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Characterization of the pharmacology of imidazolidinedione derivatives at cannabinoid CB_1 and CB_2 receptors

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Abstract

The pharmacology of 3-(2-ethylmorpholino)-5,5′-di(*p*-bromophenyl)-imidazolidinedione (DML20), 3-(1-hydroxypropyl)-5,5′-di(*p*-bromophenyl)-imidazolidinedione (DML21) and 3-heptyl-5,5′-di(*p*-bromophenyl)-imidazolidinedione (DML23) was extended by studying affinity and GTP binding modulation on cannabinoid receptor subtypes (CB_1 and CB_2) from rat tissues and human cannabinoid receptors expressed in Chinese Hamster Ovary cells. Competitive binding studies indicated that DML20, DML21 and DML23 are selective ligands for cannabinoid CB_1 receptors. In rat cerebellum homogenates, DML20, DML21 and DML23 were unable to influence [^{35}S]GTPγS binding but competitively inhibit HU 210-induced [^{35}S]GTPγS binding (pK_B of 6.11 ± 0.14, 6.25 ± 0.06 and 5.74 ± 0.09, respectively), indicating that they act as cannabinoid CB_1 receptor neutral antagonists. However, in CHO cells homogenates expressing selectively either human cannabinoid CB_1 or CB_2 receptors, they behaved as inverse agonists decreasing the [^{35}S]GTPγS binding, with similar efficacy. In conclusion, these derivatives exhibit different activities (neutral antagonism and inverse agonism) in the different models of cannabinoid receptors studied.

Keywords: Cannabinoid receptor; [^{35}S]GTPγS; 3-Alkyl-(5,5′-diphenyl)imidazolidinedione; Neutral antagonism; Inverse agonism

1. Introduction

Most of the biological effects of *Cannabis sativa* L. extract, cannabis, result from the interaction of its main active constituent, Δ^9-tetrahydrocannabinol with specific cell surface receptors (for review, see Pertwee, 1999; Howlett et al., 2002). To date, two subtypes of cannabinoid receptors have been described. The cannabinoid CB_1 receptor (Gérard et al., 1991; Matsuda et al., 1990) was first evidenced by autoradiography and radioligand binding studies using tritiated (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexan-1-ol ([^{3}H]CP 55,940; Devane et al., 1988; Herkenham et al., 1990). It was cloned from several species and was shown to be highly conserved (for review, see Lutz, 2002). It is expressed in the brain and some peripheral tissues including testis, ileum, urinary bladder and vas deferens. The cannabinoid CB_2 receptor, discovered by sequence homology, is predominantly detected in the immune system (spleen, tonsils, immune cells) and was cloned from the human (Munro et al., 1993), mouse (Shire et al., 1996) and rat (Brown et al., 2002; Griffin et al., 2000). The analysis of the amino acid sequences of both cannabinoid receptors reveals the common G-protein coupled receptors motif including a seven lipophilic transmembrane α helices structure.

Among the signal transduction associated with cannabinoid receptors described so far (Bouaboula et al., 1995; Calandra et al., 1999; Deadwyler et al., 1995; Gebremedhin et al., 1999; Mackie and Hille, 1992), the responses to agonists of the cannabinoid CB_1 and CB_2 receptors are sensitive to pertussis toxin, suggesting that both subtypes are predominantly coupled to $G_{i/o}$-type G-proteins. Consequently, non-hydrolysable GTP analogues such as 5′-guanylylimidodiphosphate (GppNHp) and guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) constitute useful tools to distinguish between ligands showing agonist or antagonist properties. Indeed, guanylyl

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nucleotides are known to disrupt the functional coupling of G-protein coupled receptors, resulting in decreased affinity of agonists (Weiland and Jakobs, 1994). On the contrary, antagonists are unaffected by such uncoupling (Bouaboula et al., 1997; Sim et al., 1995). In addition, as the radiolabelled [^{35}S]GTPγS is commercially available, a derived binding technique is now commonly used to characterize the activity of ligands at G-protein coupled receptors (Milligan, 2003), including cannabinoid receptors (Sim et al., 1995, 1996; Selley et al., 1996; Breivogel et al., 1998; for review, see Pertwee, 1999). This assay helps 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). Indeed, the inverse agonist properties of the diarylpyrazole cannabinoids, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR 141716A) (Rinaldi-Carmona et al., 1994) and *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 (SR 144528) (Rinaldi-Carmona et al., 1998), previously considered as cannabinoid antagonists, have been characterized using this approach (Bouaboula et al., 1997; Landsman et al., 1997; MacLennan et al., 1998; Portier et al., 1999; Rinaldi-Carmona et al., 1998).

Following the important discovery of the diarylpyrazole antagonists, cannabinoid receptor antagonists received a considerable interest from pharmaceutical companies with regards to their therapeutic perspectives in the control of appetite, psychosis, memory and cognition disorders. Besides the diarylpyrazoles developed mainly by Sanofi-Synthelabo, new templates have been recently proposed as cannabinoid antagonists including 4,5-dihydro-1*H*-pyrazoles from Solvay Pharmaceuticals and azetidine derivates from Aventis Pharmaceuticals (for review, see Adam and Cowley, 2002).

In this connection, we described, in previous reports, the synthesis of original cannabinoids, the 3-alkyl-5,5′ diphenylimidazolidinediones (Fig. 1, Kanyonyo et al., 1999; Ooms et al., 2002) exhibiting affinity for the cannabinoid CB_1 receptors. In these preliminary studies, three compounds, namely 3-(2-ethylmorpholino)-5,5′di(*p*-bromophenyl)-imidazolidinedione (DML20), 3-(1-hydroxypropyl)-5,5′di(*p*-bromophenyl)-imidazolidinedione (DML21) and 3-heptyl-5,5′di(*p*-bromophenyl)-imidazolidinedione (DML23), were suggested to behave as neutral antagonists of the cannabinoid receptor expressed in the rat cerebellum.

