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Src Family Kinase Inhibitor: Saracatinib as a Potential Therapeutic Agent for Epileptogenesis

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Abstract

Spontaneous recurrent seizures are the hallmark of epilepsy, a common neurological disorder. The condition is often caused by insult to the brain that spurs a prolonged seizure- *status epilepticus* (SE). Epilepsy can be highly resistant to medication in cases where brain insult is in the form of chemical nerve agent exposure. Src family kinases (SFK) have recently been identified as a mediator of neuroinflammation. This study utilizes the SFK inhibitor Saracatinib as a viable post-treatment for epileptogenesis following initiation of SE by the organophosphate nerve agent, Soman. This study tested 20 mg/kg of Saracatinib once a day for a week. Saracatinib following Soman administration minimally affected spiking activity but did not affect convulsive seizures compared to vehicle-treated animals 10 to 15 weeks post treatment. Saracatinib-treated rats had statistically significant fewer spikes on days 9, 22, 23, and 30. Vehicle-treated rats had statistically significant higher spikes than the mild group on days 30-34. A different dosing regimen for a prolonged period may have a significant outcome with respect to these parameters. Other parameters such as MRI, behavioral, and histological tests should be considered to draw meaningful conclusions of Saracatinib treatment.

Keywords: epileptogenesis, Src family kinases, chemical nerve agents, EEG recording

Introduction

Epilepsy is a common neurological disorder characterized by recurrent unprovoked seizures. Epileptogenesis, the continuing process in which a neurotypical brain becomes prone to having seizures, may be due to a genetic predisposition, brain insult, an unknown cause, or chronic neurodegenerative disease (Löscher & Brandt, 2010; Pitkänen et al., 2015). Brain insult in the form of *status epilepticus* (SE) -state of prolonged seizure- leads to irreversible neuronal damage and changes in glial cells (Trinka et al., 2015). The resulting brain damage can lead to spontaneous recurrent seizures (SRS) (Dudek & Staley, 2012; Pitkänen et al., 2015).

Unfortunately, epilepsy can be highly resistant to medical intervention. Many antiepileptic medications (AEDs) target ion channels known to contribute to neuronal hyperexcitability during seizures (Bialer & White, 2010). While effective for some, the discovery of novel AEDs is necessary to treat the substantial population who present with refractory epilepsy (~20%-44%) (Dalic & Cook, 2016; Sillanpää & Schmidt, 2006; Tian et al., 2018). Under pathological conditions, the brain's immune system can become maladaptive and invoke neuronal damage (Block, 2014; Ransohoff, 2016). One pioneering approach to epilepsy disease treatment is to target the molecular mechanistic pathways that generate and promote neuroinflammation.

Targeting Src family kinases (SFK)

SFKs, inclusive of Src and Fyn, are non-receptor tyrosine kinases that reside in both microglia and neurons. They are known for their role in cell signaling, contributing to cellular functions such as cell growth, differentiation, and apoptosis (Parsons & Parsons, 2004).

Markedly, our lab found that Fyn is upregulated in animal models of epilepsy and is implicated in neuroinflammatory pathways (Sharma et al., 2018). The KA (kainic acid) rat model for epilepsy studied the relationship between Fyn and microglial inflammation activation. Compared to Fyn knockout mice, Western blot tests revealed that Fyn positive mice (wild type) had increased levels of phosphorylated PKCδ (protein kinase C delta) following KA challenge. PKCδ can activate NFκB, a key transcription factor that mediates the neuroinflammatory response of microglia and astrocytes (Gordon et al., 2016; Liu et al., 2017).

Upon insult like SE, the brain activates glial cells, including astrocytes and microglia. These cells act as immune modulators. Astrocytes manage the extracellular concentrations of GABA, glutamate, and potassium ions (Coulter & Steinhäuser, 2015). Microglia are phagocytic cells that can release proinflammatory cytokines including IL-1, IL-6, and TNF-α as well as anti-inflammatory cytokines such as IL-10 and IL-4 (Smith et al., 2012; Wang, 2015). The presence of proinflammatory cytokines decreases seizure threshold, and therefore is considered proconvulsant (Galic et al., 2012).

In neurons, Fyn/Src can modulate N-methyl d-aspartate (NMDA, excitatory) and gamma aminobutyric acid ($GABA_A$, inhibitory) receptors (Jurd et al., 2010; Knox & Jiang, 2015). Fyn regulates NMDA receptors by phosphorylating several g-protein subunits and increasing receptor trafficking (Dunah et al., 2004; Trepanier et al., 2012). $GABA_A$ receptor subunit γ2 is known to be phosphorylated by Src (Nakamura et al., 2015; Nani et al., 2013). The γ2 subunit is integral in benzodiazepine (anticonvulsant) binding (Günther et al., 1995; Middendorp et al., 2014). Mutations in γ2 are linked to epileptic syndromes (Boehm et al., 2004; Eugene et al., 2007; Macdonald et al., 2010). SFK activity in microglia and neurons imply that they could be a therapeutic target for epileptogenesis mediation.

