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**TITLE:** Neuroprotection and Anti-Epileptogenesis with Mitochondria-Targeted Antioxidant

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Neuroprotection and Anti-Epileptogenesis with Mitochondria-Targeted Antioxidant

The goals of the project were to assess the neuroprotective and antiepileptogenic properties of a mitochondrial-targeted antioxidant, SS-31 in the pilocarpine (PILO) model of status epilepticus (SE), the kindling seizure model and the tetanus toxin (Tx) model of epilepsy. Progress on the project was limited throughout the grant period due to an inability to obtain a sufficient quantity of SS-31 to perform the proposed experiments. Our last shipment of SS-31 was obtained on May 2014. This resulted in the granting of a second no cost extensions in May 2015. We did complete the experiments proposed in Aim #1, testing the efficacy of SS-31 as a neuroprotectant-antiepileptogenic agent in the PILO model of SE. SS-31 had no effect on the latency to the onset of SE and there was no evidence of neuroprotection in hippocampal tissue assessed 3 days after SE. The insult generated by prolonged seizure activity appeared to be too severe for a single dose of SS-31 to be effective. We observed these negative results despite testing SS-31 at dose of 10mg/kg, s.c., which is higher than what has been reported to be efficacious in other models. In Aim #2 we tested the efficacy of SS-31 on hippocampal kindled afterdischarge (AD) threshold and duration. Baseline AD threshold and duration were determined for each animal. A minimum of 24hr later SS-31 (10mg/kg, s.c.; n=9) was administered 75min after the onset of SE. The brains from control and FCCP-treated rats were assessed histologically for neuroprotection 3 days after the administration of FCCP. Although FCCP (2.0-2.5mg/kg, sc.) was significantly neuroprotective in the CA3 region of the hippocampus (p < 0.05) there was a significant increase in mortality in FCCP-treated rats.
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INTRODUCTION:
A number of studies have provided evidence that reactive oxygen species play a role in the induction of seizures and seizure-induced neuronal death. The goals of this project were to test the efficacy of a novel, mitochondrial-targeted antioxidant SS-31, as a neuroprotective and antiepileptogenic agent in three experimental models of epilepsy. The pilocarpine-induced model of status epilepticus (PILO) was selected to test SS-31 as a neuroprotectant, the kindling model was selected to test SS-31 as an antiepileptogenic and anticonvulsant agent, and the tetanus toxin model (TX) was selected to test SS-31 as an anticonvulsant. If SS-31 proved to be effective in these studies future experiments would test SS-31 in models of traumatic brain injury.

KEYWORDS:
Antioxidants, antiepileptogenesis, cell death, kindling, mitochondrial dysfunction, neuroprotection, seizures, status epilepticus

OVERALL PROJECT SUMMARY:
SS-31 was created by Dr. Szeto but the rights to the drug are controlled by Stealth Peptides, Inc. Progress on the project during the initial grant period was limited due to difficulty obtaining sufficient quantities of the test agent, SS-31, to perform the proposed experiments. This led to the granting of a no-cost extension in April of 2014. At the end of May 2014 the PI was able to acquire a new supply of SS-31. In the previous grant periods we completed the experiments in Aim#1 and Aim #2. Unfortunately we did not observe evidence of a delay in the onset of SE or neuroprotection in the PILO model or a decrease in AD threshold or AD duration in the kindling model. In the case of the experiments using the kindling model we increased the test dose of SS-31 to 30mg/kg, sc, a dose of SS-31 significantly higher than what has been reported to prevent cell death in models of cardiac ischemia (Cho et al., 2007), kidney ischemia (Szeto et al., 2011) and mechanical ventilation-induced diaphragm weakness (Powers et al., 2011). Testing the drug at this elevated dose contributed to the exhaustion of our current supply of SS-31. Our continued difficulty in obtaining more SS-31 resulted in the granting of a second no-cost extension in May, 2015. Due to the failure of SS-31 to be effective in the PILO and kindling models and our inability to obtain more drug we replaced the experiments proposed in Aim #3 with experiments that would test the neuroprotective properties of carbonyl cyanide 4-trifluro-methoxy-phenylhydrazone (FCCP), a mitochondrial uncoupler in the PILO model of SE. The decision to test FCCP was agreed to by Captain Sawyer, DVM, the Scientific Officer for the project. We decided to test the neuroprotective properties of FCCP because it is commercially available and because it has been shown to be neuroprotective in an experimental model of traumatic brain injury (Pandya et al., 2009). The testing of a mitochondrial uncoupler is consistent with the theme of the proposal of developing a neuroprotective-antiepileptogenic therapy by preventing mitochondrial dysfunction.

