



# Structural determinants of the partial agonist-inverse agonist properties of 6'-azidohept-2'-yne- $\Delta^8$ -tetrahydrocannabinol at cannabinoid receptors

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**1** We have extended previous investigations of four analogues of  $\Delta^8$ -tetrahydrocannabinol ( $\Delta^8$ -THC): 6'-azidohept-2'-yne- $\Delta^8$ -THC (O-1184), 6'-azidohept-*cis*-2'-ene- $\Delta^8$ -THC (O-1238) and octyl-2'-yne- $\Delta^8$ -THC (O-584) and its 1-deoxy-analogue (O-1315).

**2** O-1184, O-1238 and O-584 displaced [<sup>3</sup>H]-CP55940 from specific binding sites on Chinese hamster ovary (CHO) cell membranes expressing CB<sub>1</sub> or CB<sub>2</sub> cannabinoid receptors, with *pK<sub>i</sub>* values of 8.28 to 8.45 (CB<sub>1</sub>) and 8.03 to 8.13 (CB<sub>2</sub>). The *pK<sub>i</sub>* values of O-1315 were significantly less, 7.63 (CB<sub>1</sub>) and 7.01 (CB<sub>2</sub>).

**3** All the analogues inhibited forskolin-stimulated cyclic AMP production by CB<sub>1</sub>-transfected CHO cells (*pEC*<sub>50</sub> = 9.16 to 9.72). Only O-1238 behaved as a full agonist in this cell line.

**4** In mouse vasa deferentia, O-1238 inhibited electrically-evoked contractions (*pEC*<sub>50</sub> = 10.18 and *E*<sub>max</sub> = 70.5%). Corresponding values for O-1184 were 9.08 and 21.1% respectively. At 1 nM, O-1184 produced surmountable antagonism of the cannabinoid receptor agonist, CP55940. However, at 0.1 nM, O-1184 did not attenuate CP55940-induced inhibition of cyclic AMP production by CB<sub>1</sub>-transfected CHO cells.

**5** In CB<sub>2</sub>-transfected CHO cells, cyclic AMP production was inhibited by CP55940 (*pEC*<sub>50</sub> = 8.59), enhanced by O-1184 and O-584 (*pEC*<sub>50</sub> = 8.20 and 6.86 respectively) and not significantly affected by O-1238 or O-1315.

**6** At 100 nM, O-1184 and O-1238 produced surmountable antagonism of CP55940 in CB<sub>2</sub> cells, decreasing the *pEC*<sub>50</sub> of CP55940 from 8.61 to 7.42 (O-1184) or from 8.54 to 7.44 (O-1238).

**7** These data support the hypothesis that increasing the degree of unsaturation of the aliphatic side-chain of  $\Delta^8$ -THC analogues has little effect on CB<sub>1</sub> or CB<sub>2</sub> receptor affinity but can reduce CB<sub>1</sub> receptor efficacy and reverse the direction of responses elicited at CB<sub>2</sub> receptors.

**Keywords:** Cannabinoids; cannabinoid CB<sub>1</sub> receptors; cannabinoid CB<sub>2</sub> receptors; inverse agonism; 6'-azidohept-2'-yne- $\Delta^8$ -tetrahydrocannabinol; O-1184; mouse vas deferens

**Abbreviations:** AM630, 6-iodopravadoline; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CP55940, (–)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; IBMX, 3-isobutyl-1-methylxanthine; O-584, octyl-2'-yne- $\Delta^8$ -tetrahydrocannabinol; O-1184, 6'-azidohept-2'-yne- $\Delta^8$ -tetrahydrocannabinol; O-1238, 6'-azidohept-*cis*-2'-ene- $\Delta^8$ -tetrahydrocannabinol; O-1315, 1-deoxy-octyl-2'-yne- $\Delta^8$ -tetrahydrocannabinol; SR144528, N-[(1*S*)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide

## Introduction

In previous experiments we have shown that the synthetic cannabinoid, 6'-azidohept-2'-yne- $\Delta^8$ -tetrahydrocannabinol (O-1184, Figure 1) behaves as a potent and selective cannabinoid receptor antagonist in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine and that it readily binds to cannabinoid receptors both on membranes obtained from this tissue and on guinea-pig forebrain membranes (Ross *et al.*, 1998). More recently, results from binding experiments with rat cerebellar membranes using [<sup>35</sup>S]-GTP $\gamma$ S or the radiolabelled cannabinoid, [<sup>3</sup>H]-CP55940, led Griffin *et al.* (1999) to conclude that O-1184 is a low-efficacy agonist at CB<sub>1</sub> receptors. Their data also suggest that replacement of the carbon-carbon triple bond in the aliphatic side-chain of O-1184 with a *cis* double bond to form 6'-azidohept-*cis*-2'-ene- $\Delta^8$ -tetrahydrocannabinol (O-1238; Figure

1) enhances CB<sub>1</sub> efficacy, whilst replacement of N<sub>3</sub> on the terminal carbon of this side-chain with C<sub>2</sub>H<sub>5</sub> to form octyl-2'-yne- $\Delta^8$ -tetrahydrocannabinol (O-584; Figure 1) has little effect either on CB<sub>1</sub> efficacy or affinity.

The present investigation was directed at characterizing the pharmacological properties of O-1184, O-1238 and O-584 more completely using Chinese hamster ovary (CHO) cells expressing human CB<sub>1</sub> or CB<sub>2</sub> receptors. This was achieved by determining the affinities of these compounds for CB<sub>1</sub> and CB<sub>2</sub> receptors and by addressing the question of whether they share the ability of the established CB<sub>1</sub>-CB<sub>2</sub> cannabinoid receptor agonist, CP55940, to inhibit forskolin-stimulated cyclic AMP production by CB<sub>1</sub> or CB<sub>2</sub> transfected cells (Pertwee, 1997). Additional experiments were performed to establish whether O-1184 or O-1238 oppose the ability of CP55940 to inhibit forskolin-stimulated cyclic AMP production in these cell lines. As there are reports that the CB<sub>2</sub> receptor affinity of certain analogues of  $\Delta^8$ -tetrahydrocannabinol can be selectively

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enhanced by removing the 1-hydroxyl group (Huffman *et al.*, 1996; 1998), it was also of interest to compare O-584 with its 1-deoxy-analogue, 1-deoxy-octyl-2'-yne- $\Delta^8$ -tetrahydrocannabinol (O-1315; Figure 1). Finally, in view of the evidence that O-1184 is a partial agonist (see above), experiments were carried out to establish whether its ability to exhibit cannabinoid receptor antagonist properties in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine (Ross *et al.*, 1998) extends to the mouse isolated vas deferens, another cannabinoid receptor-containing preparation (Griffin *et al.*, 1997; Pertwee, 1997).

