



**University of
Nottingham**

UK | CHINA | MALAYSIA

**Delivery of cannabinoids to specific
anatomical regions of the brain
following oral administration to
reach the site of action for
glioblastoma multiforme and
multiple sclerosis**

Alice Brookes, MChem, AMRSC

Thesis submitted to the University of Nottingham for the
degree of Doctor of Philosophy

December 2022

Table of Contents

Table of Contents.....	2
ACKNOWLEDGEMENTS.....	7
LIST OF PUBLICATIONS	10
LIST OF ABBREVIATIONS	11
ABSTRACT	17
LIST OF FIGURES	20
LIST OF TABLES.....	28
CHAPTER 1: General introduction.....	32
1.1 Drug delivery to the brain	32
1.1.1 The need for brain targeting	32
1.1.2 The blood-brain barrier	36
1.1.3 Oral lipid-based drug delivery.....	42
1.1.4 Effects of lipids on delivery to the brain	47
1.2 Glioblastoma multiforme	52
1.2.1 Glioblastoma multiforme treatment with temozolomide	53
1.3 Cannabidiol	58
1.3.1 Activity of cannabidiol for multiple sclerosis	60
1.3.2 Anti-cancer properties of cannabidiol	61
1.3.3 Cannabidiol <i>in vivo</i> pharmacokinetics	62
1.3.4 Cannabidiol formulations	64
1.3.5 4'-Fluoro-cannabidiol.....	65
1.4 3D OrbiSIMS	67
1.5 Project hypotheses and aims	69
CHAPTER 2: Methods	72
2.1 Materials and instruments	72

2.2 Chemical synthesis of 4'-fluoro-cannabidiol.....	73
2.2.1 General Procedures	73
2.2.2 4'-Fluoro-cannabidiol.....	74
2.3 Cell culture.....	76
2.3.1 Reviving cell stocks	76
2.3.2 Cell passaging	77
2.3.3 Cell freezing for storage	77
2.3.4 MTT assay – 3- and 6-days exposure.....	77
2.3.5 MTT combination assay – 3- and 6-days exposure.....	80
2.3.6 Cell count assay – 3- and 6-days exposure.....	81
2.3.7 Cell count combination assay – 3- and 6-days exposure	82
2.4 <i>In vivo</i> studies	84
2.4.1 Jugular vein cannulation.....	84
2.4.2 Pharmacokinetics assay	85
2.4.3 Biodistribution assay in rats	86
2.4.4 Biodistribution assay in mice	87
2.4.5 Efficacy study in experimental autoimmune encephalomyelitis (EAE) mouse model	88
2.5 <i>Ex vivo</i> studies.....	90
2.5.1 Detection of cannabidiol by HPLC-UV	90
2.5.2 Validation of the detection of 4'-fluoro-cannabidiol by HPLC-UV	91
2.5.3 Preparation of tissue samples for analysis by 3D OrbiSIMS	92
2.5.4 Preparation of cell samples for analysis by 3D OrbiSIMS	93
2.5.5 Analytical conditions for analysis of samples by 3D OrbiSIMS	94
2.6 Software and statistical analysis	96
CHAPTER 3: Anti-cancer properties of cannabinoids.....	97
3.1 Introduction.....	97

3.2 Experimental design.....	100
3.3 Results.....	101
3.3.1 Growth inhibitory activity of cannabinoids on non-tumourigenic cells	101
3.3.2 Cancer cell growth inhibition by cannabinoids.....	102
3.3.3 Synergy of cannabinoids and imidazotetrazine anti-cancer agents against GBM	105
3.3.4 Indications of anti-cancer mechanism of cannabidiol.....	112
3.3.4.1 Exposure of the cells to cannabidiol alone.....	113
3.3.4.2 Exposure of cells to a combination of cannabidiol with imidazotetrazine anti-cancer agents	115
3.4 Discussion	119
3.5 Conclusions.....	127
CHAPTER 4: Delivery of cannabidiol to the whole brain and anatomical regional brain distribution following oral administration to rats.....	129
4.1 Introduction.....	129
4.2 Experimental design.....	132
4.3 Results.....	133
4.3.1 Biodistribution of cannabidiol to the brain in rats.....	133
4.3.2 Biodistribution of cannabidiol to the brain assessed by 3D OrbiSIMS analysis.....	148
4.4 Discussion	153
4.4.1 Olfactory bulb	156
4.4.2 Frontal lobe	157
4.4.3 Striatum	158
4.4.4 Hippocampus.....	159
4.4.5 Temporal lobe	160
4.4.6 Parietal lobe.....	160

4.4.7 Occipital lobe	161
4.4.8 Medulla pons	161
4.4.9 Cerebellum	162
4.4.10 Assessment of biodistribution by 3D OrbiSIMS analysis.....	164
4.5 Conclusions.....	166
CHAPTER 5: Delivery of cannabidiol to anatomical brain regions for the treatment of multiple sclerosis.....	168
5.1 Introduction.....	168
5.2 Experimental design.....	171
5.3 Results.....	172
5.3.1 Biodistribution of cannabidiol to the brain in mice.....	172
5.3.2 Efficacy of cannabidiol in experimental autoimmune encephalomyelitis (EAE) mice model	180
5.4 Discussion	183
5.5 Conclusions.....	189
CHAPTER 6: Biodistribution of 4'-fluoro-cannabidiol to the whole brain, anatomical brain regions, and other tissues following oral administration to rats	191
6.1 Introduction.....	191
6.2 Experimental design.....	193
6.3 Results.....	195
6.3.1 Pharmacokinetics study of 4'-fluoro-cannabidiol	195
6.3.2 Biodistribution of 4'-fluoro-cannabidiol	198
6.4 Discussion	216
6.4.1 Olfactory bulb	219
6.4.2 Frontal lobe	220
6.4.3 Striatum	221
6.4.4 Hippocampus.....	221

6.4.5 Temporal lobe	222
6.4.6 Parietal lobe	223
6.4.7 Occipital lobe	224
6.4.8 Medulla pons	224
6.4.9 Cerebellum	225
6.4.10 Lymphatic system	226
6.5 Conclusions.....	229
CHAPTER 7: General discussion and conclusions	231
7.1 General discussion	231
7.2 General conclusions	237
CHAPTER 8: Future work	240
8.1 Anti-cancer properties of cannabinoids	240
8.2 Oral lipid-based cannabinoid formulations.....	242
8.3 Oral lipid-based cannabidiol formulation for multiple sclerosis treatment	243
8.4 Potential of 4'-fluoro-cannabidiol.....	244
APPENDICES	246
REFERENCES	262

ACKNOWLEDGEMENTS

Firstly, I would like to thank my parents, Adam and Nicola, who have always supported me in whatever I chose to do. I am extremely grateful for their continued encouragement through the years of my PhD work, but also throughout my life. They raised me to believe I could do anything I wanted, and so my accomplishments are also theirs. My gratitude extends to my siblings, Emily, Hannah, and Samuel, for always being there for me, their support has meant the world. To my partner, Craig Wescott, I am eternally grateful. For the never-ending support, inspiration, comfort, and encouragement he provided. Craig made both the hard days easier and the great days better, always believing in me, he is simply the best. Also, to my further family, Jasper, Lemon, and Tangerine, for making life more fun.

I would like to express my deep appreciation for my supervisor, Dr. Pavel Gershkovich. He has always provided me with the guidance, support, and encouragement I needed, whilst also allowing me the space to make my own way. Particularly during the COVID-19 pandemic, this was hugely appreciated. He always supported me to share my work at conferences and develop other skills outside of research. His trust in me helped me to trust in my work, I couldn't have asked for a better supervisor. I would also like to express my gratitude to Dr. Tracey D. Bradshaw, who provided a level of encouragement and interest in my work that every researcher should have. She encouraged me to explore interesting avenues and helped to increase the collaborative nature of my project. Her kindness and support were always appreciated, but particularly during the COVID-19 pandemic. I would also like to thank my other supervisors,

ACKNOWLEDGEMENTS

Dr. David J. Scurr, Prof. Morgan Alexander and Dr. James Butler for their support, inspiration, and feedback throughout this project. I would like to thank my collaborators at the University of Edinburgh, Dr. Emily Gwyer Findlay and Dr. Katie J. Smith for their work on the *in vivo* multiple sclerosis (MS) studies.

Next, I would like to thank my friends. To Emily Wertans, who has always been there for me and believed in me from the start of my academic journey, her support has been essential. Also, to Ami Nash, Chloe Stewart and Fiona Smith, we've gone through this journey together, and friendship has kept me going through the tough days, for their constant support I am extremely grateful.

I would like to thank everyone in the C62 lab for all the training, guidance and support throughout my PhD. Especially, thank you to Dr. Wanshan Feng and Dr. Adelaide Jewell for always taking the time to help me through the long studies, and for your friendship throughout. Also, thanks to Dr. Chaolong Qin, YenJu Chu, Haojie Chen, Abigail Wong, Liuhang Ji, Graziamarina Sinatra, Branislav Vukovic and Sarah Sulaiman. I would also like to thank those in the C64 lab, particularly to Dr. Kaouthar Bouzinab for the training and guidance, also to Dr. Isobel Holden, Dr. Shahadat Hossan, Dr. Haneen Abuzaid, Amelia Hatfield, Dr. Maria Cassioli and Rasha Khader. I would like to thank Dr. Nicholas Kindon, who was always ready to support me throughout my project, for his guidance and friendship. I would also like to thank the undergraduate students who worked with me during my PhD, Angela Klu and Jennifer Ashton.

I would like to thank everyone on the CDT in Advanced Therapeutics and Nanomedicines, especially those in the A03 office. The CDT has provided me

ACKNOWLEDGEMENTS

with a support network throughout my PhD, and friendships that will last a lifetime.

Finally, I would like to acknowledge my funding body, the UK Research and Innovation (UKRI) Engineering and Physical Sciences Research Council (EPSRC) Centre for Doctoral Training (CDT) in Advanced Therapeutics and Nanomedicines [grant number EP/L01646X/1]. I would like to acknowledge the Rosetrees Trust and Stoneygate Trust [grant number M902]. I would also like to thank the Bio-Support Unit (BSU) team at the University of Nottingham for their support and assistance in studies.

LIST OF PUBLICATIONS

1. List of publications from this thesis:

a. Published articles in peer-reviewed literature:

A. Brookes, L. Ji, T. D. Bradshaw, M. Stocks, D. Gray, J. Butler, P. Gershkovich, Is oral lipid-based delivery for drug targeting to the brain feasible?, *Eur. J. Pharm. Biopharm.* 172 (2022) 112-122.

A. Brookes, A. Jewell, W. Feng, T. D. Bradshaw, J. Butler, P. Gershkovich, Oral lipid-based formulations alter delivery of cannabidiol to different anatomical regions in the brain, *Int. J. Pharm.*, 625 (2023) 122651.

b. Manuscripts under preparation:

A. Brookes, P. Gershkovich, D. J. Scurr, M. Alexander, J. Butler, T. D. Bradshaw, Cannabidiol and fluorinated cannabidiol exhibit anti-cancer effects against glioblastoma multiforme.

A. Brookes, W. Feng, H. Chen, T. D. Bradshaw, J. Butler, P. Gershkovich, Higher delivery of fluorinated cannabidiol to the brain and anatomical regions in the brain compared to cannabidiol following oral administration in rats.

2. Additional published articles in peer-reviewed literature:

A. Jewell, A. Brookes, W. Feng, M. Ashford, P. Gellert, J. Butler, P. M. Fischer, D. J. Scurr, M. J. Stocks, P. Gershkovich, Distribution of a highly lipophilic drug cannabidiol into different lymph nodes following oral administration in lipidic vehicle. *Eur. J. Pharm. Biopharm.* 174 (2022) 29-34.

LIST OF ABBREVIATIONS

3D OrbiSIMS	3D Orbitrap secondary ion mass spectrometry
4'-F-CBD	4'-fluoro-cannabidiol
5-HT1A	5-hydroxytryptamine 1A receptor
A2A	Adenosine 2A
ABC	ATP-binding cassette
ACN	Acetonitrile
AD	Alzheimer's disease
AEA	Anandamide
AIC	5-aminoimidazole-4-carboxamide
ATP	Adenosine triphosphate
AUC	Area under the curve
BBB	Blood-brain barrier
BCECs	Brain capillary endothelial cells
BCNU	Carmustine
BCS	Biopharmaceutics classification system
BCSFB	Blood-cerebrospinal fluid barrier
BER	Base excision repair
br	Broad
cART	Combination antiretroviral therapy
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CDCl ₃	Deuterated chloroform
CED	Convection enhanced delivery

LIST OF ABBREVIATIONS

CFA	Complete Freund's adjuvant
CI	Combination index
C _{max}	Maximum concentration
CMs	Chylomicrons
CNS	Central nervous system
CpG	Cytosine-phosphate-guanine
CSF	Cerebrospinal fluid
CV	Column volumes
d	Doublet
DCM	Dichloromethane
DDT	4,4-dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DS	Dravet syndrome
DMSO	Dimethyl sulfoxide
EAE	Experimental autoimmune encephalomyelitis
EC ₅₀	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FAAH	Fatty acid amide hydrolase
FBS	Foetal bovine serum
GBM	Glioblastoma multiforme
GCIB	Gas cluster ion beam
GCTE	Glycerylcaldityltetraether
GI ₅₀	Concentration required to inhibit cell growth by 50%

LIST OF ABBREVIATIONS

GPCR	G protein-coupled receptor
GSCs	Glioma stem cells
HDL	High-density lipoproteins
HED	Human equivalent dose
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HQC	High quality control
IMS	Industrial methylated spirits
i.p.	Intraperitoneal
IS	Internal standard
ITO	Indium tin oxide
i.v.	Intravenous
<i>J</i>	Coupling constant
K_i	Inhibition constant
LC-MS	Liquid chromatography mass spectrometry
LDL	Low-density lipoproteins
LDLR	Low-density lipoprotein receptors
LFCS	Lipid formulation classification system
LGS	Lennox Gastaut syndrome
LLOQ	Lower limit of quantification
LMIG	Liquid metal ion gun
LOD	Limit of detection
LPS	Lipopolysaccharide
LQC	Low quality control

LIST OF ABBREVIATIONS

LRP-1	Low-density lipoprotein receptor-related protein 1
m	Multiplet
MALDI	Matrix-assisted laser desorption/ionisation
MDSCs	Myeloid-derived suppressor cells
MEM	Minimum essential medium
MFP	Molecular formula prediction
MGMT	<i>O</i> ⁶ -methylguanine-DNA methyltransferase
MLN	Mesenteric lymph nodes
MMR	Mismatch repair
MOG ₃₅₋₅₅	Myelin oligodendrocyte protein
MOR	μ-opioid receptor
MQC	Medium quality control
MS	Multiple sclerosis
MTIC	5-(3-methyltriazene-1-yl)imidazole-4-carboxamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVA	Multivariate analysis
NEAA	Non-essential amino acids
ND	Not detectable
NMR	Nuclear magnetic resonance
OCT	Optimal cutting temperature compound
PARP	Poly(ADP-ribose) polymerase
PBPK	Physiological based pharmacokinetics
PBS	Phosphate buffered saline
PC	Principal components

LIST OF ABBREVIATIONS

PCA	Principal component analysis
PEG	Polyethylene glycol
Pen/strep	Penicillin/streptomycin
PET	Positron emission topography
P-gp	P-glycoprotein
PK	Pharmacokinetics
PPAR γ	Peroxisome proliferator-activated receptor gamma
ppm	Parts per million
RE	Relative error
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSD	Relative standard deviation
s	Singlet
s.c.	Subcutaneous
SIMS	Secondary ion mass spectrometry
SD	Standard deviation
t	Triplet
TBI	Traumatic brain injury
THC	Δ^9 -tetrahydrocannabinol
TIC	Total ion count
TLC	Thin layer chromatography
T _{max}	Time of maximum concentration
TMZ	Temozolomide
ToF	Time of flight

LIST OF ABBREVIATIONS

TRPV-1	Transient receptor potential cation channel subfamily V member 1
TRPV-2	Transient receptor potential cation channel subfamily V member 2
TTF	Tumour treating fields
VEGF	Vascular endothelial growth factors
VLDL	Very-low-density lipoproteins
WHO	World Health Organisation

ABSTRACT

Lipid-based formulations are commonly used to improve the oral bioavailability of poorly soluble therapeutics. More recently, these types of formulations have also been explored more to alter delivery to the brain. Certain fatty acids have been reported to increase the permeability of the protective blood-brain barrier (BBB) in a reversible manner following intravascular (i.v.) administration. However, the effect of fatty acids including oleic, linoleic and myristic acids on the BBB has not been explored following oral administration. The work in this thesis explored the delivery of cannabinoids to their site of action within the brain (for example, the frontal lobe for the treatment of glioblastoma multiforme (GBM), or the temporal lobe for treatment of epilepsy) following oral administration in lipid-based formulations. The cannabinoid cannabidiol (CBD) and a fluorinated derivative of CBD, 4'-fluoro-cannabidiol (4'-F-CBD) were studied, with the hypothesis that 4'-F-CBD would exhibit similar pharmacokinetic (PK) properties to CBD but demonstrate higher delivery to the brain due to the addition of fluorine. The work herein began by exploring the anti-cancer properties of the cannabinoids against the aggressive brain cancer, GBM. Both CBD and 4'-F-CBD were shown to inhibit GBM cell growth and overcome two major resistance mechanisms to the standard treatment of GBM with temozolomide (TMZ). The cannabinoids also demonstrated a synergistic response with imidazotetrazine compounds (TMZ and analogue, T25), where high synergy was observed when cells were exposed to a combination of 4'-F-CBD with T25. Preliminary studies investigated the mechanism of anti-cancer activity of CBD, indicating that pathways including DNA-methylation are involved for the first time.

A hypothesis throughout this work was that oral administration of cannabinoids in a lipid-based formulation would increase delivery across the BBB, compared to a lipid-free formulation. This was studied in detail for CBD, with three different lipid-based formulations. Despite reports in the literature of fatty acids improving permeability across the BBB, the lipid-free formulation resulted in the highest delivery of CBD to the whole brain. However, when the individual anatomical brain regions were studied, significant differences in delivery

profiles were observed. Administration in the lipid-based sesame oil formulation resulted in the highest exposure of the rostral anatomical regions of the brain to CBD, suggesting that the high oleic acid content of the sesame oil could have contributed to increased delivery of CBD to these regions. On the other hand, the lipid-free formulation resulted in high delivery to the caudal regions of the brain. High delivery of CBD to common locations of GBM tumours was observed following oral administration, demonstrating potential of the application of orally administered CBD to treat GBM.

The work herein then built on the demonstration of differing delivery of CBD following oral administration in lipid-based formulations by investigating the difference in immunosuppressive effects of CBD against multiple sclerosis (MS) following administration in lipid-based and lipid-free formulations. It was hypothesised that the high delivery of CBD to the lymphatic system following oral administration in lipid-based formulations would result in increased efficacy of CBD. Additionally, correlation of lower severity of MS symptoms with high delivery of CBD to the anatomical brain regions relevant to the MS model used (experimental autoimmune encephalomyelitis (EAE)) could also be made. The results of the preliminary studies indicate that oral administration of CBD in the olive oil formulation resulted in fewer mice reaching the highest clinical severity score, compared to the vehicle only control group. This trend was not observed when CBD was administered in the lipid-free formulation, indicating support for the hypothesis. This study was preliminary and should be explored further in order to confirm the results.

Finally, the *in vivo* PK and biodistribution of 4'-F-CBD are reported for the first time. It was hypothesised that 4'-F-CBD would behave similarly to CBD in most cases, but due to the addition of the fluorine atom, higher delivery to the brain could be expected. Additionally, previously reported high potency of 4'-F-CBD in *in vivo* behavioural assays, compared to CBD, suggests a potential difference in the biodistribution of the cannabinoid. The PK of 4'-F-CBD following oral administration was similar to that of CBD in both lipid-free and lipid-based formulations. However, study of the biodistribution indicated that the brain was exposed to higher levels of 4'-F-CBD than CBD, supporting the hypothesis that fluorination of CBD has increased the delivery to the brain. The detailed

ABSTRACT

biodistribution of 4'-F-CBD to anatomical brain regions and other tissues is reported for the first time to the best of our knowledge, which is essential for the physiological based PK (PBPK) modelling of 4'-F-CBD, and its potential future applications.

Overall, this thesis demonstrates that the systemic biodistribution of cannabinoids following oral administration differs with the oral formulation. With the example of the anti-cancer application of cannabinoids, this thesis highlights the necessity to investigate the delivery to the specific anatomical brain regions of interest.

LIST OF FIGURES

Figure 1.1. Schematic diagram of the structure of the BBB showing the blood vessel surrounded by BCECs, supported by pericytes and astrocytes. Movement across the BBB is depicted *via* transcellular diffusion, movement through tight junctions and active transport by P-glycoprotein (P-gp). Reproduced from Lalatsa *et al* [26] with permission from Elsevier.

Figure 1.2. Different routes of transport across the BCECs of the BBB depicted by A) paracellular transport *via* tight junctions, B) passive diffusion, C) transport proteins such as P-gp, D) receptor-mediated transcytosis and E) adsorptive transcytosis. Reproduced from Lalatsa *et al* [26] with permission from Elsevier.

Figure 1.3. The biopharmaceutics classification system (BCS) showing class I-IV by aqueous solubility and membrane permeability.

Figure 1.4. Schematic diagram describing the sequential steps in the digestion of lipids and subsequent absorption following oral administration. Short and medium chain fatty acids are shown to enter the systemic circulation directly. Long chain fatty acids undergo re-esterification and associate into chylomicrons to enter the lymphatic system and then the systemic circulation *via* the thoracic duct. Reproduced from Brookes *et al* [62], with permission from Elsevier.

Figure 1.5. Common locations of GBM tumours in the brain. Produced using data from Brodbelt *et al* [90].

Figure 1.6. Chemical structure of TMZ (194.12 g/mol).

Figure 1.7. The ring-opening of TMZ at physiological pH to release the active methyldiazonium cation, based on explanation by Singh *et al* [91].

Figure 1.8. Chemical structure of T25 (258.3 g/mol), a TMZ analogue.

Figure 1.9. Chemical structures of A) THC (314.5 g/mol) and B) CBD (314.5 g/mol).

Figure 1.10. Molecular targets of CBD *in vivo*. CBD is depicted to interact agonistically with A1 receptors, 5-hydroxytryptamine 1A receptor (5-HT_{1A}) and transient receptor potential cation channel subfamily V member 1 (TRPV-1), partially agonistically with dopamine receptor 2 (D2), antagonistically with G-protein receptor 55 (GPR55), CB1 and CB2 and FAAH. CBD is a negative allosteric modulator of μ -opioid receptor (MOR) and inhibitor of equilibrate nucleotide transporter (ENT), endocannabinoid membrane transporter (EMT) and Na⁺ and Ca²⁺ channels. Reproduced from Almeida *et al* [112], with permission from John Wiley and Sons.

Figure 1.11. Plasma PK profiles of CBD following oral administration in lipid-free (propylene glycol:ethanol:water, 80:10:10 v/v/v) and lipid-based formulations at 12 mg/Kg to rats. Figure reproduced from Feng *et al* [151], with permission from Elsevier.

Figure 1.12. Chemical structure of 4'-F-CBD (332.5 g/mol).

Figure 2.1. Synthesis of 4'-fluoro-cannabidiol.

Figure 3.1. Dose-response curves of MRC-5 (non-tumourigenic control) cell line treated with test agents TMZ, T25, CBD and 4'-F-CBD after A) 3 and B) 6 days exposure. Cell growth represented as % of control (non-treated). Data measured by MTT assay and confirmed by cell count assay (Appendix 3.2). Data are presented as mean $\pm$ standard deviation (SD), three independent repeats of n = 5.

Figure 3.2. GI₅₀ of cannabinoids CBD and 4'-F-CBD compared to DNA-alkylating agents TMZ and T25 against A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after 3- and 6-days exposure. Data measured by MTT assay and confirmed by cell count assay (Appendix 3.3). Data are presented as mean $\pm$ SD, three independent repeats of n = 5. One-way ANOVA with Dunnett's multiple comparisons performed, comparing test agents to TMZ, α = 0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. The data are also presented in Table 3.1.

Figure 3.3. Isobolograms representing the combined effect of TMZ and CBD against U373-V (GBM control, -MGMT, +MMR) for A) 3 and B) 6 days exposure, U373-M (GBM, +MGMT, +MMR) for C) 3 and D) 6 days exposure and HCT116 (-MGMT, -MMR) for E) 3 and F) 6 days exposure. The blue line represents an additive response, data points above the line indicate an antagonistic response and points below the line indicate a synergistic response. Data measured by MTT assays and confirmed by cell count assays (Appendix 3.7). Dose-response curves are shown in Appendix 3.8 and 3.9. Data presented as mean $\pm$ SD, three independent repeats of $n = 5$.

Figure 3.4. Isobolograms representing the combined effect of TMZ and 4'-F-CBD against U373-V (GBM control, -MGMT, +MMR) for A) 3 and B) 6 days exposure, U373-M (GBM, +MGMT, +MMR) for C) 3 and D) 6 days exposure and HCT116 (-MGMT, -MMR) for E) 3 and F) 6 days exposure. The blue line represents an additive response, data points above the line indicate an antagonistic response and points below the line indicate a synergistic response. Data measured by MTT assay and confirmed by cell count assay (Appendix 3.7). Dose-response curves are shown in Appendix 3.10 and 3.11. Data presented as mean $\pm$ SD, three independent repeats of $n = 5$.

Figure 3.5. Isobolograms representing the combined effect of T25 and CBD against A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after 3 days exposure. The blue line represents an additive response, data points above the line indicate an antagonistic response and points below the line indicate a synergistic response. Data measured by MTT assays and confirmed by cell count assays (Appendix 3.7). Dose-response curves are shown in Appendix 3.12. Data presented as mean $\pm$ SD, three independent repeats of $n = 5$.

Figure 3.6. Isobolograms representing the combined effect of T25 and 4'-F-CBD against A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after 3 days

exposure. The blue line represents an additive response, data points above the line indicate an antagonistic response and points below the line indicate a synergistic response. Data measured by MTT assays and confirmed by cell count assays (Appendix 3.7). Dose-response curves are shown in Appendix 3.13. Data presented as mean $\pm$ SD, three independent repeats of $n = 5$.

Figure 3.7. Targeted analysis of 3D OrbiSIMS data from U373-V cells exposed to CBD, TMZ or a combination of CBD with TMZ or CBD with T25 for 3-72 h. Graphs show data as peak intensity (secondary ion counts) normalised to TIC for A) methyl-guanine, B) methyl-cytosine, C) methyl-adenine and D) methyl-thymine. ND = not detectable. Data presented as an average of $n = 3$ technical repeats. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$.

Figure 3.8. Targeted analysis of 3D OrbiSIMS data from U373-V cells treated with CBD, TMZ or a combination of CBD with TMZ or CBD with T25 for 3-72 h. Graphs show data as peak intensity (secondary ion counts) normalised to TIC for A) propargyl-guanine, B) propargyl-cytosine, C) propargyl-adenine and D) propargyl-thymine. ND = not detectable. Data presented as an average of $n = 3$ technical repeats. One-way ANOVA with Dunnett's multiple comparisons was performed, comparing treatment to the non-treated control, $\alpha = 0.05$, no significant differences were found.

Figure 4.1. CBD concentration in the whole brain vs. plasma after oral administration to rats (12 mg/Kg) in various lipid-based vehicles. Tissues were collected at plasma T_{max} . Multiple t-tests were performed, $\alpha = 0.05$. Data are presented as mean $\pm$ standard deviation (SD), brain $n = 3$, plasma $n = 6$ (collected in previous pharmacokinetics (PK) studies (Feng *et al* [151, 155])).

Figure 4.2 CBD concentration in anatomical brain regions A) olfactory bulb, B) frontal lobe, C) temporal lobe and D) brainstem vs. plasma following oral administration to rats (12 mg/Kg) in various lipid-based vehicles. Tissues were collected at plasma T_{max} . Multiple t-tests were performed, $\alpha = 0.05$,

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Data are presented as mean $\pm$ SD, brain $n = 3$, plasma $n = 6$ (collected in previous PK studies (Feng *et al* [151, 155])).

Figure 4.3. CBD concentration in the whole brain vs. plasma after oral administration to rats (12 mg/Kg) in the A) lipid-free, B) sesame oil, C) coconut oil and D) rapeseed oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, **** = $p < 0.0001$. Data are presented as mean $\pm$ SD, brain $n = 3$, plasma $n = 6$ (collected in previous PK studies (Feng *et al* [151, 155])). The PK parameters are presented in Table 4.1.

Figure 4.4. CBD concentration in anatomical brain regions vs. plasma after oral administration to rats (12 mg/Kg) in the A) lipid-free, B) sesame oil, C) coconut oil and D) rapeseed oil formulations. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Data presented as mean $\pm$ SD, brain $n = 3$, plasma $n = 6$ (collected in previous PK studies (Feng *et al* [151, 155])).

Figure 4.5. CBD concentration in anatomical brain regions A) olfactory bulb, B) frontal lobe, C) striatum, D) hippocampus, E) temporal lobe, F) parietal lobe, G) occipital lobe, H) medulla pons and I) cerebellum after oral administration to rats (12 mg/Kg) in lipid-free, sesame oil, coconut oil and rapeseed oil formulations. One-way ANOVA with Dunnett's multiple comparisons performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. Differences of lipid-based formulations to the lipid-free formulation are shown. Data are presented as mean $\pm$ SD, $n = 3$.

Figure 4.6. The expected and measured CBD [M-H]⁻ peak intensity (secondary ion counts) normalised to TIC in anatomical brain regions of rats 2 h following oral administration in a sesame oil formulation (12 mg/Kg). The expected CBD [M-H]⁻ intensities were calculated from HPLC-UV analysis. ND = not detectable. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$. The data are presented as mean $\pm$ SD. The expected intensities were calculated from triplicate biological repeats, the measured intensities were performed once.

Figure 4.7. CBD [M-H]⁻ peak intensity (secondary ion counts) normalised to the TIC in anatomical brain region homogenate tissues and brain sections of rats 2 h following oral administration in a sesame oil formulation (12 mg/Kg). ND = not detectable. Multiple t-tests were performed, $\alpha = 0.05$. The data are presented as mean $\pm$ SD, $n = 1$, apart from the frontal lobe section where $n = 2$.

Figure 5.1. CBD concentration in the whole brain vs. serum following oral administration to mice (30 mg/Kg) in the A) lipid-free, B) sesame oil and C) olive oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, *** = $p < 0.001$. Data are presented as mean $\pm$ standard deviation (SD), $n = 4$. The PK parameters of the data are presented in Table 5.1.

Figure 5.2. CBD concentration in anatomical brain regions vs. serum following oral administration to mice (30 mg/Kg) in the A) lipid-free, B) sesame oil and C) olive oil formulations. One-way ANOVA with Dunnett's multiple comparisons were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$. The data are presented as mean $\pm$ SD, $n = 4$.

Figure 5.3. CBD concentration in anatomical brain regions A) hippocampus, and B) cerebellum following oral administration to mice (30 mg/Kg) in the lipid-free, sesame oil and olive oil formulations. One-way ANOVA with Dunnett's multiple comparisons were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$. The data are presented as mean $\pm$ SD, $n = 4$. PK parameters of the data are presented in Table 5.2.

Figure 5.4. Progression of EAE as clinical score after no administration (control: no dose) or oral administration of the CBD in lipid-free or olive oil formulations, or vehicle only. When administered, CBD was dosed at 50 mg/Kg in formulations of 50 mg/mL. Animals were dosed once daily for 8-days. The data are presented as the mean clinical score.

Figure 6.1. Plasma PK profile of 4'-F-CBD following oral administration at 12.7 mg/Kg (38 μ M/Kg) to rats in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. The data are presented as mean $\pm$ standard deviation (SD), where plasma $n = 6$. The PK parameters are presented in Table 6.1.

Figure 6.2. Plasma PK profiles of 4'-F-CBD compared to CBD following oral administration at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) to rats in A) lipid-free and B) sesame oil formulations. The PK data for CBD was calculated from samples collected in previous study (Feng *et al* [155]). Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$. The data are presented as mean $\pm$ SD, $n = 6$. The PK parameters are shown in Table 6.1.

Figure 6.3. 4'-F-CBD concentration in the whole brain vs. plasma following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg) in A) lipid-free and B) sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$. The data are presented as mean $\pm$ SD, plasma $n = 6$, brain $n = 3$. The PK parameters are shown in Table 6.2.

Figure 6.4. 4'-F-CBD concentration in anatomical brain regions vs. plasma following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg) in A) lipid-free and B) sesame oil formulations. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. The data are presented as mean $\pm$ SD, plasma $n = 6$, brain $n = 3$.

Figure 6.5. 4'-F-CBD concentration in anatomical brain regions A) olfactory bulb, B) frontal lobe, C) striatum, D) hippocampus, E) temporal lobe, F) parietal lobe, G) occipital lobe, H) medulla pons and I) cerebellum following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$. The data are presented as mean $\pm$ SD, $n = 3$. The data are also shown in Figure 6.4 and the PK parameters are presented in Table 6.3.

Figure 6.6. Cannabinoid concentration in the whole brain following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) in A) lipid-free and B) sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Data are presented as mean $\pm$ SD, $n = 3$. The PK parameters are presented in Table 6.4.

Figure 6.7. Cannabinoid concentration in anatomical brain regions A) olfactory bulb, B) frontal lobe, C) striatum, D) hippocampus, E) temporal lobe, F) parietal lobe, G) occipital lobe, H) medulla pons and I) cerebellum following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. The data are presented as mean $\pm$ SD, $n = 3$. The PK parameters of 4'-F-CBD are shown in Table 6.3, and of CBD in Chapter 4, Table 4.2.

Figure 6.8. 4'-F-CBD concentration in lymph nodes vs. plasma following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg) in A) lipid-free vehicle and B) sesame oil formulation. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. The data are presented as mean $\pm$ SD, plasma $n = 6$, lymph nodes $n = 3$. The PK parameters are presented in Table 6.5.

Figure 6.9. 4'-F-CBD concentration in the A) kidneys, B) liver, C) lungs and D) spinal cord vs. plasma following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. The data are presented as mean $\pm$ SD, plasma $n = 6$, tissue $n = 3$. The PK parameters are presented in Table 6.6.

LIST OF TABLES

Table 1.1. The lipid formulation classification system (LFCS) as described by Pouton in 2000 and 2006 [54, 55].

Table 2.1. Concentrations of test agents used in MTT assay.

Table 2.2. Combinations of test agents assessed against U373-V, U373-M and HCT116 cell lines by MTT.

Table 2.3. Concentrations of test agents used for combination MTT assays.

Table 2.4. Concentration range of test agents used for cell count assays.

Table 2.5. Combinations of test agents assessed against U373-V, U373-M and HCT116 cell lines by cell count.

Table 2.6. Concentrations of test agents used for combination cell count assays.

Table 2.7. Administration groups of mice for EAE model efficacy study.

Table 2.8. Concentration of test agent exposed to cells for preparation of cells dosed in combination for 3D OrbiSIMS analysis.

Table 3.1. GI₅₀ values of test agents TMZ, T25, CBD and 4'-F-CBD individually assessed against U373-V, U373-M and HCT116 cell lines after 3- and 6-days exposure. Data calculated from MTT assays and presented as mean $\pm$ SD, three independent repeats of n = 5. The data are also presented in Figure 3.2.

Table 3.2. A summary of CIs of cannabinoids CBD and 4'-F-CBD administered in combination with TMZ or analogue, T25 after 3- or 6-days exposure against U373-V, U373-M and HCT116 cell lines. Data represented as a range for the different combination concentrations tested; full data can be found in Appendix 3.6.

Table 3.3. Targeted search for anti-cancer mechanism of action of CBD by 3D OrbiSIMS. U373-V cells were treated with CBD for 3, 6, 24 and 72 h and are compared to a non-treated control. The table shows the peak intensity (secondary ion counts) normalised to the TIC and deviation below in ppm.

Data presented as an average of $n = 3$ technical repeats. One-way ANOVA with Dunnett's multiple comparisons performed, $\alpha = 0.05$, $* = p < 0.05$. The data are also shown in Figure 3.7.

Table 3.4. Targeted search for anti-cancer mechanism of action of CBD and evidence of T25 activity by 3D OrbiSIMS. U373-V cells were exposed to CBD alone or with T25 for 3 h and are compared to a non-treated control. The table shows the peak intensity (secondary ion counts) normalised to the TIC and deviation below in ppm. Data presented as an average of $n = 3$ technical repeats. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, no significant differences were found. The data are also shown in Figure 3.8.

Table 4.1. PK parameters of CBD concentration-time profiles in plasma and whole brain after oral administration to rats (12 mg/Kg). One-way ANOVA with Dunnett's multiple comparisons was performed to compare lipid-based formulations to the lipid-free vehicle, $\alpha = 0.05$, $* = p < 0.05$, $**** = p < 0.0001$. Data are presented as mean $\pm$ SD, brain $n = 3$, plasma $n = 6$ (collected in previous PK studies (Feng *et al* [151, 155])). The data are also presented in Figure 4.3.

Table 4.2. PK parameters of CBD concentration-time profiles for the anatomical brain regions following oral administration to rats (12 mg/Kg). One-way ANOVA with Dunnett's multiple comparisons were performed, $\alpha = 0.05$, $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$, $**** = p < 0.0001$. Differences of lipid-based formulations to the lipid-free formulation are shown. Data are presented as mean $\pm$ SD, $n = 3$. The data are also presented in Figure 4.5.

Table 4.3. CBD concentration of anatomical rat brain region homogenates 2 h following oral administration (12 mg/Kg) in a sesame oil formulation (determined by means of HPLC-UV) and expected $[M-H]^-$ peak intensity (secondary ion counts) normalised to TIC. The data are presented as mean $\pm$ SD, HPLC-UV analysis was performed in biological triplicates.

Table 4.4. Fatty acid composition of the oils selected for the lipid-based formulations for *in vivo* CBD biodistribution studies, taken from the literature [151, 232].

Table 5.1. PK parameters of CBD concentration-time profiles in serum and whole brain following oral administration to mice (30 mg/Kg). One-way ANOVA with Dunnett's multiple comparisons was performed for differences between the lipid-free and lipid-based formulations, $\alpha = 0.05$. Data are presented as mean $\pm$ SD, $n = 4$, and are shown in Figure 5.1.

Table 5.2. PK parameters of CBD concentration-time profile in serum, whole brain and anatomical brain regions following oral administration (30 mg/Kg) to mice in lipid-free, sesame and olive oil formulations. One-way ANOVA with Dunnett's multiple comparisons were performed, $\alpha = 0.05$, no significant differences were found. The data are presented as mean $\pm$ SD, $n = 4$.

Table 5.3. EAE severity as percentage of mice reaching score 4.0 after no administration (control: no dose) or oral administration of CBD in lipid-free or olive oil formulations, or vehicle only. When administered, CBD was dosed at 50 mg/Kg in formulations of 50 mg/mL. Animals were dosed once daily for 8-days.

Table 5.4. Fatty acid composition of the oils selected for the lipid-based formulations for *in vivo* CBD biodistribution studies, taken from the literature [151].

Table 6.1. PK parameters of 4'-F-CBD and CBD concentration-time profiles in plasma following oral administration at 38 μ M/Kg (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) to rats. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Differences are shown between the lipid-free and lipid-based formulations of the same cannabinoid, no differences were found between 4'-F-CBD and CBD administration in the same formulation. The data are also presented in Figures 6.1 and 6.2. The CBD PK data was calculated from plasma samples collected in a previous study (Feng *et al* [155]). The data are presented as mean $\pm$ SD, $n = 6$.

Table 6.2. PK parameters of 4'-F-CBD concentration in the plasma and whole brain following oral administration at 38 μ M/Kg (12.7 mg/Kg) to rats. The data are also presented in Figure 6.3. One-way ANOVA with Dunnett's

multiple comparisons was performed, $\alpha = 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, differences between the lipid-free and lipid-based formulations are shown. The data are presented as mean $\pm$ SD, plasma $n = 6$, brain $n = 3$.

Table 6.3. PK parameters of 4'-F-CBD concentration-time profiles in anatomical brain regions following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$. The data are presented as mean $\pm$ SD, $n = 3$. The data are also presented in Figure 6.5.

Table 6.4. PK parameters of cannabinoid delivery to the whole brain following oral administration at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) to rats in both lipid-free and sesame oil formulations. The data are also presented in Figure 6.6. One-way ANOVA with Dunnett's multiple comparisons was performed, comparisons of 4'-F-CBD to CBD are shown, $\alpha = 0.05$, * = $p < 0.05$. The data are presented as mean $\pm$ SD, $n = 3$.

Table 6.5. PK parameters of 4'-F-CBD concentration-time profiles in lymph nodes following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, no significant differences between formulations were observed. The data are presented as mean $\pm$ SD, $n = 3$. The data are also presented in Figure 6.8.

Table 6.6. PK parameters of 4'-F-CBD concentration-time profiles in organs following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, no significant differences between formulations were observed. The data are presented as mean $\pm$ SD, $n = 3$. The data are also presented in Figure 6.9.

CHAPTER 1: General introduction

The review of the literature in this Chapter, section 1.1 (Drug delivery to the brain) was published in the European Journal of Pharmaceutics and Biopharmaceutics: A. Brookes, L. Ji, T. D. Bradshaw, M. Stocks, D. Gray, J. Butler, P. Gershkovich, Is oral lipid-based delivery for drug targeting to the brain feasible?, *Eur. J. Pharm. Biopharm.* 172 (2022) 112-122.

1.1 Drug delivery to the brain

1.1.1 The need for brain targeting

Drugs must reach their pharmacological site of action to be effective. If the target tissue is in the brain, the drug needs to penetrate the blood-brain barrier (BBB). Conditions such as Alzheimer's disease (AD), traumatic brain injury (TBI) and multiple sclerosis (MS), as well as many other illnesses such as brain tumours or human immunodeficiency virus (HIV) reservoirs, are examples where the drug is required to reach the brain tissue for effective treatment.

Treatment for cancers (including brain tumours) usually includes a combination of surgery, radiotherapy and chemotherapy [1]. However, surgery is not always possible, and in many cases, treatment leads to only modest survival improvements (e.g., several months to a year for glioblastoma multiforme (GBM) patients) [1]. Brain surgery to remove a tumour often allows for a one-off direct injection of chemotherapeutics into the surrounding tissue, which is advantageous compared to systemic delivery, as many chemotherapeutic agents struggle to cross the BBB [1]. There has been some development in drug delivery to brain tumours by alternative administration, including biodegradable wafers inserted during surgery for the slow release of chemotherapeutics [1]. Alternative methods of drug delivery to the brain, such as nanocarriers, naturally occurring 'cages' (e.g. apoferritin to transport agents across the BBB) [2], or the convection-enhanced approach to deliver chemotherapeutics across the BBB [3] are also being explored. Continual release by the neurosurgically placed microcatheters allows transfusion of drug through 95% of the tumour, something

not possible with systemic administration [2]. However, overall progress of new treatment approaches is slow and the prognosis for brain cancer patients unfortunately remains very poor.

HIV is treated with combination antiretroviral therapy (cART), but a complete cure cannot currently be achieved [4]. Often, during treatment, HIV ribonucleic acid (RNA) is not detectable in blood, but once treatment is stopped, viral RNA is detected in the blood again within weeks [4]. This is due to reservoirs of HIV-infected cells remaining, even under effective cART treatment. These reservoirs are present in a number of difficult to reach tissues including bone marrow, the lymphatic system, the genital tract, and the brain [4, 5]. Since many cART agents do not efficiently penetrate the BBB, it is difficult to achieve effective concentrations of antiretrovirals in the brain reservoir [4, 6]. Approaches to improve the delivery of cART agents to the brain include drug delivery systems such as liposomes, micelles, and nanoparticles. However, delivery of cART agents to the brain is only part of the problem. It is also important to ensure cART agents are effective at eradicating HIV from central nervous system (CNS)-residing cells [4]. Asahchop *et al* (2017) compared the EC₅₀ (half maximal effective concentration) values of several cART agents including darunavir, etravirine and dolutegravir in human foetal microglia and bone marrow derived macrophages [7]. They found that the EC₅₀ was significantly higher for human foetal microglia than bone marrow derived macrophages, indicating a decreased efficacy of the agents against infected microglial cells [7].

Alzheimer's Disease (AD) is an incurable, age-related neurodegenerative disorder. Much research focuses on early diagnosis for the best management of symptoms. The two main hallmarks of AD, β -amyloid plaque deposition and neurofibrillary tangles, are the focus of diagnosis [8, 9]. There has also been increased research into multi-target strategies to manage symptoms [9]. However, as most, if not all, AD treatments would need to reach the brain, appropriate drug formulation and administration should be an integral part of the drug development programme.

Traumatic brain injury (TBI) is usually the result of a blow to the head, and varies greatly in how it presents itself, from mild, temporary brain alterations to

a comatose state and even death in severe cases [10]. The desired localisation of treatment is sometimes only a small area of torn tissues but can cover the whole brain in extreme cases [11]. Depending on the extent of the damage, treatment can include daily cognitive therapy and surgery as well as preventative measures, such as anti-epileptics [11]. The future of TBI treatment looks to therapeutic neuroprotectors, administered within hours of damage, to minimise the need for invasive surgery, and prevent some of the secondary damage caused by TBI [11]. The effectiveness of such agents would rely on good BBB penetration, as well as drug potency.

MS is an autoimmune disease, in which the immune system attacks axons and myelin in the spinal cord and the brain. Symptoms include problems with movement, balance, and vision [12, 13]. Treatments help to manage symptoms, as there is currently no cure. MS is an example where improving delivery of therapeutics across the BBB is important in the treatment of symptoms and progression towards a cure [2].

As highlighted above, enhancing the delivery of therapeutic agents across the BBB is beneficial for the treatment of many diseases. Sometimes, the need to deliver drugs to tissues within the brain leads to extremely invasive approaches, such as convection-enhanced delivery, as discussed above, and direct transcranial injection [1, 14]. Another invasive method used is the deposition of an implant, commonly comprised of genetically engineered cells or polymeric matrices containing drug [15]. Implants need to be deposited close to the site of action, as drug diffusion decreases exponentially with distance (generally a 90% reduction in drug concentration at 500 μM distance)[14, 15]. This leads to inconsistencies in drug concentrations across the cells, with cells closest to the implant experiencing very high concentrations [15]. This also sometimes leads to the situation in which multiple implants are required at the same time for drug to reach all of the target tissues. Other potential options for administration include intrathecal injection [15] and the exploitation of temporary disruption to the CNS protective BBB [15]. For chronic, long-term illnesses these are not sustainable treatment options, as ideally the patient would be able to administer

their own treatments at home [15]. Two main options for non-invasive delivery are oral administration and intranasal spray, both of which are easily self-administered.

Trans-nasal delivery can lead to direct movement of drugs from the submucosal layer in the nasal cavity to the cerebrospinal fluid (CSF), entering predominantly *via* the olfactory bulb [14, 16-18]. For example, trans-nasal delivery of progesterone resulted in CSF concentrations of the drug exceeding concentrations in plasma [14, 19]. It is unclear whether many drugs could be delivered to the brain efficiently using this route due to the delivery method limitations. For instance, large doses cannot be administered this way. High variability in the amount of delivered drug can also be expected, especially as it is difficult to deliver drug consistently to the olfactory bulb. Induced minor local injury by trans-nasal delivery could also occur, meaning some molecules that would not be expected to enter the CSF are eventually able to do so *via* trans-nasal delivery, such as vitamin B12 [14]. However, this often requires delivery of high volumes, which can cause local injury at volumes >100 μ L in adults [14, 20].

Intranasal delivery appears to be a convenient option for patients to self-administer treatment at home for long-term illnesses. However, problems arise both in formulation development, and delivery itself. Drugs for intranasal delivery are usually formulated as nanoparticles, rather than as a solution [16]. As mentioned above, there is also a challenge when trying to deliver high doses *via* the intranasal route, as this could require larger volumes than are feasible, or extensive repeated administration with potential damage to tissues [14, 16, 20]. There are also limitations due to nasal-mucociliary clearance, a natural defence mechanism that is variable across humans, and in disease states [16, 17]. Therefore, whilst intranasal delivery is attractive from the perspective of patient compliance, only highly potent molecules can be delivered this way at present and there are many challenges that still need to be overcome.

1.1.2 The blood-brain barrier

The main hurdle to overcome for any approach aimed to deliver the drugs to therapeutic targets within the brain is the BBB. The BBB is a protective barrier, present in all organisms with a developed CNS, preventing the majority of compounds crossing from the blood into the CNS [14, 21, 22]. A secondary barrier, the blood-cerebrospinal fluid barrier (BCSFB), with larger openings between junctions for fluid transfer, is also able to protect the CNS from some blood-borne substances [21, 23].

The BBB protects the brain from potentially neurotoxic substances including metabolites and proteins [21]. Although cells in the CNS in early life are able to repair themselves and replicate during growth, in adults they have lower capacity for replication [21]. The CNS protective barriers deteriorate at a steady rate, as cells and neurons die without being replaced [21]. This occurs naturally in healthy brains, but the deterioration could be accelerated in disease states [21].

The structure of the BBB, shown in Figure 1.1, consists of a monolayer of brain capillary endothelial cells (BCECs) surrounding the blood capillaries [24]. The BCECs create the largest blood-brain interface for molecular exchange, with a total average area of 12 - 18 m² in adult humans [21]. BCECs have small numbers of fenestrations (pores) and much denser content of glycocalyx than other vessels (40% compared to 15% in cardiac and 3% in pulmonary vessels). This structure creates the extensive tight junctions characteristic of the BBB, preventing most large or hydrophilic molecules from interacting with, and therefore penetrating, the BBB [21, 22, 24, 25]. This results in a much lower protein content in the CSF compared to the plasma [21, 25]. Small non-ionisable molecules, including O₂ and CO₂, can easily diffuse across the barrier [22]. Transporters and receptor-mediated endocytosis are responsible for the movement of most nutrients, e.g., amino acids, glucose, insulin and iron into the brain [22, 25].

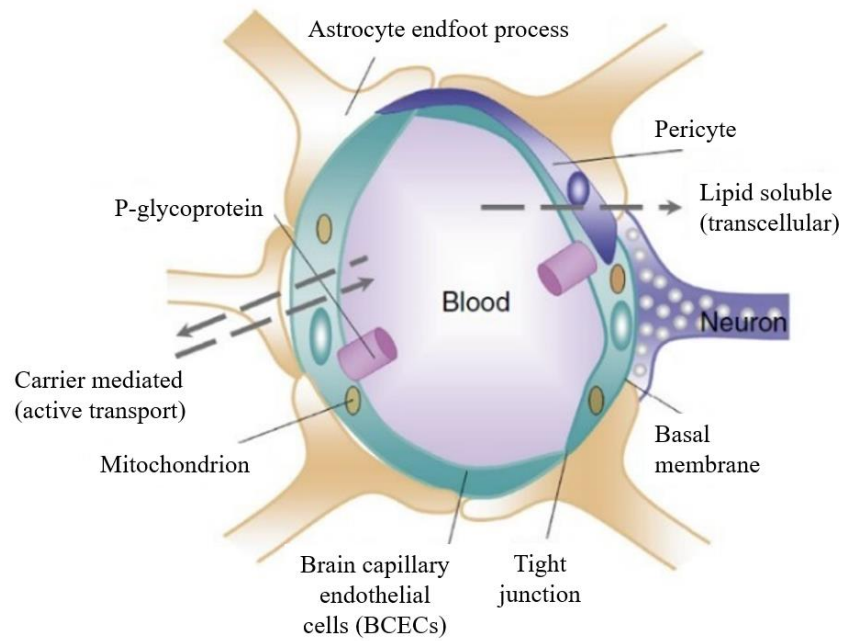


Figure 1.1. Schematic diagram of the structure of the BBB showing the blood vessel surrounded by BCECs, supported by pericytes and astrocytes. Movement across the BBB is depicted via transcellular diffusion, movement through tight junctions and active transport by P-glycoprotein (P-gp). Reproduced from Lalatsa *et al* [26] with permission from Elsevier.

Transcellular permeation is high in peripheral endothelial cells but limited across BCECs. BCECs form a more continuous lining, limiting gaps between the cells. BCECs also have low levels of transcytosis, which is the main pathway for larger molecules to cross the BBB [21, 24]. However, some large molecules are able to cross by receptor-mediated or adsorptive-mediated transcytosis, with a small number of non-specific large molecules being able to cross by non-receptor mediated mechanisms [14, 21]. A detailed depiction of the BBB and possible transport pathways across the barrier are shown in Figure 1.2.

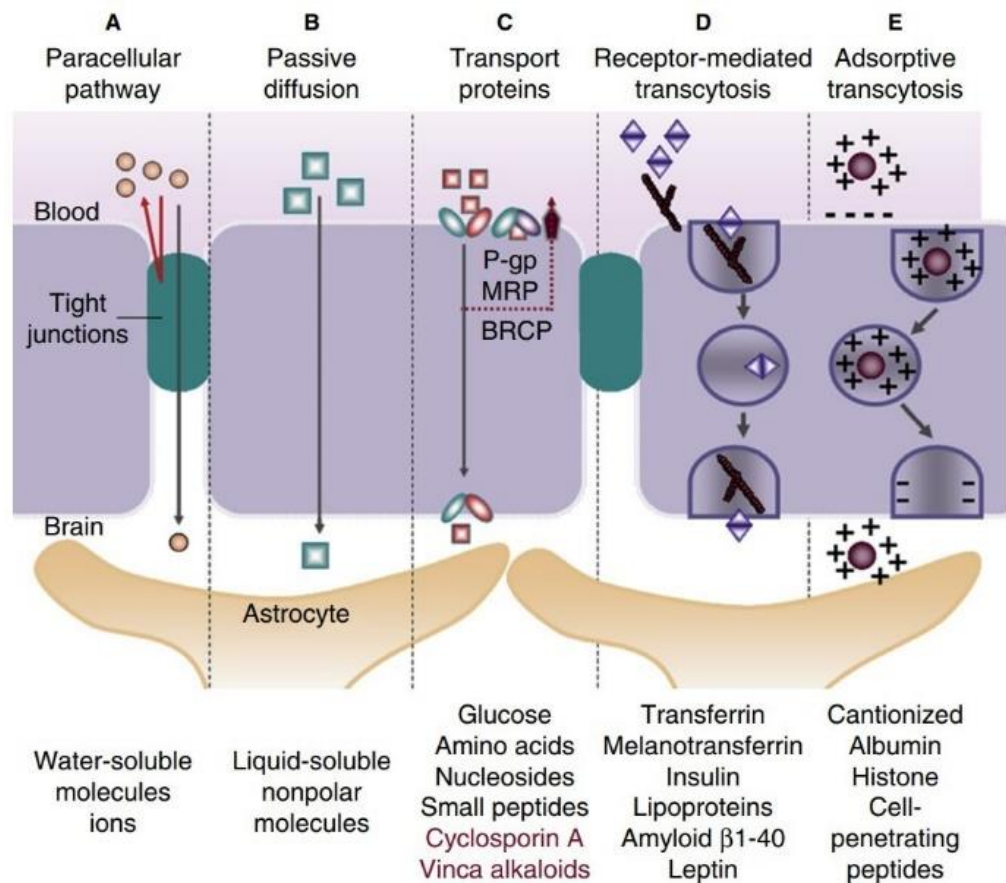


Figure 1.2. Different routes of transport across the BCECs of the BBB depicted by A) paracellular transport *via* tight junctions, B) passive diffusion, C) transport proteins such as P-gp, D) receptor-mediated transcytosis and E) adsorptive transcytosis. Reproduced from Lalatsa *et al* [26] with permission from Elsevier.

Surrounding the BCECs are astrocytes and pericytes, forming the basement membrane, which acts as a supportive structure [21, 22]. The basement membrane is essential to the key structures allowing movement across the BBB. For example, astrocytes have been shown to tighten the endothelium in leaky vessels and are suggested to be responsible for inducing BBB properties in endothelial cells [22, 27].

Tight junctions, shown in Figure 1.2 (A), are one of the first BBB features to develop, forming in the cell adhesions, creating tight gaps for very small, water-soluble molecules to move through *via* paracellular transport [21]. Tight junctions are unique in their structure and molecular composition (proteins

present in the gaps link the two cells together), creating a high-resistance electrical barrier [21, 24]. Tight junction proteins consist mainly of claudin, occludin and junction adhesion molecules, all of which are variable in number across the junctions [22]. Claudins are essential to the barrier function as they maintain the seal, or ‘tightness’, of the junction [22]. Passive diffusion across BCECs of the BBB is also possible for nonpolar molecules.

Transporters can both aid molecules entering the brain, as well as remove substances from endothelial cells back into the blood. Solute transporters carry substrates such as glucose, amino acids, and fatty acids down the concentration gradients [21, 24, 25]. Efflux, or adenosine triphosphate (ATP)-binding cassette (ABC) transporters, consume ATP to remove small molecules from the brain into the blood [21, 24]. Of particular note is drug efflux pump P-glycoprotein (P-gp; ABCB1; multidrug resistance protein-1), where enhanced expression is induced by chemotherapeutic agents, and it is therefore largely responsible for multi-drug resistance [28]. The presence of P-gp on BCEC exports and blocks the retention of many potentially efficacious drugs.

Figure 1.2 (D) and (E) describe the limited transcytosis across the BBB. Receptor-mediated transcytosis transports specific peptides and proteins, such as insulin, across the BBB. Reduced intracellular degradation is thought to be a functional feature of BCECs, allowing the transport of specific molecules through the cells. This feature is an important homeostatic requirement [26]. Absorptive-mediated transcytosis describes the uptake of cationic molecules into the brain, such as albumin.

Low-density lipoprotein receptors (LDLR) are another important aspect of the BBB, allowing transcytosis across the BBB [29]. Such receptors have led to the approach of administering statins with a LDLR targeting, encapsulated, poorly BBB-permeable drug, in order to increase permeability *via* LDLRs [30]. Low-density lipoprotein receptor-related protein 1 (LRP-1) is of particular interest, playing an important role in physiological and pathological conditions. LRP-1 is reported to regulate certain tight junction proteins, and is responsible for inducing BBB opening after ischemic attack [31].

There are some empirical rules describing which molecules are likely to permeate the BBB, similar in principle to Lipinski's rule of five for intestinal absorption [14]. A molecule should be non-polar, or have a low polar surface area ($<90 \text{ \AA}^2$) with a maximum of 5 hydrogen bond donors and 10 hydrogen bond acceptors (reducing the free energy requirement of moving from aqueous to lipid environments) [14, 21]. The molecule should also be ≤ 500 Da and should not have many rotatable bonds (<5 as the presence of rotatable bonds appears to limit permeability across the BBB) [14, 15, 21, 32]. In addition, it has been demonstrated that weak bases penetrate the BBB more readily than neutral molecules and zwitterions, with acidic molecules having the lowest CNS penetration [21, 33]. This is partly due to the interaction with the negatively charged glycocalyx as well as other factors, such as P-gp efflux [21, 33, 34]. These parameters quite successfully describe the limitation of BBB permeation for almost 98% of small molecules (and almost all large molecule) drugs [35].

As mentioned above, certain diseases, such as AD and MS, can alter the structure and integrity of the BBB [21, 36, 37]. BBB disruption from brain tumours is highly variable and is not well characterised in many disease states [36]. Disruption of the BBB is central to pathologies such as MS and epilepsy, but is often a secondary effect of disease, such as in brain cancers [24]. Therefore, barrier disruption is frequently disease dependent. For example, MS has been reported to lead to BBB breakdown by tight junction abnormalities, loss of claudin-3 and downregulation of laminin. On the other hand, HIV is reported to cause tight junction disruption as a result of leukocyte migration into the brain [21, 22]. In most cases of BBB disruption in disease states, there are changes to the tight junctions, causing their opening and allowing the permeation of larger molecules across the barrier [21]. As expected, a number of abnormalities can occur when the BBB is disrupted, leading to a range of effects [24]. For instance, disruption of glucose transporter GLUT1 leads to seizures, whereas disruption in LAT1 amino acid transporter can lead to autism spectrum-type effects [24].

BBB disruption in AD is an ongoing area of research, including as a potential early biomarker and target for treatment [24, 37]. Certain disruptions in AD

patients, such as synaptic dysfunction, are shown to be the result of pathway activation by β -amyloid [22]. Other disruptions, such as alterations in white matter lesions, have been observed but have no known definitive cause yet [23]. However, research is still ongoing into what stage in the disease progression BBB dysfunction occurs, and a full understanding of the barrier properties affected [24].

In patients with glioblastoma multiforme (GBM), the most aggressive brain cancer, loss of claudin-1 and claudin-3 from tight junctions leads to reduced BBB integrity and some functional loss of the barrier [1, 21, 22]. Exploring the possibility of exploitation of BBB disruption in cancer patients has been ongoing to selectively target chemotherapeutics to tumours [15]. For example, administration of bradykinin further increases the permeability of BBB capillaries in patients with brain tumours, with the greatest effect seen close to the tumour site, but not in healthy brains [38, 39]. This process is thought to occur by activation of B2 receptors on endothelial cells to open ATP-sensitive potassium channels [39, 40].

During a stroke, BBB disruption follows a biphasic time course, visible within hours before decreasing, and increasing again the following day [41, 42]. Stroke causes structural changes to tight junctions *via* loss of claudin-1 and increased transcytosis [22, 43]. The second phase of disruption is a result of increased cell death that occurs in the days following the stroke [24]. Approaches to reduce cell death during the second phase include poly(ADP-ribose) polymerase (PARP) inhibition (where over-activation leads to deoxyribonucleic acid (DNA) damage, ATP-depletion and necrosis), or inducing therapeutic hypothermia [44-46]. However, there are still many unknowns in the mechanism of BBB disruption during stroke, including the differences in disruption between the first and second wave.

It is important to recognise that in disease states the BBB is often disrupted, and often in different and sometimes unpredictable ways by the same disease [24]. Although some research is underway to exploit BBB disruption in disease states in order to enhance drug permeability, this is not a straight-forward option and would likely require patient- and disease stage-specific approaches [14].

Inducing reversible BBB disruption as a treatment enhancer, for example by mannitol, is also under investigation [15]. The BBB is complex, and even more so when disrupted, leaving many unanswered questions. Transport across the BBB is an important area of research, where the understanding of a molecule's interaction with the barrier is key [24].

1.1.3 Oral lipid-based drug delivery

Oral delivery of drugs is considered the easiest administration for patients, allowing convenient at home treatment, and therefore high compliance [47]. There are several other advantages associated with oral drug delivery, such as reduced costs to healthcare systems, pre-defined doses and dosing times, and the non-invasive nature of this delivery method [48]. Therefore, as highlighted above, oral administration is ideal for chronic illnesses. However, successful oral administration presents many hurdles. Firstly, as with all administrations, the delivery system needs to be biocompatible and not alter the drug's activity [47]. Factors such as low water solubility, molecular size (i.e., oral delivery of peptides and proteins is more challenging) high first-pass metabolic loss and low membrane permeability can all limit a molecule's oral bioavailability [48, 49]. Secondly, for good patient compliance, it is important that oral formulations do not have unpleasant taste. Often, taste-masking agents are used for this purpose [50].

Oral lipid-based delivery systems are a well-studied formulation approach first reported in the 1970s, with the use of lipids to enhance drug absorption [49, 51]. It is important to understand the many mechanisms leading to enhanced bioavailability of drugs administered with lipid-based delivery systems. These include: increased absorption from the gastrointestinal tract (by a combination of prolonged exposure and increased wall permeability); altered enterocyte-based transport; avoiding first-pass metabolism; and, enhanced transport to the systemic circulation by the intestinal lymphatic system [52, 53].

Most of the recently discovered drugs are classified into biopharmaceutics classification system (BCS) class II, i.e., poor aqueous solubility and high

membrane permeability, as shown in Figure 1.3 [52]. In order to improve the poor solubility of a drug, a lipid-based formulation may be used. In 2000, Pouton reported a lipid formulation classification system (LFCS) to group the different lipid-based systems used for drug delivery [54]. Formulations were divided into categories with increasing hydrophilic content from type I to type IV (type IV being added in 2006 to describe formulations not containing oils), summarised in Table 1.1 [54, 55]. Briefly, Type I formulations use 100% oils, triglycerides, or mixed mono- and di-glycerides. Type II use a combination of oils and water-insoluble surfactants. Type IIIA use predominantly oils and a mixture of water-soluble surfactants and hydrophilic co-solvents, i.e., polyethylene glycol (PEG) or propylene glycol. Type IIIB contains the same excipients as type IIIA but less oils (<20% content). Finally, type IV contains both water-soluble and water-insoluble surfactants and hydrophilic co-solvents.

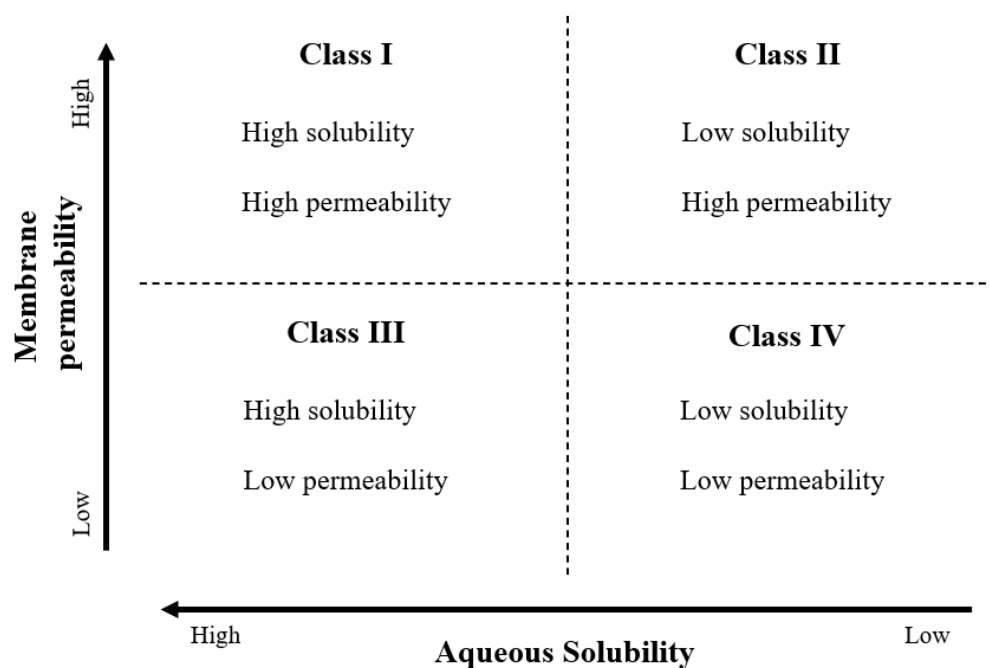


Figure 1.3. The biopharmaceutics classification system (BCS) showing class I-IV by aqueous solubility and membrane permeability.

Table 1.1. The lipid formulation classification system (LFCS) as described by Pouton in 2000 and 2006 [54, 55].

Excipients in formulation	Content of formulation (% w/w)				
	Type I	Type II	Type IIIA	Type IIIB	Type IV
Oils	100	40 - 80	40 - 80	<20	-
Water-insoluble surfactants	-	20 - 60	-	-	0 - 20
Water-soluble surfactants	-	-	20 - 40	20 - 50	30 - 80
Hydrophilic cosolvents	-	-	0 - 40	20 - 50	0 - 50

Lipid-based drug delivery systems can be advantageous not only to increase the solubility of poorly soluble molecules, but also to increase permeability, avoid first-pass metabolism and decrease food effects [49-52]. As the formulation already contains lipids, the further effects of lipids in the diet are usually limited. However, this does vary depending on the lipid formulation and drug solubility [51-53]. Birnbaum *et al* showed this with oral cannabidiol (CBD) delivery, showing 14 times higher $C_{\max}$ (maximum concentration) and 4 times higher area under the curve (AUC) in the fed state compared to control [56]. Another advantage of oral lipid-based delivery is the relative ease in the scaling-up of the product. Usually, the same (or very similar) liquid formulation that is given to animals in early pre-clinical studies can be scaled up for clinical trials [49].

Surfactants are often added excipients in lipid-based formulations, which can lead to the creation of micelles (with both hydrophobic and hydrophilic regions) in aqueous environments [50]. Surfactants are usually a secondary addition, when the use of water-insoluble oils only (such as peanut and soybean oil) does not improve bioavailability [50]. For example, the addition of d- α -tocopheryl polyethylene glycol 1000 succinate (vitamin E-TPGS) to poorly water-soluble antiretroviral drug amprenavir created micelles to stabilise the molecule, which enhanced the solubility and permeability of the drug [57]. Commonly used surfactants include Cremophor EL (polyoxyl 35 castor oil), Cremophor RH 40

(polyoxyl 40 hydrogenated castor oil), Tween 20 and 80 (polysorbate 20 and 80), TPGS, Solutol HS-15 and a number of polyglycolysed glycerides [50]. Polyglycolysed glycerides include fatty acids such as oleic acid, linoleic glycerides and mono-, di- and triglycerides [50].

Lipid nanocarriers have gained much interest in recent years as a delivery method to overcome the poor bioavailability of certain drug molecules. Some examples include the development of nanocarriers for antihyperlipidaemic drug simvastatin, which led to a 4-fold increase in oral bioavailability compared to a simvastatin suspension. Another example is polymer-lipid nanoparticles of enoxaparin, which showed a 4 - 5-fold increase in oral bioavailability, compared to an enoxaparin solution [58, 59]. Lipid liposomes have also been shown to aid in the delivery of peptides by oral administration. For example, lipid glycerylcaldityltetraether (GCTE) liposomes of antibiotic vancomycin protected the peptide structure and integrity *in vivo* after oral delivery [60]. Lipid nanocarriers are also used as new approaches against MS, showing promise in *in vitro* and *in vivo* evaluation [61].

1.1.3.1 Lipid Digestion and Transport

Oral lipid-based drug delivery systems utilise lipid digestion and transport processes. Therefore, a brief description of these processes is provided here for background (Figure 1.4).

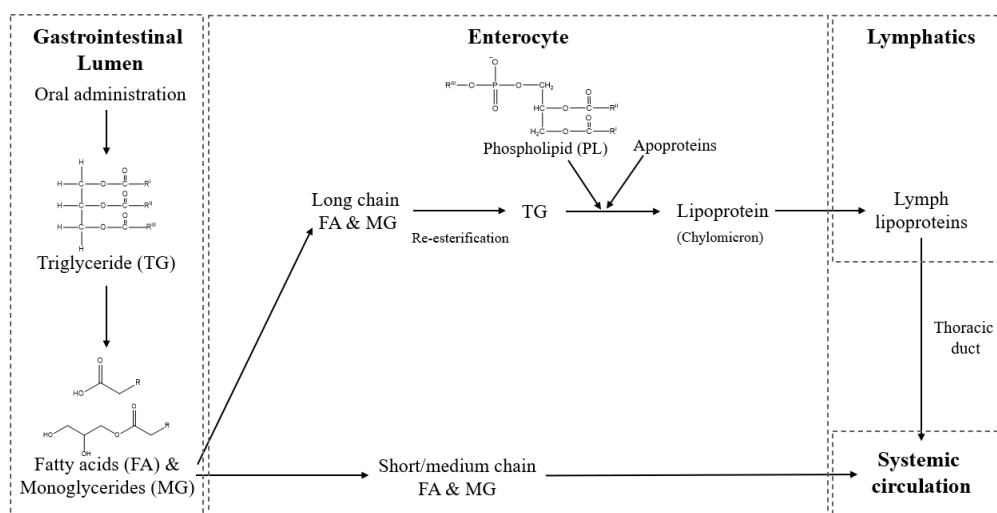


Figure 1.4. Schematic diagram describing the sequential steps in the digestion of lipids and subsequent absorption following oral administration. Short and medium chain fatty acids are shown to enter the systemic circulation directly. Long chain fatty acids undergo re-esterification and associate into chylomicrons to enter the lymphatic system and then the systemic circulation via the thoracic duct. Reproduced from Brookes *et al* [62], with permission from Elsevier.

Most dietary lipids are in the form of triglycerides, and after a series of digestive processes, the triglycerides are broken down into fatty acids and monoglycerides, which are then absorbed by enterocytes [63]. Subsequently, the triglyceride chain length determines the intracellular processing. In general, short- and medium-chain fatty acids (carbon chain length <12) diffuse through the enterocyte and then enter the blood circulation through the portal vein and liver. Longer chain fatty acids (carbon chain length >12) are re-acylated into triglycerides and assembled into chylomicrons (CMs), which are secreted into the mesenteric lymph and enter the blood circulation via the thoracic duct. CMs entering blood capillaries of certain tissues will activate the lipoprotein lipase on the surface of BCECs to generate fatty acids. These fatty acids then enter muscles and adipose tissues for storage or use. The CM remnants (mainly containing cholesteryl esters) will be recognised by the apoE receptor on the liver cell membrane and enter the liver cells.

Due to the hydrophobic nature of lipids, their transportation in the body requires lipoprotein vehicles. Lipoproteins circulate in the blood until their triglycerides are consumed by the surrounding tissues, or the lipoproteins themselves are eliminated by the liver. CMs are the largest lipoproteins found in the body, synthesised in the intestinal wall. Other lipoproteins are characterised as either very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) or high-density lipoproteins (HDL) [64]. VLDL are predominantly synthesised in the liver. VLDL's surface Apo C-II activates lipoprotein lipase, which, as described above, hydrolyses triglycerides into free fatty acids and glycerol to be absorbed by cells. LDL are rich in cholesterol and are mainly generated following VLDL metabolism in the plasma. A small proportion of LDL is synthesised by the liver and secreted directly into the blood. LDL function is mainly to transport cholesterol to peripheral tissue cells. Between 40 - 60% of LDL can be cleared by the liver (mediated by apo B and hepatic LDL receptors), and the rest is absorbed through hepatic LDL or non-hepatic non-LDL receptors. HDL is synthesised by the liver and enterocytes, and newly generated HDL does not contain cholesterol. HDL function is to obtain cholesterol from peripheral tissues and lipoproteins in order to transport it to tissues where cholesterol is needed. Additionally, long-chain fatty acids tend to exist in the form of triglyceride in lipoproteins, whereas short- or medium-chain lipids remain in the fatty acid form in the blood [65].

1.1.4 Effects of lipids on delivery to the brain

Due to the physiological characteristics of the BBB, lipophilic substances can enter the brain relatively easily. This suggests that lipids could potentially be used to facilitate the delivery of drugs to the brain. However, as the chain length of lipids and their metabolic pathways differ, not all lipids are suitable for oral administration for these purposes.

1.1.4.1 Long-chain lipids

Oleic acid (C18:1 cis-9) is abundant in human adipose tissue and is also present in animal and vegetable fats. Oleic acid circulating in the systemic blood in the free form has been shown to increase BBB permeability (the suggested mechanism of this is *via* albumin binding) [66, 67]. Sztrlha and Betz examined the effects of oleic acid infusion on BBB permeability in 1991. A sodium oleate solution was injected into the right internal carotid artery of adult rats (6 mL/min for 30 seconds) and uptake of α -aminoisobutyric acid into the brain was studied [66]. Uptake was found to be facilitated by a 1×10^{-5} M oleic acid formulation. Interestingly, and encouragingly, the effect was reversible. At 80 - 90 minutes post injection, BBB permeability had returned to normal. Kim *et al* observed a similar phenomenon when administering an oleic acid emulsion (0.05 mL oleic acid and 20 mL saline) into the carotid artery of cats (4 mL/min for 5 mins) [68]. The oleic acid emulsion was found to reversibly increase BBB permeability. The effects of oleic acid are proposed to be the result of interaction of lipids with BCEC membranes. Oleic acid allows the incorporation of fatty acids into the lipid membrane, affecting the properties and functions of the cell membrane [66]. However, there is not sufficient evidence to prove this hypothesis at this stage and further research is necessary.

