
THE CNS ENDOCANNABINOID SYSTEM: SYNTHETIC CANNABINOIDS AND METABOLIC ENZYMES

By

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ABSTRACT

Glioblastoma multiforme (GBM) is a devastating primary tumour of the central nervous system. This tumour arises from glial cells, including astrocytes and oligodendrocytes, which comprise >90% of brain volume. GBM is the most frequent and aggressive malignant brain tumour, with a poor prognosis despite available surgical and radio-/chemotherapy, prompting the necessity for alternative therapies. Recent reports indicate that cannabinoids can inhibit viability of several types of cancer cells in both in vitro and in vivo models. However, the diversity of experimental conditions and of outcomes has hindered definitive conclusions about cannabinoid efficacy. The endocannabinoid system is widely distributed in the body, and its dysregulation is associated with a variety of diseases, including various types of cancer. Anticancer activities of phytocannabinoids are mediated in glioma cells, at least in part, by the endocannabinoid receptors, triggering various cellular signalling pathways. Yet, due to the scarcity of good quality clinical trials, there remains no solid basis for the anticancer therapeutic potential of cannabis compounds.

The initial aim of this project was to study the central nervous system (CNS) endocannabinoid system and its potential involvement in neurotoxicity using cell line models, lab animals, and human brain samples. The first part of this thesis addressed the effects of cannabinoids and inhibitors of the major enzymes involved in endocannabinoid metabolism on the proliferation of the 1321N1 human astrocytoma cell line using a standard colorimetric assay. The modestly CB2-preferring agonist HU210 or CB2-selective agonist JWH015 appeared to increase 1321N1 cell numbers. The selective CB1 inverse agonist AM251 decreased 1321N1 cell viability which could suggest the involvement of the cannabinoid signalling system in the protection of human astrocytoma cells from apoptosis. However, the precise mechanism of action of AM251 was unable to be determined.

Activity-based protein profiling (ABPP) identified distinct patterns of serine hydrolases in the human astrocytoma and neuroblastoma cells. Differentiation of the SH-SY5Y cells changed the activity of multiple enzyme activities. The use of selective inhibitors suggested the presence of endocannabinoid hydrolases (MAGL and possibly ABHD6) in the astrocytoma cells, with FAAH activity in the neuroblastoma. Further characterization of these enzyme activities and their function in the two cells is warranted. ABPP in rat brain soluble and particulate fractions showed distinct profiles of serine hydrolase activities; these were largely consistently expressed across the eight brain regions examined. The use of enzyme inhibitors in the rat brain samples suggested the detection of MAGL and ABHD6, but no clear evidence for FAAH activity, indicating their involvement in brain function pathophysiology. Further work would be needed to corroborate these findings. In the rat spinal system, serine hydrolases could be detected in individual dorsal root ganglia, the pattern of which was

distinct from the spinal cord preparations. Analysis of tissue samples from animals treated with monosodium acetate as a model of arthritis showed an elevation in the activities of MAGL and ABHD6 suggesting the role they play in pain transmission. Further research is required for other serine hydrolase enzymes which as CES2 for which the activity increased on the contralateral side, suggesting a potential compensatory role in weight-bearing.

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
2-OG	2-oleoylglycerol
7HCA	7-Hydroxycoumarinyl arachidonic acid ester
AAMC	Arachidonyl 7-amino, 4-methyl coumarin amide
Δ 9-THC	Delta-9-tetrahydrocannabinol
THCV	Δ 9-tetrahydrocannabivarin
1321N1	Human astrocytoma cell line
SH-SY5Y	Human neuroblastoma cell line
ABHD6	α/β -hydrolase domain containing 6
ABHD12	α/β -hydrolase domain containing 12
AB-FUBINACA	N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide
AB-PINACA	(S)-N1(1-amino-3-methyl-1-oxan-2-yl)-1-pentyl-1H-indazole-3-carboxamide
ABPP	Activity-based protein profiling
ACEA	Arachidonyl-2'-chloroethylamide
AEA	Anandamide
AM251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
AM630	6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl(4-methoxyphenyl)methanone

ANOVA	Analysis of variance
AKB-48	N-(1-Adamantyl)-1-pentylindazole-3-carboxamide
AMP	Adenosine monophosphate
APS	Ammonium persulfate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
BNPP	Bis-4-nitrophenol phosphate
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid receptor
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CID16020046	4-[4,6-Dihydro-4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxopyrrolo[3,4-c]pyrazol- 5(1H)-yl]benzoic acid
CNS	Central nervous system
CP55,940	(-)- <i>cis</i> -3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]- <i>trans</i> -4-(3-hydroxypropyl)cyclohexanol
CP945598	1-[8-(2-Chlorophenyl)-9-(4-chlorophenyl)-9 <i>H</i> -purin-6-yl]-4-(ethylamino)piperidine-4-carboxamide hydrochloride
DAG	Diacylglycerol
DAGL α	Diacylglycerol lipase alpha
DAGL β	Diacylglycerol lipase beta
DEA	N-docosatetraenoyl-ethanolamine
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DRG	Dorsal Root Ganglia
EGF	Epidermal growth factor
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ERK	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolase
FBS	Foetal bovine serum
FDA	The United States Food and Drug Administration
FP	Fluorophosphonate (FP) Probe
HU-210	(6aR)- <i>trans</i> -3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol
GBM	Glioblastoma multiforme
GPCRs	G protein-coupled receptors
GPR18	G protein-coupled receptor 18
GPR55	G protein-coupled receptor 55
GPR92	G protein-coupled receptor 92
GPR119	G protein-coupled receptor 119
JJKK048	[4-[bis(1,3-benzodioxol-5-yl)methyl]piperidin-1-yl]-(1,2,4-triazol-1-yl)methanone
JNJ1661010	N-Phenyl-4-(3-phenyl-1,2,4-thiadiazol-5-yl)-1-piperazinecarboxamide
JWH-015	(2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenyl-methanone
JWH-018	1-naphthalenyl(1-pentyl-1H-indol-3-yl)-methanone

JZL 184	JZL184, 4-nitrophenyl 4-[bis(2H-1,3-benzodioxol-5-yl) (hydroxy)methyl] piperidine-1 carboxylat
KML29	(1,1,1,3,3,3-hexafluoropropan-2-yl 4-(bis(benzo[d][1,3]dioxol-5-yl)
KT203	4'-[1-[[2-(Phenylmethyl)-1-piperidinyl]carbonyl]-1H-1,2,3-triazol-4-yl]-[1,1'-biphenyl]-3-carboxylic acid]
MAFP	Methyl arachidonyl fluorophosphonate
MAGL	Monoacylglycerol lipase
MIA	Monosodium iodoacetate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA	messenger RNA
MUH	4-methylumbelliferylheptanoate
NADA	N-arachidonoyl dopamine
NADH	Nicotinamide adenine dinucleotide, reduced form
NAEs	N-acylethanolamines
NGF	Nerve growth factor
NAPE	N-arachidonoyl phosphatidylethanolamine
NAPE-PLD	N-arachidonoyl phosphatidylethanolamine phospholipase D
NPB	4-nitrophenylbutyrate
NSE	Neuron-specific enolase
ODA	cis-9-octadecenamide
OMDM169	(S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl formylglycinate
PEA	N-palmitoyl-ethanolamine
PBS	Phosphate-buffered saline
PPARs	Peroxisome proliferator-activated receptors
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SR144528	5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl) methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide
TEMED	(N, N, N',N'-tetramethylethylenediamine
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid-1
TRPV2	Transient receptor potential vanilloid type 2
URB597	3-(3-carbamoylphenyl)phenyl N-cyclohexylcarbamate
WIN55212-2	(R)-(+)-[2,3-dihydro-5-methyl-3-(4-Morpholinyl methyl)pyrrolo [1,2,3 <i>de</i>]1,4benzoxazin-6- yl]-1-naphthalenyl methanone mesylate
WWL70	4-(4-carbamoylphenyl)phenyl N-methyl-N-{[3-(pyridin-4-yl)phenyl]methyl}carbamate
XLR-11	[1-(5-fluoropentyl)-1H-indol-3-yl] (2,2,3,3 tetramethylcyclopropyl) methanone

CHAPTER 1

Introduction and literature review

1.1 The endocannabinoid system

The investigation of the psychotropic effects of THE Delta-9-tetrahydrocannabinol (THC), the primary active component in the plant *Cannabis sativa*, resulted in the identification of cannabinoid receptors (Devane et al., 1988, Gaoni and Mechoulam, 1966). Matsuda and colleagues discovered the cannabinoid receptor 1 (CB1) in a rat cerebral cortex cDNA library in the early 1990s (Matsuda et al., 1990), which was shown to be distributed mostly in the central nervous system (CNS) and to a lesser degree in peripheral neuronal and other tissues. This resulted in the isolation of the endogenous cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995, Sugiura et al., 1995) and cloning of the second cannabinoid receptor, cannabinoid receptor 1 (CB2) from human leukaemia (HL-60) cells in 1993 (Munro et al., 1993).

CB2 is a receptor that is abundantly expressed by immune cells, where it mediates an immunosuppressive function. It has also been reported that CB2 receptors are expressed in brain tissue; however, there is some disagreement regarding the extent to which this receptor is expressed by neuronal tissue (Carlisle et al., 2002, Onaivi et al., 2008, Walter et al., 2003).

1.1.1 Endocannabinoids

The endocannabinoid system consists of endogenous ligands called endocannabinoids, their cannabinoid receptors and proteins involved in the production and degradation of endocannabinoids. Arachidonoyl ethanolamines, such as AEA (Devane et al., 1992), and 2-AG (Sugiura et al., 1995), are the main endocannabinoids (Figure1.1). These endogenous ligands are lipid mediators that are produced on demand by membrane precursors and phospholipids, as opposed to traditional neurotransmitters which are stored in vesicles (Massa et al., 2005), included N-acyl-phosphatidylethanolamine-selective phospholipase D (NAPE-PLD), then instantly freed from cells after synthesis (Izzo and

Camilleri, 2008). The receptors are engaged to generate a biological response, which is subsequently inactivated by carrier proteins (yet to be identified at the molecular level) in the cell membrane called the endocannabinoid membrane transporters, followed by enzyme breakdown. AEA is converted into arachidonic acid and ethanolamine by fatty acid amide hydrolase (FAAH), whereas 2-AG is degraded into arachidonic acid and glycerol by monoacylglycerol lipase (MAGL) (Izzo and Camilleri, 2008). Two G protein-coupled receptors, CB1 and CB2 receptors have been identified across mammals. CB1 receptors are mainly found on central and peripheral neurons, particularly the enteric nervous system, the intrinsic neurons, the extrinsic neurons such as sensory cell bodies in ganglions of the dorsal root and ganglions and the vagal efferent cell nerves (Massa et al., 2005). In immune cells, particularly neutrophils, activated macrophages and subsets of T and B cells, and epithelial cells, the CB2 receptors are mostly expressed (Wright et al., 2005).

Other endogenous compounds show "cannabinoid-like" properties and are referred to as atypical endocannabinoid, including N-arachidonoyl dopamine (NADA), virodhamine, N-homo- γ -linolenoyl-ethanolamine(HEA),N-docosatetraenoyl-ethanolamine(DEA), palmitoylethanolamide (PEA), oleoylethanolamide and 2-AG (noladin ether), however, can change cannabinoid function possibly via entourage impacts. Using assays of rapid receptor activation n-6 NAEs (AEA, docosatetraenoyl and docosapentaenoyl derivatives) have been shown to activated both CB1 and CB2 receptors, as well as TRPV1 channels, suggesting them to be 'genuine' endocannabinoids and 'endovanilloids'. The n-3 NAEs (eicosapentaenoyl, docosapentaenoyl and docosahexaenoyl derivatives) activated CB2 receptors and some n-3 NAEs (docosapentaenoyl and docosahexaenoyl derivatives) also activated TRPV1 channels, but failed to activate the CB1 receptor (Alharthi et al., 2018).

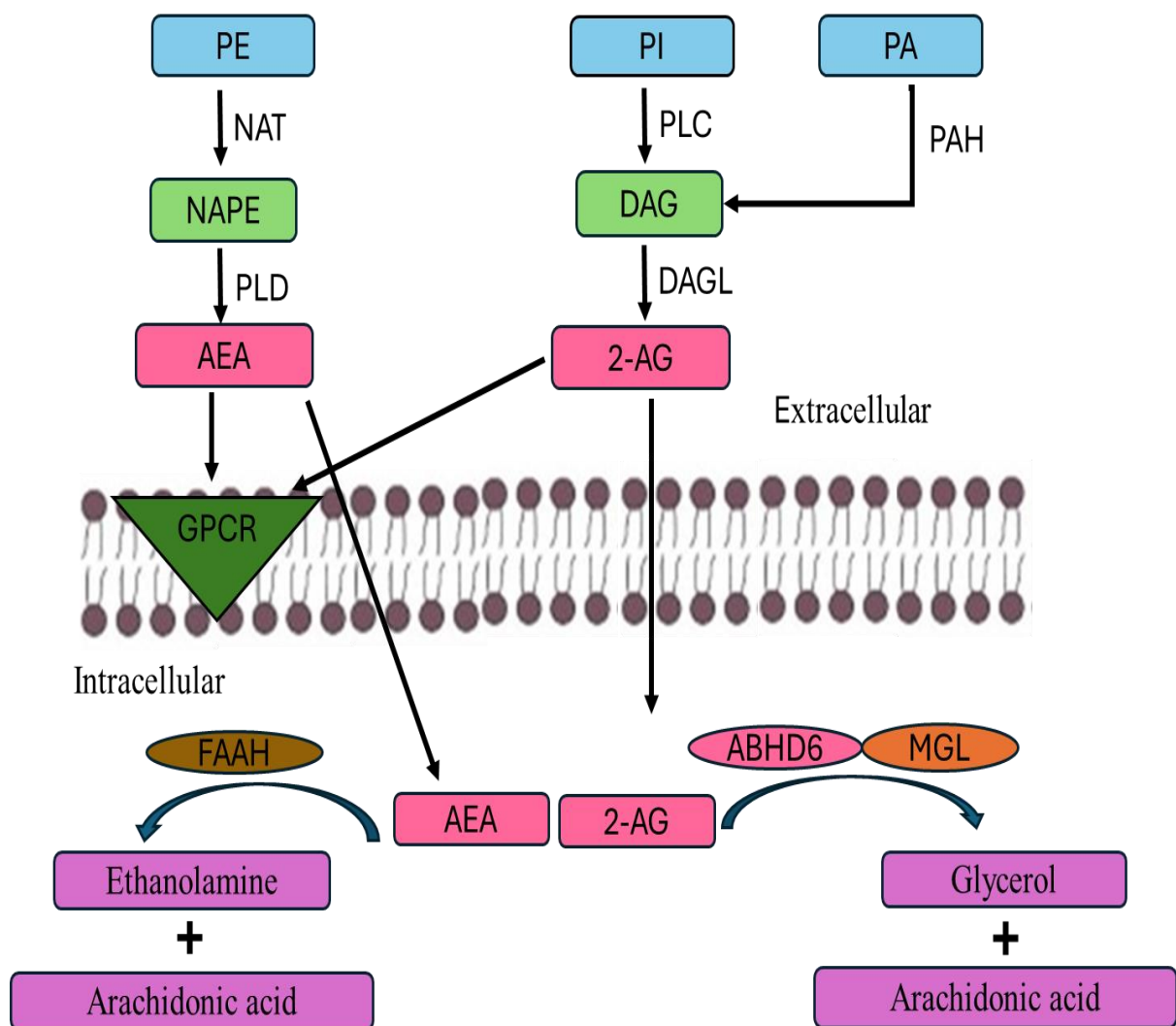


Figure 1. 1 Endocannabinoids are synthesised, transported, and degraded in the body (Sun et al.,2019). NAPE-phospholipase-D (PLD) converts N-arachidonoyl phosphatidylethanolamine (NAPE) to AEA. N-acetyltransferase (NAT) transfers an arachidonoyl group to phosphatidylethanolamine (PE). Diacylglycerol lipase (DAGL) synthesises 2-AG from diacylglycerol (DAG), which is obtained from phosphatidylinositol (PI) via phospholipase-C (PLC). Alternatively, phosphatidic acid hydrolase (PAH) hydrolyzes phosphatidic acid (PA) to form DAG. Lastly, FAAH is mostly responsible for the degradation of AEA, whereas monoacylglycerol lipase (MGL) and serine hydrolase α - β -hydrolase domain 6 (ABHD6) are primarily responsible for the hydrolysis of 2-AG. GPCR is G protein-coupled receptors.

1.1.2 Cannabinoid receptors

The classical cannabinoid receptors CB1 and CB2 have been identified as mediators of cannabinoid-induced effects after being cloned and studied in detail. An increasing body of data supports the participation of additional cannabinoid-affinity receptors, often known as non-CB1/CB2 receptors.

1.1.2.1 CB1 and CB2 Cannabinoid receptors

There are two canonical cannabinoid receptors: CB1 and CB2. CB1 regulates neurotransmission in a variety of brain areas. CB2 controls immune responses and inflammatory pathways when it is activated. When CB2 is knocked out, mice display markedly elevated signs of inflammation, which supports the notion that, in addition to its association with peripheral expression in brain microglia, CB2 also has an important role in neuroinflammation (Ashton and Glass, 2007, Palazuelos et al., 2009). Therefore, cannabinoid receptors CB1 and CB2 are the best studied. It is important to note that both of these receptors are GPCRs that mainly bind to inhibitory G proteins, Gi. They inhibit the activity of adenylyl cyclase, stimulate MAP (mitogen-activated protein) kinases and inwardly rectifying potassium channels (GIRKs) and recruit beta-arrestins. CB1 receptors have been reported to heterodimerize with other GPCRs, including dopamine D2, hypocretin, and opioid receptors, which increases the complexity of CB1 signalling (Lu and Mackie, 2021).

1.1.2.2 Non-classical cannabinoid receptors (Non-CB1/CB2 receptors)

Several studies have indicated that the effects of particular cannabinoids on the immune system, interstitial obstruction, and cardiovascular system are not mediated only by CB1 and CB2 receptors (Begg et al., 2005, Rodriguez de Fonseca et al., 2005). For example, the chemical characteristics of the cannabinoid ligands suggest antioxidant characteristics (Hampson et al., 1998), and lipophilicity, which can lead to non-specific interactions in the plasma membrane or in intracellular locations, as cannabinoids can lead to vasodilation and

lower blood pressure in blood vessels in the absence of cannabinoid receptors CB1 / CB2 (Járai et al., 1999).

The presence of non-CB1/CB2 receptors has been discovered in the CNS. AEA and WIN55212-2 have been shown to stimulate [³⁵S]- GTPγS binding in brain slices and membrane preparations from CB1-null animals, as well as in normal mice. Aside from that, WIN55212-2 and CP55940 were shown to suppress glutaminergic excitatory postsynaptic flow (EPSCs) on hippocampal CA1 pyramidal cells from CB1-null animals in an inhibitory manner (Breivogel et al., 2001, Hajos et al., 2001).

Besides the classical G protein-coupled cannabinoid receptors, GPR18, GPR55, GPR92, and GPR119 are described as cannabinoid receptor-related GPCR (Irving et al., 2017). While GPR18 and GPR55 have overlapping pharmacology with CB1 and CB2, the pharmacology of GPR92 and GPR119 is vastly different.

Cannabinoids, both naturally occurring and synthetic, have the capacity to activate and Transient receptor potential (TRP) channels in the body (Marzo and Petrocellis, 2010) as well as glycine receptors (Hejazi et al., 2006). Endocannabinoids have been shown to activate the transient receptor potential cation channel subfamily V member 1 (TRPV1) channels (Starowicz et al., 2007) and several additional receptors (McHugh et al., 2008, Offertáler et al., 2003). The transient receptor potential vanilloid 1 (TRPV1) receptor channel has been identified as a possible biological target of cannabidiol's (CBD) antihyperalgesic activity (which is used to tackle excessively elevated pain perception) (Costa et al., 2004). AEA, the archetypal endocannabinoid, acts as an agonist at the TRPV1 receptor, which is found on sensitive nerves and throughout the central nervous system (Smart et al., 2000, Zygmunt et al., 1999). A thermosensor with a high threshold has been identified as transient receptor potential vanilloid type 2 (TRPV2), which can be triggered by CBD (Qin et al., 2008, Ralevic and Kendall, 2009, Zani et al., 2007).

Some cannabinoids have also been shown to stimulate the activity of peroxisome proliferator-activated receptors (PPARs), which are a nuclear receptor family with three subtypes (α, β and γ). According to (O'Sullivan, 2007), PPARs may be activated by several compounds such as THC, HU210, WIN55212-2, CP55940, and N-arachidonoyldopamine.

The significance of these receptors in the therapeutic effects of cannabinoids and the physiological effects of endocannabinoids is still being investigated, and little is known about how their signalling is controlled by the ligands that interact with them.

1.2 Cannabinoid ligands

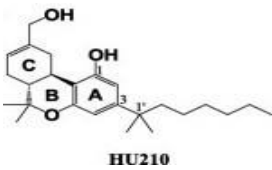
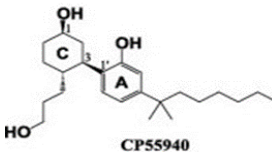
Cannabinoid ligands may be divided into many categories based on their pharmacological characteristics and chemical structures (Pertwee, 2005, Howlett et al., 2002, Pertwee, 1999).

1. Classical cannabinoids derived from tricyclic-dibenzopyran isolated from *Cannabis sativa*, such as Δ^9 -THC, or synthetic, such as HU-210 and Nabilone (Cesamet®, Lilly, authorized by the United States Food and Drug Administration (FDA) for the treatment of chemotherapy-induced nausea and vomiting (Tran, 2021)). These generally bind non-selectively to both CB1 and CB2 receptors, although modification of the THC molecule resulted in the production of selective CB2 receptor agonists such as HU-308 (Hanuš et al., 1999).
 2. Non-classical cannabinoids are structurally similar to the classical cannabinoids, but deficient a dihydropyran ring, such as CP55940, a potent cannabinoid analogue that shares biochemical and behavioural properties with natural cannabinoids such as Δ^9 -THC (Wiley et al., 1995).
 3. Aminoalkylindoles, such as WIN55212-2, are compounds which had been primarily categorised as non-cannabinoid molecules, but they then displayed cannabimimetic activity. These molecules are considered to be the most common synthetic cannabinoids found in blends, which is likely due to the fact that these molecules are less difficult to synthesise than both classical and non-classical cannabinoids (Alves et al., 2020).
- 3.1 This class is further classified into naphthoylindols (e.g. JWH-015, JWH-018, JWH-073, JWH-081, JWH-200), phenylacetylindols (e.g. JWH-251, JWH-250), naphthylmethylindoles (e.g. JWH-184, JWH-196, JWH-192), benzoylindoles (e.g., RSC-4, AM-694), indole carboxamides (e.g. APICA, 5 FAPICA), cyclopropylindoles

(e.g. UR-144, XLR-11), adamantoylindoles (e.g. AB-001, AM-1248)(Alves et al., 2020, Debruyne and Le Boisselier, 2015, Shevyrin and Morzherin, 2015).

4. Fatty acid derivatives are endogenous ligands including N-arachidonylethanolamine and 2-AG. AEA and 2-AG are generated from phospholipid precursors via the activity-dependent activation of particular phospholipase enzymes, which are synthesised from phospholipid precursors (Piomelli, 2003). Based on the AEA structure, slight chemical alterations led to the evolution of the first generation of CB1-selective agonists, R(+)-methanandamide and arachidonyl-2'-chloroethylamide (ACEA) (Hillard et al., 1999).
5. Diarylpyrazole compounds, such as the CB1 antagonist rimonabant. It has an antagonist/inverse agonist action on the CB1 receptors and was originally designed to treat chronic obesity. Unfortunately, the chronic use of rimonabant was found to have significant adverse mental effects and was thus withdrawn (Hurre et al., 2021).

Table 1.1 Showing examples of some cannabinoid ligands (classical cannabinoid and non-classical cannabinoids).

Category	Described	Examples	References	Chemical structure
Classical cannabinoids	Derived from tricyclic-dibenzopyran (TDD)	Δ^9 -THC, HU-308 and HU-210 CP 55,940	(Tran, 2021) (Hanuš et al., 1999)	
Non-classical cannabinoids	Lacking a dihydropyran ring	1-Aminoalkylindoles JWH 015, JWH-018 2- Arachidonic acid derivatives 3- Diarylpyrazole compounds	(Hurre et al., 2021, Wiley et al., 1995, Alves et al., 2020) (Piomelli, 2003) (Cichero et al., 2008)	

1.2.1 Phytocannabinoids

The plant *Cannabis sativa* has a long history of being used as a medicine, and a psychoactive recreational drug. *Cannabis* consists of over 100 individual components and is arguably one of the most versatile medicines available, but given the stigma linked with its legal status, its pharmacological impacts and curative advantages are largely unknown. Most CBs are released from the leaves and mature unseeded female flowers of the plant and can be orally ingested or smoked. Today, government controlled sites of plants, all cut from a single 'mother plant', are cloned to produce an elevated crop of desired CBs, allowing their extraction and purification and isolation by gas chromatography mass spectrometry (GC/MS) (Repetto et al., 1976), for use in medicinal research.

In 1964, delta-9-tetrahydrocannabinol, or Δ^9 -THC, the major psychoactive component of *Cannabis*, was extracted and separated by Raphael Mechoulam and his lab. Experimentally, Δ^9 -THC has been shown to exert basically the same effects as *Cannabis* preparations. Further investigations led to the determination of a number of structurally concerning compounds, including tetrahydrocannabivarin (THCV) and cannabidiol (CBD), which are also found in high amounts within cannabis but are non-psychoactive (Gaoni and Mechoulam, 1964, Izzo et al., 2009). Despite its variable legal situation and registration as a drug of abuse, *Cannabis* continues to be used medicinally for the relief of chronic symptoms that are not helped by first-line treatment drugs. Various pathological conditions, such as reduced spasticity in multiple sclerosis (MS), are gradually being recognised as potential areas where add-on drugs such as cannabinoid compounds can enhance the effectiveness of treatment. (Baker et al., 2000).

1.2.2 Synthetic Cannabinoids

Synthetic cannabinoids are lab-made compounds with much more potent effects than natural cannabinoids. These chemicals are more powerful intoxicants and are difficult to detect in standard screening procedures. Their clinical adverse effects are more severe than natural cannabinoids, and they have no recognised antidotes. They appear to work by interacting with the body's virtually ubiquitous cannabinoid receptors (CB1 and CB2), as well as additional processes not involving these receptors (Muralidhar Reddy et al., 2019). Synthetic cannabinoids have become popular as recreational drugs because they generate

comparable euphoric effects as Δ^9 -THC (Pertwee, 2008). They may cause renal impairment, seizures, irritability, delusions, hypertension and hypertension-related dizziness and vertigo, nausea and chest discomfort, cardiotoxicity, and even death (Mills et al., 2015).

1.3 Enzymes

1.3.1 FAAH

The term “fatty acid amide hydrolase” was first published in 1996, when it was definitively displayed that the enzymes formerly referred to as oleamide hydrolase and AEA amidohydrolase are the same protein (Cravatt et al., 1996, Maurelli et al., 1995). It was also clarified that FAAH promotes the hydrolysis of AEA faster than that of oleamide (Cravatt et al., 1996). FAAH has many substrates but displays preference for the long-chain amides of ethanolamine (Schmid et al., 1985). FAAH has an extensive substrate preference for fatty acid amides, including AEA and other N-acylethanolamines (NAEs), oleamide (Cravatt et al., 1996), and N-acyl taurine (Sasso et al., 2016). There are two isoforms of FAAH (1 and 2) in humans, but not rodents, that share almost 20% DNA sequence homology, despite the catalytic location is comparatively well conserved (Wei et al., 2006).

The human liver, lung, kidney and prostate express both isoforms of FAAH. However, only FAAH-1 can be detected in the human brain, small intestine, and testis, whereas in the heart FAAH-2 is the dominant form expressed (Wei et al., 2006). In subcutaneous adipose tissue, FAAH-1 and -2 are generally expressed to identical levels, with a little higher expression of FAAH-1 in lean humans (Bennetzen et al., 2011). Additionally, the two forms have various substrate characteristics, with FAAH-1 having greater hydrolytic activity on AEA than FAAH-2 (Wei et al., 2006). URB597 is a potent, relatively selective, and irreversible inhibitor of both enzyme isoforms, though it is more potent on FAAH-2 (Piomelli et al., 2006, Wei et al., 2006). FAAH is a membrane-bound enzyme (McKinney and Cravatt, 2005) and, interestingly, FAAH-

1 is thought to be expressed on the cytosolic compartment of the cell membrane, while FAAH-2 could survive on the extracellular surface (Wei et al., 2006).

FAAH is thought to be configured as a homodimer (Bracey et al., 2002). The site and structure of FAAH, linked to the cell membrane and with some channels, may permit the hydrolysis of AEA from the extracellular space without it ever accessing the cytosol (McKinney and Cravatt, 2005). For reviews on this topic, see (McKinney and Cravatt, 2005, McPartland et al., 2007).

FAAH knockdown and selective inhibitors result in antidepressant phenotypes in rats without the significant adverse effects reported with direct cannabinoid receptor agonists in humans and animals, respectively (Vinod et al., 2012) (Carnevali et al., 2015).

1.3.2 Monoacylglycerol lipase (MAGL)

MAGL was first described in fat metabolism, many years prior to the discovery of the ECS (Vaughan et al., 1964). Its role in hydrolysis of 1(3) - and 2-monoacylglycerols is necessary for the liberation of free fatty acids from adipocytes, when required, to preserve energy homeostasis. MAGL is involved in the hydrolysis of a range of monoacylglycerol compounds with different series lengths (C8 to C18) (Labar et al., 2010b). The main role of MAGL in many organs is probably in the organization of the ECS instead of in generalised fatty acid release, especially in the CNS (Dinh et al., 2002). The 33 kDa MAGL isoform, which is similar to rodents in 83% of its main structure and is present in human adipose tissue and many other organs, is shared by humans and rats (Dinh et al., 2002, Ho et al., 2002, Karlsson et al., 1997). Similar to FAAH, MAGL was shown to exist as a homodimer by X-ray crystallography. (Labar et al., 2010a).

Unlike FAAH, MAGL is mainly distributed in the cytosol. It has been displayed that in adipose tissue homogenates, the majority of MAGL activity is in the supernatant layer, in big lipid-protein aggregates (these are large structures formed by the binding of lipids (fats) and proteins. In the context of adipose tissue, these aggregates likely contain various types of fats (like triglycerides) and proteins (such as enzymes or structural proteins). Overall, "big lipid-protein aggregates" represent significant structural and functional entities formed by the

interaction of lipids and proteins in biological systems.), after centrifugation (Tornqvist and Belfrage, 1976).

MAGL is a serine hydrolase belonging to the α/β -hydrolase superfamily with a characteristic amino acid trio (Ser-122, Asp-239, His-269, and a GX SXG motif) and hydrolyzes both 1- and 2-MAGs, include 2-AG (Labar et al., 2010a). The "GX SXG motif" is a sequence pattern observed in proteins, specifically enzymes, in which X represents any amino acid, G represents glycine, and S represents serine. The "GX SXG motif" is noteworthy because it frequently suggests the existence of an active site or catalytic centre in an enzyme. The glycine residues aid in the structural integrity of the motif, but the serine residues are frequently engaged in catalytic processes, notably phosphoryl transfer reactions as illustrated in figure 1.2. Its cDNA was cloned from rat (Dinh et al., 2002), human (Karlsson et al., 2001), and mouse (Karlsson et al., 1997). MAGL is a soluble protein, which links to membranes in a peripheral method (Labar et al., 2010b) and is crystallized as a dimer (Labar et al., 2010b). MAGL is ubiquitously expressed in mouse tissues (Karlsson et al., 1997) and its expression levels in rat brain are elevated in cortex, cerebellum, and hippocampus; medium in thalamus and striatum; and lower in brainstem and hypothalamus (Dinh et al., 2002).

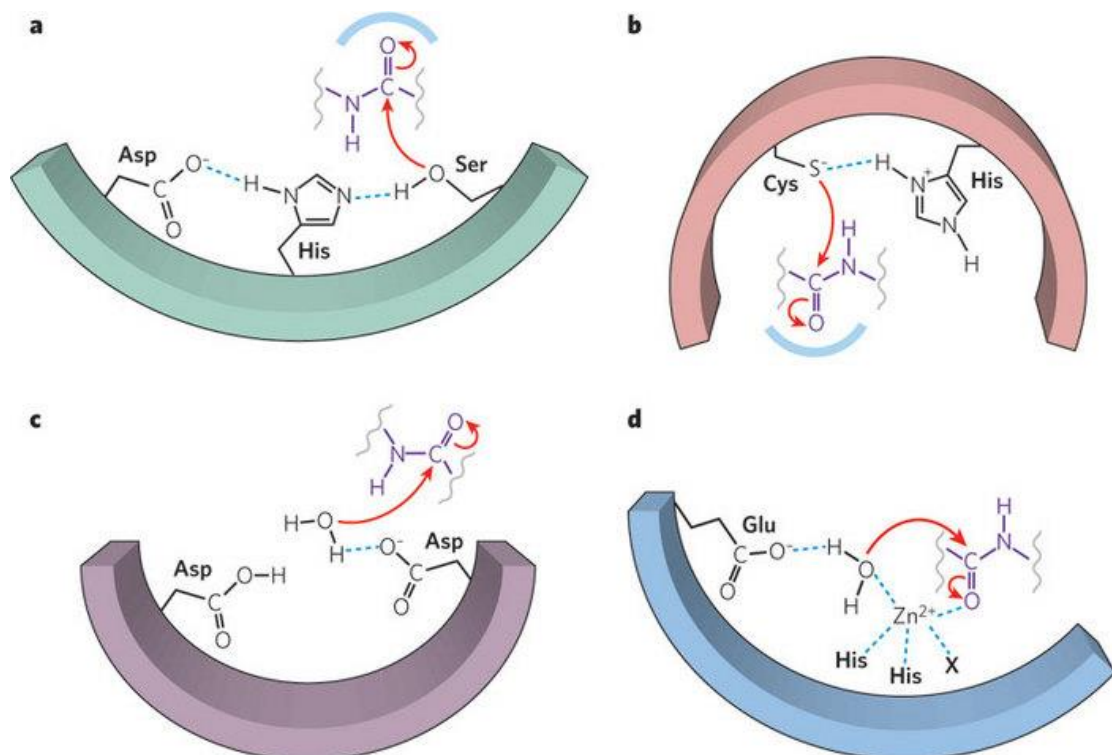


Figure 1.2 Mechanisms of Protease Action. Polypeptides can be cleaved through chemical or enzymatic means. Enzymes that facilitate the hydrolytic cleavage of peptide bonds are known as proteases, which are categorised into four primary mechanistic classes: serine, cysteine, aspartyl, and metalloproteases. In serine and cysteine proteases, the namesake residue is typically paired with a proton-withdrawing group to enhance nucleophilic attack on the peptide bond. In contrast, aspartyl proteases and metalloproteases utilize a water molecule, activated by the enzyme, as the nucleophile instead of an enzyme's functional group. Despite these differences, the overall mechanism of peptide bond cleavage remains consistent across all protease types. The main classes include soluble serine proteases (a); cysteine proteases (b); aspartyl proteases (c); and metalloproteases (d). Adapted from Neitzel et al., (2010).

A functional proteomic path exhibited that MAGL is responsible for ~85% of the 2-AG-hydrolyzing activity at pH 7.5 in mouse brain (Blankman et al., 2007). The remaining activity was largely mediated by ABHD12 and ABHD6. The 2-AG-hydrolyzing activity in the brain of MAGL deficient mice decreased by ~90% and its endogenous 2-AG level rose about 10-fold (Schlosburg et al., 2010). Similar results were noticed in different peripheral tissues such as spleen, paw skin, kidney, heart, and liver of the mice.

Importantly, repeated treatment of mice with JZL184, the MAGL inhibitor, induced analgesia through CB1 receptors, mimicking genetic MAGL disruption. Chronic MAGL blockade also resulted in physical dependence, weakness of endocannabinoid-reliant synaptic plasticity, and desensitisation of brain CB1 receptors (Schlosburg et al., 2010). However, such tolerance was not noticed by prolonged blockage of FAAH, highlighting the significance of the interplay between 2-AG and MAGL in the CB1-mediated endocannabinoid system.

High expression of MAGL resulted in malignant development of cancer cells (Nomura et al., 2010b, Nomura et al., 2011). Since MAGL inhibitors have therapeutic potential, for example, as analgesics and anticancer factors, several serine hydrolase inhibitors and sulfhydryl blockers have been identified and reported to inhibit MAGL non-specifically (Fowler, 2012, Minkila et al., 2010, Petrosino and Di Marzo, 2010).

Selective MAGL inhibitors so far reported include URB602 (Hohmann et al., 2005), CAY10499 (Muccioli et al., 2008), OMDM169 (Bisogno et al., 2009), JZL184 (Long et al., 2009a), and KML29 (Chang et al., 2012). Although repeated high doses of JZL184 caused tolerance,

repeated low doses of JZL184 reduced the inflammatory nociception over both CB1 and CB2 without obvious tolerance (Ghosh et al., 2013).

1.3.3 α/β -hydrolase domain containing 6 (ABHD6)

The enzyme α/β -hydrolase domain containing 6 (ABHD6) is from the family of serine hydrolases that include a serine-histidine-aspartate triad (Labar et al., 2010a) and controls the amounts of the endocannabinoid 2-AG in the body by hydrolysing it to arachidonic acid and glycerol. It is one of the more recently identified components of the endocannabinoid system (Cao et al., 2019). ABHD6 is responsible for approximately 4% of the 2-AG hydrolysis in mouse brain homogenates, based on its primary location at postsynaptic neurons in the brain (Blankman et al., 2007). However, despite the fact that monoacylglycerol lipase (MAGL) is the primary enzyme involved in 2-AG breakdown; at the subcellular localization of ABHD6 is also involved in regulating the 2-AG accumulation or retention. This would imply that ABHD6 and MAGL have different effects on physiological processes (Marrs et al., 2010, van Esbroeck et al., 2019) . It has been reported that inhibition of MAGL in the mouse brain (Pan et al., 2009) and ABHD6 (Marrs et al., 2010) are responsible for controlling the accumulation of 2-AG and the physiological characteristics of 2-AG through the activation of cannabinoid receptors. ABHD6 drew considerable attention because of its expression in BV-2 mouse microglial cells, which lack MAGL (Marrs et al., 2010). In adult mouse cortex, ABHD6 was mostly sited post-synaptically, in contrast to the presynaptic localization of MAGL. The suggested role of ABHD6 as a 2-AG hydrolase was to limit the intracellular accumulation of 2-AG.

Knockdown of ABHD6 in peripheral tissues protected mice from high fat diet-caused obesity, hepatic steatosis, and systemic insulin impedance (Thomas et al., 2013). ABHD6 may be a novel therapeutic target for the treatment of neuron-related illnesses (Cao et al., 2019) in particular, in the prevention and treatment of traumatic brain damage (Tchantchou and Zhang, 2013). In the CNS, ABHD6 is physically connected with AMPA receptors (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) as well (Erlenhardt et al., 2016), the hydrolase activity-independent regulation of their transport to the cell surface and synaptic functioning (Wei et al., 2016, Wei et al., 2017). By modulating 2-AG levels in the ventromedial

nucleus of the hypothalamus, ABHD6 modulates metabolic flexibility in obesity (Fisette et al., 2016).

1.3.4 Detection of endocannabinoid enzymes

Several methods have been used in the literature to measure the activities of endocannabinoid enzymes. Among those the radiometric assay is usually regarded as the 'Gold Standard' methodologies and have been used in the literature for the measurement of FAAH and MAGL activities.

The idea of this assay is based on the concept of FAAH, an intracellular enzyme responsible for the hydrolysis of endogenous AEA. This reaction terminates the biological effects of AEA with final products of arachidonic acid and ethanolamine. Researchers have been measuring FAAH activity in various models using this methodology through the use of the radioactive substrate [^{14}C -ethanolamine]-AEA and subsequent quantification of the reaction product [^{14}C]-ethanolamine which would be proportional to the activity of FAAH in the sample (Bari et al., 2022). This method constitutes an accurate and robust methodology to detect the activity of FAAH in the studied model.

Radiometric assays are also used to detect the activity of MAGL enzyme. This assay relies on the ability of MAGL to hydrolyse 2-AG. In this assay an analogue of 2-AG is used which is 2-oleoylglycerol (2-OG), this analogue does not bind to cannabinoid receptors, and its radioactive substrate [^3H]-2-OG is available commercially. MAGL hydrolyses 2-OG which removing the acyl group from the glycerol backbone. At the end of the assay, the amount of ^3H in the sample can be determined by liquid scintillation counting which can be used as a reflection of the activity of MAGL in the sample (Rademacher et al., 2008, Dinh et al., 2002). This assay may serve as a platform for studying the affinity of various MAGL inhibitors for both human and rat enzymes. Additionally, it can be utilised to identify compounds that have demonstrated potent inhibition of MAGL.

While these assays are reliable and more sensitive than previous methods using absorption or fluorescence detection, the only limitation is the cost. Therefore, finding less expensive EC enzymatic assays that can be used by a wider range of researchers is necessary.

1.4 Neuroprotection/neurotoxicity and cannabinoids

The neuroprotective impact of cannabinoids has been found both in vitro and in vivo studies. When neuronal cultures were exposed to toxic levels of glutamate, both THC and cannabidiol were found to limit glutamate toxicity and enhance neuronal viability (Hampson et al., 1998). CBD has also been shown to have mixed results, single-dose CBD administration may have neuroprotective effects in animal models of hypoxia-induced brain injury while other studies suggest that CBD exposure is associated with sensitisation of immature neuronal cells to redox-active neurotoxins and disruption of in vitro cell viability (Chye et al., 2021).

