THC mediated tolerance to the diuretic effects of cannabinoids & kappa opioids in mice

Master’s Thesis

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Title: THC mediated tolerance to the diuretic effects of cannabinoids & kappa opioids in mice

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Date to be presented: December 3rd 2014

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Abstract

Cannabinoids and opioids are two of the oldest known categories of drugs, used for their medicinal and recreational properties since ancient times. Drugs belonging to these two classes have similar molecular mechanisms of actions and thus show a number of overlapping pharmacological effects and adverse effects. One of these overlapping effects of cannabinoids and kappa opioids is dose dependent increase in diuresis in rodents, non-human primates and even humans, believed to be due to the suppression of vasopressin levels. In order to determine whether the endocannabinoid and kappa opioid system interact in any significant manner we tested the effects of chronic Δ⁹-tetrahydrocannabinol (THC) dosing on diuresis caused by CB1 agonist, AM4054 and kappa opioid, U50488 in mice. The diuretic effects in mice were measured over a period of 6 hours and diuresis was measured before chronic THC dosing (baseline) and on day 1, day 7 and day 14 post chronic 7 day THC dosing. The results showed that chronic THC tolerance caused a 3.5 to 4.5-fold rightward shift in the AM4054 diuretic dose response curve (for both the ascending and descending limbs). The baseline diuretic dose response was almost completely recovered within 14 days after cessation of THC dosing. The study of U50488 in THC tolerant mice showed an unusual flattening of the diuretic dose effect function, which was not completely reversed even at 14 days post termination of chronic THC dosing. Testing the effects of THC tolerance on diuresis caused by furosemide indicated that there was no significant difference in the diuretic effect of furosemide in normal and THC tolerant mice. From the results we concluded that AM4054 and THC are cross tolerant for their diuretic effect and further investigation of tolerance to other cannabinoid effects like antinociception and hypothermia is warranted. The flattening of the dose effect function of U50488 indicates that THC might be interfering with kappa opioid diuresis in a longer lasting manner than normal tolerance.
1. Background and Significance

1.1. The Cannabinoid System

1.1.1. History of Cannabinoids

Cannabis (Cannabis sativa) is one of the oldest known drugs to mankind. It is most prominently known for its psychoactive properties and has been used for medicinal and recreational purposes in almost all major ancient cultures. Indeed the earliest mentions of use of cannabis can be traced back to 2000 B.C. in China and possibly back to 1000 B.C. in India. There is evidence that suggests that cannabis played an important role in some of the religious rites in ancient Indian culture. Earliest evidence of cannabis use in Europe and central Asia can be traced back to around 900 to 450 B.C. [1]

Even though the use of cannabis has been commonplace for thousands of years, the mechanisms by which it elicits its pharmacological effects have been shrouded in mystery until fairly recently. Real research in the chemical components of cannabis began towards the very end of the 19\textsuperscript{th} century with the isolation of components of cannabis (1896) and the purification of cannabinol (1898), the first purified natural cannabinoid. For about 60 years after this it was believed that cannabinol was the main psychoactive component of cannabis. However, subsequent research into the metabolites and chemical analysis of cannabis and its components led to the discovery that while cannabinol did have pharmacological activity, the main active ingredient of cannabis was in fact tetrahydrocannabinol (THC) (a precursor of cannabinol). This finally culminated in the isolation and purification of $\Delta^9$ - THC in 1964. [2]
1.1.2. The Endocannabinoid System

After the discovery of ∆⁹ – THC and cannabidiol as the active ingredients of cannabis, there was a lull in cannabinoid research since it was believed that all goals regarding the chemistry and pharmacology of cannabis had been achieved. However, interest in cannabinoid research was renewed again in the 1990s when the molecular mechanisms of cannabinoids as well as endogenous cannabinoid-like compounds were discovered. The first endogenous cannabinoid compound was termed as anandamide (chemically, arachidonoyl ethanolamide) followed soon by the discovery of cannabinoid like pharmacological effects of the previously known metabolite, 2-arachidonoylglycerol. These endogenous compounds along with many others discovered later were together termed as the endocannabinoid system and their discovery resulted in the revival of cannabinoid research.

Early theories about the mechanism of action of cannabinoids postulated that they elicited their effects through membrane disruption due to the highly hydrophobic nature of the ligands. However, further research into the chemistry of these compounds led to the hypothesis of specific ligand binding sites giving rise to the idea of cannabinoid receptors.