Aim #1 – Test the neuroprotective and anticonvulsant properties of SS-31 in the pilocarpine model of status epilepticus (SE) in the rat.
In this model, prolonged seizure activity causes neuronal cell death in specific neuronal populations in the rodent hippocampus. Adult male Sprague-Dawley rats (260-405g) were used. In our initial experiments, experimental animals were pretreated with SS-31 (3 or 10mg/kg, sc) 45min before induction of SE with pilocarpine (365mg/kg, sc). One hour after the onset of SE each animal received an injection of diazepam (5mg/kg, ip) to attenuate SE and to improve survival. Control animals received an injection of saline instead of SS-31. To determine whether treatment with SS-31 affected the development of SE, the time to the onset of SE was measured for control and SS-31 treated animals. To examine neuroprotection the animals were perfusion-fixed with 4% paraformaldehyde (PAF) 1-3 days after SE. The brains were sectioned on a vibratome through the dorsal hippocampus. Sections were processed for the following histochemical and immunohistochemical stains: Nissl, Fluoro-jade C (FJ), NeuN and heat shock protein 70-72 (HSP). Nissl, FJ and NeuN stains were used to assess neuroprotection. HSP was used to detect neuronal stress but under some conditions has been shown to be neuroprotective. To pursue the possibility that PILO-induced SE was too severe an insult to observe a neuroprotective effect of SS-31 we
also tested SS-31 in a modified version of the PILO model to limit the amount of PILO-induced seizure activity. In these animals we administered a higher dose of diazepam (10mg/kg, ip) 5min after the onset of SE instead of the usual 60min. We also injected SS-31 into the experimental rats 30min before the injection of PILO instead of 45min.

Results for Aim #1 – Effect of SS-31 in the Pilocarpine model for Status Epilepticus
The administration of SS-31 (10mg//kg, sc) 30-45min before induction of SE with PILO (365mg/kg, sc) did not delay the onset of SE and histochemical/immunohistochemical staining of hippocampal tissue 1-3 days after SE provided no evidence of neuroprotection in SS-31-treated rats. A total of 42 rats were evaluated in this Aim (17 controls and 25 experimental). Three rats from each group died during SE. Our conclusion from these results is that the insult induced by SE is too severe for pretreatment with SS-31 to be effective. An additional possibility is that pretreatment with SS-31 before the onset of SE is not the optimal window for drug delivery. It is possible that delivery of the antioxidant after the degenerative process has started would yield positive results. This approach was investigated in experiments using the mitochondrial uncoupler FCCP.

Results for Aim #2 – Effect of SS-31 in the Kindling seizure model
Kindling is a seizure model where repeated, spaced delivery of an initially subconvulsive stimulus to a limbic structure results in a permanent change in brain function such that eventually the kindling stimulus regularly elicits a limbic seizure. Progression through the kindling process can be assessed by measurement of the severity of the behavioral seizure and by measurement of the threshold and duration of the electrographic afterdischarge (AD). Behavioral seizures are scored on a 1-5 scale with stages 1-2 being equivalent to partial seizures and stages 3-5 equivalent to generalized convulsions. Once animals have exhibited 3 consecutive stage 5 seizures the kindling process is considered complete the animals are considered to be fully kindled. The number of stimulations required to reach a given stage, AD threshold and AD duration can be measured to assess the epileptogenic process. AD threshold is determined by delivering a kindling stimulus at a low current intensity. If no AD is detected the delivery of the kindling stimulus is repeated at a higher current intensity until an AD is elicited. AD testing is initiated at a current intensity of 5µA and increased in 5µA increments until an AD with a duration of at least 5sec is observed.

