

Agonist-inverse agonist characterization at CB₁ and CB₂ cannabinoid receptors of L759633, L759656 and AM630

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1 We have tested our prediction that AM630 is a CB₂ cannabinoid receptor ligand and also investigated whether L759633 and L759656, are CB₂ receptor agonists.

2 Binding assays with membranes from CHO cells stably transfected with human CB₁ or CB₂ receptors using [³H]-CP55940, confirmed the CB₂-selectivity of L759633 and L759656 (CB₂/CB₁ affinity ratios = 163 and 414 respectively) and showed AM630 to have a K_i at CB₂ receptors of 31.2 nM and a CB₂/CB₁ affinity ratio of 165.

3 In CB₂-transfected cells, L759633 and L759656 were potent inhibitors of forskolin-stimulated cyclic AMP production, with EC₅₀ values of 8.1 and 3.1 nM respectively and CB₁/CB₂ EC₅₀ ratios of >1000 and >3000 respectively.

4 AM630 inhibited [³⁵S]-GTPγS binding to CB₂ receptor membranes (EC₅₀ = 76.6 nM), enhanced forskolin-stimulated cyclic AMP production in CB₂-transfected cells (5.2 fold by 1 μM), and antagonized the inhibition of forskolin-stimulated cyclic AMP production in this cell line induced by CP55940.

5 In CB₁-transfected cells, forskolin-stimulated cyclic AMP production was significantly inhibited by AM630 (22.6% at 1 μM and 45.9% at 10 μM) and by L759633 at 10 μM (48%) but not 1 μM. L759656 (10 μM) was not inhibitory. AM630 also produced a slight decrease in the mean inhibitory effect of CP55940 on cyclic AMP production which was not statistically significant.

6 We conclude that AM630 is a CB₂-selective ligand that behaves as an inverse agonist at CB₂ receptors and as a weak partial agonist at CB₁ receptors. L759633 and L759656 are both potent CB₂-selective agonists.

Keywords: Cannabinoid CB₁ and CB₂ receptors; AM630; L759633; L759656; CB₂-selective ligands; CB₂-selective agonist; CB₂-selective inverse agonist; [³⁵S]-GTPγS; cyclic AMP

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; L759633, (6aR,10aR)-3-(1,1-dimethyl-heptyl)-1-methoxy-6,6,9-trimethyl-6a,7,10,10a-tetrahydro-6H-benzo[c]chromene; L759656, (6aR,10aR)-3-(1,1-dimethyl-heptyl)-1-methoxy-6,6-dimethyl-9-methylene-6a,7,8,9,10,10a-hexahydro-6H-benzo[c]chromene; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; SR144528, N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; WIN55212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl)methanone

Introduction

Many cannabinoid effects are now known to be mediated by CB₁ receptors that are present in the central nervous system as well as in certain neuronal and nonneuronal peripheral tissues or by CB₂ receptors that are found mainly in cells of the immune system (Pertwee, 1997). Both receptor types are coupled to their effector systems through G_{i/o} proteins (Pertwee, 1997). These discoveries have led to the development of selective ligands for CB₁ and CB₂ receptors. Particularly important have been SR141716A, which behaves as a selective CB₁ receptor inverse agonist (Bouaboula *et al.*, 1997; MacLennan *et al.*, 1998) and SR144528, which behaves as a selective CB₂ receptor inverse agonist (Rinaldi-Carmona *et al.*, 1998).

A less well characterized cannabinoid receptor ligand is 6-iodopravadoline (AM630) (Figure 1). This has been shown to be a selective, competitive antagonist of certain cannabinoid receptor agonists in the mouse isolated *vas deferens* (Pertwee *et*

al., 1995) but to possess the properties of a weak cannabinoid CB₁ receptor agonist in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine (Pertwee *et al.*, 1996). There are also reports that AM630 attenuates cannabinoid-induced stimulation of [³⁵S]-GTPγS binding to mouse and guinea-pig brain membrane preparations (Hosohata *et al.*, 1997a,b) and that it behaves as an inverse agonist in Chinese hamster ovary (CHO) cells stably transfected with CB₁ receptors (Landsman *et al.*, 1998).

Although there is now good evidence that AM630 is a CB₁ receptor ligand, the question remains as to whether it also interacts with CB₂ receptor, and if so whether it is a CB₂ receptor agonist, antagonist or inverse agonist. Our prediction was that AM630 would be a CB₂ receptor ligand as it is a structural analogue of WIN55212-2, a cannabinoid receptor agonist that is already known to have some degree of selectivity for the CB₂ receptor (Felder *et al.*, 1995; Showalter *et al.*, 1996). In this investigation we first compared the ability of AM630 to bind to CB₁ and CB₂ receptors and then

