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RESEARCH PAPER

# The psychoactive plant cannabinoid, $\Delta^9$-tetrahydrocannabinol, is antagonized by $\Delta^8$- and $\Delta^9$-tetrahydrocannabivarin in mice *in vivo*

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**Background and purpose:** To follow up *in vitro* evidence that $\Delta^9$-tetrahydrocannabivarin extracted from cannabis (e$\Delta^9$-THCV) is a $CB_1$ receptor antagonist by establishing whether synthetic $\Delta^9$-tetrahydrocannabivarin (O-4394) and $\Delta^8$-tetrahydrocannabivarin (O-4395) behave as $CB_1$ antagonists *in vivo*.
**Experimental approach:** O-4394 and O-4395 were compared with e$\Delta^9$-THCV as displacers of [$^3$H]-CP55940 from specific $CB_1$ binding sites on mouse brain membranes and as antagonists of CP55940 in [$^{35}$S]GTP$\gamma$S binding assays performed with mouse brain membranes and of *R*-(+)-WIN55212 in mouse isolated vasa deferentia. Their ability to antagonize *in vivo* effects of 3 or 10 mg kg$^{-1}$ (i.v.) $\Delta^9$-tetrahydrocannabinol in mice was then investigated.
**Key results:** O-4394 and O-4395 exhibited similar potencies to e$\Delta^9$-THCV as displacers of [$^3$H]-CP55940 ($K_i = 46.6$ and 64.4 nM, respectively) and as antagonists of CP55940 in the [$^{35}$S]GTP$\gamma$S binding assay (apparent $K_B = 82.1$ and 125.9 nM, respectively) and *R*-(+)-WIN55212 in the vas deferens (apparent $K_B = 4.8$ and 3.9 nM respectively). At i.v. doses of 0.1, 0.3, 1.0 and/or 3 mg kg$^{-1}$ O-4394 and O-4395 attenuated $\Delta^9$-tetrahydrocannabinol-induced anti-nociception (tail-flick test) and hypothermia (rectal temperature). O-4395 but not O-4394 also antagonized $\Delta^9$-tetrahydrocannabinol-induced ring immobility. By themselves, O-4395 and O-4394 induced ring immobility at 3 or 10 mg kg$^{-1}$ (i.v.) and antinociception at doses above 10 mg kg$^{-1}$ (i.v.). O-4395 also induced hypothermia at 3 mg kg$^{-1}$ (i.v.) and above.
**Conclusions and implications:** O-4394 and O-4395 exhibit similar *in vitro* potencies to e$\Delta^9$-THCV as $CB_1$ receptor ligands and as antagonists of cannabinoid receptor agonists and can antagonize $\Delta^9$-tetrahydrocannabinol *in vivo*.
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**Keywords:** Tetrahydrocannabivarin; tetrahydrocannabinol; CP55940; *R*-(+)-WIN55212; cannabinoid $CB_1$ receptor antagonist; mouse vas deferens; anti-nociception; tail-flick test; ring test; hypothermia

**Abbreviations:** BSA, bovine serum albumin; CHO, Chinese hamster ovary; CP55940, (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol; $\Delta^9$-THC, $\Delta^9$-tetrahydrocannabinol; DMSO, dimethyl sulphoxide; e$\Delta^9$-THCV, $\Delta^9$-tetrahydrocannabivarin extracted from cannabis; GDP, guanosine 5′-diphosphate; GTP$\gamma$S, guanosine-5′-*O*-(3-thiotriphosphate); MPE, maximum possible effect; O-4394, $\Delta^9$-tetrahydrocannabivarin; O-4395, $\Delta^8$-tetrahydrocannabivarin; PLSD, protected least significant difference; *R*-(+)-WIN55212, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride.

## Introduction

We have demonstrated previously that (–)-$\Delta^9$-tetrahydrocannabivarin ($\Delta^9$-THCV), a constituent of cannabis and the propyl homologue of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), behaves as a competitive surmountable antagonist of (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (*R*-(+)-WIN55212), anandamide and certain other cannabinoid receptor agonists in the mouse isolated vas deferens, with a potency similar to that exhibited in this tissue by the established $CB_1$ receptor antagonist, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-

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3-carboxamide hydrochloride (SR1417161A) (Pertwee *et al*., 1995; Thomas *et al*., 2005). $\Delta^9$-THCV has also been found to antagonize *R*-(+)-WIN55212-induced stimulation of [$^{35}$S]GTP$\gamma$S binding to mouse brain membranes, albeit significantly less potently than it antagonized this agonist in the vas deferens (Thomas *et al*., 2005), and in the same investigation another $CB_1$/$CB_2$ receptor agonist, (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940), was shown to be equally susceptible to antagonism by $\Delta^9$-THCV in this assay. The potency exhibited by $\Delta^9$-THCV against *R*-(+)-WIN55212 and CP55940 in the [$^{35}$S]GTP$\gamma$S-binding assay was also similar to the potency with which it displaced [$^3$H]-CP55940 from specific $CB_1$-binding sites on mouse brain membranes, suggesting that $\Delta^9$-THCV was competing with *R*-(+)-WIN55212 and CP55940 for $CB_1$ receptors on brain membranes, but acting through some other as yet unidentified mechanism in the vas deferens.

In spite of the structural similarity between $\Delta^9$-THCV and $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), it is not unexpected that THCV can block the effects of $CB_1$ receptor agonists, even though THC is a $CB_1$ receptor partial agonist (reviewed in Howlett *et al*., 2002). Thus, data from structure–activity investigations suggest that, because its alkyl side chain (propyl) is shorter than that of $\Delta^9$-THC (pentyl), $\Delta^9$-THCV should indeed exhibit even less efficacy than $\Delta^9$-THC as a $CB_1$ receptor agonist (reviewed in Howlett *et al*., 2002). Even so, although $\Delta^9$-THCV behaves as a $CB_1$ receptor antagonist *in vitro*, it has been found to exhibit $CB_1$ agonist-like activity *in vivo*. Thus, in the first ever pharmacological experiments with $\Delta^9$-THCV, this compound was found to induce immobility in the mouse ring test (Gill *et al*., 1970), an effect that is also known to be produced by established $CB_1$ receptor agonists such as *R*-(+)-WIN55212, CP55940 and $\Delta^9$-THC acting through $CB_1$ receptors (reviewed in Howlett *et al*., 2002).