Saracatinib is an experimental drug that inhibits Src/Fyn kinase. Two phase I clinical trials of Saracatinib in cancer patients found that the drug is well tolerated (Baselga et al., 2010; Fujisaka et al., 2013). Continued trials have been met with limited success. For example, researchers discovered that in an Alzheimer's mouse model Saracatinib does enter cerebral spinal fluid, which suggests that it can cross the blood-brain barrier (Kaufman et al., 2015). However, in a study of 24 Alzheimer's disease patients there was not statistically significant therapeutic effects in Alzheimer's Disease Assessment Scale-Cognitive, Mini-Mental State Examination, Clinical Dementia Rating Scale, or 18F-fluorodeoxyglucose positron emission tomography imaging (Nygaard et al., 2015). It is notable that the trials included patients who were not in the early development stage of neurological disorder. It is left to be determined if Saracatinib could have a different effect in early-stage populations. Less explored, is the possible effect of Saracatinib on epileptogenesis. One animal trial during early stage epileptogenesis revealed statistically significant fewer CS when pretreated with Saracatinib (Sharma et al., 2018).

Current Study

We employed a Soman rat model to induce seizures in rats similar to our past studies utilizing KA and DFP (diisopropylflourophosphate) (Putra et al., 2020; Sharma et al., 2018). Soman is a chemical nerve agent that presents a threat to both civilians and military personnel. Soman was synthesized in Germany in 1944 and although it was not used in WWII, countries including Germany and the Soviet Union began stockpiling the chemical warfare agent (Szinicz, 2005). The few medical countermeasures such as 2-PAM, atropine, and diazepam counter some long-term side effects of seizurogenic neurotoxins if administered <30 min after exposure, but they do not prevent long-term neurotoxicity (Kuruba et al., 2018; McDonough et

al., 1995; Shrot et al., 2014; Wu et al., 2018). There is a remaining need for neuroprotectants that are effective for delayed treatment.

Soman is an organophosphate that inhibits acetylcholinesterase (AChE) (Bennion et al., 2015; Shih & McDonough, 1999). Acetylcholine (ACh) among other functions, binds to sodium gated ion channels on postsynaptic neurons, depolarizing and exciting that cell. Remaining ACh is degraded by AChE. Soman prevents AChE from degrading ACh, procuring hyperexcited cells which can lead to SE (Shih & McDonough, 1999). We hypothesize that after Soman induced SE, administration of Saracatinib will suppress epileptogenesis in rats.

Methodology

2.1 Animal Source and Care

A total of 24 male Sprague Dawley rats (7-8 weeks old) were purchased from Charles River (MA, USA). At MRI Global (Kansas City, MI), the animals rested for a week before Soman challenge and Saracatinib/vehicle dosing regimen. Aztra Zeneka supplied the drug Saracatinib under the Open Innovation program. One day following the termination of Saracatinib treatment, animals were then transported to the Iowa State University (ISU) Laboratory of Animal Resources. Animals were maintained under a controlled environment (19°C–23°C, 12 hr light with 12 hr dark) in both locations. Experiments were performed in accordance with protocols 18-159 and 18-160 by ISU Institutional Animal Care and Use Committee. Rats were provided *ad libitum* access to food and water. Surgical procedures and EEG recording were conducted in Dr. Thippeswamy's research laboratory at ISU. Sterile and aseptic conditions were provided, and animals were placed under general anesthesia for all

