Optimization and Development of In Vitro Bioassays to Determine Structure-Activity Relationships for Cannabinoid Receptor 1 and Cannabinoid Receptor 2

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Optimization and Development of *In Vitro* Bioassays to Determine Structure-Activity Relationships for Cannabinoid Receptor 1 and Cannabinoid Receptor 2

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Mississippi

Afeef Samir Husni

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ABSTRACT

_Cannabis_ has been around for thousands of years and has been used recreationally, medicinally, and for fiber. Over 400 compounds have been isolated from _Cannabis sativa_ with approximately 100 being cannabinoids. Of those 100 compounds, Δ⁹-THC has been determined as the primary constituent, which is also responsible for the psychoactivity associated with _Cannabis_. Along with Δ⁹-THC, cannabidiol has been studied in depth for its therapeutic effects. This has currently been the focus of many researchers since cannabidiol does not cause psychotropic effects.

Cannabinoid receptors belong to the large superfamily of G protein-coupled receptors (GPCRs). Approximately 30% of marketed drugs target GPCRs, therefore, furthering the importance of targeting the cannabinoid receptors to treat a variety of conditions such as pain, neurodegeneration, appetite, immune function, anxiety, cancer, and many others. Developing _in vitro_ bioassays to determine binding and functional activity of compounds has the ability to lead researchers to develop a safe and effective drug that may target the cannabinoid receptors. The objective is to display the therapeutic effects associated with _Cannabis_ while eliminating the unwanted effects such as psychoactivity and anxiety.

Using radioligand binding and functional bioassays, a structure-activity relationship (SAR) for major and minor cannabinoids was developed. The importance of SAR is to determine specific characteristics of a compound that allow it to bind to a specific receptor. The objective
with cannabinoid receptors is to selectively target CB2 receptors in order to avoid psychoactivity associated with CB1 receptor stimulation. Altering a compound structure based on SARs has the potential to become a lead for a novel therapeutic agent.

These radioligand assays were also used to evaluate the volatile oil of high potency Cannabis sativa. The volatile oil was subjected to bioassay-guided fractionation affording seven different fractions, three of which were active. Of the three, only one fraction did not contain $\Delta^9$-THC. The ability of the volatile oil fraction that lacks $\Delta^9$-THC to activate the cannabinoid receptors may exist due to synergistic relationships between the minor and major components.

The research presented in this dissertation confirms that compounds from Cannabis sativa have the potential for becoming novel therapeutic drugs that specifically target the cannabinoid receptors in order to treat a variety of diseases.
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CHAPTER I
INTRODUCTION

A. Cannabis

Marijuana, also known as Cannabis, is defined as any preparation of the Cannabis plant used to elicit psychoactive effects whether it is recreational or medicinal. According to the 2004 World Drug Report, 3.7% of the population 15-64 years of age consumed marijuana from 2001-2003 (2004). The use of marijuana is associated with numerous pharmacological effects; most, but not all may be attributed to $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, 1) (Figure 1.1).

![Figure 1.1. The chemical structure of $\Delta^9$-tetrahydrocannabinol.](image)

The combination of 1 and other compounds from Cannabis, such as cannabidiol (CBD, 2), may exhibit specific pharmacological effects. Since $\Delta^9$-THC is primarily responsible for the psychoactive effects of Cannabis, scientists have arned how to genetically increase the concentration of 1 within plants to produce a stronger “high.” Since 1980, the concentration of 1 within marijuana has increased from less than 1.5% to approximately 20% (ElSohly 2000).

Cannabis use has been around for thousands of years and is not only associated with recreational or medicinal use, but it is also used for fiber and seeds. Cannabis produces a durable fiber, called hemp, for the manufacturing of rope and fabric. Along with the production of hemp,
the seeds of *Cannabis* are rich in B vitamins, protein, and amino acids. The use of *Cannabis* dates back to around 200 BC when the Chinese invented hemp paper. Today, this plant serves as a recreational drug and more importantly, as a potential therapeutic treatment for numerous diseases such as wasting syndrome, obesity, and multiple sclerosis (ElSohly 2010).

**B. Cannabinoids**

Isoprene (5 carbon chain) is the basic building block of all terpenoids. A terpenoid is defined as two or more isoprene units linked together. These terpenoids can then be linked together through biosynthesis to form longer chains or cyclize to form rings. Cannabinoids are a chemical class of C$_{21}$ terpenophenolic compounds that represent a group of compounds found in *Cannabis sativa*. Isolates from *Cannabis* possessing the C$_{21}$ terpenophenolic system are known as cannabinoids (ElSohly, 2010). The cannabinoids include phytocannabinoids and endocannabinoids. Phytocannabinoids are the naturally occurring cannabinoids found in any *Cannabis* species (Pate, 1999). Endocannabinoids are compounds found endogenously that activate the cannabinoid receptors.

It is known that at least 100 cannabinoids have been derived from *Cannabis sativa* (El-Alfy et al., 2010). The first cannabinoid identified was cannabigerol, and its precursor, cannabigerolic acid, was shown to be the cannabinoid formed in the plant as well as endogenously. Likewise, all phytocannabinoids are derived from their respective carboxylic acids by methods of decarboxylation. The decarboxylation can occur via heat, light, or basic conditions (Yamauchi, 1975). Today, the most discussed phytocannabinoid is $\Delta^9$-THC and cannabidiol are the two most commonly studied naturally occurring cannabinoids.

a. **Classical Cannabinoids**

i. **Major Phytocannabinoids**
The first class of major cannabinoids is cannabigerol (CBG, 3). It is a non-psychoactive constituent found in Cannabis, and is found in higher concentrations in hemp than in the drug type Cannabis plants. It is known that cannabigerol displays antibiotic, antifungal, anti-inflammatory, and analgesic effects. Structurally, the remainder of isolates from this class have propyl side chain analogues and a monomethyl ether derivative (ElSohly 2010).

![Chemical Structures](image)

**Figure 1.2.** The chemical structures of cannabigerol.

The second class of cannabinoids is the cannabichromenes (CBC, 4). Pharmacologically, cannabichromene has been evaluated as a strong anti-inflammatory, an antibacterial, a moderate antifungal, and as a slight analgesic (Turner 1981). Recent literature validates that 4 does elicit effects in the tetrad assay, meaning it causes psychoactivity. However, administration of a CB1 antagonist did not reverse the effects of 4, which suggests the endocannabinoid system is much more complex than currently understood (DeLong, Wolf et al. 2010). There are presently five analogs of 4 with structural differences primarily in the C-5 position (ElSohly 2010).
After 1, cannabidiol (CBD, 2) is the second most discussed cannabinoid. It accounts for approximately 40% of the extracts of fiber type Cannabis (Grlic 1976), and was first isolated in 1940 (Adams 1940). However, the correct structure was not elucidated until 1963 by Mechoulam and Shvo (Mechoulam and Shvo 1963). Cannabidiol is a non-psychoactive component of Cannabis said to be responsible for anti-inflammatory, antioxidant, antipsychotic, analgesic, and neuroprotective effects. Recently, it has been discovered that 2 also inhibits cancer cell growth (Mechoulam, Peters et al. 2007).

Clinical evidence shows that 2 alone has the ability to dramatically reduce anxiety (Bornheim 1995). There is also evidence that 1 and 2 work synergistically to provide positive therapeutic effects. For instance, when 1 is taken alone the results show unpleasant side effects. However, when 1 and 2 are taken together the unpleasant side effects associated with solo administration of 1 are lacking. The reason for this phenomena is that 2 inhibits cytochrome P450 3A11, which metabolizes 1 into its more potent derivative, 11-hydroxy-THC (Mechoulam 2002).

Structurally, the analogs of 2 vary from C1 to C5 side chains for a total of seven isolated phytocannabinoids (ElSohly 2010).
Figure 1.4. The chemical structures of cannabidiol.

Although isolated in 1942, it took Gaoni and Mechoulam until 1964 to elucidate the chemical structure of 1 from the leaves of *Cannabis sativa* (Gaoni and Mechoulam 1964). Δ⁹-tetrahydrocannabinol is pharmacologically and toxicologically the best studied constituent of *Cannabis*, responsible for most of the psychoactive effects of natural *Cannabis* preparations (Grotenhermen 2002). Other than 1 being abused to achieve euphoria, it is now being used medicinally to treat patients with acquired immunodeficiency syndrome (AIDS) suffering from wasting syndrome as an appetite stimulant. It is also being used for pain management and nausea and vomiting associated with patients receiving cancer chemotherapy.

The pharmacological effects associated with 1 are thought to occur through its interaction with the human cannabinoid receptor 1 (CB1). Acting as a partial agonist at CB1, Δ⁹-THC has demonstrated various pharmacological effects such as euphoria, analgesia, appetite stimulation,
reduction of nausea, reduction of intraocular pressure, reduction of neuropathic pain and spasticity, anti-inflammatory, antioxidant, and antiemetic (Maurer, Henn et al. 1990). Along with all of these potential medicinal uses, recent literature shows promise that 1 may also be used for the treatment of Tourette’s syndrome and Alzheimer’s Disease (Ramirez 2005).

The dibenzopyran moiety is the base structure for all 1 derivatives. Furthermore, the location of the double bond at the C-9,10 position is critical for activity at the cannabinoid receptors. Current reports show there are nine different $\Delta^9$-THC type cannabinoids that have been isolated from Cannabis.

![Diagram of chemical structures](image_url)

**Figure 1.5.** The chemical structures of $\Delta^9$-THC.
Similar to 1, Δ⁸-tetrahydrocannabinol (Δ⁸-THC, 5) acts as a partial agonist at CB1 and also produces psychoactive effects. Though the C-8,9 double-bond position in 5 is thermodynamically more stable than the C-9,10 double-bond position in Δ⁹-THC, 5 is approximately 20% less active at the cannabinoid receptors than 1. In turn, this leads to similar pharmacological effects that are associated with 1, but much less potent.

Figure 1.6. The chemical structures of Δ⁸-THC.

Instead of the typical 6-membered (cyclohexane) A ring, the cannabicyclol (CBL, 6) class contains a 5-membered (cyclopentane) ring and C-1 bridge. Cannabicyclol is a non-psychoactive component of Cannabis, and is formed via heat-degradation from cannabichromene (Crombie 1968).

Figure 1.7. The chemical structures of cannabicyclol.

The seventh class of major cannabinoids is the cannabielsoin (CBE, 7) type, derived via
photochemical oxidation of cannabidiol (Kuppers, Lousberg et al. 1973). Cannabielsoin was identified in 1991 using the guinea pig *in vitro* and *in vivo* animal model (Gohda 1990).

Figure 1.8. The chemical structures of cannabielsoin.

Cannabinol (CBN, 8) was the first cannabinoid isolated from *Cannabis sativa* (Wood, Spivey et al. 1896); its structure was then elucidated in 1940 (Adams, Baker et al. 1940). The cannabinol class is very similar to 1 and 5 type classes. However, the difference is the aromatization of the A ring. This class is derived from oxidative metabolites of 1. Cannabinol does show similar pharmacological effects to 1 such as psychoactivity, sedation, antibiotic, anticonvulsant, and anti-inflammatory. Due to cannabinol having slightly weaker agonist activity for the cannabinoid receptors than 1, the pharmacological effects are rather faint.
Cannabitriol (CBT, 9) type is a class characterized by an additional hydroxyl (OH) substitution resulting in a total of three hydroxyl functional groups. Cannabitriol exists naturally in the form of both isomers and the racemate (ElSohly 1977).

Figure 1.9. The chemical structures of cannabinol.
Figure 1.10. The chemical structures of cannabitriol.

ii. Minor / Miscellaneous Phytocannabinoids

Figure 1.11. Miscellaneous phytocannabinoids.

iii. Classical Synthetic Cannabinoids
There have been many cannabinoid derivatives synthesized from the base dibenzopyran structure of compound 1. With this said, the focus in this section is to discuss some of the most potent dibenzopyran derivatives. Some of these derivatives may not equally bind to both cannabinoid receptors, but selectively bind to CB1 or CB2. This selectivity may be the benchmark for many new therapeutic drugs, which in turn lack the unwanted side effects associated with Cannabis use.

The use of marijuana is illegal in most portions of the world whether it is medicinal or recreational. The compounds within marijuana have been intensely studied over the past few years, and concluded that 1 is the main psychoactive component. Along with negative effects, such as psychoactivity, there are many beneficial effects stemming from 1 activating the endocannabinoid system. Thus, synthetic production of 1 and other analogues are currently used as treatments for wasting syndrome in AIDS patients and for chemotherapy-induced nausea and vomiting.

Two Food and Drug Administration (FDA) compounds have been approved to aid with negative side effects of cancer chemotherapy and AIDS, Marinol® (Dronabinol) and Cesamet® (Nabilone). Dronabinol is a molecularly identical version of 1, but it is produced by total synthesis rather than isolated from Cannabis. Nabilone has the dibenzopyran base structure of 1, but has minor modifications at C-3 and C-9. Both of these drugs are used for anorexia and weight loss associated with AIDS and chemotherapy-induced nausea and vomiting (Beal 1995) (Ware 2008). Although not marketed for these uses, Nabilone is also beneficial for chronic pain management associated with fibromyalgia and multiple sclerosis (Skrabek 2008). Even with all the beneficial effects currently associated with these drugs, psychoactivity still prevails.
Figure 1.12. Chemical structures of Dronabinol (left) and Nabilone (right).

Efforts have focused on the synthesis and modifications that mimic the therapeutic actions of 1 and the other beneficial phytocannabinoids without the unwanted side effects. At Hebrew University, Mechoulam et al. have synthesized numerous tetrahydrocannabinol analogues; one in particular is HU-210 (Mechoulam, Lander et al. 1990). This compound consists of the base structure of Δ⁸-THC with a hydroxy substitution at C-11 and a 1,1-dimethylheptyl chain at C-3. HU-210 shows potency of 100-800 times stronger than that of 1 (Devane, Breuer et al. 1992).

Figure 1.13. Chemical structure of HU-210.

A recent research literature report shows that mammals, including humans, are able to generate new neurons in the hippocampus. This discovery has changed the way scientists view psychiatric disorders and drug addiction (Mandyam 2004). A study performed in 2005 suggests that CB1 receptor activation by cannabinoids may promote hippocampal neurogenesis. The goal of the study was to determine if HU-210 is to able to promote neuronal generation, which leads to the anxiolytic and antidepressant effects associated with the cannabinoids. HU-210 was
administered chronically to rats over a one month time period. After one month, the rats displayed an increased number of newborn neurons in the hippocampal dentate gyrus. Also, the rats showed significantly reduced anxiety- and depression-like behaviors. Therefore, chronic administration of HU-210, which fully activates both cannabinoid receptors, does indeed promote neuronal growth (Jiang 2005).

Dexanabinol (HU-211) is the enantiomer of the previously discussed potent cannabinoid agonist HU-210 (Pop 2000). Unlike HU-210, it does not activate the cannabinoid receptors and therefore does not show psychoactivity (Mechoulam, Feigenbaum et al. 1988). However, it does function as an N-methyl-D-aspartate (NMDA) antagonist. The NMDA receptor is a glutamate receptor, which involves brain functions such as cognition, memory, movement, and sensation (Laube 1997).

![Figure 1.14. Chemical structure of HU-211, or Dexanabinol.](image)

After evaluating HU-211 for cannabinoid-like effects, proof that it acts as an NMDA receptor antagonist was needed. A study done in 1989 confirmed that HU-211 blocked tremorogenic, convulsive, and lethal effects of NMDA (Feigenbaum 1989). With these observations, more thorough pharmacological assays were performed. These tests led to the discovery that HU-211 also blocks tumor necrosis factor (TNFα). TNFα is involved in septic shock, multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis, and cachexia. To determine its therapeutic potential, HU-211 was administered in vivo to rats with closed head
injury, or brain damage. Treatment with HU-211 showed that TNFα levels were reduced by over 80%. Thus, HU-211 is a novel drug candidate for neuroprotectant diseases (Shohami 2000).

