Exploring the Bioactivity and Therapeutic Potential of Structurally Diverse Phytochemicals in Neurodegenerative and Gastrointestinal Disease

A thesis by

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Publications included in this thesis

1) Marsh, D. T. and Smid, S. D. (2021) Cannabis Phytochemicals: A Review of Phytocannabinoid Chemistry and Bioactivity as Neuroprotective Agents. *Australian Journal of Chemistry*. 74, 388-404.

Abstract

Cannabis sativa is the source of over 540 natural products, including phytocannabinoids, terpenoids and flavonoids. These natural products have been ascribed protective properites in various contexts, including neurodegenerative and gastrointestinal diseases. However, the pharmacological diversity of Cannabis is yet to be truly explored, offering exciting potential in the treatment of such diseases.

Alzheimer's Disease (AD) is the most common neurodegenerative disease, characterised by the progressive loss of cholinergic neuronal structure and synaptic function. The pathological hallmark of AD is the aggregation of amyloid β (Aβ) and its associated neurotoxicity, which result in this neruonal loss. Inflammation and oxidative stress are crucial factors not only in the progression of AD but also in inflammatory bowel disease (IBD) and gastrointestinal mucositis (GIM), resulting in reduced epithelial barrier function. Therefore, a drug which reducess the cytotoxicity of Aβ, maintains barrier function and possesses anti-inflammatory and antioxidant properties may offer wide-ranging benefits in the amelioration of AD, IBD and GIM.

Treatment of PC12 cells with Aβ results in significant loss of cell viability in the MTT assay, while also inducing key changes in neuronal cell morphology as determined by fluorescence microscopy. We identified novel bioactivity for the phytocannabinoids cannabigerol (CBG), cannabinol (CBN) and cannabichromene (CBC), which effectively prevented this loss of viability in a manner independent of either antioxidant capacity or direct interactions with Aβ, while also maintaining healthy cell morphology in the presence of Aβ. Δ^9-tetrahydrocannabinol (Δ^9-THC) significantly inhibited Aβ neurotoxicity, while cannabidiol (CBD) proved an effective antioxidant with no significant effect on Aβ-evoked neurotoxicity; cannabidivarin (CBDV) reduced neuritic projections in PC12 cells without affecting cell viability.

To assess the neuroprotective capacities of whole botannical cannabis extracts, in comaprison with pure phytocannabinoids, five proprietary chemovars were selected. The Δ^9-THC-dominant

chemovars BC-101, BC-201 and BC-401 prevented Aβ-induced neurotoxicity in NSC-34 cells without inhibiting *tert*-butyl hydroperxoide (*t*bhp)-induced lip peroxidation; no significant protection was observed following treatment with the CBD-dominant chemovars BC-301 or BC-501; heating of each extract reduced their neuroprotective capacity.

The final two studies investigated phytocannabinoids and flavonoids as inhibitors of cytokine and 7-ethyl-10-hydroxycamptothecin (SN-38)-evoked decreases in epithelial barrier function in Caco-2 cells. We identified novel roles for CBD, CBG and CBDV as inhibitors of SN-38-evoked increases in permeability, with each phytocannabinoid screened, including CBC, inhibiting cytokine-evoked increases in permeability; no antioxidant capacity was observed in the DCFDA assay. A novel role was identified for the flavonoid 2-D08 as a dual inhibitor of cytokine and SN-38-induced permeability increases, with novel protection also demonstrated for the flavonoids myricetin and transilitin against SN-38; each flavonoid significantly inhibited reactive oxygen species generation in the DCFDA assay.

In conclusion, this thesis demonstrates novel protective roles for phytocannabinoids as inhibitors of Aβ-evoked neurotoxicity and changes in neuronal morphology, as well as novel roles for select phytocannabinoids and flavonoids as inhibitors of cytokine and SN-38-evoked reductions in epithelial barrier function. These findings highlight the wide-ranging potential of natural products as protective agents in the context of AD, IBD and GIM, highlighting novel lead candidates for drug development targeting these conditions.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Dylan Thomas Marsh **Date**

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Abbreviations

2-D08	2’3’4’-trihydroxyflavone
3NP	3-nitropropionate
5-LO	5-lipoxygenase
5-HT1A	5-hydroxytryptamine receptor 1A
6-OHDA	6-hydroxydopamine
ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer’s disease
ALS	amyotrophic lateral sclerosis
APP	amyloid precursor protein
Aβ	amyloid beta
BACE	beta-secretase
BChE	butyrylcholinesterase
BSA	bovine serum albumin
CB1R	cannabinoid 1 receptor
CB2R	cannabinoid 2 receptor
CBC	cannabichromene
CBCA	cannabichromenic acid
CBD	cannabidiol
CBDA	cannabidiolic acid
CBDV	cannabidivarin
CBE	cannabielsoin
CBEA-A	cannabielsoic acid-A
CBEA-B	cannabielsoic acid-B
CBG	cannabigerol
CBGA	cannabigerolic acid
CBL	cannabicyclol
CBLA	cannabicyclolic acid
CBN	cannabinol
CBND	cannabinodiol
CBR	cannabinoid receptor
CBT	cannabitriol
CD	Crohn’s Disease
CFA	cannflavin A
CoA	coenzyme A
COX-2	cyclooxygenase 2

DCFDA	2’7’-dichlorofluorescin diacetate
DFT	density functional theory
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	dimethyl sulfoxide
DSS	dextran sulphate sodium
EGCG	epigallocatechin gallate
FAD	flavin adenine dinucleotide
FBS	foetal bovine serum
FOLFIRI	folinic acid, 5-fluoruracil and irinotecan
GI	gastrointestinal
GIM	gastrointestinal mucositis
H_2O_2	hydrogen peroxide
HD	Huntington’s disease
HNE	4-hydroxyl-2-nonenal
HO-1	heme oxygenase 1
Hsp32	heat shock protein 32
IBD	inflammatory bowel disease
IFNγ	interferon gamma
IL-1β	interleukin 1 beta
IL6	interleukin 6
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MND	motoneurone disease
mPGES	microsomal prostaglandin E2 synthase
MPP+	1-methyl-4-phenylpyridinium
MTT	thiazolyl blue tetrazolium bromide
NEAA	non-essential amino acids
NFTs	neurofibrillary tangles
NF-κB	nuclear factor kappa B
OLA	olivetolic acid
PBS	phosphate buffered saline
PCA	principal component analysis
pCB	phytocannabinoid

PD	Parkinson's disease
PPARγ	peroxisome proliferator-activated receptor gamma
ROS	reactive oxygen species
RPMI-1640	Rose Park Memorial Institute 1640
SN-38	7-ethyl-10-hydroxycamptothecin
SOD1	superoxide dismutase 1
*t*bhp	tert-butyl hydroperoxide
TEER	transepithelial electrical resistance
TEM	transmission electron microscopy
ThT	thioflavin T
TNFα	tumour necrosis factor alpha
TRPA1	transient receptor potential ankyrin 1
TRPM8	transient receptor potential melastatin 8
TRPV1	transient receptor potential vanilloid 1
UC	ulcerative colitis
UGT1A1	uridine diphosphate glucuronysyltransferase 1A1
ZO-1	zonula occludins 1
Δ^8-THC	Δ^8-tetrahydrocannabinol
Δ^9-THC	Δ^9-tetrahydrocannabinol
Δ^9-THCA	Δ^9-tetrahydrocannabinolic acid

Chapter 1: General Introduction

1.1 Dementia and Neurodegenerative Diseases

1.1.1 Overview

Dementia is a state of overall cognitive decline resulting from neurodegeneration, affecting the ability for the individual to function appropriately in social and occupational contexts. Neurodegeneration may be thought of as an umbrella term, describing a number of different disease states that directly and primarily affect the structure and function of neurons. As the term implies, neurodegeneration is progressive in nature and results in the eventual loss of specific neuronal populations within the brain, depending on the particular neurodegenerative disease. Alzheimer's disease (AD) is the most common neurodegenerative disease, representing between 60 and 70% of all dementia cases worldwide (World Health Organization, 2017). Alzheimer's-type dementia is characterised by impairment in episodic memory along with secondary impairments in word-finding skills, spatial cognition and executive functions such as behavioural control (Salmon and Bondi, 2009). Other major forms of dementia include Lewy body dementia, frontotemporal dementia and vascular dementia, although the boundaries between different forms of dementia are often indistinct (World Health Organization, 2017).

1.1.2 Dementia and Alzheimer's Disease: Prevalence and Economic Impact

Dementia represents an increasingly significant global health burden, with 1 in 10 Americans over the age of 65 suffering from Alzheimer's-type dementia (Alzheimer's Association, 2019). According to the World Health Organisation in 2015, 47 million people were affected by dementia worldwide (approximately 5% of the global elderly population), with this number predicted to increase to 75 million by 2030 and 135 million by 2050 (Prince et al., 2015a). According to the World Alzheimer Report 2015 (see Figure 1), 58% of all dementia worldwide occurred in low and middle-income countries, such as Bangladesh, Vietnam, China and India, with an expected increase to 68% by 2050 (Prince et al., 2015b). Although AD predominantly affects the elderly population, an estimated 200,000 Americans under the age of 65 are currently

suffering from younger-onset dementia (Alzheimer's Association, 2019). In 2015, an estimated 9.9 million new cases of dementia were reported worldwide, equating to approximately one new reported case every 3.2 seconds (Prince et al., 2015b).

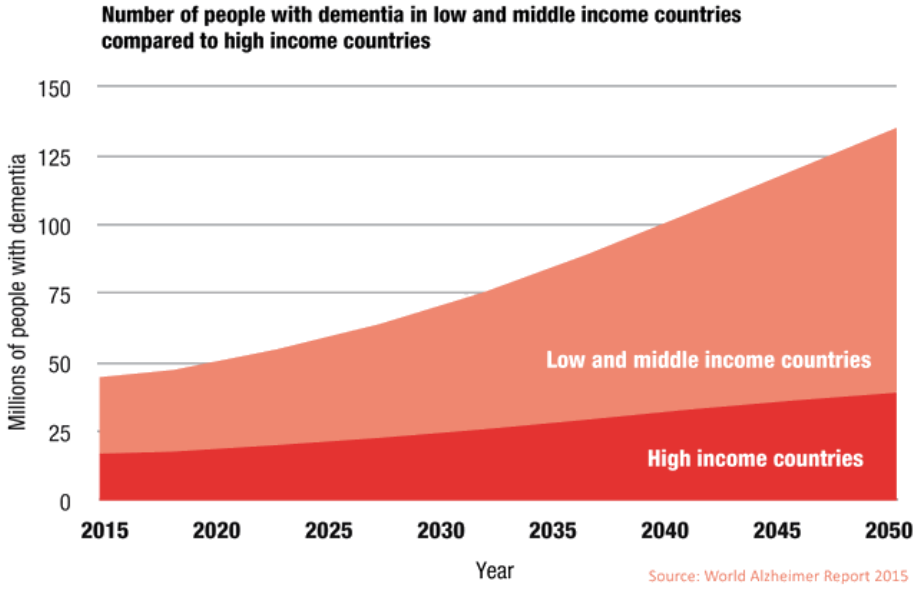

Figure 1. Comparison and future predictions for global distribution of dementia in both developing and developed countries. *Source: (Prince et al., 2015b)*

Another important aspect of the overall burden of dementia is its economic impact, both nationally and globally. In 2016 the total cost of dementia in Australia was an estimated AUD$14.25 billion, with this figure estimated to increase to more than AUD$36.8 billion by 2056 (Brown et al., 2017). The global economic burden of dementia in 2015 was an estimated USD$818 billion, which represents 1.09% of global GDP – an increase of 35% since 2010 (Wimo et al., 2017). The current economic burden of dementia has exceeded US$1 trillion and continues to rise, with global costs expected to total more than USD$2 trillion by the year 2030 (Figure 2) (Wimo et al., 2017). The following analogy serves to put these costs into perspective – if global dementia were a country, it would represent the 18th largest economy on Earth (Prince et al., 2015b).

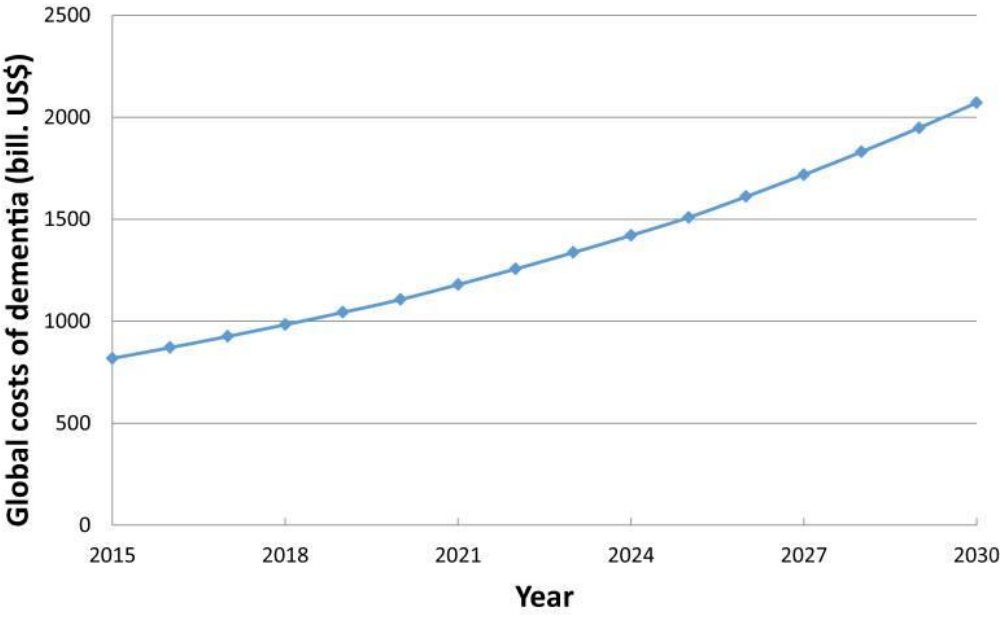

Figure 2. Predicted global costs of dementia in USD, 2015 to 2030. *Source: (Wimo et al., 2017)*

1.1.3 Neuropathology of Alzheimer's Disease

Dementia is a leading cause of death in Australia and was second only to coronary heart disease in 2017 (Australian Institute of Health and Welfare, 2019). There are two main pathological hallmarks associated with AD: senile (or neuritic) plaques and neurofibrillary tangles (NFT's) (Perl, 2010). Previous investigations have shown that these senile plaques consist primarily of a central core of beta-amyloid (Aβ) protein, surrounded by a corona of abnormally formed neuronal processes, such as dendrites or axons (Perl, 2010). Aβ itself is a 4kD protein, which displays a beta-pleated sheet configuration, is highly fibrillogenic and arranges in a radial fashion within the amyloid protein core. Microglial cells are also found at the periphery of these senile plaques along with reactive astrocytes, though the latter are typically less common (Perl, 2010). Aβ is also known to aggregate in another form, known as diffuse plaques, whereby diffuse deposits of Aβ may occur which lack the accompanying accumulation of dystrophic neurites (DeTure and Dickson, 2019). Interestingly, increases in intestinal permeability have been associated with increased amyloid deposition in AD patients, as well as increases in

systemic and neuroinflammation (Ticinesi et al., 2018, Kowalski and Mulak, 2019). Various studies have investigated the link between intestinal permeability, inflammation and AD pathology, highlighting the potential role of the gut-brain axis (Escobar et al., 2022). Therapeutics that possess the ability to inhibit amyloid toxicity while also maintaining intestinal barrier function present as attractive lead candidates in the treatment of AD.

1.1.4 The Amyloid Hypothesis

In 1906, a clinical psychiatrist and neuroanatomist named Alois Alzheimer reported the case of a 50-year-old woman whom he had observed, who displayed signs of paranoia, memory disturbance, aggression and confusion prior to her death. Alzheimer reported the presence of distinct plaques and tangles in the brain histology (Hippius and Neundörfer, 2003). In 1911, Alzheimer documented a similar case where the brain histology of another patient displaying similar symptoms identified the presence of distinct plaques, this time in the absence of neurofibrillary tangles (Alzheimer, 1911). Today, we know these plaques to consist primarily of Aβ protein aggregates. The discovery of autosomal dominant mutations in the amyloid

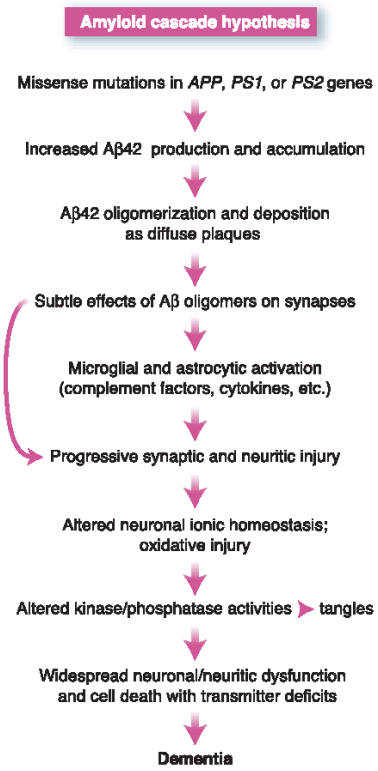

Figure 3. Sequence of events of the amyloid cascade hypothesis leading to AD. *Source: (Hardy and Selkoe, 2002)*

precursor protein (APP) gene relating to familial AD have played an important role in developing our understanding of AD pathobiology and led to the development of the amyloid cascade hypothesis (Hardy and Higgins, 1992, Goate et al., 1991). The amyloid cascade hypothesis proposes that AD is caused by an imbalance between the production and clearance of Aβ, often occurring as an initiating factor in AD progression (Figure 3). Importantly, the amyloid cascade hypothesis suggests that processes such as tau accumulation and the development of neurofibrillary tangles occur subsequently to the aggregation of Aβ (Hardy and Selkoe, 2002).

1.1.5 Mechanisms of amyloid neurotoxicity

The mechanisms of Aβ neurotoxicity have been well established and are multifarious in nature. Determining which form of Aβ is primarily responsible for its neurotoxicity has sparked a great deal of debate. However, it has been suggested that small oligomeric conformations of Aβ are more toxic than mature amyloid fibrils, due to their ability to form annular protofibrils or 'amyloid pores', which may interfere with membrane permeabilization (Lashuel et al., 2002, Bieschke et al., 2012). Moreover, oxidative stress plays a major role in the neurotoxicity of Aβ, whereby Aβ-evoked increases in reactive oxygen species (ROS) generation triggers apoptosis via the activation of apoptosis signal-regulating kinase 1 (Kadowaki et al., 2005). Aβ has also been shown to induce 4-hydroxyl-2-nonenal (HNE) production via the damage of lipid membranes, which may then induce further Aβ misfolding (Murray et al., 2007). HNE has been shown to be increased in the AD brain, indicating a specific role of lipid peroxidation in the pathology of AD (Lovell et al., 1997). Another important facet of Aβ-induced neurodegeneration is the related alterations in neuronal cell morphology. Aβ has been shown to cause dendritic spine loss, dendritic simplification and neuritic dystrophies via the activation of calcineurin (Wu et al., 2010, Umeda et al., 2015). Furthermore, Aβ has been shown to cause a decrease in neurite length and overall presynaptic integrity in primary cortical neurons; these changes in cell morphology are an important indicator of non-lethal cell injury (Nguyen et al.,

2012). It may be that an ideal candidate therapy would target both ROS generation and lipid peroxidation as well preventing Aβ-evoked changes in neuronal cell morphology.

1.1.6 Alzheimer's Disease – Current Treatments

Despite the considerable disease burden of AD, there is currently no disease-modifying treatment. Given that AD is associated with the death of cholinergic neurons and a subsequent decrease in cholinergic neurotransmission, inhibitors of acetylcholinesterase (AChE), the enzyme responsible for the synaptic degradation of acetylcholine (ACh), have been a major pharmacological focus (Vecchio et al., 2021). These AChE inhibitors include drugs such as donepezil, galantamine and rivastigmine which, along with the NMDA receptor antagonist memantine, are among the only FDA-approved treatments for AD (Atri, 2019). Memantine protects against neuronal loss caused by glutamate toxicity and has shown to be effective in this regard (Kishi et al., 2017). However, memantine does not alter the progression of the disease. Inhibitors of AChE aim to increase cholinergic neurotransmission by preventing the breakdown of ACh, thus increasing synaptic concentrations of this key neurotransmitter. This approach has been shown to mitigate cognitive decline in the early stages of AD progression (Yiannopoulou and Papageorgiou, 2020). However, these treatments have been associated with side effects including nausea, vomiting and diarrhoea and are not considered disease-modifying (Fan et al., 2020b). Other treatment options that have been explored include β-secretase (BACE) inhibitors such as lanabecestat, verubecestat and atabecestat, which aim to reduce the production of Aβ. However, these drugs have proven largely ineffective in clinical trials (Burki, 2018, Egan et al., 2019, Henley et al., 2019). In this light, there remains a clear need for the development of new, disease-modifying candidate treatments for AD that can demonstrate unequivocal efficacy and improvement in clinical outcomes

1.1.7 Benefits of natural products in drug discovery

Historically speaking, natural products have served as the basis for the development of numerous pharmaceuticals (Newman and Cragg, 2020). Given the vast structural diversity that

natural products provide, many of these compounds present as attractive lead candidates in the development of neuroprotective agents in the context of AD. Of particular importance in this regard are the multifarious mechanisms by which these natural products exert their effects. These mechanisms include the direct inhibition of Aβ aggregation, reduction in ROS generation and oxidative stress, and activity at various receptors and signalling pathways (Marsh and Smid, 2021). Plants such as cannabis sativa are known for their rich biochemical diversity and provide an extensive library of potentially bioactive small molecules. A detailed discussion of natural products as neuroprotective agents, with a particular focus on phytocannabinoids (pCBs), is provided in Chapter 2 of this thesis.

1.2 Inflammatory Bowel Disease (IBD) and Chemotherapy-induced Gastrointestinal Mucositis (GIM)

1.2.1 Overview and Prevalence of IBD and GIM

Inflammatory bowel disease (IBD) is an umbrella term that comprises two subtypes: ulcerative colitis (UC), which primarily affects the colon and Crohn's diseases (CD) which can affect any part of the gastrointestinal (GI) tract; the primary feature of these conditions being inflammation and ulceration of the gastric mucosa (Xavier and Podolsky, 2007). According to a recent study, approximately 653 patients per 100,000 in Australian general practice are suffering from some form of IBD (Busingye et al., 2021). Approximately 1.17 million people in the US suffer from IBS, with this number increasing by approximately 70,000 each year, while in the UK, rates of IBD have risen by 33.8% from 2006-2016; these data highlight the significant disease burden of IBD (Mehta, 2016, Freeman et al., 2021). In 2014, the total economic burden of IBD in the US was an estimated $14.6 to $31.6 billion USD, highlighting a considerable economic burden (Mehta, 2016). Although the mortality rate amongst IBD patients is only slightly higher than in healthy controls, IBD has a significant impact on quality of life, with patients reporting higher rates of comorbid anxiety and depression than healthy controls (Knowles et al., 2018, Card et al., 2003).

Chemotherapy-induced gastrointestinal mucositis (GIM) refers to the inflammation and ulceration of the GI mucosa resulting from the use of conventional chemotherapeutics. Chemotherapy-induced GIM is one of the most common toxicities among cancer patients, with an estimated 40% of cancer patients experiencing GIM following treatment with standard-dose, cycled chemotherapy (Cinausero et al., 2017). Chemotherapy-induced diarrhoea, a key clinical symptom of GIM, reportedly occurs in 89% of patients treated with FOLFIRI (a combination of folinic acid, 5-fluorouracil and irinotecan) for colorectal cancer (Keefe et al., 2014). Chemotherapy-induced GIM is associated with symptoms including pain, nausea and diarrhoea and has significant impacts on the quality of life of cancer patients (Dahlgren et al., 2021).

1.2.2 Pathobiology of IBD and GIM

The pathobiology of IBD involves a complex and multifaceted immune response. Key pathological features of UC include a change in microbial diversity (dysbiosis) in the intestinal lumen and impairment of epithelial barrier integrity via the disruption of tight junctions (TJs) (Yeshi et al., 2020). Cytokines such as interleukin-13 and tumour necrosis factor alpha (TNFα) play an important role in UC via their ability to synergistically regulate the expression of genes responsible for the formation of TJs (Heller et al., 2005). The associated increase in intestinal

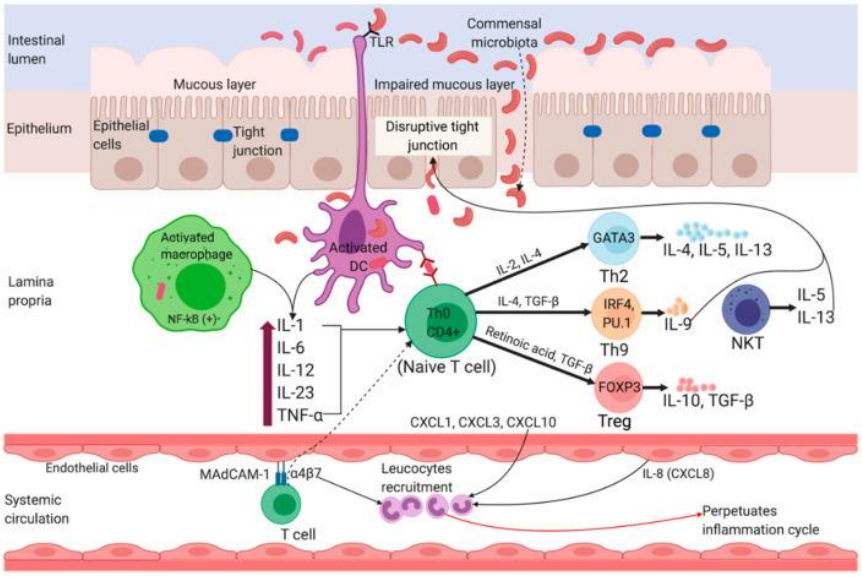

Figure 4. Schematic diagram of the pathophysiology of UC, highlighting the role of increased intestinal permeability and the associated immune response. *Source: (Yeshi et al., 2020)*

permeability allows for an enhanced influx of luminal antigens such as commensal intestinal microbiota, whereby immune cells such as macrophages and dendritic cells become activated upon recognizing non-pathogenic bacteria via Toll-like receptors (Yeshi et al., 2020). This process then triggers a further immune response resulting in the characteristic mucosal inflammation associated with UC (Figure 4). CD is also associated with an increase barrier permeability due to the disruption of tight junctions in a similar manner to UC. However, CD is associated with an increase in the expression of proinflammatory cytokines such as interferon gamma (IFNγ) and interleukin 17A, along with TNFα (Yeshi et al., 2020). Compared to UC, the clinical presentation of CD may be more subtle, often resulting in delayed diagnosis (Hendrickson et al., 2002).

Chemotherapy-induced GIM is characterised by crypt loss, villus atrophy, loss of renewal capacity and impaired intestinal barrier function (Sougiannis et al., 2021). The progression of GIM may be summarised as a five-phase sequence: 1) initiation, 2) upregulation and generation of messenger signals, 3) signalling and amplification, 4) ulceration and inflammation and 5) healing (Sonis et al., 2004). Following Stage 1, generation of ROS and activation of nuclear factor κ-B (NF-κB) results in the upregulation of numerous messengers, including the pro-inflammatory cytokines TNFα and interleukin 1β (IL-1β) (Sougiannis et al., 2021).

1.2.3 TNFα and IL-1β as modulators of IBD, GIM and intestinal barrier permeability

TNFα and IL-1β are important proinflammatory cytokines involed in the pathology of both IBD and GIM. In patients with IBD, an increase in the number of TNFα-producing macrophages and lymphocytes is observed in affected tissue, with concentrations of TNFα correlating with the grade of inflammation and disease severity (Olsen et al., 2007). The clinical efficacy of TNFα antibodies in IBD serve to highlight the importance of this cytokine in the progression of the disease (Kaser et al., 2010). Interestingly, both TNFα and IL-1β have been identified as important indictors of relapse in IBD patients (Schreiber et al., 1999). Ma and colleagues found that TNFα has the ability to induce increased barrier permeability in Caco-2 cells via the

downregulation of zonula occludins-1 (ZO-1) expression (Ma et al., 2004). Interestingly, TNFα has been shown to induce the redistribution of several TJ proteins, including ZO-1, occludins, claudins and E-cadherin to the basolateral membrane of intestinal epitheilal cells. Moreover, treatment with TNFα not only caused this redistribution of junctionl proteins, it also resulted in the shedding of whole cells from the intestinal epithelium; this event is preceded by the activation of caspase-3 due to the TNFα-induced activation of NF-κB signalling (Marchiando et al., 2011). Similarly to TNFα, expression of IL-1β is increased in IBD patients and has been shown to mediate chronic intestinal inflammation (Ligumsky et al., 1990, Mahida et al., 1989, Coccia et al., 2012). IL-1β has the ability to cause an increase in intestinal barrier permeability via the degradation of occludin mRNA, as well as via the downregulation of various other TJ proteins including claudin-1 and claudin-3 (Rawat et al., 2020, Kaminsky et al., 2021). IL-1β also has the ability to modulate intestinal inflammation via the activation of NF-κB signalling, resulting in the production of various proinflammatory modulators (Kaminsky et al., 2021). Moreover, IL-1β has been shown to modulate intestinal TJ barrier permeability in an NF-κB-dependent manner in colonic epithelial Caco-2 cells (Al-Sadi et al., 2010).

In GIM, both TNFα and IL-1β play key roles throughtout the pathogenesis of the disease. During the upregulation phase, NF-κB signalling causes an increase in the gene expression and synthesis of various proinflammatory cytokines including IL-6, TNFα and IL-1β. Next, during the upregulation phase, these cytokines act in a positive feedback loop to further promote NF-κB activation, increasing cytokine production and thus exacerbating the overall inflammatory response and increasing apoptosis (Sultani et al., 2012). This increase in cytokine production leads to the aforementioned downregulation of TJ protein expression, resulting in reduced intestinal barrier integrity. Interestingly, inflammasome activation in irinotecan-induced mucositis has been shown to be dependent on ROS generation and is mediated through the actions of IL-1β and IL-18 (Arifa et al., 2014).

1.2.4 Current treatments for IBD and GIM

There are several pharmacological treatment options available for the management of IBD and GIM. In IBD, treatment options include aminosalicylates, immunosuppressants and antibodies targeting TNFα, as well as general anti-inflammatory agents such as corticosteroids.

Aminosalicylates such as sulfasalazine and 5-aminosalicylic acid have been used in the treatment of IBD for decades (Cai et al., 2021). The proposed mechanism of action of these drugs relates to their reported ability to inhibit inflammatory modulators such as cyclooxygenase (COX), IL-1, NF-κB and TNFα, as well as their ROS-scavenging activity (Hanauer, 2004, Punchard et al., 1992). Although considered a first-in-line therapy in UC, aminosalicylates may be less efficacious in CD, with only 40-55% of patients experiencing a clinical response or remission in clinical trials focusing on mild-to-moderate CD (Hanauer, 2004). Unfortunately, there are a number of side effects associated with the use of aminosalicylates, including headaches, rashes, nausea, vomiting and abdominal pain (Shmueli and Record, 1994).

Corticosteroids such as prednisolone have also been used in the treatment of IBD, with efficacy shown in clinical trials for CD (Akobeng, 2008a). Corticosteroids possess a reported remission rate of 70-90% in mild-to-moderate CD, although this remission is not always evident by endoscopy (Modigliani et al., 1990). Corticosteroids may be used when rapid remission of inflammation in IBD is necessary. However, corticosteroids are associated with side effects such as hypertension, increased risk of infection and weight gain and are therefore not generally recommended for long-term use (Waljee et al., 2016, Gibson and Iser, 2005).

Given the role of TNFα in the pathogenesis of IBD, anti-TNFα antibodies such as infliximab, adalimumab and certolizumbab have been used as a treatment option for this disease. Infliximab is often used in patients who do not respond to corticosteroids and has been shown to induce remission in CD patients and is an effective treatment for fistulas in CD (Akobeng and Zachos, 2004, Present et al., 1999). Adalimumab has been shown to be effective in inducing remission

in moderate-to-severe CD patients and has been shown to be effective for inducing remission in patients who either cannot tolerate infliximab or do not respond to infliximab therapy (Akobeng, 2008a). However, there are several drawbacks to the use of these therapies, including a potential increased risk of hepatosplenic T cell lymphoma and increased risk of infection (Akobeng, 2008b, Vulliemoz et al., 2020).

There remains a largely unmet need for the development of effective treatments for chemotherapy-induced GIM. Currently proposed treatment options focus primarily on symptomatic relief rather than disease modification. Such treatments include probiotic therapies which focus on introducing beneficial bacteria to the gut microbiome. Although partially effective in reducing the incidence of diarrhoea and quite promising in the modulation of the gut microbiome and pro-inflammatory cytokine production, these therapies have produced mixed results in clinical trials (Touchefeu et al., 2014, Prisciandaro et al., 2011). Antioxidant therapies, such as amifostine, have also been investigated in the context of GIM. Amifostine acts as a radical scavenger and thus may act to reduce the off-target toxicity of conventional chemotherapeutics (Bensadoun et al., 2006). Anti-inflammatory agents have also been employed as a means of combatting the pathogenic inflammation associated with GIM. Interestingly, IL-1 receptor antagonists have been shown to effectively reduce 5-fluorouracil-induced GIM in mice (Dahlgren et al., 2021). Moreover, COX-2 inhibition has been shown to reduce the histopathological changes and diarrhoea associated with both 5-fluorouracil and irinotecan-induced GIM (Dahlgren et al., 2021). It therefore stands to reason that a pharmacological candidate that possesses both anti-inflammatory and antioxidant abilities may be effective in the treatment and management of GIM.

1.2.5 Natural products as inhibitors of GI inflammation

Natural products such as flavonoids and phytocannabinoids (pCBs) have been ascribed anti-inflammatory properties in various inflammatory disease contexts, including IBD. Given the vast library of natural products found in cannabis sativa, numerous pCBs have been investigated

for their anti-inflammatory properties. The most widely studied pCBs are cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC), both of which have been studied extensively in the context of inflammation (Carvalho et al., 2020). CBD has been shown to possess anti-inflammatory properties in acutely inflamed colonic tissue from IBD patients, with the ability to reduce cytokine production, with THC being shown to reduce colonic inflammation and functional disturbances associated with colitis (Couch et al., 2017, Jamontt et al., 2010). Aside from these two major players, many other pCBs have exhibited potent anti-inflammatory capacities. One such example is cannabigerol (CBG), which has been shown to attenuate murine colitis via reductions in nitric oxide production and ROS production in intestinal epithelial cells (Borrelli et al., 2013). The pCB cannabichromene (CBC) has also proven to be effective in the context of GI inflammation, with the ability to reduce inflammation-induced hypermotility in mice (Izzo et al., 2012). Along with these isolated phytocannabinoids, whole botanical extracts of cannabis have also been investigated for their anti-inflammatory capacity (Ahmed and Katz, 2016). A previous study demonstrated that a CBD-predominant cannabis extract was effective in the amelioration of inflammatory injury and hypermotility in a murine experimental colitis model (Pagano et al., 2016). Whole extracts of medicinal cannabis offer great potential due to their biochemical complexity, given the presence of various phytocannabinoids, terpenes, flavonoids and other phytochemicals. Given that many of the other major phytocannabinoids such as CBG and CBC have demonstrated anti-inflammatory effects, their inclusion in whole botanical extracts, along with the primary phytocannabinoids Δ^9-THC and CBD, allow for a more holistic, “full-spectrum” treatment approach. The use of these full-spectrum extracts over isolated phytocannabinoids has led to their investigation in various clinical trials related to IBD, with mixed results (Naftali et al., 2011, Naftali et al., 2013, Irving et al., 2018).

Flavonoids have also been studied extensively for their anti-inflammatory properties and possess the ability to reduce inflammation through various pathways, including the inhibition

of NF-κB and mitogen-activated protein kinase (Vezza et al., 2016). The flavonoid fisetin has been shown to effectively ameliorate dextran sulphate sodium (DSS)-induced colitis in mice via reductions in proinflammatory cytokine production and COX-2 expression; this effect was associated with reduced NF-κB signalling (Sahu et al., 2016). Moreover, the chalcone cardamonin, the flavonoid glycoside quercitrin and its aglycone quercetin have also been shown to inhibit GI inflammation in a DSS model of colitis via reductions in NF-κB activity (Vezza et al., 2016, Comalada et al., 2005). The flavonoid epigallocatechin gallate has been shown to reduce LPS-induced TNFα production via the inhibition of NF-κB (Yang et al., 1998). It is clear from the literature that both flavonoids and phytocannabinoids possess anti-inflammatory properties and thus present as potential candidates in the treatment of inflammatory diseases of the GI tract, such as IBD and GIM.

1.3 Research Aims

There remains an unmet need for effective treatment options for AD and IBD. Given the links between intestinal inflammation and barrier function and the development of AD, it stands to reason that a treatment which effectively inhibits amyloid toxicity and maintains intestinal barrier function may be a highly effective treatment for AD, as well as IBD. Phytocannabinoids and flavonoids have been ascribed wide-ranging therapeutic effects in various disease contexts, ranging from neuroprotective and anti-inflammatory effects to modulation of barrier permeability and gastrointestinal function. It is therefore logical to investigate the potential role of these natural products in the amelioration of these diseases. Here, we aim to investigate the broad protective capacities of these phytochemicals in the context of both neurodegenerative disease and inflammatory diseases of the gastrointestinal tract, in order to further establish their therapeutic potential as lead candidates.

Research aims addressed in Chapter 2:

1. Identify phytocannabinoids with high binding affinity for the Aβ monomer and pentamer for use in later studies
2. Identify structure-activity relationships for phytocannabinoids based on binding positions and other binding characteristics

Research aims addressed in Chapter 3:

1. Determine if the favourable binding characteristics of select phytocannabinoids from Chapter 2 translate to inhibition of $A\beta_{1-42}$ fibrillisation and aggregation.
2. Determine the protective capacity of select phytocannabinoids as antioxidant inhibitors of *tert*-butyl hydroperoxide-induce oxidative stress and cytotoxicity in PC12 cells.
3. Identify inhibitors of $A\beta_{1-42}$ neurotoxicity in an *in vitro* neuronal PC-12 cell model of AD.

    a. Determine the effect of structural differences such as alkyl side chain length and C-ring aromatisation on the neuroprotective capacity of select phytocannabinoids

4. Identify morphological changes to neuronal PC-12 cells induced by incubation with $A\beta_{1-42}$:
    a. Identify and determine morphological markers associated with $A\beta_{1-42}$ neurotoxicity
    b. Determine the protective capacities of select pCBs such as cannabigerol, cannabichromene, cannabidiol, cannabidivarin, cannabinol and Δ^9-tetrahydrocannabinol in preventing $A\beta_{1-42}$-evoked changes in neuronal cell morphology

Research aims addressed in Chapter 4

1. Compare the efficacy of five medicinal cannabis preparations as antioxidant inhibitors of *tert*-butyl hydroperoxide (*t*bhp)-evoked neurotoxicity in motorneuronal NSC-34 cells
2. Compare the neuroprotective capacity of CBD-dominant and Δ^9-THC-dominant medicinal cannabis preparation in the inhibition of $A\beta_{1-42}$ neurotoxicity in NSC-34 cells
3. Determine the relative anti-aggregatory capacity of CBD-dominant and Δ^9-THC-dominant medicinal cannabis preparations
4. Determine the effect of heat exposure on the anti-aggregatory and neuroprotective profiles of CBD-dominant and Δ^9-THC-dominant medicinal cannabis preparations
5. Determine whether whole botanical extracts of cannabis exhibit greater neuroprotection than the isolated phytocannabinoids studied in Chapter 3

Research aims addressed in Chapter 5

1. Compare the protective capacity of cannabigerol, cannabichromene and cannabidivarin with the well-characterised cannabidiol, against cytokine- and SN-38-evoked increases in barrier permeability
2. Determine the antioxidant capacity of each phytocannabinoid in inhibiting *in vitro* ROS generation
3. Determine the impact of each phytocannabinoid on the cytotoxicity of the irinotecan metabolite SN-38 in Caco-2 cells

Research aims addressed in Chapter 6

1. Compare the protective capacity of the novel flavonoids 2-D08 and transilitin with myricetin and quercetin in a model of cytokine- and SN-38-evoked increases in barrier permeability
2. Determine the antioxidant capacity of each flavonoid in the inhibition of *in vitro* ROS generation
3. Determine the impact of each flavonoid on the cytotoxicity of SN-38 in Caco-2 cells

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Contribution to the Paper	Conceptualisation, Methodology, Validation, Formal analysis, Data curation, Investigation, Writing - original draft, Writing - review & editing, Visualisation		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	24/07/22

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contr bution.

Name of Co-Author	Scott Smid		
Contribution to the Paper	Conceptualisation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition		
Signature		Date	July 25, 2022

Chapter 2. Cannabis Phytochemicals: A Review of Phytocannabinoid Chemistry and Bioactivity as Neuroprotective Agents

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Abstract

With the advent of medical cannabis usage globally, there has been a renewed interest in exploring the chemical diversity of this unique plant. Cannabis produces hundreds of unique phytocannabinoids, which not only have diverse chemical structures but also a range of cellular and molecular actions, interesting pharmacological properties, and biological actions. In addition, it produces other flavonoids, stilbenoids, and terpenes that have been variably described as conferring additional or so-called entourage effects to whole-plant extracts when used in therapeutic settings. This review explores this phytochemical diversity in relation to specific bioactivity ascribed to phytocannabinoids as neuroprotective agents. It outlines emergent evidence for the potential for selected phytocannabinoids and other cannabis phytochemicals to mitigate factors such as inflammation and oxidative stress as drivers of neurotoxicity, in addition to focusing on specific interactions with pathological misfolding proteins, such as amyloid β, associated with major forms of neurodegenerative diseases such as Alzheimer's disease.

Cannabis produces unique compounds with novel biological activity. We describe cannabis phytochemistry relative to neuroprotective properties through anti-oxidant and anti-inflammatory actions and via inhibiting aggregation of misfolded neurotoxic proteins such as amyloid β in Alzheimer's disease. Such emergent phytocannabinoid research may afford new leads in the treatment of dementia.

Introduction

In recent years, medicinal cannabis has received a great deal of attention for its potential therapeutic benefits in a wide range of human diseases. *Cannabis* is known for its rich biochemical diversity, containing a variety of phytochemicals including terpenes, flavonoids and a range of phytocannabinoids (pCBs) (Brenneisen, 2007). To date, more than 540 compounds have been isolated and identified, more than 120 of which are phytocannabinoids (Hanuš et al., 2016). A number of *cannabis* phytochemicals have been investigated for their medicinal properties in a variety of contexts, from the management of obesity to the treatment of neurological diseases (Bielawiec et al., 2020, Maroon and Bost, 2018). In particular, pCBs have been variably shown to provide neuroprotection in a number of neurodegenerative disease models, both cell and animal, via a range of mechanisms (Casteels et al., 2015, Concannon et al., 2015, de Ceballos, 2015). Research in this field aims to exploit the biochemical diversity of *cannabis* in order to extend these promising results into viable treatments for neurodegenerative diseases.

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by accelerated cognitive decline coupled with the formation of extracellular β-amyloid (Aβ) plaques and intracellular neurofibrillary tangles in the brain, as well as a significant degree of neuroinflammation. This characteristic cognitive decline has been directly linked to a reduction in cholinergic neurotransmission and neuronal cell death (Francis et al., 1999). It is clear that the likelihood of developing AD significantly increases with age, presenting an important and increasing health burden in an ageing society (Qiu et al., 2009). The Australian Institute of Health & Welfare has predicted the national prevalence of AD to reach 900,000 by the year 2050, with current global estimates in the range of 40-50 million (Health and Welfare, 2012, Nichols et al., 2019). In light of these figures, it is essential that an effective, disease-modifying treatment is developed. From a pharmacological perspective, natural products such as phytocannabinoids offer wide-ranging therapeutic potential in the context of AD via numerous

mechanisms when administered in a medicinal setting, using standardised formulations and titrated dosages. These mechanisms include direct interactions with the Aβ protein, which prevent oligomerization and aggregation, inhibition of acetylcholinesterase (AChE), prevention of tau hyperphosphorylation, inhibition of the neuroinflammatory response and more (Xiaoai et al., 2019).

