Cannabinoids as Potential New Therapeutics of Gastrointestinal Motility and Inflammatory Disorders

A thesis submitted in accordance with the conditions governing candidates for the degree of

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Presented by
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Abstract

Cannabinoids show potential as new treatments for inflammatory bowel disease (IBD), exerting several favourable effects in the gut, including anti-inflammatory and anti-motility effects. The main difficulty with cannabinoids is their psychotropic side effects, but access to the brain may be prevented by conjugating the cannabinoid to a bulky group such as a dendrimer. The aims of this thesis were to investigate the mechanism by which cannabinoids reduce gut motility and to investigate whether cannabinoids protect the intestine from inflammatory-damage. A further aim was to determine whether cannabinoids remain pharmacologically active when conjugated to a dendrimer.

All cannabinoids used (apart from arachidonoylcyclopropylamide and (-)WIN 55,212-2) caused a concentration-dependent reduction in the size of electrically-stimulated contractions in the guinea-pig ileum. The responses were not blocked by CB_1, CB_2, CB_e (putative endothelial cannabinoid receptor) or GPR55 antagonists, suggesting that none of these receptors were involved in mediating cannabinoid responses. PSN 375963 reduced carbachol-induced contraction, suggesting that the GPR119 may be present on ileum smooth muscle. (+)WIN 55,212-2 was shown to protect the guinea-pig ileum from hydrogen peroxide-induced damage but this protection was not blocked by CB_1, CB_2, CB_e or GPR55 antagonists, suggesting that the protective effects were not mediated through these receptors. Conjugation of JWH007 to a spacer (GA003) abolished activity in the guinea-pig ileum and the conjugation of JWH007 to a spacer and dendrimer (GA006) was found to be toxic in the macrophage assay.

These studies show that cannabinoid-mediated inhibition of guinea-pig ileum contractions is not mediated through the CB_1, CB_2, CB_e or GPR55 receptor. These receptors were not involved in the (+)WIN 55,212-2 mediated protection against hydrogen peroxide-induced damage in the ileum. The approach of attaching a dendrimer to JWH007 to prevent central nervous system (CNS) penetration does not appear to be a feasible approach because the cannabinoid-dendrimer was unexpectedly cytotoxic.

ACRONYMS AND ABBREVIATIONS

Abnormal CBD:	Abnormal cannabidiol
AC:	Adenylyl cyclase
ACEA:	Arachidonoyl-2’-chloroethylamide
ACPA:	Arachidonoylcyclopropylamide
2-AG:	2-Arachidonoyl glycerol
AIM:	Ascending interneuron
ANOVA:	Analysis of variance
BCA :	Bicinchoninic acid
BSA:	Bovine serum albumin
Caco-2:	Human colonic carcinoma cell line
cAMP:	Cyclic adenosine monophosphate
CHO:	Chinese hamster ovarian cell line

CM:	Circular muscle
CRE:	cAMP response element
CB_1:	Cannabinoid receptor type 1
CB_2 :	Cannabinoid receptor type 2
(-)CBD:	Cannabidiol
CB_e :	Putative endothelial cannabinoid receptor
CGRP:	Calcitonin gene-related peptide
CI:	Confidence interval
CNS:	Central nervous system
COX-2:	Cyclooxygenase-2
D_2 receptors:	Dopamine receptor type 2
DAG:	1,2-Diacylglycerol
DAGL:	DAG lipase

DIM: Descending interneuron

DMEM: Dulbecco's modified Eagle's medium

DMNX: Dorsal motor nucleus

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DNBS: Dinitrobenzene sulfonic acid

E coli **:** Escherichia coli

EC_{50}: The molar concentration of agonist which produces 50% of the maximum response for that agonist

EFS: Electrical field stimulation

EGTA: Ethyleneglycol-O, O' bis (2-aminoethyl)-N,N,N'N'-tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EM: Excitatory motor neuron

FAAH: Fatty acid amide hydrolase

FAK: Focal adhesion kinase

FBS: Fetal bovine serum

FRNK: FAK-related-non-kinase

GLP-1: Glucagon-like peptide-1

HEK 293: Human embryonic kidney cell line

HRP: Horseradish peroxidase

5-HT: 5-Hydroxytryptamine receptors

IBD: Inflammatory bowel disease

IC_{25}: The molar concentration of a drug that is required to inhibit a biological process by 25%

IC_{50}: The molar concentration of a drug that is required to inhibit a biological process by 50%

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IM: Inhibitory motorneuron

IPAN: Intrinsic primary afferent neuron

Ki: This refers to the equilibrium dissociation constant of a ligand determined in inhibition studies

K_v channels: Voltage-dependent potassium channels

LM: Longitudinal muscle

L-NAME: N^G-nitro-L-arginine methyl ester

LPI: Lysophosphatidylinositol

LPS: Lipopolysaccharide

MAG: Monoacylglycerol

MAGL: MAG lipase

MAPK: Mitogen-activated protein kinase

MP: Myenteric plexus

mRNA: Messenger ribonucleic acid

MTS: Methanethiosulphonate reagent

NAAA: N-acylethanolamine-hydrolysing acid amidase

NADA: N-arachidonoyl dopamine

NANC: Non-adrenergic, non-cholinergic

NAPE: N-acyl phosphatidylethanolamine

NAPE-PLD: NAPE phospholipase D

NO: Nitric oxide

NOS: Nitric oxide synthase

OEA: Oleoylethanolamine

PAMAM: Poly(amidoamine)

PBS: Phosphate buffered saline

PEA: Palmitoylethanolamide

PI3K:	Phosphoinositide 3-kinase
PKA:	Protein kinase A
PLC:	Phospholipase C
PMSF:	Phenylmethanesulfonyl fluoride
PPAR:	Peroxisome proliferators-activated receptor
PSN:	PSN 375963 hydrochloride
Raf:	Proto-oncogene serine/threonine-protein kinase
RAW 264.7:	Mouse leukaemic monocyte macrophage cell line
ROS:	Reactive oxygen species
SAR:	Structure activity relationship
SEM:	Standard error of the mean
SP:	Submucosal plexus
SOD:	Superoxide dismutase

T_h cells:	T helper cells
THC:	Δ^9-tetrahydrocannabinol
TNBS:	2,4,6-Trinitrobenzenesulfonic acid
TNFα:	Tumour necrosis factor α
TRP:	Transient receptor potential channel
TRPA 1:	Transient potential cation channel, subfamily A, member 1
TRPV 1:	Vanilloid receptor 1 or transient receptor potential cation channel, subfamily V, member 1
TRPV 2:	Vanilloid receptor 1 or transient receptor potential cation channel, subfamily V, member 1
VIP:	Vasoactive intestinal polypeptide
(-)WIN:	(*S*)-(-)-WIN 55,212-2 mesylate
(+)WIN:	(*R*)-(+)-WIN 55,212-2 mesylate
WR:	Working reagent

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CHAPTER 1:

INTRODUCTION

1. Introduction

1.1 Overview of thesis

Therapeutics currently available to treat inflammatory bowel disease (IBD) and Irritable Bowel Syndrome (IBS) are limited either by their lack of efficacy or their severe side effects, and there is a need for safer, more effective treatments to be developed. Cannabinoids may potentially be developed as a new class of therapeutics for IBD. They exert several beneficial effects in the gut such as reducing inflammation, motility and secretions, alleviating visceral pain (Izzo and Sharkey, 2010) and enhancing epithelial wound healing (Wright *et al*., 2005). The main barrier to a cannabinoid-based therapeutic is the resulting psychotropic side effects, but this could be overcome by conjugating the cannabinoid to a dendrimer to prevent the cannabinoid from crossing the blood brain barrier. Dendrimers are bulky polymers with tree-like branching structures. The size of the dendrimer can be selected so that it is small enough to be absorbed across the intestine but large enough to prevent central nervous system (CNS) penetration. Dendrimer properties and *in vivo* dendrimer distribution are discussed in more detail in chapter 5.

The mechanisms by which cannabinoids reduce motility in the gut have not been fully elucidated and one of the main aims of this thesis was to examine the receptors involved in these effects. Additionally, as this has not been the subject of any earlier research, these studies investigated whether cannabinoids protect the intestine against inflammatory damage. The final aim of this thesis was to test whether cannabinoids retain their pharmacological efficacy when conjugated to a dendrimer

This introduction reviews the current literature concerning the pharmacology of plant, synthetic and endogenous cannabinoids, as well as the latest evidence regarding the mechanisms by which cannabinoids modulate the immune response and reduce inflammation and motility in the gut.

1.2 Cannabis sativa

The plant *Cannabis sativa*, commonly known as marijuana, was first used for medicinal, recreational and religious purposes 5000 years ago (Pertwee, 2006) and is now the most popular illegal drug in the United States with 46.1% of 17-18 year olds being users (Klein, 2005). The plant is widely known for its psychotropic effects which include relaxation and euphoria at low doses (which accounts for its recreational use) and impairment of thinking, perceptual and psychomotor function at high doses (Smita *et al.*, 2007).

Marijuana contains at least 66 compounds known as cannabinoids, the cannabinoid Δ^9-Tetrahydrocannabinol (THC) being mainly responsible for the psychotropic effects (Pertwee, 2006). In addition to phytocannabinoids, endogenous cannabinoids and cannabinoid receptors have now been discovered in mammals and are discussed in more detail below (section 1.3).

In addition to use for its psychotropic effects, marijuana has been used to relieve the symptoms of several diseases ranging from rheumatism and epilepsy to tetanus and gonorrhoea. There is also anecdotal evidence that marijuana may be effective in relieving the symptoms of Crohn’s disease and diabetic gastroparesis (Klein, 2005).

Also, antiperistaltic, antisecretive and antiulcer activity has been exhibited by THC (Pinto *et al.*, 2002a).

1.3 The endocannabinoid system

Endogenous cannabinoid receptors and agonists have been discovered in mammals and two types of cannabinoid receptors (CB_1 and CB_2) have been cloned (Howlett *et al.*, 2002).There is also evidence for the existence of other cannabinoid receptor types (Gomez-Ruiz *et al.*, 2007). CB_1 receptors are found in the central nervous system and some peripheral tissues whereas CB_2 receptors are mainly found on immune cells, reviewed by Pertwee (2005) and Pertwee *et al* (2002). These receptors are coupled to G proteins and there is evidence that an allosteric site is present on the CB_1 receptor which modulates cannabinoid affinity for the binding site (Price *et al.*, 2005). The CB_1 receptor seems to be highly conserved across species, in contrast to the CB_2 receptors which show more variation between species (Gomez-Ruiz *et al.*, 2007).

Of the endogenous agonists that have been found, anandamide (Hanus *et al.*, 2001) and 2-arachidonoyl glycerol (2-AG) (Sugiura *et al.*, 1997; Sugiura *et al.*, 1996) have been the most extensively studied. These agonists have been found to differ in efficacy for the two receptor types (Pertwee, 1999). 2-AG is an agonist at the both receptors and is more active as a CB_2 receptor agonist than anandamide (Gomez-Ruiz *et al.*, 2007). Anandamide acts as a partial or full agonist at the CB_1 receptor, depending on the tissue and response being measured and can act as a low-efficacy agonist or an antagonist at CB_2 receptors (Gomez-Ruiz *et al.*, 2007). Other endogenous cannabinoids include noladin ether (2-arachidonoyl ether), N-arachidonoyl dopamine (NADA) and

virodhamine which vary in efficacy at cannabinoid receptors and whose biological function is unknown (Gomez-Ruiz *et al.*, 2007).

"Endocannabinoid-like" substances also exist such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA). These compounds possess low affinity for the CB_1 and CB_2 receptor (Bradshaw *et al.*, 2005; Devane *et al.*, 1992; Lambert *et al.*, 1999) but can enhance the action of endocannabinoids at their receptors, in a process known as the entourage effect (Gomez-Ruiz *et al.*, 2007). These compounds are *N*-acylethanolamines and it is thought that, in a similar way to anandamide, they are synthesised from a molecule similar to N-acyl phosphatidylethanolamine (NAPE) and broken down by fatty acid amide hydrolase (FAAH) (Alexander and Kendall, 2007). There is evidence that OEA and PEA may exert pharmacological effects through non-CB_1 and non-CB_2 receptors (Brown, 2007). OEA reduces food intake and alters metabolism, and studies have suggested that these effects may involve activation of the vanilloid receptor transient receptor potential cation channel, subfamily V, member 1 ($TRPV_1$), peroxisome proliferator - activated receptor (PPARα) (Fu *et al.*, 2003; Su *et al.*, 2006; Thabuis *et al.*, 2008) or the orphan receptor GPR119 (Ning *et al.*, 2008; Overton *et al.*, 2006). PEA, on the other hand, has anti-inflammatory effects which may be mediated through stimulation of the orphan receptor GPR55 (Ryberg *et al.*, 2007), PPARα (Lo Verme *et al*., 2005c) or potentiation of anandamide actions at several receptors (Costa *et al.*, 2008). The effect of OEA and PEA on the non-$CB_{1,}$ non-CB_2 receptors has been discussed in more detail in section 1.7.2.

1.4 Synthesis of endocannabinoids

Endocannabinoids are formed and released on demand (Elphick *et al.*, 2001; Kreitzer *et al.*, 2001; Ohno-Shosaku *et al.*, 2001; Wilson and Nicoll, 2001) in response to increased intracellular calcium (Best *et al.*, 2010) and therefore are not stored in synaptic vesicles, in contrast to other neurotransmitters. Anandamide is produced by the hydrolysis of the membrane pre-cursor NAPE, catalysed by the enzyme phospholipase D (NAPE-PLD) (Okamoto *et al.*, 2004). Prior to this, NAPE is synthesised by the transfer of arachidonic acid from phosphatidylcholine to phosphatidylethanolamine (Cadas *et al.*, 1997; see figure 1.1). Variants of this pathway have been described in the stomach and the mouse leukemic monocyte-macrophage cell line RAW 264.7 (Gomez-Ruiz *et al.*, 2007). It is now thought that there may be alternative pathways for the synthesis of anandamide as NAPE-PLD knockout mice do not have lower levels of anandamide (Leung et al., 2006; se figure 1.1). Studies have suggested that phopsho-anandamide may be formed by the subsequent hydrolysis of NAPE by a phospholipase-C like enzyme and then hydrolysed to anandamide by an enzyme such as tyrosine phophatase N22 (Liu *et al.*, 2006). Another pathway for anandamide production may be the cleavage of two acyl groups from NAPE by α/β hydrolase 4, followed by hydrolysis of glycerophospho-anandamide to anandamide by a phosphodiesterase (Simon *et al.*, 2006). It is also possible that 2-lyso-NAPE may be formed from NAPE by phospholipase A_2 and converted to anandamide by lyso-phospholipase D (Sun *et al.*, 2004).

2-AG, on the other hand, is produced from 1,2-diacylglycerol (DAG) by DAG lipase (DAGL). Two isozymes of DAG lipase, DAGL-α and DAGL-β, have been found in the adult brain (Bisogno *et al.*, 2003). DAG can be produced from phosphoinositides or from phosphatidic acid (Gomez-Ruiz *et al.*, 2007).

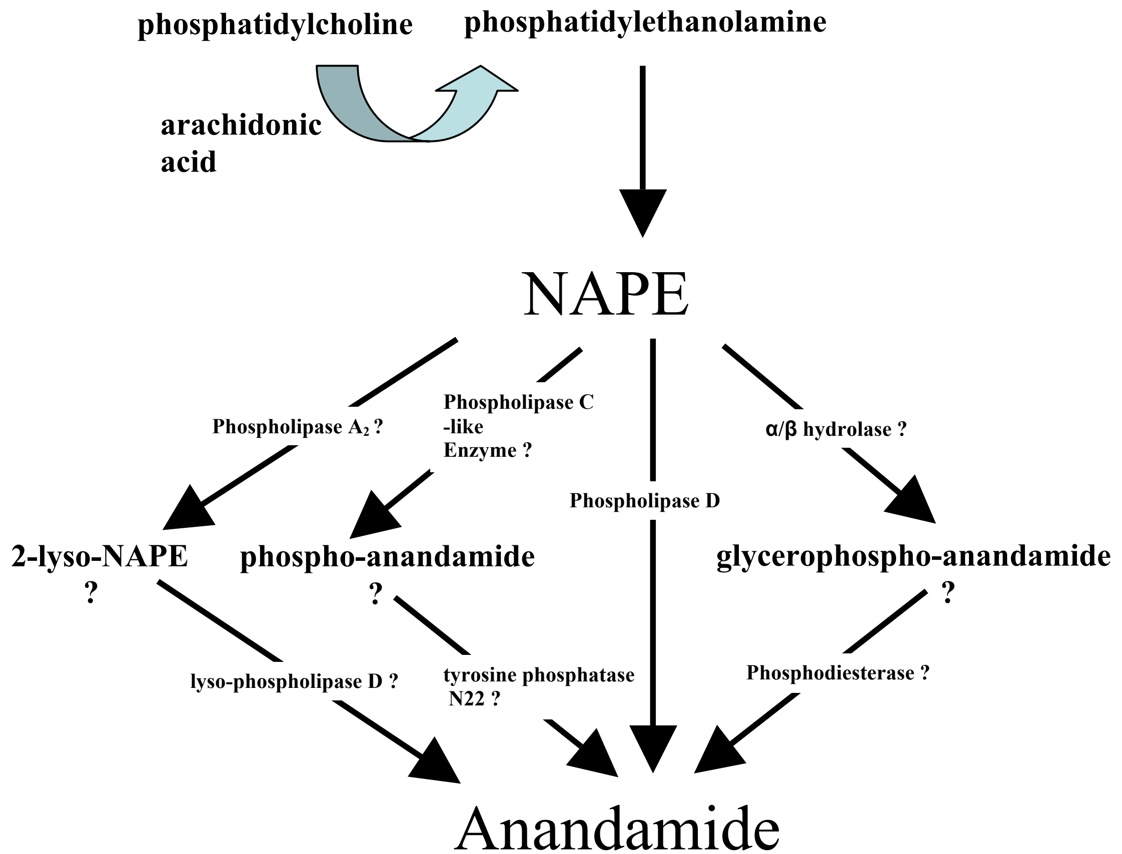


Figure 1.1 *Pathways for anandamide synthesis.*

1.5 Endocannabinoid uptake mechanisms

There is evidence that termination of anandamide's action occurs via uptake into cells. This is followed by intracellular hydrolysis to arachidonic acid and ethanolamide (Gomez-Ruiz *et al.*, 2007; see figure 1.2) which is catalysed by FAAH (Maccarrone *et al.*, 1998; Piomelli *et al.*, 1999).The mechanism of anandamide uptake is controversial as an endocannabinoid transporter has not yet been identified. Suggested mechanisms include diffusion facilitated by a membrane transporter, diffusion driven by intracellular hydrolysis of anandamide, diffusion driven by intracellular sequestration of anandamide and endocytosis (Gomez-Ruiz *et al.*, 2007).

1.6 Metabolism of endocannabinoids

Inside the cell, whereas anandamide undergoes hydrolysis to arachidonic acid and ethanolamide, catalysed by FAAH, 2-AG undergoes hydrolysis to arachidonic acid and glycerol, catalysed by a monoacylglycerol-lipase (MAGL). FAAH can also catalyse the hydrolysis of 2-AG, but MAGL is thought to be mainly responsible. Additionally, 2-AG is a substrate for α/β hydrolases, which could also be responsible for the hydrolysis of this endocannabinoid (Blankman *et al.*, 2007).

Non-FAAH amidases, the FAAH homologue FAAH-2 (Wei *et al.*, 2006) and *N*-acylethanolamide acid amidase (NAAA) (Tsuboi *et al.*, 2005) which inactivate *N*-acyl-ethanolamines, have been discovered. Anandamide and 2-AG, however, are not inactivated by these enzymes (Tsuboi *et al.*, 2005; Wei *et al.*, 2006).

O-phosphorylcholine derivatives of *N*-acylethanolamines have been discovered in FAAH null mice (Mulder *et al.*, 2006), and, although it is not clear how these are formed, this could represent a mechanism for FAAH-independent inactivation of anandamide and other *N*-acylethanolamides (De Petrocellis *et al.*, 2009).

The endocannabinoids anandamide and 2-AG are metabolised by cyclooxygenases and lipoxygenases, which are involved in the general metabolism of eicosanoids (Hampson *et al.*, 1995; Ueda *et al.*, 1995; Yu *et al.*, 1997) as well as cytochrome P450 enzymes (Bornheim *et al.*, 1995). Prostamides are formed by the oxidation of anandamide by cyclooxygenase 2 (COX-2) and subsequent conversion by prostaglandin synthases (see figure 1.2). The action of these enzymes on 2-AG, on the other hand, produces prostaglandin glycerol esters (Kozak *et al.*, 2000). The prostamide metabolites of anandamide may produce various pharmacological effects, for example the inhibition of immune cells such as macrophages (Correa *et al.*, 2008) and the increase in pulmonary

artery pressure (Wahn *et al.*, 2005). Prostaglandin metabolites of 2-AG also possess pharmacological effects, such as the mobilisation of intracellular Ca^{2+} (Nirodi *et al.*, 2004). 2-AG and anandamide may also be converted to hydroperoxy- and hydroxyl-derivatives by lipooxygenases or epoxyeicosatetraenoyl-anandamides by cytochrome p450 oxygenases (see figure 1.2). Most of these metabolites are still active at cannabinoid receptors, but there is currently no evidence that they are produced *in vivo* (De Petrocellis *et al.*, 2009).

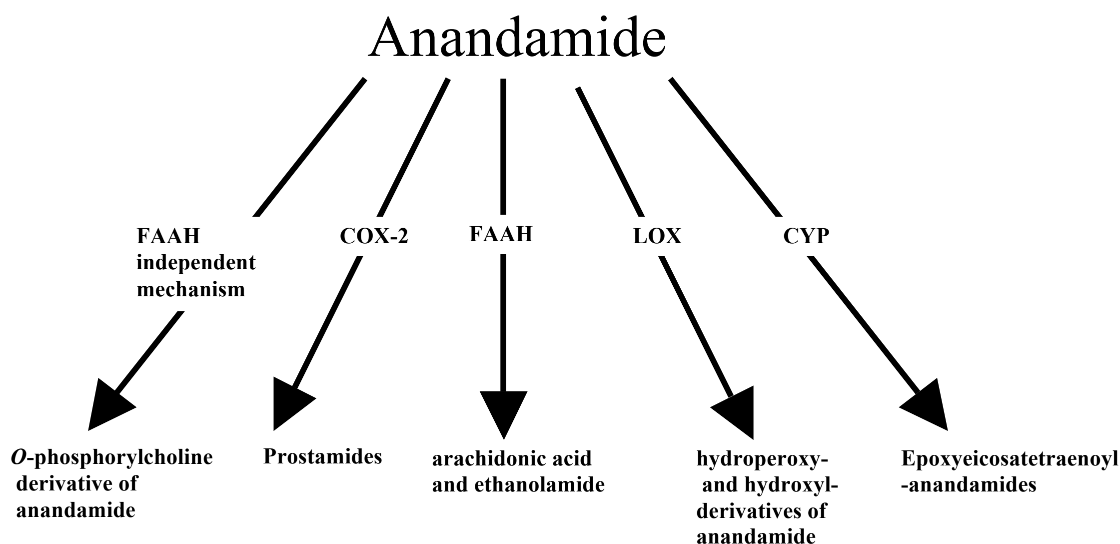


Figure 1.2 *Metabolic pathways of anandamide.*

1.7 Cannabinoid pharmacology

1.7.1 Cannabinoid receptor ligands

Cannabinoid agonists can be divided into three different classes, endocannabinoids (found endogenously in mammals), phytocannabinoids (found endogenously in plants) and synthetic cannabinoid ligands. Synthetic cannabinoid antagonists have also been designed. Some of these antagonists are neutral whereas others are inverse agonists

(reduce the constitutive activity of the receptor). Cannabinoids differ in their selectivity at the CB_1 and CB_2 receptors: the common cannabinoid agonist and antagonists and their affinities for the CB_1 and CB_2 receptors are shown in table 1.1 overleaf.

Ligand	Classification	CB_1 ki value (nM)	CB_2 ki value (nM)	Reference
ACEA	**Selective CB_1 agonist**	**1.4**	**>2000**	**(Hillard *et al.*, 1999)**
ACPA	**Selective CB_1 agonist**	**2.2**	**715**	**(Hillard *et al.*, 1999)**
Methanandamide	**Selective CB_1 agonist**	**17.9 20**	**868 815**	**(Lin *et al.*, 1998) (Khanolkar *et al.*, 1996)**
Rimonabant	**Selective CB_1 antagonist**	**11.8 12.3 5.6**	**973 702 >1000**	**(Felder *et al.*, 1995) (Showalter *et al.*, 1996) (Rinaldi-Carmona *et al.*, 1994)**
AM281	**Selective CB_1 antagonist**	**12**	**4200**	**(Lan *et al.*, 1999a)**
AM251	**Selective CB_1 antagonist**	**7.49**	**2290**	**(Lan *et al.*, 1999b)**
JWH133	**Selective CB_2 agonist**	**677**	**3.4**	**(Huffman *et al.*, 1999)**
SR144528	**Selective CB_2 antagonist**	**70 305 437 50.3 >10000**	**0.28 0.3 0.6 1.99 5.6**	**(Ruiu *et al.*, 2003) (Rinaldi-Carmona *et al.*, 1998) (Rinaldi-Carmona *et al.*, 1998) (Iwamura *et al.*, 2001) (Ross *et al.*, 1999)**
AM630	**Selective CB_2 antagonist**	**5152**	**31.2**	**(Ross *et al.*, 1999)**
JWH007	**Non-selective CB_1 / CB_2 agonist**	**9.5**	**2.9**	**(Huffman *et al.*, 2005)**
HU210	**Non-selective CB_1 / CB_2 agonist**	**0.0608 0.1 0.73**	**0.524 0.17 0.22**	**(Felder *et al.*, 1995) (Rhee *et al.*, 1997) (Showalter *et al.*, 1996)**
CP 55,940	**Non-selective CB_1 / CB_2 agonist**	**5 3.72 1.37**	**1.8 2.55 1.37**	**(Ross *et al.*, 1999) (Felder *et al.*, 1995) (Rinaldi-Carmona *et al.*, 1994)**
(+)WIN	**Non-selective CB_1 / CB_2 agonist**	**9.94 4.4 62.3**	**16.2 1.2 3.3**	**(Rinaldi-Carmona *et al.*, 1994) (Hillard *et al.*, 1999) (Felder et *al.*, 1995)**
O-1602	**Selective GPR55 agonist**	**>30000**	**>30000**	**(Ryberg *et al.*, 2007)**
(-) CBD	**GPR55 antagonist/ CB_1 antagonist/CB_2 inverse agonist**	**4350 >10000**	**2860 >10000**	**(Showalter *et al.*, 1996) (Bisogno *et al.*, 2001)**
Abnormal-CBD	**Selective CB_e agonist**	**>100000**	**>10000**	**(Showalter *et al.*, 1996)**
O-1918	**Selective CB_e antagonist**	**>30000**	**>30000**	**(Jarai *et al.*, 1999)**
PSN 375963	**GPR119 agonist**	**Reported not to bind**	**?**	**(Lambert *et al.*, 2007)**

Table 1.1 *Affinities of commonly used cannabinoid agonists and antagonists/inverse agonists at the CB_1 and CB_2 receptor.*

1.7.2 Non-CB_1 / CB_2 targets for cannabinoid receptor ligands

One of the major problems with using selective cannabinoid agonists and antagonists as tools in pharmacological studies is that they may not be as selective as first thought. It is now emerging that cannabinoids may have several other pharmacological targets apart from the CB_1 and CB_2 receptors. For example, the so-called selective CB_1 antagonist rimonabant has been shown to target a number of other receptors apart from CB_1 and has also been shown to directly alter ion channel conductance. These effects of rimonabant have been reported at concentrations used in pharmacological studies which means that results from these studies should be interpreted with caution (see table 1.5). The evidence for additional targets for cannabinoids (including putative novel cannabinoid receptors) has been discussed below.

GPR55 receptor

The orphan G protein receptor GPR55 has been proposed as a novel cannabinoid receptor as it activated by several cannabinoids (Brown, 2007). There have been some suggestions that GPR55 may be the putative CB_e receptor but the evidence is not entirely consistent with this theory (See CB_e receptor section below). Some researchers suggest that GPR55 is not a cannabinoid receptor at all, as cannabinoids do not consistently activate the GPR55 receptor (see table 1.2 a, and b). Some authors have suggested that lysophosphatidylinositol (LPI) is more likely to be the endogenous ligand as it has been reported to activate this receptor in every assay studied so far (Godlewski *et al.*, 2009).

GPR55 mRNA has been found to be expressed in the human brain (high levels have been found in the dorsal striatum, caudate nucleus and putamen) and it has been suggested that GPR55 may have a role in pain signalling and influence memory,

learning and motor functions. GPR55 transcripts have also been identified in peripheral tissues including ileum, testis, spleen, tonsil, breast, adipose tissue (Brown, 2007; Sawzdargo *et al.*, 1999) and endothelial cell lines (Waldeck-Weiermair *et al.*, 2008).

Ligand	Activity	Cell type	Measured response	Reference
Anandamide	**No effect(1µM)**	**hGPR55-HEK293**	**ERK phosphorylation**	**(Oka *et al.*, 2007; Oka *et al.*, 2009)**
	nM range	**hGPR55-HEK293**	**[^{35}S]GTPγS Assay**	**(Ryberg *et al.*, 2007)**
	µM range	**hGPR55-HEK293**	**RhoA activation**	**(Ryberg *et al.*, 2007)**
	µM range	**hGPR55-HEK293**	**Ca^{2+} mobilisation**	**(Lauckner *et al.*, 2008)**
	µM range	**HUVEC**	**Ca^{2+} mobilisation**	**(Waldeck-Weiermair *et al.*, 2008)**
	µM range	**HUVEC**	**ERK phosphorylation**	**(Waldeck-Weiermair et *al.*, 2008)**
	No effect	**hGPR55-HEK293**	**Ca^{2+} mobilisation**	**(Henstridge *et al.*, 2009b)**
2-AG	**No effect(1µM)**	**hGPR55-HEK293**	**ERK phosphorylation**	**(Oka *et al.*, 2007; Oka *et al.*, 2009)**
	nM range	**hGPR55-HEK293**	**[^{35}S]GTPγS Assay**	**(Ryberg *et al.*, 2007)**
	No effect (5µM)	**hGPR55-HEK293**	**Ca^{2+} mobilisation**	**(Lauckner *et al.*, 2008)**
	No effect	**hGPR55-HEK293**	**Ca^{2+} mobilisation**	**(Henstridge *et al.*, 2009b)**
LPI	**nM range**	**hGPR55-HEK293**	**ERK phosphorylation**	**(Oka *et al.*, 2007; Oka *et al.*, 2009)**
	µM range	**DRG neurons**	**Ca^{2+} mobilisation**	**(Lauckner *et al.*, 2008)**
	µM range	**HUVEC**	**Ca^{2+} mobilisation**	**(Waldeck-Weiermair *et al.*, 2008)**
	nM range	**hGPR55-HEK293**	**Ca^{2+} mobilisation**	**(Henstridge *et al.*, 2009b)**
	µM range	**hGPR55-HEK293**	**NFAT activation**	**(Henstridge *et al.*, 2009b)**
	µM range	**hGPR55-HEK293**	**RhoA activation**	**(Henstridge *et al.*, 2009b)**

Table 1.2a *Activity of cannabinoids at the GPR55 receptor.*

Ligand	Activity	Cell type	Measured response	Reference
(+)WIN	**No effect (1µM)**	**hGPR55-HEK293**	**ERK phosphorylation**	**(Oka *et al.*, 2007)**
	No effect (1µM)	**hGPR55-HEK293**	**[^{35}S]GTPγS Assay**	**(Johns *et al.*, 2007)**
	No effect (30µM)	**hGPR55-HEK293**	**[^{35}S]GTPγS Assay**	**(Ryberg *et al.*, 2007)**
	No effect (5µM)	**hGPR55-HEK293**	**Ca^{2+} mobilisation**	**(Lauckner *et al.*, 2008)**
AM281	**No effect (30µM)**	**U2OS cells expressing GPR55**	**B-arrestin assay**	**(Kapur *et al.*, 2009)**
	Agonism (3 to 30µM)	**hGPR55-HEK293**	**Elevation of intracellular Ca^{2+}**	**(Henstridge *et al.*, 2009a and b)**
rimonabant	**No effect (10µM)**	**GPR55-expressing microglial cells (BV2)**	**Elevation of intracellular Ca^{2+}**	**(Eldeeb *et al.*, 2009)**
	No effect (30µM)	**U2OS cells expressing GPR55**	**Phosphorylation of ERK**	**(Kapur *et al.*, 2009)**
	Agonist (EC_{50} 3.9µM)	**U2OS cells expressing GPR55**	**B-arrestin assay**	**(Kapur *et al.*, 2009)**
	Agonist (10µM)	**hGPR55-HEK293**	**G-protein dependent activation of PKCΔII**	**(Kapur *et al.*, 2009)**
	Antagonist	**hGPR55-HEK293**	**Ca^{2+} mobilisation**	**(Lauckner *et al.*, 2008)**
	Antagonist	**mouse DRG neurons**	**Ca^{2+} mobilisation**	**(Lauckner *et al.*, 2008)**
Abnormal-CBD	**nM range**	**hGPR55-HEK293**	**[^{35}S]GTPγS Assay**	**(Johns *et al.*, 2007)**
	µM range	**hGPR55-HEK293**	**[^{35}S]GTPγS Assay**	**(Ryberg *et al.*, 2007)**
	No effect(3µM)	**hGPR55-HEK293**	**Ca^{2+} mobilisation**	**(Lauckner *et al.*, 2008)**
(-)CBD	**Antagonist**	**hGPR55-HEK293**	**[^{35}S]GTPγS Assay**	**(Ryberg *et al.*, 2007)**
0-1602	**nM range**	**HEK293**	**[^{35}S]GTPγS Assay**	**(Johns *et al.*, 2007)**
	nM range	**HEK293**	**[^{35}S]GTPγS Assay**	**(Ryberg *et al.*, 2007)**
	µM range	**HEK293**	**RhoA activation**	**(Ryberg *et al.*, 2007)**
	µM range	**HUVEK**	**Ca^{2+} mobilisation**	**(Waldeck-Weiermair *et al.*, 2008)**
	No effect (10µM)	**hGPR55-HEK293**	**Ca^{2+} mobilisation**	**(Oka *et al.*, 2009)**
	No effect (10µM)	**hGPR55-HEK293**	**ERK phosphorylation**	**(Oka *et al.*, 2009)**

Table 1.2b *Activity of cannabinoids at the GPR55 receptor continued.*

GPR119 receptor

The GPR119 receptor is an orphan G protein-coupled receptor which has been proposed as a novel cannabinoid receptor .The receptor is closely related to the cannabinoid receptors (Oh *et al.*, 2006) and the endocannabinoid-like OEA is a known ligand for GPR119 (Milman *et al.*, 2006), although anandamide is only a weak agonist for this receptor (Overton *et al.*, 2006). The effects of other cannabinoids on this receptor have also been tested and are shown in table 1.3. Several selective GPR119 agonists have been designed such as PSN 375963 (EC_{50} 8.4 ± 2.7μM at human GPR119 in the yeast fluorimetric assay) and PSN 632408 (EC_{50} 5.6 ± 0.99μM at human GPR119 in the yeast fluorimetric assay) (Overton *et al.*, 2006) but unfortunately no GPR119 antagonists have been developed yet, so it is difficult to confirm the involvement of this receptor in any functional studies.

GPR119 mRNA has been found mainly in the pancreas and gastrointestinal tract (Chu *et al.*, 2007; Lauffer *et al.*, 2009) and in some brain tissues (Overton *et al.*, 2008). Immunohistochemical studies in the pancreas have shown that the GPR119 receptor is present in polypeptide-releasing cells (Sakamoto *et al.*, 2006) and co-localised with insulin in the islet of Langerhans cells (Chu *et al.*, 2007). In the intestine, the GPR119 receptor was found to be co-localised with glucagon-like peptide (GLP-1) in proglucagon positive cells of the villi (Chu *et al.*, 2007).

Studies have suggested that the GPR119 receptor is involved in controlling glucose tolerance and obesity. GPR119 agonists improved glucose tolerance by increasing insulin secretion from pancreatic cells *in vitro* and *in vivo* in diabetic mice (Chu *et al.*, 2008). OEA and PSN 632408 decreased food intake and body weight gain in mice (Lan

et al., 2009; Overton *et al.*, 2006). However, as there is no GPR119 antagonist available at present, it is not clear whether the effects of these compounds are mediated through the GPR119 receptor. The effects of OEA could be mediated by the peroxisome proliferator-activated receptor alpha (PPAR α) receptor (see 'Peroxisome proliferator-activated receptors' below), and PSN 632408 is also able to activate the GPR55 receptor (Milman *et al.*, 2006).

Ligand	**GPR119 fold induction of yeast reporter**	**Reference**
Anandamide	**2**	**(Overton *et al.*, 2006)**
PEA	**20**	**(Overton *et al.*, 2006)**
2-AG	**inactive**	**(Overton *et al.*, 2006)**
OEA	**60**	**(Overton *et al.*, 2006)**
methanandamide	**inactive**	**(Overton *et al.*, 2006)**
CP,55940	**inactive**	**(Overton *et al.*, 2006)**
(+)WIN	**inactive**	**(Overton *et al.*, 2006)**
JWH133	**inactive**	**(Overton *et al.*, 2006)**

Table 1.3 *Activity of cannabinoids at the GPR119 receptor.*

CB_e receptor

There is also evidence for a non-CB_1, non-CB_2, non-vanilloid receptor in mesenteric vasculature (Jarai *et al.*, 1999; Ralevic *et al.*, 2001; Wagner *et al.*, 1999), which has now been proposed as a novel cannabinoid receptor and is known as the 'abnormal cannabidiol' or the endothelial cannabinoid (CB_e) receptor.

Abnormal cannabidiol (abnormal-CBD) is a cannabinoid which does not bind to CB_1 or CB_2 (see table 1.1). This compound, as well as anandamide and OEA, was found to dilate isolated mesenteric artery segments and the effects of anandamide and abn-cannabidiol were antagonised by the cannabidiol analogue O-1918 (which also does not bind to CB_1 or CB_2 receptors) (Hoi *et al.*, 2006; Offertaler *et al.*, 2003). Vasorelaxation was also blocked by pertussis toxin suggesting that the receptor is Gi-protein coupled. These results lead to the suggestion that a novel cannabinoid receptor (now known as the CB_e receptor) is present in the mesentery and that abnormal-CBD cannabidiol, anandamide and OEA are agonists for this receptor whilst O-1918 is a selective antagonist (Hoi *et al.*, 2006; Offertaler *et al.*, 2003).

The CB_e receptor may also be involved in endothelial cell migration, as well as vasorelaxation. Abnormal-CBD cannabidiol was found to stimulate migration in an O-1918-sensitive manner, suggesting that this effect was mediated through the CB_e receptor (Mo *et al.*, 2004). It is important to note that the CB_e receptor has not been cloned yet and that its existence is only suggested from the results of functional studies.

It has been suggested that the orphan receptor GPR55 is the putative CB_e receptor, although the evidence is not entirely consistent with this idea. The evidence has been summarised by Ross (2009). In support of the hypothesis that GPR55 is the CB_e receptor, O-1602 and abnormal-CBD can elicit responses thought to be mediated by the CB_e receptor as well as GPR55. Furthermore, both CB_e and GPR55 receptor responses are sensitive to antagonism by rimonabant and cannabidiol. In addition, (*R*)-(+)-WIN 55,212-2 mesylate ((+)WIN) does not bind to GPR55 and also does not elicit responses thought to be mediated by the CB_e receptor. The evidence against this hypothesis is that O-1602 and abnormal-CBD exert haemodynamic effects in wild-type and $GPR55^{-/-}$

mice. Also, CP 55,940 activates GPR55 but does not act at the CB_e receptor (Ross, 2009).

More recently, the orphan receptor GPR18 has been suggested as the CB_e receptor. In one study, *N*-arachidonoyl glycine and abnormal-CBD were found to mediate cellular migration in BV-2 microglia and GPR18 transfected HEK293, effects that were blocked by O-1918. In addition, GPR18 mRNA and immunocytochemical staining was found in the BV-2 microglia. Neither *N*-arachidonoyl glycine nor abnormal-CBD produced this effect in GPR55-transfected and wild-type HEK 293 cells (McHugh *et al.,*2010*)*.

Transient receptor potential receptors

Cannabinoids are also known to act on the family of transient receptor potential (TRP) receptors. Anandamide and methanandamide activate the vanilloid receptor 1 (TRPV1), and the selective CB_1 agonist, arachidonoyl-2'-chloroethylamide (ACEA) is also a partial agonist at this receptor (Ross *et al.*, 2001; Zygmunt *et al.*, 1999). ACEA has been shown to stimulate calcitonin gene-related peptide (CGRP) release from rat ganglion sensory neurons, an effect that was blocked by TRPV1 antagonists (Price *et al.*, 2004). Rimonabant has been shown to be a mixed TRPV1 agonist/antagonist, stimulating CGRP release at high concentrations but antagonising capsaicin-stimulated CGRP release at low concentrations (Price *et al.*, 2004).

Studies have shown that the transient potential cation channel, subfamily A, member 1 (TRPA1) receptor is activated by Δ^9 -tetrahydrocannabinol (THC), (*S*)-(-)-WIN 55,212-2 mesylate (-)WIN and (+) WIN (Jordt *et al.*, 2004). Both THC and cannabidiol (-)CBD were found to increase free intracellular calcium concentrations in HEK293 cells transfected with TRPA1 receptors (Jordt *et al.*, 2004). THC and (+) WIN were found to induce inward currents in TRPA1-expressing oocytes (Hinman *et al.*, 2006) and

Chinese hamster ovarian (CHO) cells, respectively (Akopian *et al.*, 2008). THC has also been shown to activate vanilloid receptor 2 (TRPV2) receptors and induce calcium mobilisation in TRPV2-transfected HEK293 cells (Neeper *et al.*, 2007).

Cross regulation between the cannabinoid and vanilloid systems has been suggested by Gomez-Ruiz *et al* (2007). This is supported by evidence that anandamide is released following capsaicin activation of vanilloid receptors on primary sensory neurons which co-express vanilloid and cannabinoid receptors (Gomez-Ruiz *et al.*, 2007).

Peroxisome proliferator-activated receptors

Studies now suggest that cannabinoids can activate members of the PPAR family. Whereas the PPARδ (Barish *et al.*, 2006) and PPARγ1 (Auboeuf *et al.*, 1997) receptors are ubiquitously expressed, PPARγ2 is found in adipose tissue, PPARγ3 is found in macrophages (Auboeuf *et al.*, 1997) and PPARα is found in metabolically active tissues such as liver, heart and muscle (Stienstra *et al.*, 2007). All PPARs are also expressed in the brain and peripheral nervous system (Cimini *et al.*, 2005). This section focuses on PPARα and PPARγ as the effects of cannabinoids on PPARδ have not been extensively studied.

Current evidence suggests that cannabinoids can activate PPARs to affect appetite, lipid metabolism, inflammation and vasodilatation. Indeed, OEA was shown to suppress appetite (Fu *et al.*, 2003), stimulate lipolysis (Guzman *et al.*, 2004) and reduce oedema in mice (Lo Verme *et al.*, 2005). OEA was also found to be neuroprotective in a mouse model of cerebral artery occlusion (Sun *et al.*, 2006). None of these OEA-mediated effects could be reproduced in PPARα knockout mice, suggesting this receptor was responsible for its effects (Fu *et al.*, 2003; Guzman *et al.*, 2004; Lo Verme *et al.*, 2005;

Sun *et al.*, 2006). Another endocannabinoid-like compound, PEA, was found to have similar effects on inflammation as OEA.These effects were also thought to be mediated through the PPARα receptor (LoVerme *et al.*, 2006). Cannabinoids have also been shown to activate the PPARα receptor. The cannabinoid (+)WIN was shown to be neuroprotective via activation of the PPARα receptor, and the endocannabinoids anandamide, virodhamine and noladin are also known to activate PPARα. (Sun *et al.*, 2006).

There is evidence that THC, anandamide and (+)WIN activate PPARγ to dilate blood vessels (O'Sullivan *et al.*, 2005), reduce inflammation (Rockwell *et al.*, 2004) and induce apoptosis respectively (Giuliano *et al.*, 2009). Other cannabinoids which activate PPARγ are 2-AG, *N*-arachidonoyldopamine, HU210, CP 55,940, cannabidiol (O'Sullivan, 2007) and methanandamide (Eichele *et al.*, 2009).

The mechanism of action of cannabinoids at PPARs is not fully understood (O'Sullivan, 2007) – some studies suggest that cannabinoids bind directly to the receptors to alter gene expression (Fu *et al.*, 2003; (Sun *et al.*, 2006), whereas some studies suggest that cannabinoid metabolites activate PPARs (Rockwell *et al.*, 2004). Another suggestion is that cannabinoids act at receptors on the cell membrane and activate signalling pathways which lead to the activation of PPARs (O'Sullivan, 2007).