In order to consider oleic acid as a vehicle for oral lipid-based delivery to the brain, it is important to take into consideration the digestion of orally administered oil. The saturation of fatty acids plays a role in their absorption, with mono-saturated oleic acid resulting in good digestion, regardless of the oil form administered. Oleic acid is transported *via* the lymphatic system, and as discussed above, stored as triglycerides in tissues until use [69]. Studies examining the biodistribution of oleic acid have traced radiolabelled molecules to a wide range of tissues including the heart, liver, lungs, spleen, kidneys, and brain [69, 70]. Several studies have already utilised this pathway to design oral formulations for lipophilic compounds. For instance, Caliph *et al* observed a significantly higher systemic exposure of highly lipophilic substance Org 49209 using oleic acid-based formulations compared with a lipid-free vehicle in rats [71]. This substance has also been detected in the brains of rats that were orally

administered with oleic acid-based formulations, although it is not yet clear whether the presence of oleic acid increases the brain's uptake of Org 49209 [71].

Triolein emulsion (a symmetrical triglyceride containing 3 units of oleic acid) is another lipid that has been shown to increase the permeability of the BBB when administered directly into the blood circulation. Kim *et al* demonstrated that a doxorubicin loaded triolein emulsion (0.10 - 0.20 mL triolein with 10 mL saline) injected into the right carotid artery in rabbits increased BBB permeability [72]. At 2 h following administration, doxorubicin concentration in ipsilateral hemispheres increased several-fold, compared to the control of administration without triolein emulsion. Drug concentration changes in the ipsilateral brain over time were also investigated in cisplatin-treated rats after injection into the right carotid artery [72]. The highest cisplatin concentration in the ipsilateral brain was achieved 6 h post injection of 20 $\mu\text{L/mL}$ triolein emulsion (0.67 mL/Kg dose). Additionally, Ryu *et al* determined that the lowest dose of triolein emulsion that can increase the BBB permeability by intra-arterial injection in cats is 3 mL/Kg (5 $\mu\text{L/mL}$ triolein emulsion in saline) [73]. Choi *et al* repeated the study to confirm the minimal dose to increase BBB permeability is 3 mL/Kg (5 $\mu\text{L/mL}$ triolein emulsion in saline) [74].

Based on nutrition literature [75-77], it appears feasible to achieve appropriate triolein levels to affect BBB permeability in the systemic blood circulation by oral administration. Ryu *et al* calculated that the lowest dose required to increase BBB permeability in cats by intracarotid injection is ~ 13.73 mg/Kg [73]. BBB permeability was shown to still be altered 2 h post intracarotid injection of triolein [72-74]. This could indicate that the initial high plasma concentration resulted in a prolonged increase in BBB permeability, or alternatively, that a lower concentration than the C_{max} is effective in altering the BBB permeability. Triolein is well absorbed in humans [78, 79], therefore converting this dose to humans based on body weight and body surface area (human equivalent dose), the lowest dose required to increase the BBB permeability in an average adult by intra-arterial injection would be ~ 5.47 mg/Kg [80, 81]. For an adult weighing 60 Kg, as little as 330 mg triolein might be sufficient to effect BBB permeability. However, as the plasma C_{max} after oral administration is likely to be substantially lower, and some triolein would be broken down and re-assembled into different

triglycerides post absorption, it is sensible to assume a higher dose of triolein should be administered.

It should be noted that these calculations are preliminary, as additional data will be needed to prove the theoretical calculations made here. The behaviour of triolein in plasma after intracarotid vs. intravenous injection is thought to differ, and the rate of change of triolein concentration in plasma after intracarotid injection is still unclear. In addition, triolein levels in the blood following oral administration are not conclusive from reported literature. Also noteworthy is that all calculations reported here use the lowest triolein levels we found in the literature, to prevent any underestimation.

The mechanism by which triolein increases BBB permeability is not yet completely clear. To investigate the phenomenon, Sol *et al* infused triolein emulsion (3 mL of 5 μ L/mL triolein with saline) into the carotid artery of rats [82]. They report that 2 h after injection, transcellular vesicles in the BBB endothelium increased in number $\sim$ 5-fold, compared to the control group of infusion without triolein emulsion. Paracellular transport *via* tight junctions, which did not occur in the control group, was also observed after infusion with triolein. Based on this, Lee *et al* speculated that the increase in BBB permeability caused by triolein emulsion can be partially explained by enhancement of paracellular and transcellular pathways [83].

1.1.4.2 Short-chain lipids

In comparison to long-chain lipids, there are not as many studies investigating the effects of short-chain lipids on BBB, although short-chain alkylglycerols (an ether lipid abundant in shark liver oil) have been shown to increase BBB permeability after systemic administration. Erdlenbruch *et al* co-injected alkylglycerol (0.01 - 0.3 M) with cisplatin or methotrexate into the carotid artery in rats and measured drug levels in the ipsilateral brain. They found that drug concentrations increased in an alkylglycerol concentration-dependent manner (cisplatin concentration range was from 2 to 230-fold higher) [84]. Importantly, the effect was reversible, and the BBB returned to the original state within several minutes [84].

Erdlenbruch *et al* also demonstrated that different alkylglycerol chain structures affected BBB permeability in different manners after systemic administration. The increased permeability induced by 100 mM 1-O-pentylglycerol in rats returned to normal within 3 minutes, while the permeability increase caused by 50 mM 1-O-hexylglycerol remained 1 h post injection. In addition, the increased BBB permeability caused by alkylglycerols is effective for both small molecules (e.g. fluorescein and sodium) and large molecules (e.g. lissamine-rhodamine B200–albumin) [85]. Tight junction changes were observed through confocal microscopy and it was found that at least part of the enhanced BBB permeability is the result of increased paracellular transport [85].

1.2 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is a highly aggressive and common cancer of the brain. Designated as a grade IV astrocytoma by the World Health Organisation (WHO), GBM is a type of glioma originating from glial cells such as astrocytes [86, 87]. As mentioned above, and illustrated in Figure 1.1, astrocytes support the structure of the BBB, and are therefore highly abundant in the brain [21, 22]. GBM can occur as either primary or secondary tumours, typically heterogeneous in their makeup [88]. GBM are fast growing cancers, which contribute towards the poor prognosis of 5% 5-year survival in England [89]. GBM occurs more frequently in males, and is the most common astrocytoma in adults, occurring more often in adults aged 70 - 80 years [86, 87].

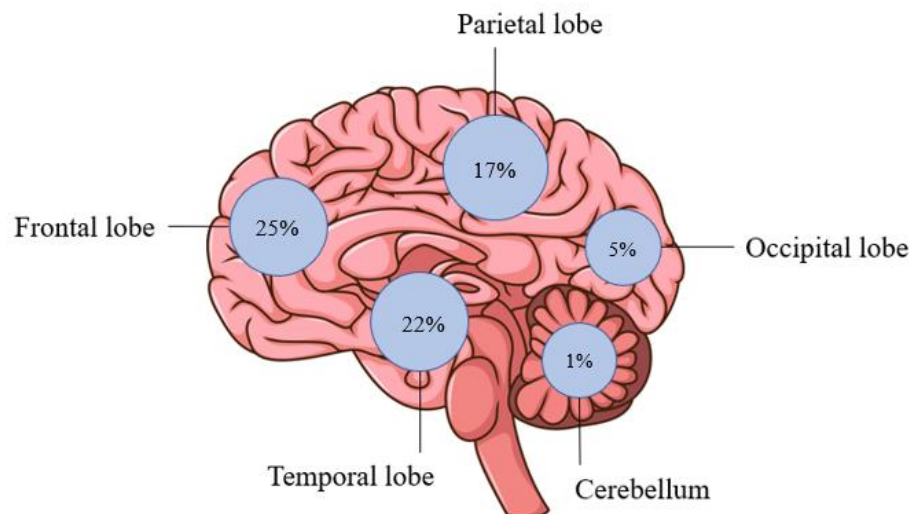


Figure 1.5. Common locations of GBM tumours in the brain. Produced using data from Brodbelt *et al* [90].

GBM tumours are most commonly located in the parietal, temporal and frontal lobes, shown in Figure 1.5 [90]. Reports of cases in England show that GBM tumours are most commonly located in the frontal lobe, making up 24.9% of GBM cases [90]. The temporal and parietal lobes are also common locations accounting for 21.8% and 16.7% of cases, respectively [90]. While the exact

estimations vary across reports, these three brain regions are consistently reported as the most common locations of GBM tumours. Symptoms of GBM depend on the tumour size and location but commonly include headaches, loss of memory and appetite, vomiting and changes to vision [86].

The standard of care treatment of GBM begins with maximal medically possible surgical resection to remove as much of the tumour as possible [87, 91]. The patient would then receive radiotherapy, followed by chemotherapy [86, 87]. 3D conformal radiotherapy is commonly used to target any remaining GBM cells post-surgery [87]. Standard post-surgical chemotherapy is 6 weeks of temozolomide (TMZ) ($75 \text{ mg/m}^2/\text{day}$), followed by TMZ ($150 - 200 \text{ mg/m}^2$) for 5 days every 28 days, for 6 cycles [87].

1.2.1 Glioblastoma multiforme treatment with temozolomide

Contributing to the poor prognosis is the common resistance of GBM to the standard of care chemotherapy treatment, TMZ. The synergistic response of TMZ with cannabinoids (discussed below in section 1.3) against GBM was studied in this work and will be discussed further in Chapter 3. TMZ (under the brand names Temodar® and Temodal®) is currently given orally to GBM patients following surgery and radiotherapy [87, 91]. TMZ is an imidazotetrazine and the structure is shown in Figure 1.6. TMZ has near 100% bioavailability following oral administration, with low protein binding of 15%. TMZ is a small, hydrophilic drug ($\log P$ of -1.15) with the ability to successfully permeate the BBB [91, 92].

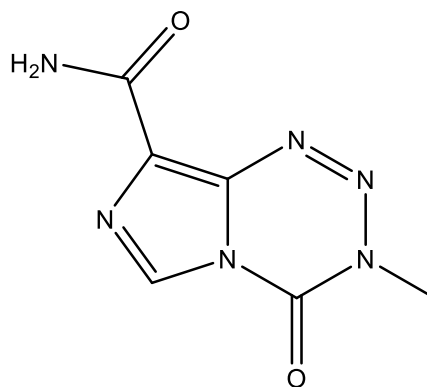


Figure 1.6. Chemical structure of TMZ (194.12 g/mol).

TMZ is a DNA-alkylating agent, predominantly methylating DNA at N^3 -adenine, N^7 - and O^6 -guanine positions. N -methylation is generally repaired quickly by base excision repair (BER), whereas O -methylation is not [93, 94]. O^6 -methylation leads to a mis-pair of guanine with thymine (rather than cytosine) during DNA replication, damaging the cells and triggering the pathway to cell death *via* apoptosis or autophagy [87, 95]. The mismatch is recognised by DNA mismatch repair (MMR) and thymine is removed before guanine is mis-paired with thymine again, and thymine is reinserted [87]. This futile cycle continues until it eventually triggers a DNA strand break, inducing cell cycle arrest at G2/M and leading to cell death by apoptosis or autophagy [87, 88, 91, 94].

DNA-alkylation by TMZ occurs following ring opening of the molecule at physiological pH to release the active methyldiazonium cation, shown in Figure 1.7 [91]. First, base-catalysed hydrolytic attack occurs at physiological pH at the C^4 -position. Then, the intermediate is protonated at the N^1 -position, opening the ring. The unstable carbamic acid decarboxylates to form the intermediate 5-(3-methyltriazene-1-yl)imidazole-4-carboxamide (MTIC), which then undergoes proteolytic degradation to form AIC and release the methyldiazonium cation [91].

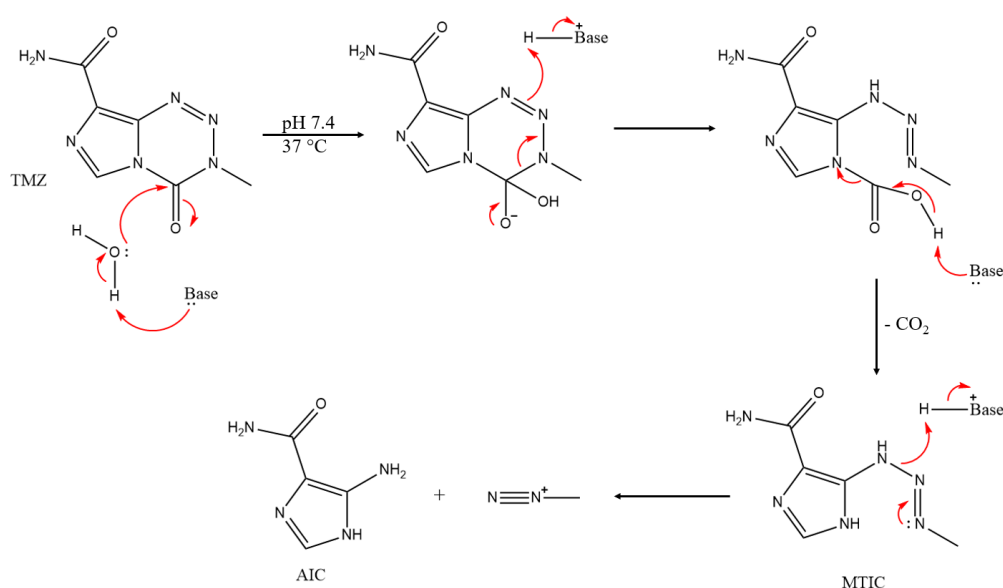


Figure 1.7. The ring-opening of TMZ at physiological pH to release the active methyldiazonium cation, based on explanation by Singh *et al* [91].

A contributing factor to the poor prognosis for GBM patients is the common resistance to treatment with TMZ, occurring in $\geq 50\%$ of cases [88, 91]. Resistance of GBM to TMZ is thought to be driven by glioma stem cells (GSCs) - a population of undifferentiated, tumourigenic stem cells [88, 91]. GSCs have the ability to regenerate tumour heterogeneity, allowing for resistance to the chemotherapy to develop [91].

There are two major resistance mechanisms to TMZ demonstrated in GBM. Firstly, an over-expression of *O*⁶-methylguanine-DNA methyltransferase (MGMT). MGMT is a DNA repair enzyme that allows the cells to repair DNA-methylation at the *O*⁶-guanine position [87, 91]. MGMT is a suicide protein, whereby for every repair of *O*⁶-methylguanine to guanine, a MGMT protein is destroyed. The methylation status of the MGMT promotor is an important factor in resistance to TMZ treatment. Methylation of the cytosine-phosphate-guanine (CpG) island of the promotor prevents expression of MGMT, and is therefore correlated with improved survival following treatment with TMZ [87, 91]. It has been demonstrated that the methylation status of the MGMT promotor can change during treatment, and is suggested that a reduction in methylation (leading to MGMT expression) is a method of acquired resistance to TMZ [91].

Secondly, a deficiency of MMR allows *O*⁶-methylguanine to be tolerated [87, 94]. MMR is an essential component of the pathway of DNA-methylation, as described above. The MMR deficiency allows the mis-pairing of *O*⁶-methylguanine with thymine, meaning the futile cycle of thymine removal and insertion does not occur. Therefore, DNA strand breaks do not occur and ultimately, cell death is avoided. MMR proficiency has been demonstrated to be inversely correlated with MGMT expression [91, 96].

There is ongoing research into chemotherapeutic alternatives to TMZ in order to overcome the common resistance mechanisms and improve outcomes. Biodegradable wafers (often containing carmustine (BCNU)) can be implanted to the tumour site during surgery for the slow release of the chemotherapeutic. As the name suggests, BCNU is a mustard compound that alkylates DNA, creating crosslinks between guanine and cytosine bases [87, 91]. However, there are other risks associated with implanting wafers, such as infection, and the diffusion distance of the chemotherapeutic agent into the tissue.

There are also alternative approaches such as targeting vascular endothelial growth factors (VEGF) by binding with bevacizumab [88]. This monoclonal antibody inhibits vascular growth within the tumour with the aim to limit the tumour growth. The combination of bevacizumab and TMZ showed no survival benefit in randomised clinical trials, however there was an observed improvement in quality of life [97, 98].

As discussed above, convection enhanced delivery (CED) is another option being researched. CED directly infuses therapeutics into the tumour localised areas, bypassing the BBB [2]. There are also new approaches for cancer treatment including immunotherapy and tumour treating fields (TTF) which are being explored for GBM treatment [88].

1.2.2 Temozolomide analogue, T25

The development of analogues of TMZ are one approach to overcome the common resistances to GBM treatment. One such analogue is T25, also referred to as N3PC8T, shown in Figure 1.8. The synergistic response in GBM cells to treatment with T25 and cannabinoids (discussed below in section 1.3) is studied herein and compared to that of combinations with TMZ. This work will be discussed further in Chapter 3 of this thesis.

T25 is a *N*³-propargyl analogue of TMZ. The addition of the propargyl group can overcome resistance by MGMT over-expression. The DNA is alkylated with a propargyl group, rather than the methyl group with TMZ, and therefore, MGMT is not able to recognise and remove the propargyl group. This effect has been evidenced *in vitro* against GBM cell lines representing the over-expression of MGMT [95, 99, 100]. Whilst efficacy work on T25 is currently unpublished, the molecule shows promise as an alternative GBM treatment to TMZ.

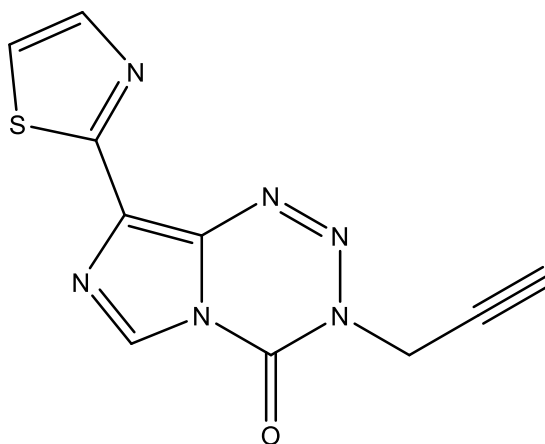


Figure 1.8. Chemical structure of T25 (258.3 g/mol), a TMZ analogue.

1.3 Cannabidiol

The *Cannabis sativa* plant has a long history of recreational and medicinal use. The most abundant cannabinoids of the plant are Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD), shown in Figure 1.9. Both THC and CBD exhibit therapeutic potential for a range of applications, however, the psychoactivity of THC has encouraged more work to focus on the non-psychoactive CBD [101, 102]. CBD has demonstrated vast activity including anti-inflammatory, anti-anxiety, anti-depressant, and anti-cancer effects [103-107]. Therefore, there is work to develop CBD treatments for diseases including MS, cancer, inflammatory bowel disease, anxiety and arthritis [103-109].

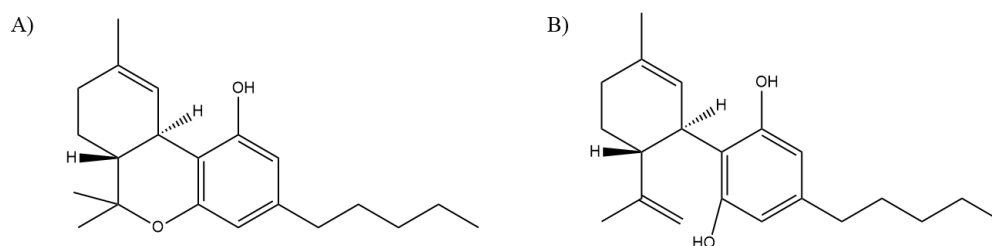


Figure 1.9. Chemical structures of A) THC (314.5 g/mol) and B) CBD (314.5 g/mol).

The endocannabinoid system is a complex network responsible for maintaining internal temperature, mood, the immune and other biological systems [110, 111]. The endocannabinoid system comprises of endocannabinoids (such as anandamide (AEA) and 2-AG), enzymes (such as fatty acid amide hydrolase (FAAH)), receptors (including cannabinoid receptors CB1 and CB2) and transport proteins [110, 111].

CB1 and CB2 are part of the G protein-coupled receptor (GPCR) membrane receptor family, distributed throughout the body. While CB1 receptors are predominantly present in the CNS, and CB2 receptors in peripheral tissues, there is also evidence of CB2 receptor expression in the brain stem [112, 113]. CBD has been shown to bind antagonistically to both CB1 and CB2 receptors, although with low affinity (inhibition constants (K_i) of 4350 – 10000 nM for

CB1 and 2860-10000 nM for CB2 receptors) [112, 114-116]. The low antagonistic binding to CB1 receptors is thought to be behind the non-psychoactivity of CBD, as agonistic binding of THC to CB1 is responsible for the psychoactivity of THC [112]. CBD has also demonstrated inhibition of FAAH, which is involved in AEA hydrolysis and uptake, therefore increasing the free endogenous cannabinoids [112, 117, 118]. A complete understanding of the interactions of CBD *in vivo* have not yet been reached, however Almeida *et al* (2020) have summarised the known interactions, shown in Figure 1.10 [112].

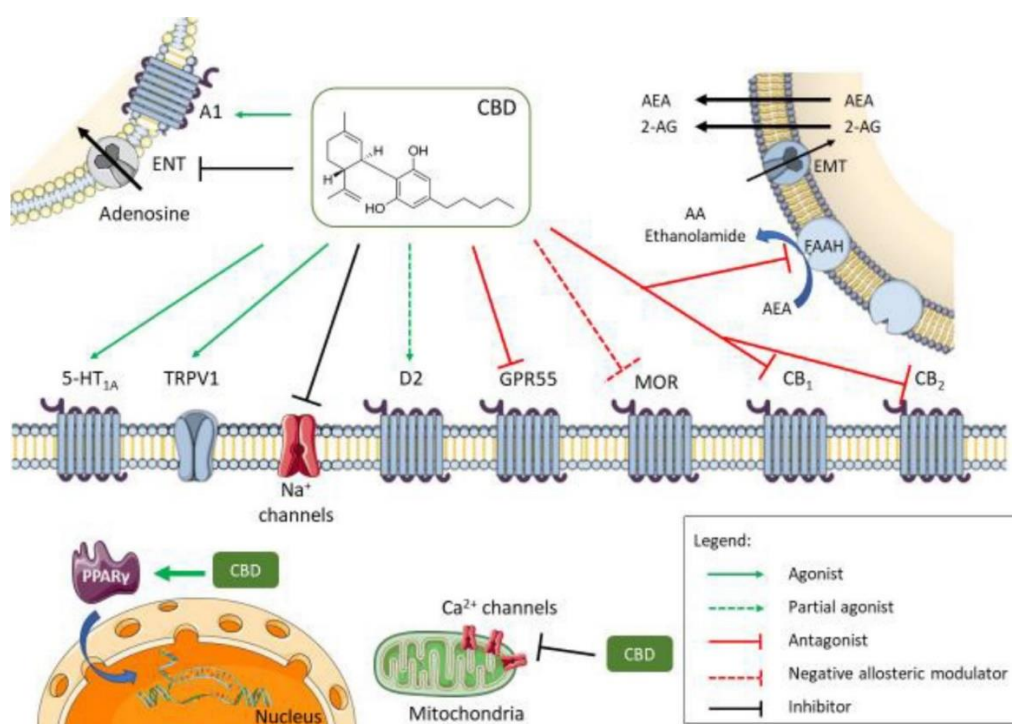


Figure 1.10. Molecular targets of CBD *in vivo*. CBD is depicted to interact agonistically with A1 receptors, 5-hydroxytryptamine 1A receptor (5-HT_{1A}) and transient receptor potential cation channel subfamily V member 1 (TRPV-1), partially agonistically with dopamine receptor 2 (D2), antagonistically with G-protein receptor 55 (GPR55), CB₁ and CB₂ and FAAH. CBD is a negative allosteric modulator of μ -opioid receptor (MOR) and inhibitor of equilibrative nucleotide transporter (ENT), endocannabinoid membrane transporter (EMT) and Na⁺ and Ca²⁺ channels. Reproduced from Almeida *et al* [112], with permission from John Wiley and Sons.

1.3.1 Activity of cannabidiol for multiple sclerosis

There is evidence CBD induces immunosuppressive effects against MS. This activity is investigated within this thesis following oral administration of CBD in lipid-free and lipid-based formulations and will be discussed in Chapter 5 of this thesis.

MS is a chronic autoimmune disease, affecting the CNS, and is one of the most common causes of disability in younger adults. In MS, the immune system attacks the myelin and axons in the brain and spinal cord [12, 13]. Due to the disease location in the CNS, the symptoms associated with MS include problems with balance, movement, vision, and cognition. It is thought that there are several potential causes of MS including both environmental and genetic factors [13]. Currently, there is no cure for MS, however, some treatments such as corticosteroids can help to manage symptoms [12].

CBD exhibits therapeutic effects against MS, often demonstrated in the *in vivo* experimental autoimmune encephalomyelitis (EAE) mouse model [119-123]. CBD has been shown to increase immunosuppressive myeloid-derived suppressor cells (MDSCs), inhibiting the T cell migration that drives the disease [120, 124]. CBD also resulted in reduced clinical scoring severity, demyelination and reduced inflammation in mice [123]. Administration of CBD after symptoms were first observed suppressed the immune response to EAE that causes neuroinflammation, demonstrated by reduced myelin oligodendrocyte protein (MOG₃₅₋₅₅) specific T cells [119]. MS progression, specifically inflammation, has been associated with CB1 and CB2 activity [111]. Intraperitoneal (i.p.) administration of CBD with THC has also been demonstrated to alter the gut microbiome - a change which reduced the lipopolysaccharide (LPS) levels in the brain that are usually seen in EAE mice [121].

1.3.2 Anti-cancer properties of cannabidiol

CBD is reported to exhibit anti-cancer properties in *in vitro* and *in vivo* animal models against a range of cancers including breast, colorectal, glioma, cervical and lung cancers [103, 125, 126]. The anti-cancer activity of CBD against GBM was investigated in this work and will be discussed in Chapter 3 of this thesis. The anti-cancer activity of CBD is often studied in combination with THC, and sometimes in combination with other anti-cancer agents. For example, the anti-cancer activity of CBD against GBM is reported in combination with THC or TMZ [103, 127-130]. The combination of CBD and TMZ has been reported as both an additive and synergistic response *in vitro* [131, 132].

When CBD was administered as a combination with THC and TMZ in a U87MG GBM mouse xenograft model, tumour growth was reduced by a larger extent than after administration of TMZ alone [127]. CBD has also been shown to be effective in other *in vivo* GBM models (U87, U251, GSC3832 and GSC387) in combination with THC and TMZ after intravenous (i.v.), i.p., subcutaneous (s.c.) and oral administration [103, 127, 133-136]. CBD has also been investigated in combination with radiotherapy against GBM in mice, showing almost 90% apoptotic levels reached [126, 137].

The reports of anti-cancer activity of CBD have led to initial clinical trials of the application against GBM. Phase I/II clinical trials in GBM patients found that Sativex® (a 1:1 mixture of CBD and THC) was safe to administer, where no drug-drug interactions with TMZ were identified [138-140]. Further clinical trials with GBM patients are currently underway to study the efficacy of combinations of radiotherapy and chemotherapy with TMZ and a 1:1 mixture of CBD and THC, as well as daily administration of CBD with TMZ [141, 142].

The molecular mechanisms through which CBD exerts its anti-cancer activity are thought to be many, due to the rich and diverse pharmacology of the cannabinoid [130, 143-145]. The role of the endocannabinoid system in cancer development is not yet fully understood, however it has been demonstrated that CB1 and CB2 receptor expression can be altered in cancers, and can be correlated with cancer cell invasion, proliferation and apoptosis [103, 129]. Inhibition of CB1 and CB2 receptors was shown to reverse some, but not all, of

the anti-cancer effects of CBD, however the pathways involved are not yet fully elucidated [103, 130].

Other suspected pathways include *via* transient receptor potential cation channel subfamily V member 2 (TRPV-2), increased reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress [103, 125, 130, 143-145]. DNA-methylation is not reported as a mechanism of anti-cancer activity of CBD as far as we are aware. However, methylation of C⁵-cytosine in CpG islands is a reported mechanism in the behavioural activity of CBD, indicating a potential DNA-methylation pathway in the anti-cancer activity of the cannabinoid [146, 147].

1.3.3 Cannabidiol *in vivo* pharmacokinetics

The metabolism and pharmacokinetics (PK) of CBD has previously been studied previously *in vivo* in animals and humans. CBD is often administered orally, and as oral administration was used in this work, the oral *in vivo* properties of CBD are discussed here. Generally, CBD is considered to be safe, following administration at up to 6000 mg/Kg with no adverse side effects observed in safety clinical trials [148].

In humans, CBD absorption has been studied following oral doses from 2 to 800 mg. While the $C_{\max}$ generally changes with the dose, the time of maximum concentration ($T_{\max}$) is not affected by dose [149]. In animal studies, the bioavailability of CBD is reported to be low, between 10 – 20% [149, 150]. However, as discussed above, the bioavailability of CBD following oral administration can be increased by using a lipid-based formulation, demonstrated in Figure 1.11 from Feng *et al* [151].

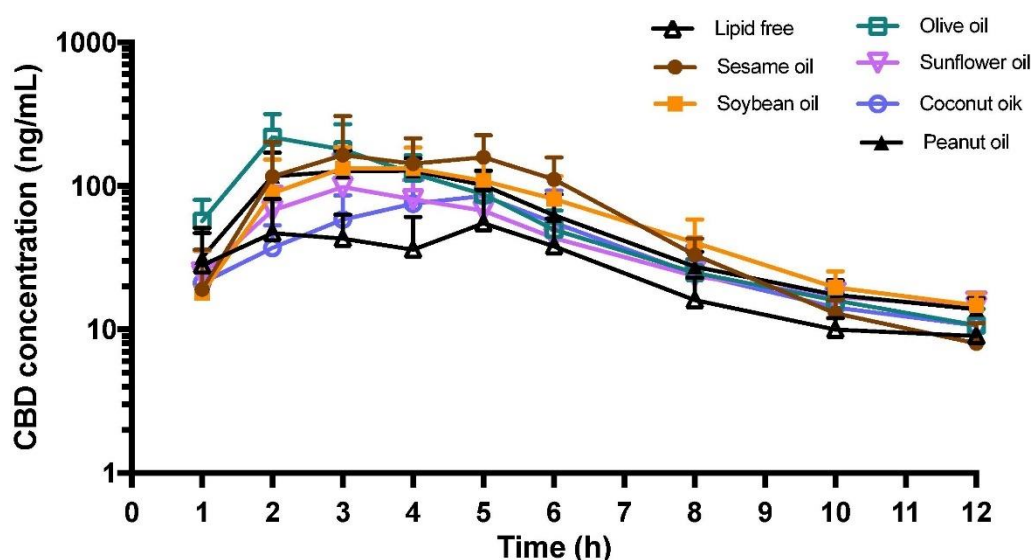


Figure 1.11. Plasma PK profiles of CBD following oral administration in lipid-free (propylene glycol:ethanol:water, 80:10:10 v/v/v) and lipid-based formulations at 12 mg/Kg to rats. Figure reproduced from Feng *et al* [151], with permission from Elsevier.

The full CBD biodistribution is not yet understood, however, reports suggest that CBD that does reach the systemic circulation is distributed throughout the body, including the brain and lymphatic system [149, 152-156].

The metabolism of CBD *in vivo* continues to be studied. For example, it has been suggested that CBD can be converted to THC under the conditions of gastric fluid [157]. However, this has not been evidenced *in vivo*, and the *in vitro* methods used to make this suggestion are not representative of *in vivo* conditions [158]. It is well understood, however, that CBD undergoes extensive first-pass metabolism [149]. Predominantly, CBD undergoes allylic hydroxylation by cytochrome P450 enzymes, where 7-hydroxy-cannabidiol is a major product of this metabolism [159]. *In vivo* receptor activity of CBD metabolites has not yet been reported [149].

Despite the high first-pass metabolism of CBD, and in line with the low bioavailability of the molecule, a good portion of CBD is excreted unchanged. CBD metabolites can also be detected in the urine. Following oral

administration, CBD elimination is generally fast (several h) than i.v. injection or inhalation (smoking), where the $t_{1/2}$ can be over 1 day [149, 151, 154, 160].

1.3.4 Cannabidiol formulations

Oral lipid-based formulations of CBD were used in this work and compared to lipid-free formulations to investigate differences in biodistribution following administration in the different vehicles. This will be discussed in Chapters 4 and 5 of this thesis.

Despite the vast therapeutic potential of CBD, there are few approved formulations. Epidyolex® is a U.S. Food and Drug Administration (FDA) approved oral treatment of seizures associated with rare forms of epilepsy (Lennox Gastaut syndrome (LGS) and Dravet syndrome (DS)). Epidyolex® is a lipid-based formulation of CBD containing sesame oil [161, 162]. Sativex® is an oral spray formulation of 1:1 CBD:THC, approved in the UK to treat muscle stiffness and spasticity associated with MS [163-165].

As discussed above in section 1.1.3, oral administration is preferred over other routes [47, 48], however CBD has low bioavailability following oral administration [166, 167]. CBD oral formulations are limited by its highly lipophilic nature and low water solubility. However, CBD is solubilised well in lipids, and administration of CBD in various oral lipid-based formulations has been reported [151, 155, 166, 168]. Our group has previously reported that administration in a sesame oil formulation resulted in a 3-fold higher bioavailability of CBD, compared to a lipid-free vehicle, and a 250-fold higher lymph fluid concentration compared to the plasma [155, 169]. Comparing different lipid-based formulations of CBD, the selection of lipid for oral administration was found to effect the lymphatic transport and bioavailability of CBD [151].

1.3.5 4'-Fluoro-cannabidiol

Fluorination of molecules is a strategy used to enhance the activity of a compound [170-172]. Fluorinated molecules are often reported to exhibit improved delivery across the BBB, compared to their parent compounds, due to the high electronegativity of the fluorine atom [173-175]. Fluorination also provides additional benefits including metabolic stability, and imaging potential is conferred by fluorinating molecules [170]. Such potential of fluorinated molecules means their use in medicinal chemistry is well recognised, with increasing fluorinated drugs in development.

4'-Fluoro-cannabidiol (4'-F-CBD), shown in Figure 1.12, is a fluorinated derivative of the cannabinoid CBD that is studied in this work. The anti-cancer properties of the molecule will be discussed in Chapter 3, and the *in vivo* investigation in Chapter 6 of this thesis. 4'-F-CBD was first reported in 2016 by Breuer *et al* [176]. Fluorination of CBD in three locations was demonstrated, at the C^4 -, C^{10} - and C^{16} -positions [176]. However, fluorination at the C^4 -position resulted in the only molecule to demonstrate significantly increased activity over CBD in *in vivo* behavioural models [176, 177].

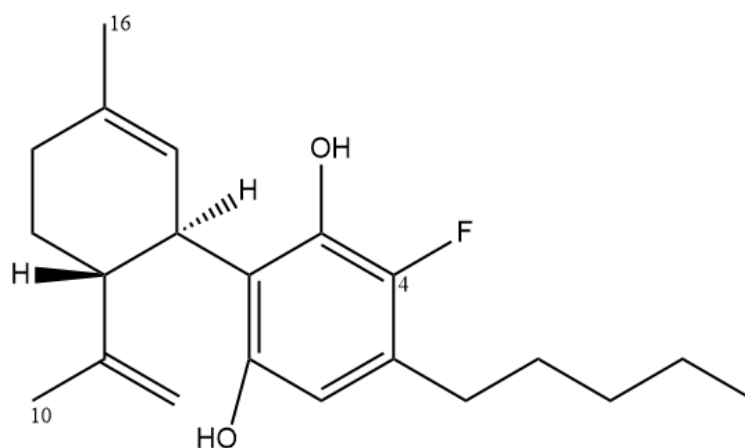


Figure 1.12. Chemical structure of 4'-F-CBD (332.5 g/mol).

Activity of 4'-F-CBD has been investigated in *in vivo* models of anxiety, depression, psychotic and compulsive behaviour. In all cases, 4'-F-CBD resulted in higher efficacy than CBD after i.p. injection at up to 10× lower doses [176, 177]. 4'-F-CBD was also reported to exhibit anti-nociceptive and neuroprotective effects *in vivo* [177, 178]. Potential anti-cancer activity of 4'-F-CBD (due to the properties of CBD) have not yet been investigated, however 4'-F-CBD has been demonstrated to prevent chemotherapy-induced pain [179].

The anti-compulsive and anti-nociceptive effects of 4'-F-CBD were demonstrated to occur *via* interaction with CB1 and CB2 receptors of the endocannabinoid system [176, 177]. Activity is also reported to involve the adenosine 2A (A2A) receptor (Figure 1.10) [180]. There have been no further reports thus far on the mechanisms of 4'-F-CBD activity, although reports to date suggest mechanisms could be similar to that of CBD.

Despite the therapeutic potential of the molecule demonstrated *in vivo*, the pharmacokinetics (PK) and biodistribution of 4'-F-CBD have not yet been investigated to the best of our knowledge. Previous reports in the literature of 4'-F-CBD activity have called for these *in vivo* properties to be investigated [176, 177].

1.4 3D OrbiSIMS

Mass spectrometry is commonly employed for label-free imaging of biological samples, in particular, secondary ion mass spectrometry (SIMS) and matrix-assisted laser desorption/ionisation (MALDI) are the most widely used. Imaging mass spectrometry allows for the detection and visualisation of the distribution of multiple molecules simultaneously throughout a sample. This provides the opportunity to monitor a drug's movement within a cell and also the pathology of the cell, as the metabolites expressed are indicative of a cell's health. In 2012, a MALDI system was reported to approach single cell resolution, visualising large metabolites inside cells [181]. High resolution, sensitivity and chemical coverage in imaging are essential to understand the behaviours of a therapeutic at the cellular level.

SIMS surface analysis was greatly advanced by the introduction of the use of gas cluster ion beams (GCIB) in 2001 by Yamada *et al* [182]. GCIBs produce less fragmentation than the traditionally used liquid metal ion gun (LMIG), allowing the detection of higher molecular signals, such as larger lipids and proteins [182].

In 2017, SIMS analysis underwent another huge advancement with the introduction of the 3D orbitrap secondary ion mass spectrometry (3D OrbiSIMS) for label-free imaging on the subcellular scale [183]. The hybrid instrument combines time-of-flight (ToF) and Orbitrap detectors for analysis with high speed, mass accuracy, spatial resolution and mass resolving power (240,000 at m/z 200) [183]. The 3D OrbiSIMS provides the capabilities of subcellular imaging with low signal-to-noise ratio [183]. The ToF analyser can acquire fast survey images with the LMIG, often used to identify areas in the sample for further analysis with the Orbitrap GCIB. Depth profiling is also possible with the Orbitrap, controlled by the GCIB sputter gun [183]. Data from the 3D OrbiSIMS can be visualised as 3D images, showing the distribution of each molecule, or fragment, within a sample. 3D images are made by measuring a set of 2D images which are reconstructed and visualised in 3D.

Sample manipulation and preparation is key to 3D OrbiSIMS analysis of biological samples. Instruments allow analysis from cryogenic to room

temperature, typically under a strong vacuum. To best retain the structures within samples, the sample should be kept hydrated, as it would be *in vivo*. Hydrated samples also don't need to go through the process of freeze-drying, which can be harsh and could cause some molecules to move, such as migration of lipids such as cholesterol to the surface. This type of migration alters the distribution of molecules within the sample and can generate over-bearing peaks in surface analysis, as well as not truly representing the structure within the sample [184]. Analysing a sample in the hydrated state, under cryogenic conditions, is considered the gold standard of sample preparation [185]. However, preparing samples as “frozen-hydrated” presents some difficulties in sample handling and loading. This type of preparation can also effect the sample aside from molecule migration. Zhang *et al* reported water crystal formation (a layer of ice on the surface) after preparing samples as “frozen-hydrated”, effecting the ion yield of their analysis [184]. Therefore, a compromise should be reached in sample preparation, taking extra care in what the sample is exposed to, often conducting analysis at RT, or on dehydrated samples.

The applications of the 3D OrbiSIMS continue to be investigated. So far, the instrument has successfully been used to study ions present in a sample, and their distribution within the sample and ion characterisation by mass spectrometry-mass spectrometry analysis in both *in vivo* and *in vitro* cell samples [186-188].

1.5 Project hypotheses and aims

There are several hypotheses tested in this thesis. Firstly, that the fluorinated CBD derivative, 4'-F-CBD, will exhibit similar anti-cancer properties as CBD. The anti-cancer properties of CBD against GBM will be investigated to understand the potential of the molecule to overcome resistance mechanisms to treatment with TMZ. Secondly, that the potential synergy of CBD with TMZ for GBM treatment that has previously been reported [131, 132], might also be observed with the TMZ analogue, T25.

It has previously been demonstrated that oral lipid-based formulations result in increased bioavailability of CBD [155, 169]. Additionally, there are reports that certain fatty acids could increase delivery of molecules across the BBB and into the brain [62, 66, 189]. Therefore, it is hypothesised that using a lipid-based CBD formulation would increase delivery to the brain following oral administration, compared to a lipid-free formulation. It is also predicted that the lipid-based formulations containing high compositions of fatty acids reported to impact BBB permeability, including oleic and myristic acids, would demonstrate high delivery to the brain.

The immunosuppressive effects of CBD in MS are documented in the literature [119-123]. As the role of the lymphatic system in the development of disease becomes increasingly better understood, our approach to treatment administration also develops. As oral lipid-based formulations of CBD have been shown to demonstrate high delivery to the lymphatic system [151], it is hypothesised that this would result in increased potency of CBD, compared to formulations with lower delivery to the lymphatic system, such as a lipid-free formulation. Due to the location of MS, it is also predicted that a formulation exhibiting high delivery of CBD to the brain could also demonstrate high potency against MS.

Finally, the properties of 4'-F-CBD are only beginning to be explored. The cannabinoid has demonstrated potential in *in vivo* behavioural models, but the PK of the molecule has not yet been investigated. Previous work on the molecule has resulted in calls for the PK properties of the cannabinoid to be studied, demonstrating the need for this in the development of potential applications of

the molecule. It is hypothesised that the PK properties of 4'-F-CBD would be similar to that of CBD, due to the small difference in the structures of the molecules. However, the addition of the fluorine atom is predicted to increase the permeation across the BBB, resulting in higher delivery of the molecule to the brain, as compared to CBD.

The aims of this thesis can be separated in the results chapters, and are summarised below:

Chapter 3: This chapter aims to investigate the anti-cancer properties of cannabinoids CBD and 4'-F-CBD. The ability of the cannabinoids to overcome two major resistance mechanisms to GBM treatment with TMZ (MGMT over-expression and MMR deficiency) will be investigated. Potential synergy of the cannabinoids with imidazotetrazine compounds TMZ and T25 will also be examined.

Chapter 4: This chapter aims to demonstrate the delivery of CBD to the brain after oral administration in lipid-free and lipid-based formulations. The brain is a complex organ responsible for a vast range of activity, and therefore the organ will be dissected to investigate potential differential delivery of CBD to specific anatomical brain regions.

Chapter 5: This chapter will examine the neuroprotective effects of CBD against MS *in vivo*. Activity of CBD against MS has previously been demonstrated, but it is hypothesised that increased delivery to the lymphatic system by oral administration in a lipid-based formulation could improve the therapeutic effects observed.

Chapter 6: This chapter will study the *in vivo* PK and biodistribution of 4'-F-CBD for the first time. It is hypothesised that *in vivo* properties could be similar to CBD, although the reported increased potency of the fluorinated derivative suggests potentially different biodistribution that is important to understand in the development of the molecule for therapeutic application.

Overall, the aim of this PhD is to investigate the *in vitro* and *in vivo* properties of cannabinoid CBD and derivative, 4'-F-CBD. The work focuses on the anti-cancer application of the cannabinoids. However, the work to investigate

differential biodistribution following oral administration in lipid-free and lipid-based formulations can be expanded to other applications, as will be shown for the application of CBD against MS. The in-depth study of the biodistribution of cannabinoids to anatomical brain regions following oral administration aims to demonstrate the importance of formulation selection for each potential application and progress the understanding of cannabinoids' complexity *in vivo*.

CHAPTER 2: Methods

2.1 Materials and instruments

Natural and synthetic cannabidiol (CBD) were obtained from THC Pharm (Frankfurt, Germany). 1-Fluoropyridinium triflate was purchased from Fluorochem (Derbyshire, UK). Isolute HM-N was purchased from Biotage (Hengoed, UK). Cell lines U373-V and U373-M were supplied by Schering Plough (NJ, U.S.A.). Cell lines HCT116 and MRC-5 were supplied from ATCC (VA, U.S.A.). RPMI-1640, minimum essential medium (MEM), foetal bovine serum (FBS), non-essential amino acids (NEAA), geneticin G418, gentamicin, L-glutamine, penicillin/streptomycin (pen/strep), sterile Hepes buffer, sterile cell culture sodium bicarbonate, ethylenediaminetetraacetic acid (EDTA), 10× trypsin-EDTA solution, temozolomide (TMZ), sesame oil, coconut oil, rapeseed oil, olive oil, 4,4-dichlorodiphenyltrichloroethane (DDT), ammonium formate, indium tin oxide (ITO) coated glass slides, dry dichloromethane (DCM), deuterated chloroform (CDCl₃) and sterile dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Dorset, UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aesar (Heysham, UK). Rat plasma was purchased from Sera Laboratories International (West Sussex, UK). Optimal cutting temperature compound (OCT) and high purity grade propylene glycol were obtained from VWR (Poole, UK). T25 was synthesised within the University of Nottingham by Helen Summers. All other chemicals and reagents used were of high performance liquid chromatography (HPLC) grade or higher, purchased from ThermoFisher Scientific (Leicestershire, UK).

2.2 Chemical synthesis of 4'-fluoro-cannabidiol

This method was used to synthesise 4'-fluoro-cannabidiol (4'-F-CBD) that was used to generate data reported in Chapters 3 and 6.

The synthesis of 4'-F-CBD was reported by Breuer *et al* (also referred to as HUF-101 and PECS-101), and this method was followed, with modifications to improve the separation of the product from any unreacted CBD [176].

2.2.1 General Procedures

A Buchi Rotavapor consisting of a V-850 vacuum controller, R-210 rotavapor and B-491 heating bath was used for drying.

A Biotage SP4 flash chromatography system was used for separation with a normal phase puriFlash (PF-15SIHP-F0004, Interchim, Montluçon, France) column cartridge. A flow rate of 5 mL/min was used with line A (hexane) and line B (20% ether in hexane). The column cartridge was equilibrated with 5% line B for 3 column volumes (CV) first. After equilibration, the product was loaded onto the column. The gradient used was 0 – 2 CV 5% line B, 2 – 12 CV 5 – 10% line B, 12 – 22 CV 10% line B, 22 – 32 CV 10 – 20% line B, 32 – 35 CV 20% line B.

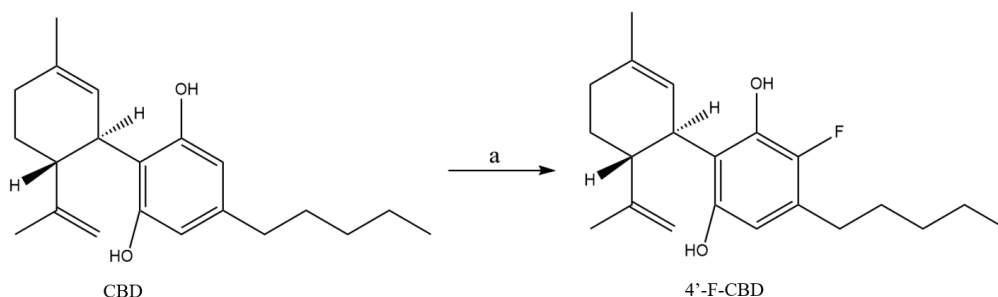
Separation was confirmed with thin layer chromatography (TLC) on silica precoated aluminium backed 60 F₂₅₄ plates (Merck, Darmstadt, Germany) using 6% ether in hexane. Compounds were visualised by an ultraviolet (UV) lamp at 254 nm.

Liquid chromatography mass spectrometry (LC-MS) was used to verify the product. A Shimadzu UFLCXR system was used with an Applied Biosystems API2000 to visualise spectra at 220 nm. Separation was achieved using a Phenomenex Gemini-NX C18 110A column (50 mm × 2 mm × 3 µm) at 40 °C. A flow rate of 0.5 mL/min was used with 0.1% formic acid in water in line A and 0.1% formic acid in acetonitrile (ACN) in line B. The gradient used was 0.0 – 1.0 min 5% line B, 1.0 – 3.0 mins 5 – 98% line B, 3.0 – 5.0 mins 98% line B, 5.0 – 5.5 mins 98 – 5% line B, 5.5 – 6.5 mins 5% line B.

A Bruker 400 Ultrashield nuclear magnetic resonance (NMR) was used to assess the product by hydrogen (^1H) NMR at 400 MHz. Deuterated chloroform (CDCl_3 , $\delta = 7.26$) (~0.5 mL) was added to dissolve a small amount (~5 mg) of product, which was then transferred to an NMR tube for analysis. MestReNova software version 14.2.2 (Mestrelab Research, Santiago de Compostela, Spain) was used to process the data. Chemical shifts (δ) are reported in parts per million (ppm). Coupling constants (J) are recorded in Hz, and the multiplicities are described as singlet (s), doublet (d), triplet (t), multiplet (m) or broad (br).

2.2.2 4'-Fluoro-cannabidiol

Synthetic CBD was used for the synthesis. 1-Fluoropyridinium triflate (79 mg, 0.3 mmol), CBD (100 mg, 0.3 mmol) and 4.5 mL dry DCM were stirred overnight in a nitrogen environment at room temperature. The yellow product was washed with (3×5 mL) aqueous sodium bicarbonate (NaHCO_3). The organic layer was then dried over sodium sulphate (Na_2SO_4) anhydrous, filtered and dried onto isolate (1 - 2 spatulas).



Reagents and conditions: (a) 1-fluoropyridinium triflate, DCM, room temperature, overnight.

Figure 2.1. Synthesis of 4'-fluoro-cannabidiol.

Separation of 4'-F-CBD from any unreacted CBD was confirmed by TLC.

Characterisation reported by Breuer *et al* [176]: total yield (27%), ^1H NMR (300 MHz, CDCl_3) $\delta = 6.17$ (s, 1H, Ar), 5.52 (s, 1H), 4.56 (s, 1H), 4.44 (s, 1H), 3.92

(s, 1H), 2.50 (br, 2H), 2.19 – 2.05 (br, 2H), 1.77 (s, 3H), 0.86 (t, 3H), LC-MS $[M+H]^+$ m/z = 332.

Characterisation found: total yield (42%), this is higher than reported due to improved separation by flash chromatography. ^1H NMR (400 MHz, $CDCl_3$) δ = 6.20 (d, J = 6.3, 1H, Ar), 5.72 (br, s, 1H, OH), 5.56 (d, J = 2.6, 1H, CH=C), 5.03 (br, s, 1H, OH), 4.60 (s, 1H, CH=C), 4.47 (s, 1H, CH=C), 3.94 (d, J = 10.1, 1H, Ar-CH), 2.69 – 2.40 (m, 3H, CH₃-C=C), 2.28 – 2.20 (br, m, 1H, CH-C=C), 2.17 – 2.07 (m, 1H, CH-C=C), 1.88 – 1.75 (m, 2H, CH₂), 1.71 (d, J = 1.3, 3H, CH₂-CH), 1.63 – 1.54 (m, 5H, CH₃, CH₂), 1.35 (dd, J = 7.3, 2.0, 2H, CH₂), 1.35 – 1.23 (m, 2H, CH₂), 0.91 (t, J = 6.8, 3H, CH₃). Whilst Breuer *et al* [176] did not report all ^1H NMR peaks, those they did report match those found, and the additional peaks could all be assigned to the structure as described. LC-MS: $[M+H]^+$ calculated m/z = 332.5, found m/z = 332.9, retention time: 3.26 mins, purity 95%. The LC-MS characterisation matches that reported.

2.3 Cell culture

The cell culture methods were used to generate data reported in Chapter 3.

Human glioblastoma multiforme (GBM) cell lines U373-V and U373-M were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% NEAA, 50 µg/mL gentamycin and 400 µg/mL G418. Human colorectal cancer HCT116 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Non-tumourigenic foetal lung fibroblasts (MRC-5) were cultured in MEM medium supplemented with 10% FBS, 1% NEAA, 1% pen/strep, 2 mM L-glutamine, 10 mM Hepes buffer and 0.075% sodium bicarbonate.

2.3.1 Reviving cell stocks

Cell lines were handled under sterile conditions and work was carried out inside a BioMat²MDH Class II microbiological safety cabinet with a laminar flow system. The cabinet interior and all materials entering the cabinet were decontaminated with 70% industrial methylated spirits (IMS). Cell batches were retrieved from liquid nitrogen storage (-196 °C). Cell vials were defrosted immediately and quickly using a 37 °C water bath. Once thawed, cells were transferred to a T25 cm² culture flask containing 6 mL of pre-warmed growth medium with 20% FBS. Cell flasks were examined for signs of infection under the Nikon Eclipse TS100 inverted light microscope and if infection free, flasks were maintained in the Sanyo carbon dioxide (CO₂) incubator (MCO-19AIC (UV)) at 5% CO₂, 37 °C. The following day, flasks were again inspected for infection and cell viability, and the medium was replaced with fresh pre-warmed medium to remove DMSO and any dead cells. Following this, medium was replaced every 2 - 3 days until cell coverage reached 70 - 80% confluency, after which time, medium with 10% FBS was used.

2.3.2 Cell passaging

Cell lines were cultured in T25 cm² culture flasks. Passaging to maintain cell growth was carried out when cells reached 70 - 80% confluency: ~once a week for U373-V and U373-M and ~twice a week for HCT116 and MRC-5. To passage cells, medium was aspirated, and cells were washed with phosphate buffered saline (PBS) before adding 0.5 mL 1× trypsin-EDTA solution to detach cells. The trypsin-EDTA solution was evenly spread across the flask surface to cover all cells and the flasks were placed in the incubator for 3 - 5 min. The trypsin was then neutralised with 4.5 mL medium, then 300 µL cell suspension for U373-V and U373-M, and 0.5 mL for HCT116 and MRC-5 were transferred to a new flask containing 6 mL of fresh pre-heated medium. Once reaching passage 30, cell flasks were disposed of, and fresh cells were revived from the liquid nitrogen storage.

2.3.3 Cell freezing for storage

To prepare cells for freezing, medium was aspirated, and cells were washed with PBS before adding 0.5 mL 1× trypsin-EDTA solution to detach cells. Trypsin was evenly spread across the flask surface to cover all cells and the flasks were placed in the incubator for 3 - 5 min. Then, 1 mL freezing medium (90% FBS, 10% DMSO) was added and the cell solutions were transferred to sterile cryogenic vials. Cells were gradually frozen in a three-step process. First, vials were placed in -20 °C storage overnight, they were then transferred to -80 °C for 24 h, and finally placed in liquid nitrogen (-196 °C) for long term storage.

2.3.4 MTT assay – 3- and 6-days exposure

The assessment of cell viability after exposure to test agents was assessed by MTT assay. MTT is readily taken up by viable cells and reduced by the mitochondria, and therefore, measurement of the formazan product can be used to assess cell viability [190, 191].

Cells were seeded into tissue culture, flat bottom polystyrene 96-well plates at the following densities: 3 days exposure: all cell lines: 3×10^3 cells/well, 6 days exposure: U373-V and U373-M cells: 650 cells/well, HCT116 and MRC-5 cells: 400 cells/well. Five replicates of each test agent concentration, ten for cell only controls and twelve for T0 control were performed on at least three separate occasions. Medium only (200 μ L) was placed in all outer cells as an additional control to minimise medium evaporation. Cells were seeded into wells at a volume of 180 μ L/well and kept in the incubator at 5% CO₂, 37 °C, for the duration of the assay. At 24 h after seeding, cells were treated with 20 μ L test agent and a MTT assay was performed to measure absorbance at the time of test agent addition (T0). Stock test agent solutions were prepared in DMSO at 50 mM, which were diluted in medium immediately before adding to cells at a concentration 10 $\times$ greater than the final required concentration in the well. Controls of DMSO only were also tested to assess any effect on cell viability from the solvent alone. The range of concentrations of each test agent used are summarised in Table 2.1.

Table 2.1. Concentrations of test agents used in MTT assay.

Test agent	Concentration range exposed to cells (μ M)			
	U373-V	U373-M	HCT116	MRC-5
TMZ	1 - 1000	1 - 1000	1 - 1000	1 - 1000
T-25	1 - 100	1 - 100	1 - 1000	1 - 1000
CBD	1 - 100	1 - 100	1 - 1000	1 - 1000
4'-F-CBD	1 - 100	1 - 100	1 - 1000	1 - 1000
DMSO	0.02 - 2.00%	0.02 - 2.00%	0.02 - 2.00%	0.02 - 2.00%

After 3- or 6-days exposure, or at 24 h post-seeding for T0 control, cell viability was assessed. MTT reagent prepared in PBS at 2 mg/mL was added to the medium in each well (50 μ L) to give a final concentration of 400 μ g/mL. The plates were then placed in the incubator for 2 h at 5% CO₂, 37 °C. The medium and any non-metabolised MTT was aspirated, and the remaining formazan

crystals were dissolved in 150 μ L DMSO. The plates were placed on an orbital shaker for 5 mins and absorbance was read at $\lambda = 570$ nm on a PerkinElmer EnVision plate reader.

Absorbance data was obtained by Wallac Envision Manager software version 1.12 (PerkinElmer Informatics, MA, U.S.A.). The concentration required to inhibit cell growth by 50% (GI_{50}) was calculated for each test agent using Equations (1) and (2).

$$GI50A = \left(\frac{(C-T0)}{2} \right) + T0 \quad \text{Equation (1)}$$

$$GI_{50} = \left(\left(\frac{(E-D)}{(X-Y)} \right) * (X - GI50A) \right) + D \quad \text{Equation (2)}$$

Where:

$GI50A$ = Absorbance at GI_{50}

C = Mean absorbance of control (cells only)

$T0$ = Mean absorbance of control ($T0$)

X = High absorbance (absorbance at data point just above $GI50A$)

Y = Low absorbance (absorbance at data point just below $GI50A$)

D = High concentration (concentration at absorbance data point just above $GI50A$)

E = Low concentration (concentration at absorbance data point just below $GI50A$)

2.3.5 MTT combination assay – 3- and 6-days exposure

Combinations of treatments were also tested for synergistic, additive, or antagonistic responses following the same MTT assay explained above in section 2.3.1 with the following change: cells were seeded into wells at a volume of 160 μL /well. Cells were treated with 20 μL of each test agent, or 40 μL medium for control wells.

The combinations of test agents assessed are shown in Table 2.2, with the concentrations of test agents used in Table 2.3. The combination index (CI) was calculated using Equation (3).

Table 2.2. Combinations of test agents assessed against U373-V, U373-M and HCT116 cell lines by MTT.

Dosing time	Test agent A	Test agent B
3 days	TMZ	CBD
	CBD	TMZ
	T25	CBD
	CBD	T25
	TMZ	4'-F-CBD
	4'-F-CBD	TMZ
	T25	4'-F-CBD
	4'-F-CBD	T25
6 days	TMZ	CBD
	CBD	TMZ
	TMZ	4'-F-CBD
	4'-F-CBD	TMZ

Table 2.3. Concentrations of test agents used for combination MTT assays.

Test agent A (µM)		Test agent B (% GI ₅₀)					
0.5	0	1.25	2.5	5	10	20	50
1	0	1.25	2.5	5	10	20	50
5	0	1.25	2.5	5	10	20	50
10	0	1.25	2.5	5	10	20	50
25	0	1.25	2.5	5	10	20	50
50	0	1.25	2.5	5	10	20	50

$$CI = \left(\frac{D1}{DX1} \right) + \left(\frac{D2}{DX2} \right) \quad \text{Equation (3)}$$

Where:

CI = Combination index

D1 = GI₅₀ of test agent A when in combination with test agent B

DX1 = GI₅₀ of test agent A alone

D2 = GI₅₀ of test agent B when in combination with test agent A

DX2 = GI₅₀ of test agent B alone

2.3.6 Cell count assay – 3- and 6-days exposure

Cell viability measured by MTT assay should also be confirmed by a second assay. Cell count assays were used as a confirmation of cell growth [192].

Cells were seeded into tissue culture, flat bottom polystyrene 6-well plates at the following densities: U373-V and U373-M cells: 4×10^4 cells/well, HCT116 and MRC-5 cells: 2×10^4 cells/well. Three replicates of each test agent concentration and cell only controls were performed on at least three separate occasions. Cells

were seeded into wells at a volume of 1 mL/well and stored in the incubator at 5% CO₂, 37 °C, for the duration of the assay. At 24 h after seeding, cells were exposed to 1 mL test agent at concentrations described in Table 2.4. Stock test agent concentrations were prepared in DMSO at 50 mM, which were diluted in medium immediately before adding to cells at a concentration 2× greater than the final required concentration in the well.

Table 2.4. Concentration range of test agents used for cell count assays.

Test agent	Concentration range cells exposed to (µM)			
	U373-V	U373-M	HCT116	MRC-5
TMZ	1 - 25	100 - 500	300 - 1000	50 - 1000
T-25	5 - 100	5 - 100	5 - 100	5 - 100
CBD	5 - 100	5 - 100	5 - 50	1 - 20
4'-F-CBD	5 - 100	5 - 100	5 - 50	1 - 100

After 3- or 6-days exposure, medium was aspirated, and cells were washed with 0.5 mL PBS. Trypsin was added (0.25 mL/well of 1× trypsin-EDTA solution) and the flasks were placed in the incubator for 3 - 5 min. The trypsin was then neutralised with 0.25 mL medium. Cells were counted using a haemocytometer under a Nikon Eclipse TS100 microscope.

2.3.7 Cell count combination assay – 3- and 6-days exposure

Combinations of treatments were also tested by cell count assay to corroborate any synergistic, additive, or antagonistic responses following the MTT assay. The cell count assay explained above in section 2.3.3 was followed. The combinations assessed are shown in Table 2.5, with the concentrations and volumes of test agent used described in Table 2.6.

Table 2.5. Combinations of test agents assessed against U373-V, U373-M and HCT116 cell lines by cell count.

Dosing time	Test agent A	Test agent B
3 days	TMZ	CBD
	T25	CBD
	TMZ	4'-F-CBD
	T25	4'-F-CBD
6 days	TMZ	CBD
	TMZ	4'-F-CBD

Table 2.6. Concentrations of test agents used for combination cell count assays.

Test agent		Volume of test agent stock solution added per well (mL)	Concentration cells exposed to
A		1	100% GI ₅₀
B		1	100% GI ₅₀
Combined well	A	0.75	25% GI ₅₀
	B	0.75	25% GI ₅₀

2.4 *In vivo* studies

All experimental procedures and animal welfare were reviewed and authorised by the United Kingdom Home Office and the University of Nottingham Ethical Review Committee in accordance with the Animals [Scientific Procedures] Act 1986. Unless stated otherwise, all animals were housed in the University of Nottingham Bio-Support Unit under regulated temperature and humidity, 12 h light-dark cycles and unrestricted access to food and water. Power calculations were used to determine the sample size for each experiment based on a Cohen's *d* effect size of 0.7 (moderate difference). The dosing of animals could not be masked for these experiments, however, all samples collected were numbered and not labelled by formulation. Therefore, the analysis of samples was masked and only unmasked after the quantification of cannabinoid in the sample had been determined.

2.4.1 Jugular vein cannulation

This method was used for the pharmacokinetics (PK) assay that was used to generate data reported in Chapter 6.

Male Sprague Dawley rats (275 - 300 g, Charles River Laboratories, UK) were used for *in vivo* PK studies. In order to reduce the pain experienced by the animal by multiple blood sampling, jugular vein cannulation is commonly performed [193-195].

First, the rats were weighed and individually placed into an anaesthetised chamber (5% isoflurane) until righting reflex was no longer observed. The rat was then placed on a warming pad with ~2% isoflurane (maintained during surgery), and the eyes were treated with eye gel to prevent drying during surgery. Warmed saline (4 mL) and meloxicam (0.01 mL) were administered by subcutaneous (s.c.) injection. The high chest and neck, back and front, were shaved and sterilised with 70% ethanol wipes. The rat was then wrapped for surgery.

With the rat on its back, a 1 - 1.5 cm incision was made in the neck skin where the right jugular vein is. Using blunt scissors, the vein was exposed by blunt dissection and isolated from the surrounding tissue using forceps and cotton tips. Two lengths of 4-0 surgical silk were placed under the jugular vein, the longer thread closest to the head. Warmed saline was added to keep the wound moist and the rat was turned onto its front.

A 0.5 cm incision was made on the back of the neck and the cannula was pulled through to the front wound using a guide. The rat was placed on its back again.

The longer thread was ligated and held stable, and the shorter thread was tied in a semi-closed loop. A small incision was made in the jugular vein surface between the two threads, and the cannula (flushed with heparinised saline) was inserted 2.5 cm into the jugular vein at the incision. The cannula function was checked by injecting a small amount of saline and then withdrawing to see blood. Once checked, the semi-closed loop was ligated. Using the longer thread, the cannula was secured along the jugular vein and to muscle tissue in the back of the neck. All open wounds were then closed with 4-0 silk suture. The cannula was checked again by injecting and withdrawing a small amount of saline. The cannula on the back of the body was cut to leave 3 - 4 cm, and a metal pin was placed in the end to prevent any blood leaking.

The rat was left to wake on the heated pad, before being placed in an individually housed cage with soft, mashed food, on heater pads for ~1 h. Rats were left for two nights to recover from surgery, where the mashed food was replaced with hard food after one night.

2.4.2 Pharmacokinetics assay

This method was used to generate data reported in Chapter 6.

PK assays were performed for 4'-F-CBD formulations. The two formulations were both prepared at 12.7 mg/mL 4'-F-CBD in either sesame oil or a lipid-free formulation (80:10:10 propylene glycol:ethanol:water). All formulations were prepared fresh on the morning of the experiment.

First, a pilot study of $n = 2$ for sesame oil and $n = 1$ for lipid-free groups was conducted to ensure the dose of 4'-F-CBD would be tolerated. The animals were fasted overnight before administration for a maximum of 16 h. The formulation was administered by oral gavage at 1 mL/Kg to give a dose of 12.7 mg/Kg 4'-F-CBD, followed by 1 mL water. Blood (300 μ L) was collected from the cannula at the pre-determined time points of 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 h post administration. Blood was placed directly into a tube containing 1% EDTA and placed in ice. After 10 mins, the blood samples were centrifuged at 2000 g, 4 °C, for 5 mins to isolate the plasma, which was stored at -80 °C until analysis.

Power calculations determined that a Cohen's d effect size of 0.7 (moderate difference) required $n = 7$ for a power >0.8 . This was the target n that could be reached by combining results of the pilot study with a further $n = 6$ per group. The same process described above was followed for the larger study of $n = 6$ per group. For PK data, Phoenix WinNonlin 6.3 Professional (Certera, NJ, U.S.A.) was applied for non-compartmental analysis of the obtained plasma concentrations.

2.4.3 Biodistribution assay in rats

This method was used to generate data reported in Chapters 4 and 6.

Male Sprague Dawley rats (275 - 300 g, Charles River Laboratories, UK) were used for *in vivo* biodistribution studies. Studies were performed using natural CBD in the following formulations; sesame oil, coconut oil, rapeseed oil and lipid-free (80:10:10 propylene glycol:ethanol:water) at 12 mg/mL. Formulations of 4'-F-CBD at 12.7 mg/mL in sesame oil and lipid-free (80:10:10 propylene glycol:ethanol:water) were also assessed. All formulations were prepared fresh on the day of the experiment.

Biodistribution of CBD was assessed at 1, 2, 3, 5, 8, 12, 15 and 18 h post administration. For each group, a Cohen's d effect size of 0.8 (moderate difference) required $n = 3$ for a power >0.8 . The animals were fasted overnight before administration for a maximum of 16 h. Formulations were administered by oral gavage at 1 mL/Kg, followed by 1 mL water. At the pre-determined end

points, animals were humanely sacrificed. Brain tissue was harvested and dissected based on Spijker (2011) [196]. With the ventral side of the brain facing down, the olfactory bulb was removed before removing the cerebral stem and isolating the cerebellum from the medulla pons. The cortex was opened from the midline by placing closed forceps between the two cortex halves and gently opening, as depicted in Spijker (2011) [196]. The cortex was moved aside to allow the removal of the hippocampus from both the left and right sides. The cortex was folded back into place and the front section of the brain was removed as the frontal lobe. This allowed the striatum to be located and part of it isolated from the frontal lobe [196]. The remaining tissue was separated into the parietal lobe, temporal lobe, and occipital lobe.

The biodistribution of 4'-F-CBD was assessed at 1, 2, 3, 5, 8, 15 and 18 h, following the procedure described for the assessment of CBD biodistribution. Brain tissues were harvested by the same method and the following other tissues were also harvested; spinal cord, heart, spleen, kidneys, liver, lungs, testes, and mesenteric, iliac, axillary, brachial, popliteal and cervical lymph nodes [197]. Additionally, after the completion of 4'-F-CBD PK studies at 12 h post administration, brain tissues and mesenteric lymph nodes (MLN) were harvested. All tissues were frozen at -80 °C until analysis.

2.4.4 Biodistribution assay in mice

This method was used to generate data reported in Chapter 5.

Male CD-1 mice (28 - 30 g, Charles River Laboratories, UK) were used for *in vivo* biodistribution studies. Studies were performed using natural CBD in the following formulations; sesame oil, olive oil and lipid-free (80:10:10 propylene glycol:ethanol:water) at 30 mg/mL. All formulations were prepared fresh on the day of the experiment.

Biodistribution of CBD was assessed at 0.5, 1 and 2 h post administration. For each group, a Cohen's *d* effect size of 0.8 (moderate difference) required $n = 4$ for a power >0.8 . The animals were fasted overnight before administration for a maximum of 16 h. Formulations were administered by oral gavage at 1 mL/Kg,

followed by 0.1 mL water. At the pre-determined end points, animals were humanely sacrificed. Brain tissue was harvested and dissected as described above in section 2.5.3. Additionally, blood samples (~0.8 mL) were collected from the posterior vena cava. Serum was isolated from the blood samples by centrifugation at 3000 g for 10 mins. All tissues were frozen at -80 °C until analysis.

2.4.5 Efficacy study in experimental autoimmune encephalomyelitis (EAE) mouse model

This method was used to generate data reported in Chapter 5.

The effect of oral administration of a lipid-based formulation of CBD on the development and severity of symptoms of multiple sclerosis (MS) was assessed in an experimental autoimmune encephalomyelitis (EAE) mouse model [198, 199]. The experimental procedure and animal welfare were reviewed and authorised by the United Kingdom Home Office, the University of Nottingham Ethical Review Committee, and the University of Edinburgh Ethical Review Body in accordance with the Animals [Scientific Procedures] Act 1986. Animals were housed at the University of Edinburgh animal facility under regulated temperature and humidity, 12 h light-dark cycles and unrestricted access to food and water.

Female C57BI/6 mice (18 - 20 g) were used for the *in vivo* efficacy study. On day 0, EAE was induced in mice by s.c. injection in both hind legs with 140 µg myelin oligodendrocyte protein (MOG₃₅₋₅₅) in complete Freund's adjuvant (CFA) [200]. Pertussis toxin (100 µL of 50 ng/mL solution) was administered by intraperitoneal (i.p.) injection on day 0 and day 1 to open the blood-brain barrier (BBB) to immune cell access.

Mice were split into the groups described in Table 2.7. CBD formulations were prepared at 50 mg/mL. All formulations were prepared on the morning of dosing. Starting on day 1, mice were administered the formulation by oral gavage at 1 mL/Kg, followed by 0.1 mL water, once daily for 8 days.

Table 2.7. Administration groups of mice for EAE model efficacy study.

Group	Formulation	n
1	Control: no dose	6
2	Control: lipid-free vehicle only	6
3	Control: olive oil vehicle only	6
4	CBD in lipid-free formulation	4
5	CBD in olive oil formulation	6

The mice were monitored every other day for the first 6 days after induction, and every day following this. Mice were weighed and their clinical scoring graded, including a record of any hunched appearance, reduced activity, and pilo-erection. Clinical scoring was done on a scale of 1.0 to 7.0, as described below. Mice were allowed to reach a score of 4.0, considered moderate disease, but once reaching this score were humanely killed as a pre-determined end point. Once mice reached a score of 2.0, they were provided with hydrated food on the floor of the cage and were monitored twice daily. Weight was also used as a measure from a score of 3.0. When mice reached 20% loss of starting body weight they were humanely killed as a pre-determined end point. The end point for any surviving animals was one month after induction.

1.0 = Healthy

2.0 = Flaccid tail

3.0 = Impaired gait and/or impaired righting reflex

4.0 = Partial hind limb paralysis

5.0 = Total hind limb paralysis

6.0 = Any sign of fore limb paralysis

7.0 = Moribund

2.5 *Ex vivo* studies

2.5.1 Detection of cannabidiol by HPLC-UV

This method was used to generate data reported in Chapters 4 and 5.

The method to detect CBD in plasma by HPLC-UV is reported in Zgair *et al* (2015) [195]. This method was assessed to also be suitable for detecting CBD in tissues.

For the preparation of tissue samples, excluding the following samples: iliac, axillary, brachial, popliteal and cervical lymph nodes, the tissues were first homogenised using a POLYTRON PT 10-35 GT (Kinematica AG, Luzern, Switzerland). To tissues weighing at least 75 mg, water was added at 500 mg/mL in a plastic tube. For smaller tissues, water was added at a concentration to reach a minimum volume of 150 μ L. The tissues were then homogenised whilst held in a beaker of ice water to remain cold.

Iliac, axillary, brachial, popliteal, and cervical lymph nodes were homogenised using a Bullet Blender 24 Gold homogeniser. Lymph node tissue was added to 1.5 mL RINO tubes, and water was added at a concentration to reach a minimum volume of 175 μ L. The tubes were placed in the bullet homogeniser for 2 mins on maximum speed. The homogenised sample could then be carefully removed from to a fresh Eppendorf tube.

Once tissues were homogenised, the preparation of samples was the same for plasma, serum, or tissues. To a glass tube, 100 μ L sample was added, along with 10 μ L DDT (internal standard (IS), 50 μ g/mL in ACN). Cold ACN (450 μ L, -20 $^{\circ}$ C) was added for protein precipitation and the sample was vortex-mixed, followed by the addition of 450 μ L water and further vortex-mixing. Liquid-liquid extraction was performed with 3 mL hexane by vortex-mixing for 5 mins. The samples were centrifuged (Eppendorf centrifuge 5810 R) at 3500 rpm, 10 $^{\circ}$ C, for 10 mins before the organic phase was evaporated until dry under nitrogen at 37 $^{\circ}$ C. The residual in the tube was reconstituted in 100 μ L ACN.

