An investigation into the targets for cannabinoid action in the ciliate *Tetrahymena pyriformis*

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Declaration

This thesis has not been submitted in support of an application for another degree at this or any other university. It is the result of my own work and includes nothing that is the outcome of work done in collaboration except where specifically indicated. Many of the ideas in this thesis were the product of discussion with my supervisor Dr. Jackie Parry and Dr. Karen Wright.

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Glossary

2-AcG	**2-Acylglycerol**
2-AG	**2-Arachidonoylglycerols**
2-EG	**2-eicosenoylglycerol**
2-GLG	**2-γ-linolenoylglycerol**
2-LG	**2-linoleoylglycerol**
2-OG	**2-oleoylglycerol**
2-PG	**2-palmitoylglycerol**
2-SG	**2-stearoylglycerol (2-SG)**
AA	**Arachidonic acid**
AEA	***N*-arachidonoyl ethanolamine, Anandamide**
CB	**Cannabinoid receptor**
CBD	**Cannabidiol**
COX	**Cyclooxygenase**
DAGL	**Diacylglycerol Lipase**
DHA	**Docosahexaenoic acid**
EEA	***N*-Eicosenoylethanolamine**
EPA	**Eicosapentaenoic acid**
FAAA	**Fatty acids amides of amino acids**
FAAH	**Fatty acid amide hydrolase**
FABP	**Fatty acids binding protein**
GLA	**γ-linolenic acid**
GLEA	***N*-γ-Linolenoylethanolamine**
GPCR	**G protein-coupled receptor**
HPD	**Haloperidol**
IC$_{50}$	**Half maximal inhibitory concentration**
LA	**Linoleic acid**
LEA	***N*-Linoleoylethanolamine**
LOX	**Lipoxygenase**
MAGL	**Monoacylglycerol lipase**
MAGs	**Monoacylglycerols**
MIC	**Minimum inhibitory concentration**
NAAA	***N*-Acylethanolamine-hydrolysing acid amidase**

NAEs	***N*-Acylethanolamines**
NAPE-PLD	***N*-Acyl-Phosphatidylethanolamine Phospholipase D**
OEA	***N*-Oleoylethanolamine**
PEA	***N*-Palmitoylethanolamine**
PPAR	**Peroxisome proliferator-activated receptor**
PPRE	**Peroxisome proliferator response elements**
PTX	**Pertussis toxin**
RXR	**Retinoid X-receptor**
SEA	***N*-Stearoylethanolamine**
TRPV1	**Transient receptor potential vanilloid type 1**
Δ^9-THC	**Delta-9-Tetrahydrocannabinol**

Abstract

The human endocannabinoid system (ECS) modulates many vital physiological and neuromodulatory functions. It comprises lipid endocannabinoids, e.g. Anandamide (AEA), which are synthesized on demand and which bind to receptors (e.g. CB_1, CB_2) to elicit a response. Single-celled protists can respond to endocannabinoids despite not possessing any of the known cannabinoid receptors suggesting they possess a rudimentary ECS with an unknown target for the endocannabinoids.

The ciliate *Tetrahymena thermophila* possesses a suite of endocannabinoids (including AEA), with *N*-palmitoylethanolamide (PEA) and *N*-oleoylethanolamide (OEA) being two of the dominant compounds. In humans, the natural receptor for the latter is the peroxisome proliferator-activated receptor alpha (PPARα) but this receptor has not been identified in the *T. thermophila* genome. It may be that *Tetrahymena* spp. possess a 'PPAR-like' receptor or they possess a different target, which mediate a reaction to these endocannabinoids.

This study examined the potential role of 'PPARs' in the ECS of *Tetrahymena pyriformis*. Cell death was examined in the presence of agonists to each of the three known PPAR types, i.e. OEA/PEA/AEA/Cannabidiol (CBD) (to PPARα), GW0742 (to PPARβ/δ), and Rosiglitazone (PPARγ). All agonists induced ciliate cell death and all, except PEA and Rosiglitazone, gave IC_{50} values similar to those recorded for human cells (GW0742, 12 µM; OEA, 45 µM; AEA and CBD, 4 µM). The negative action of some of these agonists could be alleviated by blocking PPAR isoforms with their respective antagonists (GW6471 for PPARα, GSK3787 for PPARβ/δ, and T0070907 for PPARγ). The action of AEA and GW0742 were alleviated with all antagonists, CBD was only alleviated with GW6471 (for PPARα), and OEA was alleviated by none. Results suggest that *T. pyriformis* possesses a PPAR-like protein/target for AEA, CBD, and GW0742, but not for OEA.

Since the target of OEA was not a PPAR, this study explored other potential targets by using antagonists against the Dopamine receptor (Haloperidol), Gα$_{i/o}$ (Pertussis toxin [PTX]), and Protein kinase A (H89). Only PTX slightly alleviated the effect of OEA, suggesting a Gα$_{i/o}$ receptor mechanism.

Although the results are preliminary, further study may lead to the mapping and deeper understanding of the nature of the ECS in *Tetrahymena* and these non-CB pathways may be a possible therapeutic target in key diseases such as cancer, obesity and diabetes.

1. Introduction

The human endocannabinoid system (ECS) is essential in the modulation of many fundamental regulations from cellular activities to physiological functions such as neurodegeneration, psychosis and anxiety, cardiovascular, pain, and appetite (Bih et al., 2015; Zou and Kumar, 2018). The ECS is a complex signaling pathway found throughout the body, being dominant in the brain, cardiovascular, nervous, reproductive, intestinal and the immune tissues (Zou and Kumar, 2018). This makes cannabinoids a novel therapeutic target for a range of diseases including cancer, diabetes, anxiety, addiction, epilepsy and many other neurological disorders including Parkinson's and Alzheimer's disease (Bih et al., 2015; Dariš et al., 2019).

Many years of research has been dedicated to identifying and understanding the nature and individual components of the ECS, especially the effects of cannabinoid compounds such as cannabidiol (CBD), delta-9-tetrahydrocannabinol (Δ^9-THC), 2-arahidonoyl glycerol (2-AG) and anadamide (AEA) upon it. However, studies have often been contradictory, suggesting a broad mechanism of action due to numerous possible receptors. As such, studying the endocannabinoid system in a simpler organism, which has no known receptors but can still respond to cannabinoids, may help to gain a better understanding of the nature of the ECS.

The ciliate *Tetrahymena* is a single-celled eukaryote which is widely used as a model organism in biomedical studies due to its low maintenance, rapid growth and the availability of DNA and genomic data (Ruehle et al., 2016). The study of ECS in *Tetrahymena* may reveal interesting, yet, simpler signaling pathways of the eukaryotic ECS in general.

This literature review explains the fundamental function of the ECS in humans and its main components; cannabinoid ligands, the four main receptors (both CB and non-CB), and their metabolizing enzymes. The review then provides evidence for a rudimentary ECS in single-celled eukaryotes and in particular, in the ciliate *Tetrahymena,* which despite possessing none of the four main cannabinoid receptors, responds negatively to cannabinoids. This review then further explores the most likely potential cannabinoid receptors in this ciliate; the peroxisome proliferator-activated receptor (PPAR), Dopamine (D) and orphan G protein-coupled receptors (GPCRs).

1.1.   The human endocannabinoid system

The human endocannabinoid system comprises cannabinoid receptors, together with their lipids ligand and the enzymes which synthesized and degrades them (Pertwee et al., 2010; Lu and Mackie, 2016). The system has a broad neuro-modulatory effect as well as non-neuronal effects such as the regulation of appetite through the gastrointestinal (GI) tract and fertility through the reproductive system (Izzo and Sharkey, 2010; Zou and Kumar, 2018).

The extracts of the plant *Cannabis sativa* (marijuana) have long been used for medicinal purposes to treat ailments such as cramp and provide pain relief (Mechoulam, 1986; Pertwee, 2009). However, only in the mid-1900s was the first cannabis constituent discovered, i.e., delta-9-tetrahydrocannabinol (Δ^9-THC), which is the main psychoactive phytocannabinoid of the *C. sativa* (Gaoni and Mechoulam, 1964). Then followed the discovery of the first cannabinoid receptor, now referred as CB_1 (Matsuda et al., 1990). Its identification and successful cloning then led to the discovery of the second cannabinoid receptor, CB_2, and the first 'endocannabinoid' *N*-arachidonoylethanolamine (anandamide; AEA) (Devane et al., 1992; Munro et al., 1993). This was followed shortly by the discovery of the second endocannabinoid 2-arachidonoyl glycerol (2-AG) (Sugiura et al., 1995; Mechoulam et al., 1995).

The 'endocannabinoids' are named as such because mammalian tissues are capable of producing their own cannabinoids on demand, which are released and bind to cannabinoid receptors to elicit a response. It is noteworthy that although these first two endocannabinoids (AEA and 2-AG) bind to CB_1 and CB_2, they possess distinctive properties such that 2-AG is a full agonist of both receptors with moderate to low binding affinity, whereas AEA is a partial agonist of CB_1 with high binding affinity but has almost no binding affinity for CB_2 (Pertwee et al., 2010). In addition, these agonists also induce activation of numerous non-CB receptors. For example, AEA can bind to the transient receptor potential channel vanilloid 1 (TRPV1), Dopamine, peroxisome proliferator-activated receptor (i.e. PPARα and PPARγ), and various other orphan G protein coupled receptors (e.g. GPR55) (Zygmunt et al., 1999; Ahluwalia et al., 2003a; Pertwee et al., 2010; Lee et al., 2016). Thus, in addition to the main CB receptors, cannabinoids are capable of binding to more than one receptor, depending on cell types and site of actions, which results in the broad activation of signaling pathways.

During endocannabinoid retrograde signaling, the endocannabinoids AEA and 2-AG are synthesized on demand in response to an increased intracellular Ca^{2+} concentration and which are then degraded after activation of the CB_1 and other non-CB targets (Katona and Freund, 2008) (**Figure 1.1**). Generally, AEA is synthesized from *N*-acyl-phosphotidylethanolamine (NAPE) by the enzyme NAPE-specific phospholipase D (NAPE-PLD) (Okamoto et al., 2004). On the other hand, 2-AG is synthesized from diacylglycerol (DAG) by two of the DAG lipases - DAGLα and DAGLβ (Murataeva et al., 2014). Once transported to the site of action and taken up by the cells, fatty acid amide hydrolase (FAAH) catabolized AEA into free arachidonic acid (AA) and ethanolamine, whereas 2-AG is degraded to AA and glycerol by monoacylglycerol lipase (MAGL). Catalysis of AEA and 2-AG sometimes involves the oxidative enzymes such as cyclooxygenase-2 (COX2) and other lipoxygenases (LOX) (Deutsch et al., 2002; Zou and Kumar, 2018).
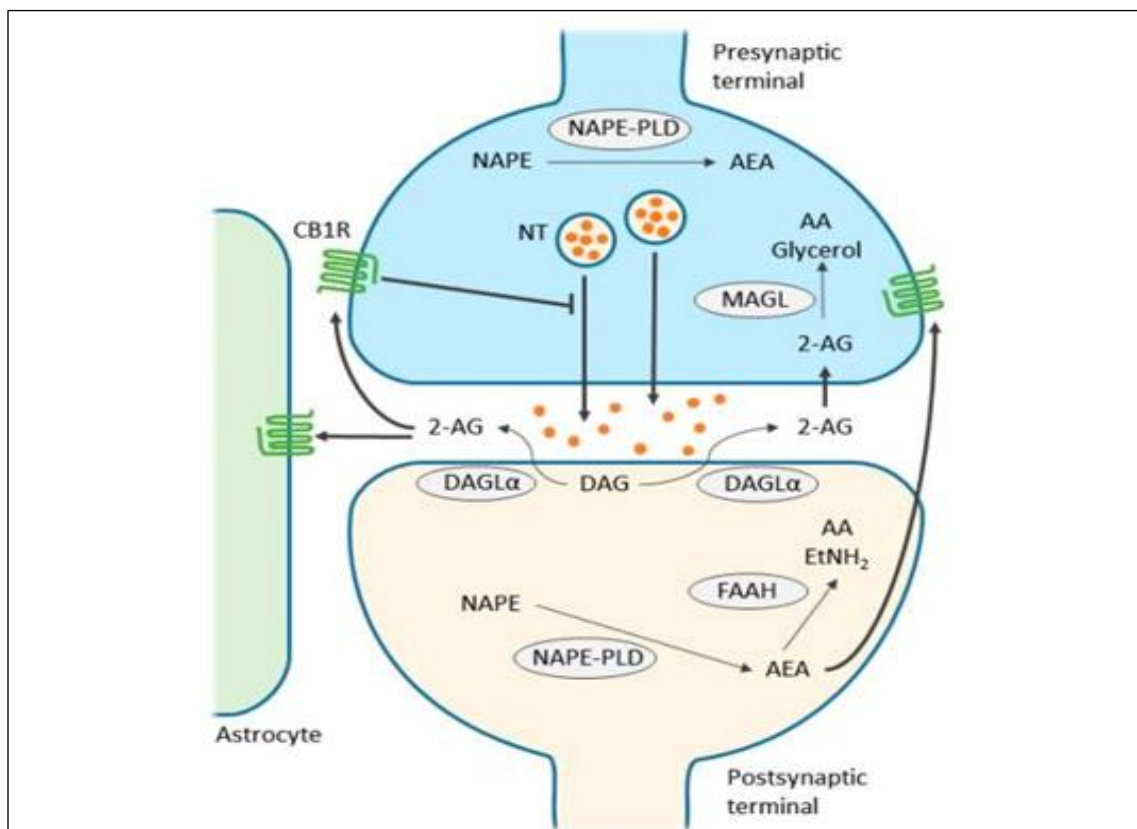

Figure 1.1: Simplified schematic representation of endocannabinoid retrograde signaling in synaptic transmission, as an example of an endocannabinoid system; comprised of the cannabinoid receptor (in this case CB_1), cannabinoid ligands (AEA and 2-AG), and their metabolic enzymes (NAPE-PLD, FAAH, DAGL, MAGL) (Zou and Kumar, 2018).

In the past few decades the study of the endocannabinoid system has gained more attention due to the promising therapeutic effects of the agonists on various diseases including cancer, Parkinson's, epilepsy and many more (Hill et al., 2012; Patil et al., 2015). The extensive study of the ECS has demonstrated that cannabinoid receptor agonists induce the activation of non-CB receptors, and at the same time, CB receptors can be activated by wide array of ligands. This indicates that the ECS is involved in broad modulatory functions and also plays a significant role in sustaining life, and therefore the understanding of this system may lead to uncovering many further therapeutic treatments.

1.2. The main cannabinoid ligands

There are many ligands involved in the activation and inhibition of the endocannabinoid system, including the endo-, synthetic- and exogenous cannabinoids. The endocannabinoid and endocannabinoid-like compounds are classified based on their acyl chain: 1) *N*-acylethanolamines (NAEs); 2) monoacylglycerols (MAGs); 3) *N*-acyldopamines; 4) *N*-acylserotonin, 5) Fatty acids amides of amino acids (FAAAs), and 6) COX2-derivatives (Piscitelli, 2015).

AEA, is a long-chain fatty acid ethanolamine and a representative of the NAEs. Other members of the NAEs include *N*-palmitoylethanolamide (PEA), *N*-oleoylethanolamide (OEA), *N*-stearoylethanolamide (SEA), and *N*-linoleoylethanolamide (LEA) (Figure 1.2). Although AEA is the most studied NAE, due to its activity and well characterized interaction with the cannabinoid receptors, it should be noted that AEA is only a minor component of NAEs whereas other NAEs, particularly OEA and PEA, are more abundant in animal tissues (Piscitelli, 2015). And, unlike AEA, OEA and PEA are better characterized as agonists of the PPARα receptor where OEA is often associated with satiety and feeding behaviour, while both OEA and PEA serve as anti-inflammatory and neuroprotective compounds (Suardíaz et al., 2007; Fu et al., 2003; Di Cesare Mannelli et al., 2013). However, evidence exists that PEA interact with the CB_2 receptor although at a much lower affinity compared to PPARα (Facci et al., 1995) and that NAEs, particularly OEA, are capable of activating several other receptors including TPRV1 and GPR119 (Kleberg et al., 2014; Piscitelli, 2015) (see section 1.4.2).

Figure 1.2: Structure of endocannabinoid-like compounds, N-acylethanolamides: *N*-arachidonoyl ethanolamide (AEA); *N*-linoleoylethanolamide (LEA); *N*-oleoylethanolamide (OEA); *N*-stearoylethanolamide (SEA); *N*-palmitoylethanolamide (PEA) (Leishman and Bradshaw, 2015).

The phytocannabinoid THC was the first cannabinoid compound identified from *C. sativa* and has long been used for medicinal and recreation purposes. Over 85 phytocannabinoids have been identified from the same plant, but the psychoactive THC and its non-psychoactive isomer, cannabidiol (CBD) still remain the most studied phytocannabinoids (El-Alfy et al., 2010; Bih et al., 2015). CBD targets both the CB_1 and CB_2 receptors but with lower affinity compared to THC (Pertwee et al., 2010), it is more likely that CBD acts as antagonist at CB_1/CB_2 rather than an agonist (Pertwee, 2008; Bih et al., 2015). Moreover, many studies have demonstrated that CBD can also target non-CB receptors such as GPR55, PPARγ, TRPV1 and those for adenosine and serotonin (Bih et al., 2015).

1.3.   The metabolizing enzymes

The metabolism of the two major endocannabinoids, AEA and 2-AG, are welled known in humans with AEA and 2-AG being synthesized with the aid of enzymes NAPE-PLD and

DAGLα/β, respectively and hydrolyzed by FAAH and MAGL, respectively (see Figure 1.1). However, multiple studies have shown that there is more than one distinct pathway involved in the synthesis and degradation of these endocannabinoids (Kano et al., 2009).
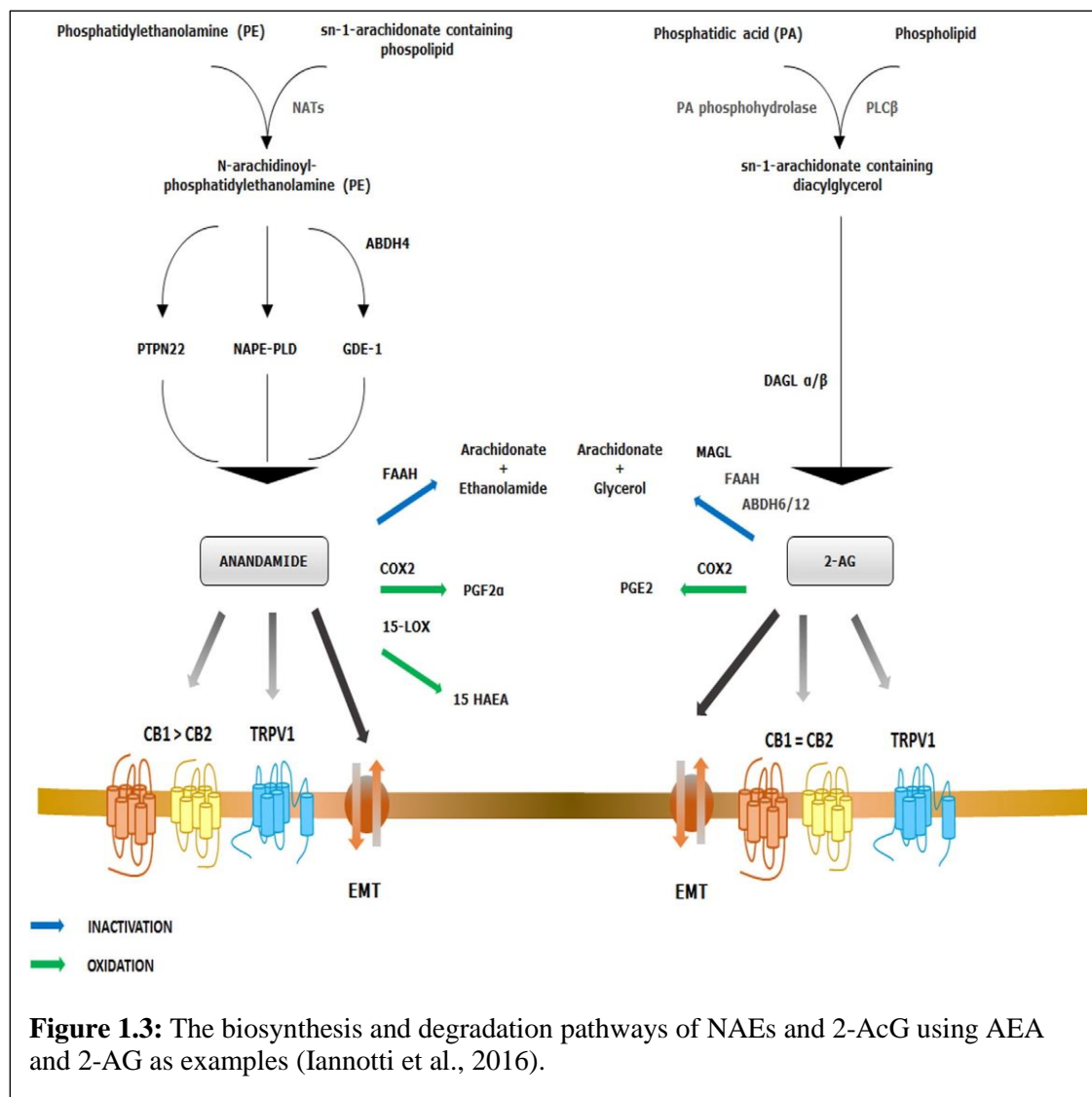
1.3.1. Biosynthesising enzymes

AEA, and its closely related long-chain NAEs, are synthesised from NAPE with the aid of the enzyme NAPE-PLD. NAPE plays a crucial role as a precursor in NAEs synthesis and can be synthesised from phosphatidylethanolamine (PE) or sn-1-arachidonate of phospholipids by the enzyme N-acyltransferase (NAT) (Iannotti et al., 2016).

