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Functional Activity and Switching of Novel Cannabinergic Ligands

Abstract

Pursuant to the discovery of the cannabinoid receptors, research in this field has grown exponentially over the last 2 decades. With their utility in various disease states such as heart disease, cancer, stroke, neurodegenerative and inflammation, cannabinoids stand poised to become a great therapeutic agent. This research seeks to better understand the functional mechanism of cannabinoids, in the hopes of ascertaining which molecular attributes confer desirable selectivity and functional activity.

Looking first at classical benzchromene core analogues, we have shown that presence of an aromatic substitution at C-1' imparts a CB1 agonist, CB2 antagonist. This is a unique mechanism and one of the first examples of an agent with dual affinity and opposing activity.

In the triaryl series of compounds, more insight is gained for the functional pharmacophore of cannabinoids. With this series of compounds we are able to learn that minor changes are able to confer profound differences, such as C-1'-gem-dimethyl derivatives are agonists while C-1' ketones are inverse agonists.

Within these triaryl series, newly synthesized derivatives help to round out the functional pharmacology for these compounds - allowing a more direct comparison to the previously synthesized classical core compounds. In total, a more thorough understanding of the function, which follows form of several cannabinergic compounds, is gained through this exercise and research.

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**FUNCTIONAL ACTIVITY AND SWITCHING OF NOVEL CANNABINERGIC
LIGANDS**

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
Bret Alan Koertge
May 2015

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DEDICATION

This dissertation is dedicated to
my loving and supportive wife,
Stephanie Nicole Koertge,
who has endured, enabled and
pushed me for all the years of
research this document represents.

Also, to my children, may you
always keep your curiosity and wit.

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ABSTRACT

Pursuant to the discovery of the cannabinoid receptors, research in this field has grown exponentially over the last 2 decades. With their utility in various disease states such as heart disease, cancer, stroke, neurodegenerative and inflammation, cannabinoids stand poised to become a great therapeutic agent. This research seeks to better understand the functional mechanism of cannabinoids, in the hopes of ascertaining which molecular attributes confer desirable selectivity and functional activity.

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CHAPTER 1. INTRODUCTION

1.1. History of Cannabinoids

Cannabinoids have enjoyed a very long and somewhat sordid history. Originally grown for its hemp fiber for use in the fashioning of clothes, ropes and paper, it has a history of therapeutic use in India and China for nearly 5000 years.¹ The use of *Cannabis sativa* preparations for treatment of a variety of pathological conditions in ancient Indian, Chinese, and Egyptian civilizations is well-documented.² This documentation and literature is rife with therapeutic indications for Cannabis with usage as an analgesic,^{3,4} an anesthetic,⁵ an appetite stimulant,⁶ an anti-inflammatory agent,⁷ and several other ailments and afflictions.² Although it took time to spread throughout the Western world for recreational and medicinal use, the true evolution of (-)-*trans*- Δ^9 -tetrahydrocannabinol (Δ^9 -THC, the active psychotropic agent of the *Cannabis sativa* plant) (**Figure 1.1**) into a therapeutic agent has a much more recent history.

Early in the nineteenth century, natural product interest and research began to gain a foothold, 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. This would hold true until Marshall and coworkers isolated a mixture of terpenes from an ether extract of Indian hemp in 1896,⁸ which produced the same pharmacological effects observed with *Cannabis sativa* use. This provided the first observational evidence that the principal active compound was not an alkaloid but a terpene. In the early 1930s Cahn and coworkers isolated cannabiol (**Figure 1.1**) and determined a partial structure after conducting extensive chemical degradation studies.⁹ This discovery was followed in rapid succession by the synthesis of several derivatives of cannabiol and $\Delta^{6a,10a}$ -tetrahydrocannabinol ($\Delta^{6a,10a}$ -THC) (**Figure 1.1**) by the Adams group^{10,11} and the Todd group.¹² The pharmacological activities of cannabiol 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 Δ^9 -THC, was reported in 1964 by Gaoni and Mechoulam.¹³ The activity of this cannabinoid was subsequently studied in a number of animal models. These studies ranged from evaluation of the cataleptic reaction in mice¹⁴ to the effects on motor activity and gross behavior in mice and rats.¹⁵ There was also extensive experimentation in rhesus monkeys¹⁶ which ultimately led to the pharmacological characterization of cannabinoids. Following these reports, several other constituents of marijuana such as cannabidiol, cannabigerol, cannabichromene, cannabicyclol (**Figure 1.1**) 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

Figure 1.1. Structures of compounds isolated from *Cannabis sativa*

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.

1.2. Cannabinoid Receptors

1.2.1. History and background of cannabinoid receptors

The beginning of the exponential growth seen in the field of cannabinoid research in the last two decades 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 via cannabinoid receptors but by dissolution into and 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.¹⁷ However, the highly stereospecific activity associated with several cannabinoids¹⁸ suggested receptor mediated action. Then, in 1988, Devane and coworkers¹⁹ utilized a tritium labeled synthetic cannabinoid to tag specific sites in the brain and on neuronal cells. This then provided strong evidence for the presence of a specific cannabinoid receptor in the rat brain. This discovery directly preceded 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).²⁰ 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.²¹ An amino terminal variant of the human CB₁ receptor has also been reported,²² in which a 167 base portion of the CB₁ coding sequence is spliced out of the mRNA – termed CB_{1A}.

The cannabinoid receptors belong to the superfamily of G-protein coupled receptors¹⁴ (GPCRs), are single polypeptides with seven transmembrane α -helices, an extracellular, glycosylated N-terminus and intracellular C-terminus. The CB₁ and CB₂ receptor subtypes each possess 68% amino acid identity within their transmembrane regions but only 48% homology throughout the entire peptide¹⁴ – a trait that is unique among the CB₁ and CB₂ receptors. Many other GPCR families share approximately 75% homology among the subtypes. Also, it is noteworthy that the CB₁ receptor is highly conserved across mouse, rat and human; however, there is only an 81% amino acid identity of the CB₂ receptor across rat and human variants.²³

Whether there are additional cannabinoid receptors is still a question that plagues many researchers in this field. This is, in part, due to the observation that the endogenous cannabinoid ligand, anandamide, induces spinal anti-nociception via a different mechanism than Δ^9 -THC.²⁴ In 2000, Di Marzo and coworkers carried out studies²⁵ using transgenic mice²⁶ 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 GPR55.²⁷⁻²⁹ This receptor is reported to be expressed in several tissues and might function in regulation of vascular tone and immune-cell migration.²⁹ Anandamide is also known to act on other classes of receptors such as the vanilloid receptor, TRPV₁. Anandamide activates TRPV₁ and produces membrane currents or increases in intracellular calcium levels.³⁰⁻³² The presence of these multiple sites for cannabinoid action (along with the aforementioned possibility for yet undiscovered sites) coupled with several signaling pathways and associated second messengers is compelling evidence for the complex pharmacology associated with cannabinoids.

1.2.2. The CB₁ receptor

The CB₁ receptor is a 52.8 kDa, 472 amino acid polypeptide, found in both the CNS and periphery in both neural and non-neural tissues. CB₁ is coupled to inhibitory G_{i/o} proteins³³ which bind to its third intracellular loop and its C-terminus.³⁴ CB₁ receptors may also exert an action on G_s proteins to stimulate cyclic AMP (cAMP).³⁵ 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.³⁶ Activation of CB₁ causes inhibition of adenylyl cyclase and a reduction in cAMP levels. Reduction of cAMP affects phosphorylation by protein kinase A which causes modulation of ion channels and other secondary messengers. Activation of voltage-sensitive outwardly rectifying K⁺ channels,³⁷ inwardly rectifying K⁺ channels³⁸ and inhibition of voltage gated N, L, P and Q-type Ca⁺² channels³⁹ are observed in response to CB₁ receptor activation.

The CB₁ receptor regulates different members of the mitogen activated protein kinase (MAP kinase) family of enzymes such as:

1. extracellular signal-regulated kinase (ERK)⁴⁰⁻⁴²
2. c-Jun N-terminal kinase^{43,44}
3. p38.^{44,45}

Activation of MAP kinase is associated with:

1. activation of immediate early genes like c-Fos and Egr1 (Krox-24)⁴¹
2. activation of focal adhesion kinase (important for integrating cytoskeletal changes associated with signal transduction events)
3. activation of phospholipases (PL), PLA, PLC, and PLD⁴⁶
4. activation of the Na⁺/H⁺ exchanger⁴⁷
5. activation of phospholipase A₂, which in turn causes release of arachidonic acid leading to the synthesis of prostaglandins.⁴⁸

There is also evidence of a second pathway initiated by CB₁ receptor stimulation coupled with sphingomyelinase activation and subsequent release of ceramide.⁴² Ceramide then causes activation of the Raf-1/MAP kinase cascade⁴⁹ and also carnitine palmitoyltransferase I in mitochondrial membranes to stimulate ketogenesis and fatty acid oxidation.⁵⁰ The elevation of intracellular ceramide have also been suggested to be

one of the possible factors responsible for the apoptotic effects of cannabinoids seen on ceramide-related cancers such as glioblastoma.⁵¹

1.2.3. The CB₂ receptor

The CB₂ cannabinoid receptor, also referred to as the peripheral cannabinoid receptor, is a 41 kDa, 360 amino acid polypeptide predominantly expressed in the immune system.^{52,53} Its amino acid sequence is quite different from that of CB₁, especially in the amino terminal domain, which is significantly shorter and not as well conserved in the CB₂ receptor when compared to the CB₁ receptor. The amino acid residues involved in ligand recognition are also found, as it was for the CB₁ receptor, in transmembrane domains 3, 5 and 6 for the CB₂ receptor.⁵⁴

Like the CB₁ receptor, CB₂ couples 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 adenylyl cyclase and therefore a reduction in cAMP levels, as it was with CB₁. 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.³⁸

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.⁵⁵ More recently, however, CB₂ expression has been confirmed in the brain on microglia,⁵⁶ blood vessels,⁵⁶ and on some neurons.^{57,58} CB₂ has a much higher prevalence than CB₁ within the immune system. The expression level of the CB₂ gene in immune tissues is 10 to 100 times the level of the CB₁ gene.⁵⁹ In fact, the CB₂ mRNA content of the spleen and tonsils is almost equivalent to the CB₁ mRNA content in the CNS.⁵⁹ This first led researchers to suggest that CB₂ receptor might play an important role in immune function – more specifically the down-regulation thereof. 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.⁶⁰ CB₂-specific ligands and specific targeting of the immune system is possible by virtue of the fact that between transmembrane domains 1 and 7, including the loop regions, the CB₂ receptor is only 44% identical to the CB₁ receptor.

1.2.4. The GPR55 receptor – A putative cannabinoid receptor

The GPR55 receptor, first identified and cloned by O'Dowd in 1999,^{61,62} found favor as a putative cannabinoid receptor by an *in silico* screen due to an amino acid sequence in the binding region that is similar. Research groups at the pharmaceutical companies Glaxo-Smith-Kline and AstraZeneca then extensively characterized the receptor in the hope that it would be responsible for the blood pressure lowering effects of cannabinoids. GPR55 demonstrates activation 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 (**Figure 1.2**).

Figure 1.2. Synthetic and endogenous cannabinoid compounds

Some of the known CB₁ and CB₂ ligands also bind to GPR55. The receptor is activated by Δ^9 -THC and cannabidiol,²⁹ synthetic cannabinoids such as CP-55,940 and the endocannabinoids anandamide (AEA), 2-arachidonoylglycerol (2-AG), and nolandin ether (**Figure 1.2**).²⁹ Recent research suggests that lysophosphatidylinositol and its 2-arachidonoyl derivative (**Figure 1.2**) may be the endogenous ligands for GPR55^{63,64} thus giving rise to it being a potential LPI receptor.⁶⁵ GPR55 appears likely to be a possible target for treatment of inflammation and pain as with the other cannabinoid receptors.⁶⁶⁻⁶⁹

While the CB₁ and CB₂ receptors couple to G $\alpha_{i/o}$ proteins, GPR55 is coupled to G α_{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).²⁷ 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.⁷⁰ Stimulation of GPR55 induces F-actin formation under the control of G α_{13} , RhoA and Rho-associated, coiled-coil containing protein kinase (ROCK).⁶³ 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).⁶³ The physiological role of GPR55 remains unclear. Mice with a target deletion of the GPR55 gene show no specific phenotype.⁷¹ 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.²⁹ Osteoblasts and osteoclasts have also been shown to express GPR55 and this has been shown to regulate bone cell function.⁷²

1.3. Cannabinoids in Disease States

In the United States of America, heart disease (**Section 1.3.1**), cancer (encompassing all variants under the auspice thereof) (**Section 1.3.2**), stroke (**Section 1.3.3**), Alzheimer's Disease (**Section 1.3.4**) and diabetes (**Section 1.3.5**) are five of the top seven causes of deaths. Many of these disease states have complex pathogenesis and are amalgamations of several years of poor habits and lifestyles of individuals; however, once diagnosed, treatment of many of these disease states is tenuous, at best. There are data that strongly point to the usefulness of cannabinoids in all of these disease states. It is for the betterment of treatment not only for these diseases, but for cannabinoid use therein that this project finds its utility and mission – to better design a cannabinergic agents we must first understand how they function. While it is well and good to understand the need for a CB₁ antagonist and a CB₂ receptor agonist to positively impact cerebral ischemia in stroke,⁷³ understanding the functional activity of drugs prior to their use *in vivo* is essential for targeted drug design and resource optimization. Furthermore, use of this information to develop a functional pharmacophore of cannabinoids helps to expedite and further hone the synthetic design of cannabinergic ligands. However, as with any project, especially one with this potential benefit, one must understand the target disease state to maximize the management thereof. Briefly, I will examine five of the top seven disease states and the positive role cannabinoids may play in each.

1.3.1. Cannabinoids in heart disease

1.3.1.1. Pathogenesis of heart disease. While the true derivation and progression of this disease is innately complex and multifactorial, there are certain key elements that lend themselves to the progression of the disease. One of these main components of heart disease is atherosclerosis, which is an inflammatory disease, at its root.^{74,75} This inflammation is a response to damage done to the blood vessels as a result of plaque deposit or rupture or small intima tears caused by high blood pressure. Cannabinoids find usefulness in treating this pathogenesis from acting as: antihypertensive agents (**Section 1.3.1.2**), anti-ischemic agents post-MI (**Section 1.3.1.3**) or anti-inflammatory agents (**Section 1.3.1.4**).

1.3.1.2. Cannabinoids as anti-hypertensive agents. While the cardiovascular effects of cannabinoids has been known since the 1960s, harnessing their power to positively impact the cardiovascular system has languished.⁷⁶ The first possible impact cannabinoids may have on this process is in the realm of blood pressure control. The peripheral effects of systemically administered cannabinoids *in vivo* seem to predominate in the overall activity of these compounds.⁷⁷ IV administration of anandamide causes a triphasic response in the blood pressure of anaesthetized rats which transitions from a transient drop, to a short-lived spike and ending in a prolonged third phase of lower blood pressure⁷⁸ – similar to the onset of higher doses of clonidine.⁷⁹ The initial phase of lowered blood pressure appears to be due to vagal output, as atropine and cervical vagotomy eradicate this initial drop.⁸⁰ The secondary, more sustained, drop in blood pressure shows signs of being CB₁-mediated through presynaptic inhibition of sympathetic output in the periphery.^{78,80} In addition to the sympathetic outflow inhibition, data exists which suggest that there is a direct vasodilatory effect of cannabinoids, as HU-210 has been shown to lower blood pressure in spite of sympathetic blockade.^{80,81}

Overall, anandamide is hypothesized to work by the aforementioned reduction of peripheral vascular resistance as well as a decrease in cardiac contractility.^{82,83} This dual mechanism for blood pressure control is consistent with many other drugs currently on the market for the treatment and management of hypertension. Additionally, mitigation of these effects occurs by administration of the CB₁ inverse agonist SR-141716A.⁸³

In humans, there is a noted tachycardia and minor pressor effect upon acute administration of cannabinoids; however, this gives way to bradycardia and hypotension with repeated dosing.^{84,85} It is worth noting, that there are differences in levels of wakefulness between the laboratory animals and the human test subjects.⁸⁶ A comparison of this effect was noted, where monkeys in a sterile, predictable laboratory environment demonstrated the same bradycardia effect noted in previous animal models after initial dosing.⁸⁷ This effect, while notable and profound for exogenously administered cannabinoids flies somewhat in the face of convention when one considers the normotensive state of CB₁ knockout mice⁸⁸ and FAAH-deficient mice had a normal hemodynamic profile.⁸⁹ Taken as a whole, these results show how endogenous cannabinoids do not exert tonic control of the cardiovascular system, under normal conditions; however, under abnormal conditions (i.e. hypertension), endocannabinoid tone may blunt the elevation of blood pressure and cardiac contractility.⁹⁰

Many *in vitro* studies have corroborated some of the current *in vivo* mechanisms, as well as generate hypotheses in their own right. First, *in vitro* experiments conducted in isolated, Langendorff-apparatus-perfused, rat hearts⁹¹ and in isolated, electrically stimulated human atrial muscle,⁹² have demonstrated HU-210's and anandamide's ability to decrease cardiac output, as was the case *in vivo*.⁹³ A second set of experiments sought to elucidate the mechanism of HU-210's vasodilator effects in spite of sympathetic blockade; however, those studies have done little to tease apart a true mechanism.^{77,94,95} This variability of mechanism also varies from species to species, where a rat aorta may experience a 20% reduction,⁹⁶ while rabbit aorta experiences an 80% relaxation.⁹⁷ It is certain that cannabinoid-induced arachidonic acid production⁹⁸ and/or nitric oxide (NO) production⁹⁹ are not mechanistic routes for the vasodilatory actions thereof in most species or vascular beds, suggesting that cannabinoid vasodilation is site-specific. A final mechanism put to *in vitro* testing is that of alternate receptor activation, more namely transient receptor potential vanilloid, type 1 (TRPV₁) and the stimulation thereof to release calcitonin gene-related peptide (CGRP) from sensory nerves. This has shown itself to be of minor consequence, as CB₁ knockout⁸⁸ and TRPV₁ knockout⁸² mice both present with normal hemodynamic profiles. The potential for involvement of TRPV₁ and GPCR do provide some insight into the sex-linked differences of vasodilation of cannabinoids – with females experiencing a higher degree of vasodilation, possibly due to higher levels of estrogens, a well-documented vasodilator.¹⁰⁰

1.3.1.3. Cannabinoids as anti-ischemic agents post-MI. Following a myocardial infarction (MI), the ischemic damage that occurs to the cardiac tissue is of top concern when evaluating a patient for long-term prognosis. Mitigation of the infarct is of the utmost importance, to ensure a full recovery with little to no loss of cardiac function. Cannabinoids, especially the endocannabinoids anandamide¹⁰¹ and palmitoylethanolamide¹⁰² have shown promise in minimizing the infarct size of experimental models of myocardial ischemia; however, there is evidence the mechanisms thereof are due to an alternate receptor pathway that has yet to be fully characterized.^{101,103} In contradiction to the novel pathway theory, there is data that suggest CB₁ and CB₂ are involved as a cohort, with or without a novel receptor site. CB₁- and CB₂-selective agents have been shown ineffective in minimizing the infarct size. Selective antagonism of CB₁ or CB₂ by SR-141716A or SR-144528, respectively abolishes anandamide's ability to influence infarct size.¹⁰¹

1.3.1.4. Cannabinoids as anti-inflammatory agents post-MI. As previously mentioned, atherosclerosis is one of the major causes of heart disease, and is itself a state of chronic inflammation.¹⁰⁴ Even at low doses, THC demonstrates a blockade to the advancement of atherosclerotic lesions in a murine model thereof.¹⁰⁵ Validating the mechanism of this being a CB₂-mediated effect on the atherosclerotic lesion,¹⁰⁶ pretreatment with SR-144528 serves to block the beneficial effects of THC in this model.¹⁰⁵ The same study that helped validate a CB₂-mediated regulation of atherosclerotic plaque formation also provided evidence of reduced T_H1-helper response and blockade of macrophage migration to the site, both of which contribute to early development processes in atherosclerosis. Further evidence to implicate CB₂ as the prime target arose from an *in vivo* murine model of established atherosclerosis and *in vitro* macrophage migration. These data, which show that peritoneal macrophages isolated

from CB₂ knockout mice as well as pretreatment of wild-type macrophages with SR-144528, did not respond to THC-induced migration.¹⁰⁵ Finally, there was evidence demonstrating the presence of CB₂ receptor expression on the atherosclerotic vessels, at the site of plaque formation, but vessels devoid of disease showed no such expression of CB₂ receptors.¹⁰⁵ Taken in totality, this suggests CB₂-selective agonists may be a desirable therapeutic agent in the treatment and/or prevention of atherosclerosis; however, there is a need for more *in vivo* work of this hypothesis before any definitive answer regarding the vitality of this as a treatment option.

1.3.2. Cannabinoids in cancer

1.3.2.1. Cancer rates in The United States of America. Cancer is an all-encompassing disease state, and it is as this cohort that it becomes the second leading killer of persons in The United States of America. Under this auspice, there are many subtypes of cancer to consider, and treatment modalities differ among them. The most recent compiled data from The American Cancer Society (2009) shows that among men and women, lung & bronchus cancer (**Section 1.3.2.2**) is the leading type of cancer for both groups – approximately 61 deaths per 100,000 males and approximately 40 deaths per 100,000 females. Looking specifically at the gender-related cancers, prostate cancer (**Section 1.3.2.3**) is the second highest killer of men at approximately 22 deaths per 100,000 males, and breast cancer (**Section 1.3.2.4**) ranks second amongst women at approximately 22 deaths per 100,000 females. The pathogenesis of each cancer is far more complex and involved of a process to cover in detail within this document. However, the crux of any cancer is that cells lose their normal apoptotic signaling and persist at an abnormal rate of cell division, eventually entering into lymphatic circulation and metastasizing to distant sites and causing similar hyperproliferative growths.

1.3.2.2. Cannabinoids in lung and bronchial cancers. Being the leading type of cancer in The U.S.A. for both men and women, it is logical that we begin the examination of cannabinoids in cancer with lung and bronchial cancers. Within lung and bronchial cancers, there are several subtypes still, with non-small cell lung cancer (NSCLC) being the most prevalent subtype.¹⁰⁷ In NSCLC, CB₁ and CB₂ receptors are overexpressed 24% and 55%, respectively, of the cases studied.¹⁰⁸ There exists several reports that point to EGFR hyperexpression and hyperactivation, which promotes resistance of lung cancer to current mainstays of chemotherapy. Given, too, that there is known cross-talk of GPCRs and EGFR, where GPCRs transactivate EGFRs,¹⁰⁹ use of CB₁ and/or CB₂ agents to inhibit the untoward effects of EGFR may present therapeutically viable option. This EGFR effect has seen positive results *in vitro* with WIN-55,212-2, a non-selective cannabinoid receptor agonist, significantly impeding the growth of A549 cancer cells by 60-70%.¹⁰⁸ The CB₂-selective agonist JWH-015 also reduced cell viability by 40-50%.¹⁰⁸

On the converse of this data lies a much more dubious section of data. In some studies, THC at low doses (100 – 300 nM) demonstrated a pro-proliferative action in NCI-H292 cells via cross-talk between cannabinoid receptors and EGFR; however, this same study showed that μ M concentrations of THC demonstrated anti-proliferative

action.¹¹⁰ Another similar study demonstrated the upregulation of Ki67, a pro-proliferative marker, in the lungs of chronic smokers of marijuana.¹¹¹ It is important to note that in both of these cases, the pro-proliferative effects occur with either smoked marijuana or at levels achievable by smoking marijuana. That is to say, when smoked, *Cannabis sativa* delivers a very small amount of THC with each dose, and that this method of drug delivery is not the most viable option. To discount entirely the use of THC and/or its derivatives from this data is not prudent – it merely highlights and emphasizes the need for more selective, potent agents with an optimized dosage route for lung and bronchial cancers.

1.3.2.3. Cannabinoids in prostate cancer. The second leading cause of cancer-related death among men is prostate cancer. With a 1 in 6 chance of developing this cancer over the course of a male's life,¹⁰⁷ it becomes of great importance to identify not only preventative measures but treatment options to positively impact persons afflicted with this disease state. In a pair of studies conducted by Sánchez, et. al. 50 nM doses of THC caused a small, but statistically significant increase in the proliferation of LNCaP and PC3 cells – an action mimicked by 2-arachidonoylglycerol (2-AG) but opposed by anandamide.^{112,113} Further justification for the use of cannabinoids in prostate cancer is that other groups have demonstrated that doses of anandamide above 2 µM have shown an anti-proliferative effect in PC3, LNCaP and DU145 cells, while 2-AG was pro-proliferative at the same concentrations.^{114,115} Similar to the process noted with THC in lung and bronchial cancer, it could be due to a dose and/or receptor affinity issue, as anandamide is a full agonist at both CB₁ and CB₂, whereas THC is only a partial agonist at both and 2-AG is a full agonist with a higher CB₁ selectivity. Thus, my hypothesis would be that an effective cannabinoid in prostate cancer would be a potent, full agonist of CB₁ and CB₂, with little or no specificity. However, due to the untoward effects of central CB₁ agonist, we would desire a peripherally administered and restricted compound. Overall, there is viability for cannabinoids in prostate cancer, and an area that will hopefully see a burgeoning of data in the near future.

1.3.2.4. Cannabinoids in breast cancer. As prostate was the second leading killer of men, breast cancer is the second leading killer of women, with a similar rate of occurrence at 1 in 8 women.¹⁰⁷ Equilibrium predominates in breast cancer, with conflicting reports of THC being pro- or anti-proliferative. Some studies in MCF-7 cells have demonstrated Δ⁹-THC as a pro-proliferative agent, up to 5 µM;^{116,117} however, this was contradicted by another group with the same cell line – showing that up to 1 mM, THC did not promote proliferation but did block 17β-estradiol-induced proliferation.¹¹⁸ Studies in MDA-MB-231 cells have also yielded a dichotomy of data, with some reports of THC being pro-proliferative¹¹⁶ and others showing inhibition of growth in this cell line.¹¹⁹ Data also exists from McAllister, et. al. of anti-proliferative effects of THC in MDA-MB-468 and EVSA-T cells.¹¹⁹

In vivo models of breast cancer show an equally confusing gamut of data. A xenotransplant of 4T1 paw cells into BALB/c mice saw an increase in tumor burden with 25 mg/kg of THC injected IP for 21 days.¹¹⁶ A separate model of MMTV-neu mice, which spontaneously develop mammary tumors, saw a reduction of not only tumor burden but also a decrease in the rate and extent of metastasis to the lungs.¹²⁰ Overall, as

is the case with cancer, data is conflicting and varies widely in the literature. This could be simply due to differences in *in vitro* or *in vivo* model systems, the drugs utilized, the dosage forms or even the cancers themselves. Moreover, there is positive data regarding cannabinoids, which would suggest pursuit of cannabinoids in breast cancer to be a fruitful endeavor, and one that commands a spot on the research docket.

1.3.3. Cannabinoids in stroke

1.3.3.1. Pathogenesis of stroke. The two major mechanisms causing brain damage in stroke are ischemia and hemorrhage. Ischemic stroke, which represents about 80% of all strokes, results from decreased or absent circulating blood which, in-turn deprives neurons of life-sustaining substrates. The effects of ischemia are fairly rapid because the brain does not store glucose, the chief energy substrate, and is incapable of anaerobic metabolism.¹²¹ Non-traumatic intracerebral hemorrhage represents approximately 10% to 15% of all strokes. Intracerebral hemorrhage originates from deep penetrating vessels and causes injury to brain tissue by disrupting connecting pathways and causing localized pressure injury. In either case, destructive biochemical substances released from a variety of sources play an important role in tissue destruction.

Formation of microscopic thrombi responsible for impairment of microcirculation in the cerebral arterioles and capillaries is a complex phenomenon. Formation of a micro thrombus results from ischemia-induced activation of destructive vasoactive enzymes released by endothelium, leucocytes, platelets and other neuronal cells. Mechanical “plugging” by leucocytes, erythrocytes, platelets and fibrin ensues.¹²²

Inflammatory response to tissue injury initiated by the rapid production of many different inflammatory mediators, tumor necrosis factor (TNF) being one of the key agents, activation of microglia and adhesion and migration of peripheral leukocytes.¹²³ Leukocyte recruitment to the ischemic areas occurs as early as thirty minutes after ischemia and reperfusion. In addition to contributing to mechanical obstruction of microcirculation, the leucocytes also activate vasoactive substances such as oxygen free radicals, arachidonic acid metabolites (cytokines), and nitric acid. The cellular effects of these mediators include vasodilatation, vasoconstriction, increased permeability, increased platelets aggregation, increased leukocyte adherence to the endothelial wall, and immunoregulation. It is through modulation of this inflammatory process that the utility of cannabinoids in stroke find their foothold as a therapeutic option.

1.3.3.2. Cannabinoids as anti-inflammatory agents post-stroke. There are several reports of the endocannabinoids, anandamide and 2-AG, released locally following neuronal damage to influence the inflammatory and cell differentiation processes.^{7,124} Of particular interest, in conjunction with this release is evidence of CB₁ expression on T-lymphocytes, which may result from endocannabinoid stimulation.¹²⁵⁻¹²⁹ This helps to bolster the proposed mechanism of anandamide, which is in abundance near the infarcted brain area, locally inhibiting T-lymphocyte proliferation.¹³⁰ While this function of CB₁ is undoubtedly important, the lion’s share of cannabinoid work as anti-inflammatory agents post-stroke is through CB₂.

CB₂ has long been known to be expressed on immune organs such as the thymus and spleen,⁵³ as well as circulating inflammatory cells such as T-lymphocytes, B-lymphocytes, NK cells, monocytes and neutrophils;^{59,131,132} however, the more recent confirmation of CB₂ on astrocytes,¹³³ microglia,¹³⁴ neural subpopulations and oligodendroglial progenitors¹³⁵ points to a more direct regulation of CB₂ in these brain inflammatory cells.¹³⁶ In microglial cells, the level of CB₂ expression is highly dependent upon the activation state of the cell as it is responding to inflammation, infection or stress.^{7,137} It is during the early inflammatory activation phase where CB₁ and CB₂ show their highest levels of upregulation,^{7,138-140} thus early treatment, within 90 minutes of symptom onset, of the post-ischemic attack is preferred for optimal outcome. In fact, use of JWH-133, a CB₂-selective agonist, demonstrated not only a reduction of microglial activation and inflammatory gene expression in a mouse middle cerebral artery occlusion and focal cerebral ischemia model but an improvement of brain infarction size and clinical outcomes.¹⁴¹ Confirmation of this mechanism bears itself out in CB₂ knockout mice, where infarct size is considerably larger in the knockout mice as compared to the wild type.¹⁴²

A final component of cannabinoids in the inflammatory process of ischemia could be via impact of the CD4+ and CD25+ T cells, as they data suggests they to play a role in neuroprotection of mice after focal cerebral ischemia.^{143,144} The cannabinoid mechanism in these cells may be due to agonist's ability to affect proliferation, polarization^{7,145} and/or cytolytic capacity¹⁴⁶ of the T cells. Studies have helped to confirm this action, with WIN-55,212-2 showing favor toward lymphocyte migration within the spleen¹⁴⁷ and 2-AG greatly minimizing mitogen-induced proliferation of mouse splenocytes.¹⁴⁸ While this data provides positive news for the use of cannabinoids as anti-inflammatory agents following a stroke, contrasting data from *in vitro* and *in vivo* models persist and pose a problem for future therapeutic use of cannabinoid agents.

1.3.3.3. Cannabinoids as anti-ischemic agents post-stroke. The ischemic process of neuronal cell death is due to the cascade of events during the first few hours after the onset of ischemia. In these early hours, reactive oxygen species (ROS), calcium (Ca⁺²) and sodium (Na⁺) increase in the cytosol and are directly proportional to necrosis and apoptosis of neurons.¹⁴⁹ The increase in Ca⁺² occurs via NMDA-mediated pathways and is due to a lack of oxygen and glucose during the ischemic attack.¹⁵⁰ Given that blockade of the Ca⁺² channels is associated with neural protection, Ca⁺² represents a pivotal target in modifying the progression of neuronal cell death during ischemia.¹⁵¹

As is the case in several facets of cannabinoid research, we do not yet fully know the exact mechanisms for neuroprotection of the endocannabinoids and synthetic cannabinoids, and data conflict in some aspects. For example, some groups report that CB₁ agonist activity is the means to neural protection,¹⁵² while others have used SR-141716A, a known CB₁ inverse agonist, to show the same neuroprotective action via a TPVR₁-mediated pathway.¹⁵³ Some things are quite clear, though, with regard to cannabinoid actions on ischemic neurons.

One such known is that activation of CB₁ causes propagation of several pro-survival pathways involving mitogen-activated protein kinase (MAPK) kinase 1/2

(MEK1/2), extracellular signal-regulated kinase (ERK1/2) and nuclear factor-kappa B (NFκB).^{154,155} In addition to potential for initiating pro-survival pathways, amelioration of both global and regional blood flow was observed with low and high doses of THC in humans;¹⁵⁶ however, more recent studies have shown a potential link between chronic cannabis use in a youth and recurrent ischemic stroke.¹⁵⁷ An even more recent, prospective study of 48 young adults with prior ischemic stroke demonstrated multifocal intracranial artery stenosis in those with cannabis consumption.¹⁵⁸

While this may seem daunting news for use of cannabinoids in the ischemic stroke setting, it is not a mutually exclusive issue. Keeping this in perspective, the human studies are utilizing dried and smoked cannabis, of which THC is the main cannabinoid present, and as previously addressed, THC is a poor agent in many of the disease states examined. In addition, smoking cannabis could by its own volition, put the person in a hypoxic state – albeit for a brief period, but this is not a clinically insignificant variable to take into account when dealing with ischemic stroke. Finally, these studies need more refinement in terms of selective CB₁ and CB₂ agents, testing the gamut of activity from agonist to antagonist to inverse agonist before making any definitive conclusions about cannabinoid use in this disease state.