With each analysis, a standard calibration curve was prepared using 100 μ L blank (untreated) tissue or plasma, spiked with 10 μ L stock solution (CBD in

ACN at a concentration 10× higher than the final required concentration) and 10 µL IS. Calibration curve samples were then prepared the same as the samples, as described above. Calibration curves were prepared at final concentrations of 10 - 1500 ng/mL.

A Waters HPLC system with 2695 separation module and 2996 photodiode array detector was used for analysis. Separation was achieved using an ACE 3 C18-PFP 150 mm × 4.6 mm, 3 µm particle, 100 µm pore size column with an ACE C18-PFP 4.0 × 4.6 mm, 3 µm particle, 100 µm pore size guard cartridge at 55 °C. The mobile phase was 62% ACN in water at a flow rate of 1 mL/min. The sample injection volume was 40 µL. Absorbance was monitored at $\lambda = 220$ nm. Data were processed using Empower 2 software (Waters, MA, U.S.A.). The retention times of CBD and IS (DDT) were 9 and 24 mins, respectively. The lower limit of quantification (LLOQ) of CBD was previously determined at 10 ng/mL [195].

2.5.2 Validation of the detection of 4'-fluoro-cannabidiol by HPLC-UV

This method was used to generate data reported in Chapter 6.

The same method reported by Zgair *et al* (2015) [195] could be used to detect 4'-F-CBD, which was validated as described below, following the U.S. Food and Drug Administration (FDA) Bioanalytical Method Validation Guidance for Industry [201]. The LLOQ, low quality control (LQC), medium quality control (MQC) and high quality control (HQC) samples were prepared at concentrations of 10, 20, 200 and 1000 ng/mL, respectively. DDT was used as the IS, and the retention time of 4'-F-CBD was 10.5 mins.

Selectivity

The selectivity of the method was assessed by comparing chromatograms of blank rat plasma and spiked samples at the LLOQ from six different batches of plasma. The method is considered to have acceptable selectivity if the blank rat plasma has no interfering peaks at the retention times of 4'-F-CBD or the IS (measured previously [195]).

Intra- and inter-day precision and accuracy

The precision and accuracy were assessed both intra- and inter-day by relative standard deviation (RSD) and relative error (RE). Intra-day precision and accuracy was assessed by analysing five replicates of blank rat plasma spiked with 4'-F-CBD at the LLOQ, LQC, MQC and HQC. Inter-day precision and accuracy was assessed by analysing five replicates of the LLOQ, LQC, MQC and HQC on three separate occasions. The acceptable limits of RSD and RE for intra- and inter-day runs are $\leq 15\%$ [201].

Recovery

Recovery during the extraction step of the sample preparation was assessed by comparing chromatograms of rat plasma spiked pre-extraction and post-extraction. Two replicates of the LLOQ, LQC, MQC and HQC were assessed. Recovery does not need to be 100%, but should be consistent and reproducible [201]. Recovery of the internal standard by this method has been assessed previously [195].

Stability

The storage stability of 4'-F-CBD in rat plasma was assessed after 6 h at room temperature and at both 24 h and 30 days at $-80\text{ }^{\circ}\text{C}$. Freeze-thaw stability was also assessed for 3 cycles from $-80\text{ }^{\circ}\text{C}$ to room temperature. Three replicates of the LLOQ, LQC, MQC and HQC were assessed for each storage condition. The acceptable limits for storage stability are $\pm 15\%$ [201].

2.5.3 Preparation of tissue samples for analysis by 3D OrbiSIMS

This method was used to generate data reported in Chapter 4.

Two types of tissue samples were assessed by 3D Orbitrap secondary ion mass spectrometry (3D OrbiSIMS); homogenate tissues and tissue sections [183].

To prepare homogenate tissue samples, brain tissue was homogenised as described in section 2.5.1. For tissues from dosed animals, samples were then placed (2 μL) onto a glass slide. Spiked homogenate samples were prepared by taking 10 μL stock (CBD or 4'-F-CBD in methanol) into 90 μL blank

homogenate tissue, before placing 2 μ L on the slide. Slides were left in a fume hood overnight to dehydrate. Homogenate samples were prepared on ITO coated glass cover slides. Conductive ITO slides were used to improve charge compensation, and therefore detection, during analysis by the 3D OrbiSIMS [183].

Tissue sections were prepared from tissue collected during *in vivo* biodistribution studies. At the pre-determined end point of the study when tissues were collected, brain tissue was harvested and placed as a whole brain into a plastic mould with a few drops of OCT at the bottom. OCT was then carefully added to ensure the tissue was entirely covered. The mould was subsequently stored at -80 °C prior to sectioning. A Leica CM3050 S research cryostat was used to prepare the tissue sections from brain samples frozen in OCT. Using OCT, the sample was attached to the chuck and placed in the holder. Excess OCT was removed from the sample using a razor. The cryostat chamber temperature was set to -20 °C. An ultramicrotome blade (Accu-Edge Sakura, Osaka, Japan) was used to cut sections at 16 μ m thickness and collected on ITO coated glass slides by holding the slides just above the tissue, allowing the section to adhere to the slide. Slides of sectioned tissue were subsequently stored at -80 °C prior to analysis.

2.5.4 Preparation of cell samples for analysis by 3D OrbiSIMS

This method was used to generate data reported in Chapter 3.

Slides of cell samples were prepared for analysis by 3D OrbiSIMS following a method based on Newman *et al* (2017) [188]. U373-V cells were cultured as described in section 2.3.2. ITO coated glass slides were used for cell samples, which were prepared by placing the ITO slide in a petri dish and seeding cells at a density of 160,000 cells/well into the dish with a total of 13.5 mL medium. Petri dishes were placed in the incubator at 5% CO₂, 37 °C.

The cells were monitored daily, and once confluency reached ~70%, the cells were treated with test agent. Cells were exposed to test agent from stock solutions prepared at $\times 10$ higher concentration than the GI₅₀ of the test agent in

medium from stocks of 50 mM in DMSO. The test agent was added to the petri dish (1.5 mL) and swirled to mix. The dish was placed back in the incubator until ready to harvest. Samples were also prepared of cells dosed in combination with CBD and TMZ or CBD and T25. These were prepared by dosing 750 μ L of each test agent stock, concentrations shown in Table 2.8, and gently mixing before returning to the incubator. Combined test agent concentrations were chosen based on combination MTT assays to represent ~75% growth inhibition.

Table 2.8. Concentration of test agent exposed to cells for preparation of cells dosed in combination for 3D OrbiSIMS analysis.

Test agent A	Test agent A concentration (μM)	Test agent B	Test agent B concentration (μM)
TMZ	2	CBD	11
T25	14	CBD	7

Slides were harvested at 3, 6, 24 and 72 h post dosing. The method used to harvest slides was based on Newman *et al* (2017) [188]. Petri dishes were removed from the incubator and the medium was aspirated. The slides were washed (3×1 mL) with 150 mM ammonium formate solution at pH 7.4. The slides were then dipped into liquid nitrogen for 10 s and placed in a benchtop freeze dryer (VirTis SP Scientific Sentry 2.0) at -50 °C for a minimum of 1 h. Once removed from the freeze drier, the slides were sealed in petri dishes with parafilm and stored at -80 °C until analysis.

2.5.5 Analytical conditions for analysis of samples by 3D OrbiSIMS

The 3D OrbiSIMS uses a Q Exactive HF for Orbitrap mass spectrometry. For acquisition of the single ion beam Orbitrap depth profile, a 20 keV Ar_{3000}^{+} gas cluster ion beam (GCIB) of 20 μ m diameter was used with a duty cycle of 4%

to produce positive and negative mode ion spectra with a mass range of m/z 75 - 1125. The profile was run on a focal area of $200 \times 200 \mu\text{m}$ using random raster mode. The injection time was set to 500 ms and 80 scans were taken for each analysis over an average of 120 s. The pressure in the main chamber was regulated using Ar gas to 9×10^{-7} mbar to enhance the charge compensation. The mass resolution was 240,000 at m/z 200.

3D OrbiSIMS data were collected using SurfaceLab 7 software (IONTOF GmbH, Münster, Germany). Peak lists were generated with minimum counts of 10,000 and combined using the 'union' function with a catch mass radius of 2 ppm. All data were normalised to the total ion count (TIC) of that analysis. Data were chemically filtered using molecular formula prediction (MFP) software, SIMS-MFP version 1.1 (University of Nottingham, Nottingham, UK) [202], into groups containing fatty acids ($\text{C}_n\text{H}_n\text{O}_2$), sulfatides ($\text{C}_n\text{H}_n\text{N}_1\text{O}_{11-12}\text{S}_1$) and glycerophospholipids ($\text{C}_n\text{H}_n\text{O}_{8/13}\text{P}$ or $\text{C}_n\text{H}_n\text{NO}_{7-10}\text{P}$) [183]. Data groups were then analysed using multivariate analysis (MVA) software, simsMVA (University of Nottingham, Nottingham, UK) [203]. The data groups were mean-centred, and the principal component analysis (PCA) function was used in algorithm mode, retaining all components. The Scores and Variance were used to find principal components (PCs) exhibiting differences between the groups, and Loadings allowed visualisation of the PCs.

2.6 Software and statistical analysis

Chemical structures and schemes were prepared using ChemDraw version 21.0.0 (PerkinElmer Informatics, MA, U.S.A.). One-way ANOVA with Dunnett's multiple comparisons, and multiple t-tests were performed in Prism version 9.3.1 (GraphPad, CA, U.S.A.) to assess the significant differences between sample groups. Differences were considered statistically significant when the p-value was < 0.05 ($\alpha = 0.05$). All data ($n \geq 3$) are represented as mean $\pm$ standard deviation (SD).

CHAPTER 3: Anti-cancer properties of cannabinoids

3.1 Introduction

It is reported in the literature that cannabinoids exhibit anti-cancer properties [103, 125, 126]. Most activity of cannabinoids in general is a result of interaction with CB1 and CB2 receptors of the endocannabinoid system. It has been demonstrated that CB1 and CB2 receptor expression can be altered in cancers, often upregulated (for example in hepatocellular carcinoma) and can be correlated with cancer cell invasion, proliferation and apoptosis [103, 129]. However, the role of cannabinoid receptor regulation in cancers is not yet fully understood. In particular, cannabidiol (CBD) and Δ^9 -tetrahydrocannabinol (THC) are often studied [125, 126, 145]. The cannabinoids are usually assessed in combination at a ratio of 1:1 CBD:THC, and sometimes in combination with other anti-cancer agents, such as temozolomide (TMZ). Cannabinoids are reported to exhibit effects against several cancers. CBD itself has demonstrated activity against colorectal, breast, glioma, cervical and lung cancers [103, 130].

There are varied reports on the anti-cancer mechanism of action of CBD [130, 143-145]. Whilst CBD is understood to have multiple targets, with a rich and diverse pharmacology, most of the pathways involved are only hypothesised. The suspected pathways involved are *via* transient receptor potential cation channel subfamily V member 2 (TRPV-2), increased reactive oxygen species (ROS) and increased endoplasmic reticulum (ER) stress, with some of the effects thought to be as a result of interaction with the endocannabinoid system [103, 125, 130, 143-145]. Additionally, the behavioural effects of CBD have been evidenced to involve deoxyribonucleic acid (DNA)-methylation, predominantly at the C⁵-cytosine in cytosine-phosphate-guanine (CpG) islands [146, 147]. DNA-methylation is not reported as a mechanism of anti-cancer activity of CBD, as far as we are aware. However, the methylation of cytosine in CpG islands indicates that nucleotide base methylation does occur as a result of CBD exposure, and therefore, DNA-methylation may be a possible mechanism of CBD anti-cancer activity [146, 147]. Inhibition of CB1, CB2 and TRPV-2

receptors has also been shown to reverse some of the anti-cancer effects of CBD, however the pathways involved are not yet fully understood [103, 130].

4'-Fluoro-cannabidiol (4'-F-CBD), also referred to as HUF-101 and PECS-101 in the literature, is a recently synthesised CBD derivative [176, 179]. 4'-F-CBD is reported to exhibit increased potency over CBD in *in vivo* behavioural assays [176, 177, 204]. Additionally, there is a recent report of 4'-F-CBD preventing chemotherapy-induced pain [179]. However, to the best of our knowledge, the anti-cancer properties of 4'-F-CBD have not yet been studied.

Glioblastoma multiforme (GBM) is an aggressive grade IV brain cancer with a poor prognosis of 5% 5-year survival [89]. Contributing to the poor prognosis is the common resistance of GBM to the standard of care chemotherapy treatment, TMZ. TMZ is a DNA-alkylating agent, predominantly methylating DNA purines at N^3 -adenine, N^7 - and O^6 -guanine positions. *N*-methylation is generally repaired quickly by base excision repair (BER), but *O*-methylation is not [93, 94]. *O*-methylation leads to a mis-pair of guanine with thymine (rather than cytosine) during DNA replication, triggering DNA mismatch repair (MMR), leading to cell death *via* apoptosis or autophagy [95]. There are two major resistance mechanisms to TMZ demonstrated in GBM. Firstly, an over-expression of O^6 -methylguanine-DNA methyltransferase (MGMT) allows the cells to repair DNA-methylation at the O^6 -guanine position. Secondly, MMR deficiency allows O^6 -methylguanine to be tolerated [87, 94].

GBM is additionally hard to treat due to the location, as the physical blood-brain barrier (BBB) protects the brain, restricting the movement of most therapeutics into the brain [87]. CBD is known to cross the BBB, as many of the observed effects of CBD are a result of interaction with the endocannabinoid system in the brain [112, 113, 152, 168, 205]. There are reports of CBD anti-cancer activity against GBM, although these are usually in combination studies with THC or TMZ [103, 127-130]. The combination of CBD and TMZ has been reported to cause both an additive and synergistic response *in vitro* [131, 132]. However, the few reports of CBD activity alone demonstrate a good response, with the concentration required to inhibit cell growth by 50% (GI_{50}) ranging from 10.67

$\pm 0.58 \mu\text{M}$ against GL216 [206] and $12.75 \pm 9.7 \mu\text{M}$ against U87MG [128, 132, 134, 137, 207] to $21.6 \pm 3.5 \mu\text{M}$ against U373MG [134].

In an *in vivo* U87MG GBM mouse model, when CBD, THC and TMZ were administered in combination, tumour growth was reduced by a larger extent than after administration of TMZ alone [127]. CBD has also been shown to be effective in *in vivo* GBM models U87, U251, GSC3832 and GSC387 at 15 - 20 mg/Kg, in combination treatments with THC and TMZ [103, 127, 133-136]. This has been demonstrated after intravenous (i.v.), intraperitoneal (i.p.), subcutaneous (s.c.) and oral administration [126, 134]. CBD has also been investigated in combination with radiotherapy in a mouse GL216 model, showing almost 90% apoptotic levels reached [126, 206].

The reports of anti-cancer activity of CBD in combination with other agents both *in vitro* and *in vivo* have led to initial clinical trials. Firstly, phase I/II clinical trials in GBM patients, which found Sativex® (a 1:1 mixture of CBD and THC, approved to treat symptoms of multiple sclerosis (MS)) to be safe, with no drug-drug interactions with TMZ identified [138-140]. Further clinical trials are underway to study the efficacy of combinations of radiotherapy and chemotherapy with TMZ and a 1:1 mixture of CBD and THC [141], as well as daily administration of CBD with TMZ [142], both in patients with GBM.

To the best of our knowledge, there are no reports investigating the activity of CBD alone against TMZ-resistant GBM. However, there is a report of CBD activity against colorectal cancer cell line, HCT116 [143]. As mentioned in Chapter 2, colorectal cancer cell line HCT116 exhibits a deficiency of MMR and is therefore used in this work as a model to represent this resistance mechanism (or tolerance) to TMZ treatment. In the study, CBD was administered alone and found to inhibit cell growth with a GI_{50} of $10.8 \mu\text{M}$ after 24 h exposure [143]. The common resistance mechanisms to GBM treatment with TMZ prevent the conversion of DNA-methylation to cell death [87, 94]. As discussed, CBD is thought to act *via* multiple pathways [103, 125, 130, 143-145], and therefore may be able to overcome the two major resistance mechanisms to GBM treatment, MGMT over-expression and MMR deficiency.

The aims of the work in this chapter start with investigating the anti-cancer activity of 4'-F-CBD against GBM. Then, the activity of cannabinoids CBD and 4'-F-CBD against GBM and colorectal cell lines resistant to the standard of care treatment, TMZ, (over-expression of MGMT and MMR deficiency) will be studied. As a synergistic response of CBD treatment with TMZ has been reported in the literature, combination treatments of cannabinoids (CBD and 4'-F-CBD) and TMZ or analogue, T25, will be studied. Finally, a new method to investigate the mechanism of anti-cancer action of CBD by 3D Orbitrap secondary ion mass spectrometry (3D OrbiSIMS) analysis will be investigated.

3.2 Experimental design

The results discussed in this chapter were generated using methods described in Chapter 2. For the assessment of effects of test agents on cell growth, methods described in sections 2.2 and 2.3 were used. For the analysis of cells by 3D OrbiSIMS, methods described in sections 2.5.4 and 2.5.5 were used. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparisons using GraphPad version 9.3.1 (GraphPad, CA, U.S.A).

3.3 Results

3.3.1 Growth inhibitory activity of cannabinoids on non-tumourigenic cells

First, the test agents were assessed for activity against non-tumourigenic foetal lung fibroblast cell line, MRC-5. TMZ, T25, CBD and 4'-F-CBD were assessed, as well as equivalent concentrations of dimethyl sulfoxide (DMSO) vehicle by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The test agents were then also assessed by cell count assay. Figure 3.1 shows the results of the MTT assay after both 3- and 6-days exposure. Cell count assay and DMSO control results can be found in Appendix 3.1. The addition of equivalent concentrations of DMSO to the cells was not found to impact cell growth or viability. TMZ was found to be the least active, only exhibiting cell growth inhibition at concentrations $>100\ \mu\text{M}$. CBD was demonstrated to be the most active, causing cell growth inhibition at $10\ \mu\text{M}$. T25 and 4'-F-CBD behaved similarly, both inhibiting cell growth at $50 - 100\ \mu\text{M}$ concentrations. The length of exposure, 3 or 6 days, did not appear to impact the results.

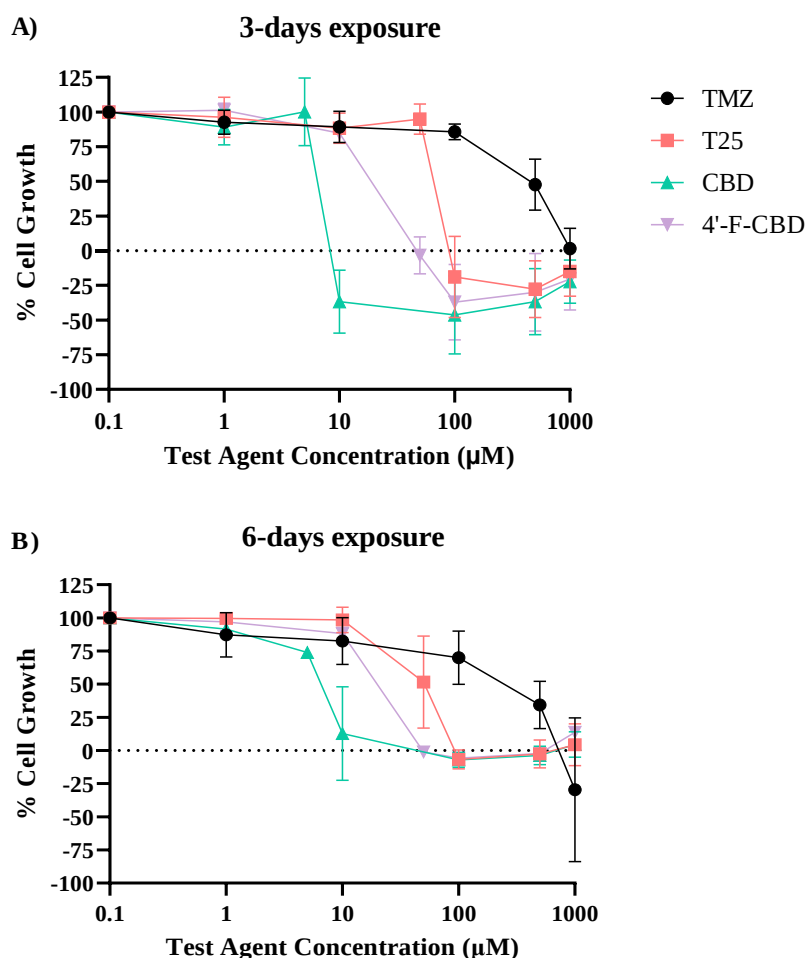


Figure 3.1. Dose-response curves of MRC-5 (non-tumourigenic control) cell line treated with test agents TMZ, T25, CBD and 4'-F-CBD after A) 3 and B) 6 days exposure. Cell growth represented as % of control (non-treated). Data measured by MTT assay and confirmed by cell count assay (Appendix 3.2). Data are presented as mean $\pm$ standard deviation (SD), three independent repeats of $n = 5$.

3.3.2 Cancer cell growth inhibition by cannabinoids

The cell growth inhibition caused by the test agents alone was assessed at both 3- and 6-days exposure first against human GBM control cell line, U373-V (-MGMT, +MMR), shown in Figure 3.2 and Table 3.1. Once administered, TMZ must undergo ring opening to the active monomethyl triazene 5-(3-methyltriazene-1-yl)-imidazole-4-carboxamide (MTIC), which reacts with water

in vivo to release the DNA-methylating compound and 5-aminoimidazole-4-carboxamide (AIC) [94]. Once the DNA is methylated at the *O*⁶-guanine position, it causes a mismatch of guanine with thymine (rather than cytosine). The mismatched thymine is removed and reinserted in the cell before triggering a DNA strand break, leading to cell death [94]. This process takes time, at least one cell cycle, and is why TMZ requires 6 days exposure to exhibit its full effect. After only 3 days exposure, the GI₅₀ is much higher, and therefore significant differences between the test agents and TMZ were observed only after 3 days exposure. All three test agents, T25, CBD and 4'-F-CBD, were found to exhibit a GI₅₀ significantly ($p < 0.01$) lower than TMZ.

Following successful inhibition of U373-V cells, all four test agents were assessed against TMZ resistant human GBM cell line, U373-M (+MGMT, +MMR) and colorectal cancer cell line, HCT116 (-MGMT, -MMR) after both 3- and 6-days exposure (Figure 3.2 and Table 3.1). Against both cell lines, T25, CBD and 4'-F-CBD were all found to exhibit GI₅₀ values significantly ($p < 0.001$) lower than TMZ, at both treatment times. These data were also confirmed by cell count assays, shown in Appendix 3.3.

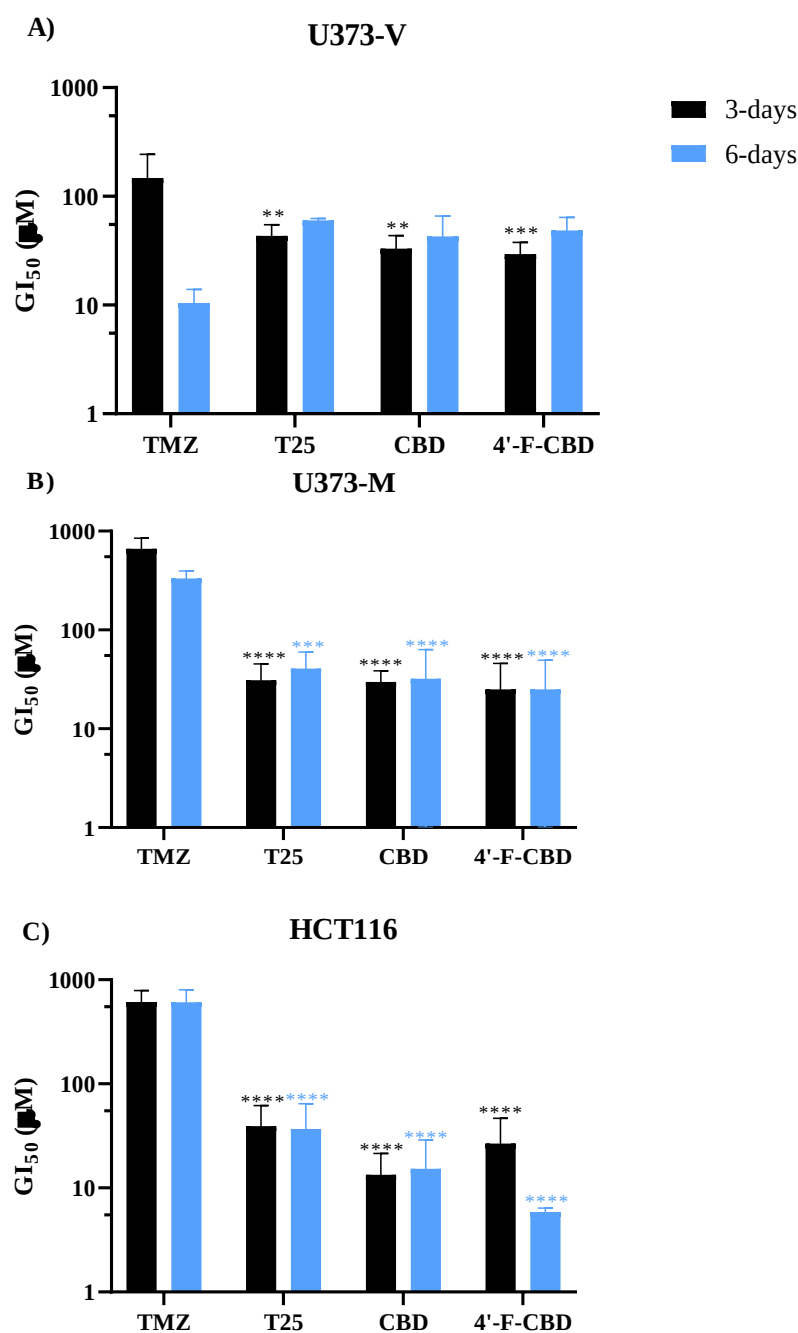


Figure 3.2. GI₅₀ of cannabinoids CBD and 4'-F-CBD compared to DNA-alkylating agents TMZ and T25 against A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after 3- and 6-days exposure. Data measured by MTT assay and confirmed by cell count assay (Appendix 3.3). Data are presented as mean $\pm$ SD, three independent repeats of $n = 5$. One-way ANOVA with Dunnett's multiple comparisons performed, comparing test agents to TMZ, $\alpha = 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. The data are also presented in Table 3.1.

Table 3.1. GI₅₀ values of test agents TMZ, T25, CBD and 4'-F-CBD individually assessed against U373-V, U373-M and HCT116 cell lines after 3- and 6-days exposure. Data calculated from MTT assays and presented as mean $\pm$ SD, three independent repeats of n = 5. The data are also presented in Figure 3.2.

Test agent	Exposure time (days)	GI ₅₀ (μ M)		
		U373-V	U373-M	HCT116
TMZ	3	147 $\pm$ 55	663 $\pm$ 106	610 $\pm$ 101
	6	10 $\pm$ 2	332 $\pm$ 36	609 $\pm$ 112
T25	3	43 $\pm$ 7	31 $\pm$ 8	39 $\pm$ 13
	6	60 $\pm$ 2	41 $\pm$ 11	37 $\pm$ 16
CBD	3	33 $\pm$ 6	30 $\pm$ 5	13 $\pm$ 5
	6	43 $\pm$ 13	23 $\pm$ 18	15 $\pm$ 8
4'-F-CBD	3	29 $\pm$ 5	25 $\pm$ 12	27 $\pm$ 11
	6	49 $\pm$ 9	25 $\pm$ 14	6 $\pm$ 0

3.3.3 Synergy of cannabinoids and imidazotetrazine anti-cancer agents against GBM

Once the test agents were studied alone, they were then administered in combination at concentrations equivalent to fractional GI₅₀ values (1.25 – 50%) to assess any potential synergy. The combinations assessed are shown in Table 2.2 but briefly, CBD was assessed in combination with TMZ after 3- and 6-days exposure. CBD was also assessed in combination with T25 after 3-days exposure. Similarly, 4'-F-CBD was studied in combination with TMZ after 3- and 6-days exposure, and 4'-F-CBD was assessed in combination with T25 after 3-days exposure. Table 2.3 in Chapter 2, section 2.3.5 demonstrates how the concentrations of test agents used for combination studies were calculated.

A summary of the concentrations used for each test agent in the combination studies after 3- and 6-days exposure are shown in Appendix 3.4 and 3.5, respectively. The combinations were assessed against cell lines U373-V, U373-M and HCT116. The combined effect was measured by combination index (CI) (calculated using Equation (3), shown in Chapter 2, section 2.3.5), a summary of which is shown in Table 3.2, and the full data can be seen in Appendix 3.6. CI values equal to 1 demonstrate an additive response between the two test agents, suggesting their effects are simply added together. CI values <1 indicate a synergistic response, suggesting that the test agents' effects are enhanced by administering them together. Finally, a CI value >1 indicates an antagonistic response, indicating that the two test agents are less effective when administered in combination.

Table 3.2. A summary of CIs of cannabinoids CBD and 4'-F-CBD administered in combination with TMZ or analogue, T25 after 3- or 6-days exposure against U373-V, U373-M and HCT116 cell lines. Data represented as a range for the different combination concentrations tested; full data can be found in Appendix 3.6.

Combination	Exposure time (days)	Combination Index (CI)		
		U373-V	U373-M	HCT116
TMZ/CBD	3	0.10 - 0.89	0.23 - 0.74	0.11 - 1.06
	6	0.12 - 0.76	0.21 - 0.74	0.05 - 0.95
TMZ/4'-F-CBD	3	0.16 - 1.01	0.13 - 0.59	0.37 - 0.74
	6	0.09 - 0.53	0.05 - 0.50	0.48 - 0.79
T25/CBD	3	0.33 - 0.65	0.22 - 0.57	0.53 - 0.93
T25/4'-F-CBD	3	0.21 - 0.52	0.19 - 0.75	0.37 - 0.79

The synergy between two test agents can also be demonstrated by isobolograms, generated using the same data as that used to calculate the CI values in Table 3.2. GI₅₀ values are plotted, with the individual GI₅₀ connecting line demonstrating where data points with an additive response would lie. Any data

points lying below this connecting line demonstrate a synergistic response, with data points above the line showing an antagonistic response.

Figure 3.3 shows the isobolograms of TMZ administered in combination with CBD. For both U373-V and U373-M cell lines, the combined GI_{50} values fall below the line, demonstrating a synergistic response. For the HCT116 cell line, most of the combined GI_{50} values are below the line, except for the high concentrations of TMZ, where the response is additive, or slightly antagonistic. It should also be noted that when assessing combinations against the HCT116 cell line, cell growth inhibition was not observed at CBD concentrations $<0.75 \mu\text{M}$, even when the highest combined concentration of TMZ ($50 \mu\text{M}$) was added.

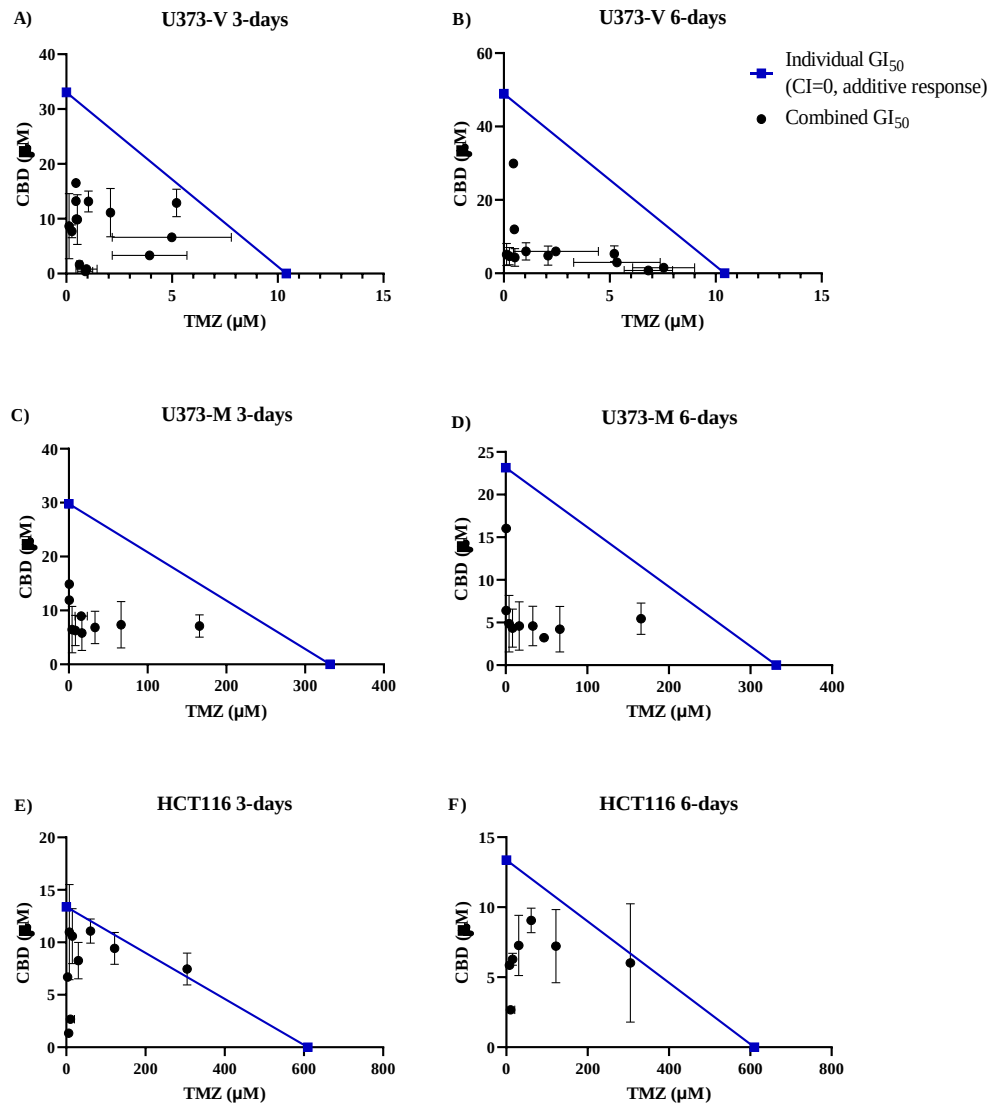


Figure 3.3. Isobolograms representing the combined effect of TMZ and CBD against U373-V (GBM control, -MGMT, +MMR) for A) 3 and B) 6 days exposure, U373-M (GBM, +MGMT, +MMR) for C) 3 and D) 6 days exposure and HCT116 (-MGMT, -MMR) for E) 3 and F) 6 days exposure. The blue line represents an additive response, data points above the line indicate an antagonistic response and points below the line indicate a synergistic response. Data measured by MTT assays and confirmed by cell count assays (Appendix 3.7). Dose-response curves are shown in Appendix 3.8 and 3.9. Data presented as mean $\pm$ SD, three independent repeats of $n = 5$.

Figure 3.4 shows the isobolograms of TMZ administered in combination with 4'-F-CBD. The combination resulted in a synergistic response against all three cell lines. Against U373-V, high concentrations of TMZ resulted in an additive response with 4'-F-CBD at both 3- and 6-days exposure. As was exhibited for the combined treatment of HCT116 with TMZ and CBD, low concentrations of 4'-F-CBD ($<0.68 \mu\text{M}$) did not result in an inhibition of cell growth with any concentration of TMZ tested against the HCT116 cell line.

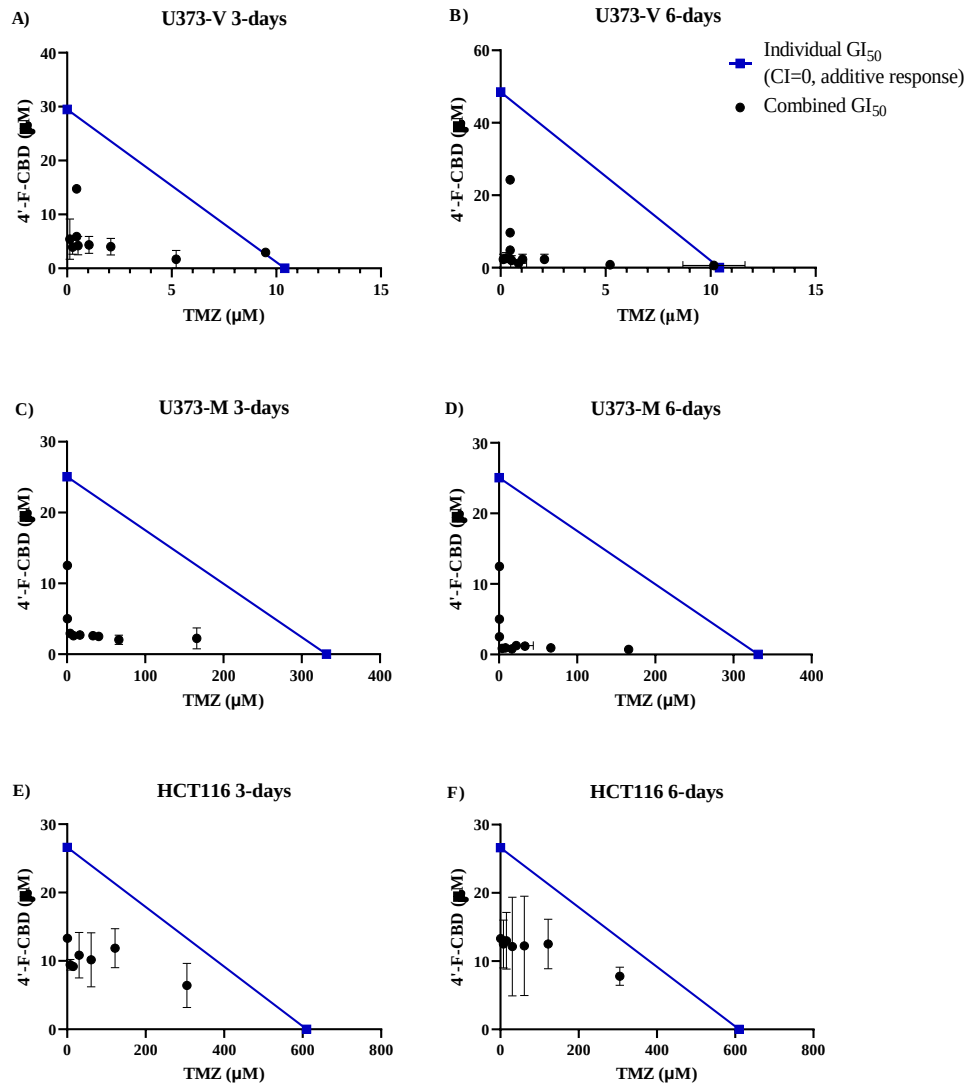


Figure 3.4. Isobolograms representing the combined effect of TMZ and 4'-F-CBD against U373-V (GBM control, -MGMT, +MMR) for A) 3 and B) 6 days exposure, U373-M (GBM, +MGMT, +MMR) for C) 3 and D) 6 days exposure and HCT116 (-MGMT, -MMR) for E) 3 and F) 6 days exposure. The blue line represents an additive response, data points above the line indicate an antagonistic response and points below the line indicate a synergistic response. Data measured by MTT assay and confirmed by cell count assay (Appendix 3.7). Dose-response curves are shown in Appendix 3.10 and 3.11. Data presented as mean $\pm$ SD, three independent repeats of $n = 5$.

All test agents other than TMZ were found to be as effective at inhibiting cell growth alone after 3 days exposure as after 6 days. Therefore, combined treatments of T25 with cannabinoids were only assessed after 3 days exposure. Figures 3.5 (T25 and CBD) and 3.6 (T25 and 4'-F-CBD) demonstrate the synergistic responses observed after treating cells with T25 combined with cannabinoids. Unlike combinations tested with TMZ, all tested combinations resulted in inhibition of cell growth.

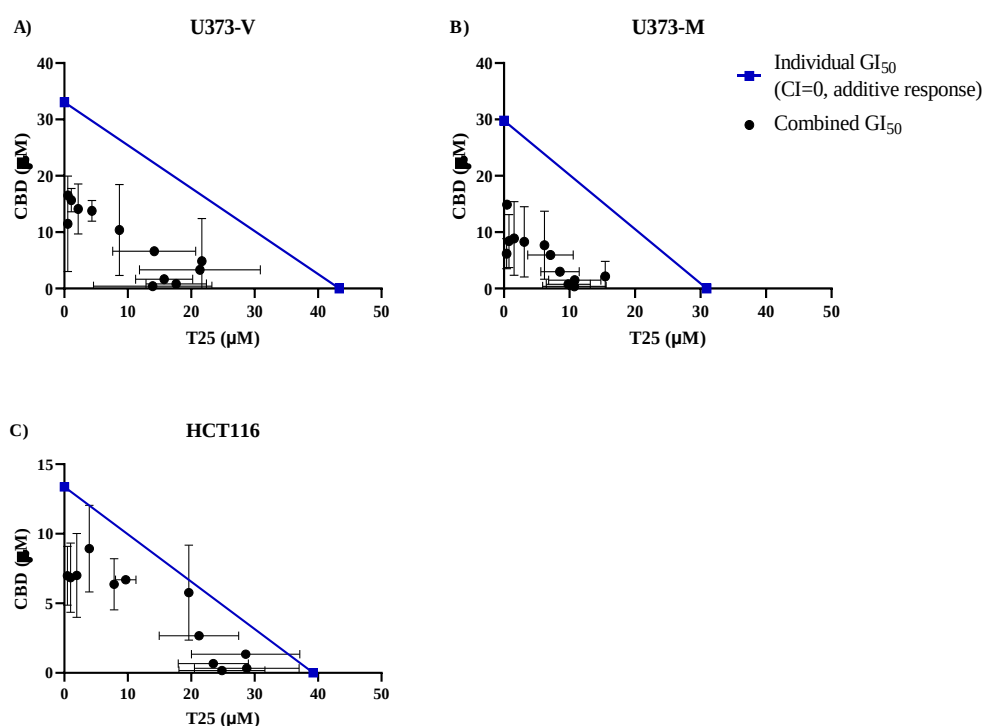


Figure 3.5. Isobolograms representing the combined effect of T25 and CBD against A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after 3 days exposure. The blue line represents an additive response, data points above the line indicate an antagonistic response and points below the line indicate a synergistic response. Data measured by MTT assays and confirmed by cell count assays (Appendix 3.7). Dose-response curves are shown in Appendix 3.12. Data presented as mean $\pm$ SD, three independent repeats of $n = 5$.

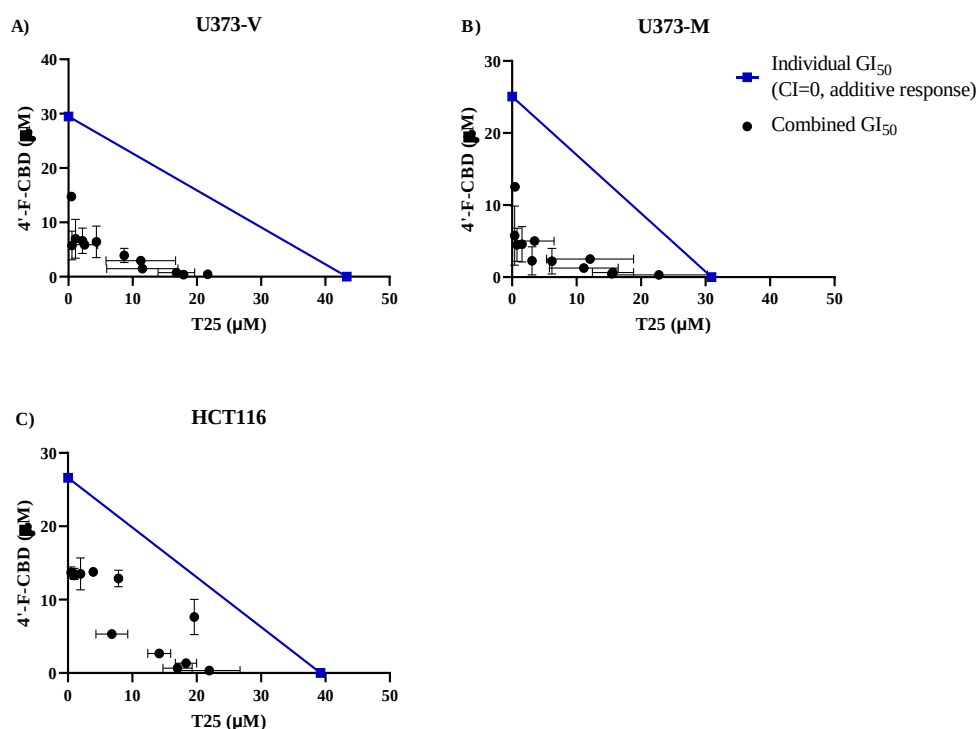


Figure 3.6. Isobolograms representing the combined effect of T25 and 4'-F-CBD against A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after 3 days exposure. The blue line represents an additive response, data points above the line indicate an antagonistic response and points below the line indicate a synergistic response. Data measured by MTT assays and confirmed by cell count assays (Appendix 3.7). Dose-response curves are shown in Appendix 3.13. Data presented as mean $\pm$ SD, three independent repeats of $n = 5$.

3.3.4 Indications of anti-cancer mechanism of cannabidiol

The anti-cancer mechanisms of action of CBD observed herein were investigated by 3D OrbiSIMS. U373-V cells were treated with either CBD alone, CBD and T25, CBD and TMZ, or TMZ alone. Control cells were not treated, but medium was added at the time of treatment instead, and samples were prepared for analysis 3 days later. As mentioned above, the anti-cancer mechanism of action of CBD is thought to be complex, involving multiple pathways. The suspected

pathways involved include *via* TRPV-2, increased ROS, and increased ER stress, although many effects are thought to be a result of interaction with the endocannabinoid system (Figure 1.10) [125, 143-145]. Additionally, the behavioural effects of CBD have been evidenced to involve DNA-methylation at the C^5 -cytosine in CpG islands [146, 147]. Therefore, methylated-DNA was also included as a possible pathway of anti-cancer activity to investigate.

3.3.4.1 Exposure of the cells to cannabidiol alone

U373-V cells were exposed to CBD, then collected for analysis at 3, 6, 24 and 72 h. Cells treated with CBD were compared to the non-treated control cells. First, a search for molecules indicative of the suspected mechanism was conducted. These included methylated-DNA, glutathione, glucose, ceramide, and anandamide. The compounds found consistently across samples were the methylated-DNA bases. The other indicators of mechanistic pathways were not found. The peak intensity (secondary ion counts) normalised to the total ion count (TIC) of the ions detected are shown in Table 3.3. Significant differences to the non-treated control were found only after 24 h exposure.

Treatment of cells with CBD alone led to an increase in the fatty acid content, as found by principal component analysis (PCA) investigation (shown in Appendix 3.14 to 3.17). It has been reported that treatment of GBM with TMZ induces increased fatty acid uptake into GBM cells [208]. Cells treated with CBD for 3 and 6 h demonstrated increased palmitic, stearic and octatriacontanoic acids, and a decrease in oleic acid, compared to the non-treated control. Cells treated for 72 h also demonstrated increased palmitic acid compared to the control.

Table 3.3. Targeted search for anti-cancer mechanism of action of CBD by 3D OrbiSIMS. U373-V cells were treated with CBD for 3, 6, 24 and 72 h and are compared to a non-treated control. The table shows the peak intensity (secondary ion counts) normalised to the TIC and deviation below in ppm. Data presented as an average of n = 3 technical repeats. One-way ANOVA with Dunnett's multiple comparisons performed, $\alpha = 0.05$, * = $p < 0.05$. The data are also shown in Figure 3.7.

Sample		Methyl- guanine	Methyl- cytosine	Methyl- adenine	Methyl- thymine
	Formula	C ₆ H ₆ N ₅ O ⁻	C ₅ H ₆ N ₃ O ⁻	C ₆ H ₆ N ₅ ⁻	C ₅ H ₅ N ₂ O ₂ ⁻
	m/z	164.0577	124.0516	148.0628	125.0357
Control		1.42 ×10 ⁻⁵ ±	6.47 ×10 ⁻⁶ ±	1.94 ×10 ⁻⁵ ±	3.09 ×10 ⁻⁴ ±
		9.29 ×10 ⁻⁶	2.02 ×10 ⁻⁶	8.28 ×10 ⁻⁶	4.99 ×10 ⁻⁵
		0.3	-0.2	0.1	0.1
CBD 3 h			3.19 ×10 ⁻⁵ ±		4.96 ×10 ⁻⁴ ±
		0	2.36 ×10 ⁻⁵	0	3.95 ×10 ⁻⁴
		-	-0.4	-	-0.3
CBD 6 h					7.86 ×10 ⁻⁶ ±
		0	0	0	4.68 ×10 ⁻⁶
		-	-	-	0.3
CBD 24 h		3.16 ×10 ⁻⁵ ±	4.79 ×10 ⁻⁵ ±	4.77 ×10 ⁻⁵ ±	1.32 ×10 ⁻³ ±
		3.26 ×10 ⁻⁶ *	1.03 ×10 ⁻⁵ *	3.92 ×10 ⁻⁶ *	2.97 ×10 ⁻⁴ *
		0.2	-0.2	0.2	-0.1
CBD 72 h					6.69 ×10 ⁻⁶ ±
		0	0	0	1.44 ×10 ⁻⁶
		-	-	-	-0.4

3.3.4.2 Exposure of cells to a combination of cannabidiol with imidazotetrazine anti-cancer agents

Exposure to the combination of CBD with TMZ or T25 was also assessed by 3D OrbiSIMS at 3 h after treatment as a preliminary study. The same peak list was used as for the cells treated with CBD alone. Methylated-DNA was detected in the samples of cells exposed to TMZ alone and TMZ with CBD at similar levels to the control sample (Figure 3.7). As with the cells exposed to CBD alone, the peaks indicating other mechanistic pathways that were searched for were not found in the samples. However, the data were also analysed by PCA, and differences in the fatty acid composition of the samples were found (Appendix 3.18). Samples exposed to TMZ and CBD in combination for 3 h were found to contain increased arachidonic acid, compared to the non-treated control.

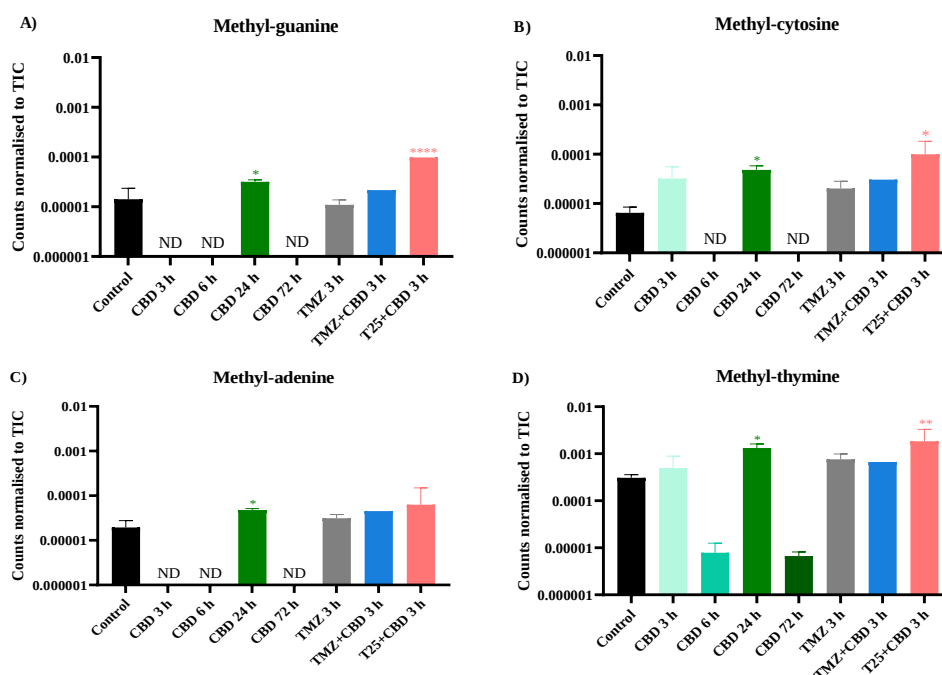


Figure 3.7. Targeted analysis of 3D OrbiSIMS data from U373-V cells exposed to CBD, TMZ or a combination of CBD with TMZ or CBD with T25 for 3-72 h. Graphs show data as peak intensity (secondary ion counts) normalised to TIC for A) methyl-guanine, B) methyl-cytosine, C) methyl-adenine and D) methyl-thymine. ND = not detectable. Data presented as an average of $n = 3$ technical repeats. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, $* = p < 0.05$, $** = p < 0.01$, $**** = p < 0.0001$.

The samples exposed to both CBD and T25 were searched for propargylated-DNA, expected to occur from DNA-alkylation by T25. These data are shown in Figure 3.8 and Table 3.4. It appears that more propargylated-DNA was found in the samples exposed to T25 and CBD than the non-treated control, however this is not statistically significant. Interestingly, propargyl-guanine and propargyl-adenine were not found to be present in the non-treated control but were detected following exposure of the cells to T25 and CBD. Methylated-DNA was also detected in samples exposed to CBD and T25, where all apart from methyl-adenine were significantly higher than the non-treated control (Figure 3.7).

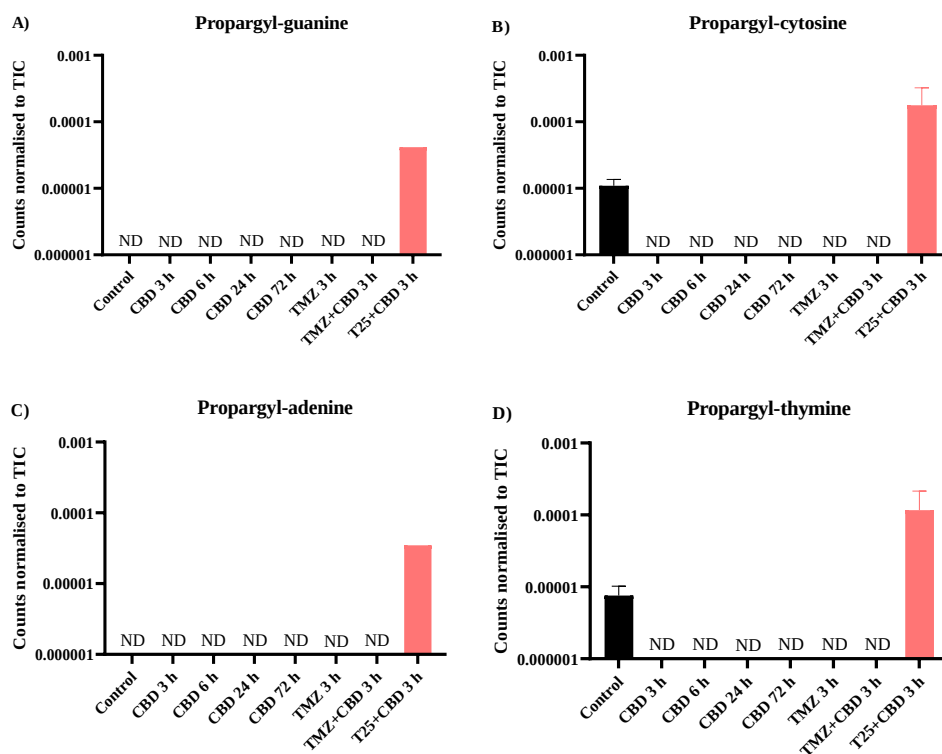


Figure 3.8. Targeted analysis of 3D OrbiSIMS data from U373-V cells treated with CBD, TMZ or a combination of CBD with TMZ or CBD with T25 for 3-72 h. Graphs show data as peak intensity (secondary ion counts) normalised to TIC for A) propargyl-guanine, B) propargyl-cytosine, C) propargyl-adenine and D) propargyl-thymine. ND = not detectable. Data presented as an average of $n = 3$ technical repeats. One-way ANOVA with Dunnett's multiple comparisons was performed, comparing treatment to the non-treated control, $\alpha = 0.05$, no significant differences were found.

Data from the samples of cells exposed to a combination of CBD and T25 were also compared to the non-treated control by PCA analysis (Appendix 3.19). Following 3 h exposure the samples were found to contain increased palmitic and octatriacontanoic acid content compared to the non-treated control.

Table 3.4. Targeted search for anti-cancer mechanism of action of CBD and evidence of T25 activity by 3D OrbiSIMS. U373-V cells were exposed to CBD alone or with T25 for 3 h and are compared to a non-treated control. The table shows the peak intensity (secondary ion counts) normalised to the TIC and deviation below in ppm. Data presented as an average of $n = 3$ technical repeats. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, no significant differences were found. The data are also shown in Figure 3.8.

Sample		Propargyl- guanine	Propargyl- cytosine	Propargyl- adenine	Propargyl- thymine
	Formula	$C_8H_6N_5O^-$	$C_7H_6N_3O^-$	$C_8H_6N_5^-$	$C_7H_5N_2O_2^-$
	m/z	188.1675	148.1431	172.1685	149.1276
Control		0	$1.09 \times 10^{-5} \pm$ 2.70×10^{-6}	0	$7.56 \times 10^{-6} \pm$ 2.65×10^{-6}
		-	0.4	-	-0.3
CBD 3 h		0	0	0	0
		-	-	-	-
CBD/T25 3 h		$4.16 \times 10^{-5} \pm$	$1.77 \times 10^{-4} \pm$	$3.48 \times 10^{-5} \pm$	$1.16 \times 10^{-4} \pm$
		0	1.47×10^{-4}	0	9.82×10^{-5}
		-0.1	0.1	0.1	-0.4

3.4 Discussion

The exploration of the anti-cancer effects of cannabinoids is a growing area of research. CBD has been shown to exhibit anti-cancer properties against cancers including breast, colorectal, lung and GBM [143, 145]. The ability of CBD to inhibit GBM cell growth *in vitro* is usually studied in combination with either THC or TMZ [127, 128]. This has led to phase I/II clinical trials in GBM patients [138-140]. Further clinical trials are underway to study the efficacy of combinations of radiotherapy and chemotherapy with TMZ and a mixture of CBD and THC, against GBM [141], as well as daily administration of CBD with TMZ [142].

The anti-cancer activity of CBD alone against GBM has been studied in cell lines including U87MG ($GI_{50} = 12.75 \pm 9.7 \mu\text{M}$) [128, 132, 134, 137, 207], GL216 ($GI_{50} = 10.67 \pm 0.58 \mu\text{M}$) [206] and U373MG ($GI_{50} = 21.6 \pm 3.5 \mu\text{M}$) [134]. These cell lines do not possess the over-expression of MGMT or deficiency of MMR that result in the major GBM resistance mechanisms to TMZ, represented in this work by human GBM cell line U373-M and colorectal cancer cell line HCT116, respectively. CBD anti-cancer activity has been studied against the HCT116 cell line previously for its effects against colorectal cancer [143]. In this work, HCT116 is used to represent the second major resistance mechanism to GBM treatment by TMZ, a deficiency of MMR. To the best of our knowledge, the anti-cancer properties of 4'-F-CBD have not been studied before. The potential advantages of treating GBM with 4'-F-CBD, compared to CBD, are, briefly, that 4'-F-CBD is reported to have increased potency in *in vivo* behavioural assays compared to CBD [176, 177, 204]. There is also the opportunity that the fluorine atom on 4'-F-CBD provides, for imaging and potentially theranostic purposes [209, 210].

First, the activity of the cannabinoids, as well as TMZ and analogue T25, were assessed against the non-tumourigenic MRC-5 cell line to indicate any cancer-selectivity. Figure 3.1 demonstrates that activity was the same after both 3- and 6-days exposure. Controls of the equivalent DMSO concentration were also assessed, finding that at 1% DMSO exposure (equivalent to the 500 μM test

agent concentration) the cell growth began to be inhibited, and cell growth was reduced by 50% at 2% DMSO exposure (equivalent to the 1000 μM test agent concentration) (Appendix 3.1). Figure 3.1 shows TMZ to be the least active test agent, only beginning to inhibit cell growth at 500 μM , although this may be from the 1% DMSO concentration in the formulation. CBD was shown to be the most active, showing a reduction in cell growth at 10 μM . 4'-F-CBD and T25 fall in the middle, inhibiting the cell growth by 100% at 50 and 100 μM , respectively.

Once the effect of the test agents on the non-tumourigenic cell line was established, their antitumour activity could be investigated against GBM cell lines. Figure 3.2 shows the calculated GI_{50} values of each test agent alone against the cell lines tested. Studying the U373-V cell line, the TMZ GI_{50} falls from $147 \pm 55 \mu\text{M}$ after 3 days to $10 \pm 2 \mu\text{M}$ after 6 days exposure. This is consistent with reported literature [3]. TMZ must undergo ring opening to the active MTIC compound, before it is able to methylate the DNA, most impactfully at the O^6 -guanine position [93, 94]. *O*-methylation leads to a mis-pair of guanine with thymine (rather than cytosine) during DNA replication, damaging the cells and triggering the pathway to cell death *via* apoptosis or autophagy [95]. This process takes time and is why 6 days exposure is required to see the full effect of TMZ treatment. Interestingly, this is not true of the other test agents studied, all of which showed inhibitory effects on the cell growth after 3 days exposure. The GI_{50} values were similar after both 3- and 6-days exposure and were similar across the test agents. The TMZ GI_{50} is high for the two cell lines representing the common resistance mechanisms. U373-M represents the over-expression of MGMT, able to recognise and repair O^6 -methyl-guanine, and HCT116 exhibits MMR deficiency, where MMR is required to recognise the mis-pair triggered by methylated-DNA to result in cell death. The high GI_{50} values for TMZ against these cells lines is expected and also demonstrated in the literature [94].

The GI_{50} values of T25, CBD and 4'-F-CBD were consistent across the cell lines studied (Table 3.1). All values were significantly smaller than that of TMZ against the two cell lines displaying TMZ resistance (Figure 3.2). This has been demonstrated previously within our group for T25 (unpublished work), as the molecule was designed to overcome resistance mechanisms associated with

TMZ treatment by creating larger DNA adducts that escape MGMT-mediated removal and tolerance following MMR-loss. The activity of CBD alone against HCT116 is consistent with that reported in the literature, supporting that CBD can overcome resistance to TMZ conferred by MMR deficiency [143]. However, this is the first time CBD has been shown to overcome the often-seen inherent resistance mechanism to TMZ treatment (although there are a few cases where this resistance has been acquired [211]) by an over-expression of MGMT. These are encouraging data, as the poor outcomes for patients diagnosed with GBM demonstrate the need for new treatments.

The mechanism of action of CBD was investigated by 3D OrbiSIMS analysis. First, a targeted approach was taken by collating information on the pathways suggested in the literature and identifying suitable targets [130, 143-145]. For example, a decrease in glutathione was searched for to demonstrate oxidative stress. Also, anandamide and oleamide were searched for to demonstrate an interaction with the endocannabinoid system. This targeted search approach yielded the finding of methylated-DNA in the samples, at significantly higher levels than the non-treated control (Figure 3.7, Table 3.3). Of particular interest is methyl-cytosine. Methyl-cytosine at the C⁵-position of CpG islands is reported in the literature to occur after CBD exposure, however the role of CpG methylation in CBD activity is not yet clear [146, 147]. Additionally, CpG islands are abundant in promoter genes, including MGMT promoter [212]. Methylated-MGMT promoter is an evidenced indicator of the prognosis of GBM [213]. MGMT promoter methylation silences the gene, meaning resistance to TMZ treatment by MGMT over-expression does not occur, and the tumours are more sensitive to TMZ treatment [214]. The methyl-cytosine evidenced herein by exposure of GBM cells to CBD could potentially occur at CpG islands on MGMT promoters. If so, this could effectively silence MGMT, possibly contributing to the synergy observed in exposure of the cells to CBD with TMZ (Table 3.2, Figure 3.3).

The presence of methylated-DNA at high intensities suggests this is one pathway of the anti-cancer action of CBD. DNA damage by methylation can result in mismatched pairs during replication and ultimately, lead to cell death [215-217]. To the best of our knowledge, this is the first report of evidence of methylated-

DNA as a potential anti-cancer mechanism of action of CBD. Although, this is not the only mechanism of action of CBD. The synergy of CBD demonstrated herein with TMZ and T25 indicates that CBD acts *via* a pathway other than DNA alkylation (as this is the mechanism of action of imidazotetrazine compounds). Combined with the presence of methylated-DNA after exposure of the cells to CBD alone, this indicates the anti-cancer activity of CBD involves a rich and diverse pharmacology, as is suggested in the literature [143-145]. The U373-M cell line exhibits MGMT over-expression, repairing methylated-DNA, specifically in the O^6 -guanine position [94]. Whilst the 3D OrbiSIMS analysis demonstrates that CBD methylates the DNA, the equivalent anti-cancer activity of CBD against U373-M to U373-V (Figure 3.2) demonstrates that other mechanisms are also perturbed, possibly including those suggested in the literature, such as ER stress, increased ROS as well as activity *via* interaction with the endocannabinoid system [126, 144].

Analysis of the 3D OrbiSIMS data by PCA also revealed some differences in the fatty acid content in the samples. Brain tissues taken from patients with GBM exhibit lipid droplets (formed predominantly of a phospholipid layer surrounded by perilipin family proteins), whereas healthy brain tissues do not [218]. Cells treated with CBD demonstrated increased palmitic, stearic and octatriacontanoic acids. An increase in palmitic acid content is shown in the literature to be associated with a decrease in GBM cell line U87 viability [219]. There is no evidence in the literature to suggest an association between stearic and octatriacontanoic acids and GBM. Cells treated with CBD were also found to contain decreased oleic acid compared to the non-treated control. Oleic acid has been shown to increase glucose utilisation and stimulate GBM cell growth [218]. As discussed in Chapter 1, oleic acid is thought to increase the permeability of the BBB, by interacting with the membranes of brain capillary endothelial cells (BCECs), which form the BBB [62, 66]. BBB opening could potentially contribute to the increased GBM growth observed when present, due to the increased access to the blood circulation.

As discussed above, synergy has previously been demonstrated between CBD and TMZ against GBM cell lines U87MG and U251 [131, 132, 220]. However, Deng *et al* reported that only certain concentrations resulted in a synergistic combination, whilst others resulted in an additive response [132]. The work reported herein confirms the reports of synergy for the U373-V cell line and investigates further in the two cell lines representing clinical resistance mechanisms to TMZ, the standard GBM treatment. Against TMZ resistant cell lines, the combination of CBD with TMZ demonstrated a synergistic response, with CI values as low as 0.21 and 0.05 against U373-M and HCT116, respectively (Table 3.2, Figure 3.3). Against the HCT116 cell line, representing a MMR deficiency, at high concentrations of TMZ, the combination resulted in an additive response, supporting the findings of Deng *et al* [132]. This is also evident in the dose-response curves, demonstrated in Appendix 3.8 and 3.9. Here, it is shown that the concentration of TMZ added does not affect the growth inhibition, and that the concentration of CBD added is driving the response. This suggests that the effects of CBD are the predominant cause of the growth inhibition, rather than equal effects from both test agents.

There have been reports that CBD enhances the uptake of TMZ into cells via the TRPV-2 pathway, therefore enhancing the effect of TMZ [131]. This may be true, however the mechanisms underpinning this activity cannot be determined from growth inhibition assays. These were investigated in the 3D OrbiSIMS analyses of the cells. However, the TMZ molecular ion ($[M-H]^+$), or an obvious fragment of TMZ, was not found in the treated samples, even when TMZ alone was administered. This may be a result of the short treatment time of only 3 h. Additionally, the 3D OrbiSIMS parameters used herein were optimised to detect CBD in complex matrices, and as the structures of the two molecules are quite different, it is likely the instrument was not set to the optimum conditions to detect TMZ. Therefore, it could not be confirmed if CBD aided the uptake of TMZ into the cells in this work. Analysis of the data by PCA did reveal that cells exposed to TMZ and CBD exhibited higher levels of arachidonic, cinnamic and palmitic acids than the non-treated control. As discussed above for CBD, palmitic acid is associated with decreased GBM cell viability [219]. Arachidonic acid is demonstrated in the literature to induce oxidative stress [221]. Cinnamic

acid has been demonstrated to reverse malignant invasive properties in cancer cells *in vitro* [222]. Taken together, these indications of fatty acid content suggest that an oxidative stress pathway is initiated when GBM cells are treated with TMZ and CBD. Additionally, as cell viability is known to be lower, demonstrated in Figure 3.3, this work is consistent with reports in the literature stating that reduced GBM cell viability is associated with palmitic and cinnamic acids.

The effects of exposure of the cells to CBD in combination with T25 were also investigated, as shown in Figure 3.5. As discussed, T25 can overcome the two major resistance mechanisms to treatment with TMZ, and a synergistic combination of CBD with T25 would likely demonstrate enhanced activity (in TMZ resistant models). The combination yielded a synergistic response at all concentrations tested. Table 3.2 also shows that the CI values are lower (<0.57 in U373-M) for this combination than the combined CBD and TMZ treatment (<0.74 in U373-M). This demonstrates enhanced synergy of this combination, likely due to the increased activity of T25 over TMZ. This combination has not been studied before, and the low CI values demonstrate promise of the combination for GBM treatment.

The combined treatment of T25 with CBD was investigated further by 3D OrbiSIMS analysis. The same markers searched for in the samples of CBD administered alone were investigated as well as propargylated-DNA, expected to occur following exposure to T25. Propargylated-DNA was found in the samples treated with T25 and CBD. Intensities of propargyl-cytosine and propargyl-thymine were not found to differ significantly from the non-treated control. However, propargyl-guanine and propargyl-adenine were not found to be present in the control. This provides evidence of the activity of T25. Evidence of other mechanisms were also searched for, and methylated-DNA was found to be present. Methyl-adenine was not found to differ from the control, whereas methyl-guanine, methyl-cytosine and methyl-thymine all were significantly higher than in the non-treated control sample. As T25 is expected, and shown here, to deposit propargyl groups on the DNA, the methylated-DNA is assumed

to be a result of CBD activity. This is also in line with the methylated-DNA seen when cells were exposed to CBD alone. Much like the samples exposed to TMZ and CBD, and CBD alone, exposure to T25 in combination with CBD led to an increase in palmitic acid present in the sample. This supports the findings from the other treated samples and reports in the literature that increased palmitic acid is associated with decreased GBM cell viability [219].

To the best of our knowledge, this work demonstrates that 4'-F-CBD exhibits anti-cancer properties for the first time. Like CBD, 4'-F-CBD is also shown to overcome the two major resistance mechanisms to the standard GBM treatment with TMZ, MGMT over-expression and MMR deficiency (Figure 3.2). While no significant differences were observed in the GI_{50} of 4'-F-CBD and CBD against cancerous cells, 4'-F-CBD requires a higher dose to inhibit non-tumourigenic cell growth than CBD, and therefore, may provide a safer treatment option to obtain the same therapeutic results (Figure 3.1). The mechanism of action of 4'-F-CBD was not investigated, however the GI_{50} values are not significantly different to CBD. As the molecular structure of the cannabinoids is similar, it is likely the activity of 4'-F-CBD is a result of similar pathway activation to that of CBD.

As the activity of 4'-F-CBD is similar to CBD when administered alone, combination treatments involving 4'-F-CBD were also investigated. Figure 3.4 demonstrates the synergistic response to 4'-F-CBD in combination with TMZ after both 3- and 6-days exposure. Only the highest concentration of TMZ tested in the U373-V cell line resulted in an additive response, all other concentrations demonstrated a synergistic response. Therefore, apart from the U373-V line, the combination of TMZ with 4'-F-CBD produced increased synergy over TMZ with CBD, (CI values as low as 0.05 with 4'-F-CBD and 0.21 with CBD, Table 3.2). The combination of 4'-F-CBD with T25 demonstrated similarly high synergistic responses. The calculated CI values are not significantly different to the combination of T25 with CBD.

The analysis of cells by 3D OrbiSIMS to investigate the mechanisms taking place in this work is a new technique adapted from the few reports in the literature [186-188]. The experiments conducted herein demonstrate the technique as useful in this scenario. The targeted analysis approach is relevant when a suspected mechanism is known, and analyses by MFP and PCA allow differences in the overall chemistries between samples to be identified, such as in the fatty acid content in samples analysed herein. This work demonstrates the use of the technique optimised for the detection of CBD in complex matrices, in this case within cells. However, there is work still to be done, as the samples analysed in this work form a preliminary study using this technique. Additionally, as mentioned, the methodology was optimised for the detection of CBD, and there are likely ions that were not detected because of this, for example, TMZ. Analysis in positive mode and with different ion source beam energies could yield different information. However, the method did allow identification of evidence of mechanisms of action, and differences between the samples, demonstrating the utility of this application.

3.5 Conclusions

The ability of CBD alone to overcome the major resistance mechanism to standard GBM treatment, MGMT over-expression, is demonstrated for the first time to the best of our knowledge. The anti-cancer properties of 4'-F-CBD are discovered to be as effective as CBD at inhibiting GBM cell growth and overcoming the two major resistance mechanisms to TMZ. Both molecules were effective after only 3 days treatment at significantly lower concentrations than TMZ. 4'-F-CBD was also demonstrated for the first time to be less active against non-tumourigenic cells than CBD.

In addition, T25 was shown to overcome the two major resistance mechanisms, MGMT over-expression and MMR deficiency. The activity of T25 was further investigated by 3D OrbiSIMS and the presence of propargylated-DNA is evidence of the mechanism of action of the molecule.

Synergy was found in combined treatments of the cannabinoids (both CBD and 4'-F-CBD) with TMZ and T25. The synergistic combination of CBD and TMZ confirms that reported in the literature and demonstrates that CBD is driving this synergy by the additional TMZ concentration not causing a significant change in the response. Lower CI values were found in combinations with T25, demonstrating increased synergy compared to TMZ across all three cell lines. This is encouraging as T25 itself can overcome the two major resistance mechanisms to TMZ treatment, and therefore treatment could be enhanced further by this combination.

The mechanism of action of CBD was also investigated by 3D OrbiSIMS analysis. The pathway of DNA-methylation was demonstrated after treatment with CBD alone. Although there is also evidence that this is not the only pathway activated, CBD activity is complex, as suggested in the literature [126, 144]. Whilst mechanisms could not be identified in cells treated with combinations, evidence of activity was demonstrated by the change in chemistries observed. The change in specific fatty acids are indicators of the treatment effectiveness, and therefore, putative biomarkers of efficacy *in vivo*. This technique to study the cells by 3D OrbiSIMS provided new insights into activity, and this preliminary work demonstrates scope for future studies.

CHAPTER 4: Delivery of cannabidiol to the whole brain and anatomical regional brain distribution following oral administration to rats

4.1 Introduction

Cannabidiol (CBD) is the most abundant non-psychoactive cannabinoid of the *Cannabis sativa* plant. CBD has high therapeutic potential, with reports of activity against conditions including anxiety, multiple sclerosis (MS) and cancer [103-107]. Chapter 3 investigated the anti-cancer properties of CBD, demonstrating its ability to overcome two common resistance mechanisms to the standard of care treatment for glioblastoma multiforme (GBM). To the best of our knowledge, this is the first time this activity has been reported for CBD. The anti-cancer properties of CBD, particularly against GBM, are promising [144]. The next quest in the development of CBD as a treatment for GBM is the *in vivo* delivery of the molecule.

