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Antihyperalgesic properties of the cannabinoid CT-3 in chronic neuropathic and inflammatory pain states in the rat

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Abstract

CT-3 (ajulemic acid) is a synthetic analogue of a metabolite of Δ^9-tetrahydrocannabinol that has reported analgesic efficacy in neuropathic pain states in man. Here we show that CT-3 binds to human cannabinoid receptors in vitro, with high affinity at hCB1 (K_i 6 nM) and hCB2 (K_i 56 nM) receptors. In a functional GTP-γ-S assay CT-3 was an agonist at both hCB1 and hCB2 receptors (EC_{50} 11 and 13.4 nM, respectively). In behavioural models of chronic neuropathic and inflammatory pain in the rat, oral administration of CT-3 (0.1–1 mg/kg) produced up to 60% reversal of mechanical hyperalgesia. In both models the antihyperalgesic activity was prevented by the CB1-antagonist SR141716A but not the CB2-antagonist SR144528. In the tetrad of tests for CNS activity, CT-3 (1–10 mg/kg, po) produced dose-related catalepsy, deficits in locomotor performance, hypothermia, and acute analgesia. Comparison of 50% maximal effects in the tetrad and chronic pain assays produced an approximate therapeutic index of 5–10. Pharmacokinetic analysis showed that CT-3 exhibits significant but limited brain penetration, with a brain/plasma ratio of 0.4 measured following oral administration, compared to ratios of 1.0–1.9 measured following subcutaneous administration of WIN55,212-2 or Δ^9-THC. These data show that CT-3 is a cannabinoid receptor agonist and is efficacious in animal models of chronic pain by activation of the CB1 receptor. Whilst it shows significant cannabinoid-like CNS activity, it exhibits a superior therapeutic index compared to other cannabinoid compounds, which may reflect a relatively reduced CNS penetration.

Keywords: CT-3; Cannabinoid; Hyperalgesia; CB1 receptor

1. Introduction

Naturally occurring and synthetic cannabinoids are highly brain penetrant compounds that exert a variety of effects in animals, mediated predominantly via neuronal CB1 and CB2 receptors which are largely restricted to cells of the immune system (Pertwee and Ross, 2002). The well established antinociceptive activity of cannabinoids in animal models of acute pain is generally accepted to be mediated via activation of supraspinal and spinal CB1 receptors. More recently it has become clear that CB agonists are also effective in persistent pain states, inhibiting hyperalgesia or allodynia in models of chronic neuropathic (Bridges et al., 2001; Fox et al., 2001; Herzberg et al., 1997) or inflammatory (Clayton et al., 2002; Jaggar et al., 1998; Martin et al., 1999; Richardson et al., 1998b) pain. In the majority of studies this activity is also CB1 receptor-mediated and is at least partly due to activation of spinal receptors (Fox et al., 2001; Martin et al., 1999; Richardson et al., 1998a). Since the behavioural and psychotomimetic side-effects associated with cannabinoids are also mediated via CB1 receptors in the CNS, the potential analgesic activity of cannabinoids is limited by these central side-effects.

Mounting evidence indicates that activation of peripheral CB receptors may also reduce hypersensitive states in conditions of chronic pain. Injection of cannabinoids into the hindpaw inhibits inflammatory hyperalgesia and oedema

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(Calignano et al., 1998; Johanek et al., 2001; Richardson et al., 1998b), and we have shown in a model of neuropathic pain that the antihyperalgesic activity of WIN55,212-2 following intraplantar injection is inhibited by systemic but not intrathecally administered CB1 antagonist, indicating a peripheral site of action (Fox et al., 2001). There is additionally increasing awareness of the role of the CB2 receptor in chronic pain processes. The activity of non-selective cannabinoids such as HU-210 and CP55940 is partially inhibited by the selective CB2-receptor antagonist SR144528 (Clayton et al., 2002; Scott et al., 2004), and AM1241, which is reported to be a selective CB2-receptor agonist, has been shown to inhibit nociceptive processes in models of acute (Malan et al., 2001), neuropathic (Ibrahim et al., 2003), and inflammatory (Quartilho et al., 2003) pain.

CT-3 (1′,1′-dimethylheptyl-THC-11-oic acid; ajulemic acid) is a synthetic derivative of THC-11-oic acid, a major metabolite of Δ^9-THC, which has been reported to have anti-inflammatory activity in models of inflammation and rheumatoid arthritis (Burstein, 2004; Zurier et al., 1998). In a recent clinical trial CT-3 was shown to be analgesic in a group of patients with neuropathic pain of mixed aetiology, in the absence of marked cannabinoid-like side effects (Karst et al., 2003). Whilst CT-3 appears to be antinociceptive in models of acute pain (Burstein et al., 1998), there are no reports of its activity in animal models of chronic pain, and its mechanism of action is uncertain. The purpose of the present study was to determine the efficacy of CT-3 in models of chronic neuropathic and inflammatory pain in the rat and assess the involvement of the cannabinoid system, as well as to explore the relative contributions of central and peripheral CB receptors.