In a rat model of intravitreal injection of N-methyl-D-aspartate (NMDA), THC and cannabidiol were found to preserve retinal neurons by mitigating the effect of peroxynitrite, believed to be formed following the NMDA effect (El-Remessy et al., 2003). The endocannabinoids AEA and 2-AG have been reported to raise cell viability in neuronal cell cultures subjected to hypoxia and glucose deficiency (Sinor et al., 2000) and 2-AG has been demonstrated to limit brain oedema in a mouse model of traumatic brain injury (Panikashvili et al., 2001). In the neonatal rat brain, exogenously-administered AEA has also been shown to decrease neurotoxicity caused by intracerebral injection of ouabain, a Na^+/K^+ -ATPase inhibitor, which perturbs ion homeostasis and induces cellular swelling and glutamate-dependent deterioration of cells (van der Stelt et al., 2001). In a diversity of brain injury models, endocannabinoids have been shown to accumulate in brain tissue. For example, brain content of AEA is high significantly following both decapitation-induced ischemia (Schmid et al., 1995) and following NMDA-induced excitotoxicity (Hansen et al., 2001). 2-AG content is raised within 5 min of an injection of the pro-convulsant picrotoxinin (Sugiura et al., 2000) and after closed head injury (Panikashvili et al., 2001). Some synthetic cannabinoids were also to have neuroprotective activities. For example, WIN55212-2 reduced hippocampal neuronal loss after transient global cerebral ischemia and decreased infarct volume after permanent focal cerebral ischemia in rats (Nagayama et al., 1999).

On the other hand, cannabinoids have also been demonstrated to trigger apoptosis in leukaemia and glioma cells through the activation of CB1 and CB2 receptors. This effect is suggested to be mediated by activation of caspase pathways and releasing reactive oxygen species (Massi et al., 2006, McKallip et al., 2006). At the cellular level, THC was reported to stimulate cell death in neuronal cell lines, cortical neurons, cultured hippocampal neurons or

hippocampal slices (Downer et al., 2003, Downer et al., 2007b, Gomez Del Pulgar et al., 2002). In vitro studies have shown that cannabinoids have both harmful and protective effects on the viability of various cell cultures through the activation of intracellular pro-death or pro-survival signalling systems, early reports claimed that activation of CB1 receptors can stimulate morphological alteration in the hippocampal dendrites (Lawston et al., 2000). Heavy cannabis smokers were reported to have a high risk of stroke (Ameri, 1999, Solowij et al., 2002).

Several factors define individual susceptibility to cannabinoid toxicity. First, some reports indicated that prolonged exposure to cannabinoids can lead to morphological alterations in brain build in experimental models, suggest tissue harm, an impact that is obvious in heavy cannabis smokers. Moreover, chronic exposure of rat to cannabinoid resulting in distinct morphological alterations in the hippocampus indicative of neurotoxicity i.e. the use of THC for 3 months reduced the mean volume of neurons and their nuclei, the synaptic density, and the dendritic length of CA3 pyramidal neurons (Scallet, 1991, Yucel et al., 2008). Second, more recently, literature has appeared displaying that cannabinoid can mediate distinctive signalling mechanisms in a single neuron, dependent on the state of the neuron (stressful conditions and age) (Kellogg et al., 2009, Roloff and Thayer, 2009). It was shown that a number of cannabinoid receptor agonists (CP55, 940, WIN 55,212-2, THC and HU-210) significantly reduced the viability of N18TG2 neuroblastoma cells that were grown under stressful circumstances (serum-free medium and glucose) but not when the same cells were grown under optimal culture circumstances (Bologov et al., 2011). THC displayed a pro-apoptotic mechanism in cerebral cortical slices from neonatal rat brain, but not from adult brain (Downer et al., 2007a).

1.5 Cannabinoid receptors in astrocytes

A relatively small number of studies have addressed the expression profile and functional importance of CB receptors in astrocytes.

The first evidence for the existence of CB-like receptors in astrocytes came from a landmark study implemented by (Venance et al., 1995). The endocannabinoid AEA, 5 μ M, but not the synthetic cannabinoids CP55940 (1 μ M) or WIN55212-2 (5 μ M), inhibited gap junctions in striatal astrocytes in culture, a response not antagonized by the CB1 receptor antagonist rimonabant (0.5 μ M). Interestingly, this AEA response was found in astrocytes from embryonic mouse striatum, but not in astrocytes prepared from embryonic mouse cortex, hippocampus and brain stem, suggesting differential expression of this CB-like receptor in these glial cells (Venance et al., 1995). In a follow-up study, the same laboratory measured cAMP generation in striatal astrocytes in culture and identified that AEA inhibited the cAMP accumulation (IC_{50} = 0.6 μ M) induced by isoproterenol, a response also insensitive to rimonabant (Sagan et al. 1999).

Astrocytes in culture prepared from varying types may vary in their phenotype, and rat astrocytes in culture were reported to express CB1 receptors, while mouse astrocytes in culture did not (Sánchez et al., 1998a). Astrocytic CB1 receptor activation governs their metabolic functions: for example, activation of CB1 receptors on rat astrocytes increases the rate of glucose oxidation and ketogenesis, two influences on the energy supply of the brain (Blázquez et al., 1999, Sánchez et al., 1998b).

Astrocyte differentiation is promoted by the activation of CB1 receptors, which also facilitates neuron-astrocyte communication (Aguado et al., 2006) important in synaptic plasticity, memory, and behaviour (Araque et al., 2014, Gómez-Gonzalo et al., 2015, Han et al., 2012, Metna-Laurent and Marsicano, 2015, Navarrete and Araque, 2008, Navarrete and Araque, 2010, Da Cruz et al., 2016). The location of CB1 receptors in astrocytes in comparison to adjacent neuronal compartments, especially at synapses, has a significant impact on the activities of astrocytes mediated by the CB1 receptor (Bonilla-Del Río et al., 2019). However, because of the low levels of CB1 receptor expression in astrocytes (Bosier et al., 2013, Han et al., 2012, Kovács et al., 2017), it is difficult to obtain a comprehensive picture of the subcellular CB1 receptor distribution in the astroglial compartments that serve as the anatomical substrate for a functional interaction with nearby synapses under normal or pathological circumstances (Bonilla-Del Río et al., 2019).

CB1 receptor expression has been reported in different human glioma cell lines and many types of human tumours with different degrees of malignancy (Bouaboula et al., 1995, Bouaboula et al., 1996). Cannabinoid receptor agonists activate ERK in human glioma cell lines in culture, in a manner sensitive to the CB1 receptor antagonist rimonabant (Bouaboula et al., 1995). A sub-clone of the rat glioma cell line C6, namely C6.9, was sensitive to the plant cannabinoid THC. However, this toxic impact was not antagonized by either rimonabant or the CB2 receptor antagonist SR144528 alone, but only by their combination, suggesting that activation of either receptor kind is enough to stimulate apoptosis in astrocytomas (Galve-Roperh et al., 2000). Autophagy and p8, a stress-regulated mediator of cell destiny, form key players in THC-induced apoptosis of these C6 cells (Carracedo et al., 2006, Salazar et al., 2009). Based on these results, Guzman *et al* (2003) examined the anti-humoral particulars of cannabinoids on C6 cells stereotactically injected into rat brain. Highly invasive tumours formed within 2 weeks of the injection of C6 cells, and untreated tumour-bearing animals did not remain alive after 3 weeks (Galve-Roperh et al., 2000). Intra-tumour treatment with either THC or the non-selective CB receptor agonist WIN55212-2 slowed the development of these malignant tumours, improving mean survival times. These results were profound: in 25% of the cannabinoid-treated situations, gliomas were entirely enucleated, and these rats survived after study observations (Galve-Roperh et al., 2000).

Moreover, cannabinoid therapy did not harm contiguous healthy tissue. Hitherto, whether these *in vivo* curative effects of THC and WIN55212-2 were really mediated by CB1 and/or CB2 receptors has not been clearly shown. Despite the majority of pharmacological data indicating that brain tumour tissue does express CB2 receptors, corresponding expression data using commercially available antibodies have been harder to interpret. To corroborate their findings, Guzman and colleagues reported that both C6 cells and human glioma tissues express CB1 and CB2 receptor mRNA and protein (Sánchez et al., 2001), at odds with an earlier study that reported no CB2 mRNA in a panel of human astrocytoma cell lines and tissues (as assessed by RT-PCR) (Bouaboula et al., 1995). A further study reported the qPCR and immunohistochemical analyses of biopsies from 37 human astrocytomas of differing malignancy (Held-Feindt et al., 2006). In summary, a major group of evidence suggests the chemotherapeutic value of cannabinoids acting on CB1 and CB2 on astrocytomas in culture, and these outcomes still want to be further investigated *in vivo*.

1.5.1 The human astrocytoma (1321N1) cell line

1321N1 is a human astrocytoma cell line isolated in 1972 as a sub clone of the cell line 1181N1 which in turn was isolated from the parent line U-118 MG (one of a number of cell lines derived from malignant gliomas by J Ponten (Ponten and Macintyre, 1968) cited in (Kraft et al., 2001). This cell line is recognised as an appropriate and reliable cell system to test, albeit indirectly, the neurotoxicity or neuroprotection potential of drugs. Astrocytoma cells from 1321N1 express several different types of receptors, including M3 muscarinic cholinergic receptors, protease-activated receptors, thromboxane A2 receptors, and epidermal growth factor (EGF) receptors (Freund et al., 2003). Previous investigations have shown that β_2 -adrenoceptors (β_2 -Ars) are expressed in glioblastomas, whether maintained as established cell lines or primary cultures obtained from human biopsies (Prenner et al., 2007, Annabi et al., 2009), as well as in the human-derived 1321N1 astrocytoma cell line, among other tumour types. The presence of β_2 -Ars in U118 cells, another human astrocytoma cell line, was also shown; however, the presence of β_2 -Ars in U118 cells was found to be lower than that seen in 1321N1 cells (Toews et al., 1983, Wakshull et al., 1985)

when 1321N1 cells were treated with forskolin, which activates adenylate cyclase, their baseline levels of intracellular cAMP significantly increased from below 0.5 nmol/mg protein to over 4 nmol/mg protein. This increase in cAMP levels indicated that the growth of these cells is affected by changes in intracellular cAMP, as shown by the cells being halted in the G1 phase of the cell cycle. Additionally, the study found that using β_2 -adrenergic receptor (β_2 -AR) agonists on these cells also raised cAMP levels and inhibited cell division in a way similar to what was observed in the control group. This suggests that both forskolin and β_2 -AR agonists can modulate cell growth by affecting cAMP levels (Toll et al., 2011).

1.6 Aim of the thesis

The aim of the research described in this thesis is to study the endocannabinoid system in the CNS. To achieve these two main objectives have been set:

1. To identify the effects of cannabinoids and cannabinoid-related compounds including endo, phyto and synthetic cannabinoids on the proliferation of human astrocytes, 1321N1 human astrocytoma. The objectives of chapter 3 were to:

- a. Validate human astrocytoma 1321N1 cells as an appropriate and reliable cell system to test the cytotoxicity or cytoprotective nature of cannabinoid agents.
 - b. Validate an experimental assay for the assessment of cell viability, the colorimetric MTT assay
 - c. Assess the effects of a panel of enzyme inhibitors that function as selective inhibitors of enzymes involved in the metabolism or biotransformation of endocannabinoid and endocannabinoid-related compounds on the cell viability of human 1321N1 cell line.
 - d. Use this glioma cell line to evaluate the potential for targeting the endocannabinoid system to reduce cell proliferation *in vitro*.
2. To study the function of endocannabinoid hydrolases in complex human, rat, and mouse proteomes. The objectives of chapters 4 and 5 were to:
- a. Gain insight in the physiological role of enzymes involved in cannabinoids turnover, An ABPP that detects the functional expression of endocannabinoid hydrolases was utilised in native biological systems for the identification and characterization of inhibitors.
 - b. Screen a targeted compound library against serine hydrolase activity in general and FAAH, MAGL, ABHD6 and ABHD12 in particular in the human, and murine brain membrane proteome. In addition to the determination of their selectivity profile across a large panel of serine hydrolases.
 - c. Study the relative activity of different hydrolases involved in endocannabinoid biosynthesis and metabolism in various murine brain regions, such as the hippocampus, cerebellum, striatum, and frontal cortex.
 - d. Compare specific serine hydrolase activities between the brain regions provided insight in which endocannabinoid metabolic pathway is important in specific brain regions.
3. To summarise the work described in this thesis and presents some future challenges.

CHAPTER 2

General Methodologies

2.1 Materials

Most chemicals used in this study were supplied by Sigma-Aldrich (Poole, UK). Some additional chemicals utilised in this study were as follows: foetal bovine serum (FBS) (Sigma), Dulbecco's Modified Eagle's Medium - high glucose (Sigma), trypsin/EDTA, phosphate-buffered saline (PBS [135 mM NaCl, 3 mM KCl, 2 mM KH₂PO₄, pH 7.5]) (Fisher Scientific), dimethyl sulfoxide (DMSO) (Sigma), trypan blue (Thermo Fisher), ethanol thiazole blue tetrazolium bromide (MTT) (Sigma) and isopropanol (propan-2-ol) (sigma).

2.1.1 Sources of substrates and enzyme inhibitors

This study involved substrates and inhibitors of enzymes associated with endocannabinoid metabolism (see Table 2.1).

Table 2. 1 Sources of compounds.

Profile	Abbreviation	Compound name	Catalogue ID	Stock solvent	Source
FAAH inhibitor	BNPP	Bis-4-nitrophenylphosphate	123943	H ₂ O	Sigma
	JNJ1661010				Sigma
	URB597	Cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl ester	U4133	DMSO	Sigma
An endocannabinoid-like molecule and substrate for FAAH	PEA	N-palmitoylethanolamine		DMSO	Synthesised by SPHA in the School of Chemistry, UoN
A surrogate substrate for FAAH	AAMC	7-arachidonoyl-aminomethylcoumarin	10005098	DMSO	Cayman chemicals
	ODA	Oleamide		EtOH	Synthesised by SPHA in the School of Chemistry, UoN
MAGL inhibitor	AEA	N-arachidonoyl ethanolamine	1339	EtOH	Tocris
	JJKK048	4-[Bis(1,3-benzodioxol-5-yl)methyl]-1-piperidinyl]-1 <i>H</i> -1,2,4-triazol-1-yl-methanone	5206	DMSO	Tocris
	JZL184	4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenyl ester	3836	DMSO	Tocris
An endocannabinoid and substrate for MAGL	2AG	2-arachidonoylglycerol	1298	EtOH	Tocris
MAGL substrate	2OG	2-oleoylglycerol	8704510	EtOH	Sigma
A surrogate substrate for MAGL	7HCA	7-hydroxycoumarinylarachidonate	62910	EtOH	Cayman chemicals
ABHD6 inhibitor	KT203	4'-[1-[[2-(Phenylmethyl)-1-piperidinyl]carbonyl]-1 <i>H</i> -1,2,3-triazol-4-yl]-[1,1'-biphenyl]-3-carboxylic acid	SML1250	DMSO	Sigma
Esterase substrate	MUH	4-methylumbelliferylheptanoate	M2514	EtOH	Sigma
	NPB	4-nitrophenylbutyrate	N9876	EtOH	Sigma
Non-selective serine hydrolase inhibitor	MAFP	Methylarachidonoylfluorophosphonate	M2939	EtOH	Sigma
A possible endocannabinoid metabolite	NAGly	N-arachidonoylglycine	1445	EtOH	Tocris
ABHD6 inhibitor	WWL70	<i>N</i> -Methyl- <i>N</i> -[[3-(4-pyridinyl)phenyl]methyl]-4'-(aminocarbonyl)[1,1'-biphenyl]-4-yl carbamic acid ester	3252	DMSO	Tocris

DMSO, dimethylsulphoxide; EtOH, ethanol

For experimental use with cells, drugs were diluted with media to the indicated final concentrations.

2.2 Basic cell culture techniques

2.2.1 Cell culture

1321N1 human astrocytoma cells were generously donated by Professor Stephen Hill from the School of Life Sciences, University of Nottingham. 1321N1 cells were cultured in 10% heat inactivated foetal bovine serum (FBS), 2 mM L-glutamine in Dulbecco's Modified Eagles Medium (DMEM). After preparation, the medium was kept in a refrigerator (4 °C) and utilised within two weeks. The cells were maintained in T75 cm² polystyrene cell culture flasks fitted with 0.22 µm filter caps (Nunc), containing approximately 20 ml of growth medium. The growth medium was removed by aspiration and the cell monolayer was washed by adding 5 ml of PBS, pre-warmed to 37 °C. The monolayer was disrupted with trypsin solution (0.5 g/l porcine trypsin and 0.2 g/l EDTA in 0.09 % (w/v) sodium chloride) in phosphate-buffered saline. Then the flask was placed in the 37 °C incubator for 5 min. After incubation, the flask was shaken to help disrupt the monolayer. 8 ml fresh medium was added to the flask and the cells were pipetted up and down several times. The resulting cell suspension was transferred to a sterile tube (30 ml Universal tube) and centrifuged at 1200 rpm (~800 g) for 5 min. The supernatant layer was removed to obtain the pellet. 5 ml fresh growth medium (pre-warmed to 37 °C) was used to re-suspend the pellet. Finally, 1 ml of the resulting suspension was transferred into T75 fresh cell culture flasks with 20 ml fresh growth medium according to the splitting ratio (1:5). The cells became confluent within 3-4 days.

2.2.2 Cell counting

Cell counting was performed using a Bio-Rad TC20 automated cell counter, using the Trypan Blue inclusion/exclusion method that identifies dead or dying cells. The chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable. An equal proportion of cell suspension and trypan blue were mixed in a microfuge tube and then 10 µl of this mixture was pipetted onto the specially designed counter slide provided by Bio-Rad. This slide was slotted into the TC20 counter which provided an instant cell count for the cell suspension.

2.2.3 Cell storage

2.2.3.1 Freezing cells

For freezing cells, the initial step was similar to that above. After obtaining a cell pellet, a freezing solution (90% FBS 10% (v/v) DMSO) at 10^6 - 10^7 cells/ml was mixed. Subsequently, 1 ml of freezing medium was transferred, and the pellet frozen in cryogenic vials. The vials were then kept at -80°C in cryogenic container (Biocision CoolCell TM). To preserve these frozen containers for an extended time, they were transferred to liquid nitrogen Dewar containers after around 24 h for long term storage.

2.2.3.2 Thawing cells

After removal from liquid nitrogen, cells were thawed in warm water (37°C) while lightly shaking the vial for less than a minute. The outer surface of the vial was cleaned with 70% (v/v) IMS before opening and mixing 20 ml of medium into a T75 flask. The pellet was mixed with 5 ml of medium and shifted to a culture flask. About 24 h, the growth medium was replaced, and two passages of cells were grown before experimentation.

2.2.4 Preparation of assay plates

2.2.4.1 Preparation of cell culture plates

1321N1 cells were maintained in T75 cm² polystyrene cell culture flasks until 90% confluent. Then the cell layers were disrupted, and cells centrifuged as described in Section 2.2.1. The supernatant layer was removed, and the pellet was re-suspended in 5 ml fresh growth medium (pre-warmed to 37°C). The TC20 automated cell counter (Bio-Rad, Hemel Hempstead, UK) was used to assess cell viability with trypan blue as described in section 2.2.2. Then, 2×10^5 or 4×10^4 cells were seeded in a 24- or 96-well plates, respectively. Cells were serum starved when they reach 80% confluency.

2.2.5 MTT cell viability assay

The MTT tetrazolium salt is transformed to the coloured formazan product by active, living cells. 1 ml PBS was used to dissolve 3 mg of MTT (thiazole blue tetrazolium bromide)

which was then kept in the fridge. The cell culture media containing 50 μ M MTT was applied to cells in 24 and 96-well plates after aspirating the previous medium. Following exposure for up to 3 h, PBS was used to wash the cells, before the same volume of isopropanol (propan-2-ol) was mixed with the cells. A micro-titre plate reader was used to assess the optical density at 570 nm of 200 μ l aliquots in 96-well plates. The number of viable cells present are reflected by the NAD(P)H-dependent cellular oxidoreductase enzymes under defined conditions (Riss *et al.*, 2004).

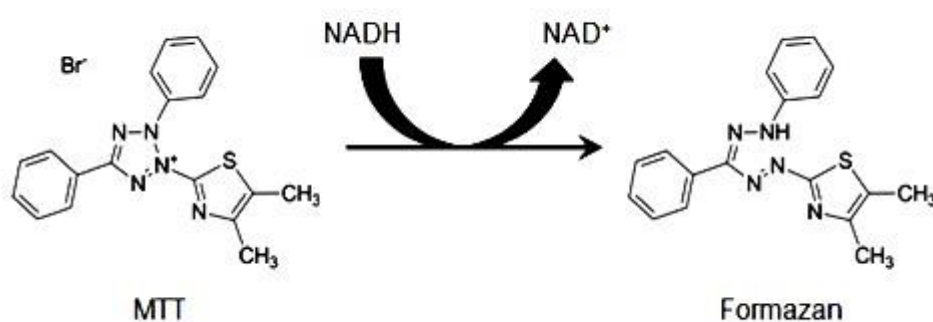


Figure 2. 1 Cell-based production of formazan Source: (Riss *et al.*, 2004).

The MTT assay and trypan blue quantification are both widely used methods for assessing cell viability, each with distinct advantages. The MTT assay is highly quantitative, sensitive, and suitable for high-throughput formats, making it ideal for detailed and large-scale studies. It measures cell metabolic activity through absorbance, providing objective and reproducible results. In contrast, trypan blue quantification is simpler, quicker, and more cost-effective, allowing for immediate assessment of viable and non-viable cells by direct counting under a microscope. It also offers additional morphological information about cells. The choice between these methods depends on the experimental requirements, with the MTT assay being preferable for precise and large-scale studies, while trypan blue is advantageous for quick and straightforward viability assessments.

2.2.6 Protein Determination

The total level of the protein in biological samples was identified using a Lowry test, which is based on measuring the reaction of proteins with Folin phenol reagent in the presence of alkaline copper conditions (Lowry et al., 1951). Bovine serum albumin (BSA, 0.05-0.40 µg/µl) was used to construct a standard curve. Standards and samples were allowed to stay at room temperature for 10 minutes, when 1 ml A: B Lowry mixture (0.2% SDS, 2% Na₂CO₃, in 1 M NaOH:1 % KNaTartarate, 0.5 % CuSO₄.H₂O) was added and the mixture vortexed. The tubes were allowed to stand for 45 min at room temperature before 100 µl of Folin's reagent (Folin Ciocalteu phenol reagent), recently diluted 1:1 in purified water, were added. A 96-well plate reader (Dynex spectrophotometer) was used to quantify the optical density at 750 nm of samples and standards, where the average optical density was calculated for triplicates of the samples. The amount of protein in every single sample was measured through a linear regression standard curve (Revelation software program).

2.2.7 Data analysis

Data were gathered from three or more independent experiments using different passages of cells and are articulated as mean ± SEM. Statistical analysis was evaluated by repeated measures ANOVA with Bonferroni's post-hoc test.

2.3 Animal procedures (conducted by Dr Peter Gowler)

A total of eight male rats (Sprague-Dawley, supplied by Charles River UK) weighing 202.1 ± 9.8 g (mean ± SD) were used. During these studies, animals were housed with free access to food and water. Procedures were conducted in compliance with local ethical rules under the licence of Professor Victoria Chapman (PPL PB3DA999F).

Rats were divided into two groups of four such that, one group were given an intra-articular injection of 1 mg monosodium iodoacetate in 50 µL 0.9% saline whereas the other group were given 50 µL of saline into the left knee. Measurements for weight bearing symmetry, knee joint diameter and paw withdrawal thresholds were recorded at baseline and three days following the injection (Figures 2.2 and 2.3).

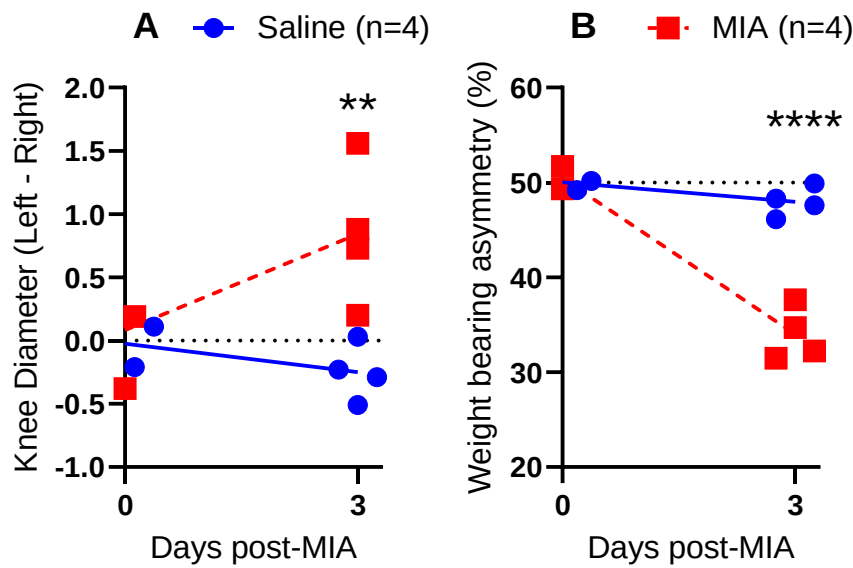


Figure 2. 2 Pain-related behaviours following unilateral MIA administration. Three days following vehicle or MIA administration, knee diameter (A) or weight-bearing (B) were assessed by Dr Peter Gowler. Data were analysed by 2-way ANOVA with Sidak's multiple comparison test. ** = $P < 0.01$, **** = $P < 0.0001$ MIA vs Saline.

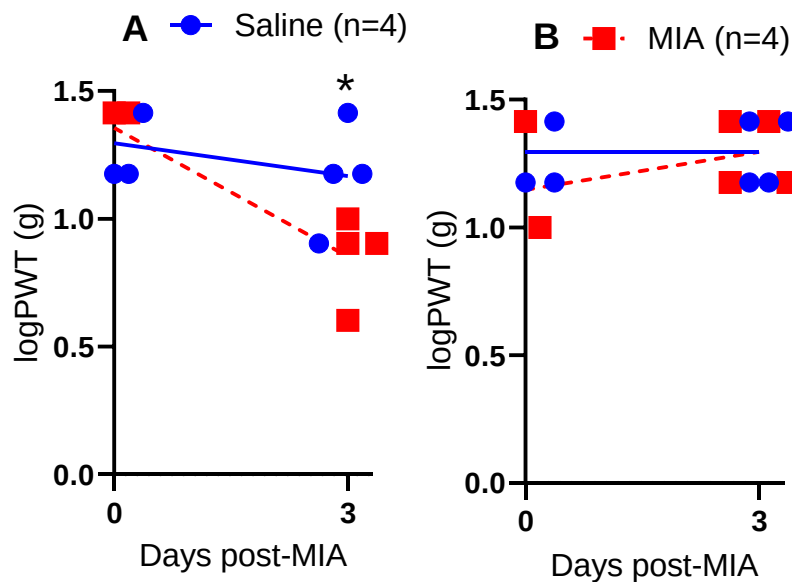


Figure 2. 2 Pain-related behaviours following unilateral MIA administration. Three days following vehicle or MIA administration, paw withdrawal thresholds to von Frey hairs were

assessed for ipsilateral (A) or contralateral (B) paws by Dr Peter Gowler. Data were analysed by 2-way ANOVA with Sidak's multiple comparison test. $*=p<0.01$ MIA vs Saline.

Animals were killed on Day 3, and a colleague (Paul Millns) dissected the spinal cord to collect dorsal root ganglia and spinal cord samples at L3, L4, and L5 levels. Tissues were snap-frozen on dry ice and then stored at -80°C .

Statistical analysis of the data revealed that there was no significant difference in terms of weight-bearing symmetry, knee joint diameter, or paw withdrawal thresholds within either of the studied groups (vehicle or monosodium iodoacetate (MIA)), whether at the baseline or three days following MIA injection. However, the administration of MIA induced a significant increase in the left knee diameter in comparison to the control group (Figure 2.2 A). while a significant decline in the weight-bearing symmetry was observed 3 days after MIA injection into experimental animals (Figure 2.2 B).

MIA was injected, and the effect of the MIA injection affecting the same side of the body (ipsilateral) or the opposite side (contralateral) of the body was examined. Results showed that the ipsilateral but not contralateral injection of MIA caused a significant decrease in paw withdrawal thresholds (Figure 2.3 A and B), indicating the induction of pain in this experimental animal group.

2.3.1 Ex vivo analysis

2.3.1.1 Tissue processing

On the day of assay, frozen samples were placed on dry ice in a microfuge tube for homogenization. A micro-pestle was employed initially to disrupt the samples in $80\ \mu\text{L}$ Tris-EDTA ($50\ \text{mM}$, $1\ \text{mM}$, $\text{pH } 7.4$) buffer while they were maintained on ice. As the results from the first gel suggested that spinal cord tissues exhibited distorted bands, subsequent tissues were disrupted using an ultrasound probe (Soniprep 150 Ultrasonic Disintegrator, MSE) three times for 10 seconds while maintaining the samples on ice. $25\ \mu\text{L}$ samples were taken for Lowry protein analysis and $16\ \mu\text{L}$ transferred to microfuges for immediate reaction with the activity probes.

Before adding the probes, the 16 μL samples were incubated at 37 °C for 15 minutes to allow the metabolism of any endogenous substrates. 2 μL of the MB064 activity probe (diluted in 50% DMSO/50% Tris/EDTA) were added (to give a final concentration of 250 nM), and the incubation continued at 37 °C for 15 min. Then 4 μL FP-rhodamine was added (to a final concentration of 500 nM) and the incubation was continued at 37 °C for 30 min. 4 μL of 6X Laemmli buffer was applied to the samples to stop the reaction and linearize the proteins. The samples were stored at -20 °C prior to gel electrophoresis.

2.4 Endocannabinoid-metabolising enzymes

2.4.1 Activity-based protein profiling (ABPP)

Activity-based protein profiling (ABPP) is an adaptable chemo-proteomic technique in drug discovery and development. The activity-based probes employed report the activity of specific enzymes or the reactivity of amino acid types in complicated biological systems. Since its inception in the early 1990s, the ABPP has helped in the identification of novel therapeutic targets and the development of many compounds in the treatment of human and infectious illnesses (Evans and Cravatt, 2006). More significantly still, as part of a persistent worldwide effort to highlight the druggable proteome, the repertoire of target classes that may be addressed using activity-based probes has significantly increased in recent years.

2.4.2 Tissue sample preparation

Charles River UK supplied male adult Sprague-Dawley rats weighing 250 to 300 g, which were housed with food and water freely available. Animals were slaughtered and Paul Millns dissected the spinal cord to isolate the dorsal root ganglia at L4, L5, and L6 and spinal cord samples, which were snap-frozen individually on dry ice. Every rat tissue was preserved in liquid nitrogen on a separate basis.

For immediate homogenization, the frozen samples were transported to microfuge tube on dry ice. Then a micro-pestle was used with maintaining samples on ice, then 20- μL of each homogenate sample transferred to fresh microfuge tube and kept on ice (4 °C) for investigation. Another 25- μL of the homogenate solution was used for the Lowry test.

2.4.3 Preparing Cells for ABPP

Cell lines (1231N1 and SHSY5Y) were cultured in Dulbecco's Modified Eagle's Medium in the T175 flasks till confluent. Monolayers were washed with 10 ml chilled phosphate-buffered saline (PBS) and then cells were scraped from the bottom of the flask. The resulting cell suspension was transferred to a sterile tube (30 ml Universal tube) and centrifuged at 1200 rpm for 5 min. The supernatant layer was removed to obtain the pellet. 1 ml ice-cold 50 mM Tris-HCl, pH 7.6 was added to re-suspend the cell precipitate for homogenization. The cell suspension was homogenised with a homogeniser (Ultra-Turrax) three times for 30 s on ice (0- 4 °C). The homogenate was centrifuged over two cycles (33000 g for 30 min at 4 °C). The supernatant layer and pellet were then investigated immediately in the ABPP assay.

2.4.4 Conducting Activity-based Protein Profiling

2.4.4.1 Reagents

Acrylamide used was purchased by Sigma-Aldrich, TEMED (N,N,N',N'-tetramethylethylenediamine; Sigma-Aldrich, cat. no. T9281), APS (ammonium persulfate; Sigma-Aldrich, cat. no. A3678). The activity-based probe TAMRA-fluorophosphonate (FP-TAMRA) was purchased from Thermo Fisher, while MB064 was synthesised by Dr Marc Baggelaar and provided by Professor Mario van der Stelt, Leiden University, the Netherlands.

2.4.4.2 Reaction with the activity probes

The 20 µL samples were incubated at 37 °C for 15 min to allow metabolism of any endogenous substrates before addition of inhibitor, where appropriate. 5 µL of the inhibitor at an appropriate concentration was added and the incubation continued at 37 °C for 30 min.

For activity analysis with a single probe, FP-rhodamine was added to a final concentration of 0.5 µM and the incubation continued at 37°C. 6 µL of 6X Laemmli buffer was applied to the samples to stop the reaction, and the mixture was linearized. After that, the samples were stored at -20 °C before gel electrophoresis

For activity analysis with two probes, the whole sample was incubated with activity-based probe MB064 (2 mM, 15 min, rt), and FP-TAMRA (500 nM, 15 min, rt) at the same time.

6 μ L of 6X Laemmli was applied to the samples to stop the reactions, and the mixture was linearized. After that, the samples were held at -20 °C before gel electrophoresis.

2.4.4.3 Gel Electrophoresis

Gels (10% acrylamide, Table 2.2) measuring 20 cm $\times$ 20 cm were utilised in these experiments. Approximately, one-third of the gel was the stacking gel, while the other two-thirds were resolving. A fixed current of 15 milliamperes was utilised until the dye front passed the stacking gel, and then it was raised to 30 milliamperes for 4-5 hours until the dye front eluted at the base of the gel.

Table 2. 1 Gel composition for making up a single 10 % acrylamide gel.

Addition	Resolving Gel	Stacking Gel
Distilled water	14.35 mL	21.35 mL
Tris buffer	8.75 mL (1.5 M, pH 8.8)	8.75 mL (0.5 M, pH 6.8)
10% SDS solution	0.35 mL	0.35 mL
30% acrylamide	11.365mL	4.34mL
TEMED (tetramethyl ethylenediamine)	17.5 μ L	35 μ L
APS (ammonium persulfate) 10% (w/v)	175 μ L	175 μ L
Total	35 mL	35 ml

2.4.4.4 Gel scanning and band quantification

The surface of the glass was patted gently dry with tissue, and the gel was placed on the designated surface of an Amersham Typhoon scanner. The filter setting was set to Cy3 (excitation wavelength of 532 nm, emission wavelength of 580 nm, duration and resolution), and the scanned gel was imaged and saved as a jpeg file.

2.4.5 Data analysis

The image that was produced by the scan was imported into Image Studio, which made it possible to distinguish the different bands and record the band intensities for each band. The background signal from an exterior area of the gel was removed from each measurement for that gel.

On each gel, a parallel sample of rat cerebral cortex soluble/particulate (20 samples) fraction (sample 264) exposed to the affinity probes was electrophoresed to allow standardization between different gels. While the molecular weight ladder was run on the gels to estimate the molecule weight of the bands on the gels.

Quantification of band intensity was conducted blind, in that the codes of which sample was being analysed were not revealed until after the intensities had been quantified. The samples were prepared with a code (Dr Peter Gowler) and organized in a non-sequential manner to ensure process and analysis was conducted including the principles of randomization and blinding.

For investigation of the effects of test compounds in vitro in the ABPP assay of rat brain samples (Chapter 5), statistical analysis was conducted using two-way ANOVA (comparing bands and test compounds) with post-hoc Dunnett's multiple comparison test, with a statistical significance cut-off set at 0.01. In these investigations, comparisons to buffer were conducted for the water-soluble compound BMP and the two solvents used for the other test compounds, ethanol and DMSO. Thereafter, the comparison for effects of the test compounds were conducted against their solvent vehicle.

For investigation of the regional variation in ABPP bands of the rat spinal system (Chapter 6), Band 17 was chosen for autologous normalization, followed two-way ANOVA (comparing bands and tissue region) with post-hoc Dunnett's multiple comparison test, with a statistical significance cut-off set at 0.05. In these investigations, activities were compared to those for the L5 DRG.

2.4.6 Molecular size estimation

On each gel, a series of standards of known molecular size (Rainbow, Amersham Catalogue number) were run in parallel with the tissue samples. Although these standards have 10 different molecular sizes, only four were evident at the wavelengths used by the

fluorescent scanner (molecular weight markers 150, 76, 52 and 31 kDa). For these standards, a migration distance from the top of the resolving gel to the peak of the band intensity was quantified using ImageJ. The ratio of the distance travelled by that band to the dye front (or more frequently, the bottom of the resolving gel) was used to express an Rf value (retardation factor). To calculate the unknown molecular weights of the tissue proteins, a plot of Log (MW) vs. Rf was generated (Figure 2.4). While many of the bands fell within the range of visible MW markers, a number were either larger than 150 kDa or smaller than 31 kDa, and so there would be less confidence in the molecular size determination for these bands.

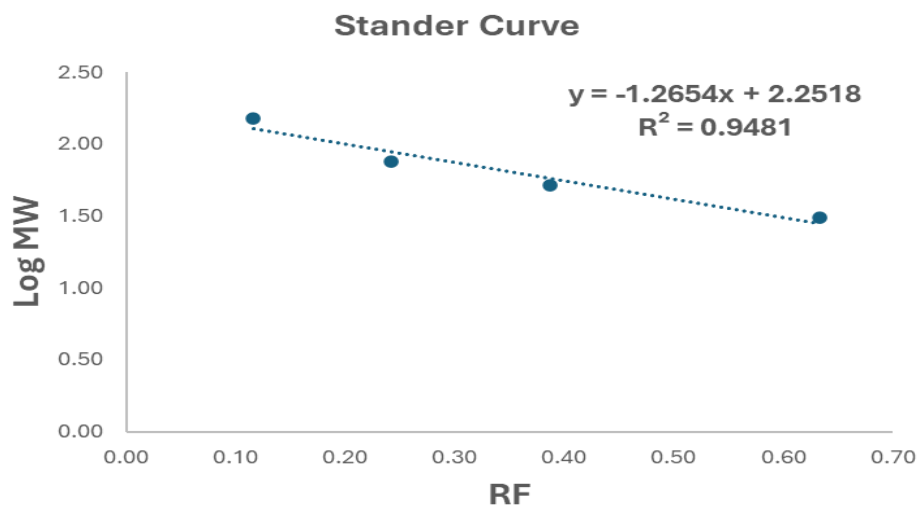


Figure 2. 3 The graph shows the migration distance (RF) of each band in the gel against the log10MW of the molecular weight markers (150, 76, 52, and 31 kDa).

CHAPTER 3

The effect of selected cannabinoid ligands on proliferation of a human astrocytoma cell line

3.1 Introduction

A variety of cell groups are present in the CNS: neurons, microglial, astrocytes and oligodendrocytes. Astroglial cells are crucial to many physiological roles of the CNS, such as immune defence, cellular support, ion homeostasis, absorption of neurotransmitters and neuromodulation (Ridet et al., 1997).

It is difficult to treat glioma, in particular, glioblastoma multiforme (GBM) as the disease has poor prognosis and because of the intra- and intertumour heterogeneity, causing the tumours to be resistance to treatment (Preusser et al., 2011, Shergalis et al., 2018). The use of glioma cell lines *in vitro* has been a crucial pillar in the study of glioma biology and therapy response (Wang et al., 2005, Higgins et al., 2010, Mullins et al., 2013). Cell models of glioma tumours have been available for decades but the extent to which these models recapitulate the human disease remains debatable (Jacobs et al., 2011). As GBM is the most malignant and highly aggressive brain tumour, GBM cell lines have given new basic insights into the therapeutic possibilities and new targets of cancer science. Related human glioma-derived lines, such as 1321N1, U87MG, U251MG, GOS-3 and GOS-10 are of malignant phenotypes and can be used in cancer research. In 1972, a human astrocytoma line isolated from the parent line U-118 MG, one of a number of lines originating from malignant glioma by J Ponten (Ponten and Macintyre, 1968). This was a subclone cell line 1, 1181N1 called 1321N1 cells (Kraft et al., 2001). This cell line is commonly used to investigate toxicity or cytoprotection of drugs as a suitable and effective cell system.

Cannabinoids have been proposed to control the protective/toxic resolution of neurons (Guzmán et al., 2002) in both *in vitro* and *in vivo* models. This neuroprotective action may occur by various mechanisms, including (i) antioxidant capacity (ii) inhibition of glutamatergic neurotransmission (iii) modulation of glial cell function and (iv) control of the

microvasculature (Maroon and Bost, 2018, Guzman, 2003). On the other hand, it was shown that cannabinoids induce apoptosis in cultured neurons (Chan et al., 1998). In addition, chronic exposure to CBs was found in some cases to result in neurotoxicity, such as in heavy *Cannabis* users (Archie and Cucullo, 2019, Hall and Degenhardt, 2009).

Synthetic cannabinoids also elicit a wide variety of physiological and pathological effects as they are similar to Δ^9 -THC on functional grounds, and they bind to the same CB1 and CB2 cannabinoid receptors in the brain and other organs like the endogenous ligands, 2-AG and AEA (Rom and Persidsky, 2013). AM251 is a synthetic biarylpyrazole cannabinoid, structurally similar to the CB1 receptor antagonist/inverse agonist, rimonabant (SR141716A) (Lan et al., 1999). This compound has also been reported to exert its effects via a novel target, as the inverse agonist properties of AM251 persisted in membranes derived from CB1-/- mice. Both Ryberg and Kapur identified AM251 as a GPR55 receptor agonist (Kapur et al., 2009, Ryberg et al., 2007), suggesting it may mediate its non-CB1 CNS effects through this cannabinoid receptor-like receptor.

There are several assays for assessment of cell viability. The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was the first homogenous cell viability assay, which measures nicotinamide adenine dinucleotide (NADH) carrying electrons from one reaction to another. When cells die, they lose their ability to convert MTT into formazan, a colour formation that gives an indication of viable cells, giving an indication of mitochondrial activity. NADH works by reducing the formazan (MTT) reagent (see Fig 2.1).

This chapter employs a set of experiments to investigate the effects of selected cannabinoid compounds including phyto-, endo- and synthetic cannabinoids on the viability and proliferation of 1321N1 human astrocytoma cells using the MTT assay. Additionally, we attempt to identify the molecular mechanism(s) through which these compounds mediate their effects to gain a better understanding of pharmacological and toxicological effects of cannabinoids and cannabinoid-related compounds on human brain cells.

3.1.1 Aim and Objectives

The aim of this study was to test the neurotoxic (neurodegenerative) and neuroprotective effects of cannabinoid ligands in human astrocytoma (1321N1) cell line. The specific objectives were to: -

1. Validate human astrocytoma 1321N1 cells as an appropriate and reliable cell system to test the neurotoxicity or neuroprotection of drugs.