Protein misfolding is a central factor in the pathogenesis of a number of neurodegenerative diseases, with perhaps the best-known example occurring in AD. This disease is associated with the aggregation of the Aβ protein, found primarily in 40 and 42 amino acid segments which form following the enzymatic cleavage of amyloid precursor protein (APP) by both β and γ secretases (De-Paula et al., 2012). Aβ displays structural polymorphisms during aggregation, resulting in a heterogeneous population of small oligomers, protofibrils and other mature fibrillary conformations. Although it is currently unclear which form of Aβ is most responsible for the toxicity associated with AD, some evidence has indicated that the acceleration of fibril formation may actually reduce Aβ toxicity, suggesting that the intermediate oligomeric conformations are more toxic than the mature fibrils (Bieschke et al., 2012). This is likely due to the propensity for oligomers to form annular protofibrils, termed 'amyloid pores', which may result in inappropriate membrane permeabilization (Lashuel et al., 2002). In similar fashion, misfolding of α-synuclein into prefibrillar and oligomeric conformations has been implicated in the progression of Parkinson's disease (PD) (Ingelsson, 2016). PD represents the second most common neurodegenerative disease behind AD, with an estimated global prevalence of 6.1 million in 2016; this figure is expected to reach 12 million by the year 2050 (Dorsey et al., 2018, Rocca, 2018). The characteristic motor symptoms of PD include rigidity, resting tremor and bradykinesia, along with significant dopaminergic neuronal loss in the substantia nigra pars compacta (Magrinelli et al., 2016). The accumulation and aggregation of α-synuclein has been directly linked to this neuronal loss via several mechanisms, including the impairment of synaptic function, diminished endoplasmic reticulum and Golgi function, impaired

mitochondrial function, promotion of tau aggregation and the inhibition of microtubule assembly (Oikawa et al., 2016, Wong and Krainc, 2017). Furthermore, the misfolding and aggregation of superoxide dismutase 1 (SOD1) is recognised as a pathological feature in the development of amyotrophic lateral sclerosis (ALS), the most common form of motorneurone disease (MND), in a subset of patients (Benkler et al., 2018). ALS is characterised by the progressive loss of motor neurons, resulting in spasticity, weakness, muscle atrophy and ultimately paralysis, often leading to death by respiratory failure within 2-5 years symptom onset (Benkler et al., 2018, Gill et al., 2019). ALS exists in two categories: familial ALS, accounting for approximately 10% of all cases, and sporadic ALS, which accounts for the remaining 90% of ALS cases (Gill et al., 2019). Mutations in SOD1 and its subsequent aggregation are often reported as occurring in 2-3% of all ALS cases, though this figure likely ranges from in 0-7.3% of sporadic ALS cases and 12-23.5% of familial ALS cases (Andersen, 2006). This aggregation has been associated with the impairment of mitochondrial function, although the exact mechanisms of mutant SOD1 toxicity remain largely unknown (Vehviläinen et al., 2014). Protein aggregation has also been identified as a central pathological feature in Huntington's Disease (HD), a condition characterised by excessive and uncontrolled motor function, as well as significant cognitive and neuropsychiatric disturbances (McColgan and Tabrizi, 2018). In HD, inherited CAG trinucleotide repeat expansions in the huntingtin gene result in abnormal polyglutamine repeats in the resultant mutant huntingtin protein, making it more prone to misfolding and aggregation (McColgan and Tabrizi, 2018, Arrasate and Finkbeiner, 2012). The aggregation of this mutant huntingtin protein is associated with detrimental effects on cellular proteostasis, axonal transport, transcription and translation, as well as synaptic and mitochondrial dysfunction (McColgan and Tabrizi, 2018). Although their core pathologies manifest in different forms, the process of protein misfolding is a common feature in each of these neurodegenerative diseases. It therefore follows that compounds with the ability to disrupt or prevent this process may offer considerable benefits in the treatment of these diseases.

Natural phenolic compounds, including cannabinoids, have been ascribed neuroprotective properties in various neurodegenerative diseases (del Rosario Campos-Esparza and Adriana Torres-Ramos, 2010, Fernández-Ruiz et al., 2010). Several mechanisms have been associated with this protective effect, ranging from free radical scavenging and antioxidant effects to inhibition of the inflammatory response and direct structural modification of pathological proteins (Manoharan et al., 2016, Devi et al., 2017, Das et al., 2016, Velander et al., 2017). Natural polyphenolics such as epigallocatechin gallate (EGCG) have been particularly implicated in this structural modification, displaying an ability to redirect amyloidogenic proteins into unstructured, off-pathway, and non-toxic protein assemblies (Ehrnhoefer et al., 2008). In the context of AD, compounds such as curcumin have proved effective in the inhibition of $A\beta_{1–42}$ oligomerization, providing neuroprotection in neuronal cells (Yang et al., 2005). Various mechanisms have been proposed for the inhibitory effect of catechol-type flavonoids, including direct hydrogen-bonding interactions and adduct formation with key amino acid residues in the structure of $A\beta_{1-42}$, particularly Lys 16 and Lys 28 (Sato et al., 2013). Furthermore, non-catechol-type flavonoids have been shown to inhibit both the nucleation and elongation of $A\beta_{1-42}$ via interactions with other residues, particularly Tyr 10-Gln 15 and Phe 19-Ala 21 (Hanaki et al., 2016). These examples serve to highlight the potential for structurally diverse natural phenolic compounds to inhibit amyloid protein misfolding through a variety of interactions, both secondary and covalent.

Several studies have aimed to determine the role of the endocannabinoid system (ECS) and cannabinoid receptors (CBRs) in neurodegenerative disease states (Aso and Ferrer, 2016, Fernández-Ruiz et al., 2015). Neuronal CB_2Rs are expressed predominantly in microglial cells and are associated with the regulation of inflammation, primarily by decreasing the production of pro-inflammatory mediators (Cabral and Griffin-Thomas, 2009). In the context of AD, significant increases in CB_2R expression have been observed in the post-mortem brain tissue of AD patients; this increase was positively correlated with increased levels of Aβ and senile

plaque score, two important molecular markers of AD (Solas et al., 2013). Furthermore, fatty acid amide hydrolase, an enzyme responsible for the degradation of several endogenous cannabinoids, is selectively overexpressed in neuritic plaque-associated glial cells, with increased activity also observed in surrounding areas (Benito et al., 2003). A recent review from Talarico and colleagues discusses the potential therapeutic benefits of modifying the ECS in AD (Talarico et al., 2019). Cannabinoid receptors have also been identified as potential therapeutic targets in the treatment of PD (Baul et al., 2019). In the initial stages of PD development, CB_1Rs are downregulated. However, as the disease progresses and characteristic Parkinsonian symptoms (such as bradykinesia) become apparent, both CB_1Rs and CB_2Rs are upregulated, as are the endocannabinoid ligands for these receptors (Garcia-Arencibia et al., 2009). Moreover, upregulation of CB_1R in the basal ganglia of PD patients and increased endocannabinoid signalling have been associated with movement suppression, providing a potential role for CB_1R modulation in the treatment of the motor symptoms associated with PD (Stampanoni Bassi et al., 2017, Fernández-Ruiz, 2009). The therapeutic potential of CBRs and the ECS in PD is further explored in a recent review from Han and colleagues (Han et al., 2020). Given the general role of CBRs and their selective overexpression in neurodegenerative disease, it is clear that the ECS represents a potential therapeutic target in the treatment of neurodegenerative disease.

Current treatment strategies for AD have focused primarily on acetylcholinesterase inhibition as a means of addressing the cognitive decline associated with AD progression (Sharma, 2019, Mendiola-Precoma et al., 2016). However, while these treatments are capable of providing a degree of symptomatic relief in AD, they have proven largely ineffective in their ability to modify or halt disease progression (Sharma, 2019, Knight et al., 2018). This has left a vital gap, which is yet to be filled by clinically viable alternatives. The use of pCBs and other natural products as inhibitors of protein misfolding and neuroinflammation provides an important avenue of exploration in this context. In this light, the current literature regarding

phytocannabinoids in AD is discussed, as well as the wider implication of these pCBs in other neurodegenerative diseases.

Phytocannabinoids

Phytocannabinoids are naturally occurring C_{21} terpenophenolic compounds common to all major species of *cannabis* (Berman et al., 2018, Morales et al., 2017b). Phytocannabinoids are divided into groups based on their structure, of which there are currently eleven conventional subclasses. These subclasses include: (1) cannabigerol (CBG)-type, (2) (-)-Δ^9-*trans*-tetrahydrocannabinol (Δ^9-THC)-type, (3) cannabidiol (CBD)-type, (4) cannabichromene (CBC)-type, (5) cannabinol (CBN)-type, (6) (-)-Δ^8-*trans*-tetrahydrocannabinol (Δ^8-THC)-type, (7) cannabicyclol (CBL)-type, (8) cannabinodiol (CBND)-type, (9) cannabielsoin (CBE)-type, (10) cannabitriol (CBT)-type and (11) miscellaneous-type (ElSohly and Gul, 2014). These pCBs generally share common structural features including a dibenzopyran ring and an alkyl side chain (Morales et al., 2017b). The general phytocannabinoid structural motif is comprised of 3 moieties: the isoprenyl residue, the resorcinyl core and the alkyl side-chain (see **Figure 1**) (Hanuš et al., 2016).

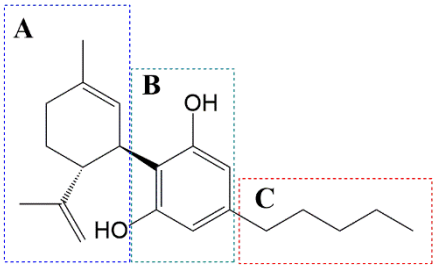


Figure 1. Schematic of the general phytocannabinoid structure, consisting of the isoprenyl residue **(A)**, the resorcinyl core **(B)** and the alkyl side chain **(C)**.

These pCBs vary greatly in the arrangements of these moieties, with key differences relating to the length of the alkyl side chain, degree of aromatization and the nature of the pyran B ring (either opened, as in CBD, or closed, as in THC), among others; these structural differences directly impact the biological and pharmacological effects of pCBs (Stern and Lambert, 2007,

Compton et al., 1993). The investigation of these structural differences and their corresponding effects on biological activity offers an exciting and important avenue for investigation into their use as therapeutic agents in the treatment of neurodegenerative diseases.

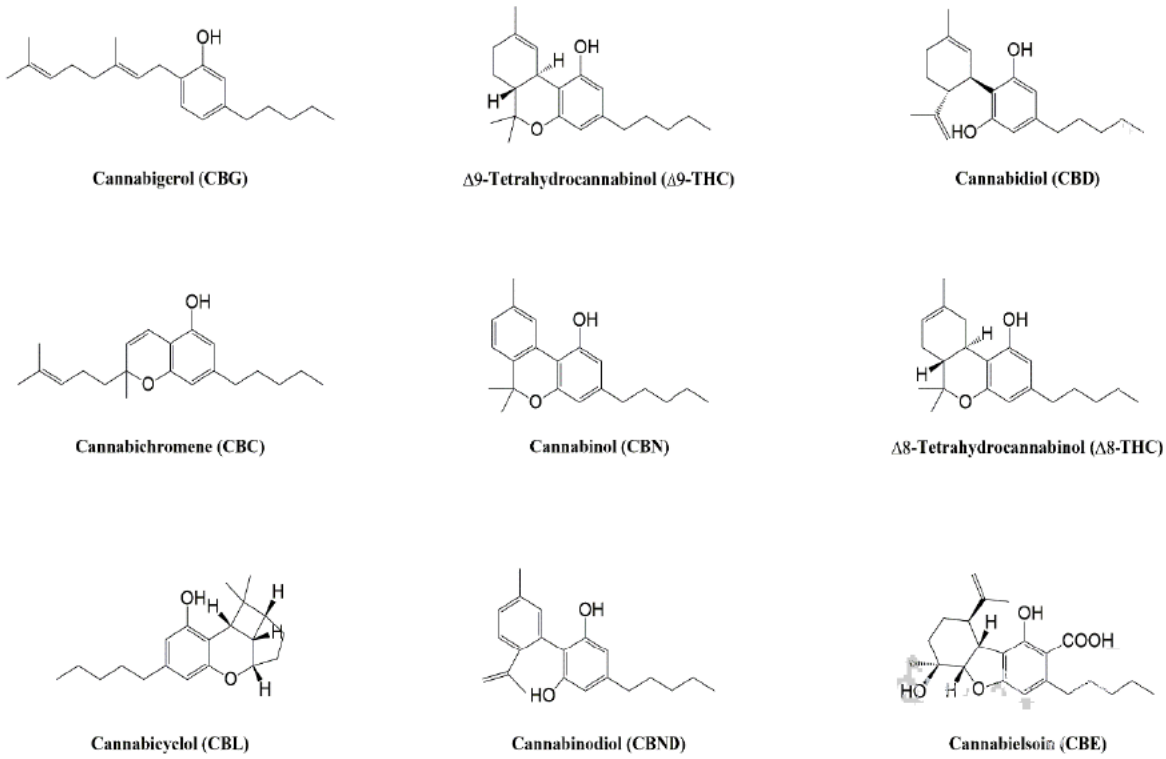


Figure 2. Structures of the major phytocannabinoids

Biosynthesis of Phytocannabinoids

The biosynthesis of phytocannabinoids begins with the formation of olivetolic acid (OLA) (ElSohly et al., 2017). Although the mechanism behind this is yet to be fully elucidated, Taura and colleagues have suggested that OLA may be synthesized by olivetol synthase from hexanoyl-CoA and three malonyl-CoA units via an aldol condensation of a polyketide intermediate (see **Figure 3a**) (Taura et al., 2009). OLA is then *C*-isoprenylated with geranyl diphosphate via the actions of geranyl-diphosphate:olivetolate geranyltransferase, resulting in the formation of cannabigerolic acid (CBGA) (Hanuš et al., 2016, ElSohly et al., 2017, Fellermeier and Zenk, 1998). CBGA may then undergo decarboxylation to form CBG, or be further processed in either of three distinct pathways, A, B or C, eventuating in the formation of each major phytocannabinoid subclass (**Figure 3b**) (Reekie et al., 2017). In pathway A,

CBGA undergoes oxidocyclization by the flavin adenine dinucleotide (FAD)-dependent oxidase cannabichromenic acid (CBCA) synthase to form CBCA, which may then decarboxylate into CBC (ElSohly et al., 2017, Flores-Sanchez and Verpoorte, 2008). Pathway A is also responsible for the formation of cannabicyclolic acid (CBLA), via UV photo-irradiation of CBCA, and cannabicyclol, either by UV photo-irradiation of CBC or via the decarboxylation of its carboxylic acid counterpart, CBLA (Lewis et al., 2017, Shoyama et al., 1972, Shoyama et al., 1968). Pathway B results in the initial formation of cannabidiolic acid (CBDA) via the oxidocyclization of CBGA by CBDA synthase (Flores-Sanchez and Verpoorte, 2008, ElSohly et al., 2017). CBDA may then undergo decarboxylation into CBD, which may be converted into CBE via photo-oxidation. Alternatively, photo-oxidation may also cause CBDA to undergo further cyclization, resulting in the formation of cannabielsoic acid-A (CBEA-A) or CBEA-B, both of which may then form CBE following decarboxylation (Lewis et al., 2017). Finally, pathway C is responsible for the formation of Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), following the Δ^9-THCA synthase-mediated oxidocyclization of CBGA (ElSohly et al., 2017, Flores-Sanchez and Verpoorte, 2008, Hanuš et al., 2016). Δ^9-THCA then undergoes a process of decarboxylation, resulting in the formation of Δ^9-THC. From here, Δ^9-THC may undergo isomerization into Δ^8-THC, or oxidative aromatization into cannabinol, which may then be photo-chemically rearranged to form cannabinodiol (Reekie et al., 2017, Lewis et al., 2017, ElSohly and Slade, 2005, Robert J.J.Ch et al., 1977).

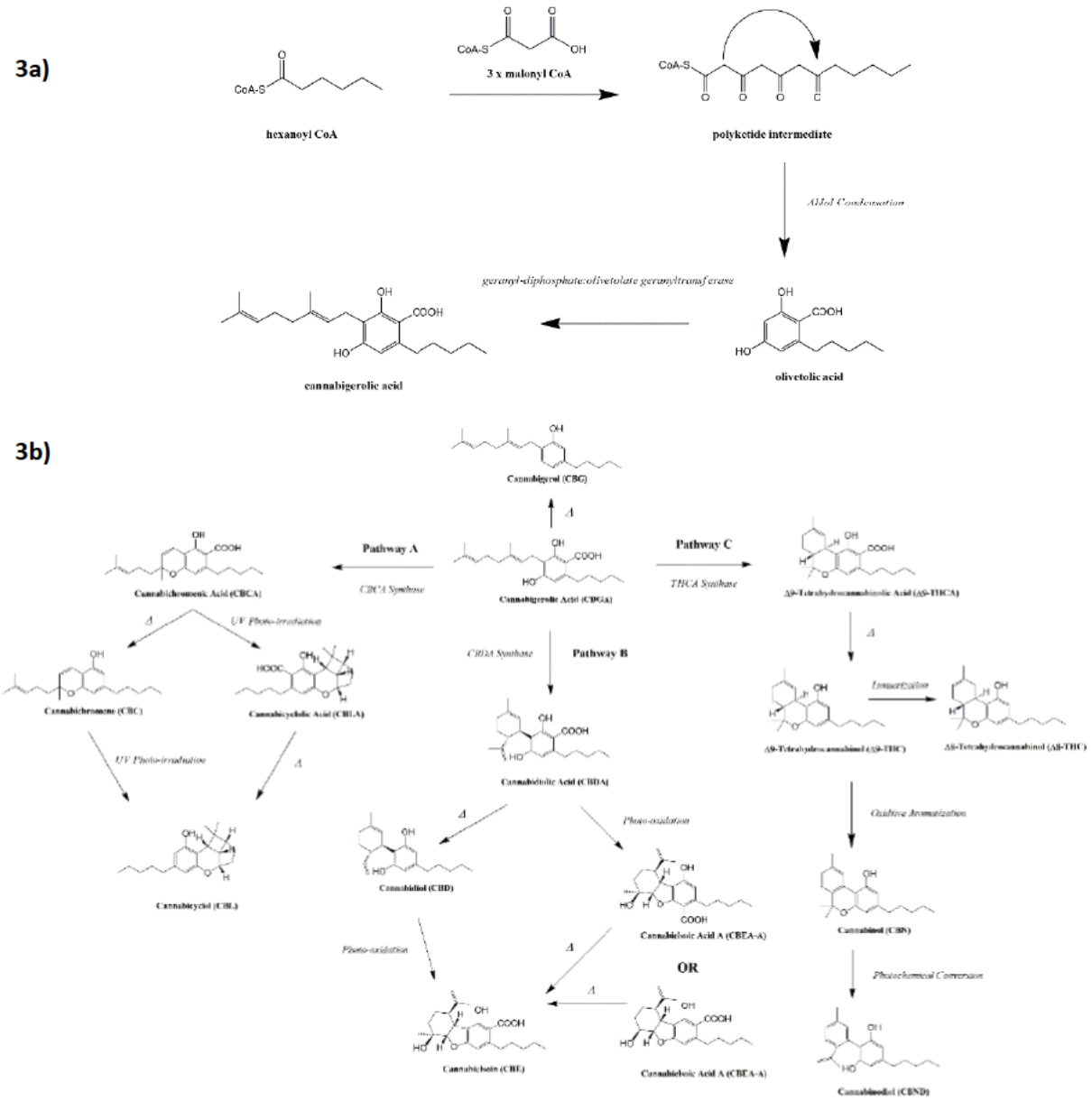


Figure 3. a) Biosynthesis of CBGA **b)** Biosynthesis of the major phytocannabinoids.

It is important to note the current assumption that all neutral phytocannabinoids originate in their carboxylated form, undergoing subsequent decarboxylation in a predominantly non-enzymatic manner, often induced by exposure to heat, light and/or atmospheric oxygen during the *cannabis* storage process (Hanuš et al., 2016, Pollastro et al., 2018). The structural changes caused by exposure to these factors are likely responsible, at least in part, for the structural diversity of the known phytocannabinoids.

Phytocannabinoids in Neurodegenerative Disease

Described by Raphael Mechoulam as a "pharmacological treasure trove", *cannabis* has been the source of a vast number of structurally diverse phytochemicals (Mechoulam, 2005). The following section introduces each major phytocannabinoid, along with a brief discussion of their general pharmacology, where known, and their applications in the treatment of neurodegenerative diseases.

Cannabigerol (CBG)

Cannabigerol is a non-psychotropic phytocannabinoid generally found only in trace amounts in cannabis. This is mainly due to the fact that its precursor, CBGA, serves as the parent compound to THC, CBD and CBC prior to its decarboxylation into CBG and is thus prone to degradation in order to fulfil this role (Russo, 2016). Structurally, CBG is characterised by the presence of a linear non-oxygenated isoprenyl residue. In general, CBG exhibits low CBR potency with low affinity for the CB_1 receptor, although it has been shown to act as a partial agonist at the CB_2 receptor (Navarro et al., 2018). Although CBG displays little affinity for the CB receptors, it is a potent Transient Receptor Potential Melastatin 8 (TRPM8) antagonist and acts to stimulate activity of both Transient Receptor Potential Vanilloid 1 (TRPV1) and Transient Receptor Potential Ankyrin 1 (TRPA1) ion channels (De Petrocellis et al., 2011, Starkus et al., 2019). CBG has also been identified as a potent α_2-adrenoceptor agonist and a moderate 5-HT_{1A} receptor antagonist, accounting for both its analgesic capacity and its ability to block the anti-emetic and anti-nausea effects of cannabidiol (Ligresti et al., 2016, Giovannoni et al., 2009, Rock et al., 2011, Cascio et al., 2010). CBG has also been studied in the context of neurodegenerative disease as a potential therapeutic, particularly in the context of PD, HD and ALS as detailed below. Despite this, the protective capacity of CBG in the context of AD has been understudied, presenting an avenue for future investigation.

One of the key pathological features common to many neurological and neurodegenerative diseases is neuroinflammation (Infante-Duarte et al., 2008). CBG has been demonstrated to target and increase the transcriptional activity of the nuclear receptor peroxisome proliferator-activated receptor-gamma (PPARγ), an attractive molecular target for the prevention and reduction of neuroinflammation due to its role as a modulator of proinflammatory cytokine expression (Granja et al., 2012, Jiang et al., 1998, O'Sullivan, 2016). Furthermore, a synthetic quinone derivative of CBG, VCE-003, has been shown to effectively ameliorate neuroinflammation in primary microglial cultures via activation of PPARγ and the inhibition of cytokine production (Granja et al., 2012). Interestingly, PPARγ activation has been associated with neuroprotection in an *in vitro* model of Aβ-induced neurotoxicity using human neural stem cells (Chiang et al., 2016). This perhaps suggests that compounds targeting PPARγ, such as CBG, may afford a degree of neuroprotection in this disease model, although this requires further experimental investigation.

CBG has been widely investigated in the context of HD, owing largely to its anti-inflammatory and antioxidant properties. Giacoppo and colleagues demonstrated that CBG was protective against hydrogen peroxide (H_2O_2)-induced oxidative stress in murine RAW264.7 macrophages, likely via modulation of the CB_2R (Giacoppo et al., 2017). The authors also identified marked reductions in a number of oxidative stress markers including iNOS, nitrotyrosine and PARP-1 via inhibition of IκB-α phosphorylation and translocation of nuclear factor-κB (NF-κB), highlighting the potent antioxidant capacity of CBG. A study investigating the protective effects of CBG in an *in vivo* model of HD found that treatment with 10mg/kg CBG significantly improved motor deficits in 3-nitropropionate-lesioned mice (Valdeolivas et al., 2015). CBG also prevented neuronal cell death in the striatal parenchyma of these 3NP-treated mice, as well as causing a significant reduction in the upregulation of inflammatory markers such as COX-2, TNFα, IL-6 and iNOS (Valdeolivas et al., 2015). Another study using a murine model of HD symptomology found that VCE-003.2, a novel synthetic, non-thiophilic derivative of VCE-003

successfully prevented quinolic acid-induced striatal neurodegeneration, while also improving motor function (Díaz-Alonso et al., 2016). Furthermore, oral administration of 10mg/kg VCE-003.2 has been shown to protect striatal neurons from mutant huntingtin-induced damage in male C57BL/6N mice while also promoting striatal neurogenesis (Aguareles et al., 2019).

CBG and its derivatives have also been investigated as potential therapeutics in PD, primarily for their anti-inflammatory properties. VCE-003.2 successfully attenuated the loss of tyrosine hydroxylase-containing neurons in the nigrostriatal neurons of LPS-lesioned mice (García et al., 2018). A recent study from Burgaz and associates confirmed the protective effect of VCE-003.2 in the same model, observing significant reductions in pro-inflammatory markers such as TNF-α, IL-1β, iNOS and COX-2 following treatment with 20mg/kg VCE-003.2 (Burgaz et al., 2019). Of particular interest in this study was the development and use of an oral lipid formulation of the drug, which may perhaps lend itself to use in clinical applications due to the ease of administration and lack of invasiveness. Several *in vitro* studies have also shown CBG to be effective in the context of ALS. One such study demonstrated that pre-treatment with CBG (2.5 and 5μM) rescued NSC-34 motor neuronal cells from neuroinflammation induced by LPS-stimulated culture medium (Mammana et al., 2019). These findings are consistent with a study by Gugliandolo and colleagues using a similar model of neuroinflammation in NSC-34 cells. In this study, pre-treatment with 7.5μM CBG caused a marked reduction in neuronal cell death following treatment with culture medium from LPS-stimulated RAW264.7 macrophages, as well as a total reduction in the expression of proinflammatory cytokines IL-1β, TNFα and IFNγ (Gugliandolo et al., 2018). Collectively, these studies demonstrate the exciting therapeutic potential for CBG in the treatment of neurodegenerative diseases. Although these studies are generally promising, much remains unknown about the broad neuroprotective capacity of CBG, highlighting the necessity for further characterisation.

(-)-Δ^9-*trans*-tetrahydrocannabinol (Δ^9-THC)

Δ^9-THC is the major psychotropic component in cannabis and is also the most abundant phytocannabinoid, readily extracted from *Cannabis Sativa* (Reekie et al., 2017). First isolated by Adams and colleagues in 1942, its structure was fully elucidated in 1964 by Gaoni & Mechoulam (Adams, 1942, Gaoni and Mechoulam, 1964). The structure of Δ^9-THC is thermodynamically unstable and readily degrades into its more stable isomer, Δ^8-THC. This occurs via the isomerisation of its double bond and the resultant shift from position 9 to position 8 (Prandi et al., 2018). Following the discovery of specific cannabinoid receptors, it was suggested that THC was an agonist at the CB_1 receptor (Felder et al., 1992). However, over the years an increasing number of *in vitro* and *in vivo* studies have suggested that Δ^9-THC is in fact a partial agonist at both CB_1 and CB_2 receptors in the low nanomolar range, displaying preferential binding at the CB_1R (Shen and Thayer, 1999, Paronis et al., 2012, Kelley and Thayer, 2004). Interestingly, it has been noted that the *n*-pentyl chain at position C-3 is the key pharmacophoric feature of Δ^9-THC's structure (Prandi et al., 2018). Modification of this side chain results in key changes to CB receptor affinity, selectivity and potency, where an increase in the number of carbon atoms (i.e. from pentyl to hexyl, heptyl or octyl) increases binding affinity and potency at CB receptors and a shorter alkyl group reduces potency (Martin et al., 1999, Razdan, 1986).

Δ^9-THC has been studied for its potential therapeutic effects in a variety of neurodegenerative disease models, including AD. One such study conducted by Eubanks and colleagues found that Δ^9-THC significantly inhibits Aβ aggregation via its interaction with acetylcholinesterase (AChE), with a degree of efficacy exceeding both donepezil and tacrine, two drugs that have been approved for the treatment of AD (Eubanks et al., 2006). Δ^9-THC has also been shown to prevent hydroperoxide-induced oxidative damage to an equal or greater degree than other common antioxidants, indicative of its antioxidant capacity (Hampson et al., 1998). It has also been suggested that Δ^9-THC may act directly on Aβ to reduce its aggregation, as evidenced by

a reduction in Thioflavin T (ThT) fluorescence (Eubanks et al., 2006). However, a subsequent study from Janefjord *et al* found via transmission electron microscopy that little to no morphological change had occurred in the Aβ aggregates following treatment with Δ^9-THC, despite observing a similar reduction in ThT fluorescence (Janefjord et al., 2014). This may suggest that Δ^9-THC interacts with ThT itself rather than the aggregating protein, a known limitation in the ThT assay shared by a number of phenolic compounds (Hudson et al., 2009). Cao and colleagues have demonstrated that THC has the ability to prevent the production of phosphorylated tau protein *in vitro*, while also demonstrating neuroprotection against $A\beta_{1\text{-}40}$ in neuronal N2A/AβPPswe cells (Cao et al., 2014). In animal studies using the 5XFAD APP transgenic mouse model of AD, once-daily treatment with 3mg/kg Δ^9-THC significantly reduced Aβ plaque formation over the course of 4 weeks, likely via an increase in neprilysin, an endopeptidase responsible for the degradation of Aβ (Chen et al., 2013). Furthermore, older mice administered 3mg/kg THC showed improvements in cognition and memory corresponding to increased synaptic plasticity in the brain when compared to younger mice on an equivalent dose, suggesting that low dose THC may restore brain functions in the absence of any pathological disease burden (Bilkei-Gorzo et al., 2017). Interestingly, the lack of effect in younger mice was further explored by a study showing that Δ^9-THC administration (also at 3 mg/kg) was associated with poorer behavioural and memory function in adolescent mice, although one that was offset by concurrent CBD administration (Murphy et al., 2017). Such distinct and contrasting age-related differences in Δ^9-THC effects in the brain require further investigation.

The protective capacity of Δ^9-THC in the context of PD has been relatively understudied. However, Δ^9-THC has been shown to be neuroprotective against 1-methyl-4-phenylpyridinium (MPP^+), lactacystin and paraquat toxicity in SH-SY5Y neuronal cells, while also reducing apoptosis and preventing oxidative stress (Carroll et al., 2012a). Although the CB_1 receptor was shown to be upregulated in response to neuronal injury, the protective

effect of Δ^9-THC was unable to be reproduced by the CB_1 agonist WIN55,212-2 and was unaltered by the addition of the CB_1 antagonist AM251, suggesting that this effect is largely CB_1 receptor-independent. Interestingly, the PPARγ antagonist T0070907 dose-dependently blocked the neuroprotective effects of Δ^9-THC, while the PPARγ agonist pioglitazone was able to protect against MPP^+-induced neurotoxicity. Furthermore, treatment with Δ^9-THC also caused an increase in PPARγ expression in SH-SY5Y cells exposed to MPP^+. A more recent study from Zeissler and colleagues has suggested that Δ^9-THC-mediated neuroprotection against MPP^+ in SH-SY5Y cells likely occurs via the PPARγ-dependent restoration of mitochondrial activity (Zeissler et al., 2016). These findings suggest that the broader neuroprotective effects of Δ^9-THC may also be mediated via activation of PPARγ, although this requires further investigation. In potential contrast to these findings, Nguyen and colleagues found that Δ^9-THC was able to ameliorate glutamate-induced toxicity and prevent apoptosis in murine primary mesencephalic cultures via the prevention of glutamate-induced mitochondrial membrane potential depolarization, in a CB_1 receptor-dependent manner (Nguyen et al., 2016). Together these findings exemplify the complexities of Δ^9-THC-mediated neuroprotection in PD, highlighting an important gap in our current understanding.

Cannabidiol (CBD)

Cannabidiol (CBD) is the second most abundant phytocannabinoid found in cannabis and the main generally non-psychotropic constituent. It has been widely studied in the context of neurodegenerative disease, primarily due to its neuroprotective, anti-inflammatory, antiepileptic, antipsychotic and analgesic capacities (Weston-Green, 2019). CBD was the first phytocannabinoid to be isolated by Adams and colleagues in 1940, yet its structure was only fully elucidated more than two decades later following the advent of NMR spectroscopy (Adams et al., 1940, Mechoulam and Shvo, 1963). Although CBD displays negligible affinity for either CB_1 or CB_2 receptors, it has been identified as a negative allosteric modulator of the CB_1 receptor (Laprairie et al., 2015). CBD readily penetrates the blood-brain-barrier following

both IP injection and oral administration in rats and mice and has an excellent safety profile with high tolerability in humans, making it an attractive lead candidate in the treatment of neurodegenerative disorders (Bloomfield et al., 2019, Deiana et al., 2012).

Numerous *in vitro* and *in vivo* studies have highlighted the neuroprotective capacity of CBD in the context of AD. *In vitro* studies have identified an ability for CBD to disrupt Aβ aggregation, as well as increasing the degradation and removal of preformed Aβ aggregates (Schubert et al., 2019). A study using an *in vitro* model of Aβ-induced neurotoxicity found that CBD was able to significantly prevent PC12 neuronal cell death in a concentration-dependent manner, while also reducing ROS generation and lipid peroxidation (Iuvone et al., 2004). A follow up study from these authors identified two distinct pathways by which CBD may exert its anti-Aβ effects. One potential pathway is via the inhibition of Aβ-induced tau hyperphosphorylation, mediated through the Wnt/β-catenin pathway (Esposito et al., 2006a). The Wnt/β-catenin pathway has been implicated in the development of oxidative stress and neuroinflammation and has been shown to be downregulated in AD (Vallée and Lecarpentier, 2016). CBD may indirectly enhance Wnt/β-catenin signalling, an effect associated with protection against Aβ-induced neurotoxicity as well as the reversal of AD-related cognitive deficits (Vargas et al., 2014, Vallée et al., 2017). Another potential mechanism of CBD-evoked neuroprotection may be through the inhibition of iNOS expression and nitric oxide production, mediated by the inhibition of phosphorylated p38 MAP kinase and TNFκB activation (Esposito et al., 2006b). This *in vitro* evidence was later confirmed *in vivo*, where CBD was able to mitigate Aβ-induced neuroinflammation in C57BL/6J mice via inhibition of iNOS and IL-1β expression, as well as nitric oxide release (Esposito et al., 2007). In line with previously mentioned studies, CBD has been shown to possess greater antioxidant potential than either ascorbate (vitamin C) or α-tocopherol (vitamin E) (Hampson et al., 1998). Other studies have also suggested that CBD may also exert its anti-amyloid effect via the downregulation of AD-associated genes, including those coding for kinases responsible for the phosphorylation of tau and secretases responsible

for the cleavage and processing of Aβ, including *BACE-1*, *PSEN1*, *PSEN2* and others (Libro et al., 2016). The authors also demonstrated that CBD is able to reduce the expression of GSK3β, an enzyme associated with the phosphorylation of tau, primarily by promoting PI3K/Akt signalling (Libro et al., 2016). Furthermore, there is *in vitro* evidence of CBD reducing the AD-associated increased in amyloid precursor protein (APP) in SH-SY5Y(APP^+) cells (Scuderi et al., 2014). The authors proposed that this occurs via the ubiquitination of APP, leading to a significant reduction in the levels of APP full length protein and a subsequent reduction in Aβ production. It was also noted that CBD could promote increased cell survival, with the authors attributing this to the selective activation of PPARγ by CBD (Scuderi et al., 2014). It has also been demonstrated that CBD is able to reduce the neuroinflammatory response and associated reactive gliosis, promote neurogenesis and prevent the development of AD-associated cognitive deficits in an *in vivo* rodent model (Watt and Karl, 2017). This study also suggested that CBD possesses the ability to antagonise the negative psychotropic effects of Δ^9-THC *in vivo*, while also showing evidence that a CBD:Δ^9-THC combination may afford a greater degree of neuroprotection that either compound in isolation (Watt and Karl, 2017). It is therefore clear that CBD represents an exciting and important candidate in the development of novel pharmacotherapies targeting AD.

Along with promising results in the field of AD, CBD has also been ascribed therapeutic potential in the treatment of the non-motor symptoms of PD. In a double-blind trial, 300mg/day CBD was shown to increase the quality of life of PD patients without psychiatric comorbidities, as measured by the PDQ-39 Questionnaire (Chagas et al., 2014). However, this same study was unable to find any significant differences in motor and general symptom scores or plasma BDNF or H(1)-MRS levels, indicating that CBD was unable to affect the primary motor symptoms associated with PD. CBD also possesses the ability to protect against MPP^+-induced neurotoxicity in PC12 cells (Santos et al., 2015). Several mechanisms have been proposed for this protective effect, including a reduction in caspase-3 activity in MPP^+-treated cells,

promotion of cellular differentiation by CBD, upregulation of axonal (GAP-43) and synaptic (synaptophysin and synapsin 1) proteins and the induction of neuritogenesis, likely via activation of trkA receptors (Santos et al., 2015). This idea is supported by the fact that CBD is unable to protect against MPP^+ toxicity in SH-SY5Y cells which do not express trkA receptors, suggesting that this receptor may play a role in CBD-mediated neuroprotection (Carroll et al., 2012a). A recent *in vivo* study also highlighted a potential role for CBD in preventing the reduction of the nociceptive threshold associated with Parkinsonism following exposure to 6-hydroxydopamine (6-OHDA) (Crivelaro do Nascimento et al., 2020). The authors suggest that this effect may be mediated via the activation of the TRPV1 receptor by CBD. Evidence from an in *in vivo* rodent model of PD found that CBD was able to attenuate dopaminergic neurodegeneration and the associated reductions in dopamine production and tyrosine hydroxylase expression following exposure to 6-OHDA (Lastres-Becker et al., 2005). A subsequent study using the 6-OHDA model of PD found that this reduction in neurodegeneration occurs in conjunction with increased mRNA expression of the antioxidant enzyme Cu,Zn-superoxide dismutase in the substantia nigra of treated rodents (García-Arencibia et al., 2007). As CBD has low affinity for CB receptors, these findings suggest that the neuroprotective effects observed in this model are likely CB-receptor independent and possibly related to antioxidant and anti-inflammatory properties instead.

The potential therapeutic effects of CBD in HD have been of considerable interest recently, with numerous studies investigating potential benefits in HD models. One such study evaluated the protective effects of CBD in PC12 cells expressing mutant huntingtin, the induction of which caused rapid cell death (Aiken et al., 2004). CBD significantly prevented mutant huntingtin-induced cell death, with the authors attributing this effect to antioxidant mechanisms. Furthermore, CBD was also able to provide protection against 3-nitropropionic acid (3-NP)-induced striatal damage *in vivo* in a CBR and TRPV1-independent manner (Sagredo et al., 2007). The authors of this study also attributed this protection exclusively to

the antioxidant properties of CBD. A subsequent preclinical study from this group investigated the potential role of Sativex (Nabiximols, 1:1 ratio Δ^9-THC:CBD) in this model. Similarly to CBD alone, administration of Sativex was found to attenuate 3-NP-induced striatal pathology independent of CBR activation and primarily through antioxidant effects (Sagredo et al., 2011). Interestingly, the authors also highlighted a protective effect of Sativex in malonate-lesioned rats via a reduction in iNOS gene expression. This promising preclinical evidence of Sativex in HD has led to several small clinical trials being conducted. Unfortunately, one pilot study of 25 HD patients found no significant improvements in HD symptomology or clinically-relevant biomarkers following daily treatment with Sativex over the course of 12 weeks (López-Sendón Moreno et al., 2016). The authors suggest that further studies with higher doses and longer treatment periods are needed.

Cannabichromene (CBC)

First isolated in 1966 by Gaoni and Mechoulam, CBC was once thought to be the second most abundant phytocannabinoid in cannabis and is considered one of the *big four* of cannabis constituents, alongside CBD, THC and CBG (Gaoni and Mechoulam, 1966, Pollastro et al., 2018). However, CBC has been vastly understudied in comparison with these other, more abundant pCBs and much remains unknown about its biological activity. CBC appears to cause hypothermia, sedation and hypoactivity in mice, while also displaying anti-inflammatory and moderately analgesic effects *in vivo* (Turner et al., 1980, Davis and Hatoum, 1983). It is also a potent TRPA1 agonist, where it acts as a non-covalent modulator with an EC_{50} value of 90nM as measured by a $[Ca^{2+}]_i$ functional assay (De Petrocellis et al., 2011). An *in* vivo study using the tail flick test as a measure of antinociceptive capacity found that CBC was able to elicit an antinociceptive response in anaesthetized rats, with a maximal response seen at 6nM (Maione et al., 2011). This antinociceptive response was blocked by the addition of a selective TRPA1 receptor antagonist, suggesting that CBC may exert its effect via the activation of this channel (Maione et al., 2011). CBC has been shown to significantly and dose-dependently reduce

carrageenan-induced rat paw oedema (Wirth et al., 1980). In line with these results, CBC also significantly reduced LPS-stimulated inflammation in the paw oedema assay by a CBR-independent mechanism (DeLong et al., 2010). A recent *in vitro* study suggested that CBC also acts as a selective agonist at the CB_2R, with greater efficacy in hyperpolarising AtT20 cells than THC (Udoh et al., 2019). Further *in vitro* studies have revealed that CBC is able to reduce the expression of proinflammatory mediators, such as macrophage and mastocyte recruiter MCP-2, while also reducing the levels of proinflammatory cytokines including IL-6 and IL-8, evidencing a potential role for CBC as an anti-inflammatory agent (Petrosino et al., 2018).

CBC has been relatively understudied in the context of neurodegenerative diseases, with very few published studies. In an *in vitro* model of AD, whereby MC65 neuronal cells were induced to generate the C99 fragment of APP, CBC proved to be a highly potent inhibitor of amyloid toxicity, with an EC_{50} of 100nM (Schubert et al., 2019). This study also highlighted the potent inhibitory effect of CBC on oxytosis, a programmed cell death pathway in which glutamate inhibits cysteine uptake into neuronal cells, causing a reduction in glutathione synthesis, which stimulates increased mitochondrial ROS production, leading to the onset of oxidative stress, intracellular calcium influx and eventual cell death (Schubert et al., 2019, Tan et al., 2001). The ability of CBC to inhibit this pathway presents an interesting avenue of exploration for future studies regarding pCBs and neurodegeneration. Another group found that 10µM CBC was able to protect against Aβ-induced toxicity in SH-SY5Y cells, while also showing that 10nM CBC significantly reduced nitrite production in Aβ-treated C6 glial cells (Iuvone et al., 2019). The reduction of intracellular nitrite levels is an important biomarker in the study of many neurodegenerative diseases including AD, PD, ALS and HD (Iuvone et al., 2019). These findings therefore suggest that CBC may afford neuroprotection in these disease models via this mechanism, though this concept requires further exploration.

Cannabinol (CBN)

Cannabinol was first the phytocannabinoid to be isolated from cannabis, a feat first achieved by Wood *et al* in 1896 following the purification of *red oil* derived from hemp (Wood et al., 1896). A partial structure for CBN was first reported by R.S. Cahn in 1933, with its structure fully elucidated by two groups in 1940 (Cahn, 1933, Adams et al., 1940, Jacob and Todd, 1940). CBN is found in large quantities in dried and aged cannabis material, owing to its nature as the primary degradation product of Δ^9-THC; this degradation is thought to occur during storage (Harvey, 1990, Pertwee, 2006). Interestingly, high amounts of CBNA is also present in dried cannabis, although this readily decarboxylates into CBN due to its heat instability (Harvey, 1990). CBN is pharmacologically active at both the CB_1 and CB_2 receptor, acting as a weak partial CB_1 agonist but binding with much higher affinity at the CB_2 receptor (Felder et al., 1995, Maurya and Velmurugan, 2018). However, there are conflicting reports on the actions of CBN at the CB_2 receptor. Firstly, Munro *et al* reported that CBN and Δ^9-THC were equipotent at the CB_2R, a finding which has since been disputed by Rhee and colleagues, who found CBN to be 2-4 times less potent (Munro et al., 1993, Rhee et al., 1997). Furthermore, in the cyclic AMP assay in COS-7 cells transfected with rat CB_2 receptors, CBN acted as a CB_2R agonist at 1μM, while in human CB2-transfigfected CHO cells, CBN acted as an inverse agonist in the GTPγS binding assay at sub-micromolar concentrations (Rhee et al., 1997, MacLennan et al., 1998). However, Turner and colleagues have suggested that this discrepancy may result from the differing concentration ranges used in each experiment, highlighting the relationship between ligand concentrations and variances in the conformational state of receptors in tissue (Turner et al., 2017, Kenakin, 2001). CBN has also been identified as a potent TRPA1 agonist, while also displaying inhibitory action at TRPM8 cation channels (De Petrocellis et al., 2011). The multifaceted receptor activity of CBN presents a vast and interesting area of exploration for future studies, with potential implications in the field of neuroprotection.

Although understudied in this context, CBN has been identified as neuroprotective in several neurodegenerative disease models. A study by Schubert and colleagues confirmed that CBN was able to afford neuroprotection via the inhibition of oxytosis, in similar fashion to CBC, with an EC_{50} of 700nM (Schubert et al., 2019). This study also determined that CBN was neuroprotective against Aβ *in vitro*, with the ability to prevent protein aggregation as well stimulating the degradation and removal of pre-formed Aβ aggregates (Schubert et al., 2019). Importantly, it was also shown that substituting the hydroxyl group at position 1 of the aromatic ring in CBN with a methoxy group rendered the compound inactive. This inactivation was also observed in JWH133, a Δ^8-THC analogue that lacks the aromatic hydroxyl group at position 1, along with the CB_1 agonists RCS-8 and MDA19 which also lack an aromatic hydroxyl group (Schubert et al., 2019). These findings suggest that the presence of an aromatic hydroxyl group is essential for neuroprotection. In a study using the SOD1 (G93A) mouse model of ALS, daily treatment with 5mg/kg CBN over 12 weeks was shown to significantly delay the onset of disease, though it was unable to prolong survival (Weydt et al., 2005). CBN has also been shown to be neuroprotective in an *in vitro* model of HD, where it acted to decrease LDH release from PC12 cells transfected with mutant huntingtin, with an EC_{50} of 30μM (Aiken et al., 2004). Overall, CBN presents itself as a promising candidate in lead development for the treatment of a number of neurodegenerative diseases, particularly where it lacks the negative psychotropism of its progenitor, Δ^9-THC.

Other pCBs and Cannabis Phytochemicals

Although major pCBs such as CBD and Δ^9-THC have been studied extensively for their neuroprotective potential, the surface has barely been scratched when it comes many of the other less abundant pCBs, with a relative paucity of studies. Below is a summary of the general findings for these pCBs as they relate to neurodegeneration, as well as emerging information regarding other non-cannabinoid phytochemicals found in the cannabis plant.

(-)-Δ^8-*trans*-tetrahydrocannabinol (Δ^8-THC)

Δ^8-THC is a mildly intoxicating isomer of Δ^9-THC which provides for increased thermodynamic stability over its precursor, afforded by a change of its double bond from position 9 to position 8 (Prandi et al., 2018). Δ^8-THC is pharmacologically active at both CB_1 and CB_2 receptors, with equivalent potency to Δ^9-THC (Stern and Lambert, 2007, Howlett et al., 2002). This CBR activity is thought to be responsible for the anti-inflammatory effects of Δ^8-THC, as made evident by Thapa and colleagues (Thapa et al., 2018). The authors induced corneal hyperalgesia via chemical cauterization of the corneal epithelium in both wild-type and CB_2R knockout mice. Topical application of Δ^8-THC effectively reduced the pain score and neutrophil infiltration in wild-type mice. Interestingly, this antinociceptive activity was blocked by the addition of the CB_1 receptor antagonist AM251, while Δ^8-THC maintained its antinociceptive capacity in CB_2R knockout mice, indicating that the antinociceptive and anti-inflammatory effects of Δ^8-THC are mediated by the CB_1 receptor (Thapa et al., 2018).