2. Methods

2.1. In vitro assays

2.1.1. Radioligand binding

Displacement of [^{3}H]-CP55,940 was measured in membranes of HEK293 cells expressing the human CB1 (hCB1) receptor, or CHO cells expressing the human CB2 (hCB2) receptor (Receptor Biology, Beltsville, MD, USA). Assays were carried out in 96-well plates using scintillation proximity beads (WGA-YSi beads, 1 mg/well) with 75 μl of membrane suspension (120–200 μg protein/ml) in assay buffer comprising: Tris–HCl, EDTA (2.5 mM), $MgCl_2$ (5 mM) and 5 mg/ml BSA at pH 7.4, 50 μl test compound in 4% (v/v) DMSO and 50 μl of 0.5 nM radioligand. All components were mixed and shaken at room temperature for 2 h, then counted on a Topcount Scintillation Counter (Perkin–Elmer). Non-saturable binding was measured in the presence of 10 μM CP55,940. IC_{50} values were calculated using a logistic fit from at least four curves carried out in triplicate. K_i values were calculated from the IC_{50} values using the Cheng–Prussoff equation ($K_i = IC_{50}/(1 + ([RL]/K_d))$ where [RL] is the radioligand concentration.

2.1.2. GTPγS binding assay

Homogenized membranes were prepared from CHO cell clones stably expressing human CB1 or CB2 receptors. Cells were grown to approximately 80% confluency on 15 cm tissue culture plates and subsequently scraped into PBS (pH 7.4), and pelleted by centrifugation. Cells were washed once with PBS and re-suspended in 20 ml of buffer A (20 mM Hepes, pH 7.4, 10 mM EDTA, and EDTA-free complete protease inhibitor cocktail). The cell suspension was homogenized on ice using a Polytron homogenizer at 25,000 rpm for three intervals of 15 s each. The homogenate was then centrifuged at 2000 rpm on a tabletop low speed centrifuge for 10 min at 4 °C. After passing through a cell strainer the supernatant was re-centrifuged at 50,000*g* for 25 min at 4 °C and the pellet re-suspended in buffer B (15% glycerol, 20 mM Hepes, pH 7.4, 0.1 mM EDTA and EDTA-free complete protease inhibitor cocktail). The protein concentration of the membrane preparation was determined using the BCA Protein Assay kit (Biorad) using BSA as standard. The membranes were aliquoted and kept frozen at −80 °C.

The GTPγS binding assay was carried out in round-bottom 96-well polystyrene assay plates (BD Falcon). The desired amount of membrane preparation was diluted with ice-cold assay buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.1% fatty acid-free BSA and 5 μM GDP) and vortexed. The assay mixture comprised 2 μl test compound, diluted as a 100× stock in DMSO, 100 μl diluted membranes (3–10 μg/well), and 100 μl [^{35}S]-GTPγS diluted 1:1000 (v/v) with assay buffer. The reaction was carried out at room temperature for 90 min before the membranes were harvested onto Perkin–Elmer Unifilter GF/B-96 filter plates using a Packard Filtermate Harvester. After several washes with wash buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$), and a rinse with 95% ethanol, the filter was dried in a 37 °C oven for 30 min. MicroScint-20 was added and the plate sealed for scintillation counting on a TopCount Scintillation Counter. Twelve different concentrations of test compound were used to generate a concentration response curve using three data points per concentration.

2.2. Animals and husbandry

Male Wistar rats were used in all experiments, housed in cages of six on a light–dark cycle of 12–12 h with free access to food and water. Rats weighing 120–140 g immediately prior to surgery were used in neuropathic studies and rats weighing 180–200 g were used for inflammatory pain studies and side-effect profiling. Animals were used in groups of six and were assigned randomly to treatment groups with the experimenter blind to treatments. All experiments were performed according to Home Office (United Kingdom) guidelines and with approval of the local Novartis Animal Welfare and Ethics Committee.

2.3. Neuropathic pain model

Hypersensitivity to noxious stimuli was assessed in a model of neuropathic pain induced by partial ligation of the left sciatic nerve (Seltzer et al., 1990). Briefly, rats were anaesthetized under enflurane and a small incision made at mid-thigh level. The left sciatic nerve was exposed and approximately one-third to one-half was tightly ligated using 7-0 silk suture. The wound was sealed with a skin clip and animals left to recover for 14–18 days prior to

experimentation. Mechanical hyperalgesia was assessed by measuring paw withdrawal thresholds to an increasing pressure stimulus placed onto the dorsal surface of the paw using an analgesymeter (Ugo-Basile, Italy) with a cut-off of 250 g. Withdrawal thresholds were measured on both the ipsilateral (ligated) and contralateral (unligated) paw prior to (predose) and then up to 6 h following compound or vehicle administration. Antagonists, where used, were administered 30 min prior to CT-3. We have previously determined that partial nerve ligation does not affect contralateral withdrawal thresholds, and sham surgery does not affect ipsilateral thresholds. Reversal of hyperalgesia at each time point was, therefore, calculated according to the following formula, which uses the contralateral paw as a reference rather than using additional groups of naïve or sham animals:

$$\%\ \text{reversal} = \frac{\text{postdose ipsilateral threshold} - \text{predose ipsilateral threshold}}{\text{predose contralateral threshold} - \text{predose ipsilateral threshold}} \times 100$$

