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Investigation in the CB_1 and CB_2 receptor binding profile and intrinsic activity of (−) and (+)-enantiomers of some naturally occurring phytocannabinoids or synthetic derivatives

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ABSTRACT

Cannabidiol (CBD) and cannabidivarin (CBDV) have shown promising clinical efficacy for the management of epilepsy, and beneficial effects have been demonstrated for CBD in a diversity of other pathologies (pain, schizophrenia, Tourette syndrome, anxiety). However, the mechanism(s) involved are still largely elusive, as are the molecular target(s) involved. CBD and CBDV do not orthosterically bind the cannabinoid type-1 (CB_1) and type-2 (CB_2) receptors, showing only modest allosteric modulation of both end-points. CBD and CBDV are biosynthesized as optically highly pure (−)-enantiomers, and most bioactivity data refer to these forms. (+)-CBD and related analogues [(+)-cannabidiolic acid (CBDA), its esters, and (+)-CBDV] can be obtained by chemical synthesis, and we present evidence that the (+)- and (−)-enantiomers of CBD, CBDV and of a selection of derivatives of CBDA have distinct binding profiles and functional activity at the CB_1/CB_2 receptors. Thus, with the single exception of the methyl ester of CBDA, all the (+)-enantiomers showed higher affinities than the (−)-isomers for both receptors, in particular for the CB_2 receptors. The affinity of the (+)-enantiomers for both CB_1 and CB_2 receptors showed a marked dependence on the nature of the alkyl residue on the aromatic ring and the esterification pattern of CBDA. Potency was rarely in the low nM value for CB_1, but generally so for CB_2. Enantiomers showing low nM activity were further investigated for their intrinsic activity using GTPγS binding assays. This proved that (+)-CBD, (+)-CBDV and the methyl ester of (+)-CBDA are agonists at the CB_2 receptor, with the β-hydroxyethyl ester of (+)-CBDA being an inverse agonist, and its β-hydroxypentyl ester behaving as an agonist at CB_1 and an inverse agonist at CB_2. Finally, we assayed *in vitro* the anti-inflammatory and neuroprotective properties of three compounds [(+)-CBD, (+)-CBDV and (+)-CBDA methyl ester] strongly activating CB_2, showing their ability to reduce the production of proinflammatory factors and protecting neurons against their toxicity. Remarkably, these benefits were eliminated by the selective blockade of the CB_2 receptor,

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highlighting its role as a (+)-CBD target. In summary, our data show that remarkable differences between (−)- and (+)-enantiomers of CBD, CBDV and related compounds exist in terms of CB_1/CB_2 receptor binding profile and intrinsic activity. The observation that the natural (−)-enantiomers do not bind CB_2 receptors suggests that their effects are associated with different targets.

1. Introduction

Cannabidiol (CBD) is a major non-psychotropic cannabis-derived cannabinoid, and has been shown to be endowed with pleiotropic properties potentially beneficial for numerous central and peripheral human disorders [1–5]. As regards CNS pathologies, CBD treatment has been associated to anticonvulsant [6,7], antiemetic [8,9], anxiolytic [10,11], antipsychotic [12,13], antioxidant [14,15], anti-inflammatory [16,17] and neuroprotective [1,18–20] activity in experimental models of epilepsy, vomiting and nausea, anxiety, schizophrenia, oxidative injury, neuroinflammation, and neurodegeneration, respectively (reviewed in Ref. [1,3–5]). However, these well-known broad--spectrum therapeutic properties of CBD contrast with the fact that its underlying cellular and molecular mechanism(s) are not yet completely clarified [1,3–5,21]. Certain activities have been related to endocannabinoid-mediated mechanisms: e.g., inhibition of endocannabinoid inactivation [22,23], allosteric modulation of classic cannabinoid receptors [24–27], activity at atypical cannabinoid receptors as G protein-coupled 55 receptor (GPR 55) [28,29] and members of the transient receptor potential (TRP) family, e.g., TRP vanilloid type-1 ($TRPV_1$) [30]. Conversely, others seem to be the result of endocannabinoid-independent processes: e.g., binding to serotonin receptor types [31–33], adenosine uptake inhibition [2,34], targeting nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) family [35], modulation of ion channels [1], among others.

None of these potential mechanisms links CBD with a direct interaction with classic cannabinoid receptors, at least with their orthosteric sites, and this is why CBD has been frequently viewed as an atypical cannabinoid as concerns its pharmacodynamics profile [1,3–5]. The only and recent exception is the possibility that CBD could bind the allosteric site in the cannabinoid type-1 (CB_1) receptor acting as a negative allosteric modulator (NAM) [24–27], then inhibiting the activity of Δ^9-tetrahydrocannabinol (Δ^9-THC) [36], something that has been recently extended to the cannabinoid type-2 (CB_2) receptor [25,37]. In both cases, the binding of CBD to classic cannabinoid receptors would be at regulatory rather than orthosteric sites, for which it shows negligible affinity [1,3–5], as confirmed by its lack of psychoactivity. The NAM activity of CBD was also demonstrated in endogenous and heterologous cell culture expression systems for ERK1/2 phosphorylation and β-arrestin-1 recruitment in the presence of orthosteric ligands such as Δ^9-THC and 2-arachydonoyl glycerol [24]. Subsequent modelling studies showed that CBD binds to putative CB_1 receptor allosteric sites [38,39]. In addition, some CBD derivatives (see Ref. [40] for a recent review) as its classic, mostly synthetic, metabolic analogues (e.g., desoxy-CBD, 7-hydroxy-CBD, cannabidiolic acid (CBDA)) [41,42], fluorinated CBD [43], CBD hydroxyquinones [44,45], abnormal CBD [46], or the (+)-CBD stereoisomers investigated in this study, have extended the pharmacodynamics profile of CBD (e.g., more potency (fluorinated CBD), activity at novel sites as GPR18 (abnormal CBD) or PPARs (CBD hydroquinones)), as well as its therapeutic potential [42–46], and could be useful also to better understand the mechanisms of action of CBD itself.

One of these derivatives is cannabidivarin (CBDV), which shows a shortened alkyl residue (3- *versus* 5-carbon length), and occurs naturally in the cannabis plant [47]. Its mechanism of action has long remained elusive (reviewed in Ref. [48]), despite its demonstrated anticonvulsant [49–51], antioxidant [52] and antitumoral [53] properties, with also beneficial effects for the management of autism-related disorders, like the Rett syndrome [51,54], and neurodegenerative disorders [55]. Overall, the biological profile of CBDV might well be independent of classic cannabinoid receptors, e.g., antioxidant effects associated to reactive oxygen species scavenging activity (also exerted by CBD [56]), activity at $TRPV_1$, $TRPV_2$ and transient receptor potential ankyrin type 1 ($TRPA_1$) receptors, as well as GPR55 antagonism (reviewed in Ref. [48]).

In the cannabis plant, CBD and CBDV exist in the (−)-enantiomer form, and the study of their biological profile has been based on these enantiomers (reviewed in Ref. [5,57]). Conversely, CBD and CBDV can be obtained by synthesis, which can generate also the non-natural (+)-enantiomeric form or the racemate [5,57]. It was early suggested that stereochemistry strongly affected the binding of Δ^9-THC and analogues to CB_1 and CB_2 receptors ([41,58–62]; reviewed in Ref. [2,5]), with the (−)-forms being more active than the (+)-forms [63]. Thus, compared to (−)-*trans*-Δ^9-THC (the major naturally-occurring isomer), (−)-*cis*-Δ^9-THC was less potent as a CB_1/CB_2 receptor partial agonist *in vitro*, although activity in the cannabinoid tetrad was retained [64]. However, (+)-*cis*-Δ^9-THC was almost inactive ($K_i > 10$ μM) at both CB_1 and CB_2 receptors, compared with the (−)-*trans*-Δ^9-THC and (−)-*cis*-Δ^9-THC [61,65,66]. The same trend was observed in Δ^9-THC derivatives, like those with a dimethylheptyl side chain, for which the (−)-enantiomer (HU-210) is highly active at CB_1/CB_2 receptors, whereas the (+)-enantiomer (HU-211 or dexanabinol) is devoid of any activity at these receptors [67], yet exhibiting interesting pharmacological properties as a non-competitive antagonist for NMDA receptors, showing potential for the management of brain trauma [68].

Overall, few studies have investigated the influence of chirality on the activity of CBD and its derivatives, and this provided the rationale for this study, where various analogues of the natural product and its native acid form (CBDA), including CBDV, CBDA-methyl ester (CBDA-Me), CBDA-glycol ester (CBDA-Gly), CBDA-hydroxypentyl ester (CBDA-Hyp), CBDA-ethyl ester (CBDA-Et) and dimethylheptyl-CBD (DMH-CBD), were investigated, also investigating if differences existed in our assays between natural and synthetic CBD, a controversial issue [69].

2. Materials and methods

2.1. Chemistry

CBD, CBDV, CBDA-Me, CBDA-Gly and CBDA-Hyp were synthesized in (−)- and (+)-*trans*-enantiomeric forms in Symrise AG, Holzminden, Germany following procedures described in Fiebich et al. [70]. CBDA-Et and DMH-CBD were synthesized also in the two enantiomeric forms at the University of Eastern Piedmont, Italy, as described in Mattoteia et al. [71].

2.2. CB_1/CB_2 receptor binding affinity

All compounds under investigation in this study were analyzed by competition studies that allow the determination of their affinity (K_i values in a range of concentrations between 10^{-11}-10^{-4} M) for both receptors against the classical cannabinoid ligand [^{3}H]-CP55940 (164.5 Ci/mmol, PerkinElmer, Boston, MA, USA). The competition studies were carried out with commercially available membranes prepared from CB_1 or CB_2 receptor-stably transfected HEK-293 cells (RBHCB1M400UA and RBXCB2M400UA; PerkinElmer Life and Analytical Sciences, Boston, MA, USA) following procedures previously published [72]. Briefly, membranes were added in assay buffer (for CB_1: 50 mM Tris-Cl, 5 mM $MgCl_2.H_2O$, 2.5 mM EDTA, 0.5 mg/ml bovine serum albumin, pH 7.4, or

for CB_2: 50 mM Tris-Cl, 5 mM $MgCl_2.H_2O$, 2.5 mM EGTA, 1 mg/ml bovine serum albumin, pH 7.5) at a final concentration of 8 μg/well and 4 μg/well for CB_1 and for CB_2 receptors, respectively. The radioligand was used at 0.4 nM for CB_1 receptors or 0.53 nM for CB_2 receptors, always in a final volume of 200 μl for both receptors. The membranes were incubated for 90 min at 30 °C with the radioligand and the different concentrations of (−)- and (+)-CBD and derivatives. Non-specific binding was determined with non-radiolabeled WIN55,212-2 (Sigma Aldrich, Madrid, Spain, 10 μM) in the presence of radioligand. 100 % binding of the [^{3}H]-CP55940 was determined by incubation of the membranes with radioligand in the absence of the compound under investigation. All plastic materials employed were siliconized with Sigmacote (Sigma-Aldrich, Madrid, Spain) to prevent possible adhesion of the compounds. After incubation, free radioligand was separated from bound radioligand, by filtration in GF/C filters, previously treated with a 0.05 % (v/v) polyethylenimine solution. Then, filters were washed nine times with cold assay buffer, using the Harvester® filtermate equipment (PerkinElmer, Boston, MA, USA). Radioactivity was measured using a liquid scintillation spectrometer (Microbeta Trilux 1450 LSC & Luminescence Counter (PerkinElmer, Boston, MA, USA)). Data were expressed as percentage of [^{3}H]-CP55940 binding and were analyzed by using GraphPad Prism® version 7 (GraphPad Software Inc., San Diego, CA, USA), for the calculation of K_i values for each receptor. They were expressed as mean ± SEM of at least three experiments performed in triplicate for each point.