1.1.3. Cannabinoid Receptors

The first cannabinoid receptor was discovered in 1990 using a radiolabelled synthetic cannabinoid-like compound. This receptor was termed as CB₁ and was found to be abundant in the central nervous system. In 1993, through a series of homology modeling experiments, another form of the CB receptors
was found. [3] This subtype was termed as CB₂ and was found to be present primarily on the cells of the immune system. [4]

Both the subtypes of the CB receptors are G protein coupled receptors coupled to the G\textsubscript{i/o} protein. [3][4][5] There is some evidence that suggests that CB\textsubscript{1} receptors are sometime also coupled to the G\textsubscript{s} protein. On a molecular level, CB receptors appear to cause a decrease in adenylyl cyclase and as a result cyclic-AMP through G\textsubscript{i/o} signaling. In addition to this, CB\textsubscript{1} receptor activation has been observed to cause a paradoxical increase in cAMP levels which supports the evidence suggesting CB\textsubscript{1} receptors sometimes couple with G\textsubscript{s} proteins. Cannabinoid receptors have also been implicated in the regulation of ion channels particularly potassium and calcium most likely through G protein signaling. Mitogen activated protein kinase (MAPK) signaling which plays a role in cellular growth, proliferation and programmed cell death, has been observed to be up-regulated with cannabinoid receptor activation. [5][6]

1.1.4. Pharmacology of CB drugs

The most well-known physiological effects of cannabinoids are their effects on the central nervous system. Prominent among these effects are the feeling of euphoria and relaxation at lower doses and depression at higher doses. These effects are usually accompanied by a dulling of the senses. Chronic exposure or high doses of cannabinoids can also cause impairment of cognitive and psychomotor functions. [7][8] Antinociceptive effects of cannabinoids are well known and are believed to be due to the effects of cannabinoids on the thalamus in the central nervous system. They have also shown some anticonvulsive effects but potential in clinical management of seizures has not been explored fully due to behavioral side effects. [8]
Peripherally, cannabinoids show a variety of effects on multiple systems. Tachycardia and vasodilation are common cardiovascular effects. CB2 receptors are primarily present on immune cells and their activation by cannabinoids results in immune suppression.\[7\]

1.1.5. Therapeutic Potential of Cannabinoids

The cannabis plant and cannabinoids have been used for medicinal purposes long before the knowledge of its active ingredients and molecular mechanisms. However the use has declined significantly since the beginning of modern medicine. This decline can be attributed to the strict regulation of the use of cannabinoids due to their psychoactive properties.

Nevertheless, cannabinoids represent a class of drugs which has tremendous potential if we can manage to separate the psychoactive effects from the peripheral and modulatory pharmacological effects. Cannabinoids have been shown to be effective as appetite stimulants and antiemetic. As such they could be possibly effective as adjuvants in chronic treatments of severe diseases like cancer and HIV. Antinociception is a major and well known pharmacological effect of cannabinoids and thus cannabinoids could find a potential role in analgesia and pain management. CB2 receptors are found to be mainly localized on immune cells and thus cannabinoids might provide relief in a number of inflammatory and autoimmune disorders. Cannabinoids are currently clinically indicated in glaucoma in order to reduce intraocular pressure. \[9\]\[10]\[11\]
1.2. **The Opioid System**

1.2.1. **History of Opioids**

The history of opioids in human civilization is quite similar to that of cannabinoids in that both classes of compounds have been in use for recreational and medicinal purposes since ancient times. As is the case with cannabinoids, the mechanism of pharmacological action of opioids was also discovered quite recently (1960s).[^12] However, the mechanism of action of opioid compounds is better understood than that of cannabinoids and this has led them to be one of the most widely used classes of drugs in modern medicine.

1.2.2. **The Opioid System**

Opioid drugs elicit their pharmacological effects by signaling through the opioid receptors (OR) which were discovered in 1970s. Opioid receptors are abundant and widely distributed in the body and elicit a wide range of physiological effects. This led to the hypothesis and subsequent finding of opioid receptor subtypes namely mu opioid receptor (MOR), kappa opioid receptor (KOR) and the delta opioid receptor (DOR). These names were derived from agonists predominantly binding to a particular subtype of OR. A fourth subtype of the opioid receptors known as the nociceptin orphanin FQ peptide receptor (NOR) has also been discovered. Endogenous peptides acting on each of the subtype of OR have also been uncovered.[^13]
All opioid receptor subtypes, like cannabinoid receptors, are membrane bound G-protein coupled receptors consisting of seven transmembrane domains. There is a high degree of sequence homology between the three subtypes of opioid receptors. Although many of the physiological effects of opioids have been characterized, the underlying mechanisms of these effects are not as well understood. It is known that all opioid receptors signal through the $G_{i/o}$ subtype of the G protein. All of the classical subtypes of opioid receptors (MOR, KOR and DOR) seem to elicit their effects on cellular function by inhibition of adenylyl cyclase – cAMP pathway as well as interaction with further downstream pathways like the PI3K and Akt pathways. These signals are believed to be caused by the $\alpha$ subunit of the $G_{i/o}$ protein. The $\beta\gamma$ subunit of the $G_{i/o}$ protein also appears to participate in the signaling process by regulating ion conduction across the cell membrane. Furthermore, recent findings suggest that apart from the shared mechanisms of signaling the different subtypes also have additional downstream signaling pathways. For instance, activation of KOR by selective agonists seem to result in activation of kinase cascades in the cells whereas, activation of MOR results in conversion of the MOR into a RTK-like unit causing additional regulation of cellular cAMP levels.