The present investigation was directed at characterizing the pharmacological properties of DML20, DML21 and DML23 in different models (rat cerebellum, rat spleen and transfected CHO cells expressing the human CB_1 or the human CB_2 receptor) by studying their affinity towards

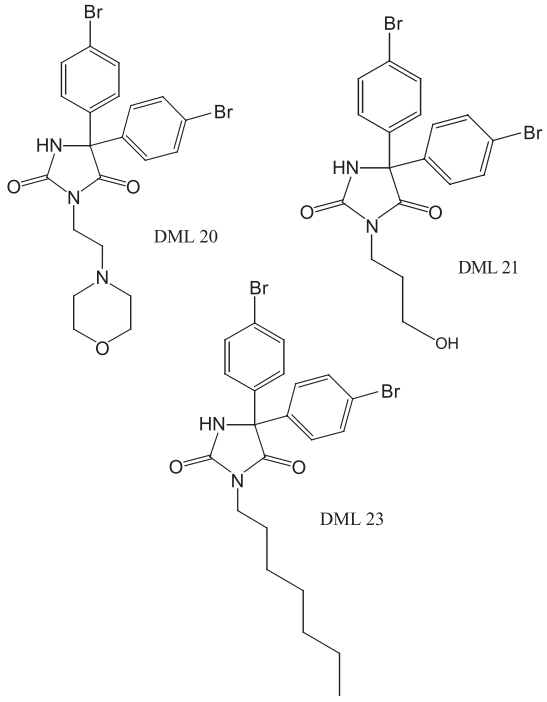

Fig. 1. Structures of the 3-alkyl-5,5′-diphenylimidazolidinediones: 3-(2-ethylmorpholino)-5,5′-di(*p*-bromophenyl)-imidazolidinedione (DML20), 3-(1-hydroxypropyl)-5,5′-di(*p*-bromophenyl)-imidazolidinedione (DML21) and 3-heptyl-5,5′-di(*p*-bromophenyl)-imidazolidinedione (DML23).

cannabinoid CB_1 and CB_2 receptors as well as their activity in [^{35}S]GTPγS binding assays.

2. Materials and animals

2.1. Drugs, chemical reagents and apparatus

Tritiated *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride ([^{3}H]SR 141716A, 52 Ci/mmol) and [^{35}S]GTPγS (1101 Ci/mmol) were from Amersham (Roosendaal, The Netherlands). Tritiated (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone ([^{3}H]WIN 55,212-2, 45.5 Ci/mmol) and tritiated (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexan-1-ol ([^{3}H]CP 55,940, 101 Ci/mmol) were from New England Nuclear (Boston, MA). Fatty acid free bovine serum albumin, dithiothreitol, GDP and GppNHp were purchased from Sigma (Boornem, Belgium). (6α*R*)-*trans*-3-(1,1-dimethylheptyl)-6α, 7, 10, 10α-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[*b,d*]pyran-9-methanol (HU 210) was from Tocris Cookson (Bristol, UK). WIN 55,212-2 was from Research Biochemicals International (Sanver Tech, Belgium). (-)-*Cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexan-1-ol (CP 55,940) was generously provided by Pfizer

(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). The 3-alkyl-5,5′-di(*p*-bromophenyl)imidazolidinediones DML20, DML21 and DML23 were synthesised in our laboratory as described (Kanyonyo et al., 1999). Glass fiber 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). Stock solutions of ligand compounds were prepared in pure dimethylsulfoxide and then further diluted. The final dimethylsulfoxide concentration was less than 0.1%.

2.2. Preparation of rat cerebellum membranes

Male Wistar rats (250–300 g) were purchased from IFFA-CREDO (Les Oncins, France). All experiments on animals were approved by the institutional ethics committee and the housing conditions were as specified by the Belgian Law of November 14, 1993 on the protection of laboratory animals (agreement no. LA 1230315). Cerebella were carefully dissected on ice. All the manipulations were performed at 0–4 °C. Rat cerebellum membranes 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 supernatant was collected and centrifuged at 39,000 × *g* for 10 min. The resulting pellet was resuspended in 50 mM Tris–HCl pH 7.4, homogenized and centrifuged again at 39,000 × *g* for 10 min. The pellet was 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 spleen membranes

Spleens from male Wistar rats (250–300 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 $MgCl_2 \cdot 6\ H_2O$, 1 mM EDTA and 0.5% serum bovine albumin. The preparation of membranes was performed according to the method described by Hillard et al. (1999).

2.4. Cell culture and preparation of hCB_1-CHO or hCB_2-CHO cells membranes

Dr. P. Nokin and Dr. M. Detheux kindly provided CHO cells stably transfected with the cDNA sequences encoding either the human cannabinoid CB_1 or CB_2 receptors, respectively (Euroscreen, Brussels, Belgium). Two different clones of CHO cells expressing the human cannabinoid CB_1 with varying receptor densities have been used: the CB_1-C1 clone with a high receptor density (44 pmol/mg prot) and the CB_1-C2 clone with a low receptor density (3.2 pmol/mg prot). Unless indicated, the experiments were performed on the CB_1-C1 clone. 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 geniticin. 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 Tris–HCl 50 mM pH 7.4, and the homogenate was centrifuged at 400 × *g* for 10 min. The pellet was resuspended in the same buffer, homogenized and centrifuged at 15,000 × *g* for 10 min. The resulting pellet (membranes) was washed twice more in the same conditions. The protein concentration was measured as indicated above.

2.5. Competitive and saturation binding assays

The competitive binding experiments were performed in the 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) or transfected CHO cells (40 μg protein/tube) resuspended in 0.5 ml (final volume) binding buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin pH 7.4). Competitors were present at varying concentrations and the nonspecific binding of the radioligands was determined in the presence of 10 μM HU 210. When indicated, GppNHp was included in the assay at a final concentration of 50 μM. After 1-h incubation, the suspension was rapidly filtered through 0.5% polyethyleneimine pretreated GF/B glass fiber filters on a 48-well Brandell cell harvester and washed twice with 3 ml ice-cold binding buffer without serum albumin. Radioactivity on filters was measured with a Pharmacia Wallac 1410 β-counter by liquid scintillation in 10 ml Aqualuma. 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 0.5–15, 0.1–10 and 0.9–30 nM, respectively.

2.6. [^{35}S]GTPγS specific binding

The [^{35}S]GTPγS binding assay was carried out at 30 °C in plastic tubes containing 20 μg protein resuspended in 0.5 ml (final volume) binding buffer (50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 1 mM disodium EDTA, 100 mM NaCl and 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 were terminated by the addition of 3 ml of ice-cold washing buffer (50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 1 mM disodium EDTA, 100 mM NaCl). The suspension was immediately filtered through GF/B filters using a 48-well Brandell cell harvester and washed twice

with ice-cold binding buffer. Radioactivity trapped on the filters was counted as mentioned above. The nonspecific binding was measured in the presence of 100 μM GppNHp.

2.7. Data analysis

Radioligand and nucleotide binding data were analyzed by nonlinear regression using the software GraphPad Prism, version 3.0 (GraphPad, San Diego, CA). Antagonist equilibrium dissociation constants (expressed as pK_B) were calculated using the following equation: $K_B = A/(CR - 1)$ where A is the concentration of antagonist used and CR is the ratio of EC_{50} values for the agonist measured in the presence or in the absence of the antagonist, respectively.