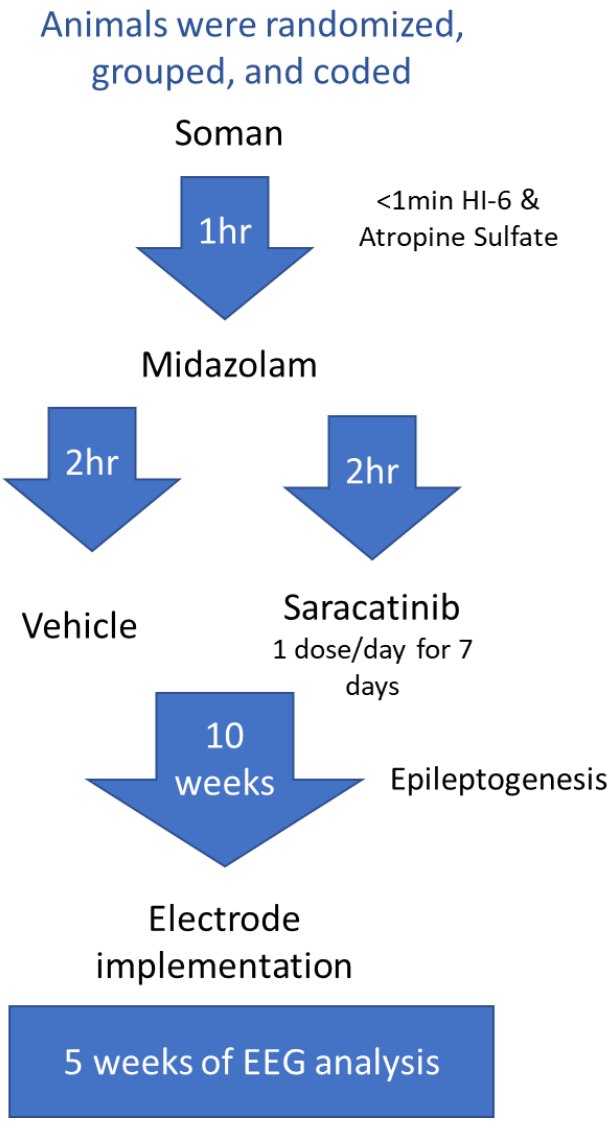
surgical procedures. Furthermore, pain and discomfort were minimized by pre- and post-operative care. Animals were observed and weighed daily after exposure to Soman (s.c.) and surgical procedures.

2.2 Experimental Groups and Drug Treatment

The 24 rats were grouped at random and coded before they were included in the experiments. The animals were placed into two groups: Saracatinib and vehicle control. At MRI global, animals were given 132 µg/kg Soman (s.c.), directly followed by the administration of 125 mg/kg HI-6 (i.m.) and 2 mg/kg Atropine Sulfate (i.m) to reduce mortality. Atropine Sulfate (i.m.) acts as a ACh receptor antagonist and HI-6 (i.m.) reactivates AChE (Kim et al., 1999). HI-6 does not cross the blood-brain barrier and therefore is unlikely to influence SE severity (Dadparvar et al., 2011). Midazolam (3 mg/kg, i.m.) was given one hour after Soman administration to halt visual seizure activity. Without telemetry implantation, we were unable to confirm the effect of midazolam on electrographic activity, though it is extremely unlikely that activity returned to baseline (Putra et al., 2020). Two hours after Midazolam exposure, 20 mg/kg Saracatinib (p.o.) or a vehicle (0.5% hydroxypropyl methyl cellulose and 0.1% tween 80) was administered with repeated doses every 24 hr (7 total doses). Over the next 10 weeks, animals were left to develop epilepsy before the implanting the telemetry device. To euthanize the animals, we gave the animals an overdose of the drug Euthanaisa (100 mg/kg), consisting of sodium pentobarbitol. A timeline of drug treatments is illustrated in **Figure 1.**

Figure 1

An Overview of the Experimental Timeline



Note. 24 animals were challenged with 132 µg /kg Soman (s.c.), followed by 125 mg/kg HI-6 (i.m.) and 2 mg/kg Atropine Sulfate (i.m). Animals were then allowed to seize for 1h before administration of 3 mg/kg Midazolam (i.m.). 2 hr following Midazolam exposure, 20 mg/kg Saracatinib (p.o.) or a vehicle (0.5% hydroxypropyl methyl cellulose and 0.1% tween 80) was administered every 24 hr (7 total doses). Over the next 10 weeks, the animals were expected to develop epilepsy proceeding telemetry device implantation. EEG recordings were collected during the following 5 weeks. The animals were then euthanized with the drug 100 mg/kg Euthanaisa.

2.3 SE Quantification

Succeeding administration of Soman, an experimenter monitored and scored animals for the initial SE severity. Video recordings were captured for additional verification as in our previous studies in the rat KA and DFP models (Puttachary et al., 2016; Sharma et al., 2018). SE staging was based on previous publications in the DFP study (Putra et al., 2020). The stages are defined in **Table 1**.

Non-convulsive seizures (NCS) were considered stage 1 and 2 while convulsive seizures (CS) were considered ≥ stage 3. Severity of SE was determined by the duration of CS (≥3 stage) in each animal: mild (<30 min), and severe (>30 min). The animals were randomly assigned Saracatinib or vehicle control so that each condition shared almost equal numbers of rats that had mild or severe SE as shown in **Table 2**. One animal died prior to Saracatinib treatment and was excluded from the study. A comparison of seizure severity and its duration is shown in **Figure 2**. Notably, the mild and severe classification are considered as the duration of CS during the 1 hr period between Soman exposure and Midazolam treatment, not the stages of SE.