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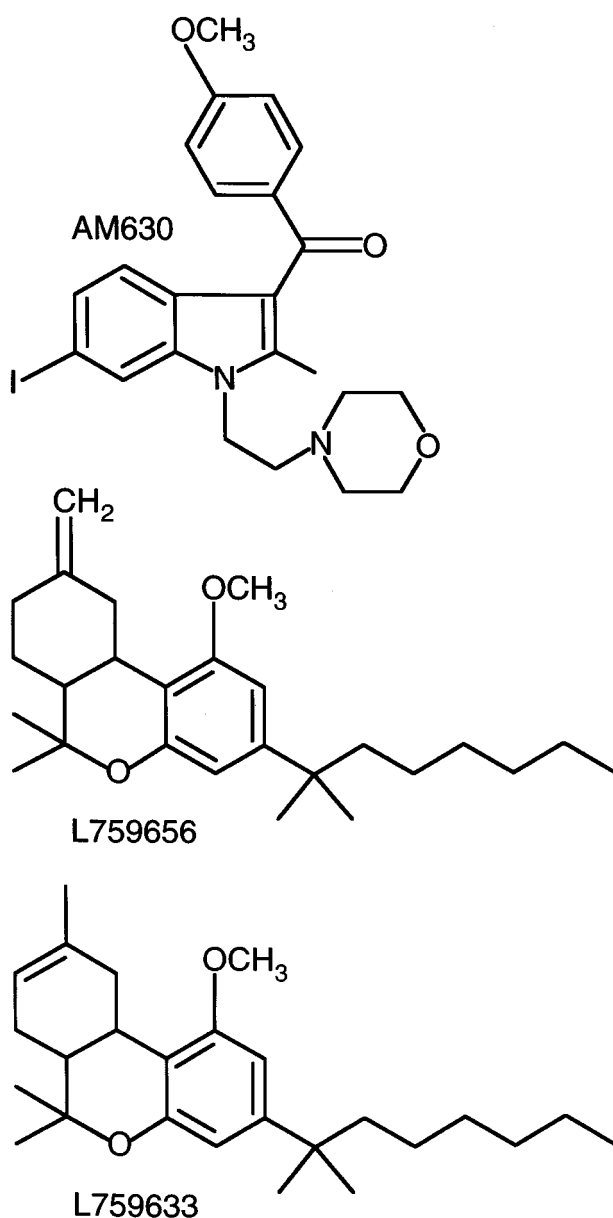


Figure 1 Structures of AM630, L759656 and L759633.

attempted to establish whether it shows agonist, antagonist or inverse agonist activity at each of these receptor types. The pharmacological properties of two other putative cannabinoid receptor ligands were also investigated in the present study. These are L759633 and L759656 (Figure 1), both of which have been reported to have a markedly higher affinity for human CB₂ than human CB₁ receptors (Gareau *et al.*, 1996). However, no other information about these compounds has yet been published. Since there is a need for CB₂-selective agonists for use as pharmacological tools, we carried out experiments to establish whether either of these compounds show significant efficacy at CB₁ and/or CB₂ receptors.

Binding assays were performed with membrane preparations of CB₁- or CB₂-transfected CHO cells. The radioactive probe used in most of our binding experiments was [³H]-CP55940, which binds equally well to CB₁ and CB₂ receptors (Showalter *et al.*, 1996). The functional assays used exploited the well-established ability of cannabinoid receptor agonists to inhibit forskolin-stimulated cyclic AMP production and of cannabinoid receptor agonists and inverse agonists to modulate [³⁵S]-GTPγS binding (Pertwee, 1997; MacLennan *et*

al., 1998). These assays were carried out with CHO cells stably transfected with CB₁ or CB₂ receptors.

Methods

CHO cells

These were stably transfected with cDNA encoding human CB₁ or CB₂ receptors. The CB₁-transfected cells used in binding assays with [³H]-CP55940 or [³H]-WIN55212-2 were supplied by Euroscreen, Brussels ($B_{\max}$ = 23.8 pmol mg⁻¹ protein) and were a gift from Organon. The CB₂-transfected cells used in binding assays with [³H]-CP55940, [³H]-WIN55212-2 or [³⁵S]-GTPγS ($B_{\max}$ = 72.5 pmol mg⁻¹ protein) and the CB₁- and CB₂-transfected cells used in cyclic AMP assays ($B_{\max}$ = 3.5 pmol mg⁻¹ protein and 50.7 pmol mg⁻¹ protein respectively) were provided by Drs G. Disney and A. Green, GlaxoWellcome R&D, Medicines Research Centre, Stevenage. Untransfected CHO cells were a gift from Dr Min Zhao, University of Aberdeen. The clones used in cyclic AMP assays were the same as those used in the sPAP reporter assay described by Green *et al.* (1998). Cells were maintained at 37°C and 5% CO₂ in DMEM (f-12 HAM) with 2 mM Glutamine, Geneticin (600 µg ml⁻¹) and Hygromycin (300 µg ml⁻¹). Because receptor overexpression may lead to the activation of effector mechanisms to which receptors in natural membranes are not normally coupled (see Kenakin, 1995), the cyclic AMP assays were performed with cells expressing fewer CB₁ or CB₂ receptors than the cells used in the binding assays.

Membrane preparation

CHO cells were suspended in 50 mM Tris buffer (pH 7.4) and 0.32 M sucrose and homogenized with an Ultra-Turrex homogenizer. The homogenate was diluted with 50 mM Tris buffer (pH 7.4) and centrifuged at 50,000 × *g* for 1 h to isolate the membranes.

Binding experiments

A filtration procedure was used to measure [³H]-CP55940 and [³H]-WIN55212-2 binding. This is a modification of the method described by Compton *et al.* (1993). Binding assays were performed with [³H]-CP55940 or [³H]-WIN55212-2, 1 mM MgCl₂, 1 mM EDTA, 2 mg ml⁻¹ bovine serum albumin (BSA) and 50 mM Tris buffer, total assay volume 500 µl. Binding was initiated by the addition of cell membranes (20–30 µg protein). Assays were carried out at 30°C for 90 min before termination by addition of ice-cold wash buffer (50 mM Tris buffer, 1 mg ml⁻¹ 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 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 µM unlabelled cannabinoid. Protein assays were performed using a Bio-Rad Dc kit. Unlabelled and radiolabelled cannabinoids were each added in a volume of 50 µl following dilution in assay buffer (50 mM Tris buffer containing 10 mg ml⁻¹ BSA). The concentration of [³H]-CP55940 or [³H]-WIN55212-2 used in displacement assays was 0.5 nM. The concentrations of cannabinoids that produced

a 50% displacement of radioligand from specific binding sites (IC_{50} values) were calculated using GraphPad Prism (GraphPad Software, San Diego, U.S.A.). Competitive binding curves were fitted with minimum values for displacement of radioligand from specific binding sites constrained to zero. Dissociation constant (K_i values) were calculated using the equation of Cheng & Prusoff (1973) and dissociation constant values of [3H]-CP55940 and [3H]-WIN55212-2 shown in the footnote to Table 1.