Studies of NAEs biosynthesising pathway suggested that they can be synthesised via 3 main routes: 1) direct hydrolysis of NAPE by NAPE-PLD; 2) a three-step catalysis, through NAPE deacylation to glycerophosphoethanolamine (GP-NAE) by α/β-hydrolase-4 (ABH4) and further to NAE by glycerophosphodiesterase-1 (GDE-1); and 3) a two-step pathway through hydrolysis of NAPE by phospholipase C, forming phospho-NAE as an intermediate and further dephosphorylate by a poorly characterised phosphatase (e.g. PTPN22) to NAE (Figure 1.3) (Kleberg et al., 2014; Iannotti et al., 2016). It was also suggested that preference for one pathway over another might also relate to cell/tissue specification and/or the availability of precursors (Muccioli, 2010).

In the case of 2-acylglycerols (2-AcG) such as 2-AG, these are generally formed as an intermediate in the synthesis of triacylglycerol and phospholipids in the central nervous system (CNS) and peripheral cells (Kleberg et al., 2014). 2-AcG is known to be produced in a stimulus-dependent manner, activating $G_{q/11}$-coupled-receptor (e.g. glutamate receptor) as a result of a strong Ca^{2+} influx, thus upregulating 2-AG production (Muccioli, 2010).

Biochemical studies have revealed two primary pathways for 2-AG production. The first one involves the combination of the enzymes phospholipase C (PLC) and diacylglycerol lipase (DAGL). Initially, PLC hydrolyses membrane phospholipids such as phosphatidylinositol, producing 1-acyl-2-arachidonylglycerol (diacylglycerol, or DAG) which is subsequently hydrolyses to 2-AG by either of the two specific enzymes DAG lipase α or β (Figure 1.3) (Kano et al., 2009).

Figure 1.3: The biosynthesis and degradation pathways of NAEs and 2-AcG using AEA and 2-AG as examples (Iannotti et al., 2016).

Besides the formation of DAG as a precursor, 2-AG can also be generated in an alternative pathway featuring a 2-arachidonoyl-lysophophatidylinositol (Lyso-PI) intermediate. This involves the sequential reactions of phospholipids by phospholipase A_1 (PLA$_1$), producing lyso-Pl intermediate, and lyso-PLC specific enzyme, producing 2-AG (Muccioli, 2010). Biosynthetic pathways for 2-AG are also considered to vary depending on a cell/tissue type and conditions of stimuli (Kano et al., 2009).

1.3.2. Catalysing enzymes

Degradation of endocannabinoids are categorised into two different pathways, hydrolysis and oxidation. The enzymes involved in the hydrolysis pathway include fatty acid amide hydrolase (FAAH) for NAEs and monoacylglycerol lipase (MAGL) for 2-AcGs (Kano et al., 2009). The oxidation pathway involves the well-known oxidative enzymes cyclooxygenase

(COX) and lipoxygenase (LOX), which regulate oxidation of many arachidonic moieties of endocannabinoids (Kano et al., 2009).

Hydrolysis: FAAH is the primary enzyme responsible for NAEs catalysis, resulting in the free fatty acid and ethanolamide (Figure 1.3). Decreased levels of FAAH via enzyme inhibition or FAAH knock out mice result in the accumulation of NAEs (Cravatt et al., 2001; Kleberg et al., 2014). FAAH is found in both the CNS and the periphery (Cravatt et al., 1996) and is an integral membrane enzyme, with a molecular mass of ~63 kDa in human, rat, and mouse cells (Giang and Cravatt, 1997; Kano et al., 2009). This serine hydrolase has an optimum alkaline pH and is able to recognise a variety of fatty acid amides, however its preferred substrate is AEA, followed by LEA and OEA. It has also been reported that FAAH can catalyse the hydrolysis of the ester bond of 2-AG *in vitro* (Di Marzo et al., 1998; Goparaju et al., 1998; Kano et al., 2009).

A second membrane-associated FAAH was identified in humans and primates, but not in rodent tissues (Wei et al., 2006). This was named 'FAAH-2'. Although both FAAHs share a common Ser-Ser-Lys catalytic triad, they only share sequence homology of ~20% and FAAH-2 only contains 524 amino acids, weighs 57.4 kDa, and is localised in cytosolic lipid droplets rather than the endoplasmic reticulum (Wei et al., 2006; Kaczocha et al., 2010). Although FAAH-2 is highly expressed throughout the periphery it is less active at hydrolysing NAEs, compared to FAAH (Kaczocha et al., 2010). Thus, it was suggested that FAAH-2 might only act as a 'rescue' enzyme in NAE hydrolysis in a decreased/inhibited FAAH condition (Kaczocha et al., 2010).

The third NAE-hydrolysing enzyme is the N-acyl ethanolamine-hydrolysing acid amidase (NAAA). In contrast to FAAH, NAAA is a member of cysteine hydrolase family, which preferentially hydrolyses PEA, with an optima at acidic pH (4.5-5) and being localised in the lysozymes (Iannotti et al., 2016). High levels of NAAA expression are seen in the immune cells, specifically macrophage, where its level can increase during inflammation in response to increased PEA levels (as anti-inflammation) (Iannotti et al., 2016).

MAGL is recognised as the main enzyme in 2-AcG degradation, hydrolysing acylglycerols to free fatty acids and glycerol (Iannotti et al., 2016). Human, rat, and mouse MAGL all contain 303 amino acids and weigh 33 kDa (Karlsson et al., 1997, 2001; Dinh et al., 2002a). A study of 2-AG hydrolysis in mouse brain homogenate clearly shows that MAGL is responsible for

~85% of 2-AG catalysis whereas the other 15% is catalysed by poorly characterised hydrolases, ABH6 and ABH12 (Blankman et al., 2007).

Oxidation: In a less common degradation route, both NAEs and 2-AcGs can be oxygenated by both COX and LOX. In mammalian systems, there are 3 forms of COX: COX-1, COX-2, and COX-3 (Kano et al., 2009). Incubation of AEA with these enzymes showed that purified COX-2, catalysed both 2-AG and AEA, as efficacious and with lower affinity compared to AA (Wilson et al., 2001). For LOX, this lipoxygenase is expressed in both mammals and plants. Several types of LOXs display active oxidation on the arachidonate positions of endocannabinoids, both AEA and 2-AG, producing various types of hydroxyeicosatetraenoic acid (HETE) (Rouzer and Marnett, 2011).

1.4. The Main Cannabinoid receptors – CB and Non-CB

1.4.1. Cannabinoid receptors: CB_1 and CB_2

The two cannabinoid receptors in mammalian systems, CB_1 and CB_2, are both primary mediators of most endocannabinoids as well as other exogenous cannabinoid ligands (e.g. THC and CBD). CB_1 is found throughout the central nervous system (CNS), with high expression in the hippocampus, olfactory bulb, basal ganglia, and cerebellum, where it is involved in retrograde signaling of endocannabinoids (Castillo et al., 2012). Research has revealed that progressive loss of CB_1 is an early sign for Huntington's disease (HD) and the worsening of the disease in an already HD patient (Blázquez et al., 2010). Although CB_1 is most abundant in the brain it can also be found in synaptic nerves, cardiac muscle, adrenal gland, ovary, testes, immune cells, as well as the GI tract where CB_1 participates in the regulation of food intake and energy balance (Zou and Kumar, 2018).

CB_2 is often referred to as 'the peripheral CB receptor' because of its relatively low expression in the CNS. CB_2 is predominantly found in immune cells particularly macrophages, and in other peripheral tissues and organs including bone, adipose tissue, liver, GI tract, reproductive and cardiovascular system (Zou and Kumar, 2018). CB_2 is also expressed by some neurons both in the brain and peripheral sites (Onaivi et al., 2006). Despite the low expression of CB_2, increased expression in the CNS was observed following tissue injury or inflammation, but the mode of action remains unclear (Maresz et al., 2005).

Both CB_1 and CB_2 are members of the superfamily of G protein-coupled receptor proteins (GCPR), and are coupled to G protein G_i and G_o (Lu and Mackie, 2016). Their activation results in, (i) the inhibition of adenylyl cyclase (AC) and voltage gated calcium channels, (ii) down regulation of cyclic AMP (cAMP), (iii) activation of the mitogen-activated protein kinase (MAPK), (iv) activation of Phosphoinositide 3-kinase (PI3K) pathway and (v) various other signaling pathways depending on the specific cell type (Lu and Mackie, 2016; Zou and Kumar, 2018). These receptors exert a broad neuromodulatory function, both in the peripheral and CNS, as well as in non-neuronal cells, in a cell type and ligand specific manner with different ligands having different selectivities and affinities for the receptors and thus exerting distinct signaling cascades (Lu and Mackie, 2016; Zou and Kumar, 2018). Studies have shown that cannabinoid receptors can be activated by endo- and exogenous ligands, including synthetic ligands such as WIN 55, 212-2 (Pertwee et al., 2010).

1.4.2. Non-CB receptors

1.4.2.1. TRPV1

The transient receptor potential vanilloid type 1 (TRPV1) belongs to the transient receptor potential (TRP) superfamily and its natural ligand is capsaicin, the natural compound found in chili pepper (Caterina et al., 1997). TRPV1 is involved in temperature sensing, pain and nociception (Caterina et al., 2000). This receptor is primarily expressed in sensory neurons where it can be activated by stimuli such as temperature change ($>43°C$), acidity ($pH<6.9$), or various other toxins (Pertwee et al., 2010).

In sensory neurons, TRPV1 co-localizes with CB_1 and CB_2, making intracellular cross-talk between several types of receptor possible (Zou and Kumar, 2018). Although AEA is an agonist of CB_1 and CB_2, under certain condition (such as during synaptic transmission and pain regulation, when CB_1 is expressed at low levels), AEA can act as a full agonist for TRPV1 (Zou and Kumar, 2018). In addition, several other endocannabinoids such as noladin ether, virodhamine, *N*-arachidonoyl dopamine (NADA), but not 2-AG, can all act as full agonists of TRPV1 (Starowiczet al., 2007). Evidence also exists for endocannabinoid-like compounds such as OEA and CBD exerting full agonist activity upon interaction with TRPV1, where CBD binds to TPRV1 almost with the same K_i as with capsaicin (Bisogno et al., 2001). On the other hand, OEA activates TPRV1 and initiates vagal neurosensory nerves excitation in the nervous control of food intake, and also inhibits intracellular Ca^{2+} uptake in cells expressing TRPV1 (Thabuis et al., 2008). The interaction of TRPV1 with wider

endocannabinoid and endocannabinoid-like compounds suggests its strong association with the ECS.

1.4.2.2. GPR55 and deorphanized GPRs

Both CB_1 and CB_2 belong to a family of Class A GPCRs and cannabinoid receptor agonists can therefore also act at other GPCRs, mainly deorphanized GPR55, GPR119, GPR3, GPR6, and GPR12 (Pertwee et al., 2010). Research is currently proposing that deorphanized GPCR is a third true cannabinoid receptor, with the most-likely candidate being GPR55 (Morales and Reggio, 2017) because it is targeted by wide variety of cannabinoids including endogenous, phytogenic, as well as synthetic cannabinoids (Pertwee et al., 2010). GPR55 is widely expressed in the brain and the peripheral site, with particularly high expression in the striatum; co-localising with CB_1 and CB_2 (Sawzdargo at al., 1999).

Although AEA targets both CB_1 and CB_2, with slightly higher affinity for CB_1 compared to CB_2, it has an even higher affinity for GPR55 compared to CB_1 (Ryberg et al., 2007). This study also demonstrated that 2-AG and THC are agonists for GPR55 while CBD is an antagonist (Ryberg et al., 2007). Despite being the target for many cannabinoid ligands, GPR55 displays low sequence identity to both CB_1 (13.5%) and CB_2 (14.4%) (Pertwee et al., 2010).

GPR3, GPR6, and GPR12 are three orphan Class A GPCRs that are mainly expressed in the CNS and involved in mediation of β-arrestin2 recruitment (Pertwee et al., 2010). They exhibit close phylogenetic relationship with cannabinoid receptors, melanocortin receptors (MCRs), lysophospholipid receptors, adenosine receptors (AR), and the GPR3/6/12 subset, forming the so-called MECA cluster (Uhlenbrock et al., 2002; Morales et al., 2018). Very little is known about their ligands. AEA and 2-AG display no significant activity at these orphan receptors, whereas CBD demonstrates activity at GPR3, GPR6 and GPR12 (Brown et al., 2017; Laun and Song, 2017). CBD acts as an inverse agonist to inhibit β-arrestin2 recruitment on GPR3, GPR6, and GPR12. This suggests that CBD may act as potential neuroprotective agent for Alzheimer's and Parkinson's disease by acting on these GPCRs (Brown et al., 2017; Laun and Song, 2017).

GPR119 is primarily expressed in the pancreatic and GI tract, and is involved in the regulation of insulin secretion (Soga et al., 2005). Although GPR119 is phylogenetically

related to cannabinoid receptors, it is only activated by fatty acid amides, including AEA, OEA and PEA, with OEA being the most efficacious (Overton et al., 2006). Despite GPR119 being closely related to cannabinoid receptors, only *N*-oleoyl dopamine and OEA display high affinity for GPR119 (Overton et al., 2006), and both of these ligands do not interact with CB_1 nor CB_2, thus GPR119 cannot yet be classified as part of the cannabinoid receptor family.
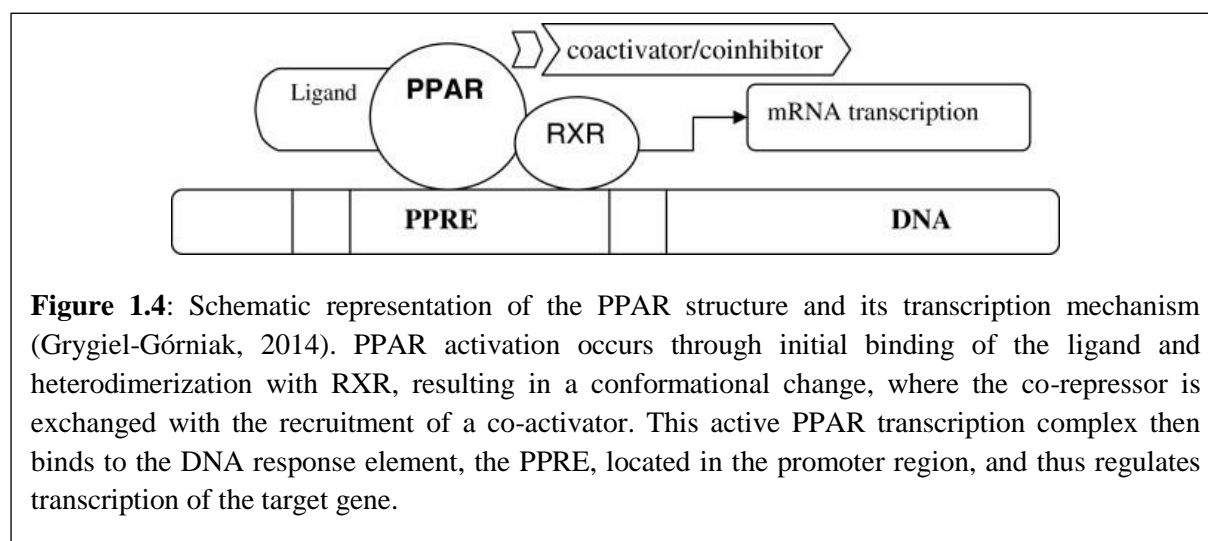
1.4.2.3. PPARs

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily, all sharing close structural homology (Tsai and O'Malley, 1994). In mammals, PPARs exist in three isoforms (α, β/δ, and γ). PPARs were first identified in 1990 and are associated with the regulation of diverse physiological and pathophysiological processes, notably lipid and glucose metabolism, inflammation, cell differentiation, and energy homeostasis (Issemann and Green, 1990; Cooper, 2000; Grygiel-Górniak, 2014).

In the nucleus, inactivated PPAR is associated to a co-repressor. The classic pathway through which PPAR activation occurs is through the formation of heterodimer complex with another nuclear hormone receptor, the retinoid X-receptor (RXR) (Grygiel-Górniak, 2014). The ligand then binds to this heterodimer, causing a conformational change in the PPAR structure and dissociation of the co-repressor and the recruitment of co-activator, simultaneously (Grygiel-Górniak, 2014). Activation and suppression of PPAR is modulated depending on the specific ligand binding to PPAR-RXR heterodimer as the exchange of co-repressor and co-activator occurs (Figure 1.4) (Grygiel-Górniak, 2014). Activated PPAR complex binds to peroxisome proliferator-response elements (PPREs) located on the regulatory regions of the target genes and carries out mRNA transcription (Chinetti-Gbaguidi and Staels, 2009; Grygiel-Górniak, 2014).

PPARs have large ligand binding domains which can be activated by various types of lipophilic ligands including synthetic compounds and plant extracts (O'Sullivan, 2016). Cannabinoids can activate PPAR through a few mechanisms: 1) direct binding of cannabinoids to PPAR, 2) indirect binding through the conversion of cannabinoid into PPAR-active metabolites, or 3) activation of cannabinoid cell surface membrane receptor which results in PPAR activation through, yet, an unclear cell signaling cascade (O'Sullivan, 2007).

Another possible activation mechanism suggested the active translocation of cannabinoids to the nucleus by intracellular fatty acid binding proteins (FABPs) (O'Sullivan, 2007).

Figure 1.4: Schematic representation of the PPAR structure and its transcription mechanism (Grygiel-Górniak, 2014). PPAR activation occurs through initial binding of the ligand and heterodimerization with RXR, resulting in a conformational change, where the co-repressor is exchanged with the recruitment of a co-activator. This active PPAR transcription complex then binds to the DNA response element, the PPRE, located in the promoter region, and thus regulates transcription of the target gene.

Ligands binding to FABPs have been shown to promote nuclear localization and PPAR interaction (Kaczocha et al., 2012; Hughes et al., 2015). Specifically, binding to FABP1 and FABP2 promotes nuclear localization and the activation of PPARα (Hughes et al., 2015). Conversely, FABP5 acts as an intracellular carrier mediating the nuclear translocation of OEA and reduces PPARα activation (Kaczocha et al., 2012). In addition, localization of AEA from plasma membrane to FAAH (for hydrolysis) was shown to be transported by FABP5 and FABP7 (Kaczocha et al., 2009). This catabolism was found to be inhibited by THC and CBD as the phytocannabinoids competed for FABP (Elmes et al., 2015).

