EVALUATION OF SIGMA LIGAND AZ66 ANALGESIC POTENTIATION OF CANNABINOIDS

by

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ABSTRACT

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Sigma receptors have become a popular subject for research the past few decades, but there is still much mystery behind these receptors and how they work. Sigma receptors, like cannabinoid-1 (CB1) receptors, have effects on the human body including appetite regulation, depression, and analgesia. AZ66 is a highly selective sigma receptor ligand that has antagonistic properties. Sigma receptor antagonists have been shown to potentiate the analgesic effects of opiates; however, there are no known literature reports about the interaction between the sigma receptor system and the endocannabinoid system, including CB1 receptors. The purpose of this study is to evaluate AZ66 for potentiation of CB1 related analgesic effects. A tetrad assay was performed for AZ66 using doses 5, 10, and 20 mg/kg (i.p.). The tetrad battery was performed one hour post drug (AZ66) administration. The potentiation study was completed using a 20 mg/kg dose of AZ66 against a 0.3 mg/kg dose of CP 55940. The AZ66 dose was administered one hour before the CP 55940 dose administration, and then the analgesic study was performed 15 minutes post CP 55940 dose administration. The tetrad assay showed that AZ66 exhibited no analgesic effects on its own compared to the control compounds. The potentiation study resulted in significant potentiation of the peripheral analgesic effects of CP 55940 with the addition of

AZ66 and insignificant potentiation of central analgesic effects. Future studies will be performed to validate the findings of this study and to further examine the interaction between the sigma receptor system and the cannabinoid system.

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I. Background

Marijuana, or cannabis, is not simply just one drug; it is a plant made up of numerous different components that all have varying effects on the human body. Cannabis has been used recreationally for thousands of years, but little was known about the plant and its capabilities up to a few decades ago [1]. Interest and research on this plant has skyrocketed due to advances in technology, starting in the 1960's with the isolation and synthesis of one of the major compounds: (-)Δ^9-tetrahydrocannabinol (THC) [1]. This compound is well-known because it is the primary psychoactive component of cannabis that gives the most effective "high" feeling sought after by recreational users [2]. Cannabidiol (CBD) is another type of cannabinoid that is well known for its positive pharmacological effects and lack of psychoactivity [2]. THC and CBD, however, are only two of 105 cannabinoids discovered so far in cannabis, all having varying psychoactive, analgesic, and behavioral effects [3]. Cannabinoids are classes of compounds that are isolated from the plant matrix and have the potential to interact with the endocannabinoid system in the body by modulating cannabinoid receptors [2]. Cannabis research took a new direction by focusing on the endocannabinoid system and the function and manipulation of cannabinoid receptors.

The endocannabinoid system was first realized in the 1980's when two separate cannabinoid receptors were hypothesized based on pharmacological

studies [1]. They were termed CB1 and CB2, and research uncovered that they are both G-coupled protein receptors (GPCRs) [1]. Research on the endocannabinoid system and the effects of endocannabinoids on its receptors has grown in popularity because of the system's effects on physical and pathological functions of the body such as motor activity, short-term memory, stress response, appetite, anxiety, and analgesia [2,4]. CB1 and CB2 receptors are located in different parts of the body and therefore produce varied effects. CB1 receptors are found in the spinal cord and the higher brain areas like the basal ganglia, cerebellum, hippocampus, and hypothalamus, and they produce the typical acute effects of cannabinoids like THC [4]. CB2 receptors, on the other hand, are found mainly in immune tissues and elicit analgesic and anti-inflammatory responses in the body [1]. Knowledge of the physiological components of the endocannabinoid system and the pharmacological effects of the system has aided the research of medical and therapeutic uses of marijuana.

There are many accounts of cannabis and its prominent usage in medicine in early Indian and Greek literature [5]. It was not until the early 20th century that the United States government classified marijuana as a Schedule 1 substance, making the usage of marijuana illegal and the research of marijuana incredibly difficult [5]. Schedule 1 drugs are identified as having a high potential of addiction with no medical benefit [4]. This classification contradicts the majority of marijuana research, and this is why the push for marijuana to be taken off of the Schedule 1 classification and legalized is gaining momentum in the United States. Due to the lack of research, the process is hindered because research

and standardization of marijuana are limited due to current policy that is difficult to change [4]. Despite the stagnant policies, there are already numerous approved medial uses of cannabinoids. The FDA has approved THC treatments for cachexia from HIV/AIDS and vomiting resulting from chemotherapy [6]. Canada and countries in Europe have also approved marijuana for treatment of spasticity from multiple sclerosis [6]. Marijuana use has been further recommended for anorexia, glaucoma, arthritis, migraines, and chronic pain [4]. There are numerous recent studies looking at the treatment of chronic pain by the suppression of hyperalgesia and allodynia using endocannabinoids or synthetic cannabinoids because the potential for addiction and abuse are vastly lower than with the use of narcotics for pain [5]. This study analyzes the interaction of the sigma receptor system with the endocannabinoid system to suppress pain.

The sigma receptor system was discovered by accident a decade after the endocannabinoid system was discovered, and the sigma receptor was initially believed to be an opioid receptor subtype [7]. This is because the first study performed used a ligand that allowed a cross reaction between the opioid and sigma receptors [7]. Almost ten years later, the two receptors were differentiated by using a ligand that did not cross react, and the new receptor was termed the sigma receptor [7]. Since then, two subtypes of receptors have been found in this system; sigma-1 receptor and sigma-2 receptor. There has been extensive research on the biological and physiological roles of sigma-1 since it was cloned from a guinea pig in 1996, but sigma-2 has still yet to be cloned or extensively

researched [8]. Sigma-1 is a chaperone molecule in the endoplasmic reticulum and plasma membrane of cells [9]. Sigma-1 receptors also are known to regulate neurotransmitter systems that are involved in numerous neuropsychiatric disorders [8]. This receptor plays a role in the addiction processes, depression, Alzheimer's disease, Parkinson's disease, Schizophrenia, and most notably for this experiment, pain [8,9]. Sigma receptors can react with a variety of compounds like opiates, neuroleptics, antihistamines, and antidepressants [10].