Effect of SS-31 on AD threshold and duration
Our first experiment was to determine if pretreatment with SS-31 altered AD threshold and AD duration. Each of the rats acted as their own control. Bipolar platinum depth electrodes were stereotaxically implanted bilaterally into the dorsal hippocampi of 13 anesthetized adult male Sprague-Dawley rats. Several screws electrodes were also implanted into the skull to allow for recording of surface EEG and to act as a ground. The electrodes were connected to a headstage which allows for connection to a stimulation/EEG recording system through a cable. Animals were allowed to recover from electrode implantation surgery a minimum of one week before entering into the study. The kindling stimulus is delivered through the same electrodes used to record EEG activity in an awake, freely-moving animal. Data were collected from 9/13 rats as 4 rats were removed from the study due to poor recording quality or loss of the electrode head stage. The experimental design consisted of initially determining baseline AD threshold and AD duration. A minimum of 24hr after the determination of baseline AD threshold and duration each animal was treated with SS-31, 30min before delivery of the kindling stimulus. This pattern was continued over a number of days. For this experiment the kindling stimulus had the following characteristics: 60Hz, 1msec biphasic pulse, delivered for 2sec. We observed no effect of pretreatment with SS-31 (10mg/kg, sc) on AD threshold or AD duration (Figure 1). When we increased the dose of SS-31 to 20mg/kg, sc (n=9) there was still no effect on AD threshold (Figure 2A) but we did observe a decrease in AD duration in 6/9 rats (Figure 2B). We repeated these experiments administring SS-31 at a dose 30mg/kg, sc (n=5). Unfortunately when SS-31 was tested at the higher dose we were unable to obtain the positive effect we observed on AD duration with a dose of 20mg/kg (Figure 3 A and B). We were unable to repeat these experiments due to our inability to obtain more SS-31. It is important to recognize that the doses of SS-31 tested in the PILO and kindling models were significantly higher than
what has been reported to be effective in other models of cell death. This raises the possibility that SS-31 does not readily crossing the blood-brain barrier.

**Results for Aim #3 – Test the neuroprotective properties of FCCP in the Pilocarpine model of Status Epilepticus.** The combination of the negative results we obtained with SS-31 treatment in the PILO and kindling models and our inability to obtain more SS-31 lead to the decision to replace the experiments proposed in Aim #3 testing SS-31 in the TX model with experiments designed to test the neuroprotective efficacy of carbonyl cyanide 4-trifluoro-methoxy-phenylhydrazone (FCCP), a mitochondrial uncoupler in the PILO model of SE. We decided to test FCCP because it is commercially available and because it has been shown to be neuroprotective in an experimental model of traumatic brain injury (TBI) (Pandya et al., 2009). The testing of a mitochondrial uncoupler is consistent with the theme of the proposal of developing a neuroprotective-antiepileptogenic therapy by preventing mitochondrial dysfunction. In the study by Pandya et al. (2009) treatment with FCCP (2.5mg/kg) administered 5min after TBI proved to be neuroprotective. The same dose of FCCP administered up to 6hr after TBI restored mitochondrial Ca$$^{2+}$$ levels to baseline levels. For this reason in our experiments using the PILO model we tested FCCP (2.5mg/kg, sc) administered 75 minutes after the onset of SE. For these experiments surviving animals underwent perfusion-fixation with 4% paraformaldehyde 3 days after SE. The brains were removed and sectioned on a vibratome (50µm) throughout the dorsal hippocampus. Sections were stained for Nissl, fluorojade, NeuN and heat shock protein (HSP 72). The following brain regions were evaluated for neuroprotection: Dorsal Hippocampus – (CA1; CA3: Dentate Hilus); and Entorhinal Cortex.

One of our initial observations was that treatment with FCCP (2.5mg/kg) decreased survival to 18% (n=11) compared to 87.5% in Control animals (n = 16) (Table 1). This increase in mortality was disappointing because in the FCCP-treated rats that survived there was evidence of neuroprotection in CA1 of the hippocampus (Figures 4 and 5) and in the entorhinal cortex at the base of the brain (Figure 6). Subsequent experiments focused on testing FCCP at lower doses with the hope that we could observe an increase in survival while retaining neuroprotection. Lowering the dose of FCCP below 2.5mg/kg did improve survival (Table 1) but even a dose of 1.25mg/kg resulted in a 40% mortality. The brains from surviving animals were evaluated after treatment with the following doses of FCCP: Control – 0mg/kg (n=10); 1.25mgkg (n=3); 2.0mg/kg (n= 4); 2.25mg/kg (n=5); 2.5mg/kg (n=2). Nissl-stained sections from the left hemisphere of each brain were evaluated for damage and scored using the following scale: 1 – no damage; 2 – some neuronal loss; 3- moderate loss; 4 – significant loss; 5- complete or almost complete loss. An one-way ANOVA was run to determine significance.