The endogenous PPAR activators are a wide class of bioactive lipids which include endocannabinoids, phytocannabinoid, and synthetic cannabinoid ligands (O'Sullivan, 2016). AEA can activate PPARα and this induces neuroprotective properties (Sun et al., 2007). Similarly, 2-AG can activate PPARα, independent of CB_1 and CB_2 (Kozak et al., 2002). There is also strong evidence that OEA and PEA act as PPAR agonists, specifically activating PPARα which affects various physiological and pathophysiological functions, notably feeding and anti-inflammatory pain (Suardíaz et al., 2007; O'Sullivan, 2016). Apart from PPARα activation, although with less evidence, studies have also shown that OEA, PEA, AEA and 2-AG are able to activate PPARγ (O'Sullivan, 2016).

The phytocannabinoids and their derivatives, including THC and CBD, do not bind to PPARα but rather activate PPARγ (O'Sullivan, 2016). Synthetic cannabinoids,

Thiazolidinedione (glitazones) compounds, a family of drugs used for Type 2 diabetes treatment, are also common PPARγ agonists, particularly Rosiglitazone, which has high affinity and specificity for this PPAR (Lehmann et al., 1995).

Only the break down products of endocannabinoids, especially of the NAEs, have been found to act as natural agonists of PPARβ/δ. For example, arachidonic acid (AA) from AEA (Yu et al., 2014), and oleamide derived from OEA (Dionisi et al., 2012), bind and increase transcriptional activity of PPARβ/δ (O'Sullivan, 2016). However, a synthetic agonist has been chemically produced (GW0742) which selectively binds to PPARβ/δ (Sznaidman et al. 2003) and which was used in the current study.

PPAR activation can therefore be achieved with phytocannabinoids, endocannabinoids and their derivatives, and synthetic endocannabinoid-like compounds.

1.5. Evidence for an endocannabinoid system in singled cell eukaryotes

The first suggestions for the existence of an ECS in single celled organisms were made in the 1970's. Bram (1976) first showed that THC and cannabinol affected the cell morphology of the slime mould *Dictyostelium discoideum*, with cells becoming round and immobile. Cells recovered within a few hours (time not stated) after THC treatment but not with cannabinol. In the same year, McClean and Zimmerman (1976) showed that THC caused the cells of the ciliate *Tetrahymena pyriformis* to become round and move in a sluggish manner; once again recovering after some hours (time not stated). THC also caused a delay in cell division in this ciliate, which was dose dependent, and with cells being most sensitive in their G_2 phase of cell division (Zimmerman et al., 1981).

Pringle et al. (1979) found that THC reduced the population growth of the amoeba-flagellate *Naegleria fowleri* and prevented transformation of trophozoites into cysts or flagellates, but there was no effect on cell shape and movement. A reduction in population growth was also reported for three other amoebae, *Acanthamoeba castellanii, Vermamoeba (Hartmannella) vermiformis, and Willaertia magna* when treated with AEA and 2-O-acylglycerol (2-O-AG) (Dey et al., 2010). This study also showed that it was the cannabinoid itself, and not a breakdown product, that elicited this response with comparable population survival being achieved with 2-O-AG and 2-O-AG ether, a nonhydrolyzable analog of 2-O-AG. Whilst all four amoebae are in very different taxonomic groups (*N. fowleri* and *W. magna* are members

of the sub-phylum Percolozoa, while *V. vermiformis* and *A. castellanii* are members of the sub-phylum Lobosa), all were affected by cannabinoids in a similar manner.

This ability to perceive and response to exogenous endocannabinoids led to the search for *N*-acylethanolamines and their metabolic enzymes in these cells. NAEs were first identified in the yeast *Saccharomyces cerevisiae* along with its phospholipid precursors NAPEs and biosynthesizing NAPE-PLD-like enzyme (Merkel et al., 2005). Then, a suite of endocannabinoids (particularly NAEs) were reported in *Tetrahymena thermophila* (Anagnostopoulos et al. 2010) and *D. discoideum* (Hayes et al., 2013). The hydrolyzing enzymes FAAH and MAGL were reported in more organisms, mainly protists, these included *D. discoideum, T. pyriformis, T. thermophila, Plasmodium falciparum,* and the yeast *S. cerevisiae* (Karava et al., 2001, 2005; McPartland et al., 2006; Evagorou et al., 2010).

Since most data available for the evidence of a presence of ECS in unicellular eukaryotes is in the ciliate *Tetrahymena*, it is worth investigating the system in this ciliate further.

1.6.   The endocannabinoid system in the ciliate *Tetrahymena*

1.6.1.   The endocannabinoid ligand

The presence of a suite of endocannabinoids has been found in the cells of *T. thermophila* (Anagnostopoulos et al., 2010). This study reported the presence of: 1) *N*-acylethanolamines (NAEs); 2) 2-acylglycerols (2-AcGs); and 3) Free fatty acids (FFAs).

The 6 main NAEs, in order of abundance, were N-γ-linolenoylethanolamine (**GLEA**), *N*-eicosenoylethanolamine (**EEA**), *N*-linoleoylethanolamine (**LEA**), *N*-palmitoylethanolamine (**PEA**), *N*-oleoylethanolamine (**OEA**) and *N*-stearoylethanolamine (**SEA**). A few other NAEs, including AEA, were also identified, but these were present at a very low concentration (Anagnostopoulos et al., 2010). Although GLEA is present at the highest concentration it is not common in nature (Anagnostopoulos et al., 2010). SEA is present at a very low concentrations compared to other NAEs in both the human and *Tetrahymena* systems (Anagnostopoulos et al., 2010; Kleberg at al., 2014). EEA can only be found in a traceable amount in the human milk and its specific function has not been characterised (Gaitán et al., 2018). LEA on the other hand, is more common and can be found primarily in the GI tract, alongside with PEA and OEA (Artmann et al., 2008) where all three NAEs exhibit similar anorexic and pharmacological functions, activating more or less at the same

receptors, mainly PPARs (α and γ) and TRPV1, and serving a crucial role in the regulation of food intake and anti-inflammatory effects (Kleberg et al., 2014).

2-AcGs corresponding to their respective NAE were 2-AcG: 2-γ-linolenoylglycerol (2-GLG), 2-eicosenoylglycerol (2-EG), 2-linoleoylglycerol (2-LG), 2-palmitoylglycerol (2-PG), and 2-oleoylglycerol (2-OG), but not 2-stearoylglycerol (2-SG). Again, 2-AG was also detected, but at much lower concentration compared to other 2-AcGs (Anagnostopoulos et al., 2010).

The free fatty acids arachidonic (AA), linoleic (LA), and γ-linolenic (GLA), were detected at sufficient concentrations, with GLA being dominant followed by LA and AA, whereas other FFAs (e.g. eicosapentaenoicacid [EPA] and docosahexaenoic [DHA]) were below the detection limit (Anagnostopoulos et al., 2010). FFAs predominated over 2-AGs and NAEs, by one to three orders of magnitude.

Anagnostopoulos et al. (2010) also tested for the substrate of FAAH enzymes using AEA, LEA, ALEA (*N*-α-linolenoylethanolamine, analog of LEA), and GHLEA (*N*-γ-homolinolenoylethanolamine, analog of GLEA). All NAEs were hydrolyzed by FAAH with the highest enzyme activity being evident with AEA, followed by LEA, ALEA and GHLEA. This suggests that AEA, although present at low abundance, is the best endogenous substrate for FAAH in *T. thermophila* (Anagnostopoulos et al., 2010).

Anagnostopoulos et al. (2010) also showed that there was a fluctuation of NAE, 2-AcG, and FFA concentrations at different cultivation temperatures; at higher temperature (33°C) NAEs significantly increased whereas FFAs and 2-AcGs decreased (Anagnostopoulos et al., 2010). Thus, it was suggested that NAEs may play a role in membrane fluidity and cellular protection in a changing environment as well as in food intake and development. The function of NAEs was not investigated further, however the authors stated that preliminary experiments showed that most NAEs affected the growth of *T. thermophila* (but no mention of positive/negative effects) (Anagnostopoulos et al., 2010).

1.6.2. Enzymes

The two main degrading enzymes, but not the synthesizing enzymes, of the endocannabinoid system have been reported to exist in *Tetrahymena*., FAAH (the NAE hydrolase) was reported in both *T. pyriformis* (Karava et al., 2001, 2005) and *T. thermophila* (McPartland et

al., 2006), whereas, MAGL (the 2-AcG hydrolase) was reported in *T. thermophila* (McPartland et al., 2006; Evagorou et al., 2010).

1.6.2.1. FAAH

Karava et al. (2001) were the first to identify membrane-bound FAAH-like activity in *T. pyriformis*. The authors demonstrated that AEA was taken up by intact *T. pyriformis* cell and was rapidly metabolized to mixed free fatty acids comprising of phospholipids (PL) (70%), neutral lipids (NLs) (20%), and free AA (6%), in descending order of cytosolic concentration (Karava et al., 2001). The presence of AA at almost undetectable level in *T. pyriformis* correlated with the study of Anagnostopoulos et al. (2010). Amide hydrolysis activity was also detected in the culture medium where almost 80% of AEA was catalyzed to free AA. The amidohydrolase was found to be specific for AEA within the cell, but once secreted it could also hydrolyse NAEs other than AEA, e.g., OEA and PEA (Karava et al., 2001).

The optimum pH for the amide hydrolase in *T. pyriformis* was pH 9-10, which is similar to FAAH activity reported in variety of tissues and cell types (Karava et al., 2001; Wei et al., 2006). It is a serine hydrolase, which does not depend on Ca^{2+}, Mg^{2+}, or sulfhydryl group for its enzymatic activity on AEA (Karava et al., 2001& 2005); also similar to the FAAH in various mammalian tissues and cell lines (Desarnaud et al., 1995; Maccarrone et al., 1998; Karava et al., 2001). Moreover, Karava et al. (2005) revealed the presence of 2 isoforms of FAAH in *T. pyriformis*: a 66 kDa (non-microsomal fraction) and a 45 kDa (in the cytosol). Interestingly, the 66 kDa isoform is close in size to that reported for human and mammalian FAAH (63 or 67 kDa) (Giang and Cravatt, 1997; Maccarrone et al., 1998). The 45 kDa on the other hand, is close to the 46 kDa of amide hydrolase in invertebrates (Matias et al., 2001).

1.6.2.2. MAGL

Using Blast and phylogenetic tree comparisons of the human and ciliate genomes, MAGL, which catalyze the breakdown of 2-AG was believed to be present in *T. thermophila* (McPartland et al., 2006). Its presence was confirmed experimentally four years later when it was found to be involved in the catalysis of 2-AG and other 2-AcGs in *T. thermophila* (Evagorou et al., 2010). In mammalian systems, the catalysis of 2-AcG involves the action of both MAGL and FAAH (see section 1.3.2) and this was also recorded for *T. thermophila* (Evagorou et al., 2010). It was also shown that 2-AG hydrolysis was pH and temperature dependent, exhibiting optimum pH at 8-9 and 37-40 °C (Evagorou et al., 2010).

Two isoforms of MAGL were found in *T. thermophila*; cytosolic (40 kDa) and membrane-bound (45 kDa) with the activity of the latter being twofold higher than the former (Evagorou et al., 2010). Two isoforms of MAGL have also been reported for several cell types (Bisogno et al., 1997; Dinh et al. 2002a).

1.6.3. The potential endocannabinoid receptor in *Tetrahymena*

A study of functional orthologs of endocannabinoid proteins, by phylogenetic tree analysis and functional mapping, showed that the main endocannabinoids receptors CB_1, CB_2, TRPV1, and GPR55 <u>were absent</u> from *T. thermophila* (McPartland et al. 2006). However, there are a few suggested (lesser-known) targets that are known to interact with NAEs, which might be the targets in *T. thermophila* (Table 1.1). Those that the current study deemed promising to investigate were PPARs, GPR6, and the Dopamine receptor.

Table 1.1: A list of receptors known to interact with cannabinoids and their presence/absence in *Tetrahymena.* [a] Pertwee et al., 2010; [b] McPartland et al., 2006; [c] Kleberg et al., 2014; [d] Lampert et al., 2011; [e] Chisa et al., 1993; [f] Ciliate.org, 2019; [g] Ud-Daula et al., 2012; [h] De Duve and Baudhuin, 1966

Receptor	Binds NAEs	Binds other cannabinoids	Presence in *Tetrahymena*
CB1 **CB2**	AEA[a]	2-AG[a], CBD[a], THC[a], WIN55212[a]	No[b]
TRPV1	AEA[a,c], OEA[a,c], PEA[a,c], LEA[a,c], SEA[a,c]	CBD[a]	No[b]
GPR55	AEA[a]	2-AG[a], THC[a], CBD[a]	No[b]
GPR119	AEA[a,c], OEA[a,c], PEA[a,c], LEA[a,c],	No	Possibly - GPR119 mainly interact with acid amides
GPR3/6/12	AEA[a]	2-AG[a], CBD[a]	Possibly - GPR6 was reported as chemo sensory receptor in *T. thermophila* [d]
Opoid	No[a]	CBD[a], THC[a]	Yes - μ subunit [e]
Glycine	AEA[a]	2-AG[a], THC[a], CBD[a]	Possibly - essential in chemotaxis
Serotonin (5-HT)	AEA[a]	CBD[a]	Yes[f]
Dopamine	AEA[a]	CBD[a], THC[a]	Yes - a present of at least D1 [g]
PPARs	AEA[a,c], OEA[a,c], PEA[a,c], LEA[a,c], SEA[a,c]	2-AG[a,c], CBD[a,c], THC[a,c], WIN55212[a]	Possibly – A presence of peroxisomes [h]

1.6.3.1. The case of PPARs

In mammalian systems, endocannabinoids and most NAEs are known to interact with PPARs, acting as both agonists and antagonists (O'Sullivan, 2016). From the list of NAEs, within *T. thermophila* (Section 1.6.1), with the exception of GLEA and EEA, all can target at least one PPAR isoform (Kleberg at al., 2014; O'Sullivan, 2016). *T. pyriformis*, although not yet reported to have PPAR receptors, do possess peroxisomes (De Duve and Baudhuin, 1966; Ciliate.org, 2019). The peroxisomes contain at least 50 different enzymes for various biochemical pathways; however, they are mainly involved in the oxidation of fatty acids, regulating the lipids biosynthesis and the major source of metabolic energy (Cooper. 2000). The peroxisomes in *T. pyriformis* are tightly associated with the mitochondria where they play a crucial role in the oxidation of fatty acids during gluconeogenesis, providing major source of metabolic energy for the ciliates (Müller et al., 1968; Hogg, 1969; Blum, 1982). This is similar to the role of PPAR in mammalian systems, e.g. PPARα activation by OEA results in lipid catabolism and homeostasis through the β-oxidation pathway. Thus, in the absence of the four main cannabinoid receptors, endogenous NAEs might target PPAR-like receptors in *Tetrahymena*.

1.6.3.2. The case for GPCRs

Another interesting receptor worth investigating is a G protein-coupled receptor (GPCR) because, 1) of their high percentage identical sequence homology to CB_1 and CB_2, sharing the same superfamily of 7-transmembrane receptor (7-TMR) (Morales et al., 2018); and 2) they are a common target of many cannabinoids including NAEs (Pertwee et al., 2010).

GPR55, which interacts with many cannabinoids, has not been identified in any species of *Tetrahymena* (McPartland et al., 2006; Ciliate.org., 2019). However, homologues to GPR6 and GPR37 have recently been identified in *T. thermophila* (Lampert et al., 2011; Zou and Hennessey, 2017). Both act as chemosensory receptors, but GPR6 responds to chemoattractants (Lampert et al., 2011) while GPR37 responds to chemorepellents (Zou and Hennessey, 2017). In mammals, both GPR6 and GPR37 are expressed in the CNS, notably in the brain, co-localizing with cannabinoid receptors (Alavi et al., 2018) and both are important regulators of the dopaminergic system, with neuroprotective effects in Parkinson's disease (PD) (Alavi et al., 2018). Of the two, GPR6 is considered a potentially interesting

endocannabinoid target in *Tetrahymena* as it shares a high amino acids sequence identity (35%) with CB_1 and CB_2 (Lee et al., 2001).

Sphingosine-1-phosphate (S1P) is the endogenous agonist of GPR6 in mammals (Uhlenbrock et al., 2002). However, further studies failed to observe specific S1P-induced responses in cells transfected with GPR6 (Yin et al., 2009). Thus, GPR6 is still considered an orphan receptor. Despite this, various endocannabinoids and phytocannabinoids have been tested for their capability to alter β-arrestin2 recruitment to GPR6 (Laun and Song, 2017). AEA and 2-AG demonstrated no significant effect at altering β-arrestin2 recruitment, while amongst the five phytocannabinoids tested, CBD significantly reduced β-arrestin2 recruitment in a concentration dependent manner. This suggests that CBD is a novel inverse agonist for GPR6 (Laun and Song, 2017). CBD has also been demonstrated to act on a variety of GCPRs. These include antagonising effects at CB_1 and CB_2 (with low affinity), GPR55, GPR18, inverse agonist at GPR3 (Laun and Song, 2017), and an agonist at Serotonin 5H1Ta (Bih et al., 2015). Nonetheless, as the presence of GPR6 (and possibly other GCPRs) has been confirmed in *Tetrahymena*, it may be possible that *Tetrahymena* elicits a response to cannabinoids through GCPRs in the absence of the four main receptors.

1.6.3.3. The case for a dopamine receptor

Dopamine is an essential neurotransmitter in the brain and is involved in many neuro-modulatory roles, including the control of movement, memory and learning, cognitive, emotions, addiction and brain reward (Covey et al., 2017). Dopamine is predominantly expressed in the brain; however, it is also found in the peripheral nervous system where it functions as a local paracrine messenger (e.g. hormone) and is involved in the control of wide physiological and pathological processes (García et al., 2015).

Cannabinoids interact with the dopamine receptor both directly (Pandolfo et al., 2011) and also indirectly through the GABA pathway in which cannabinoid binding to the CB_1 receptor causes an increase in dopamine release from dopaminergic neurons (Garbutt, 1983). Under typical conditions, dopaminergic neurons are inhibited by the activation of $GABA_B$ receptors (Figure 1.5A) (Oleson and Cheer, 2012). Consequently, during phasic dopamine events, such as when presented with a drug associated cue, intracellular Ca^{2+} abruptly rises, which results in the activation of endocannabinoid synthesizing enzymes (e.g. DAGL). This leads to the synthesis of endocannabinoids (e.g. 2-AG), which are released into the extra-synaptic cleft

where they couple to the $G_{i/o}$ of CB1 receptors on GABA presynaptic terminals and thus, GABA release is suppressed (Figure 1.5B) (Oleson and Cheer, 2012). Suppression of GABA disinhibits dopamine neurons, and at the same time promotes phasic dopamine events (Oleson and Cheer, 2012). The dopamine released then binds to dopamine receptor to elicit a response. Using these pathways, the endocannabinoids are able to tune with synaptic inhibition and stimulation of dopamine (Casadio, et al., 2011).
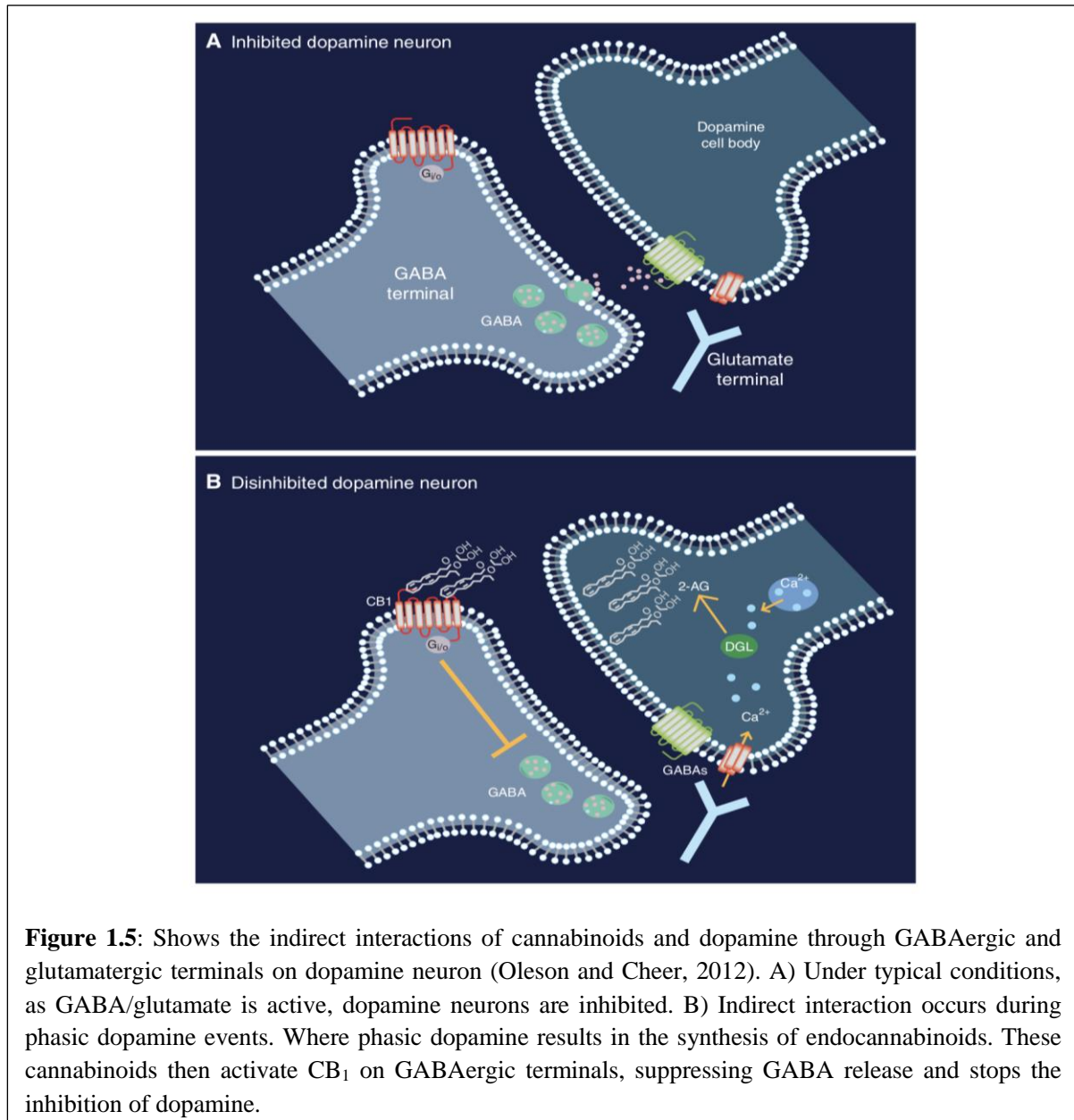

Figure 1.5: Shows the indirect interactions of cannabinoids and dopamine through GABAergic and glutamatergic terminals on dopamine neuron (Oleson and Cheer, 2012). A) Under typical conditions, as GABA/glutamate is active, dopamine neurons are inhibited. B) Indirect interaction occurs during phasic dopamine events. Where phasic dopamine results in the synthesis of endocannabinoids. These cannabinoids then activate CB_1 on GABAergic terminals, suppressing GABA release and stops the inhibition of dopamine.

