Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmaceutical Sciences 23 (2004) 233–243
EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES
www.elsevier.com/locate/ejps

Comparison of cannabinoid ligands affinities and efficacies in murine tissues and in transfected cells expressing human recombinant cannabinoid receptors

Sophie J. Govaerts[a], Emmanuel Hermans[b], Didier M. Lambert[a,*]

[a] *Unité de Chimie Pharmaceutique et de Radiopharmacie, Ecole de Pharmacie, Université catholique de Louvain, 73 Avenue E.Mounier, B-1200 Brussels, Belgium*
[b] *Laboratoire de Pharmacologie Expérimentale, Université catholique de Louvain, B-1200 Brussels, Belgium*

Received 3 April 2004; received in revised form 8 July 2004; accepted 26 July 2004
Available online 23 September 2004

Abstract

Affinities and efficacies of several reference cannabinoid ligands were investigated at central and peripheral cannabinoid receptors in three different species (rat, mouse, and human). The tested compounds belong to different chemical classes such as classical and non-classical terpene derivatives (Δ^8-THC, Δ^9-THC, HU 210, CP 55,940, CP 55,244, CP 55,243 and CP 47,947), aminoalkylindole (WIN 55,212-2, WIN 55,212-3) and diarylpyrazole cannabinoids (SR 141716A, SR 144528). As cannabinoid receptors have been shown to be mainly coupled to Gi/o type G- proteins, and by using the [^{35}S]-GTPγS nucleotide binding modulation, we characterized the functional activity of these ligands which can act as agonists (positive intrinsic activity), partial agonists (partial positive intrinsic activity), antagonists (no intrinsic activity), or inverse agonists (negative intrinsic activity). To our knowledge, some derivatives (Δ^8-THC, WIN 55,212-3, CP 55,243 and CP 47,947) have never been characterized in [^{35}S]-GTPγS binding assays and up to now, this study represents the largest survey of reference cannabinoids performed in unique experimental conditions and in the same laboratory.

Keywords: Cannabinoid CB_1 and CB_2 receptors; [^{35}S]-GTPγS; Tetrahydrocannabinol; Bicyclic cannabinoids; Aminoalkylindoles; Inverse agonism

1. Introduction

Since the discovery of Δ^9-tetrahydrocannabinol (Δ^9-THC) by Mechoulam in the 1960s (Gaoni and Mechoulam, 1964) as the main active constituent of the hemp *Cannabis sativa* L. extract, almost a quarter of the century has been needed to characterize the molecular pharmacology and the biochemistry of cannabinoid receptors. Nowadays, albeit some recent data have suggested that additional cannabinoid receptors might exist, two cannabinoid receptor subtypes have been described. The cannabinoid CB_1 receptor (Matsuda et al., 1990; Gérard et al., 1991), which was first evidenced by autoradiography and radioligand binding studies using [^{3}H]-CP 55,940 was cloned from rat, human and mouse tissues. It is expressed in the brain and some peripheral tissues including testis, ileum, urinary bladder and vas deferens. An alternative spliced form of the cannabinoid CB_1 receptor, christened CB_{1A}, has also been described, but so far, no peculiar property in terms of ligands recognition and receptor activation has been shown for this variant (Shire

Abbreviations: CHO, Chinese hamster ovary cells; CP 55,940, (−)-*cis*-3-[2-hydroxy-4-(l,l-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexan-1-ol; Gpp(NH)p, 5′-guanylylimidodiphosphate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); HU 210, (6α*R*)-*trans*-3-(l,l-dimethylheptyl)-6α,7,10,10α-tetrahydro-l-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol; PEI, polyethyleneimine; Δ^8-THC, Δ^8-tetrahydrocannabinol; Δ^9-THC, Δ^9-tetrahydrocannabinol; SR 141716A, *N*-(piperidin-l-yl)-5-(4-chlorophenyl)-l-(2,4-dichlorophenyl)-4-methyl-l*H*-pyrazole-3-carboxamide hydrochloride; WIN 55,212-2 mesylate, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[l,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone

* Corresponding author. Tel.: +32 2 764 73 47; fax: +32 2 764 73 63.
E-mail address: lambert@cmfa.ucl.ac.be (D.M. Lambert).

0928-0987/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejps.2004.07.013

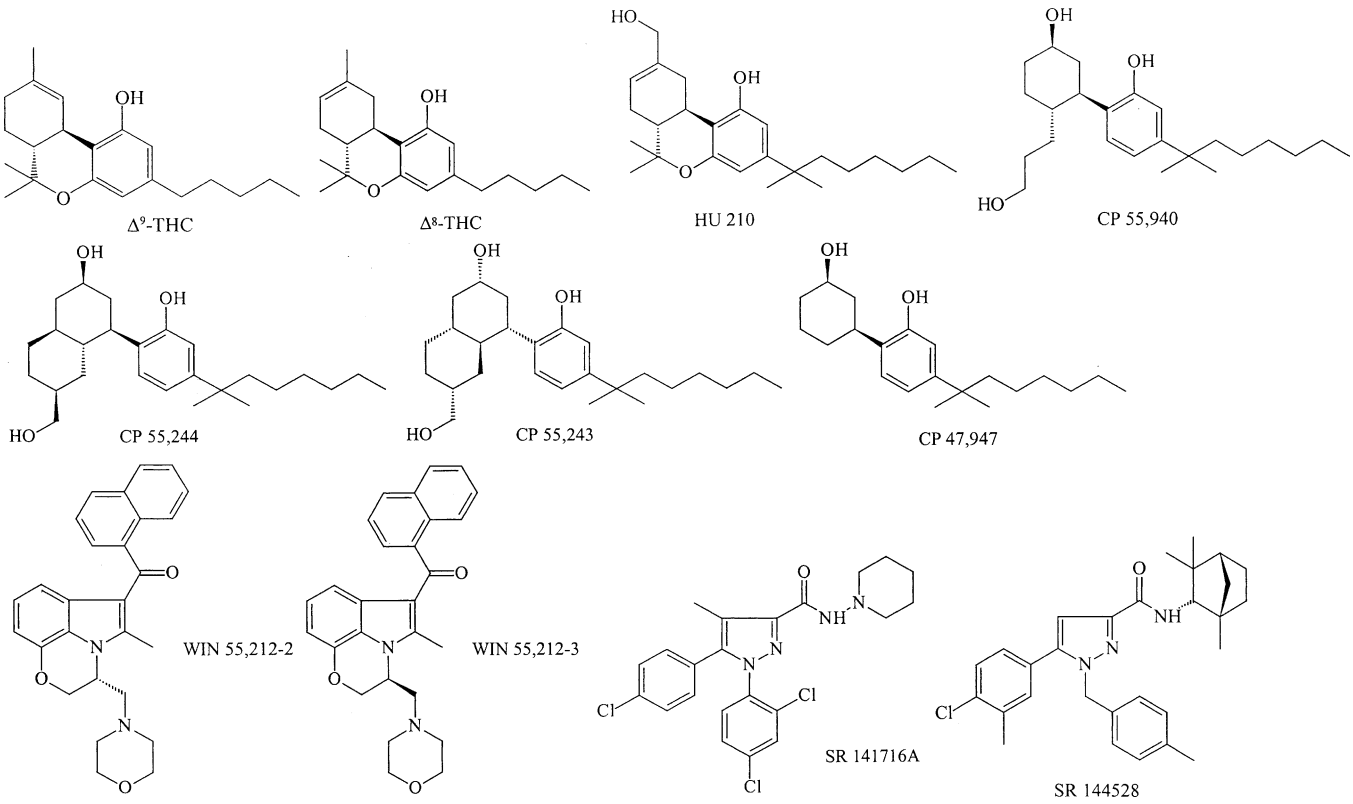

Fig. 1. Structures of the different reference cannabinoids used in this study. Δ^9-THC, Δ^8-THC, HU 210, CP 55,940, CP 55,244, CP 55,243 and CP 47,947 are classical and non-classical terpene derivatives, WIN 55,212-2 and WIN 55,212-3 are aminoalkylindole representatives and SR 141716A and SR 144528 are diarylpyrazole cannabinoids.

et al., 1995). The human cannabinoid CB_2 receptor was discovered by sequence homology (Munro et al., 1993); it was predominantly detected in the immune system (spleen, tonsils, immune cells) and further cloned from the mouse (Shire et al., 1996) and recently from the rat (Griffin et al., 2000; Brown et al., 2002).