1.2.3. Clinical uses of opioids

Although the uncovering of the mechanism of action of opioid drugs is a recent discovery, opioids have widely been used for thousands of years. Opioid class of drugs constitute one of the most potent and popular class of therapeutic agents used in pain management in modern medicine.

A common clinical indication for opioids is in acute pain and pain associated with cancer and cancer chemotherapy. The World Health Organization (WHO) has put forth guidelines for the use of
weak to strong opioid drugs in management of cancer related pain and there is evidence that this
treatment for pain is effective and well tolerated in cancer patients.\textsuperscript{[20][21]} Opioids are also used to treat
pain in arthritis when pharmacological intervention with less potent pain medication fails to provide
relief.\textsuperscript{[22]} While efficacy of opioids in management of all kinds of pain is well established, their use in
treatment of non-cancer related pain is somewhat more controversial than other clinical indications.
\textsuperscript{[23][24]} The problems of abuse potential, development of tolerance and withdrawal symptoms and major
central and peripheral side effects\textsuperscript{[25]} are the main obstacles to opioids as first line of treatment in
chronic non-malignant pain. Overcoming these issues would make opioids a very versatile class of
therapeutic agents in modern medicine.

1.3. Cannabinoids, Kappa Opioids and Diuresis

As stated above, both cannabinoid and opioid drugs exert a wide variety of pharmacological effects on
the body. The reasons for such a wide spectrum of activity of both cannabinoids and opioids can be
attributed to the widespread distribution of the cannabinoid and opioid receptors not only in the CNS but
also the rest of the body.

Studies have shown that cannabinoid receptors are present in abundance in the urinary tract of rodents
(rats and mice) as well as higher primates and humans.\textsuperscript{[26]} It was found that the CB receptors are present
in the peripheral tissues including urinary bladder of mice were prejunctional CB1 receptors.\textsuperscript{[27]}
Characterization of cannabinoid receptors in isolated rat bladder model has been done using
immunohistochemistry and functional assays and it was found that the cannabinoid receptors were
localized in the urothelium.\textsuperscript{[28]} Similar studies using immunofluorescence and RT-PCR testing done on
mouse bladder tissues led to the discovery of genes expressing CB1 receptors in the mouse urinary system. In humans and nonhuman primates, it was found that cannabinoid receptors are present in the lower urinary tract as well as the higher spinal centers involved in regulation of bladder control. In the urinary tract the receptor expression appears to be higher in the urothelium than the detrusor. Previous studies exploring role of CB1 receptors in diuresis have shown that administration of cannabinoids cause an increase in diuresis in both rats and mice. This effect shows a dose dependent biphasic dose response curve. There is evidence supporting the hypothesis that this dose dependent increase in diuresis caused by cannabinoids in intact and awake mice is caused by activation of CB1 receptors with little to no participation of CB2 receptors. One of the purposes of this project is to confirm this increase in diuresis in mice using the CB1 agonist AM4054.

After the discovery of different subtypes of opioid receptors, diuresis was one of the first effects observed with administration of the kappa opioid drugs. At the time this effect was observed in rats and was attributed to the decrease in antidiuretic hormone caused by kappa opioids. Further evidence of this diuretic effect was provided by antagonism studies carried out using nor-binaltorphimine (nor-BNI) in rats. It was found that in rats, nor-BNI, a highly selective kappa antagonist, decreased diuresis caused by kappa agonist U-50488 but beta-funaltrexamine (selective irreversible μ antagonist) did not affect U-50488 diuresis. This indicated that the diuretic effect originated purely in the kappa opioid system. Studies on the mechanism of kappa mediated diuresis have shown that while the effect is caused by central nervous system regulation of antidiuretic hormone levels, a peripheral component is also believed to be involved. The diuretic effect of kappa opioids has been documented in higher primates as well. A dose dependent increase in urine output was observed in rhesus monkeys when tested with a number of kappa opioid agonists. On the other hand morphine, a mu opioid, did not show this effect and in fact shows an antidiuretic effect. In human, it
has been observed that i.m. injections of kappa agonist U-62066 dose dependently increased volume of urine output. \cite{42} Studies with M320, a mixed kappa/mu opioid agonist has shown a bell shaped curve for diuresis in rats. \cite{39} The purpose of the following study is to verify the diuretic effect of kappa opioid U-50488 and to test whether chronic administration of THC affects the diuretic dose response to U-50488.
2. Specific Aims

The aim of the project was to determine whether tolerance develops to the diuretic effects of kappa opioids after repeated treatment with THC, and if it does occur then, to evaluate the magnitude and rate of recovery of the observed effects. Published data reveal that cannabinoids and kappa opioids both elicit a diuretic response in rodents. A number of receptor mechanisms have been suggested for this effect; however a comprehensive explanation accounting for all the features of this effect has not been established. Determining the effects of repeated cannabinoid administration on the diuresis caused by cannabinoid and kappa opioid drugs might shed some additional light in the mechanisms through which these effects occur leading to better characterization and understanding of these two classes of drugs.