As with many pCBs, the potential capacity of Δ^8-THC as a neuroprotective agent has been largely understudied. However, Δ^8-THC has been identified as an effective inhibitor of oxytosis *in vitro* with an EC_{50} of 400nM, while also effectively preventing Aβ-induced toxicity in MC65 cells with an EC_{50} of 85nM (Schubert et al., 2019). Interestingly, Δ^8-THC displayed lower EC_{50} values than Δ^9-THC in each of these settings, indicating increased potency of the isomer over its parent compound. In an *in vivo* model of experimental autoimmune encephalomyelitis, treatment with 40mg/kg Δ^8-THC for up to 21 days was shown to significantly reduce the incidence and severity of neurological deficits in both Hebrew University and Lewis rats, while also increasing glucocorticoid secretion (Wirguin et al., 1994). The authors postulate that this increase in corticosterone secretion may be the mechanism by which Δ^8-THC affords this neuroprotection, an idea supported by the work of MacPhee and colleagues (MacPhee et al., 1989). These studies indicate a range of potential mechanisms by which Δ^8-THC may provide

protection in neurodegenerative disease models, with further investigation required for confirmation and clarification.

Cannabicyclol (CBL), Cannabinodiol (CBND), Cannabitriol (CBT) & Cannabielsoin (CBE)

Cannabicyclol was first isolated by Korte and Sieper in 1964, with its structure later independently elucidated by both Clausen and colleagues and Gaoni and Mechoulam; CBL is found only in relatively minor concentrations in the cannabis plant (Korte and Sieper, 1964, Gaoni and Mechoulam, 1971, Claussen et al., 1968, Vree et al., 1972). Cannabinodiol is a derivative Δ^9-THC, resulting from the photochemical conversion of CBN. Structurally, CBND presents as the fully aromatized form of CBD and was first isolated from a hexane-ether extract of Lebanese hashish by Lousberg *et al* in 1977 (Robert J.J.Ch et al., 1977). Cannabitriol was first reported in 1966 by Obata and Ishikawa, with its structure fully elucidated more than a decade later by the work of Chan and colleagues (Obata and Ishikawa, 1966, Chan et al., 1976). Bercht *et al* identified Cannabielsoin in an ethanolic extract of Lebanese hashish in 1973, with its structure determined by Uliss and colleagues in 1974 via synthesis from cannabidiol acetate; the carboxylic acid forms of CBE, CBEA-A and CBEA-B, were isolated and synthesized by Shani and Mechoulam in 1974 (Bercht et al., 1973, Uliss et al., 1974, Shani and Mechoulam, 1974). Surprisingly, none of these pCBs have been investigated for their biological effects, with the exception of CBE. A study by Yamamoto and colleagues identified CBE as a mammalian metabolite of CBD both *in vitro* and *in vivo* in the guinea pig (Yamamoto et al., 1988). This study also investigated the pharmacological effects of CBE on body temperature and pentobarbital-induced sleep; intravenous injection of 10mg/kg CBE had little effect in each case. Given the lack of investigation into the biological and pharmacological effects of these pCBs, it is clear that a large gap remains in our understanding of the bioactivity of such minor phytocannabinoids.

Cannflavin A & Canniprene

Although the phytocannabinoids tend to garner the majority of attention, *cannabis* is also the source of a vast array of other phenolic compounds with diverse biological and pharmacological profiles. One such example is cannflavin A (CFA), a geranylated flavonoid unique to *cannabis sativa*, which was isolated from an ethanolic extract by Barrett and colleagues in 1985, though a corresponding structure was first described by Crombie, Crombie and Jamieson in 1980 (Barrett et al., 1985, Russo and Marcu, 2017, Crombie et al., 1980). The biosynthesis of CFA differs from the phytocannabinoids in that it involves the enzymatic conversion of several flavonoid precursors, namely luteolin and chrysoeriol. Firstly, luteolin undergoes regiospecific methylation via the actions of *C. sativa O*-methyltransferase 21 (CsOMT21), resulting in the formation of chrysoeriol, which may then be geranylated by *C. sativa* prenyltransferase 3 (CsPT3) to form CFA (Rea et al., 2019). The addition of a prenylated side chain, such as the geranyl side chain of CFA, is thought to increase the lipophilicity of the compound and often results in altered biological activity in comparison to other flavonoids (Šmejkal, 2014). Barrett and colleagues also identified CFA as an inhibitor of prostaglandin production *in vitro*, where CFA was able to inhibit PGE_2 production in cultured synovial cells with an almost 30-fold increase in potency over aspirin (IC_{50} values of 31ng/mL and 840ng/mL, respectively) (Barrett et al., 1985). Further investigation by Werz *et al* identified the inhibition of microsomal prostaglandin E_2 synthase (mPGES-1) and 5-lipoxygenase (5-LO) as the specific molecular mechanisms by which CFA exerts this effect (Werz et al., 2014). Although more than 30 years have lapsed since its discovery, CFA remains virtually unstudied in the context neurodegenerative disease. However, a recent study by Eggers *et al* found that 10μM CFA significantly inhibited $A\beta_{1\text{-}42}$-induced cytotoxicity in PC12 cells associated with reduction in the density of $A\beta_{1\text{-}42}$ aggregates (Eggers et al., 2019). It is apparent from this study that CFA possesses some degree of neuroprotective potential in the context of AD.

Among the myriad of *cannabis* phenolics is the dihydrostilbene canniprene, an isoprenylated bibenzyl unique to cannabis first identified by Crombie and Crombie in 1978 (Crombie et al., 1978). As is the case for many cannabis constituents, canniprene has been relatively unstudied and much remains unknown about its biological and pharmacological effects. In one of the few studies of canniprene bioactivity, Allegrone and colleagues identified the compound as an inhibitor of inflammation (Allegrone et al., 2017). The authors demonstrated that canniprene was able to inhibit 5-LO activity with greater efficacy than CFA, however it was less effective in the inhibition of mPGES-1 activity than CFA (Allegrone et al., 2017). Although this study was limited in its scope it is clear that inflammation plays a vital role in neurodegeneration. Therefore, compounds with anti-inflammatory properties such as canniprene present as attractive and novel candidates for future study.

Cannabinoid SARs in AD: Insights from *in silico* Molecular Modelling

Given that a number of studies have investigated direct phytocannabinoid interactions with Aβ as a mechanism of neuroprotection, it follows that molecular modelling can be used to provide *in silico* insights and predictions to guide *in vitro* assays and potentially further preclinical development. Molecular modelling can provide information about ligand binding interactions with proteins such as Aβ, with particular relevance to fibril and aggregate formation. Furthermore, this *in silico* approach provides information on possible protein binding domains for small molecule interactions, which may then be used to interpret the potential molecular basis for neuroprotection, allowing for efficient, iterative lead development with relatively high throughput. As Aβ is capable of adopting multiple structural conformations of varying (and contestable) neurotoxicity during fibrillogenesis, modelling is presented here for pCB ligand interactions with both the monomeric and oligomeric species of $A\beta_{1\text{-}42}$, the major isoform found in the human brain.

Molecular Modelling: Results

The ten major phytocannabinoids were docked with the $A\beta_{1-42}$ monomer (PDB ID: 1IYT) and pentamer (PBD ID: 2BEG) using CLC Drug Discovery Workbench (v2.4.1), as described previously (Marsh et al., 2017). The Δ^9-THC homologue tetrahydrocannabivarin (THCV) and the CBD homologue cannabidivarin (CBDV) were also included in this docking summary in order to identify differences in binding properties afforded by the presence of an *n*-propyl side chain at position 3 (THCV and CBDV) rather than an *n*-pentyl side chain (Δ^9-THC and CBD).

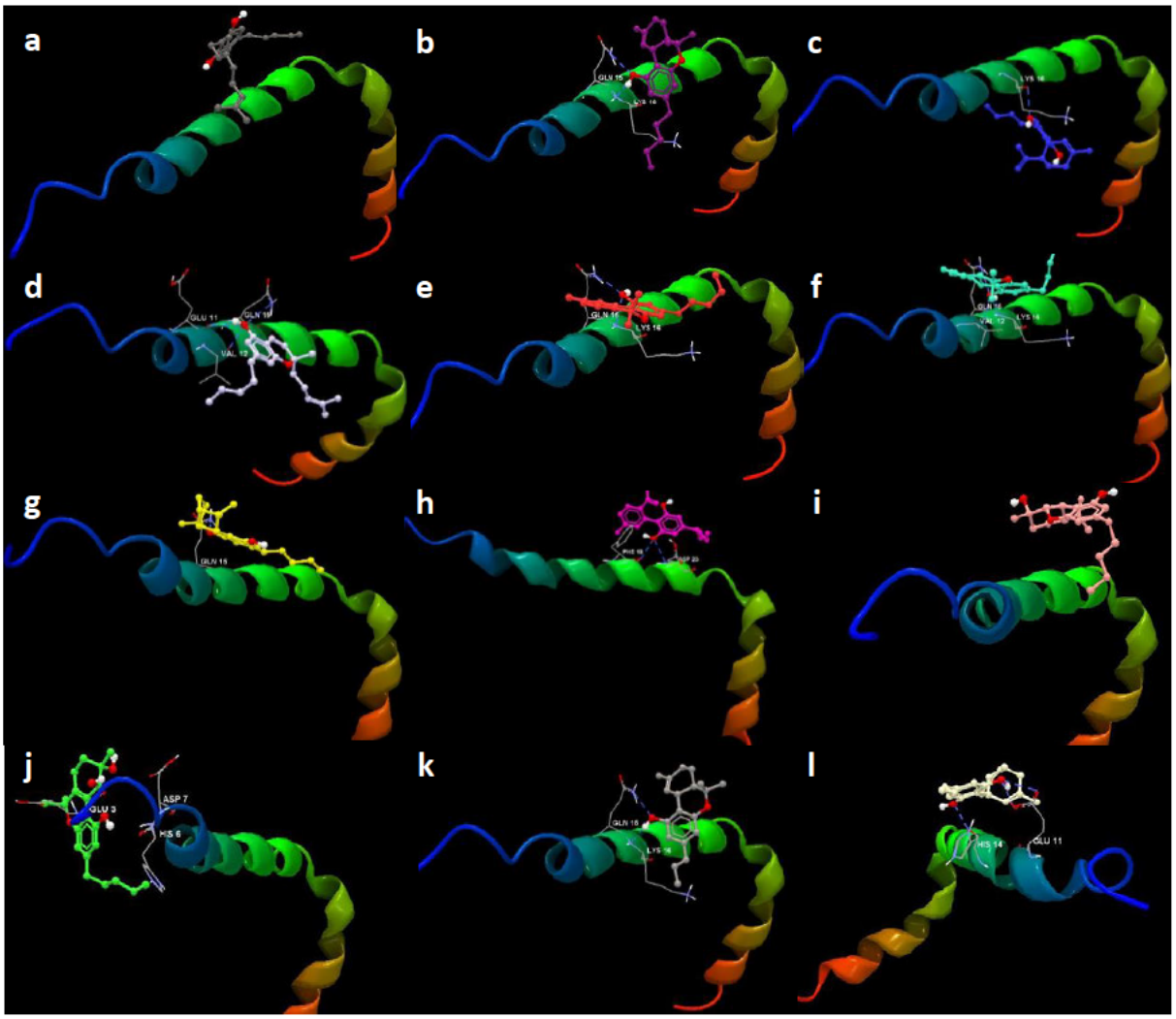
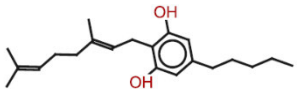
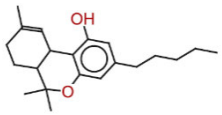
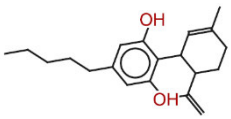
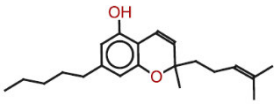
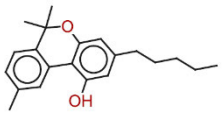
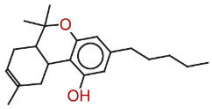
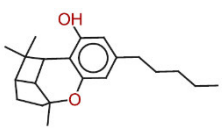


Figure 4. Docking positions for the phytocannabinoids with the $A\beta_{1-42}$ monomer (**PDB ID: 1-IYT**). **(a)** CBG, **(b)** Δ^9-THC, **(c)** CBD, **(d)** CBC, **(e)** CBN, **(f)** Δ^8-THC, **(g)** CBL, **(h)** CBND, **(i)** CBE, **(j)** CBT, **(k)** THCV, **(l)** CBDV. Most pCBs bound within the centre of the monomer, with variable hydrogen bonding and steric interactions noted.

Docking results for each pCB with the $A\beta_{1-42}$ monomer (**PDB ID: 1IYT**) are summarised in Table 1. CBG **(Fig. 4a)** possessed the highest affinity for the monomer (docking score of -55.87), followed by CBN (-50.07), with CBDV (-35.01) **(Fig. 4e and l)** displaying the lowest binding affinity of all pCBs. The majority of pCBs bound towards the centre of the monomer, displaying diverse hydrogen bonding interactions (Figure 3). Notable exceptions include CBG and CBE **(Fig. 4i)** (-48.92), neither of which displayed any degree of hydrogen bonding with the monomer. However, a high degree of steric interaction for these pCBs was noted, with scores of -57.84 and -50.13, respectively. In contrast, CBT **(Fig. 4j)** (-46.83) alone bound towards the *N*-terminus, interacting with residues Glu 3, His 6 and Asp 7. Several pCBs, such as CBN **(Fig. 4e)**, Δ^9-THC **(Fig. 4b)** (-48.28), THCV **(Fig. 4k)** (-47.51), Δ^8-THC **(Fig. 4f)** (-45.29) and CBD **(Fig. 4c)** (-44.46) formed hydrogen bonds with the key Lys 16 residue with several others, such as CBC **(Fig. 4d)** (-48.40) and CBL **(Fig. 4g)** (-45.47) binding in close proximity. CBND **(Fig. 4h)** (-42.81) bound further towards the centre of the monomer, interacting with Phe 19 and Asp 23.

pCB	Structure	Docking Score	Steric Interaction Score	H-bond Forming Residues	H-bond Score
CBG		-55.87	-57.84	None	0.00
Δ^9-THC		-48.28	-45.93	Gln 15, Lys 16	-3.48
CBD		-44.46	-43.47	Lys 16	-2.00
CBC		-48.40	-46.07	Glu 11, Val 12, Gln 15	-4.16
CBN		-50.07	-47.17	Gln 15, Lys 16	-3.30
Δ^8-THC		-45.29	-40.07	Val 12, Gln 15, Lys 16	-5.66
CBL		-45.47	-45.57	Gln 15	-2.00

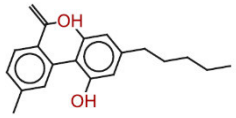
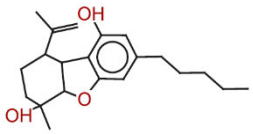
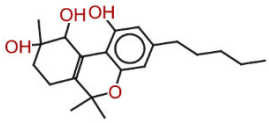
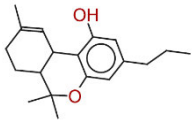
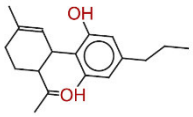
CBND		-42.81	-45.58	Phe 19, Asp 23	-4.00
CBE		-48.92	-50.13	None	0.00
CBT		-46.83	-39.01	Glu 3, His 6, Asp 7	-8.00
THCV		-47.51	-44.51	Gln 15, Lys 16	-3.38
CBDV		-35.01	-38.91	Glu 11, His 14	-6.00

Table 1. Docking profiles of phytocannabinoids with the $A\beta_{1\text{-}42}$ monomer (**PDB ID: 1-IYT**).

Docking results for the pCBs with the Aβ pentamer (**PDB ID: 2BEG**) are summarised in Table 2. This oligomeric structure is comprised of five aligned and segmented Aβ monomers (chains A, B, C, D and E), each consisting of residues 17-42 of the full $A\beta_{1\text{-}42}$ structure. In accordance with the monomer docking results, CBG **(Fig. 5a)** bound to the pentamer with the highest affinity of all pCBs by a considerable margin (docking score of -87.92), followed by CBN **(Fig. 5e)** (-76.66), with CBDV **(Fig. 5l)** (-44.46) again displaying the lowest affinity. All of the pCBs interacted with the hydrophobic groove of the Aβ oligomer, with CBG exhibiting the greatest degree of hydrogen bonding, interacting with Leu 17, Val 18 and Phe 19 of Chain D, along with Gly 38 of Chain E. CBDV, THCV **(Fig. 5k)** (-51.07) and Δ^9-THC **(Fig. 5b)** (-57.27) each bound

exclusively with Leu 17 of Chain C, with CBL **(Fig. 5g)** (-54.59) and Δ^8-THC **(Fig. 5f)** (-58.76) binding exclusively with Leu 17 of Chain E. Other pCBs including CBT **(Fig. 5j)** (-56.00) and CBND **(Fig. 5h)** (-49.17) also bound to Leu 17 of Chain C, while also interacting with Val 18 of Chain B and D, respectively. Both CBN and CBC **(Fig. 5d)** (-72.70) also bound to Val 18 of Chain D, with CBN also forming hydrogen bonds with Phe 19 of Chain D and Leu 17 of Chain E; CBC also bound to Leu 17 and Phe 19 of Chain E. CBE **(Fig. 5i)** (-60.12) formed hydrogen bonding interactions exclusively with Chain E, where it interacted with Leu 17, Phe 19 and Gly 37. Of all the pCBs, CBD **(Fig. 5c)** (-47.21) alone displayed no hydrogen bonding interactions with the Aβ pentamer, although a relatively high steric interaction score of -49.57 was observed.

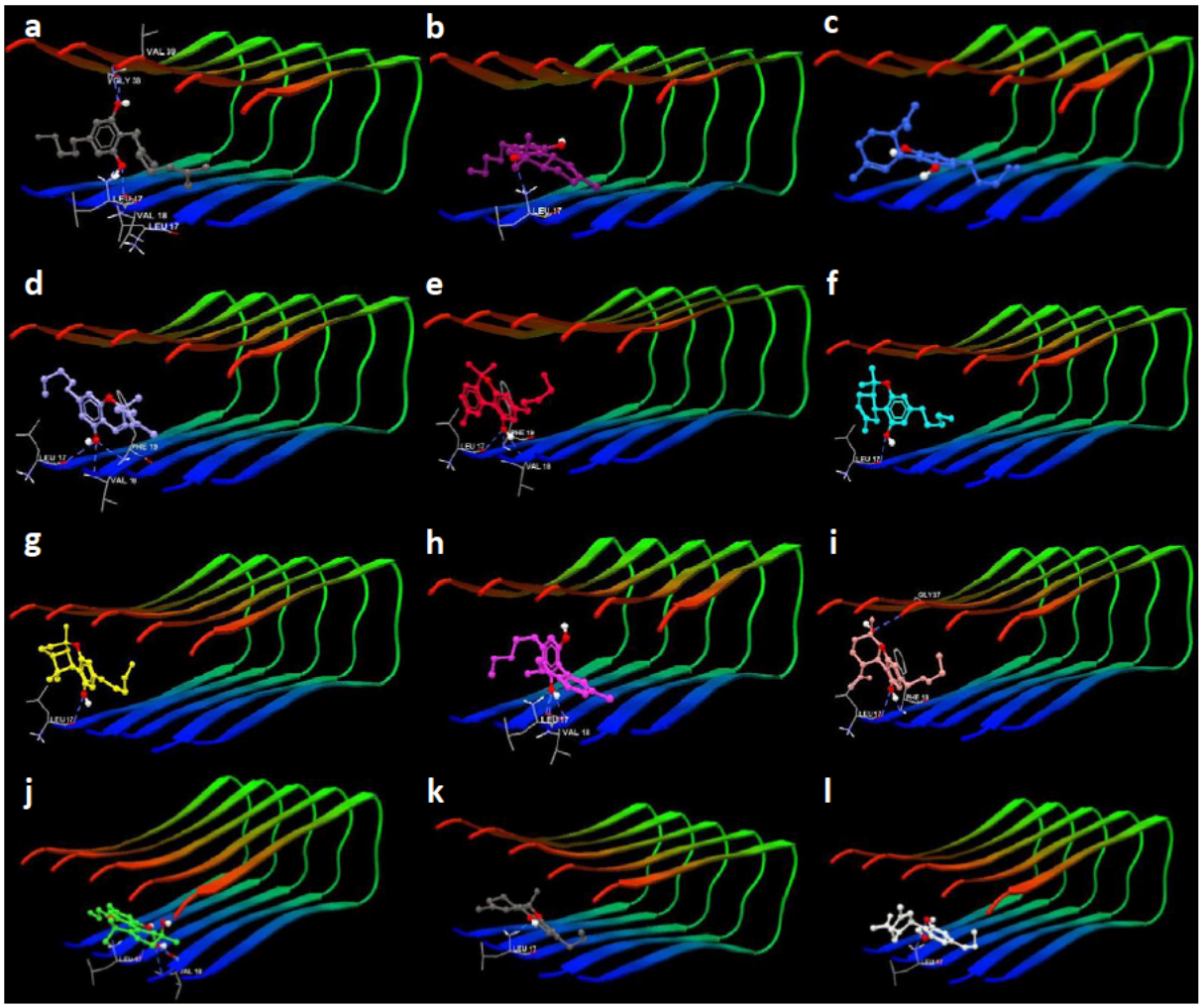
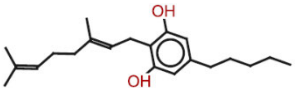
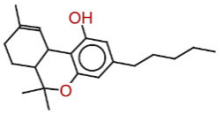
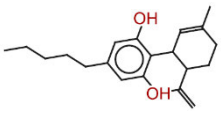
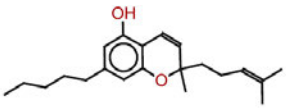
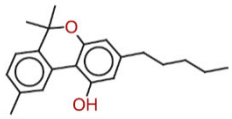
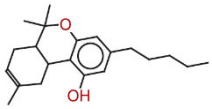
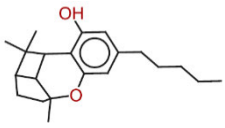
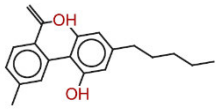


Figure 5. Docking positions for the phytocannabinoids with the Aβ pentamer (**PDB ID: 2BEG**). **(a)** CBG, **(b)** Δ^9-THC, **(c)** CBD, **(d)** CBC, **(e)** CBN, **(f)** Δ^8-THC, **(g)** CBL, **(h)** CBND, **(i)** CBE, **(j)** CBT, **(k)** THCV, **(l)** CBDV. Cannabigerol (CBG) exhibited the greatest steric interaction score, a feature noted with extensively prenylated tails. This enables an avid binding to the hydrophobic groove in the oligomeric amyloid β sheet.

pCB	Structure	Docking Score	Steric Interaction Score	H-bond Forming Residues (Chain)	H-bond Score
CBG		-87.92	-84.97	Leu 17, Val 18, Val 39 (D), Gly 38 (E)	-7.23
Δ^9-THC		-57.27	-55.81	Leu 17 (C)	-2.00
CBD		-47.21	-49.57	None	0.00
CBC		-72.70	-69.03	Val 18, Phe 19 (D), Leu 17 (E)	-5.96
CBN		-76.66	-72.92	Val 18 (D), Leu 17, Phe 19 (E)	-4.97
Δ^8-THC		-58.76	-57.59	Leu 17 (E)	-2.00
CBL		-54.59	-53.78	Leu 17 (E)	-2.00
CBND		-49.17	-51.71	Leu 17, Val 18 (C)	-5.77

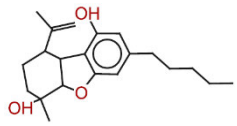
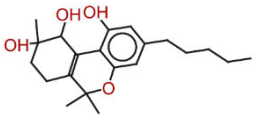
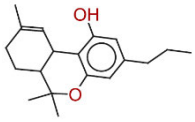
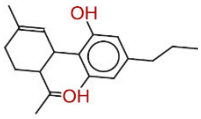
CBE		-60.12	-56.07	Leu 17, Phe 19, Gly 37 (E)	-5.56
CBT		-56.00	-51.56	Val 18 (B), Leu 17 (C)	-5.54
THCV		-51.07	-50.82	Leu 17 (C)	-0.64
CBDV		-44.46	-52.77	Leu 17 (C)	-1.60

Table 2. Docking profiles of phytocannabinoids with the Aβ pentamer (**PDB ID: 2BEG**)

Molecular Modelling: Discussion

Modelling with both the Aβ monomer and pentamer revealed that each of these pCBs interacted with the Aβ protein primarily via steric and hydrogen bonding interactions. The results showed that CBG bound with the highest affinity of all the pCBs in this study. This is likely due to the presence of a geranyl side chain in the structure of CBG, as this side chain is associated with increased lipophilicity and may therefore increase the propensity for binding within the hydrophobic groove of the amyloid β pentamer (Šmejkal, 2014). Of particular interest in this study was the relationship between alkyl side chain length and Aβ biding affinity. A consistent pattern was identified in both the monomer and pentamer modelling, whereby increased alkyl chain length was associated with an increase in binding affinity. This is made evident by the higher docking score of pCBs containing a pentyl side chain, such as Δ^9-THC and CBD, when compared to their propyl side chain counterparts, THCV and CBDV. Interestingly, this increase in binding affinity correlates with the increased cannabinoid receptor potency observed with increased side chain length (Martin et al., 1999, Prandi et al., 2018, Razdan, 1986). Increased

alkyl chain length has been associated with increased hydrophobicity in other contexts (Wang and Gao, 2018, Gao et al., 2017). This may therefore serve as a possible explanation for the higher binding affinity of the pentyl chain-possessing pCBs modelled in this study, particularly for those interactions with the hydrophobic groove of the amyloid pentamer. An interesting point to note from the docking simulation is that the degree of aromatisation appears to impact binding affinity. This is most evident in the comparison between Δ^9-THC and CBN, its fully aromatised counterpart, where CBN bound with much higher affinity, most notably with the Aβ pentamer. The increased binding affinity may perhaps be afforded by the full resonance distribution and high degree of planarity of the fully aromatised C ring, increasing hydrophobicity and thus allowing for greater propensity for binding within the hydrophobic groove of the amyloid pentamer. This effect was also noted in the comparison between CBD and the fully aromatised CBND, where CBND bound with higher affinity to the Aβ pentamer. Another point identified in this study is the fact that pCBs with closed ring structures, such as THC, THCV and CBN, bound with higher affinity than those with open-ring structures, such as CBD, CBDV and CBND; this effect remained consistent across both monomer and pentamer modelling interactions. A comparison of several studies reveals that these pCBs with closed-ring structures, particularly THC and THCV, also generally exhibit higher affinity for cannabinoid receptors than those with open-ringed structures, such as CBD and CBDV (Rosenthaler et al., 2014, McPartland et al., 2015). Although the correlation between these two observations is indirect, it does highlight the notion that alterations in the pyran B ring correlate with differences in pharmacological activity; further investigation into this relationship as it relates to Aβ binding, aggregation and toxicity is therefore warranted.

Another important insight gained from this study relates to the binding positions of particular pCBs, namely CBN, Δ^9-THC, THCV, Δ^8-THC and CBD, each of which bound directly to Lys 16. A paper from Sinha and colleagues identified Lys 16 as a key residue in the assembly and toxicity of Aβ (Sinha et al., 2012). Therefore, compounds with an ability to interact with this

residue may prevent the self-assembly of Aβ, thus preventing the associated aggregation and subsequent toxicity. Furthermore, inhibition of $A\beta_{1-42}$ nucleation and elongation by non-catechol-type flavonoids has been directly linked to interactions with the regions between Tyr10 and Gln 15, as well as Phe 19 to Ala 21 (Hanaki et al., 2016). A number of the pCBs modelled in this study, each of which lack a catechol moiety, were shown to bind to residues within these regions, perhaps suggesting that they may also possess an inhibitory effect on $A\beta_{1-42}$ fibril formation via this mechanism. However, further *in vitro* and *in vivo* investigation is required in order to explore this concept. Collectively, these points serve to highlight the exciting potential for a number for these pCBs to inhibit the assembly and aggregation of Aβ, thus providing additional avenues of investigation into the neuroprotective capacity of these compounds in the context of AD and in neurodegenerative disease more generally.

Conclusion

In any discussion regarding the potential use of a cannabis products in a therapeutic setting, it is important to distinguish this from findings in studies investigating recreational cannabis consumption, particularly the variable impacts associated with long-term and heavy adult use of smoked cannabis in the brain (Solowij et al., 2013, Jager et al., 2006, Yücel et al., 2008). Such discourse forms the basis of a narrative often juxtaposed with clinical settings under which medicinal cannabis or phytocannabinoids may be prescribed. The authors feel, however, that significant differences between the two settings make such comparisons problematic. The medicinal cannabis setting encompasses the use of pharmaceutical-grade products prescribed under clinical guidance, with standardised products and dosing, dose titration and patient monitoring for both efficacy and safety. Notwithstanding this setting, areas of caution surrounding the longer-term neurodevelopmental impacts of cannabis use should not be understated, particularly in the young and adolescent, whereas the limited preclinical evidence more promisingly suggests a relatively benign or beneficial profile when applied to dementia models with ageing.

In conclusion, phytocannabinoids are a unique class of cannabis phytochemicals that display an enormous structural diversity. These structural differences correspond with differences in biological activity, including changes not just in receptor binding affinity but also conferring activity at a broad range of cellular and molecular targets. Although initial studies into the use of these compounds in the treatment of neurodegenerative disease have been promising, extensive knowledge gaps remain, presenting opportunities for new discoveries and further exploration. As we learn more and more about the therapeutic potential of phytocannabinoids and cannabis phytochemicals more generally, further research has the potential to capitalise on the rich biochemical diversity of this plant to develop new therapeutic applications in the treatment of neurodegenerative and other diseases.

Statement of Authorship

Title of Paper	The structurally diverse phytocannabinoids cannabichromene, cannabigerol and cannabinol significantly inhibit Amyloid β-evoked neurotoxicity and changes in cell morphology in PC12 cells.
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript submitted to the Journal of Cannabis Research

Principal Author

Name of Principal Author (Candidate)	Dylan Thomas Marsh		
Contribution to the Paper	Conceptualisation, Methodology, Validation, Formal analysis, Data curation, Investigation, Writing - original draft, Writing - review & editing, Visualisation		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/07/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Chapter 3: The structurally diverse phytocannabinoids cannabichromene, cannabigerol and cannabinol significantly inhibit Amyloid β-evoked neurotoxicity and changes in cell morphology in PC12 cells.

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Abstract

Phytocannabinoids (pCBs) have been shown to inhibit the aggregation and neurotoxicity of beta amyloid (Aβ), the pathological hallmark of Alzheimer's disease (AD). However, many promising pCBs have been understudied in the context of AD, with a particular lack of research investigating their effect on Aβ-evoked changes in neuronal cell morphology. Here, we aimed to characterise the capacity of six pCBs, cannabichromene (CBC), cannabigerol (CBG), cannabinol (CBN), cannabidivarin (CBDV), cannabidiol (CBD) and Δ9-tetrahydrocannabinol (Δ^9-THC) to disrupt Aβ aggregation and protect against neuronal PC12 cell lipid peroxidation, Aβ-evoked neurotoxicity and changes in cell morphology in vitro. CBD inhibited tert-butyl hydroperoxide (*t*bhp)-induced lipid peroxidation with no significant effect on Aβ toxicity in PC12 cells, while CBN, CBDV and CBG provided modest protection against tbhp. CBC, CBG and CBN inhibited $A\beta_{1\text{-}42}$-induced neurotoxicity in PC12 cells, as did Δ^9-THC, CBD and CBDV to a lesser extent. CBC, CBN and CBDV inhibited Aβ aggregation, with CBG and CBD having no effect, while Δ^9-THC reduced aggregate density whilst increasing the preponderance of fibrils. Treatment with $A\beta_{1\text{-}42}$ resulted in significant morphological changes to PC12 cells, including a marked reduction in neuritic projections and a more rounded morphology. CBC and CBG inhibited this effect, maintaining control-like cell morphology as shown by principle component analysis (PCA). Δ^9-THC, CBD and CBDV afforded no significant alteration to the effects of $A\beta_{1\text{-}42}$ on cell morphology. These findings highlight the potential for CBC, CBG and CBN as novel cannabis-derived neuroprotective phytocannabinoids in AD.

1. Introduction

Alzheimer's Disease (AD) is the most common neurodegenerative disease characterised by accelerated cognitive decline resulting from the formation of intracellular neurofibrillary tangles and the deposition of the neurotoxic β-amyloid (Aβ) peptide. This Aβ-induced neurotoxicity has been associated with several factors including the formation of small oligomers and protofibrils, induction of oxidative stress via the generation of reactive oxygen species (ROS) and the onset of synaptic dysfunction (Carrano et al., 2011, Ono and Tsuji, 2020, Mucke and Selkoe, 2012). This characteristic Aβ-evoked loss of synaptic function has been associated with changes in neuronal cell structure, including abnormalities in dendritic branching and neurite degeneration (Tsai et al., 2004, Spires and Hyman, 2004, Spires et al., 2005). An estimated 40-50 million people are currently living with AD worldwide, with this number expected to reach 131.5 million by the year 2050 (Wu et al., 2017, Nichols et al., 2019). It is clear that the risk of developing AD increases significantly with age, presenting an important and increasing health burden in an ageing society (Qiu et al., 2009).

Oxidative stress has been implicated as a key factor in normal age-related neurodegeneration and in the pathogenesis of AD (Tönnies and Trushina, 2017) and has also been closely linked to the production and toxicity of Aβ (Zhao and Zhao, 2013). With this in mind, researchers have aimed to identify antioxidants as a means of reducing the impact of ROS generation and

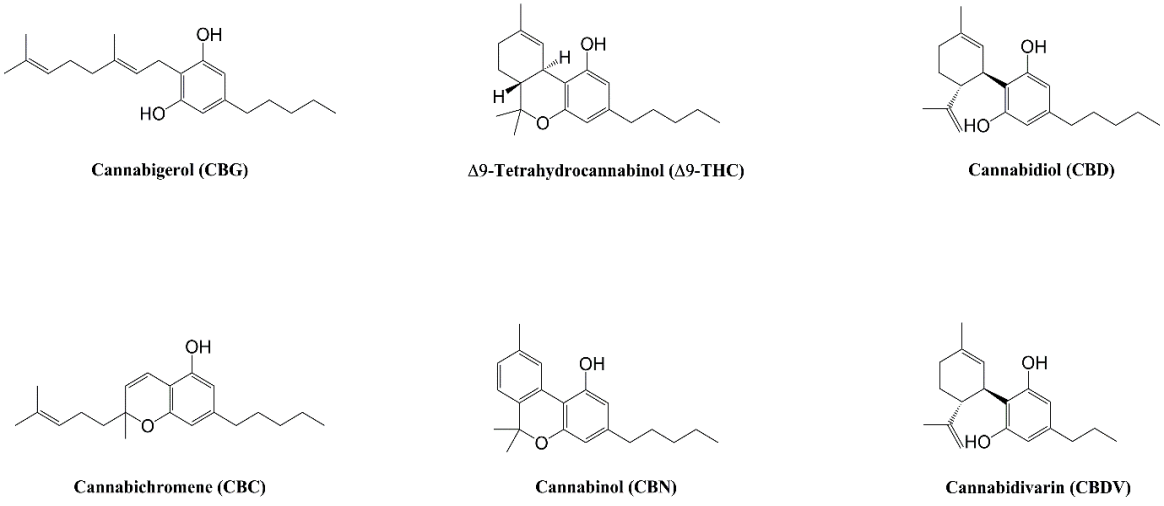


Fig.1. Structures of the phytocannabinoids used in this study

oxidative stress in the neurodegeneration associated with AD (Feng and Wang, 2012). The development of a treatment strategy focusing on compounds with a dual antioxidant and anti-amyloid effect provides a promising avenue of exploration in this context.

Natural products provide a rich source of antioxidant and anti-amyloid molecules which have been widely investigated for their therapeutic potential in AD (Bui and Nguyen, 2017, Mancuso et al., 2007, Habtemariam, 2019). Among these myriad natural products are the phytocannabinoids (pCBs), a class of naturally occurring and diverse compounds found in cannabis which have been ascribed emerging protective properties in neurodegenerative disease models but are relatively novel and understudied (Marsh and Smid, 2021).

Cannabidiol (CBD) is perhaps the most well studied in the context of AD and has been shown to protect against Aβ *in vitro* via a range of mechanisms, including activation of the nuclear peroxisome proliferator-activated receptor γ (PPARγ), inhibition of caspase-3, reduction in lipid peroxidation and more (Scuderi et al., 2014, Iuvone et al., 2004, Janefjord et al., 2014). Furthermore, CBD has been identified as an antioxidant with greater potency than both ascorbate (vitamin C) and α-tocopherol (vitamin E) and is able to effectively reduce ROS generation and lipid peroxidation in PC12 cells (Hampson et al., 1998, Iuvone et al., 2004). The most abundant phytocannabinoid, Δ^9-THC, has been shown to prevent amyloid plaque formation and improve cognition and memory *in vivo*, while also possessing antioxidant properties (Chen et al., 2013, Bilkei-Gorzo et al., 2017, Hampson et al., 1998). Other lesser studied and structurally varying compounds such as cannabigerol (CBG), cannabichromene (CBC), cannabinol (CBN) and cannabidivarin (CBDV) have been investigated to a far lesser extent.

In the present study the neuroprotective capacities of selected phytocannabinoids (**Fig. 1**) were characterised in an *in vitro* model of lipid peroxidation and amyloid β-evoked neurodegeneration in neuronal PC12 cells. Their effects on Aβ-evoked neurite damage and overall cell morphology were also assessed. Additionally, the effect of each pCB on Aβ

aggregation was evaluated as a means of identifying a potential relationship between direct structural modification of the Aβ protein by each phytocannabinoid and their neuroprotective capacity.

2. Materials and Methods

2.1 Materials and Reagents

The phytocannabinoids used in this study include cannabigerol, cannabichromene, cannabidivarin, cannabinol (Cayman Chemicals, Ann Arbor, MI, USA), cannabidiol (Australian Government National Measurement Institute, Lindfield, NSW, Australia) and Δ^9-THC (Cerilliant, Round Rock, TX, USA). Human $A\beta_{1–42}$ protein was obtained from BioLegend (San Diego, CA, USA). Thiazolyl blue tetrazolium bromide (MTT), *tert*-butyl hydroperoxide (*t*bhp), trypan blue, Rose Park Memorial Institue-1640 (RPMI-1640), uranyl acetate, phosphate buffered saline (PBS), non-essential amino acids (NEAA), penicillin/streptomycin, bovine serum albumin (BSA), 1×Trypsin-EDTA, TRITC-phalloidin and acridine orange were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from ChemSupply (Gillman, SA, AUS) and foetal bovine serum (FBS) from Fisher Biotec (Wembley, WA, AUS).

2.2 Preparation of $A\beta_{1–42}$ and pCBs

Lyophilised human $A\beta_{1–42}$ was prepared by dissolving in 100% DMSO to a concentration of 3.8mM. This stock solution was then diluted to 100µM in sterile PBS, dispensed into aliquots and frozen at -80˚C until required. CBD, CBDV and CBG arrived as powdered stocks and were initially dissolved in 100% DMSO, CBC arrived as a 5mg stock in 100µL acetonitrile, CBN as 10mg/1mL in 100% methanol and Δ^9-THC as 1mg/mL in 100% methanol. Each pCB was then further diluted to appropriate working solutions in sterile PBS and progressively lower concentrations of solvent and vortexed thoroughly as required. Final solvent concentrations

were reduced to sub-toxic levels in each assay as determined by cell viability data obtained prior to the commencement of this study.

2.3 Neuronal Cell Culture

Rat phaeochromocytoma cells (Ordway PC12) displaying a semi-differentiated phenotype were kindly donated by Professor Jacqueline Phillips (Macquarie University, NSW, Australia). Cells were maintained in complete RPMI-1640 media containing 10% foetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and 1% penicillin/streptomycin. For cell viability measurements, PC12 cells (up to passage 39) were seeded in 96-well plates at a density of 2×10^4 cells per well in complete RPMI-1640 and allowed to equilibrate for 24 hours prior to treatment.

2.4 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was used to directly visualise changes in $A\beta_{1\text{-}42}$ aggregate morphology following treatment with each pCB. Samples were prepared by incubating native $A\beta_{1\text{-}42}$ (10µM) in PBS, alone or in the presence of each of the test compounds (10µM) for a period of 48 hours at 37ºC. After incubation, a 5µL sample of each solution was taken and added onto a 200-mesh formvar carbon-coated nickel electron microscopy grid (Proscitech, Kirwan, QLD, AUS) and allowed to stand for 1 minute. Following this, the sample was blotted off using filter paper and 10 µL of a contrast dye containing 2% uranyl acetate was added. This dye was then blotted off after 1 minute and sample slides were loaded into an FEI Tecnai G2 Spirit Transmission Electron Microscope (FEI, Milton, QLD, AUS). Sample grids were scanned extensively for the presence of the target protein, with final representative images taken at 18,500× magnification.

2.5 Cell treatment and assessment of cell viability

PC12 cells were treated with each pCB at 10µM (1µM for CBG) 15 minutes prior to treatment with either *t*bhp (0-250µM) or $A\beta_{1\text{–}42}$ (0-1.5µM). Concentrations were based on concentration-response data collected prior to conducting these experiments (data not shown). Following

treatment, cells were incubated for either 24 hours (*t*bhp) or 48 hours ($A\beta_{1-42}$) at 37˚C, 5% CO_2 prior to measurement of cell viability using the thiazolyl blue tetrazolium bromide (MTT) assay. After incubation, media was removed and replaced with serum-free media containing 0.25mg/mL MTT and incubated for two hours at 37˚C, 5% CO_2. MTT solution was then removed and the cells lysed with DMSO, with absorbance values measured at 570nm using a Synergy MX microplate reader (Bio-Tek, Bedfordshire, UK).

2.6 Fluorescence microscopy

PC12 cells were seeded on 12-well chambered microscopy slides (Ibidi) in complete RPMI-1640 media at a density of 1×10^4 cells per well and left to incubate for 24 hours prior to treatment. Cells were then treated with $A\beta_{1-42}$ (1µM) ± pCBs at 10µM (CBG at 1µM) and incubated for 48 hours at 37˚C, 5% CO_2. Following treatment media was aspirated and cells were fixed with 3.7% formaldehyde for 10 minutes at room temperature. Cells were then washed twice with PBS and permeabilised using 0.1% Triton X-100 for 10 minutes at room temperature. Non-specific binding of fluorescence stains was prevented by blocking with 2% BSA for one hour at room temperature, before being washed twice with PBS. For staining, cells were exposed to 1.5µM TRITC-phalloidin for 15 minutes in the dark at room temperature. Cells were then washed with PBS before the addition of 2µg/mL acridine orange and left for a further 20 minutes in the dark at room temperature. Finally, the cells were washed once more with PBS before imaging using an Olympus FV3000 confocal microscope with a 60× oil immersion lens. Fluorophores were excited using an argon laser: TRITC-phalloidin (Ex: 540, Em: 573) and acridine orange (Ex: 503, Em: 530).

2.7 Morphological Analysis

Morphological analysis was conducted in order to quantitatively assess the effect of selected pCBs on PC12 morphology in the presence and absence of $A\beta_{1-42}$. Fluorescence images (green: acridine orange, red: TRITC-phalloidin) were separately processed to recognize the

fluorescently stained area as "masks" (**Fig. 2a**). For the nucleus mask, nuclei areas were manually lined using Image J (https://imagej.nih.gov/ij/). For the actin mask, intensity binarization was processed by original Python code using py-opencv (ver.3.4.1). From the masked area, morphological parameters were assessed and measured for individual cells. Seven morphological parameters were measured for both masks per cell: area, length, width, length-width ratio, perimeter, compactness, and solidity defined in opencv. Compactness was defined as $(\text{perimeter})^2/\text{area}$, and solidity defined as area/convex hull area. For nuclei analysis, area and

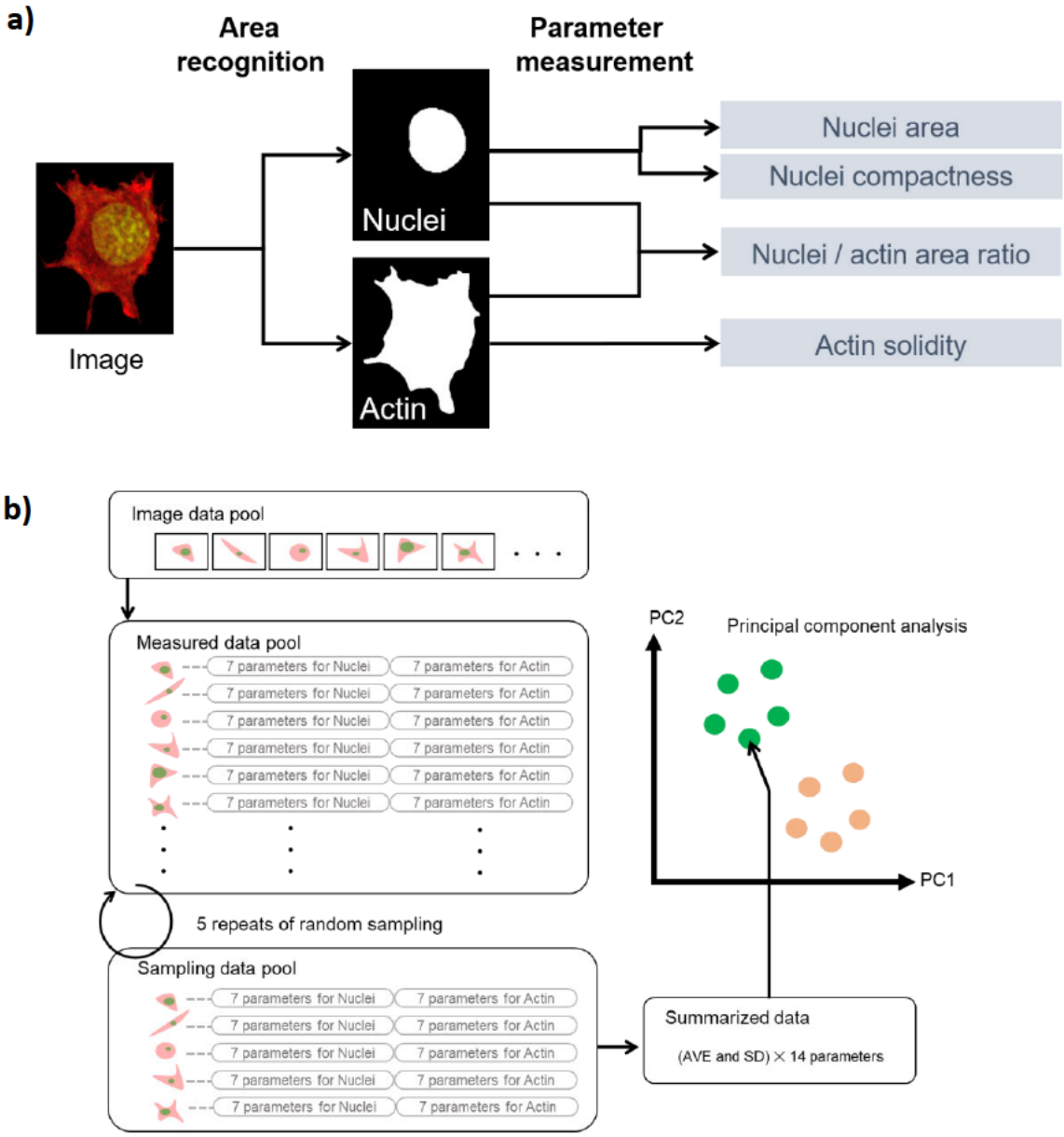


Fig.2. Schematic diagram of **(a)** fluorescence image-based morphological analysis of PC12 cells and **(b)** methodology for principal component analysis.

compactness from the nuclei masks were used. For actin analysis, solidity of the actin mask was used.