1.3.4. Cannabinoids in Alzheimer's Disease

1.3.4.1. Background of Alzheimer's Disease. Alzheimer's Disease (AD) is becoming more and more prevalent a disease as we have an increasingly aged population and this is an age-related disease. AD is hallmarked with a progressive loss of cognition and memory, and is the most common form of dementia among the elderly.¹⁵⁹ Pathological characterization of AD shows neurofibrillary tangles, amyloid-beta (Aβ) plaques and degradation of the synapses, which causes a loss of neurons specifically in the hippocampus and neighboring areas of the cerebral cortex. Evidence also exists to implicate a sustained activation of microglia in the plaque-bearing areas, causing an area of inflammation^{160,161} and oxidative stress.^{162,163}

1.3.4.2. Cannabinoids in Alzheimer's Disease. The previously known high level of CB₁ in the hippocampal and cerebral cortex regions of the brain¹⁶⁴ gave cursory probability of endocannabinoid involvement in the disease progression and pathogenesis. The microglial cells in the plaque-bearing areas have an increase in CB₁ and CB₂ expression,¹⁶⁵ while hippocampal and basal ganglia CB₁ expression overall is reduced in AD.^{165,166} Despite their high expression in the plaque regions of the brain, cannabinoid receptors are nitrosylated,¹⁶⁵ which functionally impairs the GPCRs by altering cannabinoid coupling to G-proteins. One final aspect of cannabinoid involvement is the increase of FAAH within astrocytes associated with the plaques.¹⁶⁷ As FAAH is responsible for metabolizing anandamide and 2-AG, this suggests that endocannabinoids are involved in the plaque-formation process.

There has been a good amount of pre-clinical data, which suggests that cannabinoid use is associated with positive outcomes in some of the neuropathological hallmarks of AD.¹⁶⁵ One such study demonstrated an increase in 2-AG pursuant to Aβ-induced hippocampal damage, which points to this endocannabinoid being

neuroprotective.¹⁶⁸ Another process important to the neurodegenerative process of AD, as it was in stroke, is the increase in Ca^{+2} influx which leads to neuronal damage. Currently, the FDA has approved memantadine for use in AD, and it works as an antagonist of the NMDA receptor to limit Ca^{+2} influx into the neuron to slow disease progression. Similar to memantadine, HU-211 – a synthetic, “unnatural” enantiomer of HU-210 – works as a selective NMDA receptor inhibitor and consequently helps to protect neurons from degradation.^{169,170} As AD is a very complex process, this potential crossover is but one of many ways in which cannabinoids can find usefulness.

Another possible mechanism of neural protection for cannabinoids is demonstrated when CB_2 is selectively stimulated, which leads to suppression of microglia activity and thus a decrease in the production of $\text{TNF-}\alpha$ and nitric oxide.¹⁷¹ Another study of WIN-55,212-2 and HU-210 demonstrated a similar decrease in microglia activation and $\text{TNF-}\alpha$ release.¹⁶⁵ Conclusions from these studies strongly point to the hypothesis that CB_2 overexpression in this instance is a marker and mechanism for neural protection. Another potential agent for use is cannabidiol, which has shown efficacy in blocking glutamate-induced excitotoxicity,¹⁷² reduction of proinflammatory mediators,¹⁷³ scavenging of reactive oxygen species to reduce lipid peroxidation¹⁷⁴ and inhibition of tau protein hyperphosphorylation – an additional AD neuropathological feature.¹⁷⁵

While there is currently nothing available for patients suffering from AD to completely halt or reverse the progression of the disease, cannabinoids provide a potent agent that can augment and mirror current therapies on the market. Developing cannabinoids to halt the process of neurodegeneration, or delay it much more than current therapies available, remains an ever-present goal of research in this field. Cannabinoids have already found a place in AD treatment by combatting the severe aggression that accompanies the latter stages of the disease.¹⁷⁶ Overall, AD is another exciting and useful arena for cannabinoid use and research.

1.3.5. Cannabinoids in diabetes

1.3.5.1. Endocannabinoids’ control of metabolism. There exists data which show a correlation between endocannabinoids and leptin, an adipocyte-derived signaling hormone which are proportional to the fed/satiated state of the animal (i.e. the more well-fed and satiated the animal, the higher the leptin levels). Leptin’s relationship to endocannabinoids seems to be inversely related. Studies demonstrate an increase in endocannabinoids in the hypothalamus of food-deprived rats¹⁷⁷ – specifically 2-AG levels after a 24 hour fast.¹⁷⁸ The latter of these studies, conducted by Hanus *et al.*, extend the animals out for 12 days of semi starvation and show a direct, negative correlation to 2-AG concentrations and the duration of starvation.¹⁷⁸ Taken together, one may conclude that in the acute phase 2-AG levels rise; however, they eventually decline in a prolonged fasting state due to lack of biosynthesis, thus perpetuating the cycle of maintaining a fasted state – as in anorexia. Another study shows the more direct effect of leptin on reducing anandamide and 2-AG in the hypothalamus.¹⁷⁹ As a whole, endocannabinoids show themselves as potential co-regulators for metabolism and the drive for satiety.

To that point, a variety of tissues regulates endocannabinoids, especially in the

visceral periphery. The duodenum, in periods of food deprivation, increases the local levels of anandamide and 2-AG – hypothetically, to act upon sensory and vagal nerves of the brain stem and regulate satiety.^{180,181} As with any hormone system, strict regulation of the endocannabinoid system in adipose tissue must occur. PPAR- γ stimulation can inhibit 2-AG in mature 3T3 F442A mouse adipocytes,¹⁸² and decreases CB₁ expression and increases FAAH expression in human adipocytes.¹⁸³ This data would suggest CB₁ receptors are involved in the early differentiation of adipocytes, possibly upstream of PPAR- γ , and is subsequently down regulated once PPAR- γ is at its peak and differentiation is complete. Concomitantly, PPAR- δ – itself activated via physical exertion – inhibits CB₁ receptor expression.¹⁸⁴ Lastly, there exists an inverse relationship among both insulin and leptin and endocannabinoid levels,^{182,185} which may augment FAAH expression.¹⁸⁶

The sustained increase in either endocannabinoids or CB₁, or both, in several peripheral organs and tissues of obese or hyperglycemic animals has gained widespread acceptance. Further, data indicate an increase of endocannabinoids in adipocytes and β -cells.¹⁸² The effect upon 2-AG, appears to be a PPAR- γ -mediated effect on the biosynthesis of 2-AG from mature hypertrophic adipocytes.¹⁸² In both diet-induced obese (DIO) mice and Zucker rats, compared to lean, age-matched animals, endocannabinoids are starkly decreased in subcutaneous fat,^{180,187} this data may then indicate that in obese and/or hyperglycemic rodents there is clearly a hypoactive endocannabinoid system in the adipose store, whereas in the mesenteric fat of DIO mice endocannabinoid levels are unaltered.¹⁸⁷

1.3.5.2. Endocannabinoids in type-2 diabetes and metabolic disorders. As previously mentioned, the endocannabinoid system plays an integral role in regulating the metabolic processes of the body, and its reach extends to many different organ systems. One such example demonstrates how CB₁ receptors of hypertrophic adipocytes, when subjected to constant agonist activity, suppress adiponectin expression^{182,188} – helping to explain why a CB₁ antagonist raises adiponectin in adipocytes in obese mice more readily than lean mice;¹⁸⁹ and how CB₁ antagonists restore the lean phenotype to the expression of adiponectin-dependent genes in the adipose tissue of DIO mice.¹⁹⁰ This information, coupled with the known protective role of adiponectin against insulin resistance and atherogenic inflammation, may lead one to believe that blockade of the endocannabinoid system is the key to mitigating obesity and thusly insulin resistance and atherogenic inflammation. However, as with many hormone systems – endocannabinoids, included – ubiquitous and totalitarian control may result in untoward effects. Though, recent data do show CB₁ playing a key role in the loss of visceral fat and the enhancement of thermogenesis in subcutaneous fat.¹⁹¹

Mitigation of fat accumulation and type is but one of several factors that play into metabolic disorders and into type-2 diabetes. Another hallmark of type-2 diabetes is the hyperinsulinemia resulting from insulin resistance. CB₁ stimulation has been shown to augment basal insulin release by β -cells,^{182,187,192} which may result in β -cell hypertrophy and subsequent dysfunction, perpetuating the development of type-2 diabetes. A more recent study demonstrated that rimonabant (SR-141716A) decreases basal insulin hypersecretion in Zucker diabetic rat isolated pancreatic islets, without affecting the

glucose-stimulated insulin secretion.¹⁹³ Coupled with this CB₁ overstimulation-induced β -cell damage, hyperactive CB₁ receptors in the liver of the mouse may be a root cause of insulin resistance and hepatosteatosis.¹⁹⁴ Lastly, the paramount characteristic of type-2 diabetes is the resistance of peripheral tissues, especially skeletal muscle, to insulin. Blockade of CB₁ ameliorates this trait and increases glucose uptake and AMP kinase expression of skeletal muscle.^{195,196}

As one can plainly see, dysregulation of the endocannabinoid system, more specifically the over activity thereof, shows strong ties to the development of metabolic syndrome and subsequently or concomitantly type-2 diabetes. A trio of studies involving rimonabant in Zucker rats further demonstrated this correlation. The first gave indication that with oral treatment of rimonabant at 30 mg/kg the presence of hepatic steatosis and other related metabolic syndrome features were eradicated.¹⁹⁷ Further, this study showed an improvement of hepatomegaly and markers associated with hepatic damage.¹⁹⁷ Finally, this study there was a noted improvement in blood lipids toward a more normal and healthy state.¹⁹⁷ Most notable from this study is that this was shown via pair-fed controls that the effect was not merely a result of diet, alone. The second study demonstrated the utility of rimonabant in attenuating the mortality and chronic renal failure seen in obesity and long-term diabetes, again as compared to pair-fed but untreated rats.¹⁹⁸ The study further demonstrated a sustained lowering of body weight, transient hypophagia, increased adiponectin and a confirmation of the first study's rectification of dyslipidemia.¹⁹⁸ The third study sought to examine the inflammation component, and in so doing demonstrated that rimonabant could successfully lower the neutrophil and macrophage levels in obese Zucker rats as a model for both glucose intolerance and metabolic syndrome.¹⁹⁸ As in the previous 2 studies, rimonabant successfully attenuated eating, rectified dyslipidemia and decreased atherosclerotic lesions.¹⁹⁸ This study did take the data a step further to show a decrease in proinflammatory cytokines MCP-1 and interleukin-12 (IL-12).¹⁹⁸ As a constellation, these studies and data point to the potential of the peripheral, rather than the central, endocannabinoid system as being a viable target for the treatment of obesity-related disease states.

1.3.5.3. Endocannabinoids in abdominal obesity and hyperglycemia. There is no denying that there is a positive correlation between elevated intra-abdominal obesity and type-2 diabetes – given, too, the further direct correlation to an increase in atherogenic inflammation and an increase in cardiovascular events.¹⁹⁹ As previously discussed, the endocannabinoid system lends itself to regulation and differentiation of adipose tissue when left unchecked. It has been demonstrated that 2-AG, but not anandamide, is increased in visceral, but not subcutaneous, fat;¹⁸² an increase in CB₁ expression and endocannabinoid turnover (i.e. increased biosynthetic and degradation enzyme expression) in obese individuals;¹⁸³ and a decrease in endocannabinoid synthesis in subcutaneous fat.¹⁸⁷ More recently, an observation of the subcutaneous fat of obese, type-2 diabetes patients demonstrates a ubiquitous lowering of endocannabinoid tone.²⁰⁰ These data point to the very real and very serious potential for a dysregulated endocannabinoid system to cause an increase in abdominal fat deposit in lieu of subcutaneous fat – promoting an increase in the cardiometabolic risks for patients.

Further adding to the positive association of the endocannabinoid system, intra-abdominal obesity and type-2 diabetes, a direct relationship has been shown between intra-abdominal obesity and circulating 2-AG.^{201,202} It was through these studies that a more direct correlation between intra-abdominal fat and circulating 2-AG manifest as cardiometabolic risk factors – such as low HDL, high triglycerides, low insulin sensitivity, glucose intolerance²⁰¹ and decreased plasma adiponectin.²⁰² Further causal link between hyperglycemia and increased 2-AG was observed even in non-obese individuals¹⁸² – giving more credential to a dysregulated endocannabinoid system as an independent risk factor in type-2 diabetes. This was echoed by a prospective, though small, study of viscerally obese men who underwent 12 months of therapeutic lifestyle changes to dietary habits and physical activity. The results showed an across-the-board reduction in not only body mass and fat composition, but a stark decrease in 2-AG levels.²⁰³ Further, the data showed an inverse correlation between 2-AG and HDL levels along with a direct correlation between 2-AG and insulin resistance.²⁰³ On its surface, this data suggests 2-AG as the culpable target; however, one must be mindful of the fact that endocannabinoids are not generally hormone-like in traditional function, so further study is needed to determine if 2-AG is secondary to increased peripheral production – especially in obesity.

1.3.5.4. Use of CB₁ antagonists and inverse agonists in type-2 diabetes. The central tenant in the management of type-2 diabetes is and will forever remain therapeutic lifestyle changes such as improved dietary habits and increased physical activity; however, there is always the desire to augment these changes to improve outcomes for patients. For a time, rimonabant showed great promise as this agent – successfully completing four (4) phase III and two (2) phase IIIB clinical trials²⁰⁴⁻²⁰⁷ and approved for use in the EU on June 21, 2006. Its success was short-lived, though, as it the European Medicines agency pulled the drug from the market on October 23, 2008 over concerns of serious psychiatric problems and even suicide. It was not until January 16, 2009 that the European Medicines Agency formally and completely withdrew its approval. Other potential agents, such as Merck's taranabant²⁰⁸⁻²¹⁰ and Pfizer's otenabant^{211,212} have ceased to continue with clinical trial because of similar problems arising during clinical trial.

These drugs are worth mentioning, because during their initial investigation they showed great promise themselves and provide valuable insight into the potential of cannabinergic drugs' utility in this arena. The rimonabant in obesity (RIO) trials demonstrated rimonabant's utility in reducing not only food intake but also the signs, symptoms and complications of metabolic syndrome.²¹³ Expansion of these trials into the RIO-diabetes trial showed the beneficial effects of current standard treatment with rimonabant in not only improving body composition but regulating and stabilizing glucose control.²⁰⁵ Further, the ARPEGGIO trial began to expose the weaknesses of rimonabant, highlighting the increase in anxiety and depression (both with 14% occurrence) among the treatment groups. The SERENADE study showed an increase in both anxiety and depression, though with less frequency (5.8%) than ARPEGGIO. The untoward psychotropic effects, which have long plagued cannabinoid therapies, would lead to the downfall of rimonabant, and unfortunately the entire class, both in development as well as deployment.

As is the case with many new drug classes, there are zeniths and nadirs to contend with, and with the current explosion and interest in cannabinoids, we are only now beginning to understand this intricately woven and tightly controlled system. As one can see from this brief presentation, cannabinoids present a very viable target for use in controlling metabolic syndrome and type-2 diabetes; however, there needs to be more study on separation of the beneficial and untoward effects. There are efforts underway to peripherally restrict cannabinoids,²¹⁴ but the possibility exists that compounds may be synthesized which could specifically exert beneficial effects without the need to be restricted to one compartment or the other. Given all of the aforementioned potential for cannabinoids and the relatively limited amount of understanding we have for this system, I think we have only just begun to see the utility of these compounds and how we can develop and use them to benefit and impact a variety of disease states, type-2 diabetes being one of many.

1.4. Goals of the Current Project

Knowing now how positively one may influence some of the top disease states of people via cannabinoids, this project takes on a great deal of merit. This project seeks to develop a more guided approach to the design of cannabinoid agents. This begins with the validation and refinement of an assay to ascertain functional activity (**Chapter 2**). Then, detailed look at current knowledge of structural pharmacophore data at the C-3 position of classical cannabinoids, and an exploration of a novel series of C-3-modified classical cannabinoids (**Chapter 3**). Building from the success of the C-3-modified classical cannabinoids, I examine the effects on functional activity resulting from the modification of the classical benzchromene core (**Chapter 4**). Considering all previous data, I will examine the hybridization of non-classical and classical features to result in another novel triaryl class of cannabinoids (**Chapter 5**). Finally, taking into account the current literature and body of evidence for modelling CB₁ and CB₂, coupling that with my own rudimentary modelling and functional data, a rough functional pharmacophore was developed to guide future design (**Chapter 6**).

As one can see, cannabinoids have a wide-ranging and potentially blockbuster impact to healthcare and treatment of disease states. This project seeks to gain more knowledge and insight into the function of novel cannabinergic agents synthesized in our lab. Through this work, knowledge is generated to elucidate which, if any, components of classical and triaryl-core cannabinoids can result in agonist, antagonist, inverse agonist or a mixture thereof, as defined by the cubic ternary complex model. Within this model for GPCR functional activity, there is an augmentation of the extended ternary complex model. Inherent in both of these models is the specificity of certain drugs for a particular state. The first assumption made is that the receptor is in equilibrium with an active and inactive state (i.e. ready to signal downstream or not). Agonists of this model target the active state, and thus shift the equilibrium toward the active state of the receptor. Inverse agonists target the inactive conformation of the receptor, and so the equilibrium shifts toward the inactive state. Antagonists do not discriminate nor target a particular conformation of the receptor, thus the equilibrium is not changed and the total available receptor decreases. The added component to the cubic ternary complex model is that the

receptor, in the active or inactive state – ligand bound or unbound – can bind and sequester G-proteins into a non-signaling state. This added allocation of thermodynamic activity allows for the added activity of an inverse agonist. With this model, inverse agonists could bind the inactive state and subsequently sequester G-proteins away from signaling pathways. This is actually a noted occurrence with cannabinoids, in particular.⁴⁰

Through this project's documentation, I will demonstrate the unique aspects of classical and non-classical cannabinoids which impart equally unique functional activity. Seemingly, simple interchanges at the C-1' position cause a non-classical agent to change from an agonist at CB₂ to an inverse agonist. Additionally, bioisosteric replacement of a carbocyclic ring to a heterocyclic ring in classical cannabinoids cause a similar shift from agonist to inverse agonist, and within the heterocyclics, transition from a single to a dual heteroatom heterocyclic ring yields inverse agonist at CB₁ and CB₂, respectively. Taking these unique functional activities, along with the theories presented in the cubic ternary complex model, I will attempt to reconcile and possibly implicate key residue interactions within the CB₁ and CB₂ ligand-binding pocket, which may result in these unique activities.

CHAPTER 2. ASSAY DEVELOPMENT FOR THE FUNCTIONAL ACTIVITY ANALYSIS OF NOVEL CANNABINERGIC COMPOUNDS

2.1. Introduction

As previously discussed, there exists a desire for selectively targeting CB₁ and CB₂ receptors to help maximize therapeutic benefit and minimize negative side effects – namely the psychotropic effects of CB₁ agonists. Along with that, there is a need and desire to know the functional activity of drugs (agonist, antagonist, inverse agonist) to help elucidate the downstream effect(s) of a drug. To help ascertain the functional activity of cannabinoid drugs, two assays hold the lion's share of utilization ([³⁵S]-GTPγS and β-arrestin); however, I will show the development and utility of a third assay that is currently available.

Far and away the most dominant force in functional activity analysis has been the [³⁵S]-GTPγS assay. It is useful in that it allows one to obtain the potency, efficacy and antagonist affinity without the need to have downstream amplification for agonist compounds.²¹⁵ While this is unquestionably useful, I believe, there is a deficiency inherent within that assay. The deficiency lies in the fact that it is an essentially irreversible reaction (in the presence of mM Mg⁺²)^{216,217}, such that once an agonist binds to the receptor being assayed, [³⁵S]-GTPγS binds to the Gα-subunit, forming a hydrolysis-resistant Gα-[³⁵S]-GTPγS complex, preventing hydrolysis by the GTPase of Gα and mitigating rejoining with the Gβγ-complex subunit. Measurement of activation is accomplished by filtering the membrane preparation and counting the radioactivity left on the filter(s). This does not mimic more physiologic conditions, whereupon agonist binding to the receptor GDP dissociates from and GTP binds to the Gα-subunit, eventually being hydrolyzed back to GDP by the GTPase domain of the Gα-subunit and allowing the Gα-subunit to rejoin the Gβγ-complex subunit. While the [³⁵S]-GTPγS assay is a very sensitive assay, able to detect very minute EC₅₀ values of compounds, its irreversible nature may present issues when moving drugs from the bench top into a living and dynamic system. Additionally, it has been shown that while the reaction is nearly irreversible, cannabinoids show evidence of promoting the dissociation of [³⁵S]-GTPγS from Gα,²¹⁸ thus leaving us with false data for the compounds to be tested.

Another assay utilized is the PathHunter® β-arrestin assay from DiscoverX. This assay utilizes a fragmented β-galactosidase enzyme to detect GPCR activation. In this assay system, a small fragment of the β-galactosidase enzyme is fused to the GPCR of interest; the remaining larger fragment of β-galactosidase is co-expressed as an N-terminal deletion mutant. Upon activation of the GPCR, binding of the small and large fragments forms the activated β-galactosidase enzyme. Activation of this enzyme leads to stimulation of chemiluminescent dye and subsequent detection on a plate reader. While there have been reports of this assay's use in detecting and qualifying inverse agonists;²¹⁹ however, when examining the data presented, it is evident that this assay is not as sensitive at detecting inverse agonists relative to agonists. Additionally, nowhere within the manufacturer literature nor in various other group's use of this assay has this been shown.^{220,221} While it does accomplish the aforementioned goal of more closely

mimicking physiological conditions by not relying upon an essentially irreversible activity, lack of or reduced ability to detect inverse agonist activity makes this a less-viable candidate. Additionally, while manufacturer data suggests that engineering of the GPCR of interest does not alter the binding affinity for substrate, it does impart an added variable, which should not be fully discounted in biological assays and renders this assay even less desirable.

A bridging of the gap between a need to mimic physiological conditions and to detect inverse agonist activity lies with the ACTOne assay available from Codex Biosolutions. This assay utilizes, HEK-293 cells transduced with the GPCR of interest along with a proprietary cyclic-nucleotide gated ion (CNG) channel, which opens in response to increased intracellular cAMP and allows for the influx of K^+ , Na^+ and Ca^{++} . A phosphodiesterase inhibitor (Ro 20-1724) is added to the assay well in excess (25 μ M) to prevent the degradation of cAMP and allow for its real-time detection via the CNG channel activation. Detection of this ion flow results from the presence of an extracellular cationic-dependent dye, whose fluorescence shows on a plate reader at 540 nm excitation and 590 nm emission. As the extracellular concentration of cations decreases, a result of increased intracellular cAMP and subsequent influx of cations via the CNG channel, emission detected by the plate reader begins to increase, accordingly. Thus, in the case of CB_1 and CB_2 , which are coupled to the inhibitory $G_{i/o}$, an agonist of either of these receptors will cause a decrease in intracellular cAMP and an increase in extracellular cations – this increase of cations complexes the cation-sensitive dye so that it does not emit upon excitation. Therefore, the emission detection by the plate reader directly correlates to the amount of cAMP produced in each of the wells of a plate – allowing for a real-time examination of cAMP levels, without any engineering, alteration or complexation of the GPCR of interest. It is for its simplicity, ability to detect agonist, antagonists and inverse agonists as well as a reversible, non-engineered GPCR presence that this assay found utilization. However, initial conductance of the assay was not without its problems, and refinement and optimization was paramount in assuring accuracy, consistency and comparability of the data across all drugs and receptor types of interest.

Having selected the ACTOne assay based on the aforementioned parameters, conductance of the assay proved to be problematic, with responses and EC_{50} values for well-established cannabinoid compounds waxing and waning from day to day and even from plate to plate within the same day's runs. Given that there are several variables at play in such a dynamic assay, I took the time and effort to tease apart the individual variables to regulate, stabilize and optimize each step of the assay – ensuring accuracy and consistency in the data gathered. I conducted experiments to modify the manufacturer recommended procedure (**Section 2.2.1**) to optimize: cAMP stimulants (**Section 2.2.2**), plating density (**Section 2.2.3**), plate selection (**Section 2.2.4**), plating environment (**Section 2.2.5**), pertussis toxin dosing (**Section 0**) and order of addition (**Section 2.2.6.2**), data analysis (**Section 2.2.7**) and finally arrived at a streamlined procedure (**Section 2.3**). The data presented herein shows the process of honing the assay into a tool useful in the procurement of functional activity of novel series of cannabinoids generated by my predecessors in the group.

2.2. ACTOne Assay Development

2.2.1. Manufacturer recommended procedure

2.2.1.1. Commonalities of ACTOne assay protocols. Overall, the treatment of the cells among the 3 assay protocols (CB₁, CB₂ and HEK-CNG) is the same, with growth recommended in DMEM with 10% FBS, 250 µg/mL G418 and 1 µg/mL puromycin. Each of the cell types are to be kept at 37°C with 5% CO₂, and not allowed to grow beyond 90% confluence – overgrowth may significantly reduce the cell's response to a ligand and take several passages to regenerate.

The initial recommendation for cell plating is that one should use a Poly-D-Lysine coated plate to facilitate cell adherence, and plate cells the preceding day so that they may grow overnight. For the 96-well plate, which is what I used, the manufacturer recommends plating 70,000 cells/100 µL/well; however, there is the ever-present caveat that one must optimize the cell number for each assay.

Following the overnight growth at 37°C, add ACTOne Membrane Potential Dye to each of the wells in the plate at a volume equal to the volume of cell suspension added the previous day – 100 µL, in my case. The dye is to dwell for two hours at room temperature, in a dark place. In the event a serum-free environment is required, remove the media and replace with DPBS containing 0.2% to 0.5% BSA prior to adding the dye.

The differences among the three protocols prompted an initial hypothesis that the assay needed examination with greater scrutiny. The purpose of this examination is to maintain consistency across the three protocols so that data obtained from conductance thereof is able to be cross-compared and correlated.

2.2.1.2. CB₁-specific parameters of the ACTOne assay. Differences among the three assay protocols begin with the agent used to stimulate adenylyl cyclase. In the case of the CB₁ protocol, the agent of choice is isoproterenol – exerting its effects via the constitutively expressed G_s-coupled β-adrenoreceptor stimulation. Initial stock of the compound to be tested, adenylyl cyclase stimulant and PDE-inhibitor (Ro 20-1724) are prepared at five times the final testing concentrations due to the fact that they will be diluted 1:5 when added to the assay plate (50 µL compound stock added to 200 µL of cell suspension and membrane potential dye). Consistent in all wells of the assay is Ro 20-1724 at 25 µM and isoproterenol at 300 nM, final concentrations. The compound of interest has its concentration varied from column to column in a given row to obtain a dose response curve on a logarithmic scale.

2.2.1.3. CB₂-specific parameters of the ACTOne assay. Similarly to the CB₁ protocol, the final concentration of Ro 20-1724 is 25 µM in each well; however, the adenylyl cyclase stimulant is different and at a different concentrations. For the CB₂ protocol, NECA becomes the agent of choice to stimulate adenylyl cyclase via G_s-coupled adenosine A_{2b} receptors constitutively expressed on HEK293 cells. Final concentration of NECA is 250 nM in each of the assay wells. Additionally, in the documentation from the manufacturer, CP-55,940, which is the standard I use for

non-selective CB-agonists, was at ten times higher concentrations in the CB₁ protocol tests v. the CB₂ protocol tests – though the EC₅₀ for CP-55,940 is 0.2 nM and 0.3 nM for CB₁ and CB₂, respectively, in the literature.²²²

2.2.1.4. Parental, HEK-CNG-specific parameters of the ACTOne assay. For the parental cells, since there is no over-expression of our GPCR of interest, this protocol is useful in determining the EC₅₀ of the adenylyl cyclase stimulant, as well as confirming the G_{i/o} activity observed is not due to another, constitutively expressed, GPCR of HEK293 cells. It is of an interesting note that the manufacturer reports the EC₅₀ of NECA in HEK293 cells to be 611 nM; however, as previously mentioned, the CB₂ protocol only calls for a 250 nM NECA concentration in each of the assay wells.

2.2.2. Assay stimulus standardization

Since this assay relies on cAMP as the substrate for CNG channel activation and subsequent impact upon the cation-sensitive dye, a reproducible and consistent source of adenylyl cyclase induction is essential for continuity of data and analysis. Recommended within the manufacturer's directions is the use of isoproterenol for CB₁ cells and NECA for both CB₂ and parental CNG cells to stimulate cAMP production and subsequent CNG channel activation of the cells. However, to maintain more consistency within the assay, comparing the three cells lines across multiple plates on multiple days, it was my hypothesis that the stimulus should be consistent across all runs in order for the results to be deemed comparable and consistent.

2.2.2.1. Manufacturer recommended cell stimulus. Initially, isoproterenol was employed at 50 nM, being >10X the EC₅₀ of isoproterenol at CB₁ (CB₁ = 2.56 nM; CB₂ = 12.3 nM at 50 minutes after addition) (**Figure 2.1**). As one can see immediately, the response gained from isoproterenol in the CB₂ cells was virtually nil at 50 minutes. While generally good in the CB₁ assay, there was still some inconsistency therein, coupled with a waning of the response in the CB₂ assay after 10 minutes of the kinetic run time (**Figure 2.2**) isoproterenol lost favor as a truly viable candidate. Considering the next manufacturer recommended stimulus, NECA, was tried at 100 nM, being >2X the EC₅₀ of NECA (CB₁ = 40.0 nM; CB₂ = 316 nM, 50 minutes after addition) (**Figure 2.3**). There was a glimmer of hope for NECA, inasmuch as providing a nearly equal response, albeit at an EC₅₀ ~8X that of CB₁; however, these hopes were dashed even at nearly 2X the EC₅₀ of NECA at CB₂, making this agent not readily cross-comparable (**Figure 2.4**).

2.2.2.2. CB₁ cAMP stimulant trial. In an effort to maintain a consistent stimulation of cAMP production, utilizing the knowledge that manufacturer recommended agents were adrenergic agonists, eight adrenergic agonists were screened as potentials for the CB₁ assay. The agents are: medetomidine (α -₂ agonist), norepinephrine (non-selective α - and β -agonist), clonidine (α -₂ agonist), epinephrine (non-selective α - and β -agonist), oxymetazoline (non-selective α -agonist), yohimbine (α -₂ antagonist), pseudoephedrine (non-selective α -agonist), isoproterenol (non-selective β -agonist) and NECA (adenosine receptor agonist). Evaluation of the data is relative, such

Figure 2.1. Isoproterenol EC₅₀ curve at 50 minutes post-addition

Figure 2.2. Isoproterenol-induced cAMP production at 50 nM

Figure 2.3. NECA EC₅₀ curve at 50 minutes post-addition

Figure 2.4. NECA-induced cAMP production in CB₁ and CB₂ cells

that the highest value on the assay plate corresponds to the highest possible value for all of the drugs. This analysis allows for a more direct comparison of the data, to show that isoproterenol ($EC_{50} = 14 \text{ nM}$; Lit: 123 nM^{223}), NECA ($EC_{50} = 147.5 \text{ nM}$; Lit: $A_1 = 26 \text{ nM}$,²²⁴ $A_{2A} = 26.1 \text{ nM}$,²²⁵ $A_{2B} = 1400 \text{ nM}$,²²⁶ $A_3 = 129 \text{ nM}^{227}$) and epinephrine ($EC_{50} = 110.5 \text{ nM}$; Lit: 8.2 nM^{228}) will each stimulate cAMP production in the CB₁ cells to a similar level (**Figure 2.5**).

2.2.2.3. CB₂ cAMP stimulant trial. Similar to the aforementioned trial of agonists in the CB₁ cells, evaluation of various adrenergic agonists for the CB₂ assay was conducted with: norepinephrine (non-selective α - and β -agonist), epinephrine (non-selective α - and β -agonist), oxymetazoline (non-selective α -agonist), pseudoephedrine (non-selective α -agonist), formoterol (β_2 -selective agonist), isoproterenol (non-selective β -agonist), and NECA (adenosine receptor agonist) (**Figure 2.6**). Data was calculated, as it was for the CB₁ assay, as a relative effectiveness for each of the drugs. As a result of this analysis epinephrine, formoterol, isoproterenol and NECA appear to be potential agents for further testing and elucidation of properties; however, as previously discussed isoproterenol and NECA have viability and consistency issues with the CB₂ assay.

To fully test the CB₂ assay stimulants, these potential stimulants, isoproterenol, formoterol, epinephrine and NECA, were tested at their respective literature cited EC_{50} (150 nM ,²²³ 350 nM , 6 nM^{228} and 125 nM ,²²⁷ respectively) and EC_{90} (2500 nM , 1000 nM , 1000 nM and 1000 nM , respectively) v. CP-55,940. Data from this study demonstrate that NECA, at its EC_{90} , is the only one of the 4 capable of stimulating cAMP production 50 minutes after addition to the assay plate (**Figure 2.7**); however, it still shows much evidence of variability, and this is not at all desired.

When examining the two cell lines, it is apparent that the data may not be accurately compared using equivalent doses of isoproterenol or NECA (**Figure 2.8**), nor is it possible with any of the additional adrenergic agents tested. For the CB₁ cells, a final concentration of 50 nM of isoproterenol in each well was enough to elucidate a response from the cells; however, this same dose in CB₂ cells began to decline only 10 minutes after addition to the cells (**Figure 2.8**). NECA was considered next, and at a final concentration of 100 nM in each well for CB₁, we again see a nice rise in cAMP production that is consistent over the entirety of the 60 minute kinetic run; however, in CB₂, the same dose of NECA would not elucidate a strong enough response to be detectable (**Figure 2.8**). Given the fact that isoproterenol is inconsistent with respect to time between the two assays and NECA is inconsistent with respect to dose, a more consistent and directly comparable stimulant of cAMP production was needed to help stabilize the cAMP levels in the assay and allow for more accurate cross-comparison.