In order to test the aim stated above the experiments will be organized as follows:

1) To verify that administration of CB1 agonist, AM4054 and kappa opioid, U50488 in naïve mice causes a dose dependent increase in urine output using 0.9% saline as vehicle for comparison.

2) Confirm that tolerance to the diuretic effect of cannabinoid agonists can develop after chronic administration of THC by measuring diuresis caused by CB1 agonist, AM4054 before and after chronic 7 day treatment by THC in mice.

3) Determine if mice treated chronically for 7 days with THC develop tolerance to the diuretic effects of kappa agonist, U 50488.

4) Test whether chronic administration of THC effects diuresis caused by loop diuretic, furosemide.
3. Materials and Methods

3.1. Animals

Male CD1 mice bought from Charles River Laboratories (Wilmington, MA) were used for all the diuresis studies. Weight of the mice measured at the start of each study was in the range of 25-35 g. The animals were housed in groups of 4/5 per cage in a climate controlled room in the animals care facility. The animals had access to food and water as required whenever they were not under study. Occupied cages were replaced with clean cages every week. The animals were allowed to acclimatize to the new surroundings for 7 days prior to preliminary handling. Repeated studies on same mice were avoided whenever possible but in case of unavoidable repeated testing, a minimum gap if 7 days was allowed to pass in between studies. All the studies were conducted during the light part of the animals’ day/night cycle. All the studies conducted during the research were in accordance to protocols approved by the Northeastern University Animal Care and use Committee in compliance to regulations set by the National Research Council.

3.2. Measurement of Diuresis

The diuretic effects of the drugs in mice were measured by the amount or urine voided by the mice over duration of 6 hours. Mice did not have access to food or water during this period. The mice were weighed and the pre-study weight was noted down before every study.
For the measurement of urine output, the mice were placed under plastic cups on top of a metal grate. Measuring weights were placed on top of the cup in order to prevent them from moving around. The grate was placed on top of weigh boats arranged in such a way that there was a single mouse exactly on top of one boat. The weights of the boats were measured and noted prior to placing them under the grate. Care was taken that the cups were placed in such a way as to avoid any urine from spilling into neighboring weigh boats.

Measurement of urine output was done over a period of 6 hours and in order to avoid loss of urine to evaporation, a measurement of weight of the boats was done every one or two hours. The boats were replaced by new boats (weighed beforehand). The amount of urine voided was calculated as the change in weight of the boats in two hours. The mice in the various test groups were randomized in order to avoid bias.
3.3. Drugs

**Table 1:** The drugs used in the above stated experimental protocol are listed below

<table>
<thead>
<tr>
<th>Name of Drug</th>
<th>Chemical Nomenclature</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Δ⁹ THC</td>
<td>Δ⁹-tetrahydrocannabinol</td>
<td>National Institute on Drug Abuse [(NIDA), Rockville, MD]</td>
</tr>
<tr>
<td>2. AM4054</td>
<td>9β-(hydroxymethyl)-3-(1-adamantyl)-hexahydrocannabinol</td>
<td>Center for Drug Discovery [(CDD), Northeastern University, Boston, MA]</td>
</tr>
<tr>
<td>3. U50488</td>
<td>trans-(+/-)3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]-cyclohexyl)-benzeneacetamide methane sulfate</td>
<td>Sigma Aldrich [St. Louis, MO]</td>
</tr>
<tr>
<td>4. Furosemide</td>
<td>4-chloro-2-(furan-2-ylmethylamino)-5-sulfamoylbenzoic acid</td>
<td>Sigma Aldrich [St. Louis, MO]</td>
</tr>
</tbody>
</table>
3.4. **Statistical Methods**

The urine output was measured in grams of urine voided over a duration of 6 hours of testing. Urine output was converted to g/kg using the formula \[\text{weight of urine voided (in grams)} / \text{weight of mouse (in kilograms)}\]. Mean urine output for each dose was average of urine output of each mouse in group. Standard Error Mean (SEM) was calculated using the formula \[\text{Standard Deviation of group} / \text{Square root of number of subjects in group}\].

Development of tolerance was measured by change in the ED50 values for the dose response curves. ED50 for the ascending and descending portions of the biphasic curves were calculated separately using linear regression analysis in graph pad prism. The potency ratios were calculated as a ratio of \[\text{ED50 of dose response curve after THC treatment/ ED50 of dose response curve before treatment}\] of Data analysis for significance was done using ordinary one way ANOVA with significance limit set at \(p < 0.05\). Bonferroni correction was used when more than one comparison was made.
4. Rationale, Research Strategy and Study Design

4.1. Rationale

Both cannabinoids and kappa opioids have been known to cause diuresis in mice and literature so far suggests that the type of diuresis caused by cannabinoids is similar to that caused by kappa opioids. Both classes of drugs are believed to cause diuresis by decreasing vasopressin levels.

A number of pharmacological effects (antinociception, diuresis) and more importantly adverse effects (addiction, withdrawal, tolerance) are common to both these classes of drugs.

