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Pharmacological Characterization of Cannabinoid CB2 Receptor Ligands in an In Vitro Model of Resting Microglia

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Abstract

Microglia are key regulators of central nervous system homeostasis and neuroinflammation, with cannabinoid type 2 receptors (CB2Rs) proposed as important modulators of microglial phenotype and function. This study investigated the pharmacological properties of several CB2R ligands in resting BV2 microglial cells using [^{35}S]GTPγS binding and forskolin-stimulated cAMP assays. Classical CB2R agonists (CP 55,940, WIN 55,212-2, JWH 133, and JWH 015), protean agonists ((R)-AM 1241 and GW 405833), and inverse agonists (SR144528, AM630, and JTE907) were evaluated. In the [^{35}S]GTPγS assay, WIN 55,212-2 displayed the highest intrinsic activity, while protean agonists behaved as partial agonists and inverse agonists reduced basal CB2R signalling, indicating constitutive receptor activity. In contrast, cAMP measurements revealed greater signal amplification, with (R)-AM 1241 and GW 405833 acting as full agonists and SR144528 producing marked inverse agonism. These significant differences in ligand rank, order and efficacy between assays highlighted the influence of downstream signalling and ligand-dependent signalling bias. Notably, GW 405833 exhibited a strongly biased profile, whereas SR144528 showed the most pronounced inverse agonist properties. These findings demonstrate that CB2R ligands display distinct pharmacological behaviours in native resting microglia and suggest that constitutive receptor activity and signalling bias are important determinants of CB2R function. The results support further investigation of CB2R inverse agonists, particularly SR144528, as tools to explore microglial regulation across different neuroinflammatory and neurodegenerative disease states.

Keywords: BV2 cells; microglia; CB2 receptors; cAMP; [^{35}S]GTPγS

1. Introduction

Microglia are specialized resident immune cells of the central nervous system (CNS) that continuously surveil the neural environment, protect against infectious agents, contribute to tissue repair, and participate in synaptic pruning during brain development. In addition to their protective functions, which involve the release of reactive oxygen and nitrogen species and a variety of inflammatory mediators, microglia can also exert detrimental effects on neuronal cells under pathological conditions [1]. Thus, microglia perform numerous important functions that might be targeted therapeutically such as inflammatory signalling, phagocytosis, synaptic pruning, TREM2-mediated disease response, purinergic signalling and surveillance as well as microglial proliferation and survival [2].

Resting microglia are referred to as adopting an M0 phenotype. Microglial activation is commonly classified into two further functional states: the classical pro-inflammatory activation state (M1 phenotype), which exhibits both protective and potentially neurotoxic effects, and the alternative

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anti-inflammatory activation state (M2 phenotype), which promotes tissue repair, neuronal recovery, and resolution of inflammation [2]. The transition from the M1 to the M2 phenotype is influenced by microenvironmental factors associated with different CNS pathologies and disease stages. Consequently, considerable research efforts have focused on identifying the cellular mechanisms that regulate this phenotypic switch. Among the pathways implicated in this process, the cannabinoid type 2 receptor (CB2R), which is abundantly expressed in activated microglia, has been proposed as a key regulator of microglial phenotypic shift [3–5].

Similarly to peripheral immune cells, resting microglia (M0 phenotype) express very low or undetectable levels of CB2R mRNA [6]. Upon microglial activation, CB2R expression is markedly modulated in a stimulus-dependent manner, generally being upregulated in reactive/neuroinflammatory conditions [7]; this increase has been proposed as a potential biomarker of neuroinflammation associated with several CNS disorders [2,8–10]. Notably, CB2R upregulation is not restricted to the pro-inflammatory (M1) phenotype: activation of CB2R also promotes and accompanies the anti-inflammatory (M2) polarisation, and the direction of regulation depends on the specific inflammatory stimulus [7].

In addition to expressing cannabinoid receptors, microglia are capable of synthesizing and degrading endocannabinoids (eCBs), thereby contributing to the regulation of the endocannabinoid system within the CNS [5]. Over the last decade, numerous *in vitro* studies have demonstrated an important role for the endocannabinoid system in modulating microglial polarization and regulating neuroinflammatory processes, and that CB2R activation is a major contributor to anti-inflammatory responses in the CNS [11–14]. Nevertheless, although CB2R appears to be a predominant regulator of microglial phenotype *in vitro*, its role has not been fully confirmed in animal models, where more complex regulatory mechanisms may be involved [4].

These findings suggest that CB2R may represent a promising pharmacological target for the treatment of CNS disorders in which microglial dysfunction contributes to disease pathogenesis, including Alzheimer's disease (AD) although the mechanisms involved must be further elucidated to enable effective therapeutic targeting [15].

Following the cloning and characterization of the CB2 receptor [16], numerous CB2R ligands with diverse chemical structures and pharmacological profiles have been developed [17]. However, the pharmacological characterization of CB2R ligands remains challenging because receptor constitutive activity varies considerably between native tissues and recombinant expression systems. As a result, estimates of ligand potency and efficacy may differ substantially across experimental models [18].