Even though, $CB_1$ receptor activation can induce immobility in the ring test, it remains possible that $\Delta^9$-THCV did not produce this effect by acting through $CB_1$ receptors, as certain compounds that do not activate these receptors are also known to induce immobility in this assay (Wiley and Martin, 2003). In addition, although the potency exhibited by $\Delta^9$-THCV in the ring test was 4.8 times less than that of $\Delta^9$-THC (Gill *et al*., 1970), there is little difference between the potencies of these two ligands as displacers of [$^3$H]-CP55940 from $CB_1$-binding sites (Howlett *et al*., 2002; Thomas *et al*., 2005). These findings raise the possibility that $\Delta^9$-THCV might behave as a $CB_1$ receptor antagonist *in vivo* at doses below those at which it induces ring immobility, and the main objective of the present investigation was to investigate this possibility. This was achieved by establishing whether there are any *in vivo* doses at which $\Delta^9$-THCV shares the ability of the $CB_1$-selective antagonist, SR141716A (Compton *et al*., 1996; Adams *et al*., 1998), to attenuate the production by $\Delta^9$-THC in mice of anti-nociception in the tail-flick test, immobility in the ring test and hypothermia.

For previous pharmacological experiments with $\Delta^9$-THCV (Gill *et al*., 1970; Thomas *et al*., 2005), this compound was obtained by extracting it from cannabis. As a result, the samples of $\Delta^9$-THCV used most likely contained traces of other cannabis constituents that could perhaps have contributed to the pharmacological effects that these samples produced. To avoid this possibility, the experiments described in the present paper were performed with synthetic $\Delta^9$-THCV (O-4394). To facilitate future structure–activity studies, synthetic (–)-$\Delta^8$-tetrahydrocannabivarin (O-4395) was also investigated, it being easier to synthesize analogues of $\Delta^8$- than $\Delta^9$-tetrahydrocannabivarin. Consequently, our initial experiments were directed at establishing whether O-4394 and O-4395 possess similar potencies to those found previously for $\Delta^9$-THCV extracted from cannabis as displacers of [$^3$H]-CP55940 from specific-binding sites on mouse brain membranes, as antagonists of CP55940-induced stimulation of [$^{35}$S]GTP$\gamma$S binding to mouse brain membranes and as antagonists of *R*-(+)-WIN55212-induced inhibition of electrically evoked contractions of the mouse isolated vas deferens. $\Delta^9$-THCV extracted from cannabis is referred to in the remainder of this paper as e$\Delta^9$-THCV.

The results indicate first that O-4394 and O-4395 exhibit similar potencies to e$\Delta^9$-THCV as displacers of [$^3$H]-CP55940 and as antagonists of CP55940 and *R*-(+)-WIN55212 in mouse brain membrane and vas deferens assays and second that they both can antagonize $\Delta^9$-THC *in vivo* at doses of 0.1, 0.3, 1.0 and/or 3 mg kg$^{-1}$, intravenously (i.v.).

## Methods

The methods used comply with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines for the use of experimental animals. All *in vivo* animal protocols were also approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

### *Membrane preparation*

Binding assays with [$^3$H]-CP55940 and [$^{35}$S]GTP$\gamma$S were performed with mouse whole brain membranes, prepared as described by Thomas *et al*. (2004). Protein assays were carried out using a Bio-Rad Dc kit (Bio-Rad, Hercules, CA, USA).

### *Radioligand displacement assay*

The assays were carried out with [$^3$H]-CP55940, Tris-binding buffer (50 mM Tris-HCl; 50 mM Tris Base; 0.1% bovine serum albumin (BSA)), total assay volume 500 $\mu$l, using the filtration procedure described previously by Ross *et al*. (1999b). Binding was initiated by the addition of the brain membranes (33 $\mu$g protein per well). All assays were performed at 37°C for 60 min before termination by addition of ice-cold Tris-binding buffer and vacuum filtration using a 24-well sampling manifold (cell harvester; Brandel Inc., Gaitherburg, MD, USA) and GF/B filters (Whatman, Maidstone, UK), that had been soaked in wash buffer at 4°C for at least 24 h. Each reaction well was washed six times with a 1.2 ml aliquot of Tris-binding buffer. The filters were oven-dried for at least 60 min and then placed in 5 ml of scintillation fluid (Ultima Gold XR, Perkin-Elmer Life Sciences Inc., Boston, MA, USA). Radioactivity was quantified by liquid scintillation

spectrometry. Specific binding was defined as the difference between the binding that occurred in the presence or absence of 1 $\mu$M unlabelled CP55940. The concentration of [$^3$H]-CP55940 used in our displacement assays was 0.7 nM. O-4394 and O-4395 were stored as a stock solution of 10 mM in dimethylsulphoxide (DMSO), the vehicle concentration in all assay wells being 0.1% DMSO. The binding parameters for [$^3$H]-CP55940 in the mouse brain membranes, determined by fitting data from saturation binding experiments to a one-site saturation plot using GraphPad Prism, were 2336 fmol mg$^{-1}$ protein ($B_{max}$) and 2.31 nM ($K_d$) (Thomas *et al*., 2004).

*[$^{35}$S]GTP$\gamma$S binding assay*

The procedure for measuring agonist-stimulated [$^{35}$S]GTP$\gamma$S binding to cannabinoid $CB_1$ receptors was adapted from the methods of Kurkinen *et al*. (1997) and Breivogel *et al*. (2001) as described previously (Thomas *et al*., 2005). Assays were carried out with GTP$\gamma$S-binding buffer (50 mM Tris-HCl; 50 mM Tris base; 5 mM $MgCl_2$; 1 mM ethylenediaminetetraacetic acid (EDTA); 100 mM NaCl; 1 mM dithiothreitol; 0.1% BSA) in the presence of 0.1 nM [$^{35}$S]GTP$\gamma$S and 30 $\mu$M guanosine 5′-diphosphate (GDP) and in a final volume of 500 $\mu$l. Membranes (5 $\mu$g protein per well) were preincubated for 30 min at 30°C with 0.5 U ml$^{-1}$ adenosine deaminase (200 U mg$^{-1}$) to remove endogenous adenosine. Binding was initiated by the addition of [$^{35}$S]GTP$\gamma$S to the wells. Non-specific binding was measured in the presence of 30 $\mu$M GTP$\gamma$S. The drugs were incubated in the assay for 60 min at 30°C. The reaction was terminated by a rapid vacuum filtration method using Tris-binding buffer as described previously (Ross *et al*., 1999a), and the radioactivity was quantified by liquid scintillation spectrometry. Agonists and antagonists were stored as a stock solution of 1 or 10 mM in DMSO, the vehicle concentration in all assay wells being 0.11% DMSO.

