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The Evaluation of AZ66, A Highly Selective Sigma-1 and Sigma-2 Receptor Antagonist, For Its Anti-Convulsive Effects in C57BL/6 Mice

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THE EVALUATION OF AZ66, A HIGHLY SELECTIVE SIGMA-1 AND SIGMA-2 RECEPTOR ANTAGONIST, FOR ITS ANTICONVULSIVE EFFECTS IN C57BL/6 MICE

By

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ABSTRACT

JAMIE STONE: The Evaluation of AZ66, A Highly Selective Sigma-1 and Sigma-2 Antagonist, for Anticonvulsive Effects in C57Bl/6 Mice

(Under the direction of Lisa Wilson and Dr. Christopher McCurdy)

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. Researchers have found that sigma receptor antagonists can attenuate cocaine induced convulsions, however, limited research has been conducted on the effects of these antagonists on convulsions that mimic the types of seizures associated with epilepsy. Therefore, the aim of the current study was to evaluate AZ66 (20 mg/kg i.p.), a highly selective sigma 1 & 2 receptor antagonist, against pentylenetetrazole (PTZ) (80 mg/kg s.c.) induced convulsions. The first aim of the study was to determine the optimal dose and dosing method for the administration of PTZ in our laboratories. Both subcutaneous (s.c.) and intraperitoneal (i.p.) injections were separately investigated at 50-80 mg/kg and 30-60 mg/kg doses respectively. The second aim was to find the minimum dose of AZ66 that was effective at reducing or attenuating PTZ induced convulsions. The AZ66 dose was administered one hour before the PTZ dose administration. Seizure latency and frequency were recorded. The subcutaneous 80 mg/kg dose of PTZ was chosen due to its fast response time and clear distinction between seizures. AZ66 decreased seizure latency and increased seizure frequency and resulted in death when given at the 30mg/kg dose. Further studies on the role of the sigma receptors in epilepsy are needed.

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I. Background

Epilepsy is a debilitating disease that affects over 2.4 million adults and 450,000 children in the United States alone *("Hope Through Research"*, 2016). This chronic disorder is recognized by the World Health Organization (WHO) as one of the most common neurological diseases worldwide ("Epilepsy Fact Sheet" 2016). Perhaps the most alarming thing about epilepsy is that anyone can develop it regardless of sex, age, or ethnic background. Caused by an unbalance of hyperactive electrical signals in the brain, epilepsy is commonly characterized by unpredictable and frequent seizures.

While epilepsy is a disorder of multiple seizures over a period of time longer than twenty-four hours, a seizure is a single occurrence. Seizures can be classified into two broad categories: generalized and focal/partial. Generalized seizures affect the whole brain while focal/partial seizures affect only a specific part. Generalized seizures are commonly described by a loss of consciousness and motor control. This type of seizure is further classified into either tonic-clonic, absence, or atonic seizures depending on the symptoms that manifest. Tonic-clonic seizures, formerly known as grand mal seizures, are distinguishable by their jerk-like contractions of the whole body and long recovery time. This type of generalized seizure is typically long in duration and can last for several minutes. Another type of generalized seizures is the absence seizure, also known as the petit mal seizure. This category of seizures is defined by the person being unable to control his or her body position. These seizures look similar to tonic clonic seizures, however the duration of the seizure is much shorter and the recovery time is faster. Atonic seizures refer to generalized seizures in which the patient's muscles suddenly

become limp and unexpectedly drop to the ground. These seizures are also referred to as drop attacks and often result in injury ("*Types of Seizures"*, 2016).

Unlike generalized seizures, in focal/partial seizures consciousness is not lost and the region of the brain affected is limited to a particular area. The affected area of the brain determines the classification of the simple partial seizure as well as the manifestation of the symptoms. These seizures can affect specific motor functions, blood pressure and heart rate, parts of the brain that trigger emotions, and result in sensory hallucinations. Often times, a simple partial seizure will start in one area of the brain and move to another; this results in a complex partial seizure. This type of seizure is distinguished by an altered state of consciousness not seen in simple partial seizures. People diagnosed with complex partial seizures report experiencing staring blankly into space or performing repetitive, non-purposeful tasks. As a whole, epilepsy displays a distinct combination of seizures specific to the individual patient *("Types of Seizures",* 2016). This makes each disease unique to the individual and oftentimes creates difficulties in diagnosing and treating epilepsy.

The National Institute of Neurological Disorders and Stroke (NINDS) considers a person to be epileptic if they have two or more seizures occurring over a time period longer than twenty-four hours *("Hope Through Research"*, 2016). There are many individualized aspects to epilepsy including the cause, type, and severity of the seizures that contribute to the specificity of the disease. It is imperative to understand and consider these aspects when determining the appropriate treatment plan. In the book *The Epilepsies: Seizures, Syndromes and Management,* Panayiotopoulos highlights 3 important diagnostic steps that doctors follow in order to determine the correct therapy (Panayiotopoulos 2005). First, it is important to know that the

seizures are not a result of an underlying, curable illness. Seizures can be caused by a number of disorders including a high fever, a buildup of toxins, abnormal glucose levels, or a brain infection (Shelat, 2015). If a patient is incorrectly diagnosed with epilepsy, then they are being denied lifesaving medical care. As a result, doctors need to exhaust all diagnostic tests before concluding that a person has epilepsy. If the seizures are determined to be caused by epilepsy, doctors then ascertain the type of seizures being presented. This step ensures later on that the treatment selected will be tailored for the individual patient. Finally, after the type of seizure is established, then the epileptic syndrome can be diagnosed. This ensures an accurate and personalized treatment for each patient. Currently, there is no cure for epilepsy but it can be controlled with medication and other lifestyle changes. About 50% of seizures are completely controlled using anti-epileptic drugs (AEDs) and an additional 25% of patients drastically improve (Goldenberg MM, 2010). Along with medication, doctors also implement lifestyle changes such as diet and exercise as part of a holistic treatment plan. The goal is to prevent seizures and improve the patient's quality of life.