There have been numerous studies of sigma receptors and pain using sigma-1 receptor agonist and antagonists. There has been successful research since the 1990's on the interaction with opioid receptors to mediate opioid analgesics. [8]. Sigma-1 agonists have shown attenuation of opioid analgesics while sigma-1 antagonists show potentiation of opioid analgesia [7]. Sigma-1 receptors actually increase the intrinsic activity of opioids by shifting the dose-response curve leftward for stimulation yet not affecting the maximum stimulation by the opioid or the binding affinity of the opioid to its receptor [11]. The potentiation of opioid activity is a potential answer to the age old question of how to separate the pain treatment of opioids from the abuse potential and adverse effects such as constipation and respiratory depression [11]. This thought process was applied to this experiment, except analyzing the prospective potentiation of endocannabinoid analgesia by a sigma receptor antagonist without potentiation of the adverse psychoactive effects the activation of the endocannabinoid system.

Opiates and cannabinoids are both potent analgesics, and their receptor systems have several similarities. While there are two sigma receptor subtypes, sigma-1 and sigma-2, there are four identified opioid receptor subtypes- mu (MOP), delta (DOP), kappa (KOP), and nociceptin (NOP) [12,13]. Of the four opioid receptors, three are considered to be the classical opioid receptors; MOP, DOP, and KOP. The activation of opioid or cannabinoid receptors both cause behavioral effects such as anti-nociception, sedation, hypothermia, hypotension, and motor depression, which suggests a similar mechanism of action and dispersal [12,14]. Both receptor types are also found in central nervous system (CNS) regions participating in anti-nociception, most notably localized in the superficial dorsal horn of the spinal cord where the nociceptive afferents make initial synaptic contact with the central nervous system [12,14]. Both receptor types are G-coupled protein receptors (GPCRs), so both have signal transduction properties that signal the G-protein alpha subunit to target the cAMP pathway [14]. The anti-nociception of both receptors occurs from the inhibition of cAMP production which activates MAP kinases by a second messaging system, and neurotransmitter release is inhibited by the inhibition of calcium channels and stimulation of potassium channels, causing an inhibitory post-synaptic potential [12]. This shared transduction pathway is confirmed by the location of both receptor types on presynaptic neuron terminals. [12]. Because of these anatomical and transduction pathway similarities and the confirmed potentiation of opioid analgesia by sigma ligands, the idea of the potentiation of cannabinoid analgesia by sigma ligands is examined in this experiment.

In this experiment, we examined the role of the synthetic and highly selective sigma receptor antagonist ligand AZ66 with CP 55,940, a synthetic agonist of the CB1 and CB2 receptors. CP 55,940 mimics the properties of THC, but it is ten times more potent than THC and possesses none of the depressive effects of THC. AZ66 is a highly selective antagonist to sigma receptors, and it has shown anti-convulsion and anti-psychotic effects like other sigma antagonists [10]. There will be many windows of opportunity to advance drug development with AZ66, but for this experiment the compound's potentiation of CP 55,940 analgesia was analyzed [15].

II. Methods

Synthesis of AZ66

The sigma ligand AZ66 was synthesized through a six-step process shown in Figure 1. First 4-fluorroaniline (*a*), hydrochloride was made into compound *b* through reflux with NH_4SCN in water for four hours and then recrystallization with ethanol. Compound *b* was refluxed with bromine in chloroform for two hours to form compound *c*. Compound *d* was then formed by the mixture of compound *c* with KOH. Compound *d* was refluxed with carbonyl-1,1'-diimidazole for three hours to form compound *e*, which was then alkylated with 1,4-dibromopentane in DMF for three hours to form compound *f*. Lastly, compound *f* was mixed with cyclohexyl piperazine to form the AZ66 (*g*). The compound AZ66 was converted

to the hydrochloride salt for the following *in vivo* behavioral studies.

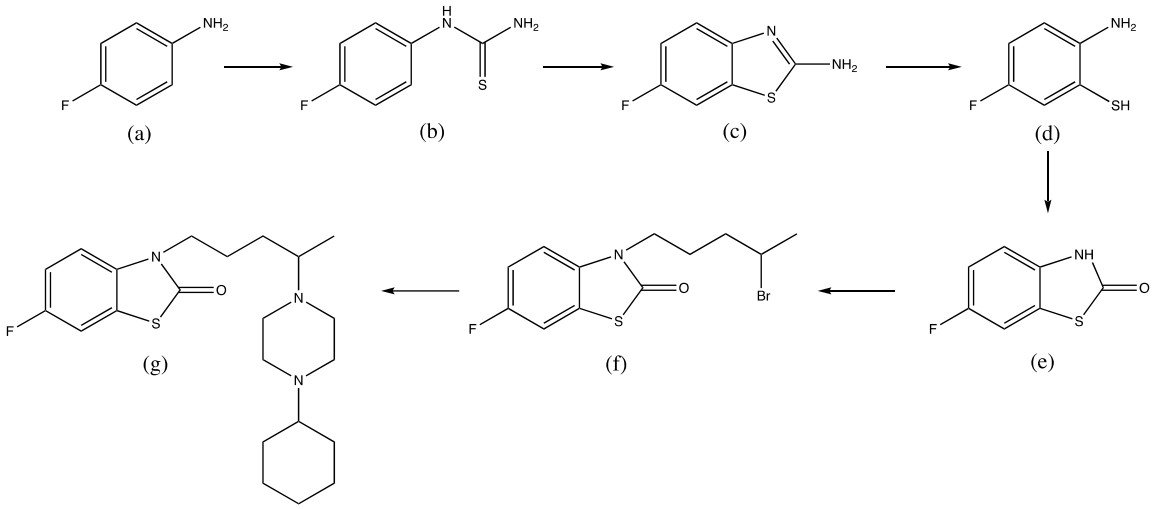

Figure 1. The synthesis of AZ66.