Dopamine was previously reported to be the major catecholamine in *T. pyriformis* (Nomura et al., 1998) and the presence of the D_1 receptor was confirmed in *T. thermophila* although they did not rule out the presence of a D_2 receptor (Ud-Daula et al., 2012). Thus, it is a worthy candidate for an endocannabinoid receptor in *Tetrahymena*.

1.7. Overall objectives and specific aims

Many studies have shown that the endocannabinoid system is involved in the regulation of many fundamental modulatory systems in both multi- and uni-cellular organisms (see sections 1.1–1.6). The ciliate *Tetrahymena* is a common model organism used in many biomedical studies due to its simplicity and availability of genome information (Ruehle et al., 2016). Moreover, there is evidence for its cells possessing a suite of cannabinoids and their metabolising enzymes (see sections 1.6.1 and 1.6.2); but its target receptor(s) is currently unknown. Therefore, this study investigated three potential cannabinoid target receptor(s) in *Tetrahymena pyriformis*.

The overall objective was to quantify and compare the lethal effect of selected PPAR agonists, OEA, PEA, AEA, CBD and two synthetic cannabinoids (GW0742 and Rosiglitazone), on *T. pyriformis* and to determine whether the response was elicited through a pathway involving PPARs, GCPRs and/or a dopamine receptor.

The specific aims were to:

a. Compare the MIC, IC_{50} and lethal doses of each agonist.

b. Determine the longevity of the negative effect on ciliate cells.

c. Using specific antagonists, to determine whether the blocking of certain receptors alleviated the negative effect of the agonists. Specifically, the following receptors/pathways were blocked:

   i. PPARα, PPARβ/δ and PPARγ receptors

   ii. Dopamine D_2 receptor

   iii. GCPR pathway inhibitors:

        i. Pertussis toxin (PTX) – targets catalysis of ADP-ribosylation of $G\alpha_{i/o}$

        ii. H89 – a Protein Kinase A inhibitor

2. Materials and Methods

2.1 Organisms and Maintenance

2.1.1. *Klebsiella aerogenes*

The bacterium *Klebsiella aerogenes* (National Collection of Type Cultures [NCTC] 9528) was used as prey for the ciliate *Tetrahymena pyriformis* (Culture Collection of Algae and Protozoa [CCAP] 1630/1W). Streak plates of *K. aerogenes* were prepared on Lysogeny Broth (LB) agar plates (see Appendix 1) and incubated at 25°C for three days. Chalkley's medium (see Appendix 1) was poured onto two plates and the cells were scraped off into suspension using a sterile spreader. The bacterial suspension was stored at 4°C.

2.1.2. *Tetrahymena pyriformis*

The ciliate was routinely cultured in 500 ml of Chalkley's medium, supplemented with *ca*. 1-2 ml of the *K. aerogenes* suspension, and incubated at room temperature (23°C) for three days prior to experiments. On the day of an experiment, the *T. pyriformis* culture was concentrated by centrifuging sixteen 15 ml samples at 2000 rpm for 15 minutes. The 14 ml supernatants were carefully removed from each tube. The remaining 1 ml *T. pyriformis* concentrates were vortexed and combined into one tube.

2.2 Cell counts

2.2.1. *T. pyriformis*

The ciliate suspension was fixed with glutaraldehyde (0.5% v/v final concentration) and loaded into haemocytometers. The number of cells in 36 large squares was determined using a light microscope (40x magnification). The average number of cells/square was multiplied by 1×10^4 to yield cell concentration (cells/ml). Experiments only used cultures that contained at least 9×10^4 cells/ml.

2.2.2. *K. aerogenes*

The bacterial suspension was diluted ten-fold down to the 10^{-3} dilution, using sterile distilled water. Each was stained with 2-3 drops of the DNA stain 4′, 6-diamidino-2-phenylindole (DAPI) for 15-20 minutes. The stained sample, of a known volume (and known dilution) was captured on a 0.2 μm filter (Whatman, Micropore) using a vacuum pump. A slide was then prepared by (i) adding one drop of immersion oil to the slide and smearing it over the slide,

(ii) placing the filter on top (cells side up), (iii) adding a drop of oil to the center of the filter, (iv) applying a coverslip and (v) adding a final drop of oil.

The slide was viewed with an epifluorescence microscope using UV excitation (cells appear blue). The number of cells in randomly selected Whipple grids was determined until at least 400 cells had been counted. The average number of cells per Whipple grid was multiply by 23,068 (as the Whipple grid is $1/23068^{th}$ the area of the filter), giving the average number of cells captured per filter. Knowing the volume filtered, and dilution used, the number of cells/ml of the undiluted bacterial suspension was deduced.

2.3 Experimental compounds

2.3.1. Agonists

Six agonists were employed in this study: *N*-oleoylethanolamine (OEA), *N*-palmitoylethanolamine (PEA), *N*-arachidonoylethanolamine (anandamide or AEA), cannabidiol (CBD), GW0742, and Rosiglitazone (all obtained from TOCRIS, Biotechne, UK). All were maintained as 10 mM stocks, except AEA which was at 14.4 mM, in ethanol, at -20°C. On the day of an experiment stock solutions were diluted to 10^{-1} and 10^{-2} (e.g. 5 µL of 10 mM stock into 45 µL Chalkley's medium for a 10^{-1} dilution). For experiments, a known volume of agonist was added to 200 µL of *T. pyriformis* to obtain the desired starting concentration (e.g. 3 µL of a 10^{-1} dilution of e.g. CBD was added to 200 µL of *T. pyriformis* suspension for a starting concentration of 15 µM).

2.3.2. Antagonists/blockers

Three types of antagonists/blockers were used in this study (Table 2.1). The first type targeted PPAR receptors, i.e., GW6471 (PPAR α blocker), GSK3787 (PPAR β/δ blocker) and T0070907 (PPAR γ blocker) (TOCRIS, Biotechne, UK). The second type, Haloperidol hydrochloride, (TOCRIS, Biotechne, UK) targeted the dopamine hormone receptor. The last type were G protein-coupled receptor pathway inhibitors, i.e., Pertussis toxin (PTX) (target catalysis of ADP-ribosylation of $G\alpha_{i/o}$) and H89 (Protein kinase A inhibitor) (TOCRIS, Biotechne, UK). All antagonists/blockers were maintained at 10 mM, in ethanol, at -20 °C, except for PTX stock which was at 100 µg/ml in distilled water and stored at 4 °C.

Table 2.1: A summary of the antagonists/blockers used in this study, together with their molecular target

Blocker	Receptor Target
GW 6471	PPARα
GSK 3787	PPARβ/δ
T0070907	PPARγ
Haloperidol	Dopamine
Pertussis toxin	Gα$_{i/o}$
H89	Protein kinase A

2.4 Experiments

2.4.1. General response of *T. pyriformis* to an agonist, using CBD as an example

Triplicate samples of *T. pyriformis* (200 µL) were treated with CBD at 0 µM, 4 µM (IC$_{50}$ value) and 8 µM (2 × IC$_{50}$ value) in the presence of 5×10^7 cells/ml of *K. aerogenes*. Tubes were incubated at room temperature (23 °C) for 72 hours. Samples were removed throughout the period, fixed with glutaraldehyde (0.5% v/v final conc.) and cells counted (see 2.2.1). Experiments were performed twice. The percentage cell survival in treated samples, compared to control, was determined for each time point, i.e. (number of treated cells at Tx/number of untreated cells at Tx) × 100, and plotted against time (h).

2.4.2. Determining the susceptibility of *T. pyriformis* to PPAR agonists – MIC and IC$_{50}$

Triplicate samples of *T. pyriformis* (200 µL) were treated with a range of concentrations of OEA, PEA, GW0742 and Rosiglitazone. Numerous experiments were performed and each included triplicate untreated samples which acted as Controls. Tubes were incubated at room temperature (23 °C) for 90 minutes after which each tube was fixed and cells counted (see section 2.2.1). The percentage survival in treated samples, compared to control, was determined and from this, the IC$_{50}$ and Minimum Inhibitory Concentration (MIC) values were determined as follows:

2.4.2.1. Determining the IC_{50}

Percentage inhibition (100% minus calculated % survival) was plotted against Log_{10} agonist concentration in QtiPlot. The Logistic function tool was used to provide information on the significance of the data (p-value), the IC_{50} (concentration at which inhibition is half maximum) and the slope of the curve.

2.4.2.2. Determining the MIC

All data on the slope of the IC_{50} curve were plotted in EXCEL as % survival vs agonist concentration. Linear regression analysis was applied and high/low points removed until the highest R^2 was achieved. The MIC (x) was then determined from the equation of the line (y = mx + c) when y = 100% survival.

2.4.3. Determining the lethal dose of PPAR agonists to *T. pyriformis*

Triplicate samples of *T. pyriformis* (200 µL) were treated with a range of concentrations of OEA, PEA, GW0742 and Rosiglitazone; above those concentrations which gave an apparent 0% survival after 90 minutes (in 2.4.2). Numerous experiments were performed and each included triplicate untreated samples which acted as Controls. Tubes were incubated at room temperature (23 ºC) for 72 hours after which the contents of each tube were examined with a light microscope (×40 magnification) for evidence of surviving cells. The lowest agonist concentration which resulted in 0% cell survival after 72 hours was considered to be the lethal dose.

2.4.4. Investigating potential molecular targets for agonists in *T. pyriformis*

Experiments were set up in the same way as described in 2.4.2, i.e. 90 minutes survival tests. The ciliate (in triplicate) was treated with the agonist (at its IC_{50} value) with and without pre-treatment with a blocker. Controls containing blocker alone were also included. The percentage survival, compared to the control, was determined at 90 minutes.

All experiments employed three replicates and were normally repeated three times (n = 9). A one-way ANOVA compared all treatments and then a post-hoc Tukey test examined differences between % survival of the (i) agonist alone vs agonist and antagonist and, (ii) the antagonist alone vs agonist and antagonist.

2.4.4.1.   PPAR receptor blocking

The action of three known PPAR agonists (OEA [PPARα], GW0742 [PPARβ/δ] and Rosiglitazone [PPARγ]) together with AEA and CBD (all at their IC_{50} value) were examined in the presence and absence of three PPAR receptor antagonists (at $2 \times IC_{50}$ value). Each blocker was added to the ciliate culture for 10 minutes prior to adding the agonists. Controls containing blocker alone were also included. All experiments were performed three times.

2.4.4.2.   Dopamine receptor blocking

The action of OEA (IC_{50}, 45 µM) was examined in the presence/absence of Haloperidol (added 10 minutes prior to adding the OEA). A concentration of 90 µM ($2 \times IC_{50}$), and even 60 µM, Haloperidol proved too toxic to the ciliates so this blocker was added at a concentration of 30 µM (see section 3.4). Controls containing blocker alone were also included. This experiment was performed twice.

2.4.4.3.   H89 and PTX

The action of OEA, AEA and CBD (at their IC_{50} values) was examined in the presence/absence of (i) H89 (at 10 µM with a 30 minutes pre-incubation time) and (ii) PTX (at 100 ng/ml with a 5 hours pre-incubation time). Controls containing blocker alone were also included. This was a preliminary experiment and only performed once.

2.4.4.4.   Dose-response

Any agonist found to be blocked by an antagonist was tested further for evidence of a dose response, i.e. agonist at IC_{50} and blocker at 10 µM, 1 µM, 0.1 µM, 0.01 µM and 0.001 µM. Controls of blocker alone and agonist alone were included. Experiments were repeated three times (with PPAR blockers) or once (with Haloperidol).

3. Results

3.1. General response of *T. pyriformis* to PPAR agonist, using CBD as example

The survival of *T. pyriformis* in the presence of 0 µM (Control), 4 µM (IC_{50} value) and 8 µM ($2 \times IC_{50}$ value) CBD was monitored over a 72 hours period (at 23 ºC) (Figure 3.1). The Control population size grew somewhat, due to the presence of *K. aerogenes* prey, hence %survival >100%. An immediate decline in cell survival was evident in CBD-treated cultures up until 90 minutes, after which no further decline was apparent (Figure 3.1a).
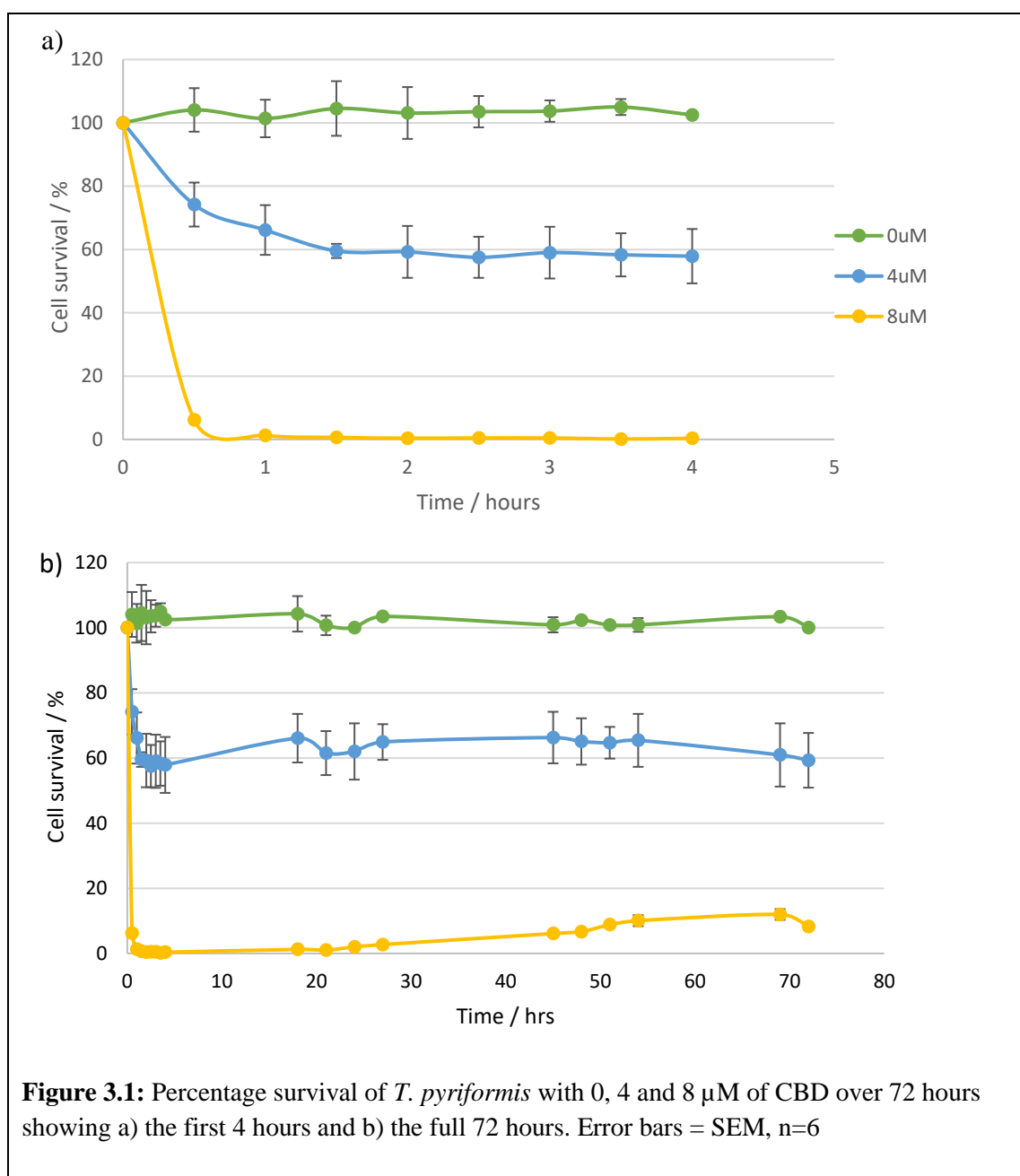

Figure 3.1: Percentage survival of *T. pyriformis* with 0, 4 and 8 µM of CBD over 72 hours showing a) the first 4 hours and b) the full 72 hours. Error bars = SEM, n=6

Cell survival was dose dependent being *ca*. 60% (4 µM CBD) and 0% (8 µM CBD) at 90 minutes (Figure 3.1a). Therefore, a standard time of 90 minutes was used to test the survival of *T. pyriformis* in the presence of different concentrations of all agonists in this study.
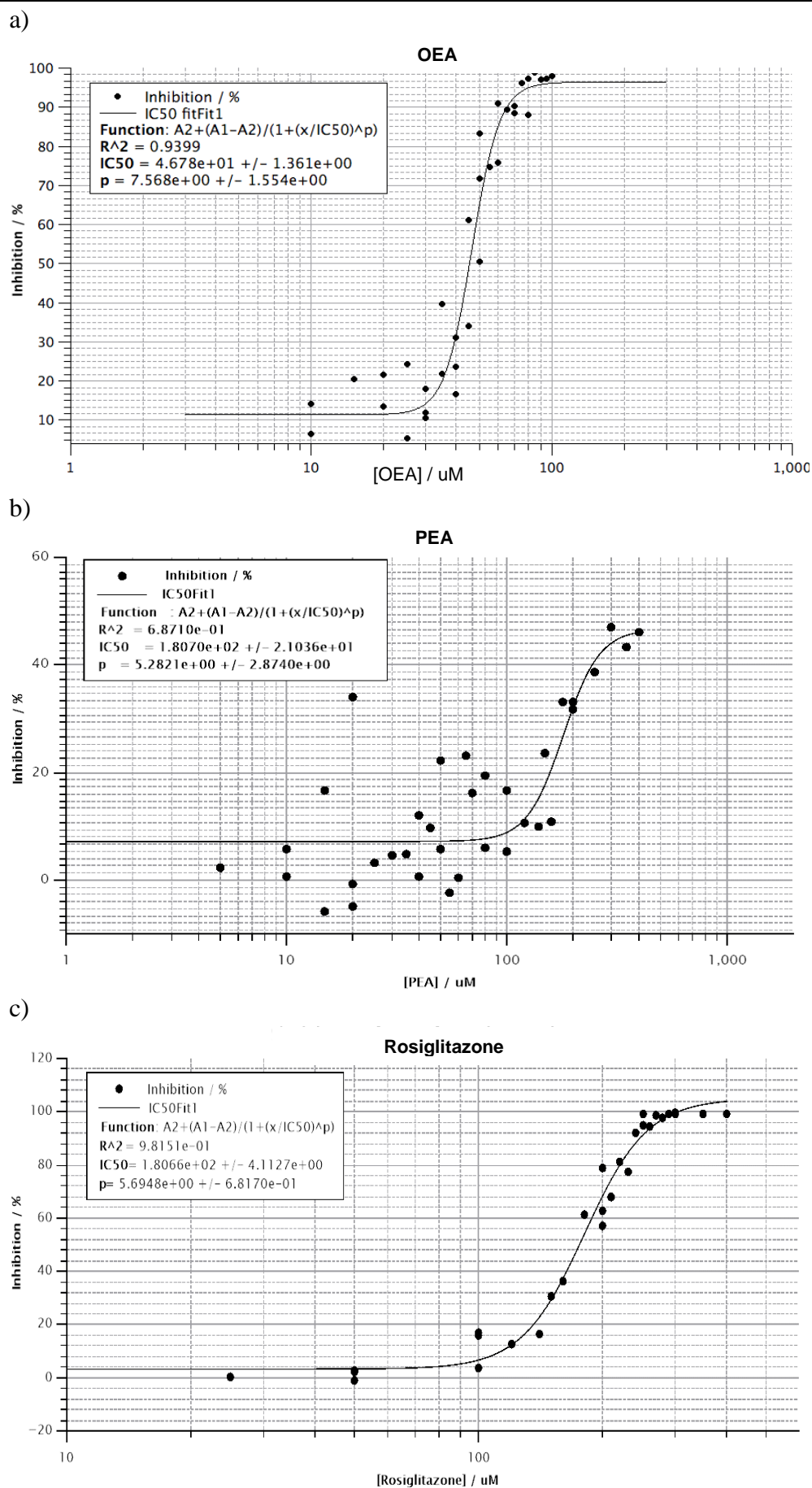
A survival of 0% at 90 minutes (with 8 µM) suggested that this concentration was lethal to the ciliate population. However, as seen in Figure 3.1b, the population recovered albeit not to the extent of the Control (even with 5×10^7 *K. aerogenes*/ml present). This ciliate concentration was therefore below the detection limit for ciliate counts (278 cells/ml) at 90 minutes, leading to an apparent 0% survival. True lethality was therefore evaluated at 72 hours for all agonists in this study.