For example, AM1241 and GW405833 (also known as L-768,242), two selective CB2R ligands, have been classified as so-called 'protean agonists' an atypical class of ligands that can behave as agonists, antagonists or inverse agonists depending on the system's constitutive activity, because of their unusual profiles in recombinant systems. In contrast, studies conducted in tissues that natively express CB2Rs have shown that these compounds behave as a full agonist and a partial agonist, respectively [18–20]. Protean agonists are characterized by their ability to produce agonist effects in systems with low constitutive receptor activity while exhibiting inverse agonist properties in systems with high constitutive receptor activity [21]. It has been proposed that these ligands stabilize inactive receptor conformations, with their functional effects depending on the proportion of receptors present in active versus inactive states within a given experimental model [22].

Our group has previously highlighted differential pharmacology of JWH 015 and JWH 133, agonists with higher CB2R than CB1R affinity, but also of CB2R inverse agonists like SR 144528, AM 630, and JTE 907 in mouse, rat and human spleen membranes [18]. However, because the spleen is a peripheral, immune-rich tissue whose CB2R pharmacology may not reflect that of the receptor in its native CNS context, it is important to compare the pharmacological profiles of these ligands directly in microglial cells. A useful experimental model for such investigations is the murine BV-2 microglial cell line, which is widely employed for in vitro studies of microglial function and, like primary glial cells, has been shown to express CB2 receptors and produce endocannabinoids [23,24].

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In the present study, we characterized the pharmacological properties of the CB2R ligands described above in BV-2 cells using cyclic AMP accumulation and [^{35}S]GTPγS binding assays [25]. The [^{35}S]-GTPγS binding assay provides a rapid and quantitative measure of G-protein activation and is particularly suitable for investigating signalling through Gi/o-coupled G protein-coupled receptors such as CB2R [26]. In addition, we examined the effects of CP55,940, a highly potent cannabinoid ligand displaying high affinity for both CB1R and CB2R and a marked degree of stereoselectivity [27], as well as WIN55,212-2, a potent cannabinoid receptor agonist active at both receptor subtypes, with a modest preference for CB2R [18,28]. Through these studies, we aimed to better define the pharmacological behaviour of CB2R ligands in resting (M0 phenotype) microglial cells and to further elucidate the role of CB2R signalling in the regulation of microglial function.

2. Materials and Methods

2.1. Culturing and Treatment of Cells

BV-2 cell culture. The murine microglial cell line BV-2 was cultured in DMEM supplemented with 3% fetal bovine serum (FBS), 10 mM HEPES, 5 mM $NaHCO_3$, penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and were passaged every 3–4 days for up to 12 passages. Approximately 24 h before the experiments, BV-2 cells were harvested for the cAMP assay and seeded into 96-well plates at a volume of 100 µL per well. Cannabinoid compounds were dissolved in DMSO as 1000× stock solutions using siliconized glass vials and siliconized pipette tips.

2.2. Membrane Preparation

Membrane preparation for [^{35}S]GTPγS binding assays. BV-2 cells were detached from culture flasks by scraping, collected by centrifugation, and stored as cell pellets at −20 °C until use. Prior to the [^{35}S]GTPγS binding assay, the cell pellets were thawed in 50 mM Tris buffer (pH 7.4) and homogenized using a handheld homogenizer fitted with a 1 mL probe. Protein concentrations were determined using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK).

2.3. [^{35}S]GTPγS-Binding Assays

Measurement of ligand-stimulated [^{35}S]GTPγS binding to cannabinoid CB2 receptors was adapted from previously described methods [29,30]. Assays were performed in GTPγS binding buffer containing 50 mM Tris-HCl, 50 mM Tris-base, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, 1 mM dithiothreitol (DTT), and 0.1% bovine serum albumin (BSA), in the presence of [^{35}S]GTPγS and GDP, in a final volume of 500 µL. Binding reactions were initiated by the addition of [^{35}S]GTPγS. Non-specific binding was determined in the presence of 30 µM unlabeled GTPγS. Test compounds were incubated for 60 min at 30 °C. Reactions were terminated by rapid vacuum filtration using Tris binding buffer, as previously described (Marini et al., 2013), and retained radioactivity was quantified by liquid scintillation spectrometry. Unless otherwise stated, all [^{35}S]GTPγS binding assays were performed using 0.1 nM [^{35}S]GTPγS, 30 µM GDP, and 40 µg of BV-2 membrane protein per well. All compounds were dissolved in DMSO and stored at −20 °C until use.

2.4. Cyclic AMP (cAMP) Assay

Intracellular cAMP levels were measured using the HitHunter® cAMP assay kit (Eurofins DiscoverX Products, Celle-L'Évescault, France) according to the manufacturer's instructions and as previously described [18,31]. Briefly, BV-2 cells were detached using a cell dissociation buffer, counted, and seeded at a density of 2 × 10^4 cells per well in 100 µL of complete culture medium in

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white 96-well plates. Cells were then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for approximately 24 h before the assay.

