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Synthesis of Novel Cannabinoid Ligands and Their Use as Anti-Glioma and Anti-Inflammatory Agents

Abstract

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Our focus in the field of cancer has been on the devastatingly lethal brain tumor glioblastoma multiforme. Due to the high expression of the CB2 receptor in human gliomas, we chose to focus on two CB2 selective lead compounds for these studies: KM-233 (CB2 antagonist) and HB-I-172 (CB2 inverse agonist). Both compounds showed *in vitro* efficacy against four human glioma cell lines ($EC_{50}s = 2.27 \pm 0.18\mu M$ to $6.34 \pm 4.10\mu M$) while HB-I-172 also showed efficacy against prostate, lung, and colorectal tumor lines ($2.38 \pm 0.75\mu M$ to $5.22 \pm 1.2\mu M$). Significant reductions in tumor load were also observed *in vivo* in both side flank and intracranial models of glioblastoma. The anti-glioma efficacies of the two lead compounds were not altered by pharmacologic blockade of the CB1 and CB2 receptors which points to a non-CB1/CB2 mediated signaling pathway. This pathway was examined in the human glioma line U-87 MG and determined to involve reductions in pMEK1/2, pERK1/2, pAkt, and p-p70S6K with increases in pGSK3B, p53, and cleaved caspases 3 and 9, and PARP. Immunohistochemical studies revealed loss of mitochondrial membrane polarization with release of cytochrome-C, DNA fragmentation and condensation, and alterations in cytoskeletal structure.

The current focus in the field of cannabinoids as anti-inflammatory agents is on CB2 selective compounds which are devoid of the CNS side-effects which could limit their therapeutic usefulness. We screened a variety of novel cannabinoids and the CB2 inverse agonist HB-I-172 emerged as a lead. *In vitro* treatment with HB-I-172 caused a significant reduction in a number of proinflammatory cytokines and chemokines in both neoplastic lung epithelial cell and macrophage models and in primary human lung epithelial and endothelial cells, macrophages, and microglia. We believe that HB-I-172 and other CB2 selective cannabinoids are promising treatments for a variety of inflammatory conditions from neuro-inflammatory disorders such as multiple sclerosis and Huntington's disease to systemic conditions such as atherosclerosis and inflammatory pain.

Finally, three new series of heterocyclic cannabinoid ligands were synthesized and screened. The hexahydro pyrimidine classical, pyrimidine nonclassical, and furanopyrimidine cannabinoids were designed with CB2 selectivity in mind. After many failed synthetic routes were abandoned, the three new classes were successfully generated. Many of these compounds showed good CB2 selectivity (77-fold) and showed efficacy in both anti-glioblastoma and anti-inflammatory studies.

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Pharmaceutical Sciences

Research Advisor

Bob M. Moore II, Ph.D.

Keywords

Cannabinoids, CB2, Glioblastoma, Heterocyclic, Inflammation

Subject Categories

Medicine and Health Sciences | Pharmacy and Pharmaceutical Sciences

Synthesis of Novel Cannabinoid Ligands and Their Use as Anti-Glioma and Anti-Inflammatory Agents

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Steven Neal Gurley
May 2010

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Dedication

This dissertation is dedicated
to my wife Amanda who has also
endured the many years of research
that this document represents.

Acknowledgements

I would like to thank my major professor, Dr. Bob M. Moore II for his support, guidance, and patience. I would also like to thank my committee members, Dr. Duane D. Miller, Dr. Isaac O. Donkor, Dr. Wei Li and Dr. Andrea Elberger for their useful suggestions and guidance.

I would like to thank my colleagues Dr. Himanshu Bhattacharjee, Dr. Suni Mustafa, and Dr. Bret Koertge for all of their support and useful discussions. I would also like to thank other members of our laboratory, both past and present, for their support and cooperation: Dr. Mathangi Krishnamurthy, Patrick Allison, Peihong Guan, Dr. Xiofeng Zhang, Erin Oliver, Dr. Supriya Bavadekar, Dr. Christopher Surdock, Dr. Galina Dulatov, Negin Nouraei, and Ammaar Abidi.

Finally I would like to thank the College of Pharmacy at The University of Tennessee Health Science Center for giving me the opportunity to pursue my research.

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List of Abbreviations

$\Delta\psi_m$	change in membrane potential
μg	microgram
μl	microliter
μM	micromolar
μmol	micromoles
μwave	microwaves
AC	adenylate cyclase
ADP	adenosine diphosphate
ATF6	activating transcription factor 6
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
BBB	blood-brain-barrier
Bcl-2	B-cell lymphoma 2
BCNU	bis-chloronitrosourea
Bp	boiling point
BSA	bovine serum albumin
C	celsius
cAMP	cyclic adenosine monophosphate
CB	cannabinoid
CB1	cannabinoid receptor type 1
CB1 _A	cannabinoid receptor type 1 A
CB2	cannabinoid receptor type 2
CCB	classical cannabinoid
cdc42	cell division control protein 42 homolog
cDNA	complementary DNA
CLSM	confocal laser scanning microscope
cm	centimeter
CNS	central nervous system
CREB	cAMP response element-binding
Ctx	cholera toxin
cytC	cytochrome C
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DIBAL-H	diisobutylaluminum hydride
DIEA	diisopropylethylamine
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EC ₅₀	half maximal effective concentration
ED ₅₀	half maximal effective dose

Egr1	early growth response protein 1
ELF2 α	E74-like factor 2 alpha
Elk-1	Ets like gene1
EMEM	Eagle's minimal essential medium
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
FAK	focal adhesion kinase
FAN	factor associated with neutral SMase activation
FBS	fetal bovine serum
FCA	Freund's complete adjuvant
G	guanine nucleotide-binding
g	grams
G-CSF	granulocyte colony-stimulating factor
G β L	G-protein Beta-subunit like protein
GBM	glioblastoma multiforme
GEF	guanine nucleotide exchange factor
<i>gem</i>	geminal
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GPR119	G protein-coupled receptor 119
GPR35	G protein-coupled receptor 35
GPR55	G protein-coupled receptor 55
GSK-3 β	glycogen synthase kinase 3 beta
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
hr	hours
I.R.	infrared spectroscopy
i.p.	intraperitoneal
i.v.	intravenous
IC ₅₀	half maximal inhibitory concentration
IFN- γ	interferon gamma
IL	interleukin
IP-10	10 kDa interferon-gamma-induced protein
JNK	c-Jun N-terminal kinase
K_d	dissociation constant
kDa	kilodaltons
kg	kilograms
KHMDS	potassium bis(trimethylsilyl)amide
K_i	dissociation constant
Lck	leukocyte-specific protein tyrosine kinase
Leu	leucine
logP	logarithm of octanol/water partition coefficient

LPI	lysophosphatidylinoitol
LPS	lipopolysaccharide
Lys	lysine
M	molar
m/z	mass-to-charge ratio
MAP	mitogen-activated protein
MCP-1	monocyte chemotactic protein-1
MEK	mitogen-activated protein kinase
MeOD	deuterated methanol
MeOH	methanol
Met	methionine
MIF	macrophage migration inhibitory factor
mg	milligrams
MHz	megahertz
min	minutes
ml	milliliters
mm	millimeters
mmol	millimoles
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MS	mass spectroscopy
N	normality
n.a.	not active
n.d.	not determined
NBS	N-bromosuccinimide
neg	negative
NFAT	nuclear factor of activated T-cells
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanograms
NK	natural killer
nM	nanomolar
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
p53	protein 53
P70S6K	70 kDa ribosomal S6 kinase
p90RSK	p90 ribosomal S6 kinase
PAK	p21-activating kinase
pAkt	phospho-Akt
PARP	poly (ADP-ribose) polymerase
pBAD	phospho-BAD
PBS	phosphate buffered saline
PCC	pyridinium chlorochromate
pERK	phospho-ERK
PERK	protein kinase-like endoplasmic reticulum kinase
PGN	peptidoglycan

pGSK-3 β	phospho-GSK-3 β
pH	potentiometric hydrogen ion concentration
Phe	phenylalanine
PI3K	phosphoinositide 3-kinase
PL	phospholipase
pMEK	phospho-MEK
pos.	positive
p-p53	phospho-p53
p-p70S6K	phospho-70 kDa ribosomal S6 kinase
ppm	parts per million
pSTAT	phospho-STAT
Ptx	pertussis toxin
PUMA	p53 upregulated modulator of apoptosis
Rac	Ras-related C3 botulinum toxin substrate
RANTES	regulated upon activation, normal T-cell expressed, and secreted
raptor	regulatory associated protein of mTOR
R _f	retention factor
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated, coiled-coil containing protein kinase
rpm	revolutions per minute
RT	room temperature
S.D.	standard deviation
SAR	structure-activity relationship
SCID	severe combined immunodeficiency
Ser	serine
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SMase	sphingomyelinase
SRT	stereotactic radiotherapy
STAT	signal transducers and activators of transcription
TBDMS	tertiary-butyldimethylsilyl
TFA	trifluoroacetic acid
T _h 1	T helper type 1
T _h 2	T helper type 2
THC	tetrahydrocannabinol
THF	tetrahydrofuran
Thr	threonine
TLC	thin layer chromatography
TNF- α	tumor necrosis factor-alpha
TRIB3	Tribbles homolog 3
Trp	tryptophan
TRPV1	transient receptor potential cation channel, subfamily V, member 1
Tyr	tyrosine
2-AG	2-arachidonoylglycerol

Chapter 1: Introduction

1.1 History

Cannabis sativa (marijuana) is one of the oldest known herbal drugs whose medicinal value has been exploited for centuries. The use of *Cannabis sativa* preparations for treatment of a variety of pathological conditions in ancient Indian, Chinese, and Egyptian civilizations is well-documented.¹ Ancient literature is rich with therapeutic indications for *Cannabis* such as its usage as an analgesic,² an anesthetic,³ an appetite stimulant,⁴ an anti-inflammatory agent,⁵ and as a treatment for hemorrhoids.¹

Early in the nineteenth century there was a rapidly increasing interest in research of natural products, and several alkaloids such as morphine, cocaine, and strychnine were isolated from plant materials. The search for the psychoactive constituent of *marijuana* began during this period of rapid growth in the field of pharmacognosy. It was the common belief for many years that the principal active constituent of *Cannabis sativa* was in fact an alkaloid. In 1896, however, Marshall and coworkers isolated a mixture of terpenes from an ethereal extract of Indian hemp,⁶ which produced the same pharmacological effects that are observed with *Cannabis sativa* use. This provided the first evidence that the principal active compound was terpenoid in nature. In the early 1930s Cahn and coworkers isolated cannabinalol (**Figure 1.1**) and determined a partial structure after conducting extensive chemical degradation studies.⁷ This discovery was quickly followed by the synthesis of several derivatives of cannabinalol and $\Delta^{6a,10a}$ -tetrahydrocannabinalol ($\Delta^{6a,10a}$ -THC) (**Figure 1.1**) by the Adams group in the US⁸ and the Todd group in Britain.⁹ The pharmacological activities of cannabinalol and $\Delta^{6a,10a}$ -THC, as tested by the Gayer areflexia test for suppression of corneal reflexes in the rabbit, were observed to be similar to those of the *Cannabis* extract, but were significantly less potent.

The isolation and structural characterization of the principal active constituent of *Cannabis sativa*, Δ^9 -tetrahydrocannabinalol (Δ^9 -THC) (**Figure 1.2**), was reported in 1964 by Gaoni and Mechoulam.¹⁰ The activity of this cannabinoid was subsequently studied in a number of animal models. These studies ranged from evaluation of the cataleptic reaction in mice¹¹ to the effects on motor activity and gross behavior in mice and rats.¹² There was also extensive experimentation in rhesus monkeys¹² which ultimately led to the pharmacological characterization of cannabinoids.

Following these reports, several other constituents of marijuana such as cannabidiol, cannabigerol, cannabichromene, cannabicyclol (**Figure 1.2**) and related cannabinoid acids were isolated and characterized. The structural characterization of Δ^9 -THC provided a springboard to stimulate interest in research on cannabinoids. This resulted in the design and synthesis of several structurally diverse classes of cannabinoids. Studies on the effects of various analogues in animal models showed a positive correlation between structure and biological activity indicating the possible involvement of a defined receptor for the cannabinoids. This variety of compounds then aided in the discovery and cloning of the cannabinoid receptors and the subsequent identification of their endogenous ligands.

Figure 1.1: Structures of cannabinol and $\Delta^{6a,10a}$ -tetrahydrocannabinol

Figure 1.2: Structures of Δ^9 -THC, cannabidiol, cannabigerol, cannabichromene, and cannabicyclol

1.2 Cannabinoid Receptors

The beginning of the exponential growth seen in the field of cannabinoid research in the last 20 years can be traced to the identification, isolation and cloning of the cannabinoid receptors. After the discovery of Δ^9 -THC, but before the discovery of the cannabinoid receptors, research on cannabinoids in the late 1970s and in the early 1980s was based on the assumption that the pharmacological effects attributed to cannabinoids were mediated not through specific receptors but by perturbation of cellular membranes. Due to their high lipophilicity, it was thought that cannabinoids behaved like general anesthetics in terms of their mechanism of action.¹³ However, the highly stereospecific activity associated with several cannabinoids¹⁴ suggested receptor mediated action. Then, in 1988, Devane and coworkers¹⁵ utilized a tritium labeled synthetic cannabinoid to tag specific sites in the brain and on neuronal cells. This then identified the presence of a specific cannabinoid receptor in the rat brain. This discovery was finally followed by the isolation of the cDNA of this receptor, its expression in Chinese hamster ovary (CHO) cells, and subsequent naming as the cannabinoid receptor type 1 (CB1).¹⁶ In 1993, a second cannabinoid receptor subtype, cannabinoid receptor type 2 (CB2), was isolated and cloned from the human promyelocytic cell line HL60 by Munro and coworkers.¹⁷ An amino terminal variant of the human CB1 receptor has also been reported¹⁸ in which, a 167 base portion of the CB1 coding sequence is spliced out of the mRNA. This amino terminal variant of the CB1 receptor was named the CB1_A receptor.

Whether there are additional cannabinoid receptors is still a hotly debated topic among several researchers in this field. This is, in part, due to the observation that the endogenous cannabinoid ligand, anandamide, induces spinal antinociception via a different mechanism than Δ^9 -THC.¹⁹ In 2000, Di Marzo and coworkers carried out studies²⁰ using transgenic mice²¹ that lack the CB1 receptor to evaluate the CB1 mediated effects of anandamide and Δ^9 -THC. They found that in these knockout mice anandamide still produced CB1-like effects such as catalepsy, analgesia, and loss of spontaneous activity whereas Δ^9 -THC did not. This suggested the presence of a new cannabinoid receptor in the brain. Recent studies have also shown that a number of the cannabinoid ligands bind to the orphan receptor GPR55.²² This receptor is reported to be expressed in several tissues and might function in regulation of vascular tone and immune-cell migration.^{22c} Anandamide is also known to act on other classes of receptors such as the vanilloid receptor TRPV1. Anandamide activates the vanilloid receptor and produces membrane currents or increases in intracellular calcium levels.²³ The presence of these multiple sites for cannabinoid action (along with the possibility for yet undiscovered sites) with several signaling pathways and associated second messengers is consistent with the complex pharmacology associated with cannabinoids.

The cannabinoid receptors belong to the superfamily of G-protein coupled receptors¹¹ (GPCRs) and are single polypeptides with seven transmembrane α -helices, an extracellular, glycosylated N-terminus and intracellular C-terminus. The CB1 and CB2 receptor subtypes possess 68% amino acid identity within their transmembrane regions but only 44% identity throughout the entire peptide. The CB1 receptor is a 52.8 kDa, 472 amino acid polypeptide, which is found in both the CNS and periphery and in both neural and non-neural tissues. CB1 is coupled to inhibitory G_{i/o} proteins²⁴ which bind to its

third intracellular loop and its C-terminus.²⁵ The amino acid residues in transmembrane regions 3, 5, and 6 of the CB1 receptor are believed to be involved in binding with cannabinergic ligands.²⁶ Activation of CB1 causes inhibition of adenylate cyclase and a reduction in cAMP levels. Reduction of cAMP affects phosphorylation by protein kinase A which causes modulation of ion channels and other second messengers. Activation of voltage-sensitive outwardly rectifying K⁺ channels,²⁷ inwardly rectifying K⁺ channels²⁸ and inhibition of voltage gated N, L, P, and Q-type Ca²⁺ channels²⁹ are observed in response to CB1 receptor activation.

The CB1 receptor is also known to regulate different members of the mitogen-activated protein kinase (MAP kinase) family of enzymes such as:

- a. extracellular signal-regulated kinase (ERK)³⁰
- b. c-Jun N-terminal kinase³¹
- c. p38.^{31b,32}

Activation of MAP kinases is associated with:

- a. activation of immediate early genes like c-Fos and Egr1 (Krox-24)^{30a}
- b. activation of focal adhesion kinase (important for integrating cytoskeletal changes associated with signal transduction events)
- c. activation of phospholipases (PL), PLA, PLC, and PLD³³
- d. activation of the Na⁺/H⁺ exchanger³⁴
- e. activation of phospholipase A₂, which in turn causes release of arachidonic acid leading to the synthesis of prostaglandins.³⁵

There is also evidence of a second pathway initiated by CB1 receptor stimulation which is coupled with sphingomyelinase activation and subsequent release of ceramide.^{30c} Ceramide then causes activation of the Raf-1/MAP kinase cascade³⁶ and also carnitine palmitoyltransferase I in mitochondrial membranes to stimulate ketogenesis and fatty acid oxidation.³⁷ This elevation in levels of intracellular ceramide have also been suggested to be one of the possible factors responsible for the apoptotic effects of cannabinoids seen on glioblastoma cells.³⁸

The CB2 cannabinoid receptor, also referred to as the peripheral cannabinoid receptor, is a 41 kDa, 360 amino acid polypeptide that is predominantly expressed in the immune system.³⁹ Its amino acid sequence is quite different from that of CB1, especially in the amino terminal domain where there is no significant conservation and which is significantly shorter in the CB2 receptor when compared to the CB1 receptor. The amino acid residues involved in ligand recognition are also found in the transmembrane domains 3, 5, and 6 for the CB2 receptor.

Like the CB1 receptor, CB2 is also coupled to the inhibitory $G_{i/o}$ proteins that bind to its third intracellular loop and C-terminus. Activation of the CB2 receptor also causes inhibition of adenylate cyclase and therefore a similar reduction in cAMP levels. Most of the known signaling pathways of CB2 are identical to those observed with CB1. However, unlike the CB1 receptor, the CB2 receptor has no effect on ion channels.²⁸

After the discovery of the CB2 receptor it was believed that it was found only in the immune system and was completely absent from the CNS.⁴⁰ Recently, however, CB2 expression has been confirmed in the brain on microglia,⁴¹ blood vessels,⁴¹ and on some neurons.⁴² CB2 is, however, expressed in much higher densities than CB1 in the immune system. The expression level of the CB2 gene in immune tissues is 10-100 times that of the CB1 gene.⁴³ In fact, the CB2 mRNA content of the spleen and tonsils is almost equivalent to the CB1 mRNA content in the CNS.⁴³ This first led researchers to suggest that CB2 receptor might play an important role in immune function. It is no surprise then that many reports have been made over the years regarding the deleterious effects of marijuana smoking on host defense mechanisms where marijuana smokers were found to be more susceptible to bacterial and viral infections.⁴⁴ Between transmembrane domains 1 and 7, including the loop regions, the CB2 receptor is only 44% identical to the CB1 receptor, making it is possible to develop selective CB2 ligands to specifically target the immune system.

GPR55 was first identified and cloned by O'Dowd in 1999.⁴⁵ It was later identified by an *in silico* screen as a putative cannabinoid receptor due to an amino acid sequence in the binding region that is similar. Research groups at the pharmaceutical companies Glaxo Smith Kline and Astra Zeneca then extensively characterized the receptor in the hope that it would be responsible for the blood pressure lowering effects of cannabinoids. GPR55 is indeed activated by endogenous, phyto-, and synthetic cannabinoids but GPR55 knockout mice generated at Glaxo Smith Kline showed no alteration in the cannabinoid induced blood pressure regulation after administration of the GPR55 agonist abnormal cannabidiol.

Many of the known CB1 and CB2 ligands also bind to GPR55. The receptor is activated by phytocannabinoids such as Δ^9 -THC and cannabidiol,^{22c} synthetic cannabinoids such as CP-55,940, and the endocannabinoids anandamide, 2-arachidonoylglycerol, and nolandin ether.^{22c} Recent research suggests that lysophosphatidylinositol and its 2-arachidonoyl derivative may be the endogenous ligands for GPR55⁴⁶ and the receptor appears likely to be a possible target for treatment of inflammation and pain as with the other cannabinoid receptors.⁴⁷

While the CB1 and CB2 receptors couple to $G_{ai/o}$ proteins, GPR55 is coupled to $G_{\alpha_{13}}$ and activation of the receptor leads to stimulation of a number of GTPases such as Ras homolog gene family, member A (RhoA), cell division control protein 42 homolog (cdc42) and Ras-related C3 botulinum toxin substrate 1 (rac1).^{22a} Members of this GTPase superfamily appear to regulate a diverse array of cellular events, including the control of cell growth, cytoskeletal reorganization, and the activation of protein kinases.⁴⁸ Stimulation of GPR55 induces F-actin formation under the control of $G_{\alpha_{13}}$, RhoA and Rho-associated, coiled-coil containing protein kinase (ROCK).^{46b} GPR55 activation also

induces intracellular calcium release and activation of the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), nuclear factor of activated T-cells (NFAT) and cAMP response element-binding (CREB).^{46b}

The physiological role of GPR55 remains unclear. Mice with a target deletion of the GPR55 gene show no specific phenotype.⁴⁹ GPR55 has been found to be widely expressed in the brain, especially in the cerebellum. It is found in the gastrointestinal tract in the jejunum and ileum and also in the adrenal glands but is apparently not highly expressed elsewhere in the periphery.^{22c} Osteoblasts and osteoclasts have also been shown to express GPR55 and this has been shown to regulate bone cell function.⁵⁰

1.3 Cannabinoids as Anti-Glioma Agents

Brain tumors are the second and fifth leading causes of cancer-related deaths in males and females ages 20 to 39, respectively.⁵¹ The most prevalent form of adult primary CNS tumors is collectively referred to as glioma, and the most common, devastating, and high-grade glioma is glioblastoma multiforme (GBM).⁵¹⁻⁵² These tumors are classically thought to originate from supporting or glial cells of the CNS, and represent roughly fifty percent of all adult primary CNS tumors. Long term survival of patients with a GBM diagnosis with the best radiological, surgical, and anti-tumor drug therapy available is extremely rare. The historical median survival for GBM patients is 3 to 12 months depending on age and other prognostic factors.⁵³

1.3.1 Current Standard of Care for Glioblastoma

CNS tumors in general are difficult to treat because of the complexities associated with removing cancerous cells from a background of normal brain tissue without introducing considerable neurological damage. Conventional therapy for malignant glioma consists of complete gross resection followed by radiation, either with or without chemotherapy. Radiation is indicated because surgical resection misses micro populations of cells that have migrated from the primary tumor site. However, currently accepted therapeutic adjuvants to surgery such as radiotherapy and chemotherapy provide only a minor improvement in the disease course and life expectancy of patients diagnosed with malignant glioma.⁵⁴ The blood-brain-barrier (BBB) prevents the use of most chemotherapeutic agents; those that are available do not significantly impact the course of disease and often have unacceptable side effect profiles.⁵⁵ Therefore, new drugs that adequately treat high-grade glioma of the brain and spinal cord are desperately needed if these tumors are going to be treated adequately in the future.

1.3.1.1 Surgical Intervention

Therapeutic treatment patterns for GBM have produced limited increases in overall survival over the last few decades. The advent of microneurosurgical techniques

along with improvements in neuroanesthesia and perioperative care has resulted in remarkable advancement in the management of benign tumors of the brain. However, current surgical technique cannot adequately rid the surrounding brain tissue of microscopic foci of high-grade glioma cells that are not visible on the post-operative MRI scan. Although surgery is not sufficient for cure of glioma, it is a necessary component of a multi modality approach that includes radiation and chemotherapy in some instances. Goals of surgery for malignant glioma include: (1) establishment of a histological diagnosis; (2) reduction in tumor cell load leading to reduced intracranial pressure; and (3) possibly changing tumor kinetics.

1.3.1.2 Radiotherapy

Surgery alone has fallen short in providing any appreciable long-term increase in life expectancy for patients with high-grade gliomas. Radiotherapy directed at the gliomatous area around the surgical cavity is considered beneficial to survival outcome, and indeed postsurgical radiation has proven to be the most effective treatment currently available for improving survival. There is also mounting evidence to suggest that additional radiation, given in the form of brachytherapy or radiosurgery, at initial diagnosis as a "boost" to standard radiation or at tumor recurrence, may provide added improvement in survival outcome.⁵⁶ With advancements in radiotherapy tools and techniques the practice of whole brain irradiation has somewhat fallen from favor as radiation therapy results in radionecrosis not only of rapidly dividing tumor cells, but also of surrounding healthy brain tissue. There are now two main radiotherapy delivery techniques: (1) external stereotactic radiotherapy (SRT) boost, administered either in one session or in fractions; and (2) brachytherapy boost by peri- or post-operative implantation of temporary catheters or permanent isotope seeds.

1.3.1.3 Chemotherapy

At present, most centers in the United States also employ adjuvant chemotherapy as part of a patient's initial treatment. Current conventional chemotherapy regimens for newly diagnosed malignant glioma include single-agent intravenous bis-chloroethyl-nitrosourea (BCNU; carmustine), single-agent oral procarbazine, or the PCV combination of procarbazine, chloroethyl-cyclohexylnitrosourea (CCNU) (lomustine), and vincristine. Since the 1980s, some but not all randomized studies have shown a small survival advantage for patients receiving one of these chemotherapy regimens in addition to surgery and RT.^{54a,b,57} A meta-analysis of published phase III randomized prospective studies showed a modest overall survival benefit and a higher proportion of long-term (> 24 months in some studies) survivors for patients with GBM who received adjuvant chemotherapy.⁵⁸ The toxicity profile varies among these treatments, with myelosuppression being the most frequent dose-limiting effect.^{55a,57} Factors limiting the use of chemotherapy are that there are no strong predictors of chemosensitivity among individual patients and the proper timing of upfront chemotherapy in relation to SRT for newly diagnosed tumors has never been studied rigorously. Empirically administered

adjuvant chemotherapy generally is begun during or shortly after completion of SRT. Despite a variety of drugs and treatment schedules, the median time to tumor progression for patients with GBM is 6 to 8 months, and the overall survival remains around 12 months. To date there has been no proven prolongation of time to progression or survival for patients with malignant glioma when other drugs are added to the "standard" BCNU or PCV chemotherapeutic regimens, including cisplatin,⁵⁹ carboplatin,⁶⁰ dibromodulcitol,⁶¹ mercaptopurine,⁶² or 6-thioguanine.⁶³

1.3.2 Cannabinoids as Chemotherapeutic Agents for the Treatment of High-Grade Gliomas

Investigational research related to the use of cannabinoids in oncology was primarily restricted to anti-emesis prior to the late 1980s. Although limited studies on Lewis lung cancer and leukemia predate this time, active research on the antineoplastic potential of cannabinoids gained increased attention following the discovery of the CB1 and CB2 receptors. Cannabinoids have now been shown to induce apoptosis in rhabdomyosarcoma,⁶⁴ colon,⁶⁵ prostate⁶⁶ and skin carcinomas,⁶⁷ glioma,^{32,68} neuroblastoma,^{68b} and lymphoma/leukemia.⁶⁹ Furthermore, a decrease in tumor size and/or cell growth inhibition has been reported in breast,^{66b,70} uterus,⁷¹ and lung cancers,⁷² and in thyroid epithelioma.⁷³ The ability of cannabinoids to modulate the cell survival and death pathways in neoplasia is extremely significant from a drug development perspective. Specifically, clinically investigated cannabinoids, in contrast to conventional cancer chemotherapies, do not exhibit the generalized toxic effects, are well tolerated by patients, and have the ability to penetrate the blood brain barrier. This fledgling strategy of cancer treatment has the potential of decreasing the often severe side effects associated with most antineoplastic agents. The future development of cannabinoids as peripheral antineoplastic agents holds great promise, however possibly the greatest impact in cancer therapy is the unprecedented opportunity for the development of antineoplastic agents capable of treating malignancies in the CNS.

The CNS and associated BBB is arguably the most difficult target organ for which drugs are developed. Despite the development of an arsenal of clinically utilized CNS drugs, e.g. anti-depressants and anti-psychotics, highly effective chemotherapeutics for the treatment of brain cancers such as glioblastoma remain elusive. One of the principle factors hindering this field of drug development is the fact that many of the otherwise efficacious natural product related antineoplastic agents are substrates for the anti-transporter proteins that are abundant in the CNS microvasculature. The established permeability of classical cannabinoids (CCBs) through the BBB combined with the demonstrated *in vitro* anti-glioma activity of several cannabinoid ligands offers an unprecedented opportunity for drug development. Often such *in vitro* data does not translate to *in vivo* efficacy; however our lab and others have demonstrated the therapeutic potential of CCBs. Specifically, KM-233 and JWH-133 were highly effective in halting the progression of human brain tumors in mice.^{68a,74} This is significant in that JWH-133 and, to a lesser extent KM-233, are selective CB2 receptor ligands and a recent report shows that primary human glioblastomas express significant levels of CB2 receptors.⁷⁵ This study was conducted prior to the emergence of GPR55 in the CB field

and as such expression levels of GPR55 were not evaluated. As stated earlier, GPR55 is widely expressed in the CNS and increased GPR55 expression is consistent with our Western blot analysis of GBM cell lines (see Chapter 2, **Figure 2.8**). Validation of GPR55 as a target for CB based anti-neoplastic agents offers a significant advancement in therapies targeted at GBM.

Further support for targeting the cannabinoid system comes from a pilot clinical study using THC, a non-selective partial agonist of the CB receptors; there was a slight improvement in survival in patients with recurrent brain cancers.⁷⁶ In this study THC was administered intratumorally which may have, in part, reduced the overall efficacy of the treatment. Specifically, in our side flank model, administration of KM-233 intraperitoneally (i.p.) resulted in a greater reduction of tumor load compared to intratumor injection.^{74b} The development of cannabinoids as anti-glioma agents represents a promising field of research into the treatment of one of the most malignant forms of cancer whose current treatment is either ineffective or only palliative.

1.4 Cannabinoids as Anti-Inflammatory Agents

It has been shown over the last forty years, initially by examining marijuana users, then in animal studies, and finally in cell culture, that cannabinoids are proven immunomodulators.⁷⁷ It has been known for a much longer period of time, however, that marijuana does possess anti-inflammatory properties. In what is perhaps the earliest literature reference to *Cannabis*, it is described as an anti-inflammatory agent. The Chinese emperor Shen-nung (ca. 2000 B.C.), in a work called *Pen-ts'ao Ching*, noted many of the effects of Cannabis in humans. Among other properties, he claimed that *Cannabis* “undoes rheumatism”, suggesting possible anti-inflammatory effects.⁷⁸

1.4.1 Cannabinoid Receptors in the Immune System

The presence of a cannabinoid receptor in cells of the immune system was known before the discovery of CB2. In 1992, Kaminsky *et al.* reported the presence of the putative cannabinoid receptor on mouse splenic cells.⁷⁹ This report was supported by the evidence of stereoselective immunomodulatory effects of cannabinoids on the splenic cells, specific binding of tritiated CP-55,940, and by the identification of mRNA for the receptor in the cells. Shortly after this discovery, the peripheral expression and distribution of CB1 was examined in human tissues. Messenger RNA for the receptor was identified in human spleen, tonsil, and peripheral blood leukocytes,⁸⁰ although at much lower levels than in the brain. The expression patterns found in the human blood cells varied significantly by type. The order of expression level was found to be B cells > natural killer (NK) cells > polymorphonuclear neutrophils > CD8+ T lymphocytes > monocytes > CD4+ T lymphocytes. CB1 mRNA at varying levels was also found in a number of monocytic and T and B leukemia cell lines, with the exception of Jurkat cells. On average, the B cell lines were found to have higher expression levels than the T cells.

After the discovery of the CB2 receptor in 1993, it was shown to be the dominant cannabinoid receptor in the immune system with (at that time) no quantifiable expression in the brain.^{17,39b} Human tissues were examined for expression levels of CB2 and it was found that levels of CB2 are 10-100X that of CB1 in human immune tissues.^{42a,81} It was shown by Galiegue and coworkers that the level of expression of CB2 in the spleen and tonsils was equivalent to the CB1 expression levels in the brain.⁴³ Knowing the physiologic function of CB1 in the CNS, the researchers drew the conclusion that the CB2 receptor must be the major subtype responsible for the immunomodulatory effects of cannabinoids. The order of expression of CB2 in human blood cells was reported to be B cells > NK cells > monocytes > polymorphonuclear neutrophils > CD8+ T lymphocytes > CD4+ T lymphocytes. Human cell lines derived from myeloid, monocytic, and lymphoid lineages were shown to have the same rank order of CB2 density as determined by mRNA levels. The high level of mRNA in the B lymphocytes also correlates with receptor expression on the cell surface. This was confirmed by Lynn and Herkenham using tritiated CP-55,940.⁸² The CB2 receptor has also been verified to be present on dendritic cells.⁸³ Its presence on these potent antigen-presenting cells suggests a possible role for cannabinoids to modulate antigen presentation. More recently, the presence of CB2 has been reported in the CNS. Here, the CB2 receptor plays a possible role in inflammation due to the high expression levels found in the macrophage-like cell type microglia.⁸⁴

With the recent identification of GPR55 as a putative cannabinoid receptor, its role in cannabinoid mediated immunomodulatory effects has not been as well established as with CB1 and CB2. While GPR55 has not been shown to be present in peripheral blood leukocytes, it has been identified in the spleen.^{22c} One possibly significant role for GPR55 in immune modulation is in the CNS. In a recent report by Pietr *et al.*, the authors claim to have discovered the presence of mRNA for GPR55 in a primary culture of mouse microglia and also in the mouse microglial cell line BV-2.^{47d,85} The possible role of this receptor in inflammation in this cell type is supported by the observation of altered levels of mRNA for the GPR55 receptor upon exposure to various inflammatory stimuli. Pietr and coworkers reported that stimulation of primary microglia or BV-2 with lipopolysaccharide (LPS) caused a concentration-dependent decrease in mRNA levels. Differing results were seen, however, utilizing stimulation with interferon gamma (IFN- γ) which caused a slight decrease in GPR55 mRNA levels in primary culture but an increase in the BV-2 cell line.^{47d} This observation is similar to the reported variation in CB2 expression on microglia based on activation state.⁸⁶

1.4.2 Cannabinoid Effects on Immunity

The effects elicited by cannabinoids on the immune system are mediated by the downstream signaling cascades of the cannabinoid receptors. This is achieved mainly by the down-regulation of cAMP formation by inhibition of adenylate cyclase (AC) activity.⁸⁷ It has been shown that lymphocyte activation and cytokine transcription in macrophages is regulated by AC activity and the cAMP cascade.⁸⁸ This is supported by the fact that cAMP analogs can stimulate or inhibit the immune response and can also antagonize the anti-inflammatory effects of cannabinoids.⁸⁷

The signaling cascade involved in the anti-inflammatory effects of cannabinoids is probably not simply due to regulation of AC. This would explain why endogenous and exogenous cannabinoids can elicit different immunomodulatory effects.

Endocannabinoids seem to have effects that are very localized, short lived, and are stimulatory in nature.⁸⁹ The endocannabinoid 2-arachidonylglycerol (2-AG), for example, has been shown to have immunostimulatory actions such as serving as a chemoattractant to myeloid leukemia cells, splenocytes, and microglia⁹⁰ and has also been shown to induce both chemotaxis and chemokinesis on microglia.⁹¹ There is some evidence, however, that certain metabolically stable analogs of endocannabinoids can be immunosuppressive. An example of this is the anandamide analog 2-methylarachidonyl-(2'-fluoroethyl) amide that has been shown to suppress immune response in a model of T lymphocyte function.⁹²

Exogenous cannabinoids, on the other hand, have effects on the immune system that are more generalized in nature, persist for longer periods of time, and tend to be immunosuppressive.⁹³ Both phytocannabinoids and synthetic cannabinoids have been shown to have immunosuppressive effects. THC, CP-55,940, and the classical cannabinoid analog HU-210 have all been shown to inhibit cell contact-dependent lysis of tumor cells by macrophages, to inhibit the ability of macrophages to process antigens, and to suppress the proliferation of both B and T lymphocytes.⁹⁴ THC has also been shown to attenuate the proliferation and maturation of cytotoxic T lymphocytes and to suppress the activity of NK cells.^{94a} Exogenous cannabinoids have also been reported to cause a switch in the T helper (T_h) cell profile from the T_h1 phenotype to the T_h2 phenotype by alteration of the cytokine / chemokine network.⁹⁵ This switching of T helper function to the anti-inflammatory T_h2 phenotype is of particular interest due to the possible therapeutic implications in autoimmune diseases. For example, blocking the T_h1 cytokines and enhancing the T_h2 pathways has shown promise in several animal models of inflammatory diseases such as experimental autoimmune encephalomyelitis,^{81,96} experimental arthritis,⁹⁷ and experimental autoimmune hepatitis.⁹⁸ Exogenous cannabinoids have also been shown to inhibit immune cell migration and recruitment,⁹⁹ in contrast to the effects of endocannabinoids such as 2-AG.