Forskolin is a well-known, direct stimulant of adenylyl cyclase in assays measuring cAMP production, with a $12 \mu\text{M}$ EC_{50} .^{229,230} To apply this to my assay, a forskolin dose response curve was conducted to ascertain the dose necessary for use in both the CB₁ and CB₂ assays. In both the CB₁ and CB₂ assays, the dose response was quite stark as the dose was increased, with an EC_{50} of 112 nM and 164 nM for CB₁ and CB₂, respectively and an EC_{90} of 292 nM and 296 nM for CB₁ and CB₂, respectively (**Figure 2.9**). The EC_{50} and EC_{90} values in both CB₁ and CB₂ are quite close and both

Figure 2.5. CB₁ adrenergic drug test for stimulation of cAMP production

Figure 2.6. CB₂ adrenergic drug test for stimulation of cAMP production

Figure 2.7. Selected adrenergic drugs in CB₂ cells vs. CP-55,940

Figure 2.8. Manufacturer-recommended cAMP stimulants

Notes: Isoproterenol is 50 nM and NECA is 100 nM for both CB₁ and CB₂.

Figure 2.9. Forskolin as a cAMP stimulant in CB₁ and CB₂ cells

achieved and held at or near maximum response within 20 minutes in both CB₁ and CB₂ (**Figure 2.10**). Given that there was not an appreciable difference in response of CB₁ or CB₂ cells between 1 μ M and the approximate EC₉₀ for both (300 nM), 800 nM was chosen as the final concentration of forskolin to use in each well of the assay across all three cell lines. Comparing equivalent (500 nM) concentrations of isoproterenol, NECA and forskolin between the CB₁ and CB₂ cells (**Figure 2.11**), we see clear evidence that any of the three cAMP stimulants work well in CB₁. However, at CB₂, isoproterenol starts to wane after only 10 minutes, NECA, while comparable to forskolin, does not have the sustained plateau and consistency needed to assure reliability within this assay.

2.2.3. Cell plating density test

Having now ascertained which stimulus to utilize in order to induce cAMP production, the next pertinent parameter to address is that of plating density. To test for activity with each of the varying cell densities, with constant levels of forskolin to serve as the stimulus for cAMP production, I selected two non-selective cannabinoid agonists (CP-55,940 and WIN-55,212-2), a CB₂-selective agonist (HU-308), a CB₁-selective inverse agonist (SR-141716A) and a CB₂-selective inverse agonist (SR-144528). In addition, as a point of reference, the manufacturer recommends plating of the cells at 70K cells/well, and in a 96-well plate (as I use) there would be 100 μ L of cell suspension present.

For the CB₁ cells, six plates with densities of 80K cells/well, 70K cells/well, 60K cells/well, 50K cells/well, 40K cells/well and 30K cells/well would serve to answer this question. Looking at the three agents used in this test (CP-55,940, SR-141716A and WIN-55,212-2), it is initially evident that there is not a great deal of difference among the varying densities (**Figure 2.12**). Looking at the EC₅₀ and R² values for each of the three compounds leaves me with 40K cells/well as the most viable option. This density strikes the best balance for all three of the known compounds for EC₅₀ and R² value, and this is the density utilized for CB₁ functional assays for the remainder of the testing.

For the CB₂ cells, six plates with densities of 70K cells/well, 60K cells/well, 50K cells/well, 40K cells/well, 30K cells/well and 20K cells/well would serve to optimize the plating density for CB₂ cells. Considering, again, the EC₅₀ and R² values for the three agents used (CP-55,940, SR-144528 and HU-308), the most viable option becomes 60K cells/well (**Figure 2.13**). Again, this gives us the strongest balance between a good EC₅₀ of all three compounds with an acceptable, though not perfect, R² value. Henceforth, 60K cells/well will be the density utilized in plating CB₂ for functional assay.

Though there is no optimization for the parental HEK-CNG cells, because it was impossible to actually generate a change in cAMP due to the lack of cannabinoid receptors, I did institute parameters to provide valid comparisons. Given that the constitutively expressed receptors are identical, I deemed it acceptable to take the average of CB₁ and CB₂ plating densities as the plating density for the HEK-CNG cells. Thus, 50K cells/well was the plating density for the HEK-CNG cell assays conducted as the control for all of the compounds tested herein.

Figure 2.10. Forskolin response in CB₁ and CB₂ vs. time

Figure 2.11. All viable cAMP stimuli at 500 nM in CB₁ and CB₂ cells

Figure 2.12. CB₁ plating density test

Figure 2.13. CB₂ plating density test

2.2.4. Assay plate selection

As stated in the manufacturer's recommendations, cells should be plated overnight on Poly-D-Lysine plates; however, cost of Poly-D-Lysine relative to more widely available polystyrene plates warranted a test. Plating at identical densities from the same batch of cells, I conducted tests of dye mixing times in polystyrene plates to compare to the Poly-D-Lysine results previously run at room temperature. Four (4) assay plates subjected to 1 hour, 45 minutes, 30 minutes or 15 minutes of dye mixing time in polystyrene plates served as the base for this test. Overall, the 1-hour dye mixing time was superior, as it was in the Poly-D-Lysine plates; however, there was a notable difference in the confluence of the cells in each well with the polystyrene plates. There seemed to be a difference in rate of growth, as plating 50,000 cells/well did not achieve the same 80-90% confluence that was noted with the Poly-D-Lysine plates. Due to this inconsistency of cell growth, the results were not directly comparable to the results of the Poly-D-Lysine plates. The decision was to forego the polystyrene plates and utilize the Poly-D-Lysine plates to help ensure consistency of results and minimize the number of cells needed to achieve confluence.

2.2.5. Plating environment optimization

Documentation from Codex Biosolutions states that once the cells are plated there is no need to feed them; however, there is a balance that must be struck between keeping the cells viable for assay and not complexing the drugs being assayed, as it is well-known that cannabinoids are highly protein bound.²³¹ Normal growth media for the cells is DMEM with 10% FBS; 1% P/S; 250 µg/mL G418 and 1 µg/mL puromycin, and is what initially was utilized for plating, minus the selection antibiotics of G418 and puromycin. The initial test was to see what, if any, difference exists between FBS and BSA and to compare different concentrations of the two against one another. Initial concentrations of FBS tested were 10%, 5% and 1%. Initial concentrations of BSA tested were 1%, 0.5% and 0.25%. In all three of the tested concentrations of BSA there was no discernible curve with any of the cAMP production stimulants (epinephrine, formoterol, isoproterenol and NECA) tested. Within the FBS concentrations tested, all three were comparable; however, 1% FBS did show slightly better across all tested cAMP stimulants, so it warranted further testing.

Using isoproterenol as the cAMP stimulant, FBS at 4 different concentrations (1%, 0.75%, 0.5% and 0.25%) were examined. The 1% FBS plate demonstrated inconsistency from the previous run, and subsequently lower concentrations of FBS proved even more erratic. So, ultimately, the decision was made to continue plating and running the assays in DMEM with 10% FBS and 1% P/S.

2.2.6. Pertussis toxin (PTx) dosing optimization

In examining CB₁ and CB₂, which are PTx-sensitive G_{i/o}-coupled receptors, one of the assays necessary to run is a PTx-sensitive G_{i/o}-inactivation of the coupling of CB₁ or CB₂ receptors to G_{i/o} prior to drug treatment and conductance of the assay. While it is

true that the HEK-CNG cells help to rule out the drug effects on any other constitutively expressed receptor, it does not validate that the actions are due to CB₁ and/or CB₂ G_{i/o} GPCR activity.

2.2.6.1. Duration of PTx pre-treatment. The first question to answer with regard to PTx treatment of the cells is how long it is necessary to pretreat the cells. I examined two methods when testing this question, both easily fitting into the normal procedure for the assay and thus their selection and utility. The first was to add PTx directly before the membrane potential dye, which would allow 1 hour of dwell time for the PTx prior to drug addition. At all 8 concentrations tested (400 ng/mL, 200 ng/mL, 80 ng/mL, 40 ng/mL, 20 ng/mL, 8 ng/mL, 4 ng/mL and 0 ng/mL), CP-55,940 was still able to exert its full effect of inhibiting forskolin-induced cAMP production (**Figure 2.14**).

The next method employed was to add the PTx into the cell suspension reservoir at the time of plating and allow the PTx to dwell in the cell suspension overnight, at least 12 hours prior to drug addition. At all 7 concentrations tested (400 ng/mL, 200 ng/mL, 80 ng/mL, 40 ng/mL, 20 ng/mL, 8 ng/mL and 4 ng/mL), CP-55,940 was unable to exert its full effect of inhibiting forskolin-induced cAMP production (**Figure 2.15**). Since literature precedence had set PTx-sensitive G_{i/o}-inactivation dosing in a range from 8 ng/mL to 100 ng/mL, I selected 4 ng/mL to conserve the most resources and still maintain a inactivation of G_{i/o} activity in the assay. Based on these data, I concluded that the optimal procedure for PTx-sensitive G_{i/o}-inactivation is 4 ng/mL with an overnight pretreatment.

To confirm these findings in CB₂, the same seven concentrations (400 ng/mL, 200 ng/mL, 80 ng/mL, 40 ng/mL, 20 ng/mL, 8 ng/mL and 4 ng/mL) of PTx were added to the CB₂ cells and allowed to sit overnight before screening against CP-55,940. As was the case in CB₁, CP-55,940 was unable to inhibit forskolin-induced cAMP production across the entire spectrum of concentrations tested (**Figure 2.16**). As a result of this study, and to maintain continuity of assay conditions across the cell types, 4 ng/mL was selected as the target concentration for future PTx-sensitive G_{i/o}-inactivation studies of the CB₂ cells.

2.2.6.2. Addition order of PTx treatment. Knowing that 4 ng/mL with an overnight dwell time is necessary before inhibition of the G_{i/o} is achieved, the question remained of how to best administer the PTx. This question became relevant due to the perception of a lack of cell adhesion to the plates when mixed into the cell suspension. To test this, 4 plates: no PTx, PTx in cell suspension, PTx in PBS added at plating and PTx in PBS added 2 hours after plating, were tested. The result was that there is no difference in how the PTx is added, just that it dwells overnight (**Figure 2.17**).

2.2.7. Assay data analysis optimization

As previously mentioned, readings of the assay plates utilizes a BioTek Synergy 2 running Gen5 software. GraphPad Prism generates the graphs and non-linear curve fitting with EC₅₀ calculations. While the Gen5 software is capable of generating graphs and EC₅₀ curves, the manipulation of the data was not as automated and high-throughput as

was desired. In addition, extraction of data from Gen5 software and importation into

Figure 2.14. CB1 cells with 1 hour pretreatment of PTx vs. CP-55,940

Figure 2.15. CB₁ cells with overnight PTx pretreatment vs. CP-55,940

Figure 2.16. CB₂ cells with overnight PTx treatment vs. CP-55,940

Figure 2.17. CB₂ cells with PTx addition order test

GraphPad Prism was a cumbersome and tedious process. Utilizing the “Power Export” function of the Gen5 software allowed me to develop custom VBA macros – visual basic applications, which allows for user-defined functions and automates processes and accessing Windows APIs – for use in Microsoft Excel, which would automate the process of data normalization and subsequent importation into and analysis by GraphPad Prism.

2.2.7.1. GraphPad Prism template building. The initial step in this process was to import manually data from one plate of an assay to construct a template in GraphPad Prism. The template contains eight XY scatter with single Y value data sheets and graphs. Analysis of all eight sheets was accomplished through non-linear regression with the built-in “log(agonist) vs. response – Variable slope” equation with automatic outlier elimination and an output of a summary EC₅₀ table. After all eight data sheets, results sheets and graphs existed, the data tables had their contents removed and the resulting shell project saved as a template, with the name “IndividualPlates.” This template resided in a subfolder in the directory utilized by Gen5 for data storage and exportation – in this case: “C:\Data\Templates\.”

Since our typical run will be for n = 6 for each drug, there needs to be data combined from across six separate plates into one master file for each compound. As such, a combined data template was necessary to create. To accomplish this task, a project was created such that contained therein was nine XY scatter with 6 replicate Y value data sheets and graphs. The data sheets were populated with data and analysis of all 9 sheets was accomplished through non-linear regression with the built-in “log(agonist) vs. response – Variable slope” equation with automatic outlier elimination and an output of a summary EC₅₀ table. Following the analysis and subsequent creation of the results sheets and population of the graphs, data tables had their contents removed, and the resultant shell project saved as a template, with the name “MultiplePlates.” This template resides in a subfolder in the directory to be utilized by Gen5 for data storage and exportation – in this case: “C:\Data\Templates\.”

2.2.7.2. Gen5 experiment design. The first task in the design of this experiment was to ensure that all data remains in a readily accessible manner for quick retrieval and obvious content. Utilizing the parameters of the Gen5 protocol options, I created a nomenclature system that automatically generates after the user inputs key, required, data at the start of an experiment. A central repository of data, simply “C:\Data\,” came to be for this task. Within that folder, a subfolder would be created for the protocol being used (CB1 Functional, CB2 Functional or HEK-CNG Functional), i.e. “C:\Data\CB1 Functional\.” Within the subdivisions of data, further compartmentalization of the experiments based on the <Barcode>_<PlateID> fields input by the user at the initial read of each plate is implemented. The <Barcode> is the notebook number of the individual conducting the assay in the format of “3-letter initials”-“notebook number”-“page in notebook”, e.g. BAK-1B-57. The <PlateID> field is the project title for that series of runs to make it more readily identifiable as to the contents thereof, e.g. “cAMP Stimulant Dose Curves.” With this nomenclature, files would be stored based on the assay run and then further segregated by the notebook number, allowing for quick callback to the experimental notes, e.g. “C:\Data\CB1 Functional\BAK-1B-57_cAMP Stimulant Dose Curves\.”

The next task was to ensure that file names followed a similarly intuitive nomenclature for ease of reference to the experimental notes in the notebook and the obvious contents of each file, were they separated from their parent folder(s). The naming scheme is set in the protocol options of each of the 3 protocols being utilized, and follows a system similar to that of the folder nomenclature: “<Protocol>_<PlateID> (autoenumeration)_<Barcode>_<Date> (YYMMDD).” The <Protocol>, as previously mentioned is CB1 Functional, CB2 Functional or HEK-CNG Functional. <PlateID> and <Barcode> are the same as previously mentioned. The (autoenumeration) field is a 4 digit, sequential series of numbers that increases with each run, so that 6 plates with identical <Protocol>, <PlateID>, <Barcode> and <Date> will all have unique (autoenumeration) values to identify which of the 6 runs it is. Lastly, <Date> is the current experiment day’s date; however, in Gen5 you cannot select the format of the date, so it always shows as YYMMDD. An example of a filename with full path for the first run in a series of 6 would be: “C:\Data\CB1 Functional\BAK-1B-57_cAMP Stimulant Dose Curves\CB1 Functional_cAMP Stimulant Dose Curves (0001)_BAK-1B-57_130516 (YYMMDD).xpt.” This nomenclature system allows for quick reference to the notebook, as well as the person responsible for the run and the content of each of the files – all aiding in expediting data acquisition and analysis.

To accomplish the task of making the data acquisition as automated as possible, the Gen5 software was customized with a unique experiment design that was copied across all three types of cell assays. The first customization was in the sequence of events when reading a plate. The sequence for each plate run in these experiments is:

1. Set Temperature: Set point 25 °C
2. Read: (F) 540/25,590/20
3. Pause
 - a. Plate In/Out - Plate Out
 - b. Popup: “Add DRUG to wells, place plate on reader and then click OK”
 - c. Plate In/Out - Plate In
4. Start Kinetic [Run 1:00:00, Interval 0:01:00]
 - a. Read: (F) 540/25,590/20
5. End Kinetic

The automation of steps helped to ensure that there was consistency among the days and persons running the assays to aid in lack of omission of steps.

At the completion of the kinetic run, the “Power Export” function was set to commence, automatically. This function a Microsoft Excel template that the user may customize to output certain parameters of each experimental run. The “Power Export” file was named exactly as the experiment from which it was generated, e.g. “C:\Data\CB1 Functional\BAK-1B-57_cAMP Stimulant Dose Curves\CB1 Functional_cAMP Stimulant Dose Curves (0001) BAK-1B-57_130516 (YYMMDD).xlsm.”

2.2.7.3. Gen5 “Power Export” template building. One of the key components in facilitating fast and accurate data analysis was the utilization of the “Power Export”

function of Gen5. Properly configured, this generated a Microsoft Excel spreadsheet that could perform all necessary data calculations and then automatically import them into GraphPad Prism utilizing templates previously mentioned for these experiments. The first step in building this template is to ensure that all pertinent data transfers on the spreadsheet that exported from Gen5 into Microsoft Excel. To accomplish this, the “Read 2” matrix migrated, beginning at cell A1. Beginning at cell A65, I placed the “Field Group,” which contained all of the experiment’s nomenclature information, i.e. protocol name, plate ID, barcode and reading date/time. Beginning at cell A70, I placed the “Well IDs” group, which listed the names of the drugs contained in each well. With these fields in place, every time the experiment performs a “Power Export” all of the data from Gen5 consistently migrates into a formatted Microsoft Excel spreadsheet.

With the data now in a more malleable form in Microsoft Excel, it is time to build the customized VBA macros that appended to the “Power Export” template inside each of the three protocols. Numerous cycles of trial and error, as well as questions asked with regard to how best to view the data led to a rough draft of the macro design. Taking this, along with how best to normalize/standardize across different plates on different days with different receptor types led to the finalized product.

Primarily, creation of a “Declarations” module is paramount, because this facilitates the passage of variables from module to module without having to worry about improper nomenclatures or pass through commands. See **Supplementary Information 1.1** for full template code of the Declarations module.

Next, I created a “switchboard” module that would be responsible for calling all of the subsequent modules for data analysis. Through this module all data is packaged, normalized to internal standards and imported into GraphPad Prism. See **Supplementary Information 1.2** for full template code of “Extract” module.

With this “switchboard” in place, it is time to start examining all of its moving parts, beginning with the first call in the process, which is to select the evaluation parameters – accomplished via the “CalcType” form. This form contains radio buttons, which allow the user to select various analytical parameters for the data, such as the time frame, X-axis component, normalization method and calculation method. After the user selects one of each of these parameters, the “Run Extraction” button is clicked, causing the global options to be updated with the new inputs and control the remainder of the data extraction and manipulation process. See **Supplementary Information 1.3** for full template code of the “CalcType” form module.

The normalization method chosen, dictates the next VBA function initialized. The “NormData” module contains all four of the data normalization methods that I had employed at one time or another; however, the one used in the analysis of all the forthcoming data was “Norm2.” Wherein the wells with the lowest concentration of drug (column 11 on the assay plate) served as the experimental max for that row at any given point in time and the “Blank” cells (column 12, rows A and B of the assay plate) serve as the experimental minimum. The use of the lowest drug concentration as the experimental maximum helped to address the drifting of data, which was noted during the early runs of

the assay. The data were then normalized values via **Equation 2.1**:

$$\frac{A_n + (x - A)(B_n - A_n)}{B - A} \quad \text{Equation 2.1}$$

Wherein A = Experimental Basal Response, B = Experimental Maximum Response, A_n = Normalized Minimum Value (0), B_n = Normalized Maximum Value (100) and x = raw data value to be normalized. See **Supplementary Information 1.4** for full template code of the “NormData” module.

The next parameter manipulated has to do with the time interval of interest. All data presented in subsequent chapters utilized the 5 minutes time interval, i.e. values from each of the concentrations of drug were examined at every 5 minutes up to 60 minutes. However, in some cases it is useful to examine based on the maximum and minimum values for the control wells (column 12, rows E and F of the assay plate) to determine the optimal time interval. See **Supplementary Information 1.5** for full template code of “FiveMin” and “ZenNadir” modules.

Within both the “FiveMin” and “ZenNadir” modules are references to necessary information for their completion. The first of which is the concentrations for each of the wells in the assay plate. If the file does not exist for this, the VBA code will call a form for the user to input the beginning concentration of each row (column 1) for the assay plate in nM. After inputting the concentrations in the form and clicking “OK,” VBA code runs to translate the nM values into $\log_{10}$ values for use in GraphPad Prism and creates a “Concentrations.txt” file so that the values do not have to be re-input by the user for subsequent runs of the same plate layout in a day. See **Supplementary Information 1.6** for full template code of the “Concentrations” module.

As part of the Gen5 experiment setup, the user should input the row names into the software; however, in the event that this step goes overlooked, a form will pop up during analysis of the data asking the user to input the row names. Upon inputting the names and clicking OK, the code runs to create a name file and apply those names to the current spreadsheet. See **Supplementary Information 1.7** for full template code of the “Names” module.

Following the time, concentration and name executions, it is now time to run the appropriate calculation module to refine the data, if need be. All functional data presented henceforth will have no additional calculations applied to it beyond the aforementioned normalization method. The 6 possible calculation methods are: take only the normalized values (the method employed in all forthcoming analysis); to subtract the Ro-containing control wells (column 12, rows C and D of the assay plate); to subtract the forskolin-containing control wells (column 12, rows E and F); to divide by the lowest concentration of drug (column 11, same row as data being evaluated and at the same point in time); to divide the drug at a given time to that well’s value at time = 0 min; or to divide each drug by the forskolin-containing control (column 12, rows E and F). See **Supplementary Information 1.8** for full template code of the different calculation modules.

After any of the calculations are completed, you will notice that there is a call to organize the raw data. This is a simple transposition of the data to ready it for importation into GraphPad Prism. See **Supplementary Information 1.9** for full template code of the “Organize” module. With the data now normalized, calculated, organized and ready for importation into GraphPad Prism, it is necessary to create individual data sheets that GraphPad will use to import into the shelled data sheets that exist in the template. See **Supplementary Information 1.10** for full template code of the “DataSheets” module. After this, the code loops continuously to await completion of the Prism importation.

After creating the data sheets for each of the 8 drugs on a plate, the VBA macro will generate a script to execute when it calls the GraphPad Prism program. See **Supplementary Information 1.11** for full template code of the “PrismScript” module. After the GraphPad Prism script completes its data importation, it creates a “Done.txt” file, which is the signal for the all-the-while-looping Microsoft Excel spreadsheet to copy itself into the master book, clean up the temporary files and close. I wanted to copy the extracted, normalized and calculated data into a master file, because I wanted a mechanism to collect all 6 runs in one file to replicate this process for the n=6 analysis of each of the drugs tested. See **Supplementary Information 1.12** for full template code for the “Copy” module.

With all of these VBA macros and forms in place and functioning, it is necessary to insert an easy mechanism to set into motion the “switchboard” and the subsequent cascade of events I’ve gone through. To accomplish this task, I inserted an object onto the main sheet of the Gen5 “Power Export” template – for this, I used an unfilled, unoutlined rectangle that I sized to the A2 cell of the sheet. After inserting this, right clicking the object and clicking “Assign Macro” allowed me to select the “Extract” macro as its designated target. Once saved, this template will be the default “Power Export” for this protocol, and the protocol need only be replicated in triplicate and assigned the 3 aforementioned names. Once the “Power Export” executes at the completion of a run, I need only click on the object previously inserted that is assigned the “Extract” macro to automate data manipulation and importation into GraphPad Prism.

2.2.7.4. Master extraction template. As each of the runs for a set of drugs completes, and as previously mentioned, the data sheet from each mirrors into a master Excel workbook located in the same folder as all of the other current data for the in-progress 8 compounds. After the completion of the sixth run, it is necessary to perform a similar, albeit more simple, action on the master workbook to extract the data from each individual run, group them together and export the data from Excel into GraphPad Prism.

As was the case with the individual run template, the first and foremost module to create is the “Declarations” module to allow for passage of variables among the different functions contained in the project. See **Supplementary Information 1.13** for details of this VBA code.

Since this workbook is merely copying already transformed data from within itself into one, centralized, sheet, the amount of VBA code necessary is quite limited relative to the previous template. Still contained within it is the scripting necessary to

generate data sheets for importation into GraphPad Prism. Therefore, with the click of a “button,” set up just as we did for the individual runs – an object assigned to the “Extract” macro – we can automate the bundling of all six runs for the eight ligands contained in each day’s runs and compile a master sheet of data. See **Supplementary Information 1.14** for details of the VBA code.

Execution of this series of macros allows for the easy bundling of 6 plates worth of data for 8 different ligands at 11 concentrations each over 60 minutes. Combination of the individual plate macros with the master workbook macros reduces data analysis time by hours, which allows for a higher, more accurate throughput of compounds.

2.3. Finalized Assay Conductance

2.3.1. Materials

Forskolin, purchased from Tocris Bioscience/R&D Systems (Minneapolis, MN). Ro 20-1724 (PDE inhibitor), G418, Puromycin, Pen/Strep (100U/100µg per mL), DPBS, Hank’s 1x Buffer, DMEM, Direct Blue 71, HEPES and pluronic acid were purchased from ThermoFisher Scientific (Waltham, MA). Fetal Bovine Serum (FBS) purchased from Atlanta Biologicals (Lawrenceville, GA). The transfected HEK-293 cells coexpressed with modified CNG channels along with Cannabinoid Receptor Type 1 (CBR-1) or Cannabinoid Receptor Type 2 (CBR-2) and their parental cells (without Cannabinoid Receptor Type 1 or 2; HEK-CNG) were purchased from Codex BioSolutions - so named ACT:One Cells (Gaithersburg, MD). Membrane potential dye was made by combining 1 L Hank’s 1x Buffer with 5.2 g HEPES (20 mM final concentration) and adjusting pH to 7.4. To the buffered solution we add 2.9 mL of DiSBAC stock – freshly prepared by adding 11.91 mg DiSBAC to 3 mL DMSO – 700 µL 10% pluronic acid and 1.874 g Direct Blue 71. Cell culture media for the CBR1 and CBR2 containing cells was made by combining 890mL DMEM, 100mL FBS, 10mL P/S, 1250 µL G418 and 100 µL Puromycin.

2.3.2. Cell culture and frozen cell preparation

HEK-CNG, CB₁ and CB₂ transfected cells were maintained in DMEM medium containing 10% FBS, 1% Pen/Strep, 250 mcg/mL G418 at 37 °C in 5% CO₂; however, the CBR-1 and CBR-2 transfected cells were cultured in medium containing an additional 1 µg/mL Puromycin. The cells, seeded at a density of 2.8 x 10⁶ cells, in a T225 flask (Corning; Corning, NY) containing 40 mL of the appropriate culture media and were allowed to grow until reaching 80% to 90% confluence.

2.3.3. CNG channel-coupled cAMP assay

Cells from 1 appropriately 85-90% confluent flask were harvested. The cells were resuspended in 10% FBS DMEM with 1% pen/strep medium at 500,000 cells/mL for

CBR-1 and HEK-CNG and 300,000 cells/mL for CBR-2. 100 μ L of resuspended cells was dispensed into each well of 6 clear, 96-well, Poly-D-Lysine plates (BD via Fisher Scientific) using a 12-channel multi-channel pipet (ThermoFisher Scientific; Waltham, MA). It is important to note that the plating method needs to be such that the cell suspension must be plated by row, rather than by column, to alleviate the false-positive change in cAMP noticed on some of my data. Thus, plate column 12, then row 1 (columns 1 through 11), row 2 (columns 1 through 11), etc. for each plate. After overnight culture at 37 $^{\circ}$ C and 5% CO₂ the cells were 75% to 80% confluent.

After the overnight incubation period, I added 100 μ L of membrane potential dye to each well of the culture plate and allowed to sit in a dark area at room temperature for 60 minutes. After the 60 minute incubation period a baseline read in a BioTek Synergy 2 plate reader with an excitation of 540/25 nm and an emission of 590/20 nm. Following the baseline read, 50 μ L of ligand stock solution containing: appropriate drug concentration (beginning at either 25 μ M or 5 μ M [5 μ M or 1 μ M, final], Table 1), 4 μ M Forskolin (800 nM, final) and 125 μ M Ro 20-1724 (25 μ M, final) in DPBS with 2.5% DMSO was added to the cell culture plate. Columns 1 through 11 contain drug, and Column 12 contains control wells. Control wells are: "Blank" (cells, media and dye), "Ro" ("Blank" + 25 μ M Ro 20-1724 [Final]), "Ro/For" ("Ro" + 800 nM Forskolin [Final]) and "CP CTRL" ("Ro/For" + 5 μ M CP 55,940 [Final]). The plate was then placed on the plate reader with 1 read per well per minute with an excitation of 540/25 nm and an emission of 590/20 nm for 60 minutes.

2.3.4. Data analysis

I analyzed primary data with customized VBA macros, developed internally, in Microsoft Excel (Redmond, WA). Column 11 (lowest [drug]), determined the experimental maximum response (100 % activity) at each individual time point, and the experimental basal response was determined by the average response given by the 2 "Blank" wells. All data were normalized with these minimum and maximum values via **Equation 2.1**. The EC₅₀ values were calculated from concentration-response curves by non-linear regression analysis utilizing GraphPad Prism Software (San Diego, CA).

2.4. Conclusions

In this chapter, I have demonstrated optimization of the ACTOne GPCR assay from Codex Biosolutions to ensure reproducible, cost-effective and reliable data in a high-throughput manner. Additionally, I have provided an example of one method to enhance the Gen5 plate reading software with Microsoft Excel's powerful VBA macro capabilities to yield a fast and effective means of analyzing large quantities of data generated from conductance of functional assays.

Future directions for this project could be a further honing of the assay to gain even more cost-savings, speed and accuracy of data acquisition. Namely, a more serious trial and investigation into the use of the more cost-effective polystyrene plates over the Poly-D-Lysine coated plates.

CHAPTER 3. FUNCTIONAL EVALUATION OF A NOVEL CLASS OF PHENYL-SUBSTITUTED SIDE CHAIN ANALOGUES OF Δ^8 -THC

3.1. Introduction

Classical cannabinoids are tricyclic compounds possessing a benzopyran core – these are of either natural origin or synthetic analogues thereof. The naturally occurring, prototypes for these compounds are Δ^9 -THC and Δ^8 -THC, which both bind to CB₁ and CB₂ receptors and exert a partial agonist activity.¹⁴ Within the classical compounds impacting the CB₁ receptor there exists novel means of dealing with anti-nociception, mitigation of chemotherapeutic-induced nausea and vomiting, decreasing intra-ocular pressure in glaucoma, increasing appetite in patients with wasting syndromes, reduction of muscle spasticity caused by diseases such as multiple sclerosis and treating diarrhea by slowing intestinal motility are present.^{232,233} With CB₂ receptors serving the body as modulators of cytokine release, agents targeting these compounds show great promise in inflammation, especially in instances of inflammation-induced organ damage.^{136,165} The drawback exists in separating CB₁ and CB₂ activity to retain or augment the desired therapeutic outcome while simultaneously mitigating the negative psychotropic effects, ascribed to CB₁, of such compounds. This desire for such potentially impactful compounds for various disease states which have favorable therapeutic use profiles has led to a bevy of research into the classical cannabinoids with regard to their pharmacology and structural activity relationships/requirements (SAR).

Current knowledge of cannabinoid SAR has centered heavily around the classical, benzopyran core, and from these studies one may gather three pharmacophoric elements exist for classical cannabinoids to exert cannabimimetic activity (**Figure 3.1**):

1. C-1 phenolic hydroxyl group
2. Trans ring junction at the B and C rings
3. Lipophilic C-3 alkyl side chain

Literature precedent shows that lengthening of the C-3 alkyl chain (**Section 3.2.1**) causes an increase in the binding affinity of the compounds, with an optimal length of 7 to 8 carbons. Additionally, unsaturation (**Section 3.2.2**), especially about the C-1',2' bond affords an even greater enhancement of binding affinity and selectivity, with the linear alkyne and *cis* 1',2' alkene showing optimal activity. Another area of exploration was the substitution of C-1' and C-2' (**Section 3.2.3**), which demonstrated the importance of this position, with 1',1'-dimethylation being optimal. Compilation of this knowledge (**Figure 3.2**) led to the synthesis of cycloalkyl²³⁴ and aromatic²³⁵ substituents (**Section 3.2.4**) introduced at C-1'.

This study seeks to utilize the ACTOne Assay (**Chapter 2**) to explore the functional ramifications of an aromatic substitution at C-1' (**Section 3.3**). Taking this functional activity data, I will then reconcile these outcomes against homology models and current literature standards' binding to propose hypothetical interactions with CB₁ and CB₂, to further develop the SAR and unique mechanism of these compounds.

Figure 3.1. Classical cannabinoid with pharmacophore elements

Figure 3.2. Design progression of the KM series of compounds

3.2. Classical Cannabinoids – Modifying C-3 Substituents to Increase Binding

For the purposes of examining this novel series of compounds, I will leave C-1 phenolic hydroxyl and the trans ring junction pharmacophoric elements intact, and I will not bring into account the optional elements of the northern and southern hydroxyl groups.

3.2.1. Varying C-3 chain length

The most logical starting point for modification of the C-3 position was to substitute the *n*-pentyl of Δ^8 -THC (**1**) (CB₁ K_i = 47.6 nM; CB₂ K_i = 39.3 nM) with varying lengths of C-3 substituents (**Figure 3.3**). This ranged from *n*-butyl (**2**) (CB₁ K_i = 65 nM) to *n*-hexyl (**3**) (CB₁ K_i = 41 nM) to *n*-heptyl (**4**) (CB₁ K_i = 22 nM) and to *n*-octyl (**5**) (CB₁ K_i = 8.5 nM).²³⁶ Data from this study show that *n*-octyl (**5**) has the highest affinity, with *n*-heptyl (**4**) a near second.