Principal component analysis (PCA) was performed using R (version 3.4.1) (R Development Core Team, https://www.r-project.org/), whereby five cells per condition were randomly selected from the image pool and their seven morphological parameters for both masks (actin and nucleus) were measured (**Fig. 2b**). The summarized profile (mean and standard deviation) was then obtained for each parameter, giving 28 total parameters which were used to describe each condition in the PCA plot.

2.8 Data and Statistical Analysis

For morphological analysis, data was analysed by one-way ANOVA using Dunnett's multiple comparisons test vs $A\beta_{1-42}$ ($p<0.05$). Data obtained from the MTT assay was analysed by two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test used to determine significance values versus control ($p<0.05$). GraphPad Prism 8.0 (GraphPad Software, San Diego, USA) was utilised for data analysis and the production of graphs.

3. Results

3.1 Effects of phytocannabinoids on *t*bhp-evoked neurotoxicity in PC12 cells

The addition of the lipid peroxidising agent *tert*-butyl hydroperoxide (*t*bhp; 0-250µM) resulted in a concentration-dependent loss of PC12 cell viability to a maximum of 29.15% (**Fig. 3**). Both CBG and CBN provided a small degree of significant protection at 150µM *t*bhp, maintaining cell viability at 80.63% and 85.83%, respectively versus control (68.92%); Δ^9-THC (**Fig. 3a**) exhibited no significant protective capacity against *t*bhp. CBD appeared to increase cell viability above 100% at 50 and 100µM *t*bhp and provided the most consistent protective effect, with significance observed at 50, 100 and 150µM *t*bhp, while CBDV was protective only at 150µM *t*bhp; CBC provided no significant protection (**Fig. 3b**).

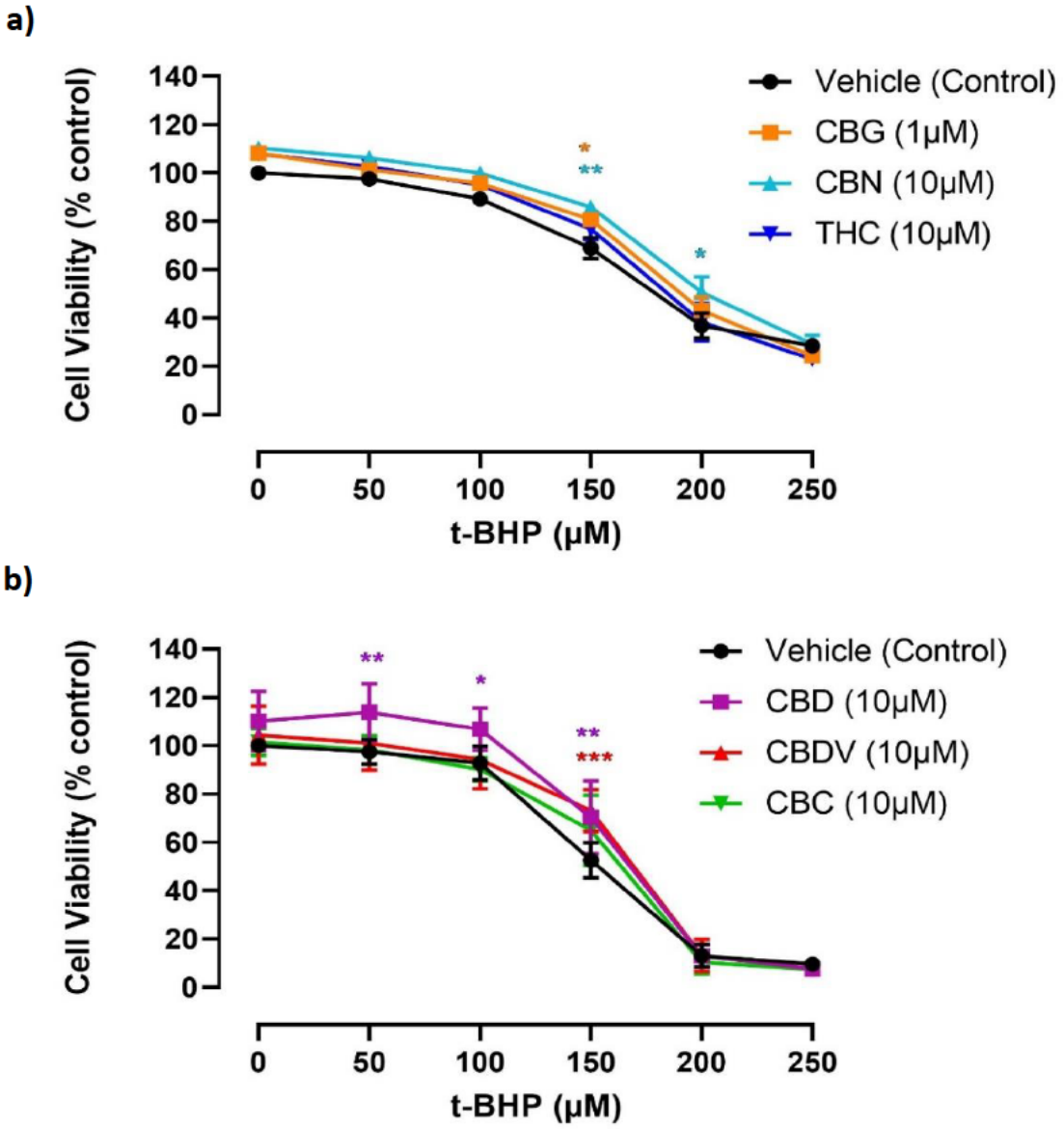


Fig.3. MTT assay of cell viability following 24hr incubation with *tert*-butyl hydroperoxide (*t*bhp) (0–250 μM), alone and in the presence of selected phytocannabinoids: **(a)** CBG (1μM), CBN (10μM), Δ^9-THC (10μM) and **(b)** CBD (10μM), CBDV (10μM), CBC (10μM). Mean (SD), n = 5 replicate plates. *p < 0.05, **p < 0.01, ***p < 0.001 vs vehicle.

3.2 Neuroprotective effects of phytocannabinoids against $A\beta_{1-42}$-induced toxicity in PC12 cells.

Incubation with $A\beta_{1-42}$ (0-1.5μM) over 48 hr produced a concentration-dependent reduction in cell viability to a maximum of 71% (Fig 4). Of the six pCBs selected for the study, CBG, CBC and CBN exhibited the greatest degree of neuroprotection, maintaining 89.74%, 92.32% and 96.5% cell viability at 1.5μM $A\beta_{1-42}$, respectively (Fig. 4a). CBD and CBDV provided only

modest neuroprotection compared to control (77.13%), maintaining cell viability at 81.78% and 81.94%, respectively; Δ^9-THC displayed a slightly greater degree of protection at 83.7% viability (**Fig. 4b**). Statistical significance was observed only at the highest concentration of $A\beta_{1\text{-}42}$ (1.5µM) in each case.

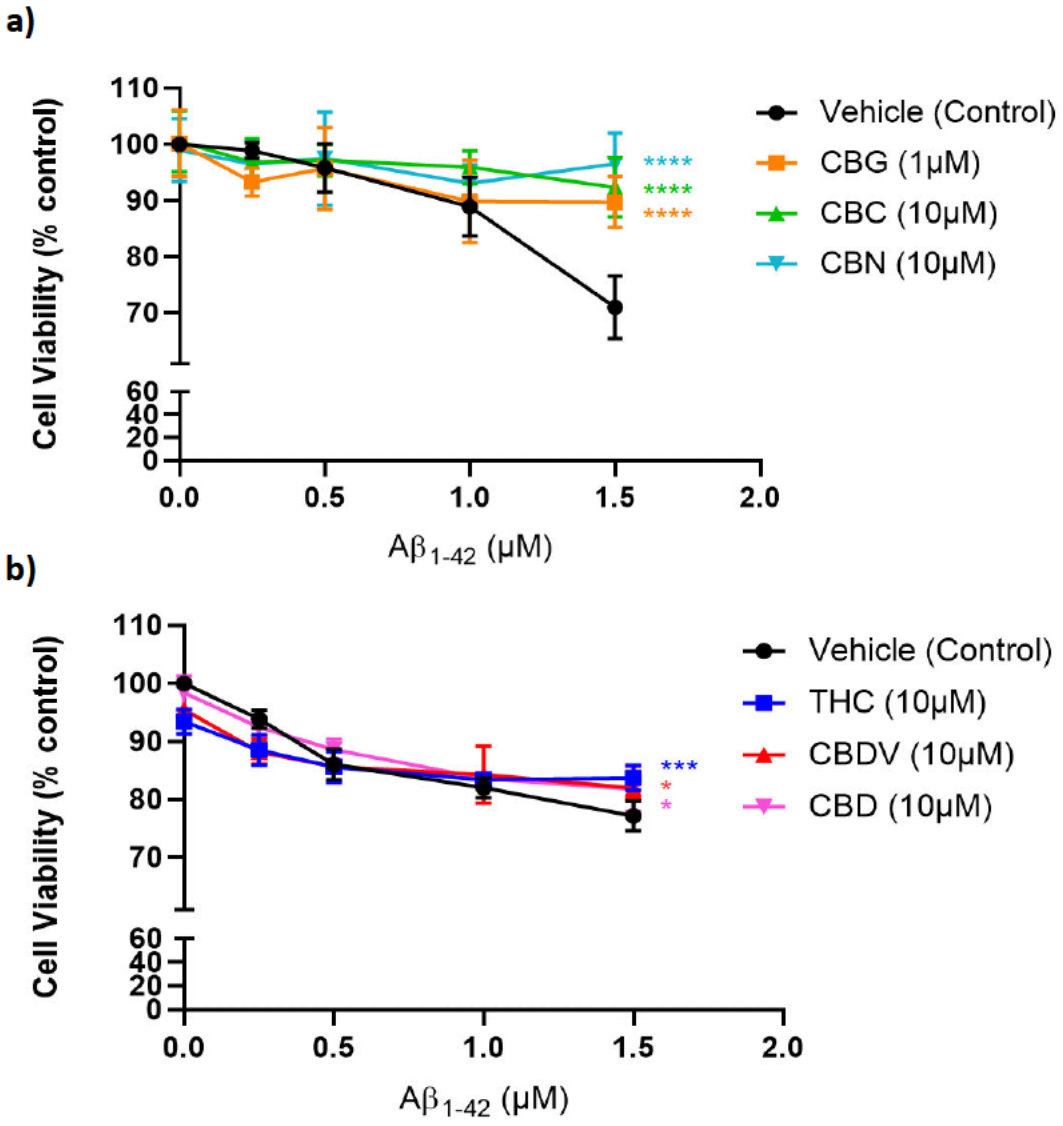
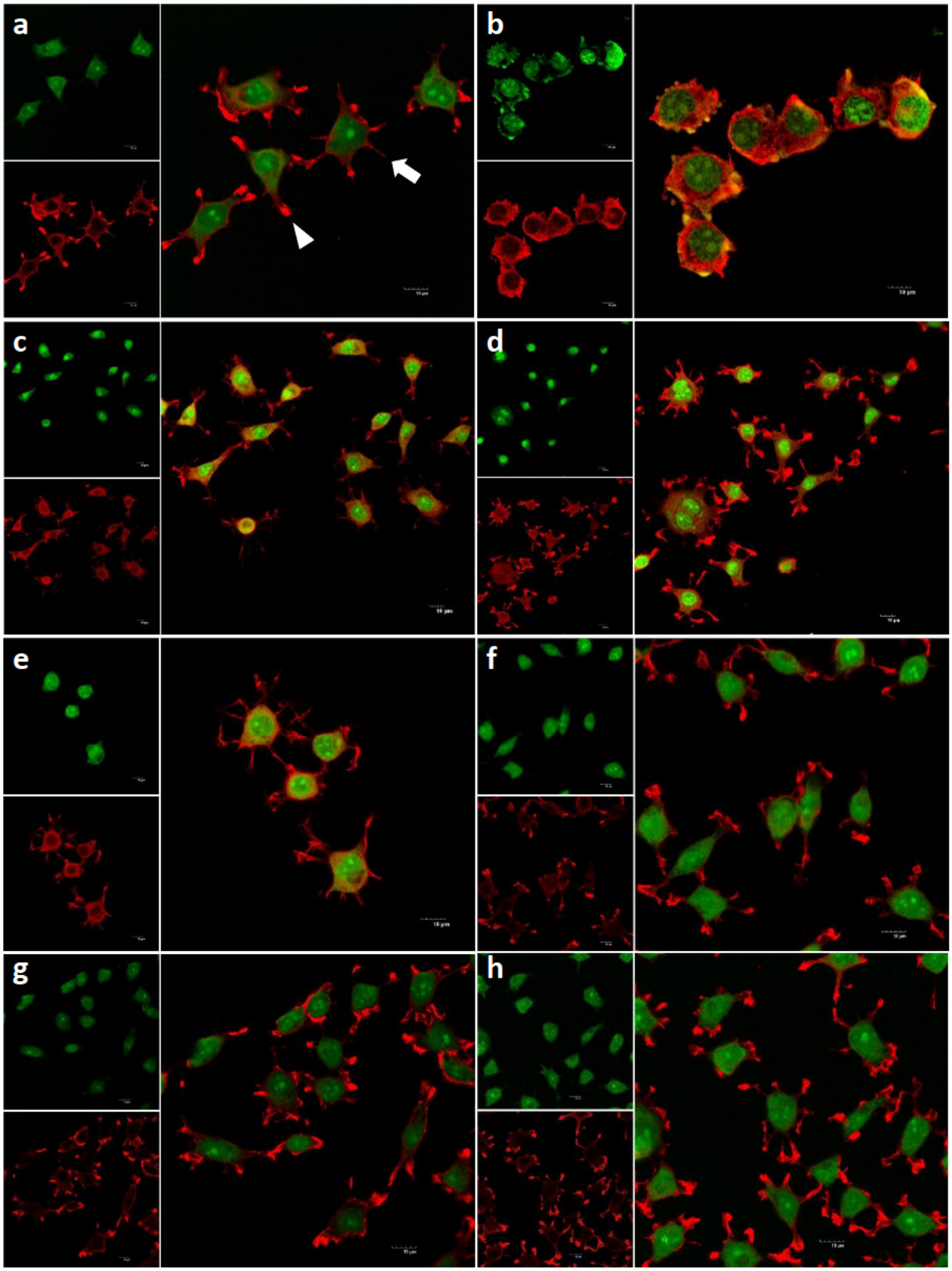


Fig.4. MTT assay of cell viability following 48hr incubation with $A\beta_{1\text{-}42}$ (0-1.5 µM), alone and in the presence of selected phytocannabinoids: **(a)** CBG, CBC, CBN and **(b)** Δ^9-THC, CBD, CBDV (each at 10µM, CBG at 1µM). Mean (SD), n = 4 replicate plates. *p < 0.05, ***p < 0.001, ****p < 0.0001 vs vehicle.

3.3 Phytocannabinoids protect against $A\beta_{1-42}$-induced morphological changes and neurite damage in PC12 cells



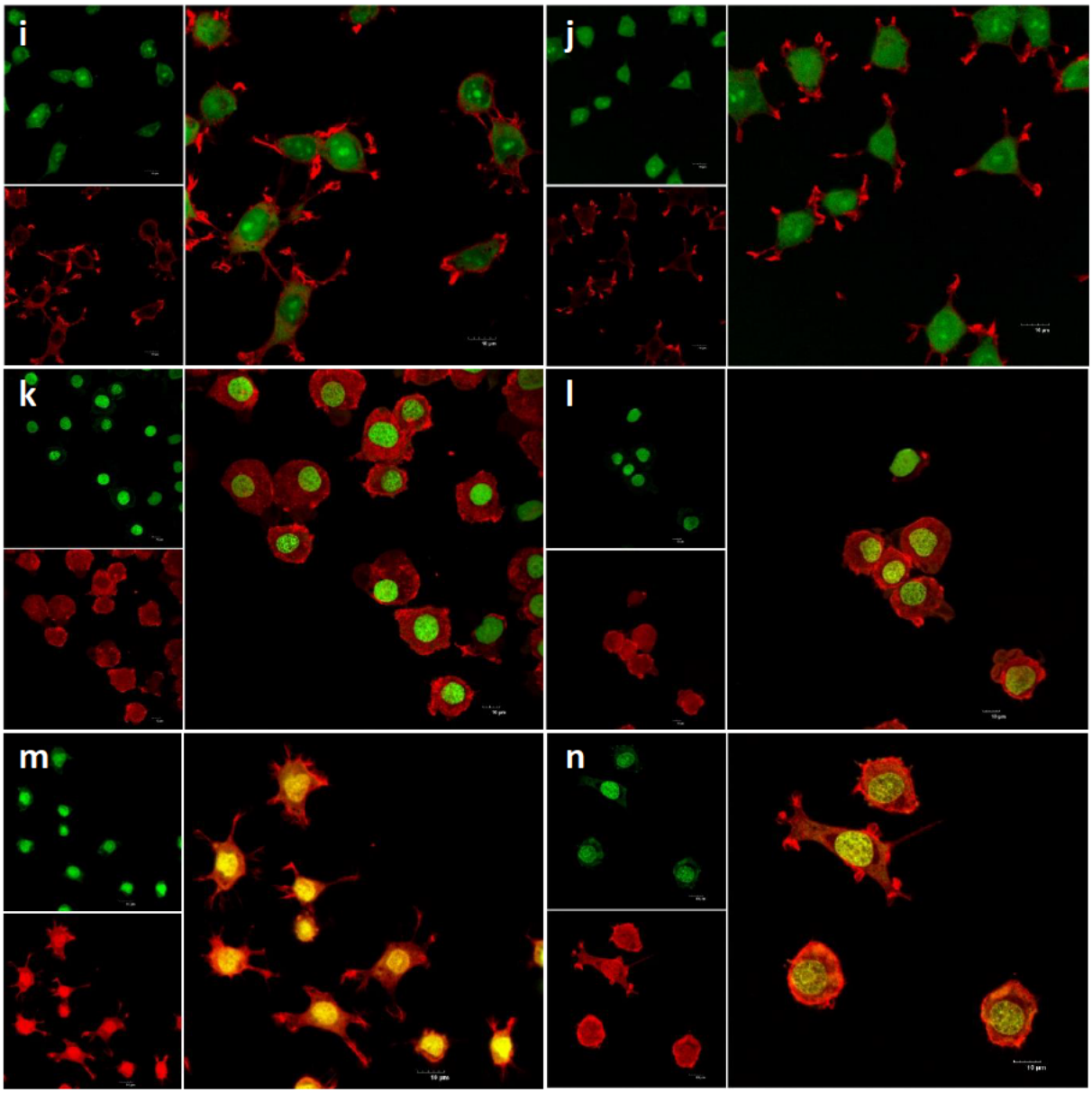


Fig.5. Fluorescence imaging with TRITC-phalloidin (red, F-actin) and acridine orange (green, nucleic acid) demonstrating the effects of selected phytocannabinoids (each at 10µM, CBG at 1µM) on $A\beta_{1-42}$-induced (1µM) neurite damage in PC12 cells following 48hr incubation. White arrow indicates example of neuritic projection/filopodium; growth cone indicated by white triangle. Each treatment condition is displayed as individual stains plus the corresponding overlap – scale bar 10µm. **(a)** control, **(b)** $A\beta_{1-42}$, **(c)** CBG, **(d)** CBG + $A\beta_{1-42}$, **(e)** CBC, **(f)** CBC + $A\beta_{1-42}$, **(g)** CBN, **(h)** CBN + $A\beta_{1-42}$, **(i)** Δ^9-THC, **(j)** Δ^9-THC + $A\beta_{1-42}$, **(k)** CBDV, **(l)** CBDV + $A\beta_{1-42}$, **(m)** CBD and **(n)** CBD + $A\beta_{1-42}$. Representative images selected from at least 3 independent replicate slides per treatment condition.

Fluorescence imaging with TRITC-phalloidin and acridine orange (**Fig. 5**) showed that incubation with $A\beta_{1-42}$ (1µM) resulted in a clear loss of neuritic projections in PC12 cells (**Fig. 5b**) versus control (**Fig. 5a**). Treatment with each pCB alone (**Fig. 5c, e, g and i**) had little effect on overall PC12 morphology with the exception of CBDV (**Fig. 5k**), which caused a loss of neuritic projections in similar fashion to $A\beta_{1-42}$. Treatment with CBC, CBG, CBN and Δ^9-THC (**Fig. 5d, f, h and j**) prevented the loss of neuritic projections caused by $A\beta_{1-42}$, with CBDV-treated cells again displaying a clear lack of neuritic projections and filopodia following co-incubation with $A\beta_{1-42}$. There was also an increase in the prevalence of growth cones in cells treated with CBC, CBG, CBN and Δ^9-THC, indicative of an increase in neurite outgrowth.

3.4 Phytocannabinoids protect against $A\beta_{1-42}$-induced morphological changes in PC12 cells

Analysis of PC12 cell fluorescence images highlighted a distinct effect of $A\beta_{1-42}$ on several key morphological parameters. Treatment with $A\beta_{1-42}$ caused significant increases in the nucleus area of PC12 cells compared to control (****p <0.0001), which was variably affected by treatment with selected pCBs (**Fig. 6a**). Of the pCBs selected, CBD proved most effective in preventing this increase (***p = 0.0009) followed by CBC (*p = 0.0336) and CBDV (*p = 0.0125), while Δ^9-THC and CBG exhibited no significant difference vs $A\beta_{1-42}$. Treatment with $A\beta_{1-42}$ also resulted in a significant increase in nucleus compactness vs control (*p = 0.0471) (**Fig. 6b**). Of all pCBs screened, only Δ^9-THC significantly inhibited this increase (**p = 0.0057).

Treatment with amyloid β also resulted in a significant increase in actin solidity compared to control (****p <0.0001) (**Fig. 6c**). Increased actin solidity indicates a reduction in the number of neuritic projections and thus a more rounded cell morphology. This increase was most significantly inhibited by treatment with CBC (****p <0.0001), CBD (****p <0.0001), and Δ^9-THC (****p <0.0001). CBG also significantly inhibited this increased actin solidity (***p = 0.0008) along with CBN (*p = 0.0435), while no significant difference was observed following treatment with CBDV.

The actin:nucleus area ratio, a measure of the area of the cell nucleus relative to the actin area, was significantly increased by treatment with $A\beta_{1\text{-}42}$ (**p = 0.0080) (**Fig. 6d**). This increased ratio indicates that the nuclei of $A\beta_{1\text{-}42}$-treated cells occupies a greater area within the cell body relative to control. This effect was inhibited by treatment with both Δ^9-THC (***p = 0.0002)

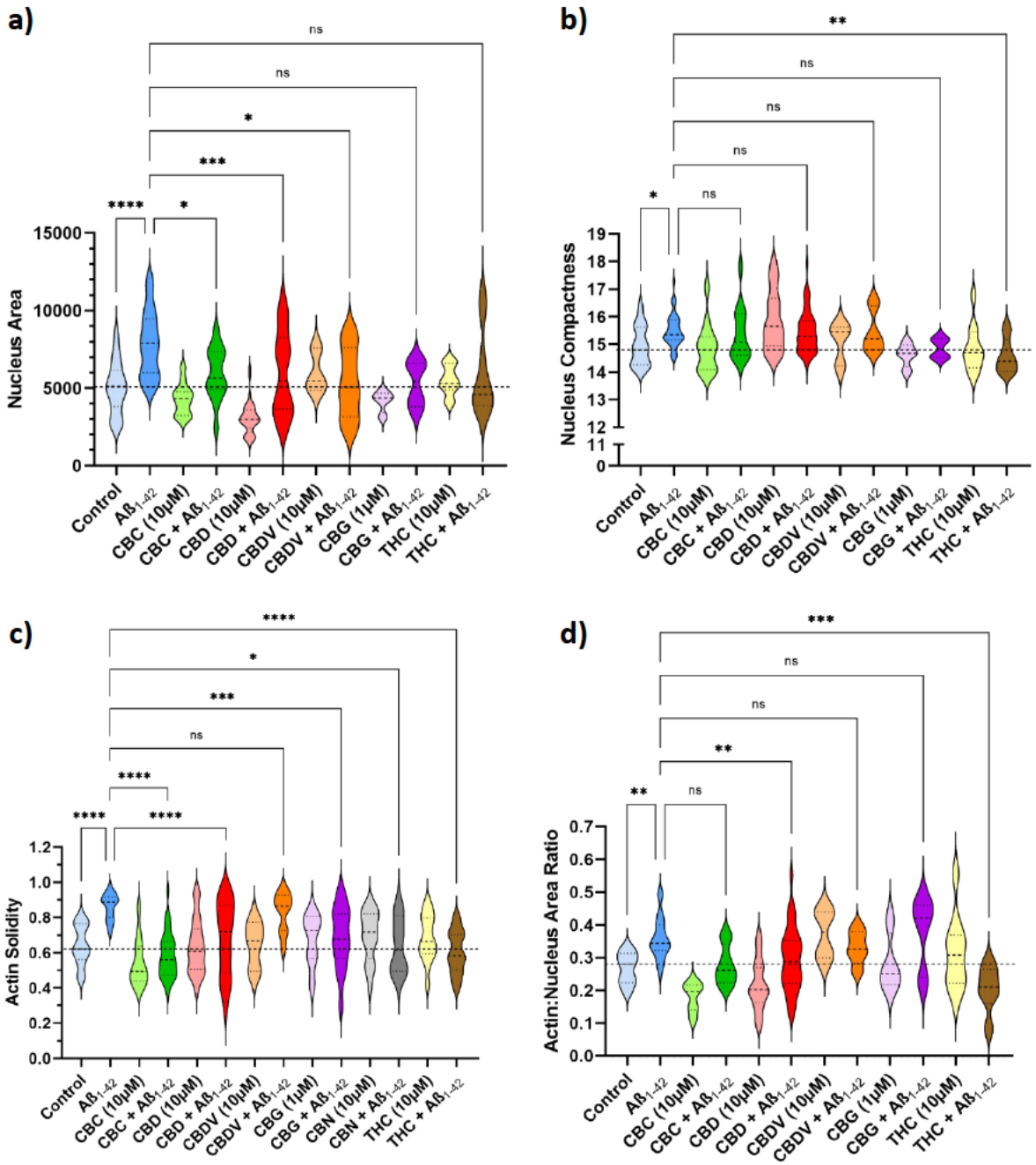


Fig. 6. Morphological analysis highlighting the effect of selected phytocannabinoids on **(a)** nucleus area, **(b)** nucleus compactness, **(c)** actin solidity and **(d)** the nucleus:actin area ratio in PC12 cells in the presence and absence of $A\beta_{1\text{–}42}$. n = at least 4 cells per treatment condition. *p < 0.05, **p < 0.01, ***p < 0.001, ****p< 0.0001 vs $A\beta_{1\text{–}42}$.

and CBD (**p = 0.0066), with no significant difference observed following treatment with either CBC, CBG or CBDV.

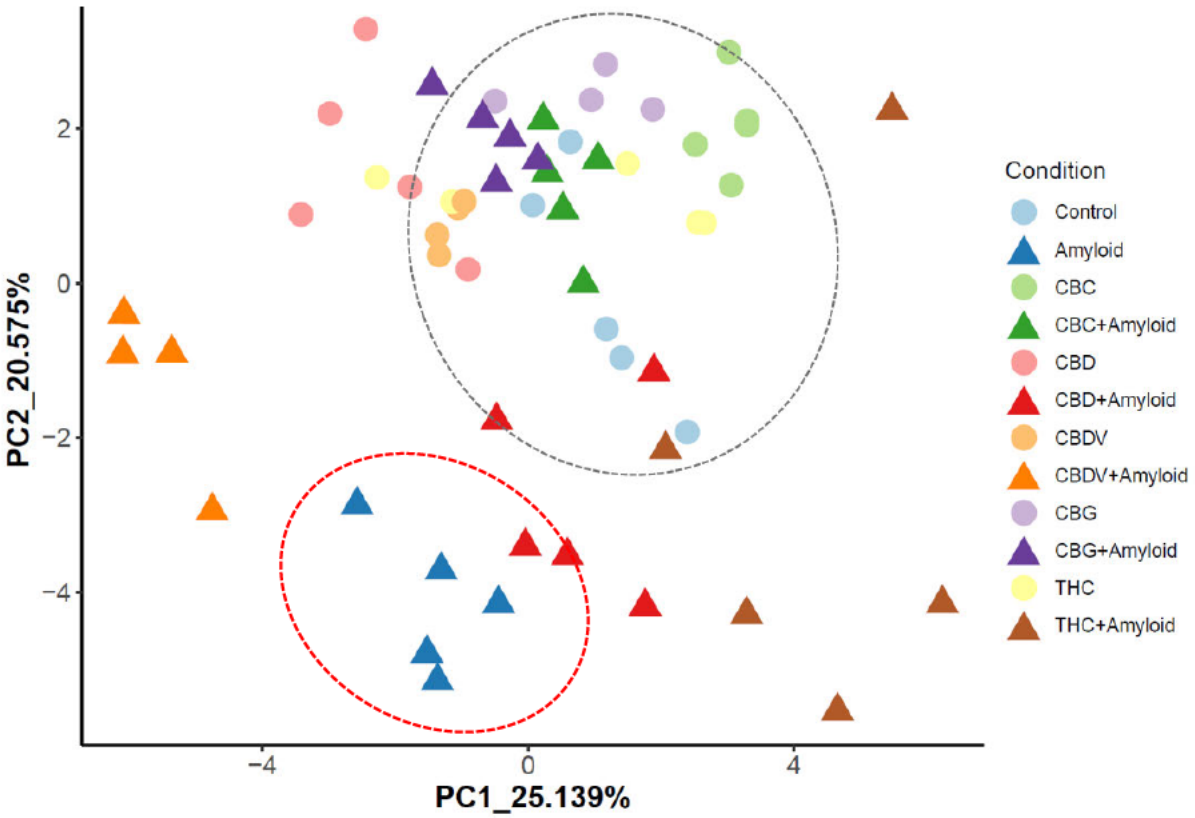


Fig. 7. Principle component analysis of overall morphological characteristics of PC12 cells following treatment with selected pCBs in the presence and absence of $A\beta_{1-42}$. Data points within the black dotted circle indicate morphological similarity with the control condition. Data points within the red circle indicate morphological similarity to the $A\beta_{1-42}$ treatment group.

Principal component analysis (PCA) was used to plot overall morphological differences between treatment conditions (**Fig. 7**). PCA showed a clear distinction between $A\beta_{1-42}$-treated cells and those in the control condition, indicating significant overall morphological differences between the two groups. The relative position of each treatment cluster may be interpreted as a measure of the degree of efficacy by each phytocannabinoid in preventing morphological changes caused by incubation with $A\beta_{1-42}$. Cells co-incubated with CBC and $A\beta_{1-42}$ displayed considerable morphological similarity to the control condition, indicative of a general

protective effect. A similar pattern was observed in the CBG + Amyloid β treatment condition, which also displayed a high degree of morphological similarity to the control condition. Cells treated with CBD and $A\beta_{1–42}$ showed a tendency to cluster between the control and $A\beta_{1–42}$ group, indicating a lesser degree of protective effect vs CBC and CBG. Finally, a lack of efficacy was observed in the Δ^9-THC + Amyloid β and CBDV + Amyloid β treatment groups, both of which displayed little to no morphological similarity to control.

3.5 Transmission Electron Microscopy (TEM) of $A\beta_{1–42}$ fibril and aggregate morphology

TEM imaging showed varying degrees of morphological change following treatment with the pCBs. CBC exerted the greatest effect on $A\beta_{1–42}$ aggregation, with a marked reduction in both the degree of aggregation and aggregate density (**Fig. 9c**). CBN-treated samples showed a similar reduction in aggregate formation and density, while also displaying a comparatively greater number of fibrils (**Fig. 9g**). Similarly, treatment with CBDV resulted in a reduction in aggregate density and an increase in the prevalence of amyloid fibrils (**Fig. 9e**). In contrast, neither CBG nor CBD had any effect on $A\beta_{1–42}$ morphology, displaying similar degrees of aggregation and aggregate density as the control (**Fig. 9a, b and d**). Treatment with Δ^9-THC resulted in the preponderance of amyloid fibrils, displaying a densely stained meshwork with limited aggregates.

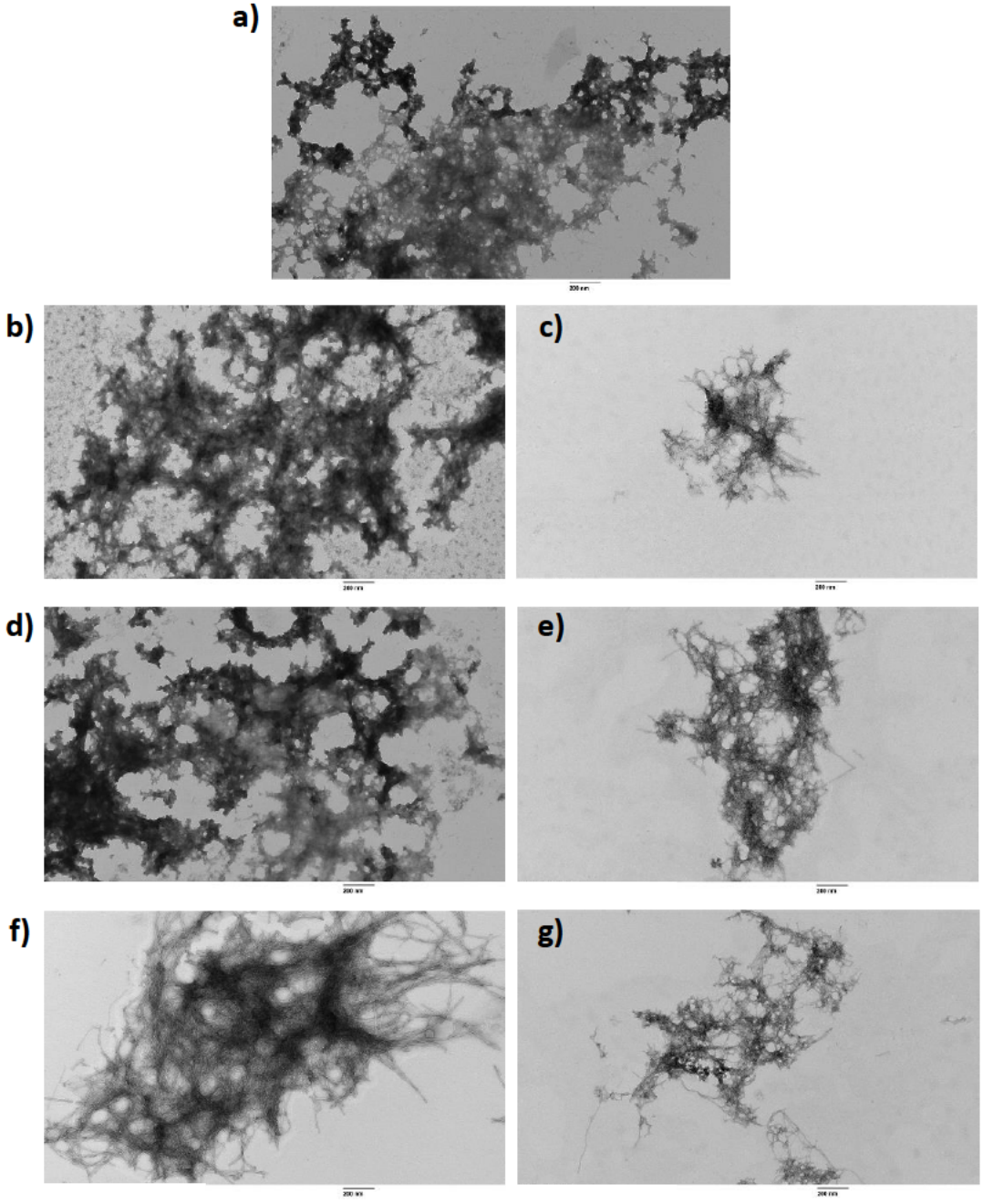


Fig.9. Transmission electron micrographs demonstrating the effects of selected phytocannabinoids (each at 10μM) on $A\beta_{1-42}$ (10μM) aggregation and morphology following 48hr incubation (scale bar = 200nm). **(a)** Control, **(b)** CBG, **(c)** CBC, **(d)** CBD, **(e)** CBDV, **(f)** Δ^9-THC and **(g)** CBN.

4. Discussion

The findings of this study highlight a novel neuroprotective role for cannabichromene (CBC), cannabinol (CBN) and cannabigerol (CBG) against Aβ-induced neurotoxicity and neurite damage, as well as a novel anti-aggregatory role for CBC, CBN and cannabidivarin (CBDV). The modest protective effect and lack of anti-aggregative capacity of cannabidiol (CBD) observed in this study is in contrast to several previous studies, which have demonstrated a protective capacity against Aβ-evoked neurotoxicity and the ability to reduce protein aggregation (Iuvone et al., 2004, Schubert et al., 2019). To our knowledge, this study presents the first direct characterisation of the anti-aggregatory capacity of CBG, CBC, CBDV and CBN, as well as the first morphological characterisation of the impact of these pCBs on Aβ-evoked neurite damage.

Cannabidiol provided the greatest degree of protection against *t*bhp-induced lipid peroxidation. This is in accordance with other studies which have demonstrated the antioxidant activity of CBD and its ability to protect against lipid peroxidation in neuronal cells (Sun et al., 2017, Atalay et al., 2019). Interestingly, the capacity for CBD to protect against oxidative stress has been linked to its ability to induce heme oxygenase-1 (HO-1) (Duvigneau et al., 2020). HO-1, also known as heat shock protein 32 (Hsp32), is an inducible form of HO that is upregulated in response to oxidative stress and inflammation and may confer neuroprotection via its antioxidant capacity (Colín-González et al., 2013, Schipper, 2004). CBD has also been shown to possess intrinsic free radical scavenging capacity (Pereira et al., 2021), which may further explain the effect observed in the present study. Cannabidivarin, cannabinol and cannabigerol were also protective against *t*bhp, although each to a lesser extent than cannabidiol and with cannabichromene providing no significant protection. These results are in accordance with a recent study which found that selected pCBs exhibited considerable *in vitro* antioxidant capacity (Dawidowicz et al., 2021). Although that study did not directly investigate protective capacity against lipid peroxidation in a cell culture model, it is clear that these compounds

possess considerable antioxidant potential. Notably, Δ^9-THC has previously been shown to possess intrinsic antioxidant capacity in cell-free assays while also protecting against cellular oxidative stress (Hampson et al., 1998, Carroll et al., 2012b). However, the results of the current study indicate a lack of efficacy of Δ^9-THC to protect against lipid peroxidation. In fact, other studies have suggested that Δ^9-THC may actually increase *in vitro* ROS generation and subsequent oxidative stress (Walker et al., 2021, Wolff et al., 2015). It is difficult to ascertain the reason behind these differing results, however it may relate to variations in the models being utilised in each study.

Interestingly, the ability for these pCBs to protect against lipid peroxidation did not correlate with the ability to protect against Aβ-evoked neurotoxicity or neurite damage. This was exemplified by the fact that CBG, CBC and CBN proved to be potent anti-amyloid compounds in this regard, while providing either modest or no protection against *t*bhp-evoked lipid peroxidation. This therefore suggests that their potent neuroprotection against Aβ is likely independent of their antioxidant effects. Conversely, CBD was highly efficacious against *t*bhp whilst providing only modest protection against $A\beta_{1\text{-}42}$. This contrasts with previous studies which have suggested that CBD possesses the ability to protect against Aβ-induced toxicity in PC12 cells (Iuvone et al., 2004, Esposito et al., 2006a). However, a previous study found CBD to be a potent inhibitor of *t*bhp-evoked neurotoxicity but with no significant neuroprotective effect against Aβ in PC12 cells (Harvey et al., 2012). It is unclear why the disparity between antioxidant capacity and anti-amyloid effect is so great, given the close association of oxidative stress and Aβ toxicity in the pathophysiology of AD (Zhao and Zhao, 2013). Previous studies have highlighted a synergistic relationship between the two, whereby Aβ deposition increases lipid peroxidation and oxidative damage, while oxidative stress further increases Aβ production by downregulating α-secretase expression and upregulating the activity of γ-secretase, β-secretase and BACE1 (Behl et al., 1994, Matsuoka et al., 2001, Smith et al., 1998, Quiroz-Baez

et al., 2009, Chen et al., 2008). However, despite the seemingly inextricable link between these factors, it is clear that antioxidant capacity alone is insufficient to surmount the toxicity of Aβ.

This study also highlighted a number of structure-activity relationships (SARs) among the selected compounds. One such example would be the reduced anti-amyloid efficacy of Δ^9-THC in comparison to its fully aromatised analogue CBN, an effect mirrored by a previous study which found CBN to be a more potent anti-amyloid compound than Δ^9-THC in MC65 cells (Schubert et al., 2019). Moreover, we have previously shown that CBN also possesses far greater binding affinity for both monomeric and pentameric conformations of $A\beta_{1\text{-}42}$ (Marsh and Smid, 2021). This may be due to the increased hydrophobicity afforded to CBN by the aromatisation of its C ring, allowing for better binding within the hydrophobic groove of the $A\beta_{1\text{-}42}$ peptide. This correlates with the inhibition of $A\beta_{1\text{-}42}$ aggregation seen in the present study, though further comparisons between the two pCBs is necessary in order to confirm this. This study also highlighted the fact that the shortening of the alkyl side chain at position 3 from an *n*-pentyl chain, as in CBD, to an *n*-propyl chain as in CBDV had no significant effect on neuroprotection against $A\beta_{1\text{-}42}$, although CBDV was able to reduce Aβ aggregation to a greater extent than CBD. Further studies examining the effect of alkyl side chain length on amyloid aggregation and neuroprotection more generally may be of interest, given that increased side chain length is associated with increased potency at cannabinoid receptors (Prandi et al., 2018, Martin et al., 1999). These findings lay a foundation for further exploration of phytocannabinoid SARs in the context of AD.

A previous study found both CBG, CBN and CBC to be significantly protective against Aβ toxicity in MC65 cells, while also clearing pre-formed aggregates (Schubert et al., 2019). This study also found that CBD was the most potent anti-amyloid compound tested, with an IC_{50} value of 30nM, two-to-three times more potent than either CBG, CBN or CBC. In direct contrast to these results, TEM imaging and cell viability data from the present study showed that CBG possesses a far greater neuroprotective capacity than CBD, despite being unable to

affect Aβ aggregation. Previous studies in other neurodegenerative disease contexts have suggested that the neuroprotective effect of CBG may be due, in part, to its anti-inflammatory effects (Giacoppo et al., 2017, Valdeolivas et al., 2015, Mammana et al., 2019). More specifically, CBG has been shown to inhibit inflammation by targeting and increasing the transcriptional activity of nuclear peroxisome proliferator-activated receptor-γ (PPARγ), which acts as a modulator of proinflammatory cytokine release (O'Sullivan, 2016, Jiang et al., 1998). This idea is further consolidated by the fact that PPARγ activation has been shown to be neuroprotective against Aβ in human neural stem cells (Chiang et al., 2016). This finding suggests that CBG may also exert its protective effect via this mechanism, although this requires further investigation.

The therapeutic potential of CBC in the context of neurodegenerative diseases has been understudied. One study found that CBC was able to protect MC65 cells against Aβ toxicity, while also protecting HT22 cells against oxytosis, a programmed cell death pathway associated with oxidative stress (Schubert et al., 2019). Furthermore, CBC was shown to be an inhibitor of inflammation in several disease models, with the capacity to reduce LPS-stimulated inflammation by a CBR-independent mechanism and reduce the expression of proinflammatory cytokines (DeLong et al., 2010, Petrosino et al., 2018, Izzo et al., 2012). This anti-inflammatory effect may be of particular relevance in AD, as the generation of Aβ has been associated with an inflammatory response which results in reductions in neuronal cell viability (Currais et al., 2016). Moreover, CBC was able to increase the viability of nestin-positive neural stem progenitor cells and is a potent TRPA1 agonist, a receptor that may play a crucial role in regulating inflammation in AD (Shinjyo and Di Marzo, 2013, De Petrocellis et al., 2011, Lee et al., 2016). Although the present study did not elucidate the mechanisms behind the observed neuroprotection, this data coupled with the relevant literature regarding CBC highlights the neuroprotective potential for CBC in the context of AD. Given that CBC, CBN and CBG also

lack the negative psychotropism associated with Δ^9-THC, the potent neuroprotective effect observed in this study warrants further exploration.