## Methods

### Mouse vas deferens

Vasa deferentia were obtained from albino MF1 mice weighing 35–52 g. Each tissue was mounted in a 4 ml organ bath at an initial tension of 0.5 g. The baths contained Krebs solution which was kept at 37°C and bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The composition of the Krebs solution was (mM): NaCl 118.2; KCl 4.75; KH<sub>2</sub>PO<sub>4</sub> 1.19; NaHCO<sub>3</sub> 25.0; glucose 11.0

and CaCl<sub>2</sub>·6H<sub>2</sub>O 2.54. Isometric contractions were evoked by stimulation with 0.5 s trains of three pulses of 110% maximal voltage (train frequency 0.1 Hz; pulse duration 0.5 ms) through a platinum electrode attached to the upper end and a stainless steel electrode attached to the lower end of each bath. Stimuli were generated by a Grass S48 stimulator, then amplified (Med-Lab channel attenuator) and divided to yield separate outputs to four organ baths (Med-Lab StimuSplitter). Contractions were monitored by computer (Apple Macintosh LCIII and Performa 475) using a data recording and analysis system (MacLab) that was linked *via* preamplifiers (Macbridge) to Dynamometer UF1 transducers (Pioden Controls). Each tissue was subjected to several 5-min periods of stimulation, the first of these beginning after the tissue had equilibrated but before drug administration. The stimulator was switched off for 25 min between each 5-min stimulation period. Concentration-response curves for O-1184 or O-1238 were constructed cumulatively by adding one or other drug immediately after each 5-min stimulation period. In some experiments, concentration-response curves for CP55940 were constructed in tissues that had been pretreated with O-1184 or O-1238. In these experiments, O-1184, O-1238 or vehicle were administered after the first 5-min stimulation period and CP55940 after the second and all subsequent stimulation periods. For addition to organ baths, each cannabinoid was mixed with two parts of Tween 80 by weight and dispersed in a 0.9% aqueous solution of NaCl (saline) as described previously for  $\Delta^9$ -tetrahydrocannabinol (Pertwee *et al.*, 1992). Drug additions were made in a volume of 10  $\mu$ l. In control experiments, Tween 80 was added instead of a cannabinoid. The control dose of Tween 80 was the same as the dose added in combination with the highest cannabinoid dose used.

### CHO cells

These were stably transfected with cDNA encoding human CB<sub>1</sub> (B<sub>max</sub>=3.41 pmol mg<sup>-1</sup> protein) or CB<sub>2</sub> receptors (B<sub>max</sub>=31.4 pmol mg<sup>-1</sup> protein). They were provided by Drs G. Disney and A. Green, GlaxoWellcome R & D, Medicines Research Centre, Stevenage, U.K. and were the same clones as those used in the sPAP reporter assay described by Green *et al.* (1999). Untransfected CHO cells were obtained from the Centre for Applied Microbiology and Research, Porton Down, U.K. Cells were maintained at 37°C and 5% CO<sub>2</sub> in DMEM/f-12 HAM with 2 mM glutamine, geneticin (600  $\mu$ g ml<sup>-1</sup>) and hygromycin (300  $\mu$ g ml<sup>-1</sup>). For binding experiments, CHO cells were removed from flasks by scraping and pellets frozen at -80°C for up to 1 month. Once defrosted the cell membranes were diluted to 1 mg ml<sup>-1</sup> in 50 mM Tris buffer (pH 7.4) and resuspended using a hand-held homogenizer.

### Binding experiments

A modification of the filtration procedure described by Compton *et al.* (1993) was used. Assays were performed with [<sup>3</sup>H]-CP55940, 1 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mg ml<sup>-1</sup> bovine serum albumin (BSA) and 50 mM Tris buffer, total assay volume 500  $\mu$ l. Binding was initiated by the addition of CB<sub>1</sub> (100  $\mu$ g protein) or CB<sub>2</sub> cell membranes (50  $\mu$ g protein). Assays were carried out at 37°C for 30 min before termination by addition of ice-cold wash buffer (50 mM Tris buffer, 1 mg ml<sup>-1</sup> BSA) and vacuum filtration using a 12-well sampling manifold (Brandel Cell Harvester) and Whatman GF/B glass-fibre filters that had been soaked in wash buffer at 4°C for 24 h. Each reaction tube was washed three times with a 4 ml aliquot of buffer. The filters were oven-dried for 60 min

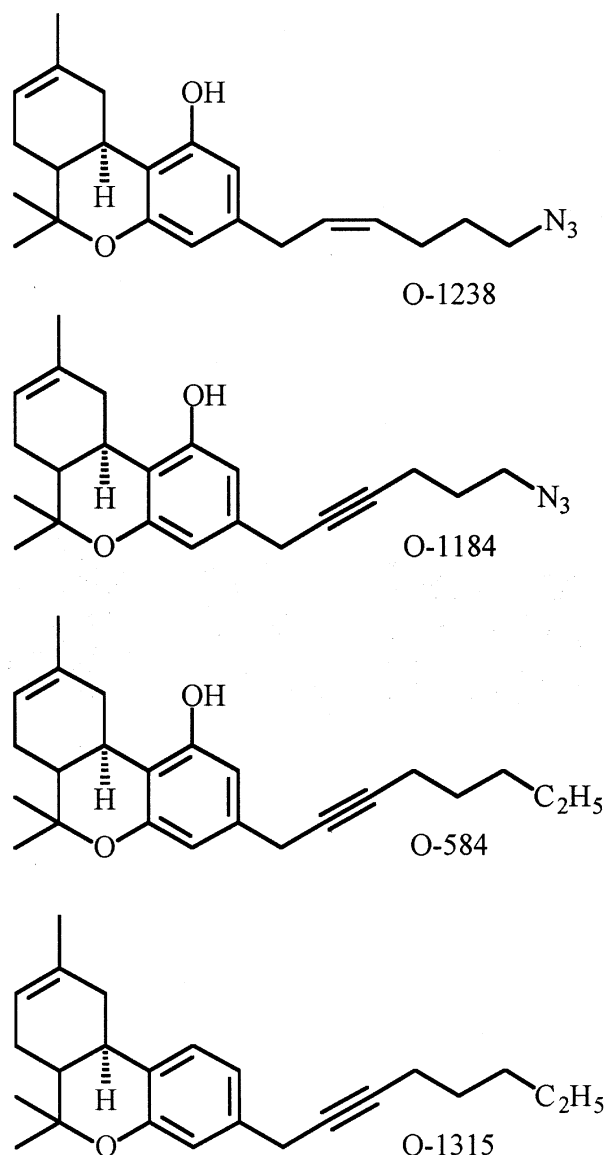


Figure 1 Structures of O-1238, O-1184, O-584 and O-1315.