2.4. Inflammatory pain

Naïve paw withdrawal thresholds of both hind paws were determined prior to injection of 25 μl Freund's complete adjuvant (FCA) into one hindpaw. Four days following FCA injection, withdrawal thresholds were measured prior to (predose) and up to 6 h following compound or vehicle administration. Antagonists, where used, were administered 30 min prior to CT-3. Reversal of hyperalgesia was calculated using the following formula:

$$\%\ \text{reversal} = \frac{\text{postdose ipsilateral} - \text{predose ipsilateral threshold}}{\text{naïve ipsilateral threshold} - \text{predose ipsilateral threshold}} \times 100$$

2.5. Tetrad

2.5.1. Rotarod

Changes in motor performance were assessed using an accelerating rotarod (Ugo Basile, Italy) in which rats were required to walk against the motion of a rotating drum, with the speed increasing from 4 to 40 rpm over 5 min. Training sessions were carried out one and two days prior to experimentation until animals were able to remain on the rotarod for at least 60 s, and latencies further determined prior to (predose) and up to 6 h following compound or vehicle administration. Data is expressed as latency(s) and percentage disruption calculated using the following formula:

$$\%\ \text{disruption} = 100 - ((\text{postdose latency} - \text{predose latency}) \times 100)$$

2.5.2. Catalepsy

Catalepsy was measured using a method modified from Pertwee (1972). Rats were hung by their front paws from a rubber coated metal ring (12 cm diameter) fixed horizontally at a height allowing their hindpaws to just touch the bench, and the time taken for the rat to move off the ring was measured, with a cut-off of 30 s. Latencies were measured immediately prior to (predose) and up to 6 h following drug or vehicle administration. Data is expressed as latency (s) and percentage maximum possible effect (% MPE) as defined below:

$$\%\ \text{MPE} = \frac{\text{postdose latency} - \text{predose latency}}{\text{cut} - \text{off (30 s)} - \text{predose latency}} \times 100$$

2.5.3. Tail-flick

The tail-flick assay measures the response to acute noxious thermal stimuli. Rats were placed inside a cotton glove to prevent gross movement and the length of the tail exposed. A radiant heat source (infra-red intensity 14) was provided by a tail-flick unit (Ugo Basile, Milan) and focussed on the rat tail approximately 3 cm from the tip. The latency measured was determined as the period of time between thermal stimuli onset and movement of the tail away from the heat source. A maximum cut-off of 15 s was used to prevent tissue damage and sensitisation in subsequent readings. Tail withdrawal latencies were determined prior to (predose) and up to 6 h post-drug or vehicle administration. Data is expressed as latency (s), and the percentage maximal possible effect (% MPE) was calculated using the following formula:

$$\%\ \text{MPE} = \frac{\text{postdose latency} - \text{predose latency}}{\text{cut} - \text{off (15 s)} - \text{predose latency}} \times 100$$

2.5.4. Hypothermia

Core temperature was assessed in rats using a RS 206-3722 thermometer (RS instruments, UK) which was lubricated and inserted into the rectum to a constant depth of 2.5 cm. Readings were taken prior to (predose) and up to 6 h post-drug or vehicle administration.

2.6. Pharmacokinetic analysis

2.6.1. Sample collection and preparation

Blood was collected by cardiac puncture into lithium–heparin containing tubes 1 and 3 h following oral administration of CT-3, or subcutaneous administration of Δ^9-THC or WIN55,212 to rats and centrifuged at 6000 rpm for 6 min to separate the plasma. Plasma samples and brains removed from the same animals were frozen at −20 °C until required for analysis. Following thawing, CT-3 was extracted from 200 μl of plasma aliquots on C18 SPE 50 mg columns (solid phase extraction). Brains were weighed and homogenized in water (1:5 w/v) for 30 s. CT-3 was extracted from 0.2 ml homogenate with 1.2 ml ethyl acetate for 1 h and then centrifuged at 3000*g* for 10 min. The supernatant was then dried under vacuum at 40 °C in Genevac (Ipswich, UK) and re-dissolved in 200 μl methanol.

2.6.2. Chromatographic separation and detection on LC/MS

Chromatographic separation of CT-3 was performed at room temperature on an HPLC-mass spectrometer system (HP1100 MSD; Hewlett Packard, UK) equipped with a vacuum degasser, autosampler, and Phenomenex C18 reversed phase column. The analysis was performed under the following conditions: eluent A: 10 mM ammonium acetate in water containing 0.3% formic acid; eluent B: acetonitrile, 100% containing 0.3% formic acid. A gradient elution of 90:10% solvent A:solvent B to 100% solvent B was used over 10 min. This was followed by elution with solvent B from 10 to 12 min and then returning to 90:10% solvent A. The sample injection volume was 25 μl and the flow rate was held constant at 1.0 ml/min. Under these conditions, the retention time of CT-3 was 10.1 min. The column efflux was directly introduced

into the ion source of the HP1100 MSD detector. Quantitative analysis was performed by selected ion recording over the respective protonated molecular ions [M+H$^+$] of CT-3 (MZ 401.2). The peak area was chosen as the chromatographic signal for quantification and integrated automatically using the WinNT ChemStation (Hewlett Packard) software package. Single samples from each animal were co-analysed along with standard curves prepared on the same day and run at the beginning of each batch of samples. The standard curves after extractions were linear, and the unknown sample concentrations were calculated from linear regression parameters of the standard curves and expressed in pmol/ml for plasma or pmol/gram weight of brain.