2. Validate the experimental assay for the assessment of cell viability, the colorimetric MTT assay.

3. Use this glioma cell line to evaluate the potential for targeting the endocannabinoid system to reduce cell proliferation *in vitro*.

3.2 Results

3.2.1 Validation of the experimental approach

To optimise the experimental conditions and select an appropriate concentration of FBS for the subsequent experiments, the effect of FBS treatment on cell viability was investigated in 1321N1 human astrocytoma cells. The cells were cultured in 24-well plates containing medium with FBS concentrations ranging from 0-10% v/v for 24 h at 37°C. Unexpectedly, the assessment of cell viability using MTT assay showed that there was no statistical difference between any of the FBS concentrations tested (1-10% v/v) compared to serum-free conditions (0% v/v FBS) (Fig 3.1).

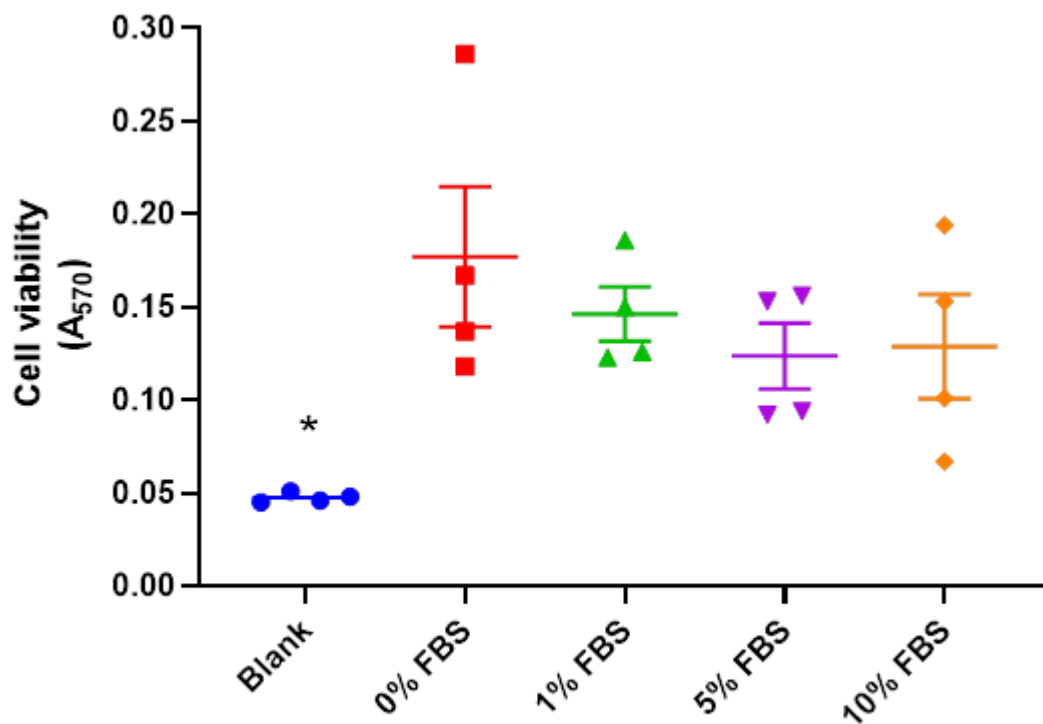


Figure 3. 1 Effects of FBS on cell viability in 1321N1 cells cultured for 24h in 24-well plates. Data are mean ± SEM. from four different cell passages with *P<0.05 compared to 0% v/v FBS, One-way ANOVA with Dunnett's post-hoc corrections.

Given the unexpected results observed in the initial experiment (Fig 3.1) using different concentrations of FBS on cell viability in 1321N1 human astrocytoma cells, we investigated whether different concentrations of FBS could affect the cell growth. Cells were cultured in 24-well plates at different cell densities, to achieve different levels of confluencies (30, 60 and 90%) following 24 h incubation with various concentrations of FBS ranging from 0-10% v/v of culture medium. Then the cell viability was assessed using the MTT assay. As before, there was no statistical difference between any of the FBS concentrations tested and the serum-free conditions (0% v/v FBS) in term of cell viability at different stages of cell growth (Fig 3.2).

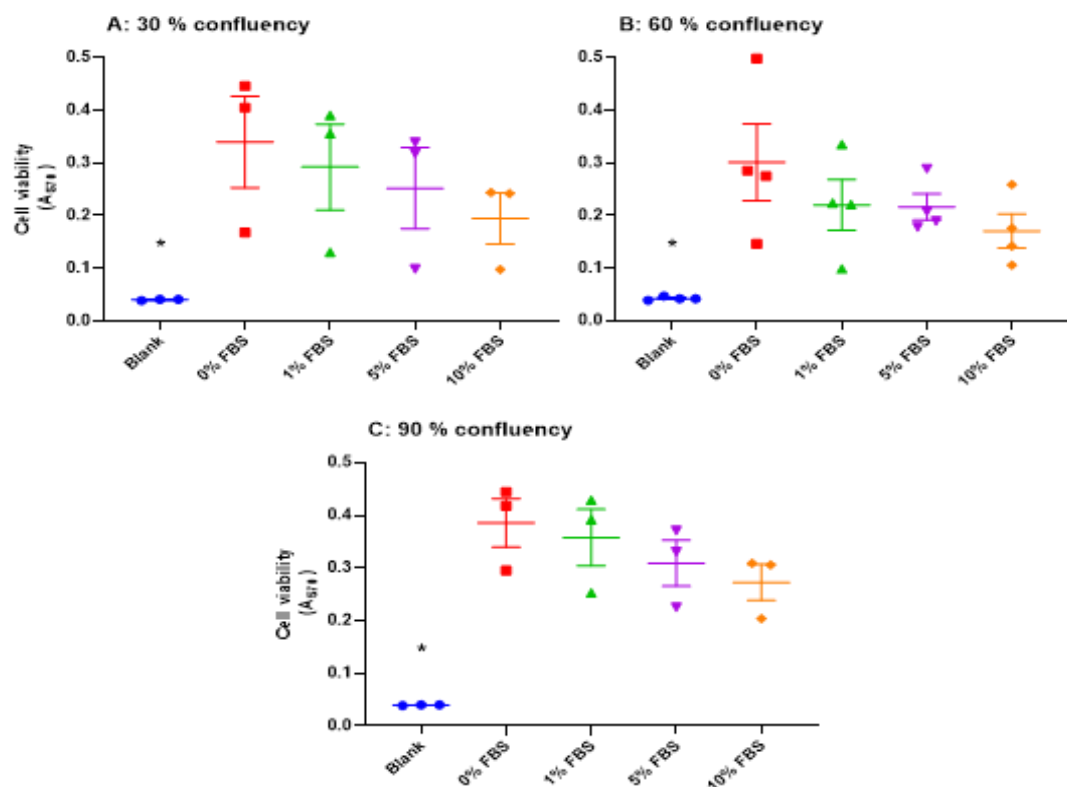


Figure 3. 2 1321N1 human astrocytoma cell viability was assessed by MTT assay at different initial densities (A) 30%, (B) 60%, and (C) 90% confluency and then cultured for 24 h in 24-well plates. The data were collected from three different cell passages and expressed as mean $\pm$

SEM. * $P < 0.05$ compared to 0 % v/v FBS, One-way ANOVA with Dunnett's post-hoc corrections.

We investigated whether increasing the time of exposure of 1321N1 cells to the tested concentration range of FBS to 48 h could change the cell viability response of these cells comparing to 24 h incubation. The cells were seeded in 24-well plates at different cell densities so that they will be at different confluencies (30, 60 and 90%) with various concentrations of FBS over 48 h, before assessing cell viability using the MTT assay. However, the results of these experiments revealed that there was no statistical difference between any of the FBS concentrations tested and serum-free conditions (0% v/v FBS) in term of cell viability at different stages of cell growth (Fig 3.3).

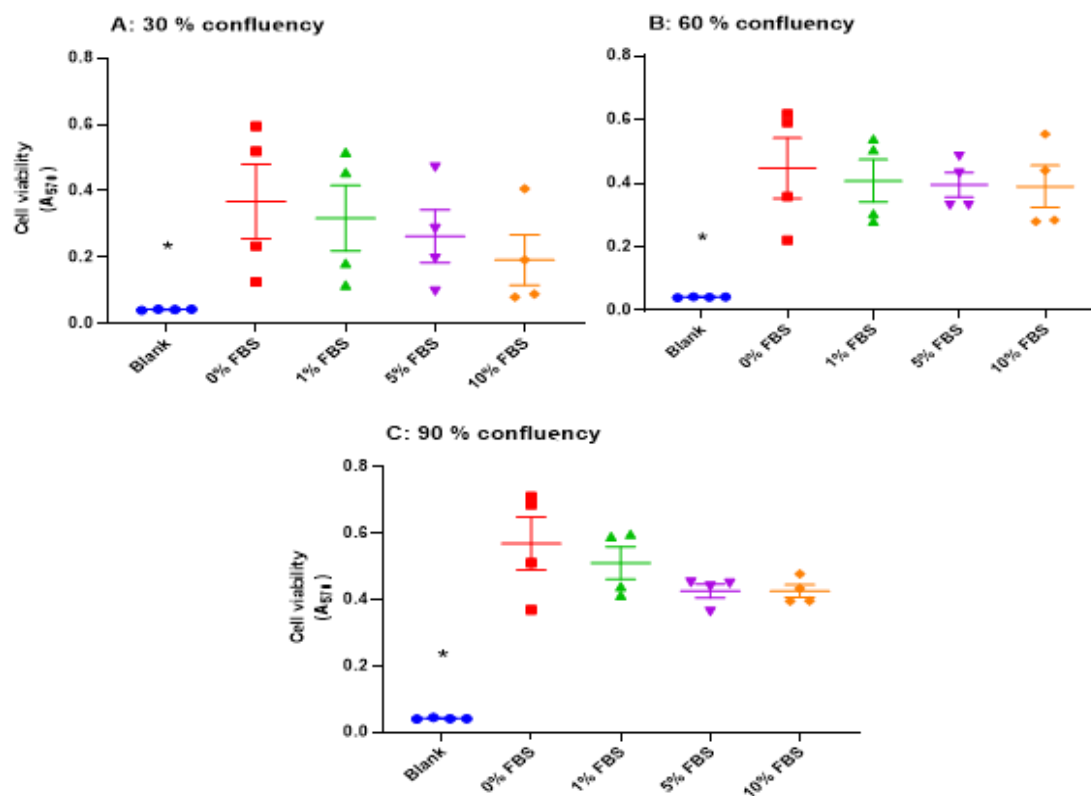


Figure 3. 3 1321N1 human astrocytoma cell viability was assessed by MTT assay at different initial densities (A) 30%, (B) 60%, (C) 90% confluency and then cultured for 48 h in 24-well plates. The data were collected from four different cell passages and expressed as mean $\pm$ SEM. *P<0.05 compared to 0 % v/v FBS, One-way ANOVA with Dunnett's post-hoc corrections.

To avoid any artefact that could result from transferring the assay buffer from 24-wells to 96-well plates we repeated the experiments to assess the effects of range of concentrations of FBS on 1321N1 cell viability using 96-well plate format. We have also considered the stage of growth at which the cell viability is assessed i.e., different confluencies, and the time of exposure of 1321N1 cells to the different concentrations of FBS tested in this study i.e., 24 h and 48 h. However, the trend of cell viability responses was very similar to that previously described using the 24-well plate format as there were no statistical differences in response to any of FBS concentrations tested comparing to the serum-free condition (0% v/v FBS) following treatment for 24 h (Fig 3.4) and 48 h (Fig 3.5).

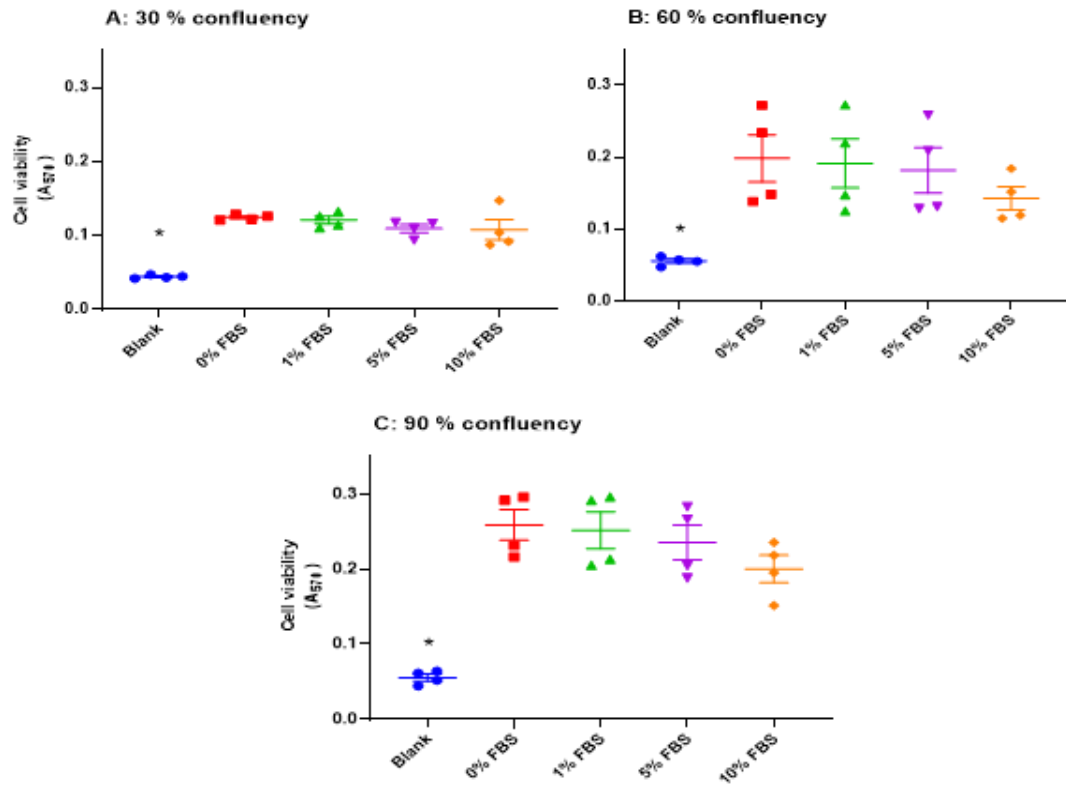


Figure 3. 4 1321N1 human astrocytoma cell viability was assessed by MTT assay at different initial seeding (A) 30%, (B) 60%, (C) 90% confluency and cultured for 24h in 96-well plates. The data were collected from 4 different cell passages and expressed as mean $\pm$ SEM. *P<0.05 compared to 0 % v/v FBS, One-way ANOVA with Dunnett's post-hoc corrections.

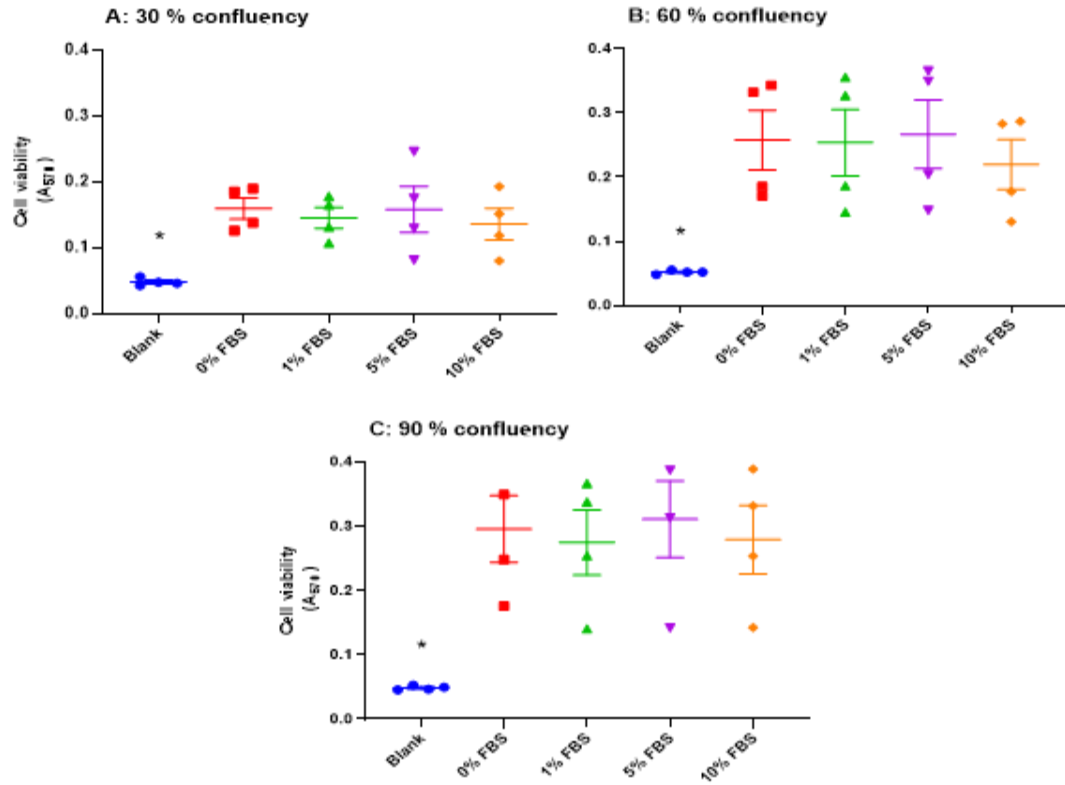


Figure 3. 5 1321N1 human astrocytoma cell viability was assessed by MTT assay at different initial seeding (A) 30%, (B) 60%, and (C) 90% confluency and cultured for 48 h in 96-well plates. The data were collected from 4 different cell passages and expressed as mean $\pm$ SEM. $P < 0.05$ compared to 0 % v/v FBS, One-way ANOVA with Dunnett's post-hoc corrections.

These experiments showed that varying concentrations of FBS had no effect on the viability of 1321N1 human astrocytoma cells, and this observation was independent of the plate type or the confluency of the cells at the start of the experiment. This is summarized in Table 3.2.

Table 3. 2 Summary of the experimental approach to 1321N1 human astrocytoma cell viability by MTT assay.

Expectations/hypotheses	Blank values should be similar for each size of cell culture plate
Observation	Blank values for 24-well plates are similar 0.04-0.048 Blank values for 96-well plates show more variation 0.044-0.0560
Interpretation	Volume of reagents used for 96-well plates is smaller – more influence of minor changes in pipetting and more residue between washing wells
Expectations/hypotheses	Blank values might change when comparing different cell culture plates
Observation	96-well plates have slightly higher blank values than the 24 well plates
Interpretation	Differences in the efficiency of washing for the 96-well plates, but these are small differences
Expectations/hypotheses	Having cells present should increase the MTT conversion, so values should be higher for 0% v/v FBS compared to the blanks
Observation	In each experiment, the presence of cells increased the absorbance, indicating an increased level of conversion of MTT to formazan
Interpretation	The assay works
Expectations/hypotheses	The more cells present, the higher the absorbance
Expectations/hypotheses	96-wells should have lower absorbance than 24 wells
Observation	In all six direct comparisons, 96-well absorbances for 0% v/v FBS are lower than in 24 well plates
Expectations/hypotheses	48 h incubations post-seeding should have greater absorbance than 24 h post-seeding incubations
Observation	In all six direct comparisons, 48 h incubation post-seeding showed higher absorbance than at 24 h
Expectations/hypotheses	Higher seeding density should have higher absorbances (90 > 60 > 30)
Observation	Three out of four comparisons, 90 % > 60 % > 30 %
Interpretation	The assay works in a (mostly) predictable way
Expectations/hypotheses	Inclusion of a cytotoxic agent should reduce absorbance through inhibiting proliferation/killing cells
Observation	Doxorubicin, employed as a positive control, evoked a concentration-dependent inhibition of evolved absorbance
Interpretation	The assay works in a predictable way

3.2.2 Effects of cannabinoid-metabolising enzyme inhibitors on 1321N1 cell viability

A set of inhibitors of various enzymes involved in endocannabinoid metabolism and biotransformation were investigated for affecting the viability of human astrocytoma 1321N1 cells cultured in medium with low concentration of FBS (1% v/v) using the MTT assay. This group included URB597 (a selective FAAH inhibitor), JNJ1661010 (a selective reversible FAAH inhibitor), MAFP (a potent and irreversible inhibitor of serine hydrolases and serine proteases), JZL184 (a potent and selective inhibitor of MAG lipase) and WWL70 (a reversible inhibitor of ABHD6) (see Table 3.2). At a low concentration (100 nM), none of the enzyme inhibitors showed significant change in cell viability relative to the control. However, increasing the concentration to 1 μ M caused significant reductions in cell viability with MAFP ($P<0.05$), JZL184 ($P<0.05$) and WWL70 ($P<0.05$) (Fig 3.6. B).

Table 3. 3 Inhibitors of endocannabinoid turnover

Inhibitor	Profile	Reference
URB597	Irreversible FAAH inhibitor	(Mor et al., 2004)
JNJ1661010	Reversible FAAH inhibitor	(Keith et al., 2008)
MAFP	Irreversible serine hydrolase and serine protease inhibitor	(Deutsch et al., 1997)
JZL184	Selectively MAG Lipase inhibitor	(Long et al., 2009b)
WWL70	Reversible ABHD6 inhibitor	(Li et al., 2007)

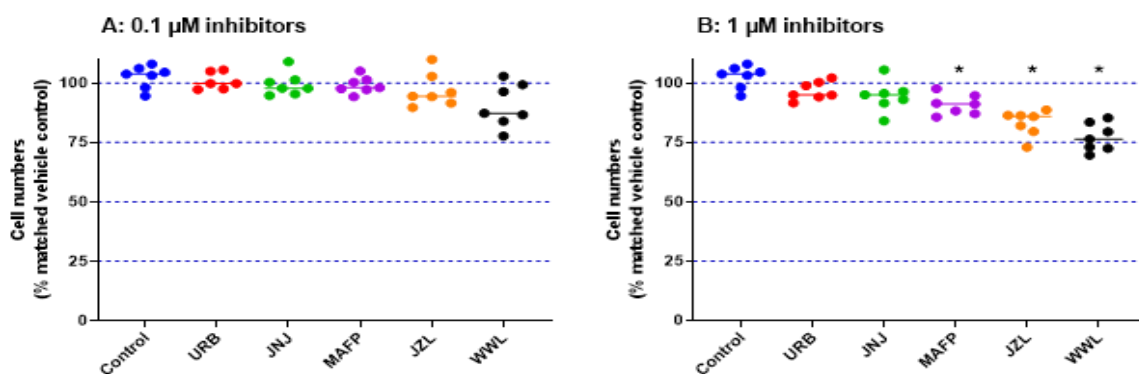


Figure 3. 6 Effects of (A) 0.1 μM or (B) 1 μM concentrations of a panel of endocannabinoid-metabolising enzyme inhibitors on cell viability in 1321N1 cells cultured in medium with FBS (1% v/v). The data were collected from 7 different cell passages and expressed as mean $\pm$ SEM and represented as percentage of matched vehicle control. $P < 0.05$ compared to 0 % v/v FBS, One-way ANOVA with Dunnett's post-hoc corrections.

3.2.3 Effects of cannabinoid receptor ligands on 1321N1 cell viability

The modest effect of the 1 μM enzyme inhibitors from the previous section might be attributed to a limited production of endocannabinoids in these cells. Therefore, direct modulation of cannabinoid receptors was examined as a regulator of cell viability.

The MTT assay was used to examine the impact on viability of 1321N1 cells in the presence of a low concentration of FBS 1 % v/v, (Fig 3.7). Vehicle controls (0.1 % v/v DMSO or ethanol) were not different to cell numbers in the absence of other ligands. The positive control, doxorubicin, at 1 μM evoked an inhibition of cell viability to 38 % of control. The GPR55 antagonist CID16020046, the CB1-selective antagonist CP945598, the CB2-selective antagonist AM630 and the CB1-selective agonist ACEA were without effect on viability of 1321N1 cells. AM251, the CB1 antagonist and GPR55 agonist, evoked an inhibition of 1321N1 cell numbers to 76 % of control. By contrast, the non-selective CB agonist HU210 and the CB2-selective agonist JWH015 evoked enhancements of 1321N1 cell numbers to 122 and 123 % control, respectively.

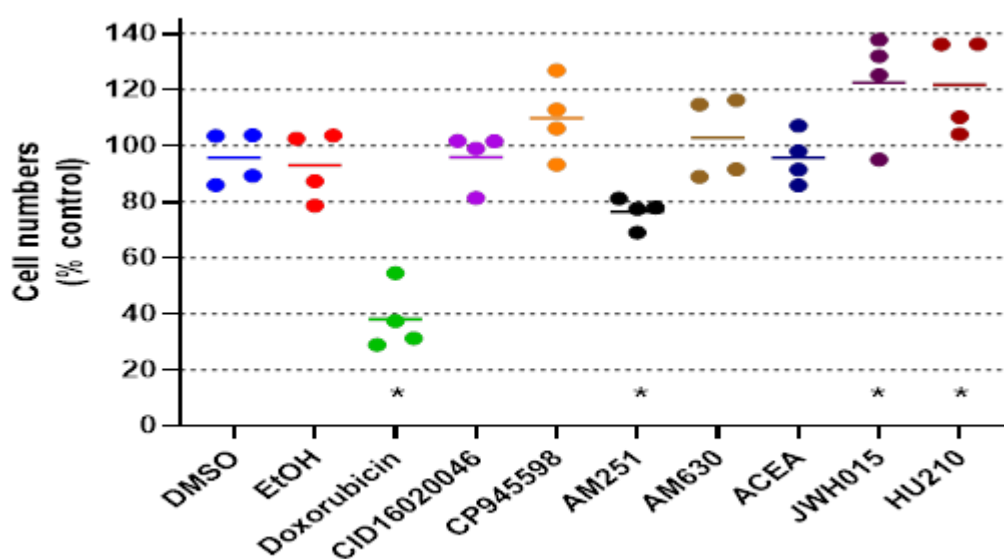


Figure 3. 7 The effects of cannabinoid compounds on the viability of 1321N1 cells. Cells were cultured in the presence of 1 % v/v FBS and 1 μ M of the indicated ligands for 72 h in 96-well plates. Data are means of four separate experiments conducted in sextuplicate, with the overall mean of the four experiments indicated by the horizontal bar. Untransformed data were analysed by one-way ANOVA and post-hoc Tukey's test.

Next, the effects of cannabinoid receptor ligands were examined in the presence of a low concentration of the cytotoxic agent doxorubicin. The MTT assay was used to examine the impact on viability of 1321N1 cells in the presence of a low concentration of FBS 1 % v/v and 200 nM doxorubicin (Fig 3.8). The intention with this investigation was to see whether the mild cytotoxicity associated with low concentration of doxorubicin could be mitigated or enhanced in the presence of cannabinoid ligands. Therefore, a side-by-side presentation was adopted to highlight whether the effects of doxorubicin were altered in the presence of the cannabinoid. Vehicle controls treated with 200 nM doxorubicin showed inhibitions of the 1321N1 cell numbers to 80%. I tested the effect of doxorubicin on a panel of cannabinoid ligands including those that showed no effect on their own to understand whether combination with doxorubicin can modify the response of cannabinoid ligands on 1321N1 cell number. The CB1-selective agonist ACEA, the CB1 antagonist and GPR55 agonist AM251 induced inhibitions of the 1321N1 cell numbers in the absence or presence of a low concentration of doxorubicin; ACEA evoked inhibition of 1321N1 cell number to 58% in the absence and 50% in the presence of doxorubicin. AM251 evoked inhibition of 1321N1 cell number to 65% in the absence and 55% in the presence of doxorubicin. The CB2-selective agonist JWH015 was without effect on viability of 1321N1 cells in the absence or presence of doxorubicin. The CB2-selective antagonist AM630 evoked inhibition of 1321N1 to 80% only in the absence of doxorubicin, while the inhibition effect of AM630 1321N1 cell viability did not significantly change in the presence of doxorubicin.

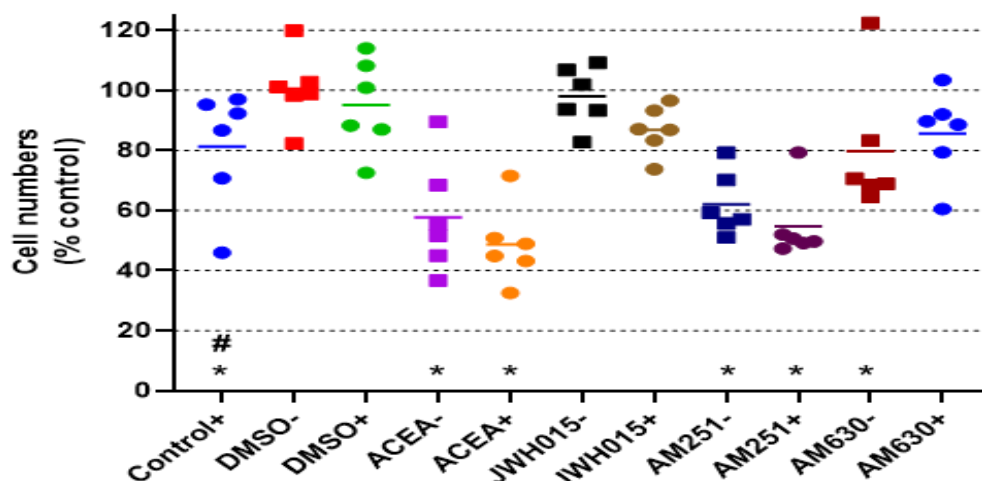


Figure 3. 8 The effects of cannabinoid compounds on the viability of 1321N1 cells in combination with 200 nM doxorubicin. Cells were cultured in the presence of 1 % v/v FBS and 1 μ M of the indicated ligands for 72 h in 96-well plates. Control cells were treated with 200 nM doxorubicin. Data are means of six separate experiments conducted in octuplicate, with the overall mean of the four experiments indicated by the horizontal bar. Untransformed data were analysed by two-way ANOVA and post-hoc Tukey's test (# P <0.05, absence vs presence of doxorubicin; * P <0.05, compared to 0.1 % v/v DMSO).

The effects of selective antagonists on agonist-evoked reductions in cell viability was assessed. The MTT assay was used to examine the impact of 1 μ M concentrations of the non-selective cannabinoid receptor agonists, HU-210 and CP 55,940 in the presence and absence of 3 μ M of selective CB1 and CB2 antagonists, namely AM251 and AM630 (Fig 3.9). Vehicle controls treated with 200 nM doxorubicin evoked inhibitions of the 1321N1 cell number to 49%. HU-210 and CP 55,940 on their own or in the presence of AM630 were without effect on viability of 1321N1 cells. In the presence of AM251, both HU-210 and CP 55,940 induced inhibitions of the 1321N1 cell numbers to 52% and 11%, respectively. This indicates that CB antagonists had a protective effect against CB agonists toxic activities in 1321N1 cell models.

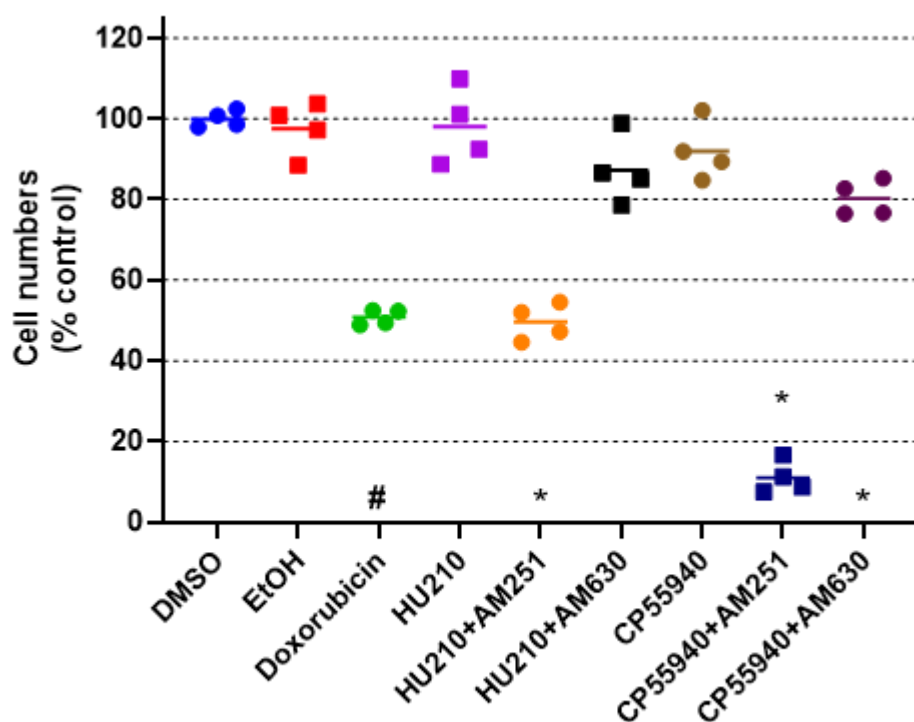


Figure 3. 69 The effects of cannabinoid antagonists on the viability of 1321N1 cells in combination with cannabinoid agonists. Cells were cultured in the presence of 1 % v/v FBS and 1 μ M HU210 or CP55940 and in the further presence of 3 μ M AM251 or AM630 for 72 h in 96-well plates. Data are means of four separate experiments conducted in octuplicate, with the overall mean of the four experiments indicated by the horizontal bar. Untransformed data were analysed by two-way ANOVA and post-hoc Tukey's test (# P <0.05, absence vs presence of doxorubicin; * P <0.05, compared to the absence of the cannabinoid antagonist).

The MTT assay was used to examine the impact of 1 μ M of cannabinoid agonists, JWH015 or ACEA in the presence and absence of 3 μ M of selective CB1 and CB2 antagonists, namely AM251 and AM630 (Fig 3.10). Vehicle controls treated with 200 nM doxorubicin evoked inhibitions of the 1321N1 cell numbers to 50%. JWH015 and ACE on their own evoked inhibitions of the 1321N1 cell numbers to 81% and 72%, respectively. In the presence of AM251, JWH015 and ACE induced further inhibitions in of the 1321N1 cell numbers to 43% and 50%, respectively. While in the presence of AM630, JWH015 and ACE induced some

inhibitions of the 1321N1 cell numbers to 24% and 25% in three and four experiments, respectively. AB-Fubinica, AB-Pinica, XLR-II and JWH015 on their own were without effect on viability of 1321N1 cells. In the presence of AM251, AB-Fubinica, AB-Pinica, XLR-II and JWH015 induced further inhibitions in of the 1321N1 cell numbers to 42% and 46%, 59% and 61%, respectively. While in the presence of AM630, all these agents were without effect on viability of 1321N1 cells.

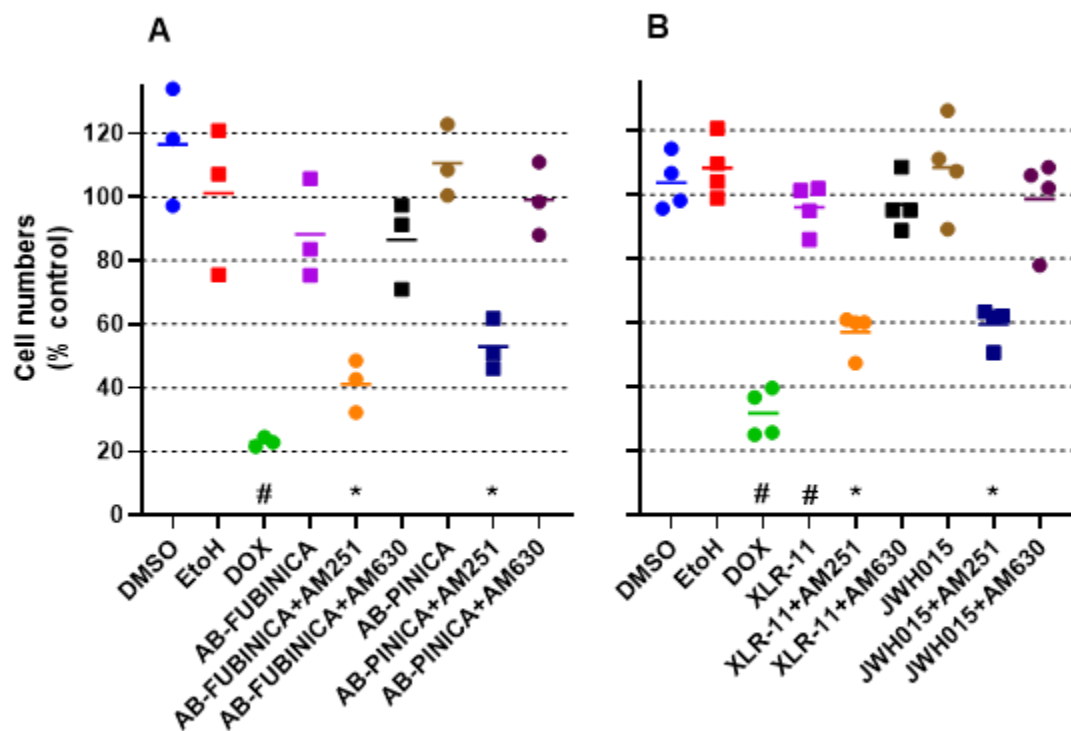


Figure 3. 8 The effects of cannabinoid antagonists on the viability of 1321N1 cells in combination with cannabinoid agonists. Cells were cultured in the presence of 1 % v/v FBS and 1 μ M AB-FUBINICA, AB-PINICA or XLR-11 and in the further presence of 3 μ M AM251 or AM630 for 72 h in 96-well plates. Data are means of three (A) or four (B) separate experiments conducted in octuplicate, with the overall mean of the three or four experiments indicated by the horizontal bar. Untransformed data were analysed by two-way ANOVA and post-hoc Tukey's test (# P <0.05, absence vs presence of doxorubicin or cannabinoid agonists; * P <0.05, compared to the absence of the cannabinoid antagonist).

We then used MTT assay to examine the effect of a range of concentrations (0.3 nM-10 μ M) of the CB1 inverse agonist AM251 on the 1321N1 cell viability over 72 h of incubation with a low concentration of FBS (1% v/v). AM251 evoked a concentration-dependent inhibition in the number of 1321N1 cells at higher concentrations, with a pIC₅₀ value of 5.7. (Fig 3.12). The lower concentrations of AM251 i.e. 5×10^{-7} M can be also repeated in the presence of other CB2-selective agonist JWH015 to understand whether the combinatory treatment might improve 1321N1 cell viability.

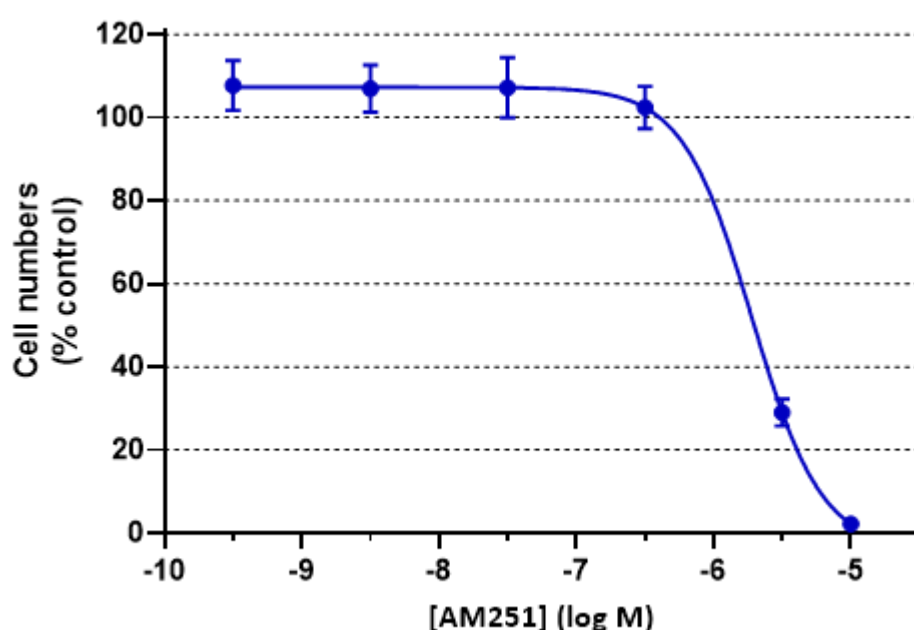


Figure 3.9 concentration dependence of AM251 effects on 1321N1 cell viability following 72h of treatment. Data are means of three separate experiments conducted in octuplicate. Error bars represent $\pm$ SEM.

The concentration-dependent effects of the CB1-selective agonist, ACEA and the non-selective cannabinoid receptor agonist, CP55940 on 1321N1 cell number cultured with low concentrations of FBS (1% v/v) for 72 h was examined in the presence and absence of the CB1 inverse agonist AM251 (3 μ M) (Fig 3.13). Both ACEA and CP55940 evoked concentration-dependent reduction in the number of 1321N1 cells with pIC₅₀ value of 5.3 in case of CP55940. Combination of 3 μ M AM251 with CP55940 induced a leftward shift in the concentration-

dependent response of CP55940 with pIC_{50} value of 6.1, indicating the higher potency of the combinatory treatment in reducing the number of 1321N1 cells. Similarly, the maximum number of 1321N1 cells in the presence of the lowest concentration of CP55940 (5 μ M) was also reduced from 97% in the absence of AM251 to 65% in the presence of AM251.