Assays and drug dilutions were prepared in a 1:1 mixture of DMEM and Ham's F-12 medium without phenol red, supplemented with 10 μM rolipram and forskolin (FSK). Prior to treatment, the culture medium was removed, and cells were washed with DMEM/F-12 medium. Cells were then exposed to the test compounds (30 μL per well) and incubated for 30 min at 37 °C and 5% CO_2.

Subsequently, cAMP standards and the appropriate detection reagents were added according to the manufacturer's instructions (Eurofins DiscoverX Products, Celle-L'Évescault, France). Plates were incubated overnight at room temperature in the dark. Chemiluminescent signals were measured using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Compounds

WIN 55,212-2, CP 55,940, JWH 133, JWH 015, (R)-AM 1241, AM 630, SR 144528, GW 405833, JTE 907, were obtained from Cayman Chemical (Ann Arbor, MI, USA). G418, Forskolin (FSK), DMSO, foetal bovine serum (FBS), HEPES, $NaHCO_3$, penicillin, streptomycin, Tris-HCl, Tris-base, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), bovine serum albumin (BSA), rolipram, DMEM/F-12 and DMEM were supplied by Sigma-Aldrich (Poole, Dorset, UK).

Statistical analysis

Values are expressed as means and variability as S.E.M. or as 95% confidence intervals (CIs), as indicated in the text. The EC_{50} values and maximal [^{35}S]GTPγS binding or FSK-induced cAMP production were determined by fitting the data to a sigmoidal concentration-response curve using nonlinear regression (Prism 5, Graph Pad Software).

3. Results

3.1. Efficacies and Potencies of Putative CB2 Receptor Agonists: CP 55,940, WIN 55,212-2, JWH 133, JWH 015 in the [^{35}S]GTPγS Assay

In the [^{35}S]GTPγS binding assay, WIN 55,212-2 behaved as a full agonist in BV-2 cell membranes, exhibiting the highest efficacy (E_{max} = 34.17 ± 1.48%) and potency (EC_{50} = 4.30 nM) among the compounds tested (Table 1; Figure 2). CP 55,940 and JWH-133 displayed similar potencies to each other, with EC_{50} values of 13.66 nM and 16.68 nM, respectively. The efficacy of CP 55,940 (E_{max} = 30.10%) was comparable to that of WIN 55,212-2, whereas JWH-133 exhibited significantly lower efficacy (E_{max} = 14.56%) (Table 1; Figure 2). JWH-015 was significantly less potent than the other agonists tested, with an EC_{50} value of 33.00 nM, and displayed intermediate efficacy (E_{max} = 20.70%), lying between those observed for CP 55,940 and JWH-133 (Table 1; Figure 1).

3.1.2. Efficacies and Potencies CB2 Receptor 'Protean' Agonists (R)-AM 1241 and GW 405833 in the [^{35}S]GTPγS Assay

The CB2 receptor 'protean' agonists, (R)-AM 1241 and GW-405833 behaved as an agonist showing EC_{50} values of 52.24nM and 128.50 nM respectively, and E_{max} value significantly lower in comparison to CP 55,940 and WIN 55,212-2 (Table 1; Figure 1).

Table 1. Effect of CB2 receptor agonists and protean agonists in the [^{35}S]GTPγS binding assay using BV-2 cells. pEC_{50} and E_{max} (maximum response, %) with S.E.M. and EC_{50}, pEC_{50} and E_{max} values with 95% C.I. were determined from GraphPad Prism.

Compound	EC_{50} (nM)	EC_{50} 95% C.I.	pEC_{50} ± S.E.M.	pEC_{50} 95% C.I.	E_{max} ± S.E.M.	E_{max} 95% C.I.
WIN 55,212-2	4.30	2.57–16.0	8.37 ± 0.11	8.60–8.14	34.17 ± 1.48	31.21–37.12
CP 55,940	13.66	6.61–28.25	7.86 ± 0.28	8.18–7.55	30.10 ± 2.16	25.79–34.41
JWH 133	16.68	4.58–60.69	7.79 ± 0.28	8.34–7.22	14.56 ± 1.87	10.82–18.30
JWH 015	33.00	6.58–158.80	7.48 ± 0.34	8.16–6.80	20.70 ± 3.41	13.88–27.52

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(R)-AM 1241	52.24	13.05–209.10	7.82 ± 0.30	7.88–6.68	23.73 ± 3.78	16.18–31.28
GW 405833	128.50	20.15–819.80	6.89 ± 0.40	7.67–6.08	12.90 ± 3.32	6.28–19.51