Cyclic AMP assay

Cells (2×10^6 cells ml^{-1}) were preincubated for 30 min at $37^\circ C$ with cannabinoid and 3-isobutyl-1-methylxanthine (IBMX; 50 μM) in phosphate buffered saline containing 1 mg ml^{-1} BSA (assay buffer) followed by a further 30 min incubation with 2 μM forskolin in a total volume of 500 μl . The reaction was terminated by addition 0.1 M HCl and centrifuged 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). Forskolin and IBMX were dissolved in DMSO.

[^{35}S]-GTP γ S assay

CB $_2$ transfected cells were removed from flasks by scraping, resuspended in homogenization buffer (0.32 M sucrose and 50 mM Tris), and homogenized using an ultra-Turrex homogenizer. The homogenate was diluted with Tris buffer (50 mM, pH 7.4) and centrifuged at 50,000 $\times g$ for 45 min. Cell membranes (20 μg) were incubated in assay buffer containing 2 mg ml^{-1} fatty acid free BSA, 20 μM GDP and 0.1 nM [^{35}S]-GTP γ S. The assay buffer contained 50 mM Tris, 10 mM MgCl $_2$, 100 mM NaCl and 0.2 mM EDTA at pH 7.4. Incubations were carried out at $30^\circ C$ for 90 min in a total volume of 500 μl . The reaction was terminated by the addition of 4 ml of ice-cold wash buffer (50 mM Tris and 1 mg ml^{-1} BSA, pH 7.4) followed by rapid filtration under vacuum through Whatman GF/B glass-fibre filters (pre-soaked in wash buffer) using a 12-tube Brandel cell harvester. The tubes were washed three times with 4 ml of wash buffer. Filters were oven dried, placed in 5 ml of scintillation fluid and bound radioactivity was determined by liquid scintillation counting. Basal binding of [^{35}S]-GTP γ S was determined in the presence of 20 μM GDP and absence of cannabinoid. Non-specific binding was determined in the presence of 10 μM GTP γ S.

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 the Kruskal-Wallis test followed by Dunn's multiple comparison 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^{-1}), 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. Drug-induced inhibition of specific [^{35}S]-GTP γ S binding was expressed as the percentage decrease below the basal level of [^{35}S]-GTP γ S binding using the equation $[100 \times (d' - d)] / d$ where d' and d are d.p.m. in the presence and absence of the drug respectively. Values for EC_{50} , IC_{50} and maximal effects (E_{max}) and the 95% confidence limits of these values have been

calculated by non-linear regression analysis using GraphPad Prism (GraphPad Software, San Diego, U.S.A.).

The ability of AM630 to antagonize CP55940-induced inhibition of forskolin-stimulated cyclic AMP production in CB $_2$ transfected cells is expressed in terms of the concentration ratio. This has been defined as the concentration of CP55940 that produces a particular degree of inhibition in the presence of AM630 at a concentration, B , divided by the concentration of CP55940 that produces an identical degree of inhibition in the absence of AM630. Since AM630 behaved as an inverse agonist at CB $_2$ receptors (see Results), it was considered inappropriate to insert concentration ratio values into the Schild equation in order to obtain a K_B value of AM630 at these receptors. Concentration ratio values and their 95% confidence limits have been determined by symmetrical (2 + 2) dose parallel line assays (Colquhoun, 1971), using responses to pairs of agonist concentrations located on the steepest part of each log concentration-response curve. This method was also used to establish whether log concentration-response curves of CP55940 constructed in the presence and absence of AM630 deviated significantly from parallelism.

Drugs

CP55940 {(-)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol} was supplied by Pfizer, WIN55212-2 {(R)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholino)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthyl)-methanone} by Research Biochemicals International, SR141716A [N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride] and SR144528 {N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide} by Sanofi Recherche and L759633 [(6aR,10aR)-3-(1,1-dimethyl-heptyl)-1-methoxy-6,6,9-trimethyl-6a,7,10,10a-tetrahydro-6H-benzo[c]chromene] and L759656 [(6aR,10aR)-3-(1,1-dimethyl-heptyl)-1-methoxy-6,6-dimethyl-9-methylene-6a,7,8,9,10,10a-hexahydro-6H-benzo[c]chromene] by Merck Frosst. AM630 (6-iodopravadoline) was synthesized in the laboratory of Dr A. Makriyannis. [3H]-CP55940 (126 Ci $mmol^{-1}$) and [3H]-WIN55212-2 (45 Ci $mmol^{-1}$) were supplied by NEN Life Science Products. Cannabinoids were stored as 1 mg ml^{-1} stock solutions in ethanol and diluted in assay buffer. At the highest concentrations used, ethanol by itself had no detectable effect on specific binding of [3H]-CP55940, [3H]-WIN55212-2 or [^{35}S]-GTP γ S or on forskolin-stimulated cyclic AMP production (data not shown).

Results

Cannabinoid binding experiments

The radioligand binding data shown in Table 1 confirm that CP55940 is a high-affinity non-selective ligand for cannabinoid receptors. The data also confirm L759656 and L759633 to be markedly CB $_2$ -selective, with much lower K_i values in CB $_2$ transfected cell membranes than in membranes of CB $_1$ transfected cells. AM630 is also CB $_2$ -selective with a CB $_1$ /CB $_2$ K_i ratio of 165 in transfected CHO cell membranes. Further experiments showed AM630 to be no less effective in displacing [3H]-CP55940 than [3H]-WIN55212-2 from CB $_2$ receptors on CHO cell membranes (Table 1). SR144528 was also equally effective in displacing [3H]-CP55940 and [3H]-WIN55212-2 from CB $_2$ receptors (Table 1).