Although individual animals in each dose group showed evidence of neuroprotection a statistical analysis of the data revealed significant neuroprotection only in area CA3 of the hippocampus at a dose of 2.0mg/kg (p< 0.05) and a dose of 2.5mg/kg (p < 0.05) (Figure 7A). There was a trend indicating neuroprotection in CA1 which would likely become significant with a larger sample size (Figure 7B).

**KEY RESEARCH ACCOMPLISHMENTS:**

- The experiments proposed in Aim #1 have been completed. Treatment with SS-31 (3-10mg/kg, sc) did not delay the onset of status epilepticus in the pilocarpine model.
- SS-31 was not neuroprotective in the pilocarpine model of status epilepticus.
- Treatment with SS-31 (30mg/kg) had no effect on kindling AD threshold or AD duration indicating SS-31 had no effect in this model.
- In both the pilocarpine and kindling models, SS-31 was tested at a dose higher than what had been reported to be effective in other models.
- Due to the failure of SS-31 to be effective in the pilocarpine and kindling models and the inability to obtain more drug we tested the neuroprotective properties of FCCP, a mitochondrial uncoupler in the pilocarpine model.
Treatment with FCCP (2.5mg/kg) resulted in a significant increase in mortality after pilocarpine-induced SE. However, there was evidence of neuroprotection in the CA1 region of the hippocampus and entorhinal cortex in the animals that survived.

Treatment with lower doses of FCCP improved survival with evidence of neuroprotection in all subfields of the hippocampus and the entorhinal cortex but statistical significance was only observed in CA3 after treatment with doses of 2.0mg/kg and 2.5mg/kg.

There was a trend towards statistically significant neuroprotection in CA1 which would likely become significant with a larger sample size.

Evidence of neuroprotection with FCCP indicates that seizure-induced mitochondrial dysfunction is one of the mechanisms underlying neuronal cell death after SE.

CONCLUSIONS:
Progress throughout the project was limited due to difficulty obtaining a sufficient quantity of SS-31 to effectively test its action. In an attempt to obtain positive results, SS-31 was tested at significantly higher doses than what was originally proposed leading to a more rapid exhaustion of the amount of drug we were able to obtain. The experiments proposed in Aims 1 and 2 were completed. Unfortunately we did not observe a positive effect of SS-31 on the latency to the onset of SE or anatomical evidence of neuroprotection in pilocarpine model of SE. We also did not observe a positive effect of SS-31 in the kindling seizure model on AD threshold or AD duration. The failure to obtain a significant positive effect in either seizure model suggests that the pilocarpine insult was too severe for this antioxidant to have a neuroprotective effect and raises the possibility that SS-31 does not cross the blood brain barrier as readily as originally anticipated. After discussions with the project’s Scientific Officer it was decided that the direction of the project would be changed. The experiments originally proposed in Aim #3 were replaced with experiments designed to test the neuroprotective properties of FCCP, a mitochondrial uncoupler, in the pilocarpine model of SE. FCCP has been reported to be neuroprotective in an experimental model of TBI (Pandya et al., 2009). FCCP was initially tested at a dose of 2.5mg/kg, sc, since this dose was reported to be effective in TBI. While this dose did result in neuroprotection it also significantly increased mortality in the PILO model. Using lower doses we were able to detect a significant neuroprotective effect in CA3 of the hippocampus at FCCP doses of 2.0mg/kg and 2.5mg/kg (p<0.05).

The observation of an increase in mortality after treatment with FCCP raises the possibility that administration of FCCP directly into the CNS, possibly through an intranasal route, would yield improved neuroprotection without the increase in mortality. These results also raise the possibility that FCCP could be administered at a lower dose in combination with neuroprotective agents that act through a different mechanism of action to yield a synergistic effect.

These results provide evidence that seizure-induced mitochondrial dysfunction contributes to seizure-induced neuronal death after SE. Our findings combined with results from studies that have examined the efficacy of mitochondrial uncouplers in models of TBI suggest that the neuronal damage that occurs after insults to the CNS that result in an increase in extracellular glutamate, independent of the source (seizures, ischemia, TBI), is partially due to mitochondrial dysfunction and that therapeutic agents that can block mitochondrial dysfunction will likely be neuroprotective after these insults.