As of now, there are only 26 anti-epileptic drugs currently on the market. These drugs produce a host of side effects that range from drowsiness to a loss of coordination or memory, and even death ("List of Anti-Epileptic Drugs", 2014). In order to generate new antiepileptic medications, NINDS created a program that tests compounds for anti-seizure activity called the Epilepsy Therapy Screening Program (ETSP) formerly called the Anticonvulsant Screening Program (ASP). One of the main focuses of this program is to identify potential therapeutic agents that could be used to treat epilepsy ("Epilepsy Therapy Screening Program" 2017). When a compound is submitted to the ETSP, its structure is compared to a large internal

database to help determine any structure-activity relationships and to ensure that the compound is chemically unique. NINDS uses well established rodent seizure models to screen in four categories: Standard Anti-Ictal Screening, Models of Pharmacoresistance, Identification/Differentiation, and Screening for Related Indications. These four areas test the compound's efficacy in treating specific seizure types, determining its effect on the seizure thresholds, and if treats related medical comorbidities.

The Maximal Electric Shock (MES) is a standard Anti-Ictal Screening model used to mimic generalized tonic-clonic seizures. This type of test involves an electric shock to see if the compound will prevent the seizure from occurring when all neuronal circuits in the brain are maximally active. In order to test a substance's ability to treat complex partial seizures, the Kindled Rat Model is employed. This model can also be modified to include a simulation of pharmacoresistance. About 30% of epileptic patients are resistant to traditional anti-seizure drugs (Brodie, Kwan, 2002), so this analysis is a useful way to identify compounds that demonstrate an ability to treat therapy resistant epilepsies. Classified under Identification/Differentiation, the Intravenous Pentylenetetrazole (Metrazol, PTZ) Seizure Threshold Test determines the compounds ability to change the seizure threshold. PTZ is intravenously injected into the rat to produce a chemically induced seizure. If the compound lowers the seizure threshold, then it is considered to be pro-convulsant because the number of seizures increases. Likewise, if the compound decreases the number of seizures it is an anticonvulsant because the substance raised the seizure threshold. The Intravenous PTZ Seizure Threshold Test is a staple in testing potential new anti-seizure medications because it quickly identifies if the compound is counter-effective. Finally, the Mouse Formalin Test of

Hyperalgesia screens for a substance's ability to also treat pain related to epilepsy. This particular model determines if the compound being tested reduces acute and inflammatory pain in the mouse. This test is important because patients, in addition to having seizures, often have to deal with the comorbidities of epilepsy or the side effects of the treatment that affect their quality of life (Kerr, 2012).

Due to the numerous side effects and the limited number of drugs on the market, many patients are seeking alternative forms of therapies. These alternative therapies are often used a last resort when approved forms of medication fail. Among these alternative therapies "herbal remedies are used as first line treatments for all illnesses in an estimated 80% of the world's population" (Kneen, Appleton, 2006). Unfortunately, these nonconventional AEDs are further encouraged by the media with little or no evidence based support and require further investigation to determine the effectiveness of such treatment plans.

One such alternative treatment for epilepsy is marijuana, also known as cannabis. Specifically, there are two compounds of interest in marijuana: cannabidiol (CBD) and tetrahydrocannabinol (THC). These two compounds are both known for their anti-convulsant effects in the MES model of epilepsy, but only THC exerts its effects through the endocannabinoid system. CBD's mechanism of action is still unclear. Interestingly, CBD and THC are suggested to have a synergistic relationship in their anticonvulsant properties due to CBD's mechanisms of action (Szaflarski et al., 2014). The ratio of CBD to THC may determine the effectiveness of treating seizures while minimizing the psychoactive effects associated with marijuana. One study found that cannabis containing high concentrations of CBD and low concentrations of tetrahydrocannabinol (THC) was very effective as an anticonvulsant in animal

models (Szaflarski et al., 2014). While medical marijuana use has been dated back to China approximately 2500 BC (Jiang et al., 2006), there is only circumstantial evidence of its effectiveness to treat epilepsy because it is still unclear how marijuana exerts its anti-epileptic properties (Szaflarski et al., 2014). Nevertheless, cannabidiol has recently gained popularity with the general public as a potential medication for drug resistant epilepsies. Capitalizing on this popularity, GW Pharmaceuticals announced a new experimental drug that, if approved by the Food and Drug Administration (FDA), will be the first marijuana based prescription drug in the United States (Pollack, 2016). Epidiolex contains cannabidiol and is currently showing promising results in treating Dravet Syndrome during phase 3 clinical trials (Pollack, 2016). During this phase, the effectiveness of the drug is statistically calculated against a placebo. After undergoing multiple tests, the FDA can then decide whether or not to approve the drug for sale. Dravet syndrome is a childhood epilepsy known for its resistance to drug therapy. If Epidiolex is approved by the FDA, it could prove to be a major breakthrough in treating intractable epilepsies. While the results of this trial look promising, it is still unknown how cannabidiol exerts its antiepileptic properties or its long term effects.