Many authors used the [^{35}S]-GTPγS nucleotide binding modulation to characterize the coupling of G-protein coupled receptors, specially when the receptors were coupled to Gi/o or Gs proteins (Milligan, 2003; Harrison and Traynor, 2003). Briefly, this derived binding technique allowed to characterize the activity of ligands at G-protein coupled receptors, including cannabinoid receptors (Sim et al., 1995; Breivogel et al., 1998; for a review, Pertwee, 1999) but also alteration in the functional properties of receptors in several diseases (Berrendero et al., 2001). This assay constitutes a functional measure of the interaction of the receptor and the G-protein, the first step in activation of the G-protein coupled receptors. In addition, it is possible to define the functional activity of ligands as agonist (positive intrinsic activity), partial agonist (partial positive intrinsic activity), antagonist (no intrinsic activity), and inverse agonist (negative intrinsic activity). The inverse agonist properties of the diarylpyrazole cannabinoids, SR 141716A (Rinaldi-Carmona et al., 1994) and SR 144528 (Rinaldi-Carmona et al., 1998), previously considered as cannabinoid antagonists were evaluated using this approach (Bouaboula et al., 1997; Rinaldi-Carmona et al., 1998; MacLennan et al., 1998; Portier et al., 1999).

The aim of this study was to characterize the affinities and efficacies of several cannabinoid ligands (Fig. 1) belonging to different chemical classes such as classical and non-classical terpene derivatives (Δ^8-THC, Δ^9-THC, HU 210, CP 55,940, CP 55,244, CP 55,243 and CP 47,947), aminoalkylindole (WIN 55,212-2, WIN 55,212-3) and diarylpyrazole cannabinoids (SR 141716A, SR 144528) and to measure their influence in the binding of [^{35}S]-GTPγS. To our knowledge, some derivatives (Δ^8-THC, WIN 55,212-3, CP 55,243 and CP 47,947) have never been characterized in this assay. Up to now, this study constitutes the largest survey of cannabinoid ligand affinities and efficacies performed in unique experimental conditions.

2. Materials and animals

2.1. Drugs and chemical reagents and apparatus

[^{3}H]-SR 141716A (1.92 TBq/mmol, 52 Ci/mmol) and [^{35}S]-GTPγS (370 MBq/ml, 1101 Ci/mmol) were from Amersham (Roosendaal, The Netherlands). [^{3}H]-CP 55,940 (3.737 TBq/mmol, 101 Ci/mmol) and [^{3}H]-WIN 55,212-2 (1879.6 GBq/mmol, 50 Ci/mmol) were

from New England Nuclear (Boston, MA). Fatty acid free bovine serum albumin, dithiothreitol, GDP and Gpp(NH)p, Δ^9-tetrahydrocannabinol (Δ^9-THC) and Δ^8-tetrahydrocannabinol (Δ^8-THC) were purchased from Sigma (Boornem, Belgium). HU 210 was from Tocris Cookson (Bristol, UK). WIN 55,212-2 and WIN 55,212-3 were from Research Biochemicals International (Boornem, Belgium).

CP 55,940, CP 55,244, CP 55,243 and CP 47,947 were generously provided by Pfizer Inc (Groton, CT, USA). SR 141716A and SR 144528 were generous gifts from Dr. F. Barth and Dr. Mossé respectively, from Sanofi-Synthelabo Research (Montpellier, France). GF/B filters were purchased from K-Lab. Aqualuma was from Lumac-LSC (Schaesberg, The Netherlands). All other reagent grade chemicals were obtained from Sigma–Aldrich–Fluka (Boornem, Belgium). All tested compounds were dissolved at 10^{-2} M in dimethylsulfoxide. The final dimethylsulfoxide concentration was less than 0.1%. The cell harvester was a 48-well Brandell cell harvester (Semat, UK).

2.2. Preparation of membranes from rat cerebellum and/or mouse brain

Male Wistar rats (250–300 g) and mice (18–27 g, OF1-IOPS) were purchased from IFFA-CREDO (Les Oncins, France). All experiments on animals were approved by the local ethical committee and the housing conditions were as specified by the Belgian Law of 14 November 1993 on the protection of laboratory animals (agreement no. LA 1230315). Cerebella and/or brains were carefully dissected on ice. All the manipulations were performed at 0–4 °C. Rat cerebellum homogenates and mouse brain homogenates were prepared in 50 mM Tris–HCl pH 7.4 with a potter and a Dounce and the suspension was centrifuged at 400 × *g* for 10 min. The supernatants were collected and centrifuged at 39,000 × *g* for 10 min. The resulting pellets were resuspended in 50 mM Tris–HCl pH 7.4, homogenized and centrifuged again at 39,000 × *g* for 10 min. The pellets were washed twice more in the same conditions. The protein concentration was measured by the Bradford assay (Bradford, 1976), using Coomasie Blue (Biorad, Belgium), with bovine serum albumin as a standard.

2.3. Preparation from rat or mouse spleen membranes

Spleens from male Wistar rats (250–300 g) and from OF1-IOPS mice (18–27 g) were carefully dissected on ice after peritoneal incision. All the manipulations were performed at 0–4 °C. The spleens were cut in several pieces and placed in a 50 mM Tris–HCl pH 7.4 solution containing 3 mM $MgCl_2$, 1 mM EDTA and 0.5% bovine serum albumin. The preparation of membranes was performed according to the method described by Hillard et al. (1999).

2.4. Cell culture and preparation of CHO-CB_1h or CHO-CB_2h transfected cells membranes

Dr. P. Nokin and Dr. M. Detheux kindly provided CHO cells stably transfected with the cDNA sequences encoding either the human CB_1 or CB_2 cannabinoid receptors, respectively (Euroscreen S.A., Brussels, Belgium). The cells were grown in Nutrient mixture Ham's F12 supplemented with 10% fetal calf serum, 2.5 μg/ml fungizone, 100 U/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml G418. At confluence, cells were trypsinized and collected by centrifugation at 100 × *g* for 10 min. The following steps were performed at 0–4 °C. Pelleted cells were lysed in ice-cold 50 mM Tris–HCl pH 7.4, and the homogenates were centrifuged at 400 × *g* for 10 min. The pellets were resuspended in the same buffer, homogenized and centrifuged at 15,000 × *g* for 10 min. The resulting pellets (membranes) were washed twice more in the same conditions. The protein concentration was measured as indicated above.

2.5. Saturation and competitive binding assays

The competitive binding experiments were performed in presence of the appropriate radioligands at 1 nM ([^{3}H]-SR 141716A, [^{3}H]-CP 55,940, [^{3}H]-WIN 55,212-2) at 30 °C in siliconized plastic tubes on membranes from rat cerebellum (20 μg protein/tube), rat spleen (80 μg/tube), mouse brain (100 μg protein/tube), mouse spleen (80 μg/tube), or transfected CHO cells (40 μg protein/tube) resuspended in 1 ml (final volume) binding buffer (50 mM Tris–HCl, 5 mM $MgCl_2{\cdot}6H_2O$, 1 mM disodium EDTA, 0.5% (w/v) bovine serum albumin, pH 7.4). Competitors were present at varying concentrations and the non-specific binding of the radioligands was determined in the presence of 10 μM HU 210. After 1 h incubation, the suspension was rapidly filtered through 0.5% PEI pre-treated GF/B glass fiber filters on a 48-well cell harvester and washed twice with 3 ml ice-cold binding buffer without bovine serum albumin. Radioactivity on filters was measured with a Pharmacia Wallac 1410 β-counter by liquid scintillation in 10 ml Aqualuma (Lumac, Schaesberg, The Netherlands). Similar binding conditions were used when performing saturation binding assays with [^{3}H]-SR 141716A, [^{3}H]-CP 55,940 or [^{3}H]-WIN 55,212-2 which were used at 0.5–50, 0.05–10 and 0.9–30 nM, respectively. Assays were made in triplicates.