*Vas deferens experiments*

Vasa deferentia were obtained from albino MF1 mice weighing 32–44 g. The tissues were mounted vertically in 4 ml organ baths. They were then subjected to electrical stimulation of progressively greater intensity, followed by an equilibration procedure in which they were exposed to alternate periods of stimulation (2 min) and rest (10 min) until contractions with consistent amplitudes were obtained (Thomas *et al*., 2004). These contractions were monophasic and isometric and were evoked by 0.5 s trains of pulses of 110% maximal voltage (train frequency 0.1 Hz; pulse frequency 5 Hz; pulse duration 0.5 ms). All drug additions were made to the organ baths after the equilibration period and there was no washout between these additions. There was an initial application of a potential antagonist or its vehicle and this was followed 28 min later by a 2 min period of electrical stimulation at the end of which the lowest of a series of concentrations of the cannabinoid receptor agonist, *R*-(+)-WIN55212 was applied. After a 13 min period of rest, the tissues were electrically stimulated for 2 min and then subjected to a further addition of *R*-(+)-WIN55212. This cycle of drug addition, 13 min rest and 2 min stimulation was repeated, to obtain cumulative concentration–response curves, only one of which was constructed per tissue (Pertwee *et al*., 1996).

In vivo *experiments*

Male ICR mice weighing 22–30 g (Harlan, Indianapolis, IN, USA) were housed in groups of five in 28 × 16 cm plastic cages with steel mesh tops in a temperature-controlled vivarium and were maintained on a 12 h light/dark cycle. Food and water were available *ad libitum*. Vehicle or 0.03, 0.1, 0.3, 1, 3, 10, 30 or 56 mg kg$^{-1}$ O-4394 or O-4395 were injected into a tail vein. In some experiments, mice were pretreated intraperitoneally (i.p.) with 3 mg kg$^{-1}$ SR141716A 10 min before the intravenous administration of 56 mg kg$^{-1}$ O-4394 or O-4395 or 3 mg kg$^{-1}$ $\Delta^9$-THC. In other antagonism experiments, mice were pretreated (i.v.) with 0.03, 0.1, 0.3, 1 or 3 mg kg$^{-1}$ O-4394 or O-4395 or with the vehicle immediately before i.v. administration of $\Delta^9$-THC either at 10 mg kg$^{-1}$, the lowest dose required to produce maximal effects in the three assays that were used, or at 3 mg kg$^{-1}$. The measured responses were tail-flick latency in the radiant heat nociceptive test (D'Amour and Smith, 1941), immobility in the ring test (Pertwee, 1972) and core temperature. For the ring test, each mouse was placed on a ring (5.5 cm diameter) that was elevated 16 cm from a table top for a 5-min observation period. The amount of time each animal remained motionless, except for respiratory movements, was recorded to the nearest second. Core temperatures were measured to the nearest 0.1°C by inserting a rectal probe connected to a telethermometer (YSI Inc., Yellow Springs, OH, USA) to a depth of 1.8 cm. As decribed previously (Varvel *et al*., 2005), control tail-flick latencies and core body temperatures were assessed in each mouse before i.v. administration of any drugs. The intensity of the stimulus from the heat lamp in the tail-flick test was adjusted to yield control latencies of 2–4 s and an automatic 10 s cut-off was used. At 20 min post exposure, tail-flick latencies were reassessed and, at 40 min mice were subjected to the ring test. Core temperatures were reassessed at 60 min. All measures were taken in each animal. The ambient temperature was approximately 22°C.

*Drugs and chemicals*

$\Delta^9$-THC was supplied by the National Institute on Drug Abuse (Bethesda, MD, USA) and O-4394 and O-4395 by Dr Raj Razdan (Organix Inc, MA, USA). SR141716A was obtained from Sanofi-Aventis (Montpellier, France) and from the National Institute on Drug Abuse (Bethesda, MD, USA) and *R*-(+)-WIN55212 and CP55940 were purchased from Tocris (Bristol, UK). For the binding experiments, [$^3$H]-CP55940 (160 Ci mmol$^{-1}$) and [$^{35}$S]GTP$\gamma$S (1250 Ci mmol$^{-1}$) were obtained from Perkin-Elmer Life Sciences Inc. (Boston, MA, USA), GTP$\gamma$S and adenosine deaminase from Roche Diagnostic (Indianapolis, IN, USA) and GDP from Sigma-Aldrich (St Louis, MO, USA). For mouse vas deferens experiments, *R*-(+)-WIN55212 was dissolved in a 50% (v v$^{-1}$) solution of DMSO and a 0.9% aqueous solution of

NaCl (saline) and all other drugs were dissolved in pure DMSO. Drugs were added to organ baths in a volume of 10 $\mu$l. For the *in vivo* experiments, O-4394, O-4395, $\Delta^9$-THC and SR141716A were dissolved in a 1:1 mixture of absolute ethanol and alkamuls-620 (Aventis, Strasbourg, France) and diluted with saline to a final ratio of 1:1:18 (ethanol/alkamuls/saline). Injections were given in a volume of 10 ml kg$^{-1}$.

*Analysis of data*

Values have been expressed as means and variability as s.e.m. or as 95% confidence limits. The concentrations of O-4394 and O-4395 that produced a 50% displacement of radioligand from specific binding sites ($IC_{50}$ values) were calculated using GraphPad Prism 4. Their dissociation constants ($K_i$ values) were calculated using the equation of Cheng and Prusoff (1973). Net agonist-stimulated [$^{35}$S]GTP$\gamma$S binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values (obtained in the presence of agonist), as detailed elsewhere (Ross *et al*., 1999a). Inhibition of the electrically evoked twitch response of the vas deferens has been expressed in percentage terms and this has been calculated by comparing the amplitude of the twitch response after each addition of a twitch inhibitor with its amplitude immediately before the first addition of the inhibitor.

Rectal temperatures have been expressed as the difference between pre- and post-injection values obtained from each mouse, and anti-nociception has been calculated by transforming the tail-flick data to the percentage of maximum possible effect (%MPE), where %MPE = 100 × ([post-injection latency−pre-injection latency]/[cutoff time−pre-injection latency]) (Varvel *et al*., 2005). Anti-nociceptive $ED_{50}$ values have been determined by least-squares linear regression and the 95% confidence limits of these values were also calculated (Bliss, 1967).

For *in vitro* data, values for $EC_{50}$ and for the s.e.m. or 95% confidence limits of these values have been calculated by nonlinear regression analysis using the equation for a sigmoid concentration–response curve (GraphPad Prism). The apparent dissociation constant ($K_B$) values for antagonism of agonists by O-4394 or O-4395 in the vas deferens or [$^{35}$S]GTP$\gamma$S-binding assay have been calculated by Schild analysis from the concentration–ratio, defined as the concentration of an agonist that elicits a response of a particular size in the presence of a competitive reversible antagonist at a concentration, B, divided by the concentration of the same agonist that produces an identical response in the absence of the antagonist. The methods used to determine concentration–ratio and apparent $K_B$ values and to establish whether log concentration–response plots deviated significantly from parallelism are detailed elsewhere (Pertwee *et al*., 2002).