The indirect effects that cannabinoids exert on immune function through alterations in the cytokine / chemokine network have been extensively studied.⁷⁷ Cytokines and chemokines are a large family of proteins that are produced by various cells of the immune system. Their function is to alter the state or the activity of other cells in the immune system or to direct the movement of immune cells to sites where they are needed. Cytokines and chemokines can be broadly classified as proinflammatory or anti-inflammatory depending upon whether their overall effect on the immune response is stimulatory or suppressive, respectively. Much of the original work in this field was with the classical cannabinoid THC and many results were confounding. For example, THC has been shown to increase the processing and release of the proinflammatory cytokine interleukin-1 (IL-1) *in vitro*¹⁰⁰ and IL-1 along with proinflammatory tumor necrosis factor-alpha (TNF- α) and IL-6 *in vivo*.¹⁰¹ On the other hand, THC has also been shown to suppress the acute phase proinflammatory cytokines such as TNF- α , for example, in both human and mouse macrophage cultures.⁷⁷

With the nearly ubiquitous expression of the cannabinoid receptors (especially CB2) in the immune system it is clear why they are ever increasing targets for the treatment of inflammatory disorders. Along with the previously mentioned cells, cannabinoids have also been shown to have immunomodulatory effects on other cell types such as mast cells,^{5,102} neutrophils,¹⁰³ astrocytes,¹⁰⁴ glia,^{95c,105} and neurons.^{95d,106} Levels of many other cytokines have been reported to be effected by cannabinoids such as decreases in the proinflammatory proteins IFN- γ , IL-2, and IL-12⁵ and increases in the anti-inflammatory factors IL-4 and IL-10.¹⁰⁷ Much of the current work with cannabinoids in this field is focused on CB2 selective compounds which are void of the psychotropic effects mediated by the CB1 receptor which could limit their usefulness. Current studies on CB2 selective ligands as anti-inflammatory agents will be covered in Chapter 5.

Chapter 2: Anti-Glioma Activity of KM-233 and Analogs

2.1 Introduction

Due to the increasing attention given to the endocannabinoid system as a target for the development of antineoplastic agents, we began to evaluate the anti-cancer potential of our novel cannabinoid ligands. The initial antineoplastic work performed in our laboratory was by Dr. Mathangi Krishnamurthy for her doctoral dissertation. She prepared a novel series of C1'-dimethyl-aryl Δ^8 -tetrahydrocannabinol analogs and evaluated the compounds for CB1 and CB2 binding.¹⁰⁸ The compound which exhibited the highest degree of CB2 selectivity was (6aR,10aR)-6,6,9-trimethyl-3-(2-phenylpropan-2-yl)-6a,7,10,10a-tetrahydro-6H-benzo[c]chromen-1-ol; designated KM-233 **1** (**Figure 2.1**). This classical cannabinoid exhibited high affinity for the CB1 and CB2 receptors with binding affinity constants of 12.3nM and 0.91nM, respectively. This corresponds to a CB1/CB2 ratio of 13.5. KM-233 showed higher affinities for both the CB1 and CB2 receptors compared to Δ^8 -THC (K_i (CB1) = 28.5nM, K_i (CB2) = 25.0nM) with 13-fold higher selectivity for the CB2 receptor. Introduction of a phenyl ring in the classical cannabinoid side chain also resulted in an increase in logP from 6.08 for Δ^8 -THC to 6.27 for KM-233, which suggests improved blood-brain-barrier (BBB) penetrating ability. Hence, this combination of lipophilicity and high CB2 affinity indicates that this compound would possess significant permeation through the blood-brain-barrier as well as good activity at the CB2 receptor and would be a viable candidate to screen for anti-glioma activity.

The cytotoxic effects of KM-233 were evaluated against the human glioblastoma multiforme cell line U-87 MG.¹⁰⁹ Cell death was analyzed after 24, 48 and 72 hours of exposure to the drug. KM-233 effectively killed glioma cells over a 3-day incubation period with maximum cytotoxic effects occurring at 5 μ M (concentrations above 5 μ M not shown) (**Figure 2.2**) and with the EC₅₀ at 3.1 μ M. The cytotoxic effect produced by KM-233 was compared with that produced by Δ^8 -THC (**Figure 2.3**) using a 5 μ M dose of each drug. Although both drugs showed similar cytotoxic effects at the end of 3 days, KM-233 was more effective than Δ^8 -THC over the first 48 hours (data not shown here). In summary, KM-233 was shown to be as efficacious as Δ^8 -THC as a chemotherapeutic agent and may have a more rapid onset of action than Δ^8 -THC.

Next, the cytotoxic effect of KM-233 was compared with that produced by BCNU, a standard glioblastoma chemotherapeutic agent. For these experiments, a 5 μ M dose of KM-233 was compared with escalating doses of BCNU. A 5 μ M dose of KM-233 was far more cytotoxic than the highest dose of BCNU tested which was 300 μ M (well above the typical 5 μ M blood level following i.v. administration in humans) (**Figure 2.4**). This led to the conclusion that KM-233 is superior to BCNU as a chemotherapeutic agent for the treatment of glioblastoma in terms of its *in vitro* cytotoxic activity.

Figure 2.1: The structure of KM-233 1

Figure 2.2: Dose response curve for KM-233 on U-87 MG

C represents untreated control, D represents untreated control with 0.5% DMSO, E represents drug treatment at concentration 0.1 μM, F represents drug treatment at 1 μM concentration, G represents drug treatment at 2 μM concentration, H represents drug treatment at 3 μM concentration and I represents drug treatment at 5 μM concentration.

Figure 2.3: Comparison of efficacies of KM-233 and Δ^8 -THC

B represents untreated control, C represents untreated control with 0.5% DMSO, D represents effect after 1 day treatment with KM-233, E represents effect after 2 days treatment with KM-233, F represents effect after 3 days treatment with KM-233 and G represents effect after 3 days treatment with Δ^8 -THC.

Figure 2.4: Comparison of efficacies of KM-233 and BCNU

B represents untreated control, C represents untreated control with 0.5% DMSO, D represents treatment with 5 μ M KM-233, E represents treatment with 100 μ M BCNU, F represents treatment with 200 μ M BCNU and G represents treatment with 300 μ M BCNU.

2.2 Murine Flank Model

The *in vitro* efficacy of KM-233 was then translated into an animal model of glioblastoma to further evaluate its potential as a chemotherapeutic agent. The first *in vivo* model performed was a mouse flank model. The flank model was performed by the Duntsch group in the Department of Neurosurgery at the University of Tennessee Health Science Center.¹⁰⁹ The mouse xenograft subcutaneous model was created by suspending 2×10^6 glioblastoma multiforme cells in PBS and injecting them subcutaneously into the flank of adult SCID mice. The cell line used was U-87 MG which had been previously transduced to express green fluorescent protein to aid in imaging of the tumor. After seeding, the tumors were allowed to develop for 5 days and the mice were then treated with KM-233 at a dose of 2mg/kg either intratumorally or intraperitoneally every 12 hours for 15 days. Control groups were treated with vehicle at the same frequencies and routes of administration. The animals were sacrificed after 15 days of treatment and the tumors removed with the aid of epifluorescence microscopy.

Once the tumors were removed, they were weighed and the weights compared between the vehicle treated and the KM-233 treated groups. The groups treated with KM-233, both intratumorally and intraperitoneally, showed a reduction in tumor size when compared to the vehicle treated control groups (**Figure 2.5**). The group treated by the intraperitoneal route exhibited a greater reduction in tumor size from control when compared to the intratumorally treated group. Clinical monitoring and daily weights were similar between groups indicating no gross deleterious effects of KM-233 when administered locally or systemically. These results, while very promising, did not answer the question of whether or not KM-233 could cross the BBB and be a viable candidate for GBM therapy.

To further assess the ability of KM-233 to cross the BBB, intracranial model studies were carried out, also by the Duntsch group. The development of a chemotherapeutic agent against GBM that crosses the BBB provides distinct advantages to existing agents; 1) most existing agents used against GBM do not cross the BBB, and to achieve therapeutic levels they are generally administered directly into the tumor; and 2) direct administration may not target distant GBM foci whereas a systematically active agent should “bathe” the brain in drug thus targeting distant foci. Therefore, the ability of KM-233 to cross the blood brain barrier and affect a reduction in tumor load was studied in the orthotopic U87 model of GBM.

Tumors were induced in SCID mice by direct intracranial inoculation of human GFP-expressing U87 glioma cells. Five days following tumor cell inoculation, animals were assigned randomly to treatment groups and injected intraperitoneally twice a day for 20 subsequent days with vehicle, 2, 4, 8, or 12 mg/kg of KM-233 formulated in 100 μ l of saline containing 3% of Cremophor and 3% of ethanol. The animals were then sacrificed on the 25th day (20th day of treatment) and the brains prepared for confocal laser scanning microscopy to measure tumor surface area using imaging software. As shown in **Figures 2.6 and 2.7**, treatment of KM-233 significantly reduced the average tumor load of each treatment group in a dose-dependent manner as compared to untreated and vehicle treated controls. Clinical monitoring and daily weights were similar between all controls and

Figure 2.5: *In vivo* efficacy of KM-233 against human U87 glioma in the murine side-pocket model

Human glioma cells were inoculated into the flanks of nude mice ($n = 4$ per group). After 5 days of tumor cell incubation, groups were treated either by i.p. injection (a) or direct injection into the tumor (b) every 12 h for 15 subsequent days with vehicle or 2 mg/kg of KM-233. Panel c depicts an epifluorescence photomicrograph of the average size of tumors dissected from control animals vs. animals treated with i.p. injection KM-233 and panel d represents vehicle control vs. treated with intertumoral injection of KM-233.

Figure 2.6: Tumor load in the U87 glioma model

Adult SCID mice bearing intracranial human U87 glioma were treated with KM-233 for 20 days. A one-way ANOVA of the untreated control vs. all treatments (symbols without parenthesis) and vehicle control vs. drug treatment (symbols with parenthesis) was conducted. * = $p < 0.05$ and # = $p < 0.001$.

Figure 2.7: Dose-dependent reduction of U87 glioma after KM-233 treatment

Adult SCID mice bearing orthotopic human U87 glioma were treated with KM-233 for 20 days. Representative fluorescence micrographs showing tumor sizes in mouse brains where: (A) untreated control (B) vehicle control; (C) 2 mg/kg KM-233; (D) 4 mg/kg KM-233; (E) 8 mg/kg KM-233; and (F) 12 mg/kg KM-233. Brains were serial sectioned (asterisks indicate areas where tumor separated from normal brain tissue) and the region of the needle track identified. Pictures represent the largest fluorescent section within an animal brain where measurements were taken.

treated groups. These studies in conjunction with the flank model described above suggest that KM-233 is safe and effective in reducing tumor load, even in the context of the BBB, and even when given systemically. Pathology on the organs of the study animals showed that there was no evidence of toxicity-related tissue damage due to KM-233 administration. The results of this study demonstrated that KM-233 is a viable candidate for further development as an anti-glioma agent.

2.3 Efficacy of KM-233 and Analogs against Multiple Glioma Lines

To further evaluate the potential of KM-233 as a chemotherapeutic for the treatment of GBM, the drug was screened against four other human glioblastoma cell lines: LN-229, T98G, DBTRG-05MG, and MT-310 (a primary GBM cell line provided by the Department of Neurosurgery at the University of Tennessee Health Science Center). We hypothesized that KM-233 initiated GBM cell death by ligation to one or more of the CB receptors CB1, CB2, and/or GPR55. The first step in this study was to determine the relative expression levels of these receptors in these cell lines along with U-87 MG. Western blot analysis of the particulate fraction, membrane bound proteins, and cytosolic fraction of cell lysates is shown in **Figure 2.8**. Consistent with earlier reports,¹¹⁰ both CB1 and CB2 receptors are present in all the human GBM cell lines with CB1 showing a lower immunohistochemical staining relative to CB2. Interestingly, the GPR55 receptor exhibited a relative increased blotting in both the particulate and cytosolic fractions. The presence of GPR55 in the cytosolic fraction may indicate active synthesis of the protein or compartmental localization in the cytosol. The qualitative analysis of the Western data was not interpreted in terms of absolute expression levels but was used to verify the presence or absence of each receptor type.

The similarities in receptor expression patterns found in each GBM cell line may account for the comparable efficacies of KM-233 in cytotoxicity assays against the human GBM cell lines U-87 MG, T98G, LN-229, DBTRG-05MG, and MT310 (**Table 2.1**). The EC₅₀ values were determined using escalating doses of KM-233 and cell viability assayed 18 hours post treatment using the CCK-8 assay which utilizes the tetrazolium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methoxy PMS. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. The amount of formazan produced is directly proportional to the number of living cells. The EC₅₀ for KM-233 was comparable between the cell lines with a mean EC₅₀ of 3.44 μ M. In comparison, the clinically relevant anti-GBM agents BCNU and temozolomide were either ineffective or manifested high micromolar EC₅₀s (**Table 2.1**). Specifically, U87 and LN-229 were sensitive to BCNU after 18 hours of exposure with EC₅₀s of 223 and 178 μ M, respectively. All cell lines responded to BCNU following 48 hours of exposure manifesting EC₅₀s ranging from 119 to 305 μ M. In contrast, the EC₅₀s of KM-233 ranged from 2.3 to 4.9 μ M and were effective in the 18 hour assay window. Temozolomide was inactive against the cell lines following 18 and 48 hours exposure

Figure 2.8: Western blots for cannabinoid receptors in human glioma cell lines

Western blots of the particulate (P) and cytosolic (C) fractions of whole cell lysates of U-87 MG, T98G, LN-229, and DBTRG-O5MG. All blots are indexed to the structural protein α -tubulin, cell lysates were divided into 3 aliquots and run on separate gels for each receptor.

Table 2.1: EC₅₀ values for KM-233 and BCNU against human GBM cell lines

Cell line	KM-233 18 hrs	BCNU 18 hrs	BCNU 48 hrs
	EC ₅₀ (μM) ± S.D.	EC ₅₀ (μM) ± S.D.	EC ₅₀ (μM) ± S.D.
U-87 MG	3.08 ± 0.30	233 ± 5.1	156.0 ± 2.4
T98G	4.68 ± 0.45	> 1000	305 ± 1.2
LN-229	2.26 ± 0.32	178 ± 2.3	119 ± 4.2
DBTRG-O5MG	2.27 ± 0.19	> 1000	124 ± 3.0
MT310	4.92 ± 1.08	n.d.	n.d.

Note: The EC₅₀ values were obtained from three independent experiments run in triplicate. Abbreviations: n.d. = not determined.

(data not shown). Temozolomide is pH activated *in vivo* and the concentration of the active compound was not assessed and therefore these results are viewed with caution.

To further evaluate the KM-233 scaffold as an anti-GBM agent we screened a series of substituted aryl analogs (**Figure 2.9**) that displayed a broad range of binding affinities for the CB1 and CB2 receptors.¹¹¹ The CB1 binding affinities ranged from 1.08 nM to 142.2 nM while the CB2 affinities ranged from 0.27 nM to 134.5 nM. The CB1/CB2 affinity ratios ranged from 0.23 to 13.5 yielding compounds with modest selectivity for the CB1 or CB2 receptor. To assess if receptor subtype selectively affected cytotoxicity in the GBM cell lines, the EC₅₀s for the 4-ethylphenyl **2**, 4-propylphenyl **3**, 4-fluorophenyl **4**, 4-chlorophenyl **5**, 2-thiophenyl **6**, and KM-233 were determined (**Figure 2.10**). This study indicates that receptor subtype selectivity does not affect cytotoxicity; however, due to the modest selectivity range it is not possible to identify a specific target, i.e. CB1, CB2, or GPR55 receptor, for KM-233 or the analogs.

2.4 Receptors Involved in the Anti-Glioma Activity of KM-233

To study the contribution(s) of the CB1, CB2, and/or GPR55 receptors to the cytotoxic effect of KM-233 we utilized pharmacological probes. The CB1 and CB2 receptors couple through G_{o*i*} whereas GPR55 couples via G_{α12/13}. To assess the possible contribution of CB1 and/or CB2 to the mechanism of action of KM-233, cytotoxicity assays were carried out using U87 cells pre-treated 18 hours before the assay with 100, 200, and 300 ng/ml of pertussis toxin (Ptx). The Ptx inactivates G_{o*i*} via catalytic ADP-ribosylation thus blocking G-protein-receptor interactions, whereas the G_{α12/13} proteins are Ptx-resistant. Increasing concentrations of Ptx caused a 0.75 fold average decrease in the EC₅₀ of KM-233 against human U87 cells (**Figure 2.11**). We have determined that KM-233 is an agonist of the CB1 and an antagonist of the CB2 receptor (**Figure 2.12**), both of which signal through G_{o*i*}. If KM-233 acted only through CB1 or CB2 it would, therefore, be predicted that Ptx would block the affects of KM-233 thus the results indicate that CB1 and CB2 do not initiate KM-233 anti-cancer activity.

The compound SR141716A is reported to be an antagonist of GPR55 that has antagonist/inverse agonist activity (depending on the type of assay) at the CB1 receptor.^{11,112} The dose response curve of KM-233 in the presence of 10 μM SR141716A (**Figure 2.11**) shifted the curve to the right increasing the EC₅₀ 7 fold. The shift in the dose response curve suggests that GPR55 is a target of KM-233; however, additional pharmacological probes need to be employed such as cholera toxin (Ctx, constitutively activates Gs), inhibition of the G_{α12/13}-RhoA-ROCK pathway (possible GPR55 mediated pathway) with Y-27632, or decoupling G_{o*i*} with Ptx and screening GPR55 selective ligands such as O-1602 or lysophosphatidylinoitol (LPI). These studies are ongoing but may not conclusively identify the mechanism(s) of KM-233 action. We have also begun to utilize shRNA to knock-down each of these receptors to compliment the pharmacological studies. Additionally, we are in the process of developing functional assays for the GPR55 receptor to aid in studying the pharmacology of KM-233 and related analogs.

Figure 2.9: C1'-dimethyl-aryl Δ^8 -tetrahydrocannabinol analogs screened against human glioblastoma cell lines

Figure 2.10: EC₅₀ values of KM-233 and analogs

Comparison of the EC₅₀ values of KM-233 and analogs as a function of the CB1/CB2 K_i ratio in five human glioma cell lines.

Figure 2.11: Effects of KM-233 on U-87 MG in the presence or absence of Ptx and SR141716A

Dose response curves for U-87 MG cells treated with KM-233 in the presence or absence of 100, 200, or 300 ng/ml Ptx or 10 μ M SR141716A. Data is derived from 3 independent experiments conducted in triplicate.

Figure 2.12: Functional activity of KM-233 at the CB1 and CB2 receptors

KM-233 does not reverse cAMP production in the CB2 assay and abolishes the activity of CP-55,940 identifying KM-233 as an antagonist at CB2.

2.5 Mechanistic Studies to Determine the Anti-Glioma Activity of KM-233

The second component of the mechanism(s) of human GBM cytotoxicity of KM-233 is the downstream pathway(s) resulting in cell death. This is where I took over the study of the anti-glioma activity of KM-233. In order to elucidate the mechanism(s) by which KM-233 elicits anti-glioblastoma activity, we employed a number of techniques including epifluorescence microscopy and protein quantification using Meso Scale Discovery (MSD) MULTI-ARRAY® microplate technology. The MSD technology platform consists of 96- and 384-well microplates in which there are 1, 4, 7, or 10 electrodes coated with capture antibodies. Cell lysates are added to the wells of the plate and analytes are captured on the appropriate electrodes. The plates are then incubated with a secondary antibody that is conjugated to ruthenium (II) tris-bipyridine-(4-methylsulfonate) NHS ester that luminesces when an electrical current is applied to each electrode within the well. The plates are imaged optically and the relative luminescent intensity of each sample is compared. We first conducted a preliminary screen of the effect(s) of KM-233 on pathways known to be over active in many cancers. Initially we examined total and phosphorylated ERK1/2, JNK, p38, and, Akt using MSD plates. The screen utilized U-87 MG cells treated with KM-233 at the EC₅₀ (8.7 μM) to evaluate signal cascade modulation over a 24 hour time course. It should be noted that the change in the EC₅₀ from the previous experiments is due to modifications made in our cytotoxicity protocol which will be discussed in chapter 3. The preliminary screen showed that ERK1/2 and Akt, but not JNK or p38, are downstream signals in U-87 MG cells following KM-233 exposure (data not shown). Based on these results we analyzed the kinetic profile of phosphorylation of MEK-ERK-STAT pathway using the MSD triplex plate for pMEK1/2, pERK1/2, and pSTAT3. A significant increase in pSTAT3 occurred 1 hour post drug treatment (**Figure 2.13**) which continued thru 18 hours followed by a rapid decline at 24 hours post drug treatment. The level of pERK1/2 (**Figure 2.13**) significantly decreased in 1 hour and increased from 12 to 18 hours then declined 24 hours post treatment. The decline in pSTAT3 is of particular interest due to its role in tumorigenesis, i.e. proliferation, angiogenesis, and inhibition of apoptosis,¹¹³ and its proposed role in immune tolerance in GBM patients,¹¹⁴ thus it provides a novel target in cancer therapy.^{113,115} This suggest that CBs may be a novel target for the regulation of STAT3 function via CB1 or CB2 regulation of G_{αo}- c-Src¹¹⁶ or via a GPR55 regulated G_{α12/13}-RhoA-ERK-STAT3 pathway.¹¹⁷ The level of pMEK (Ser 217, 221) showed a significant decline at 6 hours (**Figure 2.14**), rebounded at 18 hours (though still lower than control), and then significantly decreased at 24 hours. The increases in pMEK, pERK (Thr 202, Tyr 204, Thr 185, Tyr 187), and pSTAT3 (Tyr705) in the 6 to 18 hours time interval may represent a pro-survival response to changes in pAkt and pBAD levels. The temporal increases in pMEK and pERK from 1 to 18 hours are generally consistent with the reported effects of HU-210 against U373MG and Neuro2a cell lines¹¹⁸ while the overall decrease is consistent with studies in C6 glioma cell lines.¹¹⁹ The activation is reported to originate from CB1 activation based on the inhibition of cytotoxicity by SR141716A; however, decoupling of the signal cascade by Ptx was not studied with HU-210 thus leaving open the question of GPR55 participation. Regardless of the molecular mechanism, the Ras-Raf-MEK-ERK pathway is a potential target for the development of anti-cancer agents.¹²⁰

Figure 2.13: pERK and pSTAT3 profiles of U-87 MG cells treated with KM-233

U-87 MG cells were treated with KM-233 at the EC_{50} for the designated time intervals. Cells were scraped from the culture dishes, collected by centrifugation, lysed, and the protein concentration per lysate was determined. Total protein added to each well of the MSD plate was 20 μ g. Aliquots from this experiment were used in all the following assays. All data points represent 3 independent determinations analyzed in duplicate. * = $p < 0.02$, ** = $p < 0.01$, and *** = $p < 0.001$.

Figure 2.14: pMEK profile of U-87 MG cells treated with KM-233

U-87 MG cells were treated with KM-233 at the EC_{50} for the designated time intervals and treated as defined in **Figure 2.13**. All data points represent 3 independent determinations analyzed in duplicate. * = $p < 0.02$, ** = $p < 0.01$, and *** = $p < 0.001$.

Interplay between the ERK1/2 and Akt pathways in C6 glioma cells was previously reported by Ellert-Miklaszewska *et al.*¹¹⁹ To assess the contribution of Akt to the signal cascade associated with KM-233 we utilized the pAkt-pGSK-3 β -pP70S6K MSD assay plate. Treatment of U87 cells with KM-233 triggers a significant and rapid decrease in pAkt beginning at 6 hours and continuing through 24 hours (**Figure 2.15**). Within this time interval a significant increase in glycogen synthase kinase 3 β (pGSK-3 β , Ser9) and a decrease in 70-kDa ribosomal protein S6 kinase (pP70S6K, Ser 217/221) were manifested (**Figure 2.16**). The reduction in pAkt and pP70S6K are consistent with the recent report by Salazar *et al.* wherein U-87 MG cells were treated with THC.¹²¹ The inhibition of Akt in cancer therapy is well established; however, it has recently been shown that knock-down of p70S6K with siRNA inhibited U-251MG glioma cells by greater than 50 percent. The observed increase in pGSK-3 β is consistent with the reported increase in apoptosis observed with GSK-3 β activation¹²² although Kotliarova *et al.* reports that inhibition of GSK-3 β induces cell death.¹²³ In GBM cell lines it is generally reported that regulation of Akt pathways, as well as ERK signaling, is mediated through a G $\alpha_{i/o}$ and/or G β/γ pathways; however, it is possible that a G $\alpha_{12/13}$ -RhoA pathway, and by extension GPR55, may regulate the downstream activation/inactivation of the Akt and/or the ERK pathways. For example, G $\alpha_{12/13}$ activation of the PI3K-Akt axis has been proposed in neuroblastoma cells¹²⁴ and a pathway involving Rho-ROCK signaling and ERK activation has been proposed in the human GBM line LN-19.¹²⁵

The down-regulation of the pro-survival kinase cascades ERK1/2 and Akt raised the possibility that one downstream mediator may be the Bcl-2 family protein BAD. Specifically, phosphorylation of BAD by Akt and ERK has been previously reported,¹²⁶ therefore, KM-233 may suppress phosphorylation of BAD resulting in heterodimerization with Bcl-2 death suppressors. To evaluate the effect of KM-233 on BAD phosphorylation status we utilized the MSD pBAD (Ser 112) plate. Treatment of U-87 MG cells with KM-233 caused a significant decrease in total pBAD (**Figure 2.17**), therefore it was expected that the decrease in pBAD would correlate with mitochondrial dysfunction. One manifestation would be cytochrome C release and activation of caspase 3. To evaluate this component we employed the MSD apoptosis plate to screen cleaved caspase 3, cleaved PARP, and total- and phospho-p53. A significant increase in cleaved caspase 3 began at 12 hours with a rapid elevation at 18 hours post drug treatment (**Figure 2.18**). There were no changes in the levels of cleaved PARP or total/phospho-p53 (data not shown).

The possible contribution of mitochondrial dysfunction to the mechanism of action of KM-233 was further evaluated using DePsipher® (Trevigen; Gaithersburg, MD) to evaluate the collapse of the mitochondrial membrane potential (**Figure 2.19**). Treatment of U87 cells with 3 μ M KM-233 resulted in a significant increase in mitochondrial membrane depolarization, as indicated by the inability of the fluorescent stain, DePsipher to form red fluorescent eximers inside mitochondria with an almost complete depolarization a 6 hours. Mitochondrial involvement was further verified by the observation of cytochrome C release in U-87 MG after 18 hours of exposure to KM-233 at the EC₅₀ (**Figure 2.20**).

Figure 2.15: pAKT profile of U-87 MG cells treated with KM-233

U-87 MG cells were treated with KM-233 at the EC₅₀ for the designated time intervals and treated as defined in **Figure 2.13**. All data points represent 3 independent determinations analyzed in duplicate. *** = $p < 0.001$.

Figure 2.16: pGSK-3 β and pP70S6K profiles of U-87 MG cells treated with KM-233

U-87 MG cells were treated with KM-233 at the EC₅₀ for the designated time intervals and treated as defined in **Figure 2.13**. All data points represent 3 independent determinations analyzed in duplicate. * = $p < 0.01$, ** = $p < 0.005$, *** = $p < 0.001$, **** = $p < 0.0005$, and ***** = $p < 0.00001$.

Figure 2.17: pBAD profile of U-87 MG cells treated with KM-233

U-87 MG cells were treated with KM-233 at the EC_{50} for the designated time intervals and treated as defined in **Figure 2.13**. All data points represent 3 independent determinations analyzed in duplicate. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

Figure 2.18: Cleaved caspase 3 profile of U-87 MG cells treated with KM-233

U-87 MG cells were treated with KM-233 at the EC_{50} for the designated time intervals and treated as defined in **Figure 2.13**. All data points represent 3 independent determinations analyzed in duplicate. * = $p < 0.05$, ** = $p < 0.005$, and *** = $p < 0.001$.

Figure 2.19: Effects on membrane polarization in U-87 MG cells treated with KM-233

Change in mitochondrial membrane polarization ($\Delta\psi_m$) with DePsipher® - U-87 MG cells were treated with KM-233 at the EC_{50} for 6 hours then stained with dual fluorescent stain; Green fluorescence shifts to red emission following the formation of eximers within mitochondria as stain crosses polarized outer mitochondrial membrane. Mitochondrial depolarization by KM-233 inhibits DePsipher® permeability and subsequent red fluorescence. Images of U-87 MG cells were collected using 568 nm (Red) and 458 (Green) lines of a CLSM. Control (vehicle treated) cells are shown in the A panels and 6 hours post drug treatment is shown in the B panels.

Figure 2.20: Effects on cytochrome C release in U-87 MG cells treated with KM-233

U-87 MG cells were treated with KM-233 at the EC_{50} for 18 hours and evaluated for changes in localization of cytochrome C (red); nuclei are shown in blue. Control (vehicle treated) cells are shown in panel A and 18 hour drug treated cells are shown in panel B. Control cells exhibit localization of cytochrome C in the mitochondria (arrows) along the cytoskeletal network. KM-233 treated cells show a diffuse pattern of labeling (arrows) indicating that cytochrome C has been released from the mitochondria into the cytosol.

We also conducted a microscopic study to assess the effects of KM-233 on cytoskeleton structure based on the known activity of RhoA and the possibility of a GPR55 initiated mechanism. At 18 hours post KM-233 treatment the cytoskeleton manifests profound contraction of F-actin and α -tubulin (**Figure 2.21**). Though it is not possible to conclude a specific mechanism, the results provide a basis for further evaluating RhoA-ROCK contributions to cell death.

While many of the downstream signaling pathways have been identified, the receptor or receptors responsible for initiating the cascades are yet to be elucidated. We have utilized pharmacological knockouts in an effort to identify the target of KM-233 action. Pharmacological intervention has distinct limitations due to overlap in activity of known agonists and antagonists of the CB1, CB2, and GPR55 receptors. Our preliminary data suggest that GPR55 is the target of KM-233 however this cannot be confirmed without the selective knock-down of each CB receptor subtype. Knowledge of the target is critical for the development of a complete mechanism of action for the anti-glioblastoma activity of KM-233. The contributions of each cannabinoid receptor are currently under investigation using shRNA stably transfected cell lines.

2.6 Experimental Methods

2.6.1 Cytotoxicity Assays

Human glioblastoma cells U-87MG, T98G, LN-229, DBTRG-O5MG (American Type Culture Collection), and MT310 (primary GBM cell line, grade IV, a gift from Dr. Valery Kukekov, University of Tennessee Health Science Center, Department of Neurosurgery) were cultured in supplemented media according to the recommendations of the supplier at 37°C in an atmosphere of 5% CO₂ and 95% air. Cell lines were plated in 96-well polystyrene flat-bottom plates (7,500 cells/well) at 70% confluency in a 100 μ l total volume of supplemented media as indicated, and incubated overnight at 37°C to allow for adherence. The media was then replaced with media containing 1% FBS and the cultures were inoculated with escalating amounts of drug; cell death was analyzed at 18h using the BioTek Synergy 2 Multidetection Microplate Reader (BioTek Instruments, Inc.). The percentage of viable cells present in the culture at each time point was calculated by comparing the absorbance value at 450nm from the CCK-8 assay (Dojindo Molecular Technologies) for each condition with untreated control cells. All assays were conducted per manufacturer's protocol. All described values represent the average of three data points per determination and three independent determinations.

2.6.2 Cytotoxicity Assays with Pertussis Toxin

U-87 MG cells were seeded in 96-well polystyrene flat-bottom plates (7,500 cells/well) at 70% confluency in a 100 μ l total volume of complete growth media and incubated overnight at 37°C in an atmosphere of 5% CO₂ and 95% air to allow for

Figure 2.21: Effects on cytoskeleton structure in U-87 MG cells treated with KM-233

U-87 MG cells were treated with KM-233 at the EC_{50} and evaluated for changes in F-actin (green) and α -tubulin (red), nuclei are shown in blue. Control (vehicle treated) cells are shown in panel A and 18 hour drug treated cells are shown in panel B. KM-233 causes significant contraction of the cytoskeleton network as evidenced by the compact F-actin around the nucleus.

adherence. The media was then replaced with complete growth media containing 0, 100, 200, or 300 ng/ml pertussis toxin and the plates returned to the incubator for 18 hours. Drug solutions were prepared in DMSO at 100X concentration, and mixed 1:100 in media containing 1% FBS to yield the desired concentration. After 18 hour incubation with or without Ptx, full serum media was removed and replaced with drug-media mixtures and incubated for an additional 18 hours. 10 μ l of CCK-8 reagent was added to each well to colorimetrically assess viability. After 2-4 hours of incubation with the CCK-8 dye, absorbance was read at 450nm using a BioTek Synergy 2 plate reader.

2.6.3 CB1/CB2 Functional Assays

HEK-293 cell lines stably transfected with a cyclic nucleotide-gated channel and either human CB1 or CB2 receptors (BD Biosciences, San Jose, CA USA) were seeded in poly-D-lysine coated 96-well plates at a density of 70,000 cells per well. Plates were incubated at 37°C in 5% CO₂ overnight prior to assay. Plates were then removed from the incubator and the complete growth medium (DMEM, 10% FBS, 250 μ g/ml G418 and 1 μ g/ml puromycin) was replaced with 100 μ l DMEM containing 0.25% BSA. Next, 100 μ l membrane potential dye loading buffer (Molecular Devices) was added to each well. The plates were placed back into the incubator for 30 minutes and then the baseline fluorescence was read on a BioTek Synergy 2 multi-mode microplate reader with 540nm excitation and 590nm emission filters prior to drug addition. Drugs were added in 50 μ l DPBS containing 2.5% DMSO, 1.25 μ M 5'-(N-ethylcarboxamido) adenosine and 125 μ M Ro 20-1724. Plates were then incubated at room temperature for 25 minutes and fluorescence measured again at 540nm excitation and 590nm emission.

2.6.4 MSD Protein Quantification

U-87 MG cells were plated in poly-D-lysine coated 10cm Petri dishes at a density of 4 x 10⁵ cells/ml with 12ml of cell suspension added to each plate. The cells were grown overnight in EMEM media containing 10% FBS. The following day the media was changed to EMEM media containing 1% FBS and allowed to equilibrate for 24 hours. This step is essential to allow basal levels of signaling protein to re-equilibrate due to changes in the serum concentration. The media was then replaced at the appropriate time points with media containing 1% serum and KM-233 at the EC₅₀ or media containing 1% serum and vehicle (DMSO). After the incubation period with KM-233 or vehicle, the media was removed from the dishes and transferred to a 15ml centrifuge tube to collect any cells that were in suspension. Cells were then scraped from the surface of the dish and transferred into the same 15ml centrifuge tubes and pelleted at 1000rpm, 4°C for 5min. Supernatant was removed and 0.5ml lysis buffer was added to each pellet. The cells were suspended in the buffer and transferred to 1.5ml microcentrifuge tubes. These were incubated on ice for 30 minutes, being vortexed every 5 minutes. The lysates were then centrifuged at 10,000 x g, 4°C for 10 minutes to clear cellular debris from the lysate. The supernatant was removed and assayed for protein concentration using a Pierce BCA Protein Assay Kit (Thermo Scientific). The lysates

were then diluted with complete lysis buffer to a concentration of 20µg of protein in 25µl volume. Signaling proteins were then analyzed using MSD phosphoprotein and intracellular marker multiplex kits which were imaged on a SECTOR Imager 2400 (Meso Scale Discovery) according to the manufacturer's instructions.

2.6.5 Mitochondrial Membrane Potential Assay

U-87 MG cells were grown in 75cm² flasks in complete growth medium until 80% confluent. At the appropriate time points, the media was removed and replaced with media containing 1% FBS and either KM-233 at the EC₅₀ or vehicle (DMSO). After incubation with drug or vehicle, cells were harvested by trypsonization and pelleted by centrifugation. The cells were then dyed in suspension using DePsipher membrane potential dye according to the manufacturer's instructions. The cells were then placed on glass slides and covered with glass cover slips. The cells were then imaged using an Olympus BX50 microscope (Olympus America) fitted with a Bio-Rad MRC1024 confocal laser scanning system (Bio-Rad Laboratories).