2.3. [^{35}S]-GTPγS binding analysis

[^{35}S]-GTPγS binding was analyzed using CB_1 or CB_2 receptor-containing membranes (HTS020M2, Eurofins Discovery Services, St. Charles, MO, USA). To this end, membranes (5 μg/well) were permeabilized by addition of saponin (Sigma-Aldrich, Madrid, Spain), then mixed with 0.3 nM [^{35}S]-GTPγS (PerkinElmer, Boston, MA, USA) and 10 μM GDP (Sigma-Aldrich, Madrid, Spain) in 20 mM HEPES (Sigma-Aldrich, Madrid, Spain) buffer containing 100 mM NaCl (Merck, Madrid, Spain) and 10 mM $MgCl_2$ (Merck, Madrid, Spain), at pH 7.4. Increasing concentrations of the compounds of interest: (+)-CBD, (+)-CBDV, (+)-CBDA-Me, (+)-CBDA-Gly and (+)-CBDA-Hyp (from 10^{-12} to 10^{-5} M) were added in a final volume of 100 μl and incubated for 30 min at 30 °C. The non-specific signal was measured with 10 μM GTPγS (Sigma-Aldrich, Madrid, Spain). All 96-well plates and the tubes necessary for the experiment were previously siliconized with Sigmacote® (Sigma-Aldrich, Madrid, Spain). The reaction was terminated by rapid vacuum filtration with a filter mate Harvester apparatus (PerkinElmer, Boston, MA, USA) through Filtermat A GF/C filters. The filters were washed nine times with ice-cold filtration buffer (10 mM sodium phosphate, pH 7.4), and bound radioactivity was measured with a 1450 LSC & Luminiscence counter Wallac MicroBeta TriLux (PerkinElmer, Boston, MA, USA). [^{35}S]-GTPγS binding data were analyzed to determine the EC_{50} or IC_{50}, as well as the E_{max} values by using an iterative curve-fitting procedure with the GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego, CA, USA). Values are expressed as mean ± SEM of at least three experiments performed in triplicate for each point.

2.4. Cell-based assays for analyzing anti-inflammatory and neuroprotective effects

The anti-inflammatory and neuroprotective properties of some of the (−)- and (+)-enantiomers were investigated in two cell-based assays using cultures of the mouse induced-(or immortalized) microglial (iMG) cell line (generously provided by Dr. Isabel Lastres-Becker, Universidad Autónoma de Madrid, Spain), and of the mouse striatal neuronal cell line M213-2O (generously donated by Dr. WJ Freed, National Institute on Drug Abuse, Bethesda, MD, USA). In the first experiment, cultured iMG cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Verviers, Belgium) supplemented with 10 % fetal bovine serum (FBS, Sigma-Aldrich, Madrid, Spain), 2 mM Ultraglutamine and antibiotics (Lonza, Verviers, Belgium) in a humidified atmosphere of 5 % CO_2 at 37 °C. For experiments, cells were plated at a density of 2.25 x 10^5 cells *per* well in 12-well culture plates and incubated in DMEM with a reduction of FBS to 1 %. Three hours later, cells were treated for 24 h with 100 ng/ml LPS (from Escherichia coli 055:B5, Sigma-Aldrich, Madrid, Spain), alone or in combination with some of the (−)- and (+)-enantiomers, used at three different concentrations: 0.01, 0.1 and 1 μM, and added 1 h before LPS. Twenty-four hours after the addition of LPS, media were removed, whereas cell pellets were collected for analysing mRNA levels of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) by qPCR. In a second experiment, cultured iMG cells were again maintained in DMEM (Lonza, Verviers, Belgium) supplemented with 10 % FBS (Sigma-Aldrich, Madrid, Spain), 2 mM Ultraglutamine and antibiotics (Lonza, Verviers, Belgium) in a humidified atmosphere of 5 % CO_2 at 37 °C. Again, they were plated at a density of 2.25 x 10^5 cells *per* well in 6-well culture plates and incubated in DMEM with a reduction of FBS to 1 %. Three hours later, cells were treated for 24 h with 100 ng/ml LPS (from Escherichia coli 055:B5, Sigma-Aldrich, Madrid, Spain), alone or in combination with (+)-CBD, (+)-CBDV and (+)-CBDA-Me at the concentration of 1 μM (this was the concentration most active in reducing LPS-induced elevation of proinflammatory markers in the previous experiment) added 1 h before LPS. This experiment also included two additional groups for each (+)-enantiomer which consisted in co-treatment with the selective CB_2 receptor antagonist SR144528 (Cayman Chemical Co., Ann Arbor, MI, USA) or the selective PPAR-γ inhibitor T0070907 (Cayman Chemical, Ann Arbor, MI, USA), both at 10 μM, as well as control cells only treated with vehicle (0.1 % DMSO). Twenty-four hours after the addition of LPS, media were removed to be added to cultures of the rat M-213-2O striatal cells to induce cell death following a procedure described previously [72]. To this end, M-213-2O cells were maintained in DMEM supplemented with 10 % FBS, 2 mM Ultraglutamine, and 1 % antibiotics (Lonza, Verviers, Belgium) and under a humidified 5 % CO_2 atmosphere at 37 °C. For cytotoxicity experiments, cells were seeded at 50000 cells/well in 24-well plates and maintained under a humidified atmosphere (5 % CO_2) at 37 °C overnight. Afterwards, normal medium was completely replaced by the different conditioned media generated in iMG cell experiments. Cells were incubated for 40 h before the neuronal death was analyzed with the MTT assay (Panreac AppliChem., Barcelona, Spain). The data were normalized in relation with a control group consisting in M-213-2O cells exposed to those conditioned media generated by iMG cells in absence of LPS.

2.5. Real time qRT-PCR analysis

Cell pellets from the *in vitro* experiments were used for qRT-PCR analysis. Total RNA was isolated using TRI Reagent® (Sigma-Aldrich, Madrid, Spain) following the procedure described by manufacturers. The total amount of RNA extracted was measured by spectrometry at 260 nm and its purity from the ratio between the absorbance values at 260 and 280 nm. After genomic DNA was removed (to eliminate DNA contamination), single-stranded complementary DNA was synthesized from up to 1 μg of total RNA using the commercial kit Rneasy Mini Quantitect Reverse Transcription (Qiagen, Hilgen, Germany). The reaction mixture was kept frozen at −20 °C until enzymatic amplification. Quantitative RT-PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for TNF-α (ref. Mm99999064_m1), IL-1β (ref. Mm00434228_m1), iNOS (ref. Mm01309902_m1), COX-2 (ref. Mm00478372_m1), using GAPDH expression (ref. Mm99999915_g1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied

Biosystems, Foster City, CA, USA). Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Calculation of in silico ADME parameters

A set of 34 physicochemical descriptors was computed using QikProp version 3.5 integrated in Maestro (Schrödinger, LLC, New York, USA). The QikProp descriptors are shown in Supplementary Material 1. The 3D conformations used in the calculation of QikProp descriptors were generated using the program Spartan'08 (Wave function, Inc., Irvine, CA, USA) as follows: The structure of each molecule was built from the fragment library available in the program. Then, *ab initio* energy minimizations of each structure at the Hartree−Fock 6-31G* level were performed. A conformational search was next implemented using Molecular Mechanics, followed by a minimization of the energy of each conformer calculated at the Hartree−Fock 6-31G* level. The global minimum energy conformer of each compound was used as input for ADME studies with QikProp.

2.7. Statistical analysis

All statistical analyses were carried out using GraphPad Prism 10.2.0 for Windows (GraphPad Software, San Diego, CA, USA). Values were expressed as mean ± standard error of the mean (SEM). Data in cell-based assays were assessed for multiple comparison using the one-way analysis of variance followed by the Tukey's (data of cell viability) or Dunnett's (data of proinflammatory mediators) test.

3. Results

3.1. Binding affinity of (−)- and (+)-cannabinoids in receptor binding assays

Competition studies using CB_1 or CB_2 receptor-transfected membranes, [^{3}H]-CP55940 as a radioactive probe, and different concentrations of the compounds (always within the interval 10^{-4}-10^{-12} M) under investigation were conducted with the different (−)- and/or (+)-cannabinoids. This served to calculate their K_i values as an index of their CB_1 and CB_2 receptor affinities. In our first analysis, we compared naturally-occurring (−)-CBD (isolated from cannabis plant) and synthetic (−)-CBD for their affinity (K_i) at the CB_1 and CB_2 receptors. In both cases, (−)-CBD showed similar very low affinity values for both receptors ($K_i > 3$ μM) (Fig. 1), with an expected lack of selectivity that was visible even despite the very low affinity values (CB_1/CB_2 K_i ratio = 2.3 for synthetic (−)-CBD and 1.9 for natural (−)-CBD). This occurred in both (−)-CBD forms, then being irrespective of their natural or synthetic origin, with binding curves in Fig. 1 that reflect the characteristic hyperbole profile of those compounds having a small or negligible affinity (K_i in the μM range), which contrasts with the classic sigmoid pattern of those compounds having a high affinity (K_i in the nM range) (see below). Therefore, we can conclude that there is not any significant difference when synthetic and plant-derived (−)-CBD are analyzed for their binding at both CB_1 and CB_2 receptors (Fig. 1). This confirms that chemical synthesis *per se* does not affect the binding profile of (−)-CBD that was previously described using naturally-occurring (−)-CBD [69] and was not the reason for the differences that will be presented below when (−)-enantiomers are compared with (+)-enantiomers.