Unlike the previously discussed synthetic cannabinoids, the compound JWH-051 shows selectivity for the human CB2 receptor. The structural difference from HU-210 is the removal of the phenolic hydroxyl group. With activity in the low nanomolar range at CB2, 14nM, JWH-051 showed promising effects for the cannabinoid receptors and the cytokine network (Huffman 1996). Though it still retains slight activity for CB1, it is a very potent anti-inflammatory agent.

The scientific literature provides evidence that when mice infected with *Legionella pneumophila* were administered JWH-051, the production of interleukin-4 (IL-4) increased within the spleen. When this cytokine is stimulated, IL-4 causes a cascade of events to occur and in turn increases production of T helper cells. Therefore, this process plays a critical role in chronic inflammation and wound repair via stimulation of only CB2 receptors (Klein 1998).

![Figure 1.15. Chemical structure of JWH-051.](image)

The link between the cannabinoid system, cannabinoids, and cancerous tumor growth is unclear in *in vivo* animal models. What is known, however, is that administration of cannabinoids inhibits tumor growth. Tumor growth progresses due to the generation of a new vascular supply, or angiogenesis. Using a synthetic, potent, selective CB2 agonist, JWH-133, two mechanisms for inhibition of tumor growth *in vivo* were proposed in 2003.
To begin the study, malignant tumors were induced in immune-deficient mice. Next, CB2 selective agonist JWH-133 was administered for eight days at a dose of 50 µg/day. The results indicate that the tumors of the cannabinoid-treated mice were smaller than those of the control group. To help defend one of the proposed mechanisms of action, differences in vascular functionality were assessed. Upon further evaluation, the vascular network of the JWH-133-treated tumors were small, differentiated, and impermeable, as opposed to normal tumor growth that has a large, plastic, and leaky vascular network.

The next test was to determine whether JWH-133 inhibited tumors from secreting proangiogenic cytokines, which in turn leads to formation of a large vascular network. As a result, JWH-133 reduced expression of vascular endothelial growth factor. Vascular endothelial growth factor is a major contributor to tumor growth by mediating the prevention of vessel maturation. Thus, the second proposed mechanism provides proof that JWH-133 does suppress proangiogenic factors within the tumor. Overall, this research displays that a CB2 selective agonist such as JWH-133 has potential to become a therapeutic treatment for cancer (Blazquez 2003).

b. Non-classical Cannabinoids

The term non-classical cannabinoid refers to any compounds that stimulate the cannabinoid receptors that are not structurally related to $\Delta^9$-THC. The origin of non-classical
cannabinoids can be from natural products, endogenous, or created synthetically. There are some compounds closely related to the classical cannabinoids, such as CP-55940 and HU-308, and there are those that have structures completely different than the classical cannabinoids, such as WIN55212-2 and SR141716. Some of the most common classifications of non-classical cannabinoids are the aminoalkylindoles, diarylpyrazoles, eicosanoids, and flavonoids.

i. Non-classical Synthetic Cannabinoids

Researchers at Pfizer have synthesized new analogues of \( \mathbf{1} \) that lack the dihydropyran ring (Melvin 1984). Out of the numerous compounds synthesized, one in particular still remains as the most commonly used: CP-55940 (10). CP-55940 is a bicyclic analogue of 1, and is much less lipophilic than 1. Since 10 has binding affinity and functional activity much stronger than that of 1, it has been developed as a radiolabelled ligand. The development of \(^3\text{H}\)-CP-55940 was fundamental in the discovery and characterization of the human CB1 receptor (Devane 1988).

CP-55940 has the ability to bind to CB1 and CB2 with Ki values of 0.5 nM and 0.7 nM, respectively. Furthermore, it showed pharmacological effects \textit{in vivo} in the tetrad mouse model to range from 10 to 50 times stronger than that of 1 (Johnson 1986). CP-55940 is used in receptor assays as a control since its maximal effects exceed other cannabinoid agonists; it displays similar affinities at CB1 and CB2 and has been deemed a full agonist for both receptor subtypes (Pertwee 1999).
Although not marketed, there is a newly synthesized CB2 receptor selective ligand, HU-308. It has a selectivity of 5000x for CB2 over CB1. The success in finding a selective ligand for the CB2 receptor is the ability to devoid psychoactivity and drug abuse via stimulation of the CB1 receptor. A recent study evaluates the potency and pharmacology of HU-308 in an *in vivo* model of liver ischemia reperfusion and in human liver sinusoidal endothelial cells (HLSECs). HU-308 was injected into the femoral vein right before the reocclusion in a 10mg/kg dose. To determine the amount of tissue damage to the liver, serum transaminase alanine aminotransferase and lactate dehydrogenase activities were measured. As hypothesized, pretreatment with HU-308 significantly reduced the transaminase ALT and LDH levels, proving to be successful (Rajesh 2007). Furthermore, HU-308 has shown to promote proliferation of neuronal stem cells and inhibit TNFα (Rajesh 2007).
Most discussions about the cannabinoid receptors provide evidence that ligands at these receptors have the potential to play a critical role in drug therapeutics, whether acting as agonists or antagonists. The existence of cannabinoid receptor antagonists on the market became available in 2006 in the United Kingdom (UK) when Rimonabant (SR141716, 11) was trademarked as the first selective CB1 inverse agonist. Rimonabant is a member of the diarylpyrazole class and was primarily used to reduce appetite for the treatment of obesity, and for those who were overweight that had a high risk of type 2 diabetes (Fong and Heymsfield 2009).

Even though 11 showed major success in weight loss treatment, it was removed from the UK market. In 2009, the European Medicines Agency gave reason of removal due to high rates of deleterious side effects associated with psychiatric disorders, such as depression and suicidal thought; these adverse events outweighed the benefits of the drug. Although the relationship of CB1 receptors and psychiatric effects is not fully understood, it is known that the interaction of 11 and the CB1 receptors in the brain led to these negative side effects (Katoch-Rouse 2003).

![Chemical structures of selective CB1 inverse agonists. Rimonabant (left) and AM-251 (right).](image)

**Figure 1.19.** Chemical structures of selective CB1 inverse agonists. Rimonabant (left) and AM-251 (right).

Another member of the diarylpyrazole class is AM-251, which also acts as a selective CB1 inverse agonist. It is structurally very similar to Rimonabant, in which only a chlorine atom is replaced with an iodine atom. This substitution causes AM-251 to have almost a two-fold
increase in binding potency for the CB1 receptor, 7.5nM for AM-251 and 11.5nM for Rimonabant (Katoch-Rouse 2003).

The structure similarity to 1 for developing cannabinoids was not the case when WIN-55212-2 was developed. It is a member of the aminoalkylindole class, yet it acts as a cannabinoid receptor agonist and produces pharmacological effects similar to those of 1 (Wiley 1998). The scientific literature shows that it acts as an analgesic in rat models for neuropathic pain (Herzberg 1997). Furthermore, WIN-55212-2’s ability to inhibit adenylate cyclase also allows it to reduce intraocular pressure (Pacheco 1991).

![Chemical structure of WIN-55212-2](image)

**Figure 1.20.** Chemical structure of WIN-55212-2.

**ii. Non-classical Eicosanoids**

The eicosanoids are a class of endogenous, twenty-carbon essential fatty acids that stimulate the cannabinoid receptors. In 1992, anandamide was the first described endogenous cannabinoid neurotransmitter (Devane 1992). To date, there are five endogenous cannabinoid receptor agonists. Of the five discovered, anandamide and 2-arachidonoyl glycerol are the two most evaluated endocannabinoids in the literature.
Figure 1.21. Chemical structure of anandamide.

c. Noncannabinoid Constituents

The unique scent of Cannabis results from a group of compounds within the from Cannabis known as terpenoids (Turner 1980). There have been approximately 140 terpenoids isolated from Cannabis, and the two major monoterpenes identified are myrcene (67%) and limonene (17%). Other noncannabinoid constituents include hydrocarbons, nitrogen-containing compounds, carbohydrates, fatty acids, phenols, simple alcohols, aldehydes, ketones, acids, esters, lactones, vitamin K, and flavonoids (ElSohly 2010).

Terpenoids and flavonoids have distinct therapeutic effects that include, but are not limited to increasing cerebral blood flow, enhancing cortical activity, and eliciting anti-inflammatory activity (Di Carlo 1999). There are currently twenty-three flavonoids that have been identified in Cannabis, with cannflavins A and B (Figure 1.31) being unique to Cannabis (Barrett 1986). Humans consume flavonoids each day from fruits and vegetables in their daily diet. A review article published in 1999 states that flavonoids may be responsible for inhibition of specific enzymes, antiulcer, antispasmodic, anti-secretory, anti-inflammatory, anti-cancer, analgesic, anti-angina, anti-diabetic, anti-allergic, and vascular protection. Once flavonoids are absorbed, they influence many biological functions making them a target for a variety of diseases (Di Carlo 1999).
Figure 1.22. Chemical structures of isolated flavonoids from *Cannabis sativa*. Cannflavin A (left) and cannflavin B (right).

The use of flavonoids as anti-inflammatory agents has a long standing history (Harborne 1967), principally by inhibiting production of prostaglandins and leukotrienes (Landolfi 1984). Not only do flavonoids show anti-inflammatory activity, but also show anti-ulcerogenic activity. The mechanism in by which flavonoids exert anti-ulcerogenic properties is currently unknown. Therefore, flavonoids can be therapeutically beneficial in many ways, but their mechanism of action for some of the effects still need to be characterized (Villar 1984).

C. G Protein-Coupled Receptors

Cannabinoid receptors are G protein-coupled receptors, which are a large family of seven membered transmembrane domains that act in a second messenger fashion (Galiegue 1995). These receptors couple primarily to the G\(_i\)/G\(_o\) subtypes of G protein. When cannabionid receptors are activated, they inhibit the enzyme adenylate cyclase. Adenylate cyclase is responsible for catalyzing the conversion of adenosine triphosphate (ATP) to form cyclic AMP (cAMP). When a ligand binds to the extracellular surface of cannabinoid receptors, it causes a conformational change of the receptor. This change activates the second messenger by exchanging guanosine diphosphate (GDP) for guanosine triphosphate (GTP). Then, the G-protein’s \(\alpha\) subunit separates from the \(\beta\gamma\) subunit to cause intracellular proteins to function properly. In CB1 and CB2 receptors, cAMP acts as the second messenger. When these receptors
are activated, cAMP levels decrease within the cell. Therefore, the result of activating cannabinoid receptors leads to a decrease in cAMP levels, and in turn leads to an inhibition of function (Hwangopo 2005).

**Figure 1.23.** Example of a G Protein-Coupled Receptor.

There are currently two reported subtypes of human cannabinoid receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) (Gerard 1990). CB1 and CB2 share approximately 44% similarity in their protein sequences (Munro, Thomas et al. 1993). However, if only nucleotide sequences are considered, they share approximately 68% similarity (Galiegue 1995). Recently, there has been evidence that additional cannabinoid receptors, such as GPR18, GPR55, and GPR119, may exist (Begg 2005). Of these three potential cannabinoid receptors, GPR55 has the most similarity to CB1 and CB2 at the binding site (Ryberg 2007). The reason these receptors are believed to be cannabinoid receptors is due to their activation by known endogenous cannabinoids.

Reports show that GPR18 is the abnormal cannabidiol receptor. This research suggests
that N-arachidonyl glycine activates GPR18 and initiates microglial migration in the brain (Gantz 1997). GPR119 is expressed primarily in the gastrointestinal tract and pancreas in humans. In rat models, activation of GPR119 was shown to reduce food intake and body weight. Therefore, drugs that target this receptor have the potential to treat obesity and diabetes (Izzo 2010).

GPR55 was identified and cloned in 1999. It is expressed in the brain, small intestine, and bone (Sawzdargo 1999). Originally, GPR55 was considered the third cannabinoid receptor because *in silico* research showed similarities in the amino acid binding site when compared to CB1 and CB2 (Baker, Pryce et al. 2006). Furthermore, recent research shows that GPR55 is activated by several endocannabinoids, $\Delta^9$-THC, $\Delta^8$-THC, and CP-55940 in the low nanomolar range. The activation of the receptor by these ligands provides more evidence that GPR55 could be considered the third cannabinoid receptor (Ryberg 2007).

D. **Endocannabinoids**

Endogenous cannabinoids, or endocannabinoids, are substances produced in the body that are capable of binding to and functionally activating the cannabinoid receptors (Di Marzo 1995). Generally, neurotransmitters are stored in intracellular compartments and released presynaptically to activate the receptors on a postsynaptic cell. However, unlike most neurotransmitters, the endocannabinoids work in a reverse fashion. Endocannabinoids use retrograde signaling to achieve cannabinoid receptor activation. This means that the ligands are being produced postsynaptically, but acting presynaptically (Hanuš 2009). Another critical point in understanding the function of the endocannabinoids is that the endocannabinoid system can produce endocannabinoids “on demand” in response to an increase in intracellular calcium levels (Di Marzo 1998).
Shortly after the cloning of the cannabinoid receptors, researchers began searching for endogenous ligands that activated these receptors. As previously stated, the first endocannabinoid discovered was anandamide in 1992 (Devane 1992). Several years after the discovery of anandamide, the second endogenous ligand, 2-arachidonoyl-glycerol (2-AG), was discovered (Mechoulam, Ben-Shabat et al. 1995). Anandamide and 2-AG act as a partial agonist and full agonist, respectively, at the CB1 and CB2 receptors. Three other endogenous ligands have also been discovered: 2-arachidonyl-glyceryl ether (noladin, 2-AGE), O-arachidonyl-ethanolamine (virhodamine, OAE), and N-arachidonyl-dopamine (NADA) (Bisogno 2005). Although the structure of anandamide differs significantly from 1, both of these ligands have similar pharmacological profiles (Grotenhemen 2002).

![Chemical structure of 2-AG](image)

**Figure 1.24.** Chemical structure of 2-AG.

Although the physiological roles of the endocannabinoids are not fully defined, several pharmacological functions have been described. Studies suggest that these endogenous ligands may aid in pain relief, enhancement of appetite, blood pressure lowering during shock, embryonic development, and blocking of working memory (ElSohly 2010).

**E. Cannabinoid Receptor 1**

The CB1 receptor is encoded by the CNR1 gene, and is widely expressed throughout the brain. It is also expressed in the spinal cord, pituitary gland, thyroid gland, adrenal gland, fat cells, muscle cells, liver cells, digestive tract, lungs, kidneys, and male and female reproductive organs. Gerrard et al. cloned the rat cannabinoid receptor (Gerard 1991) and shortly after, isolation of a human CB1 receptor cDNA was reported (Matsuda 1990). The amino acid
sequence contained 472 total amino acids, one less than other mammalian species (Matsuda 1990). This receptor has been the target of much research due to the pharmacological effects associated with its activation.

As with all G protein-coupled receptors, CB1 possesses seven transmembrane domains connected by three extracellular loops and three intracellular loops, an extracellular N-terminal tail, and an intracellular C-terminal tail (Elphick 2001). Activation of the CB1 receptor can be endogenously or through administration of exogenous cannabinoids. Since CB1 receptors are primarily located in the brain, Δ⁹-THC is reported to bind to this receptor and is responsible for the psychoactivity associated with the cannabinoid receptors (Dewey 1986).