2.6.6 Microscopic Analysis of Cytoskeleton and Cytochrome C Release

U-87 MG cells were plated in complete growth medium at a density of 15,000 cells/well in poly-D-lysine coated 96-well polystyrene tissue culture plates and allowed to attach over night at 37°C in an atmosphere of 5% CO₂ / 95% air. The media was then replaced with media containing 1% FBS and either KM-233 at the EC₅₀ or vehicle (DMSO). The cells were returned to the incubator and after 18 hours exposure to the drug or vehicle, media was aspirated and the cells fixed using 4% paraformaldehyde. Cells were then stained by immunohistochemical methods using either a Cellomics Multiparameter Cell Death Detection Kit or a Cellomics Cytoskeletal Rearrangement Kit (Thermo Scientific) according to the manufacturer's instructions. Plates were then imaged using an Olympus IX71 inverted fluorescence microscope (Olympus America) fitted with a Retiga-SRV camera (QImaging).

2.6.7 Synthesis of Compounds 1 – 6

All chemicals and reagents were purchased from Sigma–Aldrich or Thermo Fisher Scientific Inc. Anhydrous solvents were prepared by distillation over sodium metal or calcium hydride prior to use. Reactions were carried out under dry conditions under an argon atmosphere. A Biotage SP1 system was used for flash chromatography purification. Routine NMR spectra were obtained on a Varian Inova 500 MHz NMR and were consistent with the assigned structures. Routine mass spectra were determined on a Bruker ESQUIRE Ion Trap LC/MS system. Compounds **1** through **6** were prepared according to the methods reported by Krishnamurthy *et al.*^{108,111} and all spectral analyses of said compounds were in agreement with published data.

Chapter 3: Anti-Glioma Activity of HB-I-172 and Analogs

3.1 Introduction

We have previously reported the synthesis of a small series of CB2 selective triaryl cannabinoids.¹²⁷ These compounds were designed with the goal of producing cannabinoid ligands that would still elicit the desired physiological effects seen with other cannabinoids (antineoplastic activity, anti-inflammatory activity, etc.) but which would be void of the unwanted CNS side effects mediated by the CB1 receptor. These triaryl compounds were developed by merging structural aspects of two previously reported classes of cannabinoids. The design utilizes the C1' aryl substitution of the classical cannabinoid core previously reported by our laboratory with compounds such as KM-233 and the introduction of an aryl ring in place of the terpene C ring in a series of biaryl compounds reported by Makriyannis and co-workers as represented by the *gem*-dimethyheptyl side chain analog compound **7**¹²⁸ (**Figure 3.1**). The design was developed with the hypothesis that the A ring of compounds **1** and **7** occupy the same region in the CB1 and CB2 ligand binding pockets.

In an effort to improve CB2 receptor affinity, the C ring of our triaryl cannabinoid core possesses a 3,5-dichloro substitution. This modification was based on the observation of Makriyannis that the 3,5-dichlo substitution pattern improves CB2 receptor affinity in the biaryl cannabinoids.¹²⁸ In addition to the abovementioned structural modifications, three types of side chain linkers: hydroxyl, carbonyl, and *gem* dimethyl were utilized in the design and four compounds were synthesized and assayed for CB1 and CB2 receptor binding affinity (**Figure 3.2**).

The compounds designed were hypothesized to be selective at the CB2 receptor sub-type. This was based not only on the contribution of the 3,5-dichlorophenyl C ring but also on the fact that the compounds comprising the phenyl series, represented by KM-233, were predominantly CB2 selective. We believed that the combined increase in electronic interactions of the substituents at both the C3 position of the phenolic A ring and the aryl C ring in the novel core would enhance the affinity and selectivity of these compounds to the cannabinoid receptor sub-type 2.

The four compounds initially synthesized with the novel triaryl core show significant binding affinity to the CB2 cannabinoid receptor subtype (**Table 3.1**). The receptor subtype affinity K_i values ranged from 1.07 to 4.77 nM at the CB2 receptor with 9–600 fold selectivity towards the CB2 receptor subtype. Although omission of the B ring from the C10 phenyl substituted classical core does not seem to affect the binding affinity to the CB2 cannabinoid receptor, there is a pronounced effect on the affinity for the CB1 receptor as evidenced by the new analogs **8-11**. The extent to which the compounds show selectivity towards the CB2 receptor appears to depend on the substitution pattern on ring A and the type of side chain linker at the C10 position. Our binding affinity data show that compounds with a hydroxyl **8** and carbonyl linker (compounds **9** and **10**) retain affinity for the CB2 receptor when compared to the *gem* dimethyl analog **11**. The presence of the *gem* dimethyl group as a linker in compound **11**

Figure 3.1: Design of the novel triaryl cannabinoid core

The design utilized elements from our C1' aryl Δ^8 classical cannabinoid analogs **1** and the biaryl cannabinoids **7** reported by Makriyannis.

Figure 3.2: Initial triaryl cannabinoids

Initial novel triaryl cannabinoids synthesized and screened for receptor binding affinity.

Table 3.1: Binding affinity (K_i) of compounds **8-11** for the CB1 and CB2 receptors compared to KM-233 **1** and Δ^8 -THC

Compound	CB1 K_i (nM)	CB2 K_i (nM)	CB1/CB2 Ratio
Δ^8 -THC	28.5 ($\pm$ 3.3)	25.0 ($\pm$ 4.8)	1.1
KM-233 1	12.3 ($\pm$ 0.61)	0.91 ($\pm$ 0.08)	13.5
8	> 1000	1.66 ($\pm$ 0.38)	> 602.4
9	> 1000	4.77 ($\pm$ 0.57)	> 209.6
10	27.0 ($\pm$ 2.30)	2.94 ($\pm$ 1.69)	9.2
11	93.66 ($\pm$ 2.33)	1.07 ($\pm$ 0.05)	87.5

Notes: Values are the mean of three experiments run in triplicate; standard deviation is given in parentheses. CB1/CB2 ratio is expressed as K_i for CB1 / K_i for CB2.

results in a CB2 selective compound with a K_i value of 1.07 nM at the CB2 receptor compared to 93.66 nM at the CB1 receptor subtype (CB1/CB2 = 87.5). Compound **8**, which was initially viewed as a synthetic intermediate, gave perhaps the most significant finding. This compound showed high binding affinity for the CB2 receptor with a K_i of 1.66 nM and exhibited a high 600-fold selectivity for the CB2 receptor. The introduction of a C10 hydroxyl or carbonyl group in classical cannabinoids has not been extensively investigated; however, previous findings by our group^{108,129} and by Papahatjis and coworkers¹³⁰ indicate that these C10 substituents reduce cannabinoid receptor affinity. Interestingly, binding affinity data for these first four compounds show that the carbonyl and hydroxyl substituents at the C10 position selectively decrease CB1 receptor affinity without altering CB2 receptor binding. The beneficial effect of the C10 equivalent hydroxyl, as well as the carbonyl, in CB2 receptor affinity may reflect that the removal of the pyran ring provides greater conformational flexibility thus allowing for more favorable receptor interactions at CB2, or that the triaryl compounds possess a different binding orientation relative to classical cannabinoids.

3.2 SAR of Triaryl Cannabinoids

To begin to develop an SAR (structure activity relationship) of this new triaryl class of cannabinoids, five more compounds were synthesized. Compounds **12-16** (**Figure 3.3**) completed the possible permutations of the three types of side chain linkers: hydroxyl, carbonyl, and *gem*-dimethyl, combined with the three substitution patterns on the A ring: dimethoxy, methoxy-hydroxy, and dihydroxy. These compounds were then screened for receptor binding affinity and functional activity at the CB1 and CB2 receptors.

After the remaining compounds were synthesized and evaluation of their affinity to the CB1 and CB2 receptors had begun, compound **10** was screened again. The results were contradictory to the previously published values. The K_i values for both CB1 and CB2 increased significantly from 27.0 ($\pm$ 2.30)nM to 6040 ($\pm$ 1477)nM and from 2.94 ($\pm$ 1.69)nM to 487 ($\pm$ 101)nM, respectively. At first, it was suspected that there was some error in the more current assay technique or analysis. However, after performing several Scatchard analyses on the membrane preparations and screening several well characterized cannabinoids such as Δ^8 -THC and CP-55,940, the current analytical techniques were validated. The remaining three original compounds (**8**, **9**, and **11**) were reevaluated for their receptor binding affinities and these values (along with compounds **12-16**) are summarized in **Table 3.2**. There has been, as yet, no sufficient explanation as to the differences between the current K_i values and those previously reported.

A general SAR concerning the side chain linker and the substitutions on the A ring for this class of compounds can be easily developed from the binding data. First, the A ring must possess at least one hydroxyl group with two being ideal for maximum affinity at both the CB1 and CB2 receptors. The three dimethoxy analogs **8**, **14**, and **15** showed very little binding at either receptor with K_i values above 10,000nM while the methoxy-hydroxy analogs **9** and **16** possessed detectable binding at the CB1 receptor with K_i values of 7235 ($\pm$ 3056)nM and 206 ($\pm$ 130)nM and at CB2 with values of 2439

Figure 3.3: Nine triaryls used to develop SAR of this new class of cannabinoids

Table 3.2: Binding affinity (K_i) of compounds **8-16** for the CB1 and CB2 receptors compared to KM-233 **1** and Δ^8 -THC

Compound	CB1 K_i (nM)	CB2 K_i (nM)	CB1/CB2 Ratio
Δ^8 -THC	28.5 ($\pm$ 3.3)	25.0 ($\pm$ 4.8)	1.1
KM-233 1	12.3 ($\pm$ 0.61)	0.91 ($\pm$ 0.08)	13.5
8	> 10,000	> 10,000	n.d.
9	7235 ($\pm$ 3056)	2439 ($\pm$ 639)	3.0
10	6040 ($\pm$ 1477)	487 ($\pm$ 101)	12.4
11	250 ($\pm$ 49)	7.67 ($\pm$ 3.14)	32.6
12	> 10,000	> 10,000	n.d.
13	> 10,000	3382 ($\pm$ 1389)	> 3.0
14	> 10,000	> 10,000	n.d.
15	> 10,000	> 10,000	n.d.
16	206 ($\pm$ 130)	66.0 ($\pm$ 17.2)	3.1

Notes: Values are the mean of three experiments run in triplicate; standard deviation is given in parentheses. CB1/CB2 ratio is expressed as K_i for CB1 / K_i for CB2. n.d. = not determined when there is no specific K_i that can be determined for either CB1 or CB2.

(± 639)nM and 66.0 (± 17.2)nM, respectively. The methoxy-hydroxy compound **12** which has a hydroxyl side chain linker, on the other hand, still possesses very low affinity for each receptor with K_i values above 10,000nM. The three compounds possessing the dihydroxy substituted A ring were the best binders at each receptor subtype. Compound **11** which has the *gem*-dimethyl linker displayed the highest affinity for CB1 ($K_i = 250 (\pm 49)$ nM) and for CB2 ($K_i = 7.67 (\pm 3.14)$ nM) while the hydroxyl side chain compound **13** possessed very low affinity for CB1 ($K_i > 10,000$ nM) and only very modest affinity for CB2 ($K_i = 3382 (\pm 1389)$ nM). Examining within each set of three compounds that have the same substitution pattern on the A ring: the dimethoxy (**8**, **14**, and **15**), the methoxy-hydroxy (**12**, **9**, **16**), and the dihydroxy (**13**, **10**, and **11**), it becomes clear that the side chain linker has a very profound effect on the binding affinity of the triaryl cannabinoids. The rank order for the highest affinity to both CB1 and CB2 is *gem*-dimethyl > ketone > hydroxyl. The negative effects on binding contributed by the hydroxyl linker is perhaps the most significant deviation from the originally reported data which showed that compound **8** retained high affinity for CB2 ($K_i = 1.66 (\pm 0.38)$ nM). It is also worth noting that while all of the compounds with detectable affinity were CB2 selective, none were as selective as the previously reported values for compounds **8** and **9** (> 602.4 and > 209.6 , respectively). Compound **11** which showed the highest affinity for each receptor also has the highest level of CB2 selectivity with a $K_i(\text{CB1}) / K_i(\text{CB2})$ ratio of 32.6. The next most CB2 selective compound was **10** with a ratio of 12.4.

3.3 Antineoplastic Activity of Triaryl Cannabinoids and Selection of HB-I-172

Due to reported efficacy of cannabinoids against a wide range of cancers,^{72a,131} it was decided to screen the nine triaryl analogs against a panel of varied human neoplastic cell lines. The compounds were tested against human lung (H69AR), prostate (DU-145 and PC-3), colorectal (HCT-15), glioma (U-87 MG, LN-229, T98G, and DBTRG-05MG) and pancreatic (BxPC-3) cancer cell lines (**Tables 3.3 and 3.4**). The dimethoxy-ketone **14** and dimethoxy-dimethyl **15** analogs were quickly eliminated due to a lack of solubility in the cytotoxicity assay conditions. All but one of the remaining seven triaryls exhibited broad spectrum antineoplastic activity against all cell lines tested. Compound **13**, the dihydroxy-hydroxy analog, was efficacious only against the PC-3 prostate carcinoma line and two glioblastoma lines: U-87 MG and LN-229. Even though compound **13** lacked broad spectrum activity, it proved to be potent against the susceptible cancers with EC_{50} s ranging from 4.63 to 5.10 μ M. Compounds **8**, **9**, **10**, **11**, **12**, and **16** were active against all cancers screened with EC_{50} s ranging from 0.30 to 6.34 μ M. These results were unexpected considering the vast range in binding affinity of these compounds for the CB1 and CB2 receptors. This finding is evidence for the possibility that the antineoplastic activity of the triaryls is mediated through a receptor other than the cannabinoid receptor subtypes 1 and 2.

To begin to elucidate the antineoplastic mechanism of action of this new class of cannabinoids we needed to pick one lead compound for the studies. We decided to evaluate their metabolic stability in order to select a compound that could potentially be evaluated *in vivo*. Phase I metabolism studies were performed in the lab of Dr. Bernd Meibohm at the University of Tennessee Health Science Center with an *in vitro* method

Table 3.3: Efficacy of three triaryl cannabinoids against a variety of human cancer cell lines

Cell Line	Compound 8 EC ₅₀ (μM) ± S.D.	Compound 12 EC ₅₀ (μM) ± S.D.	Compound 13 EC ₅₀ (μM) ± S.D.
DU-145 prostate	5.07 (± 0.30)	3.72 (± 0.83)	n.a.
PC-3 prostate	3.47 (± 1.36)	4.08 (± 0.82)	4.63 (± 0.63)
H69AR lung	6.30 (± 0.19)	5.16 (± 0.53)	n.a.
HCT-15 colorectal	5.43 (± 0.73)	3.74 (± 0.57)	n.a.
BxPC-3 pancreatic	3.28 (± 1.21)	2.49 (± 0.28)	n.a.
U-87 MG glioma	4.67 (± 0.23)	3.13 (± 0.31)	4.94 (± 0.11)
LN-229 glioma	2.57 (± 0.19)	1.93 (± 0.09)	5.10 (± 0.18)
T98G glioma	5.20 (± 0.69)	5.29 (± 2.39)	n.a.
DBTRG-05MG glioma	n.a.	5.03 (± 1.10)	n.a.

Note: The EC₅₀ values were obtained from three independent experiments run in triplicate; standard deviation is given in parentheses. Compounds **14** and **15** were not screened due to insolubility in assay conditions. Abbreviations: n.a. = not active.

Table 3.4: Efficacy of four triaryl cannabinoids against a variety of human cancer cell lines

Cell Line	Compound 9 EC ₅₀ (μM) ± S.D.	Compound 10 EC ₅₀ (μM) ± S.D.	Compound 16 EC ₅₀ (μM) ± S.D.	Compound 11 EC ₅₀ (μM) ± S.D.
DU-145 prostate	4.45 (± 0.35)	2.38 (± 0.75)	2.56 (± 0.63)	2.46 (± 0.46)
PC-3 prostate	1.96 (± 0.32)	3.83 (± 0.62)	2.90 (± 0.55)	1.83 (± 0.24)
H69AR lung	3.49 (± 0.66)	5.22 (± 1.12)	3.34 (± 0.39)	1.73 (± 0.13)
HCT-15 colorectal	2.23 (± 0.07)	4.40 (± 1.03)	2.42 (± 0.37)	2.09 (± 0.46)
BxPC-3 pancreatic	2.47 (± 0.20)	4.60 (± 0.11)	4.32 (± 0.74)	2.18 (± 0.39)
U-87 MG glioma	2.01 (± 0.27)	2.90 (± 0.27)	2.68 (± 0.74)	1.20 (± 0.58)
LN-229 glioma	1.29 (± 0.40)	4.63 (± 1.10)	1.14 (± 0.08)	0.30 (± 0.06)
T98G glioma	3.15 (± 0.75)	4.25 (± 0.26)	2.34 (± 0.49)	3.69 (± 0.74)
DBTRG-05MG glioma	6.20 (± 3.33)	6.34 (± 4.10)	4.73 (± 1.94)	2.18 (± 1.92)

Note: The EC₅₀ values were obtained from three independent experiments run in triplicate; standard deviation is given in parentheses. Compounds **14** and **15** were not screened due to insolubility in assay conditions.

utilizing rat liver microsomal preparations. The criteria used for selecting acceptable compounds would be that at least 30% of the parent drug must remain after a 90 minute incubation with the microsomes. The results of that study narrowed the compounds to three potential candidates: **10**, **12**, and **13** with 45.3, 38.7, and 42.1% remaining, respectively. Compound **13** was eliminated due to the previously mentioned lack of broad spectrum activity and compound **12** was dropped from consideration because of the presence of a chiral center in this molecule. The development of an asymmetric ligand with therapeutic potential requires efficacy and toxicology studies on each isomer along with the racemic mixture. The synthesis of each chirally pure isomer can be expensive or the attempted separation of the isomers from the mixture can be very difficult and time consuming. Therefore, compound **10** (henceforth named HB-I-172) was selected to proceed into studies to determine the mechanism of action of these compounds and to determine the receptor responsible for the antineoplastic efficacy.

3.4 Receptors Involved in the Anti-Glioma Activity of HB-I-172

Based on our previous experience with both *in vitro* and *in vivo* models of glioblastoma multiforme,^{109,111} it was decided to focus on this particular type of neoplasm for our further studies. We hypothesized that HB-I-172 initiated GBM cell death by ligation to one or more of the CB receptors CB1, CB2, and/or GPR55. The first step in our target receptor studies was to determine the relative expression levels of the receptors in the human GBM lines U-87 MG, T98G, LN-229, and DBTRG-O5MG. Western blot analysis of the particulate fraction (membrane bound proteins) and cytosolic fraction of cell lysates is shown in **Figure 3.4**. Consistent with earlier reports,^{110b,132} both CB1 and CB2 receptors are present in all the human GBM cell lines with the CB1 showing a lower immunohistochemical staining relative to CB2. Interestingly, the GPR55 receptor exhibited a relative increased blotting in both the particulate and cytosolic fractions. The presence of GPR55 in the cytosolic fraction may indicate active synthesis of the protein or compartmental localization in the cytosol. The qualitative analysis of the Western data is not interpreted in terms of absolute expression levels but is used to verify the presence or absence of each receptor type. The similarities in receptor expression patterns found in each GBM cell line may account for the comparable efficacies of HB-I-172 against the four human GBM cell lines screened.

To study the contribution(s) of the CB1, CB2, and/or GPR55 receptors to the cytotoxic effect of HB-I-172 we utilized pharmacological probes. The CB1 and CB2 receptors couple through $G_{\alpha i}$ whereas GPR55 couples via $G_{\alpha 12/13}$. To assess the possible contribution of CB1 and/or CB2 to the mechanism of action of HB-I-172, cytotoxicity assays were carried out using U87 cells pre-treated 8 hours before the assay with 100ng/ml of pertussis toxin (Ptx). The Ptx inactivates $G_{\alpha i}$ via catalytic ADP-ribosylation thus blocking G-protein-receptor interactions, the $G_{\alpha 12/13}$ proteins are Ptx-resistant. The decoupling of CB1 and CB2 from $G_{\alpha i}$ using Ptx caused an insignificant increase in EC_{50} of HB-I-172 against human U87 cells (**Figure 3.5**). We have determined that HB-I-172 is an antagonist of the CB1 receptor (binding affinity $K_i = 6040 (\pm 1477)$ nM) and an inverse agonist ($EC_{50} = 361$ nM) of the CB2 receptor (binding affinity $K_i = 487$

Figure 3.4: Western blots for cannabinoid receptors in human glioma cell lines

Western blots of the particulate (P) and cytosolic (C) fractions of whole cell lysates of U-87 MG, T98G, LN-229, and DBTRG-O5MG. All blots are indexed to the structural protein α -tubulin; cell lysates were divided into 3 aliquots and run on separate gels for each receptor.

Figure 3.5: Effects of HB-I-172 on U-87 MG in the presence or absence of Ptx

Dose response curves for U-87 MG cells treated with HB-I-172 in the presence or absence of 100 ng/ml Ptx. The EC₅₀ manifested a slight shift from 13.4 to 17.6 μ M in the presence of Ptx. Data is derived from 3 independent experiments conducted in triplicate.

(± 101)nM) coupling through $G_{\alpha i}$ (**Figure 3.6**). If the antineoplastic activity is initiated by CB1 or CB2 it would be predicted that Ptx would block the effects of HB-I-172 thus the results indicate that CB1 and CB2 do not play a role in HB-I-172 anti-cancer activity.

Selective pharmacological inhibition of the three receptors is not a viable approach to identifying the target receptors. Specifically, the CB1 antagonist AM-251 is an agonist of GPR55, and SR141716A is reported to be either an agonist or antagonist of GPR55, depending on assay conditions.⁸⁵ The selective CB2 antagonist/inverse agonist SR144528 to our knowledge has not been tested against GPR55; however, the inverse agonist activity at CB2 is the same as HB-I-172 and as such would not unambiguously help in identifying the target receptor. We are currently developing lines of U-87 MG that are stably expressing shRNA to knock-down each of the three receptors in an effort to overcome the limitations of pharmacological knock-downs.

3.5 Comparison of HB-I-172 to Standard GBM Treatments

During the course of a study utilizing immunohistochemical staining techniques to microscopically analyze the cytotoxic activities of other compounds prepared in our laboratory, it was discovered that the previously utilized method for determining cytotoxicity possessed an inherent error. In this method, cells were plated in 96-well polystyrene plates and allowed to attach overnight in an incubator. The complete growth media was then replaced with serum-free media (100 μ l) before drug addition. Small volumes of drug solutions (1 μ l) prepared in DMSO were then pipetted into the appropriate wells. The small ratio of drug solution to media did not allow for proper mixing and dilution of the drug and allowed for a much higher localized concentration of the test compounds in certain areas of the well. **Figure 3.7** illustrates this finding. This micrograph is of LN-229 that has been treated with KM-233 at the EC_{50} . It is evident that there is a disparity in the effects that the drug has on cells within this one field of view. In another set of experiments designed to evaluate the activity of caspase 3 in glioma cells treated with KM-233, we found that exposure to serum-free media during the drug incubation period increases the activity of caspase 3 separate and apart from any effect of the drug.

With the knowledge obtained from these two experiments, we developed a revised method for determining cytotoxicity of our compounds. The drug solutions are premixed in media containing 1% serum and added after the removal of the complete growth media. This allows for thorough mixing of the test compounds with the media and the small amount of serum reduces the interference with the basal metabolic activity observed in serum-free conditions.

We then screened HB-I-172 against U-87 MG and LN-229 utilizing the new method. We also tested two clinically relevant compounds, BCNU and temozolomide, under these conditions for comparison. We observed a 4.6 and 2.1 fold increase in the EC_{50} of HB-I-172 against U-87 MG and LN-229, respectively. In comparison, BCNU and temozolomide were either ineffective or manifested high micromolar EC_{50} s (**Table 3.5**). Specifically, U87 and LN-229 were sensitive to BCNU after 18 hours of exposure

Figure 3.6: Functional activity of HB-I-172 at the CB1 and CB2 receptors

HB-I-172 does not reverse cAMP production in the CB1 ActOne© assay and shifts the response curve of CP-55,940 to the right identifying HB-I-172 as an antagonist at CB1. The increase in response in the CB2 assays is indicative of inverse agonist activity.

Figure 3.7: Inefficient mixing of drug solutions with media in cytotoxicity assays

Representative immunofluorescence micrograph of LN-229 GBM cells treated with KM-233 at the EC_{50} imaged using a 10X objective. The labels are: cytochrome C (red), DAPI nuclear stain (blue), and whole cell stain (green). Cells with dysfunctional cell membranes show increased permeability so that the double stained nucleus appears cyan color (white arrows). The effects due to localized concentration of KM-233 can be easily observed in the upper right hand corner of the micrograph while the cells in the remainder of the image are relatively unaffected.

Table 3.5: Efficacy of HB-I-172 against U-87 MG and LN-229

Cell Line	HB-I-172 18 hrs	BCNU 18 hrs	BCNU 48 hrs
	EC₅₀ (μM) ± S.D.	EC₅₀ (μM) ± S.D.	EC₅₀ (μM) ± S.D.
U-87 MG	13.4 (± 0.98)	233 (± 5.1)	156.0 (± 2.4)
LN-229	9.74 (± 0.56)	178 (± 2.3)	119 (± 4.2)

Note: The EC₅₀ values were obtained from three independent experiments run in triplicate; standard deviation is given in parentheses.

with EC₅₀s of 223 and 178 μ M, respectively. Following 48 hours of exposure to BCNU the EC₅₀s decreased to 156 and 119 μ M. In contrast, the EC₅₀s of HB-I-172 were found to be 13.4 and 9.74 μ M for U-87 MG and LN-229, respectively, and were effective in the 18 hour assay window. Temozolomide was inactive against the cell lines following 18 and 48 hours exposure (data not shown). Although temozolomide is pH activated, the concentration of the active compound was not assessed and therefore these results are viewed with caution.

3.6 Mechanistic Studies to Determine the Anti-Glioma Activity of HB-I-172

3.6.1 Methods and Results

The second component of the mechanism(s) of human GBM cytotoxicity of HB-I-172 is the downstream pathway resulting in cell death. To evaluate the downstream signal cascade(s) we utilized a combination of fluorescence microscopy and protein quantification using Meso Scale Discovery (MSD) MULTI-ARRAY[®] microplate technology. We first conducted a preliminary screen of the effect(s) of HB-I-172 on total and phosphorylated ERK1/2, JNK, p38, and, Akt using MSD plates. The screen utilized U-87 MG cells treated with HB-I-172 at the EC₅₀ (13.4 μ M) to evaluate signal cascade modulation over a 24 hour time course. The preliminary screen showed that ERK1/2 and Akt, but not JNK or p38, are downstream signals in U-87 MG cells following HB-I-172 exposure (data not shown). Based on these results we analyzed the kinetic profile of phosphorylation of the MEK-ERK-STAT pathway using the MSD triplex plate for pMEK1/2 (Ser217/221), pERK1/2 (Thr202/Tyr204 and Thr185/Tyr187), and pSTAT3 (Tyr705). Cells were cultured in 10cm Petri dishes using media containing 1% serum for 24 hours to equilibrate intracellular signaling cascades. The media was replaced after equilibration with either media containing 1% serum and vehicle alone (DMSO) or media containing 1% serum and HB-I-172. The level of pMEK1/2 showed a significant decline in relative intensity 1 hour after the change in media in both control and drug treated cells (**Figure 3.8**). The control cells manifested a return to basal levels at 6 hours; however, HB-I-172 significantly reduced pMEK1/2 levels throughout the remainder of the experiment.

The levels of pERK1/2 exhibited a similar decline 1 hour post media change in both groups (**Figure 3.9**). Unlike pMEK1/2, the control group exhibited a significant increase in pERK1/2 at 6 hours that continued the remainder of the experiment. The effect of HB-I-172 on pERK1/2 however caused a significant and lasting decrease in relative levels. The Ras-Raf-MEK-ERK pathway is a potential target for the development of anti-cancer agents.¹²⁰ The precise mechanism whereby HB-I-172 inhibits ERK phosphorylation remains to be determined. Due to the observed decrease in activated MEK, it must be further upstream in the signaling cascade. We do not believe that the response is due to Ras-Raf since we would predict an increase in pERK1/2 with decreased pAkt (see below), *i.e.* pAkt negatively regulates Raf thus an increase in pERK1/2 is predicted.¹³³

Figure 3.8: pMEK1/2 profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for the designated time intervals. Cell cultures were plated for 24 hours; the media changed to 1% serum for 24 hours to equilibrate intracellular pathways, then media was changed to either media containing 1% serum or media containing 1% serum plus HB-I-172. At the designated time intervals cells were scraped from the dishes, collected by centrifugation, the pellet lysed, and the protein concentration per lysate was determined. Total protein added to each well of the MSD plate was 20 μ g. Aliquots from this experiment were used in all the following pathway assays. All data points represent 3 independent determinations analyzed in duplicate. Dashed lines indicate comparisons between control samples and solid lines between control and drug treated samples. * = $p < 0.001$.

Figure 3.9: pERK1/2 profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for the designated time intervals. See legend of **Figure 3.8** for experimental details. Dashed lines indicate comparisons between control samples and solid lines between control and drug treated samples. * = $p < 0.001$.

Interplay between the ERK1/2 and Akt pathways in C6 glioma cells was previously reported by Ellert-Miklaszewska *et al.*¹¹⁹ To assess the contribution of Akt to the signal cascade associated with HB-I-172 we utilized the MSD Akt Signaling Panel which measures pAkt, pGSK-3 β , and p-p70S6K. Treatment of U-87 MG cells with HB-I-172 triggers a significant and rapid decrease in pAkt (Ser 473) beginning at 1 hour and continuing through 24 hours (**Figure 3.10**). Within this time interval a decrease in phosphorylated 70-kDa ribosomal protein S6 kinase (p-p70S6K, Ser 217/221) (**Figure 3.11**) was observed along with a significant decrease in the Akt target glycogen synthase kinase 3 beta (pGSK-3 β , Ser 9) (**Figure 3.12**) at 1 and 6 hours followed by a highly significant increase in GSK-3 β phosphorylation beginning at 18 hours post treatment.

The reductions in pAkt and p-p70S6K are consistent with the recent report by Salazar *et al.* wherein U-87 MG cells were treated with THC.¹³⁴ The inhibition of Akt in cancer therapy is well established; however, it has recently been shown that knock-down of p70S6K with siRNA inhibited U-251 MG glioma cells by greater than 50 percent. The observed increase in pGSK-3 β is consistent with the reported increase in apoptosis observed with GSK-3 β activation¹²² although Kotliarova *et al.* report that inhibition of GSK-3 β induces cell death.¹²³ In GBM cell lines it is generally reported that regulation of Akt pathways, as well as ERK signaling, is mediated through a G $\alpha_{i/o}$ and/or G β/γ pathway; however, it is possible that a G $\alpha_{12/13}$ -RhoA pathway, and by extension GPR55, may regulate the downstream activation/inactivation of the Akt and/or the ERK pathways. For example, G $\alpha_{12/13}$ activation of the PI3K-Akt axis has been proposed in neuroblastoma cells¹²⁴ and a pathway involving Rho-ROCK signaling and ERK activation in the human GBM line LN-19.¹²⁵

The reduction in pERK1/2 could significantly impact the activity of cancer survival and gene expression pathways, for example p90RSK-BAD, p53, NF- κ B, STAT1/3, Elk-1, and/or c-fos. Three of these pathways, p53, BAD, and STAT1/3 were evaluated using MSD technology. A significant decrease in pSTAT3 occurred at 1 hour in both treatment groups (**Figure 3.13**). The levels of pSTAT3 recovered by 12 hours; however, thereafter HB-I-172 caused a significant decrease in pSTAT3 levels relative to controls. The decline in pSTAT3 is of particular interest due its role in tumorigenesis, *i.e.* proliferation, angiogenesis, and inhibition of apoptosis,¹¹³ the proposed role in immune tolerance in glioblastoma patients,¹¹⁴ and provides a novel target in cancer therapy.^{113,115} This suggests that cannabinoid receptors may be a novel target for the regulation of STAT3 function via CB1 or CB2 regulation of G α_o - c-Src¹¹⁶ or via a GPR55 regulated G $\alpha_{12/13}$ -RhoA-ERK-STAT3 pathway.¹¹⁷

The effect of HB-I-172 on the tumor suppressor protein p53 was determined using the MSD Multiplex Apoptosis Panel which measures levels of p53, p-p53 (Ser 15), cleaved PARP and cleaved caspase-3. Treatment of U-87 MG cells with HB-I-172 caused a significant increase in levels of both total p53 (**Figure 3.14**) and phospho-p53 (**Figure 3.15**) beginning 1 hour post treatment and continuing for the duration of the 24 hour experiment. This increase is probably a result of the decrease in ERK1/2 which suggests a negative regulation of p53 by ERK. This agrees with previous reports that also observed increases in p53 following suppression of ERK.¹³⁵ This increase in p53 is

Figure 3.10: pAkt profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for the designated time intervals. See legend of **Figure 3.8** for experimental details. Dashed lines indicate comparisons between control samples and solid lines between control and drug treated samples. * = $p < 0.005$, ** = $p < 0.0005$, *** = $p < 0.00001$.

Figure 3.11: Phospho-p70S6K profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for the designated time intervals. See legend of **Figure 3.8** for experimental details. Dashed lines indicate comparisons between control samples and solid lines between control and drug treated samples. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$, **** = $p < 0.00001$.

Figure 3.12: pGSK-3 β profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC₅₀ for the designated time intervals. See legend of **Figure 3.8** for experimental details. Solid lines indicate comparisons between control and drug treated samples. * = $p < 0.01$, ** = $p < 0.001$, *** = $p < 0.0005$.

Figure 3.13: pSTAT3 profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for the designated time intervals. See legend of **Figure 3.8** for experimental details. Dashed lines indicate comparisons between control samples and solid lines between control and drug treated samples. * = $p < 0.0005$, ** = $p < 0.000005$, *** = $p < 0.00000005$.

Figure 3.14: p53 profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for the designated time intervals. See legend of **Figure 3.8** for experimental details. Solid lines indicate comparisons between control and drug treated samples. * = $p < 0.005$, ** = $p < 0.0001$, *** = $p < 0.000001$.

Figure 3.15: Phospho-p53 profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for the designated time intervals. See legend of **Figure 3.8** for experimental details. Solid lines indicate comparisons between control and drug treated samples. * = $p < 0.005$, ** = $p < 0.001$, *** = $p < 0.0001$.

of interest due to its role in suppression of tumor proliferation by initiating cellular senescence¹³⁶ and its ability to initiate apoptosis.¹³⁷

The down-regulation of the pro-survival kinase cascades ERK1/2 and Akt raised the possibility that one downstream mediator of glioblastoma cytotoxicity due to HB-I-172 may be the Bcl-2 family protein BAD. Specifically, phosphorylation of BAD by Akt and ERK has been previously reported;¹²⁶ therefore, HB-I-172 may suppress phosphorylation of BAD resulting in heterodimerization with Bcl-2 death suppressors. To evaluate the effect of HB-I-172 on BAD phosphorylation status we utilized the MSD Phospho (Ser 112)/Total BAD assay. Treatment of U-87 MG cells with HB-I-172 caused a significant decrease in BAD phosphorylation at 6 hours post-exposure (**Figure 3.16**).

It was expected that the decrease in pBAD would correlate with mitochondrial dysfunction. One manifestation would be cytochrome C (cytC) release and activation of caspase 3. To evaluate this component we employed the previously mentioned MSD Apoptosis Panel. A rapid and significant increase in cleaved caspase 3 began at 6 hours post-drug treatment and persisted until the end of the experiment (**Figure 3.17**). The increase in caspase 3 activity is followed downstream by activation of caspase 9 twelve hours later at the 18 hour time point as determined by the Caspase-Glo® 9 Assay System from Promega (**Figure 3.18**). This probably accounts for the further increase in cleaved caspase 3 at the 24 hour time point as caspase 9 cleaves procaspase 3 into the active enzyme.