3.2.2. Unsaturated C-3 side chain analogs

A next logical step is to undertake the introduction of unsaturation into the C-3 side chain (**Figure 3.4**) to probe the binding pockets. Conformationally restricted aliphatic chains to help elucidate the spatial configuration of the LBP of CB₁ and CB₂ receptors about the hydrophobic C-3 binding pocket. Beginning the process of unsaturation one may turn attention first to the C-1' heptyne derivative (**6**) (CB₁ K_i = 0.65 nM; CB₂ K_i = 3.1 nM)²³⁷ and note that it has, at best, a modest proclivity for CB₁ over CB₂ but good affinity for both overall. Stepping down the unsaturation at C-1', one may examine the C-1'-heptene derivatives as the *Z*-isomer (**7**) (CB₁ K_i = 0.8 nM; CB₂ K_i = 9.5 nM)²³⁸ and *E*-isomer (**8**) (CB₁ K_i = 1.2 nM; CB₂ K_i = 5.3 nM).²³⁸ It is noteworthy that these are C-11-hydroxy compounds, but they do not differ, in the case of the *Z*-isomer at CB₁, from the C-9-methyl compounds (CB₁ K_i = 0.86 nM).²³⁶ Taking these three compounds as a group, it is evident that CB₁ affinity is unaffected among the three; however, the CB₂/CB₁ ratio changes. Most notably, this change in the ratio occurs with compound **7**, suggesting that CB₂ has less tolerance for acute angle orientation and unsaturation of C1'-C2'.

Moving more distal along the C-3 substituent chain one may examine a large number of C-2' unsaturated compounds, beginning first with the 2'-hexynyl derivative (**9**) (CB₁ K_i = 11 nM).²³⁶ 2'-octynyl (**10**) (CB₁ K_i = 4.9 nM) does well so long as there is a free phenolic hydroxyl; however, when C-1 is methoxy the affinity drops considerably (CB₁ K_i = 189 nM).²³⁶ 2'-nonyl (**11**) (CB₁ K_i = 3.7 nM)²³⁶ continues to improve upon the binding affinities of both 2'-hexynyl and 2'-octynyl, suggesting that with an optimized angle through unsaturation the binding pocket extends further. In terms of the 2'-alkenes, the *Z*-isomers of both 2'-heptene (**12**) (CB₁ K_i = 1.55 nM)²³⁹ and 2'-octene (**13**) (CB₁ K_i = 3.19 nM)²³⁶ showed the most promise, with 2'-pentene-5'-hydroxy (**14**) (CB₁ K_i = 125 nM)²³⁹ and 2'-heptene-6'-acetamide (**15**) (CB₁ K_i = 43.4 nM)²³⁹ paling in comparison. The lack of affinity in the hydrophilic derivatives points strongly to the conclusion that this is a relatively exclusively lipophilic pocket of the receptor.

Figure 3.3. C-3 chain length analogues and the effect on CB_1 binding affinity

Continuing the trek of unsaturation, one may move one carbon more distal on the C-3 chain, where relatively few modifications exist to date. 3'-butynyl (**16**) ($CB_1 K_i = 367 \text{ nM}$)²³⁶ fared much worse than 3'-octynyl (**17**) ($CB_1 K_i = 9 \text{ nM}$)²³⁶, but neither did as well as the Z-isomer of 3'-octenyl (**18**) ($CB_1 K_i = 3.36 \text{ nM}$)²³⁶ (**Figure 3.4**). This data suggests that the hydrophobic pocket of CB_1 turns, and the more rigid, straight alkynes likely have some steric issues hindering their binding affinity.

Moving next to the 4' position, there are 2 derivatives of note, 4'-octynyl (**19**) ($CB_1 K_i = 19 \text{ nM}$) and the Z-isomer of 4'-octenyl (**20**) ($CB_1 K_i = 11 \text{ nM}$)²³⁶ (**Figure 3.4**). These two suggest, again, that the binding pocket of CB_1 has a curve to it and that the more distal on the C-3 side chain the unsaturation is, especially to the alkyne (linear) state, the less likely one is to fit that curve and thus one loses binding affinity.

Next, sites of multiple unsaturation are examined, beginning with 1',6'-heptadiyne (**21**) ($CB_1 K_i = 460 \text{ nM}$)²³⁶ 2'-pentyne derivatives with a 5' substitution (**Figure 3.4**) seem to fair best as far as the multiple unsaturation derivatives go, which may seem counterintuitive given **21**'s lack of affinity. However, the presence of 5'-CN (**22**) ($CB_1 K_i = 31 \text{ nM}$)²³⁶ or 5'-NNN (**23**) ($CB_1 K_i = 6.15 \text{ nM}$)²³⁶ demonstrates that in the case of multiple unsaturation, the LBP requires a H-bond accepting terminus to bind with higher affinity. This trend continues when one looks at the 2'-hexyne derivatives containing 6'-CN (**24**) ($CB_1 K_i = 0.77 \text{ nM}$), 6'-NCS (**25**) ($CB_1 K_i = 11.5 \text{ nM}$) or 6'-NO₂ (**26**) ($CB_1 K_i = 5.34 \text{ nM}$)²³⁶. This becomes most evident and useful comparing the 2'-heptyne-6'-sulfonamide derivatives containing an *n*-methyl (**27**) ($CB_1 K_i = 2.5 \text{ nM}$; $CB_2 K_i = 0.2 \text{ nM}$), *n*-ethyl (**28**) ($CB_1 K_i = 20 \text{ nM}$; $CB_2 K_i = 1.4 \text{ nM}$) and *n*-butyl (**29**) ($CB_1 K_i = 70 \text{ nM}$; $CB_2 K_i = 86 \text{ nM}$) substituted sulfonamide.²⁴⁰ This data suggests that the CB_2 LBP has a larger hydrophobic pocket that could be targeted over the CB_1 LBP by extending the C-3 substituent length just enough without causing an eventual hindrance to both – as is the case with compound **29**. Also of interest is the affinity of **24** and the 5'-azidopent-2'-yne (**30**) ($CB_1 K_i = 2.14 \text{ nM}$)²⁴¹. The interest lies dually in the 3-fold difference of binding affinities and in that both are antagonists with 5'-azidopent-2'-yne being a partial agonist while hept-2'-ynenitrile is neutral.²⁴¹

3.2.3. C-1' modified compounds

As previously discussed in **Section 3.2.1**, optimal activity was found with *n*-heptyl (**4**) or *n*-octyl (**5**), but further derivation found those compounds with a 1',1'- or 1',2'-dimethyl substitution were more potent still.^{11,242,243} 1',1'-dimethylheptyl (**31**) ($CB_1 K_i = 0.89 \text{ nM}$; $CB_2 K_i = 1.41 \text{ nM}$) and 1',2'-dimethylheptyl (**32**) ($CB_1 K_i = 0.46 - 0.84 \text{ nM}$) provided the initial foundation of optimized chain length and substitution pattern for derivation into 1',1'-dimethyl pentyl and hexyl with 5' or 6' substituted compounds (**Figure 3.5**). As the size of the substitution increases, the affinity for CB_1 starts to numerically wane, as is exemplified by 1',1'-dimethyl-6'-hexanenitrile (**33**) ($CB_1 K_i = 0.36 \text{ nM}$), 1',1'-dimethyl-7'-heptanenitrile (**34**) ($CB_1 K_i = 0.6 \text{ nM}$) and 1',1'-dimethyl-6'-phenyl-7'-heptanenitrile (**36**) ($CB_1 K_i = 0.92 \text{ nM}$)²⁴⁴. However, these values are not a therapeutically viable or targetable difference. A similar trend is noticed when comparing the 1',1'-dimethyl-6'-N,N dimethyl hexane (**37**) ($CB_1 K_i = 0.86 \text{ nM}$) to

Figure 3.5. Acidic and basic modifications to the terminus of Δ^8 -THC C-3 substituents

1',1'-dimethyl-6'-N,N-diethylhexane (**38**) (CB₁ K_i = 13 nM).²⁴⁴ *p*-phenylsulfonamide derivatives (**39**, **40**, **41**) overall fared worse than smaller substituents, but demonstrated the size constraints of the CB₁ LBP.²⁴⁴ Interestingly, this increase in size was not as impactful on the CB₂ K_i. This tolerance is somewhat evident in the case of 1',1'-dimethyl-6'-hexanenitrile (**33**) (CB₁ K_i = 1.75 nM; CB₂ K_i = 1.1 nM) versus 1',1'-dimethyl-6'-phenyl-7'-heptanenitrile (**36**) (CB₁ K_i = 18.4 nM; CB₂ K_i = 5.75 nM) and 1',1'-dimethyl-N,N-diethylheptanamide (**38**) (CB₁ K_i = 23.9 nM; CB₂ K_i = 2.47 nM).²³⁹

In an attempt to exploit the apparent hydrophobic binding pocket that exists along with the C-3 substituent binding pocket a series of C-1' cyclic compounds were synthesized (**Figure 3.6**) for testing.²⁴⁵ This hydrophobic pocket is evidenced by the fact that 1',1'-dimethyl compounds bind with far greater affinity than their methylene brethren, and further demonstrated by the lack of binding affinity for the 1'-hydroxyl (**42**) (CB₁ K_i = 86.4 nM; CB₂ K_i = 65.6 nM) and 1'-ketone (**43**) (CB₁ K_i = 21.7 nM; CB₂ K_i = 83.7 nM). Comparing the C-3 heptyl (**4**) (CB₁ K_i = 22 nM) to 1',1'-dimethylheptyl (**32**) (CB₁ K_i = 0.83 nM; CB₂ K_i = 0.49 nM) to 1'-cyclopentyl (**44**) (CB₁ K_i = 0.45 nM; CB₂ K_i = 1.92 nM), 1'-dithiolaneheptyl (**45**) (CB₁ K_i = 0.32 nM; CB₂ K_i = 0.52 nM) and 1'-dioxolane (**46**) (CB₁ K_i = 0.52 nM; CB₂ K_i = 0.22 nM) one may see that there is evidence of a dual-lobe hydrophobic binding pocket available about C-3 substituent pocket. Interestingly, this too brings up an issue of size within both the CB₁ and CB₂ receptor LBP, as evidenced in the 1'-dithianeheptyl (**47**) (CB₁ K_i = 1.8 nM; CB₂ K_i = 3.6 nM) and 1'-benzodithioleheptyl (**48**) (CB₁ K_i = 56.9 nM; CB₂ K_i = 257 nM).²⁴⁵ This is an example, though, of a means to gain a 5-fold selection for CB₁ over CB₂ receptor subtypes. This auxiliary hydrophobic pocket is not a primary binding pocket, as evidenced in the C-1'-dithiolane derivative that is without an additional C-1' substituent (**49**) (CB₁ K_i = 168 nM; CB₂ K_i = 103 nM).²⁴⁵ There is some loss of affinity when the C-1' contains both dithiolane and cyclohexyl substituents (**50**) (CB₁ K_i = 1.86 nM; CB₂ K_i = 1.05 nM), suggesting that there exists a balance to be struck between hydrophobic binding and steric bulk too near the benzchromene core (**Figure 3.6**). However, this same C-1'-cyclohexyl in conjunction with the 1',1'-dimethyl results in one of the strongest non-selective binding compounds to date (**51**) (CB₁ K_i = 0.57 nM; CB₂ K_i = 0.65 nM).²³⁴

3.2.4. Aromatic and cyclic substituents at C-1'

As one may consider the tolerance for bulky substituents at C-1' and couple that with our previously mentioned knowledge of unsaturation, a next logical step is to introduce aromatic and bi/tricyclic substituents in at C-1'. Interestingly, much like the C-1' cyclohexyl derivatives of the previous section, the best binding is achieved when the molecule contains moieties capable of binding to both the upper and lower hydrophobic pockets of the LBP. This is evidenced by comparing *p*-substituted aryl derivatives when the aryl substituents were joined to the benzchromene ring core via direct C-3 linkage²⁴⁶ or 1',1'-dimethyl linkage²³⁵ (**Figure 3.7**). Examination of these comparator compounds one may see that the 1',1'-dimethyl compounds far exceed the binding affinity for the direct C-3 conjugation; however, the directly conjugated compounds do offer some selectivity as the *p*-substituent increases in size, but this is minimal in relation to the CB₁/CB₂ ratios achieved with the 1'-gem-dimethyl compounds.

Figure 3.6. Cyclic modifications at C-1' which affect CB_1 and CB_2 binding affinity

Figure 3.7. Aromatic modifications of Δ^8 -THC at C-3 which affect CB₁ and CB₂ binding affinity

The apparent difference in size is intriguing, as the compounds that seem to prefer CB₂ over CB₁ tend to prefer bulky C-3 substituents, whereas the CB₁-preferring ligands prefer a more compact C-3 substituent (**Figure 3.8**; **Figure 3.9**). There seems to be a breaking point in this conformational freedom where one loses both selectivity for CB₂ and overall binding affinity. If one would compare C-3 isobornyl (**54**) (CB₁ K_i = 60.2 nM; CB₂ K_i = 6.1 nM) to C-3 1-adamantyl (**53**) (CB₁ K_i = 6.8 nM; CB₂ K_i = 52.0 nM), one may see that there exists a difference between receptor preference (**Figure 3.8**).²⁴⁷ The adamantyl substituent has much less conformational space requirements compared to those of the isobornyl (**Figure 3.9**). It is noteworthy that the C-3 bornyl (**52**) (CB₁ K_i = 8.9 nM; CB₂ K_i = 7.4 nM), which bears a similar Y-axis conformational space to isobornyl, lacks the Z-axis conformational space occupied by isobornyl, and thus it is not selective for CB₁ nor CB₂, albeit quite potent. There does still exist a limit within both the CB₁ and CB₂ LBP as evidenced in the C-3 bornylmethyl (**56**) (CB₁ K_i = 32.7 nM; CB₂ K_i = 30.5 nM) and isobornylmethyl (**58**) (CB₁ K_i = 49 nM; CB₂ K_i = 49.5 nM) compounds. These compounds have a larger conformational space requirement, which appears to mitigate any preference for one receptor subtype over the other and lowers the binding affinity to near that of THC. To further support this hypothesis, consider the adamantyl derivatives of THC synthesized by Lu, *et al* (**Figure 3.8**).²⁴⁸ The C-3 1-adamantyl (**53**) has less conformational requirements (**Figure 3.9**). However, 2-adamantyl exhibits much more conformational freedom – a trait further extrapolated by the (2-adamantylidene)methyl (**57**) (CB₁ K_i = 48.6 nM; CB₂ K_i = 8.9 nM) derivative. The presence of the unsaturation within the (2-adamantylidene)methyl affords a rigid enough frame so as to maximize the Y- and Z-axes freedom while still maintaining affinity and specificity for the CB₂ receptor LBP. As one moves to a (1-adamantyl)methyl (**59**) (CB₁ K_i = 29.3 nM; CB₂ K_i = 26.9 nM) or (2-adamantyl)methyl (**60**) (CB₁ K_i = 79.7 nM; CB₂ K_i = 76 nM) the freedom afforded by the fully saturated methylene linkage causes too much freedom and a loss of both specificity and affinity for CB₁ and CB₂ occurs.

3.3. Novel Series of C-1'-Dimethyl-Aryl-Δ⁸-THC Analogs (KM Series)

3.3.1. Rationale of design

Amalgamation of all of these data gathered from previous series of cannabinoid compounds led to the work of former members of my lab group, which saw the synthesis of cycloalkyl²³⁴ and aromatic²³⁵ substituents introduced at C-1' (**Figure 3.10**). All of the compounds of this group saw marked improvement in affinity for CB₁ and CB₂ receptors over that of THC (**Table 3.1**). With such an increase in binding affinity, a need existed to establish a functional activity for these compounds.

3.3.2. Objectives of the current study

Utilizing this novel series of compounds, I explored the functional pharmacology of each in relation to CB₁, CB₂ and the parental HEK-293-CNG cell lines to understand the mechanism of action (MoA) in a cell-based reporter system. Furthermore, to aid in explanation of the observed functional data, I will reconcile known literature with regard

Figure 3.8. Bicyclic and tricyclic C-3 substituents of Δ^8 -THC that alter CB_1 and CB_2 binding affinity

Figure 3.9. Comparison of the conformational space for adamantyl and bornyl side chains of various Δ^8 -THC analogues

Notes: Accessible conformers within 8 kcal mol⁻¹ of the global energy minimum for 3-(adamant-1'-yl)- Δ^8 -THC (**53**, green), 3-bornyl- Δ^8 -THC (**54**, magenta), 3-isobornyl- Δ^8 -THC (**52**, orange), and 3-bornylmethyl- Δ^8 -THC (**58**, blue). The accessible conformational space for 3-isobornylmethyl- Δ^8 -THC (not shown) is similar to that of **58**. The global minimum energy conformer for each ligand is shown in stick display. Adapted, with permission, from: Lu, D., Guo, J., Duclos, R. I., Bowman, A. L. & Makriyannis, A. Bornyl- and Isobornyl- Δ^8 -tetrahydrocannabinols: A Novel Class of Cannabinergic Ligands. *Journal of Medicinal Chemistry* 51, 6393-6399, doi:10.1021/jm8005299 (2008). Copyright 2008 American Chemical Society.

Figure 3.10. KM series analogues

Table 3.1. Binding affinities for the KM series of compounds

Compound	CB ₁ K _i (nM)	CB ₂ K _i (nM)
61	-	-
62	0.57 (±0.05)	0.65 (±0.04)
63	12.3 (±0.61)	12.3 (±0.61)
64	3.13 (±0.37)	0.88 (±0.05)
65	2.53 (±0.54)	1.13 (±0.02)
66	34.4 (±2.84)	10.65 (±1.27)
67	11.0 (±1.67)	7.45 (±0.38)
68	1.85 (±0.16)	0.67 (±0.05)
69	1.77 (±0.20)	7.83 (±0.79)
70	76.1 (±1.55)	12.4 (±0.24)
71	5.26 (±0.94)	0.90 (±0.02)
72	18.8 (±1.39)	1.68 (±0.20)
73	2.80 (±0.05)	3.54 (±0.71)
74	5.03 (±0.39)	1.54 (±0.16)
75	1.59 (±0.16)	0.54 (±0.03)
76	9.25 (±0.23)	2.53 (±0.23)
77	2.72 (±0.29)	0.91 (±0.05)
78	1.08 (±0.04)	0.27 (±0.01)

to binding and employ a CB₁ homology model in an effort to provide some *in silico* hypothesis as to the potential residues involved in contributing to the functional activity.

3.4. Functional Pharmacology

3.4.1. ACTOne assay

Assay development and conductance details are found in **Chapter 2**. Briefly, stably transfected HEK-293 cells co-expressed with modified CNG channels along with CB₁ or CB₂ or their parental cells (without CB₁ or CB₂; HEK-CNG) were plated at an appropriate density in a clear, 96-well, Poly-D-Lysine plate and allowed to incubate at 37°C and 5% overnight. After the overnight incubation period, I added 100 µL of ACTOne membrane potential dye to each well of the culture plate and allowed to sit in a dark area at room temperature for 60 additional minutes. After the 60 minute incubation period a baseline read in a BioTek Synergy 2 plate reader with an excitation of 540/25 nm and an emission of 590/20 nm. Following the baseline read, 50 µL of drug stock solution containing (**Figure 3.11**): appropriate concentrations of ligand, Forskolin and Ro 20-1724 in DPBS with 2.5% DMSO in the cell culture plate. The plate was then placed on the plate reader with 1 read per well per minute with an excitation of 540/25 nm and an emission of 590/20 nm for 60 minutes.

I analyzed primary data with customized VBA macros, developed internally, in Microsoft Excel (Redmond, WA). Column 11 (lowest [drug]) determined the experimental maximum response (100 % activity) at each individual time point, and the experimental basal response was determined by the average response given by the two “Blank” control wells. All data were normalized with these minimum and maximum values via **Equation 2.1**. The EC₅₀ values were calculated from concentration-response curves by non-linear regression analysis utilizing GraphPad Prism Software (San Diego, CA).

3.4.2. CB₁ functional activity for the KM series

Plates were run as previously described (**Section 3.4.1**) in six replicates with CP-55,940 as a control. Compound **63** (KM-233), being the prototype, did not manifest as high of efficacy as several of the substituted aromatic ring analogs – especially those with *meta*-substitution(s). EC₅₀ values (**Table 3.2**) ranged from 34 to 516 nM with the percent cAMP inhibition ranging from 10 to 70%, 50 minutes after compound addition into the assay plate. Of particular note are the meta-substituted compounds **65**, **67**, **71**, **73**, **75** and **77** (**Figure 3.12**), which show an overall lowering of cAMP production near 70%, which is equivalent to the standard of CP-55,940. EC₅₀ values were determined to be 41.8 nM, 41.2 nM, 78.5 nM, 99.5 nM, 71.2 nM and 127 nM for compounds **65**, **67**, **71**, **73**, **75** and **77**, respectively. From the *para*-substituted derivatives, only **68** (EC₅₀ = 34.0 nM) was able to match CP-55,940 at ~70% reduction of cAMP production, all others were less efficacious at ~50% reduction (**Figure 3.13**) – highlighting the importance of *meta*-substitution in these derivatives. The remaining derivatives for this series **63**, **66** and

	1	2	3	4	5	6	7	8	9	10	11	12
A	5000 nM	2500 nM	1000 nM	500 nM	250 nM	100 nM	50 nM	25 nM	10 nM	5 nM	2.5 nM	Blank
B												Blank
C	1000 nM	500 nM	200 nM	100 nM	50 nM	20 nM	10 nM	5 nM	2 nM	1 nM	0.5 nM	Ro
D												Ro
E												Ro/For
F												Ro/For
G												CP CTRL
H												CP CTRL

Figure 3.11. Drug plate layout and concentrations

Notes: Columns 1 through 11 contain test compound, and Column 12 contains control wells. All listed concentrations are the final concentrations once added to the testing plate. In the drug plate all compounds are at 5X that of the listed concentrations in Columns 1 through 11. Control wells (Column 12) are: "Blank" (cells, media and dye), "Ro" ("Blank" + 125 μ M Ro 20-1724 [25 μ M, Final]), "Ro/For" ("Ro" + 4 μ M Forskolin [800 nM, Final]) and "CP CTRL" ("Ro/For" + 25 μ M CP-55,940 [5 μ M, Final]).

Table 3.2. EC₅₀ values for KM series functional assays

Compound	CB₁ EC₅₀ (nM)	CB₂ EC₅₀ (nM)	CNG EC₅₀ (nM)	CB₁ PTx EC₅₀ (nM)
61	n/a	-	-	-
62	14.2 ^a	-	-	-
63	204 ^a	n/a	n/a	n/a
64	350 ^a	n/a	n/a	n/a
65	41.7 ^a	n/a	n/a	n/a
66	516 ^a	n/a	n/a	n/a
67	41.2 ^a	n/a	n/a	n/a
68	34.0 ^a	n/a	n/a	n/a
69	72.3 ^a	n/a	n/a	n/a
70	356 ^a	n/a	n/a	n/a
71	78.5 ^a	n/a	n/a	n/a
72	290 ^a	n/a	n/a	n/a
73	99.5 ^a	n/a	n/a	n/a
74	237 ^a	n/a	n/a	n/a
75	71.2 ^a	n/a	n/a	n/a
76	414 ^a	n/a	n/a	n/a
77	127 ^a	n/a	n/a	n/a
78	249 ^a	n/a	n/a	n/a

Notes: n/a: Not active, based on no statistically significant change in cAMP from baseline. “-” Indicates test not performed. ^a Indicates a decrease in cAMP compared to baseline.

Figure 3.12. CB₁ functional activity for *meta*-substituted KM series compounds

Figure 3.13. CB₁ functional activity of *para*-substituted derivatives of KM series

78 only produced an approximately 30% reduction in cAMP production (**Figure 3.14**), and while **63** is our prototype for this class, it is evident that substitution greatly improves its functional activity.

3.4.3. CB₂ functional activity for the KM series

Plates were run as previously described (**Section 3.4.1**) in 6 replicates with CP-55,940 as a control for all 6 runs (**Figure 3.15; Table 3.2**). All compounds in the series demonstrated an inability to decrease cAMP production via CB₂ activation.

3.4.4. CNG functional activity for the KM series

Plates were run as previously described in 3 replicates with CP-55,940 as a control for all 3 runs (**Figure 3.16; Table 3.2**). As fully expected, all of the compounds failed to elucidate any change in cAMP production, including our standard of CP-55,940. There is a small variance among the drugs, but this is not of true consequence as one is able to gather from the graph – especially relative to the full agonist graphs noted with these compounds in CB₁ cells. This lack of activity with the parental HEK-293-CNG cells confirms that the KM series acts via CB₁, and not via any other constitutively expressed receptors present in the HEK cells.

3.4.5. CB₁ PTx-sensitive G_{i/o}-inactivation for the KM series

Plates were run as previously described (**Section 3.4.1**) in 3 replicates with CP-55,940 as a control for all 3 runs (**Figure 3.17; Table 3.2**). All of the compounds in the series failed to change cAMP production, including our standard of CP-55,940, confirming a PTx-sensitive-G_{i/o}-mediated CB₁ effect. CB₂ PTx-sensitive G_{i/o}-inactivation assays were not completed due to a lack of agonist response in the previous assay.

3.5. Discussion

3.5.1. Interactions with the CB₁ LBP

As previously discussed (**Section 1.2.2**), CB₁ receptors are seven trans-membrane G-protein coupled receptors (GPCRs).¹⁴ There exist several homology models which have sought to elucidate the residues important for binding in domains 3, 6 and 7 as well as functional activity.

To generate hypotheses, gain some insight into the possible explanations and interplay between ligand and receptor, I performed docking of the compounds. Dr. Chang, University of California at Riverdale, provided 4 models: “ACEA” – endocannabinoid model, “HU-210” – classical core model, “SR-141716A” – inverse agonist model and “WIN-55,212-2” – non-classical agonist model.²⁴⁹ The KM series of compounds, I believe, adopt a similar binding mode within the LBP to that of HU-210, thus this was the model used for docking studies.

Figure 3.14. CB₁ functional activity of misc. KM series derivatives

Figure 3.15. CB₂ functional activity of KM series compounds, 50 minutes after drug addition

Figure 3.16. CNG functional activity of KM series compounds, 50 minutes after compound addition

Figure 3.17. CB₁ PTx-sensitive G_{i/o}-inactivation functional activity, 50 minutes after compound addition

3.5.1.1. C-1 Hydroxyl. K192(3.28) plays a very important role in both receptor binding and function. Since it is a very flexible residue in the LBP, it can manifest conformations that allow it to hydrogen bond with either the C-1 hydroxyl, C-11 (“Northern”) hydroxyl, C-6 (“Southern”) hydroxyl or even the benzochromene oxygen.²⁵⁰ However, newer data suggest that K192(3.28) plays a primary role as H-bond acceptor of the C-1 hydroxyl,²⁵¹ presumably this occurs after being activated by donating a H-bond to F189(3.25) (**Figure 3.18**).²⁴⁹ S383(7.35) interacts by donating a H-bond to either the benzochromene oxygen or the C-6 hydroxyl, if present (**Figure 3.18A**).²⁵¹ Additionally, literature data suggest that the resting state of the CB₁ receptor exists when the salt bridge between K192(3.28) and D366(6.58) is intact.²⁵²

Much the same as the established CB₁ interactions of HU-210, I hypothesize that the KM series of compounds take advantage of the H-bonding with K192(3.28) with the C-1 hydroxyl and S383(7.35) with the benzochromene oxygen (**Figure 3.18B**) – as most classical cannabinoids do. Moreover than orienting the drug in the pocket, ligand interaction with K192(3.28) breaks the salt bridge between K192(3.38) and D366(6.58) – a reported key stabilizing factor for the inactive state of the CB₁ receptor.²⁵²

3.5.1.2. C-3 lipophilic side chain. A predominantly lipophilic pocket exists, seemingly as two lobes – as evidenced in the fact that 3-(1',1'-dimethylheptyl)- Δ^8 -THC binds with higher affinity than does 3-heptyl- Δ^8 -THC along with 3D-QSAR models which demonstrate this trait.²⁵³ In one homology model, residues V196(3.32), T197(3.33), F200(3.36), Y275(5.39), W279(5.43), L359(6.51) and M363(6.55) form a hydrophobic pocket which encompasses the A-ring and C-3 side chain of HU-210.²⁵⁴ More recent homology models form this pocket from I354(6.46), C355(6.47), W356(6.48), L359(6.51), L360(3.52) and M363(6.55) (**Figure 3.19A**).²⁴⁹ Interestingly, mutations studies conducted on this hydrophobic pocket show that M363A(6.55) mutation greatly impacts binding affinity for HU-210 but has virtually no impact on THC which would suggest that this is an important residue for C-1' substituted derivatives.²⁵⁵

Due to the similar CB₁ functional activity betwixt HU-210 and the KM series, it is hypothesized that the interactions of the lipophilic C-3 substituents are analogous (**Figure 3.19B**). The necessity of the M363(6.55) residue as demonstrated for HU-210 in the mutation binding studies bore itself out in the functional arena, as well. This further highlights the importance of the C-3 lipophilic side chain to both the binding affinity and the functional activity of these compounds. Residue W356(6.48) plays a key role in two ways. First, in the inactive state of the CB₁ receptor, there is a proposed interaction of W356(6.48) and F200(3.36) – a “toggle switch.”²⁵⁶ Stabilization of this interaction may lead to the specialized antagonists known as inverse agonists, and this is precisely how SR-141716A binds in some homology models – a parallel stacking between these two residues creates an aromatic micro domain for SR-141716A.²⁴⁹ However, more recent homology models do not suggest this same binding mode for SR-141716A, and thus may indicate this is not as necessary an interaction. Second, interaction of W356(6.48) with the aryl ring of the KM Series draws the aryl ring more in contact with residues flanking W356(6.48), namely I354(6.46) and L359(6.51). It is this additional hydrophobic interaction (**Figure 3.20**) that I hypothesize gives compound **67** its superior functional effect, though its binding affinity is lower than related compounds.

Figure 3.18. CB₁ LBP interactions with C-1 hydroxyl and benzchromene oxygen

Notes: Panel A depicts HU-210 H-bonding interactions as set forth in homology models. Panel B depicts the proposed interaction of Compound **67** with the LBP H-bonding groups. Homology models contributed by Dr. Chang, University of California, Riverdale.²⁴⁹

Figure 3.19. CB₁ LBP proposed hydrophobic C-3 pocket

Notes: Panel A depicts the predicted HU-210 C-3 binding pocket of CB₁. Panel B is the proposed Compound 67 C-3 binding pocket of CB₁. The arrow highlights M363(6.55), demonstrated in mutation studies to interact with the C-1'-*gem*-dimethyls. Homology models were generously supplied by Dr. Chang, University of California, Riverdale.²⁴⁹

Figure 3.20. CB₁ LBP proposed additional hydrophobic interactions of **67** with the C-3 pocket

Note: Panel A depicts the side view of **67** in the LBP. Panel B depicts the top view of **67** in the LBP. Dashed green lines indicate π - π stacking interactions Homology models were supplied by Dr. Chang, University of California, Riverdale.²⁴⁹

3.5.1.3. ABC ring system. Residues F174(2.61) and F177(2.64) are suggested to not provide direct π - π interactions with HU-210, but rather they help to stabilize the tricyclic (ABC ring) binding pocket domain of the LBP (**Figure 3.21**).²⁵⁵

Residues F200(3.36) and W279(5.43) appear to not play as important a role in terms of binding (in the case of AEA and CP-55,940) as they do in terms of receptor activation (**Figure 3.21**).²⁵⁷ That is to say, a ligand's hydrophobic substituents at the C3 position may bind well without interacting with these two residues, but agonists MUST have interaction with these residues to exert their effect.²⁵⁴ F189(3.25) is key at providing a π - π stacking (**Figure 3.21**) interaction with AEA, and is instrumental in binding of this to the pocket as its mutation to alanine causes a 7-fold drop in affinity for the receptor.²⁵⁸ F189(3.25) also seems to form the "ceiling of the anandamide binding pocket in CB₁."²⁵⁷

As has been the case with many of the other interactions within the CB₁ LBP, compound **67** shares many of the same interactions with HU-210; however, there is one key difference, which I believe helps compound **67** gain an edge. One of the key interactions within the CB₁ LBP is the simultaneous interaction of the ligand with both F200(3.36) and W279(5.43). While HU-210 is obviously able to accomplish this by being an agonist at CB₁, I believe that compound **67** is able to demonstrate a more favorable interaction with these residues via π - π stacking interactions rather than strictly hydrophobic interactions present in HU-210.

3.5.1.4. (Optional) C-11 ("Northern") hydroxyl. With none of the compounds in the KM-series having this moiety present in the parent drug, I have not included it as part of this current study; however, it is included as part of this discussion, due to the fact that the primary metabolite of the KM series is a C-11 hydroxyl.²⁵⁹ S383(7.35) shows itself to be equally important in binding of molecules along with stabilizing the LBP. S383(7.35) can hydrogen bond with the C-11 ("Northern") hydroxyl²⁵¹ (if present) or the benzochromene oxygen^{249,254}, but more importantly it helps to stabilize the LBP by maintaining the shape of the Trans-Membrane Helix 1-2-7 binding pocket.²⁵¹ The "Northern" hydroxyl group may, theoretically, H-bond with K192(3.28).

3.5.2. Interactions with the CB₂ LBP

As previously discussed (**Section 1.2.3**), CB₂ receptors are also seven trans-membrane GPCRs; however, CB₂ only shares 48% homology with CB₁ – a trait unique to the CB₁ and CB₂ receptors among GPCRs, which generally share a much higher level of homology among receptors of the same family.¹⁴ The SAR of the CB₂ LBP has yet to be fully developed, especially in terms of a reliable homology model or much less as a crystal structure. In the absence of a refined homology model, it is not possible to predict specific ligand amino acid interactions. However, very basic interpretations of the results are possible by comparing and contrasting to current literature standards. Specifically, the KM series possess high receptor affinity, and no functional activity, indicative of a neutral antagonist. Based on this, a plausible comparison between S-(-)-WIN-55,212-3 (a neutral antagonist) and R-(+)-WIN-55,212-2 (a weak agonist) and the activity of the KM series at CB₂ may be presented.