Image-based morphological analysis has been used as a predictor of numerous cell effects, including the differentiation potential of neuronal stem cells and myogenic differentiation of myoblasts (Ishikawa et al., 2019, Fujitani et al., 2017). However, using quantitative, image-based morphological analysis as a means of assessing the neurotoxicity of $A\beta_{1-42}$ has not been studied extensively. This study employed the use of a novel algorithm designed to extract morphological data from fluorescence images, allowing for the quantitative analysis of these otherwise qualitative data. Initial qualitative assessment of fluorescence microscopy images demonstrated a marked reduction in the prevalence of neuritic projections in $A\beta_{1-42}$-treated PC12 cells compared to control. Morphological analysis showed that treatment with Aβ resulted in a simultaneous increase in nucleus area along with increased actin solidity, indicating an enlarged nucleus and a reduction in the number of neuritic projections and growth cones. Growth cones are essential in the formation of axons and Aβ treatment has been shown to cause axonal degeneration, reduced numbers of neuritic projections and growth cone collapse (Dent et al., 2011, Kuboyama, 2018, Eggers et al., 2019). Principal component analysis highlighted CBC as the most efficacious of the pCBs screened in this study in preventing Aβ-evoked morphological changes. A previous study demonstrated that CBC is able to raise the viability of neural stem progenitor cells while also inducing ERK phosphorylation (Shinjyo and Di Marzo, 2013). The activation of the ERK signalling pathway is a key aspect in neurite outgrowth, with its inhibition resulting in reduced neurite outgrowth (Wang et al., 2011). The ability for CBC to induce ERK phosphorylation may therefore play a role in the protective effect observed in the present study.

Cannabigerol also proved effective in preventing Aβ-evoked morphological changes overall. A transcriptomic study by Gugliandolo and colleagues identified a role for CBG as an influencer of several synaptic pathways in NSC-34 cells (Gugliandolo et al., 2020). However, the direct

effect of CBG on neurite morphology has not been studied previously. Interestingly, actin solidity data showed that treatment with CBDV alone resulted in a significant decrease in neuritic projections, an effect that was not seen following treatment with its *n*-pentyl analogue CBD. One possible explanation for this difference may stem from the actions of these phytocannabinoids at GPR55, a cellular target generally considered to be a cannabinoid receptor, though this issue is somewhat contentious (Ross, 2009, Henstridge, 2012). GPR55 is expressed endogenously in PC12 cells and plays an important role in regulating neurite outgrowth and growth cone morphology, with antagonism associated with a loss of neuritic projections (Obara et al., 2011, Cherif et al., 2015). Previous research has shown CBDV to be a potent inhibitor of l-α-lysophosphatidylinositol (LPI)-induced ERK1/2 phosphorylation, a primary downstream signalling pathway associated with LPI-induced GPR55 activation with potency far in excess of CBD (Anavi-Goffer et al., 2012). It is also interesting to note that pCBs with an *n*-propyl side chain were consistently more potent than their *n*-pentyl counterparts, highlighting a fascinating structure-activity relationship worthy of further exploration. The combination of these two factors may explain the differential effects of CBDV and CBD observed in this study. There are inherent difficulties associated with the interpretation of morphology data with regards to inferences on neuronal cell viability and function. For example, Δ^9-THC has been shown to induce ectopic formation of filopodia and altered axon morphology (Tortoriello et al., 2014). However, surface-level assessment of morphology alone may be insufficient to detect such changes. It is therefore important to note that the morphology data presented in this study would benefit from further investigation into possible pCB-evoked alterations in protein expression and downstream signalling pathways associated with neurite outgrowth and axonal morphology, to elucidate the mechanisms behind the changes observed in the present study. However, compounds with the ability to inhibit amyloid toxicity, preserve and promote growth cone formation and prevent neurite damage such as CBG, CBC and CBN may be efficacious in the treatment of AD. This study presents the first morphological

characterisation of CBC, CBG and CBDV as protective agents against $A\beta_{1-42}$-evoked neurite damage.

Transmission electron microscopy indicated that CBC, CBN and, to a lesser extent, CBDV, were able to inhibit $A\beta_{1-42}$ aggregation, while CBD, CBG and Δ^9-THC proved ineffective in this regard. This is line with previous studies which have demonstrated a lack of efficacy for both CBD and Δ^9-THC in the inhibition of $A\beta_{1-42}$ aggregation (Janefjord et al., 2014, Harvey et al., 2012). This data is also supported by previously published molecular modelling data, where we identified CBN and CBC as high-affinity binders with various conformations of the Aβ peptide (Marsh and Smid, 2021). Interestingly, this modelling data also found CBG to be the highest-affinity Aβ binder of all the pCBs screened, yet the present study showed that it lacks the ability to prevent protein aggregation *in vitro*. This suggests that the potent neuroprotective effect of CBG is entirely independent of its ability to disrupt protein aggregation and that *in silico* predictions of binding affinity alone may not accurately predict inhibition of Aβ aggregation. Furthermore, comparisons between TEM images and cell viability data highlighted a lack of direct correlation between anti-aggregatory capacity and neuroprotection. This was perhaps most evident in the examples of CBG, which was highly neuroprotective against $A\beta_{1-42}$ but had no effect on protein aggregation and CBDV, which reduced protein aggregation but had only a modest impact on cell viability in the presence of $A\beta_{1-42}$. A similar lack of direct correlation has been observed in previous studies, suggesting that inhibition of aggregation alone may not be a particularly accurate predictor of neuroprotection against $A\beta_{1-42}$ (Eggers et al., 2019, Janefjord et al., 2014).

In conclusion, this study demonstrated that selected phytocannabinoids provide varying degrees of neuroprotection against $A\beta_{1-42}$, highlighting the efficacy of cannabigerol, cannabichromene and cannabinol in particular that is likely independent of their antioxidant capacity. Given that these pCBs proved effective in preventing Aβ-induced toxicity, Aβ-evoked neurite damage and

variably amyloid aggregation, they present as potential leads warranting further investigation and development in the treatment of AD.

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Dylan Thomas Marsh		
Contribution to the Paper	Conceptualisation, Methodology, Validation, Formal analysis, Data curation, Investigation, Writing - original draft, Writing - review & editing, Visualisation		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	24/07/22

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contr bution.

Name of Co-Author	Scott Smid		
Contribution to the Paper	Conceptualisation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition		
Signature		Date	July 25, 2022

Chapter 4: Investigating the neuroprotective capacity of medicinal cannabis extract formulations against $A\beta_{1-42}$ and lipid peroxidation in vitro

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Introduction

Cannabis sativa (*C. sativa*) has garnered considerable interest for its potential therapeutic benefits in a variety of disease contexts (Zuardi, 2006, Cassano et al., 2020, Ahmed and Katz, 2016). *Cannabis* is known for its rich biochemical diversity and is the source of hundreds of structurally diverse compounds, including various terpenes, flavonoids and phytocannabinoids (pCBs) (Brenneisen, 2007). To date, more than 540 individual compounds have been extracted and isolated from cannabis, with more than 120 of these considered to be pCBs (Hanuš et al., 2016). The classical phytocannabinoids are a group of C_{21} terpenophenolic compounds found in all major species of cannabis that share several common structural features, including a dibenzopyran ring and an alkyl side chain (Morales et al., 2017a, Berman et al., 2018). These pCBs also vary in their structure in many ways, including differences in alkyl side chain length, degree of aromatisation and the nature of the dibenzopyran B ring, whether open, as in cannabidiol (CBD), or closed, as in Δ^9-tetrahydrocannabinol (Δ^9-THC) (**Fig. 1**). Moreover, it is currently believed that all phytocannabinoids originate as carboxylated precursors which then undergo decarboxylation to 'neutral' forms in a predominantly non-enzymatic manner via

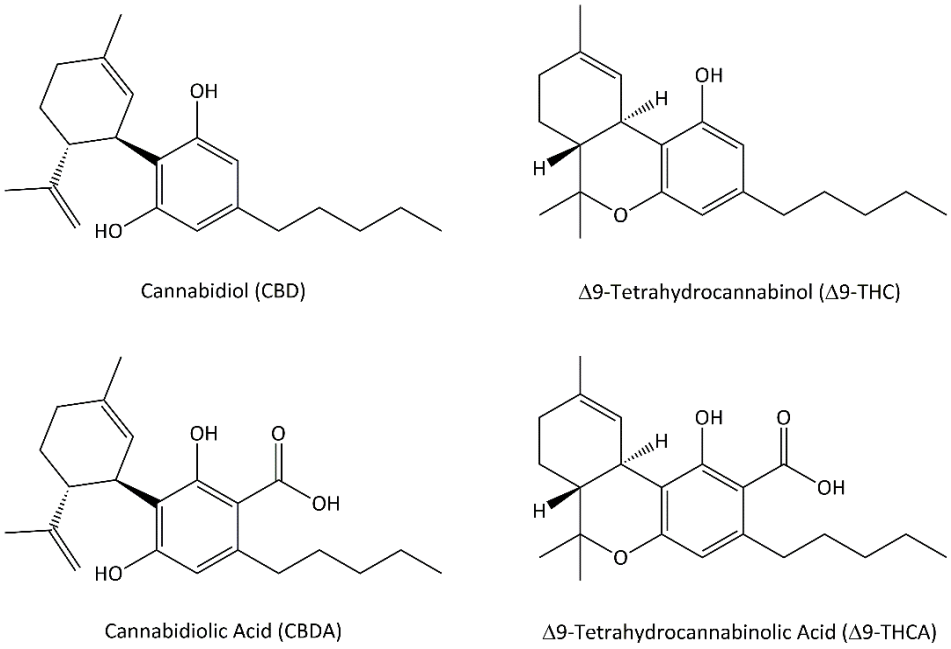

Fig.1. Structures of CBD and Δ^9-THC and their carboxylic acid precursors, CBDA and Δ^9-THCA

exposure to light, heat and/or atmospheric oxygen during the storage process (Hanuš et al., 2016, Pollastro et al., 2018). These structural changes account, at least in part, for the structural diversity of the known phytocannabinoids.

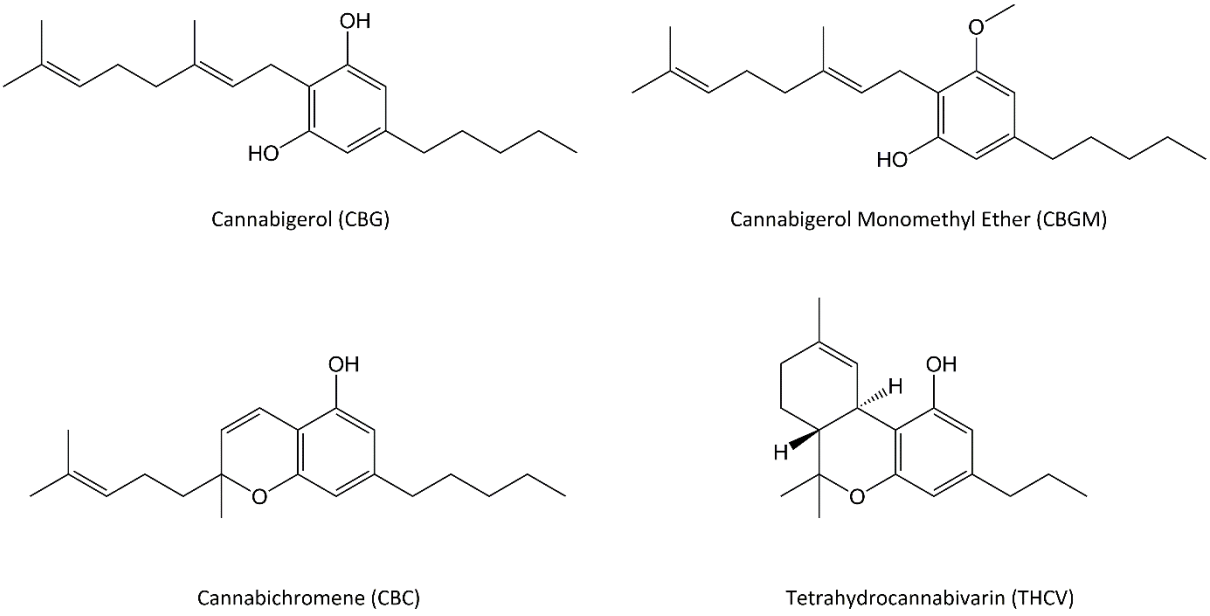

Fig.2. Structures of other phytocannabinoids present in the Bedrocan products used in this study, including the non-classical phytocannabinoids CBG, CBGM and CBC.

Phytocannabinoids such as cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC) have been shown to inhibit the neurotoxicity associated with β-amyloid (Aβ), the hallmark protein associated with Alzheimer's Disease (AD) (Cassano et al., 2020, Janefjord et al., 2014). These phytocannabinoids exert their effects via their actions at numerous receptors and pathways, including activation of PPARγ, CB_1 and CB_2 receptors, inhibition of caspase-3 and reductions in oxidative stress (O'Sullivan, 2016, Iuvone et al., 2004, Aso and Ferrer, 2016, Vallée et al., 2017). However, although there is a wealth of literature regarding the neuroprotective potential of specific phytocannabinoids, both in isolation and in combination, far less research has been conducted to determine the possible therapeutic benefits of whole botanical extracts in the context of AD and neurodegenerative diseases more generally.

While specific phytocannabinoids have been shown to possess considerable therapeutic potential in isolation, it has been suggested that these compounds may be more effective when used in combination with one another in the form of whole botanical extracts (Bonn-Miller et al., 2018, Russo, 2019). This concept is termed the "entourage effect" and refers to the manner in which combinations of phytocannabinoids and various cannabis terpenes work in concert with one another to provide a greater degree of protection than that afforded by the use of phytocannabinoids in isolation (Russo, 2011, Ferber et al., 2020). Interestingly, previous research in other neurological disease contexts has suggested that CBD-rich extracts may possess greater therapeutic potential than purified CBD, likely through the actions of this entourage effect (Pamplona et al., 2018). Furthermore, cannabis terpenes (**Fig. 3**) have been ascribed cannabimimetic properties with the ability to selectively enhance cannabinoid activity *in vivo* (LaVigne et al., 2021). Whole botanical extracts have been ascribed neuroprotective capacity, with the ability to attenuate oxidative stress in neuronal cell lines (Raja et al., 2020). Moreover, chemovars of various types have also been shown to reduce cytokine expression and alter Aβ processing in transgenic mouse models of AD, highlighting their therapeutic potential in the treatment of this disease (Aso et al., 2015). Interestingly, Sativex, a cannabis extract

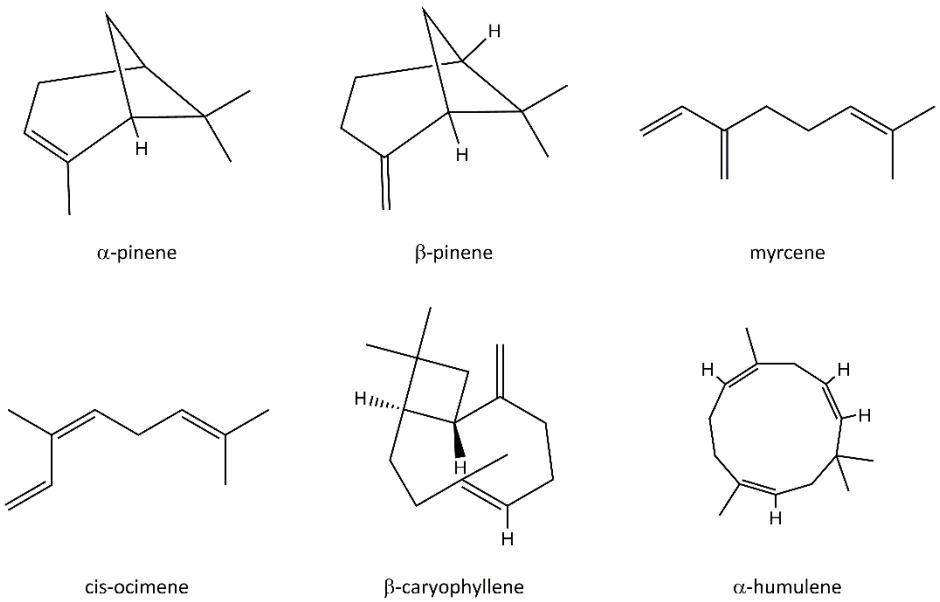

Fig.3. Structures of the primary terpenes found in each of the Bedrocan products used in this study.

containing both Δ^9-THC and CBD has been effective in the treatment of other neurodegenerative diseases including spasticity control in patients with multiple sclerosis (Patti et al., 2016). However, there is a considerable lack of data available on the general neuroprotective properties of whole botanical cannabis extracts, highlighting the need to identify and develop novel candidate chemovars.

Cannabis chemovars may be differentiated by their relative phytocannabinoid content, with type I chemovars characterised by the predominance of Δ^9-THC, type II containing both Δ^9-THC and CBD and type III being CBD-predominant (Lewis et al., 2018). Variations in the method of cultivation of medicinal cannabis chemovars are largely responsible for their diverse phytocannabinoid and terpene profiles (Lewis et al., 2018). It has previously been shown that heat exposure in cannabis extracts results in altered cannabinoid content due to the thermo-chemical conversion of phytocannabinoid acids to their neutral bioactive forms; heating may also affect the pharmacokinetic and metabolic profile of these extracts (Olejar and Kinney, 2021, Eichler et al., 2012). Moreover, heating has been shown to significantly reduce the terpene content of cannabis, given their volatile nature (Chen et al., 2021). However, very little research has been performed investigating the effects of heat exposure on the bioactivity of cannabis extracts in the context of neuroprotection.

In the present study, we characterised the protective capacity of extracts of five proprietary cannabis chemovars in an *in vitro* model of *tert*-butyl hydroperoxide (*t*bhp)-evoked oxidative damage and Aβ-induced neurotoxicity, as well as comparing the effect of heat exposure on the bioactivity of each extract. In addition, we compared the *in silico* binding profiles of the predominant phytocannabinoids in these medicinal cannabis formulations, Δ^9-THC and CBD and their carboxylated precursors THCA and CBDA, with the Aβ protein. This study presents the first characterisation of these proprietary medicinal cannabis formulations in the context of Aβ-evoked neuronal cell damage and provides a comparative insight into the variable bioactivity of these botanical extracts.

Materials and Methods

2.1 Materials

Cannabis chemovars used in this study were sourced from Bedrocan International (Veendam, Groningen, NL) and are commercially available (see Section 2.2 for more product details). Human $A\beta_{1–42}$ protein was purchased from rPeptide (Bogart, GA, USA) with thiazolyl blue tetrazolium bromide (MTT), trypan blue, *tert*-butyl hydroperoxide (*t*bhp), Roswell Park Memorial Institute 1640 (RPMI-1640) medium, uranyl acetate, phosphate buffered saline (PBS), non-essential amino acids (NEAA), penicillin/streptomycin, 1× Trypsin EDTA and foetal bovine serum (FBS) obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from ChemSupply (Gillman, SA, AUS).

2.2 Cannabis Extract and $A\beta_{1-42}$ Preparation

Lyophilised human $A\beta_{1–42}$ was prepared by dissolving in 100% DMSO to a concentration of 3.8mM. This stock solution was then diluted to 100µM in sterile PBS, dispensed into aliquots and frozen at -80˚C until required.

Cannabis from five commercial chemovars arrived in either flos or granulate form and underwent ethanolic extraction prior to use in the study. Briefly, 1g dry, macerated and powdered biomass was added per 10mL ethanol and vortexed thoroughly before being placed on a platform roller for 30 minutes. This mixture was then centrifuged for 5 minutes and decanted to remove remaining biomass; the final solution was then filtered into aliquots using a 0.45µm filter. The Δ^9-THC:CBD ratio varied between chemovars: Bedrocan® (BC-101, sativa) – 22%:<1%, Bedrobinol® (BC-201, sativa) – 13.5%:<1%, Bediol® (BC-301, sativa) – 6.3%:8%, Bedica® (BC-401, indica) 14%:<1%, and Bedrolite® (BC-501, sativa) <1%:9% (see **Table 1.**); the full terpene profile for each chemovar is listed in **Table 2** and is also available on the manufacturer's website (https://bedrocan.com/). To determine the effect of heat exposure, a portion of each chemovar was heated at 130˚C for 10 minutes to allow for the

majority conversion from carboxylated phytocannabinoid acid precursors to 'neutral', decarboxylated phytocannabinoids (Wang et al., 2016, Veress et al., 1990).

Phytocannabinoid	BC-101 (~%)	BC-201 (~%)	BC-301 (~%)	BC-401 (~%)	BC-501 (~%)
Tetrahydrocannabivarin (THCV)	0.5	0.5	–	0.5	–
Cannabidiol (CBD)	0.5	0.5	8	0.5	9
Cannabichromene (CBC)	0.5	0.5	1	0.5	1
Cannabigerol Monomethyl Ether (CBGM)	–	–	0.5	–	0.5
Δ^9-tetrahydrocannabinol (Δ^9-THC)	22	13.5	6.3	14	0.5
Cannabigerol (CBG)	2	0.5	0.5	1	0.5

Table 1. Manufacturer-reported percentages for the phytocannabinoids present in each of the Bedrocan medical cannabis products used in this study.

Terpene	BC-101 (~mg/g)	BC-201 (~mg/g)	BC-301 (~mg/g)	BC-401 (~mg/g)	BC-501 (~mg/g)
α-2-pinene	0.75	2	0.75	3.5	0.35
β-2-pinene	1.5	0.75	0.35	1	0.35
Myrcene	5	10	7.5	17.5	1.5
α-phellandrene	0.35	–	–	–	–
Δ-3-carene	0.35	–	–	–	–
R-limonene	1.5	0.2	0.35	–	0.2
Cis-ocimene	3.5	0.5	0.5	2	0.2
γ-terpinene	0.2	–	–	–	–

Terpinolene	5	–	0.75	–	0.35
(-)linalool	0.2	–	–	0.75	–
β-fenchol	0.1	–	–	–	–
Camphor	–	–	0.2	–	0.2
Borneol	0.75	–	–	–	–
α-terpineol	–	–	0.5	0.2	0.1
β Caryophyllene	1.5	1	0.75	1.5	0.75
Trans-bergamotene	0.35	–	0.2	0.2	0.1
α-guaiene	0.35	–	0.35	–	0.1
α-humulene	0.5	0.35	0.35	0.5	0.35
Trans-β-farnesene	0.35	–	0.35	0.1	0.35
γ-selinene	0.5	0.2	0.35	0.35	–
γ-cadinene	0.35	0.35	0.2	0.2	–
Eudesma-3,7(11)-diene	0.35	0.35	–	0.75	–
γ-elemene	1	0.35	0.2	0.5	–
β-caryophyllene oxide	–	–	–	–	0.2
Guaiol	–	–	–	0.5	–
γ-eudesmol	–	0.1	–	0.5	–
β-eudesmol	–	–	–	0.35	–
α Bisabolol	–	–	–	1	–

Table 2. Manufacturer-reported terpene profiles for each of the Bedrocan medical cannabis products used in this study.

2.3 Neuronal Cell Culture

Mouse neuroblastoma × spinal cord cells (NSC-34) were cultured and maintained in complete medium (Dulbecco's Modified Eagles Medium, supplemented with 10% foetal bovine serum, 1% non-essential amino acids and 1% penicillin/streptomycin) and subcultured every 2-3 days as necessary. We have previously established the use of these cells in an *in vitro* model of Alzheimer's disease (Laws III et al., 2022). For cell viability measurements, cells were seeded in 96-well plates at a density of 3×10^5 cells/well and left to equilibrate for 24 hours at 37ºC and 5% CO_2 prior to treatment.

2.4 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was used to directly visualise changes in $A\beta_{1\text{-}42}$ aggregate morphology following treatment with each extract. Samples were prepared by incubating native $A\beta_{1\text{-}42}$ (10µM) in PBS, alone or in the presence of the extracts (10µM principal component) for a period of 48 hours at 37ºC, 5% CO_2. Following incubation, a 5µL sample of each solution was added onto a 400-mesh formvar carbon-coated nickel electron microscopy grid (Proscitech, Kirwan, QLD, Australia) and left for one minute. Samples were then blotted off and 10 µL of a contrast dye containing 2% uranyl acetate was added. After 1 minute, the contrast dye was blotted off and sample loaded into an FEI Tecnai G2 Spirit Transmission Electron Microscope (FEI, Milton, QLD, AUS). Each grid was scanned extensively for the presence of the target peptide and representative images taken at 18,500× magnification.

2.5 MTT Assay

Cell viability was determined using the thiazolyl blue tetrazolium bromide (MTT) assay. After equilibration, each well was treated with cannabis extracts at 10µM principal component or a vehicle control and left to equilibrate for a further 15 minutes before being treated with $A\beta_{1\text{-}42}$ (0, 0.05, 0.1, 0.2, 0.5 & 1µM) or *t*bhp (0-150µM). After treatment the plates were incubated for

48 hours at 37ºC and 5% CO2. Following incubation, culture medium was removed from each well and replaced by serum-free medium containing 0.25mg/ml MTT. Plates were then incubated for a further 2 hours, after which MTT solution was aspirated and cells were lysed with DMSO. Absorbance readings were taken at 570nm using a Synergy MX microplate reader (Bio-tek, Bedforshire, UK).

2.6 Computational modelling of extract principal component binding with $A\beta_{1-42}$

Molecular modelling simulations were conducted as described previously (Marsh et al., 2017). Briefly, equilibrium geometries for the principal components of each chemovar, namely Δ^9-THC, THCA, CBD and CBDA, were optimised using density functional theory (DFT) utilising the Becke-Lee-Yang-Parr three-parameter hybrid functional, commonly referred to as B3LYP (Kohn et al., 1996). A large basis set, aug-cc-pVDZ was used to approximate molecular orbitals in optimised geometry for each compound, with all computations carried out using the Gaussian 09 package of codes. Optimised structures were then modelled for their binding affinity with both the $A\beta_{1-42}$ monomer (PDB ID: 1IYT) and an $A\beta_{17-42}$ oligomer comprised of parallel β-sheet structures (PDB ID: 2BEG), using CLC Drug Discovery Workbench v2.4.1. Since $A\beta_{1-42}$ contains no specific pharmacophore, a large search space covering the entire peptide was used in each case; each ligand was also kept flexible to allow for rotation of bonds during docking simulations. Docking simulations were repeated five times with 1000 iterations per simulation and the binding pose with the highest overall docking score for each ligand was selected for representative imaging.

2.7 Statistical Analysis

Cell viability data from the MTT assay was analysed via a two-way ANOVA using Dunnett's multiple comparisons test to determine significance vs treatment ($A\beta_{1-42}$ or *t*bhp). Modelling data was analysed by one-way ANOVA with significance determined by Tukey's multiple comparisons test. All data is presented as mean ± SD, with results deemed significant if $p < 0.05$.

Data analysis was performed using GraphPad Prism v.9.0.0 for Windows (GraphPad Software, San Diego, USA).

Results

3.1 The Δ^9-THC-dominant chemovars BC-101, and BC-201 provide the greatest degree of neuroprotection against $A\beta_{1-42}$ in NSC-34 cells; heat exposure generally decreases the bioactivity of cannabis extracts

Incubation with $A\beta_{1-42}$ (0-1.5µM) over 48 hours resulted in a concentration-dependent reduction in NSC-34 cell viability to a maximum of 75.38% initial viability (****p <0.0001) (**Fig. 4**). Of the five unheated extracts screened in this study, the Δ^9-THC-dominant chemovars BC-101 and BC-201 consistently provided the greatest degree of neuroprotection across the full range of $A\beta_{1-42}$ concentrations, maintaining cell viability at 86.27% and 85.96%, respectively, at 1.5µM $A\beta_{1-42}$ (****p <0.0001) (**Fig. 4a**). The Δ^9-THC-dominant BC-401 extract also significantly inhibited the cytotoxicity of $A\beta_{1-42}$ at 0.5, 1 and 1.5µM, maintaining cell viability at 88.26%, 83.36% and 83.93% initial, respectively (*p = 0.0268, **p = 0.0097 and ****p <0.0001 vs control). The BC-301 extract exhibited similar efficacy as an inhibitor of $A\beta_{1-42}$ cytotoxicity, maintaining cell viability at 88.69%, 84.34% and 82.95% initial at 0.5, 1 and 1.5µM $A\beta_{1-42}$, respectively (*p = 0.0139, **p = 0.0018 and ***p = 0.0004 vs control), while the CBD-dominant BC-501 extract displayed only modest protection, with significance seen only at the highest concentration of $A\beta_{1-42}$ (80.5% initial, *p = 0.0263).

The neuroprotective profile of each extract displayed similar trends in efficacy following heat exposure. H-BC-101 extract displayed the greatest neuroprotective capacity of all heated extracts, significantly inhibiting $A\beta_{1-42}$ cytotoxicity at each amyloid concentration. Significant neuroprotection was also observed following treatment with H-BC-201 extract, with cell viability maintained at 89.25%, 84.5% and 84.85% initial at 0.5, 1 and 1.5µM $A\beta_{1-42}$, respectively (**p = 0.0019, ****p <0.0001 and ****p <0.0001 vs control). H-BC-401 extract

provided significant protection at 0.5, 1 and 1.5μM $A\beta_{1-42}$, maintaining NSC-34 cell viability at 87.75%, 83.39% and 82.95%, respectively (*p = 0.0378, ***p = 0.0002 and ***p = 0.0001 vs control). The CBD-dominant H-BC-301 extract provided significant protection only at 1 and 1.5μM $A\beta_{1-42}$, maintaining cell viability at 81.4% and 81.56%, respectively (*p = 0.0138 and **p = 0.0032 vs control); H-BC-501 extract displayed no significant neuroprotection.

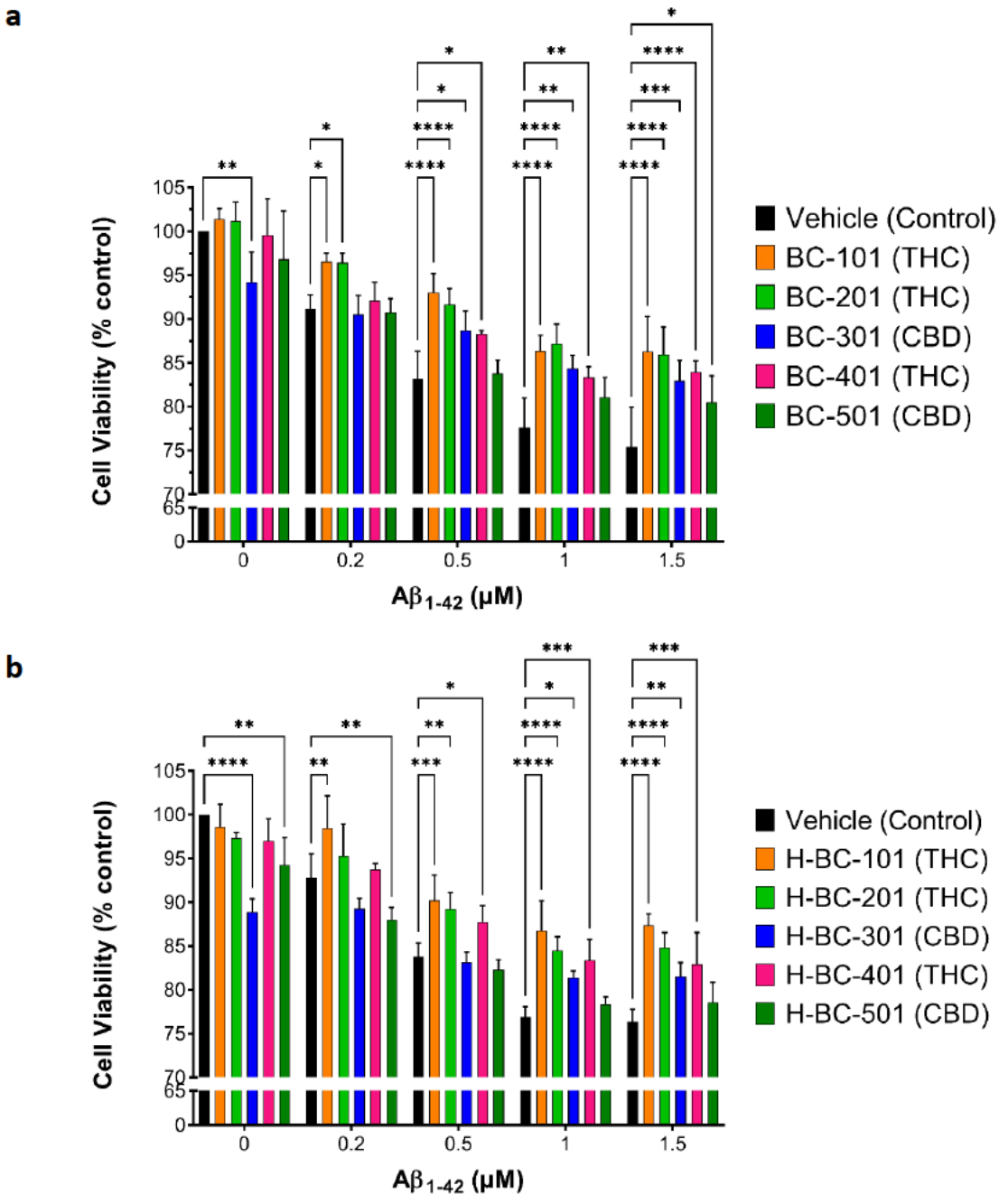

Fig. 4. MTT assay of cell viability following 48hr incubation with $A\beta_{1-42}$ (0-1.5μM) alone and in the presence of various **(a)** unheated and **(b)** heated cannabis extracts (10μM PC). Mean (SD), n = 5. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs vehicle.

Interestingly, a significant decrease in NSC-34 viability was seen after treatment with H-BC-301 extract alone (88.89% initial, ****p <0.0001); this was also observed at 0 and 0.2µM $A\beta_{1-42}$ in the case of H-BC-501 extract (94.28% and 88.03% initial, **p = 0.0011 and **p = 0.0076).

3.2 Cannabis extracts provide no significant protection against *t*bhp-induced neurotoxicity in NSC-34 cells; heat exposure increases the cytotoxicity of CBD-dominant chemovars.

Incubation with *t*bhp (0-150µM) over 24 hours resulted in a concentration-dependent decrease in cell viability to a maximum of 9.4% initial viability (****p <0.0001) (**Fig. 5** and **Fig. 6**). None of the chemovars tested provided any significant protection against *t*bhp-induced

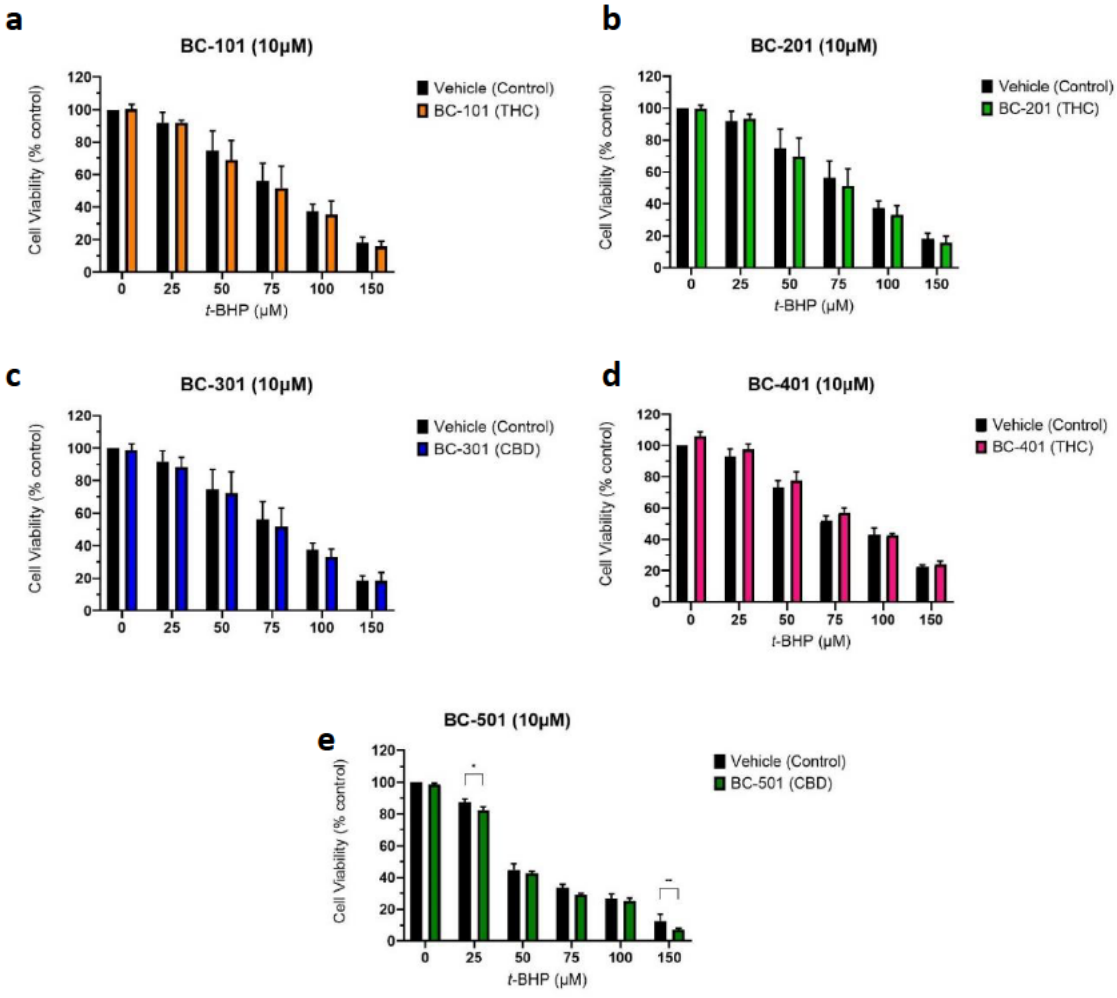

Fig. 5. MTT assay of cell viability following 24hr incubation with *tert*-butyl hydroperoxide (*t*bhp) (0-150 µM) alone and in the presence of various cannabis extracts (10µM PC): **(a)** BC-101, **(b)** BC-201, **(c)** BC-301, **(d)** BC-401, **(e)** BC-101. Mean (SD), n = 4. *p <0.05, **p <0.01 vs vehicle.

cytotoxicity. However, significant increases in toxicity were observed at 25µM (82.36% initial vs 87.13%, *p = 0.0305) and 150µM *t*bhp (7.3% initial vs 12.74%, *p = 0.01) following treatment with the CBD-dominant BC-501 extract (**Fig. 5e**).

Heat exposure resulted in a notable increase in the cytotoxicity of CBD-dominant chemovars. H-BC-301 extract displayed intrinsic toxicity while also increasing the toxicity of 25µM *t*bhp (86.95% initial vs control, ****p <0.0001 and 75.02% initial vs 87.84%, ****p <0.0001, respectively) (**Fig. 6c**). A similar pattern was observed in H-BC-501-treated cells, which also displayed intrinsic toxicity (88.24% initial vs control, ***p =0.0002) as well as reducing cell

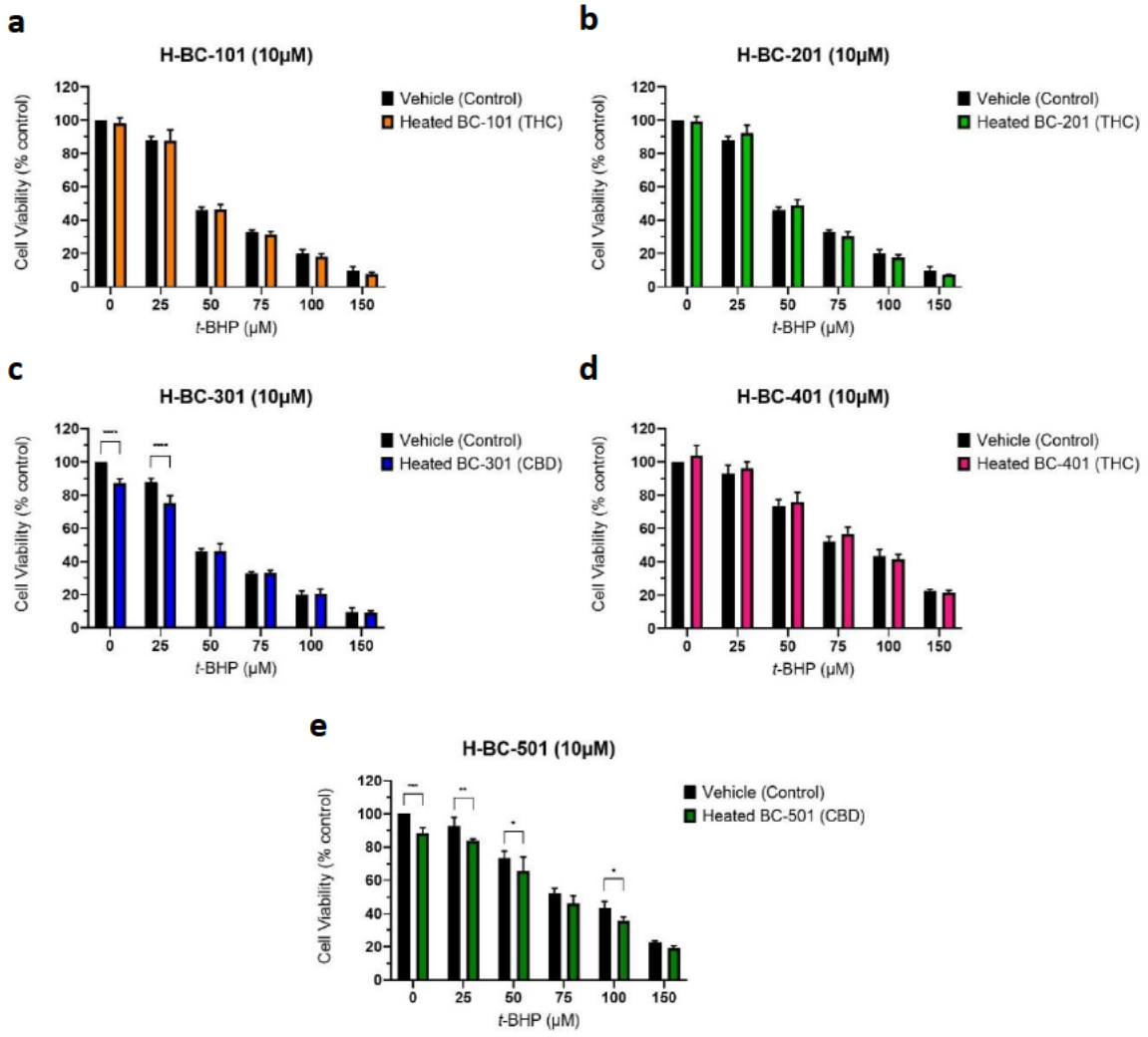

Fig. 6. MTT assay of cell viability following 24hr incubation with *tert*-butyl hydroperoxide (*t*bhp) (0-150 µM) alone and in the presence of various heated cannabis extracts (10µM PC): **(a)** H-BC-101, **(b)** H-BC-201, **(c)** H-BC-301, **(d)** H-BC-401, **(e)** H-BC-101. Mean (SD), n = 5. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 vs vehicle.

viability to 83.74%, 65.66% and 35.32% initial at 25, 50 and 100μM *t*bhp, respectively (**p = 0.0038, *p = 0.0192 and *p = 0.0145).

3.3. Cannabis extracts exert little effect on $A\beta_{1-42}$ aggregation, with modest effects on aggregate density and overall morphology

The cannabis extracts screened in this study generally exerted little effect on the overall morphology of $A\beta_{1-42}$ (**Fig. 7**). Some slight differences were observed, with BC-401 extract effectively reducing the overall degree of aggregation (**Fig. 7e**); however, the overall effect of each extract was relatively modest.

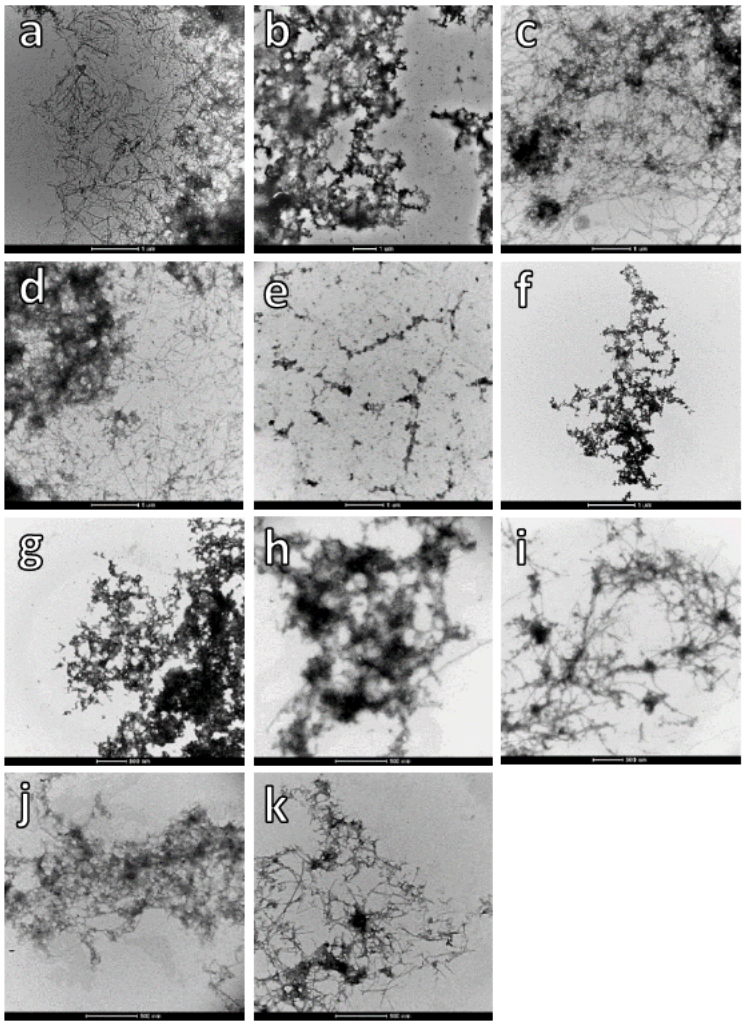

Fig. 7. Transmission electron micrographs demonstrating the effect of unheated (b-f) and heated (g-k) cannabis extracts (10μM PC) on the aggregation and morphology of $A\beta_{1-42}$ (10μM). **(a)** control, **(b)** BC-101, **(c)** BC-201, **(d)** BC-301, **(e)** BC-401, **(f)** BC-501, **(g)** H-BC-101, **(h)** H-BC-201, **(i)** H-BC-301, **(j)** H-BC-401, **(k)** H-BC-501.