Coinciding with the increase in caspase 3 activity, the same rapid increase at 6 hours was observed in the level of cleaved PARP (**Figure 3.19**). The increase in cleaved PARP is of interest due to its ability to potentiate apoptosis by depleting the intracellular supply of ATP and also to independently initiate apoptosis by production of poly(ADP-ribose) which stimulates mitochondria to release apoptosis inducing factor.¹³⁸

The possible contribution of mitochondrial dysfunction to the mechanism of action of HB-I-172 was further evaluated using DePsipher® (Trevigen; Gaithersburg, MD) to evaluate the collapse of the mitochondrial membrane potential (**Figure 3.20**). Treatment of LN-229 glioblastoma cells with HB-I-172 at the EC₅₀ resulted in a significant increase in mitochondrial membrane depolarization, as indicated by the inability of the fluorescent stain DePsipher to form red fluorescent eximers inside mitochondria with a marked effect at 6 hours and an almost complete depolarization at 18 hours post-drug treatment. Mitochondrial involvement was further verified by the observation of cytochrome C release in U-87 MG after 6 hours of exposure to HB-I-172 at the EC₅₀ as determined by immunohistochemical staining techniques (**Figure 3.21**). This same fluorescence photomicrograph also depicts cells with nuclei in which DNA has become fragmented and has begun to condense which is further evidence for an apoptotic mechanism of cell death.

The increased activity of PARP which was observed in the MSD assay was also confirmed by immunohistochemistry. In U-87 MG cells treated with HB-I-172 at the EC₅₀, the presence of PARP can be seen in the nuclei 6 hours after drug exposure (**Figure 3.22**). The presence of PARP in the nuclei further confirms that there has been DNA fragmentation as one of its major roles is to repair DNA breaks.

Figure 3.16: BAD phosphorylation of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for 6 hours. See legend of **Figure 3.8** for experimental details. Solid line indicates comparison between control and drug treated samples. * = $p < 0.00005$.

Figure 3.17: Caspase 3 activity profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for the designated time intervals. See legend of **Figure 3.8** for experimental details. Solid lines indicate comparisons between control and drug treated samples. * = $p < 0.000005$, ** = $p < 0.000000005$.

Figure 3.18: Caspase 9 activity of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for 18 and 24 hours. Cells were seeded in white, opaque 96-well culture plates at a density of 10,000 cells/well in a volume of 100 μ l media. The cells were allowed to attach overnight and the complete growth media was changed to media containing 1% serum for 24 hours to equilibrate intracellular signaling pathways, then media was changed to either media containing 1% serum or media containing 1% serum plus HB-I-172. At the designated time intervals 100 μ l of Caspase-Glo® 9 Assay Reagent (Promega) was added to each well and the plate mixed on a rotary shaker for 30 seconds. After incubation at room temperature for 90 minutes the luminescence was read on a Biotek Synergy 2 plate reader. The luminescent signal is proportional to the amount of caspase 9 activity present. All data points represent 6 independent determinations. Solid lines indicate comparisons between control and drug treated samples. * = $p < 0.01$, ** = $p < 0.005$.

Figure 3.19: Cleaved PARP profile of U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for the designated time intervals. See legend of **Figure 3.8** for experimental details. Solid lines indicate comparisons between control and drug treated samples. * = $p < 0.000001$, ** = $p < 0.0000005$, *** = $p < 0.00000005$.

Figure 3.20: Effects on membrane polarization in U-87 MG cells treated with HB-I-172

Change in mitochondrial membrane polarization ($\Delta\psi_m$) with DePsipher® - U-87 MG cells were treated with HB-I-172 at the EC_{50} for 6 and 18 hours, and then stained with dual fluorescent stain; green fluorescence shifts to red emission following the formation of eximers within mitochondria as stain crosses polarized outer mitochondrial membrane. Mitochondrial depolarization by HB-I-172 inhibits DePsipher permeability and subsequent red fluorescence. U-87 MG cells were imaged using an Olympus IX71 inverted fluorescence microscope fitted with a Retiga-SRV camera. Control (vehicle treated) cells are shown in panel A, 6 hours post drug treatment in panel B, and 18 hours post drug treatment in panel C.

Figure 3.21: Effects on cytochrome C release in U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} for 18 hours and evaluated for changes in localization of cytochrome C (red); nuclei are shown in blue. Control (vehicle treated) cells are shown in panel A and 18 hour drug treated cells are shown in panel B. Control cells exhibit localization of cytochrome C in the mitochondria (white arrow) along the cytoskeletal network. HB-I-172 treated cells show a diffuse pattern of labeling (green arrow) indicating that cytochrome C has been released from the mitochondria into the cytosol. Some of the drug treated cells show a much fainter red labeling (yellow arrow, approximate cell outline represented by dashed white line) indicating depletion of cytochrome C. Some drug treated cells also show DNA fragmentation and condensation (red arrow). U-87 MG cells were imaged using an Olympus IX71 inverted fluorescence microscope fitted with a Retiga-SRV camera.

Figure 3.22: Activity of PARP in U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 at the EC_{50} and evaluated for activity of PARP (red) in the nuclei (blue). Control (vehicle treated) cells are shown in panel A and 6 hour drug treated cells are shown in panel B. HB-I-172 causes activation of PARP as seen by the red staining in the nuclei (white arrows). U-87 MG cells were imaged using an Olympus IX71 inverted fluorescence microscope fitted with a Retiga-SRV camera.

We also conducted a microscopic study to assess the effects of HB-I-172 on cytoskeleton structure based on the known activity of RhoA and the possibility of a GPR55 initiated mechanism. At 18 hours post HB-I-172 treatment the cytoskeleton manifests profound contraction of F-actin and α -tubulin (**Figure 3.23**). Though it is not possible to conclude a specific mechanism, this result provides a basis for further evaluating the possibility of RhoA-ROCK contributions to cell death.

3.6.2 Proposed Mechanism of the Anti-Glioma Activity of HB-I-172

The ability of HB-I-172 (and possibly the other triaryl cannabinoids) to block two major pro-survival signaling pathways, namely Raf1/MEK/ERK and PI3K/Akt, may account for its broad spectrum antineoplastic activity. Activation of these pathways is essential for cell survival and over-activation of each has been shown to be common in many forms of cancer.¹¹⁹ Constitutive Akt over-activation can many times be attributed to amplification of the Akt gene or mutations in components of the upstream signaling pathway.¹³⁹ Akt plays a critical role in the regulation of cell survival and apoptosis by phosphorylation and inhibition of a number of proteins including GSK-3 β ¹⁴⁰ and caspase-9.¹⁴¹ The Raf1/MEK/ERK pathway has been shown to be over-active in nearly one-third of all human cancers. This pathway appears to play a large role in cell survival and transduction of differentiation and mitogenic signals.¹⁴² Both of these pro-survival pathways converge on the pro-apoptotic Bcl-2 protein BAD. Each pathway (Akt directly and ERK1/2 through p90 ribosomal S6 kinase (p90RSK)) phosphorylates and inhibits BAD which allows the pro-survival members of the Bcl-2 family to remain active and prevent apoptosis. Since each pathway is capable of inhibiting BAD, it would be predicted that inhibition of a single pathway would not be sufficient to bring about apoptosis. This has, in fact, been demonstrated in a recent report by Ellert-Miklaszewska, Kaminska, and Konarska in which specific inhibition of each pathway in the C6 glioma cell line did not bring about any significant cell death.¹¹⁹ These two pathways share another common signaling protein, namely GSK-3 β . Akt and p90RSK both inhibit GSK-3 β by phosphorylation. Inhibition of the two pathways leads to increases in the activity of GSK-3 β which, in turn, activates the pro-apoptotic Bcl-2-associated X protein (BAX) which leads to loss of mitochondrial membrane potential.¹⁴³ GSK-3 β also up-regulates the transcription factor protein 53 (p53) which leads to further up-regulation of BAX and also the pro-apoptotic Bcl-2 homologous antagonist/killer (BAK).¹⁴⁴ By down-regulation of both the PI3K/Akt and Raf1/MEK/ERK pathways, HB-I-172 decreases the downstream phosphorylation of BAD and allows it to dimerize with Bcl-2 in the mitochondrial membrane leading to loss of membrane potential, release of cytochrome C and activation of the caspase cascade beginning with caspase 9 (**Figure 3.24**).

The down regulation of the two pro-survival pathways in glioblastoma by HB-I-172 does not give clear evidence for the molecular target of the drug. We know that HB-I-172 is an inverse agonist at the CB2 receptor. Since CB2 signals through G_{ai}, the effects of an agonist would lead to decreases in cAMP which would decrease the activity of protein kinase A (PKA) and up-regulate the Raf1/MEK/ERK pathway. An inverse agonist, on the other hand, would cause increases in cAMP which would increase the

Figure 3.23: Effects on cytoskeleton structure in U-87 MG cells treated with HB-I-172

U-87 MG cells were treated with HB-I-172 and evaluated for changes in F-actin (yellow) and α -tubulin (red), nuclei are shown in blue. Control (vehicle treated) cells are shown in panel A and 12 hour drug treated cells are shown in panel B. HB-I-172 causes significant contraction of the cytoskeleton network as evidenced by the compact F-actin (yellow arrows) and α -tubulin (white arrows) around the nuclei. U-87 MG cells were imaged using an Olympus IX71 inverted fluorescence microscope fitted with a Retiga-SRV camera.

Figure 3.24: Proposed induction of apoptosis by HB-I-172 mediated through CB2

See text for definitions of abbreviations.

activity of PKA and down-regulate Raf1/MEK/ERK. This correlates with our observed decreases in phosphorylated MEK1/2 and ERK1/2. The inhibition of the PI3K/Akt pathway can also be explained by a CB2 mediated mechanism. Ligation of the CB2 receptor can cause an intracellular buildup of ceramide produced from sphingomyelin by the action of sphingomyelinase which is activated by factor associated with neutral sphingomyelinase (FAN) (**Figure 3.24**). The increase in ceramide inhibits the activation of both PI3K and Akt¹⁴⁵ which, again, agrees with our data.

HB-I-172 acting through CB2 alone would however not account for the observed effects on the cytoskeleton of U-87 MG cells. The formation of stress fibers following treatment with HB-I-172 is better explained through a GPR55 mediated mechanism. GPR55 signals through $G_{\alpha 12/13}$ which activates the Rho family of GTPases through the activity of guanine nucleotide exchange factors (GEFs). One of these GTPases, Rho A, then activates focal adhesion kinase (FAK) and Rho-associated, coiled-coil containing protein kinase (ROCK) which lead to cytoskeleton rearrangement and formation of stress fibers.¹⁴⁶ GPR55 can also affect both the PI3K/Akt and Raf1/MEK/ERK pathways through the action of Ras on PI3K and Raf1. For this to be a viable explanation, HB-I-172 would have to be an antagonist or an inverse agonist at GPR55 also. This would lead to decreases in the activities of Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (cdc42) and downstream decreases in p21-activated kinase (PAK) and Ras which would lead to decreases in the activity of PI3K and Raf1 (**Figure 3.25**).

The effects of either CB2 or GPR55 (or possibly both) on the two pro-survival pathways would be sufficient to initiate apoptosis in glioma cells via activation of BAD and GSK-3 β by each pathway, the activation of caspase 9 by Akt, and the activation of p53 by both GSK-3 β and ERK1/2. There, however, seems to be more to the puzzle than the inhibition of these two pathways. Our observation that caspase 3 is active approximately 12 hours before caspase 9 led us to look for alternative mechanisms for the activation of caspase 3. One such possibility is through endoplasmic reticulum (ER) stress caused by the unfolded protein response (UPR). ER stress has been shown to initiate apoptosis in a mitochondria independent manner by activation of caspase 3 via caspase 12¹⁴⁷ (**Figure 3.26**). ER stress can also initiate apoptosis via a mitochondria dependent pathway involving the release of calcium from the ER into the cytosol which causes the collapse of the mitochondrial membrane potential and leads to the release of cytochrome C.¹⁴⁸ ER stress can play further roles in the initiation of apoptosis also. The UPR can lead to activation of the transcription factor C/EBP homologous protein (CHOP) which inhibits the pro-survival Bcl-2 at the transcription level.¹⁴⁹ We observed increased translocation of active CHOP to the nuclei of HB-I-172 treated U-87 MG cells in as little as 3 hours. At the 3 hour time point, the percent of drug treated cells showing positive immunohistochemical staining for CHOP in the nucleus was nearly 8-fold higher than in control cells (data not shown). The number of CHOP positive cells in the drug treated group continued to grow throughout the length of the experiment and was 40-fold higher than control at the 18 hour terminal time point. ER stress has also been shown to inhibit Akt by activation of the tribbles homolog 3 (TRIB3) pseudo-kinase by the transcriptional co-activator p8.¹³⁴ Much like ERK1/2, ER stress can increase the activity of the tumor suppressor p53 which leads to the transcription of p53-up-regulated

Figure 3.25: Proposed induction of apoptosis by HB-I-172 mediated through GPR55

See text for definitions of abbreviations.

Figure 3.26: Proposed induction of apoptosis by HB-I-172 initiated by ER stress mediated through CB2

See text for definitions of abbreviations.

modulator of apoptosis (PUMA) and Noxa which cause cytochrome C release from mitochondria through activation of BAX and BAK.¹⁵⁰

ER stress has been shown to be caused by the accumulation of *de novo* synthesized ceramide in the ER.¹⁵¹ Considering the increase in ceramide synthesis caused by ligation of the CB2 receptor, this seems a reasonable pathway to consider. In the recent report by Salazar *et al.*, THC was shown to induce ER stress in human glioma cells which led to autophagy preceding apoptosis.¹²¹ They determined that autophagy was initiated by the inhibition of Akt by TRIB3 which leads to the down-regulation of the autophagy inhibitor mammalian target of rapamycin complex 1 (mTORC1) (**Figure 3.27**) which consists of mTOR, regulatory associated protein of mTOR (Raptor), and mammalian LST8/G-protein β -subunit like protein (G β L). Though the authors were not able to determine the exact manner in which autophagy leads to apoptosis, they did determine that it is a necessary precursor to cannabinoid induced apoptotic cell death in glioblastoma. This, in fact, does fit with our data. The human glioblastoma cell line LN-229 was treated with HB-I-172 at the cytotoxic EC₅₀ and the cells were immunohistochemically stained for the microtubule associated protein 1 light chain 3 (LC3B) which associates into membranes of autophagosomes during autophagy. The number of cells positive for LC3B increased 6-fold over control in just 3 hours with a 20-fold increase at the 18 hour terminal time point (data not shown). This corroborates the autophagy component of cannabinoid mediated glioma toxicity as reported by Salazar *et al.* The authors of the study however attributed the process to activation of the CB1 receptor. We do not believe that the CB1 receptor plays a role in the activity of HB-I-172 due to the previously mentioned finding that it is an antagonist at CB1. This same experiment needs to be repeated using U-87 MG to strengthen the evidence for autophagy in this mechanism of cell death.

I believe that there is clear evidence to support a mechanism of cell death in U-87 MG cells following HB-I-172 exposure that involves multiple targets such as CB2 and GPR55. The early intracellular events following drug exposure are most likely due to inverse agonism at the CB2 receptor. The increases in cAMP and ceramide following the ligation of CB2 by HB-I-172 would clearly lead to the down-regulation of the PI3K/Akt and Raf1/MEK/ERK pathways which is seen beginning at one hour post exposure. ER stress, as a result of ceramide production, is responsible for the activation of caspase 3 six hours before the observed activation of caspase 9. The case for ER stress is further supported by other events at earlier time points such as the activity of CHOP and the observance of autophagy as determined by LC3B staining at 3 hours. We believe that the observed changes in the cytoskeleton are due to GTPase activity resulting from ligation of the GPR55 receptor. The role that GPR55 plays in the down regulation of the PI3K/Akt and Raf1/MEK/ERK pathways remains to be determined. As previously stated, HB-I-172 could perhaps be an antagonist/inverse agonist at GPR55 which would lead to the down-regulation of Ras and the inhibition of the two pro-survival pathways. If HB-I-172 were a GPR55 agonist, an increase in the signaling of these two pathways would be expected. Cross talk and inhibition of signaling between the two receptors has been demonstrated following anandamide ligation.¹⁵² The complete proposed signaling pathway is shown in **Figure 3.28**.

Figure 3.27: Proposed induction of autophagy followed by apoptosis by HB-I-172 initiated by ER stress mediated through CB2

See text for definitions of abbreviations.

Figure 3.28: Entire proposed mechanism of glioma cell death by HB-I-172 mediated by CB2 and GPR55

See text for definitions of abbreviations.

Much remains unanswered about the signaling cascade initiated by HB-I-172 in glioblastoma. We are currently in the process of preparing for a series of assays that will evaluate the activation of the GTPases RhoA and Rac1. This will give a clearer picture of the activity, or lack thereof, of GPR55 following exposure to HB-I-172. Selective inhibition of any of a number of signaling proteins in the proposed pathways either pharmacologically or by shRNA knock-down would give further insight into the importance of each segment of the cascade initiated by HB-I-172.

While many of the downstream signaling pathways have been identified, the receptor or receptors responsible for initiating the cascades are yet to be elucidated. We have utilized pharmacological knockouts in an effort to identify the target of HB-I-172 action. Pharmacological intervention has distinct limitations due to overlap in activity of known agonists and antagonists of the CB1, CB2, and GPR55 receptors. Our preliminary data suggests that both CB2 and GPR55 are both targets of HB-I-172 however this cannot be confirmed without the selective knock-down of each CB receptor subtype. Knowledge of the target is critical for the development of a complete mechanism of action for the anti-glioblastoma activity of HB-I-172. The contributions of each cannabinoid receptor are currently under investigation using shRNA stably transfected cell lines.

3.7 Chemical Synthesis

3.7.1 General

In order to synthesize the core of the triaryl compounds **8-16**, and provide a route for future functionalization of this series, an efficient synthetic route was designed. Commercially available syringaldehyde **17** was selected to form the A ring in the triaryl series. The phenolic hydroxyl was activated for a Suzuki coupling by reaction with trifluoromethanesulfonic anhydride to yield intermediate **18** (**Scheme 3.1**). Formation of the biaryl ring system was carried out via a Suzuki coupling reaction with 3,5-dichlorophenyl boronic acid **19** in the presence of tetrakis(triphenylphosphine) palladium, and potassium carbonate in toluene and water to yield the aldehyde intermediate **20** (**Scheme 3.1**). The resulting aldehyde was reacted with phenyl magnesium bromide **21** to obtain the racemic alcohol **8**, which was oxidized using PCC, to yield the ketone **14**. The ketone **14** was dimethylated using dimethylzinc and titanium (IV) chloride to yield compound **15** (**Scheme 3.2**). The mono- and di-deprotected analogs of **14** and **15** were prepared by reaction with BBr₃ (**Scheme 3.3**). To prepare the equivalent analogs possessing the hydroxyl linker compounds **9** and **10** were reduced with sodium borohydride to give analogs **12** and **13** (**Scheme 3.4**).

All chemicals were obtained from Sigma Aldrich or Fisher Scientific Inc. Anhydrous solvents were obtained by distillation over either calcium hydride or metallic sodium and benzophenone. Final compounds and intermediates were purified using column chromatography on the Biotage SP1 system employing Flash column cartridges or by crystallization. NMR spectra were obtained on a Bruker 300MHz or Varian

Scheme 3.1: Synthesis of aldehyde intermediate **20**

(a) Triflic anhydride, 2,3,5 collidine, DCM, -20°C, 2hr; (b) $(PPh_3)_4Pd$, K_2CO_3 , toluene / water, reflux, 18hr.

Scheme 3.2: Synthesis of compounds **8**, **14**, and **15**

(a) THF, 0°C , 4hr – RT overnight; (b) PCC, DCM, RT, 18 h; (c) TiCl_4 , $\text{Zn}(\text{CH}_3)_2$, DCM, -40°C , 2hr – RT overnight.

Scheme 3.3: Synthesis of compounds **9**, **10**, **16**, and **11**

(a) BBr_3 , DCM, -78°C , 3hr – RT overnight.

Scheme 3.4: Synthesis of compounds **12** and **13**

(a) NaBH₄, MeOH, 0°C - RT, 2hr.

500MHz Inova NMR. HPLC analysis of final products was carried out by gradient elution using water/acetonitrile (0.1% TFA) with a gradient of 70% (3min), 50% (2min), and 10% (15min). A reverse-phase ODS Hypersil column, manufactured by Thermo Scientific, of dimensions 4.6 x 150mm with 5 μ m particle size was used for HPLC analyses.

3.7.2 Synthesis of 4-formyl-2,6-dimethoxyphenyl trifluoromethanesulfonate (18)

Syringaldehyde **17** (10g, 55mmol) and 2,3,5 collidine (26.7g, 28.7ml, 220mmol) were dissolved in anhydrous DCM (250ml) under an atmosphere of argon, stirred, and cooled to -20°C followed by the dropwise addition of triflic anhydride (23.4g, 14ml, 83mmol). The reaction was maintained at this temperature, with stirring, for 2 hours. The reaction was stopped by the addition of saturated aqueous NaHCO₃. The layers were separated and the aqueous layer was extracted with DCM. The organic fractions were combined and washed with saturated aqueous CuSO₄, water, and brine and dried over Na₂SO₄. The organic phase was concentrated and the product purified by column chromatography using 100% DCM as the mobile phase to yield a white to off-white solid. Yield: 14.14g (82%) (R_f = 0.648; 100% DCM). MS: m/z (ESI, pos.) = 315.1 [M + H]⁺. ¹H NMR (300 MHz, CDCl₃): δ 9.93 (s, 1H), 7.18 (s, 2H), 3.99 (s, 6H), ¹³C NMR (300MHz, CDCl₃): δ 190.52, 153.48, 136.15, 121.00, 116.52, 106.18, 56.88.

3.7.3 Synthesis of 3',5'-dichloro-2,6-dimethoxybiphenyl-4-carbaldehyde (20)

Triflate **18** (8g, 25.5mmol) along with 3,5 dichloro phenyl boronic acid (7.25g, 38mmol), Tetrakis(triphenylphosphine)palladium (1.18g, 1.02mmol), and K₂CO₃ (7.05g, 51mmol) were added to toluene (100ml) and water (33ml) in a round bottom flask fitted with a condenser. The reaction was heated to reflux for 18 hours. The reaction was then allowed to cool to room temperature and diluted with diethyl ether. The organic layer was separated and washed with saturated aqueous NaHCO₃, water, and brine and then dried over Na₂SO₄. The organic phase was concentrated and the product was purified by column chromatography using an EtOAc / hexanes gradient to yield a white solid. Yield: 4.97g (63%) (R_f = 0.402; 15% EtOAc / hexanes). MS: m/z (ESI, pos.) = 333.1 [M + Na]⁺. ¹H NMR (500 MHz, CDCl₃): δ (ppm) 9.98 (s, 1H), 7.34 (t, 1H), 7.21 (d, 2H), 7.15 (s, 2H), 3.83 (s, 6H). ¹³C NMR (500MHz, CDCl₃): δ (ppm) 191.70, 158.17, 137.66, 136.21, 134.44, 129.27, 127.73, 122.95, 105.34, 56.37.

3.7.4 Synthesis of (3',5'-dichloro-2,6-dimethoxybiphenyl-4-yl)(phenyl)methanol (8)

Aldehyde **20** (4.94g, 15.9mmol) was dissolved in anhydrous THF (100ml), stirred under argon, and cooled to 0°C. Phenyl MgBr (**21**) (3M solution, 1.67ml, 5mmol) was then added by syringe and the reaction maintained with stirring at 0°C for 4 hours and

then allowed to warm to room temperature overnight. The reaction mixture was then diluted with diethyl ether, washed with water and brine, and dried over Na₂SO₄. The organic phase was concentrated and the product was crystallized from EtOAc / hexanes to yield a white solid. Yield: 2.1g (79%) (*R_f* = 0.341; 20% EtOAc / hexanes). MS: *m/z* (ESI, pos.) = 411.1 [M + Na]⁺. ¹H NMR (500 MHz, CDCl₃): δ (ppm) 7.43 (d, 2H, *J* = 7Hz), 7.38 (t, 2H, *J* = 7.5Hz), 7.31 (t, 1H, *J* = 7Hz), 7.28 (t, 1H, *J* = 2Hz), 7.20 (d, 2H, *J* = 1.5Hz), 6.67 (s, 2H), 5.85 (d, 1H, *J* = 3Hz), 3.72 (s, 6H), 2.26 (d, 1H, *J* = 3Hz). ¹³C NMR (500MHz, CDCl₃): δ (ppm) 157.63, 145.94, 143.60, 137.24, 134.15, 129.73, 128.88, 128.15, 127.02, 126.82, 102.44, 76.65, 56.12. HPLC retention time: 10.612min; purity 98.92%.

3.7.5 Synthesis of (3',5'-dichloro-2,6-dimethoxybiphenyl-4-yl)(phenyl)methanone (14)

Alcohol **8** (2.1g, 5.4mmol), pyridinium chlorochromate (2.33g, 10.8mmol) and Celite® (2.33g) were added to anhydrous CH₂Cl₂ and stirred under argon at room temperature for 18 hours. The reaction mixture was then filtered over a plug of silica gel and the filtrate was washed with saturated aqueous NaHCO₃, water, and brine and then dried over Na₂SO₄. The organic phase was concentrated and the product was purified by column chromatography using an EtOAc / hexanes gradient to yield an off-white solid. Yield: 1.77g (85%) (*R_f* = 0.448; 15% EtOAc / hexanes). MS: *m/z* (ESI, pos.) = 409.1 [M + Na]⁺. ¹H NMR (300 MHz, CDCl₃): δ (ppm) 7.86 (m, 2H), 7.56 (m, 4H), 7.34 (t, 1H, *J* = 1.8Hz), 7.25 (t, 1H, *J* = 2.4Hz), 7.05 (s, 2H), 3.75 (s, 6H). ¹³C NMR (300MHz, CDCl₃): δ 196.18, 157.64, 139.26, 137.44, 135.62, 135.30, 132.93, 130.28, 129.26, 128.63, 128.59, 118.91, 111.49, 104.61, 56.34. HPLC retention time: 12.051min; purity 98.37%.

3.7.6 Synthesis of 3',5'-dichloro-2,6-dimethoxy-4-(2-phenylpropan-2-yl)biphenyl (15)

Anhydrous CH₂Cl₂ was added to a round bottom flask, stirred under argon and cooled to -40°C using a dry ice / acetonitrile bath. Titanium chloride (1M, 30ml, 30mmol) was added by syringe followed by dimethyl zinc (2M, 15ml, 30mmol). This mixture was allowed to stir at -40°C for 15 minutes. Ketone (14) (1.95g, 5mmol) dissolved in anhydrous CH₂Cl₂ (100ml) was then added by syringe and the reaction stirred at -40°C for 2 hours and was then allowed to warm to room temperature overnight. The reaction was then quenched by the addition of ice. The organic layer was separated and washed with saturated aqueous NaHCO₃, water, and brine and then dried over Na₂SO₄. The organic phase was concentrated and the product was purified by column chromatography using an EtOAc / hexanes gradient to yield a white solid. Yield: 1.9g (95%) (*R_f* = 0.750; 15% EtOAc / hexanes). MS: *m/z* (ESI, pos.) = 423.1 [M + Na]⁺. ¹H NMR (500 MHz, CDCl₃): δ 7.43 (m, 1H), 7.30 (m, 3H), 7.27 (t, 1H, *J* = 1.5Hz), 7.24 (d, 1H, *J* = 2Hz), 7.20 (m, 2H), 6.48 (s, 2H), 3.65 (s, 6H), 1.72 (s, 6H). ¹³C NMR (500MHz, CDCl₃): δ 157.04, 153.70, 150.12, 136.27, 135.36, 129.69, 129.54, 128.02,

126.84, 125.99, 112.49, 107.64, 102.75, 55.91, 43.50, 30.85, 30.73. HPLC retention time: 13.909min; purity 96.42%.

3.7.7 Synthesis of (3',5'-dichloro-2-hydroxy-6-methoxybiphenyl-4-yl)(phenyl)methanone (**9**) and (3',5'-dichloro-2,6-dihydroxybiphenyl-4-yl)(phenyl)methanone (**10**)

Ketone **14** was dissolved in anhydrous CH₂Cl₂ (120ml), stirred under argon, and cooled to -78°C in a dry ice / acetone bath. Boron tribromide (1M, 9ml, 9mmol) was slowly added by syringe and the reaction was allowed to stir at -78°C for 3 hours and then allowed to warm to room temperature and stirred an additional 16 hours. The reaction was then quenched by the addition of methanol and washed with saturated aqueous NaHCO₃, water, and brine and then dried over Na₂SO₄. The organic phase was concentrated and the products were purified by column chromatography using an EtOAc / hexanes gradient to yield an off-white solid **9** and a reddish solid **10**.

9 Yield: 314mg (21%) (*R_f* = 0.390; 20% EtOAc / hexanes). MS: *m/z* (ESI, neg.) = 370.9 [M - H]⁻. ¹H NMR (500 MHz, CDCl₃): δ 7.84 (d, 2H, *J* = 8Hz), 7.61 (t, 1H, *J* = 8Hz), 7.51 (t, 2H, *J* = 7.5Hz), 7.42 (d, 1H, *J* = 1.5Hz), 7.30 (d, 2H, *J* = 1.5Hz), 7.04 (s, 1H), 7.00 (d, 1H, *J* = 1.5Hz), 5.19 (d, 1H, *J* = 16Hz), 3.79 (s, 3H), ¹³C NMR (500MHz, CDCl₃): δ 196.18, 157.64, 153.36, 139.26, 137.44, 135.62, 135.30, 132.93, 130.28, 129.26, 128.63, 128.59, 118.91, 111.49, 104.61, 56.34. HPLC retention time: 10.780min; purity 99.03%.

10 Yield: 106mg (7%) (*R_f* = 0.235; 20% EtOAc / hexanes). MS: *m/z* (ESI, neg.) = 356.9 [M - H]⁻. ¹H NMR (500 MHz, CDCl₃): δ 7.84 (d, 2H, *J* = 7.5Hz), 7.61 (t, 1H, *J* = 7.5Hz), 7.48 (m, 3H), 7.37 (d, 2H, *J* = 1.5Hz), 7.02 (s, 2H), 5.42 (s, 2H), ¹³C NMR (500MHz, CDCl₃): δ 196.24, 153.77, 139.32, 137.18, 136.29, 134.46, 133.08, 130.34, 129.25, 129.18, 128.61, 117.63, 110.18. HPLC retention time: 9.618min; purity 100%.

3.7.8 Synthesis of 3',5'-dichloro-6-methoxy-4-(2-phenylpropan-2-yl)biphenyl-2-ol (**16**) and 3',5'-dichloro-4-(2-phenylpropan-2-yl)biphenyl-2,6-diol (**11**)

Dimethyl compound **15** was reacted with boron tribromide (1M, 9ml, 9mmol) in anhydrous CH₂Cl₂ (120ml) in a manner similar to the synthesis of compounds **9** and **10**. The products were purified by column chromatography using an EtOAc / hexanes gradient to yield a yellow resin **16** and a golden foam **11**.

16 Yield: 290mg (17%) (*R_f* = 0.317; 10% EtOAc / hexanes). MS: *m/z* (ESI, pos.) = 409.2 [M + Na]⁺. ¹H NMR (500 MHz, CDCl₃): δ 7.35 (t, 1H, *J* = 1.5Hz), 7.29 (m, 6H), 7.20 (m, 1H), 6.51 (d, 1H, *J* = 1.5Hz), 6.37 (d, 1H, *J* = 1.5Hz), 4.73 (s, 1H), 3.63 (s, 3H), 1.69 (s, 6H), ¹³C NMR (500MHz, CDCl₃): δ 157.04, 153.70, 153.01, 150.12, 136.27, 135.36, 129.69, 129.54, 128.02, 126.84, 125.99, 112.49, 107.64, 102.75, 55.91, 43.50, 30.85, 30.73. HPLC retention time: 11.716min; purity 99.48%.

11 Yield: 950mg (57%) (R_f = 0.167; 10% EtOAc / hexanes). MS: m/z (ESI, pos.) = 395.2 $[M + Na]^+$. 1H NMR (500 MHz, $CDCl_3$): δ 7.40 (t, 1H, J = 2Hz), 7.29 (m, 6H), 7.19 (m, 1H), 6.40 (s, 2H), 4.81 (s, 2H), 1.65 (s, 6H). ^{13}C NMR (500MHz, $CDCl_3$): δ 154.07, 153.11, 149.97, 136.07, 135.34, 129.58, 129.43, 128.68, 128.58, 128.23, 111.10, 107.27, 43.16, 30.58. HPLC retention time: 10.271min; purity 96.81%.

3.7.9 Synthesis of 3',5'-dichloro-4-(hydroxy(phenyl)methyl)-6-methoxybiphenyl-2-ol (12)

Ketone **9** (75mg, 0.2mmol) was dissolved in MeOH (5ml) and cooled to 0°C. $NaBH_4$ (9.5mg, 0.25mmol) was slowly added with stirring. The reaction was then allowed to warm to room temperature and stirred for 2 hours. The solvent was then removed by rotary evaporation and the product was purified by column chromatography using an EtOAc / hexanes gradient to yield a white foam. Yield: 63mg (84%) (R_f = 0.361; 30% EtOAc / hexanes). MS: m/z (ESI, neg.) = 372.8 $[M - H]^-$. 1H NMR (300 MHz, MeOD): δ 7.37 (m, 6H), 7.25 (m, 2H), 6.65 (s, 1H), 6.62 (s, 1H), 5.80 (d, 1H, J = 5Hz), 4.83 (s, 1H), 3.73 (s, 3H), 2.25 (d, 1H, J = 5.5Hz). ^{13}C NMR (500MHz, MeOD): δ 152.65, 153.77, 146.12, 143.03, 135.90, 135.23, 129.45, 129.40, 128.87, 128.21, 126.81, 112.96, 106.47, 105.62, 76.09, 56.54. HPLC retention time: 9.989min; purity 94.88%.

3.7.10 Synthesis of 3',5'-dichloro-4-(hydroxy(phenyl)methyl)biphenyl-2,6-diol (13)

Ketone **10** (33mg, 0.092mmol) was reacted with $NaBH_4$ (4.8mg, 0.129mmol) in MeOH (4ml) in a manner similar to compound 12. The product was purified by column chromatography using an EtOAc / hexanes gradient to yield a white solid. Yield: 18mg (54%) (R_f = 0.441; 40% EtOAc / hexanes). MS: m/z (ESI, neg.) = 358.9 $[M - H]^-$. 1H NMR (500 MHz, $CDCl_3$): δ 7.37 (t, 1H, J = 1.5Hz), 7.33 (m, 4H), 7.29 (m, 1H), 7.25 (d, 2H, J = 1Hz), 6.52 (s, 2H), 5.67 (s, 1H), 5.38 (s, 2H), 2.71 (s, 1H). ^{13}C NMR (500MHz, $CDCl_3$): δ 153.77, 146.12, 143.03, 135.90, 135.23, 129.45, 129.40, 128.87, 128.21, 126.81, 112.96, 106.47, 76.09. HPLC retention time: 9.046min; purity 96.05%.

3.8 Experimental Methods

3.8.1 Radioligand Binding Assays

Binding affinity studies were carried out using cell membranes from HEK293 cells transfected with the human CB1 receptor (K_d for tritiated CP-55,940 binding: 1.0 nM) and membranes from HEK293 cells transfected with the human CB2 receptor (K_d for tritiated CP-55,940 binding: 1.0 nM). Non-specific binding was determined using 10 μM WIN55, 212-2. Increasing concentrations of compounds to be tested were made ranging from 10^{-12} M to 10^{-4} M and were added in triplicate for each experiment and the

individual molar IC₅₀ values were determined using GraphPad Prism software. The corresponding K_i values for each drug were determined utilizing the Cheng-Prusoff equation and final data are presented as $K_i \pm$ standard deviation of $n = 3$ experiments run in triplicate.

3.8.2 Cytotoxicity Assays

Human glioblastoma cells U-87MG, T98G, LN-229, DBTRG-O5MG (American Type Culture Collection) were cultured in supplemented media according to the recommendations of the supplier at 37°C in an atmosphere of 5% CO₂ and 95% air. Cell lines were plated in 96-well polystyrene flat-bottom plates (7,500 cells/well) at 70% confluency in a 100 µl total volume of supplemented media as indicated, and incubated overnight at 37°C to allow for adherence. The media was then replaced with serum-free media and the cultures were treated with escalating amounts of drug and cell death was analyzed at 18h, using the BioTek Synergy 2 Multidetector Microplate Reader (BioTek Instruments, Inc.). The percentage of viable cells present in the culture at each time point was calculated by comparing the absorbance value at 450nm from the CCK-8 assay (Dojindo Molecular Technologies) for each condition with untreated control cells. All assays were conducted per manufacturer's protocol. All described values represent the average of three data points per determination and three independent determinations.