Mean values obtained *in vitro* have been compared with zero using the one-sample *t*-test or with each other using Student's two-tailed *t*-test for unpaired data or one-way analysis of variance (ANOVA), followed by Dunnett's test (GraphPad Prism). For *in vivo* data, the significance of any differences from controls (the vehicle group in tests of agonism and the vehicle + $\Delta^9$-THC group in tests of antagonism) was assessed by one-way ANOVA, followed by Fisher's protected least significant difference (PLSD) test *post hoc* (Bliss, 1967). A *P*-value $<0.05$ was considered to be significant.

## Results

*Experiments with brain membranes or the isolated vas deferens*

O-4394 and O-4395 each displaced [$^3$H]-CP55940 from specific-binding sites on mouse brain membranes (Figure 1). This they did in a manner that fitted better to a one-site rather than a two-site competition curve ($P<0.05$; GraphPad Prism 4). The mean $K_i$-values of both these compounds are listed in Table 1. It was also found that at 1 $\mu$M, O-4394 and O-4395 each attenuated the ability of CP55940 to stimulate [$^{35}$S]GTP$\gamma$S binding to mouse brain membranes (Figure 2), and that at 100 nM they each opposed the ability of *R*-(+)-WIN55212 to reduce the amplitude of electrically evoked contractions of the vas deferens (Figure 3). In both these tissue preparations this antagonism was surmountable. It was probably also competitive in nature, as each antagonist induced a dextral shift in the log concentration–response curves of CP55940 and *R*-(+)-WIN55212 that did not deviate significantly from parallelism (Figures 2 and 3). The mean apparent $K_B$ values of O-4394 and O-4395 for their antagonism of CP55940 and *R*-(+)-WIN55212 are shown in Table 1. These values indicate that, as found previously for e$\Delta^9$-THCV (Table 1), O-4394 and O-4395 are both markedly more potent as antagonists of *R*-(+)-WIN55212 in the vas deferens than of CP55940 in brain membranes.

Effects of O-4394 and O-4395, administered by themselves, on [$^{35}$S]GTP$\gamma$S binding to brain membranes were also investigated (Figure 2). Binding was stimulated by O-4395 at 0.1 and 1 $\mu$M, although not significantly affected by this compound at 1 or 10 nM or at 10 $\mu$M. In contrast, O-4394 did not affect [$^{35}$S]GTP$\gamma$S binding at any of these concentrations. The amplitude of electrically evoked contractions of the vas deferens was not affected by either O-4394 or O-4395 administered at the concentration that antagonized *R*-(+)-

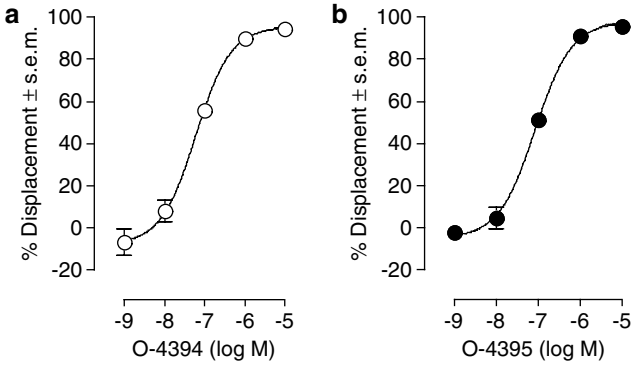


**Figure 1** Displacement of [$^3$H]-CP55940 by (**a**) O-4394 and (**b**) O-4395 from specific binding sites on mouse whole brain membranes. Each symbol represents the mean percent displacement ± s.e.m. Mean $K_i$-values for this displacement have been calculated from these data and these values are listed in Table 1.

**Table 1** $K_i$-values for displacement of [$^3$H]-CP55940 from mouse brain membranes and apparent $K_B$-values for antagonism of CP55940-induced stimulation of [$^{35}$S]GTP$\gamma$S binding to mouse brain membranes and of *R*-(+)-WIN55212-induced inhibition of electrically evoked contractions of the mouse isolated vas deferens

| *Compound* | *Mouse tissue* | *Mean values (nM)* | *95% confidence limits* | n |
|---|---|---|---|---|
| | | $K_i$ | | |
| O-4394 | Brain membranes | 46.6 | 31.3 and 69.4 | 5 |
| O-4395 | | 64.4 | 49.0 and 84.7 | 5 |
| e$\Delta^9$-THCV[a] | | 75.4 | 53.4 and 106.3 | 4–8 |
| | | Apparent $K_B$ | | |
| O-4394 (1 $\mu$M) | Brain membranes | 82.1 | 54.1 and 124.4 | 5 |
| O-4395 (1 $\mu$M) | | 125.9 | 83.1 and 195.9 | 5 |
| e$\Delta^9$-THCV (1 $\mu$M)[a] | | 93.1 | 66.5 and 130.6 | 6 |
| | | Apparent $K_B$ | | |
| O-4394 (100 nM) | Vas deferens | 4.8 | 0.3 and 30.3 | 7 |
| O-4395 (100 nM) | | 3.9 | 0.7 and 13.3 | 7 |
| e$\Delta^9$-THCV (100 nM)[a] | | 1.5 | 1.1 and 2.3 | 6–9 |

Abbreviation: e$\Delta^9$-THCV, $\Delta^9$-tetrahydrocannabivarin extracted from cannabis; *R*-(+)-WIN55212, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; CP55940, (–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol.
[a]From Thomas *et al*. (2005).

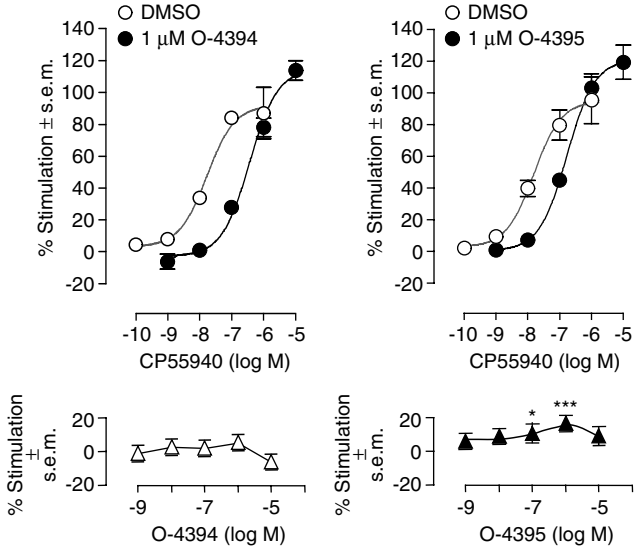


**Figure 2** The effect of 1 $\mu$M O-4394 and O-4395 on the mean log concentration–response curve of CP55940 for stimulation of [$^{35}$S]GTP$\gamma$S binding ($n=4$ or 5; upper panels) and on [$^{35}$S]GTP$\gamma$S binding in the absence of CP55940 ($n=6$; lower panels). Each symbol represents the mean percentage change in [$^{35}$S]GTP$\gamma$S binding to mouse whole brain membranes and vertical lines show s.e.m. Mean apparent $K_B$ values of O-4394 and O-4395 for their antagonism of CP55940 have been calculated from the data in the upper panels and these values are listed in Table 1. The asterisks in the lower panel denote significant differences from zero (*$P<0.05$; ***$P<0.001$; one-sample *t*-test).

