



Research article

Characterisation of inverse agonism of the orphan-G protein-coupled receptor GPR52 by cannabinoid ligands Cannabidiol and O-1918



Lisa A. Stott, Cheryl A. Brighton, Jason Brown, Richard Mould, Kirstie A. Bennett, Robert Newman, Heather Currinn, Flavia Autore, Alicia P. Higuero, Benjamin G. Tehan, Cliona MacSweeney, Michael A. O'Brien*, Steve P. Watson

Sosei Heptares, Steinmetz Building, Granta Park, Great Abington, Cambridge CB21 6DG, United Kingdom

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ABSTRACT

The identification of cannabinoid ligands Cannabidiol and O-1918 as inverse agonists of the orphan receptor GPR52 is reported. Detailed characterisation of GPR52 pharmacology and modelling of the proposed receptor interaction is described. The identification of a novel and further CNS pharmacology for the polypharmacological agent and marketed drug Cannabidiol is noteworthy.

1. Introduction

G protein-coupled receptors (GPCRs) are a large family of signalling proteins that mediate physiological responses to a variety of stimuli, and therefore serve as major targets for the development of novel drug candidates [1, 2]. Composed of seven membrane-spanning α -helical segments linked by intracellular and extracellular loops, they transmit information from the extracellular environment to the interior of the cell through conformational changes [3]. GPCRs are the largest class of membrane receptors with over 800 members [4], and a significant proportion of these GPCRs are presently targeted by drugs [5]. Of this receptor class, there are numerous orphan receptors which have not yet been linked to endogenous ligands and present an opportunity for novel drug discovery. One such orphan GPCR is GPR52, a family A Gs-coupled receptor which constitutively increases cellular cAMP levels. First cloned by O'Dowd *et al* GPR52 is an unusual family A receptor which shows little homology with any other related class A GPCRs [6] and, as reported by Stevens *et al* [7], resides in a distal branch of the GPCR phylogenetic tree with fellow orphan receptor GPR21 as its closest relative (71% sequence homology).

GPR52 is highly expressed in the striatum, exclusively on medium spiny neurons expressing dopamine D₂ receptors, and on cortical pyramidal neurons expressing dopamine D₁ receptors [8]. Agonism of GPR52 has been proposed as a possible therapeutic intervention for both the psychotic and cognitive domains of Schizophrenia [9]. Blockade of

GPR52 function has been postulated as a therapeutic approach to Huntington's disease, through modulation of soluble mutant Huntingtin protein (mHTT) levels, for example it is reported that knock-down or inverse agonism of GPR52 rescues behavioural phenotypes in a mouse model of Huntington's disease [10].

The endogenous agonist for GPR52 is unknown and may indeed not exist (see below with respect to structural evidence for activation by extracellular loop (ECL2). Table 1 shows example reported agonists and inverse agonists for GPR52. Takeda reported the first example of GPR52 agonists [11], exemplified by Compound 7m, and subsequently reported related examples such as such as Compound 17 [12] and the orally bioavailable agonist FTBMT [9], the pharmacology of which has been extensively characterised *in vitro* and *in vivo*. [13] In contrast there are only 2 reports of GPR52 inverse agonists, the natural product E7 [10], and very recently, a series of $\alpha\beta$ -unsaturated ketones [14] exemplified by compound 43. The natural product E7, also known as Isoscabertopin, derives from a family of sesquiterpene lactones from the plant *Elephantopus scaber* L. E7 and other related lactones are reported to have other biological activities, including anti-inflammatory effects and anti-tumour activity [15, 16]. Indeed E7 has recently been reported to act as a NAM (negative allosteric modulator) at an intracellular site of GPR52 forming a covalent adduct with C156^{4,40} [17].

Subsequent to the work described herein, Xu *et. al* published an x-ray crystal structure of the GPR52 receptor, both in its apo form and binding the ligand 17 (see above). The structure confirms the atypical nature of

* Corresponding author.

E-mail address: al.obrien@soseiheptares.com (M.A. O'Brien).