Table 1 K_i values of CP55940, WIN55212-2, L759656, L759633 and AM630 for displacement of [3 H]-CP55940 from CB₁ or CB₂ receptors on CHO cell membranes

Labelled cannabinoid	Unlabelled cannabinoid	CB ₁ K_i (nM)	CB ₂ K_i (nM)
[3 H]-CP55940	CP55940	5.0 ± 0.8	1.8 ± 0.2
	L759633	1043 ± 296	6.4 ± 2.2
	L759656	4888 ± 950	11.8 ± 2.5
	AM630	5152 ± 567	31.2 ± 12.4
	SR144528	> 10 μ M	5.6 ± 1.1
[3 H]-WIN55212-2	AM630	—	37.5 ± 15.4
	SR144528	—	4.1 ± 1.3

K_i values were calculated by the Cheng & Prusoff equation ($n=3$ or 4) using K_D values of 1.2 and 0.8 nM for [3 H]-CP55940 in membranes of CB₁ and CB₂ cells respectively and a K_D value of 2.1 nM for [3 H]-WIN55212-2 in membranes of CB₂ cells (Ross & Pertwee, unpublished).

Effects of CP55940, L759656, L759633 and AM630 on cyclic AMP production

Cyclic AMP concentrations in the absence and presence of 2 μ M forskolin were 5.1 ± 1.8 and 46.3 ± 13.1 pmol ml⁻¹ respectively in CB₁-transfected cells ($n=6$) and 5.6 ± 2.6 and 52.8 ± 11.7 pmol ml⁻¹ respectively in CB₂-transfected cells ($n=6$). CP55940 was highly potent in inhibiting forskolin-stimulated cyclic AMP production in both CB₁- and CB₂-transfected cells (Table 2 and Figure 2). The CB₁/CB₂ EC₅₀ ratio of 0.9 reflects the non-selective nature of this agonist. In contrast, L759633 and L759656 showed greater potency against forskolin-stimulated cyclic AMP production in CB₂-transfected cells than in CB₁-transfected cells (Table 2 and Figure 2). Indeed, forskolin-stimulated cyclic AMP production in CB₁-transfected cells was not significantly inhibited by L759656 and was only inhibited by L759633 at the highest concentration used (10 μ M) (Table 2). The maximal inhibitory effects of CP55940, L759633 and L759656 on forskolin-stimulated cyclic AMP production in CB₂-transfected cells were not significantly different (Figure 2).

AM630 produced a significant inhibitory effect on cyclic AMP production in CB₁-transfected cells, albeit only at concentrations in the micromolar range (Figure 3). More specifically, AM630 concentrations of 1 and 10 μ M caused forskolin-stimulated cyclic AMP production in this cell line to fall to $77.5 \pm 4.2\%$ ($n=4$) and $54.1 \pm 12.4\%$ ($n=8$) of the control value respectively (one-sample *t*-test). In CB₂-transfected cells, AM630 enhanced the ability of forskolin to stimulate cyclic AMP production at 0.1, 1 and 10 μ M, the highest of these concentrations provoking an increase in cyclic AMP production of 5.5 fold (Figure 3). In the absence of forskolin, AM630 had no effect on cyclic AMP production by CB₁- or CB₂-transfected cells ($n=3$; data not shown). Forskolin-stimulated cyclic AMP production in untransfected CHO cells was affected neither by AM630 nor by L759633 or L759656 (data not shown).

Effect of AM630 on GTP γ S binding and on CP55940-induced inhibition of cyclic AMP production

In CB₂-transfected cells, the ability of CP55940 to inhibit cyclic AMP production at concentrations of 1 μ M or less was abolished by 1 μ M AM630 which produced a non-parallel upward shift in the log concentration-response curve of the agonist (Figure 4). At the lower concentration of

Table 2 EC₅₀ values for inhibition of forskolin-stimulated cyclic AMP production in CHO cells stably transfected with CB₁ or CB₂ receptors

Cannabinoid	CB ₁ EC ₅₀ (nM)	CB ₂ EC ₅₀ (nM)	CB ₁ EC ₅₀ /CB ₂ EC ₅₀
CP55940	2.6 ± 1.0 (7)	2.9 ± 1.4 (9)	0.9
L759633	ca 10 μ M (6)	8.1 ± 4.5 (3)	> 1000
L759656	> 10 μ M (6)	3.1 ± 0.6 (4)	> 3000
AM630	> 10 μ M (4)	IA (4)	—
SR144528	> 10 μ M (3)	IA (4)	—

Forskolin-stimulated cyclic AMP production was normalized to 100% in the absence of cannabinoids. In CB₁-transfected cells, it fell to $52.0 \pm 10.0\%$ in the presence of 10 μ M L759633, a value significantly less than 100% ($P < 0.01$; one-sample *t*-test) and to $85.7 \pm 11.7\%$ in the presence of 10 μ M L759656, a value not significantly less than 100% ($P > 0.05$; one-sample *t*-test). Forskolin-stimulated cyclic AMP production in CB₁-transfected cells was not decreased significantly by L759633 or L759656 at concentrations of 100 nM or 1 μ M (one-sample *t*-test). IA indicates that AM630 and SR144528 behaved as inverse agonists in CB₂-transfected cells.

100 nM, AM630 produced a significant dextral shift in the log concentration-response curve of CP55940 for inhibition of cyclic AMP production by CB₂-transfected cells that did not deviate significantly from parallelism (Figure 5). The mean value of this shift, with its 95% confidence limits shown in brackets, was 21.3 (3.5 and 649.4). Also shown in Figure 5 is the ability of the established CB₂ receptor antagonist, SR144528, to oppose CP55940-induced inhibition of cyclic AMP production by the same cell line. AM630 also shared the ability of SR144528 to produce a concentration-related inhibition of GTP γ S binding to membranes from CB₂-transfected cells (Figure 6). It was 7.4 times less potent than SR144528. However, the maximum degree of inhibition produced by the two compounds was essentially the same.