3.2. *T. pyriformis* susceptibility to PPAR agonists

To test whether *T. pyriformis* might possess PPAR receptors, its susceptibility to known PPAR agonists was first tested: OEA and PEA bind to PPARα, GW0742 to PPARβ/δ and Rosiglitazone to PPARγ. The ciliate was subjected to a range of agonist concentrations and % survival determined at 90 minutes (for IC_{50}, MIC and slope) and 72 hours (for lethal concentration).

T. pyriformis was susceptible to all four PPAR agonists and IC_{50} curves (Figure 3.2) showed a classic dose response. From these graphs the IC_{50} and slope were determined in QtiPlot (Table 3.1). Linear regression analysis of the Hill slope value (Appendix 2) was used to determine the MIC (Table 3.1) whilst survival over 72 hours was used to determine the lethal dose (Table 3.1).

All data are summarized in Table 3.1. This also includes previously determined data for the action of AEA and CBD at 90 minutes on the same *T. pyriformis* strain (under the same experimental conditions) (obtained from Shruthi Sivakumar and Ashley Jones, Lancaster University).

a)

OEA

Legend:
- Inhibition / %
- IC50 fitFit1

Function: A2+(A1−A2)/(1+(x/IC50)^p)
R^2 = 0.9399
IC50 = 4.678e+01 +/− 1.361e+00
p = 7.568e+00 +/− 1.554e+00

Y-axis: Inhibition / %
X-axis: [OEA] / uM

b)

PEA

Legend:
- Inhibition / %
- IC50Fit1

Function : A2+(A1−A2)/(1+(x/IC50)^p)
R^2 = 6.8710e−01
IC50 = 1.8070e+02 +/− 2.1036e+01
p = 5.2821e+00 +/− 2.8740e+00

Y-axis: Inhibition / %
X-axis: [PEA] / uM

c)

Rosiglitazone

Legend:
- Inhibition / %
- IC50Fit1

Function: A2+(A1−A2)/(1+(x/IC50)^p)
R^2 = 9.8151e−01
IC50= 1.8066e+02 +/− 4.1127e+00
p= 5.6948e+00 +/− 6.8170e−01

Y-axis: Inhibition / %
X-axis: [Rosiglitazone] / uM

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d)

Figure 3.2: Percentage inhibition of *T. pyriformis* after 90 minutes exposure to varying agonist concentrations: a) OEA, b) PEA, c) Rosiglitazone and d) GW0742.

The MICs and IC_{50} values suggest that the most potent agonist is AEA, followed by CBD, GW0742 and then OEA. Both PEA and Rosiglitazone, although eliciting a response in the ciliate, have parameter values much higher than those previously published for other cell types (see Section 4.3). For example, not even a concentration of 400 µM PEA proved lethal to the ciliate. PEA and Rosiglitazone were removed from the study at this point. OEA was retained, even though 205 µM was required to prove lethal to the ciliate.

Table 3.1: The MIC, IC_{50}, slope of the IC_{50} curve and lethal dose values for OEA, PEA, GW0742, Rosiglitazone (PPAR agonists) with *T. pyriformis*. Data for AEA and CBD included (obtained from Shruthi Sivakumar and Ashley Jones, Lancaster University).

	Agonists					
	AEA	**OEA**	**PEA**	**GW0742**	**Rosiglitazone**	**CBD**
MIC (µM)	1.17	18.49	55.11	8.95	103.69	3.16
IC_{50} (µM)	3.78	46.78	180.39	11.88	181.08	4.38
Hill Slope	3.21	7.86	5.28	9.27	5.69	8.11
Lethal concentration (µM)	35	205	>400	30	210	40

3.3. PPAR receptors as potential targets for agonists in *T. pyriformis*

To further evaluate whether *T. pyriformis* might possess PPAR-like receptors, 90 minute survival experiments were performed in the presence of OEA, GW0742, AEA and CBD with/without a 10 minute pre-incubation of the ciliate with each of three specific PPAR-receptor antagonists: GW 6471 (PPARα blocker), GSK 3787 (PPARβ/δ blocker), and T0070907 (PPARγ blocker [T007]). Initial experiments used each blocker at a concentration twice that of the agonist's IC_{50}. Then, if a blocking of the negative effect of the agonist was recorded, the antagonist was tested at concentrations of 10, 1, 0.1, 0.01 and 0.001 µM.
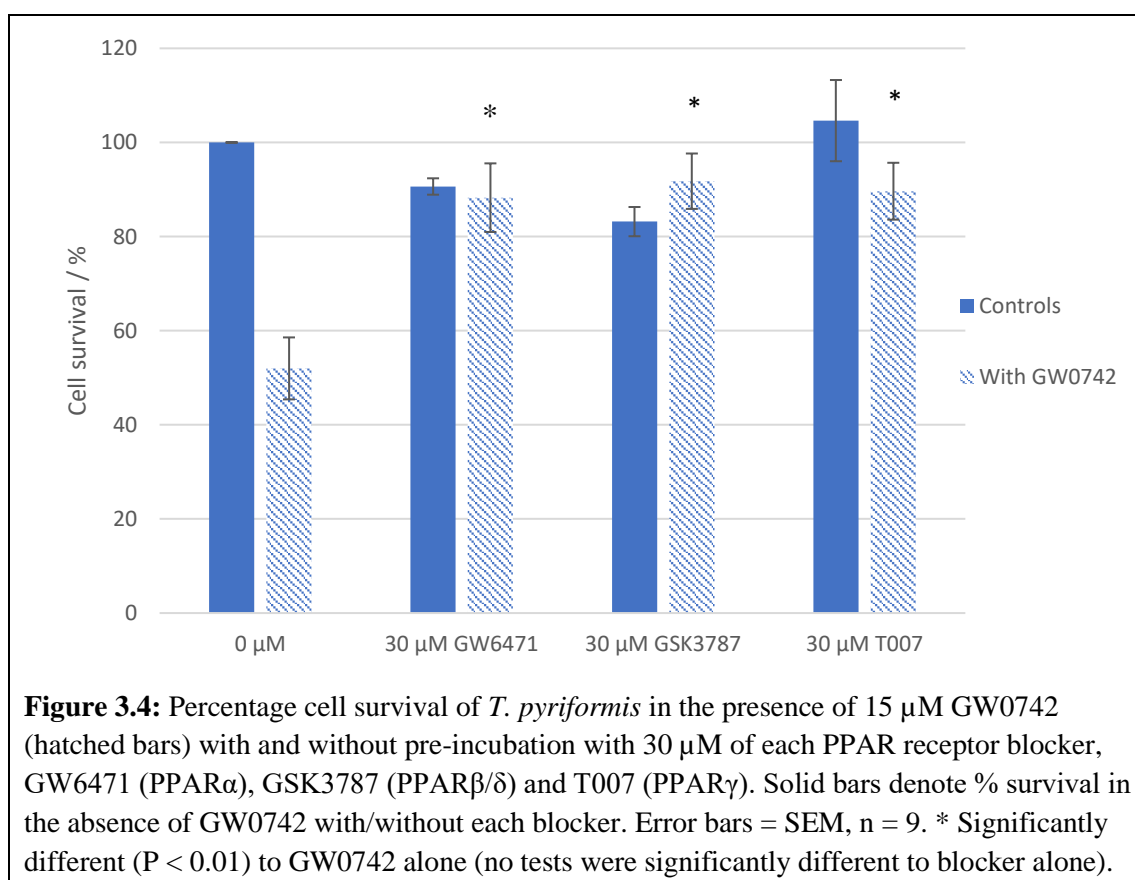
3.3.1. OEA

OEA was tested at its IC_{50} of 45 µM and antagonists at 90 µM. In the presence of OEA (Figure 3.3, hatched bars), the survival of *T. pyriformis* in the absence of blockers was 37.82 ± 12.44%, which was significantly different to the untreated control (Figure 3.3, 0 µM, solid bar) (P = 0.001). Pre-incubation with each blocker alone had no significant negative effect, compared to the control (P = 0.22 - 0.90), and did not alleviate the negative effect of OEA. The % survival value with GSK3787 [β/δ] was not significantly different to OEA alone (P = 0.90) but values were significantly lower than OEA alone with GW6471 [α] and T007 [γ] (P = 0.023 and 0.007, respectively). No dose-response experiment was carried out.

Figure 3.3: Percentage cell survival of *T. pyriformis* in the presence of 45 µM OEA (hatched bars) with and without pre-incubation with 90 µM of each PPAR receptor blocker, GW6471 (PPARα), GSK3787 (PPARβ/δ) and T007 (PPARγ). Solid bars denote % survival in the absence of OEA with/without each blocker. Error bars = SEM, n = 9. ^ Significantly different (P < 0.05) to blocker alone. *significantly different to OEA alone (P < 0.05).
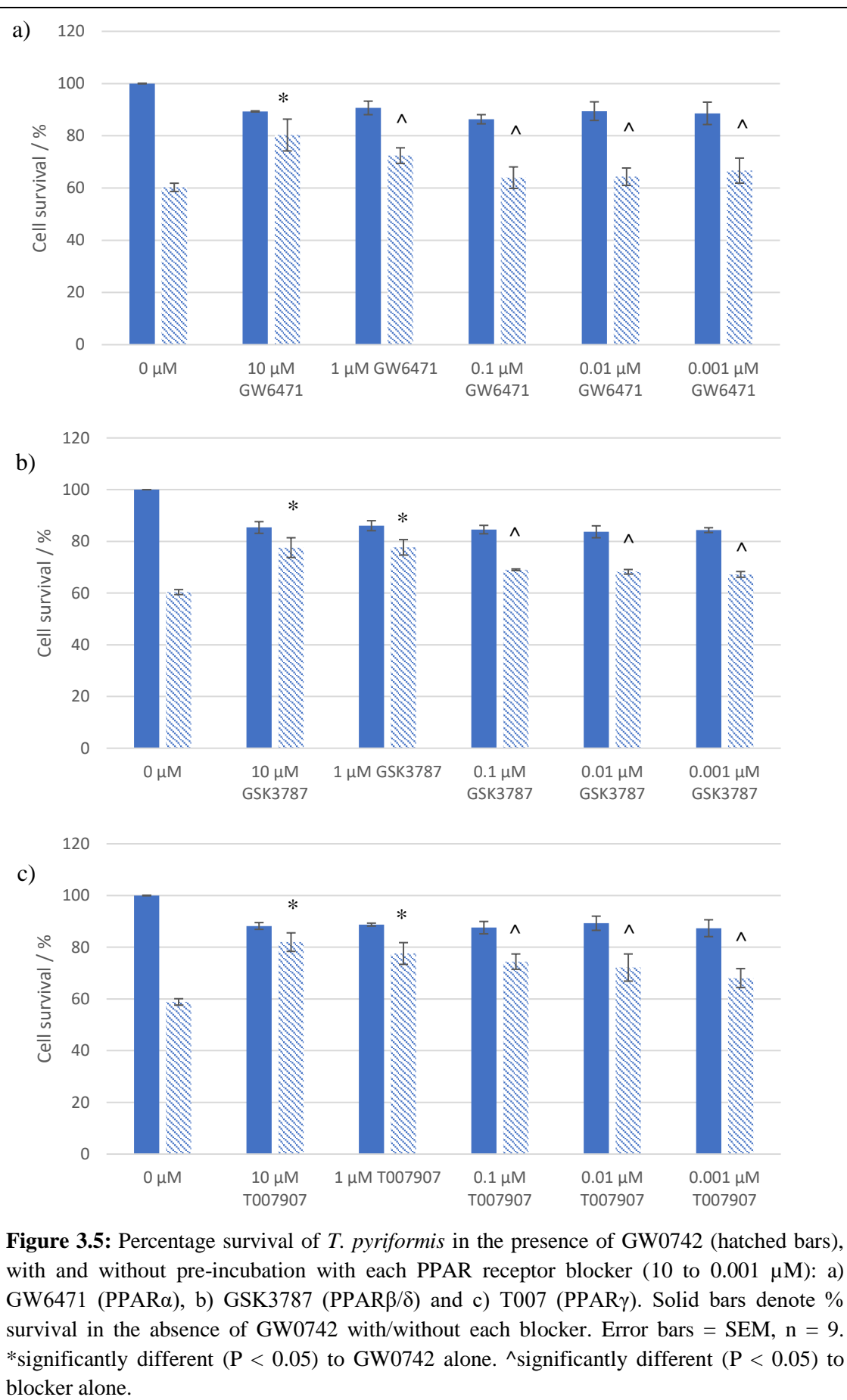
3.3.2. GW0742

GW0742 was tested at 15 µM and antagonists at 30 µM. In the presence of GW0742 (Figure 3.4, hatched bars), the survival of *T. pyriformis* in the absence of blockers was 52 ± 11%, which was significantly different to the untreated control (Figure 3.4, 0 µM solid bar) (P = 0.001). Pre-incubation with each blocker alone had no significant negative effect, compared to the control (P = 0.20 - 0.90), but totally alleviated the negative effect of GW0742 with % survival in their presence being equivalent to the control (P = 0.36 - 0.9) and significantly higher than GW0742 alone (P = 0.001 in all cases).

Figure 3.4: Percentage cell survival of *T. pyriformis* in the presence of 15 µM GW0742 (hatched bars) with and without pre-incubation with 30 µM of each PPAR receptor blocker, GW6471 (PPARα), GSK3787 (PPARβ/δ) and T007 (PPARγ). Solid bars denote % survival in the absence of GW0742 with/without each blocker. Error bars = SEM, n = 9. * Significantly different (P < 0.01) to GW0742 alone (no tests were significantly different to blocker alone).

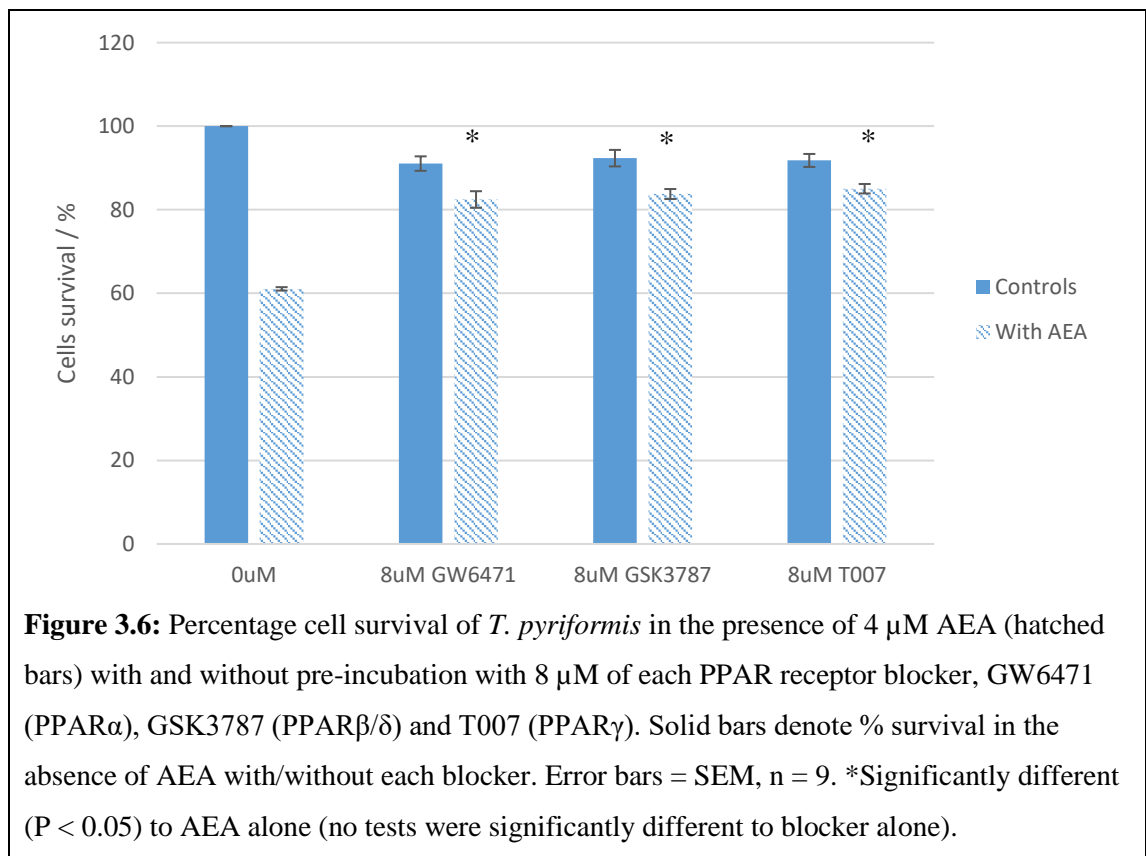Each blocker was further tested at concentrations ranging from 10 to 0.001 µM (Figure 3.5).

The PPARα blocker GW6471 completely alleviated the negative effect of GW0742 at 10 µM, i.e. 100% survival, where GW0742 with blocker was not significantly different to blocker alone, only GW0742 alone (Figure 3.5a*). No blocking effect was seen at 1 µM and lower, suggesting an MIC of >1<10 µM and that any dose-response might have been expected within these concentrations.

Figure 3.5: Percentage survival of *T. pyriformis* in the presence of GW0742 (hatched bars), with and without pre-incubation with each PPAR receptor blocker (10 to 0.001 µM): a) GW6471 (PPARα), b) GSK3787 (PPARβ/δ) and c) T007 (PPARγ). Solid bars denote % survival in the absence of GW0742 with/without each blocker. Error bars = SEM, n = 9. *significantly different (P < 0.05) to GW0742 alone. ^significantly different (P < 0.05) to blocker alone.