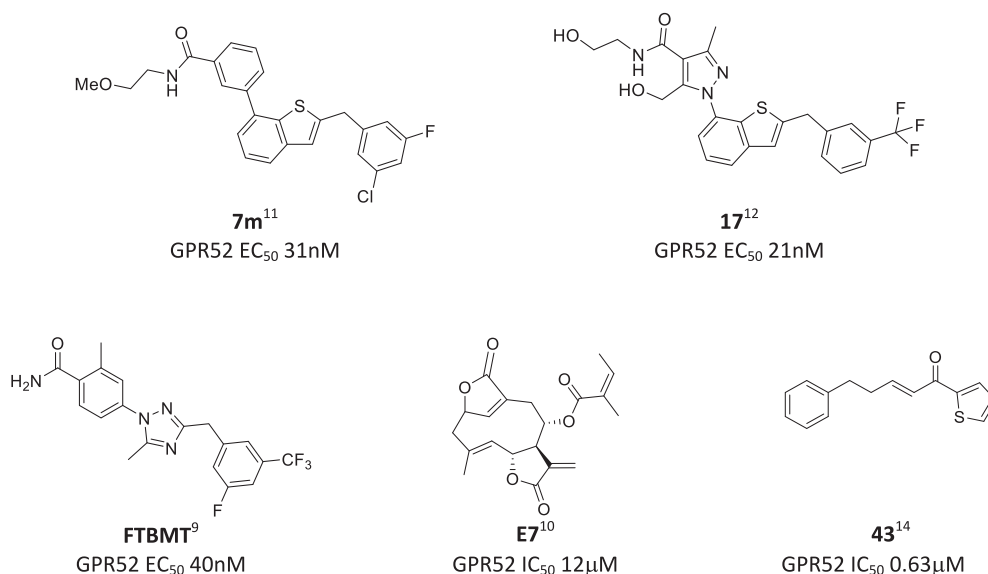


Figure 1. Literature GPR52 agonists and inverse agonists.

GPR52 and reveals a novel mechanism of ECL2 acting as an internal agonist, and accounting for the constitutive activity of the receptor [18].

In our laboratories we sought alternative GPR52 inverse agonists as start points for chemical iteration, to facilitate structural biology approaches, and to further substantiate target validation for GPR52 blockade. Screening of known GPCR ligands led to the identification of the cannabinoid ligands Cannabidiol (CBD) and O-1918 as inverse agonists of GPR52, the pharmacological characterisation of which has been described herein.

2. Materials and methods

2.1. GPR52

2.1.1. Cell line generation

CHO-K1 cells were stably transfected with the human GPR52 receptor containing a C-terminal GFP fusion (CHO-GPR52) using Gene-Juice transfection reagent according to the manufacturer's instructions and subsequently dilution cloned. Clonal CHO-GPR52 cells were maintained in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12) containing 10% fetal bovine serum and 0.5 mg ml⁻¹ geneticin to maintain selection pressure.

2.1.2. GPR52 cAMP assays

Frozen CHO-GPR52 cells were resuspended in assay buffer (Hank's Buffered Saline Solution (HBSS) supplemented with 20mM HEPES, pH

7.4) containing 0.5mM 3-isobutyl-1-methylxanthine (IBMX). Cells were seeded at 2000 cells per well onto 384-well microplates containing 50 nM test compound, stamped using an ECHO acoustic dispenser (LabCyte; San Jose, CA, USA), in a final assay volume of 10 μl. Cells were incubated at 37 °C for 30 min before cAMP was detected using cAMP Gs dynamic kit according to the manufacturer's instructions (Cisbio; Codolet, France). For agonist challenge experiments, compounds were pre-incubated for 30 min at 37 °C before addition of EC₅₀ concentration of compound 7m and a further 30 min incubation at 37 °C.

2.1.3. GPR6 cAMP assay

CHO-K1 Tet-On cells expressing human recombinant GPR6 (FAST-0701c) receptor were induced with 200 ng μl⁻¹ doxycycline for 18 h prior to experiment. Cells were seeded in assay buffer (KRH: 5 mM KCl, 1.25mM MgSO₄, 124mM NaCl, 25mM HEPES, 13.3 mM Glucose, 1.25mM KH₂PO₄, 1.45 mM CaCl₂, 0.05% BSA, supplemented with 1 mM IBMX) in a final assay volume of 10μl and incubated with compound for 30 min at room temperature. cAMP was detected as previously described.