Because CBD is a schedule 1 drug, illegal due to its high potential for abuse, no currently accepted medical use, and safety concerns, it is important to seek alternative treatments for epilepsy. Sigma receptors could prove to be that alternative treatment. Sigma receptors were once thought to be a class of opioids receptors due to the initial compounds that had opioid-like effects and unique effects that could not be reversed by standard opioid antagonists (Martin, 1976). However, it was later proven to be a separate class of receptors with a selective drug pattern and distinct anatomical distribution. For example, sigma receptors have a high

affinity for (+) benzomorphans, whereas opioid receptors selectively prefer (-) benzomorphans, and were found to produce effects unaffected by opioid antagonists. Sigma receptors interact with a diverse set of compounds including opiates, neuroleptics, antihistamines, and antidepressants. There are two distinct subtypes of sigma receptors. Sigma-1 receptors are expressed more dominantly over sigma-2 and are slightly larger in size. Sigma-1 receptors are also known to translocate during signaling and are associated with regulating intracellular secondary messengers. So far, only the sigma-1 receptor has been sequenced and cloned from a variety of different species, including humans. The identity of the sigma-2 receptor is still unknown and is under current debate. However, there is strong evidence that the sigma-2 receptor is a unique protein that resides in lipid rafts (Chu, et al. 2015). Currently, we do not know of any purely selective sigma-2 compounds. Sigma compounds that interact with receptors either non-selectively bind with either sigma-1 or sigma-2, or solely sigma-1 receptors. The sigma agonists and antagonist that are frequently utilized in research and have a higher affinity for sigma-2 receptors (over sigma-1 receptors) also bind with opioid receptors. Sigma receptors as a whole are found throughout the body. The highest concentration of sigma receptors is found in the brain and spinal cord, most notably in the brainstem motor nuclei. This suggests that sigma receptors play a significant role in motor function and control. Sigma receptors are also found throughout the body in peripheral organs suggesting that perform a necessary physiological function (Matsumoto et al., 2007). Because of their widespread distribution and concentration in motor nuclei, sigma receptors represent a potential target towards creating new medicines and in treating numerous diseases, including epilepsy (Maurice, Su, 2009).

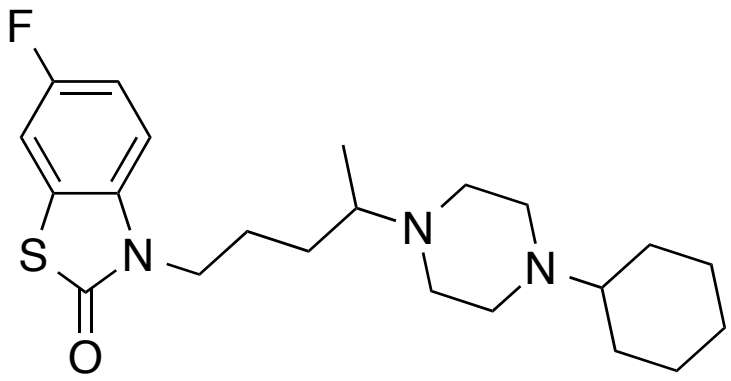


Figure 1: Structure of AZ66

AZ66 {3-[4-(4-cyclohexylpiperazin-1-yl)pentyl]-6-fluorobenzo[*d*] thiazole-2(3*H*)-one} is a highly selective sigma-1 and sigma-2 receptor antagonist that binds with almost equal affinity to each subtype. This synthetic compound was derived from CM156, also a sigma receptor antagonist. AZ66 was developed for its longer half-life and an increased metabolic stability than its precursor CM156. AZ66 has a favorable pharmacokinetic profile and is capable of being administered orally. This novel ligand has been shown to mitigate many methamphetamine-induced behaviors (Seminerio et al., 2016) as well as attenuate cocaine induced seizures (Matsumoto et al, 2002). Because of these properties, it is reasonable to assume that AZ66 could also be effective in reducing seizures produced by epilepsy. Until now, there have not been any studies to determine the effects of AZ66 on seizures that mimic epilepsy. This opens a whole new area of investigation for sigma receptor antagonists like AZ66.

This thesis aims to begin that exploration by studying AZ66's anticonvulsive effects in PTZ induced seizures that mimic epilepsy. PTZ is a $GABA_A$ receptor antagonist that is the gold standard for chemically inducing seizures in anti-epileptic drug assessments (Lüttjohann,

Fabene, & Luijtelaar, 2009). To perform this evaluation, the optimal dose and dosing method of PTZ in our laboratories needed to be assessed. From there, the minimum dose of AZ66 effective at reducing PTZ induced seizures can then be determined.

II. Methods

Subjects

Adult male black C57BL6 mice were acquired from Harlan Laboratories and were the subjects to complete the 2 aims. All animals were housed 5 to a cage and received food and water ad lib. The housing facilities were maintained on a 12-hour light schedule from 6:00am to 6:00 pm. The Institutional Animal Care and Use Committee (IACUC) approved the methods performed in this study.

Drug Preparation

All drugs were dissolved in saline solution before administration. AZ66 was prepared and synthesized in Dr. McCurdy's lab as part of the Department of BioMolecular Sciences Division of Medicinal Chemistry. Drugs were delivered to the animals by either intraperitoneal (i.p,) or subcutaneous (s.c.) injection.

Aim 1

In order to find the optimal dosing method and dose of pentylenetetrazole (PTZ), 8 mice for each dose and dosing method were brought into the procedure room to acclimate for 30 minutes. Afterwards the first mouse was injected with a single dose of PTZ, dissolved in normal saline, and placed into a Plexiglas chamber. There were no more than 2 mice in each Plexiglas chamber at one time. Normal saline was used as a negative control. Each mouse was observed

for a total time of 30 minutes, during which we watched for the appearance of tonic-clonic seizures. Tonic-clonic seizures are characterized by the stiffening of the body followed by spasms and jerks of the muscles. The latency period of the first major seizure and the number of mini and major seizures that each mouse had was recorded. We defined major seizures as having very visible tonic-clonic seizures and a slow recovery time afterwards, usually around the minute mark or longer depending on the severity of the seizure. While mini seizures were defined as very short, rapid convulsions in which the tonic-clonic seizure was difficult to observe and the mice had a very rapid recovery of a few seconds.