Other receptors

Cannabinoid agonists also target other non-cannabinoid receptors and channels, apart from TRP and PPAR, as illustrated by the cannabinoid (+)WIN and the antagonist rimonabant in tables 1.4 and 1.5 below:

Ligand	Receptor or channel	Effect	Concentration	Reference
Rimonabant	**acetylcholine (muscarinic) M_1 + M_4**	**Displacement**	**>1µM or >10µM**	**(Christopoulos *et al.*, 2001)**
	adenosine A_1	**Antagonism**	**10µM**	**(Savinainen *et al.*, 2003)**
	adenosine A_3	**Displacement**	**IC_{50} = 1.5 µM**	**(as reviewed by Pertwee, 2010)**
	α_{2A}- adrenoceptors	**Displacement**	**IC_{50} = 7.2 µM**	**(as reviewed by Pertwee, 2010)**
	α_{2C}- adrenoceptors	**Displacement**	**IC_{50} = 3.6 µM**	**(as reviewed by Pertwee, 2010)**
	angiotensin AT_1	**Displacement**	**IC_{50} = 7.2 µM**	**(as reviewed by Pertwee, 2010)**
	5-HT_6	**Displacement**	**IC_{50} = 2.8 µM**	**(as reviewed by Pertwee, 2010)**
	imidazoline	**Antagonism**	**1µM**	**(Molderings *et al.*, 1999)**
	µ opioid	**Displacement**	**IC_{50} = 3.0 µM**	**(as reviewed by Pertwee, 2010)**
	µ opioid	**Displacement**	**IC_{50} = 4.1 µM**	**(Kathmann *et al.*, 2006)**
	µ opioid	**Displacement**	**IC_{50} = 5.7 µM**	**(Cinar *et al.*, 2009)**
	κ opioid	**Displacement**	**IC_{50} = 3.9 µM**	**(as reviewed by Pertwee, 2010)**
	prostanoid EP_4	**Displacement**	**IC_{50} = 3.9 µM**	**(as reviewed by Pertwee, 2010)**
	prostanoid FP	**Displacement**	**IC_{50} = 2 µM**	**(as reviewed by Pertwee, 2010)**
	prostanoid IP	**Displacement**	**IC_{50} = 4.9 µM**	**(as reviewed by Pertwee, 2010)**
	tachykinin NK_2	**Displacement**	**IC_{50} =2 µM**	**(as reviewed by Pertwee, 2010)**
	L-type calcium(Ca_v1)channels	**Displacement**	**IC_{50} = 6.1 µM**	**(as reviewed by Pertwee, 2010)**
	T-type calcium(Ca_v3)channels	**Inhibition**	**100 nM, 1 µM**	**(Chemin *et al.*, 2001)**
	potassium TASK-1 channels	**Inhibition**	**10µM**	**(Maingret *et al.*, 2001)**
	potassium K_v channels	**Inhibition**	**10µM**	**(Van den Bossche *et al.*, 2000)**
	potassium K_v channels	**Displacement**	**IC_{50} = 2.5 µM**	**(as reviewed by Pertwee, 2010)**
	type-2 sodium channels	**Displacement**	**IC_{50} = 5.1µM**	**(as reviewed by Pertwee, 2010)**

Table 1.4 *Activity of rimonabant at other non-cannabinoid targets.*

Ligand	Receptor or channel	Effect	Concentration	Reference
(+)WIN	**imidazoline**	**activation**	**10 and 100 µM**	**(Molderings *et al.*, 2002)**
	5-HT$_3$	**antagonism**	**IC_{50} = 310 nM**	**(Fan, 1995)**
	5-HT$_{3A}$	**antagonism**	**IC_{50} = 104 nM**	**(Barann *et al.*, 2002)**
	glycine (α2)	**inhibition**	**IC_{50} = 220 nM**	**(Yang *et al.*, 2008)**
	glycine (α3)	**inhibition**	**IC_{50} = 86 nM**	**(Yang *et al.*, 2008)**
	potassium TASK-1 channels	**inhibition**	**10 µM**	**(Maingret *et al.*, 2001)**
	potassium K_v channels	**inhibition**	**20 µM**	**(Van den Bossche *et al.*, 2000)**
	sodium channels	**potentiation**	**10 nM**	**(Fu *et al.*, 2008)**
	sodium channels	**inhibition**	**10 µM**	**(Okada *et al.*, 2005)**
	type-2 sodium channels	**inhibition**	**IC_{50} = 12.2, 14.4, 21.1 µM**	**(Nicholson *et al.*, 2003)**
	type-2 sodium channels	**displacement**	**IC_{50} = 19.5 µM**	**(Nicholson *et al.*, 2003)**

Table 1.5 *Activity of (+)WIN at other non-cannabinoid targets.*

1.8 Cannabinoid receptor signalling pathways

CB_1 and CB_2 Receptor signalling

Cannabinoids can activate multiple signalling pathways through activation of the CB_1 or CB_2 receptor. The signalling pathway activated varies depending on the coupling to the receptor (for example, the CB_1 receptor preferentially couples to a subset of Gα i/o subunits) (Straiker *et al.*, 2002), the tissue type (Breivogel *et al.*, 2000; Breivogel *et al.*, 1997; Pacheco *et al.*, 1994) and also the cannabinoid activating the receptor (functional selectivity) (Berg *et al.*, 1998; Kenakin, 1995).

Cannabinoid receptors can also activate more than one signalling pathway at the same time through activation of Gα and Gβγ subunits. Cross-talk can then occur between these signalling pathways (Howlett, 2005). Cannabinoids can also dimerise with other receptors to activate specific signalling pathways. For example, the CB_1 receptor can form heterodimers with D_2 dopamine receptors (Kearn *et al.*, 2005).

G proteins coupled to the CB_1 and CB_2 receptors include members of the G i/o family (Howlett, 2005) which are involved in several signalling pathways, including the negative regulation of calcium channels, positive regulation of inwardly rectifying potassium channels, activation of protein kinases and gene induction (Howlett *et al.*, 2000).

Cannabinoids appear to mediate a number of opposing responses, including their effect on adenylyl cyclase. Usually, cannabinoids inhibit adenylyl cyclase (AC) via coupling of the receptor to Gi proteins leading to a decrease in levels of cyclic adenosine monophosphate (cAMP). However, CB_1 receptors can couple to Gs proteins instead and increase cAMP. This could be because different cannabinoid agonists induce different

conformations of the CB_1 receptor which possess different affinities for the various G proteins (Gomez-Ruiz *et al.*, 2007).

Similar cannabinoids can increase or decrease nitric oxide (NO) levels by activating constitutive nitric oxide synthase (NOS) and inhibiting inducible NOS respectively. Also, although cannabinoids decrease Ca^{2+} conductance through Ca^{2+} channels, they can also increase intracellular Ca^{2+}. Stimulation of CB_1 receptors or CB_2 receptors can produce this increase in Ca^{2+} via activation of phospholipase C (PLC), leading to increased inositol 1,4,5-trisphosphate (IP_3) levels and the release of Ca^{2+} from IP_3 – sensitive stores. This release of Ca^{2+} may be linked to the stimulation of NOS which has Ca^{2+} -calmodulin-dependent isoforms (Fimiani *et al.*, 1999). These signalling pathways have been simplified and shown in figures 1.3a and b.

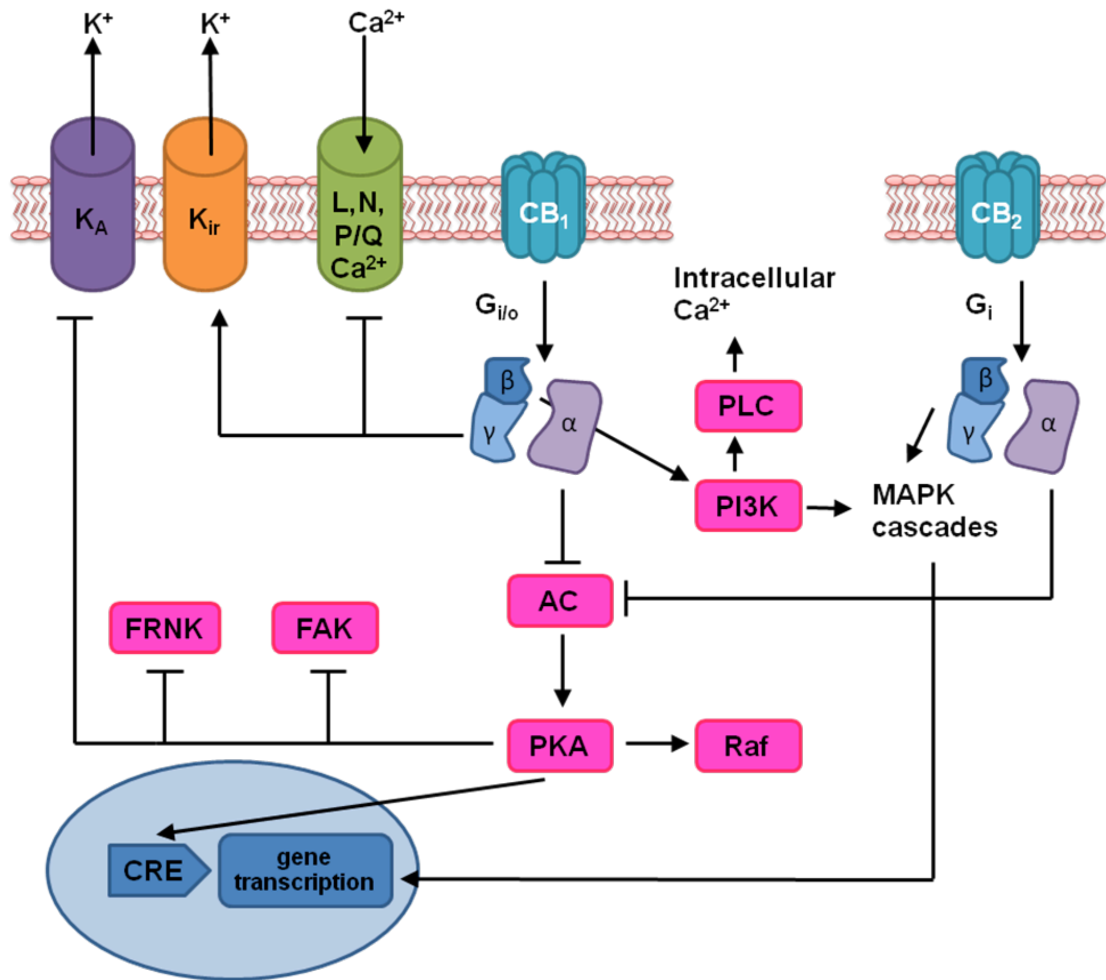


Figure 1.3a *Cannabinoid signalling at the CB_1 and CB_2 receptor (adapted from Bosier et al., 2010).*

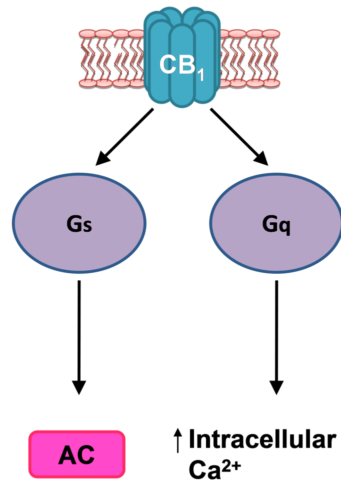


Figure 1.3b *Cannabinoid signalling at the CB_1 receptor continued (adapted from Turu et al., 2010).*

GPR55 and GPR119 receptor signalling

The GPR55 receptor does not activate Gi/o or Gs proteins, unlike the CB_1 receptor and CB_2 receptor, suggesting that it activates different signalling mechanisms (Gomez-Ruiz *et al.*, 2007**).** The GPR55 receptor seems to exhibit functional selectivity in a similar fashion to the CB_1 receptor, where different cannabinoids activate different signalling pathways. The different signalling pathways activated by GPR55 are shown (figure 1.4 overleaf).

Signalling of the GPR119 receptor may be mediated through coupling of the receptor to the G_s protein as high levels of cAMP have been found in GPR119-expressing cells (Chu *et al.*, 2007). Stimulation of AC and protein kinase A activity by GPR119 agonists has also been reported in GPR119-expressing cells (Chu *et al.*, 2007; Overton *et al.*, 2006; Reimann *et al.*, 2008; Semple *et al.*, 2008) and there is evidence for the

involvement of K^+ and Ca^{2+} channels in GPR119 signalling pathways (Ning *et al.*, 2008).

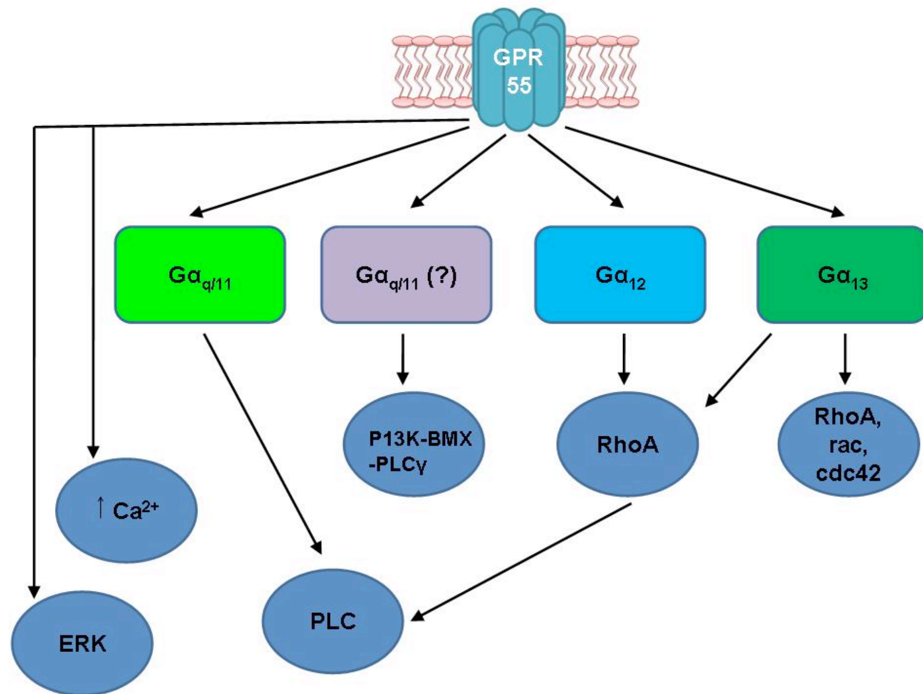


Figure 1.4 *Cannabinoid signalling at the GPR55 receptor continued (adapted from Ross, 2009).*

1.9 The therapeutic potential of cannabinoids in inflammatory bowel disease

1.9.1 Inflammatory bowel disease

The main forms of IBD are Crohn's disease and ulcerative colitis, which are chronic conditions characterised by periods of remission and relapse. The aetiology of IBD is complex, involving a combination of susceptible genes (CARD 15, IBD 2-7), environmental factors such as exposure to certain food components and microorganisms, and an overaggressive immune response. IBD develops due to an inappropriate immune response to microorganisms which enter the intestine via the leaky epithelium. The antigens bind to toll-like receptors on the epithelial cell

membrane and the epithelial cells act as antigen presenting cells to dendritic cells. Next, T helper cells become activated and stimulate macrophages to secrete pro-inflammatory cytokines such as TNFα. Natural killer cells are also activated which secrete cytokines and are cytotoxic. The inflammatory mediators released then attract circulatory leukocytes to the tissue which produce chemokines including ROS which, in turn, cause more tissue damage (Kumar and Clark, 2009).

Crohn's disease can affect any area of the gastrointestinal tract (the ileocecal region is the most commonly affected), whereas ulcerative colitis is restricted to the colon (colitis) and/or rectum (proctitis). The clinical features are similar for both conditions and include anorexia, weight loss, abdominal pain, fever and diarrhoea (Kumar and Clark, 2009). The conditions can be differentiated by their histological features which are shown below in table 1.6.

	Crohn's disease	**Ulcerative colitis**
Inflammation	**Deep (transmural)** **Patchy**	**Mucosal** **Continuous**
Granulomas	**Present**	**Rare**
Goblet cells	**Present**	**Depleted**
Crypt abscesses	**Scanty**	**Common**

Table 1.6 *Histological features of IBD (adapted from Kumar and Clark, 2009)*

1.9.2 The effects of cannabinoids on the immune response

As IBD involves activation of an inappropriate immune response, it would be important for any successful therapeutic to be able to 'dampen down' this response. Cannabinoids have shown that they are able to modulate the immune response and the evidence is summarised below.

Expression of the cannabinoid receptors CB_1 and CB_2 has been found throughout the immune system (Galiegue *et al.*, 1995; Lee *et al.*, 2001; Noe *et al.*, 2000), although the CB_2 receptor is reportedly more abundant in immune tissues than CB_1 (Croxford *et al.*, 2005). CB_2 mRNA expression has been found to vary according to immune cell type – its highest expression has been found in B cells and the lowest in T cells (Galiegue *et al.*, 1995; Lee *et al.*, 2001; Noe *et al.*, 2000).The expression of CB_1 and CB_2 receptors in the immune system has also been found to vary depending on the activation stimuli, activation state and the immune cell type used in studies (Croxford *et al.*, 2005; Klein, 2005).There is also evidence that the endocannabinoids anandamide and 2-AG are produced by macrophages, peripheral blood mononuclear cells and dendritic cells in response to stimulation by bacterial lipopolysaccharide (LPS) *in vitro* (Klein, 2005). Cannabinoids are thought to be important to maintain normal immune homeostasis and it has been found that mice that do not possess CB_2 receptors show signs of immune dysregulationulation such as developing inflammatory bowel disease (Ziring *et al.*, 2006).

The interactions between cannabinoids and immune cells are very complex and differ depending on the cell type, cell activation state, the activation stimuli, the cannabinoid ligand, and the concentration of the cannabinoid used (Croxford *et al.*, 2005; Klein, 2005). Several studies have shown that cannabinoids inhibit immune cell proliferation (Cencioni *et al.*; Klein *et al.*, 1985; Schwarz *et al.*, 1994; Yuan *et al.*, 2002), ROS and

cytokine release (Kaplan *et al.*, 2008; Ross *et al.*, 2000; Sacerdote *et al.*, 2005), antibody production (Eisenstein *et al.*, 2007; Jan *et al.*, 2007) and migration (Raborn *et al.*, 2008; Sacerdote *et al.*, 2000). Most of these inhibitory effects were reported to be CB_2 receptor mediated (Yuan *et al*., 2002; Ross *et al*., 2000; (Cencioni *et al.*; Eisenstein *et al.*, 2007; Raborn *et al.*, 2008), although it must be noted that many of the studies only used one CB_2 antagonist, and the concentrations of the cannabinoid and antagonist used were much higher than their binding affinity for the CB_2 receptor (Ross *et al*., 2000).

Some studies have suggested that CB_1, as well as CB_2 receptors, may be involved in inhibiting cytokine release (Sacerdote *et al*., 2005) and inhibiting chemotaxis (Sacerdote *et al*., 2000). Non- CB_1/ CB_2 receptor mediated mechanisms are thought to be responsible for the cannabinoid-mediated inhibition of superoxide production by neutrophils (Naccache *et al*., 1982). Studies have investigated cannabinoid signalling pathways and have found that cannabinoids inhibit immune cell response, at least in part by suppression of AC/cAMP signalling pathway (Koh *et al.*, 1995), resulting in decreased binding of transcription factors and nuclear factors to DNA, and therefore decreased gene transcription (Massi *et al.*, 2006b). This signalling pathway is shown in figure 1.1a.

Although most studies have reported cannabinoid-mediated inhibition of the immune response, cannabinoid-induced stimulation has also been observed. Cannabinoids have been shown to increase the proliferation of immune cells (Derocq *et al*., 1995), enhance migration (Croxford *et al.*, 2005) and induce cytokine release. Most of these stimulatory effects have been reported for endocannabinoids and THC, although low concentrations of CP 55,940 and (+)WIN were found to increase proliferation of B cells (Derocq et al., 1995). The CB_2 receptor has been implicated in inducing migration (Croxford *et al.*,

2005), whereas CB_2 and non-CB_1/CB_2-receptor mechanisms have been reported to be involved in the pro-proliferative effects of cannabinoids (Derocq *et al.*, 1998; Valk *et al.*, 1997).

Cannabinoids were shown to alleviate symptoms in models of inflammatory diseases and one of the reasons suggested for this is the Th_1 suppressing/Th_2 enhancing effect shown by cannabinoids. Th_1 cytokines are thought to be involved in the pathogenesis of a number of autoimmune diseases such as multiple sclerosis, where inhibition of Th_1 cytokines is thought to be therapeutic. Several studies have suggested that cannabinoids alter the balance of Th_1 to Th_2 mediated immune responses (Croxford *et al.*, 2005; Klein, 2005).

In vivo studies on mice infected with *Legionella pneumophilia* showed that cannabinoids suppressed the production of Th_1 cytokines and enhanced the production of Th_2 cytokines. CB_1 and CB_2 receptors were thought to be involved from the use of the selective cannabinoid receptor antagonists, rimonabant and SR144528 (Newton *et al.*, 1994; Klein *et al.*, 2000). It was suggested that the CB_1 receptor mediates the suppression of Th_1 activity whilst the CB_2 receptor mediates the enhancement of Th_2 activity. It was also suggested that this could be due to varying expression/ signalling of CB_2 and CB_1 receptors on Th_1 and Th_2 cells (Klein *et al.*, 2004).

The Th_2 biasing effect shown by cannabinoids is thought to occur for a number of reasons, including differences in cannabinoid receptor expression on T helper (Th) cell subsets and antigen presenting cells, the reduction of Th_1 cytokines from dendritic cells, the shift of B cell antibody production from IgM to IgE and the inhibition of Th_1 cytokines by production of Th_2 cytokines (Tanasescu *et al.*, 2010).

The cannabinoid (+)WIN reduced progression of clinical symptoms in a mouse model of chronic multiple sclerosis. This reduction was associated with a decrease in Th_1 mediated functions and inhibition of pro-inflammatory cytokine mRNA (Croxford *et al.*, 2003). Similar benefits have also been reported from the use of cannabinoids in rheumatoid arthritis (Croxford *et al.*, 2005).

Cannabinoids have also shown to be effective in models of IBD, but it is unlikely that this is due to a Th_1 suppressing/Th_2 enhancing effect as ulcerative colitis is a Th_2 driven disease and there is new evidence that Crohn's disease may be Th_{17} driven. The effects of cannabinoids in IBD models are discussed in further detail below.

1.10 Cannabinoid effects on intestinal inflammation and motility

1.10.1 Cannabinoid effects on intestinal inflammation

Studies using several animal disease models, including multiple sclerosis and rheumatoid arthritis, have demonstrated the anti-inflammatory effects of cannabinoids (Croxford *et al.*, 2003; Croxford *et al.*, 2005). Cannabinoids may also be useful as therapeutics for IBD as there is evidence from various studies that cannabinoids may produce anti-inflammatory effects in the intestine (Izzo et al., 2008; Izzo et al., 2010). They have also been shown to reduce inflammation-induced motility disturbances and protect cholinergic nerves from inflammatory damage (Jamontt *et al.*, 2010).

There is evidence that the anti-inflammatory effects of cannabinoids in the gut may be CB_1 or CB_2 receptor mediated. One study showed that selective CB_1 and CB_2 receptor agonists inhibited experimental colitis in mice induced by oil of mustard and dextran

sulphate sodium (Kimball *et al.*, 2006). However, selective antagonists were not used to corroborate the CB_1 and CB_2 receptor effects.

Another study reported that cannabinoids mediate their anti-inflammatory effects through the CB_1 receptors. Authors found that when mice were treated with DNBS, inflammation was more pronounced in CB_1(-/-) mice than CB_1(+/+) mice, and treatment of the wild-type mice with rimonabant produced the same effects as the CB_1 receptor knockout, ie stronger inflammation was produced. They also found that the non-selective cannabinoid receptor agonist HU210 or genetic ablation of FAAH protected the mice against dinitrobenzene sulfonic acid (DNBS)-induced colitis. Additionally, this study found that spontaneous oscillatory action potentials were found in circular smooth muscle cells of CB_1(-/-) but not CB_1(+/+) colons after DNBS treatment, suggesting that CB_1 receptors reduce smooth muscle irritation induced by inflammation (D'Argenio *et al.*, 2006). A caveat in the study is that no selective CB_1 agonists were used and the effect of HU210 was assumed to be CB_1 receptor mediated without confirming this effect with selective CB_1 agonists.

Several studies have suggested that cannabinoids can reduce inflammation in the gut through a non-CB_1/non-CB_2 receptor(s). Studies have been carried out using the plant-derived (-)CBD and the GPR55 agonist O-1602 in models of inflammation, neither of which binds to the CB_1 or CB_2 receptor. Firstly, a study using O-1602 showed that this cannabinoid protected against colitis and inhibited recruitment. This O-1602-mediated inhibition was still present in mice which did not express the CB_1, CB_2 or GPR55 receptor (Schicho *et al.*, 2011), suggesting this effect was non- CB_1, $CB_{2,}$ GPR55 mediated.

(-)CBD also inhibited inflammation through a non-CB_1/non-CB_2 mechanism in a mouse model of colitis. The cannabinoid reduced iNOS, IL-1β and IL-10 expression and without changing the levels of endocannabinoids. The levels of the endocannabinoids were measured as (-)CBD is a known FAAH inhibitor. The same study showed that (-)CBD reduced oxidative stress *in vitro* in caco-2 cells (Borrelli *et al.*, 2009a). This suggests that (-)CBD reduces inflammation and oxidative stress through a non-CB_1/non-CB_2 receptor(s).

Another study showed that (-)CBD reduced inflammation and associated motility disturbances in a rat model of colitis (Jamnott *et al*., 2010). (-)CBD has been shown to inhibit the release of pro-inflammatory mediators which reduce smooth muscle function and decrease neurotransmitter release from the myenteric plexus (Collins, 1996; Esposito *et al.*, 2007; Jacobson *et al.*, 1997; Kaplan *et al.*, 2008; Kinoshita *et al.*, 2006; Watzl *et al.*, 1991). This mechanism could be involved in reducing the motility disturbances. One problem with this study, however, is that it did not investigate whether (-)CBD altered the levels of endocannabinoids in the gut, so it is not clear whether (-)CBD was exerting effects indirectly by inhibiting FAAH or whether it was working through a non-CB_1/non-CB_2 receptor.

In contrast to the above studies, McVey *et al* (2003) have reported that cannabinoids may also induce intestinal inflammation as anandamide and 2-AG were found to induce ileitis in rats, an effect mediated by the vanilloid receptor TRPV1. This suggests that any cannabinoid used therapeutically for inflammatory bowel disease should stimulate cannabinoid receptors but avoid stimulating the vanilloid receptor.

1.10.2 CB_1-mediated effects on intestinal motility

Several studies have showed that cannabinoids reduce intestinal motility in animals and humans (Esfandyari *et al*., 2007; Colombo *et al*., 1998; Izzo *et al*., 1999; Izzo *et al*., 2000a; Landi *et al*., 2002; Pinto *et al*., 2000b).These effects would be beneficial in IBD which is associated with an increase in intestinal motility and is accompanied by symptoms such as intestinal cramps and diarrhoea.

The currently held view is that cannabinoids inhibit intestinal motility through activation of the CB_1 receptor. However, much of this evidence relies on the use of the CB_1 receptor antagonist rimonabant, which may not be as selective as first thought (see section 1.7.2).Also, there is now evidence for non-CB_1, non-CB_2 effects on intestinal motility (Mang et al., 2001; Smid et al., 2007). The evidence for CB_1, CB_2 and non-CB_1, non-CB_2 -mediated effects have been reviewed below and in later sections.

Firstly, studies have reported the presence of CB_1 receptor mRNA in the guinea-pig myenteric plexus (Griffin *et al.*, 1997) and in the myenteric and submucosal plexus of the rat intestine (reviewed by Pinto *et al*., 2002a). In addition, immunohistochemical studies using CB_1 receptor antibodies have detected the presence of CB_1 receptors in the neurones of the guinea-pig, rat and porcine enteric nervous system, reviewed by Pertwee (2001) and Pinto *et al*., (2002a). Studies in these animals have also shown an association between choline acetyltransferase and CB_1 receptor distribution in the myenteric plexus, suggesting that CB_1 receptors are present on cholinergic neurones within the enteric nervous system (Buckley *et al.*, 1998). More specifically, there is immunohistochemical evidence that the CB_1 receptors are present on excitatory neurones in the myenteric plexus .There is also evidence (Aviello *et al*., 2008b) for the

presence of CB_1 receptors on intrinsic sensory neurones of the enteric nervous system (see figure 1.5). However, although the immunohistochemical evidence shows that the CB_1 receptor is present in the enteric nervous system, it does not provide proof that this receptor is involved in cannabinoid-mediated inhibition of intestinal motility.

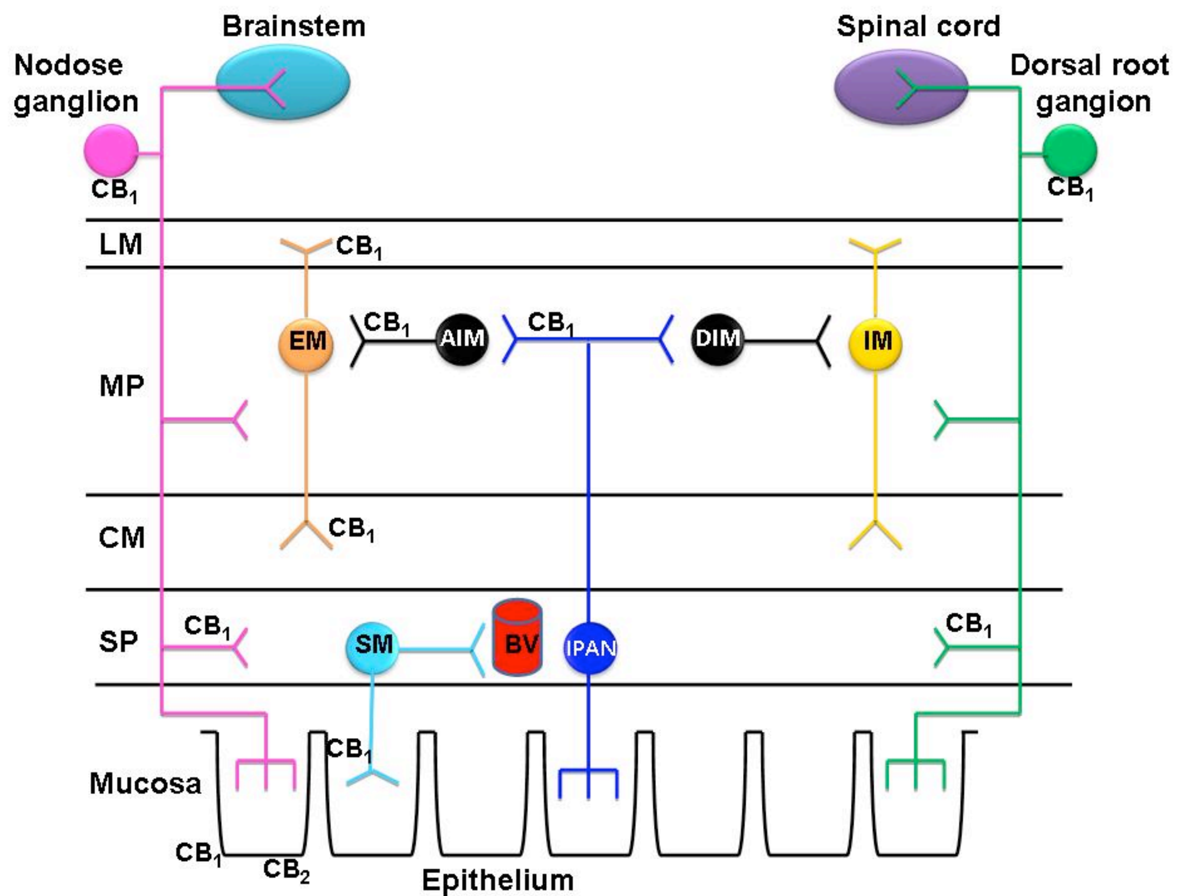


Figure 1.5 *Localisation of cannabinoid receptors in the intestine (adapted from Izzo, 2008).*

Researchers have also conducted studies on intestinal contractions *in vitro* and have demonstrated that cannabinoids can inhibit electrically stimulated contractions of guinea-pig ileum myenteric plexus-longitudinal and circular smooth muscle (Coutts *et al.*, 1997; Izzo *et al.*, 1998; Mang *et al.*, 2001; Pertwee *et al.*, 1996), as well as human ileum longitudinal and circular smooth muscle (Croci *et al.*, 1998; Manara *et al.*, 2002). The authors suggest that this inhibition is due to stimulation of pre-synaptic CB_1

receptors (as this effect is antagonised by rimonabant), which results in a decrease in acetylcholine release and hence muscle contraction (Coutts *et al.*, 1997; Croci *et al.*, 1998; Izzo *et al.*, 1998; Mang *et al.*, 2001; Pertwee *et al.*, 1996).

Studies have also shown that cannabinoids inhibit peristalsis of isolated intestinal segments, again reported to be CB_1 mediated due to antagonism by rimonabant (Grider *et al.*, 2009; Heinemann *et al.*, 1999; Sibaev *et al.*, 2009). Studies have shown that cannabinoids can inhibit all components of the peristaltic reflex, the intrinsic sensory neurons (Grider *et al.*, 2009), the ascending excitatory reflex (Grider *et al.*, 2009; Heinemann *et al.*, 1999; Sibaev *et al.*, 2009) and the descending inhibitory reflex (Grider *et al.*, 2009). The intrinsic sensory neurons are activated when the intestine is distended by food, which leads to smooth muscle contraction (ascending excitatory reflex) and relaxation (descending inhibitory reflex) which allows food to be propelled along the intestine in an anal direction (Izzo *et al*., 2010).

Cannabinoids have been shown to inhibit the ascending excitatory reflex by inhibiting cholinergic and non-cholinergic transmission (Grider *et al.*, 2009; Heinemann *et al.*, 1999; Sibaev *et al.*, 2009). Again, authors have concluded that this effect is CB_1 mediated from antagonism of the cannabinoid-mediated inhibition by rimonabant (Heinemann *et al.*, 1999; Sibaev *et al.*, 2009) or AM251 (Grider *et al*., 2009).

Much of the evidence suggests that cannabinoids reduce peristalsis by inhibiting the ascending contraction, but there are also studies that have demonstrated cannabinoid-induced effects on the descending relaxation pathway of peristalsis. One study (Grider et al., 2009) reported that cannabinoids inhibited the descending relaxation via

inhibition of vasoactive intestinal peptide (VIP) release, whereas the other (Heinemann et al., 1999) reported activation of the descending relaxation via stimulation of inhibitory motor neurones which operate through apamin sensitive K^+ channels and nitric oxide release. The former study (Grider et al., 2009) implicated the CB_1 receptor in the response due to its sensitivity to AM251. However, the presence of the CB_1 receptor on inhibitory motor neurones has not been confirmed by immunohistochemical studies (Aviello *et al.*, 2008b).

There is also evidence that cannabinoids reduce the release of CGRP from sensory neurons which usually occurs in response to distension of the intestine by ingestion of food. This was also reported to be CB_1 mediated as this was antagonised by AM251 (Grider *et al.*, 2009) .

In vivo studies from as early as 1989 have reported that cannabinoids inhibit rodent intestinal motility (Shook *et al.*, 1989). Subsequent *in vivo* studies have demonstrated that cannabinoids inhibit intestinal motility in rodents in a rimonabant-sensitive manner (Colombo *et al*., 1998; Izzo *et al*., 1999; Izzo *et al*., 2000a; Landi *et al*., 2002; Pinto *et al*., 2002b). One study has also shown that cannabinoids do not reduce motility in CB_1 knockout mice. This led the authors to the conclusion that cannabinoids inhibit intestinal motility via stimulation of CB_1 receptors.

Studies have also investigated the effects of cannabinoids on mice and rats treated with croton oil, a substance which induces a state of intestinal inflammation and diarrhoea in rodents (Izzo *et al.*, 2001b; Izzo *et al.*, 2000b). These studies (Izzo *et al.*, 2001b; Izzo *et al.*, 2000b) found that cannabinoids inhibited intestinal motility in control and croton-oil treated mice, and that this inhibition was sensitive to rimonabant. The inhibitory effect

of cannabinoids was enhanced in croton oil-treated mice compared with control mice and it was suggested that this enhanced inhibition was due to an up-regulation of CB_1 receptors, as CB_1 receptor expression seemed to be increased in the croton-oil inflamed intestine (Izzo *et al.*, 2001b).

The major problem with these pharmacological studies *in vitro* and *in vivo* is the over-reliance on one so-called selective CB_1 antagonist, usually rimonabant but sometimes AM251. Both rimonabant and AM251 have been reported to exert several non-specific effects at the concentrations used (micromolar range) in these studies (Pertwee., 2010). In addition, the studies used non-selective cannabinoids such as (+)WIN and anandamide rather than selective CB_1 agonists such as ACPA and ACEA. This, together with the fact that the antagonists may not be as selective as first thought, casts doubt on the conclusion that cannabinoids inhibit intestinal motility through the CB_1 receptor. The next section describes the evidence for the involvement of other receptors in cannabinoid-mediated inhibition of intestinal motility.

1.10.3 CB_2-mediated effects on intestinal motility

The presence of CB_2 mRNA has been reported in the rat ileum (Storr *et al.*, 2002) and human colon (Ligresti *et al.*, 2003), and immunohistochemical studies have shown the presence of this receptor in the enteric nervous system of human (Wright *et al.*, 2008) and rat ileum (Duncan *et al.*, 2008). However, evidence is conflicting for the role of CB_2 receptors in regulating intestinal motility.

In vitro studies on human and guinea-pig ileum have reported that cannabinoid-induced inhibition of intestinal motility is not affected by the CB_2 receptor antagonist, SR144528 (Croci *et al.*, 1998; Izzo *et al.*, 2000a). *In vivo* studies in mice and rats found that

cannabinoid-induced inhibition of intestinal motility was not antagonised by SR144528 (Izzo *et al.*, 1999; Izzo *et al.*, 2000a). In contrast, one study reported that a selective CB_2 receptor agonist blocked defecation, suggesting that the CB_2 receptor is present in the intestine and inhibits intestinal motility (Hanus *et al.*, 1999). There is also recent evidence that the CB_2 receptor may be involved in motility control, as anandamide stimulated VIP release in the rat myenteric plexus, and this response was antagonised by SR144528 (Kurjak *et al.*, 2008).

Another suggestion is that the CB_2 receptor may be upregulated in the gut during inflammation (Wright *et al*., 2005), and may be involved in reducing intestinal motility under these conditions (Izzo, 2007). One study showed that when rat gastrointestinal transit was increased by lipopolysaccharide (an inflammatory stimulus), a selective CB_2 agonist, but not a selective CB_1 agonist, reduced the transit back to normal level (Mathison *et al.*, 2004). This was supported by the Duncan *et al* (2008) study which showed that the selective CB_2 agonist JWH133 reduced motility in LPS-treated rats and that this was reversed by the CB_2 antagonist AM630.

1.10.4 Non-CB_1, non-CB_2 mediated effects on intestinal motility

Other studies have suggested that stimulation of non-CB_1, non-CB_2 cannabinoid receptors can alter intestinal motility. For example, in one study, anandamide inhibited acetylcholine release via stimulation of a non-CB_2, non-CB_1 receptor (Mang *et al.*, 2001). Also, there is other recent *in vitro* evidence to suggest that cannabinoids may inhibit intestinal contractions via non-CB_1, non-CB_2 mechanisms (Duncan *et al*., 2005; Makwana *et* al., 2010a). One study also showed that the non-selective cannabinoids

anandamide and 2-AG inhibited contractions of the human colon and that this was not altered by AM251, suggesting CB_1 was not involved (Smid *et al.*, 2007).

There is also evidence that the endocannabinoid-like acylethanolamides OEA and PEA reduce intestinal motility (Aviello *et al.*, 2008a; Capasso *et al.*, 2001; Capasso *et al.*, 2005; Cluny *et al.*, 2009). Both compounds are present in the gut as well as their target receptors, TRPV1 (OEA), PPARα (OEA and PEA), GPR119 (OEA) and GPR55 (PEA and OEA) (Borrelli *et al.*, 2009b). It is unclear at the moment which receptors are involved in the PEA and OEA-mediated inhibition of motility but is unlikely to be CB_1 or CB_2 as the compounds possess low affinity for these receptors and OEA reduced motility in CB_1 and CB_2 knockout mice. The PPARα receptor also does not seem to be involved as OEA also inhibited GI transit in PPARα knockout mice (Cluny *et al.*, 2009).

The endocannabinoid anandamide is also a ligand of vanilloid receptors found on primary afferent nerves, and anandamide was found to stimulate these receptors to increase acetylcholine release in the guinea-pig ileum. However, the vanilloid receptor antagonist capsazepine had no effect on inhibition of intestinal motility by anandamide *in vivo* (Izzo *et al.*, 2001a). It was, therefore, suggested by (Pinto *et al.*, 2002a) that vanilloid receptors do not have a role in the intestinal motility changes by anandamide.

1.10.5 Pre-junctional vs post-junctional effects on intestinal motility

Much of the evidence suggests that cannabinoids act only on pre-junctional CB_1 receptors to inhibit intestinal motility. Indeed, anandamide was not found to reduce contractions of guinea-pig and human ileum, guinea-pig myenteric plexus-longitudinal

and circular smooth muscle induced by exogenous acetylcholine, carbachol, substance P or histamine, as reviewed by Pertwee (2001). However, a 1997 study of cannabinoid effects on guinea-pig ileum myenteric neurones suggested that cannabinoids stimulate pre and post-synaptic CB_1 receptors to inhibit fast cholinergic transmission (Lopez-Redondo *et al.*, 1997).

Further studies are required to investigate the post-synaptic effects of a wider range of cannabinoids as most of the studies carried out relate to anandamide only (Lynn *et al.*, 1994; Mang *et al.*, 2001).

1.10.6 Cannabinoid signalling in the enteric nervous system

Cannabinoid signalling within the enteric nervous system has not been extensively investigated. However, a recent study has been carried out on guinea-pig myenteric neurones which showed that endocannabinoids are produced by myenteric neurones, in addition to other studies that have shown that cannabinoids can be produced by immune cells in the gut wall as well as vascular endothelial cells (Boesmans *et al.*, 2009). Anandamide appears to be the endocannabinoid which activates CB_1 receptors in the enteric nervous system (Boesmans *et al.*, 2009).

Cannabinoids are known to reduce neurotransmitter release by inhibiting Ca^{2+} influx into the pre-synaptic terminal (Lovinger, 2008). In the study by Boesmans (2009), it was shown that in guinea-pig myenteric neurones cannabinoids reduced the spontaneous activity of excitatory and inhibitory neurones, reducing Ca^{2+} spike frequency and mitochondrial transport. Cannabinoids were also found to alter synaptic vesicle turnover (Boesmans *et al.*, 2009). All these effects would reduce

neurotransmission (for example, the release of acetylcholine). Spontaneous activity of the neurones was also increased by the CB_1 antagonists AM251 (10µM) and rimonabant (1 µM), leading to the suggestion that enteric nerve signalling is under the control of CB_1. However, the non-specific effects of rimonabant and AM251 at these concentrations have been documented (Pertwee., 2010).

The CB_1 receptor is coupled to the inhibition of adenylyl cyclase via Gi and the activation of K^+ and inhibition of Ca^{2+} channels by Go and it has been suggested that all of these actions may contribute to cannabinoid-mediated inhibition of neurotransmission in the intestine (Galligan, 2009). However, this has not been investigated and there is no convincing evidence that CB_1 is responsible for inhibiting motility at the moment.

1.10.7 CNS effects of cannabinoids on intestinal inflammation and motility

Although the enteric nervous system can function without input from the CNS, extrinsic sympathetic and parasympathetic nerves, as well as vagal and spinal primary afferents, innervate the gastrointestinal tract and exert control over its functions *in vivo*. There is evidence for the presence of the CB_1 receptor on cell bodies of afferent neurones in the dorsal root ganglia, nodose ganglia and vagal efferents (see figure 1.3) (Duncan *et al.*, 2005).

Cannabinoids have been shown to act peripherally to reduce intestinal motility (Coutts *et al.*, 1997; Izzo *et al.*, 1998; Mang *et al.*, 2001; Pertwee *et al.*, 1996) but it has been shown that cannabinoids also alter intestinal motility through activation of cannabinoid receptors in the CNS. An *in vivo* study showed that cannabinoids were more active

when administered intracerebroventricularly than when administered intraperitoneally (Izzo et al., 2000).These effects were blocked by the CB_1 antagonist rimonabant, which led to the suggestion that the effects were CB_1 mediated (Pinto *et al*., 2002a).

Most of the studies in models of IBD reviewed above do not distinguish between the *in vivo* peripheral and central effects of cannabinoids on inflammation. However, one study showed that the peripherally-restricted CB_1/CB_2 agonist SAB378 had no effect on inflammation in mouse models of colitis, suggesting that central, as well as peripheral, cannabinoid receptor activation is required to mediate the anti-inflammatory effects of cannabinoids. SAB378 cannabinoid was found to reduce motility in these models, however, through activation of the CB_1 receptor (Cluny *et al*., 2010a). This study has important implications for the concept of a cannabinoid-dendrimer conjugate which does not enter the CNS. It suggests that such a compound would not reduce inflammation in patients with IBD as it would only act peripherally. However, this is the result of only one study and further investigations, distinguishing between the central and peripheral effects of cannabinoids in IBD, are required.

1.11 Aims of thesis

As there is a need for more effective, safer drugs to treat IBD/IBS, cannabinoids may represent a new class of therapeutics for these disorders. Indeed, cannabinoids have been shown to have several beneficial effects on the gastrointestinal tract, including anti-inflammatory and anti-motility effects.

The mechanisms by which cannabinoids reduce intestinal motility have not been fully established and so one of the aims of this thesis was to investigate the cannabinoid receptors involved in the inhibition of guinea-pig ileum contractions and to determine whether they were located pre or post-synaptically.