Subjects

For this experiment adult, male C57BL/6 mice were used, each having a weight range of 18-25 grams. C57BL/6 were optimal subjects because they are more susceptible to the effects of drugs of abuse, and male subjects are used because the estrus cycle of female mice is known to possibly interfere with behavior studies, especially analgesic studies. Eight mice were used per study performed for both the tetrad assay and the analgesic study for statistical value; less than 6 mice would not work with ANOVA analysis, and over 8 would be considered an excess of loss of life. The drugs were delivered by intraperitoneal (i.p.) injection proportional to the weight of each mouse. The mice were randomly divided into 10 groups for each the studies: 5 control groups and 5 challenge groups.

All methods performed were approved by the Institutional Animal Care and Use Committee (IACUC). Morphine was purchased from Sigma Aldrich

Procedures involving animals were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC).

Drug Preparation

CP 55,940 was acquired from Tocris Bioscience (Bristol, United Kingdom). AZ66 was synthesized by Dr. Christopher McCurdy's lab (University of Mississippi, Oxford). Cremophor and ethanol were obtained from Sigma Aldrich (Bellefonte, PA). All drugs were dissolved according to the methods of Olson et al (1973). A mixture of Ethanol, Cremophor, and Saline was prepared using a ratio of (1:1:18). Drugs were completely dissolved into ETOH before adding Cremophor and saline. Drugs were delivered to the animals using an intraperitoneal (i.p.) injection.

Tetrad Assay

The tetrad assay was developed by Martin et al. (1994). The purpose of the tetrad assay was to determine the sedative and analgesic effects of the sigma ligand AZ66 alone by analyzing behavior changes due to the drug in question. Four behaviors are analyzed in this assay; mobility, catalepsy, hypothermia, and analgesia. Cannabinoids such as THC show changes in all four behavioral aspects, so this assay is often used to screen drugs for these cannabinoid-like effects. The cannabinoid agonist, CP 55,940 was used as the positive control, and the vehicle that AZ66 was dissolved in was used as the negative control. Three AZ66 doses of 5mg/kg dose, 10mg/kg dose, and 20mg/kg were used. For each trial, the subjects were acclimated to the chamber of the hotplate 24 hours in advance for 15 minutes each. The subjects were also acclimated to the testing

room for 30 minutes on the day of the trial. The baseline readings for the hotplate, catalepsy, hypothermia, and tail flick assays were taken for each subject. The subjects were then injected i.p. with the drug dose, which took a maximum of thirty minutes to take effect. During this wait, the subjects were placed in the locomotor chambers for 30 minutes of acclimation. After these thirty minutes, the locomotor assay recorded the locomotor activity of each subject for thirty minutes. Each locomotor chamber was composed of 16 by 16 beam rays, and the computer recorded every time the subject broke a beam. These photo beam breaks were quantified as a measure of locomotor activity.

The subjects then went through the hotplate assay. This assay analyzes the perceived analgesic effect of the drug. The subjects are placed on a hotplate at 52°C, and a glass cylinder was placed over them to ensure they stay on the hotplate. The latency of pain indicators was then analyzed by manually stopping the timer when cues such as licking, tapping, or adjusting the back paw on the side of the cylinder were observed. These actions were indicators that the subject began feeling pain from the heat of the hotplate. The maximum time the mouse was allowed on the hotplate was 45 seconds to prevent heat damage to the subject's paws.

Next, the subjects were placed in the catalepsy test to analyze the psychoactive effect of the drug. The subject was positioned so that its front paws rested on a metal bar while its back paws were on the tabletop. The time it took the subject to climb on or off the bar was manually recorded. Subjects that stay in

the initial position for more than five seconds are considered cataleptic, or unaware of their surroundings.

The subjects were then analyzed for hypothermia. The core temperature of the subjects was analyzed by a rectal temperature probe. Any changes in core body temperature from the baseline reading were recorded.

The subjects were placed in the tail flick assay to analyze changes in reflex analgesia. For this assay, the subjects were placed in plastic restrainers to decrease movement that would disrupt the test. The subject was laid upside down on the surface of the machine with its tail extended in a ridge. A high-energy beam of light then hit the distal part of the tail, and the machine measured the latency of the tail flick. The beam of light and timer automatically stopped when the subject moved its tail away from the beam.

Potentiation Study

The potentiation study was conducted to specifically measure the analgesic effects of the combination of AZ66 with a low dose of CP 55,940. The goal of this study was to see if the sigma receptor antagonist would potentiate the analgesic effects of CP 55,940. The hotplate and tail flick assays were used for this study with the same procedure as above. The subjects were acclimated to the hotplate assay for 15 minutes 24 hours prior to the trial, and on the day of the trial they were acclimated to the testing room for 30 minutes. After this acclimation period, the baseline readings for the hotplate and tail flick test were taken. Then the mice were injected i.p with either the (1:1:18) vehicle or 20 mg/kg AZ66 and then placed back in their cage for an hour. The subjects were then dosed with 0.3

mg/kg CP 55,940 or 5 mg/kg morphine and placed in their cage again for 15 minutes. The 0.3 mg/kg CP 55,940 is a low enough dose to not produce any anti-nociception on its own, so any anti-nociception observed in the trial would directly be linked to the potentiation effect of AZ66. The changes in analgesia were analyzed by the hotplate and tail flick assays.

