

UNIVERSITY OF INSUBRIA

PhD in Neurobiology



**PHARMACOLOGICAL PROPERTIES
OF THE PHYTOCANNABINOIDS
 Δ^9 -TETRAHYDROCANNABIVARIN
AND CANNABIDIOL**

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Table of contents

Abstract	4
Introduction	6
Cannabis	7
Taxonomy of cannabis	7
History of cannabis	7
Cannabis constituents	11
Cannabis physiological effects	13
The endocannabinoid system	16
Cannabinoid receptors	16
Endocannabinoids (ECBs)	19
Biosynthesis and degradation of AEA and 2-AG	20
Pharmacology of selected phytocannabinoids	23
Δ^9 -tetrahydrocannabinol (Δ^9 -THC)	24
Pharmacology of Δ^9 -THC at cannabinoid receptors	25
Pharmacology of Δ^9 -THC at non-CB ₁ , non-CB ₂ receptors	27
Reported therapeutic effects of Δ^9 -THC	30
Materials and methods	35
Chemicals	36
Binding studies	36
Competitive or displacement binding assay	38
Dissociation kinetic assay	39
[³⁵ S]GTP γ S binding assay	40
Cell culture	46
Membranes preparation	47
Protein assay	52
Cyclic AMP assay	53
Statistical analysis	56

CHAPTER ONE - Pharmacological properties of the phytocannabinoid	
Δ^9-tetrahydrocannabivarin at cannabinoid receptors	57
INTRODUCTION	58
Pharmacological properties of the phytocannabinoid Δ^9 -THCV	59
AIMS OF THE PROJECT	61
RESULTS - Pharmacological properties of Δ^9-THCV at CB₁ receptors	62
Δ^9 -THCV displaces [³ H]CP55940 at CB ₁ receptors	62
Δ^9 -THCV does not stimulate [³⁵ S]GTP γ S binding at CB ₁ receptors	64
Δ^9 -THCV antagonizes CP55940 in the [³⁵ S]GTP γ S binding assay	66
Δ^9 -THCV is an inverse agonist at hCB ₁ receptors in the cyclic AMP assay	70
RESULTS - Pharmacological properties of Δ^9-THCV at CB₂ receptors	73
Δ^9 -THCV displaces [³ H]CP55940 at CB ₂ receptors	73
Δ^9 -THCV behaves as CB ₂ receptor partial agonist	75
Δ^9 -THCV activity at CB ₂ receptors: further evidences	78
DISCUSSION - Pharmacological properties of Δ^9-THCV at CB₁ receptors	81
DISCUSSION - Pharmacological properties of Δ^9-THCV at CB₂ receptors	83
CHAPTER TWO - Pharmacology of Δ^9-tetrahydrocannabivarin	
at CB₂ receptors: further evidences	95
INTRODUCTION	96
Receptor antagonist	96
Antagonists at CB ₂ receptors	98
AIMS OF THE PROJECT	101
RESULTS	102
AM630 is an antagonist/inverse agonist at hCB ₂ receptors	102
Abolition of constitutive activity at hCB ₂ receptors	103
AM630 becomes an apparent neutral antagonist in AM630-	
-preincubated hCB ₂ -CHO cells	105
AM630 antagonizes WIN55212-2 in AM630-	
-preincubated hCB ₂ -CHO cells	108
AM630-preincubation at different time points	109
Displacement binding assays in AM630-preincubated hCB ₂ -CHO	
cell membranes	111

[³⁵ S]GTPγS binding assays in AM630-preincubated hCB ₂ -CHO cell membranes	112
Displacement binding assays in AM630-preincubated whole hCB ₂ -CHO cells	113
DISCUSSION	115
CONCLUSIONS CHAPTER ONE AND TWO	120
CHAPTER THREE - Pharmacological properties of the phytocannabinoid cannabidiol at somatodendritic 5-HT_{1A} autoreceptors	122
INTRODUCTION	123
Pharmacology of CBD at cannabinoid receptors	124
Pharmacology of CBD at non-CB ₁ , non-CB ₂ receptors	125
5-HT _{1A} receptors	126
5-HT _{1A} receptors, CBD and nausea/emesis	129
AIMS OF THE PROJECT	131
RESULTS	132
CBD does not displace [³ H]8-OH-DPAT in rat brainstem membranes	132
CBD is not an agonist at 5-HT _{1A} receptors	133
8-OH-DPAT acts at 5-HT _{1A} receptors in rat brainstem	134
CBD acts at 5-HT _{1A} receptors in rat brainstem	136
CBD, at 10 μM, is an inverse agonist at CB ₁ receptors	137
CBD enhances 8-OH-DPAT dose-response curve	139
CBD is not a 5-HT _{1A} receptor allosteric modulator	142
DISCUSSION	143
CONCLUSIONS	147
References	148
List of publications	172
Abstracts	173
Acknowledgements	174

ABSTRACT

Cannabis and its derivatives have been used for medicinal purpose for thousand of years. More recently, the main cannabis constituents, cannabinoids, have been found to act and target at cannabinoid as well as other receptors. This brought particular interest around the pharmacology of phytocannabinoids and their possible clinical applications. The research object of this study focused on two phytocannabinoids, Δ^9 -tetrahydrocannabivarin (Δ^9 -THCV) and cannabidiol (CBD), and it has been divided in three individual investigations:

In the first study, we investigated the pharmacology of Δ^9 -THCV at cannabinoid type 1 (CB₁) and type 2 (CB₂) receptors. We found and confirmed that Δ^9 -THCV acts as antagonist at CB₁ receptors in experiments of [³⁵S]GTP γ S binding assay on human CB₁-CHO (Chinese hamster ovary) cell membranes. Also, in the same set of experiments, Δ^9 -THCV displayed a slight inverse agonism at CB₁ receptors, which was confirmed in experiments of cyclic AMP assay in hCB₁-CHO cells. At CB₂ receptors, we found that Δ^9 -THCV can behave as a partial agonist when the measured response is inhibition of forskolin-induced stimulation of cyclic AMP production in hCB₂-CHO cells or stimulation of [³⁵S]GTP γ S binding to membranes obtained either from hCB₂-CHO cells or from mouse spleen membranes. No such effect was displayed by Δ^9 -THCV in untransfected CHO cells, pertussis toxin (PTX)-treated hCB₂-CHO cells or CB₂^{-/-} mouse spleen membranes.

In collaboration with Dr. Barbara Costa and Dr. Dino Maione, we also showed that Δ^9 -THCV shares the ability of established selective CB₂ receptor agonist to reduce signs of inflammation and inflammatory pain (Guindon, Hohmann 2008). These experiments were performed on *in vivo* models of λ -carrageenan-induced oedema and thermal hyperalgesia, and formalin-induced nociception.

In the second investigation we brought further evidences that Δ^9 -THCV is a partial agonist at CB₂ receptors. In detail, we applied a protocol to hCB₂-CHO cells in order to convert the selective CB₂ receptor antagonist/inverse agonist, AM630, into an apparent neutral antagonist. In these experimental conditions, we found that Δ^9 -THCV still behaves as a CB₂ receptor agonist and is antagonized by AM630 in experiments

of cyclic AMP assay. Additional tests were also conducted to better understand the pharmacology of the ligand, AM630, at CB₂ receptors.

In the third study, we investigated the effect of CBD at serotonin type 1A (5-HT_{1A}) autoreceptors. This research was based on previous *in vivo* findings showing that CBD-induced anti-emetic and anti-nausea effects were mediated by somatodendritic 5-HT_{1A} autoreceptors located in the raphe nuclei (Rock et al. 2009, Parker et al. 2010). Experiments of [³⁵S]GTPγS binding conducted in rat brainstem membranes revealed that CBD, in a bell-shaped manner, is able to enhance the dose-response curve of the selective 5-HT_{1A} receptor agonist, DPAT. In addition, our results suggest that CBD does not interact directly with 5-HT_{1A} receptors, and that CBD-mediated DPAT dose-response curve enhancement might implicate the involvement of an other receptor.

INTRODUCTION

CANNABIS

Cannabis is believed to be one of humanity's oldest cultivated crops, providing a source of fibre, food, oil, medicine, and recreational drug since Neolithic times (Chopra et al. 1957, Li 1974, Fleming, Clarke 1998).

TAXONOMY OF CANNABIS

Cannabis is a genus of the dioecious annual plant, generally placed in the Hemp family Cannabaceae along with hops, *Humulus* species (spp). Recent phylogenetic studies, based on Chloroplast restriction site maps and gene sequencing, strongly suggest that the Cannabaceae should belong to the Celtidaceae clade, and that the two families should be merged to form a single monophyletic group (Song et al. 2001, Sytsma et al. 2002).

Based on morphological attributes and on systematic chemotaxonomy, cannabis was found to comprise of three different species: *sativa*, *indica* and *ruderalis* (Figure 1). All of these taxa were indigenous from Central and South Asia, and were spread all over the world by different means and at different times by human beings (Figure 2) (Hillig 2005, Russo 2007).

Morphologically, *Cannabis sativa* is a tall and thin plant, with narrow and light green coloured leaves; it grows quickly, up to 20 feet in height in some cases. *Cannabis indica* is a short thick plant, with broad and darker green leaves, flowering in 8 to 10 weeks with thick dense flower tops. *Cannabis ruderalis* is a short and unbranched roadside plant, usually weak in cannabinoids (Figure 1).

HISTORY OF CANNABIS

The use of cannabis finds its roots in Asia (China in particular), where archaeological and historical findings indicate that the plant was cultivated for fibres since 4000 B.C. Several references reported cannabis usage for the treatment of several disorders like rheumatic pain, intestinal constipation, disorders of the female reproductive system, malaria and others; and seeds, which are devoid of Δ^9 -tetrahydrocannabinol (Δ^9 -

THC), were usually reported as mainly being used for this purpose. In China, the medicinal use of cannabis never reached the heights it did in India. Here, the use of cannabis was widely disseminated, both as a medicine and recreational drug, which probably began together in around 1000 B.C. (Li 1974, Zuardi 2006).

From Asia, cannabis spread to the Western World (Figure 2) where several references reported the use of this plant in Ancient Egypt; as well as the scarce references to the use of cannabis by the Greeks and Romans, suggesting that it was little-used by these populations.

In Africa the use of cannabis began around the 15th century where it was reported useful for snake bite, to facilitate childbirth, malaria, fever, blood poisoning, anthrax, asthma and dysentery.

In the 16th Century, cannabis reached the Americas, where probably African slaves brought the plant's seeds to South America (Brazil). Here, cannabis was above all used in popular religious rituals.

The effective introduction of cannabis in Western medicine occurred in the middle of 19th century through the work of William B. O'Shaughnessy, an Irish physician, and by the book of Jacques-Joseph Moreau, a French psychiatrist. Their contributions had a deep impact on Western medicine, especially due to the scarcity of therapeutic options for infectious diseases, such as rabies, cholera and tetanus. The climax was reached in the late 19th century and the early 20th century, when various laboratories marketed cannabis extracts or tinctures, such as Merk (Germany), Burroughs-Wellcome (England), Bristol-Meyers Squibb (USA), Parke-Davis (USA) and Eli Lilly (USA) (Fankhauser 2002). During this period, the medical indications of cannabis, as reported in Sajous's Analytic Cyclopaedia of Practical Medicine (1924) (Aldrich 1997), were summarized in three areas: sedative or hypnotic, analgesic and in appetite and digestion dysfunctions.

Soon after this period, the use of cannabis significantly decreased, because of the difficulty to obtain replicable effects, due to the varying efficacy of different samples of the plant. Moreover, the introduction of many legal restrictions (e.g. Marihuana Tax Act in 1937 in the USA) limited the medicinal use and experimentation with cannabis.

In the second half of 20th century, cannabis reached great social importance as a drug for recreational use by intellectuals groups, and later spread among the younger generation throughout the Western World.

In 1964, the isolation and characterization of the active constituent of cannabis, Δ^9 -THC by Gaoni and Mechoulam (Gaoni 1964), contributed to a proliferation of studies about the active components of cannabis.

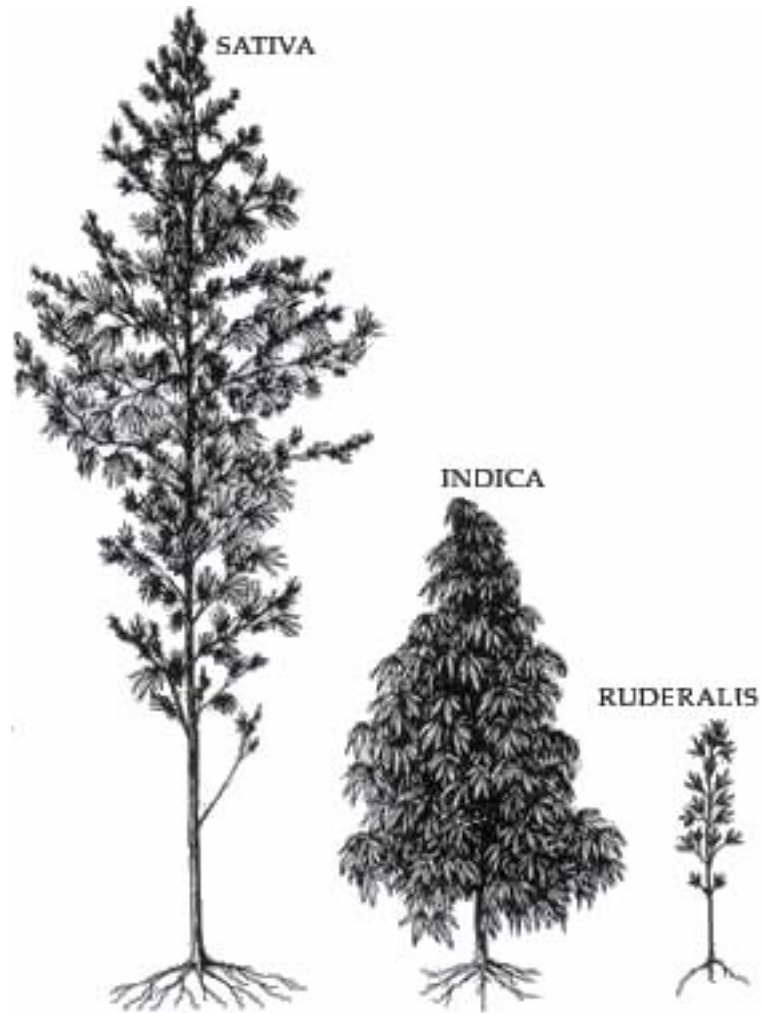


Figure 1. Morphology of Cannabis species.

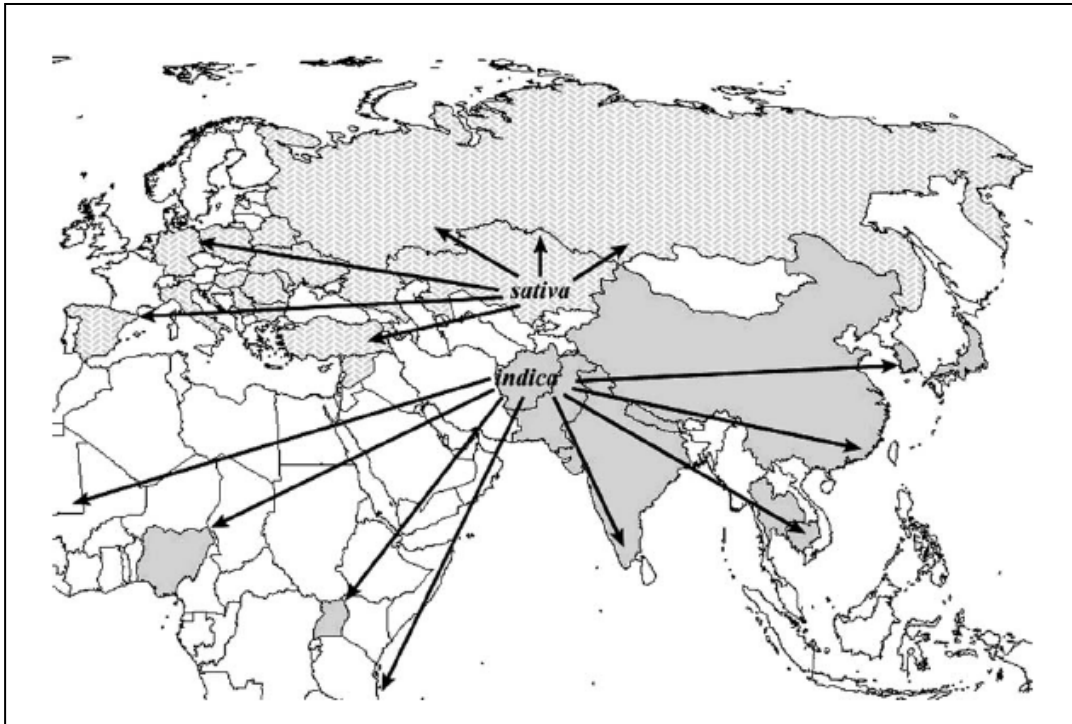


Figure 2. Map showing the countries of origin of *Cannabis indica* and *sativa*. The arrows suggest human-vectored dispersal from the presumed origin of cannabis in Central Asia. Figure taken from Hallig 2005.

So far, three different drugs have been developed in the last 30 years from cannabis or on the basis of active components derived from cannabis (Table 1):

1) Nabilone (Cesamet[®], VALEANT Pharmaceuticals International), which is a synthetic analogue of Δ^9 -THC and it is used for suppression of nausea and vomiting produced by chemotherapy.

2) Dronabinol (Marinol[®], Solvay Pharmaceuticals), which is the synthetic (-)-trans- Δ^9 -tetrahydrocannabinol; this drug finds applications as an anti-emetic and appetite stimulant, for example in Acquired Immune Deficiency Syndrome (AIDS) patients experiencing excessive loss of body weight.

3) Sativex[®] (GW Pharmaceuticals), which contains approximately equal amounts of Δ^9 -THC and the non-psychoactive plant cannabinoid, cannabidiol (CBD), and is prescribed for the symptomatic relief of neuropathic pain in adults with multiple sclerosis, and as an adjunctive analgesic treatment for adult patients with advanced cancer (Pertwee 2009).

These three drugs have been licensed in all or some of following countries: UK, Spain, Canada and USA (Table 1).

	Nabilone	Δ^9 -THC	Sativex	Sativex
Therapeutic indication	UK, Canada, USA	USA	Canada	UK, Spain
Anti-emetic	Yes	Yes	No	No
Appetite stimulant	No	Yes	No	No
Neuropathic & cancer pain	No	No	Yes	No
Spasticity due to Multiple Sclerosis (MS)	No	No	Yes	Yes
First licensed	1982	1986	2005	2010

Table 1. Summary of plant constituents or synthetic cannabinoids licensed so far. In Canada, Sativex has been approved for the treatment of spasticity in 2010. In Spain, the final approval process is expected to be completed in the 4th Quarter of 2010.

CANNABIS CONSTITUENTS

Cannabis is composed by a vast number of compounds (approximately 538), including mono- and sesquiterpenes, sugars, hydrocarbons, steroids, flavonoids, nitrogenous compounds and amino acids, among others. The best-known and the most specific class of cannabis constituents is represented by cannabinoids, which are characterized by a C₂₁ terpenophenolic constituent's structure (ElSohly, Slade 2005). There are ten main types of cannabinoids and fourteen different cannabinoids subtypes, based on their chemical structure (Figure 3)

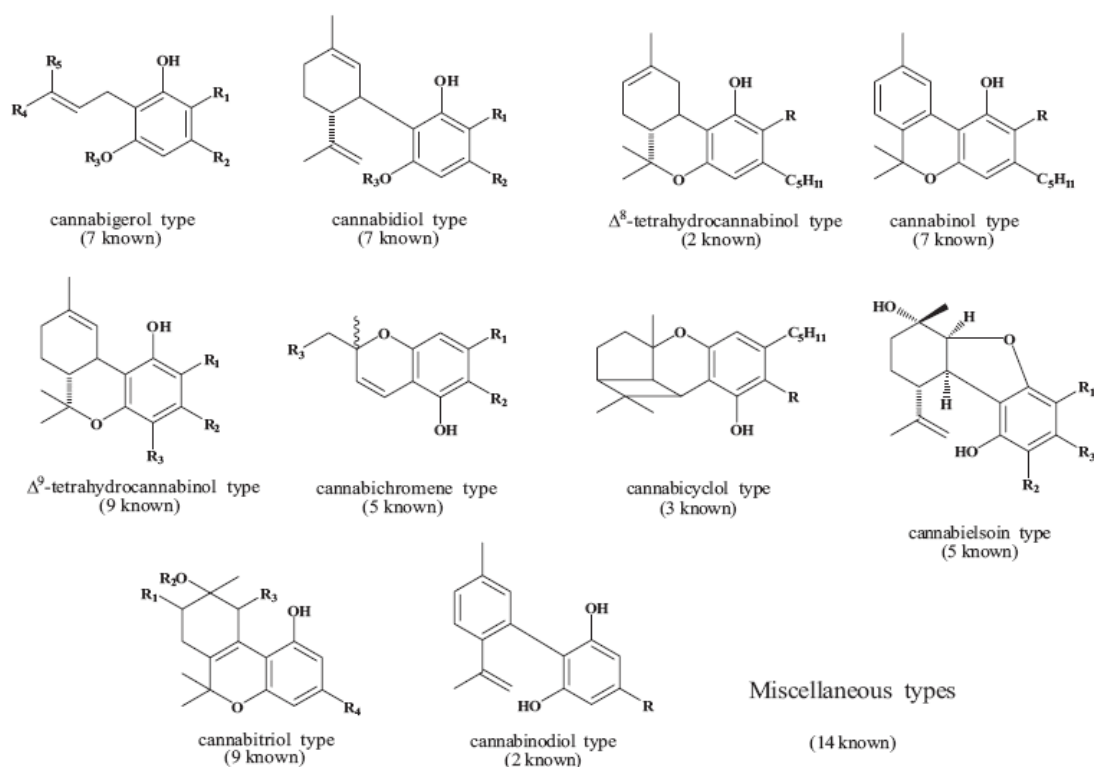


Figure 3. The phytocannabinoids can be sub-classified into 10 main groups depending on their chemical structure. Figure taken from Hanus, 2009.

The first natural cannabinoid to be discovered was cannabinol (CBN), which was extracted in 1899 (Wood 1899) and chemically characterized in 1940 (Adams, Cain & Baker 1940, Ghosh 1940). In 1963, Mechoulam's group isolated CBD, the main non-psychoactive constituent of cannabis (Mechoulam, Shvo 1963), and one year later they isolated and characterized Δ^9 -THC, the main psychoactive component of cannabis (Gaoni 1964). In more recent years, other phytocannabinoids have been isolated and characterized, but it is possible that some of them may not have been discovered yet.

Phytocannabinoids are synthesised in cannabis by glandular trichomes as carboxylic acids from geranyl pyrophosphate and olivetolic acid to yield the parent phytocannabinoid compound, cannabigerolic acid (CBGA). Subsequent reactions involving different enzymes catalyze the transformation of CBGA into other phytocannabinoids. The presence of these enzymes differs between various strains and species of cannabis, resulting in a different content of phytocannabinoids (Russo 2007).

CANNABIS PHYSIOLOGICAL EFFECTS

The acute physiological and psychological effects of cannabis as a single entity (usually in resin or dried leaf form) are well documented. Following inhalation or intravenous administration, maximal levels in the brain are reached in approximately 15 minutes, and the effects persist for around 3 hours before steadily declining. If it is administered orally, it may take up to an hour to reach maximal levels in the brain, and effects typically persist for longer, up to 5 or 6 hours, due to continuing absorption from the gut (Hollister 1986). Cannabis is primarily sought out for recreational use due to the “high” it produces, a combination of effects including euphoria, relaxation, alteration of perception, distortion of time, and enhancement of sensory modalities. Some users experience anxiety and stress instead. Impairment of short term memory, perturbation of motor function, slowed reaction times and overall impaired cognition are other common phenomena experienced by users in varying combinations and to differing degrees. Physiologically, cannabis ingestion/inhalation can cause tachycardia, drops in systemic and intraocular blood pressure, alterations to breathing rate, peripheral vasodilatation, increased cerebral blood flow, suppression of nausea, and analgesia. The multiple physiological and psychological effects of cannabis are summarized in Table 2. While recreational users seek the psychological effects of cannabis, patients using it therapeutically generally report any psychological perturbations as unwanted and sometimes disturbing side effects (Hollister 1986).

The active constituents of cannabis are highly soluble, able to cross membranes easily and can also be accumulated in fatty tissues from where they are slowly released back into the bloodstream. Hence, total elimination from the body occurs only after a considerable period of time, and certain effects may be relatively long-lasting. However, in comparison to the acute effects of cannabis use, its chronic effects are much lesser-studied, a situation no doubt partially attributable to the legal status of cannabis over the past 50 years, as well as the problems associated with studying users over several years or decades. Interpretation is also problematic as the majority of long-term recreational users smoke cannabis mixed with tobacco, and many of the reported changes associated with prolonged cannabis use are similar to those seen in tobacco smokers. Despite these difficulties, several studies have attempted to address possible effects of chronic cannabis use in both animals and humans. Reported

consequences include suppression of the immune system (both cell-mediated and humoral responses in rodents), increased risk of chronic bronchitis and respiratory carcinomas (Hollister 1986). Reproductive impairments are also thought to occur, including reduced testosterone secretion and sperm viability in males, and disruption of the ovarian cycle in females. Recent data about smoking cannabis during pregnancy show an impairment and alteration in the development of the offspring's brain (Jutras-Aswad et al. 2009). Heavy users may be subject to tolerance-dependence syndrome, and subtle cognitive impairments have been reported, which are boosted with increasing frequency/length of use. Finally, in some subjects cannabis use may exacerbate schizophrenic tendencies, and/or produce confusion, delusions, hallucinations and anxiety (Kumar, Chambers & Pertwee 2001).

<p>Central nervous system Psychological effects Effects on perception Sedative effects Effects on cognition and psychomotor performance Effects on motor function Analgesic effects Anti-emetic effects Tolerance Dependence, abstinence syndrome</p>	<p>Euphoria, dysphoria, anxiety, depersonalisation, aggravation of psychotic states Heightened sensory perception, distorted sense of space and time, misperceptions, hallucinations Generalised CNS depression, drowsiness, sleep, additive effect with other CNS depressants Fragmentation of thoughts, mental clouding, memory impairment, global impairment of performance Increased motor activity followed by inertia and lack of coordination, ataxia, dysarthria, tremulousness, weakness, muscle twitching Similar efficacy to codeine In acute doses; effect reversed with larger doses or chronic use Increased appetite To most behavioural and somatic effects including the 'high' with chronic use Rarely observed but has been produced experimentally following prolonged intoxication</p>
<p>Cardiorespiratory system Heart rate Peripheral circulation Cardiac output Cerebral blood flow Ventilation Bronchodilation Airways obstruction</p>	<p>Tachycardia with acute dosage; bradycardia with chronic use Vasodilatation, conjunctival redness, postural hypotension Increased output and myocardial oxygen demand Increased acutely, decreased with chronic use Small doses stimulate, larger doses depress Coughing, but tolerance develops Due to chronic smoking</p>
<p>Eye</p>	<p>Decreased intraocular pressure</p>
<p>Immune system</p>	<p>Impaired bactericidal activity of macrophages in lung and spleen</p>
<p>Reproductive system</p>	<p>Decreased sperm count and sperm motility in males Suppression of ovulation, complex effects on prolactin secretion, increased obstetric risks</p>

Table 2. Summary of physiological and psychological effects of cannabis. Adapted from Kumar et al, 2001

THE ENDOCANNABINOID SYSTEM

The endocannabinoid system refers to a group of endogenous lipids and their receptors that are involved in a variety of physiological processes including neurotransmitter release, motor learning, synaptic plasticity, pain-sensation, inflammation and appetite. Broadly speaking, the endocannabinoid system encompasses:

- The cannabinoid receptor type 1 (CB₁) and 2 (CB₂), two G protein-coupled receptors (GPCRs) primarily located in the central nervous system and periphery, respectively, as well as orphan receptors.
- The endogenous lipids (endocannabinoids), that are synthesized “on demand” as ligands for the cannabinoid receptors.
- Enzymes involved in the synthesis and degradation of endocannabinoids.
- Endocannabinoid membrane transporters (EMTs), which have been hypothesized to guide the endocannabinoids through the cellular membrane. However, these carriers have not been cloned yet.

CANNABINOID RECEPTORS

The first results suggesting the existence of membrane receptors for cannabinoids came from experiments performed by Howlett’s group. Here, they showed that Δ^9 -THC was able to inhibit the production of adenylyl cyclase in neuroblastoma cells, suggesting the involvement of GPCRs (Howlett, Fleming 1984). Furthermore, the same group showed that this inhibition was blocked by pretreatment with pertussis toxin (Howlett, Qualy & Khachatrian 1986), a bacterial toxin which selectively inactivates G proteins of the G_i and G_o families. These experiments strongly suggested that Δ^9 -THC produced its effects acting *via* G_{i/o}-coupled GPCRs (Howlett, Qualy & Khachatrian 1986).

The synthesis of cannabinoid compounds (i.e. CP55940) by Pfizer, and the consequently development of specific binding assays with these compounds, provided additional validation for the existence of cannabinoid receptors as member of the GPCR family, and allowed the first preliminary map distribution of the receptor

(Howlett et al. 2002). The cannabinoid receptor was eventually cloned from the rat cerebral cortex in 1990 and named the CB₁ receptor (Matsuda et al. 1990).

The CB₁ receptor is widely expressed throughout the brain, with particular emphasis to those brain regions that correlate well with the observed effects of cannabinoids, including impairment of cognition, memory, learning, motor coordination, analgesia and anti-emesis (Howlett et al. 2002). Hence, autoradiography studies show very dense receptor binding in the lateral part of the caudate nucleus and putamen, cerebellar molecular layer, innermost layers of the olfactory bulb, and the CA3 and molecular layers of the dentate gyrus of the hippocampus. The rest of the forebrain is moderately densely labelled, while the brainstem and spinal cord is sparsely labelled (Herkenham et al. 1990, Pettit et al. 1998). At the subcellular level, CB₁ receptors are highly expressed on axon terminals and pre-terminal segments, where their activation inhibits presynaptic calcium channels, decreasing calcium entry during axonal depolarization, leading to decreased neurotransmitter release (Chevaleyre, Takahashi & Castillo 2006). Peripherally, CB₁ receptors have been identified in the spleen, tonsils, intestine, bladder, vas deferens, sympathetic nerve terminals, smooth muscle cells, and at very low levels in adrenal glands, heart, prostate, uterus and ovary (Demuth, Molleman 2006).

Regarding the receptor signalling, as above-mentioned, CB₁ receptor is coupled to G_i and G_o proteins, and its activation involves the inhibition of adenylyl cyclase with a consequent decrease in cyclic AMP production (Howlett et al. 2002). A CB₁ receptor interaction with G_s has also been demonstrated *in vitro* in both striatal neurons and in Chinese Hamster Ovary (CHO) cells overexpressing CB₁ receptors (Felder et al. 1995, Bonhaus et al. 1998). Activation of the CB₁ receptor is also associated with the activation of mitogen-activated protein (MAP) kinase, which regulates many cellular functions such as cell growth, transformation and apoptosis. The exact mechanism underlying cannabinoid-mediated MAP kinase activation has yet to be fully elucidated; however this effect is both PTX- and SR141716A-sensitive (Bouaboula et al. 1995), confirming the involvement of the CB₁ receptor.

The CB₁ receptor has also been associated with the modulation of voltage-gated ion channels, in particular N- and P/Q-type calcium channels and inwardly rectifying A-type potassium channels, which are thought to underlie the cannabinoid-induced inhibition of neurotransmitter release at the presynaptic level (Howlett et al. 2002). The majority of these effects are mediated through the CB₁ receptor, although there is

evidence to suggest that cannabinoids modulate ion channel function directly (Demuth, Molleman 2006).

The second cannabinoid receptor to be discovered was the CB₂ receptor in 1993 (Munro, Thomas & Abushaar 1993). This receptor is located prevalently in the immune system, in particular in the spleen and tonsils. At the cellular level, B cells express high levels of CB₂ receptors, while moderate levels are found in natural killer cells, and low levels are found in T cells (Galiegue et al. 1995). Other tissues and cells also express CB₂ receptors including some neurons, enterocytes, osteoclasts and osteoblasts, and the liver (Mackie 2007). Interestingly, the CB₂ receptor appears to be highly inducible (Rousseaux et al. 2007), which makes this receptor an interesting tool for the treatment of diseases such as inflammation, pain, atherosclerosis, hepatic fibrosis, and bone remodelling, where CB₂ receptors have been shown to be involved (Mackie 2007).

In contrast to CB₁, the sequence of the CB₂ receptor gene shows considerable divergences between human, mouse, and rat (Howlett et al. 2002), which complicates the study of CB₂ receptor pharmacology.

The CB₂ receptor has been demonstrated to couple to G_{i/o} proteins, which implicates the inhibition of adenylyl cyclase activity and consequently the production of cyclic AMP (Felder et al. 1995, Bayewitch et al. 1995). Moreover this receptor is able to modulate the activity of MAP kinase, and cannabinoids have been shown to activate p42/44 MAP kinase *in vitro*, an effect which could be blocked by both PTX and the CB₂ receptor antagonist SR144528 (Bouaboula et al. 1996, Kobayashi et al. 2001). Conversely to CB₁, the CB₂ receptor is not able to bind to G_s proteins (Glass, Felder 1997) or modulate ion channel function.

Recently, other orphan receptors have been classified as putative cannabinoid receptors. In particular GPR55 has been found to be modulated by several plant, synthetic and endogenous cannabinoids (Ryberg et al. 2007). The pharmacology of this new putative cannabinoid receptor is still controversial, and it seems that the activation of GPR55 downstream signalling occurs a ligand- and tissue-dependent manner (Ross 2009). A possible fourth novel cannabinoid receptor is GPR119, which is strongly implicated in the regulation of energy balance and body weight, and seems to be regulated by the endocannabinoid-related compound OEA (oleoylethanolamide) (Brown 2007).

ENDOCANNABINOIDS (ECBs)

The discovery of the cannabinoid receptors suggested the existence of endogenous ligand(s), which could bind to these receptors and exert physiological effects. The first endocannabinoid to be isolated was anandamide (arachidonoyl ethanolamide, AEA), which is the amide between arachidonic acid and ethanolamine, and acts as a partial CB₁ agonist, but only as a weak CB₂ agonist (Devane et al. 1992). This finding was soon to be followed by the identification of 2-arachidonoylglycerol (2-AG), the arachidonate ester of glycerol, which activates both CB₁ and CB₂ receptors (Mechoulam et al. 1995, Sugiura et al. 1995). More recently came the discovery of other endocannabinoids, in chronological order, 2-arachidonyl-glycerol ether (noladin ether), N-arachidonoyl-dopamine (NADA)13,14, and virodhamine¹⁵ (Bisogno et al. 2000, Hanus et al. 2001, Huang et al. 2002, Porter et al. 2002).

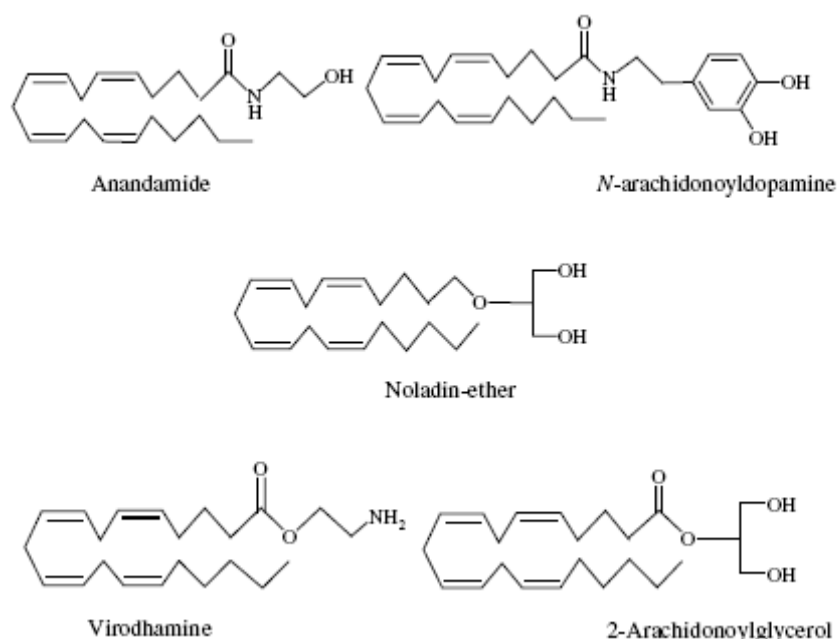


Figure 4. Chemical structure of endocannabinoids. Figure taken from (De Petrocellis, Di Marzo 2009).

Endocannabinoids are synthesized and released “on demand”, which implies a very fast degradation process through specific enzymes.

So far, anandamide and 2-AG remain the only endocannabinoids for which the pharmacological activity and metabolism have been thoroughly investigated.

BIOSYNTHESIS AND DEGRADATION OF AEA AND 2-AG

Several pathways might exist for both the formation and catabolism of anandamide and 2-AG.

In particular, anandamide originates from a phospholipid precursor, N-arachidonoyl-phosphatidylethanolamine (NArPE), formed from the N-arachidonoylation of phosphatidylethanolamine via N-acyltransferases (NATs). The N-acyl-phosphatidylethanolamine-selective phosphodiesterase (NAPE-PLD) was first identified as the enzyme responsible for NArPE catalysis of anandamide through a direct reaction which is dependent upon the concentration of Ca^{2+} . More recently other enzymes and consequently alternative pathways have been identified in the biosynthesis of AEA, through NArPE catalysis (Figure 5) (De Petrocellis, Di Marzo 2009).

Regarding 2-AG, this endogenous lipid is produced almost exclusively via the hydrolysis of diacylglycerols (DAGs), via DAG lipases (DAGLs) α and β enzymes. In turn, DAG precursors are produced either from the phospholipase-C-catalysed hydrolysis of phosphatidylinositol, or from the hydrolysis of phosphatidic acid (Figure 5) (Bisogno et al. 1999).

After their biosynthesis, endocannabinoids are released from cells through the putative endocannabinoid membrane transporter (EMT), about which there are some controversies surrounding its existence since it has been yet not cloned (Di Marzo 2008). After their release, endocannabinoids can act on cannabinoid receptors through an autocrine or paracrine mechanism, and soon after they undergo uptake by the same above-mentioned transporter (Figure 5). In neurons, the actions of endocannabinoids are peculiar, and are called “retrograde signalling”, where endocannabinoids are synthesized and released at the presynaptic level and, following a retrograde way, act at postsynaptic level where cannabinoid receptors are localized on the cellular membrane (Katona, Freund 2008).

Subsequent to their internalization, endocannabinoids are catabolised. FAAH (fatty acid amide hydrolase) is the major enzyme responsible for anandamide

degradation via hydrolysis of its amide bond (Cravatt et al. 1996). In turn, 2-AG catabolism involves an enzymatic hydrolysis reaction by the MAGL enzyme (monoacylglycerol lipase), that hydrolyzes the 2-AG ester bond (Dinh et al. 2002). However, other enzymes have been recently found to be involved in 2-AG degradation, i.e. ABH 6, 12 and FAAH, and their involvement might be tissue-specific (Figure 5) (De Petrocellis, Di Marzo 2009). Other potential catabolic pathways the endocannabinoids employ are the enzymes of the arachidonate cascade, i.e. cyclooxygenase-2 and lipoxygenases, as well as cytochrome P450 enzymes (Kozak, Marnett 2002, Hanus 2009).

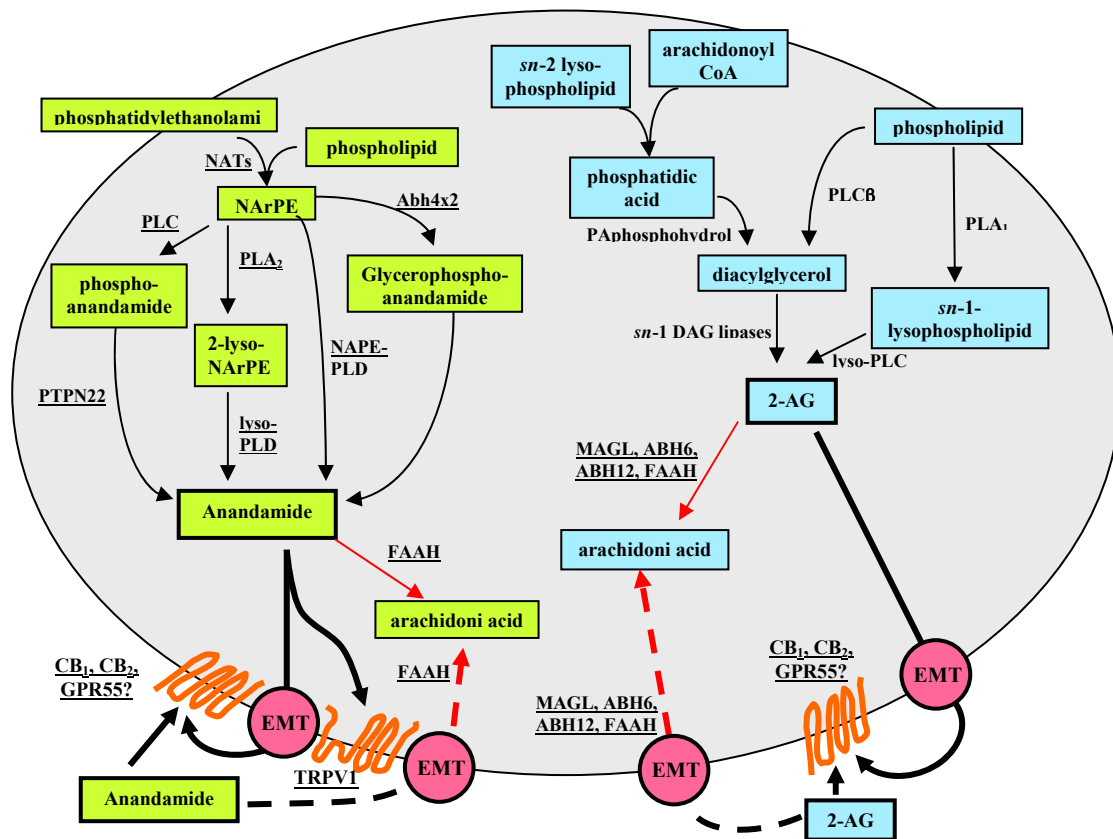


Figure 5. Biosynthesis, action, and degradation of the two best-studied endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG). Several pathways have been reported for both the formation and catabolism of anandamide and 2-AG. Anandamide is formed from a phospholipid precursor, N-arachidonoyl-phosphatidyl-ethanolamine (NArPE), originates from the N-arachidoylation of phosphatidylethanolamine via N-acyltransferases (NATs). In turn, NArPE is transformed into anandamide via four possible alternative pathways, the most direct of which is catalysed by an N-acyl-phosphatidylethanolamine-selective phosphodiesterase (NAPE-PLD). The other three pathways imply the formation of reaction intermediates, such as phopho-anandamide, 2-lyso NArPE and

glycerophospho-anandamide, through the activity of specific enzymes. 2-AG is produced almost exclusively via the hydrolysis of diacylglycerols (DAGs) via sn-1-selective DAG lipases (DAGLs) α and β . After cellular re-uptake via a specific and yet-to-be characterized mechanism (EMT), which appears to also mediate the release of de-novo biosynthesized endocannabinoids (DiMarzo, 2008), anandamide is metabolized via fatty acid amide hydrolase-1 (FAAH), and 2-AG via several monoacylglycerol lipases (MAGLs). 2-AG can also be degraded by FAAH. Both endocannabinoids activate CB₁ and CB₂ receptors with different affinities (anandamide being the one with highest affinity in both cases) and efficacies (2-AG being the one with highest efficacy in both cases). Anandamide can also activate transient receptor potential vanilloid type-1 (TRPV1) channels at an intracellular site (Zygmunt PM, 1999), whereas both compounds, at very high concentration, were recently reported to activate peroxisome proliferator activated receptors (PPAR)- α and γ (O'Sullivan SE, 2007). Both anandamide and 2-AG were also reported by some authors, but not by others, to interact with GPR55 (Ryeberg E, 2007).. Abh4,6,12, α - β hydrolases 4, 6, 12; PLD, phospholipase D; PLA1/2, phospholipase A1/A2; PTPN22, protein tyrosine phosphatase N22. Biosynthetic pathways are shown in black, degradation ones in red. Thick arrows denote movement or action. Figure adapted from (De Petrocellis, Di Marzo 2009).