Many of the therapeutic effects of CBD are likely the result of interaction with the endocannabinoid system (Figure 1.10), particularly in the brain. However, reports of quantified CBD delivery to the brain are limited, and even more so following oral administration. As discussed in Chapter 1, oral administration is a preferred route for a number of reasons including the improved patient experience, compared to other administration routes such as intravenous (i.v.) injection. The delivery of CBD to the brain following oral administration has been studied *in vivo* in rats [152, 168, 205]. These studies report the delivery of CBD to the whole brain as one tissue and at one or few time points. Therefore, the brain exposure to CBD after oral administration over time is not well understood.

The delivery of CBD to anatomical brain regions has not yet been studied in detail. It is important to consider brain delivery to specific anatomical regions, as it is known that the different regions have differing structures and chemistries, responsible for the physiological activity taking place [223, 224]. It would be reasonable to assume the biological differences between anatomical regions

could result in different delivery. Therefore, it is important to study and understand delivery to certain regions, as it is likely that delivery is not homogenous across the whole brain. The techniques usually used to study biodistribution in brain regions are various types of *in vivo* imaging. Although imaging techniques offer visual understanding, they are usually qualitative [66, 189, 225-227]. Gaining a quantitative understanding of delivery is beneficial for developing new therapeutics, in order to understand whether an effective dose reaches the site of action. A new technique with the potential to provide both visual biodistribution and quantification is the 3D Orbitrap secondary ion mass spectrometry (3D OrbiSIMS). The 3D OrbiSIMS allows for accurate mass spectrometry analysis with 240,000 resolving power at m/z 200, and spatial resolution for imaging the distribution of ions in the sample [183].

The brain is a particularly difficult area to deliver therapeutics to, due to the blood-brain barrier (BBB). The BBB is a physical, protective barrier limiting movement between the blood and the central nervous system (CNS) [14, 22, 228]. The barrier is formed of tightly packed brain capillary endothelial cells (BCECs) that surround the blood capillaries in the brain, and are supported by astrocytes [21]. Tight junctions exist between the BCECs, and the presence of specific transporters, amongst other functions, block transport of most large or hydrophobic molecules [21, 22, 24, 25]. The BBB exists to protect the brain from potentially neurotoxic substances including metabolites and proteins, but the majority of small molecule therapeutics (>97%) are also unable to penetrate the barrier [35, 228].

Lipid-based formulations are a common choice to improve the systemic availability, and possibly brain delivery, of therapeutics. Certain lipids are reported to interact with, and open, the BBB. The effects of lipids on the BBB are generally studied after i.v. administration. Following oral administration, lipid digestion becomes another factor to consider, which is different for different lipids. After digestion of the triglycerides, short and medium chain fatty acids (carbon chain length ≤ 12) enter the systemic circulation *via* the portal vein. Long chain fatty acids (carbon chain length >12) are assembled into chylomicrons following re-synthesis of triglycerides and enter the lymphatic

system before reaching the systemic circulation *via* the mesenteric and thoracic lymph duct.

Two fatty acids pertinent to this work that have been reported to increase BBB permeability are oleic and myristic acids. Oleic acid (C18:1 *cis*-9) is reported to impact BBB permeability by interacting with BCEC membranes [62, 66]. The effect has been found to be reversible 80 - 90 mins after i.v. injection. Sztriha *et al* suggested that at high concentrations, oleic acid monomers aggregate into micelle structures which can be incorporated into the cell membrane, physically disrupting the lipid-bilayer [66]. Oleic acid has also been demonstrated to interact with protein kinase C [66]. Such interactions in the BCECs can lead to BBB permeability changes in a reversible manner. Myristic acid (C14:0) is also reported to impact BBB permeability. There are reports that the C14 chain length is long enough for sufficient hydrophobic interaction with the barrier, but short enough to prevent strong binding [189]. This chain length is reported to be optimal for interaction with the BBB to increase permeability [62, 189].

There are reports in the literature of oral administration of CBD to rats in lipid-based formulations. Hložek *et al* reported the oral administration of CBD in sunflower oil, resulting in high CBD adsorption and brain delivery, in comparison to other administration routes [168]. Additionally, our group have previously reported the oral administration of CBD in various lipid-based formulations [151, 155, 166]. Administration in a sesame oil formulation was found to result in 3-fold higher bioavailability than a lipid-free vehicle, and 250-fold higher lymph fluid concentration, compared to the plasma [155, 169]. It was then reported after comparing different lipid-based vehicles, that the selection of lipid for oral administration affected the lymphatic transport and bioavailability of CBD [151].

To the best of our knowledge, there have been no reports comparing lipid-based formulations for the delivery of CBD to the brain, or the biodistribution of CBD to specific anatomical brain regions following oral administration. This chapter describes the delivery of CBD to specific anatomical brain regions following oral administration to rats in lipid-based and lipid-free vehicles. The lipid-based vehicles that were selected for the biodistribution studies were chosen based on

the CBD concentration delivered to the brain at the time of maximum concentration ($T_{\max}$) in the plasma (brain tissues collected from previous studies (Feng *et al* [151, 155])). The vehicles that exhibited high delivery of CBD to the brain or certain anatomical brain regions were chosen. Vehicles that contained different fatty acid chain lengths and degree of saturation, resulting in a different plasma $T_{\max}$, were also selected. The CBD determination in tissues following biodistribution studies were conducted using a validated high performance liquid chromatography-ultraviolet (HPLC-UV) method [195], and selected samples were also analysed by 3D OrbiSIMS in order to compare the techniques and assess the potential application of the 3D OrbiSIMS in biodistribution studies.

4.2 Experimental design

The results discussed in this chapter were generated using methods described in Chapter 2. The biodistribution of CBD was assessed in rats by the method described in section 2.4.3. The determination of CBD concentrations was performed by means of HPLC-UV, as described in section 2.5.1, based on Zgair *et al* [195]. Statistical analysis was performed by multiple-tests or one-way ANOVA with Dunnett's multiple comparisons using GraphPad version 9.3.1 (GraphPad, CA, U.S.A).

4.3 Results

4.3.1 Biodistribution of cannabidiol to the brain in rats

4.3.1.1 Delivery of cannabidiol to the brain at the plasma $T_{\max}$ following oral administration in different formulations

To select the oil formulations for biodistribution studies, rough dissection was performed on tissues that were collected in previous *in vivo* studies at the time of plasma $T_{\max}$ (Feng *et al* [151, 155]), and the CBD concentrations were determined by means of HPLC-UV. Tissues were dissected roughly because it was not possible to carry out full dissection of all anatomical brain regions reliably on frozen tissues. Delivery of CBD to the whole brain (Figure 4.1) was compared to the plasma concentrations at the plasma $T_{\max}$.

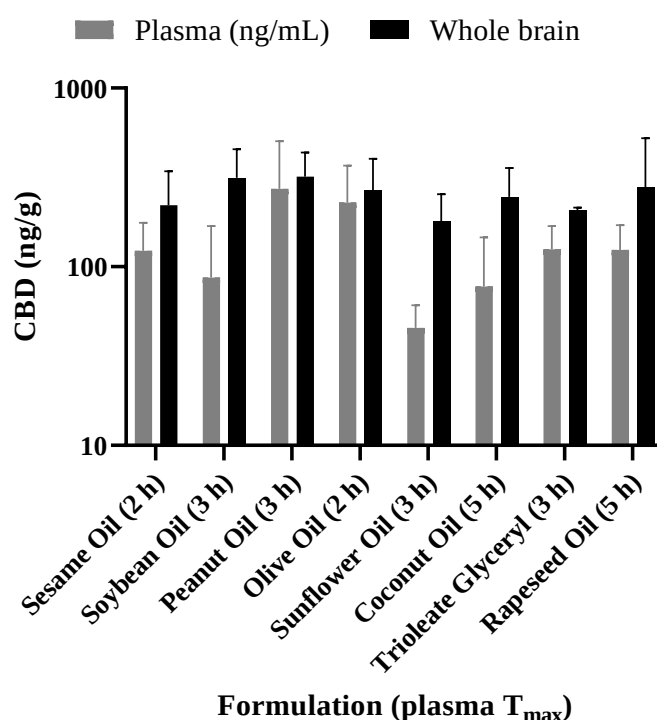
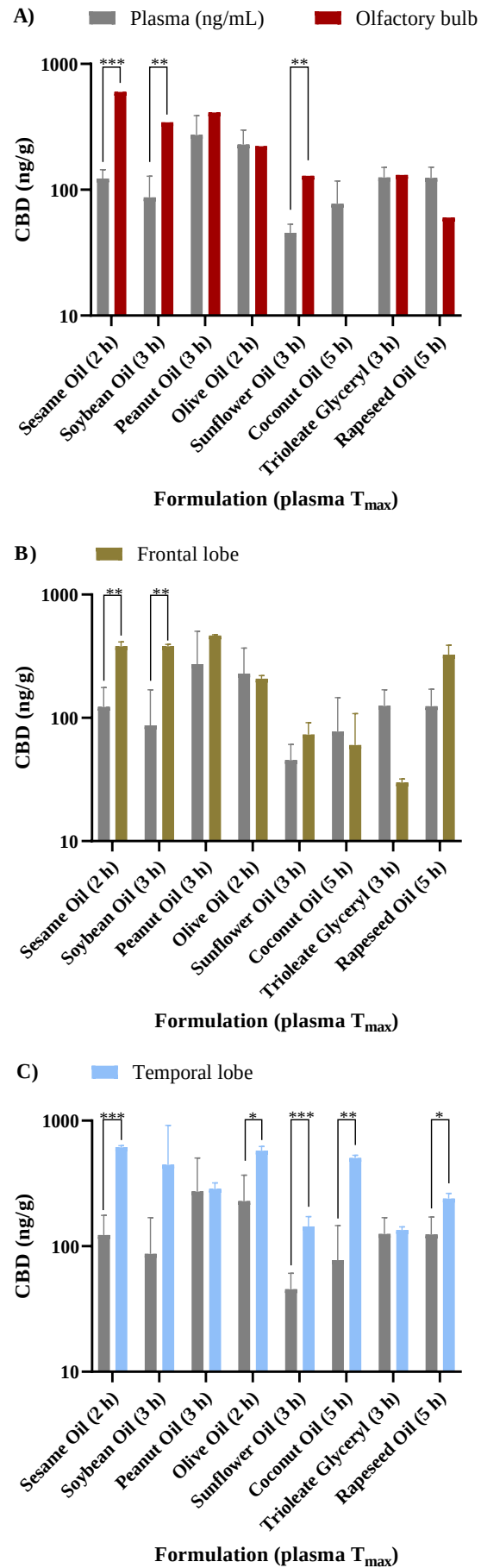


Figure 4.1. CBD concentration in the whole brain vs. plasma after oral administration to rats (12 mg/Kg) in various lipid-based vehicles. Tissues were collected at plasma $T_{\max}$. Multiple t-tests were performed, $\alpha = 0.05$. Data are presented as mean $\pm$ standard deviation (SD), brain $n = 3$, plasma $n = 6$ (collected in previous pharmacokinetics (PK) studies (Feng *et al* [151, 155])).

Figure 4.1 demonstrates that concentrations in the plasma and whole brain were similar following oral administration in each formulation, and no significant differences were observed. As described, the whole brain samples were also dissected and analysed by specific anatomical brain region, shown in Figure 4.2.

Following administration in the sesame oil formulation, the CBD concentration was significantly higher in each anatomical brain region than the plasma. The soybean oil formulation also produced higher CBD concentrations in all anatomical brain regions studied, apart from the temporal lobe. The temporal lobe was an area of particular interest because it is one of the most common locations of GBM [90, 229, 230]. As discussed above, Chapter 3 demonstrated the anti-cancer properties of CBD against GBM. The formulations that resulted in increased delivery to the temporal lobe, compared to the plasma, were the sesame, olive, sunflower, coconut and rapeseed oil formulations.

Based on the data shown in Figure 4.2, and that reported in the literature by our group of high delivery of CBD to the lymphatic system following oral administration in a sesame oil formulation [155, 166], the sesame oil formulation was chosen for full biodistribution studies. The coconut and rapeseed oil formulations were also chosen as they represent different plasma T_{max} times and contain different fatty acids, representing shorter chain lengths (coconut oil) and decreased saturation (rapeseed oil). Additionally, both formulations also resulted in higher CBD concentrations in the temporal lobe than the plasma. The olive and sunflower oil formulations were not selected to take forward as the oils have similar fatty acid compositions to sesame oil. The aim was to investigate the effects of different lipid-based formulations on delivery to the brain, therefore oils with different fatty acid compositions were chosen.



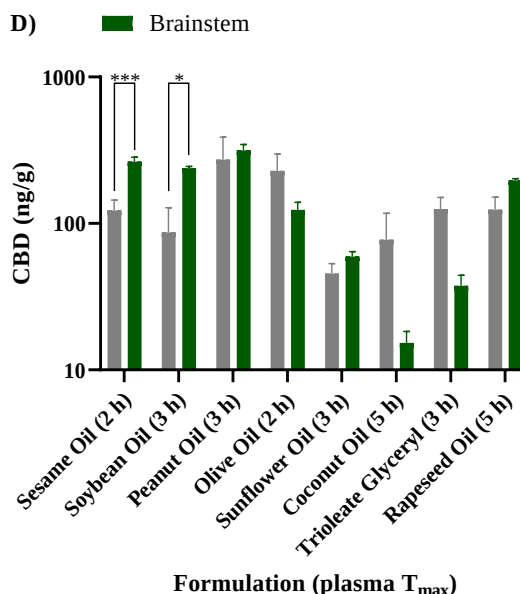
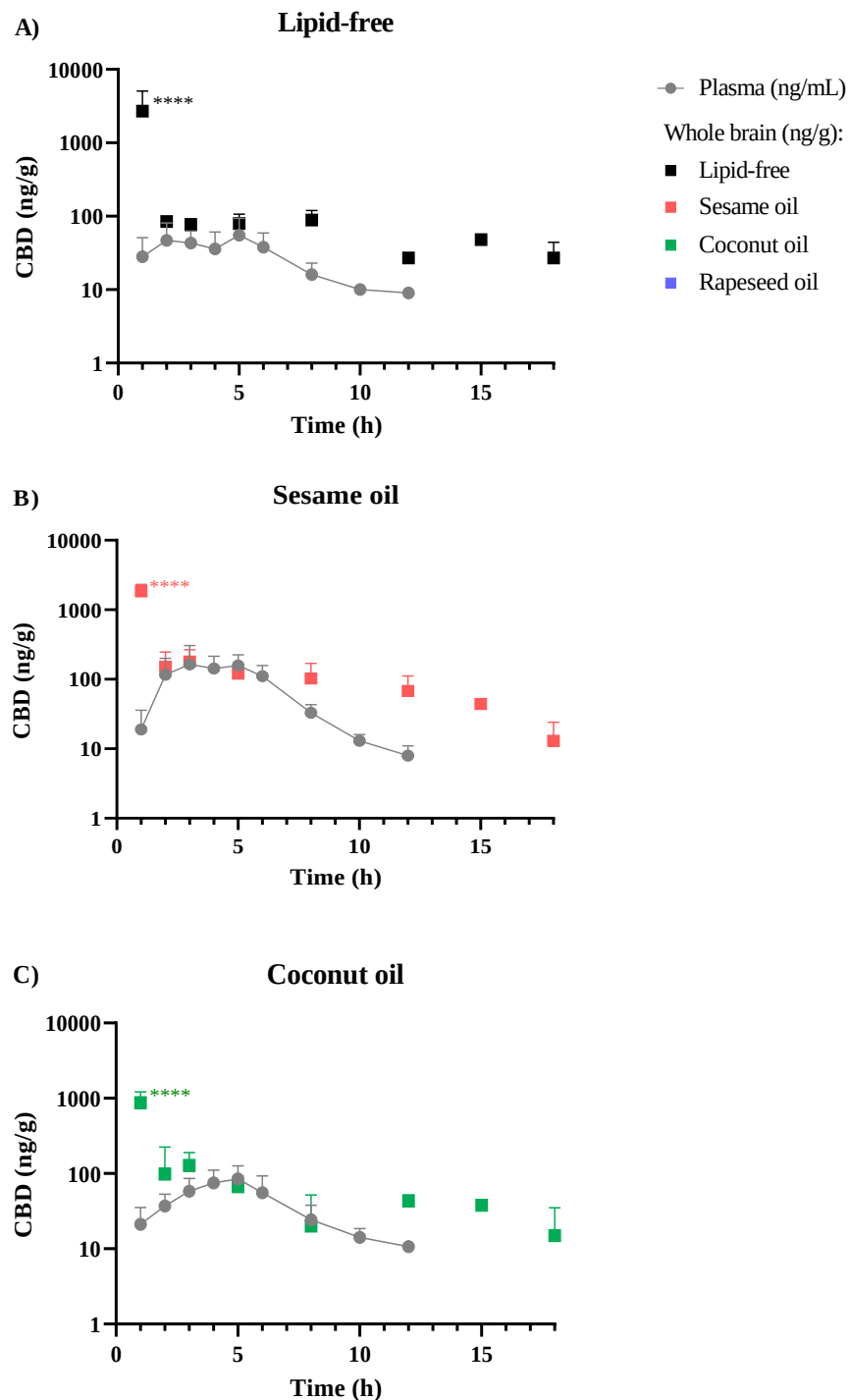


Figure 4.2 CBD concentration in anatomical brain regions A) olfactory bulb, B) frontal lobe, C) temporal lobe and D) brainstem vs. plasma following oral administration to rats (12 mg/Kg) in various lipid-based vehicles. Tissues were collected at plasma T_{max}. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, * = $p < 0.001$. Data are presented as mean $\pm$ SD, brain $n = 3$, plasma $n = 6$ (collected in previous PK studies (Feng *et al* [151, 155])).**

4.3.1.2 Biodistribution of cannabidiol following oral administration to the brain

Once the lipid-based vehicles were selected, the biodistribution of CBD to the whole brain was assessed at 1 - 18 h following oral administration at 12 mg/Kg to rats. The CBD concentration in the whole brain, compared to the plasma PK profile, of each formulation is shown in Figure 4.3. For all vehicles, the whole brain T_{max} is 1 h, as much as 2 - 4 h earlier than the plasma T_{max}. The maximum concentration (C_{max}) observed in the whole brain is significantly higher than the plasma concentration at that time (1 h) in oil formulations.

The whole brain concentrations did not fall below the plasma concentrations over the 12 h in any formulation, and no significant differences were observed after 1 h. At 12 h following administration in all vehicles, plasma CBD concentrations were close to the lower limit of quantification (LLOQ) (10 ng/mL), whereas in the whole brain, CBD was detected above 20 ng/g at 18 h.



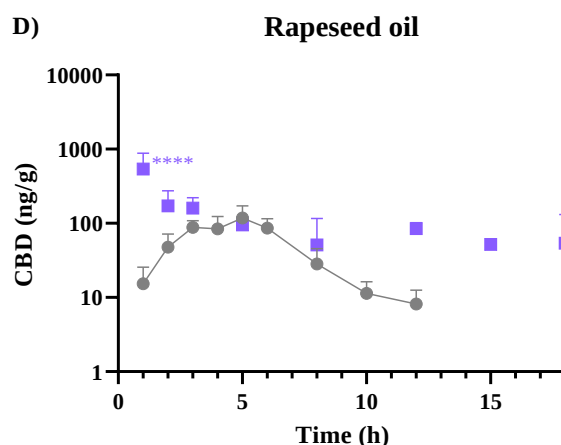


Figure 4.3. CBD concentration in the whole brain vs. plasma after oral administration to rats (12 mg/Kg) in the A) lipid-free, B) sesame oil, C) coconut oil and D) rapeseed oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, ** = $p < 0.0001$. Data are presented as mean $\pm$ SD, brain $n = 3$, plasma $n = 6$ (collected in previous PK studies (Feng *et al* [151, 155])). The PK parameters are presented in Table 4.1.**

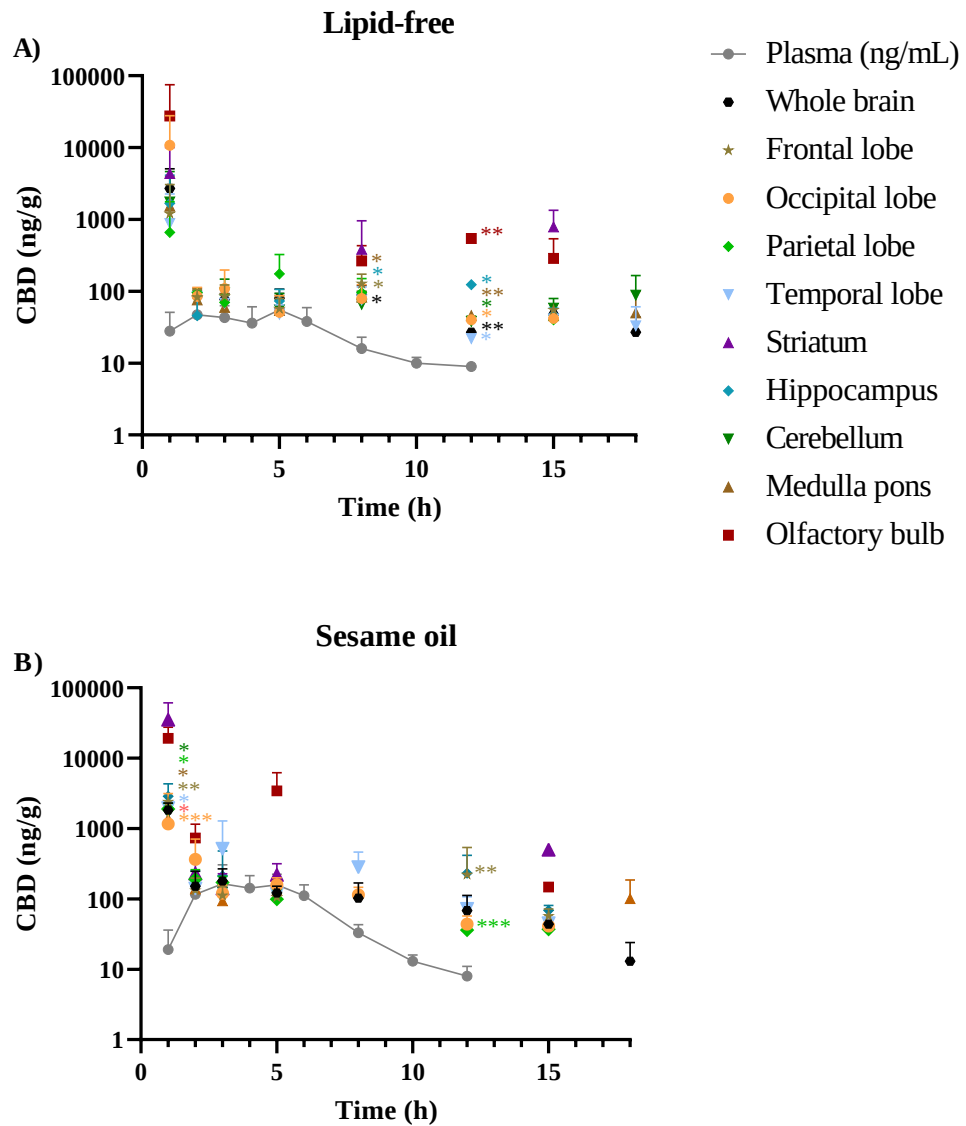
The data presented in Figure 4.3 was used to calculate the PK parameters presented in Table 4.1. Significant differences were found in the whole brain C_{max} and area under the curve ($AUC_{0-\infty}$) between the lipid-free and lipid-based coconut and rapeseed oil formulations. The sesame oil formulation resulted in a significantly different whole brain C_{max} than the lipid-free vehicle. The lipid-free vehicle produced the highest C_{max} (2712 ± 2365 ng/g), and $AUC_{0-\infty}$ (3270 ± 1890 h*ng/g), however most striking is the high brain-plasma ratio of 9.2. Of the lipid-based vehicles, the sesame oil formulation exhibited the highest C_{max} (1836 ± 473 ng/g). The $AUC_{0-\infty}$ after administration in the sesame oil formulation (3061 ± 242 h*ng/g) was higher than that of the coconut oil formulation. However, the sesame oil formulation resulted in the lowest brain-plasma ratio (3.5). The rapeseed oil formulation produced the highest brain-plasma ratio of the lipid-based vehicles at 4.6. The half-lives of CBD were longer in brain tissue than plasma, more so for the lipid-free and rapeseed oil vehicles.

Table 4.1. PK parameters of CBD concentration-time profiles in plasma and whole brain after oral administration to rats (12 mg/Kg). One-way ANOVA with Dunnett's multiple comparisons was performed to compare lipid-based formulations to the lipid-free vehicle, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.0001$. Data are presented as mean $\pm$ SD, brain n = 3, plasma n = 6 (collected in previous PK studies (Feng *et al* [151, 155])). The data are also presented in Figure 4.3.**

Formulation		T _{max} (h)	C _{max} (ng/mL or ng/g)	t _{1/2} (h)	AUC _{0-∞} (h*ng/mL or h*ng/g)	Brain- plasma ratio
Lipid-free	Plasma	5	55 $\pm$ 39	2.3	356 $\pm$ 83	9.2
	Whole brain	1	2712 $\pm$ 2365	9.6	3270 $\pm$ 1890	
Sesame oil	Plasma	3	164 $\pm$ 142	1.5	865 $\pm$ 342	3.5
	Whole brain	1	1836 $\pm$ 473 *	4.6	3061 $\pm$ 242	
Coconut oil	Plasma	5	84 $\pm$ 72	1.5	413 $\pm$ 164	4.3
	Whole brain	1	876 $\pm$ 338 ****	3.9	1794 $\pm$ 373 *	
Rapeseed oil	Plasma	5	118 $\pm$ 54	1.7	587 $\pm$ 287	4.6
	Whole brain	1	539 $\pm$ 341 ****	10.1	2726 $\pm$ 1635 *	

The CBD delivery to specific anatomical brain regions is compared to the plasma PK profile for each vehicle in Figure 4.4. In each anatomical brain region, the CBD concentrations are higher than or similar to the plasma concentrations. All brain regions exhibited a T_{max} at 1 h, the same as the whole brain. The highest CBD concentrations were observed in the olfactory bulb and striatum after administration in every formulation. Prolonged delivery at 18 h following administration was observed in different regions for each formulation. For example, after administration in the sesame oil formulation, only the medulla

pons had CBD concentrations that could be quantified at 18 h, whereas after administration in the coconut oil formulation, only the temporal lobe had quantifiable CBD concentrations.



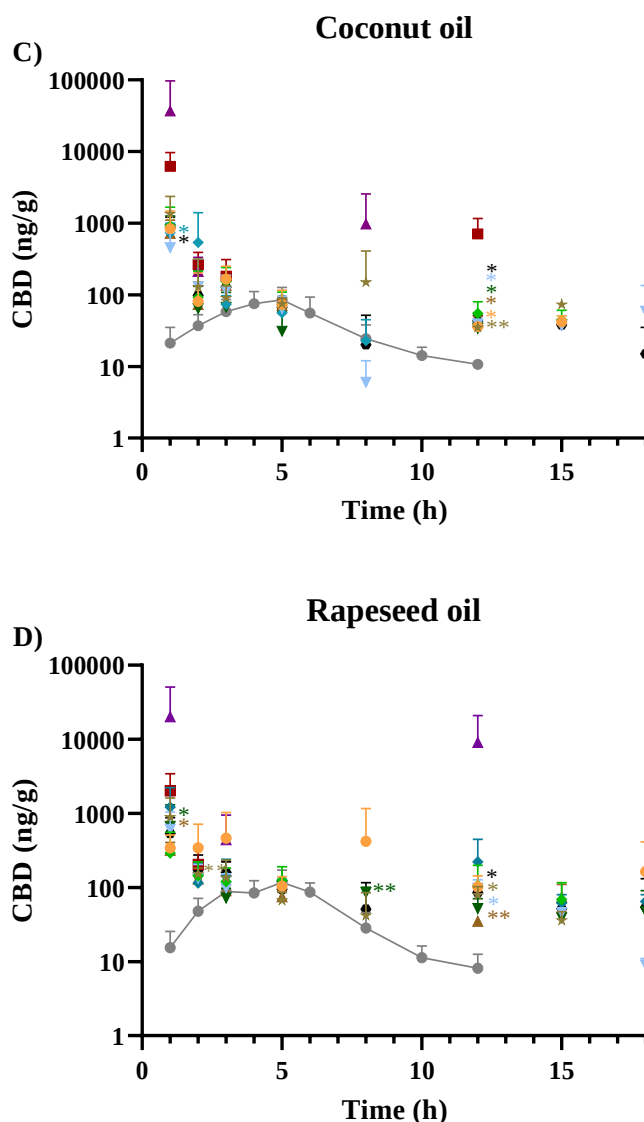
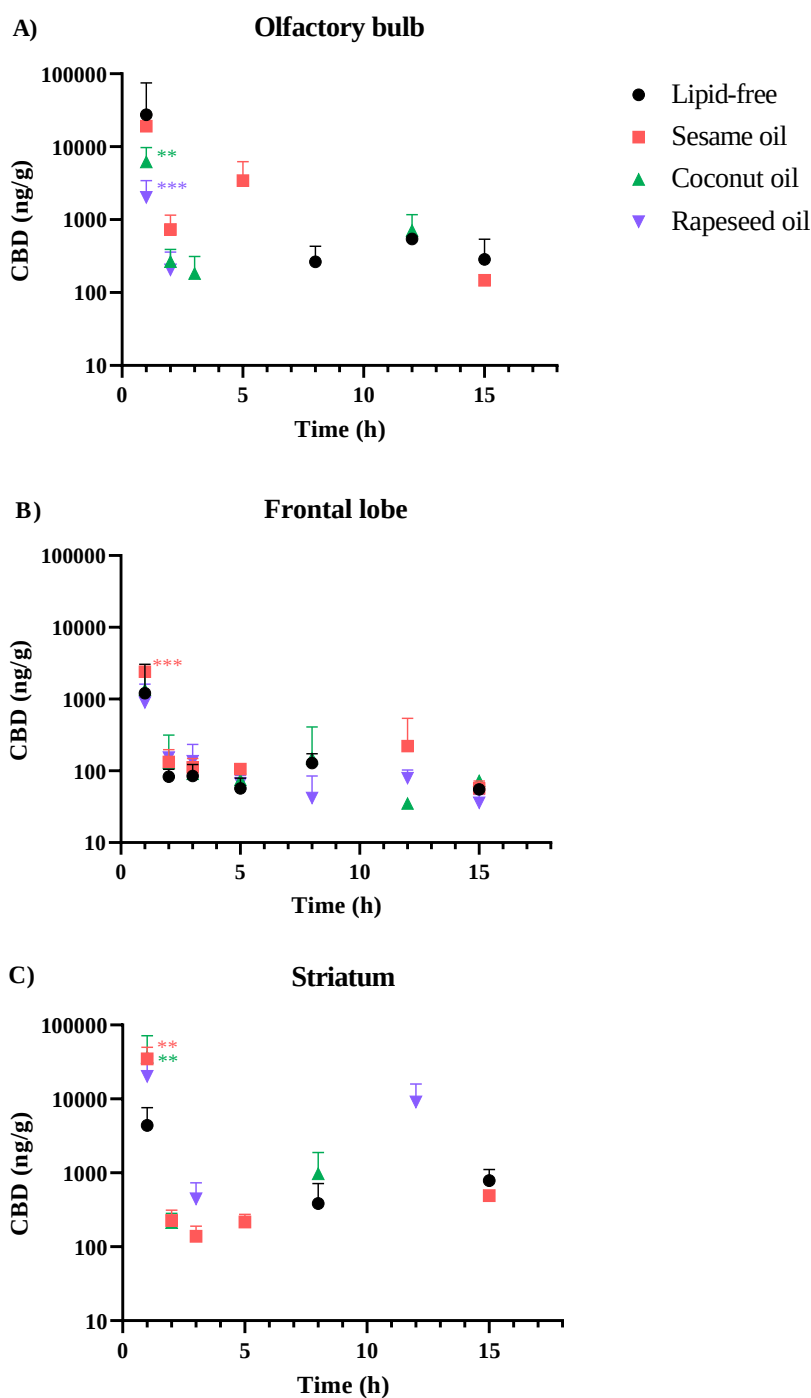
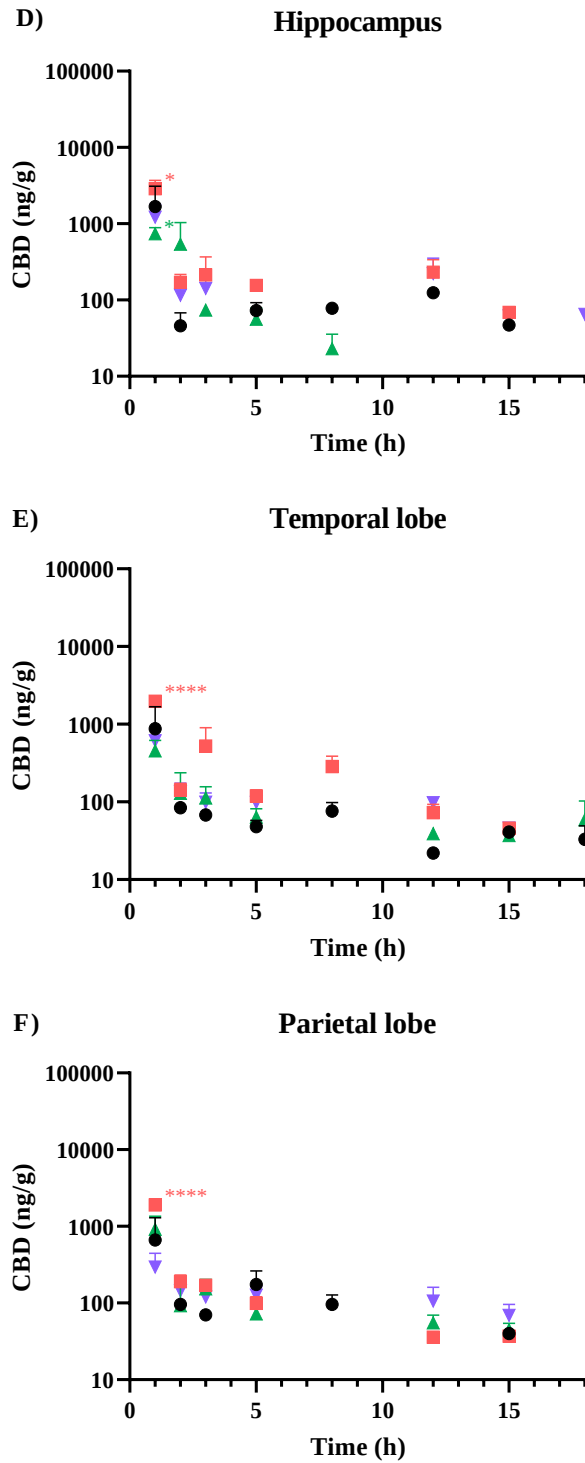


Figure 4.4. CBD concentration in anatomical brain regions vs. plasma after oral administration to rats (12 mg/Kg) in the A) lipid-free, B) sesame oil, C) coconut oil and D) rapeseed oil formulations. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, * = $p < 0.001$. Data presented as mean $\pm$ SD, brain $n = 3$, plasma $n = 6$ (collected in previous PK studies (Feng *et al* [151, 155])).**

The data presented in Figure 4.4 are also shown in Figure 4.5, separated by anatomical brain region, rather than administration vehicle. The lipid-free vehicle was found to exhibit the highest C_{max} compared to lipid-based vehicles in the olfactory bulb, occipital lobe, medulla pons and cerebellum. There were

also statistically significant differences seen in the frontal lobe, hippocampus, temporal lobe, and parietal lobe, where the sesame oil formulation resulted in the highest C_{max} . In the striatum, both the sesame and coconut oil formulations resulted in a significantly higher C_{max} than the lipid-free vehicle. The PK parameter data for the anatomical brain regions following administration in all formulations is shown in Table 4.2.





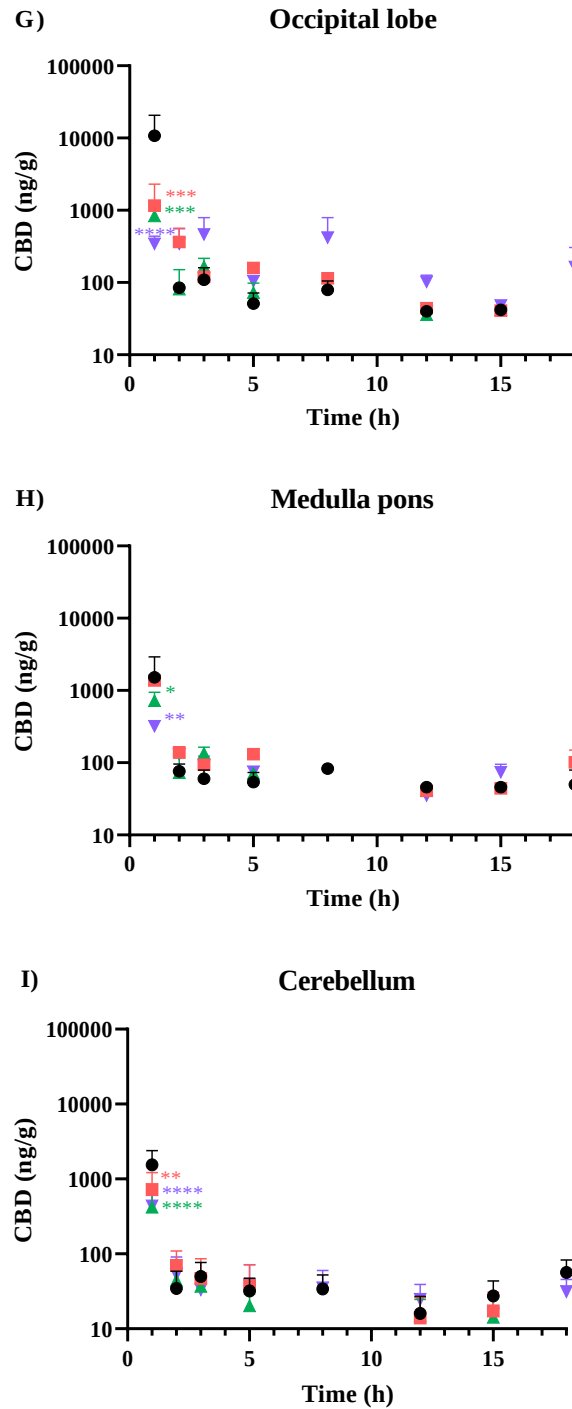


Figure 4.5. CBD concentration in anatomical brain regions A) olfactory bulb, B) frontal lobe, C) striatum, D) hippocampus, E) temporal lobe, F) parietal lobe, G) occipital lobe, H) medulla pons and I) cerebellum after oral administration to rats (12 mg/Kg) in lipid-free, sesame oil, coconut oil and rapeseed oil formulations. One-way ANOVA with Dunnett's multiple comparisons performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$,

**** = $p < 0.0001$. Differences of lipid-based formulations to the lipid-free formulation are shown. Data are presented as mean $\pm$ SD, n = 3.

Table 4.2. PK parameters of CBD concentration-time profiles for the anatomical brain regions following oral administration to rats (12 mg/Kg). One-way ANOVA with Dunnett's multiple comparisons were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, * = $p < 0.001$, **** = $p < 0.0001$. Differences of lipid-based formulations to the lipid-free formulation are shown. Data are presented as mean $\pm$ SD, n = 3. The data are also presented in Figure 4.5.**

Formulation	Brain region	T _{max} (h)	C _{max} (ng/g)	t _{1/2} (h)	AUC ₀₋₁₈ (h*ng/g)	AUC _{0-∞} (h*ng/g)
Lipid-free	Olfactory bulb	1	27542 $\pm$ 47390	2.2	53785 $\pm$ 85988	-
	Frontal lobe	1	1206 $\pm$ 1858	6.2	2066 $\pm$ 1279	2747 $\pm$ 1003
	Striatum	1	4373 $\pm$ 5637	5.7	14291 $\pm$ 6280	23480 $\pm$ 2210
	Hippocampus	1	1674 $\pm$ 2467	6.0	2270 $\pm$ 1589	3062 $\pm$ 1683
	Temporal lobe	1	874 $\pm$ 1388	12.0	1473 $\pm$ 1041	2184 $\pm$ 1045
	Parietal lobe	1	662 $\pm$ 1098	4.8	1644 $\pm$ 1411	2120 $\pm$ 1188
	Occipital lobe	1	10792 $\pm$ 17103	10.8	8157 $\pm$ 11677	8593 $\pm$ 11560
	Medulla pons	1	1525 $\pm$ 2421	14.0	2109 $\pm$ 1819	2633 $\pm$ 2383
	Cerebellum	1	1742 $\pm$ 2895	7.7	2296 $\pm$ 2373	3597 $\pm$ 3875

Sesame oil	Olfactory bulb	1	19126 ± 8473	2.7	31368 ± 18452	32556 ± 17785
	Frontal lobe	1	2413 ± 234 ***	6.1	3759 ± 1498	4505 ± 1527
	Striatum	1	34751 ± 26284 **	7.2	27831 ± 16151	33792 ± 15254
	Hippocampus	1	2873 ± 1434 *	5.3	4599 ± 636	5574 ± 876
	Temporal lobe	1	1979 ± 378 *****	2.6	4005 ± 1155	4256 ± 1025
	Parietal lobe	1	1891 ± 383 *****	5.1	2660 ± 188	2963 ± 265
	Occipital lobe	1	1163 ± 1966 ***	4.6	2532 ± 1989	2839 ± 1914
	Medulla pons	1	1371 ± 474	6.6	2429 ± 301	5929 ± 3133
	Cerebellum	1	1669 ± 495 **	6.8	2699 ± 892	3110 ± 875
Coconut oil	Olfactory bulb	1	6213 ± 3494 **	17.1	9214 ± 4330	28113 ± 6108
	Frontal lobe	1	1355 ± 1008	5.2	2565 ± 775	3112 ± 818
	Striatum	1	36810 ± 60180 **	2.8	40475 ± 4715 *	44372 ± 1205 **
	Hippocampus	1	737 ± 267	2.9	1326 ± 633	1482 ± 577
	Temporal lobe	1	455 ± 282	7.2	1270 ± 105	10327 ± 15426
	Parietal lobe	1	918 ± 383	15.2	1719 ± 1023	2215 ± 1264
	Occipital lobe	1	837 ± 646 ***	6.4	1556 ± 916	1953 ± 913

Rapeseed oil	Medulla pons	1	720 ± 383 *	5.9	1308 ± 192	1662 ± 263
	Cerebellum	1	806 ± 459 *****	17. 3	1165 ± 319	1919 ± 277
	Olfactory bulb	1	2013 ± 1422 ****	-	1785 ± 1212	-
	Frontal lobe	1	893 ± 724	7.8	1746 ± 606	2163 ± 519
	Striatum	1	20176 ± 30471	-	60381 ± 74716	-
	Hippocampus	1	1215 ± 998	3.4	3287 ± 859	4271 ± 1063
	Temporal lobe	1	611 ± 433	1.7	1883 ± 242	1965 ± 241
	Parietal lobe	1	293 ± 263	15. 7	1749 ± 737	3180 ± 1445
	Occipital lobe	3	461 ± 571 *****	8.5	3589 ± 2544	4636 ± 4173
	Medulla pons	1	319 ± 87 **	7.6	1167 ± 164	1393 ± 1897
	Cerebellum	1	665 ± 636 *****	9.4	1717 ± 592	2738 ± 1475

4.3.2 Biodistribution of cannabidiol to the brain assessed by 3D OrbiSIMS analysis

4.3.2.1 Biodistribution in rat brain homogenates

For the CBD biodistribution studies in rats, discussed above, tissue samples were analysed by HPLC-UV after they were homogenised (described in Chapter 2, section 2.5.1). Some of the tissue homogenates were also analysed by 3D OrbiSIMS to compare the information gathered from the two techniques. The samples were prepared from the cerebellum, temporal lobe and frontal cortex anatomical brain regions and the whole brain, collected at 2 h following administration in the sesame oil formulation. In order to streamline the assessment, the analysis was performed on only certain anatomical brain regions as this was a preliminary study to examine this applicability of the 3D OrbiSIMS technique to this sample type. The anatomical brain regions chosen were limited to sample homogenates large enough to perform both analyses.

First, construction of a calibration curve of rat brain homogenate tissues spiked with CBD was undertaken using the 3D OrbiSIMS. The calibration curve was made using the CBD molecular ion $[M-H]^-$ peak intensity (m/z 313.2187, $C_{21}H_{29}O_2^-$) expressed as the peak intensity (secondary ion counts) normalised to the total ion count (TIC). This would allow for calculation of the expected CBD $[M-H]^-$ peak intensity (normalised to the TIC) from the concentration determined by HPLC-UV. The calculated expected normalised peak intensities are shown in Table 4.3.

Table 4.3. CBD concentration of anatomical rat brain region homogenates 2 h following oral administration (12 mg/Kg) in a sesame oil formulation (determined by means of HPLC-UV) and expected [M-H]⁻ peak intensity (secondary ion counts) normalised to TIC. The data are presented as mean ± SD, HPLC-UV analysis was performed in biological triplicates.

Brain region	CBD concentration (ng/g)	Expected [M-H] ⁻ intensity normalised to TIC (a.u.)
Whole brain	152 ± 95	$1.52 \times 10^4 \pm 9.5 \times 10^3$
Frontal lobe	133 ± 64	$1.33 \times 10^4 \pm 6.4 \times 10^3$
Temporal lobe	142 ± 53	$1.42 \times 10^4 \pm 5.3 \times 10^3$
Cerebellum	139 ± 68	$1.39 \times 10^4 \pm 6.8 \times 10^3$

The measured CBD [M-H]⁻ peak intensities (normalised to TIC) are shown in Figure 4.6, compared to the expected areas calculated from the HPLC-UV determined concentration, in Table 4.3. The only significant difference between the expected and measured peak intensity was found in the temporal lobe sample, where the measured intensities were significantly smaller than that expected. Additionally, the CBD [M-H]⁻ was not detected in the frontal lobe or cerebellum homogenate samples, although known to be present based on detection by means of HPLC-UV. The data was also searched for fragments of CBD, the major fatty acids in sesame oil, and other indications of CBD activity. These were described in Chapter 3 including methylated-DNA, ceramide, and anandamide. However, these were either not found in the samples, or not found to differ to a blank reference sample (non-treated rat brain tissue).

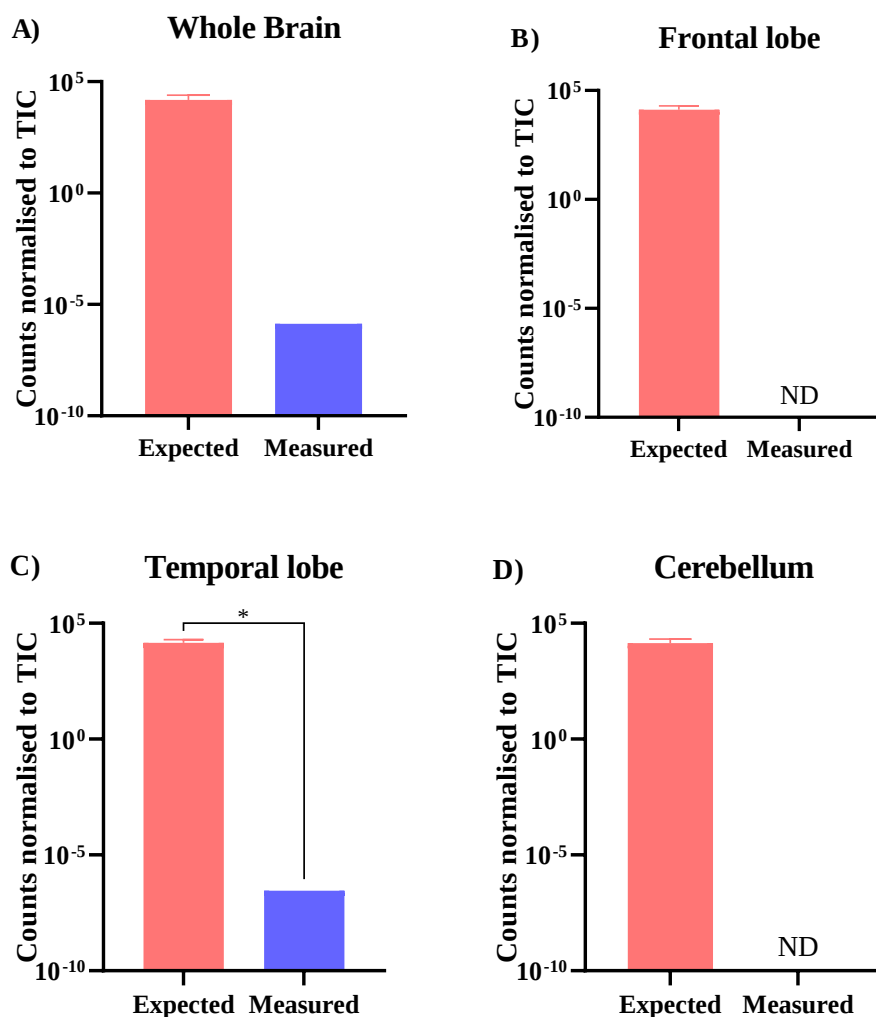


Figure 4.6. The expected and measured CBD [M-H]⁺ peak intensity (secondary ion counts) normalised to TIC in anatomical brain regions of rats 2 h following oral administration in a sesame oil formulation (12 mg/Kg). The expected CBD [M-H]⁺ intensities were calculated from HPLC-UV analysis. ND = not detectable. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$. The data are presented as mean $\pm$ SD. The expected intensities were calculated from triplicate biological repeats, the measured intensities were performed once.

4.3.2.2 Biodistribution in a rat brain section

3D OrbiSIMS analysis of a section of a dosed rat brain was performed, in addition to the analysis of tissue homogenates discussed above. As mentioned, the 3D OrbiSIMS is capable of producing images of samples, so that the distribution of an ion can be visualised. However, this can be a long process when the sample is large. When using the instrument with the Orbitrap, collecting an image takes longer than when using the liquid metal ion gun (LMIG) analyser. As discussed in Chapter 2, the Orbitrap was used for this work due to the high resolving power of the analyser. The rat brain sections were several cm in length and width, and therefore to collect an image of the section using the Orbitrap would have taken several days, which unfortunately, was not feasible for this work. Instead, “spot” analysis was performed in certain locations on the section.

The purpose of performing “spot” analysis on sections was to investigate the feasibility of biodistribution analysis by this approach, compared to the homogenate tissue samples. The section sample size is a lot larger than a 2 μL drop of homogenate tissue, which can affect the noise levels in the instrument. With more noise in the instrument, it could be harder to detect certain ion peaks, especially those that may be small, such as the CBD $[\text{M-H}]^-$. However, the reduction in sample manipulation before analysis of tissue sections, compared to homogenate samples, makes analysis of tissue sections an attractive option. Therefore, “spot” analysis of a rat brain section, compared to the homogenate tissue samples was performed, shown in Figure 4.7.

Figure 4.7 shows that in the frontal lobe and cerebellum regions of the brain, the CBD $[\text{M-H}]^-$ was detected in the section, but not in the homogenate sample. In the temporal lobe region, the peak intensities were similar in both the homogenate and section sample, with no significant difference found. The expected peak intensities, shown in Table 4.3, are in the range of 1×10^4 , and the intensities measured in both the section and homogenate samples are in the range of 1×10^{-7} . However, when statistical analysis was performed, no differences were found between the expected and measured section intensities in the frontal lobe or the cerebellum.

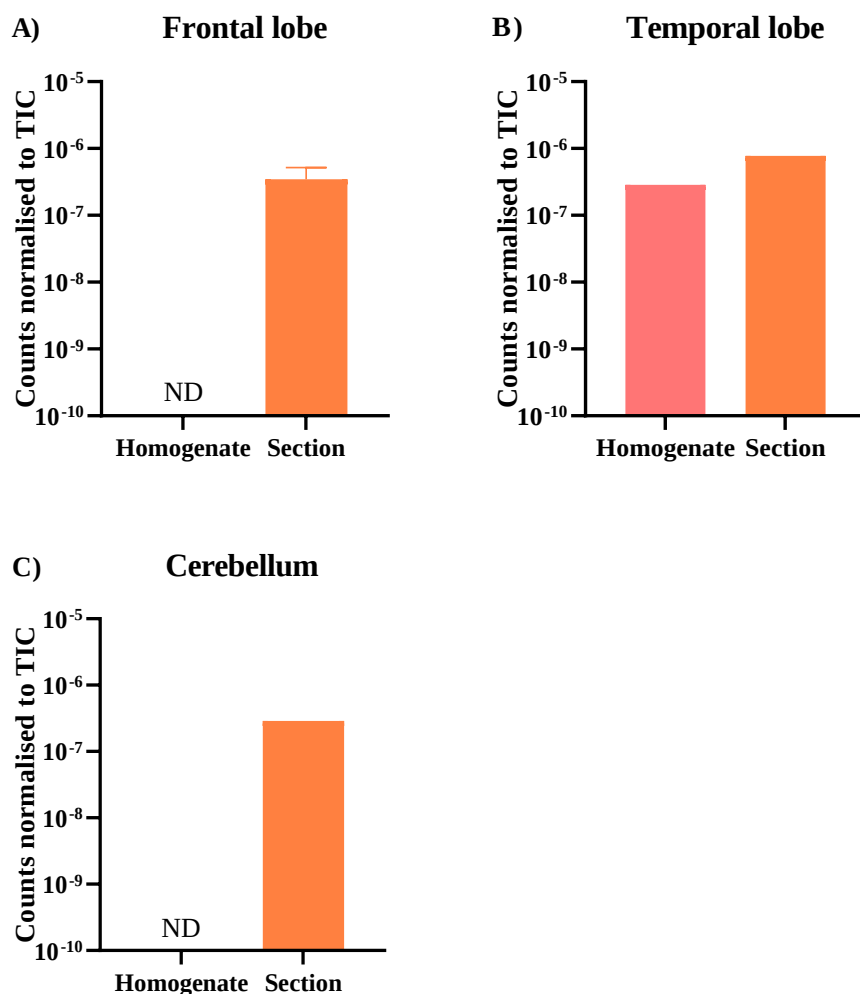


Figure 4.7. CBD [M-H]⁻ peak intensity (secondary ion counts) normalised to the TIC in anatomical brain region homogenate tissues and brain sections of rats 2 h following oral administration in a sesame oil formulation (12 mg/Kg). ND = not detectable. Multiple t-tests were performed, $\alpha = 0.05$. The data are presented as mean $\pm$ SD, $n = 1$, apart from the frontal lobe section where $n = 2$.

4.4 Discussion

The lipid-based oil formulations used for the study of the *in vivo* biodistribution of CBD in rats were selected by the whole brain concentrations at the plasma T_{max} , in Figure 4.1. No significant differences were observed between the whole brain and plasma concentrations at the plasma T_{max} . However, when separated by brain region, in Figure 4.2, statistically significant differences were observed. Administration in the sesame oil formulation resulted in significantly higher concentrations in all brain regions, and so was taken forward to biodistribution studies. The coconut and rapeseed oil formulations resulted in higher concentrations in the temporal lobe, a region of particular interest due to the prevalence of GBM tumours there [90, 229, 230]. These two formulations were also selected for biodistribution studies as the oils contain different fatty acids to sesame oil, as shown in Table 4.4. Of particular interest are myristic and oleic acids, both of which are reported to increase BBB permeability [66, 189]. Coconut oil contains myristic acid, and both sesame and rapeseed oils contain predominantly oleic and linoleic acids, with rapeseed oil containing more oleic acid. Table 4.4 also shows that coconut oil contains more short and medium chain fatty acids than sesame and rapeseed oils. Fatty acid chain length is possibly a contributing factor to BBB permeability, due to the interactions at the barrier but also the digestion of the lipid after oral administration, as discussed in section 4.1 [63].

Previous reports in the literature suggest that CBD delivery to the rat brain is slow following oral administration, exhibiting a T_{max} substantially later than in the plasma [149, 152, 231]. However, Hložek *et al* demonstrated a brain T_{max} at the same time as the plasma T_{max} after oral administration to rats in a sunflower oil formulation [168]. The data in Figures 4.1 and 4.2 show that at the sunflower oil formulation plasma T_{max} , the CBD concentration in the whole brain is similar to the plasma. Whereas the concentration in the olfactory bulb and temporal lobe are higher than in the plasma. The work herein cannot be compared to Hložek *et al*'s exactly, as only one time point was studied in this work following administration in the sunflower oil formulation. However, Hložek *et al* did report a C_{max} of ~250 ng/g at 2 h after oral administration to rats (10 mg/Kg) [168]. The plasma T_{max} after administration in the sunflower oil formulation used in this

work was previously determined to be 3 h [151] (3 h was not used in Hložek *et al*'s study). Hložek *et al* used a lower dose of 10 mg/Kg, compared to 12 mg/Kg in this work, therefore, to corroborate with the $C_{\max}$ reported by Hložek *et al*, a concentration of ~300 ng/g would be expected. Figure 4.1 shows a whole brain concentration of 180 ± 75 ng/g at 3 h. The variation in delivery was not reported in Hložek *et al*'s work, however, the CBD concentrations are similar, accounting for the likely range, this concentration would be similar to Hložek *et al*.

Table 4.4. Fatty acid composition of the oils selected for the lipid-based formulations for *in vivo* CBD biodistribution studies, taken from the literature [151, 232].

Fatty acid		Sesame oil	Coconut oil	Rapeseed oil
Saturated	Octanoic acid C8:0		4%	
	Decanoic acid C10:0		3%	
	Lauric acid C12:0		29%	
	Myristic acid C14:0		21%	
	Palmitic acid C16:0	13%	18%	4%
	Stearic acid C18:0	8%	8%	2%
Unsaturated	Oleic acid C18:1 cis-9	44%	14%	63%
	Linoleic acid C18:2 cis-9,12	30%	3%	20%
	Linolenic acid C18:3 cis-9,12,15	<1%		10%

Based on reports in the literature, and as discussed in Chapter 1, the brain $T_{\max}$ of CBD was expected to be between 5 - 8 h (at or slightly after the plasma $T_{\max}$) [149, 152, 168, 231]. Interestingly, the observed $T_{\max}$ for all vehicles herein was

1 h post administration (Table 4.1). This fast delivery to the rat brain suggests that high levels in the plasma (at the plasma T_{max}) are not required for efficient delivery to the brain. Additionally, the $t_{1/2}$ was longer in the whole brain than plasma (Table 4.1), demonstrating prolonged exposure of the brain to CBD.

The expected CBD delivery to the whole brain could be made based on Hložek *et al*'s reported C_{max} of ~250 ng/g following oral administration to rats in sunflower oil (10 mg/Kg) [168]. Sesame oil is similar to sunflower oil in fatty acid composition, with slightly more oleic acid and less linoleic acid [151]. As discussed, the dose administered in this work was higher (12 mg/Kg), therefore, a C_{max} ~300 ng/g in the whole brain would demonstrate similar brain delivery by sesame and sunflower oil-based formulations. However, the C_{max} following administration in the sesame oil formulation was earlier than Hložek *et al* (T_{max} = 1 h) at 1836 ± 473 ng/g (Table 4.1), 6× higher than expected. The differences between the two oil's fatty acid compositions are small but could be at least partly behind the difference in efficiency of brain delivery.

Following oral administration, the brain-plasma ratios are all ≥ 3.5 (Table 4.1), whereas previously these are reported in the literature at <1 for cannabinoids, apart from cannabidiolic acid (CBDA), which had a ratio of 1.9 after intraperitoneal (i.p.) injection [231]. A ratio >1 demonstrates increased accumulation of CBD in brain tissue, compared to the plasma. The highest brain-plasma ratio was observed following oral administration in the lipid-free formulation (9.2), a result of the low plasma $AUC_{0-\infty}$ (356 ± 83 h*ng/mL). Both the $AUC_{0-\infty}$ and C_{max} in the whole brain are highest after administration in the lipid-free vehicle (3270 ± 1890 h*ng/g and 2712 ± 2365 ng/g, respectively). The sesame oil formulation produced the second highest $AUC_{0-\infty}$ and C_{max} (3061 ± 242 h*ng/g and 1836 ± 473 ng/g, respectively), after the lipid-free formulation. However, the brain-plasma ratio is the lowest for the sesame oil formulation (3.5), due to the high plasma exposure ($AUC_{0-\infty} = 865 \pm 342$ h*ng/mL).

When studying the lipid-based formulations, the C_{max} and AUCs of the whole brain would suggest that the sesame oil formulation results in the highest brain exposure to CBD. However, the brain-plasma ratios of the coconut and rapeseed oil formulations are higher (4.6 and 4.3, respectively), compared to 3.5 for the

sesame oil formulation (Table 4.1). This suggests that the coconut and rapeseed oil formulations resulted in higher BBB permeability than the sesame oil formulation. A possible explanation for the high brain-plasma ratio of the coconut oil formulation is the myristic acid presence in coconut oil [151]. As discussed, myristic acid is reported to increase the permeability of the BBB by interacting with the membrane [189]. A possible reason for the high ratio for the rapeseed oil formulation is the high oleic acid content (63%, compared to 44% in sesame oil and 14% in coconut oil, Table 4.4) [151, 232]. Oleic acid is also reported to increase BBB permeability [66]. Studying the whole brain, the delivery profiles of each formulation are similar following administration to rats over the 18 h studied (Figure 4.3). Each formulation resulted in a T_{max} of 1 h and significantly higher concentration in the brain at 1 h than the plasma. Prolonged delivery up to 18 h was also observed for all formulations. However, when the brain is studied by anatomical region, different delivery profiles were observed. Figures 4.4 and 4.5 demonstrate the distribution of CBD in anatomical brain regions comparing to the plasma PK profile and each formulation, respectively. Table 4.2 shows the PK parameters of the CBD concentration-time profiles in each brain region. These data are discussed below for each of the anatomical brain regions studied.

4.4.1 Olfactory bulb

The olfactory system is an important region of study, it is effected in several neurodegenerative diseases, where dysfunction is an early symptom of both Alzheimer's disease (AD) and Parkinson's disease [233, 234]. Understanding delivery to the olfactory bulb is necessary in order to treat this dysfunction. Whilst targeting the olfactory system is possible *via* nose-to-brain delivery with a nasal spray, oral administration is also a favourable route for good patient experience [235, 236].

Following oral administration, the highest delivery of CBD in the brain was observed in the olfactory bulb and the striatum (Figure 4.4). After the T_{max} (1 h) in the olfactory bulb, the concentrations dropped significantly at 2 h (Figure 4.5). Second concentration peaks were observed after administration in the sesame oil

formulation (5 h), the coconut oil formulation (12 h) and the lipid-free formulation (12 h). The presence of two concentration peaks, most noticeable following administration in the sesame oil formulation, are possibly indicative of two mechanisms of CBD delivery to the olfactory bulb. The time of the second peak varies by the formulation, this was the fastest for the sesame oil formulation and not present at all for the rapeseed oil formulation, suggesting the second peak is determined by the formulation. This could potentially be a result of the digestion of the lipids or later, the interaction of the fatty acids with the BBB [62].

The highest CBD C_{max} in the olfactory bulb was observed following administration in the lipid-free vehicle and sesame oil formulations at 27542 ± 47390 ng/g and 19126 ± 8473 ng/g, respectively. The lipid-free vehicle also resulted in a high AUC_{0-18} of 53785 ± 85988 h*ng/g, although large variation was associated with administration in the lipid-free formulation. CBD delivery to the olfactory bulb was not prolonged, as CBD could not be detected at 18 h post administration.

4.4.2 Frontal lobe

As the largest brain region, the frontal lobe is an area of interest, responsible for a variety of functions including movement, language and attention [237]. Motor neuron disease and dementia result from brain damage located in the frontal lobe, as well as Parkinson's disease and other neurodegenerative disorders [238, 239]. In Chapter 3, the anti-cancer properties of CBD against GBM cell lines were investigated, demonstrating the ability of CBD to inhibit GBM cell growth. As well as the temporal lobe, as discussed above, GBM tumours are also commonly located in the frontal lobe, and therefore understanding delivery of CBD to the region is important [90, 229, 230].

The delivery of CBD to the frontal lobe resulted in a T_{max} at 1 h. Following this, delivery was consistent across the formulations up to 5 h post administration (Figure 4.5). After this time, the formulations produced different profiles. The sesame oil formulation produced a second concentration peak at 12 h, while the

coconut oil and lipid-free formulations produced two additional concentration peaks (at 8 and 15 h). The time of the second concentration peaks is different in the frontal lobe and olfactory bulb. This suggests that while digestion of the lipids may impact the delivery, there are also differences in reaching specific anatomical regions. The second concentration peaks at later time points resulted in higher exposure of the region to CBD. The highest AUC_{0-18} was observed following administration in the sesame oil formulation ($3759 \pm 1498 \text{ h*ng/g}$), where the highest second concentration peak (12 h) was observed. The highest C_{max} was also seen after administration in the sesame oil formulation at $2413 \pm 234 \text{ ng/g}$. The rapeseed oil formulation resulted in the lowest exposure of the frontal lobe to CBD, with an AUC_{0-18} of $1746 \pm 606 \text{ ng/g}$. As shown in Table 4.4, the two oils contain fatty acids with similar chain lengths, although with different degrees of saturation, possibly effecting the delivery [151, 232].

4.4.3 Striatum

The striatum is involved in many brain functions such as habits and decision making [240, 241]. The striatum is therefore important in many brain located diseases, including Huntington's disease, which is reported to primarily start in the striatum before eventually effecting all brain regions [242]. There are also high levels of cannabinoid receptors located within the striatum [243, 244], and CBD neuroprotective effects are reportedly linked to activity there [245].

As mentioned, the striatum exhibits the highest CBD concentrations along with the olfactory bulb (Figure 4.4). Again, the T_{max} was observed at 1 h for all formulations. The highest C_{max} was observed after administration in the coconut and sesame oil formulations. These two lipid-based formulations produced higher exposure of the striatum to CBD than the lipid-free formulation (AUC_{0-18} of 1919 ± 277 and $27831 \pm 16151 \text{ h*ng/g}$, respectively). Again, the CBD concentrations increased at later time points following administration in the sesame (15 h), coconut (8 h) and rapeseed (12 h) oil formulations. The increase observed in the rapeseed oil formulation group was significant enough to result in the highest AUC_{0-18} at $60381 \pm 74716 \text{ h*ng/g}$.

4.4.4 Hippocampus

The hippocampus is a brain region of interest for many reasons. It is where AD usually begins destroying neurons and is associated with a number of brain functions including memory processing and storage, stimulus response and behaviour [246-249]. With the hippocampus involved in so many processes, it is important to understand drug delivery to the region. In addition, CBD is often associated with the hippocampus. CBD is demonstrated to negatively correlate with the grey matter reduction in the hippocampus of cannabis users (whereas THC positively correlates), suggesting a neuroprotective effect of CBD in this region [250]. CBD is also demonstrated to activate CB1 and CB2 receptors in the hippocampus to interrupt fear memory consolidation [251]. Additionally, the anti-depressant effects of CBD are reported to be a result of activity in the hippocampus [252].

Considering the association of CBD with the hippocampus, delivery to the region was not particularly high. The sesame oil formulation resulted in the highest delivery with a C_{max} of 2873 ± 1434 ng/g and AUC_{0-18} of 4599 ± 636 h*ng/g. Whereas the coconut oil formulation resulted in the lowest delivery, with a C_{max} of 737 ± 267 ng/g and AUC_{0-18} of 1326 ± 633 h*ng/g. The difference in delivery of these two lipid-based formulations is evident in Figure 4.5, where CBD was not detected after 8 h following administration in the coconut oil formulation but was detected at 15 h after administration in the sesame oil formulation. The rapeseed oil formulation resulted in the longest exposure of the hippocampus to CBD, with detection at 18 h post administration. Prolonged exposure can be an important factor for activity, and Table 4.3 shows a trend that the AUC_{0-18} in the hippocampus was larger than other regions, particularly in the sesame and rapeseed oil formulations. Perhaps this is a result of the high oleic acid content of these two oils (Table 4.4) [151].

4.4.5 Temporal lobe

The work in Chapter 3 of this thesis described the anti-cancer properties of CBD against GBM, which is commonly located in the temporal lobe of the brain [90, 229, 230], demonstrating the need to understand delivery of CBD to the region. The temporal lobe is also a site associated with epilepsy [162]. An oral spray of CBD in a sesame oil formulation, Epidyolex®, is a current treatment of rare forms of epilepsy [161]. Therefore, high delivery of CBD to the temporal lobe is expected, due not only to products associated with the region, such as Epidyolex®, but also the high levels of cannabinoid receptors present in this region of the brain [243, 244].

The highest $C_{\max}$ in the temporal lobe was observed following administration in the sesame oil formulation at 1979 ± 378 ng/g, where the highest AUC_{0-18} was also observed (4005 ± 1155 h*ng/g). The other three formulations produced similar $C_{\max}$ and AUC_{0-18} to each other. The sesame oil formulation appears to exhibit two smaller CBD concentration peaks at 3 and 8 h, contributing to the high exposure. Whilst prolonged exposure was observed following administration in the lipid-free and coconut oil formulations (up to 18 h), the sesame oil formulation produced fast, high delivery and resulted in high exposure of the region to CBD. This work supports the choice of a sesame oil-based CBD formulation for epilepsy treatment.

4.4.6 Parietal lobe

The parietal lobe plays a critical role in both movement and behavioural response to stimuli, and therefore a number of conditions are associated with the region including AD and stroke [253-255]. Additionally, GBM tumours can also be found in the parietal lobe, along with the temporal and frontal lobes [90, 141, 229, 230]. As discussed above, Chapter 3 demonstrated the anti-cancer activity of CBD against GBM, which highlights the need to understand delivery to this region.

The delivery concentration-time profiles of CBD to the parietal lobe (Figure 4.5) appear similar across the formulations studied. The sesame oil formulation

produced the highest $C_{\max}$ and AUC_{0-18} . The other three formulations appear to result in similar delivery to each other. The rapeseed oil formulation resulted in a low $C_{\max}$, but delivery remained consistent up to 15 h, resulting in an AUC_{0-18} similar to the lipid-free and coconut oil formulations.

4.4.7 Occipital lobe

Much like other regions, epilepsy can also be linked to structures in the occipital lobe [256, 257]. As discussed above, there are current CBD treatments for epilepsy symptoms, so understanding the delivery of CBD to epilepsy-related brain regions is extremely important. The delivery of CBD to the occipital lobe was particularly interesting. It is the only brain region where the lipid-free vehicle showed much higher initial delivery, to then drop drastically at 2 h. Due to the high initial delivery of CBD from the lipid-free vehicle, this formulation produced a high AUC_{0-18} in the occipital lobe (8157 ± 11677 h*ng/g), compared to other regions. The rapeseed oil formulation showed a similar trend, with delivery to the occipital lobe higher than other regions in the caudal area of the brain (Table 4.3). The rapeseed oil formulation produced an AUC_{0-18} of 3589 ± 2544 h*ng/g, a result of the prolonged delivery to the region (CBD could only be detected at 18 h after administration in the rapeseed oil formulation).

4.4.8 Medulla pons

The medulla pons is the direct connection of the brain to the spinal cord, and is therefore a site involved in paralyses, as well as sleep cycles [258-260]. The reported therapeutic effects of CBD include delayed paralysis and improved sleep-related behaviours [261, 262]. Therefore, it is important to understand the delivery of CBD to the region. Similar to the occipital lobe, high delivery was initially observed after administration in the lipid-free vehicle, but in the medulla pons this was also observed in the sesame oil formulation. Again, the lipid-free formulation delivery profile showed a drop in concentration to be similar to the lipid-based formulations from 2 h onwards. The sesame oil and lipid-free formulations deliver similarly high exposure of the region to CBD too (AUC_{0-18}

of 2429 ± 301 and 2109 ± 1819 h*ng/g, respectively). This is also observed in Figure 4.5, which demonstrates that only the sesame oil and lipid-free formulations resulted in detection of CBD at 18 h.

4.4.9 Cerebellum

The cerebellum is the region of the brain responsible for processing motor function including sensory recognition, coordination and timing [263, 264]. CBD is reported to normalise connectivity in resting-state within the cerebellum, inducing some neuroprotective activity [265]. Similar to the medulla pons and occipital lobe, the lipid-free vehicle initially delivers the highest CBD levels, with a $C_{\max}$ of 1742 ± 2895 ng/g. Again, this drops at 2 h so that the concentration-time profiles of all formulations are similar from this time (Figure 4.5). Prolonged exposure is again seen following administration in the rapeseed oil formulation, and also the lipid-free vehicle.

It is important to study delivery to specific anatomical brain regions, as information can be obtained that otherwise would be missed from studying the whole brain as one tissue. For example, Chapter 3 described the anti-cancer properties of CBD against GBM. The most common locations of GBM tumours are in the frontal, temporal and parietal lobes of the brain [90, 141, 229, 230]. As the delivery to all three regions is described above, the sesame oil formulation resulted in the highest exposure of the regions to CBD. Therefore, for a CBD formulation to treat GBM, a sesame oil formulation would be the best choice.

Another potential application of CBD is for MS, where CBD is reported to exhibit immunosuppressive effects [119-123]. The application of CBD to slow MS progression will be explored in an *in vivo* experimental autoimmune encephalomyelitis (EAE) model in Chapter 5. Anatomical brain regions of interest for the study of MS are the hippocampus and cerebellum, where disease often localises [266, 267]. The sesame oil formulation resulted in high delivery to the hippocampus, whereas the lipid-free formulation demonstrated high delivery to the cerebellum. The rapeseed oil formulation showed prolonged

exposure in both anatomical brain regions. Therefore, the formulation choice may be dependent on the particular symptoms observed that could indicate disease location. Also, Figure 4.3 shows that when studying the whole brain, administration by the different formulations appears to produce similar concentration-time delivery profiles, with no second concentration peaks and detection of CBD in all vehicles up to 18 h post administration. However, as discussed here, studying the anatomical brain regions individually gives additional insight into the biodistribution of CBD and the effects of the formulation, outlined below.

Firstly, the regions that produced second concentration peaks were those in the rostral area of the brain (olfactory bulb, frontal lobe, striatum), whereas regions towards the caudal area of the brain (occipital lobe, medulla pons, cerebellum) resulted in a more consistent exposure of the region to CBD, after the initial $C_{\max}$. The time of these second concentration peaks varied with the formulation.

Secondly, prolonged exposure of tissue to CBD was observed in the caudal area of the brain, where CBD was detected at up to 18 h, but only up to 15 h in regions in the rostral area of the brain. It is not known from this study whether long exposure is a result of constant movement into the brain, i.e. permeability across the BBB, or of retention in the tissue. What can be taken from this work is that the rapeseed oil formulation often produces longer exposure of brain regions to CBD than the other formulations. It was discussed above that rapeseed oil contains a high oleic acid content (Table 4.4), which is known to interact with and increase the permeability of the BBB [62, 66, 232]. It is possible the oleic acid may be contributing to the prolonged exposure following administration in the rapeseed oil formulation. Alternatively, something else in the formulation could be the cause, such as other fatty acids or the degree of saturation of the oils. The oil composition is complex [151], and it is therefore likely that a combination of factors lead to the observed effect.