3.8.3 Cytotoxicity Assays with Pertussis Toxin

U-87 MG cells were seeded in 96-well polystyrene flat-bottom plates (7,500 cells/well) at 70% confluency in a 100 µl total volume of complete growth media and incubated overnight at 37°C in an atmosphere of 5% CO₂ and 95% air to allow for adherence. The media was then replaced with complete growth media containing 0 or 100ng/ml pertussis toxin and the plates returned to the incubator for 18 hours. Drug solutions were prepared in DMSO at 100X concentration, and mixed 1:100 in media containing 1% FBS to yield the desired concentration. After 18 hour incubation with or without Ptx, full serum media was removed and replaced with drug-media mixtures and incubated for an additional 18 hours. 10µL of CCK-8 reagent was added to each well to colorimetrically assess viability. After 2-4 hours of incubation with the CCK-8 dye, absorbance was read at 450nm using a BioTek Synergy 2 plate reader.

3.8.4 CB1/CB2 Functional Assays

HEK-293 cell lines stably transfected with a cyclic nucleotide-gated channel and either human CB1 or CB2 receptors (BD Biosciences, San Jose, CA USA) were seeded in poly-D-lysine coated 96-well plates at a density of 70,000 cells per well. Plates were incubated at 37°C in 5% CO₂ overnight prior to assay. Plates were then removed from the incubator and the complete growth medium (DMEM, 10% FBS, 250µg/ml G418 and 1µg/ml puromycin) was replaced with 100µL DMEM containing 0.25% BSA. Next,

100µL membrane potential dye loading buffer (Molecular Devices) was added to each well. The plates were placed back into the incubator for 30 minutes and then the baseline fluorescence was read on a BioTek Synergy 2 multi-mode microplate reader with 540nm excitation and 590nm emission filters prior to drug addition. Drugs were added in 50µL DPBS containing 2.5% DMSO, 1.25µM 5'-(N-ethylcarboxamido) adenosine and 125µM Ro 20-1724. Plates were then incubated at room temperature for 25 minutes and fluorescence measured again at 540nm excitation and 590nm emission.

3.8.5 MSD Protein Quantification

U-87 MG cells were plated in poly-D-lysine coated 10 cm Petri dishes at a density of 4×10^5 cells/ml with 12ml of cell suspension added to each plate. The cells were grown overnight in EMEM media containing 10% FBS. The following day the media was changed to EMEM media containing 1% FBS and allowed to equilibrate for 24 hours. This step is essential to allow basal levels of signaling protein to re-equilibrate due to changes in the serum concentration. The media was then replaced at the appropriate time points with media containing 1% serum and KM-233 at the EC₅₀ or media containing 1% serum and vehicle (DMSO). After the incubation period with KM-233 or vehicle, the media was removed from the dishes and transferred to a 15ml centrifuge tube to collect any cells that were in suspension. Cells were then scraped from the surface of the dish and transferred into the same 15ml centrifuge tubes and pelleted at 1000rpm, 4°C for 5min. Supernatant was removed and 0.5ml lysis buffer was added to each pellet. The cells were suspended in the buffer and transferred to 1.5ml microcentrifuge tubes. These were incubated on ice for 30 minutes, being vortexed every 5 minutes. The lysates were then centrifuged at 10,000 x g, 4°C for 10 minutes to clear cellular debris from the lysate. The supernatant was removed and assayed for protein concentration using a Pierce BCA Protein Assay Kit (Thermo Scientific). The lysates were then diluted with complete lysis buffer to a concentration of 20µg of protein in 25µl volume. Signaling proteins were then analyzed using MSD phosphoprotein and intracellular marker multiplex kits which were imaged on a SECTOR Imager 2400 (Meso Scale Discovery) according to the manufacturer's instructions.

3.8.6 Caspase 9 Assay

U-87 MG cells were seeded in white, opaque 96-well culture plates at a density of 10,000 cells/well in a volume of 100µl complete growth media. The cells were allowed to attach overnight and the complete growth media was changed to media containing 1% serum for 24 hours to equilibrate intracellular signaling pathways, then media was changed to either media containing 1% serum or media containing 1% serum plus HB-I-172. At the appropriate time intervals 100µl of Caspase-Glo® 9 Assay Reagent (Promega, prepared according to manufacturer's instructions) was added to each well and the plate mixed on a rotary shaker for 30 seconds. After incubation at room temperature for 90 minutes the luminescence was read on a Biotek Synergy 2 plate reader. The

luminescent signal is proportional to the amount of caspase 9 activity present. All data points represent 6 independent determinations.

3.8.7 Mitochondrial Membrane Potential Assay

U-87 MG cells were seeded in poly-D-lysine coated 96-well plates at a density of 10,000 cells per well. At the appropriate time points, the media was removed and replaced with media containing 1% FBS and either HB-I-172 at the EC₅₀ or vehicle (DMSO). After incubation with drug or vehicle, cells were dyed using DePsipher membrane potential dye according to the manufacturer's instructions. Plates were then imaged using an Olympus IX71 inverted fluorescence microscope (Olympus America) fitted with a Retiga-SRV camera (QImaging).

3.8.8 Microscopic Analysis Using Immunohistochemical Techniques

U-87 MG cells were plated in complete growth medium at a density of 15,000 cells/well in poly-D-lysine coated 96-well polystyrene tissue culture plates and allowed to attach over night at 37°C in an atmosphere of 5% CO₂ / 95% air. The media was then replaced with media containing 1% FBS and either HB-I-172 at the EC₅₀ or vehicle (DMSO). The cells were returned to the incubator and after the appropriate periods of exposure to the drug or vehicle, media was aspirated and the cells fixed using 4% paraformaldehyde. Cells were then stained by immunohistochemical methods using either a Cellomics Multiparameter Cell Death Detection Kit, Cellomics Cytoskeletal Rearrangement Kit, or Cellomics Cleaved PARP Activation Kit (Thermo Scientific) according to the manufacturer's instructions. Plates were then imaged using an Olympus IX71 inverted fluorescence microscope (Olympus America) fitted with a Retiga-SRV camera (QImaging).

Chapter 4: Design, Synthesis, and Testing of Novel Cannabinoid Ligands

4.1 Introduction

Classical cannabinoids are tricyclic terpenoid compounds consisting of a benzopyran core structure that are either naturally occurring in the hemp plant (phytocannabinoids) or are synthetic analogs of naturally occurring compounds. Δ^9 -THC and its isomer Δ^8 -THC (**Figure 4.1**) are the prototypical members of this class of cannabinoids. They bind without selectivity to the CB1 and CB2 cannabinoid receptors and act as agonists or partial agonists.¹¹ The classical cannabinoids are by far the most well studied of the cannabinoid compounds in terms of pharmacology and structure activity relationships (SAR) and have gained considerable attention over the years due to their potential indication in the treatment of several pathological conditions from inflammation to asthma, inflammatory bowel diseases, multiple sclerosis, Parkinson's disease, epilepsy, glaucoma, septic shock, hemorrhagic shock, and cancer.

SAR studies have determined that there are three pharmacophoric elements (**Figure 4.1**) in classical cannabinoids that are required for cannabinimimetic activity. These include:

- a. C-1 phenolic hydroxyl group
- b. lipophilic C-3 alkyl side chain
- c. trans ring junction at the B and C rings.

Many studies have been performed in an attempt to elucidate the SAR of the classical cannabinoids with respect to modifications of the tricyclic ring and side chain substituents at the C-3 position. Some of the different side chain modifications that have been reported thus far include the branched chain alkyls,¹⁵³ unsaturated alkyls,^{130,154} and alkyls that contain a 1',1'-cyclic functionality.¹⁵⁵ Each of these modifications has been directed at the goal of producing compounds that have higher binding affinities for the cannabinoid receptors and also that are selective for one subtype over the other.

The potential for important therapeutic interventions from this class of compounds has provided the impetus to develop ligands that can elicit the desired physiological effects of Δ^9 -THC (mediated through CB2) without producing the unwanted CNS side effects. In the absence of X-ray crystal structures of cannabinoid receptors, medicinal chemists have relied on chemical diversifications to develop new ligands for CB1 and CB2. Structurally diverse compounds that probe the available space in the ligand binding pocket of the cannabinoid receptors have provided important insights into the structural requirements for binding as well as for activity.

This need for diversity in the cannabinoid chemical arsenal has led to the development of compounds belonging to diverse chemical classes, both classical and non-classical. The term "non-classical" was originally used to describe compounds that

Figure 4.1: Structure of Δ^8 -THC with identification of its various structural domains

lack the B ring of the classical cannabinoids and possess an alkyl side chain such as the compound CP-55,940 (**Figure 4.2**). The term is now more widely used to describe any compound which is a ligand that binds the cannabinoid receptors which lacks the basic tricyclic benzopyran ring system. An explosion of work into the non-classical arena has led to several new structural classes of cannabinoids. These new compounds can be classified broadly into 9 distinct chemical scaffolds (**Figures 4.2 and 4.3**):

- a. Non-classical cannabinoids – exemplified by the CP-55,940 group of compounds.¹⁵⁶
- b. Aminoalkyl indoles – exemplified by the WIN 55,212-2 group of compounds.¹⁵⁷
- c. Biaryl pyrazoles – exemplified by SR14716A group of compounds.¹⁵⁸
- d. Biphenyls – exemplified by the biaryl-gem-dimethyl heptyl group of compounds.¹²⁸
- e. Triaryls – exemplified by HB-I-172.¹²⁷
- f. N-alkyl isatin acylhydrazones exemplified by MDA77.¹⁵⁹
- g. Benzimidazoles as exemplified by PF-03550096.¹⁶⁰
- h. N-arylamide oxadiazoles.¹⁶¹
- i. Quinolone-3 carboxamides.¹⁶²
- j. Triaryl bis-sulfones.¹⁶³

These compounds act via the CB1 or CB2 receptors (and possibly GPR55) and are agonists, antagonists, inverse agonists, or partial agonist/antagonists. They are collectively known as “cannabimimetic” agents, “cannabinergic” agents, or simply “cannabinoids.” These structural classes of ligands display varying affinities as well as selectivity for the cannabinoid receptor subtypes, though most of the recent work has been in improving CB2 selectivity. Although all of these cannabinoids essentially bind to the same receptors and activate or deactivate the same downstream signaling cascade, it is not surprising that there is increasing evidence to suggest that they display different binding modes. It is believed that each structural class interacts with different critical amino acids in the binding pocket of each receptor. This may explain why the differing chemical classes display very different selectivities for CB1/CB2. This growing volume of knowledge can be exploited for the further tailoring of compounds that have specific binding affinity profiles.

The reported SAR studies of cannabinoid receptor ligands indicate that there are important amino acid residues in the transmembrane domains 3, 5, and 6 of the CB1 and the CB2 receptors that are involved in the discrimination of the cannabinergic ligands.¹⁶⁴ Molecular modeling studies have also suggested the importance of specific amino acid residues in the CB1 receptor binding pocket that play a critical role in binding to classical cannabinoids (**Figure 4.4**). These residues are as follows:

Figure 4.2: Representatives of various classes of non-classical cannabinoids

Figure 4.3: Further representatives of various classes of non-classical cannabinoids

Figure 4.4: Classical cannabinoid binding interactions

Interactions between classical cannabinoids and key amino acid residues in the ligand binding pocket of the cannabinoid receptors.

- a. Lys 192 is a very important residue required for binding to classical cannabinoids. Due to the conformational flexibility of this residue, it can adopt an orientation that interacts with the phenolic hydroxyl in the A ring of classical cannabinoids.¹⁶⁵
- b. A predominantly hydrophobic pocket comprised of residues Tyr 275, Trp 279, Leu 359, Met 363, and Phe 200 is involved in binding to the C-3 side chain.¹⁶⁶
- c. Phe 189 lies in close proximity to the A ring of classical cannabinoids and may be involved in aromatic stacking interactions with the ring.¹⁶⁶

While there is a sufficient understanding of binding interactions involved between ligands and the CB1 receptor, interactions at the CB2 receptor are not as well defined. It has been demonstrated that compounds lacking the phenolic hydroxyl of the A ring or having it occupied in an ether linkage, show enhanced affinity for CB2 as well as selectivity.¹⁶⁷ The observations made with these modifications indicate the involvement of additional specific electronic and steric factors in ligand recognition at the CB2 receptor. It is also important to note that elimination of the B ring of the classical cannabinoid core with the biphenyl or triaryl core has resulted in compounds that are more CB2 selective also.¹²⁷⁻¹²⁸ Work in our laboratory has also demonstrated that classical cannabinoids with phenyl substitutions at the C3 position have affinities shifted toward CB2 selectivity.¹⁰⁸

4.2 Design and Synthesis of Novel Compounds

4.2.1 Rationale

Upon careful review of past and current literature, it became clear that there had been one major design possibility that had been overlooked. In the efforts to design compounds that were more CB2 selective, no group had reported heteroaromatic modifications to the A ring of classical cannabinoids. Replacement of the phenyl ring with a pyrimidine could take advantage of the additional electronic interactions that are involved in ligand recognition at the CB2 receptor. A logical extension of this design was the addition of the heteroaromatic motif to the triaryl scaffold as well (**Figure 4.5**). It was also speculated that the addition of the pyrimidine to these cannabinoid structures could increase water solubility thereby making it easier to formulate these compounds into dosage forms. The decrease in lipophilicity could also limit the blood-brain barrier penetration of the cannabinoids which would make them ideal candidates as therapies for systemic inflammatory conditions.

Figure 4.5: General design of pyrimidyl cannabinoids

Rationale for the pyrimidine heteroaromatic substitution in the classical cannabinoids and the triaryls.

4.2.2 Synthesis of Hexahydro Pyrimidine Classical Cannabinoids

The hexahydro pyrimidine classical cannabinoids were prepared by reacting an intermediate pyrimidine with the appropriate terpene to generate the desired compounds according to the **Scheme 4.1**. The hexahydro compounds are synthesized utilizing a modification of the Diastereoselective Domino Knoevenagel Hetero-Diels-Alder Reaction described by Tietz.¹⁶⁸ The ring formations are accomplished under microwave conditions with the appropriately substituted pyrimidine and citronellal.

Synthesis of the intermediate substituted pyrimidines proved to be more challenging. The first strategy was to form the hexahydro core with commercially available 4,6 dihydroxy pyrimidine which lacked the side chain (**Scheme 4.2**). The phenolic -OH would then be protected by a tert-butyldimethylsilyl (TBDMS) group. The addition of the side chain could then be accomplished by deprotonation of the 2 position of the pyrimidine and then reacting with an appropriate aldehyde to form the intermediate alcohol or lithiation of the same position by butyl lithium and N,N-dimethylaminoethanol and then reacting with the aldehyde to form the alcohol or a nitrile to form the ketone (**Scheme 4.3**). Neither of these methods was successful in formation of the side chain. An attempt to prime the 2 position of the pyrimidine as a Grignard substrate was also performed by reacting the lithiate with dimethylformamide to form an aldehyde in this position which could then be reacted with the appropriate magnesium halides (**Scheme 4.3**). This method was also unsuccessful.

The second strategy involved the synthesis of ethyl 4,6-dimethoxypyrimidine-2-carboxylate from malononitrile, methanol, and ethyl chloroacetate (**Scheme 4.4**).¹⁶⁹ This intermediate would then serve as the scaffold for building the side chain. The ester was then reduced to the aldehyde using diisobutylaluminum hydride (DIBAL-H) to prepare the molecule for the Grignard reaction (**Scheme 4.5**). The aldehyde was then reacted with an appropriate aldehyde and the resulting alcohol oxidized to the ketone using pyridinium chlorochromate (PCC) (**Scheme 4.5**). The ketone could then be dimethylated using tin chloride and dimethyl zinc according to the method employed by Krishnamurthy.¹⁰⁸ This method, however led to the monomethyl alcohol as did methylation using trimethyl aluminum (**Scheme 4.6**). The same product was also formed when trimethyl aluminum was paired with trimethylsilyl trifluoromethanesulfonate in an attempt to make the alcohol a better leaving group to allow for the formation of the dimethyl linker¹⁷⁰ (**Scheme 4.7**). An attempt to activate the alcohol was also made by converting it to the mesylate;¹⁷¹ this however led to the vinyl elimination product (**Scheme 4.7**).

It became clear that forming the dimethyl linker in the side chain would not be feasible using 4,6-dimethoxypyrimidine-2-carboxylate as the starting material. This would work, however, for the hydroxyl and ketone linker. Unfortunately, another problem arose. In order to form the hexahydro classical cannabinoids, citronellal must be reacted with the deprotected pyrimidine, not the dimethoxy intermediate. The differences between dimethoxy phenyl rings and pyrimidine rings caused difficulties here also. Previous methods employed by our laboratory to cleave the methyl ethers such as boron tribromide^{108,129} were unsuccessful in the deprotection of the dimethoxy pyrimidines.

Scheme 4.1: Synthesis of hexahydro pyrimidine classical cannabinoids

Hexahydro pyrimidine classical cannabinoids are synthesized from R-citronellal and a substituted dihydroxy pyrimidine. (a) piperidine, pyrimidine, EtOH, μ wave, 200 watts, 130°C , 1hr.

Scheme 4.2: Synthesis of TBDMS protected hexahydro pyrimidine classical cannabinoids

TBDMS protected hexahydro pyrimidine classical cannabinoids were synthesized from R-citronellal and substituted dihydroxy pyrimidine. (a) piperidine, pyrimidine, EtOH, μ wave, 200 watts, 130°C, 1hr. (b) TBDMS-Cl, imidazole, CH₃CN, μ wave, 150 watts, 140°C, 2hr.

Scheme 4.3: Attempts at introduction of the hexahydro pyrimidine side chain

Failed attempts at introduction of the hexahydro pyrimidine side chain to protected hexahydro pyrimidine classical cannabinoids. (a) n-butyllithium, THF, -78°C - RT, 18hr. (b) n-butyllithium, N,N dimethylaminoethanol, hexanes, THF, -78°C - RT, 18hr.

Scheme 4.4: Synthesis of 4,6-dimethoxypyrimidine-2-carboxylate

(a) methanol, HCl (gas), methyl acetate, -18°C , 3hr, cooled to 5°C . (b) DIEA, CH_2Cl_2 , 40°C - RT, 18hr.

Scheme 4.5: Introduction of the side chain to 4,6-dimethoxypyrimidine-2-carboxylate

(a) DIBAL-H, toluene, -78°C - 25min, HCl 0°C. (b) Phenyl MgBr, THF, 0°C – 4hr, RT - 18hr. (c) PCC, Celite, CH₂Cl₂, RT, 18hr.

Scheme 4.6: Attempts at dimethylation of the ketone intermediate

(a) TiCl_4 , $\text{Zn}(\text{CH}_3)_2$, CH_2Cl_2 , -40°C – 2hr, RT – 16hr. (b) Me_3Al , CH_2Cl_2 , 0°C – 1hr, RT – 16hr.

Scheme 4.7: Further attempts at dimethylation of the ketone intermediate

Me_3Al , $\text{TMSOSO}_2\text{CF}_3$, CH_2Cl_2 , 0°C – 1hr, RT – 16hr. (b) NaH , THF, 0°C – RT, 1hr, 40°C – 3hr, methane sulfonyl chloride, RT – 1hr, 40°C - 2hr.

Other methods were also employed: KOH, HBr, and methylmagnesium iodide. None of these proved successful (**Scheme 4.8**).

This combination of difficulties led to the abandonment of methods that had previously been employed successfully in our laboratory for synthesis of classical cannabinoid side chains. It was decided to form the side chain first which would contain a cyano group. This cyano group would be converted to the amidine which would be reacted with diethyl malonate to form the dihydroxy pyrimidine with the side chain in place.

The nitriles were formed either by reaction of a commercially available aryl or alkyl halide with isobutyronitrile in the presence of potassium bis(trimethylsilyl)amide (KHMDS) (**Scheme 4.9**)¹⁷² or by dimethylation of a commercially available aryl or alkyl nitrile with methyl iodide in the presence of KHMDS (**Scheme 4.10**).¹⁷³ The amidines are then formed from the nitriles by Garigipati's reaction utilizing methylchloroaluminum amide formed *in situ* from trimethylaluminum and ammonium chloride (**Scheme 4.11**).¹⁷⁴ This method, which only provided moderate yields of around 40%, was chosen due to the known unreactivity of hindered nitriles in the more common Pinner reaction. The limited availability of anhydrous ammonia at that time also made the Pinner reaction less feasible. One major drawback of this method is that it requires an extensive two day purification procedure to separate the amidines from residual ammonium chloride.

The amidines were to then be cyclized into dihydroxy pyrimidines by reacting with diethyl malonate. This reaction was first attempted in the presence of N,N-diisopropylethylamine (DIEA). This method did not lead to formation of the desired product and it was speculated that perhaps DIEA was not a strong enough base to drive the reaction forward. Sodium ethoxide was chosen as the base and commercially available solid sodium ethoxide was purchased and tried in the reaction. This also did not lead to formation of the product. The reaction was also attempted under solvent-free conditions and also under microwave irradiation. Each of these efforts remained unfruitful. Successful formation of the pyrimidines was finally achieved by *in situ* formation of fresh sodium ethoxide to deprotonate the amidines (**Scheme 4.12**).¹⁷⁵ Diethyl malonate was then added and the reactions heated to reflux overnight. The solid sodium chloride was then filtered out and the filtrate was diluted with water and acidified to pH 2 causing the dihydroxy pyrimidines to precipitate.

The pyrimidine hexahydro classical cannabinoids were then formed by reaction of the appropriately substituted dihydroxy pyrimidines with R-citronellal under microwave conditions (**Scheme 4.13**). This Domino Knoevenagel Hetero-Diels-Alder method uses ethanol as the solvent and piperidine and pyridine as co-solvents and as the base to catalyze the coupling. The final compounds are easily purified using flash chromatography and are isolated in good yields of ~80%. The side chains selected for these novel structures were the dimethyl heptyl, dimethyl cyclohexyl, dimethyl phenyl, and the dimethyl 2-thiophene. These selections were made based on the known binding affinity afforded to classical cannabinoids by the linear dimethyl heptyl and by our groups findings that the cyclohexyl has similar binding affinities to the linear alkyl and that aryl groups such as the phenyl and thiophenyl shift selectivity toward the CB2 receptor.^{153a}

Scheme 4.8: Attempts at deprotection of the dimethoxy pyrimidine intermediates

(a) BBr_3 , CH_2Cl_2 , -78°C – 3hr, RT – 16hr. (b) HBr-AcOH (1:3), 80°C , 1h, then saturated NaHCO_3 . (c) MeMgI , 1,4 dioxane, reflux, 16hr. (d) KOH , 1,4 dioxane, reflux, 16hr.

Scheme 4.9: Formation of nitrile intermediates from halides and isobutyronitrile

(a) KHMDS, toluene, 80°C, 24hr.

Scheme 4.10: Formation of nitrile intermediates from cyanides

(a) KHMDS, MeI, THF, 0°C, 10min.

Scheme 4.11: Formation of amidines from nitriles

(a) NH_4Cl , Me_3Al , toluene, 0°C - RT, 45min, reflux, 18hr, then silica gel, CHCl_3 , RT, 10min, then methanolic HCl , diethyl ether, RT, overnight, then isopropanol:acetone 4:1, RT, overnight, then diethyl ether, RT.

Scheme 4.12: Synthesis of dihydroxy pyrimidine intermediates

(a) Na metal, diethyl malonate, EtOH, reflux, 18hr.

Scheme 4.13: Coupling of pyrimidine hexahydro classical cannabinoids

(a) piperidine, pyrimidine, EtOH, μ wave, 200 watts, 130°C, 1hr.

All chemicals were obtained from Sigma Aldrich or Fisher Scientific Inc. Anhydrous solvents were obtained by distillation over either calcium hydride or metallic sodium and benzophenone. Final compounds and intermediates were purified using column chromatography on the Biotage SP1 system employing Flash column cartridges or by crystallization. NMR spectra were obtained on a Bruker 300MHz or Varian 500MHz Inova NMR. HPLC analysis of final products was carried out by gradient elution using water/acetonitrile (0.1% TFA) with a gradient of 70% (3min), 50% (2min), and 10% (15min). A reverse-phase ODS Hypersil column, manufactured by Thermo Scientific, of dimensions 4.6 x 150mm with 5 μ m particle size was used for HPLC analyses.

4.2.2.1 Synthesis of 2-methyl-2-phenylpropanenitrile (22)

To a solution of fluorobenzene (5.85mL, 62.4mmol) in 100 mL of anhydrous toluene was added isobutyronitrile (22.5mL, 250mmol) followed by 200mL (100mmol) of a 0.5M solution of KHMDS in toluene. The reaction was stirred at 80°C for 24 hours. The reaction was then allowed to cool to room temperature, diluted with diethyl ether, and washed with water and brine. The organic fraction was then dried over sodium sulfate and concentrated under reduced pressure. The product was purified by flash chromatography using an ethyl acetate/hexanes gradient to yield 4.57g (50%) of the objective compound as a brown oil. MS: (ESI, pos.) m/z 168.0 $[M + Na]^+$, I.R. (neat) nitrile 2230 cm^{-1} , ^1H NMR (500 MHz, CDCl_3): δ (ppm) 7.48 (d, 2H), 7.39 (t, 2H), 7.31 (t, 1H), 1.73 (s, 6H).

4.2.2.2 Synthesis of 2-cyclohexyl-2-methylpropanenitrile (23)

Prepared in a manner similar to compound **22** from bromo cyclohexane (0.8 ml, 6.4 mmol) and isobutyronitrile (2.34ml, 26mmol) in the presence of KHMDS (20ml, 10mmol). Purified via vacuum distillation (Bp 50-55°C at 1.1 torr). Yield: 84%, MS: (ESI, pos.) m/z 174.1 $[M + Na]^+$, I.R. (neat) nitrile 2230 cm^{-1} , ^1H NMR (500 MHz, CDCl_3): δ (ppm) 1.5 (m, 4H) 1.4-1.3 (m, 12H), 0.9 (s, 3H).

4.2.2.3 Synthesis of 2-methyl-2-(thiophen-2-yl)propanenitrile (24)

To a solution of 2-(thiophen-2-yl) acetonitrile (1g, 8.13mmol) in 4ml anhydrous THF, KHMDS (24.4mmol, 48.9ml, 0.5M in toluene) was added at 0°C. The mixture was allowed to stir for 3 minutes, after which a solution of 16.26mmol iodomethane (1.13ml in 26ml anhydrous THF) was added slowly over a period of 10 minutes. The mixture was stirred for 5 minutes and monitored by TLC. Upon completion, the reaction was quenched with aqueous ammonium chloride. The organic phase was separated with ethyl acetate and dried over sodium sulfate. The product was purified via vacuum distillation. (bp 42°C at 1 torr) Yield: 89%, MS: (ESI, pos.) m/z 174.1 $[M + Na]^+$, I.R. (neat) nitrile

2230 cm^{-1} , ^1H NMR (500 MHz, CDCl_3): δ (ppm) 7.4 ppm (d, 1H), 7.2 ppm (t, 1H), 7.0 ppm (d, 1H), 1.9 ppm (s, 6H).

4.2.2.4 Synthesis of 2,2-dimethyloctanenitrile (25)

Prepared in a manner similar to compound **24** from octanenitrile and iodomethane in the presence of KHMDS. Purified via vacuum distillation (bp 50-55°C at 1.1 torr). Yield: 84%, MS: (ESI, pos.) m/z 176.2 $[\text{M} + \text{Na}]^+$, I.R. (neat) nitrile 2230 cm^{-1} , ^1H NMR (500 MHz, CDCl_3): δ (ppm) 1.5 (m, 4H), 1.4-1.3 (m, 12H), 0.9 (s, 3H).

4.2.2.5 Synthesis of 2-methyl-2-phenylpropanimidamide hydrochloride (26)

Ammonium chloride (1.06 g, 19.8mmol) was suspended in 10 ml of anhydrous toluene, stirred under argon, and cooled to 0°C. To this suspension was added 9.2ml (18.4 mmol) of a 2M solution of trimethylaluminum in toluene. The reaction was allowed to warm to room temperature and stirring was continued until the evolution of methane ceased (~1 hour). Then, 1.6g (11mmol) of nitrile **22** in 5ml of anhydrous toluene was added and the reaction was heated to 80°C for 18 hours. The reaction was then cooled to room temperature and slowly poured into a slurry of 5.5g of silica gel in 18ml of chloroform and stirred for 5 minutes. The silica was filtered and washed with methanol. The filtrate and washings were combined and reduced to a volume of ~5ml and refiltered. Then, 4ml (12mmol) of a 3N solution of methanolic HCl was added to the filtrate followed by 300ml of diethyl ether. After 16 hours of stirring at room temperature, a white precipitate formed was subsequently filtered. This crude solid was then added to 150ml of a 4:1 mixture of isopropanol-acetone and stirred for an additional 16 hours at room temperature. Undissolved ammonium chloride was then removed by filtration and the filtrate reduced to ~5ml. After the addition of 300ml of diethyl ether the mixture was stirred for ~1 hour. The white precipitate was then filtered and dried to yield 639mg (29%) of the objective compound as the hydrochloride salt. MS: (ESI, pos.) m/z 163.0 $[\text{M} + \text{H}]^+$, ^1H NMR (500 MHz, DMSO-d_6): δ (ppm) 9.27 (br.s, 2H), 8.71 (br.s, 2H), 7.41 (m, 2H), 7.36 (m, 2H), 7.32 (m, 1H), 1.60 (s, 6H) ^{13}C NMR (500 MHz, DMSO-d_6): δ (ppm) 175.91, 143.27, 128.71, 127.42, 125.65, 44.06, 26.31.

4.2.2.6 Synthesis of 2-methyl-2-(thiophen-2-yl)propanimidamide hydrochloride (27)

Prepared in a manner similar to compound **26** from nitrile **24** (1.5g, 9.9mmol), ammonium chloride (952mg, 17.8mmol), and trimethylaluminum (8.25ml, 16.5mmol). Yield: 36% (white solid) MS: (ESI, pos.) m/z 169.0 $[\text{M} + \text{H}]^+$, ^1H NMR (500 MHz, DMSO-d_6): δ (ppm) 8.8 (br.d, 3H), 7.53 (dd, 1H), 7.13 (dd, 1H), 7.04 (dd, 1H), 1.70 (s, 1H) ^{13}C NMR (500 MHz, DMSO-d_6): δ (ppm) 174.75, 147.04, 127.18, 125.72, 125.48, 41.92, 27.20.

4.2.2.7 Synthesis of 2-cyclohexyl-2-methylpropanimidamide hydrochloride (28)

Prepared in a manner similar to compound **26** from nitrile **23** (6g, 40mmol), ammonium chloride (3.92g, 72mmol), and trimethylaluminum (33.3ml, 66.6mmol). An exception to the procedure is that after the addition of methanolic HCL and diethyl ether, the solid that formed was ammonium chloride and the amidine remained in solution. The solid was removed and the solvent evaporated to yield the product as a highly viscous yellow resin. Yield: 70%. MS: (ESI, pos.) m/z 169.1 $[M + H]^+$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 8.84 (b.d., 3H), 1.78 (m, 3H), 1.57 (m, 3H), 1.25 (s, 2H), 1.19 (t, 1H), 1.12 (s, 6H), 0.97 (m, 2H). ^{13}C NMR (500 MHz, DMSO- d_6): δ (ppm) 177.11, 44.74, 42.90, 27.47, 26.73, 26.15, 25.80, 25.73, 23.86, 21.29.

4.2.2.8 Synthesis of 2,2-dimethyloctanimidamide hydrochloride (29)

Prepared in a manner similar to compound **28** from nitrile **25** (1.5g, 9.8mmol), ammonium chloride (957mg, 17.6mmol), and trimethylaluminum (8.15ml, 16.3mmol). Product is a highly viscous yellow resin. Yield: 60.7%. MS: (ESI, pos.) m/z 171.1 $[M + H]^+$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 8.87 (b.d., 4H), 1.56 (t, 2H), 1.3-1.0 (m, 14H), 0.85 (t, 3H). ^{13}C NMR (500 MHz, DMSO- d_6): δ (ppm) 176.63, 39.97, 39.62, 31.02, 28.87, 24.80, 23.72, 22.02, 13.92.

4.2.2.9 Synthesis of 2-(2-phenylpropan-2-yl)pyrimidine-4,6-diol (30)

Sodium metal (145mg, 6.29mmol) was reacted with 8ml of anhydrous ethanol. To this was added 500mg (2.52mmol) of amidines hydrochloride **26** and the reaction was allowed to stir at room temperature for 5 minutes. Diethyl malonate (400mg, 2.5mmol) was then added and the reaction was heated to reflux overnight. The reaction was then cooled to room temperature and filtered. The solid was washed several times with absolute ethanol. The filtrate and washings were combined and diluted with 2 volumes of water and acidified with 5N HCl to a pH of ~2 and the product precipitated. The mixture was chilled and then filtered. The solid was washed several times with diethyl ether and then dried to yield 218mg (38%) of an off-white solid. MS: (ESI, neg.) m/z 228.9 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.35 (br.s, 2H), 7.33 (t, 2H), 7.23 (m, 3H), 5.19 (s, 1H), 1.62 (s, 6H)

4.2.2.10 Synthesis of 2-(2-(thiophen-2-yl)propan-2-yl)pyrimidine-4,6-diol (31)

Prepared in a manner similar to compound **30** from sodium metal (140mg, 6.11mmol), amidine hydrochloride **27** (500mg, 2.44mmol), and diethyl malonate (388mg, 2.42mmol). Product is an off-white solid. Yield: 185mg (32%). MS: (ESI,

neg.) m/z 235.1 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.39 (br.s, 2H), 7.41 (d, 1H), 6.99 (d, 1H), 6.97 (t, 1H), 5.2 (s, 1H), 1.72 (s, 6H).

4.2.2.11 Synthesis of 2-(2-cyclohexylpropan-2-yl)pyrimidine-4,6-diol (32)

Prepared in a manner similar to compound **30** from sodium metal (140mg, 6.11mmol), amidine hydrochloride **28** (500mg, 2.44mmol), and diethyl malonate (509mg, 3.18mmol). Product is an off-white solid. Yield: 185mg (32%). MS: (ESI, neg.) m/z 235.1 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.41 (br.s, 2H), 5.12 (s, 1H), 1.80 (t, 1H), 1.69 (d, 2H), 1.38 (s, 1H), 1.72 (d, 2H), 1.19 – 1.02 (m, 4H), 1.15 (s, 6H), 0.94 (dd, 2H).

4.2.2.12 Synthesis of 2-(2-methyloctan-2-yl)pyrimidine-4,6-diol (33)

Prepared in a manner similar to compound **30** from sodium metal (111mg, 4.84mmol), amidine hydrochloride **29** (400mg, 1.93mmol), and diethyl malonate (309mg, 1.93mmol). Product is a light peach colored solid. Yield: 39% MS: (ESI, neg.) m/z 236.8 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.33 (br.s, 2H), 5.13 (s, 1H), 1.62 (m, 2H), 1.22 (s, 6H), 1.20 (m, 6H), 1.04 (m, 2H), 0.83 (t, 3H).

4.2.2.13 Synthesis of (6aS,9R,10aR)-6,6,9-trimethyl-3-(2-phenylpropan-2-yl)-6a,7,8,9,10,10a-hexahydro-6H-isochromeno[3,4-d]pyrimidin-1-ol (34)

Dihydroxy pyrimidine **30** (100 mg, 0.434 mmol) was added to 3ml of absolute ethanol in a 10ml microwave reaction vessel. To this was also added 66 μ l of pyridine and 3 μ l of piperidine followed by (R)-(+)-citronellal (201mg, 235 μ l, 1.3mmol). The reaction vessel was then sealed and irradiated at 200 watts to 130°C for 1 hour. The reaction mixture was then concentrated and the product purified by flash chromatography using a methanol/methylene chloride gradient to yield 180 mg (69%) of the objective product as an off-white solid. MS: (ESI, neg.) m/z 365.0 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ 11.43 (s, 1H), 7.32 (q, 2H, $J = 7.5$ Hz), 7.21 (m, 3H), 3.06 (d, 1H, $J = 12.5$ Hz), 2.16 (td, 1H, $J = 11$ Hz and 2.5Hz), 1.76 (m, 2H), 1.56 (m, 11H), 1.37 (s, 3H), 1.01 (m, 2H), 0.87 (d, 3H, $J = 6.5$ Hz), 0.48 (q, 1H, $J = 12$ Hz). ^{13}C NMR (500 MHz, DMSO- d_6): δ (ppm) 163.44, 163.23, 145.75, 128.27, 128.23, 126.46, 126.06, 125.86, 99.47, 79.98, 47.47, 44.48, 36.86, 34.83, 33.44, 31.81, 27.19, 27.05, 26.90, 22.40, 19.46. HPLC retention time: 10.631min; purity 100%.