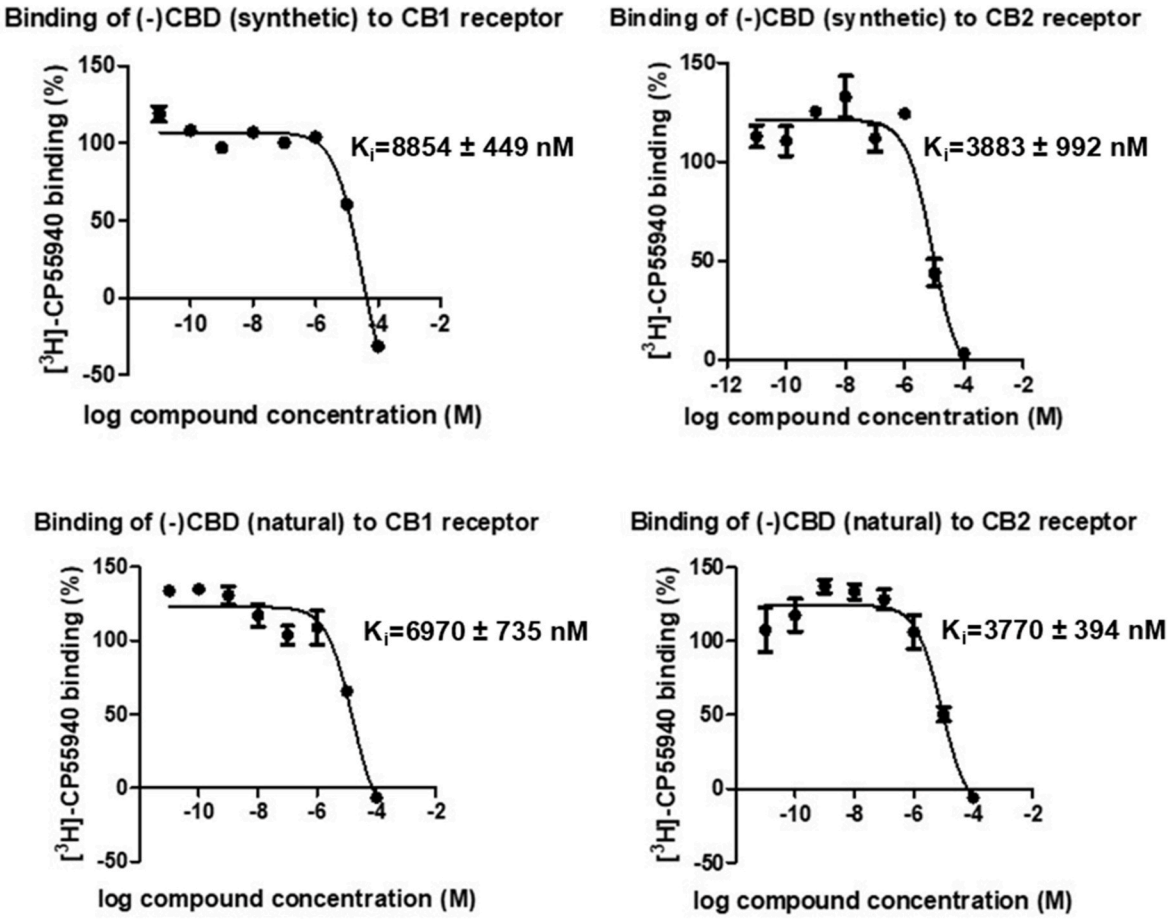

Fig. 1. Representative competition curves for the binding of plant-derived (natural) and synthetic (−)-CBD at the CB_1 and CB_2 receptors. K_i values were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

Next, we compared the two enantiomers for the seven cannabinoid derivatives under investigation in this study, all of them, (−)- and (+)-enantiomers generated always by chemical synthesis. Our data revealed, in general, a greater affinity of (+)- *versus* (−)-enantiomers of these derivatives at the CB_1 and, in particular, CB_2 receptors (Figs. 2–7). Thus, in the case of CBD, the (+)-enantiomer reached an affinity in the nM range (~100 fold higher *versus* (−)-CBD) for the CB_2 receptor, whereas the increase was lower and in the high nM range (~10 fold *versus* (−)-CBD) for the CB_1 receptor (Fig. 2). These different increases in affinity between both receptors affected the CB_1/CB_2 K_i ratio, which informs about receptor selectivity, being 2.3 for the (−)-CBD, but elevated up to 24.3 for (+)-CBD. In the case of CBDV, the (+)-enantiomer also reached an affinity in the nM range (~100 fold higher *versus* (−)-CBDV) for the CB_2 receptor, again with a lower increase, but sufficient to situate the affinity in a medium nM range (~40 fold *versus* (−)-CBD), for the CB_1 receptor (Fig. 3). The CB_1/CB_2 K_i ratio of (−)-CBDV was 3.5, whereas it elevated to 8.9 in the case of (+)-CBDV, again indicating that the (+)-enantiomer was more active at the CB_2 receptor.

CBDA-Me was the exception as both (−) and (+) enantiomer forms showed relatively similar affinities, which are in the nM range (<50 nM) for the CB_2 receptor and in a much more moderate nM range (>300 nM) for the CB_1 receptor (Fig. 4). The CB_1/CB_2 K_i ratios were 12.3 for the (+)-enantiomer and 8.1 for the (−)-enantiomer. As for CBDA-Gly, (−)- and (+)-enantiomers bind the CB_2 receptor, but the affinity of the (+)-enantiomer was again in a low nM range (~30 fold higher *versus* (−)-CBDA-Gly), whereas their affinities for the CB_1 receptor were lower, in particular in the case of the (−)-enantiomer (~10 fold lower *versus* (+)-CBDA-Gly) whose binding ability was almost negligible (K_i in the μM range) (Fig. 5). Again, the (+)-enantiomer displayed a much higher preference for the CB_2 receptor (CB_1/CB_2 K_i ratio = 27.8) compared with the (−)-enantiomer (CB_1/CB_2 K_i ratio = 10.5). CBDA-Hyp resulted in being one of the derivatives having the highest affinities for both receptors, in particular in the case of the (+)-enantiomer (~330 fold and ~80 fold higher *versus* (−)-CBDA-Hyp for the CB_1 and CB_2 receptor, respectively), which showed K_i values in a low nM range (K_i < 3 nM) (Fig. 6). Contrarily to the above compounds, the (−)-enantiomer showed much more preference for the CB_2 receptor (CB_1/CB_2 K_i ratio = 10.9) compared with the (+)-enantiomer (CB_1/CB_2 K_i ratio = 2.6), although in this case the differences turned out to be lower. All (−)- and (+)-CBDA-Hyp data (including similar data to those obtained here for (−)- and (+)-CBD) agree with previous results already published for this CBD derivative in a study addressed to investigate its benefits for the treatment of diabetic nephropathy in mice [73].

Two additional derivatives (CBDA-Et and DMH-CBD) were also analyzed for their affinity at the CB_1 and CB_2 receptors and showed again that the (+)-enantiomers resulted in being more active at these receptors than (−)-enantiomers (Figs. 7 and 8). Thus, (+)-CBDA-Et showed higher affinities (~5 fold higher *versus* (−)-CBDA-Et) for both receptors, with a CB_1/CB_2 K_i ratio also similar for the (−)- and (+)-enantiomers (13.7 and 12.6, respectively) (Fig. 7), strongly indicating their preference for the CB_2 receptor. As regards to (−)- and (+)-enantiomers of DMH-CBD, they were all highly active (K_i < 10 nM) at both receptors, with the (+)-enantiomer having a higher affinity (~20 fold and ~3 fold higher *versus* (−)-DMH-CBD for the CB_1 and CB_2 receptors, respectively) (Fig. 8). Their CB_1/CB_2 K_i ratios were 1.3 for the (−)-enantiomer, indicating no preference, but lower (0.2) for the (+)-enantiomer. This may indicate a certain preference for the CB_2 receptor despite the low K_i values which enable the (+)-enantiomer to be active at both receptors. It is important to remark that the high affinity and potency of DMH-CBD for cannabinoid receptors was already disclosed in the early 90s, showing that the presence of a dimethyl-heptyl side chain, both in Δ^9-

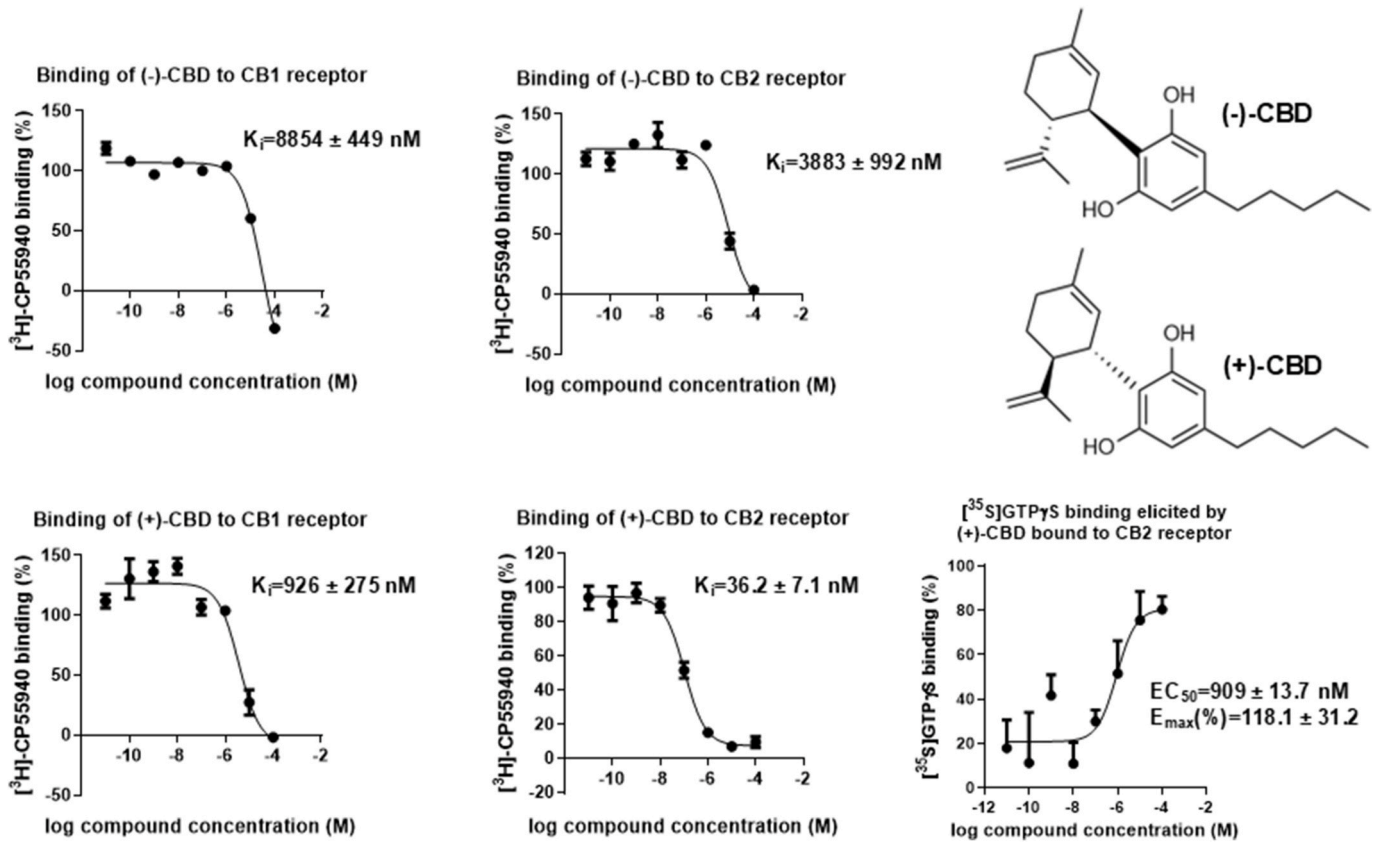

Fig. 2. Representative competition curves for the binding of synthetic (−)- and (+)-CBD at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and EC_{50} and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