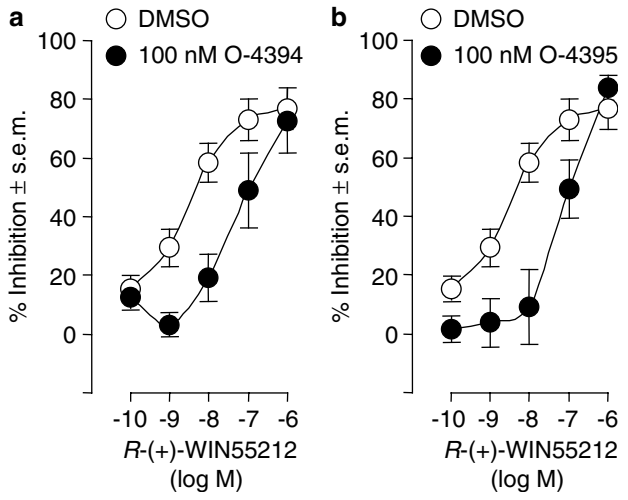


**Figure 3** The effect of pretreatment with (**a**) 100 nM O-4394 or (**b**) 100 nM O-4395 on the mean log concentration–response curve of *R*-(+)-WIN55212 in the mouse isolated vas deferens. Each symbol represents the mean value (and vertical lines show s.e.m.) for inhibition of electrically evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of *R*-(+)-WIN55212 to the organ bath. O-4394, O-4395 or DMSO was added 30 min before the first addition of *R*-(+)-WIN55212, further additions of which were made at 15 min intervals. Each log concentration–response curve was constructed cumulatively without washout ($n=7$). Mean apparent $K_B$ values of O-4394 and O-4395 for their antagonism of *R*-(+)-WIN55212 have been calculated from these data and these values are listed in Table 1.

WIN55212. Thus, the mean amplitude was no different when measured 30 min after 100 nM O-4394 or O-4395 than when measured 30 min after DMSO ($P>0.05$; ANOVA followed by Dunnett's test; $n=7$).

In vivo *experiments*

In experiments with 10 mg kg$^{-1}$ $\Delta^9$-THC, anti-nociception induced by this cannabinoid was significantly opposed by O-4394 at 3 mg kg$^{-1}$ and by O-4395 at 0.3, 1 and 3 mg kg$^{-1}$ (Figure 4). Although O-4394 also seemed to oppose the antinociceptive effect of $\Delta^9$-THC at 0.3 and 1 mg kg$^{-1}$, the apparent antagonism induced by these lower doses was not significant. In the ring test, O-4395 significantly reduced the ability of $\Delta^9$-THC to induce immobility when administered at a dose of 0.3 or 3 mg kg$^{-1}$, although not when administered at 1 mg kg$^{-1}$ (Figure 4). In contrast, O-4394 did not antagonize $\Delta^9$-THC in this bioassay at 0.3, 1 or 3 mg kg$^{-1}$.

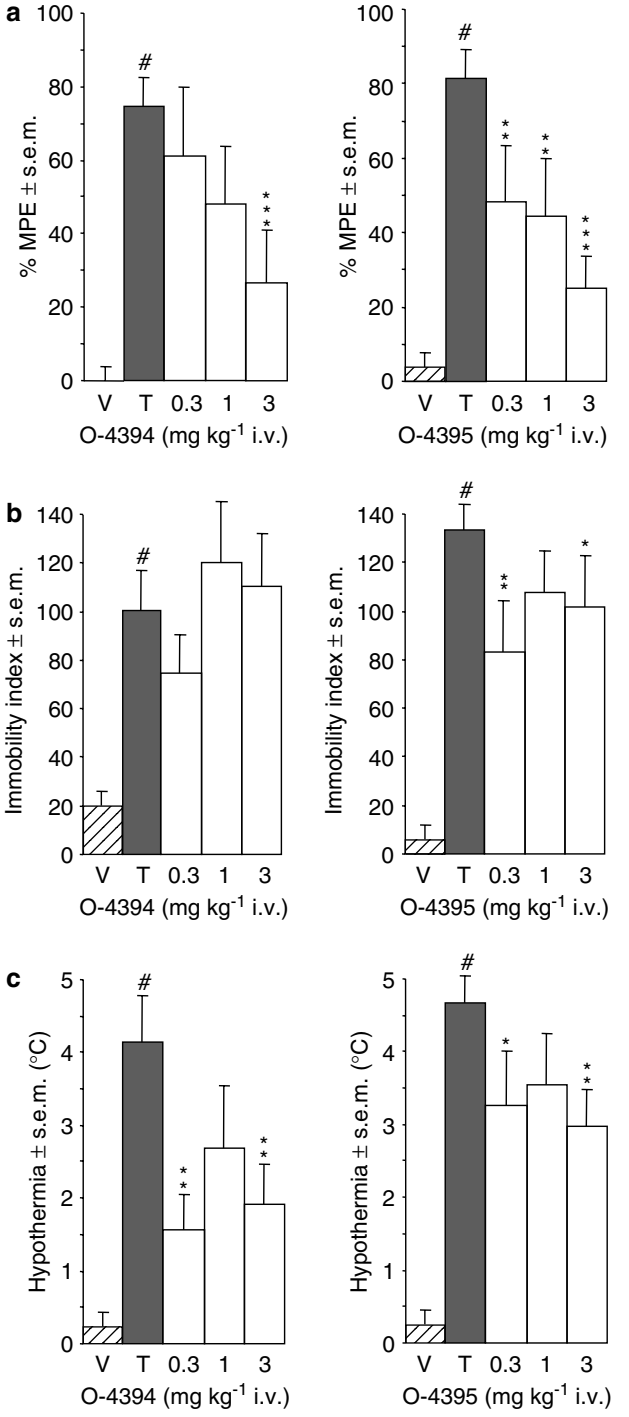


**Figure 4** Effects of O-4394 ($n=6$ or 12; left-hand panels) and O-4395 ($n=12$ or 15; right-hand panels) on the ability of $\Delta^9$-THC, 10 mg kg$^{-1}$ i.v., to induce (**a**) anti-nociception at +20 min, (**b**) ring immobility at +40 min and (**c**) hypothermia at +60 min in mice. Each column represents the mean value and vertical lines show s.e.m. O-4394 and O-4395 were injected i.v. immediately before $\Delta^9$-THC. %MPE is the percentage of maximum possible effect in the tail-flick test. The symbol # denotes a significantly greater response to $\Delta^9$-THC (T) than to its vehicle (V; $P<0.001$; ANOVA followed by Fisher's PLSD test) and asterisks indicate significant differences between responses to $\Delta^9$-THC + vehicle and responses to $\Delta^9$-THC + O-4394 or $\Delta^9$-THC + O-4395 (*$P<0.05$; **$P<0.01$; ***$P<0.001$; ANOVA followed by Fisher's PLSD test).