Thus, it was of interest to explore if the endocannabinoid system and the opioid system interact with or affect each other in any significant manner.

Such and exploration could shed additional light on the molecular mechanisms of effects associated with these drugs which in turn could be beneficial to for development of next generation of cannabinoids and opioids with fewer adverse effects.

4.2. Research Strategy

The studies undertaken to achieve the specific aims of the project were as follows

Study 1: To test the effects of chronic THC dosing on diuretic dose response of AM4054

Study 2: To test the effects of chronic THC dosing on diuretic dose response of U-50488

Study 3: To test the effects of chronic THC dosing on diuretic dose response of furosemide
4.3. Study Design

- Arrival of new mice to facility
- Preliminary Handling
- Preliminary Vehicle Data
- Vehicle Data

- At least 7 Days
- At least 4 Days
- At least 2 Days

- Baseline Measurement
- THC Injections Begin
- THC Injections End
- 24 Hour Measurement
- 7 Day Measurement
- 14 Day Measurement

- Day 0
- Day 1
- Day 7
- Day 8
- Day 15
- Day 22
5. Results

5.1. Validating Cannabinoid mediated diuresis in mice

The optimal dose of saline to be used as the vehicle control (fluid load) was fixed at 10 ml/kg based on previous findings in our lab. [34]

In order to confirm previously observed diuretic effects of cannabinoids AM4054 was administered to 5 groups of mice at the following doses 0.01 mg/kg (n = 6), 0.03 mg/kg (n = 6), 0.10 mg/kg (n = 6), 0.30 mg/kg (n = 6) and 1 mg/kg (n = 6). Urinary output over a period of 6 hours was measured. As shown in the figure, AM4054 showed a biphasic dose response curve with maximum effect observed at the dose of 0.3 mg/kg with the maximum urine output of 34.99 ± 5.73 g/kg. The dose of 0.01 mg/kg appeared to be ineffective for diuretic effect but was included to get a complete dose effect function. Using ordinary one way ANOVA, it was determined that administration of AM4054 causes a statistically significant (p = 0.0001) change in mean urine output in mice. This confirmed previous observations that cannabinoids and specifically AM4054 cause a dose dependent increase followed by a decrease in diuresis in mice.
Figure 1: Biphasic dose response curve for cannabinoid agonist AM4054 (n = 6) over a duration of 6 hours post drug administration in CD1 mice. ‘V’ represents a subcutaneous dose of 10 ml/kg volume of vehicle for comparison. * = p value ≤ 0.05 represents statistically significant differences in means of test group and vehicle group.
5.2. Validating Kappa Opioid mediated diuresis in mice

Confirmation of increased diuresis by kappa opioid receptors was done by administering 1 mg/kg (n = 6), 3 mg/kg (n = 7), 10 mg/kg (n = 7), 30 mg/kg (n = 6) and 100 mg/kg (n = 6) doses of kappa agonist U50488. 10 ml/kg saline was used as vehicle treatment and the test was carried out for 6 hours as stated above. A biphasic dose response curve for the diuretic effect was noted with a peak effect at the dose of 30 mg/kg with urine output of 34.24 ± 4.34 g/kg. ANOVA analysis of the data showed that U50488 also dose dependently caused a statistically significant change (p = 0.0005) in the urine output in mice. This confirmed that the kappa agonist, U50488 does cause a dose dependent increase followed by a decrease in urine output in mice.
Figure 2: Biphasic dose response curve for kappa opioid agonist U50488 (n = 6 or n = 7) over duration of 6 hours post drug administration in CD1 mice. ‘V’ represents a subcutaneous dose of 10 ml/kg volume of vehicle for comparison. * = p value ≤ 0.05 and *** = p value ≤ 0.001 represents statistically significant differences in means of test group and vehicle group.
5.3. Effects of Chronic THC dosing on the diuretic dose response of the CB1 agonist, AM4054

In order to determine the effects of chronic THC administration (10 mg/kg) on the diuretic response of CB1 agonist AM4054, urine output was measured before chronic THC dosing (baseline) and after chronic THC on days 1 (24 hour), 7, and 14. The doses of 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10 mg/kg AM4054 were tested as well as urine output following administration of 10 ml/kg vehicle (see figure 3). The full dose response curve for diuretic effects of AM4054 1 day after the last of the chronic THC dosing showed a significant rightward shift; both the ascending and descending parts of the biphasic curve were affected. The increases in ED50 values quantify the magnitude of the shift and are presented, along with the potency ratios, in Tables 2 and 3.

The rightward shift in the curves is indicative of tolerance to the diuretic effects of AM4054 caused by chronic THC administration. The 24 hour measurement represents the maximum tolerance that was measured. At 7 days after ending the THC treatment, both the limbs of the AM4054 dose response curve were to the left of the 24 hour dose-response curve, but to the right of the baseline dose response curve. This shows that partial recovery from the developed tolerance begins within the first 7 days after chronic THC dosing is stopped. The 14 day diuresis measurement shows an almost complete recovery of diuretic activity from the observed tolerance. The potency ratios showed an approximately 3.5 (ascending) – 4.5 (descending) fold increase in ED50 of the diuretic effect 1 day after chronic THC dosing, and these values decreased over time.