Aim 2

After a 30 min acclamation period, during which the mice were weighed and marked, the AZ66 compound was administered at either a 10, 20, or 30 mg/kg dose. After an hour pretreatment of the AZ66, the mice were then treated with a subcutaneous 80 mg/kg dose of PTZ. Then, as before in the dose response, the mice were then observed for 30 minutes and the seizure latency and the number of mini and major seizures recorded.

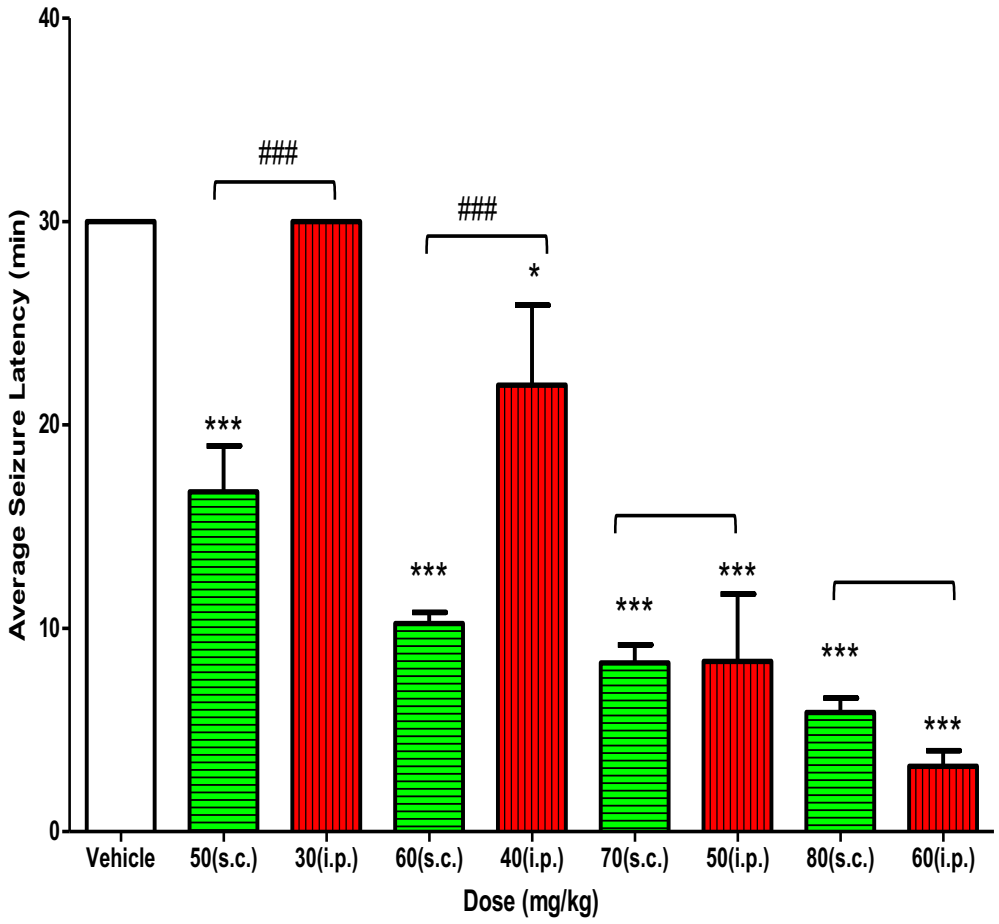
Data Analysis

Data was shown as mean ± SEM. with each group having n= 8 animals. Statistical analysis was performed using one-way ANOVA preceded by the Tukey post hoc test to define the significant different against the vehicle control at $p<.05$ for each dose of PTZ and AZ66.