However, both O-4394 and O-4395 significantly antagonized $\Delta^9$-THC-induced hypothermia at 0.3 and 3 mg kg$^{-1}$, although not at 1 mg kg$^{-1}$ (Figure 4).

When a lower dose of $\Delta^9$-THC was used (3 mg kg$^{-1}$, i.v.), i.v. injected O-4395 significantly attenuated $\Delta^9$-THC-induced anti-nociception at 0.1, 0.3 and 1 mg kg$^{-1}$ but not at 0.03 or 3 mg kg$^{-1}$; $\Delta^9$-THC-induced hypothermia at 0.1 and 0.3 mg kg$^{-1}$ but not at 0.03, 1 or 3 mg kg$^{-1}$; and $\Delta^9$-THC-induced immobility at 0.03 mg kg$^{-1}$ but not at 0.1, 0.3, 1 or 3 mg kg$^{-1}$ ($n=6$ or 10; $P<0.05$; ANOVA followed by Fisher's PLSD test; data not shown).

Effects of injecting O-4394 and O-4395 by themselves are shown in Figure 5. Neither O-4394 nor O-4395 induced a significant degree of anti-nociception at a dose of 3 or 10 mg kg$^{-1}$. However, both compounds were antinociceptive at 30 and 56 mg kg$^{-1}$. As to ring immobility, this was increased significantly by O-4395 at 3 mg kg$^{-1}$, by O-4394 at 10 mg kg$^{-1}$, and by both O-4394 and O-4395 at 30 and 56 mg kg$^{-1}$. O-4395 but not O-4394 also induced a significant degree of hypothermia at 3, 10, 30 and 56 mg kg$^{-1}$. When administered i.p. at a dose of 3 mg kg$^{-1}$, the $CB_1$-selective antagonist, SR141716A, markedly attenuated anti-nociception induced by O-4394 and O-4395 at 56 mg kg$^{-1}$ (Figure 5) and also anti-nociception, hypothermia and ring immobility induced by $\Delta^9$-THC at 3 mg kg$^{-1}$ (Figure 6). However, the same dose of SR141716A did not antagonize hypothermia or ring immobility induced by 56 mg kg$^{-1}$ O-4394 or O-4395 (Figure 5).

## Discussion

The results obtained in this investigation indicate that both O-4394 and O-4395 exhibit pharmacological properties *in vitro* similar to those demonstrated for eTHCV by Thomas *et al*. (2005). More specifically, as shown in Figure 1 and Table 1, each synthetic compound displaced [$^3$H]-CP55940 from specific sites on mouse brain membranes with a $K_i$ value that does not differ significantly from that of the corresponding $K_i$ value of eTHCV determined previously. It was also found that the ability of eTHCV to behave as a competitive surmountable antagonist of *R*-(+)-WIN55212 in the mouse isolated vas deferens and of CP55940 in the [$^{35}$S]GTP$\gamma$S binding assay performed with mouse brain membranes (Thomas *et al*., 2005) was shared by both O-4394 and O-4395. The apparent $K_B$-values of O-4394 and O-4395 for this antagonism of *R*-(+)-WIN55212 and CP55940 do not deviate significantly from the corresponding apparent $K_B$-values of eTHCV determined previously (Table 1). O-4394 and O-4395 also resemble eTHCV in being markedly more potent as antagonists of *R*-(+)-WIN55212 in the vas deferens than of CP55940-induced stimulation of [$^{35}$S]GTP$\gamma$S binding to brain membranes (Table 1). The apparent $K_B$-values of all three compounds for their antagonism of CP55940 in the [$^{35}$S]GTP$\gamma$S binding assay are not significantly different from their $K_i$-values for displacement of [$^3$H]-CP55940 from specific sites on brain membranes, suggesting that they were all antagonizing CP55940 by interacting with this ligand at cannabinoid receptors. These are most likely $CB_1$ receptors. Thus, although CP55940 and