Since CB1 receptors are not present in the medulla oblongata, part of the brain stem responsible for respiratory and cardiovascular functions, there is not a risk of overdose resulting in respiratory depression or cardiovascular failure that may be seen with abuse of other drugs, such as the opioids (Holland, Schwope et al. 2011). Among many other therapeutic uses, ligands acting as agonists at CB1 have the potential to aid with wasting syndrome associated with AIDS, nausea and vomiting associated with cancer chemotherapy, and as an analgesic for neuropathic pain associated with multiple sclerosis. Conversely, inverse agonists, have the potential to help with obesity and type 2 diabetes.

F. **Cannabinoid Receptor 2**

Shortly after characterizing and cloning the human CB1 receptor, the CB2 receptor was cloned. The CB2 receptor is encoded by the CNR2 gene (Munro, Thomas et al. 1993). The amino acid sequence contains approximately 360 total amino acids. The CB1 and CB2 receptors have approximately 44% similarity of their amino acid sequences (Cabral 2009). Using computer modeling, ligand-induced receptor selectivity between CB1 and CB2 appears to occur at CB2
receptor residues S3.31 and F5.46. It is thought that the lipophilic portion of the ligand binds to the F5.46 residue and causes a conformational change, allowing the ligand to form a hydrogen bond with the S3.31 residue, and in turn cause the pharmacological effects associated with CB2 interaction (Tuccinardi 2006).

These receptors are widely expressed throughout the peripheral tissues of the immune system, spleen, tonsils, thymus, and gastrointestinal system (Galiegue 1995). Further investigation of CB2 receptors led to the discovery that these receptors are also expressed within the brain (Onaivi 2006). However, unlike CB1 receptors that are found on neurons, the CB2 receptors are found on microglia (Cabral 2008). The CB2 receptors play a major role in inflammatory diseases due to their interaction with these receptors in the immune system. Furthermore, since the discovery of CB2 receptors in the brain, researchers believe that interaction with these receptors cause the antinociceptive effects associated with cannabinoids (Ibrahim 2006). This could be the milestone for developing novel therapeutic drugs used for pain that do not have addictive properties.

Along with anti-inflammation and antinociception, current research shows that CB2 receptors may have a role in the treatment of Alzheimer’s Disease (Benito 2003). Alzheimer’s disease forms plaques within the brain that disrupts neuronal function. These plaques are formed from aggregation of beta-amyloid proteins (Tiraboschi 2004). A CB2 selective agonist, JWH-015, has shown to induce macrophages to remove beta-amyloid proteins (Tolon 2009). The range of diseases associated with cannabinoid receptors is vast and the use of selective agonists/antagonists holds strong potential for use with these diseases.

G. Medicinal Uses

According to the United Nations, *Cannabis* “is the most widely used illicit substance in
the world” (2010). There are people who use Cannabis medicinally, and there are others who abuse Cannabis in order get “high,” or obtain a state of euphoria. Those who use marijuana regularly for medicinal purposes use strict, smaller amounts to control the strength and duration of the psychoactivity. However, those who abuse marijuana attempt to smoke or ingest as much as necessary to achieve their own personal state of euphoria.

This abuse negatively affects the people who need Cannabis to help with unwanted side effects associated with cancer chemotherapy and AIDS. Cannabis is not only used to help those suffering from cancer chemotherapy and AIDS, but it also lowers intraocular pressure for those with glaucoma, acts as a pain reliever, and more recently has been found to help with symptoms of multiple sclerosis and depression. Therefore, researchers are attempting to formulate synthetic cannabinoids that resembles the compounds isolated from Cannabis, but do not express psychotropic properties.

a. Appetite

 Patients suffering from AIDS, cancer chemotherapy, and obesity are now becoming the main target for the therapeutic use of Cannabis, or synthetic cannabinoids. The literature provides evidence that the use of marijuana does increase appetite via agonism at CB1, which can increase energy in daily life routines. Contrary to some of the therapeutic effects via agonists at CB1, an inverse agonist has the potential to treat obesity, which in turn can treat type 2 diabetes. Whether it is a natural or synthetic preparation, the use of cannabinoids has a major effect on appetite (Berry and Mechoulam 2002).

i. Stimulant – Wasting Syndrome

 Elderly patients whose kidneys fail to retain sodium (Harrigan 2001) and patients with AIDS tend to lose their desire to eat regularly throughout the day. When this occurs, the patient
becomes weak, agitated, tired, and anorexic; this occurrence is known as Wasting Syndrome. Research shows that at least 90% of patients who smoke marijuana had an immediate desire to eat (Haines 1970). With the use of Cannabis as a therapeutic drug to stimulate appetite, the suffering patients may be able to eat on a regular basis throughout the day, thus improving their quality of life.

In a study conducted by Mattes and colleagues, the appetite stimulating effects of cannabinoids, specifically Δ⁹-THC, were examined. A major focus in this study, for a means of clarification from previous research, was the route of administration of 1. The four different ways in which 1 was administered includes oral, inhaled, sublingual, and suppository. There are high levels of variability in determining if 1 does indeed stimulate appetite. Factors such as environment, age, gender, tolerance, dosage, and social influences play a role in the effect of 1 on appetite. During one study, the suppository route of administration resulted in the highest energy intake when compared to oral, sublingual, and inhaled administration of 1.

![Figure 1.25](image.png)

**Figure 1.25.** Mean data from patients dosed orally and via suppository over a 72 hour time period (Mattes, Engelman et al. 1994). License Number: 2850411374927

There is no single outcome on the effect of Δ⁹-THC on appetite stimulation no matter the form of administration. The results vary from having no effect to the possibility of having major food cravings. In some circumstances, not only did the food cravings become increased, but
during a meal the food seemed to also have an increased taste of delightfulness. The conclusion of this study indicates that 1 as an appetite stimulant produces its highest effects on healthy, adult individuals who use low dosage amounts (Mattes, Engelman et al. 1994).

ii. Suppressant – Obesity

Obesity is a medical condition in which excess body fat accumulates in a person and increases the chances of developing other, more serious conditions such as heart disease and type 2 diabetes (Jarvis 2006). Currently, there is only one drug approved by the FDA for the treatment of obesity, Xenical. This drug helps weight loss by inhibiting pancreatic lipase (1999). In Europe, Rimonabant was once used as a treatment for obesity. It stayed on the market for three years and was later removed due to psychological side effects. Rimonabant acts an inverse agonist at the CB1 receptor (Fong and Heymsfield 2009). Contrary to the appetite stimulating effects of $\Delta^9$-THC, Rimonabant displays appetite-suppressing effects.

New drugs targeting the CB1 receptors for the treatment of obesity are currently being investigated. The objective of these potential therapeutic compounds is to stimulate CB1 receptors acting as inverse agonists, but to not cross the blood-brain-barrier and act via the CNS. Thus, compounds should interact with CB1 receptors in peripheral organs and avoid the interaction with CB1 receptors in the CNS. This means that peripheral CB1 receptors may be the key to unlocking the treatment of obesity, type 2 diabetes, and dyslipidemia without having the psychological side effects seen with Rimonabant (Nogueiras 2008).
Figure 1.26. Proposed mechanism of action of CB1 receptor inverse agonists for the treatment of obesity.

b. Nausea and Vomiting

Cancer chemotherapy is ultimately defined as the treatment of cancer using an antineoplastic drug. The objective of chemotherapy is to kill the rapidly dividing cancerous cells; however, rapidly dividing healthy cells are also harmed (Skeel 2003). Along with many others, it has been reported that nausea and vomiting are the two most feared side effects of cancer chemotherapy. This fear of nausea and vomiting can be so overwhelming that a patient decides to not partake in chemotherapy (Coates, Abraham et al. 1983). Though many patients use these synthetic drugs, a handful of doctors and patients still choose Cannabis as a preferred treatment.

Contrary to popular belief, cannabinoids are not easily prescribed, but only used when patients are unresponsive to other antiemetic agents. Beside the use of the natural Cannabis plant, the synthetic cannabinoids most commonly used are Dronabinol, Nabilone, and Nabiximols (Sativex). Dronabinol is a synthetic replica of 1, Nabilone is a synthetic cannabinoid with minor structure changes of 1, and Nabiximols is a combination of 1 and 2 formulated into a buccal spray. All of these treatments interact with both CB1 and CB2 receptors. The interaction of these compounds with CB1 causes the psychoactive effects associated with Cannabis use (Pertwee 2005). The ultimate goal for cannabinoids and antiemetic drug therapy associated with
chemotherapy-induced nausea and vomiting is to develop compounds that display the beneficial pharmacological effects without the unwanted side effects, such as psychoactivity.

c. Depression

Depression may be described as a mood disorder that interferes with every day life, and is associated with feeling down, sad, angry, or lost. The most commonly associated drug categories for the treatment of depression include monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), selective-serotonin reuptake inhibitors (SSRIs), and serotonin-norepinephrine reuptake inhibitors (SNRIs). A new field of research involving Cannabis may include a potential link to the treatment of depression. However, studies show conflicting data as to whether Cannabis is beneficial (Grinspoon 1998) or detrimental for the treatment of depression (Bovasso 2001). Due to the conflicting results of these studies, Witkin switched the focus to the role of the endocannabinoid system and the treatment of depression from exogenously administered cannabinoids (Witkin 2005). Since 2005, it has been concluded that the endocannabinoid system does play a role in the treatment of depression, but differs from minor depression to major depression.

New research has found that a common characteristic of Cannabis, mood elevation, may be the link to the treatment of depression. A study published by El-Alfy and co-investigators in 2010 describes the antidepressant effects associated with administration of phytocannabinoids. The objective of this study was to isolate the major cannabinoids from Cannabis and evaluate the antidepressant effects using the mouse forced swim test (FST), followed by the tail suspension test (TST). Typically in mice, when cannabinoids are administered they exert hypothermia and catalepsy, which means that a psychoactive state is being achieved. For these depression studies, only low dosages of these phytocannabinoids were administered so that the test subjects did not
demonstrate psychoactivity.

To ensure that hypothermia and catalepsy were not achieved, the tetrad assay was completed after administration of each cannabinoid. Out of the six cannabinoids tested, only Δ⁹-THC and Δ⁸-THC showed a U-shaped dose response in the forced swim test. With this, only Δ⁹-THC showed significant antidepressant-like effects. Administration of the non-psychoactive components revealed that cannabidiol and cannabichromene displayed antidepressant-like effects in the forced swim test. However, a high dose of cannabidiol was used to display these antidepressant-like effects.

**Figure 1.27.** Effects of each phytocannabinoid on immobility time in the mouse forced swim test (El-Alfy 2010). License Number: 2850411159444

To further confirm these tests, Δ⁹-THC and cannabichromene were evaluated in the tail suspension test. Between these two phytocannabinoids, only 1 continued to exhibit these antidepressant-like effects at low doses. Therefore, the results of this study show that 1 and other phytocannabinoids administered exogenously do indeed aid with the treatment of depression (El-Alfy 2010).
Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory disease in which myelin sheaths surrounding the axons in the CNS are impaired. In turn, this leads to demyelination. There is currently little treatment for the pharmacological effects associated with MS, such as spasticity, tremors, and pain (Compston and Coles 2008). However, studies have shown promising results evaluating cannabinoids for the treatment of MS. For instance, results from experiments with animal models of MS have shown a major reduction in tremors and spasticity. These positive effects result from agonists binding to CB1 and CB2 receptors. Evidence indicates that in animals with encephalomyelitis, CB1 and CB2 agonists decrease spasticity, and that CB1 inverse agonists increase spasticity (Baker 2000).

A questionnaire was distributed to patients who were using Cannabis to self medicate themselves. The result of this questionnaire showed that over 90% of patients experiencing symptoms showed improvement after Cannabis use. Although there is evidence that Cannabis and/or cannabinoids are effective against side effects associated with MS, further research needs to be done to determine the exact mechanism of action by which these cannabinoids are working (Pertwee 2002).

e. Analgesic
The use of opioid analgesics has been around for many years. Although they work very well for pain management, they display dangerous side effects such as addiction, tolerance development, and respiratory depression leading to death. The role of cannabinoid receptors as a therapeutic treatment for neuropathic pain has significantly increased in recent years. Since the discovery of CB2 receptors in the CNS, compounds have been synthesized to selectively target these receptors (Onaivi 2006). The strong therapeutic potential for cannabinoids as antinociceptive medication arises from the development of CB2 selective compounds in order to avoid the psychoactivity associated with the CB1 receptor (Munro, Thomas et al. 1993).

Compounds such as HU-308 have been developed and evaluated as CB2 selective agonists. HU-308 exhibits anti-inflammatory and antinociceptive properties through activation of the CB2 receptors. As a potential therapeutic candidate, it was necessary to determine if HU-308 showed CNS activity in the tetrad assay. As a result, lack of CB1 activation did indeed result in the absence of CNS psychoactivity. To ensure that CB2 selective agonists are only activating CB2, administration of CB1 and CB2 antagonists are administered to determine if the effects are reversed. Thus, CB2 selective agonists have the potential to be a non-psychoactive and non-addictive treatment for pain management (Hanus 1999).

For the past several years, Cannabis has been the most commonly used drug in the United States. The use of Cannabis comes with both benefits and risks to human health. The negative side effects associated with Cannabis outweigh the beneficial effects, making it an illegal drug, without prescription, in the United States. A few of the negative effects associated with Cannabis are euphoria, heart rate changes, and impairment of cognitive function. Some of the main beneficial effects are factors that aid with pain, appetite, and inflammation associated with numerous diseases. Since there have been no reports associated with death from Cannabis
ingestion, the pursuit of *Cannabis* and cannabinoids as therapeutics may be the future for the treatment of many diseases and disorders (ElSohly 2010).
CHAPTER II
ENDOCANNABINOID SYSTEM

The discovery of the endogenous cannabinoid system, or endocannabinoid system, came shortly after the discovery of the cannabinoid receptors. The endocannabinoid system refers to the cannabinoid receptors, endogenous cannabinoids, and the proteins for their synthesis and inactivation. Endogenous cannabinoids, or endocannabinoids, are compounds produced within the body that are capable of functionally activating the cannabinoid receptors (Di Marzo 1995). To date, there have been five endocannabinoids discovered (Figure 2.1). Although the mechanism of action for all of them is not clear, they appear to have a role in appetite, pain, mood, and memory.
Figure 2.1. Chemical structures of the endogenous cannabinoids.

Most neurotransmitters are stored in vesicles until needed. Once activated, they are released presynaptically to activate the receptors on a postsynaptic cell. The endocannabinoids, however, work oppositely. They use retrograde signaling in order to achieve receptor activation. This means they are produced postsynaptically, but are acting presynaptically (Hanuš 2009). Endocannabinoids are not stored in vesicles, but are produced “on demand” in response to an increase in intracellular calcium levels (Di Marzo 1998).

N-arachidonoyl-ethanolamine (AEA) was the first endogenous ligand discovered in 1992. It was later named anandamide from the Sanskrit word *ananda*, meaning “internal bliss,” and the presence of the amide functional group (Devane 1992). It belongs to the family of N-acyl-ethanolamines (NAEs), and like all endocannabinoids, is derived from arachidonic acid (Bisogno 2000). Anandamide acts as a partial CB1 agonist and a weak CB2 agonist (Pertwee 1997). In
1995, 2-arachidonoylglycerol (2-AG) was described as the second endocannabinoid discovered (Mechoulam, Ben-Shabat et al. 1995). It acts as a full agonist at CB1 and CB2 receptors (Sugiura 1995). A few years later, 2-arachidonyl-glyceryl ether (noladin, 2-AGE) (Hanus 2001), O-arachidonoyl-ethanolamine (virodhamine, OEA) (Porter 2002), and N-arachidonoyl-dopamine (NADA) (Bisogno 2000) were discovered, and all displayed functional activity for the cannabinoid receptors. Noladin and NADA are selective CB1 agonists, while virodhamine is a CB1 antagonist and partial CB2 agonist (Bisogno 2005).