2.1.4. Docking experiment

Schrodinger 2020-3 release was used to perform the docking experiments presented here. The GPR52 publicly released structure [18] (pdb code 6LI0) was prepared with the Protein Preparation Wizard. Glide module was used to perform the dockings with SP precision and extended sampling. Dockings were done with and without water 304 from the 6LI0 structure to compare poses that could interact with that water or displace it.

Table 1. GPR52 inverse agonists potency.

Compound				
	CBD	O-1918	1	2
GPR52 pIC ₅₀	5.61 ± 0.05	5.45 ± 0.14	<4.5	5.47 ± 0.13
GPR52 pEC ₅₀	5.61 ± 0.13	5.84 ± 0.14	<4.5	5.84 ± 0.14

O-1918 and CBD are commercially available from Tocris (2288 and 1570 respectively).

Compound 1 and Compound 2 syntheses are reported in the literature [19].

3. Results and discussion

The cannabinoid ligands O-1918 and CBD were both identified as GPR52 inverse agonists, with pEC_{50} s of 5.84 ± 0.11 and 5.61 ± 0.13 respectively (Table 1). Both ligands displayed the ability to reduce cAMP levels to that of untransfected, wild-type (WT) CHO cells (Figure 1). This contrasted to the expected agonism of **7m**.

O-1918 and CBD are cannabinoid ligands with many structural similarities, but with some contrasting features, therefore **1** and **2** were also specifically chosen for investigation as they hybridise the features of CBD and O-1918. Specifically, **2** can be conceived of as CBD with the demethylated phenols of O-1918, or as O-1918 with the extended n-pentyl tail of CBD. Similarly, **1** represents the bis phenolic version of O-1918, or the alkyl-truncated counterpart of CBD. When examined in the GPR52 cAMP assay, compound **1** showed little activity, only reducing cAMP levels at the highest concentration tested (50 μ M) whereas **2** was demonstrated to be a partial inverse agonist, with lower maximal response than CBD and O-1918 and pEC_{50} of 5.84 ± 0.14 .

In order to further characterise the above compounds and the specificity of the GPR52 response, their ability to antagonise the response of the selective GPR52 agonist **7m** was investigated (Figure 2B). All 4 compounds inhibited an EC_{50} challenge concentration of **7m** with pIC_{50} s described in Table 1. The inverse agonism of CBD and O-1918 led to not only antagonism of **7m**, but reduced cAMP to below basal levels. As in the inverse agonist mode (Figure 2A), **1** only showed activity at the highest concentration tested while **2** appears to have reduced activity compared to CBD and O-1918.

To confirm the specificity of compound responses to GPR52, all ligands were examined for their ability to modulate cAMP in untransfected CHO cells (Figure 2C). All ligands were inactive in these cells, except for CBD which showed a very small decrease in cAMP concentration with pEC_{50} of 5.06 ± 0.28 . CBD is known to be a promiscuous ligand [20], so a small response is perhaps unsurprising. However, the reduced potency of this response relative to the CHO-GPR52 cells and the antagonism of the **7m** response in CHO-GPR52 cells, lends confidence to the classification of CBD as a GPR52 inverse agonist. O-1918 was also shown to be inactive at the unrelated $G\alpha_s$ -coupled orphan receptor GPR6 in the same cell background and assay format ($IC_{50} < 4.8\mu$ M, data not shown), however GPR6 could not be used as a specificity test for CBD as it is a known inverse agonist at this receptor [21, 22, 23].

The data presented here clearly demonstrate that CBD and O-1918 are inverse agonists of GPR52, however the reduced activity of the hybrid compounds (**1** and **2**) relative to CBD and O-1918 is perhaps surprising. This attenuation of activity on hybridising the features of these

compounds may suggest that the two molecules bind to GPR52 in different ways despite their apparent structural similarity (Figure 3).