Data Analysis

Data was shown as mean ± SEM. with each group having an n=8 animals. Both hotplate and tail flick were expressed as percent maximum effect (%MPE=[(post-drug latency-basal latency)/(cutoff latency-basal)]x 100 (Martin et. al, 1994). Statistical analysis was performed using one-way ANOVA preceded by the Dunnett's post hoc test to define significant different against the vehicle control at $p<0.05$.

III. Results and Discussion

Tetrad Assay

Figures 2-6 below summarize the results of the tetrad assay with the three doses of AZ66 compared to the CP 55,940 positive control and the (1:1:18) vehicle control. The point of the tetrad assay was to analyze if AZ66 had any behavioral effects on its own without the use of CP 55,940. If no effects were seen in the tetrad assay, then all analgesic effects seen in the potentiation study could be contributed to the potentiation of CP 55,940 only. Figure 2 shows the locomotor activity effects of AZ66 compared to the vehicle and 1 mg/kg dose of

CP 55,940. The 1 mg/kg dose of CP 55,940 produced statistical significance with a p value <0.001 compared to the vehicle. All three doses of AZ66 showed no statistical significance compared to the vehicle. It can be inferred that there was much more locomotor activity with the AZ66 compared to the CP 55,940 dose.

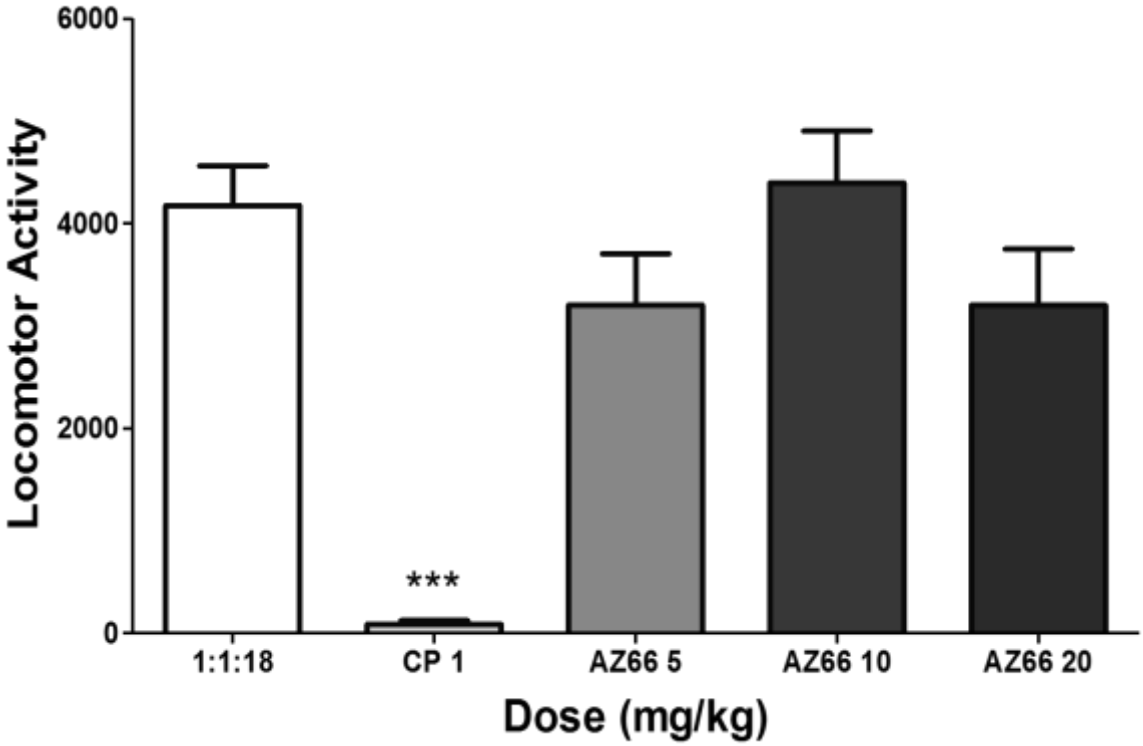

Figure 2: Locomotor activity post-injection of the three doses of AZ66 compared to CP 55,940 and (1:1:18) vehicle.

Figure 3 illustrates the catalepsy effects of AZ66 with the positive and negative control. CP 55,940 showed significant latency, while the AZ66 doses showed virtually no change in latency. With AZ66 the subjects almost always hopped off the bar or changed positions immediately, showing no psychoactive effects.