Several studies have showed that cannabinoids reduce intestinal inflammation but studies have not investigated whether cannabinoids protect the intestine against inflammatory damage. Another aim of this thesis was to determine whether cannabinoids were protective in *in vitro* models of inflammatory damage.

The major problem with a cannabinoid-based therapeutic would be the resulting psychotropic side effects, but this may be overcome by conjugating the cannabinoid to a dendrimer that it is large enough to prevent penetration of the blood brain barrier. The effect of cannabinoid conjugation on pharmacological activity has not been studied, and so the final aim of this thesis was to investigate whether novel cannabinoid-dendrimer conjugates retained activity in two pharmacological assays.

CHAPTER 2:

GENERAL METHODS

2. General methods

2.1 Introduction

The technique used most extensively throughout the thesis was electrical stimulation of the guinea-pig ileum (chapters 3, 4 and 5). Co-axial, transmural electrical stimulation of the guinea-pig isolated ileum is one of the oldest pharmacological techniques, used to examine the effects of drugs on the release of acetylcholine from cholinergic nerve endings (Scriabine *et al.*, 1970). The method was first established by Paton (1955) and has since been modified by other research groups (Scriabine *et al.*, 1970). Carbachol-induced contraction of the ileum was used instead of electrical stimulation to investigate any direct effect of cannabinoids on ileal smooth muscle. Carbachol directly stimulates muscarinic receptors on smooth muscle to produce contraction and is non-hydrolysable (unlike acetylcholine).

The Paton ileum technique was used to screen cannabinoid-dendrimer conjugates for CB_1 potency as the literature indicated that cannabinoids reduced electrically-evoked contractions through the CB_1 receptor. However, as the thesis progressed it became apparent that the CB_1 receptor was not responsible for this effect and that another receptor may have been involved.

Another assay was required to assess the potency of the cannabinoid-dendrimer conjugates at the CB_2 receptor. The assay chosen was LPS-stimulation of TNFα release from RAW 264.6 macrophages. This assay was chosen as cannabinoids have been shown to inhibit the release of inflammatory mediators (including TNFα) from RAW 264.7 macrophages (Fischer-Stenger *et al.*, 1993; Jeon *et al.*, 1996; Ross *et al.*, 2000)

and this has been shown to occur through activation of the CB_2 receptor (Ross *et al*., 2000; Hao *et al*., 2010).

LPS levels used in the literature vary (2-1000ng/ml) as well as the exposure period (between 2-24 hours) and bacterial strain (commonly *Escherichia coli* 055:B5 or 026:B6) (Fischer-Stenger *et al*., 1993., Jeon *et al*., 1996; Kim *et al*., 2008; Lahat *et al*., 2008., Peirce *et al*.,2010; Ross *et al*., 2000; Shen *et al*., 2009; Zhao *et al*.,2010; Eads *et al*., 2009; D'Souza *et al*., 1996; Zhang *et al*., 2007). The LPS level and the exposure period for the pharmacological assay used in the thesis were chosen from a pilot study (see 2.4 'Lipopolysaccharide (LPS)-stimulation of a macrophage cell line' below). LPS used was from *Escherichia coli* 055:B5 as this is commonly used in the literature and produces reproducible results (Kim *et al*., 2008; Lahat *et al*., 2008; Zhang., 2007).

2.2 Electrical field stimulation of the guinea-pig ileum

Animal husbandry

Male Dunkin-Hartley guinea-pigs (Harlan, UK) weighing 250-500g were housed and treated in accordance with the Animals (Scientific Procedures) Act 1986. The animals were subjected to 12 hour light-dark cycles. Animals received Harlan global diet complete feed for guinea-pigs 2940 (Harlan, UK) and were allowed food and water *ad libitum*.

Tissue preparation

The guinea-pigs were killed by concussion followed by exsanguination. The abdomen was dissected and the ileum was excised (14cm from the caecum). The fat and

connective tissue was removed from the ileum, which was then cleaned with Krebs' solution (see section 2.1.3 for composition).

Organ bath preparation

A 2 cm long segment of ileum was used for this procedure. Cotton was threaded through each end of the ileum and the ileum was mounted onto an electrode (through the lumen) and secured at one end (see figure 2.1). The electrode was lowered into an organ bath filled with 50mls Krebs' solution, maintained at 37°C and continuously aerated with carbogen (95% O_2 and 5% CO_2). The Krebs' solution was prepared daily and composed of (in mM) NaCl 118, KCl 4.7, $CaCl_2.6H_2O$ 1.3, $MgSO_4.7H_2O$ 1.2, K_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1. Approximately 0.5g of tension was applied to the ileum. The tissue was left to equilibrate for 10 minutes before electrical stimulation was applied.

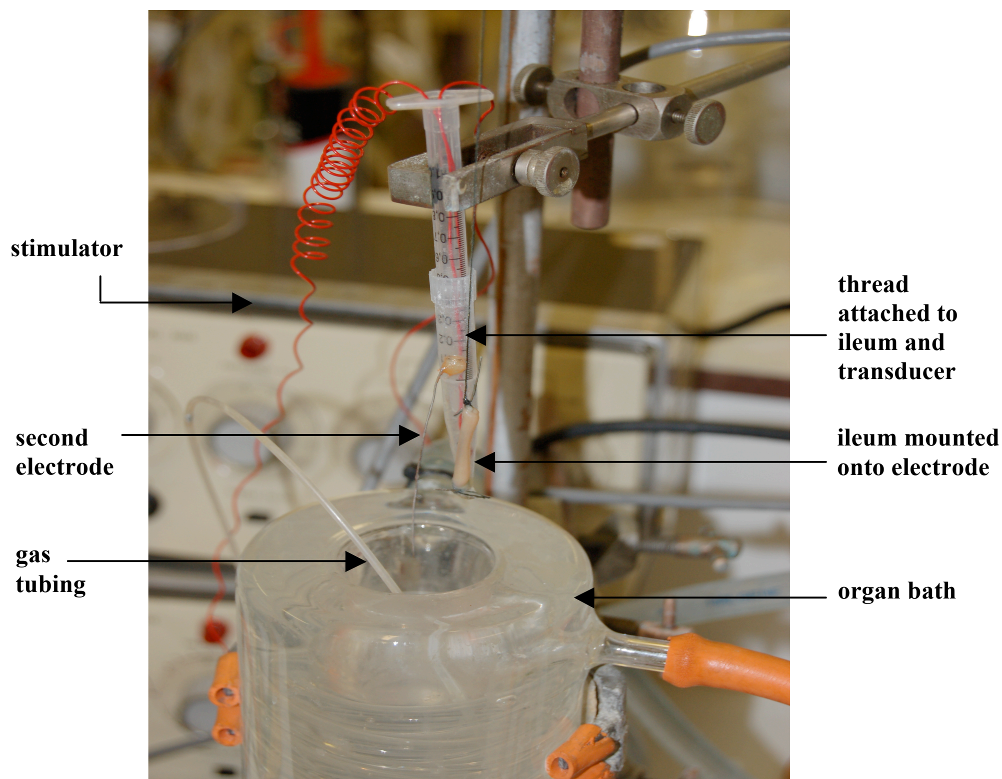


Figure 2.1 *Set-up of the ileum*

Electrical field stimulation

Electrical pulses (at a frequency of 0.1Hz, pulse width of 1.0 ms) were delivered to the electrodes via a Harvard model 6012 stimulator (Massachusetts, USA) (see figure 2.1). The electrical stimulation of the ileum causes the release of acetylcholine from cholinergic nerve endings which contracts the ileum (Scriabine *et al.*, 1970). The stimulus amplitude was found to produce strong, reproducible contractions of the ileum was 25 volts. The contractions were detected by the dynamometer UFI isometric transducer (Pioden Controls, Isle of Wight, UK) and amplified by a bridge amplifier (ADI instruments, Chalgrove, UK). The amplified signal was then displayed on a chart recorder (Labchart and Scope, ADI instruments, Chalgrove, UK). The equipment was calibrated daily.

The ileum preparation was left for 30 minutes to allow the EFS-induced contractions to stabilise. The tissue remained viable for at least 3 hours, during which time all experiments were conducted.

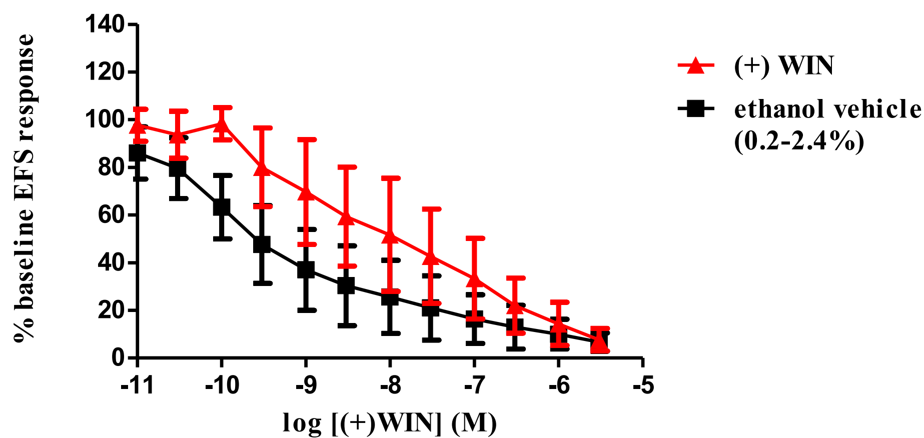
Choice of cannabinoid vehicle in the ileum

Cannabinoids are poorly soluble in water and so the recommended solvents for these drugs are ethanol or dimethyl sulfoxide (DMSO). DMSO had been previously been found by the laboratory group to significantly reduce the size of ileum contractions and so ethanol was chosen as the vehicle where possible.

During the initial experiments with the cannabinoid (+)WIN, the ethanol vehicle caused a substantial reduction in the size of EFS-induced contractions of the ileum at high bath

concentrations (0.2-2.4%) but had no effect at lower concentrations (0.02-0.33%; see figure 2.2A and B below). Ethanol (at low concentrations) was then used as the vehicle for all other cannabinoid agonists and antagonists used in the ileum (in EFS and carbachol experiments). The only exceptions were AM281 and AM630, which were not soluble in ethanol at the concentrations required, and were instead dissolved in DMSO (0.02%).

A



B

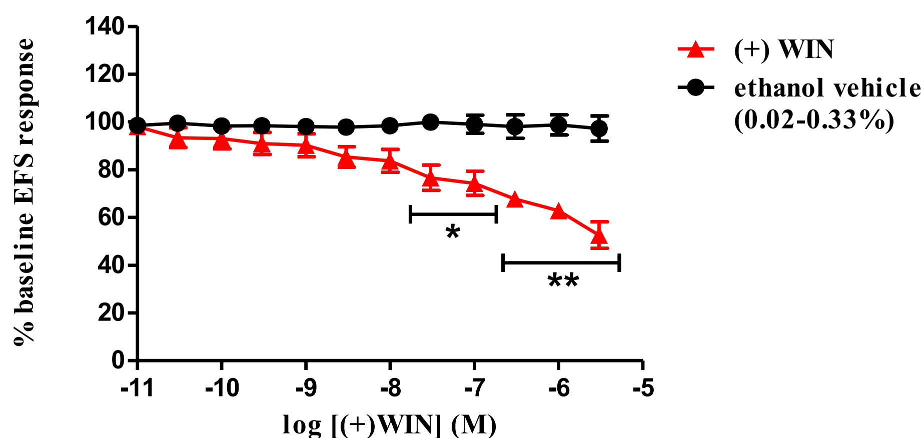


Figure 2.2A and B *The effect of (+) WIN dissolved in (A) ethanol (0.2-2.4%), (B) ethanol (0.02-0.33%) and vehicles alone on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline size was measured immediately before the addition of (+) WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05, **P< 0.01 (one way ANOVA with post-hoc Dunnett's test).*

2.3 Carbachol - induced contraction of the ileum

To investigate any post-synaptic effects of cannabinoids on the ileum, the ileum was set up as described previously (see ‘Organ bath preparation’), but carbamoyl chloride (carbachol) was used to contract the ileum instead of electrical stimulation. Carbachol directly stimulates muscarinic receptors on ileum smooth muscle to produce contraction and is non-hydrolysable (unlike acetylcholine).

A sub-maximal concentration ($3x10^{-7}M$) suitable for use in pre-contracting sections of ileum was determined from the cumulative concentration-response curve shown in figures 2.3 A and B overleaf. The tissue was left for 20 minutes subsequent to the addition of carbachol to allow the contraction to stabilise. The contraction was sustained for at least 90 minutes, during which time all experiments were conducted.

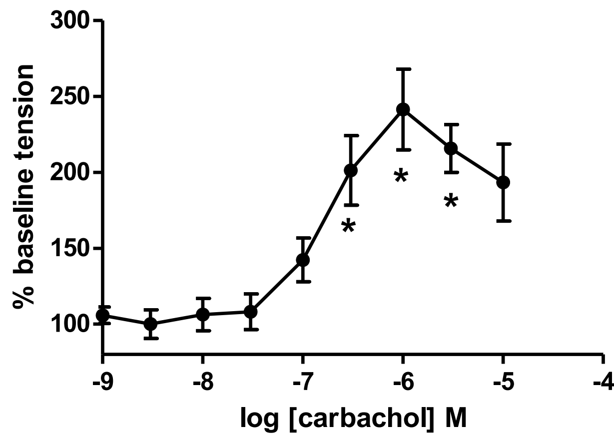


Figure 2.3A *The effect of carbachol on baseline tension of the guinea-pig ileum (n=3). Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM), data were analysed by one sample Student's t test.*

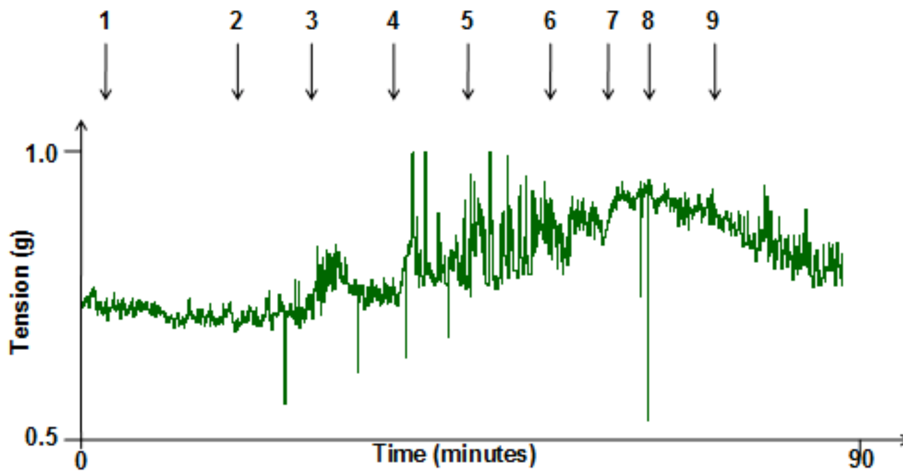


Figure 2.3B *Trace showing the effect of carbachol on baseline tension of the guinea-pig ileum. Concentrations (M) shown below:*

1 = $1x10^{-9}$	**4** = $3x10^{-8}$	**6** = $3x10^{-7}$	**9**= $1x10^{-5}$
2 = $3x10^{-9}$	**5** = $1x10^{-7}$	**7** = $1x10^{-6}$	
3 = $1x10^{-8}$	**6** = $3x10^{-7}$	**8** = $3x10^{-6}$	

2.4 Lipopolysaccharide (LPS)-stimulation of a macrophage cell line

Cell line

The RAW 264.7 mouse leukaemic monocyte – macrophage cell line (European Cell Culture Collection) was used for all experiments. Cells were supplied at passage 7.

Resuscitation of cells

Cells were thawed in a 37°C water bath and suspended in supplemented medium (pre-warmed to 37°C). The cells were then centrifuged at 150g for 5 minutes and re-suspended in supplemented medium and transferred to a T25 (25cm^2 surface area) flask. The flask was incubated at 37°c in a 95% air and 5% CO_2 humidified atmosphere. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated bovine serum and 100 units/ml penicillin and 100µg/ml streptomycin. Medium was replenished every 72 hours.

Sub - culture of cells

At 80% confluency, medium containing cells in suspension was removed from the flask and retained. The adherent cells were scraped from the bottom of the flask and suspended in the spent medium. The cells were then centrifuged at 150g for 5 minutes and re-suspended in fresh supplemented medium and split between 3 T25 flasks (1:3 ratio). The flasks were then incubated as above.

Freezing of cells

Stocks of cells were frozen at passages 8,10,11. The medium containing suspension cells was removed from the flask and retained. The adherent cells were scraped from the bottom of the flask and suspended in the spent medium. The cells were then centrifuged (as in'Subculture of cells'), re-suspended in cold freezing medium (10% DMSO, 20% FBS and 70% DMEM) and transferred to cryovials. The cryovials were frozen at -80°c overnight and then transferred to liquid nitrogen (-140°c).

Cell counting

A 100µl sample of cells was taken and transferred to a cuvette. This was made up to 10mls with isotonic solution Isoton® and the cells were counted using a Coulter® Z2 Counter set to read particles sized between 9 and 30 microns. The readings were taken three times and the average was calculated .This number was then multiplied by 200 to calculate the number of cells per ml.

LPS-induced TNFα release from RAW 264.7 macrophages

The cells were counted as above and seeded onto a 24 well plate (1.9cm^2/well) at a density of 40,000 cells/cm^2. Cells were grown for 96 hours, at which point the medium was removed from the wells and replaced with antibiotic-free medium containing LPS (5ng/ml). After an incubation period of 16 hours, the medium was removed from the wells and assayed for TNFα at a later date (see 'TNFα measurement' below). The concentration of LPS used was decided from the pilot study shown in figure 2.4.

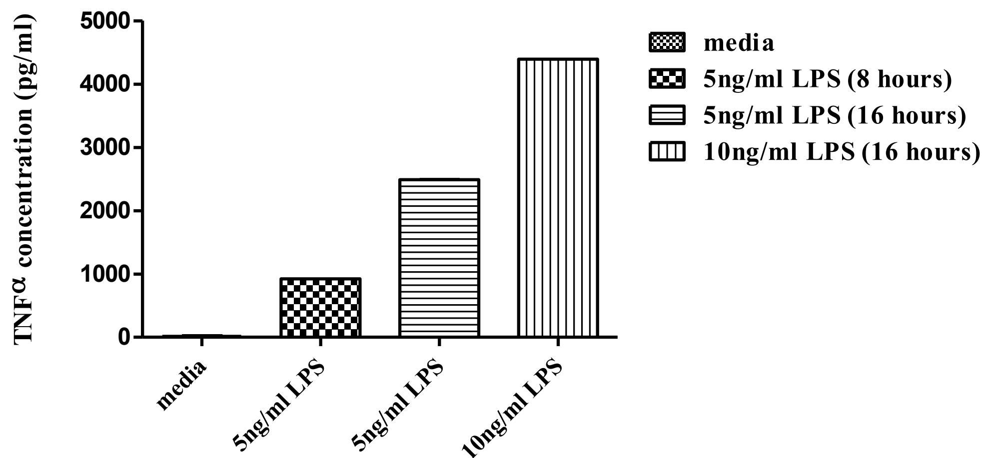


Figure 2.4 *LPS pilot study (n=1). RAW 264.7 macrophages were exposed to LPS at 5ng/ml for 8 hours and 5ng/ml and 10ng/ml LPS for 16 hours. Higher concentrations of 25-1000ng/ml LPS were also tested but the absorbance readings were too high and did not fall within the range of the standard curve.*

Cell Lysis

At the end of the LPS exposure macrophages were lysed to remove their protein content. Lysis buffer (pH 7.5) was made up on the day of lysis and kept on ice. This consisted of 0.4mM $NaVO_4$, 50mM NaF, 1mM Phenylmethanesulfonyl fluoride (PMSF), 20μM phenylarsine oxide, 10 mM sodium molybdate, 10μg/ml leupeptin and 10μg/ml aprotinin, 50mM Tris, 5mM (Ethyleneglycol-O, O' bis (2-aminoethyl) N,N,N'N'-tetraacetic acid) EGTA disodium salt, 150mM NaCl and 1% Triton dissolved in distilled water.

Before the lysis buffer was added, the cell medium was removed from the wells, centrifuged at 1000g for 10 minutes and stored in aliquots at -20˚C. The TNFα concentration in the medium was measured at a later date (see 'TNFα measurement'). The wells were then washed with 0.1M PBS to remove any remaining LPS. 125μl of lysis buffer was added to each well and left on ice for 15 minutes before scraping the cells from the bottom of the wells. The cell suspension was then centrifuged at 13000rpm at 4˚C for 15 minutes. The supernatant was then removed and stored in

aliquots at -20˚C for up to 1 week. The protein concentration in this supernatant was measured at a later date (see 'Protein measurement').

TNFα measurement

The aliquots of cell medium (see 'Cell lysis' above) were thawed and the TNFα concentration was determined using the R and D systems mouse TNFα Duoset ELISA Development kit and following the kit protocol.

Principle of assay: the assay employed the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody (capture) specific for mouse TNFα was coated onto a 96-well ELISA plate. Standards and samples were pipetted into wells and any mouse TNFα present was bound by the immobilized antibody. Another polyclonal antibody (detection) specific for mouse TNFα was then added to the wells which bound to the TNFα present. Streptavidin conjugated to horseradish peroxidise (streptavidin-HRP) was added to the wells which bound to the detection antibody. Substrate solution containing H_2O_2 and tetramethylbenzidine was added to the wells and converted to a coloured blue product by the HRP enzyme. This turned yellow when the H_2SO_4 stop solution was added. The absorbance was then measured at 450nm using a spectrophotometer (Sunset instruments, LLC, Hebron, USA). The plate was washed between steps to wash off any unbound substances.

Protein measurement

Protein quantification was achieved by using the bicinchonic acid (BCA) assay. Bovine serum albumin (BSA) standards were prepared by serially diluting the BSA stock (2mg/ml) with distilled water. Aliquots of the cell lysate (see 'Cell lysis') were thawed and 10μl of lysate or standard was pipetted in duplicate onto a 96 well plate. BCA™ working reagent (WR) was prepared by mixing 50 parts of BCA™ reagent A with 1 part of BCA™ reagent B. 200μl of the WR was added to each well and mixed by placing the plate on a plate shaker for 30 seconds. The plate was then incubated at 37°C for 30 minutes. After cooling the plate for 5 minutes at room temperature, the absorbance was read at 562nm using a spectrophotometer (Sunset instruments, LLC, Hebron, USA).

Principle of assay: When the working reagent (containing BCA™ reagents A and B) was added to the cell lysate, the protein present reduced the Cu^{2+} ions (BCA reagent B) to Cu^{1+} ions by forming light blue protein/copper chelates. The BCA™ reagent A provided the alkaline environment for this reaction to occur. In the next step, bicinchonic acid (BCA™ reagent A) reacted with the Cu^{1+} ions to form purple coloured BCA/copper complexes. The absorbance of these complexes could then be measured at the 562nm wavelength.

2.5 Data Analysis

In the ileum experiments data was reported as percentage of baseline EFS or initial carbachol contractions (mean±SEM). Responses were measured once they had reached a plateau, before the next concentration of drug was added.

IC_{25} and IC_{50} values calculated for drugs were defined as the drug concentrations required to inhibit baseline EFS/initial carbachol contractions by 25% and 50% respectively. Mean values with their 95% confidence intervals (shown in brackets) were calculated.

To calculate an IC_{25} or IC_{50} value for a drug, concentration response curves were analysed individually. The concentration of drug corresponding to 25% or 50% inhibition was calculated by linear regression between the concentrations lying either side of the 25% or 50% inhibitory value. This was performed in Microsoft Excel 2007. The average IC_{25} or IC_{50} value was then calculated for each experimental group.

95% confidence intervals (CI) were also calculated using Microsoft Excel 2007. The Excel formula was based on the equation:

$CI = x \pm t^* (s / \sqrt{n})$

Where x = sample mean (mean IC_{25} or IC_{50}), t*= test statistic, s= standard deviation, n=sample number.

In the macrophage experiments the effect of each drug was expressed as a percentage of the media control. In the experiments examining TNFα release, results were also

expressed as pg TNFα / mg protein. Data were analysed in Microsoft Excel 2007 and displayed graphically using Graphpad Prism 5.

2.6 Statistical analysis

Statistical analysis was carried out using an unpaired Student's T test (two groups) or one way ANOVA with post-hoc Dunnett's test (more than two groups) using Instat. A *P* value of < 0.05 was considered significant

2.7 Materials

(*S*)-(-)-WIN 55,212-2 mesylate (Tocris, Bristol, UK).

(*R*)-(+)-WIN 55,212-2 mesylate (Tocris, Bristol, UK).

O-1602 (Tocris, Bristol, UK).

Abnormal cannabidiol (Tocris, Bristol, UK).

O-1918 (Tocris, Bristol, UK).

Rimonabant (Tocris, Bristol, UK).

AM281 (Tocris, Bristol, UK).

AM630 (Tocris, Bristol, UK).

Carbamoyl chloride (Sigma-Aldrich Company Ltd, Gillingham, UK).

JWH007 (synthesised by Ghaith Al-Jayyoussi at the Welsh School of Pharmacy,Cardiff University, UK).

GA001 (synthesised by Ghaith Al-Jayyoussi at the Welsh School of Pharmacy Cardiff University, UK).

GA002 (synthesised by Ghaith Al-Jayyoussi at the Welsh School of Pharmacy Cardiff University, UK).

GA003 (synthesised by Ghaith Al-Jayyoussi at the Welsh School of Pharmacy Cardiff University, UK).

Tocrisolve (Tocris, Bristol, UK).

SR144528 (Cambridge Biosciences Ltd, Cambridge, UK).

BCA™ protein assay reagent A (containing sodium carbonate, sodium bicarbonate, bicinchonic acid and sodium tartrate in 0.1M sodium hydroxide) (Thermo Fisher Scientific, Northumberland, UK).

BCA™ protein assay reagent B (containing 4% cupric sulphate) (Thermo Fisher Scientific, Northumberland, UK).

Methanethiosulphonate reagent (Promega Ltd, Southampton, UK).

Lipolysaccharides from Escherichia Coli 055:B5, purified by gel filtration chromatography, gamma irradiated (Sigma-Aldrich Company Ltd, Gillingham, UK).

Mouse TNFα Duoset ELISA Development kit (R and D systems, Abingdon, UK).

DMEM (Invitrogen, Paisley, UK).

Heat inactivated fetal bovine serum (Invitrogen).

Penicillin/streptomycin, containing 5,000units/ml penicillin, 5000μg/ml streptomycin (Invitrogen, Paisley, UK).

Isoton® (Beckman Coulter Ltd, High Wycombe, UK).

All other chemicals/reagents were purchased from Fisher-Scientific, UK or Sigma-Aldrich.

CHAPTER 3:

CANNABINOID PHARMACOLOGY OF THE GUINEA-PIG ILEUM

3. Cannabinoid pharmacology of the guinea-pig ileum

3.1 Introduction

As discussed in chapter 1, the currently held view is that cannabinoids reduce intestinal contractions *in vitro* and *in vivo* via activation of the CB_1 receptor (Colombo *et al*., 1998; Coutts *et al*., 1997; Izzo *et al*., 2000a; Pertwee *et al*., 1996). The literature suggests that cannabinoids activate pre-synaptic CB_1 receptors on cholinergic nerve endings in the enteric nervous system, resulting in a reduction in acetylcholine release and hence ileum contractions (Colombo *et al*., 1998; Coutts *et al*., 1997; Izzo *et al*., 2000a; Pertwee *et al*., 1996). However, much of the evidence for the involvement of the CB_1 receptor relies on the use of the CB_1 antagonist rimonabant, which is not as selective as first thought (see 'Non-CB_1 / CB_2 targets for cannabinoid receptor ligands', Chapter 1).

Initially, the CB_2 receptor was not thought to be involved in the inhibition of intestinal motility, but it is now thought that activation of this receptor may reduce contractions during inflammation (Izzo, 2007). There is also recent evidence that the CB_2 receptor may be present in the normal gut of humans and rat, and may be involved in controlling motility (Wright *et al*., 2008; Storr *et al*., 2002; Ligresti *et al*., 2003; Duncan et al., 2008; (Kurjak *et al.*, 2008). Other studies have shown evidence that cannabinoids can activate non-CB_1/CB_2 receptors to inhibit acetylcholine release (Mang *et al.*, 2001) and inhibit intestinal contractions (Duncan *et al*., 2005; Makwana *et al*., 2010a). There is also evidence for the presence of the putative cannabinoid receptors GPR55 and GPR119 in the intestine (Brown, 2007), although their role in motility is yet to be investigated. Together, these studies suggest that cannabinoid pharmacology in the

ileum may not be as straightforward as first thought and that other receptors, apart from CB_1, may be involved in regulating intestinal motility.

In this chapter, the cannabinoid pharmacology of the guinea-pig ileum was investigated using commercially available cannabinoid agonists and antagonists (listed in the following section).

Cannabinoid agonists

WIN 55,212-2

The synthetic aminoalkylindole WIN 55,212-2 is available as two enantiomers, (*R*)-(+)-WIN 55,212-2 and (*S*)-(-)-WIN 55,212-2 and exhibits stereoselectivity in its pharmacological effects. (*S*)-(-)-WIN 55,212-2 is a low potency CB_2 antagonist and a partial CB_1 inverse agonist (Savinainen *et al.*, 2005) whereas (*R*)-(+)-WIN 55,212-2 is a potent, non-selective CB_1/ CB_2 agonist (D'Ambra *et al.*, 1992) (see table 1.1, introduction). Some non-CB_1/ CB_2 effects have also been reported for the (*R*)-(+)-isomer WIN 55,212-2 (see 'Non-CB_1 / CB_2 targets for cannabinoid receptor ligands', chapter 1).

The non-selective agonist (*R*)-(+)-isomer WIN 55,212-2, referred to as (+)WIN, was used to investigate whether CB_1 and/or CB_2 receptors are involved in the inhibition of guinea-pig ileum contractions. This agonist was chosen due to its high potency at both receptors and lack of activity at TRPV1 and GPR55 receptors (Pertwee, 2010). The stereoisomer (*S*)-(-)-WIN 55,212-2, referred to as (-)WIN, was used as a negative

control to (+)WIN in these studies, as it does not activate the CB_1 or CB_2 receptor. The chemical structures of (+)WIN and (-)WIN are shown below (see figures 3.1 and 3.2).

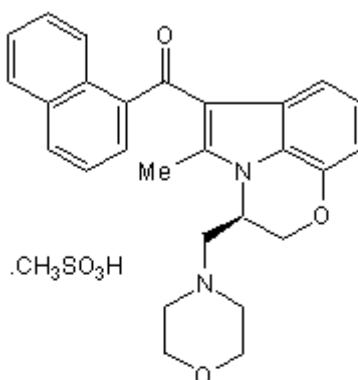


Figure 3.1 *Chemical structure of (R)-(+)-WIN 55,212-2*

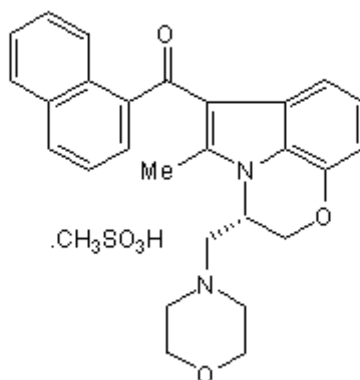


Figure 3.2 *Chemical structure of (S)-(-)-WIN 55,212-2*

Arachidonylcyclopropylamide

The synthetic analogue of anandamide, arachidonylcyclopropylamide (ACPA), is a potent, selective CB_1 receptor agonist with greater affinity for CB_1 over CB_2 receptors (see table 1.1, introduction and figure 3.3). This selective CB_1 agonist was used to investigate whether CB_1 receptors are involved in the inhibition of guinea-pig ileum

contractions. ACPA was chosen as it is one of the most potent CB_1 agonists, although it does not show resistance to hydrolysis, unlike the (*R*)-(+)-methandamide (a less efficacious CB_1 agonist; Pertwee., 2010). ACPA has not been tested for any non-CB_1/ CB_2 effects (Hillard et al., 1999; Pertwee., 1999; Pertwee 2010).

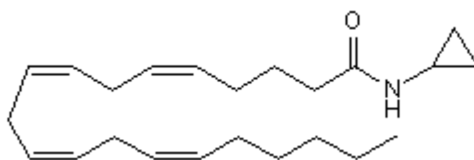


Figure 3.3 *Chemical structure of ACPA*

PSN 375963

PSN 375963 (PSN) is a synthetic oxadiazole analogue which is an agonist at the proposed novel cannabinoid receptor GPR119 (see GPR119 receptor, chapter 1). Reported EC_{50}s for the GPR119 receptor are in the micromolar range (in the yeast fluorimetric assay; Overton *et al*., 2006), suggesting it is a low potency agonist at this receptor. No activity has been reported at the CB_1 receptor (see table 1.1, introduction) but this compound may have other non-GPR119 receptor mediated effects. This compound (see figure 3.4 overleaf) was chosen to investigate whether the GPR119 receptor is involved in the inhibition of guinea-pig ileum contractions and, although it is not very potent and may not be highly selective, few GPR119 agonists are available and they all share similar limitations. As well as these issues, unfortunately no GPR119 antagonist is available to confirm any effects seen by PSN (Pertwee, 2010; Ning *et al*., 2008; Overton *et al*., 2006).

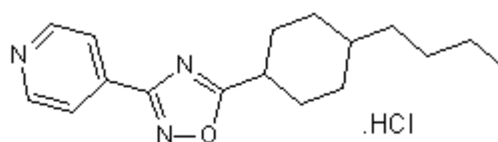


Figure 3.4 *Chemical structure of PSN 375963*

Abnormal cannabidiol

Abnormal cannabidiol (abnormal-CBD) is a synthetic regioisomer of the phytocannabinoid cannabidiol (see figure 3.5). It was chosen to investigate whether the endothelial cannabinoid receptor (CB_e) is present in the guinea-pig ileum and involved in inhibition of contractions. This compound was chosen as it is thought to be an agonist at the CB_e receptor (EC_{50} ~3μM; Offertaler *et al.*, 2003) and does not activate the CB_1 or CB_2 receptor (see table 1.1, introduction). However, it has also been shown to activate the GPR55 receptor (reported EC_{50}s 2.5μM and 2.5nM; Ryberg *et al.*, 2007 and Johns *et al.*, 2007 respectively). The GPR55 receptor is now thought to be distinct from CB_e (Ho *et al.*, 2003; Jarai *et al.*, 1999; Mo *et al.*, 2004; Ryberg *et al.*, 2007).

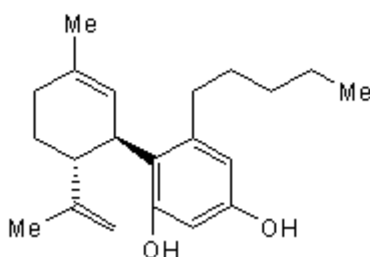


Figure 3.5 *Chemical structure of abnormal cannabidiol*

O-1602

The synthetic analogue of cannabidiol O-1602 is a selective agonist for the suggested novel cannabinoid receptor GPR55 (see figure 3.6) and was used to investigate whether GPR55 is involved in inhibition of guinea-pig ileum contractions. This compound was chosen due to its high potency at the GPR55 receptor (EC_{50}s 1.4 and 13nM; Johns *et al.*, 2007 and Ryberg *et al.*, 2007 respectively) and because it activates GPR55 in a number of different assays (Johns *et al.*, 2007; Ryberg *et al.*, 2007; Waldeck-Weiermair *et al.*, 2008). In addition, no activity at the CB_1 and CB_2 receptor has been reported (see table 1.1). There are some reports, however, that at higher concentrations (in the micromolar range) O-1602 activates the putative CB_e receptor (EC_{50} 2-3µM; Johns *et al.*, 2007)

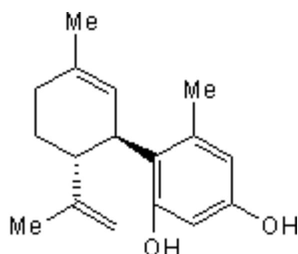


Figure 3.6 *Chemical structure of O-1602*

Cannabinoid antagonists

Rimonabant and AM281

The synthetic diarylpyrazole rimonabant and AM281 (structural analogue of rimonabant) are potent, competitive, relatively selective CB_1 receptor antagonists (Lan *et al.*, 1999a; Rinaldi-Carmona *et al.*, 1994), showing greater affinity for CB_1 over CB_2

receptors (see table 1.1). There is also evidence that these antagonists are not simply neutral antagonists but also display inverse agonism (Hosohata *et al.*, 1997; Pertwee *et al.*, 1996). Both antagonists have been shown to act at the GPR55 receptor. AM281 activates the receptor at 3-30μM (Henstridge *et al.*, 2009a and b) whereas, reports of rimonabant's effects on the receptor are conflicting (Godlewski *et al.*, 2009; Pertwee., 2010; Rinaldi-Carmona *et al.*, 1994). Several non-CB_1/ CB_2 /GPR55 effects have been demonstrated with rimonabant use (see 'Non-CB_1 / CB_2 targets for cannabinoid receptor ligands', chapter 1). The two different CB_1 antagonists were used with every agonist in this chapter to investigate if the effect was consistent regardless which CB_1 antagonist was used. Therefore, if both antagonists attenuated a response to an agonist, it would be more convincing that the agonist effect was mediated through the CB_1 receptor. The structures of AM281 and rimonabant are shown in figures 3.7 and 3.8.

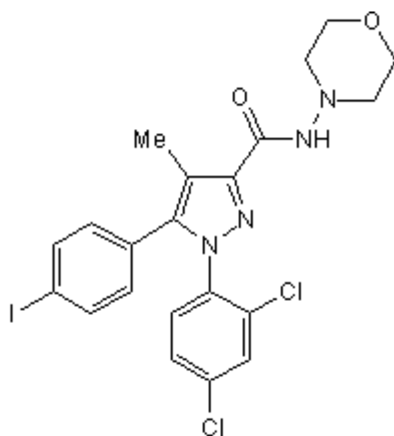


***Figure 3.7** Chemical structure of AM281*

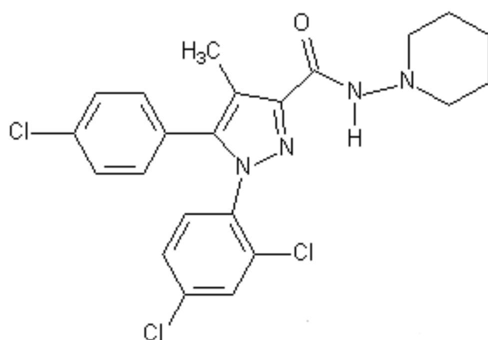


Figure 3.8 *Chemical structure of rimonabant*

SR144528 and AM630

The diarylpyrazole SR144528 and aminoalkylindole AM630 (see figures 3.9 and 3.10) are potent, competitive, selective CB_2 receptor antagonists (Hosohata *et al.*, 1997; (Rinaldi-Carmona *et al.*, 1998) showing greater affinity for CB_2 over CB_1 receptors (see table 1.1, introduction). These synthetic antagonists are also thought to act as inverse agonists at the CB_2 receptor (Ross *et al.*, 1999). Neither compound activates the GPR55 receptor (Pertwee., 2010).

Two different CB_2 antagonists were used for the same rationale as for the CB_1 antagonists; if both antagonists attenuated the agonist's response, it would be more convincing that the agonist's response was mediated through the CB_2 receptor.

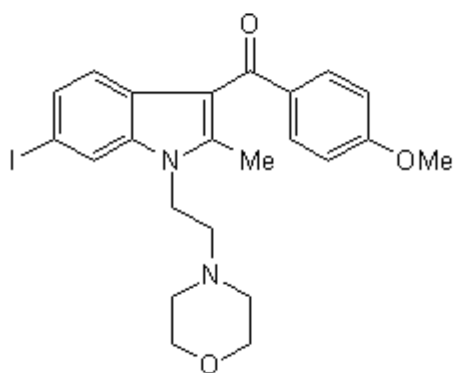


Figure 3.9 *Chemical structure of AM630*

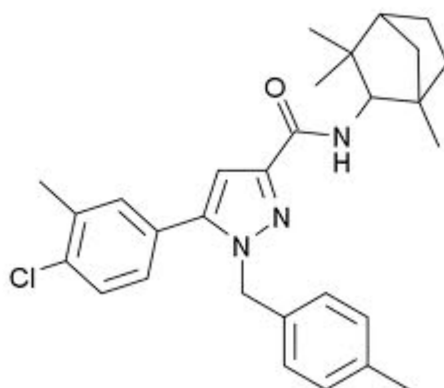


Figure 3.10 *Chemical structure of SR144528*

(-)cannabidiol

(-) cannabidiol ((-)CBD) is one of the main phytocannabinoids found in cannabis. It was used to investigate whether any of the effects of the cannabinoid agonists were mediated through the GPR55 receptor as this is the only GPR55 antagonist available. This compound is, however, not very selective; the IC_{50} for the GPR55 receptor is only slightly lower than that for the CB_1 receptor (0.4 and 3.4μM respectively; Petitet *et al.*,

1998; Thomas *et al*., 2007., Ryberg *et al*., 2007). (-)CBD is also a CB_2 receptor inverse agonist (Thomas *et al*., 2007), inhibitor of anandamide uptake (Bisogno *et al*., 2001) and an agonist at the TRPV1 receptor (Costa *et al*., 2004).

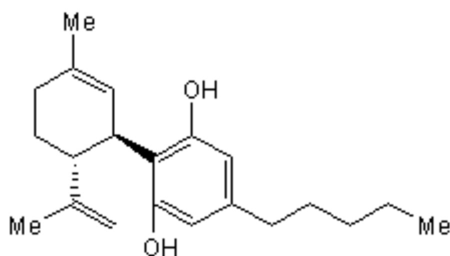


Figure 3.11 *Chemical structure of (-) cannabidiol*

O-1918

O-1918 is a synthetic analogue of CBD. It is a selective antagonist (≥ 1µM; Offertaler., 2003) at the CB_e receptor (Hoi *et al.*, 2006; Mo *et al.*, 2004; Offertaler *et al.*, 2003) and does not bind to the CB_1 or CB_2 receptor (see table 1.1; chapter 1). This was chosen to investigate whether any of the agonists mediated their effects through the CB_e receptor as this was the only CB_e receptor antagonist available.

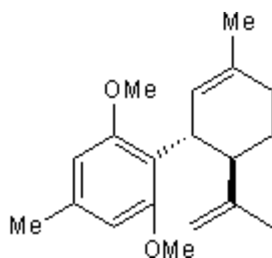


Figure 3.12 *Chemical structure of O-1918*

3.2 Aims

The aim of this chapter was to establish the identity of the receptors mediating inhibition of ileum contractions through the use of selective agonists and antagonists.

3.3 Method

Construction of cannabinoid agonist concentration-response curves

The ileum was prepared as in chapter 2 and was either subjected to EFS (see 'Electrical field stimulation of the guinea-pig ileum', chapter 2) or contracted with carbachol (see 'Carbachol-induced contraction of the ileum', chapter 2). Once contractions had stabilised, concentrations of the cannabinoids ((+)WIN, (-)WIN, abnormal-CBD, PSN or O-1602) or ethanol vehicle were added in a cumulative manner. The organ bath concentration of the cannabinoids ranged from $1x10^{-11}$M to $3x10^{-6}$ M ($1x10^{-4}$M for 0-1602 and PSN). The concentration of the vehicle (absolute ethanol) varied depending on the cannabinoid used. The volumes of vehicle used and the final bath concentrations are shown in the results section (3.4). Enough time was allowed for each response to plateau before a subsequent agonist concentration was added. Only one concentration-response curve was constructed per individual preparation.

In the antagonist studies, the ileum was incubated with the antagonist (0-1918, (-)CBD, rimonabant, AM281, SR144528 or AM630) or vehicle for 30 minutes before the first concentration of agonist was added. Rimonabant, AM281 and AM630 were administered dissolved in DMSO, whereas absolute ethanol was used as the vehicle for

0-1918, SR144528 and (-)CBD. The final concentration of DMSO in the organ bath was 0.02% where these antagonists were used.

Drug solutions

Drugs were dissolved in either absolute ethanol (WIN 55,212-2, PSN, SR144528, (-)CBD and 0-1918) or DMSO (AM630, AM281 and rimonabant). Abnormal-CBD and 0-1602 were supplied pre-dissolved in methyl acetate. Due to the large inhibition of EFS-induced contractions induced by methyl acetate, the solvent was evaporated under vacuum using a rotary evaporator and the compounds were re-dissolved in absolute ethanol. Aliquots of drug solutions were frozen at -20˚C (for up to one month) and were thawed and diluted to the desired concentration on the day of the experiment. All cannabinoid agonists were serially diluted in ethanol. ACPA was the only drug supplied in Tocrisolve™, and was serially diluted with Krebs' bicarbonate solution on the day of the experiment. The combined rimonabant and SR144528 solution was prepared by dissolving rimonabant in ethanol and then dissolving SR144528 in the rimonabant solution. This was then stored in aliquots as above and serially diluted in ethanol on the day of experiment.

Data analysis

The effect of each drug concentration on contraction size was reported as a percentage of baseline EFS or initial carbachol contraction. Data were reported as mean±SEM.

An IC_{25} or IC_{50} was calculated for each drug, defined as the drug concentrations required to inhibit baseline EFS/initial carbachol contraction by 25% and 50%

respectively. Mean values with their 95% confidence intervals (shown in brackets) were calculated.

Curve fitting was not carried out as the concentration-response curves did not fit the traditional sigmoidal shape and did not reach a maximum response at the concentrations used.