A further set of experiments with CB₂-transfected cells (Figure 7), showed that inhibition of forskolin-stimulated cyclic AMP production by 10 nM CP55940 ($85.5 \pm 3.4\%$; $n=5$) was concentration-dependently reversed by AM630. A 50% reversal was achieved by AM630 at a concentration (EC₅₀) of 128.6 ± 40.6 nM ($n=5$). This is the mean concentration of AM630 that increased forskolin-stimulated cyclic AMP production by CB₂-transfected cells to a level midway between that observed in the presence of 10 nM CP55940 and that observed in the absence of this agonist. A similar method has been used by Rinaldi-Carmona *et al.* (1998) to calculate the concentration of SR144528 that produces a 50% reversal of CP55940-induced inhibition of forskolin-stimulated cyclic AMP production by CB₂-transfected cells.

In CB₁-transfected cells, 1 μ M AM630 appeared to produce a 3 fold decrease in the potency of CP55940 for inhibition of forskolin-stimulated cyclic AMP production (Figure 4). However, this effect of AM630 was not statistically significant. Similarly, the inhibitory effect of 10 nM CP55940 on forskolin-stimulated cyclic AMP production by this cell line ($89.4 \pm 5.6\%$; $n=4$) appeared to be slightly reversed by 1 μ M AM630 although not by higher or lower concentrations of this compound (Figure 7). Again, this effect was not statistically significant. In contrast, it was possible to detect reversal of this inhibitory effect in CB₁-transfected cells by SR141716A. This compound produced a 50% reversal of the inhibitory effect of 10 nM CP55940 on forskolin-stimulated cyclic AMP production at 11.2 ± 3.1 nM ($n=5$).

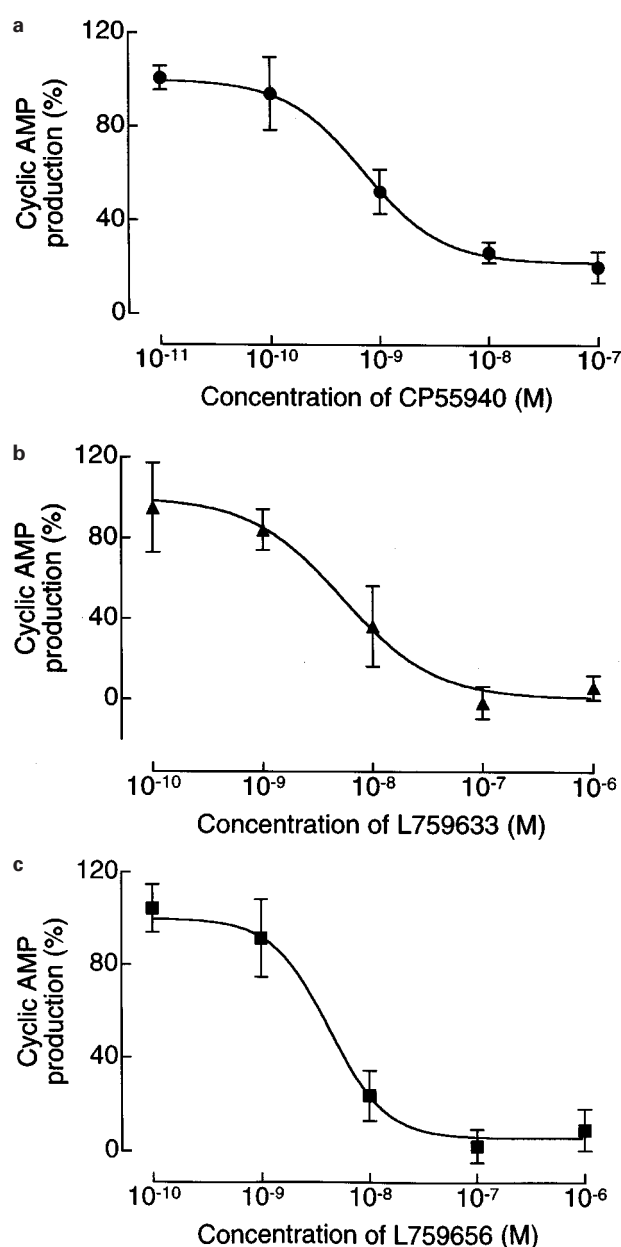


Figure 2 Inhibition by (a) CP55940 ($n=9$), (b) L759633 ($n=3$) and (c) L759656 ($n=4$) of forskolin-stimulated cyclic AMP production in CB₂-transfected CHO cells. Each symbol represents mean percentage change in forskolin-stimulated cyclic AMP production $\pm$ s.e.mean. Forskolin-stimulated cyclic AMP production in the absence of any cannabinoid has been normalized to 100%. Mean $E_{\max}$ values for CP55940, L759633 and L759656, with 95% confidence limits shown in brackets, were 21.9% (5.1 to 38.7), 0.5% (-25.3 to 26.3%) and 5.9% (-10.6 to 22.4%) respectively (GraphPad Prism).