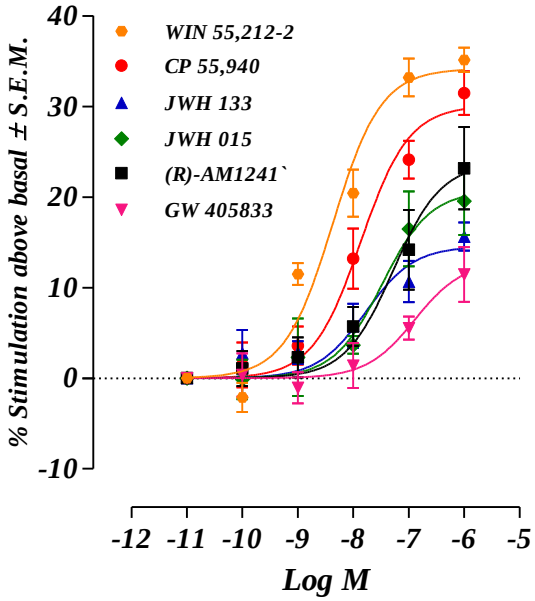

Figure 1. Concentration–response curves of CB2 receptor ligands WIN 55,212-2, CP 55,940, JWH 133 and JWH 015, (R)-AM 1241 and GW 405833, on [^{35}S]GTPγS binding. Data are expressed as the percentage stimulation of specific [^{35}S]GTPγS binding above the unstimulated basal level. Each point represents the mean ± S.E.M. of at least three independent experiments performed in duplicate.

3.1.3. Efficacies and Potencies of CB2 Receptor Inverse Agonists: SR144528, AM 630 and JTE 907 in [^{35}S]GTPγS Assay

These compounds displayed negative E_{max} values. SR144528 was the most potent (EC_{50} = 1.06 nM, pEC_{50} = 8.97) followed in order by JTE 907 (EC_{50} = 1.81 nM, pEC_{50} = 8.74) and AM 630 (EC_{50} = 15.58 nM, pEC_{50} = 7.81) but the efficacies were not significantly different between them (Table 2; Figure 2).

Table 2. Effect of CB2 receptor inverse agonists in the [^{35}S]GTPγS binding assay using BV-2 cells. pEC_{50} and E_{max} (maximum response, %) with S.E.M. and EC_{50}, pEC_{50} and E_{max} values with 95% C.I. were determined from GraphPad Prism.

Compound	EC_{50} (nM)	EC_{50} 95% C.I.	pEC_{50} ± S.E.M.	pEC_{50} 95% C.I.	E_{max} ± S.E.M.	E_{max} 95% C.I.
SR 144528	1.06	0.41–2.72	8.97 ± 0.20	9.38–8.56	−13.38 ± 1.00	−15.38–−11.38
JTE 907	1.81	0.71–4.58	8.74 ± 0.20	9.14–8.34	−12.69 ± 0.94	−14.57–−10.80
AM 630	15.58	6.78–35.82	7.81 ± 0.18	8.17–7.45	−15.02 ± 1.24	−17.50–−12.54

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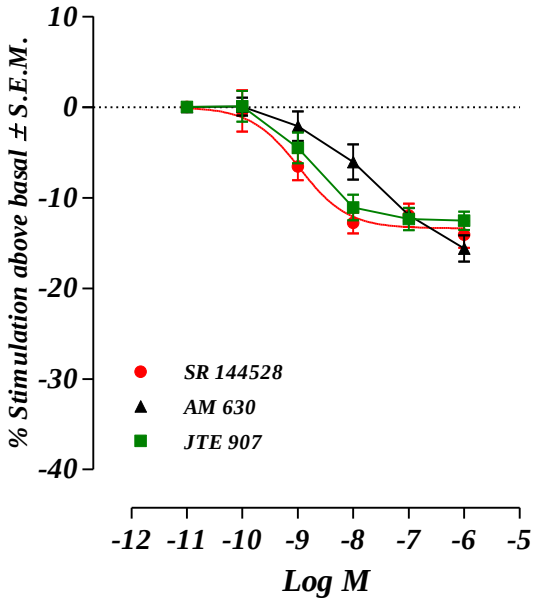

Figure 2. Concentration–response curves of CB2 receptor ligands SR 144528, AM 630 and JTE 907 on [^{35}S]GTPγS binding. Data are expressed as the percentage stimulation of specific [^{35}S]GTPγS binding below the unstimulated basal level. Each point represents the mean ± S.E.M. of at least three independent experiments performed in duplicate.

3.2. The Effects of the Evaluated Compounds Using the cAMP Assay

3.2.1. Efficacies and Potencies of CB2 Receptor Agonists: CP 55,940, WIN 55,212-2, JWH 133, JWH 015 in cAMP Assay

The efficacy (E_{max}) of CP 55,940, WIN 55,212-2, JWH 133, JWH 015 was higher than that measured in [^{35}S]GTPγS assay (68.22 ± 1.74, 60.03 ± 3.41, 47.94 ± 2.91, 39.68 ± 2.91 respectively) (Table 3; Figure 3).

3.2.2. Effects of CB2 Receptor 'Protean' Agonists (R)-AM 1241 and GW 405833 in cAMP Assay

In contrast to the [35S]GTPγS assay, in the cAMP assay, the protean behaved like full agonists showing potencies and efficacies (EC_{50} = 7.57 nM, Emax = 52.37 ± 2.40 for (R)-AM 1241) and EC_{50} = 5.04 nM, Emax = 61.93 ± 3.27 for GW 405833) similar to the classic ligands described above (Table 3; Figure 3).

Table 3. Effect of CB2 receptor agonists and protean agonists in the cAMP assay using BV-2 cells. pEC_{50} and E_{max} (maximum response, %) with S.E.M. and EC_{50}, pEC_{50} and E_{max} values with 95% C.I. were determined from GraphPad Prism.