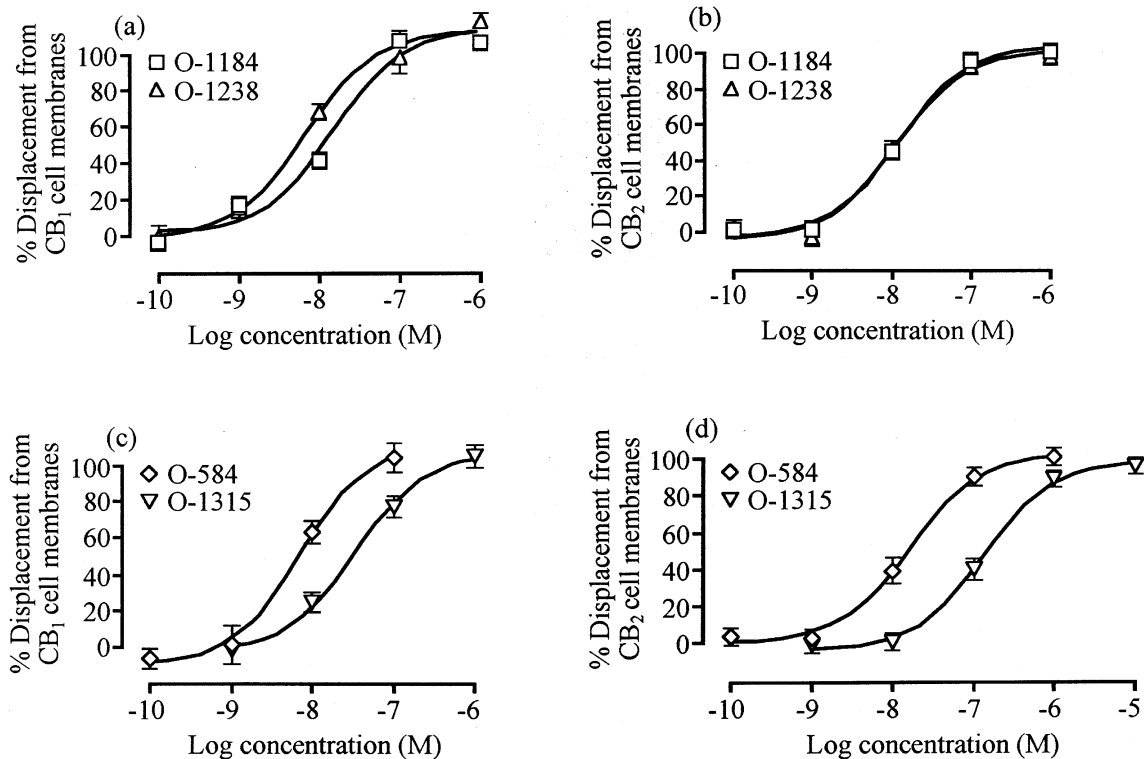
and then placed in 5 ml of scintillation fluid (Ultima Gold XR, Packard). Radioactivity was quantified by liquid scintillation spectrometry. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1  $\mu\text{M}$  unlabelled CP55940. Protein assays were performed using a Bio-Rad Dc kit. Unlabelled cannabinoids were each added in a volume of 50  $\mu\text{l}$  after serial dilution from a 1  $\text{mg ml}^{-1}$  ethanolic stock solution using assay buffer (50 mM

Tris buffer containing 5  $\text{mg ml}^{-1}$  BSA). [ $^3\text{H}$ ]-CP55940 was also added in a 50  $\mu\text{l}$  volume following dilution in assay buffer. The concentration of [ $^3\text{H}$ ]-CP55940 used in displacement assays was 0.5 nM. The concentrations of cannabinoids that produced a 50% displacement of radioligand from specific binding sites ( $\text{IC}_{50}$  values) were calculated using GraphPad Prism (GraphPad Software, San Diego, U.S.A.) Dissociation constant ( $K_i$ ) values were calculated using the equation of Cheng & Prusoff

**Table 1** Effects on forskolin-stimulated cyclic AMP production and on [ $^3\text{H}$ ]-CP55940 binding in experiments with  $\text{CB}_1$ - and  $\text{CB}_2$ -transfected CHO cells or CHO cell membranes

Cell line and direction of response	Cannabinoid	$p\text{EC}_{50}$	$E_{\text{max}}$ (%)	$pK_i$
$\text{CB}_1$ (-)	CP55940	$9.27 \pm 0.17$ (6)	$98.2 \pm 8.7$	$8.45 \pm 0.10$ (4) <sup>a</sup>
	O-1238	$9.72 \pm 0.13$ (6)	$87.3 \pm 4.2$ <sup>b</sup>	$8.45 \pm 0.13$ (3) <sup>a</sup>
	O-1184	$9.51 \pm 0.20$ (6)	$64.7 \pm 3.9$ <sup>c</sup>	$8.28 \pm 0.07$ (5) <sup>a</sup>
	O-584	$9.42 \pm 0.21$ (5)	$67.8 \pm 4.7$ <sup>c</sup>	$8.37 \pm 0.12$ (5) <sup>a</sup>
	O-1315	$9.16 \pm 0.18$ (5)	$67.4 \pm 4.0$ <sup>c</sup>	$7.63 \pm 0.08$ (5)
$\text{CB}_2$ (-)	CP55940	$8.59 \pm 0.13$ (4)	$101.9 \pm 5.2$	$8.17 \pm 0.06$ (4) <sup>d</sup>
	O-1238	— (4) <sup>f</sup>	—	$8.11 \pm 0.03$ (3) <sup>d</sup>
$\text{CB}_2$ (+)	O-1184	$8.20 \pm 0.38$ (4)	$161.0 \pm 9.7$	$8.13 \pm 0.01$ (3) <sup>d</sup>
	O-584	$6.86 \pm 0.44$ (5)	$246.0 \pm 38.2$	$8.03 \pm 0.07$ (3) <sup>d</sup>
	O-1315	— (5) <sup>f</sup>	—	$7.01 \pm 0.09$ (3) <sup>e</sup>

$pK_i$  values for displacement of [ $^3\text{H}$ ]-CP55940 from membranes of  $\text{CB}_1$  and  $\text{CB}_2$  transfected CHO cells were calculated by the Cheng-Prusoff equation. Forskolin-stimulated cyclic AMP production in the absence of cannabinoids has been normalized to 100%.  $\text{EC}_{50}$  is the concentration required to produce half-maximal inhibition or enhancement of forskolin-stimulated cyclic AMP production and  $E_{\text{max}}$  is the maximal percentage inhibition (-) or enhancement (+) produced. Number of replicates for each experiment is shown in brackets. <sup>a</sup>Greater than the  $\text{CB}_1$   $pK_i$  of O-1315 ( $P < 0.001$ ). <sup>b</sup>Greater than the  $\text{CB}_1$   $E_{\text{max}}$  value of O-1184 ( $P < 0.05$ ). <sup>c</sup>Less than the  $\text{CB}_1$   $E_{\text{max}}$  value of CP55940 ( $P < 0.01$ ). <sup>d</sup>Greater than the  $\text{CB}_2$   $pK_i$  of O-1315 ( $P < 0.001$ ). <sup>e</sup>Less than the  $\text{CB}_1$   $pK_i$  of O-1315 ( $P < 0.01$ ). <sup>f</sup>O-1238 and O-1315 did not alter cyclic AMP production significantly in  $\text{CB}_2$  cells (Figure 3). There is no significant difference between any of the  $\text{CB}_1$   $p\text{EC}_{50}$  values (analysis of variance followed by the Newman-Keuls test) or between the  $\text{CB}_2$   $p\text{EC}_{50}$  values of O-1184 and O-584 (Students' unpaired test). Other comparisons in Table 1 have also been made using Student's unpaired test<sup>f</sup> or analysis of variance followed by the Newman-Keuls test<sup>b-e</sup>.



**Figure 2** Displacement of bound [ $^3\text{H}$ ]-CP55940 (0.5 nM) by O-1184 and O-1238, (a) in  $\text{CB}_1$ - and (b) in  $\text{CB}_2$ -transfected CHO cells and by O-584 and O-1315, (c) in  $\text{CB}_1$ - and (d) in  $\text{CB}_2$ -transfected CHO cells. Each symbol represents the mean per cent displacement  $\pm$  s.e.mean ( $n=3$  or 5).

(1973) and  $K_D$  values for [ $^3$ H]-CP55940 of 1.62 (CB<sub>1</sub>) or 0.99 nM (CB<sub>2</sub>). These  $K_D$  values were obtained in saturation binding assays using membranes from CB<sub>1</sub> or CB<sub>2</sub>-transfected CHO cells as described previously (Ross *et al.*, 1999).