Table 1

Seizure Stage	Criteria
1	exorbitant salivation, lacrimation, urination and defecation (SLUD), mastication, chewing
2	tremors, wet-dog shakes, head nodding, neck jerks, kyphosis, and opisthotonus
3	forelimb clonus, Straub tail, rearing and rigid extension of forelimbs
4	rearing, forelimb clonus and loss of righting reflex
5	abducted limbs clonus/repeated rearing and generalized seizures.

Table 1 Definitions of each seizure stage congruent with DFP rat epilepsy model in Putra et al. (2020).

Table 2

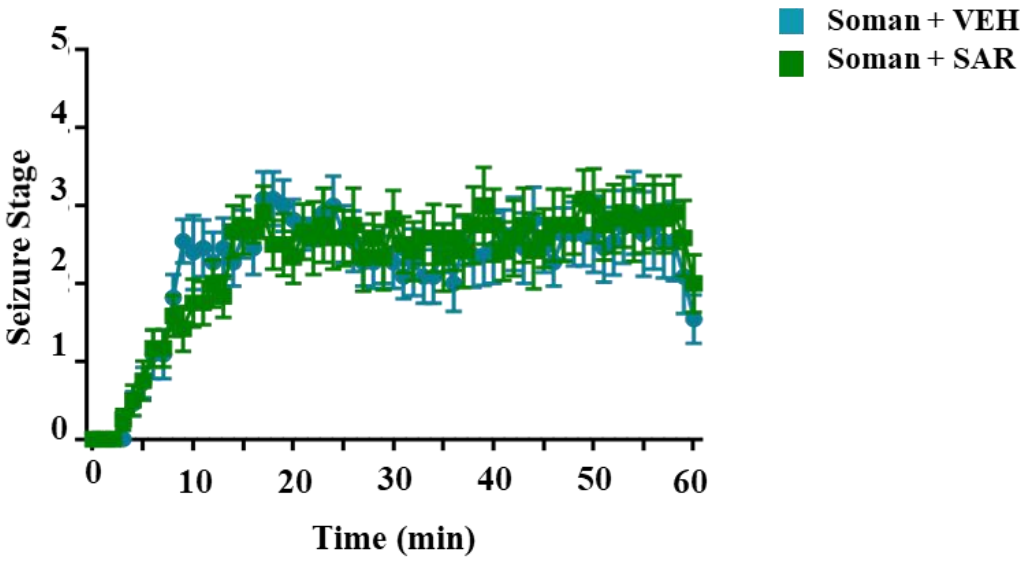
Seizure Severity	Soman + Vehicle n/group	Soman + Saracatinib n/group
Mild	4(2)	5
Severe	7(7)	7(7)
Total	11	12

Table 2 Initial SE severity and grouping were derived from the duration of CS (≥3 stage), during the 1h SE, for the vehicle or Saracatinib treatment. SE severity defined as: mild (<30 min) and severe (>30 min). Nine Vehicle rats (2 mild, 7 severe) and 7 Saracatinib treated rats (all severe) were implanted with telemetry.

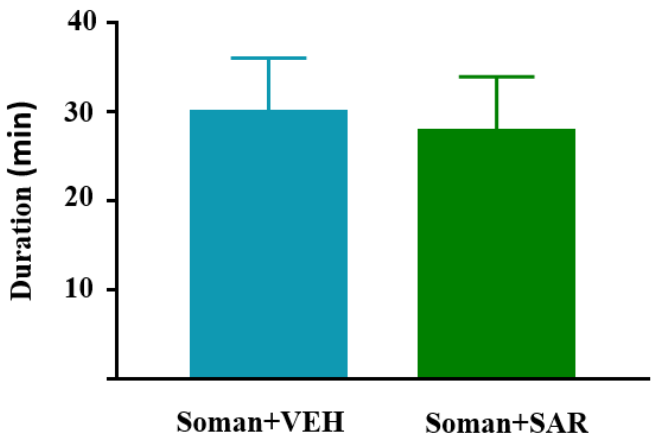
Figure 2

A Comparison of SE Severity During Soman Exposure

A



B



Note. Panel A: The mean severity of seizures following Soman administration. Panel B: The mean duration of CS (≥3 stage) per condition. The error bars represent standard error of mean; there were no statistical differences. This is important to understand the real effects of SAR.