III. Results

Aim 1

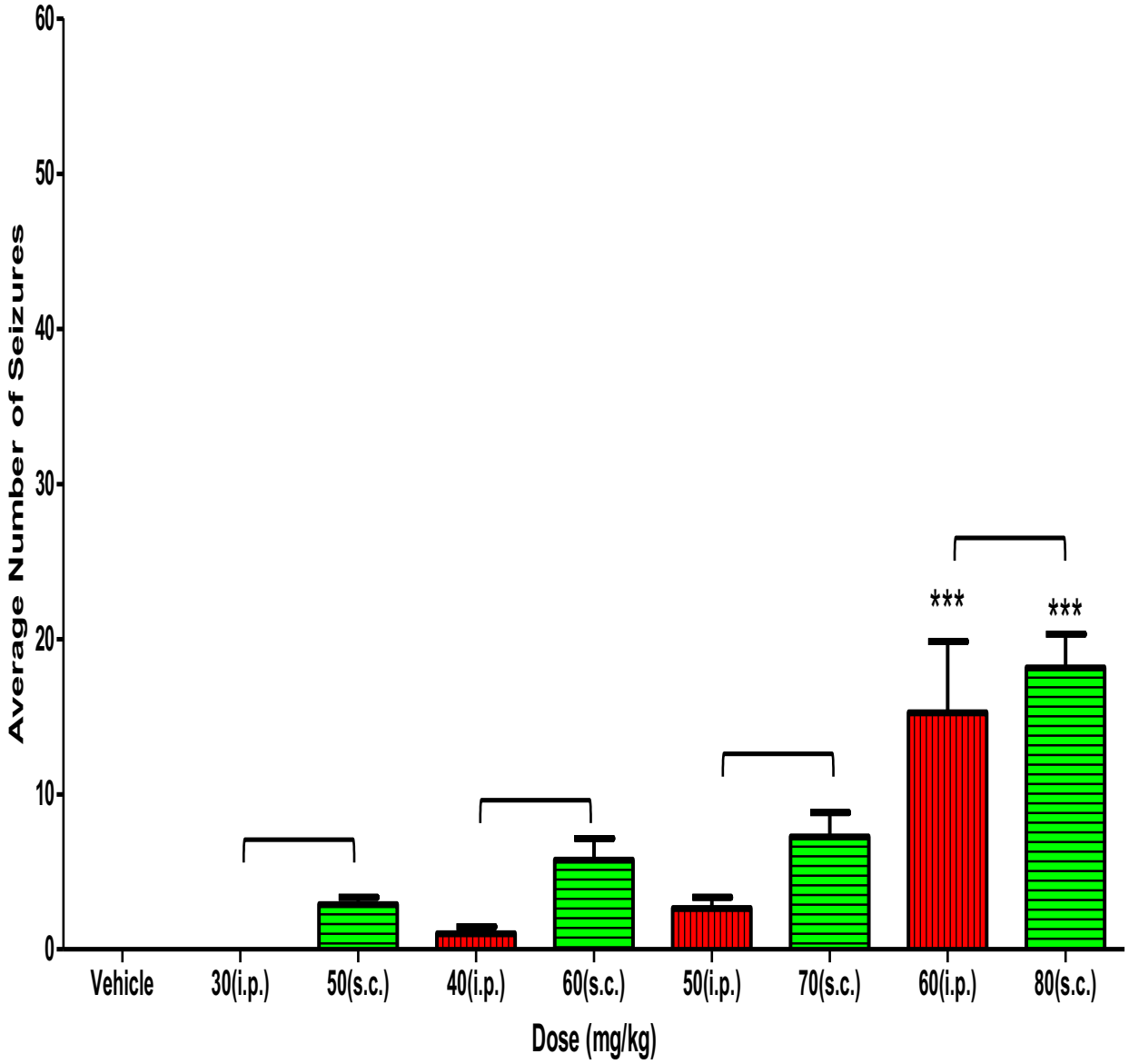
Figure 2 demonstrates the seizure latency for two dosing methods, intraperitoneal and subcutaneous, at increasing doses of PTZ. The saline solution as expected did not produce a seizure and therefore had a latency time of 30 minutes which was the total time of observation. Every dose for each dosing method was significantly different when compared to the vehicle with the exception of the intraperitoneal 30 mg/kg dose. The i.p. 40 mg/kg dose of PTZ differed from the vehicle at a p-value <.005 while the other doses, with the exception of the i.p. 30 mg/kg dose of PTZ, were significantly different with p-values < .001. The dosing methods only significantly differed between the s.c. 50 mg/kg dose compared to the i.p. 30 mg/kg dose and the 60 mg/kg dose s.c. matched to the i.p. 40 mg/kg dose of PTZ ($p<.001$). The fastest latency time was 60 mg/kg dose of PTZ administered intraperitoneally.



*p< 0.05, ***p<0.001 versus vehicle control (0 mg/kg) (Tukey's posthoc test)

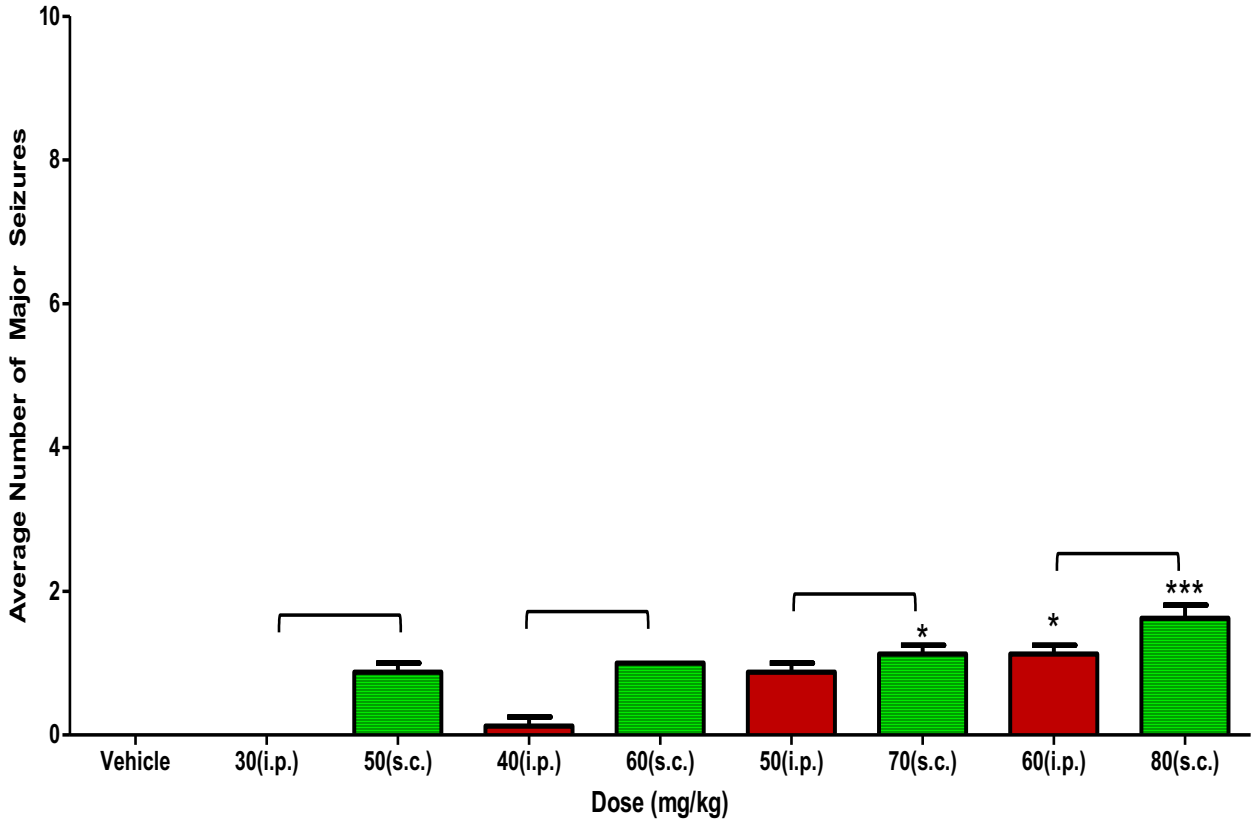
###p<0.001 versus compared group (Tukey's posthoc test)