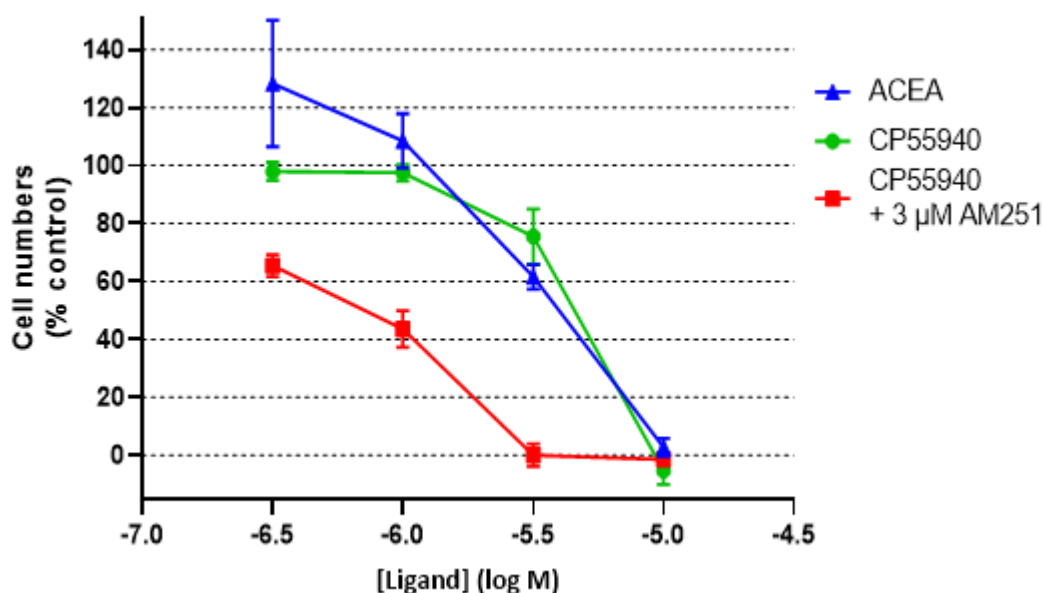


Figure 3. 10 Concentration dependence of ACEA and CP55940 in the presence and absence of 3 μ M AM251 effects on 1321N1 cell viability. Data are means of three separate experiments conducted in octuplicate. Error bars represent $\pm$ SEM.

In the previous experiments, the CB1inverse agonist AM251 consistently caused reductions in 1321N1 cell number, but it is unlikely that this effect is solely mediated by classical cannabinoid receptors, CB1 or CB2 as other antagonists did not show the same effect. Therefore, we tested the hypothesis that the effect of AM251 on 1321N1 cell viability could be mediated by cannabinoid-related or non-classical cannabinoid receptor GPR55 (Fig 3.16). Therefore, the concentration-dependent effects of AM251 on 1321N1 cell number cultured with low concentrations of FBS (1% v/v) for 72 h was examined in the presence and absence of 3 μ M GPR55 (Fig 3.14). AM251 evoked concentration-dependent reduction in 1321N1 cell number with pIC_{50} value of 5.5. However, combination of 3 μ M GPR55 was without any further effect on the 1321N1 cell viability. This indicates that AM251-mediated inhibition of 1321N1 cell viability via a distinct mechanism.

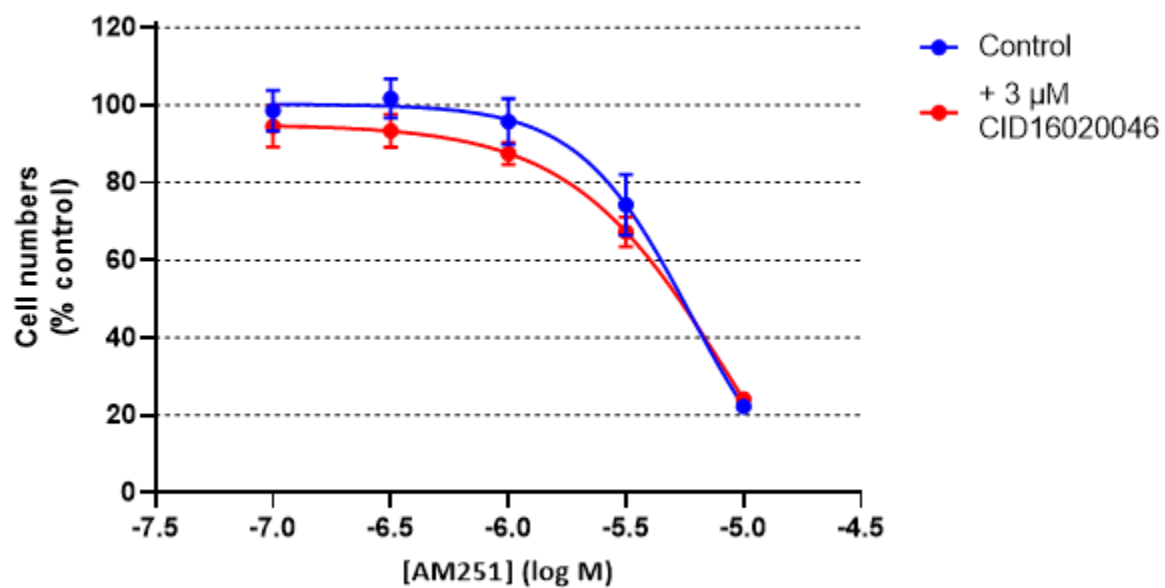


Figure 3. 11 Concentration dependence of ACEA and CP55940 in the presence and absence of 3 μ M AM251 effects on 1321N1 cell viability. Data are means of three separate experiments conducted in octuplicate. Error bars represent $\pm$ SEM.

3.3 Discussion

Malignant astrocytic tumours are the most common primary brain tumours; their growth is rapid, and they are associated with a poor prognosis, despite the use of multimodal treatment regimens. The lack of progress in the treatment of this cancer subtype has aroused the need of novel agents. In this chapter we used GBM cell line as cancer model to study malignant astrocytic tumours since it is the most malignant and highly aggressive brain tumour. The current results suggest that blocking cannabinoid receptors decreases 1321N1 cell viability indicating the involvement of cannabinoid signalling system in the protection of human astrocytoma cells from apoptosis.

1321N1 is a human astrocytoma cell line isolated in 1972 as a sub-clone of the cell line 1181N1 which in turn was isolated from the parent line U-118 MG; one of the cell lines derived from malignant gliomas by Ponten and Macintyre in 1968 and cited in 2001 by Kraft et al. This cell line is recognised as an appropriate and reliable cell system to test the neurotoxicity and neuroprotection of various cancer drugs.

To set the ground for future experiments the effects of culturing 1321N1 cells in growth media containing a range of concentrations of FBS in comparison to those cultured in serum-free medium was investigated using MTT assay. Unexpectedly the preliminary results revealed that there is no statistical difference in cell viability of 1321N1 cells cultured in the absence or presence of different concentrations of FBS (1-10% v/v) for either 24 or 48h (Figure 3.1). Likewise, 1321N1 cells seeded at different densities resulting in 30%, 60% and 90% confluency at the time of the assessment showed no effect of various concentrations of FBS (1-10% v/v) on 1321N1 cell viability (Figure 3.2). It has been reported that growth factors can be produced by glioma cells themselves (Westphal and Herrmann, 1989). Glioma cells were also found to secrete nerve growth factor (NGF), as in C6 rat glioma cells (Murphy et al., 1977) and the human astrocytoma cell lines A172 (Hamel et al., 1993) and 1321N1 (Obara et al., 1999). Therefore, since glioma cells can intrinsically produce various growth factors, the effect of serum on their viability might be minimum.

Additionally, the MTT assay measures the ability of cells to reduce the tetrazolium dye, MTT, to its insoluble formazan, resulting in purple colour. Since this requires functioning mitochondria, the MTT effectively measures the metabolic activity of live cells. It is quick,

cheap, easy to perform and relatively reliable (Mosmann, 1983), and that is why it can be used for drug screening. However, there are some limitations of this assay; first, measuring activity should be carried out at the most active part of the cell growth phase (Assanga, 2013). In addition, the MTT assay is an absorbance-based assay, which means it is not very sensitive at picking out minor changes. Most importantly, it measures metabolic activity, not necessarily the viability. Some cells can be perfectly viable but not necessarily with a lot of metabolic activity. Hence, it is always important to validate the MTT results by a complementary assay that directly measures cell apoptosis and/or necrosis like for example quantitative determination of lactate dehydrogenase (LDH) in the cell culture medium which can act as a marker for cytotoxicity, or by using other complementary biochemical and/or morphological techniques to determine whether the tested compound is cytotoxic, cytostatic or anti-adhesive.

Given that the effect of FBS on 1321N1 cell viability was not significant, we cultured our cells with low concentration of FBS (1% v/v) and apply this concentration for the subsequent experiments. We have then examined the effects of a panel of enzyme inhibitors, that selectively inhibit a set of intracellular enzymes involved in cannabinoid compounds metabolism and biotransformation on the viability of the cells of human astrocytoma 1321N1 cells cultured in a medium with a low concentration of FBS (1% v/v). Our result showed that the enzyme inhibitors namely, MAFP, JZL and WWL induced a significant reduction in 1321N1 cell viability, when they were used at a concentration of 1 μ M (Figure 3.6). This result indicated that some of the cannabinoid-metabolising enzymes may play a role in the protection of human astrocytoma cells from apoptosis. However, the expression of the enzymes involve in cannabinoid compound metabolism in this cell line is yet to be confirmed. This finding was consistent with previous research suggested that cannabinoid can protect from apoptosis in a number of neuronal cells including oligodendrocytes via CB1 and CB2 receptors through the PI3K/AKT pathway (Molina-Holgado et al., 2002), and can rescue primary astrocytes from ceramide-induced apoptosis via PI3K/PKB pathway (Del Pulgar et al., 2002). Cannabinoids have promising anti-tumoural effects in pre-clinical models. For example, THC has been reported to encourage apoptosis of transformed neural cells in culture while WIN55212-2 caused the retreatment of malignant gliomas in rats via acting at both CB1 and CB2 receptors (Galve-Roperh et al., 2000). Cannabidiol has been proposed to

have anti-tumour effects through a multitude of molecular pathways, against breast cancer, glioma, leukaemia, thyroid thyroma, lung and colon cancer (Massi et al., 2013). Cridge & Rosengren. (2013) review concluded that numerous obvious contradictions remain in the literature about the possible benefit of cannabinoid-related medicines in cancer. Clinical trials with cannabinoids in cancer are limited (Kramer, 2015, Lan et al., 1999). In a pilot clinical trial, direct intra-tumoural application of THC seemed to decrease proliferation rate on glioblastoma multiforme in patients (Guzman et al., 2006). Thus, even though there is obviously much to discover in terms of cannabinoid choice and molecular target, the poor prognosis of patients currently suffering from a diversity of cancers, combined with the positive tolerability of cannabinoids, suggest that research on anti-tumour cannabinoids should persevere.

1321N2 cell viability was then examined in the presence of panels of drugs that act as agonists or antagonists at cannabinoid receptors. Our finding showed that only AM251 which is a CB1-selective antagonist was able to decrease the 1321N2 cell viability. Nevertheless, the inhibitory effect of AM251 was not observed in when cells were treated with CP945598 which is a structurally distinct CB1 antagonist, CB2-selective antagonist (AM630) or CB1 agonist, ACEA. This suggests that the inhibitory effect of AM251 may not be mediated by its activity on CB1 antagonist. Intriguingly, the modestly CB2 preferring HU210 or CB2-selective agonist JWH015 appeared to increase the 1321N2 cell numbers (Figure 3.8), but without statistical significance. This suggests that further research is warranted to confirm the cytoprotective effect of JWH015 on glial cell growth.

We have therefore studied the effect of the synthetic cannabinoid drugs in the presence of doxorubicin to study whether they would be able to protect and maintain the cell proliferation. Doxorubicin (DOX) is an anthracycline drug that was first taken in the 1970s and routinely used in the treatment of a variety of breast, lung, gastric, renal, thyroid, non-Hodgkin and Hodgkin's lymphoma, multiple myeloma, sarcoma and childhood cancer (Arcamone et al., 1969, Weiss, 1992). DOX is an effective antibiotic anticancer agent of the anthracycline community that is available in large amounts and is used alone or together for the treatment of a variety of both haematologically and solid tumours, such as breast cancer (Jabłońska-Trypuć et al., 2017, Coldwell et al., 2010). It prevents the growth of cells, causes oxidative stress, hampers topoisomerase II and eventually contributes to cell death primarily

by apoptosis (Cao et al., 2013, Shokrzadeh et al., 2021, Sauter et al., 2010). When investigated the effect of DOX on 1321N2 cell viability in the presence or absence of panel of cannabinoid receptor agonists and antagonists. Our data indicated that in the absence or the presence of a low doxorubicin concentration, both ACEA and AM 251 evoked reductions in 1321N2 cell number. AM630 caused less reduction but its effect was more pronounced in the absence of doxorubicin, while JWH015 had no effect in the presence or absence of DOX (Figure 3.9).

Then, we studied the effect non-selective cannabinoid receptor agonists, namely HU210, CP55940 and JWH018 in the presence and absence of selective CB1 and CB2 antagonists, namely AM251 and AM630. Our analysis revealed that none of the tested compounds elicited a statistically significant response in comparison to the vehicle control. However, combining the tested ligands with AM251, but not AM630, significantly reduced the proliferation of 1321N1 cells (Figure 3.10). This effect could be solely attributed to the effect of AM251 on 1321N1 cell viability as the reduction detected in the presence of the combinatory treatment was not significantly different from that observed in the presence of AM251.

We then studied the effect of synthetic cannabinoids with high selectivity at CB1 and CB2 receptors on proliferation of 1321N1 cells in the presence or absence of 3 μ M selective CB1, AM251 or CB2, AM630 antagonists. A combined administration of THC and CBD decreased growth of U-87 MG cell-derived tumour xenografts via apoptosis (Torres et al., 2011) and inhibited cell cycle in SF126 and U-251 MG cells, but not in U-87 MG, and induced apoptosis in U-251 MG cells (Marcu et al., 2010). Synthetic cannabinoids like the agonists ACEA, JWH133 or inverse agonists AM281 and AM630 are known to be more selective than phytocannabinoids (Pertwee et al., 2010). Kapur and colleagues (Kapur et al., 2009) have reported that CP-55,940 is an antagonist of the GPR55 receptor in common with abnormal cannabidiol, O-1602, HU 210, JWH 015 and AM 251 (Baker et al., 2006, Johns et al., 2007). In here, when JWH015 were combined with AM251, it evoked an inhibition of 1321N1 cell viability. While ACEA evoked a significant inhibition, which was unaffected by AM630 but was increased in the presence of AM251 (Figure 3.11).

We have also assessed the effects of three different cannabinoid related compounds known to be abused in the UK, namely AB-Fubinic, AB-Pinica, and XLR-II on the proliferation

of 1321N1 cells and compared their effects to those evoked by the previously tested cannabinoid ligands in order to identify whether the effects of these compounds on cell viability can be modified by co-incubation with AM251 or AM630. Co-administration of these compounds with AM251, but not AM630, enhanced the inhibitory effects of these drugs on the proliferation of 1321N1 cells. This finding suggests a synergistic effect by the combination of AM251 and the three cannabinoid agonists, AB-Fubinica, AB-Pinica, and XLR-II on 1321N1 cell viability (Figure 3.11).

Evaluation of the effect of AM251 on the proliferation rate of 1321N1 cells was examined in the presence of a range of concentrations of AM251; 0.3 nM-10 μ M in culture media at low concentrations of FBS (1% v/v) for 72h. The results showed that CB1 selective antagonist, AM251 induce concentration-dependent inhibition of 1321N1 cell viability with pEC50 value of 5.7. This indicates that AM251 reproducibly caused a reduction of the 1321N1 cell proliferation. However, this effect is unlikely to be mediated by the classical cannabinoid receptors as the cannabinoid agonists i.e. ACEA was unable to reverse that inhibitory response. AM251 was also reported to be a putative GPR55 agonist since it inhibited NO induced by LPS in BV-2 at micromolar concentrations via acting at GPR55 receptors (Kapur et al., 2009, Pertwee, 2007). Hence, we raised the question whether AM251 effect could be mediated by the non-classical cannabinoid receptor GPR55. The latter is expressed in the brain and may be linked to motor behaviour (Wu et al., 2013). Recent findings showed that GPR55 modulates CB receptor signalling and forms heteromers with CB receptors (Balenga et al., 2014, Moreno et al., 2014, Sharir et al., 2012). Waksman et al. (1999) reported an inhibitory effect of the CB1/2 agonist CP55940 on endotoxin/cytokine-activated NO formation in rat cortical microglial cells mediated by CB1 receptors. CP55940 was also reported to be an agonist at GPR55 receptor (Ryberg et al., 2007). When the effect of CP55940 was examined in our system, CP55940 had no effect on cell viability at 1 μ M concentration, while increasing its concentration caused concentration-dependent reduction in 1321N1 cell viability with pIC50 value of 5.3. This could be due to activating cannabinoid receptors or GPR55 receptor. Interestingly, Combination of 3 μ M AM251 with CP55940 induced a leftward shift in the concentration-dependent response of CP55940 with pIC50 value of 6.1, suggesting that AM251 increased the potency of CP55940 in inducing inhibition of 1321N1 cell viability. In an attempt to examine the molecular mechanism by which AM251 induced inhibition of

1321N1 cell viability, the concentration-response curve of AM251 was examined in the presence of the GPR55 selective antagonist, CID16020046. We didn't have any evidence to indicate that CID6020046 doesn't adequately block GPR55, but the use of multiple different chemical structures with an overlapping pharmacophore would be the gold standard method for identifying the involvement of a particular molecular target. However, the results showed that the concentration-dependent inhibition of 1321N1 cell viability evoked by AM251 was not altered in the presence of CID16020046. This shows that GPR55 is not involved in regulating 1321N1 cell proliferation and suggests the involvement of distinct targets whereby AM251 regulates the cellular proliferation in 1321N1 cells.

3.4 Conclusions

The main conclusion of this chapter is that the human astrocytoma 1321N1 cells can survive the serum starvation for 24-48 h. To have a better understanding of the effect of serum deprivation on cell viability, the duration of incubation with a serum-free medium should be increased to 3-6 days. Our results suggest that AM251 inhibits proliferation of 1321N1 astrocytoma cells in a concentration-dependent manner; however, the molecular mechanism appears to be independent of cannabinoid receptor pathways. The cellular mechanisms involved in the response to AM251 in 1321N1 astrocytoma cells is yet to be elucidated.

Furthermore, the decline in cell viability in the presence of various inhibitors of the enzymes involved in the process of cannabinoid compounds metabolism and biotransformation namely FAAH, MAGL and ABHD6 indicates that cannabinoid signalling might play a role in the protection of the human astrocytoma cells from apoptosis. This suggests the endocannabinoid hydrolases process as useful targets for the regulation of the glioma viability.

CHAPTER 4

Endocannabinoid hydrolase profiling in two human neural cell lines

4.1. Introduction

As indicated in the Introduction, the endocannabinoid system (ECS) regulates several physiological and pathological processes in the human body, including dietary input, energy metabolism, impulse control, pain perception, memory development, and anxiety (Mechoulam and Parker, 2013, Soethoudt et al., 2018). The ECS has been the subject of much research due to its potential for medicinal use (Donvito et al., 2018, Di Marzo, 2008).

The serine hydrolase enzyme family, which accounts for 1–2% of the overall proteome, is one of the biggest functioning enzyme groups in mammals, including humans. Proteases, lipases, esterases, amidases, and transacylases are archetypal members of this enzyme family, which utilizes a conserved nucleophilic serine residue in the active site to accomplish different hydrolytic processes (Rajendran et al., 2020); Over 200 proteins employ a nucleophilic serine to hydrolyse ester, amide, or thioester bonds in small molecules and proteins through a covalent acyl-protein intermediate. Serine hydrolases are primarily responsible for the generation and degradation of endocannabinoids (Figure 4.1). Except for NAPE-PLD, and NAAA all endocannabinoid hydrolases are members of the serine hydrolase family (Long and Cravatt, 2011, Shahiduzzaman and Coombs, 2012). Diacylglycerol lipases, DAGL α and β , are the primary enzymes responsible for the production of 2-AG through diacylglycerol hydrolysis (DAG) (Gao et al., 2010, Reisenberg et al., 2012). The second endocannabinoid in mammals is N-arachidonoyl ethanolamine AEA. The primary enzyme for the hydrolysis of AEA to AA and ethanolamine is FAAH (Figure 4.1) (Cravatt et al., 1996, Patricelli et al., 1999).

Selective and in vivo active FAAH inhibitors have been available for more than a decade and have prompted numerous investigations into the metabolic and physiologic effects of augmented AEA signalling. A pharmacological agent, JZL184 was developed that potently and selectively blocks MAGL activity and elevates 2-AG levels in vivo, allowing a direct comparison between the enhanced actions of each endocannabinoid (Long et al., 2009b). It was found that WWL-70 is a selective inhibitor of α/β -Hydrolase domain 6 (ABHD6), which catalyses the hydrolysis of the endocannabinoid 2-arachidonoyl glycerol, (IC_{50} = 70 nM) (Li et al., 2007). So, focusing on these enzymes is important for understanding how hydrolases work in the body, and inhibitors could be used to change the levels of endocannabinoids in people who are sick.

Metabolic inhibitors of AEA metabolism and/or activity include methylarachidonylfluorophosphonate (MAFP) and fatty acid sulfonyl fluorides. MAFP was initially designed and developed as the active site-directed inactivator of the cannabinoid (CB1) receptor. Currently, there are several classes of FAAH inhibitors with improved selectivity and in vivo activity (De Petrocellis et al., 1997, Deutsch et al., 1997) such as 1- α -Ketoheterocycles, e.g., URB597. 3-Alkylsulfonylfluorides. In addition, selective pharmacological tools to disrupt the activity of MAGL in vivo have only become available within the last few years.

MAGL activity is sensitive to general serine hydrolase inhibitors such as arachidonoyl trifluoromethylketone, and hexadecylsulfonylfluoride. These compounds also inhibit FAAH and are therefore not suitable to distinguish between the functions of these enzymes (Minkkila et al., 2010, Petrosino and Di Marzo, 2010).

Astrocytes play a critical function in maintaining the integrity of the CNS and shielding neurons against pathogens such as viruses and bacteria (Mucke and Eddleston, 1993). Additionally, astrocytes have recently been demonstrated to control neuroinflammation by limiting microglial over-activation, which is a process known as neuroprotection (Decker et al., 2009). Therefore, we chose the 1321N1 astrocytoma and SH-SY5Y neuroblastoma cell lines as in vitro models for astrocytes and neuron respectively to study the enzyme repertoire that could be involved in cannabinoid biotransformation and find out whether targeting these enzymes would help to counteract aggressive brain tumours like glioblastoma multiforme.

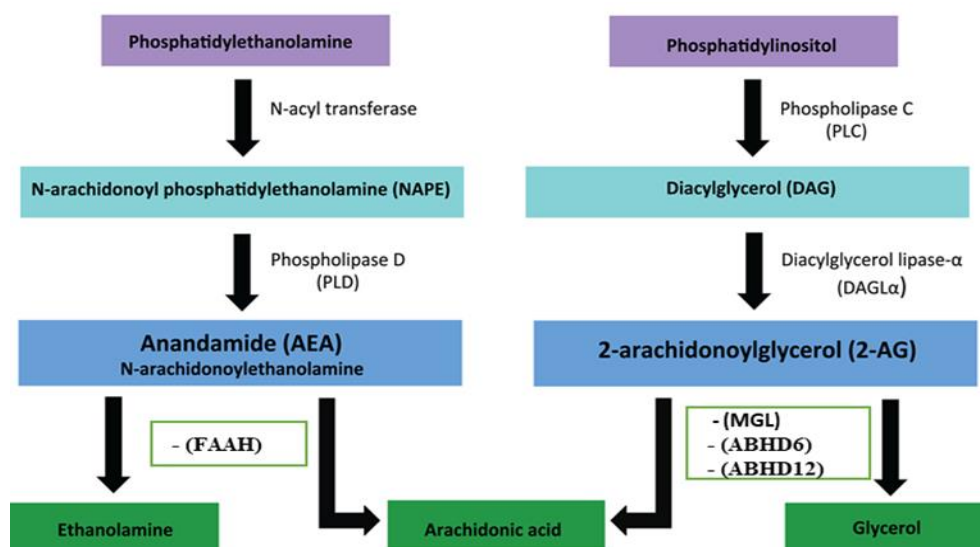


Figure 4. 1 A summary of the major metabolic routes within the endocannabinoid system Except for NAPE-PLD, all enzymes are members of the serine hydrolase protein family (Lee et al., 2016). AEA is mostly degraded by FAAH, while 2-AG is primarily hydrolyzed by monoacylglycerol lipase (MGL) and serine hydrolase α , β -hydrolase domain 6 and 12 (ABHD 6 and 12).

1321N1 cell line is recognised as an appropriate and reliable cell system to test the neurotoxicity or neuroprotection of drugs. Astrocytoma cells from 1321N1 express several different types of receptors, including M3 muscarinic cholinergic receptors, protease-activated receptors, thromboxane A2 receptors, and epidermal growth factor (EGF) receptors (Freund et al., 2003).

4.2 Measurement of endocannabinoid metabolising enzymes

4.2.1 Activity-based protein profiling (ABPP)

Activity-based protein profiling (ABPP) is an adaptable chemo-proteomic technique in drug discovery and development. The activity-based probes employed report the activity of specific enzymes or the reactivity of amino acid types in complicated biological systems. Since its inception in the early 1990s, the ABPP has helped in the identification of novel therapeutic targets and the development of many compounds in the treatment of human and infectious

illnesses (Evans and Cravatt, 2006). More significantly still, as part of a persistent worldwide effort to highlight the druggable proteome, the repertoire of target classes that may be addressed using activity-based probes has significantly increased in recent years.

4.2.2 ABPP Applications

Two major categories of experimental set-ups have been comparative and competitive ABPP (Cravatt et al., 2008). When performing comparative ABPP, the active enzyme levels in two different proteomes are measured and compared. Comparison of active protein levels in different biological samples has the goal of highlighting any differences or similarities in active protein levels in the tested samples. For example, two samples of a tissue, one of which is in a healthy state and the other of which is in a diseased state, can be used to create two different proteomes. Alternatively, comparative ABPP can be used to investigate the effects of pharmacological intervention on the activity of the enzyme under investigation.

This information can be used to identify metabolic pathways that are disrupted in disease states and help to develop treatment strategies. Eventually, this could lead to the identification of potential new drug targets. For example, it has been discovered that MAGL is more active in aggressive human cancer cell lines than in non-aggressive human cancer cell lines, resulting in the enzyme being proposed as a potential pharmacological target for cancer therapy (Nomura et al., 2010a, Nomura et al., 2010b). Many biological mechanisms, including host-virus interactions, have benefited from the use of comparative ABPP (Blais et al., 2012, Blais et al., 2010), and in addition to diet-induced obesity (Sadler et al., 2012).

Competitor ABPP experiments using broad-spectrum probes can be used to assess both inhibitor potency and selectivity at the same time (Leung et al., 2003). ABPP efficiently guides the hit and lead optimization processes, resulting in a reduction in the length of time required for drug discovery. Importantly, there is also the possibility of making unintentional discoveries, such as discovering new hits for other enzymes. To label residual enzyme activities, a sample is first treated with an inhibitor and then labelled, as in competitive ABPP. If the compound interacts with a protein, a decrease in the fluorescence intensity of the bands will indicate this. Additionally, competitive ABPP is an excellent method for confirming that an enzyme has engaged its target in a cell- or animal-based model. For example, a fluorescent probe was used to screen many compounds against many enzymes to identify compounds

that were inhibitors of a diverse set of serine hydrolases, such as the fluorophosphonate probe (Bachovchin et al., 2010b).

4.2.3 Drug Discovery Using the ABPP

Understanding the pharmacology of small molecule drug leads requires a thorough understanding of their selectivity characteristics. Small compounds that have therapeutic or investigative potential may be selected by phenotypic screening, but no information is provided on the biological targets that are responsible for the phenotype. The use of ABPP has had a positive impact on drug discovery and assay development, particularly in terms of establishing the selectivity of small molecule leads and the finding of pathways that contribute to off-target pharmacology. ABPP greatly reduces the time it takes to identify pharmaceutical targets by providing clear and unambiguous indications (Lanning et al., 2014) (Bantscheff and Drewes, 2012) in order to select protein targets, it is possible to adopt a competition-based approach (Greenbaum et al., 2002, Leung et al., 2003).

Examples include the usage of lysine-targeting sulfonyl fluoride ABPs, which aided in the identification of off-target proteins targeted by the drug dasatinib, an approved treatment for chronic myeloid leukaemia. This investigation was carried out in living cells with dasatinib at doses that were therapeutically relevant (Zhao et al., 2017). Through high throughput screening for competition for the targeted target, ABPPs can be used to identify novel small molecule leads (Adibekian et al., 2012).

In the previous chapter, the proliferation of 1321N1 cells was investigated using inhibitors of endocannabinoid hydrolases and cannabinoid receptor ligands. Specifically, the inhibitors URB597, JNJ1661010, MAFP, JZL184, and WWL70 were utilised to target endocannabinoid hydrolases, as shown in Figure 3.7. While a clear inhibitory effect was observed with a CB1 cannabinoid receptor antagonist, there were also smaller, but significant, anti-proliferative effects from the inhibitors targeting multiple endocannabinoid hydrolases. In this chapter, the ABPP technique was employed to generate corroborating evidence for the presence of these enzymes. Additionally, the SH-SY5Y cell line was used in parallel with another human cell line of neural origin widely utilised for in vitro investigations.

4.3 Objectives

The objective of this chapter was to use the ABPP technique to define the serine hydrolase activities in the 1321N1 cell line, in comparison with the SH-SY5Y cell line.

4.4 Results and Discussion

4.4.1. Estimation of the molecular weight of unknown proteins

The estimation of the molecular weight of unknown proteins was needed in order to identify individual enzyme activities. Unknown protein samples were compared to reference samples with known molecular weights. Although the MW markers were made up of 10 proteins, only four could be visualised using the fluorescent scanner settings for FP-rhodamine detection. The band intensities were identified, and the distances migrated by the standard proteins described in Table 4.1. The retardation factor (RF) was calculated using the migration distance of the protein/migration distance of the dye front. The origin was identified as the top of the resolving gel. Plotting the data as log MW versus RF allowed calculation of the unknown molecular weights of proteins in the gel using linear regression analysis (Figure 4.2).

Table 4. 1 The molecular weight marker's features used in linear regression analysis for calculating the molecular weight of unknown sample bands.

Label	Log (MW)	Length (cm)	RF value
150 kDa	2.18	3.16	0.190
76 kDa	1.88	5.32	0.320
52 kDa	1.72	7.6	0.457
31 kDa	1.49	11.16	0.671
Solvent Front	-	16.64	1.000

Data are taken from a single example of experiment.

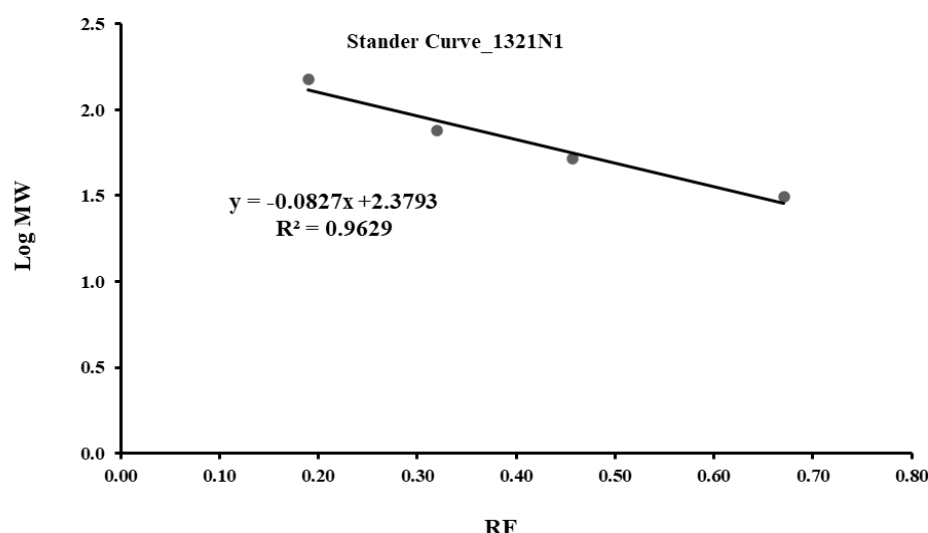


Figure 4. 2 Linear regression analysis of the log MW of molecular weight markers (150, 76, 52, and 31 kDa) versus the Rf values. The equation for the calculated line of best fit is indicated, as is the coefficient of determination.

4.4.2 Profiling membrane-bound and cytosolic serine hydrolases in 1321N1 cells

1321N1 samples of cell soluble and particulate fractions were extracted and prepared for ABPP analysis. A total of 14 bands were detected by visual inspection of gels using preparations from 1321N1 cell samples reacting with TAMRA-FP, with their molecular weights ranging from ≈ 20 kDa to ≈ 208 kDa as determined by the line of best fit Figure 4.3.

To attempt to identify endocannabinoid hydrolases among the serine hydrolase activities in the 1321N1 human astrocytoma cell line, a competitive ABPP experiment was conducted utilising the inhibitors URB597, JNJ1661010, MAFP, JZL184, and WWL70 at a final concentration of $1 \mu\text{M}$ for 30 min at 37°C (Figure 1). The membrane and soluble proteomes from samples were then treated with FP-rhodamine (500 nM, 30 min, 37°C , 2% v/v DMSO) and resolved on 10 % SDS-PAGE gels, followed by an analysis of the in-gel fluorescence. 1321N1 cells were used with FP-rhodamine, which revealed eleven distinct bands on the membrane and ten distinct bands in the cytoplasm (Figure 4.3).

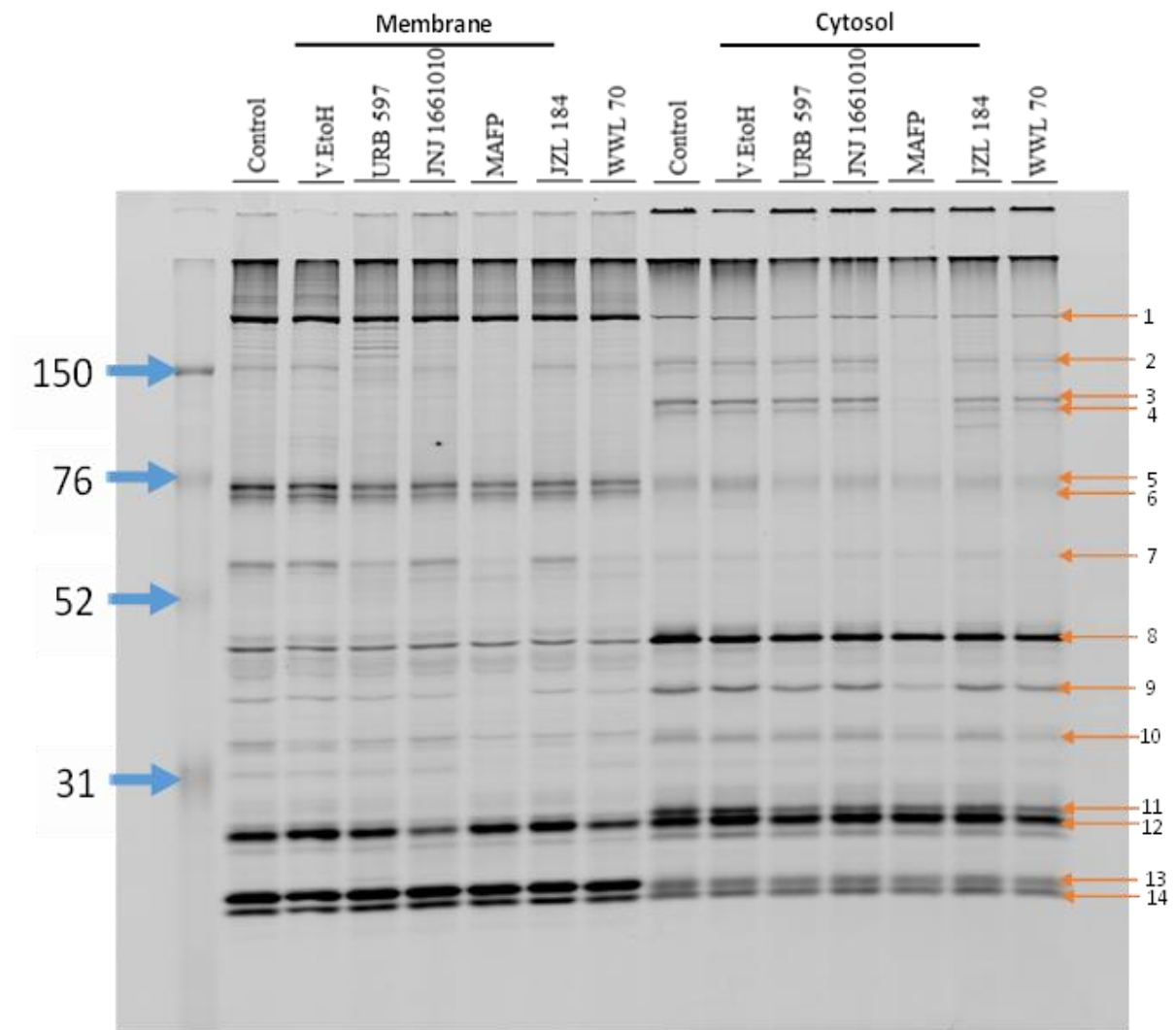


Figure 4. 3 Labelling of 1321N1cell membrane and cytosol fractions by FP-rhodamine in untreated samples and in those treated with Ethanol (Etoh) as a vehicle control of 1 μ M of URB597, JNJ1661010, MAFP JZL184 or WWL70. The gel represents a single experiment.

Table 4. 2 Serine hydrolase activities in cytosol and membrane fractions from 1231N1 cells.

Cytosolic activity (% membrane activity)					
Band	MW (kDa)	Control	EtOH control	Cell fraction*	Inhibitors
1	208	12%	11%	Membrane	
2	156	97%	76%	Both	MAFP
3	126	12814%	10917%	Cytosol	
4	114	1974%	1556%	Cytosol	
5	74	19%	22%	Membrane	
6	72	43%	34%	Membrane	
7	60	21%	29%	Membrane	URB597, JNJ1661010,MAFP,WWL70
8	45	310%	274%	Cytosol	
9	39	288%	230%	Cytosol	WWL70, MAFP
10	34	125%	96%	Both	JZL184, MAFP
11	27	2407%	4114%	Cytosol	
12	26	105%	96%	Both	JNJ1661010?
13	22	21%	37%	Membrane	
14	20	66%	72%	Both	

*A two-fold difference in enzyme activity was used as an arbitrary cutoff to identify cytosol vs membrane expression.

Activity-based profiling of the membrane and cytosolic fractions of 1231N1 cells treated with the inhibitors of the major enzymes involved in endocannabinoid biotransformation for 30 min at 1 μ M concentration revealed that the bands 2, 10, 12, and 14 were found in both cell fractions, namely, membrane and cytosol. The Bands 1, 5, 6, 7 and 13 were predominantly active in the membrane fraction while 3, 4, 8, 9 and 11 were predominantly active in the cytosolic fraction. Several bands namely, 1, 8, 11, 13 and 14 were not inhibited by any of the enzyme inhibitors utilised in this experiment including MAFP which raise the possibility of being non-serine hydrolase enzymes expressed by 1231N1 cells. URB597 inhibited bands 2, 3 and 5 while JNJ1661010 inhibited bands 5 and 12. The non-selective serine hydrolase inhibitor MAFP inhibited bands 2, 3, 4, 5, 6, 7, 9 and 10. Also JZL184

inhibited bands 5 and 6 while WWL70 inhibited bands 2, 5 and 7. Interestingly band 5 was observed to be inhibited by all enzyme inhibitors tested in this experiment.

4.4.3 Profiling membrane-bound Serine Hydrolase in differentiated and undifferentiated SH-SY5Y Human Neuroblastoma Cells

Membrane fractions from undifferentiated and differentiated SH-SY5Y cells were assessed for serine hydrolase activities (Figure 4.4) in the absence and presence of the same inhibitors as investigated in 1321N1 cells (Figure 4.3). A total of 11 bands were detected by visual inspection of gels using preparations from undifferentiated and differentiated SH-SY5Y membrane preparations reacting with TAMRA-FP, with their molecular weights ranging from ≈ 20 kDa to ≈ 203 kDa as determined by the line of best fit (Figure 4.1).

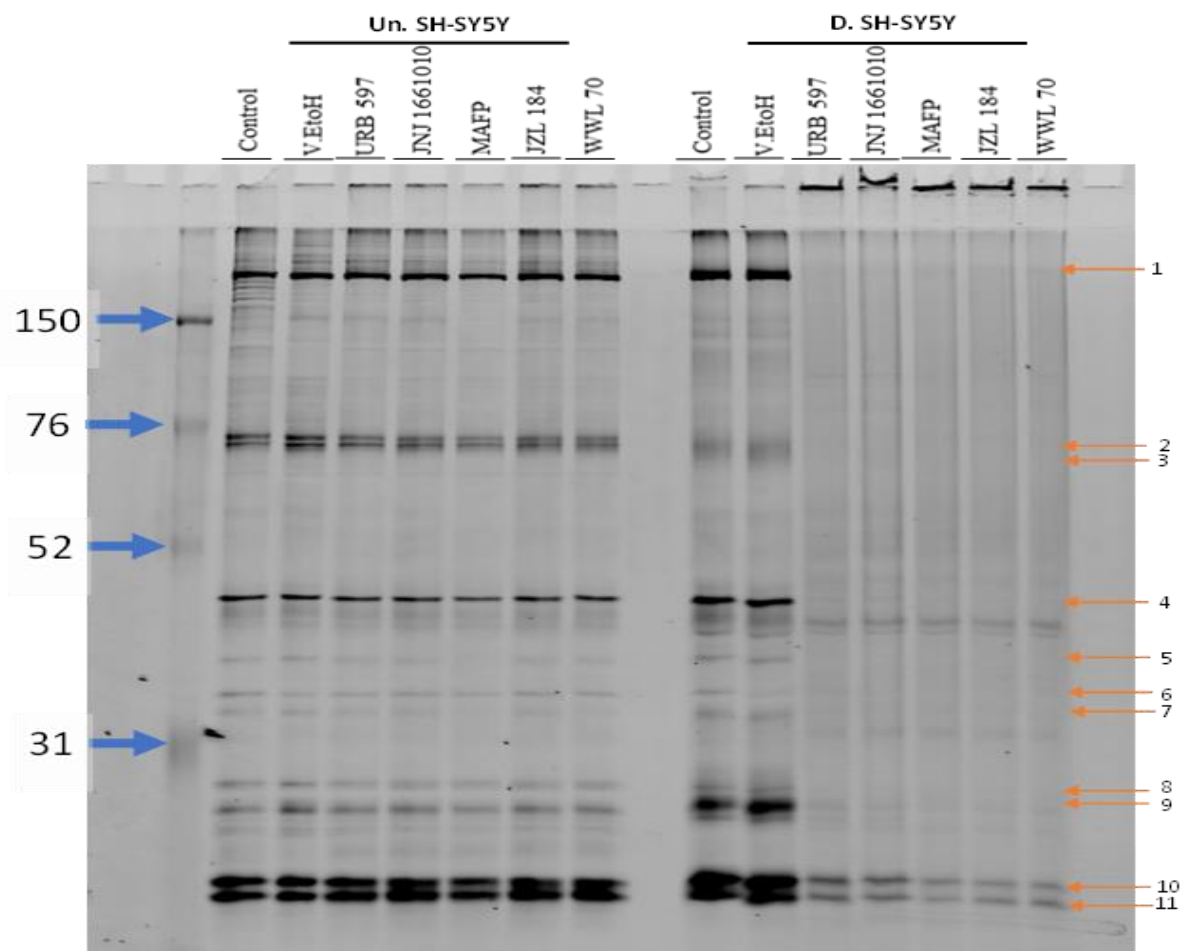


Figure 4. 4 Labelling of SH-SY5Y membranes from undifferentiated and differentiated cell by FP-rhodamine in untreated samples and in those treated with Ethanol (EtoH) as a vehicle

control of 1 μ M of URB597, JNJ1661010, MAFP JZL184 or WWL70. The gel represents a single experiment.