4.2.2.14 Synthesis of (6a*S*,9*R*,10a*R*)-6,6,9-trimethyl-3-(2-(thiophen-2-yl)propan-2-yl)-6a,7,8,9,10,10a-hexahydro-6*H*-isochromeno[3,4-*d*]pyrimidin-1-ol (35)

Prepared in a manner similar to compound **34** from dihydroxy pyrimidine **31** (23mg, 0.097mmol) and (R)-(+)-citronellal (45mg, 53μl, 0.292mmol). Yield: 46% MS: (ESI, neg.) *m/z* 371.0 [*M* – *H*][–], ¹H NMR (500 MHz, DMSO-*d*₆): δ 11.52 (s, 1H), 7.40 (d, 1H, *J* = 4.5Hz), 6.97 (m, 2H), 3.06 (d, 1H, *J* = 12Hz), 2.16 (td, 1H, *J* = 11.5Hz and 2Hz), 1.70 (m, 8H), 1.50 (m, 2H), 1.36 (s, 3H), 1.06 (m, 5H), 0.87 (d, 3H, *J* = 6.5Hz), 0.47 (q, 1H, *J* = 12Hz). ¹³C NMR (500MHz, DMSO-*d*₆): δ (ppm) 163.46, 163.07, 150.12, 126.70, 126.64, 124.86, 124.39, 99.63, 80.07, 47.42, 42.72, 36.79, 34.80, 33.42, 31.80, 28.08, 28.00, 27.02, 26.87, 22.40, 19.43. HPLC retention time: 10.580min; purity 98.04%.

4.2.2.15 Synthesis of (6a*S*,9*R*,10a*R*)-3-(2-cyclohexylpropan-2-yl)-6,6,9-trimethyl-6a,7,8,9,10,10a-hexahydro-6*H*-isochromeno[3,4-*d*]pyrimidin-1-ol (36)

Prepared in a manner similar to compound **34** from dihydroxy pyrimidine **32** (100mg, 0.42mmol) and (R)-(+)-citronellal (194mg, 228μl, 1.26mmol). Yield: 36% MS: (ESI, neg.) *m/z* 371.0 [*M* – *H*][–], ¹H NMR (500 MHz, DMSO-*d*₆): δ 11.52 (s, 1H), 3.10 (d, 1H, *J* = 12.5Hz), 2.16 (td, 1H, *J* = 11Hz and 2.5Hz), 1.73 (m, 5H), 1.52 (m, 2H), 1.34 (s, 3H), 1.26 (m, 4), 1.10 (m, 10H), 1.05 (s, 3H), 0.96 (m, 2H), 0.88 (d, 3H, *J* = 6.5Hz), 0.48 (q, 1H, *J* = 12Hz). ¹³C NMR (500MHz, DMSO-*d*₆): δ (ppm) 165.54, 163.41, 163.34, 98.96, 79.78, 47.47, 44.61, 43.13, 36.97, 34.83, 33.45, 31.83, 27.17, 27.03, 26.90, 26.39, 26.06, 22.41, 22.05, 21.99, 19.41. HPLC retention time: 11.732min; purity 97.65%.

4.2.2.16 Synthesis of (6a*S*,9*R*,10a*R*)-6,6,9-trimethyl-3-(2-methyloctan-2-yl)-6a,7,8,9,10,10a-hexahydro-6*H*-isochromeno[3,4-*d*]pyrimidin-1-ol (37)

Prepared in a manner similar to compound **34** from dihydroxy pyrimidine **33** (100mg, 0.42mmol) and (R)-(+)-citronellal (194mg, 228μl, 1.26mmol). Yield: 31% MS: (ESI, neg.) *m/z* 373.1 [*M* – *H*][–], ¹H NMR (500 MHz, DMSO-*d*₆): δ 11.57 (s, 1H), 3.10 (d, 1H, *J* = 12.5Hz), 2.16 (td, 1H, *J* = 11Hz and 2.5Hz), 1.75 (m, 2H), 1.56 (m, 6H), 1.34 (s, 3H), 1.18 (m, 12H), 1.02 (m, 5H), 0.89 (d, 3H, *J* = 6.5Hz), 0.82 (t, 3H, *J* = 7Hz), 0.48 (q, 1H, *J* = 12Hz). ¹³C NMR (500MHz, DMSO-*d*₆): δ (ppm) 163.79, 163.47, 163.14, 99.01, 79.78, 47.52, 44.58, 40.14, 36.95, 34.85, 33.44, 31.82, 30.98, 29.00, 27.03, 26.89, 25.71, 25.59, 23.87, 22.42, 22.02, 19.37, 13.91. HPLC retention time: 12.503min; purity 95.12%.

4.2.3 Synthesis of Pyrimidine Non-Classical Cannabinoids

The first attempts at synthesis of the heteroaromatic triaryl compounds utilized reactions that were successfully employed in the creation of the carbocyclic triaryl compounds.¹²⁷ The rationale was that the previously created dihydroxy pyrimidines (Compounds **30**, **31**, **32**, and **33**) could be activated for Suzuki coupling to introduce the aromatic C ring. This was accomplished by bromination of the dihydroxy pyrimidines in the 5 position by N-bromosuccinimide (**Scheme 4.14**).¹⁷⁶ This brominated compound would then be a substrate for the palladium catalyzed Suzuki reaction. This reaction, however, was not successful in formation of the triaryl coupled products (**Scheme 4.15**).

With the experience gained from the successful formation of the dihydroxy pyrimidines, it was decided to form the final compounds by cyclization of the pyrimidine ring with the side chain and the aromatic C ring already in place. This could be accomplished in a reaction similar to the formation of the dihydroxy pyrimidines by reaction of an amidine with a substituted diethyl malonate (**Scheme 4.16**). The 2-position substituted diethyl malonates can be formed by copper catalyzed coupling of an alkyl or aryl halide and diethyl malonate (**Scheme 4.17**).¹⁷⁷ Six substituted malonates were reacted with four amidine hydrochlorides to successfully yield fifteen pyrimidine nonclassical cannabinoids (**Figure 4.6**). Some of the combinations of synthons would not react to yield a substituted pyrimidine product in every permutation. The purification of these compounds was more involved than the dihydroxy pyrimidines which were precipitated by acidification. The pyrimidine nonclassical cannabinoids formed a gummy resin in the aqueous solution upon acidification. This was extracted with ethyl acetate, dried over sodium sulfate, concentrated by rotary evaporation, and purified using flash chromatography.

All chemicals were obtained from Sigma Aldrich or Fisher Scientific Inc. Anhydrous solvents were obtained by distillation over either calcium hydride or metallic sodium and benzophenone. Final compounds and intermediates were purified using column chromatography on the Biotage SP1 system employing Flash column cartridges or by crystallization. NMR spectra were obtained on a Bruker 300MHz or Varian 500MHz Inova NMR. HPLC analysis of final products was carried out by gradient elution using water/acetonitrile (0.1% TFA) with a gradient of 70% (3min), 50% (2min), and 10% (15min). A reverse-phase ODS Hypersil column, manufactured by Thermo Scientific, of dimensions 4.6 x 150mm with 5 μ m particle size was used for HPLC analyses.

4.2.3.1 Synthesis of diethyl 2-m-tolylmalonate (38)

A two-necked round-bottomed flask was charged sequentially with CuI (0.114 g, 0.6 mmol), 2-picolinic acid (0.148 g, 1.2 mmol), and CsCO₃ (5.89 g, 18 mmol). The flask was evacuated and back filled with nitrogen 3 times. Anhydrous 1,4-dioxane (10 ml) was added volumetrically followed by distilled diethyl malonate (1.9 ml, 12 mmol) and 3-iodotoluene (0.8ml, 6 mmol). The reaction was heated to 70°C for 30 hours. After monitoring the progress by TLC, the reaction was cooled to room

Scheme 4.14: Synthesis of 5-bromo dihydroxy pyrimidines

(a) NBS, DMF, RT, 40min.

Scheme 4.15: Attempt at synthesis of pyrimidine triaryl compounds

Failed attempt at synthesis of pyrimidine triaryl compounds using palladium catalyzed Suzuki coupling. (a) $(\text{PPh}_3)_4\text{Pd}$, K_2CO_3 , toluene / water, reflux, 18hr.

Scheme 4.16: Synthesis of pyrimidine nonclassical cannabinoids

(a) Na metal, EtOH, diethyl malonate, reflux, 18hr.

Scheme 4.17: Synthesis of 2-position substituted diethyl malonates

(a) CuI, 2-picolinic acid, CsCO₃, aryl or alkyl iodide, diethyl malonate, 1,4-dioxane, 70°C, 30hr.

Figure 4.6: Pyrimidine non-classical cannabinoids

temperature, diluted with ethyl acetate and washed with ammonium chloride. The organic phase was dried over sodium sulfate, concentrated, and the product purified by column chromatography using a 1:9 ethyl acetate/hexane mixture to yield the product as a colorless oil. Yield: 92%, R_f = 0.48 (ethyl acetate/hexane = 1:9). MS: (ESI, pos.) m/z 273.0 $[M + Na]^+$, 1H NMR (500 MHz, $CDCl_3$): δ (ppm) 7.12-7.3 (m, 4H), 4.62 (s, 1H), 4.20-4.28 (m, 4H), 2.27 (s, 3H), 1.22-1.25 (m, 6H).

4.2.3.2 Synthesis of diethyl 2-(3,5-dichlorophenyl)malonate (39)

Compound **39** was prepared in a manner similar to compound **38** from CuI (0.17 g, 0.89 mmol), 2-picolinic acid (0.22 g, 1.78 mmol), $CsCO_3$ (8.7 g, 26.7 mmol), diethyl malonate (2.9ml, 17.8mmol), and 1,3-dichloro-5-iodobenzene (2.4g, 8.9mmol). R_f = 0.41 (ethyl acetate/hexane = 1:9). MS: (ESI, pos.) m/z 328 $[M + Na]^+$, 1H NMR (500 MHz, $CDCl_3$): δ (ppm) 7.34 (m, 3H), 4.54 (s, 1H), 4.25 (m, 4H), 1.28 (t, 6H).

4.2.3.3 Synthesis of diethyl 2-cyclohexylmalonate (40)

Compound **40** was prepared in a manner similar to compound **38** from CuI (0.114 g, 0.6 mmol), 2-picolinic acid (0.148 g, 1.2 mmol), $CsCO_3$ (5.89 g, 18 mmol), diethyl malonate (1.9ml, 12mmol), and iodocyclohexane (0.8ml, 6mmol). Yield: 94%, R_f = 0.59 (ethyl acetate/hexane = 1:9). MS: (ESI, pos.) m/z 265 $[M + Na]^+$, 1H NMR (500 MHz, $CDCl_3$): δ (ppm) 4.22-4.14 (m, 4H), 3.12 (d, 1H), 2.14-2.05 (m, 1H), 1.75-1.63 (m, 5H), 1.34-1.24 (m, 8H), 1.20-1.20 (m, 3H).

4.2.3.4 Synthesis of diethyl 2-(pyridin-2-yl)malonate (41)

Compound **41** was prepared in a manner similar to compound **38** from CuI (0.114 g, 0.6 mmol), 2-picolinic acid (0.148 g, 1.2 mmol), $CsCO_3$ (5.89 g, 18 mmol), diethyl malonate (1.9ml, 12mmol), and 2-bromopyridine (0.59ml, 6mmol). Yield: 88%, R_f = 0.32 (ethyl acetate/hexane = 3:7). MS: (ESI, pos.) m/z 260 $[M + Na]^+$, 1H NMR (500 MHz, $CDCl_3$): δ (ppm) 8.5 (d, 1H), 7.7 (d, 1H), 7.62-7.58 (m, 1H), 7.18-7.12 (m, 1H), 4.80 (s, 1H), 4.21-4.15 (m, 4H), 1.21 (t, 6H).

4.2.3.5 Synthesis of diethyl 2-benzylmalonate (42)

Compound **42** was prepared in a manner similar to compound **38** from CuI (0.114 g, 0.6 mmol), 2-picolinic acid (0.148 g, 1.2 mmol), $CsCO_3$ (5.89 g, 18 mmol), diethyl malonate (1.9ml, 12mmol), and benzyl bromide (1.44ml, 6mmol). Yield: 78%. R_f = 0.52 (ethyl acetate/hexane = 1:9). MS: (ESI, pos.) m/z 273 $[M + Na]^+$, 1H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.24 (m, 5H), 4.15 (q, 4H), 3.66 (t, 1H), 3.22 (d, 2H), 1.18 (t, 6H).

4.2.3.6 Synthesis of diethyl 2-hexylmalonate (43)

Compound **43** was prepared in a manner similar to compound **38** from CuI (0.019 g, 0.1mmol), 2-picolinic acid (0.025 g, 0.2 mmol), CsCO₃ (0.977g, 3mmol), diethyl malonate (0.3ml, 2mmol), and 1-iodohexane (0.15ml, 1mmol). Yield: 91%, R_f = 0.44 (ethyl acetate/hexane = 1:9). MS: (ESI, pos.) *m/z* 267 [M + Na]⁺, ¹H NMR (300 MHz, CDCl₃): δ (ppm) 4.11-4.40 (m, 4H), 3.31 (t, 1H), 1.80 (m, 2H), 1.38 (m, 2H), 1.1-1.4 (m, 12H), 0.8 (t, 3H).

4.2.3.7 Synthesis of 2-(2-phenylpropan-2-yl)-5-m-tolylpyrimidine-4,6-diol (44)

Sodium metal (115mg, 5mmol) was reacted with 4ml of anhydrous ethanol. To this was added 400 mg (2 mmol) of 2-methyl-2-phenylpropanimidamide hydrochloride **26** and the reaction was allowed to stir at room temperature for 5 minutes. Diethyl 2-m-tolylmalonate **38** (500mg, 2mmol) was then added in 2ml of anhydrous ethanol and the reaction was heated to reflux overnight. The reaction was then cooled to room temperature and filtered. The solid was washed several times with absolute ethanol. The filtrate and washings were combined and diluted with 2 volumes of water and acidified with 5N HCl to a pH of ~2 and the product precipitated as a sticky resin. The product was extracted with ethyl acetate and dried over sodium sulfate. The organic phase was concentrated and the product purified on a Biotage SP-1 flash chromatography using a MeOH/CH₂Cl₂ gradient. Yield: 39% MS: (ESI, neg.) *m/z* 319.0 [M – H][–], ¹H NMR (500 MHz, DMSO-d₆): δ (ppm) 11.50 (br.s, 2H), 7.36 (t, 2H, J = 7Hz), 7.30 (d, 2H, J = 7.5Hz), 7.25 (m, 3H), 7.19 (t, 1H, J = 7.5Hz), 7.01 (d, 1H, J = 7.5Hz), 2.29 (s, 3H), 1.68 (s, 6H). ¹³C NMR (500MHz, DMSO-d₆): δ (ppm) 145.79, 135.93, 132.54, 130.96, 128.31, 127.56, 127.07, 126.75, 126.51, 126.03, 100.61, 44.64, 27.26, 21.08. HPLC retention time: 9.724min; purity 99.65%.

4.2.3.8 Synthesis of 2-(2-(thiophen-2-yl)propan-2-yl)-5-m-tolylpyrimidine-4,6-diol (45)

Compound **45** was prepared in a similar manner to compound **44** from sodium metal (140mg, 6.11mmol), EtOH (8ml), 2-methyl-2-(thiophen-2-yl)propanimidamide hydrochloride **27** (500mg, 2.44mmol), and diethyl 2-m-tolylmalonate **38** (611mg, 2.44mmol). Yield 4.3%, MS: (ESI, neg.) *m/z* 324.9 [M – H][–], ¹H NMR (500 MHz, DMSO-d₆): δ (ppm) 11.51 (br.s, 2H), 7.44 (dd, 1H, J = 5Hz and 1Hz), 7.23 (s, 1H), 7.20 (q, 2H, J = 8Hz), 7.06 (dd, 1H, J = 3.5Hz and 1.5Hz), 7.00 (m, 2H), 2.28 (s, 3H), 1.70 (s, 6H). ¹³C NMR (500MHz, DMSO-d₆): δ (ppm) 150.33, 136.00, 132.46, 130.96, 127.57, 127.14, 126.85, 126.77, 126.46, 124.91, 124.40, 123.41, 100.76, 42.88, 28.14, 21.11. HPLC retention time: 8.060min; purity 95.35%.

4.2.3.9 Synthesis of 2-(2-cyclohexylpropan-2-yl)-5-m-tolylpyrimidine-4,6-diol (**46**)

Compound **46** was prepared in a similar manner to compound **44** from sodium metal (140mg, 6.11mmol), EtOH (6ml), 2-cyclohexyl-2-methylpropanimidamide hydrochloride **28** (500mg, 2.44mmol), and diethyl 2-m-tolylmalonate **38** (796mg, 3.18mmol). Yield 21%, MS: (ESI, neg.) m/z 325 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.55(br.s), 7.26 (s, 1H) 7.23 (d, 1H, $J = 8Hz$), 7.17 (t, 1H, $J = 7.5Hz$), 6.99 (d, 1H, $J = 7.5Hz$), 2.28 (s, 3H), 1.87 (t, 1H, $J = 12Hz$), 1.72 (d, 2H, $J = 12.5Hz$), 1.44 (d, 2H, $J = 12Hz$), 1.20 (s, 6H), 1.18 to 1.06 (m, 4H), 0.99 (m, 2H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 170.36, 165.93, 135.90, 132.80, 131.01, 127.60, 127.07, 126.63, 100.04, 59.77, 44.84, 43.29, 27.22, 26.41, 26.06, 22.11, 21.13, 20.78, 14.10. HPLC retention time: 10.095min; purity 99.23%.

4.2.3.10 Synthesis of 2-(2-methyloctan-2-yl)-5-m-tolylpyrimidine-4,6-diol (**47**)

Compound **47** was prepared in a similar manner to compound **44** from sodium metal (111mg, 4.83mmol), EtOH (6ml), 2,2-dimethyloctanimidamide hydrochloride **29** (400mg, 1.93mmol), and diethyl 2-m-tolylmalonate **38** (483mg, 1.93mmol). Yield 11.2%, MS: (ESI, pos.) m/z 351.3 $[M + Na]^+$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.58 (br.s, 2H), 7.26 (s, 1H) 7.22 (d, 1H, $J = 7.5Hz$), 7.19 (t, 1H, $J = 7.5Hz$), 7.01 (d, 1H, $J = 7.5Hz$), 2.91 (s, 3H), 1.68 (m, 2H), 1.28 (s, 6H), 1.24 to 1.07 (m, 16H), 0.853 (m, 3H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 179.57, 165.84, 136.39, 133.20, 131.47, 128.08, 127.55, 127.16, 100.64, 48.48, 40.61, 31.53, 29.57, 26.21, 25.90, 24.48, 22.53, 21.61, 14.39. HPLC retention time: 9.685min; purity 91.54%.

4.2.3.11 Synthesis of 5-(3,5-dichlorophenyl)-2-(2-phenylpropan-2-yl)pyrimidine-4,6-diol (**48**)

Compound **48** was prepared in a similar manner to compound **44** from sodium metal (115mg, 5mmol), EtOH (6ml), 2-methyl-2-phenylpropanimidamide hydrochloride **26** (400mg, 2mmol), and diethyl 2-(3,5-dichlorophenyl)malonate **39** (610mg, 2mmol). Yield: 27%; MS: (ESI, neg.) m/z 372.9 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 12.48 (br.s, 2H), 7.61 (d, 2H, $J = 2Hz$), 7.49 (s, 1H), 7.31 (m, 2H), 7.27 (m, 3H), 1.68 (s, 6H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 172.33, 171.56, 141.09, 139.75, 134.08, 133.23, 129.02, 128.97, 126.78, 126.73, 110.00, 45.28, 32.74. HPLC retention time: 9.335min; purity 92.38%.

4.2.3.12 Synthesis of 5-(3,5-dichlorophenyl)-2-(2-(thiophen-2-yl)propan-2-yl)pyrimidine-4,6-diol (49)

Compound **49** was prepared in a similar manner to compound **44** from sodium metal (113mg, 4.9mmol), EtOH (6ml), 2-methyl-2-(thiophen-2-yl)propanimidamide hydrochloride **27** (400mg, 1.95mmol), and diethyl 2-(3,5-dichlorophenyl)malonate **39** (595mg, 1.95mmol). Yield: 32%; MS: (ESI, neg.) m/z 378.8 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 7.62 (s, 2H), 7.48 (s, 1H), 7.43 (d, 1H, $J = 5.5$ Hz), 7.06 (d, 1H, $J = 3.5$ Hz), 6.99 (t, 1H, $J = 5$ Hz), 1.77 (s, 6H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 172.39, 171.61, 141.14, 139.83, 134.12, 133.24, 129.13, 128.96, 126.87, 126.71, 99.19, 39.41, 28.08. HPLC retention time: 9.335min; purity 90.19%.

4.2.3.13 Synthesis of 2-(2-cyclohexylpropan-2-yl)-5-(3,5-dichlorophenyl)pyrimidine-4,6-diol (50)

Compound **50** was prepared in a similar manner to compound **44** from sodium metal (140mg, 6.1mmol), EtOH (6ml), 2-cyclohexyl-2-methylpropanimidamide hydrochloride **28** (500mg, 2.44mmol), and diethyl 2-(3,5-dichlorophenyl)malonate **39** (970mg, 3.18mmol). Yield: 7%; MS: (ESI, neg.) m/z 379.0 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 7.69 (s, 2H), 7.38 (s, 1H), 1.87 (m, 1H), 1.68 (m, 3H), 1.44 (d, 2H, $J = 11$ Hz), 1.21 (s, 6H), 1.12 (m, 3H), 0.98 (m, 2H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 172.38, 171.61, 140.65, 135.68, 133.24, 128.46, 100.25, 59.77, 44.10, 42.98, 27.22, 26.49, 26.02, 21.67, 20.34, 14.10. HPLC retention time: 8.932min; purity 94.24%.

4.2.3.14 Synthesis of 5-(3,5-dichlorophenyl)-2-(2-methyloctan-2-yl)pyrimidine-4,6-diol (51)

Compound **51** was prepared in a similar manner to compound **44** from sodium metal (111mg, 4.83mmol), EtOH (8ml), 2,2-dimethyloctanimidamide hydrochloride **29** (400mg, 1.93mmol), and diethyl 2-(3,5-dichlorophenyl)malonate **39** (589mg, 1.93mmol). Yield: 14%; MS: (ESI, neg.) 380.9 m/z $[M - H]^-$, 1H NMR (500 MHz, methanol- d_4): δ (ppm) 7.57 (s, 2H), 7.24 (s, 1H), 1.71 (m, 2H), 1.34 (m, 5H), 1.28 (s, 6H), 1.20 (m, 3H), 0.88 (t, 3H, $J = 6$ Hz). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 170.98, 169.54, 139.32, 135.79, 131.05, 129.09, 100.15, 48.48, 40.61, 30.48, 29.79, 26.92, 25.43, 23.76, 22.53, 14.41. HPLC retention time: 9.132min; purity 96.84%.

4.2.3.15 Synthesis of 5-cyclohexyl-2-(2-phenylpropan-2-yl)pyrimidine-4,6-diol (52)

Compound **52** was prepared in a similar manner to compound **44** from sodium metal (145mg, 6.3mmol), EtOH (6ml), 2-methyl-2-phenylpropanimidamide hydrochloride **26** (500mg, 2.5mmol), and diethyl 2-cyclohexylmalonate **40** (606mg, 2.5mmol). Yield: 39% (yellow waxy solid) MS: (ESI, neg.) m/z 311.0 $[M - H]^-$, 1H

NMR (500 MHz, DMSO- d_6): δ (ppm) 11.30 (br.s, 1H), 10.93 (br.s., 1H), 7.33 (m, 2H), 7.23 (m, 3H), 2.72 (tt, 1H, $J = 12.5\text{Hz}$ and 3Hz), 1.92 (q, 2H, $J = 13\text{Hz}$), 1.70 (d, 2H, $J = 12.5\text{Hz}$), 1.62 (s, 7H), 1.38 (d, 2H, $J = 12\text{Hz}$), 1.18 (m, 3H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 164.35, 145.90, 128.28, 126.46, 126.02, 104.19, 44.34, 33.28, 28.87, 27.25, 26.67, 25.75. HPLC retention time: 8.983min; purity 99.46%.

4.2.3.16 Synthesis of 5-cyclohexyl-2-(2-(thiophen-2-yl)propan-2-yl)pyrimidine-4,6-diol (53)

Compound **53** was prepared in a similar manner to compound **44** from sodium metal (140mg, 6.1mmol), EtOH (6ml), 2-methyl-2-(thiophen-2-yl)propanimidamide hydrochloride **27** (500mg, 2.44mmol), and diethyl 2-cyclohexylmalonate **40** (591mg, 2.44mmol). Yield 8%, MS: (ESI, neg.) m/z 316.9 $[\text{M} - \text{H}]^-$, ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.36 (br.s, 1H), 10.88 (br.s, 1H) 7.39 (d, 1H, $J = 5\text{Hz}$), 7.01 (s, 1H), 6.96 (t, 1H, $J = 3.5\text{Hz}$), 2.72 (t, 1H, $J = 12\text{Hz}$), 1.91 (q, 2H, $J = 12.5\text{Hz}$), 1.72 (s, 6H), 1.61 (d, 1H, $J = 10.5\text{Hz}$), 1.36 (d, 2H, $J = 12\text{Hz}$), 1.18 (m, 3H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 164.41, 126.72, 124.80, 124.29, 109.53, 104.31, 48.61, 42.60, 33.27, 28.80, 28.11, 26.63, 25.72. HPLC retention time: 8.934min; purity 100%.

4.2.3.17 Synthesis of 5-cyclohexyl-2-(2-cyclohexylpropan-2-yl)pyrimidine-4,6-diol (54)

Compound **54** was prepared in a similar manner to compound **44** from sodium metal (140mg, 6.1mmol), EtOH (6ml), 2-cyclohexyl-2-methylpropanimidamide hydrochloride **28** (500mg, 2.44mmol), and diethyl 2-cyclohexylmalonate **40** (770mg, 3.18mmol). Yield 1.3%, MS: (ESI, neg.) m/z 316.9 $[\text{M} - \text{H}]^-$, ^1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.43 (br.s, 1H), 10.64 (br.s, 1H) 2.72 (tt, 1H, $J = 12.5\text{Hz}$ and 3.5Hz), 1.91 (m, 2H), 1.81 (t, 1H, $J = 12\text{Hz}$), 1.71 (d, 4H, $J = 12\text{Hz}$), 1.61 (m, 2H), 1.37 (d, 2H, $J = 12.5\text{Hz}$), 1.23 to 0.90 (m, 16H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 164.44, 164.29, 103.69, 44.73, 42.98, 33.25, 28.94, 27.18, 26.68, 26.38, 26.06, 25.75, 22.11. HPLC retention time: 10.273min; purity 98.33%.

4.2.3.18 Synthesis of 5-cyclohexyl-2-(2-methyloctan-2-yl)pyrimidine-4,6-diol (55)

Compound **55** was prepared in a similar manner to compound **44** from sodium metal (111mg, 4.84mmol), EtOH (8ml), 2,2-dimethyloctanimidamide hydrochloride **29** (400mg, 1.93mmol), and diethyl 2-cyclohexylmalonate **40** (468mg, 1.93mmol). Yield 4.5%, MS: (ESI, neg.) m/z 319.0 $[\text{M} - \text{H}]^-$, ^1H NMR (500 MHz, DMSO- d_6): 2.72 (tt, 1H, $J = 12\text{Hz}$ and 3Hz), 1.92 (m, 2H), 1.70 (d, 2H, $J = 12.5\text{Hz}$), 1.61 (t, 4H, $J = 8.5\text{Hz}$), 1.37 (d, 2H, $J = 12\text{Hz}$), 1.23 to 1.16 (m, 16H), 1.04 (m, 2H), 0.83 (m, 4H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 164.35, 163.82, 103.74, 40.26, 33.23, 31.03, 29.04, 28.90,

26.67, 25.75, 25.71, 23.95, 22.04, 13.91. HPLC retention time: 10.304min; purity 99.06%.

4.2.3.19 Synthesis of 5-(pyridin-2-yl)-2-(2-(thiophen-2-yl)propan-2-yl)pyrimidine-4,6-diol (**56**)

Compound **56** was prepared in a similar manner to compound **44** from sodium metal (112mg, 4.88mmol), EtOH (6ml), 2-methyl-2-(thiophen-2-yl)propanimidamide hydrochloride **27** (400mg, 1.95mmol), and diethyl 2-(pyridin-2-yl)malonate **41** (463mg, 1.95mmol). Yield 1%, MS: (ESI, pos.) m/z 336.1 $[M + Na]^+$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 9.13 (d, 1H, $J = 8.5$ Hz) 8.57 (s, 1H), 8.12 (td, 1H, $J = 7.5$ Hz and 1.5Hz), 7.41 (q, 2H, $J = 7.5$ Hz), 7.59 (d, 1H, $J = 4$), 6.98 (t, 1H, $J = 4$ Hz), 1.73 (s, 6H). HPLC retention time: 8.367min; purity 97.78%.

4.2.3.20 Synthesis of 5-benzyl-2-(2-methyloctan-2-yl)pyrimidine-4,6-diol (**57**)

Compound **57** was prepared in a similar manner to compound **44** from sodium metal (111mg, 4.84mmol), EtOH (6ml), 2,2-dimethyloctanimidamide hydrochloride **29** (400mg, 1.93mmol), and diethyl 2-benzylmalonate **42** (483mg, 1.93mmol). Yield 21% MS: (ESI, neg.) m/z 327.0 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.34 (br.s), 7.21 (m, 4H) 7.11 (m, 1H), 3.57 (s, 2H), 1.63 (m, 2H), 1.22 (s, 6H), 1.21 to 1.19 (m, 6H), 1.04 (m, 2H), 0.82 (t, 3H, $J = 6.5$ Hz). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 164.70, 141.19, 128.23, 127.97, 125.49, 99.07, 54.92, 40.37, 39.77, 31.02, 29.03, 27.81, 25.77, 23.97, 22.02, 13.91. HPLC retention time: 9.716min; purity 100%.

4.2.3.21 Synthesis of 5-hexyl-2-(2-methyloctan-2-yl)pyrimidine-4,6-diol (**58**)

Compound **58** was prepared in a similar manner to compound **44** from sodium metal (140mg, 6.05mmol), EtOH (6ml), 2,2-dimethyloctanimidamide hydrochloride **29** (500mg, 2.42mmol), and diethyl 2-hexylmalonate **43** (591mg, 2.42mmol). Yield 9%, MS: (ESI, neg.) m/z 321.0 $[M - H]^-$, 1H NMR (500 MHz, DMSO- d_6): δ (ppm) 11.47 (br.s, 2H), 4.10 (q, 1H, $J = 7.5$ Hz) 3.29 (t, 1H, $J = 7.5$ Hz), 2.23 (t, 2H, $J = 7.5$ Hz), 1.71 (q, 1H, $J = 7$ Hz), 1.62 (m, 2H), 1.36 (t, 2H, $J = 6.5$ Hz), 1.24 (s, 6H), 1.21 to 1.16 (m, 9H), 1.02 (m, 2H), 0.83 (m, 6H). ^{13}C NMR (500MHz, DMSO- d_6): δ (ppm) 170.52, 169.50, 164.69, 163.88, 99.72, 60.59, 51.39, 40.40, 31.27, 31.06, 29.06, 28.73, 28.34, 27.58, 26.62, 25.81, 23.99, 22.03, 13.97. HPLC retention time: 10.616min; purity 97.38%.

4.2.4 Synthesis of Furanopyrimidine Cannabinoids

With the successful synthesis of both the pyrimidine hexahydro classical cannabinoids and the pyrimidine non-classical cannabinoids, sights were set on expansion of the pyrimidine containing series of compounds. Upon review of the current medicinal chemistry literature, a series of 4-amino-5,6-biaryl-furo[2,3-d]pyrimidines that had been developed by Amgen Inc. as inhibitors of lymphocyte-specific kinase (Lck)¹⁷⁸ caught our attention. The general structure of this series of compounds (**Figure 4.7**) has aspects of both a conformationally restricted pyrimidine non-classical cannabinoid and a ring-contracted pyrimidine classical cannabinoid in which the six-membered pyran ring has been replaced by a five-membered furan. These compounds lacked the side chain at the 2-position of the pyrimidine ring found in the previously described pyrimidine cannabinoids. This series of compounds contained two substituents on the furan ring: one being an unsubstituted phenyl and the other a substituted phenyl.

It was decided to design the furanopyrimidine with just one aryl substitution on the furan ring in order to form compounds that would occupy similar space to the previously described pyrimidine cannabinoids. These new compounds were also to be synthesized with a side chain present similar to the classical and non-classical pyrimidine cannabinoids (**Figure 4.8**). By adapting the chemistry of DiMauro *et al.*^{178a} the furanopyrimidine cannabinoids were formed by the base catalysed cyclization of a substituted dihydroxy pyrimidine with a 2-chloro-2-nitrovinyl substituted aromatic ring (**Scheme 4.18**).

The substituted dihydroxy pyrimidines used were the same ones formed for the pyrimidine hexahydro classical cannabinoids (**Scheme 4.12**) from substituted amidines and diethyl malonate. The 2-chloro-2-nitrovinyl styrenes were formed from substituted benzaldehydes, bromonitromethane, dimethylamine hydrochloride, and potassium fluoride in xylenes (**Scheme 4.19**). The reaction is fitted with a Dean-Stark trap to allow for azeotropic removal of water and heated to 125°C for 8 hours. The styrenes were then readily purified by flash column chromatography.

Four styrenes (phenyl, m-tolyl, 3,5-dichloro phenyl, and 2-thiophenyl) were cyclized with four substituted dihydroxy pyrimidines (dimethyl phenyl, dimethyl thiophenyl, dimethyl cyclohexyl, and dimethyl heptyl) to form sixteen new furanopyrimidine cannabinoids (**Figure 4.9**).

All chemicals were obtained from Sigma Aldrich or Fisher Scientific Inc. Anhydrous solvents were obtained by distillation over either calcium hydride or metallic sodium and benzophenone. Final compounds and intermediates were purified using column chromatography on the Biotage SP1 system employing Flash column cartridges or by crystallization. NMR spectra were obtained on a Bruker 300MHz or Varian 500MHz Inova NMR. HPLC analysis of final products was carried out by gradient elution using water/acetonitrile (0.1% TFA) with a gradient of 70% (3min), 50% (2min), and 10% (15min). A reverse-phase ODS Hypersil column, manufactured by Thermo Scientific, of dimensions 4.6 x 150mm with 5µm particle size was used for HPLC analyses.

Figure 4.7: General structure of Lck inhibitors

Figure 4.8: General structure of furanopyrimidine cannabinoids

Scheme 4.18: General synthesis of furanopyrimidine cannabinoids

(a) DBU, acetonitrile, μ wave, 100watts, $120^\circ C$, 15min.

Scheme 4.19: Synthesis of 2-chloro-2-nitrovinyl styrenes

(a) bromonitromethane, dimethylamine hydrochloride, potassium fluoride, xylenes, 125°C, 8hr.

Figure 4.9: Furanopyrimidine cannabinoids

4.2.4.1 Synthesis of (2-chloro-2-nitrovinyl)benzene (59)

Benzaldehyde (3.59g, 33.8 mmol), bromonitromethane (9.0g, 64.3 mmol), dimethylamine hydrochloride (24.8g, 304.2mmol), potassium fluoride (0.3g, 5.08mmol) and m- xylenes (50mL) were combined in a 250mL round bottomed flask fitted with a Dean-Stark trap. The mixture was heated at 125°C with azeotropic removal of water for 8h. The reaction mixture was concentrated and the residue was extracted with a mixture of 1:1 dicloromethane and water. After separation of the organic layer, the aqueous layer was extracted three times with dicloromethane. The combined organics were dried over anhydrous Na₂SO₄ and concentrated to a brown oil. The oily residue thus obtained was purified on a silica column (Biotage SP1 system) using a 1:9 ethyl acetate /hexane mixture to obtain the product as pale yellow needles (4.8g, 77%). *R_f* = 0.66 (1:9 ethyl acetate/hexane). ¹H NMR (300 MHz, CDCl₃): δ (ppm) 8.40 (s,1H), 7.86-7.90 (m,2H), 7.51-7.5 (m,3H).

4.2.4.2 Synthesis of 1-(2-chloro-2-nitrovinyl)-3-methylbenzene (60)

Compound **60** was prepared in a manner similar to compound **59**. ¹H NMR (300 MHz, CDCl₃): δ (ppm) 8.38 (s,1H), 7.67 (t,2H), 7.36-7.41 (m,2H), 2.45 (s,3H).

4.2.4.3 Synthesis of 1,3-dichloro-5-(2-chloro-2-nitrovinyl)benzene (61)

Compound **61** was prepared in a manner similar to compound **59**. ¹H NMR (300 MHz, CDCl₃): δ (ppm) 8.23 (s,1H), 7.74 (d,2H), 7.52 (s,1H).