3. Results

3.1. Interaction of DML derivatives with the rat and human cannabinoid CB_1 receptors

In rat cerebella membranes, saturation experiments using the radiolabelled inverse agonist, [^{3}H]SR 141716A, allowed to detect a single population of binding site with a K_d value of 3.12 ± 0.17 nM and a B_{max} value of 3.31 ± 0.12 pmol/mg protein ($n = 3$; Fig. 2A). The specific binding of [^{3}H]SR 141716A was displaced by the nonselective reference cannabinoids HU 210 and CP 55,940 (Fig. 2B). As expected for high affinity agonists of a G-protein coupled receptor, the displacement curves for HU 210 and CP 55,940 revealed the presence of two classes of binding sites. Binding proportion to the high affinity state represented approximately 42% and 23% for HU 210 and CP 55,940, respectively. The pK_i values of these compounds were in agreement with the predominance of the cannabinoid CB_1 receptor subtype in the rat cerebellum membranes (Table 1). Compounds DML20, DML21 and DML23 were found to compete with [^{3}H]SR 141716A binding and the curves best fitted with a single binding site model revealing micromolar affinities (Table 1, Fig. 2C). Similar pK_i values (5.24 ± 0.03, 5.07 ± 0.22, and 5.47 ± 0.22 for DML20, DML21 and DML23, respectively) were obtained in competition studies using the radioligand [^{3}H]CP 55,940 (K_d value of 2.08 ± 0.32 nM).

The ability of DML compounds to behave as agonists of the cannabinoid CB_1 receptor was evaluated by measuring the effect of disrupting G-protein coupling with GppNHp on their affinities in radioligand binding studies. Saturation binding studies performed in the presence of 50 μM GppNHp revealed a modest but not significant ($p = 0.09$) increase in affinity of the inverse agonist [^{3}H]SR 141716A (K_d values of 3.12 ± 0.17 and 2.49 ± 0.26 nM in the absence or in the presence of the guanylyl nucleotide, respectively). As shown in Fig. 2B, the addition of the guanylyl nucleotide shifted the displacement curves

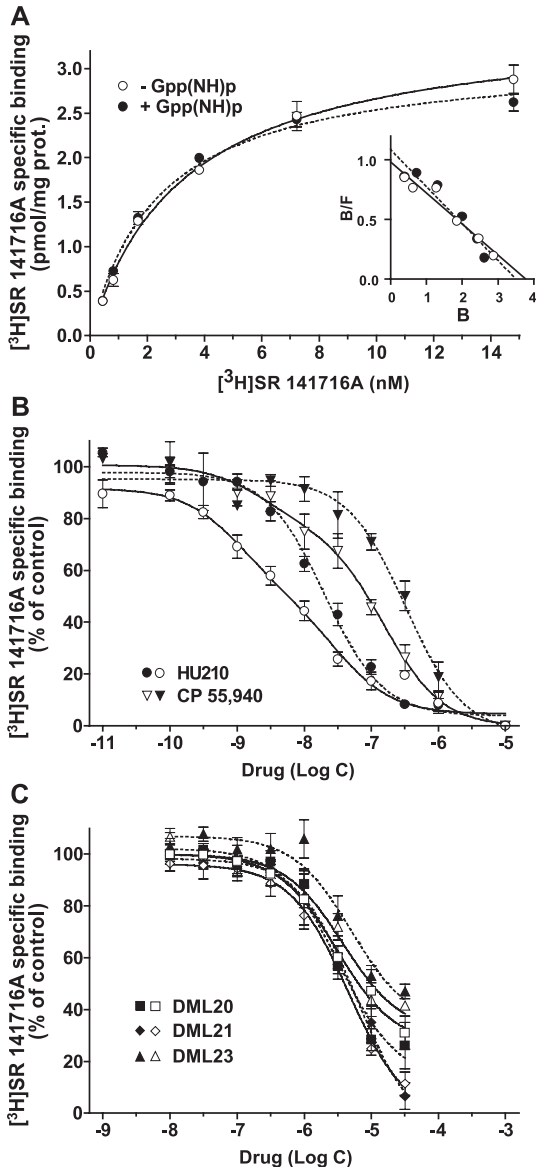

Fig. 2. (A) Saturation curves of the binding of the selective antagonist [^{3}H]SR 141716A in rat cerebella homogenates in the presence (●) or in the absence (○) of 50 μM GppNHp, a non-hydrolysable analogue of GTP. Data shown are mean ± S.E.M. from one typical experiment performed four times in triplicates. K_d and B_{max} values derived from these experiments are mentioned in Results. In the insert: Scatchard analysis of saturation curves of [^{3}H]SR 141716A in rat cerebella homogenates in the presence (●) or in the absence (○) of GppNHp. (B) Influence of GppNHp (50 μM) on the displacement of the [^{3}H]SR 141716A specific binding on rat cerebellar homogenates by cannabinoid agonists (HU 210, ●; CP 55,940, ▼), open symbols represent the data in the absence of GppNHp, filled symbols represent the data in the presence of GppNHp. (C) Absence of influence of GppNHp (50 μM) on the displacement of [^{3}H]SR 141716A specific binding on rat cerebellar homogenates by the DML20 (□), DML21 (◇) and DML23 (△). Open symbols represent the data in the absence of GppNHp, filled symbols represent the data in the presence of GppNHp.

corresponding to the agonist HU 210 and CP 55,940 to higher concentrations and nonlinear analysis revealed an interaction with a single binding site population corresponding to the low affinity component observed in

Table 1
Influence of the GppNHp (50 μM) on the affinity of reference cannabinoid ligands and DML derivatives on membranes from rat cerebellum

Compounds	Without GppNHp			With GppNHp		
	pK_{iH}	pK_{iL}	H/L (%)	pK_{iH}	pK_{iL}	H/L (%)
HU 210	8.98 ± 0.32	7.51 ± 0.25	41.6 ± 13.2	n.d.	7.70 ± 0.05	n.d.
CP 55,940	8.62 ± 0.62	6.81 ± 0.18	23.2 ± 9.8	n.d.	6.49 ± 0.09	n.d.
DML20	n.d.	5.46 ± 0.17	n.d.	n.d.	5.52 ± 0.11	n.d.
DML21	n.d.	5.69 ± 0.05	n.d.	n.d.	5.64 ± 0.04	n.d.
DML23	n.d.	5.25 ± 0.06	n.d.	n.d.	5.11 ± 0.06	n.d.

pK_i values were derived from nonlinear analysis of competition curves using [^{3}H]SR 141716A (showing K_d values of 3.12 ± 0.17 and 2.49 ± 0.26 nM in the absence or in the presence of the guanylyl nucleotide). When data best fitted with a two site competition curves, both pK_i values and the fraction of the high affinity binding component are indicated. Data shown are the mean ± S.E.M. from three to six experiments performed in triplicates.

the absence of GppNHp. In contrast, the competition curves corresponding to compounds DML20, DML21 and DML23 were not significantly modified upon addition of GppNHp. The recognition of cannabinoid CB_1 receptors by these compounds as a single population of binding sites and the absence of GppNHp-mediated changes in their pK_i values (Table 1) suggest that they are not agonists of cannabinoid CB_1 receptors in the rat cerebellum.

The interaction of DML compounds with the human cannabinoid CB_1 receptor was evaluated in membranes of stably transfected CHO cells expressing high densities of the receptor (B_{max} value of 43.79 ± 8.36 pmol/mg prot as evaluated in [^{3}H]SR 141716A saturation experiments). The specific binding of both [^{3}H]SR 141716A (K_d value 13.90 ± 2.48 nM) and [^{3}H]CP 55,940 (K_d value 4.73 ± 1.32 nM) were efficiently displaced by DML20, DML21 and DML23 with affinities similar to those found on membranes of rat cerebellum (Tables 1 and 2).