A. **Biosynthesis of AEA**

Previously reported studies indicate that AEA is biosynthesized via a phospholipid-dependent pathway consisting of the enzymatic hydrolysis of N-acyl-phosphatidylethanolamines (NAPEs) (Schmid 1983). The enzyme that catalyzes this reaction is phospholipase D selective for NAPEs. These phospholipid precursors are produced from the transfer of the acyl group from the \( sn-1 \) position of phospholipids to the \( N \)-position of phosphatidylethanolamine. This enzymatic transfer reaction is catalyzed by a \( Ca^{2+} \) dependent \( trans \)-acylase. Although other mechanisms have been reported, evidence points to this mechanism most likely responsible for AEA biosynthesis (Figure 2.2) (Ueda 2001).
Figure 2.2. Biosynthesis of AEA. NaPE: N-arachidonoylphosphatidylethanolamine, PLD: phospholipase D.

B. Biosynthesis of 2-AG

The biosynthesis of 2-AG results from the hydrolysis of diacylglycerols (DAGs) containing arachidonate in the 2 position (Figure 2.3) (Bisogno 1997). DAGs can be produced from hydrolysis of phospholipase C, phosphoinositides, or phosphatidic acid. The conversion of
DAGs to 2-AG is catalyzed by a DAG lipase selective for the \(sn\)-1 position (Bisogno 1999). Currently, there are two DAG lipases that have been cloned, DAGL\(\alpha\) and DAGL\(\beta\), and are said to be responsible for the formation of 2-AG (Bisogno 2003). Functionally, there is a number of suggestions portraying 2-AG’s function as a mediator of neuronal growth (Bisogno 2003) (Williams 2003) (Chevaleyre 2003).

![Pathways for the biosynthesis of endocannabinoid, 2-AG.](image)

**Figure 2.3.** Pathways for the biosynthesis of endocannabinoid, 2-AG.

In regard to noladin, virodhamine, and NADA, very little is known about their physiological roles.

**C. Inactivation of Endocannabinoids**

Similar to other endogenous ligands, it is critical to have a rapid and selective mechanism of inactivation. Endocannabinoids are highly lipophilic compounds, which allows for them to
diffuse easily through the cell membrane. However, removal of endocannabinoids through the plasma membrane requires methods such as facilitated transport. There are several proposed mechanisms for how anandamide is taken up by cells, but the most common mechanism is known as the anandamide membrane transporter (AMT) (Di Marzo 1994).

The AMT has not been isolated or cloned, but reports indicate that it may also be responsible for the uptake of all five endocannabinoids (Huang 2002). The process of endogenous inactivation is sensitive to factors such as saturation, temperature, and synthetic inhibitors (Beltramo 1997). Contrary to the proposed mechanism of the AMT, recent reports suggest that fatty acid amide hydrolase (FAAH) is the enzyme responsible for hydrolysis of anandamide to its inactive state. The scientific literature shows that FAAH does influence anandamide uptake, but other mechanisms of action are also necessary to enhance the rate at which endocannabinoids are metabolized (Cravatt 2003).

In 2004, a study was conducted using a synthetic compound, UCM707, which inhibits AEA uptake inactive against FAAH. The author’s results suggest that both CB1 receptors and FAAH play a role in the cellular uptake of AEA. There was also evidence suggesting that an additional protein inhibited by UCM707 also participated in AEA uptake (Ortega-Gutierrez 2004).

![Chemical structure of UCM707](Image)

**Figure 2.4.** Chemical structure of a synthetic inhibitor of FAAH.

Anandamide and 2-AG each have their own pathways of degradation once inside the cell.
However, one similar mechanism of inactivation is through hydrolysis catalyzed by FAAH. For AEA and 2-AG, degradation via FAAH results in arachidonic acid and ethanolamine or glycerol, respectively (Cravatt 1996). The first crystal structure of FAAH was published in 2002 (Bracey 2002), followed by its ability to breakdown anandamide in 2003 (Deutsch 1993). FAAH’s primary function is to catalyze the hydrolysis of long chain fatty acid amides and glycerol esters (Patricelli 2000).

One study reported that in FAAH knockout mice, levels of N-acylethanolamines were elevated in various tissues. Due to the lack of hydrolysis by FAAH, anandamide levels were significantly increased. Furthermore, reports indicated that the mice displayed extreme sensitivity to exogenous administration of cannabinoid agonists. Therefore, the ability of FAAH to regulate pain has pioneered an interest for novel antinociceptive therapeutics (Cravatt 2001).

The scientific literature indicates that FAAH may not be the only enzyme responsible for the hydrolysis of 2-AG; monoacylglycerol lipases (MAGLs) may also inactivate this endogenous cannabinoid (Ben-Shabat 1998). Currently, a MAGL that is inactive on AEA has been cloned from mouse, rat, and human. Interestingly, in rats, this MAGL has been found in the brain and is located in areas of CB1 receptor abundance. Immunohistochemical studies in the hippocampus confirmed the role of MAGL in the degradation of 2-AG as a retrograde messenger due to the presynaptic localization of the enzyme (Dinh 2002).
Esterification and oxidation are also two mechanisms in which endocannabinoids may be inactivated. For example, 2-AG can directly become inactivated, i.e. MAGLs, or it can be re-esterified into phospholipids before hydrolysis. This re-esterification is possible due to phosphorylation or acylation of its hydroxyl groups (Sugiura 2002). For all the endocannabinoids, oxidative mechanisms may also cause inactivation. The presence of the arachidonate moiety allows for high possibilities of oxidation catalyzed by lipoxygenases, cyclooxygenases, and cytochrome P450 oxidases (Kozak 2002).

D. The Role of Endocannabinoids in Potential Therapeutic Drugs

The objective in targeting the endocannabinoid system for new therapeutic drugs is to enhance endogenous half-life, or preventing their inactivation. One study shows that targeting
the endocannabinoids, specifically anandamide, may be responsible for modulation of emotional states in rats. Similar to the effects of marketed benzodiazepines for anxiety, synthetic inhibitors of FAAH have also displayed promising results in reducing anxiety. To prove these compounds were acting via cannabinoid receptors, the CB1 inverse agonist Rimonabant prevented inactivation of FAAH. Along with anxiolytic properties, administration of the FAAH inhibitors also displayed antinociceptive properties. This reveals a key role of anandamide in the regulation of emotional states and potential for novel anti-anxiety therapeutics (Kathuria 2003).

There are numerous effects observed in animal models with the therapeutic modulation of the endocannabinoid biosynthesis and degradation. Endocannabinoid inhibitors can alleviate spasticity associated with multiple sclerosis (Baker 2000), allergic encephalomyelitis (Mascolo 2002), intestinal hyperactivity caused by cholera toxin (Izzo, Capasso et al. 2003), movement associated with Parkinson’s disease and hyperactivity in glutamatergic neurons (Gubellini 2002). Furthermore, it is known that the endocannabinoid system is also involved in the pathway that leads to drug and alcohol addiction (Tanda 1997). Further clarification of the physiological role of anandamide and 2-AG, along with virdhamine, noladin, and NADA, may answer the mystery that remains with the endocannabinoid system and their use as potential therapeutic drugs.
CHAPTER III

METHODS

A. Rationale for the Development of the Bioassays

Most of the current drugs on the market that target G protein-coupled receptors were not initially designed to target these specific proteins. Functional activity was the sole determinant as to whether potential drugs of interest were pursued. It was not until years later that researchers discovered the functional activity seen correlates to a specific receptor. Currently, approximately 27% of all drugs approved by the FDA target G protein-coupled receptors (Overington 2006). Therefore, natural products that have known beneficiary pharmacological effects via the endocannabinoid system, which activate G protein-coupled receptors, should be extensively pursued in an attempt to discover novel ligands that activate their receptors. Activation of the cannabinoid receptors provides positive effects, but may also display several unwanted side effects. These negative side effects could be overcome by the development of selective compounds for each specific receptor.

The ability of ligands to activate the cannabinoid receptors and exhibit many beneficial therapeutic effects attracts further scientific research. Reports of Cannabis use for certain ailments dates back for thousands of years for numerous illnesses and diseases. In these several thousand years, there has yet to be a death attributed to solely Cannabis use (Stott 2004). Therefore, the pursuit of cannabinoid receptors as novel drug targets is of major interest in pharmaceutical industries. These receptors can mediate signal transductions associated with pain,
cardiovascular activity, cancer, gastrointestinal activity, obesity, AIDS, appetite, depression, anxiety, neurodegenerative disorders, immune function, and drug addiction (Husni 2012). The combination of excellent characteristics of Cannabis and the rise of cannabinoid receptor-based disorders warrants further investigation to help understand the unknown mechanisms of action responsible for binding and stimulation. The use of radiolabeled receptor binding and functional bioassays may lead to the discovery of novel therapeutics.

**B. In Vitro Bioassays**

**a. Cell Culture**

Human Embryonic Kidney (HEK) 293 cells (ATCC: CRL-1573) are a specific cell line commonly used in biological labs due to their ease of growth and transfection. Parental HEK293 cells were stably transfected via electroporation with full-length human recombinant cDNA for cannabinoid receptor subtypes 1 and 2. The human recombinant cDNA was obtained from Origene. During electroporation, the cells open by getting “shocked,” which allows them to accept the cDNA. Once transfected, the cells are maintained at 37°C and 5% CO$_2$ in a Dulbecco’s Modified Eagle’s medium (DMEM) nutrient mixture F-12 HAM supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 0.5% penicillin-streptomycin, and G418 (Geneticin, 600 mg/mL). The use of G418 as an antibiotic allows for only those cells that have received the cDNA to survive during the growth and maturation process. This is due to the genetic resistance of this antibiotic being incorporated into the cDNA, which includes the gene for CB1 or CB2. Those cells that survive will attach to the bottom of the cell culture plate and begin replicating.

A single cell is picked from the parental plate and forced to replicate on its own in a fresh plate with the appropriate media. The use of a single cell guarantees the overexpression of the
receptors on the cell membrane for each receptor subtype, CB1 and CB2. After the single cell multiplies, it is termed a single colony. This colony was at least 90% confluent before it is passaged into larger preparations for mass cell culture. Membranes are prepared by scraping the cells in a 50 mM Tris-HCl buffer, homogenized via sonication, and centrifuged for 40 minutes at 13,650 rpm at 4°C. These cells are stored at -80°C until used for the binding and functional assays. For clarification, the development of individual CB1 and CB2 cell lines was achieved. Protein concentration for each membrane preparation was found using the Bradford protein assay protocol reported in 1976 (Bradford 1976).

Figure 3.1. Single cell (left) replicated into a single colony (right).

To ensure the correct cDNA sequence and protein was transfected within each cell line, polymerase chain reaction (PCR) and Western blot assays are performed. PCR has the ability to amplify DNA across many orders of magnitude, which allows for generation of DNA sequencing (Saiki 1988), and the Western blot assay uses gel-electrophoresis to detect the specific proteins within a cell culture preparation (Towbin 1979).

b. **Radioligand Competitive Binding Assay**

Synthetic, endogenous, or natural cannabinoids may be tested for their binding affinity toward each of the cannabinoid receptors. Any of these compounds tested are hypothesized to
interact with the endogenous cannabinoid system, in turn causing biological effects. Two types of assays were conducted to confirm these interactions: competitive binding assay and GTPγS functional assay. A competitive binding assay is done to determine the binding affinity of a compound to each receptor. The competition is between the chosen cannabinoid and a labeled ligand, such as $^3$H- CP-55940. It is known that the tritiated-labeled ligand will tightly bind to each of the cannabinoid receptors; therefore, if a test compound shows affinity for the receptors, the amount of labeled ligand bound to the receptor will be low resulting in high binding affinity of the test compound. A compound showing strong binding affinity for either of the cannabinoid receptors, warrants further testing to determine the functional activity.

Though several methods of ligand-receptor radioligand binding for cannabinoids have been published (Pertwee 1997) (Xiong, Cheng et al. 2011) (Thomas 1998), standardization within each laboratory is required for meaningful comparisons to be made between research groups. The experimental conditions for these assays are critical as minimal changes may lead to major variations in binding and functional values. When evaluating ligands at the cannabinoid receptors, factors such as lipophilicity, solubility, and purity should be carefully considered due to the sensitivity of the assays. Since cannabinoids are generally non-polar compounds, they have a tendency to stick to glass and plastic surfaces used in ligand-receptor assays. If the cannabinoids stick to the equipment used, this could lead to inaccurate results of ligand binding and function (Console-Bram Linda 2012).

Silanization is a common technique used in laboratories to avoid cannabinoids sticking to glass and plastic. In addition to silanization, another technique is the use of Bovine Serum Albumin (BSA). BSA has been used in assays as a carrying agent and stabilizer for compounds with high lipophilicity. BSA is added to buffer recipes to create a reagent that can be absorbed
and utilized by cells (Kenna 1985). Buffer recipes for cannabinoid receptor assays have been optimized to include 50 mM Tris (23 mM Tris base and 27 mM Tris HCl to assist with pH), 154 mM NaCl, 20 mM disodium ethylenediaminetetraacetic acid (EDTA), and 0.2% BSA. The buffer pH is then adjusted to 7.4 using sodium hydroxide or hydrochloric acid. The use of this buffer and other optimized techniques, described below, allow for remarkable specific values to be obtained.

To assess binding of compounds to the cannabinoid receptors, competition binding was performed following modifications to previously published methods (Pertwee 1997). Cannabinoid binding took place under the following conditions. The binding assays were performed with 100 µL of 0.5 nM $^3$H-CP-55940, 10 µL of 10 µM test compound (unless dose-response then first well is 100 µM followed by appropriate dilutions), and 100 µL of 10 µg protein of membrane for a total assay volume of 210 µL. Binding was initiated by the addition of 10 µg protein of CB1 or CB2 cell membranes. Assays were carried out at 37°C for 90 minutes before termination via rapid vacuum filtration through Whatman GF/C glass-fiber filters, presoaked with 0.3% BSA, using a Perkin Elmer 96-well Unifilter Harvester (Perkin Elmer Life Sciences Inc., Boston, MA, U.S.A.). Each assay plate was washed seven times with ice-cold wash buffer (same as assay buffer). Filters were allowed to dry overnight at room temperature (25°C) and then radioactive counts were extracted from the filters using a scintillation cocktail before quantification using a Perkin Elmer TopCount (Perking Elmer Life Sciences Inc., Boston, Mass. U.S.A.). The cannabinoids that produced at least 50% displacement of the radioligand from specific binding sites were calculated using GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.) to obtain $K_i$ and IC$_{50}$ values.

The objective while performing in vitro cell based binding assays is to achieve an
exceptional specific binding value. For the cannabinoid assays, total binding was defined as binding in the presence of 0.1% dimethylsulfoxide (DMSO). Nonspecific binding was the binding observed in the presence of 0.1 µM CP-55940. Specific binding was defined as the difference between total and nonspecific binding. The percent binding for each assay was found with the following formula:

\[
100 - \left( \frac{\text{binding of compound} - \text{nonspecific binding}}{\text{specific binding}} \right) \times 100
\]

The development and optimization of the cannabinoid receptor competitive binding assay led to consistent specific binding values of greater than 90%.

c. **GTPγS Functional Assay**

A functional assay determines whether the test compound is acting as an agonist, antagonist, or inverse agonist. As opposed to the binding assay, an *in vitro* functional assay is not based upon competitive binding, but rather a competitive saturable process that traces the amounts of guanosine triphosphate (GTP). When the cannabinoid membranes are not stimulated, there is a pool of guanosine diphosphate (GDP) associated with the receptors. Upon stimulation, there is dissociation of the G-protein from the receptor and bound GDP is exchanged for GTP. To monitor this response, \(^{35}\text{S}\) labeled GTP is added to the assay. Therefore, an increase in GTP is directly proportional to stimulation of the receptor by labeled ligand. An agonist compound is indicated by an increase in GTP (Kenakin 2002).