Table 4. 3 Serine hydrolase activities in membrane fractions from undifferentiated and differentiated SH-SY5Y cells.

Influence of differentiation					
Band	MW (kDa)	Control	EtOH control	Summary*	Inhibitors
1	203	124%	96%		
2	73	3%	3%	Decreased	JZL184, WWL70
3	71	3%	5%	Decreased	JZL184, WWL70
4	46	135%	172%	Increased	
5	39	130%	145%		MAFP
6	35	67%	63%		
7	33	132%	131%		MAFP, WWL70
8	28	51%	38%	Decreased	
9	26	151%	154%	Increased	
10	21	97%	151%		
11	20	73%	82%		

*A two-fold difference in enzyme activity was used as an arbitrary cutoff to identify the influence of differentiation

Activity based profiling of the membrane fractions of the differentiated and undifferentiated SHSY5Y cells treated with the inhibitors of the major enzymes involved in endocannabinoids biotransformation for 30 min at 1 μ M concentration revealed that the bands 1, 5, 6, 7 and 11 were unchanged following differentiation of SH-SY5Y cells. However, the differentiation of SHSY5Y cells caused expression of some bands to be increased, namely 4, 9 and 10, while other bands were shown to be decreased including 2, 3 and 8. Several bands, namely, 1, 4, 6, 8, 9, 10 and 11, were not inhibited by any of the enzyme inhibitors utilised in this experiment including MAFP. The irreversible and reversible FAAH inhibitors, URB597 and JNJ1661010 did not elicit inhibition at all bands revealed in this experiment. The non-selective serine hydrolase inhibitor MAFP inhibited bands, 5 and 7. While JZL184 inhibited bands 2 and 3 and WWL70 inhibited bands 2, 3 and 7.

Unexpectedly the differentiated SHSY5Y cells showed an unexpected pattern of protein profiling with bands barely observed across the gel in marked contrast to the vehicle control. However, this result needs to be further investigated to decide whether the differentiation of SH-SY5Y cells interfered with the protein profiling using FP-rhodamine probe.

4.4.4 Profiling of cytosolic Serine Hydrolase in differentiated and undifferentiated SH-SY5Y Human Neuroblastoma Cells

Cytosolic fractions from undifferentiated and differentiated SH-SY5Y cells were assessed for serine hydrolase activities (Figure 4.5) in the absence and presence of the same inhibitors as investigated in 1321N1 cells and membrane fractions from SH-SY5Y cells. This study detected 15 distinct bands in cytosol fractions of undifferentiated (Un.SH-Sy5y) and differentiated (D.SH-Sy5y) SH-SY5Y Human Neuroblastoma cell line samples, with molecular weights ranging from 18 kDa to 203 kDa

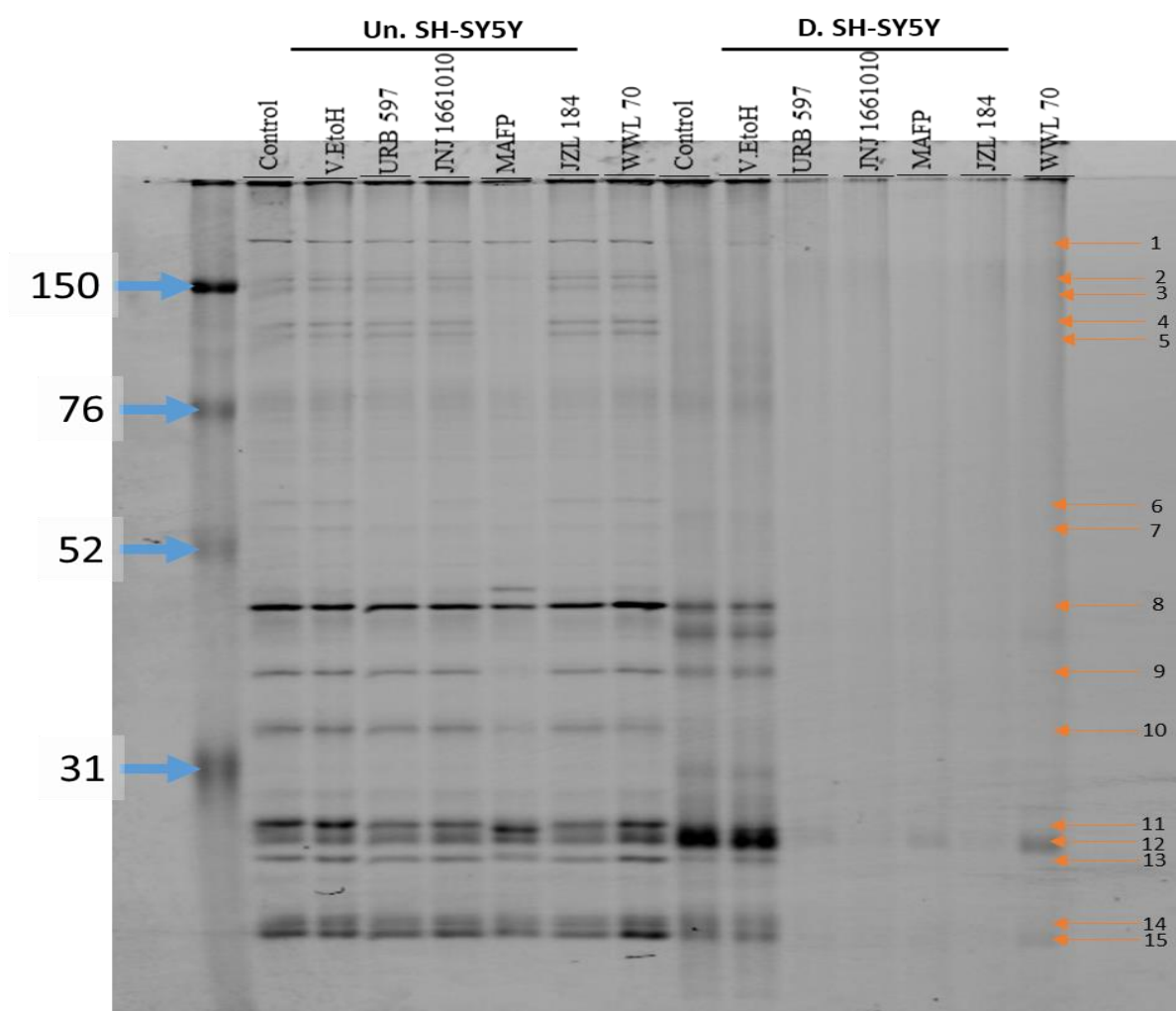


Figure 4. 5 Labelling of cytosol fractions from undifferentiated and differentiated SH-SY5Y cells by FP-rhodamine in untreated samples and in those treated with Ethanol (Etoh) as a vehicle control of 1 μ M of URB597, JNJ1661010, MAFP JZL184 or WWL70. The gel represents a single experiment.

Table 4. 4 Serine hydrolase activities in cytosolic fractions from undifferentiated and differentiated SH-SY5Y cells.

Influence of differentiation					
Band	MW (kDa)	Control	EtOH control	Summary*	Inhibitors
1	203	19%	15%	Decreased	
2	145	41%	26%	Decreased	MAFP
3	137	24%	18%	Decreased	MAFP
4	118	19%	31%	Decreased	MAFP
5	109	34%	23%	Decreased	MAFP
6	60	40%	39%	Decreased	MAFP, URB597
7	56	39%	46%	Decreased	
8	45	37%	54%	Decreased	
8a				Increased	
9	38	66%	72%		MAFP
10	33	9%	18%	Decreased	MAFP
11	24	45%	48%	Decreased	
12	23	448%	514%	Increased	URB597
13	22	44%	63%		
13	19	54%	49%	Decreased	
14	18	53%	75%		

*A two-fold difference in enzyme activity was used as an arbitrary cutoff to identify the influence of differentiation.

Activity based profiling of the cytosolic fractions of the differentiated and undifferentiated SHSY5Y cells treated with the inhibitors of the major enzymes involved in endocannabinoids biotransformation for 30 min at 1 μ M concentration revealed that the bands 9, 13 and 15 were unchanged following differentiation of SH-SY5Y cells. However, the differentiation of SHSY5Y cells caused activity of some bands to be increased, namely 8a and 12, while other bands were shown to be decreased, including 1, 2, 3, 4, 5, 6, 7, 8, 10, 11 and

14. Several bands, namely, 1, 7, 8, 8a, 11, 13, 14 and 15, were not inhibited by any of the enzyme inhibitors utilised in this experiment including MAFP. The irreversible and reversible FAAH inhibitors, JZL184, JNJ1661010 and WWL70 did not elicit inhibition of any bands in this experiment. The non-selective serine hydrolase inhibitor MAFP inhibited bands, 2, 3, 4, 5, 6, 9 and 10. While URB597 inhibited bands 6 and 12. Unexpectedly the differentiated SHSY5Y cells showed an unexpected pattern of protein profiling, identical to that observed with the membrane fractions from these cells.

4.5 Discussion

The endocannabinoid system (ECS) is principally made up of endocannabinoid receptors, endocannabinoids, and the enzymes responsible of generation and degradation of the endocannabinoid compounds. AEA and 2-AG are the most important and well-identified endogenous cannabinoids. It has been observed that the ECS plays a key role in a myriad of physiological and pathological processes including modulation of pain responses.

At the moment, it is known that endocannabinoids have a part in the pathophysiology of a variety of disorders and also act as a "protective factor" in a variety of medical situations. Numerous diseases, including pain, inflammation, epilepsy, and joint inflammation, may be treated with drugs that modulate the endocannabinoid system (Kaur et al., 2016). Research showed that In rodent models of arthritis, endocannabinoids have been shown to inhibit the progression of joint inflammation. Specifically, the anti-inflammatory potential of CB2 receptors has been confirmed in mouse models of arthritis. The protective effects of CB2 receptors include the suppression of pro-inflammatory cytokines and damaging proteinases secretion, as well as the regulation of immune cell adhesion and migration to inflamed joints. These actions collectively slow the perpetuation of disease and alleviate associated arthritic pain primarily caused by localized inflammation. Moreover, elevated levels of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) have been detected in the synovial fluid of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) but are absent in healthy controls. This suggests that local secretion of endocannabinoids may contribute to minimizing inflammation in arthritic joints (Ware et al., 2005; Malfait et al., 2000).

By employing activity-based protein profiling, this work intended to discover and characterise endocannabinoid hydrolases in the cell lines under this study pain pathway. To characterize the activity and selectivity of our work on serine hydrolase in human astrocytoma and neuroblastoma cell lines, we employed competitive activity-based protein profiling (ABPP), which is a functional proteomics technique for directly monitoring the expression of active enzymes in cell extracts and living cells. The technique relies on irreversible inhibitors provided with reactive groups (warheads) that covalently attach to the active site of enzymes and

fluorescent or affinity tags for imaging and purification purposes, respectively. It is unique in its ability to rapidly identify inhibitor activity and selectivity (van Rooden et al., 2018).

In this study, two types of cell lines samples from the human astrocytoma cell line (1321N1) and human neuroblastoma (SH-SY5Y) were tested by two separate sets of experiments. Given the essential function of astrocytes, the most common type of glial cell in the CNS, in protecting the CNS from infectious agents such as viruses and bacteria (Mucke and Eddleston, 1993), we selected the 1321N1 astrocytoma cell line as our in vitro model in this investigation.

It has been observed from studying the effects of enzyme inhibitors on the proliferation of 1321N1 cell line that MAFP, JZL184, and WWL70 at a concentration of 1 μ M caused significant reductions in cell viability with MAFP (see Fig 3.7. B in chapter 3). Interestingly these very same inhibitors at same concentration provoke a significant inhibition of several bands such that MAFP inhibited bands 2, 3, 4, 5, 6, 7, 9 and 10. JZL184 inhibited bands 5 and 6 meanwhile WWL70 inhibited bands 2, 5 and 7. This result indicated that some of the serine hydrolases (band 2, 5, 6, and 7) that are involved in cannabinoid turn over may play a role in protecting of human astrocytoma cells from apoptosis.

With SH-SY5Y cells, MAGL would be expected at ~33 kDa as a doublet, and to be in membranes and cytosol, and to be inhibited by JZL184 and MAFP, but not WWL70 or URB597. ABHD6 would be expected at ~39 kDa in membranes, and to be inhibited by WWL70 and MAFP, but not JZL184 or URB597. FAAH would be expected at ~60 kDa in membranes, and to be inhibited by URB597 and MAFP, but not WWL70 or JZL184. The off-target effects of URB597, JZL184 and WWL70 raise the question about the selectivity of these enzyme inhibitors. The absence of expression of MAG lipase corroborate the data reported by Szeremeta J. *et al.*, 2019 suggesting low MAGL expression in SH-SY5Y cells which should be taken into account when using these cells in experiments investigating the involvement of the endocannabinoid system in physiological and pathological processes.

In addition, we chose the SH-SY5Y neuroblastoma cell line as our in vitro second model in this experiment. SH-SY5Y is a well-documented in vitro model for the investigation of neuronal

biochemistry and pharmacology, as well as pathological states in healthy individuals (Kovalevich and Langford, 2013, Xicoy et al., 2017).

Cells from the SH-SY5Y strain produce dopamine-hydroxylase, choline acetyltransferase, acetylcholinesterase, and butyrylcholinesterase, all of which are neuronal enzymes (Biedler et al., 1978). In SH-SY5Y cells, mRNA and protein for the endocannabinoid system, including NAPE-PLD, FAAH, DAGL, and MAGL, are detectable (Pasquariello et al., 2009). CB1 or CB2 receptors have also been reported to be expressed in SH-SY5Y neuroblastoma cells (Lyons et al., 2020).

4.5.1 Effects of a panel of cannabinoid-metabolising enzyme inhibitors on protein profiling of human astrocytoma 1321N1 cells.

To determine their activity and selectivity profiles in the 1321N1 cell line, we performed a competitive ABPP experiment by investigating a set of inhibitors of various enzymes involved in endocannabinoid metabolism and biotransformation for influence on the protein profiling of human astrocytoma 1321N1 cells. Namely, this group included URB597, which selectively inhibits FAAH, JNJ1661010, which selectively and reversibly inhibits FAAH, and MAFP, which is a potent irreversible inhibitor of serine hydrolases and serine proteases, JZL184, which potently and selectively inhibits MAG Lipase, and WWL70, which reversibly inhibits ABHD6. The proteomes of the membrane and cytosolic compartment were loaded into wells labelled with the indicated inhibitors (Figure 4.3). The lane on the far left is the molecular weight marker lane, which has four bands and represents the molecular weights of 150 kDa, 76 kDa, 52 kDa, and 31 kDa. A sample without additives was used as a positive control and loaded into the next well labelled as "Control", and 0.1% (v/v) ethanol was used as vehicle control. 14 bands from each lane were identified and quantified. It was shown in (Figure 4.3) that the intensity and migration distance of each band in each sample were measured in the same way.

The protein profiling of both membrane and cytosolic fractions samples appears to be very similar at the level of bands 2 (156kDa), 10 (34 kDa), and 12 (26 kDa), with distinctive responses to MAFP at the level of band 2 and band 10 in either set of samples (Table 4.2).

Band intensities of the membrane samples set at the level of bands 1 (208kDa), 5 (74kDa), 6 (72kDa), 7 (60kDa), 13 (22kDa), and 14 (20kDa) were much higher than the corresponding bands of the cytosol samples group. Interestingly, URB597, JNJ1661010, MAFP, and WWL70 elicited a remarkable inhibition of the band intensity at the level of band 7 (60 kDa), suggesting the expression of membrane-bound FAAH (Table 4.2).

On the other hand, band intensities of the cytosolic fraction samples set at the level of bands 3 (126kDa), 4 (114kDa), 8 (45kDa), 9 (39kDa), 11 (27kDa), and 14 (20kDa) were much higher than the corresponding bands of the membrane samples group (Table 4.2).

4.5.2 Effects of a panel of cannabinoid-metabolising enzyme inhibitors on protein profiling of human neuroblastoma cells

4.5.2.1 Membrane

To identify the membrane-bound enzymes that have the potential to be involved in cannabinoid biotransformation, the same set of cannabinoid-metabolising enzyme inhibitors tested in the previous experiment, namely, URB597, JNJ1661010, MAFP, JZL184, WWL70 were investigated for their influence on protein profiling of differentiated and undifferentiated human neuroblastoma cells.

The proteomes of the membranes of the differentiated and undifferentiated phenotypes of human neuroblastoma SHSY5Y cells were loaded to wells labelled with indicated inhibitors (Figure 4.4). The lane on the far left is the molecular weight marker lane, which has four bands and represents the molecular weight of 150, 76, 52, and 31 kDa. The sample without additives was used as a positive control and loaded in the next well labelled as "Control" and 0.1% (v/v) Ethanol was used as vehicle control. 11 bands from each lane were identified and quantified. The bands in each sample were measured in terms of intensity and migration distance as described in (Figure 4.4).

When looking at bands 1 (208kDa), 2 (73kDa), 3 (71kDa), 4 (46kDa), 10 (21kDa), and 11 (20kDa) (Table 4.3), band intensities appeared to be higher on the left side of the gel (Undifferentiated) as compared to the right (Differentiated). This trend was observed in almost

all the bands. Suspected bands that were ambiguous and/or unclear were not quantified (e.g., possible band between bands 1 & 5 and between 5 & 10).

The ABPP system did not work consistently with SH-SY5Y cells, particularly the differentiated samples.

4.5.2.2 Cytosol

To identify the cytosolic enzymes that have the potential to be involved in cannabinoids biotransformation, the same set of cannabinoid-metabolising enzyme inhibitors tested in the previous experiment, namely, URB597, JNJ1661010, MAFP, JZL184, WWL70 were investigated for influence on the protein profiling of the differentiated and undifferentiated human glioblastoma cells.

The proteomes of the cytosolic fractions of the differentiated and undifferentiated phenotypes of human glioblastoma SHSY5Y cells were loaded to wells labelled with indicated inhibitors (Figure 4.5). The bands in each sample were measured in terms of intensity and migration distance as described in (Figure 4.5).

The general appearance of the ABPP screen reveals that the band intensities on the left side of the gel (Undifferentiated) were higher as compared to the right side (Differentiated). High band intensities were observed for bands 1 (203 kDa), 8 (45 kDa), 9 (38 kDa), 10 (33 kDa), 11 (24 kDa), 12 (23 kDa), 13(22 kDa), 14 (19 kDa), and 15 (18 kDa) with consistent response to MAFP as compared to the corresponding positive controls (Table 4.4).

URB597 and JNJ1661010 elicited a notable inhibition of band 6 (60 kDa) suggesting the expression of a cytosolic cannabinoid-metabolising enzyme.

Although we observed consistent equal distribution and expression in the membrane and cytosol of Un.SH-Sy5Y cells, just as we found with 1321N1 cells. What was most unusual was what we observed in the membrane and cytosol of D.SH-SY5Y cells, where activity was apparent in the control and vehicle-EtOH lanes, but it was completely absent in all the lanes of the enzyme

inhibitor compounds used in the study. This is something that needs to be looked at more (Figure 4.4 and 4.5).

It has been reported that a number of serine hydrolase activities, including FAAH, KIAA1363, MAGL, ABHD6, and ABHD12, have been shown in previous studies to be inhibited by the compounds MAFP (Hoover et al., 2008).

In addition, it has been revealed that URB597, the prototype of a novel family of O-arylcarbamate FAAH inhibitors, raises AEA levels in the rat and mouse brain and amplifies the effects of this endocannabinoid ligand through selective FAAH inhibition. URB597 administration increases AEA content in the brains of wild-type mice but does not boost the already high AEA levels in the brains of FAAH-null animals (Fegley et al., 2005) and support the conclusion that this enzyme represents the predominant catabolic route for AEA in the central nervous system (Cravatt et al., 2001).

It has been reported that WWL70 is a highly selective ABHD6 inhibitor (Li et al., 2007). Treatment with an ABHD6 inhibitor by WWL70 enhanced brain 2-AG levels and improved clinical symptoms of encephalomyelitis, T cell infiltration, microglia activation, and the production of activated leukocyte cell adhesion markers. The neuroprotective impact of WWL70 was established by enhanced oligodendrocyte survival, decreased demyelination, and axonal loss in the treated EAE-treated mice spinal cord. These findings imply that inhibition might be an effective therapy method for MS and other neurodegenerative illnesses (Wen et al., 2015).

It has been stated that JZL184 is a highly selective MAGL inhibitor that raises 2-AG but not AEA levels in the mouse brain (Long et al., 2009b).

While the data discussed above were informative and demonstrated some interesting differences, they were only presented one gels from a single repetition which was not ideal for any statistical analysis. Providing more replicates for these gels were not possible due to COVID19 constraints. Therefore, it is recommended to generate at least three replicate of each one of these gels in order to perform statistical analyses for these experiments and provide meaningful results that can be tested for variability and increase the producibility of the data.

4.6 Conclusion

We have employed an ABPP method that can be used to estimate the activity of multiple enzymes with endocannabinoid hydrolase activity in two cell lines, 1321N1 cell and SH-SY5Y, from humans.

In astrocytoma cells, the ABPP assay demonstrated a high degree of overlap between the membrane and soluble serine hydrolase contents. However, competitive ABPP was used to infer the identity of FAAH and other serine hydrolases. We can conclude there is a different profile in SH-SY5Y cells. This was observed regarding the number of bands identified in each model. Eleven intense bands were detected in the membrane and ten in the cytosol of 1321N1 cells, while 11 bands were observed at the membrane and 15 in the cytosol of SH-SY5Y cells. Additionally, distinct bands appeared in each model. For instance, ACOT1, ABHD12, and LYPLA1 were observed in the cytosol of 1321N1 cells, while RBBP9 was detected in the membrane of SH-SY5Y cells.

CHAPTER 5

Serine hydrolases in the rat brain determined using the ABPP technique.

5.1 Introduction

The mammalian CNS has a unique lipid composition in comparison to other organs and tissues. Numerous lipid-metabolising enzymes, which are abundantly expressed in the CNS, control and maintain this distinctive lipid composition. One such superfamily is serine hydrolases, an enzyme class that possesses the α/β hydrolase motif and a nucleophilic serine residue embedded in a Ser-His-Asp or Ser-Ser-Lys catalytic triad to facilitate the cleavage of ester, amide, or thioester bonds of protein, peptide, and small molecule substrates (Long and Cravatt, 2011). More than 200 enzymes belonging to the serine hydrolase family are categorised as metabolic serine hydrolases, whose substrates are often small molecules, including lipids. Several serine hydrolases are associated with neurologic and psychiatric diseases (Bachovchin and Cravatt, 2012). Consequently, these enzymes and their metabolic substrates or products play crucial roles in the normal activities and development of the CNS of mammals. There are various methodologies used to measure endocannabinoid-metabolising enzyme activities. These can be classified into the conventional methods and, more recently, ABPP. The conventional methods include continuous assays and discontinuous assays. Continuous assays are distinguished by their capacity to continually monitor the disappearance or appearance of a specific substrate or product, and they often depend on spectroscopic methods such as electronic ultraviolet–visible absorption and fluorescence emission (Harris and Keshwani, 2009). In the discontinuous assays, it is not possible to continuously and selectively monitor changes in the concentration of the substrate. To perform this assay, two key steps need to be conducted. Firstly, the enzymatic reaction needs to be stopped at predetermined times. This is essential to halt the progress of the reaction and prevent further substrate conversion into product. Once the reaction is stopped, it is important to efficiently separate the quantity of unreacted substrate from the reaction

mixture. This step is required to accurately measure the amount of substrate that has been converted into product during the enzymatic reaction. Following isolating the unreacted substrate, researchers can focus on analysing the concentration of the product, which provides insights into the enzyme's activity (McMaster, 2007).

ABPP is an adaptable chemo-proteomic technique in drug discovery and development. It is used to report the activity of specific enzymes or the reactivity of amino acid types in complex biological systems. It can be used for the identification of novel therapeutic targets and the development of many compounds in the treatment of human and infectious illnesses (Evans and Cravatt, 2006). In addition, this technique can be used to emphasize the druggable proteome; there has been a substantial rise in the variety of target classes that can be targeted using activity-based probes in recent years, indicating the wide range of applications for this technique in particular for determining of endocannabinoid-metabolising enzyme activities in brain samples (Janssen et al., 2018, Anajirih et al., 2023a). One common way to investigate the role of drug targets in CNS function is to divide the brain into regions. In particular, a classical tissue dissection method from the 1960s (Glowinski, 1966) has often been applied to link drug targets with particular neurotransmitters and/or neuronal pathways.

5.2 Hypothesis:

The hypothesis to be tested is that serine hydrolase activities in rat cerebral cortex would be differentially changed in the presence of various serine hydrolase inhibitors, which may be used to identify the bands on ABPP gel. In addition, the variation in individual enzyme activities in particular brain areas should reflect their association to particular functions (e.g., high activities in the hippocampus might predict an association with the acquisition of spatial memories).

5.3 Objectives:

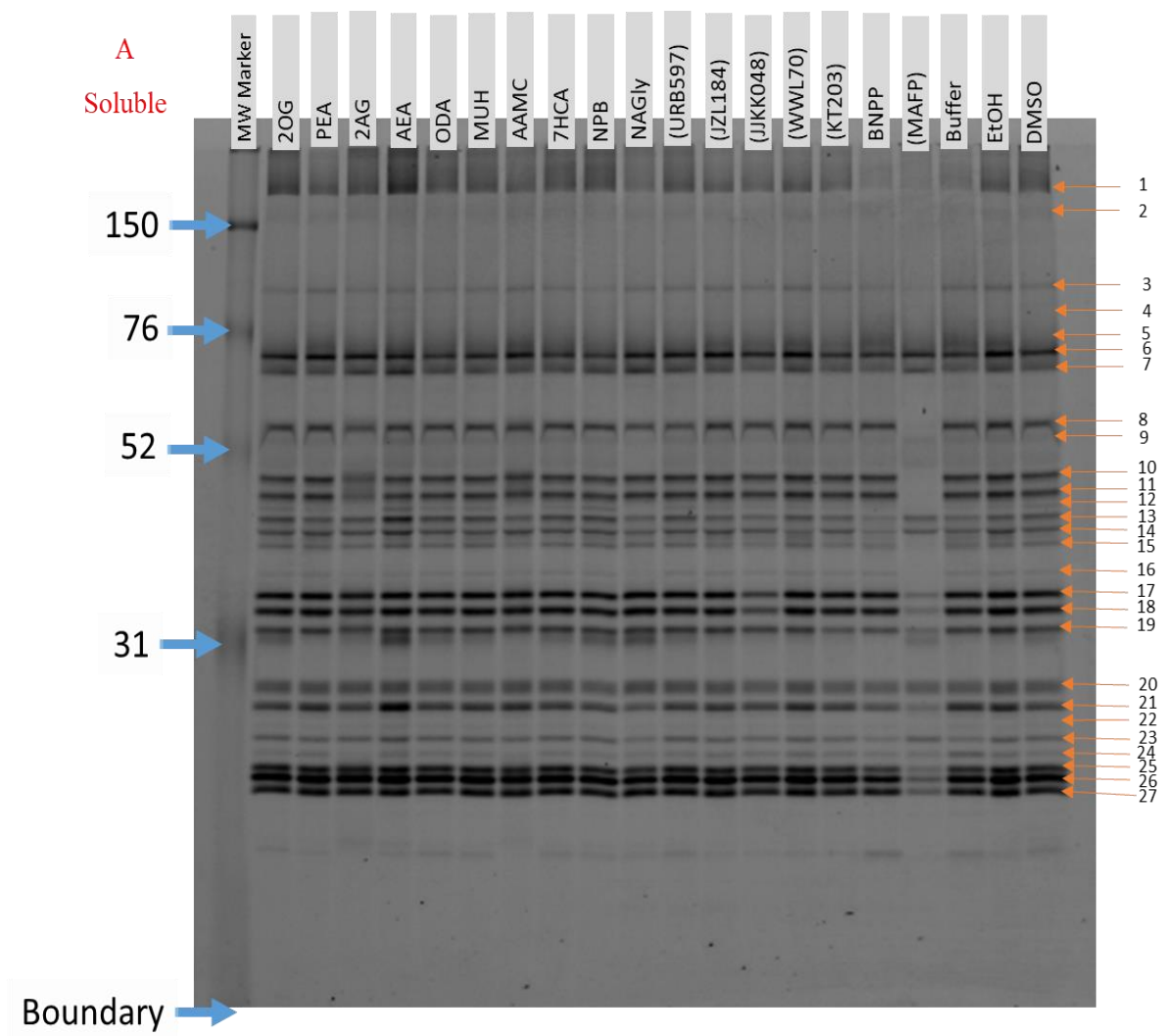
The primary objective of this study, therefore, is to analyse the pattern of serine hydrolase activities in the eight regions of the CNS described by Glowinski & Iversen and suggest their links with specific brain region functions.

5.4 Results

5.4.1 Rat cerebral cortex soluble and particulate serine hydrolases

Following homogenisation and centrifugation of preparations of rat cerebral cortex, serine hydrolase activities were estimated using FP-rhodamine exposure, gel electrophoresis and quantification of the fluorescent band densities (Figure 5.1A-B).

Any difference described in this chapter was statistically significant with a P value of <0.01 (two-way ANOVA with post-hoc Dunnett's multiple comparison test, see Section 2.4.5).



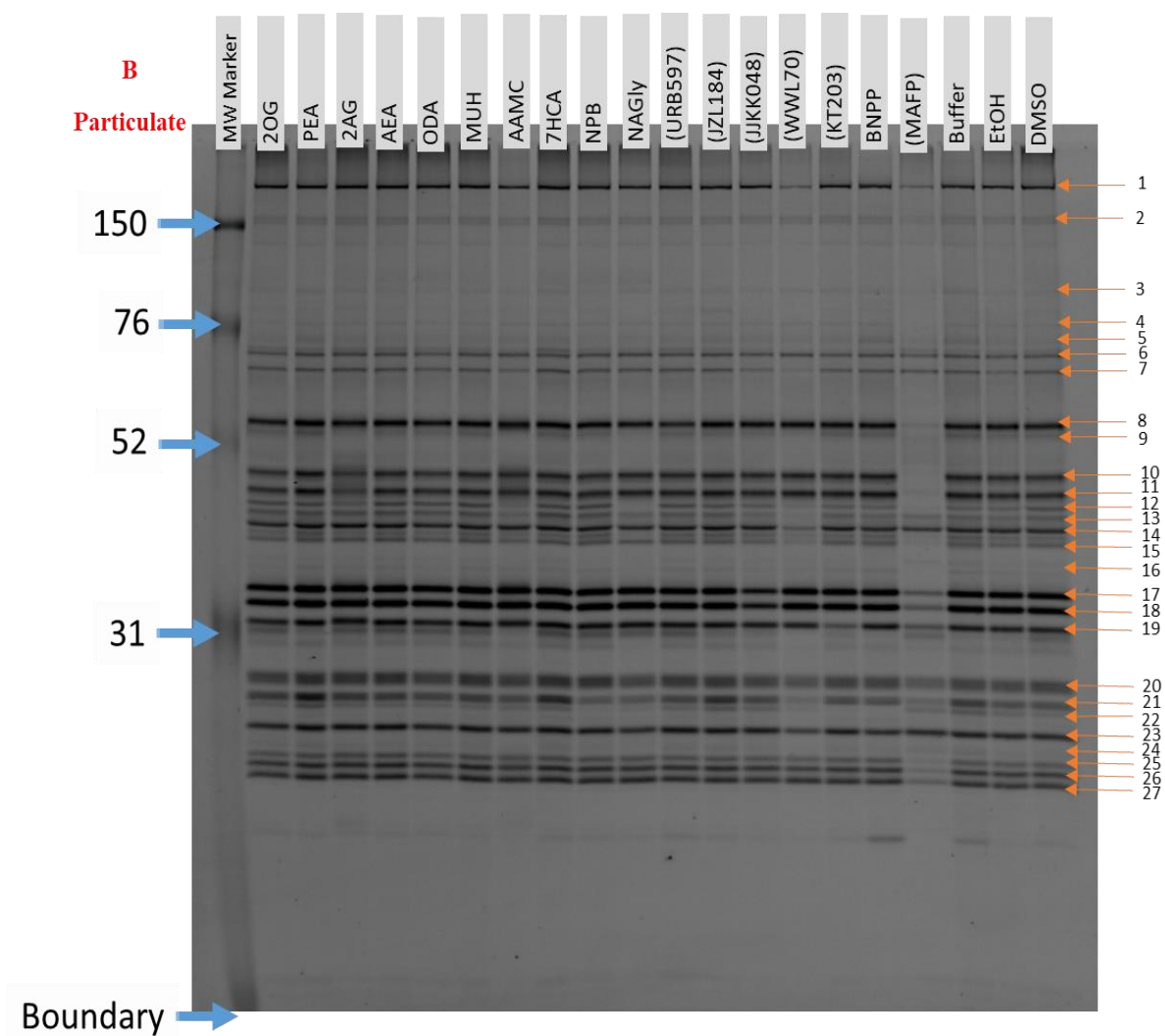


Figure 5. 1 Representative gel scans of a soluble (A) and particulate (B) fraction of rat cerebral cortex in the absence and presence of various inhibitors or alternative substrates and their vehicles. Indicated on the left-hand lane are the molecular weight markers, while the bands quantified are indicated numerically. The gels are from a single animal; tissues from five other animals were processed in an identical manner. Note that the inhibitor compounds are identified in brackets. Statistical analysis was conducted as reported in Section 2.4.5.

The bands shown on the gels in Figure 5.1 were obtained from six independent cerebral cortex samples. The intensities of these bands were analysed using densitometry and plotted in Figure 5.2. The band intensities were compared between the soluble and particulate fractions of the cortex samples.

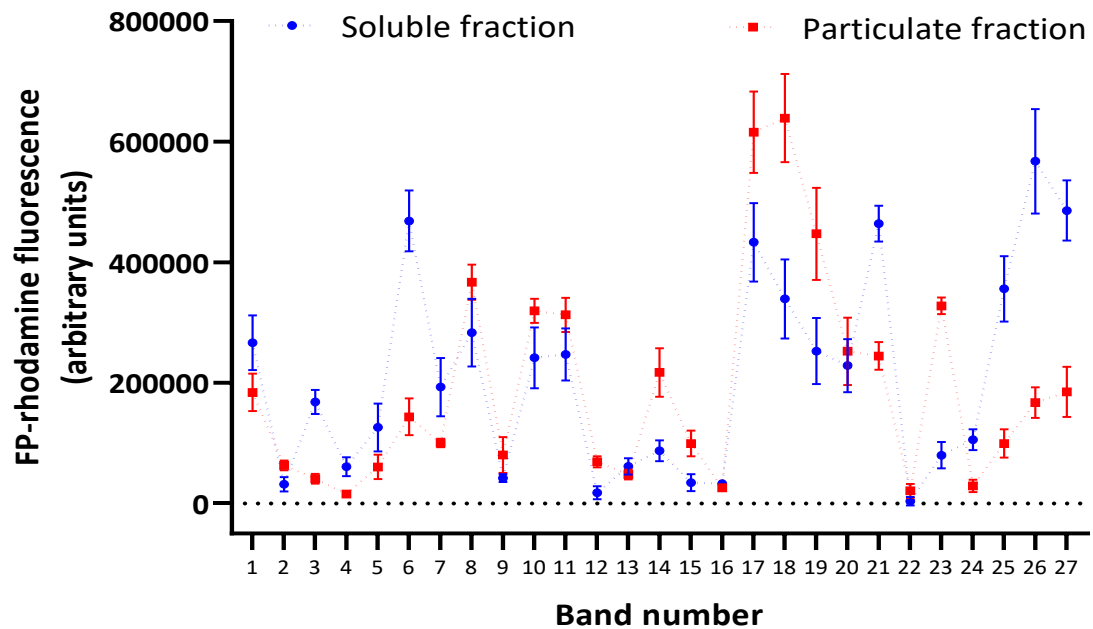


Figure 5. 2 Quantitation of band densities from ABPP gel scans of rat brain preparations. Data are means $\pm$ SEM of six independent cerebral cortex samples, expressed as the band densities (arbitrary units). These data were used to construct Table 1, where, based on an arbitrary three-fold ratio, an indication of the soluble vs particulate location was assigned.

Table 5.1 Estimation of molecular size and preliminary identification of subcellular location of individual enzyme activities.

Band	Mean travel (cm)	RF	Log MW	MW (kDa)	Cellular fraction
1	0.61	0.039	2.31	203	
2	1.22	0.078	2.21	161	
3	2.58	0.167	1.98	96	Soluble
4	3.19	0.206	1.88	76	Soluble
5	3.50	0.226	1.85	71	
6	3.69	0.238	1.84	69	Soluble
7	3.99	0.257	1.82	65	
8	4.90	0.316	1.75	56	
9	5.09	0.328	1.73	54	
10	5.85	0.377	1.68	48	
11	6.08	0.392	1.66	46	
12	6.42	0.414	1.64	43	Particulate
13	6.57	0.424	1.63	42	
14	6.80	0.439	1.61	41	
15	7.07	0.456	1.59	39	
16	7.37	0.476	1.57	37	
17	7.83	0.505	1.53	34	
18	8.06	0.520	1.52	33	
19	8.59	0.554	1.48	30	
20	9.46	0.610	1.42	26	
21	9.73	0.627	1.40	25	
22	9.96	0.642	1.33	21	Particulate
23	10.38	0.669	1.29	19	Particulate
24	10.64	0.686	1.27	18.7	Soluble
25	10.83	0.699	1.26	18.0	Soluble
26	11.10	0.716	1.23	17.1	Soluble

^abased on an arbitrary three-fold ratio, an indication of the soluble vs particulate fraction was assigned. When not indicated, the band appeared roughly equally active in both fractions.

The Rf of each band was measured by detecting migration distance (cm) from the top of the resolving gel to the peak of each band, then the ratio comparing the distance from the top of the resolving gel to the dye front (or more frequently, the bottom of the resolving gel). This Rf value was then used to calculate the unknown molecular weights of each band using the molecular weight markers to generate a standard curve. The molecular weight of each band showing on the gels in figure were illustrated in Table 5.1. The bands represent the activity of serine hydrolases in soluble and particulate rat cerebral cortex.

Table 5.1 identifies the molecular weight of the 27 bands from rat brain preparations seen in gels (Figure 5.1). All bands were detected in both fractions. However, a number of the bands appeared to be predominantly expressed in either particulate or soluble fractions of the cerebral cortex. Based on an arbitrary three-fold ratio, rat brain bands 3, 4, 6, 24, 25 and 26 appeared to be soluble activities, while bands 12, 22 and 23 appeared to be particulate activities (Figure 5.1).

5.4.2 Effect of solvent vehicles on rat cerebral cortex serine hydrolases

Preparations of soluble and particulate fractions from the rat cerebral cortex were investigated for FP-rhodamine-tagging activity in the presence of particular enzyme inhibitors and alternative substrates (Figure 5.1). Given the need for organic solvents in the dissolution of these agents, the first investigation identified whether the vehicles (DMSO or ethanol) influenced the intensity of labelling (Figures 5.1 and 5.3).

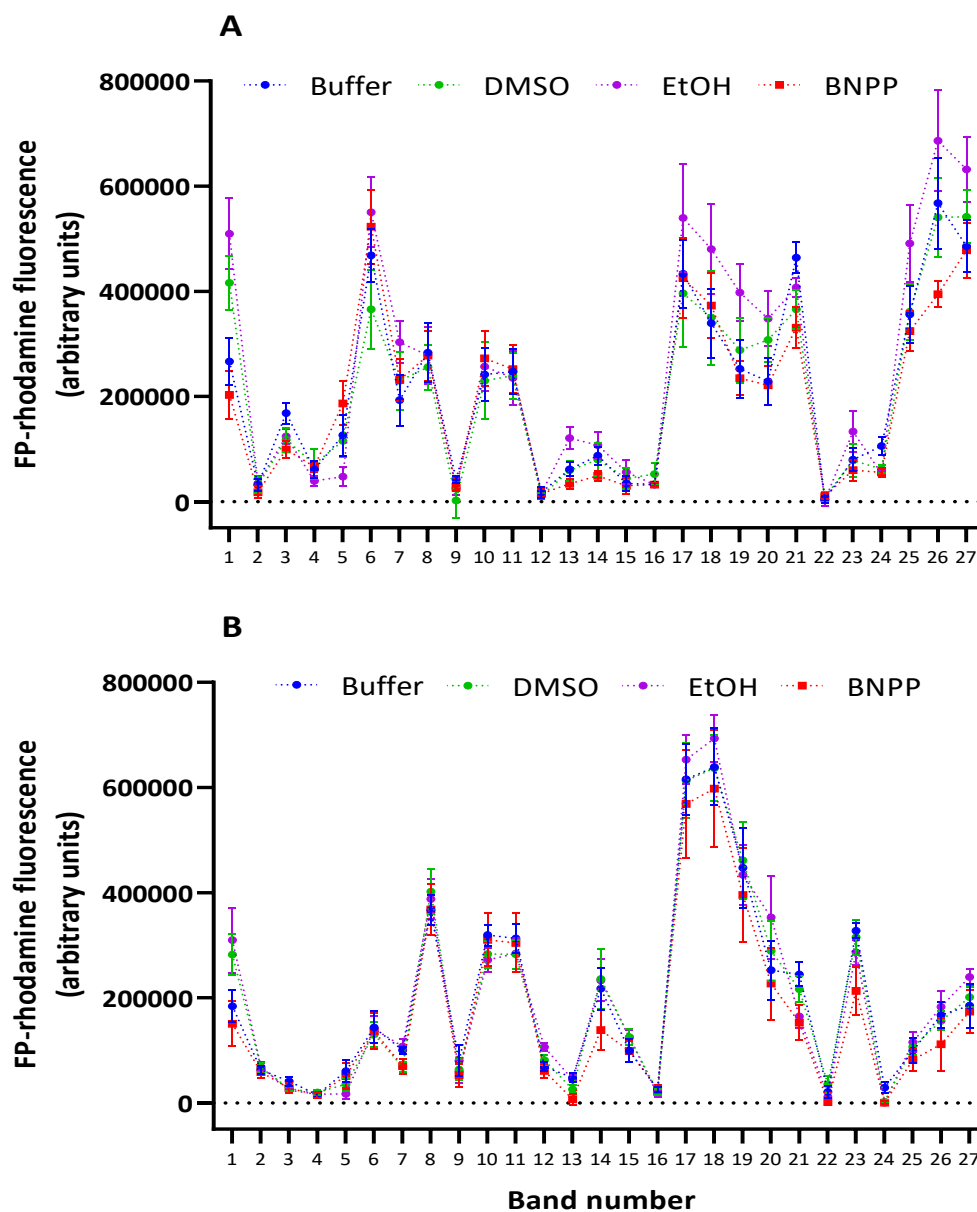


Figure 5.3 Quantitation of band densities from ABPP gel scans of rat brain preparations. Data are means $\pm$ SEM of six independent cerebral cortex samples of soluble (A) or particulate (B) fractions expressed as the band densities from gel scans (arbitrary units). Samples were co-incubated with DMSO or ethanol equivalent to the content with the inhibitors used in subsequent analyses. As BNPP is water-soluble, while the other compounds tested were dissolved in either DMSO or ethanol, the effects of BNPP (1 mM) were compared to buffer.