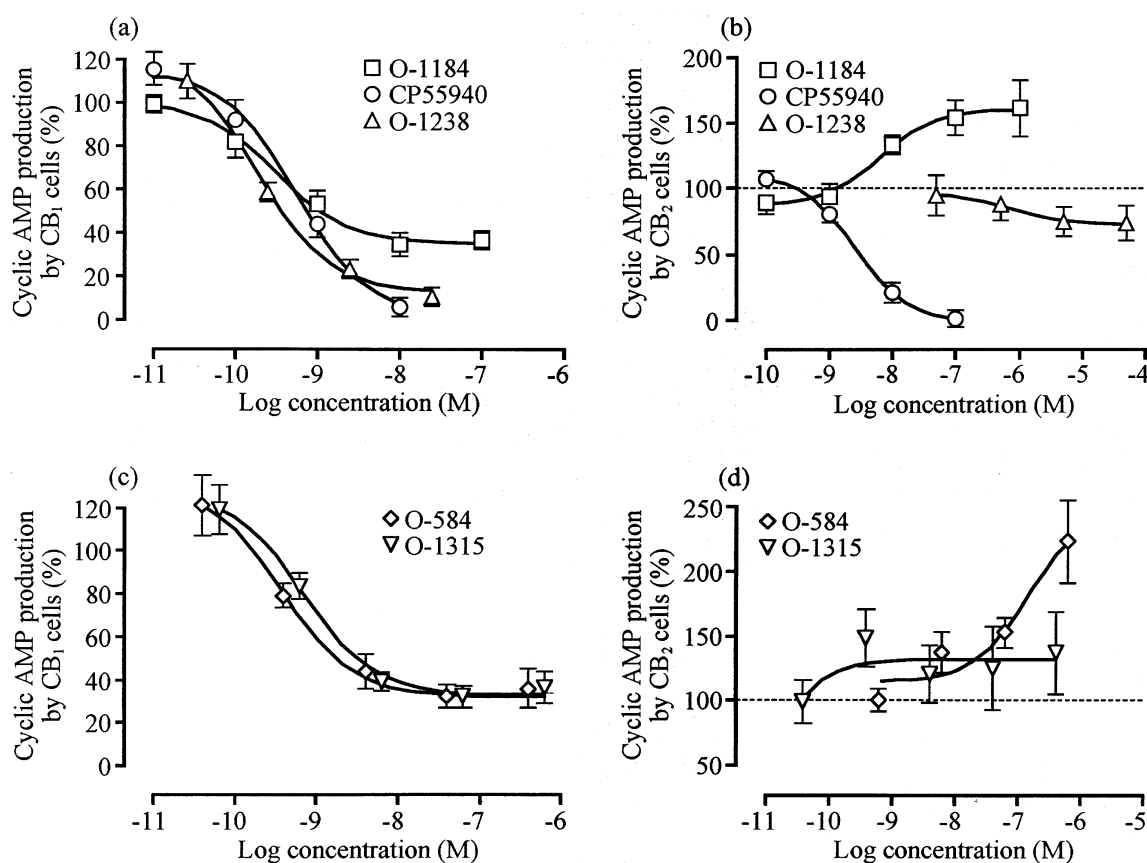
### Cyclic AMP assay

Cells ( $4-6 \times 10^5$  cells ml<sup>-1</sup>) were preincubated for 20 min at 37°C with cannabinoid agonist and 3-isobutyl-1-methylxanthine (IBMX; 50  $\mu$ M) in phosphate buffered saline containing 1 mg ml<sup>-1</sup> BSA (assay buffer) followed by a further 20 min incubation with 2  $\mu$ M forskolin in a total volume of 500  $\mu$ l. For antagonist studies, cells were preincubated for 10 min with the antagonist before agonist incubation. The reaction was terminated by addition of 0.1 M HCl and centrifugation performed to remove cell debris. The pH was brought to 8-9 using 1 M NaOH and cyclic AMP content was then measured using a radioimmunoassay kit (Biotrak, Amersham, U.K.). Cannabinoids were dissolved in ethanol as 1 mg ml<sup>-1</sup> stock solutions and diluted in assay buffer. Forskolin and IBMX were dissolved in DMSO. Cyclic AMP concentrations in CB<sub>1</sub>-transfected cells were  $21.80 \pm 6.50$  pmol ml<sup>-1</sup> in the presence of 2  $\mu$ M forskolin and  $2.83 \pm 1.01$  pmol ml<sup>-1</sup> in the absence of forskolin ( $n=11$ ). Corresponding values in CB<sub>2</sub>-transfected cells were  $18.24 \pm 5.81$  and  $3.00 \pm 1.27$  pmol ml<sup>-1</sup> respectively ( $n=11$ ). In some experiments, CB<sub>2</sub>-transfected cells were incubated with 100 ng ml<sup>-1</sup> pertussis toxin for 18 h prior to

cyclic AMP measurement in order to uncouple the CB<sub>2</sub> receptors from G<sub>i/o</sub> proteins (Pertwee, 1997).

### Analysis of data

Values have been expressed as means and variability as s.e.mean or as 95% confidence limits. Mean values have been compared using Student's unpaired *t*-test or analysis of variance followed by Dunnett's test or by the Newman-Keuls test. A *P* value <0.05 was considered to be significant. Effects of test compounds on forskolin-stimulated cyclic AMP production have been expressed in percentage terms. This was calculated from the equation  $[100 \times (f' - b)] / (f - b)$  where  $f'$ ,  $f$  and  $b$  are values of cyclic AMP production (pmol ml<sup>-1</sup>),  $f$  in the presence of forskolin and the test compound,  $f$  in the presence of forskolin only and  $b$  in the absence of both forskolin and the test compound (Ross *et al.*, 1999). Values for EC<sub>50</sub>, IC<sub>50</sub> and maximal effects ( $E_{max}$ ) and the s.e.mean or 95% confidence limits of these values have been calculated by non-linear regression analysis using the equation for a sigmoidal concentration-response curve (GraphPad Prism). In organ bath experiments, the degree of drug-induced inhibition of evoked contractions has been expressed in percentage terms. This was 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.



**Figure 3** Changes of forskolin-stimulated cyclic AMP production induced by CP55940, O-1184 and O-1238, (a) in CB<sub>1</sub>- and (b) in CB<sub>2</sub>-transfected CHO cells and by O-584 and O-1315, (c) in CB<sub>1</sub>- and (d) in CB<sub>2</sub>-transfected CHO cells. Each symbol represents mean percentage change in forskolin-stimulated cyclic AMP production  $\pm$  s.e.mean. Forskolin-stimulated cyclic AMP production in the absence of any cannabinoid has been normalized to 100%. See Table 1 for further details. None of the concentrations of O-1238 or O-1315 used caused cyclic AMP production by CB<sub>2</sub>-transfected CHO cells to deviate significantly from 100% ( $P > 0.05$ ; one-sample *t*-test).

The  $K_B$  value of O-1184 for antagonism of CP55940 in the *vas deferens* was calculated by substituting a single concentration ratio value into the equation  $(x-1) = B/K_B$ , where  $x$  (the 'concentration ratio') is the concentration of CP55940 that produced a particular size of effect in the presence of O-1184 at a concentration,  $B$ , divided by the concentration of CP55940 that produced an identical effect in the absence of O-1184 (Tallarida *et al.*, 1979). Values of the concentration ratio and its 95% confidence limits were determined by symmetrical (2+2) dose parallel line assays (Colquhoun, 1971), using responses to pairs of CP55940 concentrations located on the steepest part of each log concentration-response curve. This method was also used to establish whether the log concentration-response curves of CP55940 in the presence and absence of O-1184 deviated significantly from parallelism.