Overall, the data show that chronic (7 day) administration of THC causes development of tolerance to the diuretic effects of the CB1 agonist AM4054 and recovery from the tolerance to the baseline effect takes place within 14 days of discontinuation of THC dosing.
**Figure 3**: Diuretic dose response curves of CD1 mice with AM4054 before chronic THC dosing (blue), 1 day post chronic THC dosing (green), 7 days post chronic THC dosing (purple) and 14 days post chronic THC dosing (yellow). ‘V’ (red) represents the average urine output of the mice after 10 ml/kg vehicle administration used in the study. Each data point represents the average urine output of 3-6 mice, and vertical lines represent the SEM. All urine output measurements were measured in grams of urine per kilogram of weight.
Table 2: ED50 Values and potency ratios for the ascending part of the AM4054 dose response curves

<table>
<thead>
<tr>
<th>Curve</th>
<th>ED50 (mg/kg)</th>
<th>Potency Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM4054 - Base</td>
<td>0.08</td>
<td>1</td>
</tr>
<tr>
<td>AM4054 - 1 Day</td>
<td>0.26</td>
<td>3.31</td>
</tr>
<tr>
<td>AM4054 - 7 Days</td>
<td>0.14</td>
<td>1.79</td>
</tr>
<tr>
<td>AM4054 - 14 Days</td>
<td>0.06</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Table 3: ED50 Values and potency ratios for the descending part of the AM4054 dose response curves

<table>
<thead>
<tr>
<th>Curve</th>
<th>ED50 (mg/kg)</th>
<th>Potency Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM4054 - Base</td>
<td>0.64</td>
<td>1</td>
</tr>
<tr>
<td>AM4054 - 1 Day</td>
<td>3.03</td>
<td>4.69</td>
</tr>
<tr>
<td>AM4054 - 7 Days</td>
<td>1.38</td>
<td>2.13</td>
</tr>
<tr>
<td>AM4054 - 14 Days</td>
<td>0.18</td>
<td>0.28</td>
</tr>
</tbody>
</table>
5.4. Effects of Chronic THC dosing on the diuretic dose response of the kappa opioid, U50488

Urine output with U50488 before chronic THC dosing (baseline) and after chronic THC on days 1, 7, and 14 was measured in order to evaluate the effect of tolerance to THC on the diuretic dose response of U50488. The dose response of U50488 was determined using doses of 1, 3, 10, 30, and 100 mg/kg (see Figure 4). The baseline measurement showed a dose dependent biphasic diuretic response to U50488 in mice. Testing the diuretic effect of U50488 on mice made tolerant to THC indicated that the dose response curve lost its slope as increasing the dose U50488 did not produce diuretic responses. The ED50 values for the diuretic dose response in THC-tolerant mice could not be calculated due to this flattening of the dose response function. Statistical analysis of the diuretic response curves in tolerant mice showed that the slopes of the curves were very close to zero and that deviation of slopes from zero was not statistically significant (see Table 4). This effect was reproducible when tested with a slight modification to the method of measuring amount of urine voided, changing the interval from 2 hours to 1 hour (data not shown).

Through visual observation of the results (Figure 4), it can be seen that THC tolerant mice failed to reach the maximum urine output at any dose of U50488. It was also noted that the overall downward shift of the diuretic function remained stable over the first 7 days after THC administration and failed to recover to baseline values even at 14 days after halting the THC dosing. In order to ensure that the lack of reproducibility of the diuretic effects of U50488 could not be attributed to repeated exposure to U50488, two U50 dose-effect functions were determined in a separate group of mice which was never administered THC (see Figure 5). Results demonstrated no change in the effects of 1-30 mg/kg U50488 in mice never exposed to THC [p-value (0.4931) > 0.05].
Figure 4: Diuretic dose response curves of CD1 mice with U50488 before chronic THC dosing (red), 1 day post chronic THC dosing (green), 7 days post chronic THC dosing (purple) and 14 days post chronic THC dosing (yellow). ‘V’ (blue) represents the average urine output of all the mice after 10 ml/kg vehicle administration used in the study. Each data point represents the average urine output of 5-7 mice and vertical lines represent the SEM. All urine output measurements were measured in grams of urine per kilogram of weight.
Table 4: Linear regression analysis of diuretic dose response in THC tolerant mice