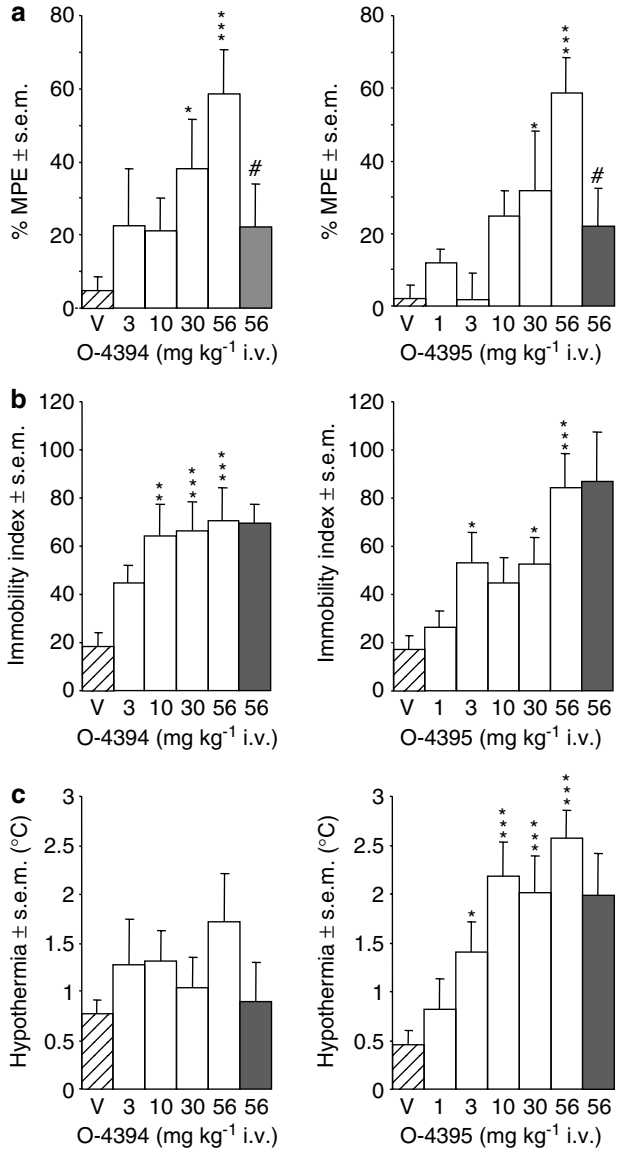


**Figure 5** Effects of i.v. injections of O-4394 ($n=6$–10; left-hand panels) and O-4395 ($n=6$–11; right-hand panels) on (**a**) anti-nociception at +20 min, (**b**) ring immobility at +40 min and (**c**) hypothermia at +60 min in mice. Each column represents the mean value and vertical lines show s.e.m. In some experiments, injection of O-4394 or O-4395 at 56 mg kg$^{-1}$ i.v. was preceded at −10 min by an i.p. injection of 3 mg kg$^{-1}$ SR141716A (solid columns). %MPE is the percentage of maximum possible effect in the tail-flick test. Asterisks denote significant differences between the effects of vehicle (V) and O-4394 or O-4395 (*$P<0.05$; **$P<0.01$; ***$P<0.001$; ANOVA followed by Fisher's PLSD test) and the symbol # denotes significant differences between responses to O-4394 or O-4395 alone at 56 mg kg$^{-1}$ i.v. and responses to this dose of O-4394 or O-4395 following pretreatment with SR141716A ($P<0.05$; ANOVA followed by Fisher's PLSD test). Mean antinociceptive $ED_{50}$ values with 95% confidence limits shown in parentheses were 43 mg kg$^{-1}$ (22 and 83 mg kg$^{-1}$) for O-4394 and 44 mg kg$^{-1}$ (23 and 82 mg kg$^{-1}$) for O-4395.

eTHCV each binds as readily to the $CB_1$ as to the $CB_2$ receptor (Howlett *et al*., 2002; Thomas *et al*., 2005), and both these cannabinoid receptor subtypes are present in the brain (Van Sickle *et al*., 2005), brain tissue is much more densely populated with the $CB_1$ subtype.

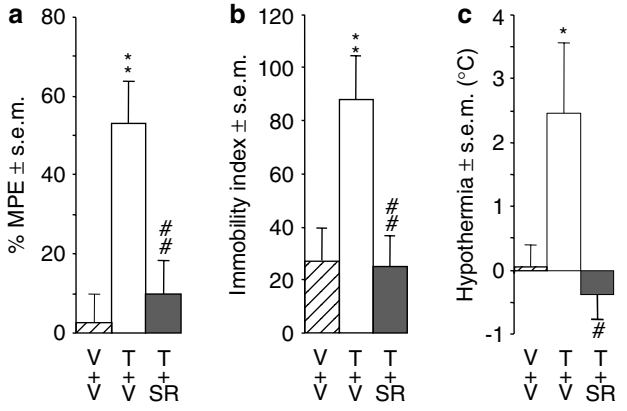


**Figure 6** Effects of i.v. injections of $\Delta^9$-THC (T) at 3 mg kg$^{-1}$ ($n=5$ or 6) on (**a**) anti-nociception at +20 min, (**b**) ring immobility at +40 min and (**c**) hypothermia at +60 min in mice. Each column represents the mean value and vertical lines show s.e.m. Injection of $\Delta^9$-THC was preceded at −10 min by an i.p. injection of either the vehicle (V) or 3 mg kg$^{-1}$ SR141716A (SR). %MPE is the percentage of maximum possible effect in the tail-flick test. Asterisks denote significant differences between the effects of vehicle and $\Delta^9$-THC (*$P<0.05$; **$P<0.01$; ANOVA followed by Fisher's PLSD test) and the symbol # denotes significant differences between effects of $\Delta^9$-THC following pretreatment with vehicle and its effects following pretreatment with SR141716A ($^{\#}P<0.05$; $^{\#\#}P<0.01$; ANOVA followed by Fisher's PLSD test).

O-4394 and O-4395 also behaved as cannabinoid receptor antagonists *in vivo*. Thus, at i.v. doses of 3 mg kg$^{-1}$ or less, both these compounds attenuated $\Delta^9$-THC-induced anti-nociception and hypothermia and O-4395 also attenuated the ability of $\Delta^9$-THC to induce immobility in the ring test. It is likely that O-4394 and O-4395 produced their antagonism of $\Delta^9$-THC by interacting with this compound at cannabinoid $CB_1$ receptors. Thus first, antagonism of these responses to $\Delta^9$-THC can also readily be induced by the $CB_1$-selective antagonist, SR141716A (Compton *et al*., 1996; Adams *et al*., 1998; see also Figure 6), and, second, as already discussed, the results we obtained in brain membrane experiments with CP55940 suggest that both O-4394 and O-4395 are $CB_1$ receptor antagonists. Moreover, previous experiments with mice have shown that the ability of synthetic cannabinoid receptor agonists to induce anti-nociception in the tail-flick test and hypothermia is not opposed by the $CB_2$-selective antagonist, SR144528 (Wiley *et al*., 2002). There are also studies showing that $\Delta^9$-THC fails to induce ring immobility and/or hypothermia in two lines of $CB_1$ knockout mice (Ledent *et al*., 1999; Zimmer *et al*., 1999), although interestingly, in one of these transgenic strains, $\Delta^9$-THC did retain the ability to display antinociceptive activity in the tail-flick test (Zimmer *et al*., 1999).

The dose range within which O-4394 or O-4395 was found to antagonize $\Delta^9$-THC *in vivo* (0.03–3 mg kg$^{-1}$; 0.1–10.5 $\mu$mol kg$^{-1}$) indicates that the potency with which these compounds induce such antagonism is of the same order as the potency exhibited by $\Delta^9$-THC as a $CB_1$ receptor agonist when this is assessed by its ability to induce anti-nociception, ring immobility or hypothermia in mice. Thus, when injected i.v., $\Delta^9$-THC has been shown to exhibit agonist activity in these assays with $ED_{50}$ values of 1.4 or 1.5 mg kg$^{-1}$

(4.5 or 4.8 $\mu$mol kg$^{-1}$; Compton *et al.*, 1992). These data lend further support to the hypothesis that O-4394 and O-4395 produced their antagonism of $\Delta^9$-THC *in vivo* by interacting with $\Delta^9$-THC at $CB_1$ cannabinoid receptors, as both O-4394 and O-4395 (Table 1) exhibit a similar potency to that of $\Delta^9$-THC ($K_i = 40.7$ nM; Compton *et al.*, 1993) as displacers of [$^3$H]-CP55940 from specific binding sites on rodent brain membranes.