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The PPARβ blocker GSK3787 and the PPARγ blocker T007 data completely alleviated the negative effect of GW0742 at 10 and 1 µM (Figure 3.5b-c*). No blocking effect was seen at 0.1 µM and lower, suggesting an MIC of >0.1<1 µM in both cases and that any dose-response might have been expected within these concentrations.

3.3.3. AEA

AEA was tested at 4 µM and antagonists at 8 µM. In the presence of AEA (Figure 3.6, hatched bars), the survival of *T. pyriformis* in the absence of blockers was $61.06 \pm 0.71\%$, which was significantly different to the untreated control (Figure 3.6, 0 µM solid bar) (P = 7.4×10^{-8}). Pre-incubation with each blocker significantly alleviated the negative effect of AEA; % survival values (with blocker) were significantly different to AEA alone (P = 0.001 in all cases) and % survival (with AEA) were not significantly different to those in the presence of blocker alone (P = 0.09 - 0.31).

Figure 3.6: Percentage cell survival of *T. pyriformis* in the presence of 4 µM AEA (hatched bars) with and without pre-incubation with 8 µM of each PPAR receptor blocker, GW6471 (PPARα), GSK3787 (PPARβ/δ) and T007 (PPARγ). Solid bars denote % survival in the absence of AEA with/without each blocker. Error bars = SEM, n = 9. *Significantly different (P < 0.05) to AEA alone (no tests were significantly different to blocker alone).
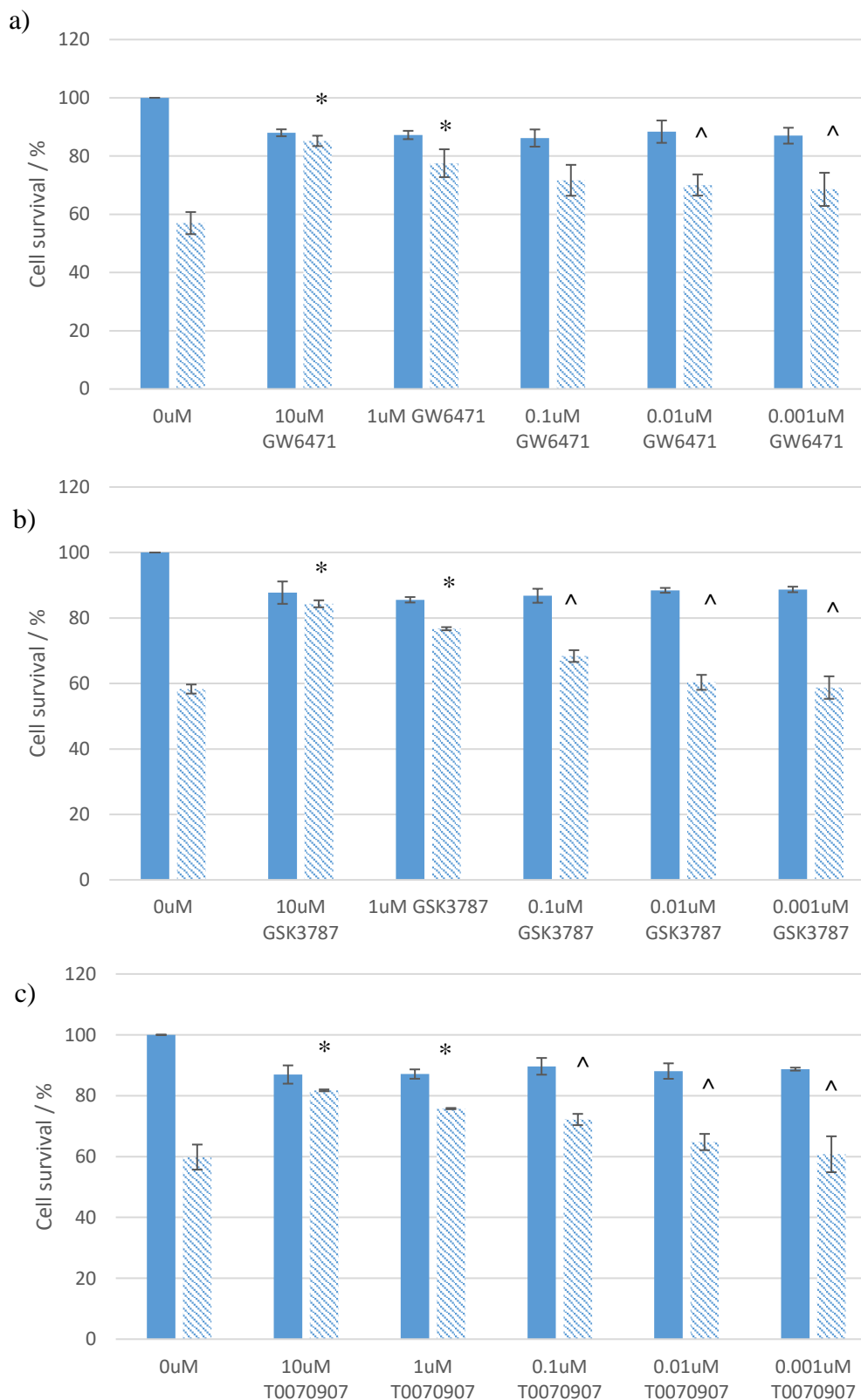
Figure 3.7: Percentage survival of *T. pyriformis* in the presence of AEA (hatched bars), with and without pre-incubation with each PPAR receptor blocker (10 to 0.001 µM): a) GW6471 (PPARα), b) GSK3787 (PPARβ/δ) and c) T007 (PPARγ). Solid bars denote % survival in the absence of AEA with/without each blocker. Error bars = SEM, n = 9. *Significantly different (P < 0.05) to AEA alone. ^Significantly different (P < 0.05) to blocker alone.
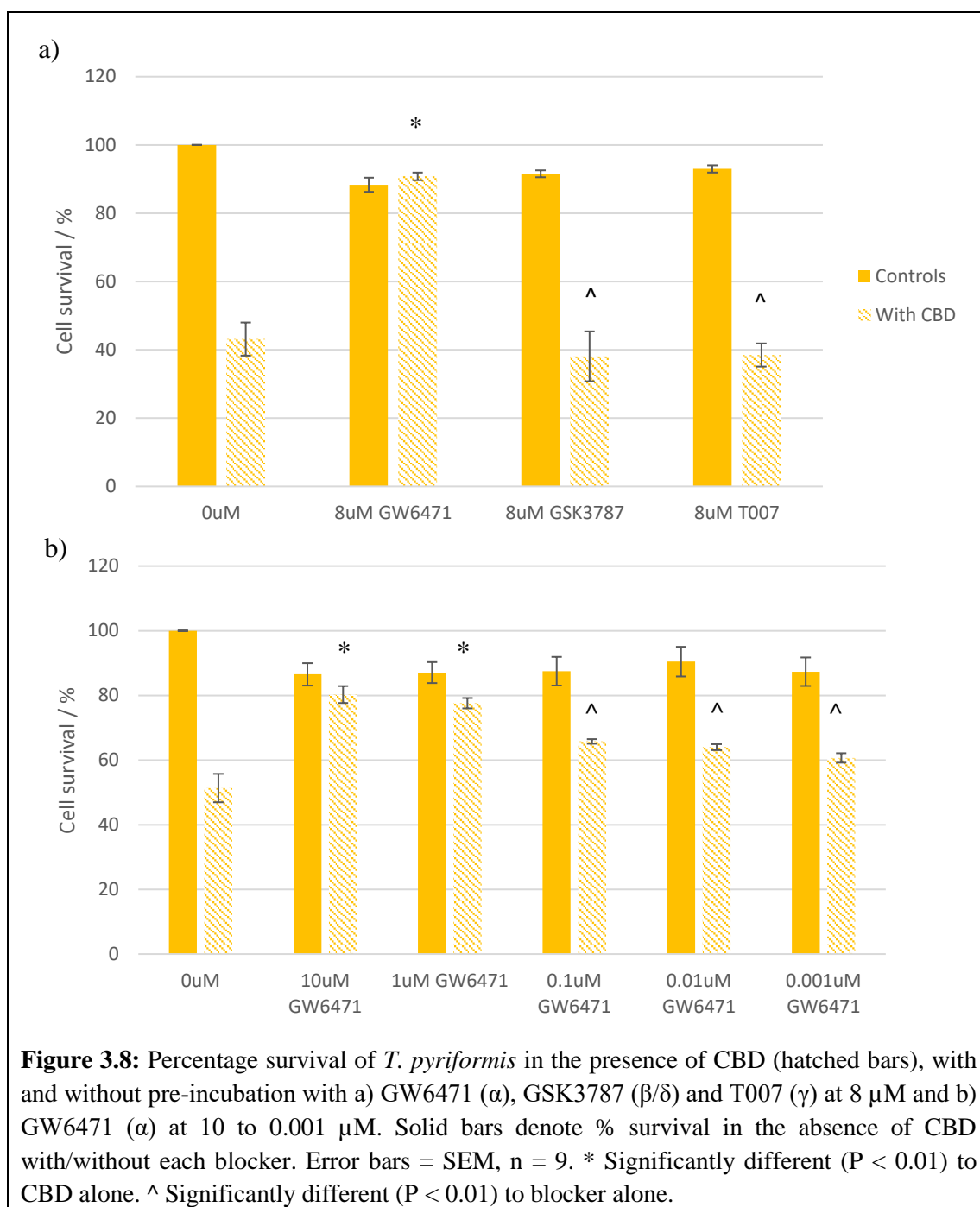
The PPARβ blocker GSK3787 and the PPARγ blocker T007 data completely alleviated the negative effect of AEA at 10 and 1 µM (Figure 3.7b-c*). No blocking effect was seen at 0.1 µM and lower, suggesting an MIC of >0.1<1 µM in both cases and that any dose-response might have been expected within these concentrations.

The PPARα blocker GW6471 completely alleviated the negative effect of AEA at 10, 1 and 0.1 µM, i.e. 100% survival, where AEA with blocker was not significantly different to blocker alone (Figure 3.7a); suggesting an MIC >0.01<0.1 µM. However, only at 1 and 10 µM was % survival with AEA and blocker significantly different to AEA alone (Figure 3.7a*); suggesting an MIC of >0.1<1 µM. It is therefore difficult to conclude what the real MIC would be in this case and it lies somewhere between 0.01 and 1 µM.

3.3.4. CBD

CBD was tested at 4 µM and antagonists at 8 µM. In the presence of CBD (Figure 3.8a, hatched bars), the survival of *T. pyriformis* in the absence of blockers was $43.14 \pm 8.39\%$, which was significantly different to the untreated control (Figure 3.8a, 0 µM solid bar) (P = 0.0003). Only pre-incubation with GW6471 (PPARα receptor blocker) alleviated the negative effect of CBD and the % survival value (with GW6471) was significantly different to CBD alone (P = 0.001). A concentration of 8 µM GW6471 (with CBD) yielded a % survival value that was not significantly different to that in the presence of GW6471 alone (P = 0.90).

In the dose response experiments (Figure 3.8b) the blocker completely alleviated the negative effect of CBD at 1 and 10 µM, i.e. 100% survival, where CBD with blocker was not significantly different to blocker alone, only CBD alone (Figure 3.8b*). No blocking effect was seen at 0.1 µM and lower, suggesting an MIC of >0.1<1 µM and that any dose-response might have been expected within these concentrations.

Figure 3.8: Percentage survival of *T. pyriformis* in the presence of CBD (hatched bars), with and without pre-incubation with a) GW6471 (α), GSK3787 (β/δ) and T007 (γ) at 8 μM and b) GW6471 (α) at 10 to 0.001 μM. Solid bars denote % survival in the absence of CBD with/without each blocker. Error bars = SEM, n = 9. * Significantly different (P < 0.01) to CBD alone. ^ Significantly different (P < 0.01) to blocker alone.

3.3.5. Summary

Table 3.2 summarizes the concentrations of antagonists required to elicit a positive alleviation of an agonist's effect on *T. pyriformis* (MIC) and that required to completely alleviate its action (100% block). Evidence for the existence of all three PPAR receptor types in *T. pyriformis* was obtained with GW0742 and AEA responding to the blocking of all three. The action of GSK3787 (β/δ) against these agonists was remarkably similar to that of T007 (γ) (Table 3.2) but variations existed with regards to their response to blocking with GW6471
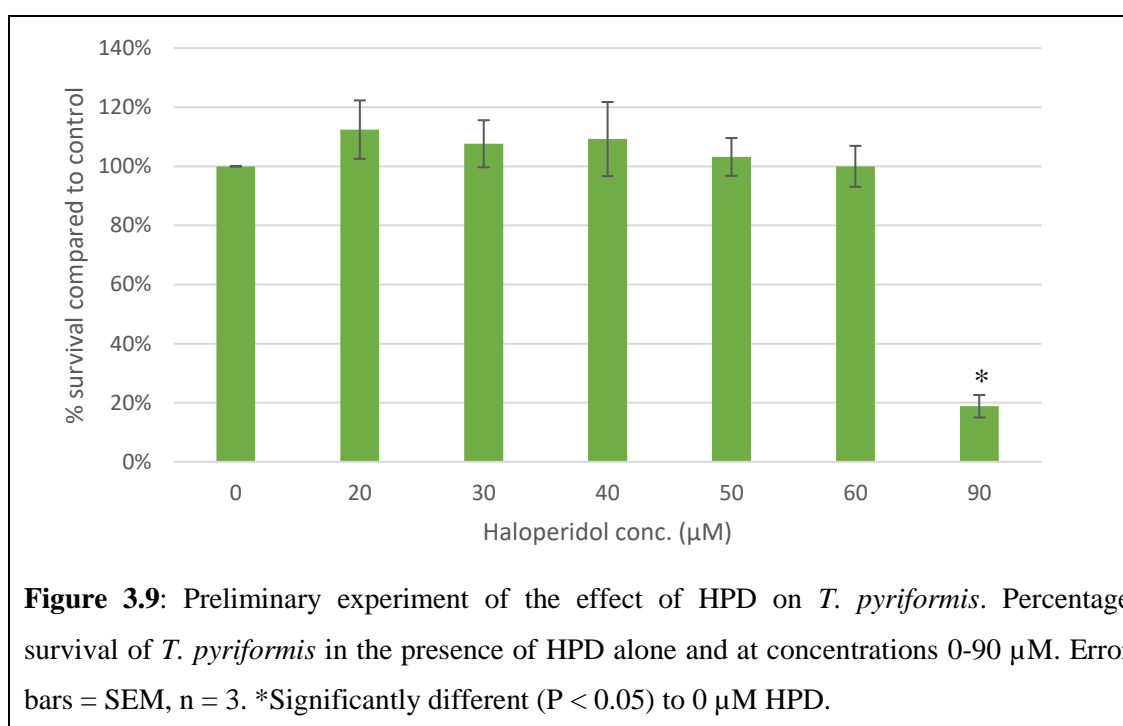
(α); AEA was more sensitive to the blocking (Table 3.2). The action of CBD was only blocked with GW6471 (α) at a comparable MIC to that of AEA.

Table 3.2: A summary of the range of Minimum Inhibitory Concentrations (MIC, µM) of each PPAR antagonist with each agonist and the concentration (100% block, µM) of antagonist required to completely alleviate the negative action of the agonist on *T. pyriformis*.
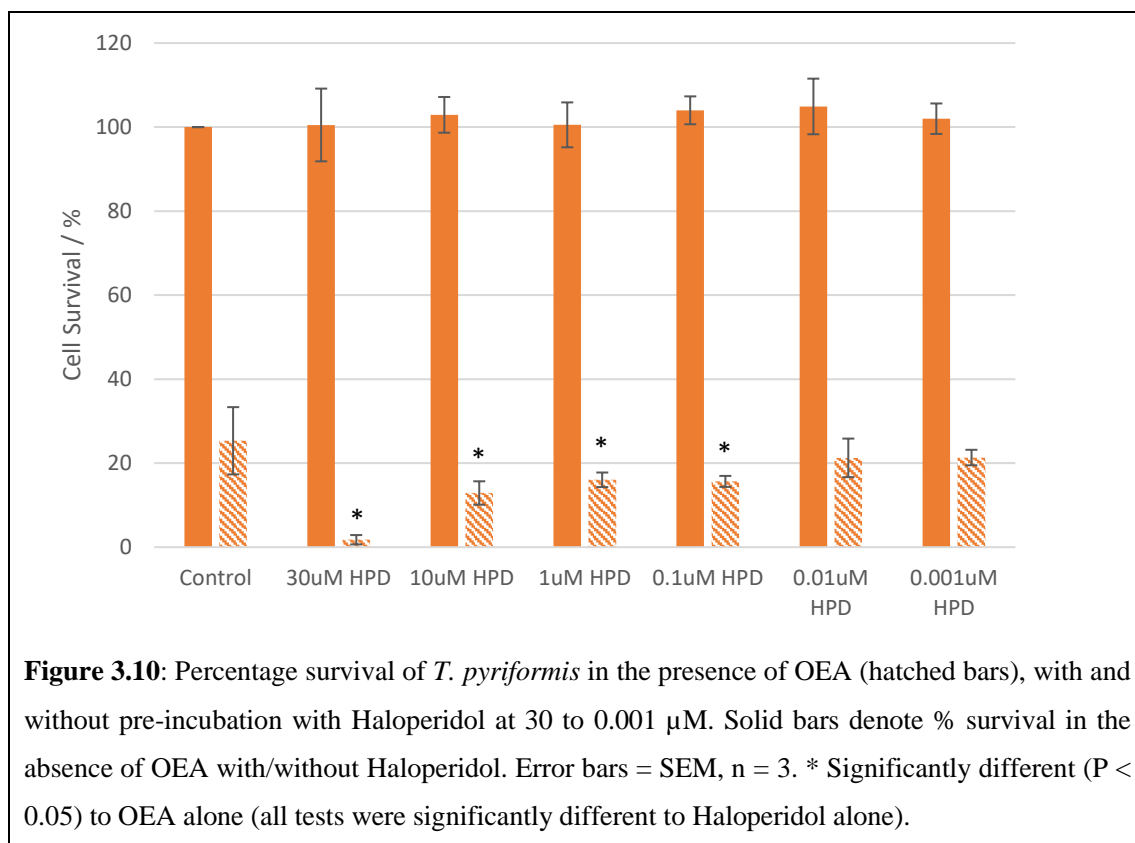
Agonist	PPAR Antagonists (µM)					
	GW6471(α)		GSK3787(β/δ)		T0070907(γ)	
	MIC	100% block	MIC	100% block	MIC	100% block
OEA	-	-	-	-	-	-
CBD	>0.1<1	>0.1<1	-	-	-	-
GW0742	>1<10	>1<10	>0.1<1	>0.1<1	>0.1<1	>0.1<1
AEA	>0.01<1	>0.1<1	>0.1<1	>0.1<1	>0.1<1	>0.1<1

3.4.   Dopamine receptor

The action of OEA (45 µM) on *T. pyriformis* was examined in the presence/absence of Haloperidol (HPD) using standard 90 minutes survival experiments, as described in Section 3.3, with the caveat that because this was a preliminary experiment it was performed twice (in triplicate, n = 6). A concentration of 90 µM HPD ($2 \times IC_{50}$) proved too toxic to the ciliate (Figure 3.9), but at 60 µM and below it was not. A concentration of 30 µM was chosen to accompany the standard dose response concentrations of 10 to 0.001 µM.

Figure 3.9: Preliminary experiment of the effect of HPD on *T. pyriformis*. Percentage survival of *T. pyriformis* in the presence of HPD alone and at concentrations 0-90 µM. Error bars = SEM, n = 3. *Significantly different ($P < 0.05$) to 0 µM HPD.