There are several terms used when discussing the functional activity of a compound. Each compound that produces an effect may act a partial agonist, full agonist, inverse agonist, or neutral antagonist. Agonists that produce a reduced level of response are termed partial agonists. Though partial agonists may occupy all of the receptors, its ability to induce G protein-receptor coupling is reduced, resulting in submaximal activity. For instance, \(\Delta^9\)-THC binds tightly to the
CB1 and CB2 receptors; however, it has a functional $K_i$ of approximately 300 nM, which means it is acting as a partial agonist, yet is still responsible for the psychoactive effects associated with Cannabis. A full agonist is defined as having the ability to bind to the receptor, and display maximal efficacy at that receptor (Brunton 2008).

![GTPγS Functional Assay](image)

**Figure 3.2.** Example of a partial agonist and full agonist in the GTPγS functional assay.

While an agonist is said to increase endogenous agonist response, an antagonist results in the blocking of endogenous agonist response and causes lack of intrinsic activity. Antagonism is sometimes referred to as competitive antagonism because the compounds causing this effect bind to the same receptor site in which the endogenous agonists bind. To detect an antagonist in the functional assay, the compound must be tested in the presence of a known agonist at that specific receptor. Due to the competition of the agonist and antagonist for the same receptor site, the antagonist blocks the ability of the agonist to fully stimulate the receptor, thus resulting in a lesser value for the agonist (U'Prichard 1977).

Another type of antagonist, also known as an inverse agonist, has the ability to reverse the biological effects associated with agonists at the cannabinoid receptors. This action occurs because affinity for the receptors is increased after uncoupling from G proteins. This type of activity was first observed in 1989 by Costa and Herz, which they described as a compound that
has “negative intrinsic activity.” It is known that of all the G protein-coupled receptor antagonists, approximately 85% are considered inverse agonists (Costa 1989). As seen with Rimonabant, compounds displaying inverse agonist activity may have promising therapeutic potential.

Figure 3.3. Example of an inverse agonist.

The correlation between in vitro and in vivo bioassays begins with evaluation of functional data in vitro to predict pharmacological effects that may be seen in vivo. Functional activity was measured in CB1 and CB2 receptors using modifications to previously published methods (Xiong, Cheng et al. 2011). The use of cell lines is preferred for these assays over tissue homogenates because the signal-to-noise ratio is improved by overexpressing the receptors. The binding affinities were not used as a guide for this assay because dissociation between affinity and efficacy is not unusual for G protein-coupled receptors (Carlsson 2010). For putative agonists and inverse agonists, full concentration curves were constructed. In the case of putative antagonists, activity was confirmed by testing increasing concentrations of the compound together with a constant concentration of a known agonist, CP-55940. The antagonist blocks the ability of the agonist to fully stimulate the receptor, thus resulting in a right shift of the agonist EC₅₀.
GTPγS functional assays were performed under slight modifications to previously published methods (Xiong, Cheng et al. 2011). The assay buffer for the GTPγS functional assay consisted of 50 mM Tris-HCl, 0.2 mM ethylene glycol tetraacetic acid (EGTA), 9 mM MgCl2, 150 mM NaCl, and 1.4g BSA. Binding took place under the following conditions: 50 µL compounds diluted to the desired concentrations in the dose response curve were mixed with 20 µg CB1 or CB2 membrane, 50 µM GDP, 0.5 nM 35S-labelled GTP, and 300 µL assay buffer for a total volume of 500 µL per well. Plates were incubated for 120 minutes at 37°C. The reaction was terminated via rapid vacuum filtration through Whatman GF/B filters using a Perkin Elmer 96-well Unifilter Harvester (Perkin Elmer Life Sciences Inc., Boston, MA, U.S.A.). Each assay plate was washed four times with ice-cold wash buffer (10mM Tris-HCl, pH = 7.4). Filter plates were allowed to dry overnight at room temperature (25°C) and then radioactive counts were extracted from the filters using a scintillation cocktail before quantification using a Perkin Elmer TopCount (Perking Elmer Life Sciences Inc., Boston, Mass. U.S.A).

Basal binding was defined as binding in the presence of assay buffer. Nonspecific binding was the binding observed in the presence of 40 µM unlabeled GTPγS salt. Emax binding was defined as binding in the presence of 1 µM CP-55940. Percent stimulation was measured by the following:

$\text{(Binding of compound-nonspecific binding) / Emax) \times 100}$

$K_i$ and EC50 values were calculated using Graph Pad Prism (GraphPad Software, San Diego, CA, U.S.A.). Cannabinoids that show promising activity in the functional assay, whether acting as an agonist or antagonist, may be tested in vivo to determine pharmacological effects.

C. Extraction, Isolation, Structure Determination

The intensive process of using natural products as lead compounds in drug discovery
requires bioassay-guided fractionation, isolation, and structure elucidation followed by a large-scale repetition for the isolated compounds with potential use. Over the past two decades, natural product research has been at a slow decline despite large amounts of success. Approximately 63% of small-molecule new chemical entities introduced between 1981 and 2006 were natural products, semi-synthetic natural product analogues, or synthetic compounds based on natural product pharmacophore (Newman 2007). This decline in natural product chemistry is due to pharmaceutical industries using combinatorial chemistry and high-throughput screening (Paterson 2005).

It was believed that pharmaceutical industries were going to shut down the use of natural product research with combinatorial chemistry and high-throughput screening; however, this is not the case. The industries found their methods not as successful as they hypothesized. Over one million compounds were synthesized for combinatorial libraries, but very few showed potential therapeutic activity. This was a mistake of the synthetic chemists who developed libraries based on accessibility and size instead of biologically relevant properties. It is critical for a library of compounds to contain chemical diversity, but it must also have “biological friendliness” and “drug-likeness” properties (Martin 2001). Even so, the few compounds that do result in hits have high potential for undesirable side effects (Paterson 2005). Thus, “a small collection of smart compounds may be more valuable than a much larger hodgepodge collection mindlessly assembled” (Borman 2002).

Natural product compounds have several common characteristics that differs their activity from synthetic compounds. Although several natural products have simple structures, most are more complex and contain more stereogenic centers than synthetic compounds. Also, natural products contain more oxygen, hydrogen, carbon, and less nitrogen atoms than synthetic
medicinal compounds. Furthermore, it is common for natural products to not follow Lipinski’s rules. In fact, Lipinski’s fifth rule states that the first four rules do not apply to natural products. Natural product chemistry is rising again due to improvements in the methods of extraction, isolation, and structure elucidation (Clardy 2004).

The concept of using natural products as a means for medicine is thriving in today’s research institutions. There are many methods that provide a way to extract, isolate, and determine the structure of novel compounds from plant material. Years of research have led to optimal methods in which the active compounds from Cannabis sativa have been extracted and purified. Once these compounds have been isolated, biological evaluation is critical for the success of novel medicines. However, since these methods are time consuming and strenuous, it is very difficult to obtain a sufficient amount of cannabinoids, especially minor cannabinoids in order to evaluate pharmacological effects (Galal 2009).

a. Extraction

To begin the extraction of compounds from Cannabis, the plant material is grown via proper cultivation in order to obtain a large amount of plant material. The Cannabis sativa plants were grown from high potency Mexican seeds in the marijuana plant garden at the University of Mississippi. Upon flowering, the male plants were removed from the field to avoid cross-pollinations and only female plants were kept for further cultivation. Cuttings were made from selected female plants for further cultivation under a controlled environment. This environment consisted of a combination of 1000 Watt full spectrum metal highlight and sodium bulbs, 25°C temperature, 55% humidity, and a hot air suction fan about 3 to 4 feet between plants and bulbs to avoid heating from high intensity discharge bulbs.

Once mature, Cannabis buds and leaves are picked and allowed an allotted time period to
dry. This may take several days since air-drying is the only process in which compounds will not decompose. Some cannabinoids are heat sensitive, therefore, using heat to dry the compounds may degrade many of the active compounds within *Cannabis* into non-active fractions, or a metabolite of the parent compound. When the buds and leaves have dried, using an organic solvent is the first step in the extraction process. In this case, the buds and leaves and are soaked in 100% hexanes overnight. Next, simple filtration using cotton is done in order to achieve only solvent containing cannabinoids. Furthermore, using a rotational evaporator under reduced pressure allows for obtainment of the extract. Using the extract, the next step is to fractionate using a vast array of solvent ratios. For example, eight different fractions are collected: 100% hexanes, 25% hexanes : 75% EtOAc, 50% hexanes : 50% EtOAc, 75% hexanes : 25% EtOAc, 25% EtOAc : 75% MeOH, 50% EtOAc : 50% MeOH, 75% EtOAc : 25% MeOH, and 100% MeOH. These fractions are collected using vacuum liquid chromatography (VLC) over a one hour time period. Using this method of VLC allows for rapid and efficient separations, which results in saving time and money. In the non-polar solvent, 100% hexanes fraction, compounds such as waxes, sterols, and oils will be isolated. The diluted fractions containing a mixture of hexanes/EtOAc or EtOAc/MeOH will contain almost all of the major and minor cannabinoids from *Cannabis sativa*. Isolation from the polar solvent fraction, 100% MeOH, will afford compounds such as polyhexanes, salts, and sugars.

b. Isolation

Each fraction of the *Cannabis* extract may contain several compounds. In order to individually separate these compounds, numerous isolation techniques can be used. Three main column chromatography methods are used for the initial isolations: silica gel column chromatography, C18 column chromatography, and Sephadex LH-20 column chromatography.
Using silica or C18 affords pure compounds from a mixture of compounds based on differences in the polarity of functional groups; Sephadex LH-20 is a liquid chromatography medium used to separate compounds based on molecular weight. Variations of these column techniques are also used, such as an open column, flash chromatography, high-performance liquid chromatography, and solid phase extraction.

An open column simply uses gravity to pull the liquid fraction through the column and into separate fractions. Flash chromatography is a very rapid form of column chromatography. It uses a smaller sized column along with an applied gaseous pressure from the top of the column allowing for rapid separation of compounds from a fraction. High-performance liquid chromatography (HPLC) is one of the most common methods of sample separation used. Today, HPLC is used for analysis of drugs in urine samples and measuring vitamin D levels in blood. This method of separation uses a pump that pushes samples through a column at very high pressure, and has a detector that identifies samples based on retention times. When dealing with smaller fractions, solid phase extraction is a commonly used technique.

c. **Structure Determination**

The final step in cannabinoid identification is to determine the structure of the isolated compound. Similar to isolation, there are many methods that can be used to determine the structure of a compound (s). The most commonly used method is gas chromatography (GC) with different detection methods, such as flame ionization detector (FID) and mass spectrometry (MS). GC/MS is used frequently due to its ability to use small sample sizes and have good limits of detection. Alone, GC is a poor qualitative tool, but has good separating ability; MS is good for confirmation of peak identity, but difficult when using complex mixtures. Together, they have become the most used technique for the identification of cannabinoids due to ease and accuracy.
The University of Mississippi has built a full library of cannabinoid data and is stored within each of the structure determining systems. With this said, GC/MS helps identify structures based on molecular weight and fragmentation. Also within the library, retention times are known for the cannabinoids that have been previously isolated and identified. Along with these methods, other methods such as infrared spectrometry (IR), nuclear magnetic resonance (NMR), high-resolution mass spectrometry (HRMS), x-ray crystallography, or ultra-violet (UV) detection are also used to aid in solving the structure of the isolated cannabinoid. The complete NMR assignments of major cannabinoids and flavonoids was published in a review in 2004 (Choi, Hazekamp et al. 2004).

D. In Vivo Bioassays

In vivo bioassays are used to determine the pharmacological effects of a potential therapeutic drug. The ability to use animal models allows scientists to know the complete effects of a potential drug, including beneficial and deleterious effects, route of administration, and dose level before going into human trials. Use of in vivo bioassays for cannabinoid research has contributed a major role to understanding their mechanism of action and potential as therapeutic drugs. In an attempt to understand the mechanism in which the endocannabinoid system can modulate pain, feeding, cardiovascular function, and other pharmacological functions, specific in vivo tests are performed to evaluate the actions that cannabinoid compounds are displaying within a test subject.

a. Tetrad Assay

In the late 1980s, Little and his colleagues began testing rodents treated with cannabinoids in a tetrad assay. The term tetrad describes a series of four different tests to help
evaluate the biological effects of a compound: 1) Spontaneous activity 2) Catalepsy 3) Hypothermia and 4) Analgesia. The spontaneous activity test allows a researcher to determine if the rodent is acting “lazy.” The rodent is placed in a box with perpendicular beams of light serving as gridlines. The test determines the amount of times the photobeams are broken in an allotted time period; an increase in the number of times broken correlates with a decrease in locomotor activity. Slight modifications of the ring test developed by Pertwee are used to determine if the drug causes cataleptic effects (Pertwee 1972). For this assay, a rodent is placed on a bar elevated off the ground surface. If the rodent remains immobile, it is considered cataleptic. Hypothermia, also know as the rectal temperature assay, is simply a measure of the rodents rectal temperature after the drug has been administered. For the last part of the tetrad assay, there are two different methods of testing for analgesic effects, hot-plate assay and tail-flick assay. In the hot-plate assay, a rodent is placed upon a hot plate and the time it takes for the rodent to react, usually a small jump, is recorded. In the tail-flick assay, the rodent is immobilized and a high temperature beam of light is sporadically placed on the tail. If the rodent feels pain, it will move its tail either left or right, hence the name tail-flick (Little 1988).

b. Feeding

It is known that the endocannabinoid system regulates appetite in humans; however, the mechanism of action is not well understood. The attempt to dissect the role of the endocannabinoid system in appetite regulation has led to novel therapies for anorexia and obesity. The interaction of compounds with the CB1 receptor is the cause of appetite stimulation/reduction seen with the endocannabinoid system. Agonists at the CB1 receptor are appetite stimulants, while inverse agonists at CB1 are appetite suppressants (Berry and Mechoulam 2002).
To determine the pharmacological effects of cannabinoids associated with appetite, in vivo feeding studies are conducted. The test compound is administered via intraperitoneal injection at specific doses. Drug administration occurs daily, along with monitoring of food intake. The animals that consume an increased amount of food in comparison to the placebo help establish that the compound is acting as an agonist at CB1. If food consumption is minimal, it is believed that the test compound is acting as an antagonist/inverse agonist at CB1. Using these in vivo animal models has led to the marketing of compounds that cause pharmacological effects associated with an interaction at the CB1 receptor.

i. Agonist at CB1

The use of Cannabis as an appetite stimulant dates back to the mid 1800s (Donovan 1845). Reports indicate that Cannabis enhanced appetite, and that it “restored the ability to appreciate food” (Birch 1889). The finding that $\Delta^9$-THC, a partial agonist at CB1, is the active ingredient in Cannabis caused researchers to evaluate its direct effects on appetite. The results indicate that $\Delta^9$-THC causes appetite stimulation by acting as an agonist at the CB1 receptors. The use of $\Delta^9$-THC as an appetite stimulant is currently available to help treat patients suffering from anorexia and wasting syndrome associated with AIDS (Beal 1995), elderly (Roubenoff 1999), and cancer (Balog 1998).

ii. Inverse Agonist at CB1

Conversely, using cannabinoid antagonists/inverse agonists has potential therapeutic benefit for the treatment of obesity. Rimonabant was the first marketed CB1 selective cannabinoid used for the treatment of obesity. It acts an inverse agonist at the CB1 receptor and therefore does not cause psychoactivity like agonists at CB1. Rimonabant causes a decrease in appetite stimulation, which in turn aids in the treatment of obesity. However, after only three
years on the market Rimonabant was removed in 2009 due to side effects associated with depression and suicide risk (Fong and Heymsfield 2009).

c. Cardiovascular

Most recent research has focused on the actions of cannabinoids on the central nervous system, now attention is shifting towards the peripheral effects cannabinoids possess including the cardiovascular effects. The in vivo studies of cannabinoids on the cardiovascular system have shown conflicting results indicating both increases and decreases in blood pressure (Stark 1980). Currently, a common underlying theme is that exogenously administered cannabinoids to animals under anesthesia cause hypotension and bradycardia. These effects are thought to be entirely CB1 receptor-mediated, as this response is absent in CB1 receptor knockout mice (Ledent 1999). In vivo hemodynamic studies are used to determine the actions of cannabinoids on cardiovascular effects. The use of these studies measures systolic and diastolic pressures, mean arterial pressure, heart rate, and contractility (Levy 1972). The fact that cannabinoids exhibit cardiovascular effects may begin to explain their participation in shock, though the exact role of the endocannabinoids on the vascular system remain unknown (Randall 2002).
CHAPTER IV
RESULTS

The development of \textit{in vitro} bioassays to determine ligand-receptor interactions of compounds for the CB1 and CB2 receptors is a necessary tool in the development of an accurate structure-activity relationship (SAR). Since the three-dimensional structures and amino acid residues at active sites of cannabinoid receptors have not been characterized, information about structural requirements for ligand-receptor interactions is obtained through development of different molecular probes (Khanolkar 2000). Along with \textit{in vitro} bioassays, ligand-receptor interactions can be determined using receptor mutants (McPartland 2003) and computer modeling (Reggio 1999).