The results showed that nearly all bands were unaffected by the solvents used to dissolve test compound compared to buffer alone (the three rightmost lanes on the gels in Figure 5.1), while two bands from the soluble fraction of cerebral cortices (Figure 5.3A) were altered in the presence of DMSO. Band 1 was enhanced (148 ± 11 % control), while Band 24 was inhibited (77 ± 6 % control). Only one band (Band 7) from the particulate fraction was inhibited by DMSO (73 ± 4 %, Figure 5.3B).

Band 1 from the soluble fraction (Figure 5.3A) was also enhanced in the presence of ethanol (175 ± 16 % control), while Band 3 was inhibited in the presence of ethanol (83 ± 3 % control). Band 12 from the particulate fraction (Figure 5.3B) was enhanced in the presence of ethanol (142 ± 7 % control), while Bands 10 (86 ± 4), 11 (92 ± 3), 16 (80 ± 4) and 21 (70 ± 4 % control) were inhibited by ethanol.

Taken together, these results indicate that most bands were unaffected by the solvents, except for two in the soluble fraction affected by DMSO; Band 1 which was enhanced while Band 24 was inhibited. Only one band from the particulate fraction was affected by DMSO, with Band 7 showing inhibition. Additionally, Band 1 from the soluble fraction was enhanced in the presence of ethanol, while Band 3 was inhibited. Band 12 from the particulate fraction was enhanced by ethanol, while Bands 10, 11, 16, and 21 were inhibited.

5.4.3 Effect of MAGL or ABHD6 inhibitors on serine hydrolases enzymes in rat cerebral cortex

Bis-4-nitrophenol phosphate (BNPP) is a substrate for carboxylesterases, which have also been reported to hydrolyse endocannabinoids (Zhou et al., 2021b). As a water-soluble agent, the effects of 1 mM BNPP were compared against the buffer control. BNPP failed to alter enzyme activities from the particulate fraction (Figures 5.1B and 5.3B) but inhibited four bands from the soluble fraction (Figures 5.1A and 5.3A); bands 1, 3, 23 and 24 were inhibited to 81 ± 3 , 73 ± 4 , 79 ± 9 and 75 ± 8 % control.

A number of the alternative substrates failed to inhibit serine hydrolase activities from either the soluble or particulate fractions. Thus, 2-OG (1 mM), PEA (100 μ M), AEA (100 μ M), ODA (100 μ M), MUH (200 μ M), AAMC (100 μ M), 7HCA (100 μ M) or NPB (500 μ M) were ineffective. 2-OG is an endogenous substrate for MAGL (Ghafouri et al., 2004), while PEA, AEA and ODA are endogenous substrates for FAAH (Thomas et al., 1997, Tiger et al., 2000, Alharthi et al., 2018). NPB is a surrogate substrate for multiple esterases/lipases (Iglesias et al., 2016, Muccioli et al., 2008), while MUH, AAMC and 7HCA are surrogate substrates for ABHD6 (Mahmood et al., 2017), FAAH (Ramarao et al., 2005) and MAGL (Wang et al., 2008), respectively. Given that tissue levels of endocannabinoids are based on assessments of large non-uniform masses, 'real' concentrations are difficult to identify. In vitro, assessments of MAGL or FAAH activities in the lab indicated that these endocannabinoid or related molecule concentrations were close to saturation of the enzyme activity.

MAGL inhibitors (JZL184 (10 μ M) and JKK048 (100 nM)), ABHD6 inhibitor (WWL70 (10 μ M)) or MAGL substrate (KT203 (10 μ M)) were compared to their vehicle (DMSO) for effects on the soluble and particulate fractions from rat cerebral cortex (Figures 5.1 and 5.4).

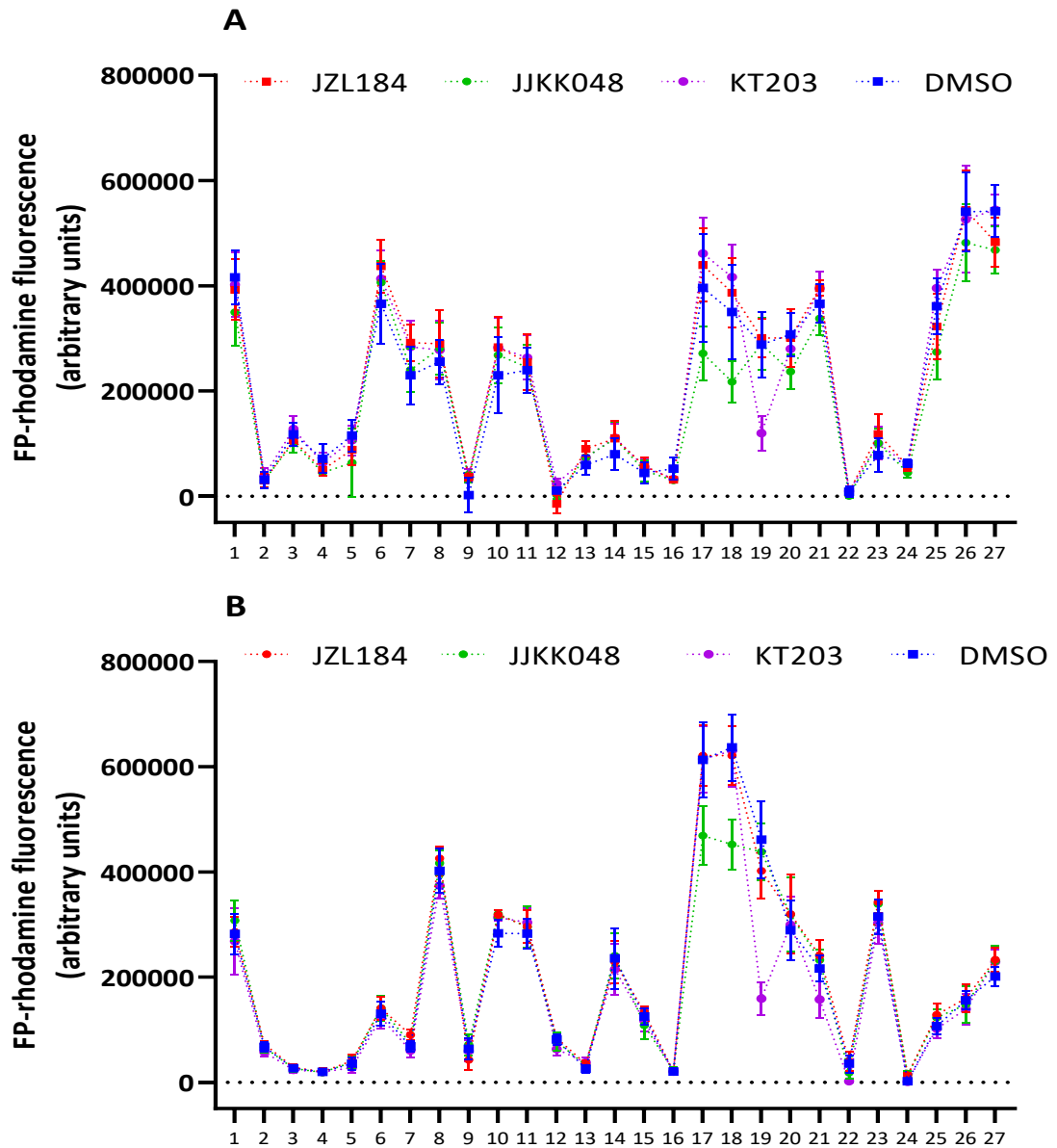


Figure 5. 4 Quantitation of band densities from ABPP gel scans of rat brain preparations. Data are means $\pm$ SEM of six independent cerebral cortex samples of soluble (A) or particulate (B) fractions expressed as the band densities from gel scans (arbitrary units). Samples were co-incubated with DMSO or JZL184 (10 μ M), JKK048 (100 nM) or KT203 (10 μ M).

JZL184 (10 μ M) and JKK048 (100 nM) failed to alter enzyme activities in the soluble phase, but JZL184 enhanced Band 7 (125 ± 7 % control), while JKK048 inhibited Bands 17 and 18 (78 ± 4 and 72 ± 3 % control, respectively) from the particulate fraction. WWL70 (10 μ M), an inhibitor of ABHD6 (Li et al., 2007), failed to alter enzyme activities in either soluble or particulate preparations from rat cerebral cortex. KT203 (10 μ M), a structurally distinct inhibitor of ABHD6 (Hsu et al., 2013), also failed to alter enzyme activities in the soluble phase (Figure 5.4A), but inhibited Band 19 (38 ± 4 % control). 2-AG (100 μ M), NAGly (300 μ M), URB597 (10 μ M) and MAFP (10 μ M) were compared to their vehicle (ethanol) for effects on the soluble and particulate fractions from rat cerebral cortex (Figure 5.5).

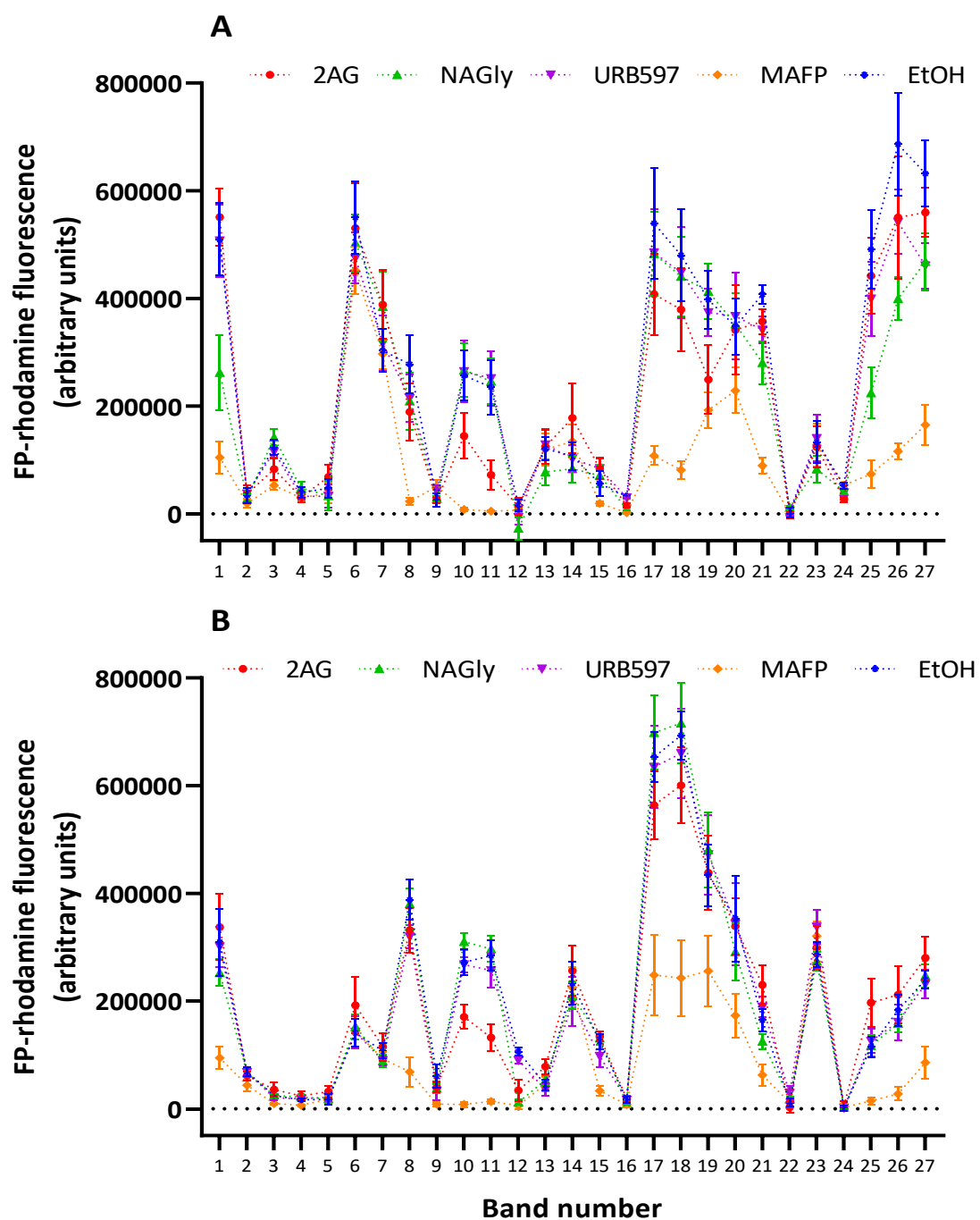


Figure 5. 5 Quantitation of band densities from ABPP gel scans of rat brain preparations. Data are means $\pm$ SEM of six independent cerebral cortex samples of soluble (A) or particulate (B) fractions expressed as the band densities from gel scans (arbitrary units) Samples were co-incubated with ethanol or 2-AG (100 μ M), NAGly (100 μ M), URB597 (10 μ M) or MAFP (10 μ M).

2-AG (100 μ M) inhibited Bands 17, 18 and 24 to 80 ± 3 , 80 ± 4 and 80 ± 6 % control in the soluble fraction (Figure 8.4A), while inhibiting only Bands 10 and 11 from the particulate fraction (76 ± 14 and 60 ± 8 % control, respectively, Figure 5.4B). NAGly (100 μ M) inhibited Bands 8, 14, 16 and 21 of the soluble phases to 77 ± 6 , 77 ± 11 , 74 ± 10 and 74 ± 6 % control, respectively. In the particulate fraction, NAGly only inhibited Band 12 (30 ± 6 % control). URB597 (10 μ M) only inhibited Band 8 from the soluble phase (81 ± 4 % control) and no activities from the particulate fraction (Figure 5.5). MAFP (10 μ M) was the most effective inhibitor tested. In the soluble phase, Bands 1 (33 ± 7), 3 (66 ± 8), 8 (29 ± 5), 10 (29 ± 4), 11 (30 ± 4), 16 (67 ± 11), 17 (33 ± 11), 18 (30 ± 3), 21 (37 ± 5), 25 (31 ± 8), 26 (30 ± 5) and 27 (36 ± 7) were all inhibited by MAFP. In the particulate fraction, Bands 8 (24 ± 6), 10 (11 ± 2), 11 (13 ± 2), 12 (24 ± 6), 15 (42 ± 12), 17 (39 ± 8), 18 (39 ± 7), 19 (59 ± 8), 25 (32 ± 6), 26 (29 ± 5) and 27 (42 ± 9 % control). This pharmacological profiling provides evidence for identifying individual ABPP bands in the rat brain, the regional distribution of which is discussed in the following sections.

5.4.4 Brain regional variation of serine hydrolase activities

Eight regions from six different rat brains, all matched for gender and weight, were homogenised and centrifuged to generate soluble and particulate fractions. Following reaction with the activity probes and electrophoresis on 10% polyacrylamide gels, 27 bands were quantified for each sample. In each gel, molecular weight markers were analysed on the leftmost side of the gel.

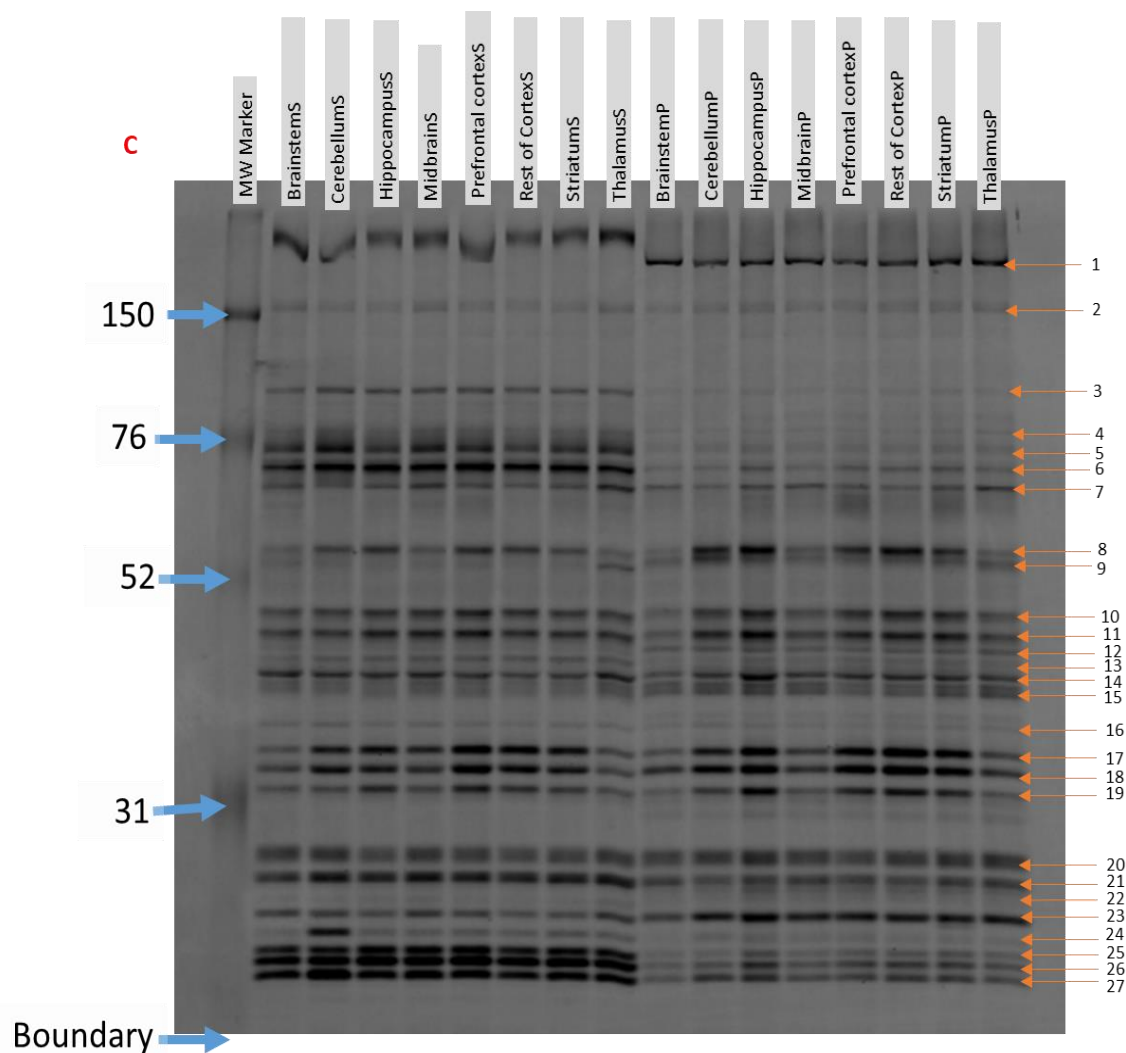


Figure 5. 6 A representative gel of FP-rhodamine-labelled serine hydrolase activities from eight different regions of the rat brain. The soluble and particulate samples were loaded in wells as indicated by the labelling above. The lane on the far left contains the molecular weight markers (150, 76, 52 and 31 kDa). 27 bands from each lane were identified and quantified. The gels are from a single animal; tissues from five other animals were processed in an identical manner.

By comparing all gels examining brain region distribution of serine hydrolases, one can see that, overall, the expression of the majority of the serine hydrolase enzymes in the soluble fractions was higher than those in the particulate. However, the expression of several bands was much higher in the particulate fractions unlike the general trend. For example, the intensities of

band 23 were higher in particulate group samples compared to soluble group samples (Figure 5.6 A), and those of bands 10, 11, 14, 15, 17 and 18 were shown to be higher in particulate fractions of samples from cerebellum, hippocampus, midbrain, prefrontal cortex, rest of cortex and striatum (Figure 5.7). On the other hand, bands 17, 18 and 20 showed the same intensities between soluble and particulate fractions (Figure 5.7). Visual inspection of each gel suggested the expression and intensities of the bands were consistent between all brain regions of the soluble and particulate fractions. However, there are some exceptions to this general statement. For example, the midbrain in the soluble fraction showed a distinctive band (band 5) at a molecular weight of about 71 kDa, as compared to the rest of the brain regions. (Figure 5.7).

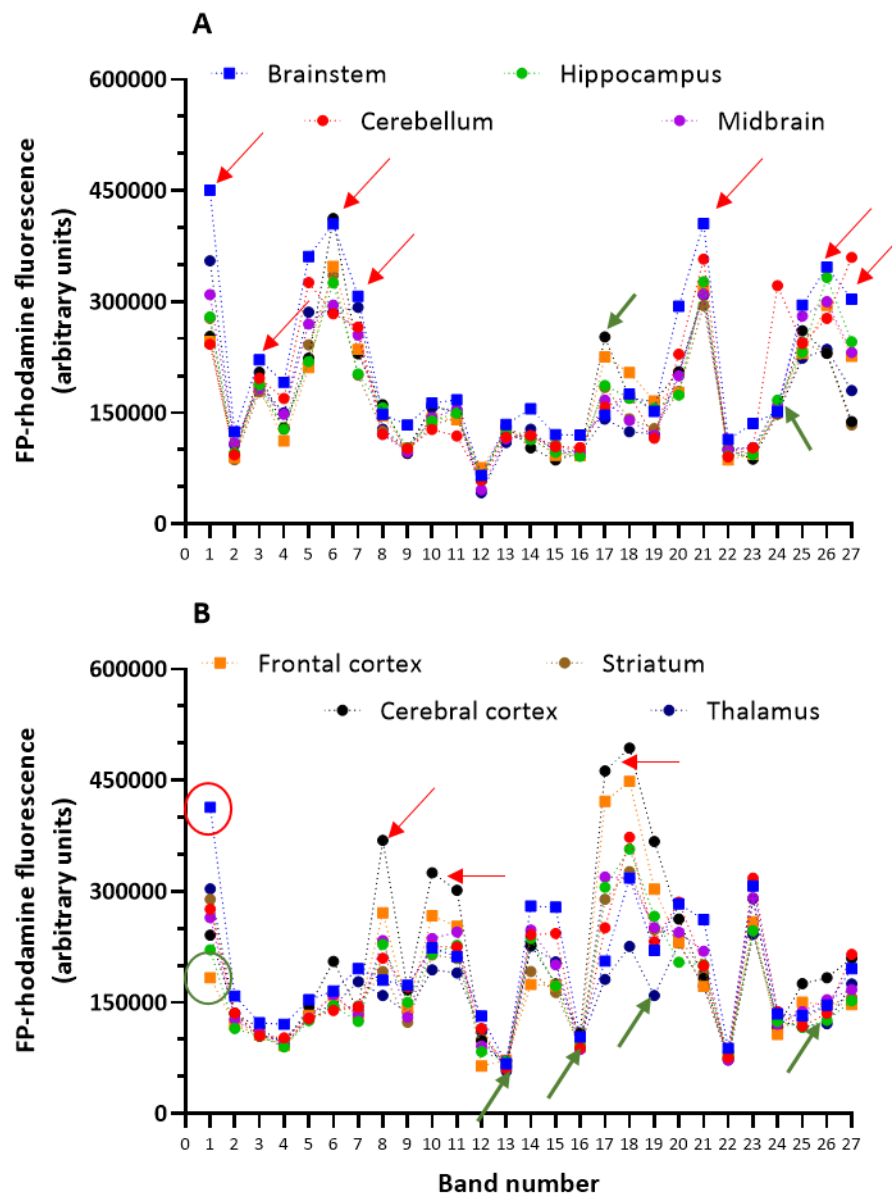


Figure 5. 7 Quantitation of band densities from ABPP gel scans of rat brain preparations. Data are means (error bars have been omitted for clarity) of six (or 5, for brainstem) independent samples of soluble (A) or particulate (B) fractions expressed as the band densities from gel scans (arbitrary units).

Each sample was measured in terms of intensity and migration distance as described in section 2.4.6.. The intensity of the bands was measured using Image Studio Lite. The analysis of variation and correlation between the bands were assessed using GraphPad Prism.

The majority of the bands showed similar levels of activity between various brain areas within each subcellular location. However, there are some exceptions for that general observation, and I am going to summarise those exceptions in here (Figure 5. 7).

In the soluble fraction, there appeared to be highest activity of many bands in the brainstem (red arrow) compared to the other brain regions. A few notable exceptions include bands where the highest activity was detected in the cerebellum preparation (green arrow).

In the particulate fractions, however, the cerebral cortex preparation appeared to have the highest activities for many bands relative to the other regions (red arrows), with the thalamus often having the lowest activities (green arrows). Notable exceptions to this pattern include band 1 of the particulate fractions, where the brainstem activity appeared highest (red circle) and the frontal cortex appeared lowest (green circle). Additionally, bands 14, 15, 20 and 21 appeared highest in the brainstem (Figure 5.7B).

5.5 Discussion

In this chapter, I describe the activities of serine hydrolyse enzymes in various regions of the rat brain. Using the combination of molecular size and pharmacological profile, a number of the bands could be provisionally identified. Previously, Blankman and others from Ben Cravatt's lab used ABPP profiling of mouse recombinant serine hydrolases labelled with FP-rhodamine to compare with the size and activity of mouse brain enzymes (Manterola et al., 2018, Blankman et al., 2007). The gel below is reproduced from their publication and shows several serine hydrolase enzymes observed in mouse brain, some of which displayed significant 2-AG hydrolase activity: MAGL, FAAH, HSL, ABHD6 and ABHD12 (Manterola et al., 2018, Blankman et al., 2007) This can be used as a framework to compare serine hydrolases between mouse and rat brain samples (Figure 5.8) (Manterola et al., 2018, Blankman et al., 2007).

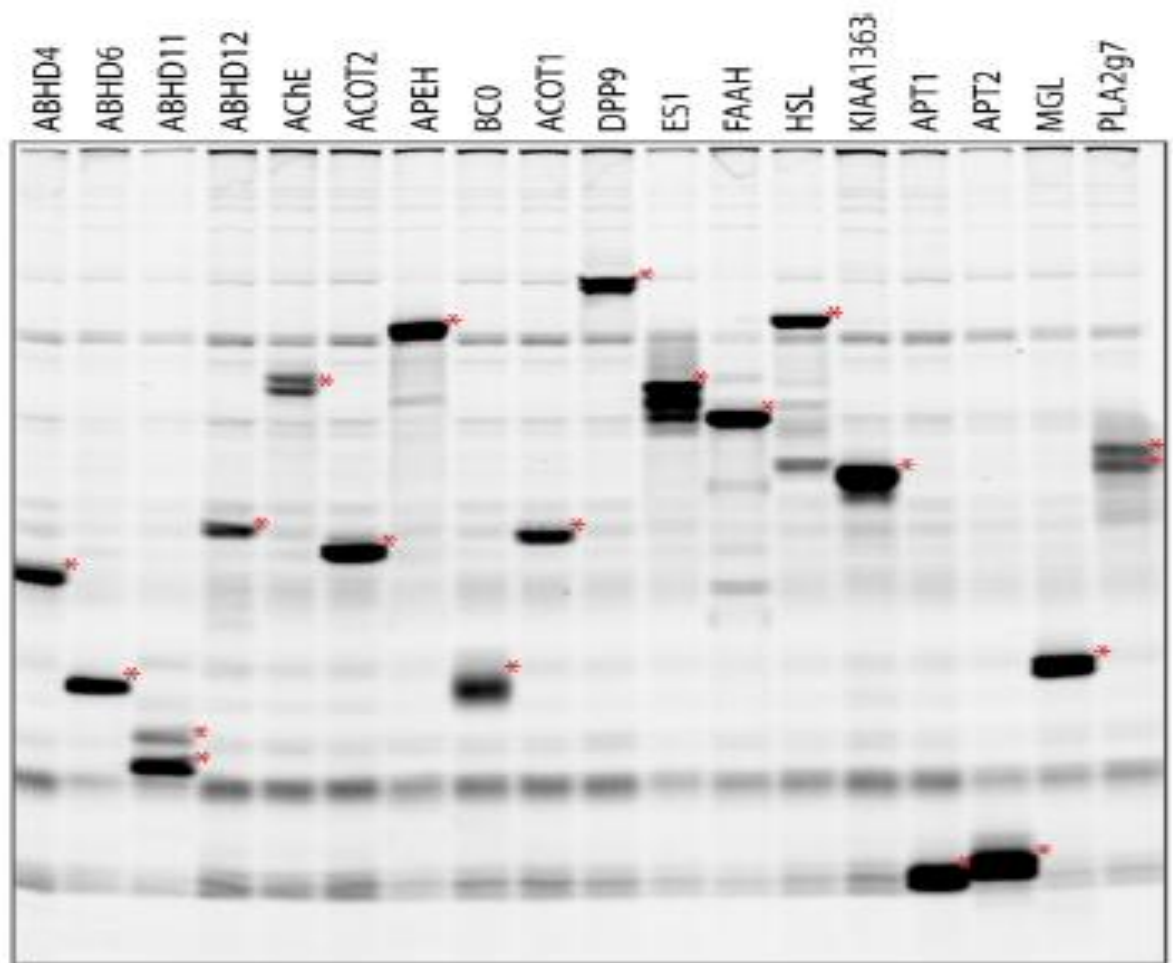


Figure 5. 8 Recombinant expression of mouse brain serine hydrolases was performed using COS-7 cells transiently transfected with mouse brain serine hydrolase cDNAs. The cells were labeled with FP-rhodamine (2 μ M for 1 hour), separated via SDS-PAGE, and analyzed using in-gel fluorescence scanning to verify the expression of active enzymes. The efficiency of active enzyme expression was determined by measuring the integrated fluorescence intensities of the marked bands. The fluorescent gel images are presented in grayscale., taken from Blankman et al. 2007.

Table 5. 2 A summary of the serine hydrolase enzyme activities in rat brain regions is given below.

Band	MW (kDa)	Cellular fraction ^a	Highest activity	Pharmacology ^b	Possible identification
1	203.3	Soluble	Brainstem (s)	↑DMSO (s) ↑EtOH (s) ↓BNPP (s) ↓MAFP (s)	
2	161.2				
3	95.8			↓EtOH (s) ↓BNPP (s) ↓MAFP (s)	
4	76.0				HSL
5	71				DAGLβ
6	69	Soluble			ACHE
7	65		Thalamus (s)	↓DMSO (p) ↑JZL184 (p)	CES2
8	56		Hippocampus (p)	↓NAGly (s) ↓URB597 (s) ↓MAFP (s,p)	FAAH
9	54				
10	48		Hippocampus (p)	↓EtOH (p) ↓2-AG (p) ↓MAFP (s,p)	LPL
11	46	Particulate	Hippocampus (p)	↓EtOH (p) ↓2-AG (p) ↓MAFP (s,p)	ACOT2
12	43			↑EtOH (p) ↓NAGly (p) ↓MAFP (p)	ABHD12
13	42				ACOT1
14	41		Hippocampus (p)	↓NAGly (s)	ACOT2
15	39			↓MAFP (p)	ACOT2
16	37			↓EtOH (p) ↓NAGly (s) ↓MAFP (s)	ABHD4
17	34		Cerebral cortex (p)	↓JJKK048 (p) ↓2A-G (s) ↓MAFP (s,p)	MAGL
18	33		Cerebral cortex (p)	↓JJKK048 (p) ↓2A-G (s) ↓MAFP (s,p)	MAGL
19	30		Cerebral cortex (p)	↓KT203 (p) ↓MAFP (p)	ABHD6
20	26.0				
21	24.9	Soluble	Cerebral cortex (s)	↓EtOH (p) ↓NAGly (s) ↓MAFP (s)	LYPLA1
22	21.3				
23	19.6			↓BNPP (s)	RBBP9
24	18.7			↓DMSO (s) ↓BNPP (s) ↓2A-G (s)	
25	18.0		Cerebral cortex (s)	↓MAFP (s,p)	
26	17.1	Soluble	Cerebral cortex (s)	↓MAFP (s,p)	

^abased on an arbitrary three-fold ratio, an indication of the soluble vs particulate fraction was assigned. When not indicated, the band appeared roughly equally active in both fractions. ^ban indication of the influence of the solvents, pharmacological inhibitors, or alternative substrates; where 's' and 'p' indicate effects on soluble or particulate fractions, respectively.

In Table 5.2, the effects of a number of non-selective and selective enzyme inhibitors were used as tools to tentatively identify some of the bands on the gels. The changes in the intensities of the bands in the presence of various inhibitors were used to determine the identity of the bands on the gel.

Some of the bands showed no difference in their intensities between various brain regions. This suggests that these serine hydrolases have a similar role in all these different regions of the brain, which may be a sign of similar, fundamental function in neurons or glia. Based on the molecular size and sensitivity of bands 17 and 18 to JKK048 and 2AG, we would tentatively characterise these bands as MAGL, while KT203 and MAFP inhibition of Band 19 shows that this band is probably ABHD6.

5.5.1 Systematic analysis of ABPP bands obtained from rat brain samples

Our hypothesis was that we could identify multiple enzyme activities based on their subcellular distribution (soluble vs particulate), distribution in different rat brain regions, but, most informatively, based on their molecular size and pharmacological profile.

A complication with analysis of these gels is the possibility that multiple enzyme activities labelled with FP-rhodamine could migrate at the same molecular size. Thus, bands of activity that are observed in both the soluble and particulate might be the same enzyme activity, or they might not. Additionally, a lack of complete inhibition or even a lack of significant inhibition might be due to multiple enzyme activities migrating at the same molecular size.

In the following section, the bands detected on the ABPP gels are discussed as three groups. The first group includes bands where there is some confidence about identifying the serine hydrolyse activity due to the effects of the pharmacological inhibitor/s. The second group is where a selective pharmacological inhibitor was not available and potential identification relied mostly on the molecular size of those enzymes as well as on some non-selective inhibitors. There is, therefore, much less confidence about their labelling. The third

group includes bands where pharmacological inhibitors were not available and the size of the enzymes were not defined in the literature, and, therefore, there is very limited confidence about those enzymes.

5.5.2 Bands where the pharmacological profile provides identification with some confidence.

Relatively few of the bands from these samples exhibited a pharmacological and subcellular location profile, which was completely consistent with established serine hydrolases.

5.5.2.1 Bands 17 and 18: MAGL

In these experiments, Bands 17 and 18 showed a profile consistent with MAGL. According to UniProt, the predicted molecular size of mouse MAGL is 33.4 kDa. The two bands were observed in mouse brain in both particulate and soluble fractions, as a doublet of 34 and 33 kDa. MAGL was shown to migrate as a doublet in mouse brain membranes which is likely due to alternative spliced isoforms (Patel et al., 2015). The finding here suggests that MAGL migrates as a doublet in samples of rat brain too.

Band 17 and 18 were shown to have higher expression in the particulate fractions of the hippocampus, cerebellum, prefrontal cortex and rest of cortex. The high activity of MAGL in the hippocampus and frontal cortex indicates a potential role in memory and learning, characteristics associated with cannabinoid signalling and MAGL inhibition (Pan et al., 2011). Pharmacological inhibition of MAGL also prevented neuroinflammation and decreased neurodegeneration, suggesting that it contributes to the development and progression of Alzheimer's disease (AD) and could be considered a potentially effective therapeutic target for both preventing and treating AD (Chen et al., 2012).

The soluble activities of Bands 17 and 18 were significantly inhibited by 2A-G and MAFP, while the particulate activity was significantly inhibited by JKKK048, a potent and selective inhibitor for MAGL (Aaltonen et al., 2013). 2-AG is a substrate for MAGL in the CNS consistent with these observations. In mouse brain, 2-oleoylglycerol and 2-AG were shown to be substrates for MAGL (Manterola et al., 2018, Blankman et al., 2007). Intriguingly there appeared to be a difference between which fraction was inhibited by JKKK048 or 2AG. MAGL

was shown to be expressed in both soluble and membrane fractions of rat brain (Ghafouri et al., 2004, Wyatt et al., 2020). While MAGL activity in brain membranes was shown to be inhibited by JNJ-42226314, a potent, selective, and reversible inhibitor of MAGL, JNJ-42226314 had no effect on MAGL activity in the soluble fraction (Wyatt et al., 2020). That is consistent with what I found in this study, as MAGL in the particulate fraction was inhibited with JKK048, while other non-selective inhibitors were only effective in the soluble fraction. This observation is worthwhile following up with a distinct MAGL assay format.

On the other hand, both URB597 and KT203 had no effect on MAGL activity in the rat brain, which is consistent with the reported literature, as URB597 is a FAAH inhibitor (Kwilasz et al., 2014), whereas KT203 inhibits ABHD6 with little effect on MAGL activity (Hsu et al., 2013).

5.5.2.2 Band 19: ABHD6

Based on the molecular weight analysis, Band 19 could potentially be identified as ABHD6. ABHD6 has an expected molecular size of 38 kDa, according to UniProt . Mouse recombinant ABHD6 was detected at a more rapid migration rate than mouse recombinant MAGL, an apparently lower molecular size (Manterola et al., 2018, Blankman et al., 2007). Band 19 was found in both soluble and particulate fractions but only the particulate activity was significantly inhibited by KT203 and MAFP (Figure 5.1). This pharmacology suggests that band 19 equates to ABHD6 (Manterola et al., 2018, Blankman et al., 2007). In mouse brain membranes, FP-rhodamine tagged activity described as ABHD6 was inhibited in the presence of 10 μ M MAFP (Manterola et al., 2018, Blankman et al., 2007).

ABHD6 is primarily a particulate enzyme since it is associated with membrane structures and lipid droplets (Poursharifi et al., 2017). In human models ABHD6 consistently migrates as doublet with MAGL (MAGL $\sim$ 33 and $\sim$ 35 kDa; ABHD6 $\sim$ 36 kDa) (Navia-Paldanius et al., 2012). In mouse brain, 2-AG was shown to be a substrate for ABHD6 (Manterola et al., 2018, Blankman et al., 2007). ABHD6 localises predominantly to the postsynaptic specialization while other enzymes such as MAGL are reported in the axons (Wei et al., 2016). Inhibition of ABHD6 activity decreased synaptic dysfunction and memory impairment in animal models of Alzheimer's (Xue et al., 2022). This implicates its role in the brain synaptic communications and suggest it as a therapeutic target to manage several brain disorders.

ABHD6 interacts with several proteins that are known to regulate its hydrolysis activity. They include brain-specific carnitine palmitoyltransferase 1C (CPT1C) and AMPA receptors (Miralpeix, et al., 2021). CPT1C was found to negatively regulate ABHD6 hydrolase activity, while ABHD6 negatively regulates the surface delivery and synaptic function of AMPA receptors in neurons (Wei, et al., 2016., Miralpeix, et al., 2021). These approaches can be used to confirm the presence of ABHD6 in my samples and to manipulate its activity to learn about its specific role in rat brain cortex.

ABHD6 can be measured using its ability to hydrolyse 4-methylumbelliferyl-heptanoate (4-MUH), a fluorogenic substrate whose hydrolysis produces the fluorescent compound 4-methylumbelliferone (4-MU). This product can be detected using a spectrophotometer (Miralpeix et al., 2021). This method could be used as an alternative methodology to confirm the presence of ABHD6 in these samples.

5.5.2.3 Bands where the pharmacology provides limited capacity for identification.

Table 5. 3 Literature descriptions of enzymes for comparison

Band	Possible identity	Predicted MW (kDa)	UniProt MW (kDa)	Subcellular distribution	Inhibitors
1	Unknown	203.3			
3	Unknown	95.8			
4	HSL	76.0			
5	DAGL β	71	73	Particulate	Orlistat
7	CES2	65	62	Soluble	Benzil
8	FAAH	56	63	Particulate	URB597, MAFP
10	LPL	48	53		NDGA
11	NCEH1	46	49		
12	ABHD12	43	45		MAFP, orlistat
14	ACOT2	41			
15	ACOT2	39			
16	ABHD4	37	39		MAFP
21	LYPLA1	24.9	24		ML348
23	RBBP9	19.6	20		ML114
24	Unknown	18.7			
25	Unknown	18.0			
26	Unknown	17.1			
27	Unknown	16.6			

On this basis, I would divide the discussion in this section to those bands for which a potential identity has been suggested (4, 5, 7, 8, 10, 11, 12, 16, 21 and 23) and those which are unknowns (1, 3, 15, 24, 25, 26 and 27) but for which there is pharmacological data.

5.5.2.4 Bands 1 and 3

The molecular size of band 1 was about 203 kDa. The expression of band 1 was shown to be similar in both fractions, but it had slightly more activity in the brainstem. Band 3 has a molecular weight of roughly 95 kDa. Band 3 expression was found to be greater in the soluble

fraction, although it was found to be similar in all brain areas studied in this chapter. Both Bands 1 and 3 were inhibited by BNPP and MAFP.

BNPP and MAFP are known to inhibit several enzymes, including the pig carboxylesterase enzymes PLE1 and PLE6, FAAH and MAGL (Pan et al., 2009, Zhou et al., 2021) and in the current study both BNPP and MAFP inhibited the activities of several enzymes in rat brain. As a result of the low specificity of those pharmacological agents, confirming the nature of these bands based on pharmacological treatment results may require alternative approaches.

