect and observed Irwin scores (s.c.) by WIN 55, 212-2 (A) and AZ11713908 (B). Data are shown as means ± SEM (n = 7–44 for A and n = 7–8 for B). Dashed lines represent 100% reversal of hyperalgesia, or 100% effect.

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A
AZ11713908 produces robust anti-hyperalgesia effect in rat carrageenan model by intra-plantar administration

B
AZ11713908 produces robust anti-hyperalgesia effect in rat FCA model by intra-plantar administration
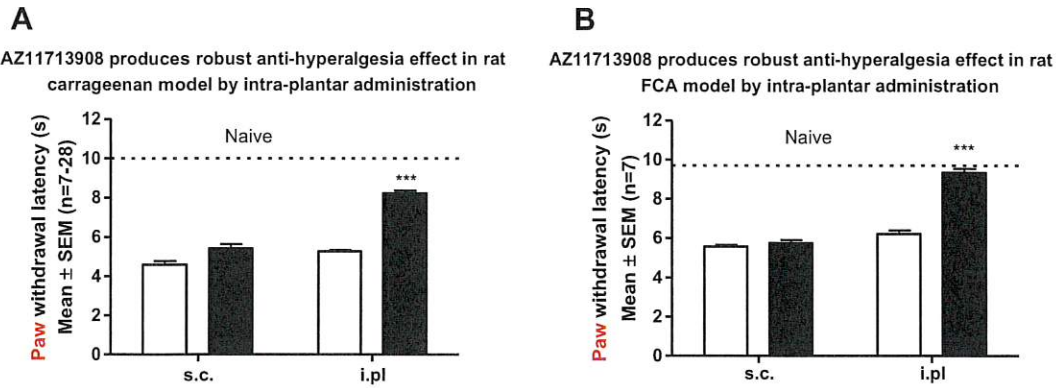

Fig. 5. Anti-hyperalgesic effect of AZ11713908 at 40 nmol/rat (i.pl.) in rat carrageenan (A) and FCA model (B). No activity after systemic injection at same dose (s.c.) was observed. Dashed lines represent 100% reversal of hyperalgesia, or 100% effect. Data were shown as means ± SEM ($n = 7$–28 for A and $n = 7$ for B). ***$p < 0.001$ vs. 20% HBC s.c. group by one-way ANOVA followed by Holm-Sidak test.

penetration, AZ11713908 was as efficacious as WIN 55, 212-2 in rodent pain models, but had a much larger side effect margin than WIN 55, 212-2 in these studies, consistent with the lack of brain exposure. Further evidence for a peripheral site of action of AZ11713908 comes from the electrophysiological study. Systemic (i.v.) administration of both AZ11713908 and WIN 55, 212-2 significantly inhibited the nociceptive responses evoked by natural stimulation in FCA-treated rats. However, WIN 55, 212-2, considerably reduced the responses to electrical stimulation of the sural nerve, whereas AZ11713908 did not, indicating a negligible spinal action of AZ11713908, compared to WIN 55, 212-2. Our data demonstrate that the anti-nociceptive effect of AZ11713908 is primarily via peripheral activation of CB1 receptors while WIN 55, 212-2 (a CNS-penetrant mixed CB1R and CB2R agonist) exhibits both spinal and peripheral analgesic sites of action, consistent with published data [31].

At analgesic doses, the free brain concentrations (based on protein binding of >90%) were calculated to be in the range of 0.8–1.7 nM, below the rat CB1R EC50 (6 nM, pEC50 = 8.2 in Table 2), consistent with the minimal CNS side-effects at these doses. However, AZ11713908 is only a peripherally restricted compound. If higher doses are administered, the free concentration in the brain would eventually be higher than the CB1R EC50 and it would act on CB1R in the brain. Thus AZ11713908 would be expected to produce classical cannabinoid CNS side-effects once brain concentrations reach sufficient levels.