Compound	EC_{50} (nM)	EC_{50} 95% C.I.	pEC_{50} ± S.E.M.	pEC_{50} 95% C.I.	E_{max} ± S.E.M.	E_{max} 95% C.I.
WIN 55,212-2	16.29	11.87–23.35	7.79 ± 0.07	7.92–7.65	68.22 ± 1.74	64.76–71.67
CP 55,940	145.90	82.53–257.80	6.84 ± 0.12	7.08–6.59	60.03 ± 3.41	53.27–66.79
JWH 133	8.99	4.27–18.94	8.05 ± 0.16	8.37–7.72	47.94 ± 2.91	42.35–53.53
JWH 015	13.19	5.30–32.99	7.88 ± 0.20	8.27–7.48	39.68 ± 2.91	33.90–45.47
(R)-AM 1241	7.57	4.18–13.70	8.12 ± 0.13	8.38–7.86	52.37 ± 2.40	47.59–57.14
GW 405833	5.04	2.47–10.36	8.30 ± 0.16	8.61–7.98	61.93 ± 3.27	55.45–68.48

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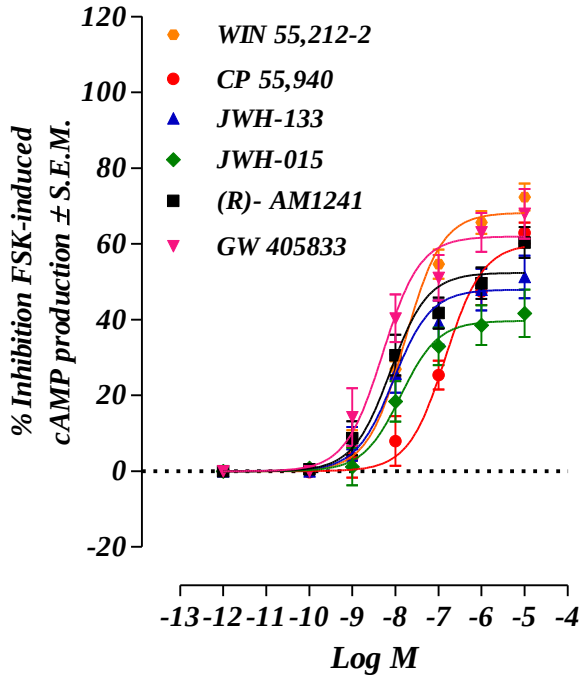

Figure 3. Effects of CB2 receptor ligands on forskolin-stimulated cAMP production in BV-2 cells. Concentration–response curves for WIN 55,212-2, CP 55,940, JWH 133, JWH 015, (R)-AM 1241 and GW 405833 are shown as percentage inhibition of forskolin (FSK)-induced cAMP accumulation. The concentration of FSK used in these experiments was 10 μM. Each point represents the mean ± S.E.M. of at least three independent experiments performed in duplicate.

3.2.3. Effects of CB2 Receptor Inverse Agonists SR 144528, AM 630 and JTE 907 in cAMP Assay

The inverse efficacy of SR 144528 was higher than that observed in the [^{35}S]GTPγS assay (−77.90 ± 5.60 vs −13.38 ± 1.00), but conversely its potency was lower (EC_{50} 141.80 nM vs EC_{50} 1.06 nM). JTE 907 showed the highest potency (EC_{50} = 1.10 nM and E_{max} −42.62 ± 2.31), whereas AM 630 displayed intermediate potency (EC_{50} = 7.35 nM) and the lowest efficacy (−27.41 ± 2.51) (Table 4; Figure 4).

Table 4. Effect of CB2 receptor antagonists/inverse agonists in the cAMP assay in BV-2 cells. pEC_{50} and E_{max} (maximum response, %) with S.E.M. and EC_{50}, pEC_{50} and E_{max} values with 95% C.I. were determined from GraphPad Prism.

Compound	EC_{50} (nM)	EC_{50} 95% C.I.	pEC_{50} ± S.E.M.	pEC_{50} 95% C.I.	E_{max} ± S.E.M.	E_{max} 95% C.I.
SR 144528	141.80	69.45–289.50	6.85 ± 0.16	7.16–6.54	−77.90 ± 5.60	−88.88–−66.92
JTE 907	1.10	0.51–2.36	8.96 ± 0.17	9.26–8.62	−42.62 ± 2.31	−47.16–−38.09
AM 630	7.35	2.27–23.73	8.13 ± 0.26	8.64–7.62	−27.41 ± 2.51	−32.33–−22.49