3.2. Interaction of DML derivatives with the rat and human cannabinoid CB_2 receptors

Albeit the mRNAs of both cannabinoid CB_1 and CB_2 receptors have been detected in the spleen, only the latter subtype has been clearly identified in this model (Lee et al., 2001). Thus, saturation binding studies using the nonselective cannabinoid radioligand [^{3}H]CP 55,940 revealed the presence of a single population of receptors with K_d and B_{max} values of 2.2 ± 0.4 nM and 696.6 ± 29.7 fmol/mg protein, respectively. In contrast, the specific binding of the cannabinoid CB_1 selective radioligand, [^{3}H]SR 141716A, was almost undetectable. Furthermore, competition studies indicated that compounds WIN 55,212-2 and HU 210 efficiently displaced the [^{3}H]CP 55,940 specific binding (pK_i values of 8.74 ± 0.09 and 7.40 ± 0.15, respectively) whereas similar displacement was only obtained with high concentrations of SR 141716A (pK_i value of 5.78 ± 0.05). In this model of rat spleen membranes, compounds DML20, DML21 and DML23 behaved as weak competitors as less than 30% displacement of [^{3}H]CP 55,940 specific binding was obtained at a concentration of 100 μM (not shown).

Similar results were obtained when studying the interaction with the human cannabinoid CB_2 receptor expressed in CHO cells. Thus, using the radioligand [^{3}H]WIN 55,212-2 on membranes of these cells (K_d value of 12.0 ± 1.3 nM and B_{max} value of 109.3 ± 10.1 pmol/mg prot), competition studies using DML20, DML21 and DML23 yielded pK_i values similar to those found in membranes from rat spleen (Table 2).

Table 2
Affinities of reference cannabinoids and DML derivatives at the cloned human cannabinoid CB_1 and CB_2 receptors expressed in transfected CHO cells

Model	Radioligand	DML20, pK_i	DML21, pK_i	DML23, pK_i
hCB_1	[^{3}H]SR 141716A, 1 nM	5.57 ± 0.21	4.94 ± 0.11	5.33 ± 0.11
hCB_1	[^{3}H]CP55,940, 1 nM	5.25 ± 0.03	4.80 ± 0.24	4.84 ± 0.07
hCB_2	[^{3}H]WIN 55,212-2, 1 nM	4.39 ± 0.08	3.98 ± 0.15	3.89 ± 0.13

pK_i values were derived from nonlinear analysis of competition curves using [^{3}H]SR 141716A, [^{3}H]CP55,940 or [^{3}H]WIN 55,212-2. Data shown are mean ± S.E.M. from three to six experiments performed in triplicates.

3.3. Modulation of [^{35}S]GTPγS binding by DML derivatives at cannabinoid CB_1 receptors

Evaluation of agonist-induced [^{35}S]GTPγS binding at the cannabinoid CB_1 receptor was optimized on membranes of rat cerebellum using the reference cannabinoid agonist HU 210. Maximal induction of nucleotide specific binding was obtained when the assay was performed with 20 μg protein resuspended in 0.5 ml (final volume) binding buffer supplemented with 20 μM GDP. The functional response to HU 210 and the other representative cannabinoids CP 55,940, WIN 55,212-2 and SR 141716A in these conditions are shown in Fig. 3A and the pharmacological data derived from these curves (maximal response and EC_{50} values) are summarized in Table 3. As expected, HU 210, CP 55,940 and WIN 55,212-2 behaved as full agonists, stimulating the [^{35}S]GTPγS specific binding to about 250% above basal. In contrast, SR 141716A showed inverse agonist properties as it decreased the nucleotide binding by 31 ± 2% as compared to basal. While the nanomolar potency of the three full agonists was in accordance with their affinity measured in competition

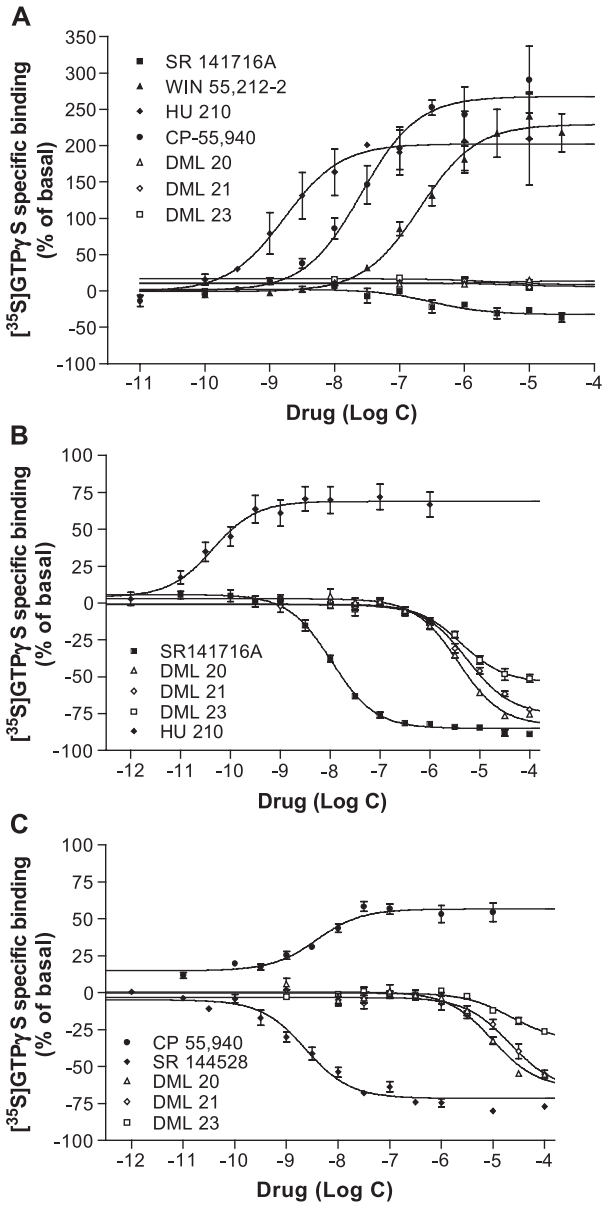

Fig. 3. (A) Effect of HU 210 (◆; full agonists), WIN 55,212-2 (▲; full agonists), CP 55,940 (●; full agonists), SR 141716A (■; inverse agonist) and DML20 (△), DML 21 (◇), DML23 (□), on [^{35}S]GTPγS binding on rat cerebella homogenates. Data are the mean from at least three separate experiments, vertical lines show S.E.M. (B) Effect of HU 210 (◆; full agonist), SR 141716A (■; inverse agonist) and DML20 (△), DML21 (◇), DML23 (□), on [^{35}S]GTPγS 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. (C) Effect of CP 55,940 (●; full agonist), SR 144528 (◆; inverse agonist) and DML20 (△), DML21 (◇), DML23 (□), 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.

binding studies, the potency of SR 141716A was in the submicromolar range (EC_{50} of 445 nM corresponding to a pEC_{50} of 6.35 ± 0.15), more than two orders of magnitude lower than its affinity. As shown in Fig. 3A and in Table 3, compounds DML20, DML21 and DML23 neither increased nor decreased the [^{35}S]GTPγS specific binding in rat cerebellum membranes.