Discussion

The results obtained showed that L759633 and L759656 bind with higher affinity to CB₂ than CB₁ receptors. Although this confirms previously reported binding data for these compounds (Gareau *et al.*, 1996), the CB₂/CB₁ affinity ratios of L759633 and L759656 were both higher in the previous investigation (793 and >1000 respectively) than reported here (163 and 414 respectively). Consistent with their binding properties, both L759633 and L759656 were markedly more potent as inhibitors of forskolin-stimulated cyclic AMP production in CB₂- than CB₁-transfected cells. Indeed, the CB₁/CB₂ EC_{50} ratios of both compounds in this functional

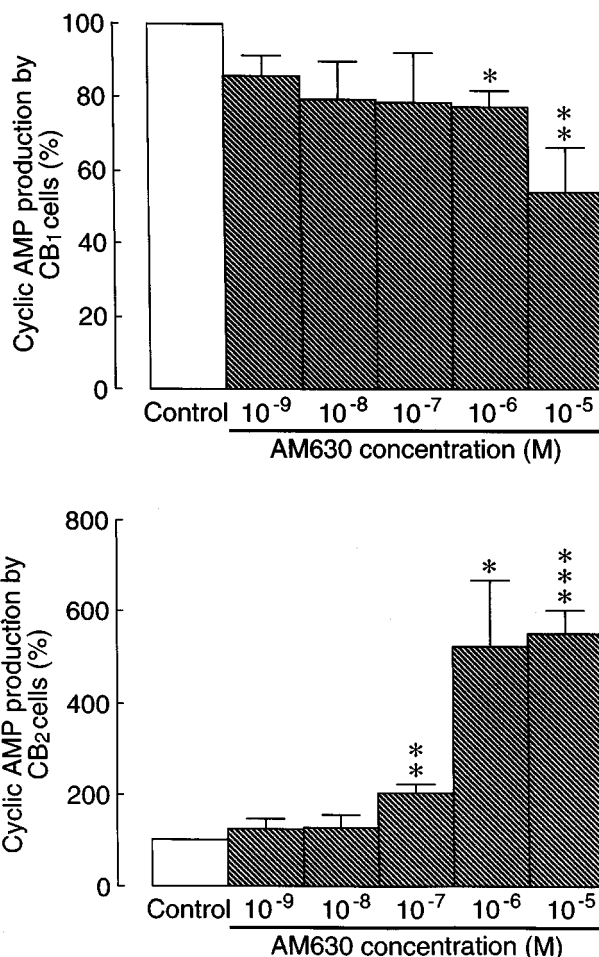


Figure 3 The effect of AM630 on forskolin-stimulated cyclic AMP production in CB₁ and CB₂-transfected CHO cells ($\pm$ s.e.mean; $n=3$ to 8). Each column represents mean percentage change in forskolin-stimulated cyclic AMP production $\pm$ s.e.mean. Forskolin-stimulated cyclic AMP production in the absence of AM630 has been normalized to 100%. Asterisks indicate values significantly less than 100% (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; one-sample t -test). The mean EC_{50} value of AM630 for enhancement of forskolin-stimulated cyclic AMP production in the CB₂-transfected cells with its 95% confidence limits shown in brackets is 230.4 nM (48.4 and 1096 nM) (GraphPad Prism).

bioassay (>1000 and >3000 respectively) were somewhat greater than their CB₂/CB₁ affinity ratios. Our results also confirm previous reports that CP55940 is a cannabinoid receptor agonist that acts with more or less equal potency at CB₁ and CB₂ receptors (Felder *et al.*, 1995). The ability of L759633 and L759656 to behave as CB₂-receptor agonists has not been reported previously. Clearly, both compounds will be useful pharmacological tools for the study of cannabinoid receptor pharmacology.

AM630 bound to both CB₁ and CB₂ receptors with a CB₂/CB₁ affinity ratio of 165, indicating it to have a similar selectivity for CB₂ binding sites as L759656. Its affinity for CB₂ receptors was 2.6 times less than that of L759656. The K_i value obtained for AM630 in these experiments with CB₂-transfected cell membranes (31.2 nM) is similar to a previously reported K_i value of AM630 (11.2 nM) for displacement of [³H]-CP55940 from a membrane preparation of mouse spleen (Pertwee, 1998), a tissue known to express CB₂ receptors (Munro *et al.*, 1993). Both these K_i values are similar to K_B values of AM630 reported by Pertwee *et al.* (1995) for antagonism of the inhibitory effects of CP55940 (17.3 nM) and WIN55212-2 (36.5 nM) on electrically-evoked contractions of the mouse

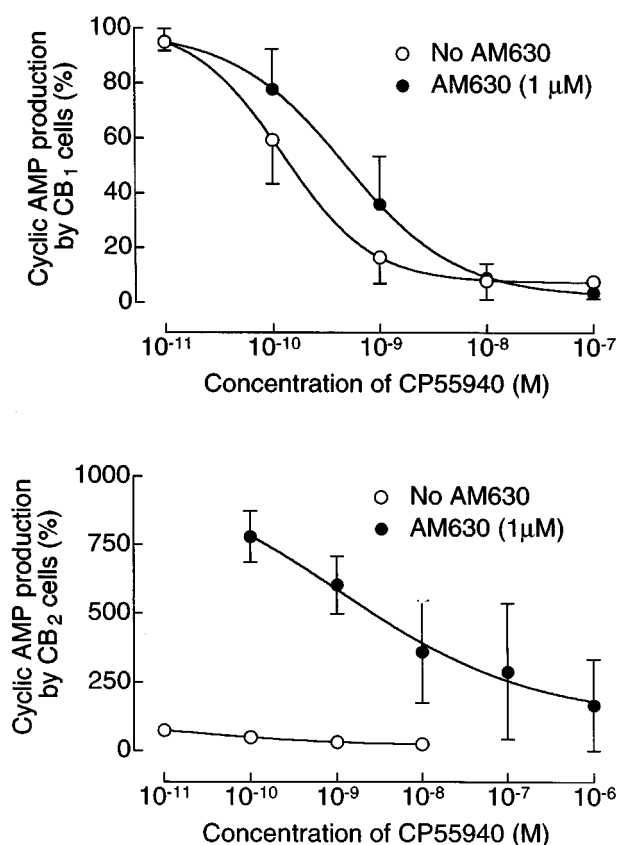


Figure 4 The effect of 1 μ M AM630 on CP55940-induced inhibition of forskolin-stimulated cyclic AMP production in CB₁ and CB₂-transfected CHO cells. Each symbol represents mean percentage change in forskolin-stimulated cyclic AMP production $\pm$ s.e.mean ($n=3$). Forskolin-stimulated cyclic AMP production in the absence of AM630 and CP55940 has been normalized to 100%. Mean EC₅₀ values of CP55940 in the CB₁-transfected cells with their 95% confidence limits shown in brackets are 0.5 nM (0.2 and 1.2 nM) in the presence of AM630 and 0.2 nM (0.1 and 0.4 nM) in its absence (GraphPad Prism).

isolated *vas deferens*, possibly reflecting the presence in this tissue of CB₂-like cannabinoid receptors that can mediate twitch inhibition (Griffin *et al.*, 1997).