Statistical analysis

Mean values for the drug and its vehicle control were compared using unpaired Student's t test (drug and vehicle) or one way ANOVA with post-hoc Dunnett's Test (multiple drugs and vehicle). One sample Student's t tests were used to determine the effects of the vehicle alone on contractions. Statistical differences between vehicle and drug were denoted with a star symbol. $P<0.05$ was considered statistically significant.

3.4 Results

3.4.1 The effect of cannabinoid agonists/antagonists on EFS-evoked contractions of the guinea-pig isolated ileum.

The effect of (+)WIN on EFS-evoked contractions

(+)WIN caused a concentration-dependent reduction in the size of EFS-induced contractions of the ileum (IC_{25}: 2.8(0.1-56) $x10^{-8}$M). (+)WIN had no effect at concentrations below $3x10^{-8}$M but caused (47.4 ± 5.5%; $P < 0.01$) inhibition at the highest concentration of 3µM (see figures 3.13 and 3.14). The ethanol vehicle had no significant effect on the size of contractions.

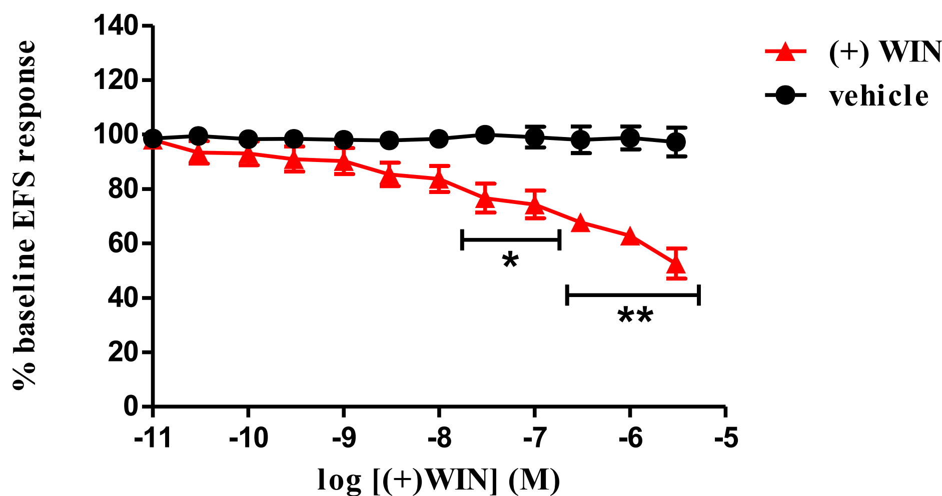


Figure 3.13 *The effect of (+)WIN (n=6) and ethanol vehicle (n=5) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline size was measured immediately before the addition of (+)WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05, **P< 0.01 (one way ANOVA with post-hoc Dunnett's test). The concentrations of ethanol vehicle used ranged from 0.02-0.33% (shown as respective (+)WIN concentrations 10^{-11}- $3x10^{-6}$M). The same vehicle control was used in several cannabinoid experiments.*

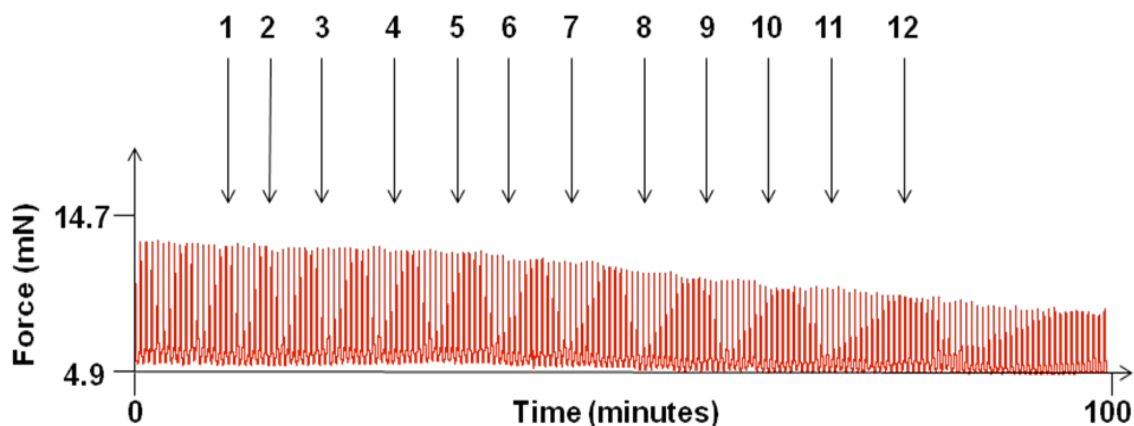


Figure 3.14 *Trace showing the effect of (+)WIN on EFS-induced contractions of the guinea-pig isolated ileum. Concentrations (M) shown below:*

1= $1x10^{-11}$	**4**= $3x10^{-10}$	**7**= $1x10^{-8}$	**10**= $3x10^{-7}$
2= $3x10^{-11}$	**5**= $1x10^{-9}$	**8**= $3x10^{-8}$	**11**= $1x10^{-6}$
3= $1x10^{-10}$	**6**= $3x10^{-9}$	**9**= $1x10^{-7}$	**12**= $3x10^{-6}$

The effect of rimonabant on (+)WIN-induced inhibition of EFS-evoked contractions

Rimonabant alone (1µM) increased contraction size by 47.3 ± 8.7% ($P < 0.05$) (see figure 3.16 and table 3.1 overleaf). The antagonist vehicle alone had no significant effect on the EFS-induced response. Rimonabant (1µM) did not antagonise the (+)WIN-induced inhibition (see figure 3.15 overleaf) of EFS-induced contractions and in fact increased the effect of 3µM (+)WIN. ((+)WIN reduced contraction size by 71.0 ± 6.2 in the presence of rimonabant compared to 40.7 ± 7.6% in the presence of antagonist vehicle, $P < 0.05$).

Drugs	*n*	% of initial EFS response
AM281	4	195.8 ± 9.0% *
rimonabant	4	147.3 ± 8.7% *

Table 3.1 *The effect of rimonabant and AM281 alone (1μM) on EFS-induced contractions of the guinea-pig isolated ileum.*

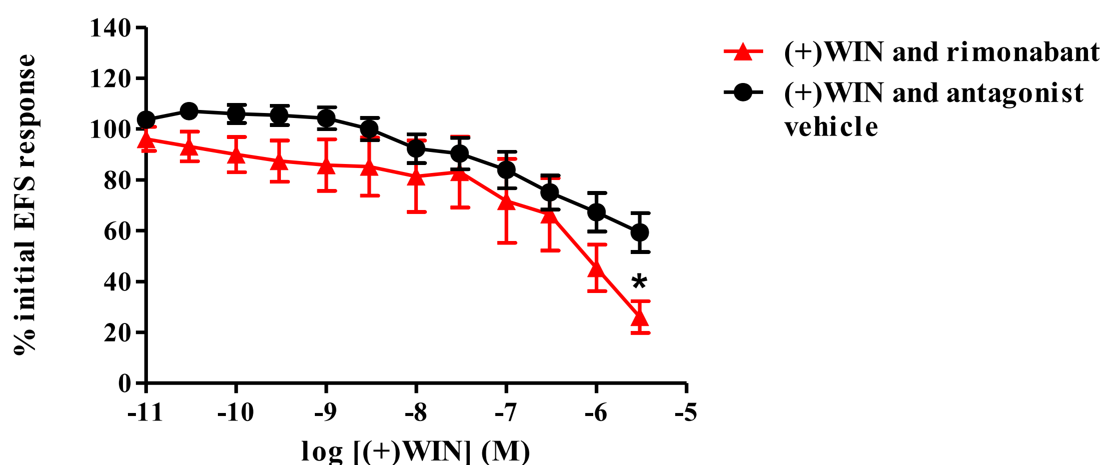


Figure 3.15 *The effect of (+)WIN on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1μM rimonabant (n=4) and antagonist vehicle (0.02% DMSO; n=5). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of (+)WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05 (one way ANOVA with post-hoc Dunnett's test). The same antagonist vehicle was used in several (+)WIN experiments.*

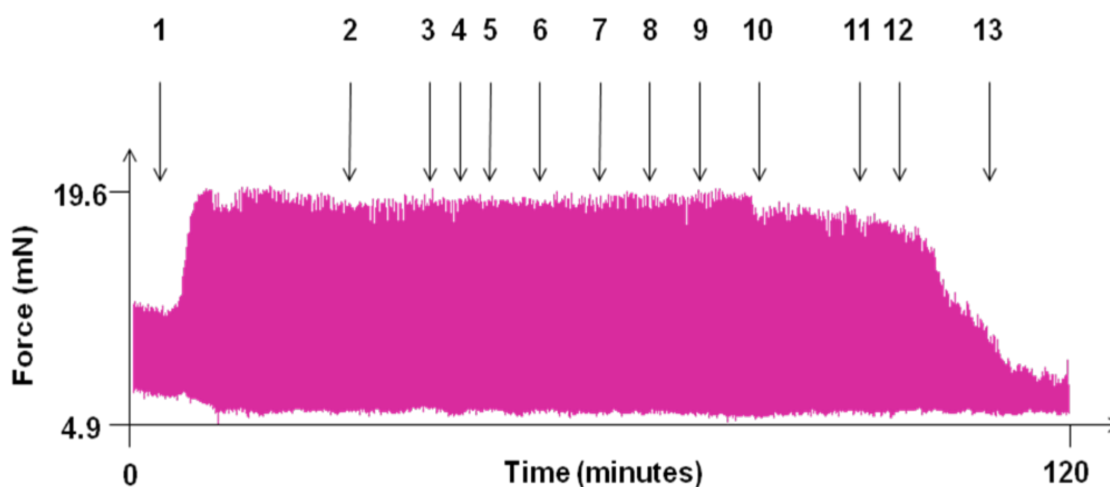


Figure 3.16 *Trace showing the effect of (+)WIN* ***(2-13)*** *on EFS-induced contractions of the guinea-pig isolated ileum in the presence of rimonabant (1µM). Concentrations (M) shown below:*

1= *rimonabant (1µM)*	**4**= $1x10^{-10}$	**7**= $3x10^{-9}$	**10**= $1x10^{-7}$	**13**= $3x10^{-6}$
2= $1x10^{-11}$	**5**= $3x10^{-10}$	**8**= $1x10^{-8}$	**11**= $3x10^{-7}$	
3= $3x10^{-11}$	**6**= $1x10^{-9}$	**9**= $3x10^{-8}$	**12**= $1x10^{-6}$	

The effect of AM281 on (+)WIN-induced inhibition of EFS-evoked contractions

AM281 alone (1µM) increased contraction size by 95.8 ± 9.0% in a similar manner to rimonabant (see table 3.1). The antagonist vehicle alone had no significant effect on the EFS-induced response. AM281 (1µM) did not antagonise the (+)WIN-induced inhibition of EFS-evoked contractions (see figure 3.17 overleaf).

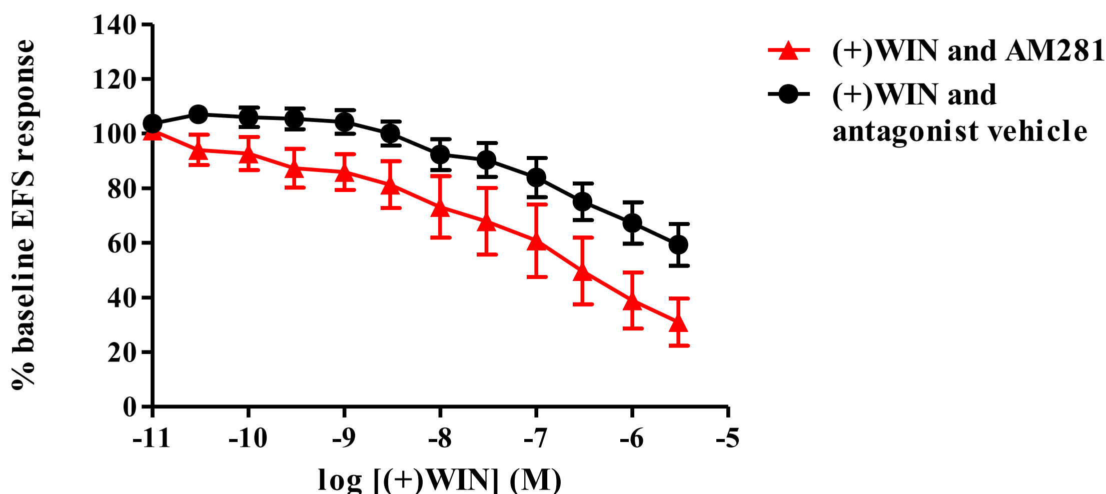


Figure 3.17 *The effect of (+)WIN on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1µM AM281 (n=6) and antagonist vehicle (0.02% DMSO; n=5). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of (+)WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by one way ANOVA. The same antagonist vehicle control was used in several (+)WIN experiments.*

The effect of AM630 on (+)WIN-induced inhibition of EFS-evoked contractions

AM630 (1µM) did not antagonise the (+)WIN-induced inhibition of EFS-induced contractions (see figure 3.18 overleaf) and, in fact, increased the response associated with low concentrations of (+)WIN ($3x10^{-11}$ to 10^{-9}M).(+)WIN at a concentration of 10^{-9}M was without effect in the absence of AM630 but caused a significant reduction in the presence of AM630, reducing contraction size by 22.2 ± 5.7% ($P < 0.05$).

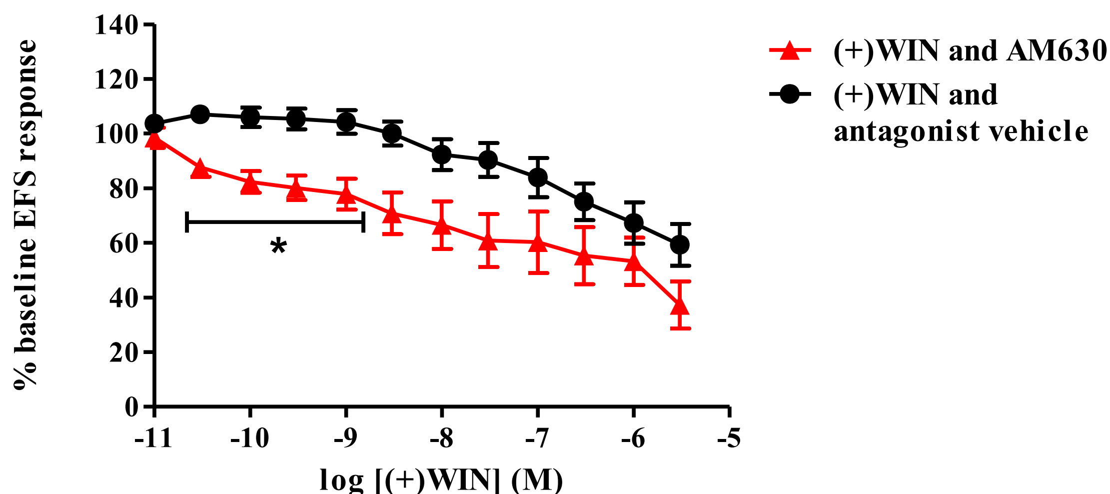


Figure 3.18 *The effect of (+)WIN on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1µM AM630 (n=5) and antagonist vehicle (0.02% DMSO; n=5). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of (+)WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05 (one way ANOVA with post-hoc Dunnett's test). The same antagonist vehicle was used in several (+)WIN experiments.*

The effect of SR144528 on (+)WIN-induced inhibition of EFS-evoked contractions

SR144528 (1µM) had no significant effect on (+)WIN-induced inhibition of EFS-evoked contractions (see figure 3.19 overleaf).

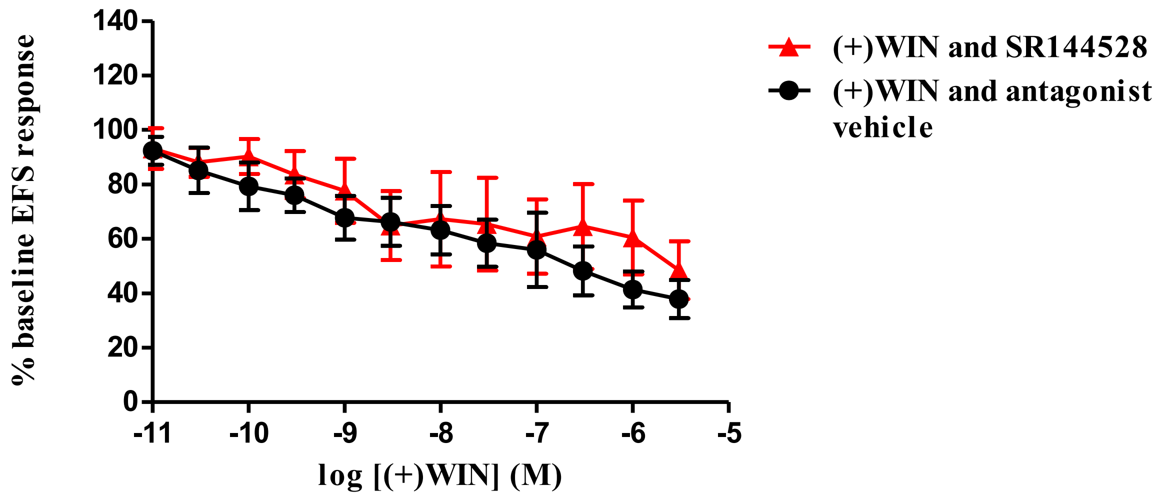


Figure 3.19 *The effect of (+)WIN on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1µM SR144528 (n=3) and antagonist vehicle (0.02% ethanol; n=5). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of (+)WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by one way ANOVA. The same antagonist vehicle control was used in several (+)WIN experiments.*

The effect of O-1918 on (+)WIN-induced inhibition of EFS-evoked contractions

O-1918 (1µM) had no significant effect on (+)WIN-induced inhibition of EFS-evoked contractions (see figure 3.20 overleaf).

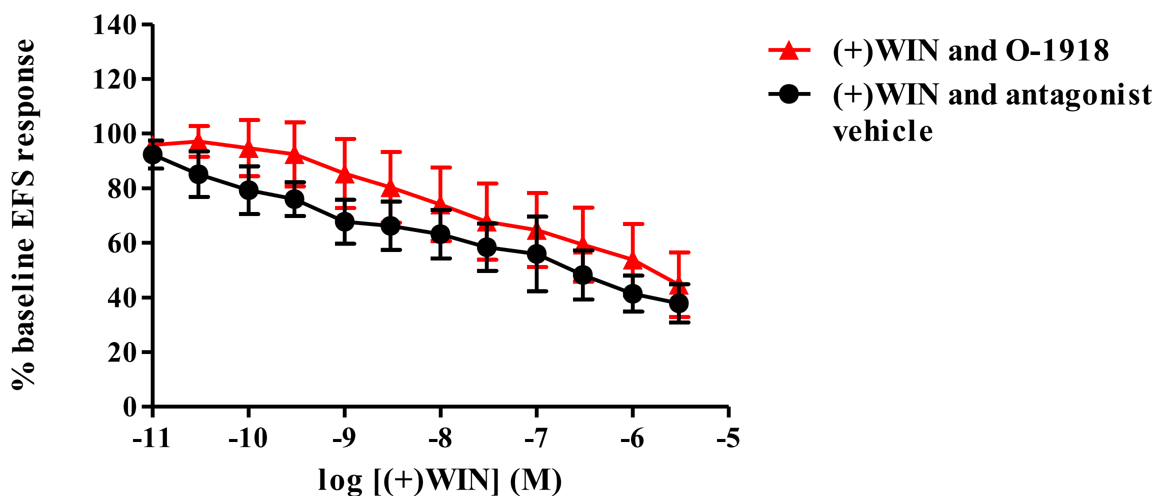


Figure 3.20 *The effect of (+)WIN on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1µM O-1918 (n=6) and antagonist vehicle (0.02% ethanol; n=5). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of (+)WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by one way ANOVA. The same antagonist vehicle control was used in several (+)WIN experiments.*

The effect of rimonabant + SR144528 on (+)WIN-induced inhibition of EFS-evoked contractions

The combination of rimonabant (1µM) and SR144528 (1µM) had no significant effect on (+)WIN-induced inhibition of EFS-evoked contractions (see figure 3.21 overleaf).

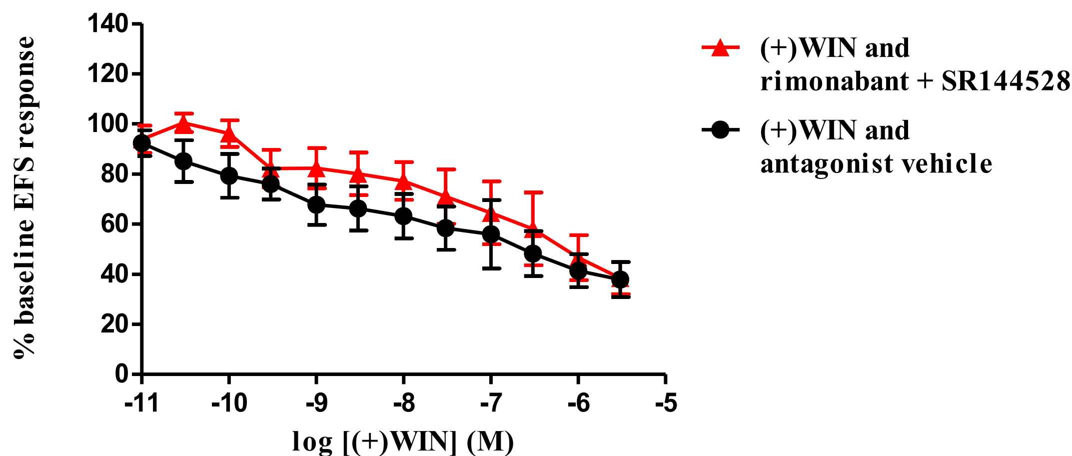


Figure 3.21 *The effect of (+)WIN on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1μM rimonabant+SR144528 (n=4) and antagonist vehicle (0.02% ethanol; n=5). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of (+)WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by one way ANOVA. The same antagonist vehicle control was used in several (+)WIN experiments.*

The effect of (-)WIN on EFS-evoked contractions

(-)WIN did not significantly reduce contraction size at any concentration (see figure 3.22 overleaf). The ethanol vehicle control caused a small reduction in contraction size at volumes required to achieve (-)WIN concentrations of $3x10^{-10}$, $1x10^{-8}$, $3x10^{-7}$, $1x10^{-6}$and $3x10^{-6}$M. The largest reduction in contraction size by the ethanol vehicle was 16.8 ± 1.5% (*P* <0.05), exerted by the volume required to achieve a (-)WIN concentration of $1x10^{-8}$M.

(-)WIN increased contraction size at $3x10^{-10}$ and $1x10^{-8}$M ($P < 0.05$) compared to the ethanol vehicle alone. Contractions size was measured as 101.2 ± 4.3% of baseline response in the presence of (-)WIN $3x10^{-10}$ M and only 85.3 ± 2.3% in the presence of the ethanol vehicle alone. Similarly, in the presence of $1x10^{-8}$M (-)WIN, contraction size was measured as 96.2 ± 3.5% of baseline response but only 83.2 ± 1.5% in the presence of ethanol vehicle alone.

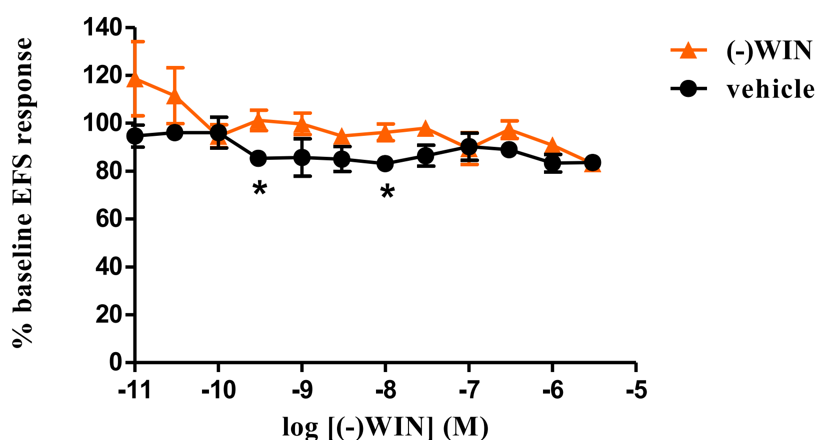


Figure 3.22 *The effect of (-) WIN (n=3) and ethanol vehicle (n=3) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of (-)WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05 (unpaired Student's t test). The concentrations of ethanol vehicle used ranged from 0.02-0.33% (shown as respective (-)WIN concentrations* 10^{-11}- $3x10^{-6}$*M).*

The effect of ACPA on EFS-evoked contractions

ACPA had no significant effect on the magnitude of EFS-evoked contractions of the ileum at concentrations of up to 10^{-4}M (see figure 3.23). Tocrisolve™ (the agonist vehicle) had no significant effect on the magnitude of EFS-evoked concentrations.

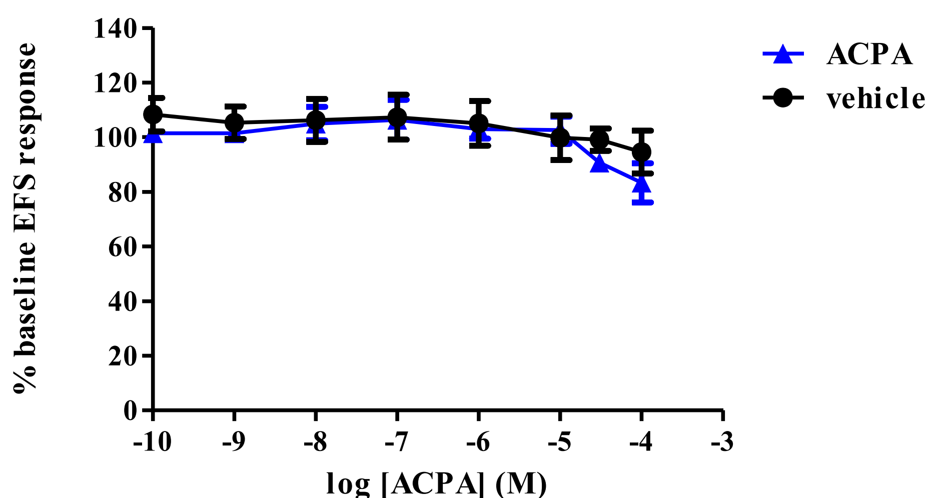


Figure 3.23 *The effect of ACPA (n=4) and Tocrisolve™ vehicle (n=4) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of ACPA. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by unpaired Student's t test. The concentrations of Tocrisolve™ vehicle used ranged from $2x10^{-6}$-0.33%. (shown as respective ACPA concentrations 10^{-10}- 10^{-4}M).*

The effect of abnormal-cannabidiol on EFS-evoked contractions

The effect of abnormal-cannabidiol (abnormal-CBD) on EFS-evoked contractions was found to vary considerably. When abnormal-CBD was initially tested, it exhibited a concentration-dependent reduction in the size of EFS-induced contractions of the ileum (IC_{25}: 9.8 (1.0 - 97) x 10^{-10}M). The compound had no effect at concentrations below $3x10^{-10}$M but caused 58.3 ± 7.3%, ($P < 0.01$) inhibition of EFS-evoked

contractions at the highest concentration of 3µM (see figures 3.24a and 3.25a overleaf). The ethanol vehicle had no significant effect on the size of contractions.

However, when abnormal-CBD was subsequently tested, it had no significant effect on EFS-evoked contractions up to $3x10^{-5}$M (see figures 3.24b and 3.25b overleaf). The ethanol vehicle had no significant effect when added in low volumes but inhibited contractions at the higher volumes required to achieve concentrations of abnormal-CBD ≥ 1µM. The ethanol vehicle reduced contractions by 22.2 ± 2.9 % (P <0.01) at the highest volume added, which was required to achieve an abnormal-CBD concentration of $3x10^{-5}$M.

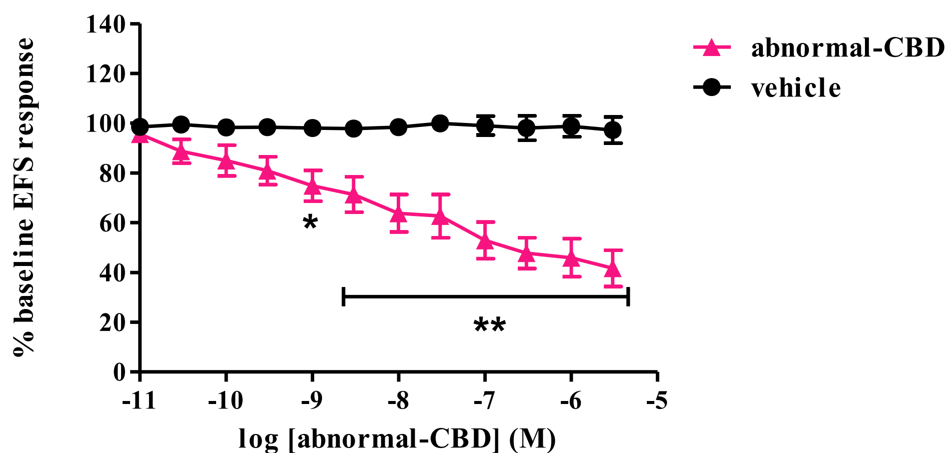


Figure 3.24a *The effect of abnormal-CBD (n=5) and ethanol vehicle (n=5) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of abnormal-CBD. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05, **P< 0.01 (one way ANOVA with post-hoc Dunnett's test). The concentrations of ethanol vehicle used ranged from 0.02-0.33% (shown as respective abnormal-CBD concentrations 10^{-11}-$3x10^{-6}$M). The same vehicle control was used in several cannabinoid experiments.*

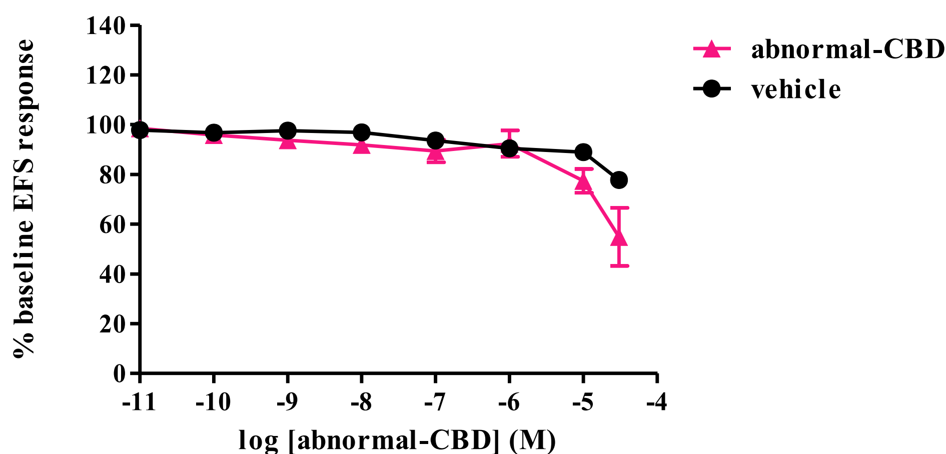


Figure 3.24b *The effect of abnormal-CBD (n=6) and ethanol vehicle (n=4) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of abnormal-CBD. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by unpaired Student's t test. The concentrations of ethanol vehicle used ranged from 0.02-0.29% (shown as respective abnormal-CBD concentrations 10^{-11}-$3x10^{-6}$M).*

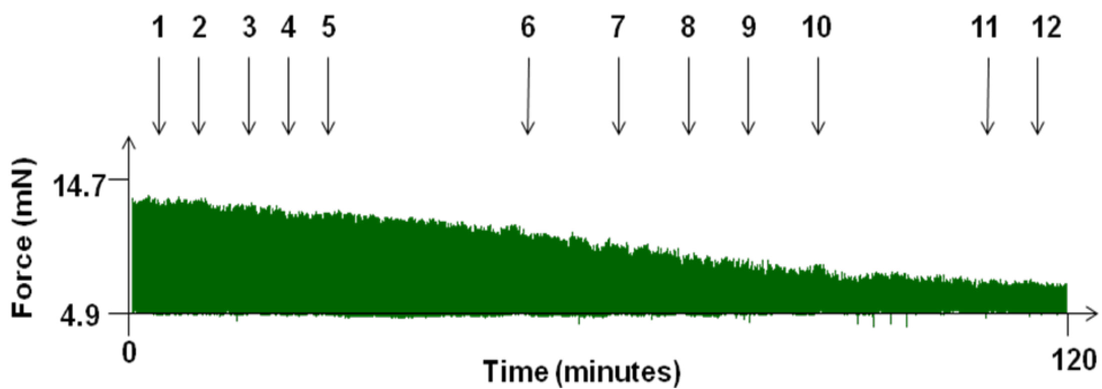


Figure 3.25a *Trace showing the effect of abnormal-CBD on EFS-induced contractions of the guinea-pig isolated ileum. Concentrations (M) shown below:*

1= $1x10^{-11}$	**4**= $3x10^{-10}$	**7**= $1x10^{-8}$	**10**= $3x10^{-7}$
2= $3x10^{-11}$	**5**= $1x10^{-9}$	**8**= $3x10^{-8}$	**11**= $1x10^{-6}$
3= $1x10^{-10}$	**6**= $3x10^{-9}$	**9**= $1x10^{-7}$	**12**= $3x10^{-6}$

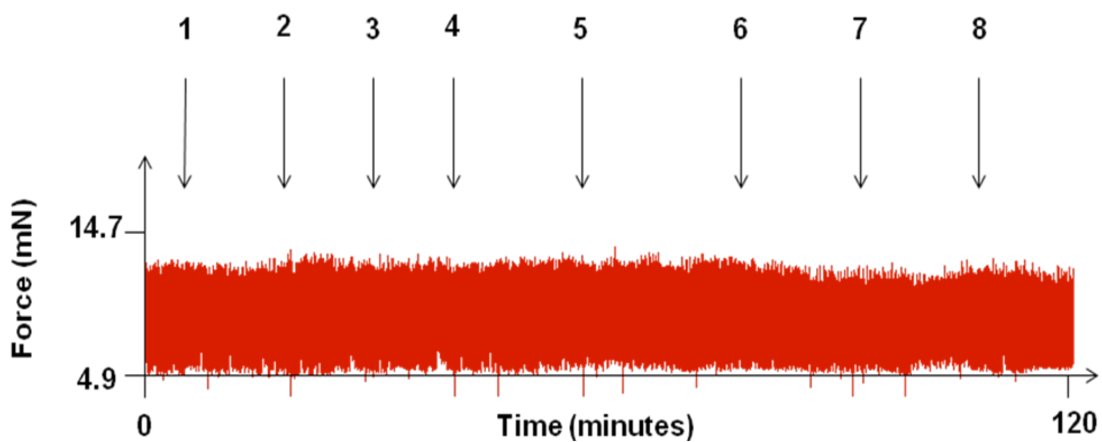


Figure 3.25b *Trace showing the effect of abnormal-CBD on EFS-induced contractions of the guinea-pig isolated ileum. Concentrations (M) shown below:*

1= $1x10^{-11}$	**4**= $1x10^{-8}$	**7**= $1x10^{-5}$
2= $1x10^{-10}$	**5**= $1x10^{-7}$	**8**= $3x10^{-5}$
3= $1x10^{-9}$	**6**= $1x10^{-6}$	

The effect of O-1602 on EFS-evoked contractions

O-1602 caused a concentration-dependent reduction in the size of EFS-induced contractions of the ileum (IC_{25}: 6.7 (0.4 - 123) 10^{-7}M and IC_{50}: 3.1 (1.4 - 7.0) x 10^{-5}M). O-1602 had no effect at concentrations below 10^{-7}M but reached (90.8 ± 1.3%, $P < 0.001$) inhibition at the highest concentration of 10^{-4}M (see figures 3.26 and 3.27). The ethanol vehicle had no significant effect on the EFS-induced response when added in low volumes but reduced contraction size by (16.0 ± 5.8%, $P < 0.05$) at the volume required to achieve an O-1602 concentration of 10^{-5}M.

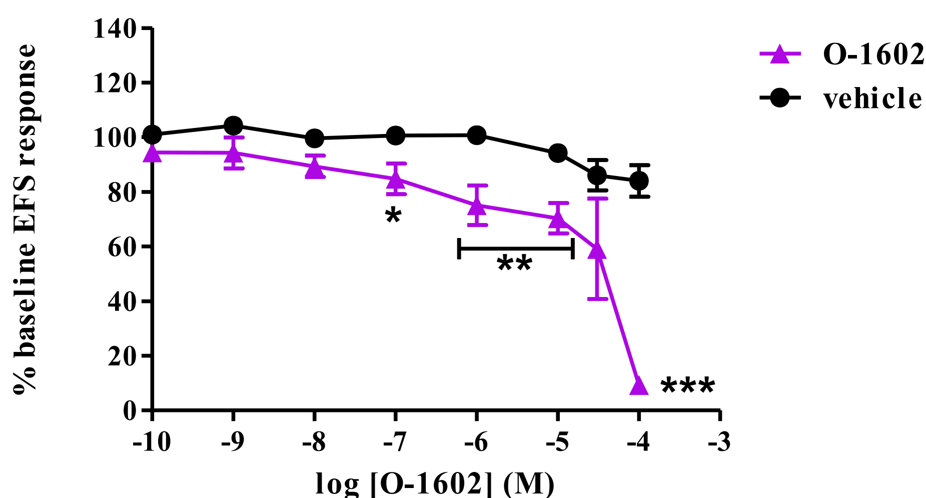


Figure 3.26 *The effect of O-1602 (n=3) and ethanol vehicle (n=4) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of O-1602. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05, **P< 0.01, ***P<0.001 (unpaired Student's t test). The concentrations of ethanol vehicle used ranged from 0.02-0.35% (shown as respective O-1602 concentrations 10^{-10}-10^{-4}M).*

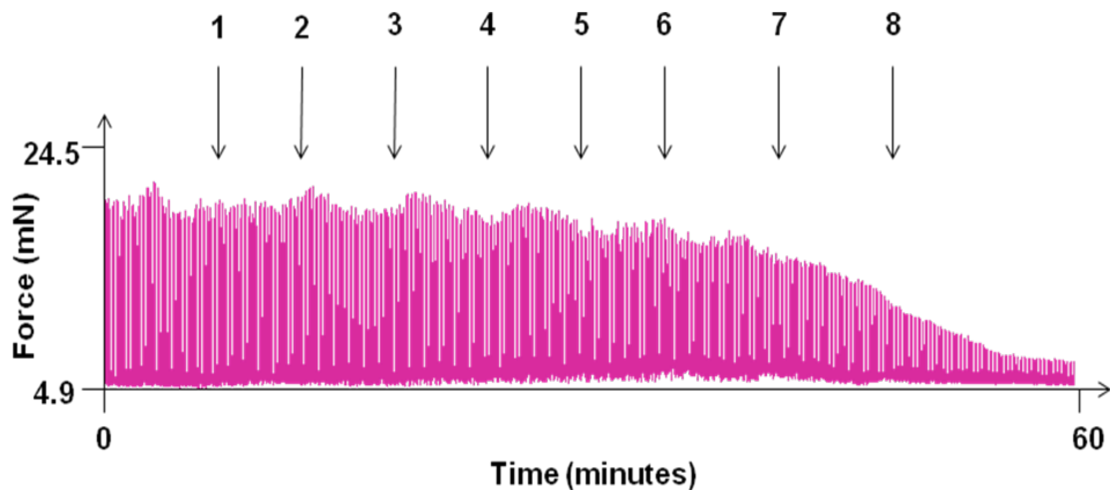


Figure 3.27 *Trace showing the effect of O-1602 on EFS-induced contractions of the guinea-pig isolated ileum. Concentrations (M) shown below:*

1= $1x10^{-10}$ ***4***= $1x10^{-7}$ ***7***= $3x10^{-5}$

2= $1x10^{-9}$ ***5***= $1x10^{-6}$ ***8***= $1x10^{-4}$

3= $1x10^{-8}$ ***6***= $1x10^{-5}$

The effect of rimonabant on O-1602-induced inhibition of EFS-evoked contractions

Rimonabant (1µM) had no significant effect on O-1602-induced inhibition of EFS-evoked contraction (see figure 3.28 overleaf).

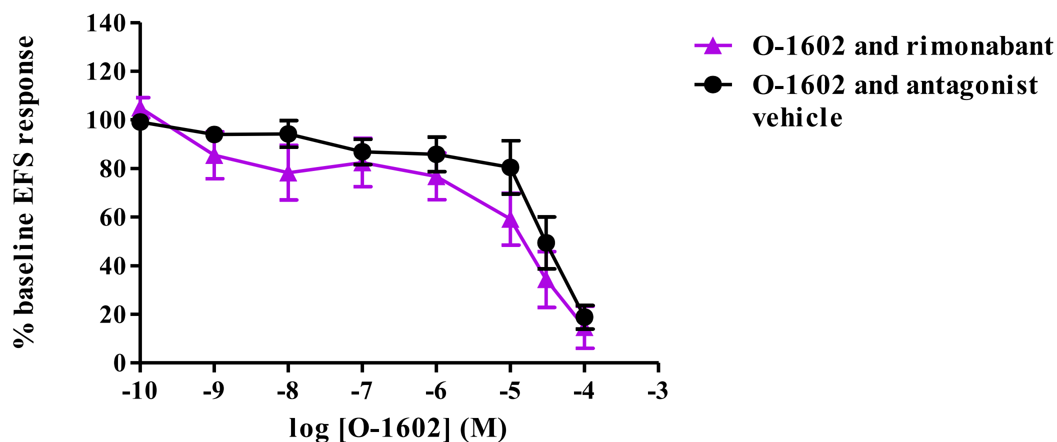


Figure 3.28 *The effect of O-1602 on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1μM rimonabant (n=4) and antagonist vehicle (0.02% DMSO; n=4). The data was expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of O-1602. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by one way ANOVA. The same antagonist vehicle control was used in several O-1602 experiments.*

The effect of AM281 on O-1602-induced inhibition of EFS-evoked contractions

AM281 (1μM) had no significant effect on O-1602-induced inhibition of EFS-evoked contraction (see figure 3.29).

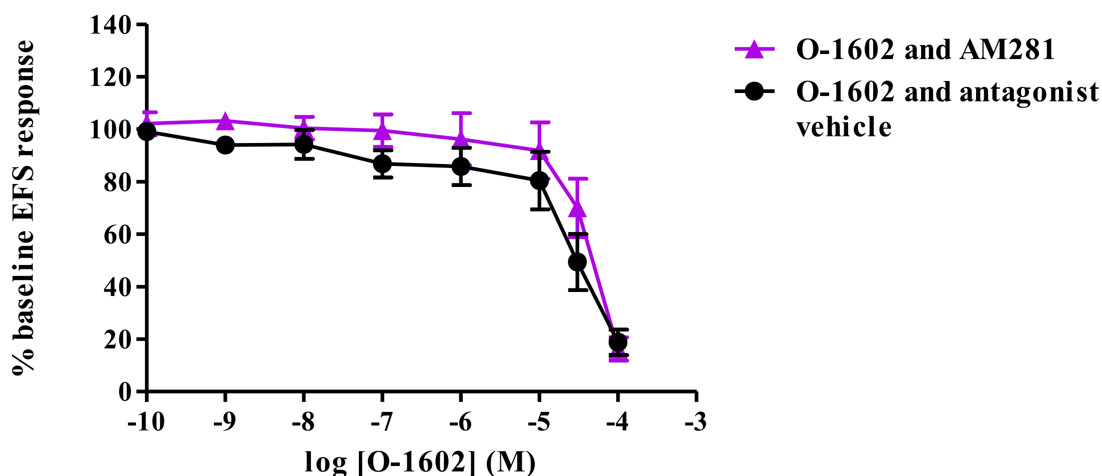


Figure 3.29 *The effect of O-1602 on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1µM AM281 (n=3) and antagonist vehicle (0.02% DMSO; n=4). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of O-1602. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by one way ANOVA. The same antagonist vehicle control was used in several O-1602 experiments.*

The effect of SR144528 on O-1602-induced inhibition of EFS-evoked contractions

SR144528 (1µM) had no significant effect on O-1602-induced inhibition of EFS-evoked contraction (see figure 3.30 overleaf).

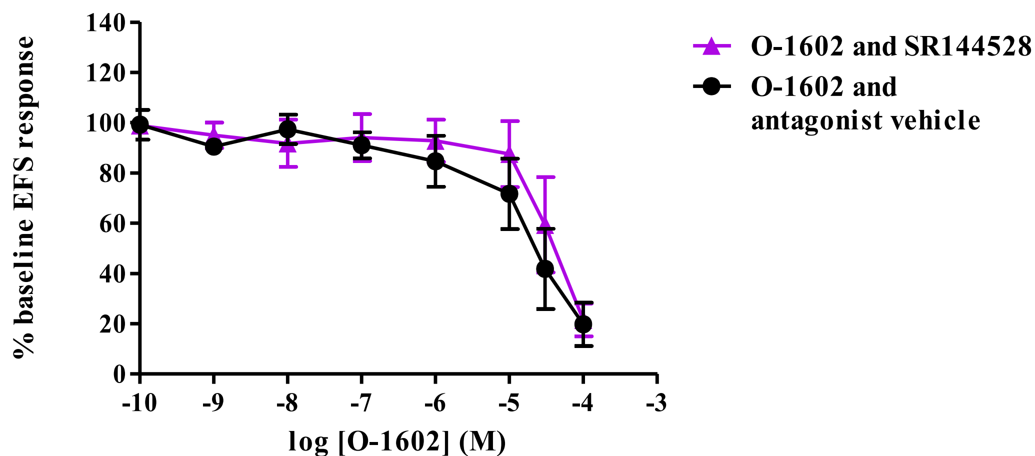


Figure 3.30 *The effect of O-1602 on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1μM SR144528 (n=3) and antagonist vehicle (0.02% ethanol; n=5). The data was expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of O-1602. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data was analysed by one way ANOVA. The same antagonist vehicle control was used in several O-1602 experiments.*

The effect of AM630 on O-1602-induced inhibition of EFS-evoked contractions

AM630 (1μM) had no significant effect on O-1602-induced inhibition of EFS-evoked contraction (see figure 3.31 overleaf).