2.6. Binding of [^{35}S]-GTPγS

The binding experiment was performed at 30 °C in plastic tubes containing 20 μg protein resuspended in 1 ml (final volume) binding buffer (50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2{\cdot}6H_2O$, 1 mM disodium EDTA, 100 mM NaCl, 0.1% (w/v) bovine serum albumin) supplemented with 20 μM GDP and 0.01 nM–100 μM agonists or antagonists. The binding was initiated by the addition of [^{35}S]-GTPγS (0.05 nM final concentration). Incubations were performed for 1 h and

Table 1
Determination of K_D and B_{max} values on membranes from rat and mouse cerebella and spleens, and from human CB_1 and CB_2 receptors expressed in transfected CHO cells

Species, source	Radioligand used (1 nM final)	B_{max} (pmol/mg proteins)	K_D (nM)
CB_1			
Rat, cerebellum	[^{3}H]-CP 55,940	1.40 ± 0.33	2.08 ± 0.32
	[^{3}H]-SR 141716A	3.32 ± 0.12	3.13 ± 0.16
Human, CB_1-CHO cells	[^{3}H]-CP 55,940	12.26 ± 4.14	4.73 ± 1.32
	[^{3}H]-SR 141716A	43.79 ± 8.36	13.90 ± 2.48
Mouse, cerebellum	[^{3}H]-WIN 55,212-2	0.81 ± 0.04	2.57 ± 0.73
CB_2			
Rat, spleen	[^{3}H]-CP 55,940	0.71 ± 0.02	2.18 ± 0.46
Human, CB_2-CHO cells	[^{3}H]-WIN 55,212-2	109.34 ± 10.02	12.02 ± 1.29
Mouse, spleen	[^{3}H]-CP 55,940	0.31 ± 0.03	3.35 ± 0.45

Data shown are mean ± S.E.M. from three to six experiments performed in duplicates.

were terminated by addition of 3 ml of ice-cold washing buffer (50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2{\cdot}6H_2O$, 1 mM disodium EDTA, 100 mM NaCl). The suspension was immediately filtered through GF/B filters using a 48-well cell harvester and washed twice with ice-cold binding buffer. Radioactivity trapped on the filters was counted as mentioned above. The non-specific binding was measured in the presence of 100 μM Gpp(NH)p. Assays were made in triplicates.

2.7. Data analysis

Radioligand and nucleotide binding data were analysed by non-linear regression with the software GraphPad Prism, Version 3.00 (GraphPad, San Diego, CA).

3. Results

3.1. Quantification of cannabinoid receptors

Before determining the relative affinities of the cannabinoid ligands, the quantification of cannabinoid receptors was performed using commercially available radioligands in the three species (rat, mouse, human). In the latter case, two recombinant human cannabinoid receptors stably expressed in the CHO cell line were used. All the data were summarized in Table 1. In adult rat cerebella membranes, where only cannabinoid CB_1 receptors are abundant, saturation experiments were performed using two radioligands: [^{3}H]-SR 141716A and [^{3}H]-CP 55,940. The antagonist, [^{3}H]-SR 141716A, labelling both the active and inactive cannabinoid CB_1 receptors (Kearn et al., 1999), allowed to detect a single population of binding site with a K_D value of 3.13 ± 0.16 nM and a B_{max} value of 3.32 ± 0.12 pmol/mg protein (n = 3). Under the same conditions, a K_D value of 2.08 ± 0.32 nM and a B_{max} value of 1.40 ± 0.33 pmol/mg protein (n = 3) were obtained when the cannabinoid radioligand agonist [^{3}H]-CP 55,940 at 1 nM was used.

Using the recombinant human cannabinoid CB_1 receptors, higher densities of cannabinoid receptors were measured using both radioligands yielding B_{max} values 9–13 times higher than the ones determined in rat cerebella, reflecting the efficacy of transfection and expression. The comparison between the data obtained using two different radioligands gave a similar situation than the one observed in the rat cerebella: the measured B_{max} values were significantly higher using the [^{3}H]-SR 141716A, compared to those obtained with [^{3}H]-CP 55,940. The density of cannabinoid receptors measured using a radioligand agonist, the [^{3}H]-WIN 55,212-2, in the mouse brain was almost similar to the one in the rat.

The cannabinoid CB_2 receptors have been studied in the three same species; the spleen was used as a source of cannabinoid CB_2 receptors for the rat and the mouse. Unfortunately, the CB_2 selective radioligand antagonist [^{3}H]-SR 144528 was not commercially available albeit previously described (Seltzman et al., 2001) and in consequence, the non-selective cannabinoid radioligand agonists [^{3}H]-CP 55,940 and [^{3}H]-WIN 55,212-2 were used (Table 1). The expression of the cannabinoid receptors in the spleen was significantly higher in the rat compared to the mouse. The density of cannabinoid CB_2 receptors expressed in CHO-cells was very high (B_{max} value = 109.34 ± 10.02 pmol/mg proteins).

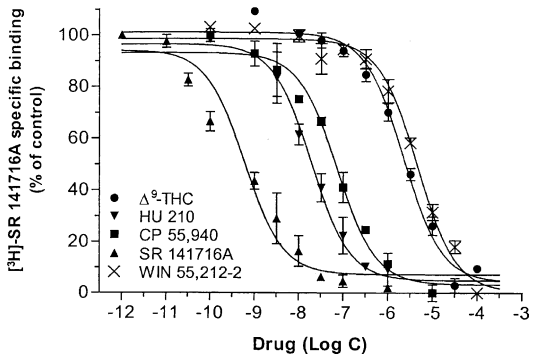

Fig. 2. Effect of HU 210 (▼), CP 55,940 (■) WIN 55,212-2 (×), Δ^9-THC (●) and SR 141716A (▲) on [^{3}H]-SR 141716A binding on rat cerebella homogenates. Data are the mean from at least three separate experiments, vertical lines show S.E.M.

Table 2
Determination of the affinities of reference cannabinoids (expressed in pK_i) on membranes from rat and mouse cerebella and spleens, and from human cannabinoid CB_1 and CB_2 receptors expressed in transfected CHO cells using [^{3}H]-SR 141716A (CB_1r), [^{3}H]-CP 55,940 (CB_1h, CB_2r and CB_2m) and [^{3}H]-WIN 55,212-2 (CB_1m and CB_2h)

	CB_1r (cerebellum)	CB_1m (cerebellum)	CB_1h (CB_1-CHO)	CB_2r (spleen)	CB_2h (CB_2-CHO)	CB_2m (spleen)	Selectivity (CB_1h–CB_2h)
Radioligands	[^{3}H]-SR 141716A	[^{3}H]-WIN 55,212-2	[^{3}H]-CP 55,940	[^{3}H]-CP 55,940	[^{3}H]-WIN 55,212-2	[^{3}H]-CP 55,940	ΔpK_i
Compounds							
HU 210	8.56 ± 0.13	9.49 ± 0.26	8.76 ± 0.06	7.35 ± 0.22	8.84 ± 0.26	7.25 ± 0.19	−0.08
CP 55,940	7.42 ± 0.08	9.52 ± 0.13	8.18 ± 0.02	N.D.	7.95 ± 0.07	N.D.	0.23
CP 55,244	N.D.	9.59 ± 0.03	8.99 ± 0.08	N.D.	8.62 ± 0.15	N.D.	0.37
CP 55,243	N.D.	N.D.	5.15 ± 0.04	N.D.	5.12 ± 0.05	N.D.	0.03
CP 47,947	N.D.	N.D.	7.21 ± 0.02	N.D.	7.04 ± 0.13	N.D.	0.17
WIN 55,212-2	6.55 ± 0.16	8.15 ± 0.28	6.89 ± 0.06	8.74 ± 0.43	7.78 ± 0.03	8.31 ± 0.42	−0.89
WIN 55,212-3	N.D.	N.D.	4.98 ± 0.08	N.D.	4.44 ± 0.38	N.D.	0.54
Δ^8-THC	N.D.	6.91 ± 0.15	6.60 ± 0.04	N.D.	6.38 ± 0.10	N.D.	0.22
Δ^9-THC	6.68 ± 0.33	7.01 ± 0.18	7.49 ± 0.13	N.D.	6.51 ± 0.15	N.D.	0.98
SR 141716A	8.91 ± 0.10	8.03 ± 0.25	7.48 ± 0.16	5.79 ± 0.74	5.67 ± 0.15	5.77 ± 0.31	1.81
SR 144528	N.D.	N.D.	6.15 ± 0.22	7.06 ± 0.21	7.46 ± 0.08	7.13 ± 0.50	−1.31

Data shown are mean ± S.E.M. from three to seven experiments performed in duplicates. N.D.: not determined. The selectivity has been determined by comparing the pK_i values obtained in CB_1h–CB_2h ($\Delta pK_i = pK_iCB_1h - pK_iCB_2h$).

3.2. Determination of the affinity on cannabinoid receptors

The affinities of a large set of cannabinoid ligands belonging to different chemical classes such as classical and non-classical terpene derivatives (Δ^8-THC, Δ^9-THC, HU 210, CP 55,940, CP 55,244, CP 55,243 and CP 47,947), aminoalkylindole (WIN 55,212-2, WIN 55,212-3) and diarylpyrazole cannabinoids (SR 141716A, SR 144528) were determined towards both types of cannabinoid receptors in the three different species (human, rat, mouse). The data are summarized in Table 2. The displacement curves obtained for these compounds in the [^{3}H]-SR 141716A radioligand competitive assay on rat cerebella homogenates, in the [^{3}H]-CP 55,940 radioligand competitive assay on the human cannabinoid CB_1 transfected CHO homogenates (hCHO-CB_1) and in the [^{3}H]-WIN 55,212-2 radioligand competitive assay on the hCHO-CB_2 homogenates are shown in Figs. 2, 3A and B and 4A and B, respectively.