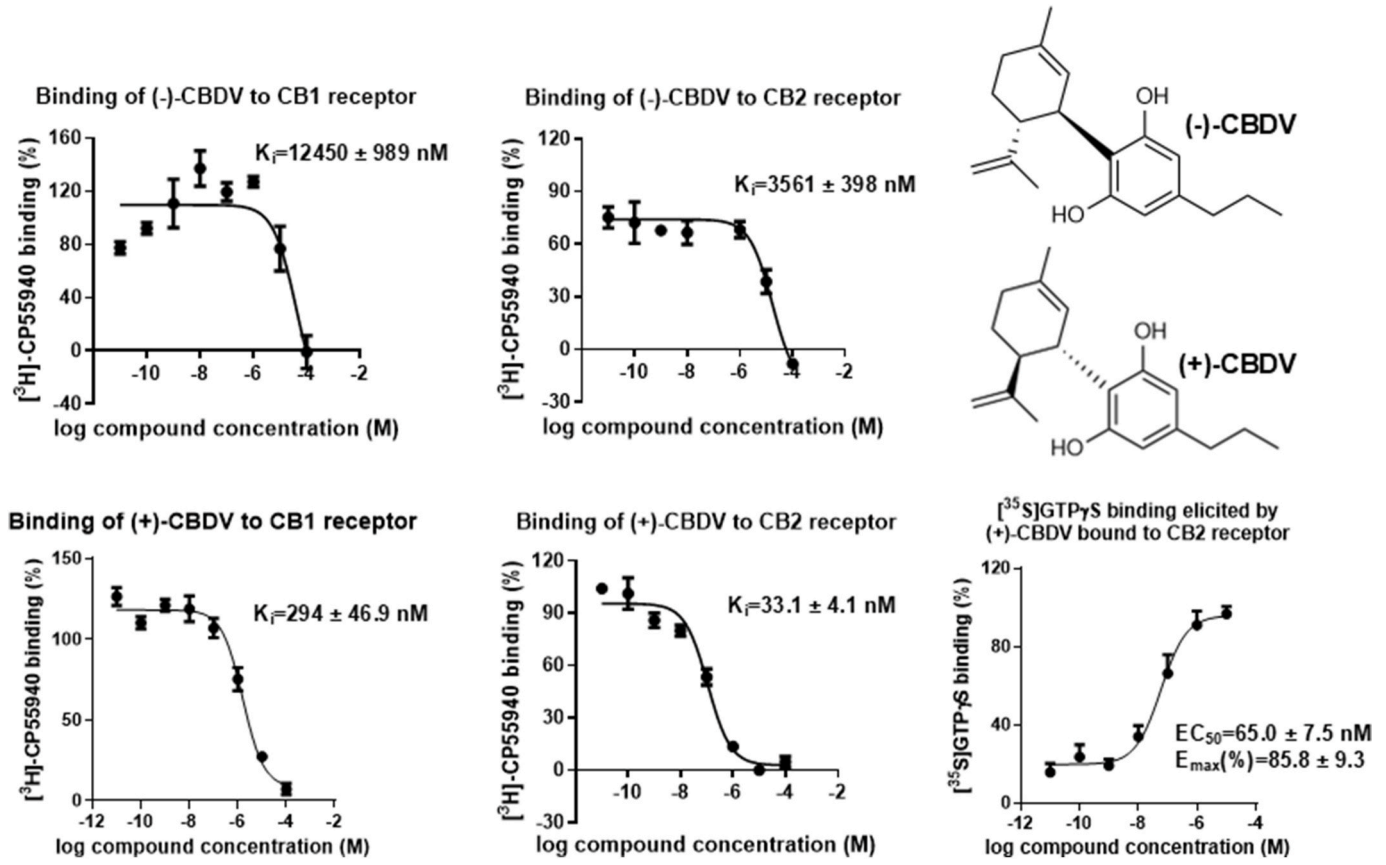

Fig. 3. Representative competition curves for the binding of synthetic (−)- and (+)-CBDV at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and EC_{50} and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

THC and some analogues, as well as in CBD, enhanced cannabimimetic activity [58–62]. As regards its (−)- and (+)-stereoisomers, our data support their activity at both CB_1 and CB_2 receptors, in contrast to data reviewed in Burstein [2] and Rao et al. [5], who concluded that (−)-DMH-CBD is equally active at both receptors, whereas (+)-DMH-CBD has a lower preference for the CB_2 receptor than for the CB_1 receptor [22,41,74].

3.2. Intrinsic activity of (−)- and (+)-cannabinoids in the [^{35}S]-GTPγS assay

Some of these novel enantiomers, in particular those showing affinities in a relatively low nM range, were furtherly investigated for their intrinsic activity (agonist, antagonist or inverse agonist) using GTPγS binding assays, focusing almost exclusively on the CB_2 receptor, based on the higher preference of all compounds for this receptor (Figs. 2–6, and Table 1). These assays proved that (+)-CBD (Fig. 2), (+)-CBDV (Fig. 3) and (+)-CBDA-Me (Fig. 4) have agonist activity at the CB_2 receptor, with (+)-CBDV having the higher potency (EC_{50} < 100 nM) followed by (+)-CBD (EC_{50} < 1 μM) and (+)-CBDA-Me (EC_{50} < 50 μM) (Figs. 2–4, respectively). On the other hand, (+)-CBDA-Gly was an inverse agonist at this receptor (IC_{50} < 400 nM; Fig. 5), whereas (+)-CBDA-Hyp behaved as agonist at the CB_1 receptor (EC_{50} < 20 nM) but as an inverse agonist at the CB_2 receptor (IC_{50} < 10 nM) (Fig. 6). DMH-CBD was excluded from these analyses as it was already investigated for its intrinsic activity in previous studies [58–62,75], whereas CBDA-Et was not investigated due to compound availability.

3.3. In silico analysis of ADME properties of (+)-CBD and derivatives

Given that previous studies have indicated that different pharmacodynamics of some of these enantiomers could be associated with differences in their pharmacokinetics (e.g., restriction at the brain-blood barrier (BBB) to enter the brain; see Ref. [41,74]), we also wanted to analyze some of the ADME properties of these compounds using *in silico* predictions. This possibility would explain why some (+)-CBD derivatives (including some of the (+)-enantiomers investigated here) bind the CB_1 receptor *in vitro*, but did not reproduce the classical cannabinoid tetrad *in vivo* [74]. This analysis was carried out with five of the (+)-enantiomers (CBD, CBDV, CBDA-Me, CBDA-Gly and CBDA-Hyp) using (−)-CBD as the reference compound. Our data indicated that ADME properties for all compounds, especially for (+)-CBD and (+)-CBDV, were, in general, within the expected ranges (see Supplementary Files 1 and 2). Of particular interest was the case of: (i) QPlogS and CIQPlogS (aqueous solubility) were higher for (+)-CBD and (+)-CBDV than for the three (+)-CBDA derivatives (Supplementary Files 1 and 2); (ii) QPlogHERG (potential cardiotoxicity problems), with some concern for (+)-CBDA-Gly and, in particular, (+)-CBDA-Hyp (Supplementary Files 1 and 2); (iii) QPPCaco, QPlogBB and QPPMDCK (crossing tissue barriers from blood including the BBB), higher for (+)-CBD and (+)-CBDV and lower for (+)-CBDA-Gly (Supplementary Files 1 and 2); and (iv) HumanOralAbsorption (human oral absorption), higher for (+)-CBD and (+)-CBDV, lower for the three (+)-CBDA derivatives. These differences were not found with the equivalent parameter PercentHumanOralAbsorption (Supplementary Files 1 and 2), although this last parameter does not have to correlate with the previous one.