3.4. Comparative molecular modelling of binding interactions between carboxylated and decarboxylated phytocannabinoids and the Aβ protein

The amyloid binding characteristics of the phytocannabinoids Δ^9-THC and CBD were compared with their carboxylated precursors THCA and CBDA (**Figs 8, 9** and **10**). In the $A\beta_{1-42}$ monomer, both Δ^9-THC and CBD bound towards the centre of the protein, interacting with the key Lys16 residue via hydrogen bonding (**Fig. 9a** and **c**), although Δ^9-THC displayed

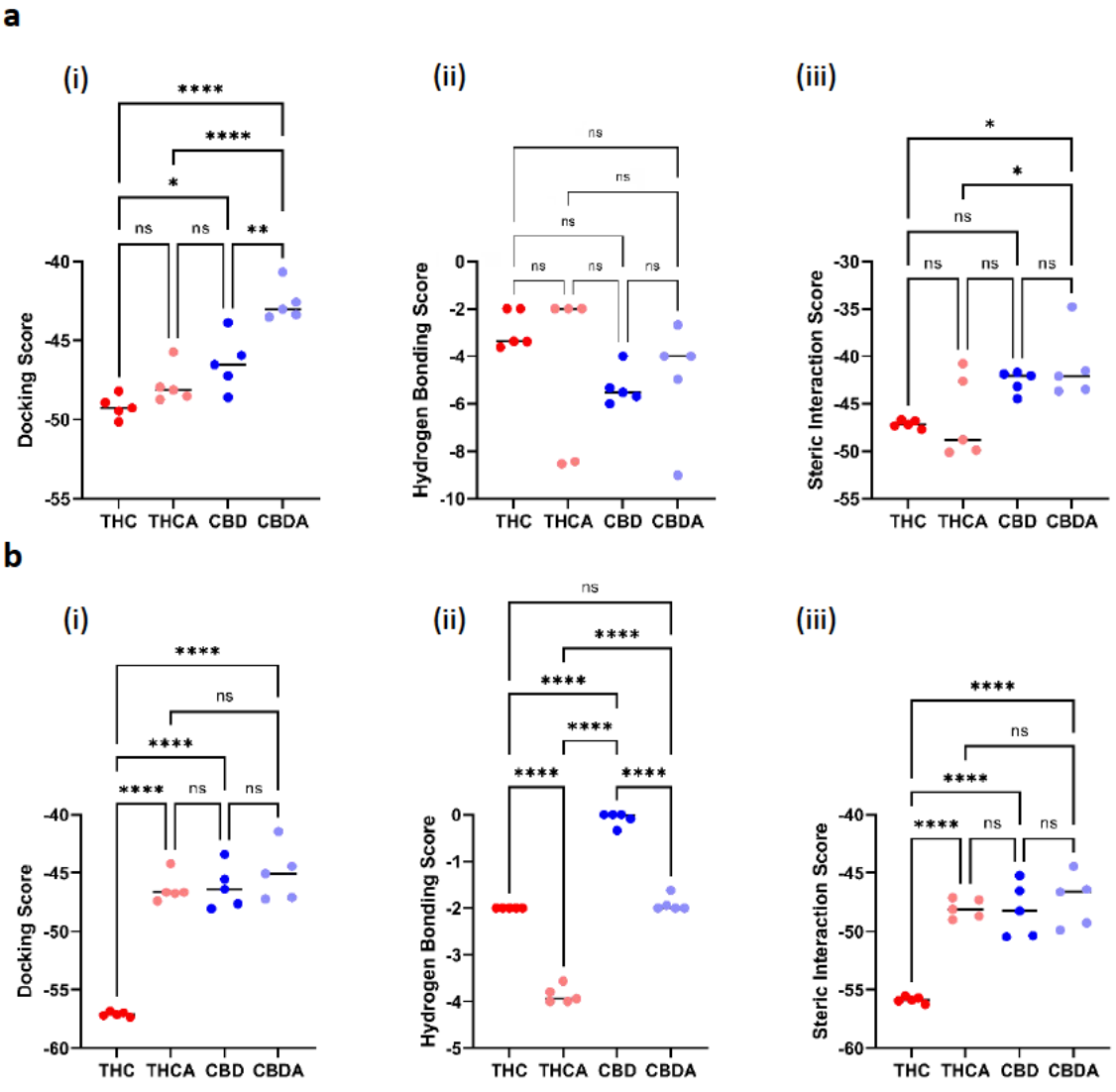

Fig.8. *In silico* modelling of binding interactions between Δ^9-THC, THCA, CBD and CBDA with Aβ **(a)** monomer (PDB ID: 1IYT) and **(b)** pentamer (PDB ID: 2BEG). Data presented as median **(i)** best docking score and the corresponding **(ii)** hydrogen bonding score and **(iii)** steric interaction score from n = 5 independent simulations. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001.

slightly greater binding affinity over CBD (*p = 0.0147) (**Fig. 8a (i)**). A similar relationship was observed between Δ^9-THC and THCA, whereby both molecules bound towards the centre of the $A\beta_{1\text{-}42}$ monomer, although THCA bound at slight opposition to Δ^9-THC and did not form hydrogen bonds with Lys16; no significant difference was observed between the docking scores of Δ^9-THC and THCA. CBD possessed greater affinity for the $A\beta_{1\text{-}42}$ monomer than CBDA (**p = 0.0011) (**Fig. 8a (i)**), despite the similarity in binding pose and position (**Fig. 9c** and **d**). Of particular note was the relationship between THCA and CBDA, whereby THCA displayed significantly greater affinity for the $A\beta_{1\text{-}42}$ monomer (****p <0.0001) (**Fig. 8a (i)**), perhaps due primarily to differences in steric interactions (*p = 0.0483) (**Fig. 8a (iii)**).

In the pentamer, Δ^9-THC displayed far greater affinity than both CBD and THCA (****p <0.0001), with no significant difference observed between CBD and CBDA nor between THCA and CBDA (**Fig. 8b (i)**). The carboxylated phytocannabinoids THCA and CBDA displayed significantly greater hydrogen bonding potential than their decarboxylated counterparts (****p <0.0001), while Δ^9-THC displayed great hydrogen bonding than CBD (****p <0.0001) and THCA significantly greater than CBDA (****p <0.0001) (**Fig. 8b (ii)**). Δ^9-THC also displayed considerably greater steric interaction scores than all other phytocannabinoids screened (****p <0.0001), likely contributing to its relative binding potency (**Fig. 8b (iii)**). Each phytocannabinoid bound in a similar position within the hydrophobic groove of the Aβ pentamer, with all but CBD displaying hydrogen-bonding interactions with Leu 17 (**Fig. 10**).

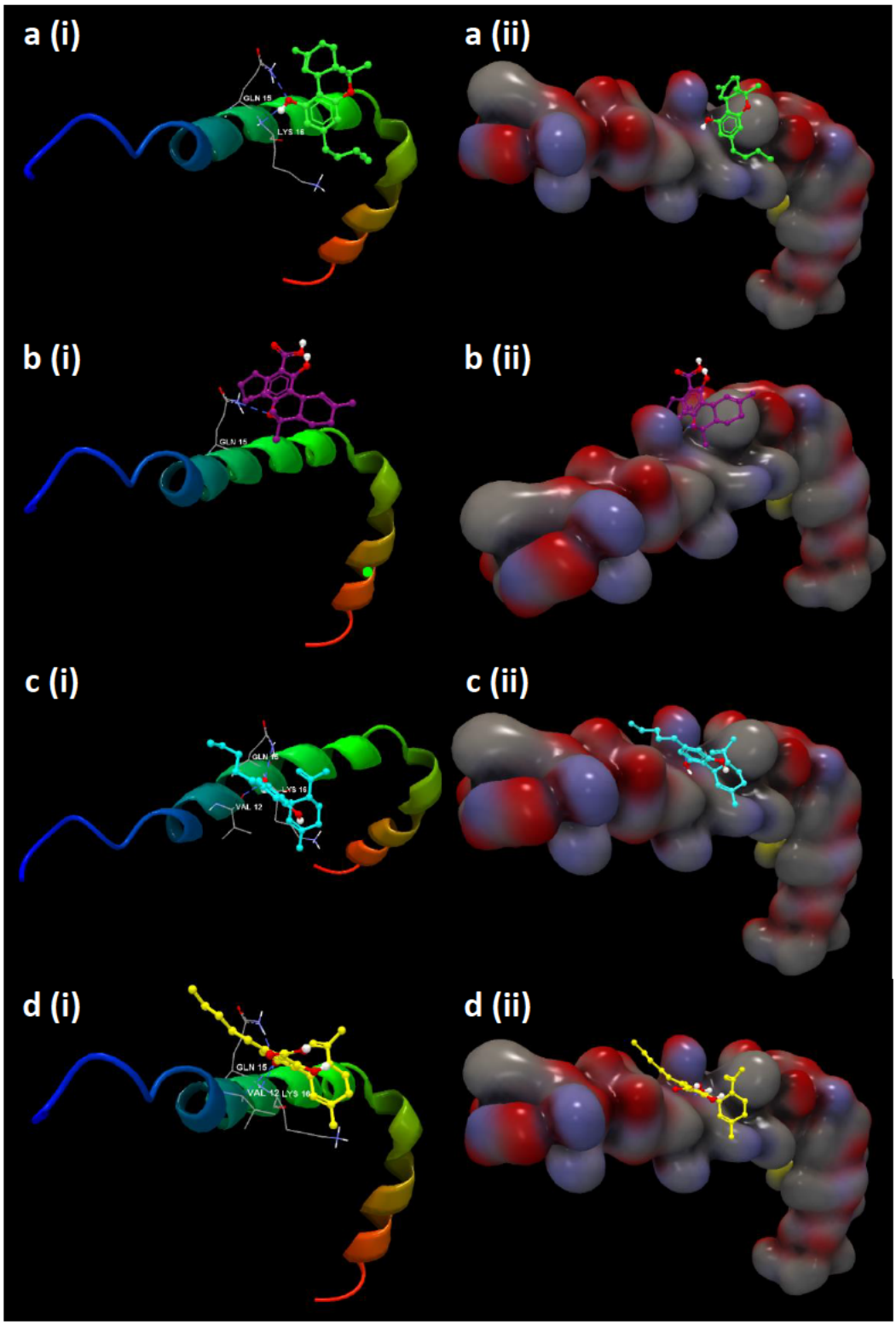

Fig. 9. Representative images of the most favourable binding interactions between **(a)** Δ^9-THC, **(b)** THCA, **(c)** CBD and **(d)** CBDA with $A\beta_{1\text{-}42}$ monomer (PDB ID: 1IYT); images presented with **(i)** ribbon structure and **(ii)** bubble.

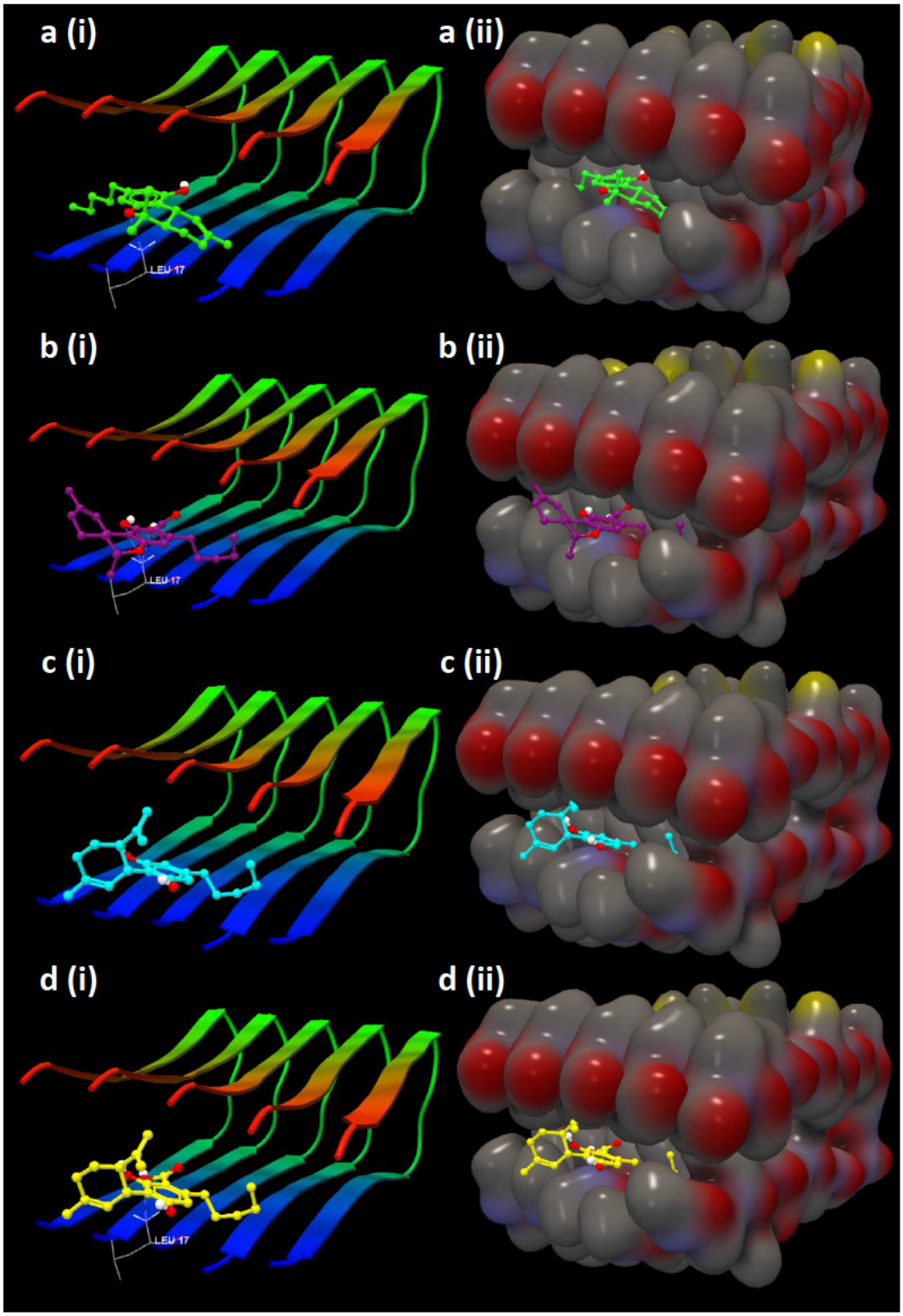

Fig. 10. Representative images of the most favourable binding interactions between **(a)** Δ^9-THC, **(b)** THCA, **(c)** CBD and **(d)** CBDA with $A\beta_{17\text{-}42}$ pentamer (PDB ID: 2BEG); images presented with **(i)** ribbon structure and **(ii)** bubble.

Discussion

The results of this study highlight the novel neuroprotective effects of ethanolic extracts of the type I cannabis chemovars BC-101, BC-201 and BC-401 against $A\beta_{1-42}$-evoked neurotoxicity. No significant protective effect was observed in *t*bhp-treated cells following co-incubation with any of the cannabis extracts screened in this study, suggesting that this neuroprotection is likely independent of antioxidant capacity. These extracts exerted minimal qualitative effects on the fibrillisation and aggregation of Aβ, indicating that this protective effect is also independent of direct interactions with the amyloid protein. This study presents the first characterisation of these five proprietary cannabis chemovars in the context of neuronal cell-based models of Alzheimer's disease neurotoxicity.

The Δ^9-THC-predominant chemovars BC-101, BC-201 and BC-401 significantly inhibited the neurotoxicity of $A\beta_{1-42}$ in NSC-34 cells, with the type II chemovar BC-301 providing a lesser degree of protection; no significant neuroprotection was observed following treatment with the type III chemovar BC-501. This pattern suggests that Δ^9-THC-predominant cannabis extracts may provide a greater degree of neuroprotection against Aβ-evoked toxicity than CBD-predominant extracts. This finding is in line with previous studies from our laboratory, which have demonstrated no significant neuroprotective capacity for CBD against $A\beta_{1-42}$ (Harvey et al., 2012)(see Chapter 3 of this thesis). Interestingly, none of the extracts screened in this study provided any protection against the lipid peroxidising agent *t*bhp in NSC-34 cells. This is in contrast to the findings of a previous study which highlighting the particular efficacy of Δ^9-THC-dominant extracts in reducing H_2O_2-induced reactive oxygen species (ROS) generation (Raja et al., 2020). This difference may, in part, be attributed to factors including the different cell types used in these studies, the different pro-oxidants stressors used and possible variations in the degree of cell differentiation. However, the authors noted the particular lack of efficacy of CBD-dominant extracts as antioxidants, a trend that was observed in the present study. Interestingly, previous research from our laboratory has shown that CBD possesses the ability

to protect neuronal PC12 cells from *t*bhp-evoked cytotoxicity, while affording no significant neuroprotection against $A\beta_{1-42}$ (see Chapter 3 of this thesis), suggesting either possible differences in pro-oxidant resilience between these phenotypically distinct neuronal cell lines or the differential expression of targets or operant pathways for CBD-based neuroprotection, whereby the definitive pharmacological target(s) for CBD's action remains unclear.

Given their lack of efficacy in the inhibition of both amyloid aggregation and *t*bhp-induced cytotoxicity, it is clear that these extracts mediate their protective effects via other mechanisms. Both Δ^9-THC and CBD have been shown to exert their neuroprotective effects via the activation of numerous receptor targets, including cannabinoid receptor 1 and 2 subtypes (CB_1 and CB_2) and peroxisome proliferator-activated receptor gamma (PPARγ) (Zou and Kumar, 2018, O'Sullivan, 2016). Interestingly, the non-selective cannabinoid receptor agonist WIN55212-2 has been shown to reduce Aβ-evoked neuroinflammation via the activation of both CB_1 and CB_2 receptors, as well as increased PPARγ signalling (Fakhfouri et al., 2012). Moreover, activation of the CB_2 receptor has been associated with the reversal of AD-associated memory impairment, with CB_2 receptor deficiency being shown to worsen the pathological progression of AD *in vivo* (Wu et al., 2013, Koppel et al., 2013). Such studies highlight the potential importance of cannabinoid and PPARγ receptors as targets in AD. Interestingly, a study from Finlay and colleagues found that the entourage effect of cannabis terpenes was not mediated by their activity at cannabinoid receptors (Finlay et al., 2020). However, future studies may employ the use of selective cannabinoid and PPARγ receptor antagonists to determine their role in the neuroprotection afforded by the particular chemovars used in this study.

Interestingly, a general trend was observed whereby heating of each extract reduced their overall neuroprotective effects, even increasing the toxicity of CBD-dominant extracts. One possible explanation for this effect is the possible decrease in terpenoids in the heated extracts, as heating of cannabis has been shown to reduce terpene retention, given their volatile nature (Chen et al., 2021). Moreover, heat exposure may result in the formation of oxidation products

such as hydroperoxides (e.g. limonene hydroperoxide) which have been associated with ROS-induced cytotoxicity via lipid peroxidation (Maggini et al., 2022). This would be consistent with the prevailing theory of the entourage effect, whereby the presence of such terpenoids may act to synergistically enhance the protective effects of cannabinoids (Russo, 2011). This effect highlights the potential therapeutic benefits of using whole botanical cannabis extracts over phytocannabinoids in isolation. It is particularly interesting to note the potential role of such cannabis terpenes in the neuroprotection observed in this study. Terpenes such as myrcene have been shown to possess neuroprotective qualities in *in vivo* models of oxidative neuronal damage and AD, with the terpenes α-bisabolol and β-caryophyllene providing significant neuroprotection against $A\beta_{1\text{-}42}$ in NSC-34 cells (Kumar et al., 2021, Ciftci et al., 2014, Laws III et al., 2022). Moreover, both α-pinene and β-pinene have been ascribed neuroprotective properties, with α-pinene providing *in vivo* neuroprotection via the suppression of the TNFα/NF-κB pathway (Khan-Mohammadi-Khorrami et al., 2022, Shin et al., 2020). Interestingly, it has been suggested that cannabis terpenes including α-humulene, linalool and β-pinene have cannabimimetic properties, with the ability to produce cannabinoid tetrad behaviours in mice suggestive of an ability to enhance cannabinoid activity (LaVigne et al., 2021). Each of these terpenes are found in the chemovars used in this study, suggesting they may play a role in the observed neuroprotection. Future *in vivo* studies may consider using standardised combinations of these terpenes in the presence and absence of Δ^9-THC and CBD, in order to further assess their therapeutic potential in AD as well investigating any possible therapeutic synergism.

Modelling with Aβ revealed that, despite binding at similar positions, Δ^9-THC possesses significantly greater binding affinity for both the monomer and pentamer than both THCA and CBD. We have previously shown that Δ^9-THC possesses greater binding affinity for both the Aβ monomer and pentamer than does CBD (Marsh and Smid, 2021). However, to our knowledge, this study presents the first comparative insights into the amyloid binding affinities

for Δ^9-THC and CBD against their carboxylated precursors. It was observed that THCA and CBDA possess significantly lower binding affinity for the Aβ protein than their decarboxylated counterparts, suggesting that unheated extracts, which likely contain a greater proportion of THCA and CBDA, should be less efficacious as inhibitors of amyloid aggregation than heated extracts. However, an interesting lack of correlation was observed between this modelling data and the morphological data obtained via TEM. Only the unheated BC-401 exerted any effect on $A\beta_{1-42}$ aggregation and morphology, an effect which was not observed following heat exposure. In any case, it is clear that the neuroprotection against $A\beta_{1-42}$ observed in this study is independent of any direct interactions with the $A\beta_{1-42}$ protein. This is consistent with previous studies which have found both Δ^9-THC and CBD to have negligible effects on $A\beta_{1-42}$ aggregation (Janefjord et al., 2014)(see Chapter 3 of this thesis). Moreover, we have found that Δ^9-THC, not CBD, is able to inhibit the neurotoxicity of $A\beta_{1-42}$ in neuronal PC12 cells without affecting protein aggregation, consistent with the results of the present study (see Chapter 3 of this thesis).

In conclusion, this study demonstrated the novel neuroprotective capacities of the proprietary type I chemovars BC-101, BC-201 and BC-401 against Aβ-evoked neurotoxicity, an effect that was reduced following heat exposure, is independent of antioxidant capacity and is unrelated to direct interactions with the $A\beta_{1-42}$ protein. Given the protective effect observed in this study and the considerable interest surrounding medicinal cannabis and the therapeutic use of whole botanical extracts, further research into the neuroprotective efficacy of medicinal cannabis extracts is warranted. As our understanding of medicinal cannabis and the entourage effect continues to grow, so too does the need for further *in vivo* studies of whole botanical extracts in the context of AD. Such studies may provide considerable insight into the true therapeutic potential of medicinal cannabis, presenting an important avenue for future exploration.

Statement of Authorship

Title of Paper	Comparing the efficacy of structurally diverse phytocannabinoids as inhibitors of SN-38- and cytokine-evoked increases in intestinal epithelial barrier permeability
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Dylan Thomas Marsh		
Contribution to the Paper	Conceptualisation, Methodology, Validation, Formal analysis, Data curation, Investigation, Writing - original draft, Writing - review & editing, Visualisation		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	24/07/22

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contr bution.

Name of Co-Author	Scott Smid		
Contribution to the Paper	Conceptualisation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition		
Signature		Date	July 25, 2022

Chapter 5: Comparing the efficacy of structurally diverse phytocannabinoids as inhibitors of SN-38- and cytokine-evoked increases in intestinal epithelial barrier permeability

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Abstract

Irinotecan and its active metabolite SN-38 have been linked to the development of gastrointestinal toxicity and inflammation, known as gastrointestinal mucositis (GIM). Phytocannabinoids have been ascribed anti-inflammatory effects in models of gastrointestinal inflammation, with the ability to reduce inflammation and maintain epithelial barrier function. However, there have been few studies regarding their potential role in ameliorating irinotecan-induced GIM. Here, we aimed to characterise the protective capacity of the phytocannabinoids cannabigerol, cannabichromene and cannabidivarin in comparison with cannabidiol in a model of intestinal epithelial barrier integrity. Caco-2 cell viability in the presence of SN-38 (1-5μM) was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay. Transepithelial electrical resistance (TEER) was measured to determine changes in epithelial permeability in the presence of SN-38 (5μM) or the pro-inflammatory cytokines TNFα and IL-1β (each at 100ng/mL). The DCFDA assay was used to determine the ROS-scavenging ability of each phytocannabinoid following treatment with 200μM *t*bhp. MTT and TEER data were analysed by two-way ANOVA with Dunnett's post-hoc test versus control; data from the DCFDA assay were analysed by one-way ANOVA with Tukey's post-hoc test.

Each phytocannabinoid provided significant protection against cytokine-evoked increases in epithelial permeability, with cannabigerol proving most effective. Cannabidiol, cannabidivarin and cannabigerol were able to significantly inhibit SN-38-evoked increases in permeability, while cannabichromene had no significant effect. None of the tested phytocannabinoids were able to significantly inhibit *t*bhp-induced ROS generation. These results highlight a novel role for cannabidiol, cannabidivarin and cannabigerol as inhibitors of SN-38-evoked increases in epithelial permeability. These results support the rationale for further investigations into the use of phytocannabinoids as potential adjunct therapeutics in the management of irinotecan-associated mucositis and intestinal inflammation more generally.

1. Introduction

Gastrointestinal inflammation plays an essential role in the pathology and progression of numerous diseases, including inflammatory bowel disease (IBD) and chemotherapy-induced gastrointestinal mucositis (GIM). In the case of GIM, treatment with conventional chemotherapeutic drugs may initiate the onset of chronic inflammation, as well as the development of severe diarrhoea, rectal bleeding and infection (Dahlgren et al., 2021, Sougiannis et al., 2021). The development of GIM symptomology often results in dose reductions and delays in treatment, which can negatively affect clinical outcomes (Lalla et al., 2014). Several chemotherapeutic agents have been implicated in the development of GIM, with perhaps the most common being irinotecan, a drug used in the treatment of various forms of cancer, including colorectal, gastric, pulmonary and ovarian (Gibson et al., 2003, Kciuk et al., 2020). Irinotecan is a prodrug which undergoes enzymatic decarboxylation by carboxylesterases 1 and 2 to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), before being inactivated via glucuronidation to SN-38 glucuronide (SN-38G) by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) (Alfirevic and Pirmohamed, 2016). Following this, SN-38G may be reactivated by bacterial β-glucuronidases, resulting in the deposition of active SN-38 into the intestinal lumen (Bailly, 2019, Wallace et al., 2010). As a result, intestinal exposure to SN-38, an established causative factor in the onset of diarrhoea following treatment with irinotecan, is dramatically increased (Sun et al., 2020). Despite its off-target toxicity, irinotecan remains a common and effective treatment for a variety of solid tumours, highlighting the need for ways to minimise its off-target toxicity and the development of GIM (Fujita et al., 2015, Pizzolato and Saltz, 2003).

Treatment with irinotecan results in increased expression of nuclear factor kappa B (NF-κB), as well as numerous pro-inflammatory cytokines, including interleukin 1-β (IL-1β) and tumour necrosis factor-α (TNFα); these cytokines have been linked to increases in intestinal permeability *in vitro* and *in vivo* (Al-Sadi et al., 2009, Chelakkot et al., 2018, M Logan et al.,

2008). Furthermore, TNFα and IL-1β have been implicated in the pathogenesis of irinotecan-induced GIM, highlighting their importance as potential therapeutic targets (Melo et al., 2008). These pro-inflammatory cytokines are also key players in the development of IBD-related symptomology, with the ability to increase epithelial permeability; increased intestinal levels of TNFα and IL-1β is also associated with increased disease severity (Al-Sadi et al., 2008, Mao et al., 2018, Sands and Kaplan, 2007, Arifa et al., 2014). Interestingly, the generation of reactive oxygen species (ROS) and the development of oxidative stress are causative factors in the development of both IBD and chemotherapy-induced GIM, with antioxidant therapies showing great promise as mucoprotective agents (Arifa et al., 2016, Al-Asmari et al., 2015, Ashcraft et al., 2015). Upstream ROS generation may activate NF-κB, resulting in the increased transcription of numerous genes, including those coding for TNFα and IL-1β, resulting in the upregulation of these cytokines (Sonis, 2004, Lingappan, 2018). The subsequent upregulation of TNFα may then activate caspase pathways and generate a feedback loop on NF-κB, leading to an exacerbated inflammatory response (Cinausero et al., 2017). It therefore follows that

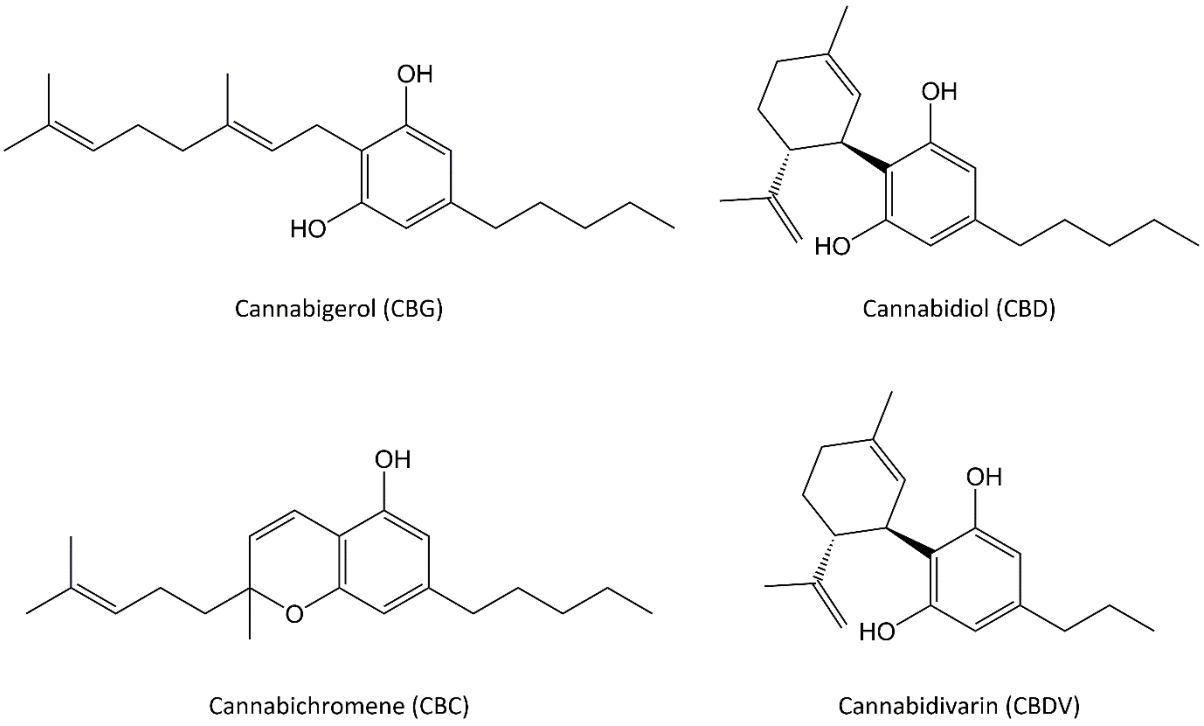

Fig.1. Structures of the phytocannabinoids cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD) and cannabidivarin (CBDV).

compounds with the ability to inhibit ROS generation and mediate the associated inflammatory response may be beneficial in the treatment and management of GIM.

Cannabis sativa (*C. sativa*) is the source of over 540 distinct phytochemicals, more than 120 of which are considered phytocannabinoids (pCBs) (Marsh and Smid, 2021). Numerous studies have investigated the therapeutic potential of *cannabis* in the treatment of GI inflammation, particularly in the context of Crohn's disease and ulcerative colitis (Naftali, 2020, Couch et al., 2018, Ahmed and Katz, 2016, Pagano et al., 2016). Phytocannabinoids possess the ability to maintain barrier integrity in models of colitis via a multitude of mechanisms, including inhibiting ROS production and reducing expression of pro-inflammatory cytokines (Borrelli et al., 2009, Cocetta et al., 2021, Pereira et al., 2021). Although pCBs have shown great promise as mucoprotective, anti-inflammatory and antioxidant agents, the majority of studies have focused on the specific actions of cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC), leaving the plethora of other pCBs largely unexplored. In this light, given the anti-inflammatory

and mucoprotective properties ascribed to pCBs in models of IBD, we hypothesise that these compounds may also be protective against SN-38-evoked reductions in barrier integrity via similar mechanisms.

In the present study, we characterised the mucoprotective capacities of the phytocannabinoids cannabidivarin, cannabigerol and cannabichromene in comparison with the well-characterised cannabidiol (**Fig. 1**). We determined the effect of each pCB on the cytotoxicity of SN-38 and assessed their ability to maintain epithelial barrier integrity in the presence of SN-38 and the proinflammatory cytokines TNFα and IL-1β. Finally, the ability for each pCB to inhibit *in vitro* ROS generation was also investigated. This study presents the first characterisation of cannabigerol, cannabichromene and cannabidivarin as mucoprotective agents against SN-38-evoked reductions in epithelial barrier integrity and aims to inform future investigations regarding the therapeutic use of phytocannabinoids in the context of chemotherapy-induced GIM.

2. Methods and Materials

2.1 Materials and Reagents

The phytocannabinoids used in this study include cannabigerol, cannabichromene, cannabidivarin (Cayman Chemicals, Ann Arbor, MI, USA) and cannabidiol (Australian Government National Measurement Institute, Lindfield, NSW, Australia). Thiazolyl blue tetrazolium bromide (MTT), trypan blue, Dulbecco's Modified Eagle's Medium (DMEM), DMEM without phenol red, tumour necrosis factor-α (TNF-α), interleukin 1-β (IL-1β), phosphate buffered saline (PBS), non-essential amino acids (NEAA), penicillin/streptomycin, 1xTrypsin-EDTA, 7-ethyl-10-hydroxycamptothecin (SN-38), 2',7'-dichlorofluorescin diacetate (DCFDA) and amphotericin B were also purchased from Sigma Aldrich (St Louis, MO, USA). Nunc™ polycarbonate cell culture inserts and carrier plates were purchased from ThermoFisher Scientific (Scoresby, VIC, AUS), black, clear-bottom 96-well plates from

Greiner Bio-One (Kremsmünster, AUT), dimethyl sulfoxide (DMSO) from Adelab Scientific (Thebarton, SA, AUS) and foetal bovine serum (FBS) from Fisher Biotec (Wembley, WA, AUS). The EVOM2 epithelial voltohmeter with chopstick electrode was purchased from World Precision Instruments (Sarasota, FL, USA).

2.2 Caco-2 Cell Culture

Human colonic carcinoma (Caco-2) cells obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in complete DMEM, containing 10% FBS, 1% NEAA and 1% penicillin/streptomycin and maintained at 37˚C, 5% CO_2. For cell viability measurements, Caco-2 cells were seeded in 96-well plates at a density of 2×10^4 cells per well in complete DMEM and left to equilibrate for 24 hours prior to treatment.

2.3 Cell Viability Measurements

Caco-2 cells were treated with each phytocannabinoid at varying concentrations and allowed to equilibrate for 15 minutes, prior to the addition of SN-38 (0, 1, 2 & 5µM). Test concentrations for SN-38 and each phytocannabinoid were selected based on concentration-response data collected prior to conducting these experiments (data not shown).

Following treatment, cells were incubated for 48 hours at 37˚C with 5% CO_2, at which time cell viability was measured using the thiazolyl blue tetrazolium bromide (MTT) assay. Briefly, culture media was removed and replaced with serum-free media containing 0.25mg/mL MTT and incubated for a further 2 hours at 37˚C, 5% CO_2. MTT solution was then removed and the cells lysed with DMSO, with absorbance values measured at 570nm using a Synergy MX microplate reader (Bio-Tek, Bedfordshire, UK).

2.4 Epithelial Permeability Measurements

Caco-2 cells were cultured until they reached 80% confluence, then washed in PBS, detached using trypsin-EDTA and centrifuged for 5 minutes at 150 × g before being resuspended in

complete DMEM. Experiments were conducted using 24-well plates containing Nunc™ polycarbonate cell culture inserts (0.4µm pore size, 0.47cm^2 culture area). The basolateral compartment was filled with 1mL of complete DMEM containing 1% amphotericin B. Cells were seeded in the apical compartment at a density of 5×10^4 cells per cm^2 in 300µL of culture medium; one insert contained media only for the purpose of normalising TEER values. Plates were incubated for 6 hours at 37˚C, 5% CO_2, before the apical media was replaced to remove non-adherent cells and prevent excessive clumping.(Hubatsch et al., 2007) Media was changed every 2 days for up to 25 days until the cells reached confluence and differentiated; all subsequent media changes were supplemented with 1% amphotericin B to avoid fungal contamination. Transepithelial electrical resistance (TEER) was measured using the EVOM2 epithelial volt/ohm meter with chopstick electrode (World Precision Instruments, Sarasota, FL, USA). Initial resistance values for each monolayer were normalised to the blank well using the formula: $R_{MONOLAYER}\ (\Omega) = R_{INITIAL}(\Omega) - R_{BLANK}(\Omega)$. Final TEER values were calculated using the formula: $TEER_{FINAL}(\Omega.cm2) = R_{MONOLAYER}(\Omega)\ x\ A_{CULTURE\ AREA}(cm^2)$. Before TEER measurements were taken, media was replaced with complete DMEM without amphotericin B. Only inserts with TEER values over 500Ω/cm^2 were used in experiments.

Once cells reached threshold TEER values, they were treated with either TNFα and IL-1β (each at 100ng/mL) or SN-38 (5µM). Cytokines were added to the basolateral compartment, with vehicle (0.2% DMSO in PBS) and SN-38 applied apically; TEER measurements were taken 2, 6, 24 and 48 hours post-treatment. Cells were treated apically with phytocannabinoids (1µM) 15 minutes prior to cytokine or SN-38 addition to allow for drug equilibration; each phytocannabinoid was also tested without cytokines/SN-38 to determine their intrinsic effects on epithelial permeability.

2.5 DCFDA fluorescence Assay

Caco-2 cells were passaged and prepared at a density of 1×10^6 cells per mL in PBS. Cells were then washed twice via centrifugation in PBS at 150×*g* for 5 minutes to remove phenol-red

containing media. Cells were then resuspended in 20µM DCFDA solution (20µM DCFDA in PBS) and incubated in the dark at 37˚C, 5% CO_2 for 30 minutes. Cells were then washed twice by centrifugation in PBS to remove remaining DCFDA solution. Cells were then resuspended at 1×10^6 cells/mL in complete DMEM without phenol red. Next, 100µL of cell suspension per well was added to a black, clear-bottom 96-well plate (1×10^5 cells/100µL). Cells were then treated with each phytocannabinoid (1µM) ± *t*bhp (200µM) and incubated in the dark for 4 hours at 37˚C, 5% CO_2. Fluorescence was measured using a Synergy MX microplate reader with excitation and emission filters set to 485nm and 535nm, respectively.

2.6 Statistical Analysis

Data from MTT and TEER assays were analysed by two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons determining significance versus control for cell viability and versus cytokine/SN-38 treatment for TEER data ($p<0.05$). Data from the DCFDA assay were analysed by one-way ANOVA with Tukey's post-hoc test ($p<0.05$).

3. Results

3.1. SN-38 causes a concentration-dependent reduction in Caco-2 cell viability that was enhanced by co-incubation with cannabidiol and cannabidivarin

Incubation with SN-38 (0-5µM) caused a concentration-dependent reduction in Caco-2 cell viability to a maximum of 25.78% (74.22% viability, *p<0.0001*), which was variably affected by co-incubation with the selected phytocannabinoids (**Fig. 2**). At 1µM SN-38, treatment with 10nM, 100nM and 1000nM CBD each significantly increased the cytotoxicity of SN-38 (83.85%, 80.12% and 80.77% viability, respectively, vs 88.74% with SN-38 alone) (**Fig. 2a**). A similar effect was observed at 2µM SN-38, with 84.85% viability observed for SN-38 alone and 79.24% and 80.07% following treatment with 100nM and 1000nM CBD, respectively; only

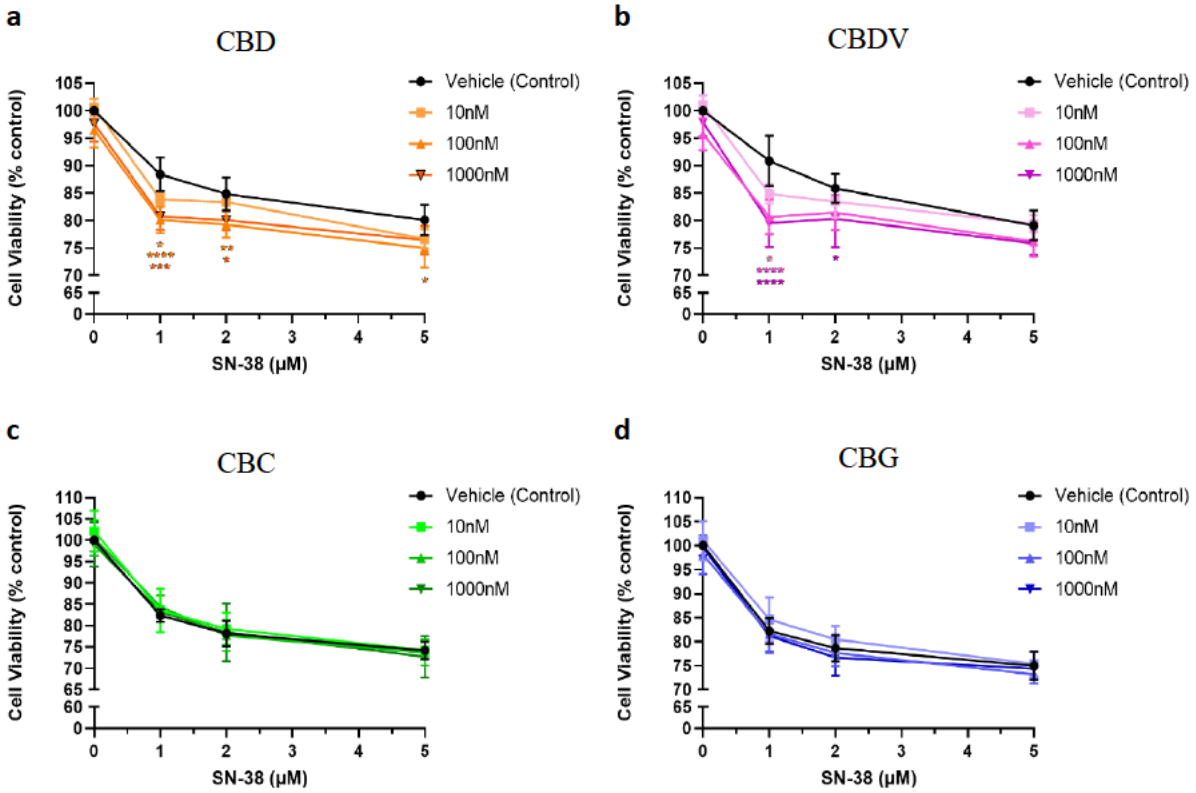

Fig 2. MTT assay of Caco-2 cell viability following 48hr incubation with SN-38 (0-5μM) alone and in the presence of selected phytocannabinoids (10-1000nM): **(a)** cannabidiol, **(b)** cannabidivarin **(c)** cannabichromene, **(d)** cannabigerol. Data presented as mean ± SD, n = 4. *p < 0.05, **p <0.01, ***p < 0.001, ****p < 0.0001 vs vehicle.

1000nM CBD showed a significant difference vs 5μM SN-38 (76.42% vs 80.12% for SN-38 alone) (**Fig. 2a**).

Treatment with each test concentration of CBDV resulted in a significant increase in the toxicity of 1μM SN-38 (90.86% viability), with 84.89%, 80.66% and 79.54% viability observed following co-incubation with 10nM, 100nM and 1000nM CBDV, respectively (**Fig. 2b**). A significant decrease in cell viability was also observed at 2μM SN-38 following co-incubation with 1000nM CBDV (80.32% viability vs 85.85% with SN-38 alone), though no significance was observed at any other concentration (**Fig. 2b**). For the CBC (**Fig. 2c**) and CBG (**Fig. 2d**) treatment groups, no significant difference was observed at any concentration of SN-38.