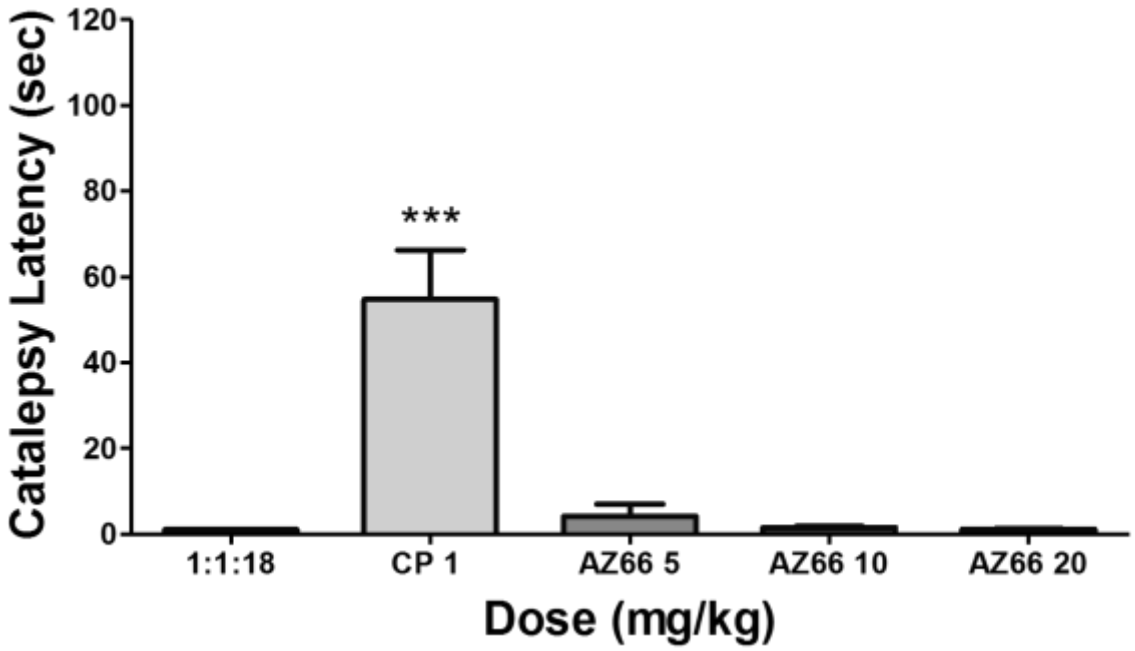

Figure 3: The catalepsy latency post-injection of three doses of AZ66 compared to the 1 mg/kg dose CP 55,940 and the (1:1:18) vehicle.

The hypothermia results are seen in Figure 4. CP 55,940 shows a significant decrease in core body temperature, whereas the AZ66 doses showed a slight, while statistically insignificant, increase in body temperature post injection compared to the vehicle. The vehicle showed mostly no change in body temperature.

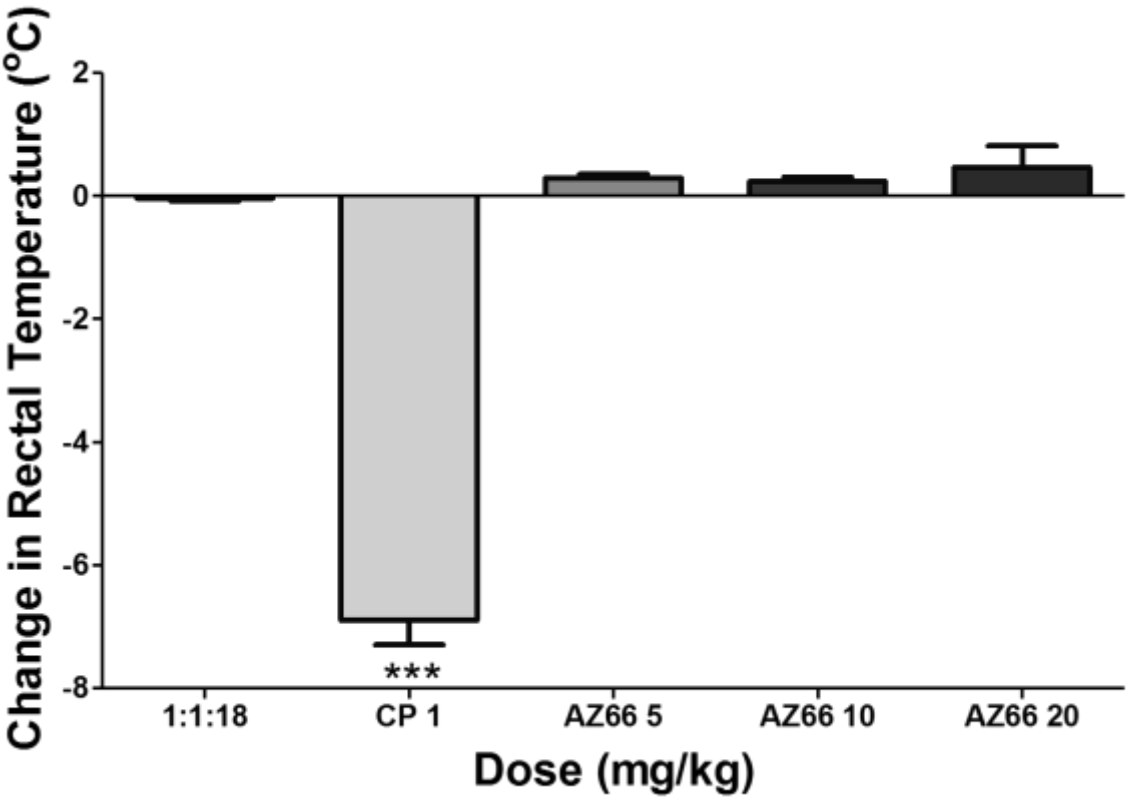

Figure 4: The hypothermia effect post-injection of three doses of AZ66 compared to 1 mg/kg dose CP 55,940 and the (1:1:18) vehicle.

Figures 5 and 6 show the analgesic effects of AZ66 alone compared to the vehicle and CP 55,940. Figure 5 shows the results of the hotplate assay with the three doses of AZ66, 1 mg/kg CP 55,940, and the (1:1:18) vehicle. Again, only the CP 55,940 dose showed significant latency, and the doses of AZ66 resembled the vehicle results. The 5 mg/kg dose of AZ66 showed a decreased latency compared to the baseline, which is inconsistent with the results of the other AZ66 doses. Therefore, AZ66 does not have a large effect on perceived analgesia as compared to CP 55,940.