4.2.4.4 Synthesis of 2-(2-chloro-2-nitrovinyl)thiophene (62)

Compound **62** was prepared in a manner similar to compound **59**. ¹H NMR (300 MHz, CDCl₃): δ (ppm) 8.65 (s,1H), 7.81 (d, 1H), 7.69 (d,1H), 7.27-7.28(t,1H).

4.2.4.5 Synthesis of 5-phenyl-2-(2-phenylpropan-2-yl)furo[2,3-d]pyrimidin-4-ol (63)

100mg (0.434mmol) of 2-(2-phenylpropan-2-yl)pyrimidine-4,6-diol **30** and 73mg (0.4mmol) of (2-chloro-2-nitrovinyl)benzene **59** were dissolved in 2ml acetonitrile in a 10ml microwave reaction vessel. 122 mg (0.8mmol) of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was then added and the vessel sealed. The vessel was irradiated in a CEM LabMate microwave reactor (CEM, Matthews, NC USA) at 100watts to 120°C for 15 minutes. The reaction mixture was then concentrated and the target compound purified by flash chromatography on a Biotage SP1 (Biotage, Charlottesville, VA USA) using a methylene chloride / methanol gradient. Yield: 42.3%. MS: (ESI, neg) *m/z* 329.0 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.03 (s, 1H), 8.24 (s, 1H), 7.96 (d, 2H, J =

7.5Hz), 7.41 (t, 2H, J = 7.5Hz), 7.33 (q, 3H, J = 7.5Hz), 7.26 (t, 3H, J = 8Hz), 1.71 (s, 6H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.49, 164.18, 159.79, 145.77, 138.41, 130.22, 128.37, 128.32, 127.94, 126.57, 129.17, 123.49, 120.92, 44.98, 27.53. HPLC retention time: 10.185min; purity 100%.

4.2.4.6 Synthesis of 5-phenyl-2-(2-(thiophen-2-yl)propan-2-yl)furo[2,3-d]pyrimidin-4-ol (64)

Compound **64** was prepared in a manner similar to compound **63** from 2-(2-(thiophen-2-yl)propan-2-yl)pyrimidine-4,6-diol **31** (100mg, 0.423mmol), (2-chloro-2-nitrovinyl)benzene **59** (73mg, 0.4mmol), DBU (122mg, 120μl, 0.8mmol), and CH₃CN (2ml). Yield: 26.8%. MS: (ESI, neg.) *m/z* 335.0 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.01 (s, 1H), 8.24 (s, 1H), 7.96 (d, 2H, J = 7.5Hz), 7.42 (q, 3H, J = 8Hz), 7.32 (t, 1H, J = 7Hz), 7.04 (d, 1H, J = 3Hz), 6.99 (t, 1H, J = 4.5Hz), 1.86 (s, 6H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.38, 162.99, 159.67, 150.22, 138.60, 130.15, 128.38, 127.94, 127.62, 126.74, 124.98, 124.56, 123.50, 103.02, 43.16, 28.42. HPLC retention time: 10.136min; purity 100%.

4.2.4.7 Synthesis of 2-(2-cyclohexylpropan-2-yl)-5-phenylfuro[2,3-d]pyrimidin-4-ol (65)

Compound **65** was prepared in a manner similar to compound **63** from 2-(2-cyclohexylpropan-2-yl)pyrimidine-4,6-diol **32** (100mg, 0.423mmol), (2-chloro-2-nitrovinyl)benzene **59** (73mg, 0.4mmol), DBU (122mg, 120μl, 0.8mmol), and CH₃CN (2ml). Yield: 43.7%. MS: (ESI, neg.) *m/z* 335.0 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.02 (s, 1H), 8.19 (s, 1H), 7.98 (d, 2H, J = 8Hz), 7.42 (t, 2H, J = 7.5Hz), 7.32 (t, 1H, J = 7Hz), 1.94 (t, 1H, J = 11.5Hz), 1.69 (d, 2H, J = 12Hz), 1.60 (d, 2H, J = 12Hz), 1.39 (d, 2H, J = 12Hz), 1.25 (s, 6H), 1.07 (m, 5H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.58, 165.54, 159.89, 138.12, 130.29, 128.36, 127.91, 127.55, 123.41, 102.42, 44.92, 43.61, 27.19, 26.39, 26.01, 22.21. HPLC retention time: 11.245min; purity 99.45%.

4.2.4.8 Synthesis of 2-(2-methyloctan-2-yl)-5-phenylfuro[2,3-d]pyrimidin-4-ol (66)

Compound **66** was prepared in a manner similar to compound **63** from 2-(2-methyloctan-2-yl)pyrimidine-4,6-diol **33** (40mg, 0.168mmol), (2-chloro-2-nitrovinyl)benzene **59** (28mg, 0.153mmol), DBU (47mg, 46.2μl, 0.308mmol), and CH₃CN (1ml). Yield: 32.6%. MS: (ESI, neg.) *m/z* 337 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.12 (s, 1H), 8.19 (s, 1H), 7.98 (d, 2H, J = 7.5Hz), 7.42 (t, 2H, J = 8Hz), 7.32 (t, 1H, J = 7.5Hz), 1.73 (m, 2H), 1.31 (s, 6H), 1.19 (m, 6H), 1.06 (m, 2H), 0.8 (t, 3H, J = 7Hz); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.65, 164.91, 159.91, 138.14,

130.28, 128.36, 127.90, 127.55, 112.34, 102.44, 40.44, 40.33, 30.98, 29.06, 25.90, 23.94, 22.01, 13.88. HPLC retention time: 11.846min; purity 100%.

4.2.4.9 Synthesis of 2-(2-phenylpropan-2-yl)-5-m-tolylfuro[2,3-d]pyrimidin-4-ol (67)

Compound **67** was prepared in a manner similar to compound **63** from 2-(2-phenylpropan-2-yl)pyrimidine-4,6-diol **30** (100mg, 0.434mmol), 1-(2-chloro-2-nitrovinyl)-3-methylbenzene **60** (79mg, 0.4mmol), DBU (122mg, 120 μ l, 0.8mmol), and CH₃CN (2ml). Yield: 29.2%. MS: (ESI, neg.) *m/z* 343.0 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 11.99 (s, 1H), 8.20 (s, 1H), 7.79 (d, 1H, *J* = 8Hz), 7.74 (s, 1H), 7.29 (m, 6H), 7.13 (d, 1H, *J* = 7.5Hz), 2.33 (s, 3H), 1.71 (s, 6H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.44, 164.12, 159.71, 145.78, 138.31, 137.32, 130.10, 128.37, 128.32, 128.26, 128.23, 126.57, 126.16, 125.30, 123.58, 102.95, 44.96, 27.53, 21.11. HPLC retention time: 10.569min; purity 100%.

4.2.4.10 Synthesis of 2-(2-(thiophen-2-yl)propan-2-yl)-5-m-tolylfuro[2,3-d]pyrimidin-4-ol (68)

Compound **68** was prepared in a manner similar to compound **63** from 2-(2-(thiophen-2-yl)propan-2-yl)pyrimidine-4,6-diol **31** (100mg, 0.434mmol), 1-(2-chloro-2-nitrovinyl)-3-methylbenzene **60** (79mg, 0.4mmol), DBU (122mg, 120 μ l, 0.8mmol), and CH₃CN (2ml). Yield: 21.5%. MS: (ESI, neg.) *m/z* 349.0 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.07 (s, 1H), 8.20 (s, 1H), 7.78 (d, 1H, *J* = 7.5Hz), 7.73 (s, 1H), 7.43 (d, 1H, *J* = 5Hz), 7.30 (t, 1H, *J* = 7.5Hz), 7.14 (d, 1H, *J* = 7.5Hz), 7.03 (d, 1H, *J* = 4Hz), 6.99 (t, 1H, *J* = 5Hz), 2.33 (s, 3H), 1.81 (s, 6H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.33, 162.94, 159.61, 150.23, 138.49, 137.33, 130.03, 127.94, 128.39, 128.27, 129.74, 125.30, 124.98, 124.55, 123.55, 103.04, 43.15, 25.42, 21.11. HPLC retention time: 10.514min; purity 100%.

4.2.4.11 Synthesis of 2-(2-(cyclohexylpropan-2-yl)-5-m-tolylfuro[2,3-d]pyrimidin-4-ol (69)

Compound **69** was prepared in a manner similar to compound **63** from 2-(2-cyclohexylpropan-2-yl)pyrimidine-4,6-diol **32** (100mg, 0.434mmol), 1-(2-chloro-2-nitrovinyl)-3-methylbenzene **60** (79mg, 0.4mmol), DBU (122mg, 120 μ l, 0.8mmol), and CH₃CN (2ml). Yield: 30.8%. MS: (ESI, neg.) *m/z* 349.0 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 11.95 (s, 1H), 8.14 (s, 1H), 7.80 (d, 1H, *J* = 7.5Hz), 7.54 (s, 1H), 7.30 (t, 1H, *J* = 7.5Hz), 7.14 (d, 1H, *J* = 7Hz), 2.34 (s, 3H), 1.93 (t, 1H, *J* = 12Hz), 1.69 (d, 2H, *J* = 12.5Hz), 1.60 (d, 1H, *J* = 12Hz), 1.40 (d, 2H, *J* = 12Hz), 1.24 (s, 6H), 1.05 (m, 5H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.53, 165.49, 138.02, 137.30, 130.17, 128.35, 128.36, 128.25, 128.19, 128.27, 125.27, 123.49, 102.45, 44.92, 43.60, 27.19, 26.82, 26.02, 22.21, 21.15. HPLC retention time: 11.680min; purity 100%.

4.2.4.12 Synthesis of 2-(2-methyloctan-2-yl)-5-m-tolylfuro[2,3-d]pyrimidin-4-ol (70)

Compound **70** was prepared in a manner similar to compound **63** from 2-(2-methyloctan-2-yl)pyrimidine-4,6-diol **33** (100mg, 0.42mmol), 1-(2-chloro-2-nitrovinyl)-3-methylbenzene **60** (79mg, 0.4mmol), DBU (122mg, 120 μ l, 0.8mmol), and CH₃CN (2ml). Yield: 36.4%. MS: (ESI, neg.) m/z 351 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.08 (s, 1H), 8.15 (s, 1H), 7.81 (d, 1H, J = 7.5Hz), 7.75 (s, 1H), 7.30 (t, 1H, J = 7.5Hz), 7.14 (d, 1H, J = 7.5Hz), 2.34 (s, 3H), 1.72 (t, 2H, J = 6.5Hz), 1.31 (s, 6H), 1.19 (m, 6H), 1.06 (m, 2H), 0.81 (t, 3H, J = 6.5Hz); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.60, 164.86, 159.85, 138.04, 137.31, 130.16, 128.34, 128.26, 128.20, 125.28, 123.48, 102.46, 40.43, 40.35, 30.99, 29.07, 25.91, 23.95, 22.02, 21.12, 13.88. HPLC retention time: 12.294min; purity 99.14%.

4.2.4.13 Synthesis of 5-(3,5-dichlorophenyl)-2-(2-phenylpropan-2-yl)furo[2,3-d]pyrimidin-4-ol (71)

Compound **71** was prepared in a manner similar to compound **63** from 2-(2-phenylpropan-2-yl)pyrimidine-4,6-diol **30** (100mg, 0.42mmol), 1,3-dichloro-5-(2-chloro-2-nitrovinyl)benzene **61** (101mg, 0.4mmol), DBU (122mg, 120 μ l, 0.8mmol), and CH₃CN (2ml). Yield: 20.4%. MS: (ESI, neg.) m/z 397.0 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.16 (s, 1H), 8.51 (s, 1H), 8.20 (s, 2H), 7.56 (s, 1H), 7.35 (t, 2H, J = 8Hz), 7.27 (m, 3H), 1.72 (s, 6H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.62, 164.63, 159.83, 145.68, 140.20, 134.13, 133.81, 128.34, 126.90, 126.61, 126.25, 126.17, 121.12, 102.55, 45.06, 27.54. HPLC retention time: 11.468min; purity 97.36%.

4.2.4.14 Synthesis of 5-(3,5-dichlorophenyl)-2-(2-(thiophen-2-yl)propan-2-yl)furo[2,3-d]pyrimidin-4-ol (72)

Compound **72** was prepared in a manner similar to compound **63** from 2-(2-(thiophen-2-yl)propan-2-yl)pyrimidine-4,6-diol **31** (100mg, 0.423mmol), 1,3-dichloro-5-(2-chloro-2-nitrovinyl)benzene **61** (101mg, 0.4mmol), DBU (122mg, 120 μ l, 0.8mmol), and CH₃CN (2ml). Yield: 32.9%. MS: (ESI, neg.) m/z 404.9 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.22 (s, 1H), 8.49 (s, 1H), 8.18 (s, 2H), 7.54 (s, 1H), 7.44 (d, 1H, J = 5Hz), 7.03 (d, 1H, J = 3Hz), 6.99 (t, 1H, J = 4.5Hz), 1.81 (s, 6H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.50, 163.42, 159.71, 150.08, 140.35, 134.13, 133.72, 126.92, 126.75, 126.26, 125.01, 124.06, 121.12, 102.64, 43.21, 28.42. HPLC retention time: 11.414min; purity 100%.

4.2.4.15 Synthesis of 2-(2-cyclohexylpropan-2-yl)-5-(3,5-dichlorophenyl)furo[2,3-d]pyrimidin-4-ol (73)

Compound **73** was prepared in a manner similar to compound **63** from 2-(2-cyclohexylpropan-2-yl)pyrimidine-4,6-diol **32** (100mg, 0.423mmol), 1,3-dichloro-5-(2-chloro-2-nitrovinyl)benzene **61** (101mg, 0.4mmol), DBU (122mg, 120μl, 0.8mmol), and CH₃CN (2ml). Yield: 24.7%. MS: (ESI, neg.) *m/z* 403.0 [M – H][–], ¹H NMR (500 MHz, CDCl₃): δ (ppm) 10.91 (s, 1H), 7.82 (s, 2H), 7.66 (s, 1H), 7.35 (s, 1H), 1.71 (m, 1H), 1.64 (m, 2H), 1.55 (d, 1H, J = 10.5Hz), 1.42 (d, 2H, J = 6Hz), 1.36 (s, 6H), 1.00 (m, 5H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.55, 160.62, 138.24, 135.21, 133.45, 128.00, 126.78, 122.63, 110.19, 102.97, 47.23, 44.38, 27.89, 26.83, 26.51, 22.97. HPLC retention time: 13.021min; purity 98.71%.

4.2.4.16 Synthesis of 5-(3,5-dichlorophenyl)-2-(2-methyloctan-2-yl)furo[2,3-d]pyrimidin-4-ol (74)

Compound **74** was prepared in a manner similar to compound **63** from 2-(2-methyloctan-2-yl)pyrimidine-4,6-diol **33** (100mg, 0.42mmol), 1,3-dichloro-5-(2-chloro-2-nitrovinyl)benzene **61** (101mg, 0.4mmol), DBU (122mg, 120μl, 0.8mmol), and CH₃CN (2ml). Yield: 19.7%. MS: (ESI, neg.) *m/z* 405.1 [M – H][–], ¹H NMR (500 MHz, CDCl₃): δ (ppm) 11.41 (s, 1H), 7.82 (s, 2H), 7.66 (s, 1H), 7.35 (s, 1H), 1.72 (m, 2H), 1.43 (s, 6H), 1.14 (m, 8H), 0.78 (t, 3H, J = 7Hz); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 166.90, 166.04, 161.08, 138.29, 135.17, 133.80, 128.02, 126.76, 122.63, 102.98, 41.90, 41.24, 31.77, 29.78, 26.55, 24.74, 22.77, 14.20. HPLC retention time: 13.910min; purity 97.51%.

4.2.4.17 Synthesis of 2-(2-phenylpropan-2-yl)-5-(thiophen-2-yl)furo[2,3-d]pyrimidin-4-ol (75)

Compound **75** was prepared in a manner similar to compound **63** from 2-(2-phenylpropan-2-yl)pyrimidine-4,6-diol **30** (100mg, 0.434mmol), 2-(2-chloro-2-nitrovinyl)thiophene **62** (76mg, 0.4mmol), DBU (122mg, 120μl, 0.8mmol), and CH₃CN (2ml). Yield: 36.1%. MS: (ESI, neg.) *m/z* 335 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.07 (s, 1H), 8.22 (s, 1H), 7.97 (d, 1H, J = 3.5Hz), 7.50 (d, 1H, J = 5Hz), 7.34 (t, 2H, J = 7.5Hz), 7.25 (d, 3H, J = 6.5Hz), 7.10 (t, 1H, J = 5.5Hz), 1.71 (s, 6H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.23, 164.49, 159.60, 145.72, 137.31, 131.43, 128.32, 128.28, 127.72, 126.58, 126.17, 125.65, 117.49, 102.49, 45.03, 27.51. HPLC retention time: 10.219min; purity 99.69%.

4.2.4.18 Synthesis of 5-(thiophen-2-yl)-2-(2-(thiophen-2-yl)propan-2-yl)furo[2,3-d]pyrimidin-4-ol (76)

Compound **76** was prepared in a manner similar to compound **63** from 2-(2-(thiophen-2-yl)propan-2-yl)pyrimidine-4,6-diol **31** (100mg, 0.423mmol), 2-(2-chloro-2-nitrovinyl)thiophene **62** (76mg, 0.4mmol), DBU (122mg, 120 μ l, 0.8mmol), and CH₃CN (2ml). Yield: 19.2%. MS: (ESI, neg.) m/z 341 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.15 (s, 1H), 8.22 (s, 1H), 7.96 (d, 1H, J = 3Hz), 7.51 (d, 1H, J = 5Hz), 7.44 (d, 1H, J = 5Hz), 7.11 (t, 1H, J = 5Hz), 7.03 (d, 1H, J = 2.5Hz), 6.99 (t, 1H, J = 4Hz), 1.81 (s, 6H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.12, 163.29, 159.49, 150.14, 137.49, 131.35, 128.30, 127.73, 126.75, 125.70, 124.99, 124.59, 117.49, 102.58, 43.21, 28.39. HPLC retention time: 10.151min; purity 98.38%.

4.2.4.19 Synthesis of 2-(2-cyclohexylpropan-2-yl)-5-(thiophen-2-yl)furo[2,3-d]pyrimidin-4-ol (77)

Compound **77** was prepared in a manner similar to compound **63** from 2-(2-cyclohexylpropan-2-yl)pyrimidine-4,6-diol **32** (100mg, 0.423mmol), 2-(2-chloro-2-nitrovinyl)thiophene **62** (76mg, 0.4mmol), DBU (122mg, 120 μ l, 0.8mmol), and CH₃CN (2ml). Yield: 40.2%. MS: (ESI, neg.) m/z 341.0 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.08 (s, 1H), 8.18 (s, 1H), 8.07 (d, 1H, J = 3.5Hz), 7.51 (d, 1H, J = 4.5Hz), 7.13 (t, 1H, J = 5Hz), 1.94 (t, 1H, J = 12Hz), 1.70 (d, 2H, J = 12.5Hz), 1.62 (d, 1H, J = 12Hz), 1.40 (d, 2H, J = 12.5Hz), 1.25 (s, 6H), 1.02 (m, 5H); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.91, 165.26, 159.71, 137.01, 131.53, 128.24, 127.71, 125.59, 117.40, 101.99, 44.95, 43.68, 27.19, 26.38, 26.01, 22.19. HPLC retention time: 11.285min; purity 100%.

4.2.4.20 Synthesis of 2-(2-methyloctan-2-yl)-5-(thiophen-2-yl)furo[2,3-d]pyrimidin-4-ol (78)

Compound **78** was prepared in a manner similar to compound **63** from 2-(2-methyloctan-2-yl)pyrimidine-4,6-diol **33** (100mg, 0.42mmol), 2-(2-chloro-2-nitrovinyl)thiophene **62** (76mg, 0.4mmol), DBU (122mg, 120 μ l, 0.8mmol), and CH₃CN (2ml). Yield: 12.3%. MS: (ESI, neg.) m/z 343.0 [M – H][–], ¹H NMR (500 MHz, DMSO): δ (ppm) 12.16 (s, 1H), 8.71 (s, 1H), 8.00 (d, 1H, J = 3Hz), 7.51 (d, 1H, J = 5Hz), 7.12 (t, 1H, J = 4Hz), 1.72 (m, 2H), 1.30 (s, 6H), 1.19 (m, 6H), 1.05 (m, 2H), 0.81 (t, 3H, J = 6.5Hz); ¹³C NMR (500 MHz, CDCl₃): δ (ppm) 165.37, 165.24, 159.73, 137.04, 131.52, 128.26, 127.72, 125.60, 117.41, 102.01, 40.50, 40.35, 30.99, 29.06, 25.08, 23.94, 22.02, 13.89. HPLC retention time: 11.881min; purity 100%.

4.3 Testing of Novel Compounds

Select compounds from each of the three series of novel pyrimidine cannabinoid ligands were tested for binding affinity to and functional activity at the CB1 and CB2

receptors. Select compounds were also tested for efficacy as anti-glioblastoma agents. The complete series are currently undergoing receptor binding assays and functional activity screens in order to develop SARs for these new structural classes of cannabinoids.

4.3.1 Pyrimidine Hexahydro Classical Cannabinoids

All four of the pyrimidine hexahydro classical cannabinoids were screened for receptor binding affinity to the CB1 and CB2 receptors (**Table 4.1**). All compounds exhibited binding affinity to both receptors with the exception of the phenyl substituted **34** which showed very little affinity for the CB1 receptor ($K_i > 10,000\text{nM}$). Compounds **34** and **35** were > 73.0 and 26.9 -fold selective for the CB2 receptor, respectively, which is greater than either KM-233 or HB-I-172. From this small series of compounds it would seem that aromatic side-chains (compounds **34** and **35**) decrease binding affinity for both receptors yet increase CB2 selectivity. Compounds **36** and **37**, which possess alkyl side-chains, displayed good binding affinity for both receptors yet lower CB2 selectivity (9.7 and 6.5 , respectively). All compounds proved to be agonists at both the CB1 and CB2 receptor subtypes. Compounds **35**, **36**, and **37** were also tested against the human glioblastoma cell line LN-229. All three compounds showed efficacy which was comparable to HB-I-172 and KM-233 (**Table 4.2**).

4.3.2 Pyrimidine Non-Classical Cannabinoids

Compounds **47**, **48**, **52**, and **55** were assayed for receptor binding affinity at CB1 and CB2. The results of the binding assays are found in **Table 4.3**. Each of the four pyrimidine non-classical cannabinoids tested has very little affinity for the CB1 receptor with all four having a $K_i > 10,000$. They do, however, retain affinity for the CB2 receptor in varying degrees. Compound **55** (dimethyl heptyl side-chain with a cyclohexyl replacement of the A ring) possessed the greatest affinity for the CB2 receptor ($K_i = 43.3$ (± 19.6) nM) with a high CB2 selectivity ratio of > 230.9 -fold. Six of the compounds from this series were tested for functional activity at the cannabinoid receptors and were found to be agonists at CB2 with little or no agonist activity at CB1 (probably due to low affinity). Two exceptions were **47** and **55** which exhibited 59 and 68% agonist activity at CB1, respectively. Three compounds **47**, **48**, and **50** were tested against LN-229 and exhibited EC_{50}s ($23.5\mu\text{M} - 55.5\mu\text{M}$) slightly higher than HB-I-172 and KM-233, but much lower than BCNU (**Table 4.4**).

4.3.3 Furanopyrimidine Cannabinoids

Five furanopyrimidines were screened for receptor binding affinity at the CB1 and CB2 receptors. All five compounds exhibited low affinity for both CB1 and CB2 ($K_i > 10,000\text{nM}$). All sixteen compounds were screened for functional activity at the cannabinoid receptors also. Despite having low affinity, several compounds did exhibit

Table 4.1: Binding affinity constants (K_i) for compounds **34-37** compared to Δ^8 -THC, KM-233, and HB-I-172

Compound	CB1 K_i (nM)	CB2 K_i (nM)	CB1/CB2 Ratio
Δ^8 -THC	28.5 ($\pm$ 3.3)	25.0 ($\pm$ 4.8)	1.1
KM-233	12.3 ($\pm$ 0.61)	0.91 ($\pm$ 0.08)	13.5
HB-I-172	6040 ($\pm$ 1477)	487 ($\pm$ 101)	12.4
34	> 10,000	137 ($\pm$ 13)	> 73.0
35	1138 ($\pm$ 227)	42.3 ($\pm$ 13.9)	26.9
36	18.8 ($\pm$ 9.3)	1.94 ($\pm$ 0.63)	9.7
37	54.6 ($\pm$ 5.4)	8.36 ($\pm$ 1.66)	6.5

Notes: Values are the mean of three experiments performed in triplicate; standard deviation is given in parentheses. CB1/CB2 ratio is expressed as K_i for CB1 / K_i for CB2.

Table 4.2: Efficacy of pyrimidine hexahydro classical cannabinoids compared to HB-I-172, KM-233, and BCNU against LN-229

Compound	EC₅₀ (μM) ± S.D.
BCNU	233 (± 5.1)
HB-I-172	9.74 (± 0.56)
KM-233	4.74 (± 0.43)
35	30.1(± 2.9)
36	10.6 (± 1.3)
37	27.3 (± 3.4)

Notes: Values are the mean of three experiments performed in triplicate; standard deviation is given in parentheses.

Table 4.3: Binding affinity constants (K_i) for select pyrimidine non-classical cannabinoids compared to Δ^8 -THC, KM-233, and HB-I-172

Compound	CB1 K_i (nM)	CB2 K_i (nM)	CB1/CB2 Ratio
Δ^8 -THC	28.5 ($\pm$ 3.3)	25.0 ($\pm$ 4.8)	1.1
KM-233	12.3 ($\pm$ 0.61)	0.91 ($\pm$ 0.08)	13.5
HB-I-172	6040 ($\pm$ 1477)	487 ($\pm$ 101)	12.4
47	> 10,000	9673 ($\pm$ 566)	> 1.0
48	> 10,000	130 ($\pm$ 38)	> 76.9
52	> 10,000	621 ($\pm$ 158)	> 16.1
55	> 10,000	43.3 ($\pm$ 19.6)	> 230.9

Notes: Values are the mean of three experiments performed in triplicate; standard deviation is given in parentheses. CB1/CB2 ratio is expressed as K_i for CB1 / K_i for CB2.

Table 4.4: Comparison of selected pyrimidine non-classical cannabinoids to HB-I-172, KM-233, and BCNU against LN-229

Compound	EC₅₀ (μM) ± S.D.
BCNU	233 (± 5.1)
HB-I-172	9.74 (± 0.56)
KM-233	4.74 (± 0.43)
47	55.5 (± 4.3)
48	23.5 (± 2.9)
50	51.8 (± 6.4)

Notes: Values are the mean of three experiments performed in triplicate; standard deviation is given in parentheses.

agonist activity. Compounds **66** and **78** proved to be agonists at CB1 and CB2 when screened at a concentration of 10 μ M. Compounds **65**, **69**, **70**, **74**, **77**, and **78** showed agonist activity at only the CB2 receptor when tested at 10 μ M. Five of the furanopyrimidines were tested against the human glioblastoma cell line T98G. When screened at a concentration of 10 μ M, five of the compounds displayed cytotoxic activity against the GBM cell line (**Figure 4.10**). Interestingly, the cells had to experience a longer exposure to these compounds (72 hours) in order to manifest the toxic effects normally seen in 18 hours with other cannabinoid ligands.

4.4 Experimental Procedures

4.4.1 Radioligand Binding Assays

Binding affinity studies were carried out using cell membranes from HEK293 cells transfected with the human CB1 receptor (K_d for tritiated CP-55,940 binding: 4.5 nM) and membranes from HEK293 cells transfected with the human CB2 receptor (K_d for tritiated CP-55,940 binding: 5.1 nM). Non-specific binding was determined using 10 μ M WIN55, 212-2. Increasing concentrations of compounds to be tested were made ranging from 10⁻¹² M to 10⁻⁴ M and were added in triplicate for each experiment and the individual molar IC₅₀ values were determined using GraphPad Prism software. The corresponding K_i values for each drug were determined utilizing the Cheng-Prusoff equation and final data are presented as $K_i \pm$ standard deviation of $n = 3$ experiments run in triplicate.

4.4.2 Cytotoxicity Assays

Human glioblastoma cells T98G and LN-229 (American Type Culture Collection) were cultured in supplemented media according to the recommendations of the supplier at 37°C in an atmosphere of 5% CO₂ and 95% air. Cell lines were plated in 96-well polystyrene flat-bottom plates (7,500 cells/well) at 70% confluency in a 100 μ l total volume of supplemented media as indicated, and incubated overnight at 37°C to allow for adherence. The media was then replaced with media containing 1% FBS and the cultures were treated with escalating amounts of drug and cell death was analyzed at 18hr or 72hr, using the BioTek Synergy 2 Multidetector Microplate Reader (BioTek Instruments, Inc.). The percentage of viable cells present in the culture at each time point was calculated by comparing the absorbance value at 450nm from the CCK-8 assay (Dojindo Molecular Technologies) for each condition with untreated control cells. All assays were conducted per manufacturer's protocol. All described values represent the average of three data points per determination and three independent determinations.

Figure 4.10: Furanopyrimidine cannabinoids screened against the human glioblastoma cell line T98G

All compounds were screened at 10 μ M for 48 hours. * = $p < 0.005$, ** = $p < 0.0005$, and *** = $p < 0.00005$.

4.4.3 CB1/CB2 Functional Assays

HEK-293 cell lines stably transfected with a cyclic nucleotide-gated channel and either human CB1 or CB2 receptors (BD Biosciences, San Jose, CA USA) were seeded in poly-D-lysine coated 96-well plates at a density of 70,000 cells per well. Plates were incubated at 37°C in 5% CO₂ overnight prior to assay. Plates were then removed from the incubator and the complete growth medium (DMEM, 10% FBS, 250µg/ml G418 and 1µg/ml puromycin) was replaced with 100µL DMEM containing 0.25% BSA. Next, 100µL membrane potential dye loading buffer (Molecular Devices) was added to each well. The plates were placed back into the incubator for 30 minutes and then the baseline fluorescence was read on a BioTek Synergy 2 multi-mode microplate reader with 540nm excitation and 590nm emission filters prior to drug addition. Drugs were added in 50µL DPBS containing 2.5% DMSO, 1.25µM 5'-(N-ethylcarboxamido) adenosine and 125µM Ro 20-1724. Plates were then incubated at room temperature for 25 minutes and fluorescence measured again at 540nm excitation and 590nm emission.

Chapter 5: Cannabinoids as Anti-Inflammatory Agents

5.1 Introduction

Pharmacologically, cannabinoids can be used to affect a variety of targets such as the central nervous system, the cardiovascular system, the immune system and/or endocrine system. More particularly, compounds possessing an affinity for either the CB1 or the CB2 receptor, and potentially GPR55, can act on the central nervous system or act as immunomodulators. In addition, these compounds are useful as anticancer agents, antiobesity agents, analgesics, myorelaxation agents and antiglaucoma agents. Such compounds can also be used for the treatment of thymic disorders, vomiting, various types of neuropathy, memory disorders, dyskinesia, migraine, multiple sclerosis, asthma, epilepsy, ischemia, orthostatic hypotension, osteoporosis, liver fibrosis, inflammation and irritable bowel disease, diabetes, and cardiac insufficiency.

However, certain cannabinoids such as Δ^9 -THC also affect normal neurotransmission, producing undesirable side effects such as drowsiness, impairment of monoamine oxidase function, and impairment of non-receptor mediated brain function. The addictive and psychotropic properties of some cannabinoids tend to limit their therapeutic value. The unwanted psychotropic effects are mediated through the CB1 receptor and are not observed upon activation of CB2. Many of the desirable effects associated with cannabinoids such as antinociception and anti-inflammation are mediated through the CB2 receptor.

Due to the effects mediated by CB2, it appears to be a promising target for the treatment of pain, inflammation, and neuro-inflammation. It is therefore no mystery as to why the current trend in the field is the development of CB2 selective compounds that will not activate the CB1 receptor and will thus be void of the unwanted psychotropic effects. The expression of CB2 was at one time thought to be limited to the periphery, being found mainly in the cells of the immune system.^{17,43} Recently, however, the CB2 receptor has also been shown to be present in the CNS in both perivascular microglial cells¹⁷⁹ and in brainstem neurons.^{42b} CB2 also appears to be up-regulated in dorsal root ganglia and peripheral neurons following injury.¹⁸⁰

CB2 has been validated as a potential target for the treatment of pain and neuro-inflammation based on preclinical data using CB2 selective agonists and receptor knockout mice. Despite the challenge that lies in developing CB2 selective compounds that maintain activity and selectivity across species, several compounds have advanced into clinical development.

5.2 Evaluation of CB2 Agonists in Preclinical Models

5.2.1 Pain

The ability of *Cannabis* to attenuate the pain response has been known for millennia in Asia. The medical use of marijuana was introduced to the West in 1839 by a physician serving with the British in India named W. B. O'Shaughnessy. He published a treatise expounding the usefulness of extracts of the plant in a wide variety of conditions including relieving the pain of rheumatism. The medical use of *Cannabis* grew rapidly in the late 19th and early 20th centuries. In the United States, the plant was even included in the *National Formulary* and *Pharmacopoeia* until 1941 when its use was virtually brought to a halt due to the Marihuana Tax Act of 1937.¹⁸¹ After the discovery of the cannabinoid receptors type I and II and their ligands, both exogenous and endogenous, research into the medical use of marijuana and cannabinoids has grown exponentially. Recently, the putative cannabinoid receptor GPR55 has been proposed as a target for the treatment of pain and inflammation, especially that of neuropathic origin. The largest body of work with cannabinoids in the field of antinociception, however, is with ligands that are selective for the CB2 receptor; therefore, the discussion will be limited to those compounds.

Several CB2 agonists such as GW405833 (**Figure 5.1**), a substituted indole derivative, have shown to be efficacious in preclinical models of neuropathic, inflammatory, and postoperative pain. GW405833 is a CB2 agonist with binding affinities of 3.92 ($\pm$ 1.58)nM for the human CB2 versus 4772 ($\pm$ 1676)nM for CB1 which translates into greater than 1000-fold selectivity.¹⁸² As with many cannabinoids, binding affinity constants and selectivity ratios are found to be variable between species. When assayed against the rat cannabinoid receptors, GW405833 was found to be only 78-fold selective for CB2. Depending on the type of assay used, the compound appears to be a partial agonist at the human CB2 receptor¹⁸² and has also been reported to be a partial inverse agonist at both human and rat CB2 receptors.¹⁸³

In a sciatic nerve partial ligation model of neuropathic pain, GW405833 showed a 63.3% reversal of pain at 1 hour following an i.p. dose of 10mg/kg with an ED₅₀ of 0.077mg/kg. This compound also exhibited a reversal of hyperalgesia with ED₅₀s of 2.58mg/kg and 0.17mg/kg in the hind paw surgical incision model and the Freund's complete adjuvant (FCA) model, respectively. The target was confirmed in a CB2 knockout mouse model in which the compound did not reverse inflammatory pain. GW405833 was also screened in a number of behavioral tests to determine if the compound would elicit unwanted effects due to interaction with CB1. The well established catalepsy assay, rotarod assay examining motor function, and tail flick and hot plate tests which test for acute analgesia were used in these studies. Only the highest dose tested (100mg/kg) showed any positive results in these assays. This suggests that at higher doses GW405833 does signal through CB1, but not at lower doses.¹⁸²

Data has been published by Abbott regarding two CB2 agonists, A-796260¹⁸³ and A-836339¹⁸⁴ (**Figure 5.1**). A-796260 is an indole derivative which possesses the same 2-

Figure 5.1: CB2 selective agonists used in preclinical studies as antinociceptive agents

morpholinoethyl substitution as GW405833. It exhibits binding affinities for the human cannabinoid receptors of 845nM and 4.37nM for CB1 and CB2, respectively, which is a selectivity ratio of 193-fold for the human CB2. Again, selectivity is lessened when assayed for affinity to the rat cannabinoid receptors. Assays obtained the values of 395nM and 13nM for rat CB1 and CB2, respectively, which is 30-fold selective for the rat CB2 receptor. Functional selectivity of A-796260 for the CB2 receptor is greater than binding affinity selectivity for both the human and rat receptors (1380-fold and 175-fold, respectively).