To explore this experimental observation in the 3D context of the receptor, we performed docking simulations to propose possible binding modes that could explain qualitatively the observed SAR and aid the future rational design to study these molecules further. Structural work by Lin et al [18] shows that although there are conformational changes in the cytoplasmic region of the receptor upon G-protein binding, the orthosteric site with the self-bound ECL2 does not change substantially. The ECL2 occupying the orthosteric site, seems to be responsible for the constitutive activity of GPR52; when Lin et al [18] delete or mutate it, they observed a significant decrease of the receptor activity. How the receptor modulates its activity is still unknown. However, small molecules agonists can be accommodated in the “side pocket” possibly by stabilising further the engagement of ECL2 in the orthosteric site and enhancing the GPR52 activity. It is reasonable then to assume that the small molecules inverse agonists, described here, bind in this “side pocket” and modulate the receptor activity from the same site. At this point, it is worth bearing in mind that this pocket also presents conformational adaptability, (in this receptor due to the flexibility of the N-terminal loop) and the challenges this carries when one uses the structure to dock other chemotypes. Figure 3 shows proposed binding modes for CBD and O-1918 inverse agonists in relation to the full agonist **17** co-crystallised in the published GPR52 structures in the Lin paper [18]. For these docking studies we have used the GPR52-17 complex crystal structure as the other GPR52 structures have narrower or inexistent side pocket. These molecules are lipophilic with a very distinct shape, in absence of the hydrogen bond donors of CBD, the shorter version O-1918 can bind in an alternate orientation that can explain the different binding modes of the analogue molecules. Nevertheless, docking alone cannot explain the mechanism of action of these inverse agonists, but it provides a 3D framework for further designs and rationalisation of the receptor modulation by these small molecules. For example, it would be interesting to explore further the role of the of the water bridge between Asp188^{ECL2} and Ser299^{7.35} and the consequences to displace it as these binding modes suggest.

CBD has recently been approved as Epidolex, for the treatment of seizures associated with Lennox-Gastaut syndrome or Dravet syndrome [20]. CBD is a low affinity antagonist for the cannabinoid CB₁ and CB₂ receptors, but also has a rich polypharmacology [22] such that characterisation of a further novel CNS pharmacology is noteworthy. Activity of CBD at, among others, the following protein targets has also been reported [20] ligand-gated ion channels (GlyR, NaV, nAch, GABAA), TRP channels (TRPV1, TRPV2, TRPA1, TRPM8), GPCRs (5-HT1A, α 1A, μ OR, δ OR, CB1, CB2, GPR18, GPR55), enzymes (FAAH, CYP450), and nuclear receptors (PPAR γ). Interestingly CBD is also reported to be an inverse agonist for orphan receptors GPR3, GPR6 (see above) and GPR12 [21]. Moreover the GPR52 inverse agonist potency of CBD is comparable to its

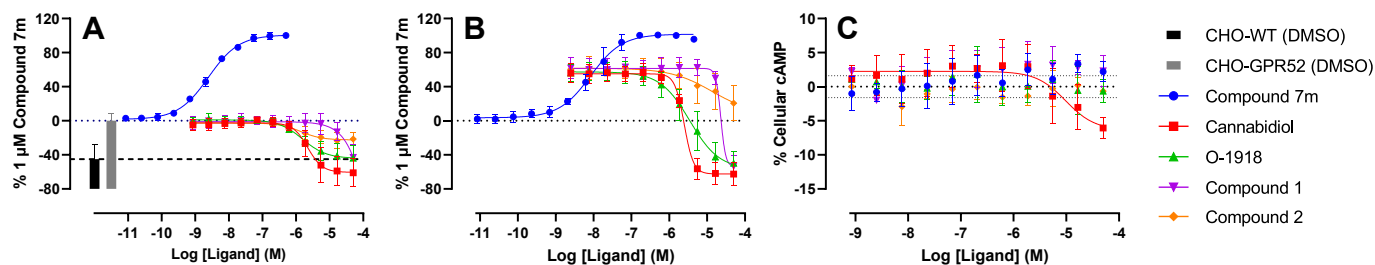


Figure 2. In vitro characterisation of CBD, O-1918 and related compounds. A. CBD, O-1918 and related compounds are inverse agonists in CHO-GPR52 cells when tested alone, reducing cAMP down to the level of CHO-WT (black bar). Basal (DMSO) response from CHO-GPR52 (grey bar) is also shown for comparison, with dotted lines to indicate response level. Data are pooled (mean \pm SD) from 4 independent experiments performed in duplicate, normalised to 1 μ M **7m** response. B. All inverse agonists inhibit an EC_{50} challenge concentration of the agonist compound **7m**, reducing cAMP levels below the basal level in line with the apparent inverse agonism in A. C. All test compounds are inactive in CHO-WT cells, except for cannabidiol, which shows a small decrease in cellular cAMP beyond the standard deviation of the DMSO response (indicated by dotted lines). Data are pooled (mean \pm SD) from 4 independent experiments performed in singlicate expressed as a percentage of the DMSO response.