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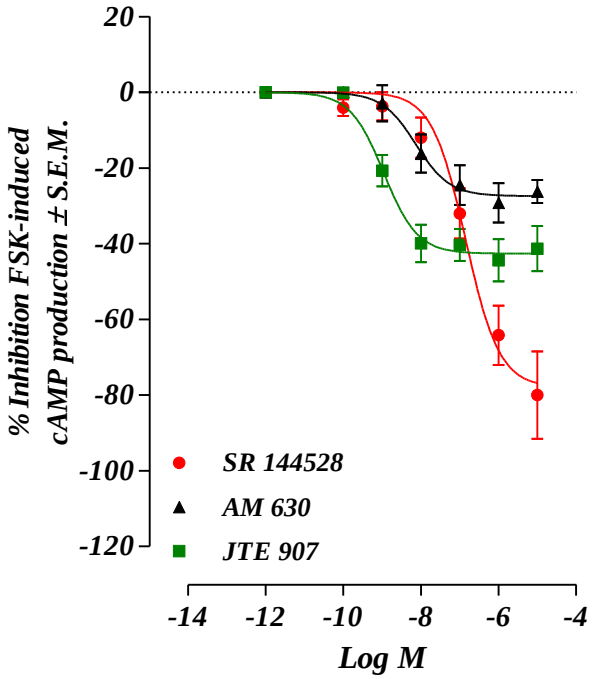

Figure 4. Effects of CB2 receptor ligands on FSK-stimulated cAMP production in BV-2 cells. Concentration–response curves for SR 144528, AM 630 and JTE 907 are shown as percentage inhibition of FSK-induced cAMP accumulation. The concentration of forskolin used in these experiments was 10 μM. Each point represents the mean ± S.E.M. of at least three independent experiments performed in duplicate.

4. Discussion

At G protein-coupled receptors, such as the CB2R used here, in the [^{35}S]GTPγS assay the EC_{50} value reflects both ligand potency and efficacy. A key advantage of the [^{35}S]GTPγS binding assay is its ability to determine classical pharmacological parameters, particularly potency, efficacy, and antagonist affinity, without the influence of signal amplification or modulation by downstream signalling pathways that commonly affect functional readouts [32]. Thus, the comparison of the non-amplified [^{35}S]GTPγS binding with the FSK-stimulated cAMP assays clearly illustrates the contribution of downstream signalling mechanisms to signal amplification. Consequently, the E_{max} values of CP 55,940, WIN 55,212-2, JWH-133, and JWH-015 were all higher in the cAMP assay than in the [^{35}S]GTPγS assay.

This pattern is consistent with findings reported in recombinant Chinese hamster ovary (CHO) cells (Soethoudt et al., 2017). However, unlike in the CHO recombinant system, the potency of CP 55,940 was markedly reduced in our cAMP assay using BV-2 microglial cells. This observation suggest the CB2 receptor binding kinetics in non-recombinant microglial cells may uniquely influence the pharmacological behaviour of this ligand [33].

The contribution of downstream pathways to signalling amplification appears to be particularly important for (R)-AM1241 and GW405833. Although both compounds behaved as partial agonists in the [^{35}S]GTPγS assay, they acted as full agonists in the cAMP assay. This differential behaviour across assays indicates that a classification of these compounds as protean agonists is appropriate in BV-2 microglial cells. To reliably compare ligand activity across assays, it is advantageous to use amplification-independent parameters such as the E_{max}/EC_{50} ratio. Although this parameter has some limitations [34], the E_{max}/EC_{50} ratio, also known as the intrinsic relative activity, reflects the combined contribution of agonist affinity and efficacy and therefore represents a practical measure of agonist activity [35]. Analysis of this index for the classical and protean agonists revealed substantial changes in rank order between the two assays. WIN 55,212-2 displayed the highest intrinsic relative activity

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in the [^{35}S]GTPγS assay (E_{max}/EC_{50} = 8.01) but was ranked only fourth in the cAMP assay (E_{max}/EC_{50} = 4.21). In contrast, CP 55,940 showed a pronounced reduction in intrinsic relative activity in the cAMP assay, yielding the lowest E_{max}/EC_{50} ratio (0.42), primarily because of an almost ten-fold increase in its EC_{50} value. To our knowledge, such a discrepancy has not previously been reported in recombinant systems expressing CB2 receptors, either for murine or human receptors, making this a novel and important observation. The most likely explanation seems to the use of the native BV-2 microglial cell line, which is closer to the native expression environment. Receptor reserve can significantly influence EC_{50} measurements, with a generally greater impact on [^{35}S]GTPγS binding assays than on cAMP-based functional assays [36]. Unlike recombinant systems, which overexpress CB2 receptors and may not faithfully reflect the *in vivo* state, BV-2 cells express a limited population of CB2 receptors and are unlikely to possess a substantial receptor reserve, a difference that not only explains the divergent observations but also underscores the importance of assaying CB2R pharmacology under near-native expression conditions.

CP 55,940 can also interact with GPR55 receptors, which are also expressed in BV-2 cells [37], so it is important to consider how this may affect the present observations. This off-target interaction may have artificially increased the apparent EC_{50} measured for CB2R-mediated responses. However, activation of GPR55 is unlikely to influence the [^{35}S]GTPγS assay, since GPR55 is primarily coupled to $G\alpha_{12/13}$ proteins rather than $G_{i/o}$ proteins, the canonical signalling partners of CB2Rs [38]. In contrast, cAMP accumulation assays are likely more susceptible to interference arising from cross-talk between these distinct intracellular signalling pathways [39].