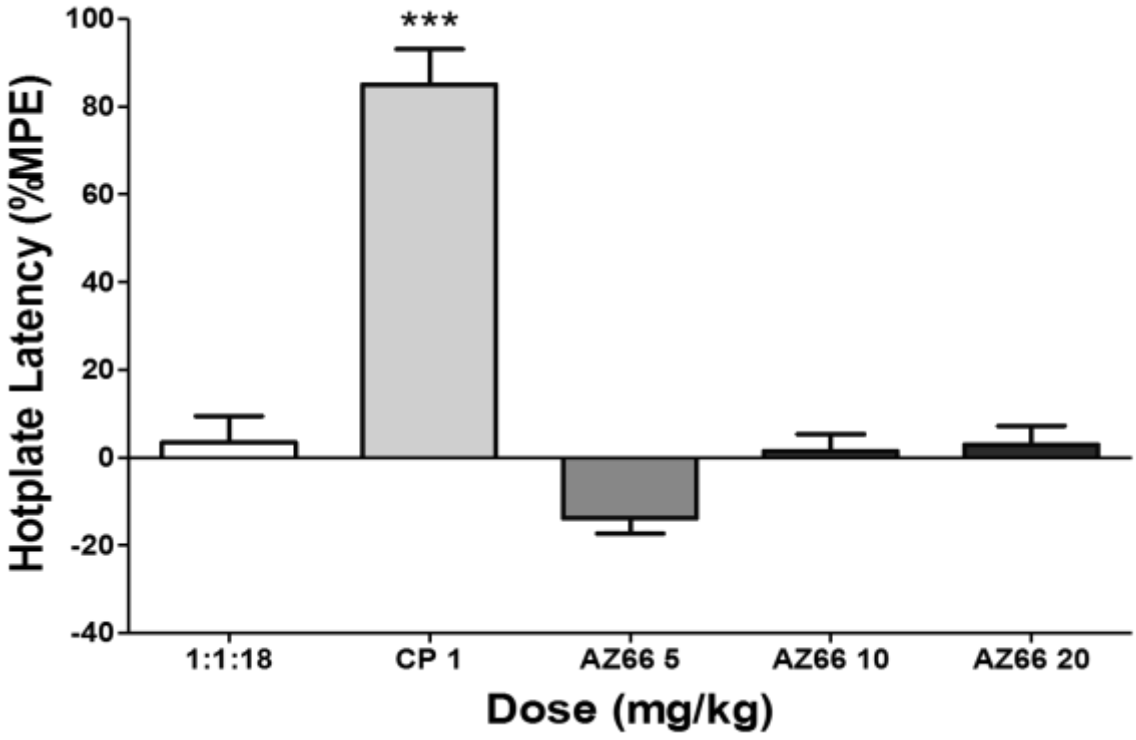

Figure 5: The hotplate latency post-injection of three doses of AZ66 compared to 1 mg/kg dose CP 55,940 and the (1:1:18) vehicle.

Figure 6 displays the reflex analgesic effects of AZ66 compared to CP 55,940 and the vehicle. The CP 55,940 dose showed significantly increased latency post injection, and the AZ66 doses showed mostly no change compared to the vehicle and baseline readings. AZ66 showed no analgesic effects on its own, as seen in Figures 5 and 6. This is important because any latency results in the potentiation study can be attributed to the potentiation of these effects from CP 55,940 and not from AZ66 itself.