As Kearn et al. (1999) have shown that [^{3}H]-SR 141716A, an inverse agonist, labelled the active and inactive cannabinoid CB_1 receptors while the agonist [^{3}H]-CP 55,940 labelled only the active cannabinoid CB_1 receptors, selected compounds (HU 210, CP 55,940, SR 141716A and WIN 55,212-2) were deeply studied using these two different radioligands, i.e. [^{3}H]-CP 55,940 and [^{3}H]-SR 141716A in the human cannabinoid CB_1 transfected CHO cells. According to these authors, the ΔpK_i obtained by the relation $\Delta pK_i = pK_{i([^3H]\text{-CP 55,940})} - pK_{i([^3H]\text{-SR 141716A})}$ gave a first indication on the function of the ligands: positive ΔpK_i were observed for agonists, while negative values ΔpK_i were observed for inverse agonists. The respective pK_i – the ΔpK_i are indicated into brackets – were: HU 210 = 8.76 ± 0.06 and 7.74 ± 0.10 [1.02]; CP 55,940 = 8.18 ± 0.02 and 7.21 ± 0.17 [0.97]; WIN 55,212-2 = 6.89 ± 0.06 and 5.37 ± 0.03 [1.52]; SR 141716A = 7.48 ± 0.16 and 8.62 ± 0.16 [−1.14].

The selectivity towards the human cannabinoid receptors has also been determined (Table 2). Considering a difference

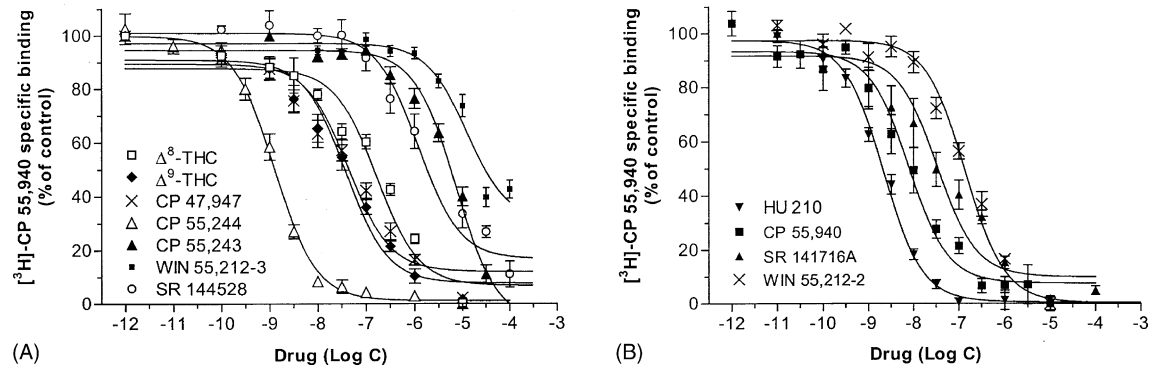

Fig. 3. (A) Effect of Δ^8-THC (□), Δ^9-THC (♦), WIN 55,212-3 (■), CP 47,947 (×), CP 55,244 (△), CP 55,243 (▲) and SR 144528 (○) on [^{3}H]-CP 55,940 binding on human cannabinoid CB_1 receptors expressed in CHO cells. Data are the mean from at least three separate experiments, vertical lines show S.E.M. (B) Effect of HU 210 (▼), CP 55,940 (■), WIN 55,212-2 (×) and SR 141716A (▲) on [^{3}H]-CP 55,940 binding on human cannabinoid CB_1 receptors expressed in CHO cells. Data are the mean from at least three separate experiments, vertical lines show S.E.M.

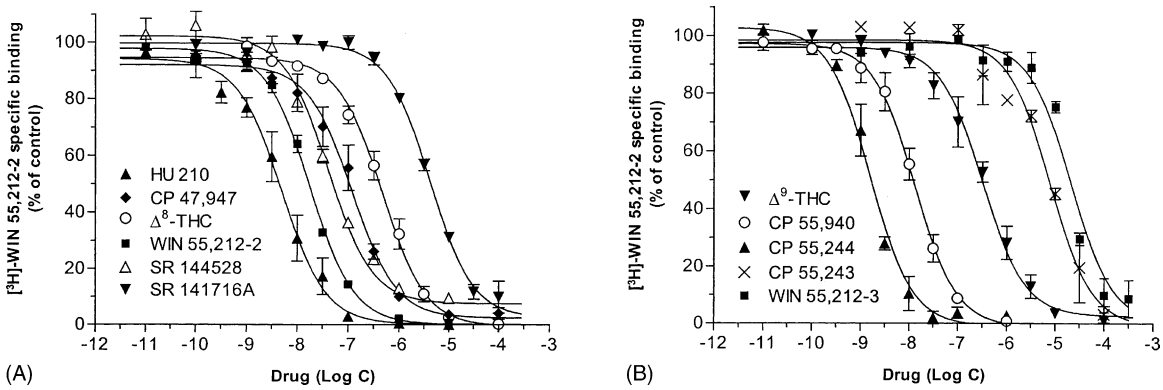

Fig. 4. (A) Effect of HU 210 (▲), CP 47,947 (♦), WIN 55,212-2 (■), Δ^8-THC (◯), SR 144528 (△) and SR 141716A (▼) on [^{3}H]-WIN 55,212-2 binding on human cannabinoid CB_2 receptors expressed in CHO cells. Data are the mean from at least three separate experiments, vertical lines show S.E.M. (B) Effect of CP 55,940 (◯), CP 55,243 (×), CP 55,244 (▲), WIN 55,212-3 (■) Δ^9-THC (▼) on [^{3}H]-WIN 55,212-2 binding on human cannabinoid CB_2 receptors expressed in CHO cells. Data are the mean from at least three separate experiments, vertical lines show S.E.M.

of one unit in the pK_i values, HU 210, CP 55,940, CP 55,244, CP 55,243, CP 47,947, and Δ^8-THC were found non selective cannabinoid ligands while Δ^9-THC and SR 141716A exhibited a CB_1, selectivity and SR 144528 showed a CB_2 selectivity (Figs. 3A and B and 4A and B). WIN 55,212-2 exhibited a slight preference for the cannabinoid CB_2 receptor with a difference of 0.89 in the pK_i values.

3.3. *Influence of cannabinoid ligands on the binding of [^{35}S]-GTPγS in rat cerebella membranes*

Optimization of cannabinoid agonist-induced [^{35}S]-GTPγS binding was performed using the reference cannabinoid agonist, HU 210. Maximal stimulation of [^{35}S]-GTPγS binding was obtained when the assay was performed with 20 μg protein resuspended in 1 ml (final volume) binding buffer supplemented with 20 μM GDP (range of GDP tested from 0 to 50 μM, data not shown). The effect of some representative cannabinoids on the binding of [^{35}S]-GTPγS to rat cerebella membranes is shown in Fig. 5A and B. The pharmacological parameters (maximal response and pEC_{50} values) obtained for a variety of natural and synthetic cannabinoid receptor agonists are summarized in Table 3. Among the drugs tested, HU 210, CP 55,940, WIN 55,212-2 and CP 55,244 behaved as full agonists, stimulating the [^{35}S]-GTPγS binding up to about 300% of basal. Partial responses were obtained using the natural cannabinoid ligands Δ^8-THC and Δ^9-THC (41.3 ± 5.9% and 47.6 ± 8.6% increase above basal). As expected, SR 141716A showed inverse agonist property as it decreased the nucleotide binding by 30.5 ± 2.1% as compared to basal, albeit this effect was modest compared to the one measured in transfected cells. However, contrasting with its nanomolar affinity for the cannabinoid receptor measured in radioligand binding assay, the potency of SR 141716A was in the submicromolar range (pEC_{50} of 6.34 ± 0.15). SR 144528, WIN 55,212-3 and CP 55,243 exhibited also inverse agonists properties.