As DML20, DML21 and DML23 were shown to interact with cannabinoid CB_1 receptors without affecting [^{35}S]GTPγS binding, the competitive antagonist property of these compounds was evaluated by measuring their ability to inhibit the functional response to the cannabinoid receptor agonist HU 210. As shown in Fig. 4A, the addition of DML20, DML21 or DML23 (30 μM) was found to cause parallel rightward shifts of the concentration–response curve for the agonist. The concentration ratios of EC_{50} values obtained in the presence and in the absence of antagonists were used to calculate their pK_B values (6.11 ± 0.14, 6.25 ± 0.06 and 5.74 ± 0.09 for DML20, DML21 and DML23, respectively; Fig. 4A). Further analysis of this competitive antagonism was performed for compound DML21. After analysis of the responses to HU 210 measured in the presence of increasing concentrations of this antagonist (1–30 μM; Fig. 4B), it was possible to estimate a pA_2 value for this antagonist of 6.16 ± 0.11 (slope of 1.11 ± 0.06).

In complement to this characterization on rat cerebellum membranes, the influence of DML derivatives on [^{35}S]GTPγS specific binding was also evaluated in membranes of transfected CHO cells expressing the human cannabinoid CB_1 receptor (Fig. 3B). These experiments have been validated using the reference agonist, HU 210 and the selective cannabinoid CB_1 inverse agonist SR 141716A. While the amplitude of the responses to these compounds was lower than in rat cerebellum membranes, they both showed a higher potency that best correlated with their respective affinities in this model. In these transfected CHO cells, compounds DML20, DML21 and to a lesser extend compound DML23 exhibit a robust inverse agonist properties almost comparable to that of SR 141716A. Thus, in the presence of these drugs, the nucleotide binding was decreased by 50% to 80% as compared to basal (Table 4).

Table 3
Functional response to reference cannabinoids and DML20, DML21 and DML23 evaluated on membranes from rat cerebellum

Compounds	E_{max} (%)	EC_{50} (nM)
HU 210	202 ± 14	3.0 (1.2–7.8)
CP 55,940	260 ± 38	33.2 (16.0–68.9)
WIN 55,212-2	232 ± 25	242 (92.2–635)
SR 141716A	− 31 ± 2	445 (50–3937)
DML20	7 ± 10[a]	–
DML21	6 ± 8[a]	–
DML23	10 ± 9[a]	–

Concentrations response curves allowed to calculate EC_{50} values and the amplitude of the maximal response (E_{max}). Results are expressed as percentages of stimulation above basal levels and EC_{50} values ± S.E.M.

[a] Percentages of stimulation at the concentration of 10 μM of the cannabinoid used.

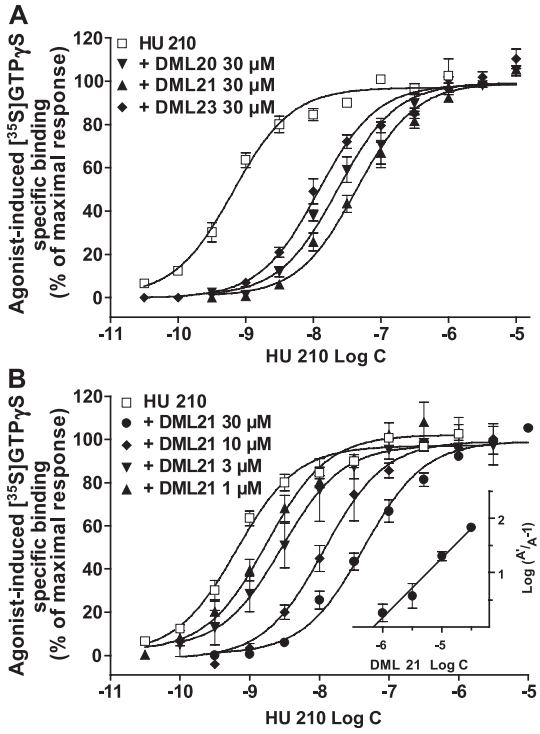

Fig. 4. (A) Determination of pK_B values of DML20 (▼), DML21 (▲) and DML23 (♦) against HU 210 (□)-induced [^{35}S]GTPγS binding in rat cerebella homogenates. The HU 210 stimulation (from 10^{-5} to $10^{-10.5}$ M) curve is presented (□), 30 μM of DML20 (▼), DML21 (▲), and DML23 (♦) induce a right-shift of the HU 210 stimulation curve. Data are the mean from at least three separate experiments, vertical lines show S.E.M. (B) Determination of pA_2 value of DML21 against HU 210-induced [^{35}S]GTPγS stimulation. The HU 210 stimulation (from 10^{-5} to $10^{-10.5}$ M) curve is presented (□), increasing concentrations of DML21 (▲: 1 μM, ▼: 3 μM, ♦: 10 μM, ●: 30 μM) induce a right-shift of the HU 210 stimulation curve; the insert is the Schild plot giving the pA_2 value of DML21. Data are the mean from at least three separate experiments, vertical lines show S.E.M.

3.4. Influence of the receptor density on the modulation of [^{35}S]GTPγS binding by DML derivatives at human cannabinoid CB_1 receptors expressed in CHO-cells

To clarify whether the discrepancy observed at rat and human cannabinoid CB_1 receptors was related to the differences of species (and consequently of receptor sequences) or to the receptor density, two different clones of CHO cells expressing distinct densities of the human cannabinoid CB_1 receptor have been compared: the CB_1-C1 clone with a high receptor density (B_{max} of 44 pmol/mg prot) and the CB_1-C2 with a low receptor density (B_{max} of 3.2 pmol/mg prot). The modulation of [^{35}S]GTPγS binding was measured in response to either HU 210, SR141716A, DML20, DML21 or DML23 (10 μM each). Whatever the receptor density, all the compounds behaved in a similar fashion and in particular, the DML20, DML21 and DML23 showed inverse agonists properties at human cannabinoid CB_1 receptors (Fig. 5A,B).