The presence of HPD enhanced the negative effect of OEA (Figure 3.10). This was significant for concentrations of 30, 10, 1 and 0.1 µM HPD whilst concentrations of 0.01 and 0.001 µM HPD had no effect. This relationship was not investigated further.
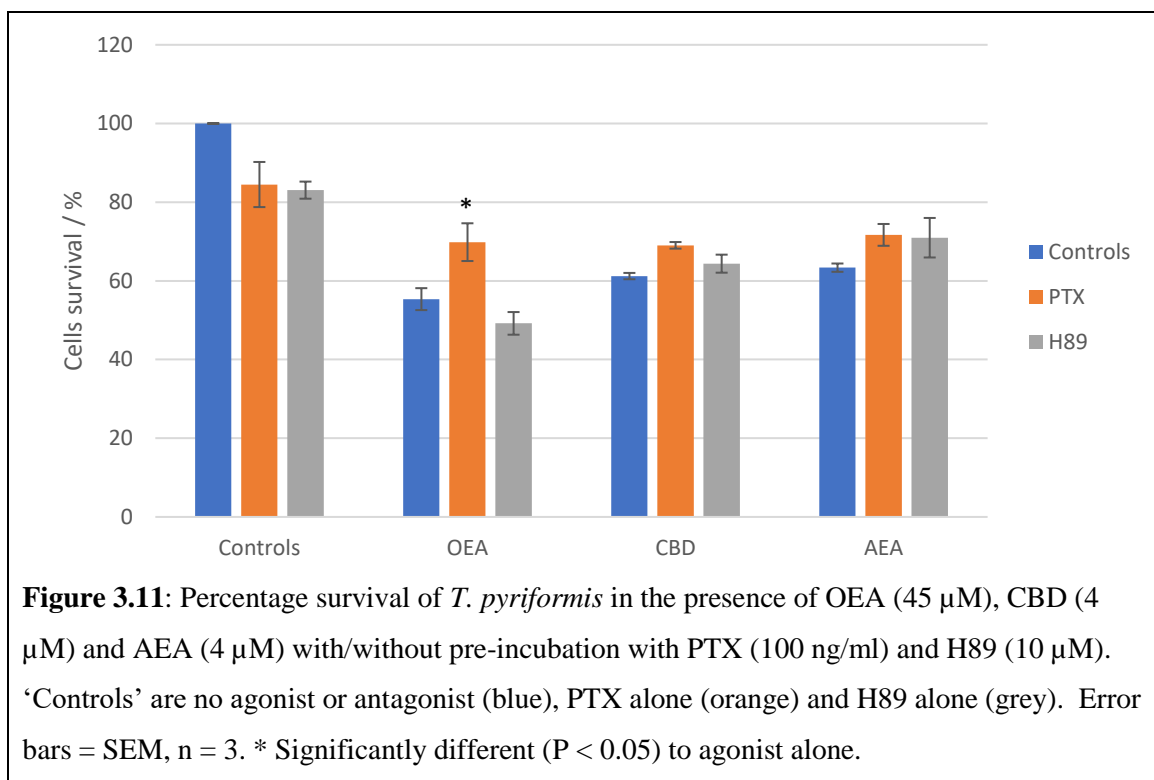

Figure 3.10: Percentage survival of *T. pyriformis* in the presence of OEA (hatched bars), with and without pre-incubation with Haloperidol at 30 to 0.001 µM. Solid bars denote % survival in the absence of OEA with/without Haloperidol. Error bars = SEM, n = 3. * Significantly different ($P < 0.05$) to OEA alone (all tests were significantly different to Haloperidol alone).

3.5. G protein-coupled receptor

The action of OEA (45 µM), AEA (4 µM) and CBD (4 µM) on *T. pyriformis* was examined in the presence/absence of H89 at 10 µM (with a 30 minutes pre-incubation time) and PTX at 100 ng/ml (with a 5 hours pre-incubation time), using standard 90 minutes survival experiments (Section 3.3). This was a preliminary experiment and was only performed once (in triplicate, n = 3).

The presence of H89 did not significantly alleviate the negative effect of any agonist (Figure 3.11) and % survival with agonist plus H89 was always significantly lower than with H89 alone. PTX alleviated the negative effect of OEA only, yielding a significantly higher survival compared to OEA only ($P = 0.005$) but still being significantly less than with PTX

alone (P = 0.014); so not completely alleviated. PTX had no significant effect on the action of CBD and AEA.

Figure 3.11: Percentage survival of *T. pyriformis* in the presence of OEA (45 µM), CBD (4 µM) and AEA (4 µM) with/without pre-incubation with PTX (100 ng/ml) and H89 (10 µM). 'Controls' are no agonist or antagonist (blue), PTX alone (orange) and H89 alone (grey). Error bars = SEM, n = 3. * Significantly different (P < 0.05) to agonist alone.

3.6. Summary of results

Table 3.3: Summary of the effect of blockers on the survival of *T. pyriformis* in the presence of cannabinoids and PPAR agonists.

Agonists	PPAR			Haloperidol	PTX	H89
	α	**β/δ**	**γ**			
OEA	Negative	No effect	Negative	Negative	**Positive**	No effect
CBD	**Positive**	No effect	No effect	N/A	No effect	No effect
AEA	**Positive**	**Positive**	**Positive**	N/A	No effect	No effect
GW0742	**Positive**	**Positive**	**Positive**	N/A	N/A	N/A

4. Discussion

4.1. Summary of major findings

The aim of this study was to investigate the effect of six cannabinoids/ligands on *T. pyriformis* and whether this involved peroxisome proliferator-activated receptors (PPARs), G protein-coupled receptor (GPCRs), and/or a dopamine receptor. Firstly, the lethal effect of the ligands was determined through four parameters 1) MIC, 2) IC_{50}, 3) Hill slope value, and 4) lethal concentration (Table 3.1). Results suggest that *T. pyriformis* was most susceptible to AEA, followed by CBD, then GW0742, then OEA. PEA and Rosiglitazone proved to be less lethal to *T. pyriformis* and were thus, eliminated from the study. As for the other cannabinoids, the slope values suggested that OEA and CBD behave similarly and might bind to the same target, whereas AEA and GW0742 might each bind a distinct target.

The study then went on to determine the ligands' target, whether PPAR, GPCR, and/or a dopamine receptor, using specific blockers to inhibit these receptors (Table 3.3). Haloperidol (Dopamine blocker) and H89 (PKA blocker) demonstrated no blocking of the effect of any agonist. In contrast, PPAR blockers alleviated the negative effects of AEA, CBD, and GW0742, but not OEA. Results suggested that AEA and GW0742 could interact with all three PPAR types (α, β/δ, and γ) whereas CBD only interacted with PPARα. In the presence of Pertussis toxin ($G\alpha_{i/o}$ blocker) only the effect of OEA was alleviated. This suggests that the ciliate might have at least two pathways for responding to these ligands; CBD, AEA and GW0742 might use a PPAR-like pathway, while OEA targets GPCRs.

4.2. Lethal effect of cannabinoids on protists

Taking CBD as an example, the ciliate population reduced to 60% (compared to the control) with 4 µM CBD, and to '0%' with 8 µM CBD, in the first 90 minutes. After this the 4 µM-treated population density remained stable while that of the 8 µM-treated population gradually increased, indicating that not all the cells in that population had been killed and that the population had been below the limit of detection at 90 minutes. The concentration of CBD found to be lethal to the whole population was 40 µM.

Previous studies have also shown that cannabinoids are toxic to protists and that the negative effect can be short-term. The treatment of *T. pyriformis* with THC elicited a dose dependent response (between 3.2 – 24 µM) with the cells showing a change in shape, having sluggish

movement and a decreased level of cellular growth and division (McClean & Zimmerman, 1976). However, even at the highest concentration (24 µM THC) this only caused an 11% reduction in cell concentration after 16 hours growth, suggesting that the IC_{50} is higher than 24 µM (McClean & Zimmerman, 1976). Comparing this to the current study suggests that *T. pyriformis* is more susceptible to CBD (IC_{50}, 4.38 µM) than THC. The toxic effect on *T. pyriformis* was also transient in both studies. In the current study, cells appeared to be in recovery by 10 hours, whereas in the study of McClean & Zimmerman (1976) cells recovered after 'several hours', although the actual time period was not stated.

THC has also been reported to inhibit the growth in several amoebae. At 60 µM THC cells of the slime mould *Dictyostelium discoideum,* which has an amoeboid form in its growth cycle, became round and immobile but after a 'few hours' (time not stated) they fully recovered (Bram and Brachet, 1976). However, at 50 µM of Cannabinol, cells rounded up but they did not recover, suggesting different effects depending on the cannabinoid used (Bram and Brachet, 1976). Even so, *D. discoideum* appears less sensitive to cannabinoids than *T. pyriformis*.

With the amoeba-flagellate *Naegleria fowleri*, the presence of phytocannabinoids prevented enflagellation and encystment but movement remained the same (Pringle et al., 1979). Three days exposure in the presence of 5 µg/mL (16 µM) THC and CBD resulted in populations which were 54% and 27% that of the control, respectively, suggesting that the IC_{50} of THC was *ca*. 16 µM while that of CBD was <16 µM (Pringle et al., 1979). This once again suggests that CBD is more toxic than THC and that *T. pyriformis* is more sensitive to CBD than these amoebae.

The endocannabinoid AEA has also been shown to inhibit growth of three other species of amoebae: *Vermamoeba* (*Hartmannella) vermiformis, Acanthamoeba castellanii,* and *Willaertia magna* with IC_{50} values (determined after 3 days exposure) of 14 µM, 17 µM, and 20 µM, respectively (Dey et al., 2010). In the current study, the IC_{50} of AEA was only 4 µM, once again showing a higher level of susceptibility in *T. pyriformis*, compared to amoebae.

4.3. Lethal effect of cannabinoids on cancer cells

Cannabinoids are considered to act more specifically to cancer cells than normal cells and much work has examined their effect on human cancerous cells (Ryberg et al. 2007; Chakravarti et al., 2014; Almada et al., 2017; Scott et al., 2017). The lung carcinoma cell line (A549) can be killed by THC at concentrations of 32 µM (IC_{50} of 24 µM) (Sarafian et al., 2002). Human leukemia cancer cell lines CEM and HL60 are more sensitive. When these cells were treated with THC, CBD and cannabigerol (CBG) the IC_{50} values for CEM cells were 13 µM, 8 µM, 11 µM for THC, CBD, and CBG, respectively while values for HL60 were slightly higher at 15 µM, 12 µM, 16 µM, respectively (Scott et al., 2017). In both cell types, CBD shown to be more toxic than THC; a similar response to that observed in amoebae (Pringle et al., 1979).

In human glioma cells, 10 µM CBD was the non-inhibitory value for growth while 25 µM CBD was 'inhibitory' suggesting an MIC >10<25 µM (Massi et al., 2006). Induction of apoptosis was via upregulation of caspase-3 activity which ultimately causes apoptotic mediated cell death (Massi et al., 2006). Human prostate carcinoma cells (PC-3, DU-145, 22RVI and LNCAP) appear to be more sensitive, being induced into apoptosis at CBD concentrations of 1 - 10 µM (De Petrocellis et al., 2000). The same study found that lower concentrations (0.5 - 6 µM) of AEA resulted in cell death; a similar response was observed with *T. pyriformis* exposed to AEA and CBD (MICs of 1.17 and 3.16 µM, respectively). Interestingly, cell death with AEA was not due to apoptosis but was instead due to AEA inhibiting the G_1/S phase of cell division (De Petrocellis et al., 2000). Although comparable studies with AEA have not been performed with protists, it is interesting to note that the cells of *T. pyriformis* were most susceptible to THC during their G_2 phase of cell division (Zimmerman et al., 1981).

The observed inhibitory effects of cannabinoids on cancer cells mirror those observed with protists. For example, the following trends are shared by both: (i) CBD is more toxic than THC, (ii) AEA is more toxic than CBD, (iii) different cell types show differences in susceptibility and, (iv) cell death occurs with cannabinoid concentrations in the lower end of the µM range. Since phylogenetic studies suggest that protists do not possess the main endocannabinoid receptors (McPartland et al., 2006), and cannabinoids can act upon mammalian cells <u>without</u> the need for receptors (Sanchez et al. 1998; De Pretrocellis et al.,

2000), it is intriguing to discover what their target is in protists and how this results in cell death.

4.4   Mechanisms of cell death in *Tetrahymena*

In the ciliate *T. thermophila*, programmed nuclear death (PND) mainly occurs during conjugation (sexual reproduction) and involves an apoptosis inducing factor (AIF), and endonuclease G (EndoG) in the mitochondria, resulting in macronucleus degeneration (Akematsu et al., 2012). Although *T. pyriformis* is not capable of sexual conjugation (Nanney, 1974) it might still possess the same PND mechanism as that exhibited by *T. thermophila*, which is similar to programmed cell death (PCD) in multicellular organisms. In mammalian cells, the macronucleus is degraded via chromatin condensation and DNA fragmentation with the help of AIF, EndoG and caspases (Akematsu et al., 2012). However, *T. thermophila* does not possess caspases (Eisen et al., 2006) and PND is considered to occur via a caspase-independent route involving either AIF or EndoG (Akematsu et al., 2012).

In a caspase independent pathway, many mitochondria move towards the parental macronucleus and AIF is released from their inter membrane space which is then translocated into the nucleus and results in nuclear condensation and large-scale DNA fragmentation (Akematsu et al., 2012). Electron microscopic studies have also revealed the presence of autophagosomes surrounding this degenerating macronucleus during *Tetrahymena* conjugation (Weiske-Benner and Eckert, 1987) suggesting that autophagy might play a direct role in PND in *Tetrahymena*.

Autophagy is a major degradative pathway which recycles cytoplasmic compartments by using the double membrane structure (the autophagosome) to fuse with the vacuole/lysosome (Yorimitsu and Klionsky 2005). Caco-2 cells have been shown to activate autophagosome arrangement between 4-6 hours after treatment with 10 µM CBD while 25 µM CBD led to over-expression of LC3-II protein which resulted in cell death (Koay et al., 2014). In protists, the degradative pathway can be upregulated by stress, with the main one being nutrient limitation. Under such conditions increased levels of autophagosomes have been recorded in both *Tetrahymena* (Nilsson, 1984; Zhang et al., 2015) and *D. discoideum* (King et al. 2011, Mesquita et al., 2013). This pro-survival characteristic of autophagy maintains tissue homeostasis and sustains cell viability under stressful conditions but when failing to restore

homeostasis, autophagy leads to cell death through the autophagic cell death (ACD) pathway (Levine & Kroemer, 2008). In *T. thermophila*, such autophagic cell death has been recorded in the presence of H_2O_2, Oligomycin and vitamin K3 but the molecular basis that regulates this, especially the genes responsible, have not been identified (Zhang et al., 2015). However, this study did correlate enhanced autophagic cell death to an accumulation of reactive oxygen species (ROS) resulting from possibly a blockage of mitochondrial electron transport (Zhang et al., 2015).

Components of the mitochondrial respiratory chain have been identified as molecular targets of cannabinoids (Bih et al., 2015). THC is a known inhibitor of NADH oxidase activity in mitochondria and above a threshold concentration (0.4 µM THC), can deplete energy (ATP) levels in cells which can lead to cellular death (Bartova & Birmingham, 1979). Human lung cancer cells (H460) treated with THC, and indeed AEA, show a concentration dependent decrease in the mitochondrial oxygen consumption with maximum effect at 20 µM (Athanasiou et al., 2007). Studies have also shown that CBD modulates several cytochrome P450 enzymes (Usami et al., 2008).

Finally, THC, AEA and 2-AG can exert their effects on human cells through inhibition of adenyl cyclase activity (Koh et al., 1997; Pisanti et al., 2013). *Tetrahymena* have been shown to contain a novel adenyl cyclase ion channel fusion protein, localized to ciliary membranes through which cAMP formation is stimulated by an ion conductance (Weber et al., 2009). Therefore, inhibition of this adenylase cyclase activity at this site might explain the lowered cAMP levels in THC treated *T. pyriformis* (Zimmerman et al., 1981), the sluggish movement of *T. pyriformis* cells in the presence of THC (McClean & Zimmerman, 1976) and CBD (Parry, personal communication), and the loss of *T. thermophila*'s avoidance response to chemorepellents (Keedy et al. 2003).

There are therefore many mechanisms by which *Tetrahymena* cells can die and also many ways in which cannabinoids can induce such death. The current study primarily examined the possible involvement of PPARs on ciliate cell death, as their interaction with cannabinoids has been well characterized in human cells (Kleberg at al., 2014; Bih et al., 2015; O'Sullivan, 2016).

4.5. Involvement of receptors in cell death

The Hill slope (IC_{50} slope factor) indicates the cooperativity/cooperative binding of the ligand. Cooperative binding occurs when binding of the first molecule (either identical or nearly identical) to a receptor changes the binding affinity for the second molecule, either positive or negative (Stefan and Le Nove`re, 2013). For example, the binding of one oxygen to the first hemoglobin's binding site increases the binding affinity (or also known as increase cooperativity) of oxygen to the three remaining binding sites (Stefan and Le Nove`re, 2013). In general, a Hill slope value of greater than 1 indicates positive cooperativity, whereas a value lesser than 1 indicates a negative cooperativity.

In this study, all agonists yielded positive slope values and so, all agonists induced a cooperative binding effect. OEA and CBD had similar slope values (7.86 and 8.11, respectively), suggesting they bind at a similar pace to the receptor, and might possess the same target(s) (Table 3.1). Although this might suggest that *T. pyriformis* possesses a PPARα-like receptor, PEA (which also binds to PPARα) did not behave similarly to OEA and its slope (5.28) was closer to that of Rosiglitazone (5.69) suggesting PEA and Rosiglitazone might share the same target(s) (Table 3.1). Thus, this suggests that there could be multiple targets for the endocannabinoid pathways in *T. pyriformis*.

4.5.1   PPARs

PPARs are mainly involved in the metabolism of fatty acids and carbohydrates, however, there is evidence that activation of PPARs is also involved in cell line proliferation and apoptosis (Stephen et al., 2004; Brunetti et al., 2019). Studies have shown that activation of PPARs can result in cytotoxic effects on various cancerous cell lines (Kim et al., 2007), and anticancer agents are being developed from this concept.

In the current study, all three PPARs were suggested to be involved in the mechanism by which AEA, CBD and GW0742, but not OEA caused cell death in the ciliate population. In the presence of GW6471 (PPARα blocker), the negative effects of CBD, AEA and GW0742 were alleviated. However, GSK3787 (PPARβ/δ blocker) and T0070907 (PPARγ blocker) were only able to block the effect of AEA and GW0742. These results bore no resemblance to the predictions made based on the IC_{50} slope values.

These results also contradict those obtained from similar studies in mammalian cells (Pertwee et al., 2010; O'Sullivan, 2016). In these systems, OEA is reported to induce activation of all PPAR isoforms (O'Sullivan, 2016) but this was not recorded in the current study. AEA is also considered an agonist of PPARα and PPARγ, but not PPARβ/δ, with the potency (IC_{50}) of $10 - 30$ µM and $8 - 10$ µM, respectively (Pertwee et al., 2010). In *T. pyriformis*, the action of AEA was 100% blocked at a much lower concentration (≤1 µM) and with all three blockers. The highly selective PPARβ/δ agonist (GW0742) is reported to interact with human PPARβ/δ, - α, and -γ, with EC_{50} values of 0.001, 1.1, and 2 µM, respectively (Sznaidman et al., 2003). The current study also found an interaction with the three PPAR types, with the effect of GW0742 being 100% blocked by all three antagonists, but the concentrations required were variable. GW0742 did not appear to be 'highly selective' for PPARβ/δ as the concentration required for 100% blocking was equivalent to that for PPARγ (≤1 µM), whereas ≤10 µM was required to block PPARα. Finally, CBD is reported to only activate PPARγ in the mammalian system (O'Sullivan et al., 2009a), with an IC_{50} of 5 µM (O'Sullivan et al., 2009b), yet in the current study its activity was 100% blocked by only the PPARα antagonist and at a lower concentration (≤1 µM).

Nonetheless, the results of the PPAR blocking study indicated that there was an alleviation of cell death in *T. pyriformis* by the PPAR antagonists and thus suggests that *T. pyriformis* either possesses, 1) all three PPAR isoforms, or 2) possibly one isoform which can bind all three agonist/antagonist types. To date, the three PPAR isoforms have only been reported in bony fish, mammals, birds, and amphibians (Zhou et al. 2015); not in invertebrates. This suggests that *T. pyriformis* might not possess all three, but a PPAR isoform that can bind all three agonists/antagonist types. PPARs are known to have a ligand-binding domain which is relatively spacious and promiscuous, binding a number of ligands at different sites (Itoh et al., 2008). It might be that even though all three agonists/antagonists can bind to this PPAR they bind at different sites. For example, CBD might bind at the site that is the same as GW6471 (the PPARα blocker) but the other two blockers do not bind to this particular site and therefore do not interfere with the binding of CBD.