Efficacy of A-796260 as an antinociceptive agent has been evaluated in the FCA-induced model of hyperalgesia using thermal stimulus. The compound exhibited dose-dependent reduction in hyperalgesia following i.p. administration with an ED₅₀ of 2.8mg/kg. In this particular model, the target was confirmed pharmacologically using the CB2 selective antagonists SR144528¹⁸⁵ and AM630.¹⁸⁶ In each case, the analgesic effects of A-796260 were blocked. The CB1 selective antagonist SR141716A, however, had no effect on the analgesia produced by the compound. To evaluate the compound's ability attenuate postoperative pain, a skin incision model was performed. In this study, A-796260 was administered i.p. 1.5 hours following surgery; it dose-dependently lowered mechanical allodynia with an ED₅₀ of 18mg/kg and with a maximum efficacy of 68 (± 7%) without the development of tolerance following repeated administration. In a study of neuropathic pain, the chronic constriction injury of the sciatic nerve model was used. In this study, A-796260 also reduced mechanical allodynia with an ED₅₀ of 15mg/kg with maximum efficacy of 66 (± 9%). This compound has also shown efficacy in an osteoarthritis pain model and performed favorably compared to celecoxib. A-796260 displayed 56% efficacy at a dose of 35mg/kg while celecoxib was 62% efficacious using a 38mg/kg dose. This compound was also screened for CNS effects and none were found up to a 35mg/kg dose.

A-836339, a dimethylthiazole carboxamide derivative, displays similar binding affinities to A-796260. The K_i for binding affinity to the human CB2 receptor is 0.64nM and a selectivity of 425-fold is observed over the human CB1. Affinity for the rat CB2 receptor is very similar to that of the human, K_i = 0.76nM, and again selectivity when compared to the CB1 receptor is lower than in the human receptors (189-fold). Like A-796260, A-836339 is a full agonist at CB2 with an EC₅₀ of 1.6nM. Both compounds have also been shown to be full agonists at CB1 but, of course, much less potent.

A-836339 has been shown to be efficacious in the FCA-induced inflammatory pain model with an ED₅₀ of 1.96μmol/kg and has also shown efficacy in the skin incision model of postoperative pain with an ED₅₀ of 12μmol/kg. The compound has also yielded promising results in a capsaicin-induced model of hyperalgesia and in the chronic constriction injury neuropathic pain model with EC₅₀s of 10.4μmol/kg and 12.9μmol/kg, respectively. A-836339 was shown to have some CB1 mediated effects as determined by decreased horizontal locomotor activity following both 15μmol/kg and 45μmol/kg doses which was reversed by SR141716A treatment. Although A-796260 has been shown to have affinity for the δ-opioid receptor, the antinociceptive effects of A-796260 and A-836339 are not due to interaction with the μ-opioid receptor as exemplified by the inability of naloxone to reverse the analgesic effects of either compound.

The phenylamino pyrimidine GW842166X (**Figure 5.2**) is a clinical candidate belonging to GlaxoSmithKline which is being studied for its anti-inflammatory potential. The compound has been reported to be fully selective for CB2, both human and rat, with EC₅₀s of 63nM and 91nM, respectively. The compound displayed no significant agonist activity at either human or rat CB1 receptors up to 30μM.¹⁸⁷ In the FCA-induced model of inflammatory pain, the compound was shown to be highly efficacious with an oral ED₅₀ of 0.1mg/kg with full reversal of hyperalgesia at an oral dose of 0.3mg/kg. As with A-796260, the development of tolerance was not observed within the timeframe of the study.

A dihydrobenzofuran developed at the MD Anderson Cancer Center, MDA7 (**Figure 5.2**), has shown to be another cross-species CB2 selective compound.¹⁵⁹ The compound has a K_i of 422 (± 123)nM for human CB2 with no affinity for the human CB1 up to 10μM. For rat cannabinoid receptors, the K_i values 2565 (± 695)nM for CB1 and 238 (± 143)nM for CB2. The compound was shown to be a weak partial agonist at the human CB1 at concentrations above 1μM. The compound has produced promising results in attenuation of mechanical allodynia due to both paclitaxel and spinal nerve ligation. The authors also reported a transient antinociceptive effect from thermal stimulus following i.p. administration of a 10mg/kg dose.

Pfizer has reported a dihydrobenzimidazole, PF-03550096 (**Figure 5.2**), that has also showed promise in preclinical research.¹⁸⁸ The compound performed well in a rat colonic pain model utilizing 2,4,6-trinitrobenzene sulfonic acid as a gastric irritant. The authors report that the compound significantly suppressed visceral hypersensitivity in this model which was reversed by the CB2 antagonist SR144528.

5.2.2 Neuro-Inflammation

CB2 selective compounds have also begun to show promise as possible therapeutic options for the treatment of neuro-inflammatory diseases such as multiple sclerosis (MS), Parkinson's disease, Huntington's disease, Alzheimer's disease, and encephalitis. Compounds that do not have CB1 activity would not have been logical choices for treatment of these disorders before the discovery that CB2 is indeed expressed in the CNS.

A preclinical model that is used for the study of MS is experimental autoimmune encephalomyelitis. CB2 was found to be a legitimate target in this model by the fact that CB2 knockout mice have a more severe disease course upon induction than do wild-type mice.¹⁸⁹ Furthermore, mice at the peak of disease development that are treated with the CB2 agonist HU-210 (**Figure 5.3**) are scored much lower on severity rating scales compared to vehicle treated controls. This efficacy is reported by the authors to be due to reduced microglia activation and reduction in the infiltration of myeloid cells.^{189a} HU-210 is an analog of cannabidiol possessing the dimethyl heptyl side chain. Receptor binding affinity for the compound at human CB1 and CB2 is > 10μM and 22.7 (± 3.9)nM, respectively.

Figure 5.2: Further CB2 selective agonists used in preclinical studies as antinociceptive agents

Figure 5.3: CB2 selective agonists used in preclinical studies as anti-inflammatory agents

5.2.3 Other Indications

CB2 selective agonists have also shown promise for indications other than the inflammatory conditions mentioned above. GW833972A, another phenylamino pyrimidine (**Figure 5.3**), is a 1000-fold selective CB2 agonist that has been shown to reduce citric acid-induced cough in a conscious guinea-pig model.¹⁹⁰ The role of CB2 in the action of GW833972A was verified by pretreatment with antagonists for the CB1 and CB2 receptors. The antitussive effects were blocked by pretreatment with the CB2 selective antagonist SR144528 while the CB1 selective antagonist SR141716A had no effect.

HU-210 has been studied in other animal models of disease also. It has been reported that observed *in vitro* effects of HU-210 are suppression of trabecular osteoclastogenesis.¹⁹¹ This effect is reported to be due to inhibition of osteoclast precursors and also inhibition of receptor activator of NFκB ligand. The authors of this report also tested this compound in a model of postmenopausal osteoporosis using ovariectomized mice. HU-210 lessened trabecular bone loss by 66% compared to untreated controls.

A potential role for CB2 agonists in the treatment of the chronic inflammatory disease atherosclerosis has also been identified. The presence of CB2 receptors was verified in both human and mouse atherosclerotic plaques.¹⁹² The non-selective cannabinoid agonist Δ⁹-THC showed significant inhibition of disease progression in a murine model of established atherosclerosis using apolipoprotein E knockouts. THC was dosed at 1mg/kg orally once a day.¹⁹² This dose is below that which is usually associated with the CB1 mediated psychotropic effects of THC. The role of CB2 in this study was verified by the observation that the CB2 selective antagonist SR144528 blocks the therapeutic effect of THC. The authors also observed *in vitro* that THC blocks macrophage chemotaxis which is crucial for the formation of atherosclerotic plaques, an effect that was also blocked by SR144528.

5.3 Evaluation of CB2 Agonists in Clinical Trials

To date, there have been reports of three pharmaceutical companies entering clinical trials with CB2 agonists for the treatment of pain.¹⁹³ Cannabinor, formerly known as PRS-211,375 (**Figure 5.4**) is a fumarate ester derivative of compounds similar to HU-308. In January 2007, Pharmos Corporation reported the results of a Phase IIa experimentally induced pain model performed with cannabinor. In this study the compound failed to meet the primary endpoint which was reduction of capsaicin-induced pain.¹⁹⁴ In this study, however, researchers made the observation that, when compared to placebo, intravenous (i.v.) administration of cannabinor did have a significant effect in the reduction of both pressure-induced and heat-induced pain. In April of that same year, Pharmos reported the results of a separate Phase IIa trial with Cannabinor. The study was designed to examine the usefulness of i.v. cannabinor to attenuate pain following 3rd molar dental extraction. The results were difficult to interpret. The lowest dose of 12mg demonstrated a significant analgesic effect while the higher doses of 24mg and 48mg

Figure 5.4: Cannabinor (PRS-211,375)

CB2 selective agonist studied in Phase II clinical trials.

were not statistically efficacious. After this unexpected pattern of results the company decided to drop further development of i.v. cannabior as an antinociceptive and retargeted the research with this compound to other possible indications and later dropped development all together. One positive note from each of these trials is that cannabior was generally well tolerated with no serious adverse events reported. Pharmos currently has other CB2 selective compounds of undisclosed structure that are currently in pre-clinical studies for the conditions of neuropathic pain, autoimmune disease, and irritable bowel syndrome.¹⁹⁵ GlaxoSmithKline has completed three Phase II clinical trials with GW842166.¹⁹⁶ One trial was for analgesia following 3rd molar extraction and the other two trials were examining the efficacy of the compound for treatment of osteoarthritis pain. The company has not reported the results of these studies and currently lists no cannabinoids in their list of pipeline drugs.¹⁹⁷ In 2009, Glenmark Pharmaceuticals announced the completion of Phase I studies in Europe of GRC10693 which is a CB2 selective agonist (> 4700-fold) that possesses good oral bioavailability and is well tolerated. The company plans to pursue the compound for the treatment of neuropathic pain.¹⁹⁸

5.4 Evaluation of Novel Cannabinoids as Anti-Inflammatory Agents

5.4.1 Initial Study

In order to exploit the therapeutic potential of our cannabinoid ligands as possible anti-inflammatory agents, we designed a preliminary *in vitro* study to determine what effects we could observe. We chose to use cytokine and chemokine production as markers for inflammation. We chose six of our novel synthetic cannabinoids (along with Δ^8 -THC) and screened them for anti-inflammatory activity against A-549 cells stimulated with tumor necrosis factor alpha (TNF- α). The six compounds chosen represented six different chemical classes (**Figure 5.5**). The previously described pyrimidine hexahydro classical cannabinoid **37** was selected along with a similar compound that is a pyridine hexahydro classical with the same dimethyl heptyl side chain **79** prepared in our laboratory by Dr. Suni Mustafa. The non-classical triaryl compounds were represented by HB-I-172, the pyrimidine nonclassical **50**, and the equivalent pyridine non-classical **80** also prepared by Dr. Mustafa. The furanopyrimidine cannabinoids had not been synthesized at this point, but Dr. Mustafa had begun on a series of furanopyridines – one of which was included in this group **81**. Cells were treated with vehicle, drug (at the toxic EC₁ or EC₁₀), TNF- α , or a combination of TNF- α and drug. The cells were allowed to incubate in the presence of the stimulus or drug for periods of 4 or 18 hours. The media was then removed from the cells and flash frozen on dry ice. The samples were then shipped to an independent laboratory for analysis of excreted cytokines and chemokines.

The results of this preliminary study were quite promising. The efficacies of the compounds were evaluated by the levels of the excreted cytokines IL-1 β , IL-6, IL-8, granulocyte colony stimulating factor (G-CSF), monocyte chemotactic protein-1 (MCP-1), macrophage migration inhibitory factor (MIF), and RANTES (Regulated upon

Figure 5.5: Seven compounds initially screened for anti-inflammatory activity

Activation, Normal T-cell Expressed, and Secreted). All of the compounds screened exhibited an attenuation of the inflammatory response manifesting as a reduction in the excretion of one or more of the pro-inflammatory cytokines assayed for (data not shown).

This positive result prompted the design of a study to pick leads from the various chemical classes in our library to screen for anti-inflammatory activity. In order to select leads we first had to define criteria which we would use to make the selections. It was decided to use functional activity as the criterion with which to narrow the compounds. We would first screen a vast number of compounds from the library for functional activity at the CB1 and CB2 receptors. We would then select compounds that matched the functional profiles of the seven compounds screened in the initial study. The list of candidates would then be reduced based on solubility and/or toxicity data. We would then examine the cytokine and chemokine release profiles of human macrophage and dendritic cell lines challenged with lipopolysaccharide (LPS, to simulate a gram negative infection) or peptidoglycan (PGN, to simulate a gram positive infection) and human epithelial and endothelial cell lines challenged with TNF- α or IL-1 β . The candidates would then be screened for their ability to modulate the excretion of cytokines and chemokines in our *in vitro* models. The one or two compounds that proved to be most efficacious would then be moved into an *in vivo* inflammatory model such as bacteremia or sepsis. The overall design of the study is summarized in **Figure 5.6**.

5.4.2 Selection of Compounds to Screen

In order to select lead compounds from our chemically diverse library of cannabinoid ligands, it was necessary to first develop functional profiles for the initial seven compounds in order to develop the paradigm used for selection. It was presumed that since all seven compounds exhibited anti-inflammatory activity that they would each have similar functional activity patterns. This proved to be an incorrect assumption. To develop the functional profiles, we screened each compound in our functional assay platform for the CB1 and CB2 receptors at 10 μ M. The assay determines a compound's ability to affect the alpha-agonist stimulated production of cAMP. Since CB1 and CB2 signal through G α_i , an agonist will decrease the amount of cAMP produced, and agonist will have no effect, and an inverse agonist will increase the production of cAMP. The profiles for each of the initial seven compounds are shown in **Figure 5.7**. The data is presented as a percentage of the cAMP production compared to a control. Higher agonist activity presents as a lower percentage. Lesser agonist activity or antagonism presents a percentage closer to 100 and inverse agonist activity will give percentages greater than 100. The compounds clearly have varied functional activity at CB1 and CB2. Δ^8 -THC along with compounds **37** and **79** has greater agonist activity at 10 μ M at the CB1 receptor than at CB2. In fact, THC appears to be an antagonist and possibly even an inverse agonist in our assay platform. HB-I-172, as previously described, is an antagonist at CB1 and an inverse agonist at CB2. The remaining compounds (**50**, **80**, and **81**) exhibited very little if any agonist activity at CB1 and moderate agonist activity at CB2. The observed differences in the functional profiles of these compounds could point to the possibility that agonists of either the CB1 or CB2 receptors are efficacious in reducing the inflammatory response, or that possibly there is another target involved such as

Figure 5.6: Anti-inflammatory study design

Figure 5.7: Functional profiles of initial seven compounds screened for anti-inflammatory activity

Compounds were screened using the ActOne© whole cell functional assay for the CB1 and CB2 receptors. The assay measures a compound's ability to alter cAMP production stimulated by an alpha agonist. The lower the percentage of control, the greater ability of the compound to suppress the formation of cAMP; therefore, the greater the agonist activity at the given receptor. Percentages closer to 100 represent lower agonist activity or antagonist activity. Percentages above 100 represent activity of an inverse agonist. Data represents a minimum of three experiments run in triplicate.

GPR55. The fact that HB-I-172 showed preliminary efficacy strengthens the possibility that CB2 inverse agonists may be viable candidates for the treatment of inflammatory disorders.

Since there was no clear pattern with which to use as a screening criterion, we chose to develop functional profiles for a large number of our compounds and select a number that had a variety of functional activities. Initially, we screened seventy-five compounds from eight distinct structural classes. The classes were the triaryls, pyridine triaryls, pyrimidine triaryls, pyridine hexahydros, pyrimidine hexahydros, benzofurans, furanopyridines, and the furanopyrimidines. Out of the seventy-five screened, forty-four compounds were carried forward. These compounds were then screened for toxicity against human dermal fibroblasts at 10, 25, and 50 μ M (data not shown). The compounds that exhibited the greatest toxicity at the lowest doses were eliminated. The remaining compounds were further narrowed based on solubility. The compounds that visibly precipitated at the 50 μ M concentration in the cytotoxicity assay were also eliminated. This narrowed down the field significantly and one compound was chosen from each class (**Figure 5.8**) to carry forward into the *in vitro* inflammation assays.

5.4.3 Cytokine and Chemokine Release Profiles

I initially decided to utilize a total of four cell lines in the anti-inflammatory study. Two cell lines were used to model cells of the immune system: THP-1 and KG-1. THP-1 is a human monocytic leukemia cell line that can be differentiated into a macrophage-like phenotype and KG-1 is a human lymphoblastic leukemia line that can be differentiated into a dendritic cell phenotype. Cells of the epithelial and endothelial lineages that also play roles in the inflammatory response were also to be studied. A-549, a human epithelial lung carcinoma cell line, was chosen to model the epithelial response and the human vascular endothelial cell line HUV-EC-C was selected to model the endothelial inflammatory response.

We decided to utilize the previously described MSD® technology to measure the cytokine and chemokine levels in-house. We selected two types of analysis plates for our studies. The first was the Human ProInflammatory 9-PlexTissue Culture Kit which measures the quantitative levels of nine cytokines, namely: IL-1 β , IL-2, IL-6, IL-8, IL-10, IL-12 p70, TNF- α , IFN- γ , and GM-CSF (granulocyte-macrophage colony-stimulating factor). To measure chemokine production, the Human Chemokine 9-PlexTissue Culture Kit was chosen which assays for Eotaxin, Eotaxin-3, IL-8, IP-10 (10 kDa interferon-gamma-induced protein), MCP-1, MCP-4, MDC (macrophage-derived chemokine), MIP-1 β (macrophage inflammatory protein-1 beta), and TARC (thymus and activation regulated chemokine).

In order to test the ability of our assay design to detect the levels of excreted cytokines and chemokines, we initially collected time-course release profiles of each cell line challenged with two different stimuli. This would also give us information as to the optimum time point to use for screening our compounds. The THP-1 and KG-1 cell lines grow in suspension and must be transformed to attach and take on the macrophage and

Figure 5.8: Eight compounds chosen for anti-inflammatory studies

dendritic cell phenotypes, respectively. This is accomplished by incubating the cells in the presence of phorbol 12-myristate 13-acetate (PMA, 10ng/ml) and ionomycin (500ng/ml) for a minimum of 5 days. The transformed cells, along with non-transformed THP-1 and KG-1 for comparison, were challenged with LPS or PGN at 1 μ g/ml and the supernatant media was assayed at 10 time points from 1 to 24 hours post stimulus for the levels of the previously mentioned cytokines and chemokines. The same procedure was performed with A549 and HUV-EC-C stimulated with TNF- α and IL-1 β . A representative graph is shown in **Figure 5.9**. This data demonstrates the release of IL-6 from transformed and non-transformed THP-1 in response to LPS over the 24 hour experiment. The entire data set is included in **Appendix A**. From the time-course experiments we were able to validate the assay platform and to draw some conclusions that helped to shape the remainder of the experiments:

1. There is a detectable, time-dependent release of cytokines and chemokines from the human cell lines THP-1, KG-1, A549, and HUV-EC-C.
2. The cytokines and chemokines reached maximum or near maximum levels at 18 hours post stimulus.
3. THP-1 exhibited a more pronounced response than did KG-1.
4. LPS elicited a greater response from THP-1 and KG-1 than did PGN.
5. The responses elicited from A549 and HUV-EC-C from TNF- α and IL-1 β were comparable.

5.4.4 Screening of Initial Eight Compounds

With the information gained from the cytokine/chemokine release profile experiments, the screening of the eight selected compounds was planned. Due to the lesser response of the transformed KG-1 cells, it was decided to use only the THP-1 cell line to model cells of the immune system and to continue with both A549 and HUV-EC-C. Each of the eight compounds was screened for toxicity against the three cell lines, the EC₅₀s were determined, and the EC₁₀s calculated. The compounds were then screened for their abilities to attenuate the inflammatory response in THP-1 stimulated with LPS or PGN and in A549 and HUV-EC-C stimulated with TNF- α or IL-1 β . The drugs were screened at the EC₁₀ or 25 μ M (whichever was less) and cytokine/chemokine levels were measured 18 hours post stimulus and drug treatment.

Each compound was capable of attenuating the inflammatory response on multiple cell lines as manifested by decreases in the levels of multiple secreted cytokines and chemokines. The entire data set from this series of experiments can be found in **Appendix B**. Significant effects were seen in a number of inflammatory markers; for example, in the transformed THP-1 cell line decreases in the cytokine IFN- γ were observed for all compounds tested in the PGN group and five of the eight compounds tested in the LPS group (**Figure 5.10**). The significance of this effect is that IFN- γ can

Figure 5.9: Time-dependent release of IL-6 from THP-1 stimulated with LPS

Figure 5.10: Effects of eight compounds on release of IFN- γ from THP-1 in response to LPS and PGN

* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.005$.

further activate macrophages and lead to an increased synthesis and release of pro-inflammatory cytokines and chemokines.¹⁹⁹ Significant results were also seen with THP-1 in the levels of IL-12p70 and IL-6 produced in response to both LPS and PGN (**Figures 5.11 and 5.12**, respectively). IL-12p70 is a cytokine that can further activate macrophages; however, its main function is the activation of NK cells²⁰⁰ and differentiation of naïve T cells.²⁰¹ Upon exposure to IL-12, T and NK cells are stimulated to produce and release the pro-inflammatory cytokines IFN- γ and TNF- α which further perpetuate the inflammatory response. IL-6 is a major pro-inflammatory mediator produced mainly by macrophages that elicits a wide range of responses such as lymphocyte activation, increased antibody production, fever, and production of acute phase proteins by hepatocytes. Overproduction of IL-6 has also been implicated in a number of autoimmune disorders such as rheumatoid arthritis.²⁰² Still further positive results were seen in the PGN stimulated THP-1 cells as evidenced by reductions in GM-CSF, IL-1 β , and TNF- α and increases in the anti-inflammatory cytokine IL-10 (**Figures 5.13 and 5.14**). GM-CSF increases the production of granulocytes and monocytes and increases monocyte adhesion.²⁰³ TNF- α and IL-1 β each produce a wide range of inflammatory responses such as increased expression of adhesion molecules on endothelial cells and increases in vascular permeability.²⁰⁴ The increased production of IL-10 following exposure to PGN and five of the eight compounds tested is of interest due to the anti-inflammatory role of IL-10 which inhibits the synthesis of pro-inflammatory mediators such as IFN- γ , IL-2, IL-3, TNF- α and GM-CSF.²⁰⁵

Attenuations in the production of cytokines were also observed in the HUV-EC-C endothelial cell line. Examples are reductions in GM-CSF and IL-6 in response to TNF- α (**Figure 5.15**) and reductions in IFN- γ , IL-12p70, and IL-2 in response to IL-1 β (**Figure 5.16**). IL-2 is a cytokine normally produced by activated T cells and stimulates the growth and differentiation of antigen-selected cytotoxic T cells and is required for the development of T cell immunologic memory.²⁰⁶ The significance of the lower levels of IL-2 produced by endothelial cells is not known.

There were also several noteworthy observations in the levels of chemokines following stimulus and drug exposure. For example, reductions in the production of MIP-1 β were detected following both TNF- α and IL-1 β challenge in both A549 (**Figure 5.17**) and HUV-EC-C (**Figure 5.18**). MIP-1 β has many varied functions in the inflammatory process. It activates neutrophils, eosinophils, and basophils. It also attracts NK, T, and dendritic cells to sites of infection.²⁰⁷ It also induces the synthesis and release of other pro-inflammatory cytokines from fibroblasts and macrophages.²⁰⁸ MIP-1 β is generally produced by macrophages and T cells, and as previously stated in regards to IL-2, the significance in the attenuation of the lower levels of MIP-1 β produced from endothelial and epithelial cells cannot be speculated. Interestingly, the production of MIP-1 β from the macrophage-like transformed THP-1 cell line was not significantly affected by any of the eight compounds tested. Significant reductions in the chemokine IP-10 were also observed for both A549 and HUV-EC-C after TNF- α challenge (**Figure 5.19**). IP-10 attracts monocytes/macrophages, NK, T, and dendritic cells to sites of infection and also promotes T cell adhesion to endothelial cells.²⁰⁹ The triaryl HB-I-172 was also found to decrease the production of IL-8 from HUV-EC-C stimulated with either TNF- α or IL-1 β (**Figure 5.20**). IL-8 is another major pro-inflammatory mediator.

Figure 5.11: Effects of eight compounds on release of IL-12p70 from THP-1 in response to LPS and PGN

* = $p < 0.05$ and ** = $p < 0.01$.

Figure 5.12: Effects of eight compounds on release of IL-6 from THP-1 in response to LPS and PGN

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$, and **** = $p < 0.0005$.

Figure 5.13: Effects of eight compounds on release of GM-CSF and IL-1 β from THP-1 in response to PGN

* = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$, and **** = $p < 0.0001$.

Figure 5.14: Effects of eight compounds on release of IL-10 and TNF- α from THP-1 in response to PGN

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, and **** = $p < 0.001$.

Figure 5.15: Effects of eight compounds on release of GM-CSF and IL-6 from HUVEC in response to TNF- α

* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.005$.

Figure 5.16: Effects of eight compounds on release of IFN- γ , IL-12p70, and IL-2 from HUVEC in response to IL-1 β

* = $p < 0.05$ and ** = $p < 0.005$.

Figure 5.17: Effects of eight compounds on release of MIP-1 β from A549 in response to TNF- α and IL-1 β

* = $p < 0.05$, ** = $p < 0.005$, and *** = $p < 0.001$.

Figure 5.18: Effects of eight compounds on release of MIP-1 β from HUVEC in response to TNF- α and IL-1 β

* = $p < 0.05$ and ** = $p < 0.005$.

Figure 5.19: Effects of eight compounds on release of IP-10 from A549 and HUVEC in response to TNF- α

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$, **** = $p < 0.001$, and ***** = $p < 0.0005$.

Figure 5.20: Effects of eight compounds on release of IL-8 from HUVEC in response to TNF- α and IL-1 β

* = $p < 0.05$.

One of its main functions in the inflammatory response is to recruit neutrophils. Upon exposure to IL-8, neutrophils are mobilized and activated which is necessary for degranulation and the respiratory burst.²¹⁰ Reductions in the chemokine MCP-1 were also observed in THP-1 (**Figure 5.21**) following LPS stimulation and in HUV-EC-C (**Figure 5.22**) following both TNF- α and IL-1 β stimulation. MCP-1 activates macrophages, recruits monocytes, eosinophils, and basophils, and triggers histamine release from basophils.²¹¹ The action of this chemokine has been implicated in allergy, asthma, and arthritis, and a treatment that could effectively reduce the levels of this protein could be useful in those conditions.²¹²

5.4.5 Dose Response of Four Lead Compounds

We decided to determine if the observed reductions in cytokines and chemokines due to the eight cannabinoids screened were dose dependant. The eight compounds were further narrowed down to four based on the compounds' overall effects of the cytokine and chemokine panels. The four that were chosen were the triaryl HB-I-172, the pyrimidyl and pyridyl hexahydros **37** and **79**, and the benzofuran **83** (**Figure 5.23**). Two cell lines were chosen for the dose response study: THP-1 and HUV-EC-C and the compounds were screened in four escalating doses based on each compound's EC₅₀ against each cell line. The highest concentration for each compound was the EC₁₀ or 25 μ M, whichever was lower.

Many of the drugs exhibited a dose dependent suppression of cytokine and chemokine release from THP-1 stimulated with LPS and HUV-EC-C stimulated with TNF- α . The complete data set from this experiment may be found in **Appendix C**. Some examples of the dose dependent reduction are shown in **Figures 5.24** and **5.25**. The first graph depicts the dose dependent reduction in synthesis and secretion of Eotaxin and MCP-4 from THP-1 cells in response to HB-I-172 treatment post LPS exposure. The second graph shows the dose dependent reduction in Eotaxin-3 and TARC from THP-1 cells in response to treatment with the benzofuran **83**. Interestingly, for some cytokines and chemokines, there was a greater reduction in levels due to the lowest concentration of certain drugs (see **Appendix C**).

5.4.6 Further Evaluation of HB-I-172

HB-I-172 was selected out of the four compounds screened due to its broad spectrum anti-inflammatory activity across the cell lines tested and also due to its novel inverse-agonist activity at the CB2 receptor coupled with being an antagonist at CB1. The previously described anti-inflammatory studies, with the exception of HUV-EC-C, utilized cancerous cell lines. The known abnormalities in the signaling cascades of cancers can overlap with the inflammatory pathways within the cell. For example the Akt and ERK pathways have been shown to be one signaling mechanism leading to NF- κ B translocation to the nucleus.²¹³

Figure 5.21: Effects of eight compounds on release of MCP-1 from THP-1 in response to LPS
 * = $p < 0.05$.

Figure 5.22: Effects of eight compounds on release of MCP-1 from HUVEC in response to TNF-α and IL-1β

* = $p < 0.05$, ** = $p < 0.001$, and *** = $p < 0.0005$.

Figure 5.23: Four compounds chosen for dose response study

Figure 5.24: Examples of dose dependent reductions in chemokines produced by THP-1 after treatment with HB-I-172

Figure 5.25: Examples of dose dependent reductions in chemokines produced by THP-1 after treatment with compound **83**

In order to test HB-I-172 in a system that more closely resembles the responses of an *in vivo* environment, three human primary cell lines were used. HB-I-172 and the thiphenyl analog SMM-I-229 (**Figure 5.26**) were screened against human lung blood microvasculature endothelial cells (HMVEC-LBI, Lonza, Inc.), human small airway epithelial cells (SAEC, Lonza, Inc.), and human monocyte derived macrophages (AllCell, Inc.). SMM-I-229 possesses the same inverse agonist activity at CB2 as HB-I-172 and is also an antagonist at CB1.

Cytokine and chemokine release profiles were determined for the three cell lines, as with the initial experiments, and EC₅₀s were then determined for the two compounds against the cell lines. See **Appendix D** for the complete set of release profiles. The anti-inflammatory assays were then carried out as before. The HMVEC-LBI and SAEC cell lines were stimulated with TNF- α and the primary macrophages were stimulated with LPS before drug treatment. The supernatant media was then assayed 18 hours later for levels of the same cytokines and chemokines. The complete set of graphs can be found in **Appendix E**. While the effects of HB-I-172 on the three primary cell lines were different than in the original cell lines tested, there were reductions in several of the pro-inflammatory cytokines and chemokines assayed for.

In order to test the possible contributions of cannabinoids in the treatment of neurological inflammatory disorders such as meningitis, Alzheimer's disease, Parkinson's disease, Huntington's disease, and neuropathic pain, we next tested HB-I-172 and the CB1 agonist/CB2 antagonist classical cannabinoid KM-233 primary human microglia (Clonexpress) challenged with LPS. Again, the first step was to determine cytokine and chemokine release profiles for the cell line due to LPS exposure (**Appendix F**). The EC₅₀s were then determined for the two drugs against the cells and the assay performed as before. HB-I-172 reduced the levels of a majority of the cytokines and chemokines assayed including IFN- γ , IL-6, and MCP-1 which have all been found to play roles in a number of neuro-inflammatory conditions²¹⁴ (**Appendix G**). KM-233 also performed well in this assay. There were, however, differences in the anti-inflammatory profile of the two drugs, probably due to the different functional activities at the cannabinoid receptors.

In order to strengthen the case for the use of CB2 inverse agonists as anti-inflammatory agents, we chose to compare HB-I-172 to the clinically utilized anti-inflammatory drug dexamethasone. HB-I-172 was compared to dexamethasone in the THP-1 cell line stimulated with LPS and in A549 and HMVEC-LBI stimulated with TNF- α . In the majority of cytokines and chemokines assayed for, HB-I-172 performed as well or better than dexamethasone (See **Appendix H** for complete data set).

In conclusion, we have demonstrated that HB-I-172 is a viable candidate for development as an anti-inflammatory agent. The compound has exhibited *in vitro* reductions in the production and secretion of a wide range of cytokines and chemokines from a variety of cell types involved in the inflammatory process. Not only have we demonstrated that the compound is efficacious in standard inflammatory models using cancerous cell lines (THP-1, KG-1, and A549) but that this activity also translates into efficacy when normal human primary cells are utilized and when compared to a standard therapy. A number of future studies are planned to further evaluate the utility of HB-I-

Figure 5.26: CB2 inverse agonists screened as anti-inflammatory agents against human primary macrophages, epithelial, and endothelial cells

172 as an anti-inflammatory agent. These include an experiment to test the compound's ability to favorably modulate the immune response of T and B cells by observing the effects on antigen receptor-dependent activation using murine splenocytes, an *in vivo* endotoxemia model, and murine models of Huntington's disease, multiple sclerosis, and neuropathic pain. Future studies are also planned to determine the exact molecular target of HB-I-172 that is responsible for the anti-inflammatory activity and the signaling pathway that this activates. Knowledge gained from the work with HB-I-172 in the treatment of glioblastoma (**Chapter 3**) gives us some clues as to possible signaling mediators involved. As stated earlier, the Akt and ERK pathways have been shown to be one signaling mechanism that leads to NF- κ B translocation to the nucleus during the inflammatory response. The profound effects of HB-I-172 on the PI3K/Akt and Raf1/MEK/ERK pathways in glioma may also be found in cells treated with the compound following inflammatory challenge. As observed in glioma, the effects of HB-I-172 on STAT3 and GSK-3 β may also play a role, as each of these proteins has been shown to be mediators of inflammation.²¹⁵ We also believe that the possible interaction of HB-I-172 with the GPR55 receptor may contribute to its action as RhoA has been shown to contribute to NF- κ B-dependent proinflammatory gene transcription.²¹⁶

5.5 Experimental Methods

5.5.1 Transformation of THP-1 and KG-1

Differentiation of Monocytes: To THP-1 human leukemia monocytes (ATCC #TIB-202) or KG-1 human leukemia myeloblasts (ATCC #CCL-246) in suspension was added phorbol 12-myristate 13-acetate (PMA Aldrich #P1585) and ionomycin (Aldrich #I0634), 10 and 500 ng/ml respectively, to induce differentiation into macrophage-like or dendritic-like cells, respectively. Cells were seeded at 30,000 cells/well in a 96-well polystyrene dish and allowed to incubate at 37°C in 5% CO₂ / 95% air for 5-10 days to complete transformation. Media was refreshed as needed until assay.

5.5.2 Cytokine and Chemokine Assays

A549 (ATCC #CCL-185), HUV-EC-C (ATCC #CRL-1730), HMVEC-LBI (Lonza #CC-2815), SAEC (Lonza #CC-2547), monocyte derived macrophages (AllCells #PB-MDM-001F), microglia (Clonexpress #HMG 030), or differentiated THP-1 or KG-1 cells were maintained according to suppliers recommendations and were seeded on 96-well polystyrene plates at a density of 300,000 cells/ml (100 μ L per well) and incubated at 37°C in 5% CO₂ / 95% air for 24 hours to allow cell attachment. Drug solutions were prepared in DMSO at 100x concentration and mixed 1:100 in media containing 1% FBS to yield the desired concentration. Plates were then removed from the incubator and the complete growth media was replaced with 50 μ L media containing 1% FBS and lipopolysaccharide or peptidoglycan at 1 μ g/ml (for macrophages, microglia, or differentiated THP-1 or KG-1), or TNF- α (10ng/ml) or IL-1 β (1 ng/ml) in the case of

A549, HUV-EC-C, HMVEC-LBI, or SAEC or without stimulus in the case of control wells. Cells were returned to the incubator for 1 hour before drug treatments. Drug-media solutions were prepared at 2x desired final concentration in media containing 1% FBS and the appropriate stimulus at the previously mentioned concentration. Control media was also prepared which contained only vehicle (DMSO). 50 μ L of media containing drug or vehicle alone was then added to appropriate wells and the plates returned to the incubator for 18 hours. Media supernatants were then assayed for cytokines and chemokines using the Human ProInflammatory 9-PlexTissue Culture Kit (MSD # K15007B-1) and the Human Chemokine 9-PlexTissue Culture Kit (MSD # K15001B-1) according to the manufacturer's protocol. The plates were then imaged on the SECTOR Imager 2400 (Meso Scale Discovery) according to the manufacturer's instructions.

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Appendix A: Cytokine and Chemokine Release Profiles of THP-1, KG-1, A549, and HUV-EC-C

Appendix B: Effects of Eight Cannabinoids on Cytokine and Chemokine Release of THP-1, A549, and HUV-EC-C

**Appendix C: Dose Response of Four Cannabinoids on Cytokine and Chemokine
Release of THP-1 and HUV-EC-C**

**Appendix D: Cytokine and Chemokine Release Profiles of HMVEC-LBI, Monocyte
Derived Macrophages, and SAEC**

**Appendix E: Effects of HB-I-172 and SMM-I-229 on the Cytokine and Chemokine
Release of HMVEC-LBI, Monocyte Derived Macrophages, and SAEC**

Appendix F: Cytokine and Chemokine Release Profiles of Primary Human Microglia

**Appendix G: Effects of HB-I-172 and KM-233 on the Cytokine and Chemokine
Release of Primary Human Microglia**

Appendix H: Comparison of the Effects of HB-I-172 to Dexamethasone on the Cytokine and Chemokine Release of THP-1, A549, and HMVEC-LBI

Vita

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