3.2. Cannabidiol, cannabidivarin and cannabigerol significantly inhibit SN-38-evoked increases in Caco-2 monolayer permeability

Exposure to SN-38 (5µM) over a period of 48 hours resulted in significant reductions in the TEER of Caco-2 monolayers, with the greatest reduction observed at 24 hours (83.57% initial, ****p <0.0001) (**Fig. 3**). Of the four phytocannabinoids selected for this study, cannabidiol (1µM) provided the greatest degree of protection against this decrease in TEER, with significant protection observed at 6 hours (98.88% initial vs 90.24% for SN-38 alone), 24 hours (99.19% initial vs 83.57%) and 48 hours (99.88% initial vs 86.89%) (**Fig. 3a**). A similar pattern of protection was observed following treatment with cannabidivarin (1µM), with significant protection observed at 6 hours (94.77% initial vs 90.24% for SN-38 alone), 24 hours (90.70%

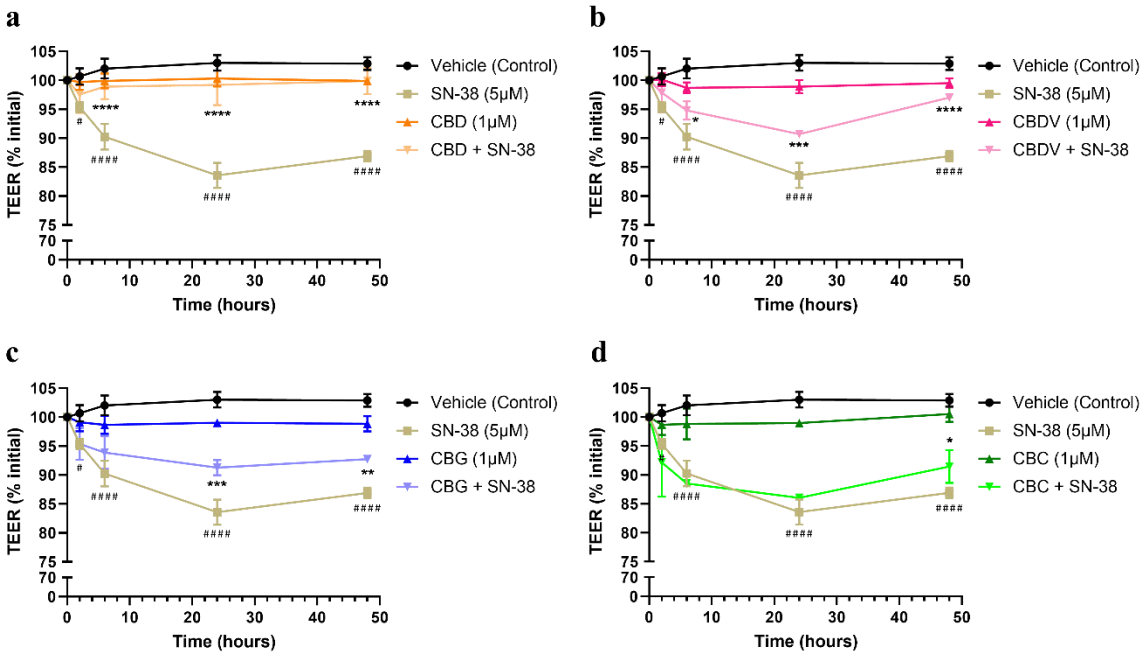

Fig 3. Caco-2 monolayer permeability as determined by TEER measurements during 48hr incubation with SN-38 (5µM) alone and in the presence of selected phytocannabinoids (1µM): **(a)** cannabidiol, **(b)** cannabidivarin **(c)** cannabigerol, **(d)** cannabichromene. Data presented as mean ± SD, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs SN-38 alone. # represents comparison to control, * represents comparison to SN-38 + pCBs.

initial vs 83.57%) and 48 hours (96.99% initial vs 86.89%) (**Fig. 3b**). A lesser degree of protection was observed following treatment with cannabigerol (1µM), with significant protection observed at 24 hours (91.26% initial vs 83.57%) and 48 hours (92.74% initial vs 86.89%) (**Fig. 3c**). Treatment with 1µM cannabichromene resulted in a modest protective effect at 48 hours (91.43% initial vs 86.89); CBC exhibited no significant effect at any other time point (**Fig. 3d**).

3.3. Phytocannabinoids exhibit potent anti-inflammatory capacity against cytokine-evoked increases in Caco-2 monolayer permeability

Incubation with the pro-inflammatory cytokines TNFα and IL-1β (each at 100ng/mL) resulted in a significant decrease in TEER over 48 hours to a maximum of 70.87% initial at 24 hours (****p <0.0001) (**Fig. 4**).

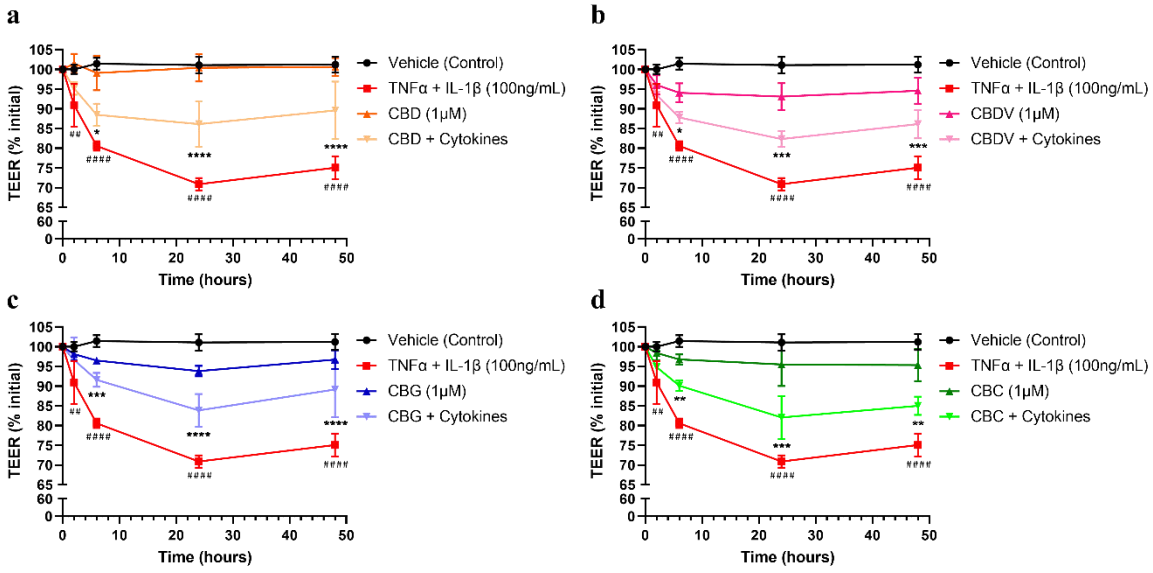

Fig 4. Caco-2 monolayer permeability as determined by TEER measurements during 48hr co-incubation with TNFα and IL-1β (each at 100ng/mL) alone and in the presence of selected phytocannabinoids (1µM): **(a)** cannabidiol, **(b)** cannabidivarin **(c)** cannabigerol, **(d)** cannabichromene. Data presented as mean ± SD, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs cytokines alone. # represents comparison to control, * represents comparison to treatment group.

Cannabidiol (1µM) offered significant protection against this decrease in TEER at 6 hours (88.48% initial, vs 80.58% for the cytokines alone), 24hrs (86.13% initial vs 70.87%) and 48hrs (89.60% initial vs 75.08%) (**Fig. 4a**). In line with the SN-38 results, a similar pattern of protection was again observed following treatment with cannabidivarin (1µM), with significance at 6 hours (87.81% initial vs 80.58%), 24 hours (82.34% initial vs 70.87%) and 48 hours (86.10% initial vs 75.08%). Cannabigerol provided the most consistent protection across the 48-hour incubation period, with significance observed at 6hrs (91.57% initial vs 80.58%), 24hrs (83.837% initial vs 70.87%) and 48hrs (89.16% initial vs 75.08%) (**Fig. 4c**). Finally, in contrast with the SN-38 results, cannabichromene (1µM) proved significantly protective, exhibiting statistical significance at 6 hours (90.10% initial vs 80.58%), 24 hours (82.05% initial vs 70.87%) and 48 hours (85% initial vs 75.08%) **(Fig. 4d)**.

3.4 Phytocannabinoids have no significant effect on ROS generation

Treatment with *t*bhp for a period of 4 hours resulted in a 477% increase in DCFDA fluorescence versus control (****p <0.0001) (**Fig. 5**). No significant reduction in ROS generation was observed following treatment with either of the pCBs screened in this study, although significant variability was observed in the CBDV treatment group. No significant increase in ROS generation was observed in Caco-2 cells following treatment with SN-38, TNFα or IL-1β either alone or in combination (data not shown).

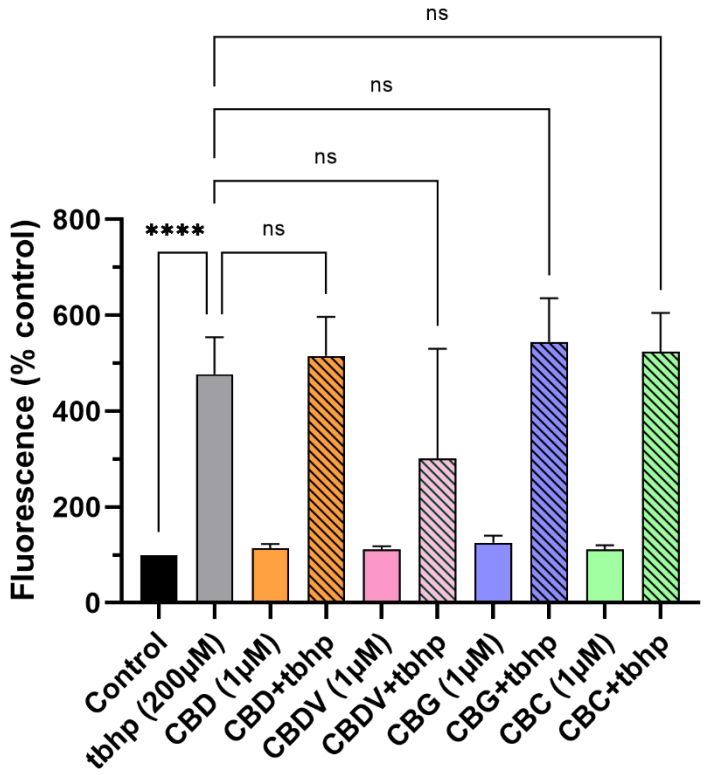

Fig 5. ROS generation in Caco-2 cells as determined by DCFDA fluorescence following 4hr incubation with *t*bhp (200µM) alone and in the presence of selected phytocannabinoids (1µM). Data presented as mean ± SD, n = 4. ****p <0.0001, ns = not significant vs *t*bhp.

4. Discussion

The anti-inflammatory capacity of phytocannabinoids has been well established in models of IBD, providing protection via numerous mechanisms (Esposito et al., 2013, Ahmed and Katz, 2016, Couch et al., 2018). A similar effect was observed in this study, whereby each phytocannabinoid maintained barrier integrity following treatment with the pro-inflammatory cytokines TNFα and IL-1β. However, the efficacy of these pCBs as anti-inflammatory and mucoprotective agents in the context of irinotecan-induced GIM has not yet been investigated. In the present study, we identified novel roles for CBD, CBDV, and CBG as inhibitors of SN-38-evoked increases in epithelial barrier permeability and confirmed the established anti-inflammatory efficacy of each test pCB. To our knowledge, this study presents the first

characterisation of CBG, CBC and CBDV as protective agents in the context of SN-38-evoked changes to epithelial barrier function.

Both CBD and CBDV significantly increased the toxicity of low micromolar concentrations of SN-38 in Caco-2 cells. This is in line with a previous study by Alsherbiny and colleagues which identified an ability for CBD to synergistically enhance the cytotoxicity of SN-38 in MCF-7 cells (Alsherbiny et al., 2021). Interestingly, the ability for CBD to enhance the cytotoxicity of anti-cancer drugs has also been noted in a model of human head and neck squamous cell carcinoma (Go et al., 2020). CBD has also demonstrated intrinsic anti-proliferative and pro-apoptotic effects, along with the ability to dose-dependently reduce viability of cancer cells but not normal human colorectal cells (Seltzer et al., 2020, Jeong et al., 2019). The cytotoxicity of CBDV in colorectal cell lines has not been studied extensively. A study from Russo and colleagues found that CBDV is less cytotoxic than CBD in a range of cell lines, including Caco-2 cells (Russo et al., 2021). This is supported by another study by Borelli et al, which showed IC_{50} values of approximately 4µM and 10µM in Caco-2 cells for CBD and CBDV, respectively (Borrelli et al., 2014). However, with the exception of the present study, there is currently no data investigating the effect of CBDV on the cytotoxicity of common chemotherapeutic drugs. Interestingly, none of the pCBs screened in this study had any significant effect on ROS generation, although previous studies have suggested that both CBD and CBDV possess a degree of antioxidant capacity (Russo et al., 2021). The lack of efficacy noted in this study likely relates to variations in drug concentrations and the different assays used in each case. However, it is clear that the inhibition of ROS generation is unlikely to have played any significant role in the effects observed in this study.

Each of the phytocannabinoids screened in this study significantly inhibited cytokine-evoked increases in epithelial permeability. This was unsurprising, as the ability for phytocannabinoids to protect against the deleterious effects of colitis-associated inflammation has been well characterised (Couch et al., 2018, Naftali, 2020). Although the majority of previous works have

focused on the efficacy of CBD and Δ^9-THC, several studies have highlighted a promising anti-inflammatory role for CBG in models of colitis (Borrelli et al., 2013, Pagano et al., 2021). Moreover, CBDV has been ascribed anti-inflammatory properties with the ability to reduce pro-inflammatory cytokine expression and overall inflammation in models of ulcerative colitis, while CBC has been shown to ameliorate murine colitis, with an ability to inhibit nitric oxide production (Pagano et al., 2019, Romano et al., 2013). Interestingly, CBG proved to be the most efficacious inhibitor of cytokine-evoked increase in epithelial permeability in the present study, further establishing its therapeutic potential in the context of GI inflammation. The results of this study emphasise the anti-inflammatory properties ascribed to these pCBs, highlighting their ability to maintain epithelial barrier function in the presence of pro-inflammatory cytokines. However, despite their established roles as anti-inflammatory agents in GI disease models, these pCBs had not yet been investigated for their potential to ameliorate the epithelial damage and consequent increase in membrane permeability associated with irinotecan-induced GIM. We therefore aimed to characterise the ability for these pCBs to maintain barrier integrity in the presence of the irinotecan metabolite SN-38.

Of the pCBs screened, all but CBC were able to maintain the integrity of Caco-2 monolayers in the presence of SN-38; CBDV proved highly effective in this regard. This effect may be related to the known activity of CBDV at transient receptor potential (TRP) channels, particularly TRPA1 (De Petrocellis et al., 2011). Interestingly, Alvarenga et al showed that carvacrol is able to reduce irinotecan-induced GIM via TRPA1 activation (Alvarenga et al., 2016). Moreover, it has been shown that TRPA1 is upregulated in response to intestinal inflammation and its activation exerts a protective effect by reducing the expression of pro-inflammatory cytokines (Kun et al., 2014). These findings indicate a potential role for TRPA1 in the observed effect of CBDV in the present study. CBD proved to be the most effective inhibitor of SN-38-evoked increase in epithelial permeability. This was somewhat predictable, given the relative abundance of studies highlighting the protective effect of CBD in models of

GI inflammation (Martínez et al., 2020, Esposito et al., 2013). The anti-inflammatory effect of CBD has been linked to a variety of mechanisms, including reductions in the expression of inducible nitric oxide synthase, inhibition of ROS generation and activation of receptors such as peroxisome proliferator-activated receptor gamma (PPAR-γ) (Cocetta et al., 2021, De Filippis et al., 2011). The results of the present study do not support the inhibition of ROS production as a causative factor in maintaining barrier integrity, given the lack of direct antioxidant capacity observed for each of the pCBs in this study. However, the potential involvement of PPAR-γ is of considerable interest in this context, given that PPAR-γ agonism has been shown to strengthen intestinal barrier function in Caco-2 cells, with its inhibition resulting in impaired barrier function (Zong et al., 2019). Furthermore, PPAR-γ is widely expressed in colonic epithelial cells and is an important mediator of intestinal inflammation, making it an attractive target in the treatment of GI inflammation (Annese et al., 2012, Decara et al., 2020). Several pCBs including CBD and CBG have demonstrated activity as PPAR-γ agonists, with CBG possessing the greatest potency at this receptor (O'Sullivan et al., 2009, Granja et al., 2012, O'Sullivan, 2016). It is also interesting to note that treatment with irinotecan has been shown to downregulate the expression of PPAR-γ in Caco-2 cells (Boeing et al., 2022). The authors noted that the flavonoid luteolin was able to inhibit irinotecan-evoked inflammation and oxidative stress via a PPAR-γ-dependent mechanism, highlighting a potential role for this receptor as a therapeutic target in irinotecan-induced GIM. In this light, further investigation is required in order to establish a potential role for PPAR-γ activation in mediating the ability for pCBs such as CBD and CBG to protect against SN-38-evoked reductions in epithelial barrier integrity.

5. Conclusions

To our knowledge, this study presents the first characterisation of CBD, CBDV, CBC and CBG in the context of irinotecan-induced GIM. We identified novel protective roles for CBD, CBDV and CBG as inhibitors of SN-38-evoked increases in epithelial permeability, highlighting the

exciting therapeutic potential for these phytocannabinoids in this context. Our results support the rationale for further investigations into the use of phytocannabinoids as potential adjunct therapeutics in the management of irinotecan-associated intestinal inflammation. These findings serve to further establish the therapeutic potential of phytocannabinoids in the treatment of chemotherapy-induced GIM and GI inflammation more generally.

Statement of Authorship

Title of Paper	The semi-synthetic flavone 2',3',4'-trihydroxyflavone (2-D08) inhibits both SN-38- and cytokine-evoked increases in epithelial barrier permeability in vitro
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript submitted to Food and Function.

Principal Author

Name of Principal Author (Candidate)	Dylan Thomas Marsh		
Contribution to the Paper	Conceptualisation, Methodology, Validation, Formal analysis, Data curation, Investigation, Writing - original draft, Writing - review & editing, Visualisation		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	31/07/22

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Scott Smid		
Contribution to the Paper	Conceptualisation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition		
Signature		Date	August 1, 2022

Chapter 6: The semi-synthetic flavone 2',3',4'-trihydroxyflavone (2-D08) inhibits both SN-38- and cytokine-evoked increases in epithelial barrier permeability *in vitro*

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Abstract

The chemotherapeutic drug irinotecan and its active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) have been linked to the development of off-target gastrointestinal toxicity and inflammation, termed gastrointestinal mucositis (GIM). Flavonoids possess antioxidant and anti-inflammatory effects in models of gastrointestinal inflammation; however, few studies have investigated their potential in ameliorating chemotherapy-induced GIM. Here, we characterised the intestinal epithelial barrier-protective and antioxidant capacity of the novel flavonoids 2',3',4'-trihydroxyflavone (2-D08) and transilitin in comparison with flavones myricetin and quercetin in vitro via viability and permeability assessments in Caco-2 epithelial monolayers exposed to SN-38. Transilitin, 2-D08 and myricetin maintained barrier function in the presence of SN-38, with 2-D08 proving most effective. 2-D08 was the most effective inhibitor of cytokine-evoked increases in epithelial permeability, with myricetin providing modest protection; quercetin afforded no significant protection against either SN-38 or cytokine-evoked reductions in barrier integrity. Each flavonoid significantly reduced *tert*-butyl hydroperoxide (*t*bhp)-induced reactive oxygen species (ROS) generation, although 2-D08 was comparatively less effective. These results highlight a novel role for 2-D08 as an inhibitor of both SN-38 and cytokine-evoked increases in epithelial permeability, with lesser barrier protective roles ascribed to transilitin and myricetin and not correlated with antioxidant capacity. Such novel flavonoids as 2-D08 may have active or adjunctive roles in ameliorating chemotherapy and inflammatory-evoked changes in intestinal barrier function.

1. Introduction

In recent years a great deal of light has been shed upon the phenomenon of off-target gastrointestinal toxicity and inflammation, commonly referred to as gastrointestinal mucositis (GIM), following treatment with conventional chemotherapeutic agents (Sougiannis et al., 2021, Dahlgren et al., 2021, Bowen et al., 2019). The symptoms of GIM include severe diarrhoea, rectal bleeding and infection, often resulting in dose reductions and delays in treatment negatively affecting clinical outcomes (Lalla et al., 2014). There are a number of chemotherapeutic agents associated with the development of GIM, with perhaps the most common being irinotecan, a drug used in the treatment of a variety of cancers including colorectal, ovarian and pulmonary (Gibson et al., 2003, Kciuk et al., 2020). Cases of clinically significant GIM symptoms following treatment with irinotecan are common, with diarrhoea reported in 60-87% of patients in Phase I and II trials, up to 40% of which were considered severe (Hecht, 1998). Irinotecan is a prodrug which is converted to its active metabolite, 7-

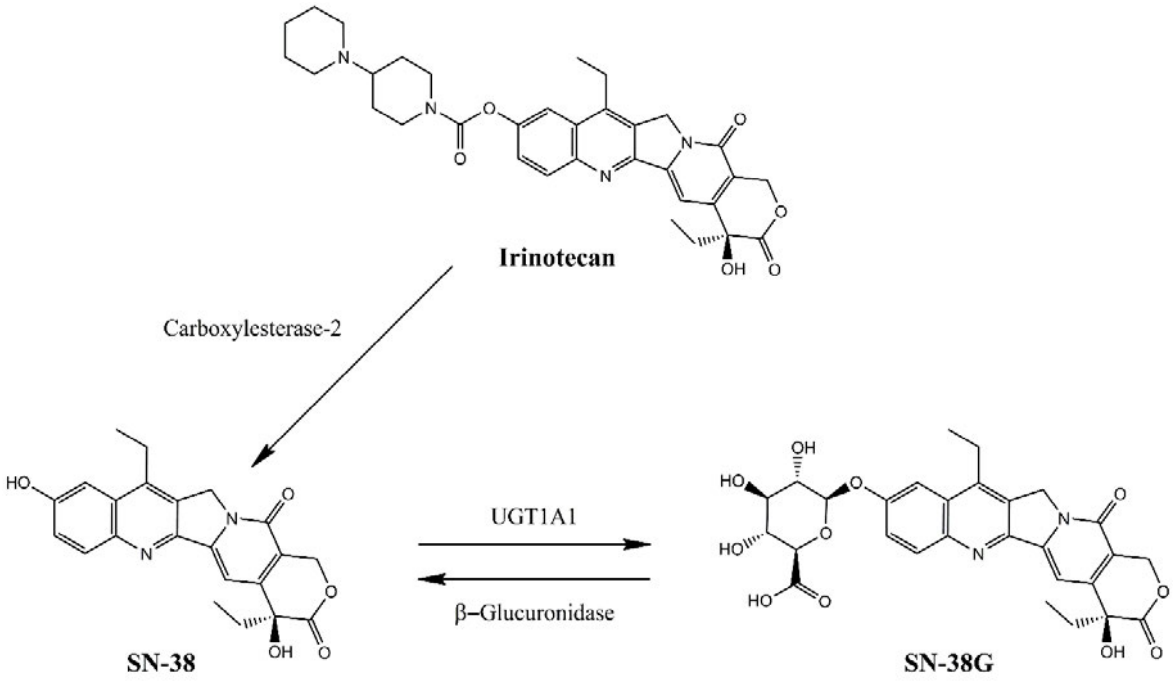


Fig.1. Metabolism of irinotecan. Irinotecan is converted to its active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) via carboxylesterases. SN-38 then undergoes glucuronidation by UGT1A1 to form the inactive metabolite SN-38G, which may be converted back to the active SN-38 by bacterial β-glucuronidase.

ethyl-10-hydroxycamptothecin (SN-38), by carboxylesterases 1 and 2, before being inactivated via glucuronidation by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) to SN-38G (see **Fig. 1**) (Alfirevic and Pirmohamed, 2016). However, bacterial β-glucuronidases may reactivate SN-38G to the active SN-38, which is then deposited into the intestinal lumen (Bailly, 2019, Wallace et al., 2010). Intestinal exposure to SN-38 has been identified as a causative factor in the onset of diarrhoea following treatment with irinotecan (Sun et al., 2020). Despite its associated toxicity, irinotecan is an effective chemotherapeutic agent and remains a common treatment for a variety of solid tumours, highlighting the need for ways to minimise the development and secondary impacts of irinotecan-induced GIM (Pizzolato and Saltz, 2003, Fujita et al., 2015).

Treatment with irinotecan results in increased serum concentrations and overall expression of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα), and these pro-inflammatory cytokines have been linked to significant increases in intestinal barrier permeability both *in vitro* and *in vivo* (Al-Sadi et al., 2009, Chelakkot et al., 2018, M Logan et al., 2008, Arifa et al., 2014). Furthermore, TNFα and IL-1β have been directly implicated in the pathogenesis of irinotecan-induced GIM, highlighting their importance as potential therapeutic targets (Melo et al., 2008). Moreover, co-administration of thalidomide, an inhibitor of TNFα, significantly reduced the toxicity of irinotecan, further emphasizing a potential role for this cytokine in the pathogenesis of irinotecan-induced GIM (Yang et al., 2006). Interestingly, oxidative stress and the generation of reaction oxygen species (ROS) have been linked to the increased expression of pro-inflammatory cytokines and may play a role in the pathogenesis of GIM, with various antioxidant therapies showing promise as mucoprotective agents (Arifa et al., 2016, Al-Asmari et al., 2015, Ashcraft et al., 2015). It is proposed that upstream ROS generation may activate NF-κB, which subsequently increases the transcriptional activity of an array of genes, including those coding for IL-1β and TNFα, resulting in the increased expression of these proinflammatory cytokines (Sonis, 2004,

Lingappan, 2018). The upregulation of TNFα may then activate caspase pathways, generating a feedback loop on NF-κB and thus exacerbating inflammation (Cinausero et al., 2017). It therefore follows that compounds with the ability to prevent ROS generation and reduce inflammation may prove effective in the management of GIM.

Many natural products have been investigated for their therapeutic potential in the amelioration of chemotherapy-induced mucositis, ranging from grape-seed extracts and traditional Chinese medicines to marine polysaccharides from the sea cucumber (Cheah et al., 2014, Zuo et al., 2015, Shi et al., 2016). Dietary polyphenols such as flavonoids have also been the focus of several studies in this area and have shown promise as lead candidates in the management of mucositis-associated inflammation and the prevention of oxidative stress (Fideles et al., 2020, Lotfi et al., 2021, Shin et al., 2013). Furthermore, flavonoids have also proven to be particularly effective in maintaining barrier function in the gut, while also preventing cytokine-evoked

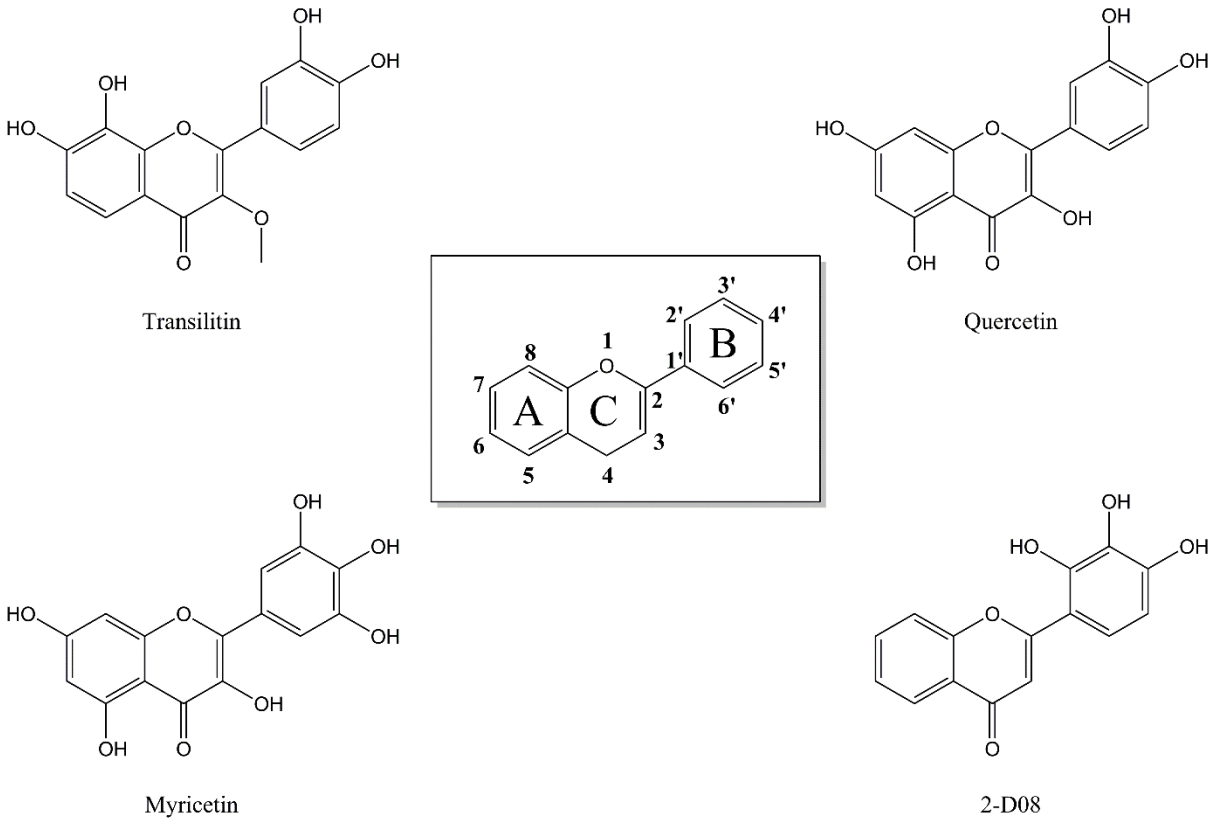


Fig.2. Structures of the flavonoids transilitin, quercetin, myricetin and 2',3',4'-trihydroxyflavone (2-D08), along with the flavonoid backbone structure.

increases in barrier permeability (Luescher et al., 2017, Bernardi et al., 2020). However, while the current literature surrounding flavonoids as mucoprotective agents in GIM has been quite promising, many studies have been focused on a rather limited set of molecules, highlighting the need for further exploration and the development of novel lead candidates.

In the present study we aimed to characterise the *in vitro* barrier protective capacity of the novel flavonoids transilitin and 2-D08 in comparison with the more common dietary flavonoids quercetin and myricetin (**Fig. 2**). We assessed the effect that co-incubation of these flavonoids had on the cytotoxicity of SN-38, their ability to protect against both SN-38 and cytokine-evoked changes in epithelial permeability and their capacity to prevent ROS generation *in vitro*. This study presents the first characterisation of both transilitin and 2-D08 as barrier-protective agents and aims to further develop our understanding of flavonoids as possible adjunct nutraceutical therapies in the management of chemotherapy-induced GIM and gastrointestinal inflammation more generally.

2. Methods and Materials

2.1 Materials and Reagents

The flavonoids used in this study include quercetin, myricetin and 2-D08 (Sigma-Aldrich, St Louis, MO, USA); Dr. Peter Duggan at CSIRO Materials Division (Clayton South, VIC, AUS) kindly provided the transilitin. Thiazolyl blue tetrazolium bromide (MTT), trypan blue, TNFα, IL-1β, Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), non-essential amino acids (NEAA), penicillin/streptomycin, 1×Trypsin-EDTA, 7-ethyl-10-hydroxycamptothecin (SN-38), *tert*-butyl hydroperoxide (*t*bhp), 2',7'-dichlorofluorescin diacetate (DCFDA) and amphotericin B were also purchased from Sigma Aldrich (St Louis, MO, USA). Nunc™ polycarbonate cell culture inserts (Cat. #140620) and carrier plates (Cat. #141002) were purchased from ThermoFisher Scientific (Scoresby, VIC, AUS), dimethyl sulfoxide (DMSO) from Adelab Scientific (Thebarton, SA, AUS) and foetal bovine serum

(FBS) from Fisher Biotec (Wembley, WA, AUS). The EVOM2 epithelial voltohmeter with chopstick electrode was purchased from World Precision Instruments (Sarasota, FL, USA). All drugs used in this study were dissolved in DSMO and diluted to desired concentrations using PBS.

2.2 Caco-2 Cell Culture

Human colonic carcinoma (Caco-2) cells obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in complete DMEM, containing 10% FBS, 1% NEAA and 1% penicillin/streptomycin and maintained at 37˚C, 5% CO_2. For cell viability measurements, Caco-2 cells were seeded in 96-well plates at a density of 2×10^4 cells per well in complete DMEM and left to equilibrate for a period of 24 hours prior to treatment. For the DCFDA assay, cells were seeded at 1×10^5 per well.

2.3 Cell Viability Measurements

Caco-2 cells were treated with each flavonoid at varying concentrations and allowed to equilibrate for 15 minutes, prior to the addition of SN-38 (0.1, 1, 5 & 10 µM); treatment for each concentration was performed in triplicate wells. Test concentrations of SN-38 and each flavonoid were selected based on concentration-response data collected prior to conducting these and all subsequent experiments (**Appendix 1)**. Following treatment, cells were incubated for 48 hours at 37˚C with 5% CO_2, after which time cell viability was measured using the thiazolyl blue tetrazolium bromide (MTT) assay. Briefly, culture media was removed and replaced with serum-free media containing 0.25mg/mL MTT and incubated for a further 2 hours at 37˚C, 5% CO_2. MTT solution was then removed and the cells lysed with DMSO, with absorbance values measured at 570nm using a Synergy MX microplate reader (Bio-Tek, Bedfordshire, UK).

2.4 Epithelial Permeability Measurements

Caco-2 cells were cultured until they reached 80% confluence, at which point they were washed in PBS and detached using trypsin-EDTA, before being centrifuged for 5 minutes at 150×*g* and resuspended in complete DMEM. Experiments were conducted using 24-well plates containing Nunc™ polycarbonate cell culture inserts (0.4 µm pore size, 0.47 cm^2 culture area). The basolateral compartment was filled with 1 mL of complete DMEM containing 1% amphotericin B. Cells were seeded in the apical compartment at a density of $5x10^4$ cells per cm^2 (23,500 cells per insert) in 300 µL of culture medium, with one insert left blank and containing medium only. Plates were then incubated for 6 hours at 37˚C, 5% CO_2, at which time the apical media was replaced in order to remove non-adherent cells and prevent excessive clumping (Hubatsch et al., 2007). Both apical and basolateral media was changed every 2 days for up to 25 days until the cells reached confluence and differentiated; all subsequent media changes were supplemented with 1% amphotericin B to avoid fungal contamination. During this growth period, transepithelial electrical resistance (TEER) was measured using the EVOM2 epithelial voltohmmeter to determine monolayer integrity. Initial resistance values for each monolayer were normalised to the blank well using the formula: $R_{MONOLAYER}\ (\Omega) = R_{INITIAL}(\Omega) - R_{BLANK}(\Omega)$. TEER values were then calculated using the formula: $TEER_{FINAL}(\Omega.cm2) = R_{MONOLAYER}(\Omega)\ x\ A_{CULTURE\ AREA}(cm^2)$. Before TEER measurements were taken, media was replaced with complete DMEM without amphotericin B. Only inserts with TEER values over 500 Ω/cm^2 were used in subsequent experiments.

Once cells reached threshold TEER values, they were treated with either the proinflammatory cytokines TNFα and IL-1β (each at 100 ng/mL) or SN-38 (5 µM) (collectively, 'stressors'); concentrations were selected based on preliminary experiments conducted prior to this study (data not shown) and previous work from our laboratory (Harvey et al., 2013). All cytokine treatments were added to the basolateral compartment, with vehicle (0.2% DMSO in PBS) and SN-38 being applied apically; TEER measurements were subsequently taken 2, 6, 24 and 48 hours after treatment. For flavonoid treatments, cells were treated apically with either transilitin

(20 µM), quercetin (10 µM), myricetin (100 µM) or 2-D08 (20 µM) 15 minutes prior to cytokine or SN-38 addition to allow for drug equilibration; the highest non-toxic concentration of each flavonoid was used as determined by preliminary cell viability studies (**Appendix 1**). Each flavonoid was also tested without stressors to determine their individual effects on epithelial permeability.

2.5 ROS Measurement Using DCFDA Assay

Caco-2 cells were cultured under normal conditions (as above), passaged and prepared at a density of 1×10^6 cells per mL in PBS. Cells were then washed twice via centrifugation in PBS at $150\times g$ for 5 minutes to ensure the removal of all phenol-red containing media. Cells were then resuspended in 20 µM DCFDA solution (20 µM DCFDA in PBS) and incubated in the dark at 37˚C, 5% CO_2 for 30 minutes. Following incubation, cells were washed twice by centrifugation in PBS to ensure the removal of any remaining DCFDA solution. After washing, cells were resuspended at 1×10^6 cells/mL in complete DMEM without phenol red. Next, 100 µL of cell suspension per well was added to a black, clear-bottom 96-well plate (1×10^5 cells/100 µL). Cells were then treated with myricetin (100 µM), 2-D08 (20 µM), transilitin (20 µM) or quercetin (10 µM) ± *t*bhp (200 µM) and incubated in the dark for a further 4 hours at 37˚C, 5% CO_2. Fluorescence measurements were then taken using a Synergy MX microplate reader with excitation and emission filters set to 485 nm and 535 nm, respectively.

2.6 Statistical Analysis

Data obtained from MTT and TEER assays were analysed by two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons used to determine significance versus control for cell viability and versus cytokine/SN-38 treatment for TEER data ($p<0.05$). Data from the DCFDA assay was analysed by one-way ANOVA with Tukey's post-hoc test used to determine significance ($p<0.05$). GraphPad Prism 8.0 (GraphPad Software, San Diego, USA) was used for data analysis and the production of all graphs.

3. Results

3.1. SN-38 causes a concentration-dependent reduction in Caco-2 cell viability that is variably affected by co-incubation with flavonoids

Incubation with the irinotecan metabolite SN-38 (0-10 µM) caused a concentration-dependent reduction in Caco-2 cell viability to a maximum of 41.2% (58.8% viability, ****p <0.0001), which was variably affected by co-incubation with the selected flavonoids (**Fig. 3**). Overall, each flavonoid was relatively innocuous across the full SN-38 concentration range, with enhanced toxicity observed only at lower concentrations of SN-38. At 0.1 µM SN-38, treatment with both 1 µM and 10 µM transilitin resulted in a significant increase in toxicity compared to

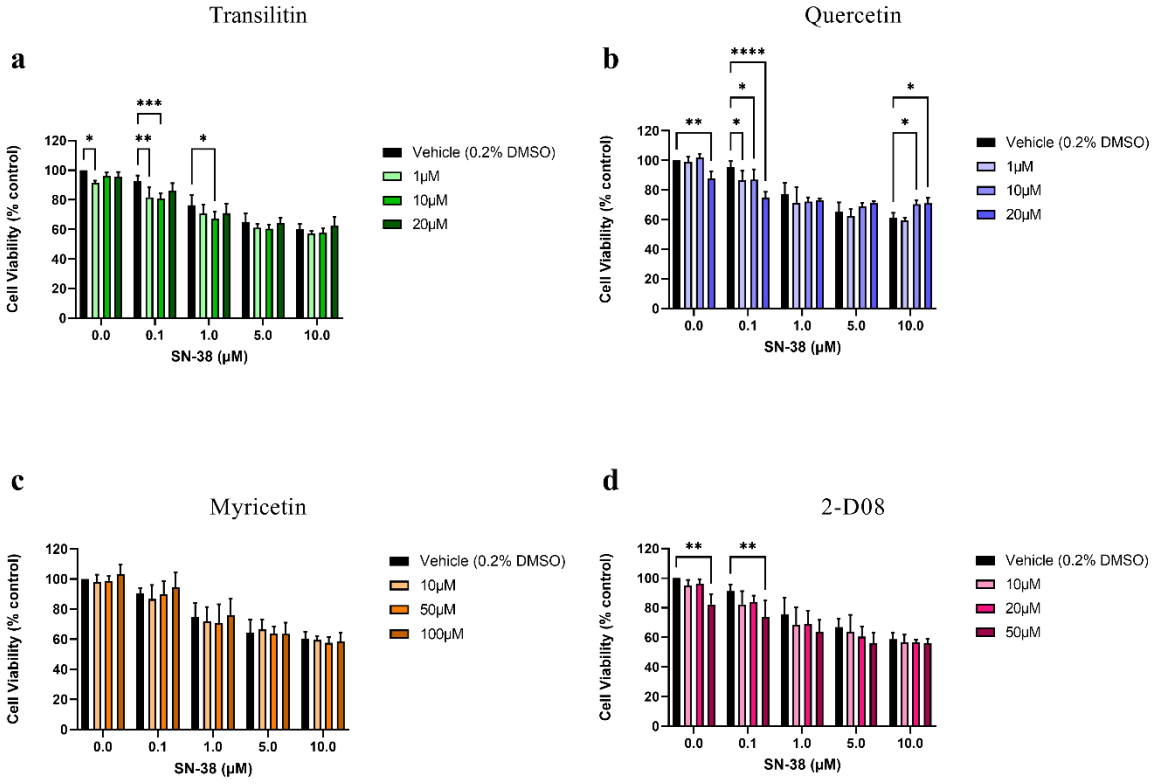


Fig 3. MTT assay of Caco-2 cell viability following 48hr incubation with SN-38 (0-10µM) alone and in the presence of varying concentrations of flavonoids: **(a)** transilitin (1-20 µM), **(b)** quercetin (1-20 µM), **(c)** myricetin (10-100 µM), and **(d)** 2-D08 (10-50 µM). Data presented as mean ± SD, n = 4 independent experiments. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 vs vehicle.

SN-38 alone (81.7% and 80.8% viability, respectively, vs 92.6% with SN-38 alone) (**Fig. 3a**). A similar effect was observed at 1 µM SN-38, with 76.4% viability observed for SN-38 alone and 67.3% with 10 µM transilitin; no significant differences were observed at either 5 µM or 10 µM SN-38 (**Fig. 3a**).

Treatment with each test concentration of quercetin resulted in a significant increase in the toxicity of 0.1 µM SN-38 (95.3% viability), with 86.8%, 87% and 75% viability observed for 1 µM, 10 µM and 20 µM quercetin, respectively (**Fig. 3b**). However, this trend was reversed at 10 µM SN-38, with significantly greater cell viability observed at both 10 µM and 20 µM quercetin (70.4% and 71.1% viability, respectively) vs control (61% viability) (**Fig. 3b**). Treatment with myricetin caused no significant changes to the toxicity of SN-38 at any of the test concentrations (**Fig. 3c**). Treatment with 50 µM 2-D08 resulted in a significant increase in the toxicity of 0.1 µM SN-38 (73.8% viability vs 91.2%), although significant toxicity was also observed at this concentration in the absence of SN-38 (82.4% viability vs control); no significant differences were observed at other test concentrations (**Fig. 3d**).

3.2 Transilitin, 2-D08 and Myricetin significantly inhibit SN-38-evoked increases in Caco-2 monolayer permeability

Caco-2 monolayers exposed to SN-38 (5 µM) over a period of 48 hours exhibited significant reductions in TEER, with the greatest reduction observed at 24 hours (86.2% initial, $p<0.0001$) (**Fig. 4**); treatment with flavonoids alone had no significant effect on TEER at test concentrations. Transilitin (20 µM) significantly prevented this decrease in TEER at 6 hours (97.5% initial vs 90.4% for SN-38 alone), 24 hours (95.5% initial vs 86.2%) and 48 hours (96.4% initial vs 86.4%) (**Fig. 4a**). Treatment with 10 µM quercetin provided no significant protection against SN-38 at any time point (**Fig. 4b**). Myricetin (100 µM) significantly inhibited the SN-38-evoked reduction in TEER at both 24 and 48 hours (92% initial vs 86.2% and 93.8% initial vs 86.4%, respectively) (**Fig. 4c**). The greatest overall protection was observed in cells treated with 2-D08 (20 µM), with significant protection observed at 2 hours (99.3% initial vs

93.5%), 6 hours (98% initial vs 90.4%), 24 hours (98.3% initial vs 86.2%) and 48 hours (99.9% initial vs 86.4%) (**Fig. 4d**).

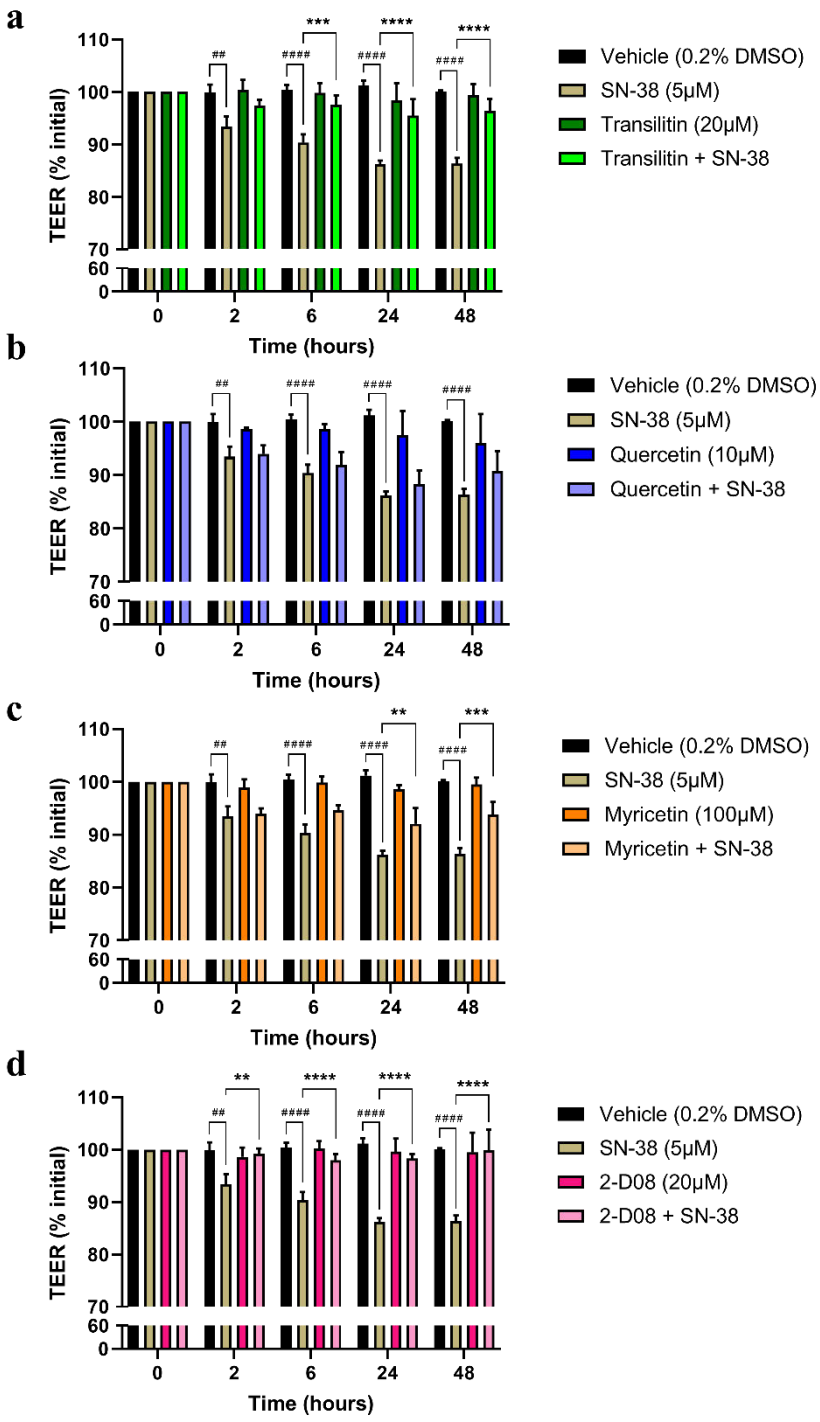


Fig 4. Caco-2 monolayer permeability as determined by TEER measurements during 48hr incubation with SN-38 (5 μM) alone and in the presence of flavonoids: **(a)** transilitin (20 μM), **(b)** quercetin (10 μM) **(c)** myricetin (100 μM), **(d)** 2-D08 (20 μM). Data presented as mean ± SD, n = 3 independent experiments. **p <0.01, ***p <0.001, ****p <0.0001 vs SN-38 alone. # represents comparison to Vehicle control, * represents comparison to SN-38.