In order to gain a better understanding of phytocannabinoids and their relationship towards the cannabinoid receptors, a SAR was developed based on \textit{in vitro} binding affinity and functional activity. The intent of developing a SAR for cannabinoid receptor ligands is to develop potential therapeutic compounds that are void of some of the unwanted side effects associated with \textit{Cannabis}. Using the relationships seen with this study of novel phytocannabinoids, known phytocannabinoids, and synthetic derivatives of phytocannabinoids, will aid in developing lead compounds to potentially treat many of the diseases associated with the regulation of the endocannabinoid system.

The objective of novel therapeutics associated with endocannabinoid regulation is to rid the unwanted side effects, such as psychoactivity. Stimulation of CB1 receptors by an agonist
causes the psychoactivity seen with Cannabis use. Whether for recreational or medicinal use, stimulation of CB1 receptors has shown to cause euphoria. Though most believe this is an “innocent high,” studies show that administration Cannabis influences reward pathways through the dopaminergic system leading to addiction. In fact, Δ⁹-THC activates dopaminergic pathways in similar ways to heroin (Tanda 1997). Similar studies conclude that Cannabis use and the endocannabinoid system serve as a link to schizophrenia (Degenhardt 2006). Current research to potentially develop safe, non-psychoactive therapeutic drugs is focusing on fully understanding the mechanism of action in which the cannabinoid receptors and endocannabinoid system work.

There are several methods to avoid the psychoactivity associated with CB1 receptors. Some examples would be to develop compounds that activate only CB2 receptors or target the CB1 and CB2 receptors located outside of the central nervous system. Developing selective ligands for CB2 have the potential to be a therapeutic treatment for neurodegenerative, cardiovascular, liver, kidney, bone, autoimmune, pain, cancer, bone, and skin diseases (Pacher 2011). Recent reports indicate that if compounds do not penetrate the blood-brain-barrier then the possibility of psychological side effects is significantly reduced (Nogueiras 2008). Thus, the development of cannabinoid receptor SAR has great potential in determining lead compounds with potential therapeutic use through effects on the endocannabinoid system.
Figure 4.1. Chemical structures of Δ⁹-THC used in the SAR study.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Affinity (nM)</th>
<th>Functional Activity (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CB1</td>
<td>CB2</td>
</tr>
<tr>
<td>1</td>
<td>18.60 ± 4.00</td>
<td>42.25 ± 9.11</td>
</tr>
<tr>
<td>16</td>
<td>1,292.00 ± 89.03</td>
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<tr>
<td>17</td>
<td>22.67 ± 5.88</td>
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<td>20</td>
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<tr>
<td>23</td>
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<td>88.45 ± 19.12</td>
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</table>

Table 4.1. Binding affinities and functional activities of Δ⁹-THC derivatives for human CB1 and CB2 receptors. All compounds acted as agonists in the functional assay.
Figure 4.2. Competitive binding curves for compound 20.

Since 1964, $\Delta^9$-THC (1) has been classified as the primary psychoactive constituent of Cannabis (Gaoni and Mechoulam 1964). Since then, it has served as lead compound for the synthesis of hundreds of analogs that have potential as pharmacological agents. It binds to and activates both cannabinoid receptors, CB1 and CB2. $\Delta^9$-THC binds tighter to CB1 than CB2 with Ki values of 18.60nM and 42.25nM, respectively. Functionally, it acts as a partial agonist at both CB1 and CB2 receptors with Ki values of 268.80nM and 327.40nM, respectively. The functionally inactive acid precursor of $\Delta^9$-THC, $\Delta^9$-tetrahydrocannabinolic acid (16), has significantly decreased binding affinity for both cannabinoid receptors.

Since the early 2000s, the lipophilic side chain at C-3 has been investigated for its role with cannabinoid ligand potency and selectivity. For $\Delta^9$-tetrahydrocannabivarin (17), the shortening of the C-3 side chain of $\Delta^9$-THC from a 5-carbon (pentyl) chain to a 3-carbon (propyl) chain retains binding affinity. Ultimately, a 1,1-dimethyl-heptyl side chain has resulted in the best binding affinity and functional activity (Liddle 2001). Accordingly, the reduction of the C-3 side chain in compound 17 did not functionally activate the cannabinoid receptors.

It is possible that some compounds may bind tightly to the cannabinoid receptors,
however, they may not functionally activate these receptors. For instance, compounds 17 and 10-α-OH-Δ⁹,¹¹-hexahydrocannabinol (20) bind to both CB1 and CB2 receptors, yet they do not functionally activate either of these two receptors. When comparing 20 and 10-β-OH-Δ⁹,¹¹-hexahydrocannabinol (21), the only structural difference is the hydroxyl substitution in the C-10 position. The α-OH substitution displays approximately 5-fold weaker binding affinity for CB1 and CB2 when compared with Δ⁹-THC; however, it still displays moderate nanomolar binding affinity. On the other hand, the β-OH substitution at C-10 does not display binding affinity for either of the cannabinoid receptors. Finally, the presence of the hydroxyl group in the C-10 position, whether α or β, completely abolishes functional activity for both receptors.

Two new isolates from Cannabis, 8-α-OH-Δ⁹-THC (22) and 8-β-OH-Δ⁹-THC (23), have the Δ⁹-THC core structure with the addition of a hydroxyl group at C-8 (unpublished). The results indicate that a hydroxyl substitution in the C-8 position of Δ⁹-THC plays a large role in ligand binding affinity. The β-OH of 23 retained strong binding affinity for CB1 and CB2 and the α-OH of 22 displayed much weaker binding affinity to both cannabinoid receptors. Functionally, both compounds acted as weak agonists for CB1. Interestingly, compound 22 displayed 8.25nM functional activity for CB2. This shows functional preference over the β-OH of 23 for the CB2 receptor. The result of the α-OH location shows that it is critical for CB2 functional activity, and displays preference over CB1. The significance in displaying preference for one receptor is the ability to retain the positive pharmacological effects associated with cannabinoid receptor activation without having negative side effects. The ability of 22 to display strong functional activity only for CB2 has the potential to aid with pain and inflammation without causing psychoactive effects associated with stimulation of CB1 receptors. Thus, this compound has the potential to be a new therapeutic for non-addictive pain management.
B. $\Delta^8$-Tetrahydrocannabinol

Figure 4.3. Chemical structures of $\Delta^8$-THC used in the SAR study.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Affinity (nM)</th>
<th>Functional Activity (nM)</th>
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<tbody>
<tr>
<td></td>
<td>CB1</td>
<td>CB2</td>
</tr>
<tr>
<td>5</td>
<td>78.11 ± 5.52</td>
<td>12.18 ± 2.01</td>
</tr>
<tr>
<td>24</td>
<td>830.10 ± 94.40</td>
<td>3,274.00 ± 515.14</td>
</tr>
<tr>
<td>25</td>
<td>31.86 ± 6.65</td>
<td>30.85 ± 4.24</td>
</tr>
<tr>
<td>26</td>
<td>4,034.00 ± 956.11</td>
<td>107.50 ± 19.08</td>
</tr>
<tr>
<td>27</td>
<td>223.60 ± 34.69</td>
<td>170.90 ± 32.00</td>
</tr>
<tr>
<td>28</td>
<td>&gt; 10,000</td>
<td>720.50 ± 122.29</td>
</tr>
<tr>
<td>29</td>
<td>3,828.00 ± 686.67</td>
<td>414.20 ± 39.39</td>
</tr>
<tr>
<td>30</td>
<td>1,812.00 ± 245.35</td>
<td>531.30 ± 24.13</td>
</tr>
</tbody>
</table>

Table 4.2. Binding affinities and functional activities of Δ8-THC analogs for human CB1 and CB2 receptors. All compounds acted as agonists in the functional assay.
Figure 4.4. Competitive binding and functional curves for compound 29.

$\Delta^8$-THC (5) is the more stable regioisomer of $\Delta^9$-THC (Galal 2009). It has a similar pharmacological profile to $\Delta^9$-THC with slightly weaker activity for CB1 and CB2 (Pertwee 2005). In comparison to 5, only one compound, 10-$\alpha$-OH-$\Delta^8$-THC (25), displayed better binding affinity for CB1 with a Ki of 31.86nM. In the $\Delta^8$-THC class of compounds, 5, 10-$\beta$-OH-$\Delta^8$-THC (24), and 25 are isolated from high potency Cannabis sativa L., and 2-nitro-$\Delta^8$-THC, 2,8-dinitro-$\Delta^8$-THC, 2-dimethylamino-$\Delta^8$-THC, 2-dibutylamino-$\Delta^8$-THC, 2-dihexylamino-$\Delta^8$-THC (26-30) are semi-synthetic analogs of $\Delta^8$-THC (unpublished). Compounds 24 and 25 contain a hydroxyl group, $\beta$-OH and $\alpha$-OH, respectively, present in the C-10 position. The hydroxy substitution influences binding affinity such that the $\alpha$-OH of compound 25 displays strong binding affinity...
for both cannabinoid receptors. The β-OH of 24 also binds to CB1 and CB2, though not as tightly. Additionally, the β-OH of 24 warrants significantly increased functional activity for CB1 over the parent molecule, Δ⁸-THC, and the α-OH substituted compound, 25.

The addition and location of different nitrogen containing functional groups plays a role in receptor binding and activity. Depending upon the synthesized location of a nitro group, binding affinity for either CB1 or CB2 may be preferred. Addition of a nitro group in the C-2 position of Δ⁸-THC shows preferential binding for CB2 with a Ki of 107.50nM. Addition of another nitro group at C-8 causes activation of CB1 receptor binding sites with approximately 200nM affinity. Looking at all of the semi-synthetic derivatives of 5, it is clear that nitrogen substitutions at the C-2 position do no play a major role in CB2 receptor binding. However, addition of a nitrogen-containing group in the C-8 position significantly increases binding affinity of C-2 nitrogen substituted Δ⁸-THC compounds for the CB1 receptor.

The addition of a nitro group in the C-2 position of 26 increased functional activity for CB1 and CB2 with Ki values of 49.21nM and 142.90nM, respectively. With a nitro group at C-2 and the addition of another nitro group at C-8, compound 27 displayed slightly stronger functional activity for both CB1 and CB2 receptors compared to Δ⁸-THC.

For compounds 28-30, three different nitrogen-containing substitutions were synthesized that only differed in the attached alkyl chain length: dimethyl, dibutyl, and dihexyl, respectively. Interestingly, these substitutions showed preferential binding for the CB2 receptor. Functionally, the results varied with 29 having the best functional activity for CB1 and CB2, 730.30nM and 52.99nM, respectively. In conclusion, it seems that an α-OH substitution at C-10 provides the best binding affinity, and a nitrogen substitution in the C-2 position yields the best functional activity for the cannabinoid receptors.
C. Cannabinol

![Chemical structure of Cannabinol](image)

**Figure 4.5.** Chemical structures of cannabinoi used in the SAR study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Affinity (nM)</th>
<th>Functional Activity (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CB1</td>
<td>CB2</td>
</tr>
<tr>
<td>8</td>
<td>75.29 ± 4.02</td>
<td>73.21 ± 4.00</td>
</tr>
<tr>
<td>31</td>
<td>8,063.00 ± 1,986.65</td>
<td>11.47 ± 1.97</td>
</tr>
<tr>
<td>32</td>
<td>565.60 ± 138.65</td>
<td>4,780.00 ± 331.00</td>
</tr>
</tbody>
</table>

**Table 4.3.** Binding affinities and functional activities of Cannabinol analogs for human CB1 and CB2 receptors. All compounds acted as agonists in the functional assay.
Figure 4.6. Competitive binding and functional curves for compound 8.

Similar to Δ⁹-THC and Δ⁸-THC, cannabinol (8) is another psychoactive constituent isolated from Cannabis. It is a metabolite of Δ⁹-THC and results in an aromatized A ring (McCallum 1975). The aromatization of this ring causes slightly weaker binding affinity and functional activity than Δ⁹-THC. A novel phytocannabinoid containing the cannabinol-base structure displayed selective binding affinity for CB2 in the low nanomolar range, 11.47nM. This isolate, 8-OH-cannabinol (31), contains a hydroxyl group at the C-8 position of cannabinol. Similarly to Δ⁹-tetrahydrocannabinvarin, analysis of cannabivarin (32) revealed that a pentyl side chain at C-3 is critical for retaining binding affinity and functional activity at both cannabinoid receptors.
D. Cannabigerol

\[
\begin{align*}
3: \quad & R = H \quad R_1 = OH \quad R_2 = \text{structure} \quad R_3 = OH \quad R_4 = H \\
33: \quad & R = \text{COOH} \quad R_1 = OH \quad R_2 = \text{structure} \quad R_3 = OH \quad R_4 = H \\
34: \quad & R = H \quad R_1 = OH \quad R_2 = \text{structure} \quad R_3 = OH \quad R_4 = H \\
35: \quad & R = H \quad R_1 = \text{OCH}_3 \quad R_2 = \text{structure} \quad R_3 = OH \quad R_4 = H \\
36: \quad & R = H \quad R_1 = OH \quad R_2 = \text{structure} \quad R_3 = \text{OAc} \quad R_4 = \text{OH} \\
37: \quad & R = H \quad R_1 = \text{O} \quad R_2 = \text{structure} \quad R_3 = H \quad R_4 = \text{O} \\
\end{align*}
\]

Figure 4.7. Chemical structures of cannabigerol used in the SAR study.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Affinity (nM)</th>
<th>Functional Activity (nM)</th>
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</thead>
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<tr>
<td></td>
<td>CB1</td>
<td>CB2</td>
</tr>
<tr>
<td>3</td>
<td>3,090.00 ± 583.22</td>
<td>2,919.00 ± 752.43</td>
</tr>
<tr>
<td>33</td>
<td>4,526.00 ± 953.53</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>34</td>
<td>&gt; 10,000</td>
<td>4,718.00 ± 87.35</td>
</tr>
<tr>
<td>35</td>
<td>&gt; 10,000</td>
<td>3,989.00 ± 772.52</td>
</tr>
<tr>
<td>36</td>
<td>1,409.00 ± 162.19</td>
<td>388.60 ± 67.00</td>
</tr>
<tr>
<td>37</td>
<td>&gt; 10,000</td>
<td>&gt; 10,000</td>
</tr>
</tbody>
</table>

Table 4.4. Binding affinities and functional activities of Cannabigerol analogs for human CB1 and CB2 receptors. All compounds acted as agonists in the functional assay.