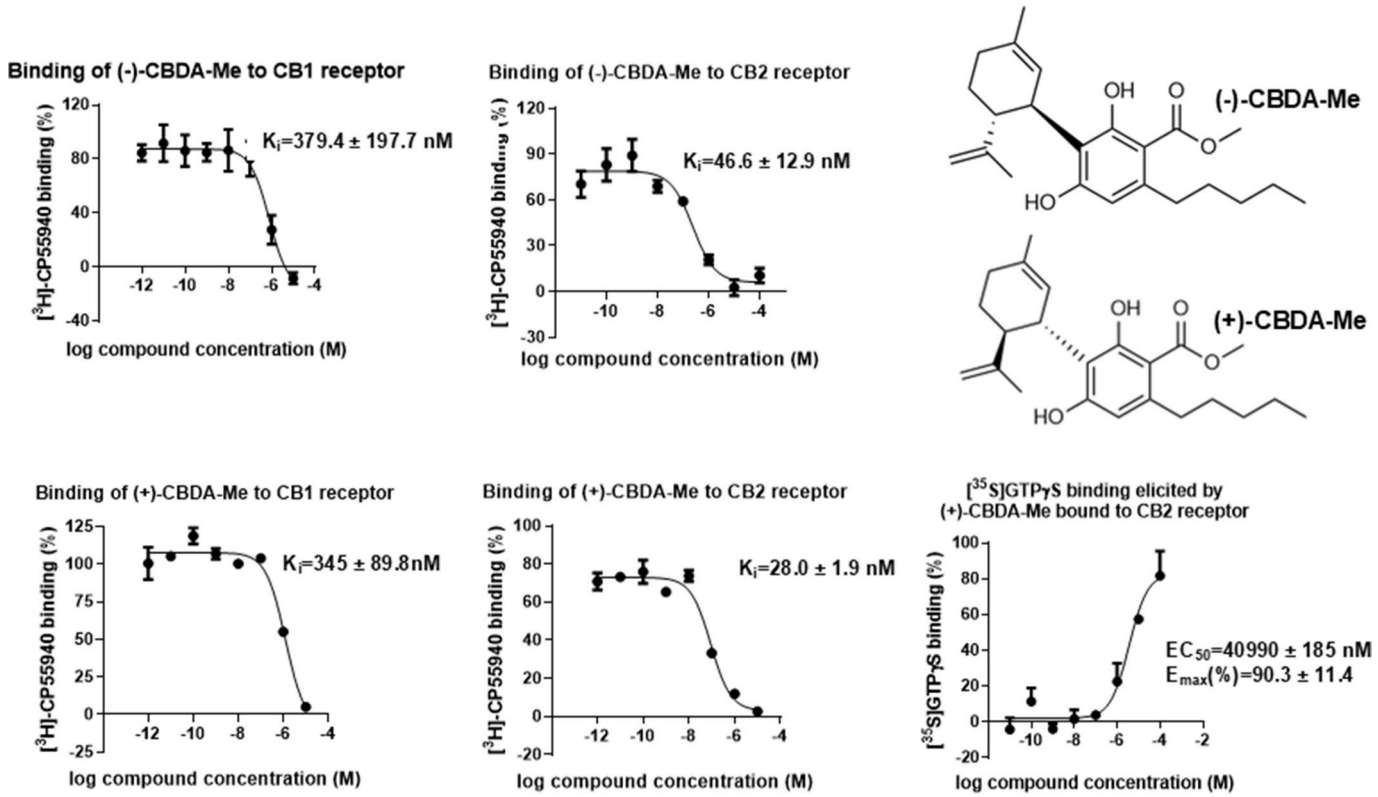

Fig. 4. Representative competition curves for the binding of synthetic (−)- and (+)-CBDA-methyl-ester (CBDA-Me) at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and EC_{50} and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

3.4. Anti-inflammatory and neuroprotective effects of some (−)- and (+)-enantiomers in cell-based assays

The last experiment in this study was aimed at exploring the ability of (+)-CBD, (+)-CBDV and (+)-CBDA-Me, the three (+)-enantiomers that showed an agonist activity at the CB_2 receptor in [^{35}S]-GTPγS binding assays, to exert anti-inflammatory and neuroprotective properties in some cell-based assays useful for this purpose. Such capability should be an expected consequence of their acquired or improved affinity and agonist activity for this receptor. To this end, we analyzed first the effect of three different concentrations of these three (+)-enantiomers against LPS-induced elevation of gene expression of some relevant proinflammatory markers, IL-1β, TNF-α, COX-2 and iNOS, in cultured iMG cells. Our results confirmed that the addition of LPS to these cells elevated the expression of the four markers analyzed (Fig. 9), whereas the co-incubation with (+)-CBD, (+)-CBDV or (+)-CBDA-Me was able to decrease (or tended to reduce) this elevated expression for all markers (except for (+)-CBDA-Me), with effects more evident for IL-1β ($F(10,37) = 2.15$, $p < 0.05$; Fig. 9), but apparently lower for TNF-α ($F(10,39) = 6.94$, $p < 0.0001$; Fig. 9) and, to a lower extent, COX-2 ($F(10,37) = 2.61$, $p < 0.05$; Fig. 9) and iNOS ($F(10,39) = 1.05$, ns; Fig. 9). In all cases, the highest concentration of 1 μM was the most active and the only being statistically significant with respect to the LPS-treated cells (Fig. 9), with the lower concentrations being inactive or remaining as mere numerical trends (Fig. 9). This is the case of the 0.1 μM which showed values of probability levels respect to the LPS-treated cells close to statistical significance for IL-1β in the case of (+)-CBDA-Me ($p = 0.09$), (+)-CBDV ($p = 0.11$) and (+)-CBD ($p = 0.18$).

In a second experiment, the aim was to demonstrate that the changes elicited by LPS (marked elevation) and by the three (+)-enantiomers (reduction or trends towards a decrease) on the gene expression of these proinflammatory markers in cultured cells would be reflected in the release of these proinflammatory cytokines or other factors in their cultured media as has been described in previous studies [72], so that their addition to cultured neurons may compromise the cell viability. To this end, we generated conditioned media from iMG cells treated with vehicle or LPS in absence or presence of the most active concentration for each (+)-enantiomer in the previous experiment (1 μM), which were added to M-213-2O cells (these cells do not express CB_2 receptors [72]) to induce cell death. Our data confirmed that conditioned media from LPS-exposed iMG cells reduced the cell viability of M-213-2O cells by approximately 40 %, whereas (+)-CBD, (+)-CBDV and (+)-CBDA-Me ($F(10,43) = 9.52$, $p < 0.0001$; Fig. 10) partially reversed the reduction in cell viability (loss of statistical significance respect LPS-treated cells), in particular in the case of (+)-CBDV and (+)-CBDA-Me (Fig. 10). In this experiment, two additional experimental groups of cells were included with the purpose to confirm that the beneficial effects elicited by (+)-enantiomers against LPS-induced cell death were dependent on the activation of CB_2 receptors by the (+)-enantiomer, as well as to discard the contribution of other related receptors (e.g., PPAR-γ). Our data demonstrated that the conditioned media generated by the co-incubation of the (+)-enantiomer and the CB_2 receptor antagonist SR144528 in LPS-exposed iMG cells eliminated the beneficial effect of the (+)-enantiomer alone in the three cases, whereas this did not happen when the co-incubation was with T0070907, an inhibitor of the PPAR-γ (Fig. 10). In fact, in the case of (+)-CBD, but not in the case of the other two (+)-enantiomers, the presence of T0070907 apparently improved the beneficial effect of (+)-CBD on cell viability (Fig. 10). A clarification of the rationale for this improvement will require further research, but *a priori* this could indicate that the activity of (+)-CBD at the CB_2 receptor may be more intense when the enantiomer has a restriction in its capability to act at the PPAR-γ due to the blockade of this receptor. However, the data showed in previous studies using T0070907 or similar covalent antagonists of PPAR-γ, which demonstrated that they

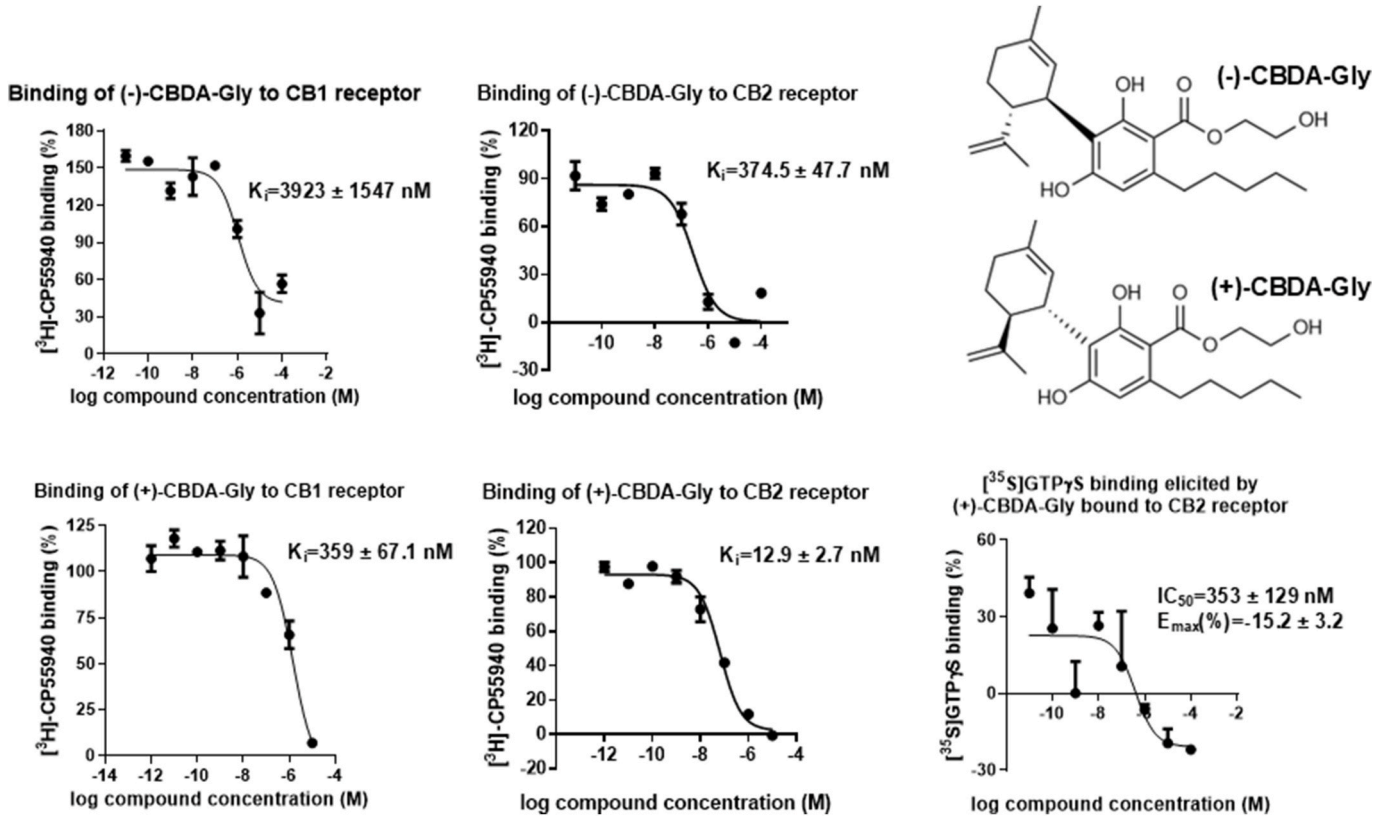

Fig. 5. Representative competition curves for the binding of synthetic (−)- and (+)-CBDA-glycol-ester (CBDA-Gly) at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and IC_{50} and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

exert only a partial blockade of the orthosteric binding site allowing that certain ligands may still activate PPAR-γ signaling through their binding to so-called omega site in the ligand binding domain [76–78], provide additional explanations based on the activity of (+)-CBD at this omega pocket. Such possibility was also claimed in our previous study carried out with a cannabigerol derivative, so-called VCE-003.2, in an experimental model of Parkinson's disease [79].