2.4 Telemetry device implantation and video-EEG recording

A CTA-F40 PhysioTel™ telemetry device (Data Science International, Minneapolis, USA) was implanted into 16 rats for video-EEG acquisition 10 weeks after Soman administration. We prioritized animals that sustained roughly >30 min of CS during SE for telemetry device implantation. Prior to surgery, animals were injected with analgesic buprenorphine (0.3 mg/kg, s.c.) followed by introduction of anesthesia- 4.0% isoflurane (flow rate at 1L/min O2) then held at 1.75-2% during the surgical operation. As described in our previous publications (Puttachary et al., 2016; Sharma et al., 2018), the operation was completed in a sterile environment. Artificial tear ointment was applied to keep the eyes lubricated during surgery, preventing corneal ulceration and dryness. The electrodes were placed epidurally over

the cerebral hemispheres with the telemetry device arranged in the subcutaneous pouch. Dental cement (A-M systems, WA, USA) was used to anchor the electrodes to the skull, and sterile surgical clips to fasten the incision closed. The surgical site was coated with Vetropolycin triple antibiotic ointment. Prior to recovery from the anesthesia, Baytril systemic antibiotic (5 mg/kg, s.c., Bayer Pharma, PA), and normal dextrose saline (1 mL) were applied subcutaneously. The animals were then housed individually for video-EEG data collection via PhysiolTel receivers (RPC-1) connected to the Data Exchange Matrix (DSI Dataquest A.R.T. system). The telemetry device senses and records locomotor activity and body temperature. Seven rats were treated with Saracatinib while 9 rats were given the vehicle control. Subsequently, the animals were monitored for 5 weeks by means of continuous video-EEG recording. Epileptiform spikes and CS were analyzed to discern the long-term efficacy of Saracatinib.

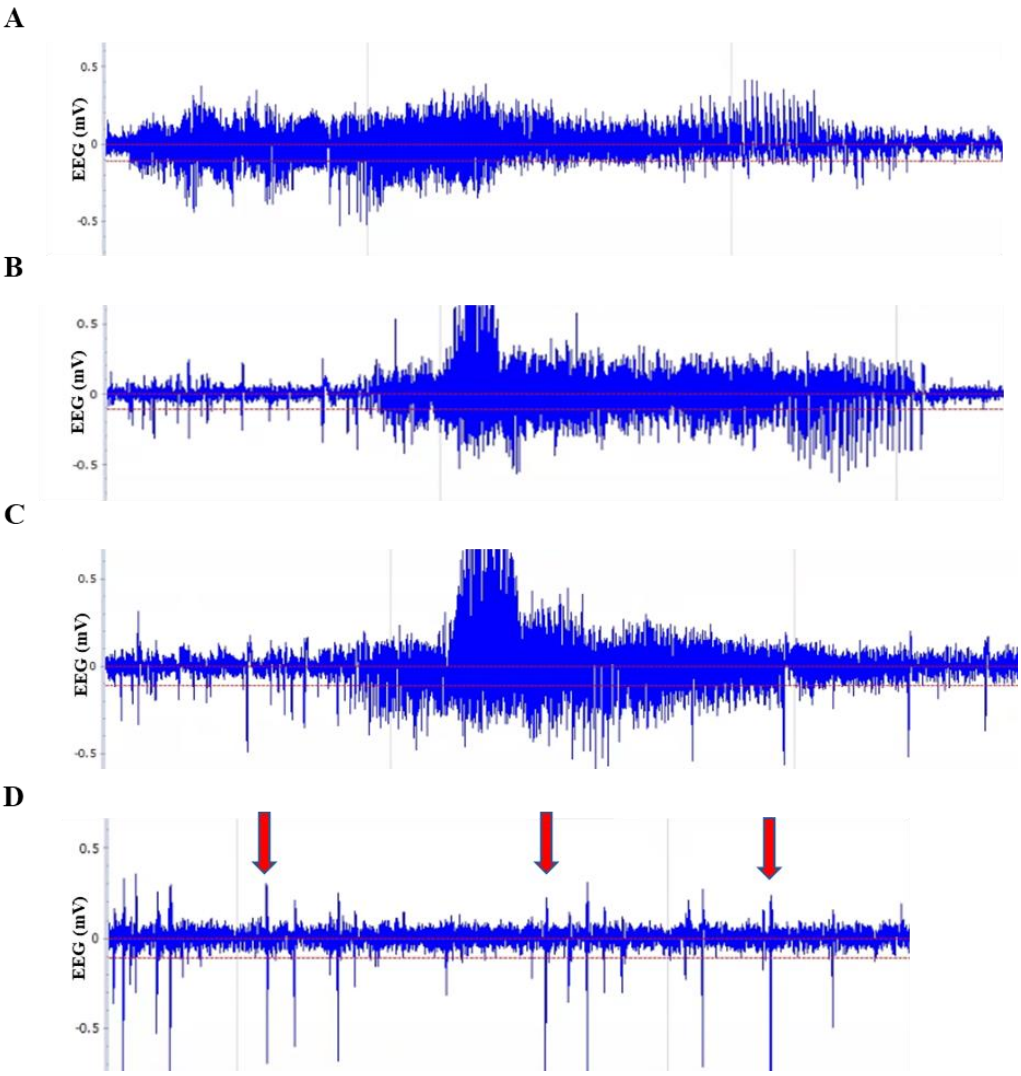
2.5 Quantification of epileptiform spikes and CS