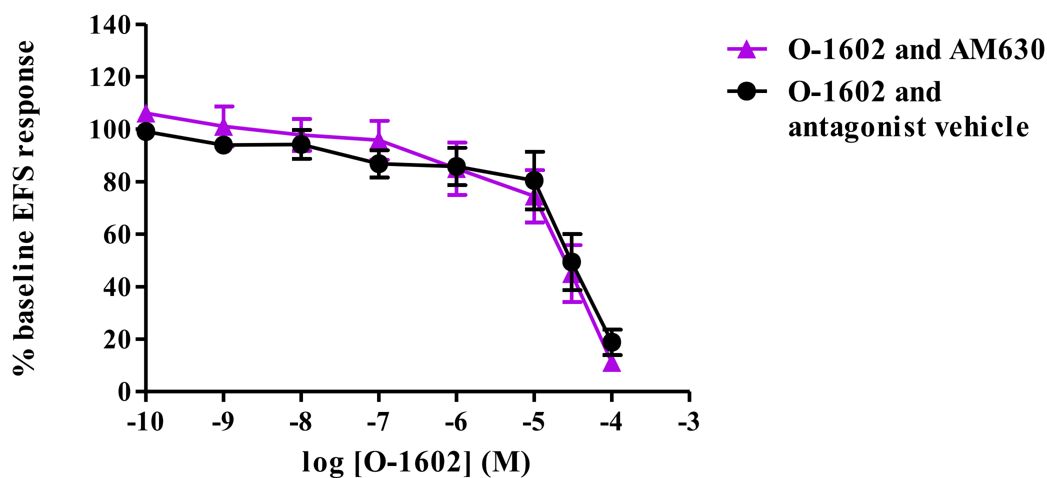


Figure 3.31 *The effect of O-1602 on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1μM AM630 (n=3) and antagonist vehicle (0.02% DMSO; n=4). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of O-1602. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by one way ANOVA. The same antagonist vehicle control was used in several O-1602 experiments.*

The effect of O-1918 on O-1602-induced inhibition of EFS-evoked contractions

O-1918 (1μM) had no significant effect on O-1602-induced inhibition of EFS-evoked contractions (see figure 3.32 overleaf).

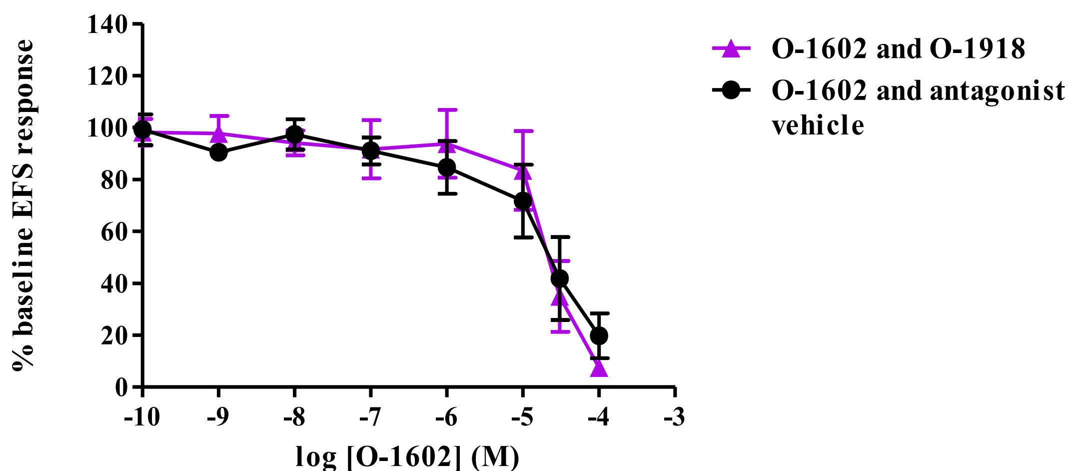


Figure 3.32 *The effect of O-1602 on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 1µM O-1918 (n=3) and antagonist vehicle (0.02% ethanol; n=5). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of O-1602. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by one way ANOVA. The same antagonist vehicle control was used in several O-1602 experiments.*

The effect of (-) CBD on O-1602-induced inhibition of EFS-evoked contractions

(-) CBD (4µM) had no significant effect on O-1602-induced inhibition of EFS-evoked contractions (see figure 3.33 overleaf).

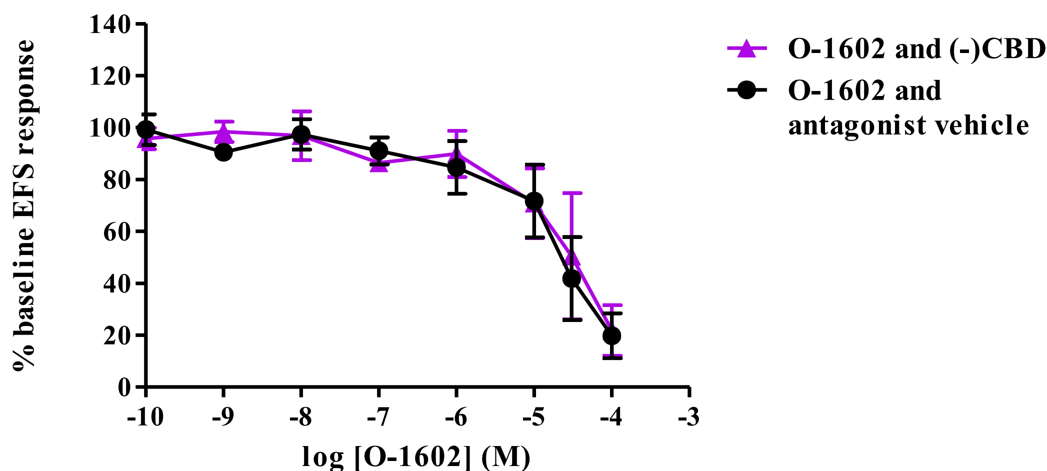


Figure 3.33 *The effect of O-1602 on the size of EFS-induced contractions of the guinea-pig isolated ileum, in the presence of 4µM (-)CBD (n=4) and antagonist vehicle (0.02% ethanol; n=5). The data were expressed as percentage of baseline EFS response. The baseline contraction size was measured after the addition of the antagonist and immediately before the addition of O-1602. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data were analysed by one way ANOVA. The same antagonist vehicle control was used in several O-1602 experiments.*

The effect of PSN on EFS-evoked contractions

PSN caused a concentration-dependent reduction in the size of EFS-induced contractions of the ileum (IC_{25}: 3.9 (2.7 – 5.5) x 10^{-5} M). PSN had no significant effect at concentrations below 10^{-5}M but caused 52.4 ± 6.9 % inhibition ($P < 0.05$) at the highest concentration of 10^{-4}M (see figures 3.34 and 3.35). The ethanol vehicle had no significant effect at any concentration used.

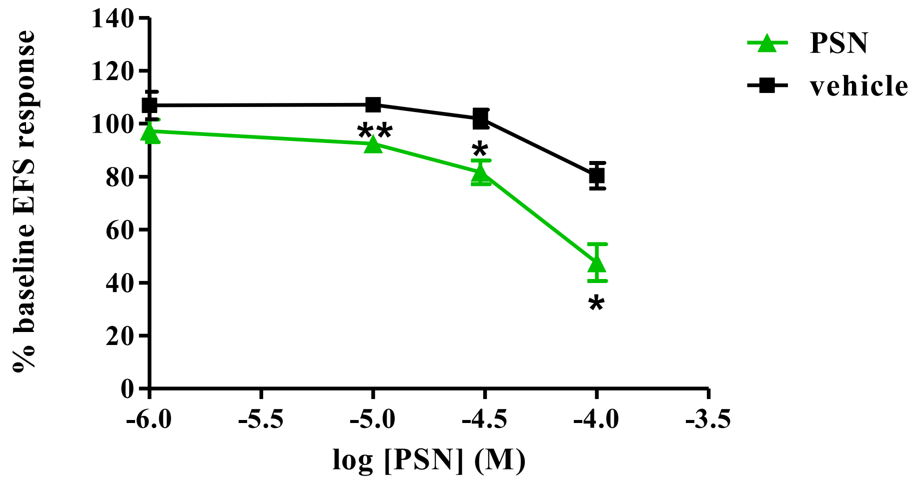


Figure 3.34 *The effect of PSN (n=3) and ethanol vehicle (n=3) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of PSN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05, **P< 0.01, (unpaired Student's t test). The concentrations of ethanol vehicle used ranged from 0.02-0.28% (shown as respective PSN concentrations 10^{-6}-10^{-4}M).*

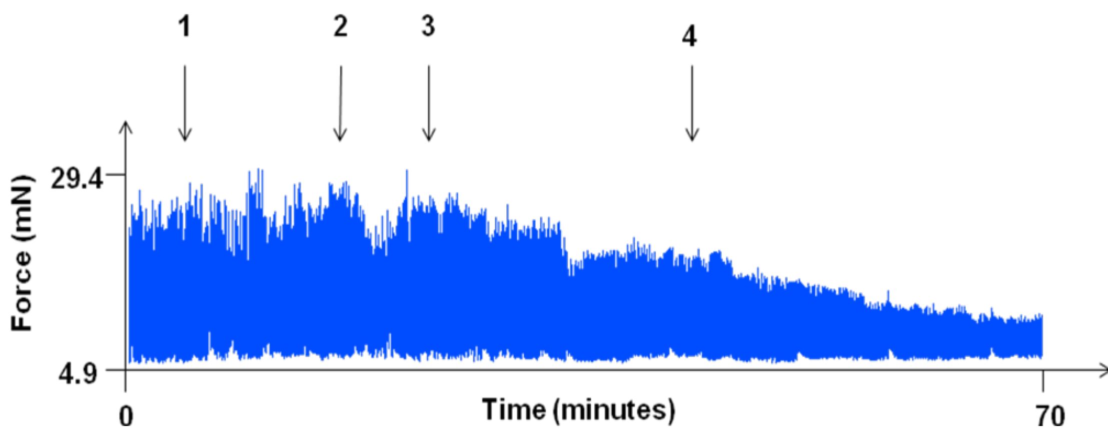


Figure 3.35 *Trace showing the effect of PSN on EFS-induced contractions of the guinea-pig isolated ileum. Concentrations (M) shown below:*

1= $1x10^{-6}$ **3**= $3x10^{-5}$

2= $1x10^{-5}$ **4**= $1x10^{-4}$

3.4.2 The effect of cannabinoid agonists/antagonists on carbachol-induced contractions of the guinea-pig isolated ileum.

The effect of (+)WIN on carbachol-induced contraction

(+)WIN had no significant effect on carbachol-induced contraction of the ileum (see figure 3.36). The ethanol vehicle had no significant effect when added in low volumes but inhibited contractions at the higher volumes required to achieve concentrations of (+)WIN ≥ 1×10^{-7}M. The ethanol vehicle reduced contractions by 37.7 ± 9.0 % (*P* <0.05) at the highest volume added, which was required to achieve 1µM (+)WIN.

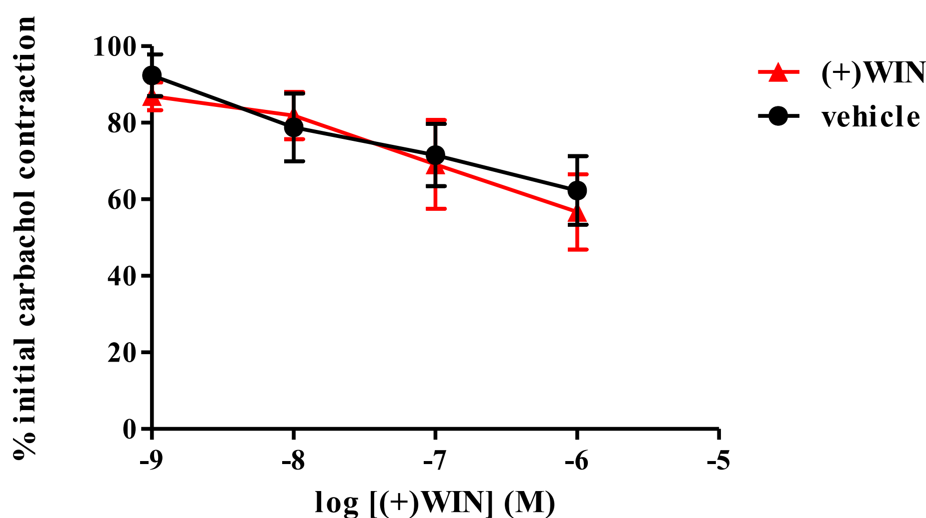


Figure 3.36 *The effect of (+)WIN (n=5) and ethanol vehicle (n=4) on the size of carbachol-induced contraction of the guinea-pig isolated ileum, expressed as percentage of initial carbachol contraction. The baseline contraction size was measured after the addition of carbachol and immediately before the addition of (+)WIN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM), data were analysed by unpaired Student's t test. The concentrations of ethanol vehicle used ranged from 0.02-0.074% (shown as respective (+)WIN concentrations 10^{-9}-10^{-6}M).*

The effect of PSN on carbachol-induced contraction

PSN showed a concentration-dependent reduction of the carbachol-induced contraction (IC_{50}: 1.2 (0.2 – 1.2) x 10^{-5} M, see figures 3.37 and 3.38). PSN showed an effect from 10^{-5}M upwards, abolishing contractions at the highest concentration of 10^{-4}M (110.0 ± 7.3% inhibition, $P < 0.001$). The ethanol vehicle had no significant effect when added in low volumes but inhibited contractions at the higher volumes required to achieve concentrations of PSN ≥ 1x10^{-5}M. The ethanol vehicle reduced contractions by 35.3 ± 7.9 % (P <0.05) at the highest volume added, which was required to achieve a PSN concentration of 1 x 10^{-4}M .

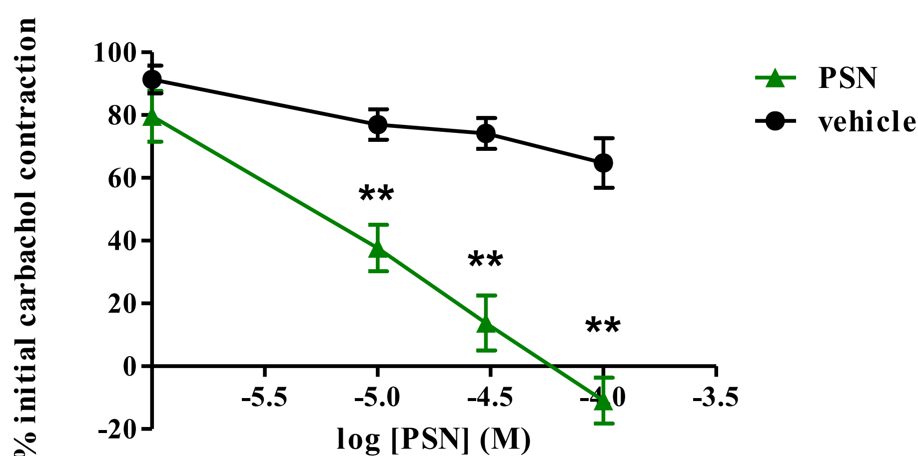


Figure 3.37 *The effect of PSN (n=4) and ethanol vehicle (n=5) on the size of carbachol-induced contraction of the guinea-pig isolated ileum, expressed as percentage of initial carbachol contraction. The baseline contraction size was measured after the addition of carbachol and immediately before the addition of PSN. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM);; * P< 0.05, **P< 0.01(one way ANOVA with post-hoc Dunnett's test).The concentrations of ethanol vehicle used ranged from 0.02-0.28% (shown as respective PSN concentrations 10^{-6}-10^{-4}M).*

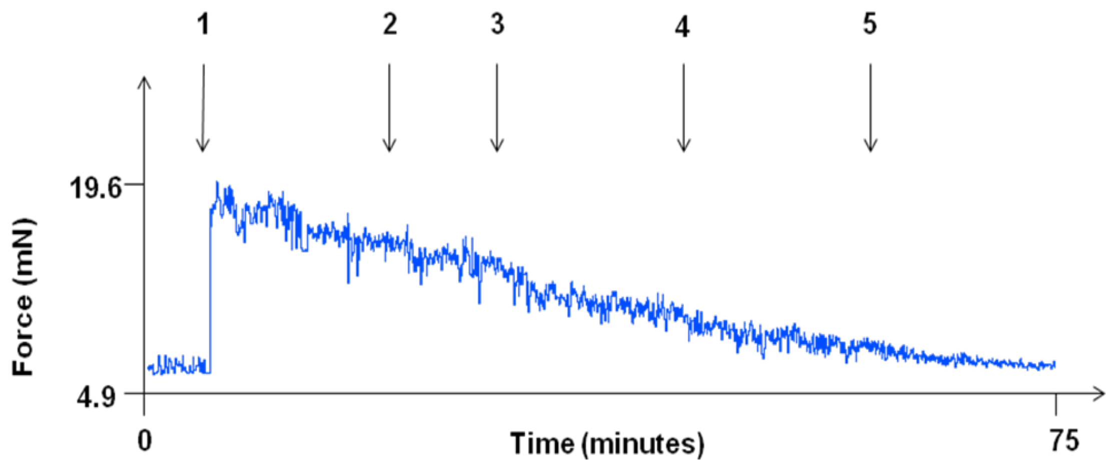


Figure 3.38 *Trace showing the effect of PSN (**2-5**) on carbachol-induced contraction of the guinea-pig isolated ileum. Concentrations (M) shown below:*

1 = *$3x10^{-7}$ carbachol* **4** = *$3\ x10^{-5}$*

2 = *$1\ x10^{-6}$* **5** = *$1\ x10^{-4}$*

3 = *$1\ x10^{-5}$*

The effect of O-1602 on carbachol-induced contraction

O-1602 had no significant effect on carbachol-induced contraction of the ileum (see figure 3.39). The ethanol vehicle had no significant effect when added in low volumes but inhibited contractions at the higher volumes required to achieve concentrations of O-1602 ≥ $1 x 10^{-5}$M. The ethanol vehicle reduced contractions by 35.3 ± 7.9 % ($P <$ 0.05) at the highest volume added, which was required to achieve an O-1602 concentration of 1×10^{-4}M .

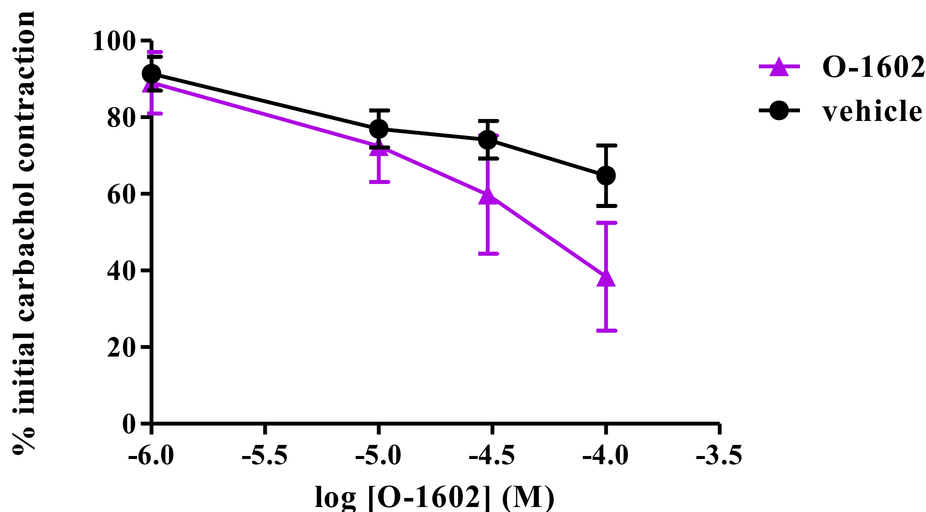


Figure 3.39 *The effect of O-1602 (n=3) and ethanol vehicle (n=5) on the size of carbachol-induced contraction of the guinea-pig isolated ileum, expressed as percentage of initial carbachol contraction. The baseline contraction size was measured after the addition of carbachol and immediately before the addition of O-1602. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM), data were analysed by one way ANOVA. The concentrations of ethanol vehicle used ranged from 0.02-0.28% (shown as respective O-1602 concentrations 10^{-6}-10^{-4}M).*

3.5 Discussion

The main finding of these studies is that cannabinoid-mediated inhibition of ileal contractions was not mediated through the CB_1 receptors, contrary to the currently held view (Colombo *et al*., 1998; Coutts *et al*., 1997; Izzo *et al*., 2000a; Pertwee *et al*., 1996). The cannabinoid responses were not blocked by either of the CB_1 antagonists, rimonabant or AM281, and the selective CB_1 agonist ACPA had no effect on EFS-evoked contractions. Although ACPA does undergo hydrolysis by FAAH (Pertwee., 2010), the concentration added to the bath (1×10^{-4}M) was 50000 times higher than the its Ki (2.2×10^{-9} M; see table 1.1, chapter 1). Therefore, even in the face of significant degradation, a response would still be expected had it been mediated by CB_1 receptors.

One effect, which was consistent with the literature, was that rimonabant or AM281 alone increased contraction size. The literature suggests that this response is due to inverse agonism or antagonism of endogenous cannabinoids at the CB_1 receptor (Pertwee *et al.*, 1996). The inability to be able to elicit a response with a selective CB_1 receptor agonist or of CB_1 selective antagonists to block cannabinoid responses would suggest that, in the guinea-pig ileum, any inverse agonism would not be mediated by CB_1 receptors.

Another important finding is that cannabinoid-mediated inhibition of contractions may be exerted through the GPR119 receptor. Although GPR119 mRNA has been discovered in the intestine, this is the first indication that the receptor may be involved in controlling motility. In this research, the GPR119 agonist PSN was found to produce a concentration-dependent reduction in EFS-induced contractions (IC_{25}: 3.9 (2.7 – 5.5) $\times 10^{-5}$ M), see figure 3.34). The response could be mediated through GPR119 as PSN

causes significant responses at 10^{-5}M, only ten times higher than the EC_{50} reported for PSN at the human GPR119 receptor (~8.4 μM EC_{50} in the yeast fluorimetric assay; Overton *et al.*, 2006). Unfortunately, this cannot be corroborated until a GPR119 antagonist becomes available.

Interestingly, PSN also reduced the carbachol-induced contraction of the guinea-pig ileum (IC_{50}: 1.2 (0.2 – 1.2) x 10^{-5} M, see figure 3.37). This response could be mediated through GPR119 as the IC_{50} is in the same range as the EC_{50} reported for PSN at the GPR119 receptor (micromolar range in the yeast fluorimetric assay; Overton *et al.*, 2006). Although this requires corroboration with a GPR119 antagonist, it suggests that the GPR119 receptor may be present on smooth muscle. This is interesting as cannabinoid receptors are not thought to be expressed on intestinal smooth muscle (Lynn *et al*., 1994; Mang *et al*., 2001; Pertwee., 2001).

The GPR119 receptor may also be present on neurones but this would need to be investigated by measuring neurotransmitter release. The GPR119 receptor does not seem to be involved in the other cannabinoid responses. O-1602 (selective GPR55 agonist) and (+)WIN (non-selective agonist) reduced the size of EFS-evoked contractions but, as (+)WIN does not bind to the GPR119 receptor (see table 1.3, chapter 1) and neither agonist reduced carbachol-induced contraction, it is unlikely that the responses were mediated via GPR119.

The CB_2 receptor does not appear to mediate cannabinoid-mediated inhibition of contractions as O-1602 and (+)WIN responses were not blocked by the selective CB_2 antagonists, AM630 or SR144528. This is consistent with the literature, which suggests that inhibition of guinea-pig ileum contractions is not mediated through the CB_2

receptor (Croci *et al.*, 1998; Izzo *et al.*, 2000a). Blocking the CB_1 and CB_2 receptors simultaneously, with the combination of SR144528 and rimonabant, also did not affect the cannabinoid response.

Similarly, there is no evidence for the involvement of the GPR55 receptor in the cannabinoid responses. In this investigation, the selective GPR55 agonist O-1602 was found to reduce EFS-evoked contractions in a concentration-dependent manner (IC_{25}: 6.7 (0.4 - 123) 10^{-7}M and IC_{50}: 3.1 (1.4 - 7.0) x 10^{-5}M, see figure 3.26). The IC_{50} was approximately 700 times higher than the reported EC_{50} for O-1602 (13 x 10^{-9}M; Ryberg *et al*., 2007) at the GPR55 receptor. Although there is little information concerning the metabolism of O-1602, even if the compound undergoes extensive breakdown, it is unlikely this could account for such a discrepancy between the observed IC_{50} and the reported EC_{50} for the GPR55 receptor. In addition, the GPR55 antagonist (-) cannabidiol did not reduce the O-1602 response. As (+)WIN does not bind to GPR55 (see table 1.2b, chapter 1), this receptor was not responsible for (+)WIN-mediated inhibition of contractions. Together, these results suggest that cannabinoid-responses are not mediated through the GPR55 receptor.

It is unclear whether the putative CB_e receptor is involved in cannabinoid-mediated inhibition of contractions. The effect of the selective CB_e agonist abnormal-CBD was found to vary considerably. When it was initially tested, it reduced EFS-evoked contractions in a concentration-dependent manner (IC_{25}: 9.8 (1.0 - 97) x 10^{-10}M, see figure 3.24a) but, when it was tested subsequently, it had no effect, even at higher concentrations (see figure 3.24b). Due to the variability of abnormal-CBD effects, O-1918 (CB_e antagonist) could not be tested, so it is unclear whether abnormal-CBD acts through the CB_e receptor. It may mediate its effect through this receptor but the

expression may vary between different guinea-pigs. The CB_e receptor is not involved in O-1602 and (+)WIN mediated inhibition of contractions as O-1918 did not block their responses. It is possible that these cannabinoids do not bind to the CB_e receptor but this can only be confirmed once that receptor has been cloned.

As the responses to (+)WIN and O-1602 do not seem to be mediated by any of the cloned or proposed cannabinoid receptors, this suggests the involvement of another receptor or a receptor-independent mechanism. It is possible that the response to (+)WIN observed is not mediated through a receptor as it does not seem to have a traditional sigmoidal concentration-response curve seen for most receptors. This was investigated using the stereoisomer (-)WIN, which does not activate the CB_1 or CB_2 receptor (activation of the receptor is stereoselective); (Savinainen *et al.*, 2005). If the response to (+)WIN was non-receptor mediated, the stereoisomer (-)WIN would have been expected to replicate this response. Instead, (-)WIN did not inhibit EFS-induced contractions of the ileum, suggesting that the response to (+)WIN was mediated by a protein target such as a receptor.

(+)WIN has been shown, at the concentrations used in this thesis, to act on a number of non-cannabinoid receptors. It has been shown to antagonise 5-HT_3 receptors (Pertwee., 2010), and this could be significant as these receptors are known to mediate contraction in the guinea-pig ileum (Fox *et al.*, 1990). (+)WIN also activates PPARα receptors (Pertwee, 2010) known to be present in the intestine (Sanderson *et al.,* 2010), although it is not know whether they affect motility. It is possible that (+)WIN could act on one or several of these receptors/channels to inhibit contractions. As O-1602 is a relatively new compound, less is known about its interactions with non-cannabinoid receptors. It would be useful to investigate the effect of pertussis toxin (which inactivates Gi and Go

G proteins) on the inhibitory effect of O-1602 and (+)WIN in the ileum to give an indication of whether their effects is mediated through a Gi/o protein-coupled receptor.

Another noteworthy result from this study was that (+) WIN was less potent (IC_{25}: 2.8(0.1-56) $x10^{-8}$M) at inhibiting contractions than in the literature (IC_{50}: 5.54 x 10^{-9}M (Pertwee *et al.*, 1996). The reason for this discrepancy is unclear. It may be due to differences in cannabinoid receptor expression between guinea-pig batches or the section of ileum used. It is unlikely that this is due to differences in guinea-pigs as the same species (Dunkin-Hartley) and similar size range was used in this research and the study by Pertwee (1996). Also, currently there is no evidence that cannabinoid receptor expression varies across the length of the ileum. The main difference between this study and the literature is that the whole guinea-pig ileum was used in this study whereas only the longitudinal muscle-myenteric plexus was used in the literature (Pertwee *et al.*, 1996). It is possible that it was more difficult for (+)WIN to access the receptors in the whole ileum as it had to cross through several layers of tissue though, again, this is unlikely as (+)WIN is lipophilic and should be able to cross the ileum. It also seems unlikely that (+)WIN was being broken down as it is resistant to hydrolysis by FAAH (Makwana *et al*., 2010b).

To summarise, this study shows that cannabinoids reduce EFS-evoked guinea-pig ileum contractions and that these effects are not mediated through the CB_1 receptor, contrary to the currently held view (Colombo *et al*., 1998; Coutts *et al*., 1997; Izzo *et al*., 2000a; Pertwee *et al*., 2006). The caveat of previous studies is the reliance on the CB_1 antagonist rimonabant, which is now known not to be as selective as first thought (see 'Non- CB_1/ CB_2 targets for cannabinoid receptor ligands'). This investigation showed

that cannabinoid responses could not be blocked by two different CB_1 antagonists and that a selective CB_1 agonist had no effect on the size of contractions.

This study provides the first evidence that cannabinoids may activate the GPR119 to inhibit contractions and that this receptor may be present on smooth muscle. This is interesting as little evidence has been found this far for any post-synaptic effects of cannabinoids (Lynn *et al*., 1994; Mang *et al*., 2001; Pertwee., 2001). This needs to be corroborated with a GPR119 antagonist, when one becomes commercially available.

Neither the CB_2 receptor nor the GPR55 receptor was involved in the cannabinoid-mediated inhibition of contractions. The CB_e receptor may be implicated but expression of this receptor may vary between animals. None of the known or putative cananbinoid receptors were involved in the responses to O-1602 and (+)WIN. However, the response to (+)WIN seemed to be mediated by a protein target such as a receptor, whereas it is not know whether this is the case for O-1602.

This study has suggested that more extensive work is required to elucidate the mechanism by which cannabinoids intestinal inhibit contractions, since CB_1 does not seem to be involved. Much of this work, however, cannot be carried out until more pharmacological tools are available, for example, a GPR119 receptor antagonist, and until more information is available concerning cannabinoid targets.

CHAPTER 4:

INVESTIGATION OF CANNABINOID-MEDIATED PROTECTION IN LPS AND H_2O_2 MODELS OF INFLAMMATORY DAMAGE IN THE GUINEA-PIG ISOLATED ILEUM

4. *Investigation of cannabinoid-mediated protection in LPS and H_2O_2 models of inflammatory damage in the guinea-pig isolated ileum*

4.1 Introduction

Inflammatory bowel disease (IBD), as discussed in more detail in Chapter one, is characterised by the infiltration of white blood cells (neutrophils and macrophages) into the intestinal mucosa. This activation of these cells results in the production of inflammatory mediators such as reactive oxygen species (ROS), proteases and cytokines which cause the intestinal tissue damage and diarrhoea associated with IBD (Kruidenier *et al.*, 2003; Saksena *et al.*, 2008).

Cannabinoids have been shown in the literature to possess several anti-inflammatory effects such as inhibiting the activity of immune cells (mainly CB_2 mediated, Croxford *et al.*, 2005) and reducing inflammation in animal models of IBD (CB_1 and CB_2 mediated, Kimball *et al.*, 2006). In addition, cannabinoids can aid tissue repair and have been shown to enhance epithelial wound healing in the colon (CB_1 mediated, (Wright *et al.*, 2005).

Cannabinoids have also been shown to protect against inflammatory damage in several tissues (Montecucco *et al.*, 2009) and nerves (Hampson *et al.*, 1998) but their potential to protect the gut has not yet been investigated. The purpose of this chapter was to investigate cannabinoid-mediated protection in LPS and H_2O_2 models of inflammatory damage in the guinea-pig isolated ileum.

H_2O_2 is a ROS which is continually produced by cells under normal conditions, 90% as a by-product of mitochondrial respiration. Leaked electrons from the respiratory electron transport train combine with oxygen to produce superoxide which spontaneously forms H_2O_2 or is converted to H_2O_2 by the enzyme superoxide dismutase (SOD). H_2O_2 is usually immediately neutralised by the antioxidant enzymes catalase and glutathione peroxidase, but the levels of these antioxidants have been reported to be impaired in IBD patients. In addition to this, under inflammatory conditions, H_2O_2 is produced in large amounts by white blood cells and bacteria as well as increased production in intestinal cells (Pravda, 2005).

H_2O_2 in excess crosses cell membranes and reacts with iron to produce the highly reactive hydroxyl radical (Fenton reaction). This chemical reacts with lipids, proteins and DNA, causing tissue damage and apoptosis/necrosis. Several papers report H_2O_2-induced damage to the intestinal epithelium and loss of barrier function (Shin *et al.,* 2010; Yamamoto, 2003). H_2O_2 has also been shown to increase the secretion of Cl^- and inhibit NaCl absorption in the colon, which may contribute to diarrhoea (Saksena *et al.*, 2008). H_2O_2 can also induce intestinal dysmotility and has been shown to inhibit contraction of the sigmoid colonic circular muscle (Cao *et al.*, 2004) and reduce excitability of myenteric neurones which control gastrointestinal motility (Pouokam *et al.*, 2009).

LPS is an inflammatory stimulus present in the cell wall of Gram-negative bacteria such as *E coli.* LPS activates macrophages through binding to toll-like receptor 4, producing inflammatory mediators such as prostaglandins, nitric oxide, cytokines and ROS (Beutler *et al.*, 2003). In the intestine, LPS produces diarrhoea and intestinal dysmotility (Duncan *et al.*, 2008).

LPS has been used to induce inflammation *in vitro* and *in vivo*. Some *in vitro* experiments involved pre-treating animals with LPS and then investigating the contractility of dissected intestinal segments in an organ bath (Gonzalo *et al.,* 2010; Gonzalo *et al.,* 2011; Rebollar *et al*., 2002). Alternatively, intestinal sections were taken from normal animals and set up in an organ bath; LPS was added to the organ bath and the contractility measured (Rebollar *et al*., 2002).

In vitro, LPS has been shown to inhibit contractions of duodenal longitudinal and circular muscle (Grasa *et al*., 2008), jejunal circular muscle (Eskandari *et al*., 1999) and ileal circular muscle (Rebollar *et al*., 2002). Prostaglandins and nitric oxide have been implicated in this inhibition (Chen *et al*., 2010; Grasa *et al*., 2008). LPS was also found to have a direct inhibitory effect on human colon smooth muscle cell contractility (Scirocco *et al*., 2010).

The effects of LPS *in vivo* are conflicting. LPS has been shown to reduce gastrointestinal transit in some studies in mice (Chen *et al*., 2010; Liang *et al*., 2005) whilst other studies have reported that LPS increases gastrointestinal transit and diarrhoea in rats (Duncan *et al*., 2008). CB_1 and CB_2 agonists were found to reduce gastrointestinal motility in mice (Li *et al*., 2010) and rats (Duncan *et al*., 2008) treated with LPS. The effect of cannabinoids was the same, regardless of whether LPS increased or decreased gastrointestinal motility.

The cannabinoids tested in the *in vitro* LPS and H_2O_2 models have already been described in chapter 3. The damaging effects of LPS and H_2O_2 and the protective effects of cannabinoids were assessed by measuring tissue contractility.

4.2 Aims

- To investigate the protective effects of cannabinoids in models of LPS and H_2O_2 inflammatory damage in the guinea-pig isolated ileum.

4.3 Method

The effect of LPS on EFS-induced contractions of the guinea-pig isolated ileum

The ileum was prepared as in Chapter 2 and was subjected to EFS (see 'Electrical field stimulation of the guinea-pig ileum', Chapter 2). Once contractions had stabilised, LPS was added to the bath and left for 2 hours before washout. Contraction size was measured before the addition of LPS and then every 20 minutes for 2 hours after the addition.

The effect of H_2O_2 on EFS-induced contractions of the guinea-pig isolated ileum

The ileum was prepared as in Chapter 2 and was subjected to EFS (see 'Electrical field stimulation of the guinea-pig ileum', Chapter 2). Once contractions had stabilised, 0.3% H_2O_2 was added to the bath for 1 minute. This concentration and time period was chosen as it was found to substantially reduce but not completely abolish contractions.

The effect of cannabinoid agonists/antagonists on H_2O_2-induced damage of the isolated ileum

In the first set of experiments, the ileum was prepared as in chapter 2 and was subjected to EFS (see 'Electrical field stimulation of the guinea-pig ileum', chapter 2). Once contractions had stabilised, the cannabinoid agonist/antagonist or ethanol vehicle was added as a 10µl or 100µl bolus dose for 30 minutes. The bath was then washed out and H_2O_2 was added to the bath for one minute before washout. The ileum was then left to recover for 2 hours (see figure 4.1). Contraction size was measured before the addition of H_2O_2 and every 20 minutes after the addition. The concentration of agonist and vehicle used are shown in the results section 4.4. Drugs were not re-added after washouts as it was shown that the drugs could not be removed even with multiple washes (see appendix 1).

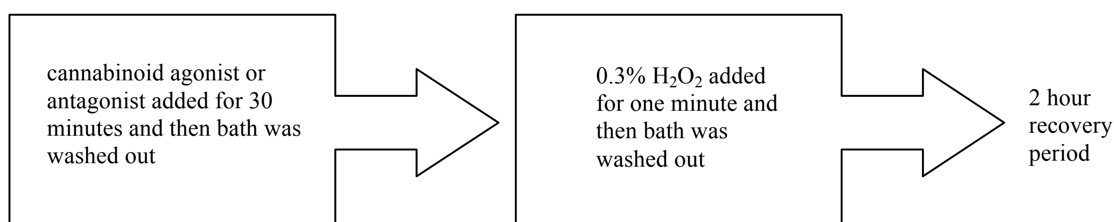


Figure 4.1 *Protocol for H_2O_2 assay with cannabinoid agonists or antagonists alone*

The second set of experiments was carried out in the same way except that the ileum was incubated with the cannabinoid antagonist for 30 minutes before the addition of the cannabinoid agonist (see figure 4.2 overleaf).

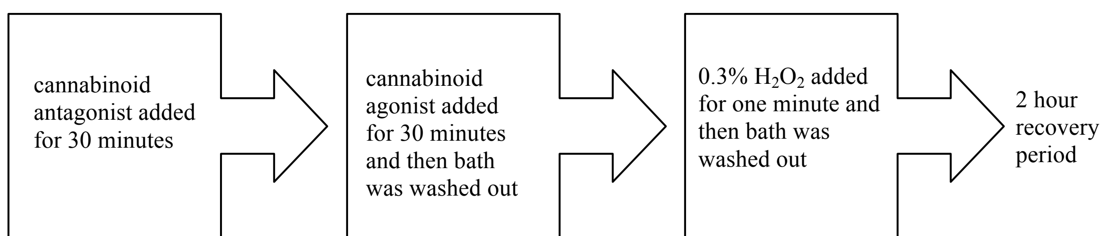


Figure 4.2 *Protocol for H_2O_2 assay with antagonist followed by agonist.*

Drug solutions

Most drugs were dissolved in absolute ethanol ((+)WIN, PSN, SR144528, rimonabant and O-1918. Abnormal-CBD and O-1602 were supplied pre-dissolved in methyl acetate. Due to methyl acetate causing significant depression of ileum contraction, the solvent was evaporated under vacuum using a rotary evaporator and replaced with absolute ethanol. Aliquots of drug solutions were frozen at -20˚C (for up to one month) and were thawed and diluted to the desired concentration on the day of the experiment.

Data analysis

The effect of each drug on recovery of contraction subsequent to H_2O_2 exposure was reported as a percentage of baseline EFS response at 20 minute intervals. Area under the curve was calculated for each drug and its vehicle. Data was reported as mean ± SEM.

Data were excluded if drugs did not produce a pharmacological inhibition of EFS-induced contractions response. The reason for this was that some cannabinoids did not always produce a pharmacological response (see abnormal-CBD cannabidiol responses, figures 3.25a and b in chapter 3.)

Statistical analysis

Mean values for the drug and its vehicle control were compared using unpaired Student's T test (drug and vehicle) or one way ANOVA with post-hoc Dunnett's Test (multiple drugs and vehicle). One sample Student's t tests were used to determine the effects of the vehicle alone on contractions. $P<0.05$ was considered statistically significant.

4.4 Results

4.4.1 The effect of LPS on EFS-induced contractions

LPS was found to have little effect on EFS-evoked contractions of the ileum (see figure 4.3).

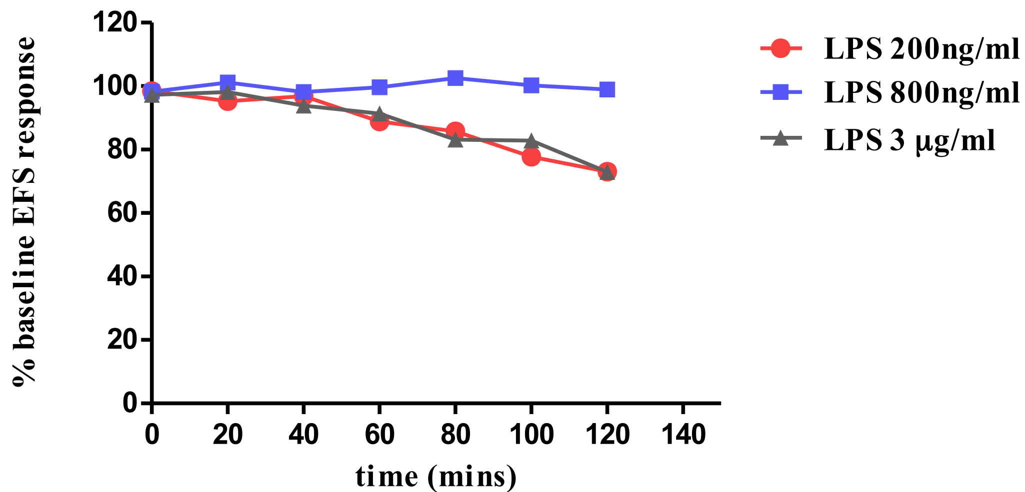


Figure 4.3 *The effect of LPS 200ng/ml (n=1), 800ng/ml (n=2) and 3µg/ml (n=2) on EFS-induced contractions of the guinea-pig isolated ileum, expressed as a percentage of initial EFS response. The baseline contraction size was measured immediately before the addition of LPS. Each symbol represents the mean percentage; data were analysed by unpaired Student's t test.*

4.4.2 The effect of H_2O_2 on EFS-induced contractions of the guinea-pig isolated ileum

Following washout (time: 0 mins), contraction size returned to 20.0 ± 8.4% in H_2O_2-treated tissues, compared to 82.2 ± 4.5% in non-treated tissues. After 60 minutes, contraction size plateaued in both treated and untreated tissues, reaching 62.1 ± 6.8% in treated tissues and 102.3 ± 6.5% in untreated tissues. The areas under the curves for

H_2O_2-treated and non-treated tissues were significantly different $P < 0.05$ (Student's unpaired t test) (see figures 4.4 and 4.5 overleaf).