### Drugs

CP55940  $\{(-)-3-[2\text{-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3\text{-hydroxypropyl)cyclohexan-1-ol}]\}$  was supplied by Pfizer, SR144528  $\{N-[1S\text{-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl}]-5-(4\text{-chloro-3-methylphenyl})-1-(4\text{-methylbenzyl})\text{-pyrazole-3-carboxamide}\}$  by Sanofi Recherche and pertussis toxin by Sigma. O-1184, O-1238, O-1315 and O-584 (Figure 1) were synthesized in the laboratory of Dr Razdan.  $[^3\text{H}]\text{-CP55940}$  ( $126\text{ Ci mmol}^{-1}$ ) was obtained from NEN Life Science Products.

### Results

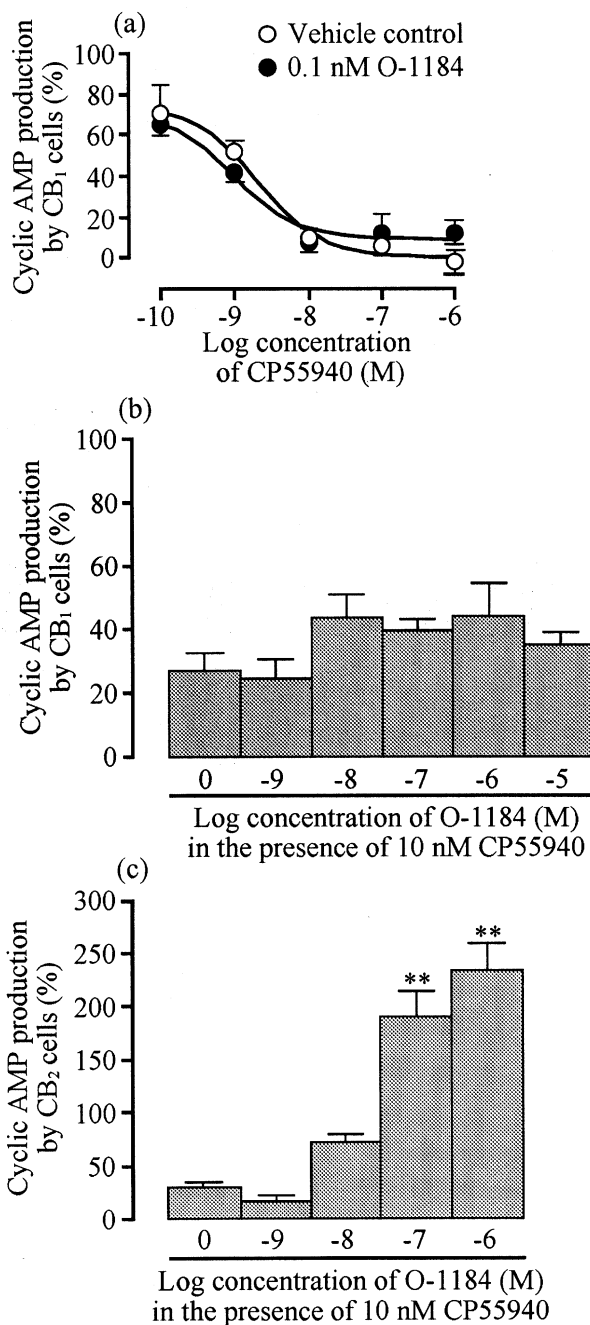
#### *Effects of O-1184, O-1238, O-1315 and O-584 on $[^3\text{H}]\text{-CP55940}$ binding and on forskolin-stimulated cyclic AMP production*

As shown in Table 1 and Figure 2, O-1184, O-1238, O-584 and CP55940 were equipotent in displacing  $[^3\text{H}]\text{-CP55940}$  from specific binding sites on both  $\text{CB}_1$  and  $\text{CB}_2$  CHO cell membranes. O-1315 also displaced  $[^3\text{H}]\text{-CP55940}$  from these binding sites but was significantly less potent than the other agents investigated (Table 1).

Cyclic AMP production in  $\text{CB}_1$ -transfected cells was potently inhibited by CP55940, O-1184, O-1238, O-1315 and O-584 (Table 1 and Figure 3). O-1184, O-584 and O-1315 were equipotent with CP55940. However, they behaved as  $\text{CB}_1$  receptor partial agonists, their maximal inhibitory effects ( $E_{\text{max}}$  values) being significantly less than the  $E_{\text{max}}$  of CP55940 (Table 1 and Figure 3). O-1238 was also equipotent with CP55940 in  $\text{CB}_1$ -transfected cells and its  $E_{\text{max}}$  did not deviate significantly from that of CP55940 (Table 1 and Figure 3). Neither O-1184 nor O-1238 affected forskolin-stimulated cyclic AMP production in untransfected CHO cells at concentrations ranging from 1 nM to 1  $\mu\text{M}$  ( $n=3$ ; data not shown).

In  $\text{CB}_2$ -transfected cells, CP55940 potently inhibited cyclic AMP production whereas O-1184 and O-584 each produced a concentration-related enhancement of this effect of forskolin (Table 1 and Figure 3). As has been reported previously (Rinaldi-Carmona *et al.*, 1998), we found that the  $\text{CB}_2$ -selective ligand, SR144528, also enhanced forskolin-stimulated cyclic AMP production in  $\text{CB}_2$ -transfected cells with an  $E_{\text{max}}$  of  $1764 \pm 468\%$  ( $n=4$ ). This value is significantly greater than the  $\text{CB}_2$   $E_{\text{max}}$  values of O-1184 and O-584 shown in Table 1 ( $P < 0.01$ ; analysis of variance followed by Dunnett's test). In the same cell line, mean responses to forskolin were increased by concentrations of O-1315 in the low nanomolar range and

decreased by micromolar concentrations of O-1238. However neither of these effects was statistically significant (Figure 3).