Futures studies should focus on the intranasal delivery of FCCP, or agents with a similar mechanism of action, directly into the CNS. From a military perspective the ability of the warfighters to carry on their person a neuroprotective agent that could be rapidly administered through an intranasal route of administration by the warfighter or their comrades could greatly improve the outcome after brain trauma in the field.

PUBLICATIONS, ABSTRACTS AND PRESENTATIONS:
None
INVENTIONS, PATENTS AND LICENSES:
None

REPORTABLE OUTCOMES:
None at this time.

OTHER ACHIEVEMENTS:
None

REFERENCES:
None

APPENDICES:

SUPPORTING DATA:

Figure 1 – Effect of SS-31 (10mg/kg, sc) on kindling AD threshold (A) and duration (B). SS-31 injected 30min before delivery of the kindling stimulus had no effect on AD threshold or duration (n=5).
Figure 2 – Effect of SS-31 (20mg/kg, sc) on kindling AD threshold (A) and duration (B). SS-31 injected 30min before delivery of the kindling stimulus had no effect on AD threshold but decreased AD duration in 6/9 animals (n=9).
Figure 3 – Effect of SS-31 (30mg/kg, sc) on kindling AD threshold (A) and duration (B). SS-31 injected 30min before delivery of the kindling stimulus did not have a significant effect on AD threshold or AD duration (n=5).
**TABLE 1 – Effect of FCCP on Survival**

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<td>Control</td>
<td>14</td>
<td>16</td>
<td>87.5</td>
</tr>
<tr>
<td>FCCP 1.25mg</td>
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<td>60%</td>
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<td>10</td>
<td>40%</td>
</tr>
<tr>
<td>FCCP 2.25mg</td>
<td>5</td>
<td>10</td>
<td>50%</td>
</tr>
<tr>
<td>FCCP 2.50mg</td>
<td>2</td>
<td>11</td>
<td>18%</td>
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**Figure 4 – Effect of FCCP on neuronal loss in CA1.** Nissl-stained section of the hippocampus from pilocarpine-treated rats. Panels A, C and E are from a rat treated with saline while panels B, D and E are from a rat treated with FCCP (25 mg/kg, sc). Arrow in A indicates damage in the CA1 region of the hippocampus which was not present in B. Panels C-F are increased magnification of the areas indicated by the arrows. Note the dying cells in E compared to the healthy cells in F. Magnification A and B – 2x; C and D -10x; E and F - 40X.
Figure 5 – Fluorojade (FJ)-stained sections of the hippocampus from a Control rat (A and B) and FCCP-treated rat (C and D). Treated rat received 2.5mg/kg. Panel A illustrates the presence of FJ positive-stained dying neurons in CA1, CA3 and the dentate hilus of the control animal. Panel C illustrates that the number of FG-positive neurons is greatly reduced in both CA1 and CA3 of the FCCP-treated rat. Evidence of FCCP-induced neuroprotection in CA1 can be observed by comparing Panel B with Panel D. Calibration bars: A and C - 500µm; B and D - 100µm.
Figure 6 - Effect of FCCP (2.5mg/kg) on neuronal loss in the entorhinal cortex (ENTO). Nissl-stained sections from a control (A and B) and FCCP-treated rat (C and D) after pilocarpine-induced SE. Note the neuronal loss in the control rat that did not receive FCCP (A and B). Magnification: A and C – 4x; B and D – 40x.
Figure 7 - Quantification of the neuroprotective effect of FCCP in the hippocampus and entorhinal cortex.
FCCP was tested at doses of 1.25; 2.0, 2.25 and 2.5 mg/kg, sc. Damage was scored on a 1-5 scale with 1 indicating no damage and 5 complete loss. Scores reported as mean +/- SEM. Statistical comparison made using one-way ANOVA. A. CA3; B. CA1; C. Dentate Hilus; and D. Entorhinal cortex. FCCP significantly protected CA3 (A) at a dose of 2.0mg/kg (p<0.05) and 2.5mg/kg (p<0.05). There was a trend towards protection in CA1 at doses of 2.0mg/kg and 2.5mg/kg (B).