Table 4
Effects of reference cannabinoids and DML20, DML21 and DML23 on [^{35}S]GTPγS binding in membranes from transfected CHO cells expressing either the human cannabinoid CB_1 or CB_2 receptors

Compounds	Human CB_1		Human CB_2	
	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}
Agonists				
HU 210	67 ± 10	10.11 ± 0.21	46 ± 6	9.22 ± 0.18
CP 55,940	60 ± 9	9.00 ± 0.58	57 ± 4	8.41 ± 0.10
Diarylpyrazoles				
SR 141716A	− 84 ± 1	8.00 ± 0.05	− 73 ± 1	5.96 ± 0.02
SR 144528	− 30 ± 3	6.42 ± 0.09	− 69 ± 4	8.67 ± 0.07
3-Alkyl-5,5′-(diphenyl)-imidazolidinediones				
DML20	− 81 ± 1	5.47 ± 0.06	− 65 ± 2	5.01 ± 0.08
DML21	− 74 ± 2	5.31 ± 0.09	− 67 ± 3	4.67 ± 0.06
DML23	− 54 ± 3	5.37 ± 0.03	− 33 ± 3	4.63 ± 0.14

Results are expressed as percentages of stimulation above basal levels and EC_{50} values ± S.E.M., $n = 3-5$.

3.5. Modulation of [^{35}S]GTPγS binding by DML derivatives at cannabinoid CB_2 receptors

Despite their poor affinity for the cannabinoid CB_2 receptor, the influence of DML20, DML21 and DML23

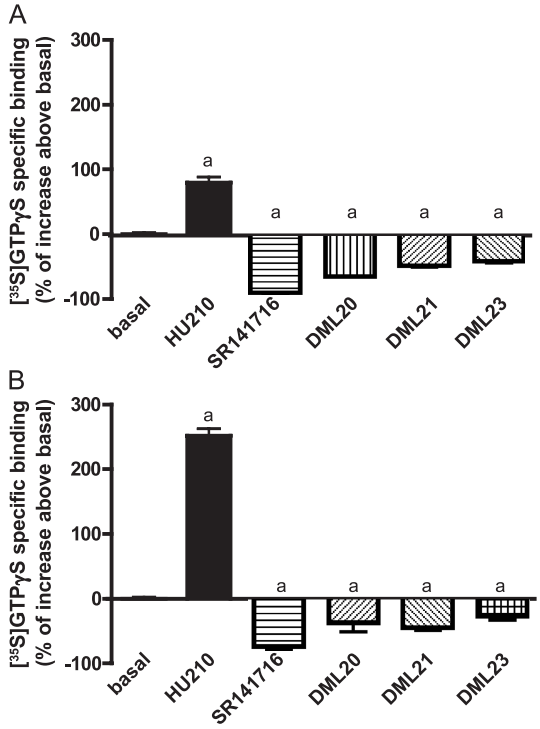

Fig. 5. Influence of the receptor density on the modulation of [^{35}S]GTPγS binding by DML derivatives at human cannabinoid CB_1 receptors expressed in CHO-cells using the CB_1-C1 clone (B_{max} of 44 pmol/mg, A) and the CB_1-C2 clone (B_{max} of 3.2 pmol/mg, B). The modulation of [^{35}S]GTPγS binding was measured in the presence of 10 μM of the compounds ($n = 3-4$). Data are the mean from at least three separate experiments, vertical lines show S.E.M. One way ANOVA followed by a Dunett post-test ($^aP < 0.01$).

on the specific binding of [^{35}S]GTPγS was evaluated on membranes prepared from the rat spleen. Whatever the GDP concentration or the protein concentration used, none of these derivatives (at concentrations up to 100 μM) were found to significantly affect the binding of the nucleotide. More intriguingly, in the same assays, we were not able to detect any increase in the [^{35}S]GTPγS specific binding by the reference cannabinoid agonists HU 210, WIN 55212,2 and CP 55,940 (not shown).

Contrasting to the lack of functional responses to cannabinoids observed in the rat spleen, a significant modulation of [^{35}S]GTPγS by reference drugs acting at the cannabinoid CB_2 receptor was detected in membranes from transfected CHO cells expressing this receptor from a human origin (Fig. 3C, Table 4). Thus, the nonselective agonist CP 55,940 and the cannabinoid CB_2 selective inverse agonist SR 144528 were shown to mediate a 60% increase and a 70% decrease in the specific binding of the nucleotide, respectively. Similarly to the results obtained at the human cannabinoid CB_1 receptor, DML20, DML21 and DML23 showed inverse agonist properties in the CHO cells expressing the human cannabinoid CB_2 receptor. The rather low potency of these drugs in this model was in accordance with their weak affinity for this receptor subtype.

4. Discussion

Diarylimidazolidinedione derivatives actions at cannabinoid receptors have been discovered in our laboratory. The interaction of DML compounds with cannabinoid receptors was initially evaluated in [^{3}H]SR 141716A competition studies performed on membranes of transfected CHO cells expressing the human cannabinoid CB_1 receptor (Kanyonyo et al., 1999). Albeit the affinity of these compounds was weaker than the highly potent synthetic cannabinoids such as HU 210 and CP 55,940, these molecules belong to a new chemical class, with an original structure, unrelated to other previously described cannabinoids. Among the diarylimidazolidinediones previously characterized (Kanyonyo et al., 1999), three of these new cannabinoid ligands—3-(2-ethylmorpholino)-5,5′-di(*p*-bromophenyl)-imidazolidinedione (DML20), 3-(1-hydroxypropyl)-5,5′-di(*p*-bromophenyl)-imidazolidinedione (DML21) and 3-heptyl-5,5′-di(*p*-bromophenyl)-imidazolidinedione (DML23)—were selected and compared to the reference cannabinoids HU 210, CP 55,940, SR 141716A and SR 144528. First, their selectivity towards the two subtypes of cannabinoid receptors and towards animal species (human and rat) was studied. In the rat, brain and spleen homogenates have been used as sources of cannabinoid CB_1 and CB_2 receptors, respectively. While the absence of cannabinoid CB_2 receptors is well documented in the central nervous system of the adult rat (Pertwee, 1999), the presence or the absence of cannabinoid CB_1 receptors in the spleen is still under debate. In our hands, in spleen homogenates, a specific binding of the nonselective radioligand [^{3}H]CP 55,940 was detected while no specific binding of the cannabinoid CB_1 selective radioligand [^{3}H]SR 141716A was measured, indicating that mostly cannabinoid CB_2 receptors are expressed in the spleen. The affinity for human cannabinoid receptors has been evaluated using transfected CHO cells expressing selectively either the human cannabinoid CB_1 or CB_2 receptors. Compared to natural tissues and especially the spleen, these cell models present the great advantage of the selectivity of the cannabinoid receptor present.

Here, we showed that in both species, DML20, DML21 and DML23 exhibited some selectivity for the cannabinoid CB_1 receptors. Indeed, whatever the radioligand used or the source of cannabinoid receptors (human or rat), the pK_i values of DML20, DML21 and DML23 were superior for cannabinoid CB_1 receptors achieving a selectivity factor varying from 9 to 27. The affinity values obtained for the human cannabinoid CB_1 receptor were lower than previously reported (Kanyonyo et al., 1999). However, we used in the present study a new hCB_1-CHO cell clone provided by Euroscreen which is much less sensitive to some cannabinoids such as WIN 55,212-2 and related compounds.