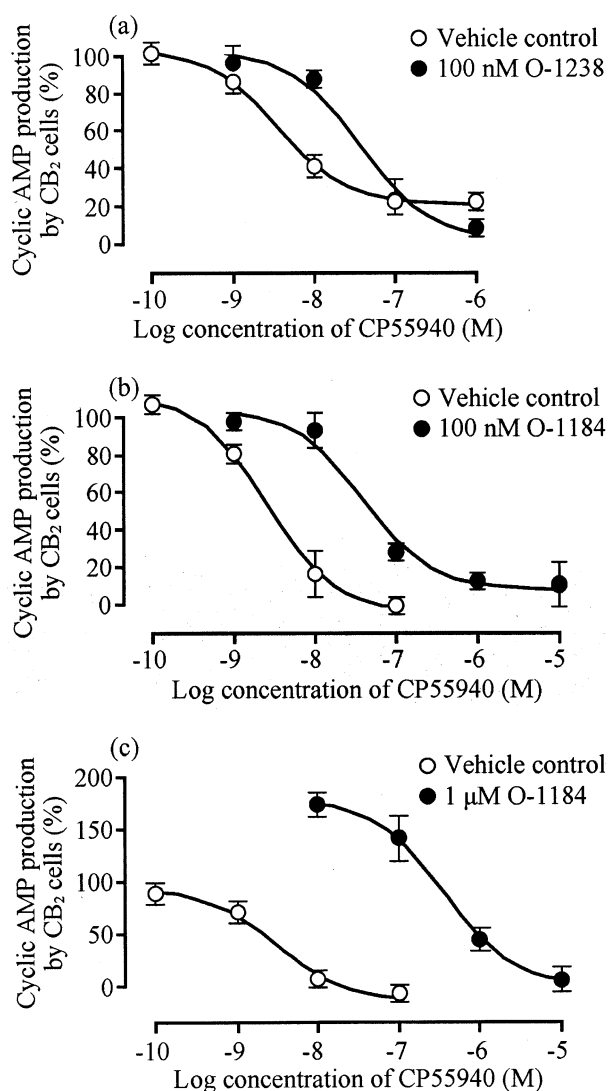


**Figure 4** The upper panel (a) shows the effect of 0.1 nM O-1184 on CP55940-induced inhibition of forskolin-stimulated cyclic AMP production in  $\text{CB}_1$ -transfected CHO cells. Each symbol in (a) represents mean percentage change in forskolin-stimulated cyclic AMP production  $\pm$  s.e.mean ( $n=3$ ). Forskolin-stimulated cyclic AMP production in the absence of CP55940 and O-1184 has been normalized to 100%. The  $p\text{EC}_{50}$  values of CP55940 in the absence and presence of O-1184 ( $8.69 \pm 0.25$  and  $9.03 \pm 0.25$  respectively) did not differ significantly (unpaired *t*-test). The lower panels (b) and (c) show the effect of O-1184 on inhibition of forskolin-stimulated cyclic AMP production by 10 nM CP55940 in CHO cells transfected with  $\text{CB}_1$  ( $n=11$ ) or  $\text{CB}_2$  receptors ( $n=6$ ). Each column represents mean percentage change in forskolin-stimulated cyclic AMP production  $\pm$  s.e.mean. Forskolin-stimulated cyclic AMP production in the absence of CP55940 and O-1184 has been normalized to 100%. Asterisks indicate significant differences from forskolin-stimulated cyclic AMP production in the presence of CP55940 alone (\*\* $P < 0.01$ ; one-way analysis of variance followed by Dunnett's test).

At concentrations ranging from 1 nM to 10  $\mu$ M, O-1184 and O-584 did not alter forskolin-stimulated cyclic AMP production in CB<sub>2</sub>-transfected cells that had been treated for 18 h with 100 ng ml<sup>-1</sup> pertussis toxin ( $n=2$ ; data not shown). Nor did concentrations of 1 nM to 1  $\mu$ M O-1184 or O-584 affect cyclic AMP production in CB<sub>2</sub> cells in the absence of forskolin (data not shown).

*Effect of O-1184 and O-1238 on CP55940-induced inhibition of forskolin-stimulated cyclic AMP production by CB<sub>1</sub> or CB<sub>2</sub> transfected cells*

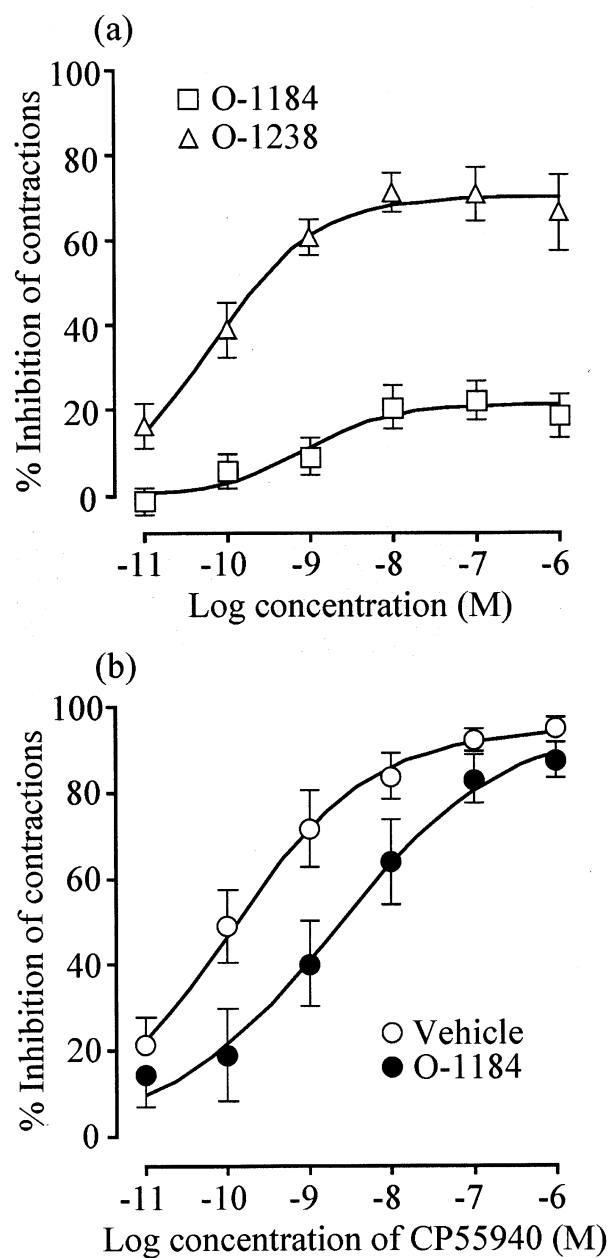
Initial experiments with CB<sub>1</sub> transfected cells were performed with a concentration of O-1184 that had little effect on cyclic AMP production when administered alone (Figure 3). This concentration of O-1184 (0.1 nM) had no effect on the position of the concentration response curve of CP55940 for inhibition



**Figure 5** Effect (a) 100 nM O-1238, (b) 100 nM O-1184 and (c) 1  $\mu$ M O-1184 on CP55940-induced inhibition of forskolin-stimulated cyclic AMP production in CB<sub>2</sub>-transfected CHO cells. Each symbol represents mean percentage change in forskolin-stimulated cyclic AMP production  $\pm$  s.e.mean ( $n=3$ ). Forskolin-stimulated cyclic AMP production in the absence of CP55940, O-1238 and O-1184 has been normalized to 100%. The upper asymptote of the log concentration response curve of CP55940 was significantly greater in the presence of 1  $\mu$ M O-1184 ( $178.8 \pm 14.3\%$ ) than in its absence ( $94.2 \pm 9.3\%$ ;  $P < 0.01$ ; unpaired  $t$ -test).

of cyclic AMP production (Figure 4). Further experiments showed that at higher concentrations, O-1184 did not reverse the inhibitory effect of 10 nM CP55940 on cyclic AMP production in this cell line (Figure 4).

In CB<sub>2</sub>-transfected cells, O-1184 induced a concentration-related reversal of the inhibitory effect of 10 nM CP55940 on cyclic AMP production (Figure 4), its pEC<sub>50</sub> for reversal being



**Figure 6** The upper panel (a) shows mean concentration-response curves for O-1238 and O-1184 in the mouse isolated vas deferens. Each symbol in (a) represents the mean value  $\pm$  s.e.mean for inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition to the organ bath of O-1238 ( $n=7$ ) or O-1184 ( $n=20$ ). The lower panel (b) shows the effect of pretreatment with 1 nM O-1184 on the mean concentration-response curve for CP55940 in the vas deferens ( $n=7$ ). Each symbol in (b) represents the mean values  $\pm$  s.e.mean for inhibition of electrically-evoked contractions expressed as a percentage of the amplitude of the twitch response measured immediately before the first addition of CP55940 to the organ bath. O-1184 or Tween 80 were added 30 min before the first addition of CP55940.