Figure 2: Seizure latency of intraperitoneal and subcutaneous dosing of PTZ



*p<0.05 and ***p<0.001 versus vehicle control (0 mg/kg) (Tukey's posthoc test)

Figure 3: Average number of total seizures induced by each dose of PTZ



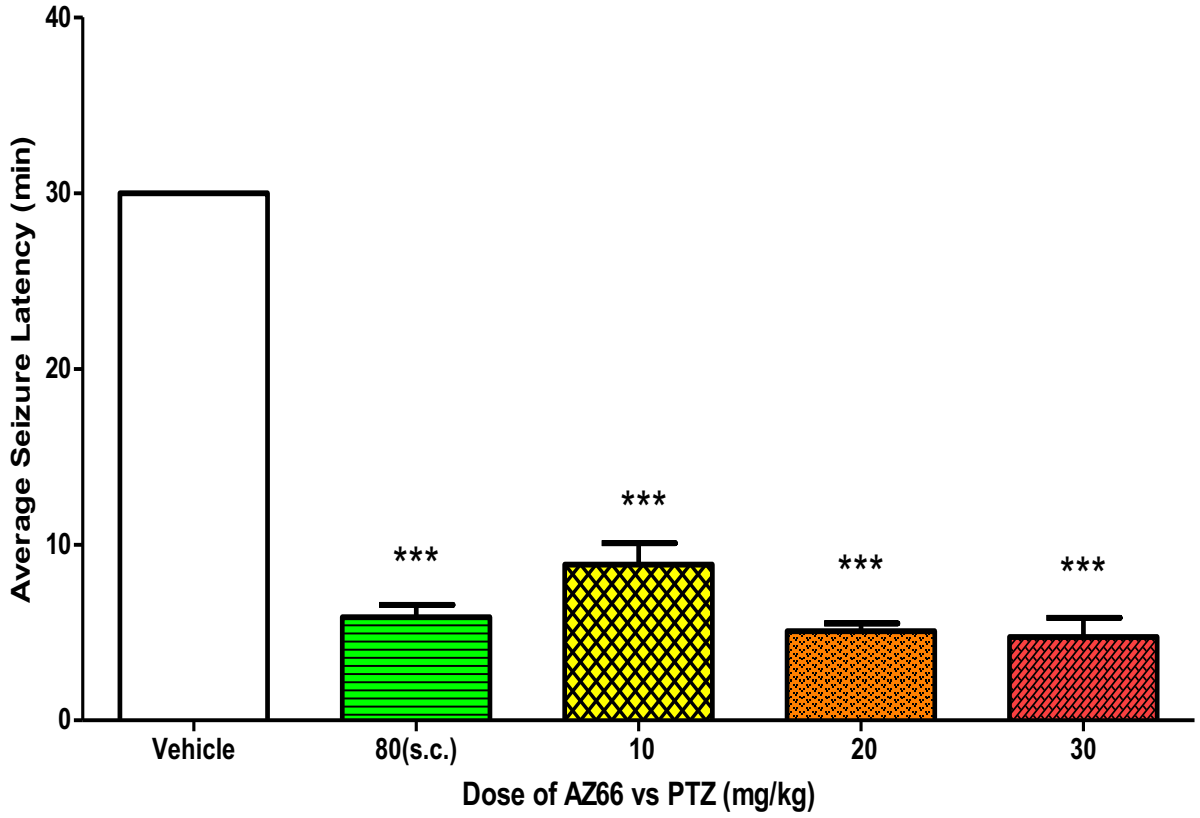
*p<0.05 and ***p<0.001 versus vehicle control (0 mg/kg) (Tukey's posthoc test)

Figure 4: Average number of major seizures for intraperitoneal and subcutaneous dosing of PTZ

Figure 3 represents the total number of seizures for each dose of PTZ and Figure 4 is the average number of major seizures for each dosing method and the dose studied. The 70 mg/kg dose administered subcutaneously and the 60 mg/kg dose administered intraperitoneally differed significantly from the vehicle at a 95% confidence interval. There was no significant difference between the dosing methods and the number of seizures for each comparison group. Both figures 3-4 reflect that the 80 mg/kg subcutaneous dose of PTZ induced the most seizures although the intraperitoneal 60 mg/kg dose was statistically comparable.