2.7. Drugs and reagents

[^{35}S]-GTPγS (250 μCi/20 μl) and [^{3}H]CP55,940 (180 Ci/mmol), were from NEN Life Sciences. Bovine serum albumin (essentially fatty acid free), GDP (sodium salt) and GTPγS (tetralithium salt) were from Sigma. EDTA-free complete protease inhibitor cocktail was from Roche Applied Science. Yttrium silicate beads coated with wheatgerm agglutinin (WGA-YSi beads) were from Amersham. WIN55,212 and CP-55,940 were obtained from Tocris Cookson (UK) and CT-3, SR141716A and SR144528 were synthesized at Novartis. CT-3 was dissolved in (v/v) 20% cremophor EL/80% water and administered orally in a volume of 1 ml. All other compounds were dissolved in (v/v) 20% cremophor EL/80% saline (0.9%) for behavioural studies except Δ^9-THC which was purchased from Sigma and diluted from stock to (v/v) 30% ethanol/70% saline (0.9%). WIN55,212, SR141716A, SR144528 and Δ^9-THC were administered subcutaneously in a volume of 0.5 ml.

2.8. Statistics and data analysis

Radioligand and nucleotide binding data were analysed by non-linear regression with the software package Origin 7.5 (Origin Lab Corporation, MA, US). For behavioural studies statistical analysis was carried out on raw data (paw withdrawal threshold, latency and temperature) using a two-way ANOVA followed by Dunnetts' test comparing time-matched drug treated groups to vehicle. In order to estimate the therapeutic index of CT-3, a D_{50} or ED_{50} is quoted. This is calculated as the effective dose resulting in 50% reversal of hyperalgesia compared with 50% disruption in rotarod performance or MPE in the catalepsy and tail-flick assays.

3. Results

3.1. In vitro activity at cannabinoid receptors

CT-3 binds with high affinity to human cannabinoid receptors, displacing [^{3}H]-CP55,940 binding from hCB1 and hCB2 receptors with calculated K_i values of 5.7±3.1 and 56.1±2.2, respectively. The affinity at both receptors was somewhat less than that of CP55,940 (Table 1, Fig. 1). In the GTPγS assay to test functional activity (Table 1, Fig. 2), CT-3 was active at both hCB1 and hCB2 receptors, increasing binding with calculated EC_{50} values of 11.6±2.2 and 13.4±2.3 nM. Although it was again slightly less potent than CP55,940, CT-3 appeared as a full agonist, and produced 101 and 83% of the maximal response to CP55,940 at hCB1 and hCB2 receptors, respectively (Fig. 2).

Table 1
In vitro activity of CT-3 and CP55,940 at human cannabinoid receptors

	Radioligand binding (K_i, nM)		GTPγS (EC_{50}, nM)	
	hCB1	hCB2	hCB1	hCB2
CT-3	5.7±3.1	56.1±2.2	11.6±2.2	13.4±2.3
CP55,940	0.8±0.1	0.3±0.02	0.4±0.08	0.5±0.7

Data show affinities measured by displacement of [^{3}H]-CP55,940 in membranes expressing hCB1 and hCB2 receptors, and functional activity measured as inhibition of binding of [^{35}S]-GTPγS. Data represent mean± SEM from at least three separate experiments, each performed in triplicate.

3.2. Pharmacokinetics

CT-3 was well absorbed following oral administration (3 mg/kg) with high plasma levels observed 1 h post-administration, and declining by 3 h (Table 2). It showed significant brain penetration which followed a similar pattern to the plasma levels, but overall brain penetration appeared limited with brain/plasma ratios of 0.3–0.4 measured 1 and 3 h following administration. In contrast, Δ^9-THC and WIN55,212, whilst showing lower plasma levels following subcutaneous administration (10 mg/kg),

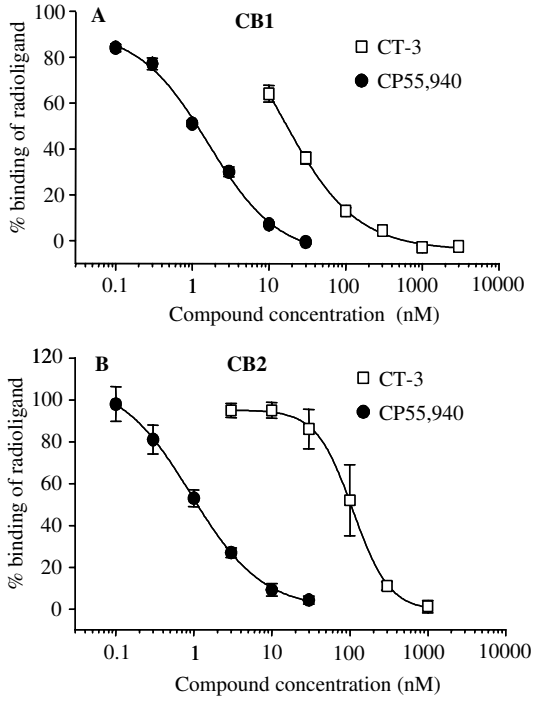

Fig. 1. Binding of CT-3 and CP55,940 to human CB receptors. Figures show displacement of [^{3}H]CP55,940 binding in (A) hCB1 receptors expressed in HEK293 cells and (B) hCB2 receptors expressed in CHO cells. Data were pooled from the mean±SEM of four individual experiments each performed in triplicate and curves fitted assuming a one-site binding model.