Another observation is that the sesame oil formulation often produced the highest $C_{\max}$ and AUC of CBD in the anatomical brain regions, for example in the frontal lobe, striatum, hippocampus, temporal and parietal lobes. However, in the caudal region of the brain (occipital lobe, medulla pons and cerebellum),

the lipid-free formulation resulted in the highest CBD concentrations. As discussed above, sesame oil contains a high content of oleic acid (Table 4.4 [151]), which could be contributing to the high delivery [62, 66], as was discussed for the prolonged exposure of regions to CBD that was observed following administration in the rapeseed oil formulation.

Finally, the coconut oil formulation often resulted in low CBD delivery, and did not produce the highest C_{max} , AUC or longest exposure in any of the anatomical brain regions. Coconut oil contains 21% myristic acid (Table 4.4) [151]. Myristic acid is reported to interact with the BBB to increase permeability [62, 189]. However, increased delivery of CBD was not observed in the coconut oil formulation (compared to the lipid-free vehicle). It is possible that coconut oil does not contain enough myristic acid to induce an obvious effect, as mentioned, the oil compositions can be complex. Therefore, it should be investigated in future studies the effect of specific fatty acids on delivery to the brain and anatomical brain regions, as this work demonstrates that the lipid-based formulation does impact CBD distribution within the brain.

4.4.10 Assessment of biodistribution by 3D OrbiSIMS analysis

The biodistribution of CBD in the brain was also studied by 3D OrbiSIMS analysis at 2 h following oral administration in the sesame oil formulation. The brain distribution was assessed by homogenate samples, to compare to HPLC-UV analysis, and brain sections. Firstly, Figure 4.6 shows a comparison of the two analytical techniques. The whole brain was the only sample that demonstrated no difference between the expected CBD $[M-H]^-$ peak intensity, and that measured in the homogenate sample. In the temporal lobe sample, the measured peak intensity was lower than expected, and in the frontal lobe and cerebellum the CBD $[M-H]^-$ peak was not detected at all.

The calibration curve of CBD $[M-H]^-$ peak intensities demonstrated detection by the 3D OrbiSIMS with a CBD limit of detection (LOD) of 10 ng/g. Therefore, as the CBD concentration in the tissue samples was around 100 - 150 ng/g (Table 4.3), it was expected the CBD $[M-H]^-$ peak would have been detected. The

inconsistency of detection by 3D OrbiSIMS between the calibration curve analysis and samples, and between the samples themselves, suggest that this technique is not a good choice for biodistribution studies. Additionally, the differences in the brain regions between the expected CBD $[M-H]^-$ peak intensities and that measured, demonstrate that the technique should not be used to quantify CBD presence in tissue samples. The performance could be different in other matrices, for example quantification in cells may be possible, and with other therapeutics, as the instrument can be optimised to detect the molecule of choice.

Figure 4.7 demonstrates the difference in the CBD $[M-H]^-$ peak intensities between the homogenate and section brain samples. When the peak was detected in both samples (in the temporal lobe), there was no difference between the peak intensities. Therefore, the CBD $[M-H]^-$ peak intensity in the temporal lobe region of the brain section was also significantly smaller than expected. In the frontal lobe and cerebellum regions of the brain section, the CBD $[M-H]^-$ was detected, at similar intensities to the temporal lobe region. For the frontal lobe, the peak intensities in the tissue section were smaller than expected. However, for the cerebellum, the measured peak intensity was not significantly different to that expected.

The analysis by 3D OrbiSIMS performed herein was inconsistent between the tissue types, and with the HPLC-UV analysis. HPLC-UV is a reliable method to quantify CBD in animal samples [195], and therefore, it can be concluded that the inconsistencies reflect on the performance of the 3D OrbiSIMS in this setting. Chapter 3 demonstrated the use of 3D OrbiSIMS analysis to investigate the anti-cancer mechanism of action of CBD. In this scenario, the technique was not used to quantify CBD, and was therefore useful in gaining an understanding of the activity. There are useful applications of the technique, however this work suggests that quantifying biodistribution is not a strength of the instrument.

4.5 Conclusions

The work presented in this chapter demonstrates the differences in delivery concentration-time profiles between the specific anatomical brain regions. This highlights the need to study the delivery of a molecule to the desired tissue region, as information could be missed from studying the tissue as one sample. In this work, CBD was found to be delivered in high concentrations for short periods of time to certain regions (olfactory bulb, striatum), and delivered at lower concentrations consistently over a long time to others (medulla pons, cerebellum).

High, prolonged delivery of CBD to the rat brain was observed following oral administration in both lipid-free and lipid-based formulations. A $T_{\max}$ of only 1 h was observed for all formulations in all brain regions, to the best of our knowledge this is the first time such fast delivery of CBD to the brain has been reported. All formulations studied resulted in brain-plasma ratios ≥ 3.5 , demonstrating the high delivery to the brain, compared to the plasma concentrations. The second concentration peaks in concentration-time profiles of tissues towards the rostral area of the brain suggest that there could be two mechanisms of CBD delivery to this region.

This chapter also demonstrates that the lipid of choice for the formulation does impact the delivery. The sesame oil formulation often produced the highest delivery and exposure of CBD in the rostral area of the brain, whereas the lipid-free formulation produced higher delivery towards the caudal area of the brain. As discussed, the fatty acid content of the lipids may impact their delivery. Oleic acid is reported to increase the permeability of the BBB, possibly contributing to the high and prolonged delivery observed following administration in the sesame and rapeseed oil formulations. The coconut oil formulation often resulted in the lowest CBD delivery, suggesting that the myristic acid content of the oil did not aid delivery. However, any impact of fatty acids on delivery is speculative, and studies of administration in the fatty acid alone should be performed to investigate these effects further.

The application of the 3D OrbiSIMS to study the biodistribution of CBD in brain tissue was found to not be a reliable technique to use for this therapeutic. The

peak intensities could not be used to quantify the CBD present in the sample, as they were inconsistent with that expected from the reliable HPLC-UV analysis. However, the 3D OrbiSIMS can be useful for qualitative analysis, and proved so in the work in Chapter 3 to investigate the anti-cancer activity of CBD.

CHAPTER 5: Delivery of cannabidiol to anatomical brain regions for the treatment of multiple sclerosis

5.1 Introduction

Cannabidiol (CBD) is the most abundant non-psychoactive phytocannabinoid of the *Cannabis sativa* plant. CBD has a vast therapeutic potential with reports including anti-inflammatory, anti-anxiety, anti-depressant, and anti-cancer activity [103-107]. The most abundant phytocannabinoid, Δ^9 -tetrahydrocannabinol (THC), also exhibits extensive therapeutic potential, however the psychoactivity of the molecule has encouraged research to focus on CBD [101, 102].

Chapter 3 demonstrated the anti-cancer properties of CBD against glioblastoma multiforme (GBM) *in vitro*. CBD effectively inhibited the GBM cell growth both alone and in combination with imidazotetrazine agents. CBD was also demonstrated to overcome the major resistance mechanism to the standard of care treatment for GBM (temozolomide (TMZ)), over-expression of *O*⁶-methylguanine-deoxyribonucleic acid (DNA) methyltransferase (MGMT), to the best of our knowledge, this is the first time this has been reported [94].

CBD is also reported to exhibit immunosuppressant effects potentially beneficial for the treatment of multiple sclerosis (MS). MS is a complex, chronic, autoimmune disease that affects the central nervous system (CNS) and is one of the most common causes of disability in younger adults. In MS, the immune system attacks the myelin and axons in the brain and spinal cord [12, 13]. Due to the location of the disease in the CNS, there are a range of symptoms associated with MS including problems with balance, movement, vision, and cognition. The cause of MS is not entirely understood, although it is thought there are several potential causes including both environmental and genetic factors, and there is mounting evidence supporting the role of Epstein-Barr virus as a trigger of MS [13, 268]. Currently, there is no cure for MS, however, some treatments (such as corticosteroids) can help to manage the symptoms [12].

Many of the observed effects of CBD *in vivo* are a result of activity in the brain, and therefore delivery to the brain is understood to occur. However, there are few reports assessing CBD delivery to the brain *in vivo* following oral administration [152, 168, 231]. To the best of our knowledge, Chapter 4 of this thesis is the first to report CBD delivery to specific anatomical brain regions *in vivo*. It was demonstrated that oral administration of CBD resulted in high delivery to all brain regions as rapidly as 1 h post administration to rats (Figure 4.4). Delivery to distinct brain regions varied depending on the formulation used, and the lipid chosen also impacted the delivery concentration-time profile and exposure of the tissue to CBD. Both the lipid-free and sesame oil formulations resulted in high delivery to several anatomical brain regions.

The biodistribution of CBD to the brain following oral administration in an olive oil formulation has not been studied in detail. One time point of CBD administered in the olive oil formulation to rats was analysed (plasma T_{max} , 2 h), shown in Chapter 4, Figures 4.1 and 4.2. The olive oil formulation was found to deliver a high concentration (compared to the plasma) to the temporal lobe, but not to other brain regions, and so was not selected for the full biodistribution studies in rats. However, recent work from our group shows the high bioavailability of CBD after oral administration to rats in an olive oil formulation [151]. The work also showed high delivery to the lymphatic system following administration in the olive oil formulation ([151], unpublished work).

MS is commonly studied *in vivo* in mice models. Experimental autoimmune encephalomyelitis (EAE) is a commonly used model in female mice. Females are used due to reports that the symptoms exhibited are more severe than in male mice [269, 270]. The *in vivo* MS model used and the method of induction in the mice, affects how the disease progresses. The method used for EAE induction in this work is described in Chapter 2, section 2.4.5, but briefly, myelin oligodendrocyte protein (MOG₃₅₋₅₅) in complete Freund's adjuvant (CFA) and pertussis toxin were administered by subcutaneous (s.c.) injection. Induction by MOG₃₅₋₅₅ results in consistent infiltration to the brain throughout the disease, with localisation in the hippocampus and cerebellum [266, 267].

CBD has been reported to exhibit therapeutic effects against the *in vivo* EAE model in mice [119-123]. CBD has been shown to increase immunosuppressive myeloid-derived suppressor cells (MDSCs), inhibiting T cell migration [120, 124]. Additionally, administration of CBD resulted in reduced clinical scoring severity, demyelination and inflammation [123]. After oral administration of CBD in corn oil to EAE mice once symptoms were observed, a suppressed neuroinflammatory immune response was reported, demonstrated by reduced MOG₃₅₋₅₅ specific T cells [119]. Reduced MOG₃₃₋₅₅ specific T cell movement to the spinal cord was not found to be dependent on CB1 and CB2 receptors [122, 124]. Intraperitoneal (i.p.) administration of CBD with THC has also been demonstrated to alter the gut microbiome, a change which reduced the lipopolysaccharide (LPS) levels in the brain that are usually seen in EAE mice [121]. These reports study the effects of CBD after administration from the onset of symptoms. The disease would begin progressing before symptoms are observed, and it is not yet known what the immunosuppressive effects of CBD would be before the onset of symptoms.

The lymphatic system is involved in the development of EAE in mice, as Th17 cells and neutrophils move from the lymphatic system into the CNS, driving the disease [271-273]. Therefore, it could be beneficial to consider targeted treatment to the lymphatic system. The delivery of CBD to the lymphatic system following oral administration has previously been demonstrated in rats, where high delivery was observed after administration in lipid-based vehicles [151, 155, 156, 169].

The work in this chapter investigates the effect of increased lymphatic and brain delivery of CBD has on the development and progression of the clinical symptoms in the *in vivo* EAE model. The delivery of CBD to the mouse brain was first studied following oral administration in lipid-based and lipid-free vehicles. This is also studied in the anatomical regions of the hippocampus and the cerebellum, prominent regions adversely affected in the EAE model. The immunosuppressive effects of CBD in the *in vivo* MS model were then studied. It is hypothesised that increased delivery of CBD to the lymphatic system following administration in the lipid-based formulations could enhance the immunosuppressive effects.

5.2 Experimental design

The results discussed in this chapter were generated using methods described in Chapter 2. The biodistribution of CBD was assessed in mice by the method described in section 2.4.4. The determination of CBD concentrations in the tissue was performed by means of HPLC-UV, as described in section 2.5.1, based on Zgair *et al* [195]. The efficacy of CBD against an *in vivo* mouse MS model, EAE, was assessed as described in section 2.4.5.

5.3 Results

5.3.1 Biodistribution of cannabidiol to the brain in mice

Before assessing the immunosuppressive effects of CBD in the *in vivo* MS model, the biodistribution of CBD to the brain was first studied in mice. Chapter 4 demonstrated the delivery of CBD to the brain of rats, but this preparatory study in mice was performed in order to confirm delivery to mouse brains. The dose was higher in mice, at 30 mg/Kg (compared to 12 mg/Kg in rats) in preparation for the higher dose administered to the mice in the EAE study. The human equivalent dose (HED) of 30 mg/Kg in mice, for a 62 Kg human adult would be 2.4 mg/Kg, shown in Equations (4) and (5) [274, 275]. CBD is generally considered safe and has been reportedly administered to humans safely at doses of 6000 mg/Kg in safety clinical trials [148]. Therefore, the 30 mg/Kg dose in mice is a low range dose of CBD when scaled up to humans (HED 2.4 mg/Kg).

$$HED = Animal\ dose \times \left(\frac{Animal\ weight}{Human\ weight} \right)^{0.33} \quad \text{Equation (4)}$$

$$HED = 30\ mg/Kg \times \left(\frac{0.03\ Kg}{62\ Kg} \right)^{0.33} = 2.4\ mg/Kg \quad \text{Equation (5)}$$

The sesame and olive oil vehicles were the lipid-based formulations chosen to compare to the lipid-free vehicle. As discussed, the sesame oil formulation produced high delivery of CBD to the rat brain, demonstrated in Chapter 4. The olive oil formulation was used due to the high delivery of CBD to the lymphatic system that was observed after oral administration to both rats [151] and mice (unpublished work) in our recent studies, compared to other lipid-based vehicles. Due to the mechanism of EAE development in the model [271-273], it was hypothesised that increased delivery to the lymphatic system could enhance the effects of CBD.

The biodistribution of CBD to the whole brain was compared to serum concentrations, rather than plasma, as the blood samples were collected after the

animals were sacrificed. While the blood was collected as quickly as possible, there are reported changes in distribution post-mortem [276-279]. Few and short time points were used for this work, as the aim was to establish delivery to the brain tissue, therefore it was not necessary to use more animals for further studies once delivery had been demonstrated.

Figure 5.1 shows the comparison of CBD concentration in the whole brain to the serum concentration, and the pharmacokinetic (PK) parameters of this data are shown in Table 5.1. Figure 5.1 shows that the lipid-free vehicle is the only formulation to produce increased CBD concentrations in the whole brain, in comparison to the serum. Both the sesame and olive oil formulations resulted in whole brain CBD concentrations not significantly different to the serum levels at most time points, and lower than the serum concentration at 0.5 h after administration in the olive oil formulation. This is highlighted particularly by the brain-serum ratio, both the sesame and olive oils formulations resulted in ratios <1 , whereas the lipid-free formulation resulted in a ratio of 7.1 (Table 5.1). However, this is likely the result of low levels of CBD in the serum following administration in the lipid-free formulation, as the C_{max} and AUC_{0-2} in the whole brain were similar for all formulations. All three formulations appear to produce similar concentration-time profiles in the whole brain, with a relatively consistent CBD concentration.

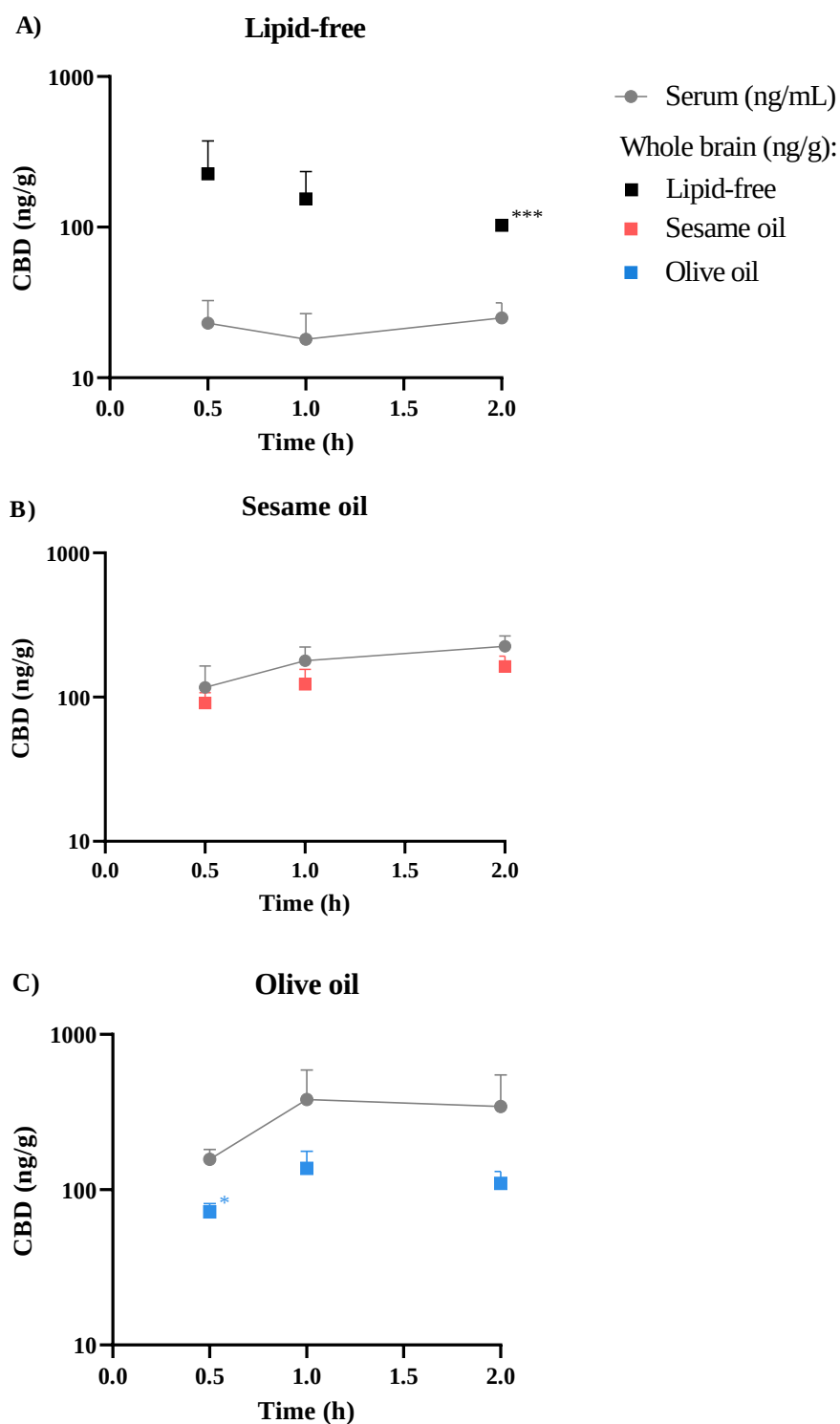


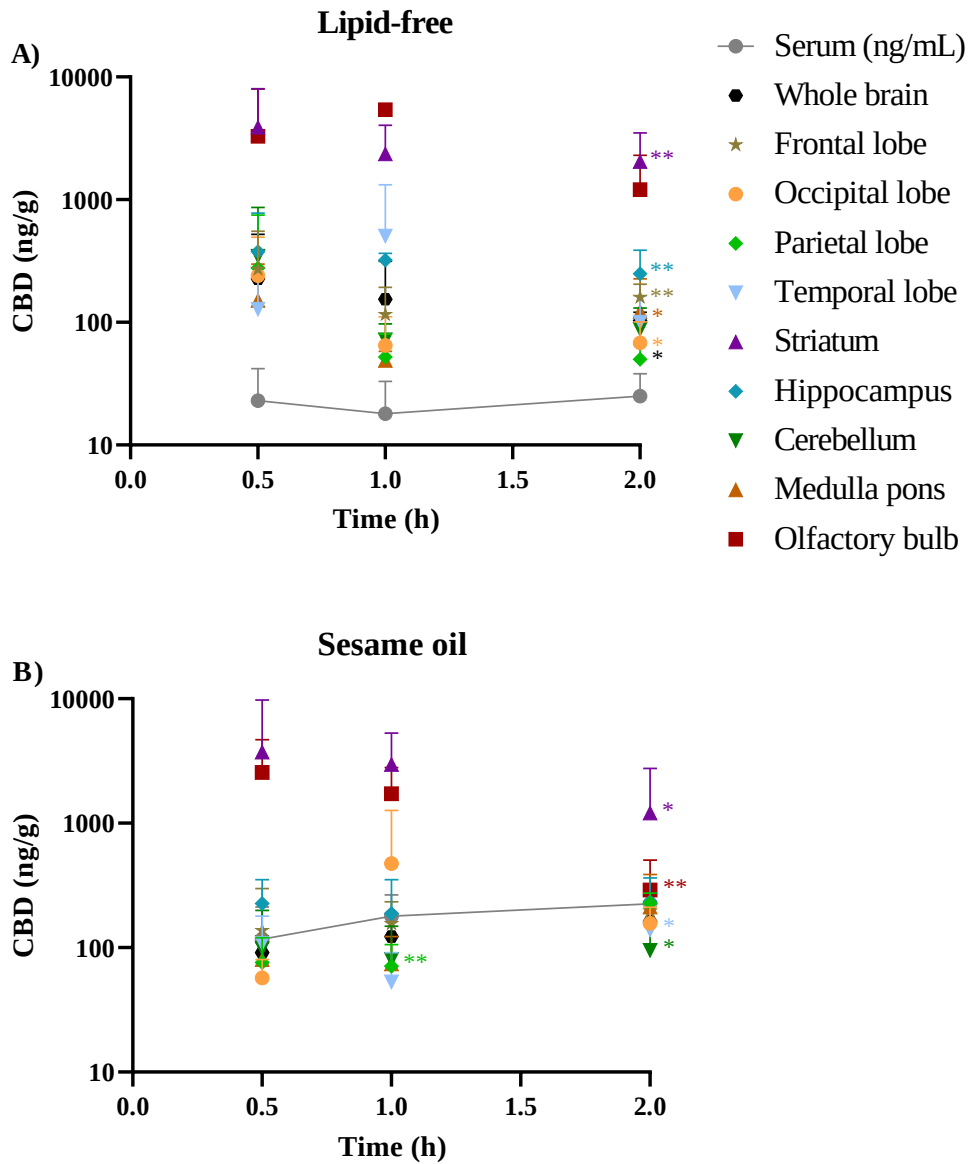
Figure 5.1. CBD concentration in the whole brain vs. serum following oral administration to mice (30 mg/Kg) in the A) lipid-free, B) sesame oil and C) olive oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, *** = $p < 0.001$. Data are presented as mean $\pm$ standard deviation (SD), $n = 4$. The PK parameters of the data are presented in Table 5.1.

Table 5.1. PK parameters of CBD concentration-time profiles in serum and whole brain following oral administration to mice (30 mg/Kg). One-way ANOVA with Dunnett's multiple comparisons was performed for differences between the lipid-free and lipid-based formulations, $\alpha = 0.05$. Data are presented as mean $\pm$ SD, n = 4, and are shown in Figure 5.1.

Formulation		T _{max} (h)	C _{max} (ng/mL or ng/g)	t _{1/2} (h)	AUC ₀₋₂ (h*ng/mL or h*ng/g)	Brain- serum ratio
Lipid-free	Serum	2.0	25 $\pm$ 13	-	38 $\pm$ 24	7.1
	Whole brain	0.5	226 $\pm$ 294	1.4	271 $\pm$ 254	
Sesame oil	Serum	2.0	225 $\pm$ 82	-	305 $\pm$ 51	0.7
	Whole brain	2.0	163 $\pm$ 59	-	219 $\pm$ 41	
Olive oil	Serum	1.0	381 $\pm$ 420	-	536 $\pm$ 102	0.3
	Whole brain	1.0	137 $\pm$ 80	-	164 $\pm$ 89	

Chapter 4 demonstrated that the CBD concentration-time profiles in rats are different in anatomical regions of the brain, compared to the whole brain. As discussed, EAE infiltration into the mouse brain is seen particularly in the hippocampus and cerebellum in this model [266, 267]. Therefore, it was important to assess the delivery of CBD to these anatomical brain regions, as well as the whole brain. Figure 5.2 shows the comparison of the CBD concentration in the anatomical brain regions to the serum concentration. CBD concentrations were found to be higher in the striatum than in the serum 2 h following administration in all formulations. Concentrations were also found to be higher in the hippocampus, frontal lobe, medulla pons and occipital lobe 2 h after administration in the lipid-free vehicle. At 2 h following administration in the sesame and olive oil formulations, the CBD concentrations in the olfactory bulb were also higher than in the serum. However, concentrations in the parietal lobe and cerebellum were found to be below those in the serum after administration in the sesame and olive oil formulations. The concentration in the

occipital lobe was also lower than the serum at 2 h post administration in the olive oil formulation.



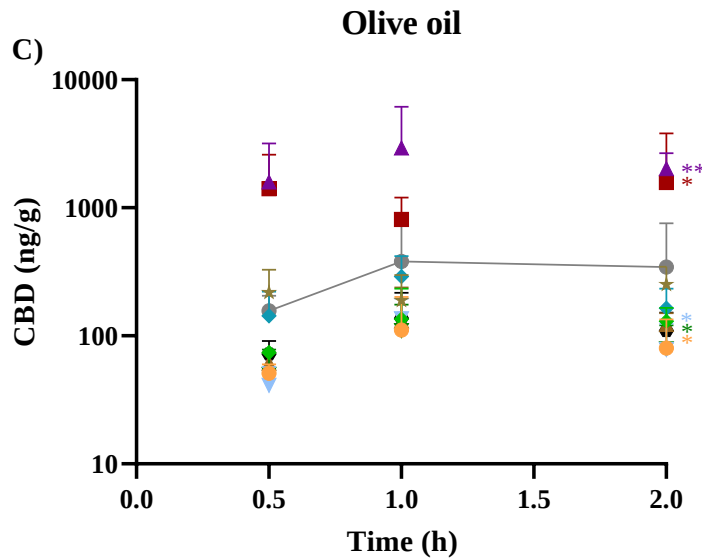


Figure 5.2. CBD concentration in anatomical brain regions vs. serum following oral administration to mice (30 mg/Kg) in the A) lipid-free, B) sesame oil and C) olive oil formulations. One-way ANOVA with Dunnett's multiple comparisons were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$. The data are presented as mean $\pm$ SD, $n = 4$.

Figure 5.3 shows the same data as Figure 5.2 but comparing delivery in each formulation to the anatomical brain regions relevant to EAE progression. Delivery in all formulations was similar to both the hippocampus and cerebellum anatomical brain regions. The only difference was found 0.5 h post administration to the cerebellum, where the lipid-free vehicle resulted in the highest CBD concentration. The T_{max} was clearly 0.5 h in the cerebellum following administration in the lipid-free formulation. Administration in the other vehicles and to the hippocampus produced consistent exposure of the tissue to CBD.

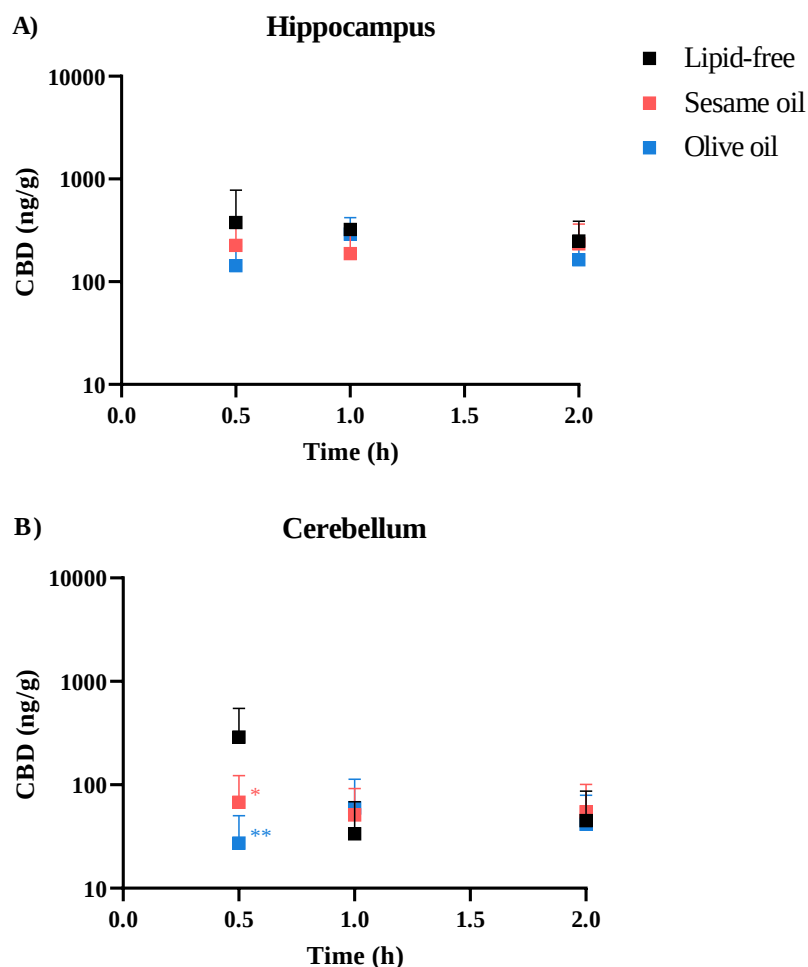


Figure 5.3. CBD concentration in anatomical brain regions A) hippocampus, and B) cerebellum following oral administration to mice (30 mg/Kg) in the lipid-free, sesame oil and olive oil formulations. One-way ANOVA with Dunnett's multiple comparisons were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$. The data are presented as mean $\pm$ SD, $n = 4$. PK parameters of the data are presented in Table 5.2.

Table 5.2. PK parameters of CBD concentration-time profile in serum, whole brain and anatomical brain regions following oral administration (30 mg/Kg) to mice in lipid-free, sesame and olive oil formulations. One-way ANOVA with Dunnett's multiple comparisons were performed, $\alpha = 0.05$, no significant differences were found. The data are presented as mean $\pm$ SD, n = 4.

Formulation		T _{max} (h)	C _{max} (ng/mL or ng/g)	t _{1/2} (h)	AUC ₀₋₂ (h*ng/mL or h*ng/g)
Lipid-free	Serum	2.0	25 $\pm$ 13	-	38 $\pm$ 24
	Whole brain	0.5	226 $\pm$ 294	1.4	271 $\pm$ 254
	Hippocampus	0.5	376 $\pm$ 402	2.5	541 $\pm$ 243
	Cerebellum	0.5	347 $\pm$ 515	0.9	245 $\pm$ 222
Sesame oil	Serum	2.0	225 $\pm$ 82	-	305 $\pm$ 51
	Whole brain	2.0	163 $\pm$ 59	-	219 $\pm$ 41
	Hippocampus	2.0	234 $\pm$ 130	-	360 $\pm$ 117
	Cerebellum	0.5	98 $\pm$ 101	-	152 $\pm$ 56
Olive oil	Serum	1.0	381 $\pm$ 420	-	536 $\pm$ 102
	Whole brain	1.0	137 $\pm$ 80	-	164 $\pm$ 89
	Hippocampus	1.0	291 $\pm$ 129	-	289 $\pm$ 152
	Cerebellum	1.0	110 $\pm$ 65	-	131 $\pm$ 72

5.3.2 Efficacy of cannabidiol in experimental autoimmune encephalomyelitis (EAE) mice model

The potential of the immunosuppressive effects of CBD to reduce or delay the progression of MS symptoms was studied in an EAE mouse model. EAE was induced by s.c. injection of MOG₃₅₋₅₅ in CFA and pertussis toxin into female mice. Differences between male and female mice have been reported, with female mice reported to exhibit more spinal cord infiltration of the disease and demyelination than male mice [269, 270]. The dose of CBD was raised to 50 mg/Kg in the ~20 g mice in which EAE was induced, in order to maximise the immunosuppressive effects of CBD that may be observed in this preliminary study. The HED of 50 mg/Kg in 20 g mice for a 62 Kg human adult would be 3.5 mg/Kg, shown in Equation (6), using Equation (4) [274, 275]. As mentioned above, this is still a low range dose, considered safe in humans [280].

$$HED = 50 \text{ mg/Kg} \times \left(\frac{0.02 \text{ Kg}}{62 \text{ Kg}} \right)^{0.33} = 3.5 \text{ mg/Kg} \quad \text{Equation (6)}$$

The clinical scoring of the mice was measured as reported in Chapter 2, section 2.4.5 over 28-days until the end of the study, or until animals reached the maximum allowed disease severity and were humanely sacrificed. The average clinical scoring of the mice for each dosing group is shown in Figure 5.4. Figure 5.4 shows that symptoms begun to appear at day 9 – 11. Figure 5.4 also shows that the CBD in olive oil group had a lower average clinical score than the olive oil only control group at the end of the study (day 28). However, presenting the data in this way does not demonstrate the number of mice surviving, and therefore the scores can be misleading. This is shown in Table 5.3, as the mice were not allowed to exceed clinical severity 4.0 and were therefore humanely killed as the predetermined study endpoint when they reached this score.

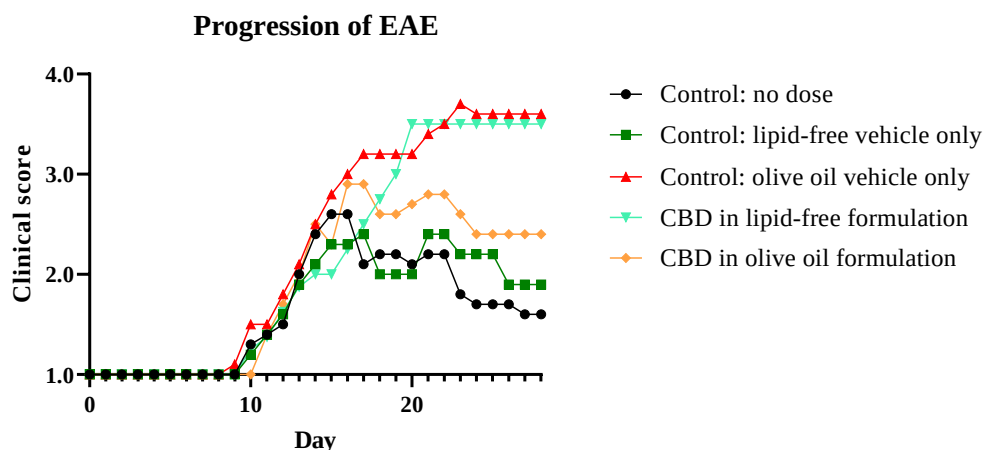


Figure 5.4. Progression of EAE as clinical score after no administration (control: no dose) or oral administration of the CBD in lipid-free or olive oil formulations, or vehicle only. When administered, CBD was dosed at 50 mg/Kg in formulations of 50 mg/mL. Animals were dosed once daily for 8-days. The data are presented as the mean clinical score.

Table 5.3 shows the percentage of mice in each group that reached the highest clinical score, 4.0. The CBD in the lipid-free formulation group had the highest percentage of mice reaching clinical score 4.0, however there was a reduced sample number in this group due to traumatic oral gavages. In the olive oil vehicle only control group, 66.7% mice reached score 4.0. For the CBD in olive oil formulation group, only 16.7% mice reached score 4.0. Comparing the percentage of animals surviving, only 33.3% of mice survived to day 28 in the olive oil vehicle control, but 83.3% survived in the CBD in olive oil formulation group.

Table 5.3. EAE severity as percentage of mice reaching score 4.0 after no administration (control: no dose) or oral administration of CBD in lipid-free or olive oil formulations, or vehicle only. When administered, CBD was dosed at 50 mg/Kg in formulations of 50 mg/mL. Animals were dosed once daily for 8-days.

	Percentage of animals reaching the highest clinical severity score (4.0)	Sample number (n)
Control: no dose	16.7%	6
Control: lipid-free vehicle only	16.7%	6
CBD in lipid-free formulation	75.0%	4
Control: olive oil vehicle only	66.7%	6
CBD in olive oil formulation	16.7%	6

5.4 Discussion

This chapter demonstrates the investigation of the immunosuppressive effects of CBD in an *in vivo* MS model. First, the biodistribution of CBD to the mouse brain following oral administration in lipid-based and lipid-free formulations was studied. MS is a condition of the CNS, located in the spinal cord and brain in humans, including the cerebellum and hippocampus [12, 13]. The location varies with *in vivo* models, for the model used in this work, EAE, the disease begins in the spinal cord and progresses into the brain. Therefore, it was important to demonstrate delivery to the mouse brain following oral administration, and specifically to the hippocampus and cerebellum brain regions, where the disease is predominantly located in this model [266, 267].

Chapter 4 demonstrated the difference in the delivery concentration-time profiles of CBD to the brain and anatomical brain regions following oral administration to rats in different lipid-based formulations. Therefore, it was considered that the sesame and olive oil formulations may produce different delivery profiles. Table 5.4 shows that the fatty acid composition of the two oils is similar in terms of chain length, but olive oil contains more oleic acid and less linoleic acid than sesame oil. If the oleic acid content impacts the delivery, as is suggested by the reports in the literature [66], then it would be expected that the olive oil formulation would result in higher CBD delivery to the brain than the sesame oil formulation. Additionally, there are reports of a role for *n*-3 and *n*-6 fatty acids, such as linoleic acid, in treating MS [281]. The doses used herein may be too small to see effects of the linoleic acid content of the formulations (doses of ≥ 5 mg/day were given to humans in clinical trials [281]), but there is potential the lipid-based formulations may offer additional therapeutic benefit against MS.

Table 5.4. Fatty acid composition of the oils selected for the lipid-based formulations for *in vivo* CBD biodistribution studies, taken from the literature [151].

	Fatty acid		Sesame oil	Olive oil
Saturated	Octanoic acid	C8:0		
	Decanoic acid	C10:0		
	Lauric acid	C12:0		
	Myristic acid	C14:0		
	Palmitic acid	C16:0	13%	12%
	Stearic acid	C18:0	8%	8%
Unsaturated	Oleic acid	C18:1 cis-9	44%	72%
	Linoleic acid	C18:2 cis-9,12	30%	5%
	Linolenic acid	C18:3 cis-9,12,15	<1%	<1%

Following oral administration, it is interesting that only the lipid-free formulation resulted in a brain-serum ratio >1 (7.1, Table 5.1). The sesame and olive oil formulations both resulted in brain-serum ratios <1, with the ratio the smallest for the olive oil formulation at 0.3. However, it is important to note here that the length of the study was only 2 h, as it is not known what happens at later time points, the findings only apply to the first 2 h following oral administration.

Table 5.1 shows the PK parameters for the data from studying the brain as one tissue. The $C_{\max}$ and AUCs in the whole brain are not significantly different across the formulations. This suggests that no immediate effect of oleic acid content in the formulation is observed, unlike that reported by Sztriha *et al* following intravenous (i.v.) injection, where the effect on the BBB had occurred and reversed back to normal after 90 mins [66]. As discussed in Chapter 4, the work in this thesis used oral administration, and therefore, due to the digestion process, any effects observed from fatty acids in the formulation are likely to be seen later than those reported after i.v. injection.

The lipid-free vehicle was the only formulation to produce a CBD concentration in the whole brain significantly higher than in the serum, observed at 2 h post administration, likely the result of low levels of CBD in the serum (Figure 5.1). The olive oil formulation resulted in a CBD concentration lower than the serum at 0.5 h following administration. The sesame oil formulation and other time points showed CBD concentrations in the brain not significantly different to the serum. Chapter 4 demonstrated the need to study biodistribution to specific anatomical brain regions, and therefore this was also performed herein.

Figure 5.2 demonstrates that at 2 h after administration in the lipid-free vehicle, CBD concentrations were higher than the serum in the striatum, hippocampus, frontal lobe, medulla pons and occipital lobe. Following administration in the sesame and olive oil formulations, this was only true of the olfactory bulb and striatum. In fact, CBD concentrations in the parietal lobe and cerebellum were below the serum at 2 h following administration in both lipid-based formulations. However, comparing the actual concentrations, these are similar for the brain regions in all formulations, demonstrated in Figure 5.3. Therefore, although the lipid-free vehicle produced higher delivery of CBD to the brain, compared to the serum, this did not result in higher concentrations of CBD in the brain.

Figure 5.3 highlights the delivery of CBD to the hippocampus and cerebellum following oral administration in the lipid-based and lipid-free formulations. In the hippocampus, delivery by all formulations is consistent up to 2 h, and no significant differences in CBD concentrations were observed after administration in the different formulations. In the cerebellum, administration in the lipid-free formulation resulted in the highest CBD concentration 0.5 h post administration but was then similar to the lipid-based vehicles at 1 and 2 h. This is similar to the concentration-time profiles observed following oral administration to rats, shown in Chapter 4, Figure 4.5. In rats, the lipid-free formulation produced the highest $C_{\max}$ at 1 h, and then was similar to the lipid-based formulations at the later time points. It was observed in Chapter 4 that following oral administration to rats, the lipid-free formulation produced the highest CBD AUC in the caudal regions of the brain. This may also be true in mice, as Figure 5.3 suggests, however the time points in mice are too few and

too short to reliably draw this conclusion, and more studies should be conducted to do so.

EAE was induced in the mice by s.c. injection of MOG₃₅₋₅₅ in CFA and pertussis toxin into the hind legs, as described in Chapter 2, section 2.4.5. Th17 cells play an important role in the development of EAE. They can move from the lymph nodes, particularly the inguinal nodes which drain the injection site, into the CNS, driving the disease as well as influencing neutrophil movement into the CNS [271-273]. The method used to induce EAE in mice has been shown to affect the movement of the disease into the brain. Induction by MOG₃₅₋₅₅ results in consistent infiltration to the brain throughout the disease, localised in the hippocampus and cerebellum, similar to common localisation observed in humans [266, 267].

The lipid-free and olive oil formulations were selected for the study of CBD efficacy in the *in vivo* EAE model due to the high delivery of CBD to the lymphatic system following administration in the olive oil formulation in rats [151, 169] and mice (unpublished work). As mentioned, in the EAE model, Th17 cells migrate from lymph nodes into the CNS to drive the disease [271-273]. Therefore, it was hypothesised that higher delivery to the lymphatic system could increase the immunosuppressive effects of CBD [120, 122]. Additionally, Figures 5.1 and 5.3 show that delivery of CBD to the brain and anatomical brain regions, the hippocampus and cerebellum, was similar following administration in both the sesame and olive oil formulations.

Figure 5.4 shows the clinical scoring of the mice over the duration of the study, demonstrating that symptoms began to appear at day 9 - 11 in all groups. Comparing the day symptoms were first observed in each group, the olive oil vehicle only control group exhibited symptoms 1-day before the not dosed control, and CBD in olive oil 2-days later. To account for any effect of the vehicle, it is most beneficial to compare the CBD dosed groups with the vehicle dosed groups. The CBD in lipid-free group ended the study with a higher clinical score than the lipid-free vehicle dosed group. However, it is important to note the reduced n number in this group, as two mice were sacrificed before the onset of symptoms due to complications with oral gavage dosing. Therefore, it may be

more representative to compare the groups by the percentage of mice reaching the highest clinical score, 4.0, as shown in Table 5.3.

The group that showed the highest percentage of mice to reach clinical score 4.0 (75%) received CBD in the lipid-free formulation. However, as stated, the low n number in this group could affect these results. The olive oil formulation demonstrated promising immunosuppressive effects. Following administration of the olive oil vehicle control, 66.7% of mice reached clinical score 4.0, whereas for the CBD in olive oil group this was only 16.7%. This can also be represented by the percentage of animals surviving until day 28, 33.3% of the olive oil vehicle control group and 83.3% of the CBD in olive oil formulation group. This difference is indicative of the immunosuppressive effect of CBD following administration in the lipid-based olive oil formulation before the onset of symptoms [120, 122]. This difference was not observed after administration in the lipid-free formulation. Administration of low concentrations of CBD does not result in the immunosuppressive effects observed at higher concentrations [282]. Therefore, the reason the effect of CBD is not observed following administration in the lipid-free formulation could be because of low delivery to the lymphatic system ([151], unpublished work). Therefore, this work supports the hypothesis that higher delivery to the lymphatic system could demonstrate increased immunosuppressive effects. However, as mentioned, the n number in the CBD in lipid-free formulation group was smaller ($n = 4$), and the study would need to be repeated to report conclusive results.

Table 5.2 demonstrates that there were no significant differences in the PK parameters of the delivery of CBD to the mouse brain between the lipid-free and olive oil formulations. The only difference found is shown in Figure 5.3 where the lipid-free vehicle produced the highest CBD concentration in the cerebellum 0.5 h after administration. However, this did not result in a higher AUC (Table 5.2). Table 5.2 does show that the lipid-free formulation resulted in CBD delivery with higher variation than the olive oil formulation. It is important to note that the biodistribution was studied over 2 h, but the mice in the EAE study were dosed daily for 8-days. Additionally, in the EAE study, the mice were given pertussis toxin in order to increase BBB permeability. This likely means very different delivery to the brain occurred in these mice. This should not negate the

possible effect of increased lymphatic delivery of CBD on the immunosuppressive effects observed in this work. Rather, this demonstrates the need for further studies of the biodistribution of CBD in mice induced with EAE.

The mice numbers used in this study were low ($n = 6$), as this was preliminary work to study any effects of CBD as well as determine the dose and methods. The study should be repeated to confirm the differences observed. This could involve higher doses of CBD so that the effects may be observed more clearly, as discussed, the 50 mg/Kg is still well within the low dose range for humans when allometrically scaled (Equation (6)), and reports in the literature have safely orally administered CBD at 3500 mg/Kg to mice [283]. It could also be interesting to study the effects of administration in the sesame oil formulation in future work, to compare any differences between the two lipid-based vehicles.

5.5 Conclusions

The delivery of CBD to the mouse brain following oral administration is demonstrated in both lipid-free and lipid-based formulations. Administration in the lipid-free vehicle was the only formulation to produce a brain-serum ratio >1 . However, biodistribution was only studied between 0.5 h and 2 h post administration to demonstrate a level of delivery. Chapter 4 demonstrated high exposure of some regions of the rat brain to CBD over 18 h after oral administration, and therefore, the PK parameters of CBD delivery to the brain in mice could be different if additional, later time points were used.

The MS model used herein, EAE in mice, causes constant infiltration of disease into the brain, with prominence in the hippocampus and cerebellum. Delivery of CBD to these anatomical brain regions following oral administration in the lipid-based and lipid-free formulations was demonstrated. Delivery was consistent in all formulations over the time period studied, apart from at 0.5 h, where the lipid-free formulation resulted in high delivery to the cerebellum.

The preliminary EAE study is indicative of the immunosuppressive effects of CBD. This can be seen in the differences between the olive oil formulation groups, where the group dosed with CBD had a lower percentage of animals that reached the highest clinical score, 4.0. This reduced clinical symptom severity was not observed following administration in the lipid-free formulation. If the immunosuppressive effects observed were a result of delivery of CBD to the brain, no difference between the formulations would be observed, as delivery to the brain was similar in both formulations. The olive oil formulation has previously been shown to result in increased lymphatic delivery of CBD, compared to the lipid-free formulation. Therefore, the work herein supports the hypothesis that high lymphatic delivery could increase the immunosuppressive effects of CBD. However, additional studies would be required to confirm this, including longer biodistribution studies in mice induced with EAE to further study the delivery of CBD to the brain and any correlation with the immunosuppressive effects observed.

CHAPTER 6: Biodistribution of 4'-fluoro-cannabidiol to the whole brain, anatomical brain regions, and other tissues following oral administration to rats

6.1 Introduction

4'-Fluoro-cannabidiol (4'-F-CBD) is a fluorinated derivative of the phytocannabinoid cannabidiol (CBD), first reported in 2016 by Breuer *et al* [176]. Breuer *et al* described the fluorination of cannabidiol (CBD) in three locations, at the C⁴-, C¹⁰- and C¹⁶-positions (Figure 1.12) [176]. Fluorination at the C⁴-position (4'-F-CBD, or HUF-101) resulted in significantly increased activity compared to CBD [176, 177]. In tests for anti-anxiety, antidepressant, antipsychotic and anti-compulsive activities, 4'-F-CBD resulted in higher effects than CBD after intraperitoneal (i.p.) injection at up to 10× lower dose. Breuer *et al* also reported that the anti-compulsive effects of 4'-F-CBD were prevented by pre-treatment with CB1 and CB2 antagonists, demonstrating that at least some activity is *via* the endocannabinoid system [176].

In 2017, Silva *et al* reported similar anti-nociceptive effects of 4'-F-CBD (HUF-101) to CBD after i.p. injection at a lower dose than CBD [177]. They also confirmed reports of the mechanism of 4'-F-CBD activity *via* the endocannabinoid system. Their findings corroborated with that of Breuer *et al* that some of the observed anti-nociceptive effects appeared to involve the CB1 and CB2 receptors, in the hot plate and carrageenan-induced inflammatory hyperalgesia tests [176, 177]. The difference in *in vivo* activity suggests that either 4'-F-CBD has increased efficacy at a molecular level, more 4'-F-CBD is delivered to the active site (the central nervous system (CNS) in these cases), or there is slower elimination of 4'-F-CBD [176, 177]. There has been one reported study of 4'-F-CBD *in vitro*, by Mithner *et al*, who reported a higher potency of 4'-F-CBD (HUF-101) compared to CBD in an Alzheimer's disease (AD) model [204]. Increased permeability of fluorinated compounds across the blood-brain barrier (BBB) and into the brain has previously been demonstrated for other molecules [173-175].

In 2021, Stollenwerk *et al* investigated the contribution of the adenosine 2A (A2A) receptor to CBD and 4'-F-CBD (PECS-101) activity *in vivo* following i.p. injection [180]. They found that 4'-F-CBD reduced exploratory behaviour in an elevated plus maze assay, and potentiated the cataleptic response to THC in a manner consistent with increased adenosine signalling [180]. In addition, 4'-F-CBD is reported to exhibit neuroprotective effects *in vivo* after i.p. injection [178].

To the best of our knowledge, the data described in Chapter 3 demonstrate the anti-cancer properties of 4'-F-CBD for the first time. 4'-F-CBD was found to inhibit glioblastoma multiforme (GBM) cell growth *in vitro*. 4'-F-CBD could also overcome the two common resistance mechanisms to the standard of care treatment, temozolomide (TMZ): O⁶-methylguanine- deoxyribonucleic acid (DNA) methyltransferase (MGMT) over-expression, and a deficiency of DNA mismatch repair (MMR). Like CBD, 4'-F-CBD also demonstrated synergy with TMZ and its analogue, T25. 4'-F-CBD (PECS-101) has also recently been reported to prevent chemotherapy-induced neuropathic pain following i.p. injection to mice [179]. Activity was found to be peroxisome proliferator-activated receptor gamma (PPAR γ) dependent, protective activity was exhibited *in vivo* if 4'-F-CBD was administered at the same time as the chemotherapeutic agent paclitaxel. Interestingly, 4'-F-CBD was found not to interfere with the anti-cancer activity of paclitaxel, and importantly, did not exhibit abuse potential [179].

The findings of Chapter 3, along with the reports of 4'-F-CBD activity *in vivo* in the literature [176-180], indicate vast therapeutic potential of 4'-F-CBD. The anti-cancer properties reported in Chapter 3 demonstrate similar activity of 4'-F-CBD to CBD *in vitro*, with no significant differences between the concentrations required to inhibit cell growth by 50% (GI₅₀). This suggests that the increased efficacy seen *in vivo* is likely a result of increased delivery to the brain, or slower elimination of 4'-F-CBD. However, the *in vivo* assays were behavioural, and *in vivo* biodistribution of 4'-F-CBD has not yet been reported. Therefore, the higher delivery of 4'-F-CBD to the site of action (the brain) compared to CBD has only been hypothesised.

Despite the therapeutic potential of the molecule, to the best of our knowledge, *in vivo* pharmacokinetics (PK) and biodistribution of 4'-F-CBD have not yet been reported. These are important to study for a potential therapeutic in order to understand the exposure in the circulation and delivery throughout the body following administration. Previous reports in the literature of 4'-F-CBD activity have called for the PK and biodistribution to be investigated in comparison to CBD, in order to understand if 4'-F-CBD exhibited increased efficacy, or if another factor, such as increased delivery, underlies the increased potency of the molecule [176, 177].

The work in this chapter will study the PK and biodistribution of 4'-F-CBD following oral administration. Biodistribution will be studied to anatomical brain regions, as well as other potentially therapeutically important organs and tissues. Chapter 4 describes the biodistribution of CBD to the brain following oral administration in lipid-free and lipid-based vehicles, where differences in delivery were observed between the formulations. The sesame oil formulation resulted in high delivery of CBD to the frontal, temporal and parietal lobes of the brain, all anatomical regions where GBM tumours are commonly located [90, 229, 230]. This was potentially a result of high oleic acid content of the sesame oil formulation, in comparison to other lipid-based formulations studied [151]. Therefore, the sesame oil formulation was selected, along with the lipid-free vehicle, for investigation of the PK and biodistribution of 4'-F-CBD following oral administration. The delivery of 4'-F-CBD to other tissues was also studied in order to understand the full body biodistribution of the molecule after oral administration. Additionally, comprehensive biodistribution data will be needed for physiological based PK (PBPK) modelling.

6.2 Experimental design

The results discussed in this chapter were generated using methods described in Chapter 2. *In vivo* studies were carried out in rats by oral gavage administration of 4'-F-CBD in both lipid-free and sesame oil formulations. The PK of 4'-F-CBD was assessed after its administration to rats by methods described in sections 2.4.1 and 2.4.2. The biodistribution was assessed by the method laid out

in section 2.4.3. The quantification of 4'-F-CBD in the plasma and tissues was assessed by a validated HPLC-UV method adapted from Zgair *et al* [195], as described in section 2.5.2. Non-compartmental analysis of the PK data was performed using Phoenix WinNonlin 6.3 Professional (Certera, NJ, U.S.A.). All other statistical analysis was performed using Prism version 9.3.1 (GraphPad, CA, U.S.A.).

6.3 Results

6.3.1 Pharmacokinetics study of 4'-fluoro-cannabidiol

The PK of 4'-F-CBD was studied up to 12 h following oral administration to rats. The time points were selected based on previous CBD PK studies [151, 155], as the PK profile of 4'-F-CBD was expected to be similar to that of CBD, and so that a comparison between the two cannabinoids could be made. A molar equivalent dose of 4'-F-CBD to that of CBD in previous studies [155] and Chapter 4 of this thesis (12 mg/Kg), 38 μ M/Kg, was used so that comparisons could be made directly.

Figure 6.1 shows the PK profile of 4'-F-CBD following oral administration in lipid-free and sesame oil formulations. Higher concentrations were observed after administration in the sesame oil formulation from 0.5 – 4 h. From 5 h onwards, the profiles for both formulations appeared similar, with concentrations close to the lower limit of quantification (LLOQ, 10 ng/mL) of the method at 12 h. The time to the maximum concentration (T_{max}) was observed to be similar for both formulations, at 3 - 4 h. The PK parameters, including the maximum concentration (C_{max}) and area under the curve (AUC) for both 4'-F-CBD and CBD are reported in Table 6.1.

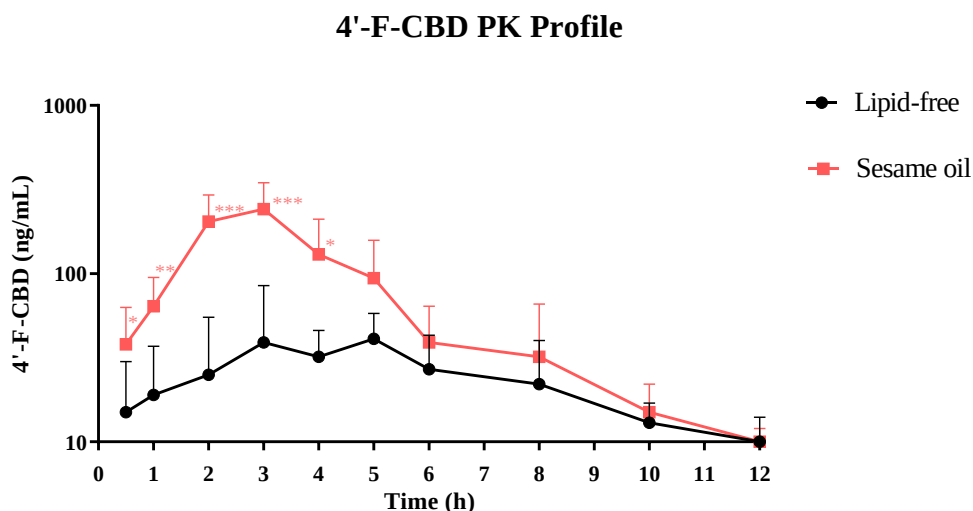


Figure 6.1. Plasma PK profile of 4'-F-CBD following oral administration at 12.7 mg/Kg (38 μ M/Kg) to rats in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. The data are presented as mean $\pm$ standard deviation (SD), where plasma $n = 6$. The PK parameters are presented in Table 6.1.

Table 6.1. PK parameters of 4'-F-CBD and CBD concentration-time profiles in plasma following oral administration at 38 μ M/Kg (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) to rats. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Differences are shown between the lipid-free and lipid-based formulations of the same cannabinoid, no differences were found between 4'-F-CBD and CBD administration in the same formulation. The data are also presented in Figures 6.1 and 6.2. The CBD PK data was calculated from plasma samples collected in a previous study (Feng *et al* [155]). The data are presented as mean $\pm$ SD, $n = 6$.

Formulation		T_{max} (h)	C_{max} (nM/mL)	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (h*nM/mL)
4'-F-CBD	Lipid-free	4	0.21 ± 0.09	3.6	1.01 ± 0.44
	Sesame oil	3	0.84 ± 0.23 ***	2.5	2.76 ± 0.86 **
CBD	Lipid-free	5	0.26 ± 0.01	2.3	1.13 ± 0.26
	Sesame oil	3	0.66 ± 0.38 *	1.5	2.75 ± 1.09 *

For both cannabinoids, the sesame oil formulation produced the higher C_{max} and $AUC_{0-\infty}$. However, comparing the two cannabinoids, the PK parameters were not significantly different. Figure 6.2 shows the data used to calculate the PK parameters in Table 6.1. Figure 6.2 demonstrates that significant differences in the plasma cannabinoid concentrations were only observed at 1 h post administration in the sesame oil formulation.

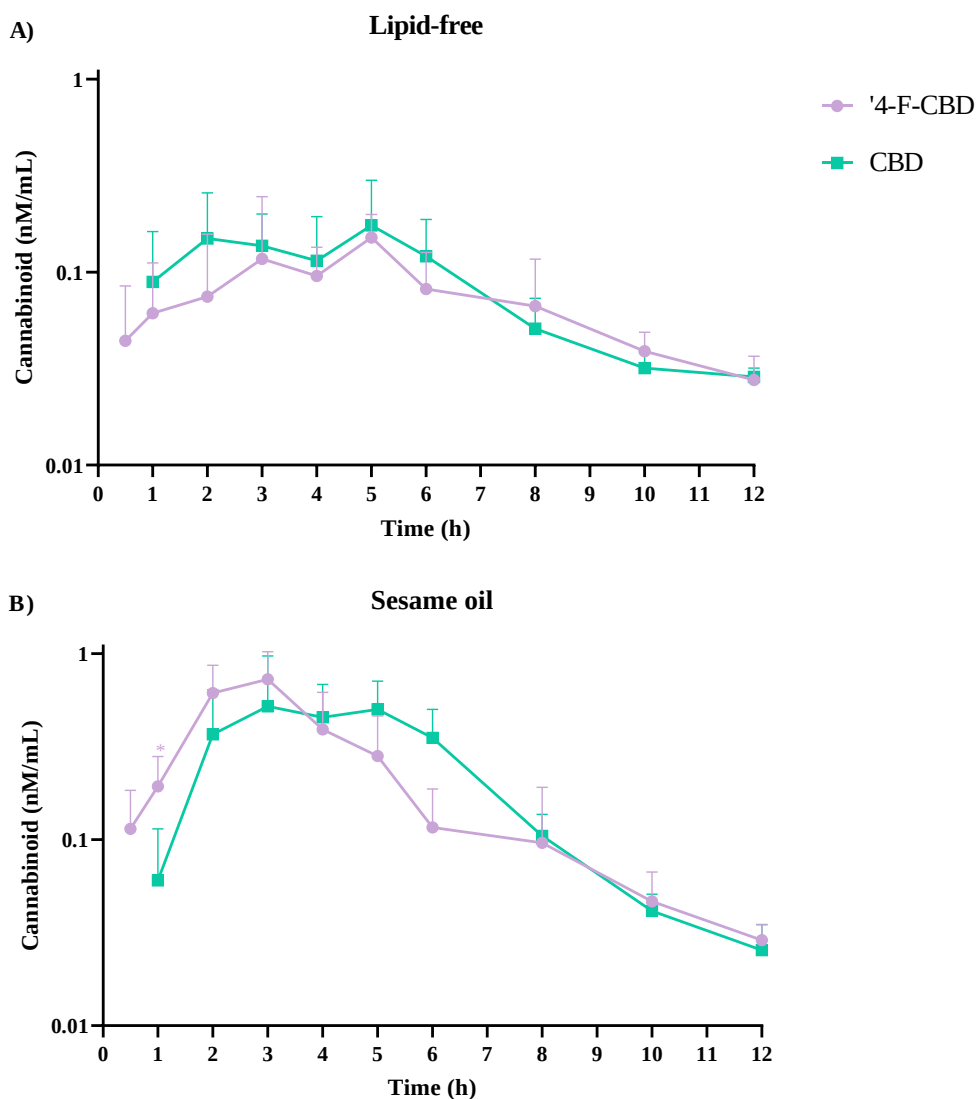


Figure 6.2. Plasma PK profiles of 4'-F-CBD compared to CBD following oral administration at 38 μ M/Kg (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) to rats in A) lipid-free and B) sesame oil formulations. The PK data for CBD was calculated from samples collected in previous study (Feng *et al* [155]). Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$. The data are presented as mean $\pm$ SD, $n = 6$. The PK parameters are shown in Table 6.1.

6.3.2 Biodistribution of 4'-fluoro-cannabidiol

6.3.2.1 Biodistribution of 4'-fluoro-cannabidiol to the brain

The PK studies demonstrated that 4'-F-CBD had similar plasma exposure to CBD (Table 6.1). The time points for the study of 4'-F-CBD biodistribution were therefore selected as the same as for the study of CBD biodistribution in rats (investigated in Chapter 4). This would allow comparison of delivery and exposure in tissues between the cannabinoids. This time range (1 – 18 h) was also expected to cover the T_{max} , based on the T_{max} of CBD in the brain at 1 h (Chapter 4), and the similar PK profiles of the molecules (Table 6.1).

Figure 6.3 shows the whole brain concentration of 4'-F-CBD following oral administration, compared to the plasma PK profile. The PK parameter data of 4'-F-CBD delivery to the whole brain is also presented in Table 6.2. Figure 6.3 shows that following administration in the lipid-free vehicle, the concentration of 4'-F-CBD in the whole brain was higher than the plasma concentration at all time points where plasma was collected (up to 12 h). After administration in the sesame oil formulation, a significantly higher 4'-F-CBD concentration was observed in the whole brain compared to the plasma at 1, 5, 8 and 12 h. At 2 and 3 h after administration in the sesame oil formulation, the 4'-F-CBD concentration was similar in the whole brain and plasma. Table 6.2 demonstrates that the T_{max} after administration in both formulations was 5 h. Also, there were no significant differences in the whole brain exposure to 4'-F-CBD or C_{max} following administration in both formulations (Table 6.2).

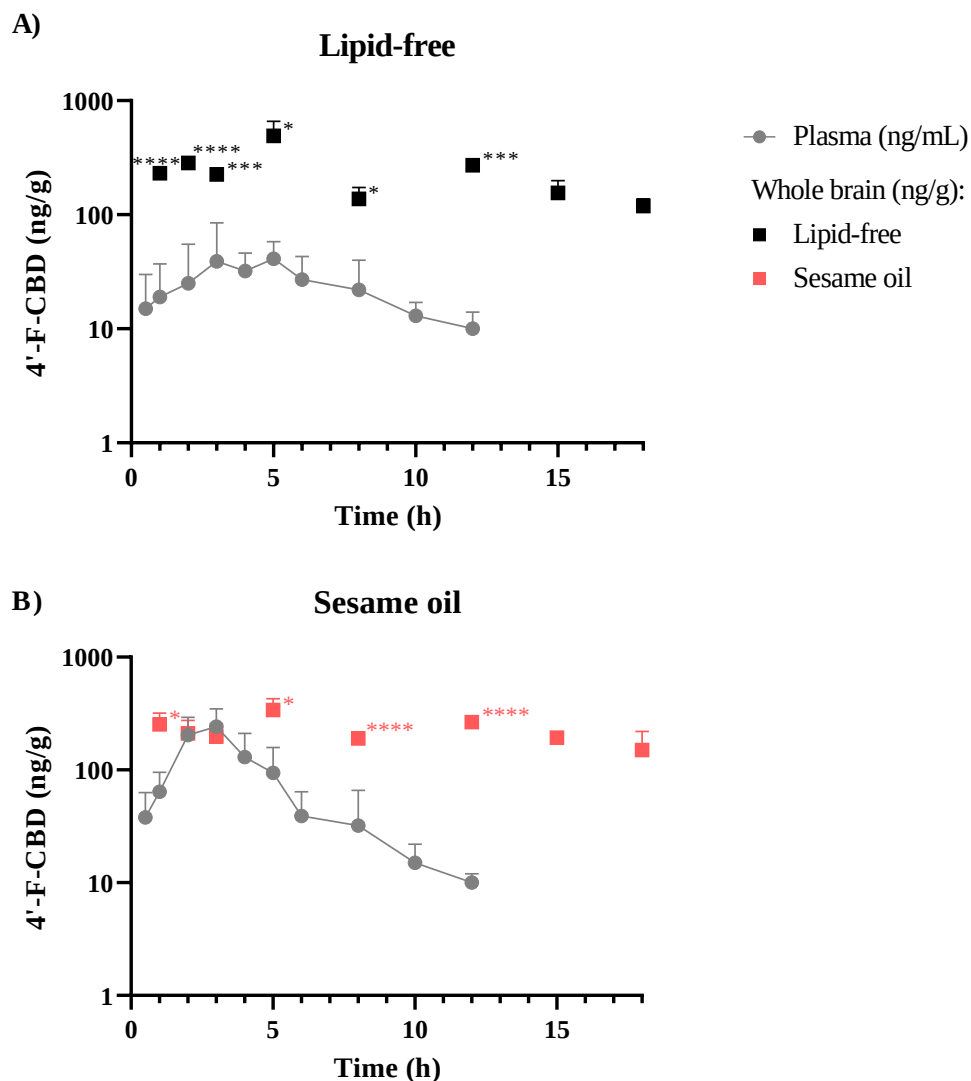


Figure 6.3. 4'-F-CBD concentration in the whole brain vs. plasma following oral administration to rats at 38 μ M/Kg (12.7 mg/Kg) in A) lipid-free and B) sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, * = $p < 0.001$, **** = $p < 0.0001$. The data are presented as mean $\pm$ SD, plasma $n = 6$, brain $n = 3$. The PK parameters are shown in Table 6.2.**

Table 6.2. PK parameters of 4'-F-CBD concentration in the plasma and whole brain following oral administration at 38 μ M/Kg (12.7 mg/Kg) to rats. The data are also presented in Figure 6.3. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, ** = $p < 0.01$, * = $p < 0.001$, differences between the lipid-free and lipid-based formulations are shown. The data are presented as mean $\pm$ SD, plasma n = 6, brain n = 3.**

		T_{max} (h)	C_{max} (ng/mL or ng/g)	t_{1/2} (h)	AUC_{0-∞} (h*ng/mL or h*ng/g)	Brain- plasma ratio
Lipid-free	Plasma	4	69 $\pm$ 31	3.6	335 $\pm$ 147	14.7
	Whole brain	5	490 $\pm$ 169	5.1	4910 $\pm$ 634	
Sesame oil	Plasma	3	278 $\pm$ 76 ***	2.5	916 $\pm$ 285 **	9.9
	Whole brain	5	352 $\pm$ 77	7.3	9100 $\pm$ 6757	

Chapter 4 demonstrated the need to study the delivery of a molecule to different anatomical brain regions of interest. In order to compare the cannabinoids, the brain regions isolated and analysed following administration of 4'-F-CBD were the same as those isolated after administration of CBD. Figure 6.4 shows the 4'-F-CBD concentration in the anatomical brain regions in comparison to the plasma PK profiles. After administration in both formulations, the olfactory bulb and striatum exhibited higher 4'-F-CBD concentrations than the plasma at every time point. At 2 and 12 h after administration in the lipid-free vehicle, the hippocampus demonstrated 4'-F-CBD concentrations higher than the plasma, as well as at 5 h following administration in the sesame oil formulation. Additionally, at 5 h after administration in the lipid-free formulation, the medulla pons, and frontal and parietal lobes also exhibited higher 4'-F-CBD concentrations than the plasma.

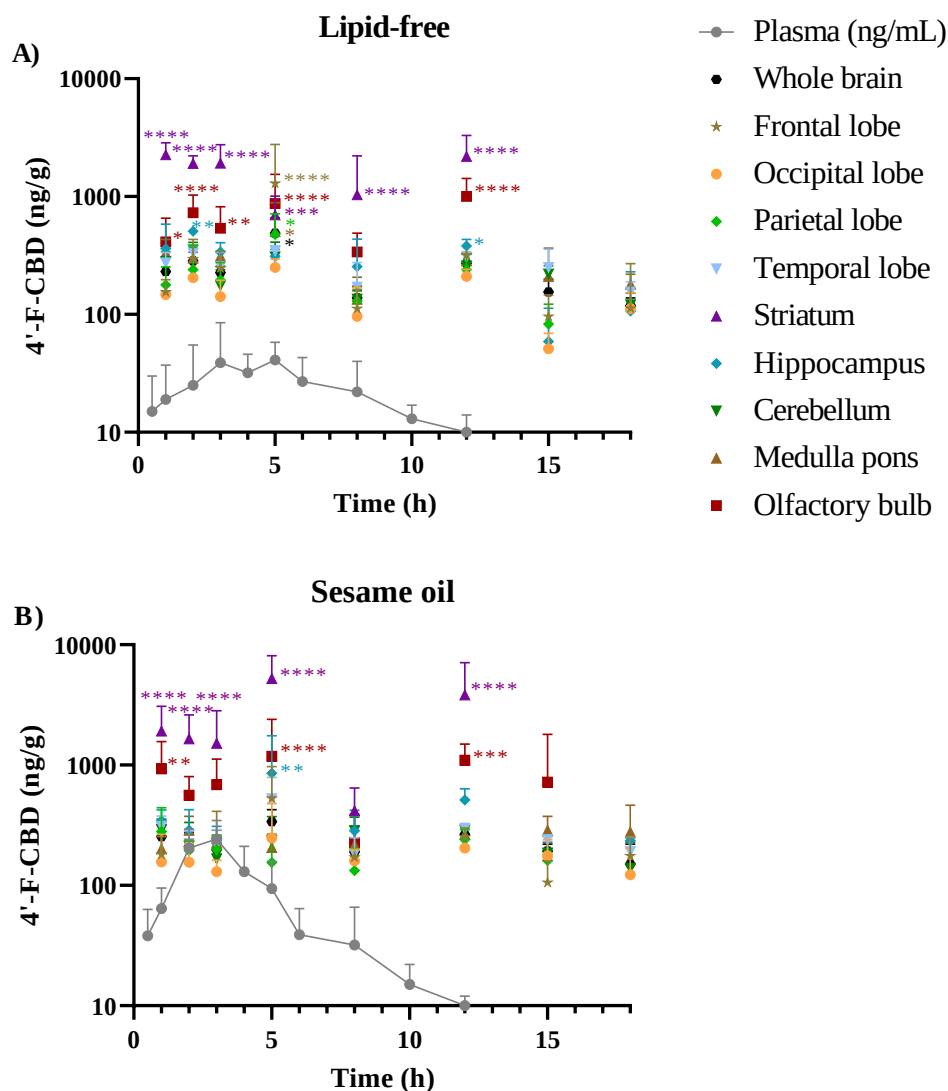
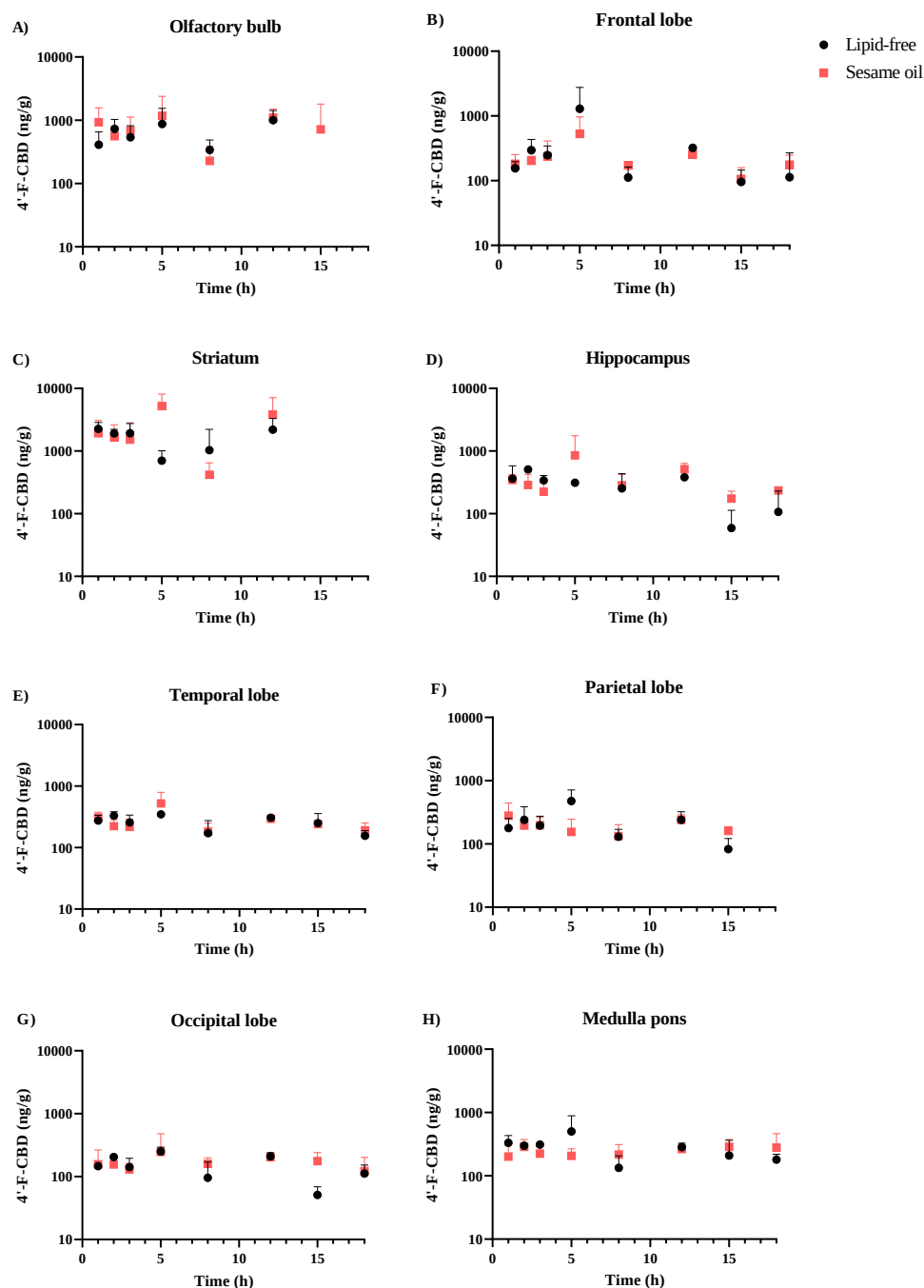


Figure 6.4. 4'-F-CBD concentration in anatomical brain regions vs. plasma following oral administration to rats at 38 μ M/Kg (12.7 mg/Kg) in A) lipid-free and B) sesame oil formulations. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, * = $p < 0.001$, **** = $p < 0.0001$. The data are presented as mean $\pm$ SD, plasma $n = 6$, brain $n = 3$.**

Figure 6.5 demonstrates the data shown in Figure 6.4 but separated into anatomical brain regions in order to compare the delivery following administration in the different formulations. In all anatomical brain regions, the delivery of 4'-F-CBD was similar after administration in both formulations at all time points. The only difference observed was in the olfactory bulb at 15 h

following administration, where no 4'-F-CBD was detected following administration in the lipid-free formulation. The PK parameter data for delivery to the anatomical brain regions following administration in both the lipid-free and lipid-based formulations is shown in Table 6.3.