The last experiment in this part of the study was aimed at investigating whether (−)-CBD and (−)-CBDV, which, according to our competition studies for the CB_2 receptor are unable to bind and activate this receptor, may, nevertheless, to be also active in reducing the LPS-induced elevation of proinflammatory factors in iMG cells, as their (+)-enantiomers, which are able to bind and activate the CB_2 receptor, did. If this were the case, the mechanism(s) for these effects should be other than the activation of the CB_2 receptor (e.g., activity at the PPAR-γ or other targets), or would imply a possible *in vivo* biotransformation of the (−)-enantiomer into the (+)-isomer. Our data open these two possibilities or others, as we recorded mostly similar patterns in the effects of (−)-enantiomers compared to (+)-enantiomers, in particular for IL-1β ($F(7,31) = 3.36$, $p < 0.01$; Supplementary File 3), COX-2 ($F(7,31) = 9.73$, $p < 0.0001$; Supplementary File 3), iNOS ($F(7,31) = 15.61$, $p < 0.0001$ Supplementary File 3) and, to a lower extent (it happened only for (−)-CBDV), TNF-α ($F(7,31) = 10.78$, $p < 0.0001$; Supplementary File 3), in all cases with the higher concentration (1 μM) being the most active, but also having statistically significant effects (or numerical trends) with the 0.1 μM concentration in some cases (e.g., (−)-CBD and (−)-CBDV for iNOS).

4. Discussion

The aim of this study was to compare the CB_1/CB_2 receptor binding profile and/or intrinsic activity of several synthetic (−)- and (+)-cannabinoids, including CBD, CBDV and some CBDA derivatives, with the aim of demonstrating the existence of important differences between (−)- and (+)-enantiomeric forms of these cannabinoids. However, before addressing this major objective, we wanted to discard the hypothesis that any enantiomer-dependent difference (see below) may be the result of the natural or synthetic origin of the different isomers. The rationale for addressing this question is that most binding data for CBD have been obtained with (−)-CBD from cannabis [58–62] and one recent study claimed that using (−)-CBD generated by chemical synthesis could result in different pharmacodynamics parameters [69]. However, our data confirmed that, independently from the origin: (i) (−)-CBD has a poor affinity at the classic cannabinoid receptors, with K_i values for both CB_1 and CB_2 receptors within the low μM range; (ii) the binding of (−)-CBD to CB_2 receptors was certainly higher (approximately 2-fold) than to CB_1 receptors, although always within the μM range; and (iii) the receptor binding affinity of (−)-CBD was always similar comparing the synthetic (−)-CBD with the plant-derived compound. This result is in agreement with a previous report showing that CBD is a partial agonist for CB_2 receptor at high concentrations [80]. In general, our results confirm previous data showing that (−)-CBD, irrespective of its origin, is a low-affinity ligand for the cannabinoid receptors [81], yet capable to modulate certain intracellular signals that are dependent on the activation of these receptors [82]. Thus, our results may also confirm previous observations on the possible activity of (−)-CBD as a negative allosteric modulator at the CB_1 receptor [69,83]. They also confirm its proposed behavior at the CB_2 receptor, in which claims for an agonist activity have been made in specific experimental conditions [84], despite other studies have also claimed for a similar antagonist/negative allosteric modulator activity [38] that has been proposed for the CB_1 receptor.

Having discarded the possible influence of the natural or synthetic origin of the different compounds under investigation in their receptor

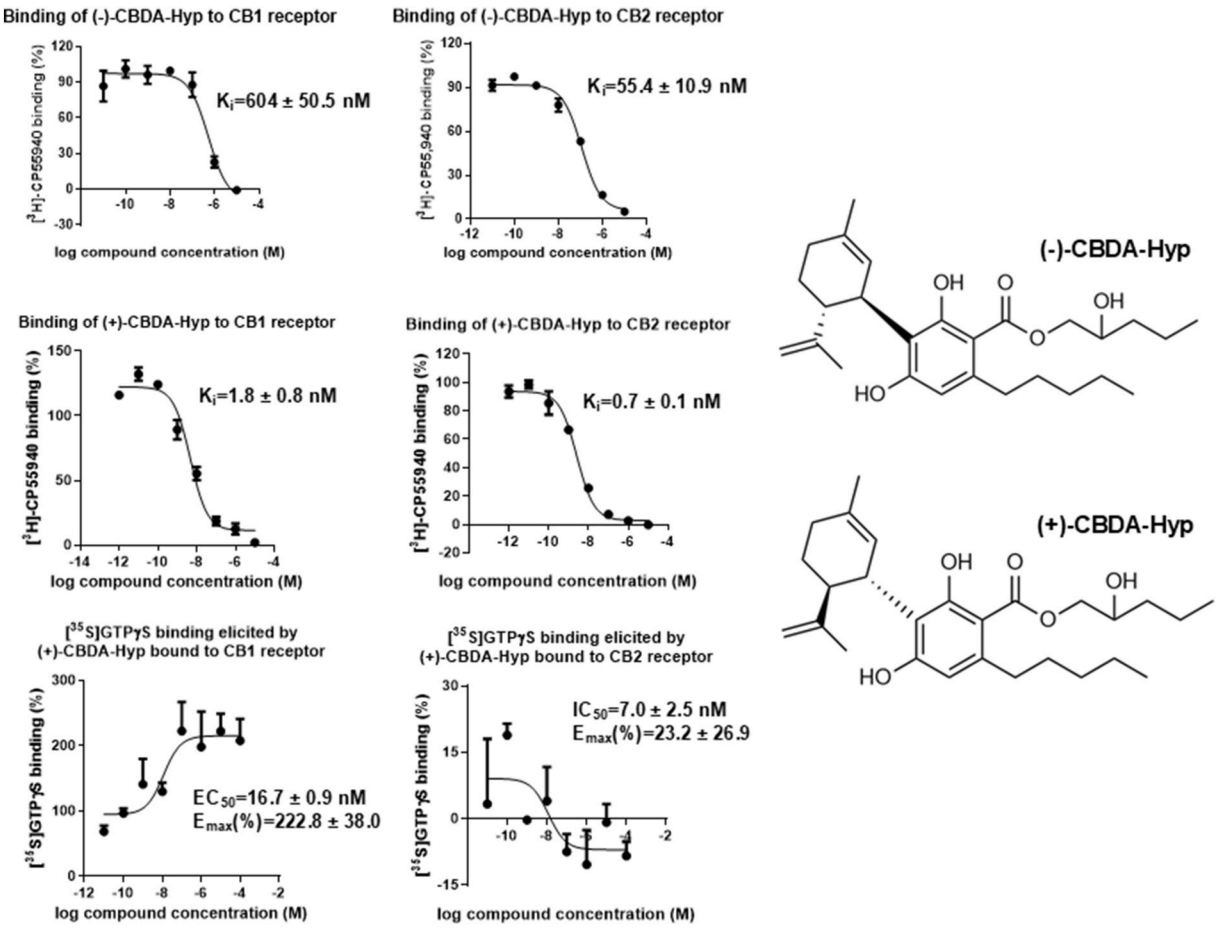

Fig. 6. Representative competition curves for the binding of synthetic (−)- and (+)-CBDA-hydroxypenthyl-ester (CBDA-Hyp) at the CB_1 and CB_2 receptors, including the analysis of the intrinsic activity, using the procedure of [^{35}S]GTPγS binding, of those derivatives showing affinity in the low nM range. K_i for receptor binding and EC_{50} (or IC_{50}) and E_{max}(%) values for [^{35}S]GTPγS binding were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

binding profile, we addressed our major objective, namely evaluating if (−)- and (+)-enantiomeric forms of various chiral cannabinoids showed differences in their receptor affinity for CB_1 and/or CB_2 receptors, as well as in their intrinsic activity at these receptors, following previous results that have been reviewed by Burstein [2] and Rao et al. [5]. Our data fully confirmed this hypothesis, with (+)-enantiomers always showing higher affinities than (−)-enantiomers for both receptors, in particular for the CB_2 receptor, except in the case of CBDA-Me. Our quantification of receptor affinities (K_i) of (+)-enantiomers for the CB_1 receptor yielded to the following order: DMH-CBD = CBDA-Hyp > CBDA-Et > CBDV = CBDA-Me = CBDA-Gly > CBD, with only DMH-CBD and CBDA-Hyp having a K_i in a low nM range. In the case of the CB_2 receptor, the order was CBDA-Hyp > DMH-CBD > CBDA-Et > CBDA-Gly > CBDA-Me = CBDV = CBD, all of them with K_i < 50 nM. These data, however, present differences from those published by Bosquez-Berger et al. [85] who reported that effectively (+)-CBD was more potent than (−)-CBD for some responses related to CB_1 receptor activity (e.g., ~5 fold higher affinity (lower K_i) at the CB_1 receptor; ~10 fold greater potency at inhibiting depolarization-induced suppression of excitation), but lower for others (e.g., CB_1 receptor-mediated suppression of cAMP accumulation). These authors also demonstrated that (+)-CBD, but not (−)-CBD, was active at some non-cannabinoid receptors as sphingosine-1-phosphate receptors [85]. In addition, Golliher and co-workers also described higher affinity to CB_1/CB_2 receptors for (+)-CBD than the natural enantiomer [57], and the same happened with some CBD derivatives as 8,9-dihydro-7-hydroxy-CBD, with the (+)-enantiomer having K_i in the nM range, but the (−)-enantiomer showing very low affinity [86]. A former study also compared several CBD derivatives in both enantiomeric forms and found higher activity of (+)-stereoisomers for the CB_1 receptor in the low nM range, whereas the differences for the CB_2 receptor binding were smaller [41]. These data showed the opposite that we found in our study, although the only common compound with our study was DMH-CBD [41].