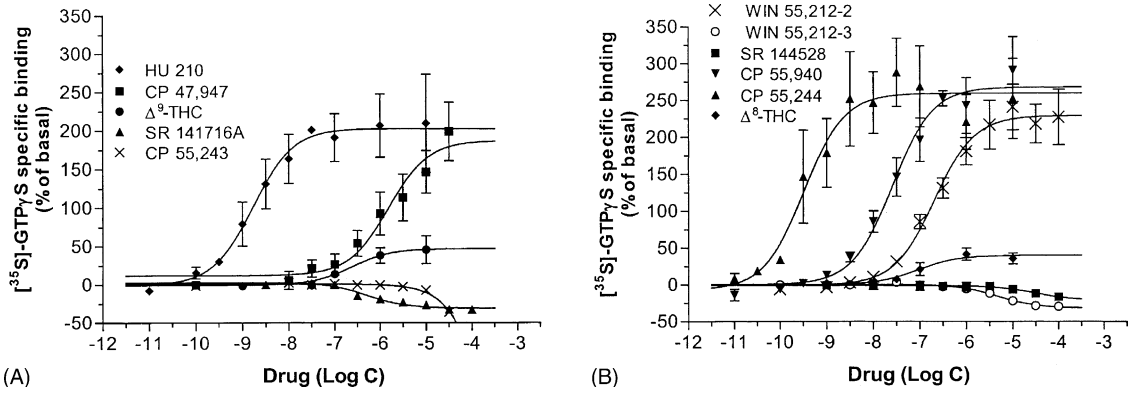

Fig. 5. (A) Effect of HU 210 (♦), CP 47,947 (■) (full agonists), Δ^9-THC (●) (partial agonist), CP 55,243 (×) and SR 141716A (▲) (inverse agonists) on [^{35}S]-GTPγS binding on rat cerebella homogenates. Data are the mean from at least three separate experiments, vertical lines show S.E.M. (B) Effect of CP 55,244 (▲), CP 55,940 (▼), WIN 55,212-2 (×) (full agonists), Δ^8-THC (♦) (partial agonist), WIN 55,212-3 (◯) and SR 144528 (■) (inverse agonists) on [^{35}S]-GTPγS binding on rat cerebella homogenates. Data are the mean from at least three separate experiments, vertical lines show S.E.M.

Table 3
Determination of potency (pEC_{50}) and percentage of basal maximal stimulation (E_{max}) of reference cannabinoids on membranes from rat cerebella and from human cannabinoid CB_1 and CB_2 receptors expressed in transfected CHO cells

Compounds	CB_1r		CB_1h		CB_2h	
	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
HU 210	8.77 ± 0.21	203 ± 14	10.11 ± 0.21	67 ± 10	9.22 ± 0.18	46 ± 6
CP 55,940	7.61 ± 0.05	260 ± 38	9.00 ± 0.58	60 ± 9	8.41 ± 0.10	57 ± 4
CP 55,244	9.03 ± 0.22	232 ± 42	10.14 ± 0.46	68 ± 16	8.97 ± 0.01	70 ± 14
CP 55,243	[a]	[a]	4.86 ± 0.19	−83 ± 10	6.34 ± 0.07	47 ± 8
CP 47,947	5.86 ± 0.17	185 ± 15	8.24 ± 0.35	30 ± 6	9.65 ± 0.12	49 ± 6
WIN 55,212-2	6.67 ± 0.10	232 ± 25	7.39 ± 0.13	68 ± 19	8.25 ± 0.12	32 ± 2
WIN 55,212-3	5.55 ± 0.16	−32 ± 2	5.40 ± 0.03	−43 ± 6	5.54 ± 0.19	−26 ± 4
Δ^8-THC	6.93 ± 0.23	41 ± 6	N.D.	N.D.	8.88 ± 0.18	−16 ± 4
Δ^9-THC	6.61 ± 0.08	48 ± 9	N.D.	N.D.	7.63 ± 0.18	−27 ± 1
SR 141716A	6.34 ± 0.15	−31 ± 2	8.00 ± 0.05	−84 ± 1	5.96 ± 0.02	−73 ± 1
SR 144528	4.56 ± 0.10	−21 ± 10	6.42 ± 0.09	−30 ± 3	8.67 ± 0.07	−69 ± 4

Data shown are mean ± S.E.M. from three to seven experiments performed in duplicates. N.D.: not determined.
[a] GraphPad Prism was not able to calculate neither pEC_{50} nor E_{max}.

3.4. Influence of cannabinoid ligands on the binding of [^{35}S]-GTPγS using transfected CHO cells expressing human recombinant cannabinoid receptors

The effects of cannabinoid ligands on the stimulation of the binding of [^{35}S]-GTPγS in homogenates of transfected CHO cells expressing a high level of either human cannabinoid CB_1 or CB_2 receptors are shown in Table 3. For the human cannabinoid CB_1 receptor (Fig. 6A and B) as well as for the rat CB_1 receptor (Fig. 5A and B), the ranking of the pEC_{50} was as follows: WIN 55,212-2 < CP 47,947 < CP 55,940 < HU 210 < CP 55,244. Except for CP 47,947, the E_{max} values of the agonists reached almost 70% of the basal maximal stimulation using the recombinant CB_1 receptor model suggesting that CP 47,947 was the only partial agonist in this assay. Four compounds behaved as inverse agonists (SR 144528, WIN 55,212-3, CP 55,243 and SR 141716A), with the highest negative effect of the binding of [^{35}S]-GTPγS observed in this study: it varied from −30 to −84%. On the human cannabinoid CB_2 receptor (Fig. 7A and B), the efficacy of stimulation (E_{max}) by agonists was variable with the following ranking WIN 55,212-2 < HU 210–CP 47,947–CP 55,243 < CP 55,940 < CP 55,244. All the remaining compounds including Δ^8-THC and Δ^9-THC behaved as inverse agonists. Together, these results indicated that a same amplitude of response was observed between the two recombinant receptors models in both maximal positive and negative stimulations of [^{35}S]-GTPγS binding.

In contrast, in the rat cerebellum, even if all the ligands kept the same function, the results were different in terms of amplitude. The positive stimulation induced by agonists was by far higher, reaching 260% in E_{max} while the negative effect of inverse agonists was less pronounced approximately −30%.

4. Discussion

The aim of this study was to compare the affinities and efficacies of several reference cannabinoids. To our knowledge, some of them have not been characterized in the [^{35}S]-GTPγS binding assays and the whole study represents the

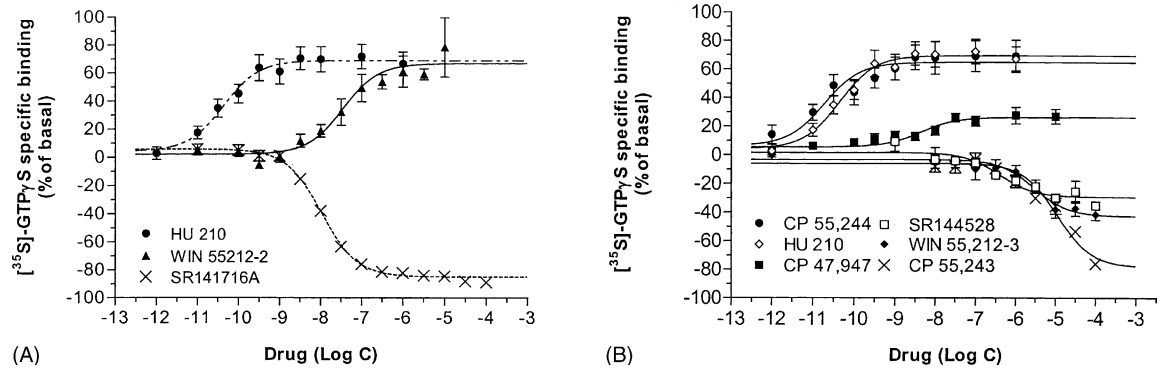

Fig. 6. (A) Effect of HU 210 (■), WIN 55,212-2 (▲) (full agonists) and SR 141716A (×) (inverse agonist) on [^{35}S]-GTPγS binding on human cannabinoid CHO-CB_1 cells homogenates. Data are the mean from at least three separate experiments, vertical lines show S.E.M. (B) Effect of HU 210 (◊), CP 55,244 (●) (full agonists), CP 47,947 (■) (partial agonist), WIN 55,212-3 (♦), CP 55,243 (×) and SR 144528 (□) (inverse agonists) on [^{35}S]-GTPγS binding on human CHO-CB_1 cells homogenates. Data are the mean from at least three separate experiments, vertical lines show S.E.M.