Figure 3.21. CB₁ LBP π - π stacking interactions and key residue interactions

Notes: Panel A (top view) and Panel B (side view) depict the interactions of HU-210 within the CB₁ LBP. Panel C (top view) and Panel D (side view) depicts the proposed interactions of **67** in the CB₁ LBP. Demonstrating the proximity of key receptor activators F200 and W279 as well as the π - π stacking residue F189. Homology supplied by Dr. Chang.²⁴⁹

Attention now turns to the *trans*-B/C ring-junction. With data relatively limited for CB₂'s LBP, especially in the arena of classical core compounds, one must make some inferences based on available aminoalkylindole derivative homology model data. One such extrapolation lies in WIN-55,212-2's biologically assumed conformation to be *s-trans*-conformer.²⁶⁰ With the *s-cis*-conformer, there are suggested steric interactions with V113(3.32) (**Figure 3.22**).²⁶¹ By extension, WIN-55,212-3, the S(-) isomer of WIN-55,212-2 and antagonist, may also orient the morpholino side chain such to prevent critical amino acids interactions in the CB₂ LBP as discussed below.

Taking WIN-55,212-2 and ascribing its moieties to those of the representative example of KM-233 (**63**) (**Figure 3.23**), one can see that there are many similarities. While the similarities are numerous, the differences are profound as well, because the steric interactions of WIN-55,212-2 with V113(3.32) are non-existent with the KM-series. The steric issue comes in with what I hypothesize to be the "northern" aliphatic hydroxyl region, or C-11 substituted derivatives of Δ^8 -THC, thus the KM-series has little to contend with in this arena. It does raise an important question, though, for future design, synthesis and study of the classical core ligands – would incorporation of bulky C-11 substituents, especially conformationally (*cis* or *trans*) restricted derivatives, impact the CB₂ selectivity and functional ability of compounds?

3.6. Conclusions

The KM Series of compounds represent a unique group of ligands for modulating the CB₁ and CB₂ receptor system. Increases in binding affinity come about from taking advantage of residues present in the binding pocket otherwise not adequately utilized, such as L359(6.51) and I354(6.46). Key interactions with the "toggle" switch at CB₁ (W356(6.48)) to hold it in the active state. Future study in this would test the hypothesis of the residue-ligand interactions via homology modeling studies. Additionally, testing the series as antagonists of CB₂ against known agonists would functionally confirm this as a mechanism.

Figure 3.22. CB₂ LBP steric hinderance of *cis* and *trans* isomers of WIN-55,212-2

Figure 3.23. Comparison of analogous functional groups of WIN-55,212-2, KM 233 and WIN-55,212-3

CHAPTER 4. FUNCTIONAL EVALUATION OF A NOVEL CLASS OF HETEROCYCLIC CORE ANALOGUES OF HEXAHYDRO Δ^8 -THC

4.1. Introduction

As previously discussed (**Section 1.1**), classical cannabinoids are tricyclic compounds possessing a benzopyran core – these are of either natural origin or synthetic analogues thereof. The naturally occurring, prototypes for these compounds are Δ^9 -THC and Δ^8 -THC, which both bind to CB₁ and CB₂ receptors and exert a partial agonist activity.¹⁴ Within the classical compounds impacting the CB₁ receptor novel means of dealing with anti-nociception, mitigation of chemotherapeutic-induced nausea and vomiting, decreasing intra-ocular pressure in glaucoma, increasing appetite in patients with wasting syndromes, reduction of muscle spasticity caused by diseases such as multiple sclerosis and treating diarrhea by slowing intestinal motility are present.¹⁴ It is well known in the literature that CB₁ receptors are responsible for causing the untoward psychotropic effects of cannabinoid compounds. With CB₂ receptors serving the body as modulators of cytokine release, agents targeting these compounds show great promise in inflammation, especially in instances of inflammation-induced organ damage.^{136,165} The drawback exists in separating CB₁ and CB₂ activity so as to retain or augment the desired therapeutic outcome while simultaneously mitigating the negative psychotropic effects of such compounds. This desire for such potentially impactful compounds for various disease states which have favorable therapeutic use profiles has led to a bevy of research into the classical cannabinoids with regard to their pharmacology and structural activity relationships/requirements (SAR).

For the classical cannabinoids it is established that substitution of the A-ring, aside from positions 1 and 3, is generally detrimental to the K_i of compounds, and substitution of the 1 position with anything other than hydroxyl is equally detrimental, though it can afford some selectivity for CB₂ (**Section 4.2.1**). Manipulation of the B-ring of the classical core improves the potency of compounds over the parent THC compound (**Section 4.2.2**). Finally, desaturating the C-ring (as in cannabinol) serves to increase affinity and impart selectivity to compounds (**Section 4.2.3**). Considering these along with the previously mentioned C-3 modifications (**Section 3.2**), the classical core is rife with opportunities to exploit increases in affinity and specificity.

The non-classical cannabinoids provide a further insight on cannabinoid action as well. From the aminoalkylindoles (AAIs) it becomes apparent that size is of concern when substituting at the N-1 position, but this can be optimized to afford highly potent compounds (**Section 4.3.1.1**). From the AAIs one may also gather that C-2 substitution does increase potency, but at the expense of CB₂ specificity (**Section 4.3.1.2**). At C-3 one gathers that conformation and size play a crucial role in cannabinoid binding and activity (**Section 4.3.1.3**). Finally, the AAI compounds demonstrate how substitution of the indole ring system can result in highly potent and specific compounds (**Section 4.3.1.4**).

The other non-classical cannabinoid class, which provides valuable information in the development of this novel series of cannabinoids, is the well-known diarylpyrazole class. Much like the AAIs, size and electron-withdrawing substituents at N-1 aid in

compound specificity (**Section 4.3.2.1**). C-3 derivatives of this class afford the knowledge that CB₁ prefers a long and flexible moiety (**Section 4.3.2.2**). C-4 and C-5 derivatives for this class are relatively few; however, derivatives thereof provide knowledge that electron-withdrawing groups are preferred (**Section 4.3.2.3** and **Section 0**). Summing all of this knowledge from classical (**Figure 4.1**) and non-classical cannabinoids gave rise to a true hybrid series of compounds, the pyridine- and pyrimidine-hexahydro-THC compounds (**Figure 4.2**).

The aim of this study is to utilize the ACTOne Assay (**Chapter 2**) to explore the functional ramifications of a heterocyclic A-ring, inconjunction with a hexahydro C-ring and C-1' substituents similar to the KM series (**Chapter 3**) (**Section 4.4**). Taking this functional data, I will then reconcile these outcomes against current literature standards' binding and homology models to propose hypothetical interactions with CB₁ and CB₂ – in an attempt to further understanding of cannabinoid SAR and the unique MoA for these compounds.

4.2. Classical Cannabinoids – Modifying the A/B/C Ring

Three pharmacophoric elements exist for classical cannabinoids to exert cannabimimetic activity (**Figure 4.1**):

1. C-1 phenolic hydroxyl group
2. Trans ring junction at the B and C rings (6aR, 10aR - required)¹⁸
3. Lipophilic C-3 alkyl side chain

For the purposes of examining this novel series of compounds, we will leave C-1 phenolic hydroxyl and the trans ring junction intact, and we will not bring into account the optional elements of the northern and southern hydroxyl groups. We will vary the A-ring with heterocyclic moieties and fully saturate the C-ring. Several sections of this chapter use a muricidal rat model as an *in vivo* characterization of cannabinoid activity. This model was utilized because several of the compounds were synthesized and tested prior to the isolation and characterization of the cannabinoid receptors, thus no K_i values are reported, but the relative biological activity is. While this method does not provide a perfect 1:1 comparison, it does give a distinct picture the rank order of the compounds

4.2.1. Modifying the A-ring.

Variations about the A-ring are one of the most widely studied modifications within the tricyclic core. Several groups have manipulated this ring in an attempt to augment LBP interactions. Substitutions within the A-ring, with exception of the C-1 hydroxyl and C-3 aliphatic, greatly reduce affinity of the compounds for CB₁ receptors (**Figure 4.3**).²⁶² C-4-bromo- Δ^8 -THC (**79**) (CB₁ K_i = 5250 nM) and C-4-nitro- Δ^8 -THC (**80**) (CB₁ K_i = 1630 nM) greatly reduce the affinity over Δ^9 -THC (**1**) (CB₁ K_i = 44 nM).²⁶² A C-2-nitro- Δ^8 -THC (**82**), C-2,4-diiodo- Δ^8 -THC (**83**) or C-2,4-dinitro- Δ^8 -THC (**84**) substitution abolishes all affinity for the compounds at CB₁ (CB₁ K_i = >10,000 nM).²⁶² A C-2-Iodo- Δ^8 -THC (**81**) (CB₁ K_i = 89 nM) substitution confers roughly a

Figure 4.1. Classical cannabinoid with pharmacophoric elements

Figure 4.2. Design progression for the hexahydro series of compounds

Figure 4.3. A-ring substituted compounds

^aJ.W. Huffman²⁶⁵

^bJ.W. Huffman²⁴²

2-fold decrease in binding affinity relative to Δ^9 -THC (**1**); however, a C-2-Iodo- Δ^8 -THC-DMH (**85**) (CB_1 K_i = 61 nM) gives almost an 80-fold decrease in binding affinity at CB_1 versus Δ^8 -THC-DMH (**32**) (CB_1 K_i = 0.77 nM).²⁶² These data point to a need for the A-ring to have a stable π -electron system for optimal π - π stacking interactions in the LBP.

The most extensive modification to the ABC core ring system of the classical series of compounds takes place at C-1 of the A-ring, which naturally exists as a hydroxyl group. Data suggest that in the $\Delta^{6a,10a}$ derivatives, it may be replaced by an amine and retain equivalent biological activity. In the muricidal rat model, used as a means of testing compounds due to the lack of CB receptor characterization at that time, to Δ^9 -THC only if the C-3 substituent contains C-1',2'-dimethylheptyl (**86**) (5 mg/kg) (**Figure 4.4**).²⁶³ Binding studies have confirmed in the Δ^9 -THC derivatives (**Figure 4.4**) that, while reduced in affinity relative to C-1-hydroxyl, there is a manner of CB_2 selectivity for C-1-amine (**87**) (CB_1 K_i = 616 nM; CB_2 K_i = 215 nM).²⁶⁴ Larger C-1-amine substituents further reduce the affinity for both CB_1 and CB_2 , but there are some instances where this garners some CB_2 selectivity – e.g. C-1-cyano (**88**) (CB_1 K_i = 1720 nM, CB_2 K_i = 387 nM); methylcarbamate (**89**) (CB_1 K_i = >3000 nM, CB_2 K_i = 1400 nM); methylsulfite (**90**) (CB_1 K_i = >3000 nM, CB_2 K_i = 1720 nM); isonicotinamide (**91**) (CB_1 K_i = >3000 nM, CB_2 K_i = 1770 nM); methoxymethanamine (**92**) (CB_1 K_i = >3000 nM, CB_2 K_i = 1400 nM); methylurea (**93**) (CB_1 K_i = >3000 nM, CB_2 K_i = 782 nM); and methylacetamide (**94**) (CB_1 K_i = >3000 nM; CB_2 K_i = 1500 nM) show this trend.²⁶⁴ This effect was even more profound for these derivatives when C-3-1',1'-dimethylheptyl was present in lieu of C-3-*n*-pentyl, especially in the case of C-3-CN, C-3-acetamide and C-3-methylsulfite (**Table 4.1**).²⁶⁴ Acidic and amide derivations at C-1 (**Figure 4.5**) were less active, with N-methylformamide (**95**) (CB_1 K_i = >3000 nM; CB_2 K_i = 1260 nM) and N,N-dimethylformamide (**96**) (CB_1 K_i = >3000 nM; CB_2 K_i = 1210 nM) showing the most promise for CB_2 selectivity and activity.²⁶⁴ The C-1 substituent may not be a thiol group, this abolishes biological activity in the muricidal rat model, again this model is used due to the predating of CB receptor characterization, with C-3-*n*-pentyl (**97**) or C-3-1',2'-dimethylheptyl (**98**) (**Figure 4.5**).²⁶³ Interestingly, though, conversion of the C-1 substituent to a methylthiol with C-3-*n*-pentyl (**99**) (**Figure 4.5**) yields a compound with lower binding affinity, but nearly a 2-fold selectivity for CB_2 over CB_1 (CB_1 K_i = 955 nM; CB_2 K_i = 465 nM).²⁶⁴

Replacing C-1-hydroxyl with C-1-F causes a staunch drop in CB_1 affinity and a moderate drop in CB_2 affinity, which would allow for selective ligands at CB_2 (**Figure 4.6**). As an example, when 3-heptyl- Δ^8 -THC (**100**) (CB_1 K_i = 22 nM) is compared to 1-fluoro-1-deoxy-3-heptyl- Δ^8 -THC (**101**) (CB_1 K_i = 1560 nM; CB_2 K_i = 45 nM) one sees the precipitous drop in CB_1 affinity and immense CB_2 selectivity present therein.²⁶⁶ It is noteworthy that there is no binding data available in current literature for 3-heptyl- Δ^8 -THC (**100**), so a true comparison of any CB_2 activity gained or lost is not possible. I noticed a similar trend when comparing C-1-hydroxy and C-1-methoxy derivatives of compounds (**Figure 4.6**). Examining 3-(6'-azido-2'-hexynyl)- Δ^8 -THC (**102**) (CB_1 K_i = 2.14 nM; CB_2 K_i = 1.12 nM) to 1-methoxy-3-(6'-azido-2'-hexynyl)- Δ^8 -THC (**103**) (CB_1 K_i = 82.5 nM; CB_2 K_i = 11.2 nM) one may note the nearly 40-fold increase in CB_1 as compared to the 10-fold increase at CB_2 .²⁶⁶ The preference of C-1-methoxy for CB_2 receptors holds true when comparing C-3-(1',1'-dimethylheptyl)-

Figure 4.4. Amino and aminomethyl derivatives

Note: ^aJ.W. Huffman²⁶⁵ M.E.D. stands for Minimum Effective Dose

Table 4.1. CB₁/CB₂ K_i ratios for selected compounds

R₁	CB₁/CB₂ Ratio R ₂ = <i>n</i> -pentyl	CB₁/CB₂ Ratio R ₂ = 1',1'-dimethylheptyl
-CN	4	13
-NH ₂	3	4
-NHCOCH ₃	>2 ^c	6
-NHCONHCH ₃	>1.5 ^c	1
>2 ^c	>3 ^c
-NHSO ₂ CH ₃	>2 ^c	7

^cValues cannot be calculated accurately due to a CB₁ affinity in excess of 3μM.

Figure 4.5. Amide and thiol derivatives

^aJ.W. Huffman²⁶⁵

Figure 4.6. C-1 deoxy and methoxy derivatives

Δ^8 -THC (**104**) (CB_1 K_i = 713 nM; CB_2 K_i = 57 nM)²⁶⁷ to C-3-(1',1'-dimethylheptyl)- Δ^8 -THC (**32**) (CB_1 K_i = 0.77 nM; CB_2 K_i = 0.27 nM).²⁴² Complete absence of a C-1-hydroxyl allows for good selectivity of CB_2 (**Figure 4.6**).^{250,267-270} This deoxy preference is exemplified by 1-deoxy-3-(1',1'-dimethylheptyl)- Δ^8 -THC (**105**) (CB_1 K_i = 22.8 nM; CB_2 K_i = 2.9 nM) – a trend augmented by addition of C-11-hydroxy, with 1-deoxy-11-hydroxy-3-(1',1'-dimethylheptyl)- Δ^8 -THC (**106**) (CB_1 K_i = 1.2 nM; CB_2 K_i = 0.03 nM) showing the highest CB_1/CB_2 ratio.^{250,267} The data show the CB_2 LBP has an inclination for C-1-deoxy or C-1-methoxy when compared to the CB_1 LBP² – which may indicate that the hydrogen bond in the LBP may not be as necessary as it is in the CB_1 LBP. Further derivation of these compounds utilizing C-1-Br have been tested and showed a similar preference for CB_2 , again downplaying the need for LBP H-bonding (**Figure 4.6**). In this modification of the C-1 substituent, 3-(1',1'-dimethylhexyl)-1-Br- Δ^8 -THC (**107**) (CB_1 K_i = 1444 nM; CB_2 K_i = 28 nM) exhibited the best CB_1/CB_2 ratio.²⁷⁰

4.2.2. Modifying the B-ring

In most synthetic and all naturally occurring cannabinoids, the B ring is a pyran; however, there are synthetic derivatives wherein this ring exists as a piperidine (**108**) (**Figure 4.7**) (CB_1 K_i = 0.6 nM) without any loss of activity compared to the pyran parent compound.^{271,272} Further derivation of the B ring into $\Delta^{6a,10a}$ derivatives or oxepane derivatives gives some insight into the binding of these compounds into the LBP. For example, $\Delta^{6a,10a}$ -lactones (**109**) (**Figure 4.7**) show inactivity at testing doses in a muricidal rat model (>10 mg/kg)²⁷³; however, the $\Delta^{6a,10a}$ -C-6-*gem*-dimethyl (**110**) (**Figure 4.7**) showed substantially better activity (0.62 mg/kg)²⁷³ over Δ^9 -THC (**1**) (**Figure 4.7**) (5.0 mg/kg)²⁷³ in the same model. This demonstrates that $\Delta^{6a,10a}$ increases efficacy; however, there is a need for hydrophobic substituents at C-6 to maximize this benefit.

Expansion of the B ring to the oxepane and substituting C-6 and C-7 with concomitant unsaturation ($\Delta^{7a,11a}$) shows the limitations of the binding pocket about the tricyclic core. In the case of $\Delta^{7a,11a}$ compounds where both C-6 and C-7 are unsubstituted (**111**) (**Figure 4.7**) (1.25 mg/kg)²⁷³, there is a gain relative to Δ^9 -THC (5.0 mg/kg) (**1**), but a loss compared to the $\Delta^{6a,10a}$ -C-6-*gem*-dimethyl pyran derivative (0.62 mg/kg) (**110**) in the muricidal rat model. Further exploration of the oxepan derivatives shows that C-6-*gem*-dimethyl (**112**) (**Figure 4.7**) (2.5 mg/kg)²⁷³ is better than a C-6-lactone (**113**) (**Figure 4.7**) (5 mg/kg),²⁷³ which further shows the need for hydrophobic substitution at this position. Interestingly, a C-7-*gem*-dimethyl substitution, with (**114**) (**Figure 4.7**) or without (**115**) (**Figure 4.7**) C-6-ketone, results in completely inactive compounds at tested doses (>10 mg/kg)²⁷³ – showing C-7 substitution may be detrimental.

4.2.3. Modifying the C-ring

As is the case for the two novel classes examined in this chapter, unsaturation of the C-ring resulting in either Δ^8 or Δ^9 compounds is not necessary for activity.²⁷⁴⁻²⁷⁶ For

Figure 4.7. B-ring modifications

^aNot Active

both the hot plate and anti-writhing test models, conversion of Δ^9 -THC (**1**) (**Figure 4.8**) (>100 mg/kg and 43 mg/kg, respectively) to a $\Delta^{6a,10a}$ derivative (**116**) (**Figure 4.8**) (9.3 mg/kg and 3.0 mg/kg, respectively) showed a dramatic decrease in EC₅₀.²⁷⁶ Complete unsaturation resulting in a benzene ring, in conjunction with a C-6 ketone moiety (**117**, **119**, **121**) (**Figure 4.8**), yields compounds near or better at CB₂ receptors than those containing C-6-*gem*-dimethyl (**118**, **120**, **122**) (**Figure 4.8**) – it is noteworthy, that the C-6 ketone compounds did bear a great deal more selectivity for CB₂ over CB₁.²⁷⁷ Along with this, conversion of the C-ring to a heterocyclic ring does not impede or improves activity.²⁷⁴⁻²⁷⁶ In fact, converting the C-ring to pyridine (**123**, **124**) (**Figure 4.9**) increased the efficacy of compounds over THC (**1**) (**Figure 4.9**) in both the rat tail flick and acetic acid writhing tests.²⁷⁴ Conversion of these pyridine C-ring derivatives to pyridine oxide rings (**125**, **126**) (**Figure 4.9**) causes a substantial drop in efficacy,²⁷⁴ suggesting that destabilization of the pi-ring system and/or size constraints about the C-ring cause an issue. In cases where the C-ring was a $\Delta^{6a,10a}$ -tetrahydropyridine (**127**, **128**) (**Figure 4.9**), compounds exhibited an activity equivalent to or worse than Δ^9 -THC (**1**) (**Figure 4.9**) except in cases where the substituent on the tetrahydropyridine nitrogen was a small aliphatic group.²⁷⁵ There are references^{278,279} and compounds synthesized that attempt to modify C-11 into esters and ethers as an attempt to garner a prodrug and/or peripherally restricted compound. I discounted these modifications in this analysis because they are inactive until converted, at which time the active drug's properties predominate.

4.3. Non-Classical Cannabinoids – Heterocyclic Ring-Containing Compounds

4.3.1. Aminoalkylindoles (AAIs)

The aminoalkylindole (AAI) compounds, originally developed in the early to mid-1990s as non-steroidal anti-inflammatory drugs (NSAIDs), sought to inhibit the pain and inflammation pathways while diverting from the ulcerogenic properties common among non-COX-2 specific NSAIDs.²⁸⁰ These compounds have found favor and infamy in the synthetic cannabis designer drug realm. Some of the compounds herein are found in trace amounts in over-the-counter “herbal incense.” Recreational use of these herbal incense products has led to serious injury and death because of the cannabinoids hallucinogenic properties. Due to these serious issues, addictive potential and non-regulated production and use, the DEA has classified many of these compounds as Schedule I controlled substances, despite their therapeutic potential. Structurally, they have no resemblance to classical cannabinoids; however, the eventual assignment of anti-nociception via cannabinoid receptors^{281,282} has cemented these compounds as part of the cannabinoid family. By far the most widely studied of these compounds is WIN-55,212-2 (**Figure 4.10**). WIN-55,212-2 is thus known to be a potent agonist at both CB₁ and CB₂. The close examination of this molecule has allowed the study of cannabinoids to draw some potential conclusions with regard to requirements and/or potential switches for functional activity.

Figure 4.8. C-ring unsaturation

^aJ.W. Huffman²⁶⁵

Figure 4.9. Heterocyclic derivatives of the C-ring

Notes: ^aNumbers in parentheses are oral doses in mg/kg, percentages are percent reduction vs. control (THC). ^bN/A: Not Active

Figure 4.10. Derivations of N-1 of aminoalkylindoles

Notes: ^aReference 225.²⁷² ^bConcentration of compound required to inhibit 50% of 0.5 nM [³H]WIN-55,212-2 binding in rat cerebellum membranes as described in reference XX. Values are the IC_{50} or percent inhibition at the highest tested dose (nM). Negative values indicate stimulation rather than inhibition. ^cReference 247.²⁸² ^dReference 248.²⁸³ ^eReference 249.²⁸⁴ ^fReference 250.²⁸⁵

Within the aminoalkylindoles, four pharmacophoric elements exist:

Study of WIN-55,212-2 led to a more refined understanding of the CB₁ and CB₂ LBP.

1. N-1 substituents
2. C-2 substituents
3. C-3 substituents
4. Indole ring substituents and derivatives

4.3.1.1. N-1 substitutions of aminoalkylindoles. Other groups have synthesized and tested a number of indole analogs bearing different aminoalkyl substituents at N-1.²⁸² One such study found the aminoethyl substitution as an optimal requirement with morpholino (**129**), thiomorpholino (**130**) and piperidino (**131**) analogs – aminopropyl morpholino (**132**) demonstrated an induced rather than inhibitory binding for [³H]-WIN-55,212-2 (**Figure 4.10**). The respective acyclic amine, deemed to be possible metabolites, and piperazine analogs demonstrated activity similar to that of **132**.²⁸²

The Sterling Winthrop and Makriyannis laboratories further explored structural requirements at the N-1 position by synthesizing novel analogs in which the aminoalkyl chain of the indole ring is attached to a heterocyclic amine through a C–C bond. These analogs are generally more potent compared to the C–N analogs and exhibit more favorable physicochemical properties. Potency was optimum for N-methylpiperidinyl-2-methyl substitution at the N-1 position (**133**, **Figure 4.10**), with activity residing predominately in the R-enantiomer.²⁸³

Mackriyannis, *et al.* developed AM-1241 (**134**, **Figure 4.10**), a highly CB₂-selective and potent agonist.^{284,286} Design of this molecule incorporated the N-methylpiperidinyl-2-methyl substituent at the N-1 position and a novel 2-iodo-5-nitrobenzoyl group at C-3. AM-1241 exhibits remarkably high peripheral analgesia *in vivo* and does not produce catalepsy, hypothermia, inhibition of spontaneous locomotor activity or impairment of performance on the rotarod apparatus. This agent could thus see higher use in experimental neuropathic pain model experiments.^{287,288}

Replacement of the aminoalkyl substituent by an alkyl chain results in N-alkyl indoles (non-aminoalkylindoles) (e.g., **135**, **Figure 4.10**). The SAR of cannabimimetic 2-methylindoles indicates that compounds with N-alkyl substituents from n-propyl to n-hexyl have good affinities for both CB₁ and CB₂ receptors and a preference for CB₂. Previous reports of the *in vivo* potencies for these compounds are consistent with their receptor affinities.^{285,289,290}

4.3.1.2. C-2 substituents of aminoalkylindoles. Analysis of the effect of C-2 substitution on cannabinoid receptor affinity in aminoalkylindoles reveals a strong preference for a small substituent at C-2. Thus, hydrogen or methyl groups are well tolerated with the C-2 H analogs (e.g., **136**, **Figure 4.11**) (CB₁ K_i = 245 nM; CB₂ K_i = 11 nM) exhibiting slightly higher affinities for the CB₂ than C-2 methyl analogs (**137**, **Figure 4.11**) (CB₁ K_i = 8 nM; CB₂ K_i = 29 nM).^{282,291,292} Recently, researchers at Bristol-Myers-Squibb reported their discovery of indazole carboxamides (e.g., **138**, **Figure 4.11**), replacing the C-2 carbon of 3-amido aminoalkylindoles (**136**) with nitrogen. The indazole analog **138** exhibits high affinity for the CB₂ receptor (K_i = 2.0 nM) compared to the

Figure 4.11. Modifications to C-2 of aminoalkylindoles

corresponding AAI analogs.²⁹² Indolopyridones (e.g., **139**, **Figure 4.11**), which are conformationally restricted C-3 amido aminoalkylindoles, exhibit increased affinities for the CB₂ receptor ($K_i = 1.0$ nM) and possess anti-inflammatory properties when administered orally in an in vivo murine inflammation model.²⁹²

4.3.1.3. C-3 substituents of aminoalkylindoles. Pravadolone (**129**, **Figure 4.10**), was originally developed as an NSAID to circumvent the gastric issues of currently available NSAIDs; however, it was found to be anti-nociceptive via CB₂, and it serves as the prototype for elucidating C-3 substituent variations.^{280,282} Additionally, this compound preceded and led to the discovery of WIN-55,212-2.^{281,293} The *p*-methoxy of **129** gives good activity; however, ortho-substitution of the benzene ring with other groups such as CH₃, OH, Cl, CN, or F (e.g. **140**, **Figure 4.12**) diminishes activity. The presence of an ethyl group at the para position (**141**, **Figure 4.12**) improves potency, but further increase in chain length results in diminished potency. The 1-naphthoyl (**142**, **Figure 4.12**) ($IC_{50} = 19$ nM) substitution at C-3 is more potent than the 2-naphthoyl (**143**, **Figure 4.12**) ($IC_{50} = 128$ nM) analog. Replacement of the naphthyl ring with an alkyl (e.g., CH₃) or alkenyl [(CH₃)₂C=CH] groups results in complete loss of CB₁ receptor affinity ($K_i > 10,000$ nM).²⁸⁹

NMR and X-ray crystallography studies of **129** and its C-2 H analog have revealed that aminoalkylindoles can exist in two distinct conformations based on the orientation of the C-3 aryl system.^{260,280} In the *s-trans* (**Figure 4.13A**) conformation, which predominates when the C-2 substitution is hydrogen, the aryl group is nearest C-2, while the carbonyl oxygen locates near C-4. In the *s-cis* (**Figure 4.13B**) conformation, which predominates when the C-2 substituent is a methyl group, the conformational preference shows the aryl ring to be located near C-4, and the carbonyl oxygen near C-2.

Naphthylidene-substituted alkylindenes (e.g., **144**, **Figure 4.12**) were originally designed to circumvent the CNS side effects of **129**.²⁹⁴ These analogs were tested as a mixture of E- and Z-isomers and exhibited higher CB₁ affinity compared to **129**. Later, it was shown that the CB₁ and CB₂ affinities and pharmacological potencies were higher for the E-isomer (**144**) compared to the Z-isomer.²⁶⁰ By removing the carbonyl oxygen of the C-3 aryl group in AAIs, having an unsubstituted C-2, one gets a moderate reduction in affinity for CB₁ relative to their carbonyl precursors.²⁹⁵ This loss of affinity is larger in the 2-methyl substituted analogs (**145**, **Figure 4.12**). Both observations support the hypothesis that the *s-trans* conformation of AAIs, such as WIN-55,212-2, is the preferred conformation for interaction at both CB₁ and CB₂ receptors, and that aromatic stacking of the ligands with aromatic residues in helices 3, 4, and 5 of both receptors may be an important interaction for AAIs at these receptors.^{260,295,296}

The spatial and electronic requirements of the C-3 substituent were further explored by Bristol-Myers-Squibb, introducing a C-3 amide group. The AAI C-3 amide ligand **146** (**Figure 4.12**) with a methoxy group at C-7, exhibited high CB₂ affinity ($K_i = 8$ nM) and selectivity (CB₁/CB₂ = 500).²⁹¹ Replacement of the amino acid moiety in **146** with the *S*-fenchylamine component resulted in slightly reduced affinity for the CB₂ receptor ($K_i = 30$ nM); however, when hydrogen replaced the 2-methyl group in indole, the resulting ligand (**147**, **Figure 4.12**) showed improved CB₂ affinity ($K_i = 11$ nM).²⁹¹

Figure 4.12. Modifications to C-3 of aminoalkylindoles

^aConcentration of compound required to inhibit 50% of 0.5 nM [³H]WIN-55,212-2 binding in rat cerebellum membranes as described in reference 238. Values are the IC_{50} or percent inhibition at the highest tested dose (nM). Negative values (**140**) indicate stimulation rather than inhibition.

Figure 4.13. Confirmations of Pravadoline (129)

Derivation of the 4-alkyloxy indole analogs (e.g., **148**, **Figure 4.12**) resulted from the translocation of the C-3 substituent of AAI to C-4 via an ether linkage. Some of these exhibited *in vivo* cannabimimetic activity, but most of them lacked cannabinoid receptor affinity.²⁹⁷

4.3.1.4. Indole Ring Substituents and Modifications. Introduction of a methyl group at C-4 or various substituents such as CH₃, OCH₃, F, Br or OH at C-5 of **129** diminishes affinity. Conversely, C-6 substitution with CH₃, OCH₃ or Br groups improves receptor affinity, but the ligands exhibit diminished agonist properties.²⁸² Incorporation of an iodo group at C-6 led to AM-630 (**Figure 4.10**), a ligand that exhibits improved affinity as well as selectivity for CB₂.^{298,299} This compound is selective inverse agonist for CB₂.³⁰⁰ I have since confirmed confirmed through use of the ACTOne assay.