As Kearn et al. (1999) have shown that [^{3}H]SR 141716A, an inverse agonist, labels the active and inactive cannabinoid CB_1 receptors, the agonist [^{3}H]CP 55,940 was also used in the characterization of DML20, DML21 and DML23. Again, in both species, these three compounds displaced more efficiently the specific binding of [^{3}H]SR 141716A rather than the specific binding of [^{3}H]CP 55,940. In addition, in the presence of 50 μM GppNHp, a non-hydrolysable analogue of GTP, the affinity of DML derivatives was unaffected. Taken together, these results indicate that DML20, DML21 and DML23 do not act as agonists of cannabinoid receptors. More interesting, the analysis of the displacement curves obtained in the rat cerebellum in the presence or in the absence of GppNHp for HU 210 and CP 55,940 revealed the presence of two classes of binding sites. This was previously observed by Sim-Selley et al. (2001) for SR 141716A. These authors proposed that SR 141716A binds to two sites of the cannabinoid CB_1 receptor: a high-affinity binding site at which SR 141716A exerts a competitive agonism and a lower affinity site at which its exerts its inverse agonism properties.

To further characterize the pharmacology of DML20, DML21 and DML23, their effect on the [^{35}S]GTPγS binding was measured to determine whether these compounds act as agonists, partial agonists, inverse agonists or neutral antagonists. In rat cerebella homogenates, HU 210, WIN 55,212-2 and CP 55,940 behaved as full agonists: their E_{max} varying from 158% to 243% above basal levels. 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 243 ± 36% and fulfils the criteria of an agonist. The inverse agonist (negative intrinsic activity) properties 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 (Griffin et al., 1998; Kearn et al., 1999) were confirmed in our study. Thus, this compound was found to decrease the nucleotide binding by 29% with an EC_{50} of 445 nM. This moderate potency of SR 141716A in inhibiting the receptor intrinsic activity contrasts with its particularly high (nanomolar) affinity measured in radioligand binding assays in the same model and in identical conditions (buffer, temperature...). 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 considerable higher potency (Pan et al., 1998). The ability of DML20, DML21 and DML23 to modulate the [^{35}S]GTPγS binding on rat cerebella homogenates was tested up to 10 μM concentration. As shown in Fig. 3A and in Table 3, DML20, DML21 and DML23 do not affect the [^{35}S]GTPγS binding, indicating that they probably act as antagonists. As the DML compounds were not able to modify the specific binding of [^{35}S]GTPγS binding, the antagonist properties of DML20, DML21 and DML23 on rat cerebella homogenates were confirmed by measuring their ability to inhibit the functional response to a full agonist HU 210. Although complete inhibition of HU 210-mediated nucleotide binding was not obtained, the rightward displacement of the dose–response curve clearly revealed the competitive antagonism of DML20, DML21 and DML23. As expected from their relative affinities for the CB_1 receptor, DML21 was the most potent antagonist among the three imidazolidinediones. In this model, DML20, DML21 and DML23 behave as neutral antagonists.

In rat spleen preparations, neither HU 210 nor WIN 55,212-2 promoted the [^{35}S]GTPγS binding despite repeated assays in several experimental conditions (varying GDP concentration, protein concentration). To our knowledge, a similar attempt has never been reported in the literature while the [^{35}S]GTPγS binding assay for cannabinoid CB_2 receptors has been performed on transfected cells only, the absence of stimulation of the [^{35}S]GTPγS binding in the spleen remains unsolved.

As neutral antagonism appears unusual, a similar [^{35}S]GTPγS assay has been performed in human cannabinoid hCB_1-CHO homogenates, a model where SR 141716A exhibits robust inverse agonist properties (MacLennan et al., 1998; Portier et al., 1999; Rinaldi-Carmona et al., 1998). DML20 and DML21 exhibited inverse agonists properties with amplitude similar to the one of SR 141716A. The E_{max} were between −54% and −81%. The E_{max} value for SR 141716A was −84%. May the discrepancy between the results obtained in rat cerebellum and in the human cannabinoid CB_1 receptors transfected cells with the DML compounds be due to the differences of species (and consequently of receptor sequences) or to the receptor density? To address this last hypothesis, the modulation of [^{35}S]GTPγS was measured using the two clones of human cannabinoid hCB_1-CHO cells with distinct receptor densities. The reference 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. In contrast, the stimulation by HU 210 was found similar in the rat cerebella homogenates and in the human cannabinoid CB_1-C2 clone. Regarding the effect of the inverse agonist SR 141716A, the opposite picture was observed: the more pronounced inverse agonist effect was observed when the receptor density was the highest. These data might suggest that, in a cell culture model where the cannabinoid receptors are overexpressed, the G-proteins prone to be activated were somewhat saturated. The levels of expression of cannabinoid CB_1 receptors were similar in the C2 clone (B_{max} = 3.2 pmol/mg prot) and in the rat cerebellum (B_{max} = 3.31 pmol/mg prot). Interestingly, in both clones of human cannabinoid CB_1 receptors, DML compounds are inverse agonists. It can be concluded that the receptor density is not the cause of the discrepancy of the behaviour of DML compounds. Probably the differences in the sequences or the transduction machinery in the expression system might be the origin of this difference in behaviour. Based on a cannabinoid CB_1 receptor mutants and on molecular docking studies, Hurst et al. (2002) proposed that the lysine residue 3.28 (192) is playing a pivotal role for the inverse agonist properties of SR 141716A. Along this line, it seems elegant to suggest that lysine environment is somewhat different in the human and in the rat cannabinoid receptor, albeit a 97% homology in sequences is known. A close examination of both rat and human sequences indicate that considering the seven transmembrane domains, only one residue in the second helix is different in the rat sequence compared to the human one. This amino acid does not seem to be close to the residues involved in the R* state of the model of SR 141716A interaction proposed by Hurst et al. (2002). However, in the extracellular or intracellular tails, major changes occur and may contribute to the difference of amplitude of the inverse agonism effect observed in rat and human cannabinoid CB_1 receptors.

Albeit their affinity for cannabinoid CB_2 receptors is very low, the sensitivity of the [^{35}S]GTPγS assay allows determining the properties of DML compounds on human cannabinoid hCB_2-CHO homogenates. Here too, DML20, DML21 and DML23 share the same inverse agonists properties with E_{max} range between −33% and −67%. The amplitude of the effect of SR 144528 was −69%.