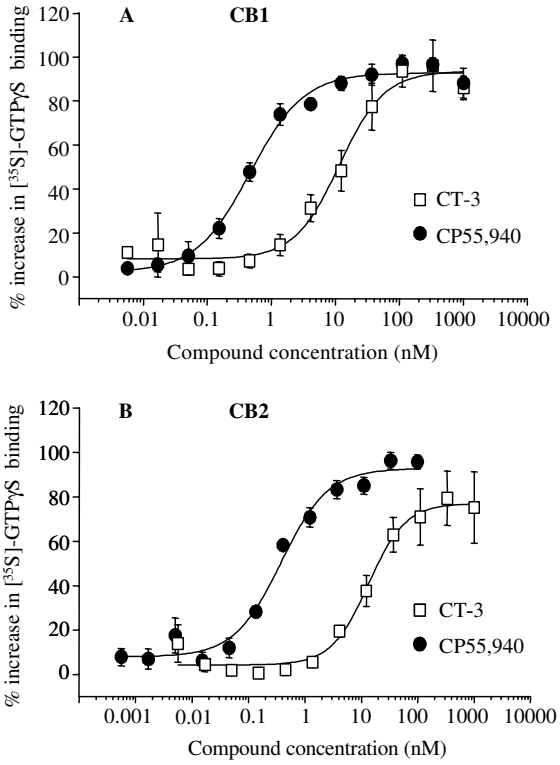

Fig. 2. Functional activity of CT-3 and CP55,940 at human CB receptors. Figures show increase in [^{35}S]-GTPγS binding in CHO cells expressing (A) hCB1 receptors and (B) hCB2 receptors. Results are normalized to the increase in binding produced by 100 nM CP55,940 assayed on the same plate. Data were pooled from the mean ± SEM of three individual experiments each performed in triplicate and curves fitted assuming a one-site binding model.

exhibited markedly higher brain penetration with brain/plasma ratios of 1.0–1.9 measured 1 and 3 h post administration.

3.3. Neuropathic pain model

Paw withdrawal thresholds measured 14–18 days post-ligation revealed a distinct mechanical hyperalgesia with ipsilateral thresholds averaging approximately 65 g compared to contralateral thresholds of approximately 105 g. Oral administration of CT-3 up to 1 mg/kg resulted in a dose-dependent reversal of hyperalgesia lasting from 3 to 6 h post-administration, with a maximal reversal of 63%, and a D_{50} value of 0.5 mg/kg calculated from data at 3 h post-dose (Fig. 3A). CT-3 did not affect contralateral paw withdrawal thresholds. Pretreatment with the CB1-receptor antagonist, SR141716A (3 mg/kg, s.c.) inhibited the anti-hyperalgesic activity of CT-3, whilst the CB2 receptor antagonist SR144528 was without effect (Fig. 3B). Neither antagonist affected paw withdrawal thresholds when administered alone.

3.4. Inflammatory pain model

Intraplantar administration of FCA caused a pronounced mechanical hyperalgesia comparable to that obtained in the neuropathic model. Ipsilateral withdrawal thresholds were reduced from approximately 105 to 60 g. Oral administration of CT-3 resulted in a dose-dependent reversal of hyperalgesia, with a maximal 57% reversal and a D_{50} value calculated from data obtained 3 h following administration of 0.7 mg/kg (Fig. 4A). Pretreatment with the CB1 antagonist SR141716A prevented the antihyperalgesic activity of CT-3, such that there was no significant effect between vehicle-treated animals and those treated with CT-3 and SR141716A. Treatment with the CB2 antagonist SR144528 produced only a slight reduction of the antihyperalgesic activity of CT-3 (Fig. 4B). Throughout inflammatory mechanical hyperalgesia experiments no changes in contralateral paw withdrawal thresholds were observed (data not shown).

3.5. Tetrad

CT-3 produced significant activity in all of the tetrad of tests for CNS-mediated cannabinoid-like activity following oral administration. Thus, it elicited up to 77% disruption of motor performance in the rotarod assay, and up to 77 and 84% maximal possible effect in the catalepsy and tail-flick assays, respectively. A significant reduction in core temperature (2.4 °C) was seen only with the highest dose of 10 mg/kg. In general, the profile of activity was similar in all these tests with maximal activity observed 3 h following administration (Fig. 5). The magnitude of effect in each assay was comparable to that of WIN55,212-2 (6 mg/kg, s.c.) included as a positive comparator, except for the hypothermia test where WIN55,212-2 was not significantly active in this experiment. CT-3 was, however, less potent in the tetrad tests than in the chronic pain models, with ED_{50}

Table 2
Plasma and brain levels of cannabinoids following systemic administration in rats

	CT-3		WIN55,212-2		Δ^9-THC	
	1 h	3 h	1 h	3 h	1 h	3 h
Plasma	1378 ± 120	936 ± 75	150 ± 17	104 ± 22	156 ± 19	178 ± 25
Brain	455 ± 103	390 ± 15	197 ± 27	203 ± 40	154 ± 13	184 ± 33
Brain/plasma ratio	0.3	0.4	1.3	1.9	1.0	1.0

Concentrations in plasma and brain samples from rats obtained following oral administration of CT-3 (3 mg/kg), or subcutaneous administration of WIN55,212-2 and Δ^9-THC (10 mg/kg) were measured by HPLC/MS. Data show mean ± SEM from three rats per time point.