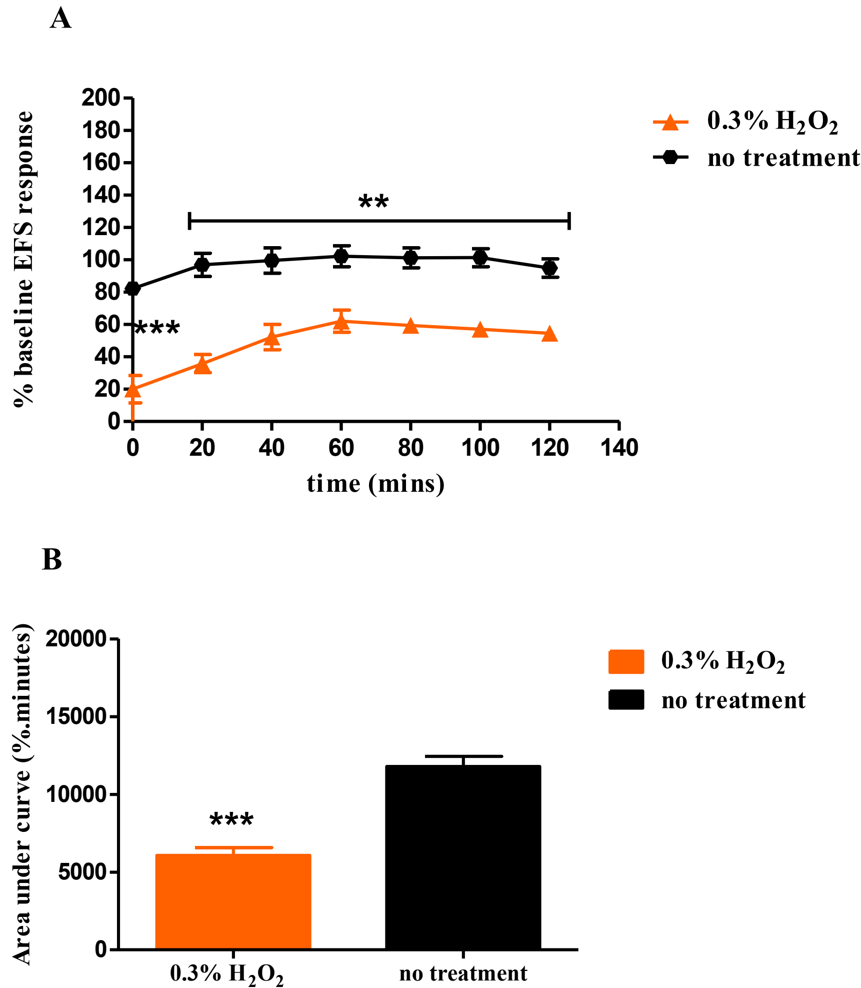


Figure 4.4 *The Recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3%* H_2O_2 *(1 minute exposure; n=3) or washout (no treatment; n=5). The size of EFS-induced contractions is expressed as percentage of initial EFS response* ***(A).*** *The baseline contraction size was measured immediately before the addition of* H_2O_2*. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)****. * P<0.05, **P<0.01, ***P<0.001 (Student's unpaired t test).*

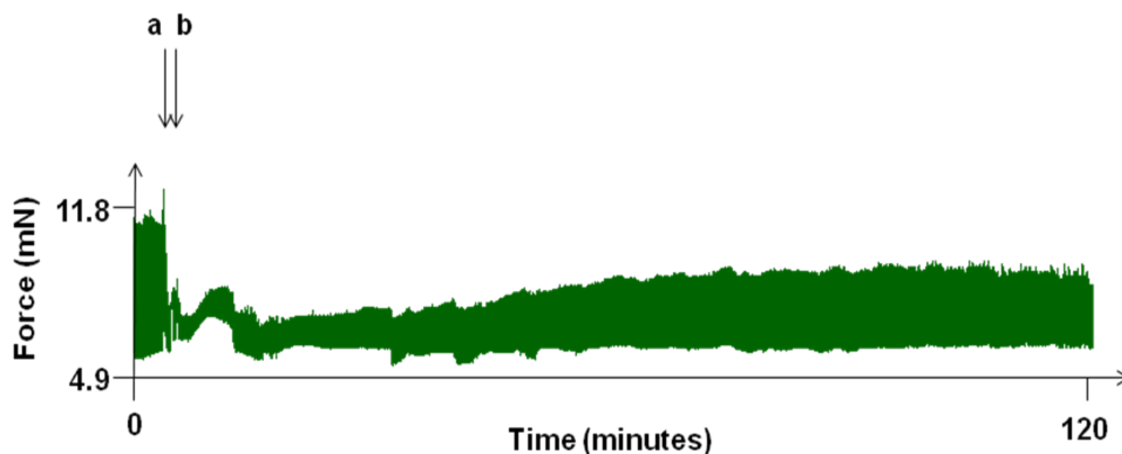


Figure 4.5 *Trace showing the recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure).*
a = *0.3% H_2O_2* , **b** = *washout*.

The effect of cannabinoid agonists/antagonists on H_2O_2-induced damage of the guinea-pig isolated ileum

The effect of (+)WIN on H_2O_2-induced damage of the ileum

Although (+)WIN had no significant effect on the total area under the curve for H_2O_2-treated tissues, at 120 minutes the recovery of contraction in the (+)WIN-treated group was significantly better than vehicle ($P < 0.05$). Contraction returned to 154.6 ± 31.0% of baseline response in the presence of (+)WIN and only 70.3 ± 16.4% in the presence of vehicle alone (see figures 4.6 and 4.7 overleaf). The vehicle alone had no effect on the total area under the curve or on the size of EFS-contractions at any time point.

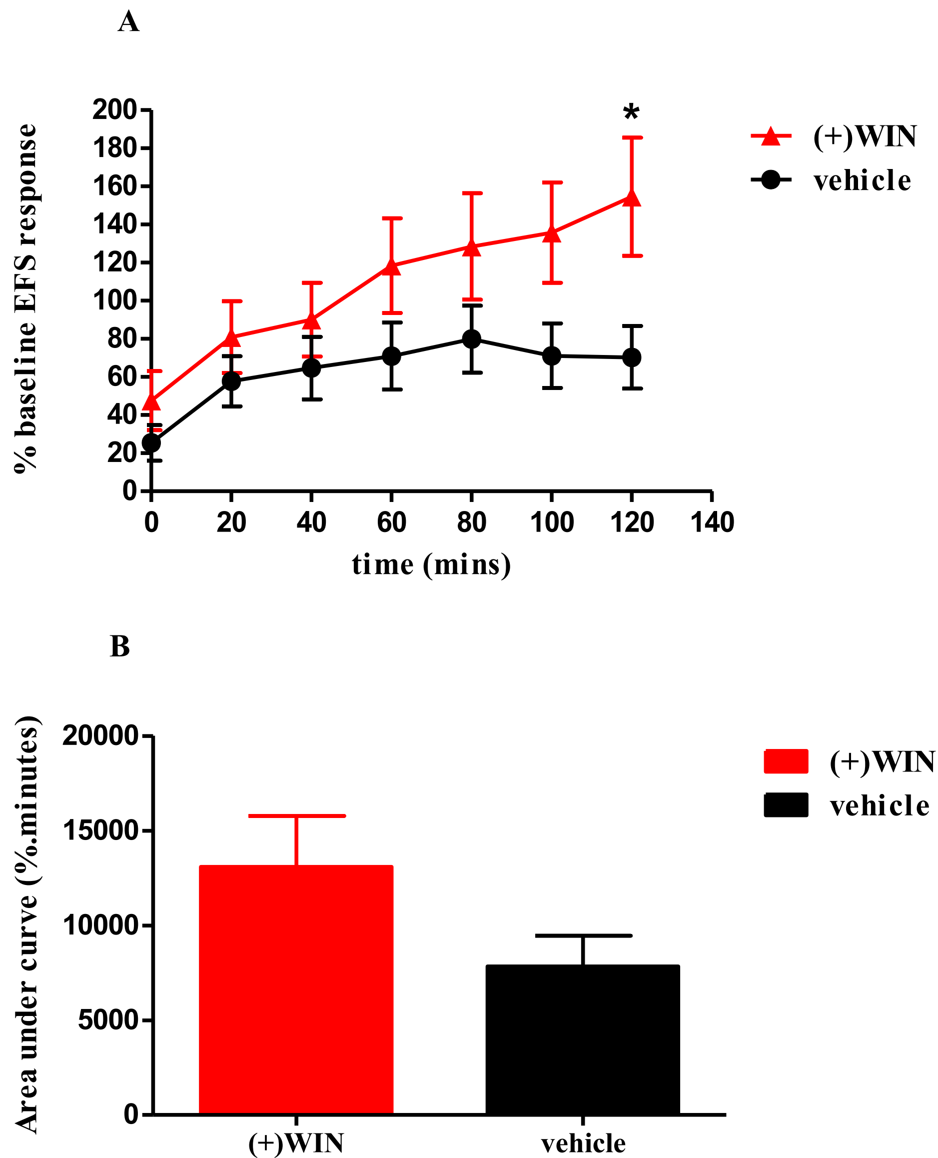


Figure 4.6 *The effect of (+)WIN* 10^{-7}*M (n=7) and ethanol vehicle 0.01% (n=7) on recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3%* H_2O_2 *(1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response* ***(A).*** *The baseline contraction size was measured after the addition of (+)WIN and before the addition of* H_2O_2*. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)****. * P<0.05 (one way ANOVA with post-hoc Dunnett's test). ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

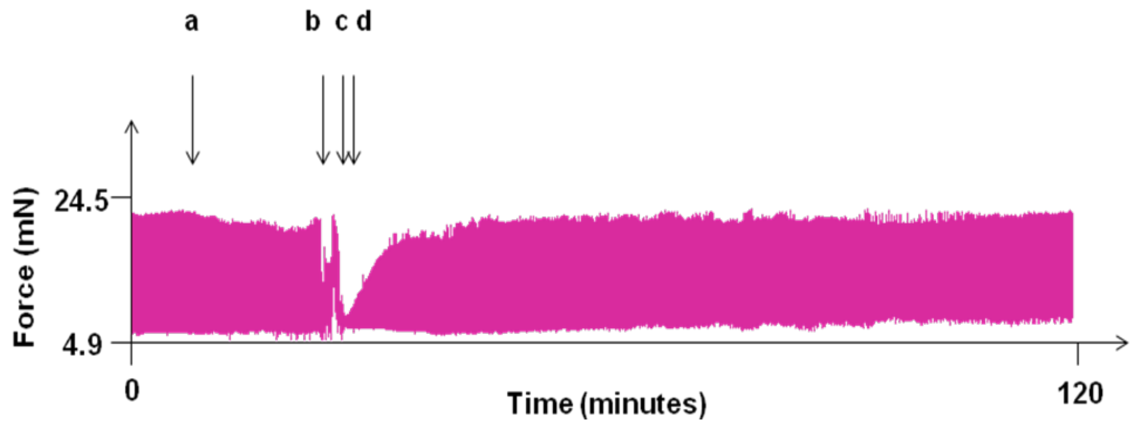


Figure 4.7 *Trace showing the effect of (+)WIN 10^{-7}M on recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure).*

a = *(+) WIN 10^{-7}M* **c** = *0.3% H_2O_2 (1 minute exposure)*

b = *washout* **d** = *washout*

The effect of rimonabant on H_2O_2-induced damage of the ileum

Neither rimonabant (1μM) nor its vehicle control had a significant effect on the total area under the curve for H_2O_2-treated tissues (see figure) or the size of EFS-contractions at any time point (see figure 4.8 overleaf).

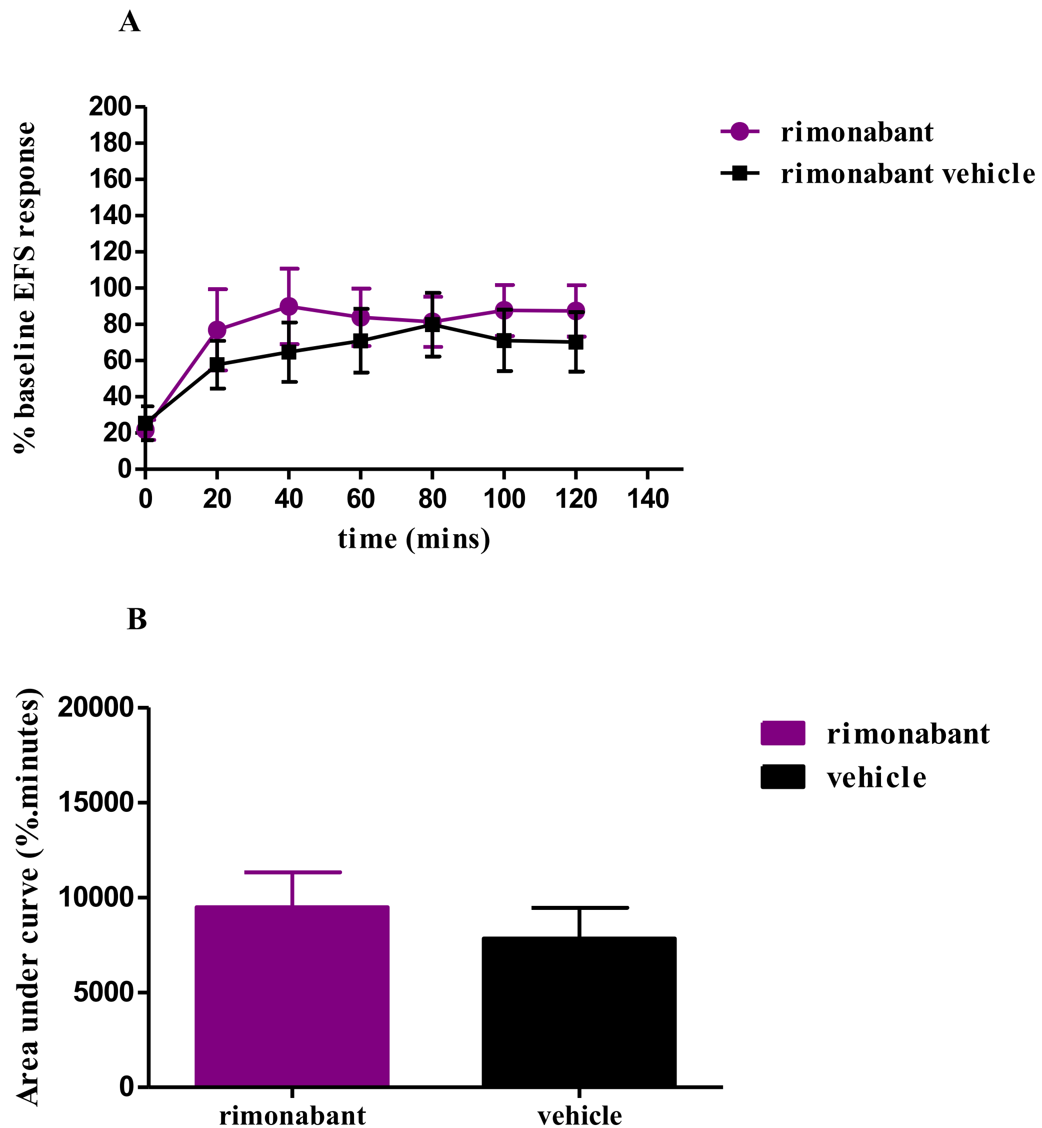


Figure 4.8 *The effect of rimonabant 10^{-6}M (n=6) and ethanol vehicle 0.01% (n=7) on recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response* ***(A).*** *The baseline contraction size was measured after the addition of rimonabant and before the addition of H_2O_2. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)****. Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

The effect of rimonabant on (+)WIN-induced protection against H_2O_2 damage

Rimonabant (1µM) had no significant effect on the area under the curve for (+)WIN-induced contraction recovery in H_2O_2-treated tissues, or on the size of EFS-induced contractions at any time point (see figure 4.9).

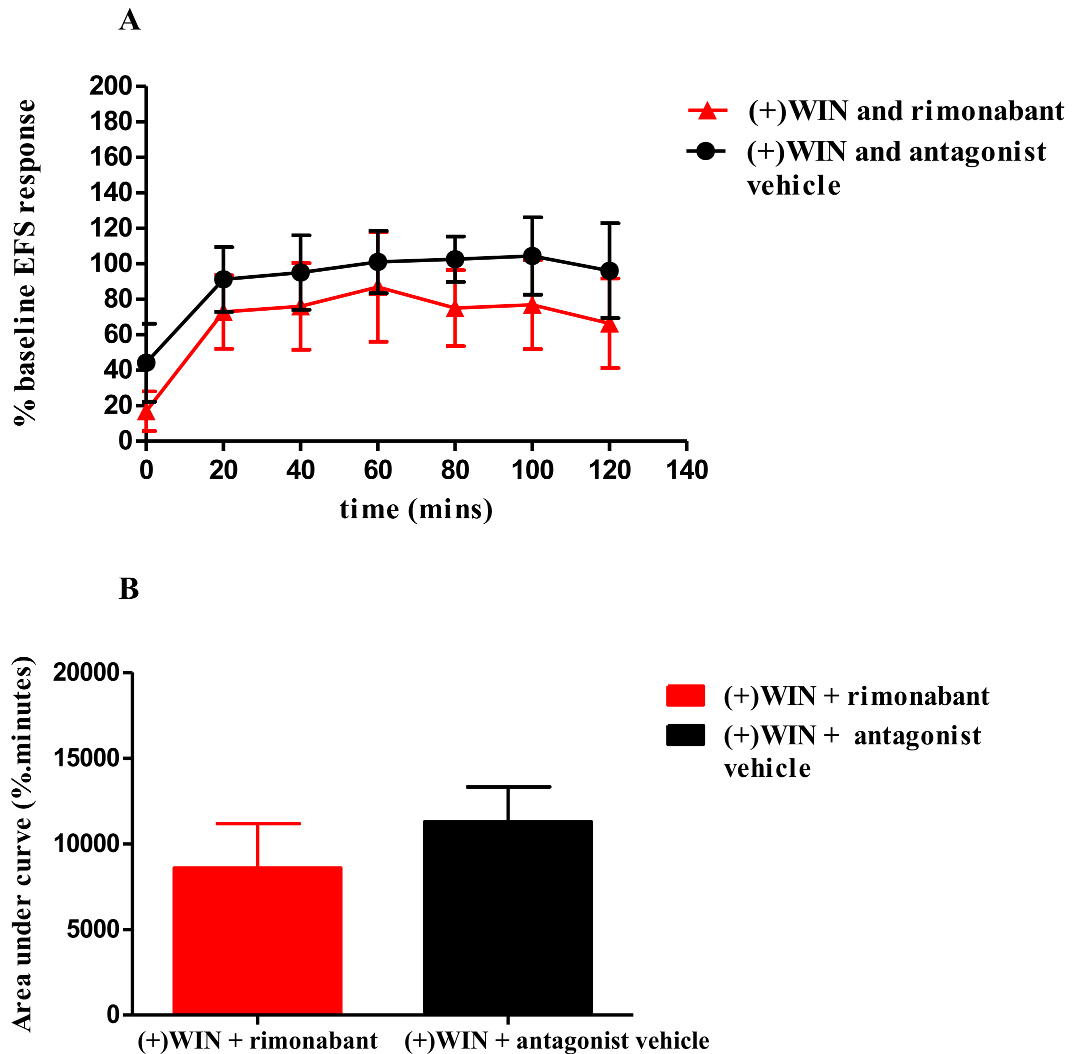


Figure 4.9 *The effect of rimonabant* 10^{-6}*M (n=5) and ethanol vehicle 0.01% (n=4) on (+)WIN-induced protection of the guinea-pig isolated ileum following washout of 0.3%* H_2O_2 *(1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response* ***(A).*** *The baseline contraction size was measured after the addition of (+)WIN and before the addition of* H_2O_2*. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)****. Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

The effect of SR144528 on H_2O_2-induced damage of the ileum

Neither SR144528 (1µM) nor its vehicle control had a significant effect on the total area under the curve for H_2O_2-treated tissues or on the size of EFS-contractions at any time point (see figure 4.10).

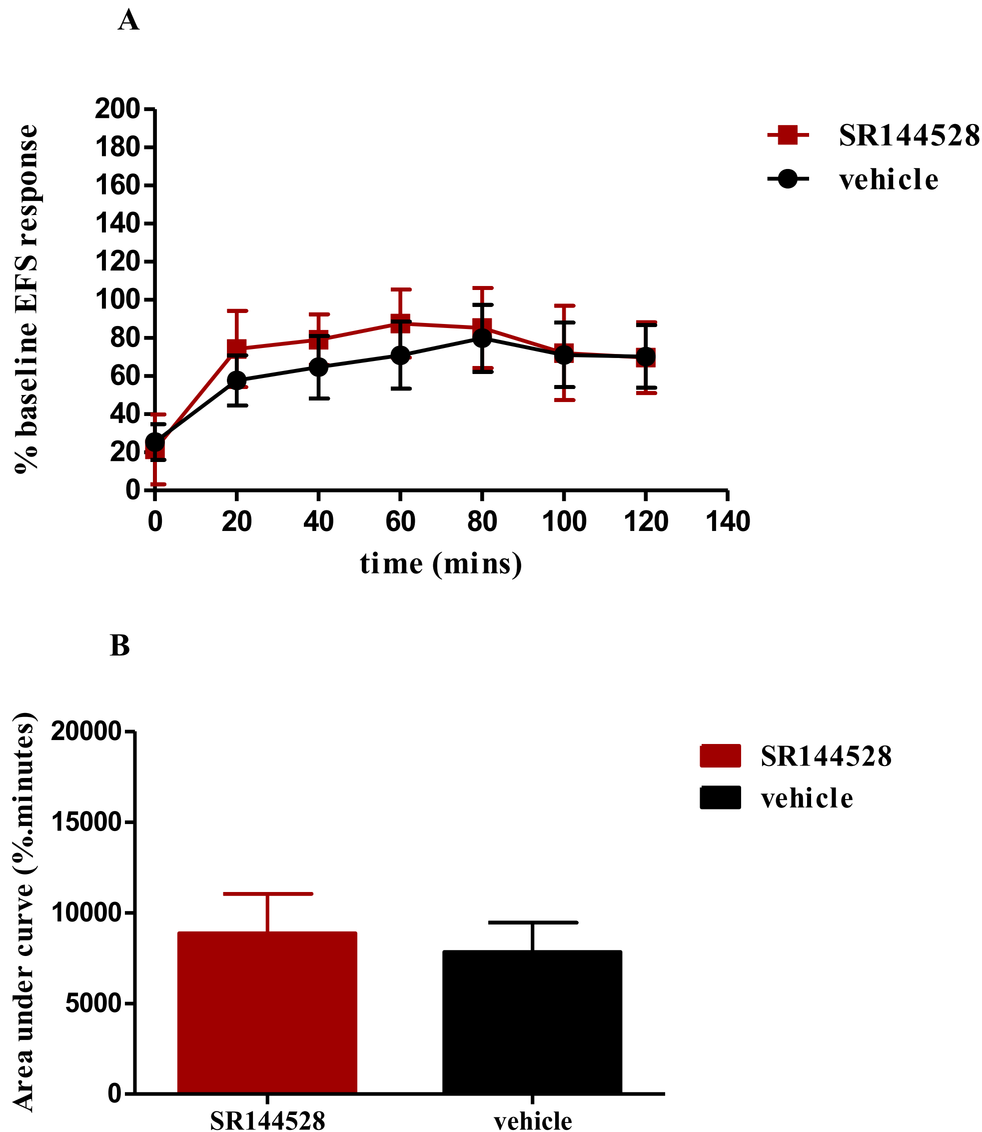


Figure 4.10 *The effect of SR144528 10^{-6}M (n=4) and ethanol vehicle 0.01% (n=7) on recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response* ***(A).*** *The baseline contraction size was measured after the addition of SR144528 and before the addition of H_2O_2. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)****. Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

The effect of SR144528 on (+)WIN-induced protection against H_2O_2 damage

SR144528 (1µM) had no significant effect on the area under the curve for (+)WIN-induced contraction recovery in H_2O_2-treated tissues, or on the size of EFS-induced contractions at any time point (see figure 4.11).

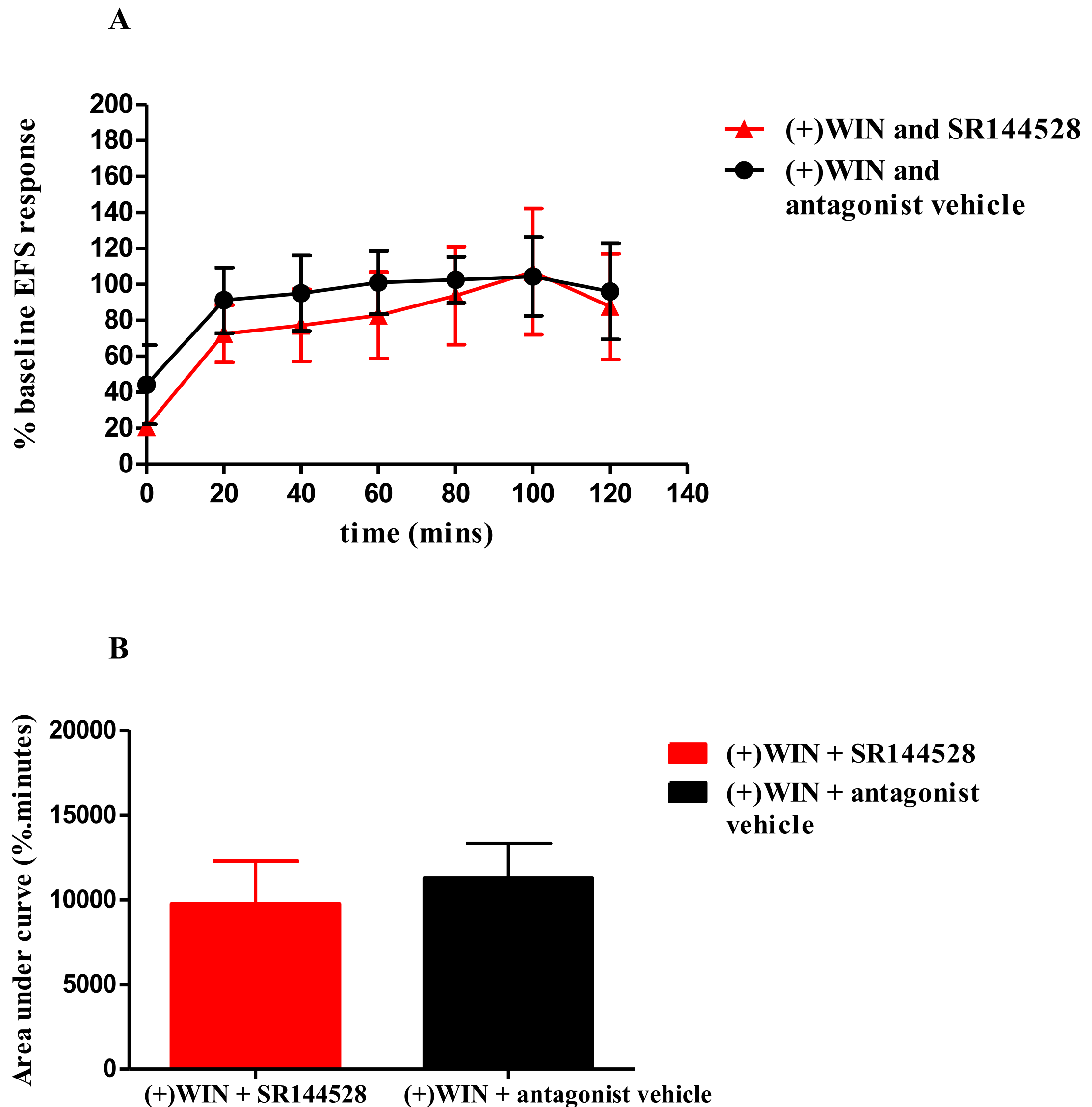


Figure 4.11 *The effect of SR144528 10^{-6}M (n=6) and ethanol vehicle 0.01% (n=4) on (+)WIN-induced protection of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response* ***(A).*** *The baseline contraction size was measured after the addition of (+)WIN and before the addition of H_2O_2. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)****. Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments*

The effect of O-1918 on H_2O_2-induced damage of the ileum

Neither O-1918 (1µM) nor its vehicle control had any effect on the total area under the curve for H_2O_2-treated tissues or on the size of EFS-contractions at any time point. (see figure 4.12).

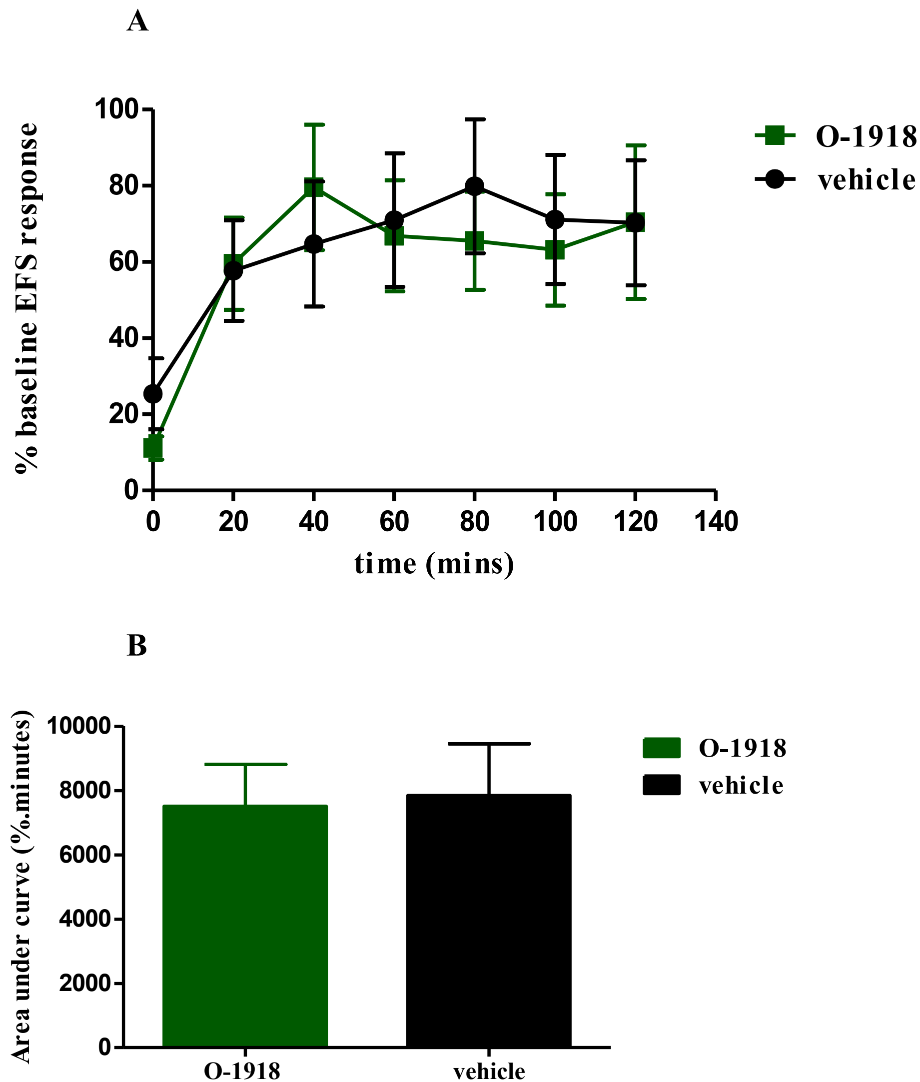


Figure 4.12 *The effect of O-1918 10^{-6}M (n=7) and ethanol vehicle 0.01% (n=7) on recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response **(A)**. The baseline contraction size was measured after the addition of O-1918 and before the addition of H_2O_2. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes **(B)**. Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

The effect of O-1918 on (+)WIN-induced protection against H_2O_2 damage of the ileum

O-1918 (1μM) had no significant effect on the area under the curve for (+)WIN-induced contraction recovery in H_2O_2-treated tissues, or on the size of EFS-induced contractions at any time point (see figure 4.13).

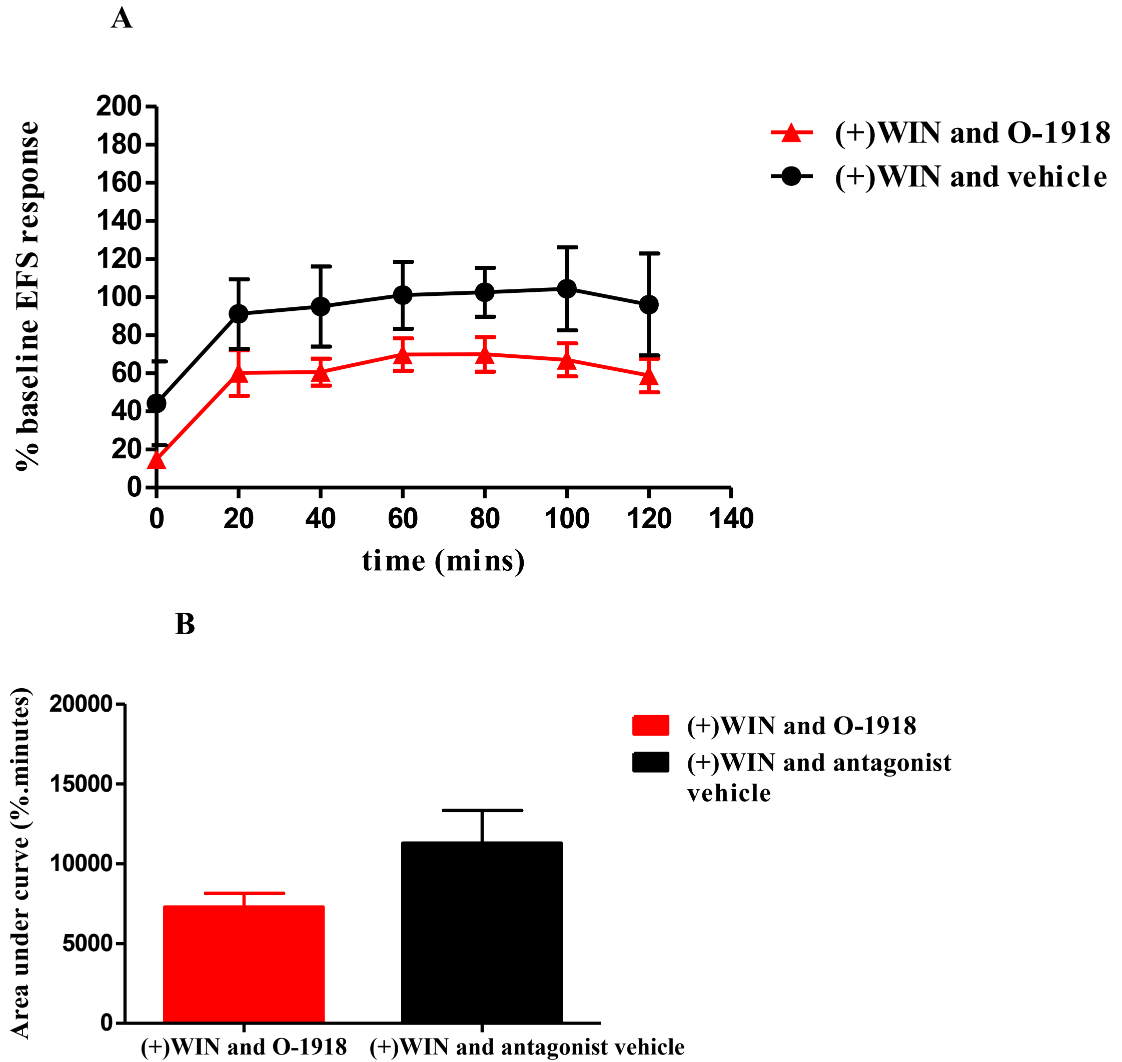


Figure 4.13 *The effect of O-1918 10^{-6}M (n=7) and ethanol vehicle 0.01% (n=4) on (+)WIN-induced protection of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response* ***(A).*** *The baseline contraction size was measured after the addition of (+)WIN and before the addition of H_2O_2. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)****. Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

The effect of ACPA on H_2O_2-induced damage of the ileum

Neither ACPA ($3x10^{-5}$M) nor its vehicle control had a significant effect on the total area under the curve for H_2O_2-treated tissues or on the size of EFS-contractions at any time point (see figure 4.14).

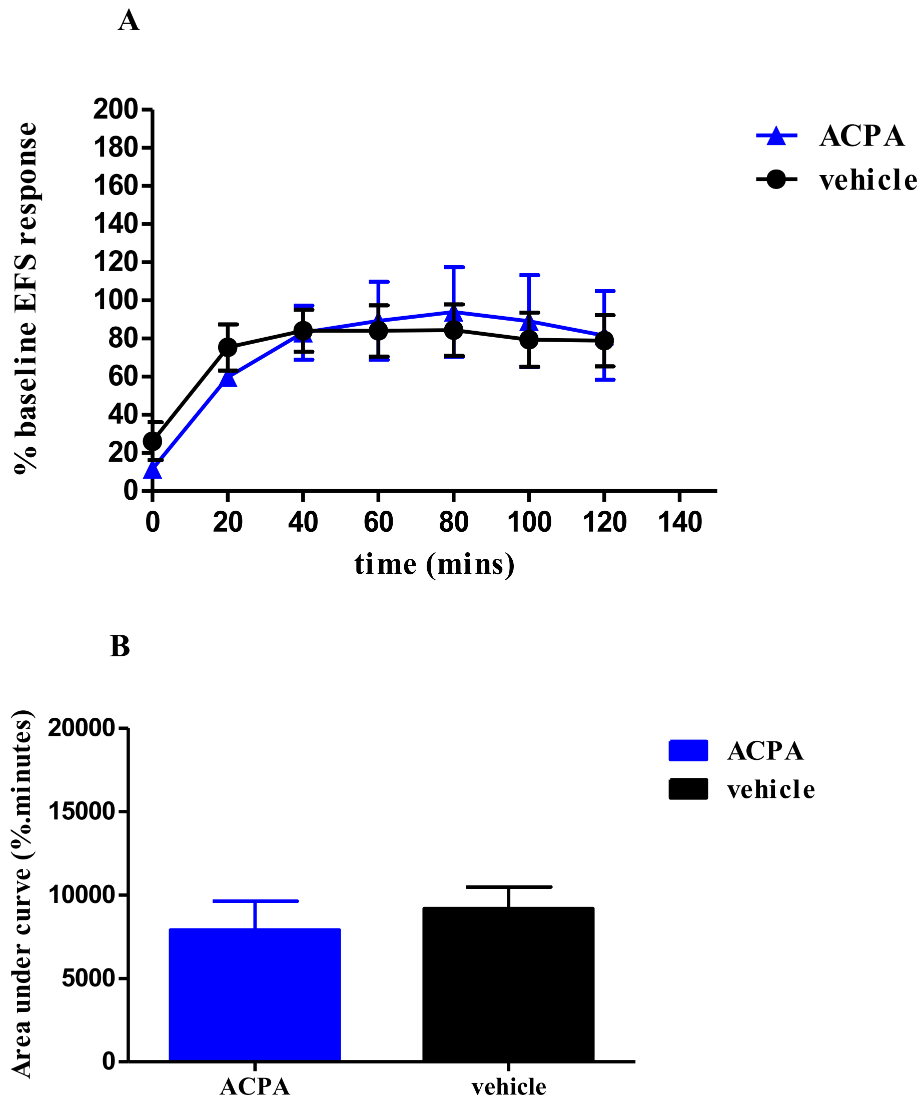


Figure 4.14 *The effect of ACPA $3x10^{-5}$M (n=4) and ethanol vehicle 0.1% (n=6) on recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response **(A)**. The baseline contraction size was measured after the addition of ACPA and before the addition of H_2O_2. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes **(B)**. Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

The effect of abnormal-cannabidiol on H_2O_2-induced damage of the ileum

Neither abnormal-CBD ($3x10^{-5}$M) nor its vehicle control had a significant effect on the total area under the curve for H_2O_2-treated tissues or on the size of EFS-contractions at any time point (see figure 4.15).

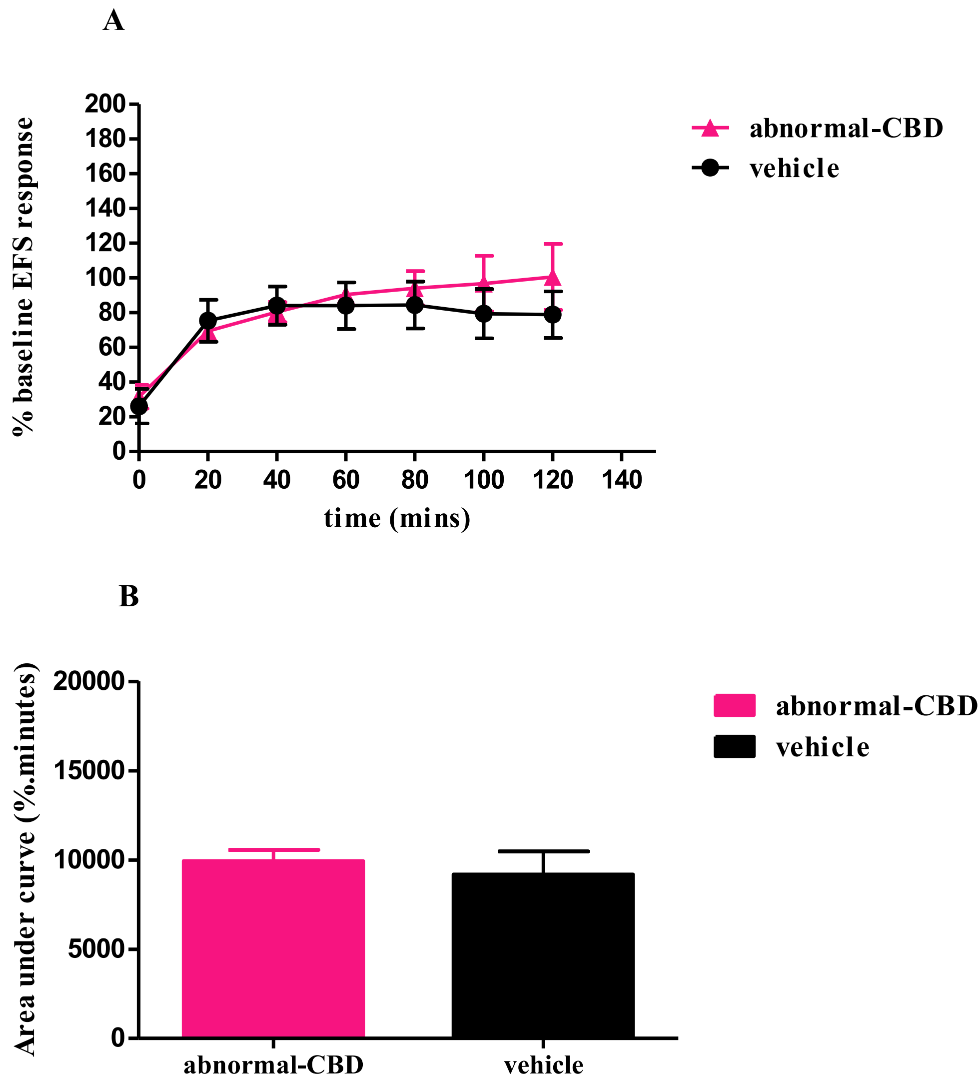


Figure 4.15 *The effect of abnormal-CBD $3x10^{-5}$M (n=3) and ethanol vehicle 0.1% (n=6) on recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response* ***(A).*** *The baseline contraction size was measured after the addition of abnormal-CBD and before the addition of H_2O_2. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)****. Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

The effect of O-1602 on H_2O_2-induced damage of the ileum

Neither O-1602 ($1x10^{-5}$M) nor its vehicle control had any effect on the total area under the curve for H_2O_2-treated tissues or on the size of EFS-contractions at any time point. (see figure 4.16).

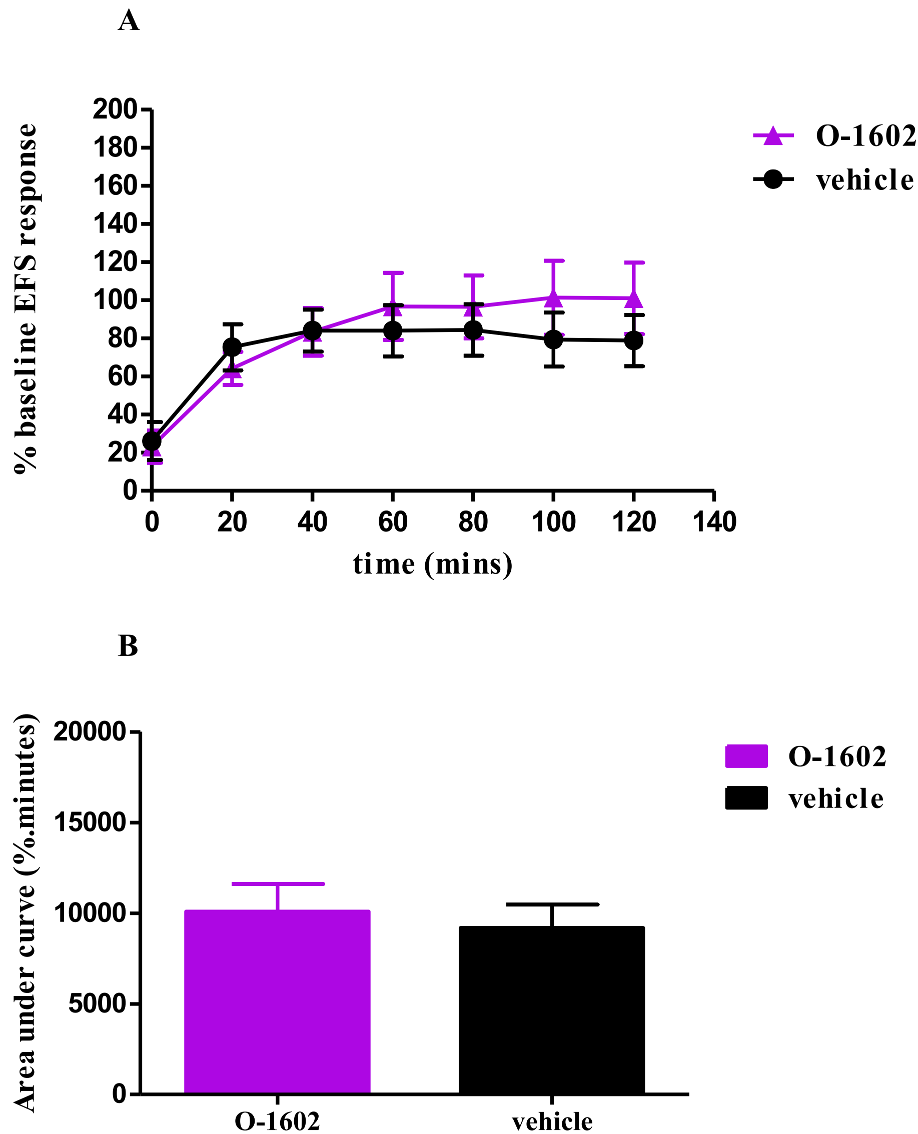


Figure 4.16 *The effect of O-1602 $1x10^{-5}$M (n=6) and ethanol vehicle 0.1% (n=6) on recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response* ***(A).*** *The baseline contraction size was measured after the addition of O-1602 and before the addition of H_2O_2. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)***. *Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

The effect of PSN on H_2O_2-induced damage of the ileum

PSN ($3x10^{-5}$M) nor its vehicle control had a significant effect on the total area under the curve for H_2O_2-treated tissues or on the size of EFS-contractions at any time point (see figure 4.17).