Cannabigerol (3) is a non-psychoactive constituent of *Cannabis* (Izzo 2009), which lacks the dibenzopyran moiety seen in $\Delta^9$-THC. Though isolated from *Cannabis*, cannabigerol acts as an $\alpha_2$-adrenergic receptor agonist and 5-HT$_{1A}$ receptor antagonist (Cascio 2010). Of the six cannabigerol-type compounds isolated, only one displayed noteworthy binding affinity for either CB1 or CB2 receptors. This compound, 4-OH-5-acetoxy-cannabigerol (36), contains an acetyl substitution off the hydroxyl group at C-5, and has a binding affinity of 388.60nM for the CB2 receptor.
Functionally, several compounds warranted notable activity at CB1 or CB2 receptors. The acid precursor of cannabigerol, cannabigeric acid (33), acts as an agonist for CB1 and CB2 receptors with Ki values of 182.50nM and 118.10nM, respectively. Methoxy-cannabigerol (35) and 36 act as partial agonists for the CB1 receptor with Ki values of 235.40nM and 618.60nM, respectively. It is also important to note that the epoxide functional group present in the aliphatic side chain of 6,7-epoxy-cannabigerol (34) abolished CB2 functional activity.

E. Flavonoids

![Figure 4.8](image.png)

12: $R = \text{OCH}_3$, $R_1 = \text{H}$, $R_2 =$  
13: $R = \text{OCH}_3$, $R_1 = \text{H}$, $R_2 =$  
14: $R = \text{OCH}_3$, $R_1 =$  , $R_2 = \text{H}$  
15: $R = \text{H}$, $R_1 = \text{H}$, $R_2 =$

**Figure 4.8.** Chemical structures of flavonoids used in the SAR study.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Affinity (nM)</th>
<th>Functional Activity (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CB1</td>
<td>CB2</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>559.50 ± 121.33</td>
<td>2,930.00 ± 752.23</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9,550.00 ± 1,588.00</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,094.00 ± 213.73</td>
<td>1,361.00 ± 192.88</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5,040.00 ± 1,134.43</td>
<td>9,799.00 ± 2,344.00</td>
</tr>
</tbody>
</table>

Table 4.5. Binding affinities and functional activities of flavonoid analogs for human CB1 and CB2 receptors. All compounds acted as inverse agonists in the functional assay.
Figure 4.9. CB1 functional curves for compounds 12-15.

The flavonoids isolated from *Cannabis* are not considered cannabinoids since they do not contain the C$_{21}$ terpenophenolic base structure. The four flavonoids tested, cannflavin A (12), cannflavin B (13), cannflavin C (14), and 5,7,4’-trihydroxy-6-prenyl-flavone (15), showed moderate to weak binding affinity and strong to moderate functional activity. Since these compounds are not cannabinoids, it was expected that these compounds would not bind to the cannabinoid receptors. This hypothesis was proved wrong as these compounds displayed both binding affinity and functional activity. The binding affinity seen with the flavonoids is most likely due to the similarities it shares with Δ$^9$-THC. For instance, they both contain lipophilic chains that are known to increase binding affinity. Also, they both contain a pyran functional
group and a phenolic hydroxy. These three major functional groups may be the reason that flavonoids bind to both cannabinoid receptors. As seen with cannflavins A and C, longer lipophilic chains are critical for binding affinity at CB1 and CB2 receptors.

Dissociation between binding affinity and functional activity is not unusual for G protein-coupled receptors (Carlsson 2010). The flavonoids showed strong inverse agonist functional activity for both cannabinoid receptors, CB1 and CB2. Cannflavin C displayed the most potent ability to reverse efficacy for CB1 and CB2 with Ki values of 98.69nM and 51.64nM, respectively. A significant loss of functional activity is present with compound 15. This loss of activity could correlate to the absence of the methoxy group in the C-3’ position.

The importance of compounds acting as inverse agonists at the cannabinoid receptors is critical for the success of novel therapeutic drugs. For example, Rimonabant is a selective CB1 inverse agonist that was marketed for the treatment of obesity. Although it has been removed from the market because of psychological side effects, it has been a strong lead compound for the novel treatments of obesity (Colombo 1998). The natural occurring flavonoids in Cannabis may potentially be used a lead treatment for obesity that could lack the undesirable psychological effects, such as suicidal thoughts.

Furthermore, these four flavonoids act as inverse agonists for the CB2 receptor as well. Inverse agonist activity at CB2 has shown broad effects on cellular protein phosphorylations in human monocytes. This has shown to modulate bone damage and block encephalomyelitis in rats (Lunn 2008). Thus, a compound displaying inverse activity at CB2 has the potential to become a novel treatment for pain and inflammation associated with multiple sclerosis (Buccellato 2011).

F. Cannabichromanone
Currently, there is little known about the pharmacological role that cannabichromanone isolates may exhibit. All three compounds evaluated in this study were isolated in 2008 by ElSohly and colleagues at the University of Mississippi. The isolated phytocannabinoids were named cannabichromanone B (38), cannabichromanone C (39), and cannabichromanone D (40) due to their structure similarity of previously isolated cannabichromanone A. Several of the
cannabichromanone-type compounds displayed antimalarial and antileishmanial activity. Furthermore, these compounds all displayed strong anti-oxidant properties (Ahmed 2008).

The in vitro binding and functional studies show that the cannabichromanone derivatives display a variety of activity at both cannabinoid receptors. It is worthy to note that cannabichromanone D has a slightly different structure than the other derivatives as the aliphatic chain cyclizes and forms a third ring with the phenolic hydroxylic. Interestingly, cannabichromanone D displayed selectivity for the CB1 receptor in the GTPγS functional assay, acting as a full agonist with a Ki value of 8.73nM. Though not as potent as cannabichromanone D, cannabichromanone B also displayed preferential activity for the CB1 receptor, acting as an agonist with a Ki value of 965.30nM. Finally, cannabichromanone C displayed moderate functional activity for the CB1 and CB2 receptors with Ki values of 483.30nM and 138.50nM, respectively.

Though the cannabichromanone derivatives have not been evaluated for their ability to induce psychoactivity, it is safe to say that these compounds will induce psychoactivity, depending on the dose, because of their ability to functionally stimulate the CB1 receptors.

G. Cannabidiol

![Chemical structure of cannabidiol](image)

**Figure 4.11.** Chemical structures of cannabidiol used in the SAR study.
Table 4.7. Binding affinities and functional activities of Cannabidiol analogs for human CB1 and CB2 receptors. All compounds acted as agonists in the functional assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Binding Affinity (nM)</th>
<th>Functional Activity (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CB1</td>
<td>CB2</td>
</tr>
<tr>
<td>2</td>
<td>151.90 ± 28.88</td>
<td>4,582.00 ± 613.42</td>
</tr>
<tr>
<td>41</td>
<td>503.00 ± 58.13</td>
<td>3,970.00 ± 976.84</td>
</tr>
</tbody>
</table>

Cannabidiol (2) is one of the major, non-psychotropic cannabinoids isolated from Cannabis sativa (Grlic 1976). It was first isolated in 1940 (Adams 1940), but the exact structure was not determined until 1963 (Mechoulam and Shvo 1963). Cannabidiol has been the most studied non-psychoactive phytocannabinoid because of its potential as a therapeutic agent for many diseases. The scientific literature shows that cannabidiol may aid in inflammation (Malfait 2000), nausea, sedation (Grotenhermen 2002), neuroprotection (Hampson 1998), cancer cell growth (McAllister 2007), and schizophrenia (Zuardi 2006). Interestingly, studies have shown that the anxiety produced from Δ⁹-THC can be blocked with the administration of cannabidiol (Zuardi 1982). Furthermore, a combination of Δ⁹-THC and cannabidiol are now marketed in Canada under the trade name Sativex, which is used to alleviate pain associated with Multiple Sclerosis (Barnes 2006). The potential that rises from the use of cannabidiol as a therapeutic drug is of importance in today’s society.

The low functional activities of cannabidiol and cannabidivarin (41) for the CB1 receptor supplement that these compounds do not display psychotropic effects. The positive therapeutic effects of cannabidiol are seen through its interaction with the CB2 receptor. Cannabidiol acts as
an agonist and has preference for the CB2 receptor. Similarly, cannabidivarin, acting as a full agonist, has selectivity for the CB2 receptor. The strong Ki value of these two compounds for the CB2 receptor correlate with studies that discuss the potential for selective CB2 agonists as treatments for pain and inflammation associated with numerous CNS diseases (Mechoulam, Peters et al. 2007).

H. Volatile Oil

The constituents of Cannabis have been commonly studied and evaluated for their pharmacological effects as pure compounds. Currently, there are new approaches associated with the evaluation of compounds from Cannabis. It is believed that the mixture of cannabinoids, terpenoids, and flavonoids may have a synergistic relationship for producing therapeutic effects since many unwanted side effects associated with pure compounds result upon administration (Hazekamp 2009). It is known that terpenoids possess a wide range of biological effects such as chemopreventive effects, skin penetration enhancement, antimicrobial, antifungal, antiviral, anti-hyperglycemic, anti-inflammatory, and antiparasitic activities (McPartland 2001). Therefore, it is of interest to identify the terpenes within Cannabis for potential therapeutic leads.

The cannabinoids isolated from Cannabis sativa are odorless compounds; however, the terpenes present in the volatile oil of Cannabis provide its unique aroma. The volatile oil contains a large percentage of terpenoids, monoterpenes and sesquiterpenes, with monoterpenes dominating the percentage. There is approximately 90% monoterpenes, 7% sesquiterpenes, and 1% other chemical classes such as simple ketones and esters. Generally, the most abundant terpenoid associated with the volatile oil of Cannabis is myrcene. The scientific literature indicates that depending upon the life cycle of the plant, distillation method, drying, and storage, percentages of specific terpenoids may be significantly increased or decreased. For instance, in
1996 Ross and ElSohly published an article discussing the composition of fresh and air-dried buds of *Cannabis sativa*. The results indicated that the method of drying the buds does not alter chemical composition of the volatile oil, but the length of storage of the dried buds does cause a decrease of terpenoids in the volatile oil (Ross 1996).

\( \Delta^9 \)-THC is known to cause negative psychological reactions such as anxiety. The scientific literature reports that the terpenoids associated with *Cannabis* may alleviate the negative effects because of their ability to act as sedatives and antidepressants (Brenneisen 2010). To date, there have been no reports of the volatile oil of *Cannabis* and its ability to bind the human cannabinoid receptors. Here we present seven different fractions from the crude volatile oil of high potency *Cannabis sativa* for their *in vitro* ability to bind to human CB1 and CB2 receptors. Due to the sensitivity of the bioassays, the volatile oil fractions containing \( \Delta^9 \)-THC are expected to show a high binding percentage for CB1 and CB2 receptors. Interestingly, some of the fractions that do not contain \( \Delta^9 \)-THC also showed binding to the cannabinoid receptors.

All of the components from high potency *Cannabis sativa* were extracted from the air-dried buds using hexanes and methanol solvents. The crude *Cannabis* extract was heated to 120°C for approximately two hours in order to decarboxylate the inactive cannabinoid acids into their respective active state. Finally, the extract was then subject to vacuum distillation using a Kugelrohr in order to prepare the crude volatile oil. The volatile oil of *Cannabis* accounts for approximately 10% total weight of the dried buds. An initial cannabinoid primary screen indicated that the volatile oil had significant binding affinity to both CB1 and CB2 receptors. Using silica gel column chromatography for separation, seven different fractions of the volatile oil were evaluated for their ability to bind at CB1 and CB2 receptors (Table 4.8).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>CB1 % Binding</th>
<th>CB2 % Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si-A</td>
<td>39.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Si-B</td>
<td>36.8</td>
<td>12.0</td>
</tr>
<tr>
<td>Si-C</td>
<td>78.6</td>
<td>94.1</td>
</tr>
<tr>
<td>Si-D</td>
<td>79.2</td>
<td>89.3</td>
</tr>
<tr>
<td>Si-E</td>
<td>86.0</td>
<td>78.3</td>
</tr>
<tr>
<td>Si-F</td>
<td>50.6</td>
<td>45.0</td>
</tr>
<tr>
<td>Si-G</td>
<td>39.1</td>
<td>29.7</td>
</tr>
</tbody>
</table>

Table 4.8. CB1 and CB2 percent binding of seven different fractions from the volatile oil of *Cannabis*.

The fractions displaying greater than 60% binding for CB1 and CB2 were deemed active fractions: Si-C, Si-D, and Si-E. Next, using gas chromatography with flame ionization detection (GC-FID) and gas chromatography with mass spectrometry (GC/MS), the major components, retention times, and percentage of compound within each fraction was determined. Table 4.9 displays the major components for each of these fractions.
Table 4.9. Major components, retention times ($T_R$), and percentages of components within the volatile oil of high potency *Cannabis sativa*.

To further support the hypothesis that those fractions containing $\Delta^9$-THC will be active, Si-C and Si-D both contain $\Delta^9$-THC and resulted as active fractions. Although these fractions showed activity, further analysis of these fractions was not performed since both contain $\Delta^9$-THC. Interestingly, Si-E also displayed binding activity for the cannabinoid receptors and does not contain $\Delta^9$-THC. Therefore, the major components in Si-E, that were isolated or available for purchase, were tested to potentially determine which compound (s) was responsible for the activity (Figure 4.8). Table 4.10 shows the CB1 and CB2 percent binding for each of the pure compounds from Si-E.
Figure 4.12. Chemical structures of pure compounds in Si-E

<table>
<thead>
<tr>
<th>Compound</th>
<th>CB1 % Binding</th>
<th>CB2 % Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-cymen-8-ol</td>
<td>-22.6</td>
<td>-14.4</td>
</tr>
<tr>
<td>caryophyllene oxide</td>
<td>-27.4</td>
<td>-13.1</td>
</tr>
<tr>
<td>cannabinol</td>
<td>82.6</td>
<td>89.8</td>
</tr>
<tr>
<td>pulegone</td>
<td>-6.0</td>
<td>-9.6</td>
</tr>
</tbody>
</table>

Table 4.10. CB1 and CB2 percent binding of the pure compounds from Si-E.

Note: not all major components were tested for CB1 and CB2 binding.

Cannabinol as a pure compound displayed a high percent binding for CB1 and CB2.

Furthermore, fractions Si-E, Si-F, and Si-G all contained a small percentage of cannabinol,
1.80%, 0.92%, and 2.13%, respectively. It is critical to note that the trace amounts of cannabinol are not responsible for the activity seen with fraction Si-E. If cannabinol were responsible for the activity associated with Si-E, fraction Si-G would have also shown activity since it has a slightly greater amount, 2.3%, than Si-E. Therefore, the minor and major components in fraction Si-E are acting synergistically to produce its effects on the cannabinoid receptors.