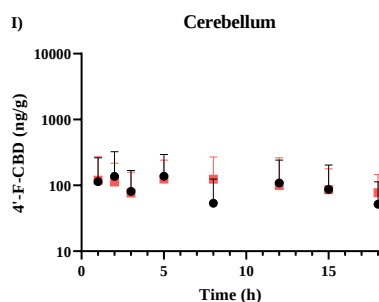


Figure 6.5. 4'-F-CBD concentration in anatomical brain regions A) olfactory bulb, B) frontal lobe, C) striatum, D) hippocampus, E) temporal lobe, F) parietal lobe, G) occipital lobe, H) medulla pons and I) cerebellum following oral administration to rats at 38 μ M/Kg (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$. The data are presented as mean $\pm$ SD, n = 3. The data are also shown in Figure 6.4 and the PK parameters are presented in Table 6.3.

Table 6.3. PK parameters of 4'-F-CBD concentration-time profiles in anatomical brain regions following oral administration to rats at 38 μ M/Kg (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$. The data are presented as mean $\pm$ SD, n = 3. The data are also presented in Figure 6.5.

Formulation	Brain region	T _{max} (h)	C _{max} (ng/g)	t _{1/2} (h)	AUC ₀₋₁₈ (h*ng/g)	AUC _{0-∞} (h*ng/g)
Lipid-free	Olfactory bulb	6	1367 $\pm$ 281	11.8	7169 $\pm$ 2259	20093 $\pm$ 3111
	Frontal lobe	5	1294 $\pm$ 1468	14.8	5124 $\pm$ 2267	10568 $\pm$ 8632
	Striatum	5	2676 $\pm$ 910	659.7	16522 $\pm$ 7551	1428394 $\pm$ 1972407
	Hippocampus	2	522 $\pm$ 58	4.9	4497 $\pm$ 215	5670 $\pm$ 1633

Sesame oil	Temporal lobe	4	351 ± 33	7.5	4381 ± 336	6084 ± 519
	Parietal lobe	5	477 ± 237	4.2	3081 ± 583	3399 ± 673
	Occipital lobe	7	261 ± 35	7.0	2442 ± 328	3675 ± 970
	Medulla pons	4	572 ± 304	11.4	4515 ± 826	7754 ± 2454
	Cerebellum	6	377 ± 49	6.9	3813 ± 376	5081 ± 967
	Olfactory bulb	11	1982 ± 525	5.3	10222 ± 2229	10192 ± 2507 *
	Frontal lobe	7	541 ± 430	11.0	3954 ± 1345	7283 ± 3589
	Striatum	4	5300 ± 2781	96.2	25231 ± 12259	310948 ± 391769
	Hippocampus	10	994 ± 739	6.0	6296 ± 1291	8345 ± 1853
	Temporal lobe	4	531 ± 258	34.2	4744 ± 600	15083 ± 7375
	Parietal lobe	8	324 ± 112	21.8	2679 ± 666	8554 ± 9080
	Occipital lobe	7	330 ± 159	4.4	3053 ± 491	3724 ± 350
	Medulla pons	16	389 ± 89	8.9	4274 ± 379	6268 ± 422
	Cerebellum	8	355 ± 54	7.1	4046 ± 542	5875 ± 2235

6.3.2.2 Comparison of biodistribution of 4'-fluoro-cannabidiol and cannabidiol to the brain

Direct comparisons of the delivery of cannabinoids 4'-F-CBD and CBD to the whole brain and anatomical brain regions were made. As for the PK analysis, this comparison is made using the units nM/g, as the dose was 38 μ M/Kg for both cannabinoids. The comparison of cannabinoid delivery to the whole brain is shown in Figure 6.6, and the PK parameters of this delivery are shown in Table 6.4.

Figure 6.6 shows that at 1 h following administration in the sesame oil formulation, the CBD concentration in the whole brain was higher than that of 4'-F-CBD. At 3, 8 and 18 h and 1, 8 and 12 h after administration in the sesame oil and lipid-free formulations, respectively, the cannabinoid concentrations were not significantly different. At all other time points following administration in both formulations, the 4'-F-CBD concentration in the whole brain was higher than CBD. Table 6.4 shows that the C_{max} of CBD was significantly higher than 4'-F-CBD following administration in the sesame oil formulation. However, the AUC_{0-18} of 4'-F-CBD was higher than CBD.

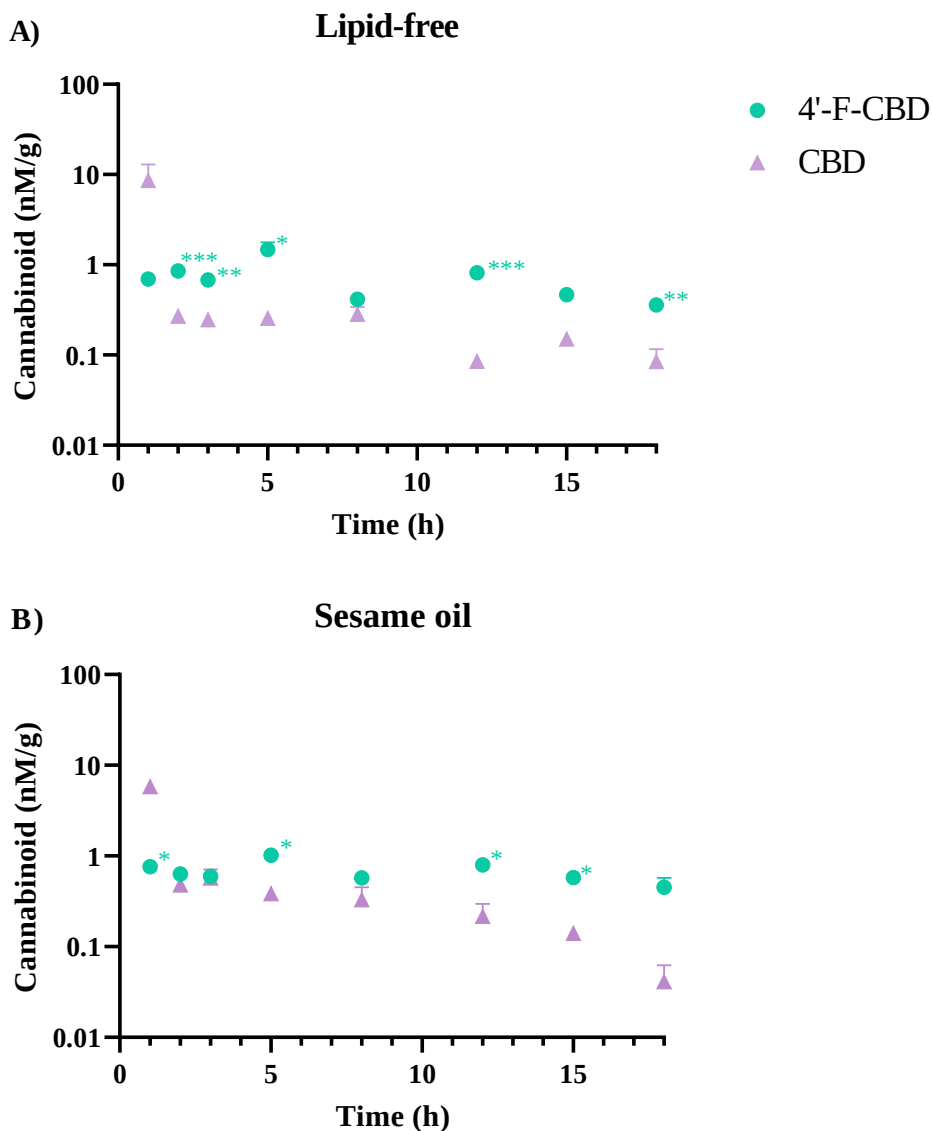


Figure 6.6. Cannabinoid concentration in the whole brain following oral administration to rats at 38 $\mu\text{M/Kg}$ (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) in A) lipid-free and B) sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Data are presented as mean $\pm$ SD, $n = 3$. The PK parameters are presented in Table 6.4.

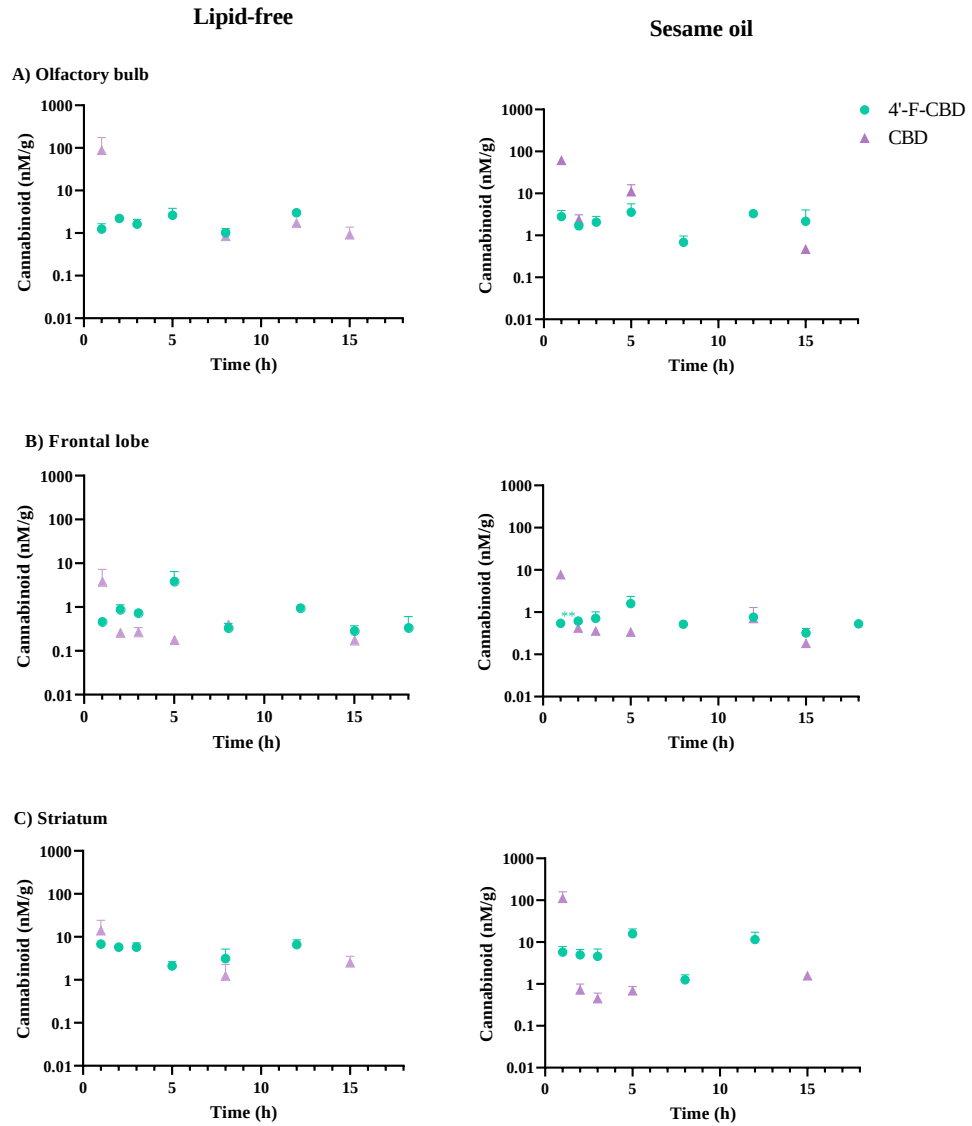
Table 6.4. PK parameters of cannabinoid delivery to the whole brain following oral administration at 38 μ M/Kg (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) to rats in both lipid-free and sesame oil formulations. The data are also presented in Figure 6.6. One-way ANOVA with Dunnett's multiple comparisons was performed, comparisons of 4'-F-CBD to CBD are shown, $\alpha = 0.05$, * = $p < 0.05$. The data are presented as mean $\pm$ SD, n = 3.

		T_{max} (h)	C_{max} (nM/g)	$t_{1/2}$ (h)	AUC_{0-18} (h*nM/g)	$AUC_{0-\infty}$ (h*nM/g)
4'-F-CBD	Lipid-free	5	1.5 $\pm$ 0.5	5.2	12.0 $\pm$ 1.9	14.8 $\pm$ 1.9
	Sesame oil	4	1.1 $\pm$ 0.2 *	17.9	11.9 $\pm$ 1.1 *	27.4 $\pm$ 20.3
CBD	Lipid-free	1	8.6 $\pm$ 7.5	9.6	9.5 $\pm$ 5.9	10.4 $\pm$ 6.0
	Sesame oil	1	5.8 $\pm$ 1.5	4.6	9.4 $\pm$ 0.8	9.7 $\pm$ 0.8

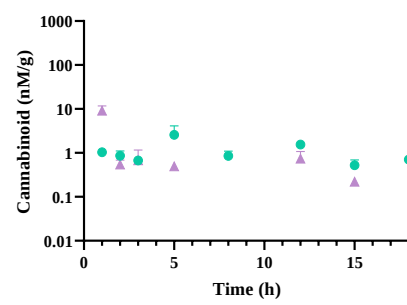
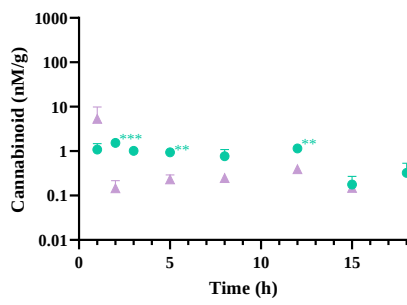
Delivery of the two cannabinoids to the specific anatomical brain regions are compared in Figure 6.7. Following administration in the lipid-free formulation, no differences were observed between the cannabinoid concentrations in the olfactory bulb, frontal lobe, striatum, parietal lobe and cerebellum. In the hippocampus and occipital lobe, the 4'-F-CBD concentration was higher than CBD at 2, 5 and 12 h following administration. The temporal lobe again showed higher 4'-F-CBD concentrations at 2, 5, 12 and 18 h. In the medulla pons, the concentration of 4'-F-CBD was consistent over the 18 h, and resulted in higher concentrations than CBD at 2, 3, 12 and 18 h post administration.

Following administration in the sesame oil formulation, differences were not observed between the cannabinoid concentrations in the olfactory bulb, striatum, hippocampus, and cerebellum. In the frontal, temporal and parietal lobes and the medulla pons, the CBD concentration was higher than 4'-F-CBD at the CBD T_{max} (1 h). In the temporal and parietal lobes and medulla pons, the 4'-F-CBD concentration was higher than CBD at 12 and 15 h following administration. In

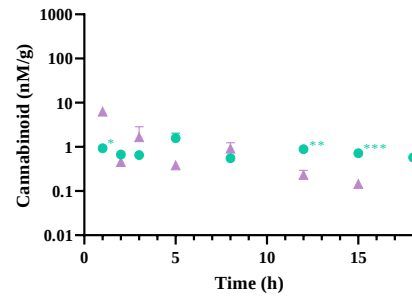
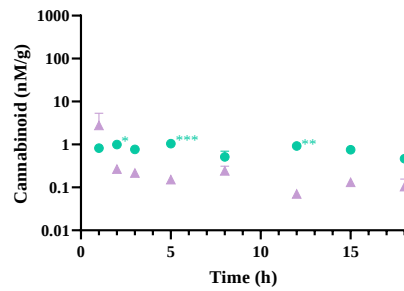
the occipital lobe, the 4'-F-CBD concentration was higher than CBD only at 12 h post administration.



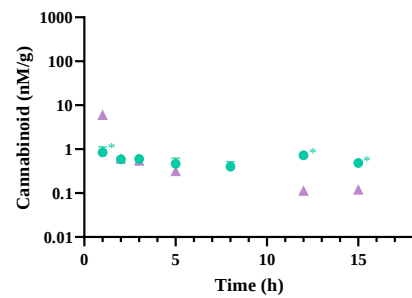
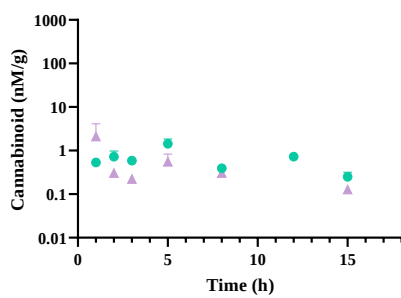
D) Hippocampus



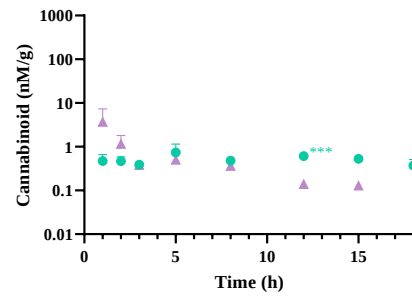
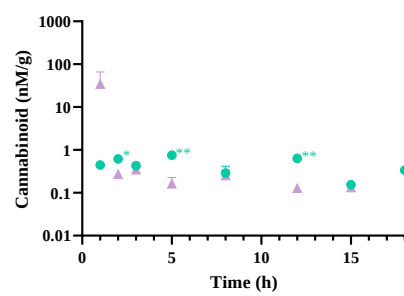
E) Temporal lobe



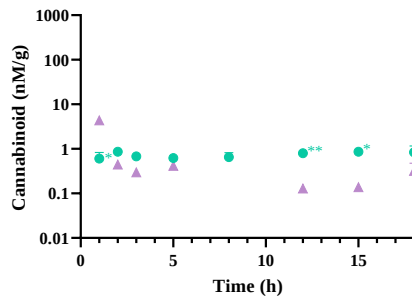
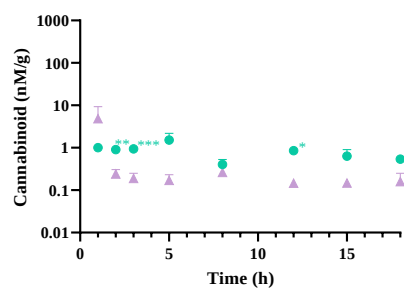
F) Parietal lobe



G) Occipital lobe



H) Medulla pons



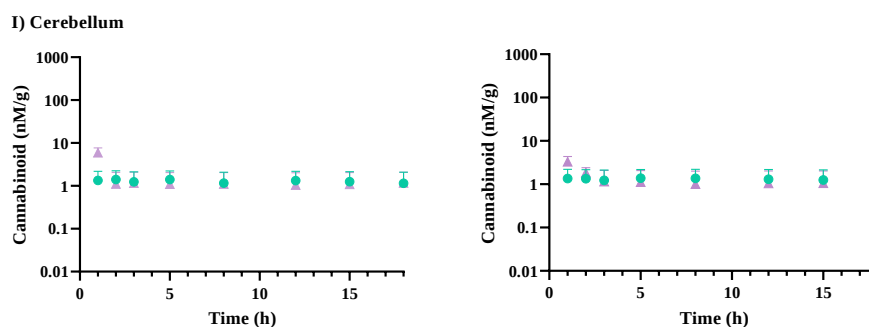


Figure 6.7. Cannabinoid concentration in anatomical brain regions A) olfactory bulb, B) frontal lobe, C) striatum, D) hippocampus, E) temporal lobe, F) parietal lobe, G) occipital lobe, H) medulla pons and I) cerebellum following oral administration to rats at 38 μ M/Kg (12.7 mg/Kg 4'-F-CBD, 12 mg/Kg CBD) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, * = $p < 0.001$. The data are presented as mean $\pm$ SD, n = 3. The PK parameters of 4'-F-CBD are shown in Table 6.3, and of CBD in Chapter 4, Table 4.2.**

6.3.2.3 Biodistribution of 4'-fluoro-cannabidiol to other tissues

To the best of our knowledge, the biodistribution of 4'-F-CBD has not previously been reported in the literature. Therefore, the biodistribution was studied to other tissues as described in Chapter 2, section 2.4.3. The biodistribution of 4'-F-CBD to the isolated lymph nodes in comparison to the plasma PK profile is shown in Figure 6.8. Following administration in both formulations, the mesenteric lymph nodes (MLN) exhibited higher 4'-F-CBD levels than the plasma at 1 - 8 h following administration. At 15 and 18 h, only the MLN and cervical lymph nodes had quantifiable 4'-F-CBD concentrations. The PK parameters of delivery of 4'-F-CBD to the lymph nodes are shown in Table 6.5.

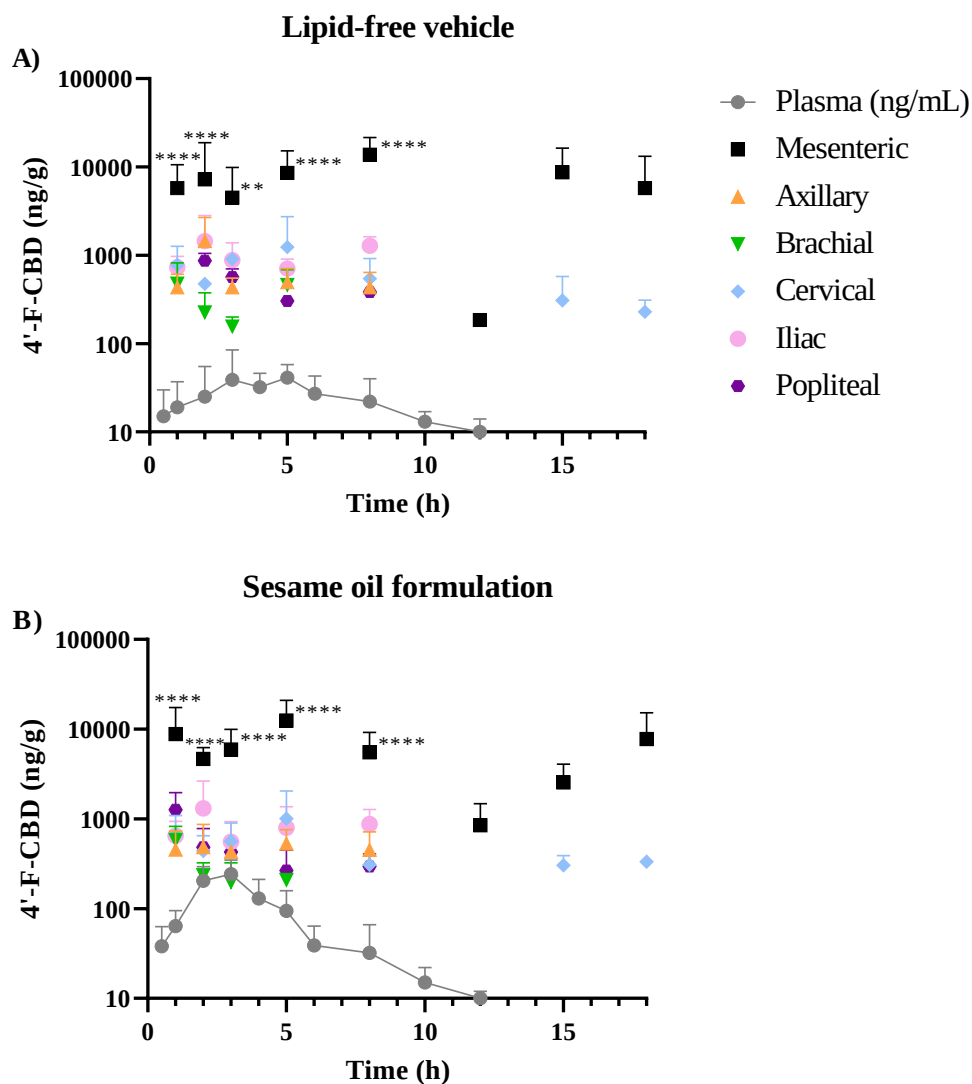
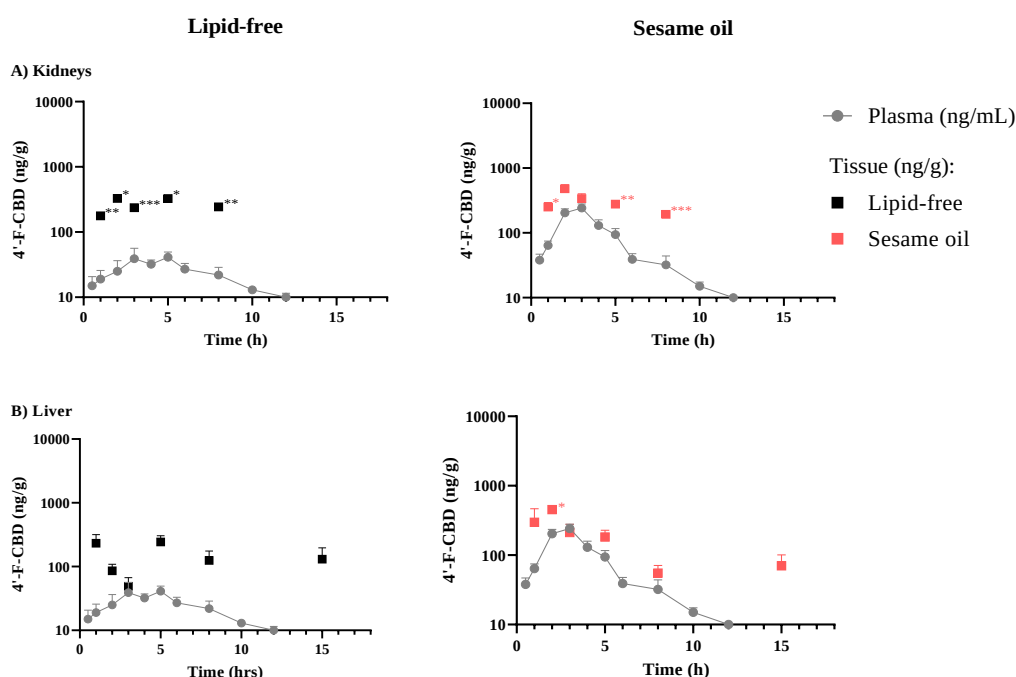


Figure 6.8. 4'-F-CBD concentration in lymph nodes vs. plasma following oral administration to rats at 38 μ M/Kg (12.7 mg/Kg) in A) lipid-free vehicle and B) sesame oil formulation. One-way ANOVA with Dunnett's multiple comparisons was performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, * = $p < 0.001$, **** = $p < 0.0001$. The data are presented as mean $\pm$ SD, plasma n = 6, lymph nodes n = 3. The PK parameters are presented in Table 6.5.**

Table 6.5. PK parameters of 4'-F-CBD concentration-time profiles in lymph nodes following oral administration to rats at 38 μ M/Kg (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, α = 0.05, no significant differences between formulations were observed. The data are presented as mean $\pm$ SD, n = 3. The data are also presented in Figure 6.8.

Formulation	Lymph node	T_{max} (h)	C_{max} (ng/g)	t_{1/2} (h)	AUC₀₋₁₈ (h*ng/g)	AUC_{0-∞} (h*ng/g)
Lipid-free	Mesenteric	5	16326 $\pm$ 4654	11.8	104392 $\pm$ 49694	287484 $\pm$ 338856
	Axillary	2	1441 $\pm$ 1243	7.3	4251 $\pm$ 1097	8341 $\pm$ 1781
	Brachial	2	641 $\pm$ 265	14.2	1376 $\pm$ 419	7231 $\pm$ 4411
	Cervical	4	1587 $\pm$ 1191	9.7	9609 $\pm$ 4761	12977 $\pm$ 2266
	Iliac	4	2010 $\pm$ 870	13.3	7032 $\pm$ 591	29775 $\pm$ 14881
	Popliteal	2	871 $\pm$ 182	5.8	3722 $\pm$ 178	7014 $\pm$ 1739
Sesame oil	Mesenteric	4	17539 $\pm$ 444	8.2	87704 $\pm$ 21053	176818 $\pm$ 100931
	Axillary	3	793 $\pm$ 99	14.0	3498 $\pm$ 545	15591 $\pm$ 16208
	Brachial	1	584 $\pm$ 241	3.9	1290 $\pm$ 388	2463 $\pm$ 255
	Cervical	4	1328 $\pm$ 778	15.5	7570 $\pm$ 1323	14964 $\pm$ 2352
	Iliac	3	1744 $\pm$ 975	4.8	5932 $\pm$ 1797	14890 $\pm$ 9445
	Popliteal	1	1325 $\pm$ 613	4.0	3309 $\pm$ 708	5375 $\pm$ 1264

Figure 6.9 demonstrates the biodistribution of 4'-F-CBD to the kidneys, liver, lungs and spinal cord. Biodistribution was also studied to the heart and testes, where 4'-F-CBD was not detected. The PK parameters of the 4'-F-CBD concentration-time profiles are shown in Table 6.6. Delivery of 4'-F-CBD to the kidneys resulted in higher concentrations than the plasma 1 - 8 h following administration in the lipid-free formulation. 4'-F-CBD concentrations were only higher in the kidneys after administration in the sesame oil formulation at 1, 5 and 8 h. In the liver, following administration in both formulations, the 4'-F-CBD concentration was similar to the plasma, apart from at 2 h post administration in the sesame oil formulation. At 1 and 2 h following administration in both vehicles, and 8 h in the sesame oil formulation, the 4'-F-CBD concentration in the lungs was higher than the plasma. The concentration of 4'-F-CBD in the spinal cord was consistent over the 18 h, with higher concentrations than the plasma observed at 1, 2, 5 and 8 h post administration in the lipid-free vehicle and at 5 h for the sesame oil formulation.



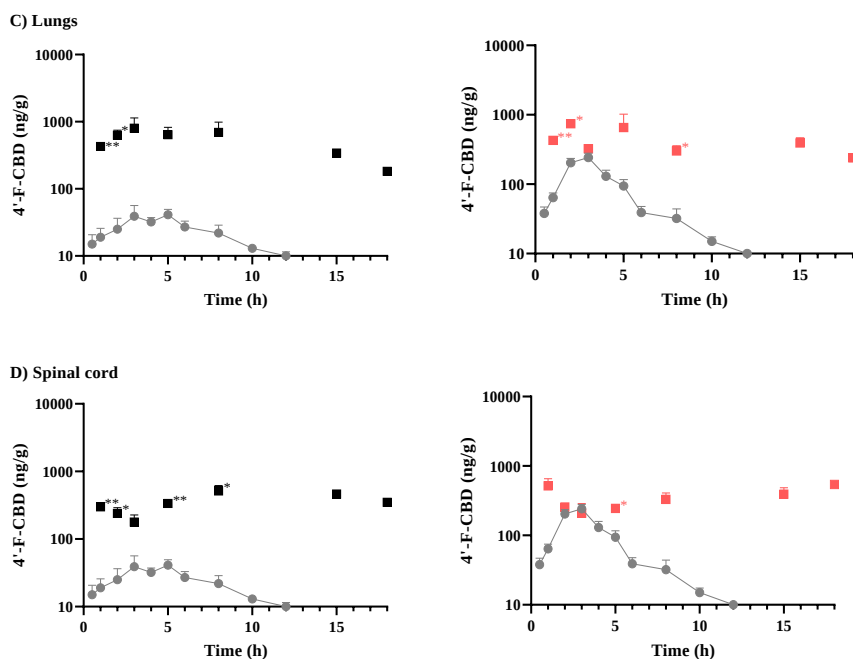


Figure 6.9. 4'-F-CBD concentration in the A) kidneys, B) liver, C) lungs and D) spinal cord vs. plasma following oral administration to rats at 38 μ M/Kg (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, * = $p < 0.05$, ** = $p < 0.01$, * = $p < 0.001$, **** = $p < 0.0001$. The data are presented as mean $\pm$ SD, plasma $n = 6$, tissue $n = 3$. The PK parameters are presented in Table 6.6.**

Table 6.6. PK parameters of 4'-F-CBD concentration-time profiles in organs following oral administration to rats at 38 μ M/Kg (12.7 mg/Kg) in lipid-free and sesame oil formulations. Multiple t-tests were performed, $\alpha = 0.05$, no significant differences between formulations were observed. The data are presented as mean $\pm$ SD, n = 3. The data are also presented in Figure 6.9.

Formulation	Tissue	T _{max} (h)	C _{max} (ng/g)	t _{1/2} (h)	AUC ₀₋₁₈ (h*ng/g)	AUC _{0-∞} (h*ng/g)
Lipid-free	Heart	-	-	-	-	-
	Kidneys	3	367 $\pm$ 36	34.0	2025 $\pm$ 143	15642 $\pm$ 19536
	Liver	4	320 $\pm$ 61	10.6	1873 $\pm$ 236	3935 $\pm$ 3130
	Lungs	3	1055 $\pm$ 393	7.0	8881 $\pm$ 2508	10734 $\pm$ 1753
	Spinal cord	10	552 $\pm$ 112	12.0	7028 $\pm$ 851	13326 $\pm$ 4119
	Testes	-	-	-	-	-
Sesame oil	Heart	-	-	-	-	-
	Kidneys	2	479 $\pm$ 133	7.6	2198 $\pm$ 267	4431 $\pm$ 1788
	Liver	2	482 $\pm$ 145	5.5	1949 $\pm$ 645	2621 $\pm$ 1224
	Lungs	3	1000 $\pm$ 355	16.1	6894 $\pm$ 1873	12569 $\pm$ 1940
	Spinal cord	12	599 $\pm$ 135	-	6085 $\pm$ 1283	-
	Testes	-	-	-	-	-

6.4 Discussion

Some of the *in vivo* effects of 4'-F-CBD (also named HUF-101 and PECS-101) have been investigated in the literature [176-180]. In addition, Chapter 3 demonstrated the anti-cancer properties of 4'-F-CBD against GBM for the first time to the best of our knowledge, and high synergy of the molecule with imidazotetrazine agents. However, to the best of our knowledge the *in vivo* PK and biodistribution of the molecule have not yet been reported. Herein, the PK of 4'-F-CBD was investigated following oral administration in both lipid-free and lipid-based formulations. Sesame oil was selected as the lipid-based formulation based on the *in vivo* biodistribution assessment of CBD (discussed in Chapter 4). The delivery of CBD to common locations of GBM tumours (the temporal, frontal and parietal lobes [90, 229, 230]) was observed to be the highest following oral administration in the sesame oil formulation. The sesame oil formulation also resulted in improved delivery of CBD to the whole brain compared to the other lipid-based formulations studied (coconut and rapeseed oils). Therefore, based on this work and that reported in the literature of the superior bioavailability of CBD following administration in a sesame oil formulation [155, 166], sesame oil was selected as the lipid-based vehicle for this work.

The PK concentration-time profiles, shown in Figure 6.1, demonstrate that the 4'-F-CBD concentration in the plasma was higher following administration in the lipid-based sesame oil formulation at 0.5 - 4 h post administration. After 4 h, the concentrations were similar for both formulations, falling close to the LLOQ of the method (10 ng/mL) by 12 h. Lipid-based formulations have previously been reported to increase cannabinoid plasma concentrations compared to lipid-free formulations [155, 166, 168, 169]. Table 6.1 shows that the lipid-based sesame oil formulation produced higher C_{max} than the lipid-free formulation at 0.84 ± 0.23 nM/mL, compared to 0.21 ± 0.09 nM/mL. The sesame oil formulation also resulted in a higher $AUC_{0-\infty}$ of 4'-F-CBD than the lipid-free formulation (2.76 ± 0.86 h*nM/mL, compared to 1.01 ± 0.44 h*nM/mL).

It was anticipated that the PK profile of 4'-F-CBD would be similar to CBD, due to the reports of similar *in vivo* activity of the two cannabinoids. Therefore, the

PK profiles observed in this work are in support of the hypothesis, which was based on the profiles of CBD following oral administration in the same formulations in previous work (Feng *et al* [155]). Figure 6.2 shows that the only difference in the plasma PK profile between 4'-F-CBD and CBD was found at 1 h following administration in the sesame oil formulation, where the 4'-F-CBD concentration was higher. Table 6.1 also demonstrates that the PK parameters, including AUC, were not significantly different between the two cannabinoids.

Silva *et al* reported a potentially improved pharmacological profile of 4'-F-CBD over CBD following i.p. injection [177]. This could be as a result of increased bioavailability (investigated in this work by PK profile), improved biodistribution to the site of action (the brain), or increased activity of the molecule due to the addition of the fluorine atom. Interestingly, this work shows that the PK profile following oral administration of 4'-F-CBD is not significantly different to that of CBD. However, the biodistribution of 4'-F-CBD to the brain was found to be different to CBD (Figures 6.6 and 6.7).

Chapter 3 demonstrated the anti-cancer activity of 4'-F-CBD against the aggressive brain cancer GBM *in vitro*. There are reports of *in vivo* activity of 4'-F-CBD in the brain including anti-anxiety and anti-compulsive behaviour, but not anti-cancer activity previously [176, 177]. These reports do however demonstrate the need to understand the delivery of 4'-F-CBD to brain tissue, which was explored in the work herein following oral administration to rats. The biodistribution to the whole brain was first studied and is shown in Figure 6.3. Following oral administration in the lipid-free formulation, the 4'-F-CBD concentration in the whole brain was consistently higher than in the plasma. After administration in the sesame oil formulation, the 4'-F-CBD concentration in the brain was consistent up to 18 h post administration. The brain and plasma 4'-F-CBD concentrations were similar at 2 and 3 h post administration, and higher in the brain at 1, 5, 8 and 12 h. Similar 4'-F-CBD concentrations in the whole brain were observed following administration in both formulations, as is shown in Table 6.2. However, the brain-plasma ratio was higher after administration in the lipid-free formulation (14.7, compared to 9.9 for the sesame oil formulation), a result of the low plasma AUC_{0-∞} observed for the lipid-free formulation.

The delivery of 4'-F-CBD to the whole brain was compared to that of CBD following oral administration in the same formulations in Figure 6.6. After administration in both formulations, the difference in the profiles of the cannabinoids can be observed: the CBD T_{max} was at 1 h, with a sharp fall, whereas the 4'-F-CBD concentration remained consistent from 1 - 18 h. Following administration in the lipid-free formulation, the 4'-F-CBD concentration was higher than CBD at 2, 3, 5, 12 and 18 h post administration. This was also observed after administration in the sesame oil formulation at 5, 12 and 15 h. However, in the sesame oil formulation, the CBD concentration was higher than 4'-F-CBD at 1 h post administration (the CBD T_{max}). Table 6.4 shows that differences between the PK parameters of cannabinoids were observed following administration in the sesame oil formulation. The higher C_{max} was observed for CBD, but the higher AUC_{0-18} was observed for 4'-F-CBD, a result of the similar concentrations of 4'-F-CBD over time seen in Figure 6.6.

Chapter 4 demonstrated the importance of studying the biodistribution to individual anatomical brain regions, as well as to the whole brain. The concentration-time profile of CBD in the anatomical brain regions was shown to be different to the whole brain. The formulations used also showed differences in the delivery, highlighting that certain vehicles would provide the optimal delivery of CBD, depending on the desired site of delivery. Therefore, the distribution of 4'-F-CBD to specific anatomical brain regions was studied herein.

4'-F-CBD has been reported to exhibit some of the same *in vivo* behavioural effects as CBD, at higher potency [176, 177]. There are also reports of activity of 4'-F-CBD to reduce chemotherapy-induced pain [179]. The behavioural effects observed are often associated with the hippocampus, temporal lobe and frontal lobe regions of the brain. Additionally, Chapter 3 described the anti-cancer properties of 4'-F-CBD for the first time against aggressive brain cancer, GBM. GBM is most commonly located in the temporal, frontal and parietal lobes of the brain [90, 229, 230]. As described for CBD in Chapter 4, it is important to understand the delivery of 4'-F-CBD to these brain regions. Therefore, the brain regions that were isolated in Chapter 4 for the study of CBD biodistribution were also isolated to study 4'-F-CBD biodistribution.

Figure 6.4 demonstrates that the 4'-F-CBD concentration in all brain regions was above or not significantly different to the plasma concentration following oral administration in both formulations. The 4'-F-CBD concentrations in the olfactory bulb and striatum were consistent up to 12 h post administration in the lipid-free formulation, and 15 h for the olfactory bulb in the sesame oil formulation. This can also be seen in Figure 6.5, which demonstrates that there were no significant differences in the 4'-F-CBD concentration in any anatomical brain regions following administration in the two formulations. The only perceived difference was in the olfactory bulb, where after administration in the sesame oil formulation, 4'-F-CBD was detected at 15 h, but only up to 12 h after administration in the lipid-free vehicle.

The olfactory bulb, striatum and parietal lobe were the only anatomical brain regions where 4'-F-CBD was not detected at all time points studied (up to 15 h, 12 h and 15 h, respectively). The delivery of 4'-F-CBD was consistent over time across all brain regions, where the frontal lobe was the only region to appear to demonstrate a concentration peak at 5 h post administration. The highest 4'-F-CBD concentrations were observed in the olfactory bulb and striatum, similar to that observed for CBD in Chapter 4, Figure 4.5. The biodistribution of the cannabinoids to the brain was compared in Figure 6.7.

6.4.1 Olfactory bulb

The delivery of the cannabinoids to the olfactory bulb was not significantly different at any of the time points studied. However, there were differences in when the cannabinoids were detected. Following administration in the lipid-free vehicle, CBD was detected at 1, 8, 12 and 15 h, but not detected at 2, 3, and 5 h, whereas 4'-F-CBD was detected at all time points up to 12 h. After administration in the sesame oil formulation, CBD was detected at 1, 2, 5 and 15 h, and again 4'-F-CBD was detected at all time points up to 15 h. Administration in both formulations resulted in consistent 4'-F-CBD concentrations over time. The 4'-F-CBD T_{max} was observed later after administration in the sesame oil formulation (11 h, compared to 6 h for the lipid-free formulation), and a shorter $t_{1/2}$ (5.3 h, compared to 11.8 h for the lipid-free formulation). However, the C_{max}

and AUC_{0-18} of 4'-F-CBD were similar following administration in both formulations. Delivery of CBD demonstrated a clear peak at 1 h, and inconsistent delivery over the 15 h. Whilst 4'-F-CBD demonstrated similar concentrations over time, the higher AUC_{0-18} was observed for CBD following administration in both formulations.

6.4.2 Frontal lobe

In the frontal lobe, the CBD T_{max} was observed at 1 h following administration, whereas the 4'-F-CBD T_{max} was 5 h, for both formulations. The C_{max} of the two cannabinoids were not significantly different to each other. However, Figure 6.7 shows again the consistent 4'-F-CBD concentrations in the frontal lobe over the 18 h studied. CBD was detected up to 15 h post administration, but not at 12 h or 8 h in the lipid-free and sesame oil formulations, respectively. The consistent delivery of 4'-F-CBD following administration in both formulations resulted in higher AUC_{0-18} than CBD.

As mentioned, GBM is commonly located in the frontal lobe, as well as the parietal and temporal lobes [90, 229, 230]. Chapter 3 demonstrated the anti-cancer properties of both cannabinoids against GBM, where significant differences in the activity of the cannabinoids were not observed (Figure 3.2). Additionally, compulsive behaviour is often associated with the prefrontal cortex in the frontal lobe [284], and 4'-F-CBD has demonstrated anti-compulsive behaviour effects [176]. The increased exposure of the frontal lobe to 4'-F-CBD, over CBD, shows that a lower dose of 4'-F-CBD could be used to reach the same cannabinoid concentration in the brain region, demonstrating that 4'-F-CBD would be a preferential treatment for GBM, compared to CBD. The improved anti-compulsive behaviour activity of 4'-F-CBD over CBD could be at least partially explained by the increased delivery of the cannabinoid to the frontal lobe. The fluorine atom also provides additional options including imaging and theranostic potential, that were not explored in this work.

6.4.3 Striatum

Delivery of the cannabinoids to the striatum showed no significant differences in the concentrations observed at each time point. The 4'-F-CBD concentration was consistent at each time point up to 12 h following administration in both formulations. This resulted in a long $t_{1/2}$ of 4'-F-CBD after administration in both formulations (Table 6.3). CBD was detected at 1, 8 and 15 h following administration in the lipid-free vehicle, and 1, 2, 3, 5 and 15 h after administration in the sesame oil formulation. While CBD was detected at a later time point, 4'-F-CBD concentrations were similar at all time points up to 12 h. The AUC_{0-18} of the cannabinoids were similar, with the AUC_{0-18} of both higher following administration in the sesame oil formulation.

As mentioned previously, there is a high density of cannabinoid receptors located in the striatum [243, 244], and the neuroprotective effects of CBD are linked to activity there [245]. Additionally, A2A receptors are also located in the striatum [180]. 4'-F-CBD activity is reported to be at least partially *via* an adenosine signalling dependent pathway, and 4'-F-CBD was also reported to exhibit neuroprotective effects *in vivo* [178, 180]. To the best of our knowledge, whether 4'-F-CBD demonstrates higher activity *via* the A2A pathway than CBD has not yet been reported. 4'-F-CBD demonstrated more consistent delivery to the striatum than CBD, which could suggest potentially higher activity of 4'-F-CBD. However, this did not translate into a higher AUC_{0-18} . Therefore, it would not be surprising to observed similar activity of the cannabinoids *via* the A2A pathway.

6.4.4 Hippocampus

As discussed, 4'-F-CBD is reported to exhibit anti-anxiety, antidepressant and anti-nociceptive effects *in vivo*, all of which are commonly associated with activity in the hippocampus [176, 177, 252, 285]. Therefore, as investigation into these effects of 4'-F-CBD continues, it is important to understand the delivery of the cannabinoid to the region. Additionally, comparing 4'-F-CBD delivery to

CBD, will further understanding, as 4'-F-CBD is reported to have increased potency over CBD [176, 177].

Following oral administration of CBD, the T_{max} in the hippocampus was observed at 1 h, and CBD was detected up to 15 h post administration. 4'-F-CBD was detected at 18 h following oral administration in both formulations. Administration in the sesame oil formulation resulted in similar concentrations of CBD and 4'-F-CBD at the time points where both cannabinoids were detected. Following administration in the lipid-free vehicle, the 4'-F-CBD concentration was found to be higher than CBD at 2, 5 and 12 h post administration. The C_{max} of CBD was higher than 4'-F-CBD for both formulations, however the consistent concentrations of 4'-F-CBD over the 18 h resulted in the higher AUC_{0-18} being observed for 4'-F-CBD. This is interesting, as it indicates that at least part of the increased potency of 4'-F-CBD could be due to the increased exposure of the brain to the cannabinoid. As discussed, it is understood that fluorinated compounds often result in increased permeation across the BBB into the brain [173-175]. However, the exposure of the hippocampus to 4'-F-CBD is 1.4× higher than to CBD, whereas the reported *in vivo* activity of 4'-F-CBD was found to be the same as CBD at 10× lower dose than CBD [176]. This suggests that while the increased activity of 4'-F-CBD observed may be partly due to increased delivery of the cannabinoid to the hippocampus, this does not fully account for this increase observed, which may in part be due to increased activity of the molecule at the receptor level.

6.4.5 Temporal lobe

As for all anatomical brain regions, and discussed in Chapter 4, oral administration of CBD led to a T_{max} at 1 h in the temporal lobe. Oral administration of 4'-F-CBD resulted in a T_{max} of 5 h for both formulations. Following administration in the sesame oil formulation, the CBD concentration was higher than 4'-F-CBD at 1 h. While the cannabinoids exhibited similar concentrations at 1 h following administration in the lipid-free vehicle, at 2, 5, 12 and 18 h the 4'-F-CBD concentration was higher than CBD. This was also true at 12 and 15 h after administration in the sesame oil formulation. Much like

other regions, CBD shows an obvious $C_{\max}$ at 1 h, whereas the 4'-F-CBD concentration is similar at all time points. Like other brain regions, the consistent delivery of 4'-F-CBD over time resulted in a higher AUC_{0-18} than CBD following administration in the lipid-free formulation. Interestingly, differences in the exposure of the temporal lobe to cannabinoids were only observed following administration in the lipid-free formulation, as administration in the sesame oil formulation resulted in similar AUC_{0-18} for both cannabinoids.

As discussed above, GBM is commonly located in the temporal lobe [90, 229, 230], and Chapter 3 demonstrated the anti-cancer properties of the cannabinoids. The improved delivery of 4'-F-CBD over CBD following administration in the lipid-free vehicle suggests that this would be a good formulation choice for GBM treatment. However, the 4'-F-CBD AUC_{0-18} in the lipid-free vehicle was not significantly higher than for the sesame oil formulation, or of CBD in the sesame oil formulation.

6.4.6 Parietal lobe

Delivery to the parietal lobe was one of the few anatomical brain regions where 4'-F-CBD was not detected at 18 h (as well as the olfactory bulb and striatum). Again, the 4'-F-CBD concentration was consistent over time, whereas CBD was not detected at 12 h following administration in the lipid-free vehicle, or at 8 h for the sesame oil formulation. At 1 h, the CBD concentration in the parietal lobe was higher than 4'-F-CBD after administration in the sesame oil formulation, but at the later time points (12 and 15 h) the 4'-F-CBD concentration was higher. As for other brain regions, the $C_{\max}$ of CBD was higher than 4'-F-CBD following administration in the sesame oil formulation, but the AUC_{0-18} of the cannabinoids were similar. GBM is also commonly located in the parietal lobe [90, 229, 230], but as for the temporal lobe, the choice of cannabinoid for treatment could be based on other factors than the exposure of the brain region to the cannabinoids, as this was similar. It could be that other treatment options, such as combination treatment (also investigated in Chapter 3) are more important, where 4'-F-CBD showed increased synergy with TMZ analogue, T25, compared to CBD.

6.4.7 Occipital lobe

The delivery of CBD resulted in an obvious $C_{\max}$ at 1 h post oral administration, however the 4'-F-CBD concentrations were consistent over time. CBD was not detected at 18 h following administration in either formulation, whereas 4'-F-CBD was, demonstrating a prolonged exposure of the occipital lobe to 4'-F-CBD, over CBD. The 4'-F-CBD concentration was also higher than CBD at 2, 5 and 12 h following administration in the lipid-free formulation, and at 12 h for the sesame oil formulation. However, the high $C_{\max}$ of CBD after administration in the lipid-free vehicle resulted in the highest AUC_{0-18} of the cannabinoids observed for this formulation. There were no significant differences observed in the delivery of 4'-F-CBD following administration in the formulations.

6.4.8 Medulla pons

Following oral administration of CBD, the $T_{\max}$ was observed at 1 h in the medulla pons, where the CBD concentration was significantly higher than the 4'-F-CBD concentration after administration in the sesame oil formulation. Following this, the CBD concentration was similar up to 18 h, except for at 8 h after administration in the sesame oil formulation, where CBD was not detected. The 4'-F-CBD concentration was consistent over the 18 h, resulting in higher concentrations than CBD in the medulla pons at 2, 3, 12 and 18 h for the lipid-free vehicle and 12 and 15 h for the sesame oil formulation. Administration in the sesame oil formulation resulted in a later $T_{\max}$ of 4'-F-CBD (16 h, compared to 4 h in the lipid-free formulation). However, Figure 6.7 shows that the concentrations of 4'-F-CBD were similar over the 18 h following administration in the two formulations. The consistent delivery of 4'-F-CBD to the medulla pons resulted in higher exposure of the region to 4'-F-CBD than CBD.

6.4.9 Cerebellum

The cerebellum was the only brain region where the cannabinoid concentrations were the same across the 18 h following administration in both formulations. The one exception is the CBD $C_{\max}$ observed at 1 h, whereas 4'-F-CBD did not have an obvious $T_{\max}$ (Figure 6.7). The clear $T_{\max}$ of CBD did not result in a significantly higher exposure of the cerebellum to the cannabinoid, as the AUC_{0-18} were similar for both cannabinoids.

While the plasma PK profile of 4'-F-CBD was not significantly different to that of CBD, the biodistribution to the brain was. Oral administration of 4'-F-CBD resulted in similar concentrations in the whole brain and anatomical brain regions up to 18 h post administration. The concentration-time profiles of 4'-F-CBD were similar following administration in both the lipid-free and lipid-based sesame oil formulations. The similar concentrations of 4'-F-CBD in the whole brain over the 18 h resulted in higher AUC_{0-18} than CBD, demonstrating higher exposure of the brain to the fluorinated cannabinoid. This was also true in the hippocampus, temporal lobe, medulla pons and cerebellum. Following administration in both formulations in the frontal lobe, and the occipital lobe after administration in the sesame oil formulation, the $AUC_{0-\infty}$ of 4'-F-CBD was higher than CBD. In the parietal lobe, there were no significant differences in the exposure of the region to the cannabinoids, and the olfactory bulb and striatum were the only regions to have a higher exposure to CBD than 4'-F-CBD.

The work herein studying the biodistribution of 4'-F-CBD to the brain demonstrates the higher exposure of the tissue to the fluorinated cannabinoid than CBD. Additionally, Chapter 3 demonstrated no significant differences in the anti-cancer activity of the cannabinoids, showing that 4'-F-CBD was not more potent than CBD *in vitro*, at least for the anti-cancer activity. Together, this suggests that the increased potency of 4'-F-CBD *in vivo* that has been previously reported [176-180] could in part be the result of increased delivery of 4'-F-CBD to the brain, over CBD. Although, as discussed, the factor of increased delivery to a region is not the same as the reported increased potency, and it is therefore

possible that 4'-F-CBD is more potent than CBD on a molecular level in some applications.

6.4.10 Lymphatic system

The delivery of 4'-F-CBD to the lymph nodes, shown in Figure 6.8, showed that prolonged delivery was observed following oral administration in both the lipid-free and sesame oil formulations in the cervical and MLN, where 4'-F-CBD was detected at 18 h following administration. The 4'-F-CBD concentration in the MLN was significantly higher than the plasma concentration at 1 - 8 h after administration in both formulations. The 4'-F-CBD concentration in the other lymph nodes studied was similar to the plasma, showing that the highest concentrations were observed in the MLN. Our group has previously reported the CBD concentration in MLN positions following oral administration in a sesame oil formulation up to 8 h post administration [156]. At 8 h, the CBD concentration was observed to be falling, and the highest CBD concentration at 8 h was ~3000 ng/g [156]. Figure 6.8 shows increased delivery of 4'-F-CBD, demonstrating concentrations up to 17539 ± 444 ng/g (Table 6.5). Whilst the 4'-F-CBD concentration in the MLN fell at 12 h, by 15 h it was higher, and similar to the $C_{\max}$. The 4'-F-CBD $C_{\max}$ in the MLN was higher than that previously reported for CBD [151, 155, 156].

The prolonged delivery of 4'-F-CBD to the cervical lymph nodes correlates to drainage from the brain into the lymphatic system (Figure 6.8). This can be seen by comparing the delivery of 4'-F-CBD to the whole brain (Figure 6.3) and the cervical lymph nodes. The concentrations were similar at all time points in both tissues, producing similar concentration-time profiles. This was also true for the exterior regions of the brain (the olfactory bulb, and frontal, parietal and occipital lobes, Figure 6.5). The cervical lymph nodes are understood to connect to the meningeal lymphatic vessels that sit between the brain tissue and the skull, providing drainage from the brain into the lymphatic system [286]. However, it can be seen that 4'-F-CBD is not present throughout the lymphatic system at later time points, as it was only detected in the axillary, iliac and popliteal lymph nodes up to 8 h post administration. The brachial lymph nodes had the shortest

exposure to 4'-F-CBD, detected up to 3 h following administration in the lipid-free vehicle, and 5 h for the sesame oil formulation. The delivery of CBD to the iliac lymph nodes has been previously reported by our group, with concentrations ~2000 ng/g at 8 h following oral administration [156]. This is similar to the C_{max} reported herein for 4'-F-CBD at 2010 ± 870 ng/g and 1744 ± 975 ng/g after administration in the lipid-free and sesame oil formulations, respectively (Table 6.5).

The delivery of 4'-F-CBD to other organs was also investigated. It is important to understand the full biodistribution of 4'-F-CBD, as investigation of potential applications in the future will begin with an understanding of the concentration delivered to the desired site in order to conduct comprehensive PBPK modelling. Exposure of the liver and kidneys to 4'-F-CBD was found to be short, where 4'-F-CBD was detected up to 15 and 8 h, respectively. In the kidneys, oral administration in the lipid-free vehicle resulted in preferential delivery, where the 4'-F-CBD concentration was consistently higher than in the plasma, resulting in an AUC_{0-18} of 2025 ± 143 h*ng/g. Delivery to the kidneys following administration in the sesame oil formulation was similar (AUC_{0-18} 2198 ± 267 h*ng/g), although the concentration was higher than in the plasma only at 1, 5 and 8 h post administration.

4'-F-CBD was not detected in either the heart or testes over the 18 h following administration. However, prolonged exposure was observed in both the lungs and spinal cord after oral administration in both formulations, where 4'-F-CBD was detected at 18 h. At 1 and 2 h following administration in both formulations, the concentration observed in the lungs was significantly higher than in the plasma, this was also true at 8 h after administration in the sesame oil formulation.

In the spinal cord, significantly higher concentrations of 4'-F-CBD than in the plasma were observed up to 5 h following administration in the lipid-free vehicle, but only at 5 h for the sesame oil formulation. However, the concentration-time profiles in the spinal cord were similar after administration in both formulations. The delivery of 4'-F-CBD to the spinal cord followed the

same profile as delivery to the medulla pons, the point where the spinal cord connects to the brain (Figures 6.5 and 6.9). This suggests that delivery occurs simultaneously to the CNS and the brain. As mentioned, 4'-F-CBD has demonstrated anti-nociceptive effects, which have been linked to activity in the spinal cord [177]. Therefore, demonstrating the delivery of 4'-F-CBD to the tissue following oral administration is important for any potential future 4'-F-CBD-based anti-nociceptive treatments.

6.5 Conclusions

The plasma PK profile of 4'-F-CBD following oral administration in both lipid-free and sesame oil formulations was demonstrated to be similar to that of CBD. As for the PK of CBD after oral administration [155, 166, 168, 169], 4'-F-CBD was also found to have a higher C_{max} and $AUC_{0-\infty}$ following administration in the lipid-based sesame oil formulation. This work demonstrates that the addition of the fluorine atom to CBD did not significantly alter the plasma PK profile following oral administration.

The biodistribution studies to the brain tissue herein did demonstrate a difference between CBD and 4'-F-CBD following oral administration. When the whole brain was studied as one tissue, the 4'-F-CBD concentrations were consistent over the 18 h, resulting in $\sim 2\times$ higher exposure of the tissue to the fluorinated cannabinoid. This was also true in most anatomical brain regions, and the olfactory bulb and striatum were the only regions with higher exposure to CBD than 4'-F-CBD. The ($1.5 - 3\times$) higher exposure of the tissue to the fluorinated cannabinoid is consistent with reports of preferential delivery of fluorinated molecules across the BBB and into the brain [173-175]. It is likely that the higher exposure of the brain to the fluorinated cannabidiol is responsible for some of the increased potency over CBD observed *in vivo* [176, 177].

Studying the biodistribution of 4'-F-CBD to other tissues demonstrated the high delivery to the MLN and cervical lymph nodes. As for the delivery to the brain, the 4'-F-CBD concentrations observed in the MLN were higher than that previously reported for CBD, demonstrating higher delivery of 4'-F-CBD to the lymphatic system. Additionally, delivery to the cervical lymph nodes could be matched to certain anatomical brain regions, demonstrating drainage of 4'-F-CBD from the brain, *via* the lymphatic system. Delivery of 4'-F-CBD to the spinal cord was also shown, demonstrating the exposure of the CNS to the fluorinated cannabinoid.

The higher exposure of the brain to 4'-F-CBD, compared to CBD, demonstrates great potential of this molecule, where lower doses could be administered in order to achieve the same therapeutic effect. Fluorinated molecules also provide

additional benefits such as the potential for radiolabelling. Also, there is the anti-cancer theranostic potential of this molecule, briefly discussed in Chapter 3.

CHAPTER 7: General discussion and conclusions

The work in this thesis aimed to investigate the *in vitro* and *in vivo* properties of the phytocannabinoid cannabidiol (CBD) and its derivative, 4'-fluorocannabidiol (4'-F-CBD). The *in vitro* investigation explored the anti-cancer properties of the cannabinoids, while the *in vivo* work considered application across the vast therapeutic potential of the molecules, including anti-cancer and immunosuppressive activity against multiple sclerosis (MS). In-depth biodistribution of the cannabinoids following oral administration in lipid-free and lipid-based formulations was also investigated.

7.1 General discussion

The anti-cancer properties of cannabinoids have previously been investigated in the literature against several cancers [103, 125, 126]. Both CBD and Δ^9 -tetrahydrocannabinol (THC) are reported to exhibit anti-cancer effects against glioblastoma multiforme (GBM), usually in combination with each other, or with the standard of care treatment, temozolomide (TMZ) [103, 127-130]. CBD has previously been reported to demonstrate anti-cancer properties against a colorectal cancer cell line (HCT116) that exhibits a deficiency of deoxyribonucleic acid (DNA) mismatch repair (MMR), a common resistance mechanism to GBM treatment with TMZ [143]. The work in Chapter 3 demonstrated the ability of CBD to also overcome a second major mechanism of GBM resistance to TMZ, over-expression of *O*⁶-methylguanine-DNA methyltransferase (MGMT). To the best of our knowledge, this is the first time this has been reported. The ability of CBD to overcome these major resistance mechanisms demonstrates the potential of the molecule for treating GBM.

As mentioned, CBD anti-cancer activity has been demonstrated *in vitro* in combination with TMZ [131, 132]. Chapter 3 confirmed the synergy between CBD and TMZ that is reported in the literature [131, 132], and also demonstrated synergy between CBD and a TMZ analogue, T25, for the first time. As an *N*³-propargyl analogue of TMZ, T25 was expected to overcome the over-expression of MGMT resistance mechanism [95, 99, 100]. T25 was also shown to

overcome GBM resistance to TMZ by deficiency of MMR. Therefore, the synergy observed between T25 and CBD represents a promising potential new treatment for GBM.

In order to further investigate the potential of CBD as a treatment for GBM, Chapter 4 studied the delivery of CBD to the site of action in the brain *in vivo* after oral administration. Delivery to the frontal, temporal and parietal lobes was observed as quickly as 1 h, and up to 18 h, following administration. Taking the temporal lobe as an example, the highest exposure to CBD was observed following oral administration in the sesame oil formulation at 4005 ± 1155 h*ng/g (0.006 h* μ M) (Table 4.2). Chapter 3 showed that the concentration required to inhibit cell growth by 50% (GI_{50}) of CBD for the non-resistant GBM cell line to be 33 μ M (Table 3.1), and so the exposure of the temporal lobe to CBD demonstrated herein would not be sufficient to reach the GI_{50} of the molecule. However, the dose used in this work (12 mg/Kg) is a low range dose, where the human equivalent dose (HED) is 2.1 mg/Kg (Equation 4). CBD has been safely administered to humans in clinical trials at doses of 6000 mg/Kg, more than $2800\times$ higher than the HED used herein. Therefore, theoretically, concentrations of 17 h* μ M could be safely reached in the temporal lobe. This is not as high as the *in vitro* GI_{50} of CBD, however, CBD demonstrated high synergy with T25 *in vitro*. CBD concentrations as low as 1.25% the GI_{50} demonstrated an inhibition of cell growth by 50% when administered with T25. Therefore, if CBD was administered with T25, the concentration required at the site of action (the temporal lobe in this example) could be lowered to 0.4 μ M, well within the safe dose range. Therefore, a low oral dose of CBD could potentially be used as a treatment for GBM. However, it is important to note these are basic calculations to estimate concentrations, and do not take the high protein binding of CBD into account ($\sim 90\%$ [287]), or the low serum volume in the medium used *in vitro* (10%). There is a further remaining question of the efficacy of CBD *in vivo*, which will be discussed further in Chapter 8.

Chapter 4 demonstrated the differences in the delivery of CBD to anatomical brain regions following oral administration. Anatomical brain regions with second CBD concentration peaks in the concentration-time profiles following oral administration were those towards the rostral area of the brain (olfactory

bulb, frontal lobe, striatum). Regions in the caudal area of the brain (occipital lobe, medulla pons, cerebellum) exhibited concentrations of CBD that were consistent over time following the initial maximum concentration ($C_{\max}$) at 1 h. The caudal area of the brain also resulted in prolonged exposure, with CBD detected up to 18 h.

A hypothesis throughout this work was that oral administration of cannabinoids in a lipid-based formulation could improve delivery across the protective blood-brain barrier (BBB). This was based on the previously demonstrated increased bioavailability, and delivery to the lymphatic system, of CBD following oral administration in a lipid-based formulation, compared to a lipid-free vehicle [155, 169]. Additionally, as discussed in Chapter 1, there are reports that fatty acids, such as oleic and myristic acid, can increase the permeability of the BBB [62, 66, 189].

The work discussed in Chapters 4 – 6 investigated the *in vivo* delivery of cannabinoids to the brain following oral administration in lipid-based and lipid-free formulations. Chapter 4 demonstrated that the highest concentrations and exposure of the whole brain to CBD were observed following oral administration in the lipid-free formulation. The delivery following oral administration in the sesame oil formulation resulted in the second highest exposure of the brain to CBD. The rapeseed oil formulation often resulted in longer exposure of an anatomical brain region to CBD than the other lipid-based formulations. Additionally, the sesame oil formulations often produced the highest $C_{\max}$ and exposure of regions in the rostral region of the brain to CBD (in the caudal region of the brain, the lipid-free formulation produced the highest CBD concentrations). Both rapeseed and sesame oils contain high oleic acid content (Table 4.4 [151]), and it was discussed in Chapter 4 that the presence of the fatty acid in the formulations may be contributing to the high delivery, as oleic acid has been reported to increase BBB permeability.

The difference in concentration-time delivery profiles between the whole brain and anatomical brain regions reported herein demonstrates the need to investigate delivery to the region of interest within a tissue. The study of CBD delivery to anatomical brain regions described in Chapter 4 provides a detailed

understanding of the biodistribution of CBD following oral administration in lipid-free and lipid-based formulations. The discussion in the chapter shows how this information is useful, especially for molecules with vast therapeutic potential such as CBD [103-109]. The importance of in-depth biodistribution studies was similarly shown in Chapter 5, following oral administration of CBD to mice, and Chapter 6, following oral administration of 4'-F-CBD to rats.

The immunosuppressive effects of CBD were investigated following oral administration in a lipid-free and lipid-based formulation in an *in vivo* MS model (experimental autoimmune encephalomyelitis (EAE)). As for Chapter 4, the work in Chapter 5 demonstrated differences in the biodistribution of CBD to the brain following administration in different formulations in mice. The lipid-free formulation resulted in the only brain-serum ratio >1, however the exposure of the brain to CBD was not significantly different to the lipid-based formulations studied.

Improved delivery of CBD to the lymphatic system following oral administration in a lipid-based formulation (compared to a lipid-free formulation) has previously been demonstrated in rats [151] and more recently in mice (unpublished work). It was hypothesised that increased delivery of CBD to the lymphatic system could improve the immunosuppressive effects of the cannabinoid due to the process of MS development and migration into the central nervous system (CNS) *via* the lymphatic system [271-273]. The work in Chapter 5 showed that following oral administration of CBD in the lipid-based olive oil formulation, 16.7% of mice reached the highest clinical score of 4.0. Comparing this to administration of the olive oil vehicle only, 66.7% of mice reached the clinical score 4.0. Additionally, following administration of CBD in the lipid-free formulation, 75% of mice reached clinical score 4.0. This suggests that administration of CBD either slowed the progression of MS, or reduced the severity of symptoms, when administered in the olive oil formulation, but not in the lipid-free formulation. Administration of low doses of CBD does not result in the same immunosuppressive effects observed following higher doses [282]. Therefore, while the study is regarded as preliminary work as small sample sizes were used, the results support the hypothesis that high delivery to the lymphatic system could enhance the immunosuppressive effects of CBD.

In 2016, Breuer *et al* reported the synthesis of the fluorinated CBD derivative 4'-F-CBD [176]. Assessment of 4'-F-CBD in *in vivo* behavioural models has shown increased potency of the molecule over CBD [176, 177]. However, to the best of our knowledge, Chapter 6 reported the *in vivo* pharmacokinetics (PK) and biodistribution of 4'-F-CBD for the first time. The PK profile of 4'-F-CBD following oral administration was similar to that of CBD (previously reported by Feng *et al* [155]), with no significant differences between the cannabinoids PK parameters observed (Table 6.1). Administration of 4'-F-CBD in the sesame oil formulation resulted in a higher C_{max} and area under the curve (AUC) than the lipid-free formulation. However, there were no significant differences in the biodistribution of 4'-F-CBD to the brain and anatomical brain regions following administration in the two formulations. This was also true of the biodistribution to the lymphatic system, kidneys, liver, lungs, and spinal cord. 4'-F-CBD concentrations observed in the mesenteric lymph nodes (MLN) were higher than that previously reported for CBD following oral administration at the same molar dose [156]. Considering this with the results of Chapter 5, that suggest high delivery of CBD to the lymphatic system could enhance the immunosuppressive effects of the molecule, if 4'-F-CBD were to demonstrate similar immunosuppressive effects to CBD, there is potential for even greater efficacy against MS with 4'-F-CBD.

Following oral administration of 4'-F-CBD in the lipid-free formulation, the delivery to the whole brain was similar to that of CBD, with no significant differences observed. However, following administration in the sesame oil formulation, CBD exhibited a higher C_{max} , but there was a higher exposure of the brain tissue to 4'-F-CBD. This is explained by the concentration-time profiles (Figure 6.6), where following administration of CBD, the peak in concentration is well pronounced, whereas for 4'-F-CBD, the concentrations remain largely the same over time. This resulted in higher exposure of the whole brain to 4'-F-CBD than CBD. This supports reports of other molecules demonstrating increased BBB permeability when fluorinated [173-175], and is likely at least partly responsible for the increased potency of 4'-F-CBD over CBD observed in *in vivo* behavioural assays [176, 177]. This pattern was also observed for the anatomical brain regions studied (Figure 6.7).

A potential application of 4'-F-CBD that has been investigated herein is against cancer. Chapter 3 demonstrated that 4'-F-CBD was as potent as CBD in inhibiting the GBM cell growth in cells sensitive to TMZ, cells exhibiting MGMT over-expression and cells with a deficiency of MMR. 4'-F-CBD also demonstrated synergy with TMZ and T25, just like CBD. However, the combination indices (CIs) of 4'-F-CBD with T25 were lower than for CBD with T25, suggesting enhanced synergy of T25 with 4'-F-CBD. Similarly, as for CBD, biodistribution to the anatomical brain regions where GBM tumours commonly occur (frontal, parietal and temporal lobes [90, 229, 230]) was studied. Again, using the temporal lobe as an example, the exposure of the region to 4'-F-CBD was $4744 \pm 600 \text{ h*ng/g}$ ($0.007 \text{ h*}\mu\text{M}$). This is slightly higher than the exposure of the region to CBD (Table 4.2), discussed above. Chapter 3 demonstrated 4'-F-CBD to have a similar GI_{50} to CBD against GBM cells ($29 \mu\text{M}$) and a higher CI value with T25, indicating that a sufficient 4'-F-CBD concentration could be reached in the region of interest (the temporal lobe) to inhibit GBM cell growth following a lower dose than CBD. However, the safety of 4'-F-CBD has not been studied in humans, and as for the discussion above for CBD, *in vivo* efficacy of 4'-F-CBD has not yet been confirmed.

The work in this thesis has used several traditional techniques such as *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and high performance liquid chromatography (HPLC). The 3D Orbitrap secondary ion mass spectrometry (OrbiSIMS) is a relatively new technique for which the applications are still being explored [186-188]. In Chapter 3, the 3D OrbiSIMS was used to investigate the anti-cancer mechanism of action of CBD, a novel application of the instrument. Chapter 4 also investigated the biodistribution of CBD in the brain by 3D OrbiSIMS, studying the correlation between the technique and HPLC analysis. Whilst the biodistribution study by 3D OrbiSIMS was inconsistent with the HPLC analysis, the use of the instrument to investigate differences between treated and non-treated cells, thereby identifying potential mechanisms of action, did prove beneficial. The analysis was able to provide information previously unknown about cancer treatment with CBD, and has provided useful direction for future work (discussed further in Chapter 8).

7.2 General conclusions

The ability of CBD to inhibit GBM cell growth alone were reported. The ability of CBD alone to overcome a major mechanism of resistance to the standard GBM treatment (TMZ), an over-expression of MGMT, was demonstrated for the first time. Synergy between CBD and TMZ, and T25, were also demonstrated against GBM TMZ-sensitive cells, and the two major resistance mechanisms (MGMT over-expression and a deficiency of MMR). This work also begun to investigate the mechanism of anti-cancer activity of CBD, where DNA-methylation was indicated as at least one of the pathways of CBD anti-cancer activity for the first time. Other changes in GBM cells following exposure to CBD were also observed, including changes in fatty acid content, which can be indicators of treatment effectiveness *in vivo*.

The ability to deliver CBD to the common locations of GBM tumours (frontal, parietal and temporal lobes) was demonstrated following oral administration at a low range dose in both lipid-based and lipid-free formulations. CBD was delivered to the brain regions as fast as 1 h following administration. The biodistribution of CBD to anatomical brain regions was also reported for the first time. Oral administration in a lipid-based sesame oil formulation often produced the highest exposure of the rostral regions of the brain to CBD, whereas administration in a lipid-free formulation resulted in high CBD concentrations towards the caudal area of the brain. The study of delivery to anatomical brain regions in this work demonstrates the importance of studying biodistribution to specific regions of tissues and is the first reporting of detailed biodistribution of CBD in the brain following oral administration.

The biodistribution of CBD in the brain following oral administration in different lipid-based formulations demonstrated that the myristic acid-containing coconut oil formulation did not result in increased delivery of CBD to the brain, compared to the lipid-free formulation. However, the sesame and rapeseed oil formulations, both of which contain oleic acid, did demonstrate increased delivery to some anatomical brain regions. This is in line with reports in the literature that oleic acid can increase the permeability of molecules across

the BBB [62, 66], however the oils used herein have complex compositions, and therefore the impact of fatty acid presence is speculative.