$7.93 \pm 0.07$  ( $n=6$ ). It was also found that O-1238 and O-1184 could both produce dextral shifts in the log concentration-response curve of CP55940 (Figure 5). At 100 nM, O-1238 decreased the  $pEC_{50}$  of CP55940 in CB<sub>2</sub>-transfected cells from  $8.54 \pm 0.23$  to  $7.44 \pm 0.23$ , a 12.6 fold potency decrease ( $P < 0.05$ ; unpaired *t*-test;  $n=3$ ).  $pEC_{50}$  values of CP55940 in the absence and presence of 100 nM O-1184 were  $8.61 \pm 0.16$  and  $7.42 \pm 0.17$  respectively, a 15.6 fold potency decrease ( $P < 0.01$ ; unpaired *t*-test;  $n=3$ ). At this concentration, neither O-1238 nor O-1184 significantly affected the position of the upper or lower asymptotes of the CP55940 log concentration-response curve (unpaired *t*-test;  $n=3$ ). However, at 1  $\mu$ M, O-1184 caused the concentration-response curve of CP55940 to become steeper and to shift upwards as well as to the right (Figure 5). This upward shift presumably reflects the ability of O-1184 to enhance forskolin-stimulated cyclic AMP production in the CB<sub>2</sub> transfected cells.

#### *Effects of O-1238 and O-1184 on CP55940-induced inhibition of electrically-evoked contractions of the mouse vas deferens*

As shown in Figure 6, O-1238 decreased the amplitude of evoked contractions in a concentration-related manner, with a  $pEC_{50}$  of  $10.18 \pm 0.20$ . O-1184 also induced detectable inhibition of evoked contractions with a  $pEC_{50}$  of  $9.08 \pm 0.46$  (Figure 6). However, its  $E_{max}$  ( $21.1 \pm 3.54\%$ ) was significantly less than that of O-1238 ( $70.5 \pm 3.9\%$ ) which, in turn, was less than that of CP55940 ( $95.1 \pm 5.3\%$ ; Figure 6) ( $P < 0.01$ ; analysis of variance followed by the Newman-Keuls test). Electrically evoked contractions were not significantly inhibited by Tween 80 ( $n=6$ ; data not shown). At 1 nM, O-1184 produced a 17.3 fold dextral shift in the log concentration-response curve of CP55940 for inhibition of evoked contractions that did not deviate significantly from parallelism (Figure 6). Insertion of this value (concentration ratio,  $x$ ) and its 95% confidence limits into the equation  $(x-1) = B/K_B$  (see Methods) gave a  $pK_B$  value for O-1184 of  $10.21 \pm 0.38$ . This value is significantly greater than the CB<sub>1</sub> and CB<sub>2</sub>  $pK_i$  values of O-1184 shown in Table 1 ( $P < 0.01$ ; analysis of variance followed by Dunnett's test).

## Discussion

O-1184 displayed a similar potency to the established cannabinoid receptor agonist, CP55940, both in its affinity for CB<sub>1</sub> binding sites and in its ability to inhibit forskolin-stimulated cyclic AMP production by CB<sub>1</sub>-transfected CHO cells. However, its maximal effect for inhibition of cyclic AMP production was less than that of CP55940, suggesting it to be a partial agonist. In line with this pharmacological classification, O-1184 exhibited mixed agonist-antagonist properties in the mouse vas deferens. Thus whilst it shared the ability of CP55940 to inhibit electrically-evoked contractions of this tissue at low nanomolar concentrations, its maximal effect was well below that of CP55940. Moreover, at a concentration of 1 nM, which induced only marginal inhibition of evoked contractions (Figure 6), O-1184 behaved as a cannabinoid receptor antagonist with a  $K_B$  value against CP55940 in the picomolar range. These results are consistent with an earlier observation that O-1184 can antagonize CP55940-induced inhibition of electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine, again with a  $K_B$  value in the picomolar range (Ross *et al.*, 1998).

The classification of O-1184 as a partial agonist at CB<sub>1</sub> receptors is supported by results from recent experiments with rat cerebellar membranes (Griffin *et al.*, 1999). These showed O-1184 to lack detectable agonist activity in the GTP $\gamma$ S binding assay and yet, as expected for a ligand that possesses at least some efficacy, to be less effective in displacing [<sup>3</sup>H]-CP55940 in the presence than in the absence of GDP, GTP $\gamma$ S and added sodium ions. Further support for this classification comes from evidence that 6'-cyanohept-2'-yne- $\Delta^8$ -tetrahydrocannabinol (O-823), which differs from O-1184 only in having CN rather than N<sub>3</sub> on the terminal carbon of the aliphatic side-chain, is a partial agonist at cannabinoid receptors in the mouse vas deferens (Pertwee *et al.*, 1996).

O-1184 did not show mixed agonist-antagonist properties in CB<sub>1</sub>-transfected cells, sub-inhibitory concentrations of this compound having no antagonistic effect on CP55940-induced inhibition of forskolin-stimulated cyclic AMP production in this cell line. This apparent discrepancy may be an indication that there were fewer cannabinoid receptors in the vas deferens than in the transfected cell line. Thus, it is generally accepted that the agonist-antagonist properties of a partial agonist show tissue-dependence, the agonist component dominating in tissues that are well-endowed with receptors for the partial agonist and the antagonist component dominating in tissues that are more sparsely populated with such receptors (Kenakin, 1993). In previous experiments with the myenteric plexus-longitudinal muscle preparation (Ross *et al.*, 1998), we found O-1184 to behave as a cannabinoid receptor antagonist at a concentration at which it behaved as an agonist in the mouse vas deferens (100 nM). This too may reflect an inter-tissue difference in receptor density, there already being some evidence that the myenteric plexus-longitudinal muscle preparation expresses fewer cannabinoid receptors than the mouse vas deferens (Pertwee *et al.*, 1996).

The mean  $pK_i$  values of O-1184 for displacement of [<sup>3</sup>H]-CP55940 from CB<sub>1</sub> and CB<sub>2</sub> binding sites shown in Table 1 (8.28 and 8.13 respectively) are of the same order as a previously reported  $pK_B$  value of this compound for antagonism of CP55940-induced enhancement of [<sup>35</sup>S]GTP $\gamma$ S binding to rat cerebellar membranes, the mean value of which is 8.53 (Griffin *et al.*, 1999). However, these  $pK_i$  and  $pK_B$  values are markedly less than the mean  $pK_B$  of O-1184 for antagonism of CP55940-induced inhibition of electrically-evoked contractions of the mouse vas deferens (10.21; see Results). This difference may stem from the use of different vehicles, Tween 80 in the vas deferens experiments and ethanol in the [<sup>3</sup>H]-CP55940 and [<sup>35</sup>S]-GTP $\gamma$ S binding assays. However, it also remains possible that in the vas deferens, O-1184 and CP55940 interact at least in part through an as yet unidentified cannabinoid receptor. Indeed, each of these cannabinoids binds avidly to both CB<sub>1</sub> and CB<sub>2</sub> receptors, demonstrating a lack of selectivity for any one cannabinoid receptor type, and there is already evidence for the presence of a novel CB<sub>2</sub>-like receptor type in the mouse vas deferens (Griffin *et al.*, 1997).

Like its affinity for CB<sub>1</sub> binding sites, the affinity of O-1184 for CB<sub>2</sub> binding sites matched that of CP55940. However, O-1184 differed from CP55940 in that it enhanced rather than inhibited forskolin-stimulated cyclic AMP production by CB<sub>2</sub>-transfected cells. In this respect O-1184 resembles the CB<sub>2</sub>-selective ligands AM630 (6-iodopravadoline) and SR144528 (Rinaldi-Carmona *et al.*, 1998; Ross *et al.*, 1999), although the maximum degree of the enhancement it produced (1.6 fold) is somewhat less than that produced in the same cell line by AM630 (4.9 fold; Ross *et al.*, 1999) and SR144528 (17.6 fold; Results). Also like AM630 and SR144528 (Rinaldi-Carmona *et al.*, 1998; Ross *et al.*, 1999), O-1184 attenuated CP55940-

induced inhibition of forskolin-stimulated cyclic AMP production by CB<sub>2</sub>-transfected cells.