3.3 The flavonoids 2-D08 and Myricetin significantly inhibit cytokine-evoked increases in Caco-2 monolayer permeability

Treatment with the flavonoids alone had no effect on TEER at test concentrations. Incubation with the pro-inflammatory cytokines TNFα and IL-1β resulted in a significant decrease in TEER over 48 hours to a maximum of 69.7% initial (****p <0.0001) (**Fig. 5**). Neither transilitin (20 μM) nor quercetin (10 μM) significantly prevented the cytokine-evoked reductions in TEER at any point over 48 hours (**Figs. 5a and 5b**). Myricetin (100 μM) was able to significantly increase TEER vs cytokines at both 24hrs (91.9% initial vs 77.9%) and 48hrs (82.6% initial vs 69.7%) (**Fig. 5c**). In line with SN-38 results, 2-D08 again provided the greatest overall protection against cytokine-evoked reductions in TEER, with significance observed at 6hrs (106.7% initial vs 94.4%), 24hrs (103.5% initial vs 77.9%) and 48hrs (96.9% initial vs 69.7%) (**Fig. 5d**).

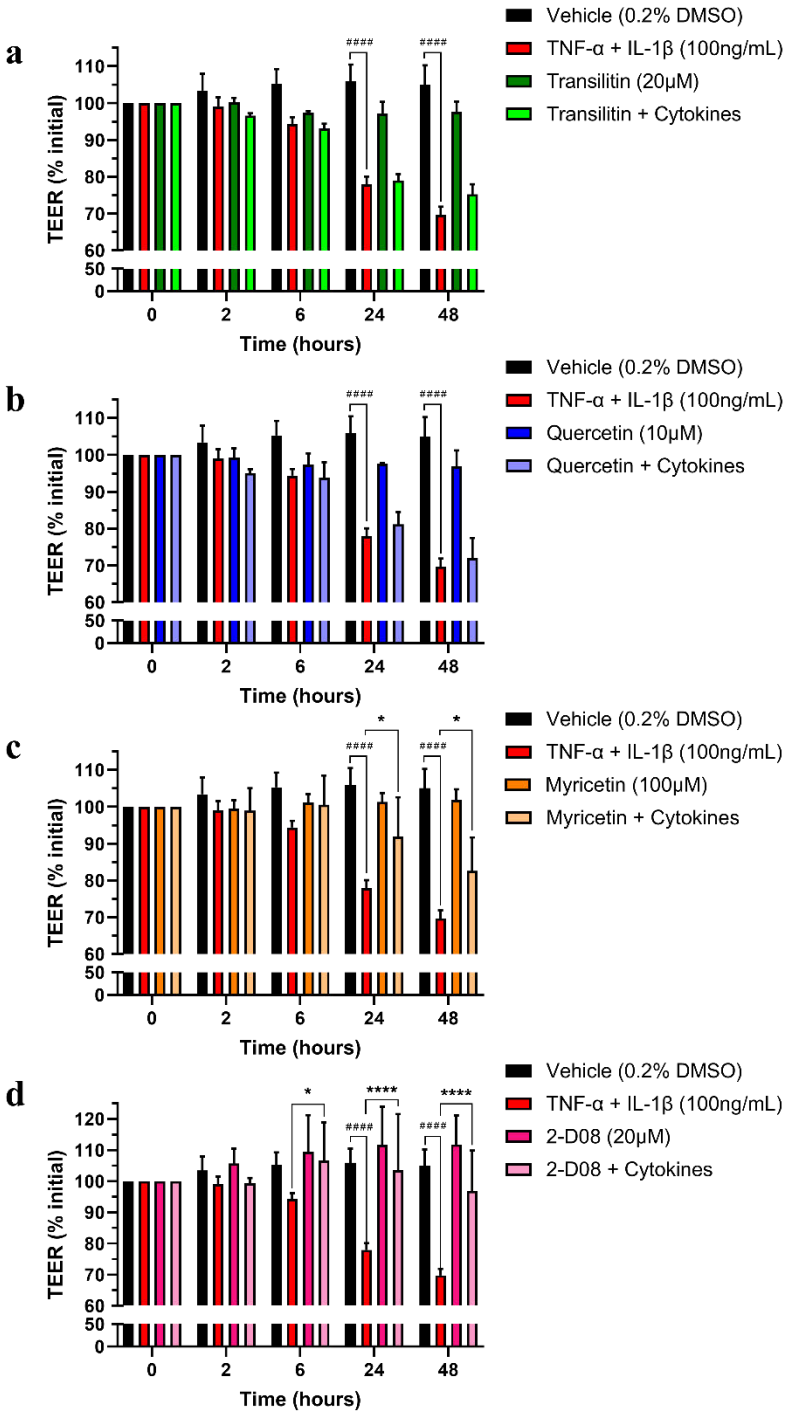


Fig 5. Caco-2 monolayer permeability as determined by TEER measurements during 48hr co-incubation with TNFα and IL-1β (each at 100 ng/mL) alone and in the presence of flavonoids: **(a)** transilitin (20 μM), **(b)** quercetin (10 μM) **(c)** myricetin (100 μM), **(d)** 2-D08 (20 μM). Data presented as mean ± SD, n = 3 independent experiments. *p <0.05, ***p <0.001, ****p <0.0001 vs cytokines. # represents comparison to Vehicle control, * represents comparison to cytokine group.

3.4 Flavonoids significantly inhibit *t*bhp-evoked increases in ROS generation

Treatment with the pro-oxidant *t*bhp caused a 466.3% increase in DCFDA fluorescence versus control (****p <0.0001) (**Fig. 6**). Each of the flavonoids used in this study significantly inhibited this increase in ROS generation, with no significant differences observed between each flavonoid alone and control. Transilitin reduced DCFDA fluorescence to 194.6% versus control (****p <0.0001 vs *t*bhp alone), with 100 µM myricetin and 10 µM quercetin limiting ROS-generated fluorescence to 228.1% (****p <0.0001 vs *t*bhp alone) and 238.3% (****p

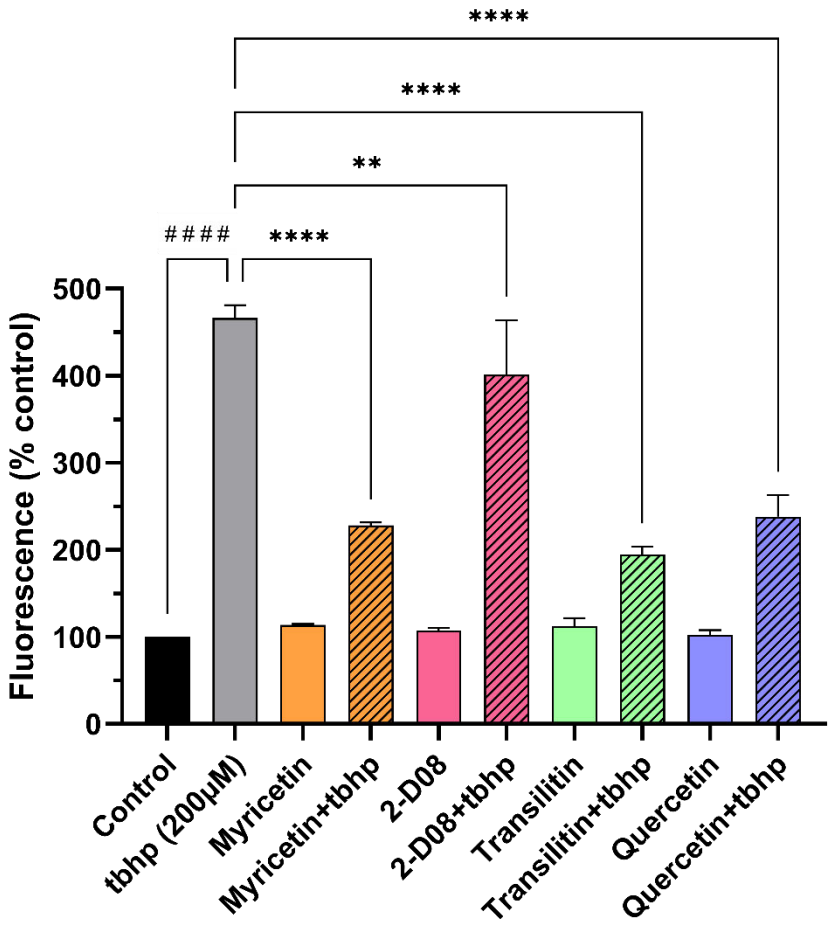


Fig 6. ROS generation in Caco-2 cells as determined by DCFDA fluorescence following 4hr incubation with *t*bhp (200 µM) alone and in the presence of flavonoids: myricetin (100 µM), 2-D08 (20 µM), transilitin (20 µM) and quercetin (10 µM). Data presented as mean ± SD, n = 4 independent experiments. **p <0.01, ****p <0.0001 vs *t*bhp. # represents comparison to Control, * represents comparison to *t*bhp.

<0.0001 vs *t*bhp alone), respectively; no significant difference was observed between these three treatment groups. Although still significantly reducing ROS generation, 2-D08 proved to be the least effective antioxidant of the flavonoids tested in this study, limiting DCFDA fluorescence to 401.1% versus control (**p =0.0088 vs *t*bhp alone).

4. Discussion

The results of the present study highlight a novel role for the semi-synthetic flavone 2-D08 as an inhibitor of both SN-38- and cytokine-evoked increases in barrier permeability, as well as identifying a novel role in reducing ROS generation *in vitro*. This study also presents the very first characterisation of transilitin in this context, revealing a novel role as a potent inhibitor of ROS generation, as well as an efficacious inhibitor of SN-38-evoked increases in barrier permeability in Caco-2 cells. The protective effect of myricetin was also assessed, representing the first direct characterisation of this common dietary flavonoid's effects on the cytotoxicity of SN-38, as well as its role in maintaining barrier integrity in the presence of SN-38 and a proinflammatory cytokine combination. Each of these compounds were compared with the well-characterised flavonoid quercetin in order to gain insights into their ability to maintain gastrointestinal barrier integrity, as well as their relative capacities as antioxidant agents. This comparison also allowed for an insight into the effect of structural differences among the test molecules and flavonoid structure-activity relationships more generally. To our knowledge, this study presents the very first characterisation of both 2-D08 and transilitin in the context of intestinal epithelial barrier permeability, while also presenting the first characterisation of myricetin's effects in the context of functional intestinal epithelial barrier integrity.

Neither myricetin nor 2-D08 had a significant effect on the toxicity of SN-38, although intrinsic loss of cell viability was observed at 20 µM 2-D08. However, both transilitin and quercetin significantly increased the toxicity of low concentrations of SN-38 in Caco-2 cells. This is in line with a previous study which demonstrated the ability for quercetin to enhance the toxicity of SN-38 in AGS cells, a human gastric adenocarcinoma cell line (Lei et al., 2018). This

phenomenon has also been exhibited in other studies, whereby co-incubation with flavonoids can increase the potency of irinotecan (Lefort and Blay, 2011, Benkovic et al., 2007). One possible mechanism behind this increase in cytotoxicity may relate to the activity of UGT1A1, the enzyme responsible for the inactivation of SN-38 to SN-38G, which has been shown to be expressed endogenously in Caco-2 cells (Xu et al., 2014). Flavonoids exert variable effects on the expression and activity of this enzyme, with the nature and location of specific functional groups playing a key role (Ma et al., 2013, Hiura et al., 2014, Walle and Walle, 2002). Of particular note is the potent inhibitory effect that quercetin exerts on UGT1A1 activity, with an IC_{50} of 7.47 µM (Liu et al., 2019). As the primary enzyme responsible for the glucuronidation of SN-38, the inhibition of this enzyme may have a substantial impact on the ratio of SN-38:SN-38G, resulting in greater exposure to the active form of the drug, thus increasing the opportunity for the development of toxicity. Although no data currently exists regarding the effect of transilitin on UGT1A1 activity, the same explanation may be applicable, given the body of literature identifying flavonoids as modulators of this enzyme (Hiura et al., 2014, Galijatovic et al., 2001). This concept requires further exploration, particularly as it relates to the cytotoxicity of SN-38 and presents an avenue for future exploration in the broader context of GIM.

None of the flavonoids in this study exerted a significant intrinsic effect on epithelial permeability. Treatment with SN-38 alone resulted in a significant decrease in TEER over 48 hours, an effect which echoes previous studies (Yue et al., 2021a). SN-38 has been shown to disrupt tight junction (TJ) formation and significantly downregulate the expression of occludin, which may explain this reduction in TEER (Yue et al., 2021a). Previous studies have indicated that quercetin can increase TEER via upregulation of TJ proteins such as occludin and claudin-1 (Suzuki and Hara, 2009, Amasheh et al., 2008). However, these studies have also used significantly higher concentrations of quercetin, which may account for the lack of efficacy observed in the present study.

The semi-synthetic flavone 2-D08 proved to be the most effective inhibitor of SN-38-evoked increases in epithelial permeability, maintaining barrier integrity over the 48-hour incubation period. Although 2-D08 remains relatively understudied, previous work has identified potential molecular targets of this flavone that may be of relevance to the effect observed in the present study. Choi and colleagues identified 2-D08 as an inhibitor of ZEB-1 expression and inducer of zonula occludens-1 (ZO-1), an important TJ protein (Choi et al., 2018). Increased ZEB-1 expression is a key factor in the progression of epithelial to mesenchymal transition (EMT), a process which has been identified as an important pathological process in inflammatory bowel disease (IBD)-associated intestinal fibrosis, where it has been directly linked with chronic inflammation; TNFα is also a notable inducer of EMT (Lovisa et al., 2018, Jiang et al., 2018, Ricciardi et al., 2015, Lamouille et al., 2014). During EMT, junctional proteins responsible for maintaining epithelial barrier integrity, such as ZO-1 and E-cadherin, are downregulated, leading to increased membrane permeability (Kalluri and Weinberg, 2009, Elamin et al., 2014). This was exemplified by Elamin et al, who showed that inhibition of the Snail protein, a repressor of E-cadherin expression and an important inducer of EMT, improved barrier integrity and reduced barrier permeability in Caco-2 cells (Elamin et al., 2014). ZEB-1 has also been shown to repress E-cadherin, suggesting that its inhibition may serve as a functional target for 2-D08 in maintaining epithelial barrier integrity (Sánchez-Tilló et al., 2010). Considering this, the role of 2-D08 as a potential inhibitor of EMT warrants further exploration in the context of gastrointestinal inflammation, particularly in the area of chemotherapy-induced GIM. In general, the ability for 2-D08 to maintain barrier function in the presence of SN-38 highlights its exciting potential as a possible adjunct to conventional chemotherapeutics.

The pro-inflammatory cytokines TNFα and IL-1β significantly increased epithelial permeability in the present study, an effect that has been widely established in the literature (Al-Sadi et al., 2009, McKay and Baird, 1999). A general trend was observed whereby flavonoids with trihydroxylated B-rings, namely myricetin and 2-D08, offered a greater degree

of anti-inflammatory protection than the dihydroxylated flavonoids quercetin and transilitin. The differing effects of these flavones is likely independent of their antioxidant potential, given that each, including quercetin, significantly inhibited ROS generation, with 2-D08 being the least effective antioxidant. Interestingly, a previous study in IEC-6 intestinal epithelial cells showed an intrinsic increase in TEER following treatment with quercetin and myricetin, an effect that was not observed in this study (Fan et al., 2020a). Previous investigations of flavonoid structure-activity relationships in the context of inflammation have suggested that the presence of 5,7-hydroxyl groups may be important structural features in affording anti-inflammatory activity (Hošek and Šmejkal, 2015, Kim et al., 2004). However, the considerable anti-inflammatory effect of 2-D08 highlighted in the present study would suggest that A and C ring hydroxylation is not a requirement for anti-inflammatory capacity. Previous research has highlighted the multifaceted anti-inflammatory properties of flavonoids, offering mucoprotection via mechanisms such as the inhibition of NF-κB activity, inhibition of ROS generation, inhibition of COX-2 activity and improving epithelial barrier function (Vezza et al., 2016, Kim et al., 2004). Recently, the post-translational modification of target proteins through processes such as SUMOylation has also gained considerable traction in the context of inflammation (Karhausen et al., 2021). Interestingly, suppression of the post-translational modifier SUMO-1 has been shown to significantly decrease the secretion of pro-inflammatory cytokines such as TNFα while also increasing TEER values in human tonsil epithelial cells under hypoxic conditions (Lin et al., 2021). Conversely, the downregulation of SUMO E2 enzyme (Ubc9) has been linked to the exacerbation of the inflammatory response in an *in vivo* model of inflammatory bowel disease (Mustfa et al., 2017). Understanding the relationship between inflammation and SUMOylation remains exceedingly complex, as SUMOylation of multiple distinct components within a single pathway may produce contradictory effects (Karhausen et al., 2021). However, the exploration of this relationship may offer a potential explanation for the observed ability for 2-D08, a known SUMOylation inhibitor, to maintain barrier integrity in the presence of both SN-38 and pro-inflammatory cytokines. Although this

concept requires considerable experimental investigation and validation, it presents an exciting conceptual pathway for future studies to explore.

Unsurprisingly, each of the flavonoids in this study proved to be potent antioxidants, although 2-D08 comparatively proved the least efficacious in this regard. This echoes numerous other studies which have highlighted the potent antioxidant capacity of flavonoids such as quercetin and myricetin (Yokomizo and Moriwaki, 2006, Shimmyo et al., 2008, Kumar and Pandey, 2013). The potent free-radical scavenging efficacy of flavonoids has been attributed to a number of structural features; namely the presence of a catechol moiety in the B ring and a 2,3-double bond conjugated with the 4-oxo group (Pietta, 2000, Bors et al., 1990). These structural features are common to each of the four flavonoids in this study and present the most likely explanation for the antioxidant effect noted here. It has also been suggested that the presence of a 3-OH group may act to further enhance the radical-scavenging capabilities of flavonoids, which may explain the reduced efficacy of 2-D08 in this regard (Pietta, 2000). A previous study has shown 2-D08 to be an effective radical scavenger in the DPPH and MDA assays, with the authors highlighting the particular importance of 2',3',4'-trihydroxylation in this effect (Cotelle et al., 1996). However, it has been noted that 2'-hydroxyflavones, which occur rarely in nature, are less effective ROS scavengers overall than other flavonoids, which would support our present findings from the radical scavenging assay (Bailly, 2021). This was also exemplified in a study by Tokalov and colleagues which found 2'-hydroxyflavone to possess less radical scavenging capability than 5,2′-dihydroxyflavone and considerably less than either 5,8-dihydroxyflavone or quercetin in HL-60 cells (Tokalov et al., 2004). Fascinatingly, 2-D08 has been shown to exhibit both antioxidant and pro-oxidant properties in various contexts related to its effects on protein SUMOylation, exemplifying the complexities of 2-D08's bioactivity (Bailly, 2021). Interestingly, it has been noted that the presence of an ortho-hydroxylation pattern in the A-ring may provide an equal degree of radical-scavenging capacity as a catechol group in the B-ring (Foti et al., 1996). This may aid in explaining the potent antioxidant capacity of transilitin,

which contains both of these structural features. Another interesting relationship noted in the present study was the apparent lack of correlation between antioxidant capacity and anti-inflammatory effect. This is perhaps best exemplified by 2-D08, which proved to be the most efficacious of the test compounds in maintaining barrier function yet was the least effective antioxidant. Conversely, quercetin proved highly effective as an inhibitor of ROS generation and yet was largely ineffective in maintaining barrier function in the presence of pro-inflammatory cytokines. It is well established that antioxidants play an important role as inflammatory modulators and may provide protection against inflammation-associated cellular damage through mechanisms such as free radical scavenging (Arulselvan et al., 2016, Griffiths et al., 2016). However, the lack of correlation observed in the present study would suggest that the ability for these flavonoids to maintain barrier integrity is independent of their antioxidant activity.

There has been a wealth of research conducted into the nature of the gut microbiome, its function in maintaining homeostasis and its role in various pathologies (Kho and Lal, 2018, Valdes et al., 2018). Altered intestinal microbiota has been implicated in the pathogenesis of inflammatory bowel disease (IBD) and plays an important role in the development of irinotecan-induced GIM (Geier et al., 2007, Khan et al., 2019, Zuo and Ng, 2018, Yue et al., 2021b, Lin et al., 2014). Dietary polyphenols such as flavonoids play an important role in maintaining intestinal homeostasis and possess the ability to modulate the gut microbiome in a variety of ways, such as influencing the abundance of various pathogenic bacteria, and may play a role in the amelioration of IBD-associated dysbiosis (Wang et al., 2021, Rodríguez-Daza et al., 2021). In this light, future studies focusing on novel flavonoids such as 2-D08, transilitin and myricetin and their effects on intestinal pathologies linked to dysbiosis are warranted. Interestingly, topoisomerase 1, the primary target of irinotecan, has been identified as a substrate for SUMOylation, with SUMOylation playing an important role in the nucleolar delocalisation of this protein (Mo et al., 2002). Given that 2-D08 is a known inhibitor of protein

SUMOylation, future studies may consider exploring this relationship as a means of ascertaining mechanistic insights into the effects observed in this study. It has been well established that downregulation of particular TJ proteins is directly associated with reductions in barrier integrity and function, and treatment with proinflammatory cytokines such as TNFα has been shown to downregulate TJ expression through various mechanisms (Buckley and Turner, 2018, Slifer and Blikslager, 2020). Moreover, treatment with both irinotecan and SN-38 have been linked to the downregulation of several TJ proteins, resulting in reduced barrier function (Wardill et al., 2014, Yue et al., 2021a). Numerous flavonoids possess the ability to maintain and even increase TJ protein expression, highlighting their therapeutic potential in this regard (Suzuki, 2020, Salaritabar et al., 2017). Future studies exploring the protective efficacy of 2-D08 and transilitin in maintaining intestinal barrier integrity should therefore investigate the effect of these novel flavonoids on TJ expression.

5. Conclusions

This study presents the first characterisation of both 2-D08 and transilitin as protective agents with the ability to maintain intestinal barrier integrity, but particularly the multifaceted functional efficacy of 2-D08 against a broader base of chemo-injurious and inflammatory stimuli. These findings also provide structure-activity insights germane to the anti-inflammatory capacity of flavones in particular and may serve as a foundation for further investigations into the use or design of novel flavonoids as adjunct nutraceutical therapies in the treatment of chemotherapy-induced GIM and inflammatory bowel disease.

Chapter 7: General Discussion

This thesis provides novel insights into the multifaceted protective capacities of a range of natural products, focusing on phytocannabinoids and flavonoids, in the context of both neurodegenerative disease and inflammatory diseases of the GI tract. Through the use of biochemical cell-based assays, TEM imaging, fluorescence microscopy, bioinformatics and molecular modelling, we have demonstrated the wide-ranging benefits of these natural products. The following discussion summarises the key findings of this thesis, their significance, study limitations and future directions.

7.1. Significant findings

In Chapter 2, a thorough review of the literature surrounding natural products as neuroprotective agents was conducted, with a particular focus on phytocannabinoids and related natural products including cannabis flavonoids. This review covered the biosynthesis of the major phytocannabinoids and their precursors and elaborated extensively on the current evidence of neuroprotection afforded by these pCBs. This chapter also included novel molecular modelling data aimed at determining the binding characteristics of various phytocannabinoids as a means of identifying high-affinity binders with various conformations of the Aβ protein. We identified CBG and CBC as particularly high-affinity binders with both monomeric and pentameric conformations of Aβ. This may be attributed largely to the alkyl side chains of these pCBs, which allows for optimal binding within the hydrophobic groove of the β-sheet structure. We also identified a number of key structure activity relationships regarding the binding characteristics of these compounds. For example, we found that pCBs with shorter alkyl side chain lengths possessed lower binding affinity than structural analogues with longer alkyl chain lengths. This was exemplified by the fact that Δ^9-THC and CBD possessed greater binding affinity for Aβ than their propyl counterparts THCV and CBDV. Moreover, the degree of aromatisation in the C ring proved to be an important factor in affording increased binding affinity. This was perhaps best exemplified by the decreased binding affinity for Δ^9-THC and

CBD compared to their fully aromatised analogues CBN and CBND. This effect may be related to the full resonance distribution and high degree of planarity afforded by the fully aromatised C ring, increasing hydrophobicity and thus allowing for greater propensity for binding within the hydrophobic groove of the amyloid peptide.

Chapter 3 demonstrated novel bioactivity of the phytocannabinoids CBG, CBC, CBDV and CBN in an *in vitro* model of AD. This study highlighted the ability for these compounds to prevent Aβ-induced neuronal cell death, while also preventing Aβ-evoked changes in neuronal morphology, as shown by fluorescence microscopy and bioinformatics-based morphological analysis. Treatment with Aβ alone resulted in a distinct loss of neuritic projections, as well as increases in the size of the cell nucleus and a more rounded overall morphology. CBG and CBC proved highly efficacious as inhibitors of Aβ neurotoxicity, with a dual role in maintaining 'healthy' neuronal morphology. A novel effect was also observed for CBDV, whereby non-toxic concentrations of the drug caused a significant reduction in the number of neuritic projections in PC12 cells. The fact that CBDV-treated cells displayed a loss of neuritic projections without any overt toxicity suggests that loss of neuritic projections may not be an ideal indicator of cytotoxicity in PC12 cells. A clear distinction was identified whereby the antioxidant effects of each phytocannabinoid were unrelated to their ability to afford neuroprotection against Aβ. For example, we found CBD to be a potent antioxidant capable of preventing *t*bhp-induced cytotoxicity, however CBD was unable to provide significant neuroprotection against Aβ. Conversely, CBG proved to be rather ineffective as an antioxidant yet was highly efficacious as an inhibitor of amyloid β toxicity. Several novel structure-activity relationships were also demonstrated in this study, with one such example being the increased neuroprotective effect of CBN over Δ^9-THC. Interestingly, CBN also proved to be both an avid binder with Aβ, as shown in Chapter 2, and a more effective inhibitor of amyloid aggregation. One possible explanation for this may again relate to the increased hydrophobicity afforded by the aromatisation of the C ring in the structure of CBN, allowing for better binding within the

amyloid β-sheet structure. A second SAR identified in this study relates to the effect that alkyl side chain length has on neuroprotection. In Chapter 2, we found that increased alkyl side chain length correlated with increased binding affinity with Aβ. However, in Chapter 3, we found that shortening of the alkyl side chain at position 3 from an *n*-pentyl chain, as in CBD, to an *n*-propyl chain as in CBDV had no significant effect on neuroprotection against $A\beta_{1\text{-}42}$, although CBDV proved to be a better inhibitor of amyloid β aggregation.

In contrast to Chapter 3, Chapter 4 aimed to investigate the potential neuroprotective effects of whole botanical extracts of various cannabis chemovars, rather than isolated phytocannabinoids. This study identified novel neuroprotective effects for extracts of several Δ^9-THC-dominant chemovars, with no significant protection afforded by CBD-dominant preparations. Moreover, it was established that heat exposure reduced the overall protective efficacy of these cannabis preparations. This reduction in neuroprotective capacity may relate to the assumed reduction in terpene concentrations, as terpenes have been shown to synergistically enhance the activity of pCBs. Moreover, heating of each extract may have increased the conversion of the phytocannabinoid acids THCA and CBDA to their decarboxylated forms Δ^9-THC and CBD, which may have also played a role in the observed loss of neuroprotection. This study also highlighted a distinct lack of anti-aggregatory capacity of these cannabis preparations against amyloid β, an effect which we have previously noted for isolated phytocannabinoids such as CBD and Δ^9-THC. Although the mechanism by which these extracts exert their neuroprotection remains inconclusive, it is clear that these are unrelated to any direct effects on Aβ aggregation.

In Chapter 5 we employed the use of a Caco-2 cell model of intestinal barrier function, given that these cells may form monolayers which exhibit many of the characteristics of intestinal epithelium (Sun et al., 2008). Treatment with the chemotherapeutic drug SN-38 resulted in significant increase in barrier permeability, as measured by TEER; this increase in permeability was also observed following treatment with the proinflammatory cytokines TNFα and IL-1β.

We demonstrated novel bioactivity for the pCBs CBD, CBDV and CBG, as each was able to prevent the increase in intestinal barrier permeability associated with treatment with both TNFα and IL-1β and SN-38 to varying degrees. CBC was a potent inhibitor of cytokine-evoked increases in permeability, however no effect was observed following treatment with SN-38. This study also highlighted the lack of correlation between inhibiting ROS generation and intestinal protection, given that none of the pCBs screened exhibited any significant reductions in ROS generation *in vitro*, as determined by DCFDA fluorescence. The broader implications of these results are discussed in Section 7.2.

In Chapter 6, we aimed to extend upon the work performed in Chapter 5 by employing the same model as a screening for flavonoid bioactivity. This study focused on the novel flavonoids 2-D08 and transilitin, along with the more common myricetin and quercetin. Novel bioactivity was discovered for 2-D08 as a dual inhibitor of both cytokine- and SN-38-evoked increases in intestinal barrier permeability, with 2-D08 also exhibiting significant ROS scavenging capabilities. Transilitin was identified as a novel inhibitor of SN-38 evoked increases in permeability, however no protective effect was observed in the presence of cytokines, indicating that this protection is likely independent of anti-inflammatory capacity. Each of the flavonoids screened in this study proved to be effective antioxidants in the DCFDA assay, although 2-D08 proved to be the least effective in this regard; this fact would indicate that the ability for 2-D08 to maintain barrier function is likely to be independent of antioxidant activity.

7.2. Exploring the Link Between Intestinal Inflammation, Intestinal Barrier Permeability and Alzheimer's Disease

In recent years, the potential role of the gut microbiota in the pathobiology of various diseases has garnered a great deal of attention. Alterations in the composition of gut microbiota result in increased gut barrier permeability and immune system activation, leading to systemic inflammation, which may result in the impairment of the blood-brain barrier and promote neuroinflammation and neurodegeneration (Kowalski and Mulak, 2019). Interestingly, the gut

microbiome of AD patients has been shown to have significantly less microbial diversity and is compositionally distinct from healthy age- and sex-matched controls (Vogt et al., 2017). Moreover, gut dysbiosis and increased permeability have been associated with increases in cerebral amyloid plaque formation and deposition (Ticinesi et al., 2018). High-fat diets have been associated with an increased risk of AD, which may be related in part to alterations in gut permeability and inflammation (D'Argenio and Sarnataro, 2019). Interestingly, the dietary intake and microbial metabolism of natural polyphenols in the form of a grape seed extract has been associated with a decrease in Aβ aggregation (Wang et al., 2015). Furthermore, urolithins, a group of gut microbiota-derived metabolites of a pomegranate extract, have been shown to cross the blood-brain barrier, disrupt Aβ fibrillisation and aggregation and protect against amyloid neurotoxicity *in vivo* (Yuan et al., 2016). Although research in this area is still in its infancy, it is clear that the gut-brain-axis plays an important role in the regulation of systemic inflammation, neuroinflammation and neurodegeneration. This presents an interesting point in relation the findings of this thesis. Compounds such as CBG that possess considerable neuroprotective efficacy in the context of AD that also effectively maintain intestinal barrier function in the presence of pro-inflammatory stimuli present as exciting candidates for drug development. Not only do such drugs have considerable therapeutic potential in AD, IBD and GIM in isolation but also in combination. This presents an exciting avenue for future exploration whereby these compounds may be explored for their protective efficacy in similar disease contexts.

7.3. Study Limitations and Future Directions

The use of *in vitro* disease models has many benefits, including low cost, ease of use and the potential for relatively high throughput. The Caco-2 cell barrier model has been employed by numerous studies looking at both drug bioavailability and changes in intestinal permeability. However, such models lack the complexity of *in vivo* models and human tissue models, which allow for more accurate determinations of true bioactivity and safety to be made. The Caco-2

cell monolayer also lacks a mucus membrane, which acts as a physical and chemical barrier within the GI tract. For this reason, the GI studies included in this thesis would benefit greatly from the use of either human gut explant models, as a means of assessing the effects of these phytochemicals in human tissue, or murine models of IBD and GIM, which have been employed by numerous other studies. The use of *in vivo* models of IBD and GIM allow for the broader involvement of the immune system to be investigated, given the essential role that immune cells such as lymphocytes play in these diseases. Another limitation was perhaps the use of the MTT assay as the central measure of cell viability. MTT is a useful and important assay, however it is only capable of measuring endpoint mitochondrial activity and is not a specific measure of cytotoxicity. The combined use of an alternative assay such as the lactate dehydrogenase assay may serve to validate and consolidate the viability findings from the MTT assay. Morphological analysis has been used as a means of detecting morphological changes associated with cell death in various cell lines. However, this relationship has not been fully explored in relation to certain neuronal cell populations such as PC12 cells. Further exploration into the potential use of morphometric analysis as a means of measuring PC12 cells death presents an exciting avenue for future exploration, which may help to elaborate on the imaging and viability data presented in this thesis.

Another general limitation of this thesis is the lack of mechanistic insight. Although we have established novel protective effects for a range of phytochemicals in several disease models, we have not fully elucidated the mechanisms by which these drugs may be exerting their effects. Future studies may use the work presented in this thesis as the foundation for further exploration into these mechanisms. Given the known activity of many of these phytochemicals at various receptor targets, employing the use of cannabinoid receptor antagonists, as well as antagonists at other key receptors such as PPARγ, may elucidate the potential role of such receptors in the protection observed in this study. We conducted a brief set of experiments aimed at probing the role of PPARγ in the neuroprotective effects against Aβ observed in this thesis (see **Appendix**

2). However, these preliminary studies demonstrated no significant effect of either the PPARγ agonist rosiglitazone or the PPARγ antagonist GW9662 on Aβ neurotoxicity in PC12 cells. Future studies may also seek to explore the potential role of relevant inflammatory modulators, such as the NF-κB signalling pathway, given that inflammation plays a central role in the pathogenesis of AD, IBD and GIM. It may be that the phytochemicals used in this thesis are acting on such inflammatory pathways; this concept warrants further investigation.

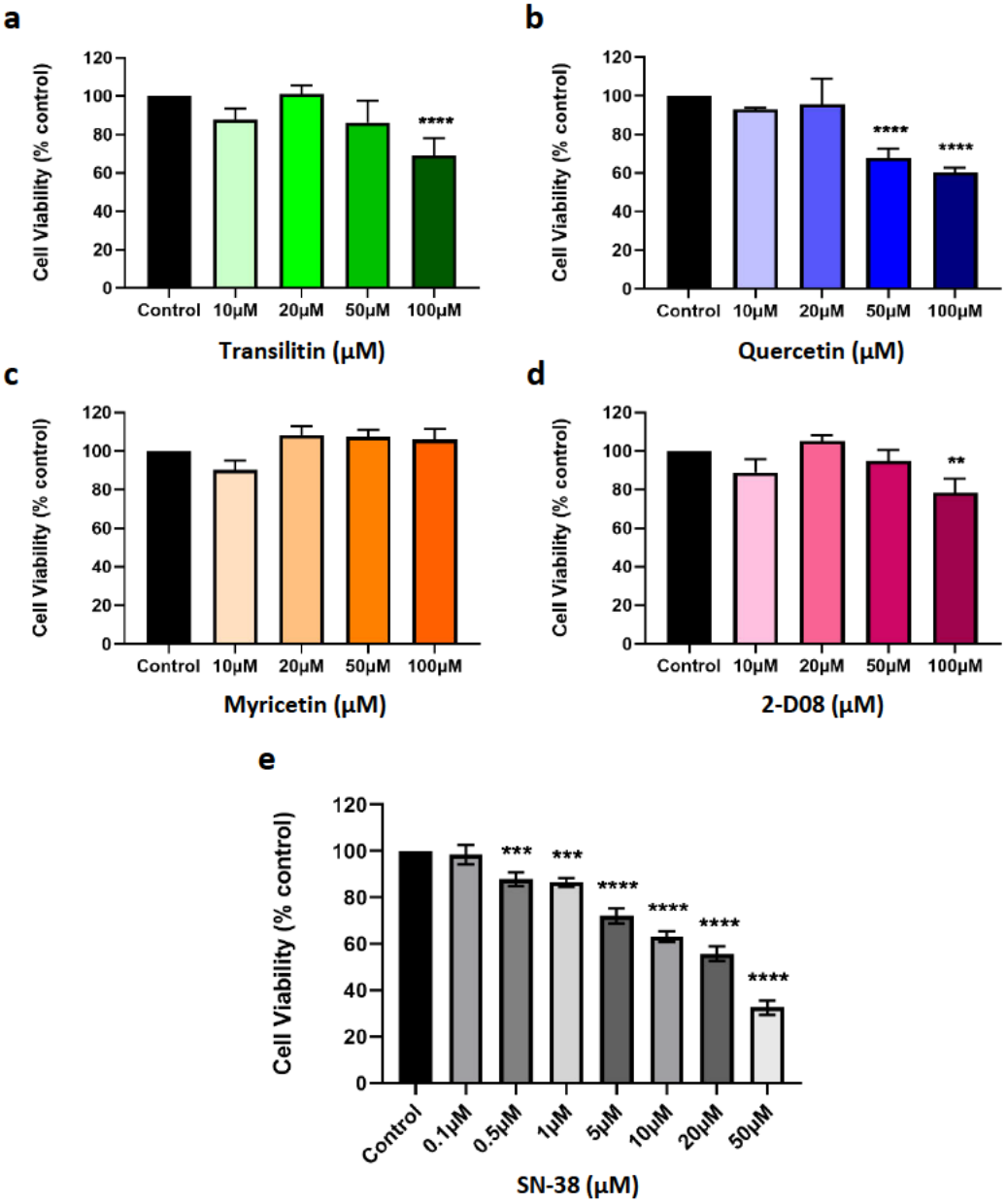
There are several factors that would have improved the quality of the study conducted in Chapter 4. For example, the study would have benefited greatly from further analysis of the extract components following extraction, rather than the use of crude extracts. This would allow for greater reproducibility as it would give an insight into the relative concentrations of phytocannabinoids and phytocannabinoid acids, as well as more well-defined terpene profiles. This would have proven particularly useful in comparing the effects of heat exposure on the bioactivity of each extract, as we would have been able to directly assess the differences in phytocannabinoid and terpene content induced by heat exposure.

Regarding the GI studies presented here, an investigation into the potential changes in TJ protein expression is warranted, given the vital role of these proteins in maintaining appropriate barrier function. Both phytocannabinoids and flavonoids have been shown to exert varying effects on the expression of these proteins, suggesting that this may be a mechanism by which these drugs maintain barrier function. Given the suggested links between intestinal barrier permeability, GI inflammation and the development of AD, further research focusing on the potential impact of dietary polyphenols and phytocannabinoids on gut permeability and inflammation is warranted. As the gut microbiome plays a key role in the regulation of GI health and inflammation, studies focusing on the use of probiotic bacterial strains, in concert with the phytochemicals used in this study, may be of use in the amelioration of IBD, GIM and perhaps also in the context of AD.

In conclusion, the studies presented in this thesis have identified novel bioactive phytocannabinoids and flavonoids that exhibit various protective effects in both Alzheimer's disease and inflammatory diseases of the GI tract. Given the established link between GI health and the development of neurodegenerative diseases such as AD, phytocannabinoids such as cannabigerol, which effectively maintain barrier function and prevent the neurotoxicity of Aβ, present as attractive lead candidates in the development of pharmacotherapies aimed at combatting AD and GI inflammation. The work presented in this thesis has broader implications in the context of drug development and may serve as a foundation for further studies into the bioactivity of the phytochemicals presented here.

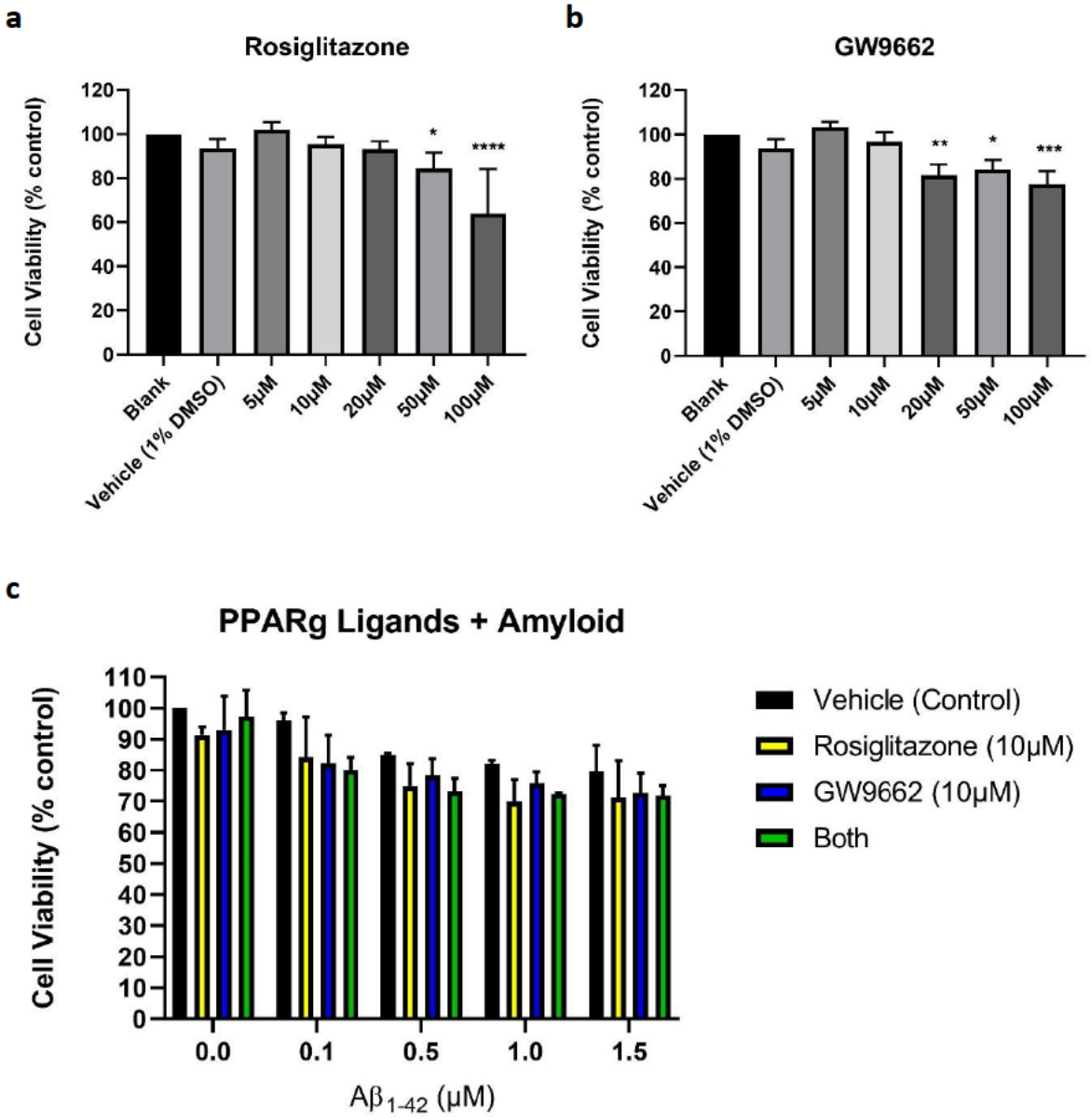
Chapter 8: Appendices

Appendix 1. Concentration-response data for effects of selected flavonoids and SN-38 on cell viability in Caco-2 cells



Appendix 1. MTT assay of Caco-2 cell viability following 48hr incubation with selected flavonoids (10-100µM): **(a)** transilitin, **(b)** quercetin, **(c)** myricetin, **(d)** 2-D08 **(e)** SN-38 (0.1-50µM). Data presented as mean ± SD, n = 4. Two-way ANOVA with Dunnett's post-hoc test vs control, **p<0.01, ***p < 0.001, ****p < 0.0001 vs vehicle.

Appendix 2. The effect of PPARγ ligands on Aβ neurotoxicity



Appendix 2. MTT assay of PC12 cell viability following 48hr co-incubation with Aβ and the PPARγ agonist rosiglitazone and antagonist GW9662: **(a)** rosiglitazone concentration-response (n=4) **(b)** GW9662 concentration-response (n=4), **(c)** co-incubation of each PPARγ ligand (10μM) with Aβ (0-1.5μM) (n=2). Data presented as mean ± SD. One-way ANOVA with Dunnett's post-hoc test vs control, *p <0.05, **p <0.01, ***p 0.001, ****p <0.0001 vs vehicle.

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