5.5.2.5 Band 4

The molecular size of band 4 was about 76 kDa. This band could equate to hormone-sensitive lipase (HSL) as the purified HSL rat enzyme was detected at a MW around 84 kDa on SDS-PAGE (Martín-Hidalgo et al., 2005). Expression of this band was shown to be similar in both fractions in all brain regions. HSL was reported to be a soluble enzyme (Jenkins-Kruchten et al., 2003) consistent with the lower activity of Band4 in the particulate sample (Figure 5.7B). Using pharmacological treatment, the soluble sample was not inhibited by any of the compounds. HSL is reported to have a 2-AG hydrolase activity in rat adipose tissue (Belfrage et al., 1977). In addition, TAG, DAG, and 2-AG were shown to be substrates for HSL in mouse brain (Manterola et al., 2018, Blankman et al., 2007). However, in the current study, 2-AG failed to have an effect on the activity of Band 4, which could argue against the correct estimation of Band 4 as HSL or identifying the lower affinity of HSL for 2AG. To confirm the identity of Band 4, one can follow the methodology used in Ben Ali et al.'s article, where products generated after the hydrolysis of the HSL substrates by HSL were identified on mass spectrometry to confirm the presence of these enzymes in rat brain samples (Ali et al., 2012).

5.5.2.6 Band 5

The molecular size of band 5 was about 71 kDa. This analysis suggested that Band 5 could be DAGL β , as rat DAGL β has a molecular size of 73, according to Uniprot. In general, the expression of band 5 was higher in the soluble fraction, but it showed higher activity in the soluble fraction of the cerebellum and thalamus, and in the particulate of the rest of cortex. DAGL β contributes to 2-AG synthesis and can be inhibited with MAFP (Patel et al., 2009).

However, there was no significant effect of MAFP on Band5 activity in the current study, which suggests that further analysis is required to confirm the identity of band 5. Previously Bisogno et al developed MAFP organophosphorus analogues which showed high selectivity for DAGL α over DAGL β and other lipases (Kohnz and Nomura, 2014). This method may be used to create inhibitors that specifically inhibit DAGL, which can then be used to confirm the nature of band 5 in this chapter. It should be noted that FP-rhodamine is not effective for detecting DAGL, which is why Mario van der Stelt created MB064 (Baggelaar et al., 2013a, Baggelaar et al., 2015).

5.5.2.7 Band 7

The molecular size of band 7 was about 65kDa. Based on the molecular weight analysis, it is possible Band7 equates to carboxylesterase type 2 (CES2). CES2 has an expected molecular size of 62 kDa, according to UniProt. Band 7 activity was shown to be higher in the soluble fraction and particularly active in the brain stem, midbrain, and the thalamus. The particulate activity of band 7 was increased in the presence of JZL184.

JZL184 and URB597 are reported to cause inhibition of CES2 as an off-target effect (Crow et al., 2012). In my analysis, neither JZL184 nor URB597 inhibited the activity of band 7, which argues against the prediction I made based on the molecular weight of CES2. Inhibitors of carboxylesterases include benzil (Hyatt, 2005), which could be used as a pharmacological tool to investigate this activity in future.

5.5.2.8 Band 8

The molecular size of band 8 was about 63 kDa, which is consistent with FAAH. FAAH was detected using FP-rhodamine ABPP in mouse brain membranes (Manterola et al., 2018, Blankman et al., 2007).

Band 8 was found in both soluble and particulate fractions, but its expression was shown to be higher in the particulate fractions of the prefrontal cortex, midbrain and rest of cortex. The soluble activity was inhibited by NAGly, URB597 and MAFP, which we would expect to be the case for FAAH. However, the particulate phase was not affected by URB597 and MAFP, which could argue against the presence of FAAH in these samples. The lack of effect of URB597 might be interpreted as a relatively reduced activity of FAAH in the brain

compared to MAGL or probably incomplete inhibition by the agents employed in the concomitant incubation of inhibitor alternative substrates with the FP-rhodamine and tissue samples. In mouse brain, AEA and 2-AG were shown to be substrates for FAAH (Manterola et al., 2018, Blankman et al., 2007). Future identification of FAAH can be achieved using JNJ-42165279 which is a novel specific inhibitor for FAAH (Schmidt et al., 2021).

This may confirm the presence of FAAH in my samples. Previous research showed that FAAH contributes to memory acquisition and that inhibition of FAAH by URB597 served to enhance memory function in rat brain through activation of PPAR-alpha nuclear receptors (Mazzola et al., 2009).

5.5.2.9 Band 10

The molecular size of band 10 was about 48 kDa, consistent with lipoprotein lipase (LPL) expression. Expression of band 10 was shown to be higher in the particulate fraction of prefrontal cortex, midbrain, and the rest of cortex. LPL activity was shown to be highest in hippocampus, this indicates its role in memory and cognition. This observation was recapitulated in the literature as Wang et al. (2012) who detected significant impairment in learning and memory in LPL-deficient mice indicating the role played by LPL in cognitive function in the CNS (Xian et al., 2009).

In Band 10, particulate activity was inhibited by 2AG, and both the soluble and particulate fractions were inhibited by MAFP. However, LPL was reportedly unaffected by either MAFP or 2-AG (Napolitano et al., 2012), while nordihydroguaiaretic acid did inhibit LPL (Napolitano et al., 2012), while nordihydroguaiaretic acid did inhibit LPL (Kang et al., 2019). This finding contradicts our prediction, indicating that further research is required to confirm the prediction for Band 10 in the particulate samples.

5.5.2.10 Band 11

The molecular size of band 11 was about 46 kDa, consistent with NCEH1 neutral cholesterol ester hydrolase 1 (NCEH1). Activity of band 11 was shown to be higher in the particulate fraction of prefrontal cortex, midbrain, and the rest of cortex. The particulate activity of Band 11 was inhibited by 2-AG and MAFP, while the soluble was inhibited only by MAFP. To my knowledge, there is no report indicated that NCEH1 could be inhibited by 2-AG

and MAFP. However, based on the mouse data from Blankman, ACOT2 migrates just below ABHD12, while KIAA1363/NCEH1 is just above ABHD12. The presence of NCEH1 which is involved in the regulation of cholesterol ester metabolism in rat brain samples can be confirmed in future using specific inhibitors for NCEH1 such as JW 480 which has shown potency and selectivity for NCEH1 enzyme (Chang et al., 2011).

5.5.2.11 Band 12

The molecular size of band 12 was about 43 kDa, consistent with ABHD12 expression. Expression of band12 was shown to be higher in the particulate fraction with similar expression in all brain regions. The particulate enzyme activity was inhibited by MAFP and NAGly. MAFP was shown to inhibit human recombinant ABHD12 and a similar-sized enzyme activity in mouse brain membranes (Parkkari et al., 2014). In ABHD12 KO brains, the level of NAGly was elevated this indicates the interplay between ABHD12 and NAGly (McHugh et al., 2010). In mouse brain, 2-AG was shown to be a substrate for ABHD12, while tetrahydrolipstatin (THL) inhibited its activity (Manterola et al., 2018, Blankman et al., 2007). In this study 2-AG had no effect on ABHD12, while I have not tested the effect of THL on ABHD12. However, THL was shown to inhibit other enzymes including ABHD16a (Manterola et al., 2018, Blankman et al., 2007). Hence, more selective inhibitors for ABHD12 are required to confirm its presence such as DO264 (Ogasawara et al., 2019). This compound showed efficacy in inhibiting ABHD12 in mouse brain using ABPP technique. Applying this compound in the current study would help to identify the nature of band 12.

5.5.2.12 Band 14

The molecular size of band 14 was about 41 kDa. Only the soluble activity was significantly inhibited in the presence of NAGly. As NAGly is a metabolite of the endocannabinoids and can be a substrate for several enzymes, it is hard to determine the nature of band 14 based on the pharmacological analysis.

5.5.2.13 Band 15

The molecular size of band 15 was about 39kDa. Only the particulate activity was significantly inhibited by MAFP. As several enzymes are known to be inhibited by MAFP, it is

hard to determine the nature of band 15 based on the pharmacological analysis. In Blankman, ACOT2 migrates below ABHD12, it might be Band 14 or Band 15 in my current study.

5.5.2.14 Band 16

The molecular size of band 16 was about 37kDa, consistent with ABHD4 expression. ABHD4 was detected using FP rhodamine in mouse brain (Manterola et al., 2018, Blankman et al., 2007). The expression of band 16 was low in both fractions but was slightly higher in all regions of particulate fractions. Only the soluble activity was significantly inhibited by NAGly and MAFP. There is no information in the literature concerning the effect of NAGly on ABHD4. However, MAFP reduced the activity of ABHD4 (Miller et al., 2016), which could suggest that my molecular weight estimation for Band16 is correct. A recent screen identified several potential ABHD4 inhibitors using the ABPP technique in mouse brain (Bononi et al., 2021). These compounds could be used to verify the identity of band 16.

5.5.2.15 Band 21

The molecular size of band 21 was about 24.8 kDa, consistent with LYPLA1 expression. Band 21 was found in both soluble and particulate fractions. It had higher expression in all brain regions of the soluble fractions. Only the soluble activity was significantly inhibited by NAGly and MAFP. There is no clear evidence from the literature to suggest that NAGly or MAFP can inhibit LYPLA1. On the other hand, ML-348 can inhibit LYPLA1 (Patel et al., 2022). ML348 was shown to be a selective inhibitor for LYPLA1 while employing ABPP in mouse brain (Adibekian et al., 2014). This inhibitor can be used to in current study to confirm the identity of band 21 (Won et al., 2016).

5.5.2.16 Band 23

The molecular size of band 23 was about 19.6kDa. It could potentially be estimated as RBBP9. RBBP9 has an expected molecular size of 20 kDa and was detected using the FP-biotin pulldown/MS assay in mouse brain (Manterola et al., 2018, Blankman et al., 2007). Band 23 was predominantly expressed in all brain regions of the particulate fraction. The activity of band 23 was inhibited by BNPP in the soluble fraction. There is no clear evidence in the literature suggesting that RBBP9 is inhibited by BNPP, and hence I cannot confirm the existence of RBBP9 based on the pharmacological evidence I had. ML114 was shown to be a

selective inhibitor for RBBP9 while employing ABPP in mouse brain (Bachovchin et al., 2010a). This inhibitor can be used to in current study to confirm the identity of band 23. The presence of RBBP9 in my samples can be determined using western blot techniques in the presence of RBBP9 antibodies, which are commercially available (Nachmany et al., 2012).

5.5.2.17 Band 24-27

Bands 24, 25, 26 and 27 were detected at 18.7, 18, 17.1 and 16.6kDa. Band 24 was predominantly a soluble enzyme activity with its highest activity in the cerebellum. It was inhibited by BNPP and 2AG. However, as the presence of these drugs decreased the activities of several bands in this study, I am unable to confirm the identity of band 24.

Band 25, 26 and 27 were predominantly soluble enzyme activity. In particulate fraction, the expression of bands 25-27 was the highest in prefrontal cortex, midbrain, and rest of cortex. Both particulate and soluble fractions were only inhibited by MAFP. As MAFP is a non-selective inhibitor of many endocannabinoid-metabolising enzymes, it is hard to identify those bands based solely on their inhibition with MAFP.

5.5.3 Bands lacking pharmacological profiling

A number of the bands from the rat brain samples labelled with FP-rhodamine were unaltered in the presence of any of the pharmacological inhibitors, which limits the capacity for identification.

Bands 2, 9, 13 and 20 were estimated to be around 161, 54, 46 and 26 kDa, respectively. While Bands 9 and 13 have molecular sizes consistent with lipoprotein lipase and ACOT1, respectively, the lack of pharmacological limits further identification.

Band 2 was shown to have low activity in both subcellular locations but slightly higher expression in the particulate, where their activity was the highest in the brainstem, cerebellum, hippocampus, midbrain, striatum, and the rest of the cortex. Band 9 showed similar activity in both locations (soluble and particulate fractions) and had higher expression in the soluble fraction of the brainstem, cerebellum, and thalamus than in the particulate fraction of the midbrain. Band 13 had higher expression in the soluble fraction and showed higher activity in the prefrontal cortex, striatum, and rest of the cortex. Band 20 had higher

activity in the particulate, and their activity was higher in the particulate of the midbrain, cerebellum, and thalamus, and in the soluble fraction of the brainstem, thalamus, and rest of the cortex.

5.6 Summary and conclusions

In conclusion, 27 bands were detected in the rat brain. Based on the pharmacological and size analysis approximately half have tentative assignment. However, there are still several enzymes where the current analysis was unable to determine the specific enzyme. Some of these enzymes are expressed in all brain regions, as is the case for DAGL β and ACHE. This indicates the ubiquitous role of these enzymes in the brain. Other enzymes, however, were predominantly expressed in some brain regions which is the case for band 8, which showed higher activities in the prefrontal cortex and the midbrain. This indicates that FAAH could play a specific role in those areas as compared to the others where it showed lower activities. The differential activity of a serine enzyme in different brain regions could be due to differences in the level of expression, localization, or post-translational modifications, among other factors. This has a direct correlation with the role the enzyme specifically plays in this region, examples of that are the high activities of ACHE in the basal ganglia region or the high activities of MAGL in the hippocampus and the relevant roles these enzymes can play in those specific regions. The information provided by this chapter could be used to target the relevant enzymes in specific brain regions in order to develop therapeutic approaches to some brain disorders i.e., movement disorders or dementia.

CHAPTER 6

An investigation of serine hydrolases in the rat spinal system and the influence of pain

6.1 Introduction

Pain is an unpleasant sensory experience which resembles real or potential tissue damage in the body. It is a complex process that is characterised by both peripheral and central neuronal alterations. A number of theories have been proposed to understand the physiological basis of pain, yet none of which are able to explain all aspect of pain perception. Due to that issue, current pain medicines have several hindrances and side effects including dependence and cognitive impairment which make further research in this area warranted. One endogenous system involved in the regulation of pain and inflammatory response is the endocannabinoid system, as ligands for CB1 and CB2 receptors are expressed almost ubiquitously throughout nociceptive pathways (Donvito et al., 2018). In the transmission of pain perception, endocannabinoids can be generated when required and act as short-term circuit breakers producing antinociceptive effects (Katona and Freund, 2008).

There are a number of reports suggesting the effectiveness of exogenous cannabinoids in the management of pain in various models, including AEA or the synthetic cannabinoid receptor agonist WIN55,212-2 (Manning et al., 2001, Vivian et al., 1998, Hohmann, 2002). In neuropathic pain models, the levels of cannabinoid receptors and their endogenous ligands i.e. AEA and 2-AG were shown to be increased in rat lumbar (L4 and L5) dorsal root ganglia (DRG) (Mitrirattanakul et al., 2006). This suggests an approach for suppressing both neuropathic and inflammatory pain. However, the effect of endocannabinoids on pain perception is also sensitive to antagonists of TRPV1 channels or PPAR α nuclear receptor modulation suggesting that multiple targets could be involved in the mechanism of their action (Adamson Barnes et al., 2016, Jhaveri et al., 2008).

The antinociceptive effect of endocannabinoids during pain is modulated by catabolic enzymes (Fowler et al., 2005) with several enzymes shown to regulate the levels of

endocannabinoids in pain models including FAAH. Inhibition of FAAH by URB597 significantly inhibited the effect of the proinflammatory molecule, carrageenan in inducing pain perception in neurons in the rat hind paw; an effect reversed by a selective PPAR- α antagonist (Sagar et al., 2008). This finding suggests that increasing the level of cannabinoids would induce an antinociceptive effects by an indirect inhibition of sensitisation of neuronal responses through PPAR- α activation. I hypothesized that changes in enzyme activities in relevant neural pathways would be altered in pain by using dual activity-based proteomic probes.

6.2 Hypothesis:

In this chapter, the hypothesis to be tested was that serine hydrolase enzyme activities in relevant neural pathways, particularly those known to regulate endocannabinoid levels, will be altered following painful stimuli.

6.3 Objectives

1. To identify whether enzyme activities measured using the ABPP technique differed between locations at the spinal level (dorsal root ganglia (DRG) L2, L3 and L4 reagrions which provide sensation to the front part of your thigh and inner side of the lower leg, dorsal spinal cord (DSC), and ventral spinal cord (VSC)).

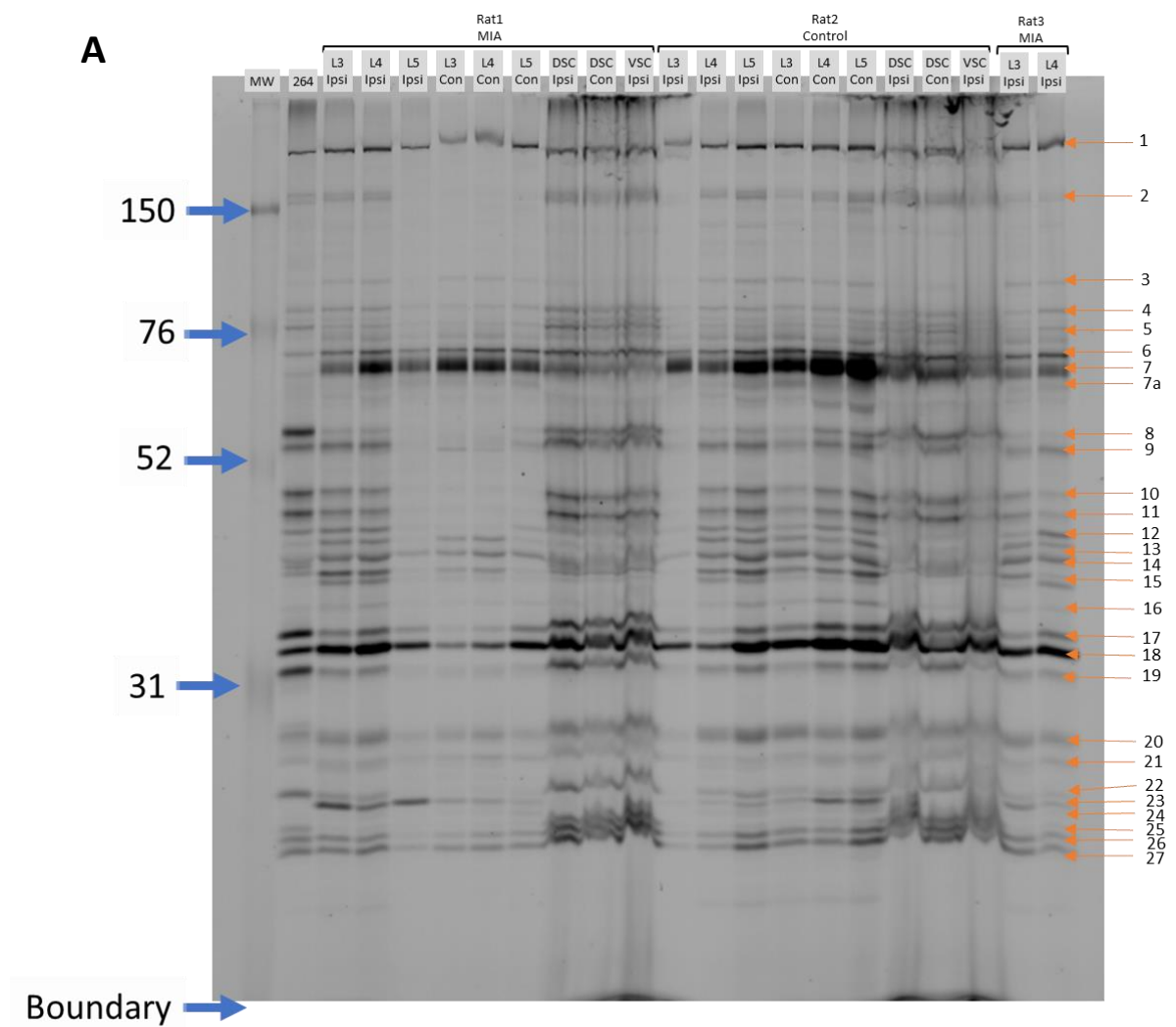
2. To identify whether enzyme activities were altered in animals experiencing a painful stimulus

For that purpose I use the Monoiodoacetate (MIA) model of pain which unilateral MIA is a widely used experimental model for studying pain and related pathophysiology in animals, particularly rodents. This model is valued for its ability to mimic the chronic pain. The model involves the intra-articular injection of MIA into the joint, usually the knee. MIA inhibits glycolysis by inactivating glyceraldehyde-3-phosphate dehydrogenase, leading to chondrocyte death and subsequent cartilage degradation, mimicking the pathophysiological changes of osteoarthritis. In my thesis, I unilaterally injected the MIA into the knee and studied the changes happened at the ipsilateral or contralateral paws.

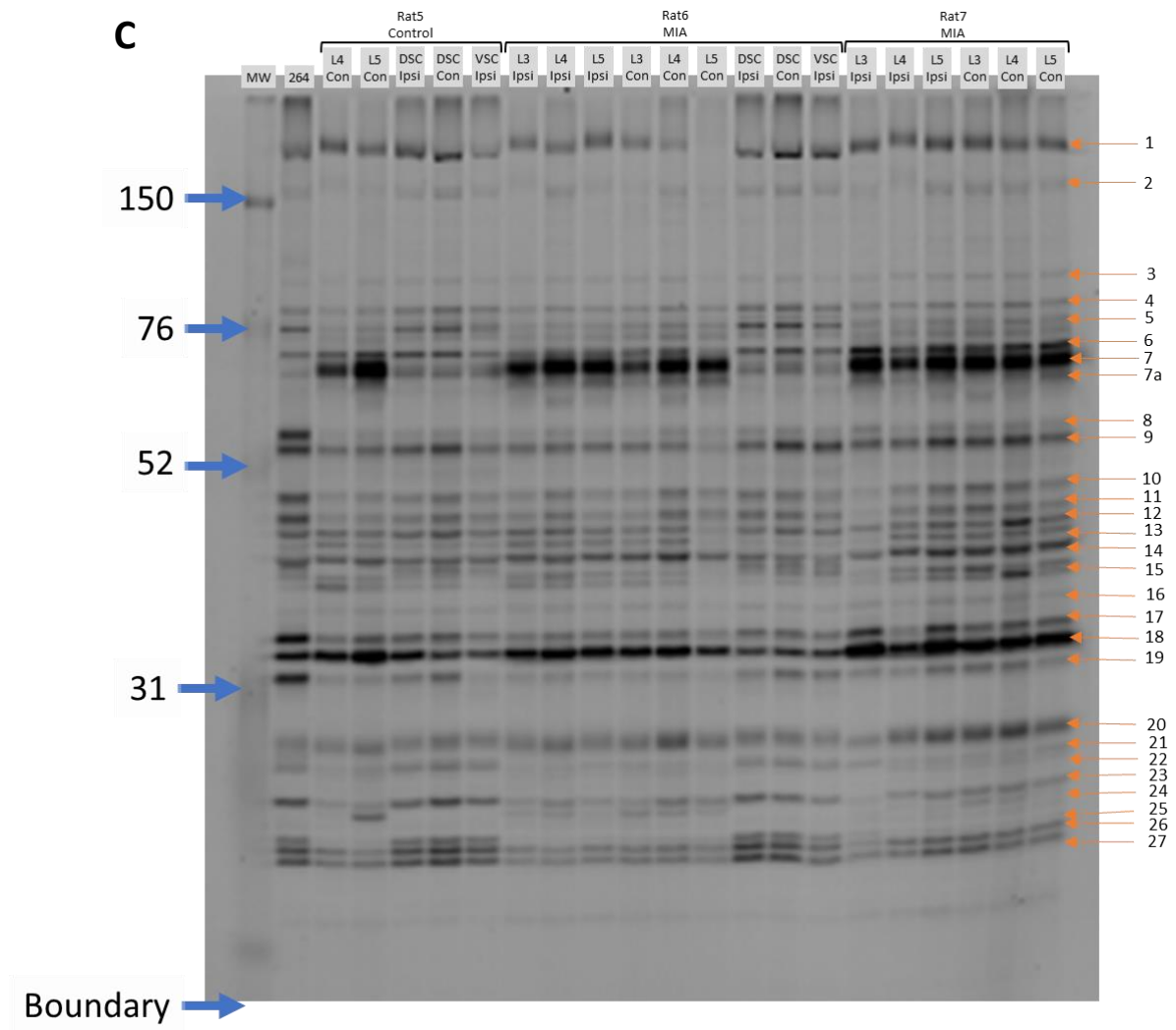
6.4 Results

6.4.1 Gel Scans

Enzyme activities were analysed from four gels (Figure 6.4A-D). Any difference described in this chapter was statistically significant with a P value of <0.05 (two-way ANOVA with post-hoc Dunnett's multiple comparison test, see Section 2.4.5).







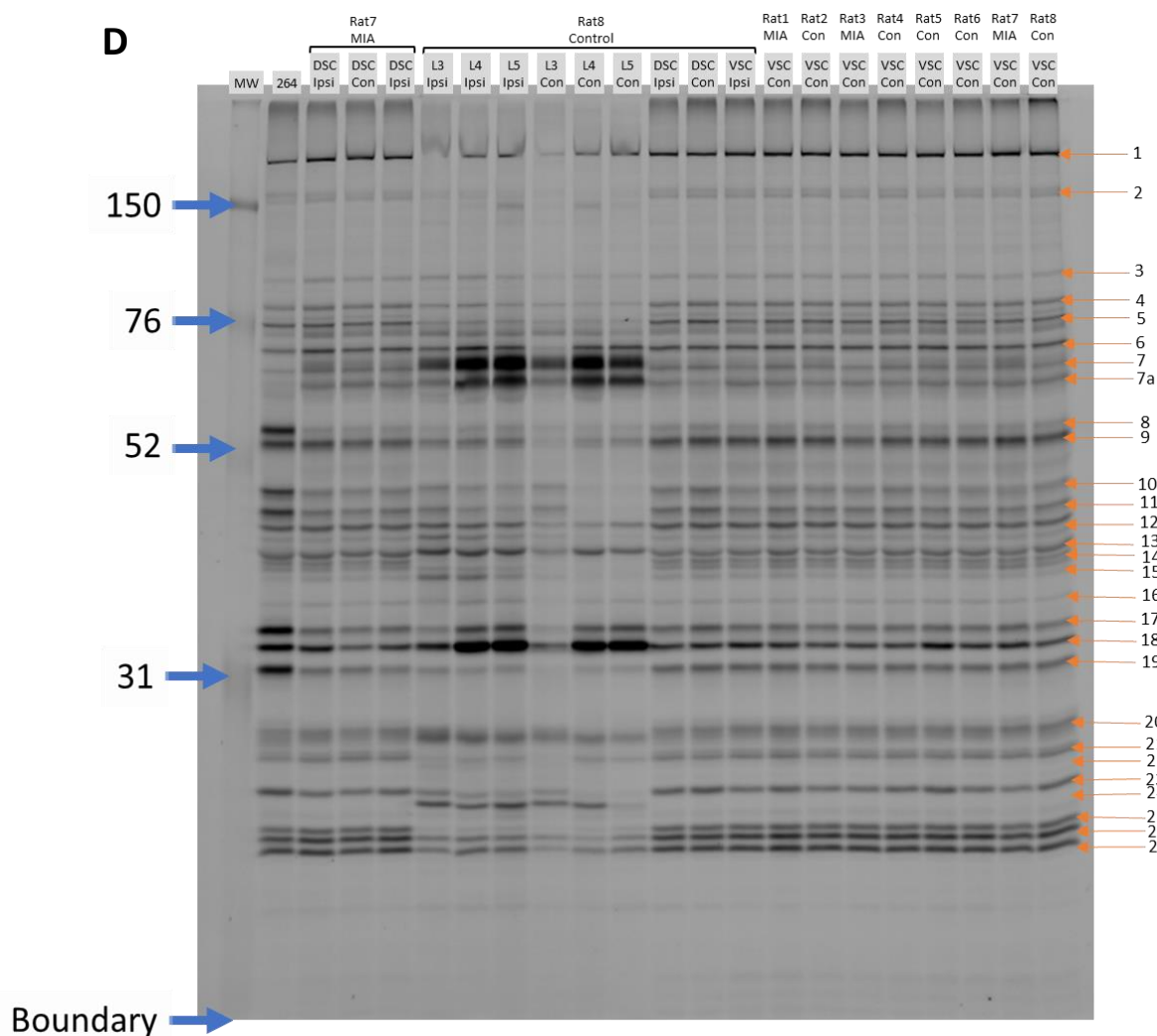


Figure 6. A-D In-gel fluorescence scan of rat DRG and spinal cord tissues probed with a combination of MB064 and FP-rhodamine. Rat brain membrane sample (264) was used as a positive control. DRG and spinal cord samples were loaded as shown in the figures.

As in the previous chapter, tentative identification of individual bands on the gels were suggested based on their estimated molecular weights which were compared against the published literature, or the known molecular weight of serine hydrolase enzymes obtained from UniProt. 28 bands were detected in spinal cord, while in rat brain, in the previous chapter, only 27 bands were detected.

The first gel run in this sequence (Figure 6.4A) exhibited distortion and blurriness of the gel (specifically with the spinal cord samples in lanes 7, 8, and 9 in the middle of the gel and 15, 16, and 17 on the right side of the gel). This may have resulted from sample

overloading or incompletely homogenized samples through using the micro-pestle. Subsequently, an ultrasound probe (Soniprep 150 Ultrasonic Disintegrator, MSE) was used to prepare samples for analysis in Figures 6.4B-D.

6.4.2 Enzyme activity analysis

Analysis of the samples to compare the activities of all enzymes (normalised to the brain fraction, band 6) was conducted using the L5 DRG as a comparison. We chose band 6 of sample 264 because its activity level was approximately median among all the activities measured. It was not at the top or bottom, but rather around the middle of the range. This middle-range activity was selected to ensure a balanced representation, with expected variations on either side—higher and lower activities. We aimed to avoid any bias by selecting an activity level at one extreme of the scale. This particular band was chosen because it was among the 27 bands analysed, positioned in the middle of the range, and sufficiently distinct from other bands, making it easy to quantify. Additionally, since the same quantity of protein was loaded onto each gel, this band served as a practical normalisation point for comparing all activities. Data were assessed using a two-way ANOVA and Dunnett's multiple comparison tests. There were no differences between L3 or L4 DRG compared to L5 DRG enzyme activities (Figure 6.2).

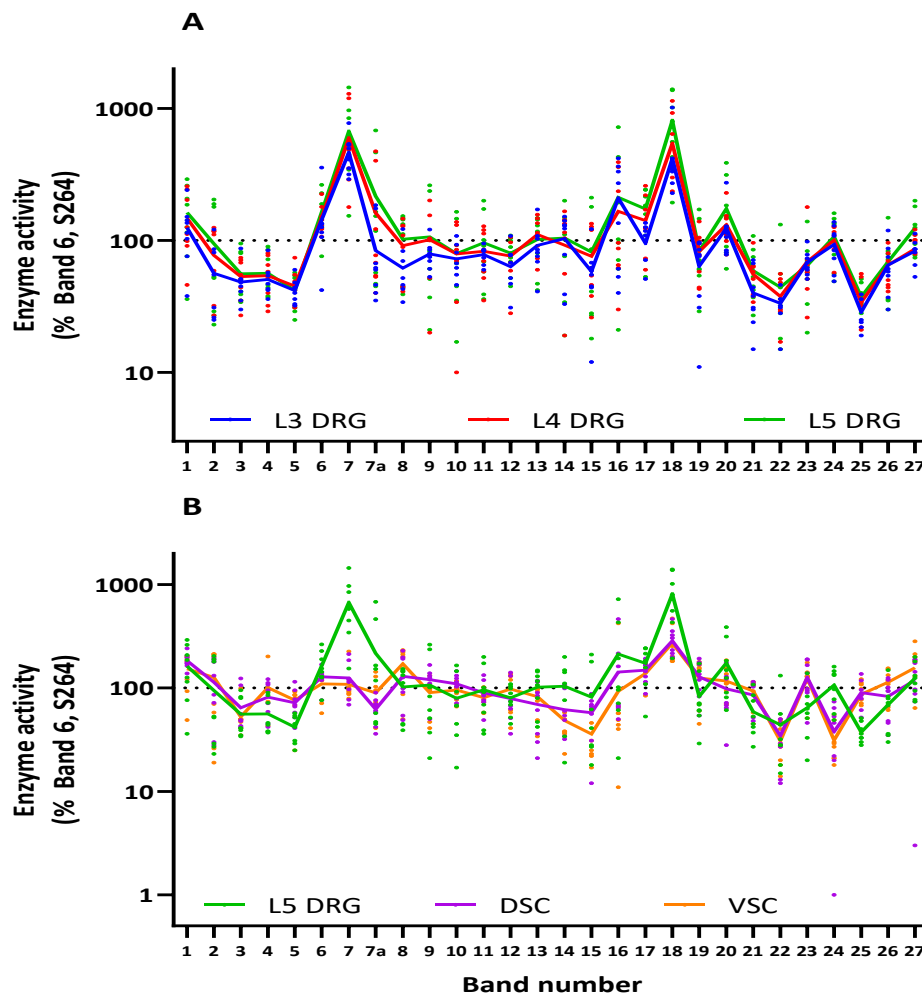


Figure 6. 2 Comparison of serine hydrolase activities in the spinal system. Data are individual band densities from (A) ipsilateral and contralateral L4, L5 and L6 DRG and (B) L5 DRG with DSC and VSC from four animals, expressed as a % of Band 6 of the rat brain 264 sample present on every gel. Note that X-axis represents the band numbers as illustrated in Table 5.1. The band number on the X-axis is in descending order of molecular size from the gels (Figures 6.1A-D). Statistical analysis was conducted as reported in Section 2.4.5.

Enzyme activities between L5 DRG and dorsal spinal cord (DSC) and ventral spinal cord (VSC) were then analysed and assessed using two-way ANOVA and Dunnett's multiple comparison tests. In contrast to the dorsal root ganglia comparisons, three bands differed between L5 DRG and dorsal spinal cord (DSC), Bands 7, 19, and 26. On the other hand, seven

bands differed between L5 DRG and ventral spinal cord (VSC), bands 5, 7, 9, 19, 22, 26, and 27 (Figure 6.2).

Although there was no statistical difference in Band 7a activities between DRG and spinal cord samples, this Band was not detectable in the rat brain (see Chapter 5 on rat brain particulate and soluble fractions).

Figure 6.3 represents the potential differences between tissues in the control animals. For each lane on the gel (tissue sample), band density was normalised to the density of Band 17, which appeared close to the median density on most gels. This allowed comparison of activities in an individual tissue relative to a well-defined serine hydrolase activity. Among the four animals, eight datapoints (from ipsi- and contralateral tissues) were compared. The datapoints representing activity in L3 or L4 DRG or dorsal or ventral spinal cord were compared to the same band in the L5 DRG samples. For example, Band 18 had mean densities of 456, 380 and 453 % of Band 17 in L3, L4 and L5 DRG, respectively. In the dorsal and ventral spinal cord, the density of Band 18 was 207 and 215 % of Band 17 density, respectively.

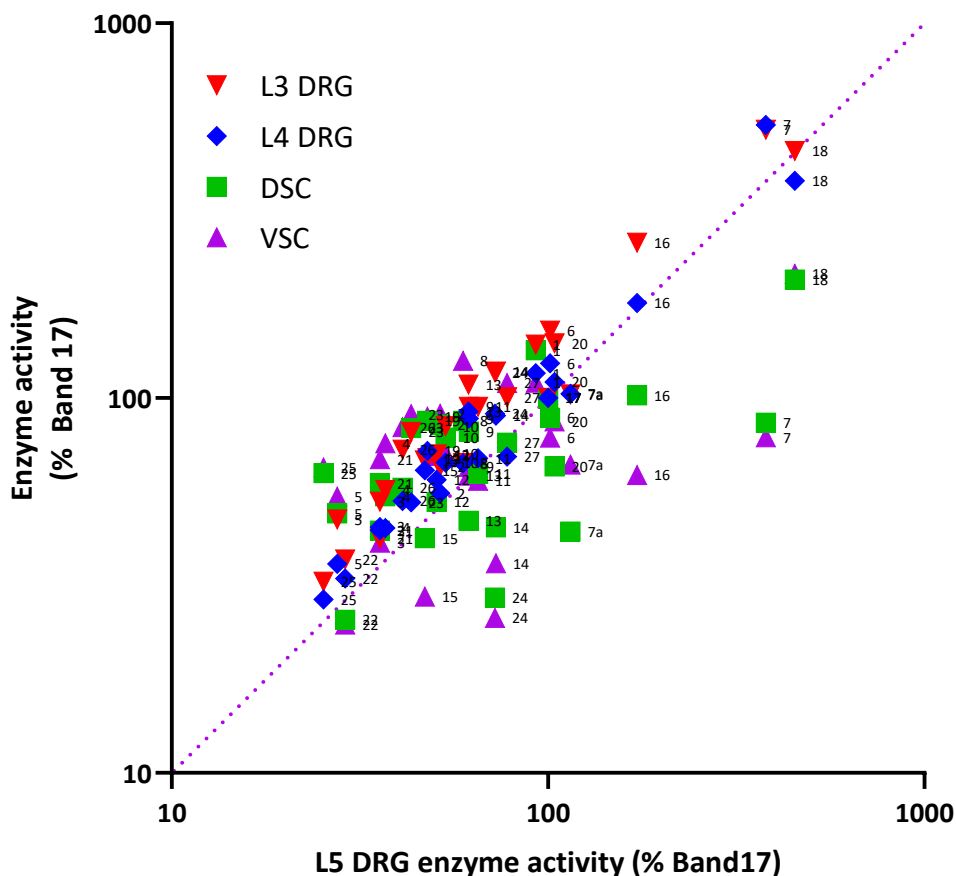
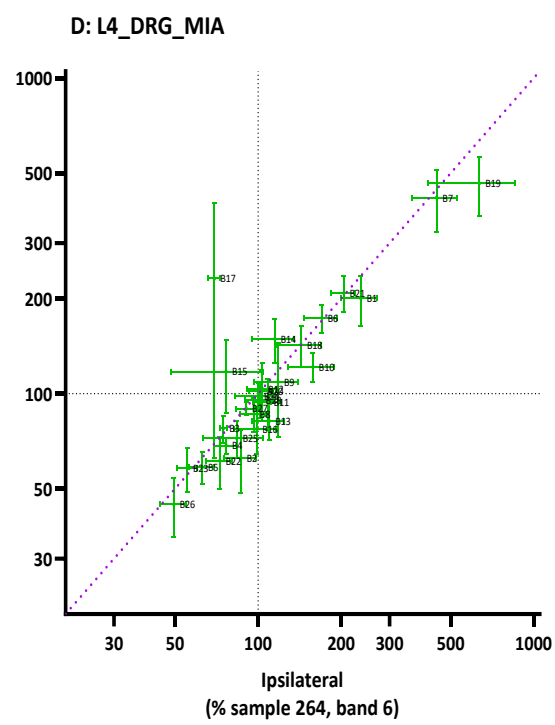
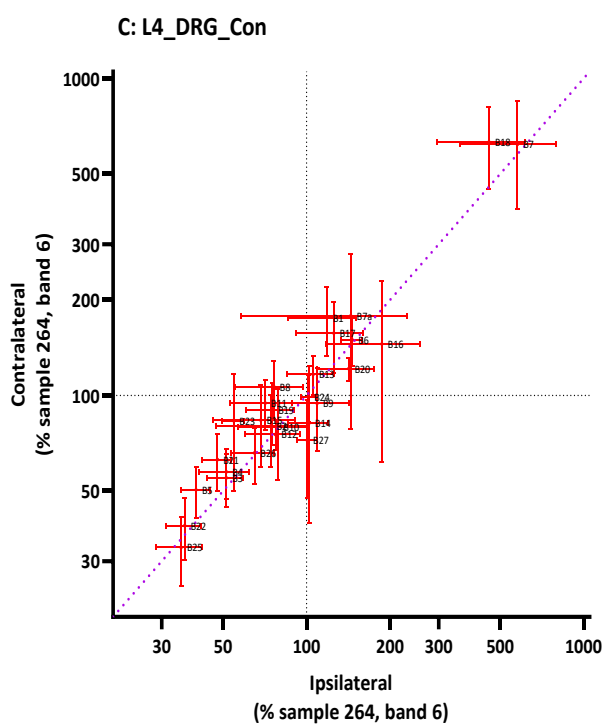
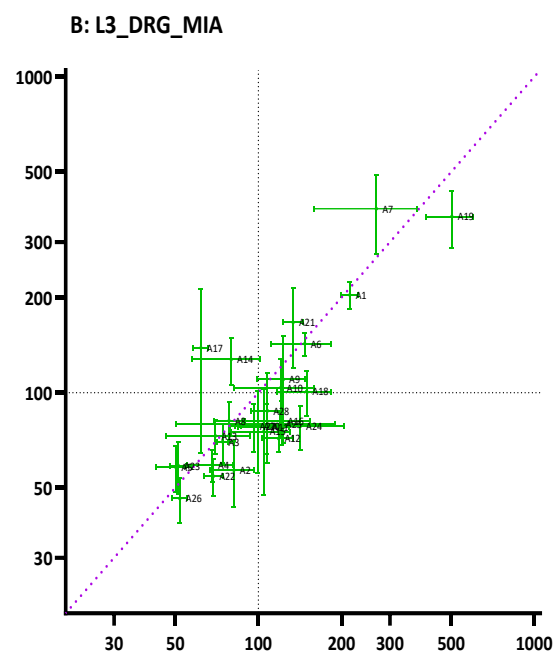
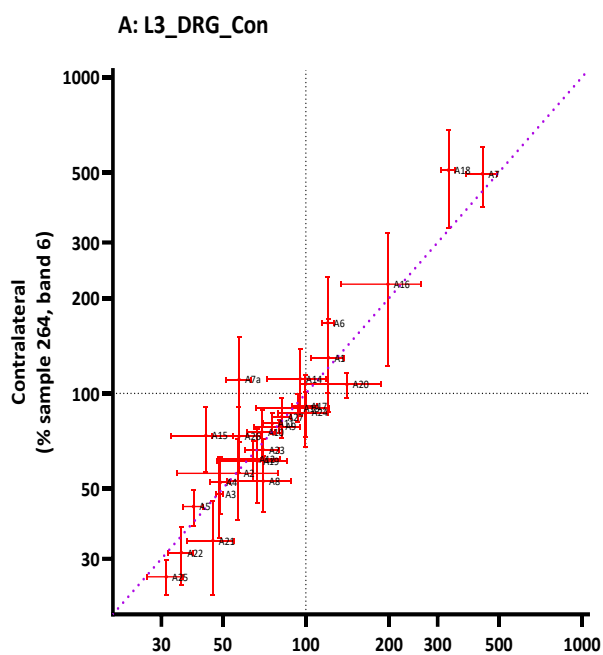
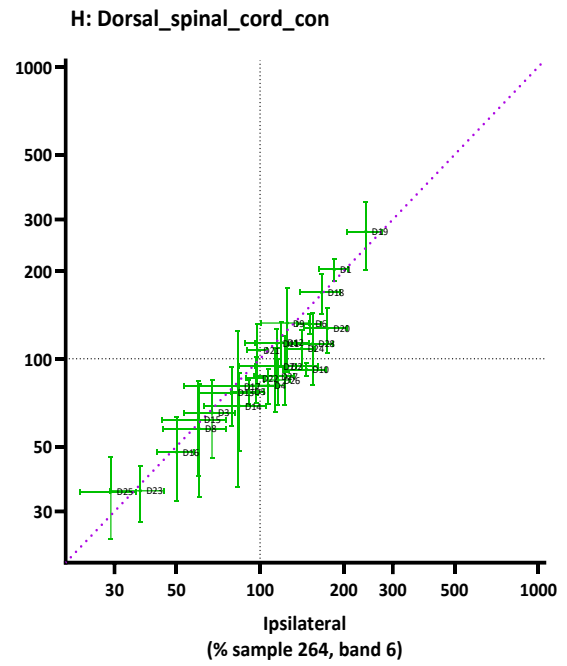
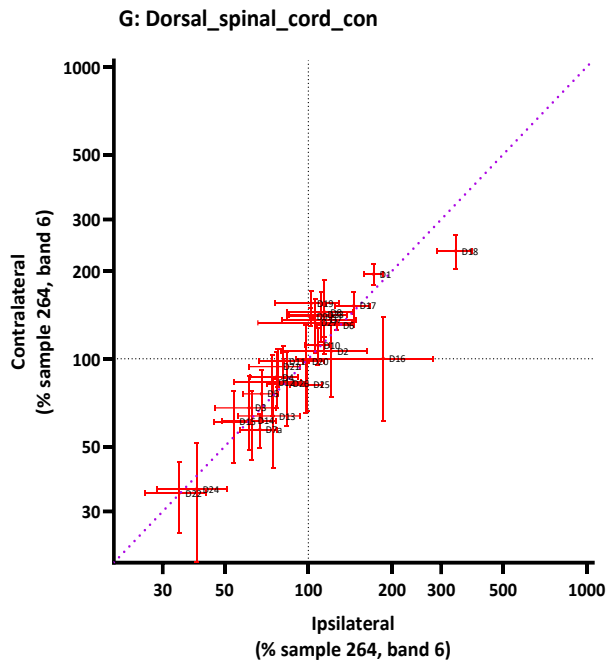
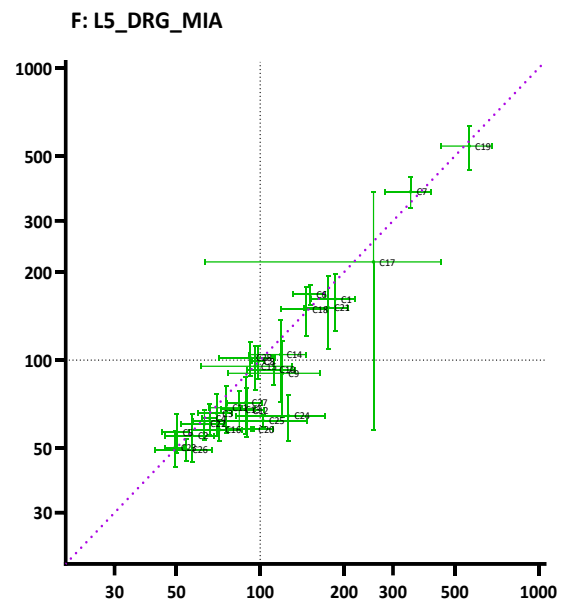
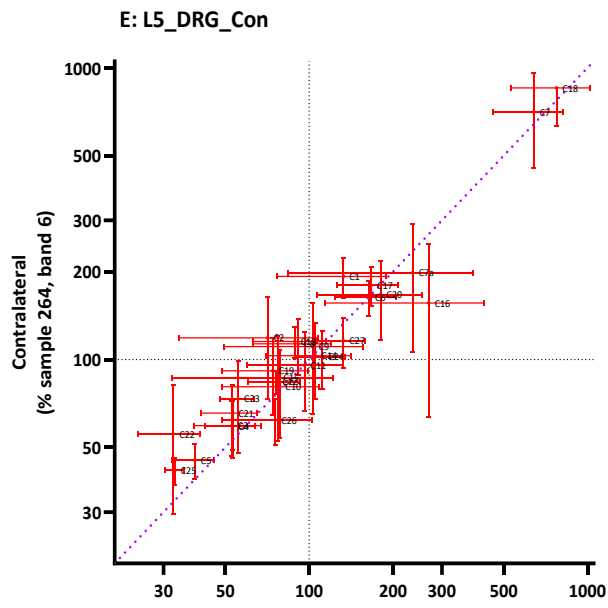


Figure 6. 3 Comparison between tissues in the control spinal system. For each lane on the gel, band density was normalised to the density of Band 17, which appeared close the median density on most gels. The datapoints represent activity in L3 or L4 DRG or dorsal or ventral spinal cord when compared to the same band in the L5 DRG samples.