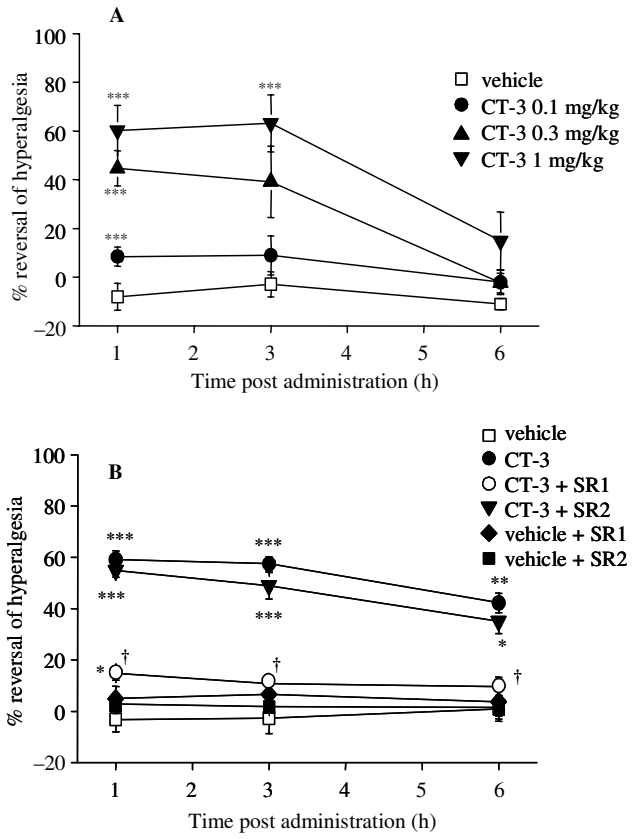

Fig. 3. Activity of CT-3 in a model of neuropathic pain in the rat. (A) Oral administration CT-3 produces a dose-related reversal of mechanical hyperalgesia induced by partial sciatic ligation. (B) Antihyperalgesic activity of CT-3 is inhibited by the CB1 antagonist SR141716A (SR1, 3 mg/kg, s.c.) but not the CB2 antagonist SR144528 (SR2, 10 mg/kg, s.c.). Antagonists were administered 30 min prior to oral administration of CT-3 (1 mg/kg). Data represent mean ± SEM from six animals per treatment group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to vehicle-treated animals, $^{\dagger}P<0.01$ compared to CT-3 treated animals, by two-way ANOVA plus Dunnett's test carried out on withdrawal threshold data.

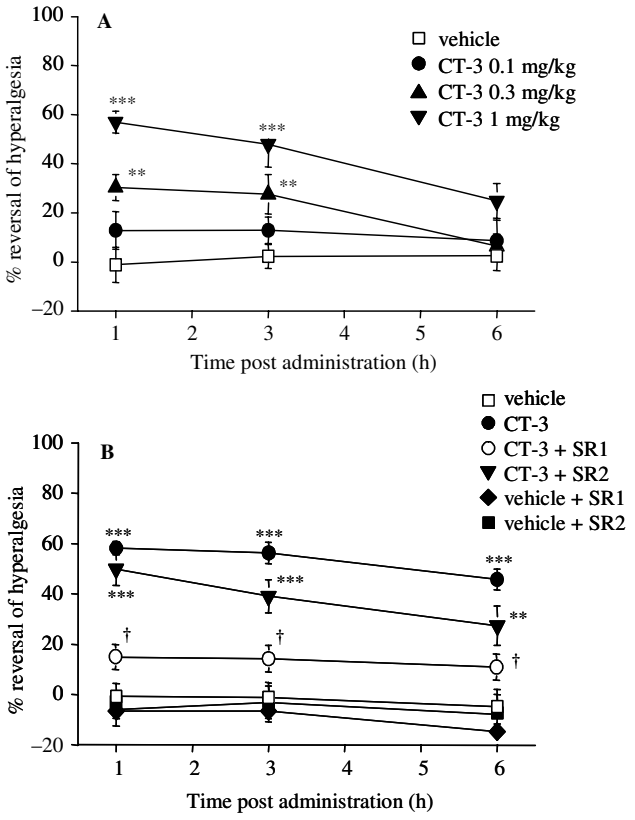

Fig. 4. Activity of CT-3 in a model of inflammatory pain in the rat. (A) Oral administration CT-3 produces a dose-related reversal of mechanical hyperalgesia measured 4 days following FCA injection into one hindpaw. (B) Antihyperalgesic activity of CT-3 is inhibited by the CB1 antagonist SR141716A (SR1, 3 mg/kg, s.c.) but not the CB2 antagonist SR144528 (SR2, 10 mg/kg, s.c.). Antagonists were administered 30 min prior to oral administration of CT-3 (1 mg/kg). Data represent mean ± SEM from six animals per treatment group. $^{**}P<0.01$, $^{***}P<0.001$ compared to vehicle-treated animals, $^{\dagger}P<0.01$ compared to CT-3 treated animals, by two-way ANOVA plus Dunnett's test carried out on withdrawal threshold data.

values of 5.3, 2.3 and 4.3 in the rotarod, catalepsy and tail-flick assays, respectively, and a minimal effective dose of 10 mg/kg in the hypothermia test. A comparison of potencies in the neuropathic pain model and in the tetrad tests indicates an estimated therapeutic index of 5–10.