Using SAR based on in vitro binding affinity and functional activity of compounds isolated from high potency Cannabis sativa has the potential to develop a lead compound to be further pursued in its path to becoming a therapeutic drug for the treatment of many physiological and pathological diseases associated with the cannabinoid receptors. There are three possible lead compounds from this study, 8-α-OH- Δ⁹-THC, 2-dibutylamino-Δ⁸-THC, and cannabidivarin, which can be further pursued by synthesizing derivatives to potentially increase potency and selectivity for CB2.
CHAPTER V
CONCLUSION

A. Summary

*Cannabis* has been around for thousands of years and has been used for recreation, medicine, and fiber. Over 400 compounds have been isolated from *Cannabis sativa* with approximately 100 being cannabinoids. Of these 100 compounds, Δ⁹-THC has been determined as the primary constituent, which also is responsible for inducing psychoactivity. Along with Δ⁹-THC, cannabidiol has been studied in depth for its therapeutic effects. This has currently been the focus of many researchers since cannabidiol does not cause psychotropic effects. The objective is to develop a drug that acts in a similar manner to *Cannabis* that lacks the unwanted side effects.

G protein-coupled receptors are the largest superfamily among all human receptors. They are said to be responsible for mediating a variety of physiological and pathological processes. These receptors play an important role in drug discovery and development because they can be used as drug targets for potential therapeutic agents. It is known that approximately 27% of drugs approved by the Food and Drug Administration target G protein-coupled receptors. The cannabinoid receptors belong the large family of G protein-coupled receptors and play an important role in a variety of processes including pain, appetite, addiction, neurodegeneration, metabolic regulation, anxiety, cancer, and immune function. Thus, advances in cannabinoid receptor receptor-based therapies have the potential to treat a variety of conditions.
The discovery of the endocannabinoid system has opened the door for scientists all over the world to further explore cannabinoid receptors and ligands that activate them. However, the realization that this system is very complex has discouraged the development of new therapeutic drugs that target the cannabinoid receptors. So, it is important to develop a structure-activity relationship for phytocannabinoids that bind to and activate the cannabinoid receptors in order to help understand the important features of a core structure that give optimal activity. This will not only help the mystery of many related CNS diseases, but also serves as a lead for new therapeutics.

In order to develop an accurate SAR, the development and establishment of in vitro bioassays is critical. The use of radioligand competitive binding and functional assays has been used for many years in the field of drug development. Full-length human recombinant cDNA for CB1 and CB2 were transfected into individual HEK293 cell lines and grown for use in the bioassays. Standardization of ligand-receptor radioligand binding and functional assays is required for meaningful comparisons to be made between research groups. When working with cannabinoids, factors such as lipophilicity, solubility, and purity are carefully investigated due the extreme sensitivity of the assay. Success with cannabinoid receptor bioassays requires optimal experimental conditions as any minimal changes may lead to major variations in binding affinity and functional activity.

Targeting the cannabinoid receptors with selective compounds is critical for developing novel treatments that lack the unwanted side effects associated with Cannabis. It is known that CB1 receptors are widely spread throughout the CNS and activation of these receptors by an agonist leads to psychoactivity. Recent research has shown that CB2 receptors are also located within the brain, but are primarily peripheral. Therefore, the ability to selectively stimulate CB2
receptors does not cause psychoactive effects and is currently the primary target for drug development (Figure 5.1).

Figure 5.1. Compounds from the SAR study that acted as agonists and showed the strongest preferential functional activity for the CB2 receptor.

Flavonoids are common in everyday life and are consumed daily in fruits and vegetables. They act as powerful antioxidants. The flavonoid compounds isolated from Cannabis sativa were the only compounds to functionally act as inverse agonists. The ability of these compounds to “reverse efficacy” at both, CB1 and CB2, receptors has the potential to become a therapeutic
target for the treatment of obesity and pain and inflammation associated with multiple sclerosis.

In conclusion, *in vitro* bioassays involving radioligand binding and function have been a useful tool in the discovery of novel agents. This dissertation highlights the feasibility of exploring new structural scaffolds, with binding affinity and functional activity for cannabinoid receptors, from high potency *Cannabis sativa*.

B. Future Direction

a. Evaluation of non-Cannabis plants

It has been concluded that natural products exhibit a vast array of pharmacological functions throughout the human body, and elicit these functions by acting in a manner very specific for their receptor. Natural products have a high chemical diversity in the compounds they may contain. The ecological role of natural products endows them with their biological function. It was the intensive study of the chemistry and pharmacology of $\Delta^9$-THC and other active cannabinoids from *Cannabis sativa* L. that led to the identification of the cannabinoid receptors, and ultimately the endocannabinoid system. Therefore, the combination of natural products and the discovery of the cannabinoid receptors offer an excellent opportunity to yield novel agents to treat cannabinoid receptor-based disorders.

Fungi-derived natural products have been very successful in drug discovery in pharmaceutical industry for many years. However, it was not until recently that fungi-derived natural products were explored for the their ability to interact with cannabinoid receptors. In 2011, twenty-nine compounds were isolated from the fungi *Eurotium repens*, *Neocosmospora* sp., *Eupenicillium parvum*, and UK-149 with the use of bioassay-guided fractionation. Of the twenty-nine compounds isolated, three displayed activity for the cannabinoid receptors (Figure 5.2) (Gao 2011). If only four different fungi were evaluated and resulted with three hits for the
cannabinoid receptors, the field of fungi-derived natural products and their effects on cannabinoid receptors holds promise to the development of novel therapeutic drugs.

![Chemical structures](image1)

**Figure 5.2.** Fungi-derived natural products that bind to the cannabinoid receptors. a) isolated from fungus *Eurotium repens*, b) and c) isolated from fungus isolated from fungus *Eupenicillium parvum*.

Different species of fungi should be pursued to find lead compounds that activate the cannabinoid receptors. Furthermore, other natural products derived from sources such as plants, marine sponges, coral, and fish, bacteria, animals, and venom should also be evaluated. The key to discovering novel therapeutics and the mystery behind the mechanism of action for the cannabinoid receptors may lie within a natural product source.
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CB1 Binding
\[\Delta^8\text{-THC}\]

-10  -8  -6  -4
log [\Delta^8\text{-THC}] [M]

% Stimulation

\begin{align*}
\text{IC}_{50} & = 3.721e-008 \\
\text{Ki} & = 1.505e-008
\end{align*}

CB2 Binding
\[\Delta^8\text{-THC}\]

-10  -8  -6  -4
log [\Delta^8\text{-THC}] [M]

% Stimulation

\begin{align*}
\text{IC}_{50} & = 3.451e-008 \\
\text{Ki} & = 4.225e-008
\end{align*}

CB1 Binding
\[\Delta^8\text{-tetrahydrocannabinolic acid}\]

-10  -8  -6  -4
log [\Delta^8\text{-tetrahydrocannabinolic acid}] [M]

% Stimulation

\begin{align*}
\text{IC}_{50} & = 2.584e-008 \\
\text{Ki} & = 1.292e-006
\end{align*}

CB2 Binding
\[\Delta^8\text{-tetrahydrocannabinolic acid}\]

-10  -8  -6  -4
log [\Delta^8\text{-tetrahydrocannabinolic acid}] [M]

% Stimulation

\begin{align*}
\text{IC}_{50} & = 3.301e-008 \\
\text{Ki} & = 1.650e-008
\end{align*}

CB1 Binding
\[\Delta^8\text{-tetrahydrocannabivarin}\]

-10  -8  -6  -4
log [\Delta^8\text{-tetrahydrocannabivarin}] [M]

% Stimulation

\begin{align*}
\text{IC}_{50} & = 4.534e-008 \\
\text{Ki} & = 2.267e-006
\end{align*}

CB2 Binding
\[\Delta^8\text{-tetrahydrocannabivarin}\]

-10  -8  -6  -4
log [\Delta^8\text{-tetrahydrocannabivarin}] [M]

% Stimulation

\begin{align*}
\text{IC}_{50} & = 2.107e-007 \\
\text{Ki} & = 1.053e-007
\end{align*}

CB1 Binding
\[10\alpha\text{-OH-}\Delta^8\text{-THC}\]

-10  -8  -6  -4
log [10\alpha\text{-OH-}\Delta^8\text{-THC}] [M]

% Stimulation

\begin{align*}
\text{IC}_{50} & = 6.587e-006 \\
\text{Ki} & = 3.293e-006
\end{align*}

CB2 Binding
\[10\alpha\text{-OH-}\Delta^8\text{-THC}\]

-10  -8  -6  -4
log [10\alpha\text{-OH-}\Delta^8\text{-THC}] [M]

% Stimulation

\begin{align*}
\text{IC}_{50} & = 5.541e-006 \\
\text{Ki} & = 2.771e-006
\end{align*}
CB1 Binding
Cannabinol

% Stimulation

log [Cannabinol] [M]

IC50 1.506e-007
Ki 7.529e-008

CB2 Binding
Cannabinol

% Stimulation

log [Cannabinol] [M]

IC50 1.684e-007
Ki 7.321e-008

CB1 Binding
8-OH-Cannabinol

% Stimulation

log [8-OH-Cannabinol] [M]

IC50 1.613e-005
Ki 8.083e-006

CB2 Binding
8-OH-Cannabinol

% Stimulation

log [8-OH-Cannabinol] [M]

IC50 2.295e-006
Ki 1.147e-006

CB1 Binding
Cannabivarin

% Stimulation

log [Cannabivarin] [M]

IC50 1.131e-006
Ki 5.856e-007

CB2 Binding
Cannabivarin

% Stimulation

log [Cannabivarin] [M]

IC50 9.559e-006
Ki 4.780e-006
CB1 Binding

Cannabigerol

% Stimulation

log [Cannabigerol] [M]

IC50 6.179e-006
K_i 3.090e-006

CB2 Binding

Cannabigerol

% Stimulation

log [Cannabigerol] [M]

IC50 5.837e-006
K_i 2.919e-006

CB1 Binding

Cannabigeric acid

% Stimulation

log [Cannabigeric acid] [M]

IC50 9.051e-006
K_i 4.526e-006

CB2 Binding

Cannabigeric acid

% Stimulation

log [Cannabigeric acid] [M]

IC50 3.768e-005
K_i 1.884e-005

CB1 Binding

6,7-epoxy-cannabigerol

% Stimulation

log [6,7-epoxy-cannabigerol] [M]

IC50 2.325e-005
K_i 1.162e-005

CB2 Binding

6,7-epoxy-cannabigerol

% Stimulation

log [6,7-epoxy-cannabigerol] [M]

IC50 9.436e-006
K_i 4.718e-006

CB1 Binding

Methoxy cannabigerol

% Stimulation

log [Methoxy cannabigerol] [M]

IC50 4.489e-005
K_i 2.245e-005

CB2 Binding

Methoxy cannabigerol

% Stimulation

log [Methoxy cannabigerol] [M]

IC50 7.979e-006
K_i 3.989e-006
CB1 Binding
4-OH-5-acetoxy-cannabigerol

% Stimulation
-50 0 50 100 150
log (4-OH-5-acetoxy-cannabigerol) [M]

IC50 2.819e-006
Ki 1.409e-006

CB2 Binding
4-OH-5-acetoxy-cannabigerol

% Stimulation
0 50 100 150
log (4-OH-5-acetoxy-cannabigerol) [M]

IC50 7.772e-007
Ki 3.886e-007

CB1 Binding
2-geranyl-5-n-pentyl-1,4-benzoquinone

% Stimulation
-50 0 50 100 150
log [2-geranyl-5-n-pentyl-1,4-benzoquinone] [M]

IC50 5.874e-005
Ki 2.937e-005

CB2 Binding
2-geranyl-5-n-pentyl-1,4-benzoquinone

% Stimulation
0 20 40 60 80 100
log [2-geranyl-5-n-pentyl-1,4-benzoquinone] [M]

IC50 ~ 0.04499
Ki ~ 0.08997
CB1 Binding
Cannflavin A

% Stimulation
vs. log [Cannflavin A] [M]

IC50: 1.119e-006
Ki: 5.595e-007

CB2 Binding
Cannflavin A

% Stimulation
vs. log [Cannflavin A] [M]

IC50: 5.859e-006
Ki: 2.930e-006

CB1 Binding
Cannflavin B

% Stimulation
vs. log [Cannflavin B] [M]

IC50: 1.910e-005
Ki: 9.550e-006

CB2 Binding
Cannflavin B

% Stimulation
vs. log [Cannflavin B] [M]

IC50: 4.888e-005
Ki: 2.444e-005

CB1 Binding
Cannflavin C

% Stimulation
vs. log [Cannflavin C] [M]

IC50: 2.187e-005
Ki: 1.094e-006

CB2 Binding
Cannflavin C

% Stimulation
vs. log [Cannflavin C] [M]

IC50: 2.726e-005
Ki: 1.361e-006

CB1 Binding
5,7,4'-trihydroxy-6-prenyl-flavone

% Stimulation
vs. log [5,7,4'-trihydroxy-6-prenyl-flavone] [M]

IC50: 1.008e-005
Ki: 5.040e-006

CB2 Binding
5,7,4'-trihydroxy-6-prenyl-flavone

% Stimulation
vs. log [5,7,4'-trihydroxy-6-prenyl-flavone] [M]

IC50: 1.900e-005
Ki: 9.708e-006
CB1 Binding
Cannabichromanone B

CB2 Binding
Cannabichromanone B

CB1 Binding
Cannabichromanone C

CB2 Binding
Cannabichromanone C

CB1 Binding
Cannabichromanone D

CB2 Binding
Cannabichromanone D

IC50 8.940e-006
Ki 3.470e-006

IC50 6.940e-006
Ki 4.371e-006

IC50 1.736e-005
Ki 9.581e-006

IC50 1.155e-005
Ki 5.769e-006

IC50 1.423e-005
Ki 7.117e-006

IC50 5.656e-006
Ki 2.828e-006
CB1 Binding
Cannabidiol

% Stimulation

log [Cannabidiol] [M]

IC50 3.036e-007
K_i 1.519e-007

CB2 Binding
Cannabidiol

% Stimulation

log [Cannabidiol] [M]

IC50 9.163e-006
K_i 4.582e-006

CB1 Binding
Cannabidivarin

% Stimulation

log [Cannabidivarin] [M]

IC50 1.006e-006
K_i 5.030e-007

CB2 Binding
Cannabidivarin

% Stimulation

log [Cannabidivarin] [M]

IC50 7.940e-006
K_i 3.970e-006
CB1 Functional
8-β-OHΔ9-THC

% Stimulation

Log (8-β-OHΔ9-THC) [M]

IC50 5.070e-006
Kd 2.535e-006

CB2 Functional
8-β-OHΔ9-THC

% Stimulation

Log (8-β-OHΔ9-THC) [M]

IC50 ~ 0.3882
Kd ~ 0.1941
CB1 Functional
Cannflavin A

% Stimulation vs Log (Cannflavin A) [M]

IC50: 2.309e-006
Ki: 1.155e-009

CB2 Functional
Cannflavin A

% Stimulation vs Log (Cannflavin A) [M]

IC50: 7.329e-007
Ki: 3.681e-007

CB1 Functional
Cannflavin B

% Stimulation vs Log (Cannflavin B) [M]

IC50: 2.075e-007
Ki: 1.037e-007

CB2 Functional
Cannflavin B

% Stimulation vs Log (Cannflavin B) [M]

IC50: 4.613e-007
Ki: 2.307e-007

CB1 Functional
Cannflavin C

% Stimulation vs Log (Cannflavin C) [M]

IC50: 1.974e-007
Ki: 9.869e-008

CB2 Functional
Cannflavin C

% Stimulation vs Log (Cannflavin C) [M]

IC50: 1.033e-007
Ki: 5.164e-008

CB1 Functional
5,7,4’-trihydroxy-6-prenyl-flavone

% Stimulation vs Log (5,7,4’-trihydroxy-6-prenyl-flavone) [M]

IC50: 4.606e-006
Ki: 2.303e-006

CB2 Functional
5,7,4’-trihydroxy-6-prenyl-flavone

% Stimulation vs Log (5,7,4’-trihydroxy-6-prenyl-flavone) [M]

IC50: 5.162e-006
Ki: 2.301e-006
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