PHARMACOLOGY OF SELECTED PHYTOCANNABINOIDS

Since the isolation of phytocannabinoids, and later the discovery of cannabinoid receptors and endocannabinoids, research and consequently the number of publications about cannabis has tremendously increased in the last 20 years (Figure 6).

In particular, the research has been focused around some of the constituents of cannabis, among these Δ^9 -tetrahydrocannabinol (Δ^9 -THC), cannabidiol (CBD), cannabinol (CBN), Δ^9 -tetrahydrocannabivarin (Δ^9 -THCV), and cannabigerol (CBG). The pharmacology of these compounds has not yet been fully elucidated. However, data obtained so far suggest a very complex pharmacological profile which involves not only cannabinoid receptors, but in some cases also other receptors, channels or intracellular targets (Pertwee 2009). The complex pharmacological profile demonstrated for some cannabis constituents has opened the way to various possible therapeutic applications. Thus, in order to introduce some of the receptors involved in the pharmacology of phytocannabinoids and the diseases in which the cannabinoid system is mainly implied, here I will mainly focus on the best-known phytocannabinoid characterized to date, Δ^9 -THC. In particular, I will explain its pharmacological profiles and the disease states in which it has been mainly characterised. In the results section I will instead focus on other two phytocannabinoids, Δ^9 -THCV and CBD.

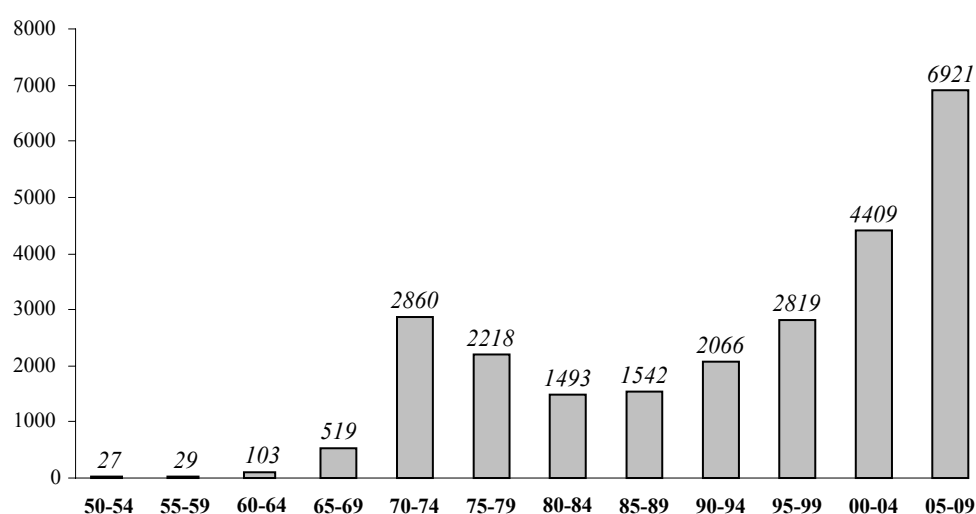


Figure 6. Histogram showing the number of cannabis-related publications (y-axis) in the last 50 years (x-axis). The source used was the “ISI Web of Knowledge” with the keywords: cannabis or marijuana or marihuana.

Δ^9 -TETRAHYDROCANNABINOL (Δ^9 -THC)

Δ^9 -THC is the main psychoactive constituent of cannabis and it was first isolated by Mechoulam and Gaoni in 1964 (Gaoni 1964). Chemically, Δ^9 -THC is a poorly water soluble, amorphous substance which is sticky, resin-like and highly viscous which makes it difficult to handle and process (Thumma et al. 2008).

As a cannabis constituent, Δ^9 -THC is assumed to be involved in self-defence, perhaps against herbivores. Besides, Δ^9 -THC has been found to possess high UV-B (280-315 nm) absorption properties, which have been speculated to protect the plant from harmful UV radiation exposure (Pate 1994).

Dronabinol is the synthetic analogue of tetrahydrocannabinol, in particular the (-)-trans-delta9-tetrahydrocannabinol isomer that is the main isomer of Δ^9 -THC in cannabis. Dronabinol is sold as Marinol (a registered trademark of Saolway Pharmaceuticals). In this formulation, the drug has limited stability and has to be stored at low temperatures (4°C). Moreover, the oral bioavailability of the drug is low (circa 6%) and inconsistent which is mainly due to its first pass metabolism and poor solubility. Furthermore, tetrahydrocannabinol has been reported to be unstable in acid solutions or when exposed to heat, air, and light. Hence, in the last few years researchers have been challenging the development of Δ^9 -THC as a prodrug, to avoid the pharmacokinetics and physico-chemical limitation properties of the pure drug (Thumma et al. 2008).

Regarding the pharmacokinetics, Δ^9 -THC is metabolized mainly to 11-hydroxy-THC (11-OH-THC) by the human body. This metabolite is still psychoactive and is further oxidized to 11-nor-9-carboxy-THC (THC-COOH). In humans and animals, more than 100 metabolites could be identified, but 11-OH-THC and THC-COOH are the predominant metabolites. Metabolism occurs mainly in the liver by cytochrome P450 enzymes CYP2C9, CYP2C19, and CYP3A4. More than 55% of Δ^9 -THC is excreted in the feces and ~20% in the urine. The main metabolite in urine is the ester of glucuronic acid, THC-COOH, and free THC-COOH. In the feces, mainly 11-OH-THC was detected (Huestis 2005a).

PHARMACOLOGY OF Δ^9 -THC AT CANNABINOID RECEPTORS

Several studies have been conducted around the pharmacology of this compound, in particular on cannabinoid receptors. Δ^9 -THC has a high affinity for CB₁ receptors (Pertwee 2008), in fact displacement binding assays performed with rat brain membranes or cell membranes transfected with rat CB₁ receptors, show mean K_i values in the nanomolar range (Rinaldi-Carmona et al. 1994). Similar affinity has been shown by Δ^9 -THC on CB₂ receptors in experiments performed in rat spleen membranes or cell membranes transfected with rat CB₂ receptors (Rinaldi-Carmona et al. 1994, Bayewitch et al. 1996). [³⁵S]GTP γ S binding assays, performed in the aforementioned membranes, showed agonistic properties of Δ^9 -THC at both CB₁ and CB₂ receptors, indicating an ability of this compound to activate both these receptors and consequently to inhibit the production of cyclic AMP, as direct consequence of G_{i/o}-mediated cannabinoid receptor signalling. However, the efficacy displayed by Δ^9 -THC in these assays is lower compared to well-known synthetic CB₁/CB₂ receptor agonists (e.g. HU-210, CP55940 and WIN55212-2), indicating it to be a partial agonist for both of these receptors (Pertwee 2008).

In vivo, the pharmacology of Δ^9 -THC on CB₁ receptors has been proved by a typical bioassay: the “tetrad test”. This test represents a series of behavioral paradigms in which rodents treated with CB₁ agonists show effects (Little et al. 1988). The four behavioral components of the tetrad are spontaneous activity, catalepsy, hypothermia, and analgesia. In particular, these bioassays are evaluated in the following way:

1. Spontaneous activity (or hypomotility) is determined by an open field test, in which a mouse is placed in a cage with perpendicular grid lines, usually spaced by approximately 1 inch. An experimenter counts the number of line crossings by the mouse in a given amount of time.
2. Catalepsy is determined by the bar test. The mouse is placed on a bar oriented parallel to and approximately 1 inch off of the ground. If the mouse remains immobile on the bar for typically > 20 seconds, it is considered cataleptic.

3. Hypothermia is determined by using a rectal probe to measure the rectal temperature.
4. Analgesia is usually determined by the hot plate or tail immersion test. In the hot plate test, the mouse is placed on a heated plate, typically between 54 and 58°C. An experimenter measures the time it takes for the mouse to jump off of the hot plate. In the tail immersion test, the mouse is immobilized and its tail is placed into a warm water bath, typically also between 54 and 58°C. An experimenter measures the time it takes for the mouse to remove its tail from the water bath.

Δ^9 -THC and other CB₁ agonists are able to induce suppression of locomotor activity, hypothermia, immobility in the ring test and antinociception in the tail immersion test or hot-plate test (Howlett et al. 2002). Tetrahydrocannabinol produces these “tetrad effects” with a potency (half-maximal effective dose = 1-1.5 mg/kg intravenous) that is consistent with its CB₁ receptor affinity. Moreover, the involvement of CB₁ receptors in all “tetrad effects” is supported by findings that these are readily antagonized by the selective CB₁ receptor antagonist, SR141716A (Rimonabant[®]), and that most of these effects are not produced by Δ^9 -THC in mice from which the CB₁ receptor has been genetically deleted (Pertwee 2008).

There is evidence to suggest that the activity of a partial agonist on a receptor is particularly influenced by the density and the coupling efficiencies of this receptor (Newman-Tancredi et al. 2000, Kenakin 2001). In other words, a partial agonist can antagonize agonists that possess higher receptor efficacy when these are administered exogenously or released endogenously. Because Δ^9 -THC has low cannabinoid receptor efficacy, and since both the density and coupling efficiencies of CB₁ receptors vary widely within the brain and in different species, several studies show that Δ^9 -THC can behave as an antagonist both *in vitro* and *in vivo* at the CB₁ receptor (Pertwee 2008). For example, it has been shown that Δ^9 -THC can oppose R-(+)-WIN55212-induced stimulation of guanosine-5'-O-(3-thiotriphosphate) ([³⁵S]GTP γ S) binding to rat cerebellar membranes (Sim et al. 1996). *In vivo*, Δ^9 -THC shares the ability of the CB₁ antagonists, SR141617A and AM251, to induce signs of anxiogenic activity in a mouse model in which CP55940 and R-(+)-WIN55212 displayed anxiolytic-like activity (Patel, Hillard 2006). Besides, it can attenuate inhibition of glutamatergic synaptic transmission induced in rat or mouse cultured hippocampal neurons by R-(+)-WIN55212 or 2-arachidonoylglycerol (Pertwee 2008).

Δ^9 -THC can also produce antagonism at the CB₂ receptor (Bayewitch et al. 1996). Thus, this compound (0.01-1 μ M) has been found to exhibit partial agonism in COS-7 cells transfected with human CB₂ (hCB₂) receptors when the measured response was inhibition of cyclic AMP production stimulated by 1 μ M forskolin. Instead, Δ^9 -THC behaved as a CB₂ receptor antagonists in this bioassay when incubated in combination with the agonist, HU-210, displaying an apparent K_B value of 25.6nM (Bayewitch et al. 1996). Another group have found that Δ^9 -THC (1 μ M) shares the ability of the CB₂-selective antagonist, SR144528, to abolish 2-arachidonoylglycerol-induced migration of human leukemic natural killer cells (Kishimoto et al. 2005). This dual partial agonism/antagonism exerted by Δ^9 -THC has a strong therapeutic impact for disorders in which the pathology increases the expression of the cannabinoid receptors, or the production and release of endogenous cannabinoids (Pertwee 2005). This potential therapeutic aspect will be evaluated with more emphasis in the next paragraphs.

PHARMACOLOGY OF Δ^9 -THC AT NON-CB₁, NON-CB₂ RECEPTORS

Some of the phytocannabinoids investigated so far have been found to interact with non-CB₁ and non-CB₂ receptor targets, amplifying the therapeutic potential of these natural compounds.

As shown in Table 4, Δ^9 -THC has been found to interact with several non cannabinoid receptors, targeting the orthosteric or allosteric site of other GPCRs, transmitter-gated channels, ion channels or nuclear receptors (Pertwee 2010).

For example, Δ^9 -THC has been found to bind the allosteric sites of opioid receptors. Thus, in equilibrium binding experiments with rat whole brain membranes tetrahydrocannabinol was found to interact in a non-competitive/allosteric manner with μ and δ opioid receptors (Vaysse, Gardner & Zukin 1987). In the same paper, Δ^9 -THC was shown to inhibit binding to μ opioid receptors with an IC₅₀ of 7 μ M. Corroborating results came several years later when another group found that in rat brain cerebral cortical membranes, Δ^9 -THC could accelerate the dissociation of [³H]DAMGO and [³H]naltrindol, presumably from μ and δ opioid receptors,

respectively. This effect was induced by Δ^9 -THC only at high concentrations (Table 3) and was no more than two-fold (Kathmann et al. 2006).

GPR55 is an additional GPCR that has been found to be activated by Δ^9 -THC. The potency and the efficacy exerted by this compound versus GPR55 are very controversial and depend on the samples and the assays used to evaluate the receptor activity (Pertwee 2010).

Δ^9 -THC acts as quite a potent antagonist on the ionotropic receptor 5-HT₃, which belongs to the serotonergic receptor family. 5-HT₃ receptors are located prevalently in the central and peripheral neurons, where they trigger rapid depolarization due to the opening of non-selective cation channels (Na⁺, Ca⁺⁺ influx, K⁺ efflux) (Hannon, Hoyer 2008). In particular, Δ^9 -THC has been found to modulate the 3A subunit of the human 5-HT₃ receptor. In HEK293 cells transfected with this receptor subunit, Δ^9 -THC was able to inhibit 5-HT_{3A}-mediated currents induced by 5-HT (serotonin), with an IC₅₀ of 38 nM (Table 3) (Barann et al. 2002).

Moderate potency has been shown by Δ^9 -THC versus glycine receptors, where this phytocannabinoid can potentiate receptor activation, possibly in an allosteric manner (Pertwee 2010). Glycine receptors are widely distributed in the central nervous system, where they mediate inhibitory transmission predominantly in the spinal cord and brain stem. Thus, it has been found that Δ^9 -THC can potentiate glycine-activated currents in both homomeric $\alpha 1$ and heteromeric $\alpha 1\beta 1$ subunits of human glycine receptors that had been transfected into *Xenopus laevis*, and in native glycine receptors expressed by neurons obtained from the ventral tegmental area of the rat brain (EC₅₀ = 115 nM) (Table 3) (Hejazi et al. 2006).

Δ^9 -THC has also been shown to interact with some ion channels, in particular T-type Calcium (Ca_v3) and Potassium Voltage (K_v1.2) channels. In both these channels Δ^9 -THC exerts inhibition with an IC₅₀ in the micromolar range (Table 3) (Pertwee 2010). Additional Δ^9 -THC-target receptors are Transient Receptor Potential (TRP) channels, which are a family of ion channels with relative non-selective permeability for cations, including sodium, calcium and magnesium. In particular, Δ^9 -THC has been found to activate both TRP Vanilloid-2 (TRPV2) and TRP Ankirin-1 (TRPA1) channels, with an EC₅₀ in the micromolar range (Table 3) (Pertwee 2010).

Finally, Δ^9 -THC has been shown to interact with PPAR γ (O'Sullivan et al. 2005). In detail, Randal's group found that Δ^9 -THC (10 μ M) can cause slowly developing

vasorelaxation in rat isolated aorta in a manner that can be antagonized by the selective PPAR γ antagonist, GW9662, but not by the cannabinoid CB $_1$ receptor antagonist, AM251. They also found that GW9662 antagonizes Δ^9 -THC-induced relaxation in rat superior mesenteric arteries and that at concentrations of 100 nM and above, Δ^9 -THC activates PPAR γ in HEK293 cells expressing this receptor (Pertwee 2010, O'Sullivan et al. 2005). PPAR γ , also known as the glitazone receptor, is a nuclear receptor which regulates fatty acid storage and glucose metabolism by controlling the expression of specific target genes involved in adipogenesis, inflammatory responses, and lipid metabolism.

Receptor or Channel	Effect	Concentration
μ opioid	Displacement	IC $_{50}$ = 7 μ M
μ opioid	Dissociation	EC $_{50}$ = 21.4 μ M
δ opioid	Dissociation	EC $_{50}$ = 10 μ M
β -adrenoreceptor	Potentialiation	3 & 10 μ M
5-HT $_{3A}$	Antagonism	IC $_{50}$ = 38 nM
glycine (α 1)	Potentialiation	EC $_{50}$ = 86 nM
glycine (α 1 β 1)	Potentialiation	EC $_{50}$ = 73 nM
Glycine	Potentialiation	EC $_{50}$ = 115 nM
T-type calcium (Ca $_v$ 3) channels	Inhibition	1 μ M
Potassium K $_v$ 1.2 channels	Inhibition	IC $_{50}$ = 2.4 μ M
TRPA1	Activation	20 μ M
TRPA1	Activation	400 μ M
TRPV2	Activation	EC $_{50}$ = 16 & 43 μ M
PPAR γ	Activation	300 nM

Table 3. List of G protein-coupled receptors, transmitter-gated channels, ion channels and nuclear receptors which have been proven to interact with Δ^9 -tetrahydrocannabinol. Table adapted from (Pertwee 2010).

REPORTED THERAPEUTIC EFFECTS OF Δ^9 -THC

Since the emerging role of the endocannabinoids in a wide variety of physiological and pathophysiological processes, Δ^9 -THC has been tested so far in different disorders, demonstrating some therapeutic effects.

Brain related disorders. Neuroprotection. Δ^9 -THC has been shown to exert protection in several models of excitotoxicity on neuronal cultures (Costa et al. 2007). Nowadays, excitotoxicity is known to be one of the main pathological processes which occurs during brain injury or diseases, such as spinal cord injury, stroke, traumatic brain injury and neurodegenerative diseases of the central nervous system (i.e. multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, alcoholism or alcohol withdrawal, and Huntington's disease). This phenomenon is caused by an accumulation of glutamate neurotransmission with a consequent hyperstimulation of glutamatergic receptors on neurons, in particular NMDA receptors, whose activation causes a massive influx of Ca^{2+} ions. In turn, the accumulation of Ca^{2+} , as consequence of NMDA over-activation, causes a series of intracellular events which lead to neuronal death by apoptosis (Manev et al. 1989). The presynaptic localization of CB_1 receptors on glutamatergic neurons inhibits the excitatory transmission, suggesting a neuroprotective effect of this cannabinoid receptor against the excitotoxicity process. During this disease state, the potential neuroprotective properties of Δ^9 -THC have been tested in several neuronal models (Costa et al. 2007). In all of these assays a protective effect of Δ^9 -THC has been demonstrated, but unfortunately the results obtained with the Δ^9 -THC treatment, imply a scenario more complicated than that suggested. In fact, in some models, the neuroprotective effects of Δ^9 -THC were blocked by CB_1 receptor antagonists, indicating a cannabinoid receptor-mediated effect; conversely, in other assays the protective action of Δ^9 -THC was not CB_1 receptor mediated, suggesting an antioxidant role of this phytocannabinoid (Costa et al. 2007). Moreover, the efficacious concentration of this compound was very close to its toxic effects in some

models, possibly due to an over-activation or desensitization of CB₁ receptors. In summary, the potential protective role and the exact mechanism of Δ⁹-THC action in this scenario have yet to be fully clarified, but open up the possibility of discovering potential new drugs with more selective activity.

Multiple Sclerosis. This neurodegenerative disease is characterised by the demyelization of axons in the brain and spinal cord, with a consequently progressive deterioration of neurological and bodily functions.

In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, Δ⁹-THC showed efficacy in improving the signs of MS, notably causing delayed onset and survival greater than 95% (Lyman et al. 1989). In the same model, a more stable and less psychoactive Δ⁹-THC analogue, Δ⁸-THC, was found to reduce the incidence and severity of the neurological deficit (Wirguin et al. 1994). These data suggested an efficacy of Δ⁹-THC and analogues in the treatment of EAE and encouraged researchers to test these ligands in patients affected by MS.

In the last 10 years several systematic, placebo-controlled, clinical trials have been conducted with the cannabis extract, Δ⁹-THC as oral medication (dronabinol), nabilone or CBME (cannabis based medicinal extract, i.e. Sativex). The results obtained with the pure Δ⁹-THC or its analogue were controversial: in a few cases, patients reported an improvement in spasticity, muscles spasms, pain, sleep quality, tremors, and general condition; on the other hand, no improvements were reported by other patients treated with Δ⁹-THC. Conversely, clinical trials conducted with CBMEs produced a statistically and clinically significant reduction in spasticity (Costa et al. 2007, Smith 2010). However, it is still not clear which therapeutic advantages CBMEs will offer over conventional medications.

The exact mechanisms underlying the reduction in cannabinoid-induced spasticity remain to be fully understood. It has been suggested that an inhibition of the excitatory transmission (glutamatergic receptors and potassium channels) through activation of pre-synaptic CB₁ receptors in the CNS, in particular the spinal cord, could be responsible for these effects (Fujiwara, Egashira 2004).

Parkinson's Disease. Parkinson's disease is one of the most common progressive neurodegenerative disorders, and is characterise by the loss of dopaminergic neurons, particularly in the *substantia nigra pars compacta*. In 2005, Fernandez-Ruiz and co-workers reported a beneficial effect of Δ⁹-THC in a Parkinson's disease animal model. In this model, Δ⁹-THC treatment was shown to rescue the neurodegeneration

induced by injection of the toxin, 6-hydroxydopamine (Lastres-Becker et al. 2005). The same neuroprotective effect was exerted by the non-psychoactive phytocannabinoid, CBD, suggesting a more probable antioxidant effect, instead of a CB₁ receptor-mediated mechanism.

A clinical trial was conducted in United Kingdom using 19 patients suffering from Parkinson's disease and levodopa-induced dyskinesia. In this trial the oral administration of a cannabis extract, composed of 2.5 mg of Δ^9 -THC and 1.25 mg of CBD, failed to show any objective or subjective improvement in parkinsonism or dyskinesia (Carroll et al. 2004).

Cancer. Antitumoral effects. The first study showing the anti-cancer effects of tetrahydrocannabinol was conducted by Munson et al. in 1975. Both Δ^9 -THC and Δ^8 -THC were able to inhibit the growth of Lewis lung carcinoma cells *in vitro* and *in vivo* after oral administration to mice (Munson et al. 1975). Since then, Δ^9 -THC and other phytocannabinoids have been tested in various cancer types (lung, glioma, thyroid, lymphoma, skin, pancreas, uterus, breast and prostate carcinoma) using both *in vitro* and *in vivo* models. The resulting anti-cancer properties of Δ^9 -THC in these models imply an anti-proliferative, anti-metastatic, anti-angiogenic and pro-apoptotic effect of this drug, suggesting the involvement of several pathways (Alexander, Smith & Rosengren 2009).

Recently, the first clinical trial has been reported, where 9 patients affected by glioblastoma multiforme were administered an intratumoral injection of Δ^9 -THC. The results showed that the intracranial administration of Δ^9 -THC was safe and did not result in any obvious psychoactive effects. Moreover, Δ^9 -THC was reported to inhibit the proliferation of the tumours *in vitro*, and reduce the Ki-67 immunostaining in two of the treated patients, confirming the anti-proliferative effect of this drug in cancer cells (Guzman et al. 2006).

Although promising anti-cancer effects of Δ^9 -THC and other cannabinoids have been reported, there are a few studies which have shown a biphasic effect of Δ^9 -THC in cancer cells, where lower concentrations result in an increase in proliferation of cancer cells and higher concentrations cause a decrease in cell proliferation (Alexander, Smith & Rosengren 2009).

Together, these results highlight the importance of further studies to truly establish the anti-cancer effects of Δ^9 -THC.

Palliative care in cancer. One of the best established effects of Δ^9 -THC in the clinic is its anti-nausea and anti-emetic properties in cancer patients. Thus, the synthetic Δ^9 -THC, dronabinol, and its synthetic analogue, nabilone, are approved in different countries for this purpose (Table 1). Two possible mechanisms have been suggested to explain the anti-emetic effects of Δ^9 -THC. In particular, the location of CB₁ receptors in cholinergic nerve terminals of the myenteric plexus of the stomach, duodenum, and colon are suggested to be responsible for the Δ^9 -THC -induced inhibition of digestive-tract motility. It has also been suggested that the presence of CB₁ receptors in the brainstem might have a role in Δ^9 -THC -induced control of emesis (Di Carlo, Izzo 2003).

Despite the encouraging results from clinical trials conducted in the early 1980's, the usage of this drug has been eclipsed by the severe incidence of side effects and the development of more-effective anti-emetic drugs (Costa et al. 2007).

Other Δ^9 -THC -palliative applications include the anti-anorexic properties of this drug. This effect is supported by the well-established role of endocannabinoids system in feeding behaviour in several animal models, where the expression of CB₁ receptors, in particular brain regions, nerve terminals, and adipocytes, might have a crucial role in the feeding behaviour produced by Δ^9 -THC.

Δ^9 -THC finds application as anti-anorexic drug not only in cancer, but also in patients affected by AIDS. In fact, dronabinol has been licensed for use in the USA for this purpose (Costa et al. 2007).

Analgesic Effects. Δ^9 -THC has been shown to have anti-nociceptive abilities in a wide range of assays, in particular in both acute (e.g. tail-flick and hot-plate tests) and tonic (e.g. stretching) nociceptive tests (Costa et al. 2007). The involvement of CB₁ receptors in Δ^9 -THC -induced antinociception is well established. In fact, CB₁ receptors are located in tissues associated with the transmission and processing of nociceptive information (spinal cord, thalamus, periaqueductal grey, rostro-ventromedial medulla, dorsal-root ganglia, afferent-fiber terminals) (Tsou et al. 1998). Moreover, the CB₁ receptor antagonist, SR141716A, was found to prevent the anti-nociceptive effects of Δ^9 -THC. Additional evidence comes from CB₁ knock-out mice, in which during the hot plate test, the antinociceptive effects of Δ^9 -THC were completely abolished. Furthermore, in the warm-water tail withdrawal test, the effects of Δ^9 -THC were strongly reduced. However, CB₁ receptors are not the only target

involved in Δ^9 -THC -induced anti-nociception. In fact, in the tail flick test using CB₁ knock-out mice, Δ^9 -THC retained the ability to induce antinociception, indicating the involvement of other targets (Zimmer et al. 1999).

Concerning neuropathic pain, only one study has been conducted in an animal model for this disease, showing an anti-hyperalgesic effect of Δ^9 -THC. Unfortunately when Δ^9 -THC was orally administered to patients suffering from neuropathic pain, no improvements in pain, or in quality of life were reported (Costa et al. 2007).

A possible important therapeutic application of Δ^9 -THC might be the treatment of cancer pain. In fact, chronic cancer pain has a nociceptive component, which originates from inflammatory reactions around the sites of injury, and a neuropathic pain component, which results from damage to the nervous system. Δ^9 -THC has been tested with this purpose in some clinical trials, showing effectiveness as analgesic drug (Costa et al. 2007).

Unfortunately the data collected so far are insufficient to support the introduction of Δ^9 -THC as analgesic drug.

MATERIALS AND METHODS

CHEMICALS

All reagents were purchased from SIGMA-ALDRICH® (Poole, UK), unless otherwise stated.

Cannabidiol and tetrahydrocannabivarin were supplied by GW Pharmaceuticals (Porton Down, Wiltshire, UK) and SR144528 was obtained from Sanofi-Aventis (Montpellier, France).

R-(+)-[2, 3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo-[1, 2, 3-de]-1,4 benzoxazin-6-yl]-1-naphthalenylmethanone (*R*-(+)-WIN55212), (–)-*cis*-3-[2-hydroxy 4-(1,1-dimethylheptyl) phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940), maprotiline hydrochloride and *R*-(+)-8-hydroxy-2-(di-*n*propylamino) tetralin (8-OH-DPAT) were purchased from Tocris (Bristol, UK).

For the binding experiments, [³H]CP55940 (160 Ci/mmol), [³H]8-OH-DPAT (180 Ci/mmol) and [³⁵S]GTPγS (1250 Ci/mmol) were obtained from PerkinElmer Life Sciences Inc. (Boston, MA, USA), GTPγS, adenosine deaminase and Roche Protease Inhibitor Cocktail from Roche Diagnostic (Indianapolis, IN, USA).

In all the assays, drugs were applied at 0,1% DMSO (v/v)

BINDING STUDIES

Introduction

Binding experiments are based on the incubation of a sample containing the receptor of interest and a labelled ligand for this receptor in controlled time, temperature and pH conditions.

During the incubation, part of the ligand X is able to bind the receptor R, resulting in the complex RX formation (Figure 7).

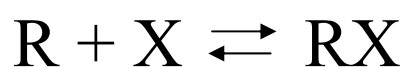


Figure 7. The formation of the Complex RX is determined by an equilibrium reaction between the receptor R and the compound X.

The incubation is followed by filtration and washing which allow the separation of the complex RX from the unbound ligand X. The radioactivity of the complex RX is then quantified by a scintillation liquid. From the elaboration of the radioactivity data is possible to obtain specific parameters (i.e. K_i , B_{max} , ...).

The ligand can be an agonist or antagonist at the receptor in question and must be labelled with a radioisotope at high specific activity (usually ^3H or ^{125}I) to ensure a sensitive assay.

The incubation time is one of the key factors for the reaction, a competitive or displacement binding assay is carried out when the equilibrium of the complex RX is reached. Conversely, the incubation time is variable in the kinetic studies where other parameters are considered (i.e. half time of the RX dissociation). In addition, radioligands may also bind to other sites, as well as the receptor of interest. The binding to the receptor of interest is called *specific binding*, whereas the binding to the other sites is called *non-specific binding* (Figure 8).

Non-specific binding is usually (but not necessarily) proportional to the concentration of radioligand (within the range it is used). *Non-specific binding* is detected by measuring radioligand binding in the presence of a saturating concentration of an unlabeled drug that binds to the receptors.

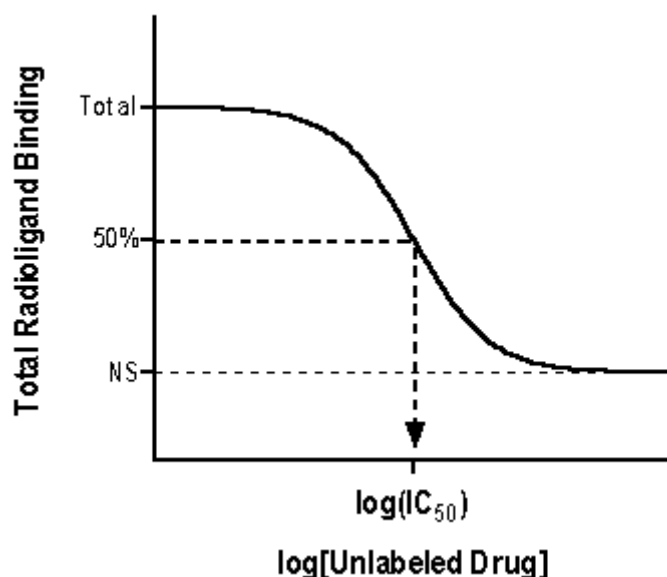


Figure 8. The graph represents a competitive binding assay. The top of the curve is a plateau at a value equal to radioligand binding in the absence of the competing unlabeled drug. The bottom of the curve is a plateau equal to *non-specific binding* (NS). The concentration of unlabeled drug that produces radioligand binding half way between the upper and lower plateaus is called the IC_{50} (inhibitory concentration 50%) or EC_{50} (effective concentration 50%).

COMPETITIVE OR DISPLACEMENT BINDING ASSAY

Competitive binding experiments measure the binding of a single concentration of labeled ligand in the presence of various concentrations of unlabeled ligand.

In a competitive binding assay, both the radioligand and the unlabelled compound can compete for the orthosteric site of the receptor, which is the effective site responsible of the receptor activation. In this case, the unlabelled ligand will produce 100% of displacement of the unlabelled compound (Figure 9A). Competition binding experiments allow the calculation of the K_i , which is the equilibrium dissociation constant and is a measure of the affinity of the unlabeled ligand for the receptor in question (Cheng, Prusoff 1973). By definition, the K_i is the concentration of the competing ligand that will bind to half the binding sites at equilibrium, in the absence of radioligand or other competitors. A ligand binding with a K_i of 1 nM or less is generally considered to have high affinity for its receptor whereas ligands binding with K_i of 1 μ M have low affinity.

However the unlabelled compound can bind to the allosteric binding site, which is a regulatory receptor binding site. In this case, the displacement of the radioligand from its receptor will be incomplete and it is possible to observe different effects (Figure 9B). In fact, the binding of the unlabelled compound to the allosteric site can decrease or increase the binding of the radioligand to the orthosteric site (Figure 9B).

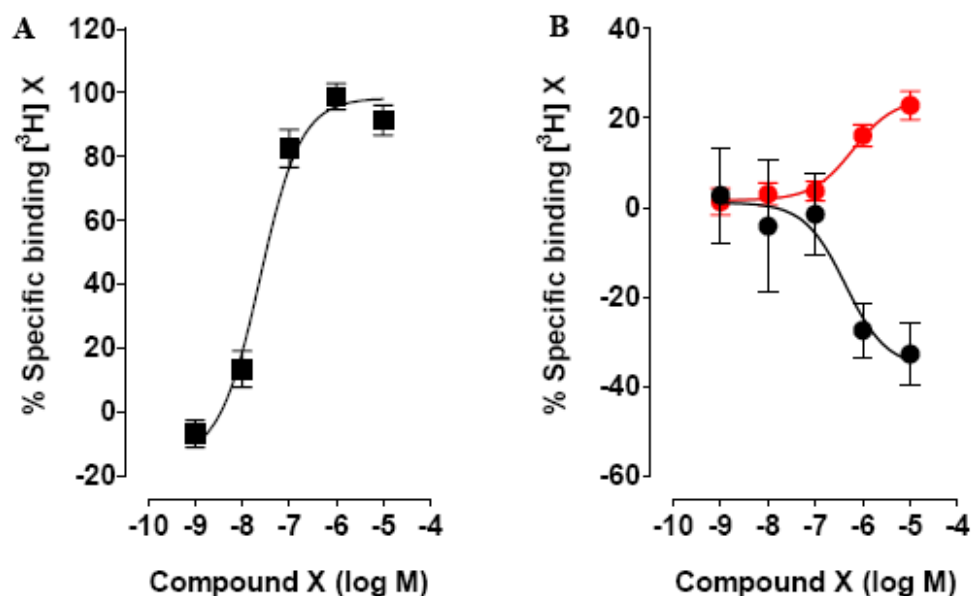


Figure 9. A) Increasing concentrations of compound X displaces [³H]X from specific binding sites of tissues or cells. B) Compounds which do not bind directly to the orthosteric site can produce a partial displacement of [³H]X (shown in black symbols), or even enhance binding of [³H]X to the orthosteric site (red symbols).

Method-procedure

The assays were carried out with 0.7 nM of [³H]CP55940 or [³H]8-OH-DPAT and Tris-binding buffer (50 mM Tris-HCl, 50 mM Tris-base, 0.1% BSA, pH 7.4) in a total assay volume of 500 μL. Binding was initiated by the addition of transfected hCB2-CHO cells (50 μg proteins per well or 31250 whole cells per well), mouse whole brain membranes (33.3 μg of proteins per well) or rat brainstem membranes (500 μg protein per well). All assays were performed at 37°C for 60 min before termination by the addition of ice-cold Tris-binding buffer and vacuum filtration using a 24-well sampling manifold (Brandel Cell Harvester) and Brandel GF/B filters that had been soaked in wash buffer at 4°C for at least 24 h (Brandel Inc, Gaithersburg, MD, USA). Each reaction well was washed six times with a 1.2 mL aliquot of Tris-binding buffer. The filters were oven dried for 60 min and then placed in 5 mL of scintillation fluid (Ultima Gold XR, PerkinElmer). Radioactivity was quantified by a liquid scintillation counter (Tri-Carb 2800TR, Perkin Elmer). Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 μM unlabelled CP55940 or 8-OH-DPAT. The vehicle concentration in all assay wells being 0.1% DMSO.

DISSOCIATION KINETIC ASSAY

Allosteric modulation is classically defined as the binding of a ligand to a site which is topographically distinct from the orthosteric site. Binding to the allosteric site of the receptor can induce a conformational change of the receptor that can enhance or decrease the affinity of ligands for the orthosteric site. Similarly the conformational change can modify the efficacy of the response produced by the orthosteric ligand. The dissociation kinetics assay measures the rate at which a single concentration of radioligand dissociates from the orthosteric site of the receptor. A dissociation rate constant which deviates to that of a control performed in the presence of an unlabelled

ligand is indicative of allosteric modulation (Motulsky, Mahan 1984). Allosteric modulators have been found for many GPCRs, such as muscarinic acetylcholine receptors, dopamine D2 receptors and metabotropic glutamate receptors (reviewed in (Gao, Jacobson 2006). An allosteric site has also been discovered on the CB1 receptor (Price et al. 2005).

Method-procedure

Dissociation kinetic assays were performed with the 5-HT_{1A} receptor agonist [³H]8-OH-DPAT (0.7 nM) and Tris-binding buffer, total assay volume 500 µl. We used the “isotopic dilution” method to measure the dissociation rate constant for [³H]8-OH-DPAT from brain stem membranes (Price et al. 2005, Christopoulos 2001). [³H]8-OH-DPAT (0.7 nM) was incubated with rat brain stem membranes (500 µg protein per well) for 60 min at 25°C. Dissociation was initiated by the addition of 1 µM unlabeled ligand in the presence and absence of test compounds. Dissociation times of 0.5 to 120 min at 25°C were used. To determine the nonspecific binding, experiments were also performed in the presence of a 1 µM concentration of the unlabeled ligand. Binding was terminated by addition of ice-cold wash buffer (50 mM Tris-HCl, 50 mM Tris-base, and 0.1% BSA) followed by vacuum filtration method.

[³⁵S]GTPγS BINDING ASSAY

The [³⁵S]GTPγS binding assay is a functional assay which measures the levels of G protein-coupled receptor (GPCR) activation. The principle of this assay exploits the physiology of GPCRs which normally occurs upon activation of the receptor by a ligand (Figure 10).

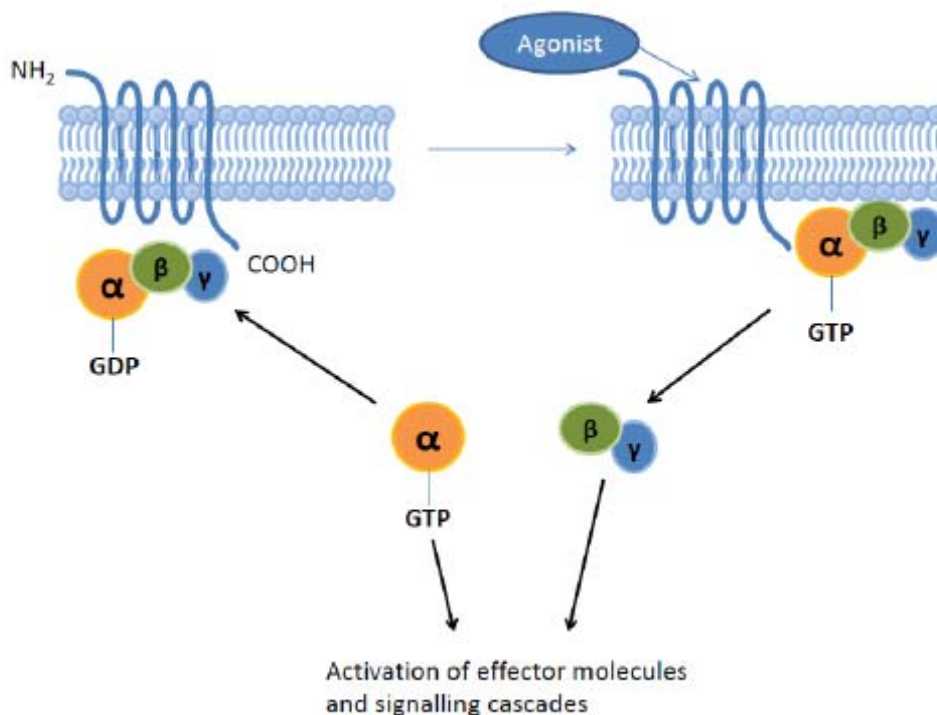


Figure 10. All GPCRs are similar in structure, consisting of a single peptide containing seven hydrophobic α -helices separated by hydrophilic regions which form intracellular and extracellular loops. When an extracellular ligand binds to a GPCR it alters the conformation of domains of the seven transmembrane helix pocket, which consequently causes a change in conformation of the intracellular domains of the receptor. Receptors interact with G proteins through the COOH terminal and intracellular loops. G proteins are in a heterotrimeric complex with a $G\alpha$ protein associated with a $\beta\gamma$ complex. The conformational change in the receptor caused by agonist binding leads to a promotion of exchange of GDP bound in the $G\alpha$ active site to GTP. The binding of GTP causes dissociation of the G protein heterotrimeric complex. The GTP bound α subunit and the $\beta\gamma$ subunit then regulate the activities of intracellular effector molecules downstream.

In the assay, [^{35}S]GTP γ S replaces endogenous GTP and binds to the $G\alpha$ subunit following activation of the receptor to form a $G\alpha$ -[^{35}S]GTP γ S species. Since the γ -thiophosphate bond is resistant to hydrolysis by the GTPase of $G\alpha$, G protein is prevented from reforming as a heterotrimer and thus [^{35}S]GTP γ S labelled $G\alpha$ subunits accumulate and can be measured by counting the amount of ^{35}S -label incorporated (Harrison, Traynor 2003). Because the $G\alpha$ subunit remains associated with the membrane this is simply done by filtering the preparation, and counting the radioactivity retained on the filters. The assay is functional and can show if a compound is behaving as an agonist or antagonist. Moreover, [^{35}S]GTP γ S binding

assay can give a direct measure of potency (EC_{50}), efficacy (E_{max}) and antagonist affinity (K_B), for test compounds, by plotting sigmoidal dose-response curves (Harrison, Traynor 2003).

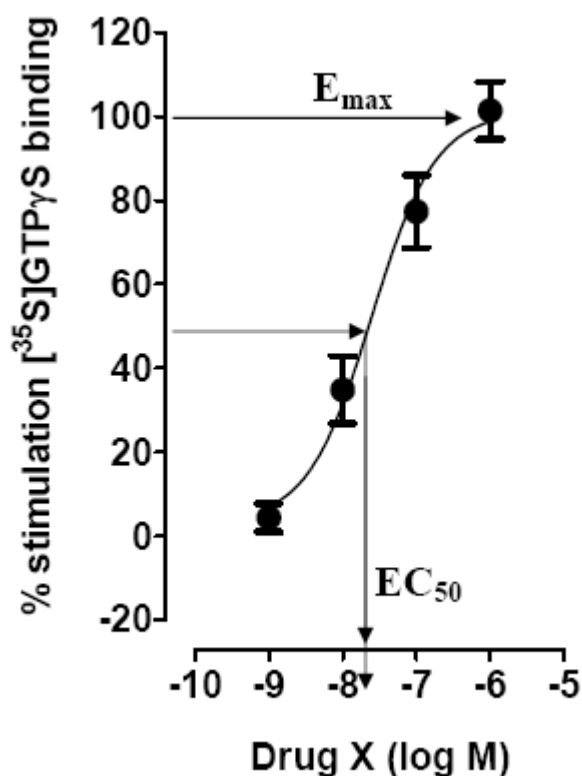


Figure 11. A hypothetical sigmoidal dose-response curve produced by Drug X in the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay. From this curve it is possible to extrapolate the mean E_{max} (maximal efficacy) and EC_{50} (potency) values.