4. Discussion

The present study demonstrates that CT-3 is a high affinity agonist for human cannabinoid receptors, and has potent antihyperalgesic activity in models of chronic neuropathic and inflammatory pain in the rat which are mediated via the CB1 receptors subtype. In addition, we provide a direct comparison of its activities in chronic pain models with those in the 'tetrad' of tests which are characteristic of central cannabinoid-like activity and show that antihyperalgesic activity can occur at doses slightly lower than those producing side-effects. This is supported by pharmacokinetic studies which, whilst demonstrating significant brain penetration, also indicate that CT-3 has a partially restricted entry into the CNS with brain levels reaching only 30–40% of peak plasma levels following oral administration.

Whilst CT-3 has previously been reported to be active in models of acute pain and inflammation, this is the first report of its activity in models of chronic pain. Earlier studies have shown that CT-3 is analgesic in the hot-plate and tail-clip tests of acute pain, as well as in the formalin test for acute inflammatory pain processes (Burstein et al., 1992, 1998; Dajani et al., 1999). In addition, it has been reported to inhibit cytokine-induced air pouch inflammation, with a reduction in cytokine release from leukocytes, and shows pronounced activity in a model of rheumatoid arthritis (Zurier at al., 1998). The exact mechanism of action of CT-3 remains unclear, and its analgesic and anti-inflammatory activity may result from different underlying cellular

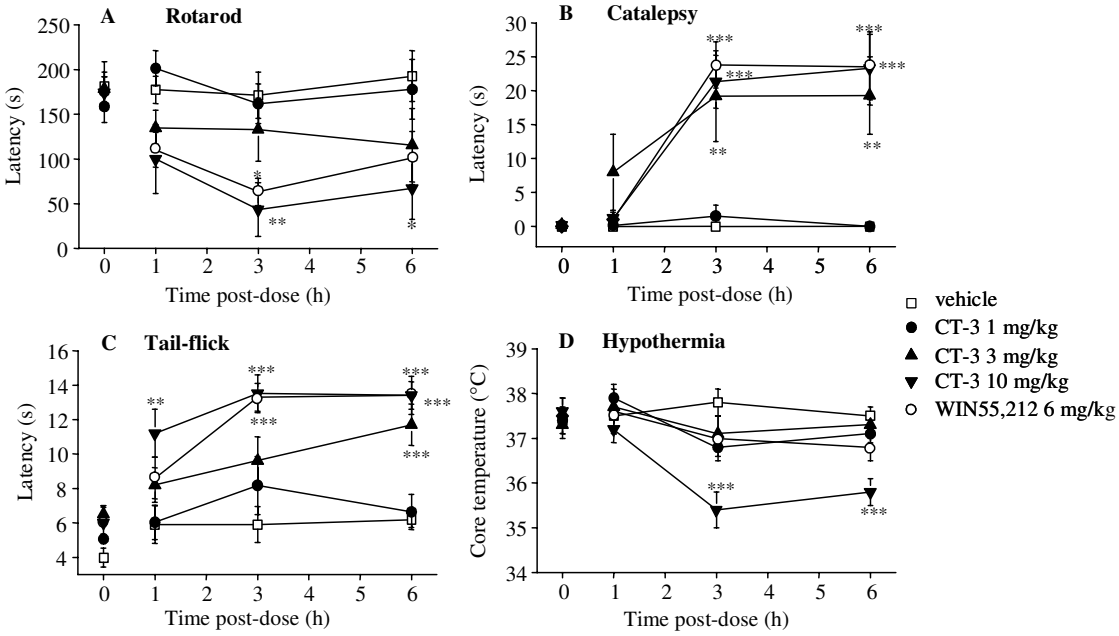

Fig. 5. Activity of CT-3 in tetrad of tests for cannabinoid-like activity in the rat. Data show activity in (A) rotarod, (B) catalepsy, (C) tail flick and (D) core temperature assays following oral administration of CT-3 or WIN55,212-2 (6 mg/kg, s.c.). Data show mean ± SEM from six animals per treatment group. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared to vehicle-treated animals by two-way ANOVA followed by Dunnett's test.

processes. There is some evidence that it inhibits COX-2 activity (Zurier et al., 1998) and most recently it has been reported to activate peroxisome proliferator-activated receptor-γ (PPARγ; Liu et al., 2003) which has been linked to inhibition of inflammatory processes such as monocyte activation and cytokine production (Ji et al., 2001; Jiang et al., 1998). Whilst PPARγ activation could, therefore, at least partially account for the anti-inflammatory activity of CT-3, it is less clear how this could result directly in its analgesic activity.