Substitution at C-7 (**Figure 4.11**; **Figure 4.12**) gives modest improvement in binding affinity. Potent analogs came to be by conformationally restricting the N-1 side chain through the formation of a six membered ring between the N-1 and C-7 substituents (not shown).²⁸¹ For N-alkyl indoles, replacement of the indole phenyl ring with a cyclohexyl ring led to an analog with reduced affinities for both CB₁ and CB₂.³⁰¹ Removal of the phenyl ring in AAI or non-AAIs led to a pyrrole class.^{290,301} Most of the pyrrole-derived analogs are less potent than the corresponding indole derivatives; however, the 4-bromopyrrole analog shows high affinity for CB₁ and CB₂ (EC₅₀ = 13.3 nM for rCB₁ and 6.8 nM for hCB₂), comparable to WIN-55,212-2.³⁰¹

4.3.2. Diarylpyrazoles

The most widely studied compound of the diarylpyrazole class is SR-141716A (Rimonabant) (**Figure 4.14**) developed by Rinaldi-Carmona and co-workers at Sanofi.³⁰² Originally touted as a “wonder drug” for use in anti-obesity treatment, it was approved for use in Europe in 2006; however, it was withdrawn from the market in 2009 for the same reason approval was never granted in the United States of America – psychiatric side effects, some quite serious.²¹¹ This highly potent and selective CB₁ receptor ligand has served as a unique pharmacological and biochemical tool for further characterization of the CB₁ cannabinoid receptor^{303,304} – serving as the prototype CB₁ inverse agonist for evaluation of the compounds presented herein (**Chapter 2**). In vitro, SR-141716A antagonizes the inhibitory effects of cannabinoid agonists on both mouse vas deferens contractions³⁰⁵ and adenylyl cyclase activity.³⁰⁶ SR-141716A also antagonizes the pharmacological and behavioral effects produced by CB₁ agonists after intraperitoneal (IP) or oral (PO) administration.³⁰²

Other diarylpyrazole ligands that have contributed to our understanding of CB₁ pharmacology are AM-251 and AM-281, both of which are specialized CB₁ antagonists known as inverse agonists (**Figure 4.14**), and capable of displacing [³H]SR-141716A and [³H]CP-55,940 in CB₁ receptor membrane preparations.³⁰³ Both AM-251 and AM-281 share the ability of SR-141716A to attenuate the responses to established cannabinoid receptor agonists like WIN-55,212-2 or CP-55,940; however, data show that AM-251 may have a more “CB₁-selective” role than SR-141716A.³⁰⁷ In addition to AM-630 (**93**), the most notable CB₂ receptor inverse agonist is SR-144528 (**Figure 4.14**), a

Figure 4.14. Known standards of diarylpyrazole class of cannabinoid ligands

diarylpyrazole developed by Sanofi, exhibiting 700-fold selectivity for the CB₂ receptor over CB₁.³⁰⁸ Structural requirements for SR-141716A-like compounds are similar to those found in the aminoalkylindoles:

1. N-1 substituents
2. C-3 substituents
3. C-4 substituents
4. C-5 substituents

4.3.2.1. N-1 substituents of diarylpyrazoles. 2,4-Dichlorophenyl (SR-141716A) is the optimal substituent for both high CB₁ affinity and subtype selectivity.^{303,309} Its replacement with (5-isothiocyanate)-pentyl (**149**, **Figure 4.15**) group decreased CB₁ affinity by a factor of four.³¹⁰ The inclusion of strictly a phenyl (**150**, **Figure 4.15**), 4-butylphenyl (**151**, **Figure 4.15**) or a 4-pentylphenyl (**152**, **Figure 4.15**) group at N-1 significantly reduces affinity^{311,312} while n-pentyl (**153**, **Figure 4.15**), n-hexyl (**154**, **Figure 4.15**), n-heptyl (**155**, **Figure 4.15**) substitution retains good affinity, relative to SR-141716A.³¹¹ A 4-methylbenzyl group as represented in SR-144528 contributes optimal selectivity for CB₂.³⁰⁸

In the 2,4-dichlorophenyl moiety of SR141716A, elimination of *p*-chloro (**156**, **Figure 4.16**) substitution of *o*-chloro with *o*-fluoro (**157**, **Figure 4.16**) or *o*-methoxy (**158**, **Figure 4.16**) groups led to low-affinity analogs.³¹³ Replacement of the 2,4-dichlorophenyl moiety of SR141716A with cycloalkyl (**159**, **Figure 4.16**) groups decreased both CB₁ and CB₂ affinities, while the 3-methyl and 4-methylcyclohexyl analogs exhibited moderate improvement in CB₂ affinity without any enhancement in selectivity compared to SR-141716A.³¹⁴

4.3.2.2. C-3 substituents of diarylpyrazoles. Alkylation of the amide group (e.g. **160**) as well as replacement of the amide group by a ketone, alcohol or ether (e.g. **161**) greatly decreases CB₁ affinity (**Figure 4.17**).^{312,315} Replacement of the piperidinyl group with the respective five- or seven-membered heterocyclic rings or by a cyclohexyl group (e.g. **162**, **Figure 4.17**) does not alter CB₁ binding affinity. While replacement of the piperidinyl group with a morpholine group (**163**, **Figure 4.17**) leads to reduction in CB₁ affinity. However, this seems to be true only in cases where the C-5-*p*-phenyl substituent is not iodine, as AM-281 retains good activity and excellent specificity.³⁰³ To probe the structural and steric requirements of this pharmacophore, alkyl hydrazines, amines and hydroxyalkylamines replace the aminopiperidinyl moiety.³¹⁶ For alkylamides, hydroxyalkyl amides and alkyl hydrazides, affinity for CB₁ increased directly with increasing chain length (across this group of compounds) from ethyl to butyl up to and including pentyl. Increasing the carbon chain length beyond pentyl reduces affinity for both receptors. Alkylamide analogs (e.g. **164**, **Figure 4.17**) exhibited enhanced CB₁ selectivity when compared to SR-141716A, conversely hydroxyalkyl amide and alkylhydrazide analogs (e.g. **165**, **Figure 4.17**) had both decreased affinities and equivalent to slightly worse selectivity for CB₁ or CB₂.³¹⁶

4.3.2.3. C-4 substituents of diarylpyrazoles. Compounds with methyl, ethyl, bromo, or iodo substituents in the 4-position of the pyrazole ring are approximately

Figure 4.15. Alkyl N-1 modifications of diarylpyrazoles

Figure 4.16. Aromatic and cycloalkyl modifications to N-1 of diarylpyrazoles

Figure 4.17. Modifications of C-3 of diarylpyrazoles

equipotent, whereas replacement of methyl with hydrogen results in a 12-fold decrease in CB₁ affinity.³¹²

4.3.2.4. C-5 substituents of diarylpyrazoles. The 4-chloro group of the phenyl ring can be replaced by bromo (e.g. **162**) or alkyl groups (e.g. **156**) but not by nitro or amino groups.^{222,303,312} Replacement of 4-chloro with a 4-iodo substituent (e.g. AM-251) leads to significant CB₁ affinity and selectivity. Conversely, replacement of the aromatic ring with alkyl groups abolishes CB₁ affinity.³⁰³

Solvay first reported some rigid SR-141716A, tricyclic CB₁-selective ligands in which the 4- and 5-substituents are conformationally restricted through the formation of a relatively rigid tricyclic system.^{272,317} In these compounds the 4-methyl group is connected with the ortho position of the aromatic 5-aryl substituent to form benzocycloheptapyrazole analogs, i.e. **166** (**Figure 4.18**) that exhibited higher CB₁ affinity than the parent, SR-141716A.^{272,317} Later Pinna and co-workers reported similar tricyclic pyrazole analogs in which the additional 7-membered ring of **166** was replaced by a five-membered ring (e.g. **167**, **Figure 4.18**).³¹⁸ Of note, most ligands in this class had high affinity and selectivity for CB₂ compared to **166** and SR-141716A.

Solvay also reported a novel class of 3,4-disubstituted pyrazoline analogs exhibiting high CB₁ selectivity (e.g., **168**, **Figure 4.18**).³¹⁹ Another novel class of CB₁ antagonists that has received only limited attention includes the 3-alkyl-5-arylhydantoins (e.g. **169**, **Figure 4.18**).³²⁰

4.4. Novel Series of C-1'-Dimethyl-Hexahydro-Pyridine/Pyrimidine-THC Analogs (Hexahydro Series)

4.4.1. Rationale of design

Previous work from our group saw the synthesis of cycloalkyl²³⁴ and aromatic²³⁵ substituents introduced at C-1'. Both groups of compounds saw marked improvement in affinity for CB₁ and CB₂ receptors (**Chapter 3**). To further expand upon this discovery, the novel pyridine and pyrimidine hexahydro compounds (**Table 4.2**) attempt to incorporate the advantages of the hexahydro compound's increased potency^{271,272,276} as well as the increase in selectivity for CB₂ added by addition of nitrogen into the ABC ring system.^{264,271,272} The binding data (**Table 4.3**) do show that the addition of the nitrogen(s) to the A-ring does not negatively impact the binding affinity relative to the carbocyclic hexahydros. In fact, this small change lends some increase and a measure of selectivity toward CB₂. With an increase in binding affinity, coupled with the presence of favorable potential H-bonding interactions within the LBP of both CB₁ and CB₂ and the potential for a more therapeutically favorable preference of CB₂ over CB₁, a need existed to establish a functional mechanism for these compounds.

Analysis of compound **172** via ¹H-¹⁵N HSQC, ¹H-¹³C HSQC and ¹H-¹H NOSTY shows that the compound exists as the acetamide form (**Figure 4.19**). A diagnostic amide peak in the IR spectra (1640 – 1690) was used to confirm that amide structure of the

Figure 4.18. Conformationally constrained and non-traditional diarylpyrazoles

Table 4.2. Hexahydro-THC derivatives

Pyridine Hexahydro-THC			Pyrimidine Hexahydro-THC		
Compound	Y	R	Compound	Y	R
170	CH		174	N
171	CH		175	N
172	CH		176	N
173	CH		177	N

Table 4.3. Binding affinities for hexahydro series

Compound	CB₁ K_i (nM)	CB₂ K_i (nM)
170	1481 (N=1)	678 (±347.1)
171	-	-
172	-	83 (±42.51)
173	-	-
174	55 (N=1)	8.33 (±1.53)
175	18.5 (±16.26)	1.67 (±1.15)
176	>10,000	137 (±13.23)
177	-	52.33 (±28.18)

Figure 4.19. NMR structure confirmation of the pyridine hexahydro compounds

Notes: Panel A shows the actual structure of compound **172** in solution, and a possible way (in an aqueous environment) it would exist when interacting with the LBP. Panel B shows the existence of the N-H and thus amide composition of the A-ring. Panel C shows that position 4 is C, and thus the N-H must be at position 2 of the A-ring. Panel D shows the NOE correlation between the C-4 hydrogen and the C-6 methyl groups (weak) and C-1' methyl groups (strong).

remaining pyridine-related structures. Initial findings via the ^1H - ^{15}N HSQC show that the nitrogen of the pyridine ring exists, at least in solvent, with a hydrogen atom attached to it; however, the question was if it remained at the 2 or 4 position of the A-ring. ^1H - ^{13}C HSQC demonstrated that the 4 position was, indeed, a carbon with a hydrogen attached - ^1H - ^1H NOSY confirmed this finding by demonstrating weak coupling into both C-6 methyl moieties and very strongly into both C-1' methyl moieties.

4.4.2. Objectives of the current study

Utilizing this novel series of compounds, I explored the functional pharmacology of each in relation to CB₁, CB₂ and the parental HEK-293-CNG cell lines (covered in greater detail in **Chapter 2**) to better understand the mechanism of action (MoA) in a cell-based functional assay. Furthermore, to aid in explanation of the observed functional data, I will reconcile known literature with regard to binding and homology models.

4.5. Functional Pharmacology

4.5.1. ACTOne assay

Assay development and conductance, discussed in detail in **Chapter 2**. Briefly, stably transfected HEK-293 cells co-expressed with modified CNG channels along with CB₁ or CB₂ or their parental cells (without CB₁ or CB₂; HEK-CNG) were plated at an appropriate density in a clear, 96-well, Poly-D-Lysine plate and allowed to incubate at 37°C and 5% overnight. After the overnight incubation period, I added 100 μL of ACTOne membrane potential dye to each well of the culture plate and allowed to sit in a dark area at room temperature for 60 additional minutes. After the 60 minute incubation period a baseline read in a BioTek Synergy 2 plate reader with an excitation of 540/25 nm and an emission of 590/20 nm. Following the baseline read, 50 μL of drug stock solution containing (**Figure 4.20**): appropriate concentrations of drug, Forskolin and Ro 20-1724 in DPBS with 2.5% DMSO was added to the cell culture plate. The plate was then placed on the plate reader with 1 read per well per minute with an excitation of 540/25 nm and an emission of 590/20 nm for 60 minutes.

Screening of compounds against CB₁ and CB₂ were completed as technical replicates of $n = 6$ each. Screenings against HEK-CNG Parental and CB₁/CB₂ PTx-sensitive G_{i/o}-inactivation were completed as technical replicates of $n = 3$ each. Utilizing the lower number of replicates for HEK-CNG Parental and CB₁/CB₂ PTx-sensitive G_{i/o}-inactivation was employed due to the expected lack of response from these compounds to those three (3) groups of assays, in an effort to maximize time and resource management without compromising data integrity or statistical significance.

Primary data were analyzed with customized VBA macros (**Chapter 2**), developed internally, within Microsoft Excel (Redmond, WA). The experimental maximum response (100 % activity) was determined by Column 11 (lowest [drug]) at each individual time point, and the experimental basal response was determined by the

	1	2	3	4	5	6	7	8	9	10	11	12
A	5000 nM	2500 nM	1000 nM	500 nM	250 nM	100 nM	50 nM	25 nM	10 nM	5 nM	2.5 nM	Blank
B												Blank
C	1000 nM	500 nM	200 nM	100 nM	50 nM	20 nM	10 nM	5 nM	2 nM	1 nM	0.5 nM	Ro
D												Ro
E												Ro/For
F												Ro/For
G												CP CTRL
H												CP CTRL

Figure 4.20. Drug plate layout and concentrations

Notes: Columns 1 through 11 contain test compound, and Column 12 contains control wells. All listed concentrations are the final concentrations once added to the testing plate, so in the drug plate all compounds are at 5x of the listed concentrations in Columns 1 through 11. Control wells (Column 12) are: "Blank" (cells, media and dye), "Ro" ("Blank" + 125 μ M Ro 20-1724 [25 μ M, Final]), "Ro/For" ("Ro" + 4 μ M Forskolin [800 nM, Final]) and "CP CTRL" ("Ro/For" + 25 μ M CP-55,940 [5 μ M, Final]).

average response given by the 2 “Ro” control wells. All data were normalized with these minimum and maximum values via **Equation 2.1**. The EC₅₀ values were calculated from concentration-response curves by non-linear regression and automatic outlier elimination analysis utilizing GraphPad Prism Software (San Diego, CA).

4.5.2. CB₁ functional activity for the Hexahydro series

Plates were run as previously described (**Section 4.5.1**) in six (6) replicates with CP-55,940 as the internal control for all six (6) replicates (**Figure 4.21**; **Table 4.4**). All compounds, with the exception of compound **176**, bind with good affinity to the CB₁ receptor.²⁵⁹ Compounds **170**, **171**, **174** and **175** (**Figure 4.22**) demonstrated good agonist activity at CB₁ with an EC₅₀ of 78.9 nM, 209 nM, 276 nM and 56.3 nM, respectively, with percent cAMP inhibition ranging from 72 to 77%, 50 minutes after compound addition into the assay plate. These three (3) compounds exhibit a decrease in cAMP production equivalent to our standard of CP-55,940, albeit at a higher EC₅₀ than for CP-55,940. Special notation should be given to compound **173**, because it shows itself to be a special type of antagonist, known as an inverse agonist – with an EC₅₀ of 48.9 nM.

4.5.3. CB₂ functional activity for the Hexahydro series

Plates were run as previously described (**Section 4.5.1**) in six (6) replicates with CP-55,940 as the internal control for all six (6) replicates (**Figure 4.23**; **Table 4.4**). All compounds in the series bind with good affinity to the CB₂ receptor.²⁵⁹ As was the case with the CB₁ functional activity assays, compounds **170**, **171**, **174** and **175** (**Figure 4.24**) demonstrated agonist activity; however, they were not equipotent in terms of EC₅₀ or percent cAMP inhibited at CB₂ compared to CB₁. EC₅₀ for compounds **170**, **171**, **174** and **175** were 333 nM, 835 nM, 682.1 nM and 66.8 nM, respectively, with percent cAMP inhibition ranging from 27 to 51%. Interestingly, compound **173** did not exhibit its inverse agonist activity against CB₂, as it had for CB₁.

4.5.4. CNG parental functional activity for the Hexahydro series

Plates were run as previously described (**Section 4.5.1**) in three (3) replicates with CP-55,940 as the internal control for all three (3) replicates (**Figure 4.25**; **Table 4.4**). As was expected, all compounds in the series failed to elucidate any change in the cAMP production of the cells, including the reference standard of CP-55,940.

4.5.5. CB₁ PTx-sensitive G_{i/o}-inactivation for the Hexahydro series

Plates were run as previously described (**Section 4.5.1**) in three (3) replicates with CP-55,940 as the internal control for all three (3) replicates (**Figure 4.26**; **Table 4.4**). All compounds failed to alter cAMP production, including the reference standard of CP-55,940. Only compounds **170**, **171**, **174** and **175** were screened in the PTx-sensitive G_{i/o}-inactivation assays due to their ability to elucidate a response in the standard CB₁ assay.

Figure 4.21. CB₁ functional activity of hexahydro series compounds, 50 minutes after compound addition.

Table 4.4. EC₅₀ values for hexahydro series functional assays

Compound	CB₁ EC₅₀ or Concentration (% baseline)	CB₂ EC₅₀ or Concentration (% baseline)	CNG EC₅₀ (nM)	CB₁ PTx EC₅₀ (nM)	CB₂ PTx EC₅₀ (nM)
170	78.9 ^a	333 ^a	n/a	n/a	n/a
171	209 ^a	835 ^a	n/a	n/a	n/a
172	25 (40%) ^b	n/a	n/a	-	-
173	250 (25%) ^b	n/a	n/a	-	-
174	276 ^a	682.1 ^a	n/a	-	n/a
175	56.3 ^a	66.8 ^a	n/a	n/a	n/a
176	100 (12%) ^b	500 (36%) ^b	n/a	-	-
177	n/a	n/a	n/a	-	-

Note: All concentrations are listed as nM, percentages indicate the change from baseline. n/a: Not active, based on no statistically significant change in cAMP from baseline. “-” Indicates test not performed. ^a Indicates a decrease in cAMP as compared to baseline. ^b Indicates an increase in cAMP as compared to baseline.

Figure 4.22. CB₁ functional activity for select hexahydro series compounds, 50 minutes after compound addition

Figure 4.23. CB₂ functional activity for hexahydro series, 50 minutes after compound addition

Figure 4.24. CB₂ functional activity for select hexahydro series compounds, 50 minutes after compound addition

Figure 4.25. HEK-CNG parental cell functional activity for hexahydro series, 50 minutes after compound addition

Figure 4.26. CB₁ PTx-sensitive G_{i/o}-inactivation of functional activity for hexahydro series, 50 minutes after compound addition

4.5.6. CB₂ PTX-sensitive G_{i/o}-inactivation for the Hexahydro series

Plates were run as previously described (**Section 4.5.1**) in three (3) replicates with CP-55,940 as the internal control for all three (3) replicates of compounds 170, 171, 174 and 175. As was expected, all compounds failed to alter cAMP production, including the reference standard of CP-55,940 (**Figure 4.27**).

4.6. Discussion

4.6.1. Interactions with the CB₁ LBP

As previously discussed (**Section 1.2.2**), CB₁ receptors are 7 trans-membrane G-protein coupled receptors (GPCRs).¹⁴ Interaction of classical cannabinoids within the LBP of CB₁ occurs primarily in domains 3, 6 and 7. Several homology models help to elucidate the residues important for binding and functional activity.

To generate hypotheses, gain some insight into the possible explanations and interplay between ligand and receptor, I performed docking of the compound. Dr. Chang of The University of California, Riverdale, kindly supplied the models for the CB₁ receptor. Dr. Chang provided 4 models: “ACEA” – to mimic the endocannabinoid binding, “HU-210” – to mimic the classical core binding, “SR-141716A” – to mimic the inverse agonist binding and “WIN-55,212-2” – to mimic the non-classical agonist binding.²⁴⁹ The hexahydro series of compounds, being similar in form to that of HU-210, I believe adopt a similar binding mode within the LBP, thus this was the model used for docking studies.

4.6.1.1. C-1 hydroxyl. K192(3.28) plays a very important role in both receptor binding and function. Since it is a very flexible residue in the LBP, it may hydrogen bond with the C-1 hydroxyl, C-11 (“Northern”) hydroxyl, C-6 (“Southern”) hydroxyl or even the benzochromene oxygen.²⁵⁰ However, newer data suggest that K192(3.28) plays a primary role as H-bond acceptor of the C-1 hydroxyl,²⁵¹ presumably this occurs after being activated by donating a H-bond to F189(3.25) (**Figure 4.28**).²⁴⁹ S383(7.35) interacts by donating a H-bond to either the benzochromene oxygen or the C-6 hydroxyl, if present (**Figure 4.28**).²⁵¹ Additionally, data suggest that the resting state of the CB₁ receptor exists when the salt bridge between K192(3.28) and D366(6.58) is intact.²⁵²

The hexahydro series of compounds sought to further the functional activity and binding affinity of the KM Series (**Chapter 3**), and therein brought about novel activity. Within this series, aromatic substitutions yielded inverse agonists at CB₁ and neutral antagonists CB₂, while aliphatic substitutions at C-1' yielded agonists at both CB₁ and CB₂. This is unique when considering the change between **KM-233 (63)**, compounds **172** and **176** are the inclusion of a pyridine and pyrimidine A-ring, respectively. NMR data shows the hexahydro compounds exist in solution as an amide at the A-ring, which may contribute to the binding modes in the LBP and change in activity compared to the KM series.

Figure 4.27. CB₂ PTx-sensitive G_{i/o}-inactivation of functional activity for hexahydro series, 50 minutes after compound

Looking at compounds **170**, **171**, **174** and **175** and considering their agonist activity, two literature-reported interactions should be present for agonist activity:

1. The K192(3.38) and D366(6.58) salt bridge must be severed.²⁵²
2. Each of these ligands has to interact with both residues F200(3.36) and W279(5.43) to exert agonist effect.²⁵⁷

The first is the severance of the salt bridge by interacting with K192(3.38). I hypothesize that this novel series of compounds binds similarly to classical cannabinoids; however, the key difference lies in the presence of the amide moiety in the A-ring. This moiety may cause the ABC ring to bind more deeply into the tricyclic (ABC ring) pocket to facilitate H-bonding between the amide N-H and K192(3.28) (**Figure 4.28B**).

4.6.1.2. C-3 lipophilic side chain. A predominantly lipophilic pocket exists, seemingly as two lobes – as evidenced in the fact that 3-(1',1'-dimethylheptyl)- Δ^8 -THC binds with higher affinity than does 3-heptyl- Δ^8 -THC along with 3D-QSAR models which demonstrate this trait.²⁵³ In one homology model, residues V196(3.32), T197(3.33), F200(3.36), Y275(5.39), W279(5.43), L359(6.51) and M363(6.55) form a hydrophobic pocket which encompasses the A-ring and C-3 side chain of HU-210.²⁵⁴ More recent homology models form this pocket from I354(6.46), C355(6.47), W356(6.48), L359(6.51), L360(3.52) and M363(6.55) (**Figure 4.29A**).²⁴⁹ Interestingly, mutations studies conducted on this hydrophobic pocket show that M363A(6.55) mutation greatly impacts binding affinity for HU-210 but has virtually no impact on THC which would suggest that this is an important residue for C-1' substituted derivatives.²⁵⁵ The hexahydro series, given that it shares a C-1'-gem-dimethyl substituent, likely share a similar interaction with M363(6.55) (**Figure 4.29B**).

The second element suggested for an agonist is the simultaneous interaction with residues F200(3.36) and W279(5.43). Compounds **170**, **171**, **174** and **175**, with their long, flexible, aliphatic C-3 substituents may still provide necessary interactions with both F200(3.36) and W279(5.43). The more compact and rigid aromatic substituents of compounds **176** and **177** may not provide these interactions as a result of the deeper binding into the tricyclic (ABC ring) pocket or stronger intra-molecular π - π interactions (**Figure 4.30**) and lack of activity.

Looking at the special case of compounds **172**, **173**, **176** and **177** within the CB₁ LBP, there is a unique situation. Given the current functional data generated from my assay conductance suggests that these compounds are members of a special class of antagonists, known as inverse agonists. Two key interactions are reported in literature to help stabilize the CB₁ receptor in its inactive state:

1. A salt bridge between residues K192(3.28) and D366(6.58).²⁵²
2. A π - π stacking, rotameric “toggle” switch between residues F200(3.36) and W356(6.48).^{256,321,322}

I hypothesize that in order for compounds to bind into the LBP, they must break the salt bridge between K192(3.28) and D366(6.58). Thus, the salt bridge represents an inactive

Figure 4.29. CB₁ hydrophobic C-3 substituent binding pocket

Note: Panel A depicts HU-210 in the CB₁ LBP, with focus given to the hydrophobic pocket. Panel B depicts Compound 170 in the CB₁ LBP, with focus given to the hydrophobic pocket.

Figure 4.30. CB₁ LBP – comparison of the aliphatic and aromatic hexahydro interactions

Note: Panel A depicts Compound 170 interacting with W279(5.43). Panel B depicts Compound 176 with a lack of interaction.

state for the CB₁ receptor; however, it does not represent a ligand-bound state of the receptor, because the act of binding ligand to receptor results in a severance of this salt bridge. The aromatic C-3 substituent of **172**, **173**, **176** and **177** would then potentially stabilize the “toggle” switch of F200(3.36) and W356(6.48) (**Figure 4.31**). The F200(3.36)/W356(6.48) stabilization is reported for inverse agonists. Given that the compounds in this series bind, but the aliphatic-substituted compounds lack of ability to stabilize the “toggle” switch points to a unique mechanism of aromatic substituents in CB₁ LBP interactions. This hypothesis would be more consistent with the work of homology modeling of SR141716A³²² and of McAllister, *et al.*²⁵⁶ – the group who thus behave as an inverse agonist. Dashed green lines indicate π - π interactions. Helix 3 shown in yellow, helix 6 shown in blue. originally proposed the salt bridge²⁵² concept. In addition, it is important to note, that in the KM series there exists a thiophene derivative **78**, which was an agonist at CB₁. Thus, stabilization of the “toggle” switch in conjunction with a lack of interaction with both F200(3.36) and W279(5.43) – which is suggested to arise from the inclusion of a heteroatom-containing A-ring, though the exact root cause is currently unknown – give the unique CB₁ mechanism.

4.6.1.3. ABC ring system. Residues F174(2.61) and F177(2.64) are not suggested to be involved in binding to HU-210, but rather they help stabilize the tricyclic (ABC ring) pocket where the ABC benzochromene ring binds the LBP.²⁵⁵ Residues F200(3.36) and W279(5.43) appear to play an important a role in both in terms of binding (in the case of AEA and CP-55,940) and receptor activation (**Figure 4.30**).²⁵⁷ That is to say, a ligand’s hydrophobic substituents at the C3 position may bind well without interacting with these two residues, but agonists MUST have interaction with these residues to exert their effect.²⁵⁴ F189(3.25) is key at providing a π - π stacking interaction with HU-210 (**Figure 4.32A**), and is instrumental in binding of AEA to the pocket as its mutation to alanine causes a 7-fold drop in affinity for the receptor.²⁵⁸ F189(3.25) also seems to form the “ceiling of the anandamide binding pocket in CB₁.”²⁵⁷ From a π - π stacking standpoint, the hexahydro series shares these interactions (**Figure 4.32B**) – though not depicted on the 2D output. However, the major difference lies in the compounds’ ability to interact with the key residues, which were addressed previously.

4.6.1.4. (Optional) C-11 (“Northern”) hydroxyl. While there is not data currently to confirm, logical extension of the known fact that C-11 hydroxylation is the primary metabolite of the KM series would suggest that this would be a. metabolite of the hexahydro series. As a result of this metabolite potentially contributing to the overall activity of the compound *in vivo*, this potential interaction is discussed and explored. S383(7.35) shows itself to be equally important in binding of molecules along with stabilizing the LBP. S383(7.35) can hydrogen bond with the C-11 hydroxyl²⁵¹ (if present) or the benzochromene oxygen^{249,254}, but more importantly it helps to stabilize the LBP by maintaining the shape of the trans-membrane helix 1-2-7 binding pocket.²⁵¹ The “Northern” hydroxyl group may also H-bond with K192(3.28); however, this is not a preferred interaction in several recent homology models.

Figure 4.31. Stabilization of the CB₁ LBP in the inactive state

Note: Panel A depicts the two (2) key residues involved in stabilizing the inactive state of CB₁. Panel B depicts these two (2) residues in the active state of the receptor. Panel C is the hypothesized interaction of 173 to stabilize the CB₁ receptor in the inactive state.

Figure 4.32. CB₁ LBP proposed π - π stacking and key residue interactions

4.6.2. Interactions with the CB₂ LBP

As previously discussed (**Section 1.2.3**), CB₂ receptors are also 7 trans-membrane GPCRs; however, CB₂ only shares 48% homology with CB₁ – a trait unique to the CB₁ and CB₂ receptors among GPCRs, which generally share a much higher level of homology among receptors of the same family.¹⁴ The SAR of the CB₂ LBP has yet to be fully developed – even less so with the classical cores. B/C ring trans junction. With data relatively limited for CB₂'s LBP, especially in the arena of classical core compounds, one must make some inferences based on available aminoalkylindole derivative homology model data. One such extrapolation lies in WIN-55,212-2's biologically assumed conformation to be *s-trans*-conformer.²⁶⁰ Docking the *s-cis*-conformer, there are steric interactions with V113(3.32) (**Figure 4.33**).²⁶¹ Existence of this clash may suggest a trans junction of the B/C ring allows for proper orientation in the LBP to circumvent this steric interaction with V113(3.32) within the classical core molecules. By extension, WIN-55,212-3, the S(-) isomer of WIN-55,212-3, may also orient the morpholino side chain such to prevent critical amino acids interactions in the CB₂ LBP as discussed below. Taking WIN-55,212-2 and ascribing its moieties to those of the representative example of **172** (**Figure 4.34**), one can see that there are many similarities. While the similarities are numerous, the differences are profound as well, because the steric interactions of WIN-55,212-2 with V113(3.32) are apparently non-existent with the hexahydro series. The steric issue comes in with what I hypothesize to be the “northern” aliphatic hydroxyl region, or C-11 substituted derivatives of Δ^8 -THC, thus the hexahydro series has little to contend with in this arena.

4.6.2.1. C-1 Hydroxyl, benzchromene oxygen and (optional) C-6 hydroxyl.

Mutation studies with HU-210, CP-55,940 and WIN-55,212-2 have shown that binding of both HU-210 and CP-55,940 experience negative consequence with a S292A mutation. This mutation-induced change of binding strongly suggests S292(7.46) is involved in H-bonding with the benzchromene oxygen – assuming a similar LBP arrangements relative to the CB₁ LBP.³²³ Though WIN 55-212-2's ketone is in suggest to be in proximity to residue S193(5.42) it does not appear to hydrogen bond.²⁶¹ In the case where amide linkages exist between the aryl groups, it is suggested that S193(5.42) does act as a H-bond donor.^{324,325}

4.6.2.2. Hydrophobic and π - π stacking. As has been the case previously, it remains so that many of the interactions derive from the aminoalkylindole derivative WIN-55,212-2. Beginning with the indole ring of WIN-55,212-2, there are reported π - π stacking interactions between it and residues F197(5.46), W258(6.48) and F117(3.36).²⁶¹ There also exists a shared π - π stacking between the indole ring and naphthyl ring with residue W194(5.43).²⁶¹ The naphthyl ring has an added π - π stack with residue F106(3.25).²⁶¹ More recent 2-pyridone derivatives add additional residues to the binding pocket for π - π stacking, namely Y190(5.39) and F281(7.35) along with a C288(7.42) residue.^{324,325} For the 2-pyridone analog containing N-butyl, there involves a hydrophobic interaction with I198(5.47).³²⁵

4.6.2.3. Proposed interactions of the hexahydro series with the CB₂ LBP. Due to their similarities, compounds **170**, **171**, **174** and **175** are the aliphatic constellation and

Figure 4.33. CB₂ LBP steric hinderance of *cis* and *trans* isomers of WIN-55,212-2

Figure 4.34. Comparison of analogous functional groups of WIN-55,212-2, 172 and WIN-55,212-3

compounds **172**, **173**, **176** and **177** are the aromatic constellation. Similar to the KM Series, I hypothesize that the binding mode for the hexahydro series is more similar to THC, in that the ABC ring occupies the tricyclic (ABC ring) pocket of the LBP and the C-3 substituents project into the major pocket. Therein the major pocket lies the key residue of W258(6.48), the rotameric “toggle” switch for CB₂ – proposed to H-bond with L255(6.45) to open place C2.57(6.47) in optimal range for agonist interaction.³²⁶ As was the case in CB₁, compounds **170**, **171**, **174** and **175**, with their long, flexible, aliphatic C-3 substituents can then still provide necessary interactions with W258(6.48) while still allowing the rotameric change from gauche⁺ to trans of the residue (**Figure 4.35**).

4.7. Conclusions

The pyridine and pyrimidine hexahydro series of compounds represent a novel series of compounds, which may also give insight into the binding mode and residue interactions of these compounds in the CB₁ LBP. The presence of the amide moiety in the A-ring causes these series of compounds to bind further into the tricyclic (ABC ring) pocket of the CB₁ LBP making interaction with the two (2) key residues, F200(3.36) and W279(5.43), possible only with a long, flexible C-3 substituent – such as that found on **170**, **171**, **174** and **175**. Unique mechanisms with regard to heterocyclic stabilization of the CB₁ receptor’s inactive state by aiding in the π - π stacking of residues F200(3.36) and W356(6.48) to yield an inverse agonist. Interactions within the CB₂ LBP can only be postulated in the absence of a refined model. However, aromatic C-3 substituents seem to stabilize the gauche⁺ rotamer of W258(6.48) in order to yield an antagonist at CB₂ – aliphatic substituents allow for rotation of W258(6.48) and thus receptor activation.

Future studies to test these hypotheses would be to conduct homology model studies with these compounds – more specifically to reconcile the divergence of binding mode for these compounds and further solidify key residues. Additionally, synthesis of other derivatives would help to determine a more exact functional pharmacophore. Lastly, testing of the antagonist compounds against known CB₁ and CB₂ agonists will confirm this mechanism and establish EC₅₀ doses for these compounds.

Figure 4.35. Proposed CB₂ LBP residues for compound 170

CHAPTER 5. FUNCTIONAL EVALUATION OF A NOVEL CLASS OF TRIARYL CANNABINERGIC LIGANDS

5.1. Introduction

As previously discussed (**Section 1.1**), classical cannabinoids are tricyclic compounds possessing a benzopyran core – these are of either natural origin or synthetic analogues thereof. The naturally occurring, prototypes for these compounds are Δ^9 -THC and Δ^8 -THC, which both bind to CB₁ and CB₂ receptors and exert a partial agonist activity.¹⁴ **Chapter 3** and **Chapter 4** have been examples of the extensive study and mining of these classical core compounds for functional pharmacophoric elements. To further the field and probe CB₁ and CB₂ further, new chemical scaffolds are essential. To move away from the classical core and parse out even more functional pharmacophoric elements, the non-classical cannabinoids came into their own right. These non-classical cannabinoids sought to simplify the overall structure of cannabinoid ligands and to augment the understanding of CB₁ and CB₂ ligand requirements.

It has been established through study of these non-classical cannabinoids that simplifying the overall structure to that of an AC-bicyclic increases the affinity for both CB₁ and CB₂; however, transition from AC-bicyclic to ACD-tricyclic allows for selectivity between CB₁ and CB₂ (**Section 5.2.1**). Merging traits of the classical with these AC-bicyclic and ACD-tricyclic compounds gave rise to a hybrid crossover series, with increased affinity for both without any appreciable selectivity (**Section 5.2**). The biaryl class of cannabinoid compounds sought to take the best of the classical C-3 substituent and merge it with the bicyclic structure of the AC-bicyclic compounds, resulting in a series with very good affinity and specificity towards CB₂ (**Section 5.2.3**). A final derivative in the non-classical cannabinoid derivatives was that of the triaryl bis-sulfones, which were the result of the diarylpyrazole derivatives and resulted in extremely potent and selective compounds at CB₂ (**Section 5.2.4**).