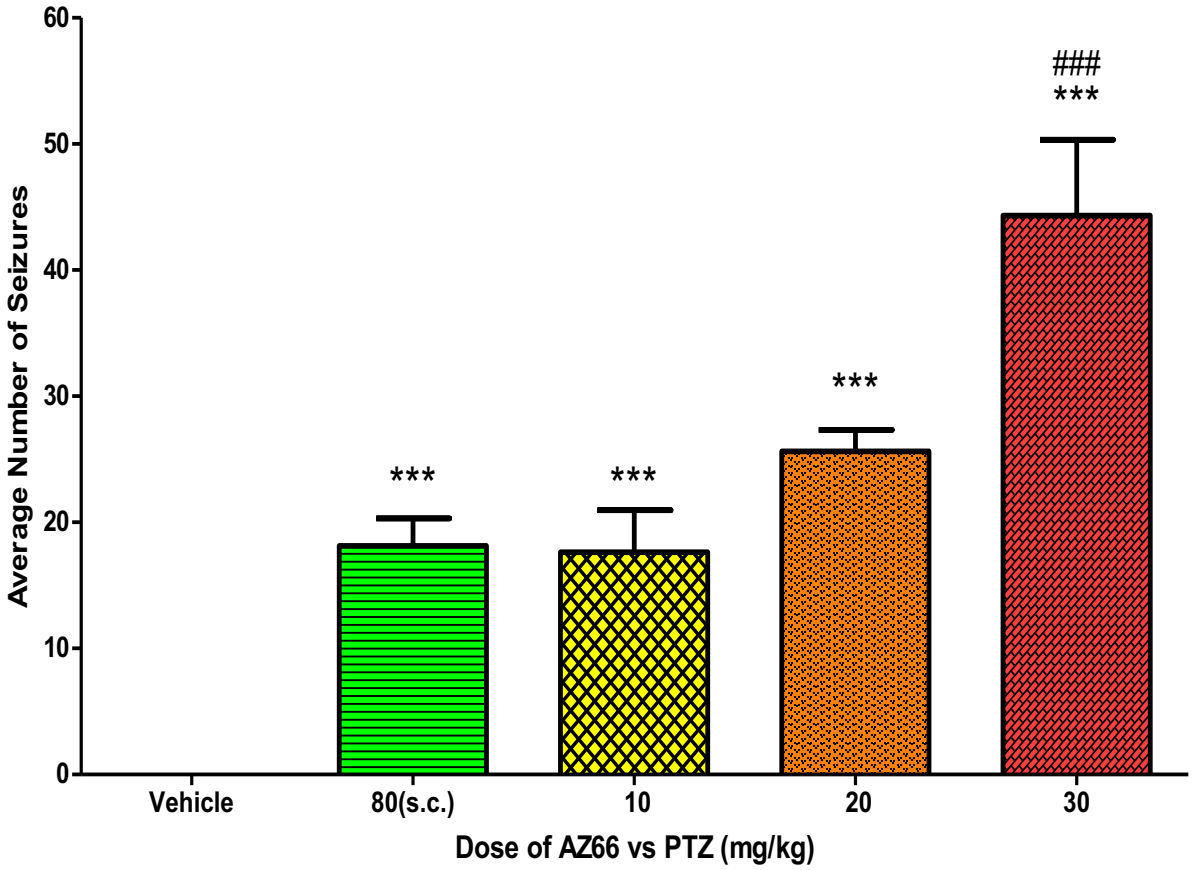
Aim 2

AZ66 did not produce any seizures or adverse side effects during the hour pretreatment. Figure 5 relates the average seizure latency time of the different doses of AZ66 compared to the lone 80 mg/kg dose of PTZ. All doses of AZ66 were statistically significant at a p-value < .001 when compared to the vehicle. As the dose of AZ66 increased, the onset of the seizures occurred more rapidly. While conducting the trial with the pretreatment of the 30 mg/kg dose of AZ66, the experiment was stopped early when it became apparent that inducing a seizure was fatal at this dose.