CT-3 is structurally a cannabinoid, being a derivative of a carboxylic metabolite of THC. The only previous report of its activity at cannabinoid receptors indicates relatively low affinity at CB1 receptors in rat brain (K_i 480 nM), although it had higher affinity at human CB1 (K_i 32 nM) and human CB2 (170 nM) heterologously expressed in cell lines (Rhee et al., 1997). Nevertheless, it is generally stated as having little activity at cannabinoid receptors and possessing no 'psychotropic' activity in animal studies (Burstein, 2004; Liu et al., 2003; Pertwee, 1997), although there is little published behavioural data on potential cannabinoid-like central effects. Here we show that CT-3 binds with high affinity to human CB1 and CB2 receptors. Furthermore, it behaves as a full agonist with high potency in a functional assay at both receptor types. In behavioural studies, CT-3 produced significant activity in all four of the tetrad of tests characteristic of cannabinoid-like activity. This included the tail-flick test of acute pain, thereby confirming previous reports of the acute antinociceptive activity of CT-3. As stated previously, this cataleptic, acute analgesic, hypothermic and hypolocomotor activity of cannabinoids is mediated via central CB1 receptors (Chaperon and Thiebot, 1999; Fox et al., 2001; Pertwee, 1997). Whilst we have not determined the affinity of CT-3 at rat CB receptors in vitro, there is little evidence for pharmacological differences between rodent and human CB receptors, and this profile, therefore, indicates that it behaves in vitro and in vivo as a typical cannabinoid.

The activity of CT-3 in chronic pain models has not previously been reported, although it has been shown to be effective recently in a clinical trial of neuropathic pain (Karst et al., 2003). In the present study we show that CT-3 reverses established mechanical hyperalgesia in models of chronic neuropathic and inflammatory pain in the rat. In both models this activity was inhibited by a CB1 antagonist but not significantly affected by a CB2 antagonist, establishing the mechanism of action as cannabinoid receptor agonism. This pattern is the same as that previously seen for synthetic cannabinoid agonists such as WIN55,212-2 and CP55,940 which also showed antihyperalgesic or antiallodynic activity in chronic pain models which was inhibited by a CB1 antagonist (Bridges et al., 2001; Fox et al., 2001). Thus, whilst CB2 selective agonists may be active in animal models of chronic pain (Clayton et al., 2002; Ibrahim et al., 2003; Quartilho et al., 2003), in general it appears that the CB1 activity predominates with agonists of mixed CB1/CB2 activity. Although this makes the assumption that both antagonists are completely selective at the doses used, data using ex vivo binding studies in the mouse support their in vivo selectivity (Rinaldi-Carmona et al., 1995, 1998), and the fact that SR144528 had no effect at all on the activity of CT-3 does indicate that it is behaving

differently to SR1441716A, and is likely CB2 selective here.

As mentioned in the Section 1, the major issue with the use of CB1 selective agonists as analgesic agents is the lack of a therapeutic window due to the central side-effects also elicited by CB1 receptor activation. This is evident in animal studies where there is little separation between the potency of centrally penetrant CB agonists in the tetrad of tests and in chronic pain models (Fox et al., 2001). A similar picture is seen in man, where increasing reports indicate that THC or cannabis extracts have analgesic activity in conditions such as neuropathic pain, cancer pain or multiple sclerosis, but their use is limited by cannabis-like adverse events such as dizziness and euphoria (Campbell et al., 2001; Svendsen et al., 2004; Wade et al., 2003). It is this issue which has driven the increased research into CB2 selective agonists as a means of harnessing the analgesic activity of cannabinoids, whilst avoiding the central side-effects, since CB2 receptors do not mediate behavioural side-effects, although they may be expressed in the spinal cord after nerve injury (Zhang et al., 2003). An alternative approach is to target peripheral CB1 receptors and there is considerable evidence to indicate that activation of CB1 receptors on peripheral sensory nerves inhibits neuronal activity with a consequent reduction in pain behaviours in models of chronic pain (Calignano et al., 1998; Fox et al., 2001; Johanek and Simone, 2004; Khasabova et al., 2004; Ko and Woods, 1999; Richardson et al., 1998b). The primary goal of the present study was to examine the role of cannabinoid receptors in the activity of CT-3 in chronic pain models. Although the site of action of CT-3 was not examined in detail, the results indicate at least a partial role of peripheral CB receptors. The activity in the tetrad test indicates that CT-3 does penetrate the CNS following oral administration, and this was confirmed by the pharmacokinetic analysis. However, maximal activity in the tetrad tests was achieved at higher doses than those reversing hyperalgesia in the pain models, and the calculated therapeutic index is larger than that previously reported for other synthetic cannabinoids (Fox et al., 2001). Moreover, the pharmacokinetic analysis indicates that although there is significant brain penetration in the rat it is restricted to a degree, with peak levels in the brain, measured at the peak pharmacodynamic timepoints, reaching only 30–40% of those seen in the plasma. This contrasts with the profile observed with WIN55,212-2 and THC which show a significantly higher relative brain penetration, with brain levels reaching 100–190% of those seen in the plasma. These data complement the recent findings in man in which CT-3 was found to reduce pain scores in neuropathic pain patients in the absence of cannabis-like psychotropic adverse events (Karst et al., 2003).

In summary, the results presented here show that CT-3 is a cannabinoid receptor agonist that is highly effective in models of chronic neuropathic and inflammatory pain in the rat and shows a superior therapeutic index to other cannabinoid agonists. Whilst the reduced psychotropic activity of CT-3 in man has been attributed to a non-CB receptor mechanism (Burstein, 2004) our data suggest that it may rather reflect a reduced brain penetration and a greater contribution of peripheral CB receptors to its mechanism of action. These findings, therefore, provide further support to the concept of peripherally restricted CB receptor agonists as analgesic agents with reduced side-effects.

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