Taking this non-classical knowledge and amalgamating it with the previous classical, KM series and hexahydro-heteroaromatic compounds, gave rise to 4 distinct classes of triaryl cannabinoid ligands: HB series, SMM series, pyridine triaryl series and pyrimidine triaryl series (**Figure 5.1**; **Section 5.3**). The aim of this study is to utilize the ACTOne Assay (**Chapter 2**) to explore the functional effects of the HB series (**Section 5.4.2**), SMM series (**Section 0**), pyridine triaryl series (**Section 5.4.4**) and pyrimidine triaryl series (**Section 5.4.5**). Following this, I will reconcile these data against current literature standards' binding and homology models to propose hypothetical interactions within the CB₁ and CB₂ LBP to further develop the SAR, functional pharmacophore and unique MoA of these compounds.

Figure 5.1. Triaryl series design flow

5.2. Non-Classical Cannabinoids

5.2.1. AC-bicyclic and ACD-tricyclic compounds

These series of compounds were developed by Pfizer and omit the B-ring of the classical cannabinoid compounds, which is generally a pyran ring. The best known of these series is CP-55,940 (**Figure 5.2**), which works to incorporate many of the previously mentioned elements of the classical cannabinoids without, itself, being a classical cannabinoid. This AC-bicyclic structure has very good affinity and no specificity for CB₁ and CB₂ (CB₁ K_i = 0.6 nM; CB₂ K_i = 0.7 nM). Closely related to CP-55,940 is CP-47,497 (**Figure 5.2**) which incorporates all of the same elements with the notable exception of a southern aliphatic hydroxyl (SAH). Lack of the SAH in CP-47,497 causes almost a 4-fold increase in affinity for the CB₁ receptor (CB₁ K_i = 2.1 nM). While this is in no way a poor compound, it does show the added benefit of the SAH moiety in these compounds. Knowing that the SAH is a useful component of the cannabinoid compounds, a more conformationally restricted ACD-tricyclic derivative was synthesized with CP-55,244 (**Figure 5.2**) (CB₁ K_i = 0.21 nM). Restricting the SAH to project into the tricyclic (ABC ring) pocket allows for a compound that is more potent than the prototype CP-55,940. Still missing was the lack of specificity for targeting CB₂ over CB₁; however, the advent of the AC"D"-tricyclic ligand of HU-308 (**Figure 5.2**) (CB₁ K_i = >10,000 nM; CB₂ K_i = 22.7 nM) helped to change that.

5.2.2. Hybrid cross-over compounds

After the advent of the non-classical cannabinoid compounds, and with good understanding of classical cannabinoids, compounds were synthesized to take advantage of useful aspects of both – more namely the C3-1',1'-dimethylheptyl of the classical and the SAH of the non-classical compounds. These compounds served a unique purpose in the understanding of cannabinoids by providing three-dimensional probes for the CB₁ and CB₂ LBP. Data from studies conducted in the Makriyannis lab group demonstrated that C-6 β -hydroxypropyl (**178a**, **Figure 5.3**) (CB₁ K_i = 70.5 nM) had a higher affinity for CB₁ than did its α -hydroxypropyl (**178b**, **Figure 5.3**) (CB₁ K_i = 1353 nM) epimer.³²⁷⁻³²⁹ Further derivation of these hybrid compounds led to unsaturation at the 1' position of the C-6 SAH substituent. The alkyne (**179**, **Figure 5.3**) demonstrates great affinity for both CB₁ (K_i = 1.2 nM) and CB₂ (K_i = 0.3 nM), with a slight affinity for CB₂. The alkene (**180**, **Figure 5.3**) demonstrates good affinity for both CB₁ (K_i = 0.7 nM) and CB₂ (K_i = 8.6 nM), with a >10-fold affinity for CB₁ – the C1'' *cis* isomer (not pictured) of **180** demonstrated a difference in activity (CB₁ K_i = 2.24 nM; CB₂ K_i = 0.33 nM).³³⁰ It is noteworthy, that the values listed for compounds **179** and **180** are for the racemic mixture, and that work by Thakur and co-workers demonstrated that the active isomer of **180** is, as drawn, the 6S, 6aR, 9R, 10aR configuration.³³¹

Figure 5.2. Non-classical cannabinoids

Figure 5.3. Hybrid cannabinoid ligands

5.2.3. Biaryl cannabinoids

As an added attempt to bridge the gap among the non-classical cannabinoids and further hone in upon a CB₂-selective compound, the biphenyl compounds were developed by Makriyannis' group. These compounds sought to build upon the work at Pfizer with the "CP" compounds, and add the selectivity of HU-308. The group, overall, has good affinity, with the prototype compound (**181**, **Figure 5.4**) having good affinity for both receptors, with a CB₁ K_i = 2.6 nM and a CB₂ K_i = 0.6 nM. Addition of more substituents to the "C"-ring (e.g. **182** and **183**, **Figure 5.4**) led to a nearly 100-fold and 300-fold preference for CB₂ over CB₁, respectively. Incorporation of a heteroaromatic (furan) ring as the "C"-ring (**184**, **Figure 5.4**) confers a great deal of CB₂ selectivity (CB₁/CB₂ ratio = 90).³³² This class of compounds has been disclosed in patent literature; however, little outside thereof has come to be known about these compounds.

5.2.4. Triaryl bis-sulfones

Initial discovery of these compounds was a direct result of work in the discovery and development of the diarylpyrazoles, such as SR-141716A. These were originally conceived as biaryl sulfone compounds (e.g. **185**, **Figure 5.5**); however, analogs of this compound and an ultra-pure sample of this compound showed poor activity. Analysis of **185** via LCMS demonstrated a tainted sample – which in light of the pure sample's failure to elucidate effect, were likely contributing to the activity *in vitro*. Of these impurities, compound **186** (**Figure 5.5**) became the new lead molecule for development.³³³ Derivation of this lead compound centered initially on the substituents attached to the B- and C-ring, where investigation demonstrated a penchant for substitution at the 2 position of the C-ring and small alkyl/halogen substitution at the 4 position of the B-ring.³³⁴ Also examined were the linkages between A- and B-rings and B- and C-rings, with sulfone being the only link tolerated between B- and C-rings and ultimately the best choice for both junctions.³³⁴ Next in the elucidation and optimization for these compounds was the amide moiety, attached to the A-ring. Modifications about this position seemed to favor small substituents, with a preference for electron withdrawing groups. The primary example being the trifluoromethyl derivative (**186**) (CB₁ K_i = 235 nM; CB₂ K_i = 0.3 nM). This trait was further exemplified by the methylsulfonamide derivative (**187**, **Figure 5.5**) (CB₁ K_i = 905 nM; CB₂ K_i = 0.4 nM) – showing inverse agonist activity in the [³⁵S]GTPγS assay, with an EC₅₀ of 0.6 nM.³³³

Further development of **187** saw modifications to the C-ring, where o-F-phenyl (**188**, **Figure 5.5**) (CB₂ K_i = 1.3 nM; CB₁ K_i/CB₂ K_i = 4387) and 2-pyridine (**189**, **Figure 5.5**) (CB₂ K_i = 2.9 nM; CB₁ K_i/CB₂ K_i = 4403) exhibited similar selectivity ratios, but o-F-phenyl demonstrated a superior CB₂ K_i.³³⁵ With a newly optimized C-ring, the amide moiety of the A-ring was re-examined, and data demonstrated that a trifluoromethylsulfonamide (**190**, **Figure 5.5**) (CB₂ K_i = 2 nM) held top honors.³³⁵ The next step in optimization of the compound was to examine the halogen substitution on the B-ring; however, Cl held its place atop the possible moieties examined.³³⁵ To complete the cycle, the C-ring was examined with the newly optimized trifluoromethylsulfonamide present on the A-ring, and as with the Cl, o-F-phenyl retained its spot.³³⁵

Figure 5.4. Biaryl cannabinoid ligands

Figure 5.5. Triaryl bis-sulfone derivatives

5.3. Novel Series of Triaryl Cannabaergic Ligands

5.3.1. Rationale of design

As previously examined in **Chapter 3**, the C-1'-dimethyl-aryl- Δ^8 -THC analogs demonstrated a unique mechanism in that they were agonists at CB₁ and antagonists at CB₂. Additionally, considering the increase in binding affinity gained with this novel series, they are good agents in their own right; however, as scientists are in the pursuit of as near perfection as one may attain, “good” is not good enough. Recall, too, the increase in binding affinity gained from the biaryl cannabinoids (**Section 5.2.3**) – though these particular cannabinoids did not lend themselves to specificity. With the combination of these two series, a third novel series was born – the HB series (**Table 5.1**).

Success of the HB series compounds in testing led one-step further in development – combine the qualities and attributes of the HB series with those of the triaryl bis-sulfones (**Section 5.2.4**). The hypothesis is that one may retain the well-established attributes of a C-1' derivative while maximizing the hydrophilic interactions within the LBP – thus the SMM series (**Table 5.2**) came to be. To further hone activity, consideration was given to the classes of the aminoalkylindoles (**Section 4.3.1**) and diarylpyrazoles (**Section 4.3.2**), which have proven repeatedly that they are a potent classes of compounds. Additionally, the successes of the hexahydro heterocyclic derivatives discussed in **Chapter 4** add another layer of consideration to the developing cannabinoid pharmacophore. Coupling all of these components with the SMM and HB series spawned two unique series to mimic the heterocyclic central core of the diarylpyrazoles and aminoalkylindoles. A heterocyclic A-ring afforded the pyridine (**Table 5.3**) and pyrimidine (**Table 5.4**) triaryl series of compounds. All were synthesized to separate affinity and/or activity to yield therapeutically viable compounds.

5.3.2. Objectives of the study

Utilizing these 4 novel series (**Figure 5.6**), I explored the functional pharmacology of each in relation to CB₁, CB₂ and the parental HEK-293-CNG cell lines (greater detail in **Chapter 2**) to better understand the functional activity. Further, reconciliation of known literature of binding and homology models helps to explain observed data and interaction of the ligands with the LBP of CB₁ and/or CB₂ receptors.

Figure 5.6. Generic triaryl structure nomenclature

Table 5.1. HB triaryl series analogues

ID	R ₁ (C-ring)	R ₂ (D-ring)	R ₃
191			H
192			OH
193			OH
194			OMe

Table 5.2. SMM triaryl series analogues

ID	R ₁ (C-ring)	R ₂ (D-ring)	R ₃	R ₄	R ₅
195			OH	OH
196			OMe	OMe
197			OMe	OMe
198			OMe	OMe
199			OMe	OMe
200			OMe	OMe

Table 5.2. (Continued)

ID	R ₁ (C-ring)	R ₂ (D-ring)	R ₃	R ₄	R ₅
201			OMe	OMe
202			OMe	OMe
203			OMe	OMe
204			OMe	OMe
205			OMe	OMe
206			OH	OMe

Table 5.2. (Continued)

ID	R ₁ (C-ring)	R ₂ (D-ring)	R ₃	R ₄	R ₅
207			OH	OH
208			OH	OMe
209			OH	OH
210			OH	OMe
211			OH	OH

Table 5.2. (Continued)

ID	R ₁ (C-ring)	R ₂ (D-ring)	R ₃	R ₄	R ₅
212			OMe	OMe
213			OMe	OMe
214			OH	OH
215			OH	OH

Table 5.3. Pyridine triaryl series compounds

ID	R ₁	R ₂
216
217
218
219
220
221

Table 5.3. (Continued)

ID	R ₁	R ₂
222
223
224
225

Table 5.3. (Continued)

ID	R ₁	R ₂
226		
227		
228		
229		

Table 5.4. Pyrimidine triaryl series compounds

ID	R ₁	R ₂
230
231
232
233

Table 5.4. (Continued)

ID	R ₁	R ₂
234		
235		
236		
237		
238		
239		

5.4. Functional Pharmacology

5.4.1. ACTOne Assay

Assay development and conductance was discussed in detail in **Chapter 2**. Briefly, stably transfected HEK-293 cells co-expressed with modified CNG channels along with CB₁ or CB₂ or their parental cells (without CB₁ or CB₂; HEK-CNG) were plated at an appropriate density in a clear, 96-well, Poly-D-Lysine plate and allowed to incubate at 37°C and 5% overnight. After the overnight incubation period, 100 µL of ACTOne membrane potential dye was added to each well of the culture plate and allowed to sit in a dark area at room temperature for 60 additional minutes. After the 60 minute incubation period a baseline read in a BioTek Synergy 2 plate reader with an excitation of 540/25 nm and an emission of 590/20 nm. Following the baseline read, 50 µL of drug stock solution containing appropriate concentrations of drug (**Figure 5.7**), Forskolin and Ro 20-2304 in DPBS with 2.5% DMSO was added to the cell culture plate. The plate was then placed on the plate reader with 1 read per well per minute with an excitation of 540/25 nm and an emission of 590/20 nm for 60 minutes.

Screening of compounds against CB₁ and CB₂ were completed as technical replicates of n = 6 each. A decrease in the number of replicates (n = 3) for HEK-CNG Parental and CB₁/CB₂ PTx-sensitive G_{i/o}-inactivation was employed due to the expected lack of response from these compounds to those three (3) groups of assays, thus maximizing resource allocation and ensuring accurate data evaluation.

Primary data were analyzed with customized VBA macros (**Chapter 2**), developed internally, within Microsoft Excel (Redmond, WA). Column 11 (lowest [drug]) determined the experimental maximum response (100 % activity) at each individual time point, and the experimental basal response was determined by the average response given by the 2 blank control wells. All data were normalized with these minimum and maximum values via Equation 2.1. The EC₅₀ values were calculated from concentration-response curves by non-linear regression and automatic outlier elimination analysis utilizing GraphPad Prism Software (San Diego, CA).

5.4.2. Functional activity for HB triaryl series

5.4.2.1. CB₁ functional activity for the HB series. Plates were run as previously described (**Section 4.5.1**) in six (6) replicates with CP-55,940 as the internal control for all six (6) replicates (**Figure 5.8**). Compounds **192** and **193** demonstrated agonist activity at CB₁ with an EC₅₀ of 993 nM, 1429 nM, respectively, with percent cAMP inhibition ranging from 46% to 57%, 50 minutes after compound addition into the assay plate (**Table 5.8**). These two (2) compounds exhibit a decrease in cAMP production equivalent to our standard of CP-55,940, albeit at a higher EC₅₀. Compound **191** was tested at a lower concentration gradient than the other three (3) compounds to examine the effects of its mono-hydroxy A-ring more directly with compound **195**. However, data do suggest that shifting the concentrations to the higher range may result in curves more similar to **192** and **193**, with a 10% decrease in cAMP at 1 µM drug concentration.

	1	2	3	4	5	6	7	8	9	10	11	12
A	5000 nM	2500 nM	1000 nM	500 nM	250 nM	100 nM	50 nM	25 nM	10 nM	5 nM	2.5 nM	Blank
B												Blank
C	1000 nM	500 nM	200 nM	100 nM	50 nM	20 nM	10 nM	5 nM	2 nM	1 nM	0.5 nM	Ro
D												Ro
E												Ro/For
F												Ro/For
G												CP CTRL
H												CP CTRL

Figure 5.7. Drug plate layout and concentrations

Notes: Columns 1 through 11 contain test compound, and Column 12 contains control wells. All listed concentrations are the final concentrations once added to the testing plate, so in the drug plate all compounds are at 5x of the listed concentrations in Columns 1 through 11. Control wells (Column 12) are: "Blank" (cells, media and dye), "Ro" ("Blank" + 125 μ M Ro 20-**2304** [25 μ M, Final]), "Ro/For" ("Ro" + 4 μ M Forskolin [800 nM, Final]) and "CP CTRL" ("Ro/For" + 25 μ M CP 55,940 [5 μ M, Final]).

Figure 5.8. CB₁ functional activity of HB triaryl series, 50 minutes after compound addition

Table 5.5. EC₅₀ values for HB triaryl series

Compound	CB₁ EC₅₀ or Concentration (% baseline)	CB₂ EC₅₀ or Concentration (% baseline)	HEK-CNG EC₅₀ (nM)	CB1 PT_x EC₅₀ (nM)	CB2 PT_x EC₅₀ (nM)
191	n/a	25 (10%) ^b	n/a	-	-
192	993 ^a	10.3 ^a	n/a	n/a	n/a
193	1429 ^a	7.4 ^a	n/a	n/a	n/a
194	1000 (10%) ^a	1000 (10%) ^a	n/a	-	n/a

Notes: All concentrations are listed as nM, percentages indicate the change from baseline. n/a: Not active, based on no statistically significant change in cAMP from baseline. “-” Indicates test not performed. ^a Indicates a decrease in cAMP as compared to baseline. ^b Indicates an increase in cAMP as compared to baseline

5.4.2.2. CB₂ functional activity for the HB Series. Plates were run as previously described (Section 5.4.1) in six (6) replicates with CP-55,940 as the internal control (Figure 5.9). All compounds in the series bind with good affinity to the CB₂ receptor.²⁵⁹ Similar to CB₁ functional activity assays **192** and **193** (Figure 5.9) demonstrated agonist activity; however, it was not as effective in terms the percent cAMP inhibited at CB₂. Compounds **192** and **193** did demonstrate an EC₅₀ that was significantly better than it had fared at CB₁. EC₅₀ for **192** and **193** was 10.3 nM and 7.4 nM, with percent cAMP inhibition of approximately 17% and 14%, respectively. Additionally, **191** and **194** showed a 10% change in cAMP in an inverse agonist and agonist fashion, respectively.

5.4.2.3. CNG parental functional activity for the HB series. Plates were run as previously described (Section 5.4.1) in three (3) replicates with CP-55,940 as the internal control for all three (3) replicates (Figure 5.10). As was expected, all compounds in the series failed to elucidate any statistically significant change in the cAMP production of the cells, including the reference standard of CP-55,940.

5.4.2.4. CB₁ PTx-sensitive G_{i/o}-inactivation for the HB series. Plates were run as previously described (Section 5.4.1) in three (3) replicates with CP-55,940 as the internal control for all three (3) replicates (Figure 5.11). As was expected, all compounds failed to alter cAMP production, including the reference standard of CP-55,940. Only compounds **192** and **193** were screened in the PTx-sensitive G_{i/o}-inactivation assays due to their ability to elucidate a response in the standard CB₁ functional assay.

5.4.2.5. CB₂ PTx-sensitive G_{i/o}-inactivation for the HB series. Plates were run as previously described (Section 5.4.1) in three (3) replicates with CP-55,940 as the internal control for all three (3) replicates (Figure 5.12). As was expected, the tested compound failed to alter cAMP production, including the reference standard of CP-55,940. Only compound **192** was screened in the PTx-sensitive G_{i/o}-inactivation assays due to its ability to elucidate a response in the standard CB₂ functional assay.

5.4.3. Functional activity for SMM Triaryl series

5.4.3.1. CB₁ functional activity for the SMM series. Plates were run as previously described (Section 5.4.1) in six (6) replicates – unless the compound was known to not bind, in which case it was run in three (3) replicates – with CP-55,940 as the internal control for all replicates (Figure 5.13). As evidenced in Figure 5.13, nearly half of the compounds had no appreciable effect on the cAMP levels. Of those with some alteration of cAMP activity, all demonstrated an increase; however, most did not result in curves with a good fit upon data analysis. Compounds **200**, **206** and **210** showed a maximum effect at 5 μ M with an increase in cAMP of 48%, 32% and 24%, respectively. Compounds **202** and **203** showed a maximum effect at 50 nM, with an increase in cAMP of 85% and 75%, respectively. Three compounds **204**, **211** and **213** (Figure 5.14), demonstrate inverse agonist activity at CB₁, with an EC₅₀ of 469 nM, 1497 nM and 153 nM, respectively (Table 5.6), with percent cAMP induction ranging from 37% to 68% over basal. These compounds failed to attain the level of cAMP induction relative to our standard inverse agonist of SR-41716A (80% increase); however, they are worth noting.

Figure 5.9. CB₂ functional activity of HB triaryl series, 50 minutes after compound addition

Figure 5.10. CNG functional activity of HB triaryl series, 50 minutes after compound addition

Figure 5.11. CB₁ PTx-sensitive G_{i/o}-inactivation of HB triaryl series, 50 minutes after compound addition

Figure 5.12. CB₂ PTx-sensitive G_{i/o}-inactivation of HB triaryl series, 50 minutes after compound addition

Figure 5.13. CB₁ functional activity of SMM triaryl series, 50 minutes after compound addition

Figure 5.14. CB₁ functional activity of select SMM triaryl series compounds, 50 minutes after compound addition

Table 5.6. EC₅₀ values for SMM triaryl series

Compound	CB₁ EC₅₀ or Concentration (% baseline)	CB₂ EC₅₀ or Concentration (% baseline)	HEK-CNG EC₅₀ (nM)	CB₁ PTx EC₅₀ (nM)	CB₂ PTx EC₅₀ (nM)
195	n/a	744	n/a	-	n/a
196	n/a	-	n/a	-	-
197	n/a	-	n/a	-	-
198	n/a	-	n/a	-	-
199	n/a	5000 (81%) ^b	n/a	-	-
200	5000 (48%) ^b	5000 (20%) ^b	n/a	-	-
201	n/a	-	n/a	-	-
202	50 (85%) ^b	-	n/a	-	-
203	50 (75%) ^b	5000 (50%) ^b	n/a	-	-
204	469 ^b	5000 (43%) ^b	n/a	n/a	-
205	n/a	-	n/a	-	-
206	5000 (32%) ^b	5000 (97%) ^b	n/a	-	n/a
207	n/a	97.6 ^b	n/a	-	n/a
208	n/a	5000 (73%) ^b	n/a	-	-
209	n/a	5000 (110%) ^b	n/a	-	-
210	5000 (24%) ^b	5000 (85%) ^b	n/a	-	-
211	1497 ^b	702 ^b	n/a	n/a	n/a
212	n/a	-	n/a	-	-
213	153 ^b	5000 (125%) ^b	n/a	n/a	-
214	n/a	5000 (200%) ^b	n/a	-	n/a
215	n/a	5000 (110%) ^b	n/a	-	n/a

Notes: All concentrations are listed as nM, percentages indicate the change from baseline. n/a: Not active, based on no statistically significant change in cAMP from baseline. “-” Indicates test not performed. ^a Indicates a decrease in cAMP as compared to baseline. ^b Indicates an increase in cAMP as compared to baseline.

5.4.3.2. CB₂ functional activity for the SMM series. Plates were run as previously described (Section 5.4.1) in six (6) replicates – unless the compound was known to not bind, in which case it was run in three (3) replicates – with CP-55,940 as the internal control for all replicates (Figure 5.15). All compounds in the series bind with good affinity to the CB₂ receptor, except **200**, **201**, **202**, **205** and **212**.²⁵⁹ Unlike the CB₁ functional assays, none of the compounds in demonstrated agonist activity at CB₂. Compounds **195** (744 nM), **206** (>5000 nM), **207** (>5000 nM), **211** (702 nM), **214** (>5000 nM) and **215** (>5000 nM) (Figure 5.16) all demonstrated induction of cAMP in excess of our standard(s) SR-144528 (and AM-630 (Table 5.5), with a percent cAMP induction ranging from 148% to 266% over baseline. It is important to note that the EC₅₀ values for these compounds are not possible to calculate due to lack of a saturation response with all of the compounds except **195** and **211**.

5.4.3.3. CNG parental functional activity for the SMM series. Plates were run as previously described (Section 5.4.1) in three (3) replicates with CP-55,940 as the internal control for all three (3) replicates (Figure 5.17). As was expected, all compounds in the series failed to elucidate any statistically significant change in the cAMP production of the cells, including the reference standard of CP-55,940.

5.4.3.4. CB₁ PTx-sensitive G_{i/o}-inactivation for the SMM series. Plates were run as previously described (Section 5.4.1) in three (3) replicates with CP-55,940 as the internal control for all replicates (Figure 5.18). As was expected, all compounds failed to alter cAMP production, including the reference standard of CP-55,940. Only compounds **204**, **211** and **213** were screened in the PTx-sensitive G_{i/o}-inactivation assays due to their ability to elucidate a response in the CB₁ functional assay.

5.4.3.5. CB₂ PTx-sensitive G_{i/o}-inactivation for the SMM series. Plates were run as previously described (Section 5.4.1) in three (3) replicates with CP-55,940 as the internal control for all replicates (Figure 5.19). As was expected, the tested compound failed to alter cAMP production, including the reference standard of CP-55,940. Only compounds compounds **195**, **206**, **207**, **211**, **214** and **215** were screened in the PTx-sensitive G_{i/o}-inactivation assays due to their ability to elucidate a response in the CB₂ functional assay.

5.4.4. Functional activity for pyridine triaryl series

5.4.4.1. CB₁ functional activity for the Pyridine Triaryl series. Plates were run as previously described (Section 5.4.1) in six (6) replicates – unless the compound was known to not bind, in which case it was run in two (2) replicates – with CP-55,940 as the internal control for all replicates (Figure 5.20). Only compounds **217**, **218**, **219** and **222** were known to bind with any appreciable affinity to CB₁, and compound **223** has no binding data available, so it was treated as a known binder.²⁵⁹ EC₅₀ values for these compounds ranged from 76.1 nM to 3238 nM, with a percent decrease in cAMP ranging from 27% to 65% (Table 5.7). Despite binding to CB₁, compounds **219** and **222** did not have any impact on cAMP and showed themselves to be antagonists, the steady rise of **222** in Figure 5.21 is attributable to the drift that is occasionally noticed within the assay, as discussed in Chapter 2.

Figure 5.15. CB₂ functional activity of SMM triaryl series

Figure 5.16. CB₂ functional activity of select SMM triaryl series compounds

Table 5.7. EC₅₀ values for pyridine triaryl series

Compound	CB₁ EC₅₀ or Concentration (% baseline)	CB₂ EC₅₀ or Concentration (% baseline)	HEK- CNG EC₅₀ (nM)	CB₁ PTx EC₅₀ (nM)	CB₂ PTx EC₅₀ (nM)
216	n/a	n/a	n/a	-	n/a
217	5000 (38%) ^a	n/a	n/a	n/a	n/a
218	5000 (63%) ^a	470.1 ^a	n/a	n/a	n/a
219	n/a	108.6 ^a	n/a	n/a	n/a
220	n/a	-	-	-	-
221	5000 (40%)* ^a	389.4 ^a	n/a	-	n/a
222	n/a	n/a	n/a	n/a	n/a
223	5000 (34%) ^a	294.8 ^a	n/a	n/a	n/a
224	123.54* ^b	-	n/a	-	-
225	131.25* ^b	-	n/a	-	-
226	364.7* ^b	5000 (43%) ^b	n/a	-	n/a
227	5000 (16%)* ^b	5000 (39%) ^b	n/a	-	n/a
228	5000 (21%) ^b	5000 (30%) ^b	n/a	-	n/a
229	5000 (21%) ^b	5000 (30%) ^b	n/a	-	n/a

Notes: All concentrations are listed as nM, percentages indicate the change from baseline. n/a: Not active, based on no statistically significant change in cAMP from baseline. “-” Indicates test not performed. ^a Indicates a decrease in cAMP as compared to baseline. ^b Indicates an increase in cAMP as compared to baseline. “*” = N=3.

Figure 5.17. CNG functional activity for SMM triaryl series

Figure 5.18. CB₁ PTx-sensitive G_{i/o}-inactivation for selected SMM triaryl series compounds

Figure 5.19. CB₂ PTx-sensitive G_{i/o}-inactivation for select SMM triaryl series compounds

Figure 5.20. CB₁ functional data for pyridine triaryl compounds

Figure 5.21. CB₁ functional activity of known binders within pyridine triaryl series

As evidenced in **Figure 5.22**, some of the compounds (**224**, **225**, **226** and **227**) demonstrated a functional effect without having any known binding affinity for the CB₁ LBP. Of great intrigue is the fact that the compounds containing either a C-1' cyclohexyl or phenyl demonstrated an inverse agonist activity at CB₁. Compound **227** and **221** did not elucidate a discernable curve upon analysis, and this could be because these non-binding compounds were not run in a large enough sample size to obtain a viable curve. These data taken in the context of the whole warrants further investigation of this compound.

5.4.4.2. CB₂ functional activity for the pyridine triaryl series. Plates were run as previously described (**Section 5.4.1**) in six (6) replicates – unless the compound was known to not bind, in which case it was run in three (3) replicates – with CP-55,940 as the internal control for all replicates (**Figure 5.23**). All compounds in the series bind with good affinity to the CB₂ receptor, except **224**, **225** and **220**.²⁵⁹ As with the CB₁ assay, there is mixed activity within this class of compounds. Compounds **218**, **219**, **221** and **223** (**Table 5.3**) all demonstrated inhibition of cAMP production, with and EC₅₀ of 470.1 nM, 108.6 nM, 389.4 nM and 294.8 nM, respectively (**Figure 5.24**; **Table 5.7**), with a percent cAMP reduction ranging from 11.5% to 45%.

Additional compounds in this series known to bind are **216**, **217**, **222**, **226**, **227**, **228** and **229** (**Table 5.3**). Within these compounds, neither **216**, **217** nor **222** (all with linear aliphatic C-3 substituents) showed any change in cAMP production – in fact, **216** and **217** were virtually identical in their lack of activity; however, **226**, **227**, **228** and **229** all demonstrated a very weak inverse agonist activity (**Figure 5.25**). The EC₅₀ for these compounds was not determined, as they are all in excess of my limit of 5 μM; however there was an approximately 40% increase in cAMP for these compounds at 5 μM.

5.4.4.3. CNG parental functional activity for the pyridine triaryl series. Plates were run as previously described (**Section 5.4.1**) in three (3) replicates with CP-55,940 as the internal control for all three (3) replicates (**Figure 5.26**). As was expected, all compounds in the series failed to elucidate any statistically significant change in the cAMP production of the cells, including the reference standard of CP-55,940.

5.4.4.4. CB₁ PTx-sensitive G_{i/o}-inactivation for the pyridine triaryl series. Plates were run as previously described (**Section 5.4.1**) in three (3) replicates with CP-55,940 as the internal control for all replicates (**Figure 5.27**). As was expected, all compounds failed to alter cAMP production, including the reference standard of CP-55,940. Only compounds **217**, **218**, and **223** were screened in the PTx-sensitive G_{i/o}-inactivation assays due to their ability to elucidate a response in the CB₁ functional assay.

5.4.4.5. CB₂ PTx-sensitive G_{i/o}-inactivation for the pyridine triaryl series. Plates were run as previously described (**Section 5.4.1**) in three (3) replicates with CP-55,940 as the internal control for all replicates (**Figure 5.28**). As was expected, all compounds failed to alter cAMP production, including the reference standard of CP-55,940. Only compounds **217**, **218**, **219**, **222** and **223** were screened in the PTx-sensitive G_{i/o}-inactivation assays due to their ability to elucidate a response in the CB₁ functional assay.

Figure 5.22. CB₁ active compounds with unknown K_i values.

Figure 5.23. CB₂ functional data for pyridine triaryls

Figure 5.24. CB2 functional agonists of the pyridine triaryl series

Figure 5.25. CB₂ functional activity of non-agonists with known binding within the pyridine triaryl series

Figure 5.26. HEK-CNG functional assay with pyridine triaryl series

Figure 5.27. CB₁ PTx-sensitive G_{i/o}-inactivation with pyridine triaryl series

Figure 5.28. CB₂ PTx-sensitive G_{i/o}-inactivation with pyridine triaryl series

5.4.5. Functional activity for pyrimidine triaryl series

5.4.5.1. CB₁ functional activity in the pyrimidine triaryl series. Plates were run as previously described (Section 5.4.1) in six (6) replicates – except the known non-binding **231**, which was run in two (2) replicates – with CP-55,940 as the internal control for all replicates (Figure 5.29). EC₅₀ values for these compounds were inconclusive due to a lack of saturation response; however, the percent increase ranged from 8% to 27%. This increase is over the non-specific increase noted in the HEK-CNG cells but the significance remains to be determined and was not treated as significant.

5.4.5.2. CB₂ functional activity in the pyrimidine triaryl series. Plates were run as previously described (Section 5.4.1) in six (6) replicates – with CP-55,940 as the internal control (Figure 5.30). Only compounds **231** and **233** are known to bind with good affinity to CB₂, all others are unknown and treated as if they bind CB₂.²⁵⁹ Contrary to the CB₁ assay, 3 compounds set themselves apart and demonstrated agonist activity. Compounds **231**, **236**, **238** and **239** showed signs of partial agonist activity with EC₅₀ values of 475.4 nM, 167.9 nM, 5 μM (21%) and 5 μM (29%), respectively, with a percent inhibition of cAMP for all compounds of approximately 30% (Figure 5.31; Table 5.8).

5.4.5.3. CNG parental functional activity in the pyrimidine triaryl series. Plates were run as previously described (Section 5.4.1) in three (3) replicates with CP-55,940 as the internal control for all three (3) replicates (Figure 5.32). As was expected, all compounds in the series failed to elucidate any change in the cAMP production of the cells, including the reference standard of CP-55,940.

5.4.5.4. CB₁ PTx-sensitive G_{i/o}-inactivation in the pyrimidine triaryl series. Plates were run as previously described (Section 5.4.1) in three (3) replicates with CP-55,940 as the internal control for all replicates (Figure 5.33). As was expected, all compounds failed to alter cAMP production, including the reference standard of CP-55,940. All compounds were tested except **231**, due to its known lack of binding affinity and all other compounds appearing to be inverse agonists.

5.4.5.5. CB₂ PTx-sensitive G_{i/o}-inactivation in the pyrimidine triaryl series. Plates were run as previously described (Section 5.4.1) in three (3) replicates with CP-55,940 as the internal control for all replicates (Figure 5.34). As was expected, all compounds failed to alter cAMP production, including the reference standard of CP-55,940. Only compounds **231**, **236** and **239** were screened in the PTx-sensitive G_{i/o}-inactivation assays due to their ability to elucidate a response in the CB₁ functional assay.