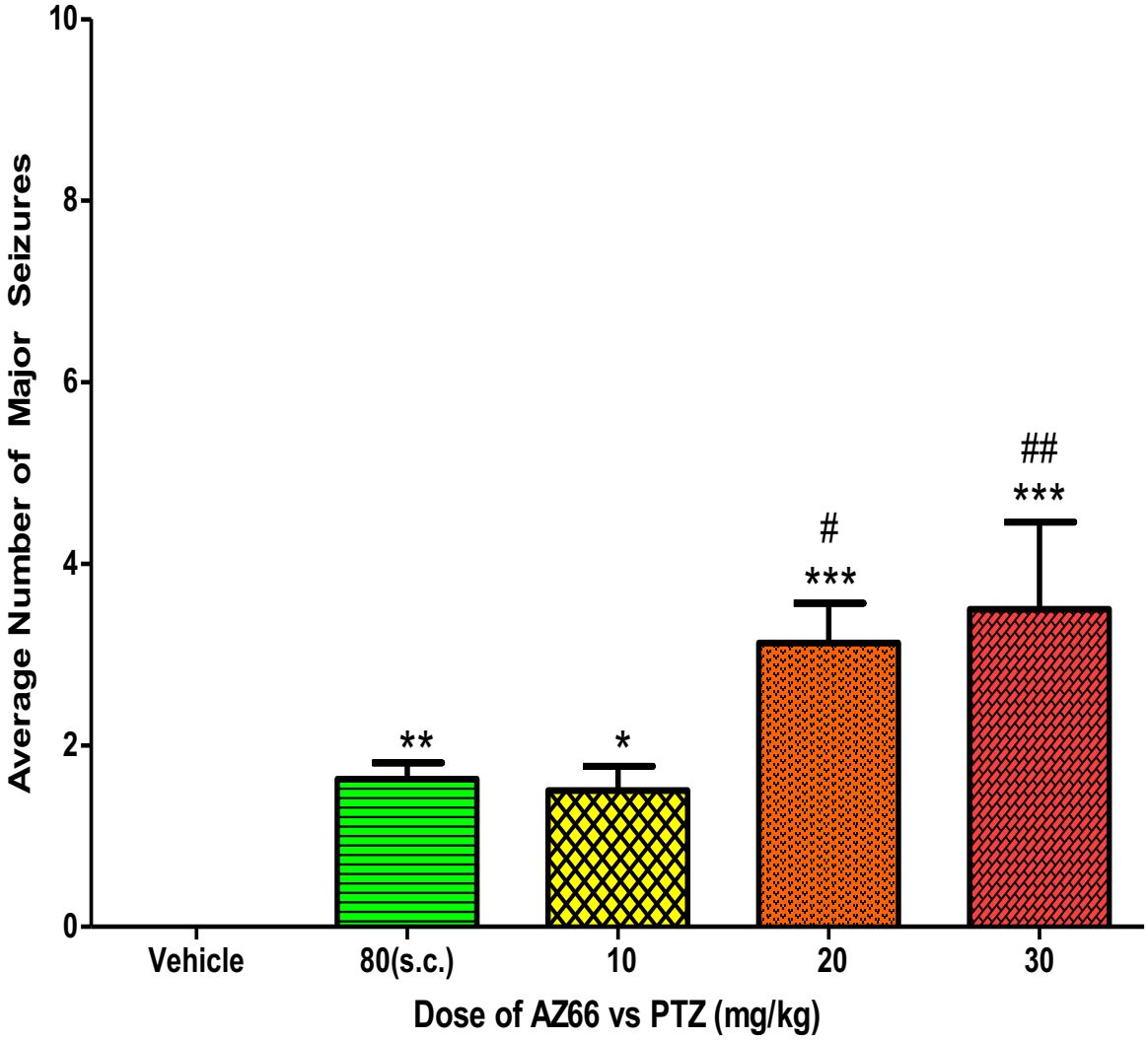
***p<0.001 versus vehicle control (0 mg/kg) (Tukey's posthoc test)

Figure 5: Average seizure latency time for doses of AZ66



*p<0.05, **p<0.01, and ***p<0.001 versus vehicle control (0 mg/kg) (Tukey's posthoc test)
#p<0.05, ##p<0.001 and ###p<0.001 versus 80mg/kg PTZ group (0 mg/kg) (Tukey's posthoc test)

Figure 6: Average number of total seizures for each dose of AZ66 with PTZ (80mg/kg s.c.)



*p<0.05, **p<0.01, and ***p<0.001 versus vehicle control (0 mg/kg) (Tukey's posthoc test)
#p<0.05, ##p<0.001 and ###p<0.001 versus 80mg/kg PTZ group (0 mg/kg) (Tukey's posthoc test)

Figure 7: Average number of major seizures for doses of AZ66 with PTZ (80mg/kg s.c.)

Figures 6 and 7 represent the dose response of AZ66 on the number of chemically induced seizures. In figure 6, all groups were significantly different at a p-value of .001 when compared to the vehicle. When compared to the lone dose of 80 mg/kg subcutaneous dose of PTZ, only the highest dose of AZ66 (30 mg/kg) proved to be significant ($p<.001$). Figure 7 demonstrates the effects of increasing the dose of AZ66 on the number of major seizures. All doses of AZ66 differed from the vehicle at a p-value $< .05$. When the number of major seizures produced when pretreated with AZ66 was compared to the lone dose of the s.c. 80 mg/kg dose of PTZ, the 20 mg/kg and the 30 mg/kg proved to be significantly different ($p<.05$). Both of these graphs illustrate that as the dose of AZ66 increased so did the frequency of the seizures increased.

IV. Discussion

The subcutaneous 80mg/kg dose of PTZ was chosen due to the quick response time, frequency of the seizures, and the clear distinctions between mini and major seizures. However, the effects of AZ66 were the opposite of what we expected. When screening AZ66, the compound actually decreased the seizure latency time while increased the number of seizures as the dose increased. These seizures were more violent and resulted in death at the 30 mg/kg dose of AZ66.

While AZ66 may attenuate seizures induced by cocaine, it does not do so for PTZ induced seizures. PTZ exerts its effects by acting as a $GABA_a$ receptor antagonist. GABA is one the most important inhibitory neurotransmitters in the central nervous system (Heard, Palmer, & Zahniser 2008) and is the target of many AEDs. Because AZ66 did not perform as expected, it is

reasonable to assume that AZ66 does not directly act on the GABA pathway. This is consistent with the fact that cocaine indirectly induces its effects on GABA receptors through neurotransmitters (Centonze, et al., 2002). It is likely that sigma antagonists are not be effective in treating GABA-mediated, chemically induced seizures. This assumption is supported by the fact that AZ66 is effective at reducing seizures induced by cocaine, but not seizures induced by PTZ. However, because cocaine induced seizures are drug refractory, AZ66 could still prove useful in other types of epilepsy.

V. Future Studies

Futures studies for this project are needed. First off, AZ66 needs to be tested for efficacy in reducing electrically induced seizures using the Maximal Electroshock seizure test. It is necessary to perform this test to rule out the possibility of AZ66's antiepileptic effects in a different seizure type. While AZ66 is not effective in attenuating seizures caused by chemical stimulation, it may be beneficiary in electrically kindled seizures. From there, PTZ and MES induced convulsion tests should be run on selective antagonists for both sigma 1 and sigma 2 receptors to find which is responsible for the increase of the convulsions seen when using the non-selective sigma antagonist AZ66.

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