If the armamentarium of cannabinoid ligands is quite large for agonists, only few so-called antagonists have been reported. They have been recently reviewed by Barth and Rinaldi-Carmona (1999) and by Adam and Cowley (2002). Beside the diarylpyrazole compounds from Sanofi-Synthelabo and their variants (Gatley et al., 1998; Stoit et al., 2002), only an aminoalkylindole 6-iodopravadoline (AM

630) (Hosohata et al., 1997; Landsman et al., 1998) was described and fully characterized. All seem to display inverse agonist properties, when such properties have been investigated. Here, we reported with DML20, DML21 and DML23 a new chemical cannabinoid template which, despite modest affinity, exhibits a selectivity for the cannabinoid CB_1 receptor, acts as an inverse agonist in human cannabinoid recombinant receptors, and could either constitute new tool for cannabinoid pharmacology or new lead compound for medicinal chemistry developments. They may provide also new pharmacological tools to investigate the molecular determinants of the inverse agonism and of the neutral antagonism at cannabinoid CB_1 receptors.

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References

Adam, J., Cowley, P., 2002. Recent advances in the cannabinoids. Expert Opin. Ther. Pat. 12, 1475–1489.

Barth, F., Rinaldi-Carmona, M., 1999. The development of cannabinoid antagonists. Curr. Med. Chem. 6, 745–755.

Bouaboula, M., Poinot-Chazel, C., Bourrie, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., Casellas, P., 1995. Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB_1. Biochem. J. 312, 637–641.

Bouaboula, M., Perrachon, S., Michigan, L., Canat, X., Rinaldi-Carmona, M., Portier, M., Barth, F., Calandra, B., Pecceu, F., Lupker, J., Maffrand, J.P., 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.

Calandra, B., Portier, M., Kerneis, A., Delpech, M., Carillon, C., Le Fur, G., Ferrara, P., Shire, D., 1999. Dual intracellular signalling pathways mediated by the human cannabinoid CB_1 receptor. Eur. J. Pharmacol. 374, 445–455.

Deadwyler, S.A., Hampson, R.E., Mu, J., Whyte, A., Childers, S., 1995. Cannabinoids modulate voltage sensitive potassium A-current in hippocampal neurons via a cAMP-dependent process. J. Pharmacol. Exp. Ther. 273, 734–743.

Devane, W.A., Dysarz, F.A., Johnson, M.R., Melvin, L.S., Howlett, A.C., 1988. Determination and characterization of a cannabinoid receptor in rat brain. Mol. Pharmacol. 34, 605–613.

Gatley, S.J., Lan, R., Volkow, N.D., Pappas, N., King, P., Wong, C.T., Gifford, A.N., Pyatt, B., Dewey, S.L., Makriyannis, A., 1998. Imaging the brain marijuana receptor: development of a radioligand that binds to cannabinoid CB_1 receptors in vivo. J. Neurochem. 70, 417–423.

Gebremedhin, D., Lange, A.R., Campbell, W.B., Hillard, C.J., Harder, D.R., 1999. Cannabinoid CB_1 receptor of cat cerebral arterial muscle functions to inhibit L-type Ca^{2+} channel current. Am. J. Physiol. 276, H2085–H2093.

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.

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.

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.

Herkenham, M., Lynn, A.B., Little, M.D., Johnson, M.R., Melvin, L.S., de Costa, B.R., Rice, K.C., 1990. Cannabinoid receptor localization in brain. Proc. Natl. Acad. Sci. U. S. A. 87, 1932–1936.

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.

Hosohata, Y., Quock, R.M., Hosohata, K., Makriyannis, A., Consroe, P., Roeske, W.R., Yamamura, H.I., 1997. AM630 antagonism of cannabinoid-stimulated [^{35}S]-GTPγS binding in the mouse brain. Eur. J. Pharmacol. 321, R1–R3.

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Mackie, K., Martin, B.R., Mechoulam, R., Pertwee, R.G., 2002. International Union of Pharmacology: XXVII. Classification of cannabinoid receptors. Pharmacol. Rev. 54, 161–202.

Hurst, D.P., Lynch, D.L., Barnett-Norris, J., Hyatt, S.M., Seltzmann, H.H., M., M., Zhong, M., Song, Z.-H., Nie, J., Lewis, D., Reggio, P.H., 2002. *N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*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.

Kanyonyo, M.R., Govaerts, S.J., Hermans, E., Poupaert, J.H., Lambert, D.M., 1999. 3-Alkyl-(5,5′-diphenyl)imidazolidinediones as new cannabinoid receptor ligands. Bioorg. Med. Chem. Lett. 9, 2233–2236.

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.

Landsman, R.S., Makriyannis, A., Deng, H., Consroe, P., Roeske, W.R., Yamamura, H.I., 1998. AM630 is an inverse agonist at the human cannabinoid CB_1 receptor. Life Sci. 62, PL109–PL113.

Lee, S.F., Newton, C., Widen, R., Friedman, H., Klein, T.W., 2001. Down-regulation of cannabinoid receptor 2 (CB_2) messenger RNA expression

during in vitro stimulation of murine splenocytes with lipopolysaccharide. Adv. Exp. Med. Biol. 493, 223–228.
Lutz, B., 2002. Molecular biology of cannabinoid receptors. Prostaglandins, Leukot. Essent. Fat. Acids 66, 123–142.
Mackie, K., Hille, B., 1992. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proc. Natl. Acad. Sci. U. S. A. 89, 3825–3829.
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.
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]-GTPS 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.
Ooms, F., Wouters, J., Oscari, O., Happaerts, T., Bouchard, G., Carrupt, P.A., Testa, B., Lambert, D.M., 2002. Exploration of the pharmacophore of 3-alkyl-5-arylimidazolidinediones as new CB_1 cannabinoid receptor ligands and potential antagonists: synthesis, lipophilicity, affinity, and molecular modeling. J. Med. Chem. 45, 1748–1756.
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.
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., Brelière, 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., Brelière, J.C., Le Fur, G.L., 1998. SR 144528, the first potent and selective antagonist of the CB_2 cannabinoid receptor. J. Pharmacol. Exp. Ther. 284, 644–650.
Selley, D.E., Stark, S., Sim, L.J., Childers, S.R., 1996. Cannabinoid receptor stimulation of guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding in rat brain membranes. Life Sci. 59, 659–668.
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.
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.
Sim, L.J., Hampson, R.E., Deadwyler, S.A., Childers, S.R., 1996. Effects of chronic treatment with delta9-tetrahydrocannabinol on cannabinoid-stimulated [^{35}S]-GTPγS autoradiography in rat brain. J. Neurosci. 16, 8057–8066.
Sim-Selley, L.J., Brunk, L.K., Selley, D.E., 2001. Inhibitory effects of SR 141716A on G-protein activation in rat brain. Eur. J. Pharmacol. 414, 135–143.
Stoit, A.R., Lange, J.H.M., den Hartog, A.P., Ronken, E., Tipker, K., van Stuivenberg, H.H., Dijksman, J.A.R., Wals, H.C., Kruse, C.G., 2002. Design, synthesis and biological activity of rigid cannabinoid CB_1 receptor antagonists. Chem. Pharm. Bull. 50, 1109–1113.
Weiland, T., Jakobs, K.H., 1994. Measurement of receptor-stimulated guanosine 5′-*O*-(gamma-thio)triphosphate binding by G proteins. Methods Enzymol. 237, 3–13.