In our study, some (+)-enantiomers, selected because their affinities were in the low nM range and also because of their availability and the absence of previous data, were also investigated for their intrinsic activity using GTPγS binding assays. These assays proved that (+)-CBD, (+)-CBDV and (+)-CBDA-Me are agonists at the CB_2 receptor and (+)-CBDA-Gly was an inverse agonist, whereas (+)-CBDA-Hyp behaved as agonist at the CB_1 receptor but as an inverse agonist at the CB_2 receptor. However, it is important to highlight that these intrinsic activities were found in agonist-stimulated GTPγS binding assays, but, in previous studies carried out using a cell-based assay to measure cAMP-mediated signaling, receptor activity for the (+)-CBDA-Hyp proved opposite behaviors with the compound acting as an antagonist at the CB_1 receptor and as an agonist for the CB_2 receptor ([73]; reviewed in Ref. [5]). These differences appear to be related to certain limitations of the agonist-stimulated GTPγS binding assay with respect to similar cell-based assays, which may be based on whether compounds bind orthosteric and/or allosteric sites, the subtype of G protein involved, the possibility of heteromer formation which modifies individual receptor activity, and even possible signaling crosstalks. Further experiments will be necessary to clarify this controversy. Irrespective of this, the fact that some of the (+)-enantiomers investigated in our study (CBD, CBDV and

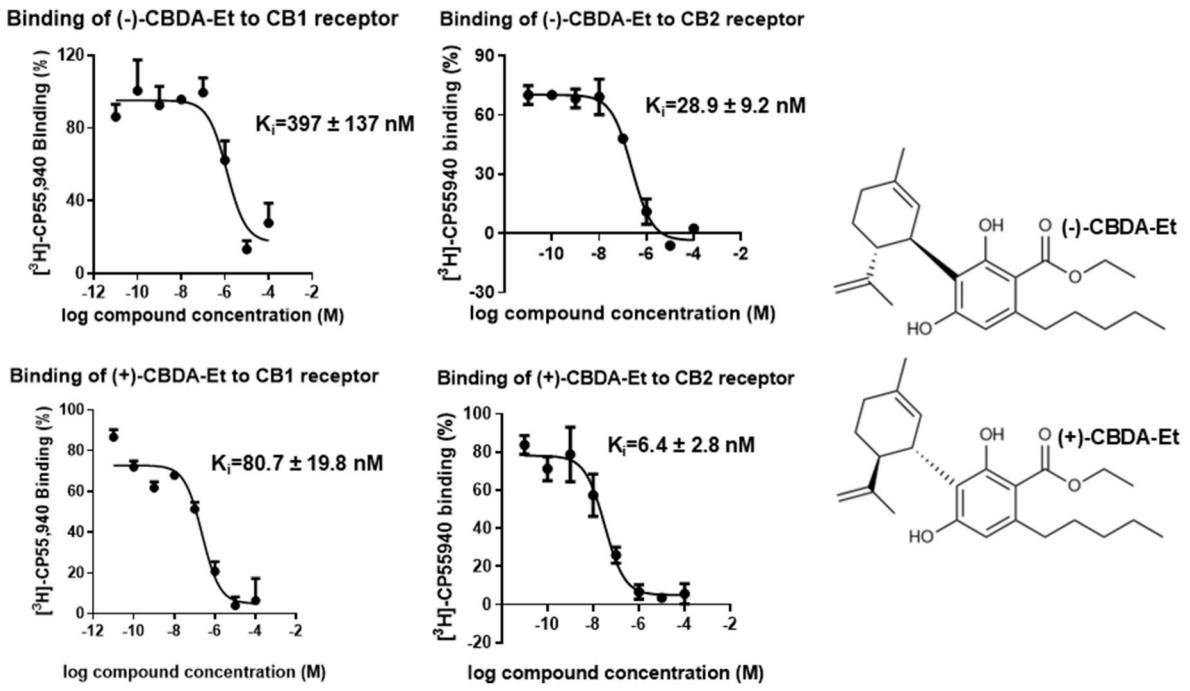

Fig. 7. Representative competition curves for the binding of synthetic (−)- and (+)-CBDA-ethyl-ester (CBDA-Et) at the CB_1 and CB_2 receptors. K_i values were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

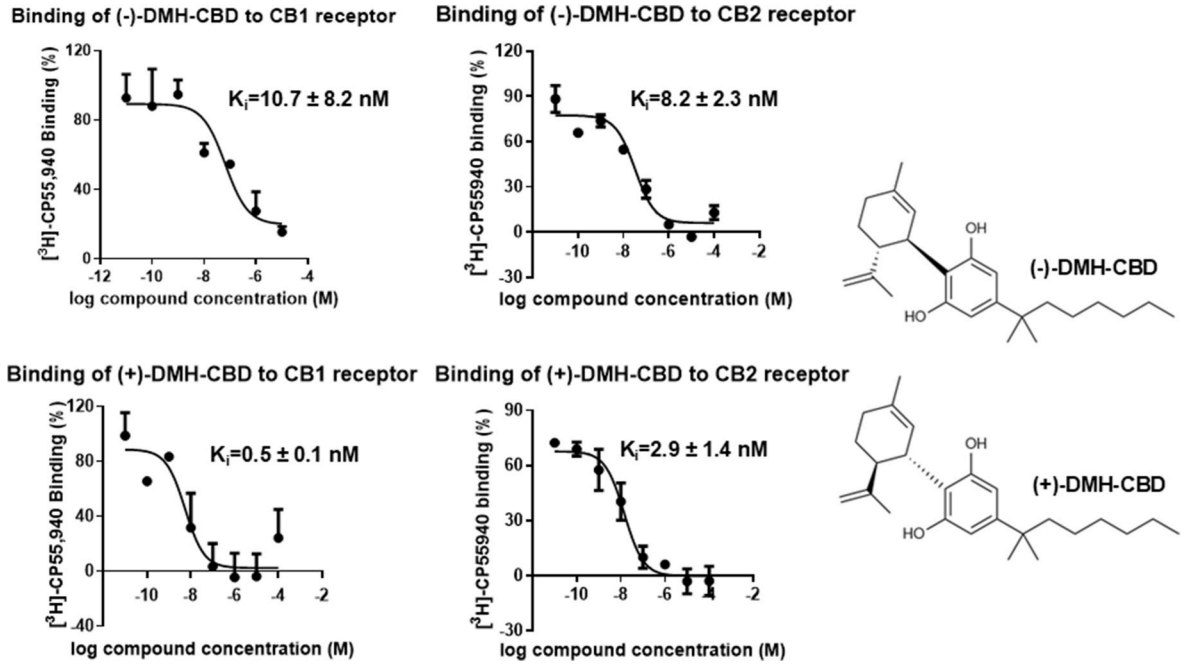

Fig. 8. Representative competition curves for the binding of synthetic (−)- and (+)-dimethyl heptyl-CBD (DMH-CBD) at the CB_1 and CB_2 receptors. K_i values were expressed as means ± SEM and were obtained from three independent experiments conducted with triplicates.

CBDA-Me) are active as agonists of the CB_2 receptors agree with the idea, recently reviewed in Rao et al. [5], that (+)-CBD isomeric derivatives show, in general, a good potential for the treatment of neuroinflammatory disorders. In fact, we investigated here this anti-inflammatory and neuroprotective profile using adequate cell-based assays already used in previous studies [72]. Our data strongly indicated that (+)-CBD, (+)-CBDV and (+)-CBDA-Me were capable of attenuating the effect of LPS exposure in cultured iMG cells on the expression of several proinflammatory cytokines (IL-1β and TNF-α) and enzymes (COX-2 and iNOS), likely reducing the proinflammatory scenario elicited by this inflammatory toxin. As previous studies have demonstrated [72], the use of conditioned media containing several factors generated in this proinflammatory scenario may result toxic for cultured neurons when added in their incubation media. We used this strategy to confirm: (i) the reduced neuronal viability observed with conditioned media derived from iMG cells incubated with LPS; (ii) the reversion of these neurotoxic effects with conditioned media derived from LPS-exposed iMG cells that were treated with the most active

Table 1
Summary of data for the affinity and intrinsic activity at the CB_1 and CB_2 receptors of (−)- and (+)-enantiomers of CBD, CBDV and some derivatives (see details in Figs. 1–8).

Compound	CB_1 receptor		CB_2 receptor	
	Affinity	Intrinsic activity	Affinity	Intrinsic activity
(−)-CBD	Negligible (K_i > 3 μM)	not measured[a]	Negligible (K_i > 3 μM)	not measured[a]
(+)-CBD	Low (K_i > 500 nM)	not measured[a]	High (K_i < 40 nM)	agonist
(−)-CBDV	Negligible (K_i > 3 μM)	not measured[a]	Negligible (K_i > 3 μM)	not measured[a]
(+)-CBDV	Moderate (K_i > 200 nM)	not measured[a]	High (K_i < 40 nM)	agonist
(−)-CBDA-Me	Moderate (K_i > 200 nM)	not measured[a]	Moderate to high (K_i > 40 nM)	not measured[a]
(+)-CBDA-Me	Moderate (K_i > 200 nM)	not measured[a]	High (K_i < 40 nM)	agonist
(−)-CBDA-Gly	Negligible (K_i > 3 μM)	not measured[a]	Moderate (K_i > 200 nM)	not measured[a]
(+)-CBDA-Gly	Moderate (K_i > 200 nM)	not measured[a]	High (K_i < 40 nM)	antagonist
(−)-CBDA-Hyp	Low (K_i > 500 nM)	not measured[a]	Moderate to high (K_i > 40 nM)	not measured[a]
(+)-CBDA-Hyp	Very high (K_i < 5 nM)	agonist[b]	Very high (K_i < 5 nM)	antagonist[b]
(−)-CBDA-Et	Moderate (K_i > 200 nM)	not measured[a]	High (K_i < 40 nM)	not measured
(+)-CBDA-Et	Moderate to high (K_i > 40 nM)	not measured[a]	High (K_i < 40 nM)	not measured
(−)-DMH-CBD	High (K_i < 40 nM)	not measured[c]	High (K_i < 40 nM)	not measured[c]
(+)-DMH-CBD	Very high (K_i < 5 nM)	not measured[c]	Very high (K_i < 5 nM)	not measured[c]

[a] not measured due to the absence of affinity in the low nM range.
[b] opposite results using cell assays instead [^{35}S]GTPγS binding [73].
[c] already described as agonists (see references in the text).

concentration of these (+)-enantiomers; and (iii) the contribution of CB_2 receptors, but not PPAR-γ receptors in the effects of (+)-enantiomers, which agree with the binding profile found for these compounds at the CB_2 receptor compared with their (−)-enantiomers. In addition, in a preliminary experiment addressed to explore an additional objective, we also demonstrated that (−)-CBD and (−)-CBDV mostly mimicked the effects of their (+)-enantiomers despite the (−)-stereoisomers do not bind and activate the CB_2 receptor. Obviously, this observation will require further research to be explained following two major lines of hypothesis: (i) that the molecular mechanism(s) for these effects should be other than the activation of the CB_2 receptor (e.g., activity at the PPAR-γ or other targets); and (ii) the possibility of an *in vivo* biotransformation of the (−)-enantiomer into the (+)-isomer by the activity of cellular racemases that may transform one enantiomer in the other and *vice versa*, something that will represent the follow-up studies generated by the experiments included in the present study. In the case that this racemic transformation may be confirmed, an additional question of high interest would be the relevance that this biotransformation may have in those pathologies in which CBD has been found to be beneficial. An important case for us is Parkinson's disease, in which we and other groups have described numerous neuroprotective effects of CBD in experimental models of this disease ([18,19,87,88]; reviewed in Ref. [89]), which have been frequently assigned to cannabinoid receptor-independent effects of CBD, frequently because the assumption of lack of affinity of (−)-CBD for the two classic cannabinoid receptors (reviewed in Ref. [1]). The present demonstration that (+)-enantiomers of CBD and derivatives are, however, able to more efficiently bind and eventually activate these receptors opens the possibility of alternative mechanisms of action derived from a possible *in vivo* biotransformation of these derivatives, something that will require to be investigated in such follow-up studies.