NeuroScore 3.2.0 software was used to identify all epileptiform spikes. The spikes were then summed across animal groups to determine the effects of Saracatinib. Spike trains were considered as spike clusters as well as individual epileptiform spikes which included pre-ictal and inter-ictal activity. Quantified epileptiform spikes included spikes in spike trains and electrographic NCS. CS were identified as high amplitude and high-frequency spikes on the EEG. In addition, CS were confirmed by fast Fourier transformation generated power spectrum and matched with real-time video. The quantification of CS instances in this study is as outlined in our previous publications (Putra et al., 2020; Puttachary et al., 2015; Sharma et al. 2018). Electrical noise, grooming and exploratory behavior, were recognized and omitted from epileptiform spike analysis as indicated in the rat and mouse KA models (Puttachary et al., 2015,

2016; Sharma et al., 2018; Tse et al., 2014). Examples of EEG epileptiform spikes are displayed in **Figure 3.**

Figure 3

EEG Epileptiform Activity



Note. Representative EEG traces showing different types of spontaneous CS: stages 3 (Panel A), stage 4 (Panel B), and Stage 5 (Panel C). Panel D: Individual epileptiform spikes (a few examples are indicated by arrows).

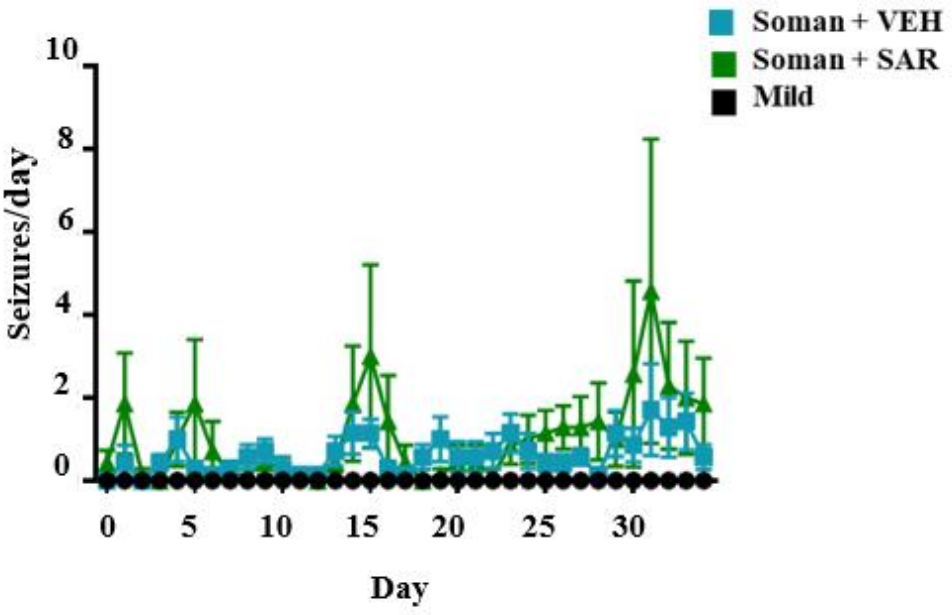
2.6 Statistical analysis

The program Graphpad was employed to conduct all statistical analyses and graph creation. Both t-tests and analysis of variance (ANOVA) were performed at which $p<0.05$ was considered as a statistically significant difference between Saracatinib and vehicle treated groups. A two-way ANOVA was conducted to compare the means of seizure stage between each group across the 60 minutes (pre Saracatinib or vehicle administration) **Figure 3.A**. A t-test then assessed if each group had an overall statistically significant difference in CS duration throughout SE **Figure 3.B**. All **Figure 4** graphs compare the conditions across the 5 weeks of EEG analysis (post vehicle or Saracatinib administration). Two telemetry implanted rats in the mild vehicle group provided a baseline to compare with the severe Saracatinib and vehicle treated groups. A two-way ANOVA was conducted to compare mean number of CS by day and week **Figure 4(A and B)**. A t-test was used to contrast the total number of CS **Figure 4.C**. Lastly, epileptiform spikes were compared by day, by performing a two-way ANOVA **Figure 4.D**.