The E_{max} represents magnitude of the maximal response or in other words the maximal possible effect of a compound (Figure 11). The EC_{50} is the potency of a test compound and is given by the concentration that is required to produce 50% of the maximal response produced by the drug (Figure 11). Full agonists are drugs with high efficacy which will elicit the maximum response that is capable of the tissue in question even at low levels of receptor occupancy or with using low concentrations of the drug. Drugs with intermediate levels of efficacy may not elicit a full response even if all of the target receptors are occupied, these drugs are termed partial agonists (Figure 12).

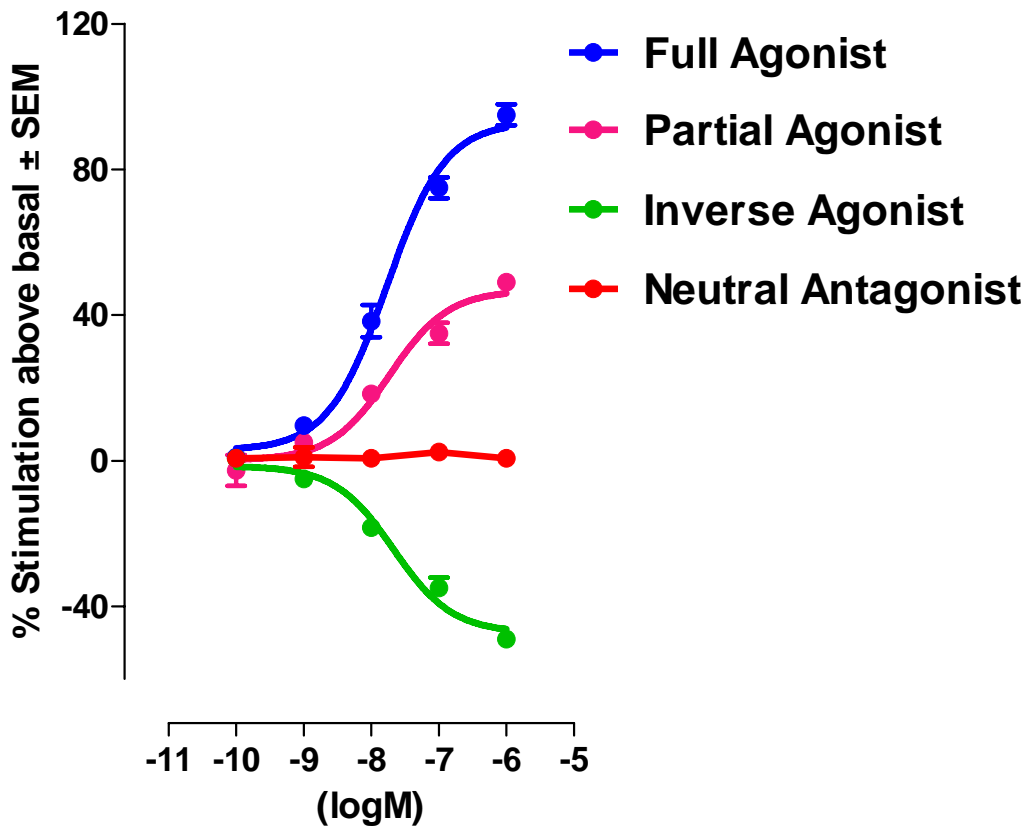


Figure 12. An illustration of typical effects seen in response to the incubation of different types of ligand in the [³⁵S]GTPγS binding assay.

Inverse agonists behave in the opposite manner to agonists and are said to have negative efficacy, they are characterised by their ability to decrease levels of [³⁵S]GTPγS binding below that of the basal levels. This is possible when the receptor is in a constitutively active state (“on” state) in the absence of any ligand (Figure 13). This aspect will be discussed in more detail in the second chapter.

Neutral antagonists have affinity for the receptor but do not stimulate levels of [³⁵S]GTPγS binding (Figure 12).

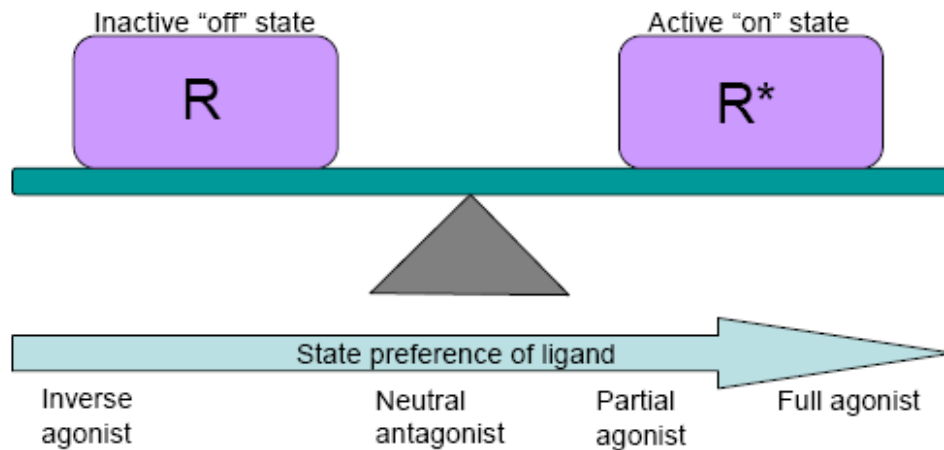


Figure 13. The two state model of receptor activation depicting how different ligands have different affinities for the two receptor states.

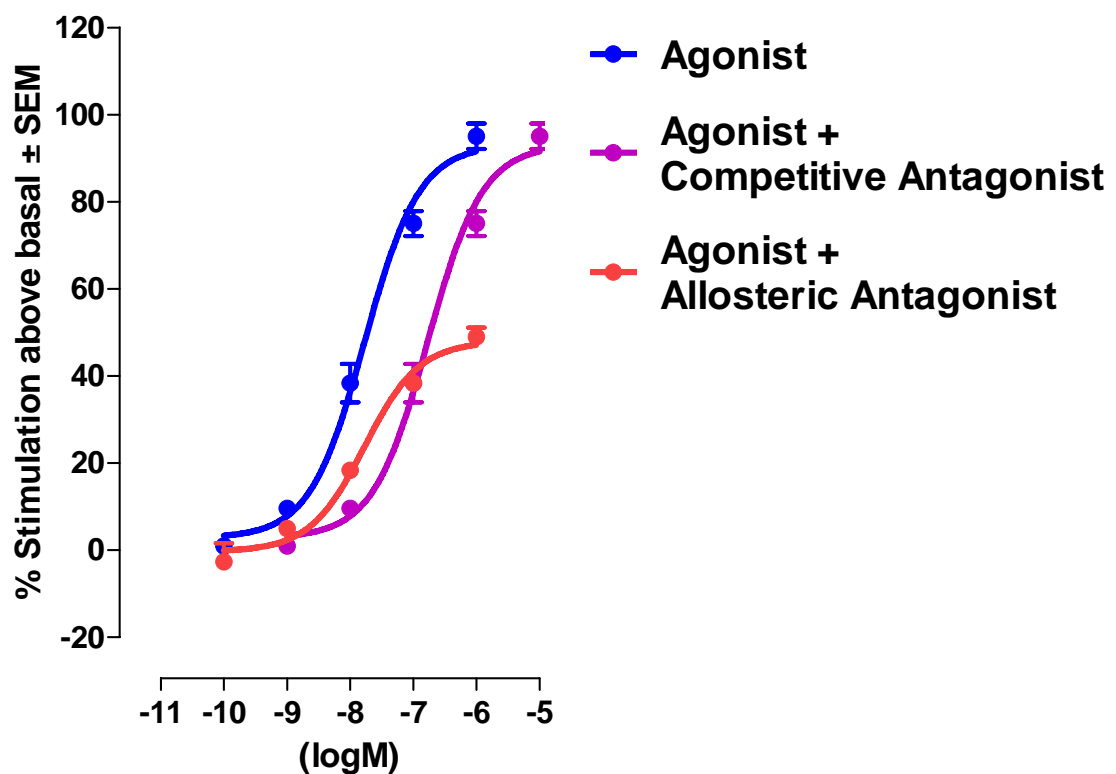


Figure 14. The differences that may be observed in levels of [³⁵S]GTP γ S activation by competitive or allosteric antagonists.

Antagonists can also be tested in the [³⁵S]GTPγS assay. When antagonism is competitive in nature a parallel rightward shift of the plotted dose response curve is observed (Figure 14). If the plotted curve appears to hinge downwards, this could indicate that the antagonism is non competitive, possibly because the level of [³⁵S]GTPγS binding being induced by the agonist is being modulated allosterically. Alternatively if an antagonist has inverse agonistic properties (Figure 12) at the concentration at which it is used as an antagonist then a downwards displacement may be observed (Thomas et al. 2007).

Method-procedure

Brain and brainstem membranes were thawed at room temperature, 500 μl of adenosine deaminase (ADA) added (0.75 units per ml), and then membranes were diluted up to 1.5 ml by adding binding buffer (Table 4) and incubated for 30 min in a 30°C water bath.

GDP (10 mM) was added to binding buffer (Table 4) in order to obtain a final concentration of 30 μM of GDP. Then, 350 μl of this solution was added into 24 wells of a 96 well plate, and 50 μl of cold GTPγS (final assay concentration 30 μM), vehicle control (0.1% (v/v) DMSO), or test compounds were added in duplicate into each well. 50 μl of 1 nM [³⁵S]GTPγS was then added to each well to give a final assay concentration of 0.1 nM, followed by 10 μg (brain membranes), 100 μg (brainstem membranes) or 50 μg (cell membranes) proteins per well. Non-specific binding (NSB) was measured using 30 μM of cold GTPγS. The assay was incubated for 1 hour at 30°C, and then filtered by rapid vacuum filtration using a 24-well sampling manifold, onto GF/B filters washed with ice-cold wash buffer (50 mM Tris-HCl, 50 mM Tris-Base and 0.1% (w/v) BSA, pH 7.4). The filters were oven dried for 60 min, soaked in 5 ml scintillation fluid (Ultima Gold XR, PerkinElmer) and the amount of [³⁵S]-label quantified using a liquid scintillation counter.

BINDING BUFFERS	Binding buffer for brain and brain stem membranes	Binding buffer for cell membranes
Tris-HCl	50 mM	50 mM
Tris-Base		50 mM
NaCl	100 mM	100 mM
MgCl ₂	3 mM	5 mM
EGTA	0.2 mM	
EDTA		1 mM
DTT		1 mM
BSA (fatty acid free)	0.1% (w/v)	0.1% (w/v)

Table 4. Composition of binding buffers, pH 7.4.

CELL CULTURE

Cells were cultivated in a sterile incubator (Galaxy S CO₂ incubator, RS Biotech) at 37°C in humidified air containing 5% CO₂. All culture procedures were performed in a sterile laminar flow hood using instruments sterilised with 70% ethanol and plasticware supplied in sterile package, which were opened within the hood.

Cell Lines

CHO (Chinese Hamster Ovary) cells either untransfected or transfected with cDNA encoding human cannabinoid CB₂ or CB₁ receptors were maintained in Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM, supplemented with 1 mM L-glutamine, 10% fetal bovine serum and 0.6% penicillin–streptomycin for all cells together with G418 (400 mg·mL⁻¹) for the hCB₂- and hCB₁-CHO cells.

Cell Maintenance

Cells were grown in monolayer and passaged twice weekly when they reached a state of 80% confluence. In summary, media was discarded and dead cells were removed by washing once with 10 ml of PBS (Phosphate Buffered Saline). After removal of the PBS, cells were then dissociated from the flask by adding 5 ml of non enzymatic

cell dissociation fluid (Cell Dissociation Solution Non-enzymatic 1X). Cells were left to incubate at 37 °C for 2 minutes in the cell dissociation fluid, and then detached by tapping the side of the flask. The dissociation fluid containing the cells was then diluted with equal amount of medium and mixed well to obtain a homogeneous cellular suspension. For passage into a medium flask (75 cm²), 1 ml of resuspended cells (approximately 1.5 millions of cells) was added to 25 ml of media. For a large flask (175 cm²), 2 ml of resuspended cells were added to 50 ml of media.

PTX-treatment

Pertussis toxin (PTX) disrupt the function of G_{i/o}-coupled receptors (Howlett, Qualy & Khachatrian 1986), and can be used to implicate G_{i/o}-coupled receptors in agonist-activated responses.

The PTX stock powder was diluted with distilled water at the concentration of 100 ng/μl. This stock solution was then diluted 1000X (100 ng/ml) in complete media. Cells grown in large flasks were washed once with 10 ml of PBS and incubated overnight with 20 ml of media containing PTX (100 ng/ml) at 37°C. Prior using the cells for experiments, cells were washed once with PBS.

MEMBRANES PREPARATION

Since the present investigation focused on some small brain regions, in order to obtain a high amount of proteins from the sample processing we decided to modify the commonly used method (“Old” Processing Method) in this laboratory. Therefore, we set up a new method for processing brain and brain regions (“New” processing method).

Animals

All mice and rats were provided with free access to food and water and were in an environment with 12/12h light/dark cycle. MF1 mice or Lister Hooded rats were obtained from Harlan UK Ltd (Blackthorn, UK).

Brain and brain regions dissection

Mice or rats were killed by CO₂ exposure followed by rapid dislocation. The brain was then collected, washed rapidly in cold HEPES buffer (Table 5) and frozen down in dry ice. To isolate the different brain regions, the brain was washed in HEPES buffer, kept on a cold support and dissected with curved forceps (Micro-serrations forceps, MORIA MC31). The brain regions were then frozen down in dry ice. The tissues were moved to -80°C and stored for no more than 3 months.

HEPES buffer	Concentration (mM)	g/500ml of DDW
NaCl	130	3.7985
KCl	5.4	0.2015
CaCl ₂	1.8	0.1325
MgCl ₂	1	0.1015
HEPES	10	1.1915
D-Glucose	25	2.252

Table 5. HEPES buffer was prepared 10X without D-Glucose, pH 7.4, autoclaved and stored at 4°C for 2-3 months. The day of the experiment, the buffer was diluted up to 1X and D-Glucose was added to the solution.

DDW (double distilled water)

“Old” Processing Method for brain

The procedure was set up for 4 mouse brains,

1. 4 brains were thawed on ice and placed in 2 tubes containing 5 ml of ice cold centrifugation buffer (Table 6).
2. Brains were homogenised using a polytron homogeniser.
3. The two tubes were centrifuged at RCF 1600 (rotor number 12158) for 10 minutes at 4°C.
4. The resulting supernatant was saved on ice and the pellet was resuspended in 5 ml of centrifugation buffer.
5. The two tubes with resuspended pellets were centrifuged as before in step 3.
6. Again the supernatant was kept and combined with the supernatant from step 4. The large pellet consisting mostly of blood was discarded.

7. The two tubes of supernatant were centrifuged at RCF 28000 for 20 minutes at 4°C.
 8. The resulting supernatant was discarded and the small pellet in each tube was resuspended in 20 ml of buffer A (Table 6).
 9. The 2 tubes were incubated in a water bath at 37°C for 10 minutes.
 10. The 2 tubes were then centrifuged at RCF 23000 for 20 min at 4°C.
 11. The supernatant was discarded and the pellet in each tube was resuspended in 20 ml of buffer A again and incubated at room temperature for 40 minutes.
 12. The 2 tubes were centrifuged at RCF 11000 for 15 minutes, at 4°C.
 13. The supernatant was discarded and the combined pellets from both tubes were resuspended in a total volume of 2 ml of buffer B (Table 6).
 14. The membrane preparation was homogenised using a glass hand-held homogeniser.
 15. The concentration of proteins was determined using a BioRad protein assay kit.
- Brain membranes to be used for displacement binding assay the membranes were stored in 1 ml aliquots of 1 mg/ml.
16. Aliquots of membranes were stored at -80°C.

“New” Processing Method for brain and brainstem

1. The brain and/or brainstem were thawed and homogenized on ice in processing buffer (Table 7) using a glass hand-held homogeniser (5 ml of processing buffer for 1 rat brain or 2 mouse brains, and 5ml of processing buffer for 3 rat brainstems).
2. The samples were then transferred into 1.5 ml tubes and centrifuged at 10.000 rpm for 15 min at 4°C.
3. After centrifugation, the resulting supernatants were discarded and the pellets were frozen down to -80°C for at least 2 h (to enhance the breaking process of the membranes).
4. The pellets were then thawed, resuspended in 300 µl/pellet of TME buffer (Table 7) and homogenized on ice using a hand motor mixer (VWR[®] pellet mixer).
5. The resulting membranes were then collected and the protein concentration was assessed using a BioRad protein assay kit.

6. The membranes were diluted in TME buffer (Table 7) at different concentrations depending on the sample and the assay of interest (Table 8).
7. The aliquots were stored at -80°C and used within 3 months.

Centrifugation Buffer	Concentration (mM)	g/500ml of DDW
Tris HCl	2	0.16
Tris Base	2	0.12
EDTA	2	0.37
MgCl ₂	5	0.24
Sucrose	320	54.77

Buffer A	Concentration (mM)	g/500ml of DDW
Tris HCl	50	3.94
Tris Base	50	3.03
EDTA	2	0.37
MgCl ₂	5	0.24
NaCl	100	2.92

Buffer B	Concentration (mM)	g/500ml of DDW
Tris HCl	50	3.94
Tris Base	50	3.03
EDTA	1	0.19
MgCl ₂	3	0.14
NaCl	100	2.92

Table 6. This Table summarizes all the buffers and their respective components used in the old processing method for brain. The pH in all the buffers is 7.4.

Choi Lysis Buffer	Concentration (mM)	g/500ml of DDW
Tris HCl	20	1.57
EGTA	0.5	0.095
EDTA	0.2	0.037
Sucrose	320	54.77

Processing Buffer	Concentration	5ml of solution
Choi Buffer		4825 μ l
Roche protease inhibitor cocktail	1:40 v/v	125 μ l
PMSF	1 mM	50 μ l

TME Buffer	Concentration (mM)	g/500ml of DDW
Tris HCl	50	3.94
EDTA	1	0.19
MgCl ₂	3	0.14

Table 7. This Table summarizes all the buffers and their respective components used in the old processing method for brain. Choi lysis Buffer pH is 7.5, TME buffer pH is 7.4.

PMSF (phenylmethylsulphonyl fluoride)

Sample	Assay	Concentration ($\mu\text{g}/\mu\text{l}$)	Aliquots (μl)	Amount of proteins (μg /well)
mouse/rat brain	$[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding	10	30	10
mouse/rat brain	Displacement binding	1	1000	33.3
rat brainstem	$[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding	10	300	100
rat brainstem	Displacement binding	10	1500	500
rat brainstem	Dissociation/association binding	10	1500	500

Table 8. This Table summarizes the sample concentrations in each assay.

PROTEIN ASSAY

Unknown protein concentrations were determined by generating a standard curve using a set of standards of known protein concentrations, starting from a stock of concentration of 1.45 mg/ml bovine serum albumin (BSA) (Table 9).

500 μl of reagent A (Bio-Rad Dc kit; Hercules, CA, USA) was added to each tube and vortexed, then 4 μl of reagent B (Bio-Rad Dc kit) added to each tube and vortexed. Tubes were incubated at room temperature for 15 min and absorbance read at 750 nm using a Novaspec III absorbance reader (Amersham Biosciences).

The known protein amount of standards was plotted against their absorbance reading (nm) using Prism 5. Data were analyzed using a linear regression analyses and the unknown value were extrapolated from the straight line originated from the standard points.

	1	2	3	4	5	Unknown
BSA (μl)	100	75	50	25	0	20 μ l of sample
Tris Buffer (μl)	0	25	50	75	100	80
Protein amount (μg)	145	107.50	72.50	36.25	0	X

Table 9. Generation of protein standard curve and calculating an unknown protein concentration. Tris buffer (50 mM Tris-HCl, 50 mM Tris-base), pH 7.4.

CYCLIC AMP ASSAY

The cyclic AMP (adenosine monophosphate) assay is a functional assay which measures the levels of the second messenger cyclic AMP. Cyclic AMP is synthesized from ATP (adenosine triphosphate) by the enzyme adenylyl cyclase located on the inner side of the plasma membrane. Adenylyl cyclase is activated by a range of signaling molecules through the activation of adenylyl cyclase stimulatory G (G_s) protein-coupled receptor and inhibited by agonists of adenylyl cyclase inhibitory G ($G_{i/o}$) protein-coupled receptor.

Since cyclic AMP is a second messenger the receptor-signaling is more amplified and consequently, assays based on cyclic AMP detection are more sensitive than [35 S]GTP γ S binding assay (Pertwee 1999).

In this investigation the cyclic AMP assays were first carried out with a kit based on radioactive cyclic AMP detection and purchased by *GE Healthcare* (Amersham cyclic AMP [3 H] assay kit). The production of this kit was interrupted in September 2009 and the cyclic AMP assays were then carried out with a kit based on luminescence technology and purchased by *DiscoverRx* (HitHunter cyclic AMP assay kit).

These experiments were performed in adherent native CHO (Chinese Hamster Ovary) cells or CHO cells overexpressing human CB1 receptor (hCB1-CHO cells) or human CB2 receptor (hCB2-CHO cells).

Importantly, since cannabinoids receptors are preferentially coupled to $G_{i/o}$ proteins, to detect a response upon activation of these receptors is essential that the basal levels of cyclic AMP in the cells are high. For this purpose, cells were treated with forskolin (SIGMA), a direct activator of adenylate cyclase, which is able to increase the intracellular cyclic AMP levels.

Amersham cyclic AMP [^3H] assay kit

The assay is based on the competition between unlabelled cyclic AMP (produced by the cells) and a fixed quantity of the tritium labeled cyclic AMP for binding to a protein which has a high specificity and affinity for cyclic AMP. The amount of labeled protein-cyclic AMP complex formed is inversely proportional to the amount of the unlabelled cyclic AMP present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cyclic AMP in the sample to be calculated.

Cells were grown in large flasks (175 cm^2) for 3-4 days and collected the day of the experiment by washing once with 10 ml of cold PBS and detached using 10 ml non-enzymatic cell dissociation solution. Cells were then collected and centrifuged at 1200 rpm (revolutions per minute) for 5 min.

After centrifugation, cells were resuspended (2×10^6 cells/ml) in assay buffer containing PBS (calcium and magnesium free), 1% BSA (bovine serum albumin) and 10 mM of rolipram (SIGMA), a phosphodiesterase inhibitor which blocks the hydrolysis of cyclic AMP. Experiments were carried out in 1.5 ml tubes containing 250 μl of resuspended cells, 190 μl of assay buffer and 50 μl of the cannabinoid under investigation and incubated for 30 min in a water bath at 37°C . In antagonism studies, the antagonist was pre-incubated with cells and buffer first for ten minutes before the addition of the cannabinoid. In this case only 140 μl of buffer was used to account for the extra 50 μl of antagonist.

A further 30 min incubation was carried out with 10 μl of forskolin (10 μM for native CHO and hCB2-CHO cells, or 5 μM for hCB1-CHO cells) in a total volume of 500 μL . The reaction was terminated by the addition of 500 μl HCl (0.1 M) and incubated for 15 min at room temperature in order to break the cellular membranes. The cellular lysate was then centrifuged at 14000 rpm for 5 minutes to remove cell debris. 200 μl of the supernatant was removed and 11 μl of NaOH (1 M) was added to bring the pH

to 8-9. 50 μ l of each sample and cyclic AMP standards were then transferred to 1.5 ml tubes, and 50 μ l of [3 H] cyclic AMP and 100 μ l of binding proteins were added. The tubes were then vortexed and incubated on ice for 2 hours. After incubation, 100 μ l of charcoal solution (supplied with the kit) was added to each tube, followed by vortex and centrifugation at 14000 rpm for 5 min at 4°C. Finally, 200 μ l of the supernatant was removed and 3 ml of scintillation liquid (Ultima Gold XR, PerkinElmer) was added. The samples radioactivity was measured using a liquid scintillation counter and the data were expressed as percentage of inhibition of cyclic AMP production.

HitHunter cyclic AMP assay kit

This assay is based on enzyme fragment complementation (EFC) technology which uses two fragments of *E. coli* β -galactosidase (β -gal): a large protein fragment (enzyme acceptor, EA) and a small peptide fragment (enzyme donor, ED). Separately, these fragments are inactive, but in solution they rapidly complement (recombine) to form active β -gal enzyme.

In this assay, cyclic AMP from cell lysates and ED-labeled cyclic AMP (ED-cyclic AMP) compete for antibody binding sites. Unbound ED-cyclic AMP is free to complement EA to form active enzyme, which subsequently produces a luminescent signal. The amount of signal produced is proportional to the amount of cyclic AMP in the cell lysate.

Cells were detached using cell dissociation buffer, counted and seeded at 2×10^4 cells per well in 100 μ l of complete medium onto white 96-well plates with clear bottom, and incubated at 37°C and 5% CO₂ for approximately 24 hours before running the experiment. In some experiments hCB₂ CHO cells were preincubated with 10 mM of AM630 for up to 24 hours and then subjected to intense washing, 6 times for 10 min each, with complete medium (Mancini et al. 2009). The assays and the drug dilutions were performed in a 1:1 mixture of DMEM and Ham's F12 medium without phenol red (D-MEM / F-12 Media), containing 10 μ M of rolipram and forskolin. Before running the assay, the medium was discarded and cells were washed once with D-MEM/F-12 Media. Then, cells were treated with the assigned drugs (30 μ l per well) and incubated for 30 min at 37°C and 5% CO₂. Finally, cyclic AMP standards and the appropriate mixture of kit components were added (as described by the manufacturer, DiscoverX). Plates were incubated overnight at room temperature in the dark.

Chemiluminescent signals were detected on a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Data were expressed as percentage of inhibition of cyclic AMP production.

STATISTICAL ANALYSIS

Values are expressed as mean and variability as standard error mean (SEM) or as 95% confidence limits (CLs).

The concentration of the compounds under investigation that produced a 50% displacement of radioligand from specific binding sites (IC_{50}) and the corresponding K_i values were calculated using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). EC_{50} and E_{max} values were calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve in GraphPad Prism 5.

The apparent dissociation constant (K_B) values for antagonists were calculated by Schild analysis in GraphPad Prism 5, constraining Schild and Hill slope equal to 1. Therefore, K_B values were calculated only from data obtained in experiments of antagonism in which the rightward shift in the agonist dose-response curve was indicated by (2 + 2) dose parallel line analysis to be statistically significant and not to deviate from parallelism (Pertwee et al. 2002).

CHAPTER ONE

Pharmacological properties of the phytocannabinoid Δ^9 -tetrahydrocannabivarin at cannabinoid receptors

INTRODUCTION

Δ^9 -Tetrahydrocannabivarin (Δ^9 -THCV or THCV) is the *n*-propyl analogue of Δ^9 -tetrahydrocannabinol (Δ^9 -THC). The discovery of this phytocannabinoid was made in 1970 by Edward Gill (Gill, Paton & Pertwee 1970), who detected it in the tincture of cannabis BPC (British Pharmaceutical Codex), then a licensed medicine in UK.

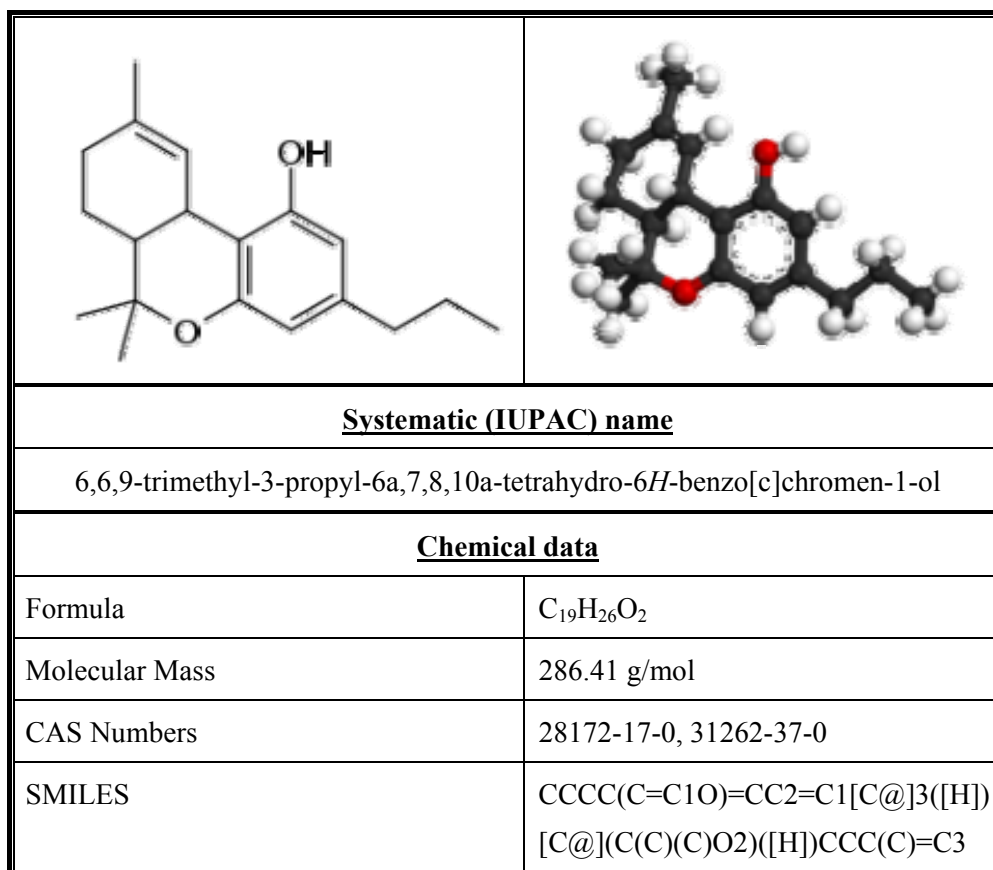


Figure 15. Δ^9 -THCV chemical structure and data.

CAS (Chemical Abstracts Service) registry numbers are unique numerical identifiers for chemical elements, compounds, polymers, biological sequences, mixtures and alloys.

SMILES (simplified molecular input line entry specification) is a specification for unambiguously describing the structure of chemical molecules using short ASCII strings.

Differently from other phytocannabinoids, the biosynthesis of *n*-propyl phytocannabinoids (or their analogues) has been suggested to use a different precursor than olivetol, namely 4-carboxy-5-propylresorcinol ((Russo 2007); see general introduction). Δ^9 -THCV is found in large amounts in *Cannabis indica*, where in some

varieties it can reach a very high percentage of the total cannabinoid content (e.g. 48.23 percent in the Afghanistan strain and 53.69 percent in the South Africa strain) (Turner, Hadley & Ps 1973).

Δ^9 -THCV is metabolized by human hepatocytes to 11-*nor*- Δ^9 -tetrahydrocannabivarin-9-carboxylic acid (THCV-COOH). Since this metabolite is excreted through urines, the content of THCV-COOH in urine specimens has been proposed as a marker to distinguish the ingestion of Marijuana versus Marinol[®] (ElSohly et al. 1999). Recently, a double-blind placebo-controlled trial, conducted with 117 patients, failed in the detection of THCV-COOH content in the urines of 50% of the patients smoking cannabis, suggesting that THCV may not be a sensitive enough measure to detect recent marijuana use. However, the different content of Δ^9 -THCV in cannabis varieties has been suggested as a possible explanation for the lack of THCV detection (Levin et al. 2010).

Pharmacological properties of the phytocannabinoid Δ^9 - tetrahydrocannabivarin

Preliminary pharmacological studies showed that Δ^9 -THCV shares the ability of Δ^9 -THC to induce catalepsy in the mouse ring test, and that it can induce Δ^9 -THC-like effects in humans, although with a potency four or five times less than that exerted by Δ^9 -THC (Gill, Paton & Pertwee 1970, Merkus 1971). These results were later confirmed with synthetic Δ^9 -THCV (O-4394), which has been shown to produce not only cataleptic behaviour in the ring test, but also antinociception in the tail-flick test, confirming the THC-like effects exerted by Δ^9 -THCV (Pertwee et al. 2007). Like Δ^9 -THCV extracted from cannabis (e Δ^9 -THCV), O-4394 exhibited less potency than Δ^9 -THC in these bioassays. Moreover, the anti-nociceptive effect of O-4394 was attenuated by the selective CB₁ receptor antagonist/inverse agonist, SR141716A, suggesting Δ^9 -THCV to be an agonist at CB₁ receptors (Pertwee et al. 2007). This finding was supported by displacement binding experiments on mouse whole brain membranes, where CB₁ receptors are higher expressed than CB₂ receptors. In these assays, both e Δ^9 -THCV and O-4394 were able to displace the non-selective cannabinoid receptor agonist, [³H]CP55940, from specific binding sites, with K_i values of 75.4 nM and 46.6 nM, respectively (Thomas et al. 2005, Pertwee et al. 2007).

While these results show that Δ^9 -THCV acts as agonist at CB₁ receptors *in vivo*, there is also evidence that it can behave as antagonist at these receptors both *in vivo* and *in vitro*. Thus, the administration of O-4394 to mice at doses below those at which it produces signs of CB₁ receptor agonism, showed the ability of this compound to block Δ^9 -THC-induced anti-nociceptive and hypothermia effects, which are thought to be CB₁ receptor mediated (Pertwee et al. 2007). Besides, Δ^9 -THCV has recently been shown to suppress food intake and weight gain in mice (Riedel et al. 2009), an effect shared by the selective CB₁ receptor antagonists, SR141716A and AM251.

In vitro, both O-4394 and Δ^9 -THCV have been found to antagonize CB₁ receptor agonists in a surmountable manner. In detail, both O-4394 and Δ^9 -THCV antagonize CP55940-induced stimulation of [³⁵S]GTP γ S binding to mouse whole brain membranes with mean apparent K_B values (82 and 93nM, respectively) that do not deviate significantly from their K_i values for displacement of [³H]CP55940 from these membranes, suggesting that the antagonism is at CB₁ receptors (Thomas et al. 2005, Pertwee et al. 2007). Moreover, Δ^9 -THCV showed antagonism also in experiments with the mouse vas deferens, a tissue in which cannabinoid receptor agonists are thought to inhibit electrically-evoked contractions by acting on prejunctional neuronal CB₁ receptors to inhibit contractile transmitter release (Pertwee 2008).

Conversely from *in vivo* data, Δ^9 -THCV (O-4394) when tested alone in the [³⁵S]GTP γ S binding assay on mouse whole brain membranes, did not produce any detectable activity (Pertwee et al. 2007). One possible explanation of the apparent discrepancy between the *in vitro* and *in vivo* data could be that Δ^9 -THCV is metabolized *in vivo* to a compound with higher efficacy as a cannabinoid receptor agonist (Pertwee 2008). Given the structural similarities between Δ^9 -THC and Δ^9 -THCV, this hypothesis is supported by two findings:

1. Δ^9 -THC exhibits less potency *in vivo* as a CB₁ receptor agonist than its 11-hydroxy metabolite (Pertwee 2008).
2. Δ^9 -THCV can be metabolized to a 11-hydroxy metabolite (Pertwee 2008).

Additional *in vitro* evidence that Δ^9 -THCV can block the activation of neuronal CB₁ receptors comes from experiments with murine cerebellar slices, in which Δ^9 -THCV has been shown to block CB₁-mediated inhibition of GABA release from basket-cell interneurons caused by R-(+)-WIN55212 (Ma et al. 2008). The same group has also

demonstrated the ability of Δ^9 -THCV to antagonize R-(+)-WIN55212 in both cerebellum and piriform cortex membranes (pA₂ values of 7.62 and 7.44, respectively) (Dennis, Whalley & Stephens 2008).

Interestingly, based on the results observed for Δ^9 -THCV at CB₁ receptors, “GW Pharmaceuticals” started, in 2007, a phase 1 clinical trial on Δ^9 -THCV as a potential drug for treatment of obesity and related metabolic disorders (<http://www.gwpharm.com/Metabolic%20Disorders.aspx>).

Regarding the pharmacology of Δ^9 -THCV on CB₂ receptors, only few experiments have been conducted so far. Specifically, displacement binding assays performed in CHO (Chinese Hamster Ovary) cells transfected with human CB₂ receptor (hCB₂-CHO cells) have shown the ability of Δ^9 -THCV to displace quite potently [³H]CP55940 from specific binding sites, with a K_i value of 62.8 nM. Moreover, in the same cell membranes, Δ^9 -THCV has been shown to antagonize the non-selective cannabinoid receptor agonist, CP55940, in a surmountable manner, with a K_B value of 10.1 nM (Thomas et al. 2005). Further investigations are required to better understand the pharmacology of Δ^9 -THCV at CB₂ receptors.

AIMS OF THE PROJECT

The present work was directed at the pharmacological characterization of Δ^9 -THCV at cannabinoid receptors.

From the few data reported in literature it emerges that there are some controversial *in vitro* and *in vivo* results regarding the pharmacology of Δ^9 -THCV at CB₁ receptors, in particular for its agonistic properties. With the aim of further characterizing Δ^9 -THCV, the present investigation focused on the *in vitro* pharmacology of this phytocannabinoid at CB₁ receptors by using several assays both in mouse whole brain membranes and in CHO cells overexpressing human CB₁ receptors (hCB₁-CHO cells).

It was also of our interest to investigate the pharmacology of Δ^9 -THCV at CB₂ receptors. Specifically, the few data present in the literature prompted us to perform experiments both *in vitro* and *in vivo*.

RESULTS

1) Pharmacological properties of Δ^9 -tetrahydrocannabivarin at CB₁ receptors

Δ^9 -THCV displaces [³H]CP55940 at CB₁ receptors

With the aim of investigating the affinity of Δ^9 -THCV for CB₁ receptors, displacement binding assays on mouse whole brain membranes, which naturally express these receptors, were first carried out. In addition, as already introduced in the Material and Methods section, a new sample processing method had been set up in order to obtain a higher amount of proteins. Hence, with the further aim of validating this new sample processing method, displacement binding experiments on mouse whole brain membranes were performed in parallel with the old and new sample processing protocols. The radioligand [³H]CP55940 was used to occupy CB₁ receptor binding sites and the displacement was quantified as a percentage of specific binding produced in the presence of 1 μ M of the unlabelled ligand CP55940.

	Old protocol	New protocol
<i>Specific Binding</i>	71.5% (\pm 0.3%)	58.3% (\pm 5.9%)
<i>E_{max}</i>	97.6% (95-100.3%)	96.7% (91-102.5%)
<i>EC₅₀</i>	40.9 nM (32.8-50.9 nM)	41 nM (25.7-65.4 nM)
<i>K_i</i>	31.4 nM (25.2-39.1 nM)	31.5 nM (19.7-50.2 nM)

Table 10. The Table reports the mean parameters resulting from the displacement binding assay in mouse whole brain membranes using the two different processing protocols. The labelled and the unlabelled compounds were [³H]CP55940 and Δ^9 -THCV, respectively. The 95% of Confidence Limits (CLs) are shown in brackets.

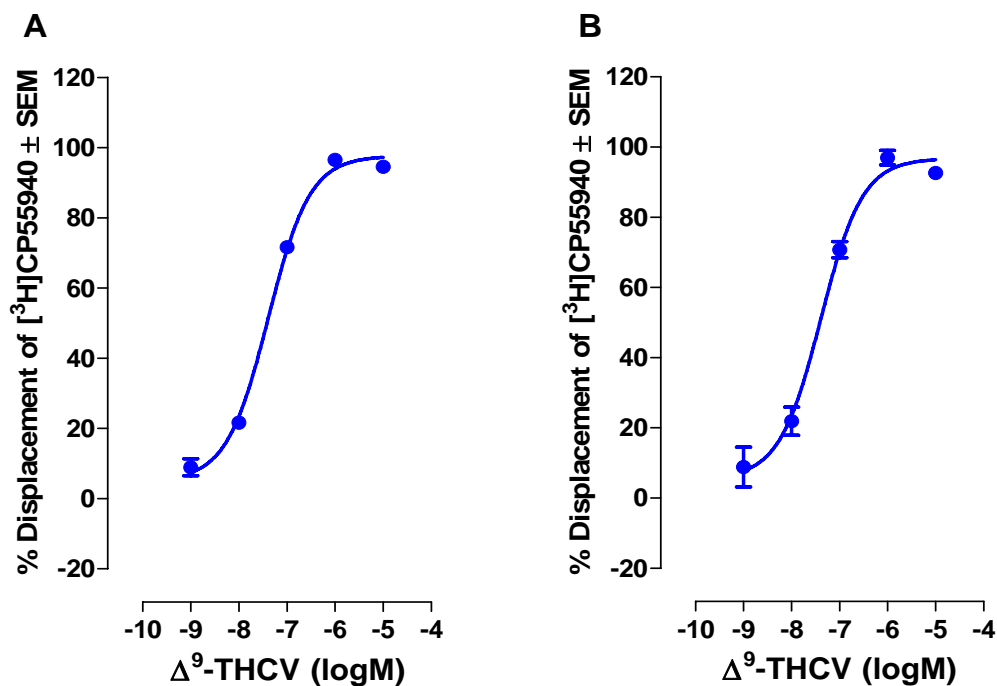


Figure 16. Displacement of [³H]CP55940 by Δ^9 -THCV on mouse whole brain membranes with (A) old protocol (n=4) and (B) new protocol (n=4). Each symbol represents the mean percentage displacement \pm SEM.

As previously published in this laboratory (Thomas, 2005), Δ^9 -THCV fully displaced [³H]CP55940 in mouse whole brain membranes (Figure 16, Table 10), with a moderately strong affinity versus [³H]CP55940 binding sites, as shown by K_i values in the nanomolar range (Table 10). Moreover, no differences were observed between the membranes obtained with the two different processing protocols (Table 10), validating the new assay method.

Since the brain expresses not only CB₁ receptors but also CB₂ receptors, and CP55940 is a non-selective cannabinoid receptor ligand, experiments of displacement binding in hCB₁-CHO cell membranes were then performed.

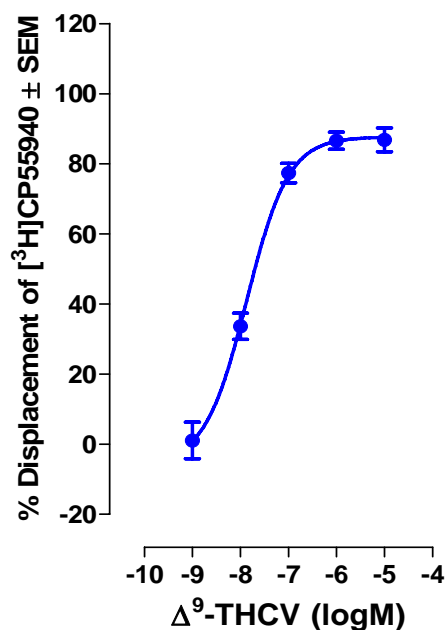


Figure 17. Displacement of $[^3\text{H}]\text{CP55940}$ by Δ^9 -THCV from specific binding sites on hCB_1 -CHO cells ($n=4$). Each symbol represents the mean percentage displacement \pm SEM.

As shown in Figure 17, Δ^9 -THCV fully displaced $[^3\text{H}]\text{CP55940}$ from specific binding sites in hCB_1 -CHO cell membranes with a K_i of 9.5 nM (95% CL 6.1 and 14.8 nM) and an E_{max} of 87.7% (95% CL 82.8 and 92.5%).

Δ^9 -THCV does not stimulate $[^35\text{S}]\text{GTP}\gamma\text{S}$ binding at CB_1 receptors

As previously published in this laboratory, Δ^9 -THCV does not stimulate $[^35\text{S}]\text{GTP}\gamma\text{S}$ binding in mouse whole brain membranes (Thomas et al. 2005). However, as above mentioned, a new sample processing protocol had been set up. Hence, in order to further confirm the activity of Δ^9 -THCV in mouse whole brain membranes, experiments of $[^35\text{S}]\text{GTP}\gamma\text{S}$ binding assay in this tissue were carried out.

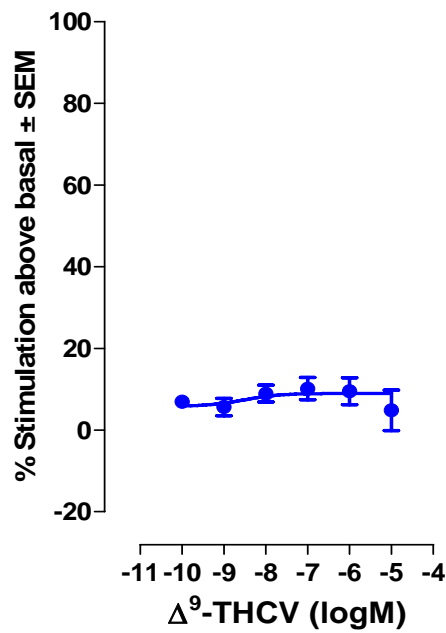


Figure 18. The effect of Δ^9 -THCV on [35 S]GTP γ S binding to whole brain membranes obtained from MF1 mice (n=8). Each symbol represents the mean percentage change in binding \pm SEM. None of the concentrations of Δ^9 -THCV tested were significantly different from zero (one sample t-test analyses).

As shown in Figure 18, Δ^9 -THCV did not display any stimulation on [35 S]GTP γ S binding to mouse whole brain membranes, confirming data already published in literature (Thomas et al. 2005).

However, since the brain expresses different types of GPCRs (G protein-coupled receptors), with the aim of establishing the specific activity of Δ^9 -THCV at CB₁ receptors, we carried out experiments using the [35 S]GTP γ S binding assay in membranes obtained from cells over-expressing the human CB₁ receptor (hCB₁-CHO cells).

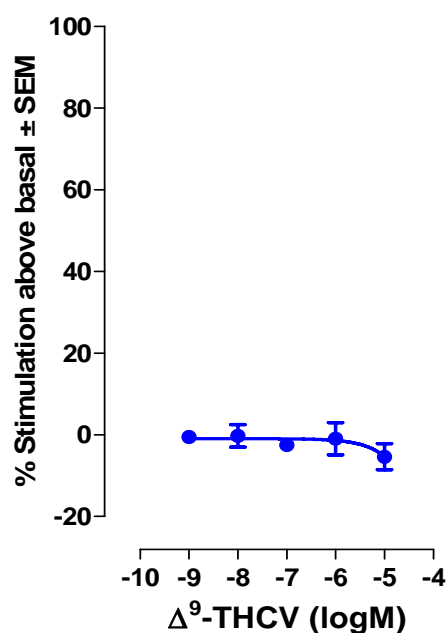


Figure 19. The effect of Δ^9 -THCV on [35 S]GTP γ S binding to hCB $_1$ -CHO cell membranes (n=4). None of the concentrations of Δ^9 -THCV tested are significantly different from zero (one sample t-test analyses).

We found that Δ^9 -THCV did not stimulate [35 S]GTP γ S binding in hCB $_1$ -CHO cell membranes (Figure 19). In addition, Δ^9 -THCV at concentrations of 10 μ M showed a slight, although not statistically significant inverse agonism in these membranes.

Δ^9 -THCV antagonizes CP55940 in the [35 S]GTP γ S binding assay

Further experiments were carried out aimed at investigating the potential antagonistic properties of Δ^9 -THCV at CB $_1$ receptors. As previously published by this group, Δ^9 -THCV has been shown to antagonize the non-selective cannabinoid receptor agonists, CP55940 and WIN55212, in both mouse brain membranes and vas deferens, with K_B values in the nanomolar range (Thomas et al. 2005). To further confirm these results, Δ^9 -THCV was tested at 1 μ M as antagonist of CP55940 in mouse whole brain membranes using [35 S]GTP γ S binding assay.

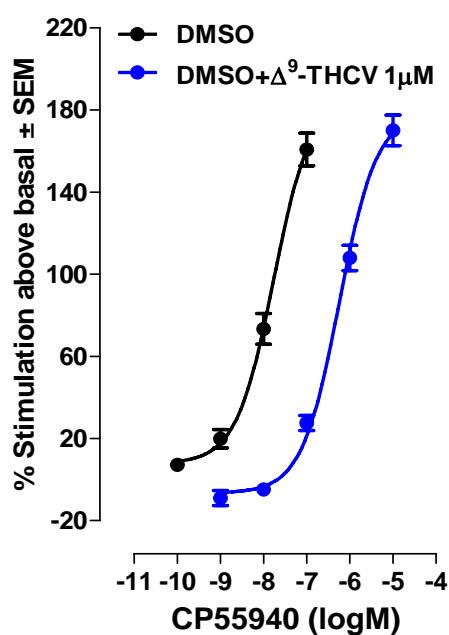


Figure 20. The effect of 1 μM Δ^9 -THCV on the mean log concentration-response curve of CP55940 for stimulation of [^{35}S]GTP γ S binding to mouse whole brain membranes. Each symbol represents the mean percentage change in binding of [^{35}S]GTP γ S to mouse whole brain membranes \pm SEM (n=8). The right-ward shift produced by Δ^9 -THCV in the log concentration-response curve of CP55940 did not deviate significantly from parallelism ($P>0.20$) (2+2 dose parallel line analysis).

The ability of CP55940 to stimulate [^{35}S]GTP γ S binding to mouse brain membranes was attenuated by Δ^9 -THCV (Figure 20), which at 1 μM produced a significant dextral shift in the log concentration-response curve of this cannabinoid receptor agonist that did not deviate significantly from parallelism. The mean apparent K_B value for this antagonism was 22.4 nM (95% CL 16.4 and 34.2 nM), which is very similar to the K_i value obtained from [^3H]CP55940 displacement binding assay in the same membranes (Table 10).

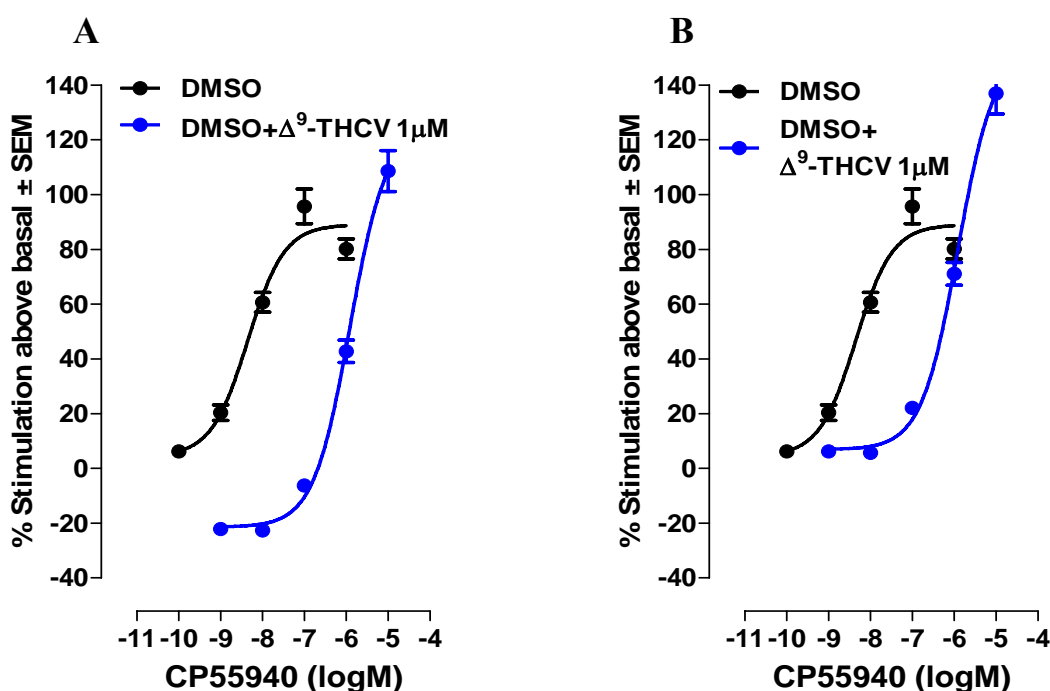
With the aim of elucidating whether the antagonism observed was CB_1 receptor-mediated, Δ^9 -THCV was then tested at 1 μM against CP55940 in the [^{35}S]GTP γ S binding assay using h CB_1 -CHO cell membranes.

The first set of these experiments showed that CP55940-induced stimulation of [^{35}S]GTP γ S binding to h CB_1 -CHO cell membranes was significantly antagonized by Δ^9 -THCV at 1 μM (Figure 21A). However, Δ^9 -THCV at this concentration appeared to produce inverse agonism as displayed by a downward shift in the log concentration-response curve of CP55940. Subtracting the inverse agonism, a

significant rightward shift in the log concentration-response curve of CP55940 was still apparent (Figure 21B). Importantly, the mean apparent K_B value calculated for the rightward shift (Table 11) did not differ significantly from the mean K_i value of Δ^9 -THCV for displacement of [3 H]CP55940 from hCB₁-CHO cell membranes (Table 10).

As shown in Figure 21C and E, Δ^9 -THCV at these concentrations was still able to antagonize CP55940-induced stimulation of [35 S]GTP γ S binding to hCB₁-CHO cell membranes. Again, Δ^9 -THCV appeared to produce both a rightward and a downward shift in the log concentration-response curve of the agonist. Importantly, after compensating for the downward shift (Figure 21D and F), it was found that the mean apparent K_B values did not differ significantly between the different concentrations of Δ^9 -THCV tested, as indicated by an overlap in the 95% confidence limits (Table 11). Besides, none of the mean apparent K_B values differed significantly from the mean K_i value of Δ^9 -THCV for displacement of [3 H]CP55940 from both brain and hCB₁-CHO cell membranes (Figure 16 and 17). Since the rightward shift did not deviate significantly from parallelism for all the concentrations of Δ^9 -THCV tested, it is likely that Δ^9 -THCV is a competitive antagonist at CB₁ receptors.

Interestingly, Δ^9 -THCV at the concentrations of 1 μ M and 100 nM displayed an increase in the E_{max} of the log concentration-response curve of the agonist tested (Figure 21A and C).



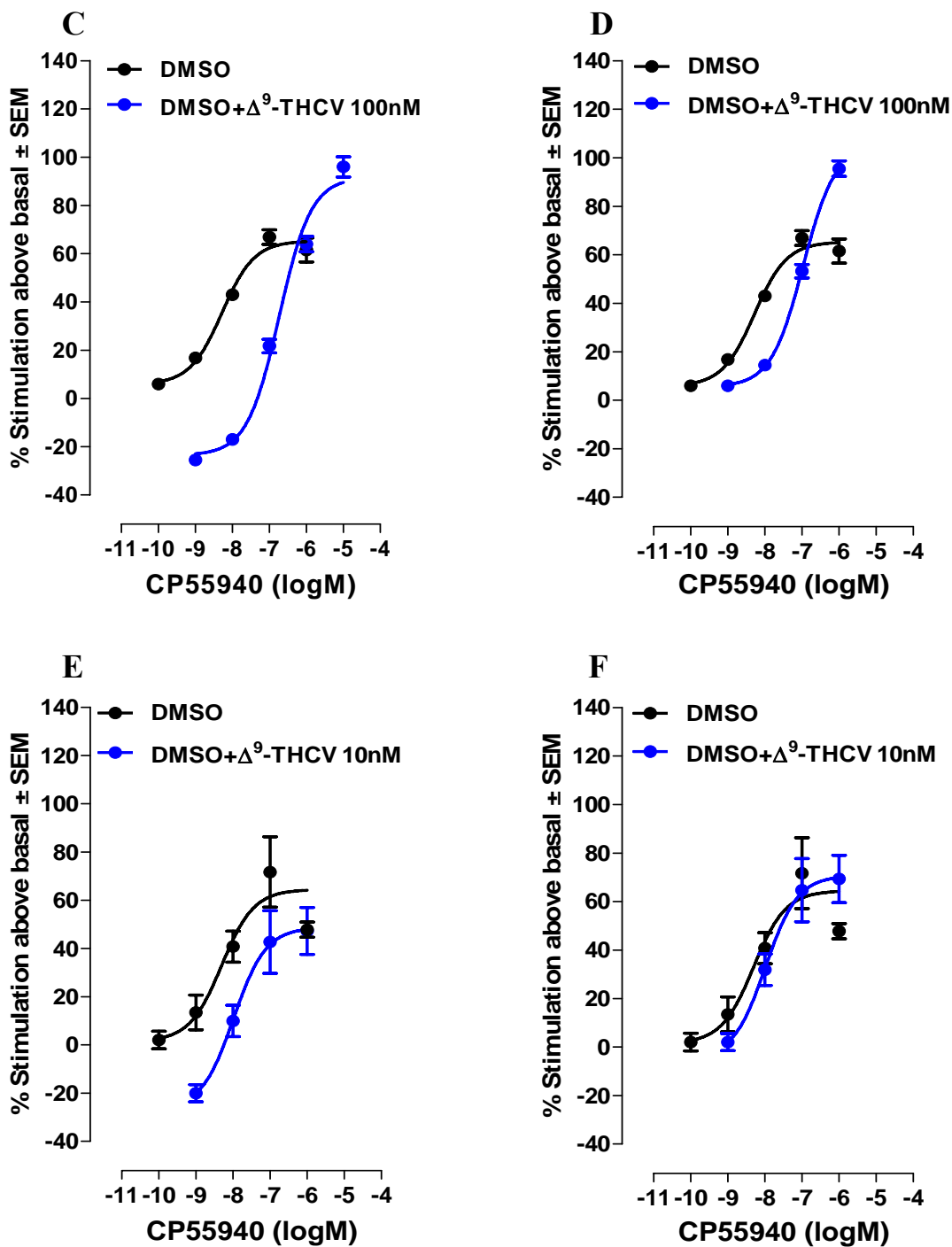


Figure 21. The effects of different concentrations of Δ^9 -THCV (A, C and E) on the mean log concentration-response curve of CP55940 for stimulation of [35 S]GTP γ S binding to hCB $_1$ -CHO cell membranes. The effect of different concentrations of Δ^9 -THCV (B, D and F) on the mean log concentration-response curve of CP55940 for stimulation of [35 S]GTP γ S binding after subtraction of the mean inhibitory effect induced by each concentration of Δ^9 -THCV in the presence of the lowest concentration of CP55940. Each symbol represents the mean percentage change in binding of [35 S]GTP γ S to hCB $_1$ -CHO cell membranes \pm SEM (n=4). The rightward shift produced by all

concentrations of Δ^9 -THCV tested in the log concentration-response curve of CP55940 do not deviate significantly from parallelism ($P > 0.05$ for B and $P > 0.2$ for D and F).

<i>Antagonist concentration</i>	<i>Mean apparent K_B (95% CLs)</i>	<i>Mean apparent K_B (95% CLs)¹</i>	<i>n</i>
Δ^9 -THCV 1 μ M	3.5 nM (1.8 and 6.9 nM)	19.6 nM (8.3 and 46.6 nM)	4
Δ^9 -THCV 100 nM	2 nM (0.9 and 4.8 nM)	67.6 nM (11.8 and 386.7 nM)	4
Δ^9 -THCV 10 nM	0.7 nM (0.2 and 3.3 nM)	9.4 nM (0.76 and 117.5 nM)	4

Table 11. The mean apparent K_B values of Δ^9 -THCV for antagonism of CP55940-induced stimulation of [35 S]GTP γ S binding to hCB $_1$ -CHO cell membranes.

¹Calculated after the subtraction of the mean inhibitory effect induced by each concentration of Δ^9 -THCV in the presence of the lowest concentration of CP55940.

CLs, confidence limits.

Δ^9 -THCV is an inverse agonist at hCB $_1$ receptors in the cyclic AMP assay

As Δ^9 -THCV displayed signs of inverse agonism when tested as an antagonist of CP55940 in the [35 S]GTP γ S binding to hCB $_1$ -CHO cell membranes, it was of our interest to investigate this phytocannabinoid in a more sensitive assay, the cyclic AMP test (Pertwee 1999). We found that, unlike CP55940 (Figure 22B), Δ^9 -THCV yielded an increase on forskolin-induced cyclic AMP production when tested using hCB $_1$ -CHO cells, thus behaving as an inverse agonist at CB $_1$ receptors (Figure 22A). As shown in Figure 23A, the ability of Δ^9 -THCV to enhance forskolin-induced stimulation of cyclic AMP production was not observed in hCB $_1$ -CHO cells when these had been preincubated overnight with pertussis toxin (PTX) in a manner expected to eliminate G $_{i/o}$ -mediated signalling (Bonhaus et al. 1998, Glass, Felder 1997, Coutts et al. 2001). In contrast, the effect of CP55940 on cyclic AMP production switched from an inhibitory to a stimulatory effect in response to such preincubation to pertussis toxin (Figure 23B), presumably reflecting the reported ability of CB $_1$ receptors to activate G $_s$ proteins in the absence of functional G $_{i/o}$ coupling (Howlett et al. 2002, Bonhaus et al. 1998, Glass, Felder 1997).

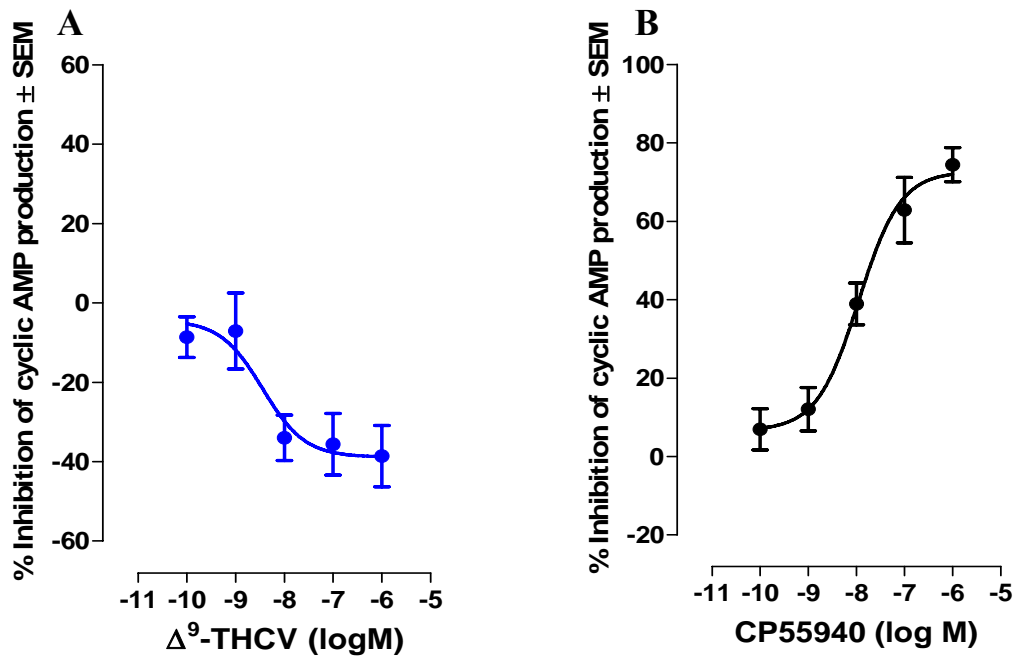


Figure 22. The effect of Δ^9 -THCV (A) and CP55940 (B) on forskolin-induced stimulation of cyclic AMP production in CHO cells transfected with hCB₁ receptor. The mean E_{max} and EC_{50} values of Δ^9 -THCV (A), with 95% CLs shown in brackets, were -38.9% (50.1 and 27.7%) and 3.6 nM (0.3 and 35 nM), respectively. The correspondent values of CP55940 (B) were 72.7% (62.6 and 82.9%) and 11.2 nM (4.4 and 28 nM). The assays were performed using a kit purchased by *GE Healthcare*.

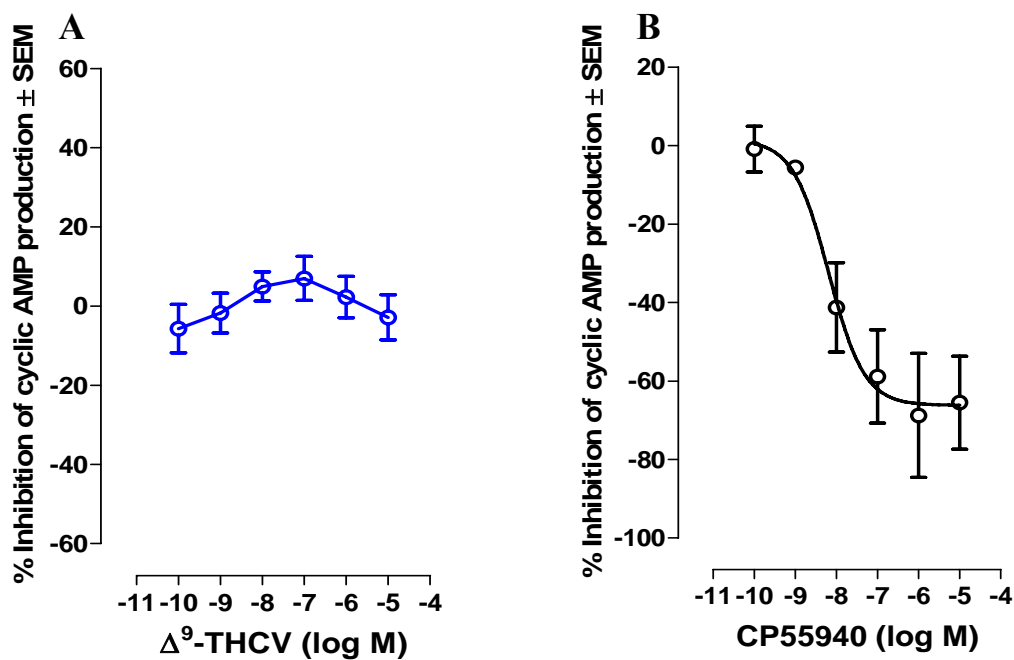


Figure 23. The effect of Δ^9 -THCV (A) and CP55940 (B) on forskolin-induced stimulation of cyclic AMP production in hCB₁-CHO cells preincubated overnight with pertussis toxin (PTX 100 ng/ml). The mean E_{max} value of Δ^9 -THCV (7 %) in A did not differ significantly from zero (one sample t-test

analyses). The mean E_{\max} and EC_{50} values of CP55940 (B), with 95% CLs shown in brackets, were -66.2% (-79.3 and -53.2%) and 6.5 nM (1.4 and 30 nM), respectively. The assays were performed using a kit purchased by *GE Healthcare*.

When cyclic AMP assays were performed in untransfected CHO cells, neither Δ^9 -THCV nor CP55940 produced any detectable effect on forskolin-stimulated cyclic AMP production (Figure 24).

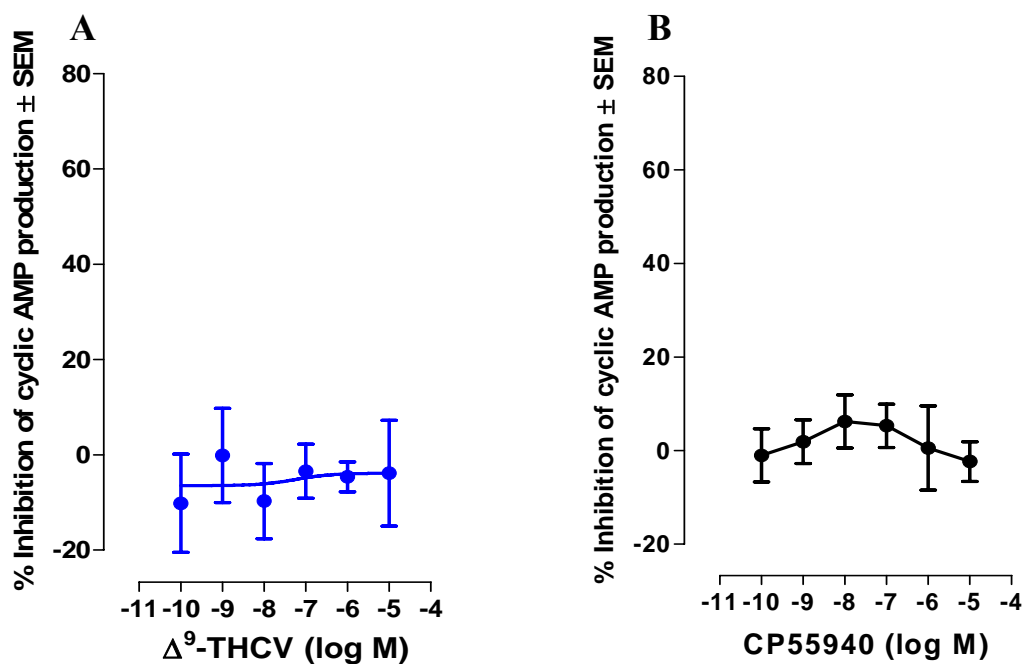


Figure 24. The effect of Δ^9 -THCV (A) and CP55940 (B) on forskolin-induced stimulation of cyclic AMP production in untransfected CHO cells. None of the concentrations of Δ^9 -THCV and CP55940 tested differ significantly from zero (one sample t-test analyses). The assays were performed using a kit purchased by *GE Healthcare*.

2) Pharmacological properties of Δ^9 -tetrahydrocannabivarin at CB₂ receptors

Δ^9 -THCV displaces [³H]CP55940 at CB₂ receptors

In order to assess the affinity of Δ^9 -THCV at CB₂ receptors, experiments of displacement binding were carried out in CHO cells transfected with human CB₂ receptors (hCB₂-CHO cells), using Δ^9 -THCV at concentrations from 1 up to 10000 nM. The displacement was quantified as a percentage of specific binding produced in the presence of 1 μ M of the unlabelled ligand CP55940.

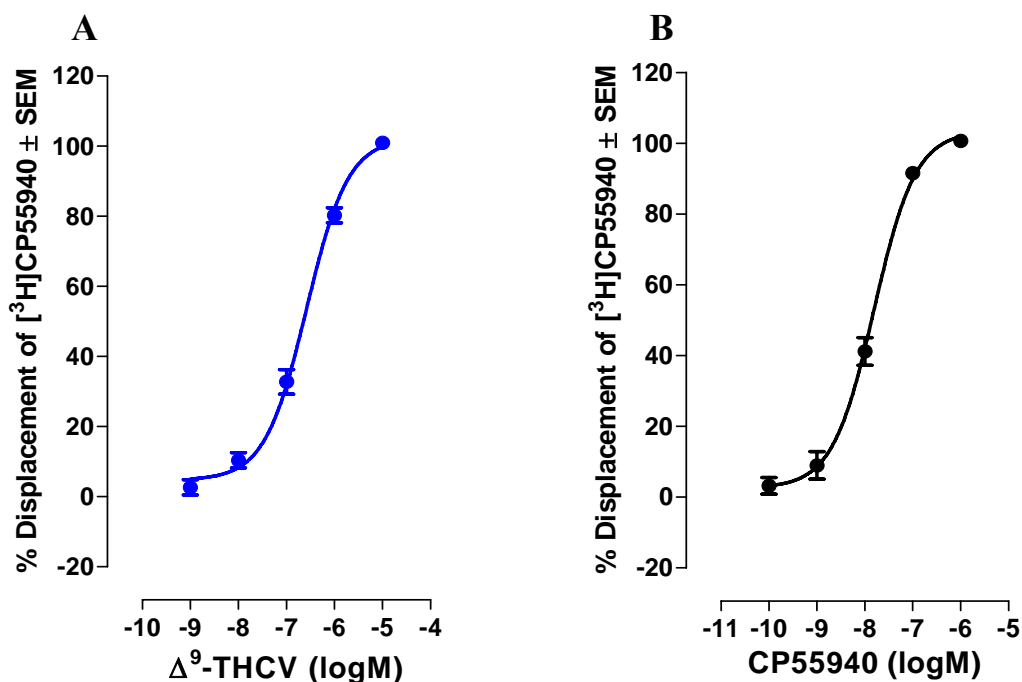


Figure 25. Displacement of [³H]CP55940 by Δ^9 -THCV (A) and CP55940 (B) from specific binding sites on hCB₂-CHO cells (n=8). Each symbol represents the mean percentage displacement \pm SEM.

Δ^9 -THCV was able to completely displace [³H]CP55940 from CB₂ receptor binding sites (Figure 25A), as shown by its E_{\max} of 102.3 % (95% CL 97.5 and 107.1%). The mean K_i value resulting from this displacement was 225.1 nM (95% CL 170.4 and 297.5 nM), which is 3.6 times higher than the mean K_i value reported previously in literature using the same method (Thomas et al. 2005).

In order to better understand the reason underlying this difference, we then carried out experiments looking at [³H]CP55940 displacement in hCB₂-CHO cells using the cannabinoid agonist, CP55940, as the cold ligand (Figure 25B). CP55940 was able to fully displace [³H]CP55940 with E_{max} and K_i values of 103.4% (95% CL 95.5 and 108.4%) and 13.4 nM (95% CL 10.1 and 17.8 nM), respectively. Importantly, the mean K_i value displayed by CP55940 was still above the K_i value reported in literature (Schire, 1996). The high expression of hCB₂ receptors in these cells (215 pmol/mg) could explain the reason of such high K_i values. In fact, one likely effect of a large receptor concentration is to reduce the potency with which a tritiated ligand is displaced from its specific binding sites by an unlabelled compound (Kenakin 1997). In light of this, we then performed experiments of displacement binding, using the same conditions as above, in CHO cells expressing lower amount of human CB₂ receptors (8.7 pmol/mg) (Figure 26).

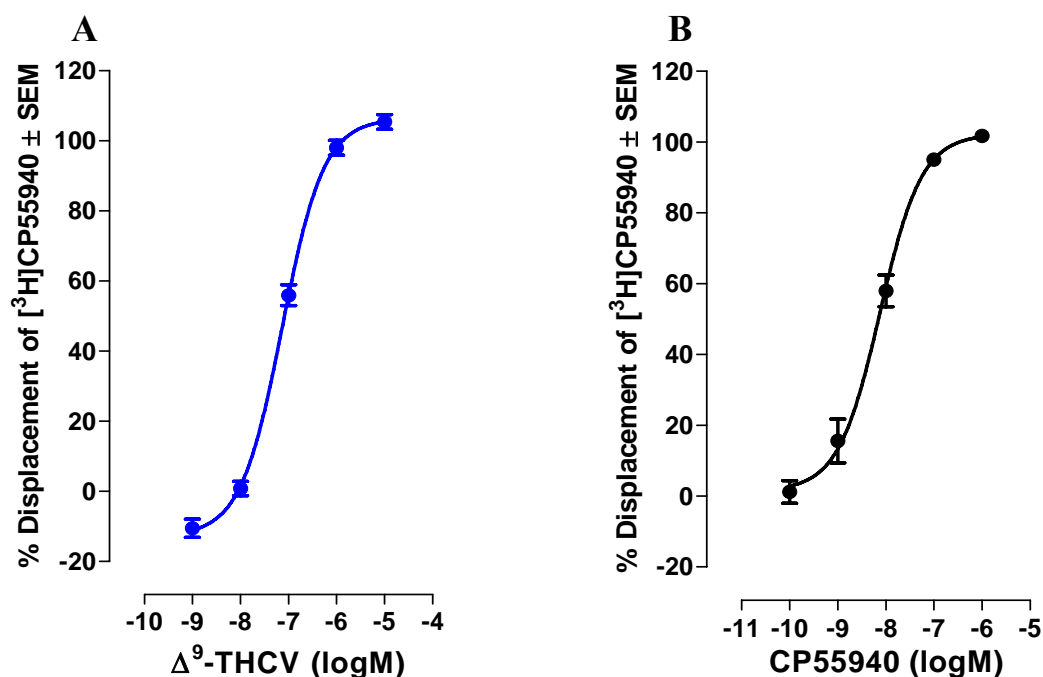


Figure 26. Displacement of [³H]CP55940 by Δ⁹-THCV (A) and CP55940 (B) from specific binding sites on CHO cells expressing lower amount of human CB₂ receptors (n=8). Each symbol represents the mean percentage displacement ± SEM. Mean K_i values, with 95% confidence limits shown in brackets, were (A) 51.6 nM (42 and 63.4 nM) for displacement with Δ⁹-THCV and (B) 5.3 nM (3.6 and 7.8 nM) for displacement with CP55940.

Both Δ⁹-THCV and CP55940 were able to fully displace [³H]CP55940 from CB₂ receptor binding sites, as the E_{max} values obtained were 106.3% (95% CL 102.4 and 110.1%) and 102.1% (95% CL 96 and 108.3%), respectively (Figure 26). The K_i

values displayed by both these compounds were in line to those reported in literature (Thomas et al. 2005, Shire et al. 1996), confirming our hypothesis.

Δ^9 -THCV behaves as CB_2 receptor partial agonist

The high affinity exerted by Δ^9 -THCV at CB_2 receptors prompted us to investigate the effect of this phytocannabinoid on [35 S]GTP γ S binding to h CB_2 -CHO cell membranes. The experiment was carried out in parallel with the non-selective cannabinoid receptor agonist, CP55940.

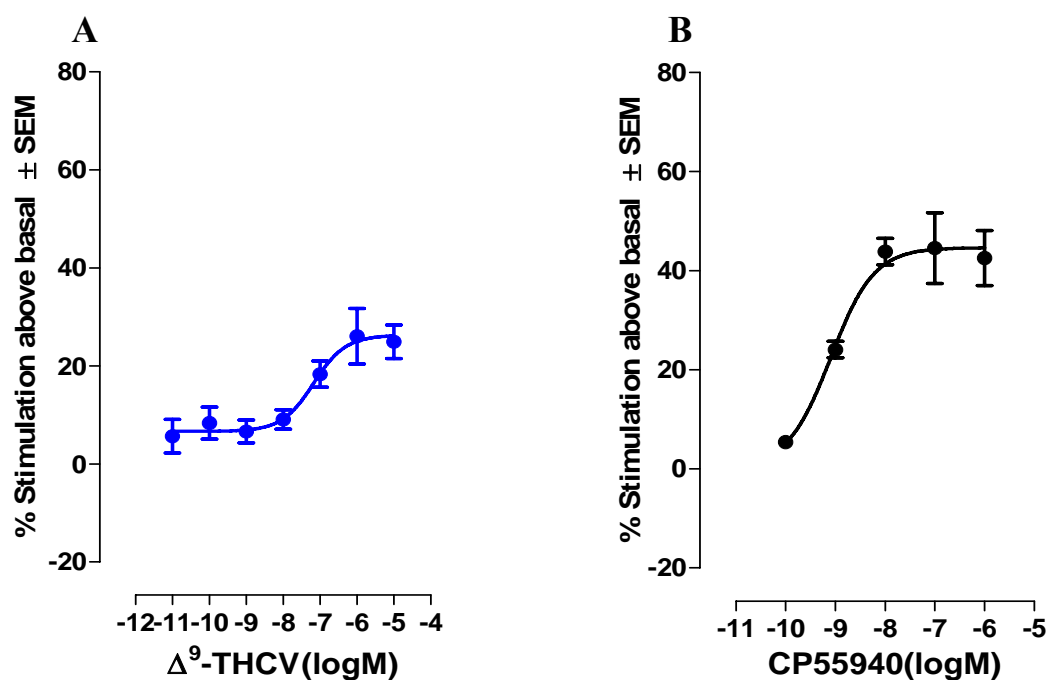


Figure 27. The effect of (A) Δ^9 -THCV (n=8) and (B) CP55940 (n=4) on [35 S]GTP γ S binding to h CB_2 -CHO cells. Each symbol represents the mean percentage change in [35 S]GTP γ S binding \pm SEM. The mean EC_{50} and E_{max} values of these cannabinoids are listed in Table 12.

As shown in Figure 27, both Δ^9 -THCV and CP55940 were able to stimulate [35 S]GTP γ S binding to membranes obtained from h CB_2 -CHO cells. In particular, the efficacy of Δ^9 -THCV in stimulating [35 S]GTP γ S binding was lower compared to that from the full agonist CP55940 (Table 12), suggesting it to be a partial agonist.

<i>Cannabinoid</i>	<i>Assay</i>	<i>E_{max} % stimulation above basal</i>	<i>EC₅₀ (95% confidence limits)</i>
Δ^9 -THCV	[³⁵ S]GTP γ S binding	26.3 \pm 2.8	66.6 nM (14.3 and 311 nM)
CP55940	[³⁵ S]GTP γ S binding	44.6 \pm 2.3	0.8 nM (0.3 and 2.1 nM)
Δ^9 -THCV	Cyclic AMP ¹	40 \pm 3.9	38.1 nM (11.7 and 124 nM)
CP55940	Cyclic AMP ¹	54.7 \pm 2.5	6.8 nM (3.5 and 13.8 nM)
Δ^9 -THCV	Cyclic AMP ²	39 \pm 4.2	23.1 nM (4.9 and 109 nM)
CP55940	Cyclic AMP ²	87.7 \pm 2.4	1.9 nM (1.2 and 2.9 nM)

Table 12. The mean E_{max} values \pm SEM and the mean EC_{50} values, with 95% confidence limits (CLs) are in brackets for the efficacy of tested cannabinoids in hCB₂-CHO cells.

¹cAMP assay kit based on radioactive reagents and purchased by GE Healthcare.

²cAMP assay kit based on luminescence reaction, patented and purchased by DiscoverX.

To confirm these data, we investigated whether Δ^9 -THCV was able to inhibit forskolin-induced stimulation of cyclic AMP production in hCB₂-CHO cells. Again, the experiment was performed in parallel with the full cannabinoid agonist, CP55940.

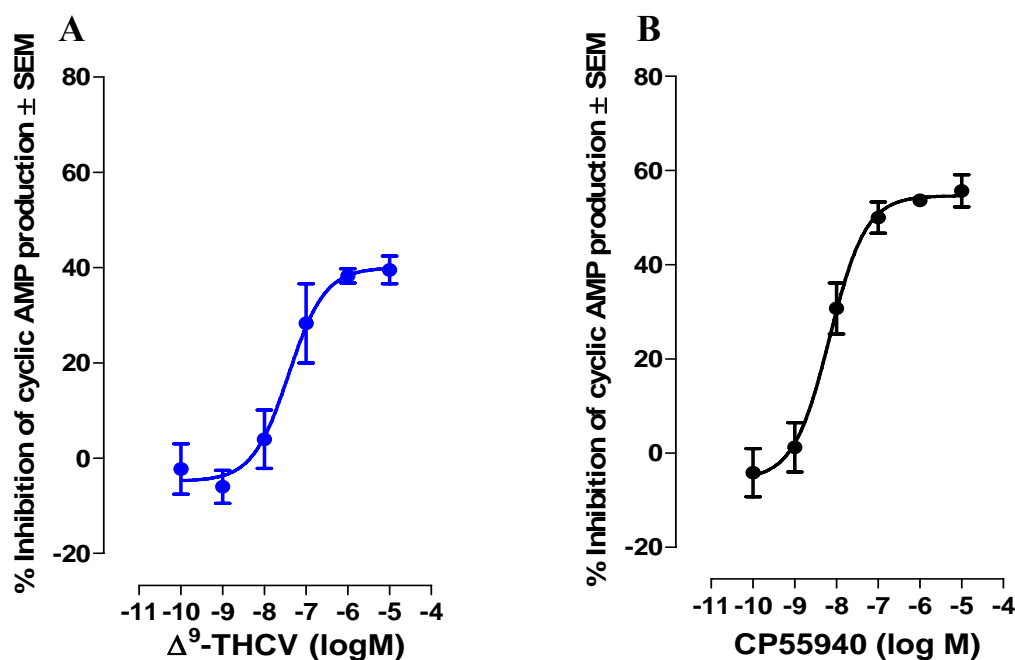


Figure 28. The effect of (A) Δ^9 -THCV (n=8) and (B) CP55940 (n=4) on forskolin-induced cyclic AMP production in hCB₂-CHO cells. Symbols represent mean values \pm SEM. The mean EC_{50} and E_{max}

values of these cannabinoids are listed in Table 12. The experiments were performed with a kit purchased by *GE Healthcare*.

As shown in Figure 28A, we found that Δ^9 -THCV at concentrations in the nanomolar range can indeed induce such inhibition in this bioassay, with an EC_{50} very similar and not significantly different to that reported in the [35 S]GTP γ S binding assay (Table 12). However, the E_{max} showed by Δ^9 -THCV in the cyclic AMP assay was approximately 10% higher compare to that shown in stimulating [35 S]GTP γ S binding (Table 12)

Differences between the two assays were indeed found for CP55940 (Table 12). In fact, although this full agonist showed an inhibition of forskolin-induced cyclic AMP production in hCB $_2$ -CHO cells (Figure 28B), CP55940 displayed an higher efficacy but a lower affinity versus CB $_2$ receptors in this bioassay (Table 12). The different E_{max} obtained between the [35 S]GTP γ S binding and cyclic AMP assay could be due by the higher sensitivity displayed by cyclic AMP assay (Pertwee 1999).

Importantly, the cyclic AMP analyses were conducted with a kit purchased by *GE Healthcare* and based on radioactive reagents. However, the sudden interruption in the supply of this kit production, forced us to move and try different technologies based on cyclic AMP detection. Among those ones available, we chose a kit patented and purchased by *DiscoverX*, based on luminescence technology. We then investigated the activity of Δ^9 -THCV and CP55940 on forskolin-induced cyclic AMP production in hCB $_2$ -CHO cells using this new kit.

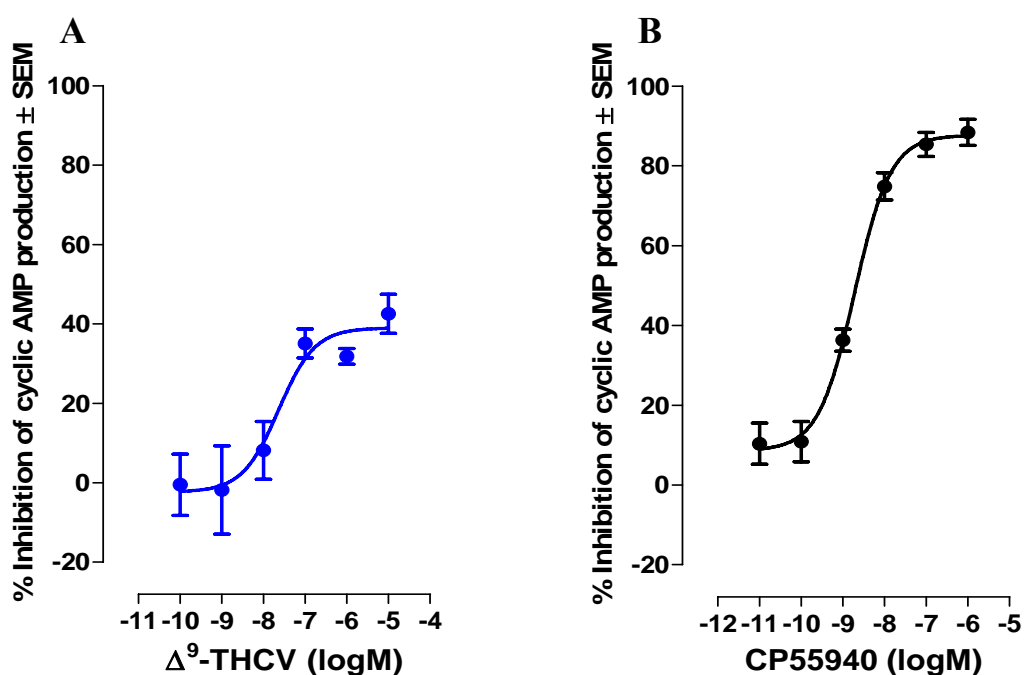


Figure 29. The effect of (A) Δ^9 -THCV (n=8) and (B) CP55940 (n=4) on forskolin-induced cyclic AMP production in hCB₂-CHO cells. Symbols represent mean values \pm SEM. The mean EC₅₀ and E_{max} values of these cannabinoids are listed in Table 12. The experiments were performed with a kit purchased by *DiscoverX*.

As shown in Figure 29, we found that both Δ^9 -THCV and CP55940 were indeed able to inhibit the production of cyclic AMP induced by forskolin. The EC₅₀ displayed by these compounds were very similar and not significantly different compared to those found in the [³⁵S]GTP γ S binding assay (Table 12).

Differences were instead found in the efficacy yielded by these compounds, which were significantly greater than those displayed in [³⁵S]GTP γ S binding assay (Table 12).

Summarizing, in relation to the efficacy window displayed by the full agonist, CP55940, in all of these assays, the log concentration-response curves displayed by Δ^9 -THCV suggest it to be a partial agonist at hCB₂ receptors.

Δ^9 -THCV activity at CB₂ receptors: further evidences

In order to establish whether Δ^9 -THCV was acting at CB₂ receptors, we first carried out experiments of antagonism using a CB₂-selective antagonist/inverse agonist, AM630, on cyclic AMP assay.

Thus, Δ^9 -THCV in the concentration range of 0.1 to 10000 nM was antagonized by AM630 at the concentration of 100 nM in hCB₂-CHO cells.

As already reported previously (Ross et al. 1999), we found that AM630 exerted a marked inverse agonism at CB₂ receptors, masking the antagonistic properties of this compound (Figure 30). Thus, the lack of a CB₂ receptor neutral antagonist (which will be the subject of the Chapter 2) prompted us to explore other strategies to assess the selective activity of Δ^9 -THCV at CB₂ receptors.

Firstly, as shown in Figure 31, we established that at concentrations of 0.1 nM to 10 μ M, neither Δ^9 -THCV nor CP55940 affected forskolin stimulated cyclic AMP production in hCB₂-CHO cells when these had been preincubated overnight with pertussis toxin in a manner expected to eliminate G_{i/o} signalling (Bonhaus et al. 1998, Glass, Felder 1997, Coutts et al. 2001).

Secondly, we tested the effects of these two compounds in the cyclic AMP assay in untransfected CHO cells. As previously shown (Figure 24), we found that none of the compounds tested were able to significantly modulate forskolin-induced cyclic AMP production.

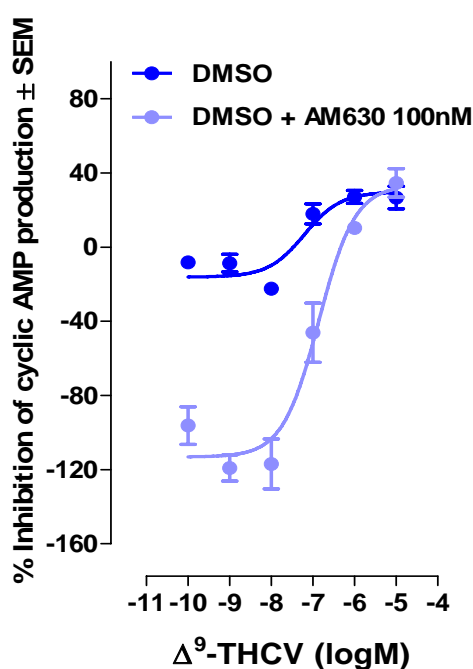


Figure 30. The effect of 100 nM of AM630 on the mean log concentration-response curve of Δ^9 -THCV in forskolin-induced stimulation of cyclic AMP production in hCB₂-CHO cells. Symbols represent mean values \pm SEM.

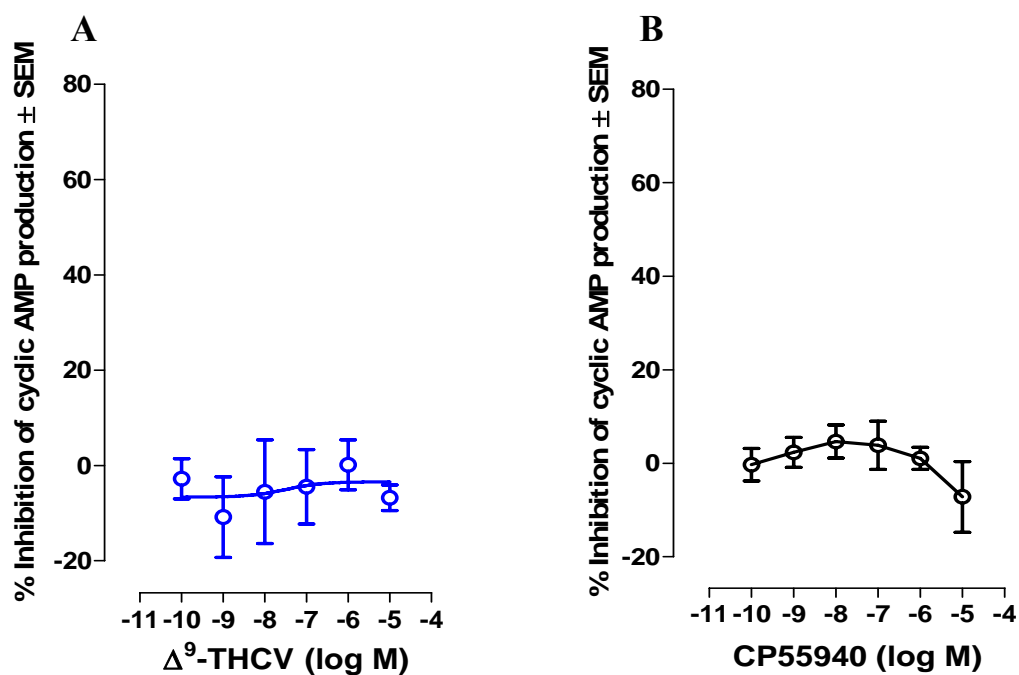


Figure 31. The effect of Δ^9 -THCV (A) and CP55940 (B) on forskolin-induced stimulation of cyclic AMP production in hCB₂-CHO cells preincubated overnight with pertussis toxin (PTX 100 ng/ml). Symbols represent mean values \pm SEM. None of the doses tested for both Δ^9 -THCV and CP55940 are significantly different from zero (one sample t-test analyses).

DISCUSSION

Pharmacological properties of Δ^9 -tetrahydrocannabivarin at CB₁ receptors

Results from our *in vitro* experiments show that Δ^9 -THCV exhibits a strong affinity and antagonism at CB₁ receptors.

We first confirmed previous data (Thomas et al. 2005) in which Δ^9 -THCV displaced [³H]CP55940 from specific binding sites on membranes prepared from brain tissue (Figure 16). We found that the mean K_i value displayed by Δ^9 -THCV (31.5 nM) was two times lower than the one reported previously (Thomas et al. 2005) in the same membranes (75.4 nM). It is not possible that Δ^9 -THCV has more affinity for CB₁ receptors because the method used to process the brain tissues was different. In fact, no differences in the K_i values were found when the tissues were processed with the same protocol reported in Thomas *et al.* (2005) (Table 10). However, one possible explanation could be the purity of Δ^9 -THCV which might be slightly different compared to the past (Thomas et al. 2005).

We also confirmed that Δ^9 -THCV does not affect [³⁵S]GTP γ S binding to mouse brain membranes (Figure 18), but instead it behaves as a reasonably potent antagonist versus the CB₁/CB₂ cannabinoid receptor selective ligand, CP55940 (Figure 20). In particular, Δ^9 -THCV displayed an antagonism at CB₁ receptors which is competitive in nature, as shown by a significant dextral shift in the log concentration-response curve of CP55940 that did not deviate significantly from parallelism. Interestingly, the K_B value displayed by Δ^9 -THCV for its antagonism of CP55940 (22.4 nM) was approximately four times lower than that previously found in this laboratory (93 nM) (Thomas et al. 2005). Again, the purity of Δ^9 -THCV might explain the difference in K_B values.

Importantly, the mean K_B value showed by Δ^9 -THCV did not differ significantly from the mean K_i value for displacement of [³H]CP55940 to mouse brain membranes, suggesting that Δ^9 -THCV is acting at CB₁ receptors. In fact, there is little evidence for the presence of a significant population of CB₂ receptors in healthy brain tissue (Howlett et al. 2002).

Further support to this hypothesis comes from results obtained in CHO cells transfected with human CB₁ receptors. First we found that Δ⁹-THCV was indeed able to displace [³H]CP55940 from specific binding sites on membranes prepared from hCB₁-CHO cells (Figure 19). The K_i value displayed by Δ⁹-THCV (9.5 nM) was 3.3 times lower than the reported K_i value found in mouse brain tissue (31.5 nM). It is likely that Δ⁹-THCV has more affinity for the human form of the CB₁ receptor compared to the mouse one, and/or the expression of different receptor populations in the brain tissue might influence the binding of Δ⁹-THCV to CB₁ receptors.

As in mouse brain membranes, Δ⁹-THCV did not affect [³⁵S]GTPγS binding on hCB₁-CHO cells at any of the concentrations tested (Figure 19). However, when this phytocannabinoid was tested as antagonist of CP55940, it was found that, in addition to a right-ward shift, the log concentration-response curve of CP55940 showed a down-ward shift (approximately 20%) at all the concentrations of Δ⁹-THCV tested (Figure 21). The ability of Δ⁹-THCV to inhibit [³⁵S]GTPγS binding when tested as antagonist of CP55940 is not surprising. Previous data reported a similar effect for the CB₂ receptor antagonist, SR144528 (Thomas et al. 2007). In this paper it was found that the inverse efficacy exerted by SR144528 on [³⁵S]GTPγS was higher when the compound was tested as an antagonist of CP55940 than by itself (Thomas et al. 2007). The authors suggested that CP55940, being an agonist and according to the two-state model, would be expected to shift the equilibrium of the receptors from R to R* and so increase the number of receptors in the constitutive R* active state. Thus, it may be possible that Δ⁹-THCV did not affect the GTPγS by itself because the amounts of CB₁ receptors in the constitutively active state were not sufficient.

Importantly, when the component of Δ⁹-THCV -induced antagonism that seemed to arise from its ability to inhibit [³⁵S]GTPγS binding to hCB₁-CHO cell membranes was excluded, significant right-ward shifts in the log concentration-response curves of CP55940 were still apparent (Figure 21). Furthermore, the mean K_B values calculated from these dextral shifts (Table 11) at all the concentrations of Δ⁹-THCV tested, did not differ significantly from the mean K_B values obtained for the antagonism of CP55940 to mouse brain membranes, further supporting the hypothesis that Δ⁹-THCV is a CB₁ receptor antagonist. In addition, the 2+2 analyses showed that the antagonism exerted by Δ⁹-THCV on CP55940 to hCB₁-CHO cell membranes was not

significantly different from parallelism (Table 11), suggesting it to be competitive in nature.

The cyclic AMP assay provided further information about the activity of Δ^9 -THCV at CB₁ receptors. In contrast to the data obtained using the [³⁵S]GTP γ S binding assay, we found that Δ^9 -THCV by itself affected forskolin-induced stimulation of cyclic AMP production in hCB₁-CHO cells, producing signs of inverse agonism (Figure 22). No Δ^9 -THCV-induced inverse agonism was observed in either untransfected CHO cells or in hCB₁-CHO cells that had been preincubated with pertussis toxin, suggesting that this effect was indeed CB₁ receptor mediated (Figure 23, 24). It is possible that Δ^9 -THCV behaved as inverse agonist in the cyclic AMP assay because CB₁ receptor signalling is amplified in this assay compared to [³⁵S]GTP γ S binding assay (Pertwee 1999). Furthermore, it should be considered that the [³⁵S]GTP γ S binding assay is performed with an excess of GDP reagent, with the aim to decrease the basal activity of the receptor and increase the receptor responsiveness. However, this approach can affect the amount of R* (constitutively active receptors) and consequently influence the response of slight inverse agonists, such as Δ^9 -THCV.

Whether Δ^9 -THCV can induce inverse agonism *in vivo* remains to be investigated. There is already evidence, however, that it can behave *in vivo* as a CB₁ receptor antagonist. Thus, Δ^9 -THCV has been reported to suppress food intake and weight gain in mice (Riedel et al. 2009) and to attenuate several *in vivo* effects of Δ^9 -THC, including Δ^9 -THC-induced antinociception in the tail flick test and catalepsy in the ring test (Pertwee et al. 2007).

Pharmacological properties of Δ^9 -tetrahydrocannabivarin at CB₂ receptors

Results from our *in vitro* experiments indicate that Δ^9 -THCV exhibits significant potency and efficacy as a cannabinoid CB₂ receptor agonist. Thus, Δ^9 -THCV shared the ability of the established CB₁/CB₂ receptor agonist, CP55940, both to inhibit forskolin-induced stimulation of cyclic AMP production by hCB₂-CHO cells and to stimulate [³⁵S]GTP γ S binding to membranes obtained from these cells (Table 12). In addition, neither Δ^9 -THCV nor CP55940 inhibited cyclic AMP production either in

untransfected CHO cells or in hCB₂-CHO cells that had been preincubated overnight with pertussis toxin in order to eliminate G_{i/o} mediated signalling (Figure 24, 31).

In contrast to our results, it was previously found in this laboratory that Δ⁹-THCV opposes CP55940-induced stimulation of [³⁵S]GTPγS binding to membranes derived from hCB₂-CHO cells (Thomas et al. 2005) and that by itself Δ⁹-THCV does not behave as a CB₂ receptor agonist in this bioassay (Thomas and Pertwee, unpublished). However, the CB₂ receptor density determined by [³H]CP55940 saturation binding was three times lower in the CHO cells used in these previous experiments ([³H]CP55940 B_{max} = 72.57 pmol mg⁻¹) than in the cells used in the present investigation (Methods). Hence, Δ⁹-THCV is most likely a hCB₂ receptor partial agonist since, as predicted by classical drug receptor theory for an agonist of this kind (Kenakin 2001, Kenakin 1997), the efficacy that Δ⁹-THCV displays at hCB₂ receptor appears to be greatly influenced by the expression levels of these receptors. Thus, Δ⁹-THCV seems to undergo conversion from an apparent neutral hCB₂ receptor antagonist (Thomas et al. 2005) to an apparent hCB₂ receptor agonist in the [³⁵S]GTPγS binding assay when the expression level of these receptors is increased (Figure 27). Moreover, the E_{max} displayed by Δ⁹-THCV for the activation of hCB₂ receptors both in the [³⁵S]GTPγS binding (Figure 27) and cyclic AMP assays (Figure 28, 29) is less than that of the full agonist, CP55940, further supporting that Δ⁹-THCV is a partial agonist at CB₂ receptors.

Importantly, the mean EC₅₀ values of Δ⁹-THCV for inhibition of cyclic AMP production (Figure 29) and for stimulation of [³⁵S]GTPγS binding (Figure 27) to hCB₂-CHO cells were significantly less than its mean apparent K_i value (225 nM) for displacement of [³H]CP55940 from specific binding sites in the same cell membranes (Figure 25). However, this is because the mean apparent K_i value we obtained in hCB₂-CHO cells, which express high levels of hCB₂ receptors (Methods), was significantly above the true K_i value of Δ⁹-THCV for CB₂ receptors. In fact, one likely effect of a large receptor population is to reduce the potency with which a tritiated ligand is displaced from its specific binding sites by an unlabelled compound (Kenakin 1997). Thus, when Δ⁹-THCV was tested in the displacement binding assay in CHO cells expressing lower amounts of hCB₂ receptors, it displayed a K_i value of 51.6 nM (Figure 26), which is in line and not significantly different to the EC₅₀ values found in the above mentioned assays, thus confirming our hypothesis.

The ability of Δ^9 -THCV to activate CB₂ receptors has also been tested in membranes obtained from mouse spleen, a tissue that is thought to express CB₁ as well as CB₂ receptors (Pertwee 1997). The experiments were performed by a colleague in my present laboratory. It has been found that Δ^9 -THCV can indeed stimulate [³⁵S]GTP γ S binding to these membranes (Bolognini et al. 2010). Its EC₅₀ and E_{max} values, with 95% CLs shown in parentheses, were 69 nM (2.6 and 1804 nM) and 23% (3.8 and 42%), respectively (n=6). Moreover, when experiments of [³⁵S]GTP γ S binding were performed in CB₂^{-/-} mice spleen membranes, Δ^9 -THCV did not show any effect (Bolognini et al. 2010), confirming the ability of this phytocannabinoid to selectively activate CB₂ receptors in this tissue.

Having obtained evidence that Δ^9 -THCV can activate naturally expressed mouse CB₂ receptors *in vitro*, we went on to investigate its ability to activate mouse CB₂ receptors *in vivo*. We began collaborations with Dr. Barbara Costa (University of Milano-Bicocca) and Dr. Sabatino Maione (University of Naples) directed at determining whether Δ^9 -THCV shares the ability of established selective CB₂ receptor agonists (Guindon, Hohmann 2008) to ameliorate signs of inflammation and thermal hyperalgesia induced in rats or mice by intraplantar injection of λ -carrageenan or formalin. The data presented were recently published in a peer reviewed journal (Bolognini et al. 2010).

Lambda carrageenan (λ -carrageenan) is a mucopolysaccharide extracted by the red alga, *Chondrus crispus*. The subcutaneous injection of this substance causes swelling and pain. In experiments conducted by Dr. Barbara Costa, 20 μ l of λ -carrageenan (2%w/v in saline) were injected in the paw of C57/black mice with successive evaluation of the oedematous effects and thermal hyperalgesia, which were measured by a plethysmometer and a plantar test, respectively. At 2 hours after λ -carrageenan administration, it was found that pretreatment with Δ^9 -THCV at 0.3 mg/kg significantly reverted the λ -carrageenan-induced oedema effect (Figure 32A). Moreover, Δ^9 -THCV, at the doses of 0.3 and 1 mg/kg, was also found efficacious in reducing the thermal hyperalgesia induced by the injection of λ -carrageenan (Figure 32B).

Because λ -carrageenan-induced oedema and thermal hyperalgesia remained at a high level throughout an observation period of 24 h (Figure 33), the anti-inflammatory and anti-nociceptive responses elicited by Δ^9 -THCV at 0.3 mg/kg were investigated at

additional time points. It was found that the anti-oedema effect of Δ^9 -THCV was still present and unreduced at 3 and 4 h after λ -carrageenan administration, slightly reduced but still present at 6 h after λ -carrageenan, and absent at 24 h after λ -carrageenan (Figure 33A). Similarly, the anti-hyperalgesic effect of Δ^9 -THCV decreased progressively over this same observation period and was no longer detected 24 h after λ -carrageenan administration (Figure 33B). The anti-inflammatory and anti-hyperalgesic properties of Δ^9 -THCV were also tested at different time points within a 4 day time period. In detail, Δ^9 -THCV was administered at 0.3mg/kg, 30 min after λ -carrageenan injection, and then again once daily over the next 3 days. As shown in Figure 34, Δ^9 -THCV significantly diminished oedema and thermal hyperalgesia after its first injection, and these anti-oedema and anti-hyperalgesic effects of Δ^9 -THCV remained undiminished after each of its subsequent injections.

The possible target/s underlying the anti-oedema and anti-nociceptive effects of Δ^9 -THCV were also investigated in the λ -carrageenan model by administration of the CB₁ receptor antagonist, rimonabant, or the CB₂ receptor antagonist, SR144528. These compounds were administered 15 min before Δ^9 -THCV (0.3 mg/kg), and evaluations of oedema and thermal hypersensitivity were made at 2 and 3 h after λ -carrageenan, respectively. Figure 35A shows that only SR144528 was able to reverse the anti-oedema effect of Δ^9 -THCV, suggesting that this phytocannabinoid induced its anti-inflammatory effect primarily through activation of the CB₂ receptor. However, rimonabant elicited a partial, although not statistically significant, reversal of Δ^9 -THCV-induced anti-hyperalgesia, whereas SR144528, at doses that have been found to block the effect of an established CB₂ receptor agonist in this assay (Guindon and Hohmann, 2008), produced no sign of any such reversal (Figure 35B). Importantly, the doses of rimonabant (0.5 mg/kg) and SR144528 (1 mg/kg) used in these experiments did not affect λ -carrageenan-induced paw oedema or thermal hypersensitivity when administered alone (data not shown). These data suggest that Δ^9 -THCV is able to decrease signs of inflammation through the activation of CB₂ receptors, since the ability of this phytocannabinoid to reverse λ -carrageenan-induced oedema effect was antagonized by the CB₂ receptor antagonist, SR144528. Conversely, the Δ^9 -THCV-mediated anti-hyperalgesic effects show a more complicated panorama, since neither CB₁ nor CB₂ receptors seem to be involved in

this effect. In order to clarify this aspect, Δ^9 -THCV was then tested in the formalin test assay, which is a model to measure the anti-nociceptive response of an established compound. In particular, the formalin test is characterized by two phases, which are separated by a transient quiescent period (Guindon, Hohmann 2008). The two phases are:

1. Early phase (0-7 min), which involves acute activation of chemo, thermo and mechano-sensitive fibers (C- and A-delta fibers).
2. Second phase (15-60 min), which is a prolonged phase of tonic pain and involves an inflammatory reaction in peripheral tissue, the development of CNS sensitization and additionally involves activation of primary afferent nociceptors.

Δ^9 -THCV was found to display dose-dependent activity against formalin-induced nociceptive behaviour (Figure 36). In detail, 30 μ l of formalin (1.25% in saline) was injected subcutaneously into the hind paw of the mice. It was found that the intraperitoneal (i.p.) injection of Δ^9 -THCV reduced pain behaviour in both phases of the formalin test at a dose of 5 mg/kg (Figure 36A), which is in contrast with previous findings with established CB₂ receptors agonists, as reported in several investigations to suppress the second phase but not the first phase of formalin-induced nociceptive behaviour (Guindon, Hohmann 2008, Whiteside, Lee & Valenzano 2007). However, Δ^9 -THCV affected only the second phase when administered at the dose of 1 mg/kg (Figure 36A). The ameliorating effect of the higher dose of Δ^9 -THCV (5 mg/kg) on the first and second phases of the formalin response (Figure 36B) and of the lower dose of Δ^9 -THCV (1 mg/kg) on the second phase (Figure 36C) was attenuated by pretreatment with both rimonabant (0.5 mg/kg) and SR144528 (1 mg/kg). As for the λ -carrageenan test, the doses of rimonabant and SR144528 used in these experiments did not affect formalin-induced pain behaviour when administered alone (data not shown). The data obtained from the formalin test suggest that Δ^9 -THCV produces its anti-nociceptive effect by activating both CB₁ and CB₂ receptors. It is noteworthy therefore that Δ^9 -THCV has been found to behave *in vivo*, though not *in vitro* (Figure 18, 19), as a CB₁ receptor agonist at doses above those at which it produces signs of CB₁ receptor blockade (Pertwee et al. 2007). Combined CB₁ and CB₂ receptor activation provides a possible explanation why Δ^9 -THCV-induced suppression of λ -carrageenan-induced hyperalgesia is not antagonized by SR144528 at a dose at which this CB₂-selective antagonist does antagonize λ -carrageenan-induced oedema. It

might also explain why the highest dose of Δ^9 -THCV tested was able to suppress both phases of formalin-induced pain behaviour. In fact, there is evidence that, in contrast to CB₂-selective agonists, established mixed CB₁/CB₂ receptor agonists such as Δ^9 -THC and CP55940 have the ability to suppress both phases of the formalin test (Pertwee 2001). Why the relatively low doses of Δ^9 -THCV would activate CB₁ receptors in addition to CB₂ receptors remains to be established, one possible explanation being that it is a consequence of λ -carrageenan- and/or formalin-induced upregulation of the CB₁ receptor in pain pathways, there already being evidence that such upregulation does occur in primary afferent neurons in at least one rodent model of inflammatory pain (Amaya et al. 2006).

In conclusion, Δ^9 -THCV has the ability to activate human CB₂ receptors *in vitro*, acting as partial agonist, and mouse CB₂ receptors both *in vitro* and *in vivo*.

Concerning CB₁ receptors, it seems that Δ^9 -THCV is able to activate these receptors at high doses only *in vivo* (Pertwee et al. 2007) and not *in vitro*, where instead it exerts inverse agonistic properties (Figure 22). As already mentioned (see introduction) it is likely that Δ^9 -THCV is metabolized *in vivo* to a more potent compound with agonistic properties at CB₁ receptors. On the other hand, it is clear that Δ^9 -THCV acts as a CB₁ receptor antagonist both *in vitro* (Figure 20, 21) and *in vivo* (Pertwee et al. 2007, Riedel et al. 2009), at doses below those at which it behaves as an agonist *in vivo*. The antagonistic properties of Δ^9 -THCV at CB₁ receptors make this phytocannabinoid an interesting drug for such diseases (e.g. chronic liver disease and stroke) in which there is evidence that symptoms can be ameliorated by a combination of CB₂ receptor activation and CB₁ receptor blockade (Mallat et al. 2007, Zhang et al. 2009).

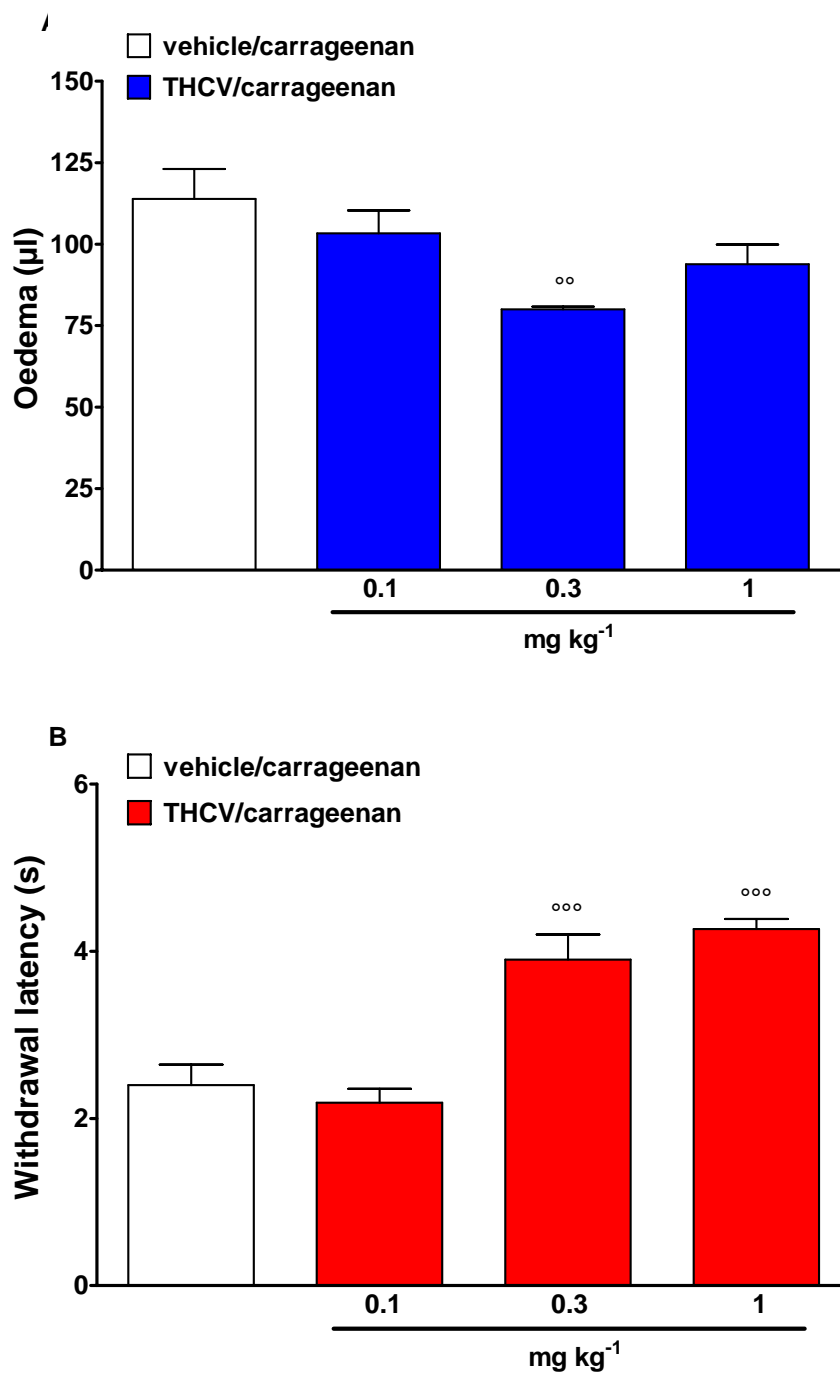


Figure 32. Effect of Δ^9 -THCV, administered i.p. 30 min before λ -carrageenan (2%, 20 mL intraplantar), on (A) oedema evaluated 2 h after λ -carrageenan, and (B) thermal hypersensitivity, evaluated 3 h after λ -carrageenan. The basal hind paw withdrawal latency displayed by vehicle-treated mice was 10 ± 0.45 s. Data represent mean values \pm SEM (n = 9). ^{oo}P < 0.01, ^{ooo}P < 0.001 versus mice treated with vehicle/ λ -carrageenan

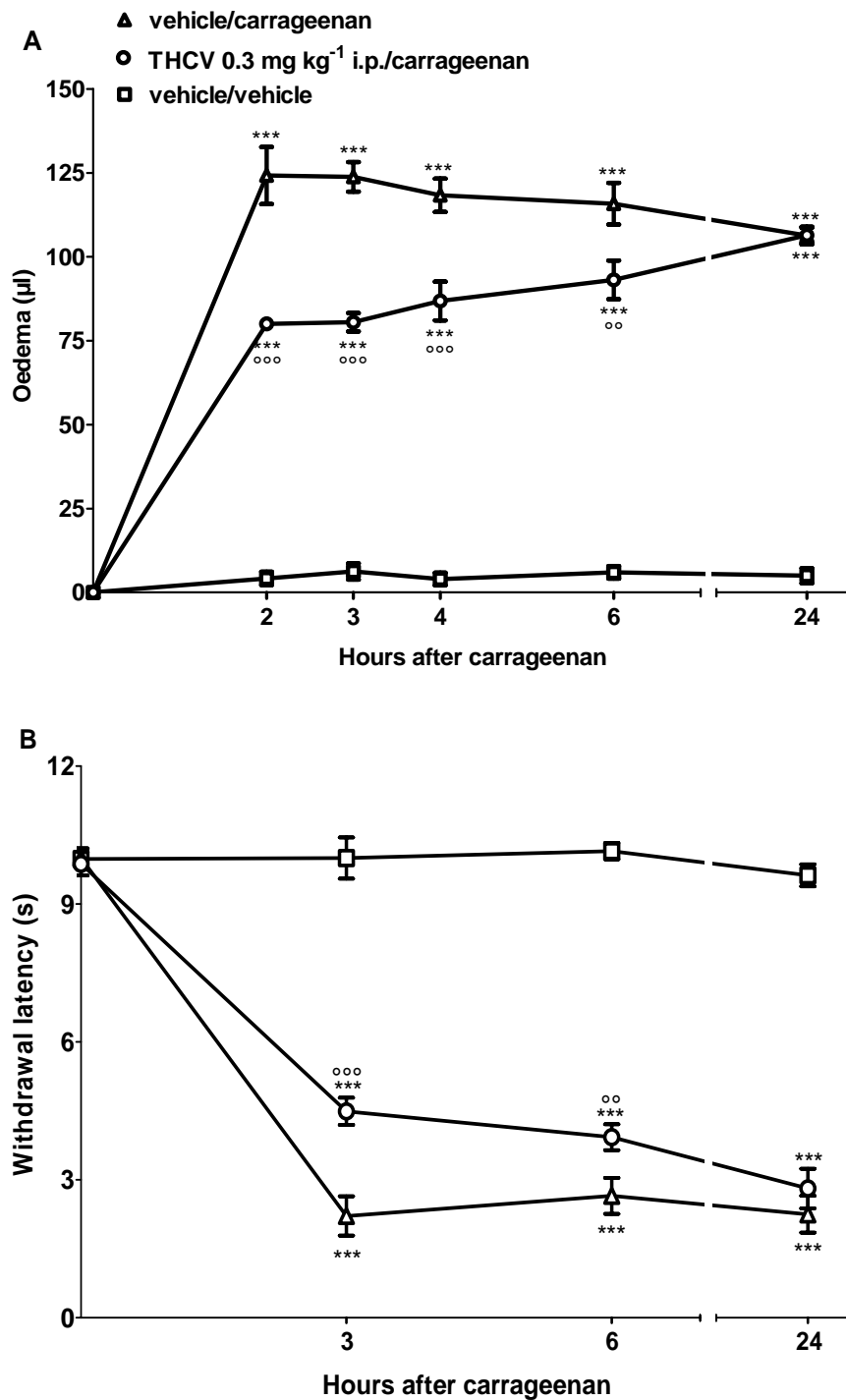


Figure 33. Effect of Δ^9 -THCV (0.3 mg·kg⁻¹ i.p.), administered 30 min before λ -carrageenan (2%, 20 mL intraplantar), on (A) oedema and (B) thermal hypersensitivity evaluated at different times after λ -carrageenan. Data represent mean values \pm SEM (n = 5). ***P < 0.001 versus mice treated with vehicle/vehicle. °°P < 0.01, °°°P < 0.001 versus mice treated with vehicle/ λ -carrageenan.

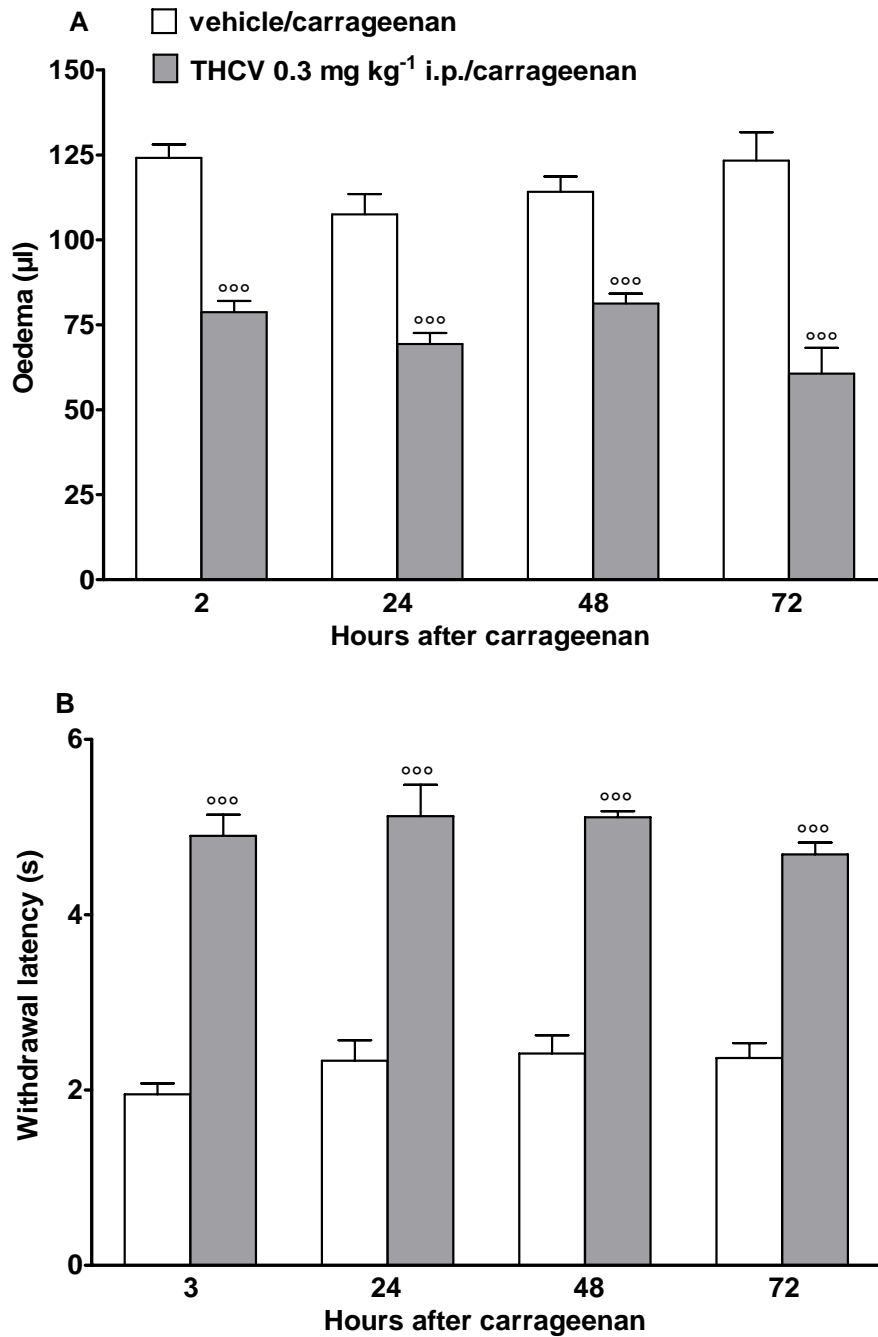


Figure 34. Effect of Δ^9 -THCV (0.3 mg·kg⁻¹ i.p.), administered after λ -carrageenan once daily for 4 days, on (A) oedema and (B) thermal hypersensitivity. Data represent mean \pm SEM (n = 6–8). ^{ooo}P < 0.001 versus mice treated with λ -carrageenan/vehicle.

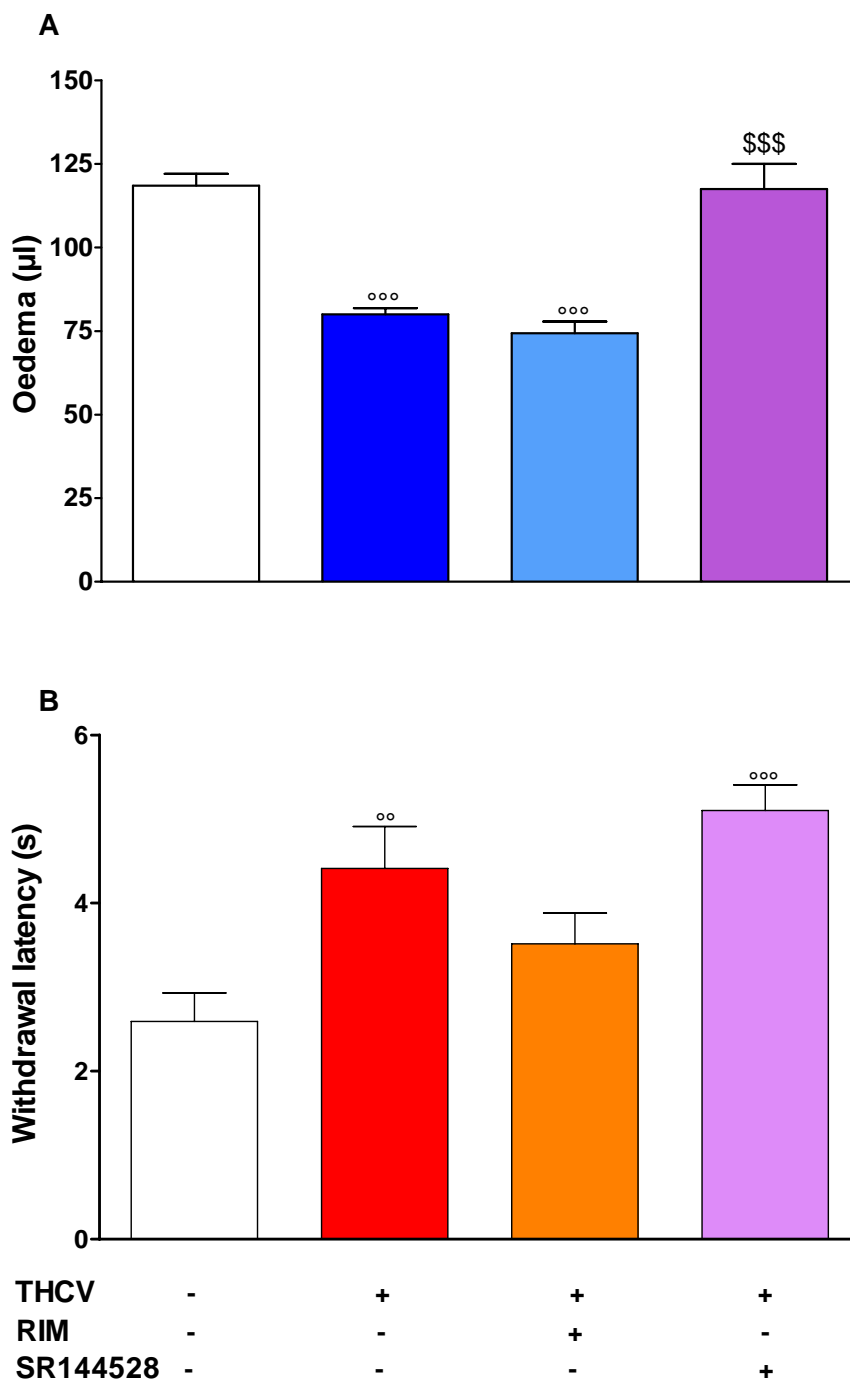


Figure 35. Effect of rimonabant (RIM; 0.5 mg·kg⁻¹ i.p.) and SR144528 (1 mg·kg⁻¹ i.p.) on (A) anti-oedema and (B) antinociceptive effects evoked by Δ⁹-THCV (0.3 mg·kg⁻¹ i.p.). Antagonists were administered 15 min before Δ⁹-THCV, and behavioural evaluations were made 2 h (oedema) and 3 h (thermal hypersensitivity), after λ-carrageenan. Data represent mean values ± SEM (n = 8–10). °°P < 0.01, °°°P < 0.001 versus mice treated with vehicle/ λ-carrageenan. \$\$\$P < 0.001 versus mice treated with vehicle/THCV/ λ-carrageenan.

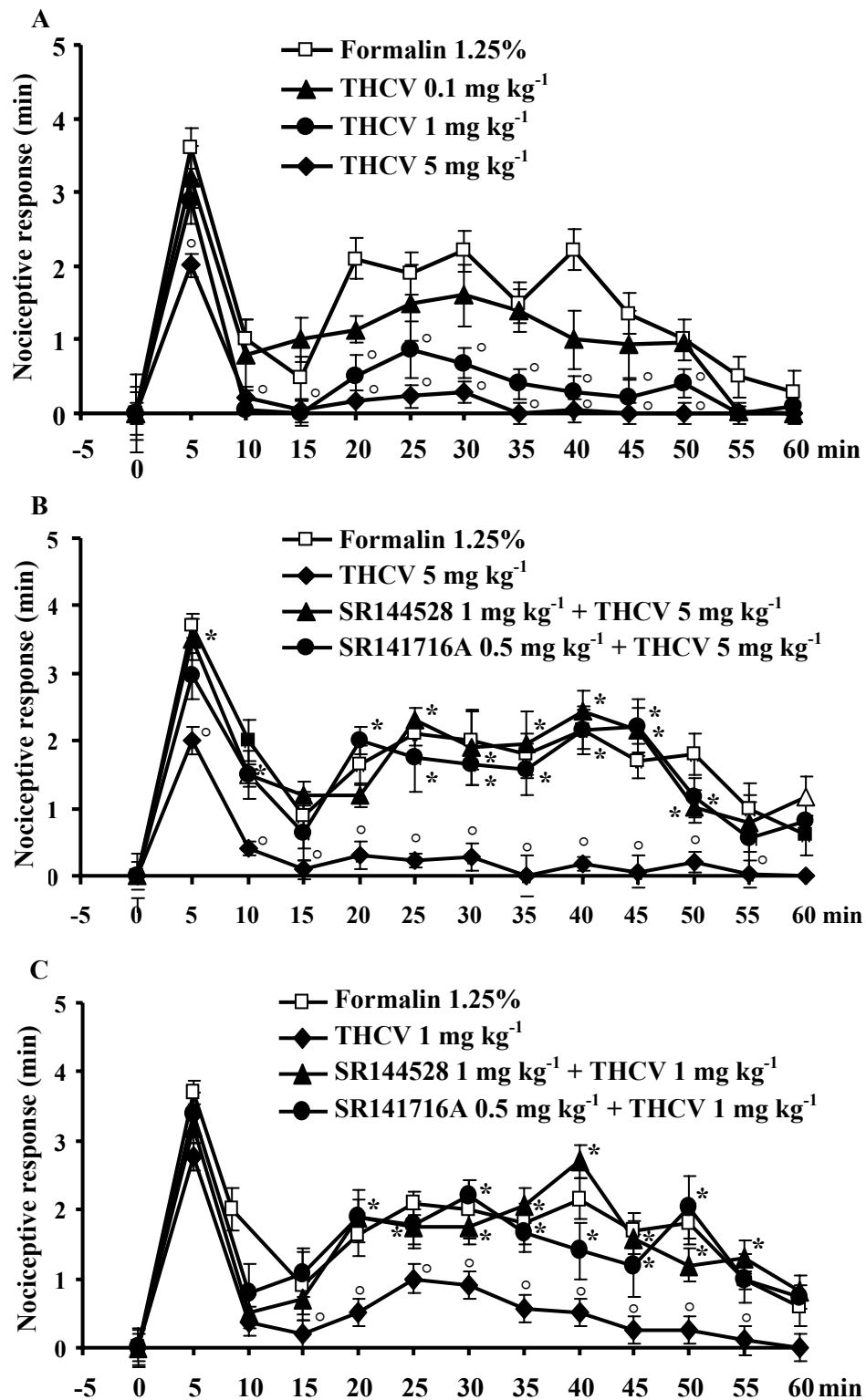


Figure 36. Effect on nociceptive behaviour evoked by formalin (1.25%, 30 mL s.c.) of Δ^9 -THCV when administered at different doses by itself (A) or at 5 mg·kg⁻¹ (B) or 1 mg·kg⁻¹ (C) in combination with SR144528 (1 mg·kg⁻¹ i.p.) or rimonabant (0.5 mg·kg⁻¹ i.p.). Δ^9 -THCV was administered intraperitoneally 15 min before formalin. Antagonists were given 15 min prior to Δ^9 -THCV. Data

represent mean values \pm SEM (n = 8). °P < 0.05 versus vehicle. *Nociceptive responses to formalin that were significantly greater after SR144528 + Δ^9 -THCV or rimonabant + Δ^9 -THCV than after Δ^9 -THCV alone (P < 0.05).

CHAPTER TWO

Pharmacology of Δ^9 -tetrahydrocannabivarin at CB₂ receptors: further evidences

INTRODUCTION

Receptor antagonist

A receptor antagonist is a ligand that is able to block or dampen the agonist-mediated biological responses. Antagonists are important pharmacological tools to evaluate the selective activity of a ligand to a particular receptor, and they can find application both *in vitro* and *in vivo*. As shown in Table 13, antagonists can be classified into two main classes (Vauquelin et al. 2002):

1) Surmountable antagonists. These ligands are competitive in nature since they reversibly bind the same binding site (orthosteric) of the endogenous ligand or agonist. Pharmacologically, surmountable antagonists produce parallel rightward shift of agonist dose-response curves with no alteration of the maximal response (Figure 37A). Considering the receptor two-state model (see material methods, Figure 13), a seven transmembrane receptor can be found in an inactive state (R) and an active state (R*). Consequently, surmountable antagonists can be divided in 3 different subclasses:

A) Neutral antagonists. These ligands can bind both the active and inactive state of the receptor without altering the equilibrium between these two forms. When tested by itself in functional assays, a neutral antagonist does not provoke any receptor response.

B) Partial agonists are ligands that induce a response in a functional assay, but their maximum response is less than the one elicited by a full agonist. Although they are agonists, partial agonists can act as a competitive antagonist in the presence of a full agonist, as they compete with the full agonist for receptor occupancy, thereby producing a net decrease in the receptor activation as compared to that observed with the full agonist alone.

C) Inverse agonists preferentially bind the inactivated form of the receptor, reducing the amount of active receptors and consequently the intrinsic activity. Depending of the percentage of constitutive active receptors in the system, they can act both as antagonist or inverse agonist (Seifert, Wenzel-Seifert 2002, Milligan 2003a).

2) Insurmountable antagonists. These ligands produce a rightward shift on agonist dose-response curves with an additional decrease in the maximal response (Figure 37B). In turn, insurmountable antagonists can be divided in:

A) Non-competitive. These ligands antagonize the activity of an agonist through an interaction with the allosteric binding site of the same receptor (allosteric antagonism) or binding to an other receptor (functional antagonism).

B) Competitive. Similar to the surmountable antagonists, these ligands bind the orthosteric site of the receptor, so they compete with the agonist to bind the receptor. However these antagonists bind the receptor irreversibly or they dissociate to it very slowly, thus depressing the agonist-induced maximal response (Figure 37B).

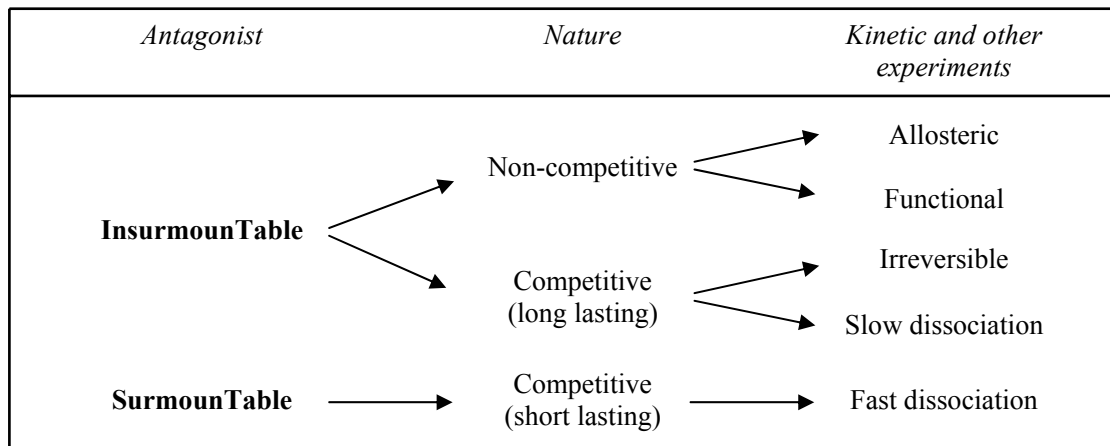


Table 13. Schematic representation of the potential mechanisms for surmountable and insurmountable antagonists. Table adapted from (Vauquelin et al. 2002)

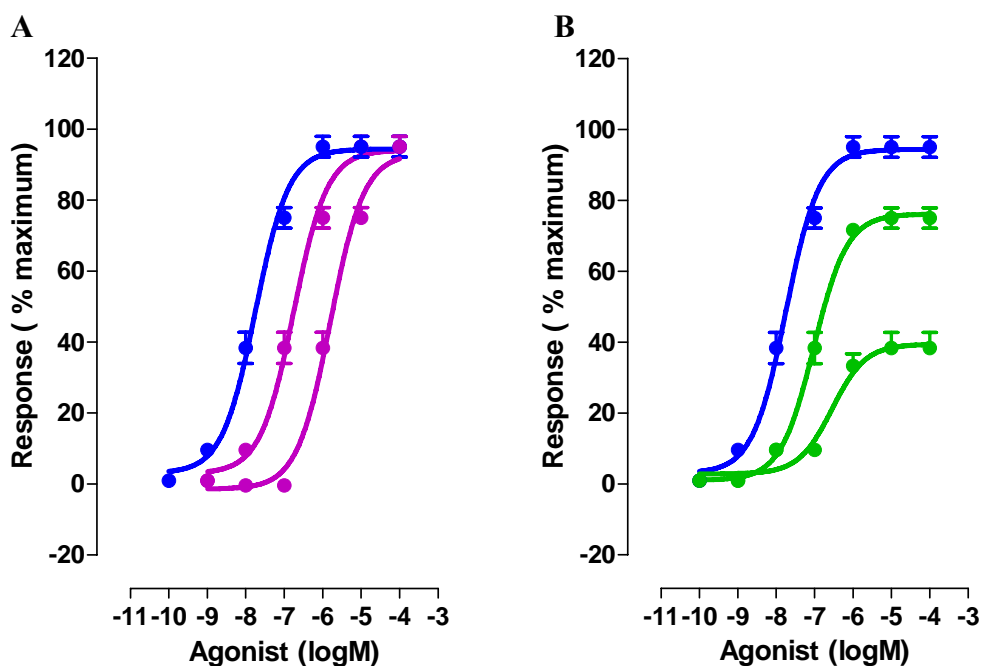


Figure 37. Theoretical agonist dose-response curves with increasing concentrations of surmountable antagonist (A) or competitive insurmountable antagonist (B).

Antagonists at CB₂ receptors

So far, the only antagonists available for CB₂ receptors are ligands with both antagonist and inverse agonist properties. In particular, the most notable CB₂-selective antagonists/inverse agonists are SR144528 and AM630 (6-iodopravolidone) (Figure 38). Both these compounds bind with much higher affinity to CB₂ receptors than to CB₁ receptors, exhibit marked potency as CB₂ antagonists and behave as inverse agonists at CB₂ receptors (Pertwee RG, Tocris Bioscience Scientific review Series). As aforementioned, inverse agonists are defined as ligands which maximally stabilize the inactive R state and reduce basal GDP/GTP exchange. In this regard, SR144528 is referred as a full inverse agonist since it maximally reduces the exchange between GDP and GTP, whereas AM630 is a partial inverse agonist as less efficient in this regard than full inverse agonists (Seifert, Wenzel-Seifert 2002).

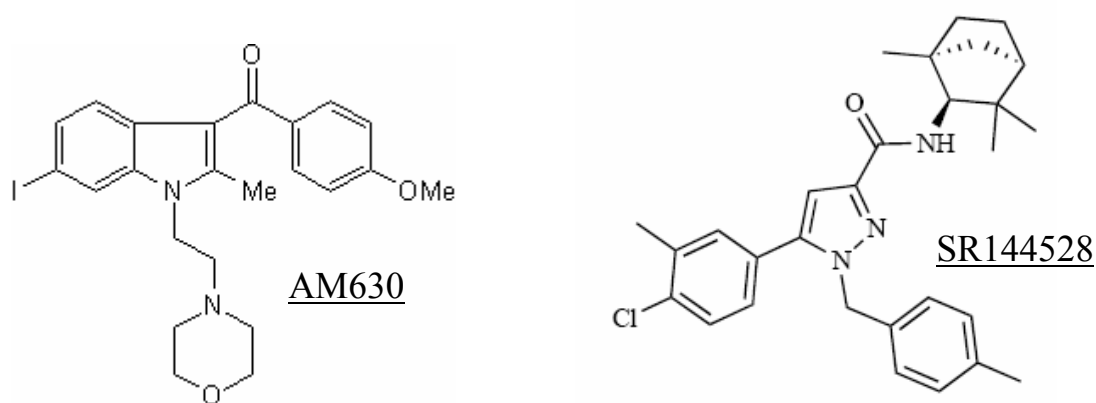


Figure 38. AM630 and SR144528 chemical structures. SR144528 is a pyrazole analogue, whereas AM630 is an aminoalkylindole analogue.

In light of this, the receptor constitutive activity is an important parameter when antagonists/inverse antagonists are tested as antagonists of endogenous ligand or agonist. In fact, in the presence of constitutive active receptors in the system, antagonists/inverse agonists can determine a marked downward shift in the agonist dose-response curve, masking the possible antagonist effect (Figure 39).

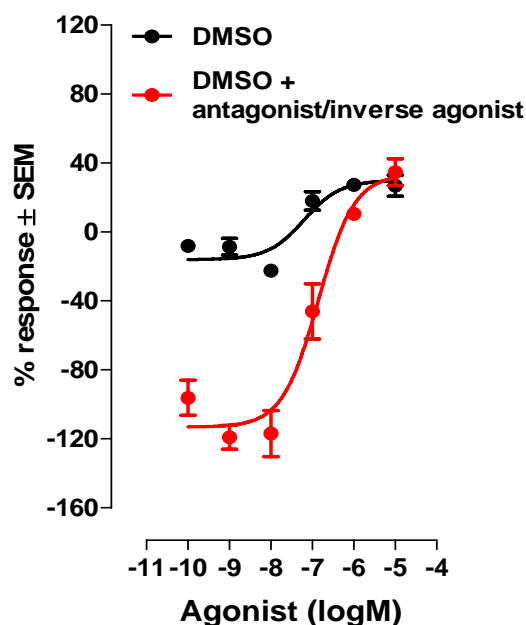


Figure 39. The effect of an antagonist/inverse agonist in the agonist dose-response curve on a biological system characterized by receptor constitutive activity.

The receptor constitutive activity is defined as the ability of a GPCR (G-protein coupled receptor) to adopt an active conformation in the absence of an agonist

(Seifert, Wenzel-Seifert 2002). Constitutive activity has been reported in both native and cellular overexpressing systems (Seifert, Wenzel-Seifert 2002). In particular, constitutive activity can be produced in a recombinant system by increasing the level of receptors expressed on the cell membranes. The dependence of constitutive activity on receptor density $[R_i]$ is given by:

$$\frac{[R_aG]}{[G_{tot}]} = \frac{[R_i]}{[R_i] + (K_G/L)}$$

Equation 1. This equation defines the relation between receptor density $[R_i]$ and constitutive active receptors $[R_aG]$. L is the allosteric constant describing the propensity of the receptor to spontaneously adopt the active state, and K_G is the equilibrium dissociation constant for the activated receptor/G-protein complex.

It can be seen from equation 1 that a hyperbolic relationship is predicted between constitutive activity and receptor concentration (Kenakin 2006).

Constitutive active CB_2 receptors in our cell system (h CB_2 -CHO cells) are documented by results reported in chapter 1. In fact, when AM630 was tested as antagonist of Δ^9 -THCV on forskolin-induced cyclic AMP production in h CB_2 -CHO cells, a marked downward shift of Δ^9 -THCV dose-response curve was observed (Figure 30). Thus, AM630 inverse agonism suggests the presence of CB_2 constitutively active receptors in our cell line.

Hence, the usage of antagonists/inverse agonists in our cell system does not represent a good tool for evaluating the selective activity of a ligand at CB_2 receptors, highlighting the importance to find a protocol to by-pass this problem.

AIMS OF THE PROJECT

The key points of this project were:

1. Eliminate the inverse agonism showed by AM630 in our cell line (hCB₂-CHO cells).
2. Evaluate the possibility that AM630 becomes a neutral antagonist at CB₂ receptors after abolishment of the receptor constitutive activity.
3. Confirm the selective activity of Δ^9 -THCV at CB₂ receptors using AM630 as selective CB₂ receptor neutral antagonist.

RESULTS

AM630 is an antagonist/inverse agonist at hCB₂ receptors

In our initial experiments, we investigated whether Δ^9 -THCV -induced inhibition of forskolin-stimulated cyclic AMP production by hCB₂-CHO cells was susceptible to antagonism by the established selective cannabinoid CB₂ receptor antagonist/inverse agonist, AM630. As expected, (Ross et al. 1999), AM630 by itself produced marked signs of inverse agonism in the cyclic AMP assay performed in hCB₂-CHO cells (Figure 41A). In light of this, the highest concentration of AM630 at which it didn't show any inverse agonism (100nM) was chosen to antagonize the CB₂ receptor partial agonist Δ^9 -THCV.

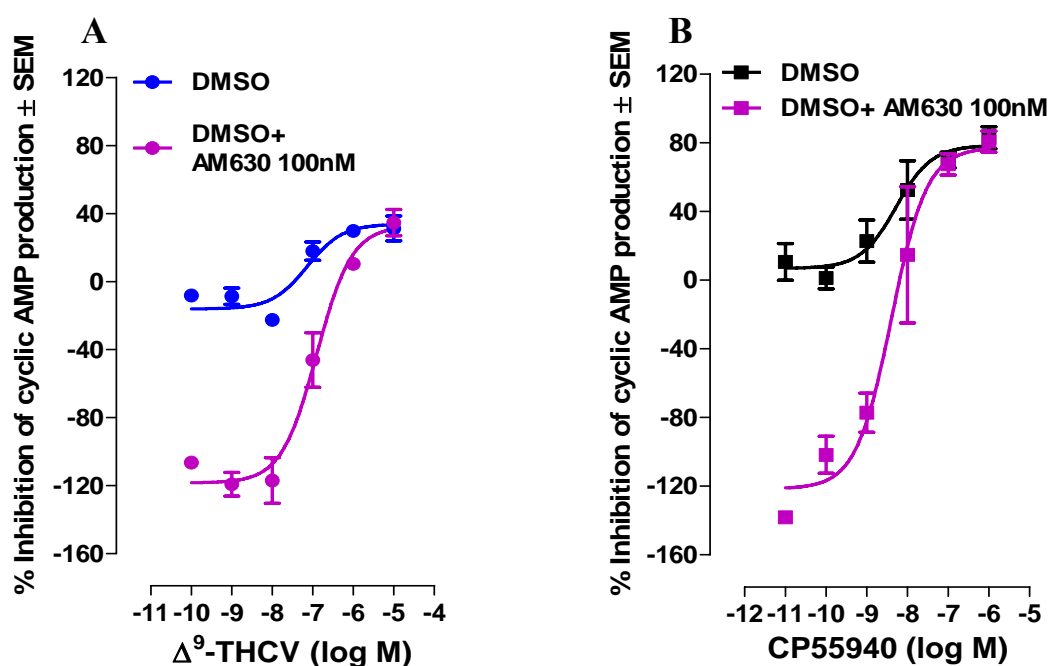
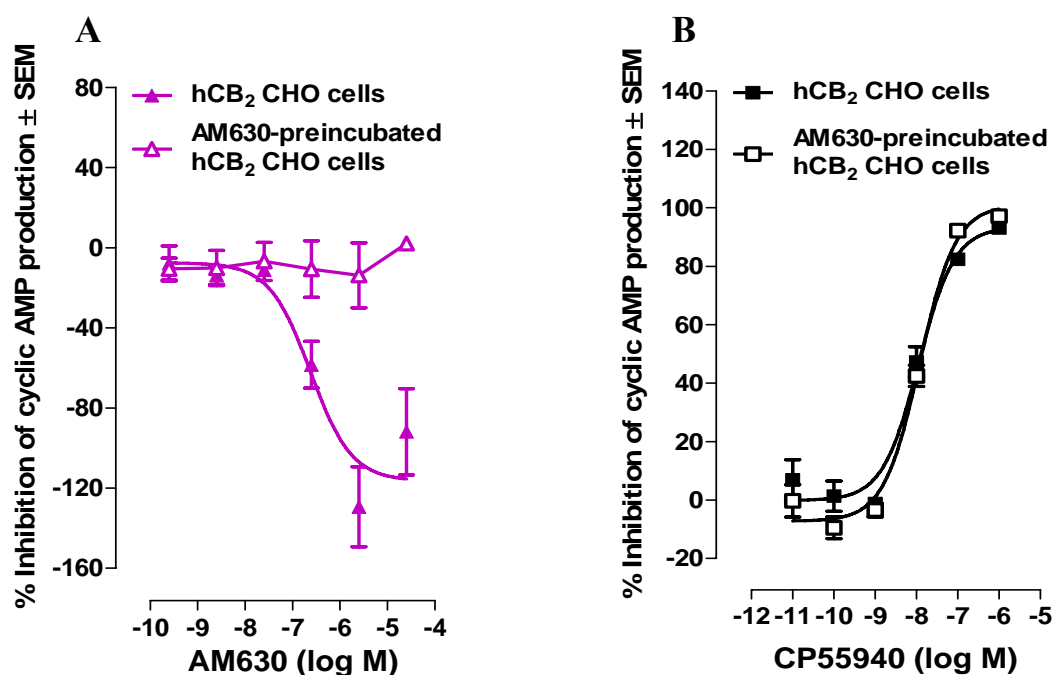


Figure 40. The effect of 100 nM of AM630 on the mean log concentration-response curve of (A) Δ^9 -THCV (n=3) and (B) CP55940 (n=4) on forskolin-induced stimulation of cyclic AMP production in CHO cells transfected with hCB₂ receptors. Symbols represent the mean values \pm SEM. Mean EC₅₀ values of CP55940 and Δ^9 -THCV in the absence of AM630, with 95 % confidence limits shown in brackets, were 5.3 nM (1.2 and 22.7 nM) and 72.7 nM (20.3 and 260.5 nM), respectively. The corresponding mean E_{max} values were 78.3 % (62.1 and 94.3 %) and 33.8 % (21.5 and 46.1 %), respectively.

As shown in Figure 40A and 30, at 100 nM, AM630 displayed a marked and significant downward shift in the log concentration-response curve of Δ^9 -THCV for inhibition of cyclic AMP production in hCB₂-CHO cells. Similar results have also been obtained when AM630 was used to antagonize the established CB₁/CB₂ receptor full agonist, CP55940 (Figure 40B).

Abolition of constitutive activity at hCB₂ receptors

In order to eliminate the inverse agonism of AM630, hCB₂-CHO cells were preincubated for 24 hours with the antagonist /inverse agonist AM630. This protocol has been previously demonstrated to abolish the receptor constitutive activity (Mancini et al. 2009, Seifert, Wenzel-Seifert 2002, Milligan 2003b, Chanrion et al. 2008). Experiments of cyclic AMP were then carried out in parallel both in unpreincubated and AM630-preincubated hCB₂-CHO cells.



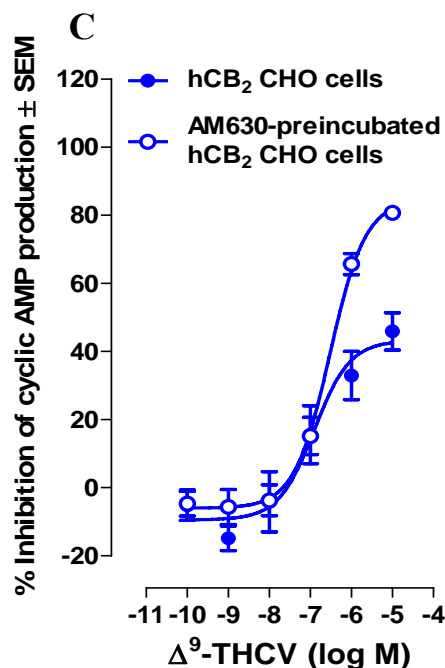


Figure 41. The effect of (A) AM630 (n=3-4), (B) CP55940 (n=6), and (C) Δ^9 -THCV (n=8) on forskolin-induced stimulation of cyclic AMP production in unpreincubated and AM630-preincubated hCB₂-CHO cells. Symbols represent the mean values \pm SEM.

As reported above, AM630 by itself, at concentrations in the range of 0.25 nM to 25 μ M, enhanced the ability of forskolin to stimulate cyclic AMP production in unpreincubated hCB₂-CHO cells (Figure 41A), with EC₅₀ and E_{max} values reported in Table 14. Conversely, when cells were preincubated for 24 hours, AM630 was no longer able to stimulate forskolin-induced cyclic AMP production (Figure 41A). Different scenario has been shown by the full agonist CP55940, which was able to inhibit forskolin-induced cyclic AMP production in both unpreincubated and AM630-preincubated hCB₂-CHO cells (Figure 41B). This it did with EC₅₀ and E_{max} values that did not differ significantly between the two experimental conditions (Table 14). Similarly, the ability of Δ^9 -THCV (Figure 41C) to inhibit forskolin-induced cyclic AMP production was maintained in cells preincubated with AM630 (Figure 41C). However, Δ^9 -THCV displayed a significant higher efficacy in absence of constitutively active CB₂ receptors (Table 14), confirming it to be a partial agonist at this receptor.

<i>Compound</i>	<i>Unpreincubated hCB₂-CHO cells</i>		<i>AM630-preincubated hCB₂-CHO cells</i>	
	EC ₅₀ (95% CLs)	E _{max} (95% CLs)	EC ₅₀ (95% CLs)	E _{max} (95% CLs)
AM630	239,1 nM (65 and 879.1)	-116.4 % (-142.6 and -90.3)	ND	ND
CP55940	11.2 nM (7 and 17.7)	93.4 % (85.9 and 100.9)	11.9 nM (8.6 and 16.6)	100.9 % (94.5 and 107.2)
Δ ⁹ -THCV	123.3 nM (37.7 and 403.6)	123.3 % (32.1 and 54.3)	298 nM (182 and 488)	84.5 % (76 and 92.9)

Table 14. EC₅₀ and E_{max} values of AM630 (n = 3), CP55940 (n = 6) and Δ⁹-THCV (n = 8) for inhibition of forskolin-induced stimulation of cyclic AMP production in unpreincubated and AM630-preincubated hCB₂-CHO cells.

ND, non determined indicates that the drug had not effect on the assay.

CLs, confidence limits.

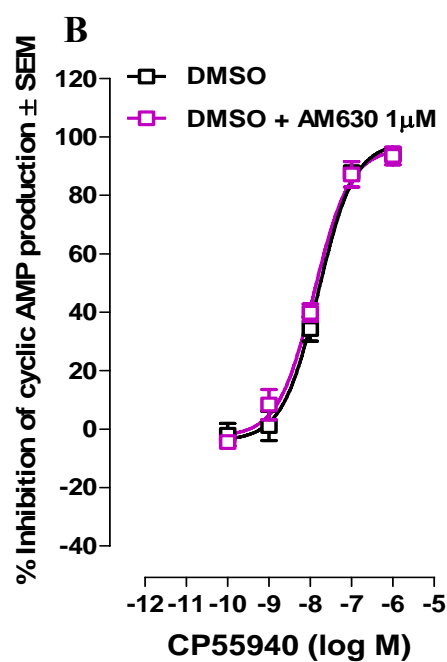
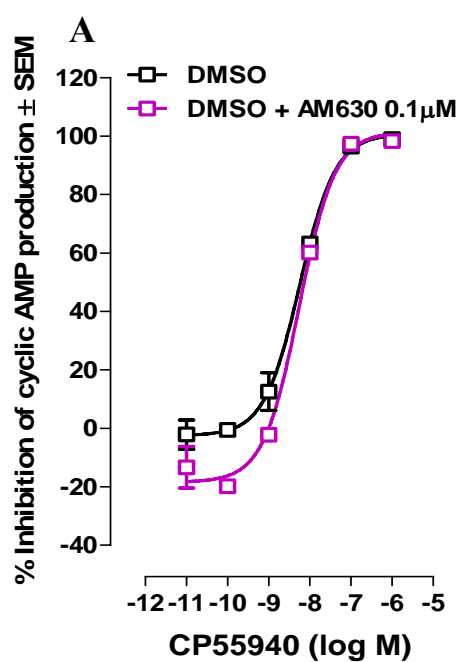
AM630 becomes an apparent neutral antagonist in AM630-preincubated hCB₂-CHO cells

The results obtained in AM630-preincubated hCB₂-CHO cells raised the possibility that AM630 might behave as neutral antagonist in absence of constitutively active CB₂ receptors. Then, to investigate this possibility, we carried out experiments of cyclic AMP to establish whether AM630 could still antagonize the CB₁/CB₂ receptor full agonist, CP55940, in hCB₂-CHO cells preincubated with AM630.

We found that AM630, at concentrations in the range of 0.1 μM up to 25 μM, did not display any downward shift on CP55940-induced inhibition of cyclic AMP production (n= 2-4). In detail, at 0.1 and 1 μM, AM630 did not display any downward and/or rightward shift in the log concentration-response curve of CP55940 (Figure 42A and B). At 10 μM, AM630 displayed a slight antagonism on CP55940 dose-response curve (Figure 42C), with a rightward shift not significantly different from 1. Again, no downward shift has been shown by AM630 at this concentration. At 25 μM, AM630 displayed a significant antagonism on CP55940-induced inhibition of cyclic AMP production (Figure 42D). This it did with a mean apparent K_B value of 5.2 μM that was significantly higher than its apparent K_i value for displacement of

[³H]CP55940 from hCB₂-CHO cells (Ross et al. 1999). Importantly, no downward shift was observed in the log concentration-response curve of CP55940, suggesting AM630 to be a neutral antagonist in absence of constitutively active CB₂ receptors (Figure 42D). In addition, as this rightward shift did not deviate significantly from parallelism, it is likely that AM630, in these experimental conditions, behaves as a competitive antagonist at hCB₂ receptors.

In AM630-preincubated hCB₂-CHO cells (Figure 42E), at 25 μM, AM630 antagonized also Δ⁹-THCV-induced inhibition of cyclic AMP production. The mean apparent K_B value for this antagonism was 2.1 μM, which did not differ significantly from the mean K_B value for the antagonism of CP55940 by AM630. Again, this rightward shift (Figure 42E) did not differ significantly from parallelism.



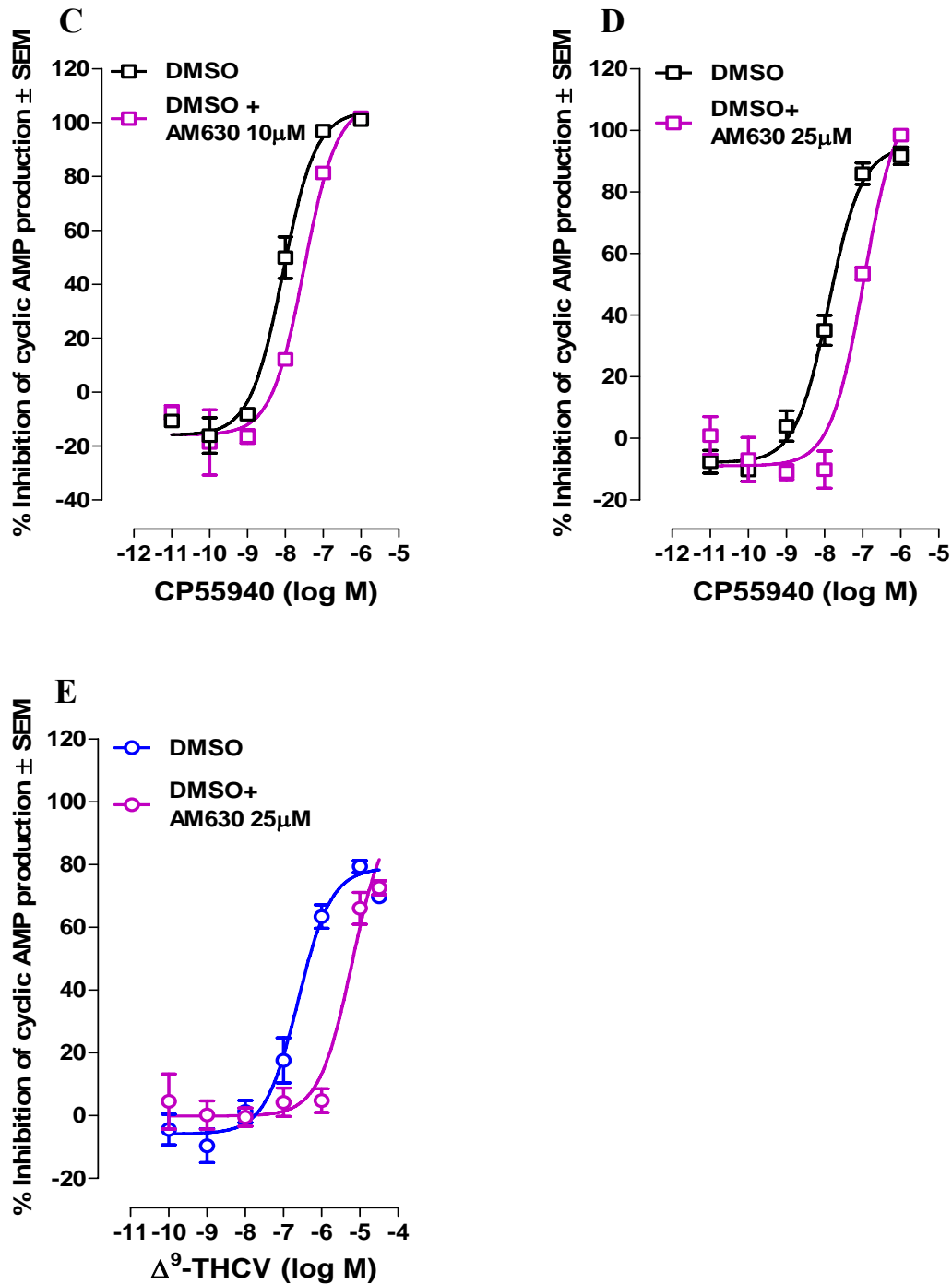


Figure 42. The effect of 0.1, 1, 10 and 25 μ M of AM630 on the mean log concentration-response curve of (A, B, C, D) CP55940 (n=2-4) and (E) Δ^9 -THCV (n=4) on forskolin-induced stimulation of cyclic AMP production in hCB₂-CHO cells preincubated with AM630. Symbols represent the mean values \pm SEM. The mean apparent K_B values of AM630 for its antagonism of CP55940 (D) and Δ^9 -THCV (E), with 95% confidence limits shown in brackets, were 5.2 μ M (2.9 and 9.2 μ M) and 2.1 μ M (1.1 and 4.1 μ M), respectively. The rightward shift produced by AM630 in the mean log concentration-response curve of both CP55940 (D) and Δ^9 -THCV (E) did not deviate significantly from parallelism ($p > 0.20$).

AM630 antagonizes WIN55212-2 in AM630-preincubated hCB₂-CHO cells

The reason why AM630 displayed low affinity as both CP55940- and Δ^9 -THCV-antagonist, in AM630-preincubated cells, is to be further investigated. One possible explanation is that AM630, unlike CP55940, is an aminoalkylindole. It could be possible that CP55940 and AM630 bind to hCB₂ receptors using different binding pockets.

With this in mind, we went on to investigate whether AM630 could antagonize more potently the CB₁/CB₂ receptor full agonist and aminoalkylindole analogue, WIN55212-2. We then carried out experiments of cyclic AMP using AM630-preincubated hCB₂-CHO cells.

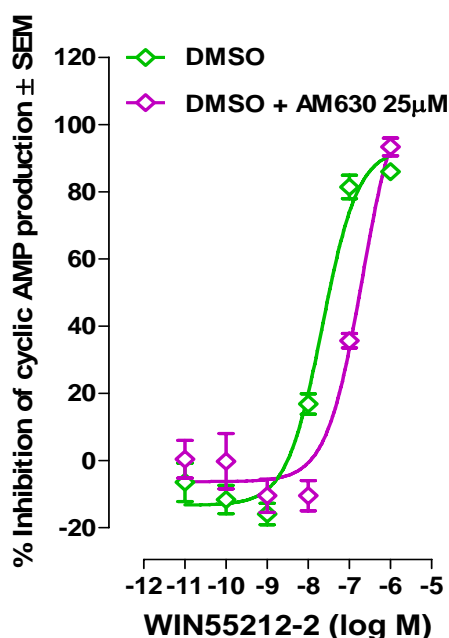


Figure 43. The effect of 25 μ M AM630 on the mean log concentration-response curve of WIN55212-2 ($n = 4$) on forskolin-induced stimulation of cyclic AMP production in hCB₂-CHO cells preincubated with AM630. Symbols represent the mean values \pm SEM. The mean apparent K_B value, with 95 % confidence limits shown in brackets, was 6.9 μ M (3.3 and 12.8 μ M). The rightward shift produced by AM630 in the mean log concentration-response curve of WIN55212-2 did not deviate significantly from parallelism ($p > 0.20$).

As shown in Figure 43, at 25 μ M, AM630 was indeed able to antagonize WIN55212-2 with a mean apparent K_B value of 6.9 μ M which was not significantly different from the one displayed to antagonize both CP55940 and Δ^9 -THCV. Again, no

downward shift was displayed by AM630 in the log concentration-response curve of WIN55212-2 (Figure 43). This result suggests that AM630 could interact and bind the same CB₂ receptor binding pocket of CP55940.

Taken together, these findings support the hypothesis that AM630 is a neutral antagonist at CB₂ receptors in absence of constitutively active receptors, albeit with much less affinity for CB₂ receptors in these experimental conditions than in unpreincubated hCB₂-CHO cells (Ross et al. 1999).

AM630-preincubation at different time points

A long exposure of cells to an inverse agonist could create alterations in receptor signalling. In order to rule this out, we carried out experiments of cyclic AMP assays in which hCB₂-CHO cells were preincubated with AM630 at different time points and treated thereafter with AM630.

Preincubation time (hours)	Unpreincubated hCB ₂ -CHO cells		AM630-preincubated hCB ₂ -CHO cells	
	EC ₅₀ (95% CLs)	E _{max} (95% CLs)	EC ₅₀ (95% CLs)	E _{max} (95% CLs)
0.5	80.3 nM (17.8 and 359.4)	-174.1% (-207 and -141.5)	137.6 nM (6.42 and 2950)	-41.5% (-58.3 and -24.8)
1	101.7 nM (46.9 and 220.6)	-195.7% (-215.3 and -176.2)	16.9 nM (1.9 and 153.1)	-36.2% (-44.3 and -28.1)
2	224.5 nM (98.7 and 510.6)	-212.7% (-242.4 and -183)	60.1 nM (3.3 and 1102)	-26.4% (-37.3 and -15.5)
6	156.8 nM (42.4 and 580.4)	-246.7% (-293.7 and -199.6)	ND	ND
12	157.2 nM (100.9 and 245)	-336.2% (-359.2 and -313.3)	ND	ND

Table 15. EC₅₀ and E_{max} values of AM630 (n = 2-4), for inhibition of forskolin-induced stimulation of cyclic AMP production in hCB₂-CHO cells preincubated at different time points with vehicle (unpreincubated) or AM630.

ND, non determined indicates that the drug had not effect on the assay.

CLs, confidence limits.

As shown in Table 15, following 0.5, 1 and 2 hours of preincubation, AM630 displayed a slight inverse agonism by enhancing the ability of forskolin to stimulate cyclic AMP production. At 6 hours, AM630 was no longer able to alter forskolin-induced cyclic AMP production (Table 15), suggesting this experimental to be sufficient to abolish the CB₂ receptor constitutive activity. Similar results were obtained after 12 hours of incubation with AM630 (Table 15).

In order to investigate whether AM630 could antagonize more potently the non selective CB₂ receptor agonist, CP55940, experiments of cyclic AMP assay were carried out in hCB₂-CHO cells preincubated with AM630 for 6 hours.

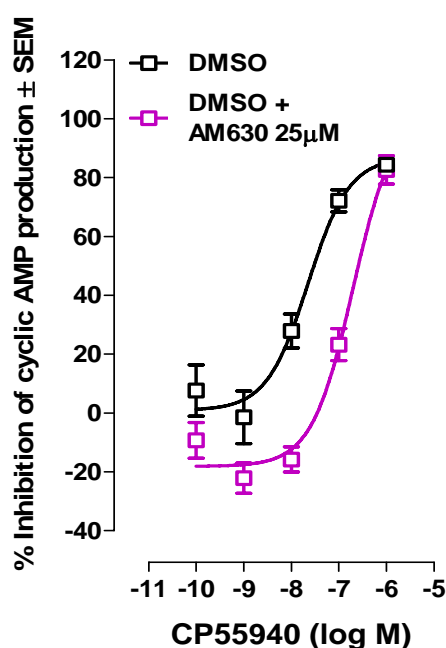


Figure 44. The effect of 25 μM AM630 on the mean log concentration-response curve of CP55940 (n = 4) on forskolin-induced stimulation of cyclic AMP production in hCB₂-CHO cells preincubated for 6 hours with AM630. Symbols represent the mean values ± SEM. The mean apparent K_B value, with 95 % confidence limits shown in brackets, was 2.6 μM (1.2 and 5.5 μM).

As shown in Figure 44, 25 μM AM630 was not more effective as antagonist of CP55940 to hCB₂-CHO cells preincubated with AM630 for 6 hours than after 24 hours of preincubation (Figure 42B). Moreover, AM630 displayed a slight but significant inverse agonism in the log concentration-response curve of CP55940 (Figure 44). These results suggested that a preincubation time less than 24 hours is not enough to abolish completely the constitutively active CB₂ receptors. In light of this, we carried on preincubating hCB₂-CHO cells with AM630 for 24hours.

Displacement binding assays in AM630-preincubated hCB₂-CHO cell membranes

The high K_B values obtained with AM630 in absence of constitutively active CB₂ receptors prompted us to investigate whether the affinity of AM630 for hCB₂ receptors could negatively have been affected by the 24 hours pretreatment. Specifically, [³H]CP55940 displacement binding assays both in unpreincubated and AM630-preincubated cell membranes have been performed. AM630 has been tested in parallel with CP55940 and Δ^9 -THCV.

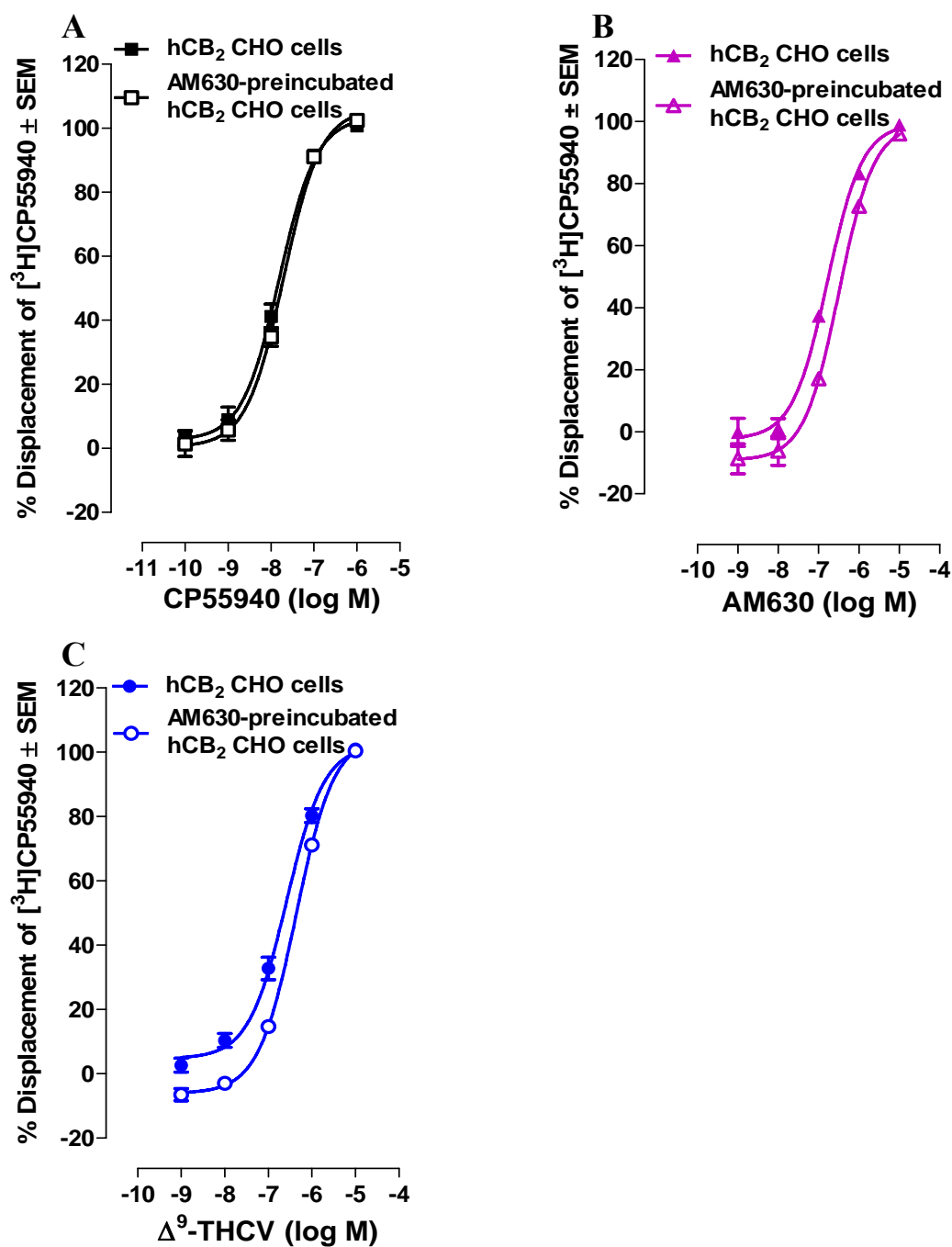


Figure 45. Displacement of [³H]CP55940 by CP55940 (A), AM630 (B), and Δ⁹-THCV (C) from specific binding sites in membranes from AM630-preincubated or unpreincubated hCB₂-CHO cells (n=4-8). Each symbol represents the mean percentage displacement ± SEM.

As shown in Figure 45A, CP55940 displaced [³H]CP55940 from specific binding sites with mean IC₅₀ values not significantly different between unpreincubated and AM630-preincubated hCB₂-CHO cell membranes (Table 16). Conversely, the potency displayed by both AM630 and Δ⁹-THCV (Figure 45B and C) in displacing [³H]CP55940 was approximately two times lower in AM630-preincubated hCB₂-CHO cell membranes compared to unpreincubated hCB₂CHO cell membranes (Table 16). However, such a difference did not justify the high K_B values obtained for AM630 on cyclic AMP assay (Figure 42 and 43).

Compound	IC ₅₀ nM (95% CLs)	
	hCB ₂ -CHO cells	AM630-preincubated hCB ₂ -CHO cells
CP55940	15.6 nM (11.7 and 20.8 nM)	19.6 nM (14.7 and 26.1 nM)
AM630	165.5 nM (124.2 and 220.5 nM)	317.4 nM (225.6 and 446.7 nM)
Δ ⁹ -THCV	261.8 nM (198.1 and 345.9 nM)	437.2 nM (387.3 and 493.6 nM)

Table 16. IC₅₀ values of CP55940, AM630 and Δ⁹-THCV for displacement of [³H]CP55940 from specific binding sites on unpreincubated (n = 4) or AM630-preincubated hCB₂-CHO cell membranes (n=4 or 8).

[³⁵S]GTPγS binding assays in AM630-preincubated hCB₂-CHO cell membranes

Since displacement binding assays were performed using hCB₂-CHO cell membranes and cyclic AMP assays using intact cells, it is likely that the pretreatment with AM630 decreased the receptor constitutive activity only on the cell surface. In light of this, experiments of [³⁵S]GTPγS binding in both unpreincubated and AM630-preincubated hCB₂-CHO cell membranes have been carried out.

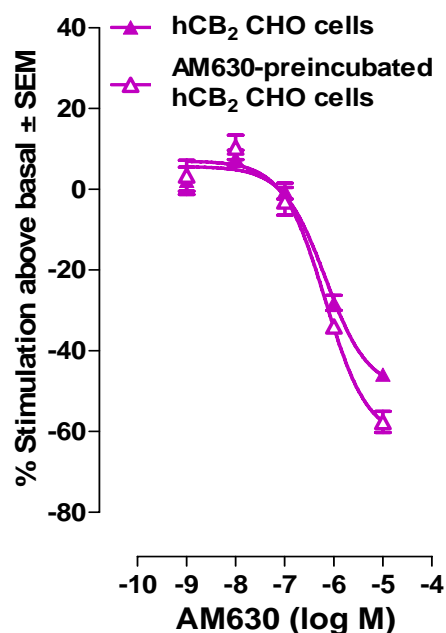


Figure 46. The effect of AM630 on [³⁵S]GTPγS binding to unpreincubated or AM630-preincubated hCB₂-CHO cell membranes (n=4). Each symbol represents the mean percentage change in [³⁵S]GTPγS binding ± SEM. The mean EC₅₀ values of AM630 in unpreincubated and AM630-preincubated hCB₂-CHO cells, with 95% confidence limits shown in brackets, were 675.5 nM (402.8 and 1133 nM) and 669.6 nM (386.7 and 1159 nM), respectively. The corresponding E_{max} values were -49.7 % (-56 and -43 %) and -61.9 % (-70.3 and -53.5 %), respectively.

We found that AM630, in both experimental conditions (Figure 46), produced a concentration-related inhibition of [³⁵S]GTPγS binding, thus behaving in both cases as inverse agonist. This it did with EC₅₀ and E_{max} values very similar in both unpreincubated and AM630-pretreated cell membranes (Figure 46).

Displacement binding assays in AM630-preincubated whole hCB₂-CHO cells

Results from [³⁵S]GTPγS binding assays raised the possibility that a preincubation time of 24 hours with AM630 could affect exclusively the constitutively active CB₂ receptors on the cell surface. We therefore carried out some additional experiments directed at testing this hypothesis. Displacement binding experiments with AM630 and CP55940 were performed in whole hCB₂-CHO cells.

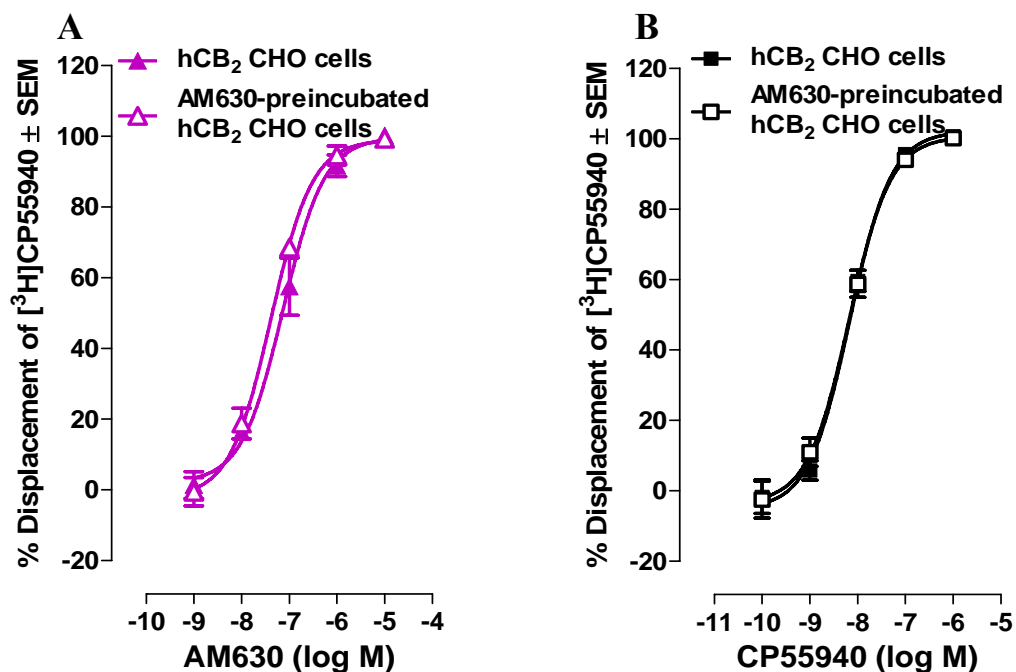


Figure 47. Displacement of [³H]CP55940 by AM630 (A) and CP55940 (B) from specific binding sites on unpreincubated and AM630-preincubated whole hCB₂-CHO cells (n = 4). Each symbol represents the mean percentage displacement ± SEM. Mean IC₅₀ values of AM630 in unpreincubated and AM630-preincubated whole hCB₂-CHO cells, with 95% confidence limits shown in brackets, were 73.6 nM (45.9 and 118.1 nM) and 41.5 nM (29.4 and 58.5 nM), respectively. The corresponding IC₅₀ values for CP55940 were 6.8 nM (5.1 and 9.1 nM) and 6.6 nM (4.7 and 9.4 nM).

As shown in Figure 47, both these compounds were able to displace [³H]CP55940 from specific binding sites with mean IC₅₀ values not significantly different between unpreincubated and AM630-preincubated whole hCB₂-CHO cells.

DISCUSSION

AM630 is a well-known selective CB₂ receptor antagonist/inverse agonist (Ross et al. 1999) that, based on the “two state model”, is able to bind preferentially to the inactive form of the receptor (R), reducing the amount of the active receptors (R*) and consequently their intrinsic activity (Seifert, Wenzel-Seifert 2002, Milligan 2003a).

In our initial experiments of cyclic AMP assays, using hCB₂-CHO cells, we have confirmed the inverse agonism of AM630 (Figure 41A), suggesting the presence of constitutively active CB₂ receptors in our cells.

When we tested AM630 as potential CP55940- and Δ⁹-THCV-antagonist (Figure 40), we found that the inverse agonism was still present at 100 nM, the highest concentration at which AM630 did not show any detectable effect by itself (Figure 41A).

These results were not surprisingly since, as we have already reported for the other selective CB₂ receptor antagonist/inverse agonist, SR144528 (Thomas et al. 2007), some inverse agonists could become more potent in presence of agonists, i.e. CP55940 and Δ⁹-THCV, that shift the equilibrium from the inactive form of the receptor (R) to the active form (R*), causing a considerable increase of the constitutively active receptors (Thomas et al. 2007, Leff 1995).

Then, in presence of constitutively active receptors, the antagonistic properties of some compounds could be masked by their inverse agonism.

So far only antagonists/inverse agonists are available for CB₂ receptors. Therefore, a strategy aimed to transform inverse agonists in neutral antagonists is fundamental. To this intention, it has been reported in literature that the receptor constitutive activity in a cell system can be decreased by inverse agonist long-term exposure (Mancini et al. 2009, Seifert, Wenzel-Seifert 2002, Milligan 2003b, Chanrion et al. 2008, Rinaldi-Carmona et al. 1998, Kenakin 2004). With this in mind, following their experimental conditions (Mancini et al. 2009), hCB₂-CHO cells have been preincubated for 24 hours with 10 μM AM630. We found that, as consequence of this long-term exposure, AM630 was no longer able to stimulate forskolin-induced cyclic AMP production (Figure 41). This is in agreement with data reported in literature (Mancini et al. 2009), showing that AM630 did not display any detectable activity both in AM630-

pretreated rat and human CB₂-CHO cells. As expected, the non selective CB₁/CB₂ receptor full agonist, CP55940, did not show any changes both in its potency and efficacy in AM630-pretreated cells compared to the unpretreated cells (Figure 41). Again, this result is in agreement with data already reported both in rat and human CB₂-CHO cells (Mancini et al. 2009). Moreover, similar results were reported in Rinaldi-Carmona et al., (1998) where both the efficacy and potency of CP55940 dose-response curve were unaltered in hCB₁-CHO cells preincubated with the selective CB₁ receptor antagonist/inverse agonist, SR141716A.

Differently than CP55940 and as we expected, the CB₂ receptor partial agonist, Δ⁹-THCV, increased its efficacy in AM630-preincubated hCB₂-CHO cells (Figure 41). This effect could be due to the fact that, the preincubation with an inverse agonist causes not only abolishment of the constitutively active receptors but also an increase in the number of receptors, in particular on the cell surface (Chanrion et al. 2008, Rinaldi-Carmona et al. 1998, Bouaboula et al. 1999, Bouaboula, Dussosoy & Casellas 1999). In support of this, Bouaboula et al (1999) reported an increase in the G protein pool after preincubating CHO cells overexpressing hCB₂ receptors with the antagonist/inverse agonist, SR144528. Then, it is likely that, following AM630 long-term exposure, the increase in Δ⁹-THCV maximal efficacy is a consequence of the highest levels of CB₂ receptors and associated G proteins. This would also confirm our previous results (Bolognini et al., 2010), that Δ⁹-THCV behaves as a partial agonist at CB₂ receptors (Newman-Tancredi et al. 2000, Kenakin 2001).

In addition, in our experiments of cyclic AMP, AM630 was found to behave as an apparent neutral antagonist in absence of constitutively active CB₂ receptors, since none of the concentrations tested displayed a downward shift in CP55940 dose-response curve (Figure 42).

Surprisingly, AM630 behaved as a reasonably weak competitive antagonist of CP55940, as indicated by the manner at which it antagonized the ability of this cannabinoid receptor agonist to inhibit forskolin-induced cyclic AMP production in AM630-preincubated hCB₂-CHO cells (Figure 42). In detail, the K_B value displayed by AM630 for this antagonism was 5.2 μM, which was approximately 100 times higher compared to the mean K_i or IC₅₀ values of AM630 for its displacement of [³H]CP55940 from specific binding sites in unpreincubated hCB₂-CHO cells (Ross, 1999, Table 16).

AM630 was also able to antagonize, in the same experimental conditions, the CB₂ partial agonist, Δ⁹-THCV, in an apparent competitive manner (Figure 42) and with a K_B value not significantly different to the one displayed for the antagonism of CP55940 (Figure 42).

The reason of such a very low potency of AM630 as both CP55940- and Δ⁹-THCV-antagonist, in AM630-preincubated cells, is not clear yet and needs further investigation. A reasonable explanation could reside in the fact that AM630, differently than CP55940 and Δ⁹-THCV, has an aminoalkylindole chemical structure. It could be possible that AM630, as already reported for the other aminoalkylindole WIN55212-2 (Reggio 1999), binds to hCB₂ receptors using a binding pocket different than that used by CP55940 and/or Δ⁹-THCV. With the aim of ruling out this hypothesis we have investigated further the ability of AM630 (25 μM) to antagonize WIN55212-2 in AM630-preincubated hCB₂-CHO cells (Figure 43). Unfortunately, our results did not confirm our hypothesis, showing that AM630 antagonizes WIN55212-2 with a rightward shift not significantly different than the one displayed for the antagonism of both CP55940 and Δ⁹-THCV (Figure 43). This result suggests that CP55940, Δ⁹-THCV, AM630 and WIN55212-2 could interact with a common CB₂ receptor binding pocket. It is also unlikely that AM630 effect is due to an interaction with different kind of GPCRs expressed in CHO cells, since in experiments of cyclic AMP performed in untransfected CHO cells, AM630 did not show any significant effect (data not shown).

Next, with the aim of confirming the results observed in cyclic AMP assays, we have also performed [³⁵S]GTPγS binding assays using membranes from both AM630-preincubated and unpretreated hCB₂-CHO cells. Surprisingly, we found that AM630 behaved as an inverse agonist in both experimental conditions (Figure 46). A possible explanation of these apparent contradictory results could be that the [³⁵S]GTPγS binding assay was performed in cell membranes, instead the cyclic AMP assay in intact cells. Several studies have reported the presence of functional intracellular GPCRs (Marrache et al. 2005, Gobeil et al. 2006, Zhu et al. 2006). In particular, Rozenfeld et al. (2008) have shown the presence of functional cannabinoid type 1 (CB₁) receptors in intracellular endosomal/lysosomal compartments where they are associated with the subunit Gα_i. Similarly, it is likely that AM630 long-term exposure increases CB₂ receptor density not only on the cell surface but also in the

endosomal/lysosomal compartments. If so, the inverse agonism displayed by AM630 on AM630-preincubated hCB₂-CHO cell membranes in [³⁵S]GTPγS binding assay could be the result of CB₂ intracellular active receptors. It is also likely that the pretreatment with AM630 decreases the CB₂ receptor intrinsic activity only on the cell surface, maintaining unaltered possible intracellular constitutively active CB₂ receptors. Unfortunately we cannot rule out this hypothesis, since [³⁵S]GTPγS binding assays in intact cells cannot be done because of the inability of the [³⁵S]GTPγS reagent to cross cell membranes (Harrison, Traynor 2003).

An alternative explanation of such a low potency of AM630 as antagonist, in AM630-pretreated cells, could be that the affinity of this compound for CB₂ receptors has been reduced by the pretreatment. To rule out this hypothesis, we have performed experiments of displacement binding in both unpreincubated and AM630-preincubated hCB₂-CHO cell membranes, using CP55940, AM630 and Δ⁹-THCV. We found that these compounds were able to displace [³H]CP55940 from specific binding sites in both unpreincubated and AM630-preincubated hCB₂-CHO cell membranes with a potency ratio of 0.8, 0.52 and 0.6, respectively (Table 16). Specifically, in hCB₂-CHO cells preincubated with AM630, CP55940 displayed a slight, although not statistically significant, lower affinity, while AM630 and Δ⁹-THCV displayed two times lower affinity for displacement of [³H]CP55940 from specific binding sites. However, these data did not explain why, in cyclic AMP assays, AM630 displayed 100 times lower potency for antagonism of CP55940 in AM630-preincubated hCB₂-CHO cells. Also, Δ⁹-THCV did not show any significant difference in the potency for inhibition of forskolin-induced cyclic AMP production in unpreincubated and AM630-preincubated hCB₂ cells.

The slight lower affinity displayed by CP55940, AM630 and Δ⁹-THCV in AM630-preincubated hCB₂-CHO cells, in displacement binding assays, could be explained by the increase of CB₂ receptor density after the pretreatment (Chanrion et al. 2008, Rinaldi-Carmona et al. 1998, Bouaboula et al. 1999, Bouaboula, Dussosoy & Casellas 1999). In fact, one possible effect of a large receptor concentration is to reduce the potency with which a tritiated ligand is displaced from its specific binding sites by an unlabelled compound (Kenakin 1997, Bolognini et al. 2010).

Conversely to the data obtained in cell membranes, experiments of displacement binding performed in intact hCB₂-CHO cells showed that CP5540 and AM630

displaced [³H]CP55940 from specific binding sites with a similar potency both in unpreincubated and AM630-preincubated cells (Figure 47). At the moment we do not know why differences in the ligand affinity were displayed in membranes and not in intact hCB₂-CHO cells in experiments of displacement binding. It could be possible that the pretreatment with AM630 causes an increase of CB₂ receptor expression in intracellular compartments more than on the cell surface.

Taken together, these results suggest that AM630 does not change significantly its affinity for CB₂ receptors after AM630 long-term exposure in displacement binding experiments. Hence, the reason why AM630 displayed a marked low affinity in experiments of cyclic AMP, after elimination of constitutively active CB₂ receptors, warrants further investigations. It is possible that AM630 has two different affinities for the CB₂ receptor, one for the inactive form (R) and one for the active form (R*). In particular, AM630 would have high affinity (nanomolar range) for the receptor inactive form (R), and this would explain its inverse agonist properties. On the other hand, AM630 would have only low affinity (micromolar range) for the receptor active form (R*), which would be responsible of the antagonism at CB₂ receptors.

Further investigations will now be directed at testing these hypotheses. In particular, in order to confirm the results obtained so far with AM630, further experiments will be performed with the other well-known CB₂ receptor antagonist/inverse agonist, SR144528.

Concluding, since neutral antagonist at CB₂ receptors have not been discovered yet and that the receptor constitutive activity is a common property for cells overexpressing a particular receptor, the inverse agonist long-term exposure method could be a useful tool to decrease the receptor constitutive activity and consequently to transform an antagonist/inverse agonist into an apparent neutral antagonist. Thus, this protocol could find application in experiments of high throughput screening to test the selectivity of compounds at CB₂ receptors.

Regarding Δ⁹-THCV, these results further confirm our previous hypothesis that this phytocannabinoid behaves as a partial agonist at CB₂ receptors.

CONCLUSIONS CHAPTER ONE AND TWO

The main findings from chapter one and two are:

- Δ^9 -THCV displayed high affinity for CB₂ receptors (K_i of 225.1 nM) in experiments of displacement binding assay on hCB₂-CHO cell membranes (Figure 25)
- Δ^9 -THCV, compared to the CB₁/CB₂ receptor full agonist (CP55940), displayed partial agonism in experiments of [³⁵S]GTP γ S binding and cyclic AMP assays on hCB₂-CHO cells (Table 12)
- Δ^9 -THCV did not show any effect in experiments of cyclic AMP assay on untransfected- or PTX-treated hCB₂-CHO cells, confirming the selective activity of Δ^9 -THCV at hCB₂ receptors (Figure 24, 31).
- Δ^9 -THCV displayed selective partial agonism in [³⁵S]GTP γ S binding experiments on wild type but not CB₂^{-/-} mouse spleen membranes, confirming the ability of Δ^9 -THCV to stimulate both human and mouse CB₂ receptors (Bolognini et al. 2010).
- Δ^9 -THCV displayed a significant higher efficacy in experiments of cyclic AMP assay on hCB₂-CHO cells after AM630 long-term exposure (Figure 41). Hence, Δ^9 -THCV is still an agonist in absence of constitutively active CB₂ receptors.
- Δ^9 -THCV was antagonized by the CB₂ receptor selective antagonist/inverse agonist, AM630, in AM630-treated hCB₂-CHO cells on cyclic AMP assays (Figure 42).
- The partial agonism of Δ^9 -THCV at CB₂ receptors has also been shown efficacious as anti-inflammatory and anti-hyperalgesic agent on *in vivo* models of λ -carrageenan-induced oedema and thermal hyperalgesia, and formalin-induced nociception (Figure 18, 19, 20, 21, 22). However, these data suggest the involvement not only of CB₂ receptors, but also of CB₁ receptors.

Thus, all these findings confirm Δ^9 -THCV to be a partial agonist at CB₂ receptors and that this activity might be implicated in Δ^9 -THCV-mediated anti-inflammatory effects. Conversely, Δ^9 -THCV mediated anti-hyperalgesic effect seems to involve also CB₁ receptors rather than only CB₂ receptors.

In vitro, future directions will be addressed at investigating the ability of Δ^9 -THCV to interact and mediate pathways involved in inflammatory states.

In vivo it will be important to establish more conclusively whether, as has been proposed for CB₂ receptor agonists (Guindon, Hohmann 2008), Δ^9 -THCV has therapeutic potential both as an anti-inflammatory agent and for the relief of inflammatory, or indeed, neuropathic pain.

CHAPTER THREE

Pharmacological properties of the phytocannabinoid cannabidiol at somatodendritic 5-HT_{1A} autoreceptors

INTRODUCTION

Cannabidiol (CBD) is the main non-psychoactive cannabis constituent. This compound was first isolated in late 1930s, and was chemically characterized 30 years later (Mechoulam, Shvo 1963). The CBD molecule is chiral, and the plant cannabis contains only the levorotatory form ((-)-CBD) (Figure 48).

Like most of the cannabis constituents, CBD is insoluble in water but soluble in organic solvents. At room temperature it is a colourless crystalline solid, and under acidic conditions it cyclizes to Δ^9 -THC.

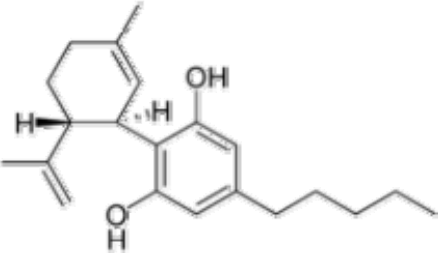
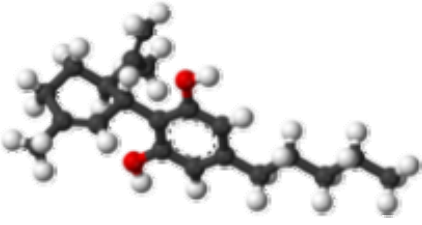
	
<u>Systematic (IUPAC) name</u>	
2-[(1R,6R)-6-isopropenyl-3-methylcyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol	
<u>Chemical data</u>	
Formula	C ₂₁ H ₃₀ O ₂
Molecular Mass	314.46 g/mol
CAS Numbers	13956-29-1
SMILES	<chem>Oc1c(c(O)cc(c1)CCCC)[C@@H]2\C=C/CC[C@H]2\C(=C)C</chem>

Figure 48. CBD chemical structure and data.

CAS (Chemical Abstracts Service) registry numbers are unique numerical identifiers for chemical elements, compounds, polymers, biological sequences, mixtures and alloys.

SMILES (simplified molecular input line entry specification) is a specification for unambiguously describing the structure of chemical molecules using short ASCII strings.

Regarding the pharmacokinetics, CBD metabolism is similar to that of Δ^9 -THC, with primary oxidation of C9 to the hydroxy and carboxylic acid moieties, and side chain oxidation (Huestis 2005b). Like Δ^9 -THC, CBD is subjected to a significant first-pass

effect, which reduces considerably the amount of CBD and its metabolites in the circulatory system. Unlike Δ^9 -THC, a large proportion of CBD is excreted unchanged in the faeces (Huestis 2005b). Moreover, it has been proven that the co-administration of CBD and Δ^9 -THC does not significantly affect the clearance, volume of distribution, and terminal elimination half-lives of Δ^9 -THC metabolites (Huestis 2005b).

Research surrounding CBD has increased dramatically in the last decade due to its lack of central side effects.

Pharmacology of CBD at cannabinoid receptors

Preliminary experiments of [3 H]CP55940 displacement binding assay conducted in specific tissues or cell transfected membranes, showed a low affinity (micromolar range) of CBD versus both CB₁ and CB₂ receptors (Pertwee 2008). These results explain the lack of central side effects by CBD.

However, recent results show that CBD can interact with both cannabinoid receptors at reasonably low concentrations when tested as antagonist of both CB₁ and CB₂ receptor agonists (Thomas et al. 2007). In detail, Pertwee's group showed the ability of CBD to antagonize both the non-selective CB₁/CB₂ receptor agonists, CP55940 and R-(+)-WIN55212-2, in the [35 S]GTP γ S binding assay using mouse whole brain membranes with mean K_B values of 79 and 138 nM, respectively (Thomas et al. 2007). However, it has been suggested that CBD-induced antagonism might be non-competitive in nature, since CBD has been found to behave as inverse agonist in the same assay at concentrations as those which exerted antagonism.

These findings are supported by previous reports:

1. CBD at 10 μ M antagonizes CP55940-induced stimulation of [35 S]GTP γ S binding to rat cerebellar membranes (Petitet et al. 1998).
2. CBD antagonizes CP55940 and R-(+)-WIN55212-2 in the mouse isolated vas deferens assay with an apparent K_B value in the nanomolar range (Pertwee 2008).
3. CBD can block various responses to Δ^9 -THC in rabbits, rats, mice and human subjects (Pertwee 2008).

Similar results were obtained in cell membranes over-expressing human CB₂ receptors (hCB₂-CHO cells), where CBD antagonized CP55940 in the [³⁵S]GTPγS binding assay with a K_B value of 65 nM, which is markedly less than its K_i value for displacing [³H]CP55940 in the same cells (Thomas et al. 2007). In mouse brain membranes, as well as in hCB₂-CHO cell membranes, CBD administered by itself inhibits [³⁵S]GTPγS binding (Thomas et al. 2007), suggesting that CBD antagonizes CP55940 at CB₂ receptors in a non-competitive way (Pertwee 2008, Thomas et al. 2007).

Further investigations are needed to elucidate the mechanism by which CBD exerts its moderately potent antagonism at both CB₁ and CB₂ receptors, in spite of its low K_i values at these receptors.

Pharmacology of CBD at non-CB₁, non-CB₂ receptors

The low affinity exerted by CBD for CB₁ and CB₂ receptors in displacement binding assays prompted researchers to seek out and characterise CB₁ and CB₂-independent modes of action for this phytocannabinoid (Mechoulam et al. 2007).

The first evidence that CBD can interact with sites different than cannabinoid receptors was offered by the observation that natural CBD is able to bind to and stimulate the type-1 vanilloid receptor (VR1) (Bisogno et al. 2001).

CBD has also been shown to interact with the adenosine system. In fact, a study conducted by the Hillard group demonstrated the ability of CBD to enhance adenosine signalling through inhibition of its uptake (Carrier, Auchampach & Hillard 2006, Castillo et al. 2010).

Recent evidences suggest CBD to be a positive allosteric modulator at a low micromolar concentration for both α1 and α1β glycine receptors (Ahrens et al. 2009). Moreover, at concentrations above 100 μM, CBD has been shown to directly activate both these receptors (Foadi et al. 2010).

In addition, several papers have also showed that CBD is able to interact with the type-1A serotonin receptor, 5-HT_{1A}. Russo *et al.* (2005) showed that, in CHO cells over-expressing human 5-HT_{1A} receptors, CBD displaced the agonist [³H]8-OH-DPAT in a dose-dependent manner, and in experiments of [³⁵S]GTPγS binding and

cyclic AMP assays CBD could act as agonist at 5-HT_{1A} receptors. However, CBD displayed these effects only at micromolar concentrations (Russo et al. 2005). *In vivo*, the interaction between CBD and 5-HT_{1A} receptors has been demonstrated to be involved in several disease models. For example, these serotonergic receptors were shown to induce the neuroprotective effects of CBD in a middle-cerebral-artery occlusion (MCAO) model (Mishima et al. 2005). In particular, CBD treatment displayed a reduction in the MCAO-mediated infarct volume by boosting the cerebral blood flow. This effect was antagonized by WAY100135, a 5-HT_{1A} receptor antagonist, suggesting that the neuroprotective effects of CBD in this model might be related to the increase in cerebral blood flow through the 5-HT_{1A} receptor (Mishima et al. 2005).

In two other papers, the involvement of 5-HT_{1A} receptors has been proposed for both the anti-stress and anxiolytic-like effects of CBD. In fact, the 5-HT_{1A} receptor antagonist, WAY100635, was able to reverse both of these effects (Campos, Guimaraes 2008, Resstel et al. 2009).

Supporting data about the interaction of these serotonin receptors and CBD come from a hepatic encephalopathy (HE) model, bile-duct ligation (BDL). This is a common model of chronic liver disease in rodents which exacerbates cerebral inflammation, mimicking human HE. CBD treatment was shown to ameliorate cognitive and motor dysfunctions, and moderated neuroinflammation in a BDL mouse model. These effects were reversed by the 5-HT_{1A} receptor antagonist, WAY100635, suggesting the involvement of these receptors in CBD-mediated HE symptoms amelioration (Magen et al. 2010).

In addition, in a recent presentation at the International Cannabinoid Research Society (ICRS), the Parker group suggested that 5-HT_{1A} receptors might be involved in the anti-emetic and anti-nausea effects of CBD (Rock et al. 2009, Parker et al. 2010). This therapeutic potential aspect of CBD will be evaluated with more emphasis later in this introduction.

5-HT_{1A} receptors

Serotonin neurons originate from raphe nuclei, which are 7 different cerebral nuclei localized in the brainstem (Figure 49). In particular, raphe nuclei are distributed in the

midbrain (where the main nucleus is dorsal raphe nucleus), in pons and medulla. The nuclei localized in the midbrain project into the brain (forebrain and cerebellum), instead the nuclei localized in the medulla project to spinal cord and brainstem (Figure 49) (Kandel, Schwartz & Jessel 2000).

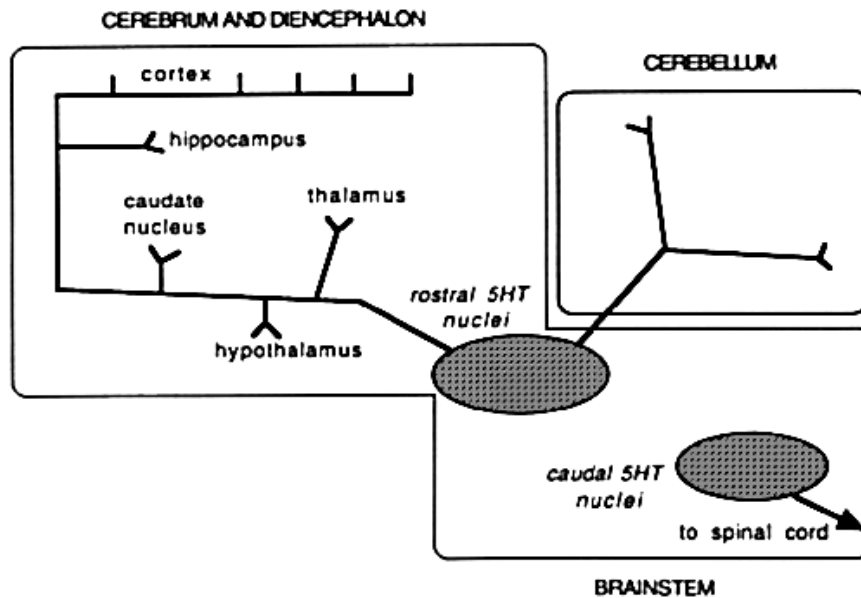


Figure 49. Distribution and organization of 5-HT neurons. Dark circles indicate the origin of serotonergic neurons.

Serotonergic receptors include 7 different receptor families, and a total number of 14 structurally and pharmacologically distinct subtypes (Figure 50). The endogenous ligand for these receptors is the neurotransmitter serotonin (5-HT) (Hannon, Hoyer 2008).

5-HT_{1A} receptors are GPCRs coupled to G_{i/o} that mediate inhibitory neurotransmission (Hannon, Hoyer 2008). These receptors are largely distributed throughout the brain, but are also present in the peripheral nervous system (PNS), especially in the gastro intestinal tract (Barnes, Sharp 1999). In the brain, 5-HT_{1A} receptors are densely expressed in limbic brain area, notably hippocampus, lateral septum, cortical areas (particularly cingulated and entorhinal cortex), and also mesencephalic and medullary raphe nuclei. Extremely low 5-HT_{1A} receptor levels are detected in the basal ganglia and cerebellum (Hannon, Hoyer 2008, Barnes, Sharp 1999, Lanfumey, Hamon 2000). At subcellular level, 5-HT_{1A} receptors are localized in the soma and dendrites in raphe nuclei, where they act as autoreceptors to inhibit cell firing. In limbic area, especially in the hippocampus, 5-HT_{1A} receptors are localized at postsynaptic level on non-5-HT

neurons (heteroreceptors) (Riad et al. 2000). The activation of 5-HT_{1A} receptors mediates neuronal hyperpolarization, through G-protein coupled K⁺ channel (GIRK channels), with the consequent inhibition of cell firing and 5-HT release (5-HT_{1A} autoreceptors) (Hannon, Hoyer 2008).

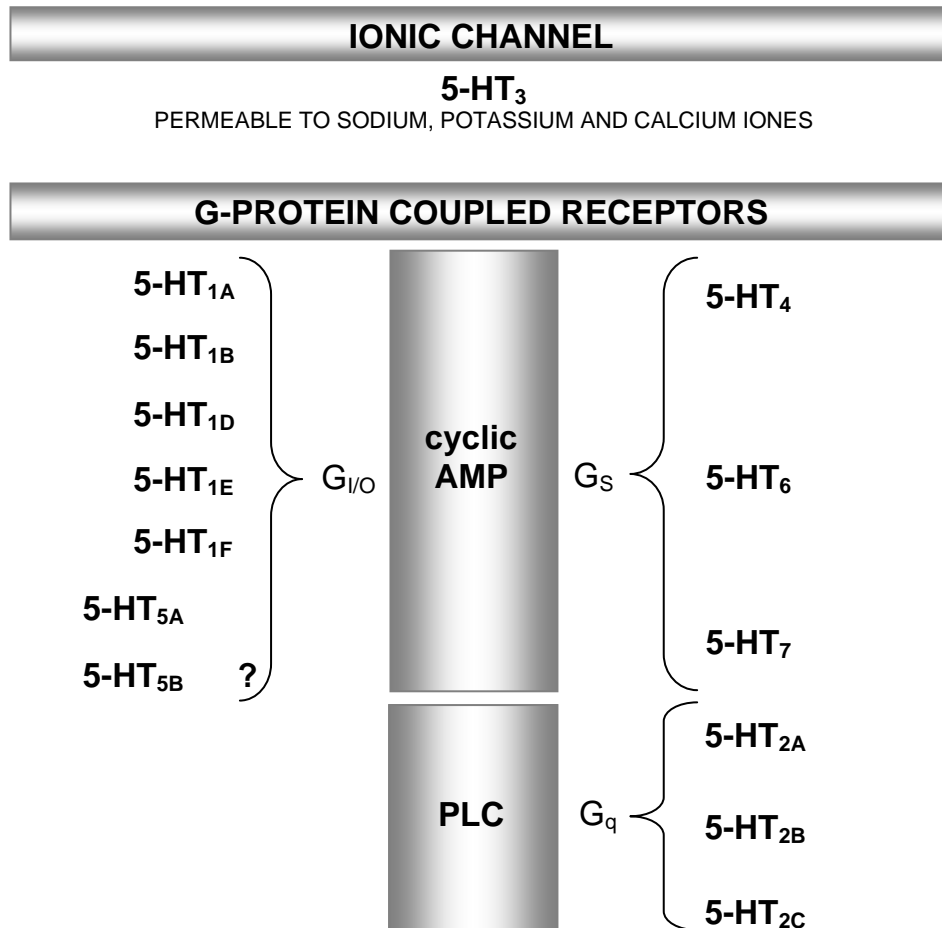


Figure 50. Classification of 5-HT receptors. The G-protein coupled to 5-HT_{5B} has not been defined yet. Phospholipase C (PLC). The picture was adapted from (Hoyer, Hannon & Martin 2002).

5-HT_{1A} receptors are involved in several physiological responses (Table 17) (Barnes, Sharp 1999). Some of these are controlled specifically by presynaptic or postsynaptic 5-HT_{1A} receptors. On the other hand, in some physiological states the involvement of these receptors is still controversial; for example it seems that presynaptic 5-HT_{1A} receptors are more involved in the anxiolytic effect of serotonin, instead postsynaptic 5-HT_{1A} receptors has been proposed as a target for antidepressant treatment (Batool 2008).

Level	Target	Response	Mechanism
Neurochemical	5-HT release	Inhibition	Presynaptic
	Noradrenaline release	Stimulation	Postsynaptic
	Acetylcholine release	Stimulation	Postsynaptic
	Glutamate release	Inhibition	?
Neuroendocrine	ACTH	Stimulation	Postsynaptic
	Prolactin	Stimulation	Postsynaptic
Behavioral	5-HT syndrome		Postsynaptic
	Hypothermia		Pre/Postsynaptic
	Hyperphagia		Presynaptic
	Anxiolysis		Pre/Postsynaptic
	Antidepressant		Pre/Postsynaptic
	Sexual behavior	Stimulation	Pre/Postsynaptic
	Discriminative stimulus		Pre/Postsynaptic

Table 17. Summary of the functional physiological responses associated with activation of the brain 5-HT_{1A} receptors. Pre and postsynaptic refer to the 5-HT_{1A} receptor localization. ACTH refers to the adrenocorticotrophic hormone. Table adapted from (Barnes, Sharp 1999).

5-HT_{1A} receptors, CBD and nausea/emesis

Nausea and vomiting are one of the most common side effects of chemotherapy. The cannabis-mediated anti-nausea and anti-emetic effects are widely known, although the exact mechanism it is still uncertain (Mechoulam, Parker & Gallily 2002). Interestingly, the observations that marijuana suppresses nausea more effectively than oral Δ^9 -THC (Grinspoon, Bakalar 1997) led a Canadian group to investigate the potential anti-emetic and anti-nausea properties of CBD. The Parker group conducted several experiments in mouse musk shrew (*Suncus murinus*), an animal model in which the injection of Lithium Chloride (LiCl) induces vomit (Parker et al. 2004). In this model, CBD produced a biphasic pharmacological profile, with lower doses (5-10 mg/Kg) producing suppression and higher doses (40 mg/Kg) producing enhancement of LiCl-induced vomiting (Parker et al. 2004). Recently, the same group presented new data supporting the anti-emetic effect of CBD, in particular the efficacy of this

phytocannabinoid has been demonstrated in nicotine-, cisplatin- and LiCl-induced vomiting in shrews (Rock et al. 2009). Moreover the anti-nausea properties of CBD have been proved in conditioned gaping in rats, an *in vivo* model to induce nausea (Parker, Limebeer 2006, Rock et al. 2008). Interestingly, even in this model CBD displayed a typical bell-shaped pharmacological profile, where only low doses of CBD were effective (Rock et al. 2008). The anti-emetic and anti-nausea effects of CBD, in these models, have been suggested to be mediated by its action at somatodendritic 5-HT_{1A} autoreceptors located in the raphe nuclei (Rock et al. 2009, Parker et al. 2010). The role of 5-HT_{1A} autoreceptors in nausea and emesis is supported by several evidences. It has been demonstrated that the 5-HT_{1A} agonist, 8-OH-DPAT, which reduces serotonin release, interfered with LiCl-induced conditioned gaping in rats (Parker et al. 2010). In addition, depletion of forebrain serotonin by lesion of median and dorsal raphe nuclei attenuated the effects of LiCl-induced conditioned gaping (Limebeer, Parker & Fletcher 2004). The proposed 5-HT_{1A} receptors-mediated anti-nausea and anti-emetic effects of CBD are supported by the capacity of the 5-HT_{1A} antagonist, WAY100135, to block these effects (Rock et al. 2009). In addition, the intracranial delivery of the non-selective 5-HT_{1A} antagonist, WAY 100635, to the dorsal raphe nuclei (DRN) has been shown to attenuate the anti-nausea property of CBD, suggesting that this phytocannabinoid is acting through somatodendritic 5-HT_{1A} autoreceptors located on the raphe nuclei (Parker et al. 2010).

AIMS OF THE PROJECT

The potential anti-nausea and anti-emetic properties of CBD prompted us to start collaboration with Professor Linda Parker (University of Guelph, Canada). In particular, we focused our attention on the possible interaction between CBD and somatodendritic 5-HT_{1A} autoreceptors. As aforementioned, CBD, at very high concentrations (16 μM), was already found to be an agonist in CHO cells overexpressing human 5-HT_{1A} receptors (Russo et al. 2005). However, since CBD displayed anti-nausea and anti-emetic effect only at low concentrations (Parker et al. 2004, Rock et al. 2008), we tested this phytocannabinoid in the concentration range of 1 nM up to 10 μM. Moreover, in order to isolate somatodendritic 5-HT_{1A} autoreceptors we chose the brainstem for our experiments. In fact, this brain region contains all the raphe nuclei and consequently it is rich of somatodendritic 5-HT_{1A} autoreceptors.

Thus, with the aim of investigating a possible interaction between CBD and 5-HT_{1A} autoreceptors we carried out experiments of displacement and [³⁵S]GTPγS binding assays in rat brainstem membranes.

RESULTS

CBD does not displace [³H]8-OH-DPAT in rat brainstem membranes

There is already evidence that CBD, albeit at the rather high concentration of 16 μM , can directly bind to and activate human 5-HT_{1A} receptors that have been transfected into CHO cells (Russo et al. 2005). However, the ability of lower concentrations of this phytocannabinoid to activate 5-HT_{1A} receptors *in vitro*, when they are expressed naturally at physiological levels in rat brainstem membranes, has not been investigated before. Accordingly, we sought for evidence that CBD can directly target 5-HT_{1A} receptors in rat brainstem when administered *in vitro* at concentrations ranging from 1 nM to 10 μM .

First, in order to investigate the ability of CBD to bind 5-HT_{1A} receptors, experiments of displacement binding were carried out in rat brainstem membranes. The radioligand [³H]8-OH-DPAT was used to occupy 5-HT_{1A} receptor binding sites and the displacement was quantified as a percentage of specific binding produced in the presence of 1 μM of the unlabelled ligand 8-OH-DPAT. Experiments were conducted in parallel with the selective 5-HT_{1A} agonist, 8-OH-DPAT (Figure 51).

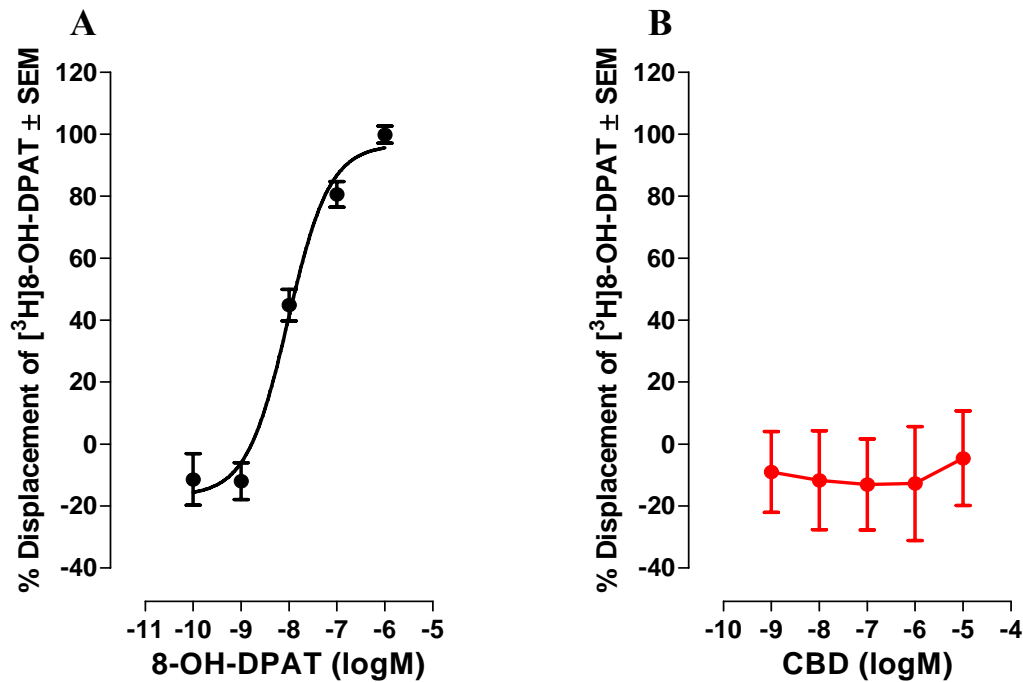


Figure 51. Effects of 8-OH-DPAT (A) and CBD (B) on specific binding of [³H]8-OH-DPAT to rat brainstem membranes (n=6). The IC₅₀ and E_{max} values of 8-OH-DPAT for its displacement of [³H]8-OH-DPAT, with 95% confidence limits shown in parentheses, were 9.6 nM (5.6 and 16.3 nM) and 96.6% (86.8 and 106.5%), respectively. Symbols represent mean values ± SEM.

As shown in Figure 51A, 8-OH-DPAT was able to fully displace [³H]8-OH-DPAT from specific binding sites in rat brainstem membranes. The mean IC₅₀ value displayed by 8-OH-DPAT was very similar to the one reported in literature for displacement of [³H]8-OH-DPAT in rat raphe area membranes (Assie, Koek 2000).

Conversely, CBD at concentrations up to 10 μM did not share the ability of 8-OH-DPAT to induce such displacement (Figure 51B). These results suggested that CBD, in this range of concentrations, is not able to bind the orthosteric site of 5-HT_{1A} receptors.

CBD is not an agonist at 5-HT_{1A} receptors

Since 5-HT_{1A} receptors signal through G_{i/o} proteins (see introduction chapter three), we also compared the ability of both 8-OH-DPAT and cannabidiol to stimulate [³⁵S]GTPγS binding to rat brainstem membranes.

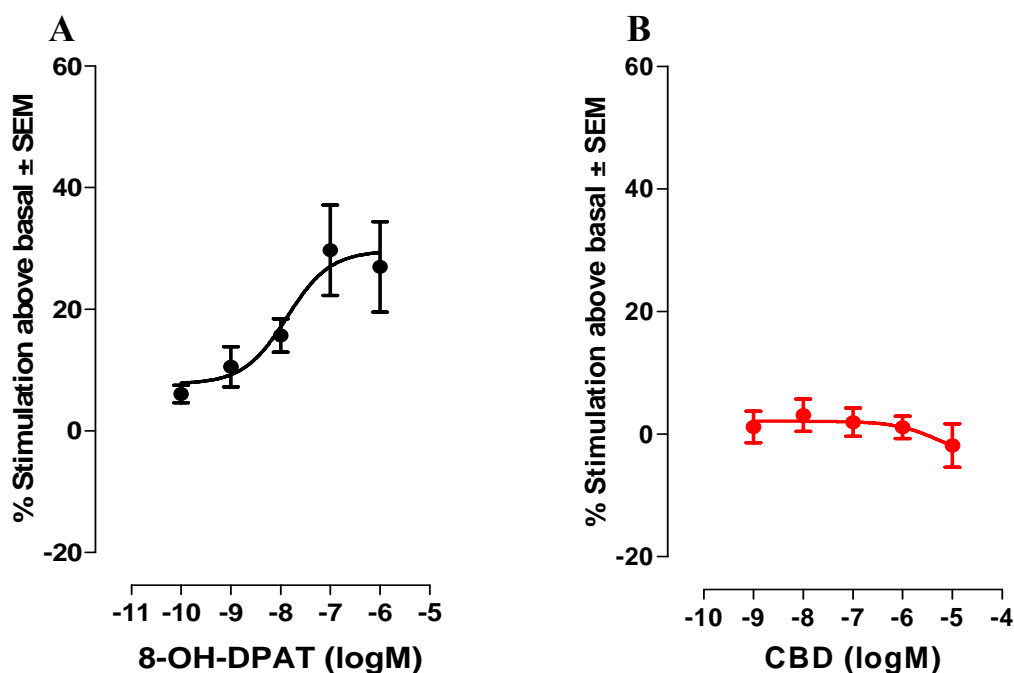


Figure 52. Effects of (A) 8-OH-DPAT (n=5) and (B) CBD (n=4) on [³⁵S]GTPγS binding to rat brainstem membranes. Mean EC₅₀ and E_{max} values for 8-OH-DPAT, with 95% confidence limits shown in parentheses, were 13.4 nM (0.91 and 197 nM) and 29.6% (19.7 and 39.5%). None of the concentrations of CBD tested were significantly different from zero (one sample T-test analyses). Symbols represent mean values ± SEM.

As shown in Figure 52A, 8-OH-DPAT, as expected, was able to stimulate the [³⁵S]GTPγS binding to rat brainstem membranes, with an EC₅₀ value of 13.4 nM, which was not significantly different to the one reported in [³⁵S]GTPγS binding experiments in rat dorsal raphe nucleus (Rossi, Burke & Hensler 2008) or hippocampus membranes (Sprouse et al. 2004, Newman-Tancredi et al. 2009).

Conversely, CBD, in the same experimental conditions, was unable to stimulate or inhibit the [³⁵S]GTPγS binding at any of the concentrations tested (Figure 52B). Together with the displacement binding data, these results rule out the ability of CBD, in this range of concentrations, to directly bind to and activate 5-HT_{1A} autoreceptors.

8-OH-DPAT acts at 5-HT_{1A} receptors in rat brainstem

Although 8-OH-DPAT is a 5-HT_{1A} receptor agonist, it has been reported that this ligand has moderate affinity also for 5-HT₇ receptors (Sprouse et al. 2004, Adham et al. 1998). In addition, 5-HT₇ receptors are expressed in the dorsal raphe nucleus,

although at low levels (Hannon, Hoyer 2008). Thus, in order to confirm that 8-OH-DPAT-induced [³⁵S]GTPγS binding stimulation was mediated by 5-HT_{1A} receptors, we then tested the ability of the 5-HT_{1A} receptor antagonist, WAY100635, to antagonize 8-OH-DPAT dose-response curve in experiments of [³⁵S]GTPγS binding in rat brainstem membranes.

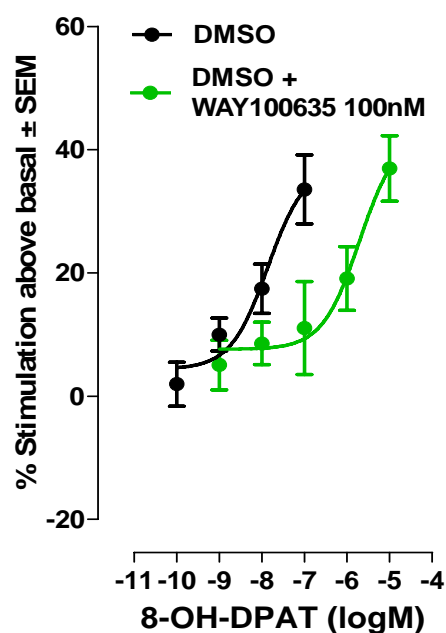


Figure 53. Effects of 8-OH-DPAT in the presence of DMSO (Vehicle; n=11) or 100 nM WAY100635 (n=11) on [³⁵S]GTPγS binding to rat brainstem membranes. Mean EC₅₀ values for 8-OH-DPAT, with 95% confidence limits shown in parentheses were 13.6 nM (2.7 and 68.8 nM), in the presence of vehicle and 1936 nM (240 and 15630 nM) in the presence of WAY100635. The rightward shift produced by WAY100635 in the log concentration-response curve of 8-OH-DPAT was significant and did not deviate statistically from parallelism ($p > 0.2$). The mean apparent K_B value of WAY100635 for this antagonism, with 95% confidence limits shown in brackets, was 1 nM (0.24 and 4.1 nM). Symbols represent mean values \pm SEM.

We found that WAY100635 did antagonize the stimulatory effect of 8-OH-DPAT on [³⁵S]GTPγS binding assay to rat brainstem membranes (Figure 53). The mean apparent K_B value of WAY100635 for this antagonism was 1 nM, which was not significantly different to the mean K_B value reported previously in this laboratory for antagonism of 8-OH-DPAT in mouse whole brain membranes (Casco et al. 2010). These results confirmed that the agonism displayed by 8-OH-DPAT in rat brainstem membranes was mediated by 5-HT_{1A} receptors.

CBD acts at 5-HT_{1A} receptors in rat brainstem

Having established that CBD *per se* had not activity on 5-HT_{1A} receptors on rat brainstem membranes (Figure 52B) and that 8-OH-DPAT was acting selectively at 5-HT_{1A} receptors on the same membranes (Figure 53), it was of our interest to investigate whether CBD could behave as 5-HT_{1A} receptor enhancer. This we did to explore the possibility that 5-HT_{1A} receptor antagonist, WAY100635, reduces CBD-induced suppression of LiCl-induced conditioned gaping in rats (Rock et al. 2009) because this cannabinoid augments activation of 5-HT_{1A} receptors in the brainstem by endogenously released serotonin. With this in mind, we tested 10 μ M CBD, the highest concentration at which it did not stimulate [³⁵S]GTP γ S binding on its own, as antagonist of 8-OH-DPAT in rat brainstem membranes using [³⁵S]GTP γ S binding assay.

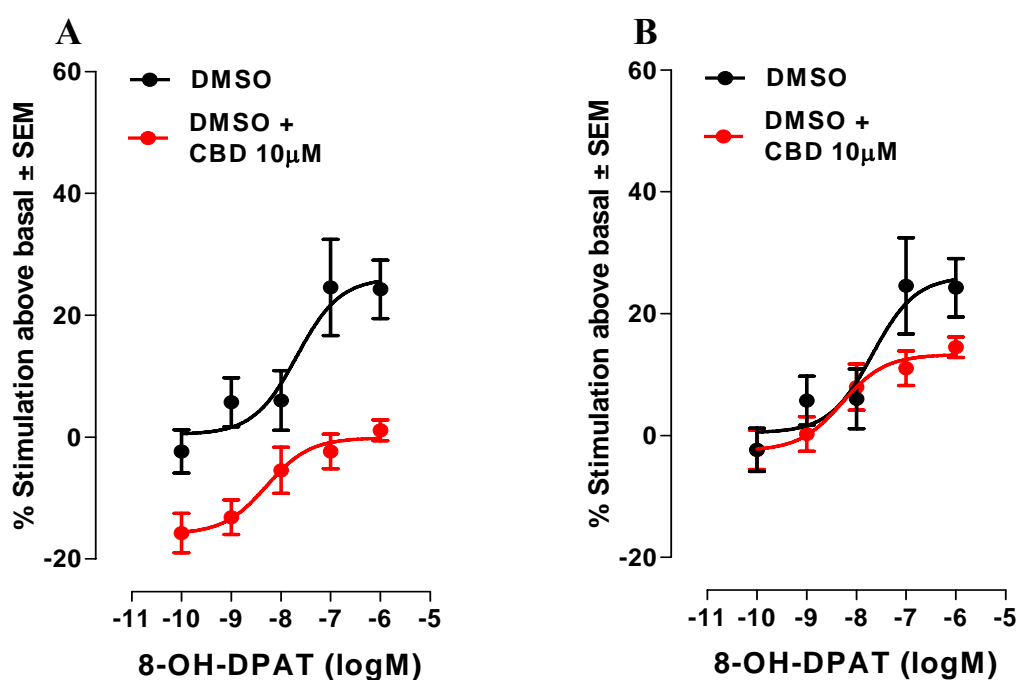
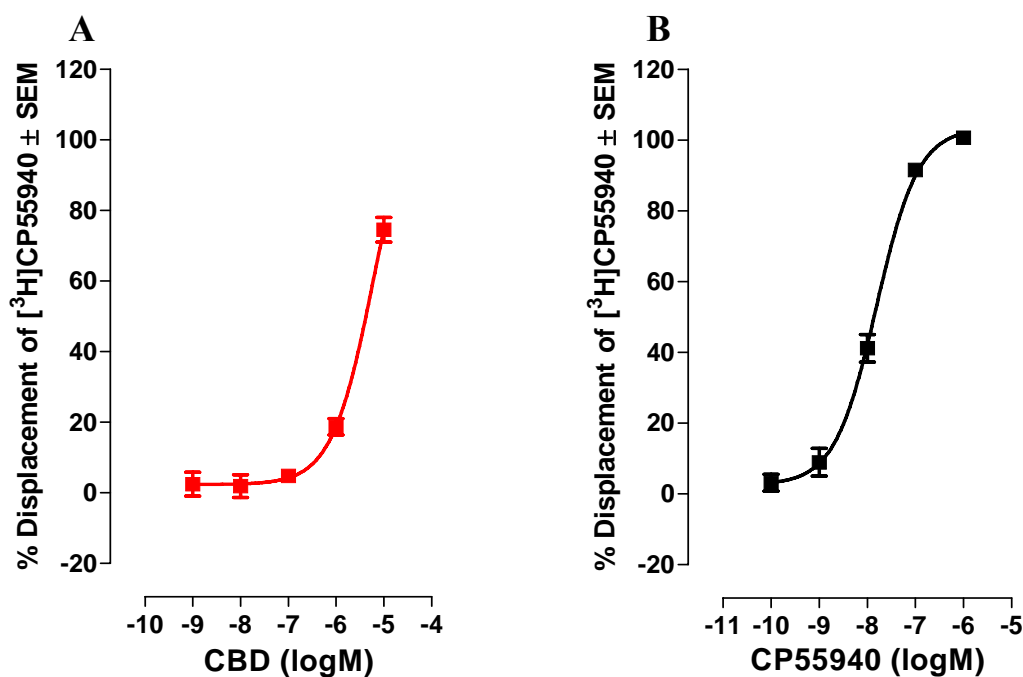


Figure 54. Effect of (A) 8-OH-DPAT on [³⁵S]GTP γ S binding to rat brainstem membranes in the presence of DMSO (Vehicle; n=4) or CBD (n=4). Effect of (B) 8-OH-DPAT on [³⁵S]GTP γ S binding to rat brainstem membranes in the presence of DMSO (Vehicle; n=4) or CBD (n=4) after subtraction of the mean inhibitory effect induced by 10 μ M CBD in the presence of the lowest concentration of 8-OH-DPAT. Mean E_{max} values for (A) 8-OH-DPAT, with 95% confidence limits shown in parentheses were 26.1% (16.4 and 35.9%) in the presence of vehicle and -0.1% (-6.9 and 6.6%) in the presence of 10 μ M CBD. Corresponding mean EC_{50} values for (A) 8-OH-DPAT were 20.8 nM (2 and 214 nM) in the presence of vehicle and 5.1 nM (0.52 and 51 nM) in the presence of 10 μ M CBD. Symbols represent mean values \pm SEM.

The ability of 8-OH-DPAT to stimulate [³⁵S]GTPγS binding on rat brainstem membranes was attenuated by CBD (Figure 54), which at 10 μM produced a downward but not a rightward shift in the log concentration-response curve of 8-OH-DPAT. Subtracting the inverse agonism, no antagonism was displayed by CBD on 8-OH-DPAT dose-response curve, suggesting that CBD is not a competitive antagonist at 5-HT_{1A} receptors.

CBD, at 10 μM, is an inverse agonist at CB₁ receptors

Data previously reported in this laboratory showed that CBD, at 10 μM, was able to displace the non-selective CB₁/CB₂ receptor radioligand, [³H]CP55940, from specific binding sites in mouse whole brain membranes (Thomas et al. 2007). Moreover, 10 μM CBD was shown to inhibit [³⁵S]GTPγS binding in the same brain membranes. In order to confirm the ability of CBD to act at CB₁ receptors we went on performing displacement and [³⁵S]GTPγS binding experiments in CHO cells overexpressing hCB₁ receptors.



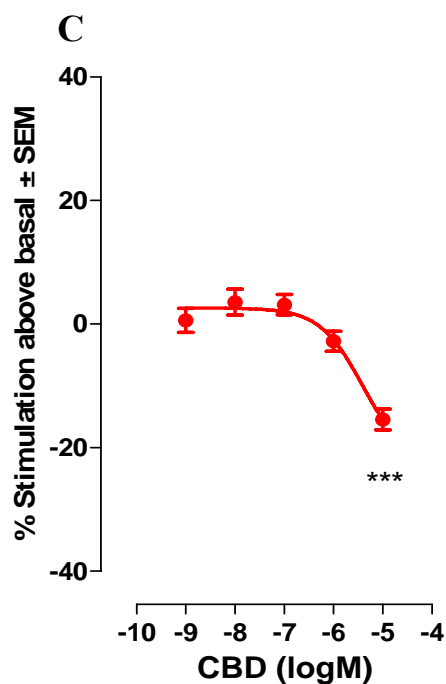


Figure 55. Displacement of [³H]CP55940 by CP55940 (A) and CBD (B) from specific binding sites on hCB₁-CHO cell membranes (n=4). The E_{max} values were (A) 103.4% and (B) 74.5%. (C) The effect of CBD on [³⁵S]GTPγS binding assay to hCB₁-CHO cell membranes (n=4). The [³⁵S]GTPγS binding-inhibition induced by CBD, at 10 μM, was statistically different from zero (one sample t-test analyses). Each symbol represents the mean percentage displacement ± SEM. *** p<0.0001.

CBD, compared to CP55940 (Figure 55B), at 10 μM was able to partially, but not fully, displace [³H]CP55940 from specific binding sites on hCB₁-CHO cell membranes (Figure 55A). In [³⁵S]GTPγS binding assay, CBD displayed significant inverse agonism only at the concentration of 10 μM (Figure 55C).

In order to confirm CB₁ receptor-mediated CBD inverse agonism, we carried on performing cyclic AMP experiments in both hCB₁-CHO cells and untransfected CHO cells.

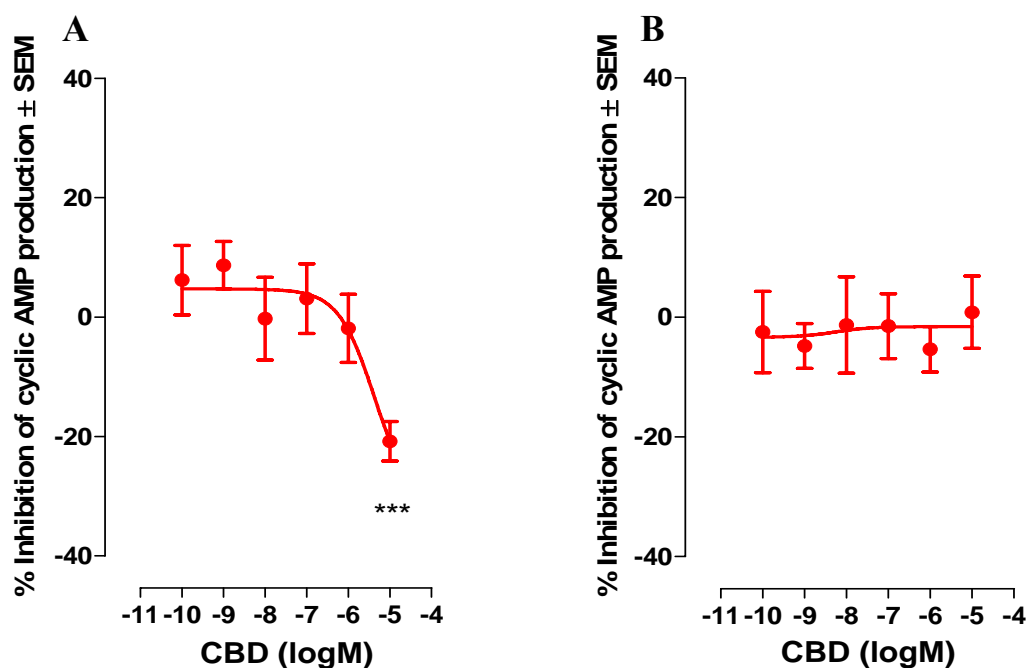


Figure 56. The effect of CBD on forskolin-induced stimulation of cyclic AMP production in hCB₁-CHO cells (A) and untransfected CHO cells (B). The inhibition induced by 10 μM CBD (A) was statistically different from zero (one sample t-test analyses). *** p<0.0001. The assays were performed using a kit purchased by *GE Healthcare*.

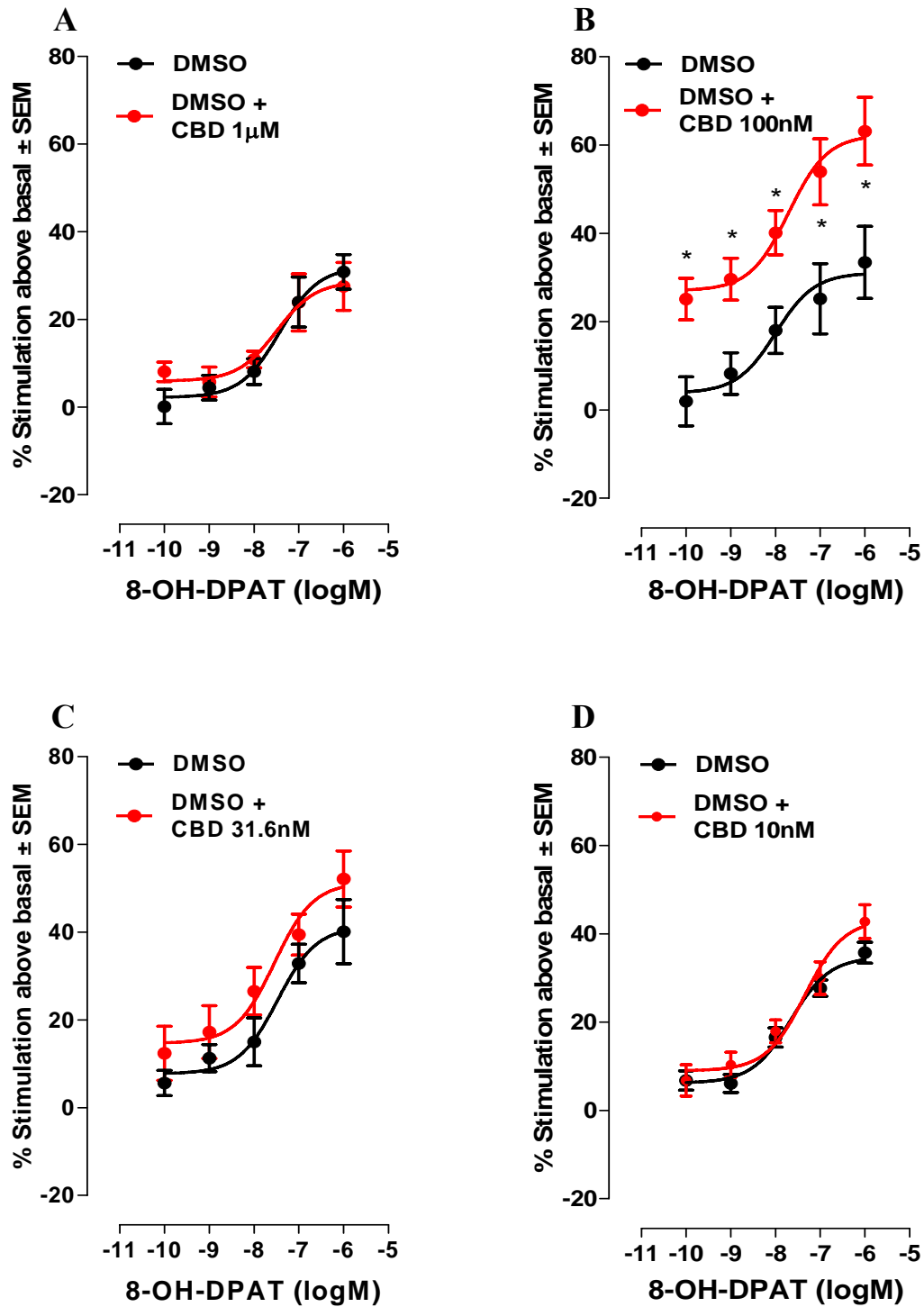
We found that in hCB₁-CHO cells, CBD, at the concentration of 10 μM, displayed a significant increase on forskolin-induced cyclic AMP production (Figure 56A). Conversely, none of the concentrations of CBD tested affected forskolin-induced cyclic AMP production in untransfected CHO cells (Figure 56B).

Taken together these results suggested that CBD at 10 μM is able to bind to and act at CB₁ receptors. Thus, it is possible that the downward shift produced by CBD on 8-OH-DPAT dose-response curve in rat brainstem membranes (Figure 54A) is the result of an interaction between CBD and CB₁ receptors.

CBD enhances 8-OH-DPAT dose-response curve

Since the anti-emetic and anti-nausea effects of CBD were displayed only at very low concentrations, as a possible enhancement in 5-HT_{1A} receptor signalling (see introduction), and that CBD at 10 μM inhibited 8-OH-DPAT dose-response curve (Figure 54A), we went on investigating whether CBD, in the concentration range of

1nM up to 1 μ M, could enhance 8-OH-DPAT dose-response curve. We then performed [35 S]GTP γ S binding experiments testing CBD as potential allosteric enhancer of 8-OH-DPAT in rat brain stem membranes.



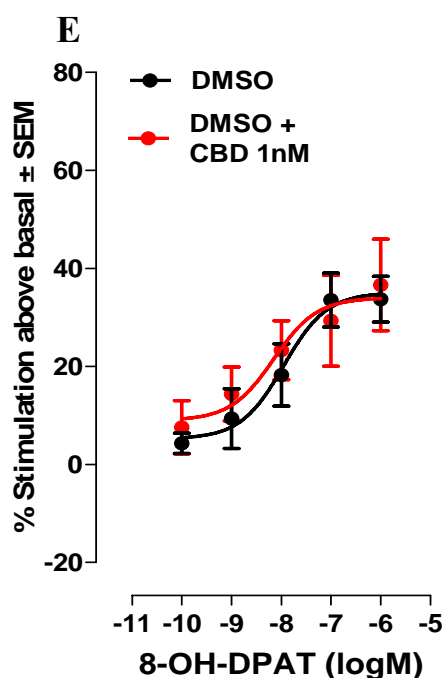


Figure 57. Effect of 8-OH-DPAT on [³⁵S]GTP γ S binding to rat brainstem membranes in the presence of DMSO (Vehicle) or different concentrations of CBD. Mean E_{max} values for 8-OH-DPAT in panels A, B, C, D and E with 95% confidence limits shown in parentheses were 32% (23.3 and 40.7%; n=9), 31% (20.2 and 42%; n=10), 41.2% (30 and 52.4%; n=6), 34.7% (30.3 and 39.3; n=8) and 35.1% (26.6 and 43.5%; n=8), respectively, in the presence of vehicle and 28.7% (19.1 and 38.3%; n=9), 62.3% (51.0 and 73.6%; n=10), 51.3% (39.3 and 63.3%; n=6), 45.3% (35.5 and 51%; n=6) and 34% (22.2 and 45.8%; n=8), respectively, in the presence of 1 μ M, 100 nM, 31.6 nM, 10 nM, or 1 nM CBD. Corresponding mean EC_{50} values for 8-OH-DPAT were 37.6 nM (7.6 and 185 nM), 10.1 nM (0.75 and 137 nM), 37.5 nM (6.7 and 168 nM), 22 nM (9.5 and 50.9nM) and 11.2 nM (1.9 and 66.7 nM), respectively, in the presence of vehicle and 31.8 nM (3.2 and 317 nM), 19.7 nM (2.8 and 139 nM), 28.6 nM (4.5 and 180 nM), 45.7 nM (14.1 and 149 nM) and 7.1 nM (0.4 and 126 nM), respectively, in the presence of 1 μ M, 100 nM, 31.6 nM, 10 nM, or 1 nM CBD. Symbols represent mean values \pm SEM. * $p < 0.1$ (one sample t-test analyses).

As shown in Figure 57A, C and E, CBD at the concentrations of 1 μ M, 10 nM and 1 nM did produce neither stimulation nor inhibition on log concentration-response curve of 8-OH-DPAT on [³⁵S]GTP γ S binding assay in rat brainstem membranes. Conversely, 100 nM and 31.6 nM CBD produced an upward shift in the log concentration-response curve of 8-OH-DPAT that resulted in an increase of the E_{max} but not EC_{50} of this 5-HT_{1A} receptor-selective agonist (Figure 57B and C). In particular, 100 nM CBD produced a significant different upward shift at all the concentration of 8-OH-DPAT tested ($p < 0.1$).

CBD is not a 5-HT_{1A} receptor allosteric modulator

The ability of 100 nM CBD to enhance the log concentration-response curve of 8-OH-DPAT raised the possibility that this phytocannabinoid might target an allosteric site on the 5-HT_{1A} receptor, there already being evidence for the presence of such a site on this receptor (Barrondo, Salles 2009). We therefore investigated the ability of a 8-OH-DPAT-potentiating concentration of CBD, 100 nM, to alter the rate at which [³H]8-OH-DPAT dissociates from specific binding sites in rat brainstem membranes.

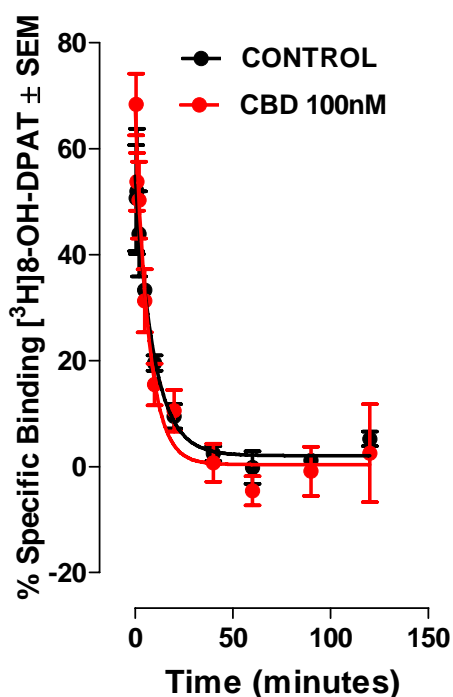


Figure 58. The dissociation of [³H]8-OH-DPAT from binding sites in rat brainstem membranes in the presence of vehicle or 100 nM CBD. These mean dissociation rates with their 95% confidence limits shown in parentheses were 7.0 min (4.3 and 18.2) and 4.8 (3.5 and 8.1), respectively (n=3). Each symbol represents the mean percentage of specific binding S.E.M.

These experiments showed that the mean dissociation rates of [³H]8-OH-DPAT in the presence of vehicle or of 100 nM CBD were not significantly different (Figure 58). These results suggested that CBD is not an allosteric modulator at 5-HT_{1A} receptors.

DISCUSSION

Anti-nausea and anti-emetic effects of cannabis are widely recognized (Mechoulam, Parker & Gallily 2002) and are an important alternative for treatment of nausea associated with chemotherapy. Thus, in the 80's the synthetic Δ^9 -THC, dronabinol, and the synthetic Δ^9 -THC analogue, nabilone, have been licensed in several countries as drug for treatment of nausea and emesis associated with cancer therapy when first-line treatments (standard therapy recommended for the initial treatment of a disease) were not effective (see general introduction). Nevertheless, the side effects mediated by these drugs caused a reduction in the usage of these agents as a medication in nausea and emesis (Costa et al. 2007). Thus, in the last decade the research on cannabis, for this and other disease states treatment, has been focused on the non-psychoactive cannabis constituent, CBD (Mechoulam et al. 2007). Recent evidences have shown the efficacy of CBD as anti-nausea and anti-emetic agent in several *in vivo* models (see introduction), where this phytocannabinoid has shown a typical bell-shaped pharmacological profile. In addition, the Parker group suggested a possible involvement of somatodendritic 5-HT_{1A} autoreceptors in CBD-mediated anti-nausea and anti-emetic effects (Rock et al. 2009, Parker et al. 2010).

Results from our *in vitro* experiments indicated and confirmed the presence of 5-HT_{1A} receptors in rat brainstem membranes. This was supported by the ability of the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT, both to displace the radioligand [³H]8-OH-DPAT from specific sites (Figure 51A), and to stimulate [³⁵S]GTP γ S binding to rat brainstem membranes (Figure 52A). In addition 8-OH-DPAT-induced stimulation of [³⁵S]GTP γ S binding was antagonized by the 5-HT_{1A} antagonist, WAY100635 (Figure 53), further confirming the presence of 5-HT_{1A} receptors in rat brainstem membranes and the ability of 8-OH-DPAT to act on these receptors.

When CBD was tested in the concentration range of 1 nM to 10 μ M neither displacement of [³H]8-OH-DPAT from specific binding sites (Figure 51B) nor stimulation of [³⁵S]GTP γ S binding (Figure 52B) were observed in rat brainstem membranes. These results suggested that CBD is not able to bind directly to 5-HT_{1A} autoreceptors in this concentration range. This partially supports data already published in literature, where the ability of CBD to directly bind to and activate

recombinant human 5-HT_{1A} receptors was observed only at the high concentration of 16 μM (Russo et al. 2005).

Then, we assessed the possibility of CBD to indirectly activate the 5-HT_{1A} autoreceptors. Nevertheless, at 10 μM, CBD behaved as a non-competitive antagonist of 8-OH-DPAT, as indicated by the downward shift displayed by this phytocannabinoid in 8-OH-DPAT dose-response curve for stimulation of [³⁵S]GTPγS binding to rat brainstem membranes (Figure 54A). Moreover, when this downward shift was subtracted no rightward shift was displayed by CBD on 8-OH-DPAT dose-response curve (Figure 54B), suggesting that this downward displacement accounts entirely for the antagonism of 8-OH-DPAT. Together with the previous results, this confirms that CBD is not able to bind to the orthosteric 5-HT_{1A} receptor binding site. Importantly, these results confirm the *in vivo* data showing the ability of high CBD concentrations to enhance LiCl-induced vomiting (Parker et al. 2004).

We then sought for the possible target underlying this CBD-mediated 5-HT_{1A} receptor inhibition. Data previously published in our laboratory showed that CBD is an inverse agonist at 10 μM in mouse whole-brain membranes (Thomas et al. 2005). Thus, we found that CBD at the concentration of 10 μM is able both to displace [³H]CP55490 from specific binding sites and inhibit [³⁵S]GTPγS binding to hCB₁-CHO cell membranes (Figure 55). The CBD-induced inverse agonism at CB₁ receptors was indeed confirmed in experiments of cyclic AMP assays, where this phytocannabinoid displayed inhibition of forskolin-induced cyclic AMP production in hCB₁- but not untransfected-CHO cells (Figure 56).

Hence, the downward shift displayed by 10 μM CBD in 8-OH-DPAT dose-response curve in [³⁵S]GTPγS binding to rat brainstem membranes could be mediated by CB₁ receptors. This would also explain why CBD on its own did not display any effect on [³⁵S]GTPγS binding in rat brainstem membranes (Figure 52B). In fact, since the expression of CB₁ receptors is low in this brain region (Howlett et al. 2002), it is possible that CBD displayed inverse agonism only in the presence of an agonist. In light of this, it is likely that a heterodimerization or cross-talk could occur between 5-HT_{1A} and CB₁ receptors in brainstem. 5-HT_{1A} receptors, in the brainstem, are localized at somatodendritic level (Riad et al. 2000). Conversely, CB₁ receptors are well-known to be localized at the presynaptic terminals (Chevaleyre, Takahashi & Castillo 2006). However, several evidences showed the presence of CB₁ receptors at

somatodendritic level in different brain regions, such as rat caudate putamen, rat nucleus accumbens, dorsal horn (Hohmann, Briley & Herkenham 1999, Rodriguez, Mackie & Pickel 2001, Pickel et al. 2004). In addition, a recent paper showed a somatodendritic distribution of CB₁ receptors in the locus coeruleus, which is localized in the brain stem, suggesting a possible heterodimerization between CB₁ and mu-opioid receptors (MOR) (Scavone, Mackie & Van Bockstaele 2010). Hence, although there are not evidences so far showing the presence of somatodendritic CB₁ receptors on 5-HT neurons in brainstem this cannot be ruled out, as well as a possible cross-talk between 5-HT_{1A} and CB₁ receptors in this brain region. Thus, it is likely that 8-OH-DPAT dose-response curve was inhibited by 10 μM CBD through an interaction between 5-HT_{1A} and CB₁ receptors.

Having established that CBD, at the high concentration of 10 μM, was not able to enhance 8-OH-DPAT-mediated [³⁵S]GTPγS binding stimulation, we moved on testing lower concentrations of CBD. Thus, as previously reported *in vivo* data show that low concentrations of CBD to be effective in the inhibition of LiCl-induced nausea and vomiting (Parker et al. 2004, Rock et al. 2008), we found that CBD, at the concentration of 100 nM, was able to enhance 8-OH-DPAT dose-response curve on [³⁵S]GTPγS binding assays in rat brainstem membranes (Figure 57). In particular, 100 nM CBD showed an enhancement of approximately 20% on 8-OH-DPAT-induced [³⁵S]GTPγS binding stimulation (p<0.1). These results strengthen the *in vivo* data, suggesting that CBD might suppress LiCl-induced conditioned gaping in rats by somehow augmenting activation of 5-HT_{1A} receptors in the brainstem produced by endogenously released 5-HT. Interestingly, CBD displayed a typical bell-shaped pharmacological profile as antagonist of 8-OH-DPAT in rat brain stem membranes. In fact, when CBD was tested at lower and higher concentrations than 100 nM in experiments of [³⁵S]GTPγS binding assay, it did not show any effect on 8-OH-DPAT dose-response curve (Figure 57). Again, these results confirm data already reported *in vivo*, where CBD displayed a biphasic profile on LiCl-induced vomiting in musk shrew model (Parker et al. 2004). Moreover, this biphasic pharmacological profile displayed by CBD has also been reported in other apparent 5-HT_{1A} receptors-mediated effects on ischemic injury (Mishima et al. 2005), anxiety (Campos, Guimaraes 2008) and depression (Zanelati et al. 2010).

The possible mechanism underlying CBD-mediated 5-HT_{1A} receptor potentiation is still under investigation. Preliminary results indicated that CBD is not an allosteric modulator at 5-HT_{1A} receptors. In fact, the 8-OH-DPAT-potentiating concentration of CBD, 100 nM, did not alter the rate at which [³H]8-OH-DPAT dissociates from specific binding sites in rat brainstem membranes (Figure 58). Hence, this result strengthens the hypothesis that CBD is not interacting directly with 5-HT_{1A} receptors, but its effect could be mediated through a possible cross-talk between these serotonin receptors and CB₁ or other receptors. Thus, the implication of heterodimers between 5-HT_{1A} receptors and CB₁ or other receptors cannot be ruled out. In fact, the ability of 5-HT_{1A} receptors to form homo- and heterodimers has been demonstrated in recombinant systems (Salim et al. 2002). In particular, 5-HT_{1A} receptors formed heterodimers with several GPCRs, such as 5-HT_{1B} and 5-HT_{1D}, Endothelial Differentiation Gene (EDG) 1 and 3 receptors (Salim et al. 2002). On the other hand, CB₁ receptors have been demonstrated to form heterodimers with:

- Adenosin 2A (A_{2A}) receptors (Carriba et al. 2007)
- Mu-opioid receptors (MOR) (Rios, Gomes & Devi 2006)
- Orexin-1 receptors (OX₁) (Ellis et al. 2006)
- Dopamine 2 (D₂) receptors (Kearn et al. 2005)

Differently from the concentration of 10 μM, CBD at 100 nM did not show any affinity for CB₁ receptors. However, data previously reported in this laboratory showed that CBD has affinity for CB₁ receptors in the nanomolar range when this phytocannabinoid is tested as antagonist of the non selective CB₁/CB₂ receptor agonists, CP55940 and WIN55212-2 (Thomas et al. 2007). Despite this, the possible implication of CB₁ in CBD-induced 5-HT_{1A} receptors potentiation is yet to be established and warrants further investigations.

Thus, experiments are now in progress to further confirm data obtained so far and to better characterize the mechanism underlying CBD effects in brainstem. First of all, in order to confirm [³⁵S]GTPγS binding assay data, 8-OH-DPAT-potentiating concentration of CBD, 100 nM, will be tested in association with 8-OH-DPAT in experiments of cyclic AMP assay in primary neuronal cultures derived from raphe nuclei.

Moreover, with the aim of further confirming the selective activity of CBD at somatodendritic 5-HT_{1A} autoreceptors in raphe nuclei, [³⁵S]GTPγS binding

experiments will be performed in hippocampus, a cerebral region rich of postsynaptic 5-HT_{1A} heteroreceptors.

An other possible experiment to uncover the involvement of other receptors in CBD-mediated 5-HT_{1A} receptors potentiation would come from functional assays (i.e. [³⁵S]GTPγS binding and/or cyclic AMP assays) in cells overexpressing 5-HT_{1A} receptors. The expression of these receptors in a system different from the brain should give us more information about the possible specific activity of CBD in the brainstem and the possible involvement of other receptors in this scenario.

CONCLUSIONS

Data here reported confirm that the anti-nausea and anti-emetic effects of CBD are mediated by somatodendritic 5-HT_{1A} receptors. Moreover, our results suggest that the effect of CBD at 5-HT_{1A} receptors might also involve the interaction with other receptors.

Thus, the lack of psychoactive effects and the efficacy showed by CBD to treat nausea and vomit, bring evidences that CBD might be a good candidate to treat these disease states associated with chemotherapy.

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LIST OF PUBLICATIONS

1. Goffredo D, Conti L, Di Febo F, Biella G, Tosoni A, Vago G, Moiana A, Bolognini D, Toselli M, Cattaneo E. *Setting the conditions for efficient, robust and reproducible generation of functionally active neurons from adult subventricular zone-derived neural stem cells*. Cell Death Differ. 2008; 15(12):1847-56.
2. Massi P, Valenti M, Bolognini D, Parolaro D. *Expression and function of the endocannabinoid system in glial cells*. Curr Pharm Des. 2008; 14(23):2289-98.
3. Cascio MG, Bolognini D, Pertwee RG, Palazzo E, Corelli F, Pasquini S, Di Marzo V, Maione S. *In vitro and in vivo pharmacological characterization of two novel selective cannabinoid CB₂ receptor inverse agonists*. Pharmacol Res. 2010 Apr; 61(4):349-54.
4. Garavaglia A, Moiana A, Camnasio S, Bolognini D, Papait R, Rigamonti D, Cattaneo E. *Adaptation of NS cells growth and differentiation to high-throughput screening-compatible plates*. BMC Neurosci. 2010 Jan 19; 11:7.
5. Bolognini D, Costa B, Maione S, Comelli F, Marini P, Di Marzo V, Parolaro D, Ross RA, Gauson LA, Cascio MG and Pertwee RG. *The plant cannabinoid Δ^9 -tetrahydrocannabivarin can decrease signs of inflammation and inflammatory pain in mice*. Br J Pharmacol. 2010 Jun; 160(3):677-87.
6. Pasquini S, Ligresti A, Mugnaini C, Semeraro T, Cicione L, De Rosa M, Guida F, Luongo L, De Chiaro M, Cascio MG, Bolognini D, Marini P, Pertwee R, Maione S, Di Marzo V, Corelli F. *Investigations on the 4-Quinolone-3-carboxylic Acid Motif. 3. Synthesis, Structure–Affinity Relationships, and Pharmacological Characterization of 6-Substituted 4-Quinolone-3-carboxamides as Highly Selective Cannabinoid-2 Receptor Ligands*. J. Med. Chem. 2010 Aug 26; 53(16):5915-28.

Abstracts

1. International Cannabinoids Research Society (ICRS) meeting 2008
Poster presentation: *Salvinorin A interaction with the cannabinoid system*.
Cinzia Guidali, Daniele Bolognini, Daniela Vigano', Mariaelvina Sala and Tiziana Rubino.
2. British Pharmacological Society (BPS) meeting 2009
Poster presentation: *Evidence that Δ^9 -tetrahydrocannabivarin can reduce inflammation in mice by activating cannabinoid CB₂ receptors*. Daniele Bolognini, Barbara Costa, Maria Grazia Cascio and Roger G. Pertwee.
3. International Cannabinoids Research Society (ICRS) meeting 2010
Poster presentation: *Conversion of AM630 into an apparent neutral CB₂ receptor antagonist*. Daniele Bolognini, Maria Grazia Cascio, Ruth A. Ross and Roger G. Pertwee. **ICRS AWARD**

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