The mechanism(s) by which CB1 receptor agonists can lead to analgesia are not completely understood at present and may well differ between peripheral and central sites. It is known that CB1 receptors, coupled to Gi/o proteins can modulate several cellular mechanisms, all of which can regulate the excitability of the neurons, e.g. closure of Ca^{2+} channels, opening of K^+ channels, inhibition of adenylyl cyclase and stimulation of mitogen-activated kinases [26]. There are several studies showing that CB1 agonists can alter excitability of nerve terminals in a significant manner. For example, a study using an in vivo model of trigeminovascular-mediated nociception demonstrated that pre-synaptic CB1 receptor activation prevents calcitonin gene-related peptide (CGRP) release from trigeminal sensory fibers [2]. A recent study using cultured rat dorsal root ganglion (DRG) cells has shown that activation of the CB1 receptor could inhibit the release of Substance P via a cyclic AMP-dependent PKA pathway [23]. In the periphery, this inhibition of terminal excitability would be expected to result in reduced generation of action potentials with a consequent reduction in nociceptive transmission.

In summary, AZ11713908 is a peripherally restricted full CB1R agonist and partial/inverse CB2R agonist that is highly effective in chronic neuropathic and inflammatory pain models in rodents via CB1R. In addition, using an electrophysiological assay, we found that AZ11713908 acts predominately in the periphery even after systemic administration. AZ11713908 shows a greatly superior therapeutic index to WIN 55, 212-2. Our findings therefore provide
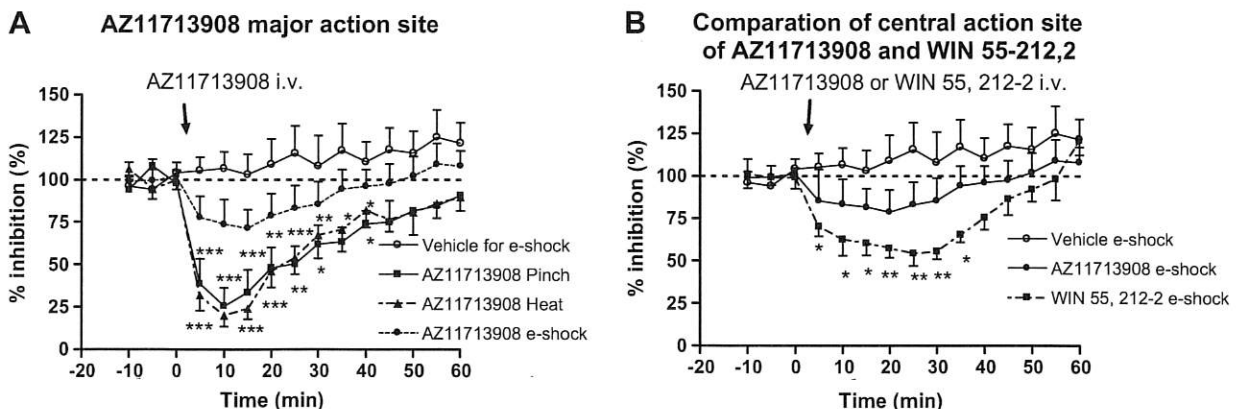
A **AZ11713908 major action site**

B **Comparison of central action site of AZ11713908 and WIN 55-212,2**

Fig. 6. Effect of AZ11713908 at 10 μmol/kg (i.v.) on (A) responses to electrical, pinch and heat stimuli in FCA rats (means ± SEM, $n = 7$–18) and (B) effects of AZ11713908 and WIN 55–212, 2 at 0.5 μmol/kg (i.v.) on responses to electrical stimuli in FCA rats (means ± SEM, $n = 6$–18). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. vehicle group by t-test.

further support for development of peripherally restricted CB1R agonists as effective analgesic agents with manageable side-effects.

Conflict of interest

There are no other conflicts of interest to declare.

Acknowledgments

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