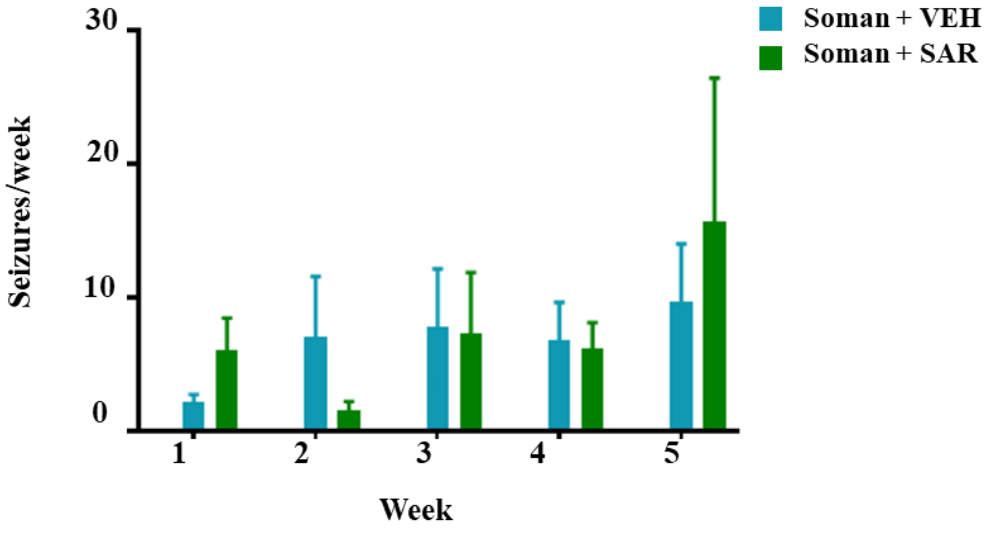
Figure 4

Number of Seizures and Spikes Over Time

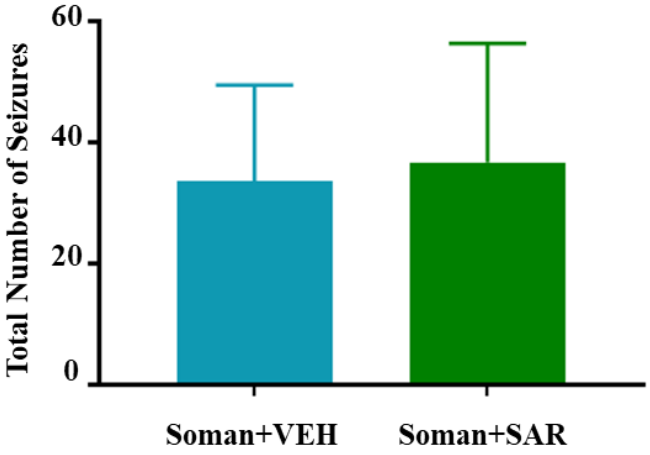
A



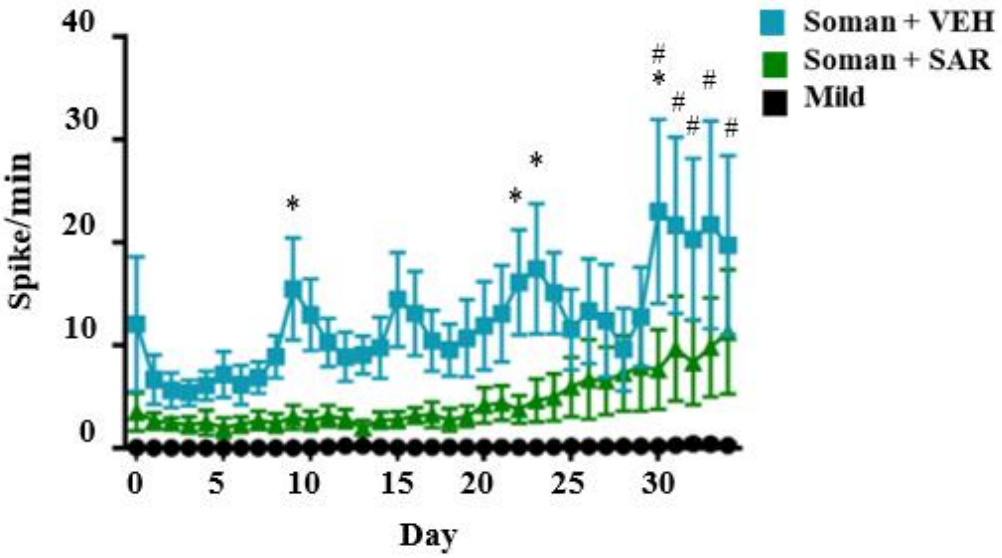
B



C



D



Note. The mean number CS, Panel A: by day, Panel B: by week and Panel C: across the total 5 weeks of EEG analysis. Panel D: The mean number of epileptiform spikes per day during the 5 weeks of EEG analysis (*p<0.05 VEH vs SAR; #p<0.05 VEH vs Mild). The error bars represent standard error of mean.