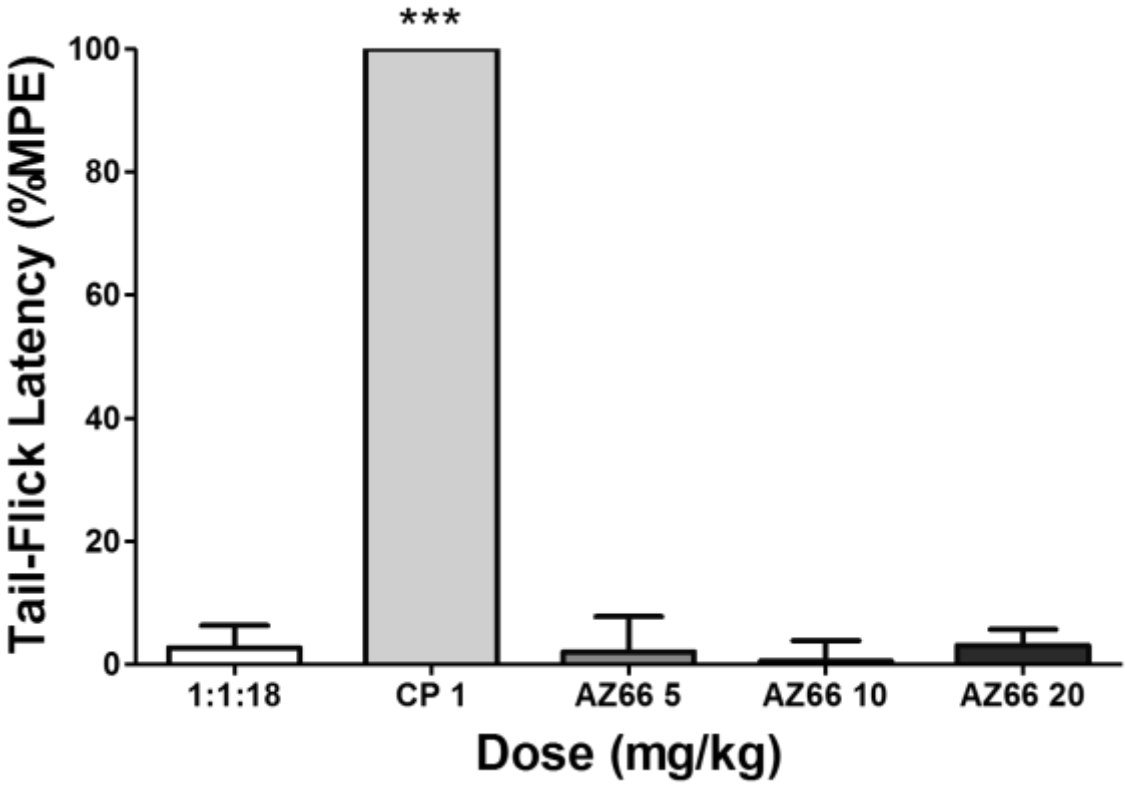

Figure 6: The tail flick latency post-injection of three doses of AZ66 compared to 1 mg/kg dose CP 55,940 and the (1:1:18) vehicle.

Potentiation Study

Figures 7 and 8 illustrate the results from the analgesic potentiation study. A 20 mg/kg dose of AZ66 was used with 0.3 mg/kg dose of CP 55,940. The 0.3 mg/kg dose of CP 55,940 would itself fail to show significant analgesic effects due to the low dosage, so any significant results can be attributed to the successful potentiation of CP 55,940 with AZ66. The negative control used was using two doses of the vehicle in the same fashion the dosage of CP 55,940 with AZ66. The vehicle was also used with CP 55,940 to contrast the effects of CP 55,940 with AZ66 to the effects of CP 55,940 itself. Results from a previous study done in

Dr. McCurdy's lab that analyzed the potentiation of a 5 mg/kg dose of morphine with AZ66 were also included. Since the potentiation of morphine with sigma receptor antagonists is already well documented, the morphine study was used to compare these potentiation results with the CP 55,940 potentiation results. Figure 7 illustrates the latency results from the hotplate assay. The perceived analgesic effect of the 0.3 mg/kg dose of CP 55,940 was not significantly higher than the vehicle negative control. The latency results of the trial with CP 55,940 and AZ66 revealed a p value of <0.01 compared to the vehicle. Although the AZ66/morphine combination showed a greater effect with a p value of <0.001, the AZ66/CP 55,940 combination's significant results infer that AZ66 did successfully potentiate the perceived analgesic effects of CP 55,940.

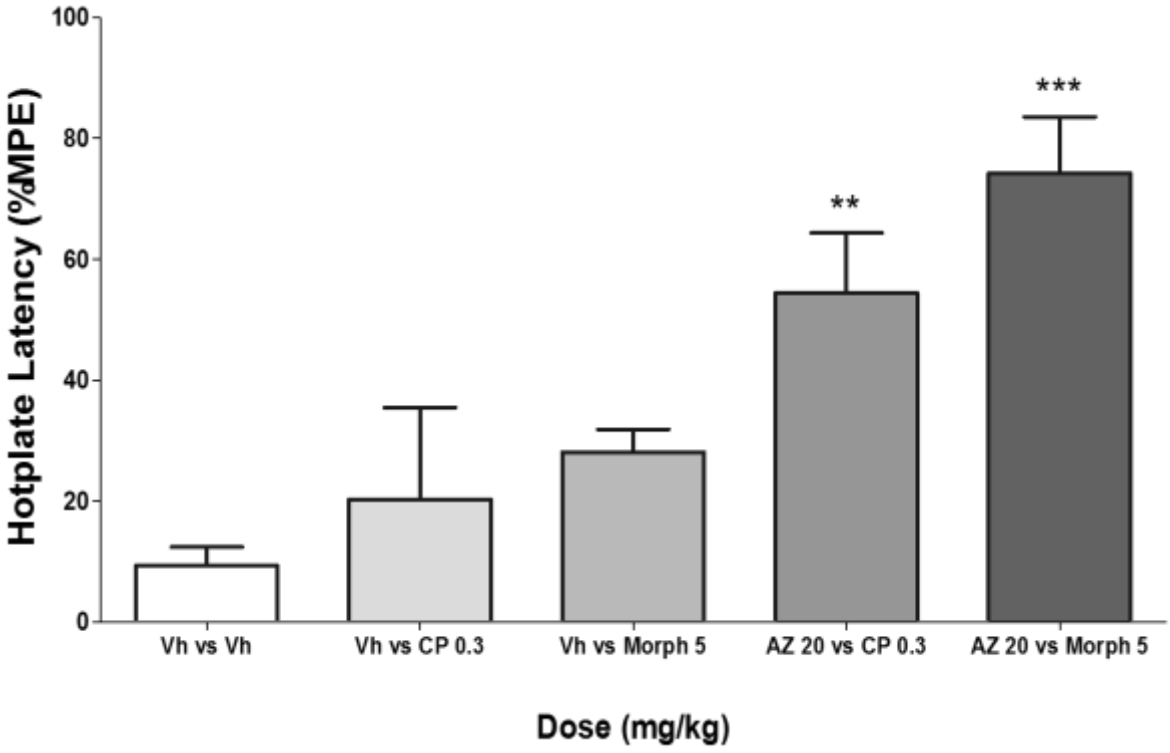

Figure 7: The hotplate latency shows the potentiation of perceived analgesic effects of 0.3 mg/kg CP 55,940 and 5 mg/kg morphine with 20 mg/kg AZ66.