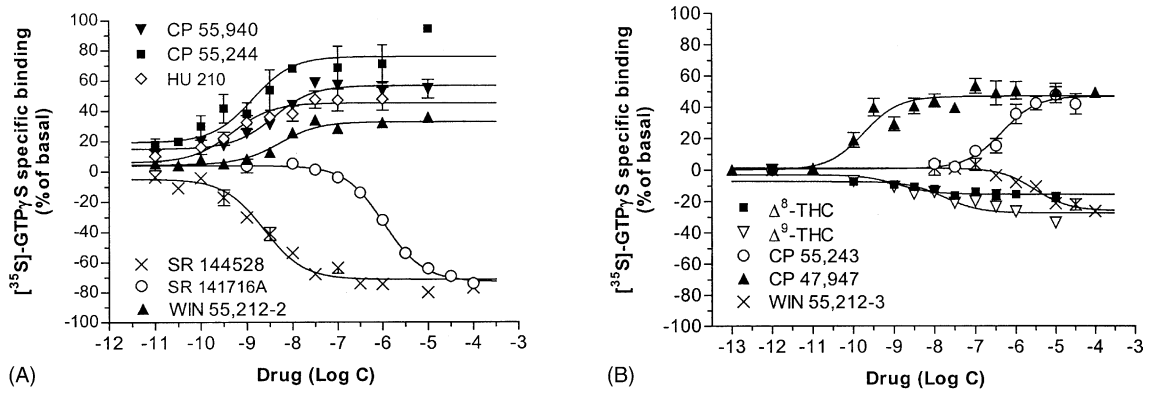

Fig. 7. (A) Effect of HU 210 (◊), WIN 55,212-2 (▲), CP 55,940 (▼), CP 55,244 (■) (full agonists), SR 141716A (◯) and SR 144528 (×) (inverse agonist) on [^{35}S]-GTPγS binding on human cannabinoid CB_2 receptors expressed in CHO cells. Data are the mean from at least three separate experiments, vertical lines show S.E.M. (B) Effect of CP 55,243 (◯), CP 47,947 (▲) (partial agonist), Δ^8-THC (■), Δ^9-THC (▽), WIN 55,212-3 (×) (inverse agonists) on [^{35}S]-GTPγS binding on human cannabinoid CB_2 receptors expressed in CHO cells. Data are the mean from at least three separate experiments, vertical lines show S.E.M.

largest comparison of reference cannabinoids performed in the same experimental conditions.

The commercial availability of different cannabinoid radioligands such as the non selective [^{3}H]-CP 55,940 and [^{3}H]-WIN 55,212-2 and the CB_1 inverse agonist [^{3}H]-SR 141716A allowed the characterization of the cannabinoid receptors in the different species. In rat cerebella homogenates, the level of cannabinoid receptors was around 1.40 pmol/mg proteins when [^{3}H]-CP 55,940 was used and 3.32 pmol/mg proteins when [^{3}H]-SR 141716A was used. In the hCHO-CB_1 preparation, the level of receptors was considerably higher and the labelling by [^{3}H]-SR 141716A was superior than the one performed with the agonist [^{3}H]-CP 55,940. Most of the ligands follow the same ranking of affinity for the cannabinoid CB_1 receptor in the mouse, rat and human. Slight differences were observed depending on the nature of the radioligand, the agonists exhibit higher pK_i values versus the agonist radioligand compared to the inverse agonist radioligand. This phenomenon was previously observed by Kearn et al. (1999). As described in the literature, most of the classical and non-classical cannabinoids do not show absolute selectivity for one subtype of cannabinoid receptor. The aminoalkylindole WIN 55,212-2 was found gently selective for the cannabinoid CB_2 receptor. At the opposite, Δ^9-THC exhibited some selectivity for the cannabinoid CB_1 receptor. The two inverse agonists SR 141716A and SR 144528 were the more selective compounds studied.

The use of high affinity, but non discriminating CB_1 and CB_2 receptor, radioligands such as [^{3}H]-CP 55,940 and [^{3}H]-WIN 55,212-2 for studying the cannabinoid CB_2 receptors in the spleen tissues merits some comments. Indeed, the mRNAs of both cannabinoid CB_1 and CB_2 receptors have been detected in the spleen, but it was reported that cannabinoid CB_2 receptors were, by far predominant in this tissue (Schatz et al., 1997). In the present study, the data strengthen this observation. Saturation binding studies using [^{3}H]-CP 55,940 revealed the presence of a single population of receptors with K_D and B_{max} values of 2.18 ± 0.46 nM and 0.71 ± 0.02 pmol/mg protein, respectively in the rat spleen; with K_D and B_{max} values of 3.35 ± 0.45 nM and 0.31 ± 0.03 pmol/mg protein, respectively in the mouse spleen (Table 1). Using the same rat spleen preparations, the specific binding of the cannabinoid CB_1 selective radioligand, [^{3}H]-SR 141716A, was almost undetectable (not shown). Competition studies indicated that compounds WIN 55,212-2, HU 210 and SR 144528 efficiently displaced the [^{3}H]-CP 55,940 specific binding (pK_i values of 8.74 ± 0.09, 7.40 ±0.15 and 7.06 ± 0.21, respectively) whereas similar displacement was only obtained with high concentrations of SR 141716A (pK_i value of 5.78 ± 0.05) (Table 2).

In the rat cerebellum [^{35}S]-GTPγS assay, as previously reported, WIN 55,212-2, CP 47,947, HU 210, CP 55,940 and CP 55,244 were characterized by a full agonist response and their potency was in agreement with those reported in the literature. The inverse agonist (negative intrinsic activity) property of SR 141716A first described in transfected CHO cells expressing the human cannabinoid CB_1 receptor (Bouaboula et al., 1997; Landsman et al., 1997) and also described in the rat (Kearn et al., 1999; Griffin et al., 1998) was confirmed in our study. Thus, this compound decreased the nucleotide binding by 30.5% with an EC_{50} of 445 nM. This moderate potency of SR 141716A in decreasing the receptor intrinsic activity was in contrast with its particularly high (nanomolar) affinity measured in radioligand binding assays in the same model and in identical condition (buffer, temperature, etc.). Indeed, some discrepancy between cannabinoid agonist affinity and potency was previously reported (Breivogel and Childers, 2000). However, previous characterization of the inverse agonist properties of SR 141716A revealed a markedly higher potency (Pan et al., 1998).

In the nucleotide binding assay, the natural constituents of hemp *Cannabis sativa*, Δ^8-THC and Δ^9-THC, showed weak efficacy, confirming that they act as partial agonists at the rat cannabinoid CB_1 receptor. This is in accordance with Sim et al. (1996) who previously showed the partial activation of the rat cerebellar cannabinoid CB_1 receptor by Δ^9-THC using

the same experimental approach, before Petitet et al. (1998) confirmed it. Burkey et al. (1997b) showed the same effect with mouse brain cannabinoid CB_1 (Burkey et al., 1997a,b). In this connection, Shen and Thayer (1999) have presented biochemical evidence that Δ^9-THC acts as a partial agonist to modulate glutamatergic synaptic transmission in rat neurones. Here, we presented data showing that Δ^8-THC, differing by the double bond position from Δ^9-THC, also acted as a partial agonist. The partial agonist properties of major active ingredients of the plant – Δ^9-THC and Δ^8-THC – might have some relevance in human clinics, regarding to its mild addiction. Martin et al. (1999) suggested that the side-chain of tetrahydrocannabinol was the key structural feature to delineate between the agonists, partial agonists and antagonists. Indeed, all the cannabinoids studied here and exhibiting a dimethylheptyl chain were able to stimulate the [^{35}S]-GTPγS binding and acted as full agonists. Even CP 55,940 previously reported by Griffin et al. (1998) as a partial agonist was, in our hands, able to robustly activate the receptor with an E_{max} of 260 ± 38% and fulfilled the criteria of an agonist. Interestingly, the so-called "inactive enantiomer" WIN 55,212-3, albeit a weak affinity, exhibited a significant inverse agonist effect.

Using the hCHO-CB_2 assay, several issues needed to be addressed. First, all the agonists approximately displayed the same efficacy with E_{max} values between 32 and 70%. Second, all the compounds exhibiting inverse agonists properties might be divided in two groups. The less active enantiomer WIN 55,212-3 and the natural cannabinoids Δ^8-THC and Δ^9-THC showed a weak negative E_{max} value around 30%, while the two diarylpyrazole SR 141716A and SR 144528 were more efficient in the suppression of the constitutive activity of the cannabinoid receptor. Interestingly, our study, to our knowledge, constituted the first report that the natural cannabinoids acted as inverse agonists at the human cannabinoid CB_2 receptor, using the [^{35}S]-GTPγS binding assay. Based on results of adenylate cyclase activities, a partial agonism of Δ^9-THC was first proposed by Bayewitch et al. (1995) for the cannabinoid CB_2 receptor. One year later, the same team reported an antagonist activity of Δ^9-THC. These authors showed that Δ^9-THC antagonised the agonist-induced inhibition of adenylyl cyclase mediated by the cannabinoid CB_2 receptor (Bayewitch et al., 1996). Utilizing CHO and COS cells transfected with the cannabinoid receptors, respectively with the rat and the human sequences for the cannabinoid CB_1 and CB_2 receptors, they showed that, even if Δ^9-THC bound to both receptors with similar affinity, it differently acted at the transduction level. However, in contrast to its capacity to serve as an agonist for the cannabinoid CB_1 receptor, Δ^9-THC was only able to induce a modest inhibition of adenylyl cyclase at the cannabinoid CB_2 receptor.