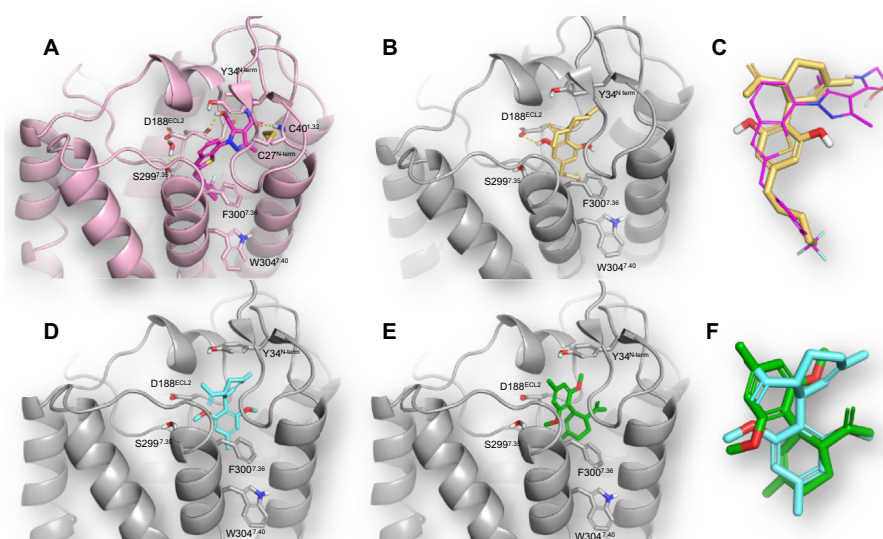


Figure 3. Proposed binding modes for CBD and O-1918. A: Crystal structure of c17 (magenta) bound to GPR52, PDB code 6L10, water bridge between D188^{ECL2} and S299^{7.35} in sticks. This structure has been used for the docking experiments. Panel A is left in magenta shade to differentiate it from docking poses in grey. B: Docked pose of CBD (pale yellow) in GPR52 (grey), hydrogen bond with Asp188^{ECL2} in dotted lines. C: Overlay of the proposed CBD (pale yellow) binding mode with the binding conformation of c17 from the GPR52 crystal structure (magenta). D: O-1918 (cyan) bound to GPR52 (grey) resembling the proposed CBD binding mode at B. E: O-1918 (green) bound to GPR52 (grey) with an alternate binding mode. F: O-1918 overlay of both proposed binding modes for O-1918, despite its distinct shape the molecule can bind in an almost symmetrical manner.

potency at many of these other targets (e.g. reported [20] affinities of CBD at GPCRs 5-HT_{2c}, μ OR, κ OR, δ OR, D1, H3, and α 2b all fall in the 1–10 μ M range). Indeed, it is possible that GPR52 inverse agonism may play a role in the *in vivo* pharmacology of CBD.

In summary we have demonstrated that O-1918 and CBD are inverse agonists of the orphan GPCR GPR52. These molecules may serve as start-points to identify suitable molecules to further substantiate the validation for this mechanism as a therapeutic opportunity and potentially initiate drug discovery programmes. Moreover, the identification of a further new pharmacology for the polypharmacological approved drug CBD is noteworthy.

Declarations

Author contribution statement

Lisa A Stott, Michael A. O'Brien: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Cheryl A. Brighton: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Jason Brown: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Richard Mould: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Kirstie A. Bennett, Robert Newman: Conceived and designed the experiments.

Heather Currinn, Flavia Autore: Conceived and designed the experiments; Performed the experiments.

Alicia Higuero: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Benjamin G. Tehan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Cliona MacSweeney: Conceived and designed the experiments; Wrote the paper.

Steve P. Watson: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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