Figure 8 shows the results from the tail flick assay. The reflex analgesic effects of CP 55,940 alone were not significantly higher than the vehicle. The CP 55,940 potentiated with AZ66 did not show a significant increase in tail flick latency compared to the negative control Vh vs CP dosage. The AZ66 vs morphine combination showed a p value of <0.001. There was not a successful potentiation of the CP 55,940 reflex analgesia with AZ66 compared to the large potentiation of morphine's reflex analgesia by AZ66.

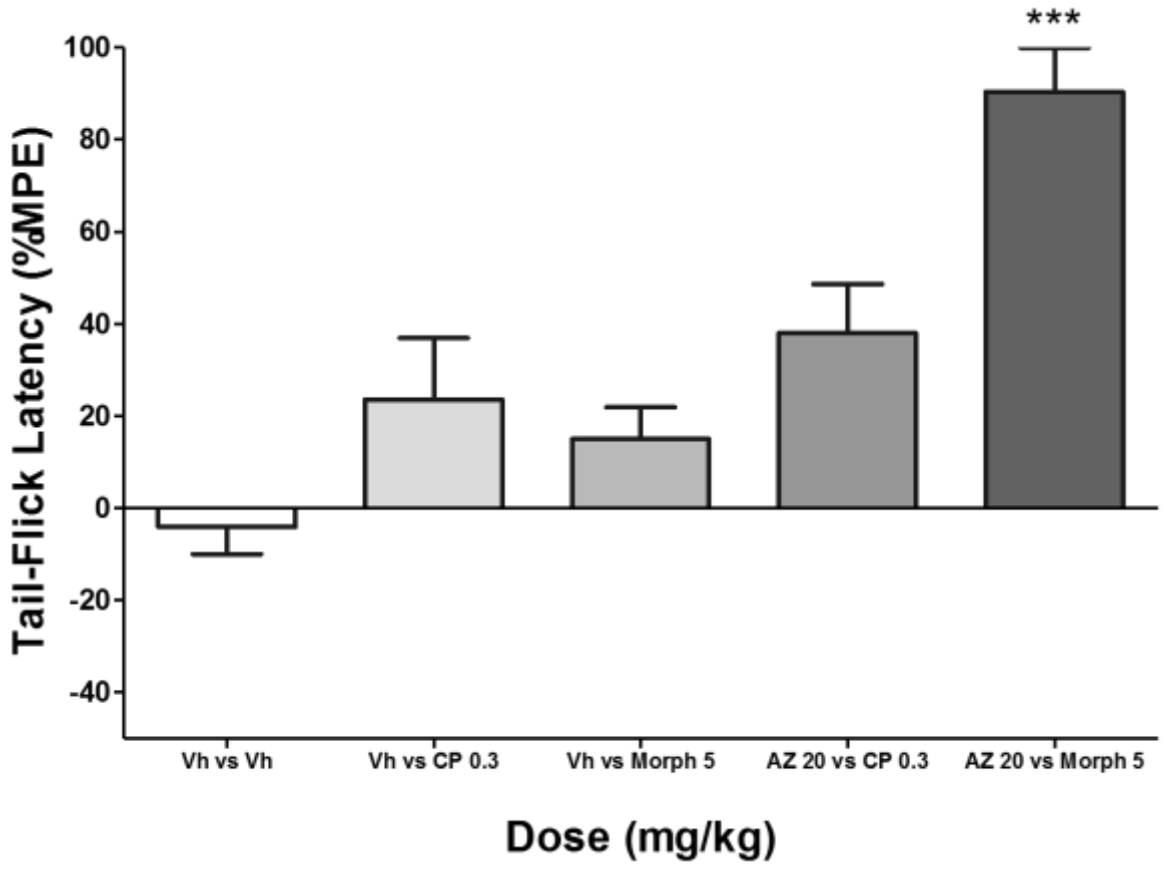

Figure 8: The tail flick latency shows the reflex analgesic effects of 0.3 mg/kg CP 55,940 and 5 mg/kg morphine with 20 mg/kg AZ66.

IV. Conclusion

Since there was no previous knowledge or literature of any interaction between the sigma receptor system and the cannabinoid receptor system, this is the first study to indicate that sigma receptor antagonists can directly enhance the analgesic effects of cannabinoids. This data can create many new opportunities for further study and use of the sigma receptor system. The use of sigma receptor antagonists with morphine is significant because this combination increases the analgesic effects of a low dose of morphine without increasing

adverse side effects such as constipation, tolerance, and addiction [11]. This logic can be applied to sigma receptor antagonists and cannabinoids because of the similarities of the opioid and cannabinoid systems. This study revealed an interaction between the cannabinoid system and the antagonism of sigma receptors, and it further indicated that the sigma receptor antagonist AZ66 potentiates the analgesic effects of low doses of cannabinoids without increasing the psychoactive effects of the drug. Specifically, the analgesic study showed that AZ66 significantly potentiated the perceived analgesic effects of CP 55940, but it did not significantly increase the reflex analgesic effects. Nevertheless, the study supported the idea of interaction between the two systems, and it gives way into a potential new field of research for the drug delivery of medical marijuana.

This study lays the groundwork for future research, but there are several studies to be performed to better understand the interaction between the cannabinoid system and sigma receptor antagonism. Because there are two types of sigma receptors, a current study is analyzing whether the analgesic potentiation of opiates and cannabinoids is from sigma-1 antagonists, sigma-2 antagonists, or both. A timed study will be performed to deduce how long the potentiated effects last, and potentiation studies using varied doses of AZ66 will also be executed to find the optimal AZ66 dose. Lastly, research will also be conducted to analyze the modification of other behaviors besides analgesia through sigma receptor antagonists. These studies will help researchers better

understand the interaction between the sigma receptor system and the cannabinoid system for further study and applications.

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