Unlike L759633 and L759656, AM630 did not inhibit forskolin-stimulated cyclic AMP production by CB₂-transfected cells. Instead, it readily reversed the ability of CP55940 to produce this inhibitory effect with an EC₅₀ (128.6 nM) approximately one order of magnitude higher than the corresponding EC₅₀ value (10 nM) of the established CB₂-selective antagonist, SR144528 (Rinaldi-Carmona *et al.*, 1998). In addition, AM630 alone produced a concentration-dependent enhancement of forskolin-stimulated cyclic AMP production by CB₂-transfected cells. Possibly, AM630 is antagonizing a CB₂ receptor agonist that is being spontaneously released by this cell line. Another possibility is that the CB₂-transfected cells contain constitutively active CB₂ receptors in equilibrium with inactive CB₂ receptors and that AM630 is an inverse agonist that binds preferentially to the receptors in the inactive state thereby decreasing the proportion of constitutively active receptors. A similar mechanism has been proposed to explain the ability of the CB₂ receptor antagonist, SR144528, to enhance forskolin-stimulated cyclic AMP production by CB₂-transfected cells (Rinaldi-Carmona *et al.*, 1998). In line with both suggested mechanisms is our finding that AM630 shares the ability of SR144528 to inhibit GTP γ S binding to CB₂ receptors. Thus

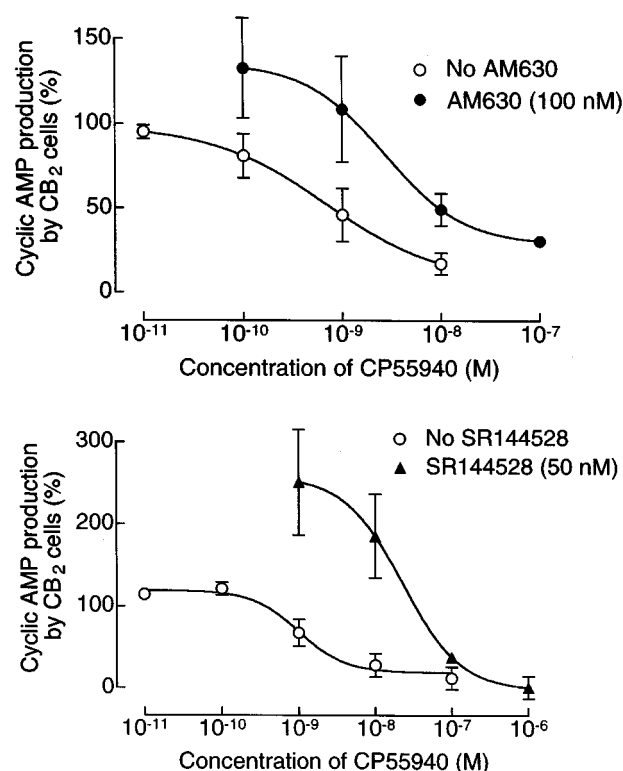


Figure 5 The effect of 100 nM AM630 or 50 nM SR144528 on CP55940-induced inhibition of forskolin-stimulated cyclic AMP production in CB₂-transfected CHO cells. Each symbol represents mean percentage change in forskolin-stimulated cyclic AMP production $\pm$ s.e.mean ($n=3$ or 4). Forskolin-stimulated cyclic AMP production in the absence of AM630, SR144528 and CP55940 has been normalized to 100%. The mean dextral shift in the log concentration-response curve of CP55940 produced by AM630 is 21.3 and its 95% confidence limits are 3.0 and 649.4 (symmetrical (2+2) dose parallel line assay). It did not deviate significantly from parallelism.

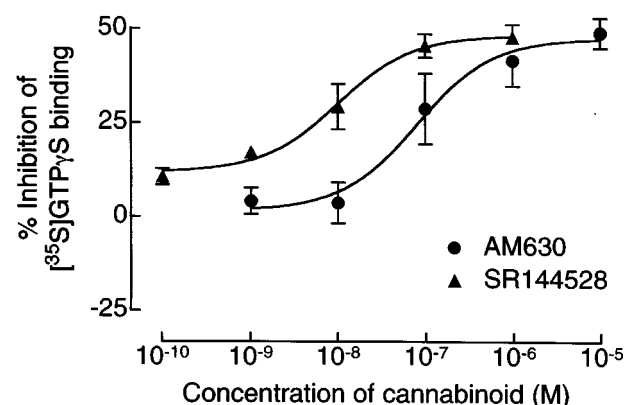


Figure 6 Effects of AM630 and SR144528 on specific binding of [³⁵S]-GTP γ S to CB₂-transfected CHO cell membranes. Each symbol represents mean percentage decrease in binding $\pm$ s.e.mean ($n=3$ to 5). The mean EC₅₀ values of AM630 and SR144528 for inhibition of [³⁵S]-GTP γ S binding with their 95% confidence limits shown in brackets are 76.6 nM (16.5 and 356.2 nM) and 10.4 nM (3.6 and 29.7 nM) respectively. Corresponding values for the maximum degree of inhibition produced are 47.2 $\pm$ 5.8% and 48.5 $\pm$ 2.9% respectively (mean E_{max} values $\pm$ s.e.mean; non-linear regression analysis; GraphPad Prism).