5.5. Discussion

5.5.1. Interactions with the CB₁ LBP

5.5.1.1. CB₁ LBP interactions with non-classical ketone moiety. Interactions within the CB₁ LBP for the non-classical cannabinoids differs among the main branches. Most differences center around the presence or absence of interactions with K192(3.28).

Figure 5.29. CB₁ functional activity for pyrimidine triaryl series

Figure 5.30. CB₂ functional activity of pyrimidine triaryl series

Figure 5.31. CB₂ functional activity for select agonists in pyrimidine triaryl series

Table 5.8. EC₅₀ values for pyrimidine triaryl series

Compound	CB₁ EC₅₀ or Concentration (% baseline)	CB₂ EC₅₀ or Concentration (% baseline)	HEK- CNG EC₅₀ (nM)	CB₁ PTx EC₅₀ (nM)	CB₂ PTx EC₅₀ (nM)
230	n/a	n/a	n/a	n/a	-
231	-	475.4 ^a	n/a	-	n/a
232	n/a	5000 (14%) ^b	n/a	n/a	-
233	n/a	n/a	n/a	n/a	-
234	n/a	5000 (23%) ^b	n/a	n/a	-
235	n/a	n/a	n/a	n/a	-
236	n/a	167.9 ^a	n/a	n/a	-
237	n/a	5000 (14%) ^b	n/a	n/a	n/a
238	n/a	5000 (21%) ^a	n/a	n/a	-
239	n/a	5000 (29%) ^a	n/a	n/a	n/a

Notes: All concentrations are listed as nM, percentages indicate the change from baseline. n/a: Not active, based on no statistically significant change in cAMP from baseline. “-” Indicates test not performed. ^a Indicates a decrease in cAMP as compared to baseline. ^b Indicates an increase in cAMP as compared to baseline.

Figure 5.32. HEK-CNG functional activity of the pyrimidine triaryl series

Figure 5.33. CB₁ PTx-sensitive G_{i/o}-inactivation for select pyrimidine triaryl compounds

Figure 5.34. CB₂ PTx-sensitive G_{i/o}-inactivation for select pyrimidine triaryl compounds

For the diarylpyrazoles (prototype, SR-141716A), K192(3.28) plays an important role in H-bonding with the carbamide oxygen (**Figure 5.35A**).^{249,252,257,336} In the case of WIN-55,212-2 there are conflicting data regarding K192(3.28) – it is held that the interaction between K192(3.28) and F189(3.25) help to hold each other in proper orientation to facilitate one, the other or both interacting with the ligand.^{249,254} Mutation of K192A(3.28) will negatively impact the binding of SR-141716A and the classical or endogenous ligands, but has a negligible effect on WIN-55,212-2³³⁷ – further cause to keep this interaction in perspective and consider it only in certain instances with the non-classical agents. Overall, this is not as integral an interaction with the non-classical agents as it was with the classical ligands. Additionally, SR-141716A literature shows interactions with N187(3.23) and S173(2.60) as H-bond interactions on the ketone.²⁴⁹ Mutagenesis S173A(2.60) has little effect on CP-55,940 binding, and thus is hypothesized to have little effect on SR-141716A,²⁵¹ though it has not been tested to date.

Within the HB triaryl series of compounds, functional data show compounds **192** and **193** exert agonist activity at CB₁, with EC₅₀ values of 993.4 nM and 2008 nM, respectively and total percent inhibition of cAMP production at around 50%. While these are not as potent as our reference compound, CP-55,940, they do achieve a similar level of efficacy and are qualified as a full agonist. Also worth noting is the fact that a methoxy group (**194**) or a mono hydroxyl substitution (**191**) present on the A ring results in a compound that has antagonist activity at CB₁. Thus, our first requirement would be for 1,5-dihydroxy on the A-ring of our triaryl series of compounds. The need for this 1,5-dihydroxy A-ring points strongly to the importance of K192(3.28) in H-bonding with these molecules (**Figure 5.35B**). The current homology model²⁴⁹ used for these cursory data show a possible involvement of S383(7.37) (**Figure 5.35B**). A similar trend shows within the SMM series, although these do not have the same impact.

The SMM triaryl series, gives us another valuable insight into the SAR of the triaryl compounds, as well as building upon previous data within the HB triaryl series. The first confirmation of SAR findings within this series is the utility of a 3,5-dihydroxy A-ring, as compounds **196**, **197**, **198**, **199**, **200**, **201**, **202**, **203**, **205**, **206**, **208**, **210** and **212** all contain either a 1,5-dimethoxy or a mono-hydroxy A-ring and lack any effect at CB₁. However, within this series, we see certain situations where a 1,5-dimethoxy is of benefit. One such case is the presence of a 3,5-dichloro C-ring with a heteroaromatic “D”-ring (**205**), as compared to a *m*-toluyl C-ring with a heteroaromatic “D”-ring (**213**). **205** does not elucidate any response of cAMP production at CB₁, whereas **213** demonstrates a weak inverse agonist activity. Di-deprotection of **205** results in **211**, showing activity similar to **213** – the mono-deprotected product, **210**, shows antagonist activity at CB₁. Conversely, di-deprotection of **213** to yield **214** results in no impact of cAMP production at CB₁. From this, I hypothesize that heteroaromatic “D”-rings coupled to electronegative C-rings require deprotection for activity at CB₁, whereas these same “D”-rings coupled to non-electronegative C-rings require di-protected A-ring hydroxyls (**Figure 5.35D**) to alter cAMP production.

5.5.1.2. CB₁ LBP π - π stacking interactions. Far and away, the π - π stacking interactions within the LBP are the most important to consider for the non-classical agents. Considering WIN-55,212-2 and SR-141716A, there exists a great deal of

common amino acids within the LBP of CB₁. Commonalities are: F174(2.61), F189(3.25), F200(3.36), Y275(5.39), W279(5.43), W356(6.48), F379(7.35) and F381(7.35). Of these shared residues, F200(3.36) and W279(5.43) show themselves to be crucial in binding, as the mutation F200A(3.36) causes a precipitous drop in binding affinity for both WIN-55,212-2 and SR-141716A, and W356(6.48) adds itself as an additional imperative interaction for SR-141716A.^{249,252,257} One additional residue, F177(2.64), shows itself as important for binding of WIN-55,212-2 but not SR-141716A. F174(2.61) and F177(2.64) have been demonstrated in classical cannabinoid SAR to be important in maintaining patency of the minor groove of the LBP; however, they have not shown themselves to be involved in the binding of those ligands.²⁵⁵ In the case of WIN-55,212-2, they coordinate with the naphthyl moiety of the molecule as a π - π stack.²⁴⁹ SR-141716A shows no additional unique π - π interactions relative to WIN-55,212-2.

5.5.1.3. CB₁ LBP additional known interactions of non-classical agents.

Remaining interactions for our prototype non-classical agents are only for SR-141716A. Remaining interactions are hydrophilic interactions with S383(7.39) and M384(7.40). These residues are suggested to stabilize the pyrazole ring of SR-141716A within the LBP, and may play an important role in augmenting the binding potential of ligands built off its scaffold.²⁴⁹ Mutagenesis S383A(7.39) has a profound effect on binding of CP-55,940 to CB₁, and so it is hypothesized that this may play a more critical role in binding of SR-141716A but not WIN-55,212-2.²⁵¹

5.5.1.4. CB₁ LBP interactions of the hydrophobic pocket. As a result of the physical nature of the non-classical agents is a more compact, aromatic nature, interactions within the hydrophobic pocket of the LBP are not discussed *per se*; however, some of the key interactions from the π - π stacking and receptor activation/stabilization are located in this region. For purposes of discussion, I will consider the classical agent of HU-210 and its interaction within the LBP. A predominantly lipophilic pocket exists, seemingly as two lobes – as evidenced in the fact that 3-(1',1'-dimethylheptyl)- Δ^8 -THC binds with higher affinity than does 3-heptyl- Δ^8 -THC along with 3D-QSAR models which demonstrate this trait.²⁵³ In one homology model, residues V196(3.32), T197(3.33), F200(3.36), Y275(5.39), W279(5.43), L359(6.51) and M363(6.55) form a hydrophobic pocket which encompasses the A-ring and C-3 side chain of HU-210.²⁵⁴ More recent homology models form this pocket from I354(6.46), C355(6.47), W356(6.48), L359(6.51), L360(3.52) and M363(6.55) (**Figure 5.36**).²⁴⁹ Interestingly, mutations studies conducted on this hydrophobic pocket show that M363A(6.55) mutation greatly impacts binding affinity for HU-210 but has virtually no impact on THC which would suggest that this is an important residue for C-1' substituted derivatives.²⁵⁵

The novel SMM triaryl series offers insight within the C-3 binding domain. One such element found within the SMM triaryl series is the function of a ketone at C-1'. Recalling that **192** and **193** are both 1',1'-*gem*-dimethyl derivatives and that they exert full agonist activity, we compare them to **214** and **211**, respectively. By exchanging the C-1' substituent, we either flip or erase the agonist activity. In the case of **192/214** we move from a full agonist (**192**) to an antagonist (**214**) at CB₁ and with **193/211** the functional activity transitions from a full agonist (**193**) to an inverse agonist (**211**).

Figure 5.36. CB₁ hydrophobic C-3 substituent binding pocket

Note: Panel A depicts HU-210 in the CB₁ LBP, with focus given to the hydrophobic pocket. Panel B depicts Compound 170 in the CB₁ LBP, with focus given to the hydrophobic pocket.

Bearing in mind that agonists at CB₁ cause the negative psychotropic effects of cannabinoids, this simple change – since **214** and **211** are the precursors to **192** and **193**, respectively – results in a very desirable therapeutic implication, as the psychotropic effects of these cannabinoid derivatives could prove to be rendered null in whole-animal studies. However, this is not the only gem gathered from the SMM series data in functional assay.

Related to the presence or absence of an electro-negative C-ring is the fact that a C-3 heptyl substituent with C-1' ketone (**204**) demonstrates inverse agonist activity, while a C-3 cyclohexyl with C-1' ketone (**203**) shows antagonist activity. The implications here are that the linear chain fares better with di-protected hydroxyls, while the cycloalkyl has no cAMP impact regardless of its state of A-ring deprotection (di-protected (**203**), mono-deprotected (**208**) or di-deprotected (**209**)). It is also of note that the C-3 heptyl (**204**) derivative is an inverse agonist only at CB₁. These trends take a slight detour with the heterocyclic derivatives.

The pyridine and pyrimidine triaryl series are a constellation due to their similarities. Looking at the C-3 aliphatic chains, we see that compounds **217**, **218**, **219**, **221** and **223** have agonist activity at CB₁. Comparatively, **222**, **224**, **225**, **226** and **227** have either an aromatic or cyclohexyl C-3 substituent and exhibit an inverse agonist activity. Though the current lack of further study and/or more robust modelling capabilities do not allow for a more precise diagnosis of mechanism, it is noteworthy and warrants further study. Inclusion of a heteroaromatic C-3 substituent seems to yield a non-binding compound with an obvious null effect on cAMP production. None of the pyrimidine compounds exerted any effect on CB₁; however, to call them antagonist would not be prudent, as we do not currently have binding data on these compounds.

5.5.2. Interactions with the CB₂ LBP

Given the varied functional activity of the triaryl compounds at CB₁, and the fact that CB₂ only shares a 48% homology with CB₁, the potential for more targeted exploitation of this receptor exists. Though the SAR of the CB₂ LBP is not as well-characterized as CB₁, there are still data which help to highlight some of the important residues and interactions within the LBP. Unfortunately, without a validated CB₂ model, insights into the triaryl series are not feasible.

5.5.2.1. C-1 Hydroxyl, Benzchromene Oxygen and (optional) C-6 Hydroxyl.

Mutation studies with HU-210, CP-55,940 and WIN-55,212-2 have shown that binding of both HU-210 and CP-55,940 experience negative consequence with a S292A mutation. This mutation-induced change of binding strongly suggests S292(7.46) is involved in H-bonding with the benzchromene oxygen – assuming a similar LBP arrangements relative to the CB₁ LBP.³²³ Though WIN 55-212-2's ketone is in proximity to residue S193(5.42) some data suggest it does not hydrogen bond.²⁶¹ In the case where amide linkages exist between the aryl groups, S193(5.42) does act as a H-bond donor.^{324,325} These interactions may help to provide insight into why di-protected triaryls do not behave as their di-deprotected counterparts.

As was the case in CB₁, so it remains in CB₂, the requirement for 1,5-dihydroxy A-ring, as **191** and **194** failed to cause a change in the cAMP production. Next, it is evident that an *m*-toluyl C-ring (**192**) shows agonist activity at both CB₁ and CB₂, and has an increased potency at CB₂, with an EC₅₀ of 18.7 nM and a 35% reduction in cAMP production. While **192** is only a partial agonist of CB₂ (not as efficacious as CP-55,940), it is in line with CP-55,940's potency.

Considering now the SMM series, again the SAR requirements for CB₁ begin to reemerge as similarly pertinent in CB₂. First, comparing **193** to **211**, we see how a C-1' ketone again allows a change to an inverse agonist from a neutral antagonist. It becomes evident within the SMM triaryl series at CB₂ that the C-1' substituent must be a ketone for this inverse agonist, as **195** is a very effective CB₂ inverse agonist, but when C-1' is a hydroxyl (the direct precursor of **195**), all activity is lost.

5.5.2.2. Hydrophobic and π - π stacking. As has been the case previously, it remains so that many of the interactions derive from the aminoalkylindole, WIN-55,212-2, and play a central role in non-classical binding. Beginning with the indole ring of WIN 55,212-2, there are π - π stacking interactions between it and residues F197(5.46), W258(6.48) and F117(3.36).²⁶¹ There also exists a shared π - π stacking between the indole ring and naphthyl ring with residue W194(5.43).²⁶¹ The naphthyl ring has a proposed added π - π stack with residue F106(3.25).²⁶¹ More recent 2-pyridone derivatives add additional residues to the binding pocket for π - π stacking, namely Y190(5.39) and F281(7.35) along with C288(7.42).^{324,325} For the 2-pyridone analog containing N-butyl, there is a proposed hydrophobic interaction with I198(5.47).³²⁵ Though little data exists, it is suggested that SR-144528 (a CB₂-selective inverse agonist) has interaction with W194(5.43) and F197(5.46),³³⁸ which may play a role in stabilizing the inactive state and impart inverse agonist activity. However, the 2-pyridone shows agonist activity and interaction with W194(5.43) and F197(5.46), so interaction with these residues does not impart inverse agonist activity. Considering the triaryl composition of this series of cannabinergic agents, it is logical to hypothesize that the π - π interactions are important to receptor recognition and function. Future modelling is necessary and will be used to identify potential interactions within the CB₂ LBP for further ligand refinement.

From the SMM series, one may speculate about another CB₂ SAR – the need for an aromatic D-ring to obtain inverse agonist activity (**Figure 5.37**). Compounds **195**, **206**, **207**, **211**, **213** and **215** all contain aromatic D-rings, and when compared to **204** with its linear aliphatic and **203**, **208** and **209** with their cycloalkyl, we see that non-aromatic compounds take on a neutral antagonist role. Interestingly, the effect of deprotection is not as staunch in the CB₂ receptor studies; however, there is a requirement for at least a mono-deprotected A-ring, as evidenced in **202** (di-methoxy), **206** (mono-methoxy) and **207** (di-hydroxy). Compound **206**, a mono-deprotected A-ring, and its successor **207**, a di-hydroxy A-ring, demonstrate the same trend, with the efficacy being similar at high concentrations, but the potency of **207** (EC₅₀ 89.7 nM) greatly exceeds that of **206** (EC₅₀ >5000 nM) (**Figure 5.37**). These data suggest the CB₂ pocket has a varied hydrophilic requirement of the CB₁ pocket and could allow for more selective targeting of compounds. However, the heteroaromatic compound data do not show the same bifurcation of CB₁ and CB₂, at least with the pyridine derivatives.

Figure 5.37. CB₂ required D-ring and A-ring elements for compound 195

At CB₂, the pyridine trend holds, with compounds **217**, **218**, **219**, **221** and **223** having agonist activity, and **222**, **224**, **225**, **226** and **227** having inverse agonist activity (**Figure 5.38**). It is at CB₂ where we begin to see the utility of the pyrimidine series, with **231**, **236** and **239**, all with aliphatic C-3 substituents demonstrating agonist activity. With the exception of **231**, these compounds have saturated aliphatic substituents at both C-3 and C-6 of the A-ring. Comparing **236** to **237** we see the effect of an aromatic substitution at C-6, with a loss of agonist activity. Looking at **231** and **235**, we see a loss of activity. This holds when comparing **236** to **238** where one sees the incorporation of a heteroaromatic ring at C-1' results in a non-active compound (**Figure 5.39**).

5.6. Conclusions

In this chapter, I have demonstrated by utilization of a functional assay how small synthetic changes help to yield more selective or opposing actions on CB₁ and CB₂. The first of which is the ability to convert from an agonist to an inverse agonist by the exchange of the C-1'-*gem*-dimethyl substituent for C-1' ketone – a trend that holds true in both CB₁ and CB₂.

Additionally, I demonstrated how to target CB₂ more effectively by mono-deprotection of the 3 and 5 position of the A-ring, as this is permissible in CB₂ assays but not in CB₁. Lastly, I provided evidence of functional interchange within a given series – incorporation of a heterocyclic A-ring allows for linear aliphatic C-3 substituents to exert agonist activity and cycloalkyl/aromatic substituents to provide an inverse agonist effect. In addition, this heterocyclic A-ring with cycloalkyl/aromatic C-3 substituent provides evidence of a non-competitive inverse agonist.

Future directions for this project are to synthesize matched derivatives within each of the four (4) triaryl series to ensure that we have a more accurate comparison of these SAR findings via functional assay validation. Additionally, further derivation of heterocyclic D-ring and C-ring, along with cycloalkyl C-ring derivatives – matched across the 4 series – will help to solidify this trait. Unsaturation along the C-3 heptyl chain, coupled with chain extension and retraction, would help to probe the depth and orientation of the pocket to optimize their activity. Finally, as we saw in the HB and SMM triaryl series, changing the pyridine and pyrimidine to C-1' ketone could further solidify this as a key interchange for activity in the CB₁ and CB₂ receptor.

Figure 5.38. CB₂ required C-1' elements for pyridine triaryls

Figure 5.39. CB₂ required C-6 and C-1' elements for pyrimidine triaryls

CHAPTER 6. FUNCTIONAL PHARMACOPHORE

As has been demonstrated, minor changes in a compound's structure – be it a bioisosteric replacement, heteroatom substitution or a change in the oxidation state of one atom – have generated functionally distinct and potentially therapeutically significant molecules. One of the first examples presented was the interaction of the KM series (**Figure 6.1**) with the CB₁ and CB₂ receptors, wherein it was shown that these compounds are CB₁ agonists and CB₂ antagonists – a trait not displayed in other compounds with a classical core scaffold. Next, compounds built directly from this series sought to incorporate one (pyridine) or two (pyrimidine) nitrogen atoms in the A-ring of the classical benzchromene core (**Figure 6.1**). From the hexahydro series it was shown that aliphatic C-1' substituents were able to retain the traditional agonist properties of classical core compounds; however, pyridine A-ring with C-1'-phenyl demonstrate CB₁ inverse agonist activity and pyrimidine A-ring with C-1'-phenyl CB₂ inverse agonists. Finally, the triaryl series (**Figure 6.1**) demonstrated another unique and potentially therapeutically useful switch. Within this series, a small change at the C-1', going from a C-1'-gem-dimethyl to a C-1' ketone, results in a switch from a full agonist to an inverse agonist. All of these minor chemical changes result in such staunch, sometimes opposing and potentially therapeutically exploitable switches and options. This leads to the question: What is happening within the respective receptors, and what residues may be involved that could be even further exploited for new therapeutic agents? Herein, I will summarize and highlight interactions, which may help to direct and drive future development. Further, with a validated CB₁ homolgy model, I will propose LBP residues and interactions, which may further highlight the potentially therapeutically exploitable facets of the CB₁ receptor. Unfortunately, I lack access to a validated CB₂ homology model, and thus the same propositions are not possible.

To generate hypotheses and to gain some insight into the possible explanations and interplay between ligand and receptor, docking of the compounds was performed. Dr. Chang of The University of California, Riverdale, kindly supplied the models for the CB₁ receptor.²⁴⁹ Compounds in the KM series and the hexahydro (pyridine and pyrimidine) series were docked and compared in the “HU-210” model. Classical and non-classical inverse agonists were docked and compared in the “SR-141716A” model. Finally, non-classical agonists were docked and compared in the “WIN-55,212-2” model. All tested/docked compounds were minimized and prepped with LigPrep, receptor grid generation and docking was completed utilizing these prepared ligands in GLIDE.

6.1. CB₁-Specific Proposed Functional Switches

Within the CB₁ “HU-210” model, one can see that HU-210 and Δ^9 -THC occupy nearly the same space (**Figure 6.2**). As previously mentioned (**Section 3.5.1**), the KM series may exhibit a binding mode in the CB₁ pocket that very closely mimics that of Δ^9 -THC (**Figure 6.3**). One notable exception is that the pentyl side chain of Δ^9 -THC projects into the hydrophobic pocket more than the aromatic xylyl of **67**. The aromatic xylyl, in this preliminary model, shows a preferential interaction with residue W356(6.48). The

Figure 6.1. Representative examples from each of the novel cannabinergic agents

Figure 6.2. CB₁ spatial relationship between HU-210 and Δ^9 -THC

Note: Panel A shows the front view. Panel B shows the side view. For ease of visualization with the stacked molecules, HU-210 is colored red and Δ^9 -THC is colored blue.

Figure 6.3. CB₁ spatial relationship between Compound 67 and Δ^9 -THC

Note: Panel A shows the front view. Panel B shows the side view. For ease of visualization with the stacked molecules, **67** is colored red and Δ^9 -THC is colored blue.

correlation of Δ^9 -THC to both HU-210 (the basis for this particular model) and **67** allow for my hypothesis about requirements and exploitations of the CB₁ LBP.

6.1.1. Common residue interactions

Literature demonstrates time and again that F189(3.25) is important, if not crucial for binding of the endocannabinoids, such as AEA. In fact, the F189A CB₁ mutant shows a 7-fold drop in affinity for AEA compared to w.t. The current model used for this examination, while rudimentary at best, does show the importance of an aromatic A-ring for interaction with this residue in the LBP (**Figure 6.4**). This interaction, I believe, is what secures and properly orients the molecule in the LBP.

For most compounds, the classical core KM series and hexahydro series included, K192(3.28) plays an integral role in binding with the C1 hydroxy or amide oxygen (**Figure 6.4**). This holds true for most of the triaryl series, as well; however, other factors begin to outweigh the need for K192(3.28) in binding and exerting a functional effect. That said, having a moiety at C1 or an analogous position is one of the first and strongest considerations to be made in the design of targeted molecules.

As previously mentioned (**Section 3.5.1.2**), M363(6.55) has an extremely profound effect on the binding affinity of HU-210 when methionine is exchanged for alanine in the M363A mutant receptor. This precipitous drop in affinity does not hold true for Δ^8 -THC, so it is a logical conclusion that the C1'-*gem*-dimethyl group interacts with this residue. This bears itself out in the non-molecular-dynamic-docking into the "HU-210" homology model, as well, with M363(6.55) showing good contact with the C1'-*gem*-dimethyl of **67**, but not so with Δ^9 -THC (**Figure 6.5**). This residue does seem to provide a viable target to impart increased CB₁ affinity, but is not necessary for function.

For receptor activation to occur, interaction with W279(5.43) is essential, according to literature. The interactions, according to the current model, coupled with the functional data suggest that the interaction may be via π - π stacking or aliphatic interactions. The constant is that a functionally active compound models with some sort of interaction with this residue (**Figure 6.4**).

6.1.2. The W356(6.48) "toggle" switch

As previously discussed (**Section 3.4.2**), the KM series of compounds has an aromatic C-1' substituent, and are agonists at CB₁. Based on the current "HU-210" homology model, the aromatic substituent interact simultaneously with W279(5.43) and W356(6.48). Spatially, W356(6.48) is flanked by W279(5.43) and F200(3.36), and it is its interaction with one or the other which seemingly determines receptor activation (W279(5.43)) or stasis (F200(3.36)). Looking at the interaction of **67**, an established CB₁ agonist, the model suggests a π - π stack which links W279(5.43) to W356(6.48) (**Figure 6.6**) – presumably leading to receptor activation. To the converse of this point, **172** is similar to **67** except for the fact that the A-ring contains a nitrogen at position 2 and the C-1 hydroxyl is now an amide oxygen. This is a relatively small change to the molecule,

Figure 6.4. CB₁ LBP common residues for classical core compound 67

Note: Depicts the common residues found in classical core interactions. Panel A is side view with F189(3.35) removed. Panel B is the top view with K192(3.28) removed. Dashed green lines indicate π - π interactions, dashed yellow lines indicate H-bond.

Figure 6.5. CB₁ M363(6.55) contact with compound 67 and Δ^9 -THC

Note: Dashed green lines indicate “good” contact between receptor and ligand.

Figure 6.6. CB₁ LBP W356(6.48) "toggle" switch presumed to be activated by compound 67

Note: Dashed green lines indicate π - π stacking interactions.

but it has a large impact on its function. Based on the model, this small change in the A-ring alters the binding and preference of the aromatic C-1' substituent to now co-localize and π - π stack with W356(6.48) and F200(3.36) (**Figure 6.7**)—resulting in an inverse agonist. I hypothesize that this shift is due to the amide in the A-ring altering how the hydrophilic interactions take place and the overall conformation in the LBP.

6.1.3. The lack of hydrophilic interaction within the LBP

When examining the triaryl series of compounds, another unique trait began to emerge, which was quite contrary to previously held belief about the rigors and requirement for binding. As previously mentioned, K192(3.28) and S173(2.60) play a key role in H-bond formation with the ligand and subsequent stabilization in the LBP and/or a conformational change induction. That said, the requirement for hydrogen bond donor groups on the A-ring is negated when C-1' is a heteroaromatic group, as in the case of **214**. Too, stabilization of the W356(6.48) “toggle” switch in the inactive state is required for inverse agonist activity. The “HU-210” model does suggest a stabilization of the “toggle” switch, it is conceivable that the interaction could be occurring with the aromatic thiol of **214** is near enough to have potential to interact in a dynamic system (**Figure 6.8**). To fully test this from a functional standpoint, one would need to synthesize a derivative with 1,5-dimethoxy and various C-1' heteroaromatic groups and potentially anoxic A-ring derivatives. Synthesis of these additional derivatives coupled with more robust modelling and molecular dynamics runs would help to further elucidate the root cause of this interesting functional switch.

6.1.4. C-1' importance beyond binding affinity

Previously, it was brought to light that M363(6.55) played a key role in increasing the binding affinity of classical core compounds, substituted with C-1'-*gem*-dimethyls. In the case of the triaryl compounds wherein the C-1' substituent is a ketone, the compound turns from a full agonist to an inverse agonist and in many cases an extremely potent one, at that. Looking at the model, it is evident that there exists a small pocket in the LBP in the vicinity of C-1' which contains a good number of cysteine residues – namely C355(5.47), C382(7.38) and C386(7.42). Further, looking at superimposed, docked ligands **192** and **214** (**Figure 6.9**), which are identical except for their C-1' substituent and functional activity, one may see that the thiophene ring of **192** projects into this pocket while the C-1' ketone of **214** occupies this space. It is my hypothesis that the ketone's interaction with this cysteine pocket contributes to the inverse agonist activity of the compound through induction of a conformational change of the receptor. However, to fully validate, confirm and test this, more detailed binding studies, in conjunction with mutation and/or more detailed modelling and dynamic simulations would be necessary and may represent a potential direction to expand upon this current project.

Figure 6.7. CB₁ LBP W356(6.48) “toggle” switch presumed to be deactivated by compound 172

Note: Dashed green lines indicate π - π stacking interactions.

Figure 6.8. CB₁ interaction of 214 and 192 with the W356 "toggle" switch

6.2. CB₂-Specific Proposed Functional Switches

6.2.1. Common residue interactions

Most of the common interactions currently well documented in CB₂ modelling literature and precedent center around WIN-55,212-2. Residues F117(3.36), W194(5.43), F197(5.46) and W258(6.48) make up the bulk of the common aromatic interactions. Additionally, as was the case with CB₁, a hydrophilic residue – usually S193(5.42), S292(7.46) or T114(3.33) – resides in the LBP and interacts with the ligand, if applicable. Currently, I am unable to utilize a validated homology model for CB₂, unlike CB₁. As a result, the key residues and/or binding sites – be they orthosteric or allosteric – cannot be given with sufficient certainty. However, there are certain moieties which seem to impart specific activity.

6.2.2. The Di-hydroxy A-ring

The first evidence of this requirement comes in the HB triaryl series. Compound **191**, a mono-hydroxy A-ring, showed a very modest 10% increase in cAMP production relative to baseline. Compound **194**, a mono-deprotected A-ring, showed an agonist activity, with an approximately 15% decrease in cAMP, but only at the upper end of the tested concentrations. Compound **193**, the di-deprotected A-ring, analog of **194** demonstrated the same 15% decrease efficacy as **193**; however, the potency of **194** (EC₅₀ 7.4 nM) greatly exceeded that of **194** (EC₅₀ 494 nM) (**Figure 6.10**). Additionally, compound **192**, another di-hydroxy A-ring analog, demonstrated an efficacy and potency nearly directly parallel to **194**. Further foray into the necessity of this moiety being present appears in the SMM series. Compound **206**, a mono-deprotected A-ring, and its successor **207**, a di-hydroxy A-ring, demonstrate the same trend, with the efficacy being similar at high concentrations, but the potency of **207** (EC₅₀ 89.7 nM) greatly exceeds that of **206** (EC₅₀ >5000 nM). Lastly, the majority of the di-protected SMM triaryl series failed to elucidate any change in cAMP production in the CB₂ assay – except for **199** and **213** at 5000 nM. Taken as a whole, the first requirement for optimal activity is the presence of a 1,3-dihydroxy A-ring.

Further, as alluded to during the introduction, one can see that making a modification to the A-ring composition has a relatively profound effect upon the functional activity of the compound. Take for example, **195**, **227** and **233** with a carbocyclic, pyridine and pyrimidine A-ring, respectively – all have a 3,5-dichloro C-ring and a phenyl D-ring; however, **227** and **233** have a C-1'-*gem*-dimethyl and **195** has a C-1'-ketone (**Figure 6.11**). The difference in these compounds is quite profound, with **195** showing great CB₂ inverse agonist activity, **227** showing appreciable activity at the highest tested concentration (5000 nM) and **233** showing virtually no activity. This raises an interesting question: is this change in functional activity due to the heteroaromatic A-ring or the C-1'-*gem*-dimethyls? Further synthesis and expansion of this series is warranted to help gain some more definitive insight into the impact of a heteroaromatic A-ring and its help or hindrance of targeting agents within the cannabinoid system.

Figure 6.10. CB₂ compounds demonstrating the requirement for di-hydroxy A-ring

Figure 6.11. CB₂ C-1' substituents and functional changes

6.2.3. The C-1' substituent effect

As was the case with CB₁, C-1' becomes extremely important when dealing with the functional switches and changes at CB₂. Comparing **192** to **214**, one can see that the only difference between the two is a ketone vs. C-1'-*gem*-dimethyl substitution (**Figure 6.12**). The functional data shows that a change from C-1'-*gem*-dimethyl to ketone results in a compound switching from an agonist to a profoundly effective inverse agonist, respectively. Further, a ketone seems to be necessary, as a C-1'-hydroxy results in no effect when all else is equal and compared to a C-1'-ketone. Though it is not clear if this effect is due to the aforementioned di-protected A-ring or the hydroxyl itself – as the current library of compounds only has concomitant expression of the C-1'-hydroxyl and di-protected A-ring. One question that does arise from this study and data is whether testing of the C-1'-ketone moiety in the known CB₂-binding KM series compounds would result in a similar or more potent/effective inverse agonist compound at CB₂.

6.2.4. The D-ring substituent effect

The last component of the functional SAR garnered from this study is the D-ring effect. From this study, it is evident that incorporation of the previous two elements – 1,5-dihydroxy A-ring and C-1'-ketone – requires, too, the presence of an aromatic D-ring for maximum potency and efficacy. Example of this comes in the form of the cycloalkyl derivative of **209** as compared to its aromatic counterpart of **195** (**Figure 6.13**). Compound **209** exerts an inverse agonist effect (111% increase over baseline) at the maximum concentration tested (5000 nM), as compared to the increase in both potency (744 nM) and efficacy (215% increase over baseline) for compound **195**.

6.3. Summary

Overall, as this data shows that very small changes in the structure of a compound can have significant impact upon its activity or affinity for CB₁ and/or CB₂. To understand this causal relationship with more certainty, a few key advances need to occur:

- A reliably predictable model (or crystal structure) for both the CB₁ and CB₂ receptor needs to be created and validated against a bevy of current literature standard cannabinoid compounds.
- For the compounds presented as products of this lab, matched sets, incorporating as similar as possible D-rings, C-1' substituents, C-rings and A-ring oxidation, across all the series need to be synthesized and tested – in doing so, it will allow more definite trends to emerge.
- A refinement of the functional assay to test for and qualify antagonists – not only from a potency standpoint, but also from a binding site standpoint, as some of these switches may be due to an all-together unique binding site.
- A validation and assimilation of the data generated from this assay to an *in vivo* model of specific disease states.

Figure 6.12. CB₂ C-1' substituent effects - switch from agonist to inverse agonist

Figure 6.13. CB₂ aromatic D-ring requirement

In summation, the field and this project has come leaps and bounds from where it originated not too long ago. It is an exciting time in the field of cannabinoid research, and emergence of better homology or crystal structure models coupled with expansion and refinement of this data will help to carry the field further. Combination of these approaches will help to ensure that cannabinoid research becomes more viable and targeted to usher it into its rightful place in therapy and treatment of various disease states.

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