6.4.3 The influence of the MIA model on serine hydrolase activities in the rat spinal sensory system

In this analysis, the hypothesis to be tested was that enzyme activities would be symmetrically distributed on ipsilateral compared to contralateral sides, while the unilateral MIA intervention would induce asymmetrical distribution in the test animals since the Pain signals can cross to the opposite side of the body through neural pathways like the spinothalamic tract. Using GraphPad Prism, the goodness of fit of the data from individual tissues to a line passing through the origin with a slope of unity was assessed.





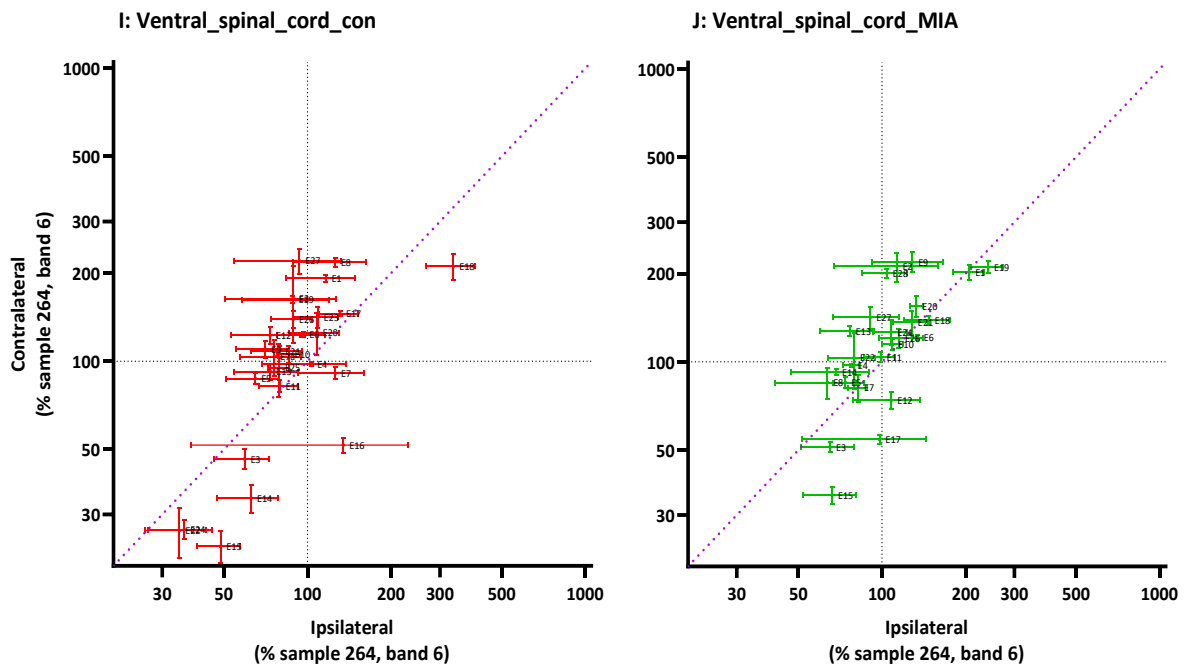


Figure 6. 4 Contralateral: ipsilateral correlation analysis of serine hydrolase activities in the rat spinal cord system in the MIA model of arthritis. Red and green circles represent data from control (A. L3 DRG, C. L4 DRG, E. L5 DRG, G. dorsal spinal cord, I. ventral spinal cord) and MIA (B. L3 DRG, D. L4 DRG, F. L5 DRG, H. dorsal spinal cord, J. ventral spinal cord) serine hydrolase activities, respectively. The purple dotted line is the line of symmetry.

Figure 6.4 demonstrates how symmetrically the activities of serine hydrolase enzymes are distributed around the line of unity and whether the hypothesis is correct for both control and MIA models. In L3, L4 and L5 DRGs of control animals, the band activities were symmetrically arranged around the line, consistent with the hypothesis. This shows that in DRGs from control animals, the band activities might increase or decrease but they would always be evenly altered on ipsi- and contralateral sides.

Table 6. 1 Symmetry analysis of serine hydrolases in the rat spinal sensory system

Control			MIA			
Tissue	Coefficient of determination (R ²)	Discrepancy (F)	P-value	Coefficient of determination (R ²)	Discrepancy (F)	P-value
L3 DRG	0.5834	0.7051	0.8511	0.4421	1.822	0.0190
L4 DRG	0.5676	0.3828	0.9973	0.4725	1.162	0.2941
L5 DRG	0.6469	0.2317	>0.9999	0.6721	0.3158	0.9995
Dorsal spinal cord	0.3097	2.101	0.0050	0.4444	0.8121	0.7282
Ventral spinal cord	0.08247	15.74	<0.0001	0.4887	16.04	<0.0001

Table 6.1 reflects the output from the Prism analysis of the data symmetry and shows that the variance in ipsilateral regions was dependent on the variance in the contralateral regions in control DRG (identified by the discrepancy value, F). That means the changes on ipsilateral and contralateral sides are matched, with P values ~1, not deviated far from the line.

However, for the spinal cord samples, the discrepancy value was increased, suggesting variance in ipsilateral regions was not dependent on the variance in the contralateral regions. The reasons for that might be because the lanes containing some spinal cord samples were distorted (see gel in Figure 6.1A). This may well have skewed the data causing some datapoints to distribute away from the line of unity. Lack of time and the small tissue samples obtained meant that these gels could not be repeated.

In tissue samples from the MIA model, the variance in ipsilateral regions was dependent on the variance in the contralateral regions in all regions, except for the L3 DRG and ventral spinal cord (Table 6.1). In the L3 DRG samples, several bands demonstrated changes which deviated from the line of unity. Examples of those bands were A7, A17 and A19 which possibly equate to bands CES2, MAGL, and ABDH6, respectively. The activity of bands A7, and A17 were shown to be higher on the contralateral as compared to the ipsilateral while that of band A19 was shown to be higher on the ipsilateral side.

6.5 Discussion

In this chapter, I describe the activities of serine hydrolase enzymes in the rat lumbar spinal system, namely in the DRG, dorsal spinal cord, and ventral spinal cord. The enzyme sizes were determined based on their molecular weights on the gel, which provides less confidence than the detection of the bands performed in Chapter 5, where pharmacological measurements were employed. In the spinal system, I detected 28 bands as compared to the 27 bands seen in the rat brain. I have also studied changes in the bands detected after pain induction in the MIA model. This analysis showed that several bands might play a role in pain perception and may be used in the future as potential targets for novel analgesics.

6.5.1 Identification of individual enzyme activities in the rat spinal system

The Rf of each band was calculated per demonstrated in section 2.4.7.1. The molecular weight of each unknown band was then calculated from the standard curve and illustrated in Table 6.2.

The process of identifying individual bands as enzyme activities relied on estimating the approximate molecular size of the band, followed by comparison with the pharmacological profile from the rat brain samples (Chapter 5) and online databases (notably UniProt). The small sample masses in the spinal system meant that analysis was conducted on the tissue homogenates rather than the soluble and particulate fractions. Table 6.2 also compared the bands between the DRG and spinal cord to those seen in the rat brain. The bands seen in the DRG and spinal cord look similar to those detected in the rat brain, with the exception of an extra band, No. 7a, which was not observed in the rat brain samples.

Table 6. 2 Estimation of molecular size and possible identification of the rat spinal system as compared to rat brain samples.

Rat brain			Rat spinal system		
Band No	MW (kDa)	Possible Identification	Band No	MW (kDa)	Comment
Band 1	203.3		Band 1	205.3	
Band 2	161.2		Band 2	162.0	
Band 3	95.8		Band 3	101.0	
Band 4	76.6	HSL	Band 4	85.5	
Band 5	71	DAGL β	Band 5	76.5	
Band 6	69	ACHE	Band 6	68.5	
Band 7	65	CES2	Band 7	65.7	More prominent in DRG than in the spinal cord
		FAAH	Band 7a	63.3	Visible in spinal samples, not rat brain
Band 8	56	FAAH	Band 8	57.2	More prominent in rat brain than in rat DRG and spinal cord
Band 9	54		Band 9	55.1	
Band 10	48	LPL	Band10	49.2	More prominent in rat brain than in rat DRG and spinal cord.
Band 11	46	ACOT2	Band 11	46.9	More prominent in rat brain than in rat DRG and spinal cord.
Band 12	43	ABHD12	Band 12	45.0	
Band 13	42	ACOT1	Band 13	42.4	
Band 14	41	ACOT2	Band 14	41.2	
Band 15	39	ACOT2	Band 15	41.0	
Band 16	37	ABHD4	Band 16	37.8	
Band 17	34	MAGL	Band 17	35.5	
Band 18	33	MAGL	Band 18	33.8	
Band 19	30	ABHD6	Band 19	32.3	More prominent in rat brain than in rat DRG and spinal cord.
Band 20	26		Band 20	24.7	
Band 21	24.9	LYPLA1	Band 21	22.4	
Band 22	21.3		Band 22	21.2	
Band 23	19.6	RBBP9	Band 23	20.1	More prominent in the spinal cord than in DRG
Band 24	18.7		Band 24	18.8	More prominent in DRG than in the spinal cord and rat brain
Band 25	18		Band 25	18.0	More prominent in the spinal cord than in DRG
Band 26	17.1		Band 26	17.4	More prominent in the spinal cord and rat brain than in DRG
Band 27	16.6		Band 27	16.8	More prominent in the spinal cord and rat brain than in DRG

6.5.2 Differential expression of enzyme activities

6.5.2.1 Bands more prominent in the spinal system

This analysis was conducted on Figures 6.1 and Table 6.2 to compare the expression of the proteins of interest between DRG and those of DSC and VSC using the rat brain (band number 264 on the gels) as a control. While all bands, except band 7a, were detected in both systems, there were some bands that were prominently active in the spinal cord. This was mainly the case for bands 23 (possibly RBBP9), 25, 26, and 27. Some other bands were more active in the DRG region as compared to the rat brain or the spinal cord system. That was the

case for band 7 (possibly CES2) and band 24. The higher activity of those bands in the spinal system as compared to the brain could suggest their involvement in pain transmission.

Interestingly, the top band of the FAAH (band 7a) was not apparent in rat brain, which was not consistent with previous literature where the FAAH was present in various areas of the human, mice and rat brains (Egertova et al., 2003, Blankman et al., 2007). Nevertheless, this result indicates that in our model, FAAH activity is more predominant in the DRG, indicating a more peripheral role for the endocannabinoid-metabolising enzyme FAAH in the rat model. A previous study investigated the distribution and activity of FAAH in the rat spinal cord and DRG. The authors found that FAAH activity was significantly higher in DRG than in spinal cord tissue, suggesting that this enzyme may play a more important role in regulating pain in the peripheral nervous system than in the central nervous system (Della Pietra et al., 2021).

6.5.2.2 Bands more prominent in the rat brain:

This analysis showed that other bands were highly active in the rat brain as compared to the spinal system. This was the case for Band 8 (a soluble activity inhibited by URB597), Band 10 (possibly LPL), Band 11 (possibly ACOT2), and Band 19 (ABHD6). The higher activity of those bands in the brain may suggest their involvement in particular brain pathways. That was the case for MAGL, which was reported to be the primary enzyme metabolising the endocannabinoid 2-AG in the brain. (Chen et al., 2012). Previous research showed that ABHD6 and ABHD12 are enzymes that hydrolyse, together with MAGL, approximately 99% of brain 2-AG activity (Savinainen et al., 2012). I found here that the activity of ABHD6 was high in the brain, while that of ABHD12 did not differ between the brain and the spinal cord. This suggests the different roles of these enzymes in CNS function.

The rest of the bands, including bands 4, 5, 6, 12–18, and 21, equate to HSL, DAGL β , ACHE, ABHD12, ACOT1, ACOT2, ABHD4, MAGL, and LYPLA1, respectively, showed similar activities between the spinal system and the brain. MAGL has been proposed to act as a unique metabolic hub connecting the endocannabinoid system with prostaglandin signalling pathways in the brain, so blocking this enzyme could offer a novel and potentially less risky approach to inhibiting the inflammatory processes responsible for neurodegenerative diseases, this was the case for animals treated with other anti-inflammatory drugs where they

exhibited spared the hemorrhaging caused by COX inhibitors in the gut(Nomura et al., 2011). When the activities of MAGL and FAAH were compared in the rat spinal system using ABPP, the activity of MAGL in the DRG and trigeminal ganglia was nine-fold higher than that of FAAH, implicating MAGL as a priority target for blocking peripheral mechanisms of pain (Della Pietra et al., 2021).

The activity of band 19 (ABHD6) was previously reported in mice DRG and spinal cord, but it is not clear whether their levels differed between these different regions (Wen et al., 2018). This finding was replicated in the current study, where the activity of ABHD6 did not differ between the DRG and spinal cord but was less than that seen in the brain.

6.5.3 Change of enzyme activities after pain induction

In MIA-treated animals, the expression of some bands was altered in the spinal system. In DRG, the expression of bands 9, 19, and 20 equates to MAGL, and ABHD6, which were elevated in the pain model, indicating the role they play in pain transmission (Figure 6.1). Figure 6.4 showed that there are three main bands that were deviated from the line of unity. Among those bands, band A19, which equates to ABHD6, increased on the ipsilateral as compared to the contralateral side, which indicates a potential involvement in pain progression. This finding suggests ABHD6 could be targeted for the future development of analgesic medications for patients with chronic pain. More research is required for other bands that only increased on the contralateral side, including A7 and A17 which possibly equate to CES2 and MAGL, suggesting a compensatory role in weight-bearing.

ABHD6 has been shown to play a role in controlling pain responses in mouse sciatic nerve. Inhibition of ABHD6 with WWL70 significantly alleviated chronic constriction injury-induced thermal hyperalgesia and mechanical allodynia, by reducing the production of the inflammatory mediators in the spinal cord dorsal horn. However, since cannabinoid receptor antagonists did not reverse its effect on pain, this indicates that ABHD6 induced an antinociceptive effect through a novel mechanism independent of cannabinoid receptors (Wen et al., 2018). Other enzymes playing a role in pain is MAGL. Research showed that blocking MAGL and FAAH caused CB1-dependent antinociceptive effects in mouse models of neuropathic pain (Kinsey et al., 2009). While pharmacological inhibition of MAGL or ABHD6

reversed periorbital allodynia associated with cortical spreading depression induction in a rat model (Liktor-Busa et al., 2023).

This could explain the elevation in the levels of MAGL in my MIA animals, as they might be among the pathways activated following the MIA injection, but this finding may very well suggest a potential therapeutic target for patients with neurotrophic pain. Cholinergic receptor activation via pharmacological agents was shown to reduce pain in animal and human models (Naser and Kuner, 2018). ACHE, which breaks down ACH, can cause worsening pain. The activity of band 24 (LYPLA1) decreased in the MIA group. LYPLA1 catalyses the thioester hydrolysis of palmitoylated cysteine residues. There was no report in the literature linked LYPLA1 to pain perception, and hence further research is required to understand the role of LYPLA1 in pain progression.

6.5.4 Other methodologies to validate the expression of serine enzymes in the model

The ABPP technique is a well-established technique and has been previously used in other models to validate the expression of various serine hydrolysis enzymes. Research conducted by colleagues in Nottingham studied the differences in serine hydrolase enzymatic activity between platelets and red blood cells. The overall finding showed that FAAH activity was highest in platelets, while MAGL activity was most abundant in erythrocytes (Anajirih et al., 2023). Using the ABPP technique, several enzymes have been detected and the study was robust and reproducible. The use of the pharmacological inhibitors selective for these enzymes confirmed the presence of their enzymes and based on this analysis several enzymes have been detected, including LYPLA2, ACHE, MAGL, and FAAH, which appeared at comparable sizes to those observed in my study (Anajirih et al., 2023b). In addition, Szafran et al. study used ABPP technique to detect the activity of carboxylesterase enzymes in lung inflammation, they also presented the activity of CES1/2 ~65kDa which is consistent to the size I detect CES2 enzymes in my samples (Szafran et al., 2022).

However, further research is needed to confirm and better understand these differences. This may be achieved by performing a radiometric assay usually regarded as the 'Gold Standard' assay for the measurement of catabolic enzymes such as FAAH and MAGL activities (Dinh et al., 2002, Bari et al., 2022). Likewise, other enzyme activities can be determined using different techniques such as ABHD6 which can be measured using its ability

to hydrolyse 4-MUH to produce the fluorescent compound 4-MU which can be detected using a spectrophotometer (Nada Mahmood, PhD thesis, 2018), (Miralpeix et al., 2021).

Additionally, expression of the enzymes can be tested using immunoblotting. This was the case for FAAH as it has been widely studied in the literature (Leung et al., 2013). Also the expression of MAGL and CES1 has been determined using immunoblotting (Szafran et al., 2018). However, while this technique is simple, rapid, cheap, and semi-quantitative finding specific antibody for specific protein was proven challenging in some cases, which could be considered as a limitation for a high scale analysis.

The use of specific further selective enzyme inhibitors would be beneficial to confirm the activity of several serine hydrolysis hydrolase enzymes; particularly the ones that were tentatively detected in the current study. I have discussed several novel inhibitors for EC hydrolysis enzymes in chapter 5.

Lastly, the expression of these enzymes in rat spinal or brain systems can be determined at mRNA level using techniques such as quantitative polymerase chain reaction (qPCR) analysis. By using the levels of mRNA encoding specific enzymes, one can confirm whether the serine hydrolysis gene expression is induced or suppressed in my rat samples.

CHAPTER 7

General Discussion and Future Studies

7.1 General Discussion

Despite encouraging antineoplastic results that have been reported since 1975 (Munson et al., 1975), the potential antitumour activity of cannabinoids remained neglected until the detection of the endocannabinoid system (ECS) in the 90s; clinical data for cannabinoids in cancer continue to be remarkably rare. The first part of this thesis addressed the effects of cannabinoids and cannabinoid-related compounds including endo, phyto, and synthetic cannabinoids in addition to a set of inhibitors of the major enzymes involved in endocannabinoid synthesis and degradation on the proliferation of a human astrocytoma (1321N1) cell line using a standard colorimetric MTT assay.

7.1.1 The involvement of endocannabinoid system in regulating glial cell proliferation

The modestly CB2 preferring HU210 or CB2-selective agonist JWH015, but not the CB1-selective agonist ACEA, appeared to increase 1321N1 cell numbers. These data suggest a potential protective role for CB2 receptors in glial cell proliferation. The involvement of CB2 receptor in this potential cytoprotective effect is suggested as the presence of the selective CB2 receptor antagonist, AM630, appeared to inhibit the JWH015-induced increase in 1321N1 cell numbers. However, this inhibition did not reach statistical significance (Fig 3-10).

In contrast to the multiple effects of CB1 receptor deletion, the reported consequences of knocking out CB2 receptors are much more subtle, primarily involving modest perturbations in the immune system (Buckley, 2008, Buckley et al., 2000). Most of what we know about CB2 receptor function comes from pharmacological studies using CB2 receptor agonists and antagonists. These studies suffer from the usual caveats of pharmacological studies—the possible confounding factors of inadequate drug selectivity. Nonetheless, there is now considerable evidence for a CB2 role in several pathologies lacking

satisfactory treatments. The pathological processes for which the evidence of CB2 involvement is strongest, and also those that are perhaps the most promising in terms of drug development are neuroinflammation and pain (Bryk and Starowicz, 2021, Smoum et al., 2022).

However, therapeutic opportunities based on the manipulation of CB2 receptors exist for many other pathological conditions, including systemic inflammation, osteoporosis, cancer, and transplantation biology, several CNS disorders including drug addiction and anxiety, and liver disease (Bab et al., 2009, Karsak et al., 2007, Mallat and Lotersztajn, 2010, Nagarkatti et al., 2010, Patel et al., 2010, Xi et al., 2011).

The selective (compared to CB2 receptors) CB1 receptor inverse agonist AM251 decreased 1321N1 cell viability which suggested a protective role for the cannabinoid signalling system in human astrocytoma cells. However, the inhibitory effect of AM251 was not mimicked when cells were treated with CP945598 (a structurally distinct CB1 antagonist), a CB2-selective antagonist (AM630) or a CB1 selective agonist (ACEA). This suggests that the inhibitory effect of AM251 may not be mediated by its activity as a CB1 antagonist.

In line with my finding, AM251 and its iodinated analogue rimonabant significantly decreased the viability and caused apoptosis in two *in vitro* models of prostate cancer; DU-145 and PC3 cells (Louka et al., 2020, Sarnataro et al., 2006). With regard to the molecular mechanism by which AM251 limited cell numbers, reports showed that treatment with AM251 led to cleavage of caspase3/8 and PARP protein which, in turn, led to DNA fragmentation, and apoptosis induction in DU-145 cells (Louka et al., 2020). In addition, rimonabant inhibited breast cancer (MDA-MB-231) cell proliferation with a mechanism involving CB1 receptors (Sarnataro et al., 2006).

On the other hand, in different cell lines such as astrocytoma cells, the expression of cannabinoid receptors was shown to be important for astrocytoma cell survival. Brain tumour (DBT) cell line treated with CP-55,940 showed an increase in ERK1/2 phosphorylation which ultimately led to a decrease in cell survival which was independent of CB1, CB2 receptors; particularly at high concentrations of cannabinoid agonist (Cudaback et al., 2010). This supports the observation that the effects of AM251 on 1321N1 cell viability might be independent of cannabinoid receptors.

AM251 and rimonabant were reported to have further targets including positive allosteric modulation of GABA_A receptors (Baur et al., 2012), TRPV1- and TRPA1-mediated responses in sensory neurons. AM251 is also suggested as potential agonist for several receptors that are related to the endocannabinoid system including GPR55 and GPR18 (Patil et al., 2011, Henstridge et al., 2010, Kapur et al., 2009, Lan et al., 1999, McHugh et al., 2012, Pryce and Baker, 2017, Ryberg et al., 2007). A role for ERK/MAP kinase signalling during microglial activation and the promotion of cancer cell proliferation by GPR55 has been proposed. Thus, the actions of AM251 and rimonabant, which have been widely interpreted as being mediated by CB1, may, in fact, include off-target cannabinoid effects, some of which might be mediated by GPR55 activation. Given that observation, the effects of a GPR55 antagonist (CID16020046) were investigated; but when I examined it in the current study this agent failed to reverse the effects of AM251, suggesting this target was not involved. However, other GPR55 antagonists with more specificity might be used such as ML192 or ML193 to confirm the involvement of the receptor in the AM251 response (Heynen-Genel et al., 2011).

To confirm the findings presented in this thesis, various behavioural tests could be conducted on an in vivo rat model treated with AM251, both with and without the presence of GPR55 antagonists like ML192. Previously, AM251 was shown to enhance memory and improved retention of contextual fear conditioning in rat models (Sink et al., 2010). The impact of AM251 on rats could be assessed alongside the GPR55 antagonist ML192 to determine if GPR55 plays a role in the AM251-induced improvement in contextual fear conditioning in rat models. In humans, rimonabant treatment was linked to an increase in anxiety, depression and suicidal ideation in some patients (Ettaro et al., 2020). In that respect the effect of GPR55 antagonist could be used to ameliorate the effects of rimonabant and decrease the anxiety and depression phenotype in obese patients.

In addition, given the complexity of responses from the agonists and antagonists used in this study, including AM251, further techniques can be employed to verify the specificity of these drugs against the receptors. For instance, novel CRISPR/Cas9 technology can be utilized to knock out the expression of GPR55 receptors. Subsequently, by testing the effect of cannabinoid receptor antagonists, such as AM251, one can confirm whether AM251 is still

capable of eliciting its effect in GPR55 knock-out model. This approach can assist determining if the GPR55 receptor is involved in the observed response to AM251 on cell viability.

Interestingly, particular enzyme inhibitors, namely MAFP, JZL184 and WWL70, but not URB597, induced a small, but significant reduction in 1321N1 cell proliferation, at a concentration of 1 μ M (but not 0.1 μ M). This result indicated that some of the cannabinoid-metabolising enzymes, notably the esterases rather than the amidase, may play a role in the protection of human astrocytoma cells from apoptosis. In this context, a previous PhD student (Nada Mahmood) in the same group observed that attempting to express MAGL and ABHD6 stably in CHO fibroblasts was unsuccessful as the cells failed to survive. This suggests that there is an optimal level of the substrates (or products) of these enzymes, whether endocannabinoid or other lipid ester, to maintain cell function.

A competitive ABPP experiment was performed to investigate a set of inhibitors of various enzymes involved in the endocannabinoid metabolism and biotransformation in human astrocytoma 1321N1 cells. This methodology is complementary and directly reports on the identity and activity of many enzymes in parallel in their native environment with all post-translational modifications in place and allows not only the possibility of hypothesis testing, but also the opportunity for hypothesis generation, since there are often less well characterized enzyme activities quantified. For this study, the purpose of the ABPP analysis was to gather biochemical evidence for the presence of the esterases, which appeared to regulate 1321N1 cell numbers. In addition, the lack of effect of FAAH inhibition might be explained either by an absence of the enzyme or the lack of influence of FAAH substrates/products.

We selected the SH-SY5Y neuroblastoma cell line as our second in vitro model for this study. SH-SY5Y cells are a well-documented in vitro model for the study of neuronal biochemistry, pharmacology, and pathological conditions in healthy humans (Kovalevich and Langford, 2013, Xicoy et al., 2017) and has been reported to express mRNA and protein for components of the endocannabinoid system in SH-SY5Y cells. These components include NAPE-PLD, FAAH, DAGL, and MAGL (Pasquariello et al., 2009).

Samples were labelled with fluorescent activity-based probes, and this allowed for the imaging of probe targets using SDS-PAGE and in-gel fluorescence scanning. ABHD6, MAGL,

and FAAH are among the numerous hydrolases labelled by FP-TAMRA (Niphakis and Cravatt, 2014, Liu et al., 1999). Since FP-rhodamine does not work well to label DAGL, Mario van der Stelt and his team synthesised MB064 instead. The customised lipase probe MB064 interacts preferentially with DAGLalpha, DAGLbeta, ABHD6, and ABHD12 proteins (van Esbroeck et al., 2019, Baggelaar et al., 2013b).

Endocannabinoid-metabolising enzymes play a vital role in generation and degradation of many signalling molecules which may involve in proliferation, migration, and invasion of a broad range of healthy and malignant cells. The expression and function of EC-metabolising enzymes should be elucidated further in other human glioma cell line models like LN 18, U87 and U251, human GBM stem cell survival in comparison to healthy tissue specimens and evaluate their role for patient's survival.

Although the investigation of inhibitors in the differentiated SH-SY5Y cells was unsuccessful, for unclear reasons, using inhibitors with the undifferentiated cells suggested weak activity of MAGL as a doublet (membrane bands 6 & 7) and singlet (cytosol band 10) sensitive to MAFP and JZL184 (at least in the membrane). This is consistent with the report that showed low MAGL expression in SH-SY5Y cells, as evidenced by the lack of sensitivity of [³H]-2-OG hydrolysis to inhibition by JZL184 (Szeremeta et al., 2019).

From a simplistic viewpoint, the use of FP-rhodamine identifies a distinct pattern of activities in the two cell lines, highlighting (presumably) distinct functional roles of serine hydrolases in astrocytes and neurons. Eleven intense bands were identified in the membrane and ten intense bands in the cytosol of 1321N1 cells. On the other hand, SH-SY5Y cells exhibited 11 bands at the membrane and 15 bands in the cytosol.

The bands unique to preparations from 1321N1 cytosol might be ACOT1, ABHD12, and LYPLA1. In contrast, the band unique to membrane-bound preparations from SH-SY5Y cells might be RBBP9. These would need to be confirmed at gene level, for example, by RT-qPCR, and their roles in the two different cell types investigated further. While pharmacological tools may not be available for all of these enzymes, molecular biological means of inhibiting enzymes may be used to characterise the activities further and substantiate potential roles in cell proliferation.

In the investigation of rat brain regions, either soluble or particulate fractions, FP-rhodamine-tagged activities appeared very similar in number. However, the pattern of intensities was distinct when comparing soluble with particulate fractions. Particulate fractions are associated with membrane-bound and structural components of the cell, while soluble fractions consist of the dissolved substances in the cytosol. The expression of the majority of the serine hydrolase enzymes in the soluble fractions was higher than those in the particulate. The lack of major variation in band intensities across different brain regions suggests that these serine hydrolases perform similar functions in these diverse areas, which may be an indication of common neuronal or glial functions. There were some exceptions to these observations for instance, the midbrain in the soluble fraction showed a distinctive band (band 5) at the level of about 71 kDa molecular weight as compared to the rest of the brain regions. Additionally, band12 appeared only in the particulate fraction with no activity seen in the soluble fraction.

The relatively consistent activities of MAGL and ABHD6 across different brain regions are consistent with the broad expression of the endocannabinoid system in the central nervous system. Given the capacity of the ABPP technique in terms of being able to measure activity in μg quantities of tissue, it will be of interest to investigate smaller areas and nuclei from the rat brain in the future. That might be subregions of the hippocampus associated with memory processing or nuclei in the hypothalamus associated with nutrient sensing or pain processing.

The preliminary identification of the bands was conducted based on the molecular weight of rat serine hydrolase enzymes obtained from Uniport or from the literature. This prediction was confirmed using non-selective or (where available) selective inhibitors for those enzymes. This was the case for certain enzymes such as MAGL where its soluble activities were significantly inhibited by 2-AG and MAFP, while its particulate activity was significantly inhibited by JKK048, which is a potent and selective inhibitor for MAGL (Aaltonen et al., 2013). However, for some enzymes, the pharmacological analysis was not conclusive, as it did not show the pattern of inhibition we hypothesized. That is the case for band 7, which may equate to CES2. Literature showed that the activity of CES2 is inhibited by JZL184 and URB597 (Crow et al., 2012). However, in my analysis neither of these drugs had an effect on band 7 activity which argue against the prediction. That is interesting and suggests that more

selective drugs need to be investigated. For example novel selective inhibitors at CES2 were described namely 1-(3,4-dimethylphenyl)-2-phenylethane-1,2-dione and 1-{4-[oxo(phenyl)acetyl]phenyl}-2-phenylethane-1,2-dione (Hatfield et al., 2010). These inhibitors and others could be used in a subsequent study to confirm the presence of CES2 in the brain samples. That was also the case for ABHD12, where more recent selective inhibitors such as DO264 can be used to confirm its expression (Ogasawara et al., 2019).

An alternative interpretation of the incomplete inhibition by the agents employed is the concomitant incubation of inhibitors or alternative substrates with the FP-rhodamine and tissue samples. While prior incubation is likely to have enhanced inhibitory effects of some of the irreversible agents employed (URB597, MAFP, etc.), this was avoided since pre-incubation would also result in hydrolysis of the alternative substrates (AEA, 2OG, etc) and a reduced inhibitory effect. Hence this approach might be the reason behind the lack of effect of some drugs used in this study on some of the enzyme's activity.

On the other hand, regarding other enzymes including ACOT2 or RBBP9, which appear to equate to bands 11 and 23, respectively, other techniques can be used to confirm their expression at the gene level such as qPCR or protein level such as immunoblot. Alternatively, products generated after the hydrolysis of some of these enzymes can be identified on mass spectrometry to confirm the presence of these enzymes in the brain samples. This approach was used previously by Ali et al. (2012) to identify products generated by HSL activity (Aaltonen et al., 2013).

Several enzymes showed higher activity in various areas of the brain including the hippocampus and frontal cortex. This was the case for MAGL and ABHD6. This finding is interesting as it may indicate that those enzymes play a potential role in memory and learning. Those enzymes may be used as targets in the future to manage conditions like cognitive impairments or memory loss.

7.1.2 The involvement of endocannabinoid system in regulating pain signalling

Several endocannabinoid metabolising enzymes can be targeted to manage neuropathic pain since the endocannabinoid system is one endogenous system involved in the regulation of pain and inflammatory response (Katona and Freund, 2008). In this thesis, I

determined the changes in endocannabinoid metabolising enzyme activities between various areas of the spinal level: dorsal root ganglia, dorsal spinal cord, and ventral spinal cord. I also investigated the changes in enzyme activities in the MIA model where several enzymes including MAGL and ABHD6 were shown to change indicating their involvement in the pain process. While my research highlighted the importance for further research into other targets, such as CES2 and MAGL, where their activities only increased on the contralateral side, suggesting a compensatory role in weight-bearing.

In agreement with that finding previous research showed that dual FAAH/MAGL inhibitors were effective in both inflammatory and mechanically evoked visceral pain (Sakin et al., 2015). This indicates the necessity of considering MAGL as a potential target for pain relief medication.

7.2 Future Work

7.2.1 Future investigating cannabinoid effects on cell viability

The selective CB1 inverse agonist AM251 provoked a concentration-dependent decrease in cell viability of human astrocytoma, 1321N1 cells, however the exact mechanism for this effect is yet to be determined. Elucidation of this mechanism in future work would be very important to find out whether the cannabinoid signalling pathway is involved in mediating this effect. The identification of cannabinoid ligand sensitivity and receptor expression by intracellular organelles involved in cell viability, like mitochondria, would be of interest.

Previous research showed that AEA and the phyto-cannabinoid Delta-9-tetrahydrocannabinol (THC) caused morphological changes characteristic of apoptosis in human lung cancer (H460) cells. While these drugs caused significant decreases in oxygen consumption and mitochondrial membrane potential in rat heart mitochondria, as well as significant increases in mitochondrial hydrogen peroxide production. This finding indicated the ability of cannabinoid agonists to induce changes in integrated mitochondrial function (Athanasίου et al., 2007). In addition, intracellular CB1 receptors and mitochondrial processes are involved in regulating endocannabinoids in the hippocampus, which impacts neuronal energy metabolism (Bénard et al., 2012). Therefore, it is important to study the relationship

between cannabinoid signalling pathways and mitochondrial complexes and their impact on 1321N1 cell viability.

Furthermore, a structure-activity relationship for analogues of AM251 (Lan et al. 1999) would be of interest, to determine whether higher potency compounds exist to regulate astrocytoma viability. Although there would need to be further validation experiments, the possibility of generating agents which could inhibit glioblastoma growth in human patients would be the ultimate aim.

This study also encompassed a preliminary protein profiling of two human cell lines namely, 1321N1 and SHSY5Y. Both cell lines showed a profile of the main enzymes for cannabinoid metabolism which was quite distinct from that observed in the rat brain proteome. This should be considered in future work involving cannabinoid metabolism and signalling pathways. Whether isolated astrocytes and neurons from the rat brain show greater similarities to the cultured cells will be of interest. Additionally, further work with the ABPP technique would be directed to identify the serine hydrolases in human proteome including samples isolated from healthy, post-mortem, and fresh biopsies from patients with various stages of glioblastoma multiforme. Indeed, the group have previously been able to quantify endocannabinoid hydrolases in human post-mortem brains and identify changes in alcoholic and schizophrenic subjects (Erdozain, 2015; Muguruza, 2019).

7.2.2 Future avenues of the ABPP enzymatic identification and pain findings

Although the pharmacological competitive ABPP approach allows preliminary identification of enzyme activities, this is obviously limited by the availability of suitably selective tools. Biotin-FP is a probe which, when coupled with proteomics/mass spectrometry, allows data to be obtained about SH enzymes in a more unequivocal manner. Using tissue samples from animal models and post-mortem/surgical material would allow the identification of individual (possibly novel) enzymes, which may develop into potential therapeutic targets for poorly treated human diseases/disorders.

In here, I attempted to use selective inhibitors for endocannabinoid metabolising enzymes; several enzymes have no commercially available, selective inhibitors, which makes it difficult to confirm their identity. A wider medicinal chemistry program should be able to

generate novel probe molecules for these enzymes (based on previous successful programs in the area). Once novel selective inhibitors are available, they can be used as leading molecules to manage disease conditions in animal models. This may be appropriate for some key enzymes such as MAGL, ABHD6 and FAAH where they potentially play a significant role in the pain process, but their activities were not confirmed with confidence due to the lack of specific enzymes in the market.

In chapter 7 the identity of bands 1, 2, 3, 9, 20 and 24-27 were not identified due to the lack of selective inhibitors. These bands might be detected via a novel technique involves combining the data-independent acquisition (DIA) mass spectrometry with ABPP to develop an efficient label-free quantitative chemical proteomic detection (Yang et al., 2022). This novel approach will enable an efficient detection of serine hydrolases bands on ABPP gel, and it can be used to confirm the identity of the bands on ABPP gels, but primarily can be used to detect those bands that I was not able to identify. By confirming the identity of these bands, one will be able to study their role in pain or other disease mechanisms such as cognitive impairments; particularly because the activity of some of these bands such as 20 and 26 increased in hippocampus which may suggest roles for those enzymes in learning and memory.

Lastly, all of this work has been conducted in rodent models or human cell lines i.e., SH-SY5Y. It would be useful to replicate this work in 'real' human samples, such as surgical or post-mortem brain and spinal system from patients with chronic pain. In addition, we can use novel technologies such as induced pluripotent stem cells derived sensory neurons or astrocyte to elucidate the changes happened in a more realistic human model system where treatment with, for example, inflammatory mediators can be explored.

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