In marked contrast to CP 55,940, GW405833 displayed the lowest intrinsic relative activity in the [^{35}S]GTPγS assay (E_{max}/EC_{50} = 0.10) yet emerged as the most active compound in the cAMP assay (E_{max}/EC_{50} = 12.36). These findings strongly support the existence of biased signalling by GW405833, in favour of the cAMP pathway. This profile differs substantially from that of (R)-AM1241, another protean agonist, which maintained a relatively stable rank order across assays, ranking third in the [^{35}S]GTPγS assay (E_{max}/EC_{50} = 1.57) and second in the cAMP assay (E_{max}/EC_{50} = 6.90).

In recombinant systems, both (R)-AM1241 and GW405833 have previously been characterized as protean agonists. These ligands can behave either as agonists or inverse agonists depending on the degree of receptor constitutive activity. For example, suppression of constitutive activity in CHO cells expressing recombinant human or rat CB2Rs unmasks the agonist properties of these compounds in cAMP assays [20]. Therefore, discrepancies between the *in vitro* and *in vivo* pharmacological profiles of CB2R ligands may arise from differences in constitutive receptor activity between recombinant systems and native microglial cells, such as BV-2 cells. Under our experimental conditions, comparing the [^{35}S]GTPγS and cAMP assays therefore likely detects different levels of constitutive receptor activity. Based on the E_{max}/EC_{50} values obtained, constitutive activity appears to be lower in the [^{35}S]GTPγS assay than in the cAMP assay. Furthermore, given the striking difference in the behaviour of GW405833 in the two assays, we hypothesize that its apparent efficacy is highly dependent on the level of constitutive receptor activity. The markedly greater activity observed in the cAMP assay further suggests an important contribution of downstream constitutive signalling events with GW405833.

The behaviour of the inverse agonists SR144528, JTE907, and AM630 in both assays confirms the presence of constitutive CB2R activity in BV-2 cells. Indeed, all three compounds reduced basal [^{35}S]GTPγS binding while increasing FSK-stimulated cAMP accumulation. Finally, the EC_{50} values for SR144528 and AM630 in the [^{35}S]GTPγS assay indicated greater potency than that previously reported [25], although their efficacy was comparable.

Previous studies have shown that, in recombinant systems, SR144528 and AM630 reduce constitutive CB2R activity by preferentially binding to the inactive receptor state [25,40]. Our results are largely in agreement, as they suggest that a similar mechanism operates in native, non-stimulated models such as BV-2 microglial cells, despite their relatively low basal expression of CB2Rs. Several studies have demonstrated that inflammation is a major determinant of both CB2R expression and activity [41,42]. More recently, CB2R expression has been shown to be dynamically regulated in both

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primary murine microglia and BV-2 cells, increasing or decreasing depending on the specific inflammatory stimulus and cellular context [43].

SR144528 exhibited a marked amplification of its inhibitory effect in the cAMP assay. This phenomenon may be due to the stimulation of adenylyl cyclase V and the inhibition of adenylyl cyclase II previously reported in transfected cells [44]. Such effects may be amplified in the presence of low CB2R expression, particularly considering that the E_{max} values observed in the [^{35}S]GTPγS assay were substantially lower. Additionally, the enhanced cAMP response may reflect the reported ability of SR144528 to inhibit G_i protein signalling directly [45].

Interestingly, the approximately 100-fold reduction in potency observed for SR144528 in the cAMP assay relative to the [^{35}S]GTPγS assay closely resembles its behaviour in transfected cell systems [46]. Accordingly, a similar mechanistic explanation may apply. SR144528 may possess greater inverse efficacy because the ratio (α) between its affinities for the active (R*) and inactive (R) receptor conformations is lower than that of other inverse agonists, such as AM630. In contrast, AM630 retains inverse agonist activity in BV-2 cells, presumably because the relative proportion of active and inactive CB2 receptor states (described by the allosteric constant L = R*/R) remains sufficiently high so that α > L. Therefore, although both AM630 and JTE907 behaved as inverse agonists in our experimental model, it is worth noting that these compounds have displayed protean agonist properties in other cellular systems [47].

A key translational aspect of the present study is that all compounds were evaluated in resting, non-stimulated BV-2 cells. Under these conditions, CB2R expression is expected to be relatively low and constitutive receptor activity minimal (i.e., in the M0 state), as suggested by the limited effects of inverse agonists in the [^{35}S]GTPγS assay. Consequently, this model may provide a useful representation of the pharmacological behaviour of agonists, protean agonists, and inverse agonists in quiescent microglia prior to inflammatory activation.

Both endocannabinoids and exogenous cannabinoid agonists promote polarization toward the anti-inflammatory M2 phenotype. In turn, M2 microglia may produce 2-arachidonoylglycerol (2-AG) following stimulation by IL-4 and IL-13, whereas transforming growth factor-β (TGF-β) promotes the production of anandamide (AEA) [7,15]. Several authors have reported that modulation of the M1-M2 balance can exert either beneficial or detrimental effects depending on the stage of disease progression, particularly in neurodegenerative disorders. These observations highlight the importance of treatment timing when targeting microglial activation therapeutically [2].