Regarding another cannabinoid, the behaviour of the 'less active enantiomer' CP 55,243 differed from the one of the 'most active' CP 55,244. In presence of rat or human cannabinoid CB_1 receptors as well as in presence of cannabinoid CB_2 receptors, CP 55,244 showed agonist properties, while CP 55,243 exhibited inverse agonist properties at the CB_1 and partial agonist properties at the CB_2.

The observation that the amplitude of the constitutive activity and consequently of the inverse agonist properties were different in the models studied here raised, once again, the question of the relevance of the inverse agonism in physiological conditions. Costa and Herz (1989) originally found that a delta opioid antagonist ICI 174864 modified the basal levels of response in NG108-15 cells, a phenomenon which was abolished by another opioid receptor antagonist. The concept of inverse agonism was born. Albeit it is not limited to G-proteins coupled receptors, it is now widely evidenced. In a recent review, Terry Kenakin surveyed the activity of 380 antagonists on 73 G-protein coupled receptors: 322 were inverse agonists and 58 were found neutral antagonists (Kenakin, 2004, see also supplemental table). Among the G-proteins coupled receptors, the inverse agonism at cannabinoid receptors has also been described and was recently reviewed (Pertwee, 2003). Therein, the author summarized the inverse cannabimimetic effects produced by SR 141716A both in vitro and in vivo and discussed the inverse agonism in terms of "two-state" and "three-state" model of G-protein coupled receptor.

Here, we showed that rat cerebellum and human cannabinoid receptors expressed in CHO cells constituted two different models for studying the cannabinoid receptors. Both models shared some characteristics: constitutive activity and inverse agonism were present, with, however, differences in amplitude regarding the positive and negatives responses. In few words, the rat cerebellum seemed to be a model where the cannabinoid receptor exhibited a relatively modest constitutive activity, estimated by the negative effects of SR 141716A (−30%) but was also prone to be highly activated by agonists (E_{max} almost reached 250%). The observation that the density of cannabinoid receptors was, by five-fold, higher when it was measured by [^{3}H]-SR 141716A rather by [^{3}H]-CP 55,940 was also in accordance with a model of modest constitutive activity. The human cannabinoid receptor expressed in CHO cells constituted a model with a high receptor constitutive activity, a pronounced negative effect induced by inverse agonists and a relatively modest stimulation of [^{35}S]-GTPγS binding induced by agonists. The inverse agonism is, at least for the human cannabinoid CB_1 receptor, a molecular property. Indeed, Hurst et al. (2002) elegantly demonstrated that the lysine residue at 3.28 (192) is playing a pivotal role for the inverse agonist properties of SR 141716A. In addition, in a study aiming to demonstrate the existence of neutral antagonists of the rat cannabinoid receptors (Govaerts et al., 2004), we showed some evidence using CHO cells expressing different densities of cannabinoid receptors that the density of receptors also played a major role in the amplitude of the responses of full agonists but did not modify, in a dramatic extent, the negative effects of SR 141716A. The cannabinoid agonist HU 210 was able to positively stimulate the [^{35}S]-GTPγS in both clones; however, the stimulation

was the highest when the receptor density was the lowest: the E_{max} expressed percentages of stimulation above basal dropped from 270% (similar to the value observed here using the rat cerebellum) to approx. 70%, when the density of receptors increased. Despite the numerous evidences of inverse agonism and constitutive activity at cannabinoid receptors, the physiological relevance of inverse agonism remains to be fully understood. Very recently, Savinainen et al. (2003) reported an optimised approach to study endocannabinoid signalling which provides evidence against constitutive activity of cannabinoid CB_1 receptors. Starting from the observation that SR 141716A acts as specific and selective antagonists of the cannabinoid CB_1 receptor at nanomolar concentrations, but in the micromolar range, these compounds were shown to inhibit basal G-protein activity, and this is often interpreted in terms of constitutive activity at the cannabinoid CB_1 receptors in native tissue, they showed that micromolar concentrations of SR 141716A and AM251 inhibit basal G-protein activity in rat cerebellar membranes, but only in conditions where tonic adenosine A, receptor signalling was not eliminated. In our rat cerebella preparations, such conditions were not used; in addition, the inverse agonism properties were more pronounced in both types of CHO cells than in the murine tissues. Further studies should be addressed to confirm the presence or not of either an endocannabinoid signalling system or an adenosine signalling.

In conclusion, about the characterization of the ligands, this study provides a large set of experimental data measured under unique and similar experimental conditions. The choice of radioligands and more specially the function of the radioligands – i.e. antagonists/inverse agonists or agonists – appeared to be a crucial parameter for the determination of the cannabinoid receptors densities. This was evidenced for rat cerebellar membranes by Kearn et al. (1999) but was extended in our study (i) to cannabinoid CB_1 receptors expressed in CHO cells as well as in mouse cerebellar membranes; (ii) to cannabinoid CB_2 receptors expressed in CHO cells and those present in murine tissues. As mentioned before, some compounds were never been characterized by the [^{35}S]-GTPγS binding assay. For instance, the so-called inactive cannabinoid enantiomers such as CP 55,243 and WIN 55,212-3 were not found as silent compounds in our hands. They were, sometimes, used as reference substances to demonstrate the implication or not of cannabinoid receptors. However, our present findings indicated that, depending the dose used, they might interfere with cannabinoid signaling.

Finally, most of the ligands studied here kept the same function at the different cannabinoid receptors: for instance, the agonists HU-210, WIN 55,212-2 and CP 55,940 on the one hand the inverse agonists SR 141716A and SR 144528 on the other hand. Some of them did not: this is the case for the natural cannabinoids Δ^8-THC and Δ^9-THC and the weak synthetic cannabinoid CP 55,943.

As natural cannabinoids are widely studied, their inverse agonist properties at the cannabinoid CB_2 receptors should be kept in mind, especially for the interpretation of results dealing with their effects on the immune responses. In addition, these two compounds, even they did not bind with high affinity to the cannabinoid receptors, constitute interesting templates for the precise understanding of the molecular origins of partial and inverse agonism properties at cannabinoid receptors.

Acknowledgements

DML wishes to thank the Université catholique de Louvain for providing a research FSR grant. SJG received a BFR grant from the Grand-Duchy of Luxemburg. E. Hermans is Senior Research Associate from the National Fund for Scientific Research (FNRS, Belgium).

References

Bayewitch, M., Avidor-Reiss, T., Levy, R., Barg, J., Mechoulam, R., Vogel, Z., 1995. The peripheral cannabinoid receptor: adenylate cyclase inhibition and G protein coupling. FEBS Lett. 375, 143–147.

Bayewitch, M., Rhee, M.H., Avidor-Reiss, T., Breuer, A., Mechoulam, R., Vogel, Z., 1996. (−)-Delta^9-tetrahydrocannabinol antagonizes the peripheral cannabinoid receptor-mediated inhibition of adenylyl cyclase. J. Biol. Chem. 271, 9902–9905.

Berrendero, F., Sanchez, A., Cabranes, A., Puerta, C., Ramos, J.A., Garcia-Merino, A., Fernandez-Ruiz, J., 2001. Changes in cannabinoid CB(1) receptors in striatal and cortical regions of rats with experimental allergic encephalomyelitis, an animal model of multiple sclerosis. Synapse 41, 195–202.

Bouaboula, M., Perrachon, S., Milligan, L., Canat, X., Rinaldi-Carmona, M., Portier, M., Barth, F., Calandra, B., Pecceu, F., Lupker, J., Maffrand, J.P., Le Fur, G., Casellas, P., 1997. A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. J. Biol. Chem. 272, 22330–22339.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Breivogel, C.S., Childers, S.R., 2000. Cannabinoid agonist signal transduction in rat brain: comparison of cannabinoid agonists in receptor binding, G-protein activation, and adenylyl cyclase inhibition. J. Pharmacol. Exp. Ther. 295, 328–336.

Breivogel, C.S., Selley, D.E., Childers, S.R., 1998. Cannabinoid receptor agonist efficacy for stimulating [^{35}S]GTPγS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. J. Biol. Chem. 273, 16865–16873.

Brown, S.M., Wager-Miller, J., Mackie, K., 2002. Cloning and molecular characterization of the rat CB_2 cannabinoid receptor. Biochim. Biophys. Acta 1576, 255–264.