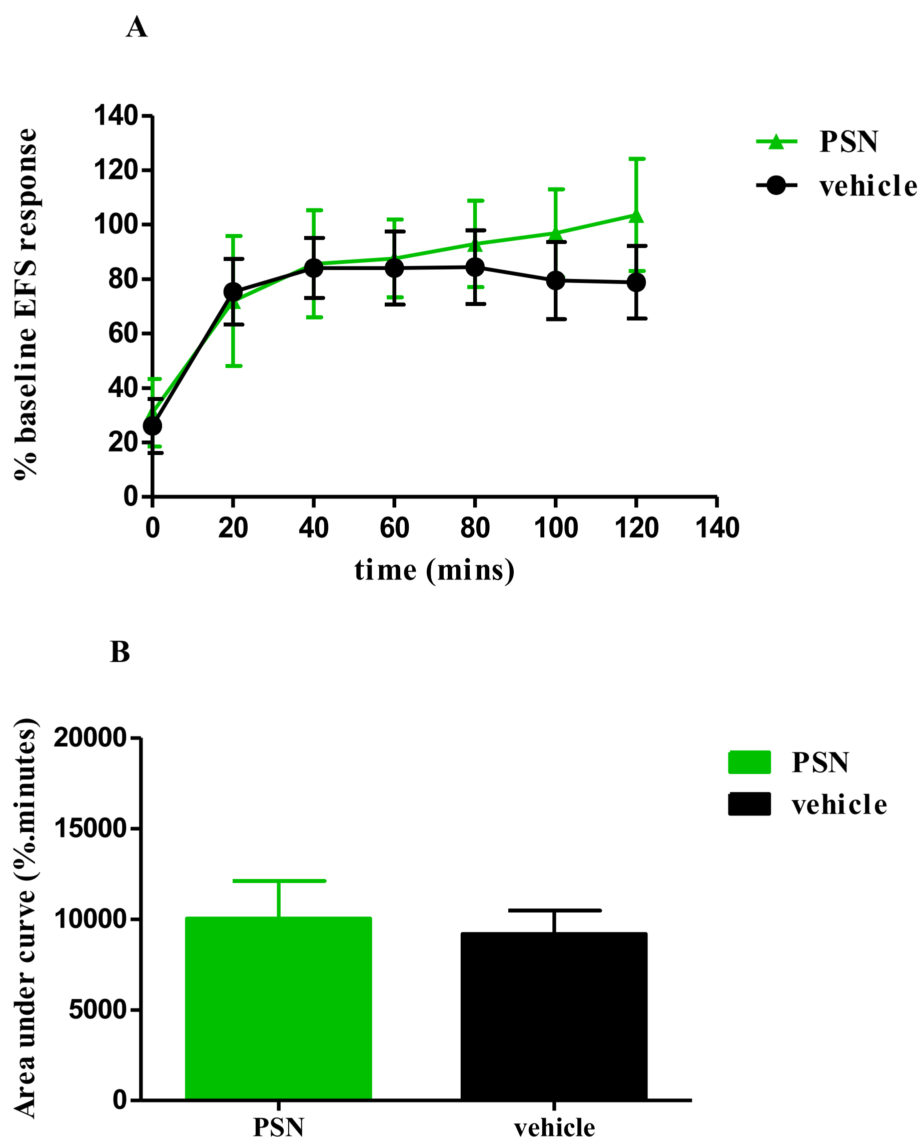


Figure 4.17 *The effect of PSN $3x10^{-5}M$ (n=5) and ethanol vehicle 0.1% (n=6) on recovery of EFS-induced contractions of the guinea-pig isolated ileum following washout of 0.3% H_2O_2 (1 minute exposure). The size of EFS-induced contractions is expressed as percentage of baseline EFS response* ***(A).*** *The baseline contraction size was measured after the addition of ACPA and before the addition of H_2O_2. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM). The areas under the curves were calculated as %.minutes* ***(B)***. *Data were analysed by ANOVA. ANOVA was used because the same vehicle control was used in several cannabinoid experiments.*

4.5 Discussion

The main finding of this chapter is that (+)WIN protects the ileum from inflammatory-type damage but that this does not appear to be mediated through any of the classical or putative cannabinoid receptors. This is the first study which shows the protective effects of cannabinoids in the intestine. However, the limitation associated with these *in vitro* models of inflammatory damage is that immune cells are not recruited from the bloodstream to the tissue (which occurs during inflammation *in vivo*).

LPS model of inflammatory damage in the ileum

Studies have shown that LPS induces intestinal dysmotility *in vivo* (Chen *et al*., 2010; Duncan *et al*., 2008; Liang *et al*., 2005) and *in vitro* (Gonzalo *et al.,* 2010; Gonzalo *et al.,* 2011; Rebollar *et al.,* 2002), although the effects *in vivo* are conflicting.Sometimes intestinal transit is decreased whereas other times it is increased (Chen *et al*., 2010; Duncan *et al*., 2008; Liang *et al*., 2005). Cannabinoids have been shown to inhibit LPS-induced production of inflammatory mediators (Fischer-Stenger *et al.*, 1993; Ross *et al.*, 2000) and attenuate LPS-induced dysmotility *in vivo* (Duncan *et al.*, 2008).

Most of the *in vitro* studies examining the effect of LPS on normal intestinal segments have been carried out on rabbit intestine. These studies showed that LPS reduced the size of intestinal contractions and suggested that this involved the production of prostaglandins and nitric oxide (Chen *et al*., 2010; Grasa *et al*., 2008). In the guinea-pig ileum, however, LPS did not have a significant effect on contraction, even though a range of LPS concentrations were used. The highest concentration used exceeded the

concentrations previously reported as effective in the rabbit intestine (Chen *et al*., 2010; Grasa *et al*., 2008). It is possible that LPS had no effect in the guinea-pig ileum because the toll-like receptor 4 was not present. LPS would probably be more effective *in vivo* as the ileum would become infiltrated with immune cells from the bloodstream which express toll-like receptor 4. In future, it may be more effective to pre-treat the guinea-pigs with LPS and then examine the contractility of the dissected ileum segments, as carried out previously in the rabbit (Gonzalo *et al*. 2010; Gonzalo *et al*., 2011; Rebollar *et al*., 2002).

H_2O_2 model of inflammatory damage in the ileum

H_2O_2 was used to produce oxidative stress, which is a major component of inflammation. Indeed, the intestinal inflammation that occurs in IBD involves excessive production of ROS. It has also been shown that the antioxidant balance is impaired in IBD patients, which leads to further oxidative damage. The impaired antioxidants include superoxide dismutases, catalase, glutathione peroxidase, glutathione and metallothionein (Kruidenier *et al.*, 2003). H_2O_2 may contribute to dysmotility in IBD. The tissue damage caused by ROS such as H_2O_2 has been described in the Introduction, as well as the reduction in excitability of myenteric neurones which control gastrointestinal motility (Pouokam *et al.*, 2009). In addition, H_2O_2 has been shown to reduce contractions of the colon (Cao *et al.*, 2004).

Cannabinoids have been shown to possess anti-oxidative properties. They have been shown to upregulate antioxidant enzymes such as superoxide dismutase (Garcia-Arencibia *et al.*, 2007) and reduce production of ROS from immune cells (Hao *et al*., 2010). Cannabinoids have also been shown to be free radical scavengers (Velez-Pardo

et al., 2010). Although much of the literature suggests that cannabinoids reduce oxidative stress, there are a few studies which suggest in certain situations cannabinoids can induce ROS production (Massi *et al.*, 2006a; Wu *et al.*, 2008).

The anti-oxidant activity have been shown to contribute to the neuroprotective (Hampson *et al.*, 1998), cardioprotective (Montecucco *et al.*, 2009) and hepatoprotective (Rajesh *et al.*, 2007) effects of cannabinoids. The CB_1 (Kessiova *et al.*, 2006) and CB_2 (Montecucco *et al.*, 2009) (Rajesh *et al.*, 2007) receptors have previously been implicated in the anti-oxidant effects of cannabinoid as well as cannabinoid receptor independent effects (Chen *et al.*, 2000; Kessiova *et al.*, 2006).

The recovery in the (+)WIN treated group from H_2O_2-induced damage was significantly better than the ethanol control group. Contraction returned to 154.6 ± 31.0% of the initial EFS response in the presence of (+)WIN compared with only 70.3 ± 16.4% in the presence of the ethanol vehicle alone. Antagonists for the CB_1 (rimonabant), CB_2 (SR144528) or the putative CB_e (O-1918) receptor had no effect on (+)WIN-induced recovery, suggesting these receptors are not involved in the protection by (+)WIN. This is supported by the lack of effect of the CB_1 agonist ACPA and the putative CB_e receptor agonist abnormal-CBD. As (+)WIN does not bind to the GPR55 receptor, this receptor would be unlikely to play a role in (+)WIN-induced protection from H_2O_2-induced damage. This is further supported by the finding that the GPR55 agonist O-1602 had no significant effect on recovery from H_2O_2-induced damage. GPR119 could be involved in the (+)WIN-induced protection but this is unlikely as the GPR119 agonist PSN had no effect in the H_2O_2 assay. Unfortunately, corroboration could not be obtained as there is currently no selective antagonist of GPR119 available.

As the protective effect of (+)WIN does not seem to be due to any of the known cannabinoid receptors, it may either be mediated through a novel cannabinoid receptor or it is cannabinoid receptor independent effect. (+)WIN cannot be acting by reducing ROS production as H_2O_2 is added directly to the bath to produce oxidative stress. It is also unlikely to be due to up-regulation of anti-oxidant enzymes as the timescale of the experiments was only two hours, whereas the up-regulation in the literature was reported after two weeks of cannabinoid treatment. It is possible that (+)WIN was acting as a free radical scavenger to prevent tissue damage. Although there was washout before and after H_2O_2 was added, (+)WIN could not be removed (see washout controls appendix 1) and would have been present to act as a scavenger. With (+)WIN reducing the amount of oxidative damage, the tissue would have been able to recover over time and contract effectively again. If (+)WIN were acting as a free radical scavenger, this would explain the cannabinoid receptor independent effect, which has been suggested in the literature (Chen *et al*., 2000; Kessiova *et al*., 2006; Underdown *et al*., 2005).

CHAPTER 5:
IN VITRO TESTING OF THE PHARMACOLOGICAL ACTIVITY OF CANNABINOID-DENDRIMER CONJUGATES

5. In vitro testing of the pharmacological activity of cannabinoid-dendrimer conjugates

5.1 Introduction

Cannabinoids have shown several pharmacological effects which may be beneficial in the treatment of inflammatory bowel disease (IBD). They have been shown to reduce gut inflammation in animal models of IBD via activation of CB_1 and CB_2 receptors (Kimball *et al.*, 2006), enhance epithelial wound healing via CB_1 activation (Wright *et al.*, 2005) and reduce gut motility via CB_1 activation (Heinemann *et al.*, 1999; Izzo *et al.*, 1998). The CB_2 receptor may be involved in reducing motility under inflammation conditions (Izzo, 2007).

The major barrier to the use of cannabinoids as therapeutics is their psychotropic effects, mediated by the CB_1 receptor in the CNS (Xiong *et al.*, 2011). However, CB_1 mediated effects are also desirable clinically, especially for the treatment of IBD (as shown above). Therefore, novel cannabinoids developed to treat IBD need to retain CB_1 and CB_2 activity, but not act in the CNS.

There are a number of strategies which could be adopted to prevent compounds from crossing the blood brain barrier. These include increasing the size of the compound, reducing its lipophilicity or creating a charged molecule. The strategy chosen in this thesis was to attach a large bulky group (dendrimer) to the cannabinoid.. Dendrimers are ideal for this purpose as they are polymers with bulky, tree-like branching structures (see figure 5.1). They differ in size depending on how many repeated branching cycles (known as the generation number) are performed during their synthesis. The more

cycles performed (the higher the generation number), the larger the dendrimer. The size of the dendrimer can be selected so that it is small enough to be absorbed across the intestine but large enough to prevent CNS penetration.

In addition to increasing the size of the compound, conjugation to a dendrimer can also increase the hydrophilicity of the molecule and introduce a charge to the compound (cationic or anionic dendrimers). These combined properties should prevent the cannabinoid from crossing the blood brain barrier.

Another reason for using the dendrimer conjugation strategy was because dendrimers have been shown to be non-toxic (Scharbin *et al*., 2010) and not to alter pharmacological activity in the literature (Khandare *et al*., 2005).

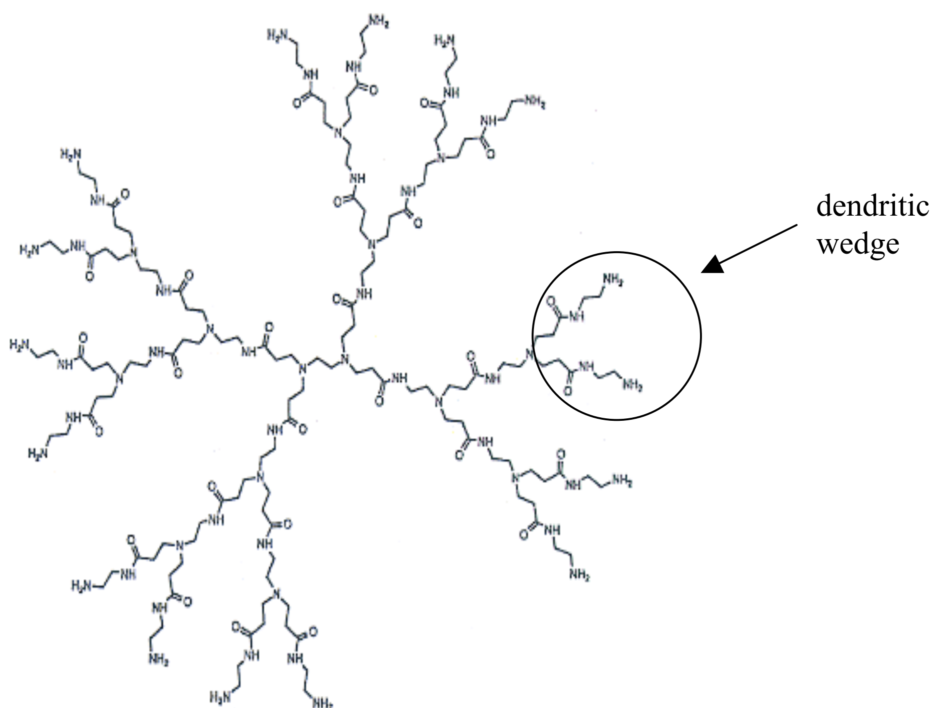


Figure 5.1 The tree-like branching structure of dendrimers.

Ghaith Al-Jayyoussi, from the Welsh School of Pharmacy has synthesised a cannabinoid-dendrimer conjugate, which consists of the non-selective cannabinoid JWH007 (see figure 5.2), with a PAMAM dendrimer attachment (Al-Jayyoussi, 2011). JWH007 was the chosen cannabinoid due to its potency at the CB_1 and CB_2 receptor (see table 1.1, chapter 1). Also, JWH007 should be resistant to hydrolysis by FAAH as it lacks the required amide linkage (Makwana *et al*., 2010). The dendrimer chosen was the anionic generation 3.5 poly(amidoamine) (G3.5 PAMAM dendrimer) as it was thought that this would be large enough to prevent access to the CNS and these molecules have been shown to be biocompatible (Scharbin *et al*., 2010) . Before testing the final cannabinoid-dendrimer conjugate, preliminary studies were carried out on JWH007 with bulky group attachments. The purpose of this was to see how the pharmacological activity of JWH007 at the CB_1 and CB_2 receptor altered with the size of the attached group.

The compounds were tested in two pharmacological assays, the Paton ileum (see 'Electrical field stimulation', chapter 2) and LPS-stimulated macrophages (see 'LPS-stimulation of a macrophage cell line', chapter 2).The Paton ileum preparation was used to screen the compounds for CB_1 activity as inhibition of ileum contractions by cannabinoids was reported to be CB_1 mediated (Izzo *et al.*, 1998). However, although cannabinoid-induced inhibition of intestinal contractions was thought to be CB_1 mediated, this thesis suggests that the inhibition is mediated through a non- CB_1, non-CB_2 receptor (see chapter 3). The Paton ileum model was still retained for the testing of novel cannabinoids, not as a CB_1 assay, but to investigate the anti-motility effects of the drugs.

The macrophages were used to screen the compounds for CB_2 activity as the reduction of inflammatory mediators from these LPS-stimulated cells by cannabinoids was reported to be CB_2 mediated (Ross *et al.*, 2000).

The first compound to be tested in the assays was JWH007; the activity of the novel cannabinoids were subsequently compared to this. GA001 was the first novel cannabinoid to be tested. This compound was identical to JWH007, apart from a methyl group attachment to a napthoylene ring (see figure 5.3). The position of the methyl group attachment was decided based on literature detailing the structure-activity relationship (SAR) of cannabinoids as well as considering the difficulty of the attachment chemistry. The position chosen for the methyl group was a site of easy attachment which avoided areas of the JWH007 molecule important for pharmacological activity. The portions of JWH007 reported to be important to be for pharmacological activity at the CB_1 and CB_2 receptor (Huffman *et al.*, 2005) have been circled in figure 5.2.

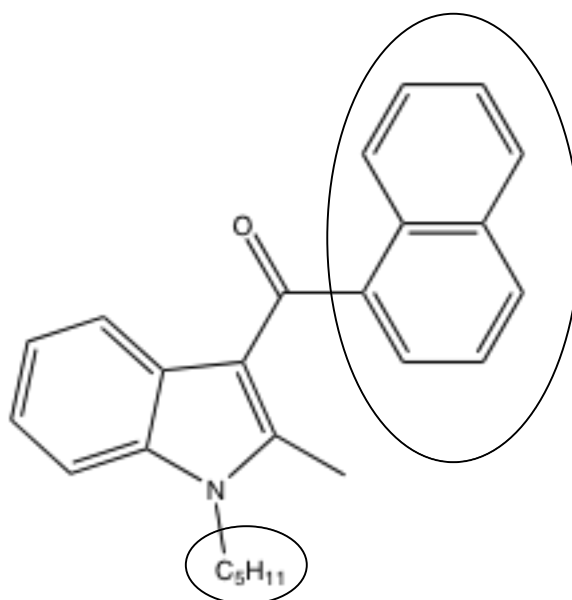


Figure 5.2 The chemical structure of JWH007. The circled regions are reported to be important for pharmacological activity (Huffman *et al.*, 2005)..

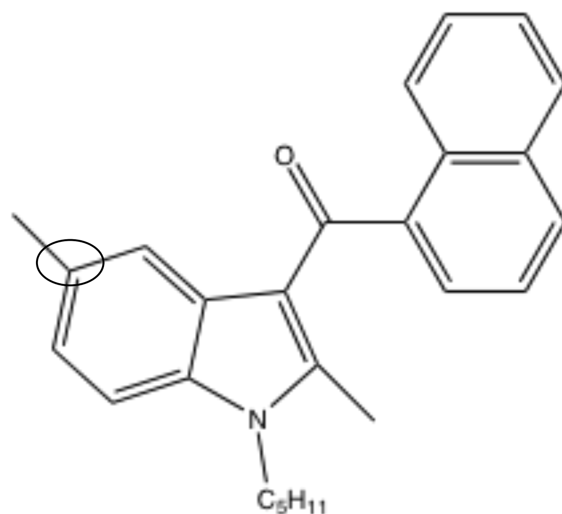


Figure 5.3 *The chemical structure of GA001. The site of the methyl group attachment is circled.*

The second compound to be tested was GA002, identical to GA001, but instead of the methyl group, a bulkier group was attached (similar to a portion of the dendrimer – a dendritic wedge). The site of attachment was the same (see figure 5.4).

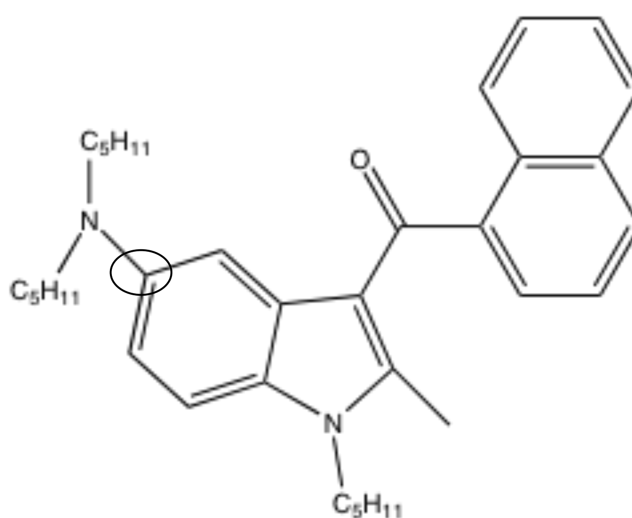


Figure 5.4 *The chemical structure of GA002. The site of the dendrimer wedge-like attachment is circled.*

The third compound to be tested was GA003. GA003 was identical to GA001 but, instead of the methyl group, a longer chain was attached (a spacer). The site of attachment was the same (see figure 5.5 overleaf). This spacer is similar to the spacer used in the final cannabinoid-dendrimer conjugate. The purpose of the spacer in the final conjugated molecule was to provide the compound with flexibility to avoid the steric hinderence which could prevent receptor binding.

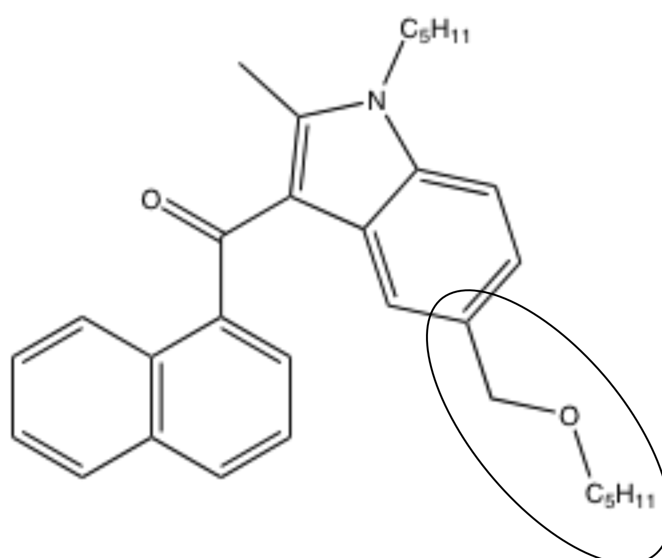


Figure 5.5 *The chemical structure of GA003. The site of the spacer attachment is circled.*

The fourth compound to be tested was GA006 which was the final JWH007-PAMAM dendrimer conjugate. GA006 was identical to GA001 but with the spacer and dendrimer attached instead of the methyl group (see figure 5.6).

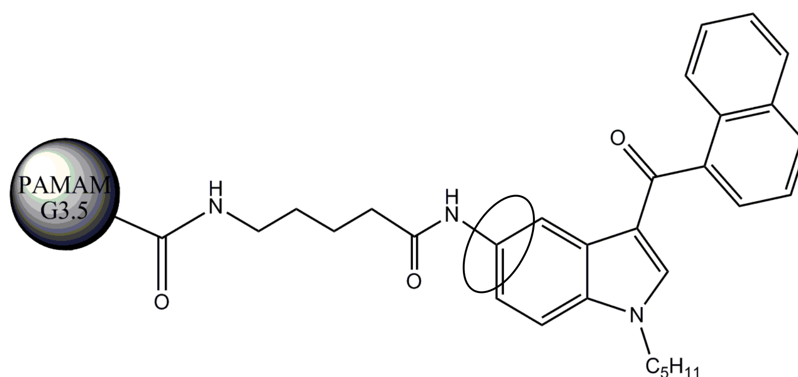


Figure 5.6 *The chemical structure of GA006. The site of the spacer-dendrimer attachment is circled.*

5.2 Aims

- To determine whether cannabinoids retain potency at the CB_1 and CB_2 receptor with the attachment of bulky groups such as dendrimers.

- To investigate whether the selectivity of cannabinoids for the CB_1 and CB_2 receptor is altered with the attachment of these bulky groups.

5.3 Method

Although JWH007 and GA001 were tested in all experiments, due to limited drug supply, GA002, GA003 and GA006 were only tested in some experiments.

Drug solutions

Ileum experiments: GA001 and GA002 were dissolved in absolute ethanol and stored at 20ºC for up to 1 month. GA003 was dissolved in DMSO and stored at 20ºC for up to 1 month. GA001, GA002 and GA003 were serially diluted in ethanol on the day of the experiment.

Macrophage experiments: The cannabinoids were dissolved in DMSO and stored at ‾20˚C for up to 1 month. On the day of the experiment, the cannabinoids were thawed and diluted in media (90% DMEM, 10% FBS) which had been pre-warmed to 37˚C.

The LPS was reconstituted in saline (0.9% sodium chloride) and aliquots were frozen at -20˚C for up to 1 month. The aliquots were thawed out on the day of the LPS experiments and serially diluted with media (90% DMEM, 10% FBS).

5.3.1 The effect of novel cannabinoid compounds (based on the structure of JWH007) on EFS-evoked contractions of the guinea-pig isolated ileum

This has been described in detail in Chapter 2. Cumulative concentration response curves for each cannabinoid was constructed (as in chapter 3 for (+) WIN).

5.3.2 The effect of novel cannabinoid compounds (based on the structure of JWH007) on LPS-stimulated TNFα release from macrophages

Cells were grown on a 24 well plate for 96 hours as in chapter 2 (see 'LPS-induced TNFα release from RAW 264.6 macrophages'). Medium was then removed from each well and replaced with 1 ml of cannabinoid or DMSO vehicle solution (see 'Drug solutions' above).The concentrations of cannabinoid ranged from 1 to 30μM and the concentration of the DMSO vehicle was 0.02%. The plate was incubated at 37˚C for 30 minutes to allow the drugs to reach equilibrium. After 30 minutes, LPS was prepared (see 'Drug solutions' above) and 10μl (500ng/ml) was added to each well to produce a final concentration of 5ng/ml. The plated was then incubated for 16 hours at 37˚C, After 16 hours, the cell media was removed from the wells to be assayed for TNFα (see 'TNFα measurement', Chapter 2) and the cells were lysed (see 'Cell lysis', Chapter 2) to remove and quantify their protein content (see 'Protein measurement', Chapter 2).

Investigation of the effect of the novel cannabinoids on RAW 264.7 macrophage viability

Cells were grown for 96 hours as in chapter 2 (see 'LPS-induced TNFα release from RAW 264.6 macrophages') but were seeded onto a 96 well plate (0.32cm^2/well) instead of a 24 well plate. Medium was then removed from each well and replaced with 200μl of cannabinoid or DMSO vehicle solution (see 'Drug solutions' above). The concentrations of cannabinoid ranged from 1 to 30μM and the concentration of the DMSO vehicle was 0.02%. The plate was incubated at 37˚C for 30 minutes to allow the drugs to reach equilibrium. After 30 minutes, LPS was prepared (see 'Drug solutions

above') and 2μl was added to each well to produce a final concentration of 5ng/ml. The plated was then incubated for 16 hours at 37˚C, after which the (Methanethiosulphonate reagent) MTS assay was performed.

The MTS assay involved adding 20μl of MTS reagent to 100μl of media in the wells and incubating for 2 hours 30 minutes. The absorbance of the plate was then read at 490nm using a spectrophotometer. The purpose of this assay was to investigate whether the cannabinoids would affect cell viability at any of the concentrations used. This was important as a reduction in cell viability could affect the concentration of TNFα in the media and make it difficult to interpret the effects of the cannabinoids.

Data analysis

Ileum experiments: The effect of each drug concentration on contraction size was reported as a percentage of baseline EFS or initial carbachol contraction. Data were reported as mean ± SEM.

An IC_{25} or IC_{50} was calculated for each drug, defined as the drug concentrations required to inhibit baseline EFS/initial carbachol contraction by 25% and 50% respectively. Mean values with their 95% confidence intervals (shown in brackets) were calculated.

Curve fitting was not carried out as the concentration-response curves did not fit the traditional sigmoidal shape and did not reach a maximum response at the concentrations used.

Macrophage experiments: The effect of each drug was expressed as a percentage of the media control. In the experiments examining TNFα release, results were also expressed as pg TNFα / mg protein.

Statistical analysis

Mean values for the drug and its vehicle control were compared using unpaired Student's t test (drug and vehicle) or one way ANOVA with post-hoc Dunnett's Test (multiple drugs and vehicle). One sample Student's t tests were used to determine the effects of the vehicle alone on contractions. $P<0.05$ was considered statistically significant.

5.4. Results

5.4.1 The effect of novel cannabinoid compounds (based on the structure of JWH007) on EFS-evoked contractions of the guinea-pig isolated ileum

The effect of JWH007 on EFS-evoked contractions

JWH007 showed a concentration-dependent reduction in the size of EFS-induced contractions of the ileum (IC_{25}: 2.0 (0.1 - 45) x 10^{-10}M and IC_{50}:1.5 (0.02 – 100) x10^{-8}M; see table 5.1). JWH007 started to exert an effect at 3x10^{-11}M, reaching 72 ± 8.07% inhibition (*P* < 0.01) at the highest concentration of 3x10^{-6}M (see figures 5.7 and 5.8). The ethanol vehicle had no significant effect when added in low volumes but inhibited contractions at the higher volumes required to achieve concentrations of JWH007 ≥ 3x10^{-7}M. The ethanol vehicle reduced contractions by 18.23 ± 5.5 % (P <0.05) at the highest volume added, which was required to achieve JWH007 3x10^{-6}M.

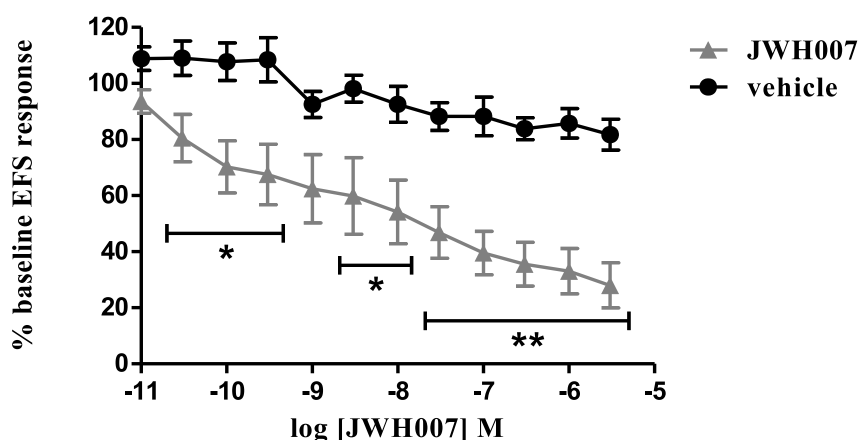


Figure 5.7 *The effect of JWH007 (n=4) and ethanol vehicle (n=6) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of JWH007 or ethanol vehicle. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05, **P< 0.01 (one way ANOVA with post-hoc Dunnett's test). The concentration of ethanol vehicle ranged from 0.02-0.33% (shown as respective JWH007 concentrations 10^{-11}-3x10^{-6}M). The same vehicle control was used in several cannabinoid experiments.*

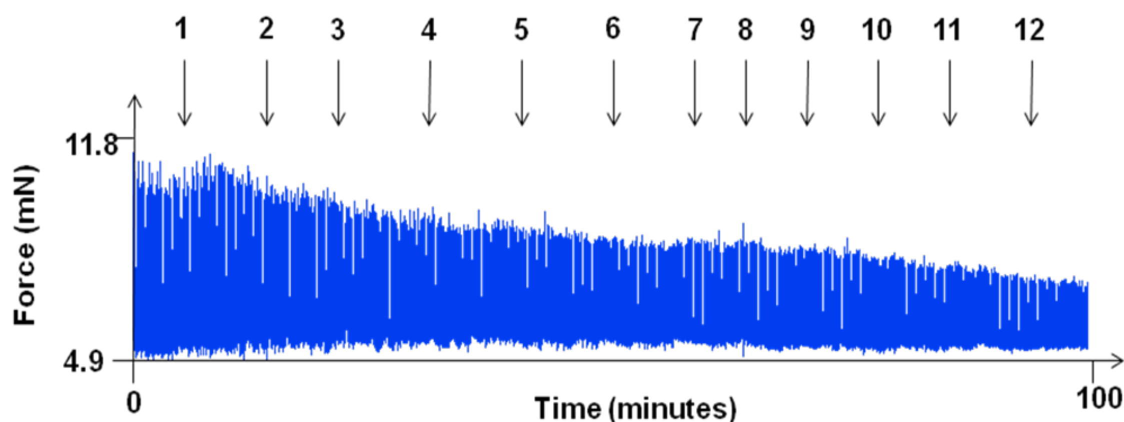


Figure 5.8 *Trace showing the effect of JWH007 on EFS-induced contractions of the guinea-pig isolated ileum. Concentrations (M) shown below:*

1= *1x10^{-11}*	**4**= *3x10^{-10}*	**7**= *1x10^{-8}*	**10**= *3x10^{-7}*
2= *3x10^{-11}*	**5**= *1x10^{-9}*	**8**= *3x10^{-8}*	**11**= *1x10^{-6}*
3= *1x10^{-10}*	**6**= *3x10^{-9}*	**9**= *1x10^{-7}*	**12**= *3x10^{-6}*

The effect of GA001 on EFS-evoked contractions

GA001 caused a concentration-dependent reduction in the size of EFS-induced contractions of the ileum (IC_{25}: 3(0.01 – 910) x 10^{-10} M and IC_{50}: 4.9 (0.03 – 700) x 10^{-9}M; see table 5.1). GA001 started to exert an effect at 1x10^{-11}M, causing 63.9 ± 2.3% inhibition ($P < 0.01$) at the highest concentration of 3x10^{-6}M (see figures 5.9 and 5.10 overleaf). The ethanol vehicle had no significant effect when added in low volumes but inhibited contractions at the higher volumes required to achieve concentrations of GA001 ≥ 3x10^{-7}M. The ethanol vehicle reduced contractions by 18.23 ± 5.5 % (P <0.05) at the highest volume added, which was required to achieve GA001 3x10^{-6}M.

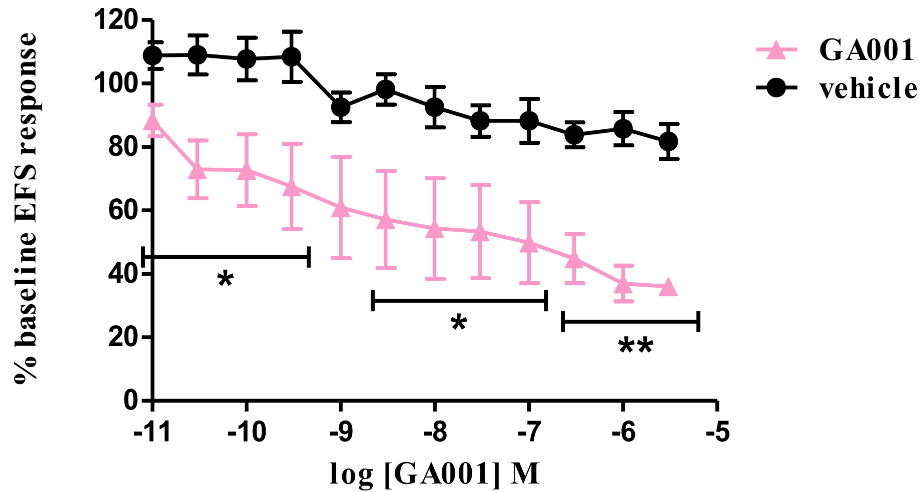


Figure 5.9 *The effect of GA001 (n=3) and ethanol vehicle (n=6) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of GA001 or ethanol vehicle. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05, **P< 0.01 (one way ANOVA with post-hoc Dunnett's test). The concentration of ethanol vehicle ranged from 0.02-0.33% (shown as respective GA001 concentrations 10^{-11}-$3x10^{-6}$M). The same vehicle control was used in several cannabinoid experiments.*

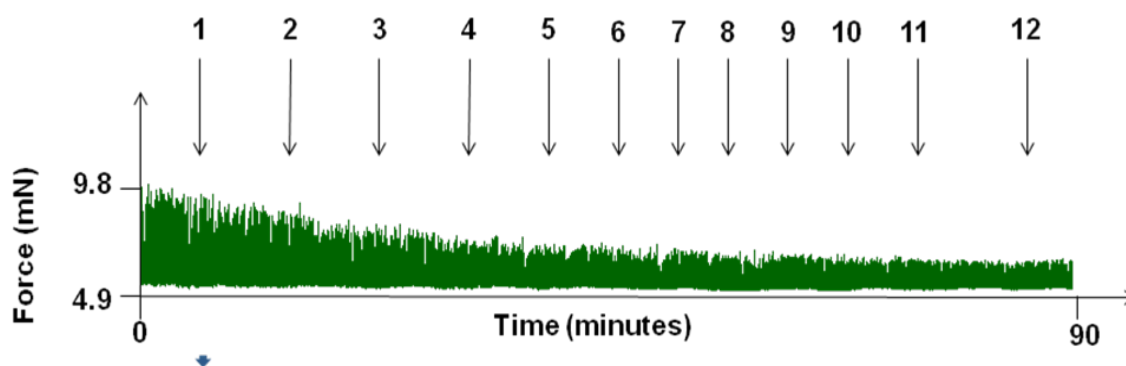


Figure 5.10 *Trace showing the effect of GA001 on EFS-induced contractions of the guinea-pig isolated ileum. Concentrations (M) shown below:*

1= $1x10^{-11}$	**4**= $3x10^{-10}$	**7**= $1x10^{-8}$	**10**= $3x10^{-7}$
2= $3x10^{-11}$	**5**= $1x10^{-9}$	**8**= $3x10^{-8}$	**11**= $1x10^{-6}$
3= $1x10^{-10}$	**6**= $3x10^{-9}$	**9**= $1x10^{-7}$	**12**= $3x10^{-6}$

The effect of GA002 on EFS-evoked contractions

GA002 showed a concentration-dependent reduction in the size of EFS-induced contractions of the ileum (IC_{25}: 1.8 (0.1 – 39) x 10^{-8} M; see table 5.1). GA002 had no effect at concentrations below $1x10^{-6}$M, but reached 51.39 ± 7.03% inhibition (*P* < 0.01) at the highest concentration of $3x10^{-6}$M (see figures 5.11and 5.12 overleaf). The ethanol vehicle had no significant effect when added in low volumes but inhibited contractions at the higher volumes required to achieve concentrations of GA002 ≥ $3x10^{-7}$M. The ethanol vehicle reduced contractions by 18.23 ± 5.5 % (P <0.05) at the highest volume added, which was required to achieve GA002 $3x10^{-6}$M.

Figure 5.14 shows the percentage inhibition of EFS-induced contractions of the guinea-pig isolated ileum by JWH007, GA001 and GA002 at the maximum concentration used (3 x 10^{-6} M).

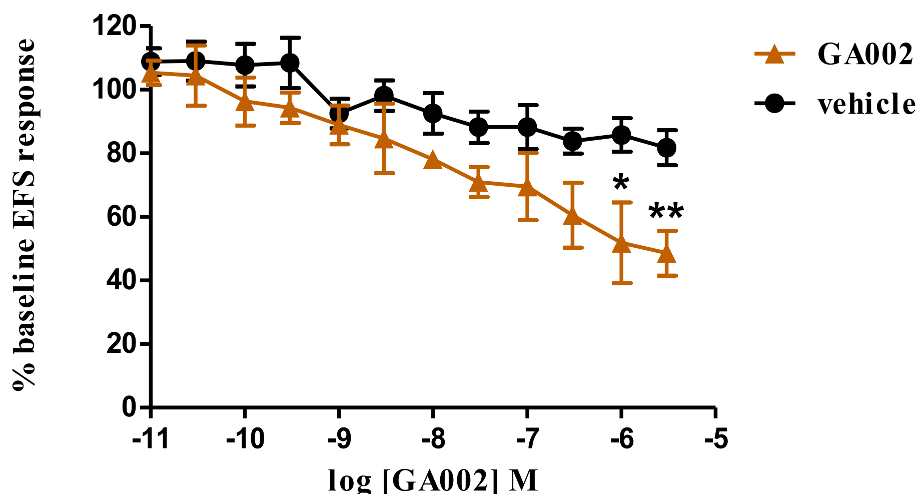


Figure 5.11 *The effect of GA002 (n=4) and ethanol vehicle (n=6) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of GA002 or ethanol vehicle. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); * P< 0.05, **P< 0.01 (one way ANOVA with post-hoc Dunnett's test). The bath concentration of ethanol vehicle ranged from 0.02-0.33% (shown as respective GA002 concentrations 10^{-11}-$3x10^{-6}$M). The same vehicle control was used in several cannabinoid experiments.*

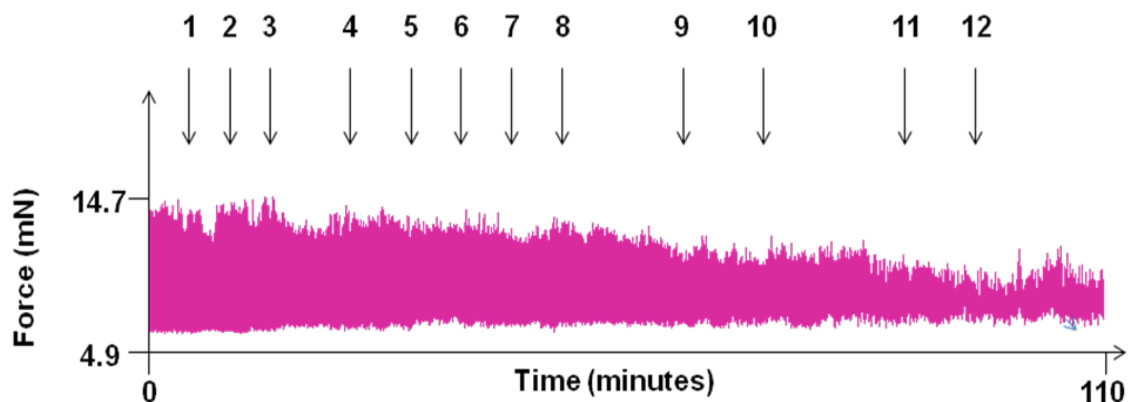


Figure 5.12 *Trace showing the effect of GA002 on EFS-induced contractions of the guinea-pig isolated ileum. Concentrations (M) shown below:*

1= $1x10^{-11}$	**4**= $3x10^{-10}$	**7**= $1x10^{-8}$	**10**= $3x10^{-7}$
2= $3x10^{-11}$	**5**= $1x10^{-9}$	**8**= $3x10^{-8}$	**11**= $1x10^{-6}$
3= $1x10^{-10}$	**6**= $3x10^{-9}$	**9**= $1x10^{-7}$	**12**= $3x10^{-6}$

The effect of GA003 on EFS-evoked contractions

Neither GA003 nor the DMSO/ethanol control affected the size of EFS-evoked contractions at the concentrations used (see figure 5.13).

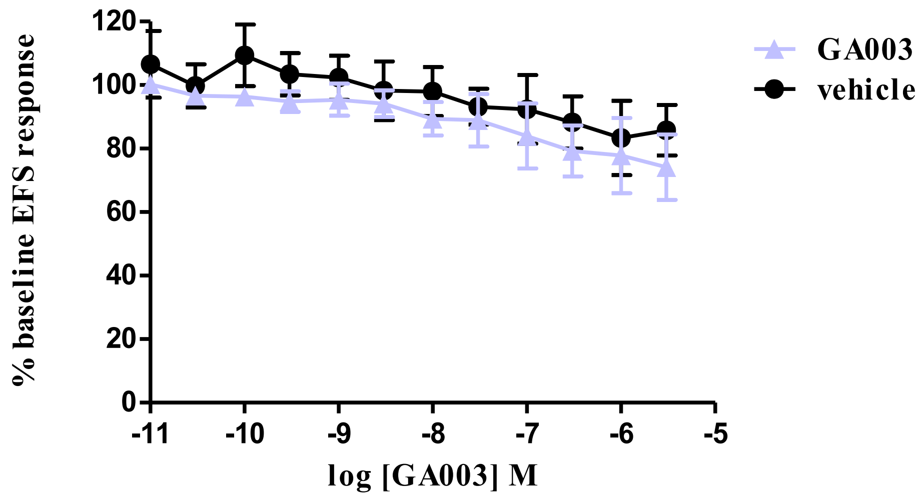


Figure 5.13 *The effect of GA003 (n=4) and mixed ethanol/DMSO vehicle (n=5) on the size of EFS-induced contractions of the guinea-pig isolated ileum, expressed as percentage of baseline EFS response. The baseline contraction size was measured immediately before the addition of GA003. Each symbol represents the mean percentage; vertical bars indicate standard error of the mean (SEM); data was analysed by unpaired Student's t test. The bath concentration of DMSO ranged from* $4x10^{-8}$*-0.012% and ethanol from 0.02-0.32% (shown as respective GA003 concentrations* 10^{-11}*-*$3x10^{-6}$*M)*

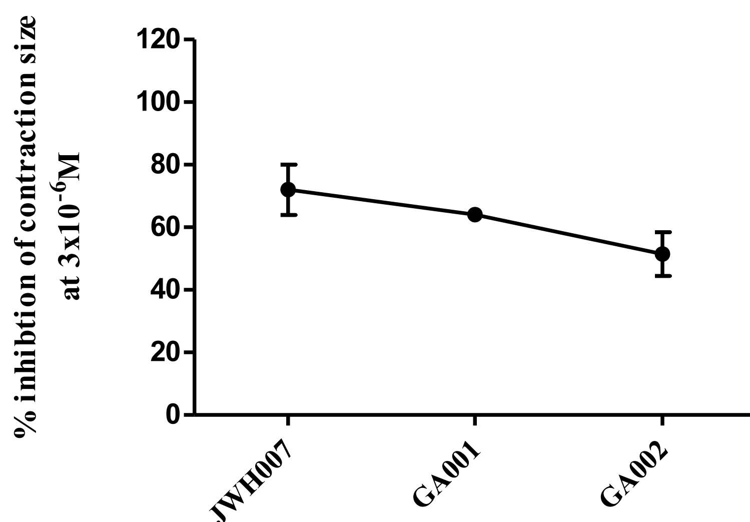


Figure 5.14 *The percentage inhibiton of EFS-induced contractions of the guinea-pig isolated ileum by JWH007, GA001 and GA002 at the maximum concentration used (3 x 10^{-6} M).*

Agonist	IC_{25} mean (M)	IC_{25} 95% confidence limits (M)	IC_{50} mean (M)	IC_{50} 95% confidence limits (M)
JWH007	2 x 10^{-10}	(0.1 – 45) x 10^{-10}	1.5 x 10^{-8}	(0.02 - 100) x 10^{-8}
GA001	3 x 10^{-10}	(0.01 – 910) x 10^{-10}	4.9 x 10^{-9}	(0.03 – 700) x 10^{-9}
GA002	1.8 x 10^{-8}	(0.1 – 39) x 10^{-8}	–	–

Table 5.1 *IC_{25} and IC_{50} values are shown for JWH007, GA001 and GA002. IC_{50}s were not calculated for GA002 because the inhibition did not reach 50% in all experiments.*

5.4.2 The effect of novel cannabinoid compounds (based on the structure of JWH007) on LPS-stimulated TNFα release from macrophages

The effect of JWH007, GA001 and GA006 on macrophage viability

JWH007, GA001 and their vehicle (DMSO 0.02%) had no effect on macrophage viability after 16 hours in the presence of 5ng.ml LPS. 5ng/ml LPS alone (GA006 control) also did not affect cell viability. 30μM GA006, however, reduced cell viability by 97.1 ± 1.4% (see figure 5.15).