When administered alone, albeit only at doses above those at which they attenuated the ability of $\Delta^9$-THC to induce anti-nociception and hypothermia in mice, both O-4394 and O-4395 elicited antinociceptive responses, and O-4395 but not O-4394 induced hypothermia. O-4395 also antagonized $\Delta^9$-THC in the ring test with greater potency than it induced ring immobility when injected by itself. Although in most of the bioassays used in this investigation O-4394 and O-4395 exhibited less potency *in vivo* as agonists than as antagonists of $\Delta^9$-THC, the highest dose at which O-4395 antagonized $\Delta^9$-THC-induced anti-nociception and hypothermia (3 mg kg$^{-1}$ i.v.) did produce a significant degree of hypothermia and ring immobility. It is likely that the antinociceptive effects of O-4394 and O-4395 were $CB_1$ receptor-mediated, as they were attenuated by SR141716A when this was administered i.p. at 3 mg kg$^{-1}$, a dose that additionally antagonized not only anti-nociception but also ring immobility and hypothermia induced in mice by $\Delta^9$-THC (Figure 6). In contrast, the ring immobility induced by O-4394 and O-4395 and the hypothermia induced by O-4395 were not significantly attenuated by SR141716A at 3 mg kg$^{-1}$ i.p. This may have been because this dose of SR141716A, an established surmountable $CB_1$ receptor antagonist, was insufficient to attenuate responses to what appears to be a supramaximal dose, at least of O-4394 in the ring test and of O-4395 for its production of hypothermia (Figure 5). Another possible explanation is that O-4394 and O-4395 did not induce their effects on ring immobility and core temperature by activating $CB_1$ receptors. It is noteworthy, therefore, that experiments with the mouse isolated vas deferens have already provided evidence that e$\Delta^9$-THCV can produce effects that are not mediated by $CB_1$ receptors. These experiments showed that, at concentrations above those at which it antagonized *R*-(+)-WIN55212, anandamide, CP55940 or $\Delta^9$-THC, e$\Delta^9$-THCV inhibited electrically evoked contractions of this tissue in an SR141716A-independent manner and reduced contractile responses to both phenylephrine and $\beta,\gamma$-methylene-ATP (Thomas *et al.*, 2005). If, on the one hand, O-4394 and O-4395 are $CB_1$ receptor antagonists and, on the other hand, they can induce ring immobility through a $CB_1$ receptor-independent mechanism, this could explain why the ability of a low dose of O-4395 to attenuate ring immobility induced by 3 mg kg$^{-1}$ $\Delta^9$-THC disappeared when higher doses of O-4395 were administered. Also, this could be the reason why none of the doses of O-4394 used in our experiments produced any detectable attenuation of the ring immobility induced by 10 mg kg$^{-1}$ $\Delta^9$-THC.

It is currently unclear why O-4394 and O-4395 behave as $CB_1$ receptor antagonists in the tail-flick assay at doses of 3 mg kg$^{-1}$ or less, and as $CB_1$ receptor agonists in this assay at doses above 10 mg kg$^{-1}$. One possibility is that O-4394 and/or O-4395 lack significant efficacy as cannabinoid receptor agonists, but can be metabolized *in vivo* to compounds that are capable of inducing signs of anti-nociception in this assay when they are present at sufficiently high concentrations. Given the structural similarities between THC and THCV, this hypothesis is supported by evidence that $\Delta^8$-THC and $\Delta^9$-THC exhibit less potency than their 11-hydroxy metabolites as antinociceptive agents in the mouse hot plate test (Wilson and May, 1975), and that $\Delta^8$-THC and $\Delta^9$-THC are also less potent than 11-hydroxy-$\Delta^8$-THC and 11-hydroxy-$\Delta^9$-THC, respectively, as inducers of tachycardia and conjunctival reddening in human subjects (Hollister, 1974). There is already evidence that O-4394 and O-4395 are metabolized to 11-hydroxy metabolites (Brown and Harvey, 1988). Clearly, however, further experiments are required to establish firstly, whether unmetabolized O-4394 and O-4395 lack significant antinociceptive activity at the doses used in this investigation, and second, whether the 11-hydroxy metabolites of these cannabinoids induce signs of anti-nociception in the tail-flick test. In the meantime, it is worth noting that the structural similarity that exists both between $\Delta^9$-THC and O-4394 and between $\Delta^8$-THC and O-4395 makes it very likely that the 11-hydroxy metabolites of O-4394 and O-4395 exhibit greater antinociceptive activity than their parent compounds. For O-4394, there is further evidence that it lacks significant activity as a $CB_1$ receptor agonist. Thus, O-4394 was found not to share the ability of CP55940 to stimulate [$^{35}$S]GTP$\gamma$S binding to brain membranes when applied at concentrations of up to 10 $\mu$M. In contrast, O-4395 did induce a small but significant stimulation of [$^{35}$S]GTP$\gamma$S binding to brain membranes at some concentrations. It is noteworthy, however, that the maximal degree of stimulation of [$^{35}$S]GTP$\gamma$S-binding produced by O-4395 was markedly less than that induced by CP55940, an indication that O-4395 may be a low-efficacy partial agonist for the $CB_1$ receptor. In view of our finding that O-4395 exhibits agonist activity over quite a narrow concentration range, it remains possible that O-4394 would also exhibit such activity if administered at concentrations other than those used in this investigation. That O-4394 should resemble O-4395 in this way would not be unexpected, given the marked similarity that exists between the pharmacology of $\Delta^8$- and $\Delta^9$-THC, the pentyl analogues of O-4395 and O-4394, respectively (reviewed in Howlett *et al.*, 2002).

In conclusion, this investigation has provided evidence that O-4394 and O-4395 exhibit similar pharmacological properties to e$\Delta^9$-THCV *in vitro* and that they can antagonize the $CB_1$/$CB_2$ receptor agonist, $\Delta^9$-THC, *in vivo*. Our results showing that O-4394 and O-4395 behave *in vivo* as antagonists in one dose range, but as agonists in another are in line with previous findings; for example, Sulcova *et al.* (1998) and Sañudo-Peña *et al.* (2000) demonstrated that some cannabinoid receptor agonists exhibit biphasic or even triphasic *in vivo* effects in a dose-dependent manner (reviewed in Pertwee, 1985; Dewey, 1986). As e$\Delta^9$-THCV exhibits unexpectedly high potency as an antagonist of anandamide and *R*-(+)-WIN55212 in the mouse vas deferens (Thomas *et al.*, 2005), it will be of interest to establish in future experiments whether there are any responses to exogenously administered anandamide or *R*-(+)-WIN55212,

or to endogenously released anandamide that are particularly sensitive to antagonism by O-4394 or O-4395, or indeed by e$\Delta^9$-THCV, when it is administered *in vivo*. The ability of O-4394 and O-4395 to antagonize $CB_1$-mediated *in vivo* effects of $\Delta^9$-THC other than anti-nociception, ring immobility or hypothermia, for example $\Delta^9$-THC-induced inhibition of intestinal motility (reviewed in Pertwee, 2001), also merits future investigation.

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## Conflict of interest

The authors state no conflict of interest.

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