Results

The effectiveness of Saracatinib as an adjunct epilepsy treatment was measured on account of the mean number of CS (≥ stage 3) and epileptiform spikes during the 5 weeks of EEG analysis. A comparison of Saracatinib and vehicle groups revealed that there was not a statistically significant difference ($p<0.05$) in the mean number of CS by day, week, or the cumulative 5 weeks **Figure 3(A, B, and C)**. The mean number of epileptiform spikes between Saracatinib, vehicle, and mild groups are denoted in **Figure 3.D**.

EEG spikes indicate an abnormal electrical discharge created by congregate neurons and are more likely to occur in animals that are predisposed to spontaneous recurrent seizures (Staley & Dudek, 2006; Zacharaki, et al., 2016). Accordingly, they provided an additional tool to evaluate epileptic behavior. Spikes between Saracatinib and vehicle groups held a statistically significant difference on days 9, 22, 23, and 30. A comparison between vehicle and mild groups identified a statistically significant difference of spikes on days 30-34. There was not a significant difference between Saracatinib and mild groups over the entire 34 days. The two rats in the mild group did not have any CS and spikes remained similar to the control after SE induction.

Discussion

In the current study, the SFK inhibitor Saracatinib was tested as an adjunct treatment for epileptogenesis. The results suggest that Saracatinib has some influence on epileptiform activity. Saracatinib had some statistically significant effects on spike rate, but not on CS. In this respect,

Saracatinib with the tested treatment regimen (20 mg/kg for a week) had a limited long-term effect on spikes and CS.

Interestingly, these results contradict the past findings of a rat KA model, in which Saracatinib as a pretreatment and post-treatment separately, significantly reduced epileptiform spikes and CS (Shama et al., 2018). The previous study had a different dosing regimen and a different source of Saracatinib (Selleckem). Unlike Soman, an anti-acetylcholinesterase, KA is a neuroexcitatory glutamate analogue (Kandratavicius et al., 2014; Lévesque & Avoli, 2013). The compounds are used interchangeably in models of temporal lobe epilepsy (TLE) however, there is no literature that directly compares the two animal models.

The timing of telemetry implantation and EEG recording is another factor to consider. EEG recording did not begin until 10 weeks after SE. Telemetry device implantation was postponed for behavioral tests (not included here). Moreover, EEG recording directly following treatment would reflect the earliest effects of Saracatinib on the epileptogenesis.

Epilepsy animal models, including the Soman model, have not measured the outcome of long-term dosing Saracatinib. Epileptogenesis is a process that typically has a marked latent period and begets chronic epilepsy (Dudek & Staley, 2012; Löscher & Brandt, 2010; Pitkänen et al., 2015). Critically, epileptogenesis does not have a set end point. The decision to administer Saracatinib solely over the first week following SE hinged upon evidence of elevated levels of Fyn and PKCδ during this period (Shama et al., 2018). Prolonging Saracatinib treatment beyond the first week of epileptogenesis could yield a different result.

A change in dose regimen would further illustrate the continued effects of SFKs throughout epileptogenesis. Dosing quantities in this study were inspired by previous literature- two cancer

targeted trials and one animal model of TLE (Green et al., 2009; Hennequin et al., 2006; Sharma et al., 2018). Given that Saracatinib has only been tested twice in TLE models, there are many questions unanswered regarding optimal dosing regimen. To better our understanding of the pharmacokinetics of this drug it is necessary to test increased or decreased amounts of Saracatinib and different time intervals between administration in different animal models.

In conclusion, there remains an obligation to discover epilepsy medications for the substantial population of epileptics that have unmanageable seizures due to unknown etiologies. The current study recognizes that the chosen Saracatinib does not significantly reduce the occurrence of CS when tested at the end of a 10 week treatment period. There was however, a minimal statistically significant reduction in epileptiform spikes. The SFK mechanism in neuroinflammation is in a preliminary phase of study and requires systemic investigations to fully grasp its role in epileptogenesis and epilepsy.

Acknowledgements

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