Burkey, T.H., Quock, R.M., Consroe, P., Ehlert, F.J., Hosohata, Y., Roeske, W.R., Yamamura, H.I., 1997a. Relative efficacies of cannabinoid CB_1 receptor agonists in the mouse brain. Eur. J. Pharmacol. 336, 295–298.

Burkey, T.H., Quock, R.M., Consroe, P., Roeske, W.R., Yamamura, H.I., 1997b. Delta^9-Tetrahydrocannabinol is a partial agonist of cannabinoid receptors in mouse brain. Eur. J. Pharmacol. 323, R3–R4.

Costa, T., Herz, A., 1989. Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. Proc. Natl. Acad. Sci. U.S.A. 86, 7321–7325.

Gaoni, Y., Mechoulam, R., 1964. Isolation, structure, and partial synthesis of an active constituent of haschich. J. Am. Chem. Soc. 86, 1646–1647.

Gérard, C.M., Mollereau, C., Vassart, G., Parmentier, M., 1991. Molecular cloning of a human cannabinoid receptor which is also expressed in testis. Biochem. J. 279, 129–134.

Govaerts, S.J., Muccioli, G.G., Hermans, E., Lambert, D.M., 2004. Characterization of the pharmacology of imidazolidinedione derivatives at cannabinoid CB_1 and CB_2 receptors. Eur. J. Pharmacol. 495, 43–53.

Griffin, G., Tao, Q., Abood, M.E., 2000. Cloning and pharmacological characterization of the rat CB_2 cannabinoid receptor. J. Pharmacol. Exp. Ther. 292, 886–894.

Griffin, G., Atkinson, P.J., Showalter, V.M., Martin, B.R., Abood, M.E., 1998. Evaluation of cannabinoid receptor agonists and antagonists using the guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate binding assay in rat cerebellar membranes. J. Pharmacol. Exp. Ther. 285, 553–560.

Harrison, C., Traynor, J.R., 2003. The [^{35}S]GTPγS binding assay: approaches and applications in pharmacology. Life Sci. 74, 489–508.

Hillard, C.J., Manna, S., Greenberg, M.J., DiCamelli, R., Ross, R.A., Stevenson, L.A., Murphy, V., Pertwee, R.G., Campbell, W.B., 1999. Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB_1). J. Pharmacol. Exp. Ther. 289, 1427–1433.

Hurst, D.P., Lynch, D.L., Barnett-Norris, J., Hyatt, S.M., Seltzman, H.H., Zhong, M., Song, Z.H., Nie, J., Lewis, D., Reggio, P.H., 2002. *N*-(piperidin-l-yl)-5-(4-chlorophenyl)-l-(2,4-dichlorophenyl)-4-methyl-l*H*-pyrazole-3-carboxamide (SR 141716A) interaction with LYS 3.28(192) is crucial for its inverse agonism at the cannabinoid CB_1 receptor. Mol. Pharmacol. 62, 1274–1287.

Kenakin, T., 2004. Efficacy as a vector: the relative prevalence and paucity of inverse agonism. Mol. Pharmacol. 65, 2–11.

Kearn, C.S., Greenberg, M.J., DiCamelli, R., Kurzawa, K., Hillard, C.J., 1999. Relationships between ligand affinities for the cerebellar cannabinoid receptor CB_1 and the induction of GDP/GTP exchange. J. Neurochem. 72, 2379–2387.

Landsman, R.S., Burkey, T.H., Consroe, P., Roeske, W.R., Yamamura, H.I., 1997. SR 141716A is an inverse agonist at the human cannabinoid CB_1 receptor. Eur. J. Pharmacol. 334, R1–R2.

MacLennan, S.J., Reynen, P.H., Kwan, J., Bonhaus, D.W., 1998. Evidence for inverse agonism of SR 141716A at human recombinant cannabinoid CB_1 and CB_2 receptors. Br. J. Pharmacol. 124, 619–622.

Martin, B.R., Jefferson, R., Winckler, R., Wiley, J.L., Huffman, J.W., Crocker, P.J., Saha, B., Razdan, R.K., 1999. Manipulation of the tetrahydrocannabinol side chain delineates agonists, partial agonists, and antagonists. J. Pharmacol. Exp. Ther. 290, 1065–1079.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Milligan, G., 2003. Principles: extending the utility of [^{35}S]-GTPγS binding assays. Trends Pharmacol. Sci. 24, 87–90.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365, 61–65.

Pan, X., Ikeda, S.R., Lewis, D.L., 1998. SR 141716A acts as an inverse agonist to increase neuronal voltage-dependent Ca^{2+} currents by reversal of tonic CB_1 cannabinoid receptor activity. Mol. Pharmacol. 54, 1064–1072.

Pertwee, R.G., 1999. Pharmacology of cannabinoid receptor ligands. Curr. Med. Chem. 6, 635–664.

Pertwee, R.G., 2003. Inverse agonism at cannabinoid receptors. Int. Cong. Ser. 1249, 75–86.

Petitet, F., Jeantaud, B., Reibaud, M., Imperato, A., Dubroeucq, M.C., 1998. Complex pharmacology of natural cannabinoids: evidence for partial agonist activity of delta9-tetrahydrocannabinol and antagonist activity of cannabidiol on rat brain cannabinoid receptors. Life Sci. 63, PL1–PL6.

Portier, M., Rinaldi-Carmona, M., Pecceu, F., Combes, T., Poinot-Chazel, C., Calandra, B., Barth, F., Le Fur, G., Casellas, P., 1999. SR 144528 an antagonist for the peripheral cannabinoid receptor that behaves as an inverse agonist. J. Pharmacol. Exp. Ther. 288, 582–589.

Rinaldi-Carmona, M., Barth, F., Héaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Maruani, J., Néliat, G., Caput, D., Ferrara, P., Soubrié, P., Breliere, J.C., Le Fur, G., 1994. SR 141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett. 350, 240–244.

Rinaldi-Carmona, M., Barth, F., Millan, J., Derocq, J.M., Casellas, P., Congy, C., Oustric, D., Sarran, M., Bouaboula, M., Calandra, B., Portier, M., Shire, D., Breliere, J.C., Le Fur, G., 1998. SR 144528, the first potent and selective antagonist of the CB_2 cannabinoid receptor. 284, 644–650.

Savinainen, J.R., Saario, S.M., Niemi, R., Jarvinen, T., Laitinen, J.T., 2003. An optimized approach to study endocannabinoid signaling: evidence against constitutive activity of rat brain adenosine A_1 and cannabinoid CB_1 receptors. Br. J. Pharmacol. 140, 1451–1459.

Schatz, A.R., Lee, M., Condie, R.B., Pulaski, J.T., Kaminski, N.E., 1997. Cannabinoid receptors CB_1 and CB_2: a characterization of expression and adenylate cylase modulation within the immune system. Toxicol. Appl. Pharmacol. 142, 278–287.

Seltzman, H.H., Foster, M.C., Wyrick, C.D., Carroll, F.I., 2001. Tritium labeling of the hydrogenolysis labile cannabinoid receptor antagonist SR 144528 with $LiAl_3H_4$. In: Pleiss, U., Voges, R. (Eds.), Synthesis and Applications of Isotopically Labelled Compounds, vol. 7. Wiley, Europe.

Shen, M., Thayer, S.A., 1999. Delta^9-tetrahydrocannabinol acts as a partial agonist to modulate glutamatergic synaptic transmission between rat hippocampal neurons in culture. Mol. Pharmacol. 55, 8–13.

Shire, D., Calandra, B., Rinaldi-Carmona, M., Oustric, D., Pessegue, B., Bonnin-Cabanne, O., Le Fur, G., Caput, D., Ferrara, P., 1996. Molecular cloning, expression and function of the murine CB_2 peripheral cannabinoid receptor. Biochim. Biophys. Acta 1307, 132–136.

Shire, D., Carillon, C, Kaghad, M., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., Caput, D., Ferrara, P., 1995. An amino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. J. Biol. Chem. 270, 3726-3731; Erratum in: J. Biol. Chem. 271 (1996) 33706.

Sim, L.J., Hampson, R.E., Deadwyler, S.A., Childers, S.R., 1996. Effects of chronic treatment with delta^9-tetrahydrocannabinol on cannabinoid-stimulated [^{35}S]GTPγS autoradiography in rat brain. J. Neurosci. 16, 8057–8066.

Sim, L.J., Selley, D.E., Childers, S.R., 1995. In vitro autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5′-[gamma-[^{35}S]thio]-triphosphate binding. Proc. Natl. Acad. Sci. U.S.A. 92, 7242–7246.