<table>
<thead>
<tr>
<th>Curve</th>
<th>Slope of Curve</th>
<th>95% Confidence Intervals of Slope</th>
<th>Is slope significantly non-zero?</th>
</tr>
</thead>
<tbody>
<tr>
<td>U50488 - Baseline</td>
<td>0.5445</td>
<td>0.04866 to 1.040</td>
<td>0.0420, Significant</td>
</tr>
<tr>
<td>U50488 - 1 Day</td>
<td>0.0549</td>
<td>-0.1285 to 0.2385</td>
<td>0.4108, Not Significant</td>
</tr>
<tr>
<td>U50488 - 7 Days</td>
<td>0.0626</td>
<td>-0.1296 to 0.2549</td>
<td>0.3760, Not Significant</td>
</tr>
<tr>
<td>U50488 - 14 Days</td>
<td>0.3114</td>
<td>-0.3954 to 1.018</td>
<td>0.1984, Not Significant</td>
</tr>
</tbody>
</table>
Figure 5: Diuretic dose response curves of CD1 mice with repeated U50488 administration with no exposure to THC dosing. Base-line 1 (red) was measured on day 1 and base-line 2 (green) was measured after a 4 day rest period. ‘V’ (blue) represents the average urine output of 24 mice after 10 ml/kg vehicle administration used in the study. Each data point represents the average urine output of 6 mice and vertical lines represent the SEM. All urine output measurements were measured in grams of urine per kilogram of weight.
5.5. Effects of Chronic THC dosing on diuretic dose response of furosemide

The effects of chronic THC administration (10 mg/kg) on the diuretic response to the loop diuretic furosemide were tested by measuring baseline urine output (before chronic THC dosing) and post chronic THC Day 1 urine output. The doses of 10 mg/kg and 30 mg/kg of furosemide were tested and vehicle urine output at the dose of 10 ml/kg was used for comparison, results are depicted in Figure 6. The diuretic effects of furosemide 1 day after the last of the chronic THC dose, were not significantly different from the baseline measurement [p-value = 0.6425 (10 mg/kg); p-value = 0.5643 (30 mg/kg)]. The results confirmed our hypothesis that chronic THC does not affect diuretic response of the locally acting loop diuretic furosemide. These findings are in line with the literature which suggests that cannabinoid mediated diuresis is regulated through a mechanism other than local action at the nephron.
**Figure 6:** Diuretic dose response curves of CD1 mice with furosemide before chronic THC dosing (red) and one day post chronic THC dosing (green). ‘V’ (blue) represents the average urine output of the mice after 10 ml/kg vehicle administration of 12 mice used in the study. Each column represents the average urine output of 6 mice. All urine output measurements were measured in grams of urine per kilogram of weight.
6. Discussion

The aim of the project was to explore the effects of THC tolerance on the diuretic effects of AM4054 (a full CB1 agonist) and U50488 (a selective kappa opioid agonist). Both cannabinoids and kappa opioids have been known to cause diuresis in mice and previous studies suggest that the type of diuresis caused by cannabinoids is similar to that caused by kappa opioids [34] [36]. A number of pharmacological effects (antinociception, diuresis) and more importantly adverse effects (addiction, withdrawal, tolerance) are common to both these classes of drugs. Thus, it was of interest to explore if the endocannabinoid system and the opioid system interact with or affect each other in any significant manner.

AM4054 is a full agonist at the CB1 receptor. It shows the full spectrum of CB1 mediated activities similar to THC. However, one significant characteristic of AM4054 is that it is more potent than other full CB1 agonists, i.e., it can elicit the same magnitude of CB1 effects at lower doses as compared to other CB1 agonists. The literature also suggests that it may have a shorter half-life than other CB1 agonists. A previous study conducted in our lab testing the diuretic effects of cannabinoids in mice has shown that AM4054 exhibits a biphasic dose responses curve [34].

Published research classifies U50488 as a highly selective kappa opioid agonist based on evidence from antagonism studies and binding studies. U50488 also shows a diuretic effect in rats at the doses of 10 mg/kg and 30 mg/kg. Tolerance to the analgesic effect of U50488 is known to develop after chronic administration of U50488 in rats and the mechanism of tolerance is believed to be through phosphorylation of the opioid GPCR [47].
Our preliminary findings confirmed previous findings, showing that both AM4054 and U50488 caused a dose dependent diuretic effect in mice. Interestingly, both these drugs showed a biphasic dose response curve (reported in previous studies and confirmed with this data) which is unusual for a diuretic dose response. The biphasic nature of the dose effect function was not explored in detail as that was beyond the scope of the project. However, visually we noted that test subjects in the higher dose groups showed a higher degree of sedation than the lower dose groups for both AM4054 and U50488. This could be one of the possible reasons for decline in urine output at higher doses. A more in depth look into the biphasic nature of the diuretic dose response might potentially help in identifying secondary messenger pathways involved in the signaling of these drugs.

THC is the principle constituent of cannabis and is responsible for most of its psychoactive properties. Like other psychoactive agents, tolerance can develop to the different effects of cannabinoids after chronic exposure. Studies have shown that THC tolerance to the antinociceptive effects starts developing about 3 days on a regimen of 10 mg/kg dose twice daily \(^{[48]}\). We modified this regimen slightly and changed the dose of THC to 10 mg/kg once daily for seven days in order to develop tolerance to THC.