CB₂ receptors are G protein coupled (Pertwee, 1997) and it is to be expected that the binding of GTP γ S to G proteins will be stimulated by agonists for G protein-coupled receptors and

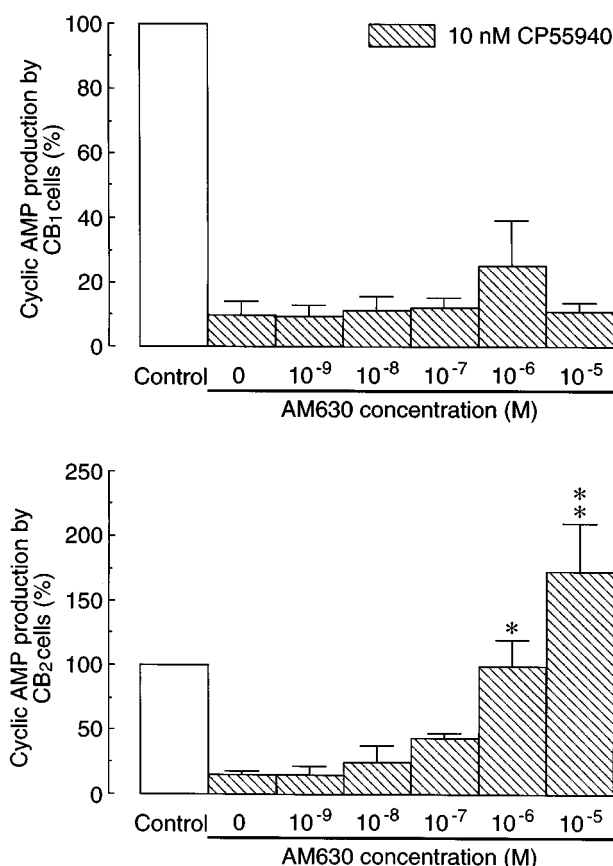


Figure 7 Effects of AM630 on inhibition of forskolin-stimulated cyclic AMP production by 10 nM CP55940 in CB₁- and CB₂-transfected CHO cells ($\pm$ s.e.mean; $n=4$ to 6). Each column represents mean percentage change in forskolin-stimulated cyclic AMP production. Forskolin-stimulated cyclic AMP production in the absence of AM630 and CP55940 has been normalized to 100%. Asterisks indicate significant differences from forskolin-stimulated cyclic AMP production in the presence of CP55940 and absence of AM630 (* $P<0.05$; ** $P<0.01$; Kruskal-Wallis test followed by Dunn's multiple comparison test).

inhibited by inverse agonists for such receptors (Bouaboula *et al.*, 1997; Breivogel *et al.*, 1997; MacLennan *et al.*, 1998). A third possibility is that AM630-induced enhancement of cyclic AMP production in CB₂-transfected cells is mediated by G_s proteins. However, AM630 had no effect on cyclic AMP production by CB₂-transfected cells in the absence of forskolin. Moreover, although there is evidence that, under certain conditions, CB₁ receptors may be coupled to adenylate cyclase through G_s proteins, there is also evidence that CB₂ receptors are not (Glass & Felder, 1997).

Although AM630 antagonizes CP55940 in CB₂-transfected cells, it shares the ability of CP55940 to inhibit forskolin-stimulated cyclic AMP production by CB₁-transfected cells,

albeit with an EC₅₀ above 1 μ M. This finding is consistent with a previous observation that AM630 behaves as a CB₁ receptor agonist in the myenteric plexus-longitudinal muscle preparation of guinea-pig small intestine with an EC₅₀ value of 1.9 μ M (Pertwee *et al.*, 1996), a potency value that is reasonably close to the K_i of AM630 for displacement of [³H]-CP55940 from CB₁-transfected cell membranes (Table 1). AM630 does not seem to behave as a CB₁ receptor agonist in all CB₁ receptor-containing preparations. Landsman *et al.* (1998) have reported that AM630 inhibits [³⁵S]-GTP γ S binding to CB₁-transfected cell membranes with an EC₅₀ of 0.9 μ M. This effect is opposite in direction to that of CB₁ receptor agonists which stimulate [³⁵S]-GTP γ S binding (Breivogel *et al.*, 1997; MacLennan *et al.*, 1998). Hosohata *et al.* (1997a,b) have found AM630 to antagonize cannabinoid-induced stimulation of [³⁵S]-GTP γ S binding to mouse and guinea-pig brain membrane preparations with K_B values of 3.1 and 9.3 μ M respectively. Thus it would seem that, depending on the CB₁ receptor-containing preparation used, AM630 can behave as a CB₁ receptor agonist, antagonist or inverse agonist. Further experiments are required to establish why this should be. It is noteworthy, however, that in the present investigation, a concentration of AM630 that inhibited forskolin-stimulated cyclic AMP production by CB₁-transfected cells when administered alone (1 μ M), appeared to produce a dextral shift in the log concentration-response curve of CP55940 for inhibition of forskolin-stimulated cyclic AMP production by CB₁-transfected cells. This dextral shift was not statistically significant. However, this observation does raise the possibility that high concentrations of AM630 may produce a significant degree of antagonism in this cell line in which case AM630 would possess the mixed agonist-antagonist properties of a CB₁ partial agonist.

In conclusion, our results confirm L759633 and L759656 to have significantly higher affinity for CB₂ than CB₁ receptors (see Introduction). In addition, they show that both these compounds are potent, high-efficacy CB₂ receptor agonists, that L759656 lacks significant activity as a CB₁ receptor agonist and that although L759633 can activate CB₁ receptors, it does so only at micromolar concentrations that are above those at which it is capable of interacting with CB₂ receptors. The pharmacological properties of AM630 are more complex. Thus, we found AM630 to behave as an inverse agonist at CB₂ receptors but as a partial agonist at CB₁ receptors. Further experiments are required to establish the structural features of AM630 responsible for each of these actions.

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