It was demonstrated herein that oral administration of CBD in an olive oil formulation resulted in a lower percentage of animals reaching the highest clinical score in an *in vivo* MS model (EAE) compared to the vehicle only control group. This trend was not observed when CBD was administered in the lipid-free formulation. While the work in the MS model was preliminary, it suggests support for the hypothesis that increased delivery of CBD to the lymphatic system results in increased immunosuppressive effects during the development of MS.

Oral administration of 4'-F-CBD in the lipid-based sesame oil formulation resulted in higher plasma exposure of the cannabinoid, compared to the lipid-free formulation, similar to CBD [155]. To the best of our knowledge, this is the first study of 4'-F-CBD PK, demonstrating similar systemic exposure to CBD following oral administration. The biodistribution of 4'-F-CBD was found to differ from CBD. The concentrations of 4'-F-CBD in the brain were consistent over the 18 h studied, resulting in higher exposure of the tissue to 4'-F-CBD, compared to CBD. This work provides an understanding of the *in vivo* PK and biodistribution of 4'-F-CBD for the first time, which will be necessary to further develop the therapeutic application of the molecule.

The anti-cancer activity of 4'-F-CBD was demonstrated for the first time. 4'-F-CBD was shown to inhibit GBM cell growth with similar potency to CBD, as well as overcoming the two major resistance mechanisms to GBM treatment with TMZ, MGMT over-expression and MMR deficiency. A synergistic response was observed when cells were treated with 4'-F-CBD in combination with TMZ or T25. The CIs of 4'-F-CBD with T25 were lower than that of CBD with T25, indicating increased synergy between T25 and 4'-F-CBD.

Overall, this PhD aimed to study the *in vitro* and *in vivo* properties of CBD and 4'-F-CBD, focussing on the anti-cancer application of the molecules. Both cannabinoids were shown to exhibit high activity against GBM *in vitro*, and good delivery to the location of common GBM tumours *in vivo*. The in-depth biodistribution of the cannabinoids to anatomical brain regions following oral

administration highlights the importance of studying biodistribution to the region of interest within a tissue. The impact of the formulation choice was also demonstrated, where the application of lipid-based oral formulations for delivery to the brain was shown to be feasible.

CHAPTER 8: Future work

8.1 Anti-cancer properties of cannabinoids

Chapter 3 discussed the anti-cancer properties of cannabinoids cannabidiol (CBD) and 4'-fluoro-cannabidiol (4'-F-CBD). While the results in Chapter 3 demonstrate the ability of the cannabinoids to inhibit glioblastoma multiforme (GBM) cell growth, the mechanism of this activity warrants further investigation. There are reports of suspected mechanisms of anti-cancer activity of CBD, including *via* transient receptor potential cation channel subfamily V member 2 (TRPV-2), increased reactive oxygen species (ROS), endoplasmic reticulum (ER) stress and C^5 -cytosine methylation in cytosine-phosphate-guanine (CpG) islands, as well as interaction with the endocannabinoid system [125, 143-147]. Chapter 3 included initial investigations into the mechanism of anti-cancer action of CBD, indicating activity *via* methylated- deoxyribonucleic acid (DNA). Chapter 3 also discussed preliminary studies investigating the mechanism of combination treatment for GBM with CBD and temozolomide (TMZ). The fatty acid content of the GBM cells treated with CBD and TMZ in combination suggest that an oxidative stress pathway is initiated in the cells [221]. However, the data in Chapter 3 only indicate this pathway, and future studies could investigate this further. Future work could involve further studies by 3D Orbitrap secondary ion mass spectrometry (3D OrbiSIMS), where the instrument could be optimised for the detection of certain fatty acids of interest. Additionally, investigation by *in vitro* techniques could demonstrate activity *via* the ROS pathway.

The synergistic activity observed for GBM cells treated with CBD and TMZ has previously been reported in the literature [131, 132]. It has been suggested that CBD enhances the uptake of TMZ into cells *via* the TRPV-2 pathway [131]. As discussed in Chapter 3, the investigation of the anti-cancer mechanism of action of CBD herein could not study the potential increased uptake of TMZ into cells. Investigation was conducted by 3D OrbiSIMS analysis, where the instrument was optimised to detect CBD. CBD is best detected in negative mode analysis, whereas the differences in the structures of CBD and TMZ (shown in Figures

1.5 and 1.8) indicate that TMZ is likely best detected in positive mode analysis [288]. Repeated 3D OrbiSIMS analysis using conditions optimised for the detection of TMZ could investigate if CBD does enhance the uptake of TMZ into GBM cells. Alternatively, a more conventionally quantitative technique, such as liquid chromatography mass spectrometry (LC-MS) could be used.

Chapter 3 focussed on the investigation of anti-cancer activity of CBD, and therefore, the subsequent study of the mechanism of action also focussed on CBD. Investigation of the mechanism of CBD activity by 3D OrbiSIMS discussed in Chapter 3 represents preliminary studies to demonstrate the potential of this technique in this application. Due to time constraints as a result of lockdowns during the COVID-19 pandemic, 3D OrbiSIMS analysis of cells treated with CBD was prioritised. Therefore, analysis of cells treated with T25 alone, to compliment the study of cells treated with a combination of CBD and T25, could not be included in this work. However, this demonstrates a clear next step for this work, to compare samples of cells treated with CBD and T25 to those treated with T25 alone, as well as investigation of the mechanism of activity, starting with the activity suggested for the combination of CBD with TMZ [131].

To the best of our knowledge, the anti-cancer properties of 4'-F-CBD had not been investigated before this work. Chapter 3 demonstrates the ability of 4'-F-CBD to inhibit GBM cell growth, overcome two common resistance mechanisms to the standard GBM treatment with TMZ, and showed a synergistic response when administered with imidazotetrazine agents TMZ and T25. As mentioned, the focus of Chapter 3 was on cannabidiol activity, and therefore the work did not investigate the mechanism of 4'-F-CBD activity. Whilst the *in vivo* pharmacokinetics (PK) of 4'-F-CBD and CBD were shown to be similar in Chapter 6, the chapter also demonstrated differences in the *in vivo* biodistribution of the cannabinoids. Reports in the literature of 4'-F-CBD activity in *in vivo* behavioural assays also demonstrate differences in the activity of the molecules [176-180], suggesting that 4'-F-CBD is more potent than CBD [176-180]. This suggests that while it is reasonable to suggest that 4'-F-CBD acts *via* similar mechanisms to CBD, it is equally reasonable to suggest that 4'-

F-CBD activity could be *via* different mechanisms. Investigation of this could be studied by techniques including 3D OrbiSIMS and *in vitro* assays.

The work discussed in this thesis demonstrates the anti-cancer activity of cannabinoids against GBM, and *in vivo* biodistribution of cannabinoids to the anatomical brain regions where GBM tumours are commonly located. In the development of cannabinoids for treatment of GBM, one of the next studies would be to investigate anti-cancer activity in an *in vivo* model. *In vivo* GBM models have been used to study activity of CBD activity previously, in combination with TMZ, Δ^9 -tetrahydrocannabinol (THC) or radiotherapy [103, 127, 133-136]. Chapter 3 demonstrated the high levels of synergy between CBD and T25, and 4'-F-CBD and T25. Therefore, proposed future work includes study of the activity of combination treatment of cannabinoids CBD or 4'-F-CBD with T25 in an *in vivo* model of GBM.

8.2 Oral lipid-based cannabinoid formulations

Chapters 4 – 6 discuss the different biodistribution of cannabinoids following oral administration in lipid-based formulations, compared to lipid-free vehicles. Chapter 5 studied a potential application of this for the treatment of multiple sclerosis (MS). Improved delivery of CBD to the lymphatic system following oral administration in a lipid-based formulation in mice (unpublished work) correlated with reduced severity of MS symptoms in an *in vivo* experimental autoimmune encephalomyelitis (EAE) model, when compared to the group that were administered vehicle only (Table 5.3). However, Chapter 4 investigated different lipid-based formulations further. High delivery of CBD to specific anatomical brain regions was correlated with fatty acid content of the lipid-based formulations, suggesting this work may support other reports of fatty acids such as oleic and linoleic acids increasing permeability of the blood-brain barrier (BBB) [62, 66, 189, 232]. However, work herein did not study oral administration of formulations containing only one fatty acid, but rather natural vegetable oil formulations, which have complex compositions [151]. Therefore, future work should investigate the different delivery of CBD to the brain in

formulations containing only the fatty acid of interest (oleic, linoleic or myristic acid), compared to a lipid-free formulation.

There is growing interest in the differences in *in vivo* studies between male and female animals. There are reported differences in the digestion of lipids, effects of cannabinoids, *in vivo* MS models and response to TMZ treatment between the sexes [269, 289-291]. The work in Chapters 4 and 6 were conducted in male rats, while work in Chapter 5 was conducted in female mice. It would be beneficial while work continues forwards to also conduct these studies in animals of the other sex. There is growing support that the inequalities in health research begins in *in vitro* and *in vivo* studies, and therefore change should occur at this point [292, 293]. Conducting the studies in this thesis in animals of the other sex would progress understanding in the differences in lipid digestion and biodistribution between the sexes, as well as efficacy of new treatments, and work towards reducing the gender health inequalities.

8.3 Oral lipid-based cannabidiol formulation for multiple sclerosis treatment

Chapter 5 investigated the effect of improved lymphatic delivery on the immunosuppressive effects of CBD against an *in vivo* MS model, EAE. The work in this chapter showed promise that increased lymphatic delivery of CBD following oral administration in a lipid-based formulation might improve the immunosuppressive effects of the cannabinoid. However, as discussed in Chapter 5, the work in the *in vivo* EAE model was a preliminary study. Therefore, there is future work to be done here to further understanding of this potential application of CBD. Firstly, the study could be repeated to increase the group sizes and confirm the differences observed. Additionally, adverse reaction to administration by oral gavage was observed, and therefore the control (no dose) group should receive oral gavage dosing of water to be a true control group. Secondly, as discussed in Chapter 5, the dose of CBD used in this work is a low range dose, and therefore, in order to maximise differences between groups (so that they may be observed more clearly) a higher dose of CBD could be used. Thirdly, biodistribution is understood to differ in disease state,

particularly the permeability of the BBB [21, 36, 37]. Therefore, it would improve understanding of the impact of biodistribution on the immunosuppressive effects of CBD observed, to study the biodistribution in animals in the disease state. Finally, the work in the *in vivo* EAE model was conducted using the lipid-free and lipid-based olive oil formulations. However, sesame oil formulations have also been demonstrated to result in high delivery of CBD to the lymphatic system and specific anatomical brain regions [151, 156]. Therefore, it would be interesting to examine the effects of administering CBD in a sesame oil formulation in the *in vivo* EAE model.

8.4 Potential of 4'-fluoro-cannabidiol

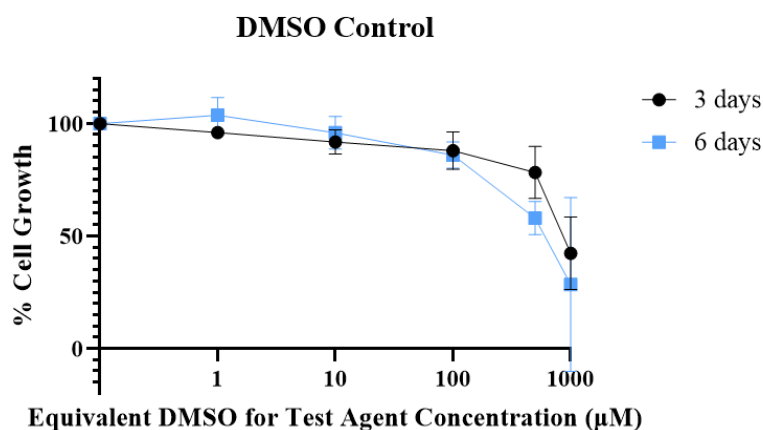
Previous reports in the literature have demonstrated the therapeutic potential of 4'-F-CBD [176-178, 204]. Chapter 3 demonstrated further potential of 4'-F-CBD for GBM treatment, and Chapter 6 showed the high exposure of the brain to 4'-F-CBD, compared to CBD, following oral administration. To the best of our knowledge, Chapter 6 is the first reporting of the *in vivo* PK and biodistribution of 4'-F-CBD, and this is a hugely important step towards developing the molecule into a therapeutic. However, the work herein investigated only oral administration of 4'-F-CBD. In order to understand the bioavailability of the cannabinoid, *in vivo* studies will need to be conducted following intravenous (i.v.) administration. When considering the range of potential applications of 4'-F-CBD (for example for cancer, Alzheimer's disease (AD), MS, anxiety, depression), it would also be beneficial to understand *in vivo* PK parameters following other administration routes, such as subcutaneous (s.c.) and intraperitoneal (i.p.).

Chapter 6 demonstrated higher exposure of brain tissue to 4'-F-CBD than CBD following oral administration. However, it cannot be understood from the studies herein whether this is a result of increased delivery across the BBB, or increased retention within the brain tissue of 4'-F-CBD. Studies of the permeability of 4'-F-CBD and CBD across an *in vitro* BBB model would begin to progress understanding of the differences between the cannabinoids, whilst minimising the *in vivo* studies that are necessary.

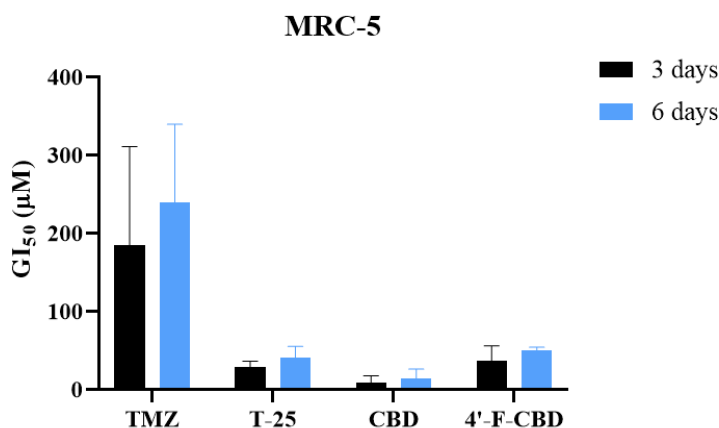
The potential of the fluorine atom in 4'-F-CBD has not been fully explored in this work. Fluorine atoms offer the option to radiolabel molecules, and study biodistribution by positron emission topography (PET) imaging *in vivo*. Future *in vivo* biodistribution studies of radiolabelled 4'-F-CBD could provide additional understanding of the biodistribution of the cannabinoid.

APPENDICES

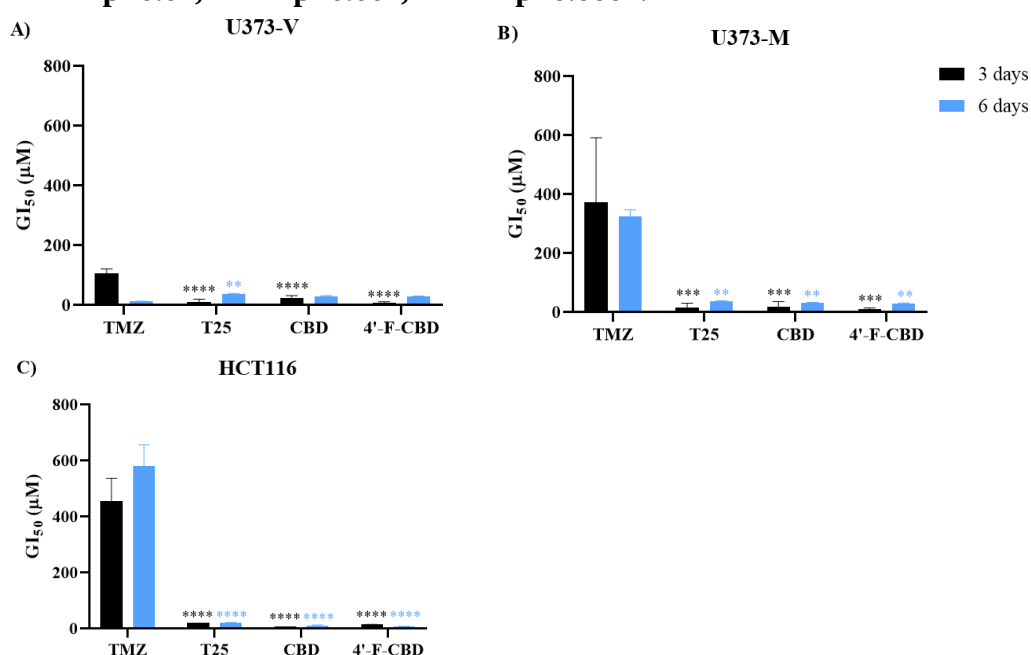
Appendix 3.1. Dose-response curve of MRC-5 (non-tumourigenic control) cell line dosed with an equivalent dimethyl sulfoxide (DMSO) percentage for the test agent concentrations tested after 3- and 6-days exposure. Cell growth represented as percentage of control (non-treated). The data are measured by MTT assay and presented as mean $\pm$ standard deviation (SD), 3 independent repeats of $n = 5$.



Appendix 3.2. Concentration required to inhibit cell growth by 50% (GI_{50}) of temozolomide (TMZ), T-25, cannabidiol (CBD) and 4'-fluorocannabidiol (4'-F-CBD) against MRC-5 (non-tumourigenic control) cell line after 3- and 6-days exposure. The data are measured by cell count assay and MTT (shown in Figure 3.1). Data presented as mean $\pm$ SD, 3 independent repeats of $n = 3$.



Appendix 3.3. GI₅₀ of cannabinoids CBD and 4'-F-CBD compared to deoxyribonucleic acid (DNA)-alkylating agents TMZ and T25 against A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after 3- and 6-days exposure. The data are measured by cell count assay (MTT assay data shown in Figure 3.2) and presented as mean $\pm$ SD, 3 independent repeats of $n = 3$. One-way ANOVA with Dunnett's multiple comparisons performed, comparing test agents to TMZ, $\alpha = 0.05$, ** = $p < 0.01$, * = $p < 0.001$, **** = $p < 0.0001$.**



Appendix 3.4. The concentrations of test agents (TMZ, T25, CBD and 4'-F-CBD) tested in the 3-days combination assays.

% GI ₅₀	Test agent (μM)			
	TMZ	T25	CBD	4'-F-CBD
U373-V				
100	147	43	33	29
1.25	1.8	0.5	0.4	0.4
2.5	3.7	1.1	0.8	0.7
5	7.4	2.2	1.7	1.5

10	14.7	4.3	3.3	2.9
20	29.4	8.6	6.6	5.8
50	73.5	21.5	16.5	14.5
U373-M				
100	663	31	30	25
1.25	8.3	0.4	0.4	0.3
2.5	16.6	0.8	0.8	0.6
5	33.2	1.6	1.5	1.3
10	66.3	3.1	3.0	2.5
20	132.6	6.2	6.0	5.0
50	331.5	15.5	15.0	12.5
HCT116				
100	610	39	13	27
1.25	7.6	0.5	0.2	0.3
2.5	15.3	1.0	0.3	0.7
5	30.5	2.0	0.7	1.4
10	61.0	3.9	1.3	2.7
20	122.0	7.8	2.6	5.4
50	305.0	19.5	6.5	13.5

Appendix 3.5. The concentrations of test agents (TMZ, T25, CBD and 4'-F-CBD) tested in the 6-days combination assays.

% GI ₅₀	Test agent			
	TMZ	T25	CBD	4'-F-CBD
U373-V				
100	10	60	43	49
1.25	0.1	0.8	0.5	0.6
2.5	0.3	1.5	1.1	1.2
5	0.5	3.0	2.2	2.5
10	1.0	6.0	4.3	4.9

20	2.0	12.0	8.6	9.8
50	5.0	30.0	21.5	24.5
U373-M				
100	332	41	23	25
1.25	4.2	0.5	0.3	0.3
2.5	8.3	1.0	0.6	0.6
5	16.6	2.1	1.2	1.3
10	33.2	4.1	2.3	2.5
20	66.4	8.2	4.6	5.0
50	166.0	20.5	11.5	12.5
HCT116				
100	609	37	15	6
1.25	7.6	0.5	0.2	0.1
2.5	15.2	0.9	0.4	0.2
5	30.5	1.9	0.8	0.3
10	60.9	3.7	1.5	0.6
20	121.8	7.4	3.0	1.2
50	304.5	18.5	7.5	3.0

Appendix 3.6. Full combination index (CI) data of cannabinoids CBD and 4'-F-CBD administered in combination with TMZ or analogue, T25, after 3- or 6-days exposure against U373-V, U373-M and HCT116 cell lines. The data are presented as mean, 3 independent repeats of n = 5.

Exposure time (days)	Test Agent A	Test Agent B	Test Agent B Concentration (% GI ₅₀)	Cell line		
				U373-V	U373-M	HCT116
3	TMZ	CBD	1.25	0.10	-	-
			2.5	0.12	-	-
			5	0.11	-	-
			10	0.48	-	0.11
			20	0.68	-	0.22

6	CBD	TMZ	30	0.34	0.35	-
			40	0.44	0.40	-
			50	0.54	0.50	0.51
			1.25	0.27	0.23	0.83
			2.5	0.26	0.24	0.82
			5	0.35	0.25	0.67
			10	0.50	0.33	0.93
			20	0.54	0.45	0.91
			50	0.89	0.74	1.06
	TMZ	CBD	1.25	0.67	-	-
			2.5	0.76	-	-
			5	0.57	-	0.05 (1 repeat)
			10	0.34	0.28	0.10 (1 repeat)
			20	0.29	0.28	0.22 (2 repeats)
			50	0.66	0.69	0.50 (1 repeat)
			1.25	0.12	0.22	0.45
			2.5	0.12	0.21	0.49
			5	0.14	0.25	0.59
			10	0.22	0.30	0.78
3	TMZ	4'-F-CBD	20	0.30	0.38	0.74
			50	0.61	0.74	0.95
			1.25	-	-	-
			2.5	-	-	-
			5	-	-	-
			10	1.01	0.22	-
			20	0.24	0.20	-
			50	0.54	0.50	0.50
	4'-F-CBD	TMZ	1.25	0.20	0.13	0.37
			2.5	0.16	0.13	0.37
			5	0.19	0.16	0.46

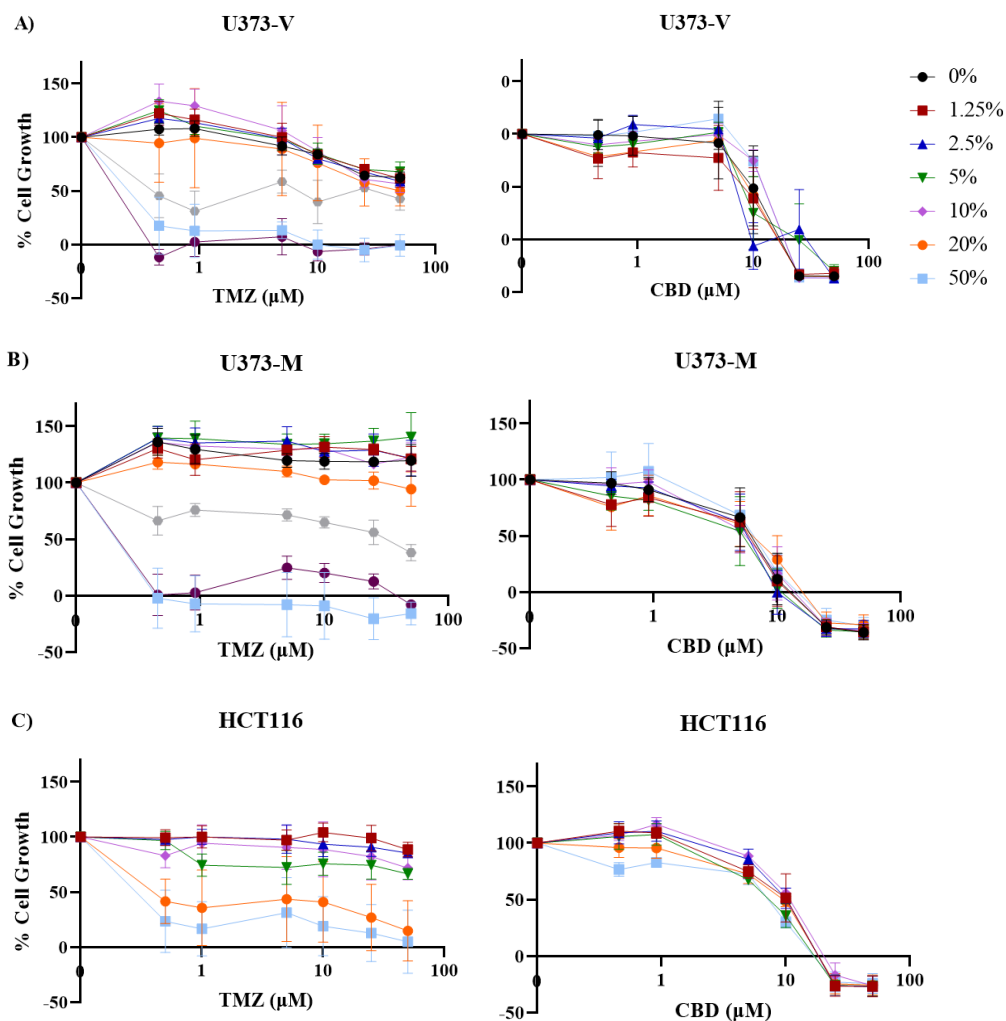
6	TMZ 4'-F- CBD		10	0.25	0.20	0.48
			20	0.34	0.28	0.65
			50	0.56	0.59	0.74
			1.25	0.99	-	-
			2.5	0.11	-	-
			5	0.09	0.12	-
			10	0.14	0.10	-
			20	0.24	0.20	-
			50	0.54	0.50	0.50
			1.25	0.09	0.05	0.48
			2.5	0.11	0.06	0.51
			5	0.12	0.08	0.51
			10	0.18	0.15	0.56
			20	0.28	0.24	0.67
			50	0.53	0.53	0.79
			1.25	0.33	0.36	0.65
			2.5	0.43	0.34	0.76
			5	0.41	0.40	0.65
3	T25 CBD		10	0.59	0.38	0.83
			20	0.53	0.43	0.74
			50	0.51	0.51	0.75
			1.25	0.36	0.22	0.53
			2.5	0.50	0.31	0.54
			5	0.48	0.35	0.57
			10	0.52	0.38	0.77
			20	0.51	0.46	0.68
			50	0.65	0.57	0.93
			1.25	0.43	0.75	0.57
			2.5	0.41	0.53	0.46
			5	0.32	0.41	0.52
3	T25 CBD	4'-F- CBD	10	0.36	0.49	0.46
			20	0.26	0.31	0.37
			50	0.51	0.51	0.52
	4'-F- CBD	T25	1.25	0.21	0.24	0.53
			2.5	0.26	0.20	0.53

	5	0.27	0.23	0.56
	10	0.32	0.19	0.62
	20	0.33	0.29	0.68
	50	0.52	0.52	0.79

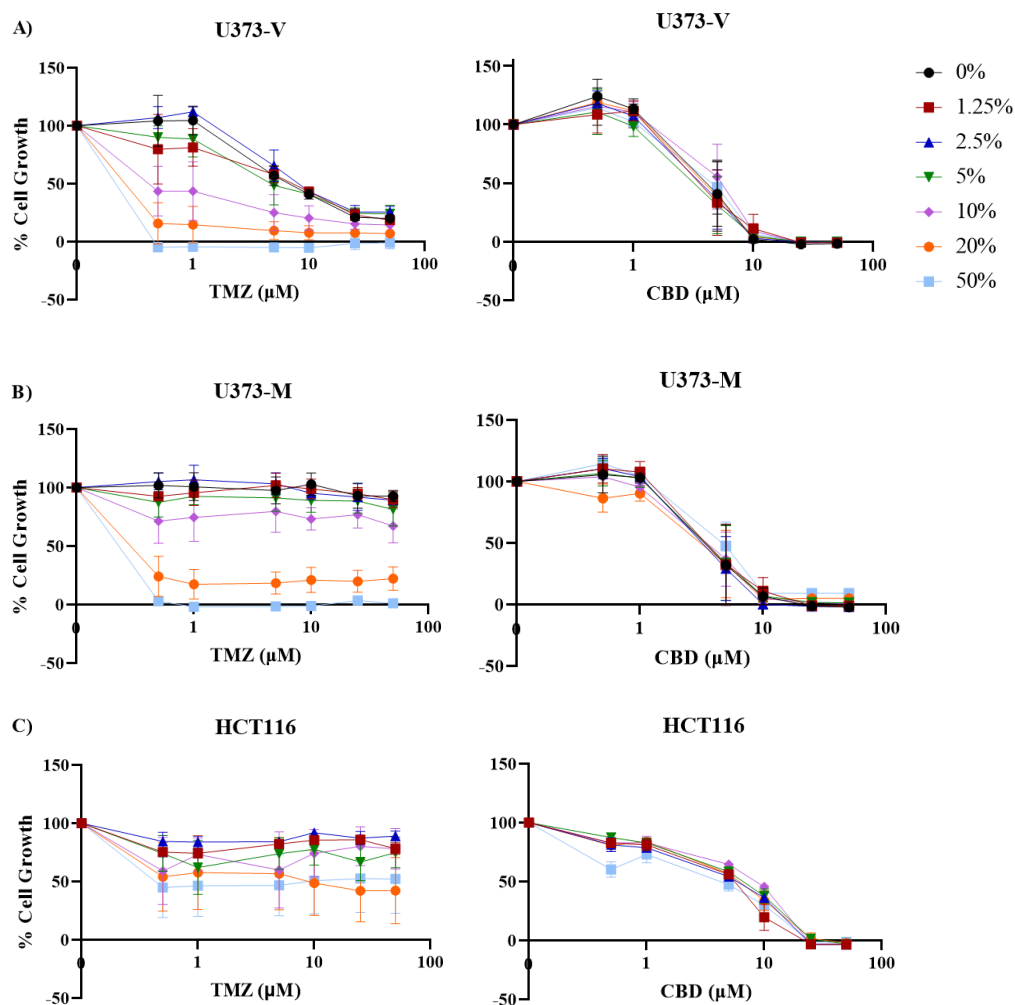
Appendix 3.7. Cell growth represented as percentage of control after treatment with cannabinoids, CBD or 4'-F-CBD, with DNA-alkylating agent TMZ or analogue, T25, after 3- or 6-days exposure. The data are measured by cell count assay at concentrations of 25% GI₅₀ and presented as mean $\pm$ SD, 3 independent repeats of n = 3.

Test Agent A	Test Agent B	Dosing time (days)	Cell line (% cell growth)		
			U373-V	U373-M	HCT116
TMZ	CBD	3	32 $\pm$ 11	40 $\pm$ 5	31 $\pm$ 13
TMZ	CBD	6	31 $\pm$ 7	50 $\pm$ 22	27 $\pm$ 5
TMZ	4'-F-CBD	3	34 $\pm$ 3	27 $\pm$ 9	19 $\pm$ 8
TMZ	4'-F-CBD	6	7 $\pm$ 2	8 $\pm$ 4	63 $\pm$ 9
T25	CBD	3	34 $\pm$ 13	27 $\pm$ 8	13 $\pm$ 12
T25	4'-F-CBD	3	28 $\pm$ 7	16 $\pm$ 8	12 $\pm$ 7

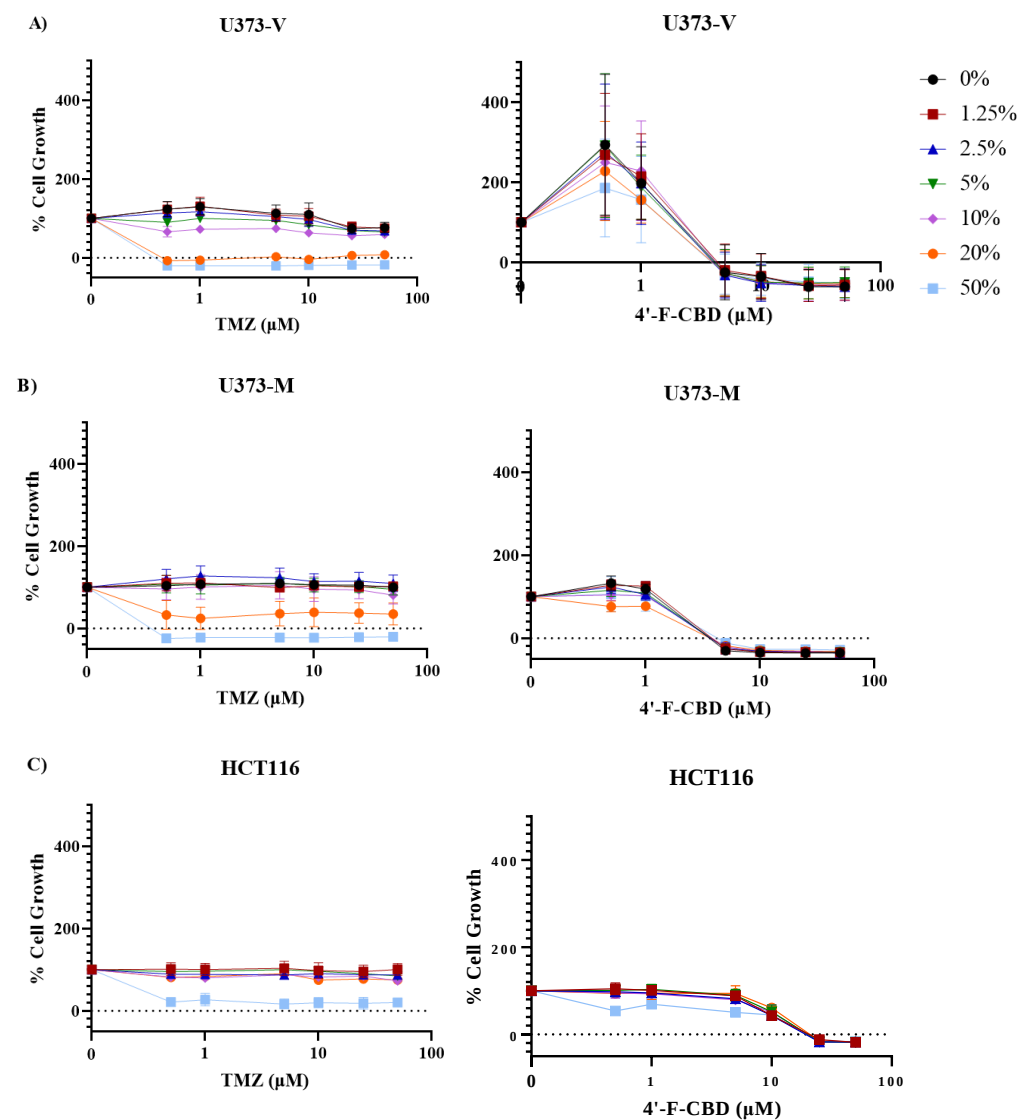
Appendix 3.8. Dose-response curves of A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after combination treatment with TMZ and CBD after 3-days treatment. Cell growth represented as percentage of control (non-treated). Test agent A shown on the x-axis and test agent B shown as %GI₅₀. Data measured by MTT assay and presented as mean $\pm$ SD, 3 independent repeats of n=5.



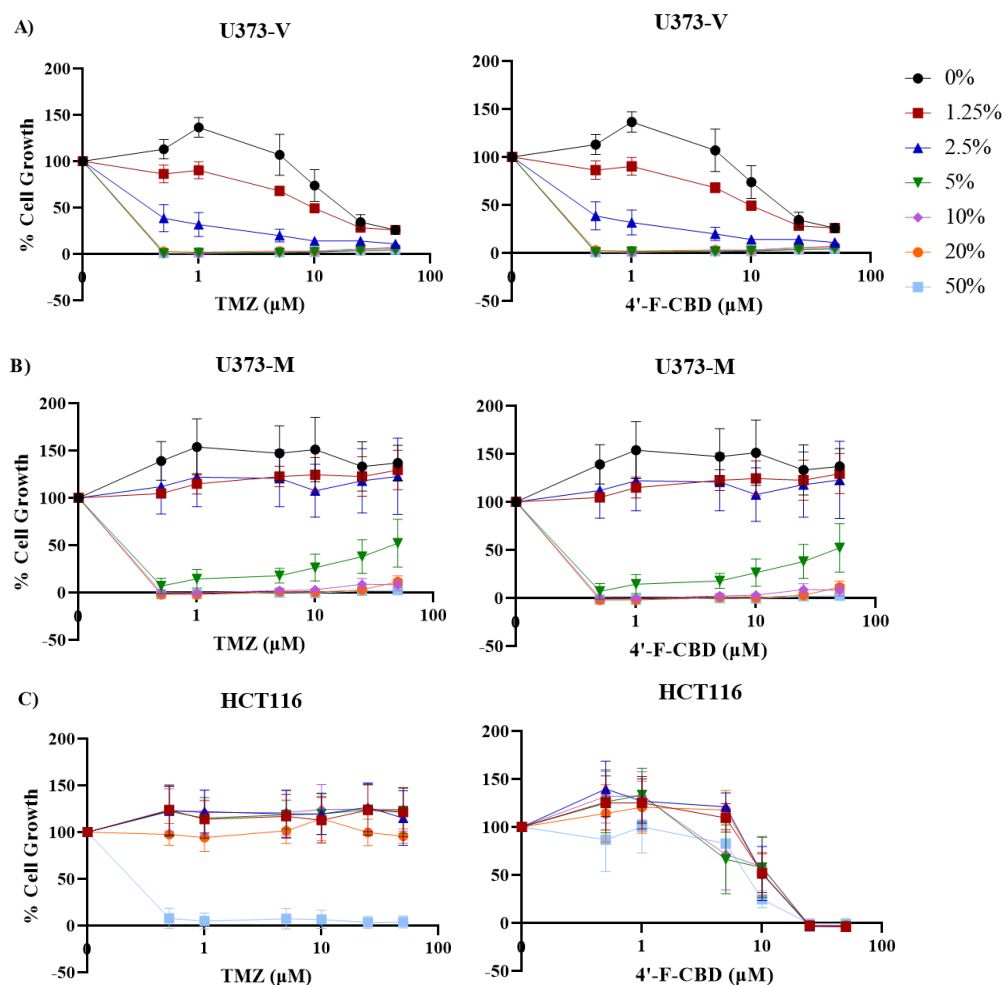
Appendix 3.9. Dose-response curves of A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after combination treatment with TMZ and CBD after 6-days treatment. Cell growth represented as percentage of control (non-treated). Test agent A shown on the x-axis and test agent B shown as %GI₅₀. The data are measured by MTT assay and presented as mean $\pm$ SD, 3 independent repeats of n = 5.



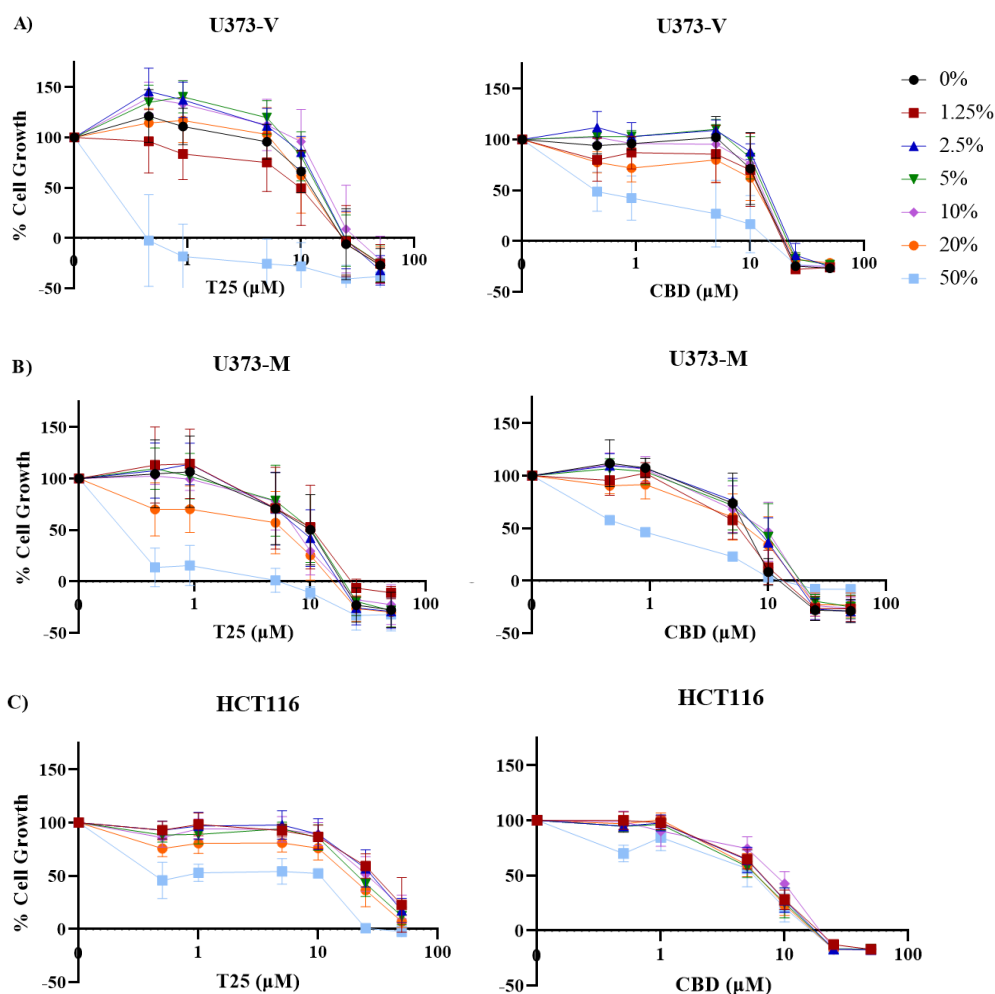
Appendix 3.10. Dose-response curves of A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after combination treatment with TMZ and 4'-F-CBD after 3-days treatment. Cell growth represented as percentage of control (non-treated). Test agent A shown on the x-axis and test agent B shown as %GI₅₀. The data are measured by MTT assay and presented as mean $\pm$ SD, 3 independent repeats of n = 5.



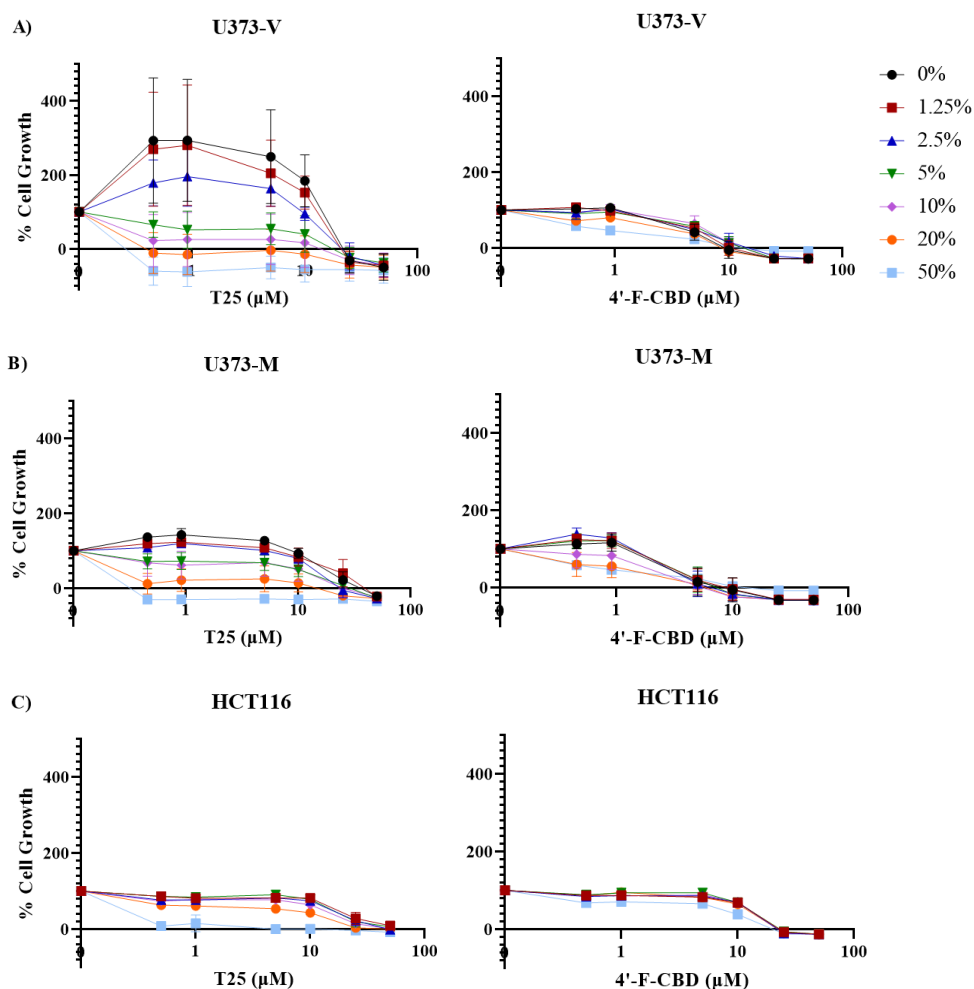
Appendix 3.11. Dose-response curves of A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after combination treatment with TMZ and 4'-F-CBD after 6-days treatment. Cell growth represented as percentage of control (non-treated). Test agent A shown on the x-axis and test agent B shown as %GI₅₀. The data are measured by MTT assay and presented as mean $\pm$ SD, 3 independent repeats of n = 5.



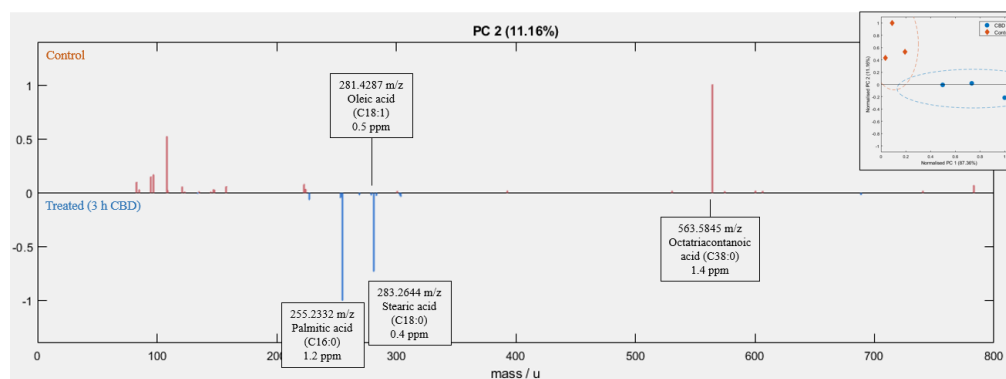
Appendix 3.12. Dose-response curves of A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after combination treatment with T25 and CBD after 3-days treatment. Cell growth represented as percentage of control (non-treated). Test agent A shown on the x-axis and test agent B shown as %GI₅₀. The data are measured by MTT assay and presented as mean $\pm$ SD, 3 independent repeats of n = 5.



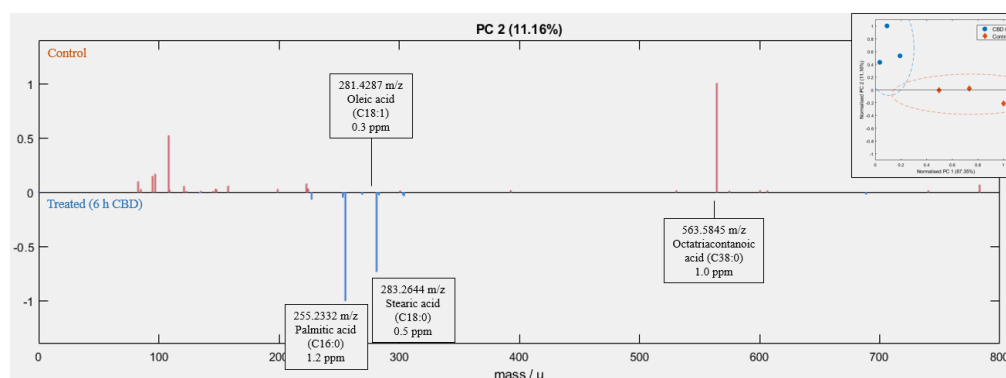
Appendix 3.13. Dose-response curves of A) U373-V (GBM control, -MGMT, +MMR), B) U373-M (GBM, +MGMT, +MMR) and C) HCT116 (-MGMT, -MMR) after combination treatment with T25 and 4'-F-CBD after 3-days treatment. Cell growth represented as percentage of control (non-treated). Test agent A shown on the x-axis and test agent B shown as %GI₅₀. The data are measured by MTT assay and presented as mean $\pm$ SD, 3 independent repeats of n = 5.



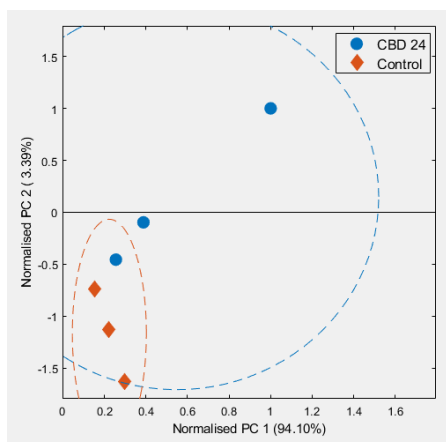
Appendix 3.14. Principal component analysis (PCA) of OrbiSIMS data of U373-V cells treated with CBD (33 μ M) for 3 h compared to the non-treated control. Principal component (PC) 2 is shown to demonstrate the largest difference between the samples (top right box). PC2 is shown with the control sample in orange and treated sample in blue. Key fatty acids are identified and their mass (m/z) and peak deviation (ppm) are shown.



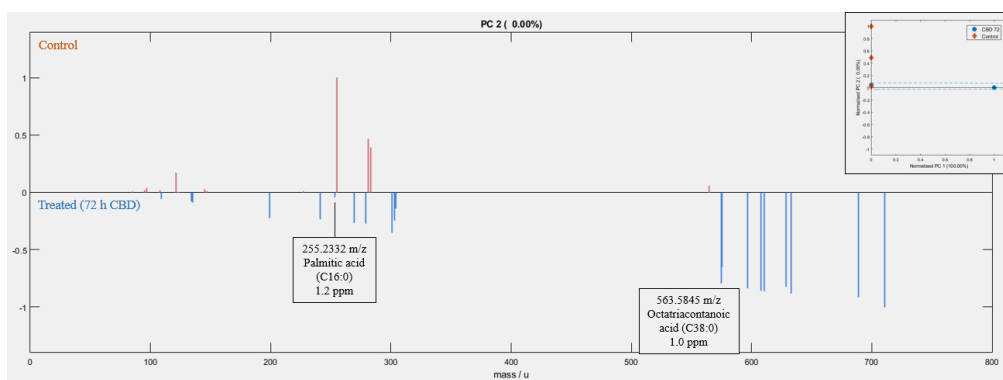
Appendix 3.15. PCA of OrbiSIMS data of U373-V cells treated with CBD (33 μ M) for 6 h compared to the untreated control. PC2 is shown to demonstrate the largest difference between the samples (top right box). PC2 is shown with the control sample in orange and treated sample in blue. Key fatty acids are identified and their mass (m/z) and peak deviation (ppm) are shown.



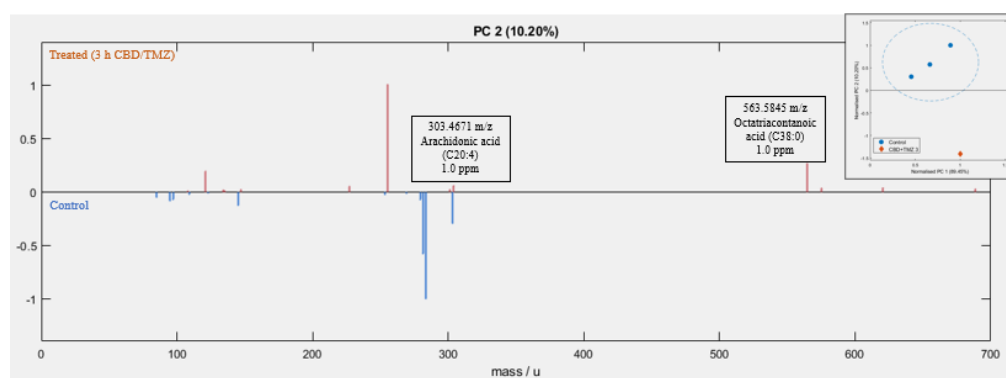
Appendix 3.16. PCA of OrbiSIMS data of U373-V cells treated with CBD (33 μ M) for 24 h compared to the untreated control. No PCs showed large differences between the two samples.



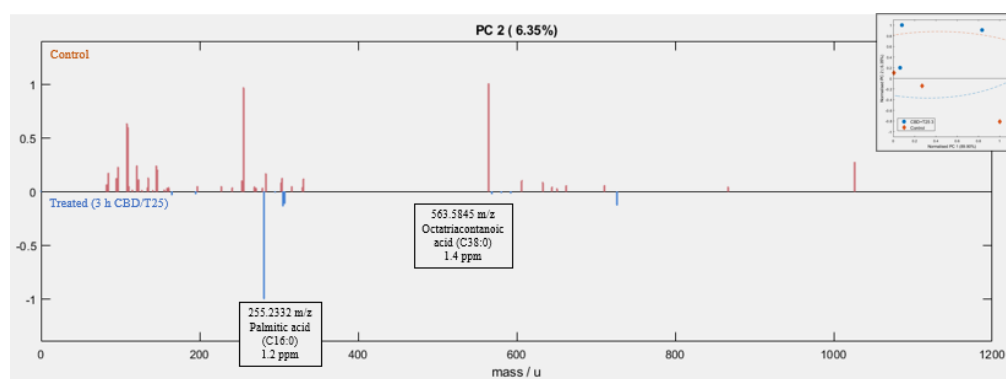
Appendix 3.17. PCA of OrbiSIMS data of U373-V cells treated with CBD (33 μ M) for 72 h compared to the untreated control. PC2 is shown to demonstrate the largest difference between the samples (top right box). PC2 is shown with the control sample in orange and treated sample in blue. Key fatty acids are identified and their mass (m/z) and peak deviation (ppm) are shown.



Appendix 3.18. PCA of OrbiSIMS data of U373-V cells treated with CBD (11 μ M) and TMZ (2 μ M) combination for 3 h compared to the untreated control. PC2 is shown to demonstrate the largest difference between the samples (top right box). PC2 is shown with the control sample in orange and treated sample in blue. Key fatty acids are identified and their mass (m/z) and peak deviation (ppm) are shown.



Appendix 3.19. PCA of OrbiSIMS data of U373-V cells treated with CBD (7 μ M) and T25 (14 μ M) combination for 3 h compared to the untreated control. PC2 is shown to demonstrate the largest difference between the samples (top right box). PC2 is shown with the control sample in orange and treated sample in blue. Key fatty acids are identified and their mass (m/z) and peak deviation (ppm) are shown.



REFERENCES

- [1] R. L. Gutman, G. Peacock, D. R. Lu, Targeted drug delivery for brain cancer treatment, *J. Control. Release*, 65 (2000) 31-41. [https://doi.org/10.1016/S0168-3659\(1099\)00229-00221](https://doi.org/10.1016/S0168-3659(1099)00229-00221).
- [2] N. U. Barua, S. P. Lowis, M. Woolley, S. O'Sullivan, R. Harrison, S. S. Gill, Robot-guided convection-enhanced delivery of carboplatin for advanced brainstem glioma, *Acta Neurochir.*, 155 (2013) 1459-1465. <https://doi.org/1410.1007/s00701-00013-01700-00706>.
- [3] K. Bouzinab, H. S. Summers, M. F. G. Stevens, C. J. Moody, N. R. Thomas, P. Gershkovich, N. Weston, M. B. Ashford, T. D. Bradshaw, L. Turyanska, Delivery of Temozolomide and N3-Propargyl Analog to Brain Tumors Using an Apoferritin Nanocage, *ACS Appl. Mater. Interfaces*, 12 (2020) 12609-12617. <https://doi.org/12610.11021/acsami.12600c01514>.
- [4] M. Céline, F. Faezeh, A. A. Amina, F. Faiza, E. M. Hala, D. Fadoua, R. Olivier, S. Christian, Targeting the Brain Reservoirs: Toward an HIV Cure, *Front. Immunol.*, 7 (2016) 3001-3013. <https://doi.org/10.3389/fimmu.2016.00397>.
- [5] C. Qin, Y. Chu, W. Feng, C. Fromont, S. He, J. Ali, J. B. Lee, A. Zgair, M. Berton, S. Bettonte, R. Liu, L. Yang, T. Monmaturapoj, C. Medrano-Padial, A. A. R. Ugalde, D. Vetrugno, S. Y. Ee, C. Sheriston, Y. Wu, M. J. Stocks, P. M. Fischer, P. Gershkovich, Targeted delivery of lopinavir to HIV reservoirs in the mesenteric lymphatic system by lipophilic ester prodrug approach, *J. Control. Release*, 329 (2021) 1077-1089. <https://doi.org/1010.1016/j.jconrel.2020.1010.1036>.
- [6] C. Gavegnano, R. F. Schinazi, Antiretroviral Therapy in Macrophages: Implication for HIV Eradication, *Antiretrovir. Chem. & Chemother.*, 20 (2009) 63-78. <https://doi.org/10.3851/IMP1374>.
- [7] E. L. Asahchop, O. Meziane, M. K. Mamik, W. F. Chan, W. G. Branton, L. Resch, M. J. Gill, E. Haddad, J. V. Guimond, M. A. Wainberg, G. B. Baker, E. A. Cohen, C. Power, Reduced antiretroviral drug efficacy and concentration in HIV-infected microglia contributes to viral persistence in brain, *Retrovir.*, 14 (2017) 1-17. <https://doi.org/10.1186/s12977-12017-10370-12975>.

- [8] J. Weller, A. Budson, Current understanding of Alzheimer's disease diagnosis and treatment, F1000Res, 1161 (2018) <https://doi.org/10.12688/f11000research.14506.12681>.
- [9] P. Zhang, S. Xu, Z. Zhu, J. Xu, Multi-target design strategies for the improved treatment of Alzheimer's disease, Eur. J. Med. Chem., 176 (2019) 228-247. <https://doi.org/210.1016/j.ejmech.2019.1005.1020>.
- [10] J. Ghajar, Traumatic brain injury, Lancet, 356 (2000) 923-929. [https://doi.org/910.1016/S0140-6736\(1000\)02689-02681](https://doi.org/910.1016/S0140-6736(1000)02689-02681).
- [11] M. Galgano, G. Toshkezi, X. Qiu, T. Russell, L. Chin, L. Zhao, Traumatic Brain Injury: Current Treatment Strategies and Future Endeavors, Cell Transplant., 26 (2017) 1118-1130. <https://doi.org/1110.1177/0963689717714102>.
- [12] M. M. Goldenberg, Multiple Sclerosis Review, Pharm. Ther., 37 (2012) 175-184.
- [13] R. Dobson, G. Giovannoni, Multiple sclerosis – a review, Eur. J. Neurol., 26 (2019) 27-40. <https://doi.org/10.1111/ene.13819>.
- [14] W. M. Pardridge, Blood-brain barrier delivery, Drug Discov. Today, 12 (2007) 54-61. <https://doi.org/10.1016/j.drudis.2006.1010.1013>.
- [15] W. M. Pardridge, Drug Delivery to the Brain, J. Cereb. Blood Flow Metab., 17 (1997) 713-731. <https://doi.org/710.1097/00004647-199707000-199700001>.
- [16] B. Chatterjee, B. Gorain, K. Mohananaidu, P. Sengupta, U. K. Mandal, H. Choudhury, Targeted drug delivery to the brain via intranasal nanoemulsion: Available proof of concept and existing challenges, Int. J. Pharm., 565 (2019) 258-268. <https://doi.org/210.1016/j.ijpharm.2019.1005.1032>.
- [17] L. Illum, Nasal drug delivery - possibilities, problems and solutions, J. Control. Release, 87 (2003) 187-198. [https://doi.org/110.1016/S0168-3659\(1002\)00363-00362](https://doi.org/110.1016/S0168-3659(1002)00363-00362).
- [18] M. Dahlin, U. Bergman, B. Jansson, E. Björk, E. Brittebo, Transfer of Dopamine in the Olfactory Pathway Following Nasal Administration in Mice, Pharm. Res., 17 (2000) 737-742. <https://doi.org/710.1023/A:1007542618378>.
- [19] T. C. A. Kumar, G. F. David, A. Sankaranarayanan, V. Puri, K. R. Sundram, Pharmacokinetics of progesterone after its administration to ovariectomized rhesus monkeys by injection, infusion, or nasal spraying, Proc. Natl. Acad. Sci. U. S. A., 79 (1982) 4185-4189. <https://doi.org/4110.1073/pnas.4179.4113.4185>.
- [20] P. Merkus, H. J. Guchelaar, D. A. Bosch, F. W. H. M. Merkus, Direct access of drugs to the human brain after intranasal drug administration?, Neurol., 60

- (2003) 1669-1671.
<https://doi.org/1610.1212/1601.WNL.0000067993.0000060735.0000067977>.
- [21] N. J. Abbott, A. A. K. Patabendige, D. E. M. Dolman, S. R. Yusof, D. J. Begley, Structure and function of the blood-brain barrier, *Neurobiol. Dis.*, 37 (2010) 13-25. <https://doi.org/10.1016/j.nbd.2009.1007.1030>.
- [22] P. Ballabh, A. Braun, M. Nedergaard, The blood-brain barrier: an overview: Structure, regulation, and clinical implications, *Neurobiol. Dis.*, 16 (2004) 1-13. <https://doi.org/10.1016/j.nbd.2003.1012.1016>.
- [23] M. Ueno, Y. Chiba, R. Murakami, K. Matsumoto, M. Kawauchi, R. Fujihara, Blood-brain barrier and blood-cerebrospinal fluid barrier in normal and pathological conditions, *Brain Tumor Pathol.*, 33 (2016) 89-96. <https://doi.org/10.1007/s10014-10016-10255-10017>.
- [24] C. P. Profaci, R. N. Munji, R. S. Pulido, R. Daneman, The blood–brain barrier in health and disease: Important unanswered questions, *J. Exp. Med.*, 217 (2020) 1-16. <https://doi.org/20190010.20191084/jem.20190062>.
- [25] S. B. Hladky, M. A. Barrand, Mechanisms of fluid movement into, through and out of the brain: evaluation of the evidence, *Fluids Barriers CNS*, 11 (2014) 1-32. <https://doi.org/10.1186/2045-8118-1111-1126>.
- [26] K. Lalatsa, A. G. Schatzlein, I. F. Uchegbu, Drug Delivery Across the Blood-Brain Barrier, *Comp. Biotechnol.*, (2019) 3rd ed. 628-637, Elsevier.
- [27] R. C. Janzer, M. C. Raff, Astrocytes induce blood–brain barrier properties in endothelial cells, *Nat.*, 325 (1987) 253-257. <https://doi.org/210.1038/325253a325250>.
- [28] T. M. Grogan, C. M. Spier, S. E. Salmon, M. Matzner, J. Rybski, R. S. Weinstein, R. J. Scheper, W. S. Dalton, P-glycoprotein expression in human plasma cell myeloma: correlation with prior chemotherapy, *Blood*, 81 (1993) 490-495. <https://doi.org/410.1182/blood.V1181.1182.1490.1490>.
- [29] B. J. Spencer, I. M. Verma, Targeted delivery of proteins across the blood–brain barrier, *Proc. Natl. Acad. Sci. U. S. A.*, 104 (2007) 7594-7599. <https://doi.org/7510.1073/pnas.0702170104>.
- [30] M. L. Pinzón-Daza, R. Garzón, P. O. Couraud, I. A. Romero, B. Weksler, D. Ghigo, A. Bosia, C. Riganti, The association of statins plus LDL receptor-targeted liposome-encapsulated doxorubicin increases in vitro drug delivery

- across blood–brain barrier cells, *Br. J. Pharmacol.*, 167 (2012) 1431-1447.
<https://doi.org/10.1111/j.1476-5381.2012.02103.x>.
- [31] Y. Zhao, D. Li, J. Zhao, J. Song, Y. Zhao, The role of the low-density lipoprotein receptor-related protein 1 (LRP-1) in regulating blood-brain barrier integrity, *Rev. Neurosci.*, 27 (2016). <https://doi.org/10.1515/revneuro-2015-0069>.
- [32] H. Pajouhesh, G. R. Lenz, Medicinal Chemical Properties of Successful Central Nervous System Drugs, *NeuroRx*, 2 (2005) 541-553.
<https://doi.org/10.1602/neurorx.1602.1604.1541>.
- [33] D. T. Manallack, R. J. Prankerd, E. Yurieva, T. I. Oprea, D. K. Chalmers, The significance of acid/base properties in drug discovery, *Chem. Soc. Rev.*, 42 (2013) 485-496. <https://doi.org/10.1039/C2CS35348B>.
- [34] A. H. Schinkel, E. Wagenaar, C. A. A. M. Mol, L.v. Deemter, P-Glycoprotein in the Blood-Brain Barrier of Mice Influences the Brain Penetration and Pharmacological Activity of Many Drugs, *J. Clin. Invest.*, 97 (1996) 2517-2524. <https://doi.org/10.1172/JCI118699>.
- [35] W. M. Pardridge, Drug transport across the blood-brain barrier, *J. Cereb. Blood Flow Metab.*, 32 (2012) 1959-1972. <https://doi.org/10.1038/jcbfm.2012.1126>.
- [36] B. J. Umlauf, E. V. Shusta, Exploiting BBB disruption for the delivery of nanocarriers to the diseased CNS, *Curr. Opin. Biotechnol.*, 60 (2019) 146-152.
<https://doi.org/10.1016/j.copbio.2019.1001.1013>.
- [37] A. Montagne, Z. Zhao, B. V. Zlokovic, Alzheimer's disease: A matter of blood-brain barrier dysfunction, *J. Exp. Med.*, 214 (2017) 3151-3169.
<https://doi.org/10.1084/jem.20171406>.
- [38] T. Inamura, K. L. Black, Bradykinin Selectively Opens Blood-Tumor Barrier in Experimental Brain Tumors, *J. Cereb. Blood Flow & Metab.*, 14 (1994) 862-870. <https://doi.org/10.1038/jcbfm.1994.1108>.
- [39] R. T. Bartus, P. J. Elliott, R. L. Dean, N. J. Hayward, T. L. Nagle, M. R. Huff, P. A. Snodgrass, D. G. Blunt, Controlled Modulation of BBB Permeability Using the Bradykinin Agonist, RMP-7, *Exp. Neurol.*, 142 (1996) 14-28.
<https://doi.org/10.1006/exnr.1996.0175>.
- [40] H. Zhang, Y. T. Gu, Y. X. Xue, Bradykinin-induced blood–brain tumor barrier permeability increase is mediated by adenosine 5'-triphosphate-sensitive potassium channel, *Brain Res.*, 1144 (2007) 33-41.
<https://doi.org/10.1016/j.brainres.2007.1001.1133>.

- [41] T. Kuroiwa, P. Ting, H. Martinez, I. Klatzo, The Biphasic Opening of the Blood-Brain Barrier to Proteins Following Temporary Middle Cerebral Artery Occlusion, *Acta Neuropathol.*, 68 (1985) 122-129. <https://doi.org/110.1007/BF00688633>.
- [42] Z. G. Huang, D. Xue, E. Preston, H. Karbalai, A. M. Buchan, Biphasic Opening of the Blood-Brain Barrier Following Transient Focal Ischemia: Effects of Hypothermia, *Exp. Neurosci.*, 26 (1999) 298-304. <https://doi.org/210.1017/S0317167100000421>.
- [43] D. Knowland, A. Arac, K. J. Sekiguchi, M. Hsu, S. E. Lutz, J. Perrino, G. K. Steinberg, B. A. Barres, A. Nimmerjahn, D. Agalliu, Stepwise Recruitment of Transcellular and Paracellular Pathways Underlies Blood-Brain Barrier Breakdown in Stroke, *Neuron.*, 82 (2014) 603-317. <https://doi.org/610.1016/j.neuron.2014.1003.1003>.
- [44] S. H. Koh, Y. Park, C. W. Song, J. G. Kim, K. Kim, J. Kim, M. H. Kim, S. R. Lee, D. W. Kim, H. J. Yu, D. Chang, S. J. Hwang, S. H. Kim, The effect of PARP inhibitor on ischaemic cell death, its related inflammation and survival signals, *Eur. J. Neurosci.*, 20 (2004) 1461-1472. <https://doi.org/1410.1111/j.1460-9568.2004.03632.x>.
- [45] K. Kurisu, M. A. Yenari, Therapeutic hypothermia for ischemic stroke; pathophysiology and future promise, *Neuropharmacol.*, 134 (2018) 302-309. <https://doi.org/310.1016/j.neuropharm.2017.1008.1025>.
- [46] M. Sun, Y. Zhao, Y. Gu, C. Xu, Anti-inflammatory mechanism of taurine against ischemic stroke is related to down-regulation of PARP and NF- κ B, *Amino Acids*, 42 (2012) 1735-1747. <https://doi.org/1710.1007/s00726-00011-00885-00723>.
- [47] S. Bertoni, D. Tedesco, M. Bartolini, C. Prata, N. Passerini., B. Albertini, Solid Lipid Microparticles for Oral Delivery of Catalase: Focus on the Protein Structural Integrity and Gastric Protection, *Mol. Pharm.*, 17 (2020) 3609-3621. <https://doi.org/3610.1021/acs.molpharmaceut.3600c00666>.
- [48] A. C. Anselmo, S. Mitragotri, An overview of clinical and commercial impact of drug delivery systems, *J. Control. Release*, 190 (2014) 15-28. <https://doi.org/10.1016/j.jconrel.2014.03.053>.
- [49] O. M. Feeney, M. F. Crum, C. L. McEvoy, N. L. Trevaskis, H. D. Williams, C. W. Pouton, W. N. Charman, C. A. S. Bergström, C. J. H. Porter, 50 years of oral lipid-based formulations: Provenance, progress and future perspectives, *Adv.*

- Drug Deliv. Rev., 101 (2016) 167-194.
<https://doi.org/110.1016/j.addr.2016.1004.1007>.
- [50] R. G. Strickley, Solubilizing Excipients in Oral and Injectable Formulations, Pharm. Res., 21 (2004) 201-230.
<https://doi.org/210.1023/B:PHAM.0000016235.0000032639.0000016223>.
- [51] W. Ma, V. Plassat, R. Savla, K. Li, The Truths and Myths of Oral Lipid-Based Drug Delivery Systems, Am. Pharm. Rev., 21 (2018) 32-35.
- [52] S. Kalepu, M. Manthina, V. Padavala, Oral lipid-based drug delivery systems – an overview, Acta Pharm. Sin. B, 3 (2013) 361-372.
<https://doi.org/310.1016/j.apsb.2013.1010.1001>.
- [53] F. Carriere, Impact of gastrointestinal lipolysis on oral lipid-based formulations and bioavailability of lipophilic drugs, Biochim., 125 (2016) 297-305.
<https://doi.org/210.1016/j.biochi.2015.1011.1016>.
- [54] C. W. Pouton, Lipid formulations for oral administration of drugs: non-emulsifying, self-emulsifying and ‘self-microemulsifying’ drug delivery systems, Eur. J. Pharm. Sci., 11 (2000) 93-98. [https://doi.org/10.1016/S0928-0987\(1000\)00167-00166](https://doi.org/10.1016/S0928-0987(1000)00167-00166).
- [55] C. W. Pouton, Formulation of poorly water-soluble drugs for oral administration: Physicochemical and physiological issues and the lipid formulation classification system, Eur. J. Pharm. Sci., 29 (2006) 278-287.
<https://doi.org/210.1016/j.ejps.2006.1004.1016>.
- [56] A. K. Birnbaum, A. Karanam, S. E. Marino, C. M. Barkley, R. P. Remmel, M. Roslawski, M. Gramling-Aden, I. E. Leppik, Food effect on pharmacokinetics of cannabidiol oral capsules in adult patients with refractory epilepsy, Epilepsia, 60 (2019) 1586-1592. <https://doi.org/1510.1111/epi.16093>.
- [57] L. Yu, A. Bridgers, J. Polli, A. Vickers, S. Long, A. Roy, R. Winnike, M. Coffin, Vitamin E-TPGS Increases Absorption Flux of an HIV Protease Inhibitor by Enhancing Its Solubility and Permeability, Pharm. Res., 16 (1999) 1812-1817.
<https://doi.org/1810.1023/A:1018939006780>.
- [58] H. A. Fathi, A. Allam, M. Elsabahy, G. Fetih, M. El-Badry, Nanostructured lipid carriers for improved oral delivery and prolonged antihyperlipidemic effect of simvastatin, Colloids & Surf. B: Biointerfaces, 162 (2018) 236-245.
<https://doi.org/210.1016/j.colsurfb.2017.1011.1064>.