However the ligand binds, it can exert an effect through both PPAR-dependent (genomic) and PPAR-independent (non-genomic) mechanisms. The classical genomic mechanism involves PPAR interacting with the retinoid X-receptor (RXR) to form a heterodimer

followed by the activation of target gene transcription (Grygiel-Górniak, 2014). The non-genomic, and more rapid, mechanism involves ligand activation of PPARs that leads to the suppression of other gene expression by antagonizing transcription factors (Campand and Tafuri, 1997).

With regards to the latter, activation of PPARα and γ has been shown to directly antagonize the NF-κβ pathway in many types of cancer cells (Camp et al., 1999; Lau et al., 2002; Chandran et al. 2016; Morinishi et al. 2019). NF-κB belongs to a highly conserved family of transcription factors which, when activated, rapidly translocate into the nucleus and induces the transcription of various cellular genes (Pahl, 1999). Ligand binding to PPARα and γ has also been shown to inhibit of the activation, and nuclear translocation, of mitogen-activated protein (MAP) kinases, leading to apoptosis in cancer cells (Chinetti et al., 1998; Su et al., 1999), with the main MAPKs being p42/p44 (ERK1/2) (Takedaet al., 2001; Chandran et al. 2016). ERK1 and ERK2 have been shown to be key mediators of signal transduction, transmitting signals from the cell surface to the nucleus in human cells (Lenormand et al. 1998; Volmat et al. 2001).

Considering protists are not known to possess RXR nor NF-κB, but *T. thermophila* possesses numerous MAPKs including putative ERK1/2 subfamily MAPKs (www.ciliate.org), it makes the latter a possible pathway by which cell death occurs in this protist.

In addition to affecting the NF-κB and MAPK pathways, and inducing apoptosis, ligand binding to both PPARα and γ has been shown to induce G_0/G_1 cell cycle arrest leading to the death of cancer cells by reducing the levels of various cell-cycle regulating cyclins, specifically cyclinD1, A and E (Aboud et al. 2013; Chandran et al. 2016). Although arrest at the G_0/G_1 phase is the most commonly reported, cell cycle arrest can occur at the G_1/S phase (De Petrocellis et al., 2000; Wakino et al. 2000).

The role of PPARβ/δ in cell death is controversial. Some studies have suggested that PPARβ/δ agonists and antagonists demonstrate anticancer effects (Zaveri et al., 2009), while others report that PPARβ/δ stimulate proliferation and suppress pro-apoptotic events in the development of colon, breast and prostate cancer (Stephen et al., 2004; Sertznig et al., 2007). Ligand activation of PPARβ/δ with GW0742 has also been shown to prevent cell cycle progression from G_1 to S phase in keratinocyte cells (Burdick et al., 2007).

PPARs are also involved in fatty acid oxidation and adipogenesis, which makes them important molecular targets in the treatment of obesity and diabetes (Grygiel-Górniak, 2014).

OEA is reported to induce activation of all PPAR isoforms in mammalian cells, with the highest affinity for PPARα (O'Sullivan, 2016) but this was not recorded in the current study. Further research examined whether GCPRs and the Dopamine receptor might be involved in the action of OEA on *Tetrahymena* cells.

4.5.2   G protein-coupled receptor

GPCRs are members of the superfamily of 7-transmembrane receptors (7-TMR) (Morales et al., 2018) and can be blocked with the Pertussis toxin (PTX). PTX is derived from bacterium *Bordetella pertussis* and it acts by modifying the natural function of GCPRs, specifically catalyzing the ADP-ribosylation of a cysteine residue on the α-subunit of $G_{i/o}$ (Reisine and Law, 1992). The subunit $G\alpha_i$ is not affected by PTX (Reisine and Law, 1992).

GPCRs are involved in many regulatory functions in humans such as growth, source of smell, taste, visual, behavioural, immune system and mood (Rosenbaum et al., 2009). Over 800 GPCRs have been identified as being specific to a particular function (Fredriksson et al., 2003). The ligands they bind range from light sensitive compounds, odours, pheromones, hormones, cannabinoids and even neurotransmitters (Rosenbaum et al., 2009), but even now some GPCR have unknown functions or unknown primary ligands, and are termed 'orphan' GPCRs. Since (i) GPCRs are involved in a wide array of regulatory functions, (ii) they can be activated by numerous ligands including cannabinoids, (iii) the main cannabinoid receptors CB_1 and CB_2 are both GCPRs (but are absent in *Tetrahymena*) and, (iv) homologues to GPR6 and GPR37 have recently been identified *T. thermophila* (Lampert et al., 2011; Zou and Hennessey, 2017), it seemed sensible to carry out a preliminary investigation to evaluate whether GCPRs might be the target for OEA in *Tetrahymena*.

Of the two GCPRs in *Tetrahymena*, GPR6 is considered a potential endocannabinoid as it shares a high amino acids sequence identity (35%) with CB_1 and CB_2 (Lee et al., 2001). CBD is considered to be an inverse agonist for GPR6 (Laun and Song, 2017) however, PTX had no effect on the interaction between CBD and *T. pyriformis* in the current study. Blocking with PTX also had no effect on the action of AEA, but this agrees with results for human cells

whereby AEA has not been shown to interact with GPR3, GPR6 and GPR12 (Brown et al., 2017). Conversely, PTX alleviated the negative action of OEA on *T. pyriformis* suggesting it employs a pathway involving GCPRs whereas CBD and AEA employ a pathway involving PPARs.

OEA can elicit a response in human cells via GPR119 which is phylogenetically related to cannabinoid receptors, and is only activated by fatty acid amides, including AEA, OEA and PEA, with OEA being the most efficacious (Overton et al., 2006). OEA binding to GPR119 leads to activation of adenylyl cyclase, increased production of cAMP, and enhanced Protein Kinase A (PKA) activity (Usdin et al., 1993). However, in the current study the blocking of PKA with H89 had no effect on OEA-induced death of the ciliate. This has also been observed in breast cancer cells, i.e., activation of GPR119 has been shown to reduce the growth of these cells and induced apoptosis by suppressing autophagosome formation but, this is not blocked with cAMP/PKA inhibitors (Im et al., 2018). The authors suggested that apoptosis induction and autophagy inhibition by a GPR119 agonist might be related to changes in cancer cell metabolism instead of canonical signaling pathway(s) of GRP119 because there was increased levels of lactate in the cells, due to glycolysis stimulation, which suppressed mitochondrial functioning (Im et al., 2018). Indeed, the fact that modulation of GPR119 is involved in glucose homeostasis in many cell types has led to the idea that GPR119 modulation might provide the basis for an anti-obesity and type 2 diabetes therapy (Overton et al., 2006).

Being a preliminary study, it is difficult to speculate further regarding the OEA pathway in *Tetrahymena* and further study is warranted.

4.5.3   Dopamine receptor

This study investigated this possibility of OEA using a pathway involving the dopamine receptor, using the well-established Dopamine blocker, Haloperidol (HPD). The result was unlike that seen with PPAR/GPCR blockers in that the presence of HDP alone proved toxic to the ciliate at concentrations above 60 µM. A toxic effect has also been reported in RAW264.7 macrophage cells, but at a lower concentration (40 µM HPD), whereby a cellular increase in nitric oxide and caspases 8 and 3 resulted in the modulation of apoptosis (da Cruz Jung et al., 2015). HPD has also been shown to promote ferroptosis in hepatocellular

carcinoma cells at concentrations as low as 10 µM (Bai et al., 2017). Ferroptosis is a form of cell death, characterised by the accumulation of reactive oxygen species (ROS), cell volume shrinking and increased mitochondrial membranes during oxidative stress metabolism. During ferroptosis, the iron-dependent accumulation of ROS induces the activation of ferroptosis through glutathione peroxidase 4 (GPX4) or glutathione (GSH) deficiency (Bai et al., 2017). Haloperidol promotes ferroptosis through binding to the Sigma 1 receptor (S1R), a protein modulator associated with a wide array of neurological diseases and increases iron accumulation and lipid peroxidation (Collina et al., 2013). S1R has not been reported in *Tetrahymena* to date and this ciliate appears to be less sensitive to HPD than mammalian cells.

When HPD was combined with OEA (45 µM) there was a significant reduction in the ciliate population, compared to OEA alone; reducing it to ~21% (with 0.001 and 0.01 µM HPD), ~17% (with 0.1 and 1 µM HPD), and to ~15% and 1% in the presence of 10 µM and 30 µM HPD. This shows that HPD somehow enhanced the effect of OEA. This might suggest that there are multiple pathways involved, with possibly the activation of multiple binding targets by HPD.

Although Haloperidol has a high affinity for the D_2 receptor in mammalian cells ($K_i = 1.2$ nM), it can target other Dopamine subtypes including D_3, D_4, D_1 and D_5 with K_i of ~7, 2.3, ~80, and ~100 nM, respectively (Seeman and Van Tol, 1994; Ilyin et al., 1996). At the same time, HPD can also act on the Serotonin receptor and subtype-selective *N*-methyl-D-aspartate (NMDA) antagonist (Lynch and Gallagher, 1996). Only D_1 and NMDA (involved in chemotaxis and Ca^{2+} signaling) has been shown to be present in *Tetrahymena* to date (Nam et al., 2009; Ud-Daula et al., 2012). Since HPD was not specific to a particular dopamine receptor (or D_2 alone), and HPD-induced death might result in multiple death pathways future work is required needed to be conducted, with the use of more specific and separate blockers for dopamine and serotonin.

4.6 Conclusions

This study aimed to quantify and compare the lethal effect of selected PPAR agonists; OEA, PEA, AEA, CBD and two synthetic cannabinoids (GW0742 and Rosiglitazone) on *Tetrahymena pyriformis*. Results showed that the ciliate was most sensitive to AEA, followed

by CBD, GW0742 and OEA, with PEA and Rosiglitazone having little lethal effect. The MICs and IC$_{50}$ values with *T. pyriformis* were of the same order as those concentrations required to elicit an effect in cancer cells. The effect on *T. pyriformis* was cell death, however, this was only observed for a 90 minutes period after which no net negative effect was recorded.

The study then aimed to determine whether the negative response was elicited through a pathway involving PPARs, GCPRs and/or a dopamine receptor. Results showed that the action of AEA, CBD and GW0742 utilized a PPAR pathway whilst OEA possibly utilizes a GCPR pathway.

Appendix I: Media Recipes

Chalkley's medium

Stock solution

NaCl	2.0 g
KCl	0.08 g
CaCl$_2$	0.12 g

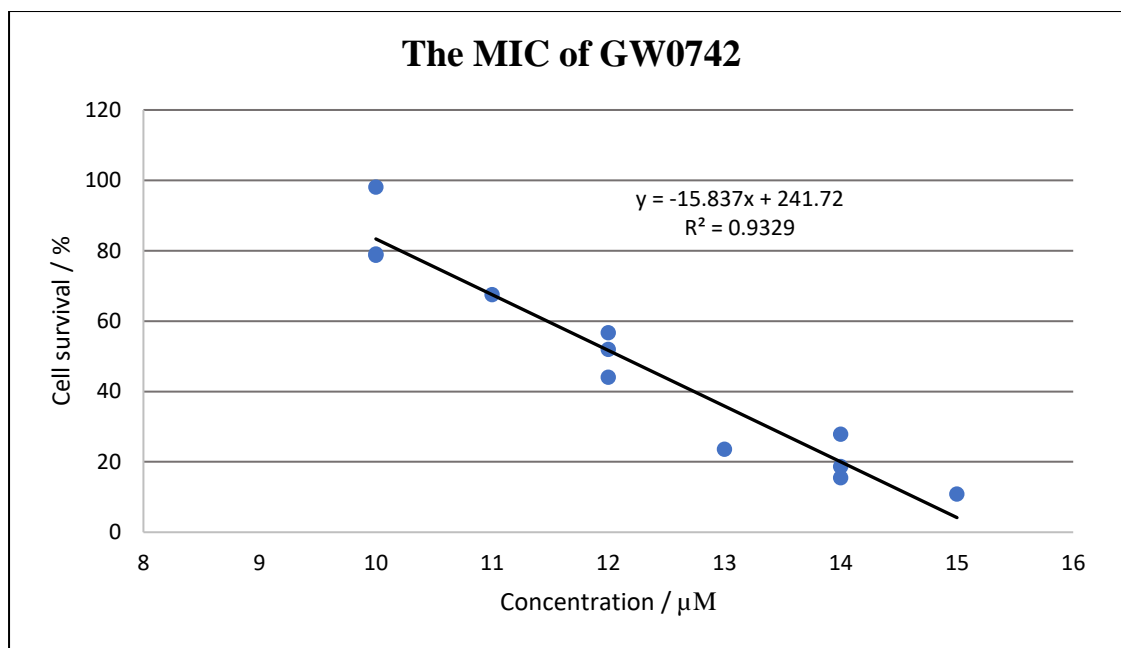Add 5ml of stock solution to 1L of distilled water. Autoclave at 121°C for 15 minutes.

LB agar

Distilled water	1 L
NaCl	10 g
Agar No.2	10 g
Tryptone	5 g
Yeast	15 g

Autoclave at 121°C for 15 minutes. Cool to 50ºC before aseptically pouring onto plates.

Appendix II: Raw data

The MIC of OEA

$$y = -1.8294x + 133.82$$
$$R^2 = 0.862$$

Cell survival / %

Concentration / µM

The MIC of PEA

$$y = -0.2016x + 111.11$$
$$R^2 = 0.8208$$

Cell survival / %

Concentration / µM

The MIC of GW0742

$y = -15.837x + 241.72$
$R^2 = 0.9329$

Cell survival / %

Concentration / μM

The MIC of Rosiglitazone

$y = -0.6692x + 169.39$
$R^2 = 0.9497$

Cell survival / %

Concentration /μM
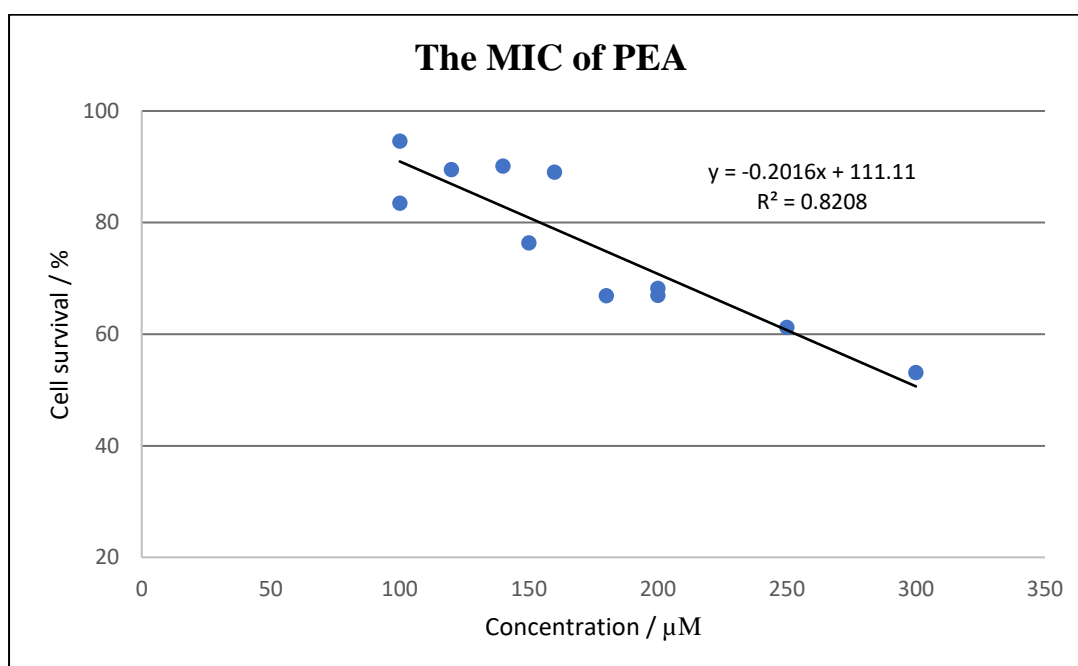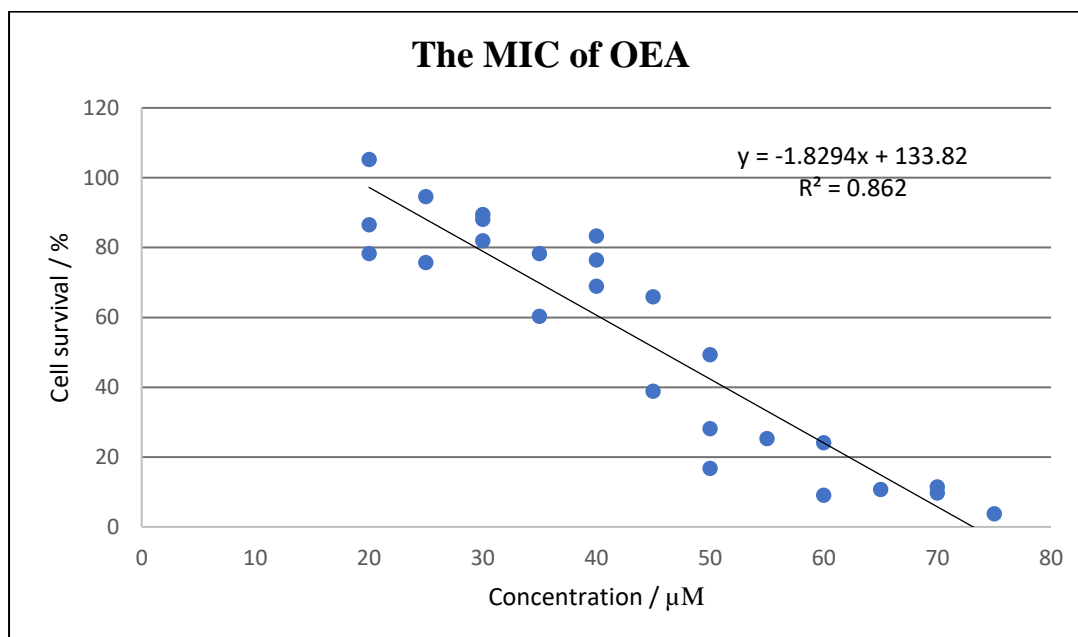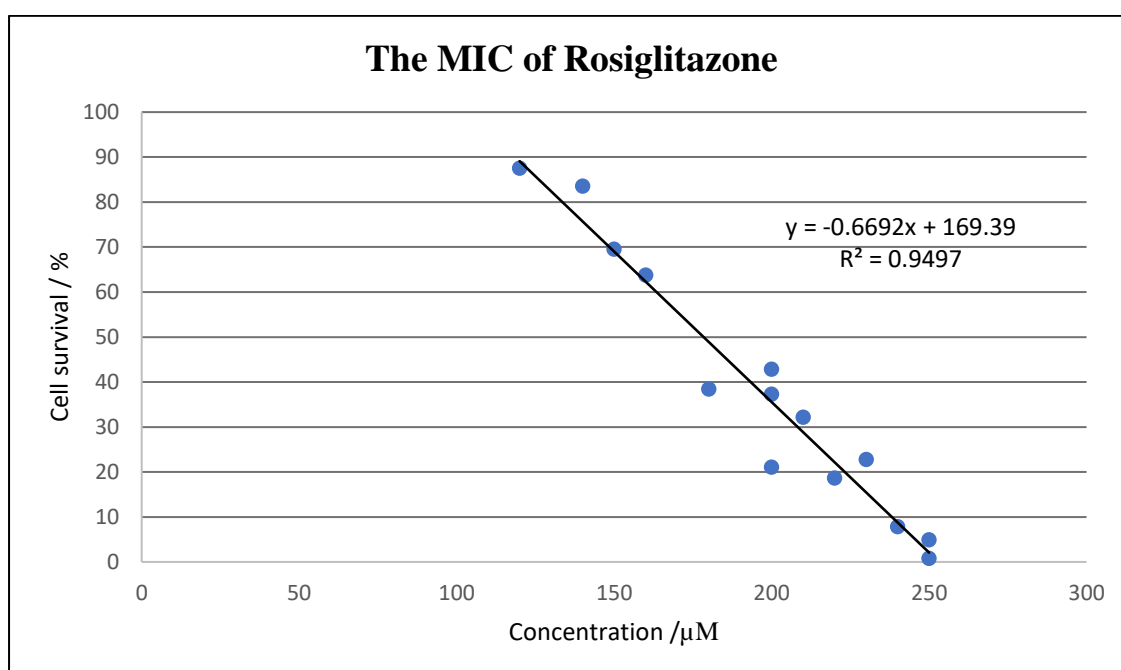
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