Finally, we also carried out some *in silico* analyses to predict possible ADME properties of the different (+)-enantiomers (CBD, CBDV, CBDA-Me, CBDA-Gly and CBDA-Hyp) using (−)-CBD as the reference compound. The rationale for this analysis was based on the fact that previous studies have remarked that the differences in pharmacodynamics of (−)- and (+)-enantiomers of CBD and derivatives could be associated with differences in their pharmacokinetics, in particular with a restriction of (+)-enantiomers to enter the brain at the BBB [74]. This fact led authors to propose (+)-CBD and derivatives (e.g., (+)-DMH-CBD) as useful peripherally-restricted CB_1 receptor agonists active against gastrointestinal disorders or peripheral pain and inflammation [74]. Therefore, our *in silico* analysis of ADME properties of (+)-enantiomers was aimed at further exploring this question. Our data indicated positive predicted ADME properties for all compounds, especially for (+)-CBD and (+)-CBDV, including some parameters (QPPCaco, QPlogBB and QPPMDCK), which allow the prediction of the ability of these compounds to cross tissue barriers from blood (including the BBB). The predictions indicated that (+)-CBD and (+)-CBDV may be apparently able to cross these barriers with values similar to (−)-CBD, which were lower for the remaining (+)-CBD derivatives, in particular for the (+)-CBDA-Gly which showed values outside the normal range. These predictions would oppose the idea of (+)-CBD derivatives as peripherally-restricted agonists [74], although they would need to be confirmed in follow-up *in vitro* and *in vivo* studies. In fact, preliminary *in vivo* experiments that are being currently in progress in our laboratory with (+)-CBD and (+)-CBDV in an experimental model of Parkinson's disease appear to indicate benefits against the motor impairment of these compounds which would be presumably caused by their activity at the brain (Rodríguez-Carreiro, unpublished data).

Taken together, the results of our investigation of the CB_1 and CB_2 receptor binding profile and intrinsic activity of CBD and its derivatives show marked differences in the biological profile of (−)- and (+)-enantiomeric forms (higher affinity). A direct consequence of this is the acquired (or enhanced) ability of (+)-enantiomers to act through these receptors, in particular through the CB_2 receptor, a fact that may help to elucidate the mechanisms of action that underlie in some pharmacological effects of these compounds as their anti-inflammatory and neuroprotective effects that have been described here. However, the fact that (−)-CBD and (−)-CBDV mostly mimicked the *in vitro* anti-inflammatory effects found for their (+)-enantiomers, despite the (−)-stereoisomers not binding to and activating the CB_2 receptor, opens the possibility that the activity of these compounds at these receptors in the two enantiomer forms may be influenced by a possible bidirectional biotransformation between these enantiomers in the body, something that will require further research as a highly relevant objective that may impact our understanding on the therapeutic use of these compounds.

CRediT authorship contribution statement

Santiago Rodríguez-Carreiro: Writing – review & editing, Investigation, Formal analysis, Data curation. **María Gómez-Cañas:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Francesca Lubrini:** Writing – review & editing, Formal analysis, Data curation. **Claudia Gonzalo-Consuegra:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Matthias Winkler:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Diego Caprioglio:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Giovanni Appendino:** Writing – review & editing, Supervision, Conceptualization. **Concepción García:** Writing – review & editing, Supervision. **Paula Morales:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Nadine Jagerovic:** Writing – review & editing, Supervision, Conceptualization. **Joerg T. Fischer:** Writing – review & editing,

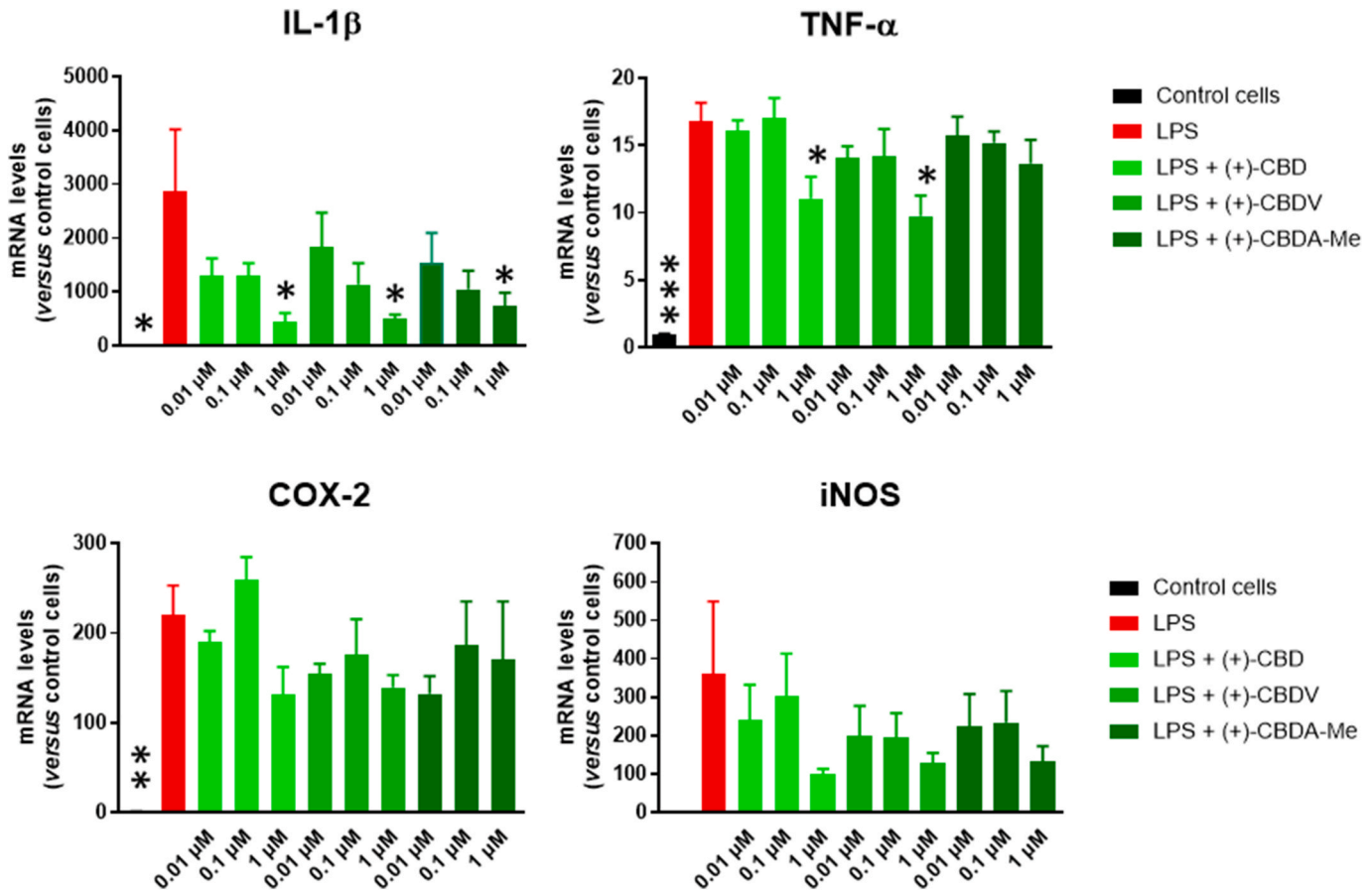

Fig. 9. mRNA levels for IL-1β, TNF-α, COX-2 and iNOS measured by qPCR in iMG cells after the exposure to LPS (100 ng/ml) in absence or presence of three different concentrations of (+)-CBD, (+)-CBDV or (+)-CBDA-Me. Results are means ± SEM of three different experiments carried out in triplicates. Data were assessed by one-way analysis of variance followed by the Dunnett's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ *vs* LPS-treated cells).

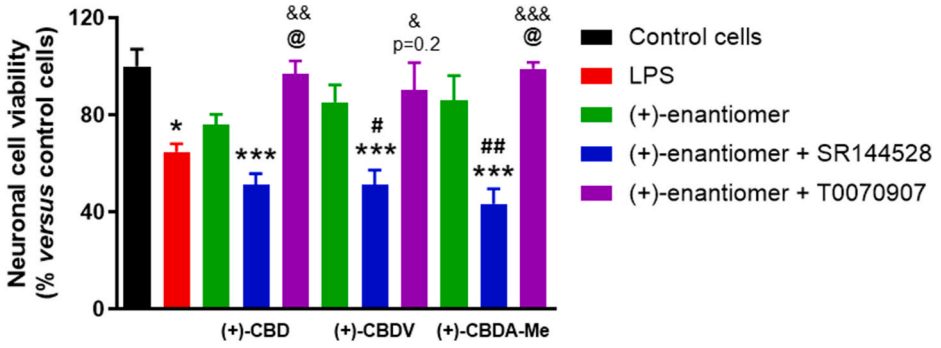

Fig. 10. Cell viability measured by the MTT assay in M-213-2O cells exposed to conditioned media generated from iMG cells after the exposure to LPS (100 ng/ml) in absence or presence of (+)-CBD, (+)-CBDV or (+)-CBDA-Me at the concentration of 1 μM and/or SR144528 (10 μM) or T0070907 (10 μM). Results are means ± SEM of three different experiments carried out in triplicates. Data were assessed by one-way analysis of variance followed by the Tukey's test (*$p < 0.05$, ***$p < 0.005$ *vs* control cells; $^{@}p < 0.05$ *vs* LPS-treated cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* LPS + cannabinoid-treated cells; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.005$ *vs* LPS + cannabinoid + SR144528-treated cells).

Investigation, Conceptualization. **Bernd L. Fiebich:** Writing – review & editing, Investigation, Conceptualization. **Marcus R. Goetz:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Eduardo Muñoz:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Javier Fernández-Ruiz:** Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Data availability statement

The datasets for this manuscript are not publicly available because of the tenure of intellectual property by Symrise AG. Requests to access the datasets should be directed to the corresponding author.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: SR-C, MG-C, FL, CG-C, DC, GA, CG, PM, NJ and JF-R declare that they have no conflicts of interest, whereas MW, JTF, BLF, MRG and EM were employees of Symrise AG or VivaCell Biotechnology at the time of study development.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmcr.2025.100262.

Data availability

Data will be made available on request.

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