Our results from comparing the diuretic response of mice made tolerant to THC with that of normal mice showed that AM4054 and THC were cross tolerant for their diuretic dose response. Unpublished observations from previous studies in our lab (Chopda Dissertation) showed that THC is capable of developing tolerance to its own diuretic effects. Mice tolerant to THC showed approximately a 3.5 to 4.5 fold rightward shift as compared to normal mice. Both the limbs of the dose response curve showed this effect. Cross tolerance between THC and AM4054 suggests that both these drugs bring about suppression in vasopressin levels by binding to the same receptors (specifically the CB1 receptors). Measurement of diuresis with AM4054 at 14 days showed an almost complete recovery from the
tolerance. This recovery was in line with literature which states that CB1 agonist binding requires about 7 to 14 days in order to recover back to normal levels from tolerance states.\(^{[49]}\). Studies have shown that down regulation of CB1 receptors and desensitization of the respective G-protein are primarily responsible for development of tolerance\(^{[50]}\). We concluded from our results that both THC and AM4054 most likely share a common binding site and common secondary signaling pathways to produce the diuretic effects. AM4054 is more potent than THC and our findings suggest that it could be a reasonable substitute or adjunct to THC in cannabinoid research. However, tolerance to effects of cannabinoids is not always apparent and does not occur equally for all effects. Hence exploration into cross tolerance between THC and AM4054 for other effects such as antinociception and hypothermia might be beneficial.

U50488 is a selective kappa opioid agonist with a proven dose dependent diuretic effect in rats. The diuretic dose response curve for naïve mice exhibited a dose dependent biphasic dose effect function. Previous studies done with U50488 and diuresis in our lab tested the dose response up to a maximum dose of 60 mg/kg\(^{[34]}\). Testing with 100 mg/kg showed a decrease in urine output which was an unexpected observation. This decrease in urination could be because of a sedative effect (observed visually) of U50488 at higher doses. Studies suggest that THC and kappa opioid agonists share a common mechanism for their antinociceptive effects\(^{[51]}\). Since both THC and U50488 elicit their diuretic response through Gi/o protein signaling and suppression of vasopressin\(^{[52]}\), we hypothesized that mice made tolerant to THC should show tolerance to the diuretic effects of U50488 as well. Interestingly we observed that chronic THC administration caused a flattening of the U50488 diuretic dose effect function. Linear regression of diuretic response curves in THC tolerant mice showed that the slopes of the curves were close to zero i.e. increasing dose of U50488 did not necessarily increase the diuretic dose response. Repeated testing with U50488 (with no exposure to THC) showed no change in
the diuretic dose response indicating that this effect observed in tolerant mice was not due to repeated administration of U50488. While these results do not indicate cross tolerance between THC and U50488 in the traditional sense, they suggest that daily THC interferes with the functioning of the kappa opioid receptors or the signaling pathways associated with them. One feature of this proposed interference was that even testing at 14 days post cessation of THC administration (suggested point of recovery from THC tolerance), the dose effect function failed to recover to baseline levels. This suggests that this effect that was observed might be a long term effect on kappa opioid signaling. Testing the nature of this interference in more detail was beyond the scope of the project but could be an interesting avenue for future studies.

Furosemide is a potent diuretic in clinical use which causes diuresis by local action at the loop of Henle of the nephron. Cannabinoids cause diuresis by centrally suppressing anti-diuretic hormone levels. It is suspected that there is a peripheral component to the diuretic effects of cannabinoids. Thus we carried out a study to determine whether THC tolerance affects the diuretic response of furosemide in mice. Our findings showed that the diuretic dose response of furosemide in mice remained unaffected by chronic THC. Since diuretic effect of furosemide remained unaffected 1 day after chronic THC, the test was discontinued as we believed taking measurements at 7 days and 14 days would serve no further purpose. From our findings we concluded that THC and furosemide have completely different molecular mechanisms of diuretic effects. Interference of AM4054 with furosemide diuresis might indicate additional molecular mechanisms of AM4054 other than CB1 signaling since THC is also a CB1 agonist and does not interfere with furosemide diuresis.
7. Conclusions

The results of the different studies undertaken as part of the project suggested the following inferences regarding the effects of THC tolerance on diuresis of other cannabinoids and kappa opioids:

- Both cannabinoids and kappa opioids show a dose dependent biphasic dose response curve in naïve CD1 mice in accordance to published literature. Molecular basis for decrease in urination at higher doses has not been studied could be an interesting aspect to explore further.

- AM4054 shows a cross tolerance with THC indicating that the mechanisms of diuretic effect share a common signaling pathway. AM4054 could be an adjunct to or substitute to THC in future of cannabinoid research.

- The flattening of the dose response function of U50488 in THC tolerant mice was unexpected and suggests that THC interferes with kappa opioid binding or signaling on some level. This opens up interesting future perspectives for studying the interaction between the endocannabinoid and opioid systems which are the two major systems involved in pain perception in animals.

- THC tolerance has no effect on the diuretic effect of furosemide indicating that the peripheral component of CB1 mediated diuresis (if it exists) does not overlap with mechanisms of loop diuretic drugs.
8. Bibliography