The currently accepted model of microglial activation suggests that inflammatory stimuli drive resting M0 microglia toward the pro-inflammatory M1 phenotype. Depending on the nature of the stimulus, this transition may be associated with either increased or decreased CB2 receptor expression [7]. Studies performed in animal models during neuroinflammatory responses have consistently reported a marked upregulation of CB2Rs in activated microglia [6]. These findings emphasize the importance of biological context when extrapolating results obtained *in vitro* to more complex *in vivo* systems. Moreover, *in vivo* studies have demonstrated that microglia rarely exist as purely M1 or M2 phenotypes; rather, they display mixed activation states characterized by varying proportions of M1- and M2-associated markers [48]. Thus, different CB2 ligands properties like those revealed in the present study could drive microglia toward a more therapeutic state at different stages of disease progression.

In Alzheimer's disease, promotion of microglial activation toward an M1-like phenotype may be beneficial during pre-symptomatic and early symptomatic stages by facilitating the clearance of amyloid-β plaques. In contrast, during later stages of disease, when tau pathology becomes prominent and neurodegeneration accelerates, suppression of excessive microglial activation and promotion of an M2-like phenotype may slow neuronal loss and reduce the rate of cognitive decline [15].

Tau is a microtubule-associated protein essential for maintaining neuronal cytoskeletal integrity. Under pathological conditions, tau can become hyperphosphorylated and aggregate into insoluble neurofibrillary tangles, a hallmark of several neurodegenerative disorders collectively termed

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tauopathies [49]. Numerous studies have demonstrated beneficial effects of suppression of microglial activation in experimental models of tauopathy [50]. Current therapeutic approaches remain unable to selectively target microglia without affecting peripheral macrophages, neurons, or other glial populations, making it difficult to determine the specific contribution of microglial modulation to clinical outcomes [2]. However, the present study suggests that timing treatment to the disease stage could promote a beneficial microglial polarization.

Among the compounds investigated, SR144528 may represent a valuable pharmacological tool for exploring CB2R function in resting microglia. In our cAMP assay, SR144528 displayed the greatest inverse agonist efficacy and, importantly, has been reported to possess one of the most selective binding profiles among cannabinoid ligands, exhibiting the fewest off-target interactions in a panel of 64 proteins associated with adverse human effects [51]. Previous studies have shown that SR144528 reverses the increase in IL-10, an established marker of the M2 phenotype, in primary murine microglia exposed to pro-inflammatory stimuli [52]. Furthermore, SR144528 partially inhibited the anandamide-induced phosphorylation of both ERK1/2 and JNK, suggesting pharmacological actions extending beyond simple CB2 receptor antagonism. It would, therefore, to have assessed the intrinsic activity of SR144528 alone in that study.

Biased agonism is an important property of a ligand to preferentially activate or inhibit specific downstream signalling pathways following engagement of the same receptor [53]. Evidence of signalling bias has been reported for SR144528. In addition to antagonizing 2-AG-induced Ca^{2+} mobilization, SR144528 reverses agonist-induced CB2R internalization and increases receptor expression at the cell surface.

Therefore, biased ligands such as SR144528 show that CB2R signalling outputs can be selectively targeted, raising the possibility of tailoring compounds to drive only the therapeutically desirable responses in microglia.

5. Conclusions

In conclusion, therapeutic manipulation of microglia in CNS disorders remains challenging due to the remarkable complexity of microglial biology, the dynamic nature of microglial phenotypes, and the incomplete understanding of microglial diversity across disease conditions, brain regions, age groups, and sexes. While most studies have focused on activated microglia, our data indicate that resting microglia represent a valuable and underexplored experimental model for investigating CB2R pharmacology. Characterization of CB2R ligands under basal conditions may help disentangle receptor-mediated effects from the numerous confounding factors associated with inflammatory activation, thereby providing novel insights into the role of the endocannabinoid system in microglial function and its potential therapeutic exploitation in CNS diseases [54,55].

Our data indicate that future studies should focus on characterizing the pharmacological effects especially of SR144528 in BV-2 microglia polarized toward M1 and M2 phenotypes. They also indicate special attention should be paid to the selection of experimental models that reflect distinct stages of disease progression, i.e., M polarization, as well as to the timing of treatment, since the consequences of microglial modulation are likely to vary according to the pathological context and disease stage.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Abbreviations

The following abbreviations are used in this manuscript:

AM 630	6-iodopravadoline
CP 55,940	(−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol
DMSO	dimethyl sulphoxide
Forskolin (FSK)	7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one
G418	3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl[oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol
GW 405833	1-(2,3-dichlorobenzoyl)-5-methoxy-2-methyl-3-(2-(morpholin-4-yl)ethyl)-1H-indole hydrochloride
JTE 907	N-(1,3-benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2-oxo-8-(pentyloxy)-3-quinolinecarboxamide
JWH 015	(2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone
JWH 133	3-(1,1-dimethylbutyl)-1-deoxy-Δ8-tetrahydrocannabinol
PMSF	phenylmethylsulphonyl fluoride
(R)-AM 1241	(R,S)-3-(2-iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1H-indole
SR 144528	N-[(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide
WIN 55,212-2	(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

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