- [59] W. Dong, X. Wang, C. Liu, X. Zhang, X. Zhang, X. Chen, Y. Kou, S. Mao, Chitosan based polymer-lipid hybrid nanoparticles for oral delivery of enoxaparin, *Int. J. Pharm.*, 547 (2018) 499-505. <https://doi.org/410.1016/j.ijpharm.2018.1005.1076>.
- [60] P. Uhl, S. Pantze, P. Storck, J. Parmentier, D. Witzigmann, G. Hofhaus, J. Huwyler, W. Mier, G. Fricker, Oral delivery of vancomycin by tetraether lipid liposomes, *Eur. J. Pharm. Sci.*, 108 (2017) 111-118. <https://doi.org/110.1016/j.ejps.2017.1007.1013>.
- [61] N. Rahiman, M. Mohammadi, S. H. Alavizadeh, L. Arabi, A. Badiee, M. R. Jaafari, Recent advancements in nanoparticle-mediated approaches for restoration of multiple sclerosis *J. Control. Release*, 343 (2022) 620-644. <https://doi.org/610.1016/j.jconrel.2022.1002.1009>.
- [62] A. Brookes, L. Ji, T. D. Bradshaw, M. Stocks, D. Gray, J. Butler, P. Gershkovich, Is oral lipid-based delivery for drug targeting to the brain feasible?, *Eur. J. Pharm. Biopharm.*, 172 (2022) 112-122. <https://doi.org/110.1016/j.ejpb.2022.1002.1004>.
- [63] J. A. Yáñez, S. W. J. Wang, I. W. Knemeyer, M. A. Wirth, K. B. Alton, Intestinal lymphatic transport for drug delivery, *Adv. Drug Deliv. Rev.*, 63 (2011) 923-942. <https://doi.org/910.1016/j.addr.2011.1005.1019>.
- [64] M. H. Davidson, Merck Sharp & Dohme Corp, M.M.P. Version, Overview of Lipid Metab., <https://www.msdmanuals.com/professional/endocrine-and-metabolic-disorders/lipid-disorders/overview-of-lipid-metabolism>, 2019 (accessed 09 July 2022).
- [65] R. P. Bazinet, S. Layé, Polyunsaturated fatty acids and their metabolites in brain function and disease, *Nat. Rev. Neurosci.*, 15 (2014) 771-785. <https://doi.org/10.1038/nrn3820>.
- [66] L. Sztriha, A. L. Betz, Oleic acid reversibly opens the blood-brain barrier, *Brain Res.*, 550 (1991) 257-262. [https://doi.org/210.1016/0006-8993\(1091\)91326-V](https://doi.org/210.1016/0006-8993(1091)91326-V).
- [67] H. J. Kim, Y. S. Pyeun, Y. W. Kim, B. M. Cho, T. H. Lee, T. Y. Moon, K. T. Suh, B. R. Park, A Model for Research on the Blood–Brain Barrier Disruption Induced by Unsaturated Fatty Acid Emulsion, *Invest. Radiol.*, 40 (2005) 270-276. <https://doi.org/210.1097/1001.rli.0000160488.0000126344.0000160485f>.
- [68] H. J. Kim, Y. S. Pyeun, Y. W. Kim, B. M. Cho, T. H. Lee, T. Y. Moon, K. T. Suh, B. R. Park, A Model for Research on the Blood–Brain Barrier Disruption

- Induced by Unsaturated Fatty Acid Emulsion, *Invest. Radiol.*, 40 (2005) 270-276. <https://doi.org/210.1097/1001.rli.0000160488.0000126344.0000160485f>.
- [69] M. A. Liebert, Final Report on the Safety Assessment of Oleic Acid, Lauric Acid, Palmitic Acid, Myristic Acid, and Stearic Acid, *J. Am. Coll. Toxicol.*, 6 (1987) 321-400. <https://doi.org/10.3109/10915818709098563>.
- [70] G. A. Dhopeswarkar, J. F. Mead, Uptake and Transport of Fatty Acids into the Brain and the Role of the Blood-Brain Barrier System, *Adv. Lipid Res.*, 11 (1973) 109-142. <https://doi.org/110.1016/B1978-1010-1012-024911-024914.050010-024916>.
- [71] S. M. Caliph, F. W. Faassen, C. J. H. Porter, The influence of intestinal lymphatic transport on the systemic exposure and brain deposition of a novel highly lipophilic compound with structural similarity to cholesterol, *J. Pharm. Pharmacol.*, 66 (2014) 1377–1387. <https://doi.org/1310.1111/jphp.12268>.
- [72] H. J. Kim, Y. W. Kim, S. H. Choi, B. M. Cho, R. Bandu, H. S. Ahn, K. P. Kim, Triolein Emulsion Infusion Into the Carotid Artery Increases Brain Permeability to Anticancer Agents, *Neurosurg.*, 78 (2016) 726-733. <https://doi.org/710.1227/NEU.00000000000001104>.
- [73] J. H. Ryu, Y. J. Jeong, H. J. Kim, D. H. Kim, D. C. Kim, M. J. Kim, MRI of the cat brain in the study of vascular permeability after intraarterial carotid injection of triolein, *Acta Radiol.*, 51 (2010) 563-568. <https://doi.org/510.3109/02841851003674538>.
- [74] S. H. Choi, H. J. Kim, L. Hwangbo, Y. W. Kim, The Minimum Percentage Of Triolein Emulsion For Studying Cerebral Vascular Permeability With Least Brain Edema, *Iran. J. Radiol.*, 11 (2014) e14887. <https://doi.org/14810.15812/iranjradiol.14887>.
- [75] N. Iritani, T. Kimura, H. Fukuda, T. Sugimoto, Differences in labelled triolein turnover after oral administration between liver and adipose tissue of rats, *Br. J. Nutr.*, 93 (2007) 53-58. <https://doi.org/10.1079/BJN20041296>.
- [76] I. Arimoto, C. Matsumoto, M. Tanaka, K. Okuhira, H. Saito, T. Handa, Surface composition regulates clearance from plasma and triolein lipolysis of lipid emulsions, *Lipids*, 33 (1998) 773-779. <https://doi.org/710.1007/s11745-11998-10269-11748>.
- [77] H. B. Lee, M. D. Blafox, Blood Volume in the Rat, *J. Nucl. Med.*, 26 (1985) 72-76.

- [78] B. Borgstrom, A. Dahlqvist, G. Lundh, J. Sjoval, Studies of Intestinal Digestion and Absorption in the Human, *J. Clin. Investig.*, 36 (1957) 1521-1536. <https://doi.org/1510.1172/JCI103549>.
- [79] G. C. Daher, D. A. Cooper, N. L. Zorich, D. King, K. A. Riccardi, J.C. Peters, Olestra Ingestion and Dietary Fat Absorption in Humans, *J. Nutr.*, 127 (1997) 1694S-1698S. <https://doi.org/1610.1093/jn/1127.1698.1694S>.
- [80] K. Wojcikowski, G. Gobe, Animal Studies on Medicinal Herbs: Predictability, Dose Conversion and Potential Value, *Phytother. Res.*, 28 (2014) 22–27. <https://doi.org/10.1002/ptr.4966>.
- [81] S. W. Mealey, Appendix H: Canine and Feline Body Surface Area Conversion Tables, In *Pharmacotherapeutics for Veterinary Dispensing*, (2019). <https://doi.org/10.1002/9781119404576.app8>.
- [82] Y. Sol, S. H. Choi, H. J. Kim, Y. W. Kim, B. M. Cho, H. S. Han, K. J. Choi, Morphologic mechanisms of increased vascular permeability of triolein emulsion to the blood-brain barrier, *Microsc.*, 66 (2017) 366-370. <https://doi.org/310.1093/jmicro/dfx1026>.
- [83] I. S. Lee, H. J. Kim, S. H. Choi, Y. W. Kim, K. J. Choi, Doxorubicin concentration in brain remains high for one day after triolein emulsion infusion induced BBB opening, *Int. J. Neurosci.*, 130 (2018) 770-776. <https://doi.org/710.1080/00207454.00202019.01705809>.
- [84] B. Erdlenbruch, V. Jendrosseck, H. Eibl, M. Lakomek, Transient and controllable opening of the blood-brain barrier to cytostatic and antibiotic agents by alkylglycerols in rats, *Exp. Brain Res.*, 135 (2000) 417-422. <https://doi.org/410.1007/s002210000553>.
- [85] B. Erdlenbruch, M. Alipour, G. Fricker, D. S. Miller, W. Kugler, H. Eibl, M. Lakomek, Opening Of The Blood-Brain Barrier To Small And Large Fluorescence Markers In Normal And C6 Glioma-Bearing Rats And Isolated Rat Brain Capillaries, *Br. J. Pharmacol.*, 140 (2003) 1201-1210. <https://doi.org/1210.1038/sj.bjp.0705554>.
- [86] Cancer Research UK, Astrocytoma and glioblastoma (GBM), <https://www.cancerresearchuk.org/about-cancer/brain-tumours/types/astrocytoma-glioblastoma-multiforme> (2019), accessed 10 October 2022.

- [87] W. Wu, J. L. Klockow, M. Zhang, F. Lafortune, E. Chang, L. Jin, Y. Wu, H. E. Daldrop-Link, Glioblastoma multiforme (GBM): An overview of current therapies and mechanisms of resistance, *Pharmacol. Res.*, 171 (2021) 105780. <https://doi.org/105710.101016/j.phrs.102021.105780>.
- [88] S. Jiapaer, T. Furuta, S. Tanaka, T. Kitabayashi, M. Nakada, Potential Strategies Overcoming the Temozolomide Resistance for Glioblastoma, *Neurol. Med. Chir. (Tokyo)*, 58 (2018) 405-421. <https://doi.org/410.2176/nmc.ra.2018-0141>.
- [89] Cancer Research UK, Brain tumours: Survival, <https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/brain-other-cns-and-intracranial-tumours> (2019), accessed 10 October 2022.
- [90] A. Brodbelt, D. Greenberg, T. Winters, M. Williams, S. Vernon, V. P. Collins, Glioblastoma in England: 2007-2011, *Eur. J. Cancer*, 51 (2015) 533-542. <https://doi.org/510.1016/j.ejca.2014.1012.1014>.
- [91] N. Singh, A. Miner, L. Hennis, S. Mittal, Mechanisms of temozolomide resistance in glioblastoma - a comprehensive review, *Cancer Drug Resist.*, 4 (2021) 17-43. <http://dx.doi.org/10.20517/cdr.22020.20579>.
- [92] National Library of Medicine, Temozolomide, <https://pubchem.ncbi.nlm.nih.gov/compound/Temozolomide> (2023), accessed 11 October 2022.
- [93] S. Y. Lee, Temozolomide resistance in glioblastoma multiforme, *Genes Dis.*, 3 (2016) 198-210. <https://doi.org/110.1016/j.gendis.2016.1004.1007>.
- [94] K. Bouzinab, H. Summers, J. Zhang, M. F. G. Stevens, C. J. Moody, L. Turyanska, N. R. Thomas, P. Gershkovich, M. B. Ashford, E. Vitterso, L. C. D. Storer, R. Grundy, T. D. Bradshaw, In search of effective therapies to overcome resistance to Temozolomide in brain tumours, *Cancer Drug Resist.*, 2 (2019) 1018-1031. <https://doi.org/1010.20517/cdr.22019.20564>.
- [95] J. Zhang, M. Hummersone, C. S. Matthews, M. F. G. Stevens, T. D. Bradshaw, N3-substituted temozolomide analogs overcome methylguanine-DNA methyltransferase and mismatch repair precipitating apoptotic and autophagic cancer cell death, *Oncol.*, 88 (2014) 28-48. <https://doi.org/10.1159/000366131>.
- [96] G. Perazzoli, J. Prados, R. Ortiz, O. Caba, L. Cabeza, M. Berdasco, B. González, C. Melguizo, Temozolomide resistance in glioblastoma cell lines: implication of

- MGMT, MMR, P-Glycoprotein and CD133 expression., *PLoS One*, 10 (2015) 1-23. <https://doi.org/10.1371/journal.pone.0140131>.
- [97] M. R. Gilbert, J. J. Dignam, T. S. Armstrong, A randomized trial of bevacizumab for newly diagnosed glioblastoma, *N. Engl. J. Med.*, 370 (2014) 699-708. <https://doi.org/610.1056/NEJMoa1308573>.
- [98] O. L. Chinot, W. Wick, W. Mason, R. Henriksson, F. Saran, R. Nishikawa, A. F. Carpentier, K. Hoang-Xuan, P. Kavan, D. Cernea, A. A. Brandes, M. Hilton, L. Abrey, T. Cloughesy, Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma, *N. Engl. J. Med.*, 370 (2014) 709-722. <https://doi.org/710.1056/NEJMoa1308345>.
- [99] D. Cousin, J. Zhang, M. G. Hummersone, C. S. Matthews, M. Frigerio, T. D. Bradshaw, M. F. G. Stevens, Antitumor imidazoj5,1-d]-1,2,3,5-tetrazines: compounds modified at the 3-position overcome resistance in human glioblastoma cell lines, *Med. Chem. Commun.*, 7 (2016) 2332-2343. <https://doi.org/2310.1039/C2336MD00384B>.
- [100] R. T. Othman, I. Kimishi, T. D. Bradshaw, L. C. D. Storer, A. Korshunov, S. M. Pfister, R. G. Grundy, I. D. Kerr, B. Coyle, Overcoming multiple drug resistance mechanisms in medulloblastoma, *Acta Neuropathol. Comm.*, 2 (2014) 57. <https://doi.org/10.1186/2051-5960-1182-1157>.
- [101] J. A. Crippa, F. S. Guimarães, A. C. Campos, A.W. Zuardi, Translational Investigation of the Therapeutic Potential of Cannabidiol (CBD): Toward a New Age, *Front. Immunol.*, 9 (2018) 2009. <https://doi.org/2010.3389/fimmu.2018.02009>.
- [102] P. Morales, P. H. Reggio, CBD: A New Hope?, *ACS Med. Chem. Lett.*, 10 (2019) 694-695. <https://doi.org/610.1021/acsmedchemlett.1029b00127>.
- [103] O. Kovalchuck, I. Kovalchuk, Cannabinoids as anticancer therapeutic agents, *Cell Cycle*, 19 (2020) 961-989. <https://doi.org/910.1080/15384101.15382020.11742952>.
- [104] S. M. Baswan, A. E. Klosner, K. Glynn, A. Rajgopal, K. Malik, S. Yim, N. Stern, Therapeutic Potential of Cannabidiol (CBD) for Skin Health and Disorders, *Clin. Cosmet. Investig. Dermatol.*, 13 (2020) 927-942. <https://doi.org/910.2147/CCID.S286411>.
- [105] M. Maccarrone, R. Maldonado, M. Casas, T. Henze, D. Centonze, Cannabinoids therapeutic use: what is our current understanding following the introduction of

- THC, THC:CBD oromucosal spray and others?, *Expert Rev. Clin. Pharmacol.*, 10 (2017) 443-455. <https://doi.org/410.1080/17512433.17512017.11292849>.
- [106] G. Watt, T. Kart, In vivo Evidence for Therapeutic Properties of Cannabidiol (CBD) for Alzheimer's Disease, *Front. Pharmacol.*, 8 (2017) 120-127. <https://doi.org/10.3389/fphar.2017.00020>.
- [107] S. Burstein, Cannabidiol (CBD) and its analogs: a review of their effects on inflammation, *Bioorg. Med. Chem.*, 23 (2015) 1377-1385. <https://doi.org/1310.1016/j.bmc.2015.1301.1059>.
- [108] T. Naftali, Is Cannabis of Potential Value as a Therapeutic for Inflammatory Bowel Disease?, *Dig. Dis. Sci.*, 64 (2019) 2696-2698. <https://doi.org/2610.1007/s10620-10019-05763-10628>.
- [109] A. M. Malfait, R. Gallily, P. F. Sumariwalla, A. S. Malik, E. Andreaskos, R. Mechoulam, M. Feldmann, The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis, *Biol. Sci.*, 97 (2000) 9561-9566. <https://doi.org/9510.1073/pnas.160105897>.
- [110] M. Rabino, S. Mallia, E. Castiglioni, D. Rovina, G. Pompilio, A. Gowran, The Endocannabinoid System and Cannabidiol: Past, Present, and Prospective for Cardiovascular Diseases, *Pharm.*, 14 (2021) 936. <https://doi.org/910.3390/ph14090936>.
- [111] H. Lowe, N. Toyang, B. Steele, J. Bryant, W. Ngwa, The Endocannabinoid System: A Potential Target for the Treatment of Various Diseases, *Int. J. Mol. Sci.*, 22 (2021) 9472. <https://doi.org/10.3390/ijms22179472>.
- [112] D. L. de Almeida, L. A. Devi, Diversity of molecular targets and signaling pathways for CBD, *Pharmacol. Res. Perspect.*, 8 (2020) 1-10. <https://doi.org/00610.01002/prp00682.00682>.
- [113] R. G. Pertwee, Pharmacology of cannabinoid CB1 and CB2 receptors, *Pharmacol. Ther.*, 74 (1997) 129-180. [https://doi.org/110.1016/S0163-7258\(1097\)82001-82003](https://doi.org/110.1016/S0163-7258(1097)82001-82003).
- [114] S. J. MacLennan, P. H. Reynen, J. Kwan, D. W. Bonhaus, Evidence for inverse agonism of SR141716A at human recombinant cannabinoid CB1 and CB2 receptors, *Br. J. Pharmacol.*, 124 (1998) 619-622. <https://doi.org/610.1038/sj.bjp.0701915>.

- [115] R. G. Pertwee, The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ^9 -tetrahydrocannabinol, cannabidiol and Δ^9 -tetrahydrocannabivarin, *Br. J. Pharmacol.*, 153 (2009) 199-215. <https://doi.org/110.1038/sj.bjp.0707442>.
- [116] S. D. Harding, J. L. Sharman, E. Faccenda, C. Southan, A. J. Pawson, S. Ireland, A. J. G. Gray, L. Bruce, S. P. H. Alexander, S. Anderton, C. Bryant, A. P. Davenport, C. Doerig, D. Fabbro, F. Levi-Schaffer, M. Spedding, J.A. Davies, The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY, *Nucleic Acids Res.*, 46 (2017) D1091-D1106. <https://doi.org/10.1093/nar/gkx1121>.
- [117] S. Maione, F. Piscitelli, L. Gatta, D. Vita, L. D. Petrocellis, E. Palazzo, V. de Novellis, V. D. Marzo, Non-psychoactive cannabinoids modulate the descending pathway of antinociception in anaesthetized rats through several mechanisms of action, *Br. J. Pharmacol.*, 162 (2010) 584-596. <https://doi.org/510.1111/j.1476-5381.2010.01063.x>.
- [118] T. Bisogno, L. Hanuš, L. D. Petrocellis, S. Tchilibon, D. E. Ponde, I. Brandi, A. S. Moriello, J. B. Davis, R. Mechoulam, V. D. Marzo, Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide, *Br. J. Pharmacol.*, 134 (2009) 845-852. <https://doi.org/810.1038/sj.bjp.0704327>.
- [119] J. M. Nichols, E. Kummari, J. Sherman, E. J. Yang, S. Dhital, C. Gilfeather, G. Yray, T. Morgan, B. L. F. Kaplan, CBD Suppression of EAE Is Correlated with Early Inhibition of Splenic IFN- γ + CD8⁺ T Cells and Modest Inhibition of Neuroinflammation, *J. Neuroimmune Pharmacol.*, 16 (2020) 346-362. <https://doi.org/310.1007/s11481-11020-09917-11488>.
- [120] D. M. Elliott, N. Singh, M. Nagarkatti, P. S. Nagarkatti, Cannabidiol Attenuates Experimental Autoimmune Encephalomyelitis Model of Multiple Sclerosis Through Induction of Myeloid-Derived Suppressor Cells, *Front. Immunol.*, (2018) 1-12. <https://doi.org/10.3389/fimmu.2018.01782>.
- [121] Z. Z. Al-Ghezi, P. B. Busbee, H. Alghetaa, P. S. Nagarkatti, M. Nagarkatti, Combination of cannabinoids, delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), mitigates experimental autoimmune encephalomyelitis

- (EAE) by altering the gut microbiome, *Brain, Behav., Immun.*, 82 (2019) 25-35. <https://doi.org/10.1016/j.bbi.2019.1007.1028>.
- [122] E. Kozela, N. Lev, N. Kaushansky, R. Eilam, N. Rimmerman, R. Levy, A. Ben-Nun, A. Juknat, Z. Vogel, Cannabidiol inhibits pathogenic T cells, decreases spinal microglial activation and ameliorates multiple sclerosis-like disease in C57BL/6 mice, *Br. J. Pharmacol.*, 163 (2011) 1507-1519. <https://doi.org/1510.1111/j.1476-5381.2011.01379.x>.
- [123] A. Rahimi, M. Faizi, F. Talebi, F. Noorbakhsh, F. Kahrizi, N. Naderi, Interaction between the protective effects of cannabidiol and palmitoylethanolamide in experimental model of multiple sclerosis in C57BL/6 mice, *Neurosci.*, 290 (2015) 279-287. <https://doi.org/210.1016/j.neuroscience.2015.1001.1030>.
- [124] N. Peyravian, S. Deo, S. Daunert, J.J. Jimenez, Cannabidiol as a Novel Therapeutic for Immune Modulation, *Immunotargets Ther.*, 9 (2020) 131-140. <https://doi.org/110.2147/ITT.S263690>.
- [125] C. Andradas, A. Truong, J. Byrne, R. Endersby, The Role of Cannabinoids as Anticancer Agents in Pediatric Oncology, *Cancers*, 13 (2021) 157-160. <https://doi.org/110.3390/cancers13010157>.
- [126] I. Kyriakou, N. Yarandi, E. Polycarpou, Efficacy of cannabinoids against glioblastoma multiforme: A systematic review, *Phytomed.*, 88 (2021) 153533. <https://doi.org/153510.151016/j.phymed.152021.153533>.
- [127] S. Torres, M. Lorente, F. Rodríguez-Fornés, S. Hernández-Tiedra, M. Salazar, E. García-Taboada, J. Barcia, M. Guzmán, G. Velasco, A Combined Preclinical Therapy of Cannabinoids and Temozolomide against Glioma, *Mol. Cancer Ther.*, 10 (2011) 90-103. <https://doi.org/110.1158/1535-7163.MCT-1110-0688>.
- [128] J. P. Marcu, R. T. Christian, D. Lau, A. J. Zielinski, M. P. Horowitz, J. Lee, A. Pakdel, J. Allison, C. Limbad, D. H. Moore, G. L. Yount, P. Y. Desprez, S.D. McAllister, Cannabidiol Enhances the Inhibitory Effects of Δ^9 -Tetrahydrocannabinol on Human Glioblastoma Cell Proliferation and Survival, *Mol. Cancer Ther.*, 9 (2010) 180-189. <https://doi.org/110.1158/1535-7163.MCT-1109-0407>.
- [129] M. Guzmán, Cannabinoids: potential anticancer agents, *Nat. Rev. Cancer*, 3 (2003) 745-755. <https://doi.org/710.1038/nrc1188>.
- [130] G. Velasco, C. Sánchez, M. Guzmán, Anticancer Mechanisms of Cannabinoids, *Curr. Oncol.*, 23 (2016) 23-32. <https://doi.org/10.3747/co.3723.3080>.

- [131] M. Nabissi, M. B. Morelli, M. Santoni, G. Santoni, Triggering of the TRPV2 channel by cannabidiol sensitizes glioblastoma cells to cytotoxic chemotherapeutic agents, *Carcinog.*, 34 (2013) 48-57. <https://doi.org/10.1093/carcin/bgs1328>.
- [132] L. Deng, L. Ng, T. Ozawa, N. Stella, Quantitative Analyses of Synergistic Responses between Cannabidiol and DNA-Damaging Agents on the Proliferation and Viability of Glioblastoma and Neural Progenitor Cells in Culture, *J. Pharmacol. Exp. Ther.*, 360 (2017) 215-224. <https://doi.org/210.1124/jpet.1116.236968>.
- [133] M. Solinas, P. Massi, A. R. Cantelmo, M. G. Cattaneo, R. Cammarota, D. Bartolini, V. Cinquina, M. Valenti, L. M. Vicentini, D. M. Noonan, A. Albin, D. Parolaro, Cannabidiol inhibits angiogenesis by multiple mechanisms, *Br. J. Pharmacol.*, 167 (2012) 1218-1231. <https://doi.org/1210.1111/j.1476-5381.2012.02050.x>.
- [134] P. Massi, A. Vaccani, S. Ceruti, A. Colombo, M. P. Abbraccio, D. Parolaro, Antitumor Effects of Cannabidiol, a Nonpsychoactive Cannabinoid, on Human Glioma Cell Lines, *J. Pharmacol. Exp. Ther.*, 308 (2004) 838-845. <https://doi.org/810.1124/jpet.1103.061002>.
- [135] E. Singer, J. Judkins, N. Salomonis, L. Matlaf, P. Soteropoulos, S. McAllister, L. Soroceanu, Reactive oxygen species-mediated therapeutic response and resistance in glioblastoma, *Cell Death Dis.*, 6 (2015) 1601-1612. <https://doi.org/1610.1038/cddis.2014.1566>.
- [136] L. Soroceanu, R. Murase, C. Limbad, E. Singer, J. Allison, I. Adrados, R. Kawamura, A. Pakdel, Y. Fukuyo, D. Nguyen, S. Khan, R. Arauz, G. L. Yount, D. H. Moore, P. Y. Desprez, S. D. McAllister, Id-1 Is a Key Transcriptional Regulator of Glioblastoma Aggressiveness and a Novel Therapeutic Target, *Cancer Res.*, 73 (2013) 1559-1569. <https://doi.org/1510.1158/0008-5472.CAN-1512-1943>.
- [137] V. N. Ivanov, J. Wu, T. K. Hei, Regulation of human glioblastoma cell death by combined treatment of cannabidiol, γ -radiation and small molecule inhibitors of cell signaling pathways, *Oncotarget*, 8 (2017) 74068-74095. <https://doi.org/74010.18632/oncotarget.18240>.
- [138] C. Twelves, M. Sabel, D. Checketts, S. Miller, B. Tayo, M. Jove, L. Brazil, S. C. Short, A phase 1b randomised, placebo-controlled trial of nabiximols

- cannabinoid oromucosal spray with temozolomide in patients with recurrent glioblastoma, Br. J. Cancer, 124 (2021) 1379-1387. <https://doi.org/10.1038/s41416-01259-41413>.
- [139] U.S. National Library of Medicine, A Safety Study of Sativex in Combination With Dose-intense Temozolomide in Patients With Recurrent Glioblastoma (2022), <https://clinicaltrials.gov/ct2/show/NCT01812603?term=CBD&cond=Glioblastoma&draw=2>, accessed 06 August 2022.
- [140] U.S. National Library of Medicine, A Safety Study of Sativex Compared With Placebo (Both With Dose-intense Temozolomide) in Recurrent Glioblastoma Patients (2022), <https://clinicaltrials.gov/ct2/show/NCT01812616?term=CBD&cond=Glioblastoma&draw=2&rank=5>, accessed 06 August 2022.
- [141] U.S. National Library of Medicine, TN-TC11G (THC+CBD) Combination With Temozolomide and Radiotherapy in Patients With Newly-diagnosed Glioblastoma (GEINOCANN) (2022), <https://clinicaltrials.gov/ct2/show/NCT03529448>, accessed 24 July 2022.
- [142] U.S., National Library of Medicine, A Study of the Efficacy of Cannabidiol in Patients With Multiple Myeloma, Glioblastoma Multiforme, and GI Malignancies (2018), <https://clinicaltrials.gov/ct2/show/NCT03607643?term=CBD&cond=Glioblastoma&draw=2>, accessed 06 August 2022.
- [143] S. H. Lee, H. S. Lee, G. Tamia, H. J. Song, C. I. Wei, Anticancer Activity of Cannabidiol (CBD) in Human Colorectal Cancer Cells: A Mechanistic Study, Curr. Dev. Nutr., 6 (2022) 246. <https://doi.org/10.1093/cdn/nzac1052.1013>.
- [144] E. S. Seltzer, A. K. Watters, D. MacKenzie, L. M. Granat, D. Zhang, Cannabidiol (CBD) as a Promising Anti-Cancer Drug, Cancers, 12 (2020) 3203-3229. <https://doi.org/10.3390/cancers12113203>.
- [145] C. G. Heider, S. A. Itenberg, J. Rao, H. Ma, X. Wu, Mechanisms of Cannabidiol (CBD) in Cancer Treatment: A Review, Biol., 11 (2022) 817-834. <https://doi.org/10.3390/biology11060817>.
- [146] A. J. Sales, F. S. Guimarães, S. R. L. Joca, CBD modulates DNA methylation in the prefrontal cortex and hippocampus of mice exposed to forced swim, Behav.

- Brain Res., 388 (2020) 112627-112636.
<https://doi.org/112610.111016/j.bbr.112020.112627>.
- [147] N. M. Wanner, M. Colwell, C. Drown, C. Faulk, Subacute cannabidiol alters genome-wide DNA methylation in adult mouse hippocampus, *Environ. Mol. Mutagen.*, 61 (2020) 890-900. <https://doi.org/810.1002/em.22396>.
- [148] L. Taylor, B. Gidal, G. Blakey, B. Tayo, G. Morrison, A Phase I, Randomized, Double-Blind, Placebo-Controlled, Single Ascending Dose, Multiple Dose, and Food Effect Trial of the Safety, Tolerability and Pharmacokinetics of Highly Purified Cannabidiol in Healthy Subjects, *CNS Drugs*, 32 (2018) 1053-1067. <https://doi.org/1010.1007/s40263-40018-40578-40265>.
- [149] K. M. Nelson, J. Bisson, G. Singh, J. G. Graham, S. N. Chen, J. B. Friesen, J. L. Dahlin, M. Niemitz, M. A. Walters, G. F. Pauli, The Essential Medicinal Chemistry of Cannabidiol (CBD), *J. Med. Chem.*, 63 (2020) 12137-12155. <https://doi.org/12110.11021/acs.jmedchem.12130c00724>.
- [150] E. Samara, M. Bialer, R. Mechoulam, Pharmacokinetics of cannabidiol in dogs, *Drug Metab. Dispos.*, 16 (1988) 469-472.
- [151] W. Feng, C. Qin, S. Abdelrazig, Z. Bai, M. Raji, R. Darwish, Y. J. Chu, L. Ji, D. A. Gray, M. J. Stocks, C. S. Constantinescu, D. A. Barrett, P. M. Fischer, P. Gershkovich, Vegetable oils composition affects the intestinal lymphatic transport and systemic bioavailability of co-administered lipophilic drug cannabidiol, *Int. J. Pharm.*, 624 (2022) 121947-121957. <https://doi.org/121910.121016/j.ijpharm.122022.121947>.
- [152] S. Deiana, A. Watanabe, Y. Yamasaki, N. Amada, M. Arthur, S. Fleming, H. Woodcock, P. Dorward, B. Pigliacampo, S. Close, B. Platt, G. Riedel, Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarin (CBDV), Δ^9 -tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive-compulsive behaviour, *Psychopharmacol.*, 219 (2012) 859-873. <https://doi.org/810.1007/s00213-00011-02415-00210>.
- [153] C. G. Stott, L. White, S. Wright, D. Wilbraham, G. W. Guy, A phase I study to assess the single and multiple dose pharmacokinetics of THC/CBD oromucosal spray, *Eur. J. Clin. Pharmacol.*, 69 (2012) 1135-1147. <https://doi.org/1110.1007/s00228-00012-01441-00220>.

- [154] A. Ohlsson, J. E. Lindgren, S. Andersson, S. Agurell, H. Gillespie, L. E. Hollister, Single-dose kinetics of deuterium-labelled cannabidiol in man after smoking and intravenous administration, *Biomed. & Environ. Mass Spectrom.*, 13 (1986) 77-83. <https://doi.org/10.1002/bms.1200130206>.
- [155] W. Feng, C. Qin, Y. J. Chu, M. Berton, J. B. Lee, A. Zgair, S. Bettonte, M. J. Stocks, C. S. Constantinescu, D. A. Barrett, P. M. Fischer, P. Gershkovich, Natural sesame oil is superior to pre-digested lipid formulations and purified triglycerides in promoting the intestinal lymphatic transport and systemic bioavailability of cannabidiol, *Eur. J. Pharm. Biopharm.*, 162 (2021) 49-49. <https://doi.org/10.1016/j.ejpb.2021.1002.1013>.
- [156] A. Jewell, A. Brookes, W. Feng, M. Ashford, P. Gellert, J. Butler, P. M. Fischer, D. J. Scurr, M. J. Stocks, P. Gershkovich, Distribution of a highly lipophilic drug cannabidiol into different lymph nodes following oral administration in lipidic vehicle, *Eur. J. Pharm. Biopharm.*, 174 (2022) 29-34. <https://doi.org/10.1016/j.ejpb.2022.1003.1014>.
- [157] J. Merrick, B. Lane, T. Seabee, Identification of Psychoactive Degradants of Cannabidiol in Simulated Gastric and Physiological Fluid, *Cannabis and Cannabinoid Res.*, 1 (2016) 102-113. <https://doi.org/10.1089/can.2015.0004>.
- [158] G. Nahler, F. Grotenhermen, A. W. Zuardi, J. A. S. Crippa, A Conversion of Oral Cannabidiol to Delta9-Tetrahydrocannabinol Seems Not to Occur in Humans, *Cannabis and Cannabinoid Res.*, 2 (2017) 81-87. <https://doi.org/10.1089/can.2017.0009>.
- [159] V. Franco, P. Gershkovich, E. Perucca, M. Bialer, The Interplay Between Liver First-Pass Effect and Lymphatic Absorption of Cannabidiol and Its Implications for Cannabidiol Oral Formulations, *Clin. Pharmacokinet.*, 59 (2020) 1493-1500. <https://doi.org/1410.1007/s40262-40020-00931-w>.
- [160] J. Atsmon, I. Cherniakov, D. Izgelov, A. Hoffman, A. J. Domb, L. Deutsch, F. Deutsch, D. Heffetz, H. Sacks, PTL401, a New Formulation Based on Pro-Nano Dispersion Technology, Improves Oral Cannabinoids Bioavailability in Healthy Volunteers, *J. Pharm. Sci.*, 107 (2018) 1423-1429. <https://doi.org/1410.1016/j.xphs.2017.1412.1020>.
- [161] European Medicines Agency, Epidyolex (2019), <https://www.ema.europa.eu/en/medicines/human/EPAR/epidyolex>, accessed 24 July 2022.

- [162] J. Engel, Introduction to temporal lobe epilepsy, *Epilepsy Res.*, 26 (1996) 141-150. [https://doi.org/110.1016/S0920-1211\(1096\)00043-00045](https://doi.org/110.1016/S0920-1211(1096)00043-00045).
- [163] J. Sastre-Garriga, C. Vila, S. Clissold, X. Montalban, THC and CBD oromucosal spray (Sativex®) in the management of spasticity associated with multiple sclerosis, *Expert Rev. Neurother.*, 11 (2014) 627-637. <https://doi.org/610.1586/ern.1511.1547>.
- [164] EMC, Sativex Oromucosal Spray (2022), <https://www.medicines.org.uk/emc/product/602/smpc#gref>, accessed 06 November 2022.
- [165] MS Society, Sativex (nabiximols) (2021), <https://www.mssociety.org.uk/about-ms/treatments-and-therapies/cannabis/sativex>, accessed 06 November 2022.
- [166] A. Zgair, J. C. M. Wong, J. B. Lee, J. Mistry, O. Sivak, K. M. Wasan, I. M. Hennig, D. A. Barrett, C. S. Constantinescu, P. M. Fischer, P. Gershkovich, Dietary fats and pharmaceutical lipid excipients increase systemic exposure to orally administered cannabis and cannabis-based medicines, *Am. J. Transl. Res.*, 8 (2016) 3448-3459.
- [167] S. A. Millar, N. L. Stone, A. S. Yates, S. E. O'Sullivan, A systematic review on the pharmacokinetics of cannabidiol in humans, *Front. Pharmacol.*, 9 (2018) 1365-1378. <https://doi.org/1310.3389/fphar.2018.01365>.
- [168] T. Hložek, L. Uttl, L. Kadeřábek, M. Balíková, E. Lhotková, R. R. Horsley, P. Nováková, K. Šíchová, K. Štefková, F. Tylš, M. Kuchař, T. Páleníček, Pharmacokinetic and behavioural profile of THC, CBD, and THC+CBD combination after pulmonary, oral, and subcutaneous administration in rats and confirmation of conversion in vivo of CBD to THC, *Eur. Neuropsychopharmacol.*, 27 (2017) 1223-1237. <https://doi.org/1210.1016/j.euroneuro.2017.1210.1037>.
- [169] A. Zgair, J. B. Lee, J. C. M. Wong, D. A. Taha, J. Aram, D. D. Virgilio, J. W. McArthur, Y. K. Cheng, I. M. Hennig, D. A. Barrett, P. M. Fischer, C. S. Constantinescu, P. Gershkovich, Oral administration of cannabis with lipids leads to high levels of cannabinoids in the intestinal lymphatic system and prominent immunomodulation, *Sci. Rep.*, 7 (2017) 14542-14554. <https://doi.org/14510.11038/s41598-14017-15026-z>.

- [170] K. L. Kirk, Fluorine in medicinal chemistry: Recent therapeutic applications of fluorinated small molecules, *J. Fluorine Chem.*, 127 (2006) 1013-1029. <https://doi.org/10.1016/j.jfluchem.2006.1006.1007>.
- [171] J. Fried, E. F. Sabo, Synthesis of 17 α -hydroxycorticosterone and its 9 α -halo derivatives from 11-EPI-17 α -hydroxycorticosterone, *J. Am. Chem. Soc.*, 75 (1953) 2273-2274. <https://doi.org/2210.1021/ja01105a01527>.
- [172] C. Heidelberger, N. K. Chaudhuri, P. Danneberg, D. Mooren, L. Griesbach, R. Duschinsky, R. J. Sshnitzer, E. Plevin, J. Scheiner, Fluorinated Pyrimidines, A New Class of Tumour-Inhibitory Compounds, *Nat.*, 179 (1957) 663-666. <https://doi.org/610.1038/179663a179660>.
- [173] B. Gaye, A. Adejare, Fluorinated molecules in the diagnosis and treatment of neurodegenerative diseases, *Future Med. Chem.*, 1 (2009) 1-15. <https://doi.org/10.4155/fmc.4109.4185>.
- [174] M. Amano, R. S. Yedidi, P. M. Salcedo-Gómez, H. Hayashi, K. Hasegawa, C. D. Martyr, A. K. Ghosh, H. Mitsuya, Fluorine Modifications Contribute to Potent Antiviral Activity against Highly Drug-Resistant HIV-1 and Favorable Blood-Brain Barrier Penetration Property of Novel Central Nervous System-Targeting HIV-1 Protease Inhibitors In Vitro, *Antimicrob. Chemother.*, 66 (2022) e01715-21. <https://doi.org/10.1128/aac.01715-01721>.
- [175] S. Sun, A. Adejare, Fluorinated Molecules as Drugs and Imaging Agents in the CNS, *Med. Chem.*, 6 (2006) 1457-1464. <https://doi.org/1410.2174/156802606777951046>.
- [176] A. Breuer, C. G. Haj, M. V. Fogaça, F. V. Gomes, N. R. Silva, J. F. Pedrazzi, E. A. D. Bel, J. C. Hallak, J. A. Crippa, A. W. Zuardi, R. Mechoulam, F. S. Guimarães, Fluorinated Cannabidiol Derivatives: Enhancement of Activity in Mice Models Predictive of Anxiolytic, Antidepressant and Antipsychotic Effects, *PLoS ONE*, (2016) 1-19. <https://doi.org/10.1371/journal.pone.0158779>.
- [177] N. R. Silva, F. V. Gomes, M. D. Fonseca, R. Mechoulam, A. Breuer, T. M. Cunha, F. S. Guimarães, Antinociceptive effects of HUF-101, a fluorinated cannabidiol derivative, *Prog. Neuro-Psychopharmacol. Biol. Psychiatry*, 79 (2017) 369-377. <https://doi.org/310.1016/j.pnpbp.2017.1007.1012>.
- [178] M. Perez, L. P. Cartarozzi, G. B. Chiarotto, S. A. de Oliveira, F. S. Guimarães, A. L. R. de Oliveira, Neuronal preservation and reactive gliosis attenuation following neonatal sciatic nerve axotomy by a fluorinated cannabidiol

- derivative, *Neuropharmacol.*, 140 (2018) 201-208.
<https://doi.org/210.1016/j.neuropharm.2018.1008.1009>.
- [179] N. R. Silva, F. I. F. Gomes, A. H. P. Lopes, I. L. Cortez, J. C. dos Santos, C. E. A. Silva, R. Mechoulam, F. V. Gomes, T. M. Cunha, F.S. Guimarães, The Cannabidiol Analog PECS-101 Prevents Chemotherapy-Induced Neuropathic Pain via PPAR γ Receptors, *Neurother.*, 19 (2022) 434-449.
<https://doi.org/410.1007/s13311-13021-01164-w>.
- [180] T. M. Stollenwerk, S. Pollock, C. J. Hillard, Contribution of the Adenosine 2A Receptor to Behavioral Effects of Tetrahydrocannabinol, Cannabidiol and PECS-101, *Mol.*, 26 (2021) 5354-5367.
<https://doi.org/5310.3390/molecules26175354>.
- [181] Y. Schober, S. Guenther, B. Spengler, A. Rompp, Single Cell Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging, *Anal. Chem.*, 84 (2012) 6293-6297. <https://doi.org/6210.1021/ac301337h>.
- [182] I. Yamada, J. Matsuo, N. Toyoda, A. Kirkpatrick, Materials processing by gas cluster ion beams, *Mater. Sci. Eng.: R: Reports*, 34 (2001) 231-295.
[https://doi.org/210.1016/S0927-1796X\(1001\)00034-00031](https://doi.org/210.1016/S0927-1796X(1001)00034-00031).
- [183] M. K. Passarelli, A. Pirkel, R. Moellers, D. Grinfeld, F. Kollmer, R. Havelund, C. F. Newman, P. S. Marshall, H. Arlinghaus, M. R. Alexander, A. West, S. Horning, E. Niehuis, A. Makarov, C. T. Dollery, I. S. Gilmore, The 3D OrbiSIMS—label-free metabolic imaging with subcellular lateral resolution and high mass-resolving power, *Nat. Methods*, 14 (2017) 1175-1183.
<https://doi.org/1110.1038/nmeth.4504>.
- [184] J. Zhang, J. Brown, D. J. Scurr, A. Bullen, K. MacLellan-Gibson, P. Williams, M. R. Alexander, K. R. Hardie, I. S. Gilmore, P. D. Rakowska, Cryo-OrbiSIMS for 3D Molecular Imaging of a Bacterial Biofilm in Its Native State, *Anal. Chem.*, 92 (2020) 9008-9015.
<https://doi.org/9010.1021/acs.analchem.9000c01125>.
- [185] S. Sämfors, J. S. Fletcher, Lipid Diversity in Cells and Tissue Using Imaging SIMS, *Annu. Rev.*, 13 (2020) 249-271. <https://doi.org/210.1146/annurev-anchem-091619-103512>.
- [186] W. Suvannapruk, M. K. Edney, D. H. Kim, D. J. Scurr, A. M. Ghaemmaghami, M. R. Alexander, Single-Cell Metabolic Profiling of Macrophages Using 3D

- OrbiSIMS: Correlations with Phenotype, *Anal. Chem.*, 94 (2022) 9389-9398.
<https://doi.org/9310.1021/acs.analchem.9382c01375>.
- [187] J. Meurs, D. J. Scurr, A. Lourdasamy, L. C. D. Storer, R. G. Grundy, M. R. Alexander, R. Rahman, D.H. Kim, Sequential 3D OrbiSIMS and LESA-MS/MS-based metabolomics for prediction of brain tumor relapse from sample-limited primary tissue archives, *bioRxiv*, (2021)
<https://doi.org/10.1101/2020.1107.1115.182071>.
- [188] C. F. Newman, R. Havelund, M. K. Passarelli, P. S. Marshall, I. Francis, A. West, M. R. Alexander, I. S. Gilmore, C. T. Dollery, Intracellular Drug Uptake - A Comparison of Single Cell Measurements Using ToF-SIMS Imaging and Quantification from Cell Populations with LC/MS/MS, *Anal. Chem.*, 89 (2017) 11944-11953. <https://doi.org/11910.11021/acs.analchem.11947b01436>.
- [189] J. Shen, M. Yu, Q. Meng, J. Li, Y. Lv, W. Lu, Fatty Acid-Based Strategy for Efficient Brain Targeted Gene Delivery, *Pharm. Res.*, 30 (2013) 2573-2583.
<https://doi.org/2510.1007/s11095-11013-11056-x>.
- [190] D. M. L. Morgan, Tetrazolium (MTT) Assay for Cellular Viability and Activity, *Methods in Molecular Biology, Polyamine Protoc.*, 79 (1998) 179-184.
- [191] J. van Meerloo, G. J. L. Kaspers, J. Cloos, Cell Sensitivity Assays: The MTT Assay, *Methods Mol. Biol.*, (2011) 237-245. https://doi.org/210.1007/1978-1001-61779-61080-61775_61720.
- [192] D. Cadena-Herrera, J. E. E. D. Lara, N. D. Ramírez-Ibañez, C. A. López-Morales, N. O. Pérez, L. F. Flores-Ortiz, E. Medina-Rivero, Validation of three viable-cell counting methods: Manual, semi-automated, and automated, *Biotechnol. Rep.*, 7 (2015) 9-16. <https://doi.org/10.1016/j.btre.2015.1004.1004>.
- [193] K. V. Thrivikraman, R. L. Huot, P. M. Plotsky, Jugular vein catheterization for repeated blood sampling in the unrestrained conscious rat, *Brain Res. Protoc.*, 10 (2002) 84-94. [https://doi.org/10.1016/S1385-1299X\(1002\)00185-X](https://doi.org/10.1016/S1385-1299X(1002)00185-X).
- [194] W. Korfmacher, Y. Luo, S. Ho, W. Sun, L. Shen, J. Wang, Z. Wu, Y. Guo, G. Snow, T. O'Shea, Utility of capillary microsampling for rat pharmacokinetic studies: Comparison of tail-vein bleed to jugular vein cannula sampling, *J. Pharmacol. Toxicol. Methods*, 76 (2015) 7-14.
<https://doi.org/10.1016/j.vascn.2015.1007.1001>.
- [195] A. Zgair, J. C. M. Wong, A. Sabri, P. M. Fischer, D. A. Barrett, C. S. Constantinescu, P. Gershkovich, Development of a simple and sensitive HPLC–

- UV method for the simultaneous determination of cannabidiol and Δ^9 -tetrahydrocannabinol in rat plasma, *J. Pharm. Biopharm. Anal.*, 114 (2015) 145-151. <https://doi.org/110.1016/j.jpba.2015.1005.1019>.
- [196] S. Spijker, Dissection of Rodent Brain Regions, *NeuroMethods*, 57 (2011) 13-26. https://doi.org/10.1007/1978-1001-61779-61111-61776_61772.
- [197] N. L. Tilney, Patterns of lymphatic drainage in the adult laboratory rat, *J. Anat.*, 109 (1971) 369-383.
- [198] A. P. Robinson, C. T. Harp, A. Noronha, S.D. Miller, Chapter 8 - The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment, *Handb. Clin. Neurol.*, 122 (2014) 173-189. <https://doi.org/110.1016/B1978-1010-1444-52001-52002.00008-X>.
- [199] C. S. Constantinescu, N. Farooqi, K. O'Brien, B. Gran, Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS), *Br. J. Pharmacol.*, 164 (2012) 1079-1106. <https://doi.org/1010.1111/j.1476-5381.2011.01302.x>.
- [200] K. J. Smith, D. Minns, B. J. McHugh, R. K. Holloway, R. O'Connor, A. Williams, L. Melrose, R. McPherson, V. E. Miron, D. J. Davidson, E. G. Findlay, The antimicrobial peptide cathelicidin is critical for the development of Th17 responses in severe inflammatory disease, *bioRxiv*, (2022) 1-33. <https://doi.org/10.1101/2022.01.27.477976>.
- [201] U.S. Food & Drug Administration, Bioanalytical Method Validation Guidance for Industry (2018), <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry>, accessed 06 June 2022.
- [202] M. K. Edney, A. M. Kotowska, M. Spanu, G. F. Trindade, E. Wilmot, J. Reid, J. Barker, J. W. Aylott, A. G. Shard, M. R. Alexander, C. E. Snape, D. J. Scurr, Molecular Formula Prediction for Chemical Filtering of 3D OrbiSIMS Datasets, *Anal. Chem.*, 94 (2022) 4703-4711. <https://doi.org/4710.1021/acs.analchem.4701c04898>.
- [203] G. F. Trindade, M. L. Abel, J. F. Watts, simsMVA: A tool for multivariate analysis of ToF-SIMS datasets, *Chemom. Intell. Lab. Syst.*, 182 (2018) 180-187. <https://doi.org/110.1016/j.chemolab.2018.1010.1001>.

- [204] N. Miltner, G. Béke, Á. Angyal, Á. Kemény, E. Pintér, Z. Helyes, T. Bíró, J. Mihály, Assessment of the anti-inflammatory effects of cannabidiol and its fluorinated derivative in in vitro and in vivo models of atopic dermatitis, *Innate Immun. Microbiol. Inflammation*, 138 (2018) S173. <https://doi.org/10.1016/j.jid.2018.1003.1032>.
- [205] C. Citti, F. Palazzoli, M. Licata, A. Vilella, G. Leo, M. Zoli, M. A. Vandelli, F. Forni, B. Pacchetti, G. Cannazza, Untargeted rat brain metabolomics after oral administration of a single high dose of cannabidiol, *J. Pharm. Biomed. Anal.*, 161 (2018) 1-11. <https://doi.org/10.1016/j.jpba.2018.1008.1021>.
- [206] K. A. Scott, A. G. Dalglish, W. M. Liu, The Combination of Cannabidiol and Δ^9 -Tetrahydrocannabinol Enhances the Anticancer Effects of Radiation in an Orthotopic Murine Glioma Model, *Mol. Cancer Ther.*, 13 (2014) 2955-2967. <https://doi.org/2910.1158/1535-7163.MCT-2914-0402>.
- [207] A. Vaccani, P. Massi, A. Colombo, T. Rubino, D. Parolaro, Cannabidiol inhibits human glioma cell migration through a cannabinoid receptor-independent mechanism, *Br. J. Pharmacol.*, 144 (2009) 1032-1036. <https://doi.org/1010.1038/sj.bjp.0706134>.
- [208] S. Caragher, J. Miska, J. Shireman, C. H. Park, M. Muroski, M. S. Lesniak, A. U. Ahmed, Temozolomide Treatment Increases Fatty Acid Uptake in Glioblastoma Stem Cells, *Cancers*, 12 (2020) 3126-3142. <https://doi.org/3110.3390/cancers12113126>.
- [209] S. I. Bukhari, S. S. Imam, M. Z. Ahmad, P. R. Vuddanda, S. Alshehri, W. A. Mahdi, J. Ahmad, Recent Progress in Lipid Nanoparticles for Cancer Theranostics: Opportunity and Challenges, *Pharm.*, 13 (2021) 840-864. <https://doi.org/810.3390/pharmaceutics13060840v>.
- [210] R. Eychenne, C. Bouvry, M. Bourgeois, P. Loyer, E. Benoist, N. Lepareur, Overview of Radiolabeled Somatostatin Analogs for Cancer Imaging and Therapy, *Mol.*, 25 (2020) 4012-4047. <https://doi.org/4010.3390/molecules25174012>.
- [211] C. Happold, P. Roth, W. Wick, N. Schmidt, A. M. Florea, M. Silginer, G. Reifenberger, M. Weller, Distinct molecular mechanisms of acquired resistance to temozolomide in glioblastoma cells, *J. Neurochem.*, 122 (2012) 444-455. <https://doi.org/410.1111/j.1471-4159.2012.07781.x>.

- [212] Q. Wu, A. E. Berglund, D. Wang, R. J. MacAulay, J. J. Mulé, A. B. Etame, Paradoxical epigenetic regulation of XAF1 mediates plasticity towards adaptive resistance evolution in MGMT-methylated glioblastoma, *Sci. Rep.*, 9 (2019) 14072-14085. <https://doi.org/14010.11038/s41598-14019-50489-14072>.
- [213] A. L. Rivera, C. E. Pelloso, M. R. Gilbert, H. Colman, C. De La Cruz, E. P. Sulman, B. N. Bekele, K. D. Aldape, MGMT promoter methylation is predictive of response to radiotherapy and prognostic in the absence of adjuvant alkylating chemotherapy for glioblastoma, *Neuro-Oncol.*, 12 (2010) 116-121. <https://doi.org/110.1093/neuonc/nop1020>.
- [214] M. E. Hegi, L. Liu, J. G. Herman, R. Stupp, W. Wick, M. W. M. P. Mehta, M. R. Gilbert, Correlation of O6-Methylguanine Methyltransferase (MGMT) Promoter Methylation With Clinical Outcomes in Glioblastoma and Clinical Strategies to Modulate MGMT Activity, *J. Clin. Oncol.*, 26 (2008) 4189-4199. <https://doi.org/4110.1200/JCO.2007.4111.5964>.
- [215] R. Ralhan, J. Kaur, Alkylating agents and cancer therapy, *Expert Opin. Ther. Pat.*, 17 (2007) 1061-1075. <https://doi.org/1010.1517/13543776.13543717.13543779.13541061>.
- [216] J. Y. J. Wang, W. Edelmann, Mismatch repair proteins as sensors of alkylation DNA damage, *Cancer Cell*, 9 (2006) 417-418. <https://doi.org/410.1016/j.ccr.2006.1005.1013>.
- [217] W. P. Roos, A. D. Thomas, B. Kaina, DNA damage and the balance between survival and death in cancer biology, *Nat. Rev. Cancer*, 16 (2016) 20-33. <https://doi.org/10.1038/nrc.2015.1032>.
- [218] B. Taib, A. M. Aboussalah, M. Moniruzzaman, S. Chen, N. J. Haughey, S. F. Kim, R. S. Ahima, Lipid accumulation and oxidation in glioblastoma multiforme, *Sci. Rep.*, 9 (2019) 19593-19607. <https://doi.org/19510.11038/s41598-19019-55985-z>.
- [219] Y. Yuan, N. Shah, M. I. Almohaisin, S. Saha, F. Lu, Assessing fatty acid-induced lipotoxicity and its therapeutic potential in glioblastoma using stimulated Raman microscopy, *Sci. Rep.*, 11 (2021) 7422-7436. <https://doi.org/7410.1038/s41598-41021-86789-41599>.
- [220] L. Soroceanu, E. Singer, P. Dighe, M. Sidorov, C. Limbad, A. Rodriguez-Brotons, P. Rix, R. W. L. Woo, L. Dickinson, P. Y. Desprez, S. D. McAllister, Cannabidiol inhibits RAD51 and sensitizes glioblastoma to temozolomide in

- multiple orthotopic tumor models, *Neuro-Oncol. Adv.*, 4 (2022) 1-11. <https://doi.org/10.1093/noajnl/vdac1019>.
- [221] A. D. Hari, N. G. Vegi, U. N. Das, Arachidonic and eicosapentaenoic acids induce oxidative stress to suppress proliferation of human glioma cells, *Arch. Med. Sci.*, 16 (2020) 974-983. <https://doi.org/910.5114/aoms.2020.92293>.
- [222] L. Liu, W. R. Hudgins, S. Shack, M. Q. Yin, D. Samid, Cinnamic acid: a natural product with potential use in cancer intervention, *Int. J. Cancer*, 62 (1995) 345-350. <https://doi.org/310.1002/ijc.2910620319>.
- [223] A. Martin, L. L. Chao, Semantic memory and the brain: structure and processes, *Curr. Opin. Neurobiol.*, 11 (2001) 194-201. [https://doi.org/110.1016/S0959-4388\(1000\)00196-00193](https://doi.org/110.1016/S0959-4388(1000)00196-00193).
- [224] S. Ackerman, *Discovering the Brain.*, Washington (DC): National Academies Press (US) (1992). <https://www.ncbi.nlm.nih.gov/books/NBK234157/>.
- [225] T. Sprenger, A. Berthele, S. Platzner, H. Boecker, T. R. Tölle, What to learn from in vivo opiodergic brain imaging?, *Eur. J. Pain*, 9 (2005) 117-121. <https://doi.org/110.1016/j.ejpain.2004.1007.1010>.
- [226] A. S. Yu, B. A. Hirayama, G. Timbol, J. Liu, A. Diez-Sampedro, V. Kepe, N. Satyamurthy, S. C. Huang, E. M. Wright, J. R. Barrio, Regional distribution of SGLT activity in rat brain in vivo, *Am. J. Physiol.*, 304 (2013) 240-247. <https://doi.org/210.1152/ajpcell.00317.02012>.
- [227] F. Sousa, S. Mandal, C. Garrovo, A. Astolfo, A. Bonifacio, D. Latawiec, R. H. Menk, F. Arfelli, S. Huewel, G. Legname, H. J. Galla, S. Krol, Functionalized gold nanoparticles: a detailed in vivo multimodal microscopic brain distribution study, *Nanoscale*, 2 (2010) 2826-2834. <https://doi.org/2810.1039/C2820NR00345J>.
- [228] N. J. Abbott, A. A. K. Patabendige, D. E. M. Dolman, S. R. Yusof, D. J. Begley, Structure and function of the blood–brain barrier, *Neurobiol. Dis.*, 37 (2010) 13-25. <https://doi.org/10.1016/j.nbd.2009.1007.1030>.
- [229] J. Gould, Breaking down the epidemiology of brain cancer, *Nat.*, 561 (2018), 240-242.
- [230] B. Jeremic, D. Grujicic, V. Antunovic, L. Djuric, M. Stojanovic, Y. Shibamoto, Influence of extent of surgery and tumor location on treatment outcome of patients with glioblastoma multiforme treated with combined modality approach *J. Neuro-Oncol.*, 21 (1994) 177-185. <https://doi.org/110.1007/BF01052902>.

- [231] L. L. Anderson, I. K. Low, S. D. Banister, I. S. McGregor, J. C. Arnold, Pharmacokinetics of Phytocannabinoid Acids and Anticonvulsant Effect of Cannabidiolic Acid in a Mouse Model of Dravet Syndrome, *J. Nat. Prod.*, 82 (2019) 3047-3055. <https://doi.org/3010.1021/acs.jnatprod.3049b00600>.
- [232] A. Lewinska, J. Zebrowski, M. Duda, A. Gorka, M. Wnuk, Fatty Acid Profile and Biological Activities of Linseed and Rapeseed Oils, *Mol.*, 20 (2015) 22872-22880. <https://doi.org/22810.23390/molecules201219887>.
- [233] J. Attems, L. Walker, K.A. Jellinger, Olfactory bulb involvement in neurodegenerative diseases, *Acta Neuropathol.*, 127 (2014) 459-475. <https://doi.org/410.1007/s00401-00014-01261-00407>.
- [234] T. G. Ohm, H. Braak, Olfactory bulb changes in Alzheimer's disease, *Acta Neuropathol.*, 73 (1987) 365-369. <https://doi.org/310.1007/BF00688261>.
- [235] U. Stockhorst, R. Pietrowsky, Olfactory perception, communication, and the nose-to-brain pathway, *Physiol. Behav.*, 83 (2004) 3-11. <https://doi.org/10.1016/j.physbeh.2004.1007.1018>.
- [236] P. W. Hellings, F. Dobbels, K. Denhaerynck, M. Piessens, J. L. Ceuppens, S. D. Geest, Explorative study on patient's perceived knowledge level, expectations, preferences and fear of side effects for treatment for allergic rhinitis, *Clin. Transl. Allergy*, 2 (2012) 1-8. <https://doi.org/10.1186/2045-7022-1182-1189>.
- [237] C. Chayer, M. Freedman, Frontal lobe functions, *Curr. Neurol. Neurosci. Rep.*, 1 (2001) 547-552. <https://doi.org/510.1007/s11910-11001-10060-11914>.
- [238] A. E. Taylor, J. A. Saint-Cyr, A. E. Lang, Frontal lobe dysfunction in Parkinson's disease: the cortical focus of neostriatal outflow, *Brain*, 109 (1986) 845-883. <https://doi.org/810.1093/brain/1109.1095.1845>.
- [239] D. Neary, J. S. Snowden, D. M. Mann, B. Northen, P. J. Goulding, N. Macdermott, Frontal lobe dementia and motor neuron disease., *J. Neurol., Neurosurg. Psychiatry*, 53 (1990) 23-32. <http://dx.doi.org/10.1136/jnnp.1153.1131.1123>.
- [240] A. M. Graybiel, S.T. Grafton, The Striatum: Where Skills and Habits Meet, *Cold Spring Harbor Perspect. Biol.*, 7 (2015) a021691. <https://doi.org/021610.021101/cshperspect.a021691>.
- [241] R. Báez-Mendoza, W. Schultz, The role of the striatum in social behavior, *Front. Neurosci.*, 7 (2013) 1-14. <https://doi.org/10.3389/fnins.2013.00233>.

- [242] P. C. Nopoulos, Huntington disease: a single-gene degenerative disorder of the striatum, *Dialogues Clin. Neurosci.*, 18 (2016) 91-98. <https://doi.org/10.31887/DCNS.32016.31818.31881/pnopoulos>.
- [243] E. M. Jansen, D. A. Haycock, S. J. Ward, V. S. Seybold, Distribution of cannabinoid receptors in rat brain determined with aminoalkylindoles, *Brain Res.*, 575 (1992) 93-102. [https://doi.org/110.1016/0006-8993\(1992\)90428-C](https://doi.org/110.1016/0006-8993(1992)90428-C).
- [244] M. Herkenham, A. B. Lynn, M. D. Little, M. R. Johnson, L. S. Melvin, B. R. de Costa, K. C. Rice, Cannabinoid receptor localization in brain, *Proc. Natl. Acad. Sci. U. S. A.*, 87 (1990) 1932-1936. <https://doi.org/1910.1073/pnas.1987.1935.1932>.
- [245] A. B. Sonogo, F. V. Gomes, E. A. Del Bel, F. S. Guimaraes, Cannabidiol attenuates haloperidol-induced catalepsy and c-Fos protein expression in the dorsolateral striatum via 5-HT_{1A} receptors in mice, *Behav. Brain Res.*, 309 (2016) 22-28. <https://doi.org/10.1016/j.bbr.2016.1004.1042>.
- [246] M. M. A. Engels, A. Hillebrand, W. M. van der Flier, C. J. Stam, P. Scheltens, E. C. W. v. Straaten, Slowing of Hippocampal Activity Correlates with Cognitive Decline in Early Onset Alzheimer's Disease. An MEG Study with Virtual Electrodes, *Front. Hum. Neurosci.*, 10 (2016) 238. <https://doi.org/210.3389/fnhum.2016.00238>.
- [247] R. J. Douglas, The hippocampus and behavior, *Psychol. Bull.*, 67 (1967) 416-442. <https://doi.org/410.1037/h0024599>.
- [248] J. D. Green, The Hippocampus, *Physiol. Rev.*, 44 (1964) 561-608. <https://doi.org/510.1152/physrev.1964.1144.1154.1561>.
- [249] H. Eichenbaum, T. Otto, The Hippocampus - What Does It Do? , *Behav. Neural. Biol.*, 57 (1992) 2-36. [https://doi.org/10.1016/0163-1047\(1992\)90724-I](https://doi.org/10.1016/0163-1047(1992)90724-I).
- [250] T. Demirkaca, A. Sartorius, G. Ende, N. Meyer, H. Welzel, G. Skopp, K. Mann, D. Hermann, Diminished gray matter in the hippocampus of cannabis users: Possible protective effects of cannabidiol, *Drug Alcohol Depend.*, 114 (2011) 242-245. <https://doi.org/210.1016/j.drugalcdep.2010.1009.1020>.
- [251] C. A. J. Stern, T. R. da Silva, A. M. Raymundi, C. P. de Souza, V. A. Hiroaki-Sato, L. Kato, F. S. Guimarães, R. Andreatini, R. N. Takahashi, L.J. Bertoglio, Cannabidiol disrupts the consolidation of specific and generalized fear memories via dorsal hippocampus CB₁ and CB₂ receptors, *Neuropharmacol.*, 125 (2017) 220-230. <https://doi.org/210.1016/j.neuropharm.2017.1007.1024>.

- [252] M. A. Abame, Y. He, S. Wu, Z. Xie, J. Zhang, X. Gong, C. Wu, J. Shen, Chronic administration of synthetic cannabidiol induces antidepressant effects involving modulation of serotonin and noradrenaline levels in the hippocampus, *Neurosci. Lett.*, 744 (2021) 1-7. <https://doi.org/10.1016/j.neulet.2020.135594>.
- [253] D. Denny-Brown, R. A. Chambers, The parietal lobe and behavior, *Res. Publ. - Assoc. Res. Nerv. Ment. Dis.*, 36 (1958) 35-117.
- [254] L. Fogassi, G. Luppino, Motor functions of the parietal lobe, *Curr. Opin. Neurobiol.*, 15 (2005) 626-631. <https://doi.org/10.1016/j.conb.2005.1010.1015>.
- [255] K. Kaida, K. Takeda, N. Nagata, K. Kamakura, Alzheimer's disease with asymmetric parietal lobe atrophy: A case report, *J. Neurol. Sci.*, 160 (1998) 96-99. [https://doi.org/10.1016/S0022-1510X\(1098\)00221-00224](https://doi.org/10.1016/S0022-1510X(1098)00221-00224).
- [256] J. E. Adcock, C. P. Panayiotopoulos, Occipital Lobe Seizures and Epilepsies, *J. Clin. Neurophysiol.*, 29 (2012) 397-407. <https://doi.org/10.1097/WNP.1090b1013e31826c31898fe>.
- [257] S. Sveinbjornsdottir, J. S. Duncan, Parietal and Occipital Lobe Epilepsy: A Review, *Epilepsia*, 34 (1993) 493-521. <https://doi.org/10.1111/j.1528-1157.1993.tb02590.x>.
- [258] S. Sciacca, J. Lynch, I. Davagnanam, R. Barker, Midbrain, Pons, and Medulla: Anatomy and Syndromes, *RadioGr.*, 39 (2019) 1110-1126. <https://doi.org/10.1148/rg.2019180126>.
- [259] F. Chen, J. J. Li, T. Liu, G. Q. Wen, W. Xiang, Clinical and neuroimaging features of enterovirus71 related acute flaccid paralysis in patients with hand-foot-mouth disease, *Asian Pac. J. Tropical Med.*, 6 (2013) 68-72. [https://doi.org/10.1016/S1995-7645\(1012\)60203-X](https://doi.org/10.1016/S1995-7645(1012)60203-X).
- [260] Y. Hishikawa, T. Shimizu, Physiology of REM sleep, cataplexy, and sleep paralysis., *Adv. Neurol.*, 67 (1995) 245-271.
- [261] Y. Zhang, H. Li, S. Jin, Y. Lu, Y. Peng, L. Zhao, X. Wang, Cannabidiol protects against Alzheimer's disease in *C. elegans* via ROS scavenging activity of its phenolic hydroxyl groups, *Eur. J. Pharmacol.*, 919 (2022) 1-8. <https://doi.org/10.1016/j.ejphar.2022.174829>.
- [262] M. H. N. Chagas, A. L. Eckeli, A. W. Zuardi, M. A. Pena-Pereira, M. A. Sobreira-Neto, E. T. Sobreira, M. R. Camilo, M. M. Bergamaschi, C. H. Schenck, J. E. C. Hallak, V. Tumas, J. A. S. Crippa, Cannabidiol can improve

- complex sleep-related behaviours associated with rapid eye movement sleep behaviour disorder in Parkinson's disease patients: a case series, *J. Clin. Pharm. Therapeutics*, 39 (2014) 564-566. <https://doi.org/510.1111/jcpt.12179>.
- [263] P. L. Strick, R. P. Dum, J. A. Fiez, Cerebellum and Nonmotor Function, *Annu. Rev. Neurosci.*, 32 (2009) 413-434. <https://doi.org/410.1146/annurev.neuro.1131.060407.125606>.
- [264] T. Ohyama, W. L. Nores, M. Murphy, M. D. Mauk, What the cerebellum computes, *Trends Neurosci.*, 26 (2003) 222-227. [https://doi.org/210.1016/S0166-2236\(1003\)00054-00057](https://doi.org/210.1016/S0166-2236(1003)00054-00057).
- [265] R. Nenert, J. B. Allendorfer, E. M. Bebin, T. E. Gaston, L. E. Grayson, J. T. Houston, J.P. Szaflarski, Cannabidiol normalizes resting-state functional connectivity in treatment-resistant epilepsy, *Epilepsy Behav.*, 112 (2020) 107297-107305. <https://doi.org/107210.101016/j.yebeh.102020.107297>.
- [266] S. Kuerten, D. A. Kostova-Bales, L. P. Frenzel, J. T. Tigno, M. Tary-Lehmann, D. N. Angelova, P. V. Lehmann, MP4- and MOG:35–55-induced EAE in C57BL/6 mice differentially targets brain, spinal cord and cerebellum, *J. Neuroimmunol.*, 189 (2007) 31-40. <https://doi.org/10.1016/j.jneuroim.2007.1006.1016>.
- [267] N. Murugesan, D. Paul, Y. Lemire, B. Shrestha, S. Ge, J. S. Pachter, Active induction of experimental autoimmune encephalomyelitis by MOG35-55 peptide immunization is associated with differential responses in separate compartments of the choroid plexus, *Fluids Barriers CNS*, 9 (2012) 1-16. <https://doi.org/10.1186/2045-8118-1189-1115>.
- [268] A. Bar-Or, M. P. Pender, R. Khanna, L. Steinman, H. P. Hartung, T. Maniar, E. Croze, B. T. Aftab, G. Giovannoni, M. A. Joshi, Epstein–Barr Virus in Multiple Sclerosis: Theory and Emerging Immunotherapies, *Trends Mol. Med.*, 26 (2020) 296-310. <https://doi.org/210.1016/j.molmed.2019.1011.1003>.
- [269] J. Wiedrick, R. Meza-Romero, G. Gerstner, H. Seifert, P. Chaudhary, A. Headrick, G. Kent, A. Maestas, H. Offner, A. A. Vandenbark, Sex differences in EAE reveal common and distinct cellular and molecular components, *Cell. Immunol.*, 359 (2021) 104242-104258. <https://doi.org/104210.101016/j.cellimm.102020.104242>.
- [270] K. A. Mifflin, E. Frieser, C. Benson, G. Baker, B. J. Kerr, Voluntary wheel running differentially affects disease outcomes in male and female mice with

- experimental autoimmune encephalomyelitis, *J. Neuroimmunol.*, 305 (2017) 135-144. <https://doi.org/10.1016/j.jneuroim.2017.1002.1005>.
- [271] G. C. Furtado, M. C. G. Marcondes, J. A. Latkowski, J. Tsai, A. Wensky, J. J. Lafaille, Swift entry of myelin-specific T lymphocytes into the central nervous system in spontaneous autoimmune encephalomyelitis, *J. Immunol.*, 181 (2008) 4648-4655. <https://doi.org/10.4049/jimmunol.181.4647.4648>.
- [272] K. Hirota, J. H. Duarte, M. Veldhoen, E. Hornsby, Y. Li, D. J. Cua, H. Ahlfors, C. Wilhelm, M. Tolaini, U. Menzel, A. Garafalaki, A. J. Potocnik, B. Stockinger, Fate mapping of IL-17-producing T cells in inflammatory responses, *Nat. Immunol.*, 12 (2011) 255-263. <https://doi.org/10.1038/ni.1993>.
- [273] M. Pelletier, L. Maggi, A. Micheletti, E. Lazzeri, N. Tamassia, C. Costantini, L. Cosmi, C. Lunardi, F. Annunziato, S. Romagnani, M. A. Cassatella, Evidence for a cross-talk between human neutrophils and Th17 cells, *Blood*, 115 (2010) 335-343. <https://doi.org/10.1182/blood-2009-1104-216085>.
- [274] A. B. Nair, S. Jacob, A simple practice guide for dose conversion between animals and human, *J. Basic Clin. Pharm.*, 7 (2016) 27-31. <https://doi.org/10.4103/0976-0105.177703>.
- [275] U.S. Food and Drug Administration, Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, Guidance for Industry (2005), <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/estimating-maximum-safe-starting-dose-initial-clinical-trials-therapeutics-adult-healthy-volunteers>, accessed 19 September 2022.
- [276] T. Hilberg, Å. Ripel, L. Slørdal, A. Bjørneboe, J. Mørland, The Extent of Postmortem Drug Redistribution in a Rat Model, *ASTM Int.*, 44 (1999) 956-962. <https://doi.org/10.1520/JFS12023J>.
- [277] M. C. Yarema, C. E. Becker, Key concepts in postmortem drug redistribution, *Clin. Toxicol.*, 43 (2005) 235-241. <https://doi.org/10.1081/CLT-58950>.
- [278] T. L. Perry, S. Hansen, S. S. Gandham, Postmortem Changes of Amino Compounds in Human and Rat Brain, *J. Neurochem.*, 36 (1981) 406-412. <https://doi.org/10.1111/j.1471-4159.1981.tb01608.x>.
- [279] T. Hilberg, S. Rodge, J. Mørland, Postmortem Drug Redistribution—Human Cases Related to Results in Experimental Animals, *ASTM Int.*, 44 (1999) 3-9. <https://doi.org/10.1520/JFS14404J>.

- [280] M. M. Bergamaschi, R. H. C. Queiroz, A. W. Zuardi, J. A. S. Crippa, Safety and Side Effects of Cannabidiol, a Cannabis sativa Constituent, *Curr. Drug Saf.*, 6 (2011) 237-249. <https://doi.org/210.2174/157488611798280924>.
- [281] L. S. Harbige, M. K. Sharief, Polyunsaturated fatty acids in the pathogenesis and treatment of multiple sclerosis, *Br. J. Nutr.*, 98 (2007) S46-S53. <https://doi.org/10.1017/S0007114507833010>.
- [282] Z. Z. Al-Ghezi, K. Miranda, M. Nagarkatti, P. S. Nagarkatti, Combination of Cannabinoids, Δ^9 -Tetrahydrocannabinol and Cannabidiol, Ameliorates Experimental Multiple Sclerosis by Suppressing Neuroinflammation Through Regulation of miRNA-Mediated Signaling Pathways, *Front. Immunol.*, 10 (2019) 1921-1938. <https://doi.org/1910.3389/fimmu.2019.01921>.
- [283] L. L. Anderson, I. K. Low, I. S. McGregor, J. C. Arnold, Interactions between cannabidiol and Δ^9 -tetrahydrocannabinol in modulating seizure susceptibility and survival in a mouse model of Dravet syndrome, *Br. J. Pharmacol.*, 177 (2020) 4261-4274. <https://doi.org/4210.1111/bph.15181>.
- [284] P. K. McGuire, The brain in obsessive-compulsive disorder, *J. Neurol., Neurosurg. Psychiatry*, 59 (1995) 457-459. <https://doi.org/10.1136/jnnp.59.5.457>.
- [285] I. Tracey, Nociceptive processing in the human brain, *Curr. Opin. Neurobiol.*, 15 (2005) 478-487. <https://doi.org/410.1016/j.conb.2005.1006.1010>.
- [286] G. A. Tavares, A. Louveau, Meningeal Lymphatics: An Immune Gateway for the Central Nervous System, *Cells*, 10 (2021) 3385-3396. <https://doi.org/3310.3390/cells10123385>.
- [287] L. Taylor, J. Crockett, B. Tayo, G. Morrison, A Phase 1, Open-Label, Parallel-Group, Single-Dose Trial of the Pharmacokinetics and Safety of Cannabidiol (CBD) in Subjects With Mild to Severe Hepatic Impairment, *J. Clin. Pharmacol.*, 59 (2019) 1110-1119. <https://doi.org/1110.1002/jcph.1412>.
- [288] J. Portnow, B. Badie, M. Chen, A. Liu, S. Blanchard, T. W. Synold, The Neuropharmacokinetics of Temozolomide in Patients with Resectable Brain Tumors: Potential Implications for the Current Approach to Chemoradiation, *Clin. Cancer Res.*, 15 (2009) 7092-7098. <https://doi.org/7010.1158/1078-0432.CCR-7009-1349>.

- [289] X. Wang, F. Magkos, B. Mittendorfer, Sex Differences in Lipid and Lipoprotein Metabolism: It's Not Just about Sex Hormones, *J. Clin. Endocrinol. Metab.*, 96 (2011) 885-893. <https://doi.org/810.1210/jc.2010-2061>.
- [290] L. Russell, C. Bolyard, Y. Banasavadi-Siddegowda, A. Weiss, J. Zhang, R. Shakya, K. Powell, B. Kaur, Sex as a biological variable in response to temozolomide, *Neuro-Oncol.*, 19 (2017) 873-874. <https://doi.org/810.1093/neuonc/nox1040>.
- [291] C. F. Moore, E. M. Weerts, Cannabinoid tetrad effects of oral Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD) in male and female rats: sex, dose-effects and time course evaluations, *Psychopharmacol.*, 239 (2022) 1397-1408. <https://doi.org/1310.1007/s00213-00021-05995-00215>.
- [292] C. M. Madla, F. K. H. Gavins, H. A. Merchant, M. Orlu, S. Murdan, A. W. Basit, Let's talk about sex: Differences in drug therapy in males and females, *Adv. Drug Deliv. Rev.*, 175 (2021) 113804-113829. <https://doi.org/113810.111016/j.addr.112021.113805.113014>.
- [293] K. Womersley, C. Hockham, E. Mullins, The Women's Health Strategy: ambitions need action and accountability, *Br. Med. J.*, 378 (2022) 1-2. <https://doi.org/10.1136/bmj.o2059>