Modification of the aliphatic side-chain of O-1184 by replacing the carbon-carbon triple bond with a *cis* double bond (O-1238) did not produce detectable changes in CB<sub>1</sub> or CB<sub>2</sub> receptor affinity or in the pEC<sub>50</sub> value for inhibition of forskolin-stimulated cyclic AMP production by CB<sub>1</sub> transfected cells. However, this structural change did cause an apparent increase in CB<sub>1</sub> efficacy, the E<sub>max</sub> of O-1238 exceeding that of O-1184 for inhibition both of cyclic AMP production by CB<sub>1</sub>-transfected cells and of evoked contractions of the vas deferens. The E<sub>max</sub> of O-1238 was similar to that of CP55940 in the cyclic AMP assay but not in the isolated tissue assay in which O-1238 behaved as a partial agonist with an E<sub>max</sub> less than that of CP55940. Again, this may reflect the presence of fewer cannabinoid receptors in the vas deferens than in the CB<sub>1</sub> cell line (see above). Evidence that O-1238 is a partial agonist at CB<sub>1</sub> receptors with greater efficacy than O-1184 has also been obtained by Griffin *et al.* (1999) from experiments in which the measured effect was agonist-induced stimulation of GTP<sub>γ</sub>S binding to rat cerebellar membranes.

Although O-1238 retained the ability of O-1184 to attenuate CP55940-induced inhibition of forskolin-stimulated cyclic AMP production in CB<sub>2</sub>-transfected cells without any reduction in potency, it did not share the ability of O-1184 to enhance forskolin-stimulated cyclic AMP production in this cell line when administered alone. Other drugs that are known to block CB<sub>2</sub> receptor-mediated responses at concentrations in the nanomolar range, for example SR144528 and AM630 (see above), resemble O-1184 in producing 'inverse cannabimimetic' effects when administered by themselves. Consequently, our O-1238 data demonstrate for the first time that it is possible for an agent to antagonize a CB<sub>2</sub> receptor-mediated response even when it lacks the ability to produce inverse cannabimimetic effects. Because O-1184 and O-1238 can both antagonize CP55940 in CB<sub>2</sub>-transfected cells but only O-1184 enhances forskolin-stimulated cyclic AMP production in this cell line, it is unlikely that O-1184 produces this enhancement by blocking the action of endogenous cannabinoids released by these cells. This conclusion is supported by evidence that no such endogenous cannabinoid release takes place in CHO cells expressing human CB<sub>1</sub> or CB<sub>2</sub> receptors (Bouaboula *et al.*, 1997; MacLennan *et al.*, 1998). A more likely explanation for our results is that the potentiation of forskolin produced by O-1184 in CB<sub>2</sub>-transfected cells reflects the presence of a mixed population of constitutively active and inactive CB<sub>2</sub> receptors and that these two sets of receptors are in equilibrium with each other. As has already been proposed for SR144528 and AM630 (Rinaldi-Carmona *et al.*, 1998; Ross *et al.*, 1999), O-1184 might then enhance forskolin-stimulated cyclic AMP production because it is an inverse agonist that reduces the proportion of receptors in the constitutively active state by binding preferentially to the inactive receptors.

In agreement with results obtained by Griffin *et al.* (1999) in experiments with rat cerebellar membranes, we found that replacement of N<sub>3</sub> with C<sub>2</sub>H<sub>5</sub> on the terminal carbon of the aliphatic side-chain of O-1184 to form O-584 had no effect on CB<sub>1</sub> receptor affinity. We also found O-584 and O-1184 to have the same affinity for CB<sub>2</sub> binding sites and the same ability to alter forskolin-stimulated cyclic AMP production in CHO cells

transfected with CB<sub>1</sub> receptors (inhibition) or CB<sub>2</sub> receptors (enhancement). Neither of these compounds enhanced forskolin-stimulated cyclic AMP production in CB<sub>2</sub>-transfected cells that had been pretreated with pertussis toxin, pointing to an involvement of G<sub>i/o</sub> proteins and hence of CB<sub>2</sub> receptors (Pertwee, 1997). That the effects of O-584 and O-1184 on cyclic AMP production by CB<sub>2</sub>- and, indeed, by CB<sub>1</sub>-transfected cells were cannabinoid receptor-mediated, is also supported by our finding that these compounds lacked detectable activity in untransfected cells. A further structural modification, in which the phenolic 1-hydroxyl group of O-584 was removed to form O-1315 did not affect either the pEC<sub>50</sub> or E<sub>max</sub> for inhibition of cyclic AMP production by CB<sub>1</sub>-transfected cells. However, this structural change did abolish the ability of the molecule to induce any significant enhancement of forskolin-stimulated cyclic AMP production by CB<sub>2</sub>-transfected cells. It also caused reductions in CB<sub>1</sub> and CB<sub>2</sub> receptor affinity of 5.0 and 10.6 fold respectively. The nature of these affinity changes was unexpected as removal of the 1-hydroxyl group from another analogue of Δ<sup>8</sup>-THC, 11-hydroxy-Δ<sup>8</sup>-THC-dimethylheptyl, or from Δ<sup>8</sup>-THC itself, has no detectable effect on CB<sub>1</sub> receptor affinity but enhances CB<sub>2</sub> receptor affinity, thereby conferring a significant degree of CB<sub>2</sub>-selectivity (Pertwee, 1999; Huffman *et al.*, 1996; 1998).

In conclusion, comparison of the pharmacological properties of O-1184 and O-1238 suggests that although increasing the degree of unsaturation of the aliphatic side-chain has little effect on CB<sub>1</sub> or CB<sub>2</sub> receptor affinity, it can markedly reduce CB<sub>1</sub> receptor efficacy and reverse the direction of responses elicited at CB<sub>2</sub> receptors. Thus at CB<sub>1</sub> receptors, the less saturated of these two agents, O-1184, appears to have the lower efficacy and to possess the mixed agonist-antagonist properties of a partial agonist. At CB<sub>2</sub> receptors the effect of Δ<sup>8</sup>-THC analogues seems to change from agonism when the side-chain is fully saturated as in 11-hydroxy-Δ<sup>8</sup>-THC-dimethylheptyl (Slipetz *et al.*, 1995), through weak partial agonism/antagonism when the side-chain contains a *cis*-double bond (O-1238) to inverse agonism when the side-chain contains a carbon-carbon triple bond (O-1184). Because O-1238 has relatively high affinity for CB<sub>2</sub> receptors but lacks significant CB<sub>2</sub> receptor efficacy or inverse cannabimimetic properties, at least in the cyclic AMP assay, it may be an important lead compound from which to develop the first pure cannabinoid receptor antagonist. Accordingly, further experiments are needed to establish whether the structure of O-1238 can be modified to yield a pure CB<sub>1</sub>-CB<sub>2</sub> antagonist through elimination of CB<sub>1</sub> efficacy or to yield a pure CB<sub>2</sub>-selective antagonist through elimination of CB<sub>1</sub> affinity. Further experiments are also needed to determine the extent to which the properties exhibited by O-1184 and its analogues in CB<sub>2</sub>-transfected cells extend to tissues in which these receptors are expressed naturally. Such studies must, however, await the development of a suitable CB<sub>2</sub> receptor bioassay.

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