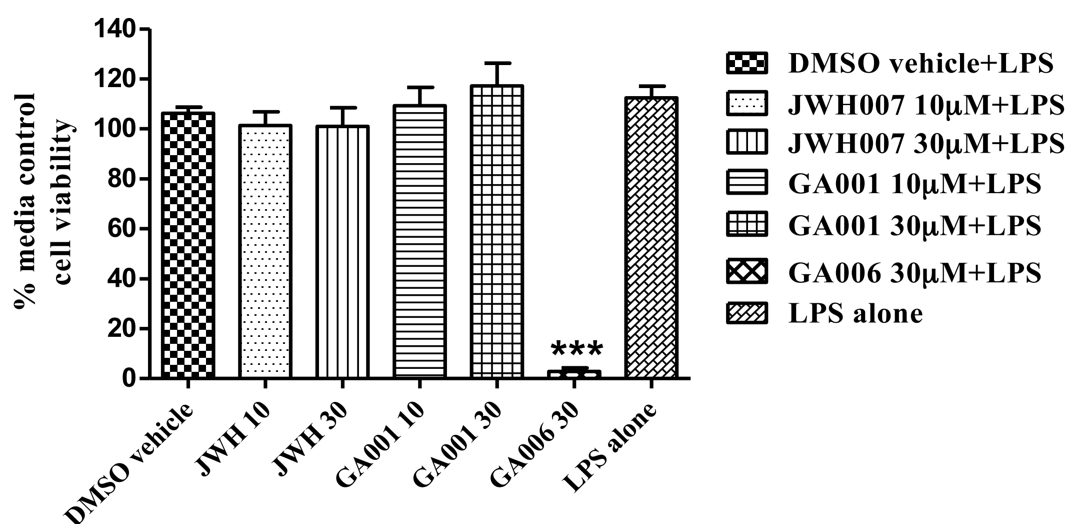


Figure 5.15 *The effect of JWH007, GA001, their DMSO vehicle and GA006 (n=3-7) on RAW 264.7 macrophage viability after 16 hours in the presence of 5ng/ml LPS. The control for JWH007 and GA001 was DMSO (0.02%) in the presence of LPS, whereas the control for GA006 (dissolved in water) was LPS 5ng/ml alone. Results are expressed as percentage of media control; vertical bars indicate standard error of the mean (SEM); *** P<0.001 (Student's unpaired t test).*

The effect of JWH007, GA001 and GA003 on macrophage protein concentration

None of the drugs or their vehicle (DMSO 0.02%) had any effect on macrophage concentration after 16 hours in the presence of 5ng/ml LPS. 5ng/ml LPS alone also did not affect macrophage protein concentration (see figure 5.16).

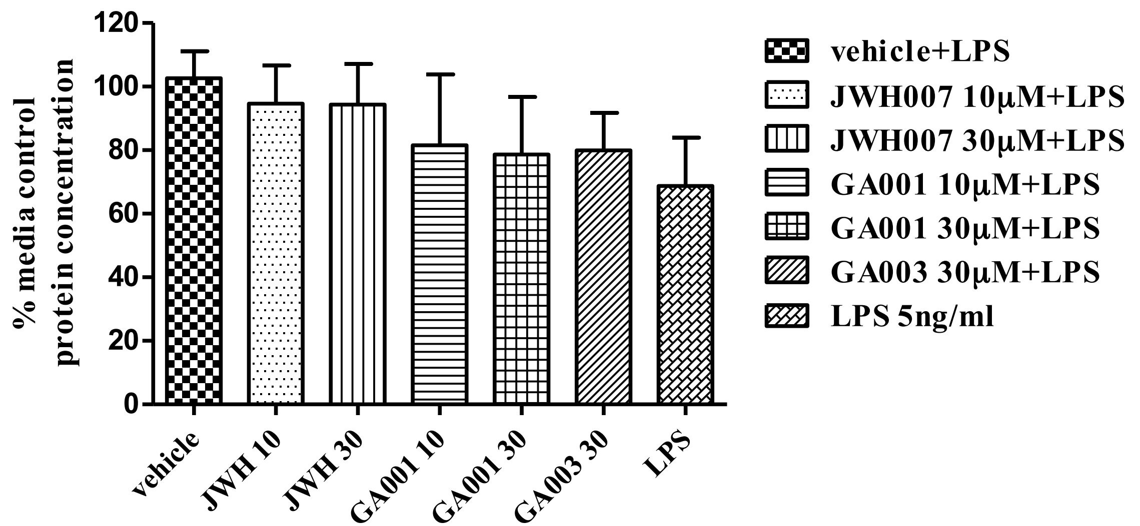


Figure 5.16 *The effect of JWH007, GA001, GA003 (n=3) and their DMSO vehicle (n=3) on RAW 264.7 macrophage protein concentration after 16 hours in the presence of 5ng/ml LPS. The effect of LPS 5ng/ml alone is also shown. Results are expressed as percentage of media control; vertical bars indicate standard error of the mean (SEM); data were analysed by one-way ANOVA with post-hoc Dunnett's test.*

The effect of JWH007, GA001 and GA003 on LPS-stimulated TNFα release from macrophages

This experiment was carried out three times, on three different passages (n=3). Due to inter-experiment variability, the results for each passage are shown separately – i.e. each graph represents n = 1. Results were expressed as TNFα release in pg/mg protein (figure A) and as percentage of LPS stimulation (figure B). Results were not collated and statistical tests were not carried out due to the inter-experiment variability.

The effect of JWH007, GA001 and GA003 on LPS-stimulated TNFα release from macrophages (results from the first passage)

Figure 5.17A overleaf shows the effects of JWH007, GA001 and GA003 on TNFα release from RAW 264.7 macrophages. The vehicle (DMSO 0.02%) seemed to have a small effect on LPS stimulation of TNFα release, reducing the LPS effect by 27.6% (see figure 5.17B overleaf). 10μM JWH007 reduced LPS stimulation by 29.6% (no more than its DMSO vehicle), whereas 30μM JWH007 reduced LPS stimulation by 54.8 %. 10μM GA001 reduced LPS stimulation by 54.9% whereas 30μM GA001 reduced LPS stimulation by 68.1 %. 30μM GA003 reduced LPS stimulation by 29.0 % (no more than its DMSO vehicle).

A

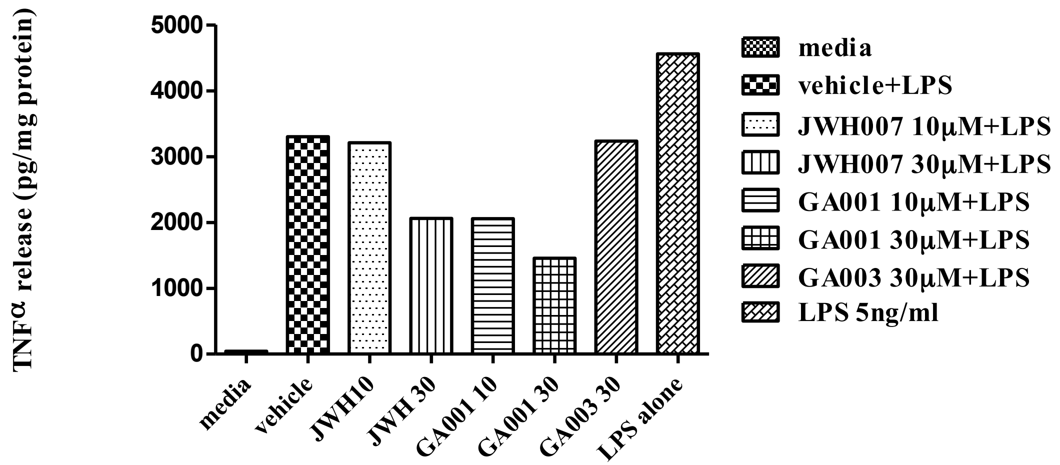


Figure 5.17A *The effect of JWH007, GA001, GA003 and their vehicle (DMSO 0.02%) (replicate number=1-3) on RAW 264.7 TNFα release after 16 hours in the presence of 5ng/ml LPS. The effect of LPS 5ng/ml alone is also showed. Results are expressed as TNFα release (pg/mg protein).*

B

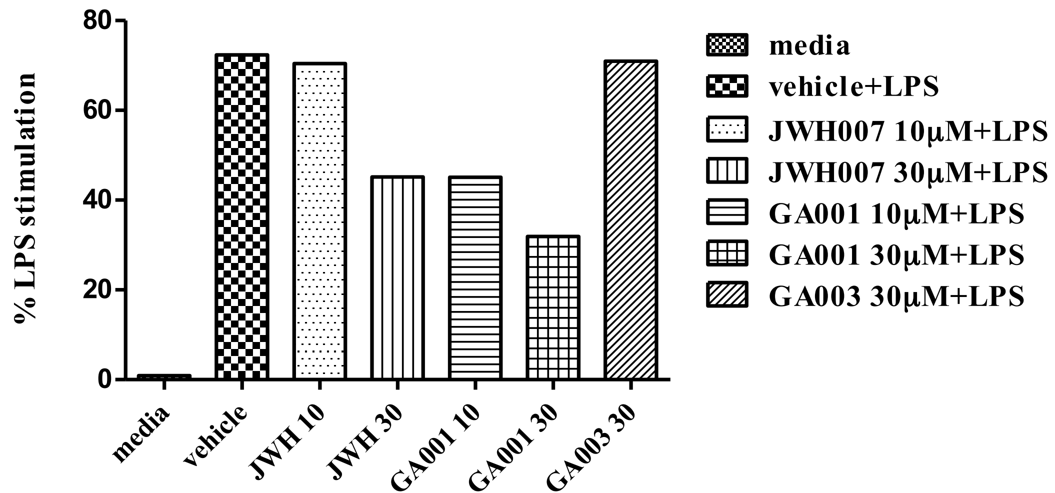


Figure 5.17B *The effect of JWH007, GA001, GA003 and their vehicle (DMSO 0.02%) (replicate number=1-3) on RAW 264.7 TNFα release after 16 hours in the presence of 5ng/ml LPS. The effect of LPS 5ng/ml alone is also showed. Results are expressed as % of LPS 5ng/ml stimulation.*

The effect of JWH007, GA001 and GA003 on LPS-stimulated TNFα release from macrophages (results from the second passage)

Figure 5.18A overleaf shows the effects of JWH007, GA001 and GA003 on TNFα release from RAW 264.7 macrophages. The vehicle (DMSO 0.02%) had a substantial effect on LPS stimulation of TNFα release, reducing the LPS effect by 72.3% (see figure 5.18B overleaf). 10μM JWH007 reduced LPS stimulation by 64.5%, whereas 30μM JWH007 reduced LPS stimulation by 52.8%. 10μM GA001 reduced LPS stimulation by 54.64% whereas 30μM GA001 reduced LPS stimulation by 56.8%. 30μM GA003 reduced LPS stimulation by 9.0%. The effect of the DMSO vehicle alone was greater than the effect of any of the cannabinoids tested.

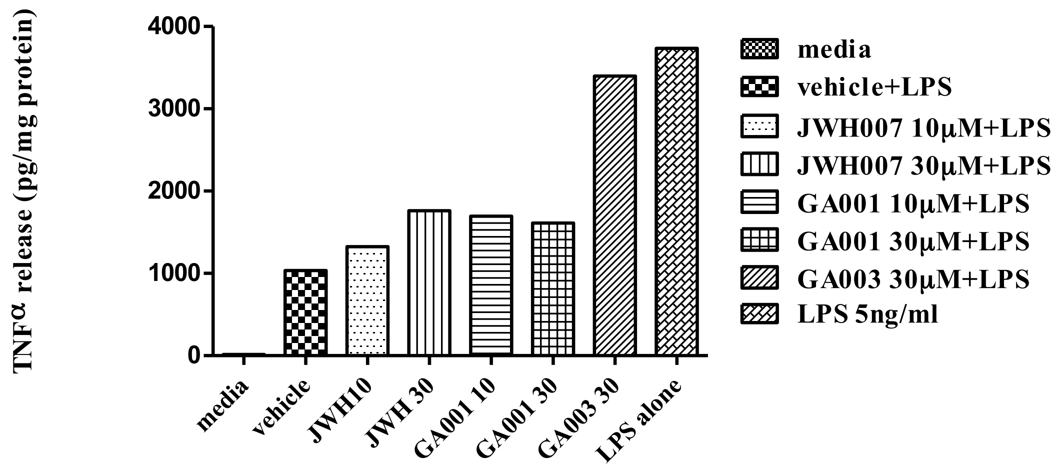


Figure 5.18A *The effect of JWH007, GA001, GA003 and their vehicle (DMSO 0.02%) (replicate number=2-3) on RAW 264.7 TNFα release after 16 hours in the presence of 5ng/ml LPS. The effect of LPS 5ng/ml alone is also showed. Results are expressed as TNFα release (pg/mg protein).*

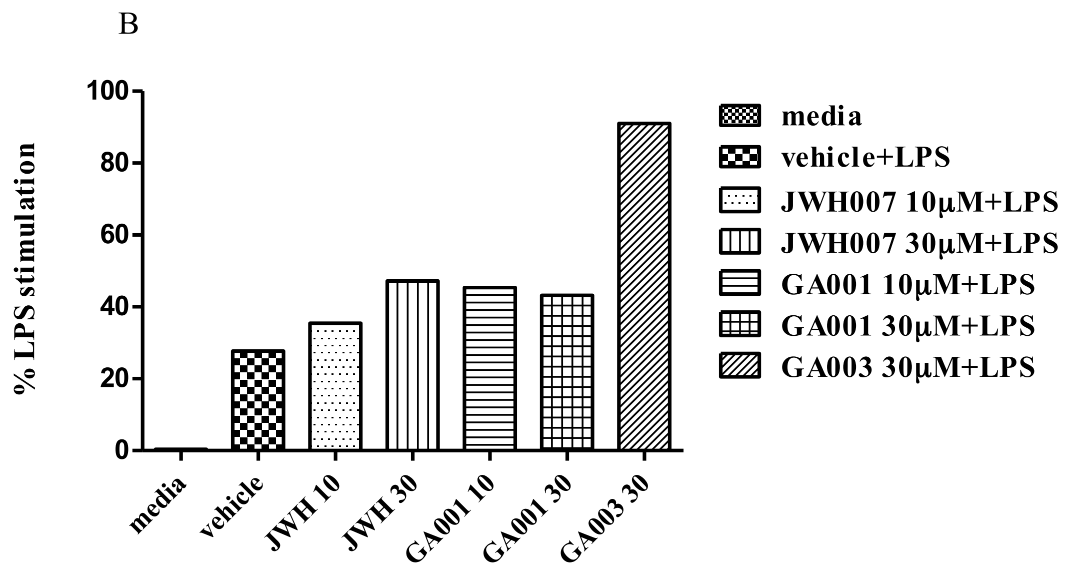


Figure 5.18B *The effect of JWH007, GA001, GA003 and their vehicle (DMSO 0.02%) (replicate number=2-3) on RAW 264.7 TNFα release after 16 hours in the presence of 5ng/ml LPS. The effect of LPS 5ng/ml alone is also showed. Results are expressed as % of LPS 5ng/ml stimulation.*

The effect of JWH007, GA001 and GA003 on LPS-stimulated TNFα release from macrophages (results from the third passage)

Figure 5.19A overleaf shows the effects of JWH007, GA001 and GA003 on TNFα release from RAW 264.7 macrophages. The vehicle (DMSO 0.02%) seemed to have a substantial effect on LPS stimulation of TNFα release, reducing the LPS effect by 84.1% (see figure 5.19B overleaf). 10μM JWH007 reduced LPS stimulation by 68.2%, whereas 30μM JWH007 reduced LPS stimulation by 63.5%. 10μM GA001 reduced LPS stimulation by 57.3% whereas 30μM GA001 reduced LPS stimulation by 34.7%. 30μM GA003 reduced LPS stimulation by 41.0%. The effect of the DMSO vehicle alone was greater than the effect of any of the cannabinoids tested.

A

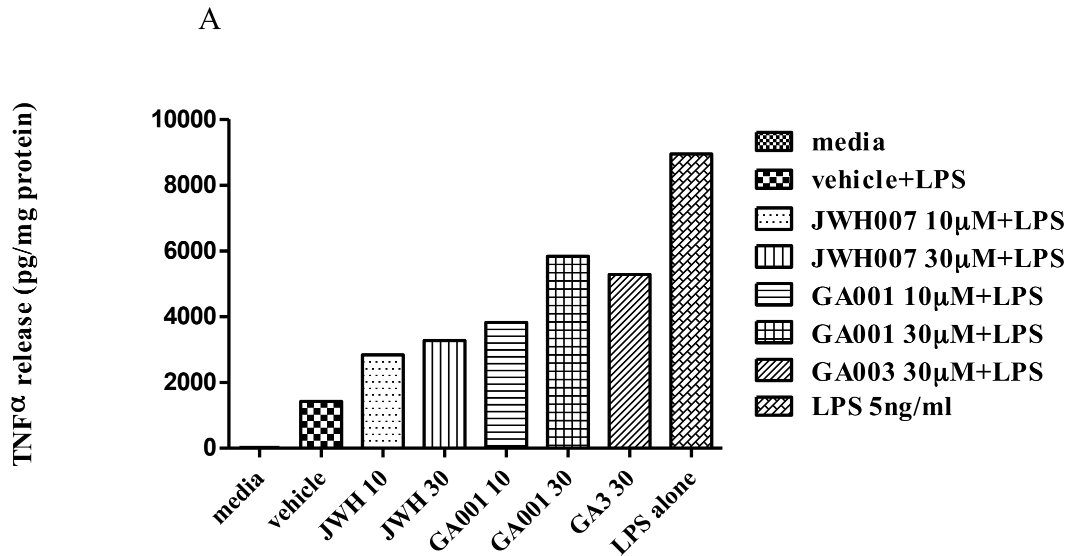


Figure 5.19A *The effect of JWH007, GA001, GA003 and their vehicle (DMSO 0.02%) (replicate number=2-3) on RAW 264.7 TNFα release after 16 hours in the presence of 5ng/ml LPS. The effect of LPS 5ng/ml alone is also showed. Results are expressed as TNFα release (pg/mg protein).*

B

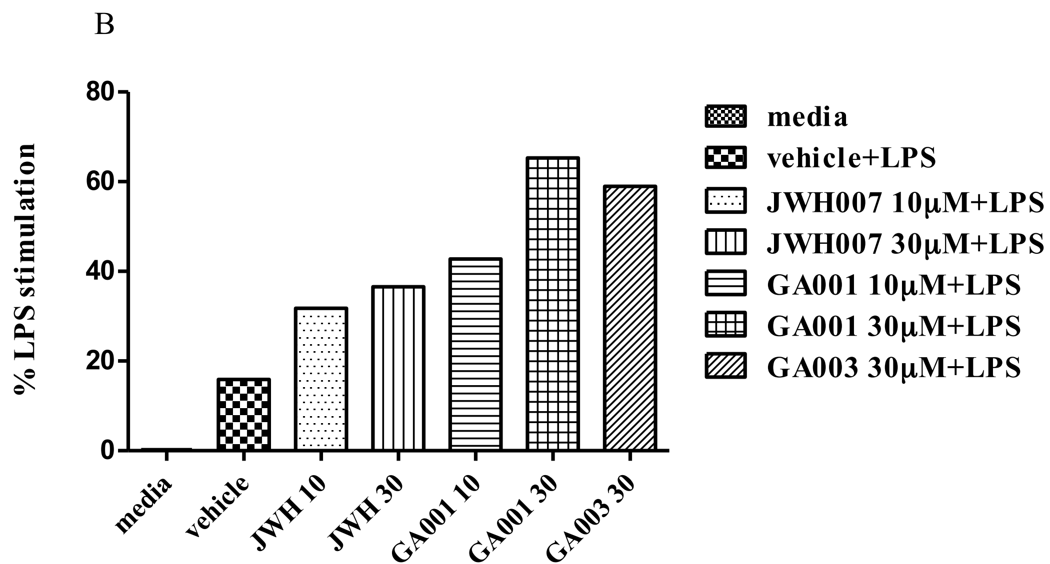


Figure 5.19B *The effect of JWH007, GA001, GA003 and their vehicle (0.02 DMSO%) (replicate number=2-3) on RAW 264.7 TNFα release after 16 hours in the presence of 5ng/ml LPS. The effect of LPS 5ng/ml alone is also showed. Results are expressed as % of LPS 5ng/ml stimulation.*

5.5 Discussion

The aim of this chapter was to set-up two reproducible pharmacological assays to test the efficacy of the novel cannabinoid compounds (based on the structure of JWH007) and compare them to the parent cannabinoid, the non-selective $CB_{1,}CB_2$ agonist JWH007. One assay was designed to test for potency at the CB_1 receptor and the other to test for CB_2 efficacy. Activity at both of these receptors was thought to be important as the literature at the time suggested that the CB_1 receptor was involved in inhibiting intestinal motility and enhancing wound healing (Heinemann et al., 1999; Izzo et al., 1998; Wright *et al*., 2005) and the CB_2 receptor was involved in reducing inflammation (Patel *et al*., 2010). All of these actions are considered to be therapeutically desirable as inflammatory bowel disease (IBD) is characterised by inflammation of sections of the GI tract, accompanied by increased intestinal motility, leading to symptoms such as cramps and diarrhoea (Kumar and Clark., 2009).

The Paton ileum model was used to investigate the CB_1 activity of the novel cannabinoids. In this model, the effects on intestinal motility were determined by measuring the size of electrically-stimulated contractions in the guinea-pig isolated ileum before and after the addition of the cannabinoids (see 'Electrical field stimulation', chapter 2). The rationale for using this as a CB_1 assay was that previous studies had reported that cannabinoids inhibited ileal contractions through activation of the CB_1 receptor (Heinemann *et al.*, 1999; Izzo *et al.*, 1998).

Although cannabinoid-induced inhibition of intestinal contractions was thought to be CB_1 mediated, this thesis has produced evidence that the inhibition is mediated through a non- CB_1, non- CB_2 receptor. The mechanisms involved, therefore, require further

investigation and this has been discussed in more detail in chapter 3. The Paton ileum model was still retained for the testing of novel cannabinoids, not as a CB_1 assay but to investigate the anti-motility effects of the drugs. It is important for a new therapeutic for IBD/IBS to reduce motility in order to alleviate intestinal cramps and diarrhoea.

In the Paton ileum model, JWH007 (the parent cannabinoid) was found to reduce the size of EFS-stimulated contractions in a concentration-dependent manner reaching 72 ± 8.07% inhibition at the highest concentration of $3x10^{-6}$M. The novel cannabinoid GA001 and GA002 found to show the same concentration-dependent inhibitory effect as JWH007 and to be of similar potency (see IC_{25}s and IC_{50}s and responses at the maximum concentration used; figure 5.14 and table 5.1). GA003 on the other hand had no effect on the size of EFS-induced contractions at any concentration used. These results show that, when JWH007 is modified with just a methyl group attachment (GA001) or a dendritic wedge (GA002), pharmacological activity in the Paton ileum model does not alter. However, activity is abolished when a longer spacer chain is attached to JWH007 (GA003), probably due to steric hinderence preventing receptor binding. Unfortunately, due to a short supply of GA006 (the final cannabinoid-dendrimer conjugate), this could not be tested in the ileum. As GA003 had no effect on the ileal contractions, this could mean that GA006 would also have no effect, since it is an even larger compound. Alternatively, the addition of the dendrimer may stabilise the spacer, prevent the interference with binding and restore activity.

The effects of cannabinoids their vehicles in the second pharmacological assay (TNFα release) are more complicated. Initially, the cannabinoids were screened to make sure they were not toxic to the macrophages.This was assessed by looking at the effects of

the cannabinoids on macrophage protein concentration and the MTS assay (cell viability) results. The results show that DMSO 0.02%, 10μM and 30μM JWH007 and have no effect on cell viability or macrophage protein concentration. GA003, though not tested in the MTS assay, had no effect on macrophage protein concentration. These results suggest that these drugs are not toxic to the macrophages. However, 30μM GA006 has a substantial effect on cell viability in the MTS assay, reducing viability by 97.1± 1.4%. This suggests that the cannabinoid-dendrimer conjugate is toxic to this macrophage cell line. This is an interesting result as JWH007 was not shown to be toxic and dendrimers are reportedly non toxic (Scharbin *et al.*, 2010). Ideally, the dendrimer alone would have been tested in the cell viability assay but, due to time constraints, this could not be carried out.

When the cannabinoids JWH007, GA001 and GA003 were tested in the LPS stimulated macrophages, there was a great deal of inter-experiment variability. The three experiments were carried out on three different cell passages at different times. Due to the variability, the results for each passage are shown separately but cannot be statistically analysed as each passage is only counted as n =1.

In the first experiment, the DMSO vehicle seemed to have a small effect on LPS stimulation of TNFα release, reducing the LPS effect by 27.6%. JWH007 and GA001 inhibited LPS-stimulated TNFα release but GA003 had no more effect than its DMSO control. GA001 seemed to be more potent than JWH007 (30μM GA001 reduced LPS stimulation by 68.1%, whereas 30μM JWH007 only reduced LPS stimulation by 54.8 %). This suggested that the methyl group attachment to JWH007 enhanced activity in this assay. However, conjugation to a longer spacer chain (GA003) abolished activity, which supports the results obtained in the ileum. As in the ileum, the spacer probably

produces steric hindrance to prevent receptor binding. It is important to note though, as this was only an *n* of 1, statistical analysis could not be carried out.

The two subsequent experiments in the macrophages, however, did not show the same results. In these experiments, the DMSO vehicle (0.02%) had a substantial effect on the LPS stimulated TNFα release, reducing the release by 73-85%. In comparison, none of the cannabinoids reduced TNFα release to the extent of the control alone.

The macrophage assay clearly needs further optimisation. The inter-experiment variability may be due to differences in cell confluency because this was estimated by observing the cells through a microscope, and is open to human error. When the assay has been optimised, the cannabinoids need to be tested again and also the cannabinoid-dendrimer conjugate, GA006 (which was not tested here). However, from the cell viability assay, it seems as though GA006 is highly toxic to cells, so this may skew the results for GA006 in this assay.

In conclusion, JWH007 was active in both assays and this activity did not seem to be adversely affected by conjugation to the small methyl group (GA001). In fact, in the cell-based assay, conjugation to the methyl group seemed to enhance the activity of JWH007. However, conjugation to the longer spacer chain (GA003) seemed to abolish activity, which may be due to steric hindrance preventing receptor binding. As GA003 had no effect on the ileal contractions or TNFα release, this could mean that GA006 would also have no effect, since it is an even larger compound. Alternatively, the addition of the dendrimer may stabilise the spacer, allow binding to the receptor (s) and restore activity.

Although GA006 still needs to be tested in the macrophage and ileum assay, the cell viability assay suggested that GA006 was extremely toxic to macrophages. Even if activity is restored, its toxicity is likely to preclude its use as a therapeutic entity.

CHAPTER 6:

GENERAL DISCUSSION

6. General Discussion

Cannabinoids show great promise in the treatment of inflammatory bowel disease. They have been found to reduce intestinal secretions, motility and inflammation (Izzo *et al*., 2010). They have also been shown to reduce visceral pain and enhance wound healing in the gut (Wright *et al*., 2005). The mechanisms involved in these effects have not been fully elucidated, however, and cannabinoid pharmacology in the gut may not be as straightforward as first thought. The major problem with a cannabinoid-based therapeutic would be the resulting psychotropic side effects. It may be possible to overcome this by conjugating the cannabinoid with a dendrimer, in order to prevent the cannabinoid from reaching the CNS whilst retaining absorption across the intestine.

The aims of this thesis were to investigate the cannabinoid receptors involved in intestinal motility and also to examine the protection afforded by cannabinoids against the type of tissue damage caused by inflammation, as this has not previously been studied in the intestine. Another aim was to determine whether cannabinoids retain their potency when conjugated to a bulky group such as a dendrimer.

There are several issues concerning the pharmacological tools available to study cannabinoid responses in the gut. Firstly, cannabinoid agonists and antagonists are highly lipophilic. As they are poorly soluble in water, organic solvents such as DMSO or ethanol must be used to dissolve these compounds. DMSO and ethanol however, were both found to inhibit EFS-induced contractions in the ileum (see 'Choice of cannabinoid vehicle in the ileum, chapter 2). Ethanol was chosen as the vehicle where possible as this had the least effect on contractions, providing the concentrations were kept to a minimum. DMSO was used as the cannabinoid vehicle in the macrophage

experiments but the effect of this vehicle was variable. In some experiments it had little effect but in others the effect was substantial. This may have been due to differences in cell confluency. Another problem with the high lipophilicity of cannabinoids is that they cannot be washed out of tissues even with multiple washes (see appendix 1). For this reason, cannabinoid was not re-added after the washout stages in chapter 5.

An additional issue is that the antagonists available are not highly selective. For example, the supposedly selective CB_1 antagonists, rimonabant and AM251 have been shown to act at several other receptors at concentrations used in pharmacological studies (see table 1.4, chapter 1 and Pertwee *et al*., 2010). Also, there are few antagonists available for the putative cannabinoid receptors. There is only one receptor antagonist for the putative CB_e receptor as this receptor has not been cloned yet. Similarly, one antagonist is available for the GPR55 receptor, (-) cannabidiol, but this is not very selective (Petitet *et al*., 1998; Thomas *et al*., 2007., Ryberg *et al*., 2007). No antagonist has been developed for the GPR119 receptor, so it was difficult to establish whether this receptor was involved in any of the effects observed in this study.

The currently held view is that cannabinoids inhibit intestinal motility through activation of the CB_1 receptor (Colombo *et al*., 1998; Coutts *et al*., 1997; Izzo *et al*., 2000a; Pertwee *et al*., 1996). However, much of this evidence relies on the use of one CB_1 receptor antagonist, rimonabant, which does not seem to be as selective as first thought (see table 1.4, chapter 1). In addition, the effects of selective CB_1 agonists on intestinal motility do not seem to have been explored. This, together with new evidence for non-CB_1, non-CB_2 effects on intestinal motility (Mang *et al*., 2001; Smid *et al*., 2007), suggests that cannabinoid pharmacology of the gut may be more complicated than first thought.

Results from this thesis suggest, contrary to current opinion, that the CB_1 receptor is not involved in cannabinoid-mediated inhibition of EFS-evoked contractions of the guinea-pig ileum. All cannabinoids were found to reduce the size of contractions in a concentration-dependent manner but this was not blocked by either of the CB_1 receptor antagonists (AM281 and rimonabant) used. This was further supported by the fact that ACPA had no effect on EFS contractions, even though the concentration added to the bath (1×10^{-4}M) was 50000 times higher than its Ki value (2.2×10^{-9} M) for the CB_1 receptor (see table 1.1, chapter 1). The CB_1 antagonists (AM281 and rimonabant) alone increased EFS-induced contraction size in this study, which supports the literature. However, researchers have suggested that this is due to inverse agonism or antagonism of endogenous agonists at the CB_1 receptor (Pertwee *et al.*, 1996). The results found in this study however, suggest that this is not the case as the CB_1 receptor does not seem to be involved in the inhibition of contraction. Therefore, AM281 and rimonabant would appear to be acting via a different mechanism other than inverse agonism of CB_1 receptors; a mechanism that is currently unknown.

This thesis provides preliminary evidence that GPR119 is present on ileal smooth muscle and that this receptor may be involved in controlling motility. This was suggested by the inhibition of EFS and carbachol-induced contractions by the GPR119 agonist PSN. These responses were observed at similar concentrations to the agonist's EC_{50} at the GPR119 receptor (in the yeast fluorimetric assay; Overton *et al.*, 2006), although the involvement of GPR119 in the responses needs to be confirmed when a GPR119 antagonist becomes available.

This study also suggests that at least one other receptor may be involved in contractile inhibition. Both O-1602 and (+)WIN caused a reduction in the size of EFS-induced contractions, but these responses were not blocked by any antagonist, suggesting that the responses were not mediated by any of the classical receptors, GPR55 or the CB_e receptor. Simultaneous blockade of the CB_1 and CB_2 receptor also had no effect on the (+)WIN-induced inhibition of contractions. GPR119 does not seem to be involved as (+)WIN does not bind to GPR119 and neither (+)WIN nor O-1602 reduced carbachol-induced contraction.

As (-)WIN had no effect on contractility of the ileum, this strongly suggests that the response to (+)WIN is mediated by a protein target such as a receptor. It is unclear whether this is the case for O-1602. In the future, the effect of pertussis toxin (which inactivates Gi and Go G proteins) on cannabinoid-mediated inhibition of ileal contractions could be investigated. This would give an indication of whether the effects of these compounds are mediated through a Gi protein-coupled receptor.

With regard to cannabinoid-mediated protection against inflammatory damage, the cardioprotection and hepatoprotection (Montecucco *et al*., 2009; Raj *et al*., 2007) is thought to be CB_2 receptor mediated whereas cannabinoid receptor-independent mechanisms may be involved in the neuroprotective effects (Hampson *et al.*, 1998). The studies reported in this thesis are the first to investigate potential protective effects of cannabinoids in the ileum.

(+)WIN effectively limited the contractile dysfunction induced by H_2O_2, demonstrating cannabinoid-mediated tissue protection. The receptors CB_1, CB_2 and CB_e do not seem to be involved in cannabinoid-mediated protection of the ileum as (+)WIN's protective

effect was not blocked by rimonabant, SR144528 or O-1918. This was supported by the lack of effect of ACPA and abnormal-CBD cannabidiol. The GPR119 and GPR55 receptors are unlikely to be involved as the GPR119 and GPR55 agonists, PSN and O-1602 respectively, had no effect on the H_2O_2-induced damage.

As the protective effect of (+)WIN does not seem to be due to any of the classical or putative cannabinoid receptors, it may either be mediated through a novel receptor or it may be a cannabinoid receptor independent effect.

(+)WIN could be acting as a free radical scavenger to prevent tissue damage. As (+)WIN could not be washed out, the cannabinoid would have been present to act as a scavenger and reduce oxidative damage. Anti-oxidant effects have been shown to contribute to the neuroprotective (Hampson *et al.*, 1998), cardioprotective (Montecucco *et al.*, 2009) and hepatoprotective (Rajesh *et al.*, 2007) effects of cannabinoids. The CB_1 (Kessiova *et al.*, 2006) and CB_2 (Montecucco *et al*., 2009; Rajesh *et al*., 2007) receptors have previously been implicated in the anti-oxidant effects of cannabinoid as well as cannabinoid receptor independent effects (Chen *et al.*, 2000; Kessiova *et al.*, 2006). If (+)WIN were acting as a free radical scavenger, this would be consistent with the cannabinoid receptor independent effect observed in this study and reported in some of the literature (Chen *et al.*, 2000; Kessiova *et al.*, 2006).

In the literature, cannabinoids were shown to be neuroprotective through non-cannabinoid-receptor mediated antioxidant effects (Hampson *et al.*, 1998). This could be the case in the gut, i.e. the antioxidant effect of (+)WIN may protect cholinergic neurones in the myenteric plexus. As it is not clear with the EFS-evoked contractions whether the H_2O_2-damage is to the myenteric neurones or smooth muscle it would be

interesting to look at the effects of H_2O_2 on carbachol-induced contractions of the ileum, to see if it affects smooth muscle directly and to look at the effect of (+)WIN in this assay.

One of the aims of the current studies was to attempt to conjugate a cannabinoid to a large dendrimer such that it would be excluded from the CNS. It was not known if such a dendrimer would retain pharmacological activity and so had to be tested in cannabinoid receptor assay systems. The Paton ileum model was still retained for the testing of novel cannabinoids, even though it was shown not to be a CB_1 assay, to investigate the anti-motility effects of the drugs. It is important for a new therapeutic for IBD to reduce motility in order to alleviate intestinal cramps and diarrhoea.

The cannabinoid JWH007 was active in both assays and this activity did not seem to be reduced by conjugation to the small methyl group (GA001). However, conjugation to the longer spacer chain (GA003) seemed to abolish activity in the assays, possibly due to steric hindrance preventing receptor binding. This could mean that the bulkier GA006 would also have no effect, as addition of the dendrimer could increase steric hindrance. It is possible, however, that activity of the cannabinoid may be restored by the addition of the dendrimer as this may stabilise the spacer and allow binding to the receptor (s). Unfortunately, due to a shortage of GA006, the pharmacological activity of this compound could not be tested. Surprisingly, GA006 was shown to be highly toxic in the cell viability assay which may limit its potential as a therapeutic. This result was unexpected as the cananbinoid (JWH007) alone did not affect cell viability and PAMAM dendrimers are reportedly non-toxic (Scharbin *et al.*, 2010), suggesting it is the conjugation of these compounds which is noxious to cells.

In summary, this thesis has shown that cannabinoids reduce contractions and protect against inflammatory damage in the ileum, which are two desirable effects in the treatment of IBD. However, contrary to the current held view (Colombo *et al*., 1998; Coutts *et al*., 1997; Izzo *et al*., 2000a; Pertwee *et al*., 1996), the CB_1 receptor was not shown to be involved in cannabinoid-mediated inhibition of contraction. This study has instead provided preliminary evidence that GPR119 may be present on smooth muscle and is involved in inhibiting contractions. This contrasts findings in the literature which suggest that cannabinoids do not act post-synaptically to exert their effects (Lynn *et al*., 1994; Mang *et al*., 2001; Pertwee., 2001). There is also evidence for a pre-synaptic receptor distinct from any of the classical or putative cannabinoid receptors.

The cannabinoid-mediated protection against inflammatory-type damage also did not seem to be mediated by any of the known or putative cannabinoid receptors which compares with cannabinoid receptor-independent effects supported in the literature. It is possible that this protection could be due to free radical scavenging, as this would be consistent with a receptor-independent effect. However, both cannabinoid-mediated protection against inflammatory damage as well as inhibition of contraction require more extensive research.

Although cannabinoids have shown effects which may be beneficial in the treatment of IBD, the psychotropic side effects are a major drawback. Conjugation to a dendrimer may prevent the psychotropic side effects associated with cannabinoids, but this thesis has shown that the toxicity of the cannabinoid-dendrimer GA006 will limit its use as a therapeutic for IBD. In the future, other conjugations could be tested, for example, using different cannabinoids, dendrimers or attachment strategy. However, as the pharmacology of cannabinoids in the gut progresses, the CB_1 receptor may emerge as a

less important target for IBD therapeutics than other receptors. It may then be possible to develop a therapeutic using a cannabinoid which does not bind to CB_1. This would avoid the psychotropic effects of cannabinoids without the need to conjugate the compounds to bulkier molecules.

CHAPTER 7:

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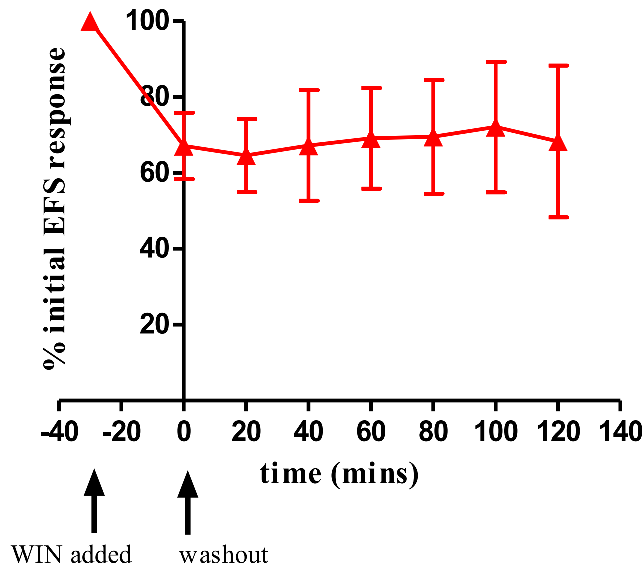
APPENDIX 1

Appendix 1

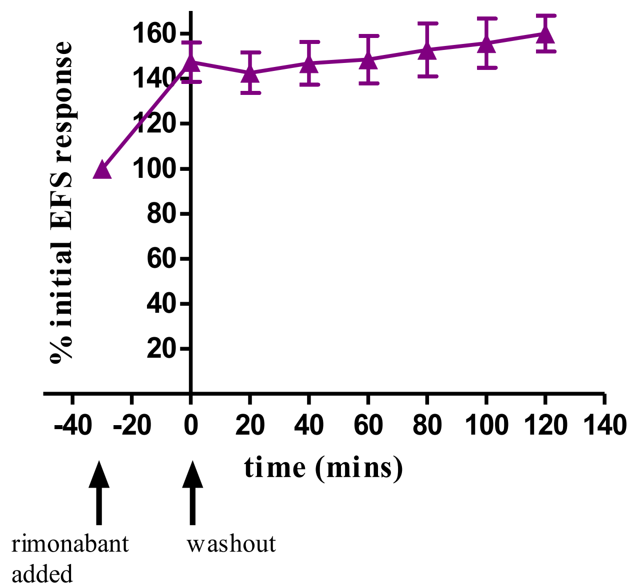
The effects of cannabinoid washout in the isolated guinea-pig ileum

The effect of washout on WIN



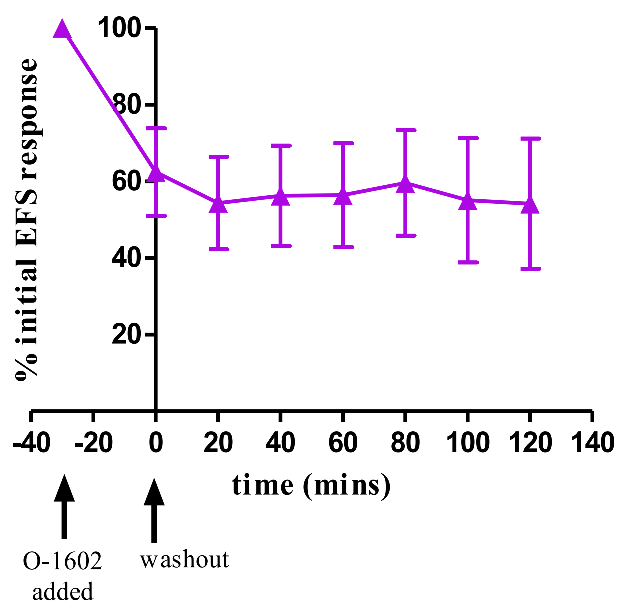
WIN 10^{-7}M was added to the bath and then washed out after 30 minutes.

The effect of washout on rimonabant



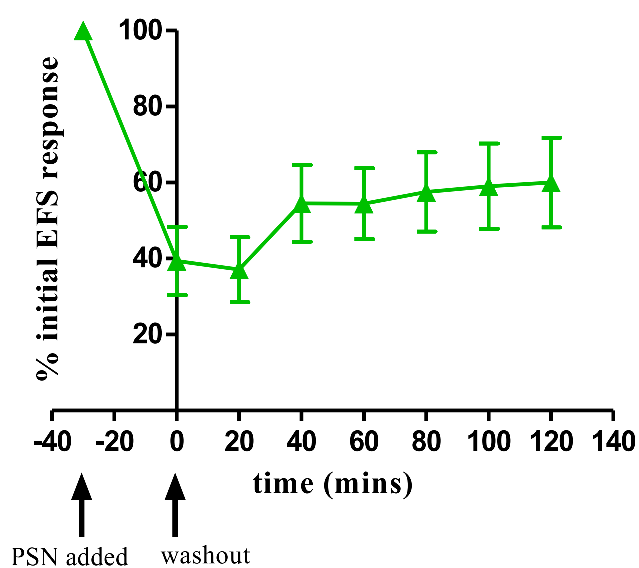
Rimonabant 10^{-6}M was added to the bath and then washed out after 30 minutes.

The effect of washout on O-1602



O-1602 1x10^{-5}M was added to the bath and then washed out after 30 minutes.

The effect of washout on PSN



PSN 3x10^{-